PEPTIDES

Synthesis - Structure - Function

Proceedings of the Seventh
American Peptide Symposium

Daniel H. Rich
Erhard Gross

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American Peptide Symposium

Edited by

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PREFACE

The Seventh American Peptide Symposium was held on the Madison Campus of the University of Wisconsin, June 14-19, 1981. Over 550 scientists from 20 countries attended to exchange ideas and review progress in the field. Topics discussed ranged from peptide synthesis and characterization to mechanism of action, internalization, and postribozomal modifications.

The meeting was organized to stress new ways drugs can be developed from peptide systems. Miguel Ondetti organized a session focusing on the design of protease inhibitors. New competitive inhibitors of angiotensin converting enzyme, acid-proteases and serine-proteases were described and evidence was developed that highly selective inhibition of proteases is possible. Betty A. Eipper organized a session to review the biosynthesis and post translational modifications of peptides. The existence of numerous proteases that process these modifications is apparent and may offer additional targets for the medicinal chemist. Competitive inhibitors of substance P, arginine vasopressin, enkephalin and glucagon were described. S. Bloom and J. Polak emphasized in their review of the distribution and significance of peripherial regulatory peptides that additional specific blockers of peptidergic transmission are needed. Thymopoietin pentapeptide, fragments of IgGl, C3a and Thymosin α, were described in a session on immunochemistry and provided new examples of small peptide segments that retain the biological activities of the parent molecule. Small peptide antigenic determinants of lactate dehydrogenase C_4, calmodulin and diptheria toxin also were reported.

The session on molecular calculations and computer graphics was highlighted by several 3-dimensional film clips plus a color stereoslide projection. That these techniques have matured into established methods for drug design was illustrated by their application to the synthesis of an orally active cyclic hexapeptide analog of somatostatin and conformationally constrained analogs of LHRH, enkephalin and TRH. The graphics session also presented attempts to calculate water hydration to amide bonds as a first step toward inclusion of solvent in calculations of peptide conformation.

The sessions on chemical synthesis of peptides illustrated the increasing dependence of the peptide chemist on organic chemistry in order to synthesize unusual amino acids, such as those required for the total synthesis of the antitumor drug bleomycin or to develop the new coupling and protection reagents used to synthesize gastrin releasing peptide and to couple peptide segments to new solid supports. The

synthesis of peptides by Recombinant DNA techniques, first described at the 1979 Peptide Symposium has already produced the successful synthesis of crystalline human insulin. Ronald Chance and Bruce Frank of Eli Lilly Company utilized the A plus B chain recombination and proinsulin approaches respectively.

Solid state NMR and Fast Atom Bombardment (FAB) mass spectrometry were two instrumental methods, described at a peptide symposium for the first time, that hold great potential for the study of peptides. FAB-MS permits mass spectral analysis of *underivatized* peptides.

The third Alan E. Pierce Award was presented to Klaus Hofmann for his contributions to peptide synthesis methodology. In the award lecture Dr. Hofmann described his new approaches to the biospecific retrieval of hormone receptors. One of the highlights of the Symposium Banquet was Klaus Hofmann's rendition of a poem by Francis Finn, written to honor the occasion. The enthusiastic response of the participants prompted us to include the poem on pages vii-ix of this volume.

Organizing this symposium was a very enjoyable experience for me. Many people willingly gave of their time to suggest program topics or to assist with the organization. In particular I want to thank the members of the local committee and their students who gratuitously helped with last minute preparations and to my students for providing critically needed manpower during the symposium. This meeting could not have succeeded without the very helpful assistance of Gudrun Sindermann and Faye A. Shivers, who coordinated activities at Wisconsin Memorial Union, George Gurda who handled all university dormatory arrangements, and Linda Frei for her help with the word processor. Bonnie J. Hagness, who worked with me for 18 months as conference coordinator, and who directed the efficient operation of the symposium office deserves special recognition for her outstanding efforts.

For the second time the Publisher of the Proceedings of this Symposium is Pierce Chemical Company of Rockford, IL. It is a pleasure to acknowledge the outstanding efforts in the Editorial Office of Melba Rinaldo. Special recognition is due Robert (Bob) Vigna who served as copy editor of this book. Bob worked extremely hard and effectively over the past 18 months to make this book a success. I am grateful to have had the opportunity to work with him.

Finally it is with sadness that I note the sudden and untimely death of Erhard Gross, my friend and co-editor of this book. A memoriam to Erhard is presented on pages xxix-xxxi.

Madison, Wisconsin; November, 1981 Daniel H. Rich

SEVENTH AMERICAN PEPTIDE SYMPOSIUM

University of Wisconsin-Madison
Madison, Wisconsin
June 14-19, 1981

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SHORT HISTORY OF PEPTIDE CHEMISTRY

It started in dem Vaterland
This stepchild of chemie
When Fisher made his first peptide
And put our ship to sea

It sailed across to Bergman's Lab
And made our lives more pleasant
We used this tool to make substrates
They're still on sale at present

Then came the work of VduVee
And peptides grew more sexy
They stimulated everything
From birth to apoplexy

The peptide field grew large and strong
New structures were unraveled
While on the strength of future gains
The peptide chemists traveled

They went to Athens, Prague and Bâle
And met with one another
They talked of coupling and protecting
And how best to recover

In Holland they were shocked to hear
Machines might take their jobs
Someone was growing peptide chains
On microporous blobs

The controversy raged for years
To solid phase or not
Some thought it much the better course
To make them in one pot

And all the while sophistication
Overcame this field
The chemists slaved with brand new ways
To help increase their yield

Gone and forgotten was poor Curtius
The azide had its foe
Its products too had racemates
This was a deathly blow

New methods sprang up all around
And soon the chemists sounded
Like people from another world
With each new group compounded

You heard MSC and HOBT
And when you'd gotten the gist
Bpoc, Fmoc, Adoc and Voc
Were added to this list

Maximum protection, minimum protection
Waxed and waned in turn
Each peptide was itself unique
A lesson one had to learn

The meetings grew, "My Lord" they cried
"The Yankees have the floor"
And so this U.S. club was formed
To open up the door

To x-ray folks, biologists
And those who cure the sick
In fact to all who wonder
What makes a peptide tick

Then came the present crisis
The plasmids were running away
With all that we held sacred
Our peptides, our future - our pay

They work like microcomputers
A protein's assembled with speed
All that's required is a program
To make whatever you need

We've looked for a fault in the system
Indeed we've even been zealous
In our search for a chink in the armor
I guess you might say we are jealous

The message decays, mutations occur
We've heard they self destroy
They tend to make their own toxins
They're really a dangerous toy

But despite our current misgivings
These genes are here to stay
And as much as we distrust them
We'll coexist in our way

The greatest problem was stated here
We'll soon have to even this score
We've discovered far more peptides
Than we have actions for

So be kind to your biochem colleagues
Pharmacologist should be respected
We need their cooperation
If this problem is to be corrected

For if this dilemma continues
If we fail to correct this gaff
We'll run out of useful employment
And Nature will have the last laugh

Frances M. Finn

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ABBREVIATIONS

A

Aala	azaalanine
ABA	azobenzene-*p*-arsonate
ABC	2′-(2-aminoethyl)-2,4-bithiazole-4-carboxylic acid
Abz	aminobenzoyl
ACE	angiotensin-converting enzyme
AcChR	postsynaptic acetylcholine receptors
Acm	acetamidomethyl
ACTH	adrenocorticotropic hormone (adrenocorticotropin)
ADH	antidiuretic hormone
Adoc	adamantyloxycarbonyl
Agm	agmatine
AHM	4-amino-3-hydroxy-2-methylpentanoic acid
Aib	α-aminoisobutyric acid
ALA	alamethicin
Aleu	azaleucine
AMC	7-amino-4-methyl coumarin
AMPA	ϵ-amidinated phospholipase A_2
ANG′	angiotensin II amide
Anle	azanorleucine
AOT	sodium diisooctylsulfosuccinate
AP	acid phosphatase
Arg-H	arginal
Asu	aminosuccinyl
AT/A II	angiotensin
AVP	8-arginine vasopressin
AVT	arginine vasotocin

B

BHI	biosynthetic human insulin
BK	bradykinin
BLM	bleomycin
BMP	2-bromo-N-methylpyridinium salt
Boc	*tert*-butyloxycarbonyl
Bpoc	2-(4-biphenylyl)propyl(2)oxycarbonyl
BSA	bovine serum albumin
*t*Bu	*tert*-butyl
Bug	*t*-butyl-glycine (β-methylvaline)
Bz	benzoyl
Bzl	benzyl

C

Cl	complement
cAMP	3′,5′-cyclic adenosine monophosphate
CCK	cholecystokinin
CD	circular dichroism
CDI	*N,N′*-carbonyldiimidazole
CFF	consistent force field
Cha	cyclohexylalanine
CI	chemical ionization
Cle	cycloleucine
CLIP	corticotropin-like intermediate lobe peptide; ACTH (18-39)
Cpm	cyclopropylmethyl

D

DADLE	D-Ala^2-D-Leu^5-enkephalin
DALEA	D-Ala^2-Leu^2-enkephalin
DAMEA	D-Ala^2-Met^5-enkephalin
DAP	diaminopimelic acid
DBU	1,5-diazabicyclo[5.4.0]undec-5-ene
DCC	dicyclohexylcarbodiimide
DCC-HOBt	dicyclohexylcarbodiimide-1-hydroxybenzotriazole
DCHA	dicyclohexylamine
dDAVP	deamino-D-arginine-vasopressin
Ddz	2-(3,5-dimethyloxyphenyl)propyl(2)oxycarbonyl
DFP	diisopropylphosphorofluoridate
Dhb	2,3-dihydroxy-N-ethylbenzamide
d.i.	days of incubation
DID	double INDOR difference spectroscopy
DIEA	diisopropylethylamine
DMAP	4-dimethylaminopyridine
DMB	3,4-dimethoxybenzyl
DMF	dimethylformamide
DMPC	dimyristoylphosphatidylcholine
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
Dnp	2,4-dinitrophenyl
Dns	dansyl, 1-dimethylaminonaphthalene-5-sulfonyl
DOPA	L-3,4- dihydroxyphenylalanine
DPC	N,N′-diisopropyl carbodiimide
DPEN	dimeric pentapeptide enkephalins
DPPA	diphenylphosphoryl azide
DSIP	delta-sleep inducing peptide
dSta	4S-amino-6-methyl heptanoic acid
DTT	dithiothreitol

E

EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDT	ethanedithiol
EEDO	N-ethyloxycarbonyl-2-ethyloxy-1,2-dihydroquinoline
EGF	epidermal growth factor
EI	electron impact
ELC	effective local concentration
Enk, EK,E	enkephalin
E.R.	endoplasmic reticulum

F

FABMS	fast atom bombardment mass spectrometry
FCA	Freund adjuvant
FDMS	field desorption mass spectrometry
FD	field desorption
FDNB	fluorodinitrobenzene
Fmoc	fluorenylmethoxycarbonyl
FNPA	4-fluoro-3-nitrophenyl azide
FSH	follicle stimulating hormone
FT	Fourier transform
FTS	facteur thymique serique (thymic serum factor)

G

GCMS	gas chromatography mass spectrometry
GH	growth hormone (somatotropin)
Gla	γ-carboxyglutamic acid
GLC	gas liquid chromatography
GLC-MS	gas liquid chromatography-mass spectrometry
Gn-RH	gonadotropin-releasing hormone (gonadoliberin)
GPI	guinea pig ileum
GRP	gastrin releasing peptide
GT	galactosyl transferase

H

HAM	human albumin microsphere
HDL	high density lipoprotein
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
HFBA	heptafluorobutyric acid
HFIP	hexafluoroisopropanol
HFM	high field magnet
hGH	human growth hormone
HMPA	hexamethylphosphoramide
HMV	α-hydroxy-β-methylvaleric acid
HNSA	4-hydroxy-3-nitrobenzene sulfonic acid
HOBT	*N*-hydroxybenzotriazole

HOSu	*N*-hydroxysuccinimide
HPLC	high pressure liquid chromatography
hPTH	human parathyroid hormone
HVPE	high voltage paper electrophoresis
HyF	α-hydroxy-β-phenylpropionic acid

I

Iaa	isoamylamide
IBCF	isobutyl chloroformate
IBTFA	iodobenzene *bis* trifluoroacetate
IEF	isoelectric focusing
IGF-I	insulin-like growth factor
INF	interferon
IR	infrared
Iva	isovaleryl
Ival	isovaline

J

J	spin-spin coupling constant

L

Lac	lactic acid
LAP	lipid association peptides
LCAT	lecithin-cholesterol acyltransferase
LDH	lactic dehydrogenase
LH	luteinizing hormone
LHRH/LRH	luteinizing hormone releasing hormone (luliberin)
β-LPH	β-lipotropin
LVP	8-lysine vasopressin

M

MA	mixed anhydride
Maq	methyleneanthraquinone
Mbh	4,4′-dimethyloxybenzhydryl
MCD	mast cell degranulating
MCY	Matsuoka, Clementi, Yoshimine potential
MDP	*N*-acetylmuramyl-L-alanyl-D-isoglutamine
MIF	macrophage inhibitory factor
MLC	mixed lymphocyte response
MNE	magnetic nonequivalence
MPO	2-mercaptopyridine-N-oxide
MS	mass spectrometry
Ms	methanesulfonyl
MSA	methane sulfonic acid
MSA	multiplication-stimulating activity

MSH	melanophore stimulating hormone (melanotropin)
Mts	mesitylene-2-sulfonyl
mvd	mouse vas deferens

N

NAL	naloxone
Napth	β-naphthylamine
NCI	negative chemical ionization
NMM	*N*-methylmorpholine
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser enhancement
NP	neurophysins
Np	nitrophenyl
Nps	2-nitrophenylsulfenyl
Npys	3-nitro-2-pyridinesulfenyl
NSE	neuron specific enolase
NT	neurotensin

O

OBzl	benzyl ester
ODS	octadecasilyl
OMPA	oxymethylphenylacetic
ORD	optical rotatory dispersion
OT	oxytocin
O*t*Bu	*tert*-butyl ester
OV	ovalbumin

P

Pac	phenacyl
PAGE	polyacrylamide gel electrophoresis
Pam	phenylacetamidomethyl
PRA	pyrimidoblamic acid
PC	phosphatidylcholine
PEG	polyethyleneglycol
Pfp	pentafluorophenyl
Pgl	propargyl glycine
PHA	phytohaemagglutinin
ΔPhe	dehydrophenylalanine
PI	protease inhibitor
PLA	phospholipase A_2
PMRI	partially modified retro-inverso
pMZ	*p*-methyloxybenzyloxycarbonyl
pNA	*p*-nitroanilide
POMC	proopiomelanocortin
Pon	phenylacetoxymethyl-3-nitrobenzamidomethyl
Pop	phenylacetoxypropionyl

PP	pancreatic polypeptide
pPP	porcine pancreatic polypeptide
PTH	parathyrine (parathyroid hormone)
PTH	phenylthiohydantoin
Pys	2-pyridinesulfenyl

R

REMA	repetitive excess mixed anhydride
RER	rough endoplasmic reticulum
RetBut	*trans*-N-retinylidene-N-butylamine
RIA	radioimmunoassay
RP-HPLC	reversed phase-high pressure liquid chromatography
RPLC	reversed phase liquid chromatography
RPS	rat parotid slices

S

SAC	6-aminocaproyl
Scm	carboxymethylsulfenyl
SDS	sodium dodecylsulfate
SMHA	N-succinimidyl-6(4′-azido-2′-nitrophenylamino)hexanoate
SP	substance P
SRBC	sheep erythrocytes
SRS-A	leucotriene D
SS	somatostatin
ST2	Stillinger-Rahmen potential
Sta	statine or 3S-hydroxyl-4S-amino-6-methylheptanoic acid
Sto	3-oxo-4S-amino-6-methylheptanoic acid
Suc	succinyl
sulfmoc	9-(2-sulfo)fluorenylmethyloxycarbonyl

T

TANDEM	Des-N-tetramethyltriostin A
TAT	trimethylammonium
TCA	trichloroacetic acid
Tce	trichloroethyl
Tcp	2,4,5-trichlorophenyl
Tcroc	2-trifluoromethyl-6-chromonylmethyleneoxycarbonyl
TdT	terminal deoxynucleotidyl transferase
TEAF	triethyl ammonium formate
TEAP	triethyl ammonium phosphate
TEMPO	2,2,6,6-tetramethyl-piperidineoxyl
TFA	trifluoroacetic acid
Tfa	trifluoroacetyl
TFMSA	trifluoromethane sulfonic acid
TFE	2,2,2-trifluoroethanol
THF	tetrahydrofuran

Tid	terephthaloyl-*bis*-iminodiacetic acid
TLC	thin layer chromatography
TLCK	N-α-*p*-L-lysylchloromethyl ketone
TMG	tetramethylguanidine
TMS	trimethylsilyl
TMV	tobacco mosaic virus
TNP	trinitrophenyl
Tos	4-toluenesulfonyl
TP	thymopoietin
TPCK	L-(1-tosylamido-2-phenyl)ethylchloromethyl ketone
TRH	thyroliberin (thyrotropin releasing hormone)
TRIS	*tris*(hydroxymethyl)aminomethane
Trt	trityl, triphenylmethyl
TSP	3-(trimethylsilyl)propionate
TTT	tetrahydro-thiazole-2-thione

U

UII	urotensin

V

VIP	vasoactive intestinal polypeptide
VP	vasopressin

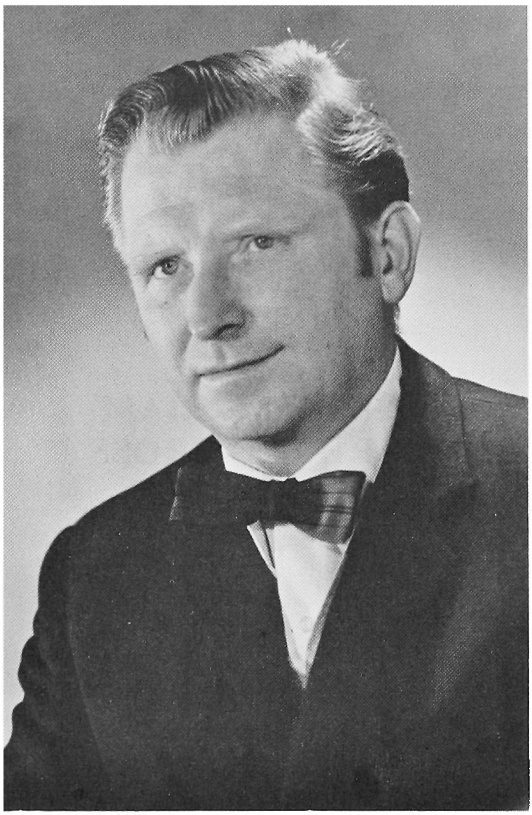
W

Wk	wasp venon

Z

Z	benzyloxycarbonyl

IN MEMORIAM
ERHARD GROSS
(1928-1981)



ERHARD GROSS
(1928-1981)

Dr. Erhard Gross, Chief of the Section on Molecular Structure at the National Institute of Child Health and Development, died September 12, 1981 in Germany as a result of an automobile accident. He was 53 years old. Born in Wenings, Germany on September 2, 1928, Erhard received the B.Sc. degree from the University of Mainz in 1953 and the Ph.D. in chemistry working with Th. Wieland in 1958 from the University of Frankfurt. He then went to the National Institutes of Health as a visiting scientist. In 1968 he was appointed section chief. Erhard was awarded the Alexander von Humboldt Prize in 1978.

Erhard made numerous research contributions to peptide chemistry, especially in the areas of analysis and synthesis, and most notably in the chemistry of sulfur containing amino acids. His work with B. Witkop on the nonenzymatic cleavage of proteins led to the discovery of the cyanogen bromide cleavage of methionine residues, which remains one on the best methods available for selectively cleaving proteins and peptides. His interest in the peptides of *B. subtilis,* developed with L.C. Craig led him to structure determination of the unusual sulfur-containing peptides subtilin, nisin, cinnamycin and duramycin, which contained unusual features such as β-methyllanthionine and dehydroamino acids. Synthetic work on these compounds led to the development of new protecting groups for peptide synthesis and new methods of forming the peptide bond. He also synthesized analogs of the naturally occurring ionophoric peptide gramicidin A to determine characteristics necessary for ion transport across lipid membranes, and contributed to the synthesis of analogs of chemotactic peptides.

Beyond his specific research contributions, Erhard will be remembered for his service in the overall development of peptide science. The community is in his debt for the 1965-66 translation into English of the Schröder and Lübke book, "The Peptides." The wider availability of this work aided the accelerating growth of peptide research in subsequent years. His recent editorial collaboration with Johannes Meienhofer to produce the open-ended treatise, "The Peptides, Analysis, Synthesis and Biology," (1979, 1980, 2 volumes in 1981) will influence and facilitate peptide research for years to come.

Erhard was an enthusiastic and active supporter of the American Peptide Symposia. He organized the highly successful 1979 Symposium at Georgetown, D.C., and expanded the scope of the meeting in remarkable ways. The Georgetown meeting had the greatest number of participants to date, the biggest industrial exhibit session yet organized and the largest proceedings issue, with 225 papers published. The continued succession of these meetings in the next years will owe much to his energetic efforts to encourage and assist volunteers to organize them.

Erhard will be missed especially at Peptide Symposia, Gordon Research Conferences, and the other meetings he attended. He was a constant contributor to the scientific discussions, formal and informal, but more, he was a warm and genial colleague, and above all a gentleman.

K.D. Kopple
D.H. Rich

Third Alan E. Pierce Award Lecture

APPROACHES TO THE BIOSPECIFIC RETRIEVAL OF HORMONE RECEPTORS

F.M. FINN and K. HOFMANN, *Protein Research Laboratory and the Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, PA, 15261*

A hormone without its receptor is an inert chemical substance. The remarkable ability of a hormone to regulate metabolic processes is only realized when the hormone combines specifically with structures, designated as receptors, that are, in the case of peptide and protein hormones, located on the cell surface embedded in a lipid matrix. The concentration of these receptors appears to be regulated by the concentration of circulating hormone. However, in general their concentration per cell is extremely low. The problem of isolating hormone receptors is akin to that faced in the isolation of neurohormones where only milligram quantities of pure hormone could be obtained from tons of starting material. From the point of view of biology certain properties of hormone receptors are well understood but from the point of view of structural understanding we are moving in largely unconquered territory. In order to gain insight into this important aspect of receptor understanding, sophisticated techniques must be developed in order to obtain the starting material for meaningful chemical analysis. Biospecific affinity chromatography appears to offer the best promise as a tool for the purification of hormone receptors since purifications in the order of several thousand fold appear to be required to obtain a homogeneous product.

A few years ago we became interested in the problem of receptor purification and selected the insulin receptor as a convenient model system for systematic studies. Considerable progress had already been made in this field and insulin receptors have been shown to be relatively stable and readily available in solubilized form. All of the current schemes for purifying insulin receptor in its native state rely on affinity chromatography. Cuatrecasas[1] pioneered in this field by using affinity columns of the type illustrated in Figure 1, where insulin is attached to Sepharose *via* a spacer arm. The attachment of the insulin to the support is not specific since unprotected insulin was coupled to the activated spacer on the resin. A family of resins was likely obtained in which the insulin molecule was attached nonspecifically through one or more of its

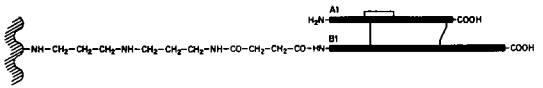


Fig. 1. The Cuatrecasas type affinity resin

three amino groups. Columns of this type bind solubilized insulin receptor but the removal of the receptor from the affinity matrix requires 4.5 M urea at a pH of 6.0, conditions that are deleterious to the insulin receptor.[2]

Immobilized anti-receptor antibodies have been employed in attempts to isolate the receptor but here again the removal of the receptor from the antibody complexes created difficulties. Harrison and Itin[3] achieved a 20-fold increase in specific insulin binding activity when they attempted to purify solubilized, partially purified insulin receptors from human placenta on an affinity resin to which antiinsulin receptor antibodies had been attached. Here again removal of the receptor from the columns was difficult and resulted in considerable loss of binding activity. The problem both in the case where the affinity column is based on insulin receptor association or where association is between an antigen and an antibody is that the association constants are high and desorption can only be achieved under rather drastic conditions that obviate the selectivity of the affinity method and frequently lower the insulin binding capacity of the receptor.

To date no one has obtained a homogeneous product and the amounts of receptor isolated have only been sufficient to permit characterization by gel electrophoretic techniques.

We were intrigued by the strong affinity of biotin for avidin (K_D 10^{-15}M) as a tool for the development of affinity resins for hormone receptor isolation. Prior to our own studies the avidin-biotin interaction had attracted the attention of workers in many different fields. Avidin-ferritin complexes had been used to stain biotinylated cell surfaces for electron microscopic visualization of surface constituents and avidin had been conjugated to fluorescein, and rhodamine for histological studies. However this is not the forum for a detailed review of the extensive literature on this subject.

Our interest involves the application of the avidin-biotin system to the isolation of peptide and protein hormone receptors. The initial plan, illustrated on Figure 2, was based on an experiment of Cuatrecasas[4] (later shown to be in error[5]) in which he described the successful removal of ^{125}I-labeled insulin from its complex with soluble insulin receptor by

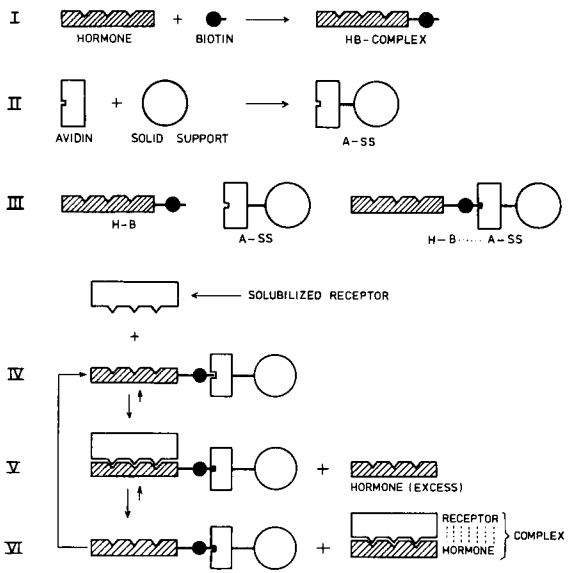


Fig. 2. Scheme for receptor isolation using affinity columns based on avidin-biotin interactions.

addition of a large excess of insulin. Our plan was as follows: Biotin was to be attached to insulin in a targeted manner that did not interfere with its biological function (line I). Next avidin was to be attached to a solid support to form immobilized avidin (line II). The biotinylated hormone was to be attached to the immobilized avidin to form an affinity resin (line III), and a solution containing solubilized receptor was to be applied to the column (line IV) to form the complex shown on Line V. The column was to be washed extensively to remove contaminating impurities and the adsorbed receptor was to be displaced in the form of the hormone-receptor complex by the addition of a large excess of hormone (line V). The regenerated column was to be reused (line VI). At the time we were pondering this approach we were invited to the laboratory of Professor Helmut Zahn in Aachen. Professor Zahn's laboratory is one of the best places in the world to learn the techniques of insulin modification and since Aachen is well endowed with rain we spent our six months sabbatical in the laboratory and synthesized N^{α},B^{1}-biotinylinsulin (biotinylinsulin).[6] The N^{α},B^{1}-attachment was selected because prior work on modified insulins had amply demonstrated that substitution at the N-terminus of the B chain exerts little effect on biological activity. Indeed, as we expected, biotinylinsulin was as active as native insulin in stimulating lipogenesis in rat epididymal adipocytes. We also established that

biotinylinsulin bound to avidin as well as to Sepharose 4B immobilized avidin. The route to N^{α},B^{1}-biotinylinsulin is illustrated on Figure 3. Geiger *et al.*[7] had shown that the reaction of insulin (I) with Boc-azide produces almost entirely N^{α},A^{1}, N^{ϵ},B^{29}-diBocinsulin (II) leaving the N-terminus of the B-chain unsubstituted. This diBocinsulin reacts readily with the N-hydroxysuccinimide ester of biotin[8] (III) to form protected N^{α},B^{1}-substituted biotinylinsulin (IV) which can be deprotected with trifluoroacetic acid to give the desired material (V). The N^{α},B^{1}-biotinylinsulin was obtained in homogeneous form by chromatography on DEAE-cellulose columns.

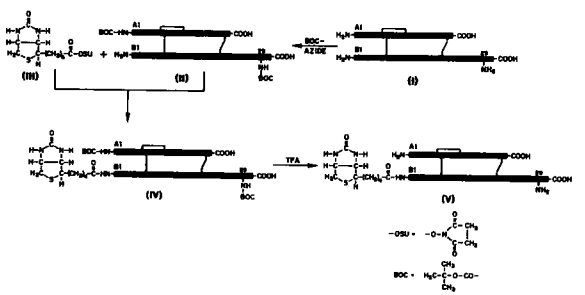


Fig. 3. Synthetic route to $N^{\alpha}B^{1}$-biotinylinsulins

Although the biotinylation of insulin in the N^{α},B^{1}-position had little effect on biological activity it became of interest to ascertain whether or not the attachment of avidin to insulin *via* the biotinyl site would interfere with binding to the insulin receptors. Furthermore it was important to determine if avidin itself interacted with biological membranes. Despite the large amount of literature on the application of the avidin-biotin system to the localization of cell surface components, there was no information available on the interaction of avidin with biological membranes. Such information was of critical importance in connection with our interests. In order to investigate this problem, labeled avidin of high specific activity was required. Avidin is composed of four identical subunits of molecular weight 17,000 each containing one binding site for biotin and a single buried tyrosine residue. Accordingly, the direct labeling of the protein with ^{125}I results in materials exhibiting low specific radioactivity. To render avidin more susceptible to iodination we introduce 3-(*p*-hydroxyphenyl)-propionyl groups by exposing the avidin to the action of N-hydroxysuccinimido 3-(*p*-hydroxyphenyl)-propionate (Figure 4).[9] The ensuing 3-(*p*-hydroxyphenyl)-propionyl avidin (pHPP-avidin) is readily iodinated to form ^{125}I-pHPP-avidin of high specific

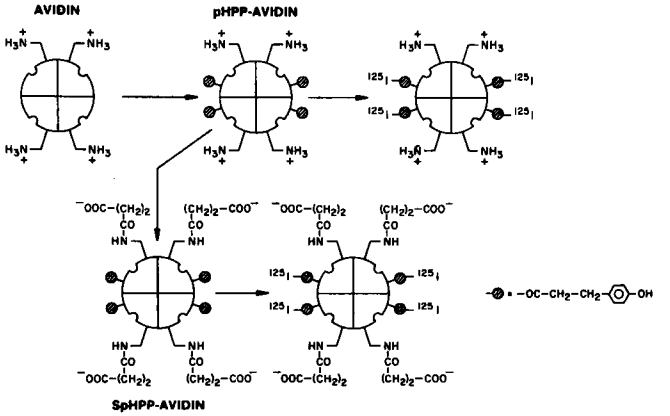


Fig. 4. Chemical modification and labeling of avidin

activity. Routinely we iodinate pHPP-avidin containing 0.8 acyl groups per subunit and employ labeled material having a specific activity of 2 mCi per nanomol. As is illustrated on Figure 5, ^{125}I-pHPP-avidin binds nonsaturably to rat liver plasma membranes. The high affinity of pHPP-avidin for biological membranes suggests that caution be exercised when interpreting results derived from studies using the avidin-biotin system.

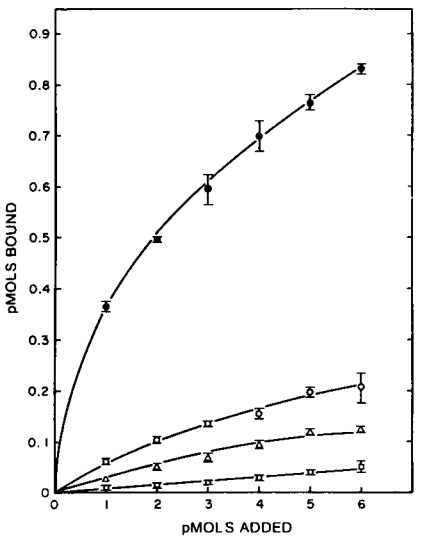


Fig. 5. Binding to rat liver membranes of ^{125}I-pHPP-avidin (●), ^{125}I-SpHPP-avidin (O), ^{125}I-SpHPP-avidin saturated with biotin (40 mol/mol) (△), and ^{125}I-SpHPP-avidin in the presence of a large excess (30 μM) of unlabeled SpHPP-avidin (□).

Avidin is a glycoprotein having an isoelectric point of 10.5. The basic nature of the molecule stems from its high content of lysine (9 residues per subunit) and arginine (8 residues per subunit). In their investigations on modified avidins, Fraenkel-Conrat *et al.*[10] observed little change in biotin binding capacity on extensive acetylation of the molecule with acetic anhydride. They also observed that iodination did not destroy this unique property of avidin. Since it appeared reasonable to assume that charge-charge interactions contribute to the binding of ^{125}I-pHPP-avidin to the membrane preparations, we reduced its basicity by exhaustive succinoylation with succinic anhydride (Figure 4). Using ^{14}C-succinic anhydride, we determined, that under the conditions used, 6 to 7 succinoyl residues were introduced per avidin subunit. Succinoylation converts positively charged ϵ-amino groups of lysine to negatively charged half-succinyl groups. Electrophoretic examination of the succinoylated material demonstrated a marked change in net charge. Succinoyl 3-(*p*-hydroxyphenyl)-propionylavidin (SpHPP-avidin) was converted to ^{125}I-SpHPP-avidin (specific activity, 2mCi per nanomol) by the Greenwood-Hunter procedure.[11] Determinations of the biotin binding capacity by separating free from bound ^{14}C-biotin by chromatography on Sephadex G-25[12] showed that pHPP-avidin bound the same amount of radiolabeled biotin as avidin; SpHPP-avidin bound 93% of the theoretical amount and avidin exposed to an amount of chloramine T sufficient to introduce 4 mols of iodine per mol of protein retained 90% of its binding capacity.

Succinoylation reduces significantly the binding of pHPP-avidin to rat liver plasma membranes (Figure 5). Unlabeled SpHPP-avidin competes with the labeled material for membrane binding sites and the binding is saturable. Saturation of the four biotin binding sites with biotin lowers still further its affinity for rat liver plasma membranes suggesting that these sites may be involved in the binding process. It must be kept in mind, however, that addition of 4 biotin molecules per mol of SpHPP-avidin introduces four additional anionic sites (biotin carboxyls) into the molecule, and this change by itself could explain the observed effect. More about biotin binding to modified avidins will be presented later.

At this point I wish to turn to a discussion of the biological activity of biotinylinsulin-avidin complexes. The biotinylinsulin avidin-complex 4:1 exhibits approximately 84% the *in vitro* activity of insulin. Addition of 5 mols of avidin per mol of biotinylinsulin lowered the activity to approximately 30% that of the parent hormone.[6] May *et al.*[13] have

prepared N^{ϵ},B^{29}-biotinylinsulin and found it to be equivalent with insulin in stimulating lipogenesis in rat epididymal adiposites. N^{ϵ},B^{29}-insulin–avidin complex (1:1) had only 5% the activity of the parent hormone. The higher activity of the N^{α},B^{1}-biotinylinsulin-avidin complex suggests that the attachment of avidin to the amino terminus of the B chain interferes less with binding of insulin to its receptors than does avidin attached to the ϵ-amino group of lysine B.[29] It should be mentioned in this context that avidin itself has no effect on the biological assay i.e. avidin does not interfere with the stimulation of lipogenesis in rat epididymal adipocytes by native insulin.

The ability of insulin, biotinylinsulin and biotinylinsulin SpHPP-avidin complexes to compete with ^{125}I-insulin for receptors on rat liver plasma membrane preparations is illustrated on Figure 6. Biotinylinsulin and biotinylinsulin-SpHPP-avidin complexes containing one or four hormone molecules attached to the modified avidin compete effectively for ^{125}I-insulin binding sites. Biotinylinsulin is equipotent with insulin, the insulin-SpHPP avidin complexes are slightly less effective. SpHPP-avidin not attached to biotinylinsulin does not compete for ^{125}I-insulin binding sites.

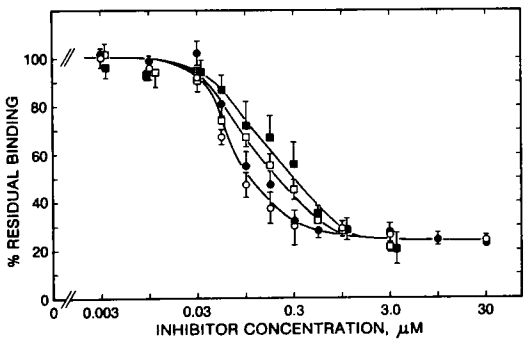


Fig. 6. Competitive inhibition of ^{125}I-insulin binding to rat liver plasma membranes by insulin (O), biotinylinsulin (●) biotinylinsulin-SpHPP-avidin (1:1) (■), and biotinylinsulin-SpHPP avidin (4:1) (□).

The insulin concentrations used in these experiments were 5μM and therefore only binding to the low affinity sites was assessed. Similar results were obtained when experiments were performed using insulin concentrations in the nM range. The ID_{50} values for insulin and biotinylinsulin-SpHPP-avidin complexes (3:1 and 1:1) were 5 and 80 nM respectively.[14]

On the basis of these results the conclusion is inescapable that the biotinylinsulin-avidin complexes can interact with their respective receptors on cells and elicit the expected response. It is also apparent that the non-specific binding of avidin or SpHPP-avidin to the cell takes place at sites unrelated to the insulin receptors.

Table I. Binding of Partially Purified Placental Insulin Receptor to AH-Sepharose-Suc-Avidin Columns

		Receptor (pmols)	
(Experimental)			
AH-Sepharose-Suc-Avidin:Biotinylinsulin			
	Applied	3.87	6.83
	Recovered	2.51	3.99
	Bound	1.36	2.84
(Control)*			
AH-Sepharose-Suc-Avidin:Biotin			
	Applied	3.87	6.83
	Recovered	3.87	6.70
	Bound	-	0.13

*The control column was also exposed to the same concentration of biotinylinsulin as the experimental after it had been saturated with biotin.

Having established that avidin attached to biotinylinsulin does not preclude its interaction with insulin receptors, we constructed an affinity resin in which biotinylinsulin was attached noncovalently to succinoyl-avidin (suc-avidin) immobilized on AH Sepharose 4B. The source of solubilized insulin receptor was human placenta. Prior to its use for studies of its binding to the affinity column, the receptor was partially purified by gel filtration on Sepharose 6B and affinity chromatography on immobilized Concanavalin A.[15] In order to assure establishment of equilibrium we rotate the affinity resin with the insulin receptor solutions. A typical experiment is shown on Table I. A control column to which biotin but no biotinylinsulin was added bound little receptor in contrast to the affinity resin which retained a sizable amount. Attempts to release the receptor from the column using 4.5M urea at pH 6 under

Fig. 7. The bifunctional nature of biotinylinsulin

conditions described by Cuatrecasas were unsuccessful. Thus we have, in biotinylinsulin, a bifunctional molecule that binds insulin receptor on one end and avidin on the other (Figure 7). However, the simultaneous strong binding to both avidin and insulin receptor appeared, at first, to preclude receptor retrieval by this method. Manipulation of the insulin insulin-receptor affinity is undesirable because weakening of the bond between insulin and its receptor will decrease the specificity of the affinity technique. Modification of the biotin side where the avidin-biotin affinity constant is some five orders of magnitude higher than the insulin insulin-receptor affinity seemed to offer a more promising solution to the problem. Thus we turned our attention to a search for biotin analogs exhibiting a lower affinity for avidin than biotin itself. Our approach is illustrated on Figure 8. The concept is akin to established procedures in the steroid hormone receptor field where the receptors are labeled with tritiated hormone and the hormone-receptor complex rather than the free receptor is isolated. Our contemplated scheme proceeds along similar lines. We envisioned the following sequence of events. Suc-avidin (A) is covalently attached to AH Sepharose 4B and ^{125}I-labeled, modified biotinylinsulin (I-H) is noncovalently attached to the resin ("sensitive bond"). The affinity resin is then rotated with an extract containing solubilized, partially purified insulin receptor (R) to form a complex. Obviously, the "sensitive bond" must be weaker than the avidin-biotin linkage to render it susceptible to cleavage. Displacement by pH manipulations or with biotin was contemplated. The systematic exploration of this scheme led to rather interesting findings.

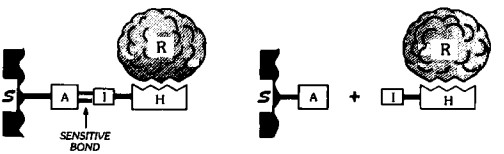


Fig. 8. Novel approach to the isolation of insulin receptors based on the avidin-biotin interaction.

In 1950 we synthesized iminobiotin, the guanido analog of biotin (Figure 9) and observed that this analog did not stimulate growth of biotin requiring organisms.[16] Green, a pioneer in the avidin field, investi-

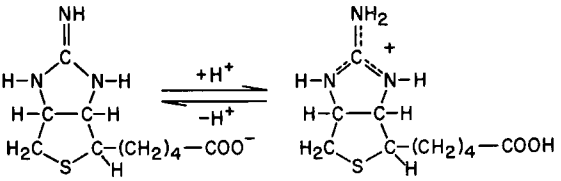


Fig. 9. The structure of iminobiotin

gated the thermodynamics of the binding of iminobiotin to avidin and found that the avidin-iminobiotin dissociation constant was pH dependent.[17] The iminobiotin affinity for avidin approached that of biotin at high pH values but was very weak at low hydrogen ion concentrations. We exploited this characteristic of iminobiotin for the isolation of streptavidin from the culture broth of *Streptomyces avidinii*.[18] Iminobiotin was attached covalently to AH Sepharose 4B and a streptavidin concentrate was applied to the column at pH 11. The column was washed extensively with pH 11 buffer and eluted with a pH 4 buffer to afford highly purified streptavidin in excellent yield. Figure 10 illustrates the purification of ^{125}I-succinoylstreptavidin on iminobiotinyl AH Sepharose 4B. Recently, iminobiotin was used by Orr[19] for the retrieval of plasma membrane components. Since iminobiotin appeared to possess some of the characteristics required for our insulin receptor studies we synthesized iminobiotinylinsulin (Figure 11, Compound III) and 6-iminobiotinylamidohexylinsulin (Figure 11, Compound IV). The former compound was prepared by the route used in the preparation of biotinylinsulin; the synthetic approach to the latter material is illustrated in Figure 12.

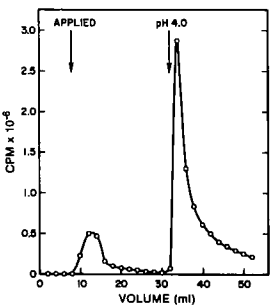


Fig. 10. Chromatography of ^{125}I-succinoylstreptavidin on iminobiotin-AH-Sepharose 4B.

Succinimido iminobiotinate hydrobromide (I) served to acylate methyl 6-aminohexanoate hydrochloride (II) and the ensuing methyl ester (III) was saponified to give the corresponding acid which was converted to the hydrochloride (IV). Reaction of (IV) with N-hydroxy-

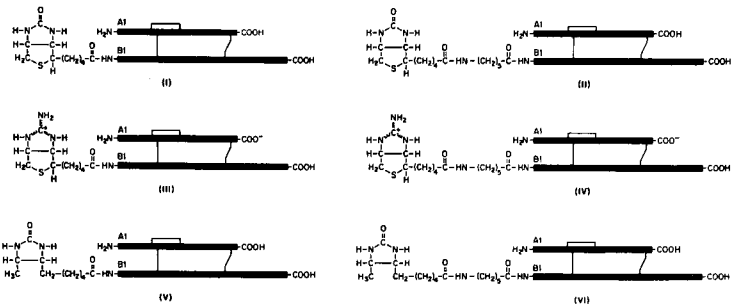


Fig. 11. Simplified structures of biotinylinsulin (I), 6-biotinylamidohexylinsulin (II), iminobiotinylinsulin (III), 6-iminobiotinylamidohexylinsulin (IV), desthiobiotinylinsulin (V), and 6-desthiobiotinylamidohexylinsulin (VI)

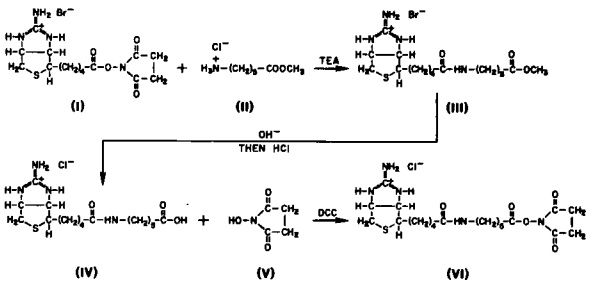


Fig. 12. Synthetic route to succinimido 6-iminobiotinylamidohexanoate.

succinimide using DCC as the condensing reagent afforded the active ester (VI) which was used to acylate N^{α},A^{1}, N^{ϵ},B^{29}-diBocinsulin as described previously.

Desthiobiotin[20] was also included in the series of compounds whose binding affinity for avidin was evaluated by Green.[21] The binding constant of this biotin analog was comparable to that of biotin, however, desthiobiotinol in which the carboxyl group of desthiobiotin is replaced by a $-CH_2OH$ group exhibited a lower binding constant. It appeared to us that desthiobiotinyl-insulin (Figure 11, Compound V) and 6-desthiobiotinylamidohexylinsulin (Figure 11, Compound VI) could be desirable ligands for our purpose and these compounds were synthesized by the procedures used to prepare the corresponding iminobiotin derivatives (Figure 12).

The structure assignments are based on the method of synthesis and the fact that the derivatized insulins differed from porcine insulin in their elution times on HPLC (Figure 13). Acid hydrolysates of the dansylated compounds did not contain Phe and acid hydrolysates of the materials

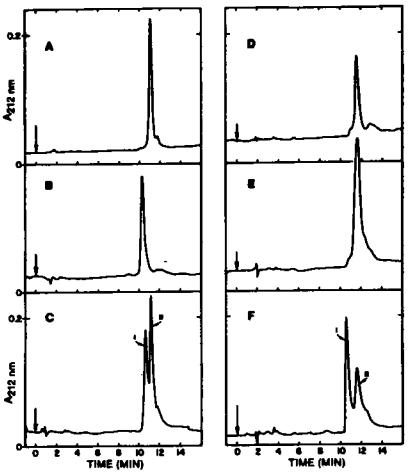


Fig. 13. HPLC of porcine insulin and derivatives. Panel A, iminobiotinylinsulin; Panel B, porcine insulin; panel C, mixture of porcine insulin and iminobiotinylinsulin; panel D, 6-iminobiotinylamidohexylinsulin; panel E, mixture of iminobiotinylinsulin and 6-iminobiotinylamidohexylinsulin; panel F, mixture of porcine insulin and 6-iminobiotinylamidohexylinsulin. A Bondapak C_{18} column with solvent system: pump A, 0.1% H_3PO_4; pump B, 50% acetonitrile in 0.1% H_3PO_4 was used. The gradient was from 50 to 80% pump B over 15 minutes with a pumping speed of 2 ml/min.

containing a spacer arm contained the theoretically expected amount of 6-aminohexanoic acid. This acid elutes from the small column of the amino acid analyzer in front of lysine (elution time 20 minutes).

Table II. Dissociation Constants of Avidin-Iminobiotin and Avidin-Iminobiotin Analog Complexes

Analogs	K_D (10^6)*
Iminobiotin	1.3±0.3
Iminobiotinyl-Gly	3.2±1.2
Iminobiotinyl-L-Phe	4.0±0.9
6-Iminobiotinylamidohexanoic acid	6.9±2.2
N^{ε}-Iminobiotinyl-L-Lys amide	42.0±4.0
Iminobiotin sulfone	3.8 ±1.6

*Constants were measured spectrophotometrically at pH 6.8[17]

At pH 9 the dissociation of the avidin-iminobiotin complex (K_D $10^{-8}M$) is several orders of magnitude higher than that of the avidin-biotin complex ($K_D \sim 10^{-15}M$). The possibility had to be considered that modifications of the carboxyl group of the weaker binding iminobiotin would alter its affinity for avidin. Table II summarizes the results of a study in which the dissociation constant of the avidin-iminobiotin complex is compared to avidin-iminobiotin analog complexes at pH 6.8. The previously discussed pH dependence of the dissociation of the avidin-

iminobiotin complex is evident. Of the complexes investigated only the avidin-N^{ϵ}-iminobiotinyllysine-amide complex exhibited a significantly different dissociation behavior. This may be attributable to repulsion between two positively charged molecules.

Rather interesting results were obtained when the dissociation of the complex suc-avidin-biotinylinsulin was compared to various suc-avidin-biotinyl-insulin analog complexes in the presence of an excess of ^{14}C-biotin (off reaction). The complexes were prepared by mixing one equivalent of suc-avidin with 8 equivalents of biotinylinsulin or the biotinylinsulin analogs (two-fold excess) and excess ligand was removed by gel filtration on Sephadex G-50. The concentration of the complex in the eluates was then determined spectrophotometrically and a 100-fold excess of ^{14}C-biotin was added. Suitable aliquots of the complex solutions were then subjected to gel filtration on Sephadex G-50 immediately after mixing or following incubation at room temperature for specified times. The radioactivity in the protein fractions provided a measure of the dissociation rate. Table III lists complexes that are significantly dissociated immediately after mixing in the first group the dissociations of iminobiotin, iminobiotinylinsulin and 6-iminobiotinylamidohexyl-insulin complexes are compared. The iminobiotin complex, at pH 9, dissociates 77%. Iminobiotinylinsulin fails to bind to suc-avidin at pH 9 indicating that the attachment of larger molecules to iminobiotin prevents the interaction of this weakly binding molecule with suc-avidin. Separating the avidin binding site from the insulin portion of the molecule by a spacer arm (6-iminobiotinylamidohexylinsulin) affords a complex that dissociates rapidly. The same situation prevails in the case of desthiobiotin analog complexes. The desthiobiotin complex dissociates rather slowly but the desthiobiotinylinsulin complex dissociates rapidly. The dissociation of the 6-desthiobiotinylamidohexylinsulin complex is significantly slower than that of the desthiobiotinylinsulin complex. In these examples the separation of the avidin binding site from the insulin decreases the off reaction rate. The rate of dissociation (off reaction) of a number of slowly dissociating biotin analog complexes is illustrated on Figure 14. The half time dissociation of biotin from avidin has been measured by Green as 200 days[21]; we determined the half time of dissociation of biotin from suc-avidin to be 127 days ($k_{-1}=3.2 \times 10^{-7}$ sec^{-1}) indicating that extensive succinoylation of avidin does not significantly alter its biotin characteristics. The suc-avidin-desthiobiotin complex is considerably less stable than the suc-avidin-biotin complex ($k_{-1} = 1.1 \times 10^{-5}$ sec^{-1}). Most surprising was the observation that the suc-avidin-

Table III. Degree of Dissociation of A-B Complexes in the Presence of Excess Biotin

Analog	pH	Dissociation %
Iminobiotin	9.0	77
Iminobiotinylinsulin	9.0	-
6-Iminobiotinylamidohexylinsulin	9.0	83
Desthiobiotin	7.6	8.5
Desthiobiotinylinsulin	7.6	88
6-Desthiobiotinylamidohexylinsulin	7.6	6
Biotin		0
Biotinylinsulin	7.6	16

biotinylinsulin complex dissociates rather rapidly (k_{-1}=3.8 x 10^{-5} sec^{-1}); the dissociation rate of the avidin-biotinylinsulin complex is identical. The suc-avidin 6-desthiobiotinylamidohexylinsulin complex dissociates at approximately the same rate (k_{-1}=3.7 x 10^{-5} sec^{-1}) as the suc-avidin biotinylinsulin complex.

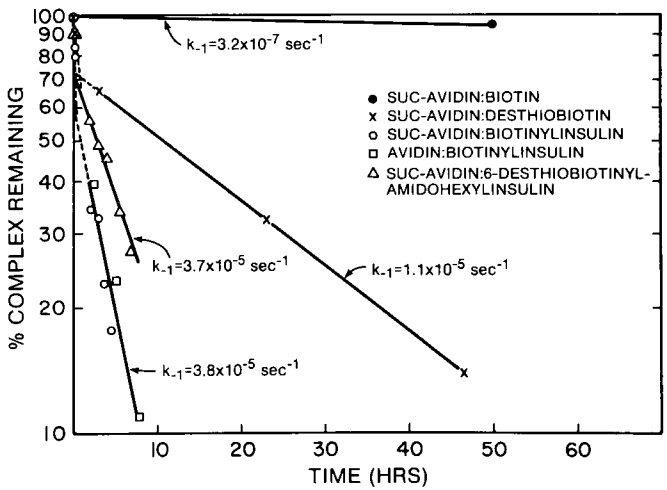


Fig. 14. Rate of dissociation of A-B Complexes. Suc-avidin-biotin (●), suc-avidin-desthiobiotin (x), suc-avidin-biotinylinsulin (O), avidin-biotinylinsulin (□), suc-avidin-6-desthiobiotinylamidohexylinsulin (△).

On the basis of these results it appears that biotinylinsulin and 6-desthiobiotinylamidohexylinsulin could be useful molecules for the retrieval of insulinreceptor-biotinylinsulin complexes.

From this study one may conclude that while the attachment of small molecules to the carboxyl group of biotin and iminobiotin exerts little influence on the stability of their complexes with suc-avidin, attachment of large molecules such as insulin lowers significantly the stability of the complexes very likely as a result of steric interference. This effect is most pronounced with weakly binding biotin analogs such as iminobiotin which fails to bind to suc-avidin when attached to insulin.

At first we were very much intrigued by the potential of the iminobiotin-avidin interaction as a tool for the retrieval of insulin receptors. However, the weak affinity of iminobiotin for suc-avidin, its sensitivity to steric effects when attached to large molecules and the rather unphysiological pH values required for maximal binding suggest that this system is of limited value for hormone receptor retrieval.

Having investigated the association behavior in solution of biotinylinsulin and biotinylinsulin analog complexes with suc-avidin, we turned to the preparation of affinity resins in which biotinylinsulin and its analogs were noncovalently attached to AH Sepharose 4B immobilized suc-avidin. The resins were prepared by reacting suc-avidin with AH Sepharose 4B using the water soluble 2-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate as the condensing reagent. The binding capacity of the affinity resin was assessed with ^{14}C-biotin and the binding of the various analogs to such resins was determined. Biotinylinsulin and the various analogs were labeled with ^{125}I for measurement of their binding to the suc-avidin resin. Solutions of these labeled materials were then percolated through standardized resin beds and the resin was washed with 250 column volumes of application buffer containing 0.1% Triton X-100. From the results presented on Table IV it can be seen that the resin retained more 6-desthiobiotinylamidohexylinsulin than any of the other derivatives. Iminobiotinylinsulin was not retained and only a small proportion of the applied 6-iminobiotinylamidohexylinsulin remained attached to the washed resin. Somewhat less desthiobiotinylinsulin than biotinylinsulin was retained. The results of these measurements, in a heterogeneous system, are in general agreement to those obtained in solution. They suggest that the iminobiotin derivatives are weak binders not suitable for receptor retrieval. The results indicate also that the small biotin molecule has access to suc-avidin sites on the resin that are not available for binding to the bulkier insulin derivatives.

Table IV. AH-Sepharose-Suc-Avidin Columns Binding Capacity for Biotinylinsulin and Analogs

Ligand	nmols BOUND/0.1ml RESIN
Biotin	10.1
Biotinylinsulin (pH 7.6)	8.3
Iminobiotinylinsulin (pH 9)	-
6-Iminobiotinylamidohexylinsulin (pH 9)	0.7
Desthiobiotinylinsulin (pH 7.6)	5.2
6-Desthiobiotinylamidohexylinsulin (pH 7.6)	9.1

I now wish to describe results of preliminary experiments dealing with the application of ^{125}I-biotinylinsulin to the retrieval of ^{125}I-biotinylinsulin-insulin receptor complex. The buffer used in these experiments was 50 mM Hepes pH 7.6 containing 0.5M NaCl and 0.1% Triton X-100. An affinity resin was prepared in which ^{125}I-biotinylinsulin was attached to AH Sepharose 4B immobilized suc-avidin. This resin was rotated with a solution of partially purified insulin receptor from human placenta and was washed with 250 column volumes of buffer. The washed resin was then rotated with buffer containing 20 mM biotin and the eluate applied to a column of Sephadex G-50 which was developed with buffer. A typical elution pattern is illustrated on Figure 15 where radioactivity is plotted against fraction number. The material corresponding to the first peak which elutes at the void volume contains ^{125}I-biotinylinsulin attached to a high molecular weight material. The second peak corresponds to the position where ^{125}I-biotinylinsulin elutes. Only the second peak is obtained when no receptor is applied (control). A similar elution pattern is obtained when an excess of cold insulin is added to the receptor solution prior to its application to the affinity resin. As was mentioned earlier we had previously established the specificity of the affinity procedure by showing (Table I) that receptor binds to biotinylinsulin affinity resins but not to AH Sepharose suc-avidin control columns.

These results, together with the findings that the high molecular weight radioactivity (Figure 15) precipitates with polyethylene glycol and cross-reacts with naturally occurring human antibodies directed against insulin receptor,[22] suggest that the insulin receptor complex can be eluted from biotinylinsulin suc-avidin affinity resins with biotin. More work will be required to characterize the high molecular weight ^{125}I-biotinylinsulin binding material and to optimize its yield.

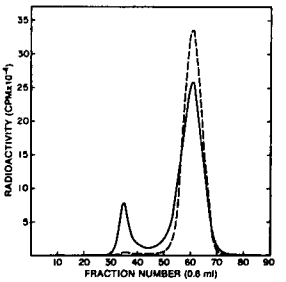


Fig. 15. Sephadex G-50 chromatography of biotin eluates of control —— and receptor ___ columns. See text for explanation.

Summary

We have shown that 1) Attachment of biotin or biotin analogs to insulin weakens their noncovalent association with suc-avidin. 2) Biotinylinsulin and 6-desthiobiotinylamidohexylinsulin bind firmly to suc-avidin AH Sepharose 4B columns and can be eluted with biotin. 3) Solubilized, partially purified human placental insulin receptor binds specifically to affinity resins in which ^{125}I-biotinylinsulin is noncovalently attached to AH Sepharose 4B immobilized suc-avidin and biotin elutes high molecular weight ^{125}I-biotinylinsulin containing material. This material is precipitable with polyethylene glycol and cross reacts with human antireceptor antibodies.

Acknowledgements

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SYNTHESIS OF TWO HEPTACOSAPEPTIDE AMIDES CORRESPONDING TO THE ENTIRE AMINO ACID SEQUENCES OF PORCINE AND CHICKEN GASTRIN RELEASING PEPTIDES

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Introduction

Recently, McDonald et al.[1] characterized the entire amino acid sequence of gastrin releasing peptide (GRP, I) from porcine nonantral tissue and clarified the chemical nature of the hitherto known bombesin-like immunoreactivity and biological activity in gastrointestinal tract.[2] The heptacosapeptide amide of GRP disclosed here has striking structural homology with bombesin.[3] Within the entire tetradecapeptide sequence of bombesin, the C-terminal decapeptide is identical with that of GRP, except for one amino acid residue. In position 8 from the C-terminus, there is glutamic acid in bombesin, histidine in GRP.

Subsequent to this finding, McDonald et al.[4] characterized a similar heptacosapeptide amide (II) from chicken intestine. The C-terminal tridecapeptide of this chicken GRP is identical with that of porcine GRP, except for one amino acid residue in position 19 as shown in Figure 1.

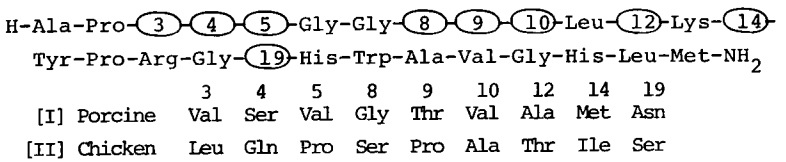

Fig. 1. Amino acid sequences of gastrin releasing peptides

We wish to report the syntheses of two heptacosapeptide amides corresponding to the amino acid sequences of these newly found gastrointestinal peptides. In these syntheses a new deprotecting procedure with trifluoromethane sulfonic acid (TFMSA)-thioanisole[5] and a new amide bond forming procedure with thiazoline-2-thione[6] were successfully employed. The scheme for the synthesis of porcine GRP is presented in Figure 2.

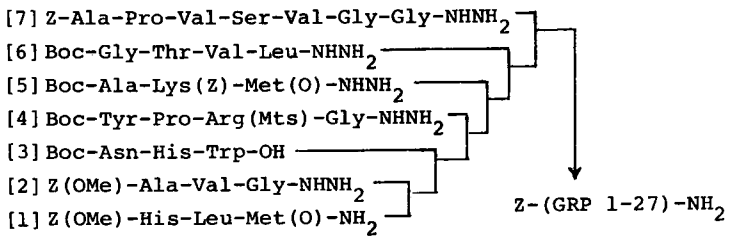

Fig. 2. Synthesis of porcine gastrine releasing peptide

Amino acid derivatives bearing protecting groups removable by TFMSA were employed, namely Lys(Z) and Arg(mesitylene-2-sulfonyl) [Arg(Mts)], the latter being a new derivative introduced by us in 1978.[7] Each Met residue was protected as sulfoxide[8] to prevent partial oxidation during synthesis and S-alkylation during deprotection.[9]

In our synthesis, the functional groups of Tyr, Ser and Thr are unprotected. In order to avoid the His-mediated over-activation[10] at these functional groups, especially at the Tyr residue, we decided to use the azide procedure as the main tool to assemble the peptide backbone. Thus, seven peptide segments, including these with COOH-terminal glycine [2],[4] and [7], were selected and synthesized as hydrazides with the exception of fragment [3] containing the Trp residue, because the azide procedure is not ideal for Trp-containing peptides. This segment was introduced via DCC-HOBT to suppress possible racemization.[11] After incorporation of the Trp residue, the Boc group was adopted as N^{α}-protecting group for segments [3] through [6], in as much as fewer side reactions at Trp are anticipated during N^{α}-deprotection with TFA,[12] than with that of the Z(OMe) group.[13] In addition, anisole containing EDT[14] was employed as a cation scavenger to minimize side reactions at Trp during the treatment with TFA.

Based on these approaches, seven segments were first prepared. In these syntheses, the usefulness of thiazoline-2-thione as effective amide forming agent was demonstrated for the first time.

3-Acylthiazoline-2-thiones are useful intermediates for the preparation of aldehydes and alcohols.[15,16] Of two alternative formulas proposed for the condensation product of thiazoline-2-thione and a carboxylic acid, X-ray crystallographic and NMR data favor formula i, instead of the thiol ester ii shown in Figure 3.

We found that the corresponding derivatives of N^{α}-protected amino acids are susceptible to aminolysis and consequently can be used for peptide synthesis. Condensation of N^{α}-protected amino acids with

thiazoline-2-thione by DCC smoothly afforded the corresponding 3-acyl derivatives as yellowish crystalline or oily compounds, depending on the amino acid employed. These N-acyl compounds react with amino acids or amino acid esters in the presence of TEA at room temperature to give the N^{α}-protected dipeptides or dipeptide esters in excellent yield. The progress of the reaction can be monitored by following the disappearance of the yellow color of the 3-acylthiazoline-2-thiones. This is an advantageous feature of the new carboxyl group-activating procedure. The new method was applied whenever it seemed feasible.

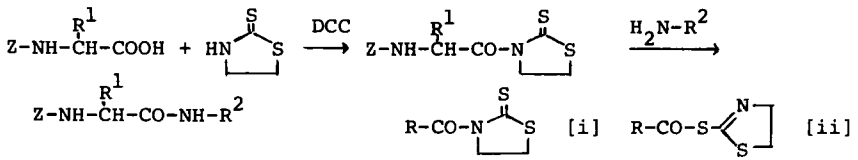

Fig. 3. Thiazoline-2-thione as carboxyl group-activating reagent

The C-terminal tripeptide, Z(OMe)-His-Leu-Met(O)-NH_2 [1], was synthesized in the sulfoxide form, an alternative to the previous synthesis of bombesin,[17] with which GRP shares the C-terminal heptapeptide sequence. The tripeptide Z(OMe)-Ala-Val-Gly-$NHNH_2$ [2] was synthesized as follows: Z(OMe)-Ala-Val-OMe obtained via the thiazoline-2-thione procedure in 70% yield, was applied as hydrazide to the subsequent condensation with H-Gly-OMe. The resulting protected tripeptide ester was converted to the hydrazide [2] in the usual manner.

The next segment, Boc-Asn-His-Trp-OH [3], was selected as one unit for reasons stated above. Z-His-Trp-OH was prepared via the azide coupling procedure.[18] The Z-group was removed by catalytic hydrogenation, thus avoiding acid treatment. The homogeneous dipeptide, H-His-Trp-OH, thus obtained, was then condensed with Boc-Asn-ONp, instead of the Z(OMe)-derivative (vide supra). The synthesis of Boc-Tyr-Pro-Arg(Mts)-Gly-$NHNH_2$ [4] was advanced as shown in Figure 4. Two dipeptide units, Boc-Tyr-Pro-OH and Z(OMe)-Arg(Mts)-Gly-OMe, were prepared via the azide and mixed anhydride[19] procedures, respectively. The former dipeptide was converted to the corresponding 3-acylthiazoline-2-thione derivative with DCC, and without characterization, allowed to condense with the TFA treated sample of the latter peptide. The reaction proceeded smoothly to give Boc-Tyr-Pro-Arg(Mts)-Gly-OMe in 69% yield, which was converted to [4] in the usual manner.

The next segment, Boc-Ala-Lys(Z)-Met(O)-$NHNH_2$ [5], was synthesized from the known dipeptide, Z(OMe)-Lys(Z)-Met-OMe,[20] via

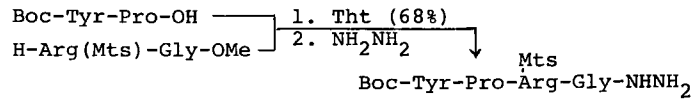

Fig. 4. Synthesis of the protected tetrapeptide [4]

oxidation with $NaIO_4$ to the corresponding sulfoxide[21] without concomitant formation of the sulfone. The thiazoline-2-thione procedure was again applied to introduce Boc-Ala-OH; the yield of the tripeptide, Boc-Ala-Lys(Z)-Met(O)-OMe, was 90%. The tripeptide was converted to [5] via established routes.

Boc-Gly-Thr-Val-Leu-$NHNH_2$ [6] was prepared starting with H-Leu-OMe. Z(OMe)-Val-OH, Z(OMe)-Thr-$NHNH_2$, and Boc-Gly-OH were successively condensed via the DCC, azide, and thiazoline-2-thione procedures, respectively. The yield in the final step was 75%.

The last segment, Z-Ala-Pro-Val-Ser-Val-Gly-Gly-$NHNH_2$ [7], was synthesized according to the approach shown in Figure 5. Z(OMe)-Val-Tht was used for the preparation of Z(OMe)-Val-Ser-OMe and Z(OMe)-Val-Gly-OMe. In addition the new procedure was applied to the synthesis of Z-Ala-Pro-OH and, in the next step, to condense this dipeptide with the TFA-treated sample of Z(OMe)-Val-Ser-Val-Gly-Gly-OMe. The yield was better than 75% in both cases. The resulting protected heptapeptide ester was easily converted to [7].

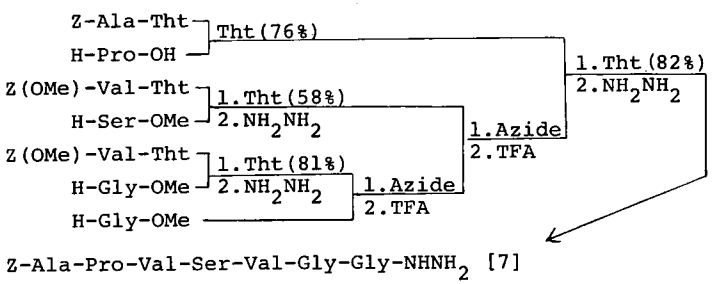

Fig. 5. Synthesis of the protected heptapeptide [7]

The seven peptide segments thus obtained were then assembled according to the scheme shown in Figure 2. Throughout the synthesis, Leu which occurs only twice and is located near the C-terminal portion was selected as diagnostic amino acid. Comparing the recovery of Leu with that of newly incorporated amino acids, satisfactory incorporation in each condensation was ascertained (Table I). With the exception of one, every reaction was performed in DMF. Due to decreasing solubility of the amine component, the final coupling with segment [7] was per-

formed in a mixture of DMSO and DMF. Following condensations most products were purified by extraction with n-butanol and precipitation from methanol with ether or isopropyl alcohol. The presence of His and Met(O) in intermediates made purification somewhat laborious, since such products were mostly water-soluble. The condensation product of [3] was purified by column chromatography on silica-gel. Purification of the condensation product of [6] was carried out by precipitation from DMF with ethanol. Protected GRP was purified by gel-filtration on Sephacryl S-200 with DMF containing 5% water as eluent[22] to remove unreacted acyl component used in excess and possible Trp-modified products. Protected GRP, thus purified, exhibited a sharp single spot on tlc and its homogeneity was further assessed by hydrolysis in 4N methanesulfonic acid (MSA)[23] and elemental analysis.

Table I. Amino Acid Ratios in 4N MSA Hydrolysates of Synthetic GRP and Intermediates

	22-27 (6)	19-27 (9)	15-27 (13)	12-27 (16)	8-27 '20)	1-27 (27)	GRP
Asp		0.98(1)	0.95(1)	1.04(1)	1.03(1)	1.08(1)	1.01(1)
Thr					0.99(1)	0.96(1)	0.96(1)
Ser						1.01(1)	0.91(1)
Pro			0.98(1)	0.95(1)	0.97(1)	1.63(2)	1.81(2)
Gly	0.96(1)	1.00(1)	1.97(2)	2.01(2)	3.11(3)	5.17(5)	4.98(5)
Ala	0.99(1)	1.00(1)	1.04(1)	2.05(2)	2.06(2)	3.11(3)	3.00(3)
Val	1.00(1)	1.04(1)	1.02(1)	1.03(1)	1.97(2)	4.22(4)	4.09(4)
Met	0.71(1)	0.95(1)	0.92(1)	1.85(2)	1.90(2)	1.83(2)	1.85(2)
Leu	1.00(1)	1.00(1)	1.00(1)	1.00(1)	2.00(2)	2.00(2)	2.00(2)
Tyr			0.96(1)	1.02(1)	1.05(1)	1.03(1)	1.08(1)
Trp		0.90(1)	0.93(1)	0.87(1)	0.97(1)	0.71(1)	0.88(1)
Lys				1.04(1)	1.04(1)	1.01(1)	1.05(1)
His	0.99(1)	2.21(2)	2.01(2)	2.04(2)	2.03(2)	2.08(2)	2.08(2)
Arg			1.01(1)	1.05(1)	0.98(1)	1.01(1)	1.06(1)
Recovery	94%	82%	83%	83%	80%	77%	89%

Finally, all protecting groups were removed from protected GRP by treatment with 1M TFMSA in TFA as shown in Figure 6. As a scavenger system, a combination of thioanisole and m-cresol was employed. The former was shown to have the ability to accelerate the cleavage of protecting groups.[24] The latter traps alkyl cations efficiently and suppresses O-sulfonation at the Tyr residue.[7] The deprotected peptide was converted to the corresponding acetate, treated with dilute ammonia at pH 10 in order to reverse the N→O shift[25] at the Ser residue and then incubated with dithiothreitol to reduce the two residues of Met(O). The reduced product was next purified by gel-filtration on Sephadex G-25, followed by column chromatography on CM-cellulose using a gradient elution with 0.1M NH_4HCO_3. The latter elution pattern is shown in Figure 7-a. When UV absorption at 280 nm was determined in each fraction, two peaks were detected. The product obtained from the main peak exhibited a single spot on tlc, but additional peaks on HPLC (Figure

Z-[14, 27- Met(O) GRP 1-27]-NH_2

↓ 1. 1M TFMSA-thioanisole in TFA
m-cresol, ethanedithiol (4°, 60 min).
2. Amberlite CG-4B (acetate form)
3. 1N NH_4OH

H-[14, 27-Met(O) GRP 1-27]-NH_2

↓ 1. Dithiothreitol (60°, 12 hr)
2. Sephadex G-25 (0.2N AcOH)

Crude H-(GRP 1-27)-NH_2 (77.2%)

↓ 1. CM-cellulose
2. Partition chromatography on Sephadex G-25.
(n-BuOH-EtOH-0.01M $AcONH_4$=4:0.5:5)

Purified H-(GRP 1-27)-NH_2 (41.2%)

Fig. 6. Deprotection and purification of synthetic gastrin releasing peptide

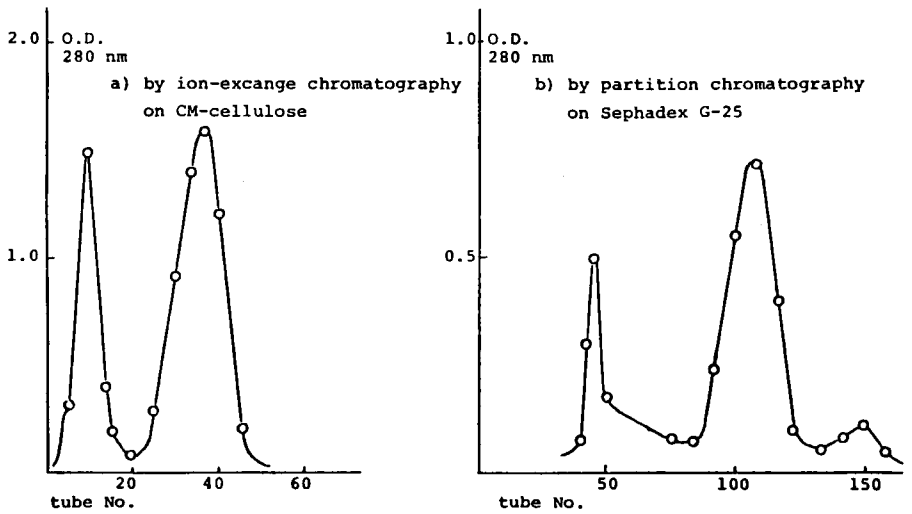

Fig. 7. Purification of synthetic gastrin releasing peptide

8-a). Partition chromatography on Sephadex G-25[26] was next employed and the solvent system of n-BuOH-ethanol-0.01M ammonium acetate (4:0.5:5) was found effective to remove this impurity which seems to be a mixture of mono and disulfoxide derivatives of GRP. When the purified product was stored in water for 2 or 3 days and reexamined by HPLC, the identical side peak was detected. The main product isolated by partition chromatography (Figure 7-b) exhibited a single peak on HPLC (Figure 8-b) and a sharp single spot on tlc. Its purity was further confirmed by acid hydrolysis and enzymatic digestion. In the latter case, in addition to

leucine aminopeptidase, papain was employed, and the presence of one residue of Asn in the synthetic GRP confirmed.

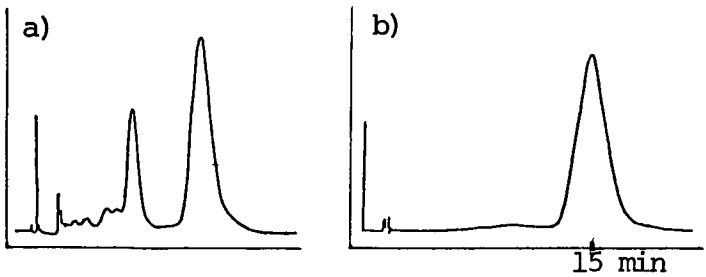

Fig. 8. High pressure liquid chromatography of synthetic gastrin releasing peptide
Column: μBondapak CN (3.9 x 30 cm) Solvent: 0.25N Et_3N-phosphate (pH 3.16):CH_3CN (85:15)

Synthesis of Chicken Gastrin Releasing Peptide

After the synthesis of porcine GRP, we approached that of GRP from chicken. Methods employed are essentially the same as those of the former synthesis (Figure 9). Of seven peptide segments, [1], [2], and [4] are identical with those employed previously. The other 4 peptide segments were newly synthesized.

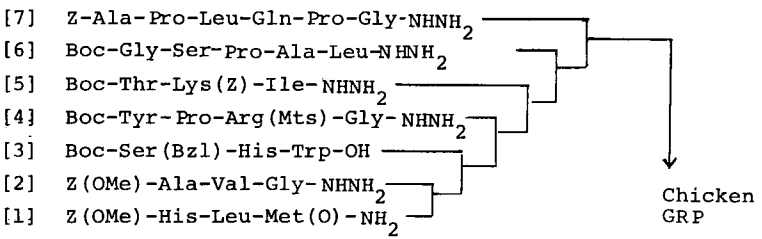

Fig. 9. Synthesis of chicken gastrin releasing peptide

Peptide [3] was prepared via azide coupling of Boc-Ser(Bzl)-His-$NHNH_2$ to Trp. In the synthesis of porcin GRP, extraction with n-butanol was required for the isolation of the His and Met(O)-containing intermediates because of their partial solubility in water. In the present synthesis, Ser was protected as Bzl ether. Each product could thus be purified by precipitation with water and subsequently with appropriate solvents. Segment [5] was prepared in step-wise manner via the thiazoline-2-thione and azide procedures, respectively. For the synthesis of segment [6], Z(OMe)-Pro-Ala-Leu-OMe was prepared via the thiazoline-2-thione and mixed anhydride procedures, respectively. Boc-

Gly-Ser was coupled via its azide. The N-terminal fragment [7] was prepared starting with H-Gly-OMe. The thiazoline-2-thione procedure was employed for the introduction of Z(OMe)-Gln-Pro-OH and Z-Ala-Pro-OH and the *p*-nitrophenyl ester method[27] for Z(OMe)-Leu-OH.

After successive condensations of 7 fragments, the resulting protected chicken GRP was purified by gel-filtration on Sephacryl S-200 as performed in the synthesis of porcine GRP. Deprotection and subsequent purification are in progress.

When administered by intravenous bolus injection into dogs and rats, synthetic porcine GRP brought about a significant increase in plasma immunoreactive gastrin levels (Figure 10). The potency of the synthetic material was judged to be equivalent to that of synthetic bombesin on a molar basis as reported by McDonald et al.[1] for natural GRP. In order to examine the relationship between the chain length and activity of GRP, four porcine GRP intermediates, (19-27), (15-27), (12-27), and (8-27) were deprotected and assayed. As predicted from the structural similarity between GRP and bombesin, these peptides, including (19-27) were as active as synthetic bombesin on a molar basis, when plasma immunoreactive gastrin was examined in dogs. The active fragment of GRP can thus be seen in the C-terminal nonapeptide sequence. The biological evaluation of synthetic chicken GRP is now under way.

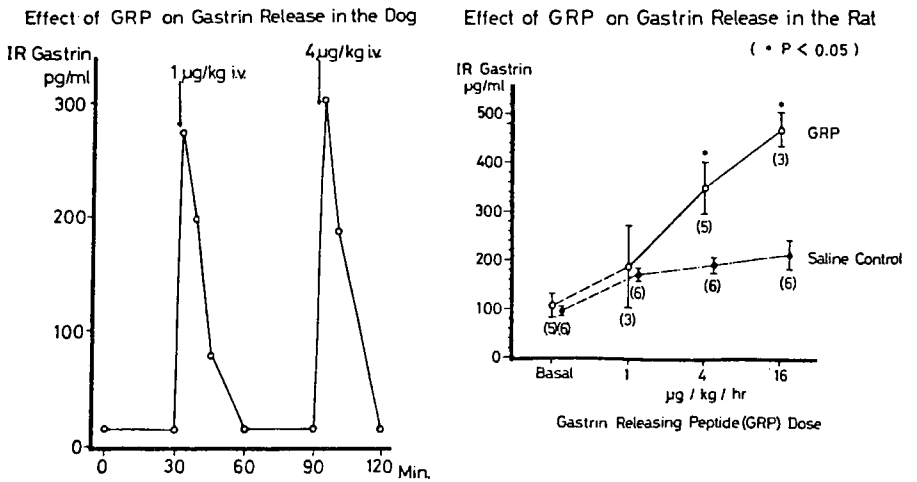

Fig. 10. Biological evaluation of synthetic porcine GRP

Acknowledgement

The authors are grateful to Dr. McDonald for his generous supply of natural porcine GRP for RIAs now under way.

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TOTAL SYNTHESIS OF BLEOMYCIN

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Bleomycin (BLM) is an antitumor antibiotic discovered by Umezawa *et al.* in 1966[1] and used clinically in the treatment of squamous cell carcinoma, malignant lymphoma *etc.* BLM is a glycopeptide consisting of a hexapeptide and a disaccharide. The total structure of BLM was determined in 1978 (Figure 1)[2]. Natural BLM is obtained as the 1:1 Cu(II)-complex with blue color. The structure of the Cu(II)-complex of BLM was proposed[3] on the basis of the structure of a biosynthetic intermediate of BLM determined by X-ray crystallography[4]. With this structure, the discussion of the mechanism of action of BLM on the molecular level became possible (Figure 2)[3].

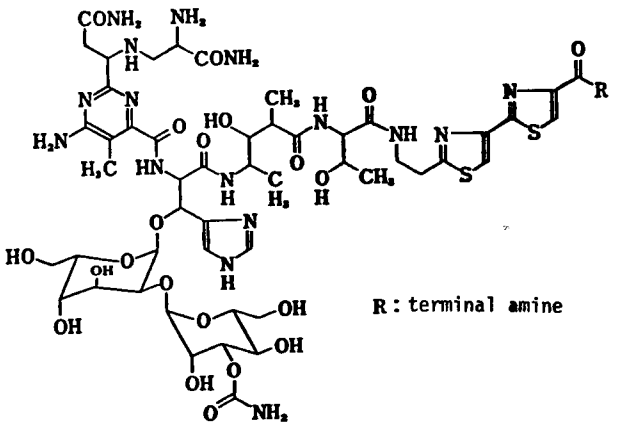

Fig. 1. The structure of bleomycin

Aspects of the Biosynthesis of Bleomycin

BLM contains four novel amino acids: pyrimidoblamic acid (PBA), *erythro*-β-hydroxy-L-histidine (β-OH-His), (2S,3S,4R)-4-amino-3-hydroxy-2-methylpentanoic acid (AHM), and 2′-(2-aminoethyl)-2,4′-

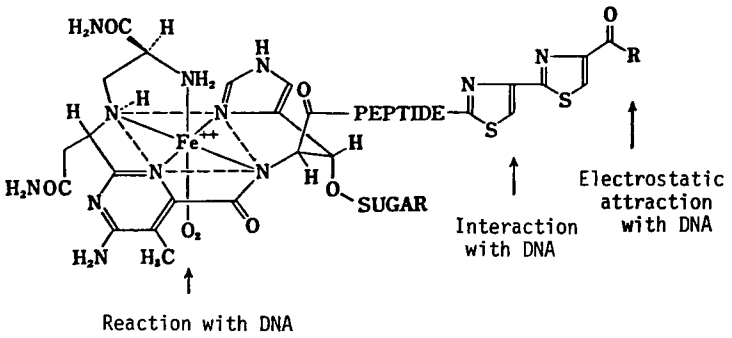

Fig. 2. The mechanism of action of bleomycin

bithiazole-4-carboxylic acid (ABC) (Figure 3). The biosynthesis of these amino acids has been studied by incorporation of ^{14}C-, ^{13}C-, and ^{3}H-labeled compounds, and by isolation of the biosynthetic intermediary peptides. Thus, PBA was suggested to be derived from serine, two moles of asparagine, and methionine-CH_3, AHM from alanine, acetic acid, and methionine-CH_3, and ABC from β-alanine and two moles of cysteine[5-7].

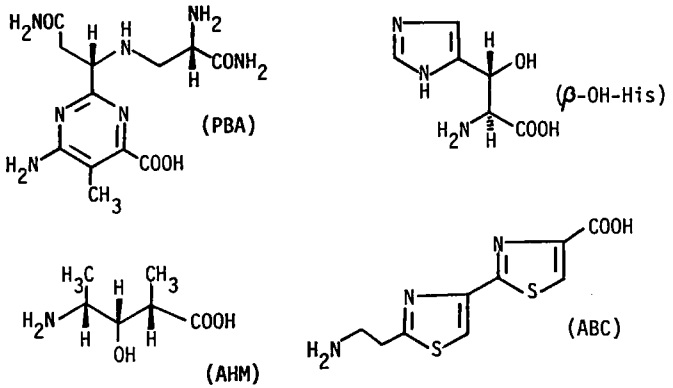

Fig. 3. Novel amino acids contained in bleomycin

The nine biosynthetic intermediates (P3~deglyco-BLM, see Figure 4) have been isolated from the fermentation broth of BLM. These structures were elucidated by chemical and spectroscopic studies (Figure 4)[5]. The isolation of a series of intermediary peptides indicates that (1) the peptide chain is elongated from the N-terminal amino acid by stepwise incorporation of common amino acids and acetic acid, (2) the novel amino acids are formed by modification of common amino acids and/or the small peptides thereof after incorporation into the peptide chain, and

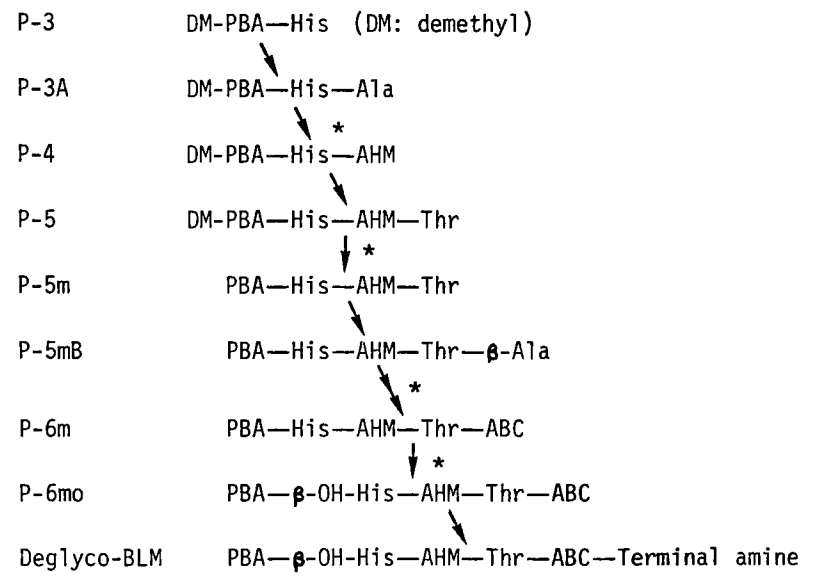

Fig. 4. Biosynthetic intermediates of bleomycin. Tilted arrow indicates elongation of peptide chain. Modification occurs at steps marked with *.

(3) the formation of AHM during peptide chain elongation suggests that the peptide part of BLM is biosynthesized by transpeptidation and transthiolation like gramicidin S, more similar to fatty acid synthesis rather than ordinary peptide synthesis[8].

By contrast to the biosynthesis, the peptide part of BLM has been chemically synthesized by elongation from the C-terminus and condensations using the pre-formed novel amino acids.

Synthesis of Novel Amino Acids

1. Synthesis of PBA[9,10] — The scheme for an improved synthesis of PBA[10] is shown in Figure 5. The Schiff's base, prepared from 2-formylpyrimidine and (S)-Nα-Boc-β-aminoalanine amide in the presence of molecular sieve, was allowed to react with vinyloxyborane to give the racemic product of the carbon skeleton of PBA. After modification of the substituent groups, the (S,S)-isomer of Boc-PBA, which has the same stereochemistry as the natural product, was isolated as crystals from the diastereoisomeric mixture upon treatment with ethanol.

Boc-PBA was derived from BLM for direct comparison with the synthetic product. Mild acid treatment of BLM (6N HCl, room temp., one month) yielded pyrimidobleonic acid, which is the tricarboxylic acid formed upon hydrolysis of two carboxamides contained in PBA. The

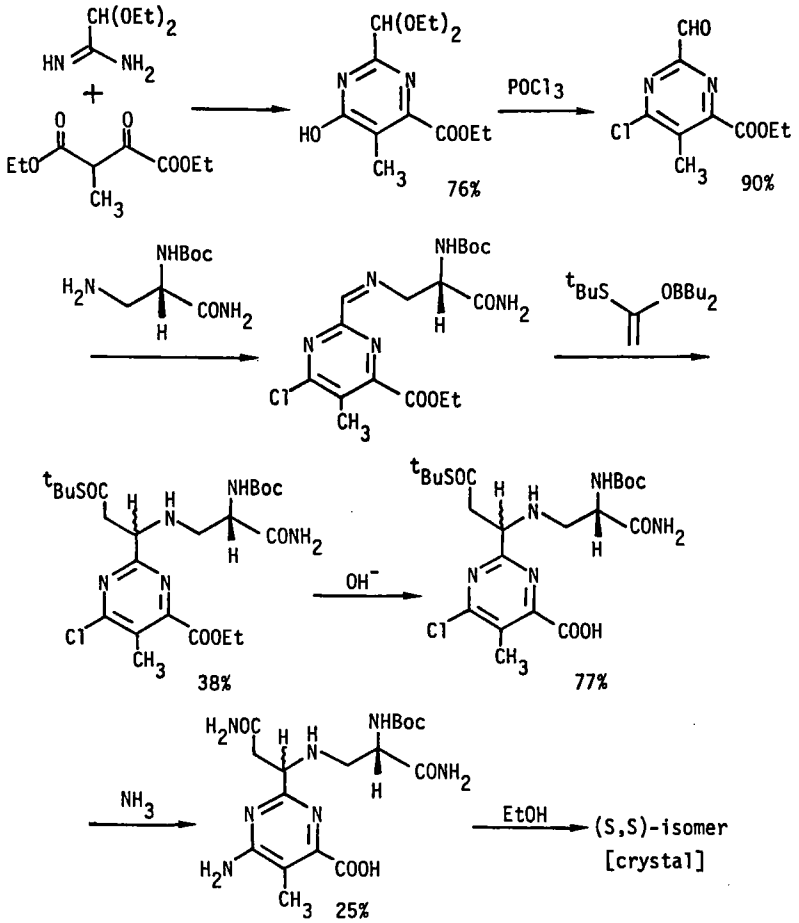

Fig. 5. The synthesis of pyrimidoblamic acid (PBA)

trimethyl ester of pyrimidobleonic acid was prepared with MeOH/HCl. Upon exposure of the trimethyl ester to $CuCO_3 \cdot Cu(OH)_2$, selective hydrolysis of the carboxylic ester attached to the pyrimidine ring occurred instantly. This may be due to involvement of the ester carbonyl in metal coordination. The resulting diester was converted to Boc-PBA by butyloxycarbonylation followed by ammonolysis. Synthetic Boc-PBA was identical with the one derived from PBA isolated from BLM for all criteria examined and used in the peptide synthesis in this form. Treatment of the trimethyl ester of pyrimidobleonic acid under mildly alkaline conditions caused the ester bond vicinal to the primary amino group to be hydrolyzed preferentially, and the desired dimethyl ester was never obtained.

2. Synthesis of AHM[11,12] — Recently the stereocontrolled synthesis of natural products with consecutive asymmetric carbons has attracted the attention of the chemist interested in their preparation. We have succeeded in the stereoselective synthesis of AHM from D-alanine and an E-vinyloxyborane (Figure 6)[12]. Among four possible diastereoisomers formed by this condensation, only two were detected in the reaction mixture, and the desired (2S,3S,4R)-isomer was isolated in a ratio of 35:1.

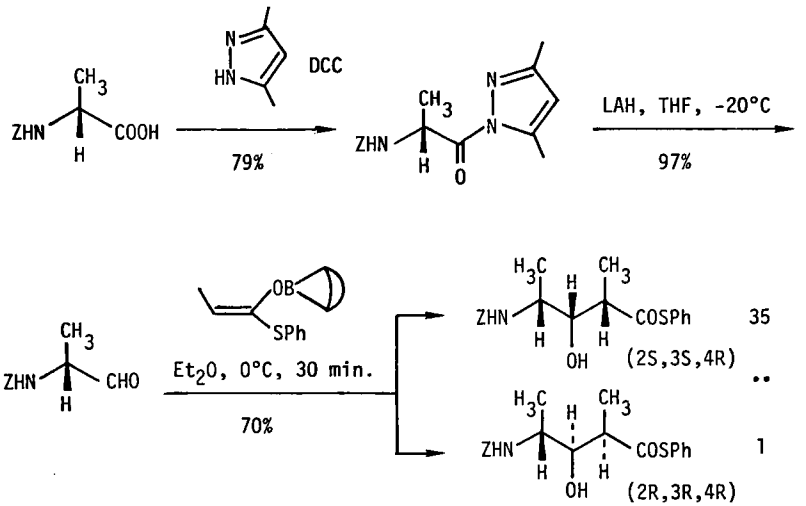

Fig. 6. The synthesis of (2S,3S,4R)-4-amino-3-hydroxy-2-methylpentanoic acid (AHM)

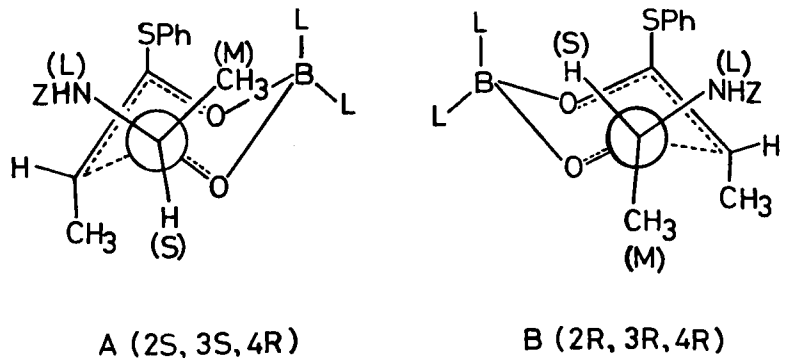

Fig. 7. Stereocontrol in the pericyclic transition state in the synthesis of (2S,3S,4R)-4-amino-3-hydroxy-2-methylpentanoic acid (AHM)

This stereoselective synthesis is explained as follows: the condensation is stereocontrolled in the pericyclic transition state (Figure

7). Two transition states, A and B, are possible. However, according to Cram's rule, transition state A is more favorable than B for the condensation. Thus, the stereoselective synthesis of AHM was achieved as predicted. Another advantage associated with this synthesis is the fact that the condensation product can be used directly as active ester in peptide synthesis.

3. Synthesis of β-OH-His — When separation of diastereoisomers and resolution of enatiomers is easily achieved, simple syntheses void of stereocontrol are of practical importance. The synthesis of β-OH-His is such a case[13]. β-OH-His was synthesized by condensation of readily available 4-formylimidazole and the glycine-Cu(II)-complex (44% yield) according to Akabori's β-hydroxy amino acid synthesis. Unexpectedly but fortunately, the desired *erythro*-isomer was formed predominantly (*erythro:threo* = 7:1). The *erythro*-isomer was isolated from the diastereoisomeric mixture by crystalization from 50% aqueous ethanol (75%). The mother liquor constituted the almost 1:1 *erythro-threo* mixture. The DL-resolution of the *erythro*-isomer was achieved by cellulose (optically active) column chromatography developed with n-BuOH-MeOH-Pyr-H_2O-AcOH (6:6:4:3:1) without derivatization [Rf-values on TLC; 0.10 (L), 0.07 (D)].

Synthesis of Deglyco-BLM A2

We chose deglyco-BLM A2, the peptide part of BLM A2, as the primary target of our synthesis of BLM, because all congeners of BLM can be chemically derived from BLM A2 by the BrCN method developed earlier[14]. At first, it is important to know the properties of the novel amino acids in peptide synthesis.

ABC, which was first synthesized by Zee-Cheng and Cheng in 1970[15], appears to pose no problem, because it is a monamino- and monocarboxylic acid without other reactive groups and void of asymmetric carbon. Protection of the secondary amine and 4-aminopyrimidine in Nα-Boc-PBA appeared to be not necessary for reasons of their poor reactivity[16]. Preliminary studies indicated that the protection of the imidazole of β-OH-His was necessary during coupling with AHM to increase the yield. Several protecting groups were examined and the Dnp group was selected for the synthesis.

The approach to the synthesis of deglyco-BLM A2 is shown in Figure 8. Strategy was established considering the supply of novel amino acids and questions of ordinary peptide synthesis. To secure optically pure peptide, two α-amino acids, β-OH-His and Thr, were coupled as

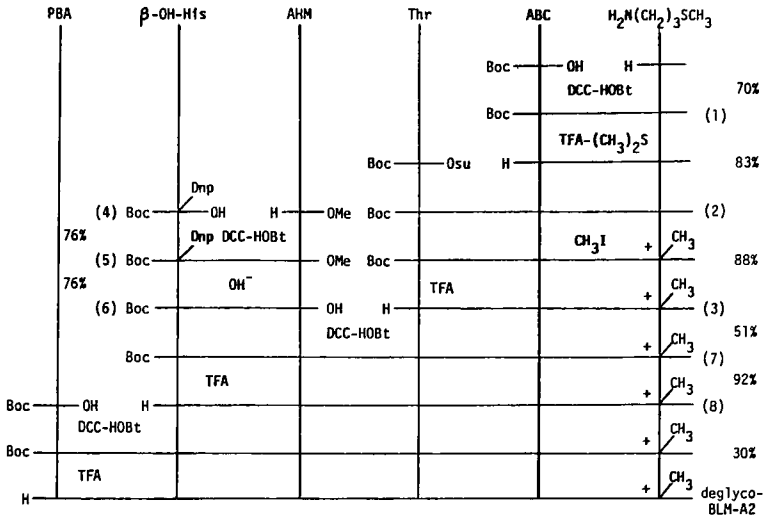

Fig. 8. The synthesis of deglyco-bleomycin A2

acid components in the early stage of the synthesis. Thus, three fragments were formed. There was no problem with the synthesis of the C-terminal tripeptide (3). The identity of 3 was confirmed by TLC, IR, and ^{1}H-NMR comparison with a sample from the natural product[17]. In the synthesis of the polyfunctional dipeptide (6), the liberation of a trace amount of the γ-lactam of AHM was observed during saponification of (5). Segment condensation of 3 and 6 was achieved with DCC-HOBt. The product (7) was purified by Sephadex LH-20 chromatography. The structure and optical purity of 7 were confirmed by TLC, ^{1}H-NMR and FDMS (M^+, m/z 840). Finally, Nα-Boc-PBA was coupled with deprotected 7 (8) using DCC-HOBt in the activation step. This was followed by deprotection with TFA to give deglyco-BLM A2. The product was purified by CM-Sephadex C-25 chromatography after Cu(II)-complex formation[18]. The purified colorless deglyco-BLM A2 free of metal was obtained from the purified Cu(II)-complex by EDTA treatment on Amberlite XAD-2 column[19]. The synthetic and natural[20] samples of deglyco-BLM A2 were identical by all criteria invoked. In particular, optical purity was ascertained by ^{1}H-NMR spectrometry at 250 MHz. Thus, the total synthesis of deglyco-BLM has been achieved for the first time[21].

Synthesis of the Sugar Moiety of BLM

The approach to the synthesis of the disaccharide of BLM, namely 2-O-(α-D-mannopyranosyl)-L-gulose, is shown in Figure 9. The

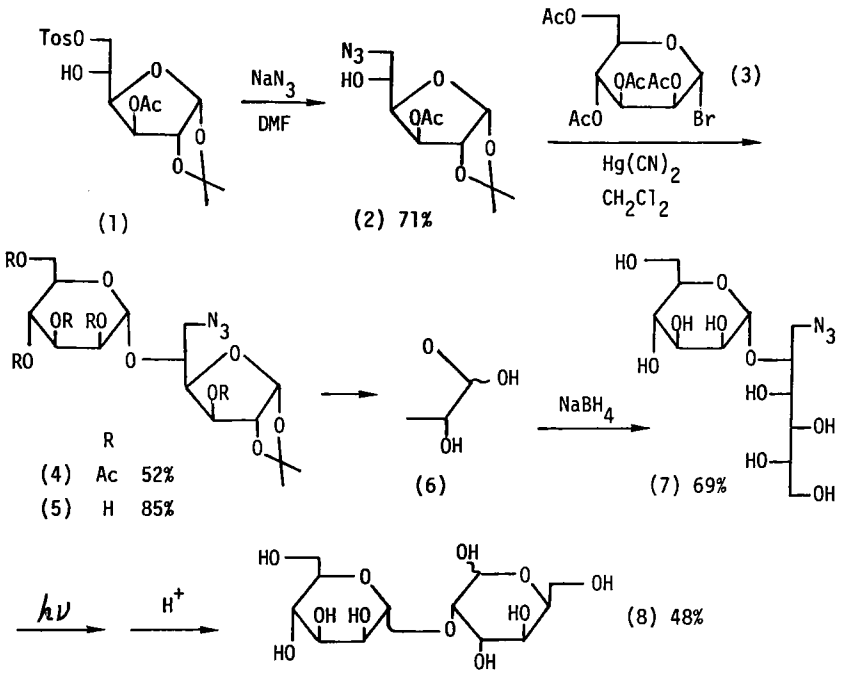

Fig. 9. The synthesis of the disaccharide of bleomycin

characteristics of this synthesis are the early-stage condensation of D-mannose to the 5-OH group of a D-glucofuranose derivative (2) and the successive head-to-tail inversion of the D-glucose moiety resulting in the conversion of C-5 of the D-glucose moiety to C-2 in the newly generated L-gulose, thus completing the synthesis of the disaccharide (8)[22]. Another feature of this synthesis is characterized by the transformation of the 6-CH_2OH group of glucose to the 1-CHO group of the gulose moiety *via* photolysis of the azide. Acetylation of 8 gave the octa-O-acetyl derivative, whose IR and ^{1}H-NMR spectra were identical with those of the compound obtained by acetylation of the natural decarbamoyl disaccharide[23].

It has already been known that the carbamoyl group can be introduced to the 3-OH group of mannose by ammonolysis of the 2,3-di-O-carbonyl derivative[24]. To secure the 2,3-di-O-carbonyl derivative of the disaccharide, the primary OH groups of 8 were first protected by preferential silylation with t-$BuMe_2SiCl$ in the presence of imidazole in DMF (9 in Figure 10). The desired cyclic carbonate (10) was obtained by treatment of 9 with carbonyldiimidazole. Deprotection of the alkylsilyl groups with Bu_4NF in THF followed by acetylation gave the anomeric

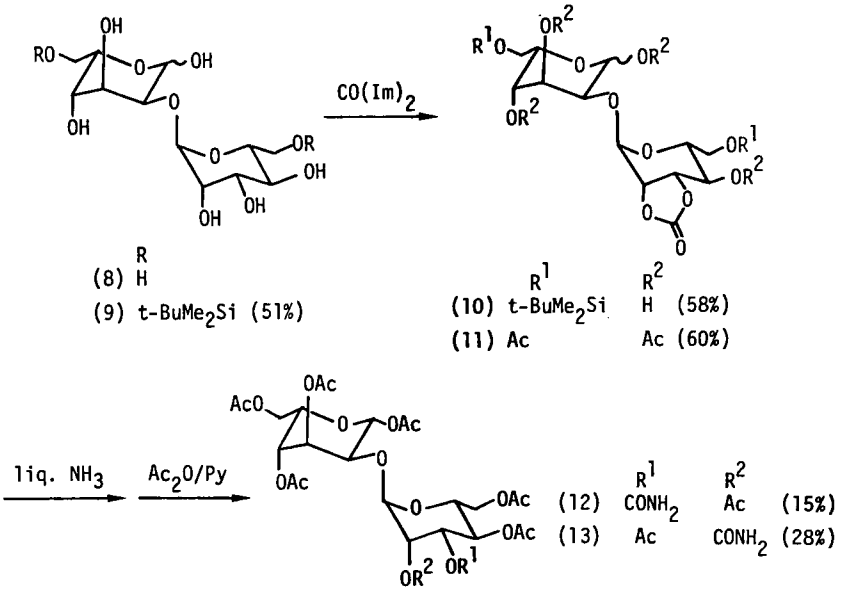

Fig. 10. Introduction of the carbamoyl group at the 3-OH group of the mannose moiety of the disaccharide in bleomycin

mixture (β/α = 6:1) of the peracetylated cyclic carbonate (11). Ammonolysis of 11 followed by acetylation yielded the 3-O- and 2-O-carbamoyl derivatives (12 & 13). Their β-anomers were separated and purified by silica gel chromatography (C_6H_6:EtOAc = 2:3) and preparative TLC. Deacetylation of 12 gave the sugar moiety of BLM, namely 2-O-(3-O-carbamoyl-α-D-mannopyranosyl)-L-gulopyranose, whose structure was confirmed by comparison of ^{1}H-NMR at 250 MHz of 12 with a sample[23] derived from BLM. Compound 13 can be transformed to 12 by isomerization of the deacetylated product[24,25]. It is obvious that use of 3-O-carbamoyl-mannose[24] as a starting material for the formation of the disaccharide in place of mannose facilitates the synthesis of the sugar moiety of BLM. Thus, the total synthesis of the sugar moiety has been achieved for the first time.

Total Synthesis of BLM A2

The final step left for the total synthesis of BLM A2 is the glycosylation of deglyco-BLM A2 with the sugar part of BLM. Several trials of the reaction with protected deglyco-BLM A2 and the bromo-disaccharide were unsuccessful. Very recently, we were successful with the total synthesis of BLM A2 by employing the following procedures:

the pentapeptide, β-OH-His-AHM-Thr-ABC-Amine(A2), protected by Boc, Dnp, and Ac (AHM and Thr) was allowed to react with the bromodisaccharide, which was prepared by treatment of 12 (Figure 10) with HBr/CH_2Cl_2 in 86% yield, in the presence of $Hg(CN)_2$ in sulfolane. The Boc-group of the reaction product, isolated by Sephadex LH-20 chromatography, was removed with TFA, and coupled with Boc-PBA using DCC-HOBt. The resulting product was treated with 0.1N NaOH-MeOH (1:1) to remove the acetyl and Dnp groups followed by deprotection of the Boc-group with TFA. The deprotected product was chromatographed on a short column of CM-Sephadex C-25 developed with a linear gradient of NaCl after Cu(II)-complex formation. The fraction of the eluate which showed antibacterial activity was collected and desalted to give the BLM A2 Cu(II)-complex. It was compared with the natural BLM A2 Cu(II)-complex by reverse-phase-HPLC, HPTLCs on silica gel and TLC on Avicel. The retention time and Rf-values detected by UV, ninhydrin and bioautography were exactly the same for both products. Thus, it is established that BLM A2 has been chemically synthesized for the first time.

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SYNTHESIS OF [MeALA^2,MeALA^6]-des-N-TETRAMETHYLTRIOSTIN A, AN ANALOG OF THE QUINOXALINE DEPSIPEPTIDE ANTIBIOTICS

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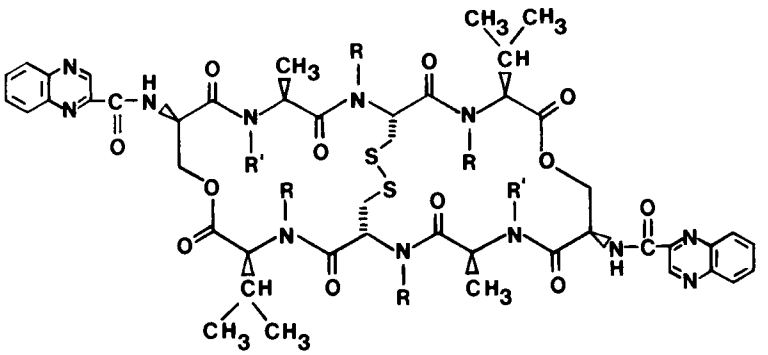
The triostin and quinomycin quinoxaline depsipeptide antibiotics are known[1] to be effective intercalating agents in binding to deoxyribonucleic acids.[2] The antibiotics have been shown[2] to bind to various natural and synthetic DNA molecules by a mechanism involving bifunctional intercalation of both quinoxaline chromophores common to the antibiotics. Solution nmr conformational studies of echinomycin (quinomycin A) and triostin A have been reported.[3,4] A model, based on conformational energy calculations, has been proposed[5] for the binding of echinomycin to DNA.

The total syntheses of triostin A (**1**) and several analogs have been accomplished.[6-8] des-N-Tetramethyltriostin A (**2**)[7], designated[9] by the acronym TANDEM, was shown to bind to DNA as a bifunctional intercalating agent and to show a high specificity for binding to poly(dA-dT).[9] The crystal structure of TANDEM recently has been determined.[10] The crystallographic results have led to a proposed model for the specificity of TANDEM in binding to poly(dA-dT), in which a key feature involves the formation of hydrogen bonds between the alanyl NH and a carbonyl oxygen of thymine.[11] The bis-N-methylalanine analog of TANDEM would lack the potential of hydrogen bond formation and would be of interest as a probe of the above model. We report in this paper the synthesis of this analog, [MeAla^2,MeAla^6]-des-N-tetramethyltriostin A (**3**).

The synthesis of **3** is given in Figures 1 and 2. N-Benzyloxycarbonyl-N-methyl-L-alanine (**4**) was esterified with 2,2,2-trichloroethanol using N,N'-dicyclohexylcarbodiimide (DCC) and 4-(N,N-dimethylamino) pyridine (DMAP); subsequent removal of the N-benzyloxycarbonyl group with HBr in acetic acid gave hydrobromide **5** in 87% overall yield. Preparation of dipeptide **6** was achieved in 88% yield by the active ester method involving reaction of the free amine generated from **5** with the 2,4-dinitrophenyl ester of Z-D-serine.

Tridepsipeptide **7** was prepared in 87% yield by esterification of Boc-Val-OH with dipeptide **6** by reaction with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and DMAP. Preparation of



1 Triostin A, R = Me, R' = H

2 Des-N-tetramethyltriostin A, R=R'=H

3 [MeAla^2,MeAla^6]des-N-tetramethyltriostin A, R=H, R' = Me

tetradepsipeptide **8** from **7** proceeded in good yield by N-deprotection with trifluoroacetic acid and coupling to Boc-Cys(Acm)-OH using EDC with 1-hydroxybenzotriazole (HOBT) as an additive.

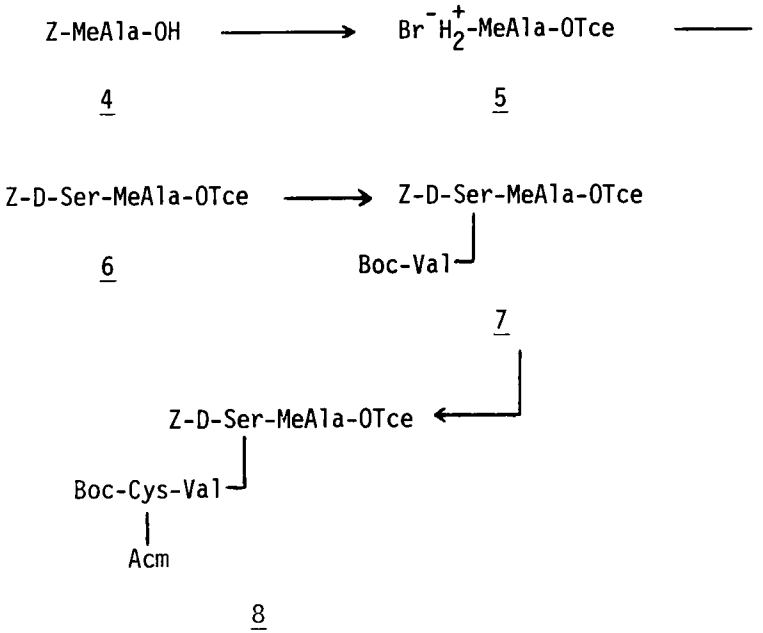


Fig. 1. Synthesis of tetradepsipeptide intermediate

The synthesis of **3** was completed (Figure 2) by the deprotective conversion of **8** to tetradepsipeptides **9** and **10,** respectively. Removal of the 2,2,2-trichloroethyl (Tce) ester with zinc in 90% aqueous acetic acid provided **9,** while deprotection of **8** with trifluoroacetic acid furnished **10.** Fragment coupling of **9** and **10** by the EDC-HOBT method gave linear octadepsipeptide **11** in 88% yield. Sequential deprotection of the amino and carboxyl termini of **11** followed by cyclization under high dilution by the slow addition of deprotected **11** to a methylene chloride solution of

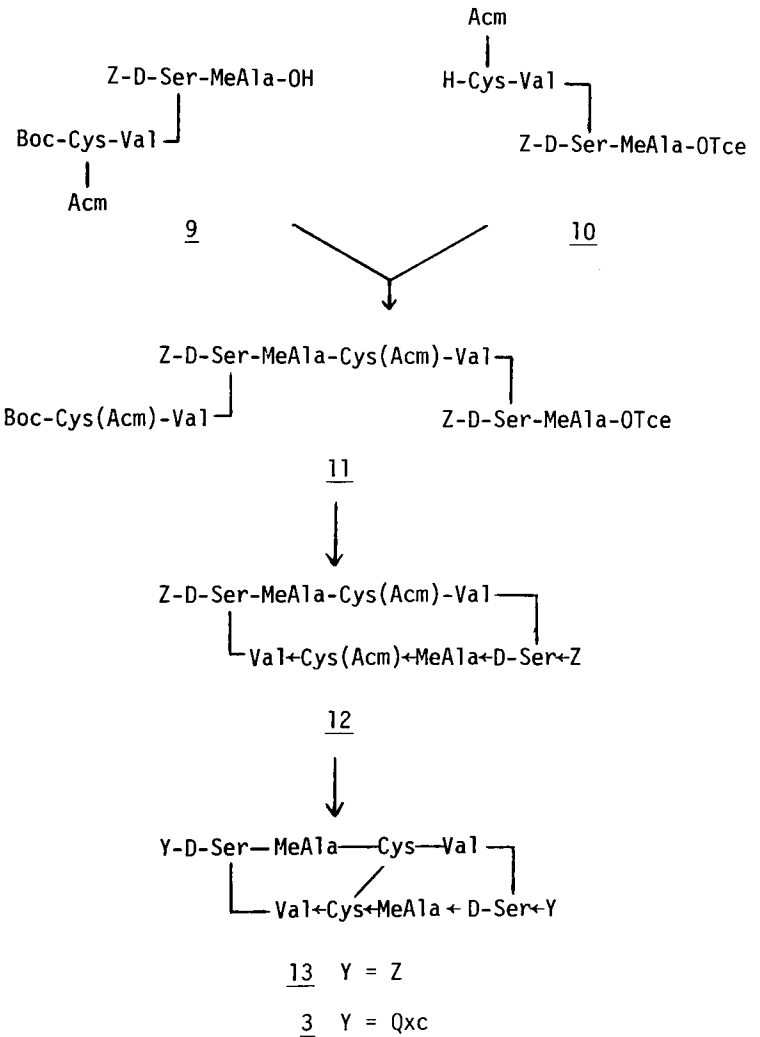


Fig. 2. Synthesis of [MeAla^2,MeAla^6]-des-N-tetramethyltriostin A.

EDC-HOBT furnished cyclic product **12** in 55% yield. Oxidative removal of the S-acetamidomethyl (Acm) groups gave (87%) disulfide **13,** which upon removal of the benzyloxycarbonyl functions of the serine units and subsequent condensation with 2-quinoxalinecarbonyl chloride gave [MeAla^2,MeAla^6]des-N-tetramethyltriostin A (**3**) in 61% overall yield from disulfide **13.**

The N-methylalanine analog **3** is being evaluated, in collaboration with Professor M. J. Waring of the University of Cambridge, with regard to its binding to DNA and any specificity that may be observed in binding to poly(dA-dT). Results forthcoming from this study will be communicated at a future date.

Acknowledgements

Appreciation is expressed to the National Institutes of Health (Allergy and Infectious Diseases, Grant AI-15759) for support of this research.

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STEREOSPECIFIC SYNTHESIS OF A meso-DIAMINOPIMELIC ACID DERIVATIVE

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Introduction

The main structural component of the cell wall in all bacterial species is a macromolecule called peptidoglycan. The polysaccharide chains of the peptidoglycan are cross-linked by a short peptide usually composed of D-glutamic acid, D-Alanine, L-Alanine and either α,α'-meso-diaminopimelic acid (meso-DAP) or lysine.

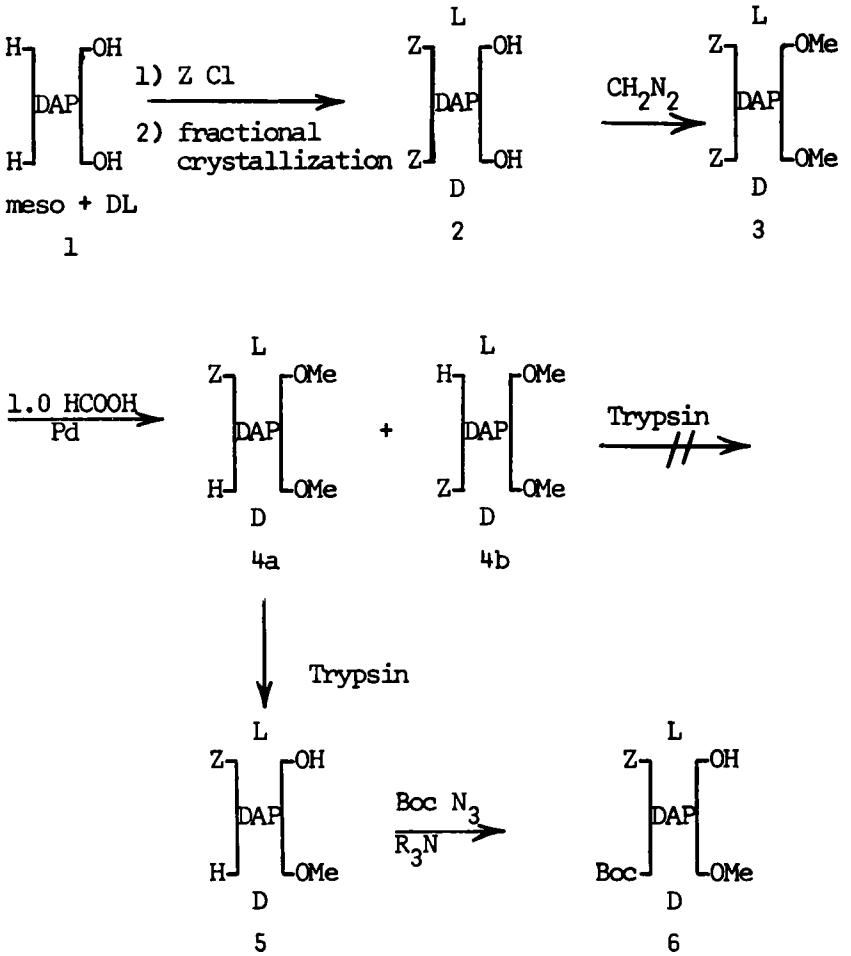
Synthesis of cell wall peptides containing meso-DAP residues requires the use of selectively cleavable protecting groups at the L- and D-centers of the molecule, and is still a challenging problem.[1]

Results

We report here the synthesis of compound 6 (Scheme 1), a trisubstituted derivative of meso-DAP, which is a suitable starting material for the synthesis of peptides having the L-configuration of meso-DAP in the peptide backbone.

Compound 1 was obtained by the procedure of Work et al.[2] except that 10% of 18-crown-6 was used to catalyze the reaction of potassium phthalimide with α,α'-dibromopimelic acid. Reaction of 1 with benzyloxycarbonyl chloride gave a mixture of meso and DL derivatives which was separated by fractional crystallization.[3] The meso compound 2 in ethyl acetate was converted quantitatively to the dimethyl ester 3, m.p. 74-75°, by diazomethane in ether. Treatment of 3 in methanol at 25° with palladium black and one equivalent of formic acid, a good hydrogen donor for catalytic transfer hydrogenation,[4] gave a mixture which was chromatographed on silica gel, eluting with 10% methanol in chloroform. Unchanged 3 (21%) was eluted first, followed by a mixture of 4a and 4b (43%). A slower-moving component (tlc), presumably the diaminodiester, remained on the column.

When a solution of the 4a,b mixture (1.3 g) in 200 ml of .05 M Tris-HCl buffer, pH 7.5, and 50 ml of methanol was stirred with 125 units of immobilized trypsin[5] at 25°, a new spot appeared on tlc ($CHCl_3$:MeOH:AcOH=90:30:5) at Rf 0.40 and reached its final intensity at 30 h. The



Scheme 1. Synthesis of Benzyloxycarbonyl (L) t-butyloxycarbonyl (D) meso-diaminopimelic acid methyl ester (D).

enzyme was removed by filtration and the filtrate concentrated *in vacuo* to remove the methanol. Solid $NaHCO_3$ was added to reach pH 8.5 and unchanged 4b was removed by extraction with ethyl acetate. The solution was readjusted to pH 7.0 with 1 N HCl and concentrated to 30 ml *in vacuo,* to obtain 5 (356 mg, 65%, m.p. 202° dec.). Compound 4b can be recycled by conversion to 3.

A solution of 5 (50 mg) in dimethylformamide (5 ml) was stirred with a mixture of tert-butyloxycarbonyl azide (0.04 ml) and diisopropylethyl amine (.025 ml) for 24 h. The solvent was evaporated and the residue,

dissolved in 20 ml of $NaHCO_3$ solution, washed with EtOAc. The aqueous layer was acidified to pH 2.0 with HCl and extracted with ethyl acetate. Evaporation of the solvent yielded 65 mg (96%) of 6 which was converted to a crystalline salt, m.p. 110-111°, with dicyclohexylamine.

Acknowledgements

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ON THE SYNTHESIS OF PHI

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Recently Tatemoto and Mutt[1,2] succeeded to isolate from porcine upper small intestinal tissue a new peptide factor with VIP-like activities, the "peptide having N-terminal histidine and C-terminal isoleucine amide", thus termed with the acronym PHI (formerly PIHIA). Its primary structure corresponds to a linear heptacosapeptide amide[3] characterized by a remarkable sequence homology with the known hormones of the glucagon family, particularly with VIP and secretin (Figure 1).

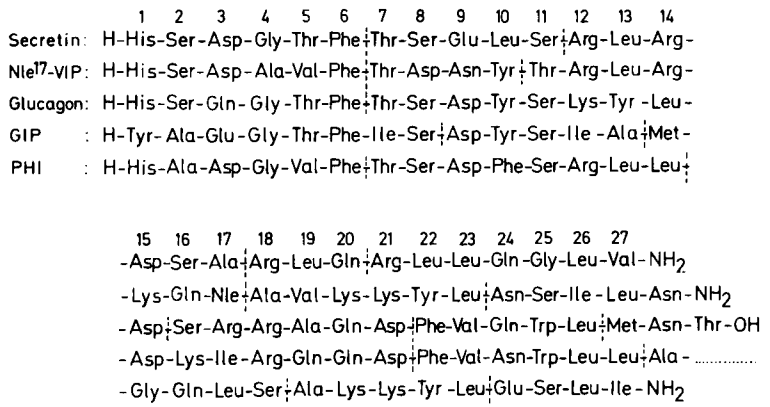

Fig. 1. Alignment of the peptide hormones of the glucagon family; vertical dashed lines indicate the segments used in our syntheses of glucagon, secretin, Nle[17]-VIP and GIP-[1-38]

Synthesis of PHI

To definitely prove the sequence proposed for PHI and to define its physiological role, the synthesis of this hormone candidate was attempted using the strategy of maximum side chain protection via *tert*-butanol and 1-adamantol derived acid labile groups in combination with benzyloxycarbonyl or 2-nitrophenylsulfenyl derivatives for the α-amino protection in the intermediate chain elongation steps, and secondly applying the fragment condensation procedure.

Taking advantage of the experiences gained in our previous syntheses of peptide hormones related to the glucagon family (Figure 1), the following six segments were prepared in suitably protected form:

I) H-Glu(OtBu)-Ser(tBu)-Leu-Ile-NH_2	H-[24-27]-NH_2
II) Nps-Ala-Lys(Adoc)-Lys(Adoc)-Tyr(tBu)-Leu-OH	Nps-[19-23]-OH
III) Nps-Gly-Gln-Leu-Ser(tBu)-OH	Nps-[15-18]-OH
IV) Z-Arg(Z_2)-Leu-Leu-OH	Z-[12-14]-OH
V) Z-Thr(tBu)-Ser(tBu)-Asp(OtBu)-Phe-Ser(tBu)-OH	Z-[7-11]-OH
VI) Adoc-His(Adoc)-Ala-Asp(OtBu)-Gly-Val-Phe-OH	Adoc-[1-6]-OH

Fig. 2. Peptide segments prepared for the synthesis of PHI

Segment I was obtained by the stepwise active ester procedure as well as using N-(2-isocyanoethyl)-morpholine as coupling reagent[4], in practically identical overall yield. For segments II-VI the Anderson method[5], *i.e.* acylation of amino acids and peptides or peptide derivatives with active esters in aqueous-organic or organic media on addition of one equivalent of base, was applied only for the respective dipeptide steps. The subsequent acylations, mainly via N-hydroxysuccinimide esters, were performed on the amino and carboxyl free peptides or peptide derivatives in dimethylformamide (in solution or suspension) without any addition of bases such as tertiary amines. This new simple condensation procedure particularly suited for a fast and clean work up, led to segments II-VI in high overall yield as analytically homogeneous materials. Additionally the danger of racemization upon overdosage of bases was bypassed.

Subsequently, the segments were assembled in the order shown schematically in Figure 3 using the dicyclohexylcarbodiimide/N-hydroxysuccinimide condensation procedure[6]. Only segment V was more efficiently coupled to segment H-[12-27]-NH_2 by using 1-hydroxybenzotriazole as additive[7]. Thus the use of 1-hydroxybenzotriazole was restricted to this single condensation step, since several reports appeared in recent years reporting remarkably high racemization rates observed with this 1,2-dinucleophile additive.

The intermediate N^{α}-deprotections were performed as indicated in Figure 3: desulfenylations proceeded smoothly with hydrogen chloride or hydrogen bromide in the presence of large excesses of 2-methylindole to lower the proton activity and thus, to suppress partial cleavage of acid labile side chain protecting groups, or by reduction with tributylphosphine[8]; the benzyloxycarbonyl groups were cleanly removed by hydrogenolysis, even in trifluoroethanol as solvent.

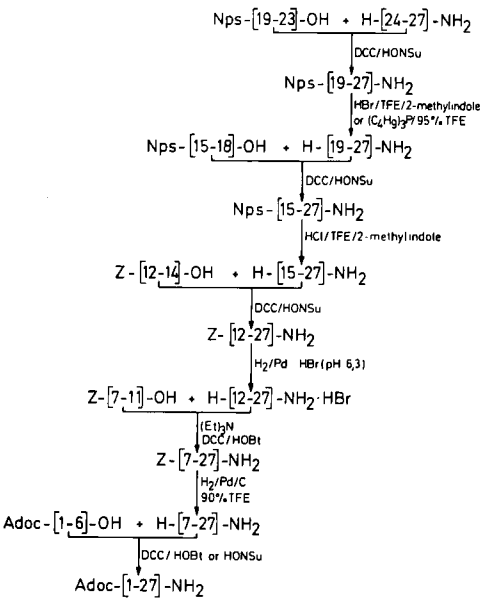

Fig. 3. Assembly of segments I-VI to the fully protected heptacosapeptide amide related to PHI.

Disruption of identity in side chain protection, successfully applied in previous syntheses[9,10], allowed us to overcome solubility problems particularly in the C-terminal region and thus to obtain the intermediate peptide derivatives as well as the fully protected heptacosapeptide amide in satisfactory yields and good analytical purity as judged from elemental analysis, amino acid composition of acid hydrolysates, and chromatographic tests.

Finally, upon deprotection by exposure to trifluoroacetic acid in the presence of conventional scavengers, such as anisole and 1,2-ethanedithiol, attempts to purify the resulting crude product by commonly employed chromatographic techniques failed thus far because of its strong irreversible adsorption on column materials. Purification on a semipreparative scale by HPLC on μ-Bondapak C-18 (eluent: acetonitrile/0.05M ammonium acetate, pH 4.9/phosphoric acid = 31/68/1), followed by desalting on Bio-Gel P-2 produced the heptacosapeptide amide contaminated to a minor extent by an impurity as revealed on HPLC (μ-bondapak C-18; solvent A: 0.1% phosphoric acid; solvent B: acetonitrile; gradient elution with solvent B from 20% to 60% in 30 min.); on TLC in several solvent systems the product behaved as homogeneous material and the amino acid analyses of the acid and enzymatic hydrolysates produced the ratios expected by theory.

Conclusion

Preliminary comparative analyses of synthetic PHI with the natural product by means of HPLC and the tryptic mapping technique suggest the identity of the two products. However, ulterior efforts particularly for the elaboration of an efficient purification step, as well as additional comparative tests by chromatographic, physico-chemical, immunological, and biological assays are necessary to definitely confirm the identity of the synthetic and natural PHI preparation.

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THE SYNTHESIS OF DELTA SLEEP-INDUCING PEPTIDE AND ITS β-ISOMER

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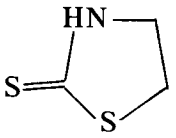
The delta sleep-inducing nonapeptide (DSIP) was isolated and synthesized by Monnier et al[1,2]. These authors reported briefly that their synthetic product was proved to be a mixture of 20% of α- and 80% of β-peptide which were separated by electrophoresis. Of the two isomers, only the α-isomer is highly active. Since these two isomers show such difference in biological activity, a more detailed study of their chemical and physiological properties as well as a more efficient method of synthesis seems to be necessary. With the purpose of obtaining a larger quantity of the α-isomer, we reported previously[3] the use of protecting groups of the tertiary butyl type, the coupling of the peptide segments being carried out in solution. The protected nonapeptide was treated with mercaptoethanol-TFA so that all the protecting groups were removed gently in one step. Almost simultaneously with us, Mikhaleva et al[4] and Hsu et al[5] also reported the synthesis of the α-peptide and its analogs via the solution and solid phase method, respectively.

The α-peptide had already been obtained by Monnier by the separation of their synthetic product, but its properties, except for the sleep-inducing action, were not mentioned in the publication. For this reason, synthesis of both isomers with minimum contamination by each other by our recently reported[6] tetrahydro-thiazole-2-thione (TTT; I) method was carried out. Synthesis of the β-isomer not only enables us to study it from different aspects but also provides an improved and more reliable route to aspartyl peptides.

The synthesis of the two isomeric peptides by the TTT method is illustrated in Figure 1. TTT was easily condensed with the carboxyl group by means of DCC or via the mixed anhydride method. The acylated derivatives are yellow crystalline substances soluble in most organic solvents and can be stored in the dark. The structure was proved by X-ray analysis to be that of 3-acyl-tetrahydrothiazole-2-thione (II) thus ruling out the isomer, i.e. the thiol ester III:



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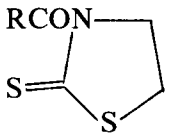

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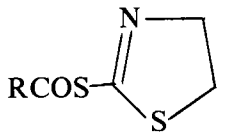

III

The molecular conformation determined from X-ray studies is shown in Figure 2.

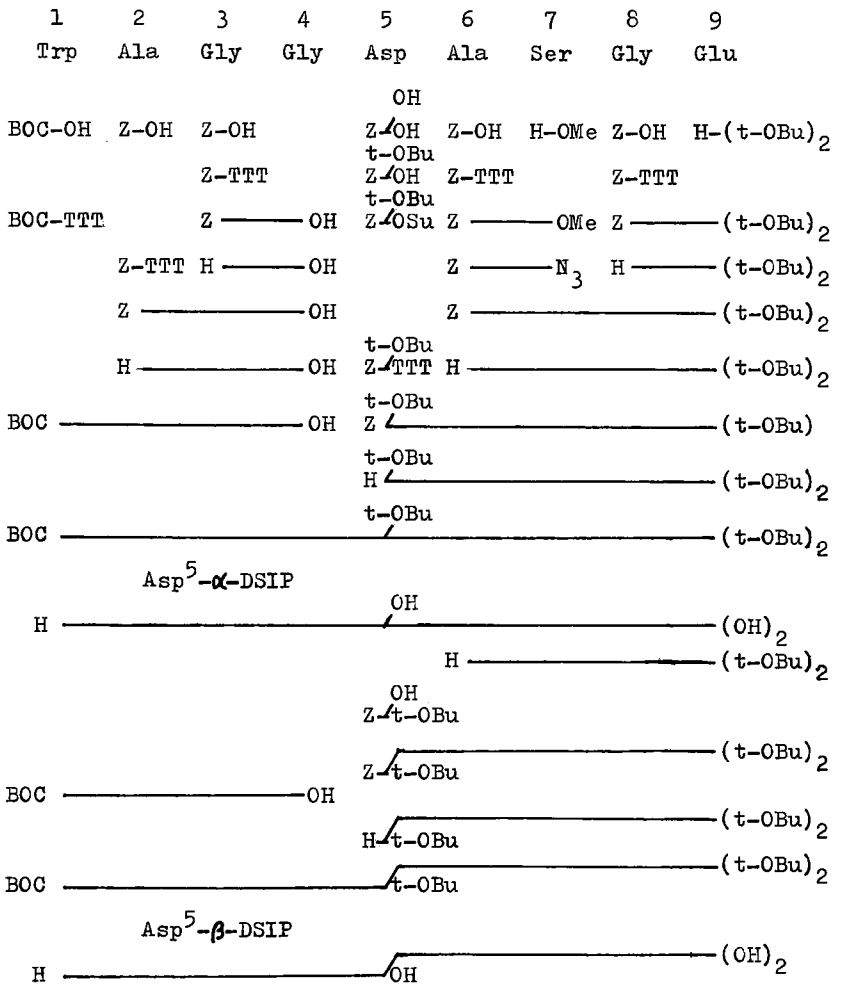

Fig. 1. The synthesis of Asp^5-α-DSIP and Asp^5-β-DSIP by the conventional and the tetrahydrothiazole-2-thione (TTT) methods.

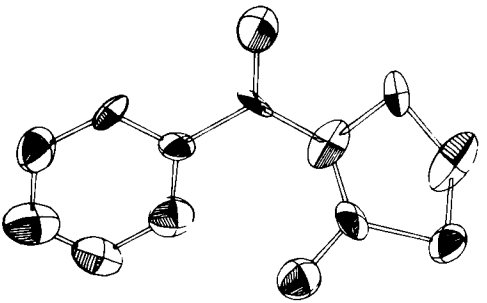

Fig. 2. Molecular conformation of 3-(N)-benzoyl-tetrahydro-thiazole-2-thione.

It is interesting to note that the C-N bond length is 1.4 Å a distance greater than that of ordinary amide bonds. Its activity is demonstrated by the fact that it reacts very rapidly with amine components forming a new amide linkage. The present synthesis shows that this reagent may be recommended as carboxyl group activiting agent for peptide synthesis. Yields observed with the TTT method are generally higher than those obtained by the mixed anhydride method, especially when the carboxyl group of the amine component is unprotected. The yellow color of the acylated TTT fades gradually during aminolysis and its disappearance may be taken to indicate the extent of reaction taking place. Since TTT is a known copper complexing reagent, its removal from the reaction mixture can be accomplished by washing with 15% copper sulfate solution. Thereafter the product is pure enough for subsequent hydrogenolysis.

Final products obtained via the TTT-method are deprotected with mercaptoethanol-TFA, filtered through DEAE-Sephadex-A25 and are proved to be homogenous by TLC, HPLC, and electrophoresis at pH 3.8. No racemization was detected in peptides synthesized via the TTT method.

The physiological effects of synthetic Asp^5-α-DSIP and Asp^5-β-DSIP on delta and sigma activity were evaluated after intravenous injection (50 ug/kg) and intraventricular infusion (5 ug/25 ul/6 min.), respectively. Experiments were performed on 25 adult rabbits of either sex. In order to avoid the influence of circadian rhythm on the animals, experiments were performed only during morning hours in a chamber with 45 lux of illumination and a constant noise background of 72 db SPL. EEG signals were recorded by unipolar or bipolar leads from frontal motor, temporal, and occipital regions. Electrophysiological recordings were carried out 20 minutes before and 1 to 2 hours after injection or infusion. Signals were recorded on magnetic tape and quantified by a Signal Processor to perform the FFT, linear power

spectrum or power spectrum array analyses. In the case of Asp^5-α-DSIP, the increase of delta and sigma activity either by intravenous injection or intraventricular infusion was found to be significant by comparison with controls. In the case of Asp^5-β-DSIP, certain effects were seen in individually tested animals, however, with regard to mean index values, the delta- and sigma-enhancing effects were not significant when compared with pre-DSIP controls. The effects of Asp^5-α-DSIP and Asp^5-β-DSIP on percent change of delta and sigma indices are presented in Figure 3.

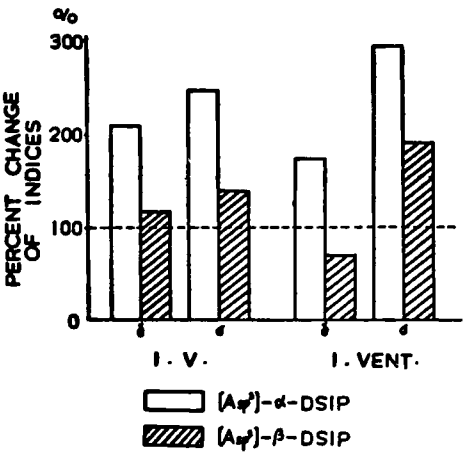

Fig. 3. Mean value of percent change of delta and sigma indices from pre-DSIP controls (100%) under the influence of Asp^5-α-DSIP and Asp^5-β-DSIP. Each bar represents the mean of a group of 5 rabbits with indepent groups of rabbits used. Measures taken before injection or infusion represent pre-DSIP controls (100%).

The data has been converted to percentages of pre-DSIP controls (100%). The results demonstrate that there are differences in the delta- and sigma-enhancing effects between Asp^5-α-DSIP and Asp^5-β-DSIP.

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TOTAL SYNTHESIS OF HUMAN PARATHYROID HORMONE (1-84) BY THE MAXIMUM PROTECTION APPROACH IN SOLUTION

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Introduction

When peptide synthesis is designed and performed for research purposes, the homogeneity of the product should predominate over any other factors, such as yield or cost of preparation. We believe that the maximum protection procedure is the best to minimize the problems which might arise in the final purification process. Since the details of our maximum protection strategy have been published elsewhere,[1] we only briefly outline its principles as follows: (1) all side chain functional groups and the C-terminal carboxyl group are protected with ordinary benzyl-type protecting groups; α-amino groups are protected by the Boc-group; (2) a longer peptide is assembled by the segment condensation method using water-soluble carbodiimide [WSCI, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide] and 1-hydroxybenzotriazole (HOBt) as coupling reagents; (3) each segment is synthesized in the form of an intermediate, Boc-peptide phenacyl (Pac) ester, which can be utilized selectivley not only as amino component but also as carboxyl component; (4) the final fully protected peptide is deprotected by the HF method; (5) the purity of the final free peptide is monitored by reversed phase high-performance liquid chromatography (HPLC). If by-products are difficult to remove by conventional purification procedures, they are eliminated by application of the HPLC technique. The total synthesis of human parathyroid hormone (hPTH)-(1-84) was carried out following the presented strategy.

Results and Discussion

The structure of hPTH was determined by Keutmann *et al.* in 1978 as shown above.[2] Fragment 1-34 is considered to be biologically active.[3] The entire molecule was assembled by segment condensation as shown in Figure 1. Each coupling reaction was continued until the fluorescamine test turned negative on TLC. To obtain Boc-peptide free acid, each Boc-peptide Pac ester was heated with Zn-dust in acetic acid.[4] The Pac

```
1                   5                  10                  15
Ser-Val-Ser-Glu-Ile-Gln-Leu-Met-His-Asn-Leu-Gly-Lys-His-Leu-
                   20                  25                  30
Asn-Ser-Met-Glu-Arg-Val-Glu-Trp-Leu-Arg-Lys-Lys-Leu-Gln-Asp-
                   35                  40                  45
Val-His-Asn-Phe-Val-Ala-Leu-Gly-Ala-Pro-Leu-Ala-Pro-Arg-Asp-
                   50                  55                  60
Ala-Gly-Ser-Gln-Arg-Pro-Arg-Lys-Lys-Glu-Asp-Asn-Val-Leu-Val-
                   65                  70                  75
Glu-Ser-His-Glu-Lys-Ser-Leu-Gly-Glu-Ala-Asp-Lys-Ala-Asp-Val-
                   80                  84
Asp-Val-Leu-Thr-Lys-Ala-Lys-Ser-Gln
```

procedure was extremely effective for the protection of C-terminal amino acid residues from unnecessary racemization during the preparation of these Boc-peptide segments.[5] Furthermore, protection of the terminal carboxyl group with Pac was effective for the selection of the best route for segment condensations, in which each product is kept soluble in ordinary solvents. For example, in the case of the synthesis of a large

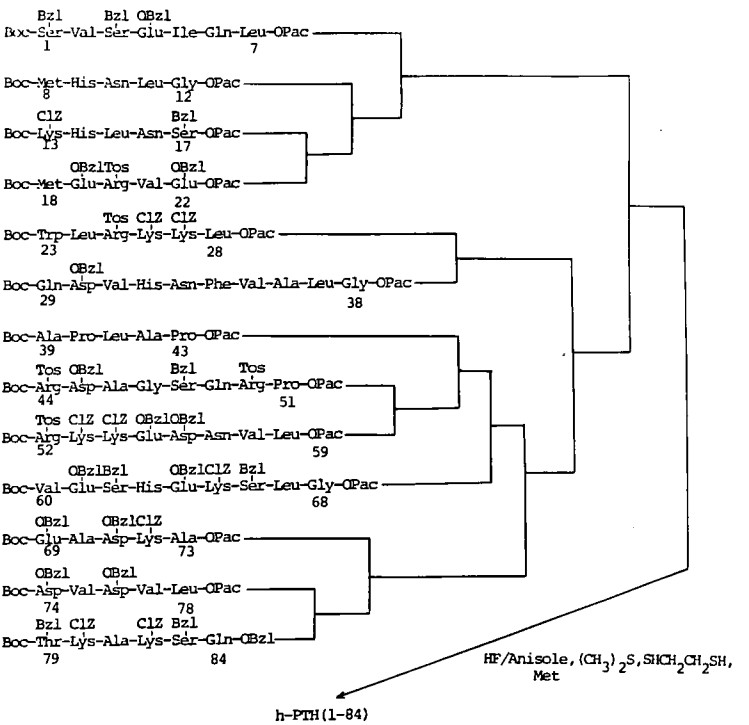

Fig. 1. Synthesis of human parathyroid hormone hPTH (1-84).

segment (39-68), we found an intermediate, Boc-(44-68)-OPac, which was obtained by the stepwise coupling of Boc-(44-51)-OH and Boc (52-59)-OH with (60-68)-OPac, to be highly insoluble in DMF. However, when Boc (39-59)-OPac was first assembled and then coupled with Boc-(60-68)-OPac, the product Boc(39-68)-OPac was reasonably soluble in DMF. Such selectivity in the approach to segment condensation is only available when both termini of each peptide are protected with the Boc and Pac group and all side chain functions are covered by stable benzyl-type groups. Similarly, all segment condensation reactions proceeded smoothly and the fully protected hPTH (1-84) was obtained in a yield of 2.4 g. To test the possibility of isolating hPTH (1-84), an aliquot of the product was deprotected by the HF method in the presence of anisole, methionine, dimethylsulfide, and ethanedithiol as scavengers. After the reaction was over, excess HF was removed under vacuum at 0° C, the residue was extracted with 1 M acetic acid, and the extract was passed through a column of Dowex 1 X2 (acetate form) to remove HF prior to lyophilization. The crude product was purified successively on CM-cellulose columns before the major component was passed over Sephadex G-50; the elution patterns are shown in Figures 2A and B. The

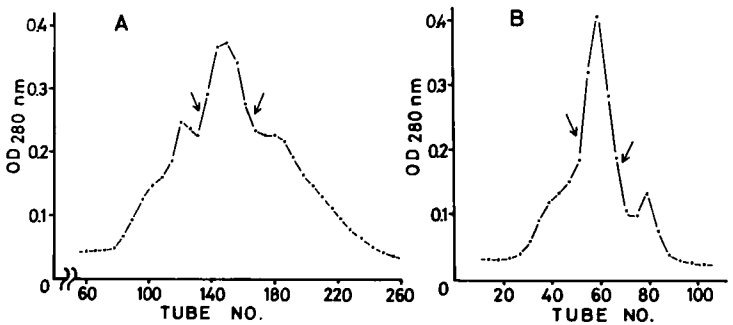

Fig. 2. Purification of deprotected hPTH (1-84) by column chromatography: A. CM-Cellulose B. Sephadex G-50.

homogeneity of the product at this stage was determined by HPLC, as shown in Figure 3A. Purification was repeated on HPLC until a major product was obtained in a single symmetrical peak (Figure 3B). The final product isolated in this way showed $[\alpha]_D^{25}$ -60° (c = 0.1, water) and amino acid ratios after acid hydrolysis: Lys, 9.18 (9), His, 3.72 (4), Arg, 5.15 (5), Asp, 10.03 (10), Thr, 1.00 (1), Ser, 6.19 (7), Glu, 10.89 (11), Pro, 3.12 (3), Gly, 4.00 (4), Ala, 7.14 (7), Val, 8.00 (8), Met, 1.58 (2), Ile, 0.94, (1), Leu, 10.00 (10), Phe 0.91 (1), Trp, 0.57 (1). The rat kidney adenyl cyclase activities[6] for the synthetic material, using WHO bovine-PTH (1-84) as

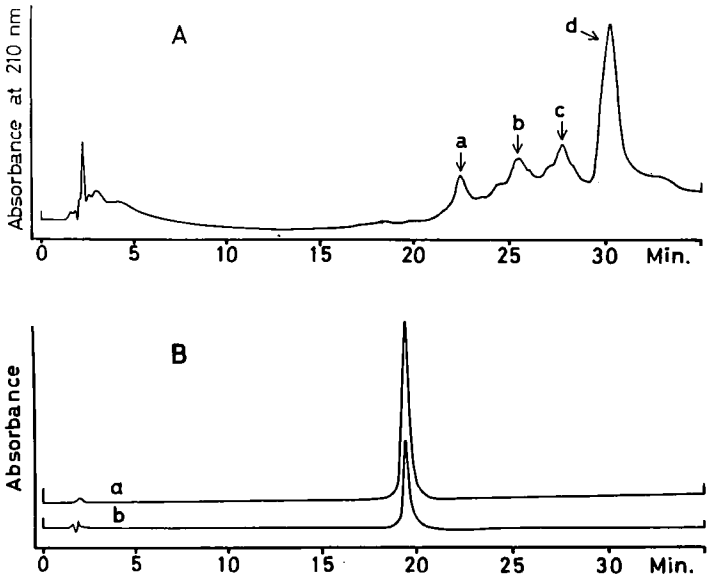

Fig. 3. Purification of synthetic hPTH (1-84) by HPLC: A. A major product separated on Sephadex G-50. Peaks a, b, and c correspond to those for peptides oxidized at Met residues, B. The final product purified by HPLC. Eluent: 10 mM phosphate buffer (pH 2.6) with Na_2SO_4 (50 mM). CH_3CN was added within 25 min in a gradient concentration: (A) 26.4% to 35.5%, (B) 25% to 60%. Detection: UV-Absorption at (a) 210 nm, (b) 280 nm.

standard, are: 3330 IU/mg for hPTH (1-34) and 1600 IU/mg for hPTH (1-44). Synthetic hPTH (1-84) was active in the same assay system. These results indicate that the present strategy is applicable to the synthesis of peptides as large as hPTH. Attempts aimed at the isolation of larger amounts of hPTH (1-84) and comprehensive physiological studies with these synthetic peptides are now in progress.

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STUDIES ON THE SYNTHESIS OF EMERIMICINS III and IV

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Synthesis of peptaibophol antibiotic peptides containing Aib residues is a continuing goal of our laboratory. This report involves the chemical synthesis of Emerimicins III and IV, which belong to the group of peptide antibiotics termed as 'peptaibophol' ionophores (Figure 1). In this report, we describe our current progress on the synthesis of these fragments of emerimicins 1-9 (Figure 2), 10-12 (Figure 3), and 13-15 (Figure 4).

Ac-Phe[1]-Aib[2]-Aib[3]-Aib[4]-Val[5]-Gly[6]-Leu[7]-Aib[8]-Aib[9]-Hyp[10]-Gln[11]-Ival[12]-Hyp[13]-x[14]-Phol[15]

x[14] = Aib, Emerimicin III
x[14] = Ala, Emerimicin IV

Fig. 1. Sequence of Emerimicins III and IV reported by Pandey *et al.*[1]

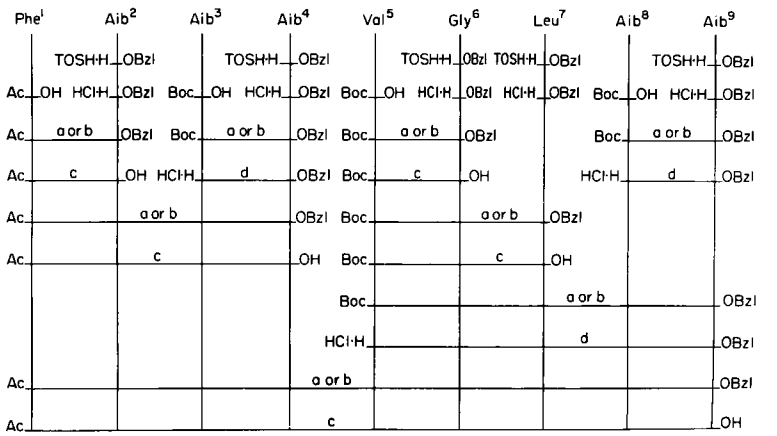

Fig. 2. Synthesis of nonapeptide 1-9.
a: DCC/HOBT, DMF; b: DPPA, DMF; c: 10% Pd/C, CH_3OH: H_2O: CH_3COOH (10:1:1); d: 4N HCl/dioxane.

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Emerimicins III and IV contain several Aib residues in their sequences like alamethicins and poses similar problems in their synthesis due to the low reactivity of these Aib residues in the coupling steps.[3] In case of alamethicin, a fragment condensation scheme in solution was employed successfully for the total synthesis of alamethicin I.[4,5,6] A similar approach was undertaken to synthesize the emerimicin peptides with special attention to improve the yields of fragment peptides. Two approaches, one involving the use of DCC with HOBT and the other using DPPA were employed in the synthesis of the nonapeptide, 1-9, of emerimicins (Figure 2) in order to evaluate their relative efficiencies in coupling Aib residues. The difficulties in the synthesis of emerimicin peptides are further enhanced by the presence of L-Ival residues in their sequences which pose additional problems due to their poor reactivities in coupling reactions.[8]

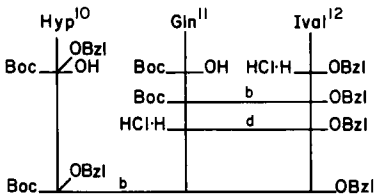

Fig. 3. Synthesis of tripeptide 10-12 derivative.

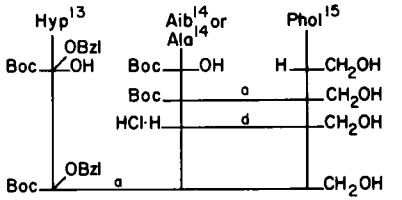

Fig. 4. Synthesis of tripeptide 13-15 derivative.

Reagents defined in Figure 1.

Table I shows the relative yields of the fragment peptides for the sequence 1-9 using DCC/HOBT and DPPA. The reaction times were approximately 48 hours at room temperature. However, in case of DPPA as the coupling reagent, the dipeptides and the tripeptides could be obtained as pure products without the use of column chromatography, whereas the use of DCC/HOBT necessitates the final products to be purified by silica gel columns. Both coupling reagents were found to be equally efficient in most of the fragment synthesis. The fragment peptides prepared by both reagents were identical in their physical characteristics. The x-ray crystal structures of several of these peptides have been elucidated and will be published elsewhere.[9]

The dipeptide, Boc-Gln-Ival-OBzl, was prepared using DPPA only since in our experience considerable dehydration of the glutamine side chain consistently occurred during the synthesis of Boc-Gln-Aib-OBzl, a fragment peptide of alamethicin[6] using DCC/HOBT. The dipeptides containing Phol were prepared using DCC/HOBT only since the use of

Table I. Physical Data on Synthetic Fragment Peptides of Emerimicins†

Compound	% Yield DCC/HOBT	% Yield DPPA	M.P. °C	$[\alpha]_D^{23}$ (C1.0, MeOH)
Ac-Phe-Aib-OBzl	67	78	175-6	+0.2
Boc-Aib-Aib-OBzl	73	79	122-3	---
Ac-Phe-Aib-Aib-Aib-OBzl	81	60	177-180	+31.7
Boc-Val-Gly-OBzl	98	65	81-3	-25.1
Boc-Val-Gly-Leu-OBzl	58	67	54-5	-33.3
Boc-Val-Gly-Leu-Aib-Aib-OBzl	67	68	73-5	-5.2
Ac-Phe^1 --> Aib^9 OBzl	77	51	220-1	+9.6
Boc-Gln-Ival-OBzl	--	83	78-9	-18.1
Boc-Hyp(OBzl)-Gln-Ival-OBzl	--	71	oil	-16.0
Boc-Ala-Phol	82	--	122-3	-51.5
Boc-Aib-Phol	80	--	115-7	-31.0
Boc-Hyp(OBzl)-Ala-Phol	40	--	116-9	-61.5
Boc-Hyp(OBzl)-Aib-Phol	67	--	52-3	-28.5

†C,H,N analyses and amino acid ratios of these peptides are found to be within normal acceptable limits; peptides are found to be homogeneous by t.l.c. in two different solvent systems.

phosphorous coupling reagents like DPPA or DEPC yielded an equimolar mixture of the desired peptide and the Phol ester of Boc-Gln, during the synthesis of a fragment peptide of alamethicin I.[6]

Acknowledgement

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SYNTHESIS OF CASEIN RELATED PEPTIDES

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Introduction

The structure of milk proteins, and primarily the caseins, is of great interest to the dairy industry. In particular, a knowledge of the phosphate bridges involved in the complex micellar structure[1] of the caseins (a mixture of three major phosphoproteins $-\alpha_s$, β, κ) would allow one to interpret the changes which occur during milk processing (*e.g.*, milk → UHT milk, milk → cheese) in chemical terms.

An area of prime importance is the heavily phosphorylated region in the α_s, and β caseins.

P P P P
-Glu-Ser-Leu(Ile)-Ser-Ser-Ser-Glu-Glu-

This region may be disrupted during heat treatment by loss of phosphate.

Our studies have been directed toward synthesis of this segment in the phosphorylated and non-phosphorylated forms. We intend to investigate its physical and chemical properties (*e.g.*, conformation, stability to heat treatment, ease of racemisation) and use this information as an aid to understanding the changes that occur during milk processing.

The approach to the synthesis of the phosphopeptide Ac-Glu-Ser(P)-Leu-Ser(P)-Ser(P)-Ser(P)-Glu-Glu-NHMe (I) is outlined in Figure 1. Both the tripeptide Ac-Glu(OtBu)-Ser(OtBu)-Leu-OMe (II) and the pentapeptide Boc-Ser(OtBu)-Ser(OtBu)-Ser(OtBu)-Glu(OBz)-Glu(OBz)-NHMe (III) were prepared using the REMA procedure.[2] All couplings proceeded in yields greater than 90%. The segment condensation of II and III was achieved in 85% yield using the mixed anhydride method after unsuccessful azide coupling attempts. Selective 'debocking' at each step in the pentapeptide sequence was carried out using 4M HCl in dioxan and the acetylation of II via the isobutyloxy mixed anhydride of acetic acid.

Intermediate peptides were analysed using ^1H and ^{13}C n.m.r. spectroscopy. Some general conclusions were that

(1) Signal assignments could be made by observation (without recourse to heteronuclear decoupling techniques) up to the tripeptide stage (see Table I).

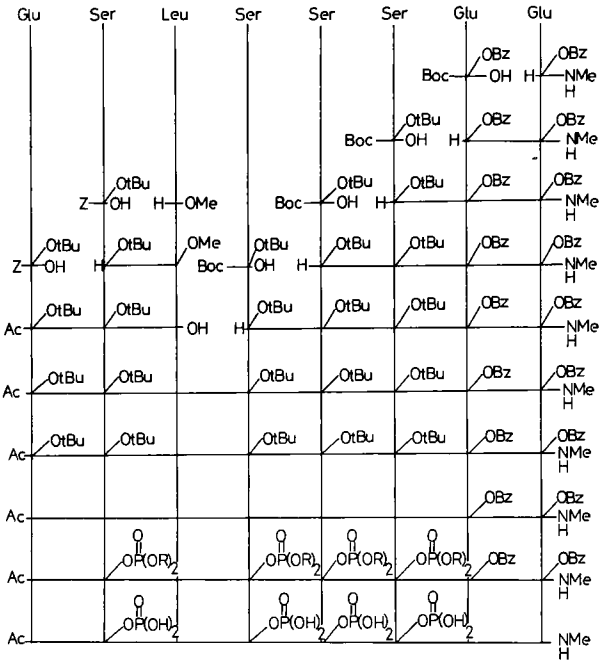

Fig. 1. Synthesis of the phosphopeptide

Table I. ^{13}C Chemical Shift Values for Amide Carbonyls. Values for the pentapeptide could not be determined owing to solubility difficulties.

Boc — Ser (OtBu)	— Ser (OtBu)	— Ser (OtBu)	— Glu (OBz)	— Glu (OBz)	— NHMe
155.68				172.17	
155.85			171.82	171.24	
155.21		170.13	170.65	171.00	
155.20	170.24	169.50	170.47	171.00	

(2) Assignment on the larger homologous peptides, and in the octapeptide were difficult or incomplete (this may be in part due to conformational changes occurring in the different solvents used in determining the n.m.r. spectra *e.g.*, the n.m.r. of Boc-Glu(OBz)-Glu(OBz)-NHMe was run in $CDCl_3$ while that of the pentapeptide (III) in DMSO-d_6.

(3) Selection of side chain protecting groups which give sharp n.m.r. resonances *e.g.*, the *tert*-butyl group allows a fuller interpretation of ^{1}H n.m.r. spectra.

The preparation of the protected phosphopeptide was readily carried out using diphenylphosphochloridate as determined by ^{1}H and ^{31}P n.m.r. The final deprotection step using Pt/H_2 was unsatisfactory, the resultant mixture containing both the desired phosphopeptide and the monophenylated phosphopeptide. Further work to bypass this problem using phosphoryltriazole as the phosphorylating agent has directly introduced the phosphate group in simpler serine containing peptides and we are now in the process of extending this to the casein peptides.

Acknowledgements

This work was supported by grants from the Australian Dairy Research Board.

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ISOLATION, CHARACTERIZATION, AND SYNTHESIS OF UROTENSIN II PEPTIDES

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Introduction

Urotensin II (UII), a peptide with fish smooth muscle stimulating and eel-pressor activity, is one of the neurohormones in the urophysis of bony fishes.[1] Isolation and structure characterization of UII are critical for the study of its physiological function, which has not been established, and of interspecies variation in hormone structure.

We describe here the isolation of a number of UII peptides of different structure, from urophyses of the carp, *Cyprinus carpio,* and the sucker, *Catostomus commersoni.* The primary structure of these peptides has been elucidated and has been confirmed by synthesis in the case of UII-α and γ from the carp.

Purification and Sequencing

Acetone-dried urophyses were homogenized in 0.1 N HCl, the resulting crude extract chromatographed on Bio Gel P-6, and UII activity determined by the trout rectum assay.[2]

Pooled bioactive fractions were chromatographed on phosphocellulose P-11 (carp) or SP Sephadex C-25 (sucker) eluting with ammonium formate or ammonium acetate. Partial separation of several UII peptides was obtained in each species by these procedures.

Final purification was achieved by reverse phase high pressure liquid chromatography (HPLC). Three distinct products of UII (classified as UII-α, -β, and -γ) were obtained from the carp; two products (UII_A and UII_B) were obtained from the sucker.

Manual and automated Edman degradations, as well as digestion with carboxypeptidases A and Y, of native and carboxymethylated peptides revealed the primary structures of five peptides as follows:

UII-α: Gly-Gly-Gly-Ala-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val

UII-β: Gly-$\frac{\text{Gly}}{\text{Ser}}$-Asn-Thr-Glu-Cys-Phe-Trp-Lys-Tyr-Cys-Val

UII-γ: Gly-Gly-Gly-Ala-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Ile

UII_A : Gly-Ser-Gly-Ala-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val

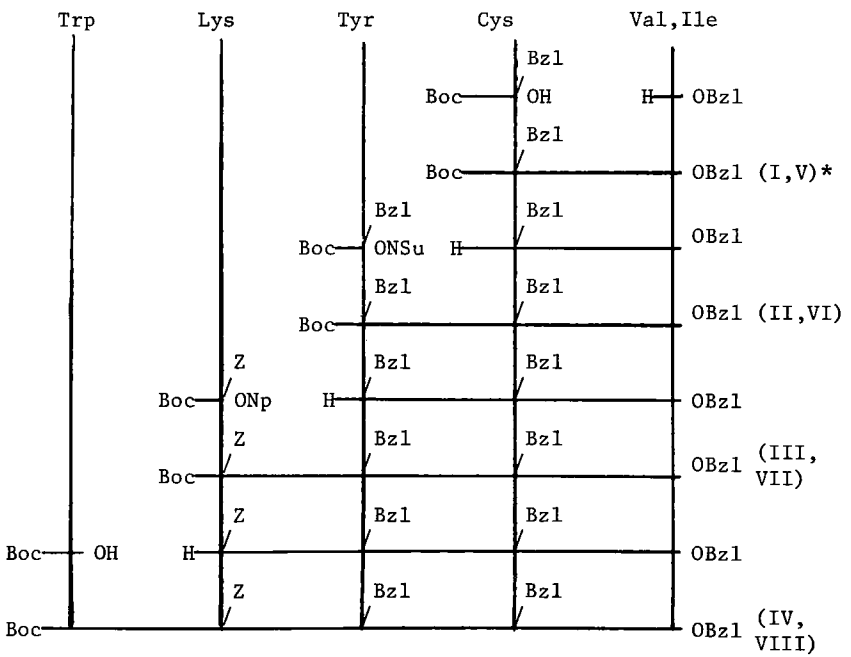
UII_B : Gly-Ser-Asn-Thr-Glu-Cys-Phe-Trp-Lys-Tyr-Cys-Val

From the results of thin layer chromatography, HPLC, amino acid analyses, and sequencing, UII-α, UII-γ, UII_A, and UII_B appear to be homogenous. UII-β, however, appears to be a mixture of two components differing only at position 2, 2-Ser-UII-β being identical to UII_B from the sucker. However, confirmation of the above awaits the separation of the components.

Synthesis of UII-α and-γ

To confirm the amino acid sequences of UII peptides, carp II-α and -γ were synthesized by the solution method.

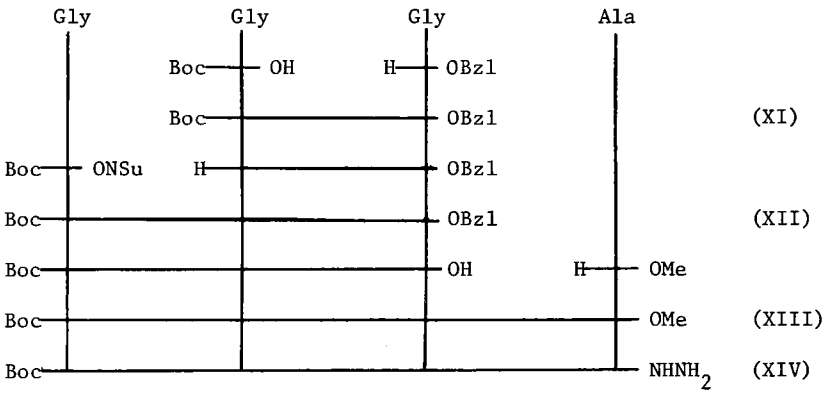
For the synthesis of the dodecapeptide derivatives, pentapeptides IV and VIII, and tetrapeptide XIV were prepared as shown in Schemes 1 and 2. Boc-Cys(Bzl)-Phe-OH(X) was prepared from Boc-Cys(Bzl)-Phe-OEt(IX) through an alkaline saponification.



Scheme 1. Synthesis of protected pentapeptides corresponding to 8-12.

*I-IV, Val-derivative and V-VIII, Ile-derivative.

The CF_3COOH treated derivatives of IV and VIII were coupled with X by WSC/HOBt to yield Boc-Cys(Bzl)-Phe-Trp-Lys(Z)-Tyr(Bzl)-Cys(Bzl)-Y-OBzl(XV: Y=Val, XVI: Y=Ile). Both protected heptapeptides were exposed to CF_3COOH in the presence of CH_3SCH_3 and mercaptoethanol, and the resulting deblocked peptides were acylated with Boc-Asp(OBzl)-ONSu to afford Boc-Asp(OBzl)-Cys(Bzl)-Phe-Trp-Lys(Z)-Tyr(Bzl)-Cys(Bzl)-Y-OBzl (XVII: Y=Val, XVIII: Y=Ile). After removal of the Boc-group from XVII and XVIII in the usual manner, both deacylated octapeptides were coupled with the N-terminal tetrapeptide moiety via azide coupling, through the activation of XIV with iso-amyl nitrite. The resulting protected dodecapeptides were exposed first to CF_3COOH and then subjected to treatment with Na in liquid NH_3, as shown in Scheme 3. Excess NH_3 was eliminated and the peptides were passed through a column of Biogel P6 with 0.1M AcOH. The products bearing the intramolecular S-S bridge were formed during the elution.



Scheme 2. Synthesis of protected tetrapeptide corresponding to 1-4.

IV or VIII $\xrightarrow[2)\ X,\ WSC/HOBt]{1)\ CF_3COOH}$ XV or XVI

$\xrightarrow[2)\ Boc\text{-}Asp(OBzl)\text{-}ONSu]{1)\ CF_3COOH}$ XVII or XVIII

$\xrightarrow[2)\ XIV,\ iso\text{-}amyl\text{-}ONO/HCl]{1)\ CF_3COOH}$ Fully protected dodecapeptides

$\xrightarrow[4)\ Sephadex\ G\text{-}25\ (BuOH\text{-}AcOH\text{-}H_2O)]{1)\ CF_3COOH,\ 2)\ Na/NH_3,\ 3)\ Biogel\ P6\ (0.1M\ AcOH)}$ Urotensin II

Scheme 3.

Final purification of the product was carried out by partition chromatography using Sephadex G-25 with the solvent system of BuOH-AcOH-H_2O.[3] The synthetic UII-α and-γ showed identical Rf-values on TLC, the same retention times on HPLC, and comparable muscle contracting activities as the natural peptides. Thus the synthetic studies confirmed that the proposed amino acid sequences of carp-UII-α and -γ are correct.

Discussion

Amino acid sequences of two forms of UII from the carp and also two forms of UII from the sucker were established. Sequence of UII-α and -γ from the carp was confirmed by synthesis. An active UIIA peptide has also been synthesized (G. Moore, D. McMaster, and K. Lederis, unpublished). A third form of carp UII appears to be a mixture, but is clearly related to UII_B of the sucker.

All the forms of UII described above differ from UII isolated from the urophysis of the goby, *Gillichthys mirabilis* (Ala-Gly-Thr-Ala-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val),[4] however, the sequence Cys-Phe-Trp-Lys-Tyr-Cys in positions 6-11 is common to all forms. The sequence Phe-Trp-Lys (positions 7-9) is common to UII and somatostatin, and it has been suggested that UII has somatostatin-like activity in fishes. No evidence has been obtained, so far, that UII is active in mammalian somatostatin bioassay systems (W. Vale; H. Friesen; personal communications).

The remarkable heterogeneity in the structures of UII peptides in different species and within a given species may imply different physiological roles. The availability of highly purified and a synthetic UII peptides will facilitate physiological studies.

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PROGRESS TOWARD A THIOL CAPTURE APPROACH TO PEPTIDE SYNTHESIS

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Introduction

Coupling of large peptide fragments remains a difficult and unreliable chemical operation, particularly when unproductive association effects are encountered, and a contemplation of the formidable difficulties that result when bimolecular reactions must be conducted at high dilution has led us to the conclusion that conventional acylation chemistry is unlikely to provide the ideal coupling reagent, either for synthesis by large fragment condensation or for semisynthesis, in which minimal side-chain protection and protic media are desirable. Recently, [1,2] we have described the acylation of cysteine derivatives by a thiol capture strategy in which an S-linkage to the acylating agent is established prior to amide bond formation, which then occurs intramolecularly, as shown in Figure 1.

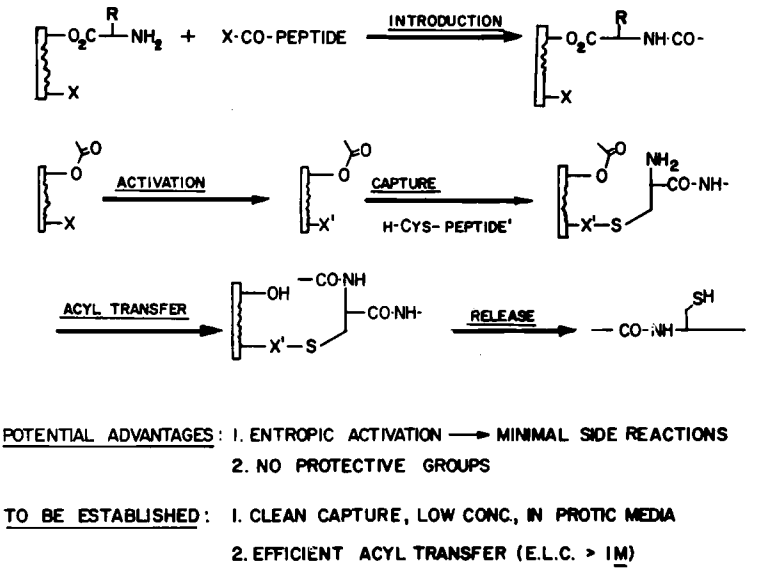

Fig. 1. Scheme for thiol capture

The potential advantage of thiol capture is the assembly of fragments by means of high and selective reactivities of the thiol function, rather than the relatively weak affinity of an activated acyl carbonyl for an amine function. (For a more detailed discussion of these issues, see reference 2).

Two points must be established before a thiol capture approach can be seriously entertained. First, a geometry must be found that permits intramolecular acyl transfer (Step 4 of Figure 1) which proceeds efficiently and rapidly with an acylating agent of the degree of activation of a simple phenyl ester, introduced as a protective group by Kenner.[3] Second, the capture step (Step 2 of Figure 1) must be shown to be rapid and complete at low fragment concentrations (10^{-3} to $10^{-4}M$) and in solvents which disrupt secondary structure and favor open, random-coil conformations.

The efficiency of an intramolecular reaction of a nucleophile can be estimated as an effective local concentration, which is defined as the concentration of an external nucleophile that is required to achieve a rate equal to that of the intramolecular reaction.[4] As seen in Figure 2, effective local concentrations decrease rapidly with ring size and for medium-sized rings have rarely been observed to exceed $0.05M$, even in cases for which the product has little torsional or van der Waals strain. However, we have previously shown that a properly chosen template, such as the xanthene of Figure 2, can permit intramolecular acyl transfer to peptide-derived amines with local concentrations considerably in excess of 1 M.[5] Our first objective, which was recently achieved and reported,[2,6] was the demonstration of a high local concentration (>1 M) of the amino function of an S-trapped cysteine residue.

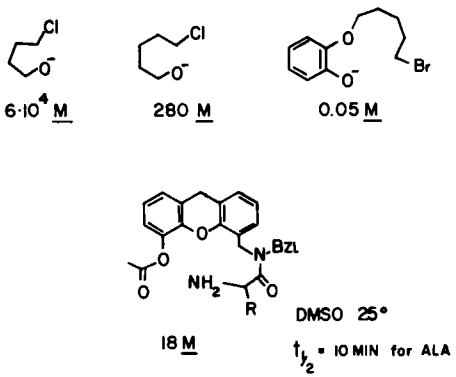

Fig. 2. ELC for acyl transfer.

Two types of capturing site (X of Figure 1) have been studied: arylmercuri derivatives, leading to formation of an Hg-S bond, and activated sulfur, generating R-S-S-R′. Each has been explored by a three-step design strategy that is not unlike the process of developing a new drug. A geometrical model or design hypothesis is used to generate candidate structures. These are screened for evidence of intramolecular acyl transfer, and finally lead structures are refined by adjustment of geometry to optimize the effective local concentration (ELC) of amine for the intramolecular acyl transfer. Since aspects of this process have been reported elsewhere,[2,5,6] only a summary of a successful search is given in Figure 3.

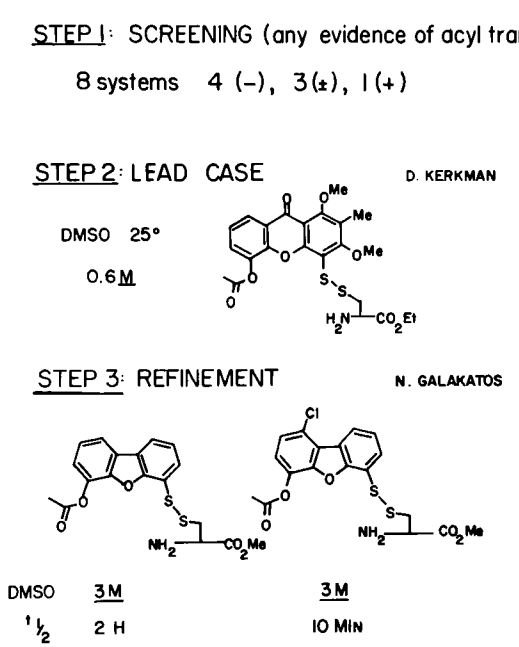

Fig. 3. Steps in capture design

As indicated in the Figure, careful inspection of molecular models indicates that the tetrahedral intermediate for acyl transfer that can result from the xanthone framework exhibits some torsional strain which is relieved if the rigid template for acyl transfer is altered to a dibenzofuran. The resulting increase in ELC supports the model. Although a lead structure has been found for mercuri-capture, we have yet to refine it.[7]

The Thiol Capture Step

With a promising unsymmetrical disulfide in hand, we next examined formation of this bond by reaction of thiols with S-carbomethoxysulfenyl (S-Scm) derivatives. First introduced by Brois,[8] the S-Scm function has been applied to the synthesis of unsymmetrical disulfides of peptides by Hiskey[9] and by Kamber.[10]

$$CH_3\text{-}O\text{-}CO\text{-}S\text{-}Cl + R\text{-}SH \longrightarrow CH_3O\text{-}CO\text{-}S\text{-}S\text{-}R$$

$$(Scm\text{-}Cl + RSH \longrightarrow Scm\text{-}S\text{-}R)$$

$$CH_3O\text{-}CO\text{-}S\text{-}S\text{-}R + R'\text{-}SH \longrightarrow R'\text{-}S\text{-}S\text{-}R + COS + CH_3OH$$

Two tactics for Scm-mediated disulfide bond formation can be contemplated for the thiol capture step -- conversion of the arenethiol of the capture site to an Scm derivative, followed by reaction with a peptide bearing an N-terminal cysteine residue, or Scm-activation of the cysteine, followed by reaction with the arenethiol. Only the latter was found to give satisfactory results. Reactions of the Scm derivatives of a variety of benzene and dibenzofuranthiols with Boc-*L*-Cys-OCH_3 in 1:1 chloroform-methanol at 25° for 30 minutes give yields of unsymmetrical disulfides in the range of 0% to 75%, and the symmetrical diarene disulfide almost invariably appears as a byproduct. By contrast, reactions of the S-Scm derivative of Boc-*L*-Cys-OCH_3 with 4-acyloxy-6-mercaptodibenzofuran proceed cleanly under comparable conditions yielding the unsymmetrical disulfides in yields of 85-90%.

The simple fluorinated alcohols are noteworthy for their tendency to break secondary structure and for their outstanding capacity to dissolve peptides and proteins.[11] Hexafluoroisopropanol (HFIP) appears to be the best solvent for the capture step that we have thus far seen. For example, 4-(Tcroc-*L*-Ala-Gly)-6-mercaptodibenzofuran reacts in HFIP with Boc-L-Cys(Scm)-OCH_3 to give unsymmetrical disulfide in 86% yield, (Tcroc = 2-trifluoromethyl-6-chromonylmethyleneoxycarboxyl).[12]

For semisynthesis, an unprotected Scm derivative of a peptide bearing an N-terminal cysteine residue is the ideal electrophilic sulfur species. Although initial experiments with sulfur capture in DMF gave low yields, the trifluoroacetate salt of H-*L*-Cys(Scm)-OCH_3 was found to react cleanly in HFIP at 0.007 *M* concentration, 0-25° C, 30 min, with an equivalent of the above thiol to yield the isolated, crystalline, unsymmetrical disulfide in 89% yield.

Hiskey and Kamber have reported formation of Scm derivatives from Acm and trityl-blocked cysteine derivatives as well as from the free thiols, which are the more reactive and selective nucleophiles. In accord with their general findings we noted that 1:1 DMF-methanol is a satisfactory solvent for the reactions of Acm-blocked cysteine derivatives with Scm-chloride. Using either Boc-Cys(Acm)-Trp-OCH_3 or a 1:1 mixture of Boc-Phe-Trp-OH and Boc-Cys(Acm)-OCH_3 and following the reactions by HPLC and UV spectra of the indole function, we found clean reactions with Scm-Cl in this solvent in the presence of N-methylmorpholine (NMM), forming the corresponding S-Scm derivatives without sulfenylation of tryptophan.

An extension of this procedure to the blocked somatostatin 3-14 sequence, Boc-Cys(Acm)-Lys(Tcroc)-Asn-Phe_2-Trp-Lys(Tcroc)-Thr-Phe-Thr-Ser-Cys(Acm)-OMaq, was carried out as shown in Figure 4, at 0.001 *M*, 0°C, in the presence of excess NMM, and with the successive addition of Scm-Cl in 1-2 equivalent amounts, monitoring the reaction by HPLC. A total of *ca* 14 equivalents of the chloride was required to achieve complete conversion to the *bis*-Scm derivative. (Other workers have noted the rapid destruction of Scm-Cl in protic solvents containing tertiary amines).

The resulting S-activated peptide was then subjected to the further operations of Figure 4. After disulfide cleavage with dithiothreitol (DTT), the thiols of the bulk product were converted to S-sulfonates to facilitate chromatographic separation. After a preliminary evaporation and trituration with ether and dichloromethane, the product was sized on an HPLC gel filtration column (Waters I-125 protein separation column) using methanol as eluant. The major peak had a retention time identical with that of independently synthesized Z-Ala-Gly-Cys(SO_3^-)-Lys(Tcroc)-Asn-Phe_2-Trp-Lys(Tcroc)-Thr-Phe-Thr-Ser-Cys(SO_3^-)-OMaq. After performic acid oxidation, the major peak gave a correct amino acid analysis, and the bulk product prior to purification gave an analysis corresponding to 80-85% of the expected values for Ala and Gly. A 250 MHz proton NMR spectrum of the product showed the expected resonances.

Obviously the coupling of a glycine-bearing dipeptide residue is only a preliminary to more stringent and realistic tests of the thiol capture strategy. Yet the experimental sequence of Figure 4 does provide first evidence for two important points. First, the activation, capture, and transfer chemistry can operate with a cysteine-bearing peptide of medium size, and second, the capture step can be carried out at low

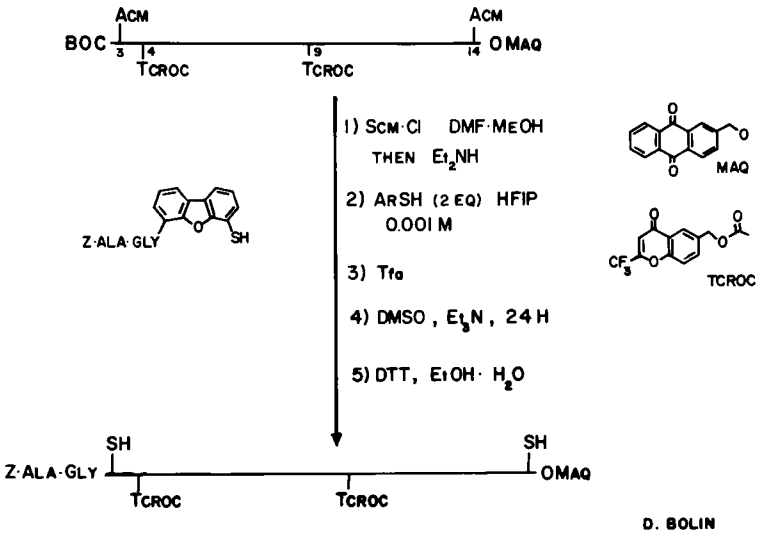

Fig. 4. Application of thiol capture

concentration in HFIP, a solvent which stands an excellent chance of achieving the important goals of facilitating end group interactions (by breaking secondary structure) and solubilizing peptide fragments.

Summary and Future Projects

Thus far, we have addressed two problems which must be resolved before a practical thiol capture strategy can be routinely employed. The first involves the acyl transfer step of Figure 1, and we have demonstrated a workable design strategy that has allowed the construction of frameworks that allow intramolecular acyl transfer to occur at effective local concentrations of the cysteine amine function of greater than 1 *M*. All the viable structures that allow efficient acyl transfer exhibit intramolecular aminolysis rates that are strongly accelerated by dipolar aprotic solvents, and DMSO (or DMF-HMPA mixtures, likely candidates, but as yet untested) is the feasible solvent.

The second problem involves the capture step. For this we have seen a working example of thiol capture using the Scm group as the sulfur-activating agent. Although the results to date at low concentrations and in the special solvent HFIP are very encouraging, further work with larger fragments and lower concentrations will be required to establish the Scm capture tactic as practical and general.

The introduction of the capture site at the C-terminus of a large peptide fragment can be envisaged as occurring late in the synthesis of

that fragment, by a conventional amide forming step involving a fully protected peptide acid and an excess of an amine component consisting of a single amino acid, esterified with the phenol of the capture site, as indicated in Figure 1. For semisynthesis, the ideal coupling would be conducted in a protic medium involving a large excess of the above amine component and a peptide that bears a single activated carboxyl at its C-terminus. Reagents that can cleave peptides in the vicinity of cysteine residues and selectively activate the carboxyl fragment are thus necessities for a versatile thiol capture strategy, and we are engaged in a search for such species.

Finally, it may be noted that the scope of thiol capture could be greatly extended if it were possible to convert the sulfur of cysteine (or selenocysteine) to hydrogen, hydroxyl, or certain other side chain functions of the common amino acids.

Acknowledgements

We wish to thank Mr. S. Drangenis for studies of the reactions of Scm derivatives of simple arenethiols with cysteine esters. Financial assistance from the National Institutes of Health (GM 13453) is gratefully acknowledged.

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SYNTHESIS OF DEHYDROAMINO ACIDS AND PEPTIDES

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Introduction

The large literature on the synthesis of dehydroamino acids (△ amino acids) has been recently reviewed.[1] It is apparent that the various methods do not yet permit the incorporation of a desired dehydroamino acid into a predetermined position in a peptide. Furthermore, the available procedures give either exclusively the Z-isomer or a Z/E ratio so high as to make the E-isomer unavailable for further use. As a result, it has not been practical to compare peptides differing only by the configuration of a dehydroamino acid.

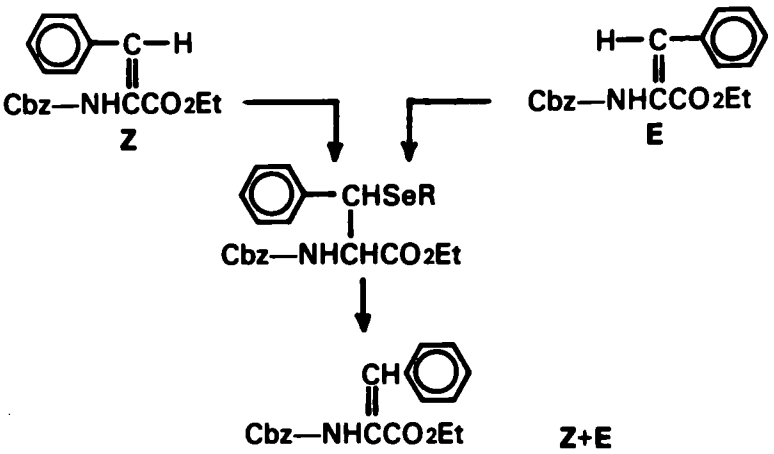
If the double bond in a dehydroamino acid is viewed as just another functional group, then what is needed is a suitable protecting group for the double bond that can be introduced and then eliminated at an appropriate point in a synthetic scheme. We selected selenium derivatives for investigation because of their property of extremely facile oxidative elimination.[2,3] Conjugate addition of selenols to α, β-unsaturated systems has practically not been explored. Among amino acids, the method was used for the synthesis of selenocystine[4] and selenomethionine.[5]

Results

First, the reaction sequence was studied using a known starting material, Cbz-(**Z**)-△Phe-OEt.[6] Conjugate addition of either benzylselenol or phenylselenol was carried out as follows: The dehydroamino acid (1 equiv.), selenol (3 equiv.) and NaOMe (0.05 equiv.) in THF were refluxed for 24 hours. Direct chromatography gave the adduct and allowed the recovery of unreacted selenium reagent. We have settled on benzylselenol for two reasons. It is less subject to air oxidation than phenylselenol which facilitates handling and storage, and it is also cheaper to synthesize. The yield of either adduct was around 80%.

The double bond was regenerated by oxidation with one equivalent of *m*-chloroperbenzoic acid in $CHCl_3$ at -20°. The reaction seemed to be instantaneous; NMR failed to reveal the selenoxide intermediate. It was found that pure Z-isomer gave a mixture, after cycling through the selenium adduct and regeneration of the double bond, that contained a

large amount of E-isomer. In all cases, the Z- and E- isomers were easy to separate by silica gel chromatography. Thus, ratios of Z- and E-isomers could be determined starting from either pure isomer (see Figure 1).

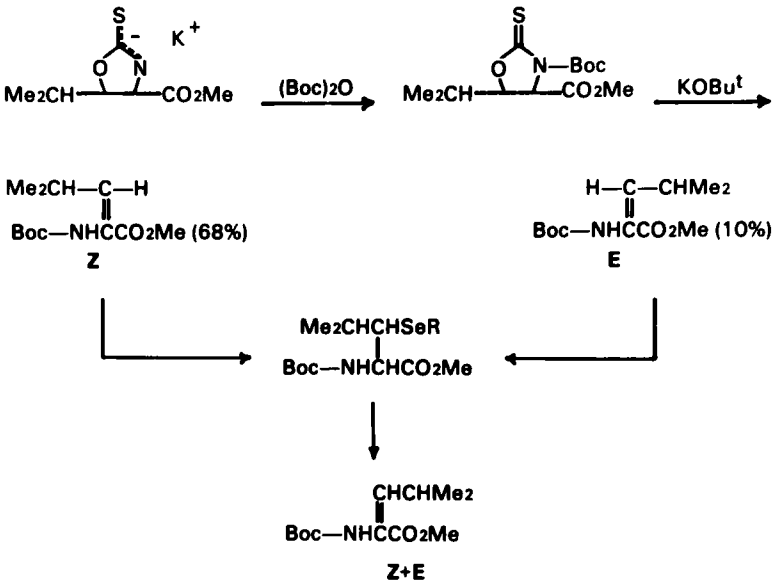


STARTING CONFIG.	R	YIELD (%)	E/Z
Z	C_6H_5	81	9.5
Z	$C_6H_5CH_2$	83	2.6
E	C_6H_5	84	2.6
E	$C_6H_5CH_2$	88	1.3

Fig. 1.

Although the method was very successful, the carbobenzoxy group was unsuitable for further synthetic work. We found that the intermediate oxazolidinethione reacted in high yield with di-*t*-butyl dicarbonate to give the N-Boc oxazolidinethione and then the Boc dehydroamino acid. The reaction sequence was investigated in detail using dehydroleucine (see Figure 2).

Boc-Dehydrophenylalanine methyl ester was prepared and the double bond protected and regenerated with yields comparable to those obtained for Cbz-ΔPhe-OEt.



Starting Config.	R	Yield (%)	E/Z
Z	C_6H_5	56	1.2
Z	$C_6H_5CH_2$	75	2.9
E	C_6H_5	54	1.0
E	$C_6H_5CH_2$	77	1.3

Fig. 2.

For coupling at the C-terminus, the Boc-dehydroamino ester was saponified and the resulting acid converted to a mixed anhydride. To couple at the N-terminus, the selenium adduct was deblocked with HCl in dioxane. The double bond was regenerated later in the synthesis. Yields in all steps were satisfactory (see Figure 3).

Conclusion

We have developed a useful protecting group for the double bond of dehydroamino acids which makes possible the synthesis of peptides having one or more dehydroamino acids in any desired position.

In addition, when a Z-dehydroamino acid is put through a cycle of protection and deprotection of the double bond, a large amount of

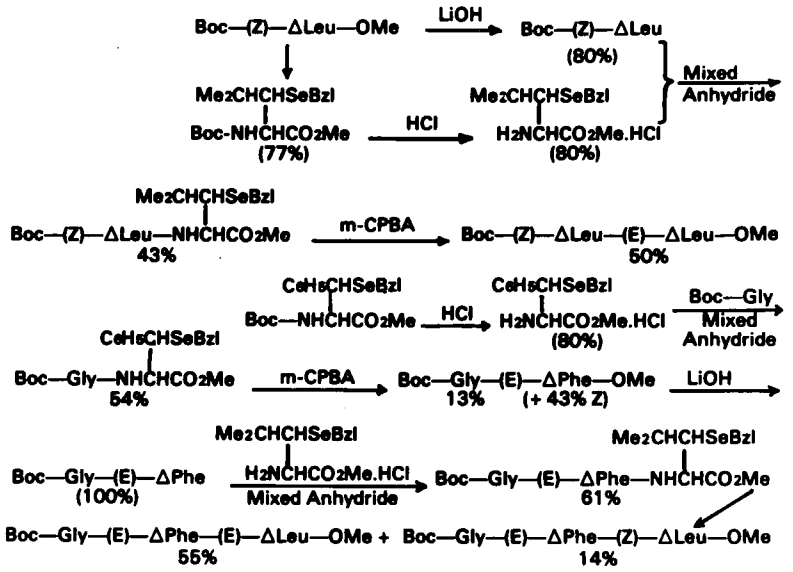


Fig. 3.

E-isomer is produced. Since the chromatographic separation of Z- and E-isomers is simple, E-isomers are now readily available for synthetic purposes.

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CONFIGURATION OF 2-SUBSTITUTED MALONYL AND *gem*-DIAMINO DERIVATIVES OF PEPTIDES

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In an effort to synthesize biologically active analogs of peptide hormones which are resistant to degradation by endopeptidases, the partial retro-inverso modification was developed.[1] The overall effect of the modification is to reverse the direction of the peptide bond between selected residues while conserving the order and spatial orientation of side chains. This is achieved by incorporating in the analog a segment whose residues are chemically identical to those in the natural peptide but connected in reverse order, using the D enantiomer of each amino acid so as to maintain the original side chain orientation. The retro-inverso segment is linked to the unmodified portions of the peptide via bifunctional bridging residues: a 2-substituted geminal diamine at one segment terminus and a 2-substituted malonic acid at the other.

The 2-substituted malonic acid residue, incorporated as a racemate, makes the resulting peptide diastereomeric. In the case of retro-inverso somatostatin and several of its precursors, we found these diastereomers to be separable by HPLC allowing the final peptides to be tested individually for biological activity. To draw conclusions about structure-activity relations in these analogs, it is necessary not only to test them separately but also to know which form of the 2-substituted malonate, the R or the S, is present in each of the separated materials.

This report describes the configurational assignment of the isomers of 2-substituted malonic acid residues in peptides. A simple model was chosen to fulfill the following requirements:

(i) The diastereomers of the compound incorporating the 2-alkyl malonate must be separable.

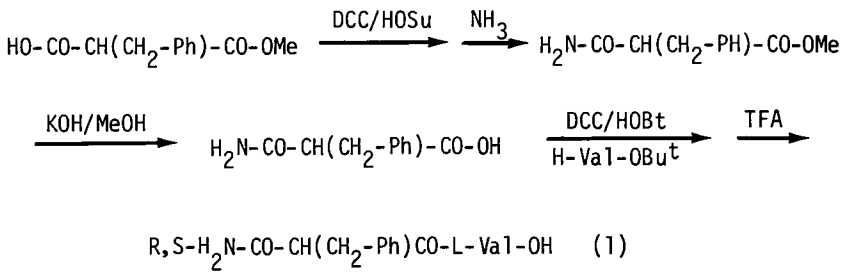
(ii) They must be convertible to materials of known configuration via a reaction whose stereochemical course is documented and which yields a chirally pure product.

(iii) The diastereomers resulting from transformation of the models must be easily separable and authentic samples of each must be available through an independent chemical route.

It was clear from the outset that the simplest models to use were derivatives of 2-substituted (racemic) malonates coupled to amino acids which would serve as precursors for the corresponding dipeptides or their

derivatives. At first the Curtius route was used to convert 2-substituted malonates through their isocyanates to the corresponding hydantoins as noted by Goldschmidt *et al.*[2] Since the hydantoins were not easily separable, this route was not pursued once a superior route was discovered. The reagent of choice for our transformation was to be iodobenzene bis trifluoroacetate (IBTFA), introduced in 1979 by Loudon[3] for the direct conversion of simple achiral amides to the corresponding amines. Since our use of IBTFA constituted its first application to peptides,[4] it was necessary to prove that it did not cause racemization. To examine racemization during IBTFA conversion of peptide amides to the corresponding amines, the reaction was carried out on each of the dipeptide diastereomers Boc-L-Phe-L-PheNH$_2$ and Boc-L-Phe-D-PheNH$_2$. The geminal diamine products were easily distinguishable by reverse phase HPLC and each peptide isomer gave rise to only one geminal diamine isomer with no racemized product detectable by HPLC (<1%).

Having thus developed a novel racemization-free route from malonamides to amino acids, criterion (ii) was satisfied, defining the starting material as a malonamidyl amino acid. It is known[5] that free dipeptide diastereomers are separable and we established that the diastereomers of the starting compound (2-benzyl-malonamidyl valine) were separable, thus both criteria (i) and (iii) were fulfilled. The synthesis of the key starting material R,S-2-benzyl-monoamidomalonyl-L-valine (1) was carried out as shown in the scheme below.



The separation of diastereomers of (1), their conversion to the corresponding dipeptide and the configurational assignment was carried out as in Figure 1.

The malonamide isomers were separated by HPLC on a semipreparative scale using 6.4 x 25 cm Lichosorb column and an isocratic elution with 80% 0.01 M ammonium acetate pH 4.05, 20% acetonitrile at a flow rate of 3 ml/min. For characterization see Table I.

The R and S forms of (1) were converted to the corresponding dipeptides by treatment with IBTFA in aqueous acetonitrile for three hours at room temperature. The resulting dipeptide isomers were compared with the authentic dipeptide diastereomers which were prepared via established methods (Figure 1). The HPLC characterization (10 → 70 gradient, A: 0.125 N TEAP, pH 2.25; B: 70% A, 30% acetonitrile) and other properties (see Table I) show that the earlier emerging isomer of (1) yielded the L,L dipeptide upon conversion while the latter gave the D,L dipeptide.

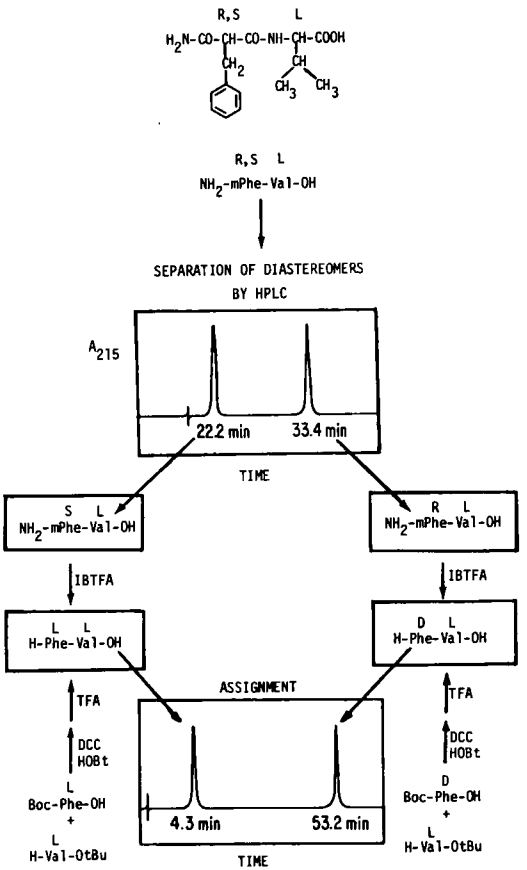


Fig. 1. Assignment of the absolute configuration of 2-substituted malonyl residues in peptides.

The assignment of the absolute configuration of 2-alkyl substituted malonyl derivatives opens the way to the incorporation of these residues with known configuration into retro-inverso peptides, and hence the

Table I. Properties of Key Compounds

Compound	HPLC, Rt[a] min	TLC R_f[b]	Mp °C	$[\alpha]_D^{22°}$ MeOH, C=1
H_2N-m-R-Phe-Val-OH	37.1	0.44 (3)	181-181.5	-33.49
H_2N-m-S-Phe-Val-OH	31.2	0.53 (3)	194-195	-7.43
TFAx-D-Phe-Val-OH	25.2	0.34 (1) 0.53 (2)	210-215 (dec)	-37.93
TFAx-L-Phe-Val-OH	10.3	0.49 (1) 0.60 (2)	174.174.5	+9.10

[a]Gradient 10 → 70% B in 30 min.; A: .125 M TEAP, pH 2.25; B: 30% CH_3CN, 70% A
[b]1: BAW (4:1:1); 2: BAEW (1:1:1:1); 3: CMA (85:10:5)

assessment of their effect on peptide conformation and biological activity becomes possible.

Acknowledgement

This work is supported by AM 15410. We wish to thank Dr. Jean Rivier of the Salk Institute for helpful discussions.

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CYCLOL FORMATION DURING TRIPEPTIDE CYCLIZATIONS. SYNTHESIS OF A SECONDARY CYCLOTRIPEPTIDE, c-(D-PHE-L-PRO-L-PRO)

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Cyclotripeptides as 9-membered rings are highly strained and should therefore possess unusual properties: (1) a very small tendency of formation from open-chain peptides, and (2) rigid conformations with *cis*- and even non-planar peptide bonds leading to special physical properties as well as to transannular reactions across the ring.

In 1965, Rothe *et al.*[2] succeeded in preparing the first cyclotripeptide, c-tri-L-prolyl. Since that time, a number of analogs containing exclusively tertiary peptide bonds of N-alkylamino acids has been obtained.

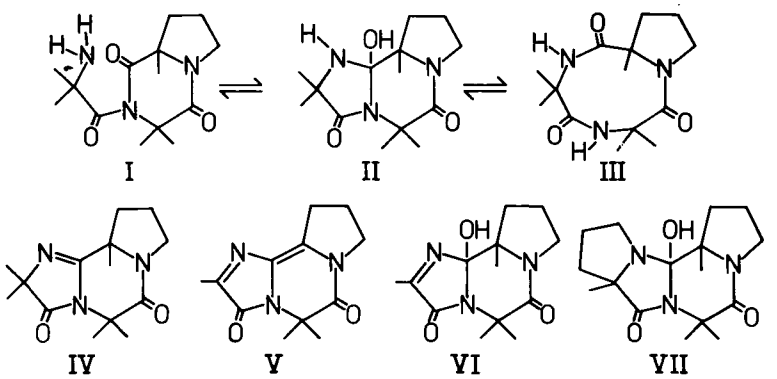
In contrast to all expectations, however, ring-closure of tripeptides with secondary CONH amide groups to yield the 9-membered peptide rings has failed up to now, even at high dilution. Cyclic dimers and cyclic oligomers are formed instead, due to conformational and van der Waals strain present in the transition state leading to the 9-membered ring.

On the other hand, remarkably high yields are obtained in the syntheses of cyclotripeptides containing N-alkylamino acids.[2] From that can be concluded that secondary cyclotripeptides can be formed indeed under appropriate conditions but are converted into consecutive products by transannular reactions as a result of the close proximity of two CONH groups in the medium-sized ring. It follows from our earlier work on medium-sized cyclopeptides[3] that tautomeric equilibria should exist between an open-chain aminoacyl diketopiperazine (I), a cyclolic peptide (II), and a cyclic tripeptide (III) which are expected to be shifted towards the first mentioned products which are far less strained.

With respect to the high reactivity and the limited stability of these types of compounds, the independent syntheses of aminoacyl diketopiperazines and of cyclols proved to be very useful. Subsequently, they were to be isomerized to give cyclotripeptides. This approach seemed to be particularly suitable since all the reactions involved proceed intramolecularly via a nearly strainless 5-membered cyclolic ring. Thus, cyclodimerization can be avoided.

Both the syntheses were performed as described previously[3] using N-protected aminoacyl diketopiperazines as intermediates. In basic medium they are unstable (except the glycine derivatives) and rearrange to the tautomeric N-protected cyclols. Deprotection of Z-Gly-diketopiperazines and Z-cyclols, respectively under mild conditions leads to free aminoacyl diketopiperazines and to free cyclols. So far, we have prepared cyclolic tripeptides (II) containing the sequence Ala-Ala-Pro, Ala-Phe-Pro, and Val-Leu-Pro.

These compounds can be stored in a refrigerator for several weeks. At room temperature and particularly in solution they turned out to be extremely unstable splitting off a molecule of water very easily to give the corresponding bicyclic acylamidines (IV). These compounds can be obtained in pure state by transfer hydrogenation of Z-protected cyclols under nitrogen. Furthermore, the acylamidines are easily oxidized by atmospheric oxygen leading to brown oils having the structure of dehydroacylamidines (V). In aqueous solution these compounds are converted into dehydrocyclols (VI) by spontaneous addition of water. With the exception of the latter, all products mentioned are easily decomposed, even during TLC. Their structures, however, have been unequivocally determined by spectroscopic means (^{1}H- and ^{13}C-NMR, IR, MS).



The investigations mentioned above have led to a complete picture of the possible reactions of secondary cyclotripeptides and of the side products formed by isomerization, dehydration, oxidation, and hydrolysis. The knowledge of their reactivity, physical properties, and chromatographic behavior proved to be extremely valuable during the study of tripeptide cyclizations.

On the other hand, not all of the products mentioned above can be formed from any given tripeptide depending on the nature and sequence

of the amino acids. In this connection, the influence of Pro, Ala, Phe, and Gly (bearing side chains at N or α-C or no side chain at all) as well as that of D-amino acids was investigated. Furthermore, previous studies of the ring-closure of tripeptides had established that the cyclization strongly depends on the conformation of the peptide chain.[2] For this reason, the following tripeptide sequences were studied with regard to their cyclization tendencies:

Gly-Pro-Pro, Pro-Phe-Pro, D-Phe-Pro-Pro;
Ala-Ala-Pro, Ala-Pro-Ala, Pro-Ala-Ala;
Ala-Phe-Pro, Phe-Pro-Ala, Pro-Ala-Phe, Ala-D-Phe-Pro;
Gly-Ala-Pro, Ala-Gly-Pro.

The cyclizations were performed under mild conditions using tripeptide *p*-nitrophenyl esters: 0.01 M, DMF/*t*-amine, 1 day, room temperature. Nevertheless, complex mixtures of cyclization products were obtained which were separated by chromatography. Cyclic peptide oligomers were separated by GPC, the brownish colored "monomeric" fraction was resolved by reverse-phase HPLC.

In the case of the sequence Pro-Phe-Pro the cyclol (VII) could be isolated; yield 20%, m.p. 67-72°; MS: M^+, $(M-18)^+$; ^{13}C-NMR: 174.7, 167.7 (C = 0), 99.2 (C-OH). This is the first example of isolation of a cyclol during cyclization of a tripeptide.

If N-terminal Pro is replaced by amino acids with primary amino groups, the cyclols (II) are formed as well although they are too unstable to be isolated. Instead, bicyclic acylamidines (IV) are obtained by elimination of water. Moreover, dark-colored oxidation products are formed among which dehydroacylamidines (V) can be detected by MS. They are easily converted into dehydrocyclols (VI) by re-addition of water which was eliminated before. At the same time, traces of dehydrocyclol methyl ethers could be detected which were formed by addition of methanol during GPC. Acylamidines and their dehydrogenation products are obtained as main products from ring-closure of Ala-Phe-Pro, Phe-Ala-Pro, and Ala-D-Phe-Pro, whereas dehydrocyclols are formed primarily from Ala-Ala-Pro and Ala-Gly-Pro. The identification of these side products of the cyclization was accomplished by chromatographic and spectroscopic comparison with authentic samples prepared by the independent syntheses mentioned above.

Cyclolic compounds are formed from tripeptides containing C-terminal Pro but not in the case of internal Pro. This points to a mechanism of formation *via* an aminoacyl diketopiperazine (I) and not *via* the cyclic tripeptide by transannular isomerization.

At the same time, cyclotripeptide oligomers up to 10 tripeptide residues (90 ring atoms) were obtained from tripeptides with C-terminal or internal Pro. They were readily separated by GPC and identified by MS. Remarkably, the well-known cyclodimerization only occurs to a small extent in these cases, whereas cyclic trimers and tetramers are formed as the main products. On the other hand, high yields of cyclohexapeptides are obtained from tripeptides containing N-terminal Pro or a D-amino acid or Gly.

Unfortunately, transannular isomerization of the cyclols (II) to give cyclotripeptides does not seem to proceed readily due to steric reasons. Treatment of the cyclol of (Ala-Ala-Pro), however, yields an isomer which we suppose to be the cyclotripeptide according to the ^{13}C-NMR spectrum in aqueous bicarbonate solution. In aqueous or organic solvent the cyclol is re-formed quickly.

Finally, we succeeded in synthesizing the first secondary cyclotripeptide, c-(D-Phe-Pro-Pro), by direct cyclization of a linear tripeptide taking advantage of the favorable effect of D-amino acids on ring formation. Using the diphenylphosphoryl azide method[4] under modified conditions we obtained 22% of a pure crystalline peptide, m.p. 176-8°, $[\alpha]_D^{22}=$ 112° (c=1, $CHCl_3$). Its structure was determined by high resolution MS (found: 341.17279, calc. for $C_{19}H_{23}N_3O_3$: 341.17395), IR in $CHCl_3$ (NH 3358, amide I 1655, no amide II (trans) at 1550/cm), and ^{13}C-NMR ($CDCl_3$) showing 3 carbonyl-C signals at 171.6, 169.2, and 167.7 ppm (similar to those of known tertiary cyclotripeptides). In protic solvents slow cyclolization occurs which is solvolyzed to give c-(D-Phe-Pro).

Acknowledgements

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CYCLOLS, CYCLODEPSIPEPTIDES, AND N-ACYL-DIKETOPIPERAZINES FROM LINEAR TRIPEPTIDES

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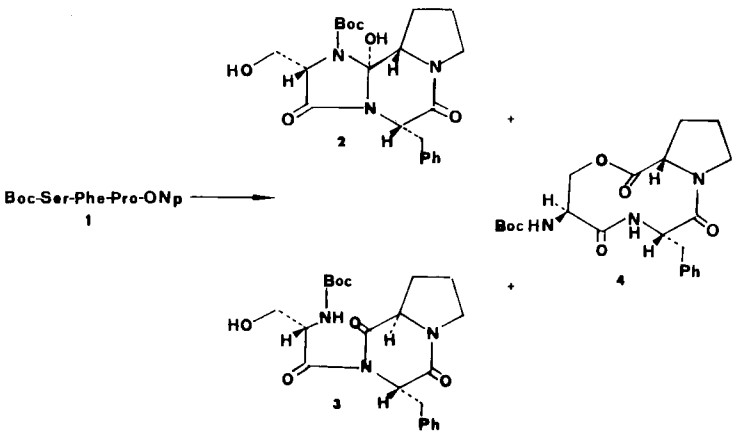
Introduction

So far no nine-membered cyclo-tripeptides or cyclo-depsitripeptides containing a free NH group in an amide bond are known; in these systems the strong trans preference of the CO-NH group and the close steric proximity of the peptide groups result in transannular interaction leading to unsaturated compounds or to five-membered cyclols. Several stable peptide cyclols, tautomeric with nine-membered cyclo-tripeptides or cyclo-depsitripeptides have been synthesized by using insertion reactions on diketopiperazines or by cyclizing activated linear peptides containing α-iminoacids as the C-terminal residue.[1,2]

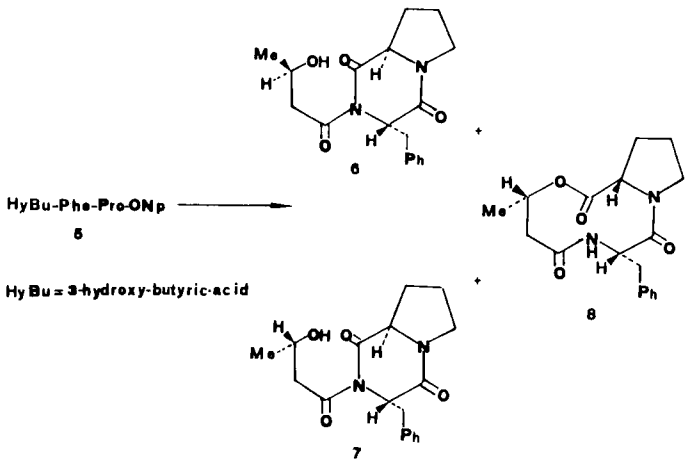
The less strained ten-membered ring homologues have received comparatively little attention. Homodetic cyclic compounds, such as cyclo-(β-Ala-Gly-Pro-) have been studied by Rothe et al.[3] These authors found that the compounds were not formed by direct cyclization of linear tripeptides but could be obtained by aminoacyl-incorporation from β-alanyl-diketopiperazines. Ten-membered cyclo-depsitripeptides or the corresponding cyclols (six-membered) seem not to have been synthesized so far. Starting from N(β-hydroxypropionyl)-cyclo(-Gly-Sar), Antonov et al.[4] reported, however, the isolation of a six-membered cyclol in which the cyclolic hydroxy group is etherified.

Results

As a continuation of our research on cyclization of linear tripeptides we report the results concerning cyclization of carboxyl activated N(β-hydroxyacyl)-dipeptides. We reasoned that Boc-Ser-Phe-Pro-ONp (1) might represent an interesting model. In fact, the sequence X-Phe-Pro has been successfully employed for the synthesis of the majority of stable five-membered peptide cyclols. Moreover, the presence of two nucleophile groups in the first residue should allow for an evaluation of the relative tendency to form the different ring systems.



Scheme 1



Scheme 2

Cyclization of the active ester **1** was performed by following two different procedures: (i) treatment with mild alkaline aqueous buffer (2.5 mmol of **1** in 50 ml dioxane, 25 ml of 0.1 M $NaHCO_3$ and 25 ml of 0.1 M Na_2CO_3 for 3.0 hours at room temperature); (ii) treatment with dimethylformamide — NaH (3.0 mmol of **1**and 3.3 mmol of NaH in 10 ml DMF for 4.0 hours at 0° C). Both procedures have been already employed to synthesize five-membered cyclols from linear tripeptides p-nitrophenylesters.[2,5]

By following procedure i (Scheme 1) only aza-cyclol (**2**) was isolated (35% yield) together with 15% of starting material. Procedure ii gave a more complex mixture from which N(Boc-Ser)-cyclo-(Phe-D-Pro) (**3**; 9% yield) and the ten-membered cyclodepsipeptide (**4**; 12% yield) were obtained in addition to aza-cyclol (**2**; 12% yield).

Since it is known that α-alkyl substituents increase the stability of oxa- and aza-cyclolic tautomers,[6,7] we synthesized and submitted to the cyclization conditions described above, (R,S)3-hydroxybutyryl-Phe-Pro-ONp (**5**). Treatment with alkaline aqueous buffer gave (Scheme 2) cyclodepsipeptide **8** (10% yield) and the two epimeric β-hydroxyacyl-diketopiperazines **6** (10% yield) and **7** (6% yield). The same three products were isolated following procedure ii; in this case the yields of **6, 7,** and **8** were 11,8, and 1-2%, respectively.

Significant spectroscopic features of aza-cyclol **2** are: ^{13}C-nmr shows only three carbonyl signals; the signal of proline C=O is replaced by a singlet at 91.4δ (TMS) consistent with a carbon bonded to three hetero atoms.[7] In the ^{1}H-nmr (DMSO), cyclolic and serine OH appear as a singlet (6.5δ) and a triplet (4.7δ), respectively. Pro CαH is found at 3.7δ in accordance with the cis arrangement with Phe CαH.

In the ^{1}H-nmr spectra of N-acyl-diketopiperazines **3, 6,** and **7,** Pro CαH is found at 2.45, 2.65, and 2.64δ, respectively; this upfield shift is consistent with ring current shielding by the benzylic side chain located cis to Pro CαH. As expected for N-acyl-diketopiperazines,[1,8] α-protons of Phe, Ser, and HyBu residues appear shifted downfield as compared to the values found for the corresponding protons in **2, 4,** and **8.**

Cyclodepsipeptides **4** and **8** show in i.r. spectra ($CHCl_3$) bands at 1750 and 1730 cm^{-1}; for compound **8** a sharp band at 3370 cm^{-1} is observed and no absorption in the range 1445-1650 cm^{-1} (amide II band). In the ^{1}H-nmr spectra, Ser CβH$_2$ and HyBu CβH are found shifted downfield as a consequence of the esterification of the OH group. Phenylalanine NH protons appear as doublets coupled to the Phe CαH with J=7.0 Hz (DMSO) and 11 Hz ($CDCl_3$) for **4** and **8**, respectively. Mass spectra of all cyclic compounds show M^+ and M^+ –18 peaks; the fragmentation patterns of all isomeric products are very similar, with the most relevant peaks related to the diketopiperazine moiety. In Figure 1 the molecular structure of **8** is shown as deduced by X-ray crystallographic analysis.

Conclusion

The results reported show that ten-membered cyclo-depsitripeptides are formed, together with β-hydroxyacyl-diketopiperazines, from the

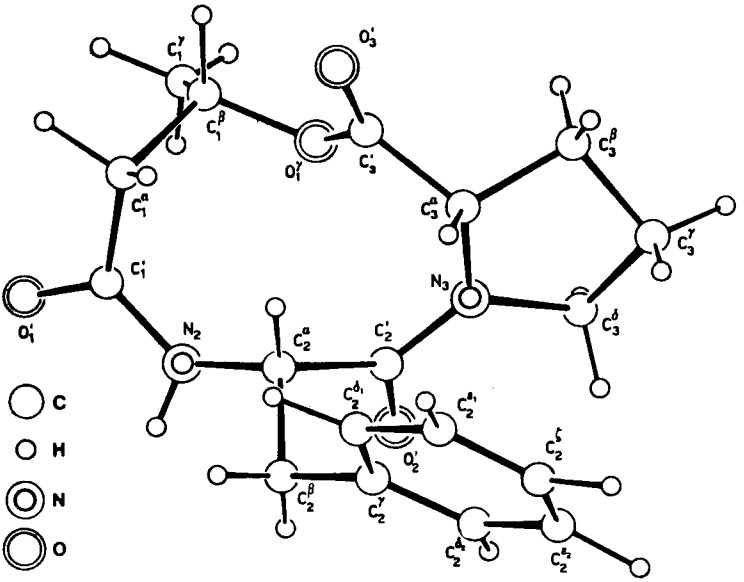


Fig. 1. X-ray crystallographic structure of compound **8**.

corresponding linear precursors containing proline as C-terminal residue; stable tautomeric six-membered cyclols have not been evidenced. Due to the presence of proline, the first step of the cyclization reaction should lead to the formation of N-β-hydroxyacyl-cis-diketopiperazines; these unstable intermediates can epimerize to the stable trans-forms[9,10] or tautomerize to give cyclodepsipeptides (through unstable six-membered cyclols) and/or (in the case of seryl peptide) aza-cyclol. Under the mild conditions adopted in procedure i, the cyclization reaction is more selective and the nucleophilic attack by urethane-NH is favored to give five-membered aza-cyclol **2.**

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WATER SOLUBLE ACTIVE ESTERS CONJUGATION OF SMALL MOLECULES TO BIO-MATERIALS

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Introduction

There is a need for improved methods of attaching small molecules, frequently peptides, to enzymes, protein carriers, cells, and virus particles. Active esters are widely used for coupling small molecules to biological materials. All too frequently such activated molecules are not soluble in physiological media resulting in the difficulty of controlling the stoichiometry of substitution. We report here the utilization of water soluble active esters based on 4-hydroxy-3-nitrobenzenesulfonic acid sodium salt (HNSA) (1,2,3).

Results

Preparation of Active Esters in Solution — N-2,4-Dinitrophenyl-6-aminocaproic acid (DNP-SAC), N-2,4,6-Trinitrophenyl-SAC (TNP-SAC), 2-bromoacetyl-SAC and biotin water soluble active esters were prepared by mixing one equivalent each of acid, dicyclohexylcarbodiimide, and HNSA in DMF. The precipitated urea was filtered; a fourfold excess of ether was added to the mother liquor. All the above derivatives were obtained as crystalline solids containing the ester and small amounts of HNSA. Thin layer chromatography was used for product analysis. In most cases it was not necessary to further purify the esters; however, purification may be achieved on silica gel columns in chloroform-acetone-acetic acid (6:3:1).

All derivatives were freely soluble in water. These esters are stable in water for several hours, provided no nucleophiles were added; all generated the corresponding amides in 1N ammonia accompanied by some hydrolysis to free acids. The esters are stable in TFA and pyridine.

The reaction of DNP-SAC-HNSA with bovine serum albumin (BSA) was investigated in greater detail and compared with DNP-SAC-N-hydroxysuccinimide ester (DNP-SAC-OSu). The two esters were added in graded amounts to BSA in borate buffer, pH 8.5, (with and without adjusting the pH) and allowed to react overnight. The solutions

were dialyzed for several days against 0.1 N ammonia, and purified by chromatography on Sephadex G-25. The optical density was read at 358 nm in borate buffer at pH 8.5; the extinction coefficient for DNP group was determined to be 1.66×10^4.

As can be seen in Figure 1, the best control and efficiency was achieved with DNP-SAC-HNSA ester (nearly a straight line; a) The efficiency of coupling in this case was 39-43%.

Mild HF Cleavage — Treatment with liquid HF resulted in the complete cleavage of DNP-SAC-OSu, DNP-SAC-HNSA, and acetyl-ONp esters. We have sought other cleavage conditions and found that these esters partially survive mild treatment with HF (see below); moreover, the mild procedure was found to be useful for the cleavage of various peptides from the resin.

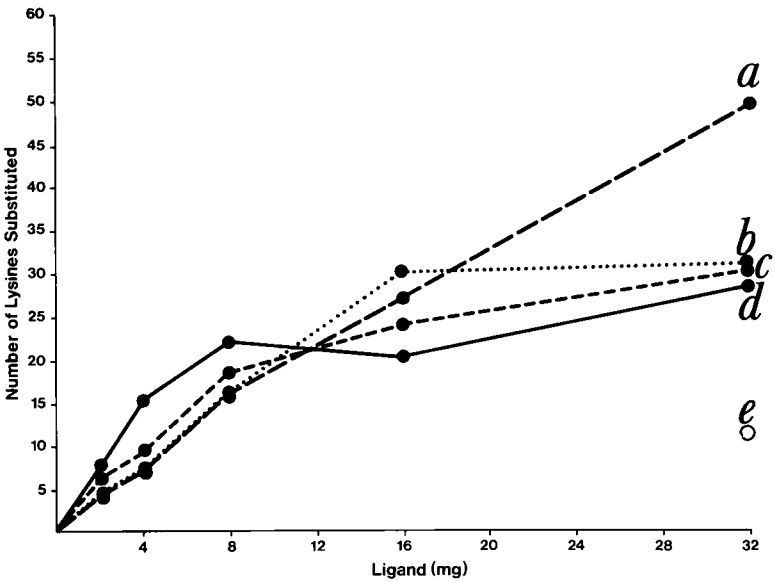

Fig. 1.a. Coupling of DNP-SAC-HNSA in indicated amounts to BSA (30 mg) in 7 ml 0.1 N borate buffer; pH was adjusted to 8.5; b. As in a; the final pH was not adjusted; c. Coupling of DNP-SAC-OSu to BSA (30 mg) in 5 ml 0.1 N borate buffer. The ligand was added in 2 ml DMF. The precipitated reagent was allowed to remain in dialysis bags for several days; d. As in c; the precipitated ligand was removed prior to dialysis; e. As in c; coupling of DNP-SAC-OSu in the absence of DMF.

The resin (or test compound) was suspended in a slurry in anisole, cooled to -70° C and exposed for one minute to HF gas. The slurry was purged with nitrogen at 0° C for 1-2 hours. The residue was washed with

ether and extracted by repeated washings of fresh aqueous/ether bilayers. The resin usually remains at the interface.

The "mild" cleavage yielded the same or slightly larger amounts of peptide from the resin as compared with the regular liquid HF cleavage. Preliminary observations with various peptides on chloromethyl resin indicate that the "mild" cleavage yields peptides in which Ser-OBzl, Thr-OBzl, Glu-OBzl, and BOC groups are cleaved, but Lys-ε-2-chlorobenzyloxycarbonyl, Tyr (O-2-bromobenzyloxycarbonyl) and Tyr (O-2, 6-dichlorobenzyl) groups are not cleaved.

Synthesis of Activated Analogs of Peptides on Resin and Their Utilization — Attachment of free peptides to carrier proteins is frequently extremely inefficient. We have sought methods to circumvent this difficulty by the synthesis of active esters of peptides directly on resin. The peptides were prepared on chloromethyl resin.

$$H_2N\text{-Peptide-CO-}\textcircled{R} \xrightarrow[\text{anhydride}]{\text{succinic}} HOOC\text{-}CH_2\text{-}CH_2\text{-CO-NH-Peptide-CO-}\textcircled{R} \longrightarrow$$

$$\xrightarrow[\text{carbodiimide}]{\text{XOH}} X\text{-OOC-}CH_2\text{-}CH_2\text{-CO-NH-Peptide-CO-}\textcircled{R} \xrightarrow{\text{cleavage}}$$

$$\longrightarrow XO\text{-CO-}CH_2\text{-}CH_2\text{-CO-NH-Peptide-COOH.}\quad X=OPNp,\text{-OSu,-PCP,HNSA}$$

The cleavage of the activated peptide was achieved by "mild" HF treatment. The slurry of 200 mg resin (substitution 0.35 meq/g) was washed with ether and suspended directly between a layer of 5 ml of 0.1 N borate buffer (pH 8.5) containing 30 mg of BSA and 10 ml of ether. The aqueous layer was dialyzed and chromatographed on a G-25 Sephadex to remove the large excess of unreacted peptide. BSA did not bind to the resin. A sample of the resin cleaved by this method was treated with aqueous ammonia to obtain the corresponding amide, H_2N-CO-CH_2-CH_2-CO-Lys-(DNP)-Ala-OH.

The amount of DNP group on BSA is shown in Table I. The extent of substitution by various active esters reflects their survival under "mild" HF cleavage conditions, not their relative reactivities.

Table I. Extent of Dinitrophenylation of BSA After Treatment With Different Esters.

ACTIVE ESTER	Moles DNP/Mole of BSA
HNSA	2
OSu	4
PNp	1
PCP	16

Since the substitutions are rather low, use of other cleavage conditions (such as HBr in TFA) is being investigated. Moreover, the FMOC system (4) which uses TFA for cleavage of the peptide from the resin, should be more applicable for cleavage of active ester peptides from the resin and is now under investigation.

Attachment of biotin-HNSA to Cells — Sheep red blood cells (SRBC) were treated with either biotin-*p*-nitrophenyl ester (Biotin-ONp) or Biotin-HNSA ester and after extensive washing, labelled with fluoresceinyl-avidin. After washing, the cells were examined on the Fluorescence Activated Cell Sorter (FACS).

A much larger increase in cell fluorescence was observed in the cells treated with Biotin-HNSA than with Biotin-ONp.

Conclusions

The water soluble active esters are useful for attaching small molecules and peptides to biological materials. It is possible to cleave peptides from resins under conditions milder than treatment with liquid HF while retaining certain protecting groups. Although limited in efficiency, the mild method of HF cleavage can be used to obtain activated analogs of peptides for direct coupling to biomaterials.

Acknowledgements

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USE OF L-PROPARGYLGLYCINE AS A PLURIPOTENTIAL AMINO ACID IN THE SYNTHESIS OF SIDE-CHAIN-MODIFIED PEPTIDE ANALOGS

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Introduction

Conventional strategy for preparing structural variants of peptide hormones involves the incorporation of a substitute amino acid at an appropriate point in a peptide synthesis, and requires that part (solution-phase) or all (solid-phase) of the synthesis be repeated for each analog being prepared. A more recent strategy facilitates synthesis of side-chain-modified peptides by making use of amino acids which contain functional groups than can easily be transformed, under mild conditions, into a variety of derivative groups. These "pluripotential" amino acids, in an approach introduced by Kemp *et al.*,[1] allow for the formation of a variety of peptide analogs from a single precursor, and can make possible the introduction of functions which normally would not survive conditions of routine peptide synthesis.

One amino acid which has been useful in a pluripotential function is propargylglycine, prepared in optically pure form by Schwyzer *et al.*[2] in 1975. L-Propargylglycine (Pgl) is stable to the conditions and reactions of routine peptide synthesis as demonstrated by its successful introduction into an enkephalin analog, BOC-Tyr-Gly-Gly-Phe-Pgl, and the ethynyl side chain has been transformed cleanly into various functions without consequence to the other peptide reactive groups. It is important to note that these are general requirements which must be satisfied by all amino acids filling the pluripotential role.

Results

Boc-Tyr-Gly-Gly-Phe-Pgl-OH was synthesized using procedures originally developed by Hoyng[3] for the synthesis of the natural enkephalins. The sequence made use of esters of 2,3-dihydroxy-N-ethylbenzamide (Dhb),[4] prepared from the free acid and N-ethyl-7-hydroxybenzisoxazolium fluoborate, for all coupling steps. Figure 1 illustrates this synthesis. We foresaw no problems in carrying the terminal alkyne group through the activation/coupling sequence, and none were encountered in either of two such couplings. We were somewhat concerned

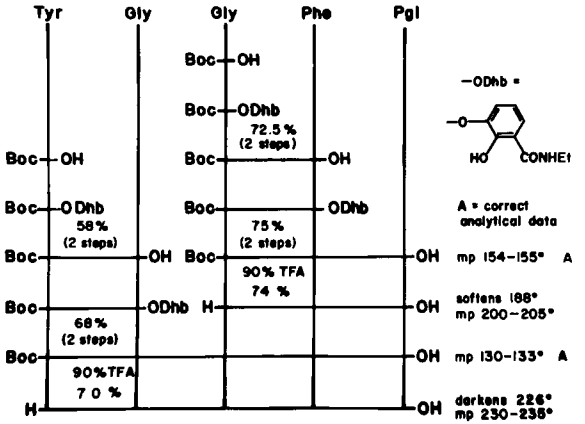

Fig. 1.

about the stability of the acetylene moiety during TFA-catalyzed cleavage of Boc groups but there were no apparent problems here either. The N-deprotected tripeptide Gly-Phe-Pgl was obtained in 74% yield from its precursor under standard conditions, and the fully deprotected analog [Pgl^5]-enkephalin was similarly obtained in a yield of 70%.

Studies on the divergent modification of the propargyl group were initially carried out using diethyl (acetamido) (propargyl)malonate as a model compound. Figure 2 shows some of the modifications which were successfully carried out on the propargyl group using conditions suitable for the peptide. Some of this chemistry is quite straightforward; however, some unusual results were obtained in our attempts to make use of the silver acetylide as a latent acetylide anion. The silver acetylide derived from diethyl (acetamido) (propargyl)malonate was prepared in 80% yield under very mild conditions. Treatment of this with lithium iodide in an inert solvent did not initially afford silver iodide but apparently resulted in the formation of some kind of complex. While it proceeded to react as expected with D_2O and with extremely soft electrophiles such as I_2 and PhHgCl, it appeared to be inert toward a variety of other electrophiles, such as acetone, CO_2, trimethylsilyl chloride, benzyl bromide, and acrolein. This was true even in the presence of strong donor solvents expected to complex the lithium cation; the protio compound was always obtained upon aqueous quenching. We are now exploring the possibility of using the phenylmercuric derivative as a latent acetylide anion.

Although in the case of the model compound, partial catalytic hydrogenation to the alkene was easily carried out, it was recognized that

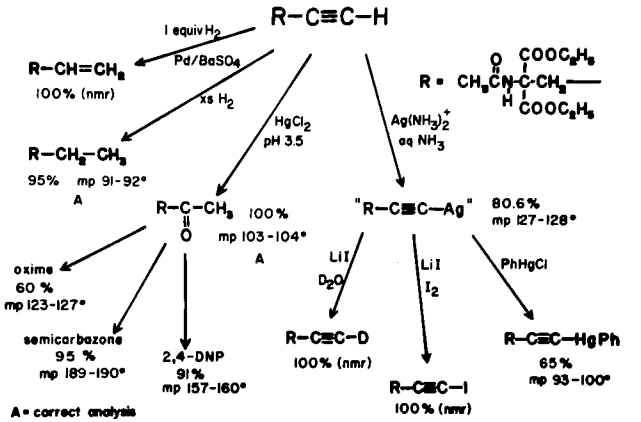

Fig. 2.

this required careful control of hydrogen consumption and could not be relied on as a clean reaction. We therefore explored the use of hindered dialkyl boranes, which have been reported to add cleanly to alkynes to give alkenylboranes which can be transformed further.[5] This reaction was fully explored with our model compound, and complex mixtures of products were invariably obtained, with evidence that the amide group underwent some reduction. Other reactions attempted without success include base catalyzed addition of R-SH across the alkene[6] (which failed to proceed) and formation of the Cu^I-acetylide (which decomposed to give complex mixtures).

The application of the reactions to the N-protected propargylglycine enkephalin analog proceeded with varying degrees of success; the results are shown in Figure 3. The silver derivative of this peptide proved to be unstable (probably due to involvement of the free carboxyl group); however, the methyl ester of the peptide provided a stable silver derivative. As expected, catalytic reduction to the alkene proved troublesome, and mixtures of alkene and alkane were always obtained under a variety of conditions employing various catalysts and catalyst poisons described in the literature as effective in limiting reduction.[7] The peptide was therefore partially hydrogenated and the mixture resolved by preparative TLC, affording the pure alkene analog in 26% yield.

Development of a repertoire of reactions for a pluripotential amino acid requires a substantial initial investment of effort. Many of the reactions introduced here would benefit from further tests before they are applied to cost-intensive situations, and many other reactions of potential utility as regards Pgl have yet to be tried out. Nevertheless, signifi-

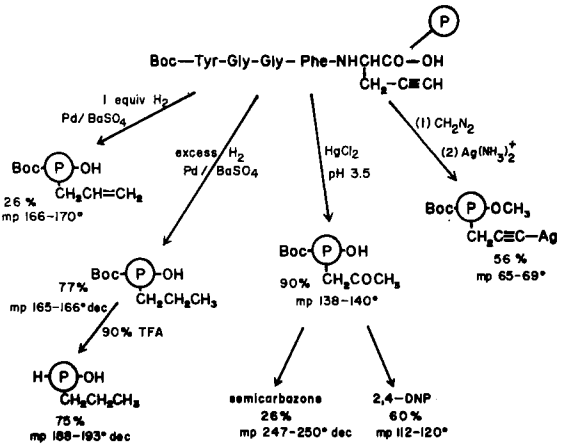

Fig. 3.

cant progress has been made toward affording for peptide chemists a capability for functional group transformation which is more akin to that enjoyed in traditional synthetic chemistry, and which we hope to be of value in the development of new peptide-derived therapeutic agents and research tools.

Acknowledgements

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THE SYMMETRICAL ANHYDRIDE AS A NEW PRECURSOR OF THE 2-ALKOXY-5(4*H*)-OXAZOLONE, AND RELATED STUDIES

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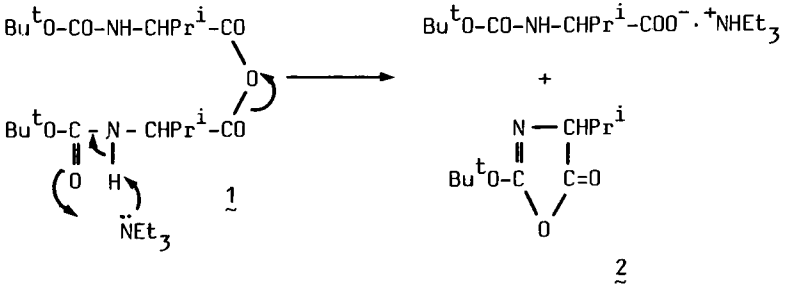
Introduction

We have recently described the isolation of the 2-alkoxy-5(4*H*)-oxazolones from the reactions of Boc-Val and Z-Val with *N*-ethyl, *N'*-(γ-dimethylaminopropyl)-carbodiimide [EDC], and have provided compelling evidence that these oxazolones are implicated in some carbodiimide-mediated reactions.[1] We believe that oxazolone originates by cyclization of the O-acylisourea.[1] The oxazolone can react with *N*-alkoxycarbonylamino acids to give anhydrides, or with amines to give amides. We report here a new precursor of the 2-alkoxy-5(4*H*)oxazolones.

Results

It is known that symmetrical anhydrides of Boc-amino acids are stable to washing with dilute aqueous acids or sodium bicarbonate.[2] We find that in non-aqueous solution, *i.e.* in dichloromethane or dimethylformamide, in the presence of tertiary amine bases, these symmetrical anhydrides undergo what apparently is the reverse of one of the reactions by which they are formed, namely, they are converted to the 2-*t*-butoxy-5(4*H*)-oxazolone and the acid as the amine salt. Detection, and quantitation of the oxazolone is readily achieved by examination of the *t*-butyl peak which has a unique chemical shift in the ^{1}H-NMR 60-MHz spectrum.[1] The amount of oxazolone present in solution when Boc-Val anhydride **1**[2] was dissolved in deuterated chloroform or dimethylformamide in the presence and absence of base is shown in Table 1. Oxazolone **2** was detected immediately in all solutions containing base. Triethylamine promoted a continuous increase in the amount of oxazolone in $CDCl_3$ over a 24-h period, reaching a yield of 80%. The same phenomenon was observed in DMF-d_7, however after reaching 50% at 4 h, the amount began to decline. Pyridine caused a more rapid conversion to oxazolone, but the amount [26%] at 1 h did not increase further. In the presence of *p*-dimethylaminopyridine [DMAP] in $CDCl_3$, one half of the anhydride is immediately transformed into the oxazolone, but the



amount present soon begins to decrease. In DMF-d_7, the amount of oxazolone [33%] declines immediately to 15% at 4 h. Some oxazolone was formed even in the absence of base, as much as 43% in DMF-d_7 at 4 h. We estimate the half-life of $(Boc\text{-}Val)_2O$ in DMF to be 3 h.

Table I. Amount of 5(4*H*)-oxazolone (**2**) Present in a Solution of $(Boc\text{-}Val)_2O$ (**1**) and Amine Base at Various Times.

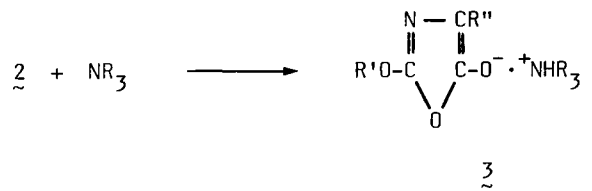
	in $CDCl_3$			
Time (h)	0	1	5	25
Base (1.2 eq)				
-	0	2	7	1.5
Triethylamine	2	17	37	72
Pyridine	11	26	27	29
DMAP	47	53	44	27
DMAP (5 eq)	63	53	36	23

	in DMF-D_7					
Time (h)	0	0.16	1	3	4	22
Base (1.2 eq)						
-	5	11		43		<5
Triethylamine	5		30		50	40
DMAP	33	26	19		15	5

In order to confirm the formation of **2** from **1**, $(Boc\text{-}Val)_2O$ [0.5 mmol] and triethylamine [0.6 mmol] were left in $CHCl_3$ for 24 h. The mixture was washed with water which removes the $Boc\text{-}Val\text{-}O^{-}\cdot{}^{+}NHEt_3$. Methylamine in CH_2Cl_2 was added after removal of solvent, and only a trace of precipitate [$Boc\text{-}Val\text{-}O^{-}\cdot{}^{+}NH_3CH_3$] appeared, demonstrating the absence of symmetrical anhydride. Seventy-five mg of $Boc\text{-}Val\text{-}NHCH_3$ [mp 112-113°] was then isolated. We estimate the amount of methylamide formed from the oxazolone to have been 80% based on the $(Boc\text{-}Val)_2O$ starting material.

$(Boc\text{-}Leu)_2O$ and $(Boc\text{-}Ala)_2O$ in the presence of Et_3N also gave oxazolone but quantitation was not possible. $(Boc\text{-}Phe)_2O$ in $CHCl_3$ with excess Et_3N gave 100% of oxazolone at 30 min; with excess DMAP, it gave 80% of oxazolone immediately but which disappeared within 15 min.

When $(Z\text{-}Val)_2O$ is left in $CDCl_3$ in the presence of Et_3N, the oxazolone cannot be detected, but a reaction does occur. An activated form of Z-Val which is not the anhydride is present. Similarly, the decline in the amount of oxazolone present in solutions of $(Boc\text{-}Val)_2O$ and DMAP is not accompanied by loss of the activated form of the amino acid. In fact, the evidence is that no decomposition occurs because the total amount of *t*-butoxy groups remains constant.



Discussion

The results demonstrate that in both a very polar and a less polar solvent in the presence of bases such as triethylamine and pyridine, anhydrides of Boc-amino acids give rise to the corresponding 2-*t*-butoxy-5(4*H*)-oxazolones. We have previously shown that a 2-alkoxy-5(4*H*)-oxazolone racemizes partially when coupled in the presence of triethylamine.[1] It therefore follows that if a Boc-amino acid anhydride is coupled in the presence of an amine base unless the coupling is much faster than the conversion to oxazolone racemization can be expected. Moreover, in any situation where the anhydride might be implicated as an intermediate in a coupling, if base is present racemization should be considered as a definite possibility. In fact, the validity of these deductions has already been amply demonstrated. *p*-Dimethylaminopyridine[3] caused racemiza-

tion when used to catalyze the esterification of *N*-alkoxycarbonylamino acids to *p*-hydroxymethylphenoxyacetylpolydimethylacrylamide resin, both when the symmetrical anhydride or the acid and DCC were used for the couplings.[4] The results presented here provide a rationalization for the racemization observed. They also demonstrate that particularly in the case where symmetrical anhydrides are used as the activated form for attachment of the first residue to hydroxymethyl polymers in peptide synthesis,[5] the reaction in the presence of DMAP[5] or other tertiary amine catalysts is not straight forward.

Related Studies

Bates *et al.* reported that the addition of 1 equiv. of *N,N'*-diisopropylcarbodiimide [DPC] to Z-Val in $CDCl_3$ leads to complete reaction with the formation of the heretofore elusive *O*-(Z-Val)-*N,N'*-diisopropylisourea.[6] Identification was on the basis of its 300-MHz ^{1}H-NMR spectrum. We were somewhat perplexed by this finding, so we decided to examine the reaction of Z-Val with DPC and then Boc-Val with DCC. On the basis of 60-MHz ^{1}H-NMR spectroscopy and isolation of products, we have come to the conclusion that the reaction of an *N*-alkoxycarbonylamino acid with 1 equiv. of either DPC or DCC in CH_2Cl_2 is exemplified by the following:

$$\text{Boc-Val-OH} + \text{RN=C=NR} = \tfrac{1}{2}\,(\text{Boc-Val})_2\text{O} + \tfrac{1}{2}\,\text{RNHCONHR} + \tfrac{1}{2}\,\text{RN=C=NR}$$

The products are the symmetrical anhydride, the urea, and unconsumed carbodiimide in equivalent amounts. Unchanged carbodiimide can be isolated after the usual work-up consisting of washing with dilute aqueous acid and bicarbonate. Our studies with DPC and the supporting data have been published.[7] Dr. J.H. Jones agrees with our conclusions [personal communication, April 17th, 1981].

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SYNTHESIS OF VERY HYDROPHOBIC ANALOGS OF THE LUTEINIZING HORMONE RELEASING FACTOR[1]

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The isolation and structure determination of the luteinizing hormone releasing factor (LRF)[2,3] has led to the synthesis of an extensive series of analogs,[4,5] some of which have exhibited enhanced agonist or antagonist potency. Of particular interest to us was the demonstration that chronic treatment with pharmacological doses of LRF or its superagonist analogs led to "paradoxical" antifertility[6] effects. Previous analog programs showed: *D*-amino acids in position 6 led to major increases in potency,[7] with an apparent preference for lipophilic amino acids (*D*-Trp most potent),[8,9] (b) NMeLeu substitution[10] in position 7 led to a small increase in activity, (c) replacement of the Gly residue in position 10 by ethylamide,[11] led to a moderate increase in activity.

We have investigated the interaction of these sites of modification while keeping the primary focus on position 6. We have synthesized analogs with very hydrophobic *D*-amino acid residues in position 6 in order to achieve prolonged biological half life and increased potency. All of these peptides were prepared[12] by the solid phase method and purified by preparative HPLC. Where enzymatic resolution of the amino acids was not possible, the *D,L* amino acids were incorporated and the diastereomeric nona- or decapeptides were resolved[13] by preparative HPLC.

The analogs were tested in an estrus suppression assay[12] designed to show the paradoxical antifertility effects of these compounds. Adult female rats were injected (s.c.) twice daily for 14 days with a solution of test compound in 0.1% BSA-0.9% saline. The percent of rats showing continuous diestrus, determined by daily vaginal lavage, was plotted against log-dose and the ED_{50} for complete suppression of estrus was calculated (Table I). The ED_{50} for the standard (**15**) was determined in each assay (0.11-0.14 μg range).

The analogs have been arranged in Table I in two groups (**1-14** and **15-21**) in order of increasing hydrophobicity, as measured by the capacity factors (k') determined by RP-HPLC (Altex Ultrasphere C-18, 40% CH_3CN/0.03 M in NH_4OAc[13], pH 7). It has been suggested by Rivier[14]

Table I. LRF Analogs — Estrus Suppression Activity

Analog		HPLC[a], k'	ED_{50} (μg/inj)	Potency[b]
1	[D-Tmo^6]LRF	0.59		1.4[d]
2	[D-Cha^6]LRF	1.45	0.20	0.5
3	[D-Pfp^6]LRF	1.73	0.12	1.2
4	[D-Nal(1)^6]LRF	1.91	0.26	0.5
5	[D-Nal(2)^6]LRF	1.95	0.07	1.9
6	[D-Mtf^6]LRF	2.05	0.13	1.1
7	[D-Ptf^6]LRF	2.14	0.11	1.4
8	[D-Tmp^6]LRF	2.18	0.07	2.0
9	[D-Bna^6]LRF	2.33	0.15	1.0
10	[D-Daa^6]LRF	3.18	0.2	0.7
11	[D-Bpa^6]LRF	3.23		1.4[c]
12	[D-Fla^6]LRF	3.50		1.0[c]
13	[D-Ana^6]LRF	4.09	0.27	0.5
14	[D-Dca^6]LRF	26.27		0.9[d]
15	[D-Trp^6,ProEA^9]LRF	1.29	standard[8]	1.0[b]
16	[D-Nal(2)^6,ProEA^9]LRF	3.55	0.16	0.9
17	[D-Nal(2)^6,NMeLeu^7]LRF	3.73	0.08	1.9
18	[D-Tmp^6,NMeLeu^7]LRF	5.13		1.1[c]
19	[D-Bha^6,ProEA^9]LRF	5.27	0.11	0.9
20	[D-Nal(2)^6,NMeLeu^7,ProEA^9]LRF	6.73	0.09	1.2

[a] $k' = (t_r - t_o)/t_o$ [b] **15** is taken as standard, potency = 1.0. [c] 1x daily injection [d] Avg. ED_{50} for **15** used.

that there is a direct correlation between potency and retention time on RP-HPLC for 6-position analogs with aromatic side chains. Very recently[15] it has been reported that a quantitative structure activity relationship (QSAR) exists between the potency of 6-position substituted LRF analogs and the calculated hydrophobicity of the amino acid side chain in position 6.

There is not a simple relationship between k′ and potency for our monosubstituted 6 position analogs. Although **4** and **5** are essentially identical in hydrophobicity (k′) there is a fourfold difference in potency. The high potency of **1** is also surprising. For the **4/5** and **6/7** pairs, the less potent analog is the one which appears to bring the side chain steric bulk closer to the peptide backbone. On the other hand, **8** is an analog with severe potential steric interaction between the side chain and the polypeptide backbone, yet it is one of our most potent analogs. In the QSAR study[15] it was suggested that side chain bulk has a slight negative effect but this appeared to be true only for [*D*-Ala^6]LRF and [*D*-Phg^6]LRF. It appears that the relatively compact, hydrophobic side chains give the most potent analogs while the larger, more hydrophobic side chains yield analogs with somewhat reduced potencies.

Analogs **15-20** were studied in an attempt to demonstrate the reported multiplicative effects[4,5] on potency of modifications in positions 6, 7, and 10. A comparison between **5** and **16** shows that the ProEA modification, which gave a 3 to 5x increase in activity for less hydrophobic analogs,[4,5] led to a substantial decrease in potency. The increase in hydrophobicity caused by the ProEA modification coupled with a decrease in potency again contradicts a simple relationship between hydrophobicity and potency.

The addition of NMeLeu to the successful Nal (2) substitution (**17**) has only a small effect on potency while its incorporation into **20** has led to a substantial increase in activity relative to **16**. When coupled with the sterically hindered Tmp (**18**) there is a substantial decrease in activity. There is no clear trend with NMeLeu for this series of analogs.

Our results show that although the dominant factor in obtaining LRF analogs of the highest potency is the hydrophobicity[14,15] of the amino acid in position 6, the relationship between hydrophobicity and biological activity is not a simple one. Modifications in positions 7 and 10 which were previously reported to enhance potency were ineffective when combined with our most potent 6-position modifications. The data reported here make it appear that **5, 8,** and **17** are among the most potent superagonists synthesized.[16]

References

1. The unnatural amino acids used in this study have been given the following abbreviations: Tmo, 3-(3,4,5-trimethoxyphenyl)alanine; Cha, 3-(cyclohexyl)alanine; Pfp, 3-(pentafluorophenyl)-alanine; Nal (1), 3-(1-naphthyl)alanine; Nal (2), 3-(2-naphthyl)alanine; Mtf, 3-(*m*-trifluoromethylphenyl)alanine; Ptf, 3-(*p*-trifluoromethylphenyl)alanine; Tmp, 3-(2,4,6-trimethylphenyl)alanine; Bna, 3[2-(1-bromonaphthyl)]alanine; Daa, 3-[9-(9,10-dihydroanthryl)alanine;

Bpa, 3-(*p*-biphenylyl)alanine; Fla, 3-(2-fluorenyl)alanine; Ana, 3-(9-anthryl)alanine; 2-(2,2-dicyclohexylethyl)glycine; Bha, 3-(benzhydryl)alanine.

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SYNTHESES AND BIOLOGICAL PROPERTIES OF "HYBRIDS" BETWEEN HUMAN INSULIN AND INSULIN-LIKE GROWTH FACTOR I

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Introduction

The amino acid sequences of human insulin[1] and insulin-like growth factor I[2] (IGF-I) show a relationship suggesting evolution from a common precursor. IGF-I is a single chain polypeptide with three intra-chain disulfide bridges; the A and B-chains are connected with a short C-peptide (residue 30-41) and the carboxyl-terminus of the A-chain is elongated with the extension peptide of residues 63-70 (Figure 1).

X-ray analysis of Rhombohedral porcine 2-zinc insulin crystals[3] has demonstrated that the hormone has a global structure shown schematically in Figure 2. Based on circular dichroism and co-crystallization with insulin, a proposed conformation model for proinsulin is suggested which is structurally related to insulin with the connecting peptide folded over the A-chain on the surface as indicated schematically in Figure 2.[4] Model building of IGF-I indicates the conservation of the insulin fold and hydrophobic core, accommodating the short connecting peptide and the extension peptide at the C-terminus of the A-chain as shown schematically in Figure 2.[5] It has been demonstrated that both insulin and IGF-I have a wide but remarkably similar spectrum of biological activities.[6] Insulin is more potent in stimulating acute metabolic effects, whereas IGF-I is more potent for growth (Figure 3). These different effects probably arise through the interaction of the polypeptides with two separate membrane receptors, one of which has a higher affinity for IGF-I, the other a higher one for insulin.[7]

It is our intention to synthesize a series of "hybrids" of human insulin and IGF-I to study: (a) their biological properties by carrying out the glucose oxidation assay[8] and the growth promoting assay for IGF-I, (b) their immunological properties by radioimmunoassay specific for insulin, and (c) their receptor binding properties by radio-receptor assay for insulin[11] and radio-receptor assay for IGF-I.[12]

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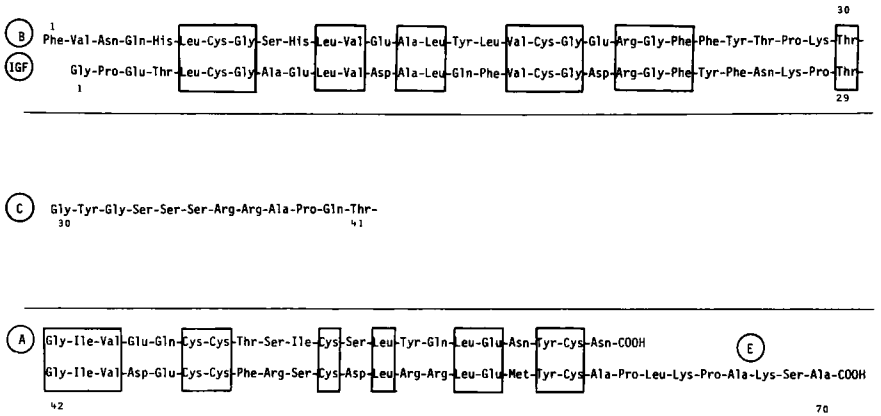

Fig. 1. Sequences of human insulin and IGF-I.

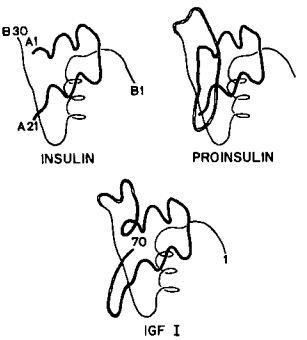

Fig. 2. Schematic representations of the structures of: (1) insulin based upon the X-ray study of 2-zinc insulin (2) proinsulin based on model-building, and (3) IGF-I based on model-building.[8]

This communication describes the synthesis, biological and immunological properties of the following preparations.

1. Human insulin extended on the C-terminus of the A-chain with the extension peptide of IGF-I (residues 63-70).
2. The threonine C-peptide of IGF-I (residues 29-41).
3. Human insulin A-chain disulfide extended on the N-terminus with residues 29-41 of IGF-I.

Results

The solution methods of peptide synthesis (segment condensation, acid labile protecting groups) and enzyme-assisted synthesis (carboxy peptidase A, trypsin) are utilized for the syntheses. The γ-carboxyl functions were protected as their tert-butyl esters, the -OH functions of tyrosine, threonine, and serine as tert-butyl ethers, the ϵ-amino groups of lysine by the tert-butyloxycarbonyl residue. The guanidine groups of

arginine were protonated. For the temporary protection of the α-amino groups, the benzyloxycarbonyl or the 2-(4-biphenylyl)isopropyloxycarbonyl groups were used. The cysteine side chains were protected by the trityl group.

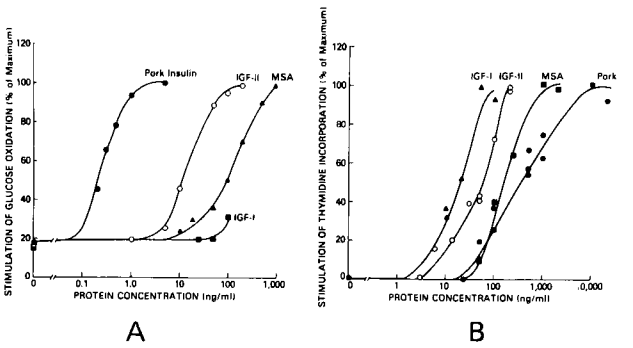

Fig. 3. (A) Stimulation of glucose oxidation in rat adipocytes by pork insulin; IGF-II, multiplication-stimulating activity (MSA) and IFG-I.[10] (B) The stimulation of [^{3}H]-thymidine incorporation by IGF-I, IGF-II, MSA and pork insulin.[10]

The synthesis of human insulin extended at the C-terminus of the A-chain with the extension peptide of IGF-I (residues 63-70) — Figure 4 illustrates the scheme for the preparation of the S-sulfonated extended insulin A-chain. The fragments were coupled either by the mixed anhydride[13] or the DCC/HOBt[14] methods. The purifications of the protected peptide intermediates were achieved either by countercurrent distribution or gel filtration on Sephadex LH 20 in dimethylformamide. Since the sequence (1-29) represents the final step of the synthesis no purification was performed on the crude coupling product. The latter was deprotected with trifluoroacetic acid using thiophenol as a scavenger[15] and converted to the tetra-S-sulfonate. The purification was achieved by gel filtration on Sephadex G-50f in 0.05M ammoniumbicarbonate buffer and ion-exchange chromatography on DEAE-cellulose at pH 5.6 with a linear salt gradient (Figure 5). The purity of the preparation was checked by amino acid analysis after acid hydrolysis, the results of which were in good agreement with the theoretically expected values, Asp 2.07 (2); Ser 2.63 (3); Glu 4.00 (4); Pro 2.00 (2); Gly 1.00 (1); Ala 1.76 (2); Val 0.37 (1); $(Cys)_2$ 1.65 (2); Ile 1.68 (2); Leu 2.89 (3); Thr 1.80 (2); Lys 1.77 (2). Paper electrophoresis at pH 2.2 and 4.8 showed a single band (Pauly positive). The net yield for the final coupling, deprotection, conversion to the S-Sulfonate and purification was 19%. Figure 6 illustrates the approach to the enzyme-catalyzed semisynthesis of [Thr^{B30}-methyl ester] B-chain

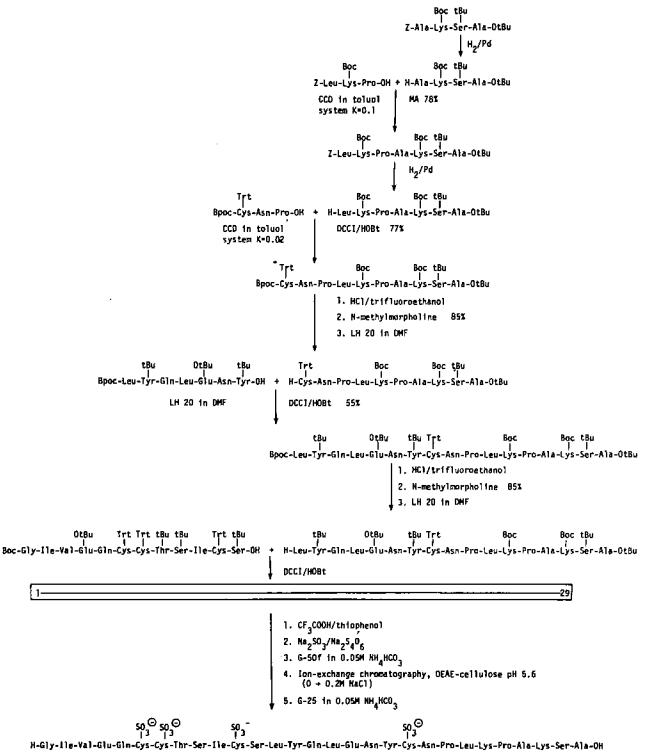

Fig. 4. Synthesis of S-sulfonated extended insulin A-chain.

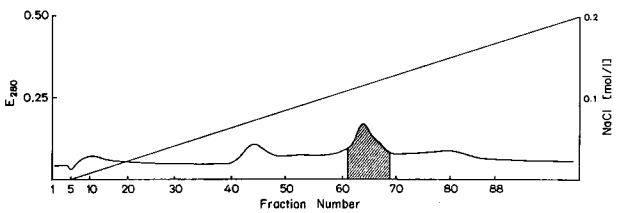

Fig. 5. DEAE-Cellulose chromatography of partially purified S-sulfonated [A^{1-29}]. Column (2 x 30 cm), 300 ml, 0.02N NaOAc/AcOH pH 5.6. Linear salt gradient of 0 → 0.2M NaCl.

disulfide. The des-Ala-di-S-Sulfonate B-chain was prepared in quantitative yield by the procedure described by Schmitt et al.[16] The des-Ala-disulfide B-chain was prepared in 70% yield by reduction with mercaptoethanol, oxidation with iodine, and gel filtration on Sephadex G-50 in 1N acetic acid. Following the procedure of Inouye et al.,[17] the des-Ala-disulfide B-chain was coupled with H-Thr-OMe in an enzyme-assisted coupling using trypsin at pH 6.5. Sephadex G-50 chromatog-

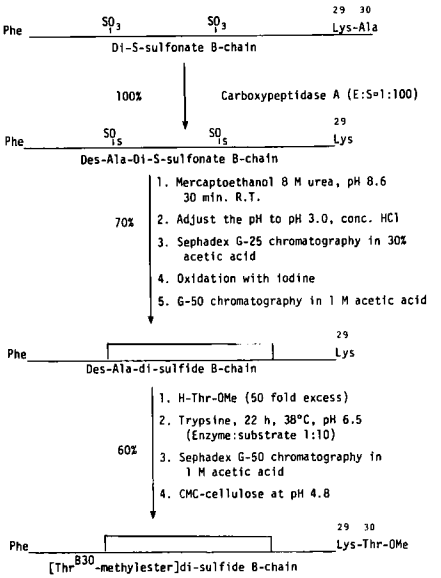

Fig. 6. Scheme for the semi-synthesis of [Thr^{B30}-methylester] di-sulfide B-chain.

raphy followed by ion-exchange chromatography on CM-cellulose yielded 60% of the [Thr^{B30}-methyl ester] B-chain disulfide. Tetra-S-sulfonate [A$^{1-29}$] A-chain was reduced with mercaptoethanol at pH 10.6. The mixture was applied to a Sephadex G-25 column of pH 10.6. The eluent was then mixed with [Thr^{B30}-methyl ester] disulfide B-chain in pH 10.6 buffer. The oxidation was complete within 48 h at 4°C. Simultaneously, the saponification of the methyl ester occurred. The crude insulin derivative was purified by gel-filtration on Sephadex G-50 in 1N acetic acid and ion-exchange chromatography on CM-cellulose at pH 4.5 using a linear salt gradient (Figure 7). The overall yield was 4%. The purity was checked by: a) amino acid analysis, Asp 3.01 (3); Thr 2.54 (3); Ser 3.76 (4); Glu 7.00 (7); Gly 3.82 (4); Ala 2.76 (3); Val 2.51 (4); Ile 0.86 (2); Leu 6.53 (6); Tyr 3.70 (4); Phe 2.83 (3); Lys 3.06 (3); His 2.00 (2); Arg 1.00 (1); b) cellulose acetate electrophoresis at pH 2.0 and 8.6; c) thin layer chromatography on cellulose sheets, and d) subtilisin digest followed by fingerprinting of the hydrolysate.

The synthesis of Threonine C-peptide of Insulin Growth Factor I (residues 29-41) — Figure 8 summarizes the route for the preparation of the protected peptide derivates Bpoc-Thr(tBu)-Gly-Tyr(tBu)-Gly-Ser(tBu)-Ser(tBu)-Ser(tBu)-Arg(HCl)-Arg(HCl)-Ala-Pro-Gln-Thr(tBu)-OMe. The same strategies and tactics were applied for this synthesis

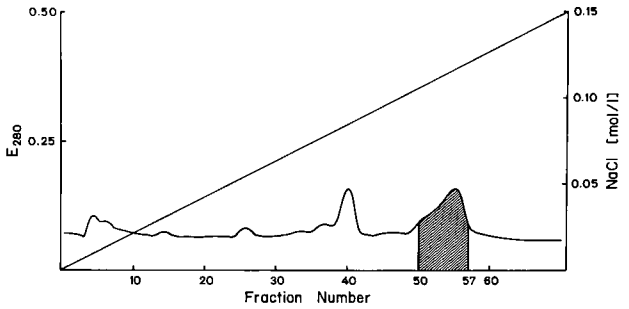

Fig. 7. CM-Cellulose chromatography of partially purified recombination mixture (column 1.5 x 16 cm), 150 ml 0.1N acetic acid/7M urea/pH 4.5, linear salt gradient of 0.15M NaCl, 5ml/fraction.

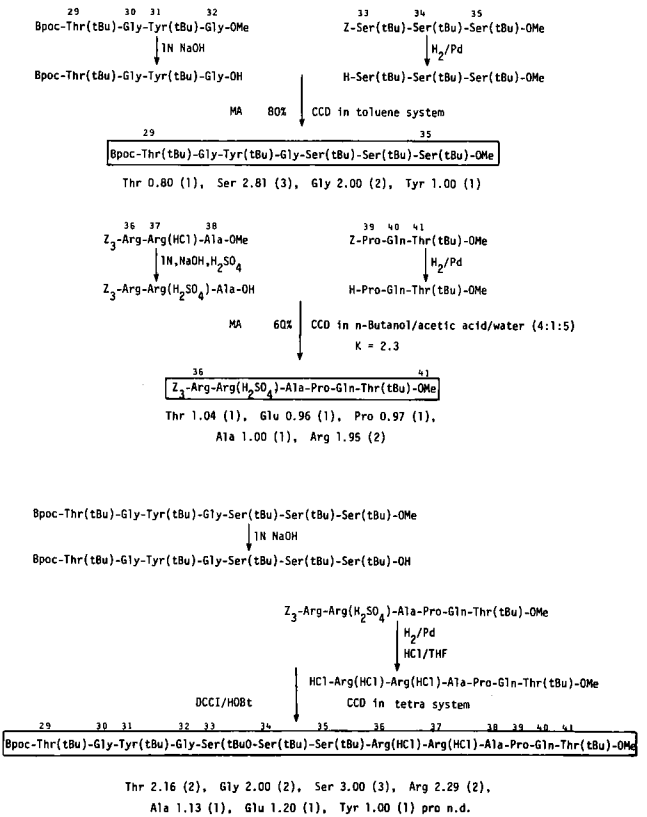

Fig. 8. Synthesis of the protected threonine C-peptide of IGF-1.

as for the previous one. The deprotection was achieved with trifluoroacetic acid with mercaptoethanol as scavenger. Purification was accomplished by gel chromatography on Sephadex G-25 in 10% acetic acid and ion-exchange chromatography on CM-cellulose in 0.01N acetic acid using an acetic acid gradient (Figure 9). The purity was checked by amino acid analysis: Thr 1.80 (2); Ser 2.71 (3); Glu 1.03 (1); Gly 1.95 (2); Ala 1.00 (1); Tyr 1.00 (1) and paper electrophoresis at pH 2.2.

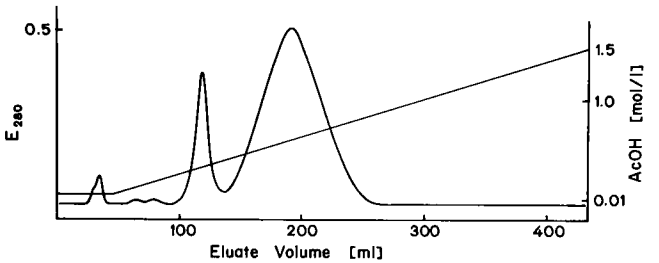

Fig. 9. CMC-Chromatography of preparation of residues (29-41) of IGF-1. Column (1 x 10 cm), 200 ml of 0.01N acetic acid, linear gradient of 0.01N → 1.5N acetic acid.

The synthesis of human insulin A-chain disulfide extended on the N-terminus with residues 29-41 of IGF-I — Figure 10 illustrates the scheme for this preparation. The protected peptide segment Bpoc-Thr(tBu)-Gly-Tyr(tBu)-Gly-Ser(tBu)-Ser(tBu)-Ser(tBu)-Arg(H_2SO_4)-Arg(H_2SO_4)-Ala-Pro-Gln-Thr(tBu)-OH was coupled by the mixed anhydride method[13] to the A-chain disulfide. Deprotection with trifluoroacetic acid, gel filtration on Sephadex G-50 in 1N acetic acid and ion-exchange chromatography on SP-Sephadex C-25 at pH 3.0 using a salt gradient yielded the pure product (Figure 11). The purity was checked by amino acid analysis and paper electrophoresis at pH 4.8 and 2.2.

Biological Data — Glucose oxidation was studied by measuring the conversion of [U-^{14}C] glucose to $^{14}CO_2$ as described by Rodbell[8] (Figure 12) growth promotion was determined by measuring [^{3}H]-thymidine incorporation into DNA of human fibroblasts as described by Rechler *et al.*[9] (Figure 13). Insulin radioimmunoassay was performed by the double antibody method as reported by Havrankova *et al.*[10] A dose-response curve of pork insulin was performed with each experiment, (variation < 10%). Table I summarizes the data for the preparations discussed in this communication.

Discussion

Utilizing the solution methods of peptide chemistry (segment condensation; acid labile protecting groups) and enzyme-assisted

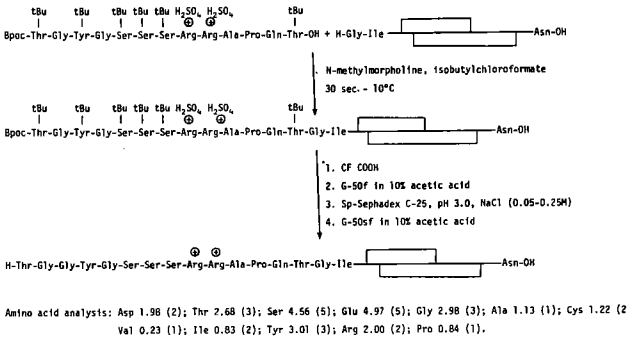

Fig. 10. Synthesis of human insulin A-chain disulfide extended at the N-terminus with residues 29-41 of IGF-I.

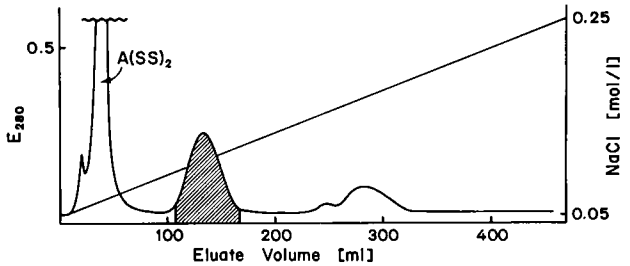

Fig. 11. SP-Sephadex C-25 chromatograph of the [C-A(SS)$_2$] preparation. Column (1 x 10 cm), 200 ml of 1.5N acetic acid/7M Urea/pH 3.0, linear salt gradient of 0.05M → 0.25M NaCl.

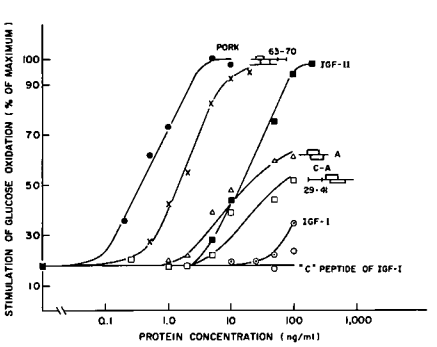

Fig. 12. Dose response curves on the stimulation of glucose oxidation in rat adipocytes of pork insulin (•), human insulin extended at the C-terminus at the A-chain with residues 63-70 of IGF-I (X), IGF-II (■), human A-chain disulfide (△), human A-chain extended at the N-terminus with residues 29-41 of IGF-I (□), IGF-I (⊙), and threonine C-peptide of IGF-I (0).

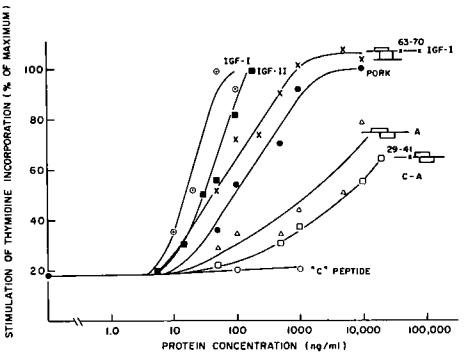

Fig. 13. Dose response curves on the stimulation of thymidine incorporations into DNA of human fibroblasts of pork insulin (•), human insulin extended at the C-terminus of the A-chain with residues 63-70 of IGF-I (X), IGF-II (■), human A-chain disulfide (△), human A-chain extended at the N-terminus with residues 29-41 of IGF-I (□), IGF-I (⊙), and threonine C-peptide of IGF-I (0).

Table I. Summary of the Biological Data of the Preparations Presented in this Communication

Preparation	Pork Radio-Immunoassay	Glucose Oxidation Rat Fat Cell	Thymidine Incorporation Human Fibroblasts
1. Human insulin extended at the C-terminus of the A-chain with the residues (63-70) of IGF-I	6%	20%	200%
2. Thronine C-peptide of IGF-I residues (29-41)	0	0	0
3. Human insulin A-chain disulfide extended at the N-terminus with the residues (29-41) of IGF-I	0.3%	0.3%	1%
4. IGF-I	0.2%	0.2%	1000%
5. IGF-II	0.3%	1%	500%
6. Human insulin A-chain disulfide	0.7%	1%	2-3%

synthesis (carboxypeptidase A, trypsin), we succeeded in synthesizing "hybrids" of human insulin and human IFG-I.

We have attempted to determine the structural differences between insulin and IGF-I that are responsible for the increase in potency for growth. The bioassay data of our first hybrid, human insulin elongated on the C-terminus with the extension peptide of IGF-I (residues 63-70), suggest that the addition of the extension peptide alone will double the insulin-type growth effect while decreasing its metabolic effect five-fold. This hybrid is behaving more like a poor analog of IGF-I. The threonine C-peptide of IGF-I (residues 29-41) is inactive in all systems studied.

Furthermore, we found that isolated human insulin A-chain disulfide did retain some immunological and biological activities for both metabolic and growth effects. These results may be due either to a small contamination of the preparation with native insulin or to retention of sufficient conformation of the refolded A-chain disulfide for recognition by antibody to pork insulin. The human insulin A-chain disulfide when extended at the N-terminus with residues 29-41 of IGF-I shows similar biological and immunological activities as the starting A-chain disulfide itself. Synthesis is in progress of another hybrid of human insulin and human IGF-I containing both the connecting peptide (residues 30-41) and the extension peptide (residues 63-70) of IGF-I in the order: insulin B-chain — IGF C-peptide — insulin A-chain — IGF extension peptide. This hybrid might exhibit growth promoting effects similar to human IGF-I.

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AQUEOUS CONDENSATION OF NATURAL AND SYNTHETIC MYOGLOBIN FRAGMENTS

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Synthetic procedures for examining the chemistry of large proteins are limited by the quantity and diversity of functional groups within proteins as well as by the need to maintain conditions which do not irreversibly denature the protein fragments. Factors which contribute to the difficulty of synthesizing proteins by a fragment condensation strategy include the differing solubility characteristics of small, protected peptides and large, minimally protected protein fragments; the task of obtaining amino component derivatives with sufficient activity to effectively couple to large fragments at the reduced concentrations encountered with high molecular weight species; and, in a scheme requiring multiple coupling steps, the need for intermediate purifications which are often ineffective in separating products differing in molecular weight by a few percent or in which the sequence or protective group strategy precludes ion exchange chromatography.

Several novel approaches to these difficulties have been taken in the development of a chemical procedure for selectively removing the N-terminal 14 residues of sperm whale myoglobin and rebuilding the native sequence by combination of the myoglobin fragment 15-153 with specific amino acid and peptide derivatives. Methoxide-catalyzed transesterification with methanol was found to be a mild and efficient method for the removal of selectively protected peptides corresponding to myoglobin residues 1-5 and 6-13 from the Merrifield resin after solid phase synthesis. A subsequent saponification reaction yielded the free C-terminal peptides which were then activated with N-hydroxysuccinimide (HOSu) and dicyclohexylcarbodiimide (DCC). A mixed aqueous solvent system utilizing N,N,N′,N′-tetra*kis*-(2-hydroxypropyl)-ethylenediamine-TFA buffer in n-propanol-H_2O (3:4 v/v) pH 9.0 (Quadrol) to adjust and maintain the condensation reaction pH at 7.2 solubilized both the active ester and protein fragment species throughout the coupling steps. Carbamylation of non-coupled products following each fragment condensation removed them from further reaction and eliminated the need for intermediate purifications. All non-native products can be removed at the end of the synthesis by the strict requirement of the heme moiety for the native structure and by ion exchange chromatography following reconstitution of the holoprotein.

Preparation of Fragment 15-153

The major component myoglobin was isolated from muscle tissue of the sperm whale[1] and the primary amino groups of the purified protein were protected by acetimidation to form the N^{α}, N^{ϵ}_{19}-acetimidyl-myoglobin.[2] Upon removal of the heme,[3] the apoprotein was dissolved to a 3% concentration in 50% acetic acid, 6 M in urea, and 180-fold molar excess of phenol was added. The solution was chilled to 4° and 2-(2-nitrophenylsulfenyl)-3-bromo-3′-methyl indolenine (BNPS-skatole) was added in a 10-fold molar excess for each tryptophan. After reaction for 48 hours at 4°, the reaction was stopped by dialysis against water. Reagent not removed by precipitation and centrifugation was eliminated by elution of the cleavage products on a G-25 column equilibrated with 8 M urea/0.1 M acetic acid. The methionine sulfoxide produced during the cleavage reaction was converted to methionine by incubation of the protein with 0.725 M β-mercaptoethanol for 62 hours at 37°.[4] Isolation of the N^{ϵ}_{19}-acetimidyl-15-153 from uncleaved N^{α}, N^{ϵ}_{19}-acetimidyl-1-153 was not necessary at this stage, as the blocked N-terminus of the latter derivative prevents participation in the fragment condensation steps.

Synthesis of Peptides

The peptides corresponding to residues 1-5 (Val-Leu-Ser-Glu-Gly) and 6-13 (Glu-Trp-Gln-Leu-Val-Leu-His-Val) of the sperm whale myoglobin sequence were synthesized by standard solid phase techniques, using DCC and 1-hydroxybenzotriazole (HOBt) as the coupling agents. All amino acids in peptide 6-13 were incorporated in the N^{α}-*t*-butyloxycarbonyl (Boc) form, and were deprotected by treatment with 4.0 N HCl in dioxane, with one exception. The peptide 7-13 with N-terminal Boc-Trp, was deprotected with 0.5 N HCl in formic acid. The formylated indole nucleus which resulted was deprotected in a later step, as noted below. Residue 12 was added as the N^{im}-dinitrophenyl derivative, which was deprotected upon completion of peptide 6-13 by treatment of the peptide (still attached to the solid phase resin) with a 1000-fold molar excess of thiophenol in dimethylformamide.[6]

Peptide 1-5 was synthesized by deprotection of Boc-glycyl-resin with HCl/dioxane and successive couplings via DCC/HOBt of the N^{α}-fluorenylmethoxycarbonyl (Fmoc) derivatives of γ-*t*-butyl-glutamic acid, β-*t*-butyl-serine, and leucine. The Fmoc group was removed with 50% piperidine in methylene chloride and the peptide completed by addition of Boc-Val.

To retain the N^{α}-Boc and β, γ-*t*-butyl protecting groups, the peptides were removed from the solid phase resin by transesterification for 24 hours at 25° in methanol, catalyzed by 0.5 equivalents methoxide ion.[7] The resulting methyl ester peptides were saponified for 24 hours at 4° to yield a free α-carboxyl group suitable for activation with N-hydroxysuccinimide (HOSu). The basic conditions also served to deformylate the indole nucleus of residue 7, tryptophan.

Purification of peptide 6-13 was accomplished by elution on an LH-20 column (100 x 2.5 cm) equilibrated with DMF. The first band eluting from the column was collected and used in the myoglobin reconstruction.

Fragment Condensation

Residue 14, tryptophan, was added to the protein fragment 15-153 as the single amino acid to avoid repetitive treatments with acid which its inclusion at the C-terminus of a solid phase synthesis of peptide 6-14 would entail. Instead, it was coupled in aqueous solution to fragment 15-153 as the N^{α}-nitrophenylsulfenyl (NPS)-tryptophan-O-succinimide active ester. The NPS group was removed from fragment 14-153 by treatment for 24 hours with 0.1 M β-mercaptoethanol in water, pH 7.0. Following coupling of residue 14, which occurred in 97% yield, and prior to N^{α} deprotection, the non-coupled fragment 15-153 was terminated from further reaction by carbamylation of the N^{α}-amino group by KCNO (180-fold molar excess) in 0.1 μ phosphate buffer pH 6.5.

The HOSu active esters of peptides 6-13 and 1-5 were coupled successively to the major protein fragment in 50% and 63% yield, respectively, and the N^{α}-Boc group removed by treatment for one hour with anhydrous trifluoroacetic acid (TFA), using anisole and dithiothreitol as scavengers. Non-coupled material was carbamylated after coupling of peptide 6-13 and prior to TFA deprotection.

The solubility of the major apoprotein fragment, which normally aggregates in the mid-pH range, was enhanced by use of the automated protein sequencer solvent "Quadrol" (N,N,N′,N′-*tetrakis*-(2-hydroxypropyl)-ethylenediamine-TFA buffer in n-propanol-H_2O (3:4 v/v) pH 9.0) to adjust and maintain the condensation reaction pH at 7.2. The peptides were dissolved in the minimal amount of methanol just prior to mixing with the protein solution. Peptide couplings proceeded 48 hours at room temperature and were ended by dialysis against water. Excess peptide non-covalently associated with the protein was removed by elution of the coupling products on a G-25 column equilibrated with 8 M urea/0.1 M acetic acid.

Conclusions

1.) Reconstruction of the sperm whale myoglobin sequence has been accomplished by a series of aqueous condensations of suitably protected myoglobin fragments. 2.) Methoxide-catalyzed transesterification with methanol produced selectively protected peptides with a free C-terminus which were suitable for activation with HOSu/DCC and for use in a fragment condensation synthesis. 3.) The solubility of the myoglobin fragments was enhanced by the use of Quadrol to adjust and maintain the condensation reaction pH at 7.2. 4.) Carbamylation of non-coupled products eliminated the need for intermediate purifications. 5.) The development of this procedure extends the fragment condensation methodology to include large, minimally protected protein fragments soluble only in aqueous systems.

Acknowledgements

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SEMISYNTHESIS OF PHOSPHOLIPASE A_2 ANALOGS

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Introduction

Phospholipase A_2 (PLA; EC 3.1.1.4) specifically catalyzes the hydrolysis of the 2-acyl ester linkage in 3-*sn*-phosphoglycerides. The pancreatic enzyme occurs as a zymogen which is converted into the active enzyme by splitting the N-terminal heptapeptide (Figure 1) upon limited proteolysis. Both proteins have low, though comparable activities towards monomeric substrate indicating the presence of a functionally active site in the zymogen as well.[1] When substrate is present as certain organized lipid-water interfaces like micelles there is a large increase in enzymatic activity for PLA but not for its zymogen. Direct binding studies with micellar substrate analogs demonstrated that PLA, but not the zymogen, binds to these lipid-water interfaces. Therefore, the pancreatic PLA's were proposed to possess a specific binding site for lipid-water interfaces called the interface recognition site (IRS). A conformational change occurs upon binding giving rise to an optimization of the active site.[1] The N-terminal region of pancreatic PLA forms an essential part of the IRS.[2] Comparison of the amino acid sequences of about 30 different PLA's shows 29 amino acid residues to be absolutely invariant, including Gln 4 and Phe 5. In addition, a Met residue is found to be present almost invariably at position 8. Occasionally Leu 8 or Val 8 have been found instead. Although Gln 4, Phe 5, and Met 8 are not part of the active site their invariant character strongly suggests that these residues fulfill important structural functions enabling the enzyme to display its optimal activity. In view of the importance of these amino acid residues we have investigated various properties of semisynthetic Nle 4-, Tyr 5-, and Nle 8-bovine PLA analogs.

Results and Discussion

Complete amidination of all ϵ-NH_2 groups in bovine pro-PLA followed by limited proteolysis yields ϵ-amidinated PLA (=AMPA); about 75% of the original catalytic activity is retained, while other properties of native PLA and AMPA are almost identical.[3] CNBr fragmentation of the ϵ-amidinated zymogen at the unique Met 8[4] produces

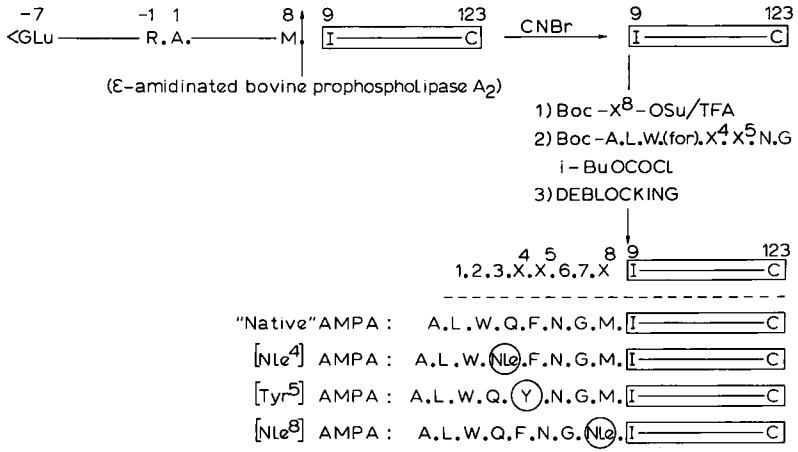

Fig. 1. Scheme depicting semisynthesis of bovine AMPA analogs.

des (Ala 1-Met 8) AMPA, a protein completely devoid of catalytic activity. Only one free NH_2 group is present *viz.* that of the N-terminal Ile 9 (Figure 1). Subsequently Boc-Met or Boc-Nle were restitched to des (Ala 1-Met 8) AMPA as their corresponding *N*-hydroxysuccinimide esters. Removal of the Boc group with TFA yields des (Ala 1-Gly 7) AMPA's (Figure 1). The various N-terminal peptide analogs (Boc-Ala 1. . .Gly 7), prepared by solid phase peptide synthesis, were then covalently linked to the des (Ala 1-Gly 7) AMPA's using the mixed carbonic anhydride method. After the usual work-up the crude Boc-AMPA analogs were deformylated and purified by ion-exchange chromatography before and after removal of the Boc group with TFA. Pure semisynthetic Nle 4-, Tyr 5-, and Nle 8-AMPA's were obtained in about 30% yield. In Figure 2 properties of semisynthetic bovine AMPA (=Asn 6-AMPA) and "native" bovine AMPA are summarized, demonstrating the feasibility of the applied procedures.

Substitution of Phe 5 by Tyr leads to the almost complete loss of enzymatic activity not only on micellar but also on monomeric substrate. However, Tyr 5-AMPA still binds micelles of the substrate analog *cis-n*-octadecenylphosphocholine with a similar affinity as "native" AMPA, indicating that the IRS is not affected by the Phe 5→Tyr substitution (Figure 2). This finding is supported by photo-CIDNP ^1H NMR spectroscopy. This method, monitoring exposed aromatic amino acid residues, reveals identical signals of the IRS residues Trp 3 and Tyr 69 for Tyr 5-AMPA as for "native" AMPA (Figure 3). Therefore, the most likely explanation for the absence of catalytic activity of Tyr 5-AMPA is

	"NATIVE" AMPA	SEMISYNTHETIC BOVINE AMPA ANALOGS [Asn6]	[Tyr5]	[Nle4]	[Nle8]
SPEC. ACT. (μequiv.min^{-1}mg protein^{-1}) (egg-yolk assay)	50	46	NO DETECTABLE ACTIVITY		56
V_{max} (μequiv.min^{-1}mg protein^{-1}) (micellar L-di-C_8 P.C.)	2300	2500	60	70	2500
K_{cat}/K_m (sec^{-1} M^{-1}) (monomeric di-thiohexanoyl PC)	172	179	5	45	228
$K_{Ca^{2+}}$ (mM)	4.6	N.D.	5.2	10	2.7
K_{MIC} (mM) (binding micellar Cis-9-octadecenylphosphocholine)	5.0	5.4	5.0	NO DETECTABLE BINDING	3.1

Fig. 2. Comparison of various properties of Nle 4- Tyr 5- and Nle 8-bovine AMPA's with "native" and semisynthetic (=Asn 6-AMPA) bovine AMPA's. (N.D. means not determined).

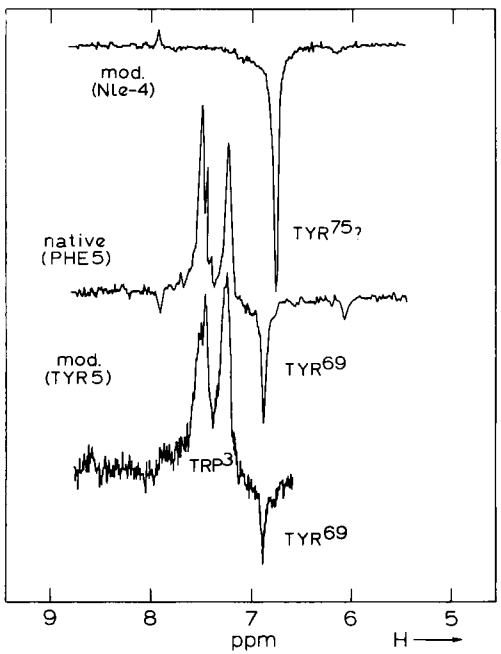

Fig. 3. 360 MHz ^{1}H NMR Photo-CIDNP difference spectra of Nle 4-AMPA (upper), "native" AMPA (middle) and Tyr 5-AMPA (bottom).

a distortion of the active site due to the aromatic group. From the 1.7 Å X-ray structure of bovine PLA it has been found that Phe 5 is part of the hydrophobic wall of the active site and it has been proposed that it partly shields the active site couple His 48-Asp 99.[5] It is therefore conceivable that the presence of the more polar Tyr residue instead of Phe considerably decreases this shielding effect resulting in the loss of catalytic properties. However, it can not be excluded that a steric constraint of the active site due to the larger Tyr residue is responsible for the loss of activity.

Nle 4-AMPA possesses about 26% of the catalytic activity of "native" AMPA using monomeric substrate. This AMPA analog, however, has lost its ability to bind to micelles of the substrate analog *cis-n*-octadecenylphosphocholine and consequently has no enzymatic activity toward micellar substrate (Figure 2). The loss of the IRS in Nle 4-AMPA becomes evident from the photo-CIDNP difference spectrum

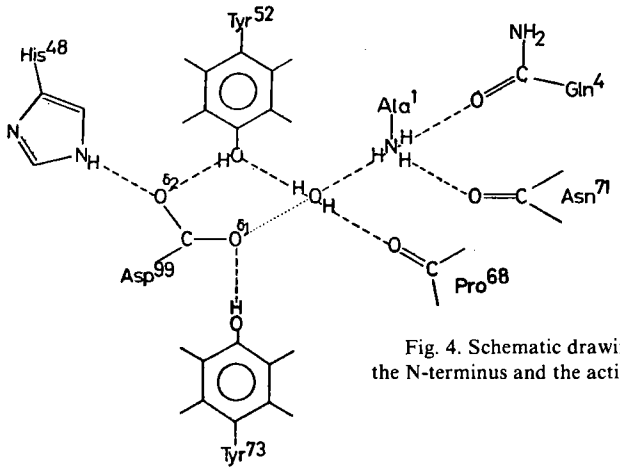

Fig. 4. Schematic drawing of the interactions between the N-terminus and the active site.[5]

in which signals originating from Trp 3 were no longer present. Moreover, a new strong Tyr signal, tentatively assigned to Tyr 75, appears in addition to that of Tyr 69 (Figure 3). These findings strongly suggest that substitution of Gln 4 by Nle results in a complete loss of a functional IRS while the active site structure is damaged only to a limited extent. From the 1.7 Å X-ray structure of bovine PLA it has been found that the N-terminus forms an α-helical structure. An extended system of H-bridges was proposed between Ala 1 and Gln 4 and between Ala 1 and the active site (Asp 99) *via* a H_2O molecule as shown in Figure 4. Previously it had already been demonstrated that binding to lipid-water interfaces requires a free α-NH_3^+ group of Ala 1.[6] Apparently the inability of Nle 4-AMPA to form a H-bond between Nle 4 and Ala 1 prevents the formation of a functional IRS.

As shown in Figure 2 substitution of Met 8, which is located in the interior of the protein near Phe 5 and Tyr 73, by Nle has no influence on the properties of the enzyme, in agreement with the fact that in some PLA's this residue is a Leu or Val.

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ENZYMATIC PRODUCTION OF PHOTOSENSITIVE PEPTIDE HORMONE DERIVATIVES

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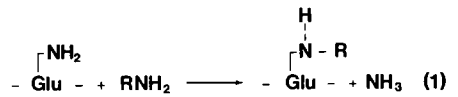
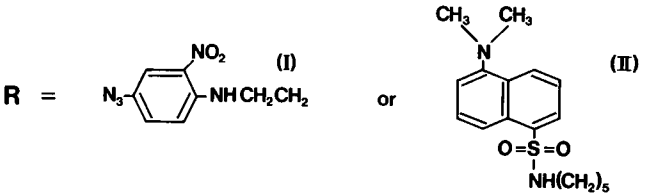
Introduction

Peptide and protein derivatives bearing aromatic azides can be used to reveal various aspects of hormone receptor interaction.[1,2] In general, chemical modification of the ϵ-amino group of lysine, the α-amino or carboxyl groups has been employed for production of photosensitive peptide derivatives. The present report describes a procedure for enzymatic addition of the 4-azido-2-nitrophenyl group to the γ-carboxamide of glutamine residues using guinea pig liver transglutaminase. This enzyme catalyses the aminolysis of peptide bound glutamine residues[3] (Reaction 1).



(1)

When a peptide hormone contains an exposed glutamine residue photoactive or fluorescent derivatives may be produced by utilizing compounds in which



Results and Conclusions

Compound I was efficiently incorporated into carbobenzoxyglutaminyl-glycine by the action of guinea pig liver transglutaminase.[4] Furthermore the photosensitive nature of the chromophore was unaltered during incorporation and isolation by HPLC. Thus I was used to

produce photosensitive derivatives of substance P and glucagon 1-6. The position of labeling of substance P was determined by isolation of fragments produced by chymotrypsin C and is indicated in Figure 1.

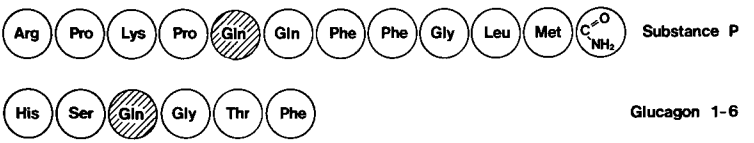


Fig. 1. Labeled residues are indicated by shading.

Both human and salmon calcitonins contain two glutamine residues and act as substrates for guinea pig liver transglutaminase as evidenced by HPLC of aliquots of incubation mixtures containing I or II (Figure 2). After purification by HPLC the products formed with II were used to identify sites of incorporation into the calcitonins. Thin layer chromatography of tryptic digests of the purified derivatives (Figure 3) showed glutamine 24 and glutamine 14 to be the reactive residues in human and salmon calcitonins, respectively (Figure 4).

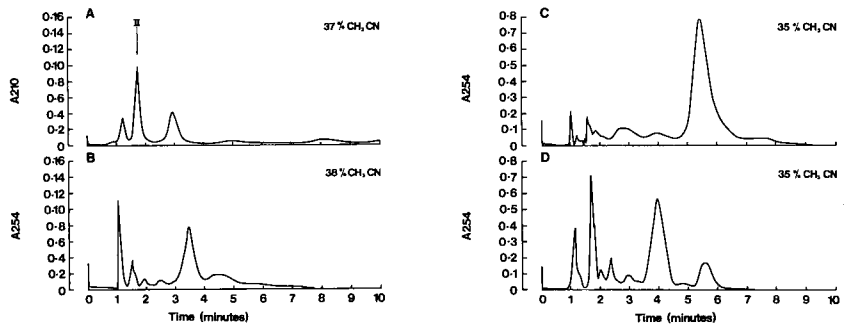


Fig. 2. High pressure liquid chromatographic separation of products formed during incubation of transglutaminase with: A, human calcitonin and II; B, human calcitonin and I; C, salmon calcitonin and II; and D, salmon calcitonin and I. With the exception of A the amine substrate was removed by gel filtration prior to HPLC which was on an analytical μBondapak C_{18} column (Waters) at 2.5 ml/min using isocratic mixtures of acetonitrile in 0.1M sodium phosphate buffer (pH 2.1). The elution position of II was determined in a separate chromatogram, the unmodified calcitonins were not retained under these conditions.

The purified derivative formed from I and salmon calcitonin retained the ability to bind to responsive target cells and to stimulate adenyl cyclase in these cells (Figure 5) as did derivatives of II and both calcitonins. It is anticipated that purified derivatives of I will permit identification of receptors for calcitonin in responsive cells.

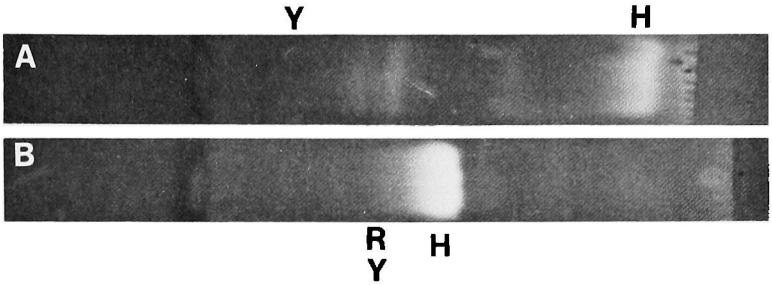


Fig. 3. Thin layer chromatograms of tryptic digests of derivatives formed between II and human (A) and salmon (B) calcitonins. Specific stains were used to identify peptides containing arginine, (R), histidine (H) and tyrosine (Y).

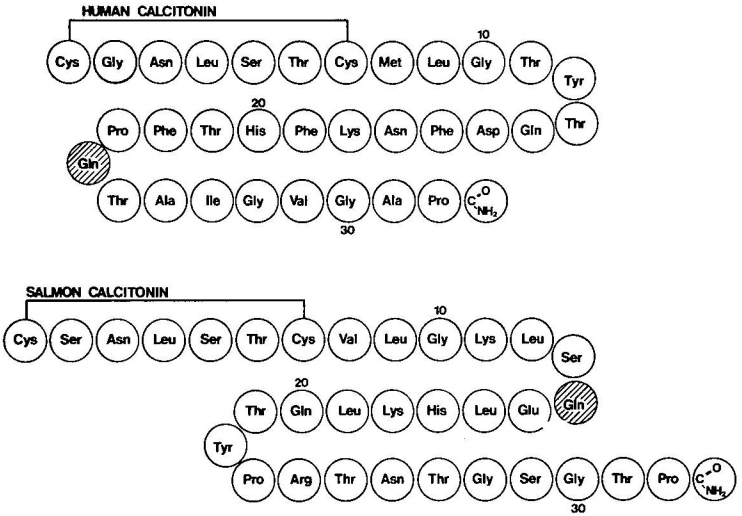


Fig. 4. Labeled residues indicated by shading.

Acknowledgements

This work was supported by the National Health and Medical Research Council of Australia. A portion of this work was carried out by J.J. Gorman in the Department of Medicine, University of Sydney, Westmead Centre, N.S.W.. Supply of human calcitonin by Dr. W. Rittel, Ciba-Geigy, Basel, and salmon calcitonin by Dr. R. Orlowski, Armour Pharmaceutical Co. Kankakee, Ill. is gratefully acknowledged.

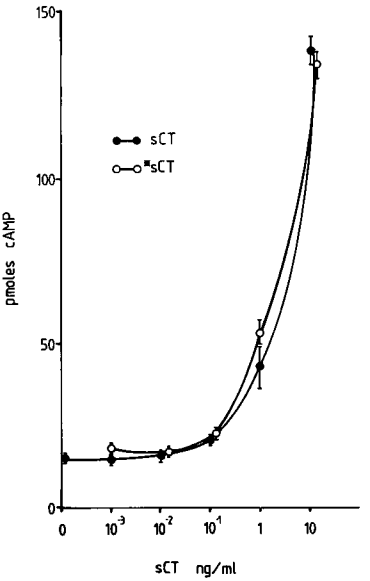


Fig. 5. Biological activity of modified (*SCT) compared with unmodified salmon calcitonin (SCT) as assayed by their ability to stimulate adenyl cyclase in T47D breast tumor cells.[5] Cyclase activity is expressed as pmoles of ^{32}p cAMP liberated from α-^{32}P ATP during a 15 minute incubation with homogenized cells.

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THE INTRODUCTION OF SULFHYDRYL GROUPS INTO GLUCAGON AND THEIR USE IN THE SEMISYNTHESIS OF NEW GLUCAGON DERIVATIVES

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Introduction

The semisynthesis of new peptide or protein derivatives can take several approaches. The approach taken here is to introduce new functional groups into the peptide and use these new functional groups for further synthesis work. Glucagon is a polypeptide hormone of 29 amino acids which contains no cysteine or cystine residues. Thus, a sulfhydryl group is an atractive new functional group to incorporate into the molecule. Sulfhydryl groups can be placed on the lysine (residue 12) and N-terminal histidine residue by reacting the native hormone with methyl-4-mercaptobuyrimidate.[1] Modification is at the alpha and epsilon or epsilon amino groups depending on the amount of reagent used. Conversion of tryptophan (residue 25) in glucagon to 2-thioltryptophan is another way to incorporate a sulfhydryl group into the hormone.[2] Thus sulfhydryl groups can be placed in both the C-terminal and N-terminal regions of the hormone and high biological potency is retained. The 2-thioltryptophan-glucagon derivative has been used in the preparation of a fluorescent analog of glucagon, S-(N-dansylaminoethyl)-2-thioltryptophan-glucagon (abbreviated dansyl-S-glucagon), and a glucagon affinity column.

Methods

Dansyl-S-glucagon was prepared by adding 1 ml of 1% solution of N-dansylaziridine (in ethanol) to a solution of 2-thioltryptophan-glucagon (10 mg) in 1 ml 4M urea containing 20 mg ammonium bicarbonate, pH 9. The mixture stood overnight at room temperature after which time it was gel filtered on Sephadex G-15 using 20% acetic acid as the eluent. Further purification was by partition chromatography using the solvent system 1-butanol/ethanol/benzene/0.2M ammonium hydroxide (5:2:1:8), pH 9.4[3]. Using this solvent system dansyl-S-glucagon had an R_f =0.85.

The glucagon affinity column was prepared by adding 2-thioltryptophan-glucagon (18 mg, 5 μmole) to Thiopropyl Sepharose 6B

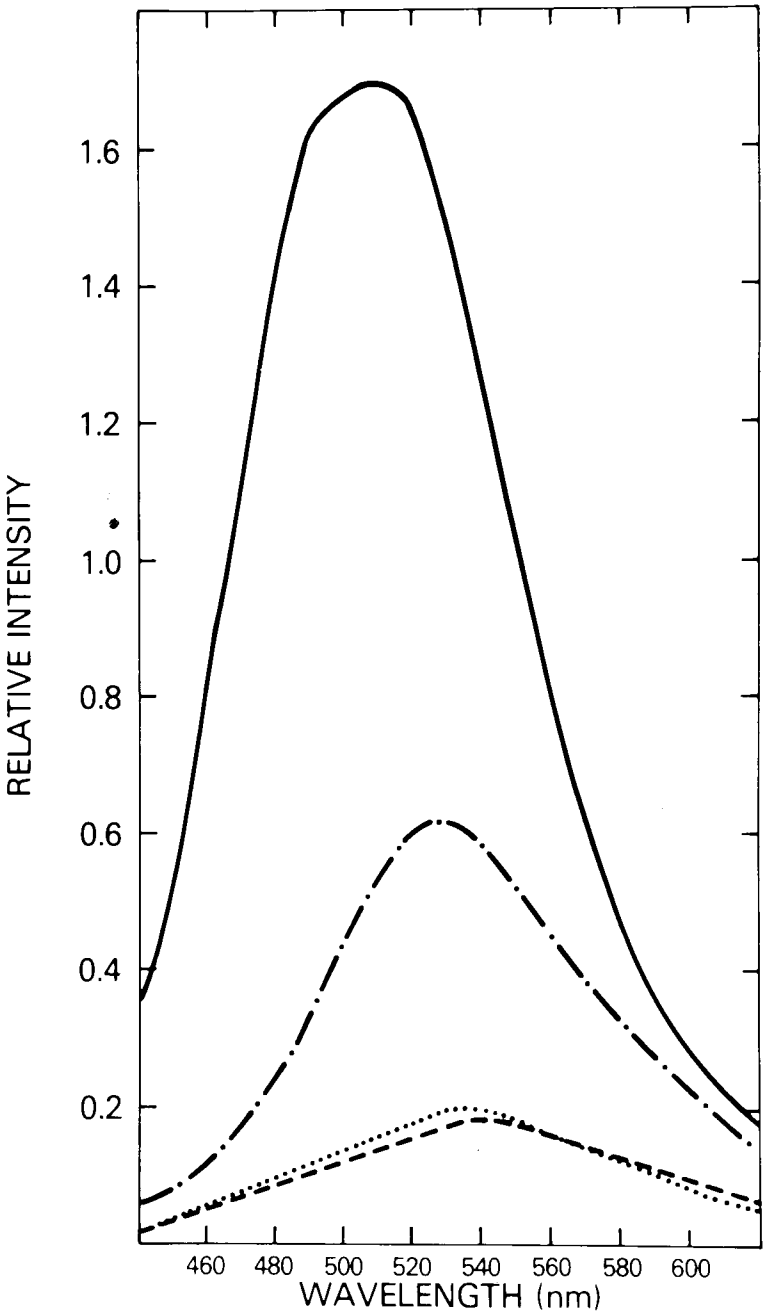

Fig. 1. Fluorescence spectra of dansyl-S-glucagon (10^{-7}M) in 20 mM Tris-HCl, pH 7.2 (. . . .); 4M guanidine-HCl/ 20 mM Tris-HCl, pH 7.4 (-----); 1-propanol/20mM Tris-HCl, pH 7.2, (2:1), (- - -) and 1-butanol/20 mM Tris-HCl, (99:1), (—).

(12 mg, 1 μmole) which was in a 4M urea/0.1M Tris-HCl, pH 7.5 buffer. After 2 h the resin was filtered and thoroughly washed with 4M urea and 20% acetic acid. Amino acid analysis gave a substitution value of 0.05 μmole/mg resin.

Hepatic adenylate cyclase and binding assays were as described previously[2] and DTT was not present in either assay.

The fluorescent measurements were made on a Perkin-Elmer model MPF-3L spectrophotometer equipped with a thermostated cell holder (30°). Emission spectra were measured with 3 cm^2 cells with slit width of 10 nm. Dansyl-S-glucagon was dissolved initially in 70 μl 0.5M Na_2CO_3 and was diluted to 2.5 ml with 20 mM Tris-HCl, pH 7.5. The fluorescent spectra shown are uncorrected and those of the fluorescent compound less that obtained with solution alone.

Results

Dansyl-S-glucagon was found to have a dose-response curve identical to that of the native hormone and 2-thioltryptophan-glucagon when assayed for the activation of hepatic adenylate cyclase. The relative affinities of native glucagon and dansyl-S-glucagon for glucagon binding sites on hepatic plasma membranes were determined by their ability to compete with the binding of ^{125}I-glucagon. Dansyl-S-glucagon showed the same affinity for the glucagon receptor as the native hormone.

The fluorescence spectra of the dansyl chromophore is sensitive to its environment producing larger quantum yields and a blue shift as it is placed in a more hydrophobic environment. These effects are seen when dansyl-S-glucagon is in solvents of different polarity. In 4M guanidine-HCl there is a slight red shift and a reduction in fluorescence.

The glucagon affinity column was tested for its ability to activate hepatic adenylate cyclase and to compete with ^{125}I-glucagon for the glucagon receptor. At a concentration of 10^{-5}M for 2-thioltryptophan-glucagon attached to the Thiopropyl Sepharose 6B beads, 50% of the maximal stimulation given by the native hormone at maximally stimulating concentrations (10^{-6}M) was obtained. The Thiopropyl Sepharose 6B beads alone did not stimulate adenylate cyclase. In competition binding studies glucagon attached to the Sepharose 6B beads was able to compete with ^{125}I-glucagon 50% as well as the native hormone at the concentrations given above. The Thiopropyl Sepharose 6B beads alone did not compete with ^{125}I-glucagon for the glucagon binding sites.

Discussion

The introduction of sulfhydryl groups into glucagon provides the versatility to make many new glucagon derivatives. Two very interesting new glucagon derivatives have been made using this approach. Dansyl-S glucagon which is environmentally sensitive should be very useful for studying concentration dependent association effects. Attachment of the dansyl group to the indole ring gives an interesting dansyl-tryptophan fluorescent system. Model studies have been made with the dansyl group attached to the alpha amino group of tryptophan,[4] but not through the indole ring as presented here. The biologically active glucagon affinity column should be of use in a strategy involving the purification of the glucagon receptor.

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THE STATE OF THE ART IN FRAGMENT COUPLING ON SOLID SUPPORT

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In the past decade careful investigations on the chemistry of transformations on polymer supports have established a realistic insight of the limits of solid phase peptide synthesis.[1] More important than all mthodological requirements for repetitive reactions in a cross-linked matrix is the supreme imperative that all chemical operations go to completion. Deviations from this requirement cause microheterogeneities which increase in number the longer the sequence. In general we can state that decapeptides can be obtained pure within a few days either as the direct result of an efficient solid phase synthesis (completed reactions in all operations) or after speedy semipreparative HPLC purification of a less perfect crude product. However, the stepwise solid phase synthesis of polypeptides of about 20 residues in length may require enormous efforts to identify and separate the desired product. Nevertheless, great successes have been reported[2] as application of modern HPLC and biological separation techniques complement the maturing solid phase techniques, to provide the synthetic peptides needed in medical research. Often for medical applications, the problems of synthetic efficiencies and yields are of minor relevance.

A true combination of all of the aspects mentioned above has led to the development of strategies for the fragment coupling in gel-phases[3] aiming at pure preparations, efficient syntheses and high yields. With great accuracy peptide fragments can be synthesized in gel phase or by conventional methods in solution as well. For pure preparations the photometric and spectroscopic monitoring of the reactions is of significant help, *e.g.* based on the uv properties of the α,α-dimethyl-3,5-dimethoxybenzyloxycarbonyl-(Ddz-) group for the temporary amino terminal protection of the intermediates.[4] The utilization of peptides for fragment condensations requires full protection of all side functions except the C-terminus. The verification of this principle in gel phases of polyamide nature is presented by Dr. Atherton, whereas Dr. Ching-I Niu in the fragment couplings used side chain functions for attachment to the polymer phase. Anchor functions have been developed which allow the smooth release of fully protected peptides from the polymer support. At

the end of all operations this linkage to the carrier can be activated by a specific reaction so that the peptides can be released fully protected. Specificity has been incorporated into anchor bonds by photolabile residues[5] or through the use of phenacyl residues.[6]

We have extensively reinvestigated the versatility of bromophenacyl anchor sites for the gel phase synthesis of fully protected peptide fragments.[7] The simplicity of the incorporation of bromophenacyl bromide into polystyrene phases as well as the reactivity of bromophenacyl sites on polymers for the reattachment of protected peptides[8] is of striking importance and offers chemoselectivity in detachment reactions without any sophisticated modification of the anchor site. Hydroxylic saponifications, hydrazinolysis, transesterifications, photolysis and aminolytic cleavages are possible with a 70-100% efficient release of fully protected peptides. We are less satisfied with the thiolytic detachment, which resulted on the average in only 50-70% of liberated peptide. The protected peptides can be purified and characterized as in conventional peptide synthesis. Of particular value is ^{1}H-NMR spectroscopic analysis to detect all protecting groups in the molecule.

The coupling of these well characterized fragments in polymer phase minimizes drastically the formation of microheterogeneities even though the building blocks usually do not completely couple to the polymer supported fragment. Enlarged differences in the molecular weights between the target sequence and the unreacted polypeptide chain effectively facilitates final chromatographic purifications of the crude product released from the support. The greatest advantage of fragment couplings in polymer phase is that the unreacted soluble portion of the condensation reaction mixture can be removed by filtration. Large excesses of these soluble fragments can favor complete transformation of the polymer-supported fragment but these are extremely precious and can easily racemize. In this respect dicyclohexylcarbodiimide plus two equivalents of 1-hydroxybenzotriazole[9] have been utilized with good success for polymer phase fragment couplings to suppress the racemization during the activation. From those activations, however, excess fragments cannot be recovered or recycled. Nevertheless, this combination of reagents was used in several cases in the total synthesis of the MCD-peptide[7] and of the insulin A-chain for fragment couplings in gel phase. Whereas the synthetic MCD-peptide showed less than 50% biological activity, semisynthetic insulin built from the synthetic[10] A-chain and natural B-chain showed 100% activity in three assays.[11]

FRAGMENTS ACTIVATED BY CARBONYL-BIS-IMIDAZOLE 1-HYDROXYBENZOTRIAZOLE	$[\alpha]_D^{25}$ prior to activation	$[\alpha]_D^{25}$ recovered after activation	yield of recovered peptide
Ddz-Lys(Z)-Glu(OBu^t)-Val-Gla(OBu^t)OH	-30.88 (c 1.0, CH_3OH)	-30.71	85%
Ddz-Arg(Tos)-Gly-Val-PheOH*1)	+ 6.00 (c 1.0, CH_3OH)	+ 5.95	83%
Ddz-Arg(Tos)OH*2)	- 7.00 (c 0.9, DMF)	- 6.89	80%
Ddz-Phe-Val-Asn(Mbh)-Glu(Mbh)-His(Dnp)-Leu-Cys(Acm)-GlyOH	-22.3 (c 0.8, DMF)	-23.4	85%
Ddz-Ser(Bu^t)-His(Dnp)-Leu-Val-Glu(OBu^t)-AlaOH	-19.3 (c 0.7, CH_3OH)	-18.8	80%
Ddz-Leu-Tyr(Bu^t)-Leu-Val-Cys(Mbzl)-GlyOH	-49.1 (c 0.8, CH_3OH)	-49.2	81%
Ddz-Glu(OBu^t)-Arg(Tos)-GlyOH	- 6.2 (c 0.8, $CHCl_3$)	- 7.0	88%

* C-terminal racemization detected by enzyme test (±4%): 1) 5% D-Phe 2) 4.5% D-Arg

Fig. 1. Optical purity comparison of fragments recovered after CDI/HOBt activation.

Very recently, we reinvestigated the utility of carbonyl-*bis*-imidazole[12] for fragment couplings in polymer phase in combination of excess 1-hydroxybenzotriazole. Imidazolides of the fragments are formed, which allow the recovery of excessively applied fragments simply by aqueous hydrolysis after filtration from the polymer. The fragments recovered from activation in all investigated cases showed the same optical rotations as before activation and hydrolysis (Figure 1). In the synthesis of the insulin B-chain[13] we utilized this method exclusively for all fragment couplings in the gel phase. In one coupling the excess fragment was recycled three times to obtain a 50% increased coupling yield in polymer phase. For the insulin B-chain the average yield in four fragment condensations by carbonyl-*bis*-imidazole/1-hydroxybenzotriazole was 75% (Figure 2). By the aid of several model syntheses including the Anderson test peptide we measured less than 5% racemization with carbonyl diimidazole/1-hydroxybenzotriazole. To our understanding of the current state of the art this new coupling procedure by carbonyl-*bis*-imidazole/1-hydroxybenzotriazole for the activation and troublefree recycling of excess fragments overcomes one of the major limitations, which to date has inhibited the general application of fragment couplings in polymer phases for the synthesis of polypeptides.

Insulin B-chain Fragment couplings in gel phase	Y I E L D, by amino acid analysis and quantitative measurement of the Ddz-fission product	
	DCC/HOBt	CDI/HOBt
IV → V	90-100%	90-100%
III → IV-V	40- 55%	30- 35%
II → III-IV-V	30- 35%	75- 85%
I → II-III-IV-V	80-100%	90-100%

Fig. 2.Comparison of fragment condensation yields resulting from DCC/HOBt and CDI/-HOBt activation in the synthesis of human insulin B-chain.

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SEGMENT COUPLING ON SOLID SUPPORTS WITH ATTACHMENTS AT SIDE CHAIN FUNCTIONS

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Introduction

Studies on the coupling of peptide segments to a solid support were initiated in our laboratory with the successful synthesis of crystalline glucagon.[1] In this approach we value highly the strategy employed since it may be considered a combination of solid phase synthesis and solution methods in a way such as to retain the merits and avoid the disadvantages inherent to both techniques. Besides, the approach features a favorable combination of Bpoc protection at the α-amino group with tertiary butyl based protection of side chains and the ordinary benzyl ester type linkage to the resin support. The milder deblocking conditions for the α-amino Bpoc group make it possible to obtain the final product rather cleanly and in readily crystallizable form. The coupling efficiency in each step is above 95% as judged by the residual amino group content after each coupling of a segment. The total amino group content of each intermediate after thorough deblocking is close to theory. However, the overall yield is not as good as one might expect considering the excellent yield of each coupling step. This is, however, not the case when we synthesize smaller peptide hormones, such as oxytocin or analogs of LHRH — both ending in amide — by the stepwise solid phase method with final deblock-ing either by ammonolysis or ethylaminolysis.[2] The difficulty here seems to rest with the HF treatment for detachment from the resin in the final stage of the synthesis. In order to overcome this problem, we have in recent years used ammonolysis as the final detachment step from the solid support even for peptides not terminating in the amide function. Attachment via ester linkage at side chain functions in solid phase synthesis of peptides with asparagine[3] or glutamine[4] would serve this purpose well. This approach has been applied to the synthesis of the C-peptide of human proinsulin,[5,6] the synthesis of peptides with asparagine, including the C-terminal peptide of the insulin A-chain, and serum thymic factor; it has been extended to the synthesis of serine and threonine containing peptides by way of linking the side chain hydroxyl group to the resin and also employing ammonolysis which could also be to detach the peptide. A survey of these studies will be given in this paper.

Attachment to Resins

Chloromethylated, aminomethylated, or bromopropionylamidomethylated 1.5% crosslinked polystyrene was used as resin support. A series of peptide segments shown in Table I was prepared in which the β-carboxyl of aspartic acid, the γ-carboxyl of glutamic acid and the hydroxyl groups of serine and threonine are points of anchorage to the support. The resin bound peptide segments belonging to the C-terminal region of target peptides were prepared as shown in Table II. Thus, for the C-terminal dipeptide of the C-peptide of human proinsulin, Leu-Gln, Bpoc-Leu-Glu-O*t*Bu was prepared, in which the γ-carboxyl is free and made to react with chloromethylated resin to form the benzyl type ester via the thioether method of Dorman and Love;[7] for the C-terminal dipeptide of serum thymic factor, Ser-Asn, a similar derivative, Bpoc-Ser (*t*Bu)-Asp-OtBu was prepared and subjected to coupling through its β-carboxyl group to form the resin bound benzyl type ester in the same manner. The amino group content of the deprotected dipeptide derivative was adjusted to about 0.5 mmole/g by varying the amount of dipeptide derivative used for coupling and constituted the basis for the calculated content of the total amino group of each intermediate. In the case of coupling Bpoc-Cys (*t*Bu)-Asp-O*t*Bu to a solid support we chose as alternative the cesium salt method, using a resin with a longer arm, namely bromopropionylamidomethylated resin. The design for attachment of the side chain functions of serine and threonine to a solid support, kindly suggested by Dr. S.S. Wang of Peninsula Laboratories during fruitful discussions by correspondence, was based on the reaction of the side chain hydroxyl group of protected serine or threonine peptides with succinic anhydride in the presence of 4-dimethylaminopyridine[8] in pyridine solution. The reaction went smoothly overnight at room temperature, and the resulting hemiester of succinic acid was easily isolated through extraction of the unreacted succinic acid by potassium bicarbonate from ether solution. The potassium salt of the heavily protected peptide hemiester of succinic acid remains in the organic solvent; the free acid was recovered therefrom by acidification and crystallization from petroleum ether. We have thus obtained two succinylated threonine peptide derivatives which were coupled to the aminomethylated resin support in nearly quantitative yield giving rise to the resin bound C-terminal peptides of human insulin B-chain and γ-endorphin. In the synthesis of γ-endorphin, two different routes have been adopted for the attachment to two different kinds of aminomethylated resin. The first is the one we have routinely used — the DCC-HOBt procedure anchoring

Table I. Peptide Segments To Be Anchored To Resin Supports

Peptide Fragment	Side chain anchoring group	Kind of resin to be attached	m.p. °C	$[\alpha]_D^{20°}$
Boc $COCH_2CH_2COOH$ Bpoc-Lys-Thr-OtBu	Succinylated β-hydroxyl of Thr	Aminomethylated poly(styrene-1.5% DVB)	58-60	-1.0 (c 1, MeOH)
$COCH_2CH_2COOH$ Bpoc-Leu-Val-Thr-Leu-OtBu	Succinylated β-hydroxyl of Thr	Aminomethylated poly(styrene-1.5% DVB)	176-8	-36.1 (c 1.33, MeOH)
		Glycyloxymethylated poly(styrene-1.5 % DVB)		
Tos $COCH_2CH_2COOH$ Boc-Ile-Arg-Ser-OMe	Succinylated β-hydroxyl of Ser	Aminomethylated poly(styrene-1.5% DVB)	60-62	-15.5 (c 0.42, MeOH)
tBu Bpoc-Cys-Asp-OtBu	β- carboxyl of Asp	Bromopropionamido-methylated poly-(styrene-1% DVB)	73-75	-14.0 (c 0.5, MeOH)
Bpoc-Leu-Glu-OtBu	γ- carboxy of Glu	Chloromethylated Poly(styrene-1.5% DVB)	84-86	-34.7 (c 1, MeOH)
tBu Bpoc-Ser-Asp-OtBu	β- carboxyl of Asp	Chloromethylated Poly(styrene-2% DVB)	80-82	-12.0 (c 0.5, MeOH)

Table II. Preparation of Resin-Bound Peptides

Resin-bound Peptide	Target Peptide	Method	Total Amino after deblocking mmole/g	Yield of Coupling %
Boc $COCH_2CH_2CONHCH_2\emptyset(R)$ Bpoc-Lys-Thr-OtBu	Insulin B chain fragment	DCC/HOBt	0.50	99
$COCH_2CH_2CONHCH_2\emptyset(R)$ Bpoc-Leu-Val-Thr-Leu-OtBu	γ-endorphin	DCC/HOBt	0.44	96
$COCH_2CH_2CONHCH_2COOCH_2\emptyset(R)$ Bpoc-Leu-Val-Thr-Leu-OtBu	γ-endorphin	RMP method	0.51	98
Tos $COCH_2CH_2CONHCH_2\emptyset(R)$ Boc-Ile-Arg-Ser-OMe	Model serine peptide	DCC/HOBt	0.46	99
tBu $OCH_2CH_2CONHCH_2\emptyset(R)$ Bpoc-Cys-Asp-OtBu	Insulin A chain fragment	Cs salt method	0.45	75
$OCH_2\emptyset(R)$ Bpoc-Leu-Glu-OtBu	Human proinsulin C peptide	Thioether method	0.56	66
tBu $OCH_2\emptyset(R)$ Bpoc-Ser-Asp-OtBu	Serum thymic factor	Thioether method	0.50	59

the protected tetrapeptide ester hemiester, Bpoc-Leu-Val-Thr($COCH_2$-CH_2COOH)-Leu-O*t*Bu, to the amino methylated resin. In the second approach, the same protected tetrapeptide ester hemiester was anchored to a glycine benzyl ester type resin via a coupling procedure involving activation with 2-bromo-N-methylpyridinium salt (BMP method), a reagent we have used to prepare certain small peptides including Leu enkephalin.[9] The compound has recently been tested for its feasibility as condensing agent in the solid phase anchoring of the model preparation Z-Thr($COCH_2CH_2CONHCH_2COOCH_2\phi$(R))-Ala-OMe.

For attachments via the side chain of serine residues we relied on a model peptide, an intermediate for the synthesis of a TMV fragment, Boc-Ile-Arg(Tos)-Ser(Bzl)-OMe. After removal of the benzyl group by catalytic hydrogenolysis, the resulting Boc-Ile-Arg(Tos)-Ser-OMe was easily succinylated to give a crystalline product. This derivative was coupled to the aminomethylated resin in the usual way and the Boc group removed with 50% CF_3COOH in CH_2Cl_2 with no loss in the total of amino group and elongated consecutively with two fragments, showing that the ester linkage on the succinic acid side is strong enough to twice withstand the conditions required for the removal of the Boc group.

Segment Coupling

Segments ranging in size from di- to pentapeptides (Table II) have been prepared by conventional solution methods, most via mixed anhydrides and several via activated esters. The purity of each fragment must be ensured through various purity tests; several of the peptides were purified by silica gel column chromatography. The coupling was done usually on a scale of 200-500 mg resin with a content of chloro, bromo, or amino group of 0.4-0.7 mmole/g. After the first coupling (attachment to the resin), the resin bound protected peptide was treated with 0.05N HCl in CH_2Cl_2 at room temperature for 1 to 1.5 hours to remove the Bpoc group. To the deblocked resin bound ester protected peptide segments were coupled consecutively with DCC and HOBt in the molar ratio of 2:2:0.8 with respect to the amine component. Coupling took place at room temperature (20-25°C) for 40-60 hours with shaking. Residual amino group was checked at each cycle by the salicylaldehyde procedure; if more than 5% of the total amino group was found to be still uncoupled, an additional run with 1 equivalent of reactants in the same proportion was carried out for 24-40 hours. The amino group content after removal of the Bpoc-group with 0.05N to 0.1N HCl was found to be a good criterion to check the completeness of the reaction, in most cases it was close to theory.

Table III. Segments To Be Coupled In The Synthesis Of Target Peptides

Target[a]	Peptide Fragments	m.p. °C	$[\alpha]_D^{20°}$
C 25-29	Bpoc-Ala-Leu-Glu(OtBu)-Gly-Ser(tBu)-OH	218d	-17.2 (c 0.8, MeOH)
C 20-24	Bpoc-Ser(tBu)-Leu-Glu(OBzl)-Pro-Leu-OH	103-5	-65.2 (c 1, MeOH)
C 15-19	Bpoc-Gly-Pro-Gly-Ala-Gly-OH	104-6	-23.5 (c 1, MeOH)
C 10-14	Bpoc-Val-Glu(OtBu)-Leu-Gly-Gly-OH	270d	-26.5 (c 0.8, EtOH)
C 5-9	Bpoc-Leu-Glu(OBzl)-Val-Gly-Glu(OBzl)-OH	210d	-15.5 (c 1, DMF)
C 1-4	Boc-Glu(OtBu)-Ala-Glu(OtBu)-Asp(OtBu)-OH	83	-22.0 (c 1, MeOH)
S 6-7	Bpoc-Gly-Gly-OH	118-20	-7.5 (c 0.1, MeOH)
S 4-5	Bpoc-Ser(tBu)-Glu(OBzl)-OH.CHA	140-42	-6.0 (c 1, MeOH)
E 12-13	Bpoc-Thr(tBu)-Pro-OH	142-43	-35.0 (c 0.5, MeOH)
E 10-11	Bpoc-Ser(tBu)-Glu(OBzl)-OH.CHA	140-42	-6.0 (c 1, MeOH)
E 8-9	Bpoc-Glu(OtBu)-Lys(Boc)-OH.CHA	122-24	-11.0 (0.43, MeOH)
E 6-7	Bpoc-Thr(tBu)-Ser(tBu)-OH.CHA	140-42	8.8 (c 0.7, MeOH)
E 1-5	Boc-Tyr-Gly-Gly-Phe-Met-OH	108-12	-12.0 (c 1, THF)
E' 4-7	Boc-Phe-Met-Thr-Ser-OH	118-20	-6.0 (c 1, THF)
E' 1-3	Boc-Tyr-DAla-Gly-OH	164-66	36.0 (c 1, MeOH)
B 24-28	Bpoc-Phe-Phe-Tyr(tBu)-Thr(tBu)-Pro-OH	138-40	-31.5 (c 1, MeOH)
M 5-7	Boc-Phe-Asp(OBzl)-Thr(Bzl)-OH	98-100	9.2 (c 1, MeOH)
M 1-4	Bpoc-Ala-Phe-Asp(OBzl)-Thr(Bzl)-OH	96-98	-13.3 (c 1, MeOH)

[a]Target peptides: C, C-peptide of human proinsulin; S, serum thymic factor; E, γ-endorphin; E', γ-endorphin analog; B, insulin B-chain C-terminal fragment; M, model serine decapeptide.

Thus far, we have completed several syntheses of the C-peptide of human proinsulin (31-peptide ending in glutamine, 6 segments); serum thymic factor (nonapeptide ending in asparagine, 5 segments); γ-endorphin (17-peptide attached at the penultimate threonine, 5 segments) and its D-Ala^2 analog by an alternate route; serine-containing decapeptide, and the C-terminal segment of the human insulin B-chain by coupling two segments to an 11-peptide terminating in threonine. The schemes of synthesis are shown in Figures 1-5; several elongation steps were carried out with Bpoc amino acids rather than with segments.

Results and Discussions

For several typical syntheses coupling efficiencies as revealed by the residual amino group content, as well as the yield given by the total amino group content after deblocking, are shown in Table IV. In the synthesis of the C-peptide of human proinsulin, the coupling efficiency of

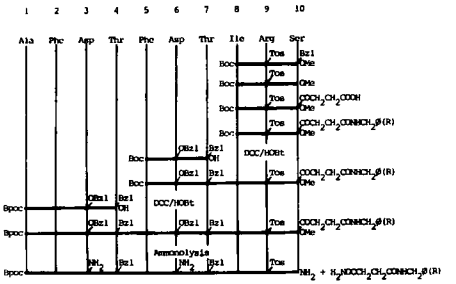

Fig. 1. Scheme of the synthesis of a serine-containing decapeptide.

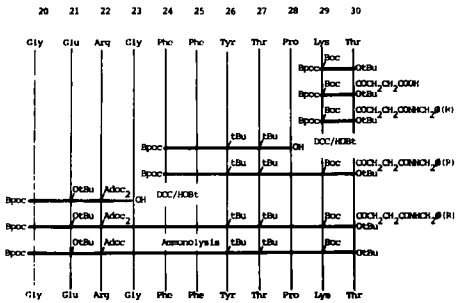

Fig. 2. Scheme of the synthesis of the C-terminal 11-peptide of the human insulin B-chain.

97% was reproduced many times; the total amino group content at the end of each deblocking step is also nearly quantitative, an exception being the third coupling with the protected pentapeptide, Bpoc-Gly-Pro-Gly-Ala-Gly-OH, where only 80% of the total amino group content was exposed. This sudden drop may be due to a side reaction during the deblocking step of this 17-peptide resin, or it is due to conformational changes that remain to be explained. An improvement in the total amino group exposure might be expected with this pentapeptide being placed at an earlier or later stage of coupling if conformational change is really involved. On the other hand, if the decline in the total amino group content after coupling of this fragment is not position dependent, then an explanation may be found in the conformation of this peptide itself a possibility now under investigation. Side reactions characteristic of this rather simple peptide structure are also under consideration.

The successful synthesis of the C-peptide is shown in the following statistics. Starting with 90 μmoles of dipeptide resin (160 mg) with a total amino group content of 0.56 mmole/g, the 27-peptide resin was isolated

Table IV. Coupling Efficiencies And Overall Yields At Various Steps In The Synthesis Of Target Peptides

Peptide Fragments	Residual Amino Group after Coupling mmole/g	Coupling efficiency %	Total Amino Group after deblocking mmole/g Found	Calcd	Overall efficiency %	Peptide Fragments	Residual Amino Group after Coupling mmole/g	Coupling efficiency %	Total Amino Group after deblocking mmole/g Found	Calcd	Overall efficiency %
C 30-31 resin			0.56			E 14-17 resin			0.44		
C 25-29	0.004	99	0.41	0.42	98	E 12-13	0.01	98	0.38	0.39	97
C 20-24	0.005	98	0.31	0.32	96	E 10-11	0.004	99	0.33	0.34	97
C 15-19	0.008	97	0.22	0.28	80	E 8-9	0.006	98	0.32[a]	0.30	-
C 10-14	0.006	97	0.20	0.25	80	E 6-7	0.006	98	0.29[a]	0.27	-
C 5-9	0.003	97	0.17	0.21	80	E 1-5	0.012	96	-	-	-
C 1-4	0.005	96	-	0.18	-	E' 14-17 resin			0.51		
S 8-9 resin			0.50			E' 13	0.058	89	-	-	-
S 6-7	0.006	99	0.46	0.47	98	E' 12	-	-	0.40	0.43	93
S 4-5	0.005	99	0.40	0.40	99	E' 10-11	0.044	90	0.285	0.33	86
S 3	0.006	99	0.36	0.36	99	E' 9	0.008	97	0.285[a]	0.26	-
S 2	0.01	98	0.35	0.35	99	E' 8	0.021	92	0.245[a]	0.22	-
S 1	0.005	99	-	0.34	-	E' 4-7	0.0135	94	0.151[a]	0.15	-
						E' 1-3	0.021	86	-	-	-
M 8-10 resin			0.45								
M 5-7	0.008	98	0.35	0.35	99	B 29-30 resin			0.50		
M 1-4	0.01	97	-	0.27	-	B 24-28[b]	0.016	97	0.35	0.36	98

[a]More exposure of total amino group than expected, presumably due to some deblocking at the ϵ-NH_2 protecting Boc or Z. [b]Protected heptapeptide ester was detached from the resin by ammonolysis, m.p. 114-6°C; $(\alpha)_D^{20}$-27.5 (c 1, MeOH). [c]Protected 11-peptide ester was detached from the resin by ammonolysis m.p. 124-126°C; $(\alpha)_D^{20}$-23.5.

with a total amino group content of 0.17 mmole/g which, after the last coupling with Boc tetrapeptide derivative, gave 360 mg of 31-peptide resin. After ammonolysis (233 mg), acidolysis, first DEAE cellulose chromatography (122 mg), second DEAE cellulose chromatography (110 mg), and final Sephadex G25 filtration, the overall yield (99 mg, 32.8 μmoles) of 36% is indeed a demonstration of the utility of the strategy of detachment from the resin in this synthesis.[10] As one of its many features, we have in the course of preparing segments, prefabricated 3 Gln residues in the form of Glu(OBzl), assuring ease of handling and total avoidance of cyano-derivative formation as a consequence of the extensive use of DCC with Asn or Gln. In the synthesis of the thymic factor, the danger of forming Z-Asn-NH_2 from Z-Asn-O*t*Bu by ammonolysis, as shown in a model experiment, has prompted us to change from the conventional order of ammonolysis preceding acidolysis. We did the acidolysis first with satisfactory results. The final product obtained in an overall yield of

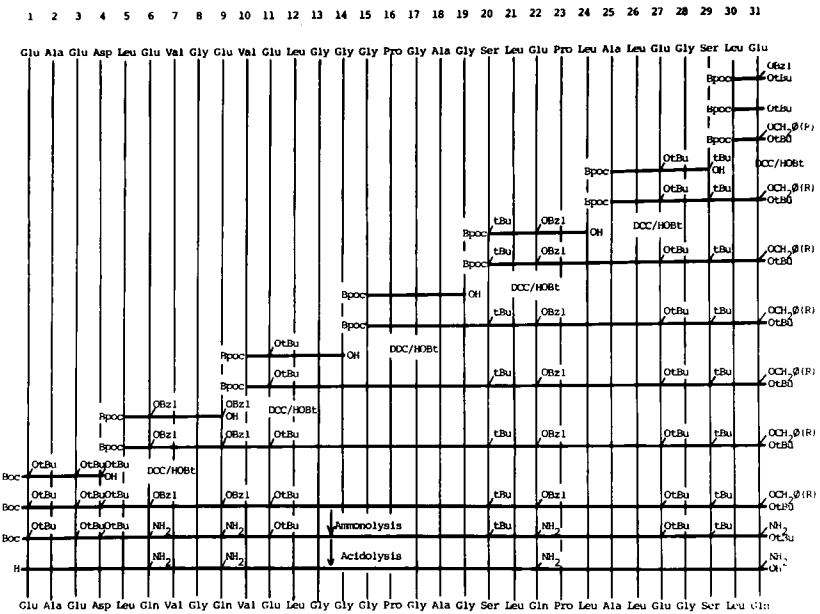

Fig. 3. Scheme of the synthesis of the C-peptide of human proinsulin.

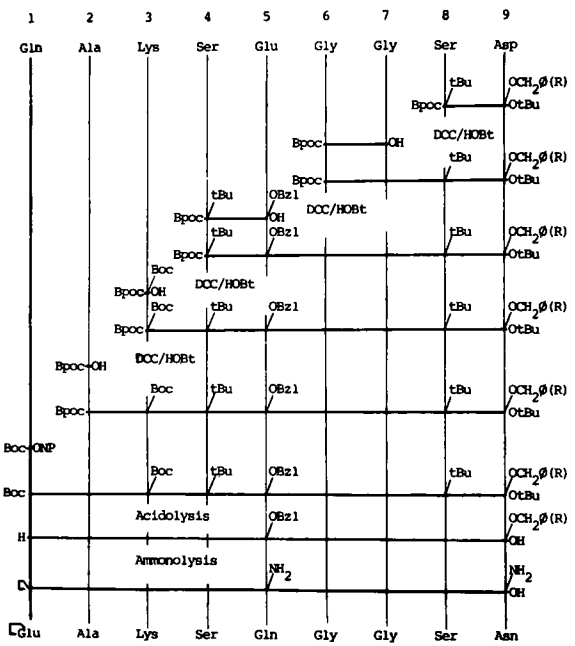

Fig. 4. Scheme of the synthesis of the serum thymic factor.

30% after two Sephadex G-15 filtrations and CMC-chromatography, was shown to be indistinguishable from an authentic sample, after comparing the amino acid composition, amide content, and the absence of isoasparagine — as ascertained by NMR spectroscopy and carboxypeptidase digestion — optical rotation, and the E-rosette test. In the meantime, we have also synthesized the same nonapeptide by the conventional solution method with approximately the same choice of segments. The characteristics of the final product were the same as those of the sample from the solid phase synthesis; the overall yield, however, was only 21%.

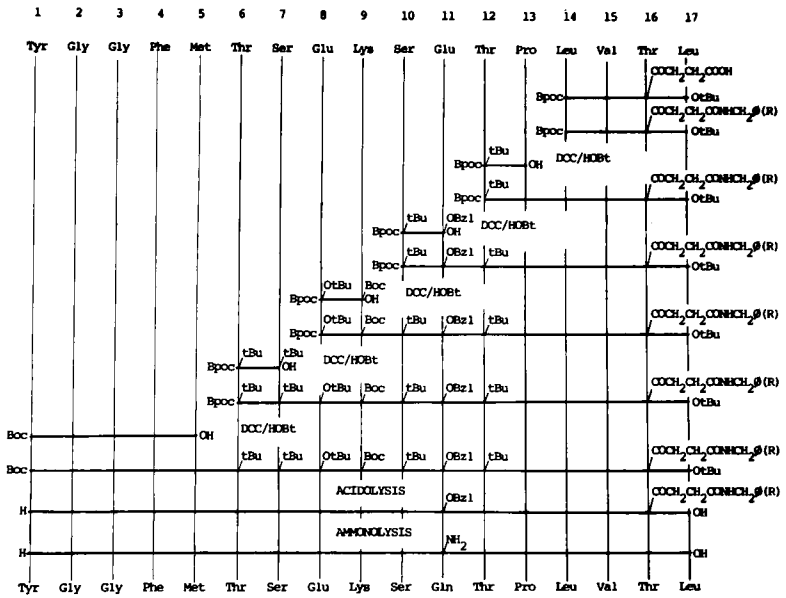

Fig. 5. Scheme of the synthesis of γ-endorphin.

The synthesis of γ-endorphin was also successful. The final product is rather clean as reflected in the amino acid composition of the final product and its various intermediates. Purification was simple, requiring only one passage over Sephadex G-15 and giving a sample with full activity in both the MVD and GPI assay. Chemical characterization data are for the sample from the DCC/HOBt method.

For peptides with physiological action this property is an important criterion in assessing the purity of the synthetic product. The reason for looking for a procedure to synthesize peptides, for instance, the B-chain of insulin via ammonolysis in the final detaching step is because we have

in mind the availability of our intermediates for biological tests. The results we have described above have indeed shed some light on a possible approach to milder processes in the synthesis of larger peptides.

Acknowledgements

We wish to thank Dr. S.S. Wang, Peninsula Laboratories, Inc., Belmont, California, U.S.A. for constructive suggestions and sending us reagents and samples of resin supports. The technical assistance of S.M. Xu, C.P. Wang, T.M. Chen, P.C. Zhu, and H.P. Li is much appreciated.

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MULTIDETACHABLE RESIN SUPPORTS

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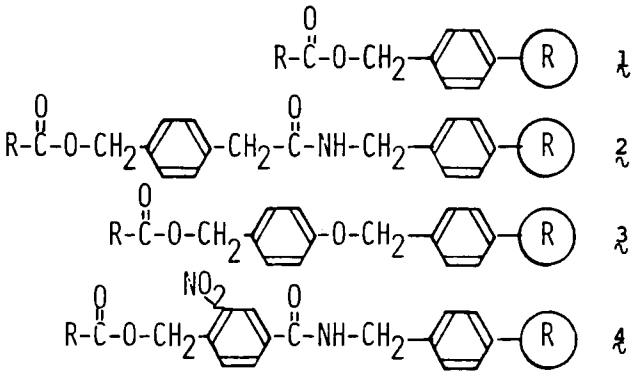
Introduction

The synthesis of peptides or small proteins, by solution or solid phase approaches,[1] can be divided into the stepwise method and the segment method. The stepwise method is usually simpler and more efficient provided that all the steps proceed quantitatively. The segment method has the advantages that all intermediates can be purified and analyzed before they are used in the next step to minimize the accumulation of impurities. Most solid phase peptide chemists favor the stepwise method while the solution peptide chemists prefer the segment method. This is exemplified in the synthesis of ribonuclease by three different groups.[2-4] A compromise approach, as demonstrated in the syntheses of bovine trypsin inhibitor[5] and glucagon,[6] is to prepare the segments by the solution method and then assemble them by the solid phase method. However, there has been no serious attempt both to generate segments and assemble them by the solid phase method. This is in part due to the inflexibility of the functionalized resin support. In this paper I wish to report the design, synthesis and chemistry of three new multipurpose, multidetachable resin supports[7,8] that can be used for both stepwise and segment condensation methods.

Nondetachable Handle Resin Support

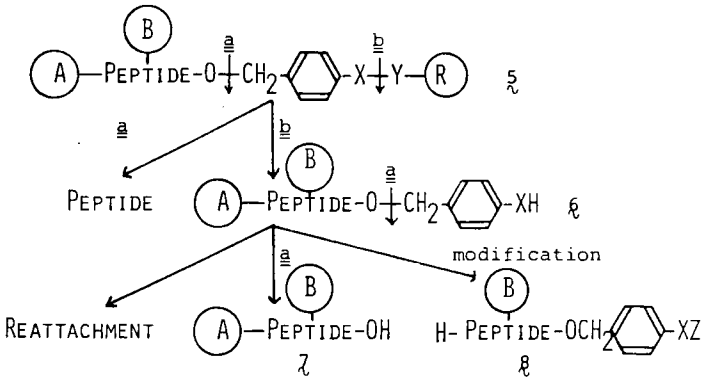
The commonly utilized chloromethyl resin is functionalized to give a resin-bound benzyl ester linkage between the peptide and the polymeric support **1**. However, it suffers from two limitations; one is flexibility and the other is acid stability. This is largely due to the fact that the benzyl ester linkage dictates that it best be used with the stepwise method together with the Boc-benzyl as α-amino and side chain protecting group strategy. The electron-donating *p*-alkyl substitution derived from the styrene-resin decreases the acid stability of this benzyl ester towards the repetitive trifluoroacetic acid treatments, resulting in an acidolytic loss of 1.4% peptide chain per cycle. These problems are partially resolved using the nondetachable handle resin support in which a spacer or handle is inserted between the peptide benzyl ester linkage and the styrene resin backbone. Thus, the use of an electron-withdrawing, nondetachable handle such as the phenylacetamidomethyl (Pam) in the Pam resin[9,10] achieves the desired result of reducing the acidolytic loss of peptide. The



p-alkoxybenyzl ester resin[11] **3** can be included as another nondetachable handle resin support, but the handle is designed to modify the electronic property of the benzyl ester. It allows the employment of an alternative protecting group strategy. The nitrobenzyl ester resin,[12] **4**, prepared as a nondetachable nitrobenzyl ester handle to the polymeric backbone provides a versatile entry for the preparation of protected peptide segments. Thus, the nondetachable handle approach for polymeric supports provides useful modifications to either electronic refinement or chemical selectivity to resin supports. It nevertheless has been a single purpose system.

Detachable Handle Resin Support

The detachable handle resin support concept has been developed to meet the requirements of better quality of resin support and greater flexibility of synthetic strategy.[7,8,13] The design of these new resins, as represented by a general structure, **5** (Scheme 1), has taken into consideration their use in both stepwise and segment methods. This is achieved with two cleavable linkages (bonds *a* and *b*) connected by a "reversible" handle (CH_2-C_6H_4-X). One of the cleavable points (bond *a*, Scheme 1) is a benzylic linkage between the peptide and the handle. This preserves the utility of the support for the stepwise method so that free, unprotected peptide can be obtained after treatment with strong acid. The other linkage between the handle and the polymeric support (bond *b*, Scheme 1) is acid stable but cleavable by an orthogonal chemical method that will leave the benzylic linkage (bond *a*) and the α-amino and side chain protecting groups intact (denoted as A and B in Scheme 1). Thus cleavage at bond *b* will give a protected peptide segment with a C^{α}-benzylic handle, **6**. After purification this completely protected peptide segment will allow three possible strategical uses: (1) reattachment to resin sup-

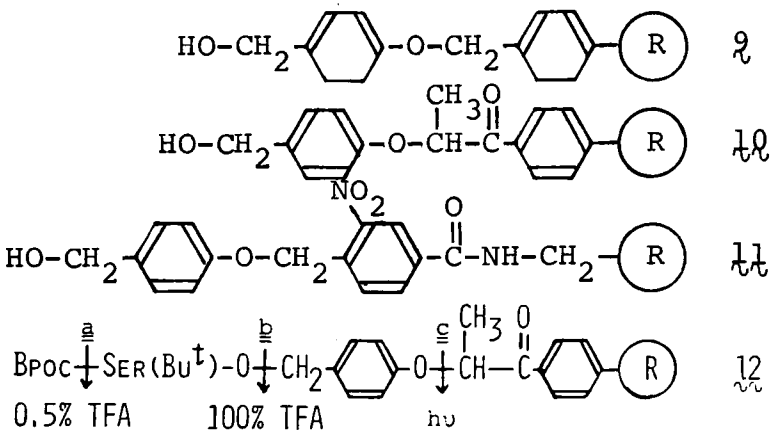


Scheme 1. Design of a Multidetachable Resin Support

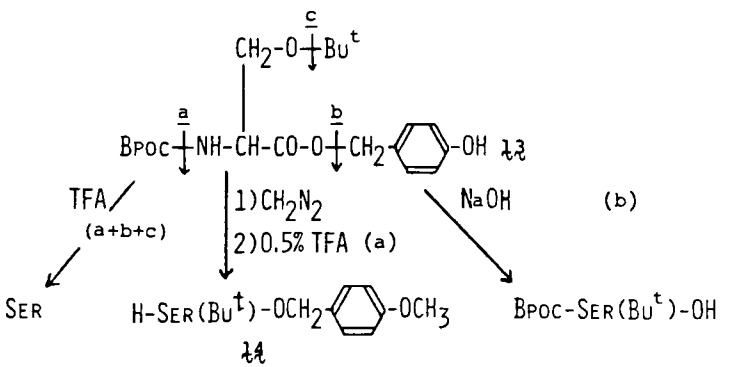
port, (2) selective removal of the reversible handle to give a N^{α}-protected peptide segment, **7**, and (3) mild chemical modification of handle and selective cleavage of the N^{α}-protecting group to give a C^{α}-protected peptide segment **8**. This design is flexible enough to be multipurpose. Furthermore, this degree of chemical flexibility has not been available in any previously reported resin support design. The success of the design depends on the proper selection of the orthogonal deprotection mechanism of the two cleavage linkages *a* and *b*. It also depends on the proper selection of the handle (e.g. CH_2-C_6H_4-X in Scheme 1) which can be easily removed without affecting the cleavage of the α-amino and side chain protecting groups. The electronic property of the handle also needs to be considered so that it can confer the correct acid stability or lability in TFA and HF. These questions are discussed in the design of three types of multidetachable resins. They are (1) photolabile *p*-alkoxybenzyl alcohol resin, (2) retrophenyl ester benzhydrylamine resin, and (3) photolabile *p*-oxyacylmethylbenzyl ester resin.

Photolabile *p*-Alkoxybenzyl Alcohol Resin Support

The normal alkoxybenzyl alcohol resin, **9**, has been useful in the stepwise synthesis since the peptide can be removed by trifluoroacetic acid. Usually, base labile Fmoc, acid sensitive Bpoc or thiolytic sensitive Nps is used as the α-amino protecting group and *t*-butyl as the side chain protecting group. For the photolabile *p*-alkoxybenzyl alcohol resin,[13] the *p*-hydroxybenzyl alcohol is attached to a photolabile resin such as the 2-propionyl resin[14] to give **10** or the nitrobenzyl resin[12] to give **11** while the normal *p*-alkoxybenzyl alcohol resin **9**, the *p*-hydroxybenzyl alcohol is attached to the chloromethyl resin. In addition to retaining the TFA-

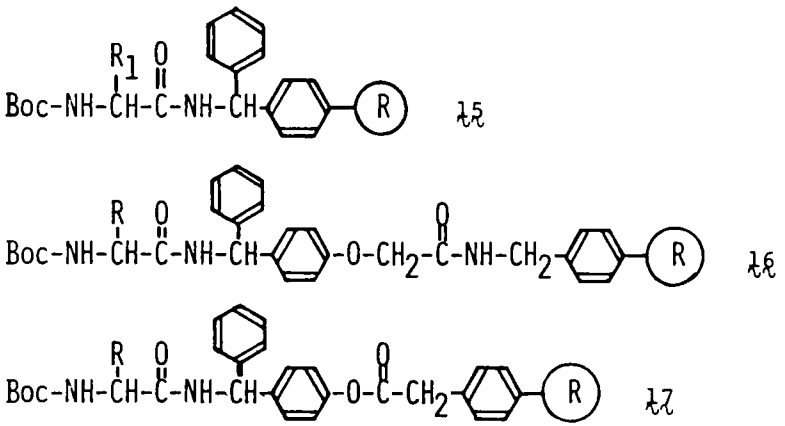


labile benzyl ester bond similar to the normal *p*-alkoxybenzyl alcohol resin, the new resin has a photolabile ether linkage. Bpoc-Ser(Bu^t)-4(2-[4-oxymethyl)phenoxy]propionyl-resin **12** was obtained after esterification of Bpoc-Ser(Bu^t)-OH to resin **10.** Thus, in TFA, bonds *a* and *b* of resin **12** were cleaved to give serine, but in 0.5% TFA, only bond *a*, the Bpoc group, was removed to allow the stepwise synthesis. Upon photolysis of **12** in DMF using a uranium filter, the *p*-hydroxybenzyl ester of Bpoc-Ser(Bu^t)-OH **13** (Scheme 2) was obtained from the cleavage of bond *c*. With photolysis in DMF alone, part of this handle was removed to give Bpoc-Ser(Bu^t)-OH, but with the addition of 1% phenol as buffer, this side reaction was suppressed. Since *p*-hydroxybenzyl ester derivative **13** is relatively unstable to the conditions of peptide synthesis, it is converted by alkylation to the *p*-methoxybenzyl ester derivative. Subsequent removal of the Bpoc-group gave a C$^\alpha$-*p*-methoxybenzyl ester



Scheme 2. Design of Photolabile *p*-alkoxybenzyl Alcohol Resin Support

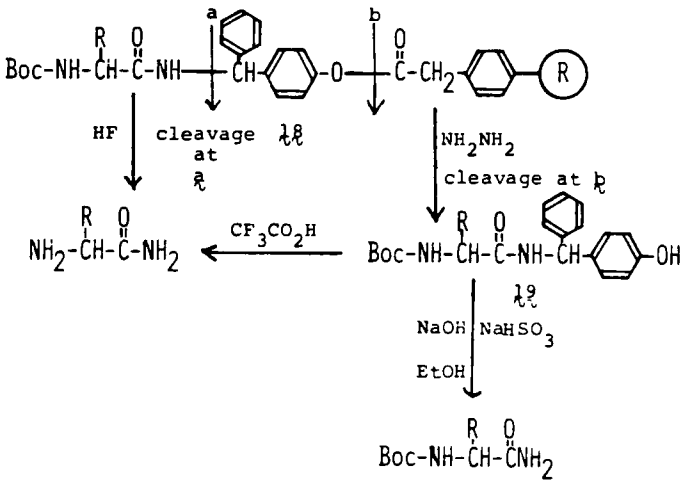
protected derivative **14** (Scheme 2). The greatest strength of this resin probably lies in the ability to generate this derivative for semi-synthesis because the reconstituted semi-synthetic product can then be obtained after TFA treatment to remove all protecting gorups (*t*-butyl and *p*-methoxybenzyl derivatives). To generate the fragment with a free C^{α}-carboxylic acid such as Bpoc-Ser(Bu^t)-OH, the *p*-hydroxybenzyl handle was removed with aqueous sodium carbonate solution and sodium bisulfite by a 1,6-elimination reaction to *p*-quinonemethide. The principle of the 1,6-elimination reaction to remove *p*-hydroxybenzylic derivatives has been used for the design of α-amino protecting groups.[15,16]



Retrophenyl Ester Benzhydrylamine Resin

The benzhydrylamide resin[17] **15** has been used to give the α-carboxamide in solid phase peptide synthesis, but very poor HF cleavage yield has been reported when the C-terminal amino acid is phenylalanine or valine. The problem is likely related to its difficulties in the preparation of the benzhydrylamine moeity on the resin. One approach to give a chemically well defined benzhydrylamide resin is by the handle approach[18] as illustrated in resin **16.** The retrophenyl ester benzhydrylamide resin,[19] **17,** is designed to provide an improved preparation of this support and to give better yields of peptide products and greater flexibility of cleavage methods. The chemically pure benzhydrylamine handle was prepared in solution as N-Boc-(4-hydroxy)benzhydrylamine from *p*-hydroxybenzophenone.[19] This was then esterified to a carboxymethyl-resin to yield the retrophenyl ester benzhydrylamine resin. After removal of the Boc-group by TFA, the new benzhydrylamine resin was coupled

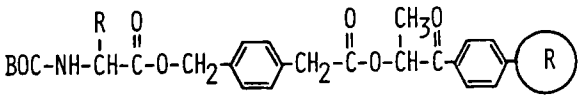
with an activated Boc-amino acid to give the Boc-aminoacyl-benzhydrylamine-4-oxycarbonyl resin, **18,** which could then be lengthened by stepwise coupling to give the desired peptide-resin. The new benzhydrylamide resin **18,** consists of two cleavable points; thebenzhydrylamide linkage *a* and the retrophenyl ester linkage *b*, connected by a *p*-hydroxybenzhydryl handle (Scheme 3). Treatment with HF/*p*-cresol (9:1, v/v) at 0° C for 1 h, provided the desired amino acid or peptide derivative in excellent yield (80-95%), even with a problematic residue, such as phenylalanine α-carboxamide. A distinctive feature of this new resin is that the ester linkage *b* is a retrophenyl ester. In normal phenyl ester, the acyl component is usually the peptide which is influenced by the nucleophile or base used in the cleavage method. but in a retrophenyl ester, the carboxyl-group is part of the resin component. This design confers the electronic properties required for the TFA stability and HF lability of the benzhydrylamine moiety. In addition a flexible cleavage pattern is achieved whether the reagent was hydrazine, cyanide or peroxide anion, the 4-hydroxybenzhydryl-peptide **19** (Scheme 3) was produced. Removal of the 4-hydroxybenzhydryl group, a *p*-hydroxybenzylic handle derivative similar to **13,** could be achieved by either TFA (because the 4-hydroxybenzhydrylamide peptide has lost the acid-stabilizing effect of the 4-acetoxy group) or by a 1,6-elimination reaction in aqueous base with $K_3Fe(CN)_6$ or with sodium bisulfite. This cleavage method will allow the preparation of a protected peptide α-carboxamide for fragment method synthesis.



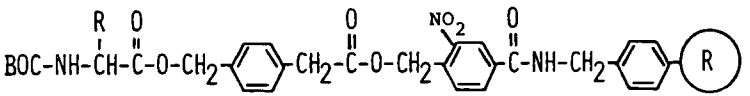
Scheme 3. Design of Retrophenyl Ester Benzhydrylamine Resin

Photolabile *p*-Oxyacylmethyl Benzyl Ester Resin

Two photolabile *p*-oxyacylmethyl benzyl ester resins,[7,8,20] Pop **20** and Pon **21** resins, have been developed to meet the design and concept of the multidetachable resin. In each case, an electron withdrawing *p*-oxyacylmethyl substitution on the peptide-benzyl ester linkage (bond *b*, scheme 4) reduces TFA loss of peptide chain 35-200 fold.[18] For example Boc-Val-OCH_2-Pop-resin is found to be about 100 times more stable than Boc-Val-OCH_2-resin in TFA, an acid stability comparable to that of the Pam resin.[7,8] This enhanced acidolytic stability is achieved without sacrificing the high cleavage yields obtained by the treatment with HF. However, unlike the Pam resin which has a nondetachable handle, Pop or Pon resin has a photolabile linkage (bond *c*, Scheme 4). Thus, photolytic treatment of Pop or Pon resin will yield a protected peptide fragment with a C^{α}-benzylic handle containing the phenylacetic acid derivative **22** which, after purification, can be used in three possible ways: (1) coupling to an aminomethyl-resin to form the Boc-peptidyl-OCH_2-Pam-resin to continue the synthesis, (2) alkylation of **22** to the methyl ester to be used in solution synthesis and (3) coupling to a primary amine carrying a tertiary amine such as [N-aminoethyl]-piperidine, which confers an ion-exchange affinity to the peptide fragment to facilitate the purification in subsequent solution synthesis.

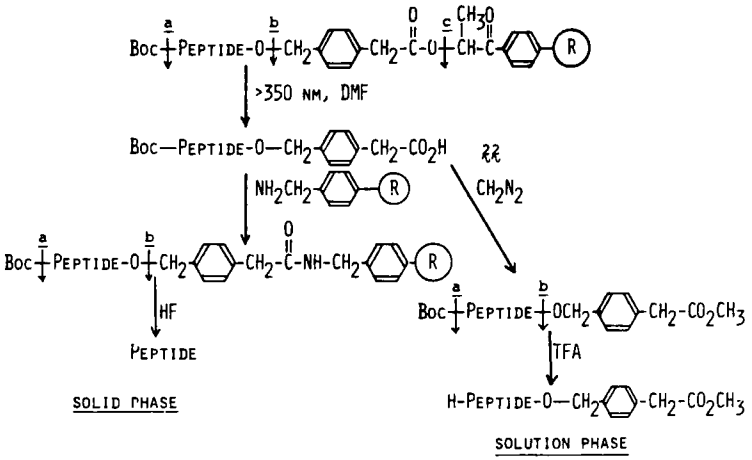


BOC-AMINOACYL-OCH_2-**POP**-RESIN **20**



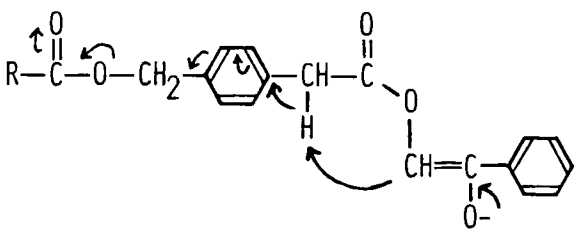
BOC-AMINOACYL-OCH_2-**PON**-RESIN **21**

In order to obtain the C^{α}-carboxylic acid fragment, the methylphenylacetic acid handle of the Boc-peptide-oxymethylphenylacetic acid **22** has to be removed selectively. This handle differs structurally from the previous *p*-hydroxybenzylic alcohol handle design (see structures **13** and **18**) and cannot be removed with a mild base by a 1,6-elimination reaction to the quinonemethide derivative. In order to obtain the C^{α}-carboxylic acid fragment the ketone moiety in the Pop resin, **20**, has to be enolized.



Scheme 4. Design of Photolabile *p*-oxyacylmethylbenzyl Ester Resin

This enolate has a 1,5-spatial relationship to the α-benzylic proton and it is envisioned that abstraction of this benzylic proton by the enolate will lead to a 1,6-elimination reaction to form a quinonedimethide derivative and to give a C^{α}-carboxylic acid peptide fragment (Scheme 5). Among many primary, secondary, and tertiary amine bases that were tested to generate this enolate, those amine bases with pKa's lower than 11.5 were totally ineffective. In reality, this is an advantage because these amines are used in the repetitive cycles of the peptide synthesis. Extensive treatment of the Pop-resin with diisopropylethylamine led to no loss of peptidyl chain from the resin. However, conjugated amine bases with pKa's 12.5 such as DBU (1,5-diazabicyclo [5.4.0]undec-5-ene) or TMG (tetramethylquanidine) effected this transformation smoothly (Table I). The amidine or quanidine base is not nucleophilic and has been used in peptide synthesis.



Scheme 5. Postulated Mechanism of Enolate Promoted 1,6-elimination Reaction

In a model study, using the solution equivalent of Pop resin **20** Boc-Val-oxymethylphenylacetic acid phenacyl ester, the base catalyzed enolate promoted 1,6-elimination reaction was rapid and completed in less than 5 min in polar aprotic solvents to give Boc-Val-OH in quantitative yields (Table I). A similar effect was also seen with unsolvated cyanide anion (e.g. tetrabutylammonium cyanide). Thus, the versatile α-methylphenacyl moiety of the multidetachable Pop-resin apparently facilitates two chemical transformations. In photolysis, it provides the α-cleavage to give the C^{α}-peptide-benzylic handle segment, **22,** and in amidine base it effects the cleavage of the benzylic ester bond that is nine atoms away to give the C^{α}-carboxylic acid segment.

Table I. Base Catalyzed Enolate Promote 1,6-Elimination of Boc-oxymethylphenylacetic Acid Phenacyl Ester to Boc-Val-OH

	99% Conversion of Boc-Val-OH (min)	
Solvent	$(Bu)_4NCN$	DBU
N-methylpyrrolidine	<5	<5
Dimethylacetamide	<5	<5
Dimethylformamide	5	5
Dimethylsulfoxide	5	5
Acetonitrile	180	180
Dioxane	>1200	>1200

Conclusion

The multidetachable resins discussed serve to provide considerable freedom and versatility in synthesis. These resins have great potential in many approaches conceivable for the peptide synthesis. So far, they have been applied satisfactorily to the synthesis of protected peptide segments of antibody binding regions, angiotensin, Leu-enkephalin, and segments related to gastrin. We are now pursuing the application of these resins to the preparation of several large peptides to demonstrate their merit in more complex situations.

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EXPLORATORY STUDIES ON SOLID PHASE SEGMENT CONDENSATION SYNTHESIS

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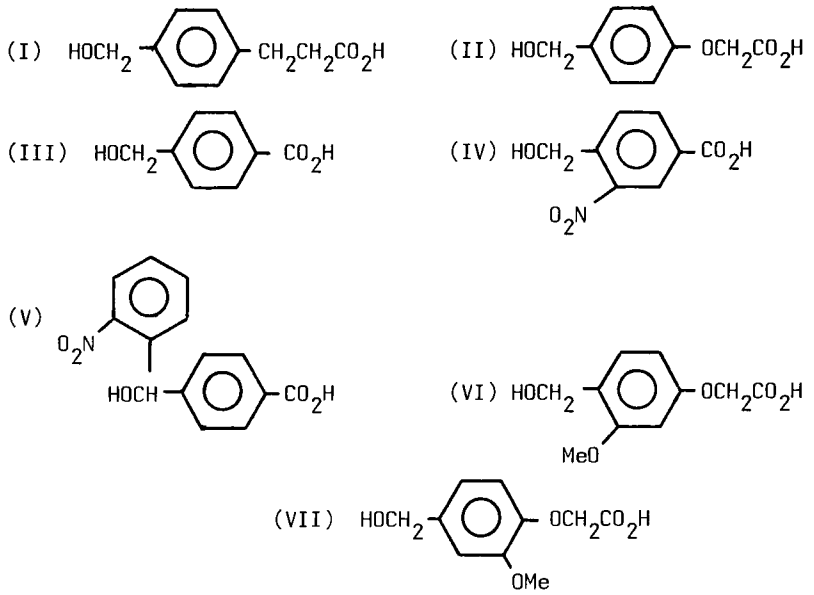
Introduction

The solid phase peptide synthesis introduced by Merrifield in 1962[1] is essentially a stepwise process in which single amino-acids are added sequentially to a resin-bound peptide chain. It has been quite remarkably successful. We all owe a great debt to Bruce Merrifield for having the imagination and courage to embark on a trail to which most organic chemists at the time would not have foreseen so successful a progression. The solid phase method has, of course, its limitations. These arise largely from the need for near quantitative reaction at every stage in order to minimize the accumulation of resin-bound impurities. In our own studies on solid phase synthesis[2] begun some ten years after Merrifield's, we sought to extend the scope of the method by further optimization of reaction conditions, minimizing impurities through greater efficiency and mildness in coupling, deprotection, and resin cleavage steps. These objectives required development of new polar polyamide supports[3] enabling reactions to be carried out in optimal polar reaction media in which both the peptide and resin were well solvated. New combinations of protecting groups and resin linkage agents were also devised,[4] the latter, e.g. (I), (II), and (III), imparting considerable flexibility and versatility. This 'polyamide method' has recently been reviewed.[5] In common with other laboratories, we feel that further development of solid phase synthesis may require that the number of sequential amino-acid additions before intermediary purification steps are limited. This might be achieved by a solid phase segment condensation strategy akin to those commonly employed in solution synthesis.

It is worthwhile drawing the distinction between solid phase assembly of solution-synthesized fragments, on the one hand, and solid phase assembly of solid phase synthesized fragments on the other. In the former process the solid phase principle is used essentially as an adjunct to conventional synthesis. It allows the use and separation of excess of the soluble component, but other advantages of the solid phase method are lost. Relatively few of the peptide bonds are formed on the resin and the



process retains the labor-intensive nature of most solution syntheses. Many laboratories using stepwise solid phase methods do so because the laboratory and human resources, experience and time needed for large scale solution peptide synthesis are not available. Under these circumstances only the second of the two fragment condensation strategies presents an alternative for increasing the scope of the solid phase method. Our studies are concerned only with this approach, and only with utilization of polar amide supports. Insofar as these supports were designed to operate efficiently in polar aprotic media such as dimethylformamide, and that solvents of this type present, in general experience, probably the best possibility for dissolving and reacting rather insoluble protected peptides, these polar supports may be particularly suitable for fragment condensation reactions. They are easily prepared in the laboratory[3b] and are now commercially available. We envisaged preparation of short readily purifiable protected fragments (up to, say, 10 residues in length), permitting use of resins with relatively high loading. Much of our preliminary work described here has been carried out on a beaded crosslinked polydimethylacrylamide functionalized with acryloylsarcosine methyl ester to the extent of about 1 meq/g. This high loading is advantageous in economic terms. High bimolecular concentrations and hence reaction rates may be maintained using only modest excesses of reactants. On the other hand, studies of fragment assembly have used a resin

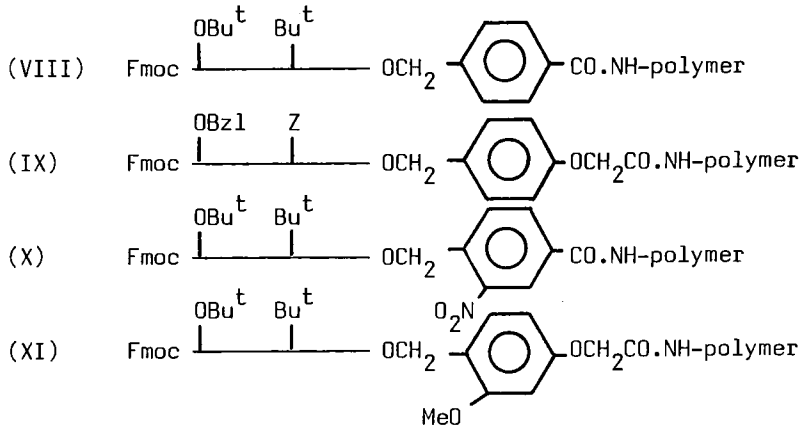
of appropriately lower loading (*ca.* 0.3 meq/g), even though the model studies reported here relate to the assembly of only short sequences.

Protecting Groups

Solid phase synthesis of protected peptide fragments requires a carboxyl-terminal peptide-resin linkage cleavable independently of protecting groups elsewhere in the molecule. Strict orthogonality (i.e. completely independent cleavage of N-terminal, side chain, and C-terminal protection) is not required, but obviously unique cleavage at the N-terminus is necessary. This last requirement is met in our current stepwise polyamide method through use of base-labile fluorenylmethoxycarbonyl-amino-acids.[4,6] Combination of this group with acid labile *t*-butyl-based derivatives for side chain protection is highly advantageous, particularly with regard to ease of final cleavage. We felt that this combination should, if at all possible form part of our fragment condensation strategy.

Three possibilities have thus far been explored for the third element -the C-terminal peptide-resin linkage. The observations in the polystyrene series of Schlatter and Mazur,[7] and of Jones,[8] suggested that under special conditions, hydrogenolysis of peptides resin-bound through simple benzyl esters might be feasible. We have confirmed that this is so using noble metal catalysts directly deposited within the resin matrix for several peptides bound to polydimethylacrylamide through the linkage agent (III). Remarkably, however, substantial cleavage of N-terminal fluorenylmethoxycarbonyl groups occurred simultaneously.[9,10] This is at variance with original observations[6] on the stability of Fmoc-derivatives to hydrogenolysis, but our results have since been confirmed elsewhere.[11] Experiments using the protecting group combination depicted in (VIII) were therefore discontinued. It is conceivable that interchange of the labilities of the side chain and C-terminal protecting groups as in (IX) would provide an alternative strategy, but this possibility has not been explored because it departs from the requirement mentioned above and also because of uncertainty regarding the final deprotection of large, benzyl-protected peptides by hydrogenolysis or by strongly acidic reagents.

Rich and Gurwara[12] showed that photolytic cleavage of *o*-nitrobenzyl linked peptides is feasible in the polystyrene series, and we have explored this possibility for polydimethylacrylamide resins using the linkage agent (IV). The protecting group combination (X) appears to provide a reasonably practical method for the asembly and detachment of protected fragments, but in our experience overall yields are not good.



The electron-withdrawing nitro group enhances the lability of the benzyl ester linkage to nucleophiles. Stability to anhydrous piperidine used in repetitive Fmoc-group cleavage is adequate, but special measures need to be taken to minimize dioxopiperazine formation at the dipeptide → tripeptide stage (see below). The Fmoc procedure itself is not ideal in this respect, providing greater opportunity for cyclization than do more conventional *t*-butoxycarbonyl-based procedures. Photolysis proceed in modest to good yields (50-70, occasionally 80%), but residual resin analysis always showed substantial uncleaved peptide. Deep reddish brown coloration of the resin accompanied photolysis. On the assumption that this coloration arose from the formation of azo compounds which might have acted as efficient light filters preventing further photolysis, we synthesized the new linkage agent (V). Barltrop[13] had already shown in solution studies that similar replacement of benzylic α-hydrogen by phenyl gave much improved yields in nitrobenzyl ester photolysis. With Boc-glycine bound to polydimethylacrylamide through (V), however, no improvement in photolytic yield was obtained and the resin again became colored. The additional phenyl group in (V) further increased the lability of the ester bond to nucleophilic attack so that appreciable cleavage by piperidine (20% in 2 hours, equivalent to 12 deprotection cycles) was now found. Thus (V) presented no advantage over the simpler linkage (IV).

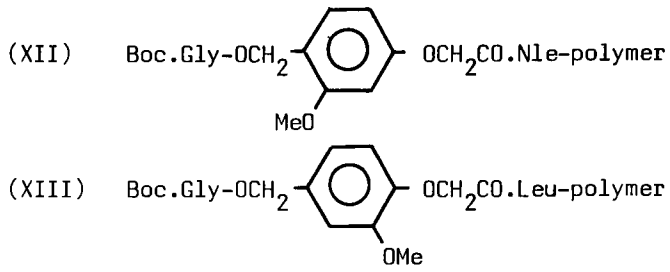
The protecting group combination (X) above is truly orthogonal but this is not a mandatory requirement. Independent cleavage of side chain protecting groups is not necessary in any practical sense. Non-orthogonal protecting group combinations imply graded lability to the same

reagent type. In considering further possibilities for fragment condensation strategies, we felt that the protecting group undergoing repetitive cleavage (i.e. the N-terminal group) should preferably not form part of any system of graded lability. Groups requiring only a single cleavage might, however, be easily differentiated. Thus a new protecting group combination suitable for the synthesis detachment, and assembly of protected peptides might be devised on the basis of acid lability of the carboxyl-terminal or peptide chain linkage substantially enhanced relative to that of the side chain *t*-butyl groups (as in XI). Such combinations do not appear to have been considered before, probably because solid phase synthesis has in the past been so firmly wedded to acid labile amino-protecting groups. Utilization of base-labile Fmoc groups for amino-protection removes this limitation.

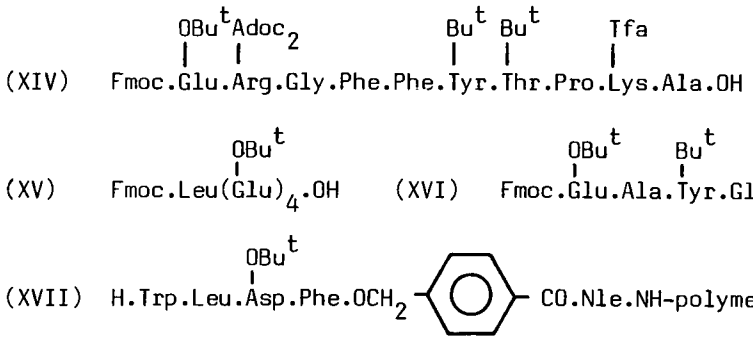
Appropriate lability of the peptide-resin bond might be achieved through linkages of the trityl, phenylisopropoxy, or polyalkoxybenzyl types. Potential ease of formation, however, strongly favors the last possibility. Simple 2,4- and 3,4-dimethoxybenzyl esters have been prepared by Photaki and her colleagues,[14] who noted the expected order of acid lability 2,4-$(MeO)_2$>> 3,4-$(MeO)_2$ > 4-MeO. We therefore prepared the new linkage agents (VI) and (VII).

Both linkage agents were coupled with polydimethylacrylamide resin, (VI) through its symmetrical anhydride* and (VII) through its 2,4,5-trichlorophenyl ester. Esterification with Boc-glycine anhydride in the presence of *p*-dimethylaminopyridine furnished the model systems (XII) and (XIII). The internal reference amino-acid residue in these derivatives enabled easy measurement of ester bond cleavage through residual resin analysis. Compound (XIII) with the 3,4-orientation of alkoxy groups was surprisingly stable (53% cleaved by 100% TFA in 30 min) and no further work was done in this series. The 2,4-dialkoxybenzyl ester in (XII) was much more labile (47, 77, 90 and 97% cleaved by 1% TFA-CH_2-Cl_2 in 5, 10, 15 and 60 min) offering good promise for differentiation from most *t*-butyl-based side chain protecting groups. Complete (16 hr) stability to acetic acid and to the weakly acidic reagents (protected amino acids and hydroxybenzotriazole) encountered in the coupling reactions of solid phase synthesis was obtained, and there was negligible cleavage (~0.02% per reaction cycle) by the piperidine reagent used in repetitive Fmoc-group removal.

* Use of immediately preformed symmetrical anhydrides of linkage agents is a new procedure. No complications have thus far been observed arising from the presence of free hydroxy groups which appear to be unreactive in the absence of basic or strongly acidic catalysts.



In separate experiments the rate of cleavage *in solution* of various *t*-butyl side chain protected Fmoc-amino-acids to 1% TFA-CH_2Cl_2 were determined using hplc methods. Derivatives of aspartic acid, glutamic acid, serine, and threonine were relatively stable ($\leqslant$3% cleavage in 3 min), but N_α-Fmoc-N_ϵ-Boc-lysine (7%) and the *O-t*-butyl-tyrosine (12%) derivative were appreciably labile. Current practice in the Fmoc-polyamide method of solid phase synthesis favors use of N_ϵ-trifluoroacetyl-lysine in place of the Boc-derivative.[15] It should be noted that the rates of side chain protecting group cleavage in free (dichloromethane) solution are not directly transferable to the solid phase situation. Slower cleavage is expected within the polar polyamide matrix, and the nature of the solid phase experiment permits rapid removal and quenching of detached peptide. Thus the tyrosine-containing tetrapeptide (XVI) was cleaved from the polydimethylacrylamide support with 1% trifluoroacetic acid in dichloromethane over 30 min with quenching of detached product after 5, 10, and 25 min. The total tetrapeptide liberated (83%) contained only 3% of des-*t*-butylated material.



Solid Phase Segment Synthesis

Trial segment syntheses have been carried out using both photolabile (IV) and acid labile (VI) linkage agents. Protected sequences prepared include the customary test tetrapeptide Leu-Ala-Gly-Val, the C-terminal decapeptide (XIV) of insulin B-chain, and a pentapeptide (XV) and tetrapeptide (XVI) from the gastrin sequence. The last two have been used as models for segment assembly studies in combination with the resin-bound tetrapeptide (XVII)[16] (see below).

The insulin decapeptide (XIV) illustrates some of the problems encountered with the photolabile linkage (IV). In a preliminary synthesis on a polydimethylacylamide resin of norleucine (internal reference) loading of 0.3 meq/g, FmocLys(Tfa) was used for incorporation of the second residue with N_{α}-deprotection by 20% piperidine in dimethylacetamide as usual. Amino-acid analysis subsequently showed a 30% loss of peptide from the resin, presumably due to dioxopiperazine formation. The assembly proceeded satisfactorily thereafter with no apparent intervention from free hydroxy groups now present on the resin. Photolysis in methanol detached 60, 63 and 75% of the total peptide in separate experiments. In dimethylformamide a cleavage yield of 50% was obtained. The synthesis was repeated, this time on a higher loading (~1 meq/g) resin. BocLys(Tfa) was used in place of the Fmoc derivative for the second residue in this dipeptide, the Boc group being cleaved with 1.5N HCl-AcOH and the hydrochloride salt neutralized after addition of Fmoc-proline anhydride. Under these conditions no appreciable loss of peptide occurred from the resin as shown by the Ala:Nle ratio. Two-fold excesses of symmetrical anhydrides were used except for coupling to the resin-bound dipeptide sequence (five-fold). Repeated acylations were required for addition of the last two residues before a completely ninhydrin-negative resin was obtained, but the final amino-acid analysis figures were excellent (Glu, 0.97; Arg, 0.95; Gly, 0.98; Phe, 1.96; Tyr, 0.99; Thr, 1.03; Pro, 1.01; Lys, 0.97; Ala, 1.02; Nle, 1.00). From 1 g of starting resin, 3.2 g of peptide-resin was obtained. Small scale photolysis (50 mg of resin) detached 70% of remarkably pure peptide. On a larger (1 g) scale, the yield was only 50% (Figure 1a).

It is central to all segment condensation strategies that individual segments should be subjected to maximum purification before reassembly. The protected decapeptide (XIV) proved to be a very sparingly soluble compound not easily amenable to chromatographic purification. Our further experiences suggest that this is likely to be a rather general situation and one that has important implications also for assembly

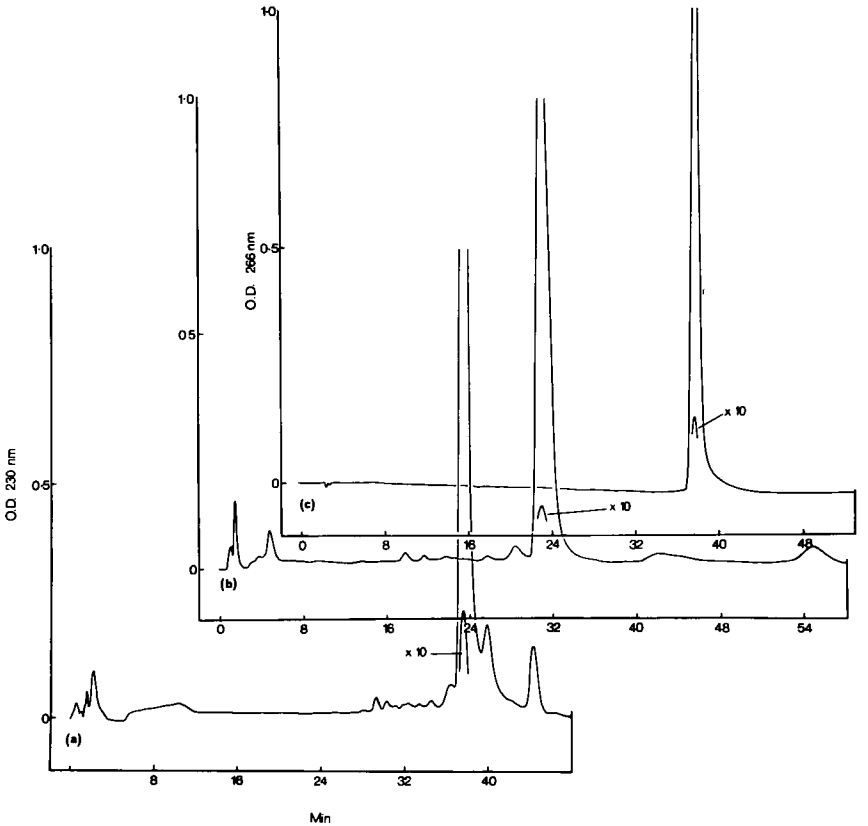


Fig. 1. Hplc of protected decapeptide (XIV). (a) Total crude photolysis product, analytical μBondapak C18 column; (b) the same using a Radial Pak ODS column; (c) purified product on μBondapak. Hplc conditions: (a and c) vessel A, 0.01M NH_4OAc, pH 4.5; vessel B, 95% CH_3CN, 5% A. Peptides were eluted using a linear gradient from 20-100% B over 40 min with a flow rate of 1.5 ml/min; (b) as for (a) with a gradient of 50-100% B over 30 min, flow rate 2 ml/minl Both (a) and (b) had similar profiles when monitored at 266 nm.

steps. The decapeptide was ultimately purified by hplc using an ODS-radial compression column which in this particular instance dramatically improved resolution (Figure 1b). This procedure has limitations of scale. The purified decapeptide was desalted on Sephadex LH20 and was then homogeneous by both tlc and analytical hplc (Figure 1c). $C_{109}H_{143}N_{14}O_{22}F_3.2H_2O$: Calculated: C, 62.46%; H, 7.12%; N, 9.36%. Found: C, 62.54%; H, 7.11%; N, 8.93%.

The shorter gastrin fragments (XV) and (XVI) were prepared using both photo-labile (IV) and acid labile (VI) linkage agents. Excess (2.5 fold) of symmetrical anhydride was used throughout together with a

highly functionalized (1.2 meq/g) resin. As before, some 30% detachment from the resin occurred for both peptides when Fmoc-amino-acids were used for the second step with the reactive photo-labile linkage. In the case of (XVI), the synthesis was repeated using BpocTyr(Bu^t) in place of the Fmoc derivative, deprotection with 0.09N HCl-AcOH, and *in situ* neutralization after addition of Fmoc-alanine anhydride. Dioxopiperazine formation was reduced to 3% but a major impurity (~20%) isolated after photolysis was shown by 400 MHz nmr to be a des-*t*-butyl form of (XVI), presumably the unprotected tyrosine derivative, since only this of the two *t*-butylated residues present in (XVI) had been exposed to acid. As noted earlier, O-*t*-butyl tyrosine is the most labile of the *t*-butyl side chain protected amino-acids. This result is, incidentally, of very poor augury for solid phase strategies based on the combination of N_α-Bpoc and side chain *t*-butyl protection.[17] Photolysis of resin-bound (XVI) cleaved 46% of the peptide (Figure 2a) which was readily purified by chromatography on silica gel (Figure 2b).

Somewhat better yields were obtained in the photolysis of the pentapeptide (XV) (Figure 3a). Purification was by chromatography on silica as before (Figure 3b).

More recently, syntheses of both gastrin fragments have been completed using the acid-labile linkage agent (VI) but under otherwise similar reaction conditions. Fmoc-amino-acids were used throughout. About 10% of the peptide was lost from the resin in both cases. This loss is surprising and needs further investigation. Cleavage of (XVI) was affected with 1% TFA-CH_2Cl_2 as mentioned previously, releasing 83% of the total peptide in 30 min. Contamination by des-*t*-butylated material was only ~3% (Figure 2c). The tetra-*t*-butyl ester (XV) was detached from the resin in 96% yield under similar conditions without detectable loss of side chain protecting groups (Figure 3c).

Fragment Condensation

Some preliminary studies have been carried out on the assembly of protected segments (XV), (XVI), onto the resin-bound tetrapeptide (XVII). Model studies are of limited value as the problems encountered are likely to be individual to each particular case as our further experience has shown. Nevertheless, these preliminary studies provide information about the likely efficacy of activation and coupling conditions. The gastrin series was selected for study only because of the availability of authentic C-terminal octa- and tridecapeptide amides from previous stepwise synthesis.[16] The products from fragment coupling were not, in

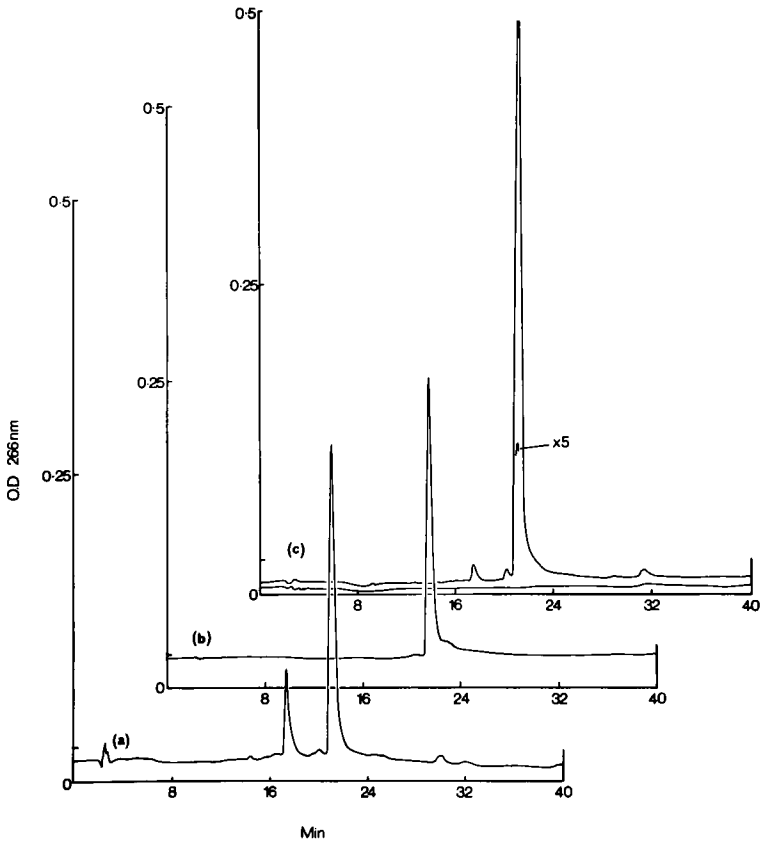


Fig. 2. Hplc of protected tetrapeptide (XVI): (a) Total crude product from photolysis; (b) after purification by silica column chromatography using a gradient of chloroform to chloroform/methanol/acetic acid (85:10:5 by volume); (c) crude product from synthesis using the acid-labile linkage agent (VI). Hplc conditions: Vessel A, 0.01M NH_4OAc pH 4.5; vessel B, 90% acetonitrile 10% A. Peptides were eluted using a linear gradient from 20-100% over 40 min at a flow rate of 1.5 ml/min.

fact, expected to match in purity and yield those from the earlier stepwise synthesis which proceeded extremely well.

All experiments were carried out on a small scale (~40 mg resin-bound tetrapeptide), initially with fragments derived from the photolysis route. fragment (XVI) was insoluble in methylene chloride, our preferred solvent for carbodiimide-mediated activation. This is likely to be rather general property of protected fragments of any size. In this case, recourse was had to the slower activation by carbodiimide in dimethylformamide in the presence of hydroxysuccinimide. After 24 h, the whole reaction mixture was added to the resin-bound tetrapeptide (XVII). The coupling

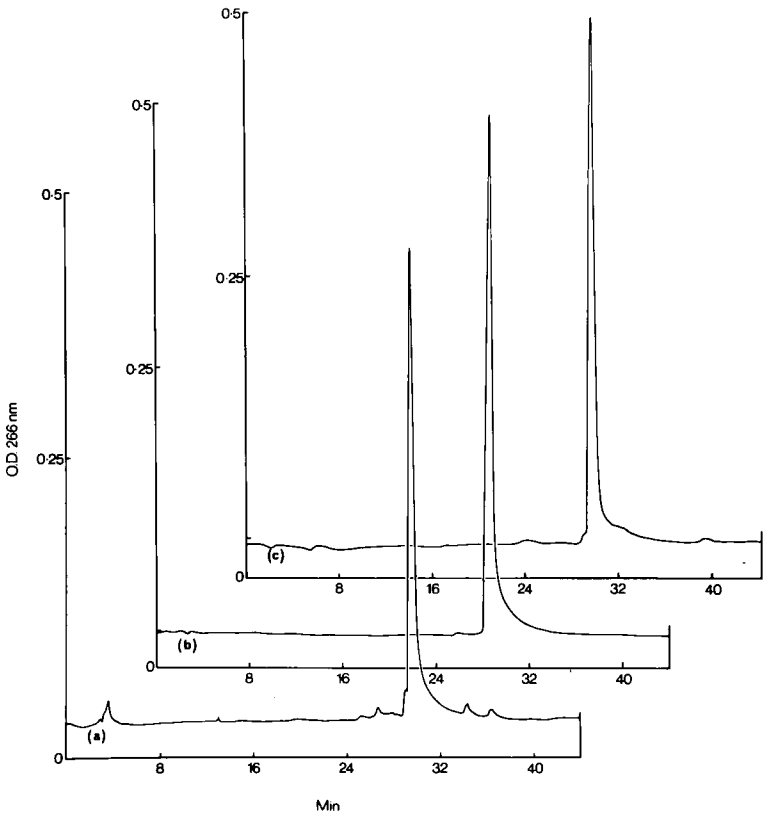


Fig. 3. Hplc of protected pentapeptide (XV): (a) Total crude product from photolysis; (a) after purification on a silica column (Lobar C) using a gradient of chloroform to chloroform/methanol/acetic acid (85:10:5 by volume); (c) crude product from synthesis using the acid-labile linkage (VI). Hplc conditions as in Figure 2.

yield after 24 h acylation was 81%. The second fragment (XV) was more soluble, and was activated in methylene chloride for 2 h at 0° with dicyclohexylcarbodiimide in the presence of hydroxybenzotriazole. The coupling reaction was carried out in dimethylformamide as before and gave a yield of 90%. Both the foregoing experiments were carried out using substantial (4-fold) excesses of acylating components. The experiments were repeated under rather similar conditions but using only 2-fold excesses of reactants and on three times the scale. Yields were 90 and 73%, respectively. These figures probably reflect some variability arising from the small scale of the experiments, but the overall results are encouraging. Products from both series were fully deprotected and cleaved from the resin by ammonolysis. The ion-exchange chromato-

gram from the first experiment is shown in Figure 4. The major peak was identified with authentic des-Trp[1]-Leu[12]-human minigastrin I by tlc and hplc (Figure 4f, insert). The yield of isolated product was 41%. In the second experiment, the yield was 59%. Good amino-acid analyses were obtained throughout.

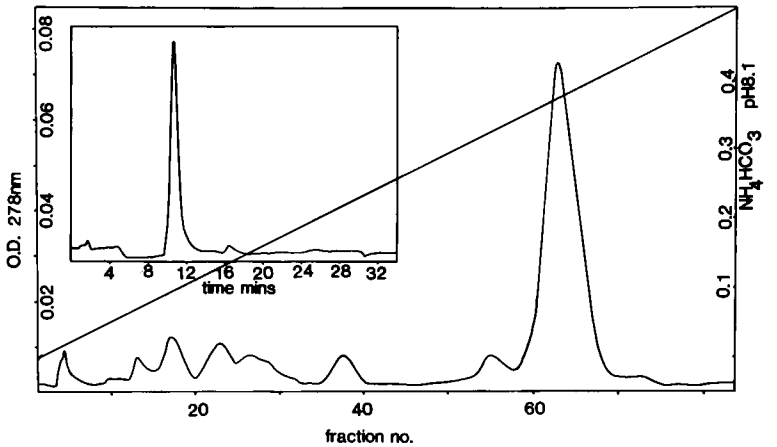


Fig. 4. Anion-exchange chromatography of des-Trp[1]-Leu[12]-human minigastrin. Insert: hplc of main peak.

Conclusion

There seems no doubt that solid phase assembly of solid phase-synthesized segments will, in cases, present a viable route. The acid-labile linkage agent (VI) presents a new opportunity for segment synthesis probably in better yield than that through photolysis. Caution will be needed in the presence of multiple *t*-butyl side chain protecting groups. Major problems in the overall procedure are more likely to arise from sparing solubility of protected segments. Solution to this problem may require development of a new generation of side chain protecting groups designed to confer increased polarity and solubility to the protected peptide chain. Such protecting groups might also be advantageous in stepwise solid phase synthesis.

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SYNTHETIC HUMAN INSULIN BY SELECTIVE DISULFIDE BRIDGING: POLYMER PHASE SYNTHESIS OF THE B-CHAIN SEGMENTS AND EXPERIMENTS TOWARDS CARRIER-SUPPORTED CONDENSATION BY CARBONYL-DI-IMIDAZOLE/ 1-HYDROXYBENZOTRIAZOLE

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Introduction

The insulin B-chain has been assembled in several laboratories by various methods of peptide synthesis.[1] In this communication we describe the preparation of the 30-residue peptide via segment condensation in polymer gel phase and the utilization of selectively removable cysteine sulfur protecting groups. Deletions and truncated peptides were suppressed by photometric control of the polymer phase synthesis with Ddz-amino acids,[2] followed by detachment, purification, and characterization of intermediates and use of pure segments for condensations in polymer phase. The five protected segments (Figure 1) were prepared on a bromophenacyl gel phase. Each of the C-terminal amino acids was attached to the gel phase by the cesium salt method.[3] The problem of Schiff's base and diketopiperazine formation was minimized by addition of preformed symmetrical anhydrides[4] to the polymer supported segments, prior to deprotonation. The fully protected peptides were released from the gel phase by treatment with a solution of 0.5 N triethylamine in methanol/dioxane (1:1, v/v) containing 0.5-5% 1 N NaOH.

Purification of the protected peptides was carried out on Sephadex LH-20 in methanol or DMF and on prepacked silica gel columns by gradient elution.

The C-terminal segment V remained on the gel phase, whereas the other four segments (I-IV) were coupled to peptide V in gel phase via two different procedures, namely activation with dicyclohexylcarbodiimide/-1-hydroxybenzotriazole (DCC/HOBt)[5] or carbonyl-di-imidazole/1-hydroxybenzotriazole (CDI/HOBt).[6]

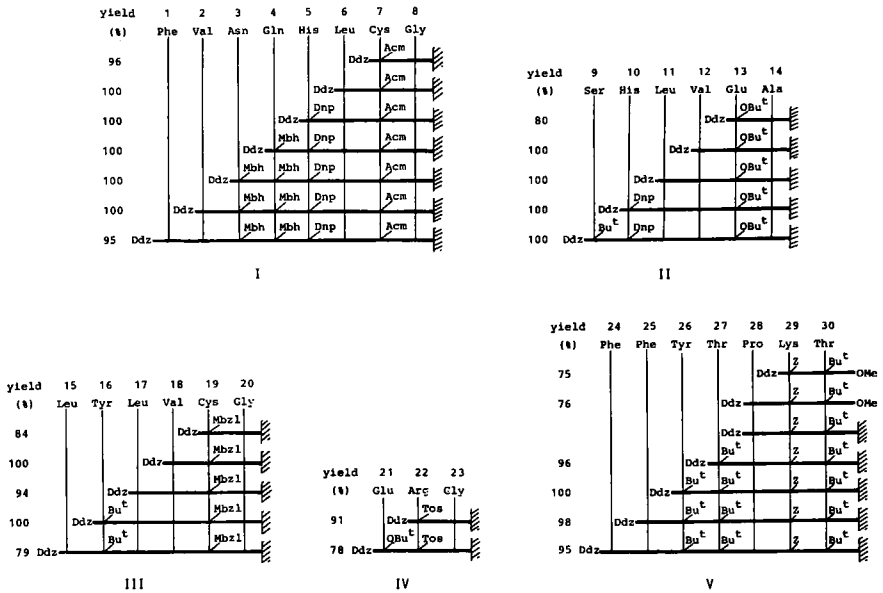

Fig. 1. Synthesis of the five insulin B-chain segments (I-V) in gel phase.

Results

The fully protected B-chain segments were synthesized in gel phase with minimal side reactions and an average yield of 93.6% (Figure 1). Peptides were released from the polymer phase within 6-8 hours and chromatographed. Pure, fully protected segments were obtained in 21% average yield and characterized by melting point, optical rotation, thin layer chromatography (TLC), amino acid and elemental analysis (Figure 2).

fragment	$[\alpha]_D^{25}$ prior to activation	$[\alpha]_D^{25}$ recovered after activation	melting point °C	elementary analysis in ( ) calcd. C H N S	amino acid analysis	TLC R_f-values*	yield (overall)
I	-22.3° c=0.8,DMF	-23.4 c=0.8,DMF	235-245	58.38 6.57 10.31 2.16 (59.70)(6.06)(11.48)(1.75)	Asn1.03(1),Gln1.01(1),Gly1.12(1) Leu1.00(1),Phe0.84(1),His0.88(1) Val0.93(1),Cys decomp.	0.90(S2) 0.31(S3)	19%
II	-19.3 c=0.7,CH_3OH	-18.8 c=0.7,CH_3OH	195-200	58.37 8.06 10.94 (58.28)(7.75)(11.33)	Ala0.95(1),Glu0.95(1),Val1.00(1) Leu1.05(1),His1.10(1),Ser0.89(1) (part. decomp.)	0.71(S1) 0.28(S2)	12%
III	-49.1 c=0.8,CH_3OH	-49.2 c=0.7,CH_3OH	182-190	61.19 7.52 7.70 3.30 (62.01)(7.57)(7.89)(3.01)	Leu2.28(2),Tyr0.91(1),Val0.94(1) Gly1.02(1),Cys decomp.	0.90(S2) 0.31(S3)	13%
IV	- 6.2 c=0.7,$CHCl_3$	- 7.0 c=0.8,$CHCl_3$	146-150	54.83 6.88 10.61 3.66 (54.53)(6.61)(10.60)(4.04)	Gly0.95(1),Glu1.04(1),Arg1.01(1)	0.41(S2) 0.78(S1)	39%

*S1=butanol/acetic acid/water 4:1:1 ; S2=chloroform/methanol/acetic acid 85:10:5 ; S3=benzene/acetic acid 7:1

Fig. 2. Analytical data of the insulin B-chain segments.

The two parallel segment coupling series using DCC/HOBt and CDI/HOBt resulted in average yields of 66% and 75% respectively (Table I). The CDI/HOBt-reaction was complete after 15 hours (for comparison: DCC/HOBt, 3 days). Excess activated segment was recovered in 70-90% yield after filtration from polymer phase and hydrolysis with water prior to recycling. No significant change in optical rotation ($<2°$) was detectable (Figure 2). The protected B-chain was cleaved from the gel phase by treatment for 20 h with a solution of 0.5 N triethylamine in methanol/dioxane (1:1, v/v) containing 1% aqueous 1 N NaOH. The yield of crude product after detachment (based upon B-chain load on the polymer support) was 95%, the yield of purified B-chain (based on crude product) was 30%.

The purification of the detached B-chain crude product was carried out by trituration with methanol, dissolution of the residue in dimethylformamide (DMF) and column chromatography on Sephadex LH-20 in DMF. The homogeneity of the product was determined by TLC (R_f = 0.82 in butanol/acetic acid/water, 4:1:1, and $R_f = 0.13$ in chloroform/-methanol/acetic acid, 85:10:5) and elution (symmetrical peak) from the LH-20/DMF column. The protected peptide was characterized by amino acid analysis: Asp 1.3 (1), Thr 2.4 (2), Ser 1.0 (1), Glu 4.1 (3), Pro 1.4 (1), Gly 2.6 (3), Ala 1.0 (1), Val 2.3 (3), Leu 3.1 (4), Tyr 1.6 (2), Phe 3.1 (3), Lys 1.4 (1), His 1.1 (2), Arg 1.2 (1), Cys 2.3 (2).

Discussion

The incorporation of Ddz-amino acids into insulin B-chain segments — prepared via gel phase synthesis — was monitored photometrically based on Ddz-cleavage. This determination of peptide bond formation needs no additional application of reagents, such as fluorescamine or ninhydrin, which, because of their bulky nature, often react incompletely with amino functions in the polymer phase.

Table I. Yields of the Segment Couplings in Gel Phase

Segment Coupling	Yield*	
	DCC/HOBt	CDI/HOBt
IV + V	90-100%	90-100%
III + IV-V	40-55%	30-35%
II + III-IV-V	30-35%	75-85%
I + II-III-IV-V	80-100%	90-100%

*Mean values from series determined by amino acid analysis and quantitative measurement of the Ddz-fission product.

The tendency of phenacyl support-bound dipeptides to form Schiff's bases (yellow-orange color change on the solid support) or diketopiperazines (determined by checking the filtrates from polymer phase reactions) was negligible (< 10%) after application of Ddz-amino acid symmetrical anhydrides in polymer phase prior to deprotonation. The purification of the protected segments on prepacked silica gel columns was found to be a convenient method to obtain pure products within 12 h using gradients from $CHCl_3$ to $CHCl_3$/ethanol.

The advantages of the newly explored carbonyl-di-imidazole/1-hydroxybenzotriazole (CDI/HOBt)-method over the commonly used dicyclohexylcarbodiimide/1-hydroxybenzotriazole (DCC/HOBt)-method are reflected in the results obtained. The possibility of recycling excess activated peptide is of great economic importance. For example, after three repetitive excess applications of recycled segment III, the coupling was increased by 50% yielding 30-35%.

To verify minimum racemization during CDI/HOBT-activation, studies with model peptides (Anderson and NMR-test) were performed showing racemization (<5%) to the same extent as with DCC/HOBt-activation. In our experience, determination of the optical rotation gives an accurate measure of the quality of the particular segment after activation. On the average, optical rotations of segments recovered differed by less than $\pm$ 2° from those of peptides prior to activation (Figure 2).

By contrast to widely used coupling procedures, such as azide, active ester, or DCC/HOBt requiring reaction times of up to one week, the CDI/HOBt-reaction is complete after 15 hours.

Purification of crude products was facilitated by the solubility in methanol of the truncated sequences. The large differences in molecular weight of the segments and the ionizeable nitrophthalic acid blocking group subsequently attached to unreacted amino functions allowed a relatively problem-free separation of the B-chain from byproducts.

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TOTAL SYNTHESIS OF HUMAN PARATHYROID HORMONE (1-84)

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Human parathyroid hormone (hPTH) is 84 amino acids long and of known sequence.[1] The native hormone is in short supply and the majority of the biological and physiological studies have been carried out using synthetic fragments of the hormone. The biological activity of hPTH is localized to the amino terminal 34 amino acids. Segments representing the full sequence of the peptide have been synthesized.[2-4] The total synthesis was considered difficult because of the limitations associated with solid phase peptide synthesis using the conventional Merrifield resin. Acidolysis of the peptide bond occurs during long stepwise synthesis and results in poor yields and numerous side products. The PAM resin designed by Merrifield and coworkers[5] possesses increased acid stability. The phenylacetamidomethyl (PAM) group inserted between the peptide and the polystyrene matrix reduces the rate of peptide loss from the resin to nearly 1% of that from conventional styrenedivinylbenzene resin[5] and enhances the applicability of this resin to the synthesis of longer peptide chains.

This report describes the total synthesis of hPTH (1-84) using the phenylacetamidomethyl (PAM) resin. The biological and immunological properties of the synthetic peptide are compared with those of the native hormone. hPTH (1-84) was synthesized by the Merrifield solid phase method[6] using a Beckman 990B peptide synthesizer and phenylacetamidomethyl (PAM) resin as the solid support. The amino acid sequence of hPTH and the various side-chain protecting groups used in the synthesis are shown in Figure 1. The Boc group was used to protect the α-amino groups of all amino acids, except arginine, where the more soluble amyloxy derivative was employed. Initially, a 25% solution of TFA in CH_2Cl_2 was used for deprotecting the α-amino groups. The acid concentration was gradually increased to 40% by step 40. Amino acids were attached to the peptide-resin by coupling equimolar ratios of dicyclohexyl carbodiimide (DCC) and the Boc-amino acid for 120 minutes. The active ester method and a coupling time of 960 minutes were used to couple the *p*-nitrophenyl esters of asparagine and glutamine to the pep-

tide. Double coupling was employed at each step and both deprotection and coupling were qualitatively monitored using the Kaiser ninhydrin test. After the second active ester coupling the peptide-resin was treated with N-acetylimidazole[7] to block unreacted amino groups. Boc-N^{in}-formyl tryptophan was used in order to protect the indole nucleus of tryptophan from oxidation[8] and avoided the necessity of using reducing agents.

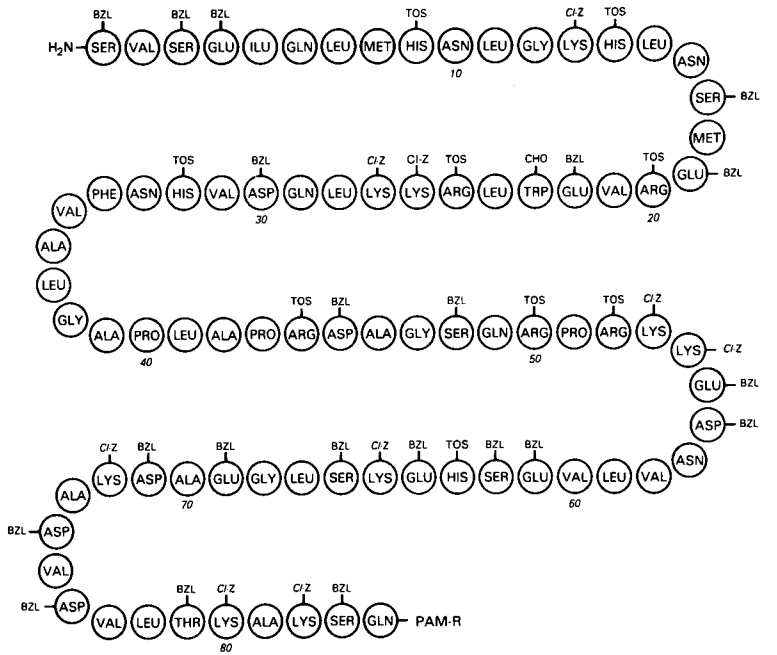

Fig. 1. Amino acid sequence of hPTH showing the side-chain protecting groups used during synthesis.

The protected peptide-resin was treated with anhydrous hydrogen fluoride in the presence of anisole and a few drops of methylsulfide at 0°C for one hour to cleave the peptide from the resin and remove all side-chain protecting groups. The N^{in}-formyl group of tryptophan was removed by treatment with 1M piperidine in 8M urea at 0°C for 45 minutes. The crude peptide was initially purified by gel filtration on BioGel P-2 in 0.1M acetic acid. This was followed by ion exchange chromatography on CM-Sephadex with ammonium acetate containing 6M urea and mercaptoethanol. Eight fractions were collected and the fraction corresponding to the elution position of the native hPTH was further characterized. Sequence analysis of the crude peptide after gel

filtration was carried out using the Edman procedure on a modified Beckman 890B sequencer.

Amino acid composition of the crude peptide as well as the various fractions from the CM-Sephadex were determined on a Beckman 120 analyzer after acid hydrolysis in 5.7 N HCl at 110° C for 24 hours in the presence of mercaptoethanol. Polyacrylamide disc-gel electrophoresis was performed in 8M urea at pH 4.4. Isoelectric focusing was carried out in polyacrylamide gels between pH 3-10.

Biological activities of the crude peptide as well as the various fractions from CM-Sephadex were determined using the adenylate cyclase assay in dog renal plasma membranes.[9-10] Standard radioimmunoassay methods[11] were used to compare the immunoreactivity of the synthetic and native hormone.

The yield of synthetic peptide following CM-Sephadex chromatography is 14% based on the total peptide after HF cleavage. Amino acid composition of the peptides from pools 4 and 5 are in excellent agreement with the theoretical values of native hPTH (1-84). Amino terminal sequence analysis of the synthetic peptide for 60 cycles was consistent with the reported sequence and no major deletions were observed. Polyacrylamide disc-gels in 8M urea at pH 4.4 of the synthetic peptide revealed a single band which was identical to the bands of both human and bovine native hormones. On isoelectric focusing the synthetic peptide had an isoelectric point comparable to the native forms of both human and bovine hormones.

The *in vitro* bioassay utilizing the activation of adenylate cyclase in dog renal plasma membrane revealed the peptide from pool 5 to be as active as the native hormone and that from pool 4 to have 95% activity of the native hormone (Table I). The crude peptide after gel filtration was only 25% as active as the native hormone. The immunoreactivity of the synthetic protein was equivalent to the native hormone. These results suggest that the synthetic hormone is virtually identical in biological potency and immunoreactivity to the native hormone. In addition, it can also be concluded that the total synthesis of a larger peptide can be achieved in good yield using the PAM resin. The long "spacer" acetamido group introduced by Sparrow[14] may not be necessary to reduce the problems associated with the solid phase synthesis. The availability of the synthetic hormone will now permit detailed biological and physiological studies on the intact hormone.

Table I. Biological Activity of Synthetic hPTH.

Sample	ng Peptide for 1/2 Max Activity	% of Native hPTH, 1-84
Native hPTH 1-84	100	-
Crude synthetic hormone	400	25
CM Sephadex, pool 1	2,000	5
pool 2	200	50
pool 3	180	60
pool 4	110	95
pool 5	100	100
pool 6	150	75
pool 7	200	50
pool 8	300	33

Acknowledgements

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SYNTHESIS OF RETINYLIDENE-PEPTIDES OF BACTERIORHODOPSIN FROM *HALOBACTERIUM HALOBIUM**

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The purple membrane from *Halobacterium halobium* contains a single protein to which retinal is bound *via* a Schiff's-base linkage to a lysine residue. On light excitation the chromophore undergoes a photocycle during which protons are pumped across the membrane (for a recent review see Stoeckenius[1]). There are three observations which distinguish the ground state of bacteriorhodopsin from that of protonated retinylidene azomethines: 1. the bathochromic shift of the absorption maximum (440 → 570nm) 2. the stabilization of the 13-*cis* over the all-*trans* conformation, and 3. the induction of chirality in the chromophore. In order to elucidate these protein-retinal interactions we synthesized retinylidene-peptides with sequences resembling the retinal binding site: TfaGly-Lys(Tfa)-Lys-Phe-Tyr-Ala (I), TfaGly-Asp-Ala-Lys(Tfa)-Lys-Phe-Tyr-Ala (II), and TfaGly-Val-Ser-Asp-Pro-Asp-Ala-Lys(Tfa)-Lys-Phe-Tyr-Ala (III).

Materials and Methods

Peptides were synthesized by the solid-phase method, using the PAM resin[2] as support. Protected amino acids were purchased from Peninsula Laboratories, San Carlos, CA, (BocAla, BocPhe, BocLys(Cl-Z), BocPro, BocSer(Bzl), Boc(Val) and Bachem (TfaGly, BocLys(Tfa)). All solvents were reagent grade. Dichloromethane and trifluoroacetic acid (TFA) were distilled from P_2O_5. TFA was redistilled from valine. BocAsp(cHex) was synthesized according to Tam *et al.*[3] The synthesis included two-hour double couplings with symmetrical anhydrides. α-Amino groups were deprotected by treatment with 50% TFA/CH_2Cl_2 for 30 minutes. The neutralization step consisted of three two-minute washes with 5% diisopropylethylamine in methylene chloride. The cleavage of the peptides from the resin was performed by hydrogen fluoride (HF) treatment at 0° C for 30 minutes. The crude peptide mixture was purified by gel-filtration (G-15) and anion-exchange chromatography (Aminex A-25). The purity was checked by high pressure liquid chromatography (HPLC) using a reverse phase column, electrophoresis, thin-layer chro-

*Dedicated to Professor H. Holzer on the occasion of his 60th birthday.

matography (TLC), and amino acid analysis. The position of the free amino group was verified by tryptic digestion of the peptides and subsequent amino acid analysis of the chromatographically-separated fragments. All-*trans*-N-retinylidene-n-butylamine (RetBut) was synthesized according to Blatz *et al.*[4] The protonated Schiff's-bases (C = NH) were prepared by adding TFA to a solution of I, II, III and RetBut in methanol.

The peptides were allowed to react with retinal in the dark and under argon using methanol and DMF as solvents with dry Na_2CO_3 and molecular sieve (4Å) present. Excess reagent was removed by gel-filtration (LH-20).

Results and Discussion

Bacteriorhodopsin, whose sequence was determined by Ovchinnikov *et al.*[5] contains seven helical segments which span the membrane. The binding site of retinal was proposed to be close to the N-terminus at (Lys-41)[6] on the cytoplasmic side of the membrane. The amino acid composition around this region includes two aspartic acid residues and one tyrosine. The rationale to synthesize peptides I, II, and III was also guided by the observation that aromatic residues play an important role in the photocycle and proton pumping.[7] Furthermore, the bathochromic shift can be explained by models containing negatively-charged groups in the neighborhood of the retinal (point-charge model).[8]

The synthesis of the peptides followed standard solid-phase procedures. To ensure the incorporation of the retinal at the correct site, a protecting group for the amino group of the N-terminus and the second lysine had to be chosen. Trifluoroacetyl derivates fulfill this condition since they are stable under the final HF-cleavage conditions but are easily removable without disrupting the Lys-retinal bond.

After HF-cleavage of the protected peptide-resins, the crude product was purified by gel-filtration (G-15) and anion-exchange chromatography (Aminex A-25). Peptides represented by the main peak (overall yield: 40-50%) were pure as judged by amino acid analysis, electrophoresis, TLC and HPLC. Thus, taking criteria such as size, composition, charge and hydrophobicity, the peptides were homogeneous.

One crucial requirement for the study of protein-retinal interaction is to know the definite position of the retinal Schiff's base. For this purpose the Tfa-peptides were treated with trypsin, and the proteolytic fragments were separated using TLC. As expected two spots were detected, one of which was ninhydrin positive and UV-active, whereas

the other was only ninhydrin positive. The amino acid analysis confirmed that the peptides were cleaved only at the C-terminal side of Lys-41, indicating the free amino-group to be in the correct position.

The Schiff's-base was readily formed after the addition of all-*trans*-retinal. Table I summarizes the absorption maxima.

Table I. Absorption Maxima (nm) of RetBut and the Retinylidene Peptides, I, II, and III and Their Protonated Schiff's-base in CH_3OH.

		RetBut	I	II	III
λ_{max}	C=N	363	362	354	359
λ_{max}	C=NH	444	445	446	447

Figure 1 shows the absorption spectra of I and III as well as their protonation product.

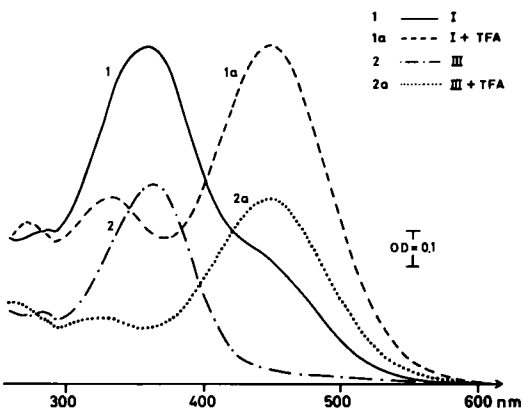

Fig. 1. Absorption spectra of I and III and their protonated Schiff's-base.

As can be seen from Table I and from Figure 1, the properties of I are comparable to ordinary Schiff's-bases of retinal like RetBut. However, in cases in which an extra charge is incorporated, as in II and III, a shoulder at 440 nm appears, which is enhanced in more hydrophobic solvents such as chloroform. The peak at 328 nm which appears on protonation is due to free retinal. Apparently, the carboxyl-group catalyses the hydrolysis of the Schiff-base.

Additional information can be obtained from difference spectra (retinylidene-peptides — retinal+peptides), which show three positive peaks at 280, 340, and 460 nm for III.

From these data it can be concluded that an intramolecular interaction of an aspartate with the protonated Schiff-base nitrogen leads to an equilibrium between unprotonated and protonated azomethine. Following this protonation of the Schiff's-base, tyrosine is repositioned such that it can interact with the chromophore. Furthermore, it could be shown by circular dichroism measurements that these interactions lead to the induction of chirality.

The initial event in the function of bacteriorhodopsin is the absorption of green light by a protonated Schiff's-base. Although peptides II and III represent only 5% of the sequence of the protein, certain features of the purple membrane are already found in these simple models.

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SYNTHESIS OF THYMOSIN β_4

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The natural thymosin β_4 was isolated from calf thymus gland as one of the components of thymosin fraction 5 and was found to exhibit several biological activities important for maturation and maintenance of the immune systems in man and in mammals.[1,2] It induces expression of the terminal deoxynucleotidyl transferase (TdT) activity in transferase-negative thymocytes both *in vivo* and *in vitro*. Thus, it was suggested that the compound controlled the early stages of the maturation process of thymus dependent lymphocytes. The primary structure was determined to be: Ac-Ser-Asp-Lys-Pro-Asp-Met-Ala-Glu-Ile-Glu-Lys-Phe-Asp-Lys-Ser-Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn-Pro-Leu-Pro-Ser-Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys-Gln-Ala-Gly-Glu-Ser-OH.[1] A synthetic material was therefore needed to confirm the proposed structure and to serve as a better source of material for studying the functional roles of this peptide hormone in biological systems and at the same time to establish that the biological activities observed were not derived from some minor contaminants that might exist in the natural product.

An automated synthesis[3,4] of thymosin β_4 was thus undertaken. Boc-Ser(Bzl)-OH was esterified onto $HOCH_2$-C_6H_4-CH_2-CONH-CH_2-resin (PAM-resin)[5] by the DCC-pyridine procedure[6] in a Beckman Model 990B automatic solid phase synthesis apparatus under manual mode operation and the synthesis was continued with Boc-Ser(Bzl)-O-PAM-resin (2.0 g, 0.15 mmol/g) obtained under automatic mode operation by sequentially incorporating one amino acid residue at a time, using 5 equivalents each of Boc-amino acid and DCC, according to the general principles of solid phase peptide synthesis,[3,4] with coupling times of 120 min. Boc-group was removed by 30 min treatment with 40% TFA containing 0.05% indole. The side chain functional groups of aspartic acid and glutamic acid were protected as benzyl esters, serine and threonine as benzyl ethers, and lysine as N^{ϵ}-2-chlorobenzyloxycarbonyl derivative. No side chain protecting group was used for glutamine and asparagine. For those couplings, N-hydroxybenzotriazole was added automatically

in order to minimize the side reactions.[7,8] Ninhydrin test[9] was performed to determine the completion of the coupling and when necessary double coupling was carried out. Acetyl moiety at the N-terminal was introduced as acetic acid using the same program (DCC, 120 min) for all the other Boc-amino acids. The acetylated tritetracontapeptide resin (3.3 g) thus obtained was then cleaved with anhydrous HF (40 ml)[10] in the presence of anisole (4 ml) and dimethylsulfide (1.5 ml) at 0° for 60 min. The crude product (0.8 g) was desalted on a Bio Gel P-6 column and then chromatographed on a DEAE-cellulose column (2.5 x 50 cm) eluted with a linear gradient of NH_4HCO_3 (pH 7.8; 0.02-0.075 M). The material under the major peak was then pooled and rechromatographed on the same column yielding 40 mg of desired product. It was found to be homogeneous on high pressure liquid chromatography performed on a Beckman Model 332 HPLC apparatus with an Ultrasphere ODS 5μ column (0.46 x 25 cm) eluted with 21% acetonitrile in 0.005 M H_3PO_4/KH_2PO_4 (pH 2.5) at a flow rate of 1.2 ml/min. Acrylamide gel isoelectric focusing (pH 3.5-9.5) showed a single band migrating identically with the natural compound. The tryptic maps of the synthetic and natural products were superimposable. Each contained nine heavy spots and one faint spot at the corresponding locations. Tryptic digestion was performed in 1% NH_4HCO_3 (pH 8.3) for 3 hr (37°) with an enzyme to substrate ratio of 1:50. Paper chromatography (n-BuOH:HOAc:H_2O = 4:1:5) was the first dimension and paper electrophoresis (pH 1.9, 60 volts/cm) was the second dimension. The amino acid composition of the synthetic peptide agreed with the structure: Lys, 8.97(9); NH_3, 4.81(4); Asp, 4.00(4); Thr, 2.82(3); Ser, 3.61(4); Glu, 11.0(11); Pro, 3.00(3); Gly, 0.98(1); Ala, 1.96(2); Met, 0.92(1); Ile, 1.82(2); Leu, 1.95(2); Phe, 0.97(1). Numbers in parentheses are theoretical values. The synthetic hormone was shown to be equally potent as natural hormone in the macrophage migration inhibitory factor (MIF) assay as well as in the *in vivo* assay for the induction of terminal deoxynucleotidyl transferase (TdT) in the hydrocortisone treated mice.[11,12] Table I summarizes the biological data for synthetic and natural thymosin β_4.

Acknowledgement

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Table I. Biological Activities of Synthetic and Natural Thymosin β_4

Assay System	Synthetic Thymosin β_4	Natural Thymosin β_4
A. MIF Assay[a]	(migration inhibition)	(migration inhibition)
0.05 nM	36.7 %	40.8 %
0.50 nM	43.5 %	47.8 %
B. *In vivo* TdT induction assay[b]	(activity increase)	(activity increase)
	41.5 %	38.0 %

[a] Macrophage migration inhibitory factor (MIF) assay was performed according to Ref. 11.
[b] *In vivo* effect of thymosin β_4 on terminal deoxyneucleotidyl transferase (TdT) activity in thymocytes of hydrocortisone acetate treated C57Bl/6J mice was assayed according to Ref. 12.

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SOLID-PHASE SYNTHESIS OF COOH-TERMINAL FRAGMENTS OF CHOLECYSTOKININ OCTAPEPTIDE

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A series of COOH-terminal fragments of Cholecystokinin (CCK) 26-33 has been synthesized by the solid-phase method. Because of the instability of O-sulfated tyrosine to strong acid,[1] the use of the β-phenacyl ester[2] (β-OPac) which can be removed with sodium thiophenoxide[3] prior to ammonolysis, seemed to be ideal. Following the synthesis (Figure 1) of CCK 30-33, treatment of the peptide resin with 1M sodium

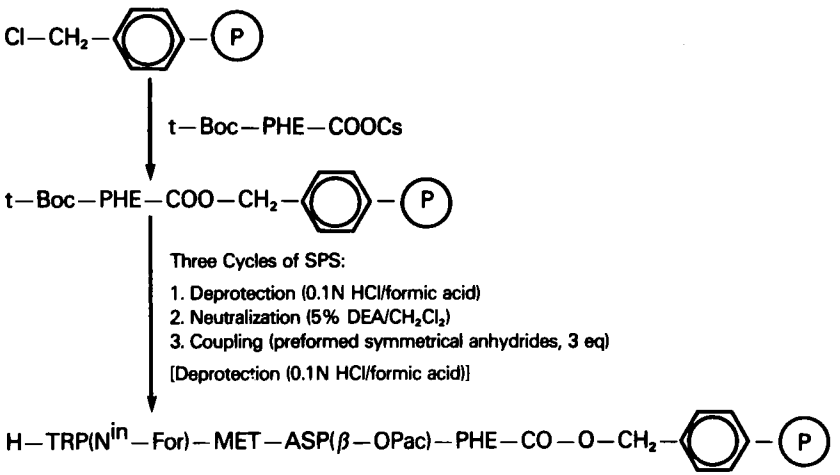

Fig. 1. Solid-phase synthesis of Trp-Met-Asp-Phe amide (CCK 30-33)

thiophenoxide in DMF (15 h, rt, argon), and ammonolysis (30% NH_3 in methanol, 48 h, 4° C), the product isolated after countercurrent distribution was shown to be a mixture of isoasparaginyl and aspartyl peptide. Analyses after acidic and enzymatic hydrolysis of the two countercurrent distribution fractions of the solid-phase synthesis peptide are shown in Table I. The presence of approximately 2 residues of ammonia and the absence of aspartic acid and of asparagine in the major component (60% of the material) indicate it to be the isoasparaginyl peptide. The second component (40% of the total) contains approximately 35% of α-aspartyl

and/or β-aspartyl peptide. These results point to the presence of aminosuccinyl (ASU) peptide prior to the ammonolysis.

The peptide Boc-Asp (β-OPac)-Phe amide was synthesized from Boc-Asp (β-OPac)-COOPfp (Pfp = pentafluorophenyl) and Phe-$NH_2 \cdot HCl$ in the presence of 1 eq of diisopropylethylamine (DIEA). The amide was prepared because of the possible increase in ASU peptide formation due to the presence of a COOH-terminal ester. The dipeptide amide was transformed to ASU peptide, at various extents, under these conditions: (a) 1M sodium thiophenoxide/DMF: total conversion in 5 min; (b) 1 eq TEA/DMF, and (c) 5% DIEA/CH_2Cl_2:$t_{1/2} \simeq 24$ h in each case. The aminosuccinyl product was isolated after treatment (5 min) with 1M sodium thiophenoxide. The parent peptide and its ASU derivative were characterized as follows: Boc-Asp(β-OPac)-Phe amide: mp 157-160°C, R_f^1 0.60, solvent system 1: ethyl acetate:acetic acid = 99:1, R_f^2 0.81, solvent system 2: ethyl acetate:pyridine:acetic acid: water = 90.0:4.9:1.5:3.6,

Table I. Characterization of Cholecystokinin 30-33 after Countercurrent Distribution

	Acidic Hydrolysis[1]					Enzymatic Hydrolysis[2]			
	TRP	MET	ASP	PHE	NH_3	TRP	MET	ASP	PHE
Major product	0.91	1.00	1.05	1.05	2.25	1.00	0.79	0.00	0.17
Minor product	0.96	1.00	1.00	1.04	1.95	1.00	0.75	0.32	0.40

Numbers indicate μmoles. Acid hydrolysis: 6N hydrochloric acid, 110° C, 24h. Enzyme hydrolysis: leucine aminopeptidase/CCK 30-33 = 1/10, 37°, 48 h.

amino acid analysis (0.63 μmole applied) values in μmoles Asp 0.63, Phe 0.59, NH_3 0.72; Anal. calc. for $C_{26}H_{31}N_3O_7$ (497.55), C 62.76, H 6.28, N 8.44%, Found C 62.52, H 6.05, N 8.29%; Boc-ASU-Phe amide: mp 84-89°C, R_f^1 0.75, R_f^2 0.87, amino acid analysis (0.67 μmoles applied) Asp 0.67, Phe 0.66, NH_3 0.70; Anal. calc. for $C_{18}H_{23}N_3O_5$ (361.40), C 59.82, H 6.41, N 11.63%, Found C 59.84, H 6.32, N 11.00%. The ASU product showed the expected electrophoretic behavior, in that no migration occurs at pH 6.7. However, after treatment with 1% triethylamine, the resulting product migrates to the anode under the same conditions. The ASU peptide is transformed further to the open form on standing for 20 h in 1M sodium thiophenoxide ($R_f^1 = 0.0$, on direct application, $R_f^1 = 0.29$ after removal of the sodium thiophenoxide; electrophoretic mobility to the anode). It seemed possible that the presence of sodium hydroperoxide resulting from the oxidation of the sodium thiophenoxide or of

sodium hydroxide resulting from adsorption of water may account for this. Therefore the preparation of the sodium thiophenoxide and its reaction with the peptide were carried out in a nitrogen flushed glove box. After 30 min. the starting peptide was completely transformed (ASU peptide, ≃70%; open form, ≃30%). A further 30 h of reaction revealed no opening of the cyclic peptide. If care is taken to exclude oxygen and water, no nucleophiles can form which are capable of hydrolyzing the ASU peptide. These observations strongly suggest an unavoidable competitive formation of ASU peptide catalyzed by the reagent itself. The only certain way to avoid these difficulties is to perform the cleavage under neutral conditions.

It seemed likely that the easily ionized SELENOPHENOL could form a complex with DMF which would allow the highly polarizable and nucleophilic selenium atom to attack the phenacyl ester. Adventitious oxygen and water would only cause some oxidation of the reagent without formation of damaging nucleophiles. The reagent (1 M in DMF) was successfully applied to Boc-Asp(β-OPac)-Phe amide and Boc-Trp(N^{in}-For)-Met-Asp(β-OPac)-Phe-resin prior to ammonolytic removal of the peptide from the resin. The resulting products were characterized as follows: Asp-Phe amide: mp 170° C (shrinkage and gas evolution) 225-227° C (melt), R_f^3 0.70, solvent system 3: 1-butanol:acetic acid:water = 3:1:1, R_f^4 0.66, solvent system 4: 1-butanol:acetic acid:pyridine:water = 30:6:20:24, amino acid analysis (0.50 μmoles applied) acid hydrolysis, values in μmoles: Asp 0.44, Phe 0.44, NH_3 0.50, enzyme hydrolysis (leucine aminopeptidase) 0.39 μmoles applied: Asp 0.36, Phe 0.35; Trp-Met-Asp-Phe amide: R_f^3 0.78, R_f^4 0.75, amino acid analysis, acid hydrolysis (0.46 μmoles applied): Trp 0.32, Met 0.41, Asp 0.44, Phe 0.44, NH_3 0.50, enzyme hydrolysis (leucine aminopeptidase) 0.46 μmoles applied: Trp 0.45, Met 0.43, Asp 0.44, Phe 0.47. Yields were 79 and 40% for the Asp-Phe amide and the Trp-Met-Asp-Phe amide, respectively.

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ENHANCEMENT OF PEPTIDE COUPLING REACTIONS BY 4-DIMETHYLAMINOPYRIDINE

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4-Dimethylaminopyridine (DMAP) is a much more powerful catalyst than pyridine in many organic reactions. It has been utilized to facilitate esterification of hindered alcohols with carboxylic anhydrides and acylation of poor nucleophiles by acylhalides.[1,2] The compound was also used as additive to dicyclohexylcarbodiimide (DCC) mediated reaction in anchoring the first amino acid[3-5] residue onto the hydroxymethylpolystyrene resins for solid phase peptide synthesis.[6,7] Coupling efficiencies in solid phase synthesis of oligonucleotides was found to be vastly elevated by the addition of DMAP.[8] Formation of thiol esters was also accelerated by this compound.[9] Applications of DMAP in many other systems have been recently reviewed.[10-12] We now wish to report the enhancement of DCC[13] and symmetrical anhydride coupling[14] by DMAP in the solid phase synthesis of peptides containing sterically hindered amino acids.[6,7]

The automated solid phase synthesis[15] of Boc-Ala-Cle-Ile-Val-Pro-Arg(Tos)-Gly-OCH_2-C_6H_4-resin (Cle: cycloleucine) was chosen as the model system for comparing the coupling efficiencies of DCC, DCC plus HOBT, symmetrical anhydride and DCC plus DMAP methods. The protected heptapeptide-resin samples obtained from each procedure were analyzed for their amino acid composition with the results shown in Table I.

It can be seen that only DCC-DMAP procedure gave the desired near quantitative coupling in the sterically hindered region of Cle-Ile-Val. The results of HPLC analyses of the crude protected heptapeptide amide Boc-Ala-Cle-Ile-Val-Pro-Arg(Tos)-Gly-NH_2 derived from ammonolytic cleavage of the peptide resin samples also indicated that there were far less impurities present in the product prepared by the DCC-DMAP method. The crude peptide from the DCC-DMAP method contained two very minor impurities, each less than 2%, and a major component with elution time of 12.5 min (Beckman 235329 Ultrasphere ODS 5 μ column; eluted with a linear gradient of 35-70% acetonitrile in 0.01 M KH_2PO_4 at a flow rate of 1.0 ml/min).

Table I. Amino Acid Composition of Boc-Ala-Cle-Ile-Val-Pro-Arg(Tos)-Gly-OCH_2-Resins Prepared by Different Methods[a]

Amino Acids	DCC	DCC-HOBT	Symm. Anhydr.[b]	DCC-DMAP
Gly	1.00	1.00	1.00	1.00
Arg	0.81	0.94	0.62	0.96
Pro	1.02	0.92	1.02	1.34
Val	0.88	0.74	0.69	1.01
Ile[c]	0.83	0.73	0.72	0.99
Cle	0.65	0.65	0.56	0.84
Ala	0.89	0.64	0.23	1.01

[a]The peptide-resin samples were hydrolyzed in conc. HCl-propionic acid (1:1), 130°, 6 hr. [b]Programmed for double coupling in each cycle. [c]Includes allo-isoleucine produced during acid hydrolysis.

To study racemization in the DCC-DMAP procedure, Boc-Ile-Val-OCH_2-C_6H_4-resin was prepared from Boc-Ile-OH and H-Val-OCH_2-C_6H_4-resin by the DCC-DMAP method and also by the standard DCC method which has been shown to proceed without significant racemization. The dipeptide resin samples thus obtained were hydrolyzed in conc. HCl-propionic acid and their allo-isoleucine content, which would reflect the extent of racemization during synthesis plus that due to acid hydrolysis, was determined.[16] It was found that the dipeptide-resin sample prepared by the DCC-DMAP method gave rise to 2.6% of allo-isoleucine whereas the sample derived from DCC method gave 2.2% of allo-isoleucine. The difference was within the experimental error of the analytical procedure used. However, significant racemization (3.5%) was observed when Boc-Phe-OH was coupled to H-Glu(OBzl)-OCH_2-C_6H_4-resin via DCC-DMAP method. In the control synthesis by the standard DCC method very little (0.4%) racemization detected. Racemization during symmetrical anhydride coupling for the same synthesis was reduced from 4.1% when one equivalent of DMAP was used, to 0.8% when 0.03 equivalent of DMAP was used. Similar reduction in the extent of racemization was observed when 0.6 equivalent of DMAP was added 20 min after the symmetrical anhydride coupling was started.

It is concluded that addition of DMAP to the reaction mixture to enhance coupling efficiency is most recommendable for steps involving hindered amino acid residues, such as Ile, Val, Cle or β-benzylmercapto-β,β-pentamethylenepropionic acid where there is little or no danger of racemization. An indiscrimate application of the reagent in every coupling raction would be unadvisable unless it is shown that there is no significant side reaction taking place in the system under study.

Acknowledgements

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A NEW APPROACH TO THE USE OF MIXED ANHYDRIDES IN SOLID-PHASE SYNTHESIS

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The mixed carboxylic-carbonic anhydride method of activation has been used infrequently in solid-phase synthesis,[1,2] probably because of the common perception that rigorously-controlled reaction conditions are required. However, because of the potential advantages of the method it was our desire to re-examine this application of mixed anhydrides (MAs). Model studies were therefore performed in solution, comparing urethane-protected MAs formed by activation with IBCF at + 20°C and -15°C.

Stability

Our initial studies with Boc-amino acids were carried out in CH_2Cl_2 using TEA as base. Although the MAs were quite stable under these conditions, most were completely formed only after warming reaction mixtures to 0°C as monitored by IR. In one case [Boc-Thr-(OBzl)] conversion to the MA was not complete until the mixture was warmed to 20°C for 40 minutes! For this reason, the use of CH_2Cl_2 and TEA in combination is to be avoided since the presence of unreacted IBCF can lead to extensive acylation of the amine component.

The remainder of our studies therefore concentrated on the use of NMM in THF or DMF as solvent. Rates of formation of the MAs were extremely rapid under these conditions (1-5 min. at -15° C). Following activation at -15°C reaction mixtures were warmed to room temperature (20-25°C) and maintained at this temperature for 1 h. After coupling with Leu-OMe or Leu-OtBu and a simple workup procedure, the yield of crude product was determined and the material analyzed by a variety of chromatographic techniques. The results of these studies are summarized in Table I.

Racemization

Lengthy activation times have been correlated with increased racemization during the MA coupling of peptide fragments.[3,4] In order to determine whether racemization was occurring under our conditions, the dipeptides TFA-Phe-Leu (prepared from Boc-Phe and Fmoc-Phe) and

HCl·Ser-Leu [prepared from Fmoc-Ser(OtBu)] were analyzed for racemization by reverse-phase HPLC. No difference was found between the samples prepared from MAs activated at room temperature and those prepared under standard, low temperature conditions.

Table I. HPLC Analysis of Dipeptides, X-Leu-OR, Prepared from Mixed Anhydrides Activated at 20°C and -15°C [a]

X	Yield (%)[b]	Purity (%)[c]	Iboc-Leu-OR (%)[c]
Boc-Phe[d]	99(97)	95(92)	0(0)
Boc-Val[d]	98(96)	- -	- -
Boc-Pro[d]	95(96)	95(96)	0(0)
Boc-Thr(Bzl)[d]	99(97)	97(99)	0(0)
Boc-Met[d]	92(99)	99(99)	0(0)
Boc-Lys(Cbz)[d]	102(101)	- -	- -
Boc-His(Tos)[d]	68(92)	89(99)	0(0)
Boc-Arg(Tos)[d]	82(91)	68(99)	0(0)
Boc-Asp(Bzl)[d]	103(103)	99(97)	0(0)
Fmoc-Asp(tBu)[e]	86(84)	>90(>90)	0(0)
Fmoc-Lys(Boc)[e]	95(98)	>95(>95)	0(0)
Fmoc-Ser(tBu)[e]	91(102)	98(95)	0(0)
Fmoc-Gly[e]	78(99)	95(95)	0(0)
Fmoc-Phe[e]	92(102)	85(95)	0(0)
Fmoc-Pro[e]	94(102)	98(98)	0(0)

[a]Activation with equimolar amounts of IBCF and NMM at -15°C for 4 min. then warmed to 20°C for 1 h. (90 min. total). After coupling with HCl·Leu-OtBu (10 min.), samples were worked up by the usual extraction procedure. Data from standard, low temperature couplings in parentheses. [b]Yields of crude product. [c]Determined by HPLC analysis (RP-C_{18}/MeOH/0.5M $HClO_4$ for Boc-X-Leu-OMe; silica/THF/hexane for Fmoc-X-Leu-OtBu). [d]In THF. [e]In DMF.

Urethane Acylation

The insertion of two amino acid residues during a coupling, which occurs via urethane acylation, has been shown to be most prevalent with urethane-protected glycine MAs.[1] The dipeptide, Fmoc-Gly-Leu-OtBu, prepared as outlined above after activation of the MA for 1 h at 20°C,

was deprotected by treatment with 50% piperidine in CH_2Cl_2, followed by 4N HCl/dioxane. Analysis of the crude product by reverse phase HPLC (RP-C_{18}/MeOH/0.07M NH_4OAc, pH 4.5) gave no detectable HCl·Gly-Gly-Leu (limits of detection $<0.05\%$).

Wrong Opening

Wrong opening of MAs (attack at the carbonate carbonyl) results in truncated sequences during solid-phase syntheses. The dipeptide products (Table I) were therefore analyzed for Iboc-Leu-OMe or Iboc-Leu-OtBu by both TLC and reverse-phase HPLC. The results of these studies are summarized in Table I. In a separate study,[5] wrong opening, which occurred on coupling of Pro-MA to Pro-O-Resin, was minimized to $\sim 2\%$.

Solid-Phase Synthesis

Based on the above results, a number of solid-phase syntheses were attempted. First, Leu-Ala-Gly-Val was synthesized on a *p*-alkoxybenzyl alcohol resin using Fmoc-amino acids. The synthesis was carried out in a pressurized flow reactor[5] using 10 min. coupling cycles and 10 min. deprotections in DMF as solvent. The final residue was added as the Boc-derivative and the tetrapeptide was cleaved in quantitative yield from the resin using 75% TFA in dichloromethane. The crude products were analyzed by reverse phase HPLC for all possible failure sequences and wrong-opening products. This analysis (Figure 1) revealed no detectable contaminants, *i.e.* ($> 99.9\%$ purity).

The method has been extended to the synthesis of the delta-sleep inducing peptide (H-Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu-OH) using N^{α}-Fmoc and *t*-butyl sidechain protection and a similar procedure, *i.e.* 10 min. coupling/deprotection cycles. The crude product was isolated in virtually quantitative yield and shown to be $>95\%$ pure by reverse phase HPLC analysis. The major impurity ($\sim 4\%$) --a des-Ala peptide – was avoided in a subsequent synthesis by increasing the coupling times of Ala_2 and Ala_6 to 20 min.

Conclusion

The results described above demonstrate that the mixed anhydride method of activation can be used successfully in solid-phase synthesis without the need for low temperatures during activation or couplings. Furthermore, use of a novel, pressurized flow reactor results in extremely rapid reactions, so that the total cycle time for addition of a single amino acid residue in a sequence is substantially shortened (<30

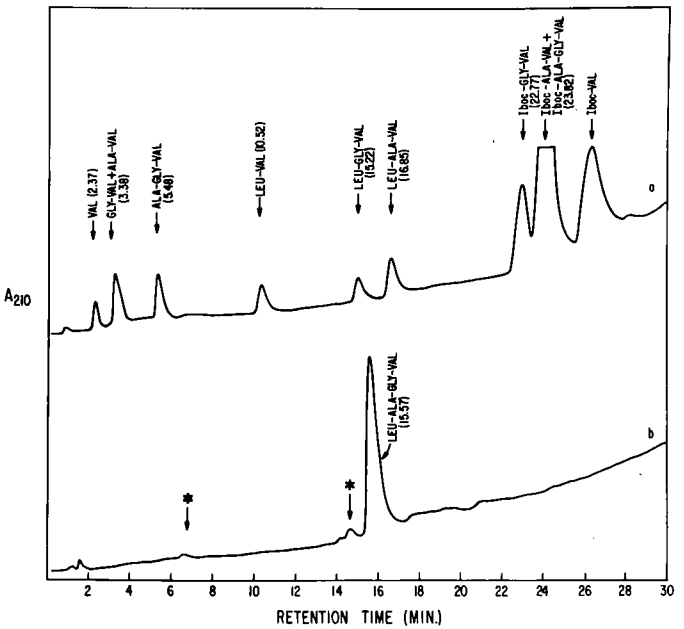

Fig. 1. Reverse phase HPLC analysis of Leu-Ala-Gly-Val and standards on Lichrosorb RP-18 using a linear gradient of 0-50% methanol in 0.5M aqueous perchloric acid; flow rate = 3 ml/min. (a) Standards; and (b) crude product after removal from the resin. Peaks indicated by (*) are due to components eluted from the resin.

minutes). The high yields and purity of the products synthesized to date encourage us to extend the method to the synthesis of larger, more complicated sequences.

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3-NITRO-2-PYRIDINESULFENYL-AMINO ACIDS IN SOLID-PHASE PEPTIDE SYNTHESIS

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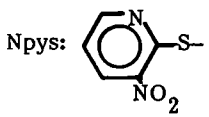
Toward the goal of employing milder deprotecting reagents in peptide synthesis, we have sought to utilize N-(3-nitro-2-pyridinesulfenyl) (Npys)-amino acids for temporary α-amino group protection in solid-phase peptide synthesis. As described by Matsueda *et al.*,[1] the Npys group offers several distinct advantages over other protecting group systems which have been proposed as alternatives to the Boc group. (1) Its removal is effected under mild conditions, by 1M 2-mercaptopyridine N-oxide(MPO) in water, for example; (2) the starting reagent, crystalline Npys-Cl, is stable for at least 6 months at 4° C; (3) Npys-amino acids, unlike Nps- or Bpoc-amino acids, are stable in TFA; (4) the N-Npys group can be activated with triphenylphosphine to form an amide bond in the presence of RCOOH.

Results

Npys amino acids were prepared with Npys-Cl using Schotten-Baumann conditions as described by Matsueda *et al.*[1]. 2-Mercapto-pyridine N-oxide was prepared from its Na-salt (Sigma) by extraction into ethylacetate from aqueous HCl and then rotary evaporation of the extract to a solid residue (mp 69-72).



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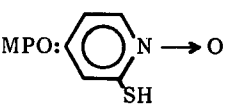

Deprotection of Npys Amino Acids — A test dipeptidyl-resin was prepared for evaluation of Npys-group deprotection. Npys-Val was coupled to α-aminobutyramidomethyl-resin (1%-cross-linked polystyrene) to give Npys-Val-Abu-NH-CH_2-resin. This protected dipeptidyl-resin was treated with a variety of deprotecting reagents after it was found that solution-phase conditions, 1M MPO in water or in resin-swelling solvents, failed to give any significant deprotection (less than

1%) as measured by the Gisin picric acid test. The control reagent, 0.2 N HCl in dioxane,[1] provided complete deprotection after 60 min. This resin sample was devoid of the yellow color which is characteristic of Npys.

Based upon the previous solution-phase work of Matsueda *et al.*,[1] deprotecting reagents containing MPO and Ph_3P were examined. As expected, a solution of 1.0 M Ph_3P, MPO, and HOAc in Ch_2Cl_2 was effective in removing the Npys group as evidenced by the complete loss of color from the test Npys-peptidyl-resin, but this was accompanied by N-acetylation (16%). To avoid this acylation, pentachlorophenol was substituted for HOAc. This reagent (#1) gave essentially complete deprotection (96%) of the test resin sample, but was considered unsuitable due to its multicomponent composition. An attempt to substitute thiourea for MPO similarly led to a complex system (reagent #2), 0.17 M thiourea in HOAc:H_2O:CH_2Cl_2(50:33:17), which was also capable of complete deprotection (97%).

The best deprotection reagent was found after examining reagents containing MPO and a proton source in CH_2Cl_2. When phenol, HOAc and TFA were used, the deprotection was 16%, 48%, and 101%, respectively. With 0.05 M MPO and 0.05 M TFA in CH_2Cl_2 a 97% deprotection was achieved. As a margin of safety, a concentration of 0.2 M for MPO and TFA was used for the subsequent experiments. This reagent (#3) was considered most effective because after a single prewash for 1 min., the resin had lost most of its yellow color. We estimate the half-life to be less than 30 sec. in reagent #3. When either MPO or TFA alone was tested, insignificant levels of deprotection were obtained (<2%).

Coupling of Npys Amino Acids — Although coupling of Npys-Val to Abu-resin with equimolar DCC proceeded without difficulty, the model peptide, Leu-Ala-Gly-Val, could not be synthesized satisfactorily with the standard DCC coupling procedure. This synthesis of LAGV gave four spots by TLC (SG using a BAW system). The product was subsequently subjected to preparative RP-HPLC (C18-μBondapak). In addition to the expected LAGV (53%), peptides containing excessive amounts of Gly (2-3 times), and even Ala, were found.

To minimize this aminoacyl-insertion side reaction, several coupling protocols were tested by coupling Npys-Gly to Val-resin. When standard DCC procedure (protocol #1) was used, a negative ninhydrin test was obtained after 90 min. The resin sample was washed with 25% TFA in CH_2Cl_2 to remove non-covalently bound Npys-Gly, then hydrolyzed in vacuo for 4 h with propionic acid:HCl(1:1) at 130° C. Amino acid analysis indicated that the Gly:Val ratio was 1.72:1. This result recon-

firmed the aminoacyl-insertion problem observed in the first LAGV synthesis. By reversing the order of addition (protocol #2), DCC being added first, the Gly:Val ratio was decreased to 1.20:1 which was still indicative of some aminoacyl-insertion.

Two other procedures which have been successfully used with Boc-amino acids failed to give a negative ninhydrin test after 90 min: when DCC was added to the salt formed between Npys-Gly and neutralized Val-resin, after washing away excess Npys-Gly (protocol #3); or when the symmetric anhydride of Npys-Gly, prepared by mixing Npys-Gly:DCC(2:1) in DMF at 0° C, was added (protocol #4).

It was reasoned that the aminoacyl-insertion as proposed by Merrifield *et al.*[2] could not occur if N-*bis*-Npys-Gly were used instead of N-mono-Npys-Gly. A four-fold excess of N-*bis*-Npys-Gly:DCC(1:1) was added to neutralized Val-resin. The ninhydrin test indicated that coupling was complete at 90 min. By amino acid analysis, the Gly:Val ratio was 0.92:1, which was consistent with the elimination of the aminoacyl insertion side-reaction. This tactic could not be used for the synthesis of LAGV because deprotection (<10%) of *bis*-Npys-Gly was incomplete under conditions which gave complete deprotection of mono-Npys-Gly. This was presumably due to the presence of two Npys groups on a single amino group.

The only coupling procedure which proved suitable was the use of the pre-formed N-hydroxybenzotriazole ester of Npys amino acid at a four-fold excess (protocol #4). A solution of Npys-amino acid:DCC:HOBt(1:1:1) in DMF was kept at 0° C for 30 min. prior to addition to the neutralized peptidyl-resin. Under these conditions, the coupling reaction was often complete within 30 min. although a coupling time of 90 min. was used routinely. Importantly, the aminoacyl-insertion problem observed with some other procedures was not detected (Gly:Val = 0.96:1.0).

Solid-phase Synthesis of LAGV with Npys Amino Acids: — Using the deprotection reagent #3 and coupling protocol #4, the solid-phase synthesis of Leu-Ala-Gly-Val with Npys amino acids was attempted and compared with a parallel synthesis in which Boc amino acids were used. Starting with 300 mg of Boc-Val-resin, prepared from HO-CH_2-resin, LAGV was assembled using CH_2Cl_2 as the solvent unless otherwise indicated. The Boc group was first removed using 25% TFA for 30 min. Neutralization was performed using 10% TEA for 5 min. with a single prewash. The resin was washed six times with CH_2Cl_2 between various reagents. Prior to neutralization, Npys Gly at four-fold excess was acti-

vated in DMF with equimolar DCC and HOBt at 0° C for 20 min. Including the time required for neutralization, a pre-activation time of 30 min was allowed for the formation of the HOBt ester. Coupling time of 90 min was used. Completeness of coupling was checked with the Kaiser ninhydrin reagents. After the incorporation of Npys-Ala and then Npys-Leu, the resin was treated with reagent #3, 0.2 M MPO and TFA in CH_2Cl_2 to remove the terminal Npys group and then dried after a methanol wash.

A sample (151 mg) of LAGV-resin was treated with 5 ml HF:anisole(9:1) for 45 min. at 0° C. After removal of the HF at 0° C by evacuation, the resin was transferred with Et_2O and eluted with 1 M HOAc. The combined HOAc fractions were lyophilized to yield 20 mg of the crude peptide. A solution (10 mg/ml) of the HF crude peptide was prepared for comparison with the control peptide, which was synthesized with Boc amino acids. By thin-layer chromatography (SG), a single spot was found when both peptide samples (100 μg) were developed with BAW(12:3:5). The identical Rf (0.57) was obtained for both peptides when either a ninhydrin or Cl_2-starch detection reagent was used. By ion-exchange chromatography (Dionex 500C), the LAGV prepared with Npys amino acids was again identical when compared with the LAGV prepared with Boc amino acids. Both tetrapeptides eluted at 49 min. In this system, the elution times of error peptides, AGV, LAV, and LGV were 46, 56, and 62 min., respectively.

Conclusion

Conditions for the complete solid-phase deprotection and coupling of Npys amino acids have been found. Using these conditions, the synthesis of a model peptide, LAGV, using Npys amino acids gave a product which was indistinguishable from the control synthesis using Boc amino acids. Experience with the solid-phase use of Npys amino acids suggests they will be compatible with *t*-butyl side-chain protection strategy along with more labile peptide-to-resin linkages offered by Wang's *p*-alkoxybenzyl-resin[3] or Stewart's *p*-alkoxy-α-phenethylamine-resin.[4] When used in conjunction with benzyl-protected side-chain functional groups and Wang's *p*-alkoxybenzyl-resin, it is clear that the synthesis of protected peptide segment intermediates will be feasible.

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A STABLE, WATER SOLUBLE POLYMER DERIVATIVE WHICH SPECIFICALLY AND REVERSIBLY ATTACHES TO THE SIDE CHAIN OF ARGININE RESIDUES

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Camphorquinone-10-sulfonic acid (Figure 1) is a bifunctional reagent in which one functional group is a specific, reversible ligand for the side chain of arginine and the second functional group can be used for covalent attachment of the molecule to chemical markers or to polymeric supports. The adducts of camphorquinonesulfonyl derivatives with the guanidino group are cleaved by *o*-phenylenediamine at pH 8-9.[1,2]

Direct acylation of polyethyleneglycol with camphorquinonesulfonyl chloride yielded a derivative in which the arginine-specific ligand was covalently linked to the polymer through a sulfonate ester bond. This linkage was susceptible to hydrolysis in aqueous solutions of pH 8-9, conditions necessary for the attachment and detachment of peptides. The problem was only partly resolved by interposition of an amino acid residue between the camphorquinonesulfonyl moiety and the polymer so that the ester bond in the linkage was a carboxylic rather than a sulfonic ester bond.[1]

Formal replacement of hydroxyl groups of polyethyleneglycol or its monomethyl ether with primary amino groups and acylation of these with camphorquinonesulfonyl chloride yielded derivatives in which the linkage was stable to the conditions of peptide attachment and detachment. Model peptides have been attached to such polymer derivatives and were subsequently cleaved from the polymer.

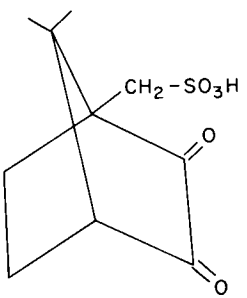

Fig. 1. Camphorquinone-10-sulfonic acid

Experimental

NH_2-(CH_2-CH_2-O-)$_n$$CH_3$ — Polyethyleneglycol monomethyl ether (50 g, nominal MW 5000) was dissolved in 300 ml warm toluene and 100 ml of the solvent was distilled off. The dried solution was refluxed overnight with 12 ml $SOCl_2$. An additional 10 ml of $SOCl_2$ was added and refluxing was continued for another 20 h. A white, crystalline solid (30-45 g) was obtained by evaporation of the solvent and crystallization from acetone. Twenty grams of the solid was stirred with 4 g potassium phthalimide in 100 ml DMF at 50° for 24 h.[3] The solution was filtered and evaporated. The residue was taken up in warm toluene, filtered, and treated with ether to precipitate a white crystalline solid. This was treated with 5 ml of hydrazine hydrate in refluxing ethanol for several hours. The solution was evaporated and the residue extracted with warm toluene. Addition of ether to the filtered solution precipitated the product as a white crystalline solid which was recrystallized from acetone. Yield: 17-19 g.

Camphorquinonesulfonyl-NH-(CH_2-CH_2-O-)$_n$$CH_3$ — One gram of amino polymer was dissolved in 5 ml $CHCl_3$ along with 0.2 g camphorquinonesulfonyl chloride. After a few minutes 5 ml of 5% Na_2CO_3 was added and the mixture stirred vigorously. After 30-40 min the ninhydrin reaction was negative. Following acidification with acetic acid, chloroform was removed by rotary evaporation. The aqueous solution was dialyzed against 0.2 M acetic acid and evaporated to dryness. The residue was dissolved in 0.2 M sodium borate, pH 9, and held at 37° for 20 h. The polymer was separated from salts on Sephadex G-50. Approximately 20-30% of the polymer represented by the trailing edge of the peak was discarded.

Attachment of Peptides to Camphorquinonesulfonyl Polymer — Samples of camphorquinonesulfonyl polymer were treated with arginyl-aspartic acid and with ribonuclease S-peptide in borate buffers of pH 8.8 at 37°. Separation of polymer-bound material from a large excess of the free dipeptide after 16-20 h left 19 nmol/mg of peptide bound to the polymer derivative. Incubation of about a 2-fold excess of RNAse S-peptide with polymer lead to the attachment of approximately one quarter to a third of the peptide. There seemed to be little difference in the degree of attachment between 15 h and 36 h of incubation in borate buffer. Treatment of the S-peptide/polymer derivative with *o*-phenylenediamine at pH 8.6 under nitrogen overnight cleaved about 70% of the peptide from the polymer and a second treatment left less than 10% of the peptide still bound. The S-peptide recovered was effective in activating S-protein

to hydrolyze yeast RNA, but has as yet not been characterized further. Other biologically active polypeptides have been recovered with full activity after derivatization with camphorquinonesulfonyl derivatives.[2]

Discussion

The content of camphorquinonesulfonyl groups in camphorquinonesulfonyl-NH-$(CH_2\text{-}CH_2\text{-}O\text{-})_nCH_3$ was low compared to expectations based on the nominal molecular weight of the starting polymer. Partly this was due to a rather broad molecular weight distribution in the starting material and a deliberate removal of material of low molecular weight. There also seemed to be an incomplete replacement of hydroxyl groups by chlorine using the method reported here. A published method[4] for a quantitative reaction with $SOBr_2$ gave considerable discoloration of the polymer. An incomplete conversion with subsequent hydrolysis of any camphorquinonesulfonyl groups attached to polymer through sulfonate ester bonds was preferred. Release of guanidino groups from the polymer with hydroxylamine at pH 7, as is possible with cyclohexanedione adducts of arginine,[5] would have been more acceptable than the treatment with *o*-phenylenediamine at pH 8-9, but camphorquinonesulfonyl derivatives are resistant to that treatment.[2]

The foregoing reservations aside, the camphorquinonesulfonyl polymer derivative described has properties which strongly recommend it as a carrier in the synthesis and semisynthesis of peptides in aqueous solution.[6] The specific and reversible binding to the side chain of a single type of amino acid residue in preformed peptides leaves the C- and N-termini free for bidirectional extension.[7,8] The linear structure of the polymer and its high water solubility are ideal for use in systems likely to require the use of enzymes on polymer-bound intermediates.[9] At the same time, the arginyl bond at the point of polymer attachment is protected against the action of trypsin which might be used to remove blocking groups elsewhere in the peptide intermediate.

Acknowledgement

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SULFUR PROTECTION WITH THE NOVEL 3-NITRO-2-PYRIDINESULFENYL GROUP IN SOLID-PHASE PEPTIDE SYNTHESIS

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Recently, the use of the 3-nitro-2-pyridinesulfenyl (Npys) group for protection of the amino and hydroxyl functions of amino acids during peptide synthesis has been reported.[1] The group was found to be resistant to TFA and 88% HCOOH, but removable by dilute HCl (e.g., 0.1N HCl in dioxane) and by triphenylphosphine or 2-pyridinethiol 1-oxide under neutral conditions. Included in the Npys derivatives of amino acids and peptides prepared was Boc-S-Npys-cysteine, characterized as the DCHA salt, but the use of this derivative in peptide synthesis was not investigated.

The objective of the work reported here was three-fold: 1. to demonstrate that the Npys group can be used for side chain protection of cysteine in conjunction with the most widely used tactic of Boc-benzyl protection during stepwise solid-phase synthesis; 2. to investigate its utility in facilitation of intramolecular disulfide bond formation by taking advantage of its selective sensitivity to free thiol; 3. to obtain further information about the chemistry of this group so that a conclusion can be reached about its potential use in extended synthesis. Lys[8]-Vasopressin (LVP) was chosen as a suitable model peptide for fulfillment of these objectives, and an initial synthesis of this using the 3,4-dimethylbenzyl (DMB) group[2] was chosen as control.

Results

The structure of the three nonapeptidyl-resins synthesized and the reasons for requiring them are shown below.

TFA·H-Cys(X_1)-Tyr(Bzl)-Phe-Gln-Asn-Cys(X_2)-Pro-Lys(ClZ)-Gly-NH-BHA

peptidyl-resin I	X_1, X_2 = DMB	control
peptidyl-resin II	X_1, X_2 = Npys	to satisfy objectives 1 + 3
peptidyl-resin III	X_1 = Npys, X_2 = DMB	to satisfy objectives 2 + 3

The syntheses were carried out manually on benzhydrylamine resin in a stepwise fashion using the appropriate Boc-amino acids and DCC or the Boc-amino acid *p*-nitrophenyl esters (Asn and Gln) for coupling. TFA: CH_2Cl_2(1:1) was employed for removal of the Boc group throughout.

With respect to objective 2, it was anticipated that the S-Npys function would be stable to HF, so that after treatment of peptidyl-resin III with HF, Cys^1 would still be protected while Cys^6 would not. This being the case, it was reasoned that a solution of the crude HF product in acidic medium could be diluted and then the pH raised to allow the free thiol on Cys^6 to displace the Npys group on Cys^1 and preferentially form an intramolecular disulfide bond.

LVP was obtained from peptidyl-resin I after HF:anisole (9:1) treatment for 45 min at 0° and $K_3Fe(CN)_6$-catalyzed air oxidation[3] of the crude, free peptide amide in dilute aqueous solution (pH 8.1). The crude oxidized product was then subjected to gel filtration on a Sephadex G-25 column in 20% HOAc. A typical elution profile of the separation obtained is shown in Figure 1. The material corresponding to the major peak (β) in the profile was purified by reverse-phase HPLC to give the final product which had the required amino acid analysis, full biological activity (adenylate cyclase activation), and co-eluted with authentic LVP (Sigma Chemical Co.) on HPLC and t.l.c. More of this product was obtained from the later eluting fraction (corresponding to peak γ) to give an overall yield of 12% (based on initial resin substitution). In addition, another component (1.6%) was isolated from this fraction and, as expected, amino acid analysis gave a very low figure for tyrosine suggesting that this product was the 3-benzyltyrosine LVP derivative from the HF-catalyzed rearrangement of the O-benzyl-tyrosine residue in the peptidyl-resin.[4] The higher molecular weight material (peaks α_1, α_2, and α_3) was shown to be fully reducible by sulfitolysis,[5] thus showing it to be the result of intermolecular disulfide bond formation.

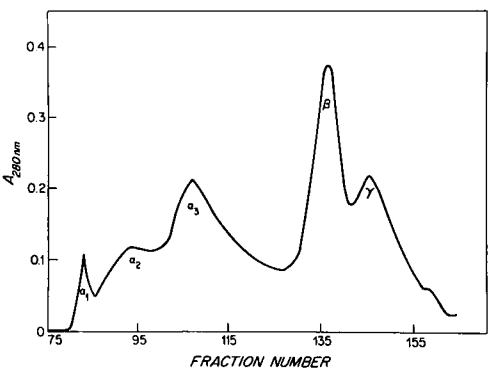

Fig. 1. Elution profile of crude LVP (equivalent to 0.0313 mmol of peptide) from peptidyl-resin I on Sephadex G-25 using 20% HOAc as eluent.

Treatment of peptidyl-resin II with HF confirmed that the S-Npys group was not cleaved under conditions as above since no yellow mate-

rial was extracted by organic solvent from the evaporated reaction mixture or from the yellow aqueous-acid extract of the crude product. This observation permitted the possibility of removing the Npys group before or after HF treatment. The former was achieved with another sample of peptidyl-resin II using a 10% solution of β-mercaptoethanol in DMF containing 0.01% NEt_3. HF treatment of the partially deprotected peptidyl-resin and subsequent oxidation and gel filtration (Figure 2) were then carried out as for peptidyl-resin I. The major component of the major peak (ϵ) crude material was isolated by HPLC and identified as LVP by co-chromatography on t.l.c. and HPLC and by amino acid analysis. Similarly, the crude material isolated from the fraction corresponding to peak η was shown to contain the 3-benzyltyrosine derivative as well as LVP. The overall yield of LVP was the same as that obtained from peptidyl-resin I. A significant amount of the higher molecular weight material, peak δ, was non-reducible by sulfitolysis, but this was not investigated further. The material corresponding to the last eluting peak was not characterized since it did not appear to be peptidic in nature.

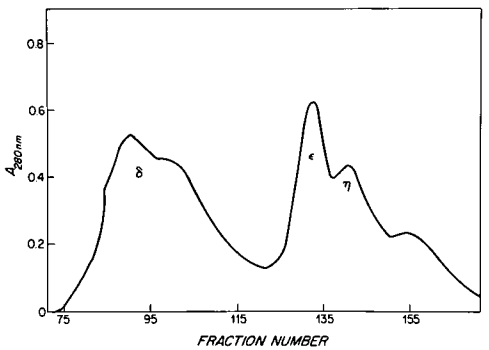

Fig. 2. Elution profile of crude LVP (equivalent to 0.0625 mmol of peptide) from peptidyl-resin II, when the Npys groups were removed before HF treatment, on Sephadex G-25 using 20% HOAc as eluent.

Removal of the S-protecting groups from the *bis*-S-Npys peptide amide resulting from HF cleavage of peptidyl-resin II was accomplished by dithiothreitol in aqueous solution (pH 8.5). The free peptide amide was then liberated from the reducing agent and yellow by-product by gel filtration on a Sephadex G-10 column in 1N HOAc. Oxidation of the resulting peptide was then carried out as before to give LVP (9%), identified by co-chromatography on t.l.c. and HPLC. The gel filtration profile was similar to that shown in Figure 2. Again, the higher molecular weight fraction was not fully reducible by sulfitolysis.

Extraction of HF-treated peptidyl-resin III with 1N HOAc gave a yellow-colored solution which was rendered almost colorless by extraction with ethylacetate. This suggested that the Npys group had been

removed during the cleavage reaction, especially as the yellow material from the organic phase co-eluted on HPLC with the major component in the yellow by-product obtained from the Sephadex G-10 column mentioned above. LVP formation from the material in the aqueous phase was achieved in this case by prolonged air oxidation in the absence of $K_3Fe(CN)_6$ to allow selective disulfide bond formation. The gel filtration profile revealed that polymer had been formed in preference to monomeric LVP, suggesting that significant disulfide bond formation occurred during HF treatment. Furthermore, the higher molecular weight fraction was fully reduced by sulfitolysis. The overall yield of monomeric LVP was calculated to be 7%.

The instability of the S-Npys group to free sulfhydryl(Cys)[6], even in strongly acidic media, was confirmed by reaction of the model pentapeptidyl-resin, H-Cys(Nps)-Leu-Ala-Gly-Val-O-CH_2-C_6H_4-®resin with HF in the absence and presence of an equimolar amount of cysteine HCl. The yellow material extracted into ethylacetate from the aqueous-acid extract of the resin in the latter case co-eluted with the corresponding material obtained from peptidyl-resin III. All colored material remained in the aqueous-acid phase in the former case.

Finally, the S-Npys group was shown to be cleavable by TFA in the presence of thiol, as evidenced by the presence of yellow-colored material in the neutralized filtrate, after peptidyl-resin II had been subjected to TFA:CH_2Cl_2 (1:1) containing 0.5% ethanedithiol.

Conclusions

The Npys group can be used successfully for side chain protection of cysteine during solid-phase synthesis when utilizing the Boc-benzyl tactic. Although the instability of the group to free sulfhydryl, even under strongly acidic conditions, does not permit the control of intramolecular disulfide bond formation, it should facilitate intermolecular disulfide bond formation between two peptides, one containing an unprotected cysteine and one containing an Npys-protected cysteine. This is currently being investigated. The Npys group can be used in extended synthesis, but not with thiols as scavengers in the TFA solution during deprotection.

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THE CONVERSION OF SOLID PHASE PEPTIDE SYNTHESIZERS TO COMPUTER CONTROL

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The noise immunity of the Schwarz-Mann synthesizer electronics is unacceptably low in certain environments, leading to unreliable operation. An evaluation of the possible methods of upgrading the control elements of the synthesizer led us to consider replacing the discrete logic found in the original controller with a microprocessor. Although other workers have employed computers to control peptide synthesizers,[1,2] no one to our knowledge has attempted an implementation with "off the shelf" components. In order to achieve a rapid implementation we chose to use a commercial microcomputer, the APPLE II+, which has a very good reputation for reliability.

Hardware

The APPLE II+ contains a 6502 microprocessor, 48K of RAM and 16K of ROM and a bus that can accommodate up to 8 peripheral circuit boards. We use a Mountain Computer clock board for real time control, an APPLE Computer high speed serial interface for printer control, a D.C. Hayes Micromodem for error control communications, and an APPLE Disk II for mass storage. For control of the synthesizers, we chose the 6522 Versatile Interface Adapter (VIA). A pair of these integrated circuits can control all the solenoids and the sensors of one of our synthesizers. While our quad 6522 VIA board was being assembled, we used a commercial interface board manufactured by John Bell Engineering which contains dual 6522's. We connected the 6522 board to an optoisolator board which provides noise immunity from the synthesizers and prevents damage to the computer in the event of component failure in the synthesizer (Figure 1). We added a small 5 volt power supply to run the quad 6522's and the optoisolator boards, and a fan to prevent premature RAM failure from overheating (approximately 4W heat is dissipated by the 6522 board). The APPLE is connected with a 50 wire ribbon cable to 4 new circuit boards which replace the original solenoid driver boards in the Schwarz-Mann controller. We chose to retain the original Schwarz-Mann power supply and solenoid switch circuitry because they are sufficiently reliable as designed. This approach allows us

to use the old controller, in the event of computer failure, simply by reinserting the original boards.

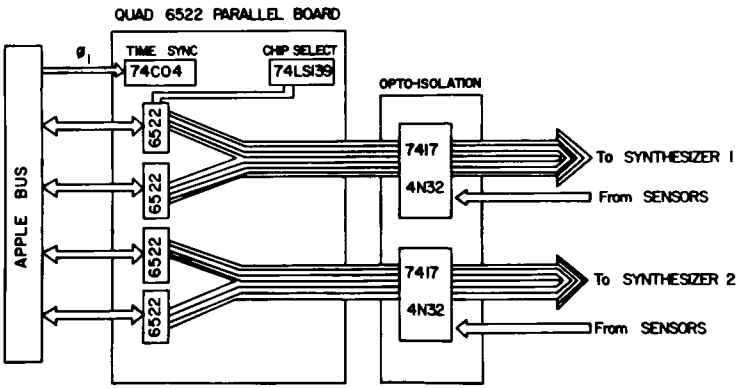

Fig. 1. Synthesizer Interface: The Apple II is connected to two Schwarz-Mann Synthesizers through a 6522 VIA based interface and an optoisolation board.

Software

The software, which is written in Basic, is divided into 3 parts: 1. the synthesis module, which actually controls the synthesizer, 2. the programming module, which allows the synthesizer to be programmed, and 3. the initialization module which allows new disks to be configured for our programs.

The synthesis module consists of a set of Basic subroutines and machine language programs. Included in this module are routines which: 1. open valves, 2. close valves, 3. examine sensors, 4. allow manual control of the synthesizer, 5. keep unauthorized personnel from changing any parameters, 6. initiate telephone calls in the event an error condition occurs, 7. print the synthesizer status on the CRT, or to a printer, and 8. store the current synthesizer parameters to disk in the event of an error condition.

Programs for the APPLE synthesis system are called Run Lists. The Run List elements, called Macros, are given single letter codes. The synthesizer module interprets these elements and calls for certain operations to be performed. Therefore, a Run List consists of a string of letters which represent the sequence of operations to be performed. When creating a new program (Run List), the user needs only type in the letters corresponding to these preformed Macros. Macros are created by choosing from a list of possible operations which the synthesizer can perform,

e.g., 1. add a certain volume of a specific reagent, 2. shake for a specific time and drain the reactor, 3. shake for a specific time and don't drain the reactor, 4. add the current amino acid, 5. set up the next amino acid to be added, involving loading in a Run List which is specific for that amino acid, thus allowing ready intermixing of types of coupling within a program, 6. wait and do nothing for a period of time, or 7. set off the alarm. Each of these choices causes a series of questions to be asked which elicit the information needed to implement the operation. Once the instructions have been entered, the new Macro is added to the list of Macros stored on disk.

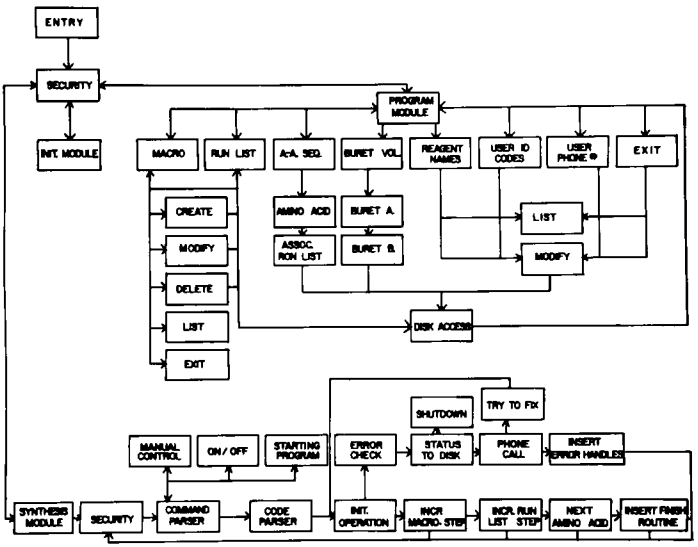

Fig. 2. Software Block Diagram. The synthesizer control program consists of 3 modules. The programming module, the synthesis module, and the initialization module. Each module occupies approximately 12.5 K bytes. Overlays are used to conserve data space.

The program module also allows the user to enter the amino acid sequence of the peptide to be synthesized, user ID codes, the names of reagent and amino acid reservoirs, a series of telephone numbers to be dialed when an alarm condition occurs, the sensor settings for the desired liquid volumes, and a series of codes which link the software to the machine language programs which turn on the solenoid valves. Typically, the user simply types in the amino acid sequence, then types a command to choose the starting Run List and finally types in one additional command to start the synthesis.

The program can currently hold 26 Macros of 100 steps each, and 50 Run Lists of 255 Macros. We have found that it is a trivial task to create new Macros and Run Lists because of the menu selection features of the program. It is difficult to make errors in programming because of the error checking features of the routines which create Macros, Run Lists, and Amino Acid Sequences. For example, if you try to type in an amino acid sequence which contains an amino acid which has not yet had a reservoir assigned to it, the program will warn you of this fact. We have found, over a period of about six months, that the only errors that have occurred have been human errors and that the computer/synthesizer hybrid has performed dependably for the synthesis of a large number of peptides.

Acknowledgement

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QUANTITATIVE MONITORING OF SOLID PHASE PEPTIDE SYNTHESIS BY THE NINHYDRIN REACTION

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Introduction

Reaction of primary amino acids in solution with ninhydrin is routinely used as a photometric method for the quantitative estimation of amino acids.[1] An understanding of the mechanism[2] of the ninhydrin reaction led us to believe that the ninhydrin reaction could be extended to the quantitative determination of amino groups of peptide chains during solid phase peptide synthesis. The crucial requirement for this usage would involve getting all of the blue color into solution, with only negligible amounts of color remaining on the solid support. During the application of the Kaiser test[3,4] to some synthetic peptides attached to our new Pam-resin support,[5] we observed that all of the color was present in solution and the beads were colorless. A side reaction leading to the blue color beads previously observed was studied and a way to overcome it was found. This has led to the development of a quantitative ninhydrin procedure for measuring the total number of amino groups on the solid support and to the extension of this reaction to the following applications in solid phase peptide synthesis.

1. To detect and quantitate the unreacted amino groups during synthesis.
2. To estimate the total number of growing chains on a solid support.
3. To study side reactions during solid phase peptide synthesis where the H_2N-terminal amino group is being lost due to cyclization or some other termination reaction.

Recommended Procedure

1. *Reagents*
   a 1. Mix 40 g of reagent grade phenol with 10 ml of absolute ethanol. Warm until dissolved. Stir with 4 g of Amberlite mixed-bed resin MB-3 for 45 min. Filter.
   2. Dissolve 65 mg of KCN in 100 ml of water. Dilute 2 ml of the KCN solution to 100 ml with pyridine (freshly distilled from ninhydrin). Stir with 4 g of Amberlite mixed-bed resin MB-3. Filter. Mix solutions 1 and 2.

b Dissolve 2.5 g of ninhydrin in 50 ml of absolute ethanol. Stopper and store in the dark under nitrogen.

2. *Samples*

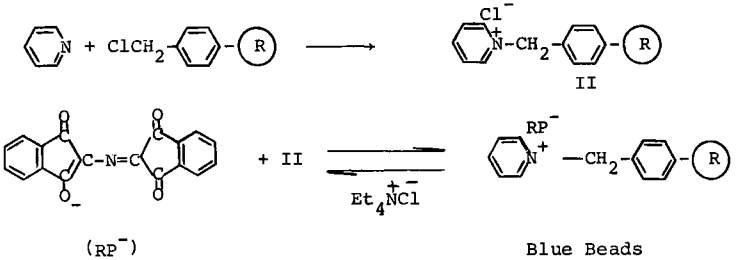
Samples of synthetic peptide-resins either after the deprotection or the coupling reaction are washed twice with 5% diisopropylethylamine in dichloromethane and three times with dichloromethane, and dried *in vacuo* at room temperature.

3. *Procedure*

Weigh a 2- to 5-mg sample of dry resin into a 10 x 75 mm test tube. Add 100 μl of reagent *a* and 25 μl of reagent *b*. Mix well. To another tube add only the reagents. Place both tubes in a heating block preadjusted to 100°. After 10 min place the tubes in cold water. Add 1 ml of 60% ethanol in water. Mix and filter through a Pasteur pipet containing a tight plug of glass wool. Rinse twice with 0.20 ml of 0.5 M Et_4NCl in CH_2Cl_2. Adjust the solution to 2.00 ml with 60% ethanol. Measure the absorbance of the sample filtrate against the reagent blank at 570 nm.

Results and Discussion

It had been observed in this laboratory that peptide-OCH_2-Pam-resin, free of any extraneous functionality, did not give blue colored beads when subjected to the ninhydrin reaction, whereas peptide chains grown on $ClCH_2$-resin gave blue beads under identical conditions. This led to the conclusion that the blue anionic chromophore (Ruhemann's purple) is held by ionic interaction with cationic groups formed on the latter resin by reaction of pyridine in the ninhydrin reagent with underivatized $ClCH_2$ groups as shown below. This blue color can be quantitatively removed from the beads by washing with a quaternary salt such as a 0.5M solution of Et_4NCl in CH_2Cl_2.



Standard samples of peptide-resin with known levels of free amine were prepared and subjected to the new ninhydrin procedure. It can be seen from Figure 1 that the absorbance at 570 nm was linear between

Table I. Variation of the Effective Ninhydrin Extinction Coefficient With The H_2N-Terminal Amino Acid and Chain Length

Compound		Amino Groups (mmol/g)	Ninhydrin Analysis A_{570}^{cm} [a]	Ninhydrin Analysis $\varepsilon_{570}^{cm} \times 10^{-4}$ [b]
NH_2CH_2-	R	0.935	1.448	1.55
H-Leu-$NHCH_2$-	R	0.217	0.375	1.73
H-Leu-OCH_2-Pam-	R	0.200	0.329	1.65
H-Val-OCH_2-Pam-	R	0.175	0.297	1.70
H-Glu(OBzl)-OCH_2-Pam-	R	0.076	0.095	1.25
H-Gly-Val-OCH_2-Pam-	R	0.156	0.186	1.19
H-Asn-Gly-OCH_2-Pam-	R	0.390	0.431	1.11
H-Lys-(ClZ)-Gln-Ala-Val-OCH_2-Pam-	R	0.180	0.303	1.68
H-(Leu-Ala-Gly-Val-OMPA)$_1$-$NHCH_2$-	R	0.196	0.319	1.63
H-(Leu-Ala-Gly-Val-OMPA)$_6$-$NHCH_2$-	R	0.210	0.335	1.60
H-(Leu-Ala-Gly-Val-OMPA)$_{10}$-$NHCH_2$-	R	0.137	0.217	1.58

a. Normalized to absorbance of 1 mg peptide-resin diluted to 10 ml and corrected for the reagent blank.

b. Calculated from the measured A_{570}^{cm} and picrate titration data.

0.0094 and 0.72 μmol of amine (0.0047 and 0.36 μmol/mg). Other results in this laboratory have shown that the linearity extends at least as low as 0.001 and as high as 1 μmol/mg.

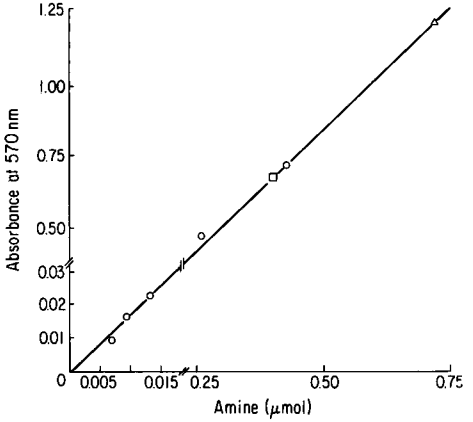


Fig. 1. Samples (2.0 mg) of H-peptide-resins with increasing substitution of peptide (0.003 to 0.36 μmol/mg) were treated by the ninhydrin procedure and diluted to 10 ml for measurement of absorbance.

To show the generality of this test, several peptide-resins with different H_2N-terminal amino acids were studied (Table I). The absorbances at 570 nm were used to calculate the effective extinction coefficient ϵ_{570} (last column Table I). An important requirement for this test to be useful is for the color yield of a particular amino acid to remain constant with increasing chain length of the growing peptide. This was studied on a series of H-(Leu-Ala-Gly-Val-OMPA)$_n$-NHCH$_2$-resins, where n = 1, 6, 10 and OMPA is oxymethylphenylacetyl.[6] The range of ϵ_{570} was only 1.58 to 1.63 x 10^4, ave, 1.60 $\pm$ 0.03 x 10^4.

The precision of the assay on replicate samples was about $\pm$ 4% over most of the analytical range. The sensitivity of the monitoring reaction under the recommended procedure was below 0.0003 mmol/g. This sensitivity is essential, since we wish to establish that the coupling reactions are at least 99.9% complete.

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NOVEL ANGIOTENSIN-II ANALOGS WITH POTENT ANTAGONISTIC EFFECT

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Introduction

The diagnosis and treatment of malignant and renovascular hypertension, caused by high renin activity, with Angiotensin-II (AII) competitive inhibitors is practiced clincally as would be the treatment of congestive heart failure secondary to renovascular hypertension.[1] The main obstacle to the practical use of potent AII analogs prepared so far has been their short biological half-life. Therefore, current research is concerned with the synthesis of analogs resistant to enzymatic degradation so that the duration of action approaches clinical expectation.[2]

Chemistry

The pentafluorophenylester technique[3] was used to synthesize about 40 new AII analogs[4] in solution by stepwise elongation. The most successful protecting group combination permitted the removal of benzyl-type, NO_2 and Z protecting groups in a single step by catalytic hydrogenation after removal of the Dnp group from His. The Boc group was used for temporary protection of the N^{α} amino groups. The synthesis of analogs containing α-hydroxyacids in position 1 was achieved by the following method: suitable α-aminooxyacids were incorporated, and upon catalytic hydrogenation to remove the protecting groups, the α-hydroxyl group was formed without configurational change by splitting the N--O bond. This is apparently a new method for the incorporation of α-hydroxyacids into the peptide chain at position 1. For the formation of the -CO-O-group in analogs containing an α-hydroxyacid in position 8, 1,-1-carbonyl-diimidazole was found to be the best condensing agent. In some cases yields were excellent. For example the intermediate Boc-Arg(NO_2)-Val-Tyr(Bzl)-Ile-His(Dnp)-Pro-Ile-ONB was obtained in 68% overall yield, which corresponds to a 93% yield for each step including coupling and deprotection.[5] The final products were purified by column chromatography on CMC 52 and their purity established by the usual methods. The structures of the compounds synthesized are summarized in Table I.

Table I. AII Analogs Substituted at Positions 1 and 8

X-Arg-Val-Tyr-Ile-His-Pro-Y

	X	Y	Number of Analogs
1. group	α-aminooxyacids α-hydroxyacids	Phe	9
2. group	α-aminooxyacids α-hydroxyacids	Leu, Ile, Thr Thr/Me/	11
3. group	α-aminooxyacids α-hydroxyacids Sar	Ile-OMe, Thr-OMe Thr/Me/-OMe, Ala-OMe	9
4. group	Sar, Ac, OGly	Ile-NH_2, OAla	5
5. group	Sar	Lac, Lac-OEt, HMW	4
6. group	Sar, HOAc	Phe-ol, Ile-ol, Leu-ol	3

Abbreviations: OGly = aminooxyacetic acid; OAla = α-aminooxypropionic acid; Lac = (+)-L-lactic acid; HMW = (+)-α-hydroxy-β-methylvalerianic acid; Phe-ol = phenylalaniol.

Biology

Preliminary biological properties of the compounds were evaluated in the following *in vivo* test. High blood-pressure was produced in anesthetized, vagotomized and ganglion-blocked cats by continuous infusion of Hypertensin® (0.5 μg/kg/min). The high blood-pressure was stabilized and then any change produced by the action of the analogs administered (i.v. and s.c.) was measured. Occasionally, carriers such as CMC or gelatine were used. Sar[1],Ile[5],Ala[8]-AII, prepared in our laboratory, served as the control. The results of these preliminary studies are summarized in Table II.

Some analogs were selected for further evaluation in three *in vitro* tests and the previously described *in vivo* test. The former were: (a) contractility of rabbit aorta strip,[6] (b) contractility of rat fundus strip[7] and (c) effect on aldosterone secretion[8] on treatment with selected AII analogs. The results, expressed in pA_2 values, are summarized in Table III. The *in vivo* test results are listed in Table IV.

Table II. Effect of AII Analogs on the High Blood-Pressure of Anesthetized Cats*

Group	Analogs		Antagonistic Effect	
	X	Y	i.v.	s.c.
I	HOAc, OAla	Phe	agonistic effect	
II	Lac	Ile	+ + +	+ + +
	D-OAla	Leu	+ + +	+ + + +
III	Sar	Ile-OMe	+ + +	+ + + + +
	HOAc	Thr/Me/-OMe	+ +	-
IV	Sar	Ile-NH_2	inactive	
	Sar	OAla	inactive	
V	Sar	Lac	+ + + +	+ + + + +
VI	Sar	Phe-ol	+ +	
	Sar^1, Ile^5, Ala^8-AII		+ + + +	+ + + +

*For abbreviations, see Table I.

Table III. pA_2 Values of Selected AII Analogs

Compound	Aorta	Fundus	Aldosterone
Sar^1, Lac^8-AII	11.74	12.08	8.85
Sar^1, Lac-OEt^8-AII	11.14	13.74	9.56
Sar^1, HMV^8-AII	12.68	11.79	8.64
Control*	11.76	11.56	8.36

*Control = Sar^1,Ile^5,Ala^8-AII (prepared in our laboratory).

Structure-Activity Relationship

From the results of these studies the following structure-activity relationships can be drawn. (1) Analogs containing α-hydroxyacids or α-aminooxyacids in position 1 and Phe in position 8, possess significant agonistic action; the high blood-pressure evoked by Hypertensin® was in some cases increased by 30%. Replacement of Phe by an aliphatic amino acid (Leu, Ile, Thr) produce the expected antagonistic effect. Consequently, it seems that the N-terminal basic group is of no importance for binding to the receptor. The duration of action of these two types of compounds was not prolonged, suggesting enzymatic degradation in the

Table IV. Effect of Some AII Analogs on the High Blood-Pressure of Anesthetized Cats

Compound	i.v.				s.c.			
	d	n	%	t	d	n	%	t
Sar^1, Lac^8-AII	10	5	23	15	50	5	29	90
	20	5	29	15	100	5	33	120
					200	5	47	180
Sar^1, Lac-OEt^8-AII	10	5	27	12	100	6	15	45
	20	8	40	25				
Sar^1, HMV^8-AII	10	5	33	45	100	5	18	30
	20	6	40	20				
Control	10	5	32	15	50	6	30	22
					100	6	29	60
					200	5	24	45

d = dose μg/kg; n = number of animals;
% = max. fall of blood-pressure expressed in %;
t = duration of action in min.

C-terminal region of the molecules. (2) Incorporation of an α-aminooxyacid[9] in position 8 extends the backbone of the peptide chain by one oxygen atom. These compounds are inactive. (3) Position 8 is very important for antagonistic action; amides are inactive while the replacement of the carboxyl group by a hydroxymethyl group diminishes the activity. (4) Remarkably potent compounds are obtained with analogs containing an α-hydroxyacid or ester group in position 8. This replacement appears not to affect the binding to the receptor, while the enzymatic degradation of these compounds is reduced. Side chains also play an important role because only analogs with aliphatic side chains possess significant antagonistic action.

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LINEAR AND CYCLIC ANALOGS OF ACTH FRAGMENTS: SYNTHESIS AND BIOLOGICAL ACTIVITY

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Earlier we discovered similarities in the structural and functional organization of ACTH (including α-MSH), wasp venom kinin (WK), and bradykinin (BK).[1] It may be anticipated that the common features of structural and functional organization of these compounds are also reflected in their three-dimensional structure. Several plausible topologies of ACTH have been described earlier.[1-4]

To study the functional role of the $ACTH_{11-24}$ sequence and the steric organization of ACTH, we have synthesized linear fragments of the parent molecule: $ACTH_{11-16}$(I), $ACTH_{11-19}$(II), $ACTH_{11-24}$(III), $ACTH_{17-24}$(IV) as well as cyclic analogs of the modified ACTH active center, i.e. cycloderivatives of 5-lysine-$ACTH_{5-10}$(V) and 5-lysine, 11-glycine-$ACTH_{5-11}$(VI) the cyclic structures being fixed by covalent bonds between the α-COOH group of C-terminal glycine and the Nϵ-amino group of lysine.

H-Lys-His-Phe-Arg-Trp-Gly (V)

H-Lys-His-Phe-Arg-Trp-Gly-Gly (VI)

Synthesis of the linear fragments I-IV was performed by DCC coupling of the appropriate segments Boc-Lys(Z)-Pro-Val-Gly-OH, H-Lys(Z)-Lys(Z)-OBzl, Boc-Arg(NO_2)-Arg(NO_2)-ProOH or H-Arg(NO_2)-Arg(NO_2)-Pro-OBzl, H-Val-Lys(Z)-Val-Tyr-Pro-OBzl in the presence of HOBT to obtain I, II, III, and IV after removal of the protecting groups by H_2/Pd and purification by partition chromotography.

Synthesis of the cyclic compound V was based on maximum side chain protection in combination with the free α-COOH group of segments. Coupling of the tripeptides (Figure 1) yielded the linear precursor; its cyclization was achieved in DMF via the corresponding Pfp ester. Synthesis of VI was based on condensation of the segments (Figure 2), although in contrast to V the choice of side chain protection for individual segments was determined by the possible selective cleavage of the

p-nitrobenzyl ester bond of protected peptides with $Na_2S_2O_4$ leaving other protecting groups (Z-, Boc-, -NO_2, -OBzl) of trifunctional amino acids (Arg, Lys, Glu) intact.[5]

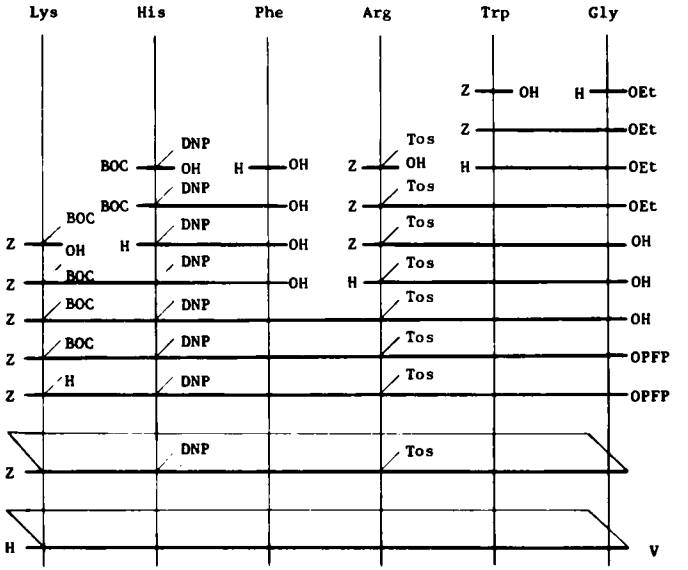

Fig. 1. Synthesis of the ACTH segment analog V

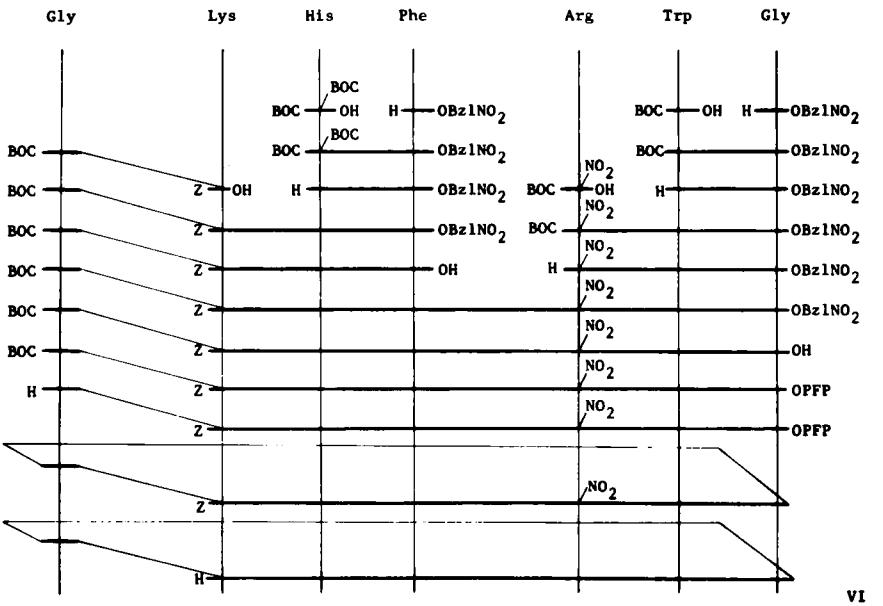

Fig. 2. Synthesis of the ACTH segment analog VI

Purification of intermediates was achieved by preparative LC on silica gel, whereas the end-products after removal of the protecting groups by HF (V) or with H_2/Pd (VI) were chromatographed on CM-cellulose.

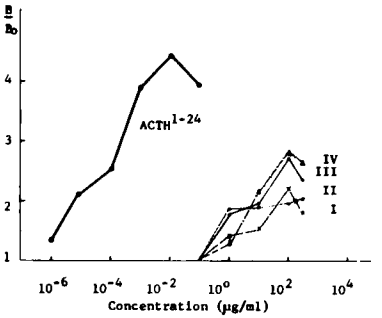

Fig. 3. Steroidogenic activity of the linear ACTH fragments I-IV

Biological studies demonstrated steroidogenic activity for the linear fragments I-IV comparable with that of 17, 18-dilysine-$ACTH_{11-18}$-NH_2[1] (Figure 3) upon their addition *in vitro* to isolated rat adrenal cells[6] obtained by collagenase treatment.[7,8] To explain the discrepancy [9-11] in the steroidogenic activity of compounds II and III we applied 17, 18-dilysine-$ACTH_{1-18}$-NH_2 to cells obtained by trypsinization[6] and to cells obtained by collagenase treatment.[8] Steroidogenic activity was observed only in the latter case (Figure 4). Consequently, trypsinization renders cells less sensitive to steroidogenic agents suggesting that the discrepancy is due to differences in methodology.

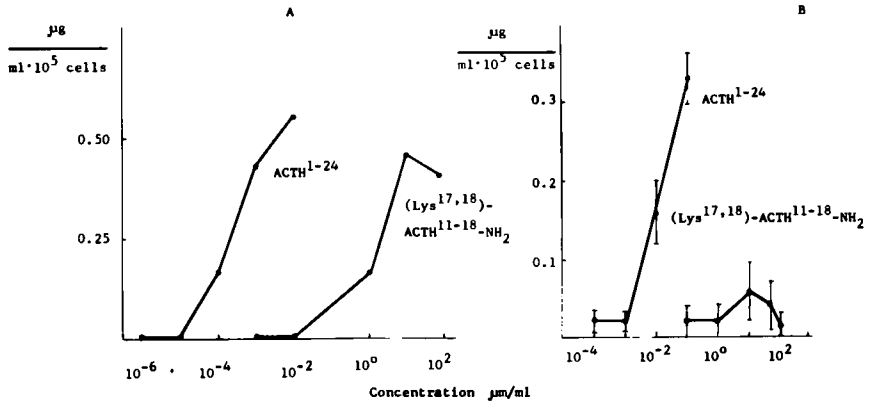

Fig. 4. Influence of $ACTH_{1-24}$ and $Lys^{17,18}$-$ACTH_{11-18}$-NH_2 on steroidogenesis in rat adrenal cells obtained by collagenase (A) and trypsin (B) treatment

Hence, the results obtained indicate the presence of a second active site of ACTH in the sequence 11-24 responsible for the manifestation of steroidogenic activity. Cyclic ACTH analogs revealed a pronounced steroidogenic activity of the 25-member cyclic structure VI over the concentration range 10-500 μg/ml (Figure 5). Unmodified linear $ACTH_{5-10}$ elicits a comparable effect only at five-fold concentrations; the 22-membered cyclic structure V is inactive. No lipolytic activity was

observed with compounds I-IV and VI within the 10-100 μg/ml concentration range when assessed *in vitro*.[12]

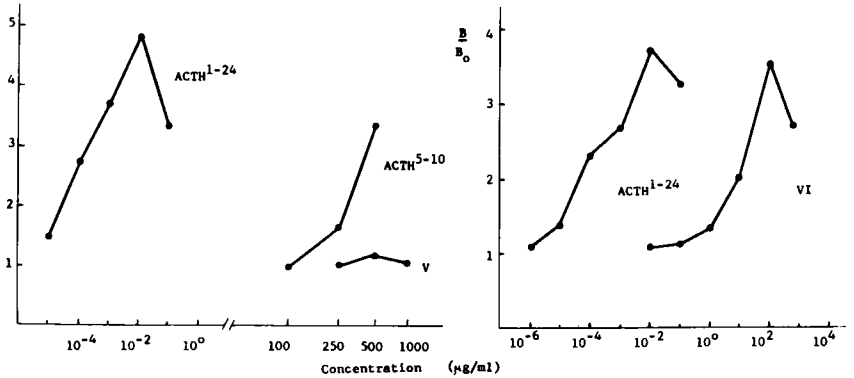

Fig. 5. Steroidogenic activity of V, VI and the linear $ACTH_{5-10}$

Thus, the steroidogenic activity of the ACTH active center is preserved by covalent bridge formation between residues 5 and 11 indicating loop formation for hormone-receptor interaction and suggesting a similar structure-function organization of ACTH and WK molecules both at primary and three-dimensional structure levels.

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THE SYNTHESIS AND ION TRANSPORT PROPERTIES OF ANALOGS OF THE LINEAR GRAMICIDINS: VALINE-GRAMICIDIN A'

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The pentadecapeptides Gramicidin A, B, and C (from *Bacillus brevis;*[1] contain D-amino acids in a unique pattern that strictly observes alternation with residues of the L-configuration.[2] This gives rise to a hydrogen bond stabilized conformation manifest in the β-helix.[3] Peptides with β-helical structure are capable of embedding in lipid bilayers and transporting univalent cations via a channel mechanism.

Analogs of the linear gramicidins with amino acid exchanges and modifications in the H_2N- and COOH-terminal regions have been synthesized and their ion transport mediating properties determined.[4,5] Here the possibility of extending the chain of the natural product by one amino acid residue of appropriate configuration and reconversion to the molecular features of valine gramicidin A is to be tested.

Methionine is the amino acid of choice for chain elongation and removal inasmuch as its peptide bond is selectively cleaved with cyanogen bromide (BrCN).[6]

The formyl group of valine gramicidin A was removed with hydrazine[7] and Boc-D-methionine attached to the des-formylated product. Deprotection with trifluoroacetic acid (TFA) gave the product required for reformylation to obtain formyl-D-Met-(des-formyl)-valine gramicidin A (HCO-D-Met-(des-HCO)-Val-G_A). Treatment of HCO-D-Met-(des-HCO)-Val-G_A with BrCN gave des-formyl valine gramicidin A, a product suitable for reformylation and the synthesis of valine gramicidin A' (Figure 1).

Single channel conductance measurements[8] confirmed that the ion transport properties of HCO-D-Met-(des-CHO)-Val-G_A (Figure 2) are closely related to those of valine gramicidin A. The single channel conductanc s of valine gramicidin A' (Figure 3) are identical with those of the natural product.[9,10]

Thus, an amino acid residue with a selectively cleavable peptide bond may be added to des-formyl valine gramicidin A to elongate the chain. This residue may be removed; the intermediate des-formyl-valine

gramicidin A may be reformylated to give a peptide with the ion transport properties of the naturally occurring valine gramicidin A.

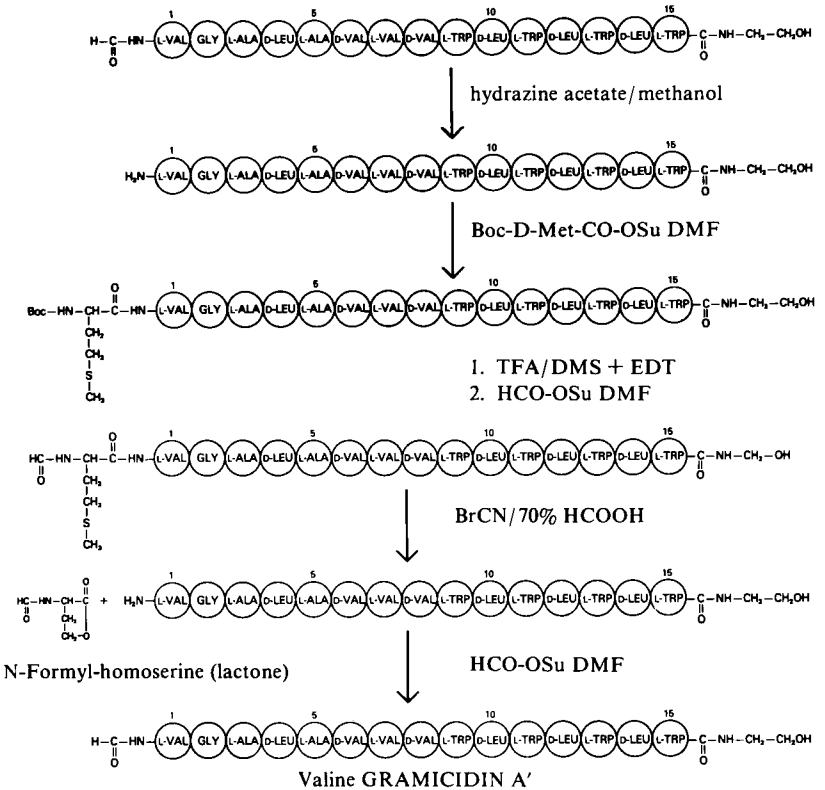

Fig. 1. The synthesis of valine gramicidin A'. Boc = *tert*-butyloxycarbonyl; TFA = trifluoroacetic acid; DMS = dimethyl sulfide; EDT = 1,2-ethane dithiol; OSu = N -hydroxysuccinimidyl ester; DMF = dimethylformamide; BrCN = cyanogen bromide.

Experimental

Valine-Gramicidin A (100 mg) was deformylated by treatment with hydrazine (0.8 ml) in 6 ml methanol plus 1 ml acetic acid.[1] Methanol and hydrazine were removed under high vacuum. The remaining powder was washed with water and freeze-dried from benzene/methanol to give 90 mg of product. R_f of des-formyl valine gramicidin A in chloroform: methanol = 8:2, 0.5 (R_f of the starting material 0.8).

des-Formyl valine gramicidin A (90 mg) was allowed to react for 18 h with Boc-D-methionine-O-succinimide ester (100 mg) in 60 ml of dimethylformamide in the presence of 0.2 ml of diisopropyl ethylamine. The solvent was removed under high vacuum, the product dissolved in 50 ml

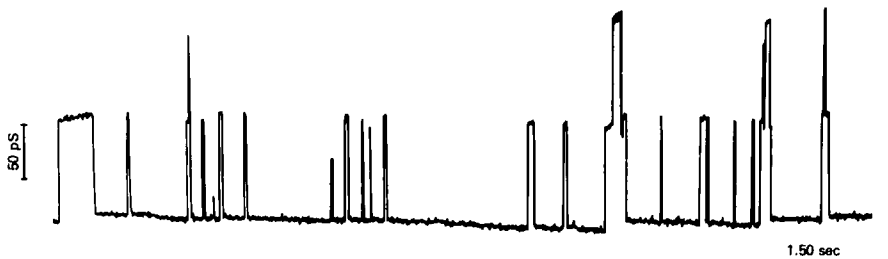

Fig. 2. Single channel conductance induced by N-Formyl-D-METHIONYL-(des-Formyl)-Valine-Gramicidine A. 1M CaCl; T = 25° C; U = 100 mV; Lipid: Monoolein (MG 18:1) 2% w/v in n-decane.

Fig. 3. Single channel conductance induced by Valine-Gramicidin A' (from HCO-D-Met-des-formyl-Valine-Gramicidin A). IM CsCl; pH 6.8; T= 25° C; U = 50 mV; Lipid: Monoolein (MG 18:1) 2% w/v in n-decane.

of ethyl acetate (EtOAc) and 0.1 ml of N-(2-aminoethyl)piperazine added to decompose the excess of active ester. The organic phase was washed with water, 0.1 N hydrochloroic acid, and water again. Following evaporation of EtOAc, 104 mg of Boc-D-Met-(des-formyl)-valine-gramicidin A (Boc-D-Met-(des-HCO)-Val-G_A was isolated. It was deblocked in 1 h with 2 ml of trifluoroacetic acid (TFA; distilled from indole) in the presence of 0.5 g of dimethylsulfide and 0.1 g of 1,2-ethane-dithiole. Solvents and by-products were removed under high vacuum to leave a powder of H_2N-D-Met-(des-CHO)-Val-G_A.

H_2N-D-Met-(des-CHO)-Val-G_A (90 mg) was dissolved in 20 ml DMF, N-hydroxysuccinimidyl formate (130 mg) and $NaHCO_3$ (300 mg) were added, and the reaction mixture allowed to stand for 48 h. DMF was removed under reduced pressure and the product dissolved in EtOAc (50 ml). N-(2-Aminoethyl)piperazine (0.10 ml) was added to decompose (20 min) the excess of active ester. The solution was washed with water, 0.1 N hydrochloric acid, and water again. After evaporation of the solvent the residue was submitted to counter-current distribution in the solvent system chloroform:toluene:methanol:water = 1320:660:1540:462. The product isolated contained traces of non-peptide material which was removed by chromatography on Sephadex LH-20 (130 x 3.5 cm) in methanol:acetic acid:water = 12:1:4. Forty milligrams of product

were isolated. An aliquot was hydrolyzed (6 N hydrochloric acid; 150° C; 2 h) and subjected to amino acid analysis (0.19 μmoles applied; μmoles found/residue numbers, 92% recovery): ethanolamine, .150/.87; Trp, .630/3.65; Gly, .173/1.00; Ala, .355/2.05; Val, .615/3.56; Met, .173/1.00; Leu, .723/4.18.

HCO-D-Met-(des-HCO)-Val-G_A (I; 130 mg) was allowed to react for 4 h at 37° with cyanogen bromide (350 mg) in 70% formic acid (50 ml). After freeze-drying the product was purified by passage over the Sephadex LH-20 column (*vide supra*).

des-HCO-Val-G_A (from I; 35 mg) was allowed to react for 2 days in 4 ml DMF with N-hydroxysuccinimidyl formate in the presence of 50 mg of $NaHCO_3$. Valine-Gramicidin A′ was isolated under the conditions given for the isolation of HCO-D-Met-(des-HCO)-Val-G_A - (II; *vide supra*) and submitted to countercurrent distribution in the solvent system used for the purification of II. The product from countercurrent distribution was passed over the Sephadex LH-20 column to give 7 mg of pure peptide. An aliquot was hydrolyzed (6 N hydrochloric acid; 150° C, 2 h) and subjected to amino acid analysis (.174 μmoles applied; μmoles found/residue numbers; 100% recovery):ethanolamine, .148/.85; Trp, .570/3.30; Gly, .176/1.00; Ala, .364/2.05; Val, .642/3.65; Leu, .718/4.07.

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[9-α-AMINOACETONITRILE] OXYTOCIN: A 9-SUBSTITUTED OXYTOCIN ANALOG WITH HIGH UTERINE ACTIVITY

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A systematic study of the structure-activity relationships of oxytocin analogs indicates that the carboxamide moiety in position 9 of oxytocin is of critical importance for hormonal activity. Deletion of the carboxamide group (as in 9-decarboxamido-oxytocin[1]), of the glycine residue (as in 9-deglycine-oxytocin[2]) or of the entire glycinamide residue (as in 9-deglycinamide-oxytocin[3]) yields, in each case, an analog of oxytocin with very low potency. The carboxamide group cannot even tolerate much modification, either on its carbonyl portion or the amino portion. Thus, analogs of oxytocin or deamino-oxytocin in which the C-terminal $CONH_2$ group was replaced by $COOH$[4-6], $CONHCH_3$[7], $CON(CH_3)_2$[7,8] or $CSNH_2$[9], had quite low biological activities. However, these substitutions do not distinguish between the relative importance of the CO and the NH_2 of $CONH_2$, so it is possible that only one of these two functional groups is required for activity. Although the replacement of $CONH_2$ by COOH might seem to distinguish between CO and NH_2, the COOH group is ionized in solution whereas $CONH_2$ is not. Replacement of $CONH_2$ by methyl- or dimethylamide changes the steric situation in the vicinity of nitrogen.

In an attempt to see whether the CO and NH_2 of $CONH_2$ are both essential for activity, we synthesized [9-α-aminoacetonitrile] oxytocin, an analog in which the α-aminoacetonitrile group ($NHCH_2CN$) replaced the glycinamide residue ($NHCH_2CONH_2$) in position 9 of oxytocin. The analog has neither the CO nor the NH_2 portion of the carboxamide in position 9. However, the nitrile group should simulate the carbonyl portion of glycinamide, and its π-electron system makes it a potential hydrogen bond acceptor like the carbonyl. For the synthesis of this analog, the protected octapeptide Boc-Cys(Bzl)-Tyr(Bzl)-Ile-Gln-Asn-Cys(Bzl)-Pro-Leu-OBzl was prepared by stepwise addition, in solution, of protected amino acids to H-Leu-OBzl. The protecting benzyl groups were removed with sodium and liquid ammonia[10,11] and the disulfhydryl

compound so produced was oxidized by an aqueous solution of potassium ferricyanide.[12] The product was purified by partition chromatography followed by gel filtration and was coupled with α-aminoacetonitrile by the use of dicyclohexylcarbodiimide/1-hydroxybenzotriazole to yield Boc-[9-α-aminoacetonitrile] oxytocin. Removal of the Boc-group with trifluoroacetic acid gave the analog, [9-α-aminoacetonitrile] oxytocin. This was purified by partition chromatography and characterized by amino acid analysis.

The analog was tested for some of the biological activities characteristic of oxytocin. It exhibited 662 ± 45 U/mg and 658 ± 40 U/mg of uterotonic activity[13] *in vitro* in the absence and in the presence, respectively, of Mg^{2+}, 389 ± 24 U/mg of milk-ejecting activity[14] and 5.2 ± 0.2 U/mg of pressor activity.[15]

The C-terminal carboxamide of oxytocin seems not to be essential for biological activities — α-aminoacetonitrile can replace the glycinamide residue with retention of high activities. Like carboxamide, nitrile is a nonionizable, neutral group; also the nitrile group is electrophilic, as is the carbonyl portion of the carboxamide. The results do suggest that the carbonyl portion of carboxamide is of greater importance than the amino portion. Possibly the amino portion is not necessary at all. Observations similar to ours were made with hexeledoisin, hepteledoisin and their analogs. It was found that the C-terminal carboxamide group of hexeledoisin is not essential for biological activity; replacement of C-terminal methioninamide by methionine nitrile affected the activity only marginally.[16] On the other hand the hepteledoisin analog with a C-terminal carboxyl group (methioninamide was replaced by methionine) had very low biological activity.[17]

[9-α-Aminoacetonitrile] oxytocin is the first 9-carboxamide substituted analog synthesized which retains full biological activity; it will be of interest to substitute nitrile for carboxamide in other biologically active peptides.

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SYNTHETIC STUDIES OF THE MODE OF ACTION OF ERABUTOXIN B.

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Among the pharmacologically active compounds found in many snake venoms is a class of small proteins known as curaremimetic neurotoxins. These proteins bind to the nicotinic acetylcholine receptor (AcChR) present in postsynaptic neuromuscular membranes; acetylcholine binding is inhibited and muscular paralysis ensues. The dissociation constant measured for the interaction of alpha-bungarotoxin, a neurotoxin from *Bungarus multicinctus* venom, and *Torpedo californica* electroplax AcChR is 7.7 to 9 x 10^{-9} M.[1] Dissociation constants measured for other variant toxin-receptor complexes have been reported to be as small as 6 x 10^{-10}M. These interactions are, therefore, among the strongest observed between protein molecules. Clues as to which structural elements in the neurotoxin are critical for binding began to emerge when amino acid sequences from a number of species were compared and several invariant residues were identified.[2] Significant consolidation of this knowledge occurred when the three-dimensional structure of the sea snake neurotoxin, erabutoxin b, was determined by X-ray diffraction.[3,4,5] Tsernoglou *et al.*, noting that several functionally invariant residues are closely grouped along the sequence (stippled residues in Figure 1), have pointed out that a hydrogen-bonded ion pair between the guanidinium group of Arg-37 and the side chain carboxylate of Asp-31 is sterically possible and results in a structure with a charge distribution and stereochemical features resembling acetylcholine.[6] A similar analysis has been presented by Low.[7]

We report here the synthesis of the hexapeptide, Trp-Ser-Asp-Phe-Arg-Gly, and the determination of the apparent dissociation constant between it and purified, detergent-solubilized AcChR from *Torpedo californica* electroplax.

The synthesis was achieved using solid-phase procedures.[8,9] Boc-glycine was esterified to chloromethylated 2%-crosslinked polystyrene by reaction in refluxing ethanol for 72 h in the presence of 0.95 equivalent of triethylamine. A 15-g sample of the Boc-glycyl resin (0.265 meq/g) was deprotected by reaction with 40% TFA in CH_2Cl_2 (2% anisole) and the remaining residues were added in a stepwise fashion with the coupling

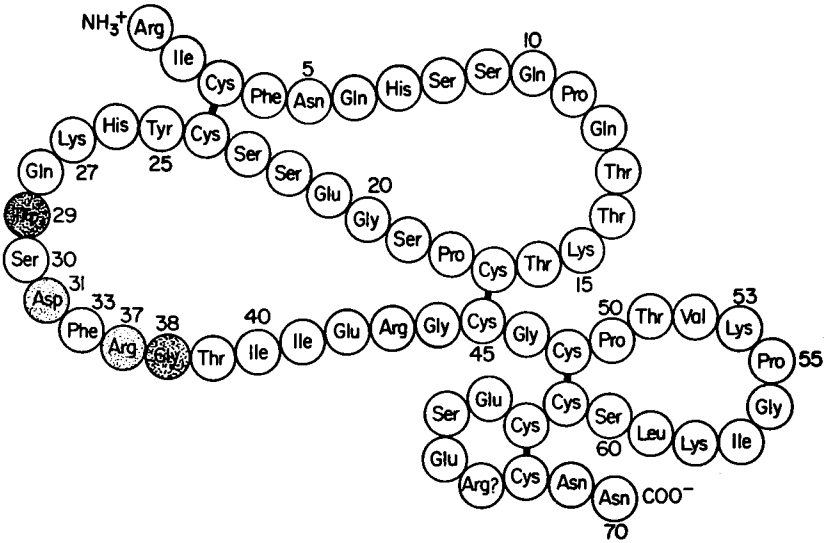

Fig. 1. The amino acid sequence of erabutoxin b.[15,16] "Homology sequence" numbering has been used.[17]

step mediated by DCC. The intermediates used were the Boc-derivatives of N^G-tosyl Arg, Phe, β-benzyl Asp, O-benzyl Ser and N^{in}-formyl Trp. Two coupling cycles with a 1.5-molar excess of amino acid were adequate as judged by the Kaiser ninhydrin test,[10] except in the case of Boc-Asp(Bzl) where three cycles were required. Cleavage from the resin and partial deblocking of the completed hexapeptide were achieved by treatment of the stirred suspension in TFA (10% anisole) with scrubbed, gaseous HBr for 90 min at 23°. Workup of the filtrate provided 3.5 g of crude product, aliquot samples of which were dissolved in 0.5 M pyridine acetate, pH 3.2, and gel-filtered through a column of Sephadex G-25 equilibrated with the same buffer. The amino acid composition of the gel-filtered material was close to theory with the exceptions of Trp and Arg (Table I). N^{in}-Formyl Trp is reported to hydrolyze to Trp in strong aqueous acid[11] and extensive destruction of Trp may occur under the hydrolytic conditions employed. Quantitative recovery of Arg from N^G-tosyl Arg is also not to be expected under these conditions.

The N^G-tosyl group was removed by treatment of the peptide with thioanisole (50 equiv) and trifluoromethanesulfonic acid (5 equiv) in TFA at 23° for 6 h according to the procedure of Kiso *et al.*[12]

After removal of solvent the oily residue was dissolved in 1.0 ml of ethyl acetate and injected into 40 ml diethyl ether; the washed and dried

white powder (45 mg) was dissolved in 2 ml 50 mM ammonium formate, pH 3.2, and purified by LPHPLC chromatography on a column (3 x 30 cm) of C_{18} Porasil B (37-75 microns) (Waters Assoc.). The instrumentation was modeled upon that described by Gesellchen *et al.*[13] Two major and six minor bands were present; center cuts of the major bands, A and B, were lyophilized and the residues dissolved in water to provide the stock solutions used in binding assays. Amino acid analysis of acid hydrolysates of aliquot samples of these stock solutions established the concentration of the peptides as well as indicating the mole ratio of amino acid residues (Table I). No difference between A and B is apparent from these data; however, retention of certain blocking groups or the formation of a diastereoisomer by racemization would not be detected. Experiments are underway to examine these possibilities.

The N^{in}-formyl group was removed from samples of stock solutions of A and B by raising the pH of the stirred solution to 11.3, allowing the solution to stand at this value for 3 min. and then acidifying to pH 4.5.[9] A comparison of the UV spectra of the solutions before and after this treatment indicated that quantitative deformylation had occurred.[11]

Table I. Amino Acid Compositions of Gel-filtered N^{in}-Formyl, N^{G}-Tosyl Hexapeptide and LPHPLC-Purified N^{in}-Formyl Hexapeptides[1]

Amino Acid	N^{in}-Formyl, N^{G}-Tosyl Hexapeptide	Band A from HPLC	Band B from HPLC
Trp	-[2]	0.78 (0.90[5])	0.75 (0.81[5])
Ser	1.04[3]	1.03	1.01
Asp	1.01	1.01	1.01
Phe	0.97	1.00	1.02
Arg	0.32[4]	0.99	0.97
Gly	0.97	0.97	0.99

[1]Hydrolysis conditions: 6 N HCl, crystal of phenol, *in vacuo*. 110°, 22 h.
[2]Not determined.
[3]Ser values are corrected for 10% hydrolytic destruction.
[4]Quantitative recovery of arg from N^{G}-tosyl arg is not expected under the hydrolytic conditions used.
[5]Values calculated using the molar absorptivity of trp at 278 nm after deformylation of stock solutions of known concentration.

The cholinergic binding activity of the peptides was determined using the procedures of Schmidt and Raftery[14] in which the inhibition of the rate of formation of the ^{125}I-alpha bungarotoxin-AcChR complex is measured as a function of peptide concentration. The results are shown in Table II.

It appears that the structural elements responsible for more than half the binding energy between erabutoxin b and AcChR are contained within the hexapeptidyl segment, -Trp-Ser-Asp-Phe-Arg-Gly-, of the

Table II

	AcChR Binding Activity
N^{in}-Formyl, N^{G}-Tosyl Hexapeptide	Not detectable
Band A from HPLC	" "
Deformylated Band A	Approx. 10^{-3} M
Band B from HPLC	5×10^{-5} M
Deformylated Band B	5×10^{-5} M

major loop although, as noted, the possibility that band B still contains a blocking group (not the N^{in}-formyl or N^{G}-tosyl groups, however) or a residue of the D-configuration has not been ruled out. The remaining increment of binding energy may be derived from interactions of the two functionally invariant lysine residues (27 and 53) with the receptor, from additional conformational constraints imposed upon the hexapeptidyl segment by its placement within the entire sequence or a combination of both elements.

Acknowledgements

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ANGIOTENSINS II AND III: THE FUNCTIONAL ROLE OF THE PENULTIMATE PROLINE RESIDUE

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Introduction

Angiotension II (ANG II), Asp-Arg-Val-Tyr-Ile-His-Pro-Phe, acts at specific receptors to elicit numerous contractile and secretory responses. The heptapeptide, angiotension III (ANG III), Arg-Val-Tyr-Ile-His-Pro-Phe, exhibits similar biological properties to ANG II, although careful scrutiny of the pharmacological properties of octapeptide and heptapeptide analogs has revealed that subtle differences exist between the two classes of peptides.[1] It has been known for over a decade that replacement of the C-terminal Phe residue of ANG II or ANG III with a non-aromatic hydrophobic amino acid, *e.g.* Ile, produces antagonists. In addition the potency of the octapeptide, whether it be an agonist or an antagonist, is enhanced by the substitution of a secondary amino acid such as Sar in position 1.

A few years ago, we decided to embark on the quest for a potent angiotensin antagonist which had negligible intrinsic pressor activity. At that time, practically every conceivable substitution for the "response producing" Phe residue at the C-terminus of the molecule had been tried, and we therefore elected to substitute the penultimate Pro residue, in the hope that the subtle changes in conformation produced near the C-terminus might provide the magic ingredient which would result in the "ideal" angiotensin antagonist. Earlier work had already shown that replacement of the Pro residue with Gly or Ala produced analogs with very weak activities,[2] and we therefore decided to make substitutions which would conserve the essential secondary structure at the C-terminus of the molecule. The structures of these secondary amino acids, namely sarcosine, N-methyl-L-alanine, DL-nipecotic acid, 3,4-dehydro-L-proline, thio-L-proline, and D-proline, are shown in Figure 1.

Materials and Methods

Peptides were synthesized and purified (CM-cellulose chromatography) by described methods.[3,4] Final purification for pharmacological investigations was achieved by subjecting peptides (1-2 mg) to reverse-

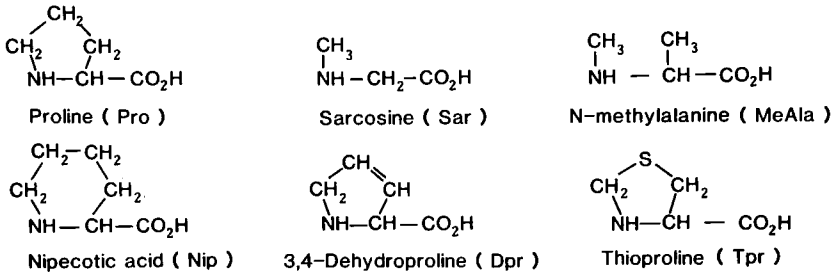

Fig. 1. Structures of proline analogs.

phase HPLC (Spectra-Physics SP 8000) on a column (25 x 0.46 cm) of LiChrosorb RP-8 using a stepped linear gradient of acetonitrile in 0.2 M ammonium acetate, pH 4.0 (0→1 min, 0→10% CH_3CN; 1→20 min, 10→55% Ch_3CN) at 40° C and a flow rate of 1.5 ml/min. Elution times for the analogs ranged between 9 and 13 min. The purity of the peptides was assessed by amino acid analysis after acid hydrolysis and by TLC in two solvent systems: *n*-butanol:pyridine:acetic acid:water = 15:10:3:6, v/v (R_f range 0.31-0.47) and chloroform:methanol:acetic acid:water = 15:10:2:3, v/v (R_f range 0.03-0.08). The assay on the isolated rat uterus was carried out on paired uteri from diethylstilbestrol-primed female Sprague-Dawley rats (150-250 g) as described previously.[5]

Results

As might be anticipated, based on the known steric problems associated with the synthesis of peptides containing secondary amino acids,[6] difficulties were encountered with the coupling of both the penultimate secondary amino acid to the C-terminal residue anchored to the resin, and with the coupling of Boc-His(Tos) to the secondary amino acid. Except in cases where the penultimate amino acid was Pro or Sar, the DCC-mediated coupling reactions were found to be incomplete even after 16 hours. Repeat coupling reactions in the presence of 1-hydroxybenzotriazole did not rectify the situation. However, in all cases, the coupling reactions could be expedited by using 2.5 equivalents each of the Boc-amino acid and EEDQ in methylene chloride for 16 hours. Based on these observations, we now use the EEDQ coupling method as a standard repeat coupling procedure in all syntheses in our laboratory. Nevertheless, we were unable to synthesize angiotensin analogs containing the constrained 4-membered ring of azetidine; this was due to failure of the coupling reaction, rather than cyclization of the dipeptide to form the diketopiperazine with concomitant removal from the resin.

The biological activities of the various ANG II and ANG III analogs synthesized are shown in Table I. The first group of peptides illustrated in the table contain Phe as the C-terminal residue and were therefore designed as agonists. In all cases these peptides turned out to be agonists, as evidenced by the pharmacological properties. In summary, it was found that the penultimate Pro residue in both the octapeptide and the heptapeptide can be replaced by a variety of different proline analogs without severely disrupting the function of the two molecules; the only exception was substitution by D-Pro which resulted in very low myotropic activities. Substitution of Sar caused a moderate decrease in the agonist potencies of the octapeptide and heptapeptide, whereas substitution by Dpr essentially produced no change and the substitution by Tpr resulted in an increased agonist potency for both classes of peptide.

All peptides containing Ile as the C-terminal residue were predicted to be antagonists of angiotensin and, for the most part, the pharmacological properties illustrate that this is the case. Interestingly, replacement of the proline residue in the octapeptide antagonist [Sar^{1}Ile^8]ANG II, with every secondary amino acid investigated in this study except D-Pro, resulted in a moderate decrease in antagonist potency, indicating that the analogs retained a high binding affinity for uterine smooth muscle receptors (pA_2 7.2-8.2). In contrast, substitution of the same secondary amino acids in the heptapeptide, *i.e.* [Des Asp^{1}Ile^8]ANG II, resulted in a much more drastic decrease in the antagonist potency of this molecule. Thus, only the substitution of MeAla or Dpr in the heptapeptide resulted in analogs with viable antagonist activity (pA_2 6.7-6.9). The substitution by Sar resulted in an analog with very low antagonist activity (pA_2 6.1), and the substitution by DL-Nip, Tpr or D-Pro abolished the antagonist activity ($pA_2 < 5$).

Conclusion

The data show that the proline residue in octapeptide analogs of the type [Sar^{1}Ile^8]ANG II can be replaced with a variety of secondary amino acids without severely affecting the antagonist potency of the molecule, whereas the equivalent heptapeptide ANG III analogs exhibit drastically reduced antagonist activities with these same substitutions. In contrast, replacement of the proline residue of "agonist" analogs of ANG II and ANG III, *i.e.* analogs retaining the C-terminal phenylalanine residue, had moderate and quantitatively equivalent effects on the activities of both the octa- and heptapeptides. These findings suggest that different criteria may be operant (regarding the functional role of the penultimate

Table I. Agonist and Antagonist Activities of Analogs of Angiotensins II and III on the Isolated Rat Uterus.

PEPTIDE	AGONIST ACTIVITY (% of ANG II)	ANTAGONIST ACTIVITY (pA_2 versus ANG II)	PEPTIDE	AGONIST ACTIVITY (% of ANG II)	ANTAGONIST ACTIVITY (pA_2 versus ANG II)
[Sar^1]ANG II	160 ± 21	-	[Sar^{1}Sar^{7}Ile^8]ANG II	<0.1	8.1 ± 0.1
[DesAsp^1]ANG II	22 ± 3	-	[DesAsp^{1}Sar^{7}Ile^8]ANG II	<0.1	6.1 ± 0.2
[Sar^{1}Sar^7]ANG II	22 ± 4	-	[Sar^{1}MeAla^{7}Ile^8]ANG II	<0.1	8.2 ± 0.1
[DesAsp^{1}Sar^7]ANG II	4 ± 0.5	-	[DesAsp^{1}MeAla^{7}Ile^8]ANG II	<0.1	6.7 ± 0.2
[Sar^{1}Tpr^7]ANG II	238 ± 14	-	[Sar^{1}DL-Nip^{7}Ile^8]ANG II	<0.1	7.8 ± 0.2†
[DesAsp^{1}Tpr^7]ANG II	47 ± 7	-	[DesAsp^{1}DL-Nip^{7}Ile^8]ANG II	<0.1	<5
[Sar^{1}Dpr^7]ANG II*	157 ± 18	-	[Sar^{1}Tpr^{7}Ile^8]ANG II	0.05	7.2 ± 0.2
[DesAsp^{1}Dpr^7]ANG II*	31 ± 3	-	[DesAsp^{1}Tpr^{7}Ile^8]ANG II	0.05	<5
[Sar^{1}D-Pro^7]ANG II	0.1	<5	[Sar^{1}Dpr^{7}Ile^8]ANG II*	<0.1	7.9 ± 0.2
[DesAsp^{1}D-Pro^7]ANG II	0.02	<5	[DesAsp^{1}Dpr^{7}Ile^8]ANG II*	<0.1	6.9 ± 0.2
[Sar^{1}Ile^8]ANG II	<0.1	8.6 ± 0.1	[Sar^{1}D-Pro^{7}Ile^8]ANG II	0.1	<5
[DesAsp^{1}Ile^8]ANG II	<0.1	7.9 ± 0.2	[DesAsp^{1}D-Pro^{7}Ile^8]ANG II	0.02	<5

NOTE: For the sake of clarity, all peptides are expressed as analogs of ANG II, although peptides lacking aspartic acid in position 1 are analogs of ANG III. *Dehydroproline derivatives racemize in solution and therefore the optical integrity of peptides containing this amino acid is questionable. †Probably represents L-isomer, since D-proline analogs are inactive.

proline residue) for the binding of heptapeptide antagonists, compared to octapeptide antagonists and hepta- and octapeptide agonists, to angiotensin receptors in uterine smooth muscle. Preliminary investigations in the rat and dog *in vivo* suggest that this may also hold true for angiotensin receptors mediating blood pressure responses.[1] Thus it may be necessary to regard heptapeptide antagonists as a special case. Whether or not these findings are related to the known multiplicity of angiotensin receptors in smooth muscle[7] remains to be established.

Acknowledgements

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PORCINE RELAXIN: SYNTHESIS AND STRUCTURE ACTIVITY RELATIONSHIPS

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Introduction

During pregnancy the pig ovary synthesizes and secretes the peptide hormone relaxin.[1] The hormone softens the cervix and lengthens the interpubic ligament thus facilitating the birth process. Relaxin also inhibits uterine contractions and may influence the timing of parturition.

Relaxin is composed of two non-identical peptide chains covalently linked by one intra- and two inter-chain disulfide bonds in a manner exactly analogous to that of insulin. These structural similarities suggest that relaxin and insulin may have evolved from a common ancestral gene.[2,3] The complete amino acid sequence of relaxin from the pig[3-6] and the rat,[7] have been reported and partial sequence information is known for the shark hormone.[8] In contrast to insulin there is considerable sequence variation between species. Several structural variants of the relaxin molecule have been isolated from pregnant pig ovaries. Sherwood and O'Byrne[9] have described three forms of pig relaxin of equivalent biological activity known as CMB, CMa′ and CMa, which are characterized by their elution position following carboxymethyl cellulose chromatography. Structural analyses of these forms of pig relaxin have revealed that they all have identical A chains of 22 amino acids but differ in the amino acid sequence at the carboxyl terminus of the B chain.[6] Thus CMB is A22 B28, CMa′ A22 B29 and CMa A 22 B31 (Figure 1). Recent studies by Walsh and Niall[10] suggest that the major form of the hormone stored in the pig ovary is the A22 B31 or CMa variant while the other structural forms arise as a result of proteolysis during the isolation procedures. The nature of the secreted and circulating form of the hormone is not yet known.

In this report we describe the solid-phase synthesis of some of the structural analogs of pig relaxin which have enabled us to define certain aspects of the structure of the hormone important for biological activity.

Experimental Procedures

The A and B chains of pig relaxin were assembled separately on phenylacetamidomethyl-1% cross-linked polystyrene resins[11] using standard solid-phase procedures and with the side-chain protecting groups previously described.[12] The crude peptides were S-sulfonated, purified by gel filtration and DEAE-Sephadex ion exchange chromatography and assessed for homogeneity by amino acid analysis, TLC, HPLC and paper electrophoresis. Relaxin A and B chains were combined by reducing a mixture of the purified S-sulfonated peptides (A:B 1.5:1) at pH 8.3 under nitrogen for 6 min. The pH was then adjusted to 5 with dilute acetic acid and the reduced peptides precipitated with acetone and washed with ethyl acetate and ether. Oxidation was performed at pH 10.6 for 40 hr at 5° C in the presence of 0.5M NaCl. The efficiency of the chain combination was monitored by radioimmunoassay, bioassay, circular dichroism and HPLC. Final purification of the combination mixture was by ion-exchange chromatography on SP-Sephadex and HPLC. The biological activity of the synthetic relaxin peptides was tested in the rat uterine contractility assay.[13]

Results and Discussion

The purified separate chains of pig relaxin were obtained in approximately 10% yield (based on crude). The major impurities in the synthetic peptides appear to have arisen through side-reactions of the cysteine residues occurring during the HF cleavage step. In more recent syntheses improved yields have been obtained using the HF stable S-ethyl mercapto protecting group for cysteine. The relaxin B chains were particularly difficult to purify. The solubility in aqueous solution was poor and the peptides adsorbed irreversibly to chromatographic media. Detailed studies of the conformation of the relaxin B chains by CD revealed that the configuration of the B31, B29 and B28 peptides in solution were largely β-structure. Shortening of the B chain at the carboxyl terminus to 25 residues resulted in a change to an unordered conformation. This conformational change was reflected in an enhanced solubility of the peptide in aqueous solution, a loss of the adsorptive behavior and an improvement in the combination yield with A chain.

Conditions for the efficient combination of the relaxin A and B chains were found to differ from that of the structurally related insulin molecule. Precipitation of the peptide chains with acetone to remove reducing agent and the addition of 0.5M NaCl during the oxidation step were essential requirements. Combination yields ranged from 0.7% for

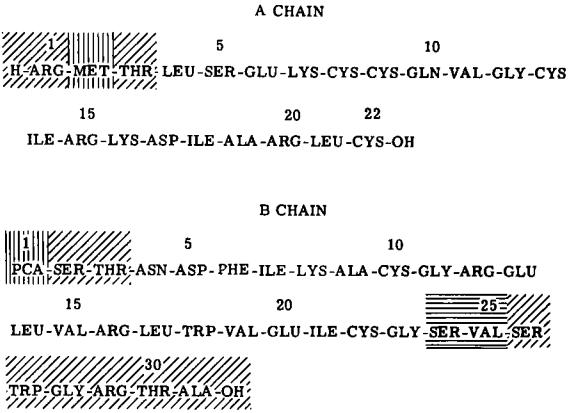

Fig. 1. Porcine relaxin A22 B31. The shaded sequence region is not required for biological activity in the uterine assay.

the A22 B28 peptides to 10% with the A22 B25 analog. In the latter case, the specific activity of the recombination mixture in the rat uterine contractility assay was 33%. Purification of the A22 B25 peptide resulted in an increase in specific activity to 93% compared with the native pig relaxin molecule (A22 B31). Thus, shortening at the carboxyl terminal region of the B chain by 6 amino acid residues does not affect the biological potency of pig relaxin in the uterine assay. Figure 1 summarizes the structural analogs of the pig relaxin molecule synthesized to date. Shortening is tolerated at the amino terminus of both the A and B chains as well as at the carboxyl terminus of the B chain. The synthetic peptide A(4-22)-B(4-23) still retains significant biological activity (1%) in the uterine assay. Assessment of the activity of these synthetic peptides in the mouse pubic symphysis assay is in progress.

Acknowledgements

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THE THERMODYNAMICS OF LIPID-PROTEIN ASSOCIATION AND THE ACTIVATION OF LECITHIN:CHOLESTEROL ACYLTRANSFERASE BY SYNTHETIC LIPOPEPTIDES

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Our long term goal has been to develop a relationship between the primary structure of apolipoproteins and their affinity for phospholipid and capacity to activate some of the enzymes involved in lipid metabolism.[1-7] We have used solid phase methods to synthesize a series of peptides that are models for the native human plasma apolipoproteins. The peptide sequences were selected such that when placed in an α-helical conformation the residues on one side of the helical axis are polar and the residues on the opposite side are non-polar; this design is similar to the putative amphipathic structure of the lipid-association regions of human plasma apolipoproteins.[8] Using techniques described previously,[2,4,5,9] we have synthesized the Lipid Associating Peptides (LAP) in Figure 1.

VSSLKEYWSSLKESFS	LAP-16
VSSLLSSLKEYWSSLKESFS	LAP-20
VSSLLSSLLSSLKEYWSSLKESFS	LAP-24

Fig. 1. The amino acid sequence of peptides synthesized and used in this study.

LAP-20 and LAP-24 are identical to LAP-16 except that they contain an insertion of Ser-Ser-Leu-Leu. These peptides were tested as functional analogs of apolipoproteins by well established physical techniques. In Figure 2, we show the fluorescence behavior of each of the peptides in Tris buffered saline (TBS), pH 7.4. Both LAP-16 and LAP-20 (165 μM) have fluorescence maxima near 350 nm but that of LAP-24 appears at 346 nm. As the insert to Figure 2 shows, the spectral maxima of the peptides at low concentration all appear at 350 nm. We assign the blue shift of LAP-24 at higher concentrations to its self-association, a process that has been identified in most apolipoproteins. Addition of dimyristoylphosphatidylcholine (DMPC) to LAP-20 and LAP-24 produced a large blueshift in the peptide fluorescence; this shift was assigned to the association of DMPC and LAP (Table I). This effect was not observed with LAP-16 except in the presence of high concentrations

Table I. Thermodynamic and Spectral Properties of Model Apolipoproteins

Peptide	ΔG_a, kcal/mole	Fluorescence, λmax
LAP-16		350 nm
LAP-16 + DMPC		350 nm
LAP-20	-8.9	350 nm
LAP-20 + DMPC		334 nm
LAP-24	-9.6	350 nm
LAP-24 + DMPC		338 nm

(>1.0M) of structure making salts. The enhancement of the association by structure making salts suggests that the association is hydrophobic.

The effect of dilution on the fluorescence and LCAT activity of complexes of LAP-20 and LAP-24 with DMPC and cholesterol (1:20:0.04 M/M) are plotted in Figure 3. These data show that dilution of the complexes results in a shift in the fluorescence of the peptides from that characteristic of a complex to one that is identical to the emission of the peptide in dilute aqueous solution. We assign this change to the dissociation, LAP·DMPC→LAP + DMPC.

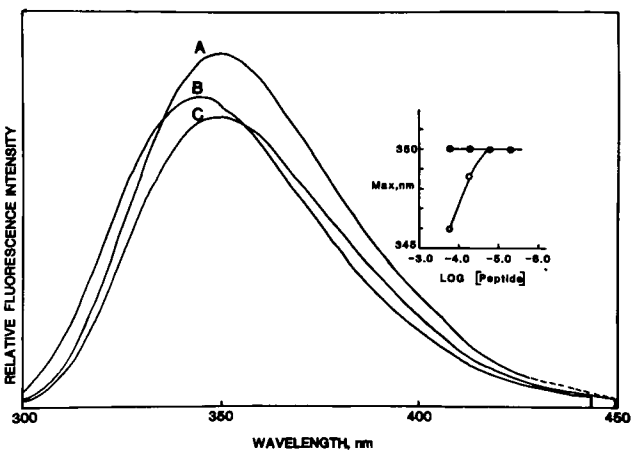

Fig. 2. Fluorescence spectra of the Lipid Associating Peptides. Spectra were collected at ambient temperature at a concentration of 165 μM in Tris buffered saline. A. LAP-20; B. LAP-24; C. LAP-16. The insert shows the concentration dependence of the fluorescence maxima; LAP-16,20 (●); LAP-24 (O).

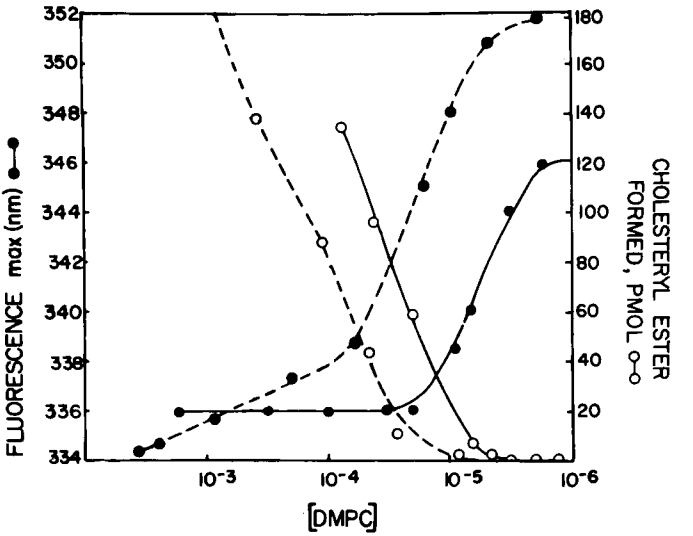

Fig. 3. Effect of complex concentration on LCAT activity and fluorescence. A complex of LAP-20 (-----) or LAP-24 (———) was prepared which contained peptide, DMPC and ^{3}H-cholesterol in molar ratios of 1:20:0.4. A stock solution of each was diluted to concentrations extending to 10^{-6} DMPC and allowed to stand until no further change in fluorescence was observed, 2 h. For LCAT assays,[7] a 25 μl aliquot of the enzyme was added after 2 h. The reaction was allowed to proceed for 1 h and then the amount of cholesteryl ester formed was determined.

From the midpoint of the shifts, we calculated the unitary free energies of association, ΔG_a, which are given in Table I. Since LAP-16 does not associate with DMPC in the absence of high salt concentrations, it was not tested. We have previously reported that LAP-20 is a potent activator of lecithin:cholesterol acyltransferase (LCAT).[7] Over the same concentration range, activity disappeared in a concentration range similar to that where the fluorescence shifts appeared. Both the fluorescence shift and disappearance of LCAT activity were at lower concentrations with LAP-24 than with LAP-20. We assigned the coincidence of the spectral shifts and the disappearance of LCAT activity to a requirement of the enzyme for an activator that is associated with the substrate. The lack of association and activation with LAP-16 supports this interpretation. Moreover, the larger ΔG_a for LAP-24 must be due to its higher hydrophobicity provided by its higher content of leucine.

Collectively, these data show that this series of peptides are good structural and functional analogs of apoA-I, the native activator of LCAT. In this respect, LAP-24 is particularly good since it self-associates, has a high affinity for lecithin, and activates LCAT. Our data

suggest that these peptides must have a high affinity for the lipid substrate to be effective as activators for LCAT and that activation only occurs with those peptides that are combined with the substrate under conditions of ionic strength where a stable complex is formed.

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DESIGN OF MORE POTENT ANTAGONISTS OF THE ANTIDIURETIC ACTION OF ARGININE VASOPRESSIN

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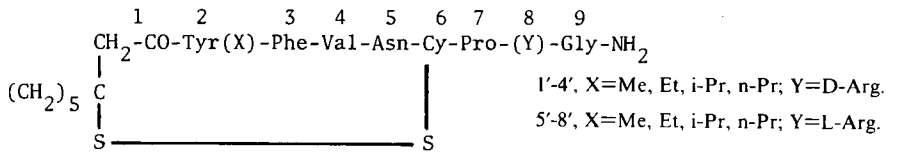
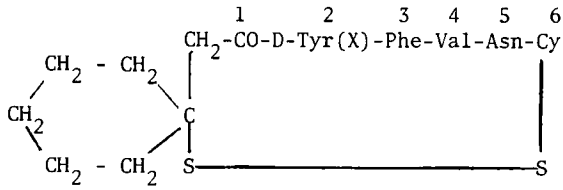
We recently reported the first known effective antagonists of *in vivo* antidiuretic responses to both exogenous and endogenous arginine vasopressin (AVP).[1,2] These analogs were designed by incorporating O-alkyltyrosine substitutents[3] at position two in [1-(β-Mercapto-β,β-cyclopentamethylenepropionic acid), 4-valine, 8-D-arginine]vasopressin [d(CH_2)$_5$VDAVP] and in its L-arginine isomer d(CH_2)$_5$ VAVP.[5] The eight analogs so designed have the following general structures:[1,2]



In both the D-Arg and the L-Arg series the O-ethyltyrosine containing analogs are the most potent.[1,2] All four of the L-arginine containing analogs are more potent than any of the four D-arginine containing analogs.[1,2] The following structural features have also been shown to be essential for antidiuretic antagonism in these analogs.[5] 1. The β,β-cyclopentamethylene group on the β-carbon at position one. 2. The O-alkyl substituents at position two. 3. Phenylalanine at position three. 4. Valine at position four.

In attempting to enhance the potency of these antidiuretic antagonists, the above eight analogs have been modified by the incorporation of the respective D-Tyr(alkyl) substituent at position two in each of the L-Arg and D-Arg series. Following initial findings on these analogs we decided to incorporate the unalkylated D-Tyr residue at position two in d(CH_2)$_5$VDAVP[4] and in d(CH_2)$_5$VAVP.[5]

The ten new analogs designed in this fashion have the following general structures:



1-4, X=Me, Et, i-Pr, n-Pr; Y=D-Arg. 9, X=H Y=D-Arg. 5-8, X=Me, Et, i-Pr, n-Pr; Y=L-Arg. 10, X=L-Arg.

Peptide Synthesis

The Boc-O-alkyl D-tyrosine derivatives were prepared using a crown ether.[6] All peptides were synthesized by the solid phase[7,8] and active ester[9] methods using previously described modifications.[2,10,11] Nitrophenyl-β-(S-benzylmercapto)-β,β-cyclopentamethylene propionate[12] was used in each final coupling step. Deblocking was carried out with Na in NH_3[13] and cyclization was effected with $K_4[Fe(CN_6)]$[14] using a modified oxidation procedure.[2] The peptides were purified by gel filtration on Sephadex G-15.[15]

Table I. Effects of D-Tyr(alk)^2 Substitution on Antiantidiuretic Potencies

No.	Antagonists	Anti-antidiuretic Effective dose[a] nmol/kg	pA_2[b]	pA_2[d]
1	d$(CH_2)_5$D-Tyr(Me)VDAVP	4.9±1.3	7.19±0.12(4)[c]	(6.68)
2	d$(CH_2)_5$D-Tyr(Et)VDAVP	1.8±0.02	7.59±0.04(4)	(7.10)
3	d$(CH_2)_5$D-Tyr(iPr)VDAVP	2.2±0.3	7.51±0.06(4)	(6.88)
4	d$(CH_2)_5$D-Tyr(nPr)VDAVP	1.7±0.2	7.60±0.05(4)	(6.67)
5	d$(CH_2)_5$D-Tyr(Me)VAVP	1.2±0.3	7.77±0.07(6)	(7.35)
6	d$(CH_2)_5$D-Tyr(Et)VAVP	1.1±0.2	7.81±0.07(5)	(7.57)
7	d$(CH_2)_5$D-Tyr(iPr)VAVP	1.6±0.4	7.66±0.11(4)	(7.32)
8	d$(CH_2)_5$D-Tyr(nPr)VAVP	1.7±0.2	7.61±0.06(4)	(7.29)

[a]The effective dose is defined as the dose (in nanomoles per kilogram) that reduces the response seen with 2x units of agonist to equal the response seen with x units of agonist administered before antagonist. [b]Estimated *in vivo* pA_2values represent the negative logarithms of the "effective doses" divided by the estimated volume of distribution (67 ml/kg). [c]Means ± S.E. number of assay groups in parenthesis. [d]Values for each of the corresponding O-alkylated L-tyrosine antagonists (1'-8' reported in Reference 2).

Bioassay Methods

The antidiuretic agonistic and antagonistic potencies of these analogs were measured by previously described methods.[1,2,16] Antagonistic potencies were determined and expressed as a) "effective doses" and as b) pA_2 values.[17] For definitions of a) and b) see Table 1 footnote[a,b]. Details of the synthesis and pharmacological properties of analogs **1-10** will be presented elsewhere.[18]

Results and Discussion

The anti antidiuretic potencies of the O-alkyl D-tyrosine analogs, *i.e.* analogs **1-8** together with the pA_2 values for the corresponding L-tyrosine analogs,[2] are presented in Table I. The antidiuretic properties of the unsubstituted D-tyrosine analogs, *i.e.* analogs **9** and **10** together with those of their respective L-tyrosine parent analogs $d(CH_2)_5VDAVP$[4] and $d(CH_2)_5VAVP$,[5] are presented in Table II. All ten D-tyrosine analogs possess transient weak antidiuretic activities (0.004-0.05 U/mg). Subsequent doses of AVP are reversibly antagonized for 1-3 h, depending on the dose of antagonist.

Effects of D- Versus L-(O-Alkyl) Tyrosine Substituents on Antidiuretic Antagonism

Comparisons of the pA_2 values for the O-alkylated L- and D-tyrosine analogs in Table I show clearly that the latter are more potent antagonists. The enhancements are by no means consistent, however, especially in the D-Arg series. In this series they range from a two fold enhancement for the O-methyl D- and L-Tyr analog pairs to almost a ten fold enhancement for the O-n-propyl D- and L-Tyr analog pairs. In the L-Arg series the enhancement is about three to four fold in all cases. Also, the size of the alkyl substituents does not appear to be as critical in the D-Tyr(alk) analogs as in the L-Tyr(alk) analogs. In addition, although the L-Arg analogs are more potent than the D-Arg analogs, these differences are not nearly as striking as those found earlier for the L-Tyr(alk) analogs. Nonetheless, it is interesting that the O-ethyl-tyrosine/L-arginine analog, *i.e.* $d(CH_2)_5$D-Tyr(Et)VAVP, is the most potent of these new analogs and is also, in fact, the most potent antidiuretic antagonist reported to date.

Table II. Unalkylated D-Tyr[2] of Weak Antidiuretic Agonists are Potent Antidiuretic Antagonists.

No.	Peptide	Antidiuretic units/mg	Antiantidiuretic Effective dose[a] nmol/kg	pA_2[b]
	$d(CH_2)_5$VDAVP	0.10±0.02[d]	agonist	-
9	$d(CH_2)_5$D-TyrVDAVP	antagonist	6.3±0.8	7.03±0.05(4)[c]
	$d(CH_2)_5$VAVP	0.2[e]	agonist	-
10	$d(CH_2)_5$D-TyrVAVP	antagonist	2.2±0.2	7.51±0.08

[a,b,c]See Table I. [d]Value from Reference 4. [e]Value for Reference 5.

Unsubstituted D-Tyrosine Also Confers Antidiuretic Antagonism

Both $d(CH_2)_5VDAVP$[4] and $d(CH_2)_5VAVP$[5] are weak antidiuretic agonists and do not exhibit antidiuretic antagonism *in vivo*. The data

given in Table II clearly shows that the substitution of D-tyrosine for L-tyrosine in both of these molecules converts these antidiuretic agonists into fairly potent antagonists of *in vivo* antidiuretic responses. This is a striking finding for it shows clearly that alkylation of the tyrosine residue at position 2 *per se* is not a prerequisite for antidiuretic antagonism.

Conclusion

We have shown that the replacement of L-Tyr(alk) by D-Tyr(alk) where alk=Me, Et, i-Pr, and n-Pr in our previously reported antidiuretic[1,2] antagonists leads to enhancements of anti antidiuretic potencies in all cases. In addition, we have shown that an unalkylated D-Tyr/L-Tyr interchange converted two weak antidiuretic agonists, $d(CH_2)_5VDAVP$ and $d(CH_2)_5VAVP$ into potent antidiuretic antagonists. All of these antidiuretic antagonists are potentially useful as pharmacological tools and as clinical agents. Furthermore, these findings have obvious potential for the design of even more potent antidiuretic antagonists.

Acknowledgements

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THE USE OF CONFORMATION-LOCUS DIAGRAMS IN CONFORMATIONAL ANALYSIS

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Introduction

We and others have studied the neurohypophyseal hormone oxytocin in D_2O by ^1H NMR spectroscopy and reported the values of the coupling constants between vicinal pairs of H^α and H^βs for various residues.[1-6] These coupling constants, designated by $^3J_{H^\alpha-H^\beta}$, are of particular interest because they reflect the state of the conformation about the C^α-C^β bond (the torsion angle χ^1).[7]

The most general assumption is that there is rotational isomerism about this bond and that interconversion among the conformations that we designate *base states* is rapid on the NMR time scale—i.e., the time scale is such that the observed values of $^3J_{H^\alpha-H^\beta}$ reflect averages with each base state contributing in proportion to its relative population. We and others[1-6] have made the assumption that the base states for this bond in various residues of oxytocin in D_2O are the three staggered conformations shown in Figure 1 and have calculated the relative populations of these states, designated rotamers I-III, by standard methods.[8-10] In addition, Boicelli *et al.*[2] and we[4,6] have tested the hypothesis that χ^1 for each hemi-cystyl residue is restricted to lie in the neighborhood of a single value—i.e., that there is only one base state, which is not necessarily one of the staggered conformations.

In this paper, we (1) review the original method employed by us[4] to test for the possibility of a restricted conformation (the *intersection-of-sets* method) and (2) discuss an improved method for this test (the *conformation-locus* method).

The Intersection-Of-Sets Method

Figure 2 shows the Karplus relationships adopted from Kopple *et al.*[11] that express the values of $^3J_{H^\alpha-H^{\beta 2}}$ and $^3J_{H^\alpha-H^{\beta 3}}$ as functions of χ^1. The values of $^3J_{H^\alpha-H^{\beta 2}}$ and $^3J_{H^\alpha-H^{\beta 3}}$ reported by us[4] for the hemi-Cys[1], Tyr[2] and hemi-Cys[6] residues of oxytocin in D_2O at pD 3.8 and 25°C are shown as horizontal lines in this figure. One set of up to four different restricted conformations is generated by the crossover points between the

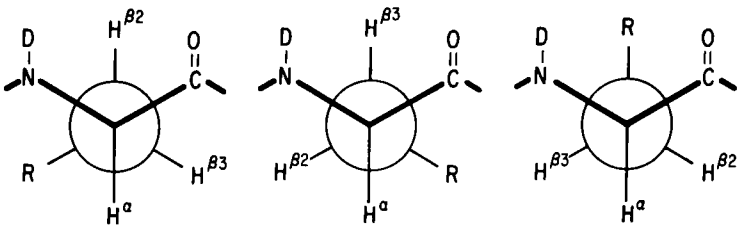

Fig. 1. The staggered conformations for amino acids of an L configuration. The stereochemical definitions of $H^{\beta 2}$ and $H^{\beta 3}$ are given in this figure. The torsion angle between H^{α} and $H^{\beta 2}$ is approximately (χ^1 - 120°), and that between H^{α} and $H^{\beta 3}$, χ^1.

Karplus curve for $^3J_{H^{\alpha}-H^{\beta 2}}$ and the horizontal line (——) expressing the experimentally determined value of this coupling constant for a particular residue, and another set, by crossover points between the curve for $^3J_{H^{\alpha}-H^{\beta 3}}$ and the line (——) expressing the value of this latter coupling constant for this residue. If these two sets (one for $^3J_{H^{\alpha}-H^{\beta 2}}$ and one for $^3J_{H^{\alpha}-H^{\beta 3}}$) contain an element in common, the data are compatible with a single, restricted conformation. For example, the value of $^3J_{H^{\alpha}-H^{\beta 2}}$ for hemi-Cys[6] (see □ in Figure 2) is compatible with the set of values of χ^1 [~+120°, ~−90°, ~−30°], and the value of $^3J_{H^{\alpha}-H^{\beta 3}}$ for this residue (■) is compatible with the set [~+120°, ~−120°, ~−60°]. These two sets have the element ~+120° in common, and so the data are compatible with a restricted conformation—i.e., a single base state—in the neighborhood of ~+120°. Since the mathematical operation for determining the common elements in different sets is known as an *intersection,* this approach for searching for restricted conformations is termed the *intersection-of-sets* method. Also note in Figure 2 that the data for hemi-Cys[1] but not Tyr[2] are compatible with a restricted conformation (for hemi-Cys[1], χ^1 = ~−120°).

The Conformation-Locus Method[12]

The intersection-of-sets method suffers from the following disadvantages: (1) it is cumbersome in that several steps must be taken in the analysis (first, horizontal lines expressing the experimental data must be superimposed on the Karplus curves, and second, a search must be made for an intersection of sets) and (2) it is often difficult to assess the closeness of an intersection (e.g., see intersections for hemi-Cys[1] and hemi-Cys[6] in Figure 2). To avoid these disadvantages we have devised a

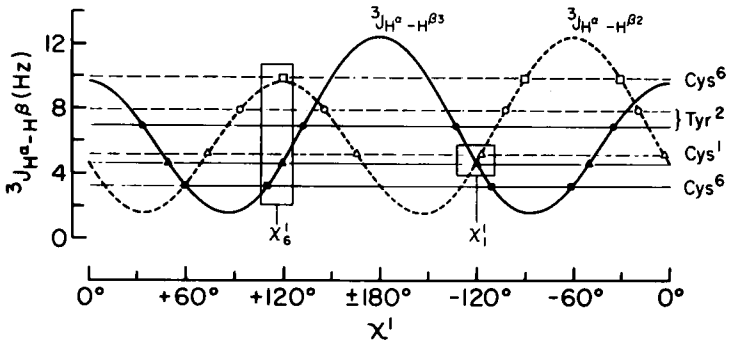

Fig. 2. An illustration of the *intersection-of-sets* method for determining restricted conformations. See text for details. Stereochemical assignments of $H^{\beta 2}$ and $H^{\beta 3}$ for hemi-Cys^1 and hemi-Cys^6 are taken from Fischman *et al.*,[6] the assignments for Tyr^2 are tentative.

new method for testing the hypothesis that data are compatible with a restricted conformation. This method makes use of a closed curve generated by a set of parametric equations that express the coupling constants $^3J_{H^\alpha-H^{\beta 2}}$ and $^3J_{H^\alpha-H^{\beta 3}}$, which can be represented on orthogonal axes in a two-dimensional space, as functions of χ^1 over a 360° range—i.e., from -180° through 0° to +180°. This curve defines the loci of permissible restricted conformations—hence, the term *conformation-locus diagram.*

Figure 3 shows the transformation of the Karplus relationships shown in Figure 2 to the form of a conformation-locus diagram. This particular version of the diagram may be termed a ^3J *vs.* ^3J *diagram.* This figure also shows the data, represented as the points ($^3J_{H^\alpha-H^{\beta 2}}$, $^3J_{H^\alpha-H^{\beta 3}}$), for hemi-Cys^1, Tyr^2 and hemi-Cys^6 taken from the horizontal lines of Figure 2. It is readily apparent that the points for hemi-Cys^1 and hemi-Cys^6 lie on the loci of restricted conformations, whereas the point for Tyr^2 lies off the loci. Note that the analysis of data is much more straightforward by the conformation-locus than by the intersection-of-sets method inasmuch as the former method requires but a single step—namely, plotting the point ($^3J_{H^\alpha-H^{\beta 2}}$, $^3J_{H^\alpha-H^{\beta 3}}$) that represents the experimentally determined values of $^3J_{H^\alpha-H^\beta}$ for a particular residue.

The conformation-locus method has several other advantages over the intersection-of-sets method, for the former method is useful even when conformational isomerism among various base states is manifest. Base states may be located on the conformation-locus diagram and joined by straight lines to construct an *allowable region* in ^3J space. This region is analogous to a *phase diagram* used in a physical chemical description of

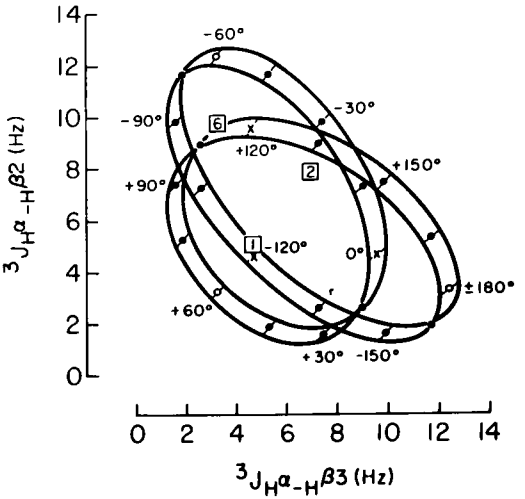

Fig. 3. An illustration of the *conformation-locus* (^3J *vs.* ^3J) method for determining restricted conformations. See text for details. The two closed curves correspond to limiting Karplus relationships given by Kopple *et al.*[11] Staggered and eclipsed conformations are indicated by o and ×, respectively. Size of boxes representing data (1, 2 and 6) indicates estimated error in experimental determination.

a mixture of components. If an experimentally determined point falls in the allowable region, the relative populations of the base states can be quickly calculated by methods similar to those used to analyze phase diagrams.

Analysis of data on a two-dimensional conformation-locus diagram often leads to equivocal results. For example, an allowable region can be formed by joining the three points (o) within the closed ribbon in Figure 3 that correspond to the staggered conformations. The experimentally determined points for hemi-Cys[1] and hemi-Cys[6] fall within this triangular region, and so the data are compatible with isomerism among the three staggered conformations as well as with restricted conformations. Further ambiguities may arise at the three crossover points (crunodes) of the cloverleaf-shaped curve. These ambiguities may be eliminated—or at least be significantly reduced—by utilizing other conformationally related observables such as heteronuclear coupling constants between the H^βs and either $^{13}C'$ or $^{15}N'$ to generate conformation-locus diagrams in a higher dimensional space. In addition, observables such as conformation-dependent components of chemical shifts, relaxation rates or nuclear Overhauser enhancements (NOEs) may, in theory, be used in the construction of these diagrams. With the proper choice of a sufficient number of observables, crunodes in the diagram can be eliminated and a clear distinction between possibilities such as a restricted conformation and isomerism among the three staggered conformations can be made. The *R*-factor analysis of Hamilton[13,14] can be used as a quantitative basis for choosing among various possibilities (e.g., see Fischman *et al.*[6] and references therein).

Another advantage that the conformation-locus method has over the intersection-of-sets method is the use of conformation-locus diagrams as aids in choosing base states. If the general assumption is made that conformational isomerism is manifest, but that a limited number of base states are involved, then sets of base states that are compatible with the experimentally determined point are also limited because this point must fall within the allowable region formed by joining the base states with straight lines (see above). If several of the base states are known, then the experimentally determined point can place a high degree of constraint on the remaining unknown base states, which, in some cases, can be chosen graphically on a conformation-locus diagram. Details will be given in a later, expanded publication.

Acknowledgements

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SOLID STATE NMR OF PEPTIDES

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Introduction

High resolution NMR spectroscopy is well established as a method for describing the conformations and dynamics of peptides in solution. Recent developments in solid state NMR spectroscopy indicate that peptides can be analyzed with equal facility as polycrystalline or amorphous solid materials.[1,2] Direct comparisons of peptides in solution and in solid state are possible with a combined spectroscopic approach.

Background on Solid State NMR

NMR spectroscopy of liquids is so widely employed that it needs no introduction. However, the techniques and instrumentation for high resolution NMR of solids are relatively recent developments,[3] and are still somewhat foreign to chemists and biochemists. NMR of liquids is much simpler than that of solids because the rapid molecular motion of liquids averages the strong spin interactions. In solids, these interactions cause severe line-broadening that obscures spectral features of interest as well as causing sensitivity problems. High resolution solid state NMR relies on radiofrequency pulses and mechanical sample spinning to average out the line-broadening mechanisms and to enhance sensitivity.[4,5]

Heteronuclear dipolar couplings are generally the strongest interactions present in solids. These are easily removed in the case of dilute spins (^{13}C, ^{15}N, ^{31}P, etc.) that are coupled to abundant spins (^{1}H), by high power radiofrequency irradiation of the ^{1}H spins.

In a proton decoupled spectrum, the lineshapes of the resonances of spin ½ nuclei are determined by the chemical shift interaction, which reflects the electronic environment. This interaction is anisotropic, and is described by a symmetric second rank tensor whose trace, or average value, is the isotropic chemical shift, as seen in liquids. Rapid mechanical rotation of microcrystalline or amorphous samples at the magic angle (θ = 54.7° relative to the applied magnetic field) averages the broad chemical shift pattern to its isotropic value. By combining high power proton decoupling and magic angle sample spinning, the two dominant sources

of line-broadening in solids are effectively removed and spectra equivalent to those for solutions result.

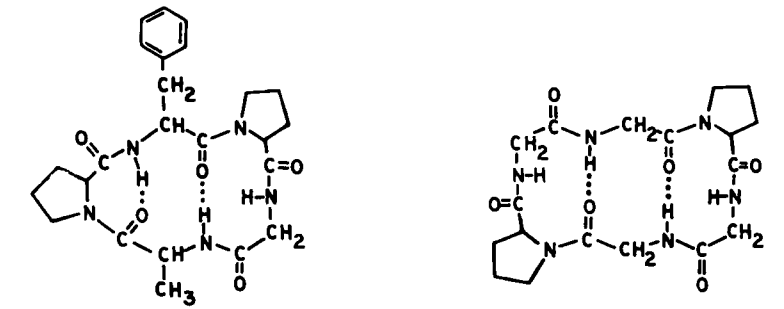
Sensitivity of ^{13}C and other dilute spin resonance signals is poor in both solutions and solids. However, the extremely long relaxation times of solids do not allow signals to be generated by pulsing the spins of interest as effectively as in liquids. Instead it is necessary to perform cross-polarization of the ^{13}C spins from the attendant ^{1}H spins, which increases the ^{13}C sensitivity by a factor of four and causes them to follow the shorter ^{1}H relaxation times.

The use of high power proton decoupling and magic angle sample spinning removes the largest of the line-broadening interactions. However, other spin interactions are not completely removed by these two procedures, especially when spin $I>½$ quadrupolar nuclei are involved. The ^{14}N-^{13}C dipolar couplings that are not averaged by magic angle sample spinning are particularly valuable sources of spectroscopic information, even though in some cases they cause a loss of resolution.[6]

Intramolecular motions that are rapid on the spectroscopic time scales of the NMR experiments strongly affect the chemical shift and dipolar interactions. In favorable cases, these motions alter the powder patterns from specific, usually isotopically labelled, sites. Molecular dynamics can be described from motionally averaged powder patterns. A convenient way of studying molecular motions in this manner is by labelling specific sites with ^{2}H and obtaining ^{2}H spectra.[7] The spin $I=1$ ^{2}H nucleus gives lineshapes dominated by the nuclear quadrupole interaction. The resulting powder patterns are highly dependent on molecular motions involving the labelled site.

NMR of Peptides in the Solid State

Interpretation of Isotropic ^{13}C Chemical Shifts — When an X-ray structure is available for a peptide, NMR data in a solid sample, which has been prepared so as to yield the same crystal form as studied by X-ray, can be interpreted in terms of the known structure. Conformational features can be definitively associated with the observed isotropic chemical shifts. Examples which we have found to be particularly informative have involved proline residues. Two cyclic peptides (see Figure 1) whose solid state structures have been determined by X-ray diffraction, and whose solution conformations have been studied in detail, serve as examples of different situations.[2] The cyclic pentapeptide cyclo(D-Phe-Pro-Gly-D-Ala-Pro) takes up the same rigid conformation, containing both a β and a γ turn, in solution[8] and in crystals.[9] Its solid



A. Cyclic (D-Phe-Pro-Gly-D-Ala-Pro)

B. Cyclic (Gly-L-Pro-Gly)$_2$

Fig. 1. Structures of the two cyclic peptides discussed.

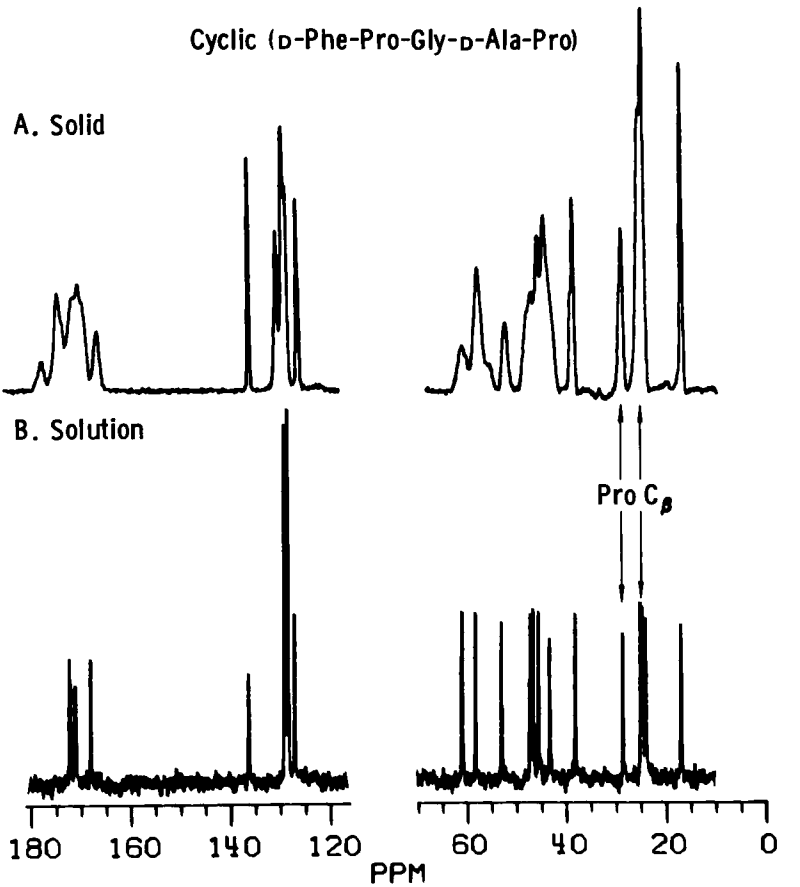


Fig. 2. ^{13}C NMR spectra of cyclo(D-Phe-Pro-Gly-D-Ala-Pro) at 38 MHz with chemical shifts relative to external Me_4Si. Top: Polycrystalline peptide, 300 mg, with magic angle spinning, high power decoupling and cross-polarization. Bottom: Peptide in $CDCl_3$, 300 mg in 10 ml, proton decoupled, 9000 accumulations.

state ^{13}C NMR spectrum yields isotropic chemical shifts that are essentially superimposable on those in solution (see Figure 2), including an unusually high field Pro Cβ resonance arising from an eclipsing interaction with the C=O in the γ turn. The region of the spectrum where Cα's resonate looks quite different in the solid state spectrum and in solution because of coupling between ^{13}C and ^{14}N. By contrast, the cyclic hexapeptide cyclo(Gly-Pro-Gly)$_2$ adopts an asymmetric conformation stablized by one intramolecular hydrogen bond in crystals,[10] but appears to take up a C_2 symmetric conformation in solution.[11,12,13] The isotropic shifts of the carbons in the solid clearly reflect the asymmetry, especially the Pro Cβ (see Figure 3). The molecule contains one type I and one type II β turn in the solid. The observation of two Pro Cβ signals, separated by 1.8 ppm, suggests that there may be characteristic shifts for

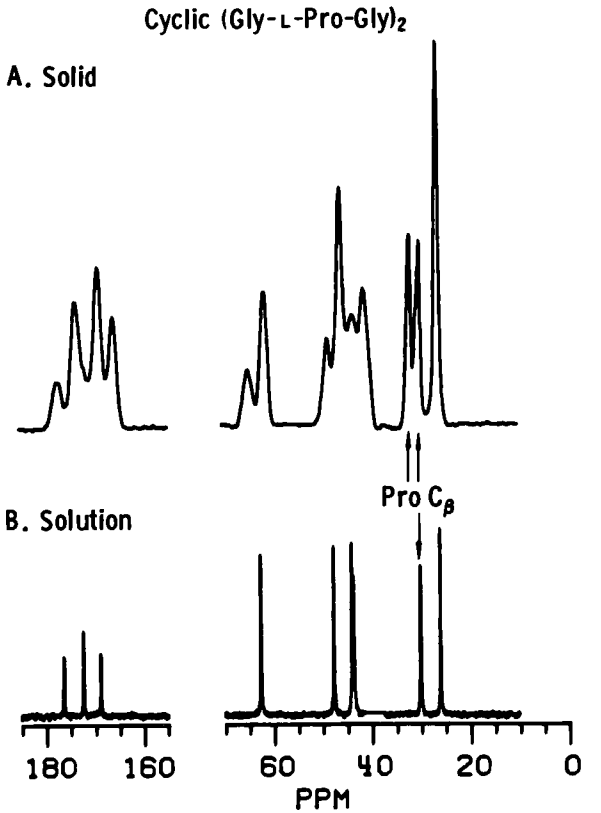


Fig. 3. ^{13}C NMR spectra of cyclo(Gly-Pro-Gly)$_2$ at 38 MHz with chemical shifts relative to external Me_4Si. A. Polycrystalline, as in Figure 2. B. Peptide in Me_2SO/H_2O solution, as in Figure 2. Solvent resonances near 40 ppm have been zeroed.

the two types of turn. The shift in solution is between the two in the solid,and may be a result of averaging between the two forms. Variable temperature experiments both in the solid and in solution may reveal whether this is the case.

We have collected data for proline-containing cyclic and linear peptides to correlate isotropic shifts with ring geometry and turn occurrence.[14] The $\Delta\delta\beta\gamma$ are related to the ψ angle in a similar fashion in the solid state and in solution. More data are needed to establish a quantitative relationship as has been described for solution.[15]

Examination of peptides with no known solid state structure illustrates the utility of solid state NMR as a means of defining conformation. For example, the solid state spectrum of the cyclic pentapeptide cyclo(D-Phe-Gly-Ala-Gly-Pro) reveals the presence of a γ turn, with a Pro in the i+1 position, by the very small $\Delta\delta\beta\gamma$ (0.7 ppm) (see Figure 4). In solution, an intermediate value of $\Delta\delta\beta\gamma$ (2.5 ppm) suggests a dynamic average between two conformers, one with the Pro in a γ turn and one with the Pro in a β turn (see Figure 5).

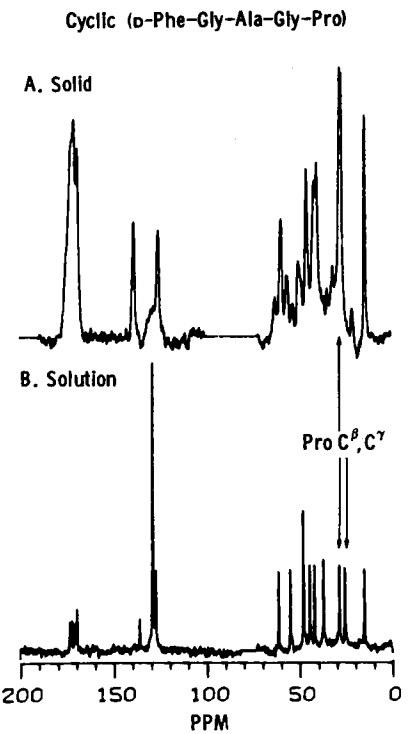


Fig. 4. 62.5 MHz ^{13}C NMR spectra of cyclo(D-Phe-Gly-Ala-Gly-Pro). Top: Polycrystalline sample. Bottom: Peptide in $CDCl_3$, saturated, *ca.* 40 mg/10 ml. Conditions same as Figures 2 and 3.

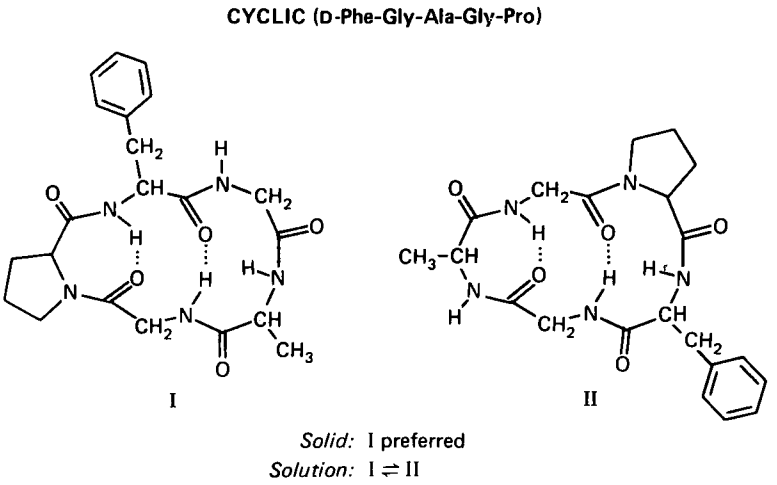


Fig. 5. Probable conformations, based on ^{13}C NMR data, of cyclo(D-Phe-Gly-Ala-Gly-Pro) in solution and in the solid.

Cis-trans isomerism around an X-Pro bond is indicated in solution by Pro Cβ and Cγ resonances separated by *ca.* 10 ppm. The solid state spectrum of Ala-Pro shows that the same correlation obtains in solids (see Figure 6). This amorphous solid appears to have a cis:trans ratio of 1:2.

In addition to the static conformational interpretations described above, aspects of molecular dynamics can be deduced from solid state NMR spectra. The absence of a component of motion due to overall molecular tumbling simplifies analysis of internal motions such as side chain rotations and backbone puckering. For example, the spectrum shown in Figure 4 of cyclo(D-Phe-Gly-Ala-Gly-Pro) has an unusual appearance in the aromatic region. No discrete δ or ϵ carbon signals are seen; instead, a broad, ill-defined resonance occurs. A reasonable interpretation is that the aromatic ring rotates or flips about the β-γ bond with a rate that is near to the chemical shift difference between the two δ's and the two ϵ's in their equilibrium positions (*i.e.* an intermediate exchange rate). This value is about 100 sec^{-1}. Hence, the peaks are actually coalesced. If the rate of flip were fast compared to the chemical shift difference, then a set of two signals, one for the two δ's and one for the two ϵ's, would be seen, as is typical of solution spectra. For slow flips, up to four lines might be discerned. Figure 7 shows the various possibilities drawn from ^{13}C spectra of peptides studied. An increase in spectrometer field strength (from a carbon frequency of 37.5 MHz to one

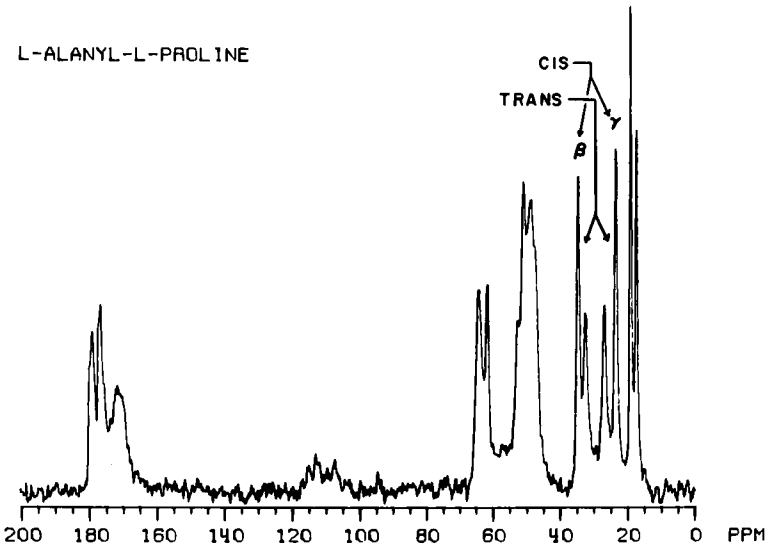


Fig. 6. ^{13}C NMR spectrum in the solid state of Ala-Pro as an amorphous solid, 38 MHz, conditions as in Figures 2, 3 and 4. Note the pattern of lines in the 20 to 40 ppm range corresponding to the proline ring carbons and indicating the presence of two different isomers around the Ala-Pro peptide bond.

of 62.5 MHz) did not significantly alter the observed pattern. To probe the ring dynamics, deuterium NMR lineshape analysis is being used.[16]

Interpretation of Deuterium Resonances — Deuterium NMR spectra have been obtained for specifically labeled cyclic pentapeptides to exploit the different time scale of motions affecting the lineshape of the quadrupolar nucleus: namely, rates of *ca.* 10^5 to 10^6 sec^{-1}. Two cyclic pentapeptides were synthesized with phenylalanine-d_5 in their sequences. Because only L-Phe-d_5 was available, the peptides were synthesized as enantiomers of the two Phe-containing pentapeptides described above; conformational interpretations should not be influenced. As can be seen in Figure 7, on the 10^6 Hz time scale of ^2H NMR the rings are static in both peptides. Combining results of both ^{13}C and ^2H NMR brackets the rate of ring motion in cyclo(L-Phe(d_5)-Gly-D-Ala-Gly-D-Pro) between 10^2 and 10^6 sec^{-1}. The other peptide has a ring which is static in both experiments, and hence rotates more slowly than 10^2 sec^{-1}. Variable temperature lineshape analysis is being used to determine the barriers to rotation in these systems.

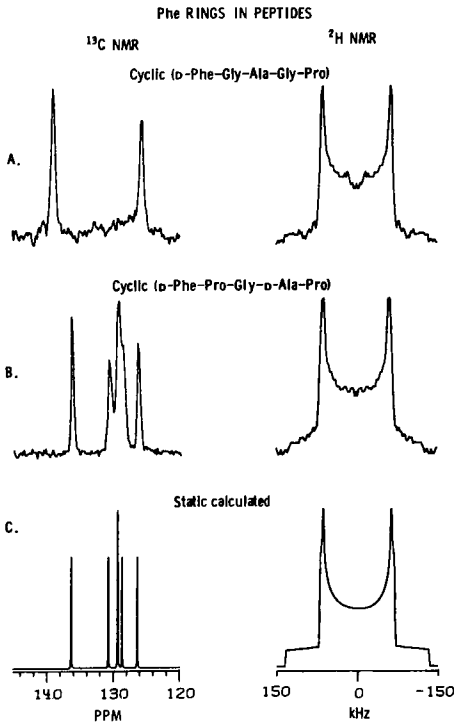


Fig. 7. ^{13}C and ^{2}H NMR spectra of aromatic rings in cyclic peptides. ^{2}H spectra were run on enantiomers of named compounds, 75 mgs, nonspin, 38 MHz. A. Cyclic (D-Phe-Gly-Ala-Gly-Pro), proposed to have a slowly flipping ring. B. Cyclic (D-Phe-Pro-Gly-D-Ala-Pro), with a static ring. C. Theoretical spectra for static ring.

Prospectus for Solid State NMR of Peptides

Solid state NMR spectroscopy makes studies of crystalline and amorphous samples feasible. The direct comparisons of spectral parameters between solution and the solid state are therefore possible. In addition, studies of peptides in media that do not allow rapid isotropic motion of the molecules can be done; both peptide-DNA binding and interactions of phospholipids with hydrophobic membrane peptides are in this category.

Structural information is available from many sources in solid state NMR. Only a few, most notably carbon isotropic shifts, have been looked at to date. Certainly methods of extracting dipolar couplings will give bond lengths with unprecedented accuracy and enable hydrogen bonds to be reliably characterized. Geometrical arrangements of sites can also be determined from appropriate experiments, such as ^{13}C-^{14}N coupling measurements.

All of the static spin interactions are characteristically averaged by intramolecular motions. The directions, amplitudes, rates, and frequencies of dynamical events can be obtained from a variety of NMR experiments.

Acknowledgement

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CONFORMATIONAL TRANSITIONS OF γ-CARBOXYGLUTAMIC ACID-CONTAINING PEPTIDES

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Introduction

The independent demonstration by Stenflo[1], Nelsestuen[2], and Magnusson[3] that functional prothrombin contained γ-carboxyglutamic acid residues and that the presence of this amino acid resulted from a post translational carboxylation reaction mediated by vitamin K has been the subject of several excellent reviews[4-7]. The continuing contributions of the laboratories of Nelsestuen, Jackson, Mann and others to our understanding of the functional role of Gla residues have been summarized in the proceedings of a recent symposium on vitamin K-dependent proteins[8].

It is now widely accepted that under conditions of normal biosynthesis, ten Glu residues located in the first 33 residues of the amino terminus of prothrombin are converted to Gla. The Gla residues seem to occur in pairs at positions 7,8; 15,17; 20,21; 26,27; and 30,33 (Figure 1). Prothrombin will interact with phospholipid surfaces possessing a net negative charge only in the presence of calcium ions. Surface-bound prothrombin molecules are much more efficiently converted to thrombin by the "prothrombinase" complex than the corresponding X_a-catalyzed process. Esmon et al.[9] in a classic study demonstrated that the catalytic effect of the phospholipid surface on the conversion of prothrombin to thrombin is observed only when Gla residues bind calcium ions and as a result the prothrombin-calcium complex binds to the negatively charged phospholipid surface. The protein comprising the *N*-terminal 156 residues of prothrombin (fragment 1) exhibits identical Ca^{2+} and phospholipid binding behavior; thus fragment 1 is often utilized as a prothrombin model for studies involving the role of Gla in the metal ion-protein phospholipid interaction.

Results

In order to ascertain the chemical events involved in the binding of calcium ions to Gla-containing proteins we have utilized a variety of spectroscopic methods. Certain methods utilize signals directly derived from the metal ion as it is bound to the Gla-peptide or protein. These include the Eu^{3+} luminescence decay rate technique devised by Horrocks and Sudnick[10] and multinuclear nmr techniques[11] using $^{43}Ca^{2+}$ and $^{25}Mg^{2+}$. Alternatively we have used methods which indirectly report on metal ion binding via structural responses of the protein and concomitant spectroscopic changes; these include circular dichroism and fluorescence measurements.

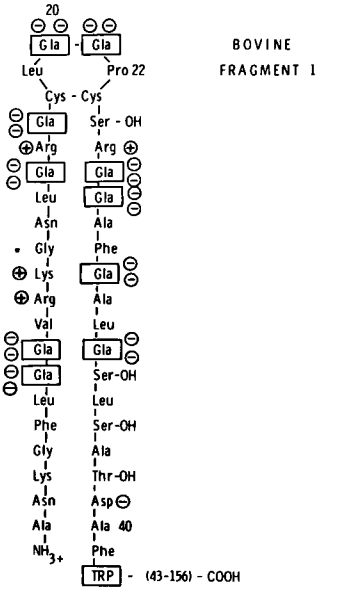

Fig. 1.

Effects of Peptide and Protein Ligands on the Metal Ion — The utilization of Eu^{3+} luminescence lifetime measurements in Gla-containing peptide model systems[12,13] yields (a) the number, ΔN, of the nine bound water molecules in the coordination sphere of the Eu^{3+} ion which are displaced by the peptide ligand ($\Delta N_{max}=9$); (b) the stoichiometry of the complex; and (c) the pH-dependence of metal ion:peptide complex formation. These results (Table I) suggest that peptides containing a single Gla residue tend to form 2:1 (peptide:Eu^{3+}) complexes while peptides with a Gla residue adjacent to another residue containing oxygen ligands prefer a 1:1 complex. In peptides such as the 19-22 sequence, the 1-10 sequence, or H-Phe-Leu-Gla-Gla-Leu-OMe 1:1 complexes predominate and ΔN in each case is about 4 (two water molecules lost from Eu^{3+} per Gla residue).

Examination of Eu^{3+} binding to peptides containing larger portions of the *N*-terminal region such as the 1-39 sequence or fragment 1 indicated a quite different behavior[13,14]; the 1-39 peptide exhibited a $\Delta N=6$ and fragment 1 a $\Delta N=7$. Another striking difference in behavior between the Gla peptide models and the 1-39 or fragment 1 peptides is

Table I: Parameters Characterizing Peptide:Eu^{3+} Complexes

Ligand	H_2O Lost Per Gla	ΔN	Model[d]	K_1^{dissoc}	K_2^{dissoc}
Gla	1	2.2	1M:2P	33μM	5.9μM
Z-Gly-Gla-Gly-OEt	2	3.9	1M:2P	4.9μM	1.5μM
Z-D-Gla-Ser-OMe	2	3.7	1M:2P	9.1μM	20μM
Z-D-Gla-D-Gla-OMe	4	3.9	2M:1P	0.6μM	1.1μM
Z-Leu-Gla-Gla-Pro-OMe	2	4.0	1M:1P	0.5μM	-
H-Phe-Leu-Gla-Gla-Leu-OMe	2	4.0	1M:1P	4.1μM	-
H-Phe-Leu-Gla-Glu-Leu-OMe	2	2.4	1M:1P	4.3μM	-
H-Phe-Leu-Glu-Glu-Leu-OMe	no Eu^{3+} binding observed				
H-Ala-Asn-Lys-Gly-Phe-Leu- -Gla-Gla-Val-Arg-OMe	2.5	5.0	1M:1P	-	-
1-39		6.0[a,c]	-	-	-
Fragment 1 (1-156)		7.0[b,c]	-	-	-

[a]Eu^{3+} binding curve is dependent on 1-39 concentration.
[b]Eu^{3+} binding curve is not dependent on fragment 1 concentration.
[c]Slow or non-exchanging Eu^{3+} binding site. [d]M = metal; P = peptide.

revealed by studies involving competition of La^{3+} or Ca^{3+} for peptide-bound Eu^{3+} ions. Under fast exchange conditions La^{3+} or Ca^{2+} should displace Eu^{3+} if the metal ions are competing for the same sites. In the case of the small Gla peptide models this is indeed the case since luminescence decay rates increased upon the addition of La^{3+} to a Eu^{3+}-containing peptide sample (Figure 2). Such was not the case when either La^{3+} or Ca^{2+} was added to Eu^{3+}-containing samples of either 1-39 or fragment 1. In these cases a decrease in Eu^{3+} luminescence decay rates was observed. Subsequent studies involving varying the order of addition of the ions indicate that the exchange rate of Eu^{3+} between free (aquo) and bound (Eu^{3+}-1:39 or fragment 1 complex) is very slow. Thus these studies and experiments involving rabbit anti-bovine fragment 1:Ca^{2+} complex antibodies indicate that the first metal ion added to fragment 1 or 1-39 is only slowly exchanged with subsequently added metal ions[14]. These data do not in themselves demonstrate that in the detailed model for positively cooperative metal ion binding it is the first metal ion binding site filled

which is slowly exchanging. Reducing the pH to around 4 results in the release of all bound metal ions indicating the dependence of the system on intact tertiary structure.

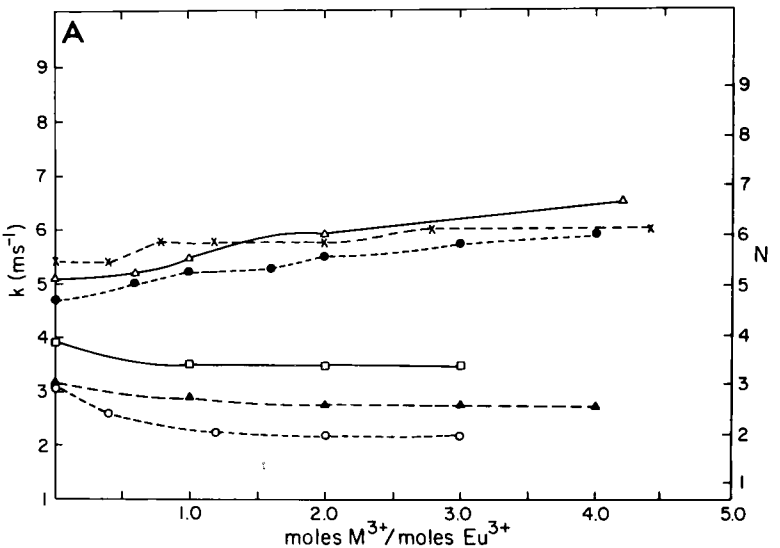

Fig. 2. Plot of the rate constant for Eu^{3+} luminescence decay, k, *vs.* molar ratio of M:Eu^{3+} where M represents La^{3+} or Gd^{3+}.

X Z-Gla-Ser-OMe:Eu^{3+} 2:1, M = La^{3+}
△ Z-Gla-Gla-OMe:Eu^{3+} 1.5:1, M = La^{3+}
● Phe-Leu-Gla-Gla-Leu-OMe:Eu^{3+} 1.5:1, M = La^{3+}
□ Eu^{3+}:fragment 1 4.2:1, M =La^{3+}
▲ Eu^{3+}:1-39 1.7:1, M =Gd^{3+}
○ Eu^{3+}:1-39 2.5:1, M = La^{3+}

Studies involving the displacement of $^{43}Ca^{2+}$ from fragment 1 by Mg^{2+} ions and displacement of $^{25}Mg^{2+}$ from fragment 1 by Ca^{2+} ions provide another method of study of competition between ions for binding sites in the Gla region of the protein[11]. It is important to note that in these studies *only* rapidly exchanging sites (on the nmr time scale) are examined. The method is blind to non-exchanging sites because of: (a) the low concentration of such sites, and (b) the large line width of the bound metal ion. Calcium displacement of $^{25}Mg^{2+}$ (Table II) reveals the process to be cooperative with a Hill coefficient of about 2. This value is lower than that obtained from fluorescence quenching measurements (n=3) of Ca^{2+} titrations of fragment 1. This difference *may* reflect the non-exchanging site to which nmr measurements should be blind but to which fluorescence titrations or Eu^{3+}/Ca^{2+} luminescence decay measurements should not. The existence of a non-exchanging metal ion binding site in fragment 1 or 1-39 requires the occupation of other, exchanging sites by

metal ions. Thus, reverse titrations involving additions of EDTA to a Ca^{2+}-saturated protein should not and do not indicate the presence of such a site.

The exchange data (Table II) also show that Ca^{2+} and Mg^{2+} are not fully competitive since about 30% of exchanging Mg^{2+} are not displaced from fragment 1 by Ca^{2+} ions and about 40% of exchanging Ca^{2+} ions are not displaced by high Mg^{2+} concentrations. Thus sites exist in a metal ion-fragment 1 complex to which other metal ions have only limited access.

Table II. Calcium: Magnesium Competition for Binding to Fragment 1 [Hill parameters (n) and fractional displacement as determined by NMR]

pH	n	Midpoint	% not displaced
Magnesium-25 displacement from fragment 1 by calcium ions			
8.2	1.96	7.1×10^{-4}	24[a]
7.0	1.37	6.9×10^{-4}	27[a]
6.0	0.93	5.3×10^{-4}	27[a]
Calcium-43 displacement from fragment 1 by magnesium ions			
7.0	0.82	5.5×10^{3}	40[b]

[a]Fraction not displaced was constant above 5 mM calcium chloride
[b]Fraction not displaced at 30 mM magnesium chloride

Effect of Metal Ions on the Protein Ligand. Metal Ion-Mediated Quenching of Fragment 1 Fluorescence — Nelsestuen[15] studied the fluorescence of bovine fragment 1 in the presence of Ca^{2+} ions. At equilibrium fragment 1 fluoresence is 40% quenched in the presence of Ca^{2+} ions at neutral pH. Upon addition of saturating Ca^{2+} it was observed that following a rapid (<1 msec) initial fluorescence decrease of about 25% of the total equilibrium quenching, a relatively slow first order decay of fluorescence to an equilibrium level occurred. Nelsestuen further demonstrated that the phospholipid binding behavior of bovine prothrombin and fragment 1 paralleled the observed fluorescence behavior. Immediately upon addition of Ca^{2+} ions about 25% of fragment 1 bound to phospholipid followed by a slow phase which exhibited the same kinetic and thermodynamic parameters as those which described the slow fragment 1 fluoroescence quenching induced by Ca^{2+} ions.

The rate of slow fluorescence quenching and the activation energies determined in the presence of Mg^{2+}, Ca^{2+} and Ba^{2+} were identical. Nelsestuen suggested that this biphasic fluorescence quenching process

was due to either two sequential protein transitions or to the presence of two populations of fragment 1 molecules.

Our kinetic and thermodynamic studies[16] of bovine fragment 1 intrinsic fluorescence indicate that in the absence of metal ions bovine prothrombin exists in two forms. Only one of these populations can interact with metal ions with *rapid* quenching of fluorescence. Forms A and B are conformational isomers of fragment 1; Ca^{2+} ions interact only with the B isomer to produce, in a fast step, isomer C which is capable of binding to the phospholipid surface or in which intrinsic fluorescence is quenched. In view of the absence of a rate dependence on the Ca^{2+} concentration the conversion of A to B must be rate limiting.

$$A \underset{\text{slow}}{\rightleftharpoons} B \underset{-Ca^{2+}}{\overset{+Ca^{2+}}{\rightleftharpoons}} C \quad \text{(fast)}$$

The energy of activation (Arrhenius plots) for the slow step (A → B) suggested that the isomerization of a proline residue was involved[17]. Since human prothrombin and bovine Factor X do not exist in two slowly interconverting forms (and hence exhibit only a rapid metal ion-induced fluorescence quenching[18]) we speculated that the *trans-* to *cis-*isomerization of Pro^{22} in bovine prothrombin was the trigger for the conversion of A → B. Human prothrombin contains a Thr and Factor X an Ala at position 22. Thus we suggeset that perhaps the sole structural difference between the two conformational isomers lies in the isomer distribution of Pro^{22} within the constrained conformation of the 18-23 cystine loop containing $Gla^{20,21}$. Further since isomer A and isomer B surely exhibit similar ionizing groups in solution (hence are both equally likely to bind Ca^{2+} ions) and since the difference between isomers A and B involves the *trans-* to *cis-*isomerization of Pro^{22}, it follows that the calcium ion:fragment 1 complex which is functional *vis-a-vis* phospholipid binding also involves a *cis-*Pro^{22} residue. Since the $Gla^{20,21}$ residues are a part of the conformationally rigid cystine loop containing *cis-*Pro^{22} the involvement of these residues at an early stage of Ca^{2+} binding by prothrombin is also suggested.

Immunological Studies on the Conformation of Fragment 1 Induced by Calcium Ions — We have isolated a population of rabbit anti-(bovine fragment 1) antibodies which exhibit a complete dependence on the presence of Ca^{2+} ions for their interaction with ^{125}I-labeled bovine fragment 1[19]. Bovine prothrombin exhibits similar behavior toward this

antibody population[20]. The antibody assay employed for the interaction of the antibody population with the bovine fragment 1: Ca^{2+} complex was sufficiently rapid so that the isomerization of A → B was rate limiting. The kinetic parameters characterizing the slow Ca^{2+}-induced conformational change in bovine fragment 1 obtained by this immuno-kinetic method at several temperatures were in excellent agreement with those obtained by fluorescence quenching studies[19]. Thus the observed kinetics of metal ion-induced fluorescence quenching of bovine fragment 1, the observed kinetics of phospholipid binding of fragment 1 after addition of Ca^{2+}, and the Ca^{2+} dependent conformational change as determined by the kinetics of the fragment 1: Ca^{2+} complex interactions with antibodies involve the same rate limiting step.

Effect of pH on Metal Binding by Gla Ligands — From our earlier studies[11,21] of the effect of pH on the intrinsic fluorescence quenching of fragment 1 by Ca^{2+} ions we concluded (a) tight binding of Ca^{2+} ions to fragment 1 is pH dependent and cooperative (n=3.0); (b) protonation of ionizing groups with apparent pK_a's of 4-4.5 reduce the affinity of fragment 1 for both Ca^{2+} and Mg^{2+} ions; and (c) groups with apparent pK_a's of 4-4.5 appear to be critical for cooperative Ca^{2+} binding and thus for the establishment of the Ca^{2+} binding conformation (isomer C).

The pH-dependence of metal ion binding to Gla-containing ligands using spectroscopically active metal ions ($^{25}Mg^{2+}$, $^{43}Ca^{2+}$, Eu^{3+}, La^{3+}) are given in Table III.

Magnesium ion binding to malonic acid, simple Gla peptides, and fragment 1 reveal a dependence of metal ion-ligand complex formation of pK^{app} in the range 4.2-4.8. Lanthanum ion binding similarly exhibits an inflection at pH 4.2 and an indistinct inflection at 5.3. In addition fragment 1 binding to both $^{25}Mg^{2+}$ and $^{43}Ca^{2+}$ exhibits a second inflection at pH 7.5 or greater in addition to the pH 3.8-4.2 inflection. Since these values involve determination of binding via nmr line width measurements, properties related to non-exchanging sites will not necessarily be apparent. Thus the effects of pH on exchange rates could tend to obscure the involvement of the ionizing groups. In contrast the Eu^{3+}:fragment 1 binding study reveals inflections at pH values of 3.0 and 5.3 (Eu^{3+}:fragment 1, 4.:1). We tentatively conclude that the nmr results indicate properties of freely exchanging metal ions (slow or non-exchanging sites not apparent) while the Eu^{3+} luminescence studies may indicate properties of binding sites weighted to include the tight, slowly exchanging ion binding site we believe is present in fragment 1.

Table III. pH-Dependence of Metal Ion Binding to Gla-Ligands

Gla-Peptide or Protein	Position of Titration Inflection Point			
	$^{25}Mg^{2+}$[a]	$^{43}Ca^{2+}$[a]	Eu^{3+}[b]	La^{3+}[b]
Z-D-Gla-D-Gla-OMe	-	-	3.7	-
Z-L-Arg(NO_2)-D-Gla-D-Gla-OMe	4.6-4.8	-	-	-
Z-L-Arg-D-Gla-D-Gla-OMe	4.6-4.8	-	-	-
H-Phe-Leu-Gla-Gla-Leu-OMe	-	-	3.7	-
H-Phe-Leu-Gla-Glu-Leu-OMe	5.1-5.2	-	4.1	-
Z-Gla-Ser-OMe	4.8	-	4.8	-
Fragment 1	4.2,7.5	3.8,>7.5	3.0,5.3	4.2,5.3
1-39	-	4.3	-	-
Malonic Acid	4.2	-	-	-

[a]Metal Ion NMR [b]Metal Ion Luminescence

Conclusion

The observation of a kinetic barrier to the formation of Ca^{2+}-fragment 1 complexes which will subsequently interact with a phospholipid surface is of minor significance in itself since in plasma prothrombin has unlimited time in which to interact with Ca^{2+} or Mg^{2+} ions. Nevertheless the behavior of the system indicates that only one conformation of the cystine$^{18-23}$ loop sequence (involving a *cis*-Pro^{22}) is involved in the formation of the Ca^{2+}-fragment 1 complex that is able to bind to the phospholipid surface. The interaction of Ca^{2+} ions with bovine fragment 1 arranged in the correct conformational array is rapid (B → C transition), exhibits positive cooperativity (n=3), and leads to a state (isomer C) that exhibits antigenic behavior that is different than A or B. In addition isomer C interacts with negatively charged phospholipid surfaces and also exhibits a reorganized secondary and tertiary structure in which the intrinsic fluorescence of one or more tryptophan residues (perhaps Trp^{42}) is internally quenched.

Examination of CPK molecular models of the 16-25 region of bovine prothrombin reveal the following differences in the ligand array imposed by the sulfur-sulfur bond of the Cys$^{18-23}$ loop, the Gla^{20,21} residues and a *trans*- and *cis*-Pro^{22}. The loop sequence containing *trans*-Pro^{22} is essentially planar; Gla^{20,21} easily accommodate a single complexed metal

ion in much the same manner as the complex resulting from a single metal ion and the tetra-peptide, Z-Leu-Gla-Gla-Pro-OMe. In this model both Gla^{17} and Ser^{24} are maximally removed from the space occupied by $Gla^{20,21}$ and the interacting metal ion. Thus the loop containing a *trans*-Pro^{22} within the 18-23 loop would be predicted to bind a metal ion with a specificity similar to that of a linear Gla-Gla model peptide (eg. Z-Leu-Gla-Gla-Pro-OMe).

The model of the 18-23 cystine loop containing a *cis*-Pro^{22} is much more constrained. Incorporation of a *cis*-prolyl residue forces the peptide backbone out of the plane observed for the *trans*-Pro^{22}-containing loop. The consequence of this fold is to place $Gla^{20,21}$ in juxtaposition to Gla^{17}; the metal ion in the *cis*-Pro^{22} cystine loop model is surrounded by 16 oxygen ligands (12 Gla, 4 amide oxygen atoms). We suggest that this array ($Gla^{20,21,17}$) represents the initial Ca^{2+} ion binding site; rapid sequestering of two additional Ca^{2+} ions in a positively cooperative process by the remainder of the Gla residues and the other oxygen ligands in the 1-40 sequence would effectively zipper the 1-16 and the 23-40 portions of the chain held roughly parallel by the 18-23 loop.

This model is supported by the data of Furie *et al.*[22] who reported nmr studies using $^{153}Gd^{3+}$ bound to the 12-44 peptide. They noted that a "high affinity" Gd^{3+} binding site was lost when the 12-44 peptide was reduced at the 18-23 sulfur-sulfur bond and carbomethoxymethylated. We speculate that the slowly or nonexchanging site we have observed involves $Gla^{20,21,17}$, and *cis*-Pro^{22} and the constraints of the intact 18-23 cystine loop rather than simply the intrachain interaction of two Gla residues, $Gla^{15,26}$, as suggested by Furie *et al.* In this model the zippering action of the three cooperatively-bound Ca^{2+} ions should place the indole ring of Trp^{42} close in space to a group near the amino terminus that is capable of quenching the intrinsic fluorescence (Figure 3). Synthetic studies designed to evaluate these possibilities are in progress.

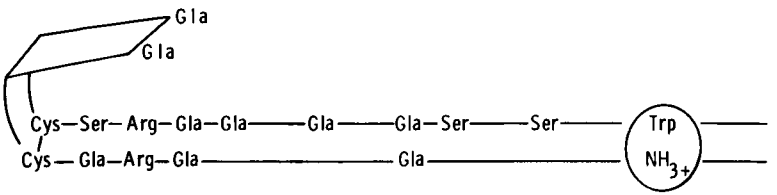

Fig. 3.

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DIFFERENCE ^{1}H NMR UTILIZING DEUTERIUM SUBSTITUTIONS: APPLICATION TO STUDIES OF BINDING OF PEPSTATIN TO PEPSIN

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Introduction

^{1}H NMR is well established as an important tool for studies of peptide conformation in solution. But for many peptides, whose biological activities are expressed through interaction with a macromolecular receptor or protein active site, the conformation of the bound state holds far more interest. Unfortunately the NMR spectrum of the complex is usually overwhelmed by protein resonances, yielding little information about the bound effector.

The solution to this problem is difference spectroscopy where the only change between two otherwise identical samples of peptide-protein complex is the replacement of certain peptide protons in one sample by deuterium. Then the difference spectrum should contain only peaks from specific protons in the bound ligand. The general technique apparently originates with a study by Sykes and coworkers[1] who used a dimer of N-acetylglucosamine (with methyls deuterated or protonated) binding to hen egg white lysozyme.

In the work reported here, we synthesized specifically deuterated analogs of pepstatin, Iva-Val-Val-Sta-Ala-Sta where Sta is statine, 4-amino-3-hydroxyl-6-methylheptanoic acid.[2] In separate experiments Val^1 and Val^2 were replaced by L-Val-d_8, and D-Val-d_8 was substituted for Val^1. Isotopic difference spectra clearly revealed peaks for the γ-CH_3's and α protons of these peptides bound to porcine pepsin, an enzyme of 327 residues.[3] Different chemical shifts were found in each of the three cases; multiple peaks provided evidence of slow exchange between bound conformers.

Experimental

Starting with deprotected Boc-Val-Sta-Ala-isoamylamide,[4] proteopeptides Boc-Val-Val-Sta-Ala-Iaa were prepared from symmetrical anhydrides of optically pure Boc-Val, the L- and D-isomers

respectively. The deuterio peptides were prepared from D,L-Val-d_8 (Merck and Co., 98%) in the same way and incorporated at the Val^1 and Val^2 positions; the D- and L-diastereomers of the deuterio peptides were separated by silica gel chromatography of the synthetic products.

Peptides were judged to be pure on the basis of TLC and NMR. The proteopeptides gave satisfactory elemental analysis. Isotopic purity was checked by NMR and estimated to be >95%.

For NMR difference spectra stock solutions of peptides (10mM) were made in CH_3OH-d_4 (99.5% d, Aldrich). Porćine pepsin was prepared by activation of pepsinogen (Sigma) at low pH followed by separation of activation peptides from the enzyme by column chromatography.[5] Stock solutions of pepsin (0.5 mM, pH* = 4.3) were made from the lyophilized protein in 100 atom % ^2H_2O (Aldrich) immediately prior to NMR runs.

NMR samples were prepared by pipetting 500μl of protein stock into each of 2 vials and then adding 25μl of peptide stock, deuterated to one and the corresponding protonated peptide to the other. Close attention to accurate quantitation and solvent purity were vital for successful difference spectra.

^{1}H NMR spectra of the peptide-protein solutions were run at 270 MHz in the FT mode with an ^{2}H lock on the solvent peak. 500 scans were collected at 2.5s intervals (90° pulses). 16K data point time domain FID's were zero filled to 32K before Fourier transformation; frequency domain spectra were subtracted. Small adjustments of relative phase, offset, and amplitude of the 2 spectra improved the difference spectra in many cases.

Results and Discussion

Difference Spectra — When spectra of .5 mM pepsin plus .5 mM specifically deuterated pepstatin analog (Boc-Val-d_8-Val-Sta-Ala-Iaa) are compared to the analogous sample containing fully protonated peptide plus pepsin, they appear identical (Figure 1, A & B). But the difference spectrum, expanded vertically in scale, reveals peaks for the bound inhibitor. Two peaks, undoubtedly methyl groups, are seen at .95 ppm and 1.27 ppm in an area ratio of approximately 3:2. For the peptide alone in ^2H_2O or CH_3OH-d_4 these methyls are near 0.88 ppm. A peak, with a splitting of $7 \pm .5$ Hz, is found at 4.35 ppm and is assigned to the α proton. The β proton does not show clearly in this spectrum but appears consistently as a broad band near 2.1 ppm in other difference spectra with the same inhibitor.

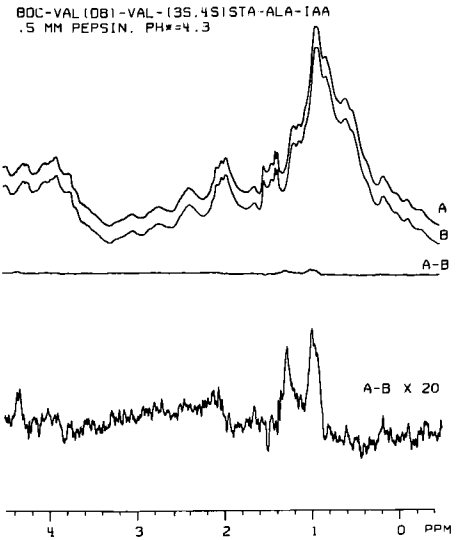

Fig. 1. A) Spectrum of pepsin plus fully protonated peptide in ^2H_2O. Aliphatic region displayed. B) Same as A except with Val^1-d_8. A-B) Difference spectrum displayed at same vertical scale as A and B. Bottom trace: Difference spectrum displayed with increased gain.

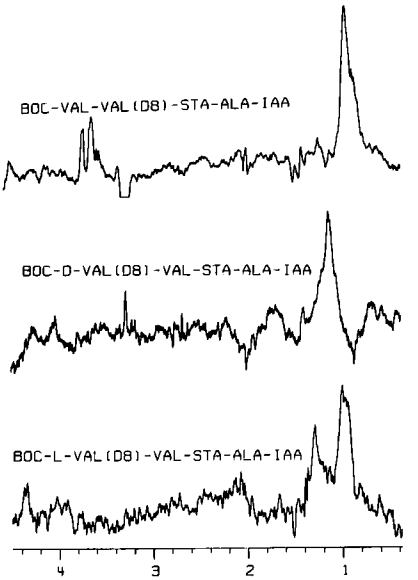

Fig. 2. Difference spectra generated as in Fig. 1 for each of the peptides indicated.

The finding of 2 methyl peaks is tentatively interpreted to indicate restricted motion of the Val^1 side chain. A small fraction of the bound peptide is in a conformation where both methyls resonate near 0.95 ppm. Other spectra with this peptide show variable ratios of methyl peak areas (high field always greater), but the α proton peak at 4.35 ppm is approximately 1/3 the area of the downfield methyl peak. Our finding of a resolved α proton peak is surprising in light of the slow molecular tumbling and consequently broad lines expected for protons in pepsin. But calculations[6] show that narrow lines (<2 Hz wide) are predicted if the α and β protons are approximately trans. A value of $^3J\alpha\beta$ = 7 Hz corresponds to a dihedral angle of $\pm 30°$ or $\pm 140°$; we tentatively choose the latter. More work is needed to determine whether resolved α protons can be found by difference spectra in other protein-peptide systems.

Other Peptides — When D-Val-d_8 or h_8 is substituted for Val^1 and the deuterium difference spectrum run with pepsin, a single, broadened methyl peak appears at 1.15 ppm (Figure 2). Two α peaks of approximately equal area are seen at 4.05 and 4.30 ppm, indicating slow exchange between bound conformations.

Substitution of L-Val-d_8 at the Val^2 position results in a methyl peak whose shift is close to the free peptide position (Figure 2). This spectrum does not show an α peak, possibly because of a shift downfield obscured by the large residual water peak.

Implications for Conformation of Pepstatin in Pepsin Active Site — One of the methyl groups of Val^1 experiences an unusually large downfield shift (0.4 ppm) upon binding. At the least this implies a lack of rotational averaging of the side chain, and it may indicate that one methyl group lies close to a source of diamagnetic anisotropy in the protein, such as an aromatic ring. The NMR data correlate well with steady-state kinetic inhibition constants for a series of pepstatin analogs.[7] Very strong binding ($K_i < 10^{-8}$M) and a time lag in development of full inhibitory power require that analogs have an isopropyl group in the position of the Val^1 side chain, suggesting an important and precise interaction for that group. Preliminary x-ray diffraction data for pepstatin bound to the acid protease of *Rhizopus chinensis* show Val^1 pointing toward the interior of the protein while the Val^2 side chain appears to point toward the surface (R. Bott and D. Davies, private communication). Again the x-ray data are in accord with the NMR where Val^2 showed little chemical shift for the γ-CH_3's, while Val^1 was strongly shifted.

Conclusion

Difference NMR with deuterium substitution has revealed peaks for single peptide α protons and methyl groups in a large molecular weight peptide-protein complex, namely pepstatin-pepsin. Assignments were automatic, and important structural information was derived from the spectra, information obtainable in no other way. The technique promises to be of general use for studying the conformation of bound peptides.

Acknowledgements

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STUDIES ON DETECTION OF INTRAMOLECULAR HYDROGEN BONDS BY HYDROGEN-CHLORINE EXCHANGE METHOD

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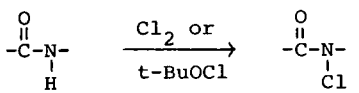
One of the most useful applications of NMR for the conformational analysis of peptides is the separation of peptide N*H* protons into two groups which are exposed to solvent and shielded from solvent either sterically or through intramolecular hydrogen bonding. Several ^{1}H-NMR techniques have been widely used for the detection of hydrogen-bonded peptide N*H*'s. These techniques are, e.g., (a) the H-D exchange of peptide N*H*'s, (b) the temperature and solvent dependences of peptide N*H* chemical shifts, and (c) the addition of a free radical broadening peptide N*H* resonances.

Previously, we reported a new method for the detection of intramolecularly hydrogen-bonded peptide N*H* by chlorine replacement reaction, which was initiated by adding a peptide to a solution containing Cl_2 or adding t-butylhypochlorite (t-BuOCl) to a solution of a peptide.[1]



The replacement reaction of peptide N*H* with chlorine was applied to a conformational analysis of the peptide antibiotics, gramicidin S and tuberactinamine N. The rates of chlorination followed by ^{1}H-NMR spectra demonstrated that intramolecularly hydrogen-bonded peptide N*H*'s were much more susceptible to the chlorination than solvent-exposed peptide N*H*'s in both antibiotics.[2] The present work confirms these new findings and extends this hydrogen-chlorine replacement reaction to other series of compounds, CH_3CONH-R (or -Ar). The apparently different reactivity of various amide N*H* protons in the chlorination is also discussed.

Results and Discussion

The amide N*H* proton of N-methylacetamide **1** was replaced by deuterium or chlorine by use of D_2O, CD_3OD, t-BuOCl, and Cl_2 as replacing reagents. The rate of the replacement was estimated by plotting the residual amide N*H* resonance (%) against time. Figure 1 shows the time course of H-D exchange and H-Cl replacement reactions of **1** in methanol. Similar experiments were carried out in $CDCl_3$, CF_3CH_2OH, and DMSO-d_6. The estimated rates (T/2) of H-Cl replacement were within 1 min. (2.5 eq. Cl_2 in DMSO-d_6), 2 min. (5 eq. t-BuOCl in $CDCl_3$) and 8 min. (5 eq. t-BuOCl in CF_3CH_2OH). On the other hand, the T/2 values of H-D exchange were 10 min. (5 eq. CD_3OD in $CDCl_3$) and over 60 min. (5 eq. CD_3OD in CF_3CH_2OH and in DMSO-d_6). The rate of H-Cl replacement reaction of **1** was much faster than that of H-D exchange reaction.

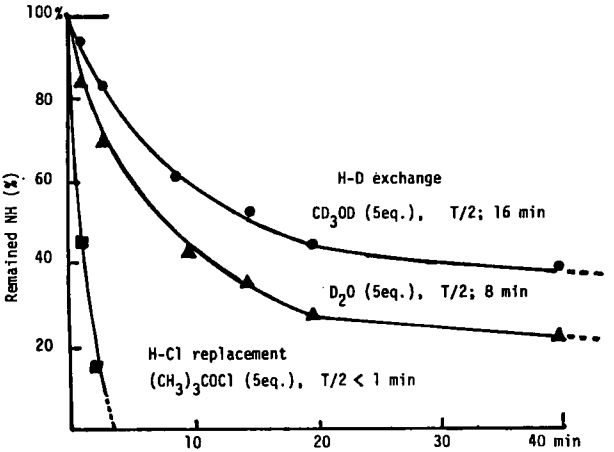


Fig. 1. Reaction of Amide N*H* of $CH_3CONHCH_3$ in MeOH.

Substitution effects of alkyl group (-R) of CH_3CONH-R on H-Cl replacement reaction in $CDCl_3$ were also examined. As shown in Table I, approximate rates (T/2) for compounds substituted by an electron-withdrawing cyanomethyl group **2** and an electron-donating isobutyl group **5** well explain the difference in the reactivity of the two amide N*H* protons: the more easily the amide nitrogen releases the proton (viz., the more downfield the N*H* proton signal appears), the more susceptible the N*H* proton is to chlorination.

As an extension of the chlorination studies, the behavior of amide N*H* proton in *o*- and *p*-substituted acetanilides was investigated. Table II shows solvent dependences of chemical shifts of the substituted acetanilides in three solvents.

Table I. Approximate Rates (T/2) of H-D Exchange and H-Cl Replacement Reactions of Amide N*H* of 0.67 M CH_3CONH-R in $CDCl_3$

No.	-R	Chemical Shift in ppm	CD_3OD (1eq)	CD_3OD (5eq)	t-BuOCl (1.2eq)	t-BuOCl (2eq)
1	$-CH_3$	6.25	18 min	5 min	10 min	5 min
2	$-CH_2CN$	7.25	2	1	4	3
3	$-(CH_2)_2CH_3$	6.10	5	2	8	5
4	$-(CH_2)_6CH_3$	6.09	—	9	—	4
5	$-CH_2CH(CH_3)_2$	6.10	20	5	20	12

Table II. Solvent Dependence of Amide N*H* Chemical Shifts (δ) of 0.4 M CH_3CONH-Ar

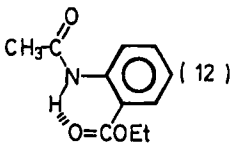
No.	Ar-	δ in ppm			Shift Difference, $\Delta\delta$		
		$CDCl_3$	MeOH	Me_2SO-d_6	1-3	1-2	2-3
11	p-EtOOC-C_6H_4-	8.25	10.10	10.26	2.01	1.85	0.16
12	o-EtOOC-C_6H_4-	11.12	11.12	10.62	0.50	0	0.50
13	p-NO_2-C_6H_4-	—	—	10.54	—	—	—
14	o-NO_2-C_6H_4-	10.35	10.30	10.27	0.01	0.05	0.04
15	p-CH_3O-C_6H_4-	7.89	9.80	9.75	1.86	1.91	0.05
16	o-CH_3O-C_6H_4-	7.85	9.10	9.06	1.21	1.25	0.04

The small solvent responses exhibited by *o*-substituted acetanilides indicate that the amide N*H*'s, especially the amide N*H* in ethyl *o*-acetaminobenzoate **12**, is strongly shielded from the solvent through six atom hydrogen bonding. The presence of intramolecular hydrogen-bonding was also supported by physicochemical properties such as lower melting points (**11**, mp 110°C; **12**, mp 61°C) and lowfield chemical shifts of amide N*H* (**11**, 8.25 ppm; **12**, 11.12 ppm).

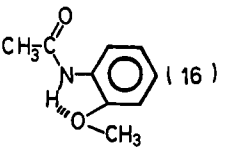
The very slow H-D exchange for solvent-shielded N*H* of **12** gave additional evidence for the existence of intramolecular hydrogen-bonding (Table III). Moreover, the T/2 for the chlorination of ortho compounds (**12, 14,** and **16**) were within 2 min. This phenomenon shows that the rapid hydrogen-chlorine exchange selectively occurred in hydrogen-bonded N*H* in the presence of one equivalent of chlorine in DMSO-d_6.

Table III. Approximate Rates (T/2) of H-D Exchange and H-Cl Replacement Reactions in Amide N*H* of 0.4 M CH_3CONH-Ar in DMSO-d_6

No.	Ar-	-NH ppm	D_2O (1.5eq)	D_2O (5eq)	Cl_2 (1eq)
11	p-EtOOC-C_6H_4-	10.26	60 min	2 min	60 min
12	o-EtOOC-C_6H_4-	10.62	200	22	2
13	p-NO_2-C_6H_4-	10.54	1	1	20
14	o-NO_2-C_6H_4-	10.27	1	1	2
15	p-CH_3O-C_6H_4-	9.75	18	—	8
16	o-CH_3O-C_6H_4-	9.06	21	—	2



(12)



(16)

The mechanism of chlorination in the aromatic rings of anilines and related amines has been postulated,[3] and has conclusively given the evidence for intermediates of N-chloroaniline in chlorination of anilines by reagents of positive chlorine source such as N-chlorosuccinimide and calcium hypochlorite.

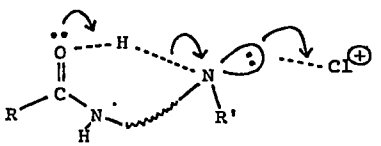


Fig. 2. Postulated mechanism for H-Cl replacement reaction.

Based on these studies, we postulate a mechanism to account for H-Cl replacement reaction (Figure 2). The mechanism also explains the rapid chlorination of peptide NH, which is connected with carbonyl oxygen through intramolecular hydrogen bonding, using reagents of positive chlorine, t-BuOCl and chlorine. This means that the H-Cl exchange method is applicable to identify readily peptide N*H* moieties which are involved in intramolecular hydrogen bonding.

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PEPTIDE BACKBONE FOLDING IN LHRH AND ANALOGS

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The proton magnetic resonance spectrum of luteinizing hormone releasing hormone (LHRH) at 270 MHz was reported on in 1973 by Wessels *et al.*[1] No indication of definite chain folding appeared in the H-N-C-H coupling constants, or in the N-H proton chemical shift and temperature coefficient data. The increased frequency separation between N-H lines obtainable at 600 MHz makes it practical to search for evidence of chain folding in LHRH and analogs using the line broadening effects of a nitroxyl cosolute. Four peptides have been examined:

I. LHRH, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

II. [D-Ala^6]-LHRH

III. [D-Phe^2, D-Ala^6]-LHRH

IV. pGlu-His-Trp-Ser-Tyr-NH-CH$\langle^{CH_2CH_2}_{CO}\rangle$N-CH(iBu)-Arg-Pro-Gly-$NH_2$

[D-Ala^6]-LHRH is somewhat more potent than LHRH itself, which has suggested that there are Tyr^5-Xxx^6 or Xxx^6-Leu^7 β-turns in the biologically active conformation. Peptide IV is a highly active analog prepared by Freidinger et al.[2] in which a Type II′ β-turn at 6-7 is favored by a bridge of appropriate stereochemistry between C^6_α and N^7, holding ψ_6 near $-120°$.

N-H proton resonances were assigned on the basis of decoupling experiments, comparisons among peptides, and effects of pH. Spectra were obtained at 600 MHz using the rapid scan cross correlation mode for the most part, although some decoupling studies on solutions of less than 15% H_2O content were done using the pulse-Fourier transform mode, and/or at lower fields.

Considerable parallelism in the N-H and α-proton resonances was found in the four compounds, but no unusual coupling constants were observed for the backbone protons, and the chemical shift ranges of the peptide and α-protons were narrow. It is probable, then, that single very stable conformations are absent.

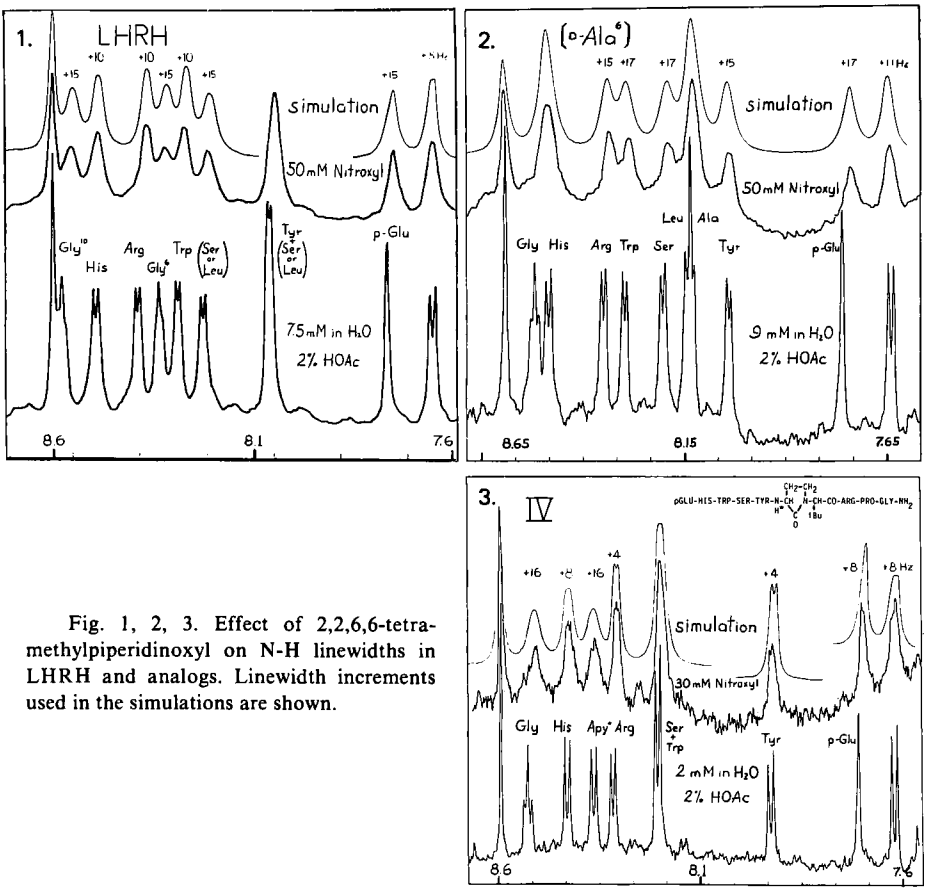

Fig. 1, 2, 3. Effect of 2,2,6,6-tetramethylpiperidinoxyl on N-H linewidths in LHRH and analogs. Linewidth increments used in the simulations are shown.

Figures 1-3 show the N-H regions of the proton spectra in the absence and in the presence of an indicated amount of 2,2,6,6,-tetramethylpiperidinoxyl, plus simulations from which the radical-produced line broadening was estimated. Spectra for I, II, and IV are shown; the N-H lines of III overlap extensively.

Previous work[3-5] has shown that in folded peptide chains N-H protons directed out of the folded chain are more sensitive to dipolar broadening by the nitroxyl paramagnetism than are N-H protons directed into the fold. In a β-turn, $N\text{-}H_{i+1}$ would be exposed to the nitroxyl and $N\text{-}H_{i+3}$ would be sequestered. In gramicidin S, *e.g.*, the exposed Phe and Orn N-H are 8-10 times more sensitive to nitroxyl than are the transannularly hydrogen bonded N-H of Val and Leu.

The spectra of LHRH (Figure 1) show that the N-H of Gly^6 is exposed to nitroxyl and the N-H of Arg^8 is relatively shielded. This is consistent with a β-turn having Gly^6 as residue i+1 and Arg^8 as residue i+3 in the most important conformations in aqueous solution. That there are distinctions among the other residues as well indicates that there is a preferred average conformation for much of the molecule.

In contrast, the spectra of [D-Ala^6]-LHRH (Figure 2) show very little distinction in line broadening among the separated N-H resonances, so that no individual mode of chain folding can be identified. This negative result also indicates that nitroxyl line broadening is sensitive to conformation, not simply to side chain size, a necessary assumption for studies like this.

The experiment with [D-Phe^2, D-Ala^6]-LHRH, not shown, reveals only that Tyr^5 N-H is more sequestered than Gly^{10} N-H.

The analog IV shows greater differentials in line broadening (Figure 3) than LHRH itself, indicating a stronger preference for one type of chain folding. This is to be expected if the covalent constraint in IV is in the direction of the already preferred folding of LHRH. The N-H of the aminopyrrolidinone, corresponding to the Gly^6 N-H of LHRH, is exposed to the nitroxyl, and the N-H of Arg^8 is very much sequestered, again consistent with the β-turn at residues 6 and 7. The N-H of Tyr^5 is also buried, agreeing with a β-structure extending beyond the immediate region of the 6-7 turn itself, possibly a hydrogen bond, Tyr^5 N-H $\rightarrow$ Arg^8 C=O. Overlaps prevent observation of the Tyr^5 N-H in LHRH for comparison.

Momany[6] has calculated a favored conformation for LHRH. The observations in Figures 1 and 3 are consistent with that conformation around Tyr^5-Gly^6-Leu^7-Arg^8 and in the corresponding part of IV. In Momany's structure there are intrachain hydrogen bonds, Arg^8 N-H $\rightarrow$ Tyr^5 C=O and Tyr^5 $\rightarrow$ Arg^8 flanking a 6-7 β-turn. In view of the enhanced activity of the constrained peptide IV, this probably does represent the biologically active folding in this region. [D-Ala^6]-LHRH is also more potent than LHRH itself, and the same chain conformation is perfectly possible for it, but the biologically active folding of [D-Ala^6]-LHRH apparently competes less effectively in solution with other chain foldings.

Other N-H protons that can be observed to be less exposed are that of Trp^3 in LHRH and those of His^2 in LHRH and IV. The first observation is consistent with a Trp N-H $\rightarrow$ Tyr O-H bond proposed by Momany, but shielding of the His N-H protons is not consistent with the calculated structure.

Acknowledgements

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FOLDED FORMS OF N-METHYLATED MODEL DIPEPTIDES

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The study of relationships between the conformations of peptides and their biological activities is often built upon observations of the effect that altering the primary structure or the chiral sequence has on the biological activities. In this scope, we have concentrated our attention on determining the influence of N-methylation on conformational features of dipeptide model compounds (Table I) with the general formulae:

I : Bu^tCO-L-Pro-Me-X-NHMe and II : Bu^t-CO-L-Pro-Me-X-OMe (X=Gly,L-Ala,D-Ala) in comparison with those of the homologous desmethylated species I'.

Experiments have been carried out in organic solvents (CCl_4, $CHCl_3$, DMSO . . .) by IR spectroscopy and ^1H and ^{13}C-NMR techniques, and in the crystal state by X-ray diffraction. N-Methylated species have been obtained through the classical procedure using BOC as NH-protecting group and BOP as coupling reagent.[1] The N-methyl group has been introduced on BOC-X-OH according to Cheung and Benoiton.[2]

Solution Conformational Studies

The percentage of *cis*-conformers depends strongly upon the solvent, the chiral sequence and the C-terminal function (Table I). In CCl_4 and $CHCl_3$ practically all the NH groups of the three derivatives I are intramolecularly hydrogen bonded to the pivaloyl CO group ($4 \rightarrow 1$ interaction) as evidenced by the low NH and CO stretching frequencies (Table I). This result is corroborated by the variation of the NH proton chemical shift against the composition of $CHCl_3$/DMSO mixtures (Figure 1). Weakly affected signals correspond to intramolecularly bonded NH groups as for the *cis*-conformers of I(LL) and I(LG) and the *trans*-conformers of I(LD) and I(LG). In contrast, the rapid variation for the minor *trans*-conformer of I(LL) is characteristic of a free NH group.[3]

In N-methylated peptides, the usual vicinal proton coupling constant $J(NH\text{-}C^{\alpha}\text{-}H^{\alpha})$ related to the rotational state θ of the $N\text{-}C^{\alpha}$ bond[4] is not available. Thus we have established a similar correlation[5] for the vicinal coupling constant $J(^{13}C,H^{\alpha})$ in the fragment $^{13}CH_3\text{-}N\text{-}C^{\alpha}\text{-}H^{\alpha}$:

$$J(^{13}C,H^{\alpha}) = 3.05 \cos 2\theta - 2.55 \cos \theta + 3.2.$$

Table I: Percentage of *cis*-conformers and ir data for the N-methylated peptides I and II.

Compound	*cis* (%)[a]			Stretching Frequency (cm^{-1})[b]	
	CCl_4	$CHCl_3$	DMSO	NH	CO
I(LL)	90	76	48	3330	1608
II(LL)	# o	5	9	-	1623
I(LD)	# o	# o	24	3362	1607
II(LD)	30	40	32	-	1624
I(LG)	35	16	27	3356/3325	1608
II(LG)	33	24	28	-	1622

[a]estimated from the N-methyl signal [b]solvent : CCl_4, concentration : 0.001 mol/l.

The constants $J(^{13}C,H^{\alpha})$ for the major *cis* or *trans* conformers (Table II) together with the existence of a $4 \rightarrow 1$ interaction in derivatives I show that both *trans* I(LD) and I(LG) accommodate the well-known βII-folded mode whereas both *cis* I(LL) and I(LG) adopt a folded form (Figure 2) similar to that denoted βVI for *cis* X-L-Pro sequences by Chou and Fasman.[6]

Table II: $J(^{13}C,H^{\alpha})$ coupling constants for peptides I and II[a]

Compound	Conformer	$J(^{13}C,H^{\alpha})$ (Hz)	ϕ_2(°)
I(LL)	*cis*-folded	5.1	- 75
II(LL)	*trans*-open	4.8	- 75
I(LD)	*trans*-βII-folded	5.3	+ 80
II(LD)	*trans*-open	5.1	+ 75
II(LD)	*cis*-open	4.3	+ 70
I(LG)	*trans*-βII-folded	2.8;5.9	+ 85

[a]Solvent : $CCl_4/CHCl_3$ 4/1 (v:v)

The influence of DMSO on the pivaloyl CO stretching frequency[3] reveals that DMSO has a poor destabilizing effect on the *trans*-βII-folded form of I(LD). In contrast, the *cis*-folded form of I(LL) is appreciably destabilized and its percentage decreases noticeably from CCl_4 to DMSO (Table I).

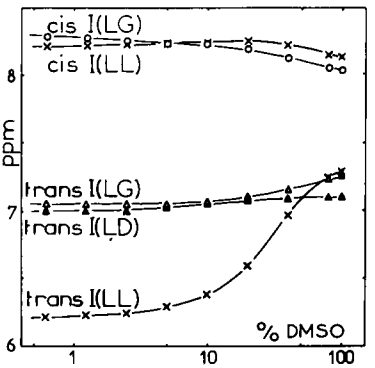

Fig. 1. Variation of the NH proton chemical shift for the *cis* and *trans* conformers of I(LL), I(LD) and I(LG) against the composition of $CHCl_3$/DMSO mixtures.

Solid State Structures

The crystal structures of I(LL), II(LL) and I(LD) have been solved by X-ray diffraction and compared with those of the homologous desmethylated sequences I'[8] (Table III). The N . . . O distance for the eventual 4 → 1 interaction is also specified. It results that the major folded conformers of I found in solution are retained in the solid state.

In the monohydrated state of I(LD), the water molecule gives rise to the N-H . . . W-H . . . O=C sequence.[9] Then the N . . . O distance increases up to 5.00 A but the general folded shape of the molecule is retained (Table III). This crystal structure is the first example evidenced by X-ray diffraction of the possible influence of water upon β-bends.

Table III. Solid State Conformation of N-methylated Peptides I and II and Homologous Desmethylated Sequences I'.

Compound	Conformation	Internal Angles (°)					N...O (Å)
		ϕ_1	ψ_1	ω_2	ϕ_2	ψ_2	(4→1)
I(LL)[a]	*cis*-folded	-62	135	-13	-119	60	2.79
II(LL)	*trans*-open	-70	153	176	-92	157	
I(LD)	*trans*-βII-folded	-58	136	-178	97	-19	2.97
I(LD),H_2O	*trans*-folded	-69	164	172	139	-35	5.00[c]
I'(LL)[b]	βII-folded	-59	136	180	66	14	3.05
I'(LD)[b]	βII-folded	-62	137	180	96	3	3.10

[a]isopropyl C-terminal group. [b]isopropyl N and C-terminal groups[8] [c]the water molecule is inserted between the NH and CO terminal groups

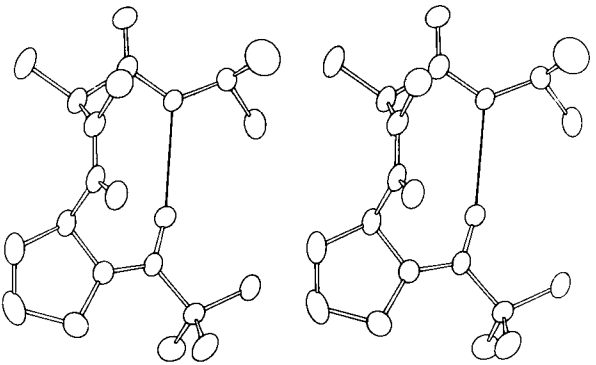

Fig. 2. Stereoview of the molecular conformation of I(LL) in the crystal state.

Conclusion

Our results attest that N-methylation has very little influence on the heterochiral L-Pro-D-Ala sequence which accommodates principally the βII-folding mode in both solution and crystal state. The same holds true for the L-Pro-Gly sequence confirming that Gly mainly acts as a D-residue when placed after proline.[3]

In contrast, N-methylation of the homochiral L-Pro-L-Ala sequence reduces the conformational freedom[3] and induces a *cis*-folded form already found in Ilamycin B_1.[10] The strong 4 → 1 interaction (Table III) seems to be necessary for the stability of this conformer since II(LL) accommodates a *trans*-open conformation.

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STERICALLY-HINDERED AMINO ACIDS. DIRECTORS OF PEPTIDE CONFORMATION

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Introduction

Peptides synthesized from sterically-hindered amino acids may be locked into particular conformational states. Such peptides will be valuable in associating thermodynamic parameters and bioactivities with defined conformational features. We are synthesizing model dipeptides with methyl groups added in sterically-hindered positions. Herein, we will discuss acetyl, methylamide derivatives of prolines with methyl substituents in the α and β positions as well as the corresponding β-methylvaline peptide. Through spectroscopic studies, we have established the main features of the conformational distributions of these peptides in a variety of solvents.

Results and Discussion

Acetyl-methylproline methylamides — Carbon-13 NMR spectra reveal that as in the case of AcProNHMe[1] the *syn*- and *anti*-3-methyl derivatives (*syn:* the methyl is on the same side of the ring as the carboxamide group; *anti:* is on the opposite side) have 15-25% *cis* amide isomer (acetyl methyl *cis* to C^{α}) for the range of solvents investigated. In contrast, for Ac-2-MeProNHMe there is no detectable (<5%) *cis* amide isomer.

The chemical shift difference between C^{β} (C^3) and C^{γ} (C^4) seems to be a reliable indicator of the proline Ψ angle.[1,2] Methyl substitution at the β position shifts both C^{β} and C^{γ} downfield. Changes in $\Delta\delta_{\beta\gamma}$ for the *anti*-3-methyl peptide parallel those of AcProNHMe. For the *trans* amide isomer of the methyl derivative, $\Delta\delta_{\beta\gamma}$ increases from 2.42 to 6.38 ppm in going chloroform to water compared to a change from 2.01 to 5.81 for AcProNHMe (Table I). For the three peptides $\Delta\delta_{\beta\gamma}$ is nearly constant for the *cis* isomers. In the case of Ac-*syn*-3-MeProNHMe, $\Delta\delta_{\beta\gamma}$ is relatively constant for the *trans* isomer as well. For Ac-2-MeProNHMe, $\Delta\delta_{\beta\gamma}$ values are larger due to the position of substitution. The variation of $\Delta\delta_{\beta\gamma}$ with solvent is intermediate. The difference in going from chloroform to water being 1.24 ppm for the 2-Me peptide compared to

0.08, 3.96 and 3.81 for *syn*-3-Me, *anti*-3-Me and AcProNHMe, respectively.

Table I. $^{13}C^{\beta}$-$^{13}C^{\gamma}$ Chemical Shift Difference for N-Ac-MeProNHMe in Various Solvents

Solvent	$\Delta\delta_{\beta\gamma}$ (ppm)						
	2-Me	syn-3-Me		anti-3-Me		Pro[1]	
	trans	trans	cis	trans	cis	trans	cis
$CHCl_3$	15.37	3.88	6.85	2.42	9.49	2.01	--
dioxane	16.05	3.90	6.91	3.97	9.61	3.13	9.03
CH_3CN	16.62	3.42	7.01	5.42	9.77	4.72	9.08
CH_3OH	16.69	3.83	7.37	6.02	10.09	5.52	9.28
H_2O	16.61	3.75	7.08	6.38	9.98	5.81	9.05

The CD spectra of the *anti*-3-methyl derivative also closely parallel those of AcProNHMe (Figure 1, Table II). The *syn*-3-methyl peptide has CD similar to the former two peptides in water, but has completely different spectra in non-polar solvents. Note particularly that in chloroform the ellipticity at 230 nm of the *syn*-3-methyl peptide is about 1/5 that of the *anti*-3-methyl derivative (Figure 1). In dioxane the *syn* peptide has zero ellipticity while the *anti* peptide has a large negative value (Table II). Ac-2-MeProNHMe has a CD spectrum in chloroform similar to those of AcProNHMe and Ac-*anti*-3-MeProNHMe, but about 20% greater in magnitude. In aqueous solution, the CD spectrum of Ac-2-MeProNHMe is unique. Especially notable is the large negative ellipticity at 215 nm.

Table II. CD for N-Ac-MeProNHMe in Various Solvents

Solvent	M_θ (225 nm) X 10^{-3}			
	syn-3-Me	anti-3-Me	Pro[1]	2-Me
Chloroform	-9	-44	-48	-60
Dioxane	0	-24	-31	-38
Acetonitrile	-2	-12	-17	-23
Methanol	+2	-4	-4	-14
Water	0	-4	-4	-15

We deduce from these data that the conformations of Ac-*anti*-3-MeProNHMe are nearly identical to those established for AcProNHMe. The C_7 conformer (Ψ *ca.* 80°) dominates in chloroform, P_{II} (Ψ *ca.* 150°) dominates in water and in other solvents there is a mixture of C_7, P_{II} and α_R (Ψ *ca.* −50°) conformers.[1,3] For Ac-*syn*-3-MeProNHMe, we infer that there is little C_7 conformer in any of the solvents reported herein. This is apparently due to steric interference between the *syn*-methyl group and the carbonyl oxygen. The CD spectrum in water is a somewhat different shape than those of AcProNHMe and the *anti* derivative. Nevertheless, we tentatively conclude that P_{II} is the major conformer in water. The small variation of $\Delta\delta_{\beta\gamma}$ may indicate only minor conformational changes with solvent, but conversions between P_{II} and α_R would not be detected.

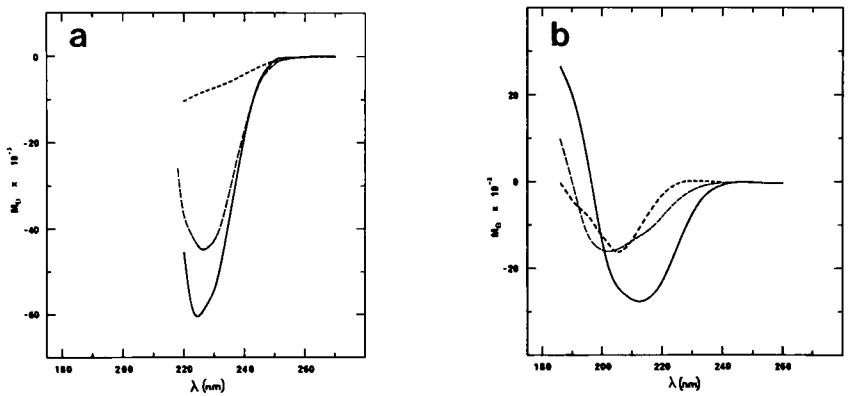

Fig. 1. CD spectra of N-Ac-MeProNHMe: 2-Me (solid line), *anti*-3-Me (long dashes) and *syn*-3-Me (short dashes) in A, Chloroform and B, Water.

The large negative CD spectrum of Ac-2-MeProNHMe in chloroform indicates the C_7 conformer. The small variation of $\Delta\delta_{\beta\gamma}$ may indicate minor conformational variation with solvent. Energy computations show steric interference of the methyl group with both the α_R and P_{II} conformers. The negative ellipticity at 215 nm is consistent with retention of a significant amount of C_7 in water.

Acetyl-β-methylvaline methylamide — Spectroscopic parameters for Ac-β-MeValNHMe are similar to those for AcValNHMe (Table III). The proton-proton coupling constant $^3J_{HN^{\alpha}H}$ is 9.0-9.5 Hz for Ac-β-MeValNHMe in all of the organic solvents, but decreases to 7.9 Hz in water. There is somewhat greater variability for AcValNHMe. A minimum in the CD spectra occurs near 220 nm in non-polar solvents.

The magnitude of this band is small for AcValNHMe and even smaller for Ac-β-MeValNHMe (compare –12,000° at 220 nm in chloroform for AcValNHMe to –50,000° to –60,000° for the proline peptides which form the C_7 conformer, Tables II and III).

The proton coupling constants in conjunction with a Karplus-like relationship[4] indicate Φ *ca.* –120° for Ac-β-MeValNHMe in organic solvents. The lower value in water indicates an average change in Φ of ± 20°. The CD spectra indicate the absence of a hydrogen-bonded C_7 conformer. Energy computations suggest that Ψ may be in a region near 100°.

Table III. Coupling Constants and CD Minima for Two Dipeptides

Solvent	$^3J_{HNC^\alpha H}$ (Hz)		$M_{\theta\,(220\ nm)} \times 10^{-3}$	
	β-MeVal	Val[3]	β-MeVal	Val[3]
Chloroform	9.5	9.5	--	-12
Dioxane	9.6	9.2	-7	-12
tert-Butyl Alcohol	--	9.2	-8	-15
Acetonitrile	9.2	8.5	-2	-5
Dimethylsulfoxide	9.6	9.0	--	--
Methanol	9.0	8.7	-5	-6
Water	7.9	7.5	-3	-3

Conclusions

The methyl group of Ac-*anti*-3-MeProNHMe does not change the conformational distribution from that of AcProNHMe. For Ac-*syn*-3-MeProNHMe there is steric interference with the C_7 conformer. In Ac-2-MeProNHMe there is steric interference with the P_{II} and α_R conformers and with the *cis* isomer. The C_7 conformer appears to be stabilized even in polar solvents. Ac-β-MeValNHMe has greater conformational restrictions than AcValNHMe. C_7 is not formed.

Acknowledgements

We thank NIH for support (GM22514) and K. Kopple for NMR data.

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CONFORMATIONAL ANALYSIS OF PEPTIDES USING SOLUBLE SPIN LABELS

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Introduction

Nitroxide radical effects on amide proton line widths have been used as criteria for the extent of solvent exposure.[1,2] Here we report: a) the increment of the proton relaxation rate of peptides in the presence and absence of spin labels by pulsed NMR; b) extension of these measurements to all protons, including aliphatic and aromatic C-H protons and amide protons; and c) the use of nitroxide enhanced spin-lattice relaxation rates as quantitative criteria for conformational moieties and solvent exposure of hydrogen atoms attached to carbon and nitrogen atoms of the backbone and side chains.

Results

The effects of nitroxide radical, 2,2,6,6-tetramethyl-piperidineoxyl (TEMPO), on the partially relaxed 270 MHz ^{1}H-NMR spectra of gramicidin S protons in DMSO-d_6 are shown in Figure 1. TEMPO at this concentration produced significant and accurately measurable enhancements of the nonselective relaxation rates of all amide, Hα and side chain protons; nitroxide effects on tyrocidine A are similar. Semilogarithmic plots yielded the ^{1}H spin-lattice relaxation rates, R_1, of most classes of proton and many individual protons in tyrocidine A and gramicidin S. These experiments were performed as a function of TEMPO concentration and plots of the enhancement of spin-lattice relaxation rates, R_{1P}, versus TEMPO concentration for gramicidin S amide and Hα protons are shown in Figure 2. The enhancement of relaxation rate, R_{1P}, for tyrocidine A amide and Hα protons are listed in Table I.

These data were used to ascertain the major factor governing the extent of nitroxide enhancement of relaxation rate of each proton in a given solution. Since conformations of gramicidin S and tyrocidine A are well-defined,[3,4,5,6] they were chosen as model compounds. Figure 3 shows the proposed conformation for tyrocidine A, consisting of an anti-parallel β-pleated sheet and two β-turns..

The small relative enhancements (S_{1P} = 52-54 $sec^{-1}M^{-1}$) of Val^{1}NH, Leu^{3}NH and Asn^{8}NH in tyrocidine A correspond to innerpointing,

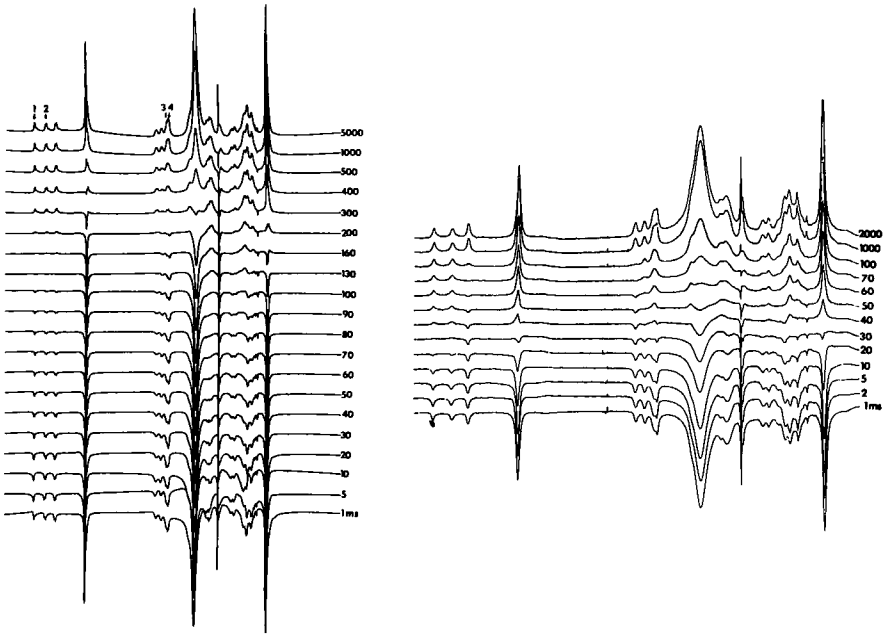


Fig. 1. Left: Partially relaxed 270 MHz^1 H-NMR spectra of gramicidin S(10 mM) in DMSO-d_6. Right: the same sample after addition of nitroxide (50 mM TEMPO). Note the selective effects of soluble spin label of proton relaxation rates of 1. PheNH 2. Orn NH 3. Phe Hα and 4. ProHα.

hydrogen-bonded amides of the anti-parallel β-pleated sheet moiety whereas the large S_{1P} values (90-125 $sec^{-1}M^{-1}$) of Orn^{2}NH, DPhe^{4}NH, DPhe^{7}NH and Gln^{9}NH correspond to outer-pointing, solvent exposed amides. Although Tyr^{10}NH is axial to the tyrocidine ring in the βI-turn, its S_{1P} value (36 $sec^{-1}M^1$) seems inconsistent with this configuration. As previously proposed,[7] it is either solvent shielded by the side chains of Gln^9 and Tyr^{10} or involved in side chain-backbone hydrogen bonds. No data were obtained for Phe^{6}NH due to spectral overlap.

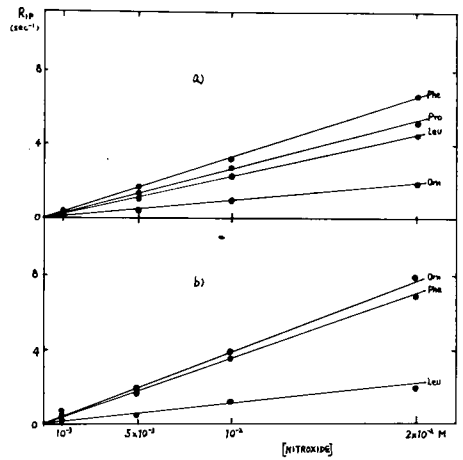
The S_{1P} values for all alpha protons also support the proposed conformation. The equatorial, inner-pointing alpha protons, Orn^{2}Hα and DPhe^{7}Hα, have low S_{1P} values (43 and 26 respectively) and the Hα protons of residues 1,3,4,5,6,8,9,10 being relatively more solvent exposed, have S_{1P} values of 63-132. The S_{1P} values of side chain protons are in the range from 70 to 125 $sec^{-1}M^{-1}$, corresponding to their greater solvent exposure than shielded backbone protons. These data are consistent with the proposed structure shown in Figure 3. Data for gramicidin S in DMSO-d_6 and CD_3OD are also fully consistent with proposed structure.

Table I. Nitroxide Effects on Tyrocidine A Backbone Proton Relaxation Rates.

Proton	Chemical Shift[a] (ppm)	Relaxation Rate for a Given Nitroxide Concentration, R_1 (sec^{-1}) Nitroxide Concentration (mM) 0	12.8	38.4	S_{1p} ($sec^{-1}M^{-1}$)
$Dphe^4NH$	9.26	3.1	4.4	6.4	90
$Dphe^7NH$	9.01	3.8	5.1	8.3	115
Asn^8NH	9.00	3.7	4.4	5.7	52
Gln^9NH	8.71	4.7	6.3	9.6	125
Orn^2NH	8.85	3.9	5.5	8.6	122
$Tyr^{10}NH$	8.40	4.4	4.9	5.7	36
Leu^3NH	7.85	4.2	5.0	6.2	54
Val^1NH	7.37	2.8	3.5	4.8	52
$Dphe^7\alpha$	5.55	3.4	3.7	4.4	26
$Orn^2\alpha$	5.26	3.3	3.8	4.9	43
$Val^1\alpha$[b]	4.52	2.3	3.5	5.8	94
$Leu^3\alpha$[b]	4.51	2.3	3.5	5.8	94
$Asn^8\alpha$[b]	4.46	2.3	3.5	5.8	94
$Phe^6\alpha$[b]	4.45	2.3	3.5	5.8	94
$Tyr^{10}\alpha$	4.27	2.1	2.9	4.6	64
$Dphe^4\alpha$	4.26	2.9	4.6	8.2	132
$Pro^5\alpha$	4.03	1.7	2.5	4.3	68
$Gln^9\alpha$	3.78	1.6	2.4	4.1	63

[a]M. Kuo, W.A. Gibbons.[6]

[b]These relaxation rates were calculated for all four unresolved Hα proton multiples.



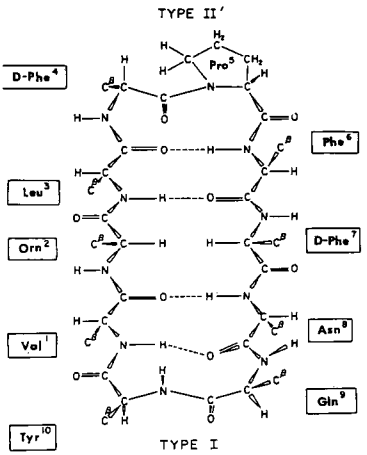


Fig. 3. Proposed conformation for tyrocidine A.[6]

Fig. 2. Dependence of spin-lattice relaxation rate enhancement, R_{1P}, on nitroxide concentration added for gramicidin S a) alpha protons and b) amide protons. Gramicidin S sample concentration was 40 mM in DMSO-d_6 299° K.

Conclusions

The enhancement of spin-lattice relaxation rate of individual protons per mole of nitroxide added to the solution quantitatively reflects the conformation of the peptide. We therefore propose their use as criteria for conformational moieties (such as β-pleated sheet, β-turns) to delineat amide hydrogen bonding patterns in peptides and for general conformational analysis of peptides.

Acknowledgements

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X-RAY STRUCTURE AND SPECTROSCOPIC PROPERTIES OF Boc-L-ALA-(AIB-ALA)$_2$-GLU(OBZL)-ALA-(AIB-ALA)$_2$-OME, A MODEL OF THE ALAMETHICIN HELIX

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The helical segment in the polypeptide antibiotics alamethicin[1], suzukacillin[2], and trichotoxin[3,4] is essential for their pore forming properties in lipid membranes. CD and ^{13}C NMR on membrane active natural and synthetic analogs[5,6], on isolated natural fragments, and model peptides[7,8] revealed more than 15 well characterized helical Aib peptides.

The electric field dependent gating processes[9] are associated with changes in dipole moment. Dipole moments of 60 and 120 Debye, assuming dimeres with parallel or antiparallel dipoles were determined in octanol corresponding to an alamethicin spheroid of 35 Å in length and 13 Å in diameter[10].

Recently the 3_{10} helix type has been emphasized, even for longer Aib peptides, on the basis of X-ray data of very short Aib peptides which adopt β-turns[11]. Indeed, we too found β-bends for Aib oligopeptides, e.g. in crystals of Boc-Gly-Ala-Aib-OMe (hydrogen bond Boc-CO to Aib-NH, type III) or of Ac-Ala-Aib-Ala-OMe (Ac-CO to Ala-NH)[12]. However, as discussed elsewhere[7,8] all results of CD studies on helical Aib peptides[1-8] are in full agreement with those of normal α-helical oligopeptides of comparable lengths. Further hints for the α-conformation of helical Aib peptides are given by ^{13}C-NMR (Figure 1). Via conventional segment condensations various Aib deca- and undecapeptides were synthesized[13]. The crystals of the undecapeptide Boc-(L-Ala-Aib)$_2$-Ala-Glu(OBzl)-Ala-(Aib-Ala)$_2$-OMe were suitable for X-ray crystallography, although they decomposed in the absence of mother liquor (dichloromethane/hexane). The solution of the structure ($P2_1$, 90 nonhydrogen atoms, Z = 2) with direct methods was achieved with a novel program of G.M. Sheldrick. Nine residues of the undecapeptide are part of an α-helix (Figure 2). Only ^{10}Aib and ^{11}Ala form a β-turn-like C-terminus. Therefore the carbonyl groups of ^{8}Aib and ^{9}Ala can act as hydrogen acceptors for the NH groups of ^{1}Ala and ^{2}Aib of a neighboring helix. Chains of head to tail linked α-helices are arranged with antiparallel helical axes.

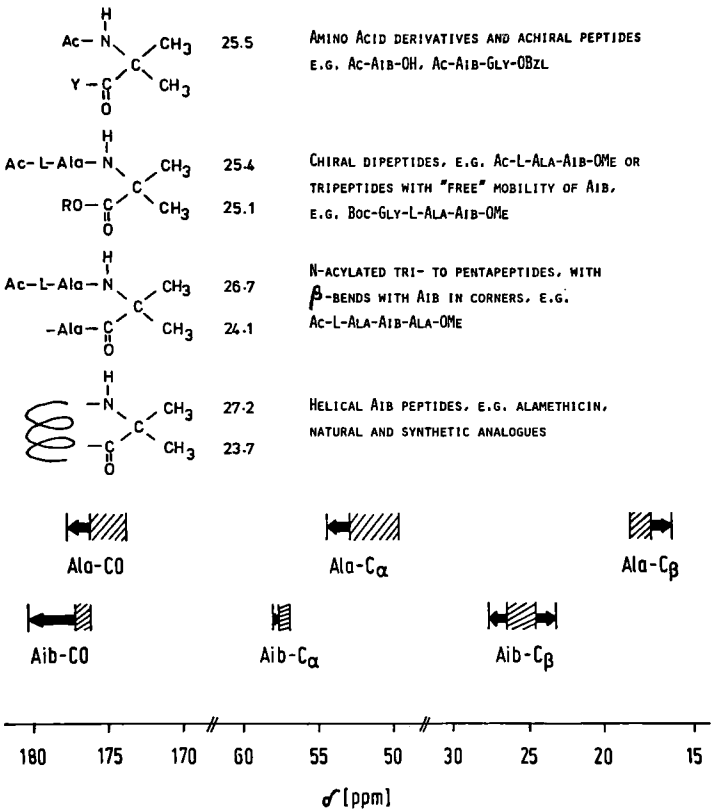

Fig. 1. Magnetic and nonequivalence (MNE) of the geminal Aib methyl groups in the Aib peptides and typical helix induced downfield shifts for CO and Cα signals; Ala-Cβ and one geminal Aib-Cβ shift upfield, the other Aib-Cβ downfield.

We assume, that the α-helix is also present in solution. ^{13}C NMR in methanol shows 2 signal groups of Aib-Cβ (23.2 - 24.1 and 26.7 - 27.1, MNE up to 4 ppm). At least 4 alanine residues are helical (4 Ala-Cα at 53.3 to 53.9 ppm), 2 alanines are not (51.1 and 49.4 ppm). 2 Ala-CO are not shifted downfield (174.3, 174.6 ppm), 4 others are at 175.9 to 177.2 ppm. Since only 2 Aib-CO are at low field (178.2, 178.3), the other 2 Aib-CO must be exposed to solvent (177.2, 177.4).

On the basis of this X-ray structure, we can confirm that alamethicin, trichotoxin, and suzukacillin possess a high content of α-helical conformation[1-8]. The N-terminus is shielded by a β-turn of Ac-Aib-Pro-Aib or Ac-Aib-Gly-Aib. For the C-terminal region we suggest a sequence of β-turns capable of adopting the ion carrier function in the aggregated pore state via its exposed carbonyl groups. The α-helical segment is not

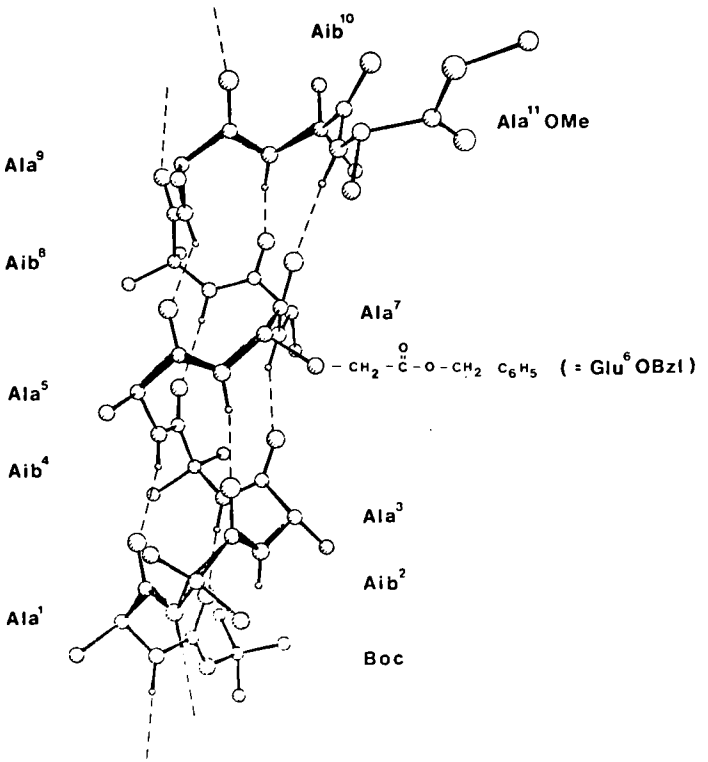

Fig. 2. The α-helical structure of Boc-L-Ala-Aib-Ala-Aib-Ala-Glu(OBzl)-Ala-Aib-Ala-Aib-Ala-OMe[13,14]. The unit cell contains two antiparallel helices and 6 solvent molecules, CH_2Cl_2. The length of the helix is approximately 14 Å and the diameter is 7 Å.

only a lipophilic anchor in the membrane. The electrical helix field [15] may act cooperatively, first by attracting charged molecules to the peptide from the surrounding solvents, and secondly by properly orienting polar substrates and the C-terminal region of alamethicin for pore formation. Indeed, the addition of C-terminal fragments to the bilayer system results in a prolongation of the pore states, whereas the addition of α-helical fragments lowers the mean life time of pores[4].

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THE CRYSTAL AND MOLECULAR STRUCTURE OF A VALINOMYCIN ANALOG, CYCLO-[(VAL-HYI-D-VAL-D-HYI)-(D-VAL-HYI-VAL-D-HYI)$_2$]

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Introduction

The cyclic dodecadepsipeptides, of which valinomycin is a member, form an important class of compounds which are involved in the transport of ions across both natural and synthetic membranes. Valinomycin,[1] cyclo-(D-Val-Lac-Val-D-Hyi)$_3$, and a variety of analogs have been studied by X-ray crystallographic techniques. These analogs include meso-valinomycin,[2] in which Lac has been replaced by Hyi, isoleucinomycin,[3] in which all of the valine residues have been replaced by isoleucine residues, and the structure described here which contains a reversal of the chirality of a pair of valine residues in positions 1 and 3 of meso-valinomycin, producing the sequence cyclo-(LLDD)-(DLLD)$_2$.

Structure Determination and Refinement

The title compound crystallizes in space group P1, cell constants a = 10.701A, b = 11.016A, c = 18.458A, α = 83.786°, β = 66.415°, γ = 87.826°, Z = 1 and with a calculated density of 1.10 g/cm^3. The structure was solved through the use of the direct methods program, QTAN[4], and has been refined by full-matrix least-squares. Hydrogen atom positions were inferred on the basis of idealized geometry. Two independent acetone molecules were subsequently located from a difference map and were refined. The large values of the thermal parameters and the long carbon-oxygen bond distances for both acetone molecules suggest that each solvent molecule may be rotationally disordered and that furthermore each position may be less than 100% occupied. The final residual for 3873 independent data with $F>4\sigma(F)$ was 0.081 and the residual for all data (4322) was 0.113.

Discussion

The conformation of the molecule is illustrated in Figure 1. All peptide bonds are *trans.* Bond distances and angles are within the

expected ranges. The side chain orientations for the L residues are equally distributed between the most commonly observed values of $\chi^1 = 180°$ and $\chi^1 = -60°$, but for the D residues, three of these torsion angles are found to have the value of -60°, two are +60° and one torsion angle is found to have the rarely observed value, for D-residues, of 180°.

The overall shape of the molecule is best described as greatly elongated with a β bend at each end of the molecule. The semiextended sections of the peptide chain connecting the two chain reversals are nearly parallel. The chain reversal at one end of the molecule is a Type II β bend, with residues L-Val[11] and D-Hyi[12] at the corners, and is stabilized by a 4 → 1 hydrogen bond (N-O, 2.92A). The chain reversal at the other end of the molecule consists of a Type I′ β bend with residues D-Val[3] and D-Hyi[4] at the corner and is also stabilized by a 4 → 1 hydrogen bond (N-O, 2.88A). Interpenetrating with this latter Type I′ bend is a weak 5 → 1 hydrogen bond between the nitrogen of L-Val[7] and the carbonyl oxygen of D-Val[3] (N-O, 3.22A). Although the chirality of the residues in the center of this bend are DDL, the same as observed in isoleucinomycin[3] and in valinomycin,[1] the ϕ and ψ torsion angles of the central residue (D-Val[5]) are quite different.

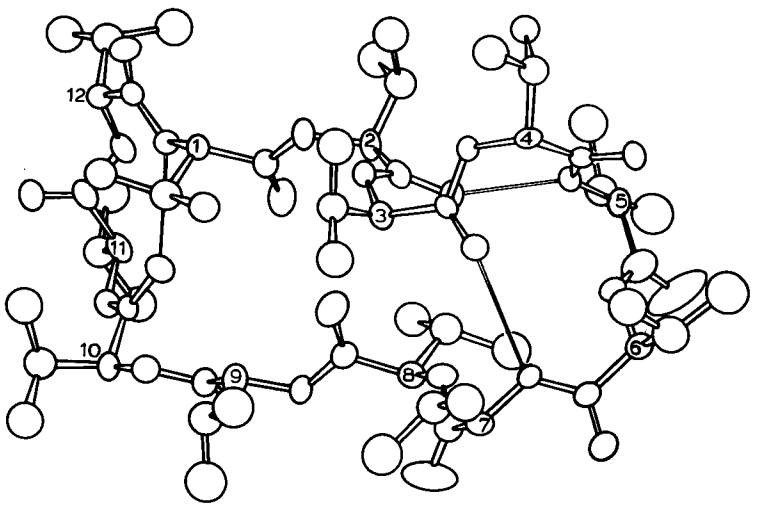

Fig. 1. Observed conformation of cyclo-[(Val-Hyi-D-Val-D-Hyi)-(D-Val-Hyi-Val-D-Hyi)$_2$]. Alpha carbon atoms are numbered and thermal ellipsoids are scaled at 30% probability.

Two intermolecular hydrogen bonds produce an infinite plane of hydrogen bonded depsipeptide molecules. A most unusual situation exists for the amino group of D-Val[3] as this nitrogen is not involved in any hydrogen bonds.

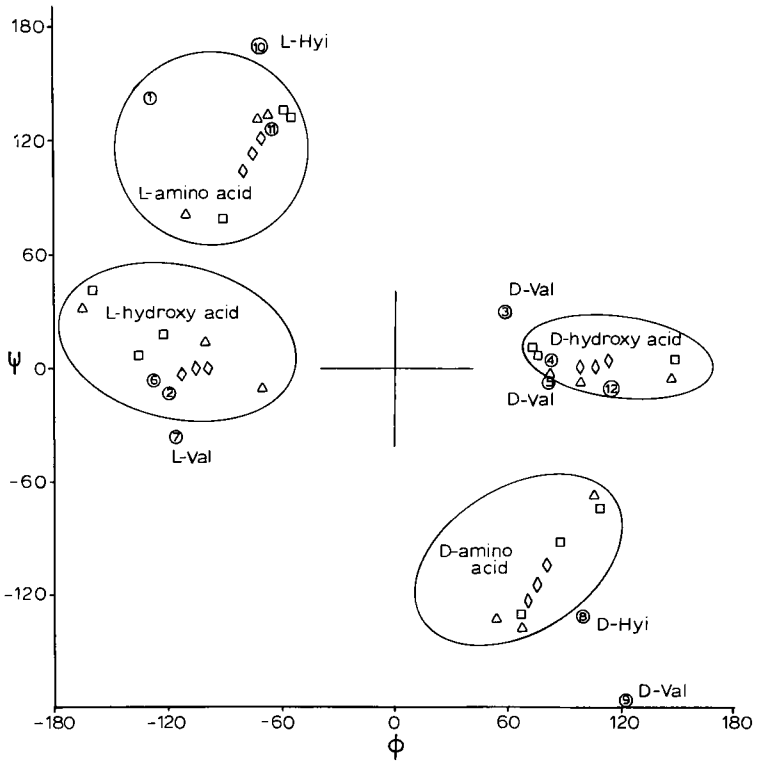

Fig. 2. ϕ,ψ torsion angle plot for valinomycin (△), meso-valinomycin (◇), isoleucinomycin (□), and the present study (○).

A comparison of the ϕ and ψ torsion angles for the uncomplexed form of valinomycin[1], meso-valinomysin[2], and isoleucinomycin[3], and the present study is illustrated in Figure 2. Valinomycin, which has an elongated conformation, is stabilized by four 4 → 1 and two 5 → 1 hydrogen bonds; meso-valinomycin has a conformation which is very similar to that of the complexed form of valinomycin and possesses six 4 → 1 hydrogen bonds; and isoleucinomycin can be thought of as adopting half of the conformation of uncomplexed valinomycin and half of meso-valinomycin, giving rise to five 4 → 1 hydrogen bonds and a single 5 → 1 hydrogen bond. In spite of these differences in conformation, four well-defined regions are found on the ϕ,ψ plot and each corresponds to a particular type of residue regardless of the compound being considered. However, in the present case, the interchange of chiralities of the two amino acid residues has altered the appearance of the plot. L-Val[1] is somewhat displaced from the region most commonly occupied by a L

amino acid, L-Val[7] has adopted a conformation normally observed for a hydroxy acid, and L-Hyi[10] has the conformation which would be expected for a L-valine residue. D-Val[3] and D-Val[5] occupy the D-hydroxy acid region but only D-Hyi[8] is found in the D-amino acid region. Furthermore, D-Val[9] is considerably removed from the region one would expect the D-amino acid residues to occupy. A consequence of this is that no D-amino acid in the present study has a conformation comparable to that observed in either valinomycin, meso-valinomycin, or in isoleucinomycin.

The effect of interchanging a single pair of D and L valine residues results in not just a kink or local change in conformation, but produces a considerable change in the overall conformation of the entire cyclic dodecadepsipeptide. A comparison of the observed conformations of these four depsipeptides also shows the degree of conformational freedom these compounds possess and how the conformation can be altered by changes in side chain composition or by changes in the chiralities.

Acknowledgements

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CRYSTALLINE CONFORMATION OF THE 1:2 COMPLEX BETWEEN Mg^{++} AND CYCLIC(GLY-L-PRO-L-PRO-GLY-L-PRO-L-PRO)

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A complex of Mg^{++} with two molecules of cyclic(Gly-L-Pro-L-Pro-Gly-L-Pro-L-Pro) is formed by mixing $Mg(ClO_4)_2$ with the peptide in an CH_3CN solution.[1] If the Mg^{++} is coordinated octahedrally (with accompanying three-fold symmetry), then the problem concerns the conformation of the cyclic peptides in which the *formula* has two-fold symmetry. Nmr analyses of the complex in solution[1] indicated that the peptides in this complex assume two *cis* Pro-Pro linkages and two *trans* Gly-Pro linkages and that the number of resonances show a lack of symmetry.

X-ray data for a crystal of (GPPGPP) $_2Mg(ClO_4)_2$ grown from an CH_3CN solution were collected from a crystal bathed in the mother liquor* and yielded the structure of the complex[2] as shown in Figure 1. Although the whole complex occupies a general position in a trigonal cell, and no symmetry is required, the upper and lower peptides are related by an approximate 2-fold rotation axis that passes horizontally through the Mg^{++} (in the orientation shown in the figure). The conformations of the two hexapeptide moieties are nearly identical; although each cyclic hexapeptide is quite asymmetric.

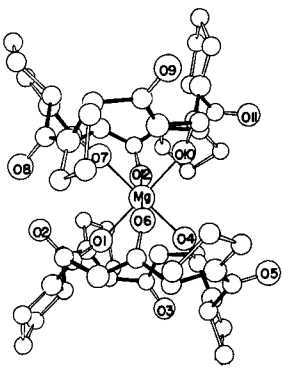

Fig. 1. The complex

The Mg^{++} forms ligands to six carbonyl oxygens: O(1), O(4) and O(6) from one hexapeptided and the comparable O(7), O(10) and O(12) from the other hexapeptide. The six-fold coordination about the Mg^{++} is nearly perfectly octahedral. A comparison of the perfect MgO_6 octahedron in the MgO mineral periclase[3] and the MgO_6 octahedron in the present complex, drawn from experimentally determined coordinates, is shown in Figure 2. The

*Space group $P3_1$, a = b = 15.744(4) A, c = 24.002(6) A, $\gamma = 120°$, V = 5153 A^3, Z = 3.

Mg—O and O—O distances in the complex, 2.02-2.11 A and 2.73-3.02 A, are near the values of 2.10 A and 2.97 A, respectively, found in the MgO crystal. Such and octahedral arrangement in a metal ion-peptide complex is not necessarily expected. In the Na^{+}-antamanide complex, e.g., the Na^{+} has 5-fold coordination in a pyramidal arrangement.[4]

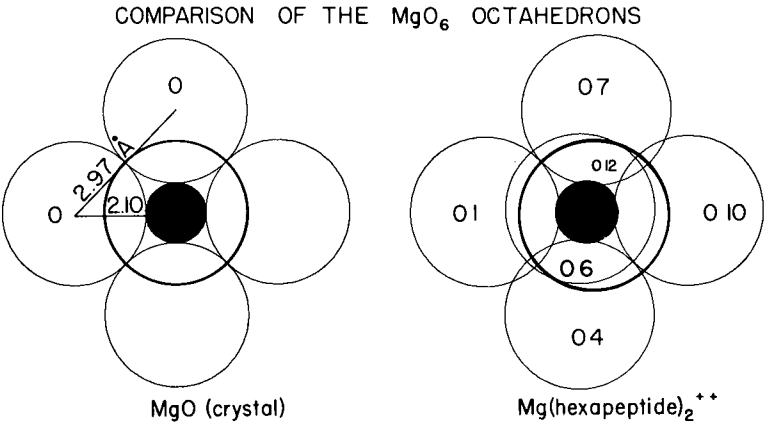

Fig. 2. Octahedral coordination

The octahedral arrangement of carbonyl oxygen atoms about the Mg^{++} imposes a strong condition on the conformation of the peptides, each of which contribute three oxygens to the octahedron. Figure 3 shows the conformation of one of the cyclic(Gly-L-Pro-L-Pro-Gly-L-Pro-L-Pro) molecules of the complex. Three oxygen atoms are directed toward the Mg^{++} ion and the remaining three are directed to the outside of the cyclic backbone. In addition to the lack of symmetry, and lack of any internal NH· · ·O bonds, there are two *cis* peptide bonds, each occurring between a pair of prolyl residues. Each peptide bond is approximately planar, within 6° of 180° for the *trans* bonds and within 15° of 0° for the *cis* bonds. The ϕ and ψ values are shown on a Ramachandran type plot in Figure 4. Gly^1 and Gly^4 occur in fully allowed regions for glycyl residues, Pro^2 and Pro^5 (*trans* peptide bonds) occur in a fully allowed region for L-residues, while for Pro^3 and Pro^6 (*cis* peptide bonds) Pro^6 occurs in a fully allowed region while Pro^3 occurs in a less favorable area of the plot for *cis* bonds.[5]

The conformation of the two peptide molecules in the complex is different than that observed for any other cyclic hexapeptide.[6] Moreover, the conformation changes drastically between the complexed and uncomplexed states. A figure of the uncomplexed (Gly-L-Pro-L-Pro-

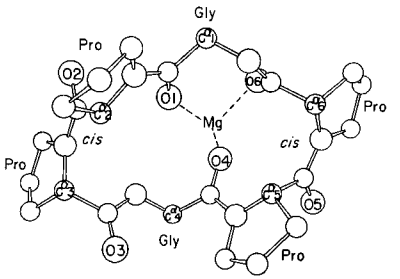

Fig. 3. One of the cyclic hexapeptide molecules in the complex.

Gly-L-Pro-L-Pro) (drawn from experimentally determined coordinates by x-ray diffraction that were provided by M. Czugler, K. Sasvári and M. Hollósi, Budapest) has been oriented to provide maximum comparability with the complexed peptide, see Figures 5 and 3. Major differences in conformation occur at both "ends" of the molecule. While the complexed molecule has a pair of *cis* peptide bonds between Pro^2-Pro^3 and Pro^5-Pro^6, the uncomplexed molecule also has two *cis* peptide bonds but they occur in tandem between Gly^4-Pro^5-Pro^6. At the other end, the uncomplexed form has a normal 4 → 1 type hydrogen bond that encompasses two Pro residues. In the uncomplexed form, the carbonyl oxygen atoms O(1) and O(6) are still directed toward the interior of the cyclic backbone but O(4) is turned to the exterior. The conformation of the uncomplexed form, as found in the crystalline state is not in readiness to accept a Mg^{++} ion. The conformational changes that must take place on complexation attest to the flexibility of cyclic peptide molecules.

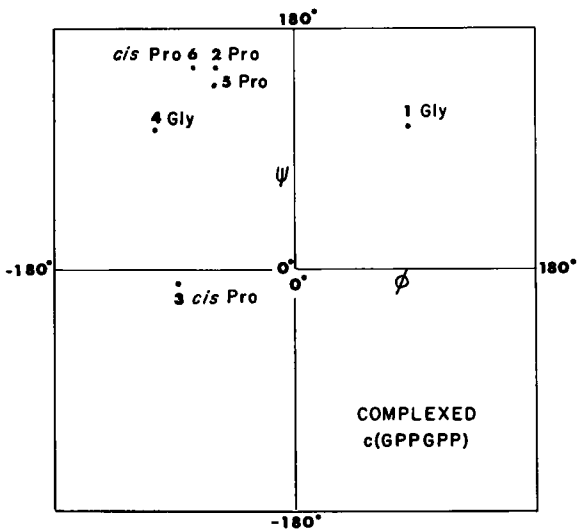

Fig. 4. Ramachandran plot of ϕ and ψ values of the molecule in Figure 3.

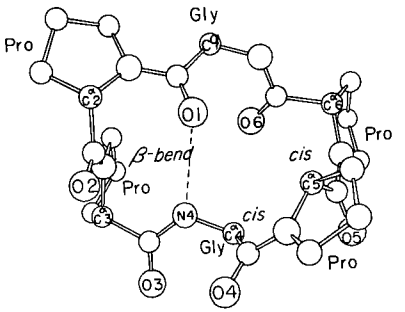

Fig. 5. Uncomplexed c(GlyProProGlyProPro). Coordinates provided by M. Czugler et al.

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RAMAN STUDIES ON BRADYKININ AND A CYCLIC BRADYKININ ANALOG

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The conformation of bradykinin, Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg (BK), has been probed by several spectroscopic methods, including circular dichroism (CD) and proton magnetic resonance (NMR).[1,2] These studies indicate that BK interconverts between an unordered structure and a partially ordered one which seems to contain an intramolecular 3 → 1 type hydrogen bond (γ-turn) bridging the Pro-7 residue.

In this study the structures of BK and a cyclic cysteine containing BK analog, Cys-Arg-Pro-Pro-Gly-Phe-Cys-Pro-Phe-Arg (cBK), were examined in aqueous solution and in the solid state by laser Raman spectroscopy.

The solid phase synthesis, purification, and biological activity of cBK[3] and BK[4] are described elsewhere. The Raman spectra of the peptides were made at room temperature with the 514.5 nm line of an Argon ion laser. The Raman system was interfaced with a microprocessor and the spectra shown are the result of a minimum of 10 computer averaged scans. The sulfate ion symmetrical stretching band at 981 cm^{-1} was used to calibrate the monochrometer. For the D_2O exchange studies the peptides were dissolved in D_2O, allowed to stand 2 hours at room temperature and then lyophilized.

The Raman spectrum of BK in the solid state, in H_2O, and of the D_2O exchanged peptide in D_2O are shown in Figure 1. The spectrum of cBK in H_2O is shown in Figure 2.

In Raman spectroscopy the amide-I and amide-III bands are generally used for the assignment of protein and peptide secondary structure[5,6]. Since there are often vibrational modes in the region in which the amide-III bands are observed, the peptide can be subjected to D_2O exchange which causes the amide-III bonds to shift to lower wavenumbers. A comparison of the native peptide spectrum with the D_2O exchanged spectrum allows for identification of those amide-III bands which are affected.

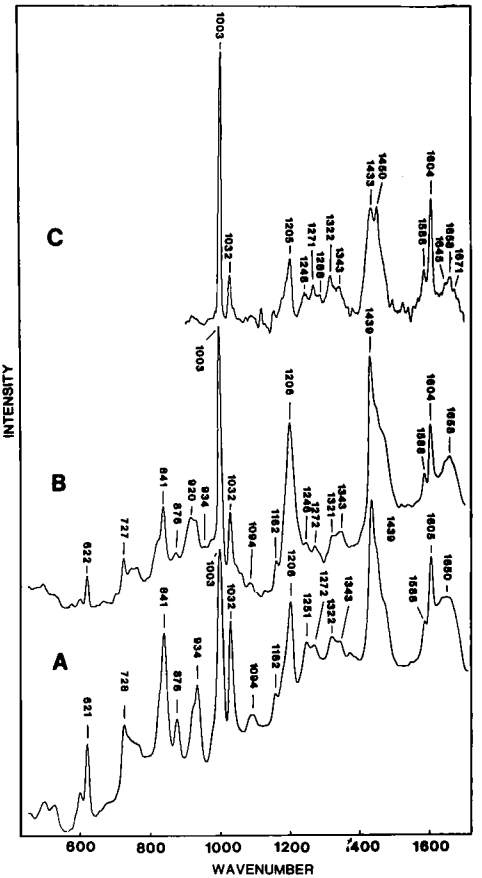

Fig. 1. Spectra of BK in H_2O(A), in D_2O(B), and as solid(C).

In aqueous solution BK shows a broad band in the amide-I region with maxima at 1640 cm^{-1} and 1650 cm^{-1}. The 1640 cm^{-1} band is assigned to the H_2O bending vibration, and the underlying 1650 cm^{-1} band is the major amide-I band which is visible. This H_2O bending band is shifted to around 1200 cm^{-1} by deuteration (Figure 1B). This shift of the water band allows for a more precise evaluation of the BK amide-I band which appears at 1658 cm^{-1} with a shoulder at 1649 cm^{-1}. In the amide-III region of BK in H_2O (Figure 1) three bands are observed at 1251 cm^{-1}, 1272 cm^{-1}, and 1322 cm^{-1}. Comparison of the spectra of BK in H_2O and D_2O shows that the 1251 cm^{-1} band is absent in the D_2O spectrum, and that the intensities of the 1272 cm^{-1} and 1322 cm^{-1} bands are reduced by deuterium exchange. These two bands are therefore assigned as amide-III bands.

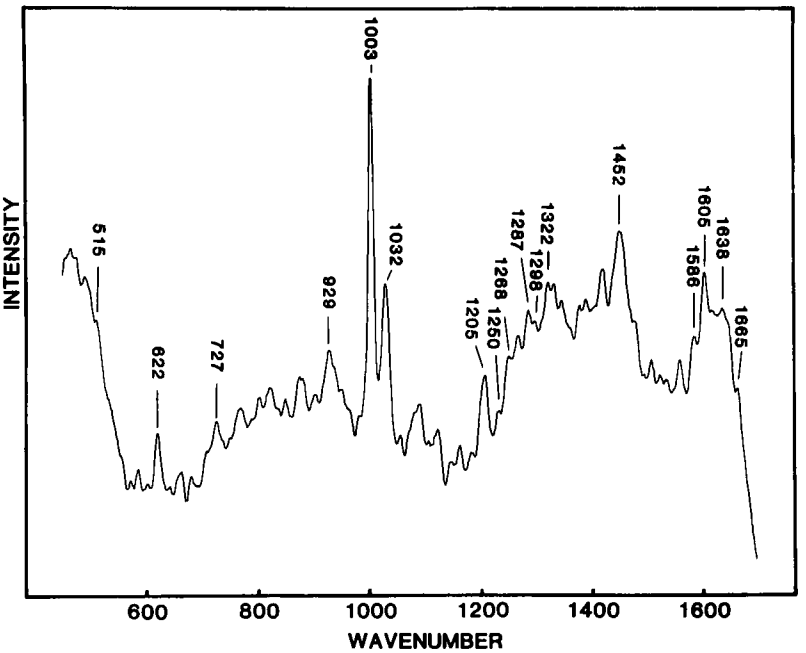

Fig. 2. Spectrum of cyclic-BK (cBK) in H_2O.

The spectrum of solid BK shows a major amide-I band at 1685 cm^{-1} with shoulders at 1645 cm^{-1}, 1671 cm^{-1}, and 1680 cm^{-1}. Several amide-III bands, all of which are affected by deuterium exchange, can be seen at 1248 cm^{-1}, 1271 cm^{-1} and 1322 cm^{-1}.

In the spectrum of cBK in water (Figure 2) the amide-I region is obscured by the broad H_2O bending band at 1638 cm^{-1}, but a shoulder attributed to an amide-I vibration can be seen at 1665 cm^{-1}. In the amide-III region the bands affected by D_2O exchange are located at 1250 cm^{-1}, 1268 cm^{-1}, 1287 cm^{-1}, 1298 cm^{-1} and 1322 cm^{-1}.

It appears from the Raman spectra of BK that the peptide contains more than one structural form. The amide-III bands at 1251 cm^{-1} for BK in water and at 1248 cm^{-1} for solid BK suggest the presence of unordered structure. The amide-III bands at higher wavenumber indicate that defined structure also exists in the peptide, probably in the form of a reverse turn[5,7]. Additionally, if one assumes that the amide-III band at 1322 cm^{-1} for BK in both the solid state and in water is the result of turn structure, then the intensity ratio of these bands demonstrates an enhanced turn structure for BK in the solid state.

The spectrum of cBK in water looks very similar to that of BK in water, and a conformational interpretation similar to that for BK applies.

In addition to information on peptide backbone conformation, the Raman spectrum of cBK also yields information on the conformation around the disulfide link. According to Van Wart and Scheraga[6] the S-S stretching band at 516 cm^{-1} results from several conformations about the C-S bonds with the C-S-S-C dihedral angles within 20° of ± 85°.

Previous CD and ^{13}C NMR studies[1,2] indicate that BK spends a maximum of 20% of its time in a partially ordered conformation which contains a γ-turn with the Pro at position 7 as the second residue. It has also been demonstrated that non-polar solvents favor the stabilization of ordered conformations in the BK molecule. From this Raman study it appears that the turn structure of BK is enhanced as the molecule goes from solution to the solid state. This is reasonable since dissolution of BK in water causes solvent competition for intramolecular hydrogen bonds and thereby increases the conformational flexibility of the molecule leading to an increase of unordered structure.

These results support earlier conclusions[1,2] that BK contains both ordered and unordered structure, and that the ordered structure is most likely turn structure. Unfortunately, the position of Raman active amide bands which are indicative of γ-turn structure could not be unequivocally identified in the spectra of BK or cBK.

Finally, it seems that the restrictions imposed on the cBK analog by the intramolecular disulfide bond must maintain a conformation which is acceptable to the BK receptors in various tissues since the cBK exhibits BK-like biological activity in the various BK assays.

Acknowledgements

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PEPTIDE BACKBONE BINDING THROUGH HYDROGEN BONDS TO PYRIMIDINE NUCLEOSIDES STUDIED BY NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY: AN APPROACH TO THE ORIGIN OF THE GENETIC CODE

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Introduction

The evolutionary origin of the genetic code is a scientific riddle: How did the present relations of particular codons to particular amino acids arise? It is our hypothesis that at the beginning of evolution peptides and primitive nucleic acids were directly bound to each other as product and template in a double-stranded structure.[1,2] In this structure every third base of the nucleic acid, always a pyrimidine, formed a pair of hydrogen bonds to the C=O and NH groups of an aminoacyl residue of the peptide (Figures 1 and 2). The specific recognition of an amino acid side chain by the nucleic acid would then involve two additional bases plus other groups.[1,3] A first approach to the problem of side chain recognition and a demonstration of hydrogen bonding between uracil and the peptide backbone (Figure 1) have been made.[3,4] We now report on hydrogen bonding to the peptide backbone by cytosine (Figure 2). Later reports will deal with the interaction of the cytosine C(2)=O with the C(β)OH of serine or threonine residues, which provides an evolutionary basis for their cytosine-centered codons.

Experimental

2′,3′-O-Isopropylidene-5′-O-t-butyldimethylsilylcytidine was synthesized by the general procedure of Ogilvie for t-butyldimethylsilyl-protected deoxynucleosides.[5] The $^{15}N(4')$ compound was prepared by the same procedure with 2′,3′-O-$^{15}N(4')$isopropylidene cytidine made by a method combining the procedures of Ikehara *et al.*[6] and Miles *et al.*[7]. [^{1}H]NMR spectra were recorded on a Varian HR-200 instrument, using 5-mm sample tubes. The constant *t*-Boc-Gly-Gly-Gly-OBz concentration in deuterated chloroform was 8.7 mM. ^{13}C and ^{15}N data were obtained on a Brucker 270 (NIH-modified) spectrometer equipped with a Nicholet

Computer 1180 with a 10-mm sample tube, using constant peptide or base concentrations of 21 mM in deuterated chloroform.

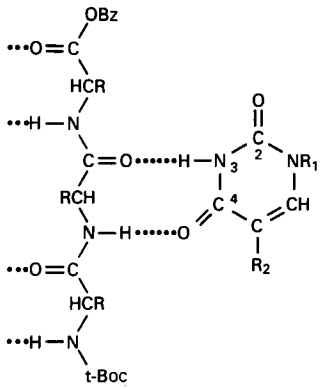

Fig. 1. Hydrogen bonds (dots) between the peptide backbone and uracil demonstrated in previous work.[4]

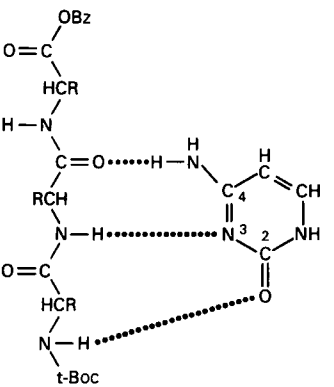

Fig. 2. Hydrogen bonds (dots) between the peptide backbone and cytosine indicated by the present experiments.

Results and Discussion

The $^{15}N(4')$cytosine derivative showed a single sharp line at 64.68 ppm (2.9 M $^{15}NH_4Cl$ in 1 N HCl) in its [^{15}N]NMR spectrum, and the undecoupled spectrum showed a triplet with a coupling constant of about 90 Hz. Thus this group exists preferentially in the amino form in chloroform. On addition of t-Boc-Gly-Gly-Gly-OBz, the [^{15}N]cytosine resonance shifted downfield, reaching a plateau at three peptide equivalents per unit of base (Figure 3), indicating participation of the $^{15}N(4')H_2$ group in peptide-base hydrogen binding. Assuming a simple 1:1 triglycine-cytosine complex, an apparent association constant was calculated to be 136.5 M^{-1}, considerably larger than the corresponding value found for guanine-cytosine under comparable conditions, 66.8 M^{-1}. A corresponding value for uracil-triglycine is 9.8 M^{-1}.

The downfield shift of the ^{13}C resonance of the C(2)=O of cytosine on addition of peptide (Figure 4) implicates this group also in hydrogen bonding to the peptide, in marked contrast to the C(2)=O group of uracil in a corresponding experiment, in which the ^{13}C resonance moved upfield due to a dissociation of uracil-uracil bonds.[4]

On addition of the cytosine derivative to the triglycine compound (Figure 5), the resonances of all three imide protons shifted downfield, implicating all of them in peptide-cytosine hydrogen bonding. The

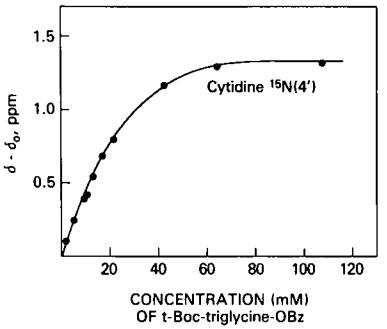

Fig. 3. Downfield shift of the ^{15}N resonance of a ^{15}N(4') cytosine derivative on addition of *t*-Boc-Gly-Gly-Gly-OBz.

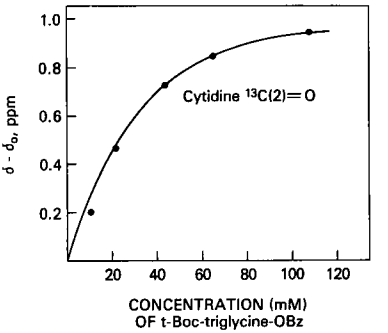

Fig. 4. Downfield shift of the ^{13}C(2)=O resonance of a cytosine derivative on addition of *t*-Boc-Gly-Gly-Gly-OBz.

apparent association constant based on the NH(II) shift is 52.7 M^{-1}. With the aid of ^{13}C-enriched carbonyl groups in three separate triglycine derivatives,[4] the three carbonyl group resonances also were found to shift downfield on addition of the cytosine derivative, as shown in Figure 6, implicating all of them too in hydrogen bonding. Because the glycine II imide is the most effective of the peptide donors, and the glycine II carbonyl is the most effective peptide acceptor, it is concluded that this pair of groups forms a pair of hydrogen bonds with the N(3) and N(4')H, respectively, of the cytosine ring, as illustrated in Figure 2. On the basis of molecular models we assume that the cytosine C(2)=O binds to the glycine I imide (Figure 2). Results from current experiments suggest that in the case of H_2N-terminal serine or threonine, the cytosine C(2)=O preferentially binds to the side chain hydroxyl group rather than to the imide.

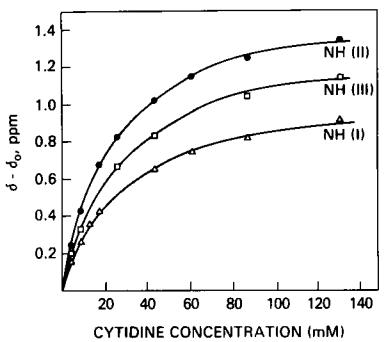

Fig. 5. Downfield shifts of the three NH proton resonances of *t*-Boc-[^{2}H]Gly-[^{2}H]Gly-[^{2}H]Gly-OBz on addition of a chloroform-soluble cytosine derivative.

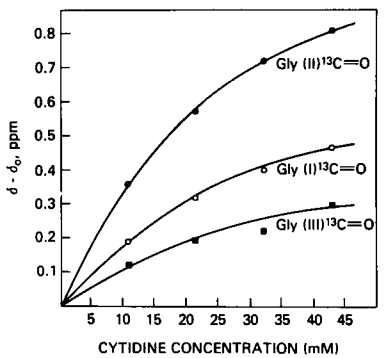

Fig. 6. Downfield shifts of the ^{13}C=O resonances of three different preparations of *t*-Boc-Gly-Gly-Gly-OBz, each enriched with ^{13}C in one carbonyl position, on addition of a chloroform-soluble cytosine derivative.

Although this work was undertaken for its evolutionary implications, there may be some hydrogen bonding interactions between the peptide backbone and nucleic acids in present organisms, as proposed by Gursky et al.[8]

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CALCIUM COMPLEXATION AND TRANSPORT BY SYNTHETIC CYCLIC OCTAPEPTIDES

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We have designed and synthesized a series of calcium-binding cyclic peptides, both to discover the structure prerequisites for calcium binding and transport in biological membranes by proteins and peptidic materials, as well as to develop new calcium ionophores as reagents for probing biochemical events linked to calcium fluxes.[1-4] These cyclic octapeptides, typified by the structure cyclo-(Glu(OBzl)-N(R_1)Gly-Gly-N(R_2)Gly)$_2$, have desirable properties with respect to hydrophobicity, array of potential liganding atoms (*e.g.*, peptide carbonyls), (cation) cavity dimensions, and the possibility for systematic variation of N-R substituents in the alkylglycine residues.[5] In the present work, we describe the transport properties in phospholipid membranes of one such peptide (I, R_1 = methyl; R_2 = cyclohexyl)[6] and report conformational details in organic solution of the calcium complex of an analogous peptide (II, R_1 = R_2 = methylcyclohexyl) using nuclear magnetic resonance (NMR) techniques.

Cation transport mediated by peptide I ("CYCLEX-2E") in sonicated phosphatidylcholine (PC) vesicles is illustrated in Figure 1. In these experiments, the vesicles are prepared in the presence of the desired radioisotope, unincorporated isotope is separated by gel filtration through Sephadex G-50 columns, the vesicles are resuspended, and treated with CYCLEX-2E. Figure 1A illustrates the ability of this peptide to remove calcium from the vesicles as shown by the residual level of radioactivity in the lipid fraction. $^{45}Ca^{2+}$ remains inside the vesicles in the absence of peptide (not shown). In Figure 1B, a further control experiment demonstrates that the vesicles retain ^{14}C-sucrose molecules during the course of the incubation, thus establishing that addition of peptide caused no nonspecific membrane damage.

In experiments aimed at determining which ions in the system act to balance the efflux of positively-charged Ca^{2+} cations, Na^+ or Ca^{2+}-loaded vesicles were incubated overnight in the presence of external $^{22}Na^+$. The vesicles were found to accumulate $^{22}Na^+$ as shown in Figures 1C and D. For 50.9 nmoles of Ca^{2+} efflux (calculated from the initial internal [Ca^{2+}]

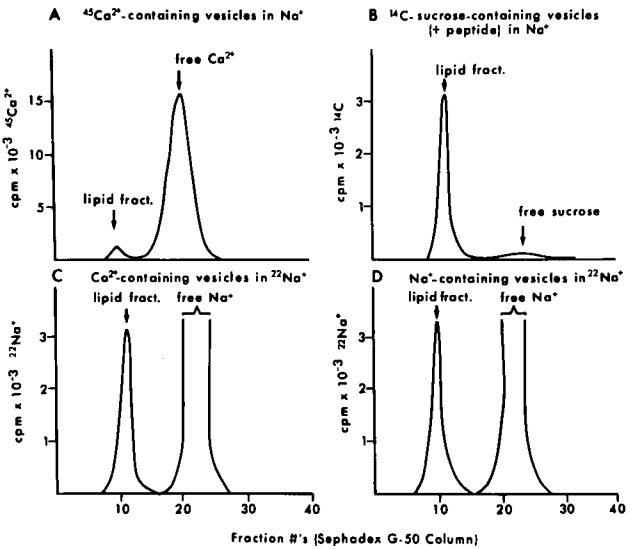

Fig. 1. Peptide-mediated $^{45}Ca^{2+}$ efflux and $^{22}Na^+$ influx into phosphatidylcholine (PC) vesicles. Vesicles (2.5 mg PC) in A, B, and C contain initially 50 mM $CaCl_2$ (and 5 mM ^{14}C-sucrose in B), 2 mM Hepes (pH 7.4); vesicles in D contain 50 mM NaCl instead of $CaCl_2$. In each experiment, vesicles are suspended in 1 ml 100 mM NaCl (+ 30 μC $^{22}NaCl$ in C and D) and treated with CYCLEX-2E (I) (150 μg/2.5 mg PC). After incubation (18-22 hr), the vesicles are passed down G-50 columns and the radioactive content of lipid fraction is determined.

and internal volume of the vesicles) 86.3 nmoles of $^{22}Na^+$ are influxed, a ratio of 1.7/1 $^{22}Na^+/Ca^{2+}$. In the $^{22}Na^+$/"cold" Na^+ case, (Figure 1D), 93.4 nmoles of $^{22}Na^+$ (calculated from the specific activity of $^{22}Na^+$) are influxed for 50.9 nmoles initial internal "cold" Na^+, a ratio of 1.8/1. In both instances, the internal and external sodium concentrations are essentially equilibrated. However, CYCLEX-2E was also found to influx $^{45}Ca^{2+}$ in the absence of any intravesicular metal ions (data not shown) suggesting either that transport of calcium and sodium is not directly coupled and/or alternative mechanisms (such as fluxes of OH^-, Cl^-, or H^+ ions) must exist for CYCLEX-2E-mediated transport of individual cations.[7]

In efforts to obtain further details concerning the peptide/calcium complex species responsible for the observed transmembrane calcium transport, we have studied the proton NMR spectra (360 MHz) of II in chloroform solution before and after addition of calcium (Figure 2). In the free form, the peptide exists in a group of conformational states, probably related by *cis* and *trans* peptide bond isomers around Glu-(N-R)Gly and Gly-N(R)Gly peptide bonds. The broadness of spectrum 2A also suggests the possibility of some "slow" interconversions about

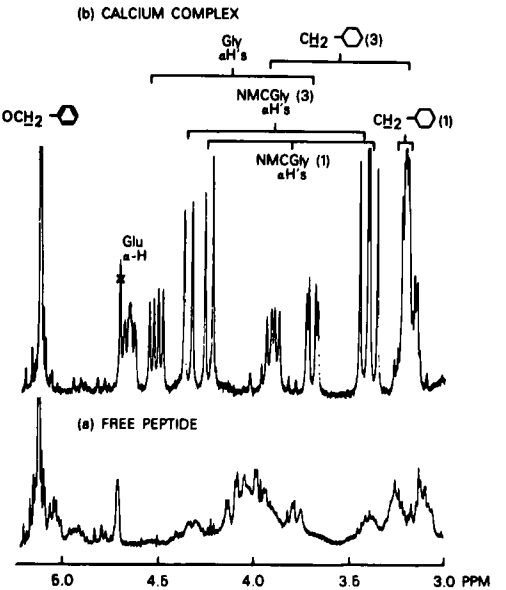

Fig. 2. Portions of the 360 MHz ^{1}H nmr spectra of the cyclic octapeptide cyclo-(Glu(OBzl)-(N-methylcyclohexyl)Gly-Gly-(N-methylcyclohexyl)Gly)$_2$ (II), 0.01 M in $CDCl_3$ solution. (a) Free peptide; (b) peptide/calcium complex formed by dissolution of one equivalent of solid calcium perchlorate. NMCGly (1) = N-methylcyclohexyl-Gly residue following Gly; NMCGly (3) = corresponding residue following Glu.

backbone single bonds (e.g. *cis'-trans'*) in the N(R)Gly-Glu and N(R)Gly-Gly regions of the molecule. Figure 2B is produced when the peptide has dissolved one equivalent of $Ca(ClO_4)_2$ into chloroform solution. The sharpness and resolution of spectrum 2B, indicative of a unique conformer, permitted assignment of most individual resonances and measurements of most geminal and vicinal coupling constants (the latter collected in Table I). Results of the overall NMR analysis on peptide II

Table I. Selected coupling constants measured from 360 MHz ^{1}H nmr spectra for the complex of cyclo (Glu(OBzl)-(N-methylcyclohexyl)Gly-Gly-(N-methylcyclohexyl)Gly)$_2$ (II) with calcium perchlorate in $CDCl_3$ solution.

Residue	Coupling	J, Hz
Gly	$^3J_{NHC^{\alpha}H}$	8.2, 4.2
Glu	$^3J_{NHC^{\alpha}H}$	7.8
Glu	$^3J_{C^{\alpha}HC^{\beta}{}_1H}$	12.1
Glu	$^3J_{C^{\alpha}HC^{\beta}{}_2H}$	1.9

and analogous peptides (e.g., $R_1 = CH_3$, R_2 = n-hexyl)[8] — using a Karplus-Bystrov[9] analysis in conjunction with molecular model-building studies — indicate that the calcium ion is coordinated in a central binding cavity to the (four) Glu-N(R)Gly and Gly-N(R)Gly peptide carbonyl oxygen

atoms in a coplanar arrangement; an octahedral-type coordination shell could be completed through further binding of Ca^{2+} to perchlorate or water molecules above or below the plane of the cyclic molecule. Sets of (ϕ,ψ,ω) backbone rotational angles[10] describing a proposed conformation are: (80°, 150°, 180°) for Glu and Gly residues, and (–60°, 150°, 180°) for (N-R)Gly residues.

A salient feature of the data is the occurrence of one large (12.1 Hz) and one small (1.9 Hz) coupling constant between the Glu-CαH and its side chain Cβ methylene protons. Analysis of the categories of rotational side chain conformers compatible with the experimental J-values[8] indicates that formation of the calcium complex has resulted in a restricted conformational state about Glu side chain Cα-Cβ bonds (χ_1 = –70°). Models suggest that upon formation of the complex, the Glu β-methylene protons and the protons of the adjacent N-alkyl substituent (i.e., the N-CH_2 methylene protons of the methylcyclohexyl substituent in the residue following Glu) become proximal. These two protons occur at 3.91 and 3.18 ppm, respectively, as compared with the corresponding protons on the N-methylcyclohexyl-Gly residue which follows Gly (3.23 and 3.19 ppm). The downfield shift of 0.7 ppm of one of these four methylene protons *vs.* the other three could occur if its environment is perturbed by steric proximity to atoms in the Glu side chain. It is reasonable to suppose the conformation deduced for this peptide/calcium complex in chloroform solution will have structural features in common with the corresponding complex in the interior of the phospholipid membrane.

Acknowledgements

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NMR ANALYSIS AND CONFORMATION OF CYCLOTRIPROLINES

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Cyclic tripeptides are characterized by two different backbone conformations separated by about 20 kcal/mol: a rigid crown and a flexible boat.[1-3] If the cyclotripeptide contains only achiral amino acids (cyclo[Sar_3],[4,5] cyclo[N-Bzl.Gly_3][2,3,5]) two degenerate crowns and six rapidly interconverting degenerate boat conformations are involved in equilibrium but stepwise introduction of proline reduces this manifold.[2,3] Finally, cyclo[L-Pro_3] (**1**) exists in one crown conformation[6] but cyclo[L-Pro_2-D-Pro] (**2**) in a twisted boat conformation.[7] X-ray structures of both peptides are known.[7-9] Here we present the NMR analysis of both peptides to answer the following questions:

— Is there a correspondence between conformation in solution and crystal?

—Does the rather rigid backbone allow flexibilities within proline rings?

For this reason full assignment of all proton and carbon signals in **1** and **2** was performed by modern NMR-techniques at 270 and 500 MHz (2D NMR: J-resolved, 2D-correlated; relaxation time measurements and double INDOR difference (DID) spectroscopy.[10]

Proton-NMR-Spectra

cyclo[L-Pro_3] — A computer simulation of the 270 MHz NMR spectrum with the previously reported NMR parameters obtained at 220 MHz[6] gave only partial agreement with experiment. The reanalysis[11] of the 270 and 500 MHz spectra in $CDCl_3$ and in $CDCl_3/C_6D_6$ mixture and reassignment[12] of the protons result in the parameters given in Table I. The bond angles obtained from the coupling constants correspond to an α^+-ring puckering[11] close to the mean value of the six different proline conformations found in the x-ray crystal structure. Another interesting result was the observation that the long range coupling constant $^4J_{cis}$ is always larger than $^4J_{trans}$ in the pyrrolidine ring.

cyclo[L-Pro_2-D-Pro] (2) — The ^{1}H-NMR spectrum of **2** is a strongly coupled 21 spin-system but in first approximation it is a superposition of three 7-spin-systems, which were separated via decoupling techniques, especially the very selective double INDOR difference spectroscopy

(DID).[10] [^{1}H]^{13}C-selective decoupling allows a full assignment of carbon connectivities.

Assignment of the three different proline rings in the sequence was performed as follows:

1. Synthesis of a derivative deuterated at Cα of D-Pro.[13]
2. Comparison of ^{13}C and ^{1}H chemical shifts with the spectra of cyclo[Pro$_2$Sar] and cyclo[Pro$_2$Bzl.Gly].[1,2,7] In these peptides the Sar and Bzl.Gly residue can only substitute for the D-Pro residue in the boat.
3. The unusual downfield shift of one Cα and one Cβ signal is caused by the unusual ψ angle at Pro^2 (model and x-ray structure).[1,7]
4. Decoupling of the α-proton signals removes the long range coupling across the peptide bond to the δ-protons of the proline ring following in the sequence.

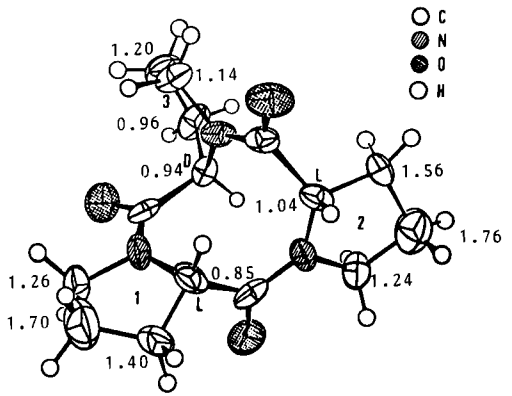

Fig. 1. X-ray structure of **2** and NT_1 values in $CDCl_3$ (see text)

Hα-decoupling is also necessary to aid the analysis of the proton spectra to remove long range couplings into the adjacent proline rings. By stepwise α-decoupling a "synthetic" spectrum was obtained, which contains three separated analyzable 7 spin systems. The results are given in Table I. Interpretation of vicinal coupling constants yield χ angles close to the x-ray structure indicating a similarity between the crystal structure and the most stable conformation in solution.

Carbon Spectra

The conformationally most important result of the C-13 NMR spectrum is the information about proline flexibility from relaxation data (NT_1; N = number of protons attached to the C, T_1 = spin-lattice relaxation time). Since the T_1-relaxation is pure dipolar (all NOE effects

Table 1. Vicinal coupling constants ^3J[Hz] and χ-angles

method			cyclo[L-Pro$_3$]		cyclo[L-Pro^1-L-Pro^2-D-Pro^3]					
					Pro^1		Pro^2		Pro^3	
		nuclei	J	χ	J	χ	J	χ	J	χ
	χ_1	$\alpha\beta^c$	7.3	28	7.2	-17	7.4	-27	6.5	-39
		$\alpha\beta^t$	0.7	30	7.7	-27	8.6	-24	0.7	-30
	χ_2	$\beta^c\gamma^c$	9.7	0	7.1	39	5.9	45	8.3	32
		$\beta^c\gamma^t$	10.0	-30	3.5	56	4.9	5	11.9	40
NMR		$\beta^t\gamma^c$	2.4	-15	10.8	33	8.3	19	1.1	19
		$\beta^t\gamma^t$	8.6	-18	7.1	39	7.1	38	7.3	37
	χ_3	$\gamma^c\delta^c$	9.2	14	7.9	-33	6.9	-39	9.1	-20
		$\gamma^c\delta^t$	4.7	1	10.0	-28	7.6	-16	3.3	- 2
		$\gamma^t\delta^c$	6.9	13	3.2	- 3	4.9	- 7	9.0	-23
		$\gamma^t\delta^t$	10.2	0	6.8	-36	8.0	-28	10.2	-13
	χ_1			31		-27		-28		-36
x-ray[15]	χ_2			-24		39		35		25
	χ_3			7		-35		-26		- 5

between 2.87 and 2.93), the NT_1-values vary according to the intramolecular mobility, assuming isotropic molecular tumbling. Comparison of the x-ray structure and the NT_1 values shows a clear correlation of the size of the thermal ellipsoids (mobility in crystal) with the relaxation data (mobility in solution) (Figure 1). It can be seen that the mobility increases in the order of $C\alpha < C\beta \approx C\delta < C\gamma$ as it was found earlier in **1**[14] and that the flexibility is small in the D-Pro-residue but high in Pro.[2]

Acknowledgements

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15. Mean values from different crystal conformations.[7-9]

CONFORMATIONS OF MODEL PEPTIDES IN MEMBRANE-MIMETIC ENVIRONMENTS

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The influence of a membrane-like environment on polypeptide conformation has been studied by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) and circular dichroism (CD) of peptides in normal and reversed micelles (see Figure 1). The types of interactions that may be operative in these systems are the same ones that are important in protein-lipid associations in membranes, namely: surface interactions due to head groups or counterions, interfacial absorption at the micelle-water boundary, partitioning into the hydrophobic region, or co-micellization due to both hydrophobic and hydrophilic interactions.[1,2]

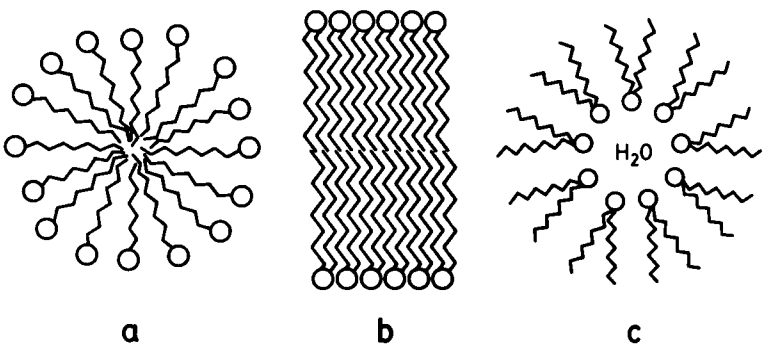

Fig. 1. Ordered assemblies of amphiphiles. Head groups indicated by circles, alkyl chains by zig-zag lines. a) normal micelle, b) bilayer (as in biological membrane), and c) reversed micelle.

Four peptides which are insoluble or only slightly soluble in water have been studied in aqueous sodium dodecylsulfate (SDS) micellar solutions: *t*-Boc-Gly-Pro-Gly-OBz, *t*-Boc-D-Phe-Pro-Gly-OBz, *t*-Boc-D-Phe-Pro-Ala-Pro-Gly-OBz, and cyclo(Ala-Pro-Gly-D-Phe-Pro). Enhanced solubilization, broadening of NMR signals (due to the slower overall tumbling time), and changes in conformational parameters for peptides in SDS solutions relative to water indicate association of the peptides with micelles. ^{1}H NMR spectra of *t*-Boc-D-Phe-Pro-Gly-OBz in several bulk solvents and in SDS solution are shown in Figure 2. Spectral parameters indicative of the adoption of a γ turn conformation (low field Pro Hα and Hβ resonances)[3,4] are observed in $CDCl_3$, while bulk solvents

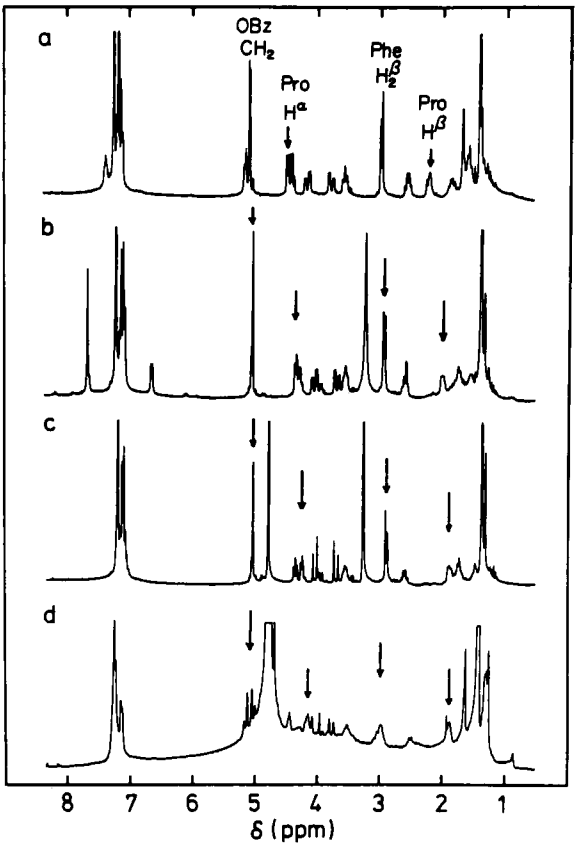

Fig. 2. 0.02 M *t*-Boc-D-Phe-Pro-Gly-OBz in a) $CDCl_3$, b) 25% DMSO-d_6: 75% $CDCl_3$, c) CD_3OD, and d) 0.025 M SDS-d_{25} in D_2O.

of increasing polarity appear to disrupt the turn. The NMR spectrum in SDS solution is similar to that in methanol in conformational parameters, except that resonances are somewhat broader. Also, the methylene resonances of the benzyl ester and the phenylalanine become more AB in character in the micellar solution, suggesting decreased aromatic ring mobility. Consistent with this apparent reduction in conformational freedom is the observation that CD spectra for the peptides in SDS micellar solutions displayed greater ellipticities and narrower bandwidths.

A solvent-dependent equilibrium ratio between all-trans and one-cis conformers of cyclo(Ala-Pro-Gly-D-Phe-Pro) reflected in ^{13}C spectral resonances of proline Cβ's and Cγ's was used as a probe of the polarity of the peptide's microenvironment (see Figure 3). This peptide in $CDCl_3$,

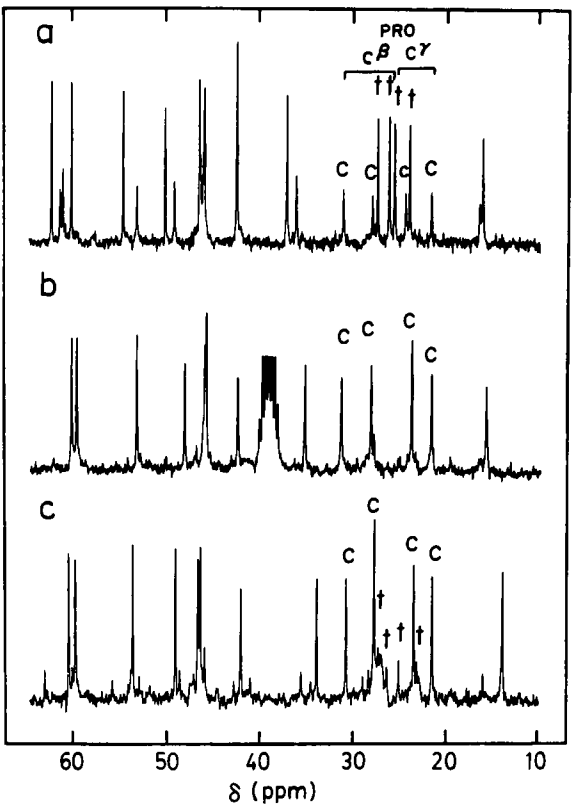

Fig. 3. 0.02 M cyclo(Ala-Pro-Gly-D-Phe-Pro) in a) $CDCl_3$, b) DMSO-d_6, and c) 0.025 M SDS-d_{25} in D_2O.

CD_3OD, DMSO-d_6, and SDS solutions exhibited all-trans to one-cis ratios of 70:30, 35:65, 0:100, and 20:80 respectively, suggesting that the effective polarity experienced by the peptide in SDS micelles is similar to that of bulk methanol.

Reversed micelles, formed from sodium diisooctyl sulfosuccinate (AOT) in heptane with small amounts of water present, solubilized the peptides Gly-Gly-Tyr and cyclo(Gly-Pro-Gly-D-Ala-Pro) which are otherwise insoluble in heptane. At low water concentrations, the CD spectra for these peptides were distinct from those in bulk water, but approached bulk water spectra as the water content increased (see Figure 4). CD spectral changes for cyclo(Gly-Pro-Gly-D-Ala-Pro) in AOT solutions resemble those reported for this peptide upon cation binding[4] suggesting that, of the possible factors affecting peptide conformation in

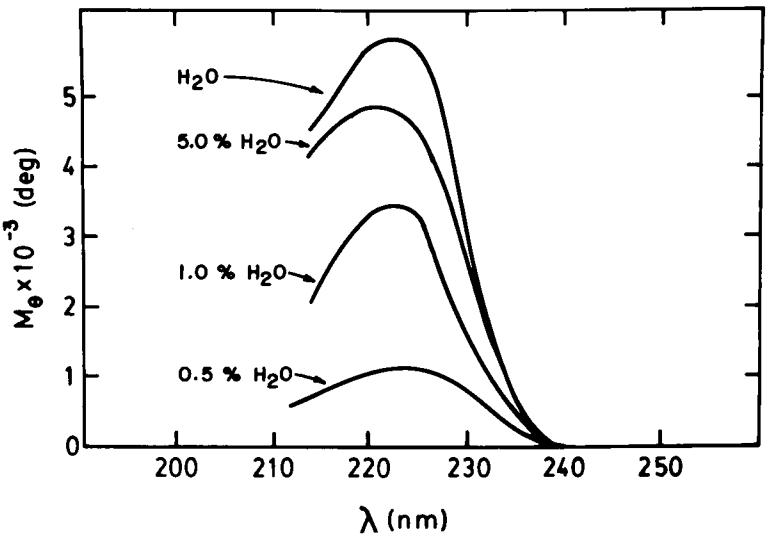

Fig. 4. Gly-Gly-Tyr (1 mM) in reversed micelles (3% AOT w/v in heptane) with varying amounts of H_2O v/v.

reversed micelles (see above), peptide-counterion interactions may be the principal ones.

These results direct attention to novel aspects of peptide-amphiphile interactions in ordered systems: namely, the association of small, hydrophobic peptides with the interfacial region and the unique conformational impact of the water adjacent to amphiphile head groups where high effective counterion concentrations exist.

Acknowledgements

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ESTIMATION OF PROLINE RING NONPLANARITY IN CYCLIC PENTAPEPTIDES FROM PROTON SPIN-SPIN COUPLING CONSTANTS

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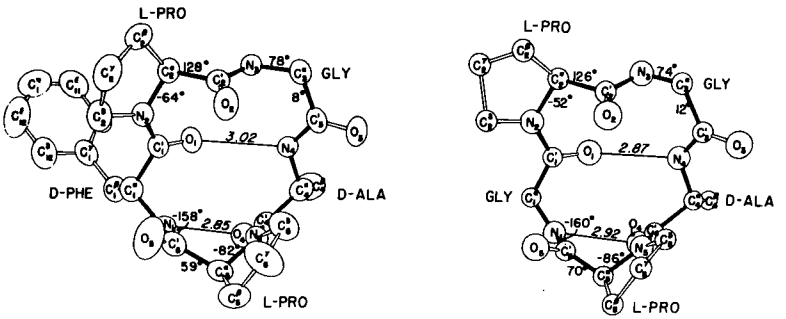
A complete spin-spin analysis of the proline rings in two model peptides, cyclo(D-Phe[1]-Pro[2]-Gly[3]-D-Ala[4]-Pro[5]),(I) and cyclo(Gly[1]-Pro[2]-Gly[3]-D-Ala[4]-Pro[5]),(II),[1] (see Figure 1) has been performed using data from 600 MHz ^{1}H nuclear magnetic resonance (NMR) spectra (see Figures 2 and 3). The results have been interpreted in terms of proline ring geometry and "puckering", which have been compared to structures of the crystalline peptides previously determined by X-ray diffraction.[2,3] We have found a correlation of ring nonplanarity with vicinal coupling constants that should be of general utility in assessing whether proline rings are rapidly interconverting between two forms or are present as a single conformer.

In a system of bonded methylenes such as exists in a proline ring, four vicinal coupling constants (two cis, ^3J_c, and two trans, ^3J_t) are related to the dihedral angle, (χ_i), between the methylenes. Population weighted Karplus relations[4] appropriate for these systems are[5]:

$$\langle {}^3J_c \rangle = K_0 + K_1\langle \cos \chi_1 \rangle + K_2\langle \cos^2\chi_1 \rangle \qquad \text{Eqn. 1}$$

$$\Delta\langle {}^3J_t \rangle = \sqrt{3}\, K_2/2\langle \sin 2\chi_1 \rangle - \sqrt{3}\, K_1\langle \sin \chi_1 \rangle \qquad \text{Eqn. 2}$$

with $K_0 = +1.0$, $K_1 = -2.33$, $K_2 = +11.3$ Hz; $\Delta^3J_t = J_t - J_{t'}$.[5] For small χ_i, $\langle\cos^2\chi_i\rangle = \langle\cos \chi_i\rangle^2$, and equation 1 can be solved directly, allowing computation of an average $\hat{\chi}_i$ from $\hat{\chi}_i = \cos^{-1}\langle\cos \chi_i\rangle$. $\hat{\chi}_i$ gives a measure of nonplanarity (or "puckering"), but no indication of direction of twist, because cos χ_i is an even function of χ_i. Another average dihedral angle, $\tilde{\chi}_i = \sin^{-1}\langle\sin \chi_i\rangle$, can be computed from equation 2, again assuming small χ_i such that $\langle\sin 2\chi_i\rangle = \langle\sin \chi_i\rangle$. Sin χ_i is an odd function of χ_i, and hence gives both magnitude and direction of twist.



I II

Fig. 1. X-ray structures of model pentapeptides analyzed.

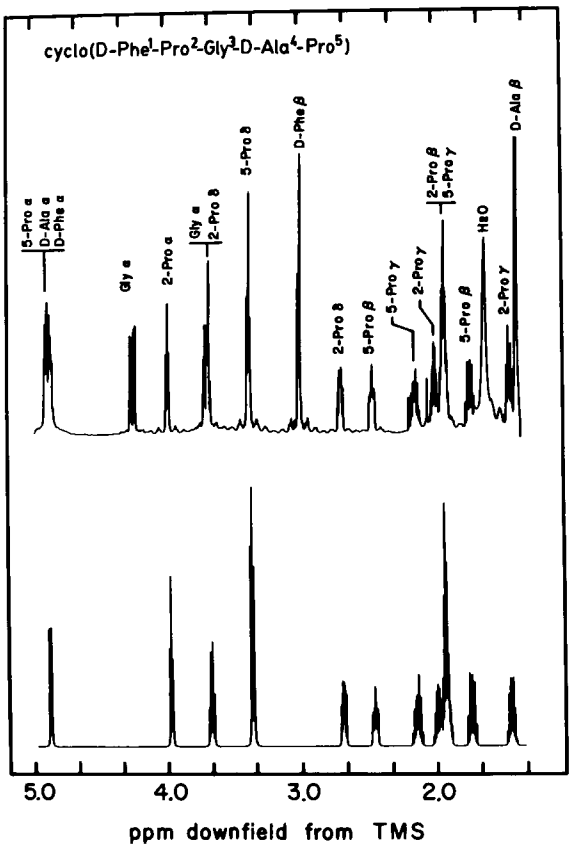


Fig. 2. 600 MHz ^{1}H NMR spectrum of compound I and simulation of spectrum of prolines.

Table I.

Compound	Proline ring	Methylene pair	$\hat{\chi}_1$	$\tilde{\chi}_1$	χ_1(x-ray)
I	2	(αβ)	35°	- 3°	+25°
		βγ	33°	+ 3°	-35°
		γδ	31°	- 3°	+31°
	5	(αβ)	25°	+22°	+28°
		βγ	25°	-13°	-26°
		γδ	11°	+ 8°	+14°
II	2	(αβ)	33°	- 2°	-27°
		βγ	37°	+ 1°	+39°
		γδ	31°	+ 4°	-36°
	5	(αβ)	23°	+23°	+28°
		βγ	24°	-14°	-24°
		γδ	26°	+ 8°	+ 9°

Results for the two pentapeptides (see Table I) imply that the Pro^2 rings of both compounds are markedly nonplanar ($\hat{\chi}_i$ clearly non-zero), but rapidly interconvert between Ramachandran A and B forms ($\tilde{\chi}_i \simeq 0$).[6] The Pro^5 rings in both compounds are less puckered (smaller $\hat{\chi}_i$), but the signs of $\tilde{\chi}_i$ indicate that the B form is preferred ($\hat{\chi}_i \simeq \tilde{\chi}_i$). This interpretation agrees well with X-ray derived χ_i's for the Pro^5 rings, but indicates that the Pro^2 rings are more constrained in the crystalline form than in solution.

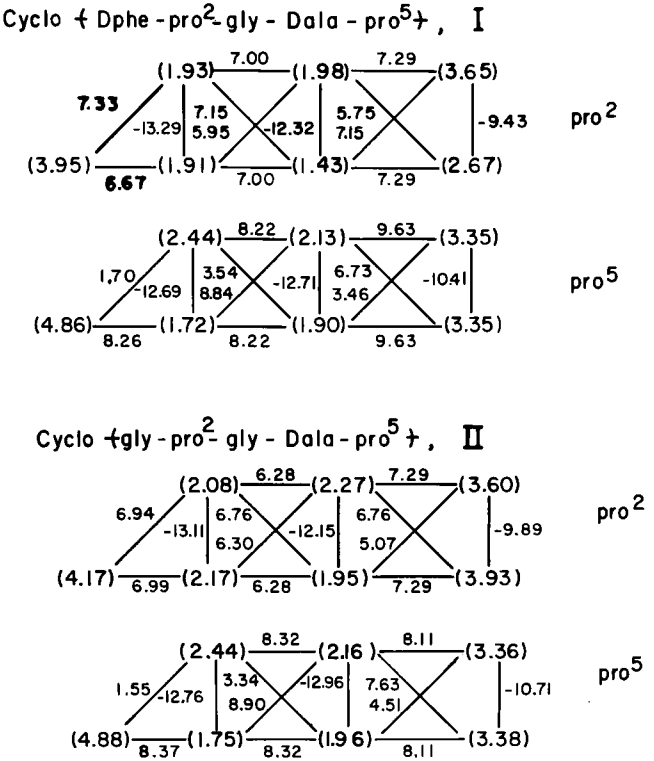


Fig. 3. Chemical Shifts (δ) and coupling constants, shown by lines, for compounds I and II. α proton on left; δ proton on right.

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THE CONFORMATION OF OXYTOCIN IN AQUEOUS SOLUTION: ORIENTATION OF THE SIDE CHAIN OF Asn^5 OF THE ANALOG [Ala^2]OXYTOCIN

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Introduction

The neurohypophyseal hormone oxytocin possesses only one aromatic residue—namely, a tyrosyl residue in position 2. To investigate the effect of the magnetic anisotropy of the aromatic ring of the side chain of this residue on the chemical shifts (δs) of neighboring protons, we synthesized the analog [Ala^2]oxytocin (2-AOT), which, unlike oxytocin (OT), does not possess an aromatic residue, and studied it in D_2O by ^1H NMR spectroscopy, comparing the values of the chemical shifts of selected protons with the corresponding protons of the parent hormone OT studied under similar conditions.

In OT the two H^βs of Asn^5 appear to be magnetically equivalent (i.e., to possess the same chemical shift), at least in ^1H NMR studies performed at 360 MHz or less, and as a consequence, values of the $^3J[H^\alpha\text{-}H^\beta]$s, the individual coupling constants between each of these H^βs and its vicinal H^α, cannot be obtained (only the sum of these $^3J[H^\alpha\text{-}H^\beta]$s can be found). On the other hand, in 2-AOT the two H^βs of Asn^5 are nonequivalent, and the values of the individual $^3J[H^\alpha\text{-}H^\beta]$s can be obtained. These values are of particular interest because they reflect the orientation of the side chain of Asn^5 about the $C^\alpha\text{-}C^\beta$ bond (the torsion angle χ^1).[1]

In this paper, we obtain values of individual $^3J[H^\alpha\text{-}H^\beta]$s for Asn^5 of 2-AOT, calculate the side-chain orientation of this residue, and discuss the long-range effect of the aromatic ring of the side chain of Tyr^2 on the chemical shifts of the H^βs of Asn^5 of OT.

Results and Discussion[2]

Values of δ — An aromatic ring is introduced in position 2 in going from 2-AOT to OT. The magnetic anisotropy of this ring can lead to shielding or deshielding of neighboring nuclei (i.e., to respective upfield or downfield shifts). The short-range effects of this ring on the chemical

shifts of protons in residue 2 (the site of substitution) and residues 1 and 3 (adjacent to this site) will be discussed in a later, expanded publication. In this paper we address ourselves only to the long-range effect of this ring on the chemical shifts of the H^{β}s of Asn^5, three positions from the site of substitution. These H^{β}s become nonequivalent by 0.057 ppm in going from OT, where they are equivalent, to 2-AOT (see below).

Values of $^3J[H^{\alpha}\text{-}H^{\beta}]$ — Unequivocal values of $^3J[H^{\alpha}\text{-}H\beta]$ for Asn^5 of isotopically unenriched OT cannot be obtained because the H^{β}s of this residue are magnetically equivalent, although the sum of these values can be determined (see below). On the other hand, unequivocal values of $^3J[H^{\alpha}\text{-}H^{\beta}]$s for Asn^5 of the analog 2-AOT can be obtained because the $H\beta$s of this residue are nonequivalent (see above). Part A of Figure 1 shows the resonances of these H^{β}s. Spectral analysis of these resonances and those of the H^{α} of Asn^5 (not shown) yields the following values: $\delta(H^{\alpha})$, 4.77 ppm; $\delta(H^{\beta D})$, 2.864 ppm; $\delta(H^{\beta U})$, 2.807 ppm; $^2J[H^{\beta D}\text{-}H^{\beta U}]$, −15.7 Hz; $^3J[H^{\alpha}\text{-}H^{\beta D}]$, 6.1 Hz; $^3J[H^{\alpha}\text{-}H^{\beta U}]$, 8.1 Hz. Part B of Figure 1 shows the spectrum simulated on the basis of these values of δ and J. The sum of $^3J[H^{\alpha}\text{-}H^{\beta D}]$ and $^3J[H^{\alpha}\text{-}H^{\beta U}]$ is 14.2 Hz. For Asn^5 of OT *per se*, values that range from 12.9 to 15.4 Hz have been reported for this sum.[3-7]

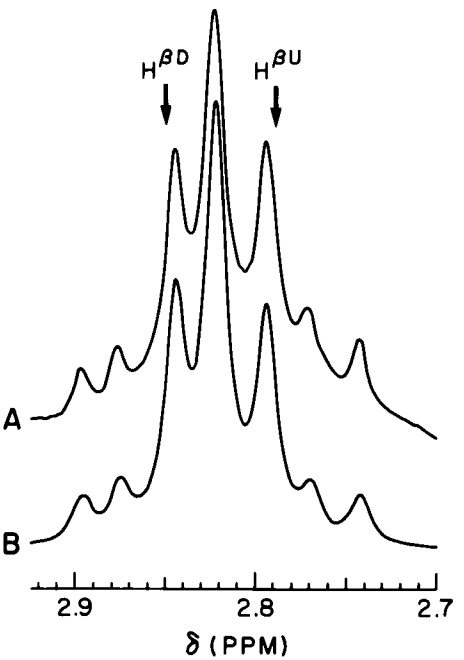

Fig. 1. The resonances of the H^{β}s of Asn^5 of [Ala^2]oxytocin (2-AOT) in D_2O at pD 3.2 and 22°C obtained at 300 MHz. Arrows indicate δs of the downfield (D) and upfield (U) H^{β}s relative to (2,2,3,3-$^2H_4$)-3-(trimethylsilyl)propionate (TSP), which was used as an internal reference. Part A is the actual spectrum, and Part B, the simulated spectrum. In oxytocin (OT) studied under similar conditions, the resonances appear as a doublet, at least at 360 MHz or less, because of magnetic equivalence of these two H^{β}s.

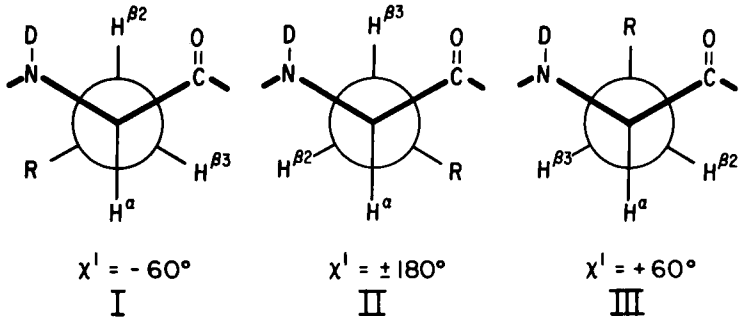

Fig. 2. The staggered conformations for amino acids of an L configuration. The stereochemical definitions of $H^{\beta 2}$ and $H^{\beta 3}$ are given in this figure.

Side-Chain Orientation — If it is assumed that conformational isomerism about the C^{α}-C^{β} bond of Asn^5 is manifest and that this isomerism is among the three staggered conformations shown in Figure 2, then the relative populations (*p*s) of these conformations can be calculated from the values of individual $^3J[H^{\alpha}H^{\beta}]$s, which are designated $^3J[H^{\alpha}$-$H^{\beta 2}]$ and $^3J[H^{\alpha}$-$H^{\beta 3}]$, by the method of Pachler.[8]

If $H^{\beta U}$ and $H^{\beta D}$ of Asn^5 of 2-AOT are arbitrarily assigned to $H^{\beta 2}$ and $H^{\beta 3}$, respectively, then we calculate the following values of *p* at pD 3.2 and 22°C from the values of $^3J[H^{\alpha}$-$H^{\beta}]$ shown above: p_I, 0.57; p_{II}, 0.32; p_{III}, 0.11. If the stereochemical assignments are incorrect, the values of p_I and p_{II} must be interchanged. In any case, the side chain spends an appreciable fraction of time in both states I and II if the assumption of isomerism among the three staggered conformations is indeed correct. On the other hand, the side chain spends only a small fraction of time (~0.1) in state III—i.e., this state is practically excluded

Long-Range Effect of the Aromatic Side Chain of Tyr^2 of Oxytocin — We tentatively assume that the conformational state of the side-chain of Asn^5 is approximately the same in OT and 2-AOT[9] and that the equivalence of the two H^{β}s of Asn^5 of OT is a result of a differential effect of the aromatic ring of Tyr^2 on these H^{β}s—i.e., that the magnetic anisotropy of the ring fortuitously creates an environment for the H^{β}s in which they are magnetically equivalent. This observation is quite striking because it indicates that the aromatic ring has a long-range effect on another residue located three positions away. Among our goals for the future are to determine (1) which side-chain conformations bring the aromatic ring of Tyr^2 and the H^{β}s of Asn^5 into fleeting proximity (e.g., Tyr^2 in state II and Asn^5 in state I) and (2) if there is any correlated motion

between the side-chain conformations (e.g., is there a higher probability of finding Tyr^2 in state II when Asn^5 is in state I than when Asn^5 is in one of the other states or is this probability independent of the state of Asn^5).

Acknowledgements

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9. This assumption can be tested by obtaining values of individual $^3J[H^{\alpha}\text{-}H^{\beta}]$s for Asn^5 of OT and comparing them with those for Asn^5 of 2-AOT. Such values can be obtained in a ^1H NMR study of an isotopic isomer of OT incorporating asparagine labeled seterospecifically with a deuteron in one of the H^{β} positions (e.g., see Kainosho, M. and Ajisaka, K. (1975) *J. Am. Chem. Soc.* **97,** 5630-5631).

FREQUENCY DEPENDENT PROTON MAGNETIC RESONANCE STUDIES OF THE PEPTIDE IONOPHORE, LINEAR GRAMICIDIN

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Several helical conformations have been proposed[1,2,3] for this family of peptide ionophores, the linear gramicidins. The sequences of these pentadecapeptides which differ by one or two amino acid residues, respectively are:

‡	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
A	CHO.	Val.	Gly.	Ala.	Leu.	Ala.	Val.	Val.	Val.	Trp.	Leu.	Trp.	Leu.	Trp.	Leu.	Trp.	EA*
	CHO.	Ile										Trp					EA*
	CHO.	Val										Phe					EA*
B	CHO.	Ile										Phe					EA*
	CHO.	Val										Tyr					EA*
C	CHO.	Ile										Tyr					EA*

Components of this mixture were separated by HPLC on a C_{18} reverse phase column using a methanol/water system. NMR studies which were carried out mainly on Val-gramicidin A and Val-gramicidin B are consistent with a highly symmetric conformation at the ϕ, ψ and χ^1 levels. A spectrum of gramicidn B at 470 MHz is shown in Figure 1.

Temperature Studies — Temperature coefficients of the amide protons, which have also been measured by other workers independently,[3] ranged from -4 to -6.8 ppm $x10^{-3}/°C$ with most of them lying between -4 and -5 ppm $x\ 10^{-3}/°C$. These uniform and borderling temperature coefficients are consistent with hydrogen bonded amides in a rather flexible structure like a helix.

Deuterium Exchange Experiments — Although accurate exchange rates were not measured, it was observed that the exchange process, when D_2O was added to the DMSO solution, was very slow (on the order of days). However, all the amide protons did eventually exchange out. Again, this is consistent with the hydrogen bonded, but rather flexible structure of a helix.

Scalar Coupling Constant Studies — With the exception of glycine, $^3J_{NH\text{-}H\alpha}$ scalar coupling constants do not allow for distinguishing

between the possible helical forms. The rather large coupling constants do exclude the α helix.

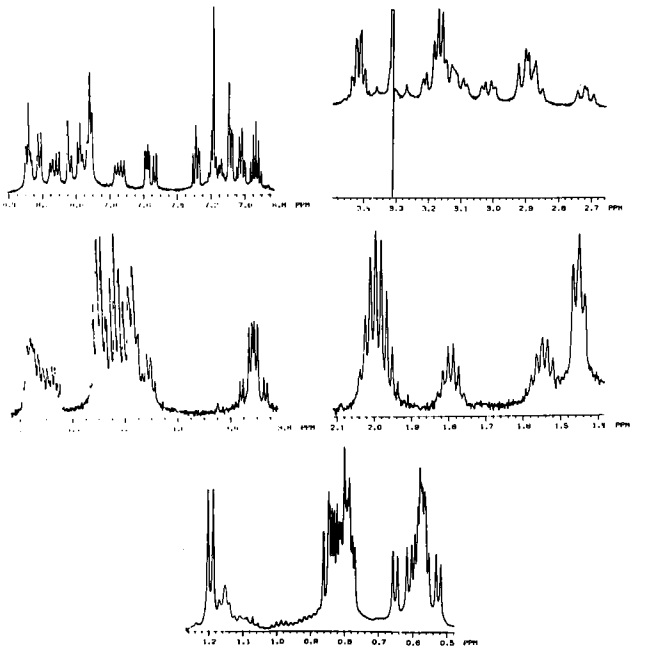

Fig. 1. 470 MHz proton spectrum of Val-gramicidin B in DMSO-d_6.

Proton Relaxation Studies — Non-selective relaxation experiments were performed at 200 MHz, 270 MHz, and 470 MHz on both a non-exchanged and an exchanged sample. An interesting feature of the experiment performed on the non-exchanged sample--Figure 2--is that regardless of their position along the chain, what amino acid residue they belong to or whether they belong to an L or D residue, the amide protons relax at practically the same rate. In the alpha region, the protons relax at almost the same rates. However, the aromatic residue alpha protons are slowest and collectively relax at the same rate and the leucine alpha protons relax slightly faster than the other aliphatic alpha protons. Thus contributions of the different side chains to the relaxation pathways of the alpha protons are seen here. This experiment is consistent with symmetry at the level of interaction of alpha protons with amide protons.

The relaxation experiment done on the exchanged sample is shown in Figure 3. Again, alpha protons relax at similar rates with the differences reflecting differences in the side chains.

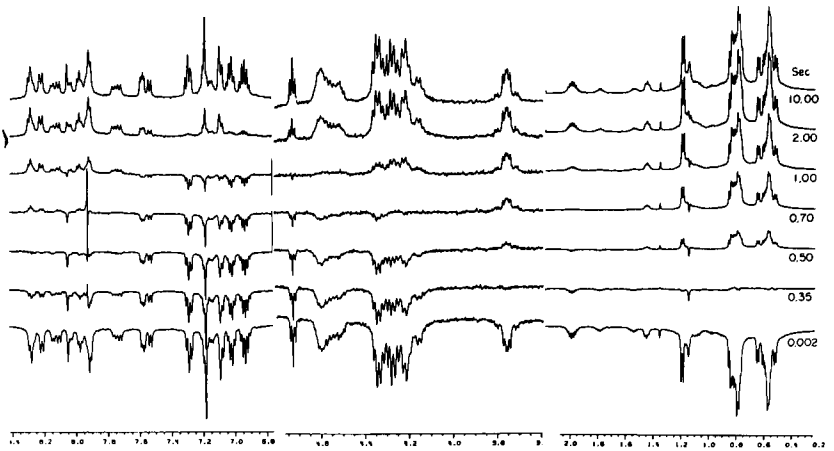

Fig. 2. Non-selective relaxation experiment of the non-exchanged sample of Val-gramicidin B performed at 470 MHz.

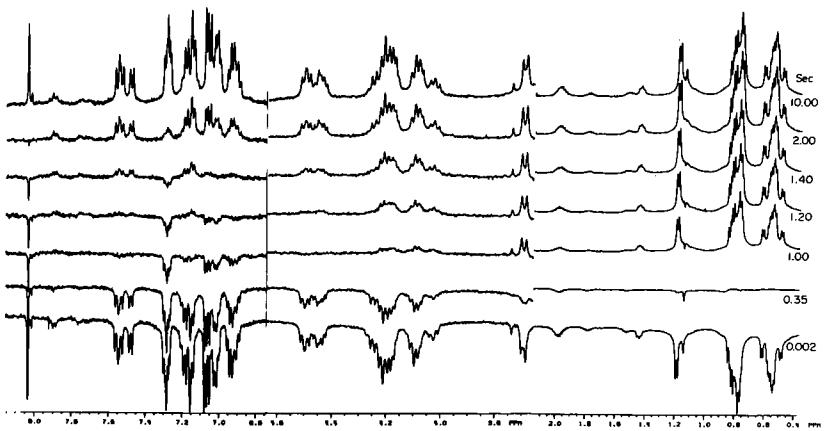

Fig. 3. Non-selective relaxation experiment of exchanged sample of Val-gramicidin B performed at 470 MHz.

From the results of the above experiments, some conclusions can be drawn on the structure of linear gramicidin in DMSO, even though its conformation cannot yet be determined unequivocally.

Conclusions

1) The hydrogen bonding studies carried out eliminate the random coil in DMSO. 2) The rather high scalar $^3J_{NH-H_\alpha}$ coupling constants rule out the α helix, but do not give ϕ values that are sufficiently precise to distinguish among the other helices. 3) Contributions of amides to the

relaxation of alpha protons is the same regardless of the position and chirality of the residue along the chain.

Acknowledgements

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PROTON NMR STUDIES OF ALAMETHICIN

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Alamethicin (ALA) is a hydrophobic peptide-antibiotic produced by the fungus *Trichoderma viride.*[1] It is of interest because of its ability to alter the permeability and electrical properties of lipid membranes by forming voltage-dependent, cation-conducting channels.[2-4] Also of interest is the description of its covalent structure, which by contrast to that of its electrical properties has gone through several stages of revision and refinement.[5-9] This situation is due in part to the fact that natural ALA is a mixture whose two major components (70 and 20%) are 20-residue peptides differing by a single amino acid.[5,10] Current evidence based on solid-phase synthesis[8,10] and independently, mass spectrometry,[9] indicate that the linear peptide (I) represents the structure of the major component: ALA-I.

Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu-Gln-Phol (I)
5 10 15 20

This compound has been purified to a high degree of homogeniety by reversed-phase HPLC[10] and examined by high field (600MHz) proton NMR spectroscopy. As described below, analysis of the spectra confirms the correctness of (I) as the structure of ALA-I. In addition, spectra of the minor component, ALA-II shows that the resonance assigned to Ala[6] in I is missing and that resonance lines assigned to several C-terminal residues are selectively broadened.

Results

Natural ALA (kindly supplied to us by Dr. G.B. Whittfield of the Upjohn Co.) was purified by reversed-phase HPLC and ^{1}H-NMR spectra of the two major fractions (*ca* 2 mg/ml of methanol-d_4) were taken at 600.6MHz on the MPC-600 system (Carnegie-Mellon University, Facility for Biomedical Research). Portions of the ALA-I spectra are shown in Figure 1. Resonances which were clearly resolved were assigned chemical shifts directly. The shifts of overlapping C_β and C_γ protons were determined indirectly by noting the positions of residual images formed in double-resonance difference spectra (Figure 1B). These experiments also established connectivities among coupled spins. Assignments to specific

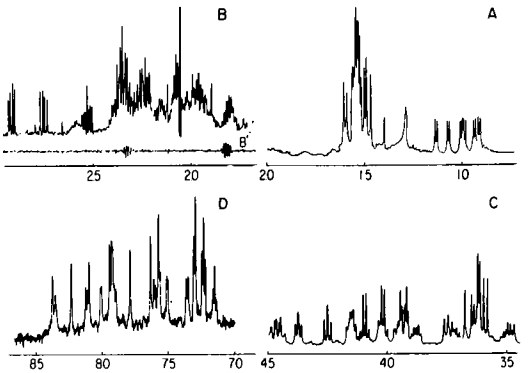

Fig. 1. 600 MHz ^{1}H-NMR spectra of ALA-I. (A) Side chain CH_3's of Aib, Ala, Leu and Val residues, (B) Side chain C β, γ, δ protons, (B') Difference spectra, Pro^{14} C_αH irradiated, (C) C_α and Pro C_δ protons (amide N's deuterated), (D) Amide NH and Phol ring (*ca.* 30 min after dissolving in methanol -d_4). All scales are in ppm from internal TMS.

proton groups were then made by considering chemical shift values, spin-spin splitting patterns and connectivities between multiplets. Further refinement of the assignments to specific amino acids (Table I) was effected by comparing these spectra with those of the synthetic 1-17 residue fragment (ALA-17) and ALA-II, the minor (ca. 20%) component of natural ALA (See Figure 2). For example, the spectra of the C_α protons of synthetic 17-ALA are essentially identical to ALA-I except that the C-terminal Glu-Gln-Phol lines are missing and the C_α^{Val-15} multiplet is shifted *ca.* 0.5 ppm downfield (Figure 2C) All the other C_δ lines can be identified by their splitting patterns after amide ^{1}H-^{2}H exchange or, in the case of Gln^7 and Leu^{12}, by location of their Cβ lines via double resonance.

Discussion and Conclusions

Resonance lines in the ^{1}H-NMR spectra of ALA-I have been assigned to nearly every proton group in the molecule. This analysis is clearly consistent with structure I. Moreover the correspondences between the spectra of ALA-I and the synthetic 17-ALA fragment confirm the correctness of the structure of the first 17 residues. As to the arrangement of the last three residues, the NMR evidence is more circumstantial. The C_αH lines of these residues do not shift upon acidification of the solution to 10^{-3} M in HCl thus suggesting that the conventional α-linkage is correct. Secondly the Gln^{19} and Phol^{20} NH's exchange with solvent ^{2}H at rates that are typical for solvent exposed NH's, but the Glu^{18} NH exchanges out about 5 times more slowly. This is consistent

Table I
Chemical Shifts of Proton Groups in Alamethicin [a,b]

	N	C_α	C_β	C_γ	C_δ
Pro^2	-	4.248	1.80 2.35	2.08 1.99	3.955 3.490
Ala^4	7.560	4.095	1.496	-	-
Ala^6	7.912	4.018	1.538	-	-
Glu^7	8.002	3.926	2.27 2.13	2.540 2.35	-
Val^9	7.500	3.588	2.24	1.136 1.069	-
Gly^{11}	8.375	3.932 3.661	-	-	-
Leu^{12}	8.110	4.457	1.96	1.91	0.937 0.914
Pro^{14}	-	4.373	1.82 2.33	2.08 1.83	3.881 3.725
Val^{15}	7.593	3.747	2.38	1.069 0.981	-
Glu^{18}	7.91 [c]	4.148	2.00 2.08	-	-
Gln^{19}	7.899[c]	4.026	2.25	-	-
$Phol^{20}$	7.351[c]	4.150	2.932 2.746	3.616	-

[a] in ppm from TMS [b] accuracy is ± 0.003 (4 sig. fig., directly observed) or ± 0.01 (3 sig. fig., via double resonance) [c] tentative assignment

with the sequence: -Gly-Gln-Phol. Further NMR evidence that (I) is the structure of ALA-I is provided by the comparison of the ALA-I spectra with the spectra of the synthetic version[8,10] of ALA-I — they are essentially identical in every respect.

The spectra of ALA-II (Figure 2B) documents the substitution of Ala^6 by another residue. Since no new $C_\alpha H$ line appears in the spectra, the substitution is most likely by an Aib and accordingly is consistent with amino acid analysis. It is also interesting to note that this addition of an extra CH_3- group produces marked broadening but no shifts in the lines of sequentially-remote residues (Pro^{14} and $Phol^{20}$). The source of broadening is presently unknown.

These NMR studies provide information about the secondary structure of ALA-I as well. First, the NH resonances of all but the N-terminal

Aib[1] and the C-terminal Glu-Gln-Phol sequence persist in methanol-d_4 for tens of hours or longer depending upon the pH. Such slow solvent-isotopes exchange rates are characteristic of solvent-shielded NH's. Secondly the ϕ-torsional angles (as estimated from the $^3J_{NC}$ coupling constants) of Ala[6], Gln[7] and Val[9] are $65 \pm 10°$. The torsional angle for an ideal α-helix is -60°.

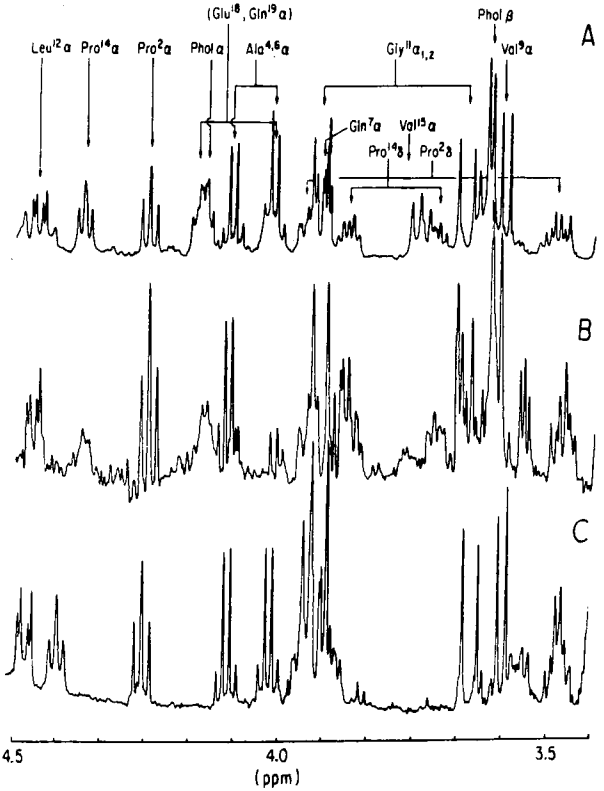

Fig. 2. Comparison of the $C_\alpha H$ region of the (A) ALA-I (B) ALA-II and (C) 17-ALA. ALA-II contains *ca.* 20% ALA-I. Note the reduced Ala[6] peak and the broadened Pro[14], Glu[18], Gln[19] and Phol[20] C_α lines in (B).

Acknowledgements

NMR specta were taken at the Carnegie-Mellon University Facility for Biomedical Research (NIH Grant RR-00292). This work was supported by NIH Grants GM24047 (BFG) and GM-242676 (DGD).

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HYDROGEN EXCHANGE KINETICS AND PARAMAGNETIC METAL BINDING OF NEUROHYPOPHYSEAL HORMONE DERIVATIVES

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Introduction

Our laboratories have been employing the neurohypophyseal hormones as models for developing improved NMR methods for delineating the solution conformations of peptides, particularly in aqueous solution. Our recent efforts have focused on the use of stereospecific paramagnetic shift and relaxation probes and on the measurement of amide hydrogen exchange rates, which serve as indices of solvent exposure. As models we have employed two oxytocin derivatives which bind lanthanide metals — [Glu^4]oxytocin (Cys^1-Tyr^2-Ile^3-Glu^4-Asn^5-Cys^6-Pro^7-Leu^8-Gly^9-NH_2) and tocinoic acid (Cys^1-Tyr^2-Ile^3-Gln^4-Asn^5-Cys^6). As a basis for extending N*H* exchange studies to primary amide components of peptides and proteins, we have measured the exchange rates of the carboxamide hydrogens of N-α-acetylglutamine methylamide (Gln′), N-α-acetylasparagine methylamide (Asn′), and acetylglycine amide (Gly′-NH_2).

Materials and Methods

Tocinoic acid and [Glu^4]oxytocin were synthesized by the methods of Hruby *et al.*[1] and Photaki *et al.*,[2] respectively. Preparation of Gln′ and Asn′ is described elsewhere.[3] Acetylglycinamide was purchased from Vega Biochemicals (Tucson, AZ).

Fourier transform ^{1}H NMR spectra were measured on a Bruker WH-400 spectrometer interfaced to an Aspect 2000 computer. Spectra in H_2O were measured by the "2-1-4-1-2 long pulse technique."[4] Amide hydrogen exchange rates were measured by the solvent saturation transfer/saturation recovery method.[5] Cross saturation between *cis* and *trans* hydrogens was taken into account in experiments performed on

primary amides.[6] Lanthanide titrations were performed in H_2O solution employing tetramethylammonium chloride as a chemical shift reference.[7]

Results and Discussion

Neurohypophyseal Hormones and Their Derivatives — Titration of [Glu^4]oxytocin and tocinoic acid with diamagnetic La(III) produces only minor changes in N*H*-C$^{\alpha}$*H* vicinal coupling constants ($J_{N\alpha}$) indicating that complexation of lanthanides is accompanied by minimal if any perturbation of the peptide backbone.[7,8] In the case of [Glu^4]oxytocin the metal is bound to the Glu^4 carboxyl group and to the carbonyl oxygen of the Asn^5 side chain.[7] In tocinoic acid binding occurs to the Cys^6 carboxyl and to the Asn^5 carboxamide oxygen.[8] Analysis of Gd(III) induced relaxation enhancements has yielded estimates of *average* distances from the metal ion of various protons of [Glu^4]oxytocin (Table I).[7] Similar experiments will soon be undertaken for tocinoic acid. The shift reagent Yb(III) has been bound to both derivatives and dissociation constants have been obtained (6.3×10^{-3} M at pH 5.8, and 3.0×10^{-3} M at pH 5.6 for [Glu^4]oxytocin and tocinoic acid, respectively).[7,8]

Table I. Average Gd(III)-Nuclear Distances of [Glu^4]Oxytocin in H_2O

Resonance	r_{AV}(Å)	Resonance	r_{AV}(Å)
m-Tyr^2 CH	7.0 ± 0.5	Asn^5 trans NH	6.3 ± 0.4
o-Tyr^2 CH	5.5 ± 0.5	Cys^6 NH	6.2 ± 0.5
Tyr^2 NH	>8	Leu^8 NH	>8
Ile^3 NH	-	Gly^9 NH	>8
Glu^4 NH	<4.5	Gly^9 cis NH	>8
Asn^5 NH	4.8 ± 0.5	Gly^9 trans NH	>8
Asn^5 cis NH	4.7 ± 0.5		

Comparison of the spectroscopic parameters of peptide N*H* resonances: chemical shifts, temperature coefficients of chemical shifts and $J_{N\alpha}$ of tocinoic acid, oxytocin, [Glu^4]oxytocin and 8-lysine vasopressin (LVP) in H_2O solution indicates a remarkable similarity between these spectral parameters of all four compounds, suggesting that their average conformations are probably quite similar.[8] All four compounds exhibit a small coupling constant for Gln^4 (or Glu^4) ($J_{N\alpha}$ = 4-5 Hz). All the other values of $J_{N\alpha}$ are 6-7 Hz. For all four compounds the rate of peptide hydrogen exchange of Cys^6 is significantly lower than the exchange rate of a fully solvated Cys peptide N*H* (Table II). There is a

Table II. Comparison of the Ratios of Calculated to Observed Peptide N*H* Exchange Rates for Oxytocin and Its Analogs

Residue	Oxytocin (pH 6.5)	LVP (pH 6.1)	[Glu^4]oxytocin (pH 6.3)	Tocinoic Acid (pH 6.2)
Tyr^2	a	0.7	a	a
Ile^3 (Phe^3)	2.4	0.4	1.2	1.0
Gln^4 (Glu^4)	2.4	b	1.3	0.7
Asn^5	8.4	1.9	5.6	2.0
Cys^6	15	7.7	7.4	3.7
Leu^8 (Lys^8)	3.4	1.1	1.3	-
Gly^9	2.8	2.1	b	-

[a]Too rapid to measure. [b]Resonance overlap.

smaller diminution in the exchange rates of the Asn^5 peptide N*H*'s of oxytocin and [Glu^4]oxytocin but not of tocinoic acid or LVP. All the other exchange rates agree with model compound data on the corresponding solvated peptides.[9] These exchange data suggest that for all these neurohypophyseal hormones a rapid equilibrium exists between folded and unfolded conformations and that a significant contribution is made by (a) conformer(s) having a hydrogen bond involving the Cys^{6}N*H*.[5,10] An additional but weaker (i.e. less probable) hydrogen bond involving the Asn^5 peptide NH may occur in oxytocin and [Glu^4]oxytocin.

A possible conformation consistent with the N*H* exchange data and the small $J_{N\alpha}$ of Gln^4 contains a β-turn in the tocin ring involving the sequence Ile^3-Gln^4-Asn^5-Cys^6, and has a hydrogen bond between the Ile^3 C*O* and the Cys^6 N*H*. Studies are now in progress to further define the conformation of neurohypophyseal hormones in aqueous solution.

Primary Amide Exchange Rates — The pH dependence of the hydrogen exchange rates of the primary amide hydrogens of Gln′, Asn′ and Gly′-NH_2 has been analyzed yielding second order rate constants associated with acid (k_H) and base catalysis (k_{OH}) (Table III).

As has been observed for other primary amides,[6,11] base catalyzed exchange of *trans* amide hydrogens is consistently more rapid than that of the corresponding *cis* hydrogen. This trend is attributed to repulsion between the nitrogen and oxygen lone pair electrons in the amide anion intermediate. For Gln′ and Gly′-NH_2 the acid catalyzed exchange rate of the *cis* amide hydrogen equals that of the corresponding *trans* hydrogen,

Table III. Rate Constants (M^{-1} s^{-1}) of Primary Amides

Compound	k_H		$k_{OH} \times 10^{-5}$	
	cis	trans	cis	trans
Gly'-NH_2	333 ± 50	327 ± 61	787 ± 1.6	1348 ± 2.6
Asn'	762 ± 57	1097 ± 146	220 ± 0.2	939 ± 1.0
Gln'	1095 ± 320	1134 ± 303	136 ± 0.3	552 ± 0.9

whereas for Asn' acid catalyzed of the *trans* hydrogen is slightly more rapid than that of the *cis* hydrogen. As has been noted by Perrin[11] and by Redfield and Waelder,[6] these rate data in conjunction with the observation of 25%-50% *cis-trans* cross saturation argue in favor of a mechanism of acid catalyzed exchange in which the amide nitrogen rather than the amide oxygen is protonated. In the case of Gln' and Gly'-NH_2 internal rotation about the sp^2-sp^3 C-N bond of the protonated amide intermediate is rapid compared to deprotonation, whereas in Asn' internal rotation and deprotonation may occur at comparable rates.

The exchange rates of these model compounds will be compared with the exchange rates of the primary amide groups of the neurohypophyseal hormones.

Acknowledgements

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DYNAMIC PROPERTIES OF AROMATIC RINGS OF OXYTOCIN AND ARGININE VASOPRESSIN ON HORMONE BINDING TO NEUROPHYSINS DETERMINED BY ^{13}C AND ^{19}F NMR

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The problem of mobility in biological macromolecules has received much study recently. It is often found that a variety of motions exist, in addition to the overall tumbling of the macromolecule. In globular proteins, exposed side chains, such as lysine, often display mobility which is at least one hundred fold greater than the rest of the protein.[1] Internal, bulky aromatic side chains have been found to undergo 180° rotations at rates varying from undetectably slow to greater than 10^9 sec^{-1}.[2,3]

We have been studying the binding of the neurohypophysial hormones, oxytocin and arginine vasopression to the neurophysins (NP) (reference 4, and other references therein). We have synthesized several hormone derivatives specifically labeled with 90% ^{13}C in a number of positions. By using ^{13}C NMR spectroscopy, we have been able to delineate many features of the protein-hormone interaction. Various enriched derivatives have been prepared with ^{13}C in the 3',5' positions of aromatic residues in positions 2 or 3. Using these compounds, it has been possible to determine rotation rates of these side chains when the hormones are bound to NP, and thereby gain a greater insight into the role of these residues in the hormone-NP interaction.

In work reported previously,[5] we have shown that the 3' and 5' carbons of tyrosine in position 2 of oxytocin become nonequivalent on binding to NP and give rise to distinct resonances. This allows the rate of rotation of the aromatic ring to be measured, and this rate is about $10^2 sec^{-1}$ at room temperature. The same rate is observed for the tyrosine ring of position 2 in AVP. We have now observed hormones enriched in the phenylalanine ring located in position 2 or 3 and the results are very different from those with tyrosine in position 2.

With (2-phenylalanine)oxytocin, which binds to NP with about 50% of the affinity of the native hormone,[6] the 3',5' carbons remain equivalent on binding. There is no chemical shift difference between free and bound

forms, and the linewidth of the bound hormone peak is about 50hz. This is about the same width as peaks due to α-carbons in residues 1-4 of the bound hormone. These carbons tumble at the same rate as the hormone-NP complex. The rotation rate of the phenylalanine ring in position-2, while faster than the rotation of the corresponding tyrosine ring, is thus slower than the overall tumbling rate of the complex, indicating a rate of 10^4-$10^8 sec^{-1}$.

We have also studied the binding to NP of AVP in which the phenylalanine ring in position 3 was enriched with ^{13}C. As was true for the (2-phenylalanine)oxytocin, the bound AVP gives rise to one unshifted resonance. In this case, however, the width of the peak was less than the width of the bound α-carbons of residues 1-4. This means that the rotation of the phenylalanine ring in position-3 of bound AVP is faster than the overall tumbling of the hormone NP complex, indicating a ring rotation rate of greater than $10^9 sec^{-1}$.

The interaction of (2-metafluorotyrosine)oxytocin with NP was monitored by ^{19}F NMR. The binding constant to NP was found to be about the same as that of oxytocin. The ^{19}F resonance of bound hormone was unshifted from the position of the free resonance, but its linewidth increased from 3Hz to 30Hz on binding.

The rotation rates are summarized in Figure 1.

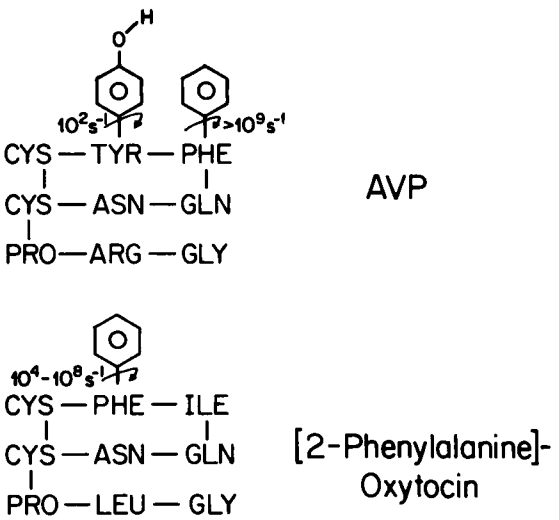

Fig. 1. Rotation rates of aromatic rings of hormones bound to neurophysin.

Discussion

The different rates of rotation of the aromatic rings in position 2 and 3 of the bound hormones are indicative of different interactions between these rings and NP. The tyrosine ring of position 2 rotates rather slowly due to a strong interaction with the protein. Replacement of the tyrosine with phenylalanine results in a somewhat reduced binding constant for the hormone, and a much more rapid rate of ring rotation, indicating that the tyrosine hydroxyl is important in mediating ring-NP interaction. The rotation rate of the phenylalanine ring in position 2 is, however, still affected by the NP interaction. The phenylalanine in position 3 of AVP must interact very weakly with NP, as the ring rotation is little affected by protein binding. The metafluorotyrosine ring in position 2 is constrained by interaction with NP, but the environment of the ^{19}F atom does not appear to differ significantly from that present in the free hormone. Studies are underway to explore this point further.

Acknowledgements

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CONFORMATIONAL PARAMETERS OF SOMATOSTATIN ANALOGS RESULTING FROM FLUORESCENCE STUDIES

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Introduction

Studies by CD[1] and NMR[2,3] spectroscopy have led to various proposals regarding the conformation(s) of somatostatin in aqueous solution and insight into the receptor-bound conformation was obtained by synthesis and pharmacological evaluation of bicyclic somatostatin analogs[4]. Structure-activity studies revealed that des[Ala^1,Gly^2]-desamino[Cys^3]-descarboxy[Cys^{14}]somatostatin (I)[5] and its dicarba-analog show activity comparable to that of native somatostatin. Furthermore, it was shown that substitution of tyrosine for phenylalanine in position 6 or 11 of the somatostatin sequence does not appreciably affect biological activity[6]. The tyrosine analogs permit the measurement of the average intramolecular distance between the phenol ring of Tyr^6 or Tyr^{11} and the indole moiety of Trp^8 by evaluation of singlet-singlet resonance energy transfer[7,8]. The Tyr-Trp distances in conjunction with the fluorescence quantum yield of Trp^8 obtained in dilute aqueous solution represent interesting conformational parameters for comparison with proposed conformations of somatostatin in solution and when bound to its receptor(s). Therefore, the syntheses of analogs of I with tyrosine substituted in position 6 and with Trp^8 in either L-configuration (II) or D-configuration (III), as well as its dicarba-analog with tyrosine substituted in position 11 and with Trp^8 in D-configuration (IV) were undertaken (Figure 1).

Somatostatin analogs were synthesized in solution by the fragment condensation method essentially by the same synthetic scheme as that described for the synthesis of native somatostatin[9]. Tyrosine and tryptophan fluorescence quantum yields were measured as described[10]. For the computation of the Förster critical distance, R_o, the tyrosine quantum yields in absence of transfer, ϕ_D^o, were determined with the fragments H·Lys-Asn-Tyr-Phe·OMe and H·Lys-Thr-Tyr-Thr-Ser·OMe. Transfer efficiencies, E, were obtained from the relative enhancement of acceptor fluorescence[10,11] and intramolecular Tyr-Trp distances, r, were calculated according to Equation 1: $r = (E^{-1}-1)^{1/6}R_o$ **Eqn. 1.**

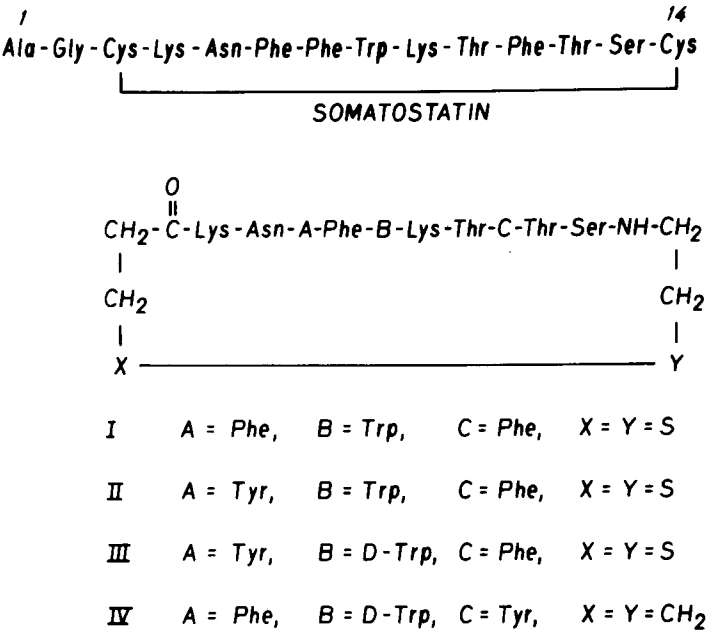

Fig. 1. Shortened somatostatin analogs

Results and Discussion

Similar average intramolecular distances of approximately 12 Å between Tyr^6 and Trp^8 were observed for analogs II and III (Table I). This result indicates that inversion of the configuration of Trp^8 does not greatly affect the Tyr^6-Trp^8 separation. The biologically active conformation proposed by Veber et al.[4,12] is characterized by a β-turn at positions 7-8-9-10 and a hydrophobic contact between the aromatic rings of Phe^6 and Phe^{11}. Furthermore, the side-chains of Trp^8 and Lys^9 are both equatorial relative to the ring structure of somatostatin in the latter model. Inspection of a CPK-model of analog II in this proposed receptor-bound conformation reveals a Tyr^6-Trp^8 distance of 12 Å in good agreement with the experimentally observed value. Shorter Tyr^6-Trp^8 separations ranging from 9 to 12 Å are obtained with this model if the orientation of the tryptophan side-chain is altered. The solution conformation of somatostatin proposed by Holladay et al.[1] features a β-turn involving residues 8-9-10-11. Since in the latter model the conformations of the side-chains in positions 6 or 8 are not defined, intramolecular distances between the phenol ring in position 6 and the indole moiety in position 8 in the relatively large ranges from 6 to 12 Å

(analog II) and from 6 to 13 Å (analog III) are observed depending on side-chain orientation. The upper limits of these ranges are compatible with our experimental findings.

Table I. Förster Parameters and Intramolecular Tyr-Trp Distances of Somatostatin Analogs[a]

Analog	ϕ_D^o	E	R_o[Å]	r[Å][b]
II	0.041	0.43 ± 0.05	11.5	12.1 ± 0.4
III	0.041	0.40 ± 0.01	11.5	12.3 ± 0.1
IV	0.037	0.52 ± 0.06	11.3	11.2 ± 0.5

[a]Peptide concentration = 3 x 10^{-5}M, solvent = 1 mM Tris-buffer (pH 7.0).
[b]Experimental error reflects uncertainty in E.

The average intramolecular D-Trp[8]-Tyr[11] distance of 11.2 Å determined with analog IV is slightly lower than the distance (13 Å) observed in the conformation proposed for somatostatin when bound to its receptor[4,12] with the side-chains of D-Trp[8] and Lys[9] in equatorial position. For other orientations of the tryptophan side-chain values ranging from 10-13 Å are obtained from inspection of this model. Depending on side-chain orientations D-Trp[8]-Tyr[11] separations in the range from 9 to 12 Å are determined with the CPK-model of somatostatin in the proposed[1] solution conformation.

Tryptophan fluorescence quantum yields, ϕ^{Trp}, of 0.033, 0.023, 0.071 and 0.078 were obtained for analogs I, II, III and IV, respectively. This result indicates that ϕ^{Trp} of analogs with Trp[8] in D-configuration is consistently 2 to 3 times higher than that of analogs with Trp[8] in L-configuration. The increase in quantum yield is accompanied by a blue-shift of 1.5 nm. These findings demonstrate that the indole ring in the D-Trp[8]-analogs is in a more hydrophobic environment than in the corresponding L-Trp[8]-analogs. The hydrophobic environment most likely results from interaction with the side-chain of Lys[9] which is more favorable in the D-Trp[8]-analogs. This observation is in agreement with the increase in the upfield shift of the γ-methylene protons of Lys[9] observed in the NMR spectra of D-Trp[8]-analogs[12]. The upfield shift has been interpreted in terms of a close proximity between the side-chains of Trp[8] and Lys[9] which is considered an important feature of the receptor-bound conformation of somatostatin[12]. The present study thus provides for the first time a correlation between a change in a NMR parameter (chemical shift) and a fluorescence parameter (quantum yield) monitored in a biologically active peptide.

Acknowledgements

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CONFORMATIONAL STUDY OF THE LANTHANIDE ION COMPLEXES OF THYMOPOIETIN$_{32-36}$: A PROTON NMR INVESTIGATION

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Introduction

Our laboratories have been studying the conformation-activity relationships of the pentapeptide, Arg-Lys-Asp-Val-Tyr (TP5), the active fragment[1] corresponding to positions 32-36 of the thymic hormone thymopoietin.[2,3] Like its parent hormone, TP5 also induces selective differentiation of T-lymphocytes. Its clinical potential in the treatment of various immunodeficiency related diseases is currently being evaluated in various laboratories. We have previously reported some main features of the free solution conformation of TP5 in aqueous solutions.[4] This pentapeptide binds metal ions naturally and in the present NMR investigation we report the use of lanthanide ions as conformational probes of Ln^{3+}-TP5 complexes. We have also examined the Asp^3 methyl ester and the C-terminal methyl ester analogs of TP5.

NMR spectra of nuclei in paramagnetic complexes exhibit a shift and/or line broadening of resonances compared to the corresponding diamagnetic complex. The dipolar contributions[5,6] to the observed shifts (δ_D) and to the relaxation enhancements ($1/T_{2M}$) are given by

$$\delta_D = K_1 [(3\cos^2\theta - 1)/r^3] + K_2 (\sin^2\theta\cos 2\phi)/r^3 \quad (1)$$

$$1/T_{2M} = D_2 f_2(\omega, \tau_c)/r^6 \quad (2)$$

In the above equations, r, θ, and ϕ are the polar coordinates of a given nucleus in the principal magnetic axis system. K_1, K_2, and D_2 are constants, and $f_2(\omega, \tau_c)$ is a function of the electron and nuclear Larmor frequencies and a resultant correlation time. The relaxation enhancements are much easier to interpret than the shifts since the latter also contain angular terms.

Results

The formation of the metal ion complex of TP5 is accompanied by a change in the orientation of the Asp^3 sidechain. This is reflected as shown in Figure 1, as a change in the Asp-CβH_2 multiplet structure upon addition of excess La^{3+}. Table I gives a rotamer population analysis[7] of the CαH-CβH_2 coupling constants for Asp^3. The Tyr^5 sidechain did not experience any such perturbation. The backbone NH-CαH coupling constants of TP5 also remained invariant upon complexation.[8] Some of the proton resonances exhibited complexation shifts due to La^{3+}. For free TP5 we have previously proposed the existence of a hydrogen bond between Arg guanidino NϵH and the Asp^3 carboxylate group.[4,8] Upon the addition of excess La^{3+}, the NϵH resonance shifts upfield, indicating a disruption or weakening of this hydrogen bond in the complex.

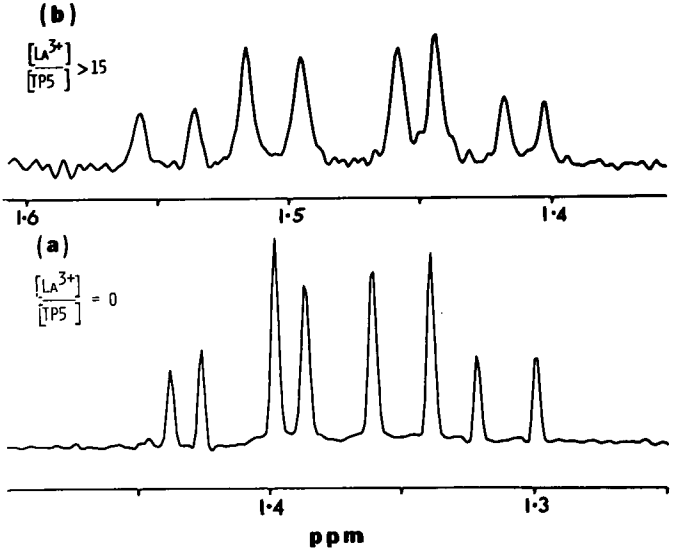

Fig. 1. Variation of Asp CβH_2 multiplet structure of TP5 with La^{3+} concentration.

Table II gives some limiting shifts induced by Pr^{3+} and the line broadenings ($1/T_{2M}$) due to Gd^{3+} in the lowfield NH and CH resonances of TP5. The stoichiometry of the complexes has been established[8] as 1:1. The measured dissociation constants, K_D, from the Pr^{3+} data were: 9.5×10^{-3} M for TP5; 1.3×10^{-1} M for Asp methyl ester TP5; and >0.5 M for C-terminal methyl ester TP5. The relative magnitudes of these K_D values clearly suggest that TP5 binds Pr^{3+} much more strongly than either of the analogs, and this observation is compatible with a bidentate model of TP5 in which both the Asp^3 as well as the C-terminal carboxylates

Table I. Rotamer Populations[a] About the Asp^3 Cα-Cβ Bond of TP5 vs La^{3+} Concentration

$[La^{3+}]$ / [TP5]	J_{ax}	J_{bx}	J_{ab}	$P_I(180^\circ)$	$P_{II}(-60^\circ)$	$P_{III}(60^\circ)$
0.00	4.64	9.04	-15.86	0.19	0.59	0.22
>15.0	8.47	6.02	-16.44	0.53	0.31	0.16

[a]Determined using standard Pachler analysis and the assumed values of $J_g^{HH} = 2.56$ Hz and $J_t^{HH} = 13.6$ Hz. The B proton resonance was assumed to occur at higher field.

participate simultaneously in metal binding. The formation of such a bidentate model is also facilitated by a change in the predominant rotamer of Asp^3 sidechain from -60° to 180° in TP5. This conclusion is also corraborated by our observation that the Asp^3 sidechain does not undergo any reorientation in either of the methyl ester analogs (each with one carboxylate group) upon complexation with La^{3+}. Nevertheless, a significant retention of the -60° and +60° rotamers even in the fully complexed state of TP5 suggests that there may be a significant amount of monodentate complex also present in solution. Figure 2 shows a variation in the linebroadening of a few select hydrogens of TP5 as a function of Gd^{3+} concentration. The Gd^{3+} induced relaxation enhancements, $1/T_{2M}$, in Table II, were calculated using an estimated $K_D = 7.1 \times 10^{-3}$ M from the shifts induced by an adjacent lanthanide, Eu^{3+}. It can be shown through detailed considerations[8] that the resultant correlation time, τ_c, in Equation 2 is primarily governed by the rotational correlation

Table II. Pr^{3+} Induced Limiting Shifts (δ_D) and Gd^{3+} Induced Relaxation Enhancements ($1/T_{2M}$) in Some Select Resonances of TP5 (25°C, pH = 5.0).

Resonance	δ_D (ppm)[a]	$1/T_{2M}$ (Hz)	Absolute r_{avg} (Å)[b]
Lys NH	0.168	59	10.0 ± 0.4
Asp NH	0.947	773	6.5 ± 0.1
Val NH	1.458	1190	6.0 ± 0.3
Tyr NH	6.023	2046	5.6 ± 0.3
Arg Guanidino NH	-0.257	67	9.8 ± 1.0
Tyr m-CH	1.436	503	7.0 ± 0.1
Tyr o-CH	0.265	160	8.5 ± 0.5

[a]Positive numbers mean down field shifts; [b]Metal-proton distances.

time(τ_r) of TP5. Using a value of $\tau_r \simeq 1.6 \times 10^{-10}$ s estimated from ^{13}C T_1 data, we have computed absolute proton-Gd^{3+} distances[8] and some of these are listed in Table II. These distances are only average distances in view of a conformational averaging between monodentate and bidentate models of TP5 (*vide supra*), each of which could further experience significant internal rotation of the sidechains. These distances provide some of the boundary conditions necessary to construct detailed molecular models of Ln^{3+}-TP5 complexes. Attempts to construct such models based on shift and relaxation data are in progress.

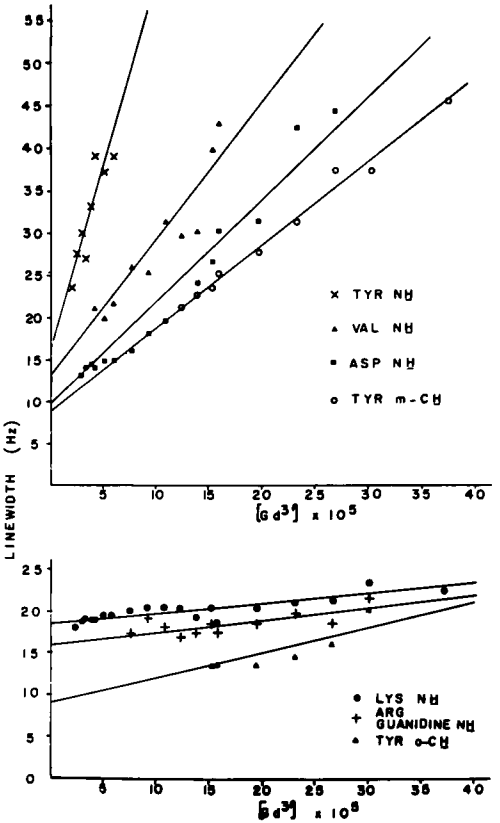

Fig. 2. Gd^{3+} induced linewidth variations in the low field ^1H resonances of TP5/H_2O.

Acknowledgements

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CONFORMATIONAL STUDIES OF THE POTENT OXYTOCIN ANTAGONISTS, [1-PENICILLAMINE, 4-THREONINE]-OXYTOCIN AND [1-PENICILLAMINE, 2-PHENYLALANINE, 4-THREONINE]-OXYTOCIN

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Introduction

Previous studies in this laboratory on the conformational and dynamic features of the potent oxytocin antagonists, [Pen^1]-oxytocin (S-C(CH_3)$_2$CH-(NH_2)-CO-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-GlyNH_2) and [Pen^1, Leu^2]-oxytocin, have shown that the 20-membered disulfide-containing rings of these compound are conformationally restricted compared with the tocin ring of oxytocin.[1-4] Certain features were proposed to be key elements of the antagonistic behavior of these analogs, it being suggested that conformational flexibility of oxytocin, upon binding to the receptor, was required to facilitate transduction.[2,5] The recent finding[6] that the oxytocin analogs, [Pen^1, Thr^4]-oxytocin and [Pen^1, Phe^2, Thr^4]-oxytocin are considerably more potent antagonists than [Pen^1]-oxytocin and [Pen^1, Leu^2]-oxytocin prompted the present study of the conformational features of the former two peptides.

Methods

[Pen^1, Thr^4]-Oxytocin and [Pen^1, Phe^2, Thr^4]-oxytocin were synthesized and purified as previously reported.[6] Proton NMR samples in 100% D_2O solution were prepared by dissolving of peptide previously lyophilized in D_2O to replace labile protons with deuterons. The pH was adjusted to approximately 3.5 (direct meter reading) with CD_3COOD. Samples for NMR in H_2O solution were prepared similarly without previous D_2O exchange and were either 20% H_2O/80% D_2O (270 MHz) or 90% H_2O/10% D_2O (250 MHz). Spectra were obtained on Bruker HX-270 and WM-250 spectrometers.

Results and Discussion

Best fit parameters of LAOCN 3 simulations[7] of the ^{1}H NMR spectra of [Pen^1, Thr^4]-oxytocin and [Pen^1, Phe^2, Thr^4]-oxytocin are shown in Table I. The near interchangeability of all the corresponding chemical shifts and coupling constants strongly suggests that the solution

Table I. Best Fit ^{1}H NMR Parameters for [Pen^1, Thr^4]-Oxytocin (open values) and [Pen^1, Phe^2, Thr^4]-Oxytocin (values in parentheses), T ~25°, pH ~3.5

Residue	δ	$J_{NH-\alpha CH}$	$J_{\alpha\beta}$	$J_{\alpha\beta'}$	$J_{\beta\beta'}$
Pen^1	δ_{NH} = ---	---	---	---	---
	δα = 3.89 (3.89)				
	δγ = 1.64 (1.65)				
	1.47 (1.47)				
Tyr^2	δ_{NH} = 9.01 (9.01)	5.9	5.8	10.8	13
(Phe^2)	δα = 4.58 (4.66)	(5.9)	(6.4)	(9.9)	(13.4)
	δβ = 3.15 (3.22)				
	2.97 (3.08)				
Ile^3	δ_{NH} = 7.84 (7.94)	2.0	5.5		
	δα = 3.98 (4.00)	(2.0)	(5.9)		
	δβ = 1.77 (1.79)				
Thr^4	δ_{NH} = 6.78 (6.80)	~5.7	4.1	$J_{\beta\gamma}$ = 6.2	
	δα = 4.09 (4.07)	(5.1, 50°C)	(4.8)	(6.6)	
	δβ = 4.27 (4.24)				
	δγ = 1.30 (1.30)				
Asn^5	δ_{NH} = 7.77 (7.80)	5.9	2.9	10.3	15
	δα = 4.67 (4.68)	(5.9)	(2.9)	(9.8)	(14.4)
	δβ = 3.13 (3.13)				
	2.93 (2.94)				
Cys^6	δ_{NH} = 8.69 (8.65)	9.6	4.2	10.9	15.2
	δα = 4.94 (4.94)	(8.8)	(4.2)	(10.9)	(15.6)
	δβ = 3.03 (3.04)				
	2.79 (2.79)				

Table I (Continued)

Residue		δ	$J_{NH-\alpha CH}$	$J_{\alpha\beta}$	$J_{\alpha\beta'}$	$J_{\beta\beta'}$
Pro^7	δ_{NH} =	---	---	5.3	8.2	---
	δα =	4.47 (4.46)		(5.1)	(8.4)	
	δβ =	2.29 (2.31)				
	δβ' =	1.94 (1.91)				
	δγ =	2.04 (2.04)				
	δδ =	3.72 (3.71)				
Leu^8	δ_{NH} =	8.45 (8.45)	6.6	3.7	10.3	---
	δα =	4.30 (4.30)	(6.6)	(4.8)	(10.3)	
	δβ =	1.65 (1.65)				
$GlyNH_2^9$	δ_{NH} =	8.38 (8.38)	5.9; 5.9		$J_{\alpha\alpha'}$ =	17.3
	δα =	3.94 (3.94)	(5.9; 5.9)			(17.3)
		3.86 (3.86)				

conformations of these two analogs are virtually identical. The corresponding parameters for invariant residues in [Pen^1]-oxytocin are also quite similar, except for Ile^3(δ_{NH} = 7.33; $J_{NH-\alpha CH}$ = 3.0). We infer from this that the overall conformations of [Pen^1]-oxytocin and the two analogs of the present study are similar with local differences in position 3 and position 4. This is consistent with previously reported ^{13}C NMR experiments[6] which also indicated general similarities but suggested greater conformational restrictions for Thr^4 compared with Gln^4.

The temperature dependence of the amide proton chemical shifts for [Pen^1, Thr^4]-oxytocin and [Pen^1, Phe^2, Thr^4]-oxytocin is presented in Table II. Again the similarity of the parameters for the two compounds is striking. Values of $-10^3 d\delta/dT$ of $\lesssim 3$ ppm/°K for backbone amide protons are generally taken as indicative of participation in an intramolecular hydrogen bond.[8] The data of Table II suggest that the peptide amide protons of both Thr^4 and Asn^5 participate in such intramolecular hydrogen bonds. This can be compared with [Pen^1]-oxytocin which also has two peptide amide hydrogen bond donors[2] in Ile^3 and Asn^5. The different hydrogen bonding features of the Ile^3 and

Table II. Temperature Dependence of Peptide Amide Proton Chemical Shifts for [Pen^1, Thr^4] Oxytocin (open values) and [Pen^1, Phe^2, Thr^4]-Oxytocin (values in parentheses)

Amino Acid	-10^3 (dδ/dT) ppm/°K
Tyr^2 (Phe^2)	7.4 (6.8)
Ile^3	7.5 (6.6)
Thr^4	-3.5 (-3.6)
Asn^5	0 (0)
Cys^6	5.3 (5.0)
Leu^8	9.7 (9.6)
Gly-NH$_2^9$	7.5 (7.6)

position 4 residues of [Pen^1]-oxytocin compared with those of [Pen^1, Thr^4]-oxytocin and [Pen^1, Phe^2, Thr^4]-oxytocin are consistent with the differences noted in Table I and provide further evidence of conformational and dynamic differences at these positions. These differences may be the source of the increased antagonist potency of the Thr^4 containing analogs. A more detailed analysis of these and other considerations (for example, the strongly *positive* temperature dependence of the Thr^4 amide proton chemical shift) is in progress.

Acknowledgements

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CONFORMATION-ACTIVITY RELATIONSHIP IN ANGIOTENSIN II: ANALOGS SUBSTITUTED IN POSITION 5

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Introduction

Pharmacological studies have shown that whereas the triggering of the initial biological response of the pressor hormone Angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) at the membrane site needs the presence of the aromatic side chain of Phe^8, the two residues Tyr^4 and His^6 are essential for the binding to the physiological receptors in smooth muscle preparations. In these, the observed response to angiotensin II analogs closely follows the structure-activity relationships seen in the pressor assay system.[1] Our earlier studies[2] clearly indicate that the side chains of Tyr^4 and His^6 behave interdependently in space and that their time averaged distance and orientation depend upon the ϕ and ψ values of the β-branched residue in position 5 (Ile or Val).[3]

In order to quantify these statements and to study the problem in greater detail, we prepared and analyzed 4 model peptides of the central A-II sequence, i.e. Ac-Tyr-X-His-NH_2 where X = Val, Leu, Ala and Gly. These residues were chosen in order to simulate the A-II analogs of position 5, i.e. Leu^5, Ala^5 and Gly^5 for which biological data are known to decrease in that order.

Results

a) Circular Dichroism: The CD spectra of all compounds studied tripeptides and A-II analogs in aqueous solution were recorded at various pH values; the titration curves at 275 nm are shown in Figure 1. Both in the tripeptides and in the corresponding A-II analogs the magnitude of the histidine titration effect on the Tyr^4 side chain signals (275nm) increases in going from X=Gly to X=Val. Clearly the effect is strongest in the case where the side chain at position X is β-branched (Val) and diminishes with the decrease in steric hindrance. The results suggest that the value of $\Delta[\theta] = [\theta]^{pH\ 4} - [\theta]^{pH\ 8}$ is a measure of the through-space influence of the His^6 titration on Tyr^4, i.e. a measure of the time averaged

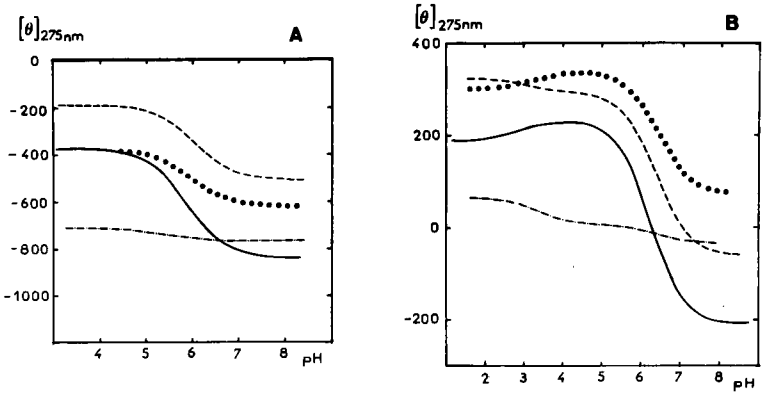

Fig. 1. CD titration curves of tripeptides (A) and A-II analogs (B) with X: Val (——), Leu (——), Ala (·····), Gly (—·—).

distance between the two chromophores. This could be due either to the variation of the time dependent orientation of the two aromatic groups, i.e. differences in the distribution of rotamers in His and Tyr, or to an opening of the peptide backbone especially at position X, increasing the distance between the two aromatic side chains in space. It seems to us that the most plausible explanation is: the greater the steric hindrance of the residue X side chain, the greater is the kink in the backbone at this site (ϕ and ψ rotation restricted), thus the greater influence of the His titration on the Tyr signal. This does not exclude the possibility that some changes in the average side chain rotamer distribution occur simultaneously.

b) NMR studies: We have focused our attention on rotamer distribution in side chains and on the backbone arrangement around position X. In aqueous solutions of the two series of compounds, the chemical shifts of i) the α protons of His and Tyr, ii) the β and β' protons of Tyr and iii) the β and β' protons of His reflect more or less the titration of the imidazole group as well as the ionization of the vicinal phenolic group. The magnitude of the observed effects follow a graduated variation from X=Val to X=Gly.

These results point to specific organization and flexibility of Tyr and His side chains which vary as a function of the size of the intermediate X residue side chain. Clearly the β and β' chemical shift nonequivalence at a given pH as well as the magnitude of the shift variations against pH suggest reciprocal effects of Tyr and His which are largest when X=Val. Since these effects decrease from X=Val to X=Gly we may assume that the side chain of X, when present exerts a strong influence on the backbone.

The pattern of the vicinal coupling constants $^3J\alpha\beta$ and $^3J\alpha\beta'$ in tyrosine examined against pH is about the same as found for the β and β'

Table I.

Tripeptides	DMSO/imid$^+$ $^3J_{NH-C^\alpha H}$	DMSO/imid$^+$ ϕ^1	DMSO/imid$^+$ ϕ^2	DMSO/imid$^+$ $\Delta\phi$	^2H_2O (pH 7.3) Tyrosine R I $\chi^1=-60°$	Tyrosine R II $\chi^1=180°$	Tyrosine R III $\chi^1=60°$	$\Delta\{\theta\}_{275}$ (CD)	biol. act. %
X = Val	8.3	-154°	-86°	68°	.44	.48	.08	460	-
X = Leu	7.5	-158°	-81°	77°	.50	.38	.12	310	-
X = Ala	6.8	-162°	-77°	85°	.51	.38	.11	240	-
X = Gly	6.2	-165°	-74°	91°	.54	.38	.08	70	-
	5.8	-168°	-72°	96°					
		58°	62°						
[Val^5] A II	8.8	-151°	-89°	62°	.46	.50	.04	450	100
[Leu^5] A II	8.0	-156°	-84°	72°	-	-	-	350	24
[Ala^5] A II	7.4	-160°	-80°	80°	.58	.31	.11	250	5
[Gly^5] A II	5.6	-169°	-71°	98°	-	-	-	50	<2
	5.6	49°	69°						

chemical shifts. This concerns both nonequivalence and sensitivity to imidazolium and phenolic group titration. Nonequivalence increases and titration effects decrease from X=Val to X=Gly.

Concerning the His residue we observe that the vicinal coupling constants $^3J\alpha\beta$ and $^3J\alpha\beta'$ remain quite constant all along the pH scale, while for a given pH the nonequivalence of $^3J\alpha\beta$ and $^3J\alpha\beta'$ is again smallest for the peptide with X=Val; it gradually increases in the series from X=Val to X=Gly.

The ^{1}H-NMR spectra in DMSO-d_6 solution show a tremendous increase of the nonequivalence of β and β' protons and their $^3J\alpha\beta$ and $^3J\alpha\beta'$ coupling constants in Tyr. There is no similar effect observed in His. The main aspect concerns the backbone: the vicinal coupling constants $^3J_{NH-C\alpha H}$ show a gradual decrease from X=Val to X=Gly in the X residue, whereas in Tyr and His no remarkable variations are observed for the corresponding coupling constants. Therefore the substitution of the side chain X results in a progressive conformational change of the ϕ and probably ψ dihedral angles mostly in position X. Such local changes entail variations in the central part of A-II such that, e.g. the average distance between Tyr and His side chains increases from [Ile^5] A-II to [Gly^5] A-II as already clearly shown by the CD titration curves.

Conclusion

In the present work we show, using CD and NMR techniques that successive substitutions of Ile^5 or Val^5 by Leu, Ala and Gly lead to a gradual increase of the time averaged distance between the Tyr^4 and His^6 side chains in A-II and in the model tripeptides Ac-Tyr-X-His-NH_2, consequently to a variation of the ϕ and ψ dihedral angles of the X residue. These conformational modifications — gradual opening of the backbone in the central sequence — attended by a very weak modification of the rotamer distributions in His and Tyr — follow perfectly well the variations of the binding properties and myotropic activities of the hormone and its X^5 substituted analogs. Since [Pro^5] A-II still has $\sim$ 10% of biological activity (close to the one of [Ala^5] A-II) we may assume that the ϕ^5 angle in A-II is close to $-90°$.

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600 MHZ AND 270 MHZ SCALAR COUPLING ANALYSIS OF SOMATOSTATIN

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In conjunction with our earlier work assigning the aqueous solution 270 MHz proton magnetic resonance spectrum of the 14 amino acid peptide hormone somatostatin[1]

$Ala^{1}Gly^{2}Cys^{3}Lys^{4}Asn^{5}Phe^{6}Phe^{7}Trp^{8}Lys^{9}Thr^{10}Phe^{11}Thr^{12}Ser^{13}Cys^{14}$

we have investigated the conformation through scalar coupling analysis at 270 and 600 MHz. The scalar coupling parameters ($^{3}J\alpha\beta$, $^{2}J\beta\beta$) were obtained for all residues except Lys^{4} through double and triple resonance, difference spectroscopy and computer simulations.[2] From these parameters we calculated spectra for each residue at both fields and from the sum of these individual residue simulated spectra obtained 270 and 600 MHz simulated somatostatin spectra. The correctness of our analysis was confirmed by the agreement between these simulated somatostatin spectra and the experimental spectra at both fields. Figure 1 shows the individual residue simulations and the experimental spectrum at 270 MHz. The observed and experimental spectra of the β region are shown in Figure 2 (270 MHz) and Figure 3 (600 MHz).

With the assumption of classical staggered side chain rotamers (χ^{1} = +180°, −60°, +60°) we calculated the populations of the three χ^{1} values for the residues from the $^{3}J\alpha\beta$. Figure 4 shows the relative populations in diagram form. It can be seen from Figure 4 that if the classical assumption is valid there exists a fair degree of averaging among the three χ^{1} values in somatostatin.

Full presentations of the assignment procedure[3] and of the scalar coupling analysis[4] have been submitted.

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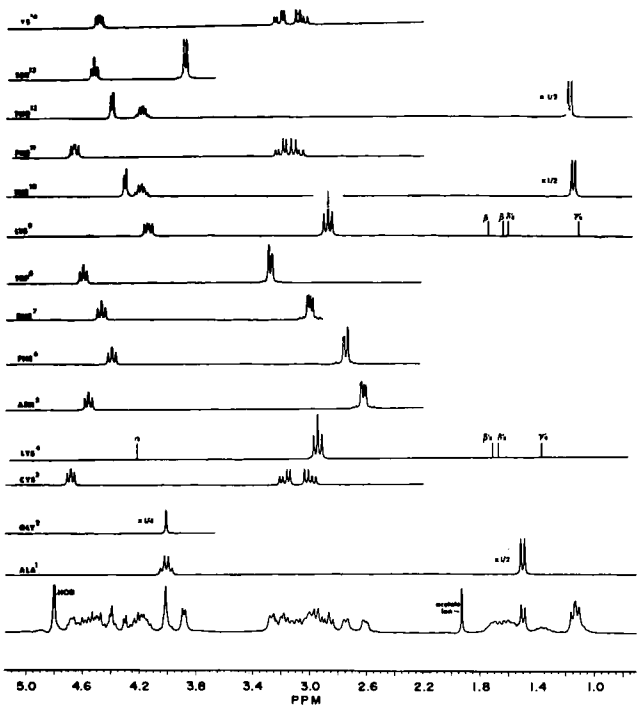

Fig. 1. Simulated spectra for protons of the individual amino acid residues of somatostatin at 270 MHz. The experimental spectrum is the lower spectrum.

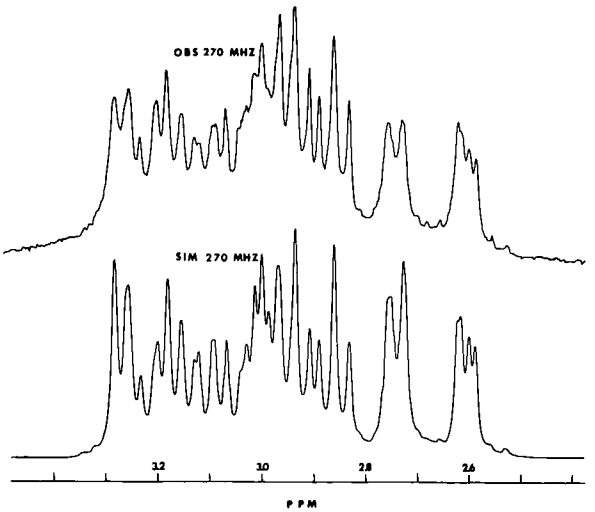

Fig. 2. β proton region, 270 MHz Upper: observed somatostatin spectrum. Lower: simulated somatostatin spectrum.

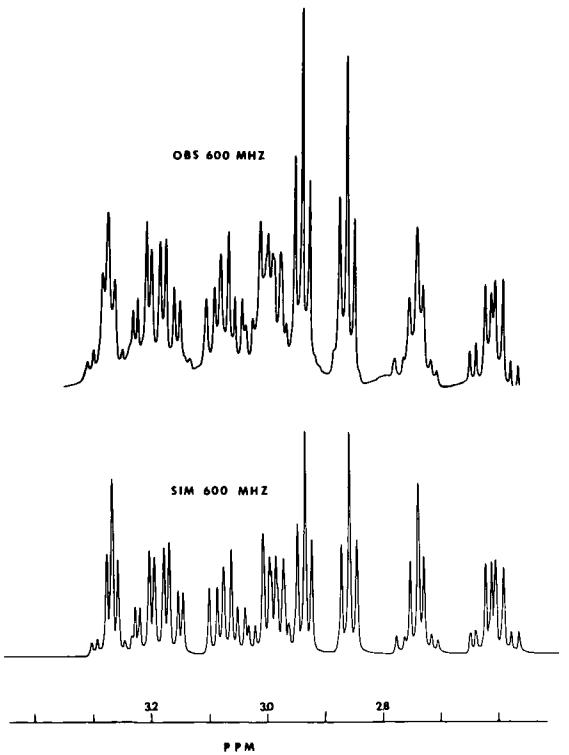

Fig. 3. β proton region, 600 MHz. Upper: observed somatostatin spectrum. Lower: simulated somatostatin spectrum.

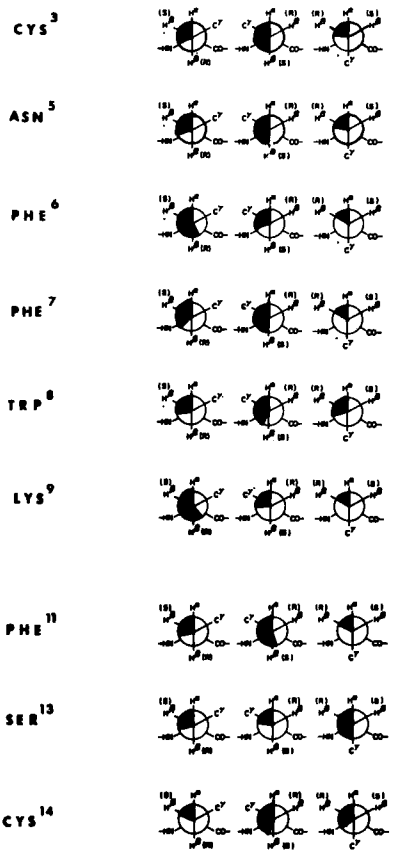

Fig. 4. Side chain rotamer populations of somatostatin amino acid residues. Shaded portion is proportional to percentage of rotamers with that χ^1 value (column 1, χ^1=180°; column 2, χ^1=–60°; column 3, χ^1=+60°).

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CONFORMATION-ACTIVITY STUDIES OF α-MELANOCYTE STIMULATING HORMONE: SYNTHESIS OF A CYCLIC α-MELANOTROPIN EXHIBITING HIGH POTENCY AND FULL BIOLOGICAL ACTIVITY

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Introduction

α-Melanocyte stimulating hormone (α-MSH, α-melanotropin) is a *linear* tridecapeptide (Table I) which reversibly darkens amphibian skins by stimulating melanosome dispersion within melanophores. α-MSH also affects mammalian melanocytes, both normal and transformed (melanoma) cells, by stimulating adenylate cyclase activity, tyrosinase activity and melanin production.[1] Our current research on a α-MSH-receptor interaction has been directed at three major goals: 1) preparation of highly potent α-MSH agonists and antagonists which may have medicinal utility; 2) determination of the biologically-active conformation of α-MSH at its receptor; and 3) determination of the molecular mechanism of α-MSH-receptor binding and transduction of hormonal stimuli at the cell membrane.

We report here the synthesis of a cyclic α-melanotropin, [half-Cys^4, half-Cys^{10}]-α-MSH, and document its superagonist biological activity. The comparative melanotropic activities of a number of other structurally- and/or stereochemically-modified α-MSH peptides are described. A conformational model which may provide new insight into the functional roles and spatial disposition of key amino acid residues comprising α-MSH as related to receptor binding and/or transduction events is discussed.

Results and Discussion

According to the literature[2], α-MSH apparently contains two active sites, (Glu)-His-Phe-Arg-Trp and Gly-Lys-Pro-Val-NH_2, each of which can independently stimulate amphibian melanophore dispersion *in vitro*. On the frog (*Rana pipiens*) skin bioassay (Table I), α-MSH_{4-10} which is structurally devoid of the putative C-terminal active site (Gly-Lys-Pro-Val-NH_2), is a very weak agonist with about 1/100,000 the potency of

α-MSH. A significant increase in melanophore-dispersing activity (about 200-fold relative to α-MSH_{4-10}) is observed following structural modifications at the 4- and 10-positions of this fragment to yield Ac-[Nle^4]α-MSH_{4-10}-NH_2 (Table I). Furthermore, substitution of Phe-7 by its *D*-enantiomer to give Ac-[Nle^4, *D*-Phe^7]-α-MSH_{4-10}-NH_2, results in the most potent α-MSH_{4-10}analog reported to date (about 20,000-fold more potent than α-MSH_{4-10}, Table I). Similarly, [Nle^4, *D*-Phe^7]-α-MSH_{4-10} was shown to exhibit higher melanotropic potency (about 60-fold) relative to α-MSH (Table I). In addition, [Nle^4, *D*-Phe^7]-α-MSH has been reported to possess other exceptional *in vitro*[4] and *in vivo*[5] biological properties on both normal and transformed melanophores.

With respect to developing an understanding of the chemical-physical basis of α-MSH-receptor interaction, interpretation of these results provides a challenging task. Based on theoretical considerations of the possible conformational effects of introducing *D*-amino acids into small peptides, and examination of 3-dimensional molecular models of these stereoisomeric α-MSH peptides, we concluded that a β-turn or other peptide chain reversal region within the central active site [(Glu)-His-Phe-Arg-Trp] of α-MSH might be functionally related to its bioactive conformation.

To evaluate the effect of covalently locking α-MSH into a cyclic or bent conformation, we substituted Met-4 and Gly-10 by cysteine residues (essentially an isosteric replacement, Figure 1) into α-MSH by total synthesis, and oxidized the intermediate free-disulfhydryl analog to form its intramolecularly disulfide-bridged derivative, [half-Cys^4, half-Cys^{10}]-α-MSH. It should be noted that both active sites were structurally conserved in the design of this cyclic conformationally-restricted α-melanotropin.

On the frog skin bioassay, [half-Cys^4, half-Cys^{10}]-α-MSH exhibited superagonist biological activity (Table I) and was about 10,000-fold more potent than the native hormone, α-MSH. This dramatic increase in potency *in vitro* indicates that the introduction of the disulfide-bridged "isosteric" constituent (cystine) near the proposed β-turn region of α-MSH, (Glu)-His-Phe-Arg-Trp, results in a conformationally-constrained analog affecting more favorable hormone-receptor interaction. On the mouse melanoma adenylate cyclase assay, [half-Cys^4, half-Cys^{10}]-α-MSH was about 3 times more potent than α-MSH (Table I). These results suggest that similar, but not identical, conformational requirements exist for α-MSH on both of these distantly-related vertebrate pigment cell systems.

Table I. Relative Potency of α-MSH Analogs in Frog Skin and Melanoma Adenylate Cyclase Assays

Peptide	Structure	Relative potency[a]	
		Frog Skin Assay[b]	Adenylate Cyclase[c]
α-MSH	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2 (1 4 7 10 13)	1.0	1.0
$\alpha\text{-MSH}_{4\text{-}10}$[d]	H-Met-Glu-His-Phe-Arg-Trp-Gly-OH	0.00001	---
Ac-[Nle^4]-$\alpha\text{-MSH}_{4\text{-}10}$-$NH_2$	Ac-Nle-Glu-His-Phe-Arg-Trp-Gly-NH_2	0.002	0.09
Ac-[Nle^4, D-Phe^7]-$\alpha\text{-MSH}_{4\text{-}10}$-$NH_2$	Ac-Nle-Glu-His-D-Phe[e]-Arg-Trp-Gly-NH_2	0.2	7.7
[Nle^4, D-Phe^7]-α-MSH	Ac-Ser-Tyr-Ser-Nle-Glu-His-D-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	60.0	8.6
[½-Cys^4, ½-Cys^{10}]-α-MSH	Ac-Ser-Tyr-Ser-Cys-Glu-His-Phe-Arg-Trp-Cys-Lys-Pro-Val-NH_2	10,000.0	2.9

[a] Relative potency = concentration of α-MSH at 50% response/concentration of peptide at 50% response. [b,c] Melanophore dispersing activity was determined by the frog (*Rana pipiens*) skin bioassay, and Cloudman S-91 mouse melanoma adenylate cyclase activity was determined as previously described.[3,4] [d] $\alpha\text{-MSH}_{4\text{-}10}$ was obtained from Peninsula Laboaratories. All other peptides reported here were synthesized by solid-phase procedures and purified by methods previously described.[3,4] [e] *Phe* denotes *D*-phenylalanine.

[Half-Cys^4, half-Cys^{10}]-α-MSH has a 23-membered ring structure, and examination of a 3-dimensional molecular model of this peptide indicates several intramolecular conformation-stabilizing features which may possibly be related to the bioactive conformation of α-MSH at the melanotropin receptor. These include: 1) H-bonding between the amide C=O of His-6 and the amide N-H of Trp-9 (this is compatible with 4 → 1 β-turn structure for the central amino acid sequence, His-Phe-Arg-Trp), 2) ionic or H-bond interaction between the side-chain γ-CO_2H group of Glu-5 and the side-chain- ε-NH_2 group of Lys-11 (this may be functionally related to the multiplicative "cooperative" melanophore dispersing activity reported[2] when the two individual active sites of α-MSH, (Glu)-His-Phe-Arg-Trp and Gly-Lys-Pro-Val-NH_2, are covalently combined [(Glu)-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2], and 3) hydrophobic interaction of lipophilic side-chains of Lys-11, Trp-9 and Phe-7.

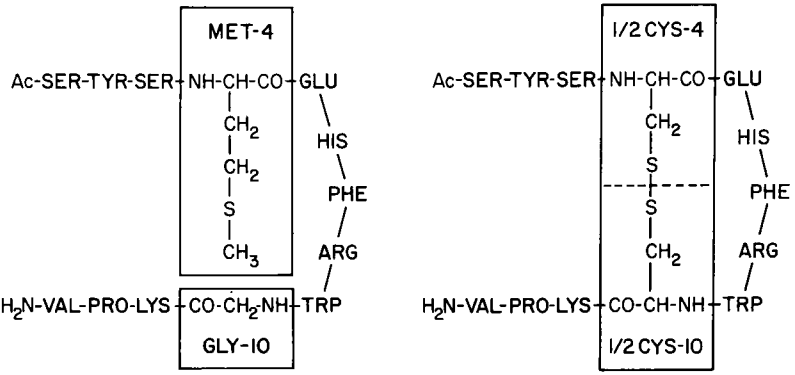

Fig. 1. "Isosteric" substitution of Met-4 and Gly-10 in α-MSH (left) by cystine in [half-Cys^4, half-Cys^{10}]-α-MSH (right).

The design of new synthetic semirigid cyclic isomers of α-MSH and analysis of their biological and physico-chemical properties, including further studies on [half-Cys^4, half-Cys^{10}]-α-MSH, will provide more detailed insight into the functionally important amino acid constituents of α-MSH interaction with its receptor as discussed here, and provide new understanding of the conformation-activity relationships of α-MSH. In the future, such highly potent and metabolically stable α-melanotropins may be useful for the detection or treatment (drug-delivery agents) of melanoma (skin cancer) in man, and for evaluation of the role of α-MSH in brain and in fetal development.

Acknowledgements

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SYNTHETIC ELASTASE INHIBITORS AND THEIR ROLE IN THE TREATMENT OF DISEASE

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Introduction

Proteases (protein hydrolyzing enzymes) are involved in many important biological processes. Some of the better known examples are blood coagulation, fibrinolysis, complement activation, protein processing fertilization, digestion and phagocytosis. Many proteases are highly specific and will cleave only one or a few peptide bonds in their natural substrate or substrates. Peptide bond cleavage often results in the activation or inactivation of proteins or enzymes, and is basically an irreversible process since there are no biochemical pathways for the resynthesis of a cleaved peptide bond in a protein. Thus, proteolysis is a prompt and irreversible method of turning on or off many physiological processes such as coagulation, and proteases and their natural inhibitors act as regulators.

A major component of all cells is protein and outside their natural environment, proteases could be very destructive if they were not carefully controlled or compartmentalized. The potential seriousness of uncontrolled proteolysis can be recognized by the fact that *ca* 10% (by weight) of the proteins found in human plasma are protease inhibitors. These include α_1-protease inhibitor (α_1-PI, an elastase inhibitor), antithrombin III (an inhibitor of coagulation proteases), and α_2-macroglobulin (an inhibitor of all classes of proteases). It is now believed that many diseases such as emphysema result when there is an imbalance between specific proteases and their natural inhibitors.

Emphysema

Emphysema is a respiratory disease which afflicts a large number of Americans especially smokers. Emphysema and bronchitis kill an estimated 35,000 people in the U.S. each year and contribute to the deaths of an equal number. Pulmonary emphysema is one disease where the linkage between proteolysis and disease is now fairly well understood.

The lung is composed of tiny flexible sacs called alveoli. Alveoli are rich in the elastic connective tissue protein elastin and also contain

collagen and various proteoglycans. In emphysema, the alveoli become enlarged and lose their ability to expand and contract. Lung degradation is thought to be caused by proteases released from human leukocytes.

The granule fraction of human polymorphonuclear leukocytes contains large amounts of the serine proteases elastase and the chymotrypsin-like enzyme cathepsin G. These proteases are normally involved in phagocytosis in the lung and contribute to the turnover of damaged lung cells and the digestion of invading bacteria. In normal individuals, α_1-PI protects the lung from any proteases which may leak from leukocytes. Some individuals are genetically deficient in α_1-PI and are not protected. Proteolysis of lung elastin leads to the development of emphysema at an early age. Most individuals with emphysema are not deficient in α_1-PI, but are smokers.

α_1-PI — The sequence at the reactive site of α_1-PI has recently been determined and shown to contain a Met-Ser bond.[1] This Met-Ser bond is cleaved when complexes of α_1-PI with proteases are dissociated with nucleophiles or at high pH. The mechanism by which α_1-PI inhibits proteases is not yet known, but it certainly involves interaction of the reactive site amino acids of α_1-PI with the active site of elastase. It is possible that the active site serine of elastase reacts covalently with the Met-Ser bond of α_1-PI to form a tetrahedral complex or an acyl enzyme, but this has not been established.

We have synthesized and investigated a number of linear peptides with sequences corresponding to the sequence at the α_1-PI reactive site.[2] The reactive site of α_1-PI and two of the longer peptides are shown below. The Met octapeptide is not an inhibitor of human leukocyte (HL) elastase, but is an excellent substrate instead and is cleaved at the Met-Ser bond.

-Ala-Ile-Pro-Met-Ser-Ile-Pro-Pro	α_1-PI reactive site	
	K_M (mM)	k_{cat}/K_M ($M^{-1}s^{-1}$)
Ac-Ala-Ile-Pro-Met-Ser-Ile-Pro-Pro-NH_2	1.6	10,000
Ac-Ala-Ile-Pro-Met(O)-Ser-Ile-Pro-Pro-NH_2	3.6	27

Oxidation of the Met in the octapeptide to methionine sulfoxide resulted in a 370 fold decrease in k_{cat}/K_M at pH 7.5. Similar results have been obtained with other Met containing peptides [3] and oxidation of α_1-PI itself has been shown to diminish its ability to inhibit most proteases.[4] Ozone and NaOCl are some of the agents that have been shown to be able to oxidize α_1-PI.

Cigarette smoke produces a functional deficiency of elastase inhibitors in the lower respiratory tract of humans[5,6] and it is quite resonable to assume that this is due to the oxidation of the essential methionine residue in α_1-PI. In fact, feeding the oxidant NaOCl to dogs results in the induction of emphysema, which can also be induced by installation of elastase into the lungs of emperimental animals. Cigarette smoke contains free radicals and a number of oxidants and so it is possible that smoke itself is directly oxidizing α_1-PI. However other possible mechanisms must be considered. Leukocyte granules contain myleoperoxidase which is an enzyme also capable of oxidizing α_1-PI. Cigarette smoke could be activating myleoperoxidase or stimulating its release. In any case, smokers can have an imbalance between proteases and protease inhibitors in the lung and emphysema can result (Figure 1).

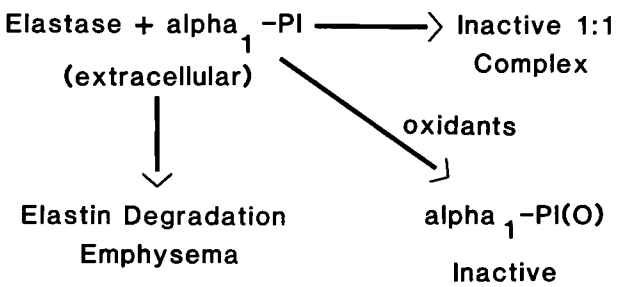

Fig. 1. Relationship between elastase, α_1-PI and emphysema.

Synthetic Elastase Inhibitors

It is evident that selective elastase inhibitors could be used in the treatment of emphysema. The inhibitors could be natural inhibitors such as α_1-PI itself isolated from blood fractionation or synthetic materials. At present it is possible to obtain only enough α_1-PI to treat a small percentage of patients with emphysema. On the other hand, synthetic inhibitors could be obtained in large amounts by synthesis, could be rationally designed to increase reactivity and selectivity and would likely be orally active in contrast to α_1-PI.

Cyclic Peptide Analogs of α_1-PI — Crystallographic data has not yet been obtained on the plasma protease inhibitor α_1-PI, but x-ray structures have been obtained on several protein protease inhibitors from plant, microbial and mammalian sources.[8] X-ray structures have also been obtained on two trypsin-trypsin inhibitor complexes. Only a small portion of the two inhibitors interact with the enzyme and most of the major contacts occur with the 6 or 7 amino acid residues in the immediate

vicinity of the inhibitor reactive site. Comparison of the various protease inhibitor structures revealed a surprising degree of similarity in the conformation of the peptide chain at the various reactive sites even though the inhibitors inactivated proteases with quite different specificities.[8] Therefore it is reasonable to assume that the conformation at the reactive site of α_1-PI bears some similarity to that of the other protease inhibitors. We have designed a number of inhibitors based on this premise.

We choose to use the reactive site of the soybean trypsin inhibitor as the model for the design of inhibitors.[9] A model was constructed of the P_4-P_3' amino acids at the reactive site of the soybean trypsin inhibitor and various ways of bridging the ends of the reactive site were considered. The bridging groups varied from a very flexible ϵ-aminocaproyl group to the very rigid *m*-Abz-*m*-Abz (*m*-Abz = *m*-aminobenzoyl) group (Figure 2).

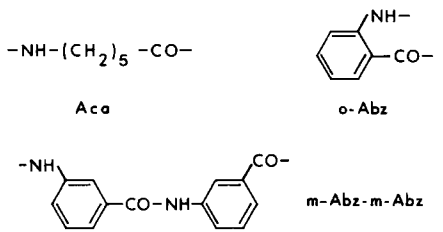

Fig. 2. Bridging Groups

The cyclic peptides synthesized and their inhibition constants (K_I) with HL elastase are listed in Table I. Peptides **1**, **2** and **3** are quite similar to the α_1-PI reactive site. The inhibitors contain a P_1' Thr residue because at the time they were designed, the α_1-PI reactive site sequence was thought to contain either a Thr or Ser at this position. We substituted Leu for Ile since we decided it would not be a significant change and would make the synthesis a little more practical. The latter two peptides **4** and **5** were synthesized to replace the P_1 Met of α_1-PI with a Val since HL elastase prefers P_1 valine residues in its substrates. At the same time, we substituted the P_1' Thr with Ser.

All of the cyclic peptides were moderate inhibitors of HL elastase with the lowest K_I value being 0.39 mM for peptide **3**. The linear peptide Ac-Ala-Ile-Pro-Met-Ser-Ile-Pro-Pro-NH_2 is a substrate for HL elastase and has a K_M of 1.6 mM. All the cyclic peptides with the exception of **1**, bind more tightly than the linear peptide. In addition none of the cyclic peptides was cleaved by HL elastase even after long incubation times. The cyclic peptides also showed good specificity and would not inhibit porcine pancreatic elastase.

Table I. Inhibition of HL Elastase by Cyclic Peptides[a]

	Peptide	K_I (mM)
	P_4 P_3 P_2 P_1 P_1' P_2' P_3' P_4'	
	-Ala-Ile-Pro-Met-Ser-Ile-Pro-Pro	α_1-PI reactive site
1	cyclo(-Aca-Pro-Met-Thr-Leu-Pro-Pro-)	17
2	cyclo(-o-Abz-Leu-Pro-Met-Thr-D-Leu-Pro-Pro-)	0.89
3	cyclo(-Ala-Leu-Pro-Met-Thr-Leu-m-Abz-m-Abz-)	0.39
4	cyclo(-Ala-Leu-Pro-Val-Ser-Leu-m-Abz-m-Abz-)	0.80
5	cyclo(-D-Ala-Leu-Pro-Val-Ser-Leu-m-Abz-m-Abz-)	0.48

[a] 0.1 M Hepes at pH 7.5 containing 0.5M NaCl and 9.8% dimethylforamide at 25° C.

The cyclic peptides can probably occupy a number of different conformations one of which would be similar to the reactive site conformation of the soybean trypsin inhibitor. The bridging groups reduce the number of possibilities when compared to the linear peptide. Interestingly, the cyclic peptide with the most rigid group **3** is the best inhibitor while the peptide **1** with the most flexible group is the poorest inhibitor. A change of a L-Ala to a D-Ala (compare **4** and **5**) improved inhibition by twofold. Other such changes which would further restrict the conformation possibilities might be expected to result in further improvement in inhibition.

A number of other cyclic peptide analogs of trypsin or chymotrypsin inhibitor reactive sites have been synthesized and some have K_I values as low as 0.75 μM. In contrast to the inhibitors reported here, most were hydrolyzed upon incubation with either trypsin or chymotrypsin.[10]

Azapeptides — Aza-aminoacids are analogs of amino acids in which the α-CH has been replaced by a nitrogen atom. This substitution has a profound effect on the reactivity of azapeptides toward serine proteases.[11] The effect of replacing each alanine residue in the good elastase substrate, Ac-Ala-Ala-Ala-ONp, with an aza-alanine residue (Aala,-NHN(CH_3)CO-) is shown in Table II. In every case the k_{cat}/K_M value for the azapeptide is considerably lower than that of the peptide substrate. The azapeptides with a P_3 or P_2 Aala have K_M values which are similar to that of Ac-Ala-Ala-Ala-ONp, but the k_{cat} values are much lower.

Table II. Reaction of Azapeptides with HL Elastase[a]

Peptide P_3 P_2 P_1	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)
Ac-Ala-Ala-Ala-ONp	1.0	150	1.5×10^5
Ac-Aala-Ala-Ala-ONp	1.7	45	2.8×10^4
Ac-Ala-Aala-Ala-ONp	2.1	2.3	1.1×10^3
Ac-Ala-Ala-Aala-ONp	0.016	0.0065	2.5×10^3

[a] pH 6.0, 0.1M citrate buffer containing 5% CH_3CN, HL elastase 0.066-0.32 μM.

Azapeptides with a P_1 aza-amino acid residue react with elastase to form carbazyl enzymes (Figure 3) which are much more stable than the acyl enzymes formed from ordinary peptide substrates and are thus elastase inhibitors. With 4-nitrophenyl esters of azapeptides, formation of the carbazyl enzyme is essentially instantaneous upon mixing the reagent with the enzyme. The stability of the carbazyl enzyme formed is dependent on the nature of the interaction between the primary substrate binding site of the enzyme and the side chain of the P_1 aza-amino acid residue. Table III lists the reactivation rates (k_{cat}) for the carbazyl enzymes formed by reaction of elastase with a number of azapeptides. The Azaleucyl (Aleu) and Azanorleucyl (Anle) analogs in particular form quite stable carbazyl derivatives. The 4-nitrophenol leaving group can be replaced by other phenols or alochols, and both Ac-Ala-Ala-Anle-OPh and Ac-Ala-Ala-Anle-OCH_2CF_3 are effective inhibitors of HL elastase.

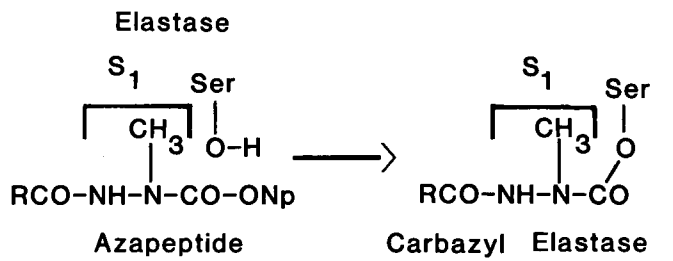

Fig. 3. Reaction of RCO-Aala-ONp with Elastase. The primary binding site (S_1) of the enzyme is shown interacting with the side chain of the azapeptide. The active site serine of elastase is acylated forming a cabazyl enzyme.

It is evident that the stability of the carbazyl enzymes (Figure 3) are affected by several factors. First, the carbazyl derivatives themselves are

Table III. Reaction of Azapeptides with a P_1 Aza-Amino Acid Residue with HL Elastase[a]

Peptide P_1	k_{cat} (s^{-1})
Ac-Ala-Ala-Aval-ONp	0.35
Ac-Ala-Ala-Aile-ONp	0.0059
Ac-Ala-Ala-Anva-ONp	0.0014
Ac-Ala-Ala-Aleu-ONp	<0.00019
Ac-Ala-Ala-Anle-ONp	<0.00019

[a]pH 6.0, 0.1M citrate

inherently more stable due to the electronic effect of the α-nitrogen on the adjacent carbonyl group. Compared to the acyl enzymes (esters) formed from ordinary peptide substrates, the carbazyl derivatives are much less susceptible to nucleophilic attack and enzyme catalyzed deacylation. The second factor which affects stability of the carbazyl enzyme, is the interaction of the side chain substituent of the aza-amino residue with the S_1 pocket of enzyme. Possibly this interaction is twisting the carbonyl group of the carbazyl group away from an orientation suitable for deacylation in the Anle and Aleu analogs.[12]

Peptide Chloromethyl Ketones — Peptide chloromethyl ketones are active site directed irreversible inhibitors for serine proteases. The inhibitors bind to the extended substrate binding site of the enzyme and the reactive chloromethyl ketone functional group is then placed in the proper position to alkylate the active site histidine residue. In addition, the serine OH reacts with the inhibitor carbonyl group to form a hemiketal.[13]

The most effective chloromethyl ketone HL elastase inhibitor found thus far is MeO-Suc-Ala-Ala-Pro-ValCH_2Cl($k_{obs}/[I]$ = 1560 $M^{-1}s^{-1}$, MeO-Suc = $CH_3OCOCH_2CH_2CO$—).[14] This will not inhibit the other major leukocyte protease cathepsin G, but is an alkylating agent and will react slowly with nucleophiles such as glutathione ($k_{obs}/[I] = 0.88\ M^{-1}s^{-1}$). This rate is quite slow and the inhibitor would discriminate in favor of HL elastase over glutathione by a factor of 1700 if the concentrations were equivalent.

The major problem with the use of peptide chloromethyl ketones *in vivo* is the potential for reaction at other sites. One possible approach to

obtaining specificity involves the attachment of the inhibitor to a suitable carrier that could be targeted to the desired site of action. This has been accomplished with human albumin microspheres (HAM).[15] HAM are nontoxic, nonantigenic and biodegradable, and because of their unique size are trapped in the pulmonary capillary bed after intravenous injection. An effective HL elastase inhibitor has been linked to HAM (shown below) and found to be capable of inhibiting elastase. When the inhibitor-HAM were injected into rats, they were rapidly and exclusively taken up by the lungs. Half of the modified HAM remained in lung with a half life of *ca* 17 days. Thus HAM offer a method for the delivery of elastase inhibitors to the lung.

HAM-CONH-Spacer-Ala-Ala-Pro-ValCH_2Cl

Animal Studies and Perspectives

Synthetic elastase inhibitors have considerable potential for the treatment of emphysema. Two peptide chloromethyl ketone elastase inhibitors Ac-Ala-Ala-Pro-AlaCH_2Cl and Suc-Ala-Pro-ValCH_2Cl have been shown to significantly diminish the extent of experimental elastase-induced emphysema in hamsters.[16] MeO-Suc-Ala-Ala-Pro-ValCH_2Cl has been shown to be orally active in providing protection against induced emphysema in rats.[17] However, there is considerable question whether such reactive alkylating agents could be used in the treatment of emphysema in man. The animal studies have however shown that elastase inhibitors can be used to treat emphysema.

At present better elastase inhibitors are needed. They should have a high degree of selectivity and minimal side reactions or toxic side effects. In addition, more studies on the effect of elastase inhibitors on a variety of animal emphysema models are needed. However, the overall prospects for the development of a synthetic elastase inhibitor for use in humans in the near future are quite good. In general the full potential of protease inhibitors has not yet been reached and the future will likely see the application of protease inhibitors to the treatment of variety of diseases and disorders.

Ackowledgements

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PEPTIDYL CHLOROMETHYL KETONES AND DIAZOMETHYL KETONES AS PROTEASE AFFINITY LABELS

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In the early days of the study of proteolytic enzymes it became apparent that the specificty of these enzymes could be determined by their action on simple peptides, possibly more readily than on proteins.[1] Even this simplification of a biological problem offered difficulties initially because of the primitive state of peptide chemistry, but it led to a major improvement, the introduction of the benzyloxycarbonyl-protecting group[2] and made possible the rapid expansion of peptide chemistry itself. In the intervening years many new proteases have been identified but the ability to define specificity is, in each case, an important consideration and simple substrates are still an important tool.

Later concerns with the mechanism of action of proteases led to the development of affinity labeling reagents. The conversion of a substrate carboxyl group to a bromo- or chloromethyl ketone group led to a new class of inactivators of serine proteases[3,4] which act in part like substrates forming initially a reversible complex with the protease. Within the complex an active center histidine residue normally positioned near the carboxyl group of the substrate to take part in proton transfer steps associated with catalysis becomes alkylated instead by the chloromethyl ketone.

Serine proteases are subdivided according to substrate specificity and it became evident that, although all serine proteases have an active center histidine[5], chloromethyl ketone derivatives of amino acids or peptides inactivate only the serine protease whose specificity is satisfied.[4,5,7,8] The possibility that affinity labeling reagents might be made so selective that they could provide new classes of therapeutic reagents was appreciated early in the application of this type of chemical modification to a variety of enzymes.[9,10]

Selective Inactivation of Trypsin-like Proteases

In the protease field, studies of trypsin-like enzymes in coagulation, fibrinolysis, hormone synthesis, and fertilization have recently provided a wealth of information which one can utilize to design affinity-labeling

inhibitors in the hope of achieving selectivity. These physiologically important proteases generally act on native protein substrates with relatively few sites of proteolysis. Some of these are indicated in Table I. We have synthesized and examined a number of tripeptides with a C-terminal chloromethyl ketone in which the amino acid sequence corresponds to a physiological cleavage site, as well as many variations, to determine to what extent one can selectively inactivate one of these and other trypsin-like enzymes.

Table I. Sequence at Substrate Cleavage Sites of Trypsin-like Proteases

Enzyme	Physiological Substrate	Sequence
Thrombin	Fibrinogen (human)	
	A-Chain	Gly-Gly-Val-Arg↓Gly-
	B-Chain	Phe-Ser-Ala-Arg↓Gly-
	Prothrombin (bovine)	Val-Ile-Pro-Arg↓Ser-
	Factor XIII (human)	Val-Pro-Arg↓Gly-
Plasma Kallikrein	Kininogen	Ser-Pro-Phe-Arg↓
Urokinase	Plasminogen	Cys-Pro-Gly-Arg↓Val-
Factor Xa	Prothrombin	Ile-Glu-Gly-Arg↓Ile-
Factors IXa and VIIa	Factor X	Gln-Val-Val-Arg↓Ile-
Proinsulin Converting Enzyme (unidentified)	Proinsulin	Pro-Gln-Lys-Arg↓Gly-

Thrombin

This important protease of coagulation produces as least four cleavages that have been characterized. Two of these have a Pro-Arg sequence at the cleavage site whereas the fibrinopeptides released have a Val-Arg- or Ala-Arg- sequence. Ile-Pro-ArgCH_2Cl inactivates thrombin readily, as shown in Figure 1 which represents a typical affinity-labeling experiment.[11] The enzymes and inhibitor are incubated in reaction mixtures and aliquots removed for assay, in this case with Z-Lys thiobenzyl ester[12] to observe the loss of activity as the enzyme is alkylated. The time-dependent decay is characteristic of affinity-labeling but in some cases may be due to other transformations.[13] In analyzing structure-function relationships of inhibitors of this type it is convenient to convert the observed rate constant for inactivation, k_{app}, to a 2nd order rate constant of inactivation, k_{app}/I, which permits comparison of experiments carried out under different conditions. It is important to avoid saturation effects where k_{app} is not proportional to changes in I concentration and to maintain I in excess over enzyme levels. Ile-Pro-ArgCH_2Cl has some

selectivity in the inactivation of thrombin since k_{app}/I is 20-fold that for kallikrein and 135-fold the rate for plasmin.

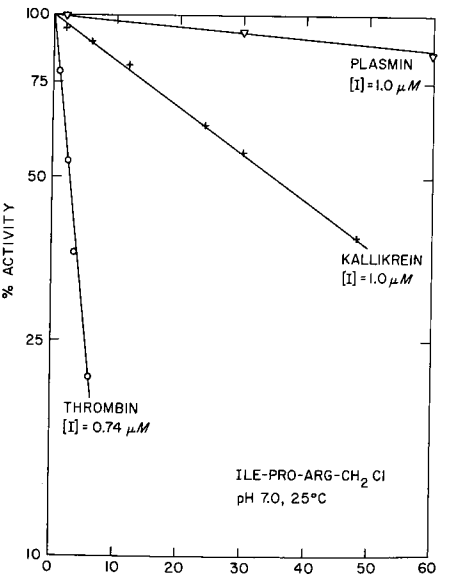

Fig. 1. Comparison of the susceptibility of thrombin, plasmin, and plasma kallikrein to inactivation by Ile-Pro-Arg-CH_2Cl at pH 7.0 and 25°C.

In comparing a number of reagents for their effectiveness in inactivating thrombin it can be seen (Table II) that the ones with a penultimate proline are more effective than the one with valine. In the case of the first pair the difference is $54/1.9 = 28$ fold in favor of Val-Pro-ArgCH_2Cl. To understand the basis for this difference a more detailed kinetic analysis is needed. In affinity labeling, the following expression is generally adequate to explain the observed phenomena:

$$E + I \rightleftharpoons \underset{\text{Complex}}{EI} \xrightarrow{k_2} \text{Inactive Enzyme}$$

The rate of the alkylation step, k_2, cannot be observed directly but is deduced from the dependence of the overall rate on inhibitor concentration analyzed by a graphical method which provides k_2 and K_i, the dissociation constant of the EI complex.[14] These analyses are not routinely made but were in the case described and the results (Table III) showed that k_2 was relatively constant and that the variation in sequence had largely influenced K_i. The reagent with proline is more tightly complexed to thrombin, accounting for the difference in reactivity, k_{app}/I, observed. Further exploration of structural changes involved the third residue and departed from natural sequences to some empirical

Table II. Relative Effectiveness of Chloromethyl Ketones Derived from Thrombin Substrates in the Inactivation of Thrombin

Affinity Label	Conc. of inhibitor (M)	t½ (min)	k_{app}/I $(min^{-1}M^{-1})10^{-4}$
Gly-Val-Arg-CH_2Cl	2.0×10^{-6}	18	1.9
Val-Pro-Arg-CH_2Cl	7.5×10^{-8}	17	54
Ile-Pro-Arg-CH_2Cl	7.5×10^{-8}	22	42
Val-Ile-Pro-Arg-CH_2Cl	7.5×10^{-8}	13	73

Table III. Comparison of Kinetic Constants of Gly-Val-Arg-CH_2Cl and Val-Pro-Arg-CH_2Cl in the Inactivation of Thrombin

Affinity Label	K_i (μM)	k_2 (min^{-1})
Gly-Val-Arg-CH_2Cl	13	0.30
Val-Pro-Arg-CH_2Cl	0.67	0.31

changes. We have observed that D-Ala-Ala-LysCH_2Cl was 20 times more effective than Ala-Ala-LysCH_2Cl in work carried out before arginine chloromethyl ketones became accessible and turned again to the introduction of a D-residue in the new series. D-Phe was chosen on the basis of its favorable influence in peptides containing a C-terminal arginine aldehyde described by Bajusz and coworkers.[5] D-Phe-Pro-ArgCH_2-Cl was found to be an extremely effective inactivator of thrombin with a rapid action on the pure enzyme even at 10^{-10} M (Figure 2).[16] It is active against other trypsin-like enzymes also, but the rates are at least three orders of magnitude less (Table IV); thus this inhibitor has a high degree of selectivity. Kinetic analysis of the interaction with thrombin has not been easy. The 2nd order rate constant for inactivation is a very reproducible observation but the determination of k_2 and K_i are more difficult. However, we have evidence that this inhibitor differs from the other affinity labels for thrombin in having an elevated k_2 (4.5 min. $^{-1}$) as well as an increased affinity ($K_i = 6.3 \times 10^{-9}$ M) by one method of analysis. On acetylation of the free amino group, a considerable loss of activity resulted amounting to 99.5%. This derivative is still active, comparable to the thrombin-directed inhibitors with a natural configuration in this position. A great loss in activity has not been encountered on elongation of peptides or aginine chloromethyl ketone not containing a terminal D-residue and the results suggest a possible conformation change in the complexed enzyme increasing the reactivity of the histidine residue. In

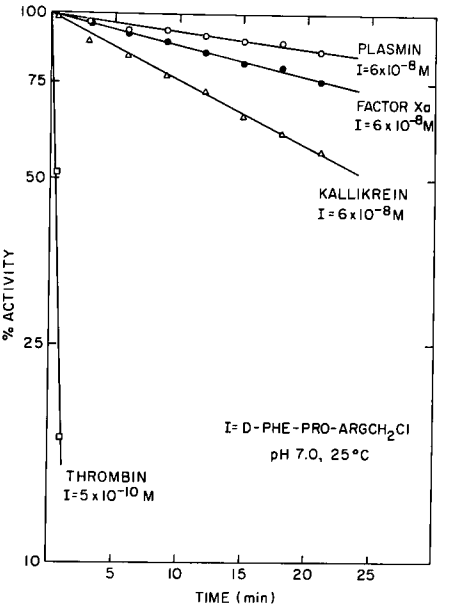

Fig. 2. Selective Inactivation of Thrombin by D-Phe-Pro-ArgCH$_2$Cl

the case of the corresponding arginine aldehyde derivative, acylation (Boc- or Bz-) has a minor effect,[15] indicating a lack of parallelism in the structure-activity relationships between the two series of inhibitors

Table IV. Selectivity of D-Phe-Pro-ArgCH$_2$Cl as a Protease Inhibitor

Protease	Concentration of affinity label	$t½$ (min)	$k_{app/I}$ (M^{-1} min^{-1} x 10^{-4})	Relative rate of inactivation
Thrombin	5×10^{-10}	1.2	115000	185000
Plasma kallikrein	6×10^{-8}	24.3	47.5	77
Factor Xa	2×10^{-7}	23.25	14.9	24
Plasmin	5×10^{-7}	32.3	4.30	6.9
Urokinase	5×10^{-6}	22.3	0.62	1

Some preliminary pharmacological observations have been made on the properties of D-Phe-Pro-ArgCH$_2$Cl by Collen and coworkers.[17] The LD_{50} in mice was found to be more than 50 mg per kg (I.V. administration). A very small fraction of this dose (10^{-4}) is capable of rapidly inactivating thrombin *in vivo* and producing a powerful anticoagulant effect. Studies in rabbits defined conditions in which the defibrination caused by initiating coagulation with a thromboplastin infusion could be prevented by the inhibitor. The prothrombin level dropped. Therefore the inhibitor was acting *in vivo* to block the thrombin that was formed in agreement with its *in vitro* action. The inhibitor disappears rapidly from the blood after a single injection. It was con-

cluded that the substance might be clinically useful in acute defibrination syndromes or in patients with low antithrombin-III levels likely to render heparin ineffective.

Plasma Kallikrein

The kallikreins (plasma and tissue enzymes exist) act on kininogens to liberate bradykinin and related hormones that have in common a C-terminal Pro-Phe-Arg sequence. Consequently Pro-Phe-ArgCH_2Cl is an alkylating agent targeted for this class of enzymes. The plasma enzyme is extremely sensitive to the reagent (Figure 3) while other trypsin-like enzymes are less so.[18] The effect of sequence on selectivity is clear since thrombin is now considerably less reactive; therefore the results described earlier cannot be ascribed to a unique susceptibility of thrombin to arginyl chloromethyl ketones. A kinetic analysis of the selectivity of Pro-Phe-ArgCH_2Cl indicated that it is due largely to a greater affinity for plasma kallikrein than other enzymes, that is, selectivity is a reflection of K_i.

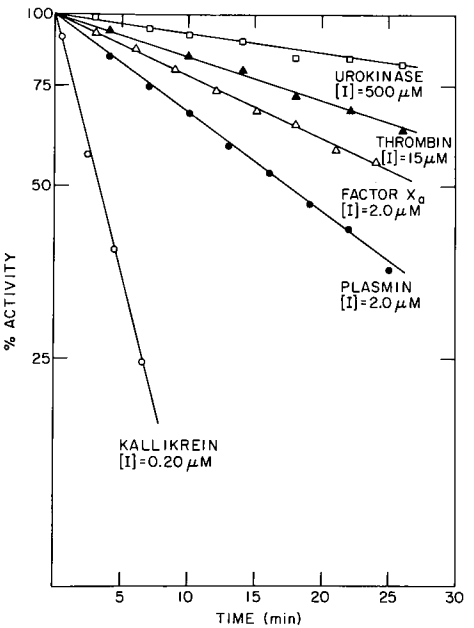

Fig. 3. Selective Inactivation of Plasma Kallikrein by Pro-Phe-ArgCH_2Cl

Other Trypsin-like Enzymes

The approach described has been applied to other trypsin-like proteases including urinary kallikrein[19], Factor Xa [20], urokinase[21], tissue

plasminogen activator[22], and acrosin.[23] In some cases the degree of selectivity was not as great as achieved above but in many cases the range of reactivities amounted to 10^5. It is this range that makes selectivity possible. The reagents are often a million-fold more effective than the parent trypsin inactivator, TLCK (tos-LysCH_2Cl).[6] K_i values of 10^{-6} or better have been encountered for the reversible phase of binding and further improvement can undoubtedly be achieved in this step.

The development of chromogenic substrates, tripeptide aldehydes, and peptidyl chloromethyl ketones have in common the utilization of normal biological recognition for complex formation. However, the subsequent steps, that is, catalysis, hemiacetal formation, or histidine alkylation have different requirements and undoubtedly are influenced by the configuration of site chains within the complex. Consequently an occasional lack of correlation as noted above and in reference 24 is probably not remarkable.

Thiol Proteases

In discussing even in this limited treatment some of the prospects for the usefulness of proteinase inhibitors, attention should be paid to a lack of specificity in a mechanistic sense. Peptidyl chloromethyl ketones and aldehydes act on both serine and thio proteases[25,26] although the peptidyl portion can modulate the reactivity within each class. Occasionally, before enzymes are characterized, their importance can be deduced from the favorable effects of such inhibitors as in the observations on blocking tumor promotion[27] or radiation-induced malignant transformation.[28] However, the enzymes involved may be either serine or thiol proteases if they are proteases at all. Because of this ambiguity and the emergence of a growing number of thiol proteases of interest in their own right, we have given attention to the development of inhibitors which act only on thiol proteases. For this purpose the diazomethyl ketone group was chosen for the covalent bond forming step on the basis of earlier work.[29] This group acts by a different mechanism than the chloromethyl ketones, the pH dependence for alklylation of the thiol group shows an increasing rate with a drop in pH[29] in contrast to chloromethyl ketones.[26] Apparently a proton is picked up from the thiol group generating the unstable alkyl diazonium ion which alkylates the thiol group with loss of nitrogen. It was shown with the plant protease, papain, that the reaction with Z-Phe-PheCHN_2 occurs stoichiometrically at the thiol group.[29] Z-PheCHN_2 which, having a phenylalanine ring, satisfies the specificity of chymotrypsin, does complex with this serine protease, $K_i = 2.5 \times 10^{-4}$, but

without inactivation. The proton transfer may take place, judging from spectral changes but no alkylation step occurs.[30] In fact, in the case of other diazomethyl ketone derivatives whose amino acid portion satisfies the specificity of other serine proteases such as trypsin or elastase, the reagents are ineffective on these enzymes although capable of inactivating thiol proteases.[31] This type of inhibitor has been found to inactive only thiol proteases.[31] As one might expect, the peptide portion influences the reactivity of the inhibitor. Thus, Z-Phe-AlaCHN$_2$ rapidly inactivates cathepsin B even at 10^{-7} M[32]; this sensitivity is in accord with the specificity of cathepsin B which positions its substrates with a hydrophobic residue penultimate to the cleavage site. The reagent has a K_i of 1.7 μM for the enzyme. Additional examples have now been accumulated and are presented in Table V. These demonstrate that a reagent that satisfied specificity as in the cases of clostripain, cathepsin B, cathepsin C, or the low M.W. post-proline endopeptidase[33] is very effective in contrast to a second reagent which is relatively ineffective. These reagents and other members of this class should help clarify the role of these enzymes *in vivo*.

Table V. Affinity Labeling of Thiol Proteases with Peptidyl Diazomethyl Ketones

Thiol Protease	Proteolytic Action	Inhibitor	K_{app}/I $M^{-1}\,min^{-1}$	Reference
Clostripain	-Arg↓-X-	Z-LysCHN$_2$	28,000	a
		Z-Phe-AlaCHN$_2$	3	a
Cathepsin L	Endopeptidase	Z-Phe-PheCHN$_2$	4×10^6	b
Cathepsin B	-Phe-Ala↓-	Z-Phe-AlaCHN$_2$	75,000	a
		Z-Phe-PheCHN$_2$	11,600	a
		Z-Ala-Ala-ProCHN$_2$	70	a
Cathepsin H	Aminoendopeptidase	Z-Phe-AlaCHN$_2$	36	b
Cathepsin C	H$_2$N-X-Y↓-	H$_2$NGly-PheCHN$_2$	1×10^6	a
		Z-Phe-AlaCHN$_2$	10^3	a
Post-Proline	X-Pro↓-Y-	Z-Ala-Ala-ProCHN$_2$	3.5×10^{-5}	c
Endopeptidase		Z-LysCHN$_2$	0	c

(a) Ref. 31; (b) Kirschke and Shaw, submitted; (c) Green and Shaw, submitted.

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THE DESIGN OF A PEPTIDE AFFINITY LABEL FOR BOVINE CARDIAC MUSCLE ADENOSINE 3′, 5′-MONOPHOSPHATE DEPENDENT PROTEIN KINASE

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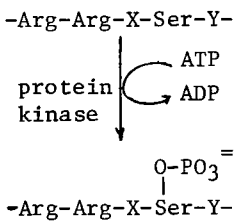
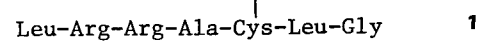
Protein kinases are known to be key enzymes involved in the initiation of many important biological processes, including hormone expression, glycolysis, and possibly, cell division and viral transformation. We are currently interested in developing specific modifying and inactivating agents for the kinases. In our initial studies we have chosen to work with 3,5′-cyclic adenosine monophosphate (cAMP)-dependent protein kinase since it is among the best characterized and most easily purified. This enzyme is a tetramer with a molecular weight of approximately 200,000, consisting of two catalytic subunits and a regulatory dimer. Addition of cAMP activates the enzyme and allows the isolation of the catalytic monomers (MW $\simeq$ 40,000) as a result of dissociation of the holoenzyme. The latter subunits appear to be capable of expressing the full catalytic activity of this kinase. Therefore, to simplify the purification of the enzyme and the analysis of the modification of catalytic activity, the isolated catalytic subunit was used in the studies which we report.

Protein kinase catalytic subunit phosphorylates peptides containing the sequence -Arg-Arg-X-Ser-Y- (where X and Y can be a variety of amino acids) according to the following scheme.[1,2,3]



By the systematic replacement of the amino acid residues of Arg-Arg-X-Ser-Y- with potentially labile amino acid analogs, it may be possible to create different types of affinity labels or suicide substrates for protein kinase. In the present article, we report the design and synthesis of peptide **1** which is capable of selectively modifying a single cysteine

residue of protein kinase and contains the group 3-nitro-2-pyridinesulfenyl (Npys) which has been used previously as a protecting group in peptide chemistry.[4]



1

Experimental Section

Leu-Arg-Arg-Ala-Cys(Npys)-Leu-Gly was synthesized by two methods. In the first, Leu-Arg-Arg-Ala-Cys-Leu-Gly, prepared by the Merrifield solid phase method, was allowed to react with 3-nitro-2-pyridinesulfenyl chloride. In the second method, Cys-(Npys) was allowed to react as the symmetric anhydride of its Boc derivative with the growing peptide chain during the solid phase synthesis. Cys(Npys) is stable to the conditions necessary for the Merrifield solid phase synthesis and to HF treatment at 0° C. Leu-Arg-Arg-Ala-Cys(Pys)-Leu-Gly was prepared by the reaction of Leu-Arg-Arg-Ala-Cys-Leu-Gly with 2,2′-dithiodipyridine. Pys is the 2-pyridinesulfenyl group.

The N and C-protected peptides Ac-Leu-Arg-Arg-Ala-Cys (Npys)-Leu-Gly-OEt and Ac-Leu-Arg-Arg-Ala-Cys(Npys)-Leu[^{14}C]Gly-OEt were synthesized by DCC/HOBt coupling in DMF of Ac-Leu-Arg-Arg-Ala to Cys(Npys)-Leu-Gly-OEt. The arginine residues of Ac-Leu-Arg-Arg-Ala were protected as the hydrochlorides during the segment coupling. Ac-Leu-Arg-Arg-Ala was prepared by Merrifield solid phase synthesis. Boc-Cys(Npys)-Leu-Gly-OEt and Boc-Cys(Npys)-Leu-[^{14}C]Gly-OEt were prepared using the polymer-bound *p*-nitrobenzophenone oxime resin according to the methodology described by DeGrado and Kaiser.[5] However, removal of the Boc group from Boc-Leu was effected with 4N HCl in dioxane rather than 25% trifluoroacetic acid in CH_2Cl_2, and Boc-Cys(Npys) was attached by direct DCC coupling rather than through the symmetric anhydride method. The Boc group was removed from the purified tripeptide with 4N HCl in dioxane.

All heptapeptides were purified by gel filtration and ion exchange chromatography. They were pure by the criteria of amino acid analysis and thin layer chromatography.

Boc-Cys(Npys)-Leu-Gly-OEt and its radioactive equivalent were purified by crystallization. Their purity was assessed by amino acid analysis, thin layer chromatography, 500 MHz NMR spectroscopy, optical rotation, and melting point.

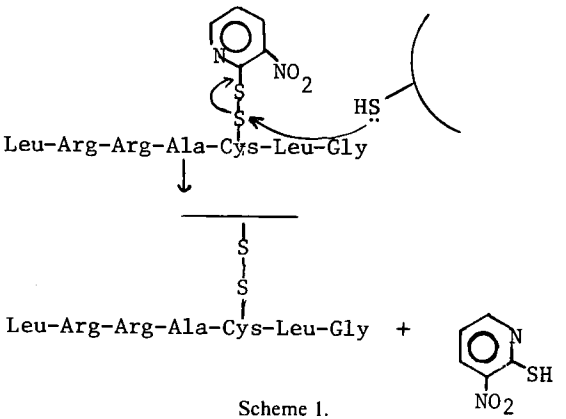
All modifications were done in Eppes[7] buffer (pH 8.5) with 1M KCl. Enzyme solution was incubated at 20° C and the reaction was initiated by

the addition of the modifying reagent. All spectra were recorded on a Cary 219 spectrophotometer.

Enzyme activity was assayed at 20° C as described in Armstrong *et al.*[6] Enzyme aliquots taken from a modification mixture were diluted 40-fold prior to assay.

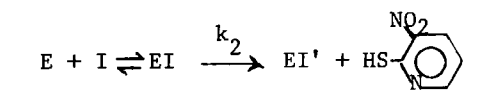
Results

Protein kinase is believed to have two active site sulfhydryl groups both of which can be modified by 2,2′-dithiobis (5,5′-dinitrobenzoic acid) (DTNB).[6] Peptide **1**, in which the modified Cys residue occupies the site of the reactive Ser residue of the usual peptide substrates, reacts with one sulfhydryl per catalytic subunit, probably according to Scheme 1.



Scheme 1.

The release of the Npys moiety is followed at 310.4 nm ($\Delta\epsilon = 3750$) and under conditions of peptide **1** in excess follows first-order kinetics. The reaction observed can be described by the kinetic scheme of equation 1 which leads to the rate expression of equation 2 and the relationship of the observed pseudo-first-order rate constant k_{obs} to the concentration of the peptide given in equation 3. In these equations $K_I = [E][I] / [EI]$, I represents the concentration of peptide **1**, and E_o is the initial concentration of the protein kinase catalytic subunit. k_{obs} was determined graphically.



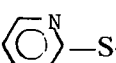
Eqn. 1

$$V = \frac{k_2[I_o]}{[I_o] + K_I} ([E]_o - [EI']) \qquad \text{Eqn. 2}$$

$$k_{obs} = \frac{k_2[I_o]}{[I_o] + K_I} \qquad \text{Eqn. 3}$$

Least squares analysis of a plot of $1/k_{obs}$ *vs* $1/[I_o]$ over the peptide concentration range 7 to 50 μM yielded values of $k_2 = 0.025 \pm 0.005$ sec^{-1} and $K_I = 47 \pm 12 \mu$M at pH 8.5 and 20.0°C. When the modification reaction was monitored by the loss of the catalytic subunit's phosphotransferase activity instead of the release of Npys, we obtained $k_i = 0.023 \pm 0.002$ sec^{-1} and $K_I = 37 \pm 6 \mu$M over the peptide concentration range 10 to 35μM. We were unable to detect any catalytic activity for the catalytic subunit of protein kinase after modification with peptide **1**, and the inactivation reaction can be completely reversed by treatment with dithiothreitol for one hour at 37° C. This observation suggests that a cysteine residue in protein kinase is modified.

While it seems possible that both cysteine residues of protein kinase are being modified by peptide **1** and that modification of either sulfhydryl group blocks access to the other sulfhydryl and causes inactivation of the enzyme, the following facts argue against this interpretation. The binding of peptide **1** appears to be tight and probably specific. The kinetics of the modification reaction are monophasic. Under identical conditions, the reaction of the catalytic subunit with excess DTNB is biphasic. After modification of the catalytic subunit with peptide **1**, only one sulfhydryl is observed to react at pH 8.5 with DTNB and the reaction obeys monophasic kinetics.

We have also prepared Npys-SCH_3,  —S-SCH_3 (Pys-SCH_3), and Leu-Arg-Arg-Ala-Cys(Pys)-Leu-Gly (peptide **2**). Peptide **2** reacts with both sulfhydryls of the catalytic subunit of protein kinase demonstrating that at least with this reagent, modification of one sulfhydryl does not prevent modification of the other. The release of the 2-thiopyridine group can be monitored at 343 nm ($\Delta\epsilon = 7060$), and the kinetics are rather complex. Thus, the kinetics of reaction have not yet been analyzed in detail, but the initial phase of the modification process occurs at roughly the same rate as that seen for peptide **1**.

Npys-SCH_3 and Pys-SCH_3 react analogously to peptides **1** and **2** giving in these cases the thiomethylated catalytic subunit. In both instances the reaction kinetics are biphasic with the Pys-containing compound reacting some ten-fold faster than that containing Npys.

We have also synthesized the labelled peptide Ac-Leu-Arg-Arg-Ala-Cys(Npys)-Leu-[^{14}C]Gly-OEt and its unlabelled analog. With this peptide we plan to monitor the stoichiometry of peptide incorporation onto the catalytic subunit and to identify which cysteine in the primary sequence is modified. Having developed selective peptide modifying agents for the sulfhydryl groups of the catalytic subunit of protein kinase,

we intend now to broaden the scope of peptide based modifying agents to include other types of affinity labels and/or suicide substrates.

Acknowledgements

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STRUCTURE-ACTIVITY RELATIONSHIPS AMONG THE TRIPEPTIDE ALDEHYDE INHIBITORS OF PLASMIN AND THROMBIN

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Introduction

The thrombin catalyzed transformation of soluble fibrinogen into fibrin polymer and the proteolytic degradation of fibrin clots by plasmin are two key events involved in hemostasis.

In medical treatment the coagulation step is frequently required to be inhibited but the process of fibrinolysis, rather stimulated. According to our previous observations[1,2] the thrombin-fibrinogen reaction could effectively be inhibited by D-Phe-Pro-Arg-H(GYKI-14166) and Boc-D-Phe-Pro-Arg-H(GHKI-14451). Further studies have revealed that the Boc derivative inhibits also fibrinolysis while the free peptide aldehyde has practically no effect on the plasmin degradation of fibrin gel formed in its presence. In order to find some explanation for these phenomena the structural requirements for peptide inhibitors of plasmin and thrombin were investigated.

Results and Discussion

Thrombin triggers the polymerisation of fibrinogen by cleaving the Arg-Gly bond in the Gly-Val-Arg-Gly-Pro-Arg-Val fragment, thereby releasing fibrinopeptide A and opening up the N-terminal Gly-Pro-Arg sequence of fibrin alpha-chain which is apparently essential for polymerization (cf.[1]).

D-Phe-*Val-Arg*-H, which contains the P_2-P_1 subsites of fibrinogen alpha-chain, shows 10 times lower potency than D-Phe-*Pro-Arg*-H which relates to the uncleaved N-terminal fragment of fibrin alpha-chain. The high affinity of the latter tripeptide aldehyde to thrombin could be explained by the fact that its Pro-Arg portion is identical with the P_2-P_1 subsites of two other native substrates of thrombin, i.e. factor XIII and prothrombin in which the Val/Ile-Pro-Arg--Gly/Ser fragments are cleaved.

Further information about the significance of the side chains and functional groups of D-Phe-Pro-Arg-H is obtained from the data given in Table I. Comparison of the inhibiting activity of Boc-D-Phe-Pro-Arg-H

Table I. Inhibition of the Thrombin-fibrinogen Reaction

Peptide inhibitor	Relative potency[a]
Boc-D-Phe-Pro-Arg-H	100
D-Phe-Pro-Arg-H	100
Gly-Pro-Arg-H	1
D-Phe-Gly-Arg-H	0.1
D-Phe-Pro-Agm	51
Boc-D-Phe-Pro-Agm	6

[a]Based on the concentration of D-Phe-Pro-Arg-H (0.2 μM) required for doubling the thrombin time of fibrinogen.

and its agmatine (Agm) analog revealed the importance of the aldehyde group but only in the presence of proper side chains since the two Gly-containing tripeptide aldehydes possessed extremely low activity. The relative high potency of D-Phe-Pro-Agm and the poor one of its Boc derivative pointed out the significance of the free amino terminus. It is very likely that D-Phe-Pro-Agm has only a minor effect on the proteolytic action of thrombin but can inhibit the polymerization of fibrin monomers by blocking the action of the amino terminal Gly-Pro-Arg sequence of fibrin involved in this step. In this way the free tripeptide aldehyde D-Phe-Pro-Arg-H may have dual action on the thrombin-firinogen reaction while Boc-D-Phe-Pro-Arg-H may only inhibit the cleavage of the Arg-Gly bond. In this context we may mention that an extremely loose gel is formed in the presence of D-Phe-Pro-Arg-H.

In contrast to thrombin and other "trypsin-like" plasma enzymes, plasmin has broad specificity.[3] Nevertheless, only about 20 of the 362 Arg/Lys-X bonds of fibrinogen are principally cleaved by plasmin and even these are hydrolysed at different rates as exemplified in Table II (cf. reference 4). Differences in the susceptibility of peptide bonds to plasmin may mainly be due to differences in accessibility, but some correlation can be observed between the hydrolysis rate of peptide bonds P_1-P_1' and the subsites P_2 and P_1. Lys at P_1 seems to be somewhat more susceptible than Arg since most of the bonds cleaved during 10-min digestion are Lys-X, and most of the fragments attacked by plasmin first contain an aromatic residue at P_2.

Table II. Principal Sites for Attack by Plasmin in Fibrinogen

Fragment P_3——P_2—P_1–/–P_1'				Digestion stage[a]	Fragment P_3——P_2—P_1–/–P_1'				Digestion stage[a]
Asn	Phe	Lys	Ser		Leu	Ile	Lys	Ala	middle
Glu	Trp	Lys	Ala		Thr	Gln	Lys	Lys	middle
Gly	Tyr	Arg	Ala	early	Thr	Leu	Lys	Ser	late
Ser	Tyr	Lys	Met		Gly	Val	Arg	Gly	late
Leu	Ile	Lys	Met		Gly	Pro	Arg	Val	very late

[a]Early, 10 min; medium, 30-60 min; late, 2 h or more.

Based on this interpretation and the difference between the antiplasmin activity of Boc- and H-D-Phe-Pro-Arg-H, we examined Boc protected tripeptide aldehydes in which the fragments of fibrin(ogen) cleaved during different stages were represented by P_2-P_1 portions: Phe-Lys(early), Leu-Lys(late) Pro-Arg(very late) (Table III). Of these, Boc-D-Phe-Pro-Arg-H, the peptide related to the least susceptible (or least vulnerable) fragment, proved to be the best inhibitor, while the second-best compound, Boc-Gln-Phe-Lys-H, is derived from one of the most sensitive (or most accessible) fragments, namely Asn-Phe-Lys-Ser. Among the Leu-Lys-H containing peptides we found extremely poor and rather good inhibitors depending on the N-terminal residues, which also influence the activity of the aldehyde containing Phe-Lys-H and Pro-Arg-H portions. Thus, any influence brought about by replacements is markedly affected by neighboring residues. Replacement of Gln by D-Phe next to Leu-Lys, Phe-Lys and Pro-Arg sequences altered the inhibiting activity by factors of 1579, 3.1 and 0.05, respectively. On the other hand, Arg and Lys at P_1 are equally suitable in the presence of Boc-Gln-Leu but Arg is 31 times better than Lys when preceeded by Boc-D-Phe-Pro. These findings give evidence that some interdependence exists among the side chains of peptides (cf. reference 5). Consequently, no generally valid structure-activity relationships can be formulated. According to this study the aromatic residue Phe is about five times more favorable than Leu in the sequence Boc-Gln-X-Lys-H, but we cannot exclude the existence of an inhibitor, *O*-Y-Leu-Lys-H, possessing much higher potency than the corresponding *O*-Y-Phe-Lys-H. Nevertheless, it

Table III. Inhibition of the Plasmin-fibrin Reaction

Peptide aldehyde	I_{50}^{a}	Relative potency
Boc-D-Phe-Leu-Lys-H	834	0.01
Boc-D-Gln-Leu-Lys-H	24	0.3
Boc—Gln—Leu-Lys-H	0.528	12.9
Boc—Gln—Leu-Arg-H	0.548	12.4
Boc-D-Phe-Phe-Lys-H	0.349	19.5
Boc—Gln—Phe-Lys-H	0.113	60.2
Boc-D-Phe-Pro-Arg-H	0.068	100
Boc—Gln—Pro-Arg-H	1.408	4.8
Boc-D-Phe-Pro-Lys-H	2.148	3.2
D-Phe-Pro-Arg-H	10	0.7

[a]Molar concentration (μM) required for doubling the hydrolysis time of fibrin gel.

is very likely that the plasmin-fibrin reaction can only be retarded by acyl (e.g. Boc) tripeptide aldehydes while the thrombin-fibrinogen reaction can effectively be inhibited by a free tripeptide aldehyde, too. Due to this difference in structural requirements D-Phe-Pro-Arg-H is a rather selective inhibitor of the latter process.

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PEPTIDE SUBSTRATES AND INHIBITOR OF COMPLEMENT ENZYMES

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The Complement Enzyme System of the Inflammatory Response typically operates in a complex environment of cells, their products, and plasma (or other body fluids).[1] In order to define the role of a particular complement enzyme in such an environment, it is necessary to develop simple, rapid, selective methods of assaying and/or inhibiting its activity, without affecting other enzymes in the mixture. We have recently shown that a tripeptide derivative, Boc-Leu-Gly-ArgAMC, based on the structure of the complement protein C5 (Table I), is a substrate for the complement enzymes C4b2a, CVFBb, and Cls.[2] It is possible to assay these purified complement enzymes by following the fluorescence (ex. 380 nm em. 460 nm) or absorbance (370 nm) of the liberated 7-amino-4-methyl coumarin (AMC). The fluorescence assay is more rapid than complex hemolytic assays for complement components,[3] and more sensitive than following the release of methanol from the previously-available peptide substrate[4] for the C3/5 cleaving enzymes C4b2a and CVFBb.

Table I. Sequences of Peptide and Protein Substrates and Inhibitor

human C3	His	Leu	Gly	Leu	Ala	Arg	Ser	C3 Convertase
human C5	Asp	Met	Gln	Leu	Gly	Arg		C5 Convertase
prothrombin	Ala/Ser	Ala/Tyr	Ile	Glu	Gly	Arg	Thr/Ile	Xa
		Boc	Leu	Gly	Arg	AMC		
	Boc	Nle	Gln	Leu	Gly	Arg	AMC	
leupeptin		Ac	Leu	Leu	Arg	CHO		

Leupeptin Inhibits CVFBb

The availability of this fluorescence assay has made it practical for us to begin a line of investigation to identify effective inhibitors for CVFBb. We have chosen to focus our initial attention on this C3/5

cleaving enzyme as cleavage of C3 is the first common step following the two major pathways of complement activation.[1] The first inhibitor we tested was the arginal-containing peptide leupeptin[5] (Table I), which, at 30 mM, had been found to inhibit Alternative Pathway-mediated lysis of paroxysmal nocturnal hemoglobinuria-like cells.[6] From the intersection of the lines on the Dixon Plot[7] (Figure 1) we can see that the K_I for leupeptin with CVFBb is 40 μM. This low K_I is surprising in light of the reported inability of 1 mM leupeptin to inhibit the Alternative Pathway.[8] This discrepancy may be due to the involvement of the Alternative Pathway enzyme C3bBb in a positive feedback loop in plasma which continually generates more active C3bBb.

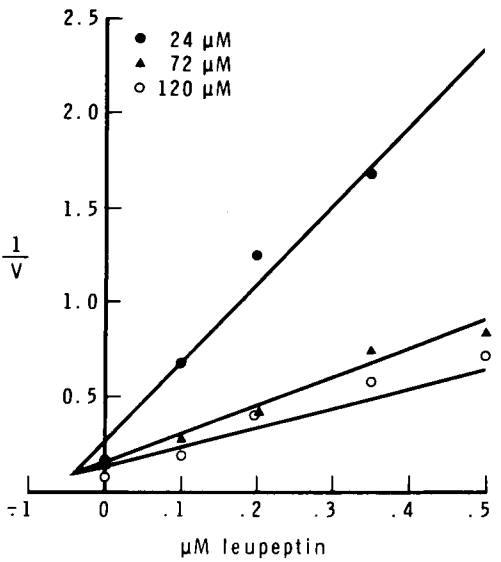

Fig. 1. Dixon plot for leupeptin inhibition of .13 nM CVFBb and 24, 72 and 120 μM Boc-Leu-Gly-ArgAMC.

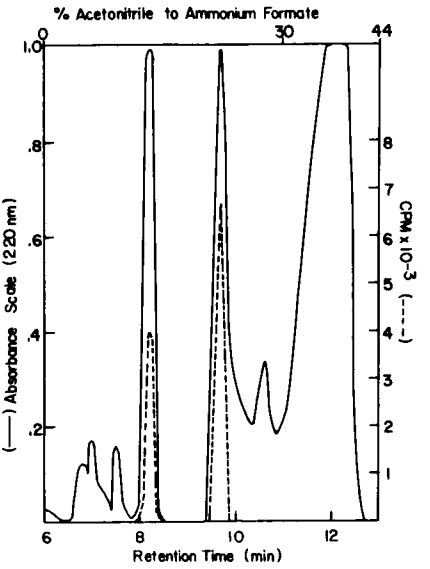

Fig. 2. HPLC of Boc-Nle-Gln-Leu-Gly-ArgAMC. 40° C Varian C_8 .01 N NH_4HCO_2 pH 4.2, 0-30% acetonitrile.

An Improved Substrate for CVFBb

A screening of some of the coagulation enzymes revealed that Boc-Leu-Gly-ArgAMC has a Km for Xa of approximately 125μM (Reference 9 and unpublished observation), which is comparable to the Km for CVFBb. Therefore, we decided to work to increase the specificity of the substrate for CVFBb by lengthening it to Boc-Nle-Gln-Leu-Gly-ArgAMC. As can be seen in Table I, this pentapeptide mimics the sequence of C5, yet bears no further similarity to Xa's natural substrate,

prothrombin. Thus we hoped that the longer substrate would have improved interactions with the active site of CVFBb, but not with Xa.

Synthesis of Boc-Nle-Gln-Leu-Gly-ArgAMC

Boc-Nle-Gln^{3}H-Leu-Gly was synthesized stepwise on the solid phase, and cleaved with HF.[10] (Hydroxybenzotriazole was added to protect the Gln sidechain). The peptide was purified using LH-20 in 10-50% methanol, Altex C_8 in .01 N $(NH_4)_3PO_4$ pH 4.2 in 0 to 15% acetonitrile, and desalted on P-2 in 10% methanol. The Boc group was reintroduced at the amino terminus using "Boc-on".[11] Boc-NleGly-Leu-Gly was dissolved in acetonitrile containing a small amount of triethylamine. The carboxyl group was activated by the addition of equimolar amounts of N,N′ disuccinimidyl carbonate and pyridine.[12] Arg-AMC was dissolved in a 1:1 v/v mixture of water and acetonitrile. An equimolar amount of *p*-toluene sulfonic acid was added to aid solubility of the unprotected arginine derivative (unpublished). The two solutions were mixed, carefully adjusted to pH 9.5 (2N NaOH) and reacted 1 hour at room temperature (for workup see Figure 2).

Comparison of the Tri and Penta Peptide Substrates

It was hoped that the longer sequence would bind more tightly to, and exhibit a lower Km for, CVFBb but not for Xa. As can be seen in Table II, this was in fact the case; the ratio of the Km for Boc-Nle-Gln-Leu-Gly-ArgAMC with CVFBb to the Km of Boc-Leu-Gly-ArgAMC with CVFBb was .03. The longer peptide provided the added bonus that it was actually a worse substrate for factor Xa than was Boc-Leu-Gly-ArgAMC as the Km rose by a factor of 100. Thus, the longer substrate provides a 3000 fold decrease (improvement) in the ratio of the Km values for CVFBb/Xa.

Table II. Comparison of Km and K_{cat}

		Xa	CVFBb
Km	$\frac{\text{BocNleGlnLeuGlyArgAMC}}{\text{BocLeuGlyArgAMC}}$	90	.03
k_{cat}	$\frac{\text{BocNleGlnLeuGlyArgAMC}}{\text{BocLeuGlyArgAMC}}$	.96	.10
k_{cat}/Km	$\frac{\text{BocNleGlnLeuGlyArgAMC}}{\text{BocLeuGlyArgAMC}}$	.01	3.

Lengthening the peptide had no effect on the turnover number (k_{cat}) with Xa; however, the longer substrate turned over only one-tenth as

rapidly with CVFBb as did the shorter substrate. Due to this loss in turnover the net gain in the specificity parameter k_{cat} / Km upon lengthening the peptide was 300 times. This gain was made up of a large decrease for the competing enzyme Xa (.01), and a small net increase for CVFBb.

Summary

By following the liberation of the fluorescent compound 7-amino 4-methyl coumarin (AMC) from Boc-Leu-Gly-ArgAMC, we were able to demonstrate that leupeptin is an effective inhibitor of CVFBb (a stable form of the Alternative Complement Pathway enzyme C3bBb).

Although such as assay using the tripeptide is very useful for studying the purified complement enzymes, it could not be used to assay the enzyme in plasma as the coagulation factor Xa also cleaves this substrate. Therefore, a pentapeptide with a sequence based on that of CVFBb's natural substrate C5 was synthesized stepwise on the solid phase, and coupled to ArgAMC in solution; *p*-toluene sulfonic acid was added to help dissolve the unprotected arginine derivative.

Lengthening the substrate to the pentapeptide resulted in a 300 fold improvement in the specificity for CVFBb over Xa as assayed by the specificity parameter k_{cat} / Km. Therefore, the pentapeptide has a greatly improved ability to distinguish between these two enzymes.

Acknowledgements

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STUDY OF CRUCIAL COMPONENTS IN ENKEPHALINASE INHIBITORS AND SYNTHESIS OF PHOTOAFFINITY LABELS AND TRITIATED DERIVATIVES

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The dipeptidylcarboxypeptidase, enkephalinase, which cleaves the endogenous enkephalins, Met-E (Tyr-Gly-Gly-Phe-Met) and Leu-E (Tyr-Gly-Gly-Phe-Leu) between Gly^3 and Phe^4 seems to be specifically involved in the enkephalinergic transmission.[1] Indeed, this new metalloprotease appears located in the vicinity of enkephalins binding sites and its regional distribution parallels that of these opiates receptors.[2] These features were not found in the case of both brain aminopeptidases and angiotensin converting enzyme (ACE). This latter carboxydipeptidase also cleaves enkephalins at the Gly^3-Phe^4 level but the apparent affinity of [^{3}H]-Leu-E is almost 100 times lower for ACE than for enkephalinase.[2] In order to unambiguously demonstrate the biological significance of enkephalinase we have recently synthesized highly potent and specific inhibitors of this enzyme.[3,4] One of these compounds, THIORPHAN, (D,L,3-mercapto-2-benzylpropanoyl)-glycine, displays antinociceptive properties after intravenous administration in mice.[4] Therefore such inhibitors represent a new approach in the development of antinociceptive and psychotropic agents. The rational design of enkephalinase inhibitors have required: i) the precise determination of the subsites specificity of the enzyme allowing to select the more appropriate structures for specific enkephalinase recognition. So, very large differences between enkephalinase and ACE have been evidenced;[5,6] ii) the introduction of strong ligands of Zn into such structures[6] following the strategy already used in the case of ACE inhibitors.[7] In this work, some enkephalinase inhibitors were synthesized in order to design new peptidase-resistant compounds and to obtain probes for both purification of enkephalinase

and *in vitro* and *in vivo* studies on its brain localization and turn-over regulation.

Synthesis and Biological Properties of *retro-inverso* Dipeptides and of *retro*-Thiorphan

The Phe[4]-Leu[5] (or Met[5]) peptide bond of enkephalins is crucially involved in enkephalinase-recognition.[8] This was clearly established using modified dipeptides like L-Phe(Me)L-Ala and H_2N-CH($CH_2\phi$)-CH_2-S-CH_2-COOH. These compounds exhibit very low inhibitory potencies $IC_{50} > 100\ \mu M$ as compared to L-Phe-L-Ala, $IC_{50} \sim 1\ \mu$. In contrast the mixture of the diastereoisomers R,S and R,R of the *retro-inverso* analogues of L-Phe-Ala display an IC_{50} value $\sim 10\ \mu M$. This was interpreted by a topological analogy between the R,R isomer and the natural L-Phe-L-Ala substrate.[6] This hypothesis was now explored using two *retro-inverso* analogs of Phe-Gly synthesized by the method of Chorev *et al.*[9] As expected the (R) isomer ($IC_{50} \sim 15\ \mu M$) exhibits a potency 7 times greater than the (S) isomer ($IC_{50} \sim 100\ \mu M$). This provides evidence that the crucial components (Phe side-chain, NH_3^+ and COO^- groups) and the CONH or NHCO bonds are in similar spatial disposition in both (S) L-Phe-Gly ($IC_{50} \sim 3\ \mu M$) and its (R) *retro-inverso* analog. Starting from these findings, we have prepared the *"retro-Thiorphan"* which exhibits only a 3 to 4 times lower potency than its parent compound. [3] This is an interesting result according to the enhancement of resistance to proteolytic degradation of *retro-inverso* derivatives.[10] The structure of *retro*-Thiorphan is easily established using NMR spectroscopy because, as described for dipeptides[10], large changes occur on backbone protons in the *retro* derivative as compared to Thiorphan.

Synthesis of a Photoaffinity Label for Enkephalinase

Photoaffinity labels could be useful for both purification of enkephalinase and visualization of the enzyme in brain, a very important experiment for the study of the physiological implication of this enkephalin-degrading enzyme. The synthesis of the azido-Thiorphan is reported in Figure 1. It can be observed that the synthetic pathway used could permit the preparation of labelled photoaffinity probes with only two "hot" steps. The reversible inhibitory potencies of such derivatives ($IC_{50} \sim 5$ nM for Leu in place of Gly) are near those of Thiorphan itself. Preliminary results seem to indicate a photocovalent binding of such probes after irradiation.

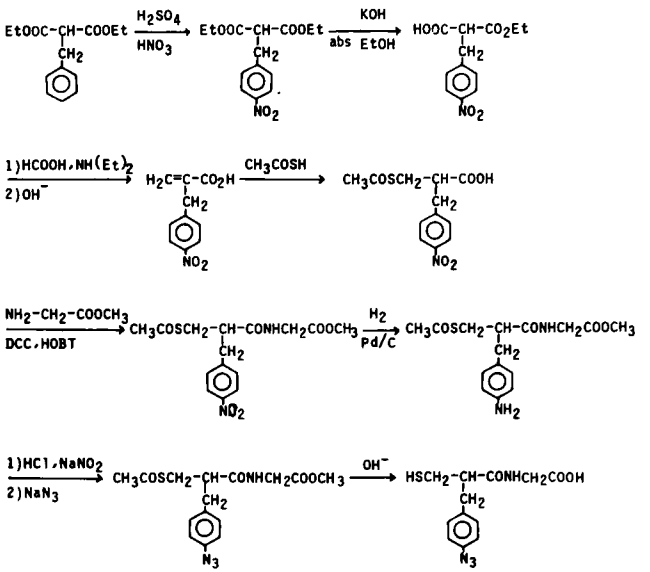

Fig. 1. Synthesis of azido Thiorphan: A photoaffinity label for enkephalinase.

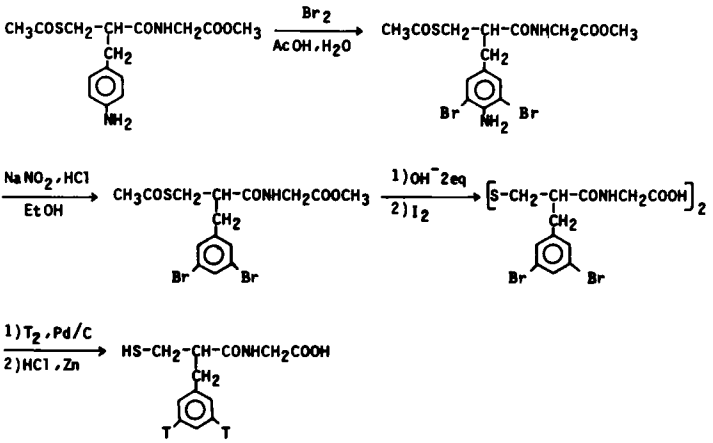

Fig. 2. Synthesis of tritiated Thiorphan.

Synthesis of Tritiated Thiorphan

The synthesis of radiolabelled Thiorphan reported in Figure 2 is a prerequisite to the study of enkephalinase at the molecular level. The bromination of the *p*-aminophenyl ring was performed as in reference.[11]

Furthermore the use of the disulfide analog as penultimate precursor allows simultaneous breakdown of S-S bond and tritiation in the last step.

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NONHYDROLYZABLE TRIPEPTIDE ANALOGS AS ANGIOTENSIN-CONVERTING ENZYME INHIBITORS

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Introduction

Angiotensin-converting enzyme (ACE), a zinc containing exopeptidase cleaves the C-Terminal dipeptide from a variety of peptides.[1] On the basis of a similarity with carboxypeptidase A, its mode of binding with peptide substrates has been considered[2] to be as shown schematically in Figure 1.

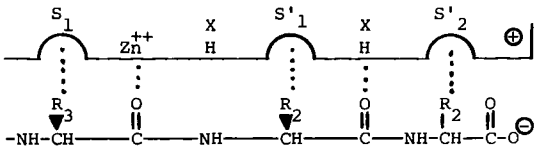
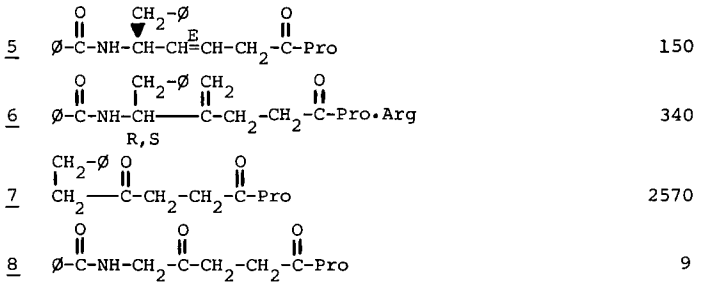


Fig. 1

In an ongoing program of exploration of the various ways of inhibiting ACE, one of our approaches has been the modification of the scissile amide bond of a tripeptide or its derivative to a nonhydrolyzable moiety.[3] Proceeding along the same line of thinking, Almquist *et al.*[4] have recently demonstrated that replacement of the scissile amide bond of substrate **1** by a nonscissile ketomethylene function leads to a potent ACE inhibitor (**2**). This paper describes other modifications (Table I) of the scissile amide bond of suitable tripeptides to nonhydrolyzable moieties.

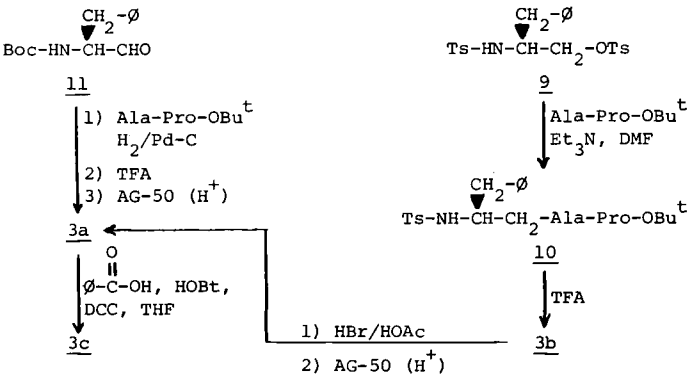
Table I

No.	Compound		I_{50} (μM)
1	Ø-C-NH-CH(CH$_2$-Ø)-C-NH-CH$_2$-C-Pro[5]		28
2	Ø-C-NH-CH(CH$_2$-Ø)-C-CH$_2$-CH$_2$-C-Pro		0.01
3	R-NH-CH(CH$_2$-Ø)-CH$_2$-NH-CH(CH$_3$)-C-Pro	3a: R=H	1350
		3b: R=-SO$_2$-(C$_6$H$_4$)-CH$_3$·TFA	560
		3c: R=-CO-Ø	150
4	R-NH-CH(CH$_2$-Ø)-CH$_2$-S-CH(CH$_3$)-C-Pro	4a: R=H	400
		4b: R=-CO-Ø	910



Synthesis

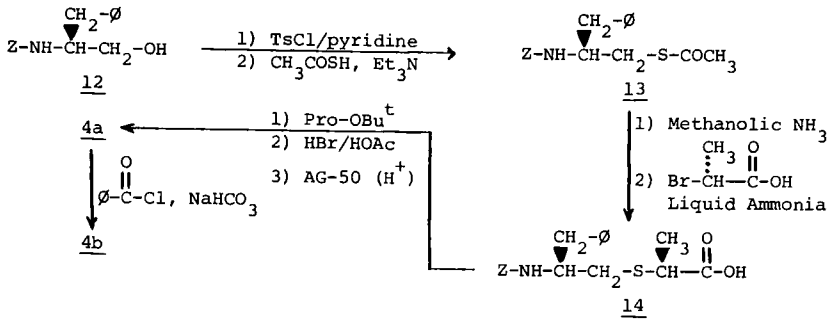
Modification of the scissile amide bond to a secondary amine to produce a "reduction analog" has been explored with substrates of several enzyme systems.[6-9] In order to explore the feasibility of such an approach for ACE inhibition, compounds **3a-c** were synthesized (Scheme I).



Scheme I.

Model experiments using L-alanine *t*-butyl ester showed that successful displacement of the tosylate, **9**, was critically dependent on the nature of the N-protecting group.[10] Displacement did not occur with both N-protection and as a leaving group (**9**) the secondary amine, **10**, was rotection; however, when the tosyl group was used for formed in good yield. This suggests that the reaction may involve an aziridine intermediate. X-ray crystallographic analysis of the product confirmed that the stereochemical disposition of the reduced phenylalanyl moiety was unchanged. In the other route, aldehyde **11** was prepared by the activated DMSO oxidation of *t*-butyloxycarbonyl-L-phenylalaniol.[11] Selective benzoylation of **3a** yielded **3c**.

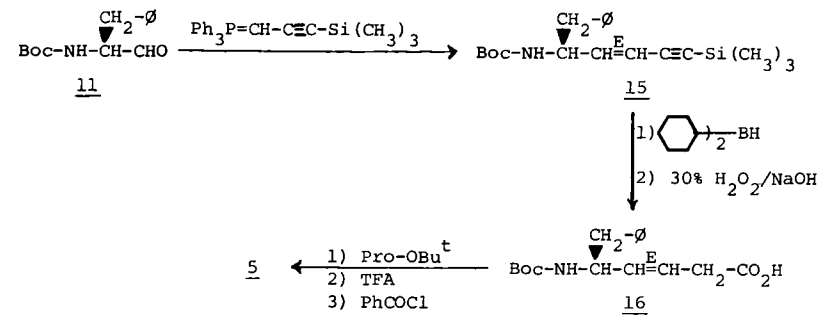
Stabilization of peptide analogs towards enzymatic hydrolysis by replacement of the amide bond with $-CH_2-S-$ are referred to as gap inhibitors.[12] The preparation of such analogs is depicted in Scheme II.



Scheme II

This route should be applicable to the preparation of peptide gap inhibitors of various sequences.

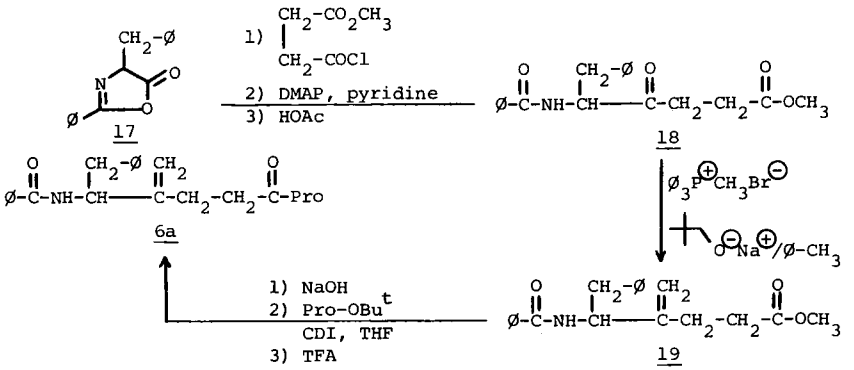
Additional examples of nonhydrolyzable analogs which also maintain the geometry of the peptide bond are represented by the olefins **5** and **6**. Use of *trans* olefins as isosteric replacements for scissile amide bond has been recently applied in the area of enkephalin analogs.[13] The route for the synthesis of the *trans* olefin (**5**) is depicted in Scheme III. The synthesis of the key intermediate, **16** is very similar to the approach used by Sammes *et al.*[13]



Scheme III

A second type of olefinic analog, exomethylene derivative, **6**, was synthesized as shown in Scheme IV. Ketone, **18**, was synthesized based on a recently published modification of the Dakin-West reaction.[14] Oxazolone, **17**, derived from benzoylphenylalanine undergoes O-acylation under kinetic conditions which rearranges to the thermody-

namically more stable C-acylated intermediate. Without isolation this intermediate is hydrolyzed and decarboxylated by brief treatment with acetic acid to yield **18**. Further elaboration yielded **6a** which was isolated as its L-arginine salt, **6.**



Scheme IV

This facile synthesis of the ketomethylene derivative **18** also led to a very convenient synthesis of compound **2** which was earlier synthesized by a very tedious route.[4] Compound **18**, after hydrolysis, was coupled to L-proline *t*-butyl ester. Acidolyis and separation of isomers by fractional crystallization yielded the ketomethylene derivative (**2**).

Biological Results and Discussion

The modification of the hydrolyzable amide bond to a ketomethylene moiety gave rise to a very potent ACE inhibitor. The increased inhibitory potency of this analog can be interpreted as due to (a) resistance to enzymatic hydrolysis by ACE; (b) specific interaction between the carbonyl group and functional residues at the active-site of the enzyme; or (c) a combination of both. The nonhydrolyzable analogs described in this study retain a number of the enzyme-binding functional groups of the peptide substrate (Figure 1), *e.g.*, C-terminal carboxyl, ultimate amide bond, side chains for secondary interactions (R_1, R_2, R_3), etc. In the case of reduction analogs (**3a-c**) some of the potential enzyme binding interactions of the scissile amide bond in the transition state, *e.g.*, a tetrahedral nitrogen are present. The olefinic analogs by retaining the sp^2 geometry at the carbonyl carbon site might facilitate the interactions of the functional groups with the enzyme beyond the sicissile amide bond. Ketomethylene analogs **7** and **8** which are synthesized to study the nature of the interaction of compound **2** with ACE exhibited very poor

inhibitory activities. The contribution to activity from the benzamido moiety (**8**) is relatively more important than that of the phenylmethyl substitution (**7**).

Since none of these nonhydrolyzable peptide analogs[15] studied display inhibitory activity similar to that of the ketomethylene analog **2**, it is tempting to speculate that in this analog there is a specific interaction between the ketone carbonyl and residues at the active-site of ACE, *e.g.*, carboxylate group and zinc ion. The presence of the phenylmethyl group, and particularly the benzamido moiety aid in this interaction.

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15. I_{50} values for Phe-Ala-Pro and Bz-Phe-Ala-Pro were determined to be 4.2 μM and 3.2 μM, respectively. The enzyme used in our study was an extract of ACE isolated from rabbit lung. I_{50} values represent the concentration inhibiting 50% of the activity of ACE at pH 8.3 in 100 mM potassium phosphate buffer containing 300 mM NaCl with the substrate Hip-His-Leu at a concentration of 5 mM.

RENIN INHIBITION BY CONFORMATIONALLY RESTRICTED SUBSTRATE ANALOGS

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Introduction

Renin is a very specific enzyme, being able to hydrolize only the Leu-Leu bond in its plasma protein substrate or in synthetic peptides containing at least the (6-13)-octapeptide sequence His-Pro-Phe-His-Leu-Leu-Val-Tyr.[1] This strict requirement for a minimum substrate size suggests the possibility of conformational factors playing a role in renin specificity. We have previously explored the solution conformation of the N-terminal tetradecapeptide segment of renin substrate through electrometric titrations, infrared and circular dichroism spectroscopy and spectrofluorometry, and found evidence for antiparallel β structure.[2] A β-turn involving the sequence His-Pro-Phe-His was proposed, which was, besides being energetically favored,[3] stabilized by interaction between the two antiparallel β-nucleating sequences 3-5 (Val-Tyr-Ile) and 10-12 (Leu-Leu-Val), and by electrostatic interactions between the N-and C-terminal ends of the molecule. The importance of this conformation for renin specificity is suggested by kinetic data with synthetic substrate analogs.[1] The removal of the N-terminal residues Asp-Arg of the tetradecapeptide substrate led to an increase in K_m from 3.7×10^{-6}M to 2.8×10^{-5}M. Removal of the next two amino acids had no effect, while in the (6-14)-nonapeptide, K_m increased to 4.7×10^{-5}M. Further shortening by removal of residue 6 caused a very large loss in the substrate ability of the peptide. Thus, the features that would be expected to stabilize the proposed conformation of the substrate also are important for renin action.

In an attempt to obtain more information about the importance of substrate conformation for renin activity, we have synthesized conformationally restricted analogs of substrate peptides and investigated their interaction with the enzyme. To stabilize the proposed β-turn, a covalent bridge between residues 5 and 10 was introduced by replacing Ile^5 and Leu^{10} by a cystine residue, following the strategy used by Veber *et al.*[4] for somatostatin.

Materials and Methods

The compounds (Table I) were prepared by solid phase synthesis using the methoxybenzyl protection for cysteine. Cleavage with anhydrous hydrogen fluoride was followed by oxidation with potassium ferricyanide and purification by ion exchange chromatography and gel permeation until products were obtained that behaved homogeneously on paper electrophoresis (at three pH values) and thin layer chromatography (with three solvent systems) and, upon amino acid analysis, yielded amino acid ratios within 6% of the expected values. Hog renin (11 Goldblatt units/mg protein) and human renin (10.5 units/mg protein) were purified preparations kindly supplied by Dr. E. Haas. Enzyme incubations were done at 37° C in 0.1M phosphate buffer of pH 6.0 or 7.5 with the (1-14)-tetradecapapetide as substrate, in concentrations varying from 1 to 8 x 10^{-5}M. The final enzyme concentration was 0.087 units/ml for hog renin and 0.005 units/ml for human renin. The reaction course was followed by the TNBS reaction,[5] performed on aliquots removed from the reaction mixture at regular intervals. K_m values were obtained by the weighted regression method of Wilkinson,[6] from plots containing at least five data points, and K_i values were calculated in a similar way from plots obtained in the presence of the inhibitor under study.

Table I. Renin Substrate Analog Peptides

(1-14)-tetradecapeptide:
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser
(5-14)-decapeptide:
Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser
(Cys^5,Cys^{10})-(5-14)-decapeptide:
Cys-His-Pro-Phe-His-Cys-Leu-Val-Tyr-Ser
(Lys^4,Cys^5,Cys^{10})-(4-14)-undecapeptide:
Lys-Cys-His-Pro-Phe-His-Cys-Leu-Val-Tyr-Ser

Results and Discussion

The conformationally restricted analog (Cys^5, Cys^{10})-(5-14)-decapeptide was not a substrate for either hog or human renin and was a good inhibitor of both enzymes. In experiments done at pH 6.0, it inhibited hog renin with a K_i that was closer to the K_m value obtained with the

Table II. Kinetic Parameters for the Interaction of Hog Renin with Substrate and Inhibitor, pH 6.0

Compound	K_m ($\times 10^{-6}$ M)	K_i ($\times 10^{-6}$ M)
(1-14)-tetradecapeptide	8.2	—
(5-14)-decapeptide	48.0	—
[Cys^5,Cys^{10}]-(5-14)-decapeptide	—	17.1

(1-14)-tetradecapeptide than to that of the (5-14)-decapeptide (Table II). The same was observed in experiments done with human renin at pH 6.0 (Table III). However, the (Cys^5, Cys^{10})-decapeptide had very low solubility in water in neutral medium, and it was impossible to get good kinetic data with this peptide in experiments done above pH 6.0.

In an attempt to obtain a more soluble inhibitor, we have synthesized an analog containing an additional lysine residue in position 4. This was done on the basis of the great increase in solubility observed when a lysine residue was added to the C-terminus of the inhibitory analog (Pro^5, Phe^{10}, Phe^{11})-(5-13)-nonapeptide.[7] We have found that (Lys^4, Cys^5, Cys^{10})-(4-14)-undecapeptide also has good solubility in water, and it could be tested without difficulty as a renin inhibitor at pH 7.5, with an inhibitory constant that was also of the same order of magnitude as the K_m for the (1-14)-tetradecapeptide substrate (Table III)

Table III. Kinetic Parameters for the Interaction of Human Renin with Substrate and Inhibitor Peptides

Compound	pH	K_m (10^{-5} M)	K_i ($\times 10^{-5}$ M)
(1-14)-tetradecapeptide	6.0	4.0	—
	7.5	2.5	—
(5-14)-decapeptide	6.0	33.4	—
	7.5	35.0	—
[Cys^5,Cys^{10}]-(5-14)-decapeptide	6.0	—	5.3
[Lys^4,Cys^5,Cys^{10}]-(4-14)-undecapeptide	7.5	—	3.3

Our results are in agreement with the hypothesis that the β-turn proposed for the tetradecapeptide renin substrate is a favorable factor for

its interaction with the enzyme. However, other structural features may be important for promoting binding. This is indicated by the fact that replacement of the two leucine residues in positions 10 and 11 by phenylanine residues[7,8] yield inhibitory analogs whose K_i values are one order of magnitude lower than the K_m for the (1-14)-tetradecapeptide and the K_i for the disulfide analogs described here.

Acknowledgement

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INHIBITION OF PEPSIN BY A KETONE PEPSTATIN ANALOG. IMPLICATIONS REGARDING THE CATALYTIC MECHANISM

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Introduction

Pepstatin (**1**) (Table I), a pentapeptide isolated by Umezawa *et al.*[1] is a reversible, tight-binding inhibitor of carboxyl (acid) proteases. Pepstatin inhibits pepsin, renin, and cathepsin-D with dissociation constants on the order of 10^{-10} - 10^{-11} M.

The 3S hydroxyl group of the central statine residue has been shown to be essential for tight-binding, time dependent inhibition of pepsin by pepstatin and synthetic peptide analogs. The importance of this 3S hydroxyl group for binding led us to synthesize the keto-statine analog **3**, which has the 3S hydroxyl group of **2** replaced by a ketone functionality. Analog **2** serves as the ideal standard for analyzing the kinetic behavior of **3** because **2** shows tight-binding (K_I = 3 x 10^{-9} M), [2] time dependent inhibition analogous to that observed for pepstatin.

Table I

Structure[a]	K_I	Time Dependent Inhibition
(1) Iva-Val-Val-Sta-Ala-Sta[2]	5.7 x 10^{-11}M	yes, 0.02 sec^{-1}
(2) Iva-Val-Sta-Ala-Iaa[2]	3 x 10^{-9}M	yes, ~0.02 sec^{-1}
(3) Iva-Val-Sto-Ala-Iaa	6 x 10^{-8}M	No
(4) Iva-Val-dSta-Ala-Iaa[2]	> 3 x 10^{-6}M	No

[a] Abbreviations: Iva, isovaleryl; Iaa, isoamylamide; Sta, statine or 3S-hydroxy-4S-amino-6-methyl-heptanoic acid; Sto, 3-keto-statine or 3-oxo-4S-amino-6-methyl-heptanoic acid; dSta, deoxy statine or 4S-amino-6-methyl-heptanoic acid.

Keto-statine analog **3** was expected to serve as a probe of the catalytic mechanism for amide bond hydrolysis. A reasonable mechanism for the action of pepsin on substrates is given in Figure 1. Related mechanisms, with an enzyme bound water molecule acting as a nucleophile via general acid, general base catalysis to generate a tetrahedral intermediate, have been proposed for other acid proteases based on

X-ray diffraction studies.[3,4,5] It was of interest to determine if analog **3** could act as a mechanism based inhibitor. As a pseudosubstrate ketone **3** could be catalytically converted to a gem diol intermediate analogous to the transition state or tetrahedral intermediate for amide bond hydrolysis (Figure 1).

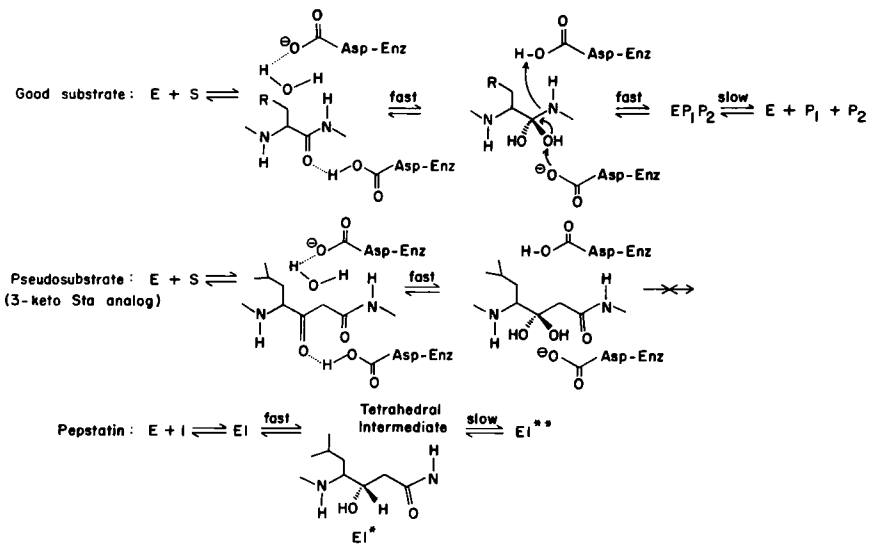

Fig. 1. Schematic representation of the relationships between proposed catalytic and inhibitory kinetic mechanisms. Top line, catalytic chemical and kinetic events associated with substrate hydrolysis by pepsin. Middle line, possible mechanism of inhibition by ketone analog 3. Bottom line, kinetic events associated with the inhibition of pepsin by pepstatin.

Results

The keto-statine analog **3** was synthesized from Boc-Val-(3S,4S)-Sta-Ala-Iaa[7] using activated dimethyl sulfoxide[6] as outlined in Figure 2.[8] Inhibition of pepsin by keto-statine analog **3** was studied using a synthetic heptapeptide[9] as described previously.[2] Analysis of the inhibition data[10] revealed that **3** is a potent competitive inhibitor of pepsin ($K_I = 6 \times 10^{-8}$ M).

The kinetic behavior of **3** is compared to the corresponding statine containing peptide **2** in Figure 3. The reaction traces represent the formation of a chromaphoric peptide product by the action of pepsin on substrate. The data were generated by addition of enzyme to the reaction mixture. Figure 3 shows that ketone **3** binds rapidly to pepsin to produce a linear (steady state) initial velocity. By contrast the statine-containing analog **2** exhibits a lag-transient or slow approach to an apparent steady state indicated by the dashed line. Keto-statine analog **3** is therefore not a

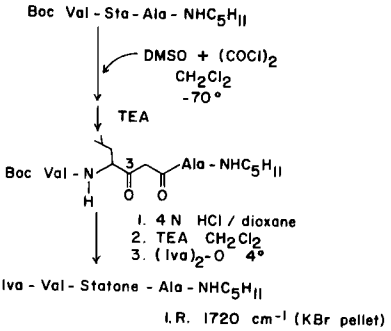

Fig. 2. Synthetic scheme used to prepare ketostatine analog **3**.

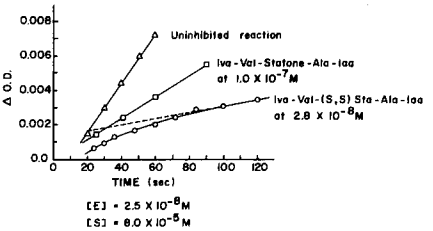

Fig. 3. Time course of inhibition of pepsin by analogs **2** (O) and **3** (□) versus control (△). Ketone **3** and control show a linear (steady state) initial velocity between 20-60 seconds. Analog **2** requires about 80 seconds to reach an apparent steady state designated by the dashed line. (Porcine pepsin, pH 4.0, 0.04 M formate, 25°).

time dependent inhibitor while the statine containing model peptide **2** is time dependent in its binding to pepsin ($k_{obs} \simeq .02$ sec^{-1}).[2] Recently an additional but much faster first order process ($k = 600$ sec^{-1}) was observed by stopped flow kinetics[11] for the binding of streptomyces pepsin inhibitor (a naturally occurring pepstatin analog in which the N-terminal Iva group of pepstatin is replaced by an acetyl group) to pepsin. The available kinetic data discussed here, along with kinetic studies of additional synthetic peptide analogs of pepstatin[12] and difference spectroscopy techniques,[13] are consistent with the minimum kinetic mechanism given by Equation 1. In this mechanism pepstatin and synthetic peptide analogs bind to pepsin as multiple enzyme-inhibitor complexes.

$$E + I \rightleftharpoons EI \overset{\text{fast}}{\rightleftharpoons} EI^* \overset{\text{slow}}{\rightleftharpoons} EI^{**} \qquad \text{(Eqn. 1)}$$

collision intermediate tightened

The ~ 20 fold tighter binding to pepsin of **2** over ketone **3** can be interpreted in terms of Equation 1. Analog **2** can achieve a "tightened complex," EI**, by a slow, first order process (Figure 3) while ketone analog **3** does not proceed beyond a "intermediate complex," EI*.

The ketone analog **3** then appears to bind to pepsin as a stable "intermediate complex." Two possible structures for this complex are the ground state, ketone form which is hydrogen bonded to a catalytic aspartic acid, or a gem idol form which clearly mimics the transition state or tetrahedral intermediate proposed for amide substrates (Figure 1). The latter possibility is preferred because (a) the K_I of **3** is much lower (~ 500X) than K_M values observed for the best known pepsin substrates,[14]

(b) analog **3** is only about 20 fold less active than 3S hydroxyl analog **2** which can mimic the tetrahedral intermediate proposed for substrates (Figure 1), and (c) ketone **3** is a 50 fold stronger inhibitor than the deoxy statine analog **4** (Table I). Carbon 13 NMR experiments and X-ray diffraction studies are in progress to establish the nature of the bindng of **3** to pepsin.

The molecular events associated with the slow binding step of pepstatin and analogs are not well understood but it is reasonable to speculate that they may mimic events accompanying product release, the rate determining step for good pepsin substrates.[14]

In summary, we have synthesized a new ketone analog of pepstatin. It is a potent competetive inhibitor of pepsin. It appears likely that the ketone **3** inhibits pepsin in a mechanism based manner by acting as a pseudosubstrate or stable isosteric replacement of the scissle amide bond of substrates.

Acknowledgements

This work was supported in part by grants from NIH (AM 20100) and Merck Sharp and Dohme.

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8. i. DMSO, CH_2Cl_2, oxalyl chloride, -70°, 10 min. ii. Boc-Val-(3S, 4S)-Sta-Ala-Iaa, CH_2Cl_2, 5 min. addition, 20 min., -60°. iii. Triethylamine added at -60° and allowed to warm to room temperature. iv. Chromatography over silica gel. v. 4 N HCl, dioxane, 40 min. vi. Triethylamine, isovaleric anhydride, CH_2Cl_2, 5-10°, 18 hr. vii. Chromatography over silica gel: m.p. 182-185°, ^1H NMR (270 MHz), IR (KBr) 1720^{-1}, $[\alpha]_D^{24} = -92°$ (C = 0.1, MeOH).
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AMINO ACID SEQUENCE OF ANGIOTENSINOGEN AS A BASIS FOR THE SPECIES SPECIFICITY OF RENIN

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Renin (EC 3.4.99.19) is an aspartyl protease elaborated by the kidney which cleaves circulating angiotensinogen to yield angiotensin I. Subsequently this peptide is converted to biologically active angiotensin II. The role of angiotensin both in the maintenance of normal blood pressure[1] and the genesis of some types of hypertension[2] is well known.

Renin shows a marked specificity for substrates. It cleaves angiotensinogen[3] and a few analogs of this sequence,[4,5] but other proteins, such as hemoglobin or insulin, are not hydrolyzed. In addition, while human renin will cleave angiotensinogen from a variety of species, it has been known for 35 years that the reverse is not true. Only primate renin will generate angiotensin I from human renin substrate.[6]

A full understanding of the role of renin in the maintenance of blood pressure requires insight into the exquisite specificity of this enzyme. Research by Tewksbury and co-workers[7] has demonstrated that the amino acid sequence in the C-terminal region of the cleavage site is different from that of the equine or porcine[8] substrate (Figure 1). The leucylvaline sequence at the cleavage site of human substrate could account for the lack of cleavage by non-primate renin. Presumably, these renins will accommodate the leucylleucine sequence found in equine, porcine, or ratine[9] angiotensinogen but are unable to cleave the human sequence. Earlier researches of Poulsen *et al.*,[5] however, demonstrated that porcine renin will cleave the leucylisoleucine peptide bond found in some substrate analog inhibitors. To identify the sequence features responsible for the specificity of human and animal renins, a series of three peptides was prepared and tested as substrates for purified human[10] and dog[11] renin.

Human:	Asp-Arg-Val-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-
Horse:	——————————— Leu-Val-Tyr-Ser
Pig:	——————————— Leu-Val-Tyr
Rat:	——————————— Leu-Tyr-Lys

Fig. 1. N-Terminal amino acid sequences of angiotensinogen from various species.

Peptides were synthesized by solid-phase techniques[12] and purified to homogeneity by gel filtration and high pressure liquid chromatography. Peptides were homogeneous by HPLC, TLC and electrophoresis.

After cleavage of the peptide by the appropriate renin, the assay mixture was analyzed for angiotensin I by radioimmunoassay.[13] Cross reactivity with uncleaved peptide (0.5-1%) required cleavage of 10-15% of the synthetic substrate for accurate analysis. Data showed that all peptides were substrates for renin with normal enzyme kinetics. Data were analyzed by a Lineweaver-Burk plot and results are shown in Tables I and II.

Table I. Kinetic Parameters for the Cleavage of Synthetic Renin Substrates by Human Renin (pH 7.5)

		K_M (μM)	V_{max} *
1	ANGIOTENSIN I — Val-Ile-His	29	1.05
2	Leu-Ile-His	15	.89
3	Leu-Val-His	16	1.06
4	Leu-Val-Tyr-Ser	32	1.00

*Relative to the rate of cleavage of TDP (4) by human renin.

Table II. Kinetic Parameters for the Cleavage of Synthetic Renin Substrates by Dog Renin (pH 7.5)

		K_M (μM)	V_{max} *
1	ANGIOTENSIN I — Val-Ile-His	13	.005
2	Leu-Ile-His	9	.098
3	Leu-Val-His	39	.002
4	Leu-Val-Tyr-Ser	74	1.00

*Relative to the rate of cleavage of TDP (4) by dog renin.

Human renin cleaves both the tridecapeptide from human angiotensinogen (**1**) and the tetradecapeptide (TDP, **4**) from the equine sequence at about the same rate. Values for K_M and V_{max} are almost identical. Dog renin, however, cleaves the two substrates at markedly different rates. V_{max} for the human tridecapeptide (**1**) is less than 0.5 percent of that for TDP (**4**). Specificity of the human substrate for human renin seems to reside within the N-terminal 13-residues.

Sequences which are intermediate between human and equine angiotensinogen (**2,3**) are also cleaved much more slowly than TDP by dog renin. Sequence differences between TDP and **3** are the histidyl residue at position-13 and the C-terminal serine. Skeggs and co-workers showed that removal of the C-terminal seryl residue from TDP does not effect K_M

for porcine renin.[4] The histidyl-residue at position-13 thus is responsible for the resistance of human angiotensinogen to cleavage by non-primate renins.

Acknowledgements

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COMPETITIVE INHIBITORS OF RENIN: LIPOPHILICITY RELATIONSHIPS

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The renin angiotensin system is thought to play a role both in the maintenance of normal blood pressure and in some types of hypertension. Over the past decade, potent inhibitors such as Saralasin,[1] which blocks binding of angiotensin by cellular receptors, and Captopril,[2] which prevents formation of angiotensin II from the precursor angiotensin I have been developed. These peptide analogs are useful for both the diagnosis and therapy of many forms of hypertension.

Inhibitors of the enzyme renin which are effective *in vivo* have also recently become available.[3] These are based on the amino acid sequence found between positions six and thirteen of equine angiotensinogen (His-Pro-Phe-His-Leu-Leu-Val-Tyr). Addition of a prolyl residue to the N-terminus of the octapeptide increases solubility and renders the peptide specific for primate renin. Replacement of the leucyl residues on either side of the renin cleavage site with phenylalanine improves binding to renin almost two orders of magnitude, and addition of a lysine to the C-terminus improves solubility and increases *in vivo* half-life (Figure 1).

Compound	Position 1	2	3	4	5	6	7	8	K_I (μM)	Solubility (μM)	Half-life in vivo (sec.)
1	His	Pro	Phe	His	Leu	Leu	Val	Tyr	40	160	--
2	Pro	—	—	—	—	—	—	—	39	320	--
3	Pro	—	—	—	Phe	Phe	—	—	1	100	~15
4	Pro	—	—	—	Phe	Phe	—	— Lys	2	840	225

Fig. 1. Modifications which convert an equine angiotensinogen sequence into an *in vivo* renin inhibitor.

The potential of the substrate analog inhibitors for treatment of renin-related forms of hypertension will be greatly increased if the inhibi-

tory constant (K_I) of the peptides can be improved. Less inhibitor would be required to block circulating renin. This design objective requires that the forces which cause the inhibitors to bind to the enzyme be identified and enhanced.

Hydrophobic interactions have long been thought to be important for binding peptides to acid proteases.[4,5] Substrate analog renin inhibitors in which the lipophilicity of the aromatic residues at positions 3,6, and 8 has been systematically varied have been synthesized. The substitutions are, as nearly as possible, isosteric. Measurement of the inhibitory constant of the analogs shows how lipophilicity is correlated with binding to renin at different positions in the peptide. Peptides listed in Table I were prepared by solid-phase techniques[6] and purified to homogeneity by gel filtration, chromatography on Biogel P-2, and semi-preparative reversed-phase HPLC. K_I valves were determined with partly purified human renin using previously described methods.[6] The peptides are all competitive inhibitors of renin.

Replacement of phenylalanine-3 with the more lipophilic *p*-chlorophenylalanine (**7**) or less lipophilic tyrosine (**6**) increases K_I for renin from 3 to 13 or 208 μM, respectively (Table I).

Table I.

Compound	Position 1 2 3 4 5 6 7 8	K_I (μM)
5	Pro-His-Pro-Phe-His-Leu-Phe-Val-Tyr	3
6	———Tyr———	208
7	———Phe(Cl)———	13
8	———Tyr———	12
9	———Phe(4Cl)———	1
10	———Phe	3
11	———Phe(Cl)	5

Since both analogs bind renin less well than the parent compound, there is no relationship between lipophilicity and inhibitory power at position-3. One possible explanation for the poorer binding is that the *p*-substituents on the aromatic ring prevent some type of interaction between the inhibitor and the enzyme.

At position-6, replacement of phenylalanine with tyrosine (**8**) increases K_I to 12 μM while substitution with *p*-chlorophenylalanine (**9**) improves binding to the enzyme. These data fit the quantitative lipophilicity relationship: $pK_I = 0.72\Delta\pi + 2.1$. $\Delta\pi$ is the change in lipophilicity relative to phenylalanine.[7] At position-6 K_I is quantitatively related to changes in the lipophilicity of the various renin inhibitors.

At position-8, replacement with phenylalanine (**10**) or *p*-chlorophenylalanine (**11**) does not change activity.

Identical changes in the lipophilicity of the substrate analog renin inhibitors affects activity in different ways at different positions. At the aromatic residue closest to the N-terminus both more and less lipophilic analogs bind renin more poorly than the parent inhibitor. At the C-terminus changes in lipophilicity do not greatly affect inhibitory power. At the cleavage site, a quantitative relationship between lipophilicity and binding to renin is observed. This "lipophilicity map" will serve as a guide for the development of more effective renin inhibitors.

Acknowledgements

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7. π-values for the amino acids were obtained from Prof. E. Jorgenson.

PRODUCTION OF α-N-ACETYL-β-ENDORPHIN (1-26) FROM α-N-ACETYL-β-ENDORPHIN (1-27)

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For the small bioactive peptides where data are available, biosynthesis begins with a precursor which is substantially larger than the bioactive peptide itself; the relatively large size of some of these precursors compared to the size of the product peptide may reflect the length of peptide needed to ensure sequestration in cisternae of rough endoplasmic reticulum and subsequent processing in Golgi and release from granules.[1] Many different arrangements of bioactive peptides within their precursors are known, and some are diagrammed in Figure 1: the bioactive peptide may be in the middle or at the extreme COOH-terminus of the precursor; there may be multiple copies of identical peptides in one precursor; there may be several different bioactive peptides within one precursor; an inactive fragment may separate peptides at both termini of the precursor that are required for activity. For proinsulin and pro-ACTH/endorphin, the non-hormone parts of the precursor are secreted together with the bioactive peptides.[2,11] Thus, as our knowledge of hormone precursors increases, we can expect to learn of many more peptides (possibly some with actions of their own) secreted by endocrine and nervous tissue.

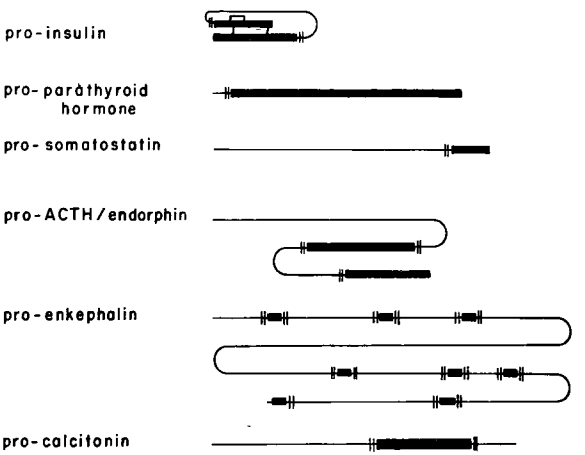

Fig. 1. Structures of some precursors. Lengths of peptide backbone are drawn approximately to scale; paired basic amino acids marking proteolytic cleavage sites around peptides of known importance are indicated by vertical lines. Data from References 2-11.

The number of post translational modifications to which hormone precursors are subject is extensive and includes proteolysis, glycosylation, acetylation, amidation, sulfation and phosphorylation.[12] Many proteolytic cleavage sites for prohormone processing are marked by pairs of basic amino acids (Lys and Arg) (Figure 1); trypsin-like and carboxypeptidase B-like activities can produce the observed products. However, not all pairs of basic amino acids are always cleaved, cleavages occur in a particular order and different tissues show different cleavage patterns. Thus anterior pituitary cells produce primarily ACTH(1-39), β-lipotropin, β-endorphin, γ-lipotropin and 16 K fragment while intermediate pituitary cells produce smaller peptides by cleaving at paired basic amino acids within ACTH, β-lipotropin and 16K fragment (Figure 2). Similarly, acetylation of ACTH, α-melanotropin and β-endorphin is specific to intermediate pituitary.

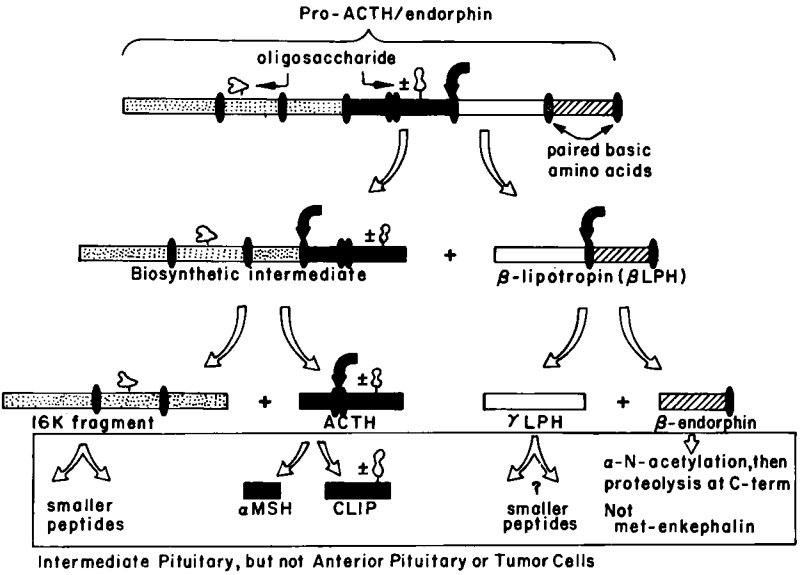

Fig. 2. Structure and processing of pro-ACTH/endorphin in pituitary tissue. Paired basic amino acids in rat molecule are indicated[7] (●).

As mentioned above, β-endorphin-sized peptides are major end products of pro-ACTH/endorphin proteolytic processing in rat intermediate pituitary. Smyth and Zakarian[13] identified several forms of β-endorphin in pituitary and brain (Figure 3). In our studies of β-endorphin biosynthesis in rat intermediate pituitary we observed an additional form of β-endorphin, namely α-N-acetyl-β-endorphin (1-26).[14] In order to determine whether α-N-acetyl-β-endorphin (1-26) could be

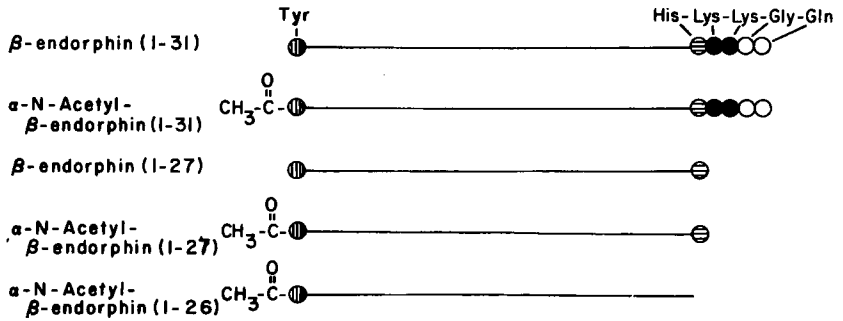

Fig. 3. Some forms of β-endorphin in intermediate pituitary.[13,14] Note pair of Lys residues at 28 and 29.

produced by the trypsin- and carboxypeptidase B-like activities already thought to be involved in prohormone processing, the following experiment was done. Rat β-endorphin (1-31) contains one methionine residue (position 5) and one histidine residue (position 27).[7] Rat intermediate pituitary cells were incubated in medium containing [^{3}H]histidine and [^{35}S]methionine and labeled α-N-acetyl-β-endorphin(1-27) was prepared by ion exchange chromatography on SP-Sephadex.[15] Synthetic α-N-acetyl-β-endorphin(1-27) was added, the sample was treated with carboxypeptidase B, desalted and analyzed on SP-Sephadex (Figure 4). All of the [^{35}S]methionine and 50% of the [^{3}H]histidine counts remained with peptides eluting in the void volume of the gel filtration column. (Figure 4A). Based on ion exchange chromatography (Figure 4B , lower), 47% of the [^{35}S]methionine counts eluted at 0.22M NaCl, coincident with a peak of [^{3}H]histidine counts and thus remained as α-N-acetyl-β-endorphin(1-27). Carboxypeptidase B treatment converted 40% of the [^{35}S]methionine counts to material eluting at the position of α-N-acetyl-β-endorphin(1-26) (0.18M NaCl); this peak of material was not labeled with [^{3}H]histidine, consistent with its identification as α-N-acetyl-β-endorphin(1-26). Similarly, based on β-endorphin immunassay, carboxypeptidase B treatment converted 41% of the synthetic α-N-acetyl-β-endorphin(1-27) into material eluting with α-N-acetyl-β-endorphin(1-26); 55% of the synthetic α-N-acetyl-β-endorphin(1-27) remained intact (Figure 4B, upper).

The biosynthesis of β-endorphin-sized material in rat intermediate pituitary is diagrammed in Figure 5; all the steps can be mimicked by the trypsin-like and carboxypeptidase B-like activities thought to be involved in earlier steps of pro-ACTH/endorphin processing. Note that rat anterior pituitary cells produce primarily β-endorphin-(1-31) and do not carry out the further processing steps characteristic of intermediate

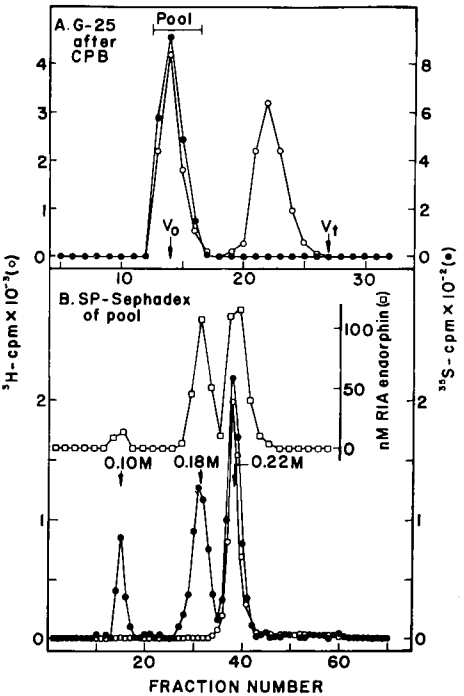

Fig. 4. Carboxypeptidase B treatment of α-N-acetyl-β-endorphin(1-27). Rat intermediate pituitary cells were incubated in medium with [^{35}S]methionine and [^{3}H]histidine for 70h followed by a 2h chase in unlabeled medium.[14] Cells were extracted and immunoprecipitable β-endorphin-sized material was fractionated by SP-Sephadex chromatography. The peak of labeled peptide eluting with α-N-acetyl-β-endorphin (1-27) at 0.22M NaCl was pooled and desalted. Synthetic camel α-N-acetyl-β-endorphin (1-27) (2μg) and bovine serum albumin (40 μg) were added to the labeled material and the sample (200 μl in 0.2M sodium phosphate, pH 7.6) was treated with carboxypeptidase B (0.5 μg) for 30 min at 22°. **A.** Large peptides were separated from free histidine on Sephadex G-25 in 50% acetic acid, 20 μg/ml bovine serum albumin; material in the void volume (V_o) was pooled and fractionated on SP-Sephadex. (shown in **B**). Overall recovery of immunactivity was 83%; recovery of [^{35}S] and [^{3}H] counts was 85%.

pituitary cells. Knowledge of the precise structure of β-endorphin-related material in a tissue is important since acetylation eliminates opioid activity[13] and the forms of β-endorphin shortened on their COOH-termini have altered biological activity compared to β-endorphin(1-31).

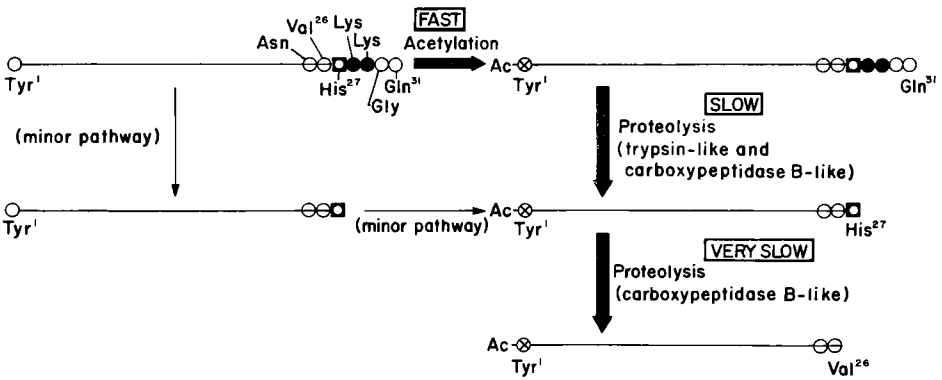

Fig. 5. Major pathway for biosynthesis of β-endorphin-related peptides in rat intermediate pituitary.

Acknowledgements

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POLYPROTEIN PRECURSORS OF REGULATORY PEPTIDES

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Most, if not all, polypeptide hormones are synthesized by way of co-and post-translational processing of larger precursor molecules (Figure 1). These biosynthetic processes include, solely or in combination, proteolytic cleavages[1,2], glycosylation, phosphorylation, amidation, acetylation, and (or) other derivatizations of amino acids,[3] and formation of intra- and inter-molecular disulfide linkages. Analyses of the products of cell-free translations of mRNAs encoding polypeptide hormones and of the products formed during pulse and pulse/chase labeling studies in intact cells have shown that cleavages, glycosylation, and assembly of polypeptides occur during their passage through the endoplasmic reticulum and transport through the Golgi complex. The modifications of the polypeptides initially synthesized are often extensive, resulting in substantial alterations in the physicochemical properties of the molecules. It is likely that these modifications alter the biologic activities of, and, in turn, may lend biologic specificities to, the polypeptides.

Studies of co- and post-translational processing of hormones have brought to light several functions that these precursors appear to fulfill in biologic systems. Two of these proposed functions, for which there is considerable supporting evidence are a) intracellular signalling, by which the cell distinguishes among specific classes of proteins and directs them to their specific sites of action (segregation of proteins) and b) the generation of multiple biologic activities from a common gene product, or polyprotein, by regulated, cell-specific variations in the co- and post-translational modifications. In addition to the processes of post-translational modifications of proteins biologic diversification of proteins may be generated at other levels of gene expression. For example, by gene duplication or multiplication it becomes possible for individual members of multigenic families to be differentially expressed in different tissues. The formation of mature mRNAs by the use of alternative splicing patterns may also alter the coding information of the messenger.

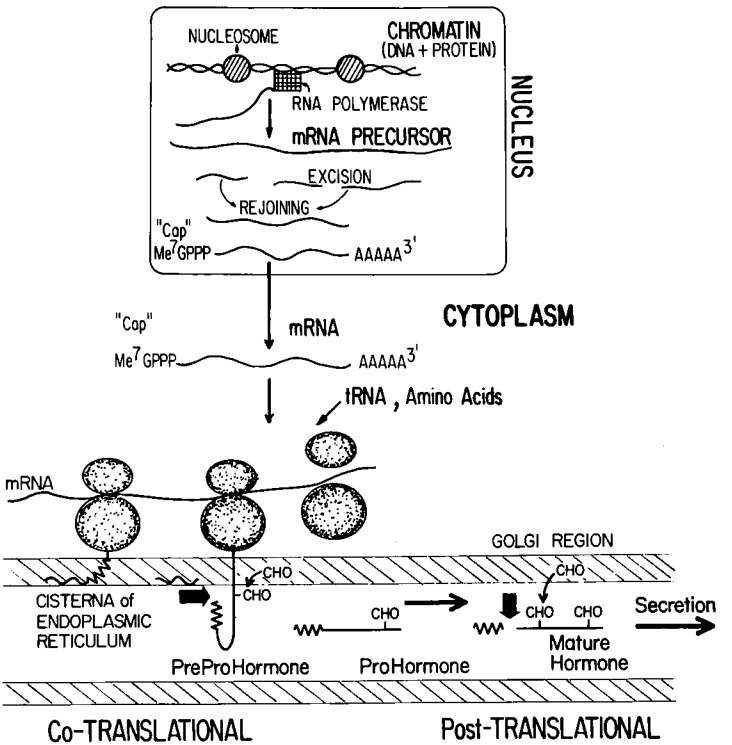

Fig. 1. Scheme depicting the cellular events in the biosynthesis of polypeptide hormones. Genetic information is initially expressed by the transcription of information in DNA into an mRNA precursor. Inasmuch as coding sequences in the gene are often interrupted, the mRNA precursor is processed by excision of intervening sequences (introns) and the mature mRNA is formed by ligation of the coding sequences (exons). Mature mRNAs are transported into the cytoplasm where they are translated into protein. Nascent polypeptides are vectorially discharged across the membrane bilayer into the cisterna of the rough endoplasmic reticulum. Cleavages of NH_2-terminal leader sequences and glycosylation (CHO) of the nascent polypeptide are shown. Further post-translational modifications of the polypeptide take place in the Golgi region (from Habener).[52]

Below we review briefly the evidence leading to the formulation of the "signal hypothesis" which operates in the early stages of protein synthesis in the segregation of exported proteins (hormones) into the cellular secretory pathway. We also present the structures of biosynthetic precursors to glucagon, calcitonin and somatostatin as examples of polyproteins. We have recently determined the structures of these precursors, or pre-prohormones, by decoding of the nucleotide sequences of cloned recombinant cDNAs.

Intracellular Signalling and Segregation

The problem of how proteins are segregated within the cell after they are synthesized has occupied the attention of cellular biologists for many

years. It has been estimated that a typical eukaryotic cell synthesizes about 20,000 different proteins at some time during its cycle.[4] Present evidence suggests that these many different proteins produced by a cell are synthesized by a common pool of polyribosomes. Each of the different proteins synthesized is directed to a specific location where its particular biologic function is expressed. For example, specific groups of proteins are transported to the nucleus and to other subcellular organelles, whereas other groups of proteins are synthesized specifically for export from the cell, *e.g.*, immunoglobulins, blood coagulation factors, serum albumin, and the protein and peptide hormones. It is clear that the cellular mechanisms involved in this process of directional transport of proteins must involve a highly sophisticated set of informational signals. Inasmuch as the information for this transport can only reside either wholly or in part within the primary structure or conformational properties of the protein itself, post-translational modification (Figure 1) may be important for specificity of protein transport; i.e., for accurate targeting of the protein to specific cellular locations.

A number of studies point to a role of precursor sequences and post-translational modifications of polypeptides in the processes of intracellular signalling and directional transport. Initial studies involved the discovery of NH_2-terminal "leader", "signal," or "pre" sequences on the products resulting from the cell-free translations of mRNAs coding hormones and other secretory proteins[1,2,5] and the demonstration that these sequences are present on nascent polypeptides during their synthesis in intact cells. It became evident that such NH_2-terminal sequences probably serve as signals that recognize specific sites on the rough endoplasmic reticulum (RER), resulting in transport of the nascent polypeptides into the secretory pathway of the cell[1,2,5-10].

Cell-free translation products containing NH_2-terminal leader sequences, termed pre-proteins (pre-hormones), or pro-proteins (pro-hormones), in instances in which intermediate precursor forms exist, have been found for numerous protein hormones including parathyroid hormone, insulin, growth hormone, prolactin, thyroid-stimulating hormone, calcitonin, somatostatin, glucagon, and adrenocorticotropin, as well as the non-hormonal secreted proteins such as pancreatic enzymes, egg-white proteins, serum albumin, immunoglobulins, mellitin (bee venom), and several membrane-associated bacterial proteins (see references 1, 2, 6 and 10 for reviews). A characteristic of the precursor-specific sequences is that they vary in length from approximately 15 to 30 amino acids; hydrophobic regions of 10 to 12 amino acids are found within the central portion of the sequences. This high degree of hydrophobicity is

found in proteins known to be specifically associated with membranes. One exception to the rule that the precursors of secreted proteins contain NH_2-terminal leader sequences is ovalbumin in which the hydrophobic sequences may be either located within the actual sequence of ovalbumin itself or if it exists at the NH_2 terminus it is not cleaved during processing.[11]

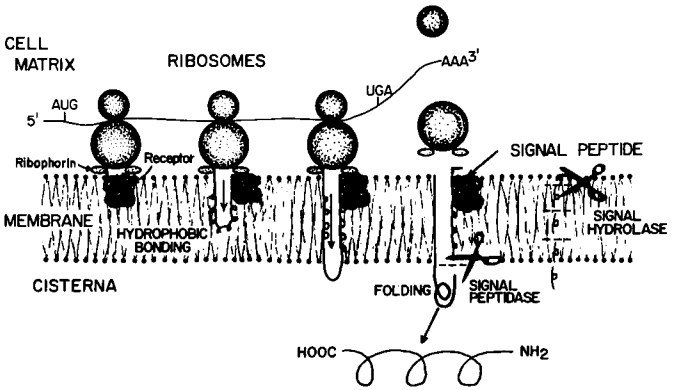

Fig. 2. Hypothetical model of molecular processes that may be involved in the transport of nascent secretory proteins (polypeptide hormones) across the membrane bilayer of the rough endoplasmic reticulum. The model incorporates elements of both the signal hypothesis[8] and the membrane-triggered hypothesis.[10] Scanning from left to right on the diagram it is proposed that the NH_2-terminal "signal" sequences of the nascent protein recognizes and binds to a receptor located in the cytoplasmic (cell matrix) face of the membrane. After specific recognition of nascent secretory proteins is achieved via this receptor-ligand interaction, the ribosome is anchored to the membrane face by both the nascent chain and by ribophorins. The growing nascent chain transgresses the membrane bilayer in the form of a loop with hydrophobic bonding between the hydrophobic residues of the signal sequence (α helix) and the intensely hydrophobic interior of the membrane providing the principle source of energy for movement of the polypeptide. As the progressing loop of the nascent peptide enters the cisterna folding of the polypeptide into secondary conformation may "pull" the remainder of the polypeptide through the membrane. The polypeptide is released from the membrane by cleavage of the NH_2-terminal signal sequence by a "signal peptidase" presumably located on the cisternal face of the membrane. The signal peptide, once cleaved from the protein, is rapidly destroyed in the membrane by putative "signal hydrolases." Although not clearly shown in the model, evidence indicates that cleavages of signal sequences occur on nascent proteins during, and not after, translation is completed.

Recent views favor the probability that precursors play a role in the post-translational processes that lead to the intracellular transport and compartmentalization of hormones in the secretory pathway.[1,2,5,6] This hypothesis, known as the signal hypothesis, was introduced by Milstein *et al.*[9] and by Blobel and Sabatini[8] to explain, at a molecular level, the mechanisms by which proteins destined for secretion from cells are able to selectively obtain access to the membrane-enclosed subcellular organelles involved in the transport, packaging, and secretion of the proteins. The amino-terminal signal, or leader, sequence of nascent proteins recognizes putative attachment sites (receptors) in the membrane of the

endoplasmic reticulum[7,12,13] (Figure 2). The polyribosome-nascent polypeptide complexes form junctions with the membrane, and the growing polypeptides are vectorially transported across the membrane into the cisterna of the RER. Co-translationally, during the transport of the nascent polypeptides, the leader sequences are removed, presumably by the action of specific peptidases located on the inner face of the membrane. After completion of the polymerization of amino acids, the polypeptides are released into the cisterna.

Experimental support of the signal hypothesis has come from studies carried out both in cell-free systems and in intact cells. Translation of mRNAs in cell-free systems supplemented with microsomal vesicles results in the translocation of the secretory proteins into the interior of the microsomal vesicles concomitant with the removal of the leader sequence[5,14] and, in certain circumstances, glycosylation[14,15] of specific sites on the polypeptide. In addition, "read-out" experiments conducted under cell-free conditions showed that the removal of leader sequences and glycosylation occur co-translationally.[5,14] Pulse labeling (for 30-60 sec) of nascent proteins in intact parathyroid gland slices using [35S]methionine revealed the presence of labeled proparathyroid hormone before the appearance of pre-proparathyroid hormone, a finding indicative of co-translational rather than post-translational processing of the nascent polypeptide.[16] Additional studies in intact parathyroid gland slices demonstrated hydrolysis of the NH_2-terminal leader sequence cleaved from the nascent preproparathyroid hormone.[17] From these observations it appears reasonable to postulate that the basic function of the leader sequence is in the early stages of protein synthesis and to provide the means for transmembrane movement. Thus, as a result of the specialized nature of the precursor sequences, proteins destined for export from the cell are selected from a great many other cellular proteins for sequestration and subsequent transport and packaging within the secretory pathway of the cell.

Precursors as Polyproteins: Sources of Diverse Biological Activities

A function that appears to be fulfilled by certain biosynthetic precursors is that of a prohormonal polyprotein that provides a means to generate multiple biologic activities by cleavages from a single gene product. Inasmuch as all of the many mRNAs thus far identified from eukaryotes are monocistronic, *i.e.,* code for only a single protein, multiple but separate biologic activities appear to be generated at the level of post-translational cleavages of precursors which, as such, serve as polyproteins. As many as seven different proteins are derived from a single precursor coded for by

polio virus RNA.[18] A prototypic hormonal precursor is the $M_r = 31,000$ protein, proopiomelanocortin, that contains within its sequence (in addition to a leader sequence) the sequences of the hormones ACTH, LPH, β-MSH, α-MSH, the endorphins, and enkephalins, as well as a $M_r = 16,000$ "cryptic" sequence in which resides a sequence corresponding to MSH.[19] The proopiomelanocortin is glycosylated and phosphorylated in various combinations at specific sites in the molecules, and, furthermore, the $M_r = 31,000$ precursor appears to undergo acetylation under certain circumstances (see Eipper, this volume). One can appreciate that different combinations of cleavages, glycosylation, phosphorylation, and acetylation provide the possibility for the generation of a large number of chemically different peptides and, as a result, the potential for further diversification of biologic activities of the peptides so generated. It is likely that the specific processing of the precursors may vary from one tissue to the next and thereby provide different, tissue-specific arrays of active peptides.[20]

Processing by proteolytic cleavages of the prohormones of parathyroid hormone (proparathyroid hormone) and of insulin (proinsulin) was shown to occur in the Golgi complex.[21,22] The prohormones are synthesized in the RER, after co-translational cleavages of leader sequences, and are transported to the Golgi complex from the RER via the smooth endoplasmic reticulum. Unlike the situation with pre-hormones, in which the amino acids at the site of cleavage between the leader sequence and the remainder of the molecule (hormone or prohormone) vary from one pre-hormone to the next,[1,2] the cleavage site(s) of the prohormone intermediates uniformly, consists of the basic amino acids lysine or arginine, or both, usually two or three together. This substrate is readily and preferentially attacked by trypsin-like endopeptidase cleavage; the basic residues are susceptible to selective removal by exopeptidases with activity resembling that of carboxypeptidase B. It is quite likely that all proproteins are cleaved by a similar enzymic process within the Golgi complex of cells of diverse origins.

Next we describe the structures of polyprotein precursors to the small peptide hormones glucagon, calcitonin and somatostatin. Our laboratory is examining the biosynthesis of a number of small regulatory peptides that appear to serve both hormonal and paracrine functions in the regulation of energy metabolism and, possibly, neurotransmission. Our studies have involved the cloning of recombinant cDNAs encoding precursors of the peptide hormones, glucagon, calcitonin and somatostatin. We have determined the nucleotide sequences of these cDNAs and, as a consequence, have deduced the amino acid sequences of the precursors by decoding of the nucleotides sequences. From the primary structures of the precursors

we can make predictions about the sites that are cleaved and glycosylated during the cellular processing of the precursors to the smaller regulatory peptides.

Pre- ProGlucagon

Glucagon is a nonacosapeptide found in the pancreas. Along with insulin, glucagon regulates carbohydrate metabolism.[23] Immunoreactive peptides similar to glucagon have been found in intestine and brain.[24] By virtue of its primary structure and biologic activities glucagon belongs to a family of peptides which includes secretin, vasoactive intestinal peptide and gasric inhibitory peptide.[25] We isolated glucagon-specific cDNAs[26] from a cloned *(E. coli)* cDNA library which we prepared from the poly(A) RNA[27] of anglerfish islets.[28] We sequenced cDNAs encoding islet precursors to the glucagons (pre-prolucagons) using the base-specific chemical method of Maxam and Gilbert.[29] The nucleotide sequence of the pre-proglucagon cDNA encoding the precursor of 14,500 daltons begins with an N-terminal methionine residue followed by a signal sequence estimated to be 24 amino acids, an NH_2-terminal extension pro-peptide of 28 amino acids, a Lys-Arg dipeptide and 29 amino acids that comprise the glucagon polypeptide respectively[30] (Figure 3). Carboxyl-terminal to the glucagon sequence is a

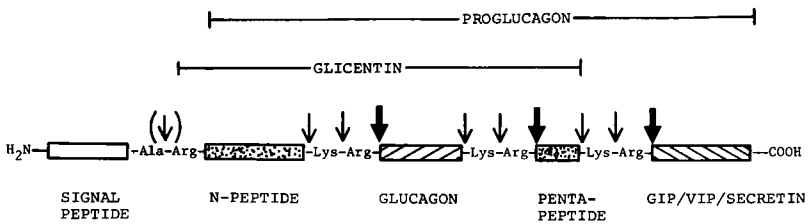

Fig. 3. PRE-PROGLUCAGON. Diagram of the primary structure of anglerfish islet pre-proglucagon I.[30] The pre-proglucagon is 124 amino acids in length and consists of an NH_2-terminal signal sequence (open box), intervening N- and pentapeptides (stippled bars), a glucagon of 29 residues and a peptide of 34 residues highly homologous to GIP, VIP and secretin (cross-hatched bars). The COOH-region four peptides are all linked by Lys-Arg sequences typical of sites that are cleaved during the post-translational processing of prohormones. Heavy arrows indicate sites of primary cleavage by trypsin-like activity, light arrows denote additional cleavages by carboxypeptidase-B-like activity, and arrow in parentheses indicates the estimated, but not proven, site of cleavage of the signal peptide. The sequence that probably corresponds to a glicentin-like peptide is indicated.

Lys-Arg dipeptide followed by the residues Ser-Gly-Val-Ala-Glu, and another Lys-Arg sequence following which there are 34 additional codons before the stop codon is reached. It is likely that four separate peptides are formed (and secreted) during the cellular processing of the pre-proglucagon inasmuch as each peptide is flanked by Lys-Arg sequences characteristic of sites that are cleaved during the post-translational processing of prohor-

mones. A remarkable feature of the C-terminal extension is its pronounced homology to the preceding glucagon sequence and to the sequences of other peptide hormones in the glucagon family, particularly that of gastric inhibitory peptide.[25] It will be of interest to determine whether this C-terminal peptide extension possesses biological activities associated with the glucagon family of peptides or is simply an evolutionary relic of an ancient gene duplication. Chemical synthesis of this polypeptide extension is currently in progress and biologic studies using antisera prepared to this peptide may provide an answer to these questions.

Pre- Procalcitonin

Medullary carcinomas of the thyroid, tumors of the thyroid C-cells of neural crest origin, synthesize large amounts of calcitonin, a hormone of 32 amino acids that lowers blood calcium levels by its actions on bone.[31] As a consequence of the high levels of production of calcitonin, the tumors contain large amounts of mRNA encoding a precursor of calcitonin.[32,33] Cell-free translation of the poly(A)-containing RNA fraction isolated from the tumor shows a polypeptide of a molecular weight 15,000 daltons, precipitable by antisera to calcitonin.[32] When translated in the presence of EDTA-stripped rough microsomes prepared from canine pancreas, the polypeptide is co-translationally processed by both cleavage of the NH_2-terminal signal sequence and by glycosylation.[34] The addition of carbohydrate to the polypeptide is accompanied by an increase in its size, sensitivity to cleavage of the carbohydrate by endoglycosidase H and affinity for concanavalin A, a lectin known to bind specifically to mannose residues.[34] As with pre-proglucagon, we identified recombinant cDNAs containing coding sequences for calcitonin in a cDNA library prepared from the medullary thyroid carcinoma by using the method of hybrid-arrested translation of carcinoma messenger RNA. We determined the nucleotide sequence which encodes pre-procalcitonin. The coding sequence consists of 136 codons[35] (Figure 4). The primary structure of the pre-procalcitonin consists of several clearly defined regions. These regions include the hydrophobic leader sequence of 24 amino acids and cryptic peptides of 58 and 16 amino acids that flank calcitonin at its amino acid and carboxyl termini, respectively. The basic amino acid sequence Lys-Arg precedes the calcitonin sequence of the NH_2-terminus, and the sequence Gly-Lys-Lys-Arg follows the calcitonin sequence at its COOH-terminus.[35] Calcitonin is known to contain a proline amide at its C-terminus.[31] The nucleotide sequence of the calcitonin, however, ends with codons for proline and glycine. Thus, it appears that the glycine, immediately following the C-terminal proline and followed by the sequence of three basic residues, is

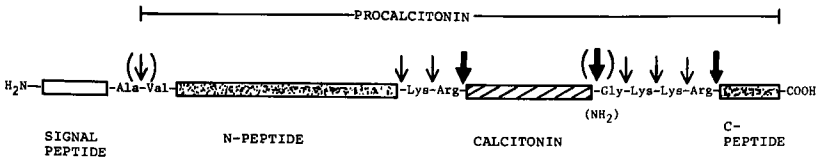

Fig. 4. PRE-PROCALCITONIN. Diagram of the primary structure of rat pre-procalcitonin.[35] Note that the sequence of calcitonin of 32 residues resides within the precursor of 136 residues and is flanked by short sequences of basic amino acids. The glycine immediately following the COOH-terminus of calcitonin is involved in the amidation (NH_2) of the COOH-terminal proline (prolinamide) of calcitonin. Arrows and shading of bars are as described in legend to Figure 3.

involved in some, as yet not understood, manner in the amidation of the COOH terminus of calcitonin during its formation from the precursor, pre-procalcitonin. This enzyme presumably acts in concert with the trypsin-like and carboxypeptidase B-like enzymes that cleave the procalcitonin at the basic residues.

Pre-Prosomatostatin

Somatostatin is a small peptide hormone of fourteen amino acids that was discovered initially in the hypothalamus and shown to inhibit the release of pituitary growth hormone.[36,37] Subsequently, the tetradecapeptide was found to inhibit many different bioactive substances in many different organs through both endocrine and paracrine influences.[38] Inasmuch as both the pancreatic islets of the anglerfish[39] and the rat medullary thyroid carcinoma[40] contain somatostatin we screened each of the libraries of cloned cDNAs that we had prepared from these two tissues and isolated cDNAs containing coding sequences for pre-prosomatostatins. We determined the nucleotide sequences of cDNAs encoding both the largest (16,000 daltons) of the two fish pre-prosomatostatins (the smaller is 14,000 daltons)[41,42,43] and a single rat pre-prosomatostatin. Decoding of these sequences gave the complete amino acid sequence of the fish islet pre-prosomatostatin[28] and a nearly-complete sequence of the rat pre-prosomatostatin[44] (Figure 5).

The sequence of the angler fish somatostatin tetradecapeptide resides at the COOH-terminus of a larger precursor of 121 amino acids.[28,45] The NH_2-terminal sequence of the precursor consists of a leader (signal) sequence containing a sequence of consecutive hydrophobic residues followed by a long "cryptic" sequence of amino acids. The COOH-terminal tetradecapeptide is preceded by the dipeptide Arg-Lys, a sequence of basic residues that is typical of sites cleaved during the post-translational processing of prohormones. In turn, the Arg-Lys sequence in the rat precursor is preceded by 12 amino acids that in continuity with the Arg-Lys and the

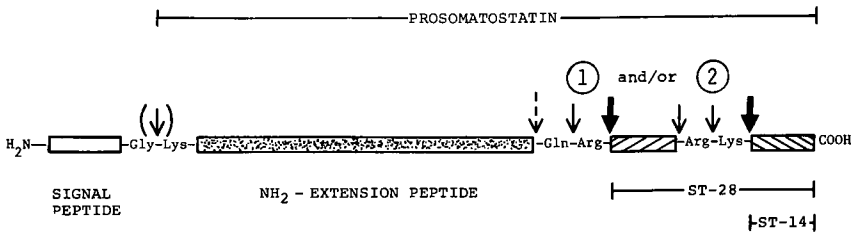

Fig. 5. PRE-PROSOMATOSTATIN. Diagram of the primary structure of pre-prosomatostatin of 121 residues. The structure is a composite determined from the nucleotide sequence of cloned recombinant cDNAs prepared from the pancreatic islets of the angler fish[28] and from a rat medullary carcinoma of the thyroid.[44] The Gln-Arg sequence flanking the NH_2-terminus of somatostatin-28 (ST-28) is from the rat precursor; the corresponding sequence in the angler fish precursor is Glu-Arg. ST-14 = somatostatin-14. Post-translational processing of prosomatostatin can occur by cleavages at (1) Gln-Arg and/or at (2) Arg-Lys. Arrows and shading of the bars are as described in legend to Figure 3.

somatostatin tetradecapeptide sequence (somatostatin-14) constitute the octacosapeptide, somatostatin-28, a super-potent, variant form of somatostatin isolated recently from extracts of intestine,[46] hypothalamus[47,48] and medullary thyroid carcinoma.[49] Notable is the finding that the somatostatin-28 sequence is flanked at the NH_2-terminus by either Glu-Arg (fish) or Gln-Arg (rat), sites not usually associated with post-translational processing. These findings suggest that the formation of somatostatin-28 from the precursor may occur via an enzymic process that differs from that involved in the formation of the somatostatin-14. Alternatively, somatostatin-14 may be the predominant, if not the sole, product formed by post-translational processing of pre-somatostatin in the anglerfish islet and the rat medullary carcinoma of the thyroid that we have studied. Tissue-specific differential processing of prosomatostatin to either somatostatin-14 or somatostatin-28 by independently regulated systems would be consistent with the recent observations that the tetradecapeptide and octacosapeptide are found in markedly different ratios of amounts in different organs of the rat,[50] and that the somatostatin-28 is more potent than somatostatin-14 in some receptor systems.

It is also remarkable that the sequences of the fish and mammalian somatostatin tetradecapeptide are identical, an indication that during the 400 million years since these two species diverged in evolution there has been a strong selective pressure to conserve the sequence of the somatostatin tetradecapeptide.[25] The NH_2-terminal region of the somatostatin precursor that lies between the signal sequence and the sequence of somatostatin-28 may also have some biologic function apart from those of somatostatin-28 and somatostatin-14. This sequence may simply serve as a "spacer" to provide sufficient length to enable the nascent pre-prosomato-

statin to span the lipid bilayer of the endoplasmic reticulum during its transport into the secretory pathway.[1] However, the marked homology of these regions in the fish compared to the rat pre-prosomatostatin again indicates that there has been a strong evolutionary pressure to conserve these sequences.[44] The sequences of the cDNAs encoding the intestinal[51] and hypothalamic somatostatin precursor and the analyses of the corresponding genomic sequences, currently in progress in our laboratory, should provide information regarding the complexities and the genetic diversity of the family of somatostatin peptides.

Clearly, a great deal of work remains to be done to fully characterize the complexity of the structures of these polypeptide precursors before it will be possible to understand in detail the exact nature of their cellular cleavages and processing as well as the physiologic actions of the component peptides formed as a result of the cellular processing of the precursors. Investigations in the future will involve analyses of the structure, organization and complexity of the genes encoding the polypeptide precursors, determinations of the different ways in which the precursors are processed within different tissues and studies to correlate the structures and processing of the polypeptides with their specific biologic functions.[52]

Acknowledgements

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THE PRIMARY EVENTS IN THE BIOSYNTHESIS AND POST-TRANSLATIONAL PROCESSING OF DIFFERENT PRECURSORS TO SOMATOSTATIN

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Introduction

Somatostatin is a tetradecapeptide that originally was isolated from hypothalamic tissue[1,2] and somatostatin immuno-reactivity has now been detected in many organs including pancreatic islets, thyroid and the intestinal tract.[3] This hormone prevents the release of growth hormone from the pituitary gland, and also inhibits the secretion of insulin and glucagon from the islets of Langerhans. Since its discovery, it has become apparent that somatostatin is synthesized as part of a larger precursor molecule, termed prosomatostatin.[4] It appears that the size of the prosomatostatin molecule is approximately 12,000-15,000 daltons in the islet of Langerhans[4] and hypothalamus.[5,6] Furthermore, a precursor-product relationship between these prosomatostatin molecules and the mature secreted hormone has been suggested.[4,5]

In order to understand why a small polypeptide hormone, such as somatostatin, or indeed any secreted polypeptide is synthesized as part of a larger precursor, we suggest that there is a minimum size requirement which is necessary to facilitate transfer of the polypeptide chain across the membrane of the endoplasmic reticulum (ER). Proteins destined for secretion are sythesized on polyribosomes bound to the ER membrane and in the cell, the nascent polypeptide chain is translocated into the ER cisternae. The mechanism of ribosome attachment to the ER membrane, and the events leading to segregation of the nascent polypeptide chain can be explained on the basis of the "Signal Hypothesis".[7] However, in attempting to understand the synthesis and segregation of a small polypeptide, such as somatostatin, one is faced with explaining how a molecule consisting of only 14 amino acids can traverse the large ribosomal sub-unit and the lipid bilayer of the ER, a distance which has been estimated to require approximately 70 amino acids.[7] We propose therefore, that any secreted polypeptide would have to be synthesized as part of a molecule which would have a minimum size of 70-80 amino acids in order for it to be translocated into the ER cisternae. Indeed, mellitin, a 26

amino acid peptide present in the venom of honey bees, was shown to be synthesized as a larger precursor, prepromellitin, which consists of 70 amino acids.[8] Consequently, we predicted that the minimum size for the primary translation product of somatostatin messenger RNA (mRNA) would be approximately 8,000.

To investigate the primary events in the biosynthesis of somatostatin, we utilized the wheat germ cell-free protein synthesizing system supplemented with mRNA isolated from the islets of Langerhans of angler fish *(Lophius americanus).* Angler fish was chosen as a source of islet tissue because its endocrine pancreas is entirely separate from the exocrine tissue and it is therefore possible to obtain large amounts of pure islet material. We demonstrated[9,10,11,12] that angler fish islet mRNA is extremely active in protein synthesis and that four major translation products ranging from 11,000 to about 18,000 molecular weight are synthesized (Figure 1, Lane A). The smallest translation product was shown to be preproinsulin[9] containing a signal sequence of 24 amino acids.[10] When this cell-free system was supplemented with microsomal preproinsulin was co-translationally processed to proin sulin.[9] By employing this same cell-free system, our laboratory and others[11,12,13] identified the primary translation products of somatostatin mRNA, these molecules have been termed preprosomatostatins. We showed[12] that there are two distinct preprosomatostatin molecules: a major species of 18 K dalton and a minor form of 19 K dalton, similar results were obtained by Goodman *et al.*[13] When the cell-free system was supplemented with microsomal membranes, the precursors were converted to prosomatostatin (~15,000 daltons) by the co-translational removal of a 25 amino acid signal peptide. Peptide mapping experiments established that the mature somatostatin sequence is located at the carboxyl terminus of each preprosomatostatin[11,12] and is preceded by at least one basic amino acid - a similar structure has been found for rat prosomatostatin.[14]

By using recombinant DNA technology, the complete amino acid sequence of two preprosomatostatin molecules from angler fish islets was determined in two laboratories.[15,16] These results confirmed the structure proposed from peptide mapping experiments and demonstrated that the mature somatostatin sequence is preceded by two basic amino acids Arg -Lys. The data also confirmed that there are two separate mRNA's coding for each of the preprosomatostatin molecules, and suggested that these mRNA's are derived from two distinct genes.[15] Hobart *et al.*[15] showed that the two somatostatin precursors (termed I and II) consist of 121 and 125 amino acids, respectively. They also showed that the mature

hormone in preprosomatostatin I (which may correspond to the 18 K dalton cell-free translation product) had the same sequence as mammalian hypothalamic somatostatin. In contrast, somatostatin II (equivalent to the 19 K dalton preprosomatostatin) differed at two residues within the authentic sequence: Tyr_7-Phe and Gly_{10}-Tyr. Furthermore, there was little homology in the amino acid sequence of the 'proregion' of these two polypeptides except for a short pentapeptide preceeding the mature hormone (see below). Goodman *et al.*[16] also determined the complete sequence of 119 amino acid preprosomatostatin from angler fish. This molecule had exact homology to somatostatin I[15] except for a continuous stretch of 19 amino acids in the proregion (position -11 through -29). Although the relationship between these molecules remains to be determined, the results implied that there could be three separate mRNA's coding for distinct preprosomatostatin molecules. To investigate this possibility we have extended our earlier observations and now report the existence of 8-10 different forms of preprosomatostatin which are probably coded for by 8 separate mRNA species.

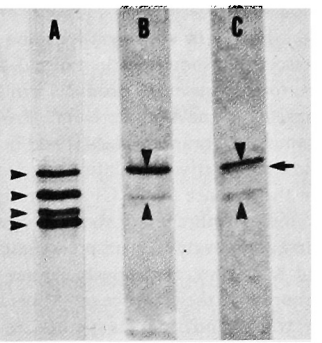

Fig. 1. *In vitro* biosynthesis of islet preprosomatostatin (ppSRIF). The translation products were prepared for electrophoresis directly (Lane A) or subjected to immunoprecipitation with antisomatostatin antibodies (Lanes B and C). Analysis was by SDS-PAGE,[11] an autoradiograph of the dried gel is shown. Lane A: total islet translation products; arrows indicate (in descending order) ppSRIF (18,000); preproglucagon and ppSRIF (16,000); preproglucagons (14,000) and preproinsulin (13,000). Lane B: somatostatin immunoreactive polypeptides: downward pointing arrow 18 K dalton ppSRIF, upward pointing arrow 16 K ppSRIF. Lane C: as lane B except that the gel contained 7 M urea, the minor 18 K dalton ppSRIF is indicated by the arrow.

Results

Translation of angler fish islet mRNA *in vitro,* followed by treatment of the products with an antiserum directed against somatostatin, resulted in precipitation of two somatostatin immunoreactive products upon analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Several criteria including antibody competition experiments and tryptic peptide analysis have previously demonstrated[11,12] that these polypeptides correspond to preprosomatostatin molecules of 18 K and 19 K daltons, respectively. Treatment of the translation products with another antisomatostatin serum (a gift of Dr. P. Davies of this Institution) revealed a third form of immunoreactive preprosomatostatin, of apparent molecular weight 16,000 (Figure 1, Lane B). Rather surprisingly,

when the immunoprecipitated translation products were analyzed upon SDS-polyacrylamide gels containing urea, the 18 K dalton preprosomatostatin band split into two separate polypeptides a major and minor species of precursor (Figure 1, Lane C).

When dog pancreas microsomal membranes were present during translation at least three additional polypeptides were evident (Figure 2, Lane B). We previously demonstrated[11] that the 18 K dalton preprosomatostatin was co-translationally cleaved to prosomatostatin of molecular weight 16,000 (Figure 2, Lane D). Analysis of the translation products, synthesized in the presence of membranes, upon SDS-gels containing 7M urea demonstrated the processing of the 16 K dalton preprosomatostatin to a molecule of about 14 K daltons (Figure 2, Lanes G and H). Surprisingly, the membrane dependent processing of the 18 K dalton precursor was not apparent in the presence of 7M urea. Clearly the ability to detect several of the preprosomatostatin molecules and at least one processed form is dependent on the presence or absence of urea in the SDS-PAGE system.[17]

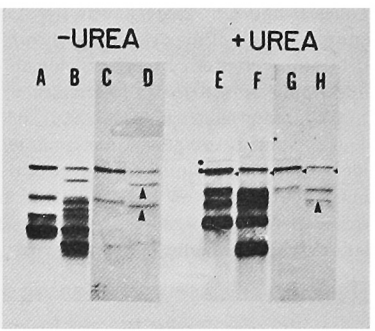

Fig. 2. *In vitro* synthesis and processing of nascent prosomatostatin molecules. Lanes A and B: total translation products synthesized in the absence (Lane A) and presence (Lane B) of microsomal membranes. Lanes C and D: somatostatin immunoreactive translation products synthesized in the absence (Lane C) and presence of microsomal membranes (Lane D); upward pointing arrows indicate nascent prosomatostatin, 16 and 14 K daltons, respectively. Lanes E-M: electrophoresis in the presence of 7M urea. Lanes E and F: translation products synthesized in the absence (Lane E) and presence (Lane F) of microsomal membranes. Lane G: minus membranes, Lane H: plus membranes. Upward pointing arrow indicates the 14 K dalton prosomatostatin. The dots (Lane E) indicate the migration of the 19 K dalton and 18 K dalton ppSRIF, respectively. The arrow head (Lanes E-H) indicates the minor species of 18 K dalton ppSRIF.

In view of the cryptic nature of the islet precursor somatostatin molecules, and their somewhat anomalous migration upon SDS-PAGE, we analyzed the somatostatin immunoreactive products on both isoelectric focusing gels (IEF) and by two-dimensional gel electrophoresis:[18]

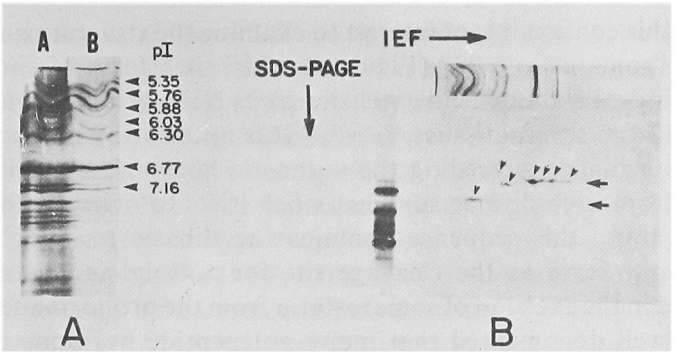

Fig. 3. A. Analysis of islet mRNA translation products by isoelectric focusing. Lane A: total products. Lane B: somatostatin-immunoreactive polypeptides. B. Analysis of somatostatin-immunoreactive products by two-dimensional gel electrophoresis. Arrow heads indicate the different preprosomatostatin molecules, arrows the migration of the 18 K and 16 K ppSRIF.

Seven major somatostatin immunoreactive polypeptides were detected by IEF (Figure 3A), which range in isoelectric point from approximately 5.3 to 7.0. All the preprosomatostatins appear to migrate in the acidic region of the IEF gel. When these molecules are subjected to two-dimensional gel electrophoresis (Figure 3B) at least 8 forms of somatostatin immunoreactive polypeptides are evident. Furthermore, the different preprosomatostatin molecules fall into two major "species": (i) those with an apparent molecular weight of about 18,000 (3-4 forms pI range 6-7); (ii) those of approximately 16 K daltons of which 3-4 different isoelectric forms were detected (pI 5.4-6). The 19 K dalton preprosomatostatin molecule was also evident upon analysis by two-dimensional gels (Figure 3B) but only one form was detected. It might be argued that the different isoelectric forms of preprosomatostatin molecule represent non-specific modifications of the primary translation products, however, this is unlikely because the same products were obtained from the wheat germ system and the rabbit reticulocyte lysate cell-free system.[17]

Discussion

Synthesis and Processing of Nascent Preprosomatostatin — Based on their apparent molecular weight, all 8 forms of preprosomatostatin are of sufficient size to ensure translocation and segregation of nascent prosomatostatin into the cisternae of the ER. Furthermore, we postulate that one possible function of the proregion of prosomatostatin, could be to serve as a "spacer" ensuring that the polypeptide chain would be of sufficient length to span both the large ribosomal sub-unit and the lipid bilayer of the endoplasmic reticulum.

In this context it is of interest to examine the structure and possible role of "somatostatin-28" (Figure 4) which is a form of somatostatin possessing a 14-amino acid extension at its NH_2-terminus that has been isolated from several tissues.[19,20,21,22] It is noteworthy that a pentapeptide, immediately preceding the authentic hormone sequence, is conserved from such diverse sources as fish islets to ovine hypothalamus. Furthermore, this sequence contains the dibasic residues 'Arg-Lys', which may serve as the cleavage site for a Golgi-associated enzyme involved in the excision of somatostatin from the prohormone. Indeed it is now well documented that many polypeptide hormone precursors possess dibasic amino acids flanking the mature hormone sequence.[23,24] In view of the presence of the dibasic amino acids and because the pentapeptide Pro-Arg-Glu-Arg-Lys is conserved in at least six species, we propose that this part of the precursor molecule may serve as a recognition or "Clip-site" for an enzyme whose function would be to cleave mature somatostatin from the precursor. The relationship of a somatostatin-like polypeptide, recently isolated from catfish islets[25] to these six forms of "S-28" (Figure 4) is unclear at present

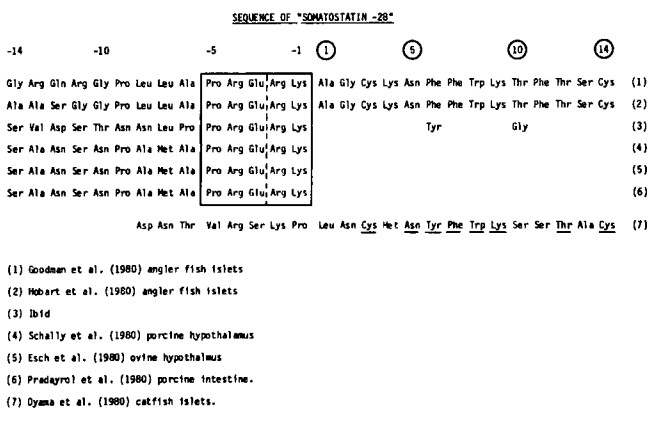

Fig. 4. Amino acid sequence of "somatostatin-28"

If this model is correct, is "S-28" a 'prosomatostatin intermediate' along the processing pathway of a larger prosomatostatin molecule? In the case of the angler fish islet prosomatostatin, we feel that "S-28" would not represent a precursor molecule for several reasons: (i) we have demonstrated that 16 K and 14 K dalton molecules are the primary prosomatostatin molecules; (ii) the presence of the identical pentapeptide sequence containing two basic amino acids immediately preceding the

mature hormone in all three different prosomatostatin molecules, suggests that this region of the molecule serves a specific function, such as a cleavage site for a processing enzyme; (iii) the amino acid at position -15 of prosomatostatin is Arg^{15} hence cleavage by a non-specific serine protease during isolation, would generate "S-28"; (iv) no prosomatostatin of 3,000 K daltons has been detected during pulse-chase experiments in isolated islet or hypothalmic tissue.[4] Even though "S-28" has been shown to be physiologically active,[20,21,22] we suggest the possibility the "S-28" isolated from ovine and procine tissues[19-22] may represent a proteolytic artefact that occurred during tissue extraction. However, until the complete sequence of the primary translation product of somatostatin mRNA is determined in these species, we cannot exclude the possibility that "S-28" may be a physiological form of somatostatin. The following general scheme for the biosynthesis and post-translational processing of somatostatin precursors is proposed.

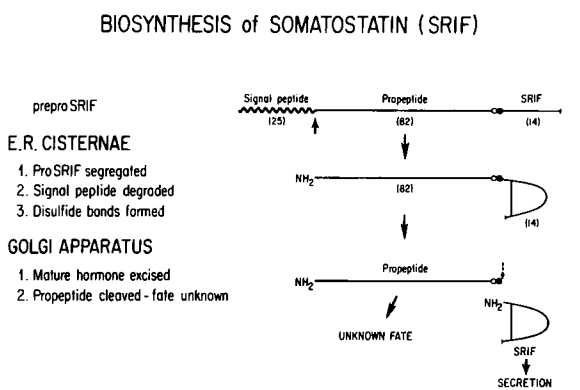

Fig. 5. Model for the biosynthesis of islet somatostain, via the "18 K dalton" preprosomatostatin. Open dot: arginine. Closed dot: lysine.

Somatostatin mRNA is translated on polyribosomes attached to the ER membrane, during translocation of the nascent polypeptide chain into the ER cisternae, a 25 amino acid signal peptide is proteolytically cleaved resulting in the segregation of only prosomatostatin. Either during co-translational segregation or shortly thereafter, the disulfide bond of the mature hormone is formed by oxidation of Cys residues 3 and 14 at the carboxyl terminus of the precursor. The folded nascent prosomatostatin is then transferred to the Golgi apparatus,[26] and during passage through the Golgi cisternae the mature hormone sequence is cleaved from the precursor at the dibasic residues. The hormone is subsequently packaged into secretory granules prior to secretion.

Multiple Precursors to Somatostatin — The two-dimensional gel electrophoresis data, suggests that multiple precursors of somatostatin fall into two major "families"-one of molecular weight 18,000 which has four isoelectric forms and the other of 16K dalton possessing at least three different forms of preprosomatostatin. In addition there appears to be only one form of the 19 K dalton preprosomatostatin. At present we do not know the physiological significance for 8 different forms of preprosomatostatin molecule. However, it is clear that each precursor is the result of translation of an active mRNA species and hence each preprosomatostatin represents the product of an "actively" transcribed gene. It is possible that the different forms of somatostatin precursor reflect the rather ubiquitous nature of the hormone and that other organs might express only a particular sub-set of these mRNAs. In this context, Goodman *et al.*[27] showed that only one form of preprosomatostatin was present in angler fish intestinal mucosa. This suggests that there is tissue specific expression of somatostatin genes or that a major species of precursor in one tissue could be a minor form in another. It will therefore be of considerable interest to investigate the biosynthesis of somatostatin in the hypothalamus, for example, to determine which forms of precursor are present.

The finding of multiple polypeptide hormone precursors is not restricted to somatostatin, we have recently shown that there are at least three distinct forms of preproglucagon molecule.[28] It is possible that the existence of multiple precursors for these polypeptide hormones represent a cellular "back-up" system which ensures that there is a sufficient cytoplasmic pool available to ensure the rapid processing and secretion of the hormone in response to various stimuli. However, whatever may be the explanation for the existence of multiple precursors for somatostatin and other polypeptides, it is probable that each precursor represents the differential expression of several closely related genes. Indeed the two-dimensional gel electrophoresis suggests the existence of a multigene family coding for preprosomatostatin and it is possible that the different mRNA's coding for somatostatin may be the result of differential post-transcriptional splicing events. Determining the factors which activate and repress these putative gene sequences should provide some vital clues to solve this engimatic problem.

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THE N-TERMINAL FRAGMENT OF SOMATOSTATIN-28 IS PRESENT IN BOTH RAT PANCREAS AND HYPOTHALAMUS

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Introduction

Two molecular forms of somatostatin have been characterized in mammals:[1-5] somatostatin (SS14) and an N-terminally extended form of SS14, somatostatin-28 (SS28). The two somatostatins can be envisioned as peptide fragments generated *in vivo* after processing of a protein precursor whose molecular weight has been estimated to be 12.5 K daltons.[6] By analogy with the peptides derived from proopiomelanocortin, different fragments generated *in vivo* from a protein precursor of somatostatin may be involved in different physiological functions. To initiate characterization of these other peptidic fragments derived from pro-somatostatin, we generated an antiserum (S298) directed against the N-terminal region of synthetic SS28 and analyzed extracts from brain and pancreas for SS28-N-terminus-like immunoreactivity (SS28-Nt-LI).

Purification from Pancreas of Peptide with SS28-Nt-LI

In preliminary studies, immunoreactivity was found in an acid extract of 80 rat pancreata which had been chromatographed on a Sephadex G-75 column (Figure 1). The SS28-Nt-LI material was recovered in a minor peak, ~ 2500 daltons in molecular weight (compatible with the presence of SS28-LI previously observed in rat pancreas[7]) and in a major peak of ~ 1500 daltons containing 2.13 μg SS28-Nt-LI. Further characterization of this latter component was undertaken using the acid extract of 439 rat pancreata chromatographed on an immunoaffinity column composed of an SS14 antiserum covalently coupled to Sepharose 4B. While the material bound to the column was used for rat SS14 characterization,[7] the unbound material was selected for purification of the SS28-Nt-LI peptide and de-salted on a C_{18}-bonded silica gel column (2.5 x 20 cm, LRP-2, Whatman Co.) as described by Böhlen *et al.*[8] The immunoreactive material was recovered by elution with 60% propanol in 0.36 M pyridine, adjusted to pH 3 with formic acid to yield 14.7 μg

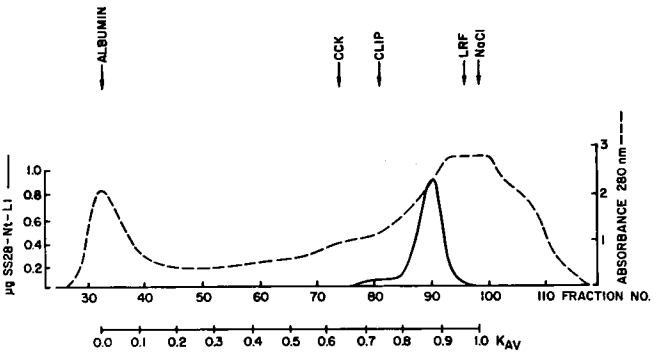

Fig. 1. Sephadex G-75 gel filtration of an acid extract of 80 rat pancreata containing SS28-Nt-LI. Column dim. = 2.7 x 105 cm, V_t = 610 ml, Vo = 198 ml. Flow rate 22 ml/h. Elution with 1 N HOAc. Fraction size: 6.3 ml. Calibration done with Albumin: molecular weight: 68,000; Cholecystokinin (CCK-33): mol wt 3834, CLIP: mol wt 2463, LRF mol wt 1181. Load 400 mg dry weight.

SS28-Nt-LI in 1.26 g total product. This was chromatographed on a Sephadex G-75 column (5 x 150 cm) in 1N HOAc. The 1500-dalton immunoreactive zone was rechromatographed on a Sephadex G-50 Fine column (1.6 x 100 cm) equilibrated in 30% HOAc. This procedure yielded a revised estimated molecular weight of ~ 1250 for the SS28-Nt-LI material. The immunoreactive zone (135 mg dry wt) from the G-50 column was further purified with a Model 332 Altex liquid chromatograph, an Altex Ultrasphere ODS HPLC column, an automatic stream-sampling fluorescamine detection system and the pyridine formate/n-propanol buffer system.[9] Figure 2A shows the HPLC results of an 80-mg load of the Sephadex G-50-purified immunoreactive material. A second load of 55 mg gave similar results. A total of 16 μg of SS28-Nt-LI was recovered from this HPLC step and further purified using a shallower n-propanol gradient (0 to 5% in 90 min.). Two peaks of immunoreactive material were obtained. The more hydrophilic component eluted at 2% propanol and contained 9.7 μg of SS28-Nt-LI, while the other contained only 6 μg of SS28-Nt-LI. The former component was further chromatographed using a second HPLC buffer system (TFA 0.1%, acetonitrile 15.8%), an Altex Ultrasphere RP-8 (5μm particles) HPLC column (4.6 x 250 mm), and a model 332 Altex liquid chromatograph with variable-length UV detector (Schoeffel). The immunoreactive substance eluted in a single fraction, which preceded the elution zone of synthetic SS28(1-14). That fraction was then rechromatographed isocratically at 0.1% TFA, 15.2% acetonitrile, but a 78% loss occurred at this step. Cation exchange chromatography of the 2.1 μg immunoreactive material recovered showed that it was more acidic than a SS28(1-14) synthetic standard.

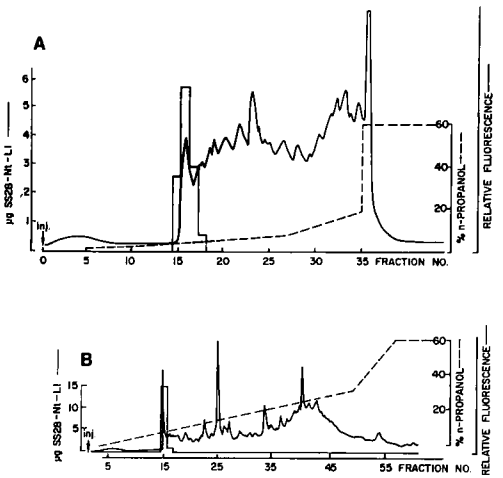

Fig. 2. Two reverse-phase HPLC runs performed with a semi-preparative column: Altex Ultrasphere 25 cm x 1 cm. ODS 5 μm. Mobile phase 0.36 M pyridine formate/n-propanol pH 3. Flow rate: 48 ml/hr. Fraction size: 3 ml. 5% of the column efluent was used for fluorescent peptide detection and 0.5% for RIA determination. In A: load of 80 mg pancreatic material from G-50 gel filtration. In B: load of 52 mg hypothalamic material not retarded on affinity chromatography to SS14 (3000 rat hypothalami equivalent).

In summary, a peptide smaller but more hydrophilic and acidic than SS28 (1-14) is present in rat pancreas. It is conceivable that this peptide corresponds to SS28 (1-12) generated after enzymatic clevage at the site of the double-basic sequence of SS28.

Purification and Characterization of SS28 (1-12) from Hypothalamus

Whole rat brains, when extracted by the method of Bennett;[10] show three peaks of SS28-Nt-LI after Sephadex G-50 Fine gel filtration. The most important of these peaks elutes - as for pancreatic tissue - in a zone of 1300 daltons ($K_{av} = 0.64$). Since brain somatostatin is highly concentrated in the hypothalamus, we chose an acid extract of 3000 rat hypothalami, purified as described above for the 439 rat pancreata, in order to characterize the SS28-Nt-LI peptide. Fractionation on HPLC (Figure 2B) showed a unique peak of SS28-Nt-LI eluting at the onset of a propanol gradient. This peak (fraction 15), containing 14 μg of SS28-Nt-LI, was rechromatographed on an Altex Ultrasphere RP-8 (5 μm) HPLC column (4.6 x 250 mm) with a 0.5% TFA/acetonitrile buffer system (10 to 20% acetonitrile gradient in 2 h) and an automatic stream-sampling fluorescamine detection system. Two peaks of immunoreactive material emerged. The more hydrophobic component, containing 6.1 μg SS28-Nt-LI, was further purified in another HPLC step using an acetonitrile gradient from 14 to 17%. The material eluted at 16% acetonitrile, but only 1.16 μg was recovered. Isocratic elution with 0.5% TFA/15% acetonitrile yielded pure material suitable for amino acid analysis.

Values from 3 analyses of this purified material gave: Asx 1.79 ± 0.09, Ser 1.57 ± 0.06, Glx 1.24 ± 0.19, Pro 2.23 ± 0.34, Ala 2.88 ±0.33, Met 1.08 ± 0.10, Arg 1.21 ± 0.16. This amino acid composition corresponds to SS28(1-12) *i.e.* Ser-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu-OH.

Conclusions

SS28(1-12) is present in rat hypothalamus. The same peptide, or one closely related, is also present in rat pancreas.

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PURIFICATION AND PARTIAL CHARACTERIZATION OF γ-MELANOTROPINS FROM BOVINE PITUITARY

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Introduction

The NH_2-terminal fragment of pro-opiomelanocortin has been identified as the 16,000-dalton glycopeptide in AtT-20 mouse pituitary tumor cell products.[1] The complete mRNA sequence coding for pro-opiomelanocortin in the bovine intermediate pituitary has revealed, in the NH_2-terminal cryptic region, a peptide fragment, His-Phe-Arg-Trp, which is also contained in α- and β-melanotropin (MSH).[2] This fragment, named γ-MSH, is located between pairs of basic amino acids at Arg^{57}-Lys^{56}, Arg^{43}-Arg^{42} and Lys^{28}-Arg^{27} (Nakanishi's numbering system[2]) and hence could be processed *in vivo* at these basic amino acid pairs to yield γ-MSH-like peptides in analogy with the biosynthesis of β-endorphin, γ-lipotropin, β-lipotropin and adrenocorticotropin (ACTH), all pro-opiomelanocortin-derived products.[1]

Radioimmunoassays (RIAs) were developed for the possible pro-opiomelanocortin-derived γ-MSH fragments, γ_1-MSH (Tyr^{55}- Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe^{45}-NH_2), γ_2-MSH (Tyr^{55}-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Phe-Gly^{44}-OH) and γ_3-MSH (Tyr^{55}-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-Arg-Arg-Asn-Gly-Ser-Ser-Ser-Ser-Gly-Val-Gly-Gly-Ala-Ala-Gln^{29}-OH).[3,4] Using these three RIAs, we have reported the presence of at least two glycosylated γ_3-MSH-like peptides, with molecular weights of 8,800 and 4,500 daltons respectively,[5] in the anterior and intermediate lobes of bovine pituitaries. The intermediate lobe alone also contains two forms of γ_1-MSH-like peptide, with molecular weights of 5,600 and 1,600 daltons respectively.[3] All γ_1-MSHs by definition have the structure -His-Phe-Arg-Trp-Asp-Arg-Phe-NH_2 at their COOH-terminus,[3] while all γ_3-MSHs carry His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-Arg-Arg-[4] as an antigenic determinant. From bovine intermediate pituitaries we have now isolated and characterized one of the 1,600-dalton γ_1-MSHs: Lys-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-NH_2[6]. In this paper we

report the isolation and partial characterization of the 4,500-dalton γ_3-MSH from whole bovine pituitaries.

Materials and Methods

Tissue Extraction — Bovine pituitaries were collected from a local abattoir and immediately frozen on dry ice. Batches of frozen pituitaries were homogenized with cold 1 M HOAc containing 20 mM HCl, 0.01% phenylmethylsulfonyl fluoride, 0.001% pepstatin A and 0.1% mercaptoethanol (10 ml/g tissue). The homogenate was centrifuged at 2,000 g for 30 min at 4°C, and the supernatant lyophilized.

Affinity Chromatography — The γ-globulin (IgG) fraction from a pool of 95 ml of γ_3-MSH antisera was prepared by ammonium sulfate precipitation.[7] The resulting IgG, reconstituted to its original serum volume with 0.02 M sodium phosphate, 0.15 M NaCl, 0.01% NaN_3, pH 7.5 (PBS), was coupled to 70 ml of Affi-Gel 10 (Bio-Rad Lab.) in a 3 x 9.5 cm column according to the manufacturer's suggested procedure. Extract of the whole pituitary (0.5 g) was homogenized in 20 ml of PBS with a Polytron homogenizer and filtered. The filtrate was pumped upwards through the affinity column at a flow-rate of 30 ml/hr. The unabsorbed material was washed out with two column volumes of PBS and the adsorbed γ_3-MSH-like peptides eluted with 1 M HOAc. The column was then equilibrated with PBS for reapplication.

Results and Discussion

In a control experiment, Sephadex G-75 chromatography of the whole bovine pituitary extract gave four peaks of immunoreactive (IR) γ_3-MSH at >30,000, 18,500, 10,200 and 4,500 daltons (data not shown). The >30,000-dalton material could correspond to pro-opiomelanocortin; the 18,500 dalton material is probably the prohormone after cleavage of β-lipotropin, similar to the 21,000-dalton ACTH.[1] The 10,200-dalton species is the intact NH_2-terminal fragment, and the 4,500-dalton peptide is a fragment from the NH_2-terminal region containing the γ_3-MSH antigenic determinant. As reported earlier,[3] two IR-γ_1-MSH peptides of 5,600 and 1,600 daltons were also detected. Gel filtration chromatography of the affinity-purified γ_3-MSH-like peptides, however, showed only three IR-γ_3-MSH peaks, with the 4,500 dalton material being present in the highest concentration (Figure 1). Apparently the larger γ_3-MSHs were not retained well on the affinty column. 5.4 g of whole pituitary extract yielded 78 μg (17 nmol based on a molecular weight of 4,500) of the 4,500-dalton IR-γ_3-MSH after affinity chromatography and Sephadex G-75 gel filtration. HPLC[8] subsequently

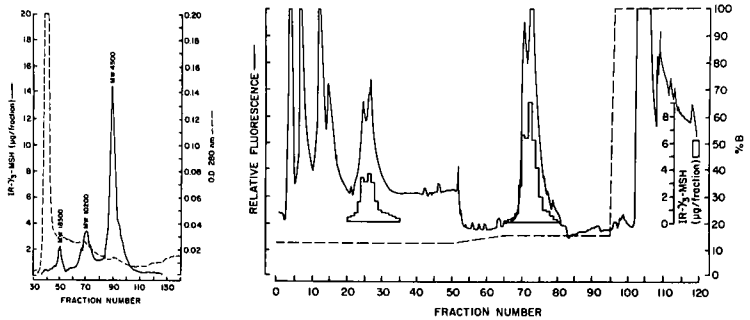

Fig. 1 left. Sephadex G-75 chromatography of the affinity-purified γ_3-MSH. 5.4 g of whole bovine pituitary extract were purified by affinity chromatography as described in the text. The IR-γ_3-MSH so obtained was chromatographed on 150 x 5 cm Sephadex G-75 column equilibrated in 1 M HOAc and previously calibrated with synthetic peptides. Fractions of 20 ml were collected at 140 ml/hr.

Fig. 2 right. Purification of the [affinity + Sephadex G-75]-purified 4,500-dalton γ_3-MSH on a Beckman Model 322 HPLC System. 75 μg of the IR-γ_3-MSH were chromatographed on a 0.46 x 25 cm RP-18 (5 μ) RPLC column protected with a 0.46 x 3 cm RP-18 guard column (Brownlee Labs). Pyridine formate/1-propanol buffers and an automatic stream-sampling fluorescamine detection system were used as described[8] to resolve the material into four peaks of IR-γ^3-MSH.

purified four IR-γ_3-MSH species from this material, as seen in fractions 25, 27, 71 and 73 of Figure 2. Dansylation[9] of the material in each of these four peaks showed that they all contained NH_2-terminal lysine residues. Amino acid analyses[8] (Table I) revealed that all four species are identical in amino acid composition. This composition is in agreement with the pro-opiomelanocortin sequence Lys[56]-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-Arg-Arg-Asn-Gly-Ser-Ser-Ser-Ser-Gly-Val-Gly-Gly-Ala-Ala-Gln[29] predicted by Nakanishi *et al.*[2] However, the calculated molecular weight of this 28-residue peptide is 3,068, which is ~ 1500 daltons less than 4,500. Since we have already determined that all IR-γ_3-MSHs contain carbohydrate[5], we tentatively proposed that the higher molecular weight and microheterogeneity among these four 4,500-dalton γ_3-MSHs are due to differences in the carbohydrate moieties that are linked to the glycosylation site,[10] Asn[29]-Gly-Ser, in the sequence. It is interesting that the 1,600-dalton γ_1-MSH peptide from the intermediate pituitary and the 4,500 dalton γ_3-MSHs all have NH_2-terminal lysine residues.

Table I. Amino Acid Composition of HPLC-Purified 4500-Dalton γ^3-MSHs

	F25	F27	F71	F73	Nearest Integer
ASX	1.9	1.9	1.7	2.1	2
SER	3.9	3.9	3.8	3.8	4
GLX	1.2	1.1	1.0	1.1	1
GLY	5.5	5.9	5.8	6.0	6
ALA	2.2	2.2	2.0	2.3	2
VAL	2.1	2.0	1.9	1.9	2
MET	1.0	0.9	1.0	1.0	1
TYR	0.9	1.0	1.0	0.9	1
PHE	2.1	2.0	2.0	1.9	2
HIS	1.0	1.0	1.0	0.9	1
TRP	0.6	0.5	0.8	0.8	1
LYS	1.2	1.3	1.4	0.9	1
ARG	4.4	4.3	4.6	4.3	4

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COMPLETE SEQUENCE OF A NOVEL PITUITARY PEPTIDE RELATED TO PRO-OPIOMELANOCORTIN: EVIDENCE FOR HIGHLY SPECIFIC ENZYME CLEAVAGE OF PRO-HORMONES

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Introduction

The pituitary hormones corticotropin (ACTH) and β-lipotropin (β-LPH) are biosynthesized from a large glycoprotein named pro-opiomelanocortin (POMC).[1] The primary structure of the bovine homologue of this precursor, including its 26 residue signal peptide, has been deduced by cDNA sequence determination[2,3]. Pulse and pulse-chase experiments have shown that during the maturation of POMC the N-terminal portion of the molecule is released as a 16-17 kilodaltons glycopeptide[1,4]. Preliminary characterization of this N-terminal peptide, isolated from human and porcine pituitary glands,[5,6] indicated that it represents the segment from the N-terminus tryptophan up to almost the ACTH molecule. It was found that considerable sequence homology is maintained between species and in all of them a melanotropin stimulating hormone (MSH) like structure, named γ-MSH[2], is conserved. Two possible sites of glycosylation within this N-terminal peptide have been described[5-7], namely, an N-glycosylation at "Asparagine 65" and an O-glycosylation at "Threonine 45". We have completed the chemical characterization of this novel peptide which was isolated from whole human pituitaries and the anterior lobe of porcine pituitaries. It contains 76 (human) and 80 (porcine) amino acids and its sequence is homologous to the corresponding amino-terminal region predicted from the cDNA sequence of bovine POMC.[2]

Materials and Methods

The peptides were isolated either from whole human pituitaries[6,7] or from freshly dissected porcine anterior pituitary lobes[5] and purified by HPLC. Cysteines 2, 8, 20, and 24 were located by microsequencing of the (^{14}C)-iodoacetamide labeled peptides[5-7]. Sequencing was performed in a Beckman 890B and the phenylthiohydantoins separated by HPLC.

Results and Discussion

Amino acid composition of the reduced and carboxymethylated N-terminal peptide is shown in Table I. The results confirmed the length of the peptide as being 76 and 80 residues for human and porcine, respectively, instead of 103 as previously suggested.[5,6]

Table I. Amino Acid Analysis of Human and Porcine N-Terminal Peptide Following 24, 48, and 72 hrs Hydrolysis in 5.6 M HCl at 105° C

Amino Acid	Human*	Porcine*
Trp	2**	2**
Lys	2	2
His	1	1
Arg	6	6
SCMC	4***	4***
Asx	8	8
Thr	4	3
Ser	10	8
Glx	10	8
Pro	5	5
Gly	7	14
Ala	3	5
Val	1	2
Met	2	1
Ile	1	1
Leu	6	6
Tyr	1	1
Phe	3	3
Total	76	80

* Both contain glycosamine and galactosamine
** Determined using 4N methane sulfonic acid for hydrolysis
*** Confirmed by sequence

Direct sequencing of the entire molecule allowed the identification of more than 50 residues with a few exceptions. The sequencing of the other CNBr fragments from both molecules made it possible, along with overlapping with tryptic peptides to determine the complete sequence of the molecule (Figures 1 and 2).

From this work, it is apparent that the γ-MSH sequence (residues 51-62) (Figures 1 and 2) is identical for the human,[7,8] rat,[9] and bovine[2] species.

The function of glycosylation at Thr 45 and Asn 65 remains to be explained adequately. This is especially important since N-acetyl-galactosamine is commonly present in O-glycosidically linked carbohydrate units; its presence in the N-glycosidic linkages has been discovered only recently in pituitary luteinizing hormone.[10] Such carbohydrate

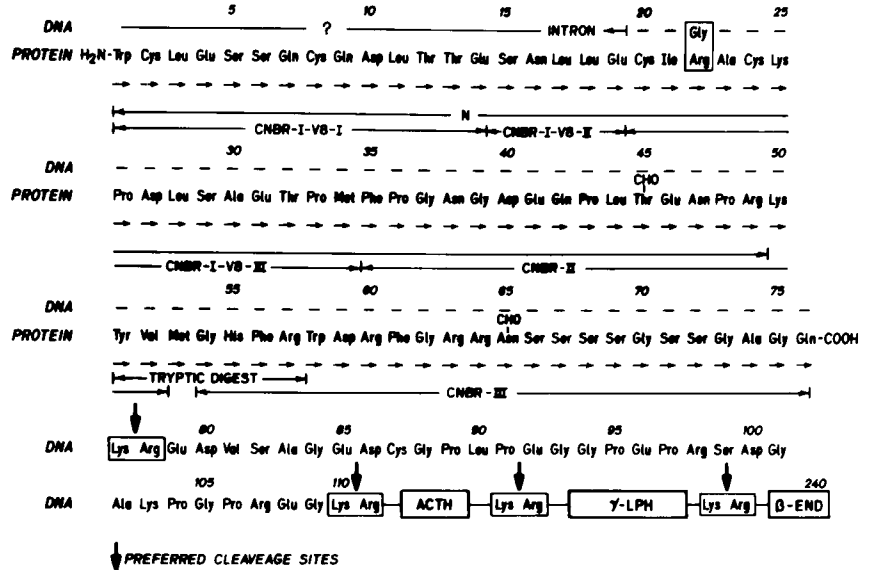

Fig. 1. Amino acid sequence of human glycopeptide compared to the reported human genomic DNA sequence.

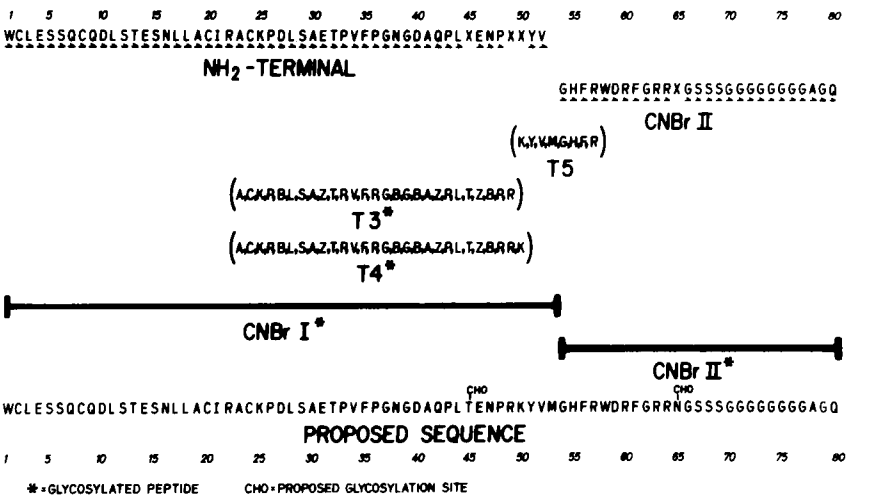

Fig. 2. Proposed sequence of porcine anterior pituitary glycopeptide. This figure shows the alignment of CNBr peptides and the amino acid composition of the T3, T4, and T5 tryptic peptides.

structures may well be involved in guiding the maturation of pro-secretory proteins.

In view of the finding that the N-terminal peptides with glycosylated amino acids migrate on SDS with an apparent molecular weight of about 18,000, it is tempting to speculate whether a peptide of similar C-terminal length represents the 16K variant in mouse AtT-20 tumor cells and in rat pars intermedia.[1,4] Since all pulse-chase experiments relied on either N-terminal sequence analysis and/or peptide mapping, that possibility remains to be clarified.

While investigating biological activities of the new glycopeptide, we found that it is 100 and 10 times more potent than angiotensin II and ACTH, respectively, in stimulating the aldosterone secretion in a human aldosterone secreting tumor.[11]

In human pituitary, the only products known to be secreted, are the N-terminal peptide (containing residues 1 to 76), ACTH, β-LPH, γ-LPH, and β-endorphin. It is thus clear that in the human pars distalis the only pair of basic amino acids cleaved in pro-opiomelanocortin is Lys-Arg (Figure 1). This remarkable selectivity may well speak for an as yet uncharacterized but highly specific maturation enzyme or enzymes.[7,12]

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ISOLATION FROM PITUITARY GRANULES AND PROPERTIES OF A PROTEASE SPECIFIC FOR CONSECUTIVE BASIC RESIDUES

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Introduction

β-Lipotropin (βLPH), its C-fragment (βLPH residues 61-91; β-endorphin), corticotropin and α-melanocyte-stimulating hormone are fragments derived from a common precursor of about 30,000 molecular weight, by stepwise processing involving proteolysis, glycosylation, amidation and acetylation.[1,2] Patterns of processing are different in the anterior lobes and in the intermediary lobes of rat and mouse pituitary.[3] In pig anterior pituitary, the COOH-terminal portion of the precursor is processed principally to βLPH and the opiate-active C-fragment, whereas in intermediary cells, the relatively inactive C'-fragment (βLPH residues 61-87), and N-acetyl derivatives of C-fragment and C'-fragment are formed.[4] Differences in the spectrum of final products could be due to tissue-specific differences in the processing enzymes.

The proteolytic steps have features in common with the processing of other prohormones and proproteins; cleavage occurs at sequences of consecutive basic residues, followed by removal of those residues by exopeptidases. Extracts of pituitaries have been examined for proteolytic activity against substrates designed to differentiate cleavage at various sites containing basic residues within the prohormone molecule.

Results and Conclusion

Subcellular fractions were prepared by differential centrifugation of homogenates of separated anterior and pooled intermediary and posterior lobes of bovine pituitaries, followed by centrifugation on gradients of sucrose. Samples obtained from separated fractions were fixed and stained, and examined under the electron microscope. Large and small granules were found to migrate between 40% and 60% sucrose in homogenates from both tissues, but were more concentrated in anterior pituitary preparations. Estimation of the rates of hydrolyses of a 'paired-basic' substrate, acetyl-L-arginyl-L-arginyl-β-naphthylamide (AcArgArgNapth) by proteases released by lysis of anterior lobe subcellular fractions sonicated at 0°C in 0.01M Tris (pH 7.4) showed highest

specific activity in the granule-rich fraction, while succinate dehydrogenase and acid phosphatase were more concentrated in other fractions. In subcellular fractions obtained from intermediary/posterior lobes, 'paired-basic' activity was more evenly distributed.

Rates of hydrolysis of AcArgArgNapth were proportional to protein concentration, showing that polypeptide fragments present in the granules did not inhibit proteolysis (Figure 1).

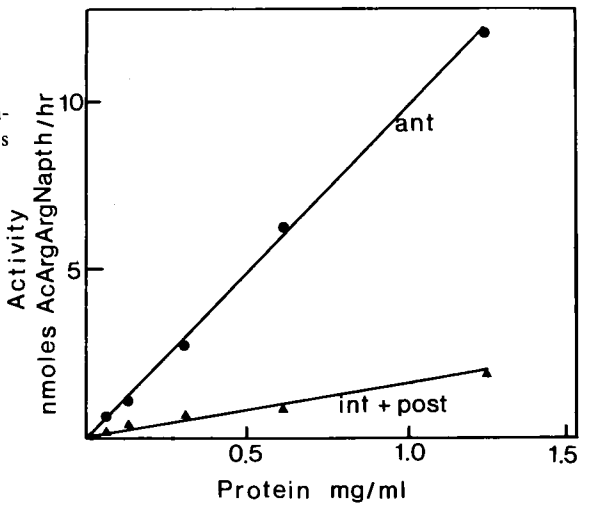

Fig. 1. Linear protein concentration curve in lysates of fractions containing secretory granules.

Specific activity in lysates of fractions banding between 40% and 60% sucrose obtained from anterior tissue was six-fold higher than that in analogous material obtained from combined intermediary and posterior lobes, whereas specific activities of cleavage of a single basic substrate acetyl-L-arginyl-β-naphthylamide (AcArgNapth) were equal in these fractions.

Anterior lobe granules possessed a major protease of about 60,000 molecular weight, with high activity against AcArgArgNapth; it exhibited little activity against the single basic substrate AcArgNapth (Figure 2A). A protease of smaller molecular weight possessed appreciable activity against AcArgArgNapth, and it was found that the proportion of the 'paired-basic' protease to the 'single-basic' protease was lower in granule-rich fractions from intermediary/posterior lobes (Figure 2B). Similar elution profiles were obtained with succinylated peptideamides, although these were poorer substrates and the hydrolysis rates were about one-third the rates of acetylated arginyl napthylamides.

Peptides Ala-His-Lys-Lys-Gly-Gln, resembling the COOH-terminal sequence of pig C-fragment, and DAla-Lys-Asp-Lys-Arg-Tyr-Gly, resembling the activation sequence in βLPH (residues 57-62 plus DAla to

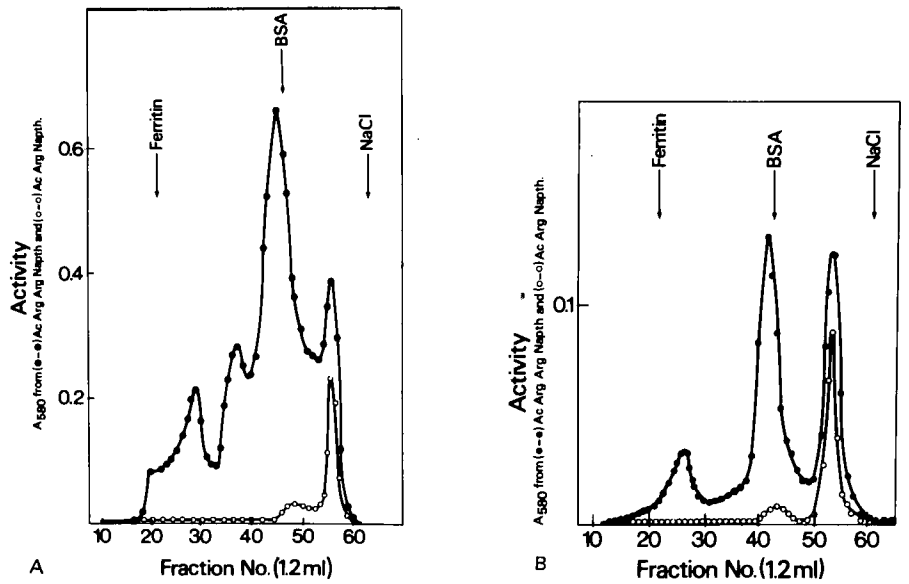

Fig. 2. Gel filtration on Bio-Gel A1.5m (200-400 mesh) in 0.01M sodium phosphate-0.1M NaCl (pH 7.4) of lysates of granule-containing fractions from beef pituitary anterior (A) and intermediary/posterior (B) lobes. The column was calibrated as indicated.

protect against aminopeptidase attack), were synthesized on 1%-cross-linked chloromethylated polystyrene resin, with Boc α-protection, and Bzl, Z- and Tos side protection. Peptides were removed and deprotected by HBr in TFA and Na in NH_3, and purified by gel filtration and ion-exchange chromatography. These substances (0.5 mM) were incubated with peak 'paired-basic' fractions from anterior lobe granules for 10 hr at 37°C and pH 8.5, and products isolated on an amino acid analyzer. The dipeptide, Tyr-Gly, which eluted just after Tyr on the short column, was produced in 22% yield, whereas no Gly-Gln, a standard sample of which was found to elute just after the buffer change to pH 4.25, was released, indicating preferred specificity for the Lys-Arg sequence. Pig βLPH was incubated with the peak fractions, and products analyzed by electrophoresis in 6M urea. Results (Figure 3) showed that C-fragment (β-endorphin) was formed; a much more complex pattern was given by products of mild tryptic digestion of β-LPH. There was no evidence of formation of C'-fragment, which has lower mobility than C-fragment in this electrophoresis system.

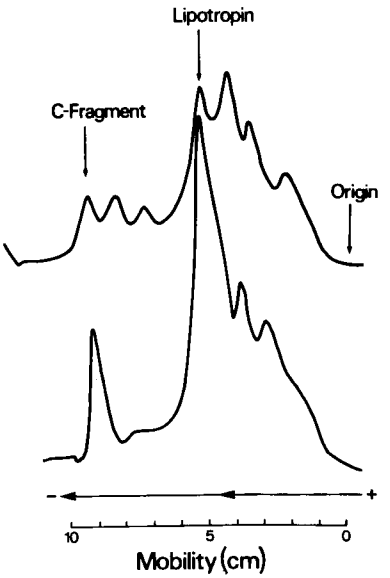

Fig. 3. PAGE of products from βLPH by incubation with Bio-Gel fractionated paired-basic cleavage enzyme (lower curve) and trypsin (upper curve) in 15% polyacrylamide and 6M urea at pH 4.3. Standards C-fragment, C'-fragment and βLPH were also run.

These results show that a protease with preferred specficity for paired-basic residues, and Lys-Arg or Arg-Arg sequences rather than Lys-Lys sequences, resides in lysates of fractions which are rich in storage granules. As this protease was found to liberate the opiate-active C-fragment from βLPH, it is possible that this enzyme is responsible for activation *in vivo*. The different patterns of processing in anterior and intermediary lobes[3,4] may reflect the different relative concentrations of this protease and a second protease that preferentially cleaves a single-basic substrate in the two lobes. A protease other than the 'paired-basic' protease may be required to release C'-fragment.

A homologous protease, also of about 60,000 molecular weight has been purified 132-fold from pig pituitaries by ammonium sulphate fractionation, anion and cation-exchange chromatograpy and gel filtration. The pH optimum of this enzyme has been found to lie between pH 8.0 and pH 8.5; it is completely inhibited by DFP, partially by sulphydryl reagents, and is not affected by EDTA or Ca^{2+}.

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A SYNTHETIC DODECAPEPTIDE SUBSTRATE FOR TYPE C RNA TUMOR VIRUS ASSOCIATED PROTEOLYTIC ENZYME

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The structural proteins of RNA tumor viruses are processed by proteolytic cleavage from high molecular weight precursor proteins. In previous studies,[1] we have determined amino- and carboxyl-terminal sequences of the internal structural proteins of several mammalian type C viruses and have postulated a model for the post-translational processing of their proteins encoded by the *gag* gene. In the avian system, the *gag* gene encoded precursor protein designated $Pr76^{gag}$ of both avian myeloblastosis virus (AMV) and Rous sarcoma virus (RSV) is cleaved during virus maturation into polypeptides designated p19, p7, p27, p12 and p15, which occur in that order from the N-terminus to the C-terminus in the precursor.[2] Our aim is to utilize synthetic peptide substrates for the characterization of proteolytic enzyme(s) responsible for processing of RNA tumor virus precursor proteins. An initial approach was to use a dodecapeptide, Pro-Ala-Val-Ser-Leu-Ala-Met-Thr-Met-Glu-His-Lys, which corresponds to the amino acid sequence spanning the cleavage site (-Ser-Leu-) between p12 and p15 of AMV and RSV $Pr76^{gag}$.[2]

Experimental

The dodecapeptide was synthesized by the solid phase Merrifield procedure in a semi-automated Vega peptide synthesizer, Model 96, using a 1% cross-linked polystyrene-divinylbenzene support. Boc-amino acids with protecting groups of Bzl for Ser, Thr, and Glu; Tos for His and 2-Cl-Z for Lys, and standard DCC couplings were employed. Completeness of coupling was monitored by the ninhydrin assay. Following attachment of the last residue, an aliquot of the protected peptide-resin was treated with 40% TFA in CH_2Cl_2 to remove N-terminal Boc group and the peptide-resin was sequenced in an LKB solid phase sequenator, Model No. 4020, to ensure that the correct peptide has been assembled. HF/10% anisole severed the side chain protecting groups and the peptide from the resin. The crude peptide mixture was desalted on Bio-Gel P2 -0.1 N acetic acid column and purified by high performance liquid chromatography (HPLC) on a preparative μBondapak C_{18} column using an aqueous TFA-CH_3CN gradient.[3] Purified dodecapeptide showed the

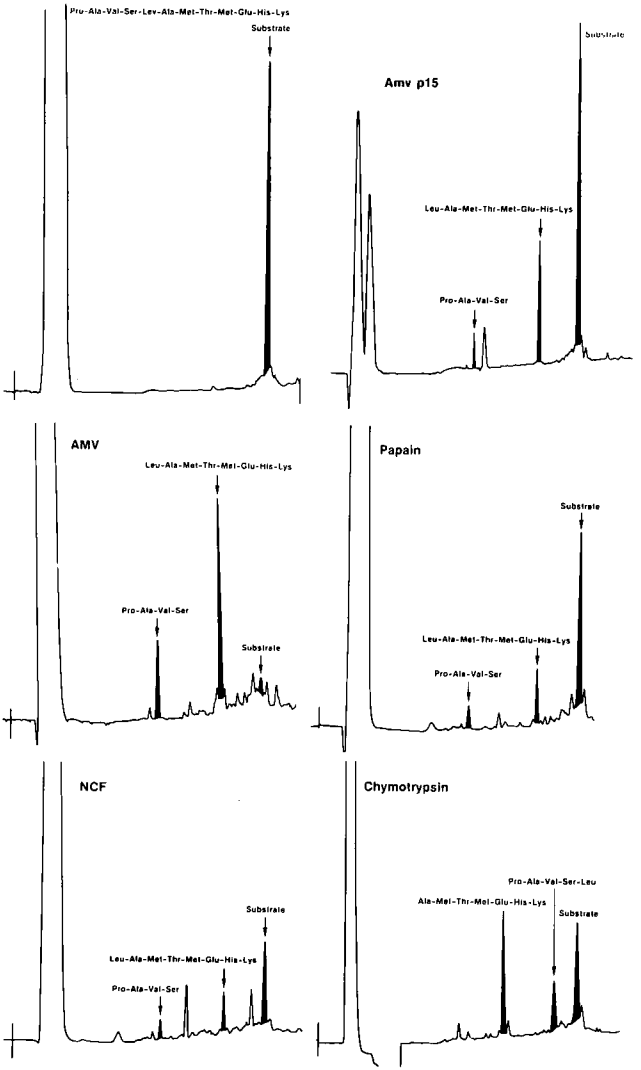

Fig. 1. HPLC profile of the dodecapeptide substrate and fragments generated by various enzymes as indicated (see text)

following correct analysis: Thr 0.94, Ser 0.87, Glu 1.14, Pro 1.04, Ala 2.03, Val 0.94, Met 1.87, Leu 1.09, His 1.00 Lys 1.02. Approximately 20 nmol of the purified dodecapeptide was sequenced in a spinning cup sequenator utilizing established procedures.[4] Due to the C-terminal lysine residue, the dodecapeptide could be sequenced to the end. At each cycle the expected amino acid was identified. No "previews" were

detected. Thus, taking together the amino acid composition and sequence obtained, we conclude that the correct peptide was synthesized and purified.

To study the suitability of the synthetic peptide as substrate for proteolytic activity, 15-20 μg of the dodecapeptide was incubated at 37° C with different enzymes for varying times and over a range of pH. After incubation, the pH was lowered to 1.5 and the extent of cleavage was assayed by HPLC on a μBondapak C_{18} column (Figure 1) followed by amino acid analysis of each separated fragment. As expected, digestion by chymotrypsin at pH 9.5 cleaved the Leu-Ala bond and resulted in two fragments: a pentapeptide Pro-Ala-Val-Ser-Leu and a heptapeptide Ala-Met-Thr-Met-Glu-His-Lys, as shown by the elution profile in Figure 1, and the amino acid composition: Ser 0.77, Pro 0.98, Ala 1.00, Val 0.94, Leu 0.99 and Thr 1.10, Glu 1.08, Ala 1.00, Met 1.90, His 0.97, Lys 1.00 respectively for each fragment. Limited (30 min) digestion with papain at pH 6 generated the tetrapeptide Pro-Ala-Val-Ser and the octapeptide Leu-Ala-Met-Thr-Met-Glu-His-Lys as seen in Figure 1. These fragments showed the following correct analysis: Ser 0.82, Pro 1.05, Ala 1.00, Val 1.03 and Thr 0.91, Glu 1.00, Ala 1.19, Met 2.00, Leu 1.19, His 1.00, Lys 1.00. After establishing that known endopeptidases were capable of cleaving the synthetic dodecapeptide, the effect of AMV associated proteolytic activity was studied. One of the *gag* gene encoded structural proteins, p15, of AMV has been implicated as being a processing enzyme responsible for the cleavage of $Pr76^{gag}$.[5] Partially purified AMV p15 (kindly provided by Dr. Ronald Luftig) known to cleave viral precursor protein[6] was assayed at various pHs using the synthetic dodecapeptide as substrate. As shown in Figure 1, at pH 6 this enzymatic activity (AMV p15 profile) generated the same two fragments as those obtained with papain. Amino acid analysis confirmed that the cleavage by AMV p15 associated enzyme occurred accurately at the Ser-Leu bond. Furthermore, the pH optimum for the cleavage of the dodecapeptide was found to be at pH 6, the previously established pH optimum for the cleavage of the natural precursor protein.[5] Similar results were obtained with sucrose density gradient purified virus (Figure 1, AMV profile) which was partially disrupted by freezing and thawing. Moreover, extracts of virus-free, normal chicken fibroblasts were also capable of cleaving the Ser-Leu bond at pH 6 (Figure 1, NCF profile).

Summary of Results and Discussion

A synthetic dodecapeptide spanning a proteolytic cleavage site in a viral precursor protein was synthesized by the solid phase Merrifield method and purified to give the expected amino acid composition and sequence. An HPLC assay combined with amino acid analysis was employed to test the dodecapeptide as substrate for enzymatic activity.

The synthetic substrate was specifically cleaved at the authentic cleavage site (-Ser-Leu-) by proteolytic enzymes found in AMV, normal chicken cells, by partially purified AMV p15 and by papain, but not chymotrypsin. These results confirmed previous findings that by its specificity AMV associated enzyme is similar to the thiolprotease papain.[5] However, it should be noted that a similar protease is also present in extracts of normal chicken cells. This raises the possibility that the cellular enzyme copurifies with AMV p15. Application of the synthetic dodecapeptide and/or its fragments to isolate the proteolytic enzyme by affinity chromatography is underway. Synthetic peptides corresponding to authentic cleavage sites in the mammalian type C virus precursor protein have also been synthesized and are being studied.

Acknowledgements

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BIOSYNTHETIC ORIGIN OF NEUROPHYSINS

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Introduction

Biochemical study of the neurophysins (NP's), hypothalamo-neurohypophyseal carriers of the neuropeptide hormones oxytocin (OT) and vasopressin (VP), has focused on fundamental questions about the biosynthetic and molecular events by which these proteins are formed and stabilized into noncovalent peptide-protein complexes. While interacting complexes can be formed between native protein and hormone in their isolated forms, it has become evident from several properties of these molecules that biosynthetic assembly is less straightforward. Both pulse-chase biosynthesis[1] and folding[2] analyses have led to predictions that NP's represent processed derivatives of originally biosynthesized forms and, further, that their incorporation into noncovalent complexes with hormones ensues in some way from these post-synthetic processing events. However, since these types of data provided largely circumstantial evidence for NP biosynthetic precursors, we have sought more direct chemical evidence that such molecules are made containing NP amino acid sequences. Implications of precursor synthesis on the origin of properties of mature NP's are considered.

Products of *in vitro* Translation of Bovine Hypothalamic mRNA

Neurophysin biosynthesis has been addressed in our laboratory mainly by *in vitro* translation.[3,5] The basic tactic has been to use wheat germ extracts and rabbit reticulocyte lysates as cell-free systems to express all proteins coded by bovine hypothalamic poly A+ mRNA and then to isolate any products that are NP-related using affinity-purified, specific antibodies elicited by the major, authentic bovine NP's (I or II). From both cell-free systems, two translated proteins are identified, with apparent molecular weights of 17-19,000 (NP I-related) and 23-25000 (NP II-related) daltons. These NP-related proteins have been defined as translation products (TP's) I and II, respectively.

Since antibody recognition of TP's does not provide rigorous proof of their identification as NP-containing, efforts have been made to establish this chemically by peptide mapping of the ^{35}S-Cys-labelled TP's

using reverse phase high performance liquid chromatography.[4] As shown in Figure 1, co-migration is observed of all cysteic acid-containing peptides of authentic NP's with those from ^{35}S-Cys-containing TP's. TP I shows some contamination with TP II, as indicated by the labeled peak corresponding to peptide 1_{IIB}. For both TP maps, the relative areas of peaks for ^{35}S-containing products assignable as peptides 1, 2, 3, and 4 correlate well with the contents of ^{35}S expected from the cysteic acid contents of the authentic peptides, namely 5, 4, 2, and 3 residues per peptide molecule, respectively. Ignoring possible background, radioisotope ratios for peaks 1, 2, 3, and 4 are 5.2:5.0:1.5:2.4 for the TP II case and 4.8:3.8:2.7:3.7 for the TP I case.

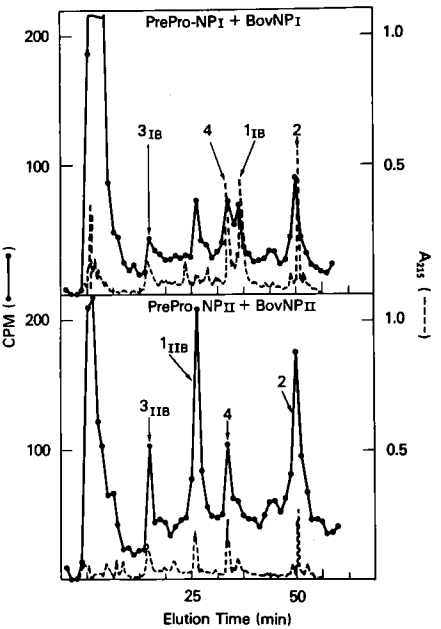

Fig. 1. Reverse phase HPLC peptide maps of TP I (top) and II (bottom) from rabbit reticulocyte lysate translation with [^{35}S]Cys. Column: Zorbax CN (Dupont, 0.46 x 25 cm); elution conditions described elsewhere.[4] Each sample: anti-I or -II immunoprecipitate from a 200 μl translation +0.5 mg bovine NP I or II; each mixture performic acid oxidized and trypsin digested. CPM from TP; A_{215} from added authentic NP. Peptides assigned by amino acid analysis; numbering as before;[4,6] subscripts: NP species (Roman numerals) and bovine source (B).

The mapping data substantiate the occurrence of the NP I and II sequences in TP's I and II. Since TP's I and II are the only major NP-related proteins isolated using hypothalamic poly A+ mRNA and since the hypothalamus is the site of NP biosynthesis, these proteins are identifiable as prepro-NP's. Interestingly, the TP's can be obtained from two different size classes of mRNA separated from hypothalamic poly A+ mRNA by electrophoresis on methylmercuric hydroxide agarose gels.[5] The smaller of these is in the range of .4-.7 kilobases and likely represents mature mRNA. The larger, of 4-9 kilobases depending on the TP, is of as yet unknown significance.

Peptide Mapping of Rat "Pro" Forms

Reverse phase HPLC mapping was used to examine pro-NP forms produced by pulse-labelling in rats[7] and provided to us for collaborative study by Drs. H. Gainer and J. Russell (NICHD, NIH) and M. Brownstein (NIMH). Maps obtained for the trypsin-digested performic acid oxidized pro-NP's I and II (ArgVP- and OT-associated, respectively, in the rat; both about 20,000 daltons) were compared with those for similarly treated authentic rat NP's, bovine NP's, OT and ArgVP. Unlike the bovine case, rat NP's have not been sequenced fully. Nonetheless, based on existing sequence information[8] and analogy with the bovine proteins, tentative assignments have been made for cysteic acid-containing peptides 1, 2, and 3. The comparisons thus made indicate the presence of all three peptides in the pro-NP's, showing chemically that the pulse-labelled pro forms also contain NP sequences.[9]

Future Directions

The study of biosynthetic precursors should help to provide an expanded framework for understanding how self-assembly operates in generating biologically active peptides and proteins. The availablity of precursor forms which are chemically verified to contain NP sequences allows several central questions to be addressed regarding the ultimate function of the mature NP's as carriers of the neuropeptides, including (Figure 2) the relationship of NP precursors to neuropeptide biosynthesis, the nature of precursor processing to mature forms, and the biosynthetic generation of both neuropeptide-binding and peptide-mediated self-association properties of the NP's. The view of a common neuropeptide-NP precursor is arguable from considerable non-chemical evidence.[1,7] As yet, though, peptide mapping data have yielded no direct support that the peptides are contained in NP precursor sequences. The octapeptide of residues 1-8 of ArgVP, which would be produced if there were a trypsin-susceptible bond at the α-amino group of Cys 1 or if Cys 1 were NH_2-terminal, is not found in maps for either prepro or pro forms. Based on peptide mapping results, tryptic fragments are being sought that contain neuropeptide sequences with extensions. Further, the lack of production of Arg VP-(1-8) by trypsin suggests that processing probably involves at least some non-trypsin-like proteases. Protease activities of hypothalamic and posterior pituitary extracts are being studied in our laboratory. Finally, mature NP's have been found to self-associate in a manner mediated by neuropeptide binding. The interrelated binding-

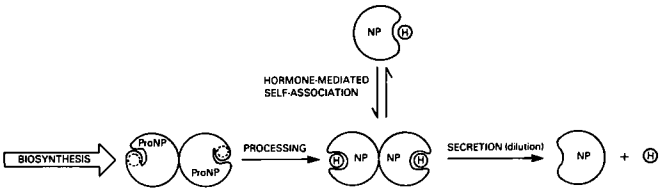

Fig. 2. Schematic drawing relating biosynthetic precursor of NP with precursor processing, hormone-mediated self-association, and ultimate dissociation of hormone-NP complex after secretion. Hormone is viewed here as a possible covalent component of the NP precursor.

association properties are likely to affect the state of neuropeptides and NP's, and perhaps precursors also, during storage in neurosecretory granules. The genesis of these interaction properties during biosynthesis remains to be defined at the molecular level.

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SYNTHESIS AND PROPERTIES OF RHODOPSIN CARBOXYL-TERMINAL PEPTIDES

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Introduction

We have prepared peptides from the carboxyl terminus of rhodopsin, a surface-exposed region of the protein which includes sites of enzymatic phosphorylation and sites of limited proteolysis. Rhodopsin, the photoreceptor protein of rod cells in the retina, is an integral membrane protein. Its structure, function and topography have been studied by chemical, physical, and enzymatic techniques including limited proteolysis. Short-term proteolysis of rhodopsin with thermolysin releases peptides from the carboxyl-terminal region, removing part of the phosphorylation[1] site. Longer-term proteolysis excises a surface-exposed region internal to the sequence, forming a two-fragment complex designated F1-F2. We have synthesized peptides comprising parts of the carboxyl-terminal 25 amino acids of rhodopsin: $Gly^{25'}$-Lys-Asn-Pro-Leu-$Gly^{20'}$-Asp-Asp-Glu-Ala-Ser-Thr-Thr-Val-Ser-$Lys^{10'}$-Thr-Glu-Thr-Ser-Gln-Val-Ala-Pro-$Ala^{1'}$-COOH. These and other peptides will be used for the cellular immunochemical localization of these regions of rhodopsin and other immunochemical studies, and a study of the requirements for the site of rhodopsin phosphorylation.

Results

Peptides 1'-12' and 1'-25' of the rhodopsin sequence were synthesized by the solid phase technique using Pam resin.[2] Protected amino acids used were N^{α}-BocSer(Bzl), N^{α}-BocThr(Bzl), N^{α}-BocAsp(OBzl), N^{α}-BocGlu(OBzl), and N^{α}-BocLys(ClZ). Peptide 1'-12' was prepared on a 1 mmol scale, and peptide 1'-25' on a 0.5 mmol scale. All solvents used were at a ratio of 13-19 ml/g resin. The protocol for a typical cycle included (1) 25% CH_3COOH-CH_2Cl_2 + 0.1% indole, 1.5 min, then repeat for 30 min, (2) CH_2Cl_2, 6X, 1.5 min, (3) 10% DIEA-CH_2Cl_2, 2X, 1.5 min, (4) CH_2Cl_2, 6X, 1.5 min, (5) 2.5 equiv. Boc-aa-CH_2Cl_2, 5 min, followed by 2.5 equiv. DCC, 120 min, (6) CH_2Cl_2, 6X, 1.5 min. For Gln coupling we used the DCC/HOBt method and CH_2Cl_2-DMF (1:1, v/v). Coupling

efficiency was monitored by the Kaiser test[3] or, for proline, by the chloranil test.[4] For synthesis of peptide 1′-25′, step 5 employed 2.5 equiv. Boc-aa and 2.5 eqs HOBt in CH_2Cl_2-DMF (1:1, v/v), and steps (3), (4), (5), and (6) were repeated. Synthesis was monitored periodically by acid hydrolysis of the peptide-resin[5] and amino acid analysis, and by solid phase sequence analysis.[6] Both resin-bound peptides were found to be free of preview.

Peptides were cleaved from the resins by treatment with 10 ml HF (without drying) + 0.1 ml H_2O, at 0° C, for 45 min. Following evaporation of HF, peptides were extracted with glacial acetic acid and desalted on BioGel P-2 in 50% acetic acid. The crude peptide 1′-12′ was shown to contain one major component when analyzed by HPLC (Figure 1). Peptide 1′-12′ was shown to contain one major component when analyzed by HPLC (Figure 1). Peptide 1′-12′ was chromatographed using polystyrene sulfonic acid ion exchange resin (BioRad Aminex A5) and a pH gradient of pyridine-acetate buffers from pH 2.40 to 3.75. Fractions were monitored by TLC (silica gel G, 15 cm, 2-BuOH/CH_3COOH/H_2O, 10:35:25). Fractions containing the main component (R_f = 0.55) were collected and found to contain the peptide in ~95% purity (Figure 1, inset) and 42% overall yield. Amino acid composition: Thr = 1.94 (2), Ser = 1.86 (2), Glu = 2.00 (2), Pro = 0.99 (1), Ala = 1.93 (2), Val = 1.82 (2), Lys = 1.00 (1).

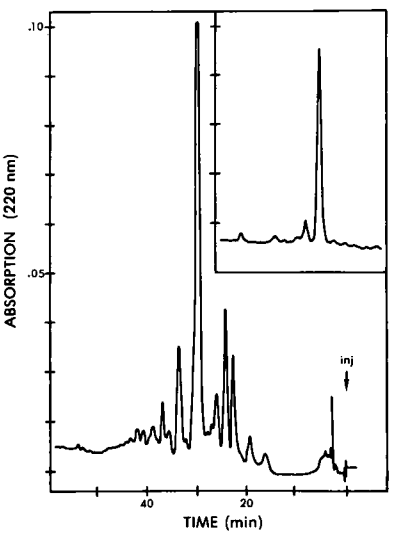

Fig. 1. HPLC analysis of peptide 1′-12′. Crude peptide was chromatographed on Waters μBondapak C_{18} (0.39 x 30 cm) with a linear gradient 0-25% B over 40 min at 2 ml/min (A = 0.1% H_3PO_4/H_2O; B = 0.1 % H_3PO_4/CH_3CN). INSET. HPLC analysis of peptide 1′-12′ following purification by Aminex A5 chromatography.

Peptide 1′-25′ was prepared with [^{3}H]-Gly in position 25′. Purification was monitored by radioactive counting. The mixture obtained following HF cleavage from the resin was more complex than that found for peptide 1′-12′ as evaluated by HPLC. Purification by ion exchange chromatography proved ineffective, but the major component was readily purified by preparative HPLC

Synthetic peptides were evaluated for their ability to compete with [^{125}I]rhodopsin in a radioimmunoassay (Figure 2). Peptide 1′-12′ competes [to an extent of $1/(5 \times 10^4)$ compared to rhodopsin, at the level of 20% inhibition] and is surprisingly a better competitor than the longer 1′-25′.

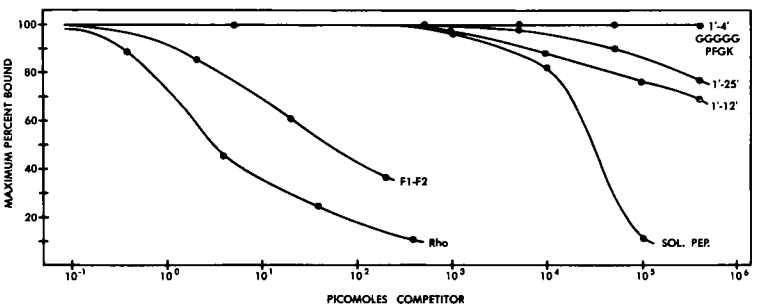

Fig. 2. Competition of synthetic peptides with [I^{125}] rhodopsin in radioimmunoassay. Tests were made with the two-fragment complex resulting from rhodopsin proteolysis (F1-F2), soluble peptides from the digestion (sol. pep.), synthetic carboxyl-terminal sequences 1′-4′, 1′-12′, 1′-25′, and the non-rhodopsin peptides pentaglycine (GGGGG) and Pro-Phe-Gly-Lys (PFGK).

Peptide 1′-12′ can be phosphorylated by rod cell kinase. Using 100X more peptide and 2X more kinase (compared to a control experiment using rhodopsin as substrate), peptide 1′-12′ was phosphorylated to an extent 62% that of rhodopsin.

Acknowledgements

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BIOSYNTHESIS OF GLYCOSYLATED AND PHOSPHORYLATED FORMS OF CORTICOTROPIN-LIKE INTERMEDIARY LOBE PEPTIDE

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Introduction

The biosynthesis of the ACTH and β-LPH family of peptides has been intensively studied in the last few years. A common 30,000 dalton precursor is processed by proteolytic cleavages and other post-translational modifications to yield ACTH and β-LPH in the anterior pituitary[2] and α-MSH, β-endorphin and corticotropin-like intermediary lobe peptide (CLIP) in the intermediary pituitary.[3] We have been studying the heterogeneity of CLIP in the rat neurointermediary lobe (NIL) by using a very efficient extraction procedure involving octadecylsilyl silica (ODS-silica)[4] and reversed-phase high performance liquid chromatography[5] (RP-HPLC). We have isolated the two major forms of rat CLIP, and have demonstrated that one of these peptides is unmodified rat $ACTH_{18-39}$.[6] In addition, phosphorylated forms of rat $ACTH_{1-39}$ and the rat 30,000 dalton precursor were also identified.[6]

Table I. Amino Acid Analysis of the 8 Isolated Rat CLIPs.

	1 PG-CLIP 18-38	2 G-CLIP 18-38	3 P-CLIP 18-38	4 CLIP 18-38	5 PG-CLIP 18-39	6 G-CLIP 18-39	7 P-CLIP 18-39	8 CLIP 18-39	Theor. CLIP 18-39
Asx	1.9	2.0	2.0	1.9	1.8	1.9	1.8	1.9	2
Ser	1.0	1.0	0.8	0.9	1.0	1.0	0.8	1.0	1
Glx	3.8	3.9	4.0	3.8	3.7	3.7	3.8	3.8	4
Pro	2.5	2.5	2.9	2.7	2.9	2.5	2.8	2.7	3
Ala	2.9	2.9	3.0	3.0	3.0	3.0	2.9	2.9	3
Val	2.3	2.8	2.9	2.8	2.4	2.4	2.8	3.0	3
Leu	1.0	0.9	1.0	0.9	0.9	1.0	1.0	0.9	1
Tyr	1.1	1.0	1.3	1.1	1.2	1.0	1.0	1.1	1
Phe	1.1	0.9	1.2	1.1	1.9	2.0	1.7	2.1	2
Lys	0.9	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1
Arg	1.1	1.0	1.1	1.0	1.2	1.0	1.0	1.0	1

It has been well recognized that rat ACTH and rat CLIP are both found in glycosylated and non-glycosylated forms,[1] and the site of glycosylation has been thought to be the asparagine at position 29.[1] In order to investigate the relationship between the glycosylated and phosphorylated forms of rat CLIP, ^{3}H-labeled N-acetyl glycosamine and ^{32}P-labeled potassium phosphate were incorporated into rat NILs and the resultant radio-labeled forms of rat CLIP were isolated and identified. Some of the findings of these experiments are described in this paper.

Results

NILs from 20 male Sprague-Dawley rats were incubated for 18 hours at 37° C in Dulbecco's Modified Eagle's Medium with monopotassium [^{32}P] phosphate (2mCi) and [^{3}H] N-acetyl glucosamine (1mCi). The tissue and medium were homogenized and extracted using ODS-silica[6] and the resultant peptide fraction was chromatographed by RP-HPLC using a linear gradient of 20-40% aqueous acetonitrile containing 0.1% trifluoroacetic acid throughout (Figure 1). The details of the RP-HPLC procedure are described elsewhere in this volume.[7] Aliquots were taken from each fraction for determination of ^{3}H and ^{32}P by scintillation counting, and also for determination of CLIP by radioimmunoassay.[5] Multiple areas of CLIP immunoreactivity, ^{32}P labeling and ^{3}H labeling were found (Figure 1). CLIPs 7 and 8 corresponded to the previously identified phosphorylated rat $CLIP_{18-39}$

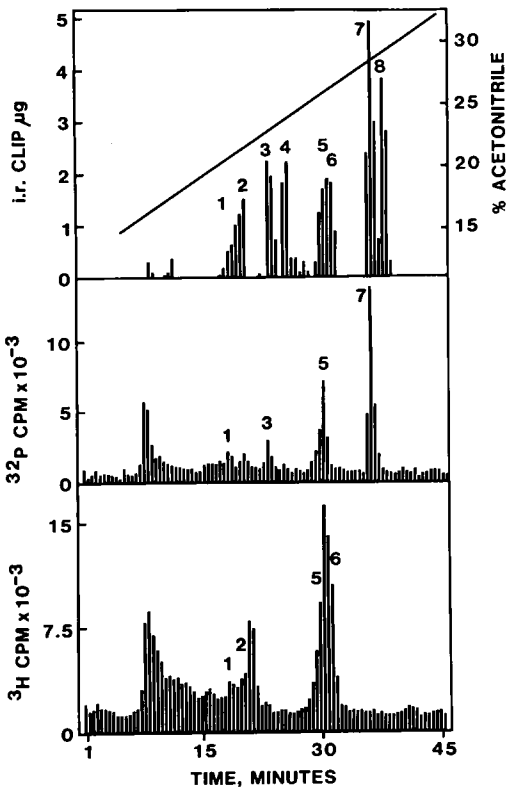

Fig. 1. RP-HPLC separation of an extract of rat NILs after an 18 hour incubation with [^{32}P] phosphate and [^{3}H] N-acetyl glucosamine.

and non-phosphorylated rat $CLIP_{18-39}$ respectively.[5,6] CLIP 7 clearly corresponded in elution position to the largest peak of ^{32}P, whereas CLIP 8 did not contain ^{32}P. Neither CLIPs 7 nor 8 corresponded to peaks of [^{3}H] N-acetyl glucosamine, confirming that they are not glycopeptides.

The largest peak of ^{3}H counts corresponded to a pair of CLIPs (5 & 6) which were not well resolved (Figure 1). Each of these could be easily purified by a second chromatography using heptafluorobutyric acid as the counter ion instead of trifluoroacetic acid. It was then clear that both CLIPs 5 and 6 contained ^{3}H counts, but only CLIP 5 contained ^{32}P. Thus CLIPs 5 to 8 corresponded to a phosphorylated, glycosylated (5), a non-phosphorylated, glycosylated (6), a phosphorylated, non-glycosylated (7) and a non-phosphorylated, non-glycosylated CLIP (8) respectively. A similar analysis revealed that the same pattern of glycosylation and phosphorylation in the same elution order was present for CLIPs 1 to 4. In order to understand the nature of these eight peptides more fully, an extract of 150 fresh rat NILs was prepared, and all 8 CLIPs were isolated by the RP-HPLC method.[5] Amino acid analyses revealed that CLIPs 1 to 4 differed from CLIPs 5 to 8 by having one phenylalanine instead of two (Table I). Carboxypeptidase digestion showed that the carboxyl terminal amino acid was glutamic acid for CLIPs 1 to 4 and phenylalanine for CLIPs 5 to 8. Thus the eight forms of rat CLIP are in order of elution: CLIP 1 phospho-glyco-$CLIP_{18-38}$; CLIP 2 glyco-$CLIP_{18-38}$; CLIP 3 phospho-$CLIP_{18-38}$; CLIP 4 $CLIP_{18-38}$; CLIP 5 phospho-glyco-$CLIP_{18-39}$; CLIP 6 glyco-$CLIP_{18-39}$; CLIP 7 phospho-$CLIP_{18-39}$; CLIP 8 $CLIP_{18-39}$.

Conclusions

Post-translational modifications of rat CLIP have produced a heterogeneous mixture of peptides. The three modifications described here (phosphorylation, glycosylation and loss of C terminal amino acid) give rise to the 8 peptides which are the product of all combinations of the presence or absence of these modifications. The phosphorylation of $serine_{31}$ and glycosylation of $asparagine_{29}$ is of interest, as the sequence Asn-Glu-Ser ($ACTH_{29-31}$) is a classical recognition sequence of glycosylation. It may be that phosphorylation and glycosylation are related events, and that asparagine at position 29 is necessary for phosphorylation of $serine_{31}$. The finding of the $CLIP_{18-38}$ peptides may reflect a general heterogeneity of the carboxyl terminus of ACTH, especially as $ACTH_{1-38}$ has been isolated from bovine pituitaries.[8] The relevance of all of these modifications to any putative biological role for CLIP remains to be discovered and is the subject of current investigation.

Acknowledgements

We thank Susan James and Susan Parkinson for their skilled technical assistance and Dr. Michel Van der Rest and Mr. E. Wan for performing the amino acid analyses. The work was supported by MRC of Canada (Grant No. MT-1658) and the U.S. Public Health Service (Grant No. HDO-4365).

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SYNTHESIS OF A NAPHTHOQUINONE TRIPEPTIDE WHICH INHIBITS VITAMIN K-DEPENDENT CARBOXYLASE

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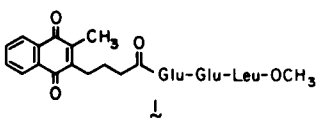
Vitamin K is a cofactor in the postribosomal conversion of glutamyl to γ-carboxyglutamyl residues in the liver microsomal precursors of the blood coagulation proteins, prothrombin and Factors VII, IX and X.[1] This O_2-dependent, ATP- and biotin-independent, carboxylation reaction is necessary for the calcium-mediated phospholipid binding required for the physiological action of these proteins. More recently, proteins containing γ-carboxyglutamate residues or the vitamin K-dependent carboxylase have been demonstrated in a number of non-hepatic organs or tissues, and this enzyme appears to have a rather wide distribution.[2]

A number of low-molecular-weight peptide substrates of the microsomal carboxylase have been synthesized.[3] However, inhibitors of the peptide-binding site, despite their value in mechanistic studies and enzyme purification, have been more difficult to obtain. The two competitive inhibitors developed to date[4,5] do not bind the enzyme tightly enough to be useful in advanced work.

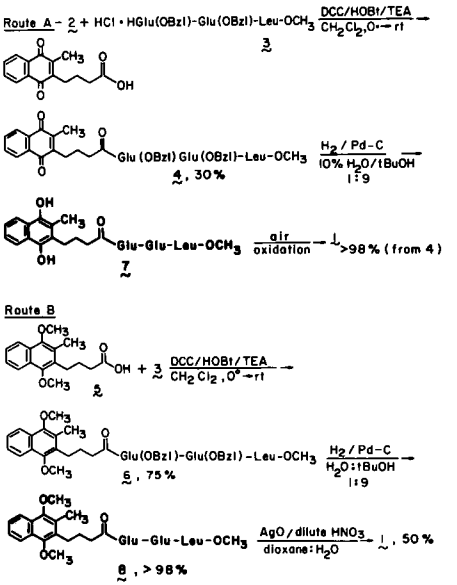
Most reaction mechanisms proposed for this unique carboxylase require that vitamin K be in close proximity to the substrate glutamyl residues.[6] If the vitamin functions in this manner, a compound in which the 2-methylnaphthoquinone nucleus of vitamin K is covalently attached to a good substrate, *i.e.*, -Glu-Glu-Leu-OCH_3,[3] could competitively inhibit the enzyme at both subsites. Multisubstrate analogs of this type have yielded important information about enzyme mechanisms.[7] We have therefore synthesized the naphthoquinone tripeptide **1** as a potential collected substrate inhibitor of vitamin K dependent carboxylase,



and report here that it is the first peptide shown to inhibit vitamin K dependent carboxylation of endogenous blood coagulation precursors as well as peptide substrates.

Results

Scheme 1. The Synthesis of Naphthoquinone Tripeptide **1** and Related Compounds.



4-(2-Methyl-1,4-naphthoquinonyl-3)-butyric acid **2**, was prepared by literature procedure[8] and coupled with HCl·HGlu(OBzl)-Glu(OBzl)-Leu-OCH_3 (3) in 30% yield using established methods for preparing substrate peptides.[3] The low yield of **4** probably is caused by reaction of the free amine of tripeptide **3** with the naphthoquinone ring either at the carbonyl or side chain methyl groups.[9] To circumvent this side reaction, a dimethyl quinol ether derivative **5** was synthesized,[10] and coupled with tripeptide **4** to give the naphthoquinol ester **6** in 75% yield (Scheme 1). Hydrogenolysis of **4** and **6** over 10% Pd/carbon[11] gave peptides **7** and **8** respectively in quantitative yield. This reagent catalyzes the reduction of the naphthoquinone system only to the dihydronaphthoquinone and not to a 5,6,7,8 tetrahydronaphthoquinone system.[12] Removal of the methyl groups from the diether **8** by treatment with AgO in dilute acid gave inhibitor **1** (50%).[13,14] The overall yield from either procedure is 30 to 40% but Route A is preferred because of the reduced number of steps involved. Compound **1** also has been obtained in modest yield (10-15%) by coupling the naphthoquinone acid **2** to H-Glu-Glu-Leu-OCH_3 using the mixed anhydride method.

Tripeptides **1**, **7** and **8** were assayed in a standard assay system[5] to see if they are substrates for vitamin K-dependent carboxylase. Compound **1** is carboxylated to the same extent as the standard peptide, H-Phe-Leu-Glu-Glu-Leu-OH, at 0.25 mM (Figure 1). At higher concentrations, apparent substrate inhibition is observed and the activity drops to baseline levels. Compound **8**, by contrast, is a poor substrate at all concentrations. When tested as potential inhibitors, compounds **1** and **7**, but not **8** inhibit carboxylation of microsomal precursor proteins 18% and 57% at 1 and 3 mM concentrations respectively (Figure 2). Added standard peptide had no effect on this inhibition of protein carboxylation.

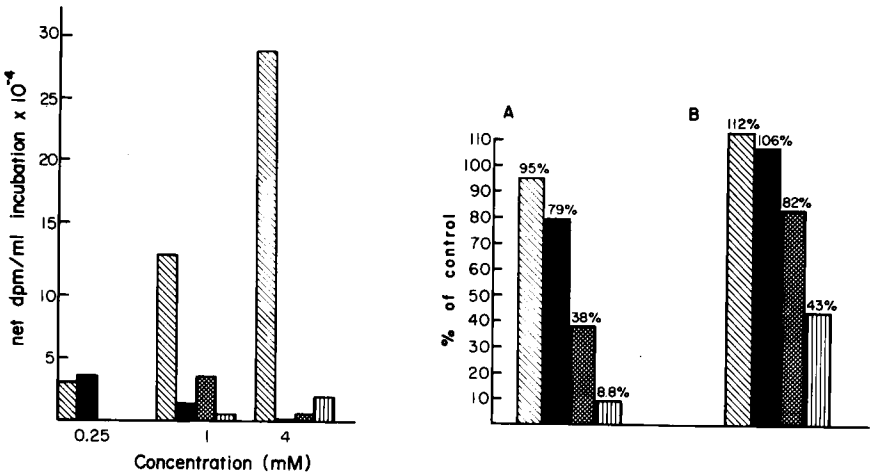


Fig. 1. (Left) Carboxylation of naphthoquinone tripeptides **1**, **7** and **8** by vitamin K-dependent carboxylase.

▨ = H-Phe-Leu-Glu-Glu-Leu-OH; ■ = **1**; ▩ = **7**; ▥ = **8**.

Fig. 2. (Right), Inhibition of peptide and protein carboxylation by naphthoquinone tripeptide **1**. A. Carboxylation of 0.5 mM H-Phe-Leu-Glu-Glu-Leu-OH in the presence of varying concentration of **1**. B. Carboxylation of endogenous proteins in the presence of varying concentration of **1**.

% of control = carboxylation of substrate in the presence of **1**/carboxylation of substrate plus carboxylation **1**. All incubations contain 133 μg/ml reduced vitamin K. ▨ = 0.0625 mM; ■ = 0.25 mM; ▩ = 1.0 mM; ▥ = 3.0 mM

Discussion

The naphthoquinone tripeptides were synthesized to see if the combination of the vitamin nucleus with a good substrate sequence would produce an effective inhibitor of the carboxylase. Both compounds **1** and **7** are substrates for vitamin K-dependent carboxylase at concentrations much lower than found for any substrate evaluated to date and this clearly establishes that the peptide portion of **1** binds to the carboxylase at the peptide binding site. Surprisingly we found that both compounds **1** and **7** are inhibitors at high concentrations, and both compounds also inhibit carboxylation of endogenous protein precursors. It remains to be established if **1** is a multisubstrate analog inhibitor[7] or if it inhibits by some other mechanism. Nevertheless, compounds **1** and **7** are the first peptides related to substrates of the carboxylase which have been found to inhibit carboxylation of the endogenous protein precursors. Further studies of these compounds to obtain improved inhibitors are in progress.

Acknowledgement

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AMINO ACID SEQUENCE COMPARISON OF RAT TONIN WITH NERVE GROWTH FACTOR-γ-SUBUNIT AND WITH SERINE PROTEASES

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Introduction

Recently, a novel serine-protease, tonin, was isolated in homogeneous form from rat submaxillary gland.[1] Substrate specificity studies revealed the protease to be able to release angiotensin II from angiotensinogen, angiotensin I and also from a synthetic tetradecapeptide comprising the first 14 residues of angiotensinogen. Furthermore, it can produce some biologically interesting peptides from β-lipotropin (β-LPH), from corticotropin (ACTH) and from ACTH/LPH common precursor, the pro-opiomelanocortin (POMC) molecule.[2-3] Initial work done on tonin showed that it is a single polypeptide chain of molecular weight 28,700 having Ile and Pro at NH_2- and COOH-termini, respectively.[4] The present report describes more work done on sequence determination of tonin (representing more than 50% of the molecule). Moreover, this report even though preliminary, will describe the residues making up the active site identified by homology localization and the close relationship of tonin with rat submaxillary gland kallikrein and surprisingly with the recently available sequence of murine nerve growth factor-γ-subunit.[5]

Methods

Rat submaxillary gland tonin and kallikrein were reduced and alkylated with iodoacetic acid in denaturating solvent using known procedures. The sequence done with approximately 85 nmoles using an updated Beckman 890B sequencer fitted with a cold trap, polybrene and 0.3M Quadrol buffer, permitted the identification of the first 65 residues together with the identification of a minor contaminating sequence arising from autolysis of the tonin and kallikrein molecule.

Other sequence data were obtained from fragments produced by cyanogen bromide cleavage on the reduced and alkylated tonin molecule.

Because of the limited amount of protein, purification was accomplished by HPLC on μC_{18} column using either acetonitrile or propanol gradient in triethylammonium formate buffer. Identification of the fragments was accomplished using amino acid analysis (on 10-30 nmoles samples) while localization was done by homology with known sequences.

Results and Discussion

It was possible to extend the previously reported sequence[4] up to 65 cycles as shown in Figure 1. Comparison between the sequence of γ-subunit, tonin, and kallikrein proved to be quite striking since it reveals more than 75% homology between these three proteins. Furthermore, the sequence V-()-()-A-A-H-C, common to all serine proteases except to haptoglobin[6] is present in all three. The presence of the histidine residue in this region together with the presence of the invariant cysteines at position 24 and 40 (tonin numbering) reinforces the close relationship with the serine proteases family since the histidine is part of the charge-relay network while the cysteines are crucial to maintain the proper orientation of the histidine.

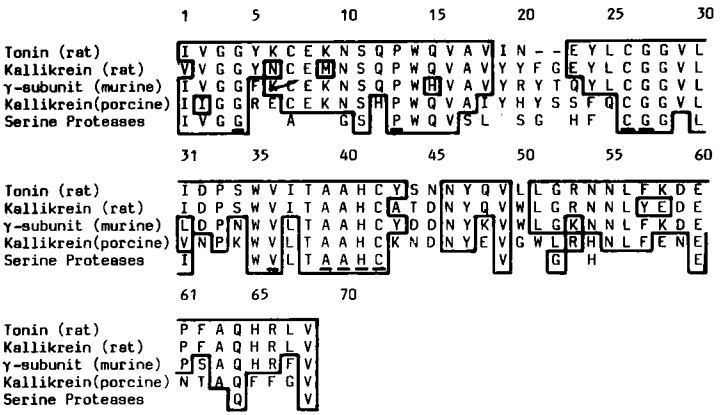

Fig. 1. Comparison of the NH_2-terminal sequence of reduced and alkylated tonin and kallikrein isolated from rat submaxillary gland with NGF-γ-subunit,[5] kallikrein[7] and conserved segments of serine proteases according to Dayhoff.[7] The residue underlined are invariant in known sequences.[7] Gaps (-) have been introduced in the sequence of tonin to maximize homology; identical residues are enclosed in boxes.

The sequences of fragments A and B illustrated in Figure 2 were obtained while looking for cyanogen bromide fragments. It is clear that fragment B is in a highly conserved region since it contains all the serine proteases invariant residues together with the aspartic residue at position 11 and the serine residue at position 17 responsible for the substrate

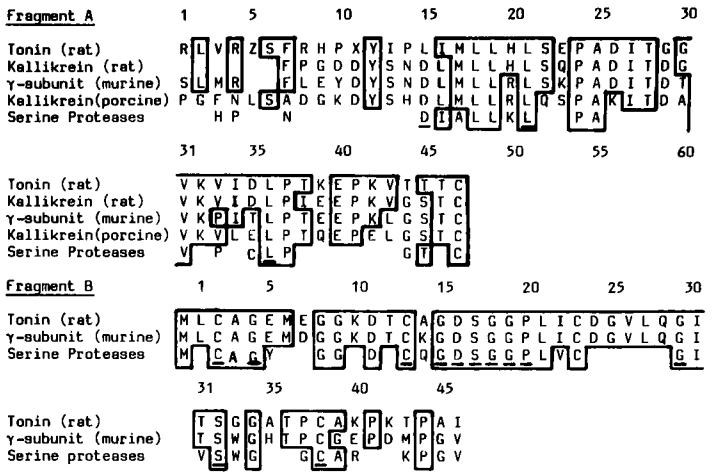

Fig. 2. Comparison of the NH_2-terminal sequence of two internal peptides with corresponding peptide aligned by homology of NGF-γ-subunit,[5] kallikrein[7] and conserved segments of serine proteases according to Dayhoff.[7] The residues underlined are invariant in known sequences[7]. Identical residues are enclosed in boxes. X denotes undetermined residue.

specificity and for the active site, respectively. On the other hand, in the sequence of fragment A, the first 16 residues representing a minor overlapping sequence of an autolysed portion of the molecule, reveal more substitutions but contain all of the serine proteases invariant residues. The only exception is the leucine residue at position 15 instead of the aspartic acid; this position is of the utmost importance since it is part of the active site and could thus, if confirmed by further studies, play a role in the enzymatic activity of tonin.

As shown in Table I, comparison of these available sequence data with the serine protease family reveals an interesting aspect of the tonin biology since γ-subunit, kallikreins, trypsinogen, and plasminogen all have an homology of more than 40%. It is too early to conclude that tonin represent a variant of γ-subunit because nerve growth factor was never detected in the submaxillary gland of the rat[11] and the substrate-specificity of tonin, as reported before[2-3], seems to be quite different from the arginine-specific activity of γ-subunit; nevertheless this assumption cannot be ruled out. It is hoped that the completion of the sequence studies will answer some of the questions raised by this close relationship with the γ-subunit and the other proteases concerning the biological role and the substrate specificity of tonin.

Table I. Homologies Between Serine Proteases and Tonin (Rat)

Enzymes	Species	No. of Residues		% Homology
		Compared	Identical	
Kallikrein	Rat	107	80	75
γ-subunit[5]	Murine	156	113	72
Kallikrein[7]	Porcine	158	88	56
Trypsin[7]	Bovine	154	70	46
Plasmin[7]	Human	148	68	46
Factor D[8]	Human	39	15	38
Chymotrypsin[7]	Bovine	153	57	37
Elastase[7]	Porcine	156	51	33
Prothrombin[7]	Bovine	155	50	32
Factor X[7]	Bovine	152	48	32
Crotalase[9]	Reptile	56	16	29
Factor B[10]	Human	140	36	26
Haptoglobin[6]	Human	147	37	25

The sequences used to determine homologies were taken from the references corresponding to the superscript number.

Acknowledgements

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ANTIGEN RECOGNITION AND THE IMMUNE RESPONSE

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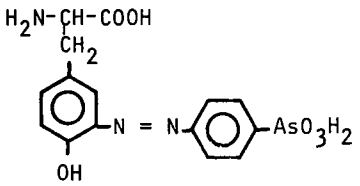
The cells involved in the immune response are lymphocytes, which are generally morphologically indistinguishable and arise from a common precursor in bone marrow. Sometime during the maturation and differentiation of the stem cells, those responsible for cellular immune reactions have been influenced (or educated) by the thymus and are, hence, designated as T cells. The cells involved in the humoral response are called B cells; these synthesize and secrete circulating antibody. Thus, the primary functional distinction between T and B cells in the immune response is that T cells do not produce antibody whereas stimulated mature B cells synthesize and secrete antibody. A third cell, the macrophage, is involved in capture and presentation of immunogen to the T cell. Macrophages are not specific with respect to determinant structure; that is, they are not genetically programmed to recognize one specific determinant the way T and B cells are. It should be pointed out that there are immunogens that circumvent the T cell compartment entirely and speak directly to B cells. (The bacterial polysacharides are examples of T-independent antigens).

In recent years it has become clear that both T and B cell populations are a very mixed bag of functionally distinguishable lymphocytes. In the T cell population, some T cell function as helpers; that is, they produce a signal or signals that tell the specific B cell to differentiate and produce antibody. There also exist cytotoxic or killer T cells capable of destroying other cells. A third distinct set are T suppressor cells which regulate the immune system; that is, they suppress the responses of B cells and other (*e.g.*, helper) T cells. In order to produce a full immune response, that is, antibody as well as cellular immunity, the immunogen molecule must possess at least two functional structures (or determinants). One determinant provides immunogenicity and stimulates the T cell compartment (with its component T helper cells): this is called the carrier or the immunogenic determinant. The second determinant induces the B cells to produce antibody directed against it; this functional structure is called hapten.

Investigation of antigen structural requirements for lymphocyte activation showed that L-tyrosine-azobenzene-*p*-arsonate, Tyr(ABA),



activates T lymphocytes without yielding any detectable antibody in guinea pigs, while the same animals, when immunized with azobenzene-*p*-arsonate groups conjugated to a suitable protein carrier, are perfectly capable of producing anti-ABA antibody.[1] This T cell activation is retained (albeit with altered specificity) if arsonate is replaced by sulfonate or trimethylammonium (TAT) groups; replacement by sulfonamide, carboxylic acid, acetamide, nitro or dimethylamino group abrogated the immunogenicity entirely.

Similarly, the alkyl portion of tyrosine (*i.e.*, the amino, carboxy, alpha and beta carbons) can be replaced by other polar groups such as hydroxyethyl, but not by hydrocarbon substituents, such as methyl, ethyl or propyl groups. Thus, for example, 4-*n*-propyl-2-azobenzene-*p*-arsonate-1-phenol is not an immunogen although it contains the entire aromatic system of Tyr(ABA). It is possible that the polar group is necessary for binding by macrophages in antigen presentation to the T cell.[2]

The Tyr(ABA) is able to induce T helper cells in cooperation with B cells when coupled with an appropriate hapten, *i.e.*, it can act as a carrier.[3] Immunization of animals with N-DNP-Tyr(ABA) and N-DNP-$(SAC)_{1-3}$-Tyr(ABA) molecules yielded cellular immunity to the Tyr-(ABA) moiety and circulating anti-dinitrophenyl antibody. The spacer 6-aminocaproyl, (SAC), was not necessary although DNP-Tyr(ABA) yielded somewhat lower anti-DNP response than the other three immunogens.

A series of antigens Tyr(ABA)-spacer-Tyr(ABA) were tested for "self-help" effect, *i.e.*, the ability of one immunogenic determinant to cooperate in the production of antibody to a second identical determinant.[4] The molecules containing flexible spacers comprised of SAC units (1-3) yielded only cellular immunity, without detectable anti-ABA antibody. Investigation of UV spectra of these molecules in 0.1 N NaOH has shown the presence of two chromophores, as expected, per molecule, but at physiological pH the extinction coefficients decreased by as much as 30%, indicating intramolecular sandwich-type stacking, probably by ionic interaction between positively charged azo and negatively charged

arsonate groups. Bifunctional molecules containing rigid spacers between the two Tyr(ABA) determinants were synthesized: acetyl-Tyr(ABA)-Pro_{10}-Tyr(ABA) induced both cellular and humoral (antibody) responses to the same ABA determinant. Thus, intramolecular association in flexible bifunctional immunogens presented a barrier to self-help by compromising the bifunctionality of such molecules.

To explore this question further, tyrosine-azobenzene-*p*-trimethylammonium derivatives, Tyr(TAT), were synthesized. In this case, both azo and trimethylammonium are positively charged and intramolecular stacking was not expected.[5] Thus, all four of the following antigens yielded anti-TAT cellular and antibody responses, although not with equal efficiency: the flexible spacer, [H-Tyr(TAT)-NH-CH_2-CH_2-CH_2-]$_2$ acetyl-Tyr(TAT)-SAC-Tyr(TAT)-OH,[-CH_2-CH_2-CO-Tyr(TAT)-OH]$_2$, and the rigid spacer containing acetyl-Tyr(TAT)-Pro_{10}-Tyr(TAT)-OH.

The first molecule can contain only positive charges in physiological solutions and this was the best immunogen of the three flexible bifunctionals for antibody responses. The next molecule has one free carboxyl and therefore one negative charge; this yielded an intermediate response. The last flexible bifunctional contains possible two negative charges and four positive at physiological pH; this immunogen yielded the lowest antibody response of the three. Thus, here too, there seems to exist a correlation between helper activity and chemical integrity of the determinants, and intramolecular association seems to compromise the bifunctionality of these antigens.

We have demonstrated earlier that DNP-Tyr(ABA)-OH is a complete immunogen. Obviously, the two determinants, hapten (DNP) and carrier [Tyr(ABA)] can be linked very closely together. We investigated the significance of distance between the two epitopes, separated by rigid poly-proline spacers.[6] A series of immunogens was synthesized and tested (Table I).

Table I. Antibody Responses to Bifunctional Antigens.

Antigen	Spacer Length, A (a)	Anti-DNP Antibody (b)
DNP-PRO_{10}-Tyr(ABA)	31	203 ± 50
DNP-Pro_{22}-Tyr(ABA)	69	143 ± 14
DNP-Pro_{31}-Tyr(ABA)	97	0
DNP-Pro_{40}-Tyr(ABA)	125	0

(a) Based on axial translation of 3.12 Å per proline residue in poly-L-proline. (b) Three weeks after immunization, 3-6 guinea pigs per group, 1.0 μM immunizing dose.

The molecules with Pro_{10} and Pro_{22} spacers yielded substantial anti-DNP antibody responses, whereas the Pro_{31} spacer produced a negative response. Thus, the cut-off distance for effective T and B cell cooperation seems to lie between 69 and 97 Å. To check this further, we synthesized a molecule with a Pro_{30} spacer containing a flexible "kink" in the middle, *i.e.*, DNP-Pro_{15}-SAC-Pro_{15}-Tyr(ABA) and compared it to the rigid DNP-Pro_{30}-Tyr(ABA). The flexible 6-aminocaproic acid in the middle should allow the two terminal determinants to approach each other in a V-type folding. Immunological testing in guinea pigs yielded anti-DNP antibody with the "kinked" molecule (unpublished results).

A comparison of the response in mice to immunization with bifunctional DNP-spacer-Tyr(ABA) and trifunctional DNP-spacer-Tyr(ABA)-spacer-Tyr(ABA) established the necessity for two T cell determinants to induce the switch from IgM antibody to IgG antibody.[7]

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SYNTHETIC STUDIES OF SERUM COMPLEMENT

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The C1q-Binding Site of IgG

The classical pathway of the complement system of serum proteins is activated by the binding of an antigen-antibody complex to the first component of complement (C1), a calcium-dependent complex of three subcomponents.[1] The first event in the activation of C1 is binding of C1q, the recognition subcomponent, to the Fc regions of two or more antibody molecules, which initiates the proteolytic activation of the serine proteases C1r and C1s. The IgG-binding sites of C1q reside in the COOH-terminal globular heads.[2,3]

The C1q-binding sites of IgG are located in the second constant domains (Cγ2) of the two heavy (gamma) chains. When aggregated on polystyrene latex, the Cγ2 fragments comprising the 253-314 region[4] of mouse IgG2a and the 253-306 region[5] of human IgG1 each exhibit about 3% of the complement activation of 7S monomeric IgG. The latter fragment, but not the adjacent 307-376 fragment, inhibits C1-mediated immune hemolysis.[5] Several studies have implicated the presence of a hydrophobic region and a cationic region at or near the C1q-binding site. Chemical modification studies[6,7] and solvent perturbation studies[8] have suggested the presence of a tryptophan residue, whereas inhibition studies with organic diamines[9,10] have indicated the presence of positively charged residues. X-ray diffraction studies[11,12] of the three-dimensional structure of the Fc fragment of human IgG show that in both Cγ2 domains the hydrophobic residues Phe-275, Trp-277, and Tyr-278 are buried in the interior and the positively charged residues His-285, Lys-288, Lys-290, and Arg-292 are exposed on the exterior.

Dr. Prystowsky, Dr. Lukas, and Mr. Muñoz have demonstrated the ability of synthetic peptides from the 275-292 region of the human IgG1 protein Eu to inhibit C1-mediated immune hemolysis.[13,14] Several peptides corresponding to segments of this region were synthesized by the solid-phase method and purified to homogeneity by reverse-phase liquid chromatography. These IgG1 segments were examined for their capacity

to inhibit the binding of C1 to EAC4 cells, which are sheep erythrocytes bearing human C4b and rabbit IgG antibodies directed against sheep erythrocytes. After incubation of a solution of the peptide and a limiting amount of C1, the amount of free C1 remaining was measured by adding the solution to a suspension of EAC4 cells, washing the cells to remove the peptide and unbound C1, adding C2 followed by C3-C9 and EDTA, and observing spectrometrically the amount of heme released by cellular lysis.[15,16]

The primary structures and activities of six synthetic peptides from the 275-292 regions of the Cγ2 domains of human IgG1 are shown in Table I. Activity is expressed as the 50% inhibitory concentration (I_{50}), which is the molar concentration of peptide or protein needed to inhibit C1-mediated immune hemolysis by 50%. This is a convenient measure of the ability of a peptide or protein to bind to C1 and thus inhibit hemolytic action of the classical complement pathway. The 7S monomeric form of the human IgG1 myeloma protein Bah and the Fc fragment of pooled human IgG served as standards for the inhibition assay. Monomeric IgG1 inhibits by 50% the hemolysis at a concentration of 74 μM and the Fc fragment produces half inhibition at 2/3 of that concentration.

The IgG peptides 281-290 and 282-292, which bear three and four positively charged sidechains, respectively, are both about 40% as active as monomeric IgG on a molar basis. Peptide 275-290F, however, which bears three aromatic sidechains in the 275-278 region in addition to the three positively charged sidechains in the 285-290 region, is within experimental error no more active than peptide 281-290. Thus, the aromatic sidechains of Phe-275, N^{i}-formylated Trp-277, and Tyr-278 evidently contribute little to the C1-binding activity of peptide 275-290. This result is not surprising because these aromatic residues are buried in the hydrophobic interior of the Cγ2 domain[11,12] and are not available for interaction with C1q.

The cation peptides 281-290 and 282-292 are about half as active as the 7S form of human IgG1. Since the latter contains two Cγ2 domains, these monomeric peptides are each about as active as one Cγ2 domain in inhibiting C1-mediated immune hemolysis. A dimer of peptide 281-290 or 282-292 should be twice as active as the monomeric peptide on a molar basis and just as active on a site basis if the sites bind to C1q noncooperatively. In order to test this prediction, a dimer of peptide 282-292 was synthesized by the scheme shown in Figure 1. Two copies of the peptide were cross-linked while still covalently bound to Pam-polystyrene resin by treatment with dicyclohexylcarbodiimide and terephthaloyl-*bis*-

Table I. Inhibition Of C1-mediated Immune Hemolysis By Synthetic Peptides From The Cγ2-Domain Of Human IgG

Inhibitor	Inhibitor structure	I_{-50}	Relative molar activity
	275 277 280 285 288 289 290 292	μM	%
IgG, Fc fragment	····Phe-Asn-Trp-Tyr-Val-Asp-Gly-Val-Gln-Val-His-Asn-Ala-Lys-Thr-Lys-Pro-Arg-····	47	157
Monomeric IgG1	····Phe-Asn-Trp-Tyr-Val-Asp-Gly-Val-Gln-Val-His-Asn-Ala-Lys-Thr-Lys-Pro-Arg-····	74	[100]
$Tid(282\text{-}292)_2$	Tid< Val-Gln-Val-His-Asn-Ala-Lys-Thr-Lys-Pro-Arg-OH / Val-Gln-Val-His-Asn-Ala-Lys-Thr-Lys-Pro-Arg-OH	77	96
282-292	H-Val-Gln-Val-His-Asn-Ala-Lys-Thr-Lys-Pro-Arg-OH	175	42
281-290	H-Gly-Val-Gln-Val-His-Asn-Ala-Lys-Thr-Lys-OH	180	41
275-290F	H-Phe-Asn-Trp(For)-Tyr-Val-Asp-Gly-Val-Gln-Val-His-Asn-Ala-Lys-Thr-Lys-OH	150	49
$Tid(289\text{-}292)_2$	Tid< Thr-Lys-Pro-Arg-OH / Thr-Lys-Pro-Arg-OH	430	17
289-292 (tuftsin)	H-Thr-Lys-Pro-Arg-OH	720	10

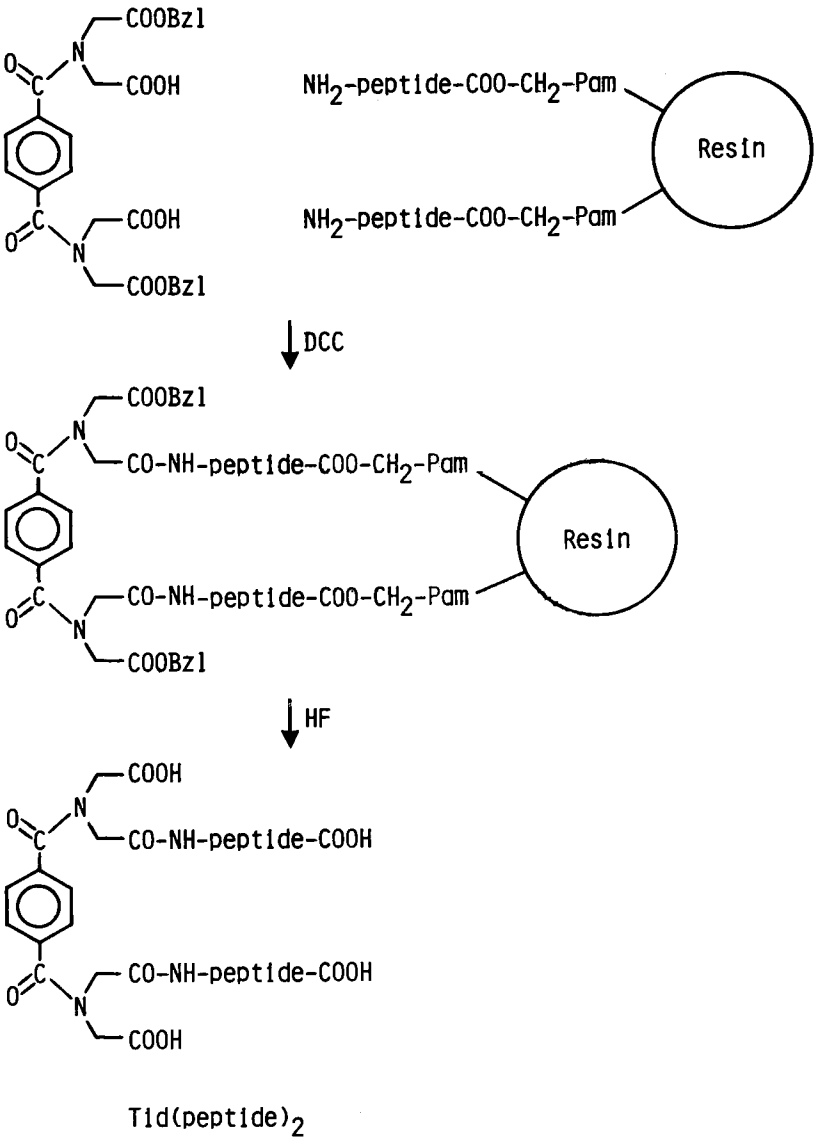

Fig. 1. Net cross-linking of two peptide chains on a solid support with terephthaloyl-*bis*-(iminodiacetic acid) (Tid).

(iminodiacetic acid monobenzyl ester).[14] Subsequent treatment with HF/anisole and purification by reverse-phase liquid chromatography furnished the desired Tid(282-292)$_2$ dimer. This new cross-linking agent was designed to minimize nonspecific binding of the peptide dimer to the

negatively charged surfaces of cells through the presence of two negatively charged carboxylate groups in the cross-linking moiety.

Dimeric peptide Tid(282-292)$_2$ is indeed about twice as active as monomeric peptide 282-292 on a molar basis and just as active on a site basis (Table I). This 22-residue peptide is as active as the 1300-residue IgG1 protein on both molar and site bases. About 1280 residues (98%) of the human immunoglobulin G1 molecule can be omitted without diminishing the ability of the remaining 22 residues to bind to C1q and inhibit C1-mediated immune hemolysis.

These results suggest that the positively charged residues His-285, Lys-288, Lys-290, and Arg-292, which lie on the outer surface of the Cγ2 domains,[11,12] may be important structural features of the C1q-binding sites of human IgG. This proposal is consistent with several previous studies suggesting that the C1q-binding site of IgG is cationic in nature. For example, 1,4-diaminobutane competitively inhibits the binding of C1 to antibody-coated erythrocytes at a concentration of 5 mM,[9] to bind to human C1q at concentrations above 3mM,[10] and to half inhibit the binding of C1q to insoluble immune aggregates at 1.6 mM.[17] Polylysine samples with average molecular weights of 3,400 and 4,000 are reported to inhibit by 50% the binding of C1q to insoluble immune aggregates at concentrations of 0.06 mM and 0.5 mM, respectively.[18,19] In addition, the decreasing binding of C1q to insoluble rabbit IgG aggregates with increasing ionic strength suggested that three charged groups are present at the C1q site that binds to the aggregates.[18] This is consistent with the presence of precisely three positively charged residues (Arg-285, Arg-288, Arg-292, human IgG1 Eu numbering) in the 280-292 region of pooled rabbit IgG.[20]

The possibility that a shorter peptide from the 282-292 region of human IgG might be as inhibitory as peptide 282-292 was explored by examing the activities of the monomeric peptide 289-292 (tuftsin) and its dimer Tid(289-292)$_2$. Tuftsin, which contains two residues with positively charged sidechains (Lys-290, Arg-292), is only about 1/4 as active as the monomeric peptides 281-290 and 282-292. The tuftsin dimer is about twice as active as tuftsin on a molar basis and as active on a site basis.

The C1q-binding site was recently proposed to reside in the 317-340 region of IgG.[21] This proposal is not consistent with the observation that, although the 253-306 fragment of human IgG does inhibit C1-mediated immune hemolysis, the 307-376 fragment does not.[5] Our proposal[13,14] that the cationic 285-292 region of human IgG is a major part of the C1q-binding site supports the previous suggestion[22] based on sequence

comparison that the positively charged residues Lys-290 and Arg-292 may be a part of this site.

The Inflammatory Sites of C3a and C4a Anaphylatoxins

The classical complement pathway involves three successive cleavages by a serine protease of an inactive precursor protein into a small activation peptide and a large activated protein.[1] First $\overline{Cls}$ cleaves C4 into the peptide C4a and the protein C4b. Later, the enzyme complex $\overline{C4b2a}$ cleaves C3 into the peptide C3a and the activated protein C3b. Finally, the ternary complex $\overline{C4b2a3b}$ cleaves C5 into the peptide C5a and the activated protein C5b. The nascent proteins C4b and C3b each contain a metastable binding site that allows covalent attachment of the protein to receptive biological particles. Successful attachment of these proteins to a cellular membrane initiates the nonproteolytic assembly of the membrane attack complex C5b-9, which causes disruption of the cellular membrane and cell death. Dr. Khan has synthesized several peptides containing the macrocyclic thiolester ring thought to be an important feature of the metastable binding site of human C3b and has studied their reaction kinetics with water and amines.[23]

The activation peptides C4a, C3a, and C5a are primary mediators of inflammation known for historical reasons as anaphylatoxins.[24] Both C3a and C4a interact with the same type of receptor site on mast cells and basophils to induce noncytolytic release of vasoactive amines, such as histamine.[25] C5a, however, exerts its vasoactive effects through interaction with a different type of receptor site. Each of these anaphylatoxins contains 74-77 amino acid residues in a single polypeptide chain cross-linked by an internal disulfide knot. Partial sequence analysis has shown that these peptides and thus their protein precursors are evolutionarily related.[26]

The inflammatory site of C3a resides at the COOH-terminus. A synthetic octapeptide (Ala-Ser-His-Leu-Gly-Leu-Ala-Arg, residues 70-77), which corresponds to the last eight residues of human C3a, contracts guinea pig ileal and uterine muscle, releases vasoactive amines from rat mast cells, and increases the vascular permeability of guinea pig and human skin.[27] The COOH-terminal pentapeptide, which is common to the C3a anaphylatoxins of rat, pig, and man, is the shortest peptide with significant capacity to contract ileal smooth muscle and produce a wheal and flare response in human skin.[28] The octapeptide and pentapeptide exhibit about 2% and 0.2%, respectively, of the molar contractile activity of native human C3a. At sufficiently high doses, each cross-desensitizes

ileal tissue to subsequent contraction by human C3a but not by human C5a. Thus these synthetic peptides specifically act by binding to the cellular C3a/C4a receptor. These peptides are about 20% and 10% as active as human C3a, respectively, in inducing the release of histamine from human basophils and are able to displace radiolabeled C3a from human leukocytes.[29]

Our previous synthetic studies[28,30] of the structural features that contribute to the inflammatory activities of C3a have shown that the hydrophobic sidechains of Leu-73 and Leu-75 and the guanidinium group of Arg-77 are functionally important. The remainder of this paper highlights several further studies by Dr. Fok, Dr. Unson, Dr. Volk-Weiss, and Mr. Molinar, who collaborated with Dr. Hugli to characterize the important structural features of the active sites of the C3a and C4a anaphylatoxins and conversely of the cellular C3a/C4a receptor.

Table II summarizes the relative molar activity of several replacement analogs of the C3a active-site pentapeptide **1** for contraction of smooth muscle. Substitution of Leu-73 by other large hydrophobes (**2-4**) maintains activity, but substitution by the smaller residue Ala (**5**) decreases activity about 15-fold. In contrast, replacement of Leu-75 by the larger residues Met and Phe (**10,11**) or the smaller residue Ala (**12**) decreases activity at least 50-fold. Even the isomeric Ile-75 analog (**9**) is 10-fold less active. Both of the leucine residues evidently make hydrophobic contacts with the cellular C3a/C4a receptor, but Leu-75 is much more sensitive than Leu-73 to structural changes. The Ala-74 analog **6** is fully active, but replacement of Gly-74 by the larger residues Leu and Gln (**7,8**) decreases activity 20 and 35 times, respectively. Similarly, substitution of Ala-76 by the larger residues Ser and Gln (**13, 14**) decreases activity 150 and 50 times, respectively. The small sidechains of residues Gly-74 and Ala-76 may avoid bad steric interactions with the cellular C3a/C4a receptor and may serve to space the hydrophobic leucine residues at favorable distances from Arg-77.

As observed for C3a,[28] the inflammatory site of human C4a anaphylatoxin resides at the COOH-terminus. The COOH-terminal octapeptide (Lys-Gly-Gln-Ala-Gly-Leu-Gln-Arg, residues 70-77) is about 0.2% as active in the ileal assay as human C4a. Since the triply substituted analog *Ala-Ala-Ala*-Ala-Gly-Leu-Gln-Arg is just as active as the C4a octapeptide, the sidechains of Lys-70 and Gln-72 are not important for activity. Although the C4a COOH-terminal pentapeptide **15** is at least 1000 times less active than the C3a active-site pentapeptide **1**, this result is roughly in accord with that expected from multiplying the 15-fold decrease in

Table II. Contraction Of Smooth Muscle Strips From Guinea Pig Ileum: Relative Molar Activity Of Synthetic C3a Peptide Analogs

Code	Structure[a]	Activity[b]
	73 74 75 76 77	
1 (C3a)	Leu-Gly-Leu-Ala-Arg	[100]
2	Phe-Gly-Leu-Ala-Arg	80
3	Ile-Gly-Leu-Ala-Arg	60
4	Met-Gly-Leu-Ala-Arg	45
5	Ala-Gly-Leu-Ala-Arg	7
6	Leu-Ala-Leu-Ala-Arg	120
7	Leu-Leu-Leu-Ala-Arg	6
8	Leu-Gln-Leu-Ala-Arg	3
9	Leu-Gly-Ile-Ala-Arg	10
10	Leu-Gly-Met-Ala-Arg	1.2
11	Leu-Gly-Phe-Ala-Arg	<0.9
12	Leu-Gly-Ala-Ala-Arg	<2
13	Leu-Gly-Leu-Ser-Arg	0.6
14	Leu-Gly-Leu-Gln-Arg	2
15 (C4a)	Ala-Gly-Leu-Gln-Arg	<0.1

[a]Residues different from those in the C3a active-site pentapeptide **1** are underlined.
[b]Relative to peptide **1** (minimum effective dose = 21-29 nmole); "<X" indicates that no contraction was seen at X, the largest dose tested.

activity observed for replacement of Leu-73 by Ala (**5**) by the 50-fold decrease seen for replacement of Ala-76 by Gln (**14**).

The COOH-terminal arginine of C3a contributes several structural features that are important for activity in the ileal assay. These features have been elucidated through substitution analogs of CH_3CO-Ala-Leu-Gly-Leu-Ala-Arg, another model peptide of the C3a active site.[28] First, the natural L chirality is important because the D-Arg analog is at least

400 times less active. Second, the negatively charged carboxylate group is important because the isosteric but neutral Arg-NH_2 analog is at least 1000 times less active. Third, three methylene groups in the sidechain are important because either increasing or decreasing this number by one decreases the activity 7 times. Fourth, the positive charge of the guanidinium group is important because the isosteric but neutral citrulline analog is at least 65-fold less active. Finally, the ability of both outer nitrogen atoms to form hydrogen bonds with a bidentate ligand (perhaps carboxylate or phosphate) on the cellular C3a/C4a receptor is probably important because the N^{δ}-acetimidoyl-ornithine analog is only about 2% as active as the model hexapeptide.

In summary, the cellular C3a/C4a receptor evidently contains a hydrophobic region for binding to the sidechains of Leu-73 and Leu-75 and negative and positive (bidentate) ligands for electrostatic interaction with the positive guanidinium and negative carboxylate groups of Arg-77.

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THYMOPOIETINS: STRUCTURAL STUDIES OF THESE IMMUNOREGULATORY POLYPEPTIDES

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Thymopoietins I and II are two closely related polypeptides isolated from bovine thymus. The bioassay used to isolate these molecules was the detection of delayed neuromuscular impairment in mice following the injection of thymic extracts or purified polypeptides.[1] This effect was restricted to thymic extracts and was not found in control extracts or other tissues. This assay was derived from basic experimental studies related to myasthenia gravis, a human disease in which a thymic hormone affecting neuromuscular transmission had been detected.[2] Two biochemically homogeneous thymic polypeptides, thymopoietin I and II (TP-I and TP-II), were isolated and both were active in this system.[1] These purified polypeptides subsequently proved to be active in the induction of early T cell differentiation, inhibition of B cell differentiation and modulation of mature lymphocytes.[3] The complete amino acid sequence of TP-I and TP-II (Figure 1) was determined[4] and biological activity was shown to reside in segment 29-41 by chemical synthesis.[5] Subsequently the pentapeptide TP-5, corresponding to residues 32-36 of boal activity of thymopoietin and thus probably corresponds to a biologically in-I and -II, was shown to retain the biologicactive site of the parent molecule.[6] TP-5 has immunoregulatory effects in a number of model systems in animals[6,7] and is presently being evaluated as an immunoregulatory therapeutic agent in man.[8]

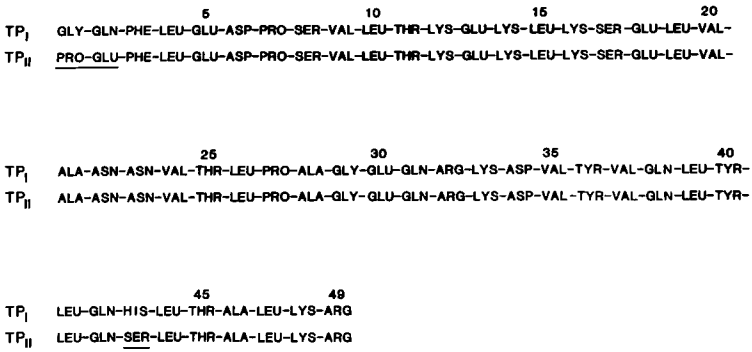

Fig. 1. Thymopoietin I and II

The synthesis and biological evaluation of fragments of TP-5 indicate that the pentapeptide is the smallest fragment that exhibits T-cell differentiating activity.[9]

Conformational studies of the pentapeptide and synthetic analogs of it have led to some interesting conclusions about the structural and functional requirements for activity.

Krishna *et al.*[10] have investigated the aqueous solution conformation of TP-5 by proton nuclear magnetic resonance (NMR). All resonances have been assigned unambiguously based on chemical shift variation with pH and coupling constant data. From the vicinal NH-C^{α}-H coupling constants, various possible backbone torsional angles (ϕ) were calculated based on the assumption that a predominant conformation is preferred for TP-5.

One conformation which appears to be consistent with all experimental data is a 1←3 bend (γ turn) for the aspartic acid residue. This is characterized by a backbone hydrogen bond between the carbonyl of lysine and the N-H of valine and is consistent with the observed shielding of the valine N-H from solvation. A low field shift titration (pK 3.57) for the $N^{\epsilon}H$ of arginine is explicable by the presence of a hydrogen bond between the $N^{\epsilon}H$ and one of the carboxylate groups.

In order to determine whether the aspartic acid β-carboxyl or the tyrosine carboxyl was hydrogen bonded to the $N^{\epsilon}H$ of arginine, we synthesized the pentapeptide methyl esters of both carboxylic acids. Proton nuclear magnetic resonance studies of lanthanide (III) complexes of these compounds and of TP-5 indicate that it is the aspartic acid β-carboxyl which is hydrogen bonded.[11]

If a guanidine ion carboxylate interaction is in some way related to the activity of TP-5, then it would be expected that the aspartic acid-β-carboxyl is the one involved since the tyrosine carboxyl is not free in native thymopoietin-I and -II. This is consistent with analogs of the pentapeptide which we have synthesized but in opposition to the results of others.[12]

We synthesized a series of TP-5 analogs in which each residue was substituted by alanine or sarcosine. From these, we have determined that neither the lysine nor valine side chains are necessary for activity while the side chains of arginine, aspartic acid and tyrosine are required. Preliminary theoretical calculations[13] generated a family of conformations for TP-5 based on the minimization of negative interaction. One conformation, with an 86.8 percent probability, was highly favored above all others. The ϕ angles of this conformation were consistent with

the angles determined by NMR studies.[10] A projection for this conformation is shown in Figure 2.[14] In this structure the side chains of the arginine, aspartic acid and tyrosine are positioned on one face of the molecule, while the lysine and valine side chains are on the opposite face. This structure allows for both the lysine-valine and arginine-aspartic acid hydrogen bonds detected from the NMR studies.

We have concluded that the structural features of TP-5 necessary for activity are the arginine, aspartic acid and tyrosine side chains in a spatial arrangement approximating Figure 2.

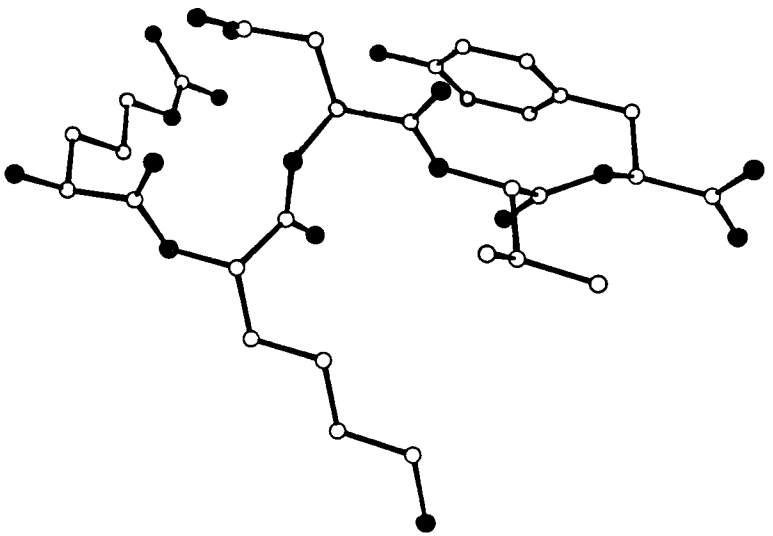

Fig. 2. Most probable conformation of TP-5 based on theoretical minimization of negative interactions.

Thymopoietin III

Thymopoietins have been readily detected in the thymus while absent from control tissues such as liver, kidney, thyroid and muscle. This analysis was determined using a radioimmunoassay, developed for thymopoietin-II, which proved to be wholly crossreactive with thymopoietin-I.[15] Recently, the radioimmunoassay detected thymopoietin-like material in spleen and lymph node extracts. To determine the nature of this material, which was subsequently termed thymopoietin-III (TP-III), a radioimmunoassay was utilized to isolate TP-III from extracts of bovine spleen and its sequence was determined (Figure 3).[4b]

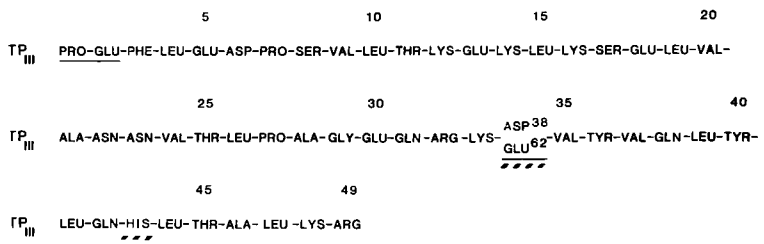

Fig. 3. Thymopoietin III, solid and dashed lines indicate non-homology with TP-I and -II, respectively.

The amino acid sequence of thymopoietin-III and -II are identical except for amino acid substitutions at position 43, Ser (TP-II) versus His (TP-III), and at position 34, Asp (TP-II) versus Glu (TP-III), albeit with some microheterogeneity between Asp and Glu at this position in TP III. Similarly, TP-I is closely related in that it differs from TP-II in only three positions, these being position 1 (Gly), position 2 (Gln) and position 43 (His), this latter residue being identical to residue 43 of TP-III.

The close amino acid sequence similarities between thymopoietin-I, -II and -III clearly imply a recent common ancestral gene with at least three copies giving rise to the three distinct amino acid sequences described. It is intriguing that two of the thymopoietins were isolated from the thymus (TP-I and TP-II) whereas the third (TP-III) was isolated from the spleen. The finding that Glu predominates at position 34 in TP-III is particularly provocative since this occurs in the putative active site region of TP-I and TP-II, and preliminary functional studies suggest that the biological activity of TP-III is distinct from that of TP-I and TP-II.

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SYNTHESIS AND PROPERTIES OF IMMUNOSTIMULATING PEPTIDES II

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Thymosin-α_1, a component of the thymus derived polypeptide mixture thymosin fraction 5, has been shown to exhibit various activities in *in vitro* immunological assays.[1-4] *In vivo,* thymosn-α_1 seems to play an important regulatory role in the late stages of T-cell differentiation.[1,5] The therapeutic potential of thymosin fraction 5 and thymosin-α_1 in the treatment of primary immunodeficiencies and some cancers has shown promise[6] and is currently under evaluation in various laboratories. Due, in part, to the non-uniformity of the biological test systems employed and the probably multifunctional role of thymosin-α_1 in lymphocyte differentiation, a relationship confining a specific biological function to a partial sequence or active site has not been established as it has been for the related thymic factor, thymopoietin.[7] However, recent studies in our laboratories[3] and also by Abiko and coworkers[4,8,9] have established that the C-terminal region of thymosin-α_1 possesses some of the immunostimulatory properties of the parent peptide.

We have shown that the C-terminal sequences 20-24 and 25-28 exhibit distinct activity in the allogenic one-way mixed lymphocyte response (MLC) and α-amanitine inhibited E-rosette assay systems.[3] Abiko and coworkers have also demonstrated that longer C-terminal sequences (14-28, 19-28) express partial activity when compared to the complete sequence in assay systems utilizing lymphocytes from patients with disease impaired immunological function.[4,8,9] In elaboration of our earlier investigations of segment sequences of thymosin-α_1, we report here the preparation of several partial sequences of the C-terminal region. Nine new peptides along with two previously reported segments were studied, the syntheses and sequences of which are illustrated in Figure 1.

Protected peptides I-III were obtained by stepwise elongation of a previously prepared common pentapeptide IV utilizing the mixed anhydride (MA) method in combination with the 2-(3,5-dimethoxyphenyl) propyl (2) oxycarbonyl (Ddz) protecting group.[10] Protected peptides V-VIII were prepared by condensation of the same common segment IV,

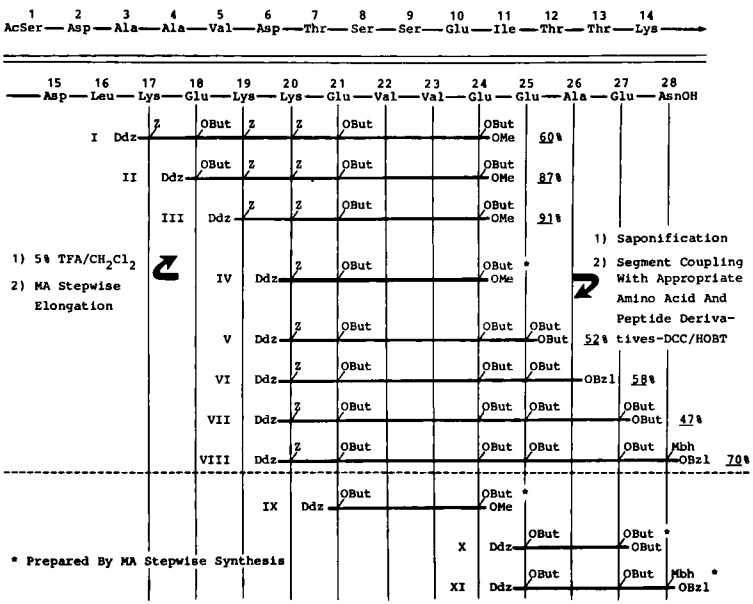

Fig. 1. Syntheses of C-terminal thymosin-α_1 segments

after saponification, with the appropriate amino acid and peptide derivatives using the dicyclohexylcarbodiimide/1-hydroxybenzotriazole (DCC/HOBt) technique.[11] Peptide derivatives required for the segment condensations were synthesized using the MA method. Compounds IV, IX and XI were also prepared by this method as previously described.[2,3] All protected peptides showed satisfactory analytical data. Following saponification and/or hydrogenolysis where necessary, protecting groups were removed by treatment with anhydrous trifluoroacetic acid (TFA) in the presence of anisole. The free peptides were purified by ion exchange chromatography using DEAE-Sephadex A-25 (AcO–) and aqueous acetic acid gradients. The resulting peptides were homogenous on thin layer chromatography in three solvent systems and provided satisfactory amino acid analyses (Figure 2).

The C-terminal peptides were tested in the allogenic one-way mixed peripheral lymphocyte response assay (MLC).[12] In this assay, human peripheral T-lymphocytes (responder cells) from a single donor are incubated with α-amanitine resulting in an inhibition of protein synthesis. These cells, when stimulated by T-lymphocytes from a second donor (stimulator cells, mitomycin-C blocked) exhibit a diminished response, as determined by the rate of ^{3}H-thymidine uptake, when compared to

Peptide	Activity[a]	TLC: R_f x 100[b]			Amino Acid Analysis[c]				
		1	2	3	Ala	Asp	Glu	Lys	Val
17-24	58	6	9	0	-	-	3.00(3) 3.00	3.06(3) 2.75	1.38(2) 1.78
18-24	60	14	18	2	-	-	3.00(3) 3.00	2.00(2) 2.10	1.80(2) 1.69
19-24	64	14	17	3	-	-	2.00(2) 2.00	2.07(2) 1.87	1.83(2) 2.07
20-24	56	23	38	6	-	-	2.00(2) 2.00	0.96(1) 0.92	1.74(2) 1.89
21-24	49	40	61	28	-	-	2.00(2) 2.00	-	1.76(2) 2.07
20-25	28	19	29	5	-	-	3.00(3) 3.00	1.03(1) 0.90	1.85(2) 1.71
20-26	90	20	31	5	0.69(1)	-	3.00(3) 3.00	0.98(1) 1.02	1.72(2) 1.60
20-27	34	18	25	3	0.95(1) 1.41	-	4.00(4) 4.00	1.07(1) 0.93	1.97(2) 1.71
20-28	51	14	20	2	1.06(1) -	1.06(1) -	4.00(4) -	1.03(1) -	1.84(2) -
25-27	94	21	29	1	0.98(1) 1.26	-	2.00(2) 2.00		
25-28	88	18	22	2	1.05(1) 1.03	1.25(1) 0.98	2.00(2) 2.00		

Fig. 2. Immunological and analytical data of thymosin-α_1, C-terminal segment sequences. [a]Activity as determined by the rate of incorporation of ^{3}H-thymidine and expressed as percent of the stimulatory activity displayed by synthetic thymosin α_1 (100%) in the same assay system. Values are an average of three determinations. Each assay consisted of 10^5 stimulator cells (blocked by mitomycin-C) and 10^5 responder cells (α-amanitine inhibited) in the presence of 2 μg/ml peptide in 240 μl final culture volume. [b]Solvent System: 1) n-Butanol/Pyridine/Acetic Acid/Water, 5:5:1:4; 2)Ethyl Acetate/Pryidine/Acetic Acid/Water, 15:20:6:11; 3) n-Pentanol/Pyridine/2-Butanone/Formic Acid/Water, 40:28:11:5:15. [c]Hydrolysis Conditions: Upper Values; 12N HCl/propanoic Acid, 1:1, 30-50 min, 160°; Lower Values; 6N HCl, 48h, 110°.

non-inhibited responder lymphocytes. When inhibited responder cells are treated with immunostimulatory peptides, such as thymosin-α_1, the α-amanitine inhibition is partially overcome and the rate of ^{3}H - thymidine uptake is greater than in untreated cells.

The MLC activities of thymosin-α_1 C-terminal segment peptides are shown in Figure 2 expressed as percent of the stimulatory activity exhibited by synthetic thymosin-α_1 in the same assay.

It is evident that all peptides tested had stimulatory activity and partially overcame the inhibitory effect of α-amanitine on the responder cells. None of the peptides showed as great an activity as synthetic thymosin-α_1, however, segments 20-26, 25-27, and 25-28 were nearly as active (90, 94, and 88%, respectively). The remaining C-terminal peptides showed stimulatory activities ranging from 28 to 64% of that of synthetic thymosin-α_1. From this data it is not possible to identify a specific sequence responsible for activity in the MLC assay, although it strongly suggests that the extreme C-terminal region (25-28) plays an important

role. It must be stressed that the MLC assay sytem is highly concentration and donor dependent. The rations of T-lymphocyte sub-populations and the present prevailing immune status of the donor are also of major importance. We conclude that activities in assay systems of this type can be compared only when determined in the same test system with the same donors. They should be taken as qualitative expressions only.

Preliminary data indicate that the C-terminal segment peptides studied here also exhibit stimulatory activity in the α-amanitine inhibited E-rosette assay.

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SYNTHESIS AND PROPERTIES OF IMMUNOSTIMULATING PEPTIDES, III: POTENTIATED IMMUNOMODULATION BY VARIED SEQUENCES OF THYMOSIN-α_1

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Introduction

In several immunological investigations on low molecular weight isolation products from thymic tissue, e.g. thymosin fraction 3-8 and thymic factor (THF), as well as on synthetic peptides comprising partial sequences from thymic hormones of established structure, e.g. thymopoietin, facteur thymic sérique (FTS), and thymosin-α_1, immunomodulatory potencies have been observed.[1] Following our total synthesis of thymosin-α_1[2], we also investigated the synthetic intermediates, comprising the entire structure of the hormone. The optimized syntheses of five thymosin-α_1 fragments together with some of their immunological *in vitro* activities have recently been described.[3] Here, we wish to report on analogs of fragments of thymosin-α_1 containing structural features for which we have indications of immunomodulatory *in vitro* activity. These structural elements are N-terminal lysine and γ-carboxy-glutamic acid.

Syntheses

All peptides were synthesized by stepwise N-terminal sequence elongation with excess mixed anhydrides (MA) of Ddz-amino acids as described earlier[4] [Ddz = 2-(3,5-dimethoxyphenyl) propyl (2) oxycarbonyl]. Related to the useful acid lability of the temporary N-terminal Ddz-protection all side functions are masked by the *t*-butyl moiety with the exception of the ϵ-amino group of lysine, which was protected by the benzyloxycarbonyl group. The procedures employed have been established to be of particular utility, because of their reproducibility, ease, and efficiency. Typical yields for our preparations on the average are 80%. This is demonstrated in Figure 1 and also in our preceding publication.[5] Deprotection of the peptides was performed by treatment for one hour with trifluoroacetic acid subsequent to catalytic hydrogenolysis (Pd/H_2) in trifluoroethanol of the N^{ϵ}-benzyloxycarbonyl group of lysine in positions 1 and 20.

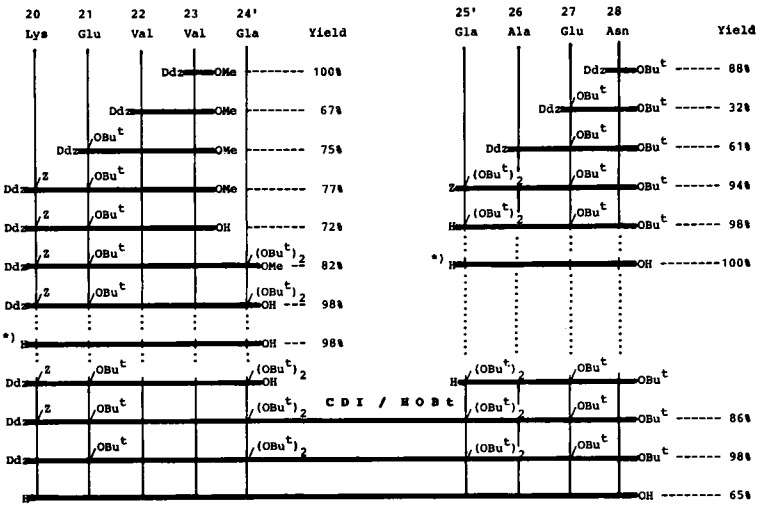

Fig. 1. Synthesis of C-terminal fragment analogs of thymosin-α_1 containing γ-carboxyl-glutamic acid. * Sequence entirely deprotected for immunological assays.

Assays

The *in vitro* immunomodulatory activity was studied in the α-amanitine inhibited E-rosette test and in the α-amanitine inhibited allogenic one-way mixed lymphocyte culture (MLC) on human peripheral T-lymphocyte populations of healthy donors. MLC: From two donors 10^5 responder cells and 10^5 stimulator cells (mitomycin-C blocked) in a total volume of 240 μl lymphocyte culture medium were incubated at 37°C for 120 hours in the presence (or absence) of 100 μg α-amanitine and 2μg thymosin-α_1 fragment/ml culture. Both additives are applied each in 20 μl aliquots/ culture prior to the admixture of the stimulator cells. For the last 8 hours of incubation 1 μCi ^{3}H-thymidine/culture is added. After isolation of the lymphocytes the radioactivity uptake is measured by liquid scintillation counting. All determinations are performed in triplicate.-E-ROSETTE TEST: $5 \cdot 10^6$ T-lymphocytes/ml buffer (Hanks solution), $5 \cdot 10^8$ sheep erythrocytes (SRBC)/ml buffer. Culture: 100 μl T-cell suspensions containing 0.2 μg α-amanitine + 10 μl human AB serum (heat stabilized) are incubated at 20°C for 13.5 hours (parallel cultures without α-amanitine). Addition of thymosin-α_1 fragment (2 μg/100 μl Hanks buffer), or of a blank of 100 μl/ total culture volume 210 μl. Incubation for 1 hour at 20°C. Addition of 100 μl SRBC suspension, incubation for 5 min. at 37°C., cooling in ice to 4°C and

centrifugation (4°C) at 100 g for 5 min, then incubation for 30 min. at 0°C followed by counting of the E-rosettes. All determinations are performed at least in triplicate. For the determination of optimal thymic peptide concentrations, 20 μg peptide/ 100 μl buffer is the stock solution, which is diluted bisectionally.

Results

Exchange via MA synthesis of acetyl-serine by lysine in the N-terminal fragment Ac (1-6) introduced two additional positive charges. Immunologically this resulted in an increased potency (93%, 16 μmolar; compared to thymosin-α_1, 1.6 μmolar) to restore the normal formation of E-rosettes in the α-amanitine inhibited assay. In the MLC described above, Lys (2-6) more strongly suppresses the response than Ac (1-6). As a consequence of these findings, we investigated the immunomodulatory action of several synthetic non-varied thymosin-α_1 segments, starting with lysine N-terminally.[5] We detected immunostimulatory potency in the C-terminal region of thymosin-α_1 in both assay systems.[3,5] In the standard MLC, responses on stimulation by fragment (20-24) and (25-28) were more pronounced than those of thymosin-α_1.[3] These results were expressed in percent of the MLC response (^{3}H uptake) without any modulating thymosin-α_1 fragment added. Assays were performed at different times on cells combined from different donors. In this paper (Figure 2) we compare these fragments with varied sequences Lys (2-6), (20-23) Gla, Gla(26-28), and (20-GlaGla-28), the latter three examples also in the presence of calcium (Gla = γ-carboxy-glutamic acid). We evaluated an α-amanitine inhibited MLC response where all samples were tested at the same concentration in cell combinations from one pair of donors. For a more uniform evaluation we are introducing a relative scale also in this assay. The stimulatory excess response caused by thymosin-α_1 after α-amanitine inhibition of the responder cells is taken as 100%, relative to the α-amanitine-inhibited MLC response without any additive modulator (0%). The data in Figure 2 indicate that Lys (2-6), also by this approach, has the lowest activity. The strongest stimulator again is (25-28), followed by (20-24) and (20-28). However, after the exchange of Glu for Gla in positions 24 and 25 of the small C-terminal fragments, the stimulatory activity vanishes due to the increase in acidity. In the fragment (20-GlaGla-28) the increase in acidity is compensated by the structural elements (lysine; hydrogen bonding) of the larger fragment, which is significantly more stimulatory than its original non-varied form. In (20-GlaGla 28) this effect is scarcely changed in the presence of one equivalent of calcium, whereas the smaller Gla-containing fragments

significantly gain in stimulatory activity in the presence of calcium. The possible influence of the Ca^{++}-binding Gla -containing fragments on parts of the T-cell membrane during activation[6] is currently under investigation.

Peptide	MLC[a]	TLC[b]	Amino Acid Analysis[c]				
			Ala	Asp	Glu	Lys	Val
Lys(2-6)	28	18	1.96(2)	2.04(2)	-	0.67(1)	1.00(1)
20-24	56	23	-	-	1.93(2)	0.83(1)	2.00(2)
25-28	88	21	1.05(1)	1.00(1)	2.04(2)	-	-
20-28	51	14	1.00(1)	1.00(1)	3.78(4)	0.97(1)	1.73(2)
20-24[d]	33	17	-	-	2.00(2)	1.16(1)	1.54(2)
25-28[e]	33	22	1.08(1)	1.00(1)	1.85(2)	-	-
20-28[f]	70	12	1.23(1)	1.00(1)	4.15(4)	0.85(1)	1.87(2)
20-24[d++]	53						
25-28[e++]	43						
20-28[f++]	66						

Fig. 2. Immunological and analytical data of thymosin-α_1 varied sequences in comparison to the original fragments. [a]Activity as determined by the rate of incorporation of ^{3}H-thymidine and expressed as percent of the excess stimulatory activity displayed by synthetic thymosin α_1 (100%) in the same assay system. Values are an average of three determinations. Each assay consisted of 10^5 stimulator cells (blocked by mitomycin-C) and 10^5 responder cells (α-amanitine inhibited) in the presence of 2μg/ml peptide in 240μl final culture volume. [b]R_f x 100 in n-Butanol/Pyridine/Acetic Acid/Water, 5:5:1:4.[c]Hydrolysis System: 12N HCl/Propanoic Acid, 1:1, 30-60 min, 160°. [d]γ-Carboxy-Glutamic Acid (Gla) in position 24.[e]Gla in position 25.[f]Gla in positions 24 and 25. [++]With equimolar $CaCl_2$

Conclusion

The data reported are qualitative expressions only as assay responses show great variations with blood donor, peptide or inhibitor concentration. The non-determined sub-set composition of the peripheral T-cell population (immune status of the donor) appears strongly to influence the results. The immunomodulatory potency of fragments of thymosin-α_1 - found despite temporary immunological variations in the assay systems - appears to be due to the possible activation of specific subpoplulations in the cell culture by distinct peptide signals for the regulation of immune responses, at least *in vitro*. This working hypothesis will be further elucidated in our laboratory.

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SYNTHESES AND CONFORMATIONAL PROPERTIES OF INTERFERON SEGMENTS

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Highly purified uniform interferons are not available so far in quantities for structural and biochemical studies. Therefore chemical syntheses of several segments of the known interferon sequences were performed mainly in order to obtain antigens for radioimmunochemical studies. Furthermore the pure synthetic segments were investigated by circular dichroism and ^{13}C-NMR. The results of conformational analyses were compared to predictions of conformations obtained according to Chou and Fasman.[1]

Firstly, we synthesized by the solid-phase method the N-terminal tridecapeptide of the sequence of human fibroblast interferon:[2] H-Met-Ser-Tyr-Asn-Leu-Leu-Gly-Phe-Leu-Glu-Arg-Ser-Ser-OH (Hu IFN-β(Fi)1-13). Boc-Ser(Bzl)-O$^-$Cs$^+$ was reacted with chloromethylated polystyrene-1% divinylbenzene yielding a loading of 0.25 mmol/g. The side chain functions of the Boc-amino acids were protected as follows: O-benzyl-serine, O-(2,6-dichlorobenzyl)tyrosine, N^g-tosyl-arginine. Coupling reagents and Boc-amino acids were used in six-fold excess, and 1-hydroxybenzotriazole was added to the second DCC coupling and to *p*-nitrophenyl-glutaminate and asparaginate couplings. After acetylation with Ac_2O/N-methylmorpholine the Boc group was removed by trifluoroacetic acid/dichloromethane (1:1). The amino group was neutralized by triethylamine/chloroform. All reactions were carried out twice using somewhat shorter reaction times than the standard method.

The N^g-Tosyl-tridecapeptide has been obtained by HBr/CF_3COOH. It has been repetitively purified by precipitations, and it showed only a minor impurity. The dry peptide was detosylated via Na/NH_3. The free tridecapeptide was purified by various precipitations from acetic acid/water, chromatography on Sephadex G-25, precipitation by acetic acid/ether, and DMF/acetone. Finally multiplicative counter-current distribution in butanol/5% acetic acid/propanol (5:5:1) yielded a tridecapeptide Hu IFN-β(Fi)1-13, which was found to be pure in various TLC and electrophoresis systems, amino acid analysis after total hydrolysis and aminopeptidase degradation. The GLC analysis of the pentafluoropropionyl-amino acid propyl esters on the chiral phase Chirasil-

Val showed practically no racemization. The ^{13}C-NMR data were in full agreement with the expected ppm values. All signals were assignable with minor ambiguities in the carbonyl region. The conformational analysis by circular dichroism showed the typical α-helical Cotton effects in trifluoroethanol (Figure 1). In more aqueous systems the helix content decreased considerably. 8 of 13 residues of Hu IFN-β(Fi)1-13 are helix formers, and the predicted conformation according to Chou and Fasman is α-helical (Table I).

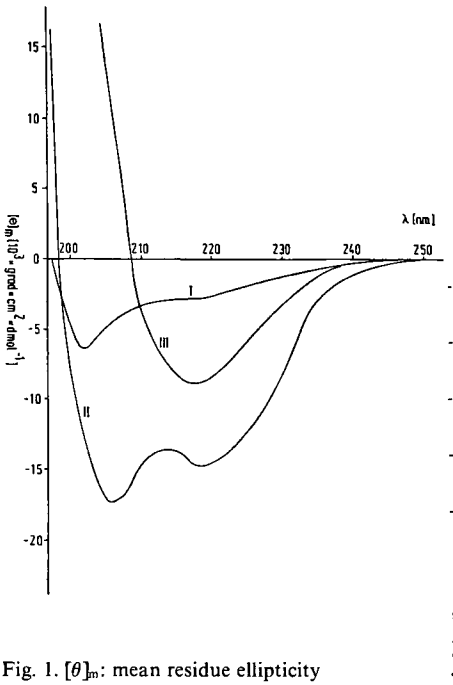

Fig. 1. $[\theta]_m$: mean residue ellipticity
I HuIFN-β(Fi)1-13 in dry ethanol
II HuIFN-β(Fi)1-13 in trifluoroethanol
III HuIFN-β(Fi)1-13 in water

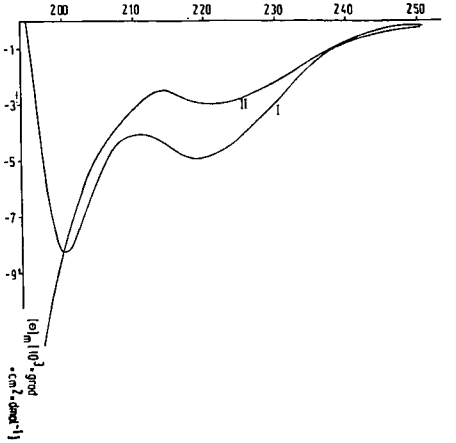

Fig. 2.
I HuIFN-α(Ly)1-10 in trifluoroethanol
II HuIFN-α(Ly)1-10 in hexafluoroacetone

Secondly, the N-terminal decapeptide of human lymphoblast interferon Hu IFN-α(Ly)1-10 Ser-Asp-Leu-Pro-Gln-Thr-His-Ser-Leu-Gly (Ser in position 1 has recently been replaced by Cys) has been synthesized under similar conditions as the segment Hu IFN-β(Fi).[2] Boc-amino acids with the following side chain protection were incorporated in double coupling steps each with five fold excess of reagents: Ser(Bzl), His(Dnp), Thr(Bzl). Aspartic acid was introduced as Fmoc-Asp(OtBu)-OH, and glutamine was coupled as Boc-Gln-ONp in the presence of 1-hydroxy-

Table I

Sample/	Greenfield[4]			Rosenkranz[5]			Chen[6]			realistic			Prediction			
Solvent	α	β	rc	α	β	rc	α	β	rc	α	β	rc	α	β	rc	
Hu IFN-β(Fi)1-13/ Ethanole	-1	6	8	0	0	13	1	3	9	0	5	8	0	6	7	or
Hu IFN-β(Fi)1-13/ Trifluorethanole	3.5	3	6	4	-3	12	5	1	8	5	0	8	5	0	8	or
Hu IFN-β(Fi)1-13/ Water	-4	-13	1	0	?	?	1	13	-5	0	13	0	0	10	3	
Hu IFN-α(Ly)1-10/ Trifluorethanole	0	5	5	0.5	-1	10	1.5	3.5	5	0	5	5	0	0	10	
Hu IFN-α(Ly)1-10 Hexafluoracetone	-1	5	5	1	3	6	0	0	10	0	5	5	0	0	10	

Figures record the number of residues in the corresponding conformation. α-helical and β-sheet segments smaller than four residues are not stable and can be neglected favoring the other conformational segments. In column "realistic" we tried to give a sensible interpretation of the evaluations.

benzotriazole. The Boc group was removed by CF_3COOH/CH_2Cl_2(1:1) and the Fmoc protection by diethylamine.

The decapeptide was split off via hydrazinolysis in DMF. It was purified by precipitations, chromatography on Sephadex LH-20 in methanol, reversed-phase chromatography on silica gel RP-8 in CH_3OH/H_2O (9:1). The protected decapeptide hydrazide Boc-Ser(Bzl)-Asp(OtBu)-Leu-Pro-Glu-Thr(Bzl)-His-Ser(Bzl)-Leu-Gly-N_2H_3 was then found to be pure according to TLC, ^{13}C-NMR spectroscopy, amino acid analysis and chiral phase gas chromatography.

The His(Dnp) decapeptide was obtained by cleavage via HBr/CH_3COOH, purification by precipitation of the Dnp group by mercaptoethanol. After removal of mercaptoethanol the free decapeptide was chromatographed on Sephadex G-15 in 0.1 M CH_3COOH and on Lichroprep RP-8 in CH_3OH/H_2O (9:1). Amino acid analysis before and after digestion with papain and aminopeptidase M gave the expected values. The ^{13}C-NMR spectrum (measured in water) was in full agreement with the expected ppm values. TLC and electrophoresis revealed trace amounts of an impurity, which can be removed by chromatography on a silica gel column. For the decapeptide Hu IFN-α(Ly)1-10 a random coil (rc) conformation is calculated, however, a quantitative evaluation of high rc contents is not possible (Table I).

CD spectra were recorded on a Roussel-Jouan-Dichrograph CD185 by two independent measurements under N_2-protection. The concentration of the solutions was 1-2 x 10^{-3} mol/l in different solvents.

As we pointed out earlier,[3] the conformational analysis of CD data yields good approximations of the conformation of peptides in solution, although all conformation determining and predicting methods have been developed for proteins and not for small oligopeptides, which may cause a lack of accuracy. Despite these problems we performed a comparative CD conformational analysis according to the methods of (1) Greenfield *et al.*,[4] (2) Rosenkranz *et al.*,[5] and (3) Chen *et al.* [6]. The results of the approximation using the least-square method for (1) and (3), respectively, and of the evaluation using simple linear equations for (2), are listed in Table I.

These results were compared to the conformational predictions we had performed according to Chou and Fasman.[1] Firstly we made the predictions for a larger segment within the two interferon molecules: 1-50 for Hu IFN-β(Fi) and 1-20 for Hu IFN-α(Ly), secondly, by reduction to the segments synthesized, we tried to obtain the most probable conformations. For Hu IFN-β(Fi), no unequivocal prediction can be given. For Hu IFN-α(Ly) no α-helical or β-sheet assignments can be made, thus only a rc-conformation is probable.

In Table I we record the results of the analyses and predictions. It illustrates well the lack of accuracy occurring on analysing CD spectra of oligopeptides: methods (1)-(3) yield results differing widely. The isodichroic method of Rosenkranz *et al.*[5] has a larger error by itself in particular for β- and rc-structures, which is due to the positions of the isodichroic points in wavelength regions with steep CD curves (202 nm and 200.5 nm, respectively).

As pointed out before, a coherence between evaluations and predictions can be obtained in the case of Hu IFN-β(Fi)1-13, whereas Hu IFN-α(Ly)1-10 requires a different data base for the evaluations than the ones used according to Greenfield *et al.* and Chen *et al.*

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SYNTHETIC PEPTIDES CONTAINING THE MACROCYCLIC THIOLESTER Cys-Gly-Glu-Glu: MODELS OF THE METASTABLE BINDING SITE OF HUMAN COMPLEMENT PROTEIN C3b

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The serum complement system is capable of attacking and killing cells and of marking particles for ingestion by phagocytes. These functions are mediated by proteolytic cleavage of C3, the third complement protein, into two fragments, the inflammatory peptide C3a ($M_r = 8,900$) and the activated protein C3b (M_r 171,000). Nascent C3b contains a metastable binding site that can react with a hydroxyl group on the surface of a receptive cell or other biological particle to form a covalent ester bond.[2] Reaction of native C3 with small nucleophiles (water, hydroxylamine, methylamine) inactivates its latent metastable binding site.[3,4] For example, reaction of human C3 with methylamine generates an N^{ω}-methylglutamine residue and liberates a thiol group. Since the amino acid sequence near the methylamine-labile site is Gly-Cys-Gly-Glu-Glu($NHCH_3$)Asn,[3] an important structural feature of the metastable binding site may be an internal thiolester bond between the sidechains of the Cys and second Glu residues, Cys-Gly-Glu-Glu. This 15-membered macrocycle contains a thiolester linkage and three peptide bonds.

We have synthesized three peptides containing this thiolactone ring, namely, H-Cys-Gly-Glu-Glu-Asn-NH_2 (**1**), H-Gly-Cys-Gly-Glu-Glu-Asn-NH_2 (**2**), and CH_3CO-Gly-Cys-Gly-Glu-Glu-Asn-NH_2 (**3**). These syntheses were carried out in solution by two different strategies involving stepwise chain elongation and segment coupling (Figure 1). Pentapeptide **1** was assembled by the strategy of forming the thiolester bond followed by closing the ring at a peptide bond. Specifically, the sidechain carboxyl group of Boc-Glu(OBzl)-Glu-Asn-NH_2 was activated with dicyclohexylcarbodiimide and 1-hydroxybenzotriazole and coupled to the thiol group of Z-Cys-Gly-OH in 77% yield. After subsequent activation of the Gly carboxyl group as the 2,4,5-trichlorophenyl ester and removal of the Boc group from Glu, the amino ester cyclized to yield the protected cyclic pentapeptide in 45% yield. In contrast, hexapeptide **2**

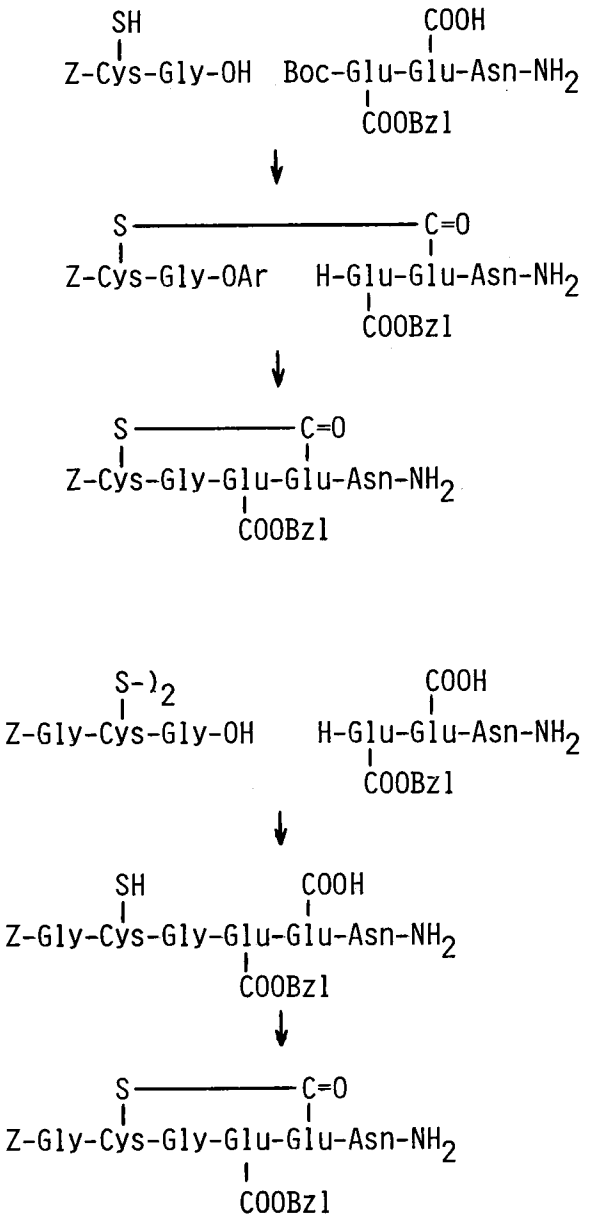

Fig. 1. Two strategies for synthesis of macrocyclic peptide thiolesters as models of the metastable binding site of human complement protein C3b.

was assembled by the strategy of forming the central peptide bond followed by closing the ring at the thiolester bond. Specifically, the disulfide dimer of Z-Gly-Cys-Gly-OH was converted into the mixed anhydride with isobutyl chloroformate and coupled to H-Glu(OBzl)-Glu-Asn-NH_2 in 84% yield. After subsequent reductive cleavage of the disulfide bond with tributylphosphine and activation of the sidechain carboxyl group of the resulting thiol acid with dicyclohexylcarbodiimide and 1-hydroxybenzotriazole, the activated thiol acid cyclized to give the protected cyclic hexapeptide in 50-65% yield. In both strategies, the desired peptides were obtained by deprotection with HF/anisole and were purified by high-pressure liquid chromatography. The acetylated hexapeptide **3** was assembled by the second strategy using the disulfide dimer of CH_3CO-Gly-Cys-Gly-OH.

In preliminary kinetic studies, we have measured the reactivity of hexapeptide **2** and acetylated hexapeptide **3** with hydroxide ion, methylamine, and imidazole in veronal-buffered saline (10 mM veronal/140 mM NaCl) at 37°C and pH 7.3. Each rate of cleavage was measured spectrophotometrically by continuously recording the 343-nm absorbance of the 2-pyridinethiolate formed *in situ* by reaction of the liberated peptide thiol group with 2,2′-dipyridyldisulfide. The apparent first-order rate constants for cleavage of these cyclic thiolesters are compared in Table I with the corresponding rate constants for cleavage of the model acyclic thiolester, CH_3CO-Cys($COCH_3$)-$NHCH_3$ (**4**). Hydrolytic ring cleavage was about 2000 times faster for the macrocyclic thiolesters than for the acyclic thiolester, whereas cleavage by methylamine and or imidazole was only about 50 times faster.

Table I. Net First-order Rate Constants For Cleavage Of Three Thiolesters In Veronal-Buffered Saline at 37° And pH 7.3[a]

Reagent; calcd. net conc. M	Acyclic Thiolester 4	Cyclic Thiolester 2		Cyclic Thiolester 3	
	k_4[b] $10^{-6}s^{-1}$	k_2 $10^{-3}s^{-1}$	$\frac{k_2}{k_4}$	k_3 $10^{-3}s^{-1}$	$\frac{k_3}{k_4}$
Hydroxide; 2.0×10^{-7}	0.60	1.54	2570	1.06	1770
CH_3NH_2; 1.3×10^{-4}	16.7	1.05	63	1.28	77
Imidazole; 6.7×10^{-2}	7.5	0.32	43	0.34	45

[a]The net rates for cleavage in 0.30 M methylamine and 0.10 M imidazole were obtained by subtraction of the background rate for cleavage in 55 M water (pH 7.3).

[b]Values for k_4 at pH 7.3 were extrapolated from the following values measured at pH 9.0: $3.0 \times 10^{-5}s^{-1}$ for hydroxide, $8.4 \times 10^{-4}s^{-1}$ for methylamine, and $3.7 \times 10^{-4}s^{-1}$ for imidazole.

The half-time for thiolester hydrolysis in veronal-buffered saline (37°C, pH 7.3) is 321 h for the acyclic thiolester **4**, 186 h for native C3, 0.20 h for cyclic thiolester **3**, and approximately[5] 10^{-8} h (60 μs) for nascent C3b. In preliminary experiments, tritiated cyclic thiolester **3** failed to bind covalently to particles of the polysaccharide zymosan. Since the macrocyclic thiolester **3** is hydrolyzed about 10^3 times faster than native C3 but about 10^7 times slower than nascent C3b, the 15-membered thiolactone ring present in peptide **3** is probably necessary but is not sufficient to explain the biological reactivity of the metastable binding site of human C3b.

Acknowledgements

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IMMUNOLOGICALLY ACTIVE PEPTIDE FRAGMENTS OF THE SPERM-SPECIFIC LACTATE DEHYDROGENASE C_4 ISOZYME

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Introduction

The C_4 isozyme of Lactate Dehydrogenase is the most abundant LDH of spermatozoa. Mouse LDH-C_4 has been purified to crystalline homogeneity.[1] Partial amino acid sequences have been reported[2,3] and the structure has been determined by x-ray crystallography.[4] LDH-C_4 is a potent antigen in males and females of homologous and heterologous species. Further, this immune response reversibly inhibits fertility.[5,6] To exploit this phenomenon in the development of a contraceptive vaccine requires that the natural product antigen be replaced with a synthetic analog. To this end we are mapping the antigenic determinants of LDH-C_4.

Materials and Methods

For tryptic digestion at 37° C, reduced, carboxymethylated LDH-C_4 (5 mg/ml in 0.1 M NH_4HCO_3, pH 8.3) was made 2 M in urea. TPCK-trypsin (Worthington) was added in 1% (w/w) increments at one hour intervals to a total of 4%. After 4 hours total, the peptides were separated from urea on Sephadex G-10 and lyophilized.

Cation exchange chromatography was performed on a 0.9 x 30 cm column containing Beckman PA-35 resin at 52° C. Gradient development was performed with 0.01 M pyridine-acetate, pH 3.1 as starting buffer and 2.0 M pyridine-acetate, pH 5.0 as the limit buffer. The gradient was generated at 0.41 ml/min with a 6-chamber Buchler Varigrad. Chambers 1 and 2 contained 200 ml starting buffer, chambers 3,4 and 5 136 ml starting buffer and 44 ml limit buffer, and chamber 6 200 ml of limit buffer. The gradient was preceded by 100 ml starting buffer and followed by 200 ml limit buffer. For detection of peptides, a 50 μl aliquot was dissolved in 3 ml 0.15 M $NaPO_4$ pH 8.6, and 200 μl of 3 mg/ml fluorescamine in dioxane was added with vortexing. Fluorescence was excited at 390 nm and read at 485 nm.

Peptides were separated by HPLC on a Waters μBondapak C-18 column. A ternary gradient was generated using three pumps and a Waters model 720 system controller. Pump C delivered a constant 0.8

ml/min aqueous trifluoroacetic acid, pH 2.00. Pumps A and B delivered a total of 1.2 ml/min of water and acetonitrile respectively. Elution was effected by a gradient in which the proportion of acetonitrile was increased. Chromatography was performed at 35° C and detection was at 214 nm with a Perkin-Elmer LC-65T detector. For preparative purposes, peak fractions were collected manually after injecting 60 nmoles of digest. The purity of each peak was determined in the same system with isocratic elution adjusted to yield $K' = 5\text{-}7$. Mixtures were repurified isocratically in this way.

Amino acid sequences were determined by Edman degradation,[7] and antibody binding with a solid-matrix radioimmunoassay.[8]

Results

The tryptic digest of mouseLDH-C_4 has been separated by cation-exchange chromatography (Figure 1) and by reverse phase HPLC (Figure 2). Neither technique completely resolves the 38-40 peptides expected from the amino acid composition of the protein. However, the HPLC separation is considerably more practical because of the relatively short run time of 65 minutes including reequilibration compared to 49 hours for chromatography on PA-35. In addition, HPLC is more amenable to final purification of partially resolved peptides by re-chromatography under isocratic conditions.

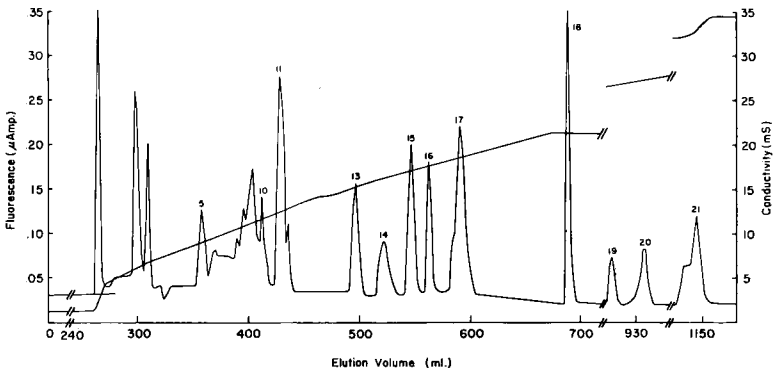

Fig. 1. PA-35 cation exchange chromatography of LDH-C_4 tryptic peptides. Representative fractions are designed by the numbers above the peaks

The immunological activity of the peptides recovered in these separations was assessed by their ability to bind rabbit anti-mouse LDH-C_4. This binding was measured with ^{125}I-goat anti-rabbit IgG. On the PA-35 column, most of the antibody binding activity is concentrated in the poorly resolved material eluting between 350 and 440 ml (Figure 1) For

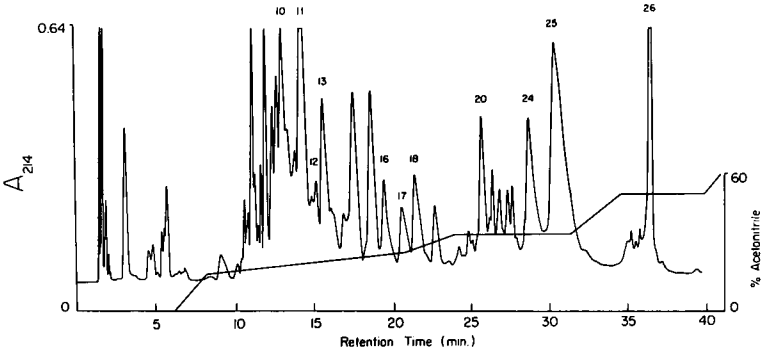

Fig. 2. HPLC elution profile of LDH-C_4 tryptic peptides. Representative fractions are designated by the numbers above the peaks.

example, fraction 5 contains 10 peptides, as judged by two dimensional thin-layer peptide mapping, and binds 340 cpm. The 3 peptides of peak 10 bind 300 cpm while the 4 peptides in fraction 11 have an activity of 430 cpm. In contrast, the pure peptides in peaks 16, 18, 19, 20 and 21 bind only 50-90 cpm. Fraction 13 contains a single, well-resolved peptide which binds 240 cpm. The amino acid sequence of this immunologically active peptide was determined to be: ISGFPVGR. It represents residues 152-159 of the native protein, and has been designated $MC_{152-159}$.

Antibody binding ranging from 1840 to 3620 cpm was observed for 10 HPLC fractions. Nine of these contained pure peptides. Peak 10 was a mixture of 3 peptides. Most of the pure peptides could be identified in the total structure from their amino acid sequences. Fractions 18, 20, 24, 25, 11, and 26 are, respectively, MC_{5-16}, MC_{44-58}, MC_{61-77}, $MC_{180-210}$, $MC_{211-220}$, and $MC_{282-317}$. Peaks 12 and 16 have not yet been conclusively identified. These immunologically active peptides may be compared to five inactive HPLC peptides which bound an average of 560 cpm. The difference in cpm observed between HPLC and PA-35 peptides reflects the difference in specific radioactivity of the two preparations of labeled second antibody.

Discussion

Immunologically active peptides have been isolated from the tryptic digest of mouse LDH-C_4 by cation-exchange chromatography and by reverse phase HPLC. This general approach can be used to identify antigenic determinants of this protein. Although the unfractionated tryptic digest binds antibody,[5] this activity can now be associated with specific fragments. Presumably, these peptides contain the molecular

structures responsible for specific antibody-antigen interaction in the native protein.

Further, a synthetic analog of $MC_{152-159}$. conjugated to BSA, elicits antibodies in rabbits. Some of these antibodies specifically bind to mouse LDH-C_4.[9] Thus, this peptide fragment is immunologically active both in binding antibody directed against the parent protein and in eliciting antibody to LDH-C_4. It seems likely that such synthetic peptide fragments can replace the natural product antigen in the development of a contraceptive vaccine based on the immunosuppression of fertility by the sperm-specific LDH-C_4 isozyme.

Acknowledgements

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SYNTHETIC PEPTIDES THAT DEFINE AN IMMUNOREACTIVE SITE OF CALMODULIN

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Calmodulin is an ubiquitous 148-residue calcium-binding protein that modulates the action of numerous enzymes in response to elevated calcium concentrations.[1,2] In order to study the role of calmodulin in cellular function, Van Eldik and Watterson[3] have used specific rabbit sera directed against performic acid-oxidized calmodulin to develop a sensitive and reproducible radioimmunoassay for calmodulin. Based on the full immunoreactivity of a tryptic peptide and an overlapping cyanogen bromide peptide, they suggested[3] that a major immunoreactive site lies within an 18-residue region (residues 127-144) in the COOH-terminal domain of calmodulin.

In order to explore which of these residues are necessary for full immunoreactivity, we synthesized by the solid-phase method[4,5] a series of peptide amides corresponding to segments from this region. The immunoreactivity of each peptide was evaluated by competition radioimmunoassay. The results are expressed in Table I as the percentage of immunoreactivity with one particular antiserum relative to that of unlabeled vertebrate calmodulin. The smallest peptide capable of complete reactivity is the heptapeptide **8** corresponding to calmodulin residues 137-143. The presence of additional residues at the NH_2-terminus (peptides **6** and **7** or COOH-terminus (peptide **4**) confers no change in immunoreactivity.

To further define the minimal structural requirements for immunoreactivity, a series of peptides lacking one or more of the COOH-terminal residues present in peptide **8** was examined. Peptides **12-14**terminate at Val-142 and peptides **15-17** end at Phe-141. The absence of Gln-143 results in decreased immunoreactivity. For example, peptide **13** was 20-fold less reactive than peptide **7** and peptide **14** was 30-fold less reactive than peptide **10**. The absence of both Val-142 and Gln-143 produces a more substantial loss of reactivity. For example, peptide **15** was about 2,100-fold less reactive than peptide **12**, and peptide **17** was approximately 760-fold less reactive than peptide **13**. These results indicate that the presence of Gln-143 is required for complete immunoreactivity and that the absence of both Val-142 and Gln-143 results in negligible immunoreactivity.

Table I. Immunoreactivity of Calmodulin Peptides

Peptide code	Peptide structure[a]	Relative Immunoreactivity[b]
	134 137 140 143 145	%
	chicken gizzard calmodulin-(1-148)	100
1	H-Glu-Glu-Phe-Val-Gln-Met(O)-Met(O)-NH_2	<0.024
2	H-Gly-Glu-Val-Asn-Tyr-Glu-Glu-Phe-Val-Gln-Met(O)-NH_2	100
3	H-Val-Asn-Tyr-Glu-Glu-Phe-Val-Gln-Met(O)-NH_2	100
4	H-Asn-Tyr-Glu-Glu-Phe-Val-Gln-Met(O)-NH_2	100
5	H-Tyr-Glu-Glu-Phe-Val-Gln-Met(O)-NH_2	0.63
6	H-Gly-Glu-Val-Asn-Tyr-Glu-Glu-Phe-Val-Gln-NH_2	100
7	H-Val-Asn-Tyr-Glu-Glu-Phe-Val-Gln-NH_2	100
8	H-Asn-Tyr-Glu-Glu-Phe-Val-Gln-NH_2	100
9	CH_3CO-Asn-Tyr-Glu-Glu-Phe-Val-Gln-NH_2	100
10	H-Tyr-Glu-Glu-Phe-Val-Gln-NH_2	0.13
11	CH_3CO-Tyr-Glu-Glu-Phe-Val-Gln-NH_2	1.7
12	H-Gly-Glu-Val-Asn-Tyr-Glu-Glu-Phe-Val-NH_2	40
13	H-Val-Asn-Tyr-Glu-Glu-Phe-Val-NH_2	4.1
14	H-Tyr-Glu-Glu-Phe-Val-NH_2	0.004
15	H-Gly-Glu-Val-Asn-Tyr-Glu-Glu-Phe-NH_2	0.019
16	H-Glu-Val-Asn-Tyr-Glu-Glu-Phe-NH_2	0.008
17	H-Val-Asn-Tyr-Glu-Glu-Phe-NH_2	0.005
18	Ala-Tyr-Glu-Glu-Phe-Val-Gln-NH_2	0.7
19	Asn-Ala-Glu-Glu-Phe-Val-Gln-NH_2	<0.005
20	Asn-Phe-Glu-Glu-Phe-Val-Gln-NH_2	14
21	Asn-Tyr-Ala-Glu-Phe-Val-Gln-NH_2	0.022
22	Asn-Tyr-Gln-Glu-Phe-Val-Gln-NH_2	0.041
23	Asn-Tyr-Glu-Ala-Phe-Val-Gln-NH_2	1.6
24	Asn-Tyr-Glu-Gln-Phe-Val-Gln-NH_2	2.1
25	Asn-Tyr-Glu-Glu-Ala-Val-Gln-NH_2	0.13
26	Asn-Tyr-Glu-Glu-Phe-Ala-Gln-NH_2	2.1
27	Asn-Tyr-Glu-Glu-Phe-Val-Ala-NH_2	0.47

[a] Met(O) = Methionine sulfoxide; substitutions are underlined.
[b] Immunoreactivity relative to that of chicken gizzard calmodulin.

The relative importance for immunoreactivity of Asn-137, the NH_2-terminal residue of heptapeptide **8**, was studied by comparing a pair of peptides that lack this residue. For example, peptide **5** lacking Asn-137 is 140-fold less reactive than peptide **4**. Similarly, hexapeptide **10** lacking Asn-137 is about 670-fold less reactive than peptide **8**. In addition, acetylated hexapeptide **11** is 150-fold less reactive than heptapeptide **8**,

which shows that the presence of just the alpha carbon and carbonyl group of Asn-137 is not sufficient for full immunoreactivity. These results indicate that Asn-137 is required for complete immunoreactivity and that the minimum peptide length required for full calmodulin immunoreactivity with this antiserum is seven residues.[6]

The possibility that another seven-residue segment whose amino acid sequence overlaps that of peptide **8** might also exhibit complete immunoreactivity was tested by examining four closely related heptapeptides containing 5 or 6 of the residues present in heptapeptide **8**. Synthetic peptides related to peptide **8** by shifting one residue to the left (peptide **13**) or to the right (peptide **5**) retain only 4% and 0.6% of the immunoreactivity of vertebrate calmodulin, respectively. The heptapeptides related to peptide **8** by shifting two residues to the left (peptide **16**) or two residues to the right (peptide **1**) retain less than 0.02% of the immunoreactivity of calmodulin. These results demonstrate that peptide **8** not only has the minimal peptide length but also is the only seven-residue segment from the 135-145 region that gives full immunoreactivity.

In preliminary experiments, a series of single-substitution analogs of heptapeptide **8** has been examined for immunoreactivity with the anti-calmodulin antiserum. Replacement of the negatively charged sidechain carboxylate group of Glu-139 or Glu-140 by the isosteric but noncharged carboxamide group of Gln reduces the immunoreactivity. Shortening these sidechains to the methyl group of Ala produces essentially no further decrease in reactivity.

Replacement of Tyr-138 by Phe (peptide **20**), which is equivalent to removal of the phenolic oxygen atom, decreases immunoreactivity about 7-fold. Replacement of Tyr-138 or Phe-143 by Ala substantially decreases reactivity, so the aromatic rings of both residues are quite important for immunoreactivity. Since replacement of Asn-137, Val-142, or Gln-143 by Ala also reduces immunoreactivity significantly, all seven sidechains are quite sensitive to truncation to a methyl group.

Except for the modest contribution of the phenolic oxygen of Tyr-138, all seven sidechains of heptapeptide **8** corresponding to the 137-143 segment of vertebrate calmodulin are important for full expression of calmodulin immunoreactivity. Tyr-138 is particularly sensitive to loss of its aromatic ring and the adjacent Glu-139 is quite sensitive to loss of negative charge.

Acknowledgements

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CONFORMATION AND ANTIGENICITY OF THE SYNTHETIC (24-33) FRAGMENT OF STAPHYLOCOCCAL NUCLEASE

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Introduction

Recent studies on cross reactivities among closely related proteins and their peptide fragment using polyclonal and (more recently) monoclonal antibodies have led to new views on the nature of antigenicity in proteins. Thus, it now seems likely that, given the appropriate pairs of species for immunogen and host, most of a protein surface can be antigenic.[1,2,3,4,5] The numerous regions of the protein surface which form the interfaces with the antibody combining sites may be described as topographic determinants or domains.[4,6,7,8] These determinant domains comprise side chains contributed by amino acids which are spatially close but not necessarily close in linear sequence. Nevertheless, small linear sequences of peptides can sometimes be isolated and shown to have sufficient binding affinity to be demonstrably antigenic. Notably, Atassi[9] delineated 5 such sequences in sperm whale myoglobin.

An accelerated method for delineating suspected antigenic peptide sequences in proteins was developed by Smith *et al.*[10] and applied by Hurrell *et al.*[11] to show that the 5 peptide determinant sequences in sperm whale myoglobin are spatially (though not sequentially) conserved in a plant leghaemoglobin; they are present also in beef myoglobin.[5] This method involves peptide synthesis on a large-pore resin support which then also serves for solid phase radioimmunoassay. As each amino acid is linked, the resulting peptide is tested for antigenicity. Completion of a linear peptide determinant is indicated when the ^{125}I-antibody (specific to the intact protein) binds to the peptide-resin adduct.

The conformational requirements for antigen-antibody recognition, and the relationship between β-bends and antigenic determinants is not well understood. Staphylococcal nuclease, which has 12 chain reversals, of which 11 are at or near the surface of the protein,[12] suggests itself as a useful model protein for studying such relationships. There is little

detailed information on the location of antigenic sequences in nuclease, though regions (18-47), (99-149) and (127-149) have all been implicated.[13,14,15,16] We found that of all the chain reversal sequences in nuclease,[17] only one bound anti-nuclease antibodies to a significantly greater extent than normal (non-specific) IgG. This sequence was 20-31. (G[20]DTVKLMYKGQP[31]GG). Binding of antibodies commenced at residue 27 (Tyr) and increased to a plateau at 25 (Leu), suggesting that the double bend located between residues 26 and 30 is part of an antigenic domain in the native protein.

Peptide (24-33) was therefore synthesized by the solid phase procedure of Merrifield[18] to examine its structural and antigenic properties in solution and also when re-attached to a solid support. Being flanked by a short segmet of antiparallel β-sheet for three residues on either side of the (26-33) turn, some residual structure might be expected in solution. After chromatographic purification, the free peptide was pure by TLC, amino acid analysis, high voltage electrophoresis, HPLC and amino acid sequencing (9 cycles on an Illitron protein sequenator using Polybrene to stabilize the peptide film on the cup walls). The latter showed no "preview" PTH amino acids and therefore the absence of deletion peptides. Hydrogenation of Tyr 27 produced a non-UV absorbing and homogeneous peptide free of tyrosine by amino acid analysis.

The CD spectra of the peptide and its hydrogenated products in water, methanol and trifluoroethanol are shown in Figures 1(a) and (b).

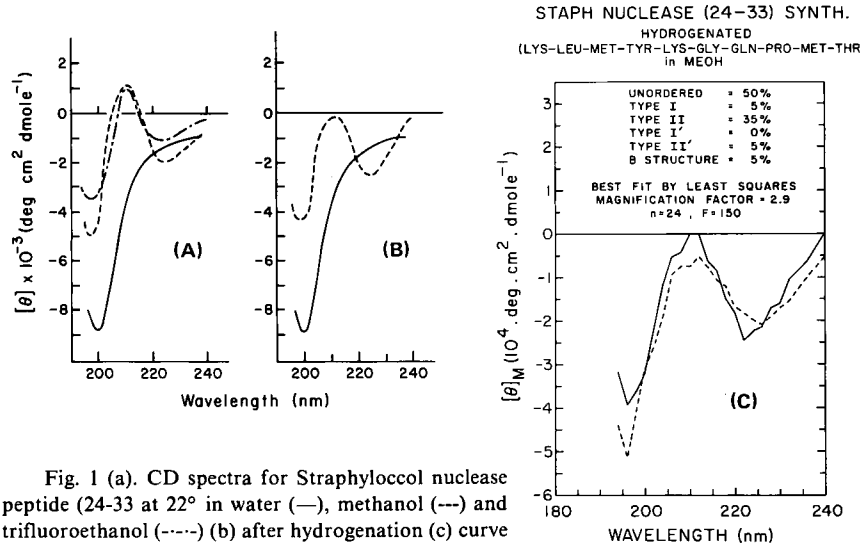

Fig. 1 (a). CD spectra for Straphyloccol nuclease peptide (24-33 at 22° in water (—), methanol (---) and trifluoroethanol (-·-·-) (b) after hydrogenation (c) curve fitting the spectrum in methanol.

The spectrum in water is consistent with that of a completely unordered peptide. Curve fitting the spectrum in methanol [see Figure 1 (c)] by the method of Leach *et al.*[19] suggested the induction of 40-50% of β-bend forms in this solvent. The integrity of the "hairpin" conformation of this sequence in the protein thus depends in large part on interactions between the two legs of the U and these are disrupted in water.

Solid-phase radioimmunoassay of the (24-33) peptide after reattachment to large-pore resin beads showed strong binding to ^{125}I antibodies raised to staph. nuclease (Table I). The substitution levels of the two peptides, nuclease (24-33) and the control peptide $(Val)_2$-Asn-Pro-Gly-Gln-$(Val)_2$ on the polymeric support are nearly equal (60 and 58 μmoles per g resin respectively) so that the cpm data may be directly compared. The control peptide (chosen for its putative U-conformation as a model for β-keratin[19] bound the specific and the control antibody populations weakly and with equal preference. In contrast, nuclease (24-33) bound specific anti-nuclease antibodies strongly by a factor of 10 times that for the same antibodies with the control peptide.

Table I. Assessment of Binding of ^{125}I Labelled Antibodies to Resin-bound Nucleases (24-33).

Resin-bound	^{125}I anti-staphylococcal nuclease bound[b] (cpm per mg beads)	^{125}I anti-myoglobin bound[b] (cpm per mg beads)
nuclease (24-33)	2702	537
control peptide[a]	261	276

[a] $(Val)_2$-Asn-Pro-Gly-Gln-$(Val)_2$. [b] Average of triplicate analyses.

The presence of a determinant between residues 24 and 33 in staph. nuclease therefore seems fairly certain. Containing a central sequence (24-33) which, in the native protein appears in a very low energy conformation (i.e. two overlapping β-bends[20]) one might expect this peptide to retain an above-average fraction of its native format when isolated from the protein and therefore to exhibit a substantial antibody-antigen affinity.[21]

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SYNTHETIC PEPTIDE INHIBITORS WHICH PREVENT INACTIVATION OF SECRETORY IMMUNOGLOBULIN BY PATHOGENIC BACTERIA

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Introduction

Immunoglobulin A (IgA) is the principal form of antibody found in mammalian external secretions such as colostrum and milk and fluids of the respiratory and gastrointestinal tracts.[1] IgA antibodies, the basis for immune defense in these secretions, are synthesized by plasma cells lying within the mucosal lining of these tissues. These mucous membranes are the site of colonization by pathogenic bacteria and are the portal of entry for infections.

Human IgA has the basic four-polypeptide chain structure characteristic of immunoglobulins, two light and two heavy (α) chains. There are two isotypic forms of IgA designated IgA1 and IgA2 and each antibody synthesizing plasma cell synthesizes only one of these isotypes. The main difference in primary structure of the two isotypes is in the hinge region between the C_H1 and C_H2 domains, where $\alpha2$ chains have a 13-amino acid deletion that involves a large part of a unique, replicated octapeptide sequence,[2] as shown in Figure 1.

IgA proteases are extracellular, neutral endopeptidases of bacterial origin which, of more than 50 proteins tested, cleave only human IgA.[3,4] The bacteria known to release these enzymes are all human pathogens and include *Streptococcus pneumoniae, Streptococcus sanguis, Neisseria meningitidis, Neisseria gonorrhoeae* and *Hemophilus influenzae;* notably, species within these same genera that do not cause human infections are IgA protease-negative. The enzyme may be conveniently purified from the cell-free supernatant of bacterial broth cultures.[3] The single peptide bond in the IgA1 heavy chain hydrolyzed by each IgA protease has been identified by amino acid sequence of Fcα fragments, and in each case proline contributes the carboxyl group (Figure 1). All bonds cleaved lie in the duplicated octapeptide sequence of the $\alpha1$ chain; IgA2 proteins are enzyme-resistant because the hinge region deletion includes all the protease-susceptible bonds. Cleavage of IgA1 markedly

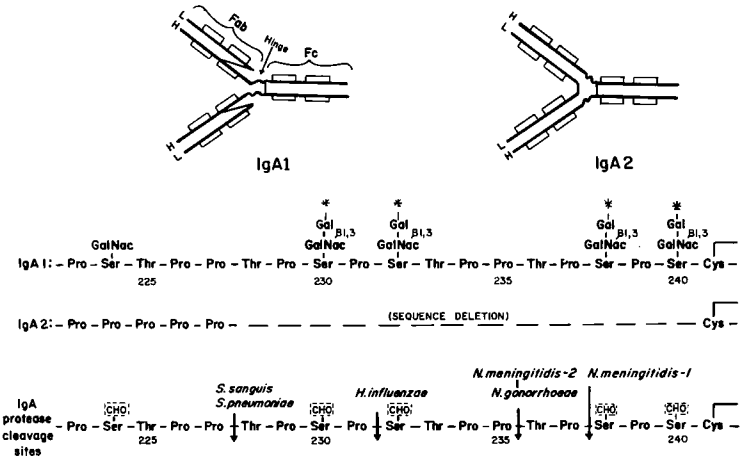


Fig. 1. Diagram showing the polypeptide chain assembly of human immunoglobulin A and the primary structure of their heavy chain hinge regions; numbering system is from Reference 7. The duplicated octapeptide T-P-P-T-P-S-P-S and its oligosaccharide side chains are largely deleted in IgA2 proteins; in addition, all peptide bonds cleaved by the various IgA proteases (shown at the bottom) are deleted in IgA2, rendering this isotype enzyme-resistant. Note that each enzyme cleaves a single peptide bond; *N. meningitidis* strains each produce one of two enzyme specificities.[2]

impairs its biological function, lowering titre several orders of magnitude and causing the loss of effector function of the Fc region.

All quantitative data on IgA protease activity have been obtained using intact human serum IgA as substrate; we have, therefore synthesized peptide analogs of the IgA1 hinge region to obtain a more useful lower molecular weight substrate. Other potential advantages of such substrates is their value in enzyme purification and the study of the properties of native IgA that contribute to protease specificity. Also, such substrates can be used as models for the design of IgA protease inhibitors.

Methods

IgA protease of *N. gonorrhoeae* was purified from cell free culture filtrates by salt precipitation and column chromatography. Human monoclonal serum IgA1 protein, purified from the plasma of a patient with multiple myeloma, was radio-labelled with [^{125}I] by the chloramine-T method. Peptide synthesis was by solid phase method of Merrifield,[5] residues being added sequentially from the carboxy-terminus. The completed peptides containing 8, 16 or 24 residues were cleaved from the

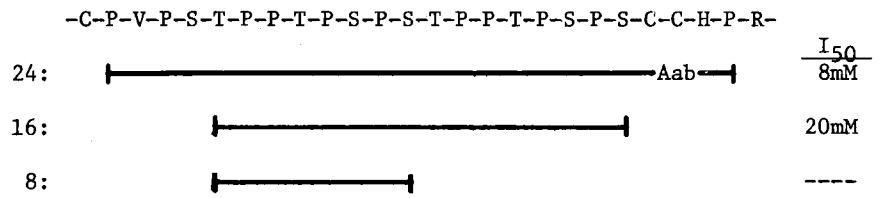
support and deprotected by hydrogen fluoride and purified by molecular sieve chromatography (G-50) and high pressure liquid chromatography (HPLC).

The capacity of the three peptides to inhibit IgA protease cleavage of native IgA was measured by dissolving varying amounts of each peptide in Tris-HCl buffer, pH 7.5, 0.05M, containing 1.6μM [^{125}I]-IgA and unlabelled IgA, and starting hydrolysis by the addition of 0.34 units gonococcal IgA protease. After resolution of the hydrolytic products of IgA on polyacrylamide gel electrophoresis the [^{125}I] Fabα material was excised and counted. Control assays in buffer free of peptide showed that IgA hydrolysis was linear with time under the conditions used.

Results

Table 1 shows that the 16 and 24-peptides, but not the octapeptide, inhibited cleavage of IgA. 50% inhibition required final concentrations of 20mM and 8mM of the 16 and 24-residue peptides, respectively. Using molecular weight markers the Fabα and Fcα hydrolysis products of IgA were the same in the presence or absence of peptide, indicating that peptides did not alter the bond cleaved in native substrate. We have not as yet definitively shown that peptides are cleaved, but the 16-residue peptide examined by HPLC after exposure to gonococcal IgA protease showed changes in elution position; these fragments have not yet been isolated and characterized.

Table I. Structure of Synthetic Peptide Analogs 8, 16 and 24 Residues in Length Based on IgA1 Hinge Region Sequence (top). I_{50} for each peptide using native human IgA substrate is shown to the right. Aab is alpha-amino butyric acid.



These experiments indicate that peptide analogs of the human IgA1 alpha chain act as substrates for microbial IgA proteases, but that the native hinge region of IgA1 is a more favored substrate. The relatively weak binding of the peptides indicates that conformational or biochemical characteristics of the native IgA1 hinge are of importance in binding, and that length alone is not the sole requirement for a suitable substrate. Unlike the synthetic peptides, the native IgA hinge is glycosylated,

although the influence of this on IgA protease specificity is not clear.[6] Further, native IgA alpha chain is dimeric, bound at cysteine residues close to the protease cleavage site. Although we are currently synthesizing dimeric peptides to examine this point, we and others have shown that isolated IgA heavy chain monomers are adequate substrates for these proteases.[6]

In summary, microbial IgA proteases are a unique family of enzymes whose only known natural substrate is human IgA of the IgA1 isotype. The distribution of activity among a diverse group of bacteria infecting human mucosal tissues indicates that the proteases are virulence factors, and the importance of IgA antibodies in human mucosal immunity favors this hypothesis. Further studies are aimed at understanding this novel microbial-host relationship.

Acknowledgement

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IMMUNOLOGICALLY ACTIVE SEQUENCE OF DIPHTHERIA TOXIN SYNTHESIZED

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Introduction

Diptheria toxin is a single polypeptide of 62000 Daltons with one of its disulfide bridges located between Cys 186 and 201 (Figure 1) [1,2]. Toxin action prevails following proteolytic cleavage within the loop (186-201), into parts A and B. Fragment A is less toxic than the original toxin, whereas fragment B, administered separately, is atoxic. Fragment B attaches to the cell membrane and acts as a hinge for facilitating the entry of A into the cell, eliciting destruction of the enyme system(s) of the cell.

The sequence of five tetrapeptides inside the loop (198-201) are not encountered in other peptides or proteins[3]: they may act as immunological determinants able to raise specific antibodies. We report here the syntheses of different fragments of the loop and the results obtained concerning their antigenicity and immunogenicity.

Synthesis

Ala-Ala-Cys-^{14C}Ala-Gly-Asn-Arg-Val-Arg-Arg-Ser-Val-Gly-Ser-Ser-Leu-Lys-Cys-
186 190 195 201

Fig. 1. Amino acids in the sequence 184-201 of diphtheria toxin.

Three different polymers were used: *p*-hydroxy-phenyl propionic resin (C_6H_5OH-R[4], *m*-nitrobenzhydrylamine resin (*m*-NO_2BZH)[5], and benzhydrylamine resin (BZH).

Protection of functional groups in amino acid side chains: Ser (OBz), Arg (NO_2), Lys (ϵ ClZ). Sulphydryl groups of Cys were protected by ACM when peptides were synthesized with BZH or C_6H_5OHR or in a solution synthesis and with MeOBz when *m*-NO_2-BZH was used. DCC/HOBt was the coupling reagent for the solid phase method. The REMA method was employed in solution synthesis. Peptides prepared on BZH were split from the polymer by means of HF at 0° C and obtained as their amides. Peptides prepared on C_6H_5OH-R were obtained with free carboxyl termini after transesterification with DMAE, hydrolysis, and treatment with HF to unblock the side chain functional groups.

After HF cleavage, peptides synthesized with *m*-NO_2-BZH were obtained as peptidyl-resin complexes, those prepared in solution were obtained with free carboxyl termini; the latter appeared to be identical with those synthesized by the solid phase method.

Treatment of the ACM peptides with iodine resulted in the formation of the disulphide linkage between Cys 186 and Cys 201.[6]

Purification of peptides was accomplished by gel filtration on Bio-Gel P_4(.1N AcOH as eluent), and ion exchange chromatography. Elutes were monitored at 206 nm. Homogeneity of the peptides was checked by TLC (SiO_2: BuOH: Pyr: AcOH: H_2O = 15:10:3:12) HPLC (μ Bondapak C_{18} with a gradient elution from solvent B O % to solvent B 40% (solvent A = CH_3CN 5 %, $AcONH_4$ 0.05 M 95%, pH 7.5, solvent B = CH_3CN 95%, $ACONH_4$ 0.05 M 5%, pH 7.5), gel electrophoresis, and amino-acids analysis.

Peptides 194-201, 192-201, 190-201, 188-201 and 186-201 were synthesized on BZH and *m*-NO_2BZH. Sequences 188-201 and 186-201 were prepared on C_6H_5OH-R and by the REMA method. The best conditions for peptide coupling to the carrier proteins or synthetic polymer for raising antibodies were investigated with the peptide 186-201 synthesized with 14C Ala 187. Radiolabelling the peptide was useful in monitoring its purification. Finally two extra Ala were added to the loop to facilitate anchorage on the carrier.

Immunological Properties

Antigenicity — Peptides deprotected but still linked to the *m*-NO_2-BZH were tested for their capability of binding anti-diphtheria toxin antibodies. Results are shown in Table I. Antibodies were labelled with ^{125}I, percent of binding is given by the number of cpm bound by the entire toxin, zero percent bindings by the number of cpm bound by the resin alone. A non-related nonapeptide (thymic serum factor, FTS) was used as a negative control.

Table I. Binding of Antidiphtheria Toxin Antibodies to Peptides of the Loop

	Percentage of binding
Diphtheria toxin	100
Hexadecapeptide (186-201)	12
Dodecapeptide (190-201)	1.6
Decapeptide (192-201)	2
Octapeptide (194-201)	0
FTS	0
Resin alone	0

Immunogenicity — Peptides were covalently linked to various carriers by their amino groups using glutaraldehyde (192-201 and 188-201 to bovine serum albumin BSA, Ala-Ala-186-201 to ovalbumin OV, and synthetic poly D-L-alanyl-poly-L-lysine, A-L). Conjugates were injected into guinea pigs under the conditions described in Table II. Sera have been tested for their ability (a) to agglutinate toxin coated sheep red blood cells, (b) to bind ^{125}I labelled diphtheria toxin, (c) to neutralize both the dermonecrotic activity of the diphtheria toxin and its lethal effect.

Table II. Immunogenicity of Peptides with Partial Sequence of Diphtheria Toxin

Immunization	Agglutination	RIA	Protection against Dermonec-test	Lethal
(192-201)-BSA + FCA	+	+	NT	NT
(188-201)-BSA + FCA	++	++	+	+
(Ala-Ala-186-201)-OV+ FCA	+	+	NT	NT
(Ala-Ala-186-201)-A--L + MDP	+	+	NT	NT

On day 1 guinea pigs received the conjugates at the dosages indicated either in complete Freund Adjuvant (FCA) or in saline with MDP (N-acetyl muramyl-L-alanyl-D-isoglutamine, muramyl dipeptide) a synthetic adjuvant glycopeptide analogous of mycobacterial cell wall. They were bled two weeks after a boost given on day 30.

Conclusions

Data reported here demonstrated: (a) that *m*-NO_2-BZH resin allows for rapid screening of the antigenicity of peptides during the course of their synthesis, since it was shown that resin linked diphtheria peptides were able to bind anti toxoid antibodies; (b) that immunization by a peptide of appropriate short sequence as present in a biologically active protein, led to the synthesis of antibodies capable of binding the native molecule observed previously by others,[7] and, moreover the neutralisation of its toxic effects.[8]

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DISTRIBUTION AND SIGNIFICANCE OF PERIPHERAL REGULATORY PEPTIDES

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When a number of peptides were first found to be present in both the brain and the gut, there was little idea of the existence of a large and potent regulatory peptide-containing diffuse neuroendocrine system, with a total body-wide distribution. It is now well established that these regulatory peptides are present in both nerves and endocrine cells of most organ systems and that the peptide-containing nerves form the largest and most important component of the autonomic nervous system.[1] Useful information on the characteristics of the regulatory peptides has been provided by the considerable advances made in a variety of relevant disciplines during the past few years. These include the determination of their genetic code (mRNA),[2] mode of action, multiple molecular forms, abnormalities in disease states[3] and their precise tissue localization, shown at both light and electron microscopical levels. The main features of some of the regulatory peptides are summarized in Tables I and II.

Neuron Specific Enolase: A Tool for the Visualization of the Diffuse Neuroendocrine System in its Integrity

In the mid-sixties a group of highly acidic, soluble proteins were found to be present exclusively in the central nervous system.[4] One of these proteins, designated 14-3-2 protein at the time, was shown to be specifically localized to neurons.[5] In view of the fact that it was the neuronal form of the glycolytic enzyme enolase, Marangos renamed the protein "neuron specific enolase (NSE)". NSE is a dimeric protein composed of two apparently identical subunits with a molecular weight of 78,000 daltons. Far from being exclusively localized to central neurons, NSE has recently been found in both neural and endocrine components of the diffuse neuroendocrine system of most organs, including that of the gut and pancreas,[6] adrenals,[7] lung[8] and skin.[9] NSE is also present in all classes of neuroendocrine neoplasias (APUDomas), including islet cell tumors, phaeochromocytomae and lung carcinoids[10] (Figure 1).

Individual Organ Systems

We shall now discuss the distribution and tissue localization of

Table I. Gut Hormones and Brain Peptides[a].

PEPTIDE	MAIN DISTRIBUTION	MAIN ACTIONS	MODE/ACT.
Gastrin	Antrum	Gastric acid secretion	Endoc.
CCK	Nervous system Gut	↑ Gall bladder contractions & Pancreatic secretions	Endoc.NT
Secretin	Gut/Brain	Pancreatic bicarbonate secretions	Endoc.
Glucagon + EG	Pancreas;Gut;Brain	Metabolic(gluc.)Trophic(GLI)	Endoc.
PP	Pancreas	Pancreatic Enzyme secretions	Endoc.
Motilin	Upper intestine	Motility	Endoc.
GIP	Upper intestine	Ins. Gastric acid secretions	Endoc.
Dynorph.	Pituitary;Brain	Opiate actions	Endoc.NT
TRH	Brain;Gut;Pancreas	Thyrotropin gastric acid	Endoc.
ACTH	Pituitary;Brain;Gut	Adrenal & corticoid	Endoc.
Endorph.	Pituitary;Brain	Opiate actions	Endoc.NT
MSH	Pituitary	Pigmentation	Endoc.

PEPTIDE	MAIN MOLECULAR FORM	SEQUENCE SIMILARITIES
Gastrin	G14-17-34	Gastrin CCK family
CCK	CCK-8-33-39	
Secretin	Secr. & prosecretin	Glucagon, PHI.VIP.GIP.
Glucagon	Progluc.-glucogon	Secretin.VIP.PHI.GIP.
PP	2 known 2.5-3KD/8.0-10KD	Not known
Motilin	2 known (larger-smaller)	Not known
GIP	Major & minor comp	Glucagon, secretin, VIP, PHI
TRH	One only	Not known
ACTH	Man 4.5, 17, 28 & 37 KD	Not known
Endorph.	β-LPT$^{61\text{-}76,61\text{-}91,61\text{-}77}$	β-Endorph.$^{1\text{-}16,61\text{-}76,61\text{-}91}$
MSH	ACTH$^{1\text{-}13,1\text{-}18}$	ACTH$^{1\text{-}13}$
Dynorph.	One only	Not known

[a]Abbreviations used: NT, neurotransmitter; secr., secretion; panc., pancreatic; gas., gastric; dynorph., dynorphin; gluc., glucagon.

regulatory peptides in individual organ systems, where their presence is fully described.

Respiratory Tract and Heart — In spite of Feyrter's earlier recognition of pulmonary endocrine cells, the realization that the respiratory

Table II. Brain/Gut Peptides[a]

PEPTIDE	MAIN DISTRIBUTION	MAIN ACTIONS	MODE/ACT.
VIP	Central & peripheral NS	Vaso.Dilation;Muscle Relaxation;Secretion	NT
Substance P	Central & peripheral NS	Pain NT;Nociceptor;Muscle contraction	NT
Enkephalin	Gut;Brain	Opiate actions	Endoc.NT
Somatostatin	Brain;Gut;Pancreas	Inhibitory hormone	Paracrine NT
Neurotensin	Gut;Brain	Gastric acid secretion; Vaso.dilation	NT
PHI	Gut;Brain	Insulin release	Unknown
PYY	Gut;Brain	Insulin release	Unknown

PEPTIDE	MAIN MOLECULAR FORMS	SEQUENCE SIMILARITIES
VIP	Large pro-VIP	Secretin,glucagon.PHI,GIP
Subs.P	One only	Not known
Enkephalin	Leu^5-Met.	Dynorphin 1-5, α,β,γ-endorphin
SRIF	SRIF = 4	SRIF 28^{15-28}
Neurotensin	Variants	Unknown
PHI (Hist.Isol.res.)		With VIP, Secretin, Glucagon, GIP
PYY (Tyro.res.)		With PP & neurotensin

AMPHIBIAN PEPTIDES

PEPTIDE	MAIN DISTRIBUTION	MAIN ACTIONS	MODE/ACT.
Bombesin	Gut;Brain;Lung	Releaser peptide	Paracrine NT
Sauvagine	Not known	↑tensive;Pituitary; modulator	Unknown
Dermorph.	Not known	Opiate actions	Unknown

PEPTIDE	MAIN MOLECULAR FORMS	SEQUENCE SIMILARITIES
Bombesin	Several, from 14-27 a.a.	Not known
Sauvagine	One only	New peptide family
Dermorph.	One only	Not known

[a]Abbreviations used: See Table I; NS, nervous system.

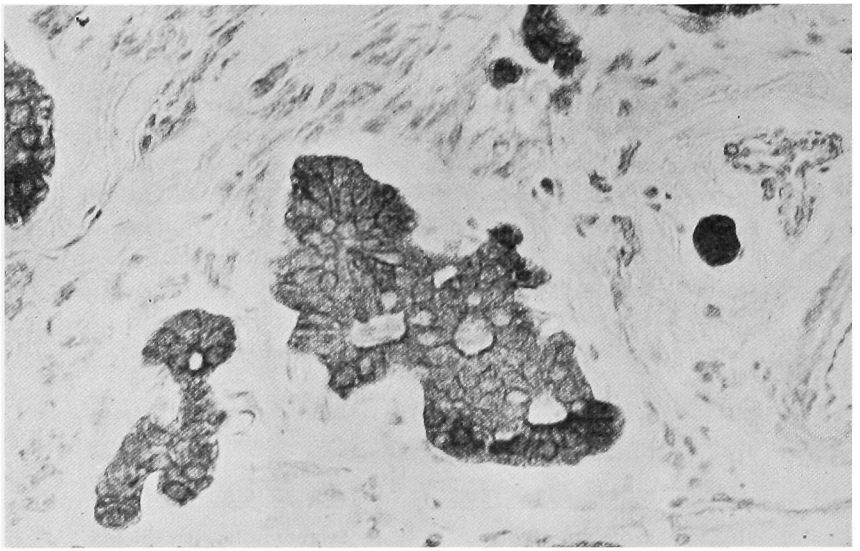

Fig. 1. Gut carcinoid immunostained for neuron-specific enolase. (x 220)

tract is an important member of the diffuse neuroendocrine system came only recently. In 1978 the localization of a regulatory peptide (bombesin) was reported for the first time (Figure 2).[11] It is now known that a variety of these active peptides and amines are found in both components, neural (substance P and VIP) (Figure 3) and endocrine (bombesin/serotonin) of the system.[12]

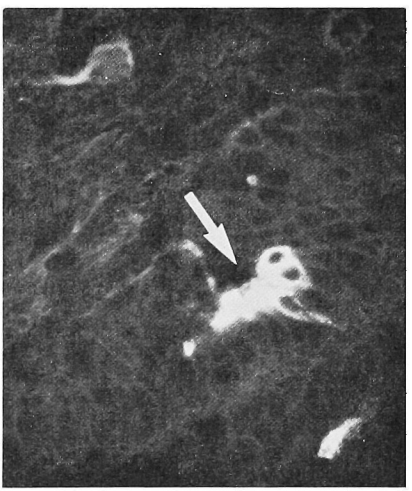

Fig. 2. Bombesin cells in bronchus of human foetal lung. (x 450)

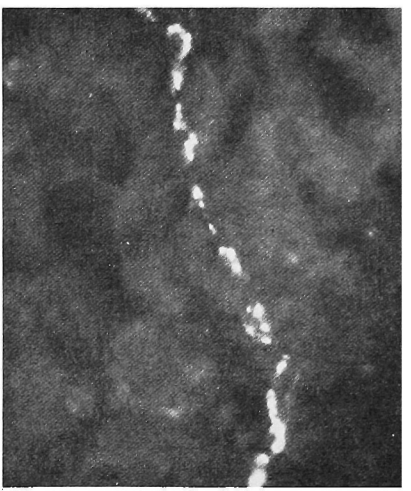

Fig. 3. A substance P nerve fiber in guinea pig lung. (x 650)

The heart is richly innervated by substance P-containing autonomic nerves, predominantly found around the large coronary vessels and in the conducting system.[13]

Central Nervous System — Biologically active peptides are widely distributed throughout the central nervous system and extensive mapping studies of their exact distribution have been made (Table III).[14] Although the peptides overlap in many areas, each peptide system has a unique distribution pattern of cell bodies, fibers and terminals. For example, although enkephalin- and neurotensin immunoreactive cell bodies have been described in many brain areas they overlap only slightly and even when present in the same nuclei the cells are separate. Our studies have shown that, generally, the following conditions hold for the

Table III. Distribution of Peptides in the Central Nervous System.[a,b]

PEPTIDE	D.HORN	V.HORN	BR.STEM	H-THAL.	AMYGD.	H-CAMP.	CORTEX
ACTH	(+)	(+)	(+)	++0	++	–	–
ANGIOTEN.	++	(+)	+	++0	+	–	–
CCK	+++ 0	++	++ 0	+++ 0	+++ 0	+++.0	++0
β-ENDORPH.	–	–	+	++ 0	++	–	–
ENK.	+++ 0	++	++ 0	+++ 0	++ 0	+	(+)
LHRH	–	–	(+)	++ 0	++	–	–
α-MSH	–	–	(+)	++ 0	++	–	–
NEUROTEN.	+++ 0	(+)	++ 0	++ 0	+++ 0	+ 0	+
OXYTOCIN	+	(+)	+	+++ 0	++	+	–
SOM.	++ 0	+	+ 0	++ 0	++ 0	+ 0	++
SUB.P	+++ 0	++ 0	++ 0	+++ 0	+++ 0	+	(+)
TRH	–	+	++ 0	++ 0	++	+	(+)
VASOPR.	+	(+)	+	++ 0	+	+	–
VIP	+	(+)	+	++ 0	+++ 0	+++ 0	+++ 0

[a]Fibers: +++ = Dense, ++ = Moderate, + = Low. O = Cell Bodies. [b]Other abbreviations defined in Tables I and II.

distribution of peptides in the central nervous system: a) No two neuropeptides have exactly the same distribution of cell bodies, fibers and terminals. Evidence has recently shown that ACTH, β-endorphin and MSH coexist in the same neurones and fibers. However, their fiber and terminal distributions are not identical.[15] Perhaps this is accounted for by different post-translational processing of the common peptide precursor within the axons. b) Cell bodies containing different neuropeptides in the same anatomical region (*e.g.* amygdala) consistently have slightly different localization.[14] c) Many peptide-containing cell bodies give rise to fibers which terminate in and innervate distant brain areas.[14] d) Where different peptide-containing fibers share a common pathway (*e.g.* stria

terminalis) each peptide component terminates in a different region of the target structure or nucleus.[14]

The spinal cord also possesses a wide variety of neuropeptides (see Table III), many of which are present in primary afferent sensory neurons of the dorsal root.[16] Substance P is an example of a peptide which is very well represented in the dorsal horn and originates from primary sensory neurons. Since the early observations of a significant depletion of this putative sensory neurotransmitter after dorsal rhizotomy, changes have been demonstrated after surgical manipulations of either the dorsal root or the sciatic nerve.[17] These manipulations include sectioning (Figure 4) or ligation of nerves as well as local applications of capsaicin.

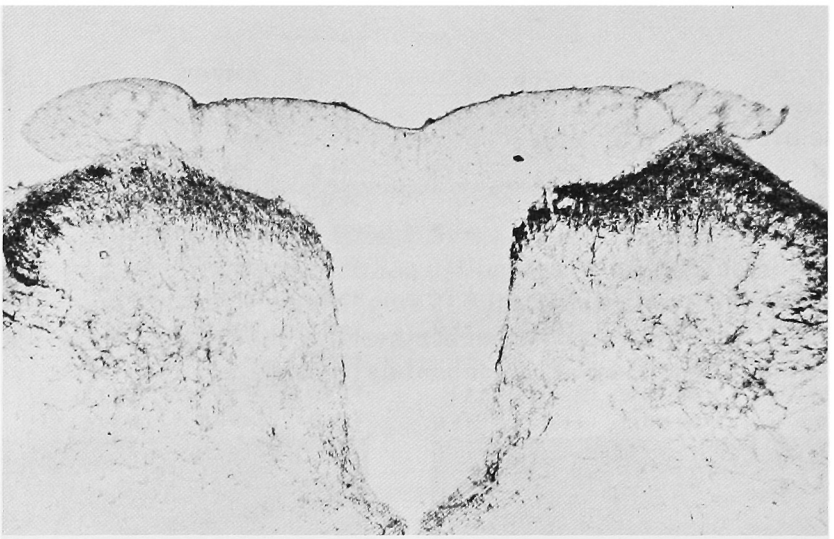

Fig. 4. Substance P immunoreactive fibers in dorsal horn of L4 segment of rat spinal cord 14 days after unilateral sciatic nerve section. Note decrease of immunoreactivity in the left horn. (x 120)

The Gastrointestinal Tract and Pancreas — The gastrointestinal tract and pancreas are the organs with the richest supply of regulatory peptides, which are found both in typical endocrine cells and in autonomic nerves.[18] Vasoactive intestinal polypeptide (VIP), substance P, enkephalin, bombesin, gastrin/CCK, thyrotropin releasing hormone (TRH), somatostatin and neurotensin (only in birds and once reported in rat), have been found to be present, exclusively or otherwise, in autonomic nerves of the gut and pancreas.[19] The distribution of peptides contained in autonomic nerves is far more widespread than that of the peptide hormones acting via the circulation. (See Tables I and II). Most of the nervous system peptides are found in different concentrations in all

layers and areas of the gut wall. However in spite of this extensive distribution, the peptidergic innervation of the gut wall shows a remarkable degree of organization. Some interesting points have recently emerged concerning these gut regulatory peptides.

Secretory Granules — Electron microscopical investigation of the secretory granules which store regulatory peptides, particularly those found in endocrine cells, has been going on for some considerable time. The shape, size, electron density and appearance of the limiting membranes of these granules are some of the many features which have enabled their classification and, hence, that of their corresponding cell types. (Figure 5). Association of a particular type of secretory granule with the production of specific peptide was based, in the early days, on purely morphological observations. The procedure has now been facilitated by the use of immunocytochemistry at the electron microscopical level which has reinforced the concept that most peptides are produced by a separate endocrine cell type. The latest classification of endocrine cells was agreed on by an international panel of experts in September 1980.[20] In contrast, little is known about the various secretory granules which form the storage site of peptides in autonomic nerves. This is in spite of the fact that the concept of a bi-partite (cholinergic-adrenergic) autonomic nervous system has been increasingly and, lately, repeatedly challenged by the realization that within this system there exists an important and heterogeneous peptidergic component.[21] It is, however, possible to predict that classification of the peptidergic neurosecretory granules of the gut will follow that of the endocrine cells. Evidence is already available that one population of the large peptidergic (p-type) neurosecretory granules is immunostained by substance P antibodies, whereas others remain unstained.[22]

The Intrinsic Neuronal Origin of Gut Peptidergic Nerves — It is now well established that most, if not all, peptidergic nerves originate from neuronal cell bodies present in either or both of the main ganglionated plexuses of the gut wall. This has been determined not only as a result of direct observations (which are, on many occasions, difficult to make, due to the content of peptide stored in the soma being below the limits of immunocytochemical detection) but, most importantly, using a number of experimental procedures. These include organotypic gut explants, extrinsic denervation and separate cultures of the two ganglionated plexuses.[23]

Involvement of Regulatory Peptides in Gut Pathology — Regulatory peptides of the gut and pancreas, present either in autonomic nerves or in endocrine cells, have been shown to be involved in a number of

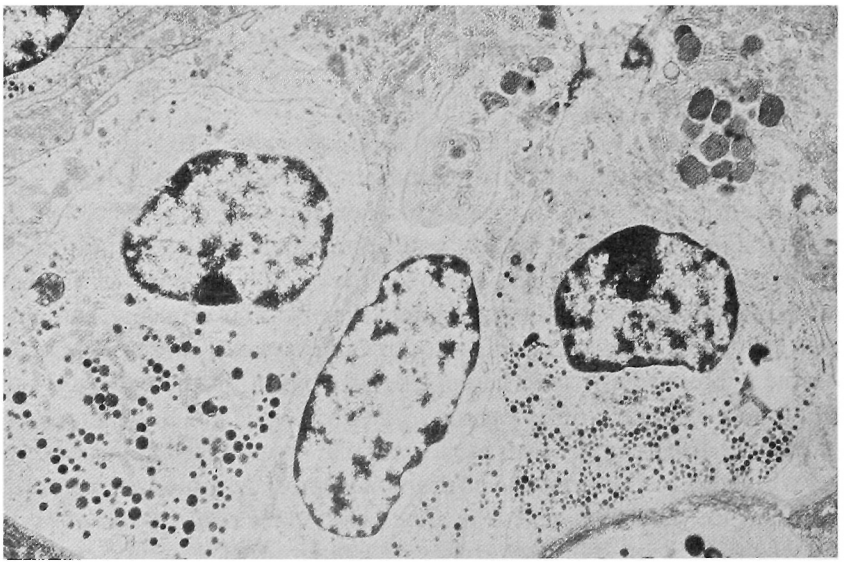

Fig. 5. Ultrastructure of human gut. Note two separate cells containing different secretory granules. (x 3,000) A) Granule size = 300 nm B) Granule size = 140 nm

gastrointestinal diseases. For the sake of brevity the reader is referred to review articles that describe instances where the profiles of gut hormones or peptidergic nerves have been shown to be abnormal.[3] Brief mention will be made here of only a few of these conditions. i) Autonomic nerves in gut diseases: Peptidergic nerves, in particular those containing VIP and substance P, are significantly decreased in two diseases of the bowel, Chagas' and Hirschsprung's disease associated with intractable chronic constipation and absence or degeneration of intrinsic neuronal cell bodies. In contrast, peptidergic nerves are normal in a generalized autonomic neuropathy like that found in the Shy-Drager Syndrome which shows no involvement of the gut neuronal cell bodies. Unlike Chagas' or Hirschsprung's disease, Crohn's disease is characterized by strikingly increased and highly abnormal VIPergic innervation in the bowel, Figure 6. Interestingly, these changes are particularly marked in the mucosa and submucosa of both the granulomatous and nongranulomatous areas, thus making the examination of endoscopic biopsies a potentially useful diagnostic tool.[24] ii) Nesidioblastosis: Nesidioblastosis is the term used to describe islet cell hyperplasia, often associated with severe and intractable hypoglycemia and hyperinsulinism. Systematic comparative analysis of neonatal pancreas from children with or without intractable hypoglycemia and hyperinsulinism led to the realization that an increase in islet cell tissue is a common feature of both normal and hypoglycemic

babies. However, the remarkable decrease in both the number of D cells and the somatostatin content of pancreas taken from hypoglycemic babies proved to be the differentiating feature.[25] In the pancreas somatostatin regulates the release of insulin and other pancreatic hormones. A depletion of this important regulatory peptide will therefore lead inevitably to abnormal and inappropriate release of insulin.

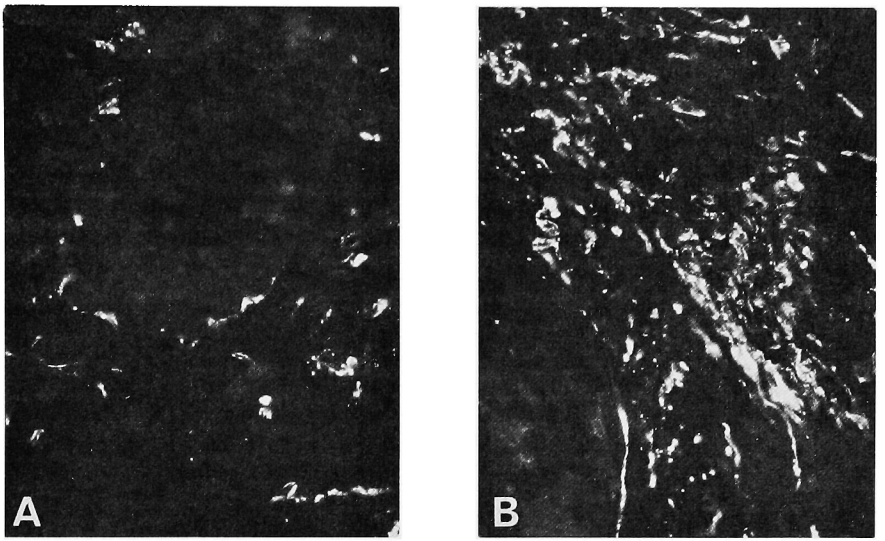

Fig. 6. VIP containing nerve fibers (A) and (B) (x 350)

Evidence of a Neurotransmitter Role for the Peptides Contained in Autonomic Nerves — A number of the criteria which determine the neurotransmitter nature of a substance have already been fulfilled for certain peptides found in autonomic nerves. These include: a) the elevation of local neuropeptide (*e.g.* VIP) concentrations following parasympathetic nerve stimulation, giving rise to a corresponding atropine resistant tissue response *e.g.* gastric relaxation, or salivary gland vasodilation; (b) the reproduction of these actions by micro-injections of pure peptide in the experimental animal.[26] This neurophysiological evidence is further supported by the finding of particularly high densities of peptidergic nerves close to the structures responsible for the specific tissue responses. For instance, VIP, which is known to be responsible for the atropine resistant vasodilation observed after parasympathetic nerve stimulation, is found in nerves in close association with blood vessels of the corresponding area.[27]

The Male and Female Genital Tract

Two regulatory peptides, VIP and substance P, are found in significant concentrations in both the male and female genital tracts. VIP, a potent vasodilatory substance and modulator of secretion and muscle tone, is found in autonomic nerves in close contact with blood vessels and smooth muscle of the uterine cervix[28] and around the pudendal arteries and in the erectile tissue of the corpus cavernosum of the penis (Figure 7). In addition, VIP nerve fibers are also seen innervating the uterine cervical glands and the vas deferens and epididymus. Conversely, substance P, a sensory neurotransmitter, is found in areas involved in sensory perception, such as the vagina or the sensory corpuscles of the glans penis.

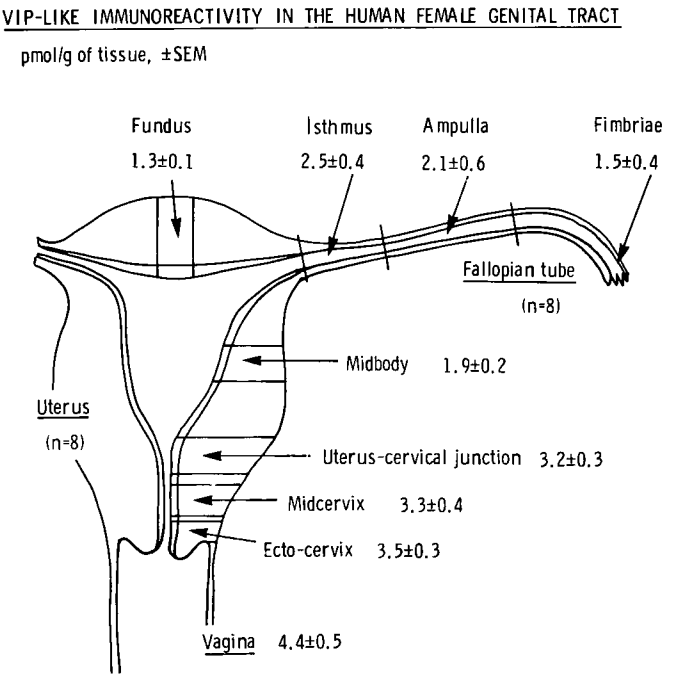

Fig. 7. Diagrammatic representation of human female genital tract showing VIP distribution as immunoreactive material.

Conclusion

The finding of potently active peptides in both the endocrine and nervous systems emphasizes the essential unity of these two main controlling systems in the body. A number of neurophysiological inconsistencies, such as the atropine resistant tissue responses which follow nerve

stimulation, can now be explained in the light of the recent findings of a large and complex peptidergic component of the autonomic nervous system. The discovery of specific blockers of the transmission of these peptidergic nerves, which are quite distinct from the classical neurotransmitter- (acetylcholine noradrenaline)- containing nerves, is anxiously awaited. These powerful peptides, known to control most bodily functions, are now being found to be involved in a growing number of diseases, including those of the gastrointestinal tract and pancreas, respiratory tract and central nervous system. It is clear that we have only touched on the edges of highly fascinating and rapidly expanding scientific field, which embraces a multitude of disciplines and will undoubtedly aid the further understanding of the workings of mankind in health and disease.

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BEHAVIORAL EVIDENCE THAT SUBSTANCE P MAY BE A SPINAL CORD NOCICEPTOR NEUROTRANSMITTER

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Introduction

Although the principal neurotransmitters for the motor nerves leaving the spinal cord have been known for more than 30 years, none of the neurotransmitters for sensory fibers entering the spinal cord have yet been identified. In 1953, Lembeck[1] found substance P (SP) in dorsal roots, and hypothesized that SP was a sensory neurotransmitter. Since identification of the SP structure[2] (ArgProLysProGlnGlnPhePheGly LeuMetNH_2), evidence has supported a neurotransmitter role for SP in primary pain afferents.[3,4,5] If SP is a neurotransmitter responsible for sensation, then it should be possible to produce a sensory experience by stimulating the spinal cord with SP. Recently, when we injected a few picomoles of SP into the spaces surrounding mouse spinal cords, the mice began to bite their toes and scratch their backs and ears.[6] This behavior occurs within a few seconds of the injection and is extremely intense. The mice direct their behavior alternately to the skin of one side of the body and then the other. A wide variety of intraspinally injected agents have not elicited this behavior, although both somatostatin and kainic acid elicit weaker one-sided scratching syndromes similar to those elicited by low SP doses. The behavior is certainly sensory in nature since the mice precisely direct their mouths and paws to the skin surfaces.

Since SP may be a nociceptor transmitter, we wondered if biting and scratching were appropriate reactions of mice to noxious cutaneous stimulation. Capsaicin, the active ingredient of Hungarian red peppers, is known to produce a sensation of intense inflammatory pain when applied to human skin.[7] When alcoholic solutions of capsaicin were applied to mouse feet, the mice would vigorously bite their feet, which were inflamed. When it was applied to their shaven backs, the mice would vigorously scratch their backs. Foot-biting was never observed with ethanol, and only a few weak wiping motions were ever detected when ethanol was put on the animals' backs. Thus, the scratching and biting

initiated by intraspinal SP is a behavior that mice utilize as a response to chemogenic inflammatory pain. Interestingly, capsaicin is known to release SP from pain afferents,[5] a property which may account for its noxious properties.

To prove that the capsaicin sensation is mediated by SP, it is necessary to block the sensation through a specific antagonism of spinal cord SP receptors. Recently, DPro^{2}DPhe^{7}DTrp^9-SP was detected as a SP antagonist on the guinea pig ileum test (Folkers and Rossel, unpublished). We have found that DPro^{2}DPhe^{7}DTrp^9-SP is also a specific antagonist to SP receptors of the isolated rat colon. In Figure 1, DPro^{2}DPhe^{7}DTrp^9-SP is shown to cause a dose-related depression of SP contractions, but not the acetylcholine contractions (compare middle trace with upper one). When DPro^{2}DPhe^{7}DTrp^9-SP was intraspinally injected, it blocked the SP-induced scratching syndrome, but not the weaker somatostatin syndrome. No animal responded to SP when it was co-administered with 10 μg of the antagonist. At 1.5 μg, the antagonist blocked the responses in half of the mice. A dose of 10 μg was not effective in depressing the tail flick and hot plate responses, traditional assays for detecting narcotic analgesics. At 30 μg, the antagonist did sometimes block these responses, but this dose also caused motor effects observed as hindlimb flaccidity.

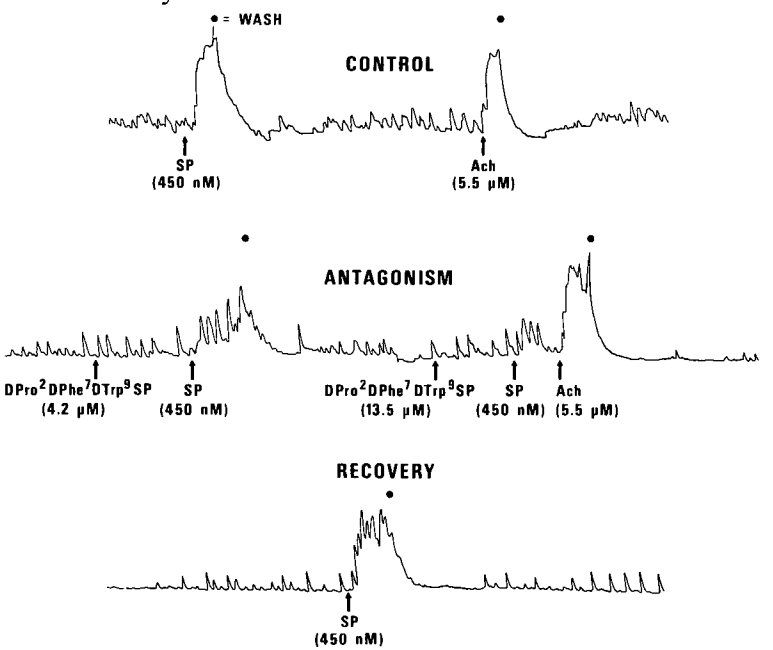

Fig. 1. Antagonism of substance P contraction of the isolated rat colon by DPro^{2}DPhe^{7}DTrp^9-SP. Ach = Acetylcholine.

Although the antagonist was not active on thermal analgesic assays, we wished to see if it would block responses to inflammatory pain. As shown in Figure 2, 10 μg of DPro^{2}DPhe^{7}DTrp^9-SP dramatically reduced capsaicin-induced foot-biting when injected intraspinally, but not intravenously. A similar antagonism of capsaicin back-scratching was also observed. These data provide the strongest support to date for SP's role as a spinal cord neurotransmitter mediating chemogenic inflammatory pain. Interestingly, chemogenic pain is thought to elicit the release of SP from nociceptor terminals in the skin;[8] this release evokes an "axon reflex" whereby inflammation spreads beyond the skin areas directly stimulated by the irritants. Thus, these data confirm the 1935 suggestion of Sir Henry Dale[9] that the mediator of the "axon reflex" should be the spinal cord nociceptor transmitter.

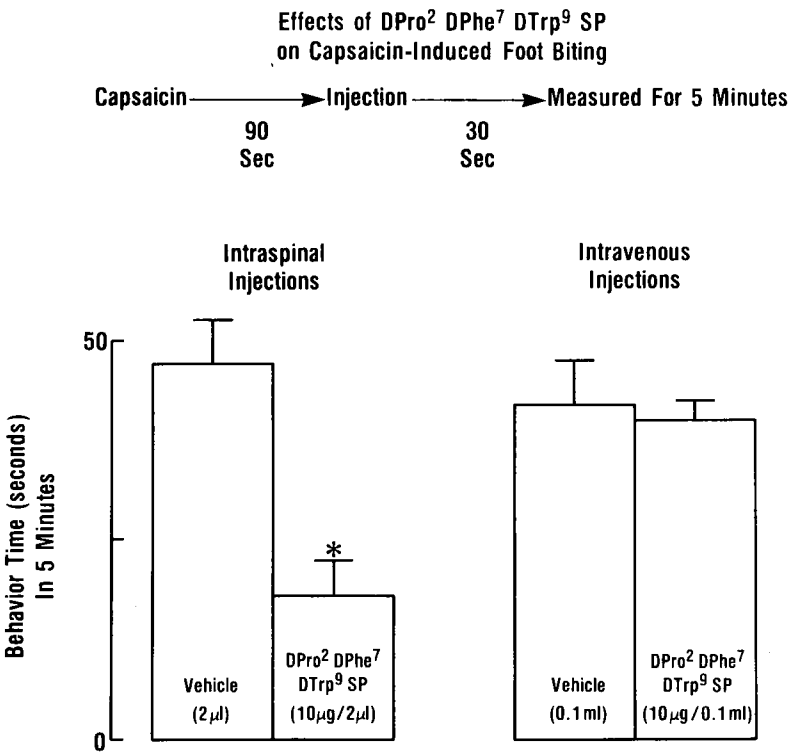

Fig. 2. Effects of DPro^{2}Phe^{7}DTrp^9-SP on capsaicin-induced foot biting. Asterisk denotes a value significantly different from control ($p < 0.05$, t test).

Conclusions

Substance P appears to be the neurotransmitter mediating chemogenic inflammatory pain. Furthermore, as previously suggested,[5] SP antagonists are likely to be novel analgesic agents. The reasons for the antagonist's lack of activity in acute thermal pain are not yet clear, but it could be because (a) the strength of the stimuli in acute pain tests are too high, (b) it was not possible to raise the antagonist doses to sufficient levels because of the untoward effects, or (c) the neural systems responsible for inflammatory pain are anatomically and/or neurochemically distinct from those responsible for thermal pain. Finally, the effects of $DPro^2DPhe^7DTrp^9$-SP on muscle movement suggests that spinal cord SP also plays a role in controlling motoneuron outflow, an observation consistent with the fact that a SP system distinct from the sensory one is known to exist in the ventral horn.[4]

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ENZYMATICALLY STABLE, PARTIALLY MODIFIED RETRO-INVERSO ANALOGS OF SUBSTANCE P — SYNTHESIS AND BIOLOGICAL ACTIVITY

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A hypotensive spasmogenic agent isolated from equine brain and intestinal extracts by von Euler and Gaddum[1] and named Substance P (SP) was found by Leeman and Hammerschlag to be identical with a potent sialogogic component isolated from bovine hypothalamic extract.[2] The sequence of the undecapeptide was found to be : H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2,[3] and later confirmed by synthesis.[4] The finding made by Pernow[5] that much higher concentration of SP are found in the dorsal or sensory root of spinal cord than in the ventral or motor root led Lembeck to postulate that SP is involved in the transmission of primary sensory neurons.[6] Carboxyl terminal fragments of SP such as SP_{6-11} and SP_{5-11} were found to retain full biological activity.[7] Otsuka and Konishi[8] reported that hexa- and heptapeptide analogs of C-terminal SP fragments [pGlu^6]SP_{6-11} and [pGlu^5]SP_{5-11}, respectively, are more potent than SP causing contraction of guinea pig ileum (GPI),[9] and depolarization of rat spinal motoneurons. The hexapeptide analog [pGlu^6]SP_{6-11} was also found to be as active as SP in releasing K^+ from rat parotid slices.[10]

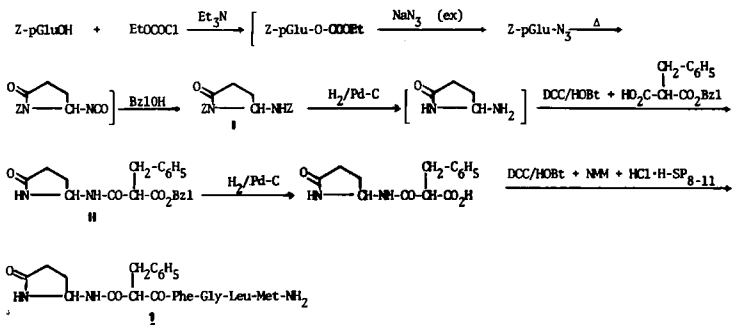
Friedman and Selinger have demonstrated that the transient release of K^+ from rat parotid slices stimulated by SP is due to inactivation of the peptide.[11] Various SP degrading enzymes have been studied.[12] Benuck and Marks[13] suggested that the peptide bonds undergoing cleavage by cytosolic enzyme isolated from rat brain are Gln^6-Phe^7, Phe^7-Phe^8 and Gly^9-Leu[10]. The first two peptide bonds were found also to be cleaved by neutral endopeptidase purified from bovine hypothalamus[14] and by a membrane bound purified enzyme from human brain.[12] The latter enzyme cleaved also the Phe^8-Gly^9 peptide bond.

Various routes were studied for the preparation of enzyme-resistant analogs of SP. We[10] as well as Sandberg et al.,[15] have prepared N-methylated agonists of SP and found some of them to be resistant to proteolytic degradation. In this paper we report an application of a novel topochemical approach suggested by Goodman and Chorev.[16] Following

this approach Chorev et al.[17] were able to prepare highly potent metabolically stable partially modified retro-inverso analogs (PMRI analogs) of enkephalin. We report here, the preparation and biological studies of PMRI analogs of [pGlu^6]SP$_{6-11}$ in which either the peptide bond pGlu^6-Phe^7 or the Phe^8-Gly^9 are reversed, leading to [gpGlu^6,(RS)-mPhe^7]SP$_{6-11}$ (1) and [pGlu^6, gPhe^8, mGly^9]SP$_{6-11}$ (2), respectively.

Synthesis

Most of the synthetic methods practiced in the preparation of retro-inverso analogs of peptides are summarized in a recent review.[18] The routes for the preparation of analogs (**1**) and (**2**) are outlined in Scheme 1 and 2, respectively. Peptides were synthesized in gram quantities by solution techniques. The excess carboxyl-carbonic mixed anhydride coupling technique was used for the formation of normal peptide bonds. In the preparation of analog **1**, the N-benzyloxycarbonyl-L-pyroglutamyl azide was obtained *via* reacting the appropriate mixed anhydride with excess sodium azide. The resultant intermediary isocyanate obtained *via* Curtius rearrangement was trapped by benzyl alcohol yielding the fully protected N,N′-*bis*-benzyloxacarbonyl 5-amino-2-pyrrolidone (I). Deprotection of I followed immediate coupling with benzyl (RS)-2-benzyl malonate gave the modified segment II (Scheme 1).

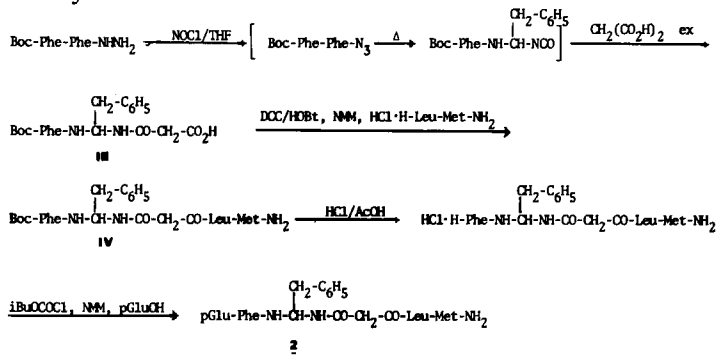


Scheme 1. Synthesis of PMRI analog of [pGlu^6]SP$_{6-11}$ in which the direction of the peptide bond pGlu^6-Phe^7 was reversed. In the resultant analog [gpGlu^6,(RS)-mPhe^7]SP$_{6-11}$ (1), the L-configuration of pGlu residue is retained during the rearrangement. The incorporation of (RS)-2-benzyl malonic acid residue results in a mixture of two diastereoisomers.

Another approach was employed in the preparation of analog **2**. The azide was obtained by nitrosation of the N-*t*-butyloxycarbonyl-L-phenylalanyl-L-phenylalanyl hydrazide with nitrosyl chloride. The corresponding isocyanate was trapped by excess of malonic acid yielding directly the carboxyl free N-protected fragment III. Coupling with H-

Leu-Met-NH_2 resulted in the modified pentapeptide IV from which analog **2** was obtained (Scheme 2).

Final products were purified by column chromatography on silicagel using chloroform-methanol mixtures as eluents. The purity of the compounds was examined by TLC, HPLC, microchemical analysis, amino acid analysis, and K^+ cationized field desorption mass spectrometry.[19]



Scheme 2. Synthesis of PMRI analog of [pGlu^6]SP$_{6-11}$ in which the direction of the peptide bond Phe^8-Gly^9 was reversed. In the resultant analog [pGlu^6, gPhe^8, mGly^9]SP$_{6-11}$ (2), the L-configuration of Phe^8 residue is retained during the rearrangement.

Biological Results and Discussion

The potencies of analogs **1** and **2** relative to the potency of the parent peptide [pGlu^6]SP$_{6-11}$ which was taken as 100% were studied by two *in-vitro* assays: The contraction of isolated guinea pig ileum (GPI)[20] and the K^+ release from rat parotid slices (RPS).[11] The latter assay may also furnish information on the proteolytic susceptibility of the peptides in the incubation media by measuring the rate of K^+ reuptake attributed to elimination of the peptides *via* proteolytic cleavage provided no desensitization occurs. Analog **1** had no agonistic nor antagonistic activity either in the GPI or in the RPS assays irrelevant of the concentrations employed ($3x10^{-7}$M to $1.5x10^{-5}$M). Analog **2** was a full agonist having 22% relative potency with ED_{50} of $9x10^{-10}$M in the GPI assay and 15% relative potency with ED_{50} of $2x10^{-7}$M in the RPS (ED_{50} for [pGlu^6]SP$_{6-11}$ was $2x10^{-10}$M in the GPI and $3x10^{-8}$M in the RPS assays). The stability of [pGlu^6]SP$_{6-11}$ and analogs **1** and **2** toward enzymatic cleavage was assessed by measuring the time course of degradation by several purified endopeptidases. Both analogs, **1** and **2**, resist degradation by pepsin whereas only analog **2** was also resistant to degradation by thermolysin, pronase, and α-chymotrypsin (Figure 1).

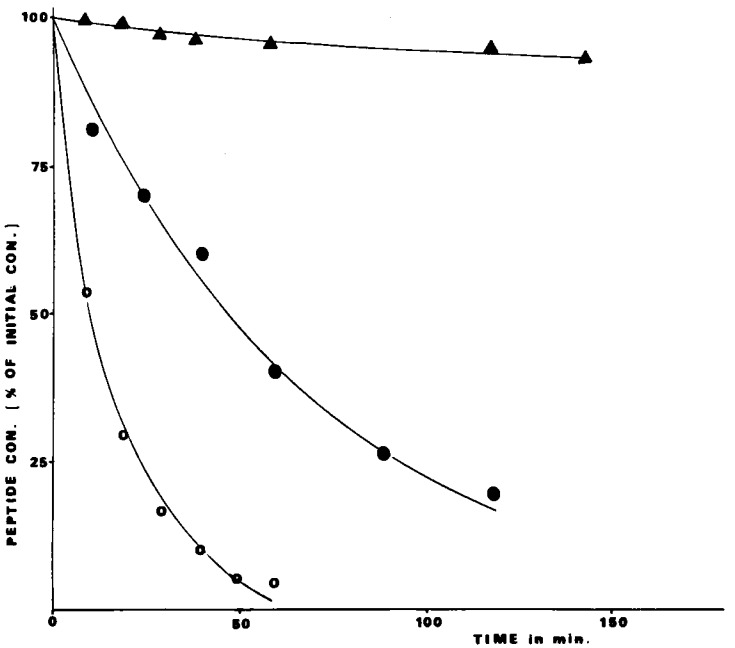


Fig. 1. Time course of digestion of [pGlu]SP_{6-11} O—O [gpGlu^6,(RS)-mPhe^7]SP_{6-11} ●—● and [pGlu^6, gPhe^8, mGly^9]SP_{6-11} ▲—▲ with α-chymotrypsin at 37°C. Peptide concentrations were obtained from peak heights of the nonhydrolyzed hexapeptide as measured by C_{18}-HPLC of the reaction mixture at various intervals of incubation.

The PMRI agonist [pGlu^6, gPhe^8, mGly^9]SP_{6-11} (2) was found to be highly resistant to proteolytic degradation. The modified residues Phe^8 and Gly^9 not only stabilize the bonds in which they are participating but also impose stability to peptide bonds away from the modified sequence. This long range effect must involve changes of the overall conformation, making it unappropriate for interaction with the active site of proteolytic enzymes. Interestingly enough, the retro-inverso modification of the N-terminal peptide bond pGlu^6-Phe^7 yielded an analog devoid of SP-like activity and partially susceptible to proteolytic degradation. We have reported similar results with an analog N-methylated at the same peptide bond, i.e., [pGlu^6, N-Me-L-Phe^7]SP_{6-11}.[10] It seems that the pGlu^6-Phe^7 peptide bond plays a very important role in the induction of biologically active conformation and cannot suffer any structural modification. On the other hand, stabilization of this peptide bond to proteolytic degradation either by N-methylation or retro-inversion does not confer further stability on other peptide bonds in the molecule.

Studies are carried on to assess the metabolic stability of analogs **1** and **2** toward membrane preparations from rat parotid and brain.

Acknowledgement

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SYNTHESIS AND BIOLOGICAL ACTIVITY OF SUBSTANCE P ANALOGS

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Introduction

Substance P (SP), discovered by von Euler and Gaddum,[1] has the sequence Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2.[2] It is widely distributed in vertebrates, exhibits a variety of biological actions,[3] most of which have been ascribed predominantly to the C-terminal part of the molecule, and is of special interest as a putative neurotransmitter. A limited number of SP-analogs have already been synthesized and their biological actions studied. The solution conformation of SP has been investigated.[4] So far little light has been thrown on the structure of the SP receptor(s).

This communication describes the first results from a structure-activity study of SP initiated recently in our laboratories. Early discouraging experiences with synthetic peptides related to the C-terminal, hydrophobic part of the molecule led us to prepare full-size analogs of SP. Because of the presence of the N-terminal, hydrophilic amino acids, the complete homologs are easier to handle during purification and testing.

Methods

Chemistry — All peptides were synthesized by the solid-phase method[5] using a benzhydrylamine resin to which either Boc-Met or Ppoc-Met was coupled using DCC. The Boc group was removed with 33% and the Ppoc group with 4% TFA, both in the presence of 2% dimethyl sulfide. Further elongation was accomplished exclusively with Ppoc-amino acids[6] and DCC. Side chain protection included NO_2 (Arg), Z (Lys) and Bzl (Glu). Glutamine was incorporated with or without Mbh-protection. Every amino acid was coupled twice. All syntheses were performed in a Beckman Model 990 Peptide Synthesizer. After the synthesis was completed, the peptides were cleaved from the support with HF in the presence of anisole. The crude products were purified by reverse-phase HPLC on C_{18} semipreparative, or more recently, prepara-

tive columns in systems containing 0.1 M triethylammonium formate, pH 3.0, and 25-35% ethanol. Amino acid analyses were performed after hydrolysis in HCl without phenol. With phenol present the methionine recovery was always low.

Pharmacology — To test smooth muscle activity, the longitudinal muscle of the guinea-pig ileum was used. A piece of the terminal part of the ileum was used after the 10 cm segment nearest the ileo-cecal junction had been discarded; the longitudinal muscle was gently peeled off and a strip 1.5-2 cm in length was mounted in a 2.5 ml organ bath containing a Krebs bicarbonate buffer. Contractions were recorded isotonically. Every preparation was tested with Substance P (Bachem, Bubendorf, Batch No. 10133) as standard. To avoid development of tachyphylaxis, the exposure time was minimized and peptide additions were appropriately spaced. The ED50 value for SP under these conditions was about 8×10^{-9} M. The activity of each analog was compared with that of standard SP.

Results and Discussion

Analogs **1-6** were prepared in attempts to change the conformation of SP in solution and/or its induced active conformation upon binding to the SP-receptor. In globular proteins, every amino acid with the exception of arginine has a significant preference for a particular type of secondary structure.[7] Assuming these data apply also for peptides, exchange of Leu-10 for Val would thus increase the β-sheet and decrease the α-helical potential of SP. Replacement of Gly-9 by Ala would preferentially induce helical conformation and replacement of Gln-6 by Pro should efficiently reduce both helical and sheet structure. Substitution of Gln-6 or Gln-5 by Glu favors helix formation

The smooth muscle activities of our analogs are presented in Table I. The significantly diminished activity of peptide **1** was unexpected and seems difficult to explain in terms of a change in hydrophobicity and is therefore presumably due to reduced ability to assume an active conformation. Peptide **2** was recently prepared by Fournier *et al.*[8] who also found it to have full activity. Peptides **3** and **5** are both modified in position 6 and have nearly full activity. The influence of modifications in this position in C-terminal fragments has recently been studied.[9,10] The activity of peptide **3** was higher than anticipated. Peptides **4** and **6** were modified outside the C-terminal region that is known to be essential for activity.[11] In some early assays peptide **6** behaved like a weak partial agonist, but more careful examination demonstrated that this was not the case.

Analogs **5-11** are all less basic than SP. As seen from Table I, two molecules of glutamic acid in positions 5 and 6 significantly lower the activity. Replacement of either arginine or lysine by a single glutamic acid residue does not seem to have much effect. Finally, the presence of glutamic acid instead of proline in position 2 or 4 also has little or no influence on smooth-muscle activity.

Table I. Smooth-muscle Activity of SP-analogs (SP = 1.0).

No.	Analog	Rel. activity
1	[Val^{10}]-SP	0.36
2	[Ala^{9}]-SP	1.2
3	[Pro^{6}]-SP	0.69
4	[Phe^{4}]-SP	0.74
5	[Glu^{6}]-SP	0.86
6	[Glu^{5}]-SP	1.0
7	[$Glu^{5,6}$]-SP	0.43
8	[Glu^{4}]-SP	0.78
9	[Glu^{3}]-SP	0.80
10	[Glu^{2}]-SP	0.90
11	[Glu^{1}]-SP	0.80

Acknowledgements

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EVALUATION OF THE BIOLOGICAL IMPORTANCE OF THE THREE PRIMARY AMIDES IN SUBSTANCE P HEPTAPEPTIDES 5-11

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Introduction

Substance P, (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2, SP), an undecapeptide which might act as neurotransmitter and/or tissue hormone contains three primary amide groups, one in position 5 (Gln), another in position 6 (Gln), and a third one in the C-terminal position (Met-NH_2). Because amide groups can interact in different ways with their environment (e.g. by hydrogen bonding to proton donors and -acceptors, or, due to the amphiphilic character, both with polar and non-polar groups) it was of interest to study the influence of these amides on receptor-hormone interactions.

For most biological tests the heptapeptide-sequence (5-11)SP is as active as SP itself or even more active; we therefore preferred to build up a series of heptapeptide analogs with modifications on the amide groups: -omission of the carboxamide, -alkylation, -extension and -replacement by esters or other polar groups.

Synthesis

The following fourteen analogs have been prepared by the stepwise solution method (see scheme).

Various alkylated analogs of Gln and Asn were prepared as already described,[1] Boc-Met(methyl-amide) and Boc-Met(dimethyl-amide) were synthesized from Boc-Met-OH and the corresponding amine by the mixed anhydride method with ethyl chloroformate. The protected peptides were purified by recrystallisation and deprotected with 1.75 N hydrochloric acid in glacial acetic acid, these peptide hydrochlorides were dried, redissolved, neutralized and subjected to a last purification by gel filtration or partition chromatography. Yields based upon the Boc-protected heptapeptides were 40-50%. The physical properties of the peptides are summarized in Tables I and II. All peptides had satisfactory amino acid and elemental analyses as Boc protected peptides and as free peptides.

Table I. Physical Constants of SP 5-11 Analogs.

Substitution of			Boc-Xxx-Yyy-Phe-Phe-Gly-Leu-Zzz						H-Xxx-Yyy-Phe-Phe-Gly-Leu-Zzz					
Xxx	Yyy	Zzz	MP*	α_D		Yield %	Rf A	Rf B	MP*	α_D		Rf A	Rf B	Rf C
Val	Gln	Met-NH_2	248-252	-34.4°	0.5% DMF	78	0.63	0.74	252-257	-193°	0.1% DMF	0.37	0.74	0.32
Ser	Gln	Met-NH_2	218-220	-43.5°	0.5% DMF	81	0.75	0.84	208-213	-29°	0.1% DMF	0.44	0.84	0.40
Thr	Gln	Met-NH_2	233-236	-36°	1% DMF	55	0.80	0.87	216-221	-22.3°	0.1% DMF	0.41	0.82	0.38
Glu(OCH_3)	Gln	Met-NH_2	246-249	-34.5°	1% DMF	63	0.86	0.93	238-242	-22.1°	0.1% DMF	0.44	0.78	0.39
Glu[$N(n\text{-}C_3H_7)_2$]	Gln	Met-NH_2	220-223	-36.4°	0.5% DMF	57	0.70	0.85	219-224	-21.7°	0.1% DMF	0.14	0.75	0.08
Glu[$N(i\text{-}C_3H_7)_2$]	Gln	Met-NH_2	223-226	-40°	0.5% DMF	68	0.83	0.93	211-216	-5°	0.1% DMF	0.11	0.70	0.07
Val	Val	Met-NH_2	248-254	-49°	0.5% DMF	62	0.85	0.91	253-258	-35.5°	0.1% DMF	0.57	0.85	0.52
Glu[$N(CH_3)_2$]	Glu[$N(CH_3)_2$]	Met-NH_2	206-209	-31°	0.5% DMF	79	0.65	0.75	212-216	-15°	0.1% DMF	0.31	0.64	0.22
Gln	Gln	Met-$N(CH_3)_2$	222-226	-17.2°	0.5% DMF	65	0.81	0.73	226-231	-27.7°	0.1% DMF	0.28	0.64	0.20
Gln	Gln	Met-$NHCH_3$	246-250	-29.4°	0.5% DMF	50	0.74	0.87	252-257	-24°	0.1% DMF	0.17	0.80	0.14
Gln	Gln	Cys(SC_2H_5)-NH_2	234-237	-39.2°	0.5% DMF	52	0.64	0.80	225-230	-34°	0.1% DMF	0.26	0.68	0.08

A: n-butanol/acetic acid/water = 4:1:1. B: n-butanol/acetic/water/pyridine = 30:6:24:20. C: n-butanol/acetic acid/water 4:1:5; upper phase.
*Decomposition.

Table II

No	Compound	In vivo BIOLOGICAL ACTIVITIES Mean arterial pressure (rat) R.B.P.	n	In vitro Guinea pig ileum α^E	pD_2	R.A.	n	AMINO ACID ANALYSIS	Glx	Phe	Gly	Leu	Met	No
0	hepta (5-11)-SP	100	97	1.0	8.77	100	108	-	-	-	-	-	-	0
1	[Val^5]-hepta (5-11)-SP	27	6	1.0	8.25	30	10	0.99 (Val)	0.98	2.09	1.00	1.00	0.91	1
2	[Ser^5]-hepta (5-11)-SP	40	6	1.0	8.62	71	8	0.92 (Ser)	0.95	2.18	1.00	1.00	0.85	2
3	[Thr^5]-hepta (5-11)-SP	55	6	1.0	8.20	27	9	0.84 (Thr)	0.86	2.30	1.00	1.05	0.83	3
4	[$Glu(OMe^5)$]-hepta (5-11)-SP	27	10	1.0	8.27	32	9	-	1.97	2.11	1.00	1.03	0.90	4
5	[($N^{5,5}$-dimethyl)-Gln^5]-hepta (5-11)-SP	30	10	1.0	8.38	41	11	-	-	-	-	-	-	5
6	[($N^{5,5}$-dipropyl)-Gln^5]-hepta (5-11)-SP	12	8	1.0	8.17	25	9	-	2.05	2.04	1.00	1.02	0.91	6
7	[($N^{5,5}$-diisopropyl)-Gln^5]-hepta (5-11)-SP	30	6	1.0	8.17	25	11	-	2.07	2.08	1.00	0.97	0.88	7
8	[($N^{4,4}$-dimethyl)-Asn^5]-hepta (5-11)-SP	68	4	1.0	8.32	36	6	-	-	-	-	-	-	8
9	[($N^{5,5}$-dimethyl)-Gln^6]-hepta (5-11)-SP	157	7	1.0	8.77	100	7	-	-	-	-	-	-	9
10	[Val^5],[Val^6]-hepta (5-11)-SP	27	8	1.0	8.49	52	9	1.87 (Val)	-	2.00	1.00	1.00	0.87	10
11	[(N^5,N^5-dimethyl)$Gln^{5,6}$]-hepta (5-11)-SP	210	6	1.0	8.47	50	9	-	2.08	1.98	1.00	1.01	0.93	11
12	[Met(methylamide)^{11}]-hepta (5-11)-SP	5	7	0.85	6.77	1	9	-	1.94	2.00	1.00	0.96	0.87	12
13	[Met(dimethylamide)^{11}]-hepta (5-11)-SP	0.2	4	-	-	<0.01	10	-	2.02	2.06	1.00	0.98	0.92	13
14	[(S-ethyl)Cys^{11}]-hepta (5-11)-SP	4	9	0.85	7.48	5	9	Present Cys(SC_2H_5)	1.98	2.04	1.00	0.97	-	14

R.B.P.: Relative blood pressure in % of (5-11)SP. R.A.: Relative affinity in % of (5-11)SP. α^E: Intrinsic activity. pD_2: Negative logarithm of a dose of agonist which causes half maximal contraction. n: Number of experiments.

Biological Activities

All peptides were tested *in vivo* on the blood pressure of anaesthetized rats and *in vitro* on strips of guinea pig ileum. Despite the fact that the effect of SP and its analogs on the guinea pig ileum is at least partially mediated by acetylcholine[2] this preparation is the most widely used *in vitro* bioassay for substance P. The biological activities, expressed by the pharmacological parameters, intrinsic activity α^E, pD_2, and relative affinity, are presented in Table 2.

Discussion

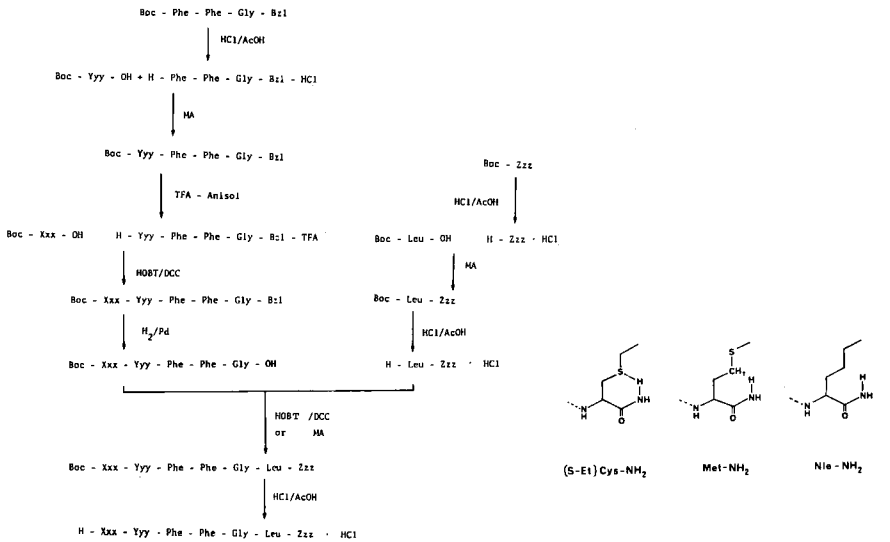
All modifications of the Gln residue in position 5 caused only minor changes of *in vivo* and *in vitro* activities; all analogs showed a certain decrease of affinity but no dramatic changes were observed. This clearly indicates that the sidechain amide in position 5 does not play a very important role: modifications which increase or decrease lipophilicity and/or size, only moderately reduce the hormone-receptor alignment.

The Gln-residue in position 6 appears, however, to be functionally more active than the residue in position 5. In the *in vitro* assay, the dimethyl-Gln analog showed full activity and the doubly substituted [$Val^{5,6}$](5-11)SP or [(N^4,N^4-dimethyl)$Gln^{5,6}$](5-11)SP had higher relative affinity than the peptides containing a single modification in position 5, namely [Val^5](5-11)SP and [(N^4,N^4-dimethyl)Gln^5](5-11)SP. In the *in vivo* assay, however, the dimethyl-Gln modifications are more potent than (5-11)SP itself. Due to the facts that (a) these modifications enhance the lipophilicity of the analog and that (b) the double substitution is even more potent (210% R.A.) than the single substitution in position 6 (157% R.A.), and (c) that the *in vitro* assay gives an opposite trend for the double substitution, it may be assumed that reduced metabolism of the hormone together with lipophilic interaction at the receptor level is responsible for the enhanced potency of these analogs *in vivo*[3].

The last three analogs were modified at the C-terminal Met-amide. The dramatic loss of activity deriving from methylation of the COOH suggests the importance of the amide and especially the amide protons for biological activity. It appears that both amide protons are essential, because the monomethylated analogs show already in both bioassays strongly reduced relative affinity.

However, the low activity of the analog with (S-ethyl)Cys was rather surprising, the sulfur of the Met-side chain has changed place with the γ-methylene group, an exchange that should not have a serious effect because the replacement of the sulfur with a methylene produces Nle^{11}-SP, a quite active analog. A possible explanation for this finding is the

Scheme



favored interaction of the amide with the sulfur in (S-ethyl)Cys analog but not with Met and Nle (cf. Figure) in the respective peptides. Such a proposed intramolecular hydrogen bond would disturb the necessary interaction of the amide hydrogens for ideal hormone-receptor alignment. This hypothesis is actually under investigation. The detailed analysis of the C-terminal amide is presented in the communication that follows.

Conclusion

The amides on the sidechains of the Gln-residues in position 5 and 6 of SP do not participate in an important way in the hormone-receptor interaction. The C-terminal amide, however, is of importance for hormone binding and deserves further investigation.

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THE DISCOVERY AND PHARMACOLOGICAL CHARACTERIZATION OF ENKEPHALIN-DERIVED OPIOID RECEPTOR ANTAGONISTS

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Introduction

Although the structural requirements for inducing antagonist character into morphine-derived opiates are well established,[1,2] incorporation of these features into enkephalin-derived structures does not result in the generation of analogs with demonstrable antagonist character.[3] Incorporation of a typical antagonist pharmacophore — an N-cyclopropylmethyl (Cpm) group — onto the tyrosine of a number of enkephalin analogs results simply in loss of potency: typically N-Cpm-Tyr-D-Ala-Gly-MePhe-$NHCH_2CH_2CHMe_2$ is a pure agonist with 1/100 the potency of the N-methyltyrosyl analog.

Alternative structural substituents were investigated as a means of inducing antagonist character. An antagonist need not be restricted to interaction with what may be defined as the 'agonist receptor' — it may also interact with complementary structural features adjacent to the agonist receptor. Antagonist "activity", therefore, may be viewed as a reflection of the ability of a molecule to compete with the agonist in *binding to the receptor environment.* Thus, it was perceived that antagonist character might be observed in enkephalin analogs bearing additional groupings which would be capable of modifying the interaction of structural features necessary for agonist activity.

Methods

Chemistry — The analogs were synthesized by solution methods utilizing a strategy similar to that described previously.[3,4] A typical synthesis is shown in Figure 1. The C-terminal amines were synthesized by reaction of a suitable amine and acid chloride followed by reduction of the resulting amide with diborane. Mixed anhydride couplings were carried out using pivaloyl chloride and N-methylmorpholine, however, in certain cases where the amino component contained bulky N-alkyl groups, the diphenylphosphinyl chloride procedure[5] was found to be

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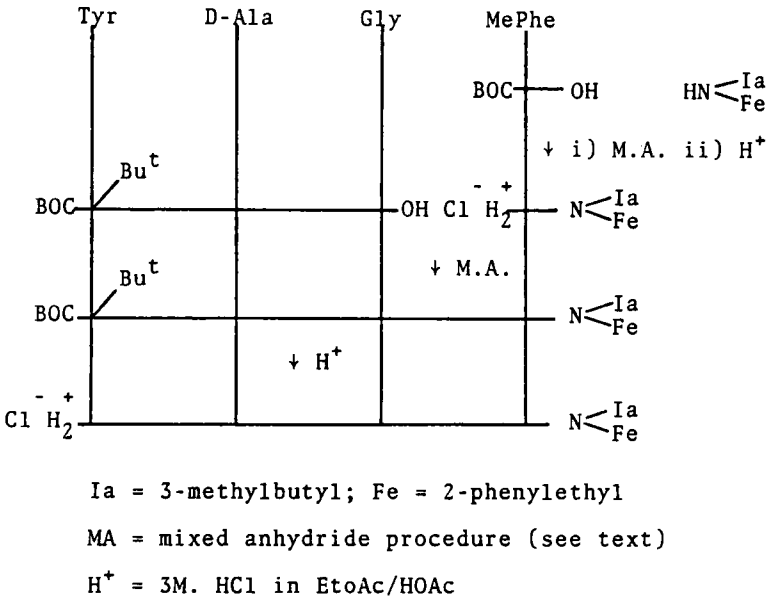


Ia = 3-methylbutyl; Fe = 2-phenylethyl

MA = mixed anhydride procedure (see text)

H^+ = 3M. HCl in EtoAc/HOAc

Fig. 1. Synthesis of Tyrosyl-D-alanylglycyl-N-methylphenylalanine-(3-methylbutyl-2'-phenylethyl)amide.

superior. The deprotected peptides were purified by absorption chromatography on silica gel followed by ion-exchange chromatography on carboxymethylcellulose using gradient elution with aqueous pyridine acetate buffers. The purified peptides were homogenous on thin-layer chromatography in three solvent systems and showed compatible NMR spectra and amino acid analysis data: analogs were lyophilized from 0.1N HCl and then water prior to biological testing.

Biology: Mouse vas deferens(mvd) — Opioid agonist potencies were determined using the method of Hughes *et al.*[6] with the modification that a 50 ml organ bath was used and that cumulative dose response curves were constructed. Agonist potencies were calculated relative to Met[5]-enkephalin (Met[5]-E). Ke values, that is, the concentration of antagonist in the presence of which the ID_{50} dose of agonist is doubled, were calculated by the formula employed by Kosterlitz and Watt.[7]

Guinea-pig ileum(gpi) — Opioid agonist potencies were determined by using the method of Kosterlitz and Watt[7] with the modification that dose response curves were obtained using a cumulative dosing method. Agonist potencies were calculated relative to Met[5]-E.

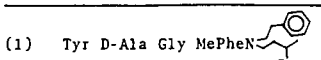
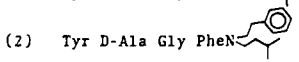
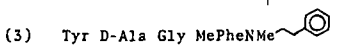
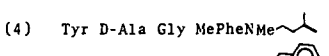
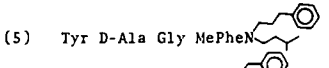
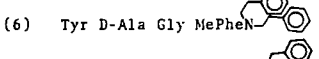
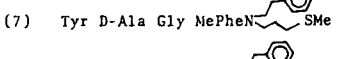
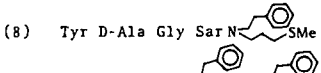
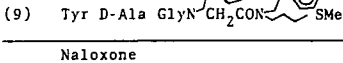
Receptor binding assays — The ability of compounds to displace ^{3}H-naloxone from whole rat brain (less cerebellum) homogenates was

investigated using the method of Pert and Snyder[8] with the modification that ice cooling after equilibration was omitted.[9] IC_{50} were values determined by log probit analysis and K_i values obtained from the relationship: $IC_{50} = K_i (K_D + S) / K_D$, where K_D is the affinity constant and S the concentration of tritiated ligand.

Results and Discussion

During the course of a program to synthesize enkephalin tetrapeptideamides it was discovered that tyrosyl-D-alanylglycyl-N-methylphenylalanyl-(3-methylthiopropyl)-(2-phenylethyl)amide (**7**, Table 1) did not exhibit measurable agonist effects in either the gpi or

Table I. Pharmacology of Analogs *in vitro*.

		mouse vas deferens			guinea-pig ileum
			antagonist		
		agonist*	vs $Met^5E^\dagger$	vs $NM^\dagger$	agonist*
(1)	Tyr D-Ala Gly MePheN 	< 0.02	566	44	< 0.1
(2)	Tyr D-Ala Gly PheN 	0.5	--	--	92
(3)	Tyr D-Ala Gly MePheNMe 	< 0.001	2530	725	~ 1
(4)	Tyr D-Ala Gly MePheNMe 	3	--	--	65
(5)	Tyr D-Ala Gly MePheN 	0.01	--	--	4
(6)	Tyr D-Ala Gly MePheN 	< 0.005	1110	83	< 0.1
(7)	Tyr D-Ala Gly MePheN 	< 0.01	724	52	< 0.1
(8)	Tyr D-Ala Gly Sar N 	< 0.01	16000	2700	< 0.1
(9)	Tyr D-Ala GlyN 	< 0.1	1500	163	~ 10
	Naloxone	< 0.01	10.05 ±2.3(6)	0.51 ±.36(8)	< 0.1

* Potency relative to Met^5-E= 100. † Ke (nm)

mvd assays, but blocked the effect of an ID_{50} dose of Met^5-E in the mvd. Its ability to antagonize the effect of Met^5-E in the mvd is shown in Figure 2. The agonist potencies and Ke values for **1** and a related series of analogs are shown in Table 1. Antagonist activity is restricted to a relatively limited range of structures; both the Phe^4 nitrogen and the C-terminal amide nitrogen must be fully substituted and the latter must bear a 2-phenylethyl group. Removal of the Phe^4 N-methyl as in (**2**), gives a pure agonist. Substitution of the phenylethyl group by methyl (**4**) gives a pure agonist as does increasing the chain length to 3-phenylpropyl (**5**).

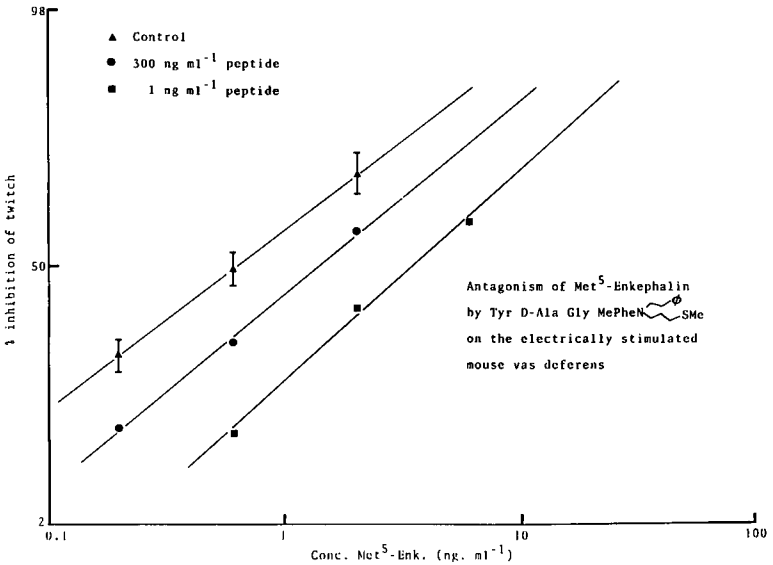
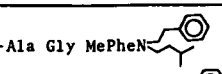
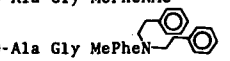
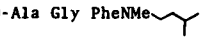
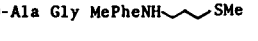


Fig. 2. The antagonistic effect of Tyr-D-Ala-Gly-MePheN($CH_2CH_2CH_2SMe$)CH_2CH_2-C_6H_5on Met^5-Enkephalin on the electrically stimulated mouse vas deferens.

Greater structural flexibility is tolerated in the C-terminal alkyl group: isoamyl (**1**) 3-methylthiopropyl (**7**) phenylethyl (**6**) substitution at this position yield essentially pure antagonists of approximately equal activity, however, the methyl analog (**3**) at this position is a weak agonist in the gpi and a weak antagonist in the mvd. As in the agonist series, the Phe^4 side chain is necessary for receptor interaction as the Sar^4 analog (**8**) has poor affinity: however, attachment of the "side chain" phenylalkyl to the nitrogen rather than the α-carbon yields (**9**), a compound with activity both as an agonist and an antagonist. It is of interest to note that the pure antagonists (**1**), (**6**) and (**7**) all reverse the effects of morphine (a μ-selective agonist) at least ten times more easily than they reverse the effects of Met^5-E (a δ-selective agonist). Therefore, they may be defined as μ-selective antagonists: in this respect their selectivity appears similar to that of naloxone.[10]

Sodium ion has been found to diminish the binding of "alkaloid" opiate agonists, but to have little effect on antagonist binding.[8] It was of interest to determine whether this finding extended into peptide-derived antagonists. From Table 2 it may be seen that in the presence of sodium ion, peptides which show either agonist (**10, 11**) or antagonist (**1, 6, 7**) character on isolated tissues have similar K_i values; however, in the absence of sodium ion only agonist binding increased, yielding 'sodium

Table II. Binding Assay Data: Displacement of ^{3}H-Naloxone.

	Ki (nM.)		
	Absence Na^+	100 nM Na^+	Na index
(1) Tyr D-Ala Gly MePheN 	42.0	31.3	0.74
(3) Tyr D-Ala Gly MePheNMe 	6.17	57.6	9.34
(6) Tyr D-Ala Gly MePheN 	74.1	44.4	0.60
(7) Tyr D-Ala Gly MePheN 	11.1	21.4	1.93
(10) Tyr D-Ala Gly MePheNH 	0.91	30.5	33.5
(11) Tyr D-Ala Gly PheNMe 	0.58	22.6	39.0
(12) Tyr D-Ala Gly MePheNH 	0.51	18.9	37.1
Naloxone	1.03	0.99	0.96
Morphine	1.52	36.2	23.8

indices' of 30-40 for the peptide pure agonists and indices of ~1 for peptide pure antagonists. The peptide partial agonist (**3**) showed an intermediate sodium index (~ 10).

Conclusions

We have shown that novel structural substitutions in a series of enkephalin analogs have resulted in the discovery of the first examples of peptide opiate receptor antagonists. These antagonists have been characterized *in vitro* as selective for the μ-class of opiate receptors. Their sodium indices support and extend the hypothesis that sodium ion selectively diminishes the binding of opiate agonists.

It is hoped that further work in this and related series will lead to the discovery of δ-selective antagonists: the availability of such compounds will be an invaluable aid to the pharmacological and physiological characterization of multiple opioid receptors.

Acknowledgements

It is a pleasure to acknowledge the contributions of Mr. S. Downing, Ms. D.M. Richards, Ms. S.J. Stacey, and Ms. A. Wilkes in this study.

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CHIRAL AMIDE BOND SURROGATES IN LH-RH AND LEUCINE ENKEPHALIN ANALOGS

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Introduction

Among the peptide backbone modifications, 1) amide bond replacements, 2) changes in the α-carbon or 3) cyclic derivatives, the D-amino acids have been most popular by virtue of their relatively free access and because of accompanying conformational changes that often lead to improved activity beyond that expected from resistance to enzymatic biodegradation alone. Since amide bond replacements of the $\psi[CH_2S]$ variety gave derivatives of LH-RH with high *in vitro* potencies,[1] a new program was initiated to introduce chirality within the amide bond framework and to explore for the first time the biological effects of varying these new asymmetric centers. Analogs of both luteinizing hormone releasing hormone antagonists and leucine enkephalin have been examined.

Experimental

Our synthetic strategy has involved the preparation of dipeptide analogs containing the $CHCH_3S$ unit as an amide bond replacement (our proposed nomenclature for such structures designates the term Glyψ[$CHCH_3S$]Gly as the dipeptide analog of Gly-Gly containing the amide replacement $CHCH_3S$ for the normal -CONH- moiety).[2] Following preparation of the various pseudodipeptides as their N-Boc or N-Cbz derivatives, they were incorporated into the requisite LH-RH or enkephalin structures by traditional SPPS or solution methods. In general, the sulfoxide derivatives $\psi[CH_2SO]$ and $\psi[CHCH_3SO]$, which also contain a new asymmetric center by virtue of the configurational stability of sulfoxides, were prepared by peroxide oxidation following purification of their sulfide precursors. The resulting diastereomers were readily identifiable and separable by semi-preparative HPLC as seen in Table I.

Using the commercially available 1-amino-2-propanol and following the basic strategy described earlier,[3] analogs of the Glyψ[$CHCH_3S$]X variety were prepared.

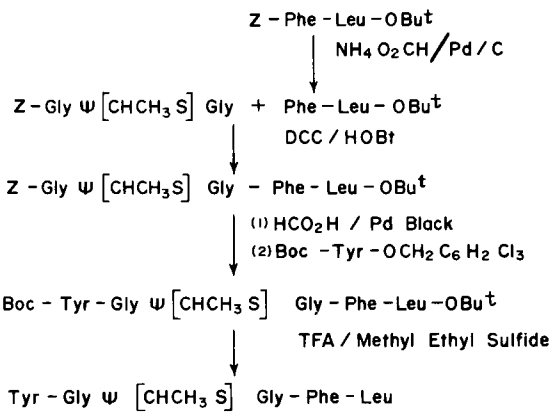
The solution synthesis of Glyψ[$CHCH_3S$]Gly$^{2-3}$] Leu-enkephalin (Scheme I) involved the use of catalytic transfer hydrogenation for

Table I. HPLC Parameters for $\psi[CHCH_3S]$ and $\psi[CHCH_3SO]$ Diastereomeric Analogs.

Peptide	Chiral Center	k' Isomer I	k' Isomer II	System
LH-RH Antagonist	$\overset{*}{C}HCH_3S^{1-2}$	6.3	6.6	A
LH-RH Antagonist	$\overset{*}{C}HCH_3\overset{*}{S}O^{1-2}$	4.2	5.5	A
Leu-Enkephalin	$\overset{*}{C}HCH_3S^{2-3}$	6.8	8.1	B

Solvent Systems: A-0.25 M TEAP/Acetonitrile; pH 3.5; 25-45% gradient (20 min); B - 0.25 M NH_4OAc/CH_3OH; pH 4.1; 30-40% gradient (20 min).

removal of Cbz groups. Note that this deprotection was successful in spite of the presence of the "poisoning" sulfide linkage in the protected tetrapeptide.



Scheme I. The solution synthesis of [Glyψ[$CHCH_3S$]Gly^{2-3}] Leu-enkephalin.

Results and Discussion

Among chiral amide bond-containing peptides prepared in this study, those with a $\psi[CHCH_3S]$ replacement include [Glyψ[$CHCH_3S$] Gly^{2-3}] Leu-enkephalin and the LH-RH antagonist [Ac-Glyψ[$CHCH_3S$]D-*p*ClPhe^{1-2}, D-$Trp^{3,6}$]LH-RH (based on a highly potent amide counterpart).[4] Both of these pairs of diastereomers were baseline separated by HPLC (k'values for these and one sulfoxide pair are given in Table I). At this time the absolute configurations of the new asymmetric centers remain unknown.

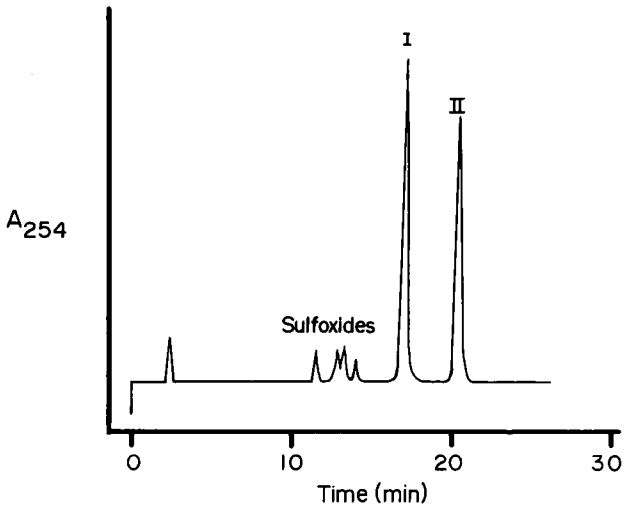


Fig. 1. HPLC of [GLYψ[CHCH$_3$S]Gly$^{2-3}$] Leu-enkephalin and sulfoxides

During solution synthesis of Glyψ[CHCH$_3$S]Gly$^{2-3}$] Leu-enkephalin, partial oxidation to the sulfoxides apparently occurred. Deliberate oxidation of the two Glyψ[CHCH$_3$S]Gly product peaks with H_2O_2 resulted in the enhancement of the four HPLC peaks labelled "sulfoxide" in Figure 1. These were later identified as the new pairs of Glyψ[CHCH$_3$-SO]Gly derivatives by separately oxidizng the two Glyψ[CHCH$_3$S]Gly-containing enkephalin diastereomers.

The purified LH-RH antagonist diastereoisomers were subjected to both *in vitro* and *in vivo* tests, while the enkephalins were examined for their ability to inhibit the naloxone-reversible electrically stimulated contraction of a guinea pig ileum strip.

Table II. Biological Potencies of Peptides Containing Chiral Amide Bond Replacements.

	LH-RH Antagonists			Leu-Enkephalin Analogs
	[Ac-Glyψ[CHCH$_3$S]D-pClPhe$^{1-2}$,D-Trp^{3,6}]			[Glyψ[CHCH$_3$S]Gly$^{2-3}$]
	In vivo Rat AOA[a]	In vitro Pituitary[b]	In vivo IV Rat[c]	Guinea Pig Ileum[d]
I	5/10	88	7.6	weak antagonist
II	7/10	72	10.5	10%

a) Rat antiovulatory assay at 250 μg; control gave 10/10 rats ovulating; b) potency relative to [Ac-Gly^1, D-*p*ClPhe^2, D-Trp$^{3-6}$] LH-RH = 100; c) LH release (ng/ml serum) in immature female rats following 0.1 μg LH-RH and 10 μg antagonist by IV injection; control = 26.8 ng/ml; d) contraction of electrically stimulated guinea pig ileum strip; relative to Leu-enkephalin agonist activity; reversible by naloxone.

In each case, variations in activity were observed, apparently due to differential conformational or receptor-active interactions for each of the isomers. The more lipophilic Leu-enkephalin isomer II (by HPLC) was not readily soluble in the buffer medium and yet proved reasonably potent in the GPI assay. Leu-enkephalin isomer I on the other hand was quite water-soluble and yet was inactive or possibly aweak antagonist in the same assay. The differences between isomers in the LH-RH antagonists were smaller, but were apparent in both *in vitro* and *in vivo* results.

Conclusions

The 1-2 LH-RH and 2-3 leucine enkephalin amide bonds are not essential for eliciting their respective antiovulatory or GPI activities. The introduction of chirality within the normal amide bond site may lead to changes in the bioactivity profiles of the resulting diastereomers. Chiral amide bond surrogates may thus provide advantageous conformation-altering properties while serving as impediments to biodegradation.

Acknowledgements

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STRUCTURE-ACTIVITY STUDIES OF ENKEPHALIN TETRAPEPTIDES

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Introduction

In the enkephalin series, Tyr-D-Ala-Gly-Phe-NH_2 contains the minimal structural requirement necessary for potent opiate-like analgesia. We have synthesized a number of tetrapeptide enkephalin analogs. These analogs were modified by (1) substitution of various alkyl groups on the nitrogen of phenylalanine, (2) substitution of fluoro or phenyl at the *para* position of the aromatic ring of phenylalanine, and (3) substitution of various alkyl groups on the nitrogen of tyrosine. The pharmacological activities of these analogs were investigated *in vitro* in the mouse vas deferens and *in vivo* in the mouse hot plate-jump test systems.[1]

Experimental

The synthesis of N-alkyl phenylalanines was accomplished by use of either of two routes (Figure 1). In the first route, the dianion was generated from Boc-Phe by treatment with potassium hydride in the presence of a catalytic amount of 18-crown-6 and then treated with the appropriate alkyl iodide.[2-4] The second method involved the alkylation of phenylalanine amide. This reaction generally gave a mixture of mono- and di-alkylated products. The desired mono substituted compound was isolated in pure form after silica gel chromatography. The peptides were prepared by classical solution-phase peptide chemistry (Figure 2) and purified by reversed-phase liquid chromatography.[5]

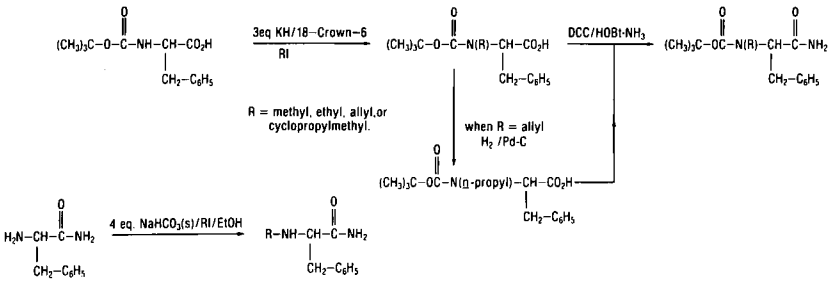


Fig. 1. Synthesis of N-alkylphenylalanines.
R = octyl, fluoroethyl, propargyl, dimethylallyl, ethylthiomethyl, or methyl acetate.

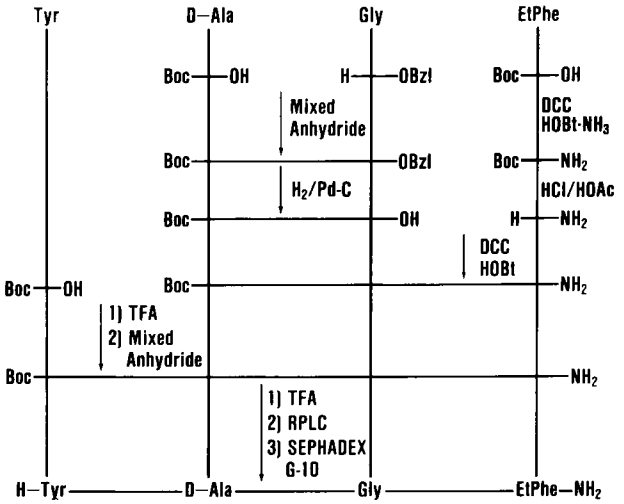


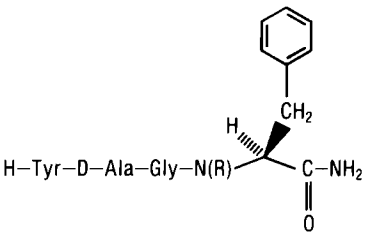
Fig. 2. Representative synthesis of enkephalin tetrapeptides.

Discussion

The structure-activity relationship studies have indicated that improvements in analgesic efficacy can be achieved by alkylation of the nitrogen of phenylalanine in enkephalin tetrapeptides. (Table I). Only two substitutions, *n*-octyl and methylacetyl, resulted in analogs possessing decreased analgesic potency when compared to H-Tyr-D-Ala-Gly-Phe-NH_2. The improvement in analgesic potency reaches a maximum with the N-ethyl substitution, which is 150 times more potent than the corresponding N-methyl analog. Analgesic potency diminishes with the larger, more lipophilic substituents.

In addition, the following general observations were made concerning analagesic efficacy: (1) substitution of a methyl group on the amino terminus of tyrosine results in improved oral activity (see also reference 6), (2) substitution of fluorine at the *para* position on the phenylalanine residue results in an increase in subcutaneous activity,[7] and (3) substitution of an oxygen for the nitrogen of phenylalanine or placement of a phenyl group at the *para* position of phenylalanine results in a dramatic decrease in all opiate-like effects (Tables II and III).

Table 1. Pharmacology of Enkephalin Tetrapeptides Containing N-Alkyl Phenylalanines.



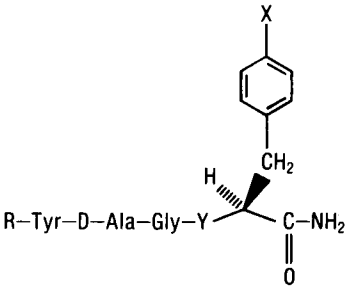
R	Mouse Vas Deferens IC_{50} (nM)	Mouse Hot Plate — Jump ED_{50} (mg/kg — s.c.)
H	190	0.85
CH_3	120	0.47
$CH_2–CH_3$	2.1	0.003
$CH_2–CH_2–CH_3$	11.0	0.15
$CH_2–(CH_2)_6–CH_3$	26.7	11.70
$CH_2–CH = CH_2$	8.50	0.01
CH_2 	1.62	0.012
$CH_2–C \equiv CH$	5.32	0.08
$CH_2–CH = C(CH_3)_2$	6.47	0.4
$CH_2–CH_2–S–CH_3$	4.25	0.05
$CH_2–CO_2–CH_3$	207	1.4
$CH_2–CH_2–OH$	50	0.33
$CH_2–CH_2–F$	3.86	0.013

Table II. SAR of N-Alkyl Tyrosine Enkephalin Tetrapeptides.

$R(R')–Tyr–D–Ala–Gly–EtPhe–NH_2$

R	R'	Mouse Vas Deferens IC_{50} (nM)	Mouse Hot Plate — Jump ED_{50} (mg/kg) s.c.	p.o.
H	H	2.1	0.003	26.
CH_3	H	17.0	0.021	4.0
CH_3	CH_3	363	1.7	NT
CH_2CH_3	H	89.6	1.44	145

Table III. Pharmacology of Enkephalin Tetrapeptides.



R	Y	X	Mouse Vas Deferens IC_{50} (nM)	Mouse Hot Plate — Jump ED_{50} (mg/kg) s.c.	p.o.
H	O	H	359	>30	>>30
H	NH	C_6H_5	150	30.0	>>30
H	NH	H	190	0.85	>30
CH_3	$N(CH_2CH_3)$	F	1.87	0.012	3.55
H	$N(CH_2CH_3)$	H	2.1	0.003	26.0
H	$N(CH_2CH_3)$	F	2.4	0.00018	19.0

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STRUCTURE-ACTIVITY RELATIONSHIPS OF ENKEPHALIN ANALOGS

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Introduction

The enkephalin analog H-Tyr-D-Ala-Gly-Phe-MeMet-NH_2 (LY127623, metkephamid) is a minimally modified analog of methionine enkephalin that possesses high analgesic efficacy and low physical dependence producing potential.[1]

Metkephamid is currently undergoing clinical trials as an analgesic agent. Preliminary data indicate that metkephamid (i.m.) produces analgesia in man.[1,2] We now report the *in vitro* (mouse vas deferens) and *in vivo* (mouse hot plate) opiate-like activities of structural analogs of metkephamid as well as other potent enkephalin analogs.

Materials and Methods

Peptides were synthesized either by solid-phase peptide synthesis on a benzhydrylamine resin using a Beckman 990 automated peptide synthesizer or by classical techniques using DCC/HOBt or mixed anhydride couplings in solution. Ester bonds were formed with carbonyldiimidazole. Amino acids that were obtained in racemic form were incorporated in the peptide without resolution. The resultant pair of diastereomeric peptides were then separated and purified by reversed-phase liquid chromatography (RPLC) followed by Sephadex G-10 chromatography.[3] Purified peptides were characterized by elemental analysis, amino acid analysis, optical rotation at two wavelengths, analytical RPLC, and tlc in four solvent systems. The peptides were tested *in vitro* on single vas deferens from mature mice (Cox, 30 to 40 g). The hot plate test for analgesia was conducted using an apparatus with an electrically heated, thermostatically controlled (52°C) metal plate. The time from contact with the plate until an escape jump occurred was recorded as a response latency.

Results and Discussion

Twenty-three analogs of metkephamid are reported in which changes have been made at the second, third, fourth, or fifth residues (Table I).

Table I: Opiate-like Activities of Metkephamid Analogs

Compound*	Mouse Vas Deferens IC_{50} (nM)	Mouse Hot Plate-Jump ED_{50} (mg/kg-s.c.)
H - Tyr - D-Ala - Gly - Phe - MeMet - NH_2	12.2	0.363
H - D-Alg - NH_2	5.8	1.21
H - L-Alg - NH_2	290	>9
H - D-Ser - NH_2	5.0	1.7
H - D-Thr - NH_2	1.2	0.30
H - D-Met - NH_2	–	2.0
H - D-Ala - β-Ala - NH_2	>10,000	>30
H - γ-Abu - NH_2	>10,000	>30
H - δ-Ape - NH_2	>10,000	>30
H - ϵ-Ahx - NH_2	>10,000	>30
H - Gly - Phe(F) - NH_2	0.77	0.022
H - Phe(Cl) - NH_2	2.2	0.67
H - D-Phe(Cl) - NH_2	180	>30
H - Phe(Br) - NH_2	2.2	1.6
H - Phe(I) - NH_2	13	>30
H - Phe(CF_3) - NH_2	1.5	0.43
H - Phe(NO_2) - NH_2	0.64	0.16
H - Phe(OH) - NH_2	380	>10
H - β-MePhe - NH_2	31	1.4
H - D-β-MePhe - NH_2	1,500	–
H - Cha - NH_2	410	49
H - hPhe - NH_2	>1,000	>30
H - Phe - OH	0.97	1.25
(H - $NH\text{-}CH_2\text{-}CH_2)_2$	0.71	9.8

*Abbreviations: Alg = 2-amino-4-pentenoic acid, δ-Ape = δ-aminopentanoic acid, ϵ-Ahx = ϵ-aminohexanoic acid, Phe(F) = *para* fluorophenylalanine, Cha = cyclohexylalanine, hPhe = homophenylalanine.

Substitutions at the second position with a D-amino acid residue result in an increase in vas deferens activity; however, only [D-Thr^2]-metkephamid gives an analgesic potency equal to or greater than the standard D-Ala substitution. This emphasizes the importance of *in vivo* assay systems to confirm efficacy improvements measured in the *in vitro* systems. Addition of one, two, three, or four methylene units to the backbone of metkephamid at the third position virtually eliminates opiate-like effects. The fourth residue has proven to be the most amenable to structural manipulation. Substitution on the aromatic ring of phenylalanine at the para position generally results in an analog with increased *in vitro* activity; however, only the fluorine substitution results in a corresponding significant increase in analgesic potency. This improvement in analgesic potency has proven general for all enkephalin analogs tested to date.[4]

A dimer of metkephamid has been synthesized using a diamino butyl group to link two metkephamid moieties at the C-terminus. While the analog is 17 times more potent than metkephamid in the *in vitro* test system, it is 25 times less potent when tested *in vivo*. These facts suggest that the dimer possesses a decreased permeability to the blood-brain barrier.

Enkephalin analogs containing ester bonds instead of amide bonds (enkephalin depsipeptides) have been synthesized (Table II). Replacement of the amide bond between the second and third residues of metkephamid with an ester bond resulted in virtually complete loss (<0.2%) of opiate-like activities. The metkephamid analog containing *S*-α-hydroxy-β-phenylpropionic acid (HyF) instead of the phenylalanine residue at position four possessed 7% of the *in vitro* activity and 1% of the *in vivo* activity of metkephamid.

Table II. Opiate-Like Activities of Enkephalin Depsipeptides

Compound[a]	Mouse Vas Deferens IC_{50} (nM)	Mouse Hot Plate-Jump ED_{50} (mg/kg–s.c.)
H · Tyr · D-Ala · Gly · Phe · Met · NH_2	8.8	>10
H — HyM — NH_2	58	4.1
H — Leu — NH_2	15	>10
H — HyL — NH_2	31	4.3
H — HyF — NH_2	–	>30
H — Phe — Phe — NH_2	76	ca 9
H — HyF — NH_2	270	4.3
H — MeMet — NH_2	12.2	0.363
H — HyF — NH_2	169	40
H — HyG — Phe — NH_2	9,400	>30
H — HyG — Gly — NH_2[b]	–	–
H — D,L-HyA — NH_2[b]	–	–
H — D-Ala — OH	410	7.0
H — OH	10,000	>30

[a]α-hydroxyacids are abbreviated using Hy(hydroxyl) plus the one letter symbol for the corresponding α-aminoacid, *eg*/ HyG = HO-CH_2-CO_2H [b]Compound unstable to purification procedures.

Substitution of an ester bond for the amide bond between the fourth and fifth residues resulted in analogs with a 2-7 fold decrease in the *in vitro* activity; however, these depsipeptides exhibited increases in analgesic potency when compared to the corresponding peptides. Although it was established that H-Tyr-D-Ala-Gly-Phe-HyM-NH_2 was stable at pH 7.4 for at least 24 hours, the extent to which the increased analgesic potency of this and similar depsipeptides is due to esterase induced hydrolysis to the tetrapeptide acid has not been established. Finally, substitution of either HyG for Gly or HyA for Ala at the second position of metkephamid gave depsipeptides that were unstable to the purification procedures utilized and, therefore, could not be tested for opiate-like activities.

A series of enkephalin analogs with very potent opiate-like activities has been synthesized which contain a phenylglycine residue at the fifth position[5] (Table III). This substitution results in enkephalin analogs that are one to two orders of magnitude more potent as analgesic agents than corresponding enkephalin analogs substituted with amino acids such as Phe, D-Phe, D-Tyr, Met, or Leu. Further improvements in analgesic and vas deferens efficacy are achieved with [MeTyr[1]], [Phe(F)[4]], or [N-alkyl Phe[4]] substitutions in phenylglycine containing enkephalins.[4,6]

Table III. Opiate-like Activities of Phenylglycine Substituted Enkephalin Peptides

Compound*	Mouse Vas Deferens IC_{50} (nM)	Mouse Hot Plate-Jump ED_{50} (mg/kg-s.c.)
H - Tyr - D-Ala - Gly - Phe - Phe - NH_2	76	ca 9
H - Tyr - D-Ala - Gly - Phg - NH_2	1.2	0.40
H - Tyr - D-Ala - Gly - MePhe - NH_2	25	0.188
H - Tyr - D-Ala - Gly - EtPhe - NH_2	5.9	0.064
H - Tyr - D-Ala - Gly - PrPhe - NH_2	6.3	0.148
Me - Tyr - D-Ala - Gly - MePhe - NH_2	12	0.21
Me - Tyr - D-Ala - Gly - EtPhe - NH_2	1.9	0.005
H - Tyr - D-Ala - Gly - Phe(F) - NH_2	0.19	0.063
H - Tyr - D-Ala - Gly - D-Phe(F) - NH_2	180	–
H - Tyr - D-Ala - Gly - Phe - D-Phe - NH_2	–	17
H - Tyr - D-Ala - Gly - D-Phg - NH_2	4.3	0.23
H - Tyr - D-Ala - Gly - MePhe - NH_2	21	3.8
Me - Tyr - D-Ala - Gly - Phe - NH_2	25	0.51
H - Tyr - D-Ala - Gly - Phe(F) - NH_2	0.54	0.103
H - Tyr - D-Ala - Gly - Phe - D-Tyr - NH_2	–	>9
H - Tyr - D-Ala - Gly - D-Phg(OH) - NH_2	–	1.0
H - Tyr - D-Ala - Gly - Phg(OMe) - NH_2	2.7	3.0
H - Tyr - D-Ala - Gly - Phe(F) - NH_2	0.45	1.0

*Phe = phenylglycine

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DIMERIC ENKEPHALINS: SYNTHESIS AND RECEPTOR BINDING ACTIVITY

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Elucidation of the physiological role of 'δ' (peptide) and 'μ' (alkaloid) opiate receptors will require agonists and antagonists of high affinity and selectivity.[1] It should be possible to increase the affinity of a ligand by increasing the number of "subsites" interacting with the receptor. Analogously, if receptors are clustered in a small portion of the plasma membrane, a dimeric lignd may be able to bind two receptor molecules simultaneously, with a potential gain in affinity and selectivity compared to the monomeric analog.[2] On this background we have synthesized dimeric analogs of enkephalin.

Synthesis of Dimeric Pentapeptide Enkephalins (DPEn): We have synthesized a series of dimers of [D-Ala^2, Leu^5]-enkephalin amide (DALEA) linked at their C-termini with methylene chains of varying length. DALEA was chosen as a 'monomer' because of its stability. We used the C-terminus as the linkage point because amino group modifications in the N-terminal residue are known to inactivate the molecule,[3] and cross-linking via lateral residues would impose serious conformational constraints. The methylene chain as connecting bridge was preferred becasue of its stability to enzymatic attack and flexibility. Syntheses were carried out by two-step coupling: 1) Cross-linking with Boc-Phe-Leu-OH; 2) Elongation with Boc-Tyr-D-Ala-Gly-OH (Figure 1).

The dipeptide acid Boc-Phe-Leu-OH was linked with the diaminoalkanes, NH_2-$(CH_2)_n$-NH_2 (n = 2,4,. . .,12), by the EDC-HOBt method. After deprotection (TFA) the resulting cross-linked dimeric dipeptides were coupled with a tripeptide acid Boc-Tyr-D-Ala-Gly-OH by EDC-HOBt. Dimeric enkephalin analogs (DPEn) were liberated by TFA and purified by gel filtration on Sephadex G-25 eluting with 30% AcOH. Amino acid analysis and elemental analysis were consistent with the structure shown in Figure 1. Dimeric structure was proven on the basis of molecular weight and fragmentation obtained by ^{252}Cf plasma desorption mass spectrometry.[4]

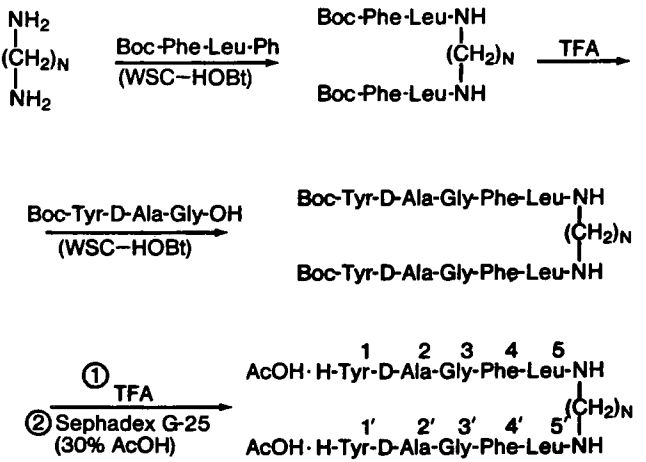

Fig. 1. Structure and synthesis of dimeric pentapeptide enkephalins (DPEn).

Three binding assays using tracers of different selectivity were used to assess the activity of DPEn: [^{3}H]naloxone (^{3}H-NAL), which labels μ receptors; [^{125}I]-[D-Ala^2,D-Leu^5]-enkephalin (^{125}I-DADLE), which labels δ; and [^{3}H]-[D-Ala^2,Met^5]-enkephalin amide (^{3}H-DAMEA), which labels both (μ and δ). Binding was studied in rat brain membranes at 26° C in the presence of bacitracin (100 mg/ml) in the absence of sodium ion (Table 1).

Table I. ED_{50} Values for DPEn in Three Radioligand Assays: 'μ', Using ^{3}H-Naloxone, 'δ', Using ^{125}I-DADLE; or 'Mixed δ and μ', Using ^{3}H-DAMEA.

Enkephalins	ED_{50} (*n*M)		
	^{3}H-NAL	^{3}H-DAMEA	^{125}I-DADLE
(Monomer)			
DALEA	1.74	1.99	2.17
(Dimers)			
DPN2	1.78	1.01	0.27
DPN4	2.28	1.40	0.33
DPN6	2.61	1.53	0.43
DPN8	3.37	2.06	0.83
DPN10	5.82	3.99	1.95
DPN12	8.51	6.50	9.12
Relative % error	± 22%	± 28%	± 37%

The ED_{50} values of dimeric enkephalin are clearly lower in the ^{125}I-DADLE binding assay than in the ^{3}H-NAL assay, suggesting that their affinity for δ receptor is very high. If the potency of DPEn is normalized to that of the parent monomer (standard DALEA=1), then the activities of dimeric enkephalins in the three binding assays can be compared as a function of the chain length (Figure 2, left panel).

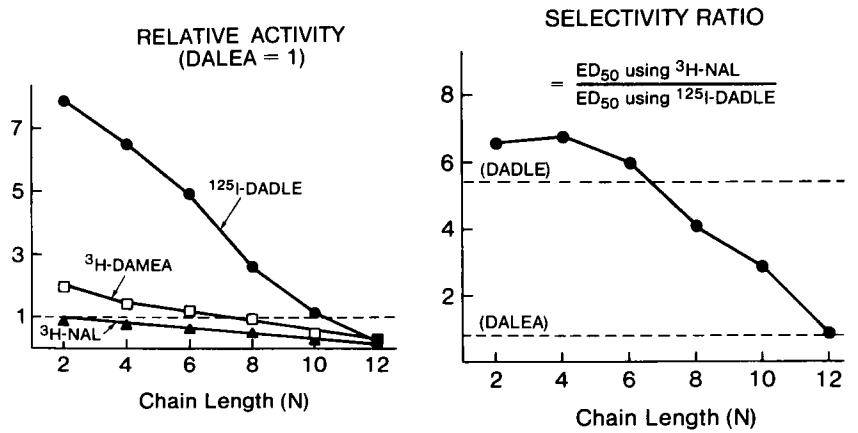

Fig. 2. Left: Relative activity of DPEn in three radioligand assays, and Right: Selectivity Ratio for DPEn, as a function of chain length (n)

The most potent analog, DPE2, is seven times more potent than the monomer standard in binding to δ receptors (^{125}I-DADLE), but equivalent to monomer when binding to μ sites (^{3}H-NAL). Elongation of the methylene chain reduces activity in all the assays. For $4 < n < 10$, dimeric enkephalins are more active than DALEA in a δ assay but less potent than DALEA in the μ assay. A chain length of 12 produces a severe drop of potency in both cases. In the ^{3}H-DAMEA radioligand assay, the activity-length relationship is intermediate.

The ratio of the ED_{50} values for any peptide when using ^{3}H-NAL as a tracer relative to its ED_{50} when using ^{125}I-DADLE furnishes a measure of the selectivity of peptides for δ and μ receptors.[5] A completely nonselective compound with the same potency in both the assays would have a ratio of 1. The shorter the chains, the higher the selectivity of dimeric enkephalins for δ receptors (Figure 2, right panel). Compounds DPE2, DPE4, DPE6 show greater selectivity for δ than DADLE itself. DPE12 shows no selectivity for δ relative to μ receptors.

Conclusions

DALEA, the monomer precursor of DPEn, is a nonselective ligand. But, when two molecules of this amide are linked together, new compounds with extremely high δ selectivity are produced, with a large increase in activity for the δ receptor, and a loss of binding activity for the μ receptor. The simplest and most attractive explanation of these data would be that dimeric enkephalins specifically cross-link δ but not μ receptors. Other explanations are unlikely, because: 1) Enhanced hydro-

phobicity cannot account for the increase in affinity, because the potency of the dimer DPEn analogs is inversely related to the length of the chain in all the assays examined. Furthermore, a nonspecific increase of affinity should not confer δ selectivity; 2) Structural change of the C-terminus in monomeric enkephalin with abolition or modification of the free carboxyl group invariably leads to loss of δ selectivity and gain of μ activity.[6,7].

Enkephalin dimers of the type described here should be useful probes of the delta opiate receptor.

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MINIMAL STRUCTURE ENKEPHALIN ANALOGS: DELETION TETRAPEPTIDES WITH HIGH OPIATE ACTIVITY

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Until quite recently it was believed that a fundamental requirement for potent naloxone-reversible opioid activity in peptide analogs of the endogenous opiates Met- and Leu-enkephalin was an intact pentapeptide sequence. This led to the synthesis and biological testing of many peptide analogs containing modifications of the primary sequence in attempts to understand the structure-activity relationships necessary for opiate-like activity. The three-point model for opiate receptor interaction[1] postulates strict spatial requirements for the free amino group and aromatic ring of Tyr in position 1 of the pentapeptide enkephalins, and for an additional hydrophobic function within the molecule which can stabilize a receptor-active conformation. In the enkephalins this second hydrophobic function is provided by the Phe in position 4. The backbone spacing between the Tyr residue at position 1 and the aromatic residue at position 4 was thought to be invariant until it was shown that several enkephalin tetrapeptides in which an amino acid residue was deleted between these two positions had remarkably high naloxone-reversible enkephalin-like activity[2] (See Table I).

The substitution of a D-Ala residue in position 2 of the deletion tetrapeptide analog of Met-enkephalin, Tyr-Gly-Phe-Met-NH_2 (**4**), which has only 2% of the activity of Met-enkephalin (**1**),led to Tyr-D-Ala-Phe-Met-NH_2 (**5**) which is more active than Met-enkephalin. This 60-fold increase in opiate potency is a significantly greater degree of increase in activity than that seen when the same substitution is made in the pentapeptides (compare Met-enkephalin amide **2** with D-Ala-2-Met-enkephalin amide **3**). This suggests that the D-Ala residue may increase the potency of the tetrapeptides by a mechanism different from that operating in the pentapeptides, and that the effect may be primarily on the conformation of the peptide rather than on any enhancement of metabolic stability.

The six new deletion peptides listed in Table I were made by standard solid phase methods on a p-methybenzhydrylamine resin[3], purified by CCD, and assayed on the stimulated guinea pig ileum[2].

Table I: Relative Potencies of Enkephalin Analogs on the Stimulated Guinea Pig Ileum (Met-enkephalin = 100).

1. Tyr-Gly-Gly-Phe-Met	100	7. Tyr-D-Ala-Phe-Leu-NH_2	120*
2. Tyr-Gly-Gly-Phe-Met-NH_2	160*	8. Tyr-Ala-Phe-Met-NH_2	9
3. Tyr-D-Ala-Gly-Phe-Met-NH_2	140*	9. Tyr-Aib-Phe-Met-NH_2	10
4. Tyr-Gly-Phe-Met-NH_2	2*	10. Tyr-Pro-Phe-Met-NH_2	37
5. Tyr-D-Ala-Phe-Met-NH_2	120*	11. Tyr-D-Pro-Phe-Met-NH_2	0
6. Tyr-Ala-Phe-Leu-NH_2	4	12. Tyr-Aib-Phe-Leu-NH_2	6

(*Data taken from reference 2. Due to a typographical error the potency of Tyr-D-Ala-Phe-Leu-NH_2 relative to Met-enkephalin is incorrectly listed in reference 2 as 20; it should be 120 *vs.* Met-enkephalin = 100.)

Pentapeptide enkephalin analogs in which the Aib residue is substituted for Gly at positions 2 and 3 are reported to show "high" enkephalin-like biological activity[4]. While there is some suggestion that the substitution of either the severely restricting Aib residue or the D-Ala residue in the 2 position of the pentapeptide enkephalins permits a more favorable receptor active conformation than the L-Ala residue, which is reflected in both receptor binding and in bioassays, this does not seem to be the case with the deletion analogs. The Ala (**8**) and Aib (**9**) substituted tetrapeptides are equipotent (about 10 times less active than the D-Ala analog **5**), indicating the possibility that the overall conformation is similar enough for the receptor to see them equally.

With the exception of a D-Pro substitution, D-amino acids in position 2 of the pentapeptides tend to increase their enkephalin-like activity[5]. The same can be seen in the deletion tetrapeptides (see peptide **11**).

Pentapeptide analogs with Pro in position 2 exhibit very low enkephalin-like activities, with the Pro-2 analogs of Met-enkephalin, Leu-enkephalin and Met-enkephalin amide having less than 1% of Met-enkephalin activity in the mouse vas deferens assay and in receptor binding studies[5]. However, the substitution of Pro in position 2 of the deletion tetrapeptides gives a highly active analog (**10**) with 37% of the activity of Met-enkephalin. The similarity between Tyr-Pro-Phe-Met-NH_2 (**10**) and the N-terminal sequence of beta-casomorphin (Tyr-Pro-Phe-Pro-Gly-Pro-Ile)[6] is striking, especially when Tyr-Pro-Phe-Met-NH_2 is compared with morphiceptin (Tyr-Pro-Phe-Pro-NH_2), a synthetic analog reported to be highly selective in binding to the putative μ opiate receptor[7].

The enkephalin-like activities of both the pentapeptide analogs and the deletion tetrapeptide analogs are generally increased by protecting the C-terminal residue (as an amide or as an alcohol), and by substituting a D-amino acid in position 2. Since the deletion analogs are 20% smaller than the pentapeptides it should be safe to assume that structural and/or functional changes in the smaller biologically active peptides will influence activity to a much greater degree, and may lead to more potent and more selective enkephalin analogs for the study of opiate receptors.

Acknowledgements

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ONTOGENY OF LEU- AND MET-ENKEPHALIN IN CHICK BRAIN: AN ENKEPHALIN-LIKE SUBSTANCE(S) PRESENT IN EMBRYONIC, BUT NOT ADULT BRAIN

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Introduction

Although several years have elapsed since the isolation and characterization of Leu- and Met-enkephalin (Enk), the functional relationship between these two peptides is still unclear. The fact that the ratio of Met-to Leu-Enk varies in different brain regions,[1-4] led to speculation that these peptides exist in separate neuronal systems. Recent immunocytochemical studies have in fact provided support for this concept,[5] although other such studies have given no certain evidence for separate Leu- and Met-Enk neurons.[6] Because an understanding of the developmental characteristics of each of these peptides should help to resolve this issue, we used specific radioimmunoassays (RIAs) to study the ontogeny of Leu- and Met-Enk in several regions of chick brain.

Methods

Brain tissues from embryos, newly hatched (NH), and 4-week-old chickens were homogenized in 10 volumes of ice-cold 0.1 N HCl. Duplicate aliquots of each homogenate were removed for protein determination. The remainder was centrifuged and an aliquot of the supernatant lyophilized for determination of Leu- and Met-Enk by RIA[4] with carboxyl specific antisera. The Leu-Enk antiserum, raised in our lab against a thyroglobulin conjugate of Leu-Enk, showed a 1.4% cross-reaction with Met-Enk and 0.34% with Tyr-Gly-Gly-Phe. The Met-Enk antiserum purchased from Immunonuclear, Stillwater, MN showed a 1.5% cross-reaction with Leu-Enk, 14% with Met-Enk sulfoxide and 0.82% with Met-Enk-Arg^6-Phe^7. Cross-reactivities of these antisera with other degradative fragments of the enkephalins, with other extended enkephalins such as the hexa- and heptapeptides, and with β-endorphin and dynorphin (1-13) were negligible. Tissue extracts to be fractionated by HPLC were treated with TCA to a final concentration of 10% and the resultant precipitates removed by centrifugation. After removal of the

TCA by ether extraction, the samples were lyophilized, the residues dissolved in buffer and applied to a Micropak MCH-10 column (0.4 x 30 cm, Varian Assoc.) which was eluted at 30 ml/hr with 0.5 M formic acid-pyridine, pH 4.0, and a linear gradient of 0-20% 1-propanol for 120 min; 0.7 ml fractions were collected. Aliquots of each fraction were dried under vacuum and taken up in RIA buffer for assay.

Results and Discussion

Met- and Leu-Enk were detected by RIA in the embryonic chick brain at 5 days of incubation (d.i.) (Figure 1). Thereafter, the concentration of Met-Enk increased consistently in all brain regions and reached adult levels at about 17 d.i. In contrast, there was little change in the concentration of Leu-Enk in the cerebrum and optic tectum over the developmental period of study. In the medulla-pons, the concentration of Leu-Enk increased 2-fold to a maximum at 17 d.i. There was, in all brain regions examined, an apparent 4- to 5-fold increase in the ratio of Met- to Leu-Enk over the developmental period of study. This increase was greatest between 5 and 13 d.i. These results appear to support the view that Leu- and Met-Enk are in separate neurons. In fact, this was the interpretation made by Patey *et al.*[7] who found independent developmental profiles for Met- and Leu-Enk in rat brain cortex and striatum. The results of our characterization of the enkephalin immunoreactivity by HPLC show, however, that it is premature to draw such conclusions from RIA data alone.

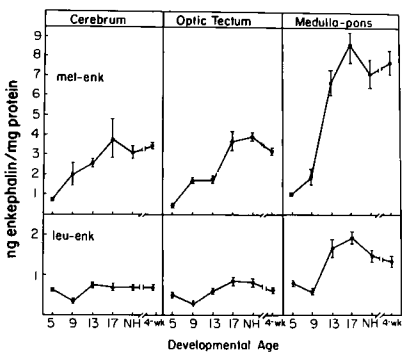

Fig. 1. Development of Met- and Leu-Enk in various regions of chick brain. Mean ± S.E. (n = 3-6).

When an HCl extract of adult chick brain was fractionated by HPLC, all of the Leu-Enk immunoreactivity eluted with the retention time characteristic of authentic Leu-Enk. Similarly, Met-Enk immunoreactivity was found only in the position of authentic Met-Enk or its sulfoxide (Figure 2). In contrast, the HPLC profiles of acid extracts of 5-day embryonic chick brain revealed two peaks of Leu-Enk immuno-

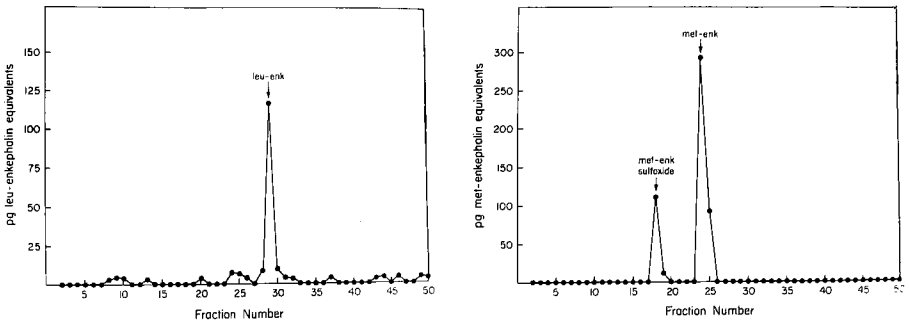

Fig. 2. Chromatography of Leu-Enk (left panel) and Met-Enk (right panel) immunoreactivity in adult chick brain.

reactivity; one emerged at the position of authentic Leu-Enk, the other eluted with about one-third the retention time (Figure 3). This early eluting material was also recognized by two other Leu-Enk antisera (data not shown). Four peaks of Met-Enk immunoreactivity were obtained when embryonic brain was fractionated by HPLC (Figure 3).

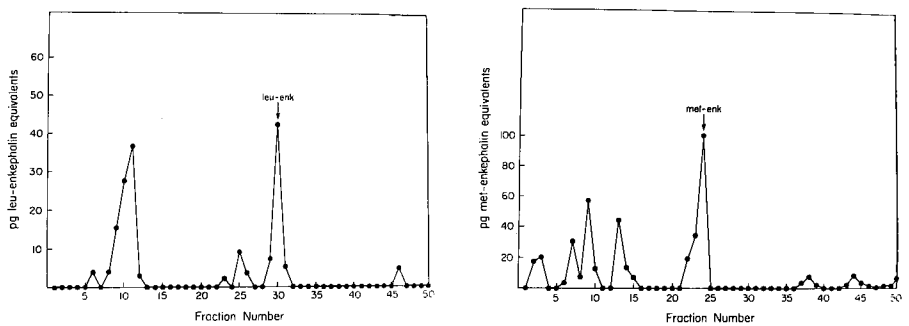

Fig. 3. Chromatography of Leu-Enk (left panel) and Met-Enk (right panel) immunoreactivity in 5-day embryonic chick brain.

Thus, the apparent developmental increase in the ratio of Met- to Leu-Enk is at least partly due to a cross-reacting substance or substances present in embryonic, but not in adult brain. Characterization of these substances is in progress. Our results call attention to the dangers inherent in assigning physiological significance to changes in levels of peptides determined solely by RIA. In addition, the different profiles of enkephalin immunoreactivity obtained in embryonic and adult brain suggest that differential processing of enkephalin precursors may occur during development.

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A NOVEL METHOD FOR ANALYSIS OF THE *IN VIVO* STABILITY OF OPIOID PEPTIDES

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Introduction

The naturally occuring enkephalins, H-Tyr-Gly-Gly-Phe-Met-OH and H-Tyr-Gly-Gly-Phe-Leu-OH, do not have measureable analgesic activities when administered systemically (i.v., s.c., i.m., i.p., or p.o.) and possess only transient analgesic properties when injected centrally (i.c.v.). Structure-activity relationships have established that substitution of a D-amino acid residue (most commonly D-alanine) for the glycine residue at position two of an enkephalin produces a peptide with improved resistance toward aminopeptidase activity. Improvement in aminopeptidase resistance results in analogs with systemic analgesic activities. While a detailed knowledge of the *in vivo* stability of the Tyr-D-AA bond (where AA is an amino acid) is of valuable assistance in the design of opioid peptides with increased systemic activity, the required analyses are often time consuming and necessitate highly specialized techniques.

The enkephalin analog, H-Tyr-D-Ala-Gly-Phe-MeMet-NH_2 (LY127623, metkephamid), a minimally modified analog of methionine enkephalin, is currently undergoing clinical trials as an analgesic agent.[1] Radiorespirometry studies in rats using metkephamid containing a carbon-14 label at the carboxyl carbon of the tyrosine residue have indicated that 10 minutes after subcutaneous administration of the peptide, the animals begin to respire $^{14}CO_2$ (Figure 1).

These data imply that there is an *in vivo* cleavage of the H-Tyr-D-Ala- amide bond to liberate free tyrosine which then undergoes further metabolism to generate the $^{14}CO_2$. We now report a new method for the analysis of the *in vivo* stability of the H-Tyr-D-AA bond of enkephalins and other opioid peptides.

Materials and Methods

Solid-phase peptide synthesis was performed on a Beckman 990 automated peptide synthesizer using *tert*-butyloxycarbonyl derivatives of the appropriate amino acids and a benzhydrylamine resin. The 3-fluorotyrosine [Tyr(3F)] was incorporated as a racemic mixture. After

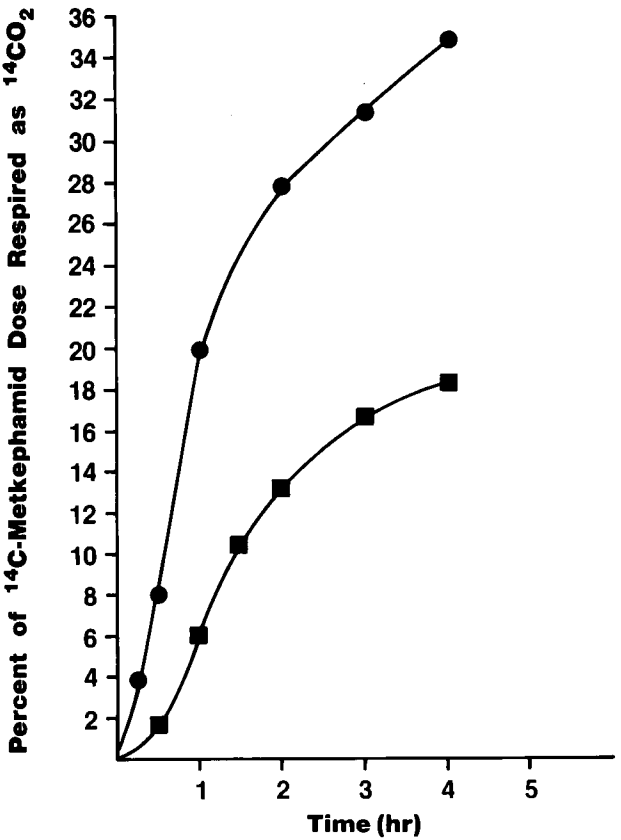

Fig. 1. Respiration of $^{14}CO_2$ following s.c. (■) or i.v. (●) administration of ^{14}C-metkephamid to rats (male, 200 g).

removal of the peptides from the resin with liquid HF at 0° C the diastereomeric peptides were separated and purified by reversed-phase liquid chromatography (RPLC) over C_{18}-silica gel followed by chromatography over Sephadex G-10 to remove salts.[2] Purified peptides were characterized by elemental analysis, amino acid analysis, optical rotation at two wavelengths, NMR spectroscopy, analytical RPLC, and TLC in three solvent systems. Aminopeptidase M digests of the peptides were performed at pH 7.0 and 37° C for 16 hours at a 1:10 enzyme to substrate ratio. The peptides were tested *in vitro* on single vas deferens from mature mice (Cox, 30 to 40 g). The hot plate test for analgesia was conducted using an apparatus with an electrically heated, thermostatically controlled (52° C) metal plate. The time from contact with the plate until an escape jump occured was recorded as a response latency.

Results and Discussion

It is known that Tyr(3F) will cause convulsions and death when administered to mice[3] or rats.[4] This toxicity is not stereospecific as both the D- and L- (R and S) isomers have equivalent effects.[5] The mechanism of this toxicity is presumed to proceed by initial degradation of the L- or D-amino acid with tyrosine transaminase or D-amino acid oxidase to give 3-fluoro-4-hydroxyphenylpyruvic acid. This keto acid is then metabolized further to ultimately give the very toxic fluoroacetate.[6]

We have synthesized metkephamid analogs containing both D- and L-Tyr(3F).[7] The configuration of the Tyr(3F) present in each diastereomeric peptide was established by digestion of the peptides with aminopeptidase M and followed by amino acid analysis to detect the liberated L-Tyr(3F).

The *in vitro* and *in vivo* assays confirmed this assignment as the L-diastereomer possessed 25-98% of the opiate-like activity of metkephamid while the D-diastereomer was inactive (Table I). Thus, substitution of L-3-fluorotyrosine for the tyrosine in metkephamid had little or no effect on the opiate-like pharmacology of metkephamid. However, toxic effects (convulsions and death) similar to those produced by either L- or D-3-fluorotyrosine are readily observable after the administration of [L-Tyr(3F)[1]]-metkephamid but not after administratoin of the diastereomeric [D-Tyr(3F)[1]]-metkephamid (Table II). The onset of toxicity produced by [L-Tyr(3F)[1]]-metkephamid is delayed relative to that produce by the free amino acid. This suggests that the peptide must first be hydrolyzed to give Tyr(3F) which can then be metabolized further to display the toxic effects. The intensity and time course of these toxic effects are thus a measure of the *in vivo* stability of the H-Tyr(3F)-D-Ala- amide bond. The fact that [D-Tyr(3F)]-metkephamid produces no convulsions over a five-hour period verifies the inability of aminopeptidase to cleave a H-D-Tyr(3F)-D-Ala- amide bond.

We can now assess the resistance of opioid peptides to *in vivo* animal terminal degradation without the requirement of a radiolabeled peptide or extensive metabolite characterization.

Table I. Opiate-like Effects of Metkephamid Analogs

	Mouse Vas Deferens		Mouse Hot Plate-Jump	
Structure	IC_{50} (M)	Relative Potency	ED_{50} (mg/kg-s.c.)	Relative Potency
H-Tyr-D-Ala-Gly-Phe-MeMet-NH_2	1.22×10^{-8}	1.00	0.363	1.00
H-L-Tyr(3F)-D-Ala-Gly-Phe-MeMet-NH_2	1.25×10^{-8}	0.98	1.45	0.25
H-D-Tyr(3F)-D-Ala-Gly-Phe-MeMet-NH_2	2.94×10^{-6}	0.004	>30.	<0.01

Table II. Time Course of Analgesic[a] and Toxic[b] Effects

	H-D,L-Tyr(3F)-OH		[H-L-Tyr(3F)[1]]-Metkephamid		[H-D-Tyr(3F)[1]]-Metkephamid	
Time (Min)	Analgesia (%)	Toxicity[c] (%)	Analgesia (%)	Toxicity[d] (%)	Analgesia (%)	Toxicity (%)
15	0	0	100	0	0	0
90	—	100	10	0	0	0
150	—	—	—	100	0	0
300	—	—	—	—	0	0

[a]Mouse hot plate-jump assay at 30 mg/kg-s.c.
[b]Hyperirritability and convulsive seizures followed by death
[c]LD_{50} = 9.9 mg/kg-s.c. A. Weissman and B.K. Koe, (1967) *J. Pharmacol. Exp. Ther.*, **155**, 135.
[d]Metkephamid LD_{50} = 1,840 mg/kg-s.c. in mice

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PIPERAZINONE ENKEPHALIN ANALOGS

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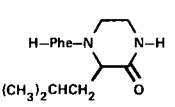
Introduction

In a search for enkephalins with improved analgesic activity we have prepared conformationally restricted analogs where the amino acid residues are cyclized to form a piperazinone ring. Enkephalins where the tyrosine residue is cyclized to a piperazinone have already been described.[1,2] In this paper, we describe the synthesis and biological evaluation of a series of enkephalin analogs in which piperazinones replace the glycine, phenylalanine, and carboxyl terminal amino acid residues.

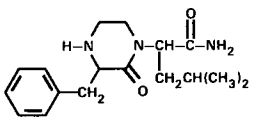
Chemical Synthesis

Structures of the enkephalin analogs initially prepared are shown in Table I. The piperazinone intermediates required for the synthesis of



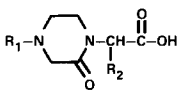
Ia L,L isomer

b L,D isomer



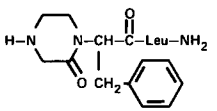
IIa L,L/D,D isomers

b L,D/D,L isomers



IIIa R_1 = BOC, R_2 = H

b R_1 = Z, R_2 = -CH_2–



IVa L,L isomer

b D,L isomer

these compounds are I-IV. The synthesis of compounds I and II has already been described.[3] Compound IIIa was prepared by reacting the sodium salt of 4-(*t*-butyloxycarbonyl)piperazinone with ethyl chloroacetate, followed by hydrolysis of the ester initially formed. Compound IIIb, prepared by the same general method, was coupled with leucine amide and then hydrogenolyzed to give IV. The methods used to synthesize compounds I, II, and IV afforded mixtures of diastereoisomers. These mixtures were separated by chromatography on silica gel. As in the case of closely related peptides, e.g. H-Phe-D, L-Leu-NH_2, the L,L isomer

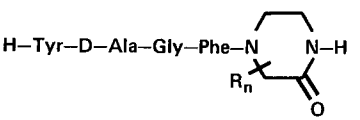
Table I. Structures and Activities of Piperazinone Enkephalin Analogs

Structure	Piperazinone Intermediate			Enkephalin Product			
	Compound	Isomer	HPLC Retention Time (min)	HPLC Retention Time (min)	Analgesic Activity ED_{50} (mg/kg)	Analgesic Activity $t_{1/2}$ (min)	^3H Etorphine Binding IC_{50} (nM)
H–Tyr–D–Ala–Gly–Phe–N(CH$_2$)$_2$CHCH$_2$–N–H (C=O)	Ia	L,L	2.5	6.3	0.4	13	15
	Ib	L,D	5.0	12.9	11.0		54
H–Tyr–D–Ala–Gly–N(CH$_2$C$_6$H$_4$)–N–CH(CH$_2$CH(CH$_3$)$_2$)–C(=O)–NH$_2$	IIa	L,L/D,D	3.1	4.6	6.2		450
	IIb	L,D/D,L	3.9	12.2	>12.5		$>10^4$
H–Tyr–D–Ala–N(CH$_2$C$_6$H$_5$)–N–CH–C(=O)–Leu–NH$_2$	IVa	L,L	3.0	5.8	>12.5		3500
	IVb	D,L	5.7	11.3	>12.5		2500
H–Tyr–N(CH$_2$)$_2$–N–CH$_2$–C(=O)–Phe–Met–NH$_2$	IIIa			7.2	>12.5		$>10^4$
H–Tyr–D–Ala–Gly–Phe–Leu–NH$_2$	H–Phe–Leu–NH$_2$		2.2	4.8	>12.5		6.5
	H–Phe–D–Leu–NH$_2$		4.1				
H–Tyr–D–Ala–Gly–Phe–Met–NH$_2$				3.4	0.9	1.7	16
Morphine					1.8	29	21

was eluted from the column ahead of the L,D isomer. The configuration of Ia has been confirmed by X-ray structure determination.[4]

Compounds I-IV were converted to the enkephalins by standard procedures. The analogs listed in Table II were prepared as described by Moon.[3] The enkephalins and intermediates were pure by reverse phase HPLC (Waters 3.9 mm x 30 cm μ-Bondapack C_{18} column using a solvent of CH_3CN:0.01 M NaH_2PO_4 pH 2.9 (1:4) and a flow rate of 2 ml/min). Enkephalins derived from IIa and IIb each contain two diastereoisomers which could not be separated by chromatography.

Table II. Structures and Activities of Piperazinone Enkephalin Analogs



R_n	Piperazinone Configuration	HPLC Retention Time (min)	Analgesic Activity ED_{50} (mg/kg)	^3H Etorphine Binding IC_{50} (nM)
3-$CH_2CH(CH_3)_2$	L	6.3	0.4	15
3-$CH_2CH_2SCH_3$	L	4.2	1.2	1.3
3-$CH_2CH_2SCH_3$	D	6.0	10	2.9
H		2.6	>25	45
3-CH_3	L	3.0	9	27
3-CH_3	D	3.0	>12.5	211
3,3-$(CH_3)_2$		3.6	8	71
6,6-$(CH_3)_2$		3.8	>12.5	18
3-$CH_2CH(CH_3)_2$-1-CH_3	L	7.5	>12.5	80
3-$CH_2CH(CH_3)_2$-6,6-$(CH_3)_2$	L	18	>12.5	14

Biological Methods

Analgesic ED_{50}'s and half lives were determined subsequent to intravenous injection using the mouse tail flick method as described by VonVoigtlander and Lewis.[5] The tabulated ED_{50} values are derived from measurements made two minutes after injection. Binding studies were carried out as described by Lahti and Collins[6] using 0.1 nM ^3H-etorphine (38 ci/mmol) as the radioactive ligand. Samples were filtered on GF/B filters, and non-specific binding was determined using naltrexone (1.0 μM).

Results and Discussion

Analgesic and ^{3}H-etorphine binding activities of enkephalins with a piperazinone ring incorporated at different positions in the molecule are shown in Table I; activities of two enkephalin standards and morphine in these tests are also presented. Enkephalins where the leucine residue was cyclized to a piperazinone were active in both tests. The L-isomer, prepared from Ia, was the more active having an analgesic half life considerably longer than D-Ala^2-Met^5-enkephalinamide. Analgesic activity was found for the cyclic phenylalanine analog derived from IIa, but cyclization of either glycine residue gave inactive products.

As the most active compounds were those where the carboxyl-terminal amino acid was cyclized, we have prepared additional analogs of this structure type with various substituents in the piperazinone ring (Table II). All of these were active in the binding assay, but a 3-L-substituent in the piperazinone ring was important for good analgesic activity. In the most active compounds the 3-substituent corresponded to a leucine or methionine side chain.

In summary, we have found that incorporating the carboxyl-terminal amino acid into a piperazinone further improves the analgesic activity of the stabilized enkephalins D-Ala^2-Leu^5-enkephalinamide and D-Ala^2-Met^5-enkephalinamide.

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DEHYDRO-ENKEPHALINS, SYNTHESIS AND BIOLOGICAL ACTIVITY

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Introduction

The synthesis of enkephalin analogs has clarified the structure activity relationships of these endogenous opioid peptides.[1] Our current interest in the incorporation of dehydroamino acid residues into peptide hormones led to the synthesis of the highly potent [D-Ala^2, ΔPhe^4, Met^5]-enkephalin amide.[2] Simple α,β-dehydrogenation of an amino acid residue results in the formation of a more rigid, hydrophobic and reactive moiety. In general, such unsaturated sites in peptide hormones may act to enhance activity by virtue of an increased receptor binding affinity or by irreversibly reacting with a nucleophile on the receptor surface. In particular, a recent hypothesis put forth by Walter[3] states that a peptide messenger (hormone) most probably contains both "binding" and "active" elements in its amino acid sequence. The placement of a double bond into an amino acid residue in the "binding" element should increase binding and elicit a stronger bioresponse.

The small pentapeptide hormone, enkephalin, is an excellent model peptide in which to investigate the effect of a dehydroamino acid moiety. To this end, we have synthesized two series (D-Ala^2 and Gly^2) of "dehydro-enkephalins" (ΔEK), which contain ΔAla$^{2 \text{ or } 3}$, ΔPhe^4 or ΔLeu^5 residues (Figure 1). Attempts to prepare ΔTyr^1-enkephalins failed because of the instability to hydrolysis of the N-terminal ΔTyr residue.

1 2 3 4 5

H-Tyr - ΔAla-Gly - Phe-Leu-OH (1)
H-Tyr-D-Ala-ΔAla-Phe-Leu-OH (2)
H-Tyr-D-Ala-Gly-ΔPhe-Leu-OH (3)
H-Tyr-D-Ala-Gly - Phe-ΔLeu-OH (4)
H-Tyr - ΔAla-Gly - Phe-OMe (5)
H-Tyr - Gly-ΔAla-Phe-Leu-OH (6)
H-Tyr - Gly-Gly-ΔPhe-Leu-OH (7)
H-Tyr - Gly-Gly-Phe-ΔLeu-OH (8)

Fig. 1. Structures of dehydro-enkephalins

Peptide Synthesis

All peptides were synthesized by conventional solution methods. The methods used for dehydroamino acid syntheses are illustrated in Figure 2. All ΔPhe and ΔLeu peptides had the Z-configurations, as determined by ^{1}H-NMR.[4]

Binding Assay

Receptor binding assays using rat brain membrane and [^{3}H]-[D-Ala^2, D-Leu^5]-enkephalin (^{3}H-DADLE) and [^{3}H]dihydromorphine (^{3}H-DHM) as tracers were performed as described by Pert and Snyder.[5] Potencies expressed in ED_{50} (dose which produces a 50% inhibition of binding) have been estimated using an RIA computer program.

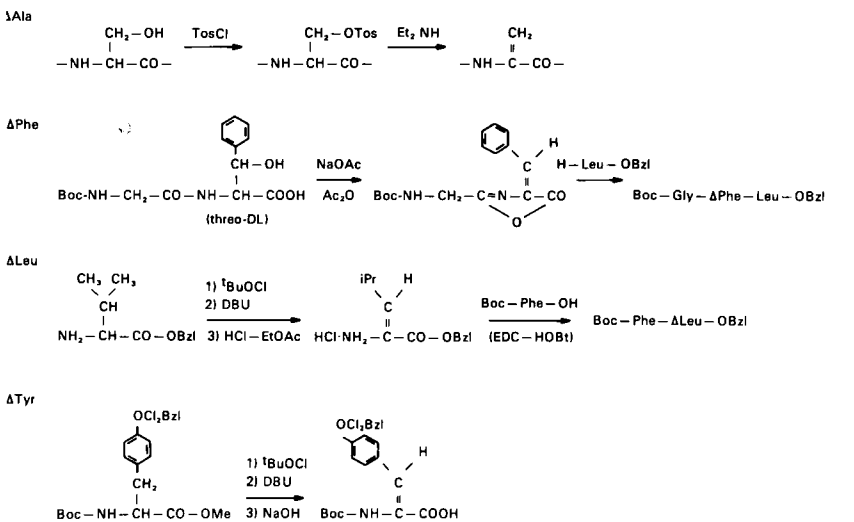

Fig. 2. Syntheses of dehydroamino acids

Results and Discussion

In the enkephalin H-Tyr-D-Ala-Gly-Phe-D-Leu-OH (ST_1), the substitution of D-Ala^2 by ΔAla^2 (**1**), L-Phe^4 by ΔPhe^4 (**3**) and D-Leu^5 by ΔLeu^5 (**4**) have maintained almost full activity (80-120%) as shown in Table I. The activity of ΔEKs in this binding assay cannot be explained by increased resistance to peptidases, because the addition of bacitracin at chosen incubation times minimized hydrolyses.[6]

The influence of the ΔAA moiety on the peptide affinity for two types of opiate receptors[7] has been examined by using ^{3}H-DADLE for peptide receptors (δ) and ^{3}H-DHM for opiate receptors (μ) as tracers.

Table I. Binding Activity of (D-Ala²)-dehydro-enkephalins.

ΔEK	ED_{50} [a] vs ^{3}H-DADLE		ED_{50} vs ^{3}H-DHM		$\frac{ED_{50} \text{ vs } ^3\text{H-DHM}}{ED_{50} \text{ vs } ^3\text{H-DADLE}}$
(1)	2.8	(68)[b]	6.1	(125)	2.2
(2)	4.5	(42)	26	(29)	5.8
(3)	1.9	(100)	6.4	(119)	3.4
(4)	2.5	(76)	8.7	(87)	3.5
(5)	180	(1)	76	(10)	0.4
(ST_1)	1.9	(100)	7.6	(100)	4.0

[a]nM [b]relative potency (%) against ST_1.

The ratio of potencies in these two assays, ED_{50} vs ^{3}H-DHM / ED_{50} vs ^{3}H-DADLE suggests preferences for one of the two types of receptors (Table I, II, Figures 3-A,B). β-Endorphin has almost the same affinity for both receptors, while enkephalin analogs generally have a greater affinity for δ receptors. The ΔAla²-tetrapeptide (**5**) is exceptional since it shows a higher selectivity for μ receptors. This result could be due, however, to the removal of the C-terminal residue.[8] As shown in Figure 2, the D-Ala² series (ST_1, **3** and **4**) prefers μ sites as compared with the Gly² series (ST_2, **7** and **8**, respectively), and furthermore, the replacement of D-Ala² by ΔAla² facilitates the shift of selectivity towards μ receptors (Figure 3-B). These results indicate that an increase in hydrophobicity at position 2 causes a preference for the μ receptors.

Table II. Binding Activity of (Gly²)-dehydro-enkephalins.

ΔEK	ED_{50} [a] vs ^{3}H-DADLE		ED_{50} vs ^{3}H-DHM		$\frac{ED_{50} \text{ vs } ^3\text{H-DHM}}{ED_{50} \text{ vs } ^3\text{H-DADLE}}$
(6)	62	(4)[b]	80	(19)	1.3
(7)	3.8	(63)	21	(71)	5.5
(8)	6.6	(36)	32	(47)	4.8
(ST_2)	2.4	(100)	15	(100)	6.2
β-Endorphin	2.3	(104)	2.8	(540)	1.2

[a]nM [b]relative potency (%) against ST_2.

Figure 3-A shows that the D-Ala² and Gly² analogs containing ΔPhe⁴ and ΔLeu⁵ residues have a preference for δ sites as do the parent saturated enkephalins. Therefore, increased hydrophobicity in positions 4 and 5 does not facilitate selective interactions with the receptors. Since D-Phe⁴-enkephalins have no binding activity, position 4 apparently provides the required conformation for receptor binding and the ΔPhe⁴

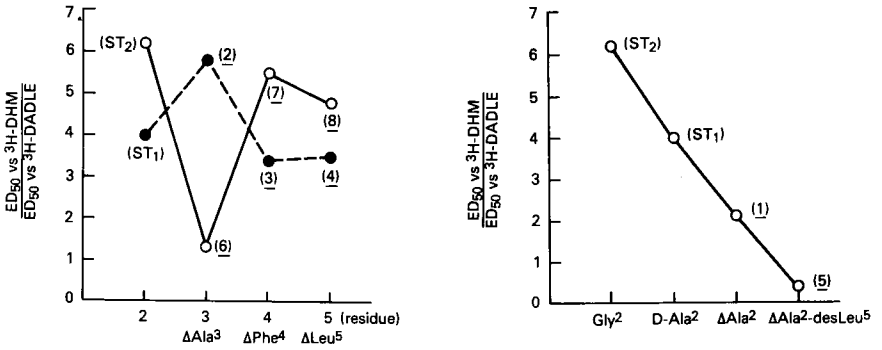

Fig. 3. Potency ratio: (A) as a function of unsaturated site in Gly^2 (o) and $D\text{-}Ala^2$ (•)-EK's, and (B) for different substituent in position 2. ratio > 1, more potent for δ; ratio < 1, more potent for μ receptors; ratio = 1, equipotent.

moiety in **3** maintains this stereochemical requirement and sustains full binding activity. On the other hand, the considerable activity of [$D\text{-}Ala^2$, ΔLeu^5]-enkephalin (**4**) is presumably due to the insensitivity of the opiate receptors to chirality in position 5. The ΔAla^3-enkephalins are significantly potent (20-40%) and [$D\text{-}Ala^2$, ΔAla^3, Leu^5]-enkephalin (**2**) has a higher affinity for the δ than for the μ receptors.

Acknowledgements

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SYNTHESIS OF NEW ACTIVE ANALOGS OF THE C-TERMINAL HEPTAPEPTIDE OF CCK 27-33, ROLE OF THE RESIDUE IN POSITION 32 IN BIOLOGICAL ACTIVITIES

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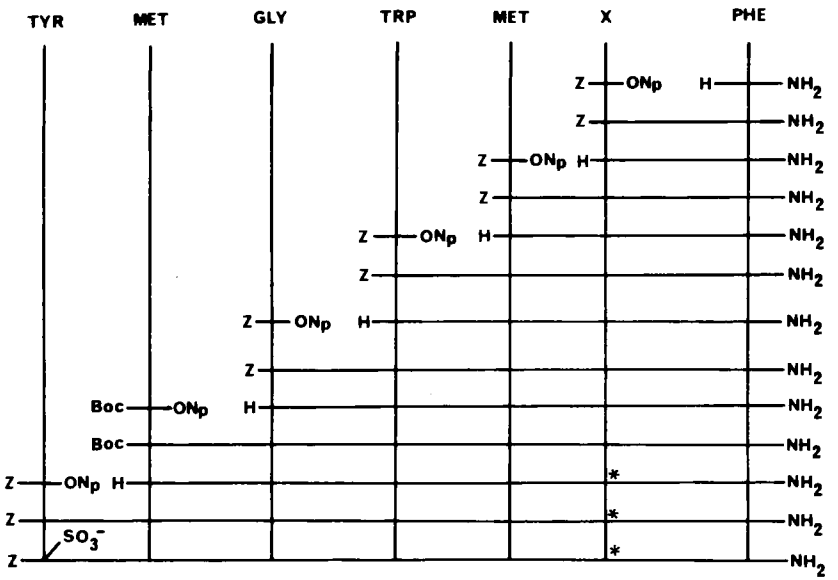
Introduction

Cholecystokinin (CCK), a peptide containing 33 amino acids,[1-3] is generally considered to be the hormone that causes the increase in pancreatic enzyme secretion and gall bladder contraction following ingestion of a meal. While some work has been done showing the importance of the sulfate ester of tyrosine 27,[4] relatively little attention has been given to the potential significance of aspartic acid in position 32 of CCK. This is surprising in as much as the C-terminal pentapeptide of CCK is identical to that of gastrin; in gastrin, the aspartic acid residue corresponding to Asp 32 of CCK has been shown to be of very great importance for gastrin-like activity.[5] The only analog that has been described to examine the role of the amino acid in position 32 is (Ala 32) CCK 26-33.[6,8] Previous results dealing with the N-protected derivative of the 32 β-Asp analog of CCK 27-33 showed that this peptide had definitely more pancreozymin-like activity that cholecystokinin-like activity.[7] A similar separation of the two types of activities had already been observed with the des-amino analog of CCK 27-33,[9] although here the difference was not so remarkable. This analog was as potent as CCK 26-33 in the stimulation of the contraction of the gall bladder and less potent in the release of amylase from pancreatic acinar cells. In cholecystokinin, the sulfate ester of tyrosine in position 27 is a critical determinant for both biological activities.[8] All observations indicate that cholecystokinin-like activity is related to the structure of the N-terminal region of CCK 27-33 and pancreozymin-like activity is more dependent on the structure of the C-terminal region. To further explore the necessary features for activity as contributed by the residue in position 32 in N-protected CCK 27-33, we have undertaken the synthesis of N-Z-(β-Ala 32)-CCK 27-33 and N-Z-(Glu 32)-CCK 27-33.

Synthesis

The two analogs Z-(β-Ala 32)-CCK 27-33 and Z-(Glu 32)-CCK 27-33 were synthesized according to Scheme I. Sulfation was performed using the SO_3-pyridine complex as previously described[7] replacing ammonium hydroxide by sodium hydrogen carbonate to neutralize the sulfuric acid gradually formed from the unreacted SO_3-pyridine complex. Final products were purified by chromatography on silica gel (ethyl acetate: pyridine: acetic acid: water 60:20:6:11) and pure fractions were lyophilized. Chromatographically homogeneous compounds gave correct amino acid analyses after acid hydrolysis and were found pure by HPLC (sharp single peak, μ-Bondapak C-18 Waters, flow rate 1.5 ml/min, detection at 254 nm, MeOH/H_2O as solvent, Waters instrument). Their structural composition was ascertained by elemental analysis (C,H, N). IR spectra of the two analogs showed the characteristic sharp band at 1040 cm^{-1} which indicates the presence of the sulfate ester group.



Scheme I. The synthesis of analogs of cholecystokinin 27-33: Z-(β-Ala 32) CCK 27-33 and Z-(Glu 32) CCK 27-33. x = β-ALA or GLU (OBu^t) ; x* = β-ALA or GLU

Amylase Secretion

Amylase secretion by dispersed acini prepared from guinea pig pancreas was determined using the techniques previously described.[10] With Z-(β-Ala-32)-CCK 27-33 and Z-(Glu-32)-CCK 27-33 the shape of the dose response curve for stimulation of enzyme secretion was the same as that obtained with Boc-CCK 27-33.[11] Replacing aspartic acid 32 by glutamic acid caused a 1000-fold decrease in potency but no change in the efficacy.

Stimulation of Guinea Pig Gall Bladder Contraction

Contractile activities were compared according to the procedure of Ljungberg.[12] The gall bladder contracting activity of 3.3 μg of Z-(β-Ala 32) CCK 27-33 was found to be lower than 0.2 Ivy dog units (IDU), (*i.e.* the activity of 1 μg is lower than 0.06 IDU). The activity of 6.7 μg was, however, higher than 0.2 IDU (i.e. 1 μg has an activity higher than 0.03 IDU). Consequently, the activity of Z-(β-Ala 32) CCK 27-33 is between 30 and 60 units per milligram. Since CCK 33 has an activity of 3000 IDU per mg, the activity of Z-(β-Ala 32) CCK 27-33 is between 1 and 2% of that of CCK 33 on a weight basis. The same applies to Z-(Glu 32) CCK 27-33. There seems to be no certain difference between the activities on the stimulation of contraction of the gall bladder between these two analogs of CCK 27-33.

Discussion

Replacement of Asp 32 by Glu, which increases the distance between the side chain carboxyl group and the peptide backbone caused a decrease in potency (1000-fold by comparison with Boc-CCK 27-33) on amylase release but did not alter the efficacy. Replacement of Asp 32 by β-Ala which eliminates the carboxyl group in the side chain and increases the length of the peptide backbone had similar effects, still leading to a more potent analog (300-fold decrease as compared to Boc-CCK 27-33).

The results obtained illustrate that the aspartic acid residue in position 32 of CCK is important but not essential for stimulation of amylase release from acini. Replacement of aspartic acid 32 by β-Ala or Glu altered the potency of the peptide but did not alter its efficacy.

Aspartic acid 32 seems to be very important but not essential for the stimulation of gall bladder contraction. Both Z-(β-Ala 32) CCK 27-33 and Z-(Glu 32) CCK 27-33, showed very little effect on gall bladder stimulation.

Conclusion

Analogs prepared by replacing Asp 32 in N-protected CCK 27-33 either by Glu or β-Ala differed in the degree of pancreozymin-like activity and cholecystokinin-like activity. These results, indicating separation of the two kinds of activities, show that cholecystokinin-like activity may be more dependent on the structure of the N-terminal region of N-protected CCK 27-33, whereas pancreozymin-like activity appears to be less demanding on the structural features of the residue in position 32.

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SYNTHESIS AND BIOLOGICAL ACTIVITY OF SOMATOSTATIN ANALOGS OF REDUCED RING SIZE

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A cyclic heptapeptide analog of somatostatin, cyclo-(Aha-Phe-Phe-*D*-Trp-Lys-Thr-Phe) (**1**), which is as potent as the natural hormone for inhibition of the release of growth hormone, insulin, and glucagon, has been designed and synthesized.[1] Believing that the four amino acids of the β-turn segment (residues 7-10) in somatostatin contribute most to inhibition of the release of these three hormones, we chose these residues in analog **1** for systematic variation. We hoped that biological evaluation of our analogs would help us assess the relative influence of residues 7-10 on such factors as metabolic stability, molecular rigidity, and side chain contribution to receptor binding.

We found that, in parallel to the behavior of somatostatin, our *L*-Trp[8] analog **14** (see Table) was considerably less active than the *D*-Trp[8] isomer **1**.[1] Thus, in order to maintain the highest possible potency, we decided to incorporate *D*-residues at position 8; and in light of the inactivity of *D*-Phe-[7], *D*-Lys-[9], and *D*-Thr-[10] somatostatin,[2] as well as the low activity of analog **14,** we chose to evaluate a number of analogs as diastereoisomeric mixtures (incorporating the substitute amino acid as a racemic mixture).[3]

The general approach used for synthesis of these analogs has been detailed elsewhere.[1,4] The protected linear precursors were prepared by the solid phase method of peptide synthesis; N^{α}-Boc amino acids were obtained from commercial sources or prepared by standard methods from the corresponding amino acids.[5]

Direct chemical modification of certain analogs has led to a series of novel residues at position 9. The *p*-NH_2-Phe[9] analog (**29**) was readily secured by reduction of the *p*-NO_2-Phe[9] analog (**28**) using zinc in acetic acid. The Arg[9] (**23**) and *homo*-Arg[9] (**24**) analogs were conveniently prepared, respectively, from the Orn[9] analog (**22**) and **1,** employing 1-guanido-3,5-dimethyl pyrazole[6] in DMF. In similar fashion, the Lys[9] N-acetimido analog **28** was prepared from **1** using methyl acetimidate hydrochloride[7] in DMF. This analog was of special interest because

Table: Biological Properties of Analogs.[a]

cyclo-(Aha-Phe-Phe-D-Trp-Lys-Thr-Phe) 1
7 8 9 10

Inhibition of release (relative potency of somatostatin = 1)

Cmpd	Residue Substitution[b]	Glucagon	Insulin	GH (in vitro)
1	None	0.86 (0.44-1.53)	0.88 (0.30-2.45)	0.93 (0.69-1.2)
2	Tyr^7	0.42 (0.09-0.85)	0.69 (0.37-1.20)	0.96 (0.78-1.17)
3	Trp^7	0.30 (0.09-0.80)	1.06 (0.64-1.84)	0.06 (0.04-0.09)
4	His^7	low act.[c]	0.27 (0.09-0.69)	0.28 (0.19-0.36)
5	*p-F-Phe^7	0.07 (0.01-0.40)	0.04 (0.02-0.06)	0.05 (0.02-0.13)
6	p-Cl-Phe^7	0.65 (0.15-1.52)	1.08 (0.57-2.11)	0.72 (0.61-0.85)
7	p-NO_2-Phe^7	2.91 (0.52-31.9)	0.63 (0.4-0.99)	0.29 (0.15-0.49)
8	p-NH_2-Phe^7	0.47 (0.12-1.56)	0.68 (0.39-1.06)	0.029 (0.024-0.036)
9[d]	*Thi^7	0.52 (0.11-1.62)	0.42 (0.19-1.02)	—
10[e]	*Thz^7	0.93 (0.1-5.18)	0.99 (0.65-1.52)	0.20 (0.09-0.38)
11[f]	*$Pyrz^7$	0.94 (0.21-4.1)	1.4 (0.55-3.8)	0.92 (0.82-1.03)
12	Leu^7	0.33 (0.05-1.05)	0.63 (0.39-0.99)	0.95 (0.84-1.07)
13	Ala^7	0.68 (0.06-6.25)	0.25 (0.17-0.38)	0.16 (0.10-0.26)
14	Trp^8	0.041(0.004-0.15)	0.10 (0.05-0.19)	0.14 (0.12-0.16)
15	*1-Me-Trp^8	0.14 (0.05-0.32)	0.26 (0.11-0.56)	0.56 (0.47-0.66)
16	*5-Me-Trp^8	<0.02	0.04 (0.03-0.08)	0.07 (0.02-0.15)
17	*5-F-Trp^8	0.95 (0.11-7.80)	0.26 (0.10-0.64)	0.15 (0.07-0.33)
18	*6-F-Trp^8	0.41 (0.32-0.52)	0.21 (0.09)-0.41)	0.41 (0.32-0.52)
19	*5-Br-Trp^8	low act.[c]	<0.07	0.02 (0.01-0.03)
20	*5-MeO-Trp^8	<0.02	0.17 (0.06-0.40)	0.02 (0.01-0.04)
21	*7-Aza-Trp^8	<0.02	0.07 (0.02-0.18)	0.07 (0.05-0.1)

Table: Biological Properties of Analogs[a] (continued).

Cmpd	Residue Substitution[b]	Glucagon	Insulin	GH (in vitro)
22	Orn^9	<0.02	<0.02	0.05 (0.02-0.10)
23	Arg^9	<0.07	<0.07	<0.07
24	homo-Arg^9	<0.07	<0.07	0.26 (0.17-0.41)
25	$^{\epsilon}$MeLys^9	<0.01	<0.01	—
26	$^{\epsilon}$Me$_2$Lys^9	<0.07	<0.07	<0.05
27[g]	$^{\epsilon}$AcimLys^9	<0.02	<0.02	0.10 (0.04-0.25)
28	p-NO$_2$-Phe^9	<0.02	<0.02	<0.05
29	p-NH$_2$-Phe^9	<0.07	—	0.011 (0.009-0.014)
30	Ser^{10}	<0.07	<0.07	—[h]
31	*βOH-Val^{10}	0.44 (0.18-1.96)	ca. 0.2[c]	0.62 (0.39-1.07)
32	Val^{10}	—[h]	—[h]	—[h]
33	Ile^{10}	—[h]	—[h]	<0.05
34	α-Me-Ala^{10}	<0.07	<0.07	0.02 (0.001-0.02)
35[i]	Acpc^{10}	<0.07	<0.07	0.15(0.12-0.17)

[a]See ref. 4 for a detailed description of the biological methods; 95% confidence limits in parentheses (all analogs showed <10% the potency of somatostatin for inhibition of gastric secretion). [b]Numbering as in **1** (somatostatin numbering); unless otherwise noted, all residues are of L-configuration; asterisk indicates *DL*-residue (*ca.* 50:50 mixture). [c]Poor dose-response relationship. [d]β-(2-thienyl)-Ala. [e]β-(4-thiazolyl)-Ala. [f]β-(1-pyrazolyl)-Ala. [g]Acetimido. [h]Lack of solubility prevented meaningful bioassay. [i]1-amino-1-cyclopentanecarbonyl.

amidines have approximately the same pK as amines.[8] Reductive methylation of **1** with formaldehyde/sodium borohydride in isopropanol-water[9] proved to be a convenient approach to both the Lys^9 N-monomethyl (**25**) and N,N-dimethyl (**26**) analogs (*ca.* 3:1 ratio), which were readily separated by silica gel chromatograhy. Catalytic reductive methylation[10] was less suitable, affording only the dimethyl analog **26.**

The biological properties of our analogs are presented in the Table. Biological effects were determined by methods previously described.[1,4] Thus, we note the wide range of aromatic nuclei allowed at position 7 without marked effect on potency. The fact that deletion of the aromatic ring only slightly lowers activity (see analogs **12** and **13**) suggests a minimal role for the position 7 side chain in receptor interaction.

At position 8, little latitude exists for change without drastic lowering of activity. The indole 5-substituted analogs, save for the 5-fluoro (**17**), show marked loss of activity relative to **1**. Since the various tryptophan analogs have differing electronic charge distributions, their reduced activity is most likely due to increased steric bulk (fluoro analog **17**, least bulky, preserves bioactivity). Interestingly, these results stand in contrast to the high level of activity seen when the same position 8 substitutions are incorporated into somatostatin itself.[11]

Results from variation at position 9 show a very stringent requirement for the lysine side chain; any derivatization of the ϵ-amine, even replacement with a comparably basic side chain (*cf.* **27**), eliminates activity. Chain length from backbone to amino group is also important (*cf.* **22**). Moreover, the inactivity of the two N-methylated analogs **25** and **26** suggests the importance of having *primary* amine.

Analogs at position 10 which represent deletion (Ser[10]) and addition (*DL*-β-OH-Val[10]) of methyl (**30** and **31**, respectively), and α-substitution (**34**) exhibit greatly diminished solubilities in the biological test systems. Low measured potencies may reflect this low solubility, rather than intrinsic activity. That such seemingly minimal structural changes so markedly affect physical properties suggests that threonine plays a key role in determining the favored conformation for peptide **1**.

Acknowledgements

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FURTHER DATA ON THE STRUCTURE-ACTIVITY RELATIONSHIP OF NEUROTENSIN

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Introduction

Previous studies underlined the major contribution of the COOH-terminal sequence (e.g. H-Arg-Pro-Tyr-Ile-Leu-OH) of the tridecapeptide neurotensin (NT) to the biological activity of this peptide.[1,2,3,4,5,6] The prominent role played by the Tyr[11] residue in the process of receptor activation by NT was emphasized.[5,6,7] NT analogs in which Tyr[11] was replaced with Ala, Leu, D-Tyr or D-Phe were found to be weak agonists in several isolated tissues, but devoid of inhibitory properties toward NT.[5,6,7] On the other hand, the replacement of Tyr[11] with L-Tyr(Me) or D-Trp resulted into selective antagonists of NT.[6,7] Unfortunatley, the latter two NT antagonists suffered three major disadvantages: first, their affinity for NT receptors was low; second, they retained intrinsic activity; third, their antagonistic properties could not be observed in all the tissues in which NT produces an action. These results led us to pursue our effort toward the development of new, more potent NT antagonists.

Methods

Several NT analogs in which Tyr[11] was replaced alternatively with L-dihydroxyphenylalanine (Dopa), *p*-iodo-Phe, cyclohexylalanine (Cha), N-MePhe, *p*NO_2Phe, His, Thr, Tyr(Me), Phe and D-Trp were prepared using the solid-phase method[8] according to procedures described previously.[9] Other NT analogs synthesized were NT-amide and a few derivatives of [D-Trp[11]]-NT. All these NT analogs were evaluated for their ability to contract rat isolated portal veins and stomach strips and for their possible inhibitory action toward NT. The biological activity or inhibitory action of NT derivatives was measured as described previously.[5,6,7]

Results and Conclusions

As shown in Table I, the presence of an additional hydroxyl (OH) group in the *meta* position of the Tyr[11] aromatic ring did not alter markedly the potency of NT (see compound **3**). On the other hand, removing the OH group of Tyr[11] (e.g. [Phe[11]]-NT) or replacing it with a

Table 1. EC_{50} Values and Relative Potency (R.P.) of Neurotensin (NT) and NT Derivatives as Measured *in Vitro* Using Rat Isolated Portal Veins and Stomach Strips.

No.	Peptide	RAT PORTAL VEIN EC_{50} ($\times 10^{-8}$M)	R.P (%)	RAT STOMACH STRIP EC_{50} ($\times 10^{-8}$M)	R.P (%)
1	NT-COOH	1.6 ± 0.1(40)	100	1.1 ± 0.1(50)	100
2	NT-$CONH_2$	1100.0 ± 160.0(8)	0.15	96.0 ± 26.0(7)	1.2
3	[Dopa^{11}]-NT	1.7 ± 0.1(8)	94	1.1 ± 0.1(8)	100
4	[Phe^{11}]-NT	17.0 ± 1.0(8)	9.4	6.7 ± 1.8(8)	16.4
5	[p-I-Phe^{11}]-NT	150.0 ± 10.0(4)	1	18.0 ± 3.0(4)	6
6	[Cha^{11}]-NT	310.0 ± 20.0(6)	0.5	360.0 ± 30.0(6)	0.3
7	[N-Me-Phe^{11}]-NT	280.0 ± 30.0(4)	0.6	110.0 ± 30.0(5)	1.0
8	[Tyr(Me)^{11}]-NT	610.0 ± 80.0(8)	0.3	240.0 ± 10.0(6)	0.5
9	[p-NO_2-Phe^{11}]-NT	3300.0 ± 300.0(4)	0.05	1000.0 ± 100.0(4)	0.1
10	[D-Trp^{11}]-NT	790.0 ± 90.0(8)	0.2	160.0 ± 10.0(6)	0.7
11	[His^{11}]-NT	6400.0 ± 900.0(4)	0.03	460.0 ± 50.0(4)	0.2
12	[Thr^{11}]-NT	6000.0 ± 500.0(6)	0.03	5000.0 ± 500.0(6)	0.02

The primary structure of NT is as follows: pGlu^{1}-Leu^{2}-Tyr^{3}-Glu^{4}-Asn^{5}-Lys^{6}-Pro^{7}-Arg^{8}-Arg^{9}-Pro^{10}-Tyr^{11}-Ile^{12}-Leu^{13}-OH. EC_{50} = Effective concentrations of NT or NT derivatives producing 50% of the maximum response to NT. EC_{50} values are expressed as means ± the standard error of the mean. The number of individual determinations is given in parentheses. The relative potency of NT-COOH was arbitrarily fixed to 100%

methoxyl (CH_3O) group (e.g. [Tyr(Me)^{11}]-NT greatly reduced the potency of NT but did not affect its intrinsic activity (e.g. ability to elicit the same maximum response at NT); the maximum decrease of potency being observed with the latter chemical modification (compound **8**). These results either suggest the major contribution of the *p*-OH group of Tyr^{11} to the affinity of NT for its receptors or the important conformational role of an intramolecular hydrogen bond involving the phenolic proton. They also emphasize that this group is not essential for triggering the biological event (e.g. contraction of smooth muscle). The replacement of the *p*-OH group of Tyr^{11} with an electron-withdrawing, bulky group (e.g. NO_2) or atom (e.g. iodine) also reduced markedly the affinity,

but not the intrinsic activity, of NT for its receptors. The largest reduction of affinity was observed with the former modification (e.g. OH replaced by NO_2). The reduction of potency observed with NT derivatives in which Tyr^{11} was replaced with Cha, His or Thr (compounds **6**, **11** and **12**) gives further support to our previous hypothesis suggesting that a properly space-oriented aromatic side chain in the eleventh position of NT optimizes the affinity for the receptors.[5,6,7] The latter compounds exhibited no inhibitory properties toward NT in the rat stomach strip or portal vein. The low potency of [N-Me-Phe^{11}]-NT compared to [Phe^{11}]-NT either precludes the stiffening of the backbone near the aromatic residue or suggests the involvement of the substituted N-H amide proton in an important intramolecular hydrogen bond. Moreover, [N-Me-Phe^{11}]-NT did not inhibit NT-induced contractions in the two tissues used. Blocking the C-terminal carboxyl function of NT with an amide group reduced the potency of NT by factors of 700 (portal vein) and 80 (stomach strip), respectively. This result suggests the major contribution of the free COOH-terminal group of Leu^{13} to the affinity of NT for its receptors[2,10] and/or the involvement of the negatively charged carboxylate ion into an electrostatic bond with the positively charged guanidino group of Arg.[9] The latter possibility arises from the study of CPK models. The intrinsic activity of NT-amide could not be measured accurately, but there was no clear tendency for the intrinsic activity of NT-amide to be decreased.

The replacement of Tyr^{11} with D-Trp reduced the affinity of NT for its receptors both in the rat portal vein and stomach strip (Table I). The intrinsic activity of this compound was slightly reduced ($\alpha^E = 0.9$) in the portal vein but not in the rat stomach strip. The most interesting result concerning this compound arose from the fact that relatively low concentrations of [D-Trp^{11}]-NT selectively inhibit the constrictor effect of NT in the rat portal vein[7] and perfused rat heart[6] without affecting its contractile effect in the rat stomach strip or its inotropic effect on the guinea pig atria.[6,7] Removing the sequence $pGlu^1$ through Pro^7 reduced the inhibitory potency of [D-Trp^{11}]-NT_{8-13} by a factor of 4. Since N-acetyl-[D-Trp^{11}]-NT_{8-13} is almost as potent an antagonist as [D-Trp^{11}]-NT, we are optimistic that potent NT antagonists containing 6 amino acids and less could be designed in the near future. The intrinsic activity exhibited by [D-Trp^{11}]-NT or [D-Trp^{11}]-NT derivatives clearly suggests that D-Trp is not an ideal substituent for Tyr^{11} if pure (e.g. devoid of intrinsic activity) NT antagonists are to be developed. We believe however that further chemical modifications of [D-Trp^{11}]-NT derivatives will lead to the development of pure NT antagonists.

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COMPARISON OF NATURAL AND SYNTHETIC PREPARATIONS OF VASOACTIVE INTESTINAL POLYPEPTIDE (VIP) BY HPLC AND IMMUNOASSAY WITH VARIOUS ANTIBODIES

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Introduction

Conflicting immunometric data obtained with antibodies to VIP in different laboratories have been attributed[1] to possible heterogeneity of VIP preparations used for assay or to varying regional specificity of antibodies. Antibodies raised against the natural hormone and synthetic preparations are widely employed. In this study, natural porcine VIP and synthetic preparations are compared by HPLC and by interaction with various antibodies raised against natural and synthetic VIP.

Materials and Methods

0.05 M NaH_2PO_4 containing 0.2% human serum albumin (AB Kabi Diagnostica, Stockholm, Sweden) is referred to as solvent A. Synthesis of VIP preparation I and of partial sequences is already described.[2] Synthetic VIP preparation II was supplied by Peninsula Laboratories, Inc., San Carlos, California, and natural VIP was supplied by AB Kabi Diagnostica.

Natural VIP and synthetic preparation II (2μg) were iodinated[3] during incubation for 30 sec with $Na^{125}I$ (1 mCi; New England Nuclear, Boston, Mass.) and chloramine T (10 μg) for 30 sec in 0.2 M NaH_2PO_4-Na_2HPO_4 buffer (45 μl total volume) pH 7.4. A solution (20 μl) of 0.12% sodium metabisulphite was added followed after 30 sec by a solution (0.5 ml) of 0.02% KI in solvent A. The mixture was applied to a column (25 cm x 1 cm) of SP Sephadex C25 equilibrated with solvent A. Radiolabelled VIP was isolated following irrigation with solvent A (120 ml) containing a gradient of 0 - 1.0 M NaCl.

Antibodies against natural VIP, A and B were provided by K.D. Buchanan, Queen's University, Belfast, Ireland, and C is described previously.[4] Antibody D raised against synthetic VIP was provided by T.M. O'Dorissio, Ohio State University, Columbus, Ohio.

HPLC was carried out using equipment from Waters Associates, (Milford, Mass.): M-45 solvent delivery systems, model 720 system controller, V6K injector and model 450 variable wavelength detector. VIP (100 μg) solutions in 0.1% TFA (0.5 ml) were applied to a reverse phase C_{18} column (10 cm x 4.1 mm, spherical particle size 7.5 microns, pore size 300 Å; Synchrom Inc., Linden, Indiana) equilibrated with 0.1% TFA. The column was irrigated with a linear binary gradient of 0 to 100% methanol in 0.1% TFA (200 ml; 1 ml/min). Fractions (1 ml) were collected into 0.04 M phosphate buffer pH 7.4, containing 0.3% human serum albumin.

Results and Discussion

Natural VIP appears to be essentially homogeneous whereas synthetic preparation II has a major contaminant (Figure 1). Radioiodinated natural VIP appears to be more immunoreactive than radioiodinated preparation II with all antibodies (Table I).

Table I. Reaction of ^{125}I-labelled VIP (5 pg) with Antibodies under Conditions of Radioimmunoassay.[4] Antisera were diluted 1:5000.

Antibody	% ^{125}I-labelled VIP bound	
	Synthetic	Natural
A	50	67
B	63	79
C	71	82
D	51	63

Results shown in Figure 2 suggest that antibody C binds mainly to region 1-10 of VIP whereas antibody B appears to have some dependence on region 10-16 in addition to 1-10. Antibodies A and D were similar to B.

The difference in properties of the VIP preparations observed here could be due to deamidation or oxidation of methionyl residues but are not likely to give rise to major inter-assay discrepancies with the antibodies available. This might occur, however, with antibodies specific for the central to C-terminal region of the hormone.

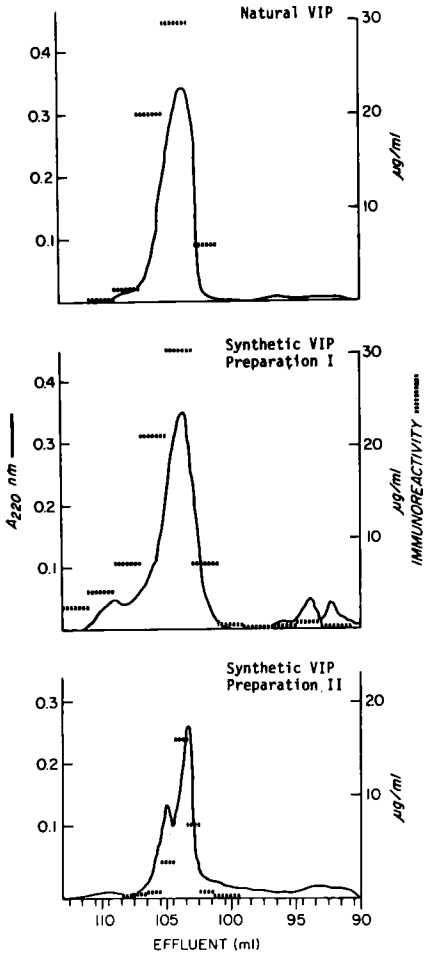

Fig. 1. HPLC of VIP preparations.

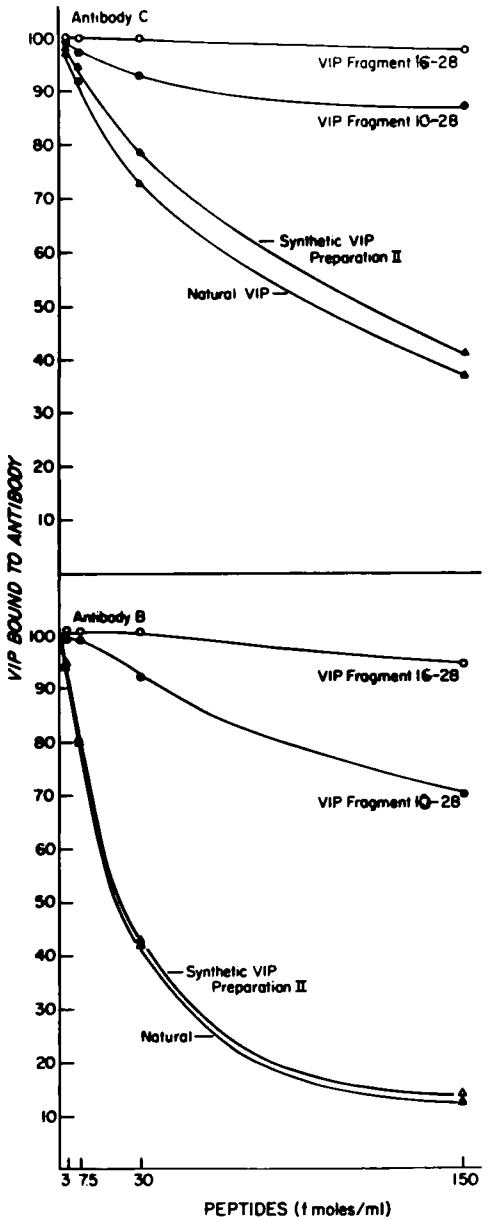

Fig. 2. Interaction of antibodies with VIP preparations and fragments under radioimmunoassay conditions. Radiolabelled natural VIP was used as tracer.

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STUDIES ON BOVINE AND HUMAN GROWTH HORMONE-RELEASING FACTORS

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Introduction

The secretion of growth hormone (GH) from the pituitary gland is regulated by a dual neural control mechanism, comprising both stimulatory[1] as well as inhibitory hypophysiotrophic factors[2] although the major mediator of GH at the anterior pituitay level is postulated to be GH-releasing factor (GHRF).[1,3,4] Experimental proof for the existence of GHRF activity in hypothalamic extracts was presented by Schally and his coworkers.[5,6] Although the chemical characterization of a GHRF peptide H-Val-His-Leu-Ser-Ala-Glu-Glu-Lys-Glu-Ala-OH purified from porcine hypothalami has been reported,[7] the failure of the native material or its synthetic equivalent to show any release of radioimmunoassayable GH,[8] poses the problem of a still unidentified GHRF. Recently, after systematic purification of bovine hypothalamic extracts, we have obtained a GHRF active peptide which releases immunoreactive GH from rat anterior pituitaries.[9] We also observed GHRF activity in the plasma of certain acromegalic patients (AM) with very high circulating levels of GH, when compared with plasma from age and sex-matched normal subjects. In this paper we briefly describe the purification and characterization of GHRF from bovine hypothalamic extracts and also from human acromegalic plasma.

Experimental Subjects, Methods and Materials

After obtaining informed consent, blood (50 ml at one time) was collected in EDTA from 4 acromegalic pateints who were admitted to the Specialized Diagnostic and Therapeutic Unit of the VA Medical Center. The plasma was separated by centrifugation, the high mol. wt. proteins precipitated by treatment with methanolic AcOH (MeOH:.1M AcOH, 1:1), the supernatant collected after centrifugation, MeOH removed in vacuuo and lyophilized at -75°C. The resultant dry powder containing the low mol. wt. polypeptides was subsequently fractionated on a G-25 column (2.6 x 100 cm) at 4°C.

Bovine hypothalamic extracts were prepared, lyophilized and fractionated initially on Sephadex G-25 and tested for GHRF or GHIF activity by ascertaining its capacity to stimulate or inhibit the GH-release, either *in vitro* in a perfusion system using rat anterior pituitary halves, or *in vivo* in adrenalectomized rats.[9] The rGH levels in the medium or in the rat serum (at 0 and 15 min) were determined by a double antibody radioimmunoassay (RIA) as described previously.[9]

In Vitro Perfusion

Sprague-Dawley, male or female rats (150-200 g, b.wt., 7 wks old) were used for collection of anterior pituitaries which were sliced into 2 equal halves and separated into groups consisting of 12 halves. One group of 12 slices was used for the peptide perfusion while the other 12 halves served as control receiving blank buffer. The tissue after incubation for 30 min in Krebs-Ringer bicarbonate buffer was transferred to the sintered disc of the special glass perfusion cell[10] through which the test solution containing the GHRF peptide (1 ng/ml) in the buffer or the buffer alone was perfused in presence of 95% O_2 + 5% CO_2. The known secretagogues K^+ (56 mM) and Ca^{++} (3mM) were allowed to mix with the buffer and perfuse through the tissue at the 2nd (K^+) and 8th (Ca^{++}) min onwards. One ml aliquots were collected each minute and the medium was assayed for rGH levels by RIA.

Further purification of GHRF active peptide fractions obtained from the bovne hypothalamic extracts was effected by affinity chromatography[9] followed by counter current distribution using BuOH:AcOH:H_2O = 4:1:5 two phase system.[11,12] Preliminary thin-layer (TLC) chromatographic separation on cellulose of the acromegalic GHRF and GHIF peptides was carried out as described previously.[13,14] Enzymatic, Edman, and mass spectral degradation studies were performed according to reported methods.[9,13,14]

Results and Discussion

The fractionation of the low molecular weight peptides from the acromegalic plasma yielded 4 major polypeptide peaks. The first was in the exclusion volume of G-25 and had a GH-content of 1067.5 ± 542.4 ng % for acromegalics and 30.0 ng% for normals, as determined by RIA or densitometry. The peak III peptides from 3 acromegalic patients showed significant GHRF activity, 309.7 ± 59.2 ng rGH/ml/pit., in the medium: control perfusion, 38.4 ± 24.3 ng rGH/ml/pit. ($p<0.01$). Corresponding fractions from normal plasma did not show any GHRF activity (Figures 1A, B) but inhibited GH-release (Peak III-peptides showed 47% and

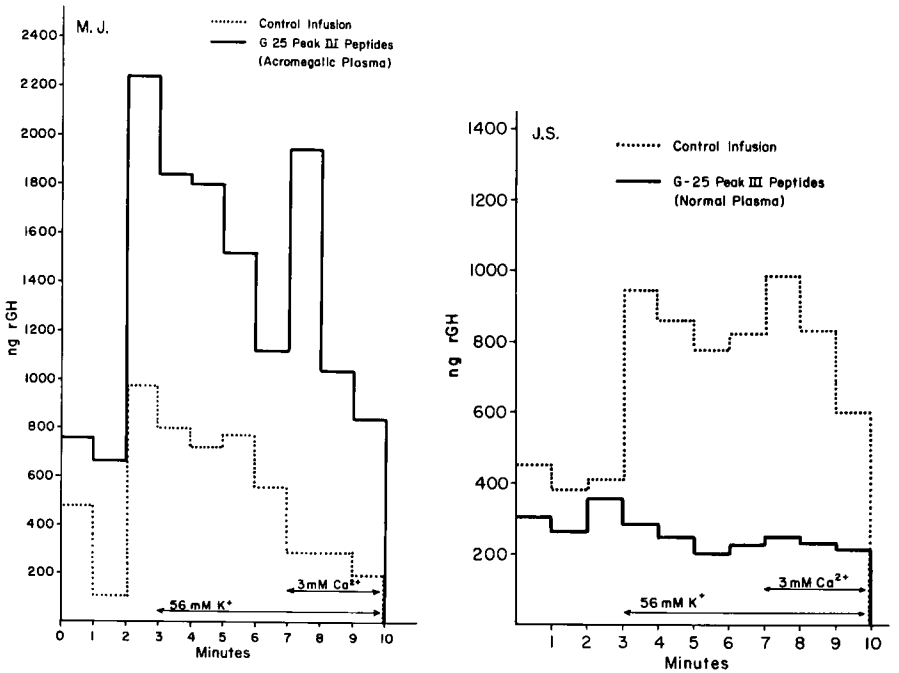

Fig. 1A, GH-RF activity (AM), peak III peptides and Fig. 1B, GHIF activity in terms of ng rGH/ml (from 6 rat ant. pituitaries).

Peak II, 57% inhibition). Peptides of peak III from the plasma of a 4th acromegalic did not show any GHRF activity (63.5 ± 10.8 ng rGH/ml/pit.). The bGHRF showed a ninefold elevation of rGH in the same test system.

Thin-layer chromatographic and electrophoretic comparison of the human GHRF peptides with authentic synthetic samples of other known hypothalamic peptides and the bGHRF indicated that hGHRF and bGHRF are nonidentical. The GH-inhibitory fraction obtained from the normal and from the 4th acromegalic's plasma, resembled somatostatin. Amino acid analysis on acid hydrolyzates of the bGHRF yielded Lys 3, His 1, NH_3 2, Arg 1, Thr 1, Ser 1, Glu 4, Gly 5, Cys 2, Tyr 1, Phe 1. Various enzymatic degradation studies and subsequent testing indicated destruction of biological activity by pyroglutamyl peptidase, trypsin and carboxypeptidase. Edman-dansyl degradation and aminopeptidase M treatment yielded negative results. Mass spectral fragmentation showed high intensity peaks at 111 ms units (m/e) in addition to others.

We have presented evidence for the existence of GH-RF activity in bovine hypothalamic extracts and human acromegalic plasma. The bGHRF is not chromatographically identical to hGHRF. It possesses a blocked N-terminus probably occupied by a pyroglutamyl moiety. The C-terminal amino acid of bGHRF is free. Degradation by trypsin indicates Lys and/or Arg. This is also confirmed by the amino acid analysis.

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PEPTIDE BOND MODIFICATION AND ITS EFFECT ON CONFORMATIONAL MIMICRY

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Introduction

One major objective in current research on peptides is the determination of their receptor-bound conformation. Numerous studies have shown that interaction with the receptor, an asymmetric environment, induces or captures a conformer which is not predominate in solution.[1] A well-characterized prototype of this situation is seen with the S-peptide of ribonuclease which shows two turns of alpha helix in its crystalline complex with S-protein and no helix detectable in the free peptide.[2] One strategy to determine the receptor-bound conformation is the systematic introduction of chemical modifications with predictable conformational effects. Retention of biological activity implies that the conformational constraints imposed by the modification are tolerated by the receptor.

An alternative use of conformational analysis is the evaluation of conformational effects prior to synthesis in order to help rank potential modifications. One difficulty in interpreting structure-activity data is that each chemical modification affects multiple parameters. For example, replacement of the amide bond with a trans-double bond changes steric and electronic (dipole) parameters, hydrogen bonding, lipophilicity, and enzymatic stability as well as modifying the geometry. In order to determine which parameter is dominant in determining the resulting biological activity, one must be able to evaluate these parameters for a variety of analogs in order to separate variables. One parameter which we feel to be useful in evaluating potential modifications is conformational mimicry. By this, one means the degree to which conformers available to the parent are available to the analog.

Methods

In order to evaluate the conformational mimicry parameter for the many amide bond modifications under study,[3] one must establish criteria for comparison of potential conformers. This is not straightforward as the normal approach, *i.e.* comparison of torsional angles, is not appropriate due to changes in bond angles and bond lengths introduced by the amide modifications. In some cases, an extra degree of torsional freedom

has been introduced by replacement of the amide with a bond not partially conjugated. Comparison of the orientation of the sidechain and subsequent peptide chain following the modification was chosen as the criterion. For each sterically allowed conformer of the dipeptide, acetyl-Ala-Ala-methylamide, a vector corresponding to the carbon alpha-carbon beta bond of the second alanine residue was stored. This resulted in a three-dimensional vector map containing the locus of alpha-beta bonds available to the parent compound at the selected step size for the torsional angles. An example of such a map is shown in Figure 1 where the amide between the alanine residues is *cis*. Using this locus map as a reference, possible alpha-beta bond positions for the analog were evaluated. If a similar vector was found, then the appropriate vector in the locus map was scored for mimicry.

An additional tally of number of allowed conformations was kept in order to provide a rough indication of the effect on entropy of the proposed modification.

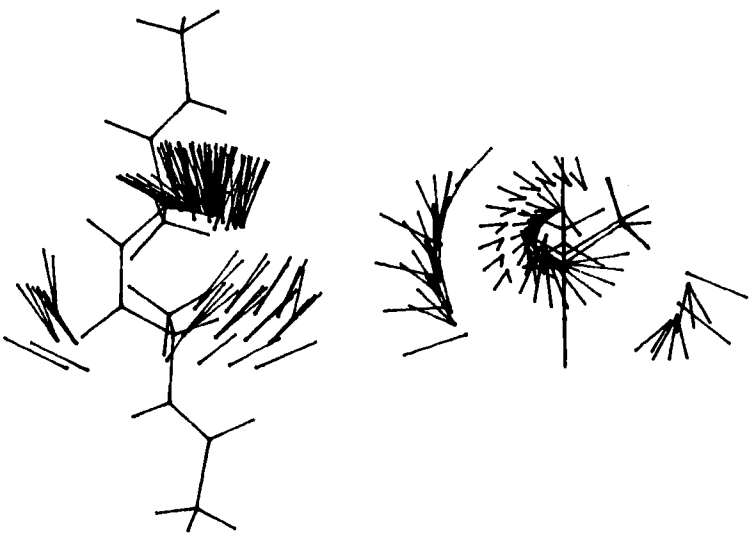

Fig. 1. Two orthogonal views of the alpha-beta bond locus map for a *cis*-peptide bond superimposed on the peptide.

Results

Two case studies will be presented in order to illustrate the approach. First, we have prepared an analog of the *cis*-amide bond in order to evaluate its role at the receptor in TRH and angiotensin. In both peptides, NMR studies have suggested[4] a possible correlation between

the presence of a *cis*-proline conformer and biological activity. Conversion of the amide to the tetrazole of Z-Phe-Ala-methyl ester and subsequent addition of pGlu and conversion to the amide gave pGlu-Phe-*tet*-Ala-amide, an analog of [Phe^2]-TRH in which the amide corresponding to that of Pro^3 is fixed in *cis*-orientation. A comparison of the crystal structures of *cis*-peptide bonds and a tetrazole showed almost identical values for C-N bond lengths, and the carbon alpha-carbonyl-nitrogen and carbonyl-nitrogen-carbon alpha bond angles were nearly the same as the analogous angles in the tetrazole. Geometrical differences were, therefore, minimal and except for an increase in steric bulk, the tetrazole is an excellent analog of the *cis*-peptide bond. This analog was found to be devoid of biological activity in stimulating the release of TSH from rat pituitary cultures.[5]

These results could be interpreted simply that the *cis*-amide is not a prerequisite for biological activity in TRH. Comparison of the mimicry parameter for the tetrazole using the *cis*-amide as parent shows that only 22% of the conformers available to the parent are available to the analog. Figure 2 shows the vectors available to the tetrazole which are also available to the *cis*-amide. Comparison with Figure 1 shows the sharp reduction in alpha-beta vectors which reflects the limited mimicry due to the conformational effects of the increased steric bulk of the tetrazole. Obviously, other analogs such as the *cis*- and *trans*-double bond replacing the His-Pro amide should be prepared in order to unambiguously resolve this issue.

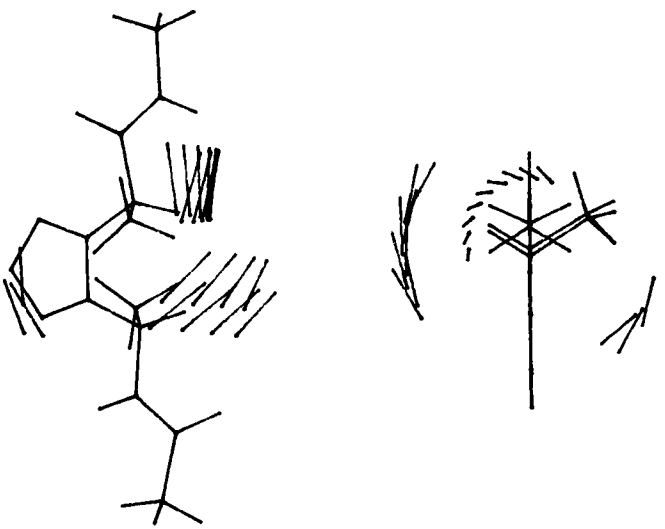

Fig. 2. Two orthogonal views of the locus map of vectors in common between the *cis*-amide and tetrazole superimposed on the analog.

A second case involves a comparison of two candidate modifications of the *trans*-amide bond which have been suggested to prevent enzymatic degradation. The retro-amide bond has resulted from topochemical arguments[6] and the *trans*-double bond is a rather obvious choice, although both require considerable synthetic effort. By the mimicry criterion, the *trans*-double bond is clearly preferred with 29% overlap in possible conformers while the retro-amide shows only 8% overlap. These numbers are based on an accuracy in vector overlap of 0.25 angstrom. If this accuracy is relaxed to 0.40 angstrom, then the overlap of the *trans*-double bond is essentially complete while that of the retro-amide bond remains near 10%. Variation of the accuracy is useful as changes in bond length and bond angle prevent complete identity and vary from case to case with the geometrical changes between the parent and the analog. These examples should illustrate the use of the mimicry parameter as a criterion for selection of analog when the receptor-bound conformer has not been elucidated.

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COMPUTER GRAPHICS AND CHEMICAL SYNTHESIS IN THE STUDY OF CONFORMATION OF BIOLOGICALLY ACTIVE PEPTIDES

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As part of an ongoing effort to define the role of peptide conformation in the expression of biological activity, we have studied novel conformationally constrained analogs. Such compounds incorporate modifications in the peptide backbone which limit or modify the conformational possibilities available to the peptide.[1] A number of these changes coupled with their effects on biological activity can provide much information about the biologically active conformation[2] at a specific receptor.[3] Conformational modifications may also result in more potent, longer acting, and more selective analogs by stabilizing a bioactive conformation or eliminating metabolized conformers or those producing unwanted activities.[4]

Interactive computer graphics and chemical synthesis are used as complementary tools in this approach to studying peptide conformation. The Merck Molecular Modeling System[5] is designed so that a synthetic chemist, with minimal training in computers, can utilize the system for his particular research problems. Computer experts are available for consultation as needed. Several computer capabilities have proven of particular value in our studies. We are able to display structures and study their three dimensional properties, as illustrated in Figure 1, by acetyl-alanyl-alanine methylamide. Currently, we generate such structures from X-ray coordinates, by putting suitable torsion angles into the ECEPP program,[6] or by inputting all bond lengths, angles, and dihedral angles. The structures may be displayed and studied in two dimensions or as stereo pairs, and as stick or spacefilling models. It is possible to view all or any part of a structure and to look at it in any orientation.

Once a structure is created, a number of measurements and manipulations can be carried out. Various molecular distances and angles can be determined precisely due to the fixed nature of the structure. The computer structure has a distinct advantage over hand held models in this respect. The conformation can be modified as desired by rotating about any of the torsion angles not in a ring. For example, rotation about ϕ_2, ψ_2, and ϕ_3 in the Type I β-turn of Figure 1 by 120°, -90°, and 10° produces a new conformation, a Type II′ β-turn.[7] Smaller structures may

be strain energy minimized[8] to find low energy conformations and to compute their relative energies. Energies may be calculated for specific conformations of larger peptides. Plots of energy versus dihedral angle can be generated for rotation about individual bonds in a structure.

A particularly useful feature of the Merck Modeling System is the ability to compare two molecules by three dimensional superposition. The two structures are superposed by matching at least three pairs of atoms and calculating the least squares fit. Comparisons may be performed on two rigid structures, or one rigid and the other having one or more freely rotating torsion angles. When the best match is obtained, deviations for individual matches and the average deviation for the complete match are measured. This process is highly interactive, and many different comparisons are often made between two structures in order to focus on a "best fit". Superposition of structures has been applied to the design of conformational constraints for testing conformational hypotheses and to the design of new analogs based on active peptide leads. This technique has also been used to show that peptides and their retro enantiomers are not topologically identical due to differences in bond lengths and angles.[9] This kind of understanding may serve as a step in the eventual design of useful D-retro analogs having modifications which might correct these differences. The superposition of the two β-turns in Figure 1 illustrates the usefulness of this capability for comparing structures.

Consideration of the computer models in Figure 1 suggests ways in which conformational constraints could be incorporated to stabilize these turns either covalently or noncovalently. It can be seen that the α-nitrogen and the β-carbon of Ala^2 in the Type I turn could be connected through a ring with minimal distortion of the conformation. For example, proline in the i + 1 position is known to stabilize this turn. Similarly, the β-methyl of D-alanine in the i + 1 position of the Type II' turn would occupy a pseudo equatorial location resulting in a stabilization effect. Also N-Me-Ala^3 in combination with D-Ala^2 would be favorable.[10] Secondary amino acids such as Pro or N-Me-Ala also introduce the new possibility of conformations with *cis* peptide bonds.

Computer modeling studies have often suggested conformational constraints which are not composed of known amino acids or simple derivatives. In such instances, we have been challenged to go beyond clauctures. One example of such a conformational restriction is the lactam synthesis in order to obtain the novel constrained strin which a covalent bridge is introduced into the peptide structure from the α-

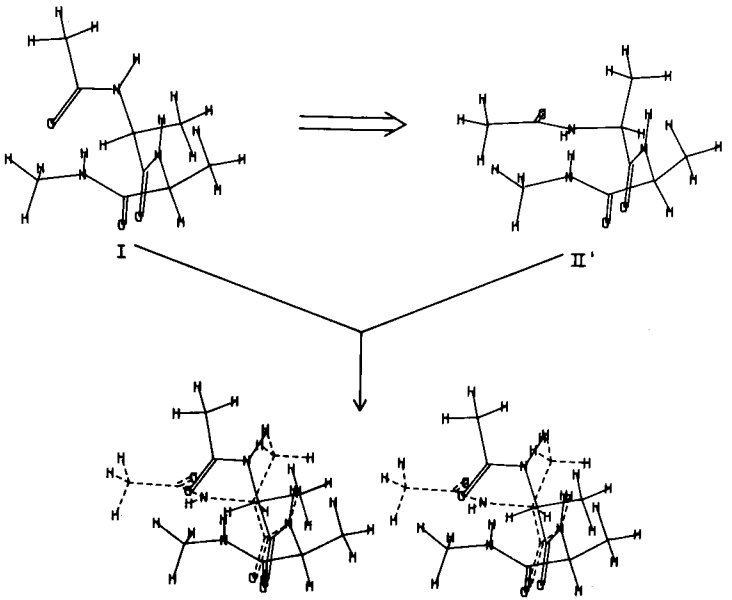


Fig. 1. Comparison of Types I and II 'β-turn conformations of Ac-Ala-Ala-NHMe.

carbon of one residue to the α-nitrogen of the next amino acid. An example showing the fit of a γ-lactam constraint with a Type II' β-turn is shown in Figure 2. We have investigated the use of γ, δ, and ϵ-lactams, and each of these has necessitated the development of new synthetic methods.

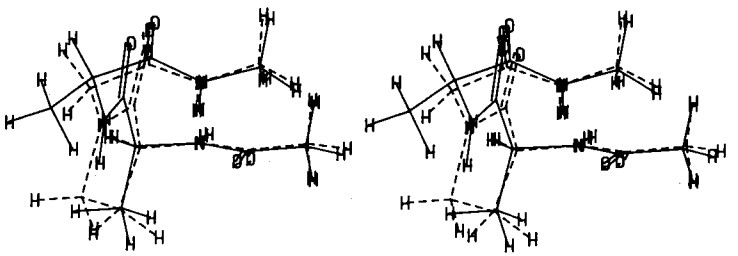
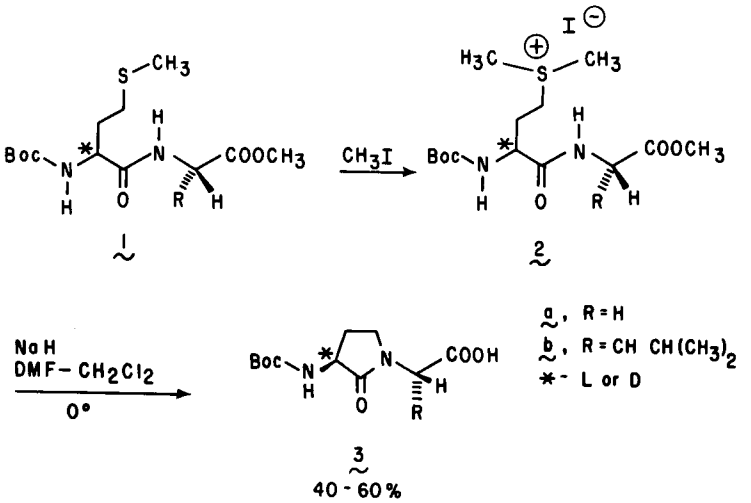


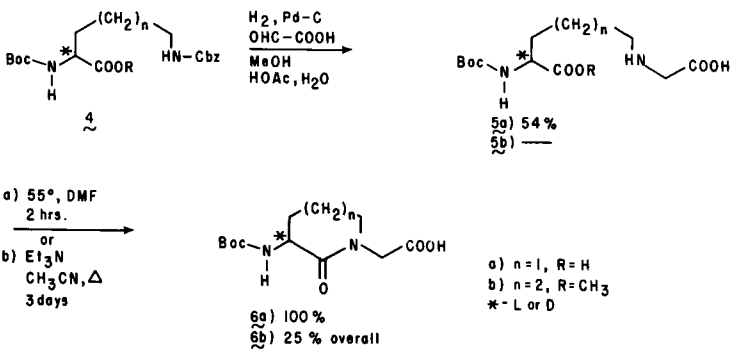
Fig. 2. Fit of a γ-lactam conformational constraint (dashed) with a Type II ' β-turn (solid).

The synthetic routes to these compounds are illustrated in Schemes 1-3.[11] The γ-lactams **3** are produced by a novel intramolecular alkylation of a methionine sulfonium salt. Intramolecular acylation provides δ- and ϵ-lactams **6**. The thiazinone **9** is synthesized by condensation of an acetamidomethyl protected β-thio amide with formaldehyde. Except for

the last case, protected chiral amino acids are converted stereospecifically into chiral lactams. The products are prepared in good yield and have protecting groups making them suitable for incorporation into higher peptides by methods commonly used. These procedures make available a variety of lactam dipeptide derivatives for use as conformational constraints in biologically interesting peptides.

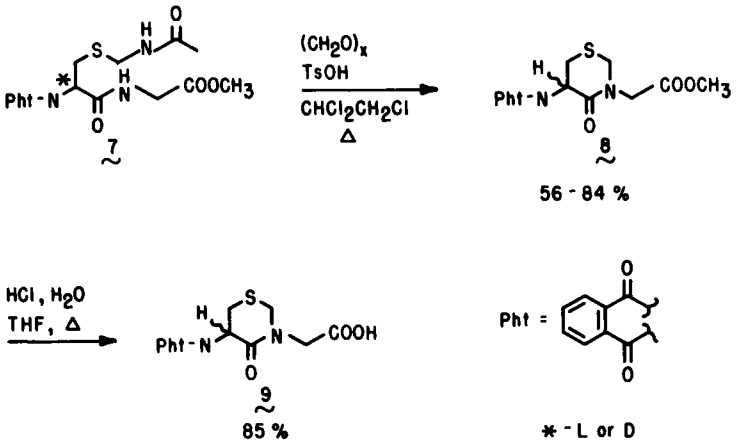


Scheme 1



Scheme 2

One of our early applications which demonstrated the utility of lactam conformational constraints was in an analog of luteinizing hormone-releasing hormone (LH-RH).[12] Based on the enhanced biological potency of D-amino acid 6-position[13] and N-methyl leucine 7-position[14] analogs as well as theoretical calculations,[15] a bioactive con-



Scheme 3

formation involving a type II′ β-turn for residues 5-8 seemed reasonable. This β-turn appears to be ideally suited to the use of a lactam conformational constraint as illustrated in Figure 2.

Computer superposition (Figure 3) illustrates the good correspondence between the backbones of the proposed LH-RH β-turn and the γ-lactam (average deviation for 6 matches, 0.13 Å). The new ring should stabilize the β-turn conformation by restricting rotation about the dihedral angle ψ_6 and forcing the Gly-Leu peptide bond to remain *trans* (ω_6). The lactam also affects ϕ_6 and ϕ_7 as a noncovalent constraint. A key point about this lactam is that the 6-position α-carbon in the ring must have the L configuration to fit the proposed β-turn. Since L amino acids at position 6 normally reduce biological potency,[13] this case is an excellent test for the utility of the lactam constraint as a conformational probe.

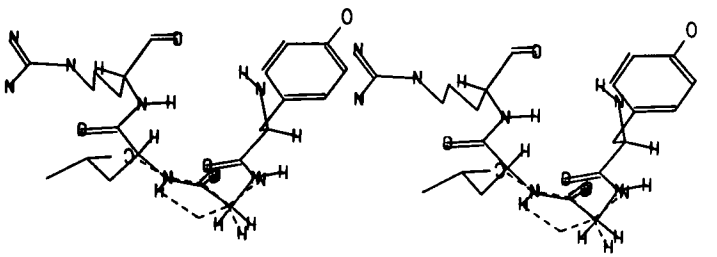
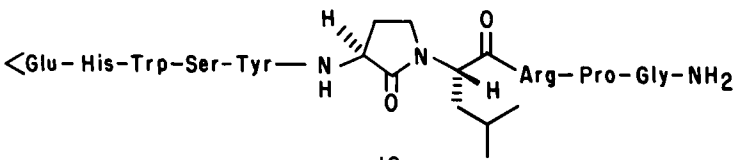


Fig. 3. Computer superposition of the proposed β-turn segment of LH-RH (Tyr-Gly-Leu-Arg) (solid) with γ-lactam conformational constraint (dashed). Average deviation of least squares fit of matched atoms is 0.13 Å.

The required analog **10** was prepared according to standard procedures using the γ-lactam derived from Boc-Met-Leu-OMe for positions 6 and 7. The compound was found to be 8.9 times as potent *in vitro* and 2.4 times *in vivo* in rats as LH-RH. This data provides additional support for a receptor-bound conformation of LH-RH which contains a Tyr-Gly-Leu-Arg Type II′ β-turn. Other types of β-turns known to exist in proteins have also been examined by computer superposition with the lactam peptide. All of these accommodate the lactam ring less well. The enhanced potencies obtained with three different conformational constraints (D amino acids, N-methyl amino acids, and lactams), all of which would stabilize a turn structure, argue against a non-turn receptor-bound conformation. The results also indicate that the loss of activity with L amino acid substitution at position 6 was due to destabilization of the favored conformation rather than some steric interaction with the receptor. This example successfully demonstrates the utility of a lactam as a conformational constraint in peptides providing inference of bioactive conformation and increased biological potency.



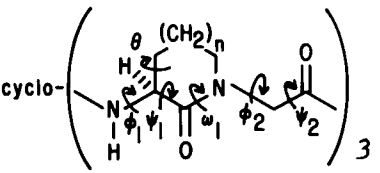
10

A second application of lactams suggested by computer modeling studies involved a series of cyclic hexapeptide analogs **11** of c-(Ala-Sar)$_3$ which have the property of altering rumen fermentation.[16] In certain conformations, the α-methyl of alanine and the N-methyl of sarcosine are close in space. A covalent bridge between these two groups will place restrictions on ψ_1 and force ω_1 to remain *trans* and should yield information about the bioactive conformation. Lactam rings of 5-, 6-, and 7-members were investigated. The lactams provided a synthetic advantage in that the cyclic hexapeptides **11** were prepared by cyclotrimerization of the lactam dipeptide amino acids.

In order to assess the conformational consequences of the lactam constraints, energy minimization calculations have been carried out on the N-acetyl methyl amides of the three lactam amino acids. The low energy conformations of the rings are listed in Table I. In addition, low energy regions for ϕ_1 and ϕ_2 have been determined for each of the conformations. These calculations show that unique peptide backbone conformations are available for each ring size.

Information on the solution conformations of compounds **11** was obtained from proton NMR spectra. The cyclic trimers have single C_3 symmetric conformations even under conditions where c-(Ala-Sar)$_3$ is assymmetric or has multiple conformations on the NMR time scale. This reduction in conformational mobility improves the probability that solution measurements will yield information about the bioactive conformation. The H-N-C$^{\alpha}$-H coupling constant can be measured directly to obtain values for ϕ_1. By comparing possible values for the H-C$^{\alpha}$-C$^{\beta}$-H dihedral angles with corresponding values for the conformers in Table I,

Table I. Low Energy Conformations Of L-Five, Six- And Seven-Membered Lactams.



II (n=0, 1, or 2)

Conformation No.	Lactam ring size	ψ_1	θ^a	Strain Energy[b] (Kcal/mol)	Ring Conformation
1	5	−111	−14, 106	23.99	Slight pucker
2	5	−144	15, 134	–	Slight pucker
3	6	−108	−41, 76	24.15	Half chair
4	6	−135	47, 165	24.21	Half chair
5	6	− 79	−45, 70	25.63	Boat
6	6	−173	53, 170	26.71	Boat
7	7	167	81, −162	26.84	Chair
8	7	− 67	−66, 48	26.95	Chair
9	7	− 98	34, 149	30.60	Twist boat
10	7	160	42, 159	30.77	Boat

[a]H-C$^{\alpha}$-C$^{\beta}$-H dihedral angles. [b]It is only valid to compare strain energies within a given ring size where the total number of bonded and nonbonded interactions will be the same.

the lactam ring conformations can be established. These results then provide ψ_1 values for the cyclic hexapeptides. Only lactam ring conformations 2, 4, 6, and 7 were compatible with the coupling constant data. It can be seen by comparing these conformations that each of the three cyclic hexapeptides is capable of assuming a different backbone conformation in solution.

The lactam cyclic hexapeptides were compared with c-(Ala-Sar)$_3$ for ability to inhibit formation of methane in the fluid taken from the stomach of a sheep. Only the six-membered lactam analog shows significant activity and is comparable to c-(Ala-Sar)$_3$. The low activity of the other analogs must relate to the changes in peptide backbone conformation, particularly differences in ψ_1. Differences in the size of the lactam ring *per se* being the determining factor for activity or lack of it is unlikely since the inactive five-membered ring analog presents less bulk than the six-membered analog while the inactive seven-membered ring presents greater bulk.

These results place considerable restriction on the cyclic hexapeptide conformation making *cis* peptide bonds unlikely and focusing attention on a small number of conformations available to δ-lactams, but determination of a unique bioactive conformation will require additional studies. This study does show that the small differences in conformation of peptides constrained by five-, six-, and seven-membered lactams is sufficient to dramatically alter biological activity. The introduction of these constraints into peptide structures can therefore be a very sensitive probe for obtaining information about the bioactive conformation of that peptide.

Based on these findings, a similar study of enkephalin has been carried out. The seven analogs containing lactam dipeptides of D and L configuration and including five-, six-, and seven-membered rings as replacements for Gly^{2}Gly^3 of methionine enkephalinamide are listed in Table II. These compounds were prepared by solid phase synthesis of tetrapeptide intermediates followed by coupling of Boc-tyrosine-N-hydroxysuccinimide ester. NMR measurements in methanol of N-C$^\alpha$-C$^\beta$-H coupling constants for the four analogs of D configuration indicate that the lactam rings exist in the same conformations (enantiomeric) in solution as were found for lactam-constrained cyclic hexapeptides. The two six-membered lactams (**14** and **16**) have very similar conformations. The biological activity of these analogs in both guinea pig ileum and naloxone binding assays was dependent on lactam optical configuration and ring size. The six-membered latams of D configuration were consid-

erably more active than the other analogs and had 2-10% the activity of methionine enkephalin.

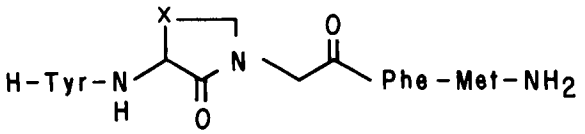


Table II. Biological Potency Of Lactam-constrained Enkephalin Analogs

Compd	X	Lactam Configuration	Naloxone binding (ED_{50},nM)[a]		Guinea pig ileum (EC_{50},μM)[b]
			$-Na^+$	Na^+	
12	CH_2	D	4500	33000	I[c]
13	CH_2	L	I	I	I
14	$(CH_2)_2$	D	80	2500	2.6
15	$(CH_2)_2$	L	6100	4500	I
16	CH_2-S	D	780	10500	8.9
17	CH_2-S	L	I	I	I
18	$(CH_2)_3$	D	I	I	I

[a] Methionine enkephalin: 10 ($-Na^+$), 250 (Na^+). [b] Methionine enkephalin: 0.24. [c] Inactive; < 1% the activity of Met Enkephalin.

As in the cyclic hexapeptide case, these activity differences are most likely due to differences in backbone conformation. The very low activity of γ-lactams **12** and **13** would argue against either a Tyr-Gly-Gly-Phe Type II or II′ β-turn in spite of the increased activity of the D-Ala[2] analog.[17-18] Other conformations are ruled out by the inactive ε-lactams. The δ-lactams **14** and **16** must be capable of most closely approaching the bioactive conformation. There are several possible explanations of their low activity. There is some evidence that N-alkylation of Gly[3] has a negative effect on potency,[19] and introduction of the lactam may negate any beneficial stabilization of conformation. A second explanation could be that in all of these cases a non-bioactive conformation may be preferentially stabilized while the higher energy bioactive conformation is present as only a small component of the equilibrium mix of conformers.

With certain muscarinic agonists, an analogous situation has been reported for a piperidine system. In rigid analogs having only the boat conformation, activity was 513 fold higher than observed when boat and chair were freely equilibrating.[20] The low activity of the δ-lactam analogs **14** and **16** may be because a higher energy conformation such as the mirror image of 5 or 6 in Table I is close to the bioactive conformation. For example, Conformation 5 would be expected to contribute less than 10% to the conformational equilibrium. Model building of a highly active cyclic enkephalin analog,[21] in fact, indicates that a two position ψ value of 79° as found in the enantiomer of Conformation 5 would be reasonable. Such a possibility would suggest that additional analogs designed to preferentially stabilize this conformation would have greater activity.

It is clear that lactam substitutions in peptides do not always lead to potent analogs or allow formulation of a proposed bioactive conformation. These constraints do complement other types of conformational modifications and often provide insights into very subtle conformational differences which can suggest additional constrained structures. Lactams provide one example of the utility of a computer graphics system for studying peptide conformation, both in initial phases when proposals are formulated and in later stages for assistance in interpretation of results. A strong synthetic capability and application of all available physical methods are also essential parts of this approach. Continued application of all of these tools, in addition to more classical methodology, is expected to yield further unique insights into peptide conformation and to the design of useful biologically active peptides.

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DESIGN OF A HIGHLY ACTIVE CYCLIC HEXAPEPTIDE ANALOG OF SOMATOSTATIN

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In spite of key biological functions and numerous possible applications to therapy, few peptides have yet had a major impact on human medicine. Notable exceptions are the important use of insulin in diabetes and of ACTH in inflammatory disease. If the problem of rapid metabolism of peptides could be overcome through structure modification to yield analogs of long duration of action and oral activity, then the peptides could show the way to new therapies in many areas of human health. With the discovery of the inhibitor peptide hormone somatostatin, we were challenged to solve the problem of a short duration of action in order to apply its useful biological properties.[1]

Early structure-activity studies on somatostatin analog showed that the two amino acids outside of the ring are not required[2] and that the existence of a ring contributes to the biological activity.[3] Initial structure modification studies within the ring presented a confusing picture not simply interpreted in terms of receptor interactions involving the altered amino acids. Deletion of residues 4,5 or 13 or replacement of residues 13 or 14 by the corresponding D-amino acid, led to the loss of only selected activities or no loss of activity at all.[4] Changes such as these which either delete or drastically alter the position of the side chain of the individual amino acid also have the potential to alter the preferred conformation of the entire 38 membered ring. Analysis of its NMR spectrum led us to believe that somatostatin was probably an equilibrating mixture of conformers at room temperature, a conclusion also reached in more detailed studies.[5,6] The D-Trp^8-diastereomer of somatostatin and analogs of somatostatin having D-Trp in position 8 appeared to show an increase in the amount of bioactive conformer which we correlated with the increased biological potency.[7] The chirality modifications at other positions mentoned above might also be selecting out specific and different conformers yielding the special selectivities observed, but this possibility has never been studied.

Since changes in chirality and amino acid deletion at single residues are capable of producing changes anywhere and everywhere else in the molecule, conclusions about these observations directed at analog design are difficult. We undertook an approach which we hoped would impose

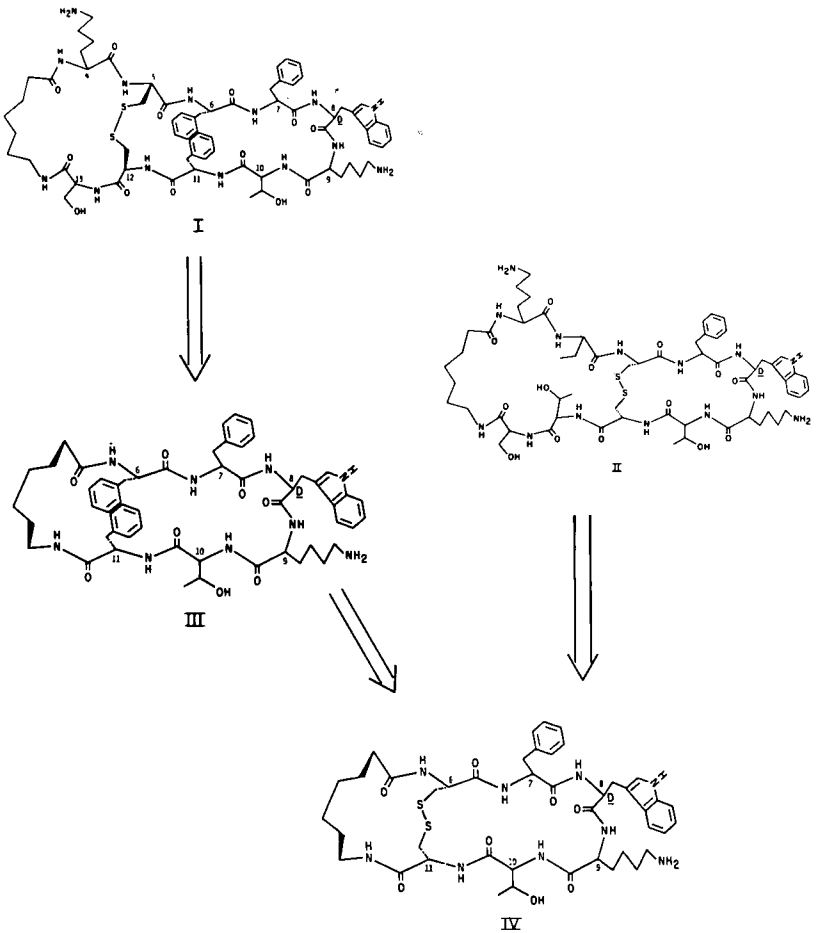

Fig. 1.

order on the molecule, allowing us to generate understanding of the structure-activity relationships and eventually allow the development of rational analog design. A bioactive conformation of somatostatin was theorized[8] based on our own and published studies of systematic replacement of the individual amino acids by the corresponding D- or α-methyl amino acids or by proline or alanine. Using this model, proximity of apparently remote side chains was recognized. Highly active bicyclic analogs were then synthesized, building a covalent link between the side chain of residues 5 and 12 (I) or residues 6 and 11 (II).[8] Rational interpolation from analog I led us to synthesize the active smaller ring

analog III and subsequently the bicyclic analog IV.[9] IV proved to be a potent inhibitor of the release of insulin, glucagon and growth hormone.

Analog IV also showed distinctive features in the NMR spectrum which led us to believe that it is less conformationally flexible than somatostatin. We used studies of the NMR spectrum to derive a solution conformation which gives a better picture of the active β-turn portion than could be obtained through solution studies of the conformation of somatostatin.[10] This solution conformation of IV was precisely defined using our Merck molecular modelling system[11] and a stereoscopic view of that model appears in refrence 10.

The high activity of IV led us to believe that the β-turn portion (residues 7-10) could supply all the elements needed to express the full activity of somatostatin for inhibition of the release of growth hormone, insulin and glucagon.[7] We therefore imagined that the left hand bicyclic portion of IV could be replaced by other simpler bridging units. Our computer system[11] was used to break apart IV after Cys-6 and before Cys-11 and the "active" tetrapeptide was isolated in imaginary form still in its fixed conformation. New bridging units were then examined which could span the distance between the free ends. An advantage of the computer system is the absolute fidelity to the defined conformation unless it is intentionally altered. Unintended alterations inevitably occur in real molecular models. One effective class of bridging unit involved acyldipeptide N-methyl amides held in various types of β-turn placed to form a cyclic hexapeptide. The ends of the "active"tetrapeptide unit both with and without simultaneous rotations about some of the bonds in the tetrapeptide unit which were equivocal in our NMR study of reference 8. One example of a superposition using a type I β-turn is shown in Figure 2. The average deviation of the 5 pairs of matched atoms in this case was 0.31 Å after allowing rotations about the bonds indicated by curved arrows.

Most other types of β-turn also gave good matches. Because these studies predicted that the active conformer of somatostatin could exist within a cyclic hexapeptide having two β-turns, we prepared a series of cyclic hexapeptides having the tetrapeptide sequence -Phe-D-Trp-Lys-Thr- and completed by systematically varied dipeptide units (Compounds **1-7**). The dipeptide combinations represent pairs that we thought should produce a β-turn either because they exist in a β-turn in some peptide X-ray analysis or because they appear compatible with a β-turn on evaluation of models. In most of the synthesized analogs (Table I) only low biological potency or no activity was detected. Among those

Table I

	Compound A	Compound B	δ Lys γ-CH_2^5	Inhibition of GH Release Rel. Pot.[b] (95% C.L.)
1	Ala	Pro	0.39, 0.55	0.06 (0.03, 0.12)
2	Pro	Pro	0.33, 0.53	0.008 (0.003, 0.02)
3	D-Ala	D-Pro	0.2-0.7[a]	0.006 (0.003, 0.01)
4	D-Ala	Pro	0.30, 0.45	<0.002
5	Pro	Ala	0.23, 0.45	<0.002
6	Pro	D-Ala	0.43, 0.60	<0.002
7	Aib	Pro	0.20, 0.43	<0.002
8	Phe	Pro	0.40, 0.55	1.74 (1.31, 2.32)
9	Phe	Phe	0.23, 0.44	0.27 (0.22, 0.33)
10	Phe	D-Phe	0.59, 0.68	0.22 (0.20, 0.25)
11	D-Phe	Pro	0.30, 0.44	<0.002
12	Pro	D-Phe	0.45, 0.61	0.03 (0.03, 0.03)
13	D-Phe	D-Pro	0.56, 0.71	<0.002
IV	Cys Aha Cys		0.32, 0.48	1.24 (0.81, 1.88)

[a]Two conformers. [b]Inhibition of spontaneous growth hormone release was evaluated by incubation of isolated pituocytes with somatostatin or analog at graded doses (at least 6 doses per analog) ranging from 10^{-10} M-10^{-5} M. Three replicate plates were used at each dose level. After 4 h incubation, growth hormone levels released into the medium were determined by a double antibody radioimmunoassay for rat growth hormone. Potency of the analogs relative to somatostatin (=1) were calculated using a relative potency formula for parallel line bioassays. [D.J. Finney "Statistical Methods in Biological Assay" Charles Griffin and Co. Ltd., London, Chap. 4, pp. 99-138 (1964).] 95% confidence limits are given in parenthesis. This bioassay is essentially as has been described by W. Vale and G. Grant in "Methods in Enzymology", Eds. B.W. O'Malley and J.G. Hardman (Academic Press, New York) Vol. 37, pp. 5-93, 1980. The ED_{50} for somatostatin in this test is 5×10^{-8} M.

having only Ala and Pro as the A-B unit (**1-7**), the most active (1) showed 6% the potency of somatostatin for the inhibition of growth hormone release *in vitro*. We interpreted low potency with a complete biological response as a consequence of deletion of some important binding element. The conformation of all other elements in the analog was assumed correct. A loss of 1-2 orders of magnitude in potency corresponds to about 2-3 Kcal of binding energy. This is only a portion of the total binding energy required for somatostatin based on an association constant to receptors[12] of about 10^{10} ($\Delta G = RT\ln K \cong 14$ KCal, where K is the receptor binding constant). Analog 1 thus has a large proportion of the binding energy of somatostatin even though it has "only" 6% the potency of somatostatin. Phenyl rings were added to the β-positions of either the A or B unit to generate analogs **8-13**, thereby adding a hydrophobic element in varied geometric relations which we hoped would mimic those

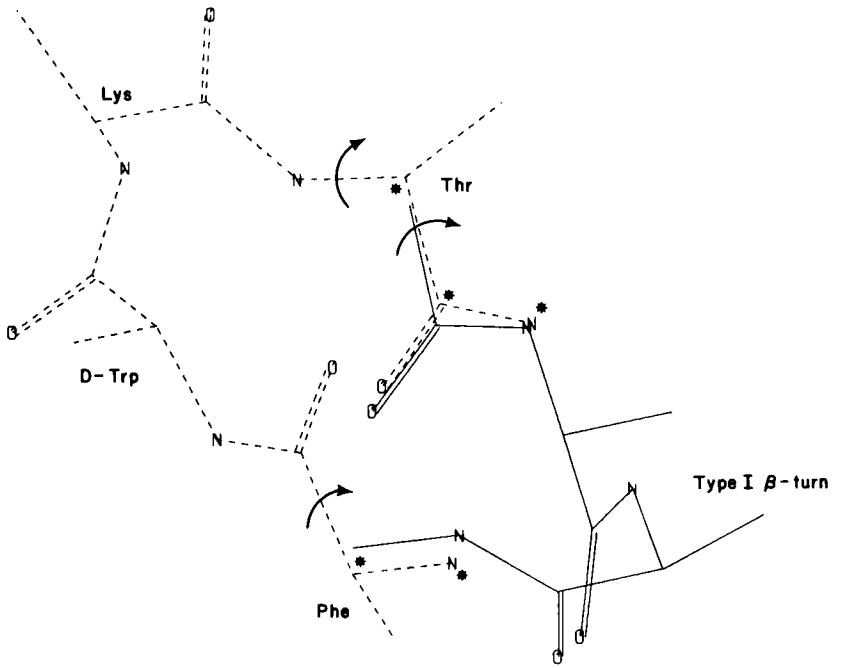

Fig. 2. Computer matching of the termini of a tetrapeptide unit and an acetyl dipeptide-N methylamide unit having the conformation of a type I β-turn. The tetrapeptide unit has the conformation proposed for the -Phe-D-Trp-Lys-Thr-portion of the proposed bioactive conformation of somatostatin analogs.[2] A least squares fitting of the atoms marked by a * was carried out while simultaneously allowing rotation about the bonds marked by arrows. The average deviation of the matched atoms in this case was 0.31 Å.

in somatostatin. Most striking was the nearly 20 fold increase in potency on adding a phenyl ring to **1** to form analog **8**.The nearly identical CD spectra of **1** and **8** in the region 190-230 nm indicates that the increased potency is contributed by the benzene ring and not by a change in conformation of the cyclic hexapeptide unit. All of the analogs of Table I appear to have a similar conformation in the region of the D-Trp-Lys bond as indicated by the upfield shift of the γ-CH_2 of lysine, a measure of the proximity of the side chains of these two amino acids.[7] Changes in the conformation of the rest of the molecule must be a source of reduced potency in some analogs. The analog **9**, which represents cyclization of residues 6-11 of somatostatin, is about 0.27 as potent as somatostatin; about 0.13 as potent as **8**. The CD spectrum of **9** is different from that of **8** in the 190-230 nm region suggesting a conformational difference (for CD spectra see reference 13).

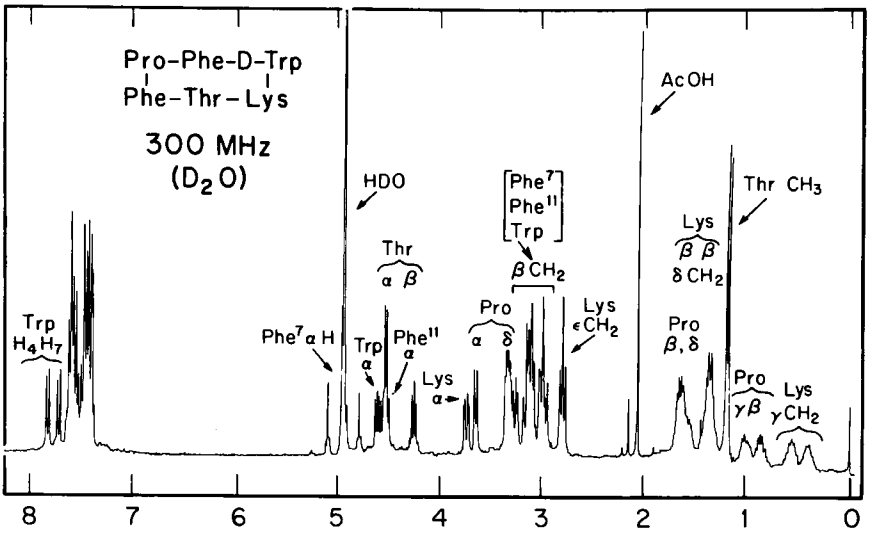

Fig. 3. NMR spectrum of cyclo(Pro-Phe-D-Trp-Lys-Thr-Phe), **8**, in D_2O at 300 MHz.

Analog **8** is also a potent inhibitor of insulin and glucagon release *in vivo* (rats) showing 5.2 (2.4, 11) and 8.0 (1.4, 60.2) the potency of somatostatin (=1). The analog is long acting and shows a response after oral administration.[13] The analog is as resistant to hydrolysis by trypsin as is the analog IV, both of which are much more stable than somatostatin.[9] The cyclic hexapeptide, **8**, therefore represents a novel class of somatostatin analog which retains most of the properties of somatostatin, shows long duration of action and oral activity, and is relatively easily synthesized.[13] Representatives of this class should serve well to test hypothesized uses of somatostatin as an adjunct to insulin therapy in juvenile diabetes.[1,14]

Table II. N-H, C_α-H Coupling Constants and Dihedral Angles for **8**.

N-H	ppm/°C*	J N-H_3C_α-H**	θ(HN,CH) Used in Model[15]
Thr	0.0003	9.2	-152
Lys	0.0061	5.9	-149
Phe-7	0.0016	7.8	161
Phe-11	0.0037	2.0-2.3	-95
D-Trp	0.0056	4.9	135

*DMSO **D_2O

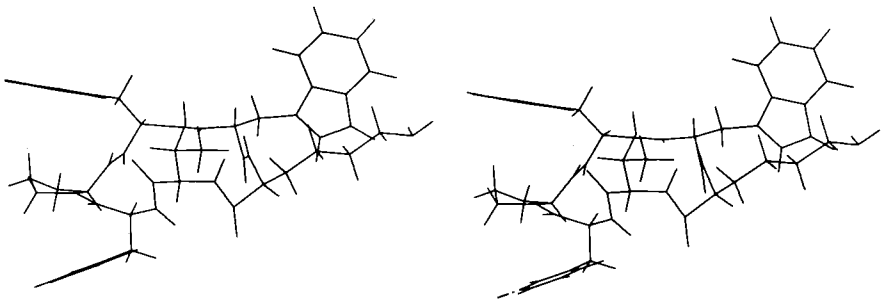

Fig. 4. Stereo view of the model of **8** based on the solution conformation derived from NMR parameters.

The NMR spectrum of **8** suggests a high degree of molecular rigidity with exceptional upfield shifts of several protons. Figure 3 shows a fully assigned PMR spectrum of **8**. This spectrum, along with studies of the N-H chemical shift exchange rates, temperature dependence and coupling constants, have given information about the solution conformation. In particular, the N-H's of Phe-7 and Thr-10 were found to be involved in intramolecular hydrogen bonds as indicated by slower exchange rate in D_2O and reduced temperature dependence of the chemical shifts compared to the other NH's. The upfield shift of the γ-CH_2 of lysine reflects proximity of the lysine side chain and the indole nucleus.[7] Three protons on the proline ring are shifted upfield, the α, one β and one γ. Analysis of the coupling constants of protons in the proline ring requires that the upfield β-proton is on the same side of the ring as the α-proton and the upfield γ-proton is on the opposite side with an angle of 153° between the upfield β- and upfield γ-protons. This is only possible when two aromatic rings are the sources of anisotropy. A conformation having the proline ring sandwiched between the phenyl rings of Phe-7 and Phe-11 is required (see model, Fig. 4). The peptide bond between Phe-11 and Pro is *cis* because the observed chemical shifts of C_β (31.6) and C_γ (22.3) fall within the ranges expected for a *cis* peptide.[16] All of the NMR data has been sufficient to generate an unequivocal solution conformation of *cyclo*(Phe-D-Trp-Lys-Thr-Phe-Pro) **(8).** A stereo view of this conformer is shown in Figure 4. The various structural features discussed can be recognized in this model. The Type II' β-turn for residues 7-10 is now much closer to the classical definition of this turn[17] than was the case for our model of IV.[10] It is also fairly close to the β-turn calculated as an energy minimum for a structure related to IV.[18] The type VI β-turn involving Phe-Pro and having a *cis* peptide bond is similar to a β-turn in ribonuclease S at residues 91-94[19,20] (Lys-Tyr-Pro-Asn).

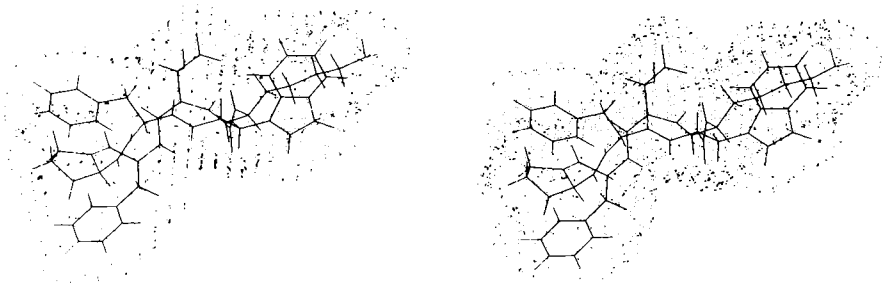

Fig. 5. A stereoview of the model of **8** with the contact surface added to indicate the possible nature of somatostatin receptors.

In Figure 5, the surface of the molecule has been superimposed on the model in order to suggest the nature of the receptor surface with which it must interact. It is noteworthy that one face is almost fully hydrophobic except for the presence of the ϵ-amine group of lysine. A complementary hydrophobic surface at the receptor having a carboxyl group could supply sufficient interaction to give the high potency (strong binding) observed. The geometric relationships of the receptor binding elements are likely to be the same as they are in the bioactive form of D-Trp^8 somatostatin and in turn of somatostatin.

We have used the model of IV to generate a model for D-Trp^8-somatostatin. Coordinates for residues 7-10 remain as in IV. The positions of phenylalanines 6 and 11 were placed by replacing the cystine of IV by two phenylalanines. The 2 and 3 carbons of 7-aminoheptanoic acid (Aha) formed the α-and β-carbons of asparagine-5 while the 6 and 7 carbons were used to establish the position of the β- and α-carbons respectively of threonine-12. These choices for modeling were based on the relation of the Aha to the cystine of I and in turn to asparagine-5 and threonine-12. The rest of the model was placed in such a way that the ring could be completed and is not based on experimental data except that it is compatible with proline at residues 5 and 13.

Several important conclusions about the function of the amino acids of somatostatin can be drawn based on comparison of the three dimensional structure of **8** with that of the derived model of D-Trp^8 somatostatin (Figure 6). Residues 7-10 form the main portion of receptor recognition. There is nothing in the cyclic hexapeptide that corresponds to the phenylalanines 6 and 11 of somatostatin, reinforcing the past conclusion that their role relates to stabilization of the active conformation rather than direct receptor interaction.[8] Most unexpected is the observation that the phenyl ring of Phe-11 in **8** corresponds to the combined side

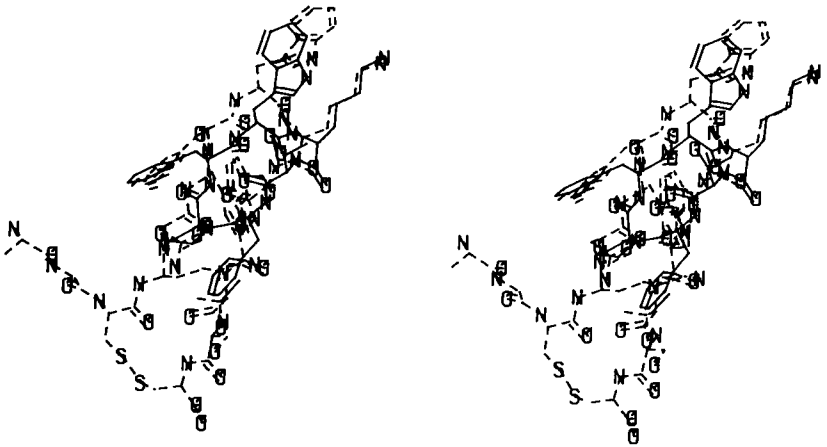

Fig. 6. A stereo view of a superposition of a model of D-Trp^8-somatostatin derived as discussed in text with that of compound **8** as derived from solution NMR data.

chains of Asn-5 and Thr-12. These two amino acids normally thought of as hydrophilic thus contribute a hydrophobic interaction great enough to correspond to the difference in activity (20 fold) between **1** and **8.** Lysine-4 and Serine-13 have no known role and may be only "spaces" between the active portions and the cysteines 3 and 14 which play a role in maintaining the active conformation.

Our studies show an approach to the design of peptide analogs of increased duration of action and oral activity. Precise definition of the structure by distilling out the components important for receptor binding and holding them in the correct steric relationship through a rigid molecular framework. Modern instrumental and computer techniques have been an important aid in the design process.

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A CONFORMATIONAL STUDY OF THE PEPTIDE HORMONE SOMATOSTATIN (SRIF) BY HIGH RESOLUTION NMR SPECTROSCOPY AND ENERGY CALCULATIONS

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Introduction

Our study on the conformations of Somatostatin by combining energy calculations and NMR spectroscopy shows that only the use of several methods for establishing conformation might be successful when so many conformational variables are involved.

Calculation Procedure and Results

By four different strategies based mainly on the linear fragment approach about 30 million backbone conformations were generated. By applying the distance constraint for Cys 3-14, to mimic the disulfide bridge, 500,000 structures were left. These were submitted to energy calculations and 3,835 backbone conformers were retained and divided in 5 sets (A,B,C,D,E,).

A first type (type A) concerns the sets with a helical region on Cys^3-Trp^8 (A_1,A_2), on the Phe^6-Thr^{10} (A_3,A_4) and on Asn^5-Lys^9(A_5). Such backbone foldings allow the proximity of non-neighboring side chains Lys^4 and Trp^8 with the aliphatic chain entering the shielding cone of the aromatic ring.

The conformers of the type B, present a bend of the backbone at Trp^8-Lys^9 such that interactions may arise between side chains of these residues. For set B_1 the aromatic ring of Trp^8 gives rise to an upfield shift mainly on the γ hydrogen of Lys^9 while for set B_2 and B_3 other aliphatic hydrogens of Lys^9 also undergo the same influence. In type C the foldings of the backbone may sometimes favor the spatial proximity of Lys^9 with a Phe side chain. In C_1 the H γ of Lys^9 is in the shielding area of Phe^{11}. For all other types, Lys^9 is not influenced by the aromatic ring currents.

Many conformers allow the spatial proximity of Phe^6 and Phe^7. In many cases Phe^{11} may be close to the Thr^{10} or Thr^{11} methyl-group. Only conformers in sets A_1, A_2, B_2 give rise to a distance of about 6 Å for C_6 -

C_{11} and only one (B_2) presents a good orientation of the two Cβ atoms as compared with the active bicyclic analog of D. Veber.[2]

From the analysis of the calculated models no clear preference emerges for a single or one group of closely related conformations. Discrimination between closely related conformations can be done with data obtained from NMR measurements.

NMR Methods and Results

The following assignment techniques were used: 1) Analysis of the 270 MHz proton spectrum. Separation of the overlapping signals through the use of J resolved NMR for the α and β region, and decoupling experiments (including spin tickling). Also the spin system was greatly simplified by the use of 500 MHz proton NMR, and the total analysis of all spin systems was achieved.

2) Assignment to individual amino-acids. To overcome the difficulties in assigning signals for individual amino acids, e.g., there were ambiguities for 2 Lys, 2 Thr, and 8 ABX systems: 2 Cys, 3 Phe, 1 Trp, 1 Ser, 1 Asn, analysis of synthetic analogs was carried out. The analog [Phg^{11}]-SRIF was used to identify Phe^{11} and thereby confirm the ring current effect on Thr^{10}.

The analog, [HSer^{12}]-SRIF, permitted the distinction between Thr^{12} and Thr^{10}. The use of fragment molecules [Ala^{14}]-SRIF$^{9-14}$ and SRIF$^{8-11}$ allowed the assignment of the Lys^9 signals. The effect of pH (pH 2 and pH 6) on ^{13}C spectra produced charge effects on C=O and α,β^{13}C signals of Cys^{14} coupled with selective off-resonance ^{13}C-H decoupling experiments allow the chemical shifts of the Cys^3, Cys^{14} and Asn^5 protons to be assigned.

Photo-CIDNP experiments[1] permitted identification of the Trp^8 β protons and the 2,4,6 protons of the indole ring, and the deuterated analog: [β,β^2H_2 Phe^7, α^2H_1 Phe^{11}]-SRIF enabled the assignment of the three Phe α,β proton systems. By these experiments we were able to assign all the signals to the individual amino-acids. By spectral simulation at 270 MHz and 500 MHz the exact values for the chemical shifts and coupling constants (except for the aromatic Phe signals) were obtained.

3) Additional experiments were carried out. The effect of temperature on the N-H region (the T coefficient, $\Delta\delta/\Delta T$ all between 0.4 and 1.5 ppb/°C) did not justify the existence of any stable β or γ turn in the molecule although the region 8-12 seems to be the most stable in this respect.

The effect of temperature on the non-amide protons indicated important conformational effects on the upfield shifted signals: Phe^6 α,β; Phe^7 α; Asn^5 α,β; Lys^9 complete side chain; Lys^4 α,γ; Cys^{14} α; Phe ring protons occurred upon raising the temperature.

The rotamer population of the 8 ABX systems also was analyzed. In unperturbed systems n_{II} dominates usually; this is the case for Phe^6 and Phe^{11}. The Phe^7 and Trp^8 rotamer population differs markedly from the values in smaller peptides. These features correlate with the presence of upfield ring current shifts.[4]

Conclusions

Only one family of calculated conformations with 3-5 extended, 8-5 βI turn, 10-7 βII or 12-9 βII turn (set B_1) is able to explain the ring current shifts on Lys^9 and Phe$\alpha\beta$ induced by the influence of the Trp^8 and Phe^7 aromatic rings, respectively. It seems most likely from the NMR results on the [Phe^{11}]-SRIF analog that there is interaction between Phe^6 and Phe^7. It is also clear that the B_1 set is *certainly not* the only group of conformations in the equilibrium. At elevated temperature the set B_1 no longer dominates.

It is very interesting to find among the low energy conformations several sets corresponding to the constraints that exist in the bicyclic SRIF analogs synthesized by D. Veber et al.[2] (βI turn in region Phe^7-DTrp^8 Lys^9-Thr^{10}). The upfield shift on Lys^9 γ does exist in SRIF itself but it is less pronounced than in the rigid bicyclic analogs due to a larger contribution of other sets of conformers. Veber has reached similar conclusions from a temperature study in CH_3OH.[3]

From the studies of D. Veber and our results it seems likely that the biologically active conformation is among the conformers found in set B_1.

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A GRAPHICS AND ENERGETICS SIMULATION OF AQUEOUS PEPTIDE CONFORMERS

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Introduction

Conformational variance is an essential factor in imparting diverse biological activities to various peptides which have similar chemical constituents. In inert media, such as the interior of biomembranes or in non-polar solvents, intramolecular interactions dictate the conformation. These interactions are well simulated by semiempirical potential functions. By contrast, in aqueous media there are strong peptide-water interactions which must be considered in addition to the intramolecular peptide interactions. These intermolecular interactions include both bulk water and specifically bound water molecules. A number of computations have been carried out on the interactions between bulk water and peptides.[1] Specific hydration is more difficult to simulate precisely. As an approximation of effects of specific hydration on peptide conformation, we consider only the waters hydrogen bonded to the polar peptide groups. Optimum water clusters have been computed for methylacetamide and various dipeptide conformers. We coupled the computation of hydration energies from potential functions to computer-graphics displays of the molecular configurations. Thus the experimenter can interactively guide energy minimization and rapidly examine alternative arrangements of the water molecules.

Computational Methods

The potential energies of clusters of water molecules around peptides were minimized using two potential functions. Each of these has steric and electrostatic terms. A dielectric constant of one was used. The potential of Rossky *et al.*[2] includes a special hydrogen-bonding term for interactions with the carbonyl oxygen and the Stillinger-Rahman ST2 potential[3] for water-water interactions. In the ST2 potential only the oxygen atom of water is given steric bulk. Charges are placed tetrahedrally around the oxygen, at the protons and at positions for the lone pairs.

A second potential function was that developed from molecular quantum mechanics by Clementi and co-workers.[4-6] The water-water potential is the configuration interaction result of Matsuoka, Clementi

and Yoshimine (MCY).[4] Beveridge *et al.*[7] have reported that this potential gives an improved simulation of bulk water over that using the ST2 potential. One aspect of this improvement is in giving less rigid clusters of water molecules. The MCY potential assigns steric bulk to the hydrogens and the oxygen atoms of water. Positive charges are placed on the hydrogen nuclei and a negative charge on the bisector of the HOH angle. Parameters for peptide-water interactions are from a study of formyltriglycylamide and water.[5] Steric parameters are given for interactions of each peptide atom with the oxygen or hydrogens of each water. Charges are placed on the nuclei both in the peptide and in water. For the peptide-water interaction the negative charge is not displaced from the oxygen nucleus. Furthermore, different charges are used than for the water-water interactions. We could reproduce the energies of Ref. 5 using a charge of +0.31 electron on each hydrogen of water (–0.62 on oxygen). This compares with a charge of +0.3321 electron which had been reported.[6]

Intramolecular peptide energies *in vacuo* were computed using the consistent force field (CFF) method of Lifson and co-workers.[8] In a previous report,[9] we utilized the potential of Rossky *et al.*[2] and rigid peptide geometry for intramolecular peptide energies.

In this study, the reported energy minima were reproduced to within 0.2 kcal/mol upon reminimization of structures which had been randomized by displacements of 0.1-0.2 A° and rotations of 10-20° about one of the Cartesian axes. This reminimization lowered some of the energies reported previously.[9]

Hydration Energies for Methylacetamide

The energies of individual hydrogen bonds computed with the two potential functions are given in Table I. The smaller water-water hydrogen bond energy of the MCY potential is more realistic.[7] However, the smaller NH-water energy of the ST2 potential may be in better accord with experimental data,[2,10] but there is no direct experimental measure of the hydration energy for an isolated NH. The CO-water energies are comparable for the two potentials and are in reasonable accord with expectations from experimental data.

Various clusters of water molecules around the NH and CO groups of methylacetamide were optimized. Two hydration layers were considered; primary (1°) waters are those directly hydrogen bonded to the peptide groups, while secondary (2°) waters are those which hydrate the primary layer. A dielectric constant of four was assumed for interactions amongst the secondary waters as they are generally well

Table I. Hydrogen Bond Energies for Two Potential Functions

H Bond	Energies (kcal/mol)	
	ST2	MCY
W---W	-6.8	-5.7
N-H---W	-5.9	-8.6
C=O---W	-7.0	-7.3

separated and would interact with additional hydration layers which were not included in these computations.

The interaction energies of the primary waters with the peptide (E_{Pep}) and the total interaction energies (E_{Tot}) of the primary waters (with the peptide, the other primary waters and the secondary waters) will be reported. The interaction energies amongst secondary waters, totaling 2-3 kcal/mol, were ignored. Proper accounting of the energies of the secondary waters would require inclusion of a third hydration layer.

Only one primary water can be attached to the NH of methylacetamide. No stable low-energy structure was found with two primary waters. The primary water on the NH is well hydrated by three secondary waters (Table II). For the MCY potential, attachment of secondary waters only to the two hydrogens of the primary water also gives a stable cluster. This latter arrangement is less stable for the ST2 potential since it would leave one of the lone pairs of the primary water without a hydrogen bond.

Table II. Hydration Energies of NH of Methylacetamide

Number of Waters		Energies (kcal/mol)			
1^{o}	2^{o}	ST2		MCY	
		E_{Pep}	E_{Tot}	E_{Pep}	E_{Tot}
1	0	-5.9	-5.9	-8.6	-8.6
1	2	--	--	-10.2	-21.5
1	3	-6.6	-26.7	-10.3	-26.4

A number of water clusters form stable arrangements around the peptide CO (Table III). Of course, the total energy continues to decrease as more waters are added. As a guide to optimum hydration, we require that the last water molecule added to the cluster lowers the energy by at least as much as a single water-water hydrogen bond. By this criterion, for primary waters only, either two (MCY) or three (ST2) waters are favored.

Table III. Hydration Energies of CO of Methylacetamide[a]

Number of Waters		Energies (kcal/mol)					
1^{o}	2^{o}	ST2			MCY		
		E_{Pep}	E_{Tot}	E_{Last}[b]	E_{Pep}	E_{Tot}	E_{Last}[b]
1	0	-12.6	-12.9	--	-15.3	-15.7	--
2	0	-18.1	-20.6	-7.7	-20.3	-22.3	-6.6
3	0	-23.0	-29.7	-9.3		c	
4	0	-27.6	-35.0	-5.3		c	
2	4	-19.1	-50.0	-7.4	-21.5	-47.2	-6.2
2	5	-20.8	-59.7	-9.7	-23.0	-54.2	-7.0
2	6	-22.3	-66.2	-6.5	-24.8	-62.8	-8.6
3	3	-25.6	-50.6	-7.0	-23.7	-47.6	--
3	6	-28.0	-82.8	-10.7	-26.8	-73.3	-8.6
3	7	-26.1	-89.5	-6.7	-24.4	-76.4	-3.1
3	8	-27.7	-88.0	+1.5	-27.9	-73.8	+2.6
4	6	-29.7	-75.4	-6.7	-23.8	-68.6	--

a. The energies include one primary water on the NH.
b. Energy gained by adding the last water compared to the preceding cluster in the table. For adding more than one secondary water the average energy is given comparing (# primary, # secondary) as follows: 2,4 to 2,0; 3,3 to 3,0 and 4,6 to 4,0. c. Unstable cluster.

A cluster of three primary waters around the CO is not stable for the MCY potential unless secondary waters are added.

With two primary waters, the cluster having six secondary waters is most stable (Figure 1). In this cluster each of the primary waters has two hydrogen-bonded waters and they share two more. Each primary water participates in five hydrogen bonds.

With three primary waters, both potentials predict that the cluster with six secondary waters is particularly stable (Figure 2). E_{Pep} is the lowest for this cluster. Adding an additional water lowers the energy by 3.1 kcal/mol (MCY) or 6.7 kcal/mol (ST2, this latter energy is nearly equal to the water-water hydrogen bond energy). The 3 primary, 6

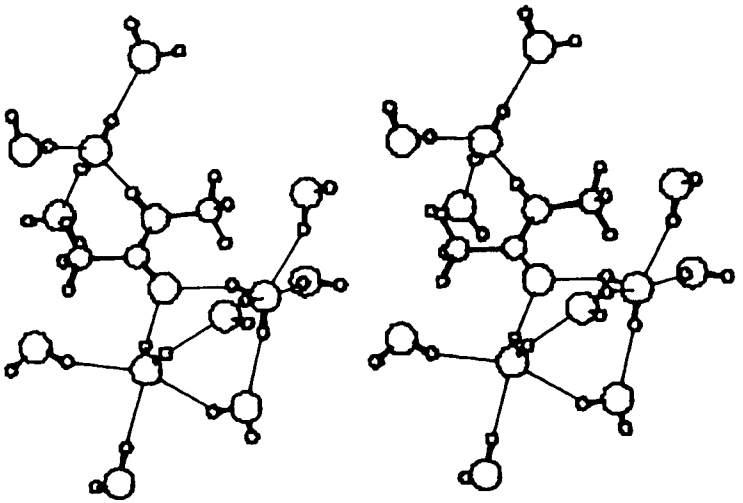

Fig. 1. Minimum energy cluster of water molecules on methylacetamide using MCY potential (2 primary, 6 secondary on CO).

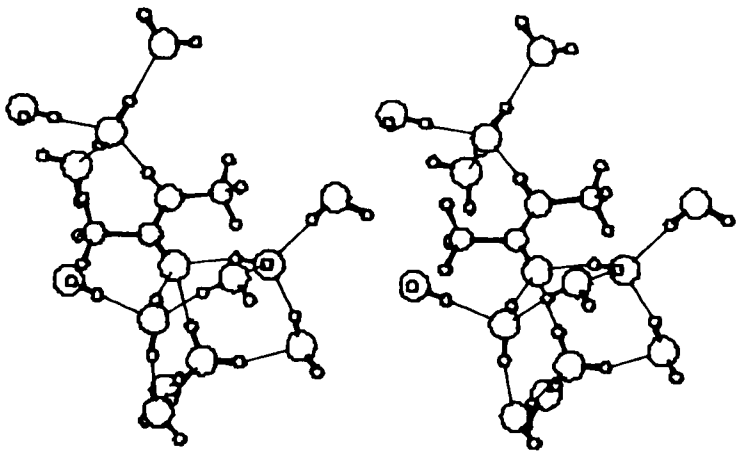

Fig. 2. Minimum energy cluster of water molecules on methylacetamide using ST2 potential (3 primary, 6 secondary on CO).

secondary cluster is 10.5 (MCY) or 16.6 kcal/mol (ST2) more stable than the 2 primary, 6 secondary cluster with one less water. In the 3 primary, 6 secondary cluster there is a symmetric arrangement of the primary waters, three secondary waters linking pairs of primaries and three secondaries hydrating individual primaries. Even though another water is added, the cluster with 4 primary and 6 secondary waters has higher energy than the 3 primary, 6 secondary cluster.

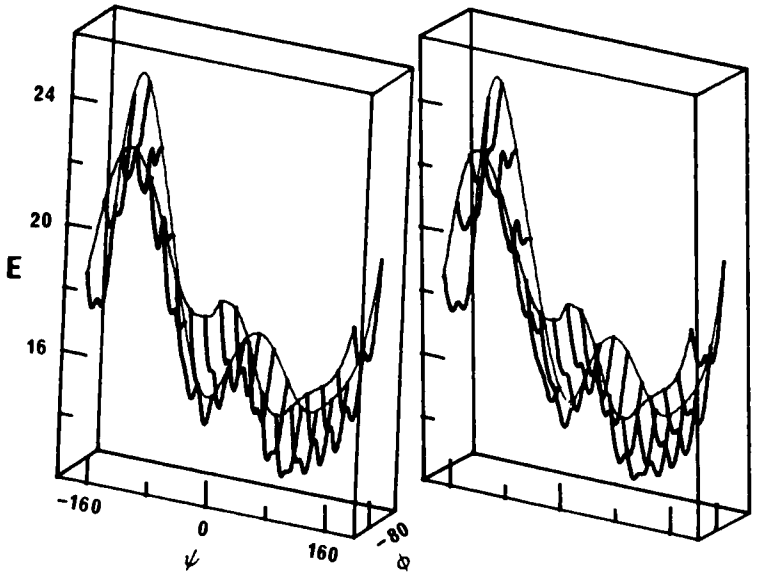

Fig. 3. Stereo view of CFF energy surface for AcProNHMe *in vacuo*.

Hydration Energies for Acetylproline Methylamide

Hydration energies were computed for AcProNHMe to determine whether hydration energies are conformationally dependent and to test predictions of preferred conformers in solution. First the intramolecular peptide energy was computed *in vacuo* by the CFF method (Figure 3). There are three regions of low energy near $\Psi = 150$, 77 and $-47°$ which will be referred to as P_{II}, C_7 and α_R, respectively. For the hydration computations, Ψ was fixed at one of the above values with $\Phi = -68°$.[11] The bonded geometry was also fixed.

Hydration energies for the three proline conformers are given in Table IV. For reference, energies for similar water clusters hydrating the single CO and NH groups in methylacetamide are given as E_{Ace}. For the P_{II} and α_R conformers with only primary waters, the hydration energies (E_{Tot}) are considerably lower than those estimated from methylacetamide (E_{Ace}). This is due to interactions among waters on adjacent peptide groups and/or bridging of the two carbonyls by the same water. Due to the peptide-peptide hydrogen bond in the C_7 conformer, the hydrating clusters are suboptimal and any decreases of E_{Tot} relative to E_{Ace} are smaller. As in the case of methylacetamide, a number of clusters containing only primary water molecules are unstable for the MCY potential. For the clusters which include secondary waters, E_{Tot} is

Table IV. Hydration Energies of AcProNHMe

Con-	Number of Waters		Energies (kcal/mol)					
former	1^{o}	2^{o}	ST2			MCY		
			E_{Pep}	E_{Tot}	E_{Ace}[a]	E_{Pep}	E_{Tot}	E_{Ace}[a]
P_{II}	5	0	-34.1	-49.7	-35.3	-36.6	-50.0	-36.0
	7	0	-42.2	-61.7	-53.5		b	
	7	13	-50.8	-172.8	-180.5	-47.8	-155.5	-155.8
α_R	5	0	-30.6	-47.5	-35.3	-27.1	-42.2	-36.0
	7	0	-41.5	-63.8	-53.5		b	
	7	13	-50.9	-178.3	-180.5	-39.3	-153.0	-155.8
C_7	4	0	--	--	--	-20.6	-28.9	-29.6
	5	0	-27.3	-38.5	-36.7		b	
	6	0	-30.5	-48.5	-44.4		b	
	6	12	-40.5	-152.6	-157.4	-39.2	-138.5	-136.7

a. Energy for hydrating single CO and NH groups of methylacetamide in a similar pattern. For instance, the fully hydrated structures have patterns based on 3 primary, 6 secondary per CO and 1 primary, 3 secondary on the NH for P_{II} and α_R conformers. For these $E_{Ace} = 2\,E_{3,6} + E_{1,3} - 2\,E^{NH}_{1,0}$ (the last term corrects for double counting of the primary water on the NH). For the 6 primary, 12 secondary structure of the C_7 conformer, $E_{Ace} = E_{3,6} + E_{2,5} + E_{1,3} - 2\,E^{NH}_{1,0}$. Similarly the patterns were matched for the other clusters. b. Cluster not stable.

generally somewhat less than E_{Ace}. This is due to sharing of waters in adjacent clusters so that the individual clusters are slightly displaced from the optimum.

The comparison between E_{Tot} and E_{Ace} is similar for C_7 and the other two peptide conformers, but in this case E_{Ace} reflects the choice of suboptimal hydration schemes. The interacting water clusters including some of the secondary water molecules are shown in Figures 4-6. The network of hydrogen bonds can be seen in these views. Note that much of the space around the peptide is occupied by waters attached to the two CO's and the one NH along with their secondary waters.

Preliminary computations indicate that the computed hydration energies are not sensitive to minor variations of the ring puckering for the α_R conformer nor to changes in Ψ of $\pm 10°$ for the P_{II} conformer. Clusters

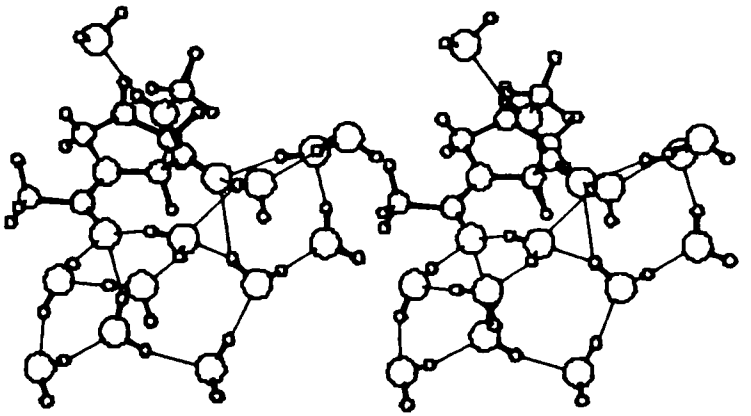

Fig. 4. Minimum energy cluster (ST2) of water molecules for α_R conformer of AcProNHMe. Peripheral waters have been stripped from the cluster of 7 primary, 13 secondary waters.

in which one of the water molecules bridges the two CO's in the P_{II} conformer (as in the reported five primary cluster) should be explored more fully with additional water molecules in the cluster.

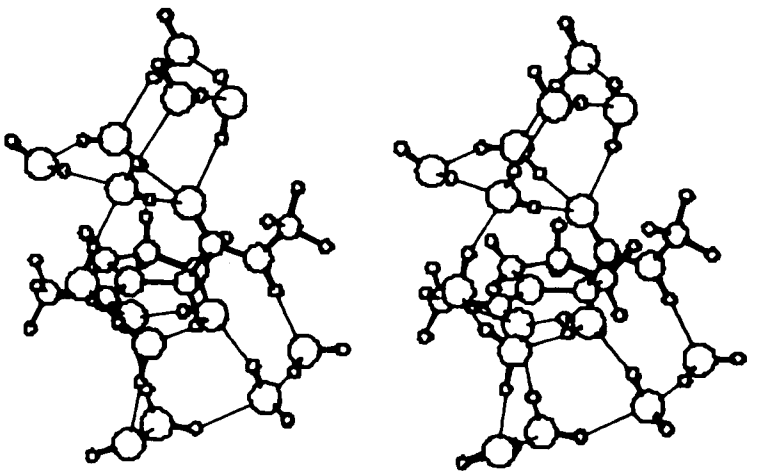

Fig. 5. Minimum energy cluster (ST2) of water molecules for P_{II} conformer of AcProNHMe. Peripheral secondary waters have been stripped from cluster of 7 primary, 13 secondary.

The relative energies of the three peptide conformers *in vacuo* and with the same number of hydrating water molecules are given in Table V. It is evident that the C_7 conformer is favored *in vacuo* as is observed in non-polar solvents.[1] In aqueous media the C_7 conformer is predicted to be depopulated, again as is observed.

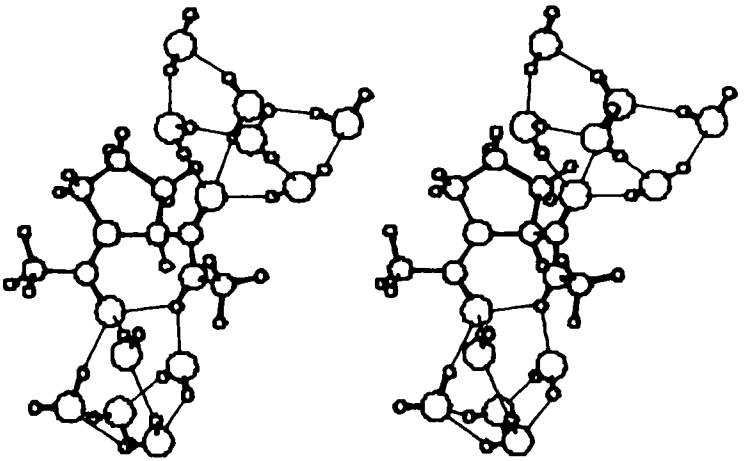

Fig. 6. Minimum energy cluster (ST2) of water molecules for C_7 conformer of AcProNHMe. Peripheral waters have been stripped from cluster of 6 primary, 12 secondary.

Table V. Intramolecular Plus Hydration Energies of AcProNHMe

Con-	Number of Waters		Energies (kcal/mol)					
former	1^{o}	2^{o}	CFF		ST2		MCY	
			E_{Vac}	E_{Rel}	E_{Sum}[a]	E_{Rel}	E_{Sum}[a]	E_{Rel}
P_{II}	5	0	14.5	1.5	-35.2	0.0	-35.5	0.0
	7	0			-47.2	2.5	--	--
	7	13			-158.3	5.9	-141.0	0.0
α_R	5	0	14.1	1.1	-33.4	1.8	-28.1	7.4
	7	0			-49.7	0.0	--	--
	7	13			-164.2	0.0	-138.9	2.1
C_7[b]	4	0	13.0	0.0	--	--	-15.9	15.6
	5	0			-25.5	9.7	--	--
	6	0			-35.5	8.3	--	--
	6	12			-139.6	9.1	-125.5	1.6

a. $E_{Sum} = E_{Vac} + E_{Tot}$ (E_{Tot} from Table IV).

b. For the C_7 conformer E_{Sum} was normalized by the ratio of the number of waters in the other clusters to the number for C_7.

A clear choice between the α_R and P_{II} conformers is not possible from the computations. Experimentally, P_{II} is found to dominate.[12] The computed energies depend on the potential function and the water cluster chosen. For the same type of water cluster the relative energies of the P_{II} and α_R conformers may differ by up to 6 kcal/mol. Energy differences must be known within 0.5 kcal/mol to get a rough estimate of the conformational distribution. The hydration energy must be computed within less than 1% to achieve this accuracy.

Conclusions

In the areas for which there is agreement between predictions from the two potential functions, we can be reasonably confident of the results. First, there is agreement on the type of water clusters which are stable around methylacetamide. In addition, the methods both predict that C_7 is disfavored for AcProNHMe in aqueous solution. Subtle distinctions between hydration energies cannot presently be made. If the competing interactions amongst water molecules could be better assessed, perhaps hydration energies could be estimated using only a few water molecules.

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COMPUTER-ASSISTED MODELING OF RIBONUCLEASE S-PEPTIDE BASED ON STRUCTURAL AND LOCAL INTERACTION ANALYSIS

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Introduction

In spite of numerous studies on structure prediction, there has been little direct experimental utilization of experimentally- and theoretically-derived secondary structure probability constants for the design of new synthetic analogs of biologically active peptides which retain specification for a defined folded structure. In recent years, many reports on synthetic analog preparations have been devoted to peptide hormone systems where the main attention has been to alter intermolecular interactions by amino acid residue replacement. This approach often disregards possible peptide backbone conformational alterations that are introduced by sequence variation, especially for linear peptides. We have used the peptide-protein noncovalent complex of bovine pancreatic ribonuclease S to examine correlates between amino acid sequence and properties of folding and function. In the present study, we have sought to define a core set of essential chemical and structural factors that must be present for the S-peptide to produce a viable noncovalent complex with S-protein through analysis of the crystal structure of RNase-S by computer and to demonstrate the validity of such basic factors through design and synthesis of a new simplified peptide that retains the essential properties of native S-peptide. Thereby, we assessed to what extent a peptide could be designed with the specified preference for a folded structure and function.

Local Interaction Analysis

In order to evaluate the minimum information necessary for the formation of RNase-S complex in terms of specific local interactions among side chains, a local interaction analysis[1] was applied to the complex. This involved the identification and evaluation of the free energy contribution of each side chain contact present between S-peptide and S-protein in the native complex. A similar analysis was applied to a series

of hypothetical conformations of RNase-S where S-peptide was systematically displaced about its native position with the aid of a computer using crystallographic coordinates of RNase-S.[2] The latter was carried out in order to identify S-peptide residues that provide a significant stabilization energy not only at native but also at some hypothetical positions. Reasonableness of such hypothetical positions, i.e. there are no serious van der Waal close contacts or overlaps of atoms, was checked by visually using computer graphics and numerically calculating interatomic distances. For each position a new set of pairwise side chain contacts is identified. The magnitude of the van der Waals stabilization free energy associated with specific side chain contact can be assessed on a pairwise basis by means of a set of interaction parameters, ξ_i, one characteristic for each type of side chain. The following equation was used to calculate the free energy change for forming the i-j side chain pair contact by removing these side chains from water and replacing i-water and j-water contacts with i-j contacts.[3]

$$\Delta G_{i-j}=\left(\frac{2V_iV_j}{V_i+V_j}\right)(\xi_i-\xi_j)^2-\left(\frac{2V_iV_w}{V_i+V_w}\right)(\xi_i-\xi_w)^2-\left(\frac{2V_jV_w}{V_j+V_w}\right)(\xi_j-\xi_w)^2 \qquad (1)$$

where $V_{i,j}$ and V_w are the molar volumes of side chain types (i and j) and water. The values of V and ξ are reported by Krigbaum and Komoriya.[3] Assuming that the sum of local interaction free energies equals the global stabilization free energy, a comparison of the total stabilization free energies associated with a given hypothetical position of S-peptide with that of the native position should provide useful information regarding the energetics of the stabilizing contacts in native RNase-S complex.

When one examines the contribution of all local side chain contacts between S-peptide and S-protein, one finds that nearly 50% of the total free energy stabilizing the RNase-S complex is contributed by only two residues, Phe-8 and Met-13. The i-j side chain contacts that stabilize native complex are Phe-8 with Val-108 and Phe-120, and Met-13 with Val-47, Val-54 and Leu-51.

Structural Analysis

An examination of the crystal structure of RNase-S indicates that Phe-8 and Met-13 are in the amino terminal α-helix (residues 3 to 13). Further, the side chain environment of Phe-8 and Met-13 suggest a necessity of a stable helical conformation for proper orientation of the two residues toward S-protein for optimum contacts for complex stability. Proper orientation of these two residues will, in turn, result in a proper orientation of catalytic residue His-12 in the RNase-S active site.

Hence, the essential structural features necessary for S-peptide to produce a viable noncovalent complex S-protein are a helix and the residues Phe-8, His-12, and Met-13. The importance of a stable helix has been shown by previous S-peptide synthetic variation of Glu-9.[4] In the present study, a model pentadecapeptide was designed comprising a polyalanine sequence with nonalanine residues at positions 2, 7, 8, 10, 12, and 13, which are Glu, Lys, Phe, Arg, His, and Met, respectively. Alanine residues were chosen based on ease of handling in chemical synthesis as well as on high helix-propensity. A comparison of native S-peptide (1-15) and model peptide (1-15) with respect to helical conformational propensity is given in Table I.

Table I. Average Single Residue Helical Potential

Region averaged	Peptide	
	Native S-peptide(1-15)	Model S-peptide(1-15)
All residues	.63	.82
N-terminal 4 residues	.74	.99
C-terminal 4 residues	.52	.96

Profiles of estimated stabilization free energies for α-helical conformation, assuming that all of the short range contacts between pairs of side chains are of the characteristic helical contacts $i \pm 3$ and $i \pm 4$ types, are illustrated in Figure 1. The profile for the model peptide shows more negative values than that for native S-peptide and the average single residue potential in all three regions indicates that the model peptide sequence has at least equal if not better propensity for helical conformation. The conclusion of conformational relatedness of the model and native S-peptide was visually checked by computer graphics by assigning S-peptide crystal structural coordinates to model peptide atoms.

The pentadecapeptide was synthesized by the solid phase method. The peptide formed an enzymatically active complex with S-protein with 36% of the specific activity of RNase-S; the affinity of model peptide for S-protein was found to be only 12 fold lower than that of native S-peptide. Further, we have found significant enzymatic activity with [Glu^2,Glu^{10}], [Arg^2,Glu^{10}], [Ala^2,Glu^{10}], and [Ala^7] analogs of model peptide, indicating that Glu-2, Lys-7, and Arg-10 can be further simplified in S-peptide modeling.

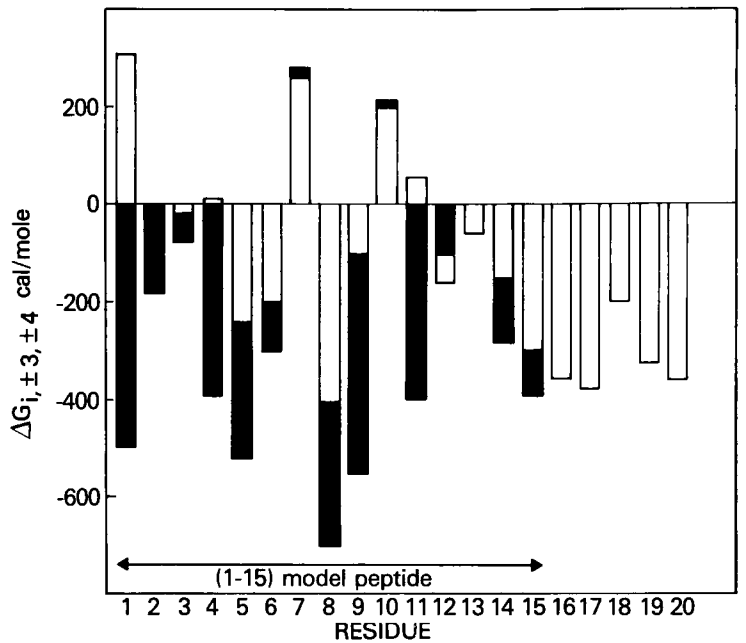

Fig. 1. Profiles of estimated local interaction free energy of stabilization for helical conformation of native (open bar) and model S-peptide (solid bar). Values for average free energy of stabilization are calculated according to Equation 1.

Discussion

The successful use of sequence modeling of S-peptide suggests the potential applicability of the general tactic in synthesizing analogs of other polypeptides. The type of modeling used here is based on the principle of retaining basic conformational potential to provide a framework on which a minimal set of important chemical details can be placed. Such conformational potential can be defined for native polypeptides of known sequence using a variety of lists of probability constants as well as three-dimensional (usually crystallographic) structures when available. This conformational approach in designing peptide and protein sequences could be a more rational way to define the functional conformations of these molecules. Further, designing amino acid sequence for controlling specification of folded structure and function seems feasible.

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ON THE STRUCTURE OF SUBSTANCE P BY CONFORMATIONAL ENERGY CALCULATIONS

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Substance P (SP) is an undecapeptide with the sequence H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2 and acts mainly as an excitatory neurotransmitter.[1] In an attempt to find the active structure of this molecule, conformational energy calculations have been carried out using the ECEPP program[2] and search and minimization routines.[3]

To reduce the computational time, the molecule was divided into fragment A [SP (1-5)] and fragment B [SP (6-11)]. From a set of starting conformations for each fragment by search and energy minimization techniques. The combinations of such low energy conformers obtained for each fragment were then used as the starting configurations to find low energy structures of the complete molecule.

Fifty-five low energy conformers for fragment A were found ranging in energy from -55.5 to -45.0- kcal/mol. However, several conformations were found to have similar side chain orientations even with different backbone dihedral angles. This observation made it possible to classify fragment A conformers into four configurational types, namely, IA, IB, IC and ID. For each configuration two distinct backbone classes of conformers were found and Table-I shows the ϕ,ψ regions for the middle three residues of segment A and the lowest energy found for each con-

Table I. The Conformational Regions of Middle Three Residues for the Conformational Classes of Fragment A of Substance P

	(ϕ, ψ) Regions			
Code Name	Pro_2	Lys_3	Pro_4	E(kcal/mol)
IA1	-,+	-,+	-,+	-54.5
IA2	-,-	+,+	-,+	-49.8
IB1	-,+	-,+	-,-	-52.7
IB2	-,-	+,+	-,-	-47.4
IC1	-,-	-,+	-,+	-53.7
IC2	-,+	+,+	-,+	-55.5
ID1	-,-	-,+	-,-	-51.8
ID2	-,+	+,+	-,-	-50.9

formational class. The conformations of the first and last residues do not have any effect on the spatial orientations of the side chains. Thus, the conformers that belong to the same configurational type are similar with respect to their side chain orientations but differ in backbone angles in the Pro[2] and Lys[3] positions (e.g. see conformer types IA1 and IA2). The details of the conformational features of each configuration will be described elsewhere.[6]

The low energy structures of fragment B may also be divided into two major groups, IIA and IIB. The conformers that belong to IIA have (ϕ, ψ) values for Gly[9] in the (—, +) or (—, —) regions, whereas, for group IIB these regions are (+, —) and (+, +). The lowest conformational energies found in groups IIA and IIB are -12.1 kcal/mol and -8.4 kcal/-mol respectively. Most conformers in group IIA preferred the α_R conformation at Gly,[9] while in group IIB it is the C_7^{ax} (+, —) region which is preferred. In both groups a number of conformational classes were found which have similar side chain orientations but differ in their backbone angles at the Phe[8] and Gly[9] positions. One such low energy structure will be described here.

The low energy structures of the complete molecule (SP), were obtained by combining the low-energy structures of fragments A and B. Conformer (SP1) is a result of combining IC with IIA, using the lowest energy conformer among the group IIA conformational types. SP1 also has an equivalent structure (SP2), in which group IIB is combined with IC. Except for the residues Phe[8] and Gly,[9] the backbone conformational regions for the remaining residues of these two conformers are the same and they are (—, +) for Arg,[1] (—, +) for Pro,[2] (+, +) for Lys,[3] (—,+) for Pro,[4] (—, +) for Gly,[5] (—, —) for Gln,[6] (—, +) for Phe,[7] (—, +)for Leu[10] and (—, —) for Met.[11] The (ϕ, ψ) values for the residues Phe[8] and Gly[9] are in the α_R region for conformer SP1 and the C_7^{eq} and C_7^{ax} regions for conformer SP2. Conformer SP2 is the lowest energy structure (E = -76.4) and SP1 is only 0.2 kcal/mol higher than the lowest energy value. Figure 1A-B shows the conformational features of these two structures.

In fragment A (configurational type IC), the side chains Pro,[2] Lys[3] and Gln[5] are on one side of the molecule and Arg[1] and Pro[3] are on the opposite side. In fragment B, Phe[7] and Leu[10] are close to each other, and the side chains of Phe[8] and Met[11] point in the opposite direction to the Phe[7], Leu[10] pair. Fragment B is further stabilized by hydrogen bonding between the amide group of the Gln[6] side chain and the carbonyl oxygen of Met.[11] A number of other low energy SP conformers were found which differed either in fragment A or in fragment B or in both. These will be reported elsewhere.[6]

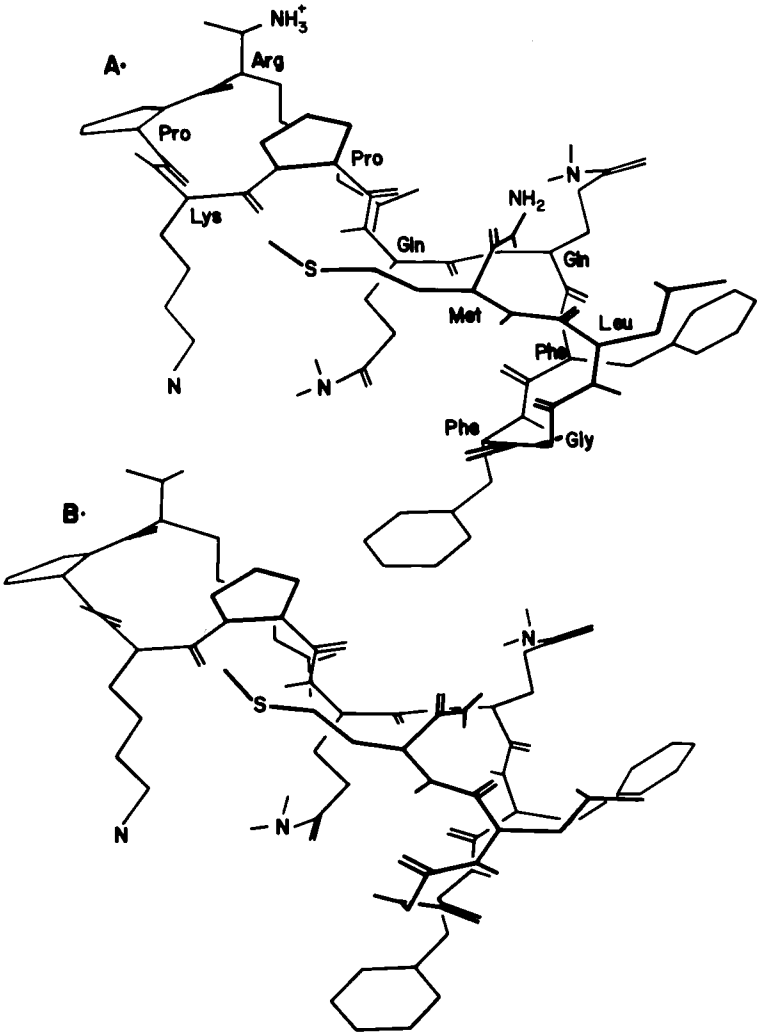

Fig. 1. Low energy conformers of substance P (A) conformer SP1; (B) conformer SP2.

Upon examining the structure-activity data of a number of analogs of SP, many low-energy structures could be eliminated. For example, it has been shown that the analog (L-Ala^9)SP^7 is as active as the native SP. This implies that the residue at position 9 may prefer to have its conformation either in the (-, +) or (-,-) regions. Also the high activity of the analogs^8 [Sar^9]SP and [(N-Me)Phe^8, Sar^9]SP indicate that an α_R conformation may not be favorable at the 7th and 8th positions. When these

results are considered in choosing the active structure of SP, one low energy structure (SP3) is found that explains the available structure-activity data. This structure which is about 3 kcal/mol higher than the lowest energy value has a IB configuration [(-, +),[1], (-, +),[2] (-, +),[3] (-, -),[4] (-, +),[5]] for fragment A. For fragment B the conformational regions are (-, +),[6] (-, +),[7] (-, +),[8] (-, -),[9] (-, .),[10] and (+, +)[11] and hence, belong to the conformation group IIA. In fragment A the Arg and Lys side chains are parallel to each other and Pro,[2] Pro[4] and Gln[5] are in the opposite direction to the Arg-Lys pair. For fragment B the Leu and Met side chains are in parallel orientations. Residues Phe[7] and Phe[8] have no apparent interaction with any other side chains. Further, the amide group of the Gln[6] side chains interacts with the carbonyl oxygen of the Met[11] residue. Even though some agreement with analog data is found with the calculated structures, other analogs must be studied before a final receptor structure is proposed.

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4-SUBSTITUTED ANALOGS OF DEAMINO-D-ARGININE-VASOPRESSIN: BIOLOGICAL POTENCIES AND STRUCTURE-ACTIVITY RELATIONSHIPS RELATED TO POSITION 4

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[Mpa^1, D-Arg^8]-vasopressin (deamino-D-arginine-vasopressin, dDAVP) is known as a peptide with a strongly elevated antidiuretic activity, and a deeply suppressed vasopressor activity. As such, it was assumed for a long time to be the most specific and also the strongest antidiuretic substance within the group of neurohypophyseal peptides. Some findings on recently synthetized peptides, however, seem to necessitate a revision of the latter statement. In particular, 4-threonine[1] and 4-valine[2] analogs of dDAVP possess distinctly higher antidiuretic activities than the parent substance itself and turned *eo ipso* the attention to position 4 in this and perhaps also other groups of neurohypophyseal peptides. Two physicochemical features of the 4-substituent are most likely involved in the generation of a hormonal stimulus: its hydrophobicity[3] and its molecular volume.[4] It was already suggested that the hydrophobicity of this position causes a regular but non-monotonic change of antidiuretic activity, with several activity extremes.[5] Relevant effects of substituent volume are assumed rather than firmly proven. We have therefore attempted to find a quantitative rule which would define biological potencies of the 4-substituted dDAVP analogs as a sum of fractional contributions of these two parameters.

To this end, we have investigated biological activities of a series of such analogs synthetized recently in our Swedish laboratory.[5] Purified peptides were assayed for their antidiuretic activity (hydrated rat), vasopressor activity (rat arterial blood pressure, dibenzyline premedication) and *in vitro* uterotonic activity (rat in natural estrus, assay in Mg^{2+}-free medium). The results, together with earlier reported data on similar analogs, are summarized in Table I.

The hydrophobicity of the side chain is best characterized by the hydrophobicity constant π recently estimated for amino acid side chains.[6,7] The substituent volume can be represented by molar refrac-

Table I. Biological Activities of 4-Substituted dDAVP Analogs

amino acid in pos. 4[a]	activity (relative to dDAVP) antidiuretic (AD)	vasopressor (VP)	uterotonic	ratio AD/VP
Gln	1.00	1.00	1.00	1.00
Val	1.44	<0.15	0.90	>9.60
Asn	1.26	0.90	0.09	1.40
Ala	0.74	<0.30	<0.10	>2.74
Ile	0.20	<0.15	<0.10	>1.33
Leu	0.10	<0.30	<0.10	>0.33
Chg	0.06	0.08	0.04	0.08
Gly	0.05	<0.30	<0.10	>0.17
Bug	0.04	1.00	0.01	0.04
Thr[1]	0.57	<0.05	0.68	>11.20
Val[2]	1.03	<0.03	1.21[b]	>39.60
Abu[5]	0.65			
Ser[5]	0.02			

[a]Abbreviations according to the IUPAC-IUB Commission on Biochemical Nomenclature. Additional abbreviations: Chg, cyclohexylglycine; Bug, *tert*-butylglycine; Abu, 2-methylalanine. Superscript numbers indicate literature reference.

[b]Obtained with our reference value for uterotonic activity. When using author's value, the relative uterotonic activity is 5.33.

tions R_m calculated from the group contributions listed in the literature.[8] Antidiuretic, vasopressor and uterotonic potency p was expressed as

$$p = \log a_A - \log a_S, \qquad \text{Eqn. 1}$$

where a_A, a_S are relevant biological activities of the analog and of dDAVP, respectively. At first inspection, no linear correlation exists between any potency and any of the two structural parameters: the correlation coefficients are low and insignificant. We have therefore assumed a multiparametric relation in which π and R_m are combined in the following way:

$$p = c_o + c_1\pi + c_2R_m + c_3\pi^2 + c_4R_m^2 + c_5\pi R_m; \quad \text{Eqn. 2}$$

quadratic terms account for possible non-monotonic relations between p, π and R_m (the meaning of the π-square term follows from the model of Hansch). The product πR_m has been introduced here as a rough measure

of "interaction" between the two features. It should be noticed, however, that the parameters π and R_m are not fully independent and that a certain degree of correlation can be detected by the regression analysis. The testing of statistical significance of individual terms proceeded by a stepwise reduction of terms in Equation 2: individual terms were omitted and the significance of the increase in multiple correlation coefficient was assessed by means of an F-test procedure. The results of these tests show

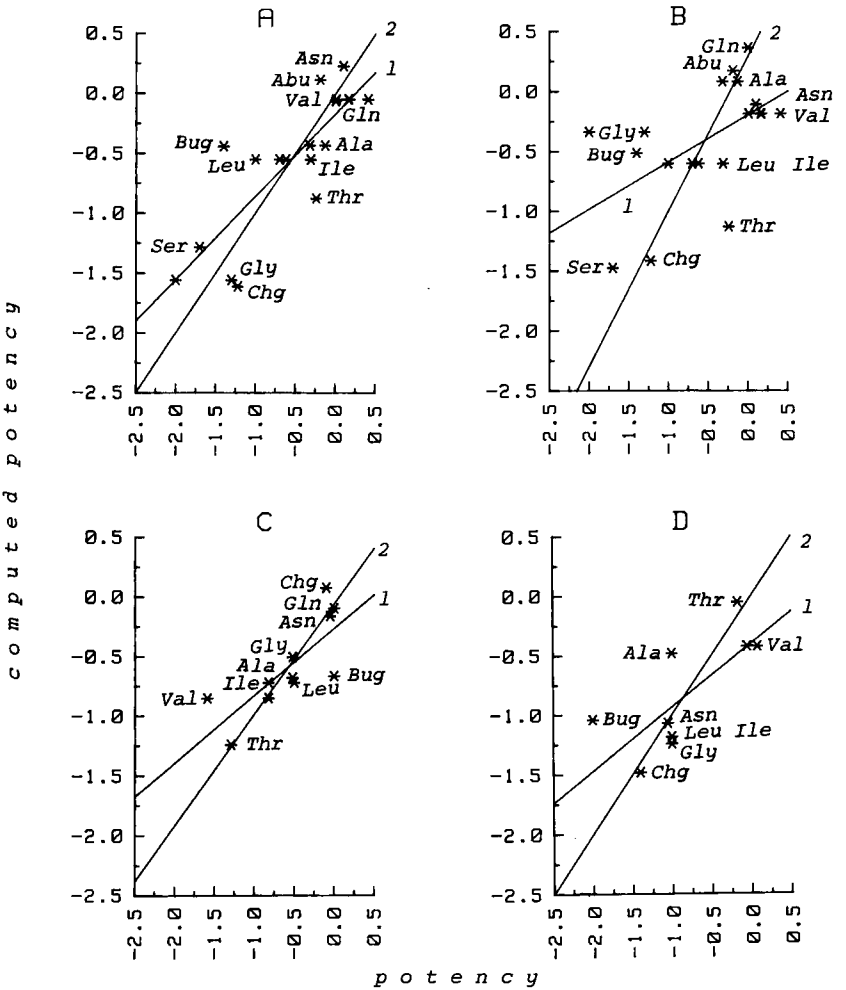

Fig. 1. Correlation (Eqn. 2) between experimental (abscissa) and computed (ordinate) potencies of 4-substituted dDAVP analogs. Panels A and B: antidiuretic potency (B after omission of the constant term c_o in Eqn. 2). Panel C: vasopressor potency. Panel D: uterotonic potency. 1: regression line $y = a_{yx} + b_{yx}x$. 2: regression line $x = a_{xy} + b_{xy}y$. Substituents in position 4 indicated at each point (abbreviations see Table I).

a clear-cut dependence of the antidiuretic potency upon the terms containing R_m. It is very striking, however, that the correlation comprising the second to sixth terms without the constant c_o is insignificant. When this constant term is considered, the multiple correlation coefficient increases from 0.55 to 0.84 (19 entries). This is shown in Figure 1 in form of a relation between values from measurements and those predicted by correlation. Apparently, p cannot be expressed solely as a sum of fragmental contributions of the two physicochemical features. So far the residue c_o has no physical interpretation. Further analysis of numeric values of coefficients in Equation 2 indicates that an increasing hydrophobicity and molar volume exercise an influence upon p, but the interaction of the two diminishes this positive effect. Thus, antidiuretic potency displays a minimum appearing at $\pi \simeq 0.4$ (corresponding to Ala) and $R_m \simeq 13.4$. Virtually the same applies for vasopressor and uterotonic activities. Although the multiple correlation coefficients show a fair degree of correlation in these latter instances, the statistical significance is low, due to a limited number of available data. A large assay error, which is a consequence of the low absolute values of these activities in the dDAVP series may account for another source of low significance.

By way of a preliminary comment, we would like to mention that these conclusions are not applicable solely for dDAVP analogs: very similar relations have also been found for other vasopressin series documented in the literature.

Acknowledgements

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THE PRODUCTION OF HUMAN INSULIN USING RECOMBINANT DNA TECHNOLOGY AND A NEW CHAIN COMBINATION PROCEDURE

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Introduction

Specific polypeptides such as insulin can now be made by microbial fermentation owing to numerous advances in molecular biology and synthetic nucleotide chemistry.[1-9] The preparation of human insulin by recombinant DNA technology not only is another significant chapter in the illustrious history of insulin, it also promises to provide an important, alternative source of insulin. We have chosen to call this insulin Biosynthetic Human Insulin (BHI).

BHI can be prepared either by combining the two independently synthesized A and B polypeptide chains or by converting proinsulin to insulin.[3] The latter approach is described elsewhere in this symposium.[10] In the chain combination procedure described below, the two insulin chains are obtained from separate *E. coli* K12 fermentations utilizing plasmids containing synthetic A- or B-chain genes linked to an appropriate leader DNA (e.g., tryptophan synthetase gene) via a methionine codon. The chains are therefore released from the respective chimeric protein translation products by reaction with cyanogen bromide. The cleaved chains are subsequently converted to the corresponding S-sulfonates, purified by chromatographic methods, and chemically combined. The resulting insulin is purified by conventional chromatographic and crystallization procedures. We have extensively tested this BHI using chemical, biological, physicochemical, and immunological methods, and have found it to be equivalent to the appropriate standards of purified pancreatic human insulin and purified porcine insulin.[8,9] Additional *in vitro* studies on BHI have confirmed our findings;[11,12] furthermore, BHI is effective in clinical testing.[13,15]

The main purpose of this paper is to describe an improved chain combination procedure which avoids the complex, multi-step methods used previously to prepare insulin from synthetic chains.[16,18] In addition,

we describe an HPLC "fingerprint" analysis of BHI using the enzyme *Staphylococcus aureus* V8 protease.

Results and Discussion

Combination of Human Insulin A- and B-Chain S-Sulfonates — The optimal conditions that we have established for preparing human insulin by the chain combination route are outlined in Figure 1. A predetermined amount of dithiothreitol (DTT) to give an $SH:SSO_3^-$ molar ratio of approximately 1.2 is dissolved in a 0.1 M glycine buffer, pH 10.5, and quickly added to a chilled chain solution (5-10 mg/ml) containing a 2:1, A:B ratio, by weight, of S-sulfonated chains in the glycine buffer. The resulting solution is stirred for 24 hours at 4° C in an open vessel to permit air oxidation. Under these conditions, the insulin yield is approximately 60% of theory relative to the amount of B chain used.

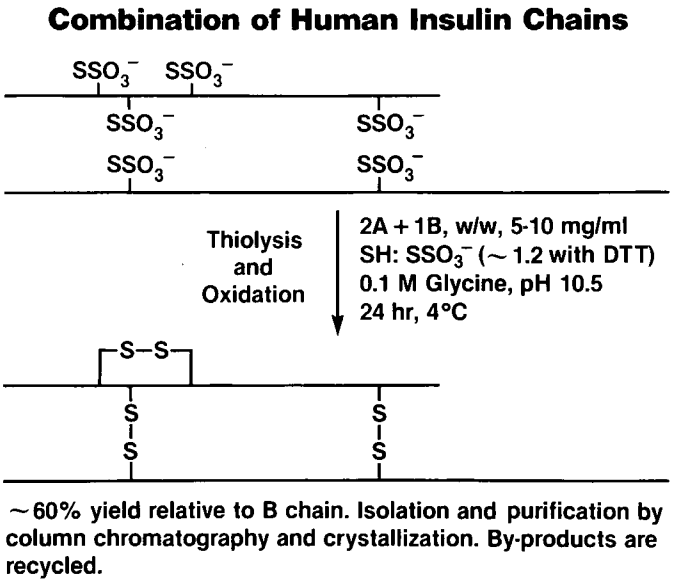
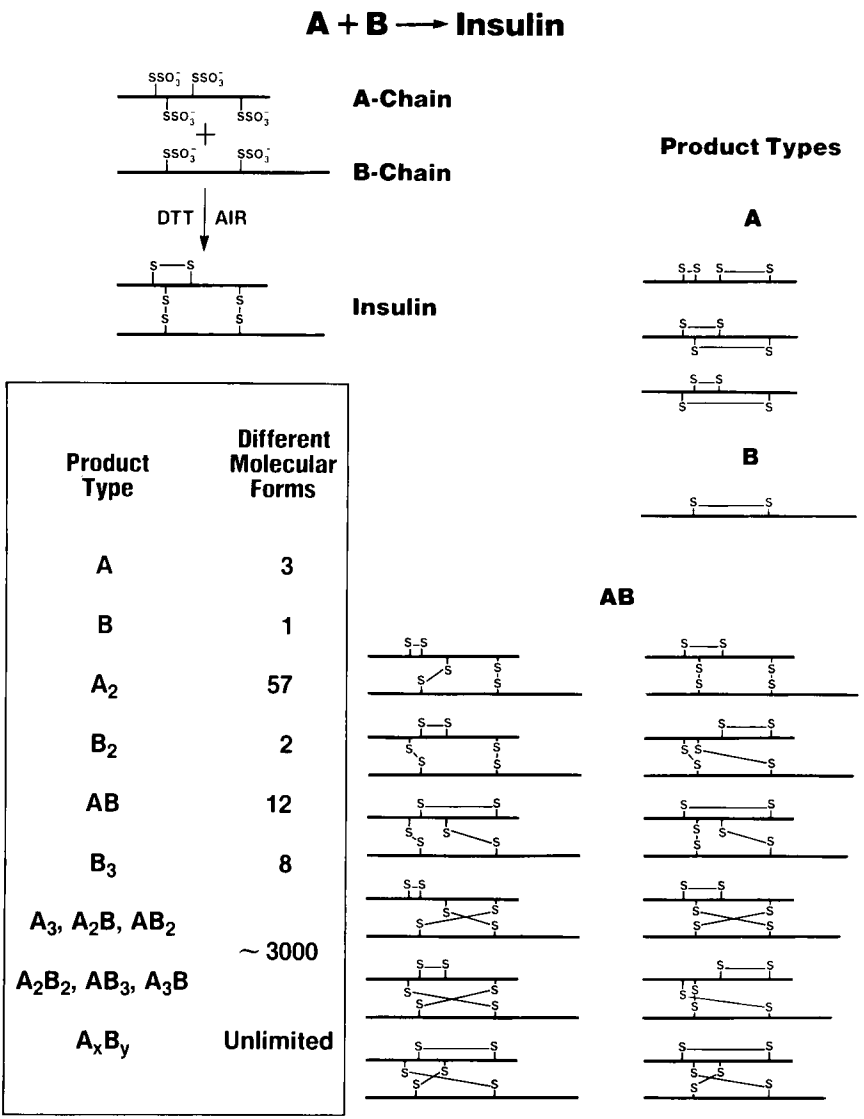


Fig. 1. Summary of a new insulin chain combination method.

An important ingredient in this single-step, single-solution combination reaction is the thiol reducing agent DTT. With the use of DTT, only slightly more than stoichiometric amounts of thiol are required for the thiolysis reaction. This is in contrast to the relatively large excesses of β-mercaptoethanol used in earlier chain combination studies.[16,18] An attractive feature of DTT is that it readily oxidizes to a stable six-



Product Type	Different Molecular Forms
A	3
B	1
A_2	57
B_2	2
AB	12
B_3	8
A_3, A_2B, AB_2	~ 3000
A_2B_2, AB_3, A_3B	
A_xB_y	Unlimited

Fig. 2. Potential products that can occur during the combination of insulin chains.[19]

membered ring and does not participate further in the combination reactions; furthermore, the oxidized DTT is easily removed from the combination solution by the subsequent insulin purification steps.

An extraordinary variety of products may result from the chemical combination of the two insulin polypeptide chains,[19] particularly if the disulfide bonds are formed randomly (see Figure 2). In fact, a reversed-

phase, high performance liquid chromatography (HPLC) analysis of a typical combination mixture displays considerable heterogeneity (see Figure 3). However, the high yield of insulin shows clearly that the combination reaction does not proceed in a totally random fashion. In addition, our purification studies indicate that most of these side products are related to monomeric A or B chains, which is consistent with recent studies that suggest such products are favored.[20] Some of the factors leading to a high yield of insulin include specific, sequence-governed side chain interactions, facile disulfide interchanges, and the apparent conformational stability of the insulin molecule.[17,19,21]

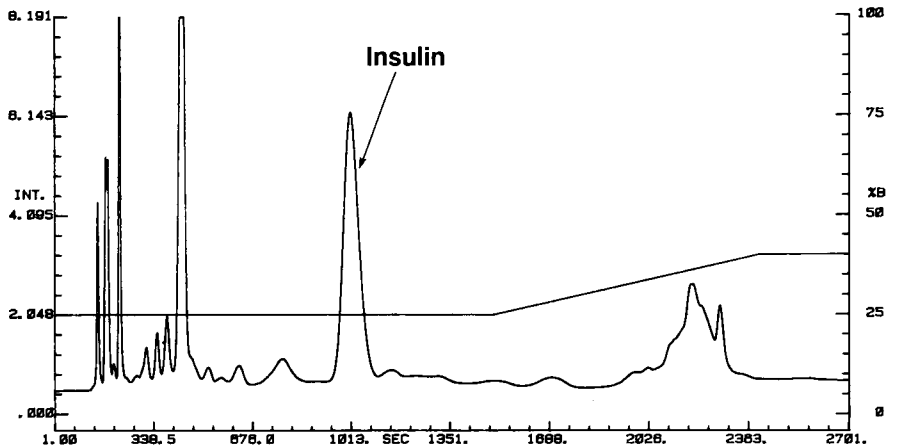


Fig. 3. HPLC profile of a completed chain combination solution as described in the text and in Figure 1. The HPLC conditions were essentially the same as described previously.[8,9] The chromatographic separation was conducted on a 4.6 x 250mm Zorbax TMS column (DuPont) thermostatted at 40° C with a column block heater. The column eluent was derived from solvents A and B, which were mixed in a ratio of 75% A/25% B for 25 minutes followed by a 15-minute gradient formed by increasing solvent B 1% per minute (see gradient line and right ordinate legend). Solvent A consisted of 0.1 M sodium phosphate (monobasic) in 95% water/5% 2-methoxyethanol adjusted to pH 2.0 with phosphoric acid. Solvent B was 95% acetonitrile/5% 2-methoxyethanol. A sample of chain combination solution was acidified with acetic acid, lyophilized, and reconstituted in an equal volume of 0.01N HCl. A 10 μl sample (~90 μg) was injected. The flow rate was 1.0 ml per minute and the column effluent was monitored at 210 nm using 0.5 AUFS (left ordinate). Numbers on abscissa are seconds.

Several variables were studied in detail to optimize the insulin yields, including pH, protein concentration, temperature, the A:B ratio and the $SH:SSO_3^-$ ratio. Of these, the nearly stoichiometric $SH:SSO_3^-$ ratio is the key factor permitting a single-step, single-solution combination reation to work successfully (see Figure 4). Since this ratio may vary from about 1.0 to about 1.3, we have found that it is crucial to determine the precise thiol requirement prior to each major combination reaction.

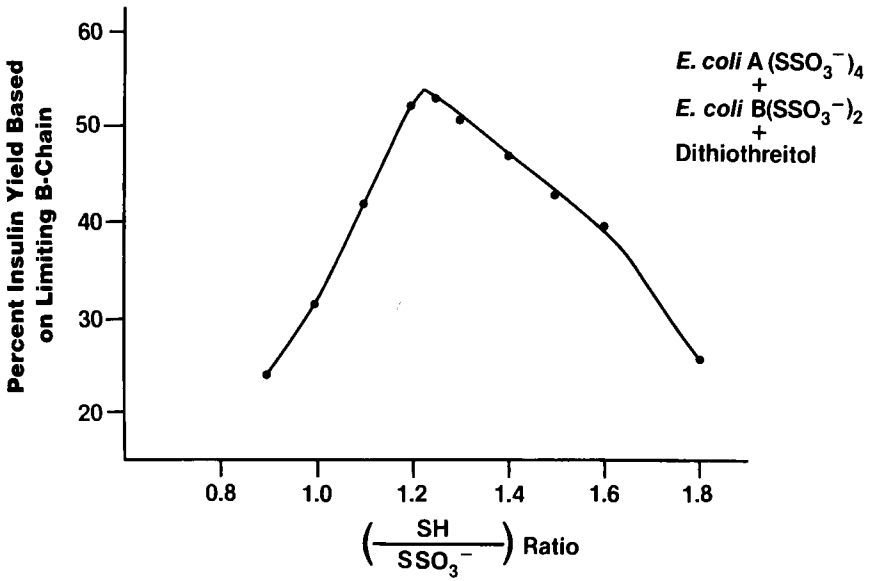


Fig. 4. The effect of different molar ratios of SH:SSO_3^- on the yield of insulin in the chain combination method (see Figure 1 for conditions). Insulin yield was based on an HPLC anlysis as described in Figure 3.

Although DTT has been the primary thiol reducing agent employed in these studies, several other reagents have also been evaluated. The SH:SSO_3^- profiles for all of these thiol reagents are similar to the DTT profile in Figure 4, although their maximal human insulin yields vary considerably. Approximate maximal insulin yields relative to DTT as giving 100% include dithioerythritol, 100%; cysteine 90%; mercaptoacetic acid, 70%; β-mercaptopropionic acid, 65%; β-mercaptoethanol, 45%; *o*-mercaptobenzoic acid, 40%; mercaptoethylamine, 35%; 1,3-dimercapto-2-propanol, 30%; and mercaptosuccinic acid, 10%.

The optimum conditions for each variable studied thus far appears to be independent of the other variables. For example, the SH:SSO_3^- optimum and the pH optimum (pH 10.5) were unchanged over a wide range of protein concentrations even though the insulin yields varied considerably. Some of the optimal combination conditions found in our work for this reaction have been used in earlier studies (e.g., pH, [16,17] A:B ratio,[17,22] temperature,[23] and protein concentration[17]). This suggests that some common mechanism may be involved both in the earlier, multi-step combination procedures and in this new, single-step chain combination method.

HPLC "Fingerprint" Analysis of Biosynthetic Human Insulin — Even though our previous studies clearly demonstrated that BHI is structurally equivalent to appropriate native insulin standards,[8,9] we have conducted additional experiments to provide evidence that the three disulfide bonds are in the correct configuration. For this work, we utilized a "fingerprint" analysis technique using HPLC (see Figure 5) to evaluate the peptide fragments resulting from a *S. aureus* V8 protease digest. This enzyme specifically hydrolyzes peptide bonds on the carboxyl side of glutamic acid residues,[24] which provides fragments containing the various disulfide bonds. In addition, we also determined that an unexpected cleavage occurred at the Ser^{12}-Leu^{13} bond in the A chain.

The enzyme reactions were conducted on crystalline zinc forms of BHI and a semi-synthetic human insulin that was derived from porcine insulin.[25,26] A two-mg sample of each human insulin preparation was dissolved in 0.2 ml of 0.01N HCl followed by the addition of 0.8 ml of 0.05 M NH_4HCO_3 containing 100 μg of enzyme (Miles Laboratories, Ltd., Lot 0590). The final pH was 7.9. A control sample containing only 100 μg of the enzyme was also included. After incubation at 37° C for 24 hours, the proteolysis was terminated by lowering the pH to 2.5, and the products were then analyzed by HPLC as shown in Figure 5. The resulting profiles are essentially indistinguishable from each other, which implies that the structures of the two insulins are identical. Chromatographic analysis of the enzyme control sample indicated that none of the peaks observed in Figure 5 was due to the enzyme itself. The fragment profiles are much more complex than expected, indicating that some cleavages were incomplete. We have isolated several of these fragments from comparable large-scale digests of BHI and porcine insulin, and have identified them using amino acid analyses, partial sequence analyses (Beckman 890C sequenator), and HPLC peak matching. One surprise was the finding of the A^{13-17} pentapeptide (see Figures 5 and 6). Either Staphylococcal protease has a unique specificity not previously reported, or there is another enzyme activity associated with this enzyme preparation. Nevertheless, the fragment profiles were identical for both human insulin digests.

Conclusion

We have prepared human insulin with an improved chain combination method using A- and B-chain S-sulfonates derived from *E. coli* modified by recombinant DNA technology. An important aspect of this single-step, single-solution combination reaction is the use of nearly

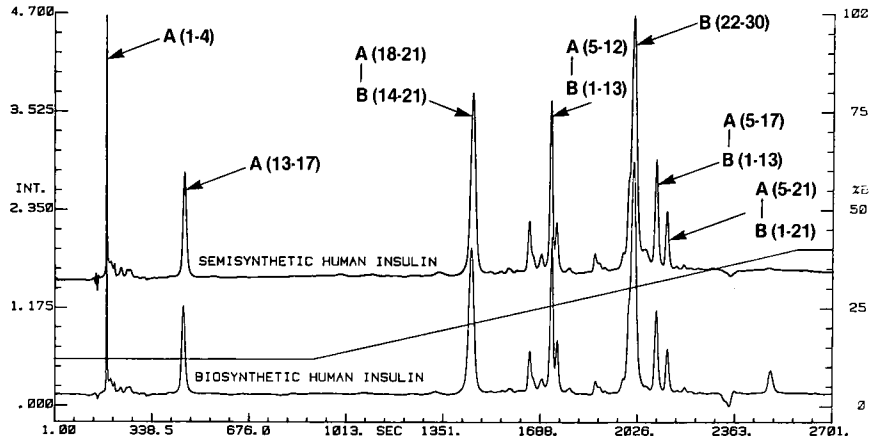


Fig. 5. HPLC profiles for the *S. aureus* V8 protease digests of BHI and semi-synthetic human insulins. The chromatography was accomplished with a Zorbax C-8 column (DuPont) using a combination of isocratic and gradient elution (see gradient line and right ordinate legend). The A solvent consisted of 0.1 M $(NH_4)_2HPO_4$ in 95% water/5% methoxyethanol and the B solvent was 95% acetonitrile/5% methoxyethanol. For the first 15 minutes of each chromatographic run, isocratic elution with 12% B/88% A was used. Thereafter, the B solvent was increased linearly at the rate of 1%/minute for 28 minutes. The final solvent composition, 40% B/60%A, was maintained for an additional two minutes. The column was thermostatted at 40° C and a solvent flow rate of 1.0 ml/minute was used. The column effluent was monitored at 210 nm using a detector setting of 1.0 AUFS (left ordinate). Sample injection volumes were 20 μl, which gave a sample load of ~40 μg.

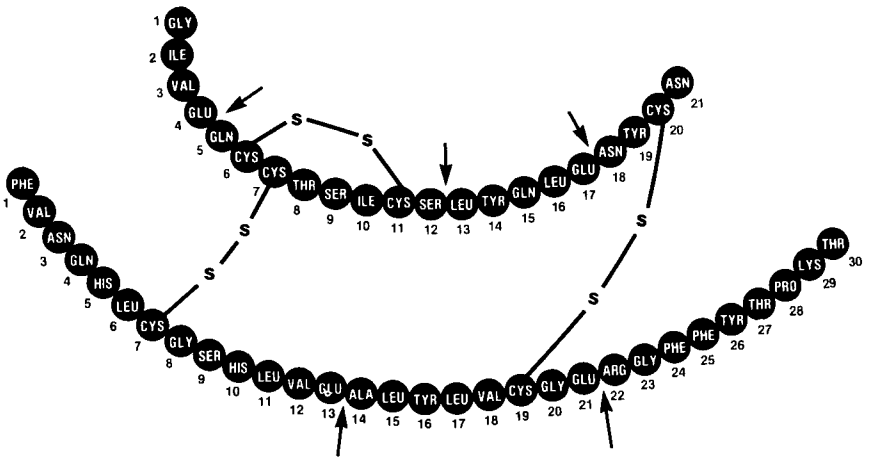


Fig. 6. The primary structure of human insulin. Arrows indicate peptide bonds hydrolyzed by a *S. aureus* V8 protease preparation. See text for experimental conditions.

stoichiometric amounts of thiol reducing agent (dithiothreitol) for the thiolysis part of the thiolysis - air oxidation reactions. The disulfide bonds in the purified human insulin prepared by this route are in the

correct configuration as determined by several criteria, including an HPLC "fingerprint" analysis using *S. aureus* V8 protease digests.

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THE PRODUCTION OF HUMAN PROINSULIN AND ITS TRANSFORMATION TO HUMAN INSULIN AND C-PEPTIDE

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Introduction

The synthesis of human insulin using recombinant DNA technology can be achieved by either of two general schemes. The first of these involves the separate preparation of the A and B chains of insulin followed by their combination to obtain insulin. The details of this scheme have been described in this symposium and elsewhere.[1,2] The second scheme for synthesis of human insulin, the proinsulin route, is analogous to the process by which insulin is produced in the pancreas. The biosynthesis of insulin in the pancreas occurs via the single chain precursor molecule proinsulin[3] (see Figure 1).

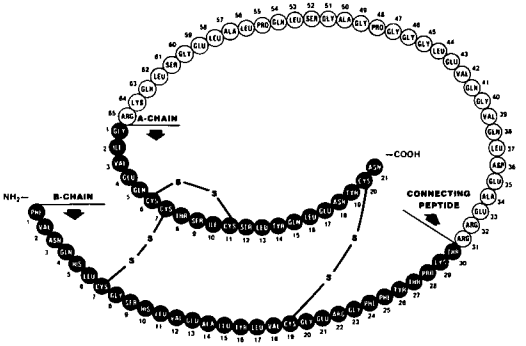

Fig. 1. The amino acid sequence of human proinsulin

The proinsulin molecule folds and forms the proper disulfide bonds and is then enzymatically converted to insulin. The obvious advantages of the proinsulin route to human insulin, using recombinant DNA technology, are that only a single fermentation and a single isolation scheme are needed in order to obtain the insulin. However, the proinsulin route requires that either the proinsulin folds and forms its proper disulfide bonds *in vivo* (within the *E. coli* cells) or that an efficient *in vitro* process be established for this step. In addition the enzyme or enzymes that are responsible for the *in vivo* conversion of human proinsulin to insulin

have not been isolated. The *in vitro* conversion of bovine proinsulin to insulin on a small scale has been reported by Kemmler *et al.*[4] using a combination of the enzymes trypsin and carboxypeptidase B. The dibasic amino acids that are the enzymatic cleavage sites in the connecting peptide of bovine proinsulin are the same in human proinsulin. However, human proinsulin does differ significantly in sequence as compared to bovine proinsulin, and the connecting peptides of the two species are particularly different [~40% of the amino acid residues are substituted or deleted in bovine versus human connecting peptide[5]]. The studies in our laboratories have been focused primarily on the proinsulin folding-disulfide bond formation and the enzymatic conversion reaction in order to establish the feasibility of the proinsulin route to human insulin. The results of these studies are described along with their application to preparing human insulin via human proinsulin.

Results

Proinsulin Folding and Disulfide Bond Formation — The earliest studies[6,7] on the refolding of proinsulin were directed towards providing experimental evidence that, under physiological conditions, reduced proinsulin was more efficient than a mixture of A and B chains in oxidizing to form the three disulfide bonds found in insulin. However, the optimal conditions found for reoxidation were not practical for large scale synthesis (see Table I). This was due primarily to the use of a very low protein concentration (20 μg/ml) in the oxidation mixture. Later investigations, using both proinsulin and insulins which had artificial crosslinks between the A and B chains, were directed towards finding more practical disulfide bond formation conditions.[8-11] Although higher protein concentrations were used in these investigations, the basic procedures were still unacceptable for large scale synthesis in two respects. First, the procedures required reducing proinsulin to its sulfhydryl form in the presence of a large excess of mercaptan. The excess mercaptan then had to be either physically removed (i.e., by gel filtration) or oxidized along with the protein. That is, the procedures required a reducing step followed by transfer to an oxidizing environment. Second, the oxidation process could lead to oxidation of the Cys-SH to forms other than the desired disulfide bond forms, for example, cysteic acid. As a result, the side-products formed in the oxidative refolding procedure, such as proinsulin dimers and higher polymers, could not be recycled to form good proinsulin because the cysteic acid could not be reduced to Cys-SH. Because these kinds of limitations existed with the oxidative types of refolding procedures, we decided to attempt to find a non-oxidative,

single step process that would efficiently transform proinsulin-S-sulfonate directly to proinsulin. The decision to use proinsulin-S-sulfonate as a starting material anticipated the type of isolation procedure that would be used for human proinsulin and that is described below.

A reverse-phase HPLC system (C_{18}) was established which provided a sensitive and rapid assay for properly refolded proinsulin. Using the HPLC assay to examine products from a variety of reaction conditions, we discovered an efficient disulfide interchange procedure for obtaining proper disulfide bond formation with proinsulin. The procedure is a single step, non-oxidative transformation of proinsulin-S-sulfonate at basic pH. The solution is allowed to stand for ~ 18 hours at 4° C and the resulting material collected. Under the conditions shown in Table II, approximately 50% of the proinsulin-S-sulfonate is isolated as properly refolded proinsulin. Because the reaction conditions are non-oxidative, the non-monomeric side-products can be recycled to the proinsulin-S-sulfonate form and subjected once more to the interchange reaction to obtain more of the desired product. Experiments using beef proinsulin-S-sulfonate gave overall yields of approximately 70% when the side-products of the first refolding were recycled in the manner outlined above. At lower concentrations (~ 100 μg/ml), single cycle yields as high as 90% have been observed. Although our initial detailed studies all were performed on beef proinsulin-S-sulfonate, the procedure was shown to be applicable to other proinsulin species.

Table I

Oxidative Refolding[a]
0.02 mg/ml Proinsulin
0.4mM Mercaptoethanol
0.05 M tris, pH 8.6
25°C, 18-24 hours, Oxygen
0.4mM Dehydroascorbic Acid

Table II

Disulfide Interchange Refolding
2 mg/ml Proinsulin-SSO_3
1.5 moles $\frac{\text{Mercaptoethanol}}{SSO_3}$
0.05 M Glycine, pH 10.5
4°C, 6-18 hours

[a] Conditions reported in reference 7

The basic reaction steps involved in this process would appear to be those shown in Figure 2.

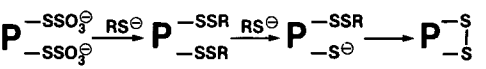

Fig. 2. Disulfide interchange reaction sequence

When $^{35}SO_3^=$ labelled proinsulin-S-sulfonate was subjected to the interchange reaction conditions, within 15 minutes less than 2% of the label could still be found on the proinsulin. At the same time, less than 3% of the cysteine residues in the proinsulin were measureable using Ellman's reagent.[12] A similar free SH value was obtained using iodoacetic acid and subsequent determination of S-carboxymethylcysteine by amino acid analysis.[13] At 30 minutes essentially no $^{35}SO_3^=$ remained on the proinsulin, and less than 1% of the potential SH groups in the protein could be found by either direct titration or by reaction with iodoacetic acid. These results are in strong contrast to the slower rate at which the native disulfide form of proinsulin appears in the reaction mixture (see Figure 3). All of these data are readily explained based on the reaction sequence outlined in Figure 2.

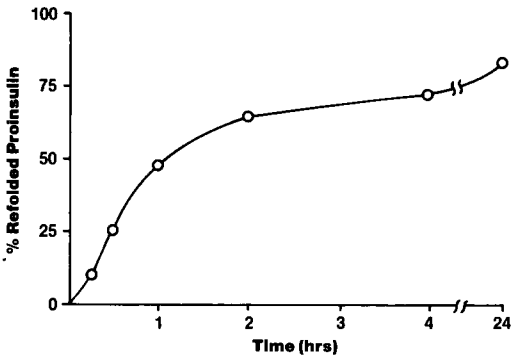

Fig. 3. Formation of proinsulin from proinsulin-S-SO_3(4°C).

Transformation of Proinsulin to Insulin — As indicated earlier, Kemmler and coworkers had transformed small amounts (< 1 mg) of bovine proinsulin to intact bovine insulin using a combination of trypsin and carboxypeptidase B.[4] In their studies, the insulin was isolated and subjected to amino acid analyses. The results of these analyses demonstrated that to within experimental error (~5%) only intact insulin was formed under their reaction conditions. Using the more sensitive analytical technique of reverse-phase HPLC, we were able to verify Kemmler's results with native bovine proinsulin. Further, the enzymatic treatment of refolded bovine proinsulin (isolated from the folding of bovine proinsulin-S-sulfonate as described above) yielded the same results as were found with native bovine proinsulin. For example, the reaction was complete within 30 minutes in both cases; the isolated bovine insulin had the same amino acid composition, CD spectrum, and biological potency in the insulin receptor assay[14] as pancreatic bovine insulin. These results

also indicated that the disulfide interchange reaction led to a proinsulin monomer which had folded properly and had formed the proper disulfide bonds. When porcine proinsulin was treated with the same enzyme combination, this enzymatic transformation procedure yielded porcine insulin of similar quality.

The initial reaction step involved in the proinsulin transformation is a trypsin cleavage at the dibasic amino acid residue sites yielding Insulin-Arg and Insulin-ArgArg. The carboxypeptidase B removes the arginine residues in the B31 and B32 positions to yield intact insulin. In the presence of carboxypeptidase B, trypsin does not appear to cleave the Lys(B29) Ala(B30) peptide bond at a significant rate. This is probably because of the rapid removal of the arginine residues by the carboxypeptidase B, since in the absence of this enzyme, trypsin will transform proinsulin to desAla(B30) insulin. With insulin alone, trypsin has a greater tendency to cleave at the B22 arginine site than at the B29 lysine site, particularly under neutral pH conditions.[15] Thus the rapid carboxypeptidase B catalysed removal of the extra basic residues on the end of the B-chain, causes trypsin to revert to its normal pattern of cleavage of insulin.

Preparation of Human Insulin from Human Proinsulin — Ross[16] has previously described the use of recombinant DNA technology to produce human proinsulin as a chimeric gene product (trp E) in *E. coli.* We isolated the chimeric protein, LE'-Met-Proinsulin, in crude form from fermented *E. coli* and then subjected it to cleavage with CNBr. The cleaved product was then converted to crude proinsulin-S-sulfonate using oxidative sulfitolysis.[17] The isolation of human proinsulin-S-sulfonate was accomplished by using ion exchange chromatography on DEAE-Sephadex, followed by gel filtration on G50 Sephadex. The isolation was monitored using reverse-phase HPLC (see Figure 4). The purified human proinsulin-S-sulfonate was folded using the disulfide interchange method described earlier. The reaction was again monitored using reverse-phase HPLC. The biosynthetic human proinsulin-S-sulfonate folded in the same manner and with the same yield as pancreatic bovine proinsulin-S-sulfonate. The folding mixture was then chromatographed on Sephadex G-50SF, and the resulting elution profile is shown in Figure 5A. The monomer region shown in Figure 5A was then subjected to final purification using preparative reverse-phase HPLC (see Figure 5B).

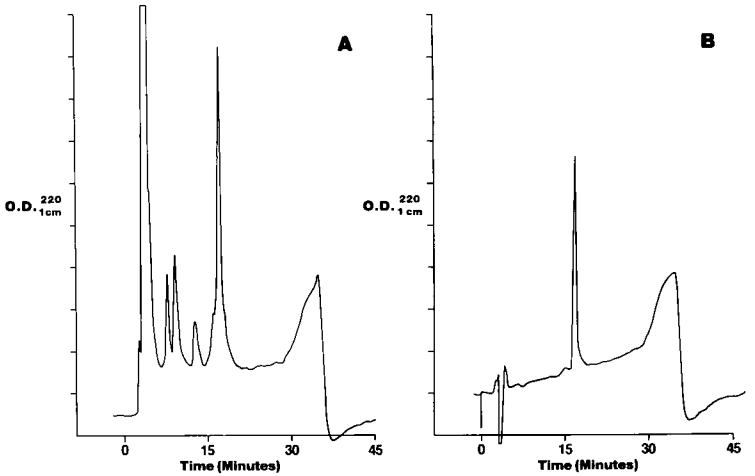

Fig. 4. HPLC (C_8) of human proinsulin-S-sulfonate. a) crude, sulfitolysis mixture; b) purified

The major peak was the biosynthetic human proinsulin. The amino acid analysis of the product is shown in Table III (control-bovine proinsulin) and is in excellent agreement with the known composition of human proinsulin.[5] End group analyses indicated the expected single phenylalanine residue at the B1 position. In order to verify that the correct disulfide bonds had formed in the folding procedure, the biosynthetic human proinsulin was subjected to cleavage with Staphlococcal Protease followed by fragment analyses on HPLC (see Chance *et al.* this volume).

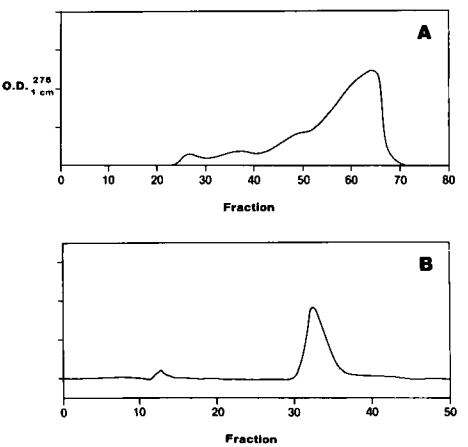

Fig. 5. Human proinsulin purification. Elution profiles obtained from chromatography.
a) Sephadex G-50SF and
b) HPLC (C_{18})

Figure 6 indicates the expected cleavage sites in proinsulin for this enzyme, and obviously the fragments containing the disulfide bonds are the same as those generated for human and pork insulin. Figure 7 contains the HPLC fingerprints of the Staphlococcal Protease cleavages of human proinsulin and human insulin. The expected disulfide fragments are clearly present in the "proinsulin" fingerprint.

Table III. Proinsulin Amino Acid Analyses.

	Bovine	Human
ASP	3.00 (3)	4.07 (4)
THR	0.94 (1)	2.82 (3)
SER	2.62 (3)	4.35 (5)
GLU	13.3 (13)	15.8 (15)
PRO	5.03 (5)	2.94 (3)
GLY	12.0 (12)	11.3 (11)
ALA	6.00 (6)	4.00 (4)
CYS	5.71 (6)	5.89 (6)
VAL	6.73 (7)	5.90 (6)
ILE	0.72 (1)	1.66 (2)
LEU	9.01 (9)	12.3 (12)
TYR	3.86 (4)	3.87 (4)
PHE	2.93 (3)	2.96 (3)
HIS	1.90 (2)	1.91 (2)
LYS	1.94 (2)	1.96 (2)
ARG	3.94 (4)	3.95 (4)

Residue values are an average of 3 determinations. 30 hr hydrolyses. Molar unity is based on alanine. Figures in brackets are known values.

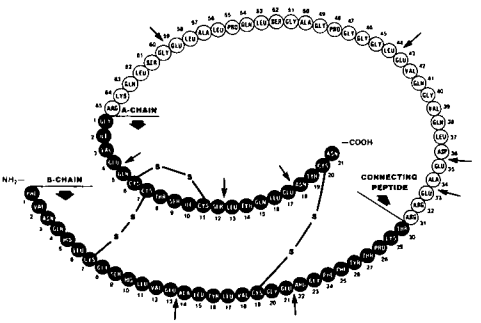

Fig. 6. Staphlococcal protease cleavage sites

In addition, fragments such as the B22-B30 nonapeptide which is present in the "insulin" fingerprint, is, as expected, absent in the "proinsulin" fingerprint. The biosynthetic human proinsulin cross-reacted to the expected degree with antibodies directed to either insulin or to human C-peptide, had the expected electrophoretic mobility on polyacrylamide gel electrophoresis, and the proper isoelectric point on electrofocusing gels.

The enzymatic conversion of biosynthetic human proinsulin was carried out using the trypsin-carboxypeptidase B procedure described earlier. At the end of the reaction, the insulin fraction was precipitated and the C-peptide isolated according to the method of Oyer *et al.*[3] The amino acid sequence of the isolated C-peptide was determined using the Beckman Sequenator and was in exact agreement with the known

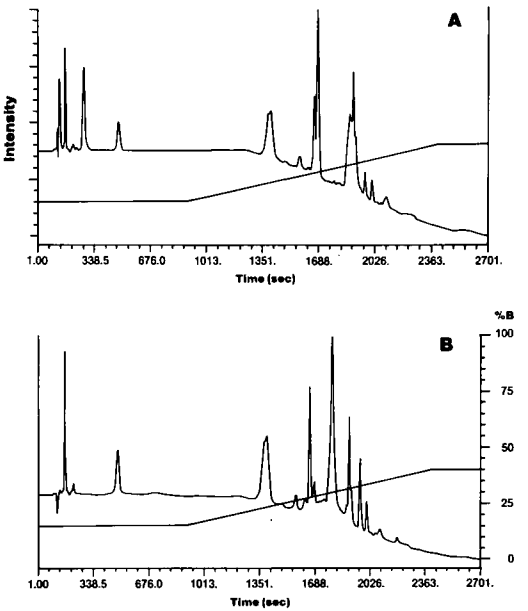

Fig. 7. HPLC "fingerprint" of proinsulin (A) and insulin (B).

sequence for this peptide. The biosynthetic human insulin was isolated using cation and anion exchange chromatography, gel filtration on Sephadex G50F and finally subjected to zinc crystallization. The human insulin isolated in this manner exhibited an amino acid composition (see Table IV), in excellent agreement with expected values. On reverse-phase HPLC, the human insulin derived from biosynthetic human proinsulin coeluted with pancreatic human insulin. The biological potency of this biosynthetic human insulin was compared to that of pancreatic human insulin in the insulin receptor assay and was 106 ± 4% that of the standard. The correct disulfide bond linkage was again verified using the HPLC "fingerprint" method discussed above.

Conclusion

We have discovered a disulfide interchange method that will efficiently yield proinsulin directly from proinsulin-S-sulfonate. The proinsulin produced by this method exhibits the same physiochemical and biological properties as pancreatic proinsulin. Further we have demonstrated that intact insulin can be generated by enzymatic treatment from either native or refolded proinsulin. This insulin is indistinguishable from insulin isolated from the pancreas. Finally, the application of these

Table IV. Human Insulin Amino Acid Analyses

	Biosynthetic	Pancreatic
ASP (3)	3.00	3.00
THR (3)	2.76	2.78
SER (3)	2.68	2.61
GLU (7)	7.17	7.21
PRO (1)	1.06	0.96
GLY (4)	4.02	4.02
ALA (1)	0.97	0.95
CYS (6)	5.47	5.41
VAL (4)	3.73	3.78
ILE (2)	1.62	1.65
LEU (6)	6.03	6.09
TYR (4)	3.85	3.95
PHE (3)	2.96	2.96
HIS (2)	2.12	2.03
LYS (1)	0.94	0.95
ARG (1)	1.01	0.96

Residue values are an average of 3 determinations. 30 hour hydrolysis. Molar unity is based on aspartic acid. Figures in brackets are known values.

procedures to human proinsulin has clearly demonstrated the feasibility of producing human insulin via human proinsulin made using recombinant DNA technology.

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PRIMARY STRUCTURE AND SYNTHESIS OF AN EGG-RELEASING PEPTIDE (ERH) FROM THE ATRIAL GLAND OF *APLYSIA CALIFORNICA*

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The atrial gland[1] and the bag cell of the parietovisceral ganglion[2] both play an important role in the neuroendocrine control of egg-laying behavior in the marine mollusc, *Aplysia*. Chiu, *et al.* have determined the structure of a bag cell peptide (ELH)[3] that induces egg-laying while Heller *et al.* established the structure of an atrial gland peptide[4] which induces both bag cell afterdischarge and egg-laying. Recently the structure of another atrial gland peptide (ERH) capable of inducing egg-laying independently and in the absence of bag cells has been determined.[5] In this communication we describe the sequence determination and chemical synthesis of ERH and peptide segments of ERH which show sequence identity to the structures, determined by Chiu[3] and by Heller.[4]

Materials and Methods

Isolation, Purification and Structure Determination of ERH — Atrial glands from 200 *Aplysia* were dissected away from the large hermaphroditic duct, homogenized and the peptide (ERH) purified by ion exchange and gel permeation chromatography as previously described.[5] Purification of ERH was monitored by UV absorbance and egg-releasing activity. Homogeneity of ERH was assessed by PAGE in the presence and absence of SDS in 8M urea, isoelectric focusing, and sequence analysis. The sequence of ERH was determined by manual analysis[6] on intact ERH and peptide fragments of ERH obtained following cleavage separately with chymotrypsin and cyanogen bromide. Identification of the PTH amino acids resulting from sequence analyses were made by thin layer[7] and high pressure liquid chromatography.[8,9]

Synthetic Methods — a. Solid Phase Peptide Synthesis. Boc-isoleucine was attached to the chloromethylated polystyrene resin via $CsHCO_3$-mediated esterification.[10] Peptides were synthesized[11] using a Vega (Tucson, AZ) automated synthesizer. The protected peptide resin was subjected to hydrogen fluoride treatment (0°C, 60 min in the pres-

ence of 1.2 ml anisole/g resin). The crude products were purified by either partition chromatography (n-butanol: toluene: HOAC: 1.5% aqueous pyridine (10:15:12:18, v/v) or gel filtration in 2N HOAC.

b. Characterization and purity criteria for ERH peptides. Purity of synthetic peptides was assessed by thin layer chromatography (system A: n-butanol: HOAC: pyridine: H_2O (15:3:10:12), system B: n-propanol: conc. NH_4OH (140:60), amino acid analysis[12] after acid hydrolysis and sequence analysis on a Beckman model 890 sequencer using a DMAA peptide program. Failure sequences and the absence of side chain protecting groups were determined on a Hewlett Packard HPLC instrument.[9]

Results

Primary Structure Determination — Following isolectric focusing, PAGE and sequence analysis, only one band appeared on the gels with a pKI of about 8.0 and only one N-terminal residue was obtained following one cycle of the Edman degradation.

The sequence of ERH was established by manual analysis on intact peptide (through 24 residues), two cyanogen bromide, and three chymotryptic fragments, and by the time-dependent release of amino acids from the C-terminus of the parent molecule (Figure 1) as previously reported.[5]

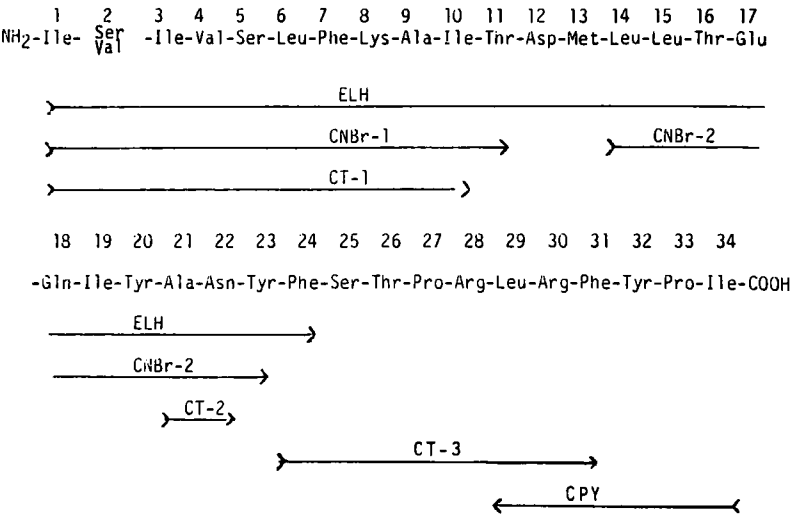

Fig. 1. The complete sequence of the atrial gland egg-releasing peptide (ERH). Arrows to the right indicate residues identified by manual Edman degradations. Arrow to the left indicates residues determined by the time dependent release of amino acids during carboxypeptidase Y digestion. CNBr indicates peptides obtained by cleavage with cyanogen bromide and CT indicates peptides produced by digestion with chymotrypsin.

Synthesis — Both N- and C- segments of the atrial gland ERH structure show sequence identity with those of the bag cell ELH[3] and the atrial gland peptides[4] (Figure 2). The following segments in the ERH structure were synthesized: (1-19, Val_2), (1-19, Ser_2), (1-34, Val_2), (1-34, Ser_2), (8-34) and (23-34).

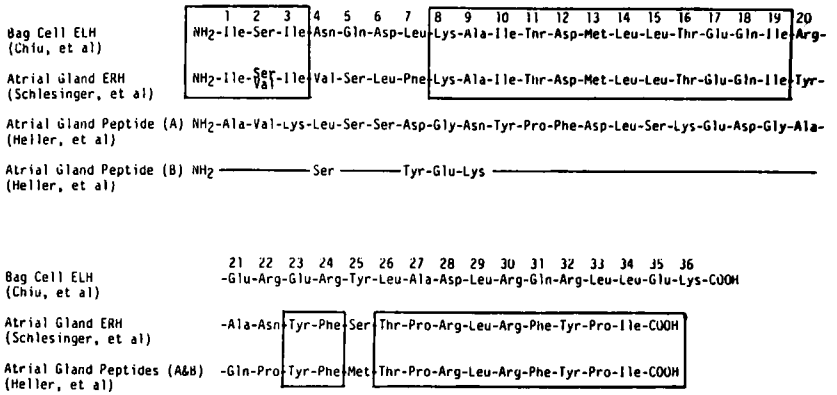

Fig. 2. Sequence homology between the bag cell egg-laying hormone (ELH)[3] and the atrial gland peptides A and B.[4] Residues within the box represent residues of amino acid identity.

The purified peptides were tested for homogeneity in two solvent systems (Table I), characterized by amino acid analysis (Table II) and N-terminal sequence analysis, the latter being a more stringent test of homogeneity.[13] The maximal amount of error peptide detected in the 1-19 and 1-34 synthesis was 2.8 and 4.4%, respectively. In addition there was no evidence of any Edman-stable side chain protecting groups remaining on the purified peptides.

Discussion

The structure of this atrial gland ERH molecule represents a genetic hybrid between the bag cell ELH[3] and the atrial gland peptide.[4] All three molecules induce egg-laying. Purified ERH induced egg-laying even in the absence of bag cells.[5] Therefore ERH acts on the ovotestis directly and independently of bag cell hormone. ERH and bag cell ELH may act similarly on the muscle fibers surrounding the follicles.[14,15] Thus, *Aplysia* may have multiple mechanisms to induce egg laying, a response essential to their survival.

Synthetic ERH and the synthetic segments of ERH provide valuable material to study the mechanism of egg release from the ovotestis and bag cell afterdischarge and will help define the segments in ERH responsible for these fundamental and essential biological activities.

Table I. Thin Layer Chromatography of the Atrial Gland ERH Synthetic Peptide Fragment.

Peptide Segment	Solvent System A		Solvent System B	
	Major Component	Minor Component(s)	Major Component	Minor Component
(23-34)	0.78		0.68	
(8-34)	0.25		0.23	
(1-34,Ser_2)	0.86	0.77	0.62	
(1-34,Val_2)	0.86	0.77	0.62	0.68
(8-19)	0.68	0.47,0.58	0.54	0.19
(1-19,Ser_2)	0.75	0.81,0.68	0.70	
(1-19,Val_2)	0.75	0.81,0.68	0.70	

Table II. Amino Acid Composition of Atrial Gland ERH Synthetic Peptide Fragments.

	23-34	8-34	1-34,Ser_2	1-34,Val_2	8-19	1-19,Ser_2	1-19,Val_2
Asp	---	2.15(2)	2.11(2)	1.99(2)	1.00(1)	1.00(1)	1.00(1)
Thr	0.97(1)	2.92(3)	2.90(3)	2.72(2)	1.96(2)	1.93(2)	1.73(2)
Ser	0.88(1)	1.34(1)	2.48(3)	1.70(2)	---	1.63(2)	0.65(1)
Glu	---	2.21(2)	2.16(2)	2.00	1.85(2)	2.30(2)	2.23(2)
Pro	1.98(2)	2.11(2)	2.21(2)	2.14(2)	---		---
Ala		1.86(2)	1.88(2)	1.85(2)	0.99(1)	0.97(1)	0.86(1)
Val			0.78(1)	1.61(2)	---	0.79(1)	1.75(2)
Met		0.81(1)	0.87(1)	0.81(1)	1.02(1)	0.99(1)	0.85(1)
Ile	1.00(1)	3.00(3)	4.78(5)	4.53(5)	2.20(2)	3.71(4)	4.20(4)
Leu	1.09(1)	3.21(3)	4.00(4)	3.67(4)	2.23(2)	3.15(3)	3.05(3)
Tyr	1.82(2)	2.95(3)	3.29(3)	3.25(3)	---		---
Phe	1.92(2)	2.15(2)	3.21(3)	3.22(3)	---	0.95(1)	0.79(1)
Lys	---	0.86(1)	0.80(1)	0.78(1)	0.97(1)	0.96(1)	0.92(1)
NH_2			2.00(2)	2.22(2)	1.90(1)	1.35(1)	1.80(1)
Arg	1.98(2)	2.21(2)	2.29(2)	2.23(2)	---		---

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FAST ATOM BOMBARDMENT AND OTHER NEW DEVELOPMENTS IN MASS SPECTROMETRIC PEPTIDE SEQUENCING

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Introduction

Protein sequence analysis remains an important but arduous task in many branches of biochemical research including enzymology and neurochemistry. The structure elucidation of biologically active peptides, the identification of post-synthetic modifications in proteins and structure/function correlations are but a few examples demanding amino acid sequence analysis. Even where DNA sequencing is applicable, a rapid definition of partial protein sequence is invaluable in locating the reading frame or defining 'introns'.

The most widely used classical approaches to protein and peptide sequencing have of course been based upon Edman degradation. During the past decade however, a radically different approach - Mass Spectrometry - has come of age and is rapidly making an impact in the field of biological structure determination.[1,2] Variants of this technology have included GC-MS studies on small peptides and, more commonly, analysis of peptides and peptide mixtures as the N-acetyl-N,O-permethyl derivatives.[3] It is the latter approach which, in a complementary role to classical studies, has proved most successful for sequencing protein-derived peptides. Strategies have been developed and demonstrated for the complete sequence analysis of proteins. In this laboratory conjoint mass-spectrometric/classical structure elucidation studies have included the proteins Ribitol Dehydrogenase (RDH),[1] Chloramphenicol Acetyltransferase (CAT)[4] and methotrexate resistant Dihydrofolate Reductase MTX-R DHFR).[1,2] The sequence analylsis of DHFR was achieved solely by mass spectrometric sequencing of peptide mixtures derived by digestion with non-specific proteases.

Mass spectrometry is probably best known for its contribution to the structure elucidation of biologically active substances where some unusual feature is either known or suspected to be present. Some examples of this type where MS has played a key role, are shown in Figure 1, which shows results, achieved in this laboratory, from the structure

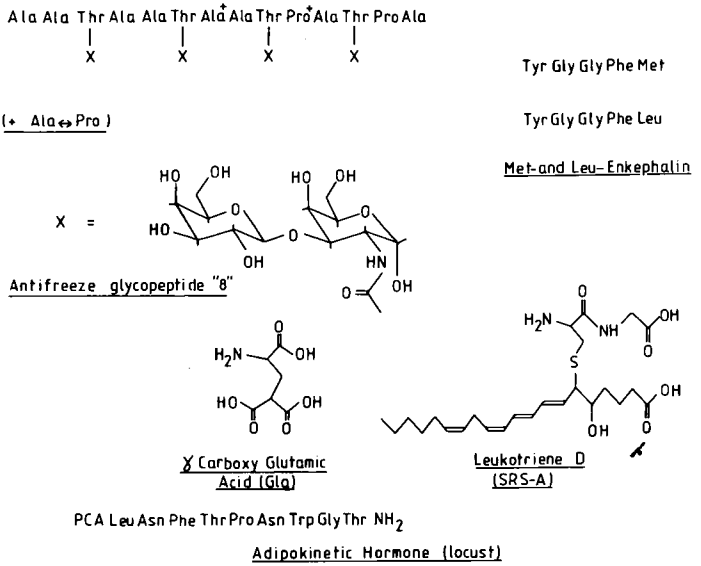


Fig. 1. Important biological substances whose structures were determined by MS.

elucidation of the Enkephalins, Leukotriene D (Slow-Reacting Substance of Anaphylaxis), γ-Carboxy Glutamic Acid (Gla) in blood coagulation zymogens, 'Antifreeze Glycopeptide 8' and Adipokinetic Hormone from locust corpora cardiaca.[1,2] All of this work has been carried out on materials of unknown structure, usually at the low nanomole level of sensitivity. In this sense the MS methods developed have proven to be equal to classical methodologies and have demonstrated the further advantage of being able to solve structures not amenable to classical techniques.

In this paper we illustrate and describe the power of modern MS methods in the solution of difficult problems in the area of biological chemistry; we do so by reference to examples from a number of fields of study both currently and formerly undertaken in this laboratory, including new data in the neuropeptide, glycopeptide and peptidolipid areas. The major features of the basic MS method are (1) structure elucidation capability and (2) quantification capability. These two features are illustrated by describing two powerful new developments originating from research on the basic instrumentation - these are High-Field Magnet-Fast Atom Bombardment (FAB) -MS and Negative Chemical Ionization (NCI)-MS.

Protein/Peptide Sequencing

The mass spectrometer is not an obvious choice for sequencing polar, high mass species as, in general, samples must be volatile and of relatively low molecular weight. The success of MS in this field has arisen from developments in methodology in the generation of suitably sized, volatile protein fragments. The mass spectrometer is uniquely endowed with an ability to analyze mixtures. The sample is fractionally volatilized into the ion source at discrete temperatures over a range of 100-350° C; components in the mixture vaporize (and are thus ionized) at different source temperatures giving rise to distinct ion abundance ratios. By comparing the spectra produced at each temperature - observing the concomitant increase and decrease in ion intensity as each compound volatilizes - the 'pure' spectrum (and thus the structure) of each component of a mixture of up to five peptides can be determined.

To make use of this "mixture analysis" capability a method is required to produce peptides of 2-15 residues suitable for MS analysis. Digestion of proteins by non-specific proteases (elastase, subtilisin etc.) has proved ideal. For MS analysis the peptides must be converted to volatile derivatives. The N-acetyl-N,O-permethyl derivative is the one of choice because under electron impact conditions this derivative fragments to give "sequence ions" from which the sequence can be deduced (see Reference 1 for a more detailed discussion). Using such procedures both total protein sequences and innumerable peptide sequences have been determined in this laboratory.

Glycopeptide Analysis

Glycoproteins play a major role in living systems with such diverse functions as disease control, cell surface recognition, blood clotting etc.. Despite many years of research the determination of their structure has proven an especially difficult problem requiring the solution of (1) protein sequence (2) carbohydrate sequence (3) nature of the protein-carbohydrate linkage and (4) nature and configuration of the carbohydrate linkages. Mass spectrometry offers great advantages in glycopeptide sequencing because it is not restricted to a particular compound class. This was clearly demonstrated in the structure determination of an antifreeze glycopeptide "AF8" from antarctic fish blood.[1] Analysis of the N-acetyl-N,O-permethyl derivative in both electron impact (N-terminal information) and chemical ionization (C-terminal information) modes gave the (heterogeneous) peptide sequence, defined the sugar as a disac-

charide and specified linkage positions. Analysis of the sugar as a trimethylsilyl derivative showed it to be β-D-galactosyl (1-3) α-N-acetylgalactosamine (Figure 1). Extension of these studies has resulted in structural data on the carbohydrate portion of prothrombin;[5] new data on the glycopeptide antibiotics, the bleomycins, will be described later (FAB/FD).

Novel Structures/Blocked Proteins

Perhaps the most obvious strength of MS is in the area of new structures where no reference compounds exist. Such a situation arose in sequencing studies on the blood coagulation zymogen Prothrombin. Peptides isolated from the N-terminal region migrated on HVPE with a mobility inconsistent with the classically determined structure. Mass spectrometric analysis confirmed the presence of an anomalous structure in these peptides; full interpretation of the spectra resulted in the structure determination and location in prothrombin of the novel residue γ-carboxyglutamic acid (Gla) (Figure 1). Gla has also been detected and located by this MS method in the N-terminal portions of Factors X_1 and X_2.[2]

In a similar manner, the identification and location of other modified amino acids (such as N-methyl lysine) reduces to an almost trivial problem. Blocked N-terminal peptides can cause major problems in classical sequencing studies. In contrast, MS is fully capable of handling such problems, since the peptide is deliberately 'blocked' during derivatization prior to analysis. By an extension of the strategies outlined earlier, including isotope labelling studies and enzyme digestion, the N-terminal regions of many blocked peptides and proteins have been sequenced by MS using only 10-50 nmol of material.[1,2,6]

Because MS is independent of sample class it is particularly well suited to the investigation of compounds of unknown structure. A recent example of this was the structure elucidation of slow-reacting substance of anaphylaxis (SRS-A) from lung (SRS-A is a bronchoconstrictor, released in picomole quantities, which is associated with the asthma crisis).[1] Following protein chemical studies on the purified material, a mass spectrum was obtained on the trimethylsilyl acetyl methyl ester derivative of 5 μg of material. To enable interpretation of the SRS-A spectrum (by differentiating significant ions from the background impurities) mixture analysis was used on the 1:1 CH_3CO:C^2H_3CO derivative; ions containing this isotopic label appeared as 1:1 doublets 3 m.u. apart (demonstrating the presence of *one* free amino group in the natural

product) and could be readily located in the spectrum. A detailed analysis of the spectra of labelled SRS-A allowed the structure assignment of the novel peptidolipid 5-hydroxy-6-cysteinylglycinyl-7,9,11,14-eicosatetraenoic acid (Figure 1). This was later termed Leukotriene D. Recent studies on this and related substances will be discussed later (FAB/FD).

Neurochemistry

MS has already played a major role in structural analysis in the neuropeptide field. Perhaps the most striking example was the structure elucidation of the endogenous opioid peptides Met- and Leu- enkephalin.[1] At around the same time (1976), in the first characterization of a hormone of insect neuroendocrine origin, the structure of locust adipokinetic hormone was determined.[1] This blocked decapeptide, PCA-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH_2, could not be sequenced classically but presented few difficulties for MS.

Problems of quantity and purity of neuropeptides in biological fluids generally preclude MS from playing a wider role in neurochemical analysis. To ameliorate these problems, and to aid characterization of these substances, a high resolution reverse phase HPLC system was developed in this laboratory.[1] Using this system 18 neuropeptides of general interest can be separated in one forty minute HPLC run, with invariant retention times and in high yields. This system has found wide use in our studies on opioid peptides and precursors and our investigations into the role of neuropeptides in disease states. In addition it was of great value in a recent study on pituitary derived biologically active substances which cause the release of aldosterone from the adrenal zona glomerulosa.[7] HPLC purification split the biological activity into 3 peaks (A,B,C). Peak A was analyzed using protein chemistry techniques (e.g. enzyme digestion, amino acid analysis) and by EI MS of the N-acetyl-N,O-permethyl derivative of the product of tryptic digestion, and was shown to have the structure Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2 which is identical to α-melanocyte stimulating hormone (α-MSH). This was the first evidence for α-MSH having a role in pituitary regulation of the adrenals. Data on peaks B and C will be discussed later (FAB/FD).

Our studies in the brain area on chemical imbalances associated with disease states (in which HPLC plays an important part) and our searches for correlations between opioid peptide levels and pain relief, demand the specific measurement of neuropeptides in the picomole range. The sensitivity obtainable using the technique of Negative Chemical Ionization

(NCI) MS[8] makes this method particularly promising for neurochemical studies. We have achieved nanomole sensitivity (with full structural information) for perfluoroacyl derivatives of the enkephalins, and provided a careful choice of derivative and running procedures is made, the sensitivity can be improved further into the picomole range. This is exemplified in Figure 2 which shows the NCI spectrum of the N-acetyl (1:1 CH_3CO:C^2H_3CO) perfluorobenzyl ester derivative of Met-enkephalin. Confidence in assignments is aided by the isotope label which results in all ions containing the N-terminus exhibiting a 1:1 doublet separated by 3 m.u.. The major high mass signal is the intense doublet at m/z 614/617 corresponding to loss of the perfluorobenzyl group from the molecular ion (resulting in a stabilized carboxyl anion). The intensity of these signals indicated that it should be possible to monitor them for quantitative purposes. In preliminary experiments involving narrow scanning across the mass range spanning this doublet of peaks we have shown that the derivative can be readily detected at the 50 picomole level. We are now extending these studies by making use of the increased sensitivity resulting from specifically monitoring the ion current at the appropriate mass (selected ion monitoring). In earlier EI-MS studies of a Leu-enkephalin derivative we were able to successfully detect and specifically quantitate, in the presence of other peptides, 25 picomoles of the neuropeptide using selected ion monitorng procedures. Hence we are confident that when this procedure is fully exploited in the NCI mode on derivatives such as that shown in Figure 2 picomole sensitivity will be realizable for many neuropeptides.

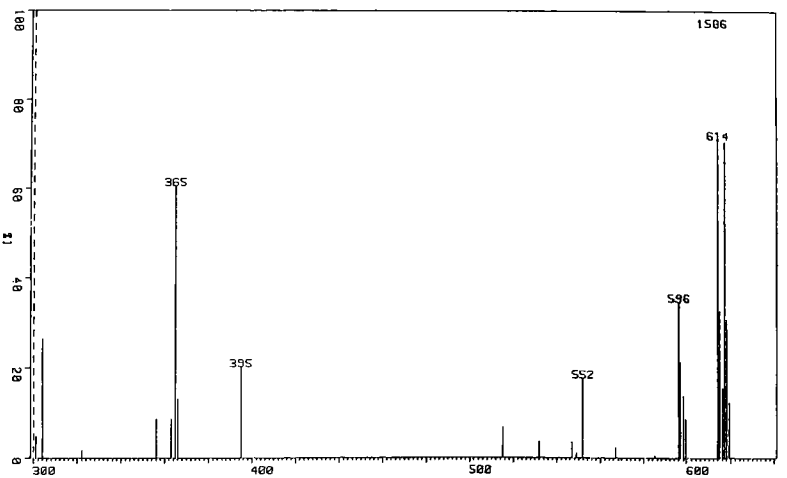


Fig. 2. NCI spectrum of the N-acetyl (CH_3CO:C^2H_3CO 1:1) perfluorobenzyl ester derivative of Met enkephalin.

High-Field Magnet/Fast Atom Bombardment/FD MS

There is a continuing need in many areas of biochemical research for reliable data on substances in the mass range 2000-4000 m.u.. With this in mind a High-Field Magnet (HFM) was commissioned for this laboratory enabling an extension of the mass range of conventional mass spectrometers from 1000 to 3000 m.u. at full accelerating voltage and thus full sensitivity. Apart from the obvious advantages obtained from the HFM (notably increased sensitivity at high mass), the development of the HFM initiated the production of extended range instruments (up to 1800 m.u.) now available from several manufacturers.

The HFM has proved invaluable in our EI and CI studies on peptides and carbohydrates, but perhaps the most successful application of the technology has been in the analysis of high mass, polar and thermally labile substances by Field Desorption (FD) MS. Under FD conditions the sample is ionized and desorbed from an activated tungsten wire by the action of an electric field gradient. This method imparts little internal energy to the sample and results in abundant quasimolecular ions and very little fragmentation.

FD MS has been used in this laboratory to obtain molecular weight data on compounds previously thought to be not amenable to MS analysis. These include the antibiotics Vancomycin and Echinomycin, vitamin B_{12} and its biosynthetic precursors and, more recently, the bleomycin family of glycopeptide antibiotics. The bleomycins are particularly intractable molecules for MS due to their polarity and thermal instability. In addition their complexity precludes the use of simple chemical procedures for the preparation of volatile derivatives suitable for EI or CI analysis. Recently we have reported the first successful characterization by FD MS of bleomycin B_2 and related phleomycins.[10] We have now extended this work to other bleomycins and have obtained excellent FD spectra on bleomycin A_2, demethylbleomycin A_2, tallysomycin A, and Blenoxane (both free and Cu complexed) both as the native materials and as acetylated derivatives. The success of these studies is partly due to the HFM facility - FD data being obtained at full sensitivity - and partly due to the application of sample handling procedures suitable for the FD analysis of thermally fragile and polar molecules. In our hands FD spectra can be obtained routinely; nevertheless considerable skill and experience is required.

In contrast the new ionization technique of Fast Atom Bombardment (FAB) MS[11] yields data on the bleomycins with relative ease. For FAB MS the sample is loaded in a glycerol matrix on a metallic target

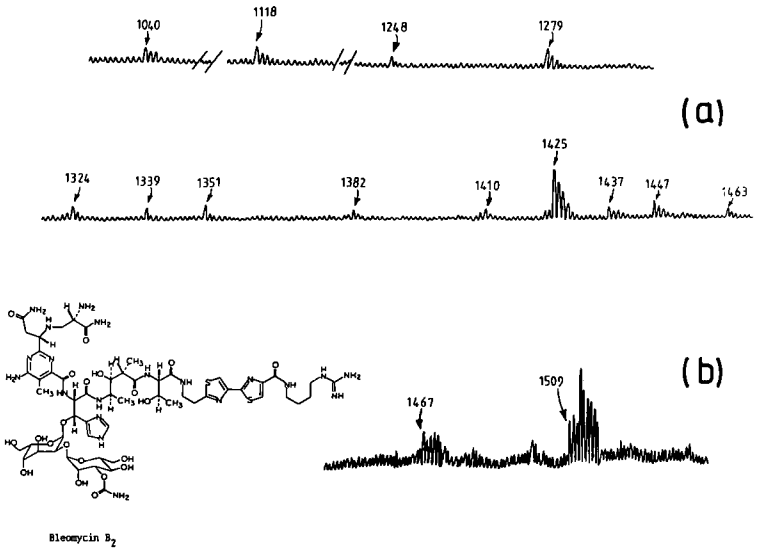


Fig. 3. FAB spectra of bleomycin B_2 (a) and its acetyl derivative (b).

and bombarded with a beam of accelerated argon atoms. The sample is ionized by the impinging atoms and long lived ion beams are generated which are then mass analyzed. Both molecular weight and fragment information can be obtained by FAB MS. We have examined a variety of bleomycins by this procedure and all have yielded excellent spectra with abundant quasimolecular ions. A typical example is given in Figure 3a. This shows the FAB spectrum of bleomycin B_2; note the $M+H^+$ species at m/z 1425. Simple chemical modifications can be peformed in the glycerol matrix and monitored by FAB MS. Figure 3b shows the spectrum obtained from bleomycin B_2 after the addition of $(CH_3CO)_2O/(C^2H_3CO)_2O/CH_3OH$ (1:1:4) to the matrix. The sample is converted mostly to the diacetyl derivative within one minute as shown by the 1:2:1 pattern at m/z 1509, 1512, 1515.

We are currently applying this powerful new technique in our continuing studies in the peptide area. Peptides have been analyzed by FAB MS in both positive and negative modes at the low μg level and have included the following (in some cases as unknown samples): γGlu-Cys-Gly, AcAla-Ser-Phe, Phe-Gly-Gly-Phe, Pro-Phe-Gly-Lys, Ala-Asn-Asn-Lys, Trp-Met-Asp-PheNH_2, Tyr-Leu-Pro-Glu-Phe, Tyr-Leu-Gly-Glu-Phe, Lys-Phe-Ile-Gly-Leu-MetNH_2, Val-Gly-Leu-Ala-Pro-Val-Ala, Gln-Tyr-Tyr-Thr-Val-Phe-Asp-Arg, Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg, Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe, α-MSH, and α-

endorphin. All peptides studied give intense quasimolecular ions, together with, in many cases, fragmentation from the N- and C-terminus. If fragmentation takes place it occurs via chemical ionization pathways; no N-C cleavage ions are observed. To facilitate interpretation of the fragmentation pattern the N- (acetyl:^{2}H-acetyl 1:1) derivative is prepared. N-terminal ions appear as 1:1 doublets 3 m.u. apart whereas ions arising from the C-terminus are singlets unless they contain Lys or Arg which may also acetylate. We have applied this method to assist colleagues at Cambridge (Dr. J. Walker, MRC Molecular Biology Lab.) to complete the sequence of a bovine ATPase derived peptide. Consideration of the FAB spectra of both the free material and its 1:1 acetyl: ^{2}H-acetyl derivative allowed the assignment of the partial structure H...Asp-Ala-Thr-Thr-Val...OH (Figure 4). In contrast to the classically derived data the FAB sequence is homologous with an ATPase peptide from E. coli which strongly suggests the FAB-derived sequence is correct.

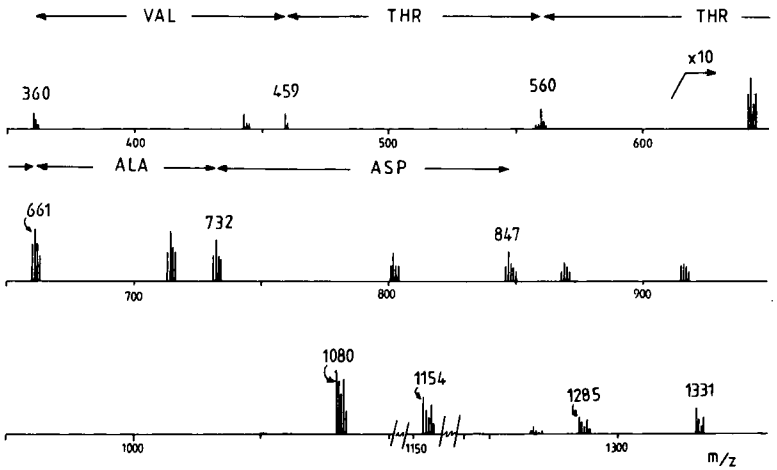
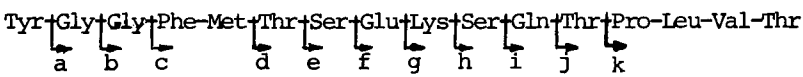


Fig. 4. FAB spectrum of the N-(1:1 acetyl:^{2}H-acetyl) derivative of bovine ATPase peptide. Structural information from C-terminal fragmentation (singlets).

FAB MS also shows great promise in the neruopeptide field. We have demonstrated the sensitivity of the technique by the production of intense quasimolecular ions from Lys-vasopressin (M_+H^+, 1056; M_+Na^+, 1078; M_+K^+, 1094; 2 nmol), α-endorphin (M_+H^+, 1745; 300 pmol), and α-MSH (M_+H^+, 1664; 300 pmol). Somewhat higher loadings of α-endorphin resulted in the production of C-terminal ions at m/z 1582, 1525, 1468, 1190, 1089, 1002, 873, 745, 658, 530 and 429 (a-k):



FAB MS has also proven invaluable in our pituitary/adrenal work described earlier. Peak B gives an intense quasimolecular ion at m/z 1706, 42 m.u. higher than that obtained for peak A (α-MSH). Further, peak B is readily converted into peak A on mild base treatment and is indistinguishable from A in its amino acid content and EI spectrum. From these data we conclude that B is an O-acetyl derivative of α-MSH. The location of the additional acetyl group is being established by FAB MS of enzyme digests. Peak C gave the quasimolecular ions m/z 1193 ($M-H^-$) and 1195 ($M+H^+$) but no fragment information. The partial sequence Val-Val-Tyr-Pro(Trp,Glx present) was obtained from the EI spectrum of the acetyl permethyl derivative of peak C. From earlier work we recognized this sequence as being part of the β-chain of hemoglobin. From these data it was deduced that peak C contained the peptide Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg. Investigations into the biological role of this peptide, if any, are in progress.

We have applied FAB MS, in an extension of our studies in the asthma field, to Leukotrienes C and D. Quasimolecular ions were observed on 1 μg in both positive and negative modes ($M+H^+$, 626, 497; $M-H^-$, 624, 495 respectively). No fragmentation was obtained, with the ion current being carried by the quasimolecular species; this is particularly promising for future low level quantitative studies. Interestingly FAB showed clearly that N-acetylation of Leukotrienes C and D results in the formation of a lactone; only the M minus H_2O species being observed. The lactone is, however, readily opened.

The mixture analysis capability of MS has already been extensively exploited in EI studies of peptides. Our FAB studies of peptide mixtures have demonstrated that selective ionization of components can occur. For example FAB of a four component mixture yielded molecular weight and sequence information from Val-Gly-Leu-Ala-Pro-Val-Ala, molecular weight data only from PCA-Tyr-Tyr-Thr-Val-Phe-Asp-Arg and the corresponding Gln peptide, while no data was obtained on the fourth component Ala-Asn-Asn-Lys except under extreme conditions (a weak quasimolecular ion was obtained from the acetyl peptide after total glycerol evaporation). This illustrates the dangers of placing full reliance on any one technique in structural studies.

In the very brief time since the introduction of FAB MS the technique has already solved a number of important problems. We believe it will become increasingly important in biological structure determination as a complementary technique to EI/CI/FD.

Conclusion

Mass spectrometry has already played a major role in the structure elucidation of substances of biological interest such as neuropeptides, peptidolipids, glycopeptides, antibiotics and proteins. As the technique develops both in terms of mass range and sensitivity MS promises to allow the solution of many important problems only just beyond the scope of present technology. For biological mass spectrometry the next decade should be as exciting and fruitful as the last.

Acknowledgements

We thank the MRC and SRC for financial support and gratefully acknowledge the loan of a FAB source from M-Scan Ltd, 5 The Ridgeway, Iver, Bucks, U.K.. The pituitary/adrenal work was carried out in collaboration with Dr. G. Vinson, St. Bartholomew's Hospital Medical College, London and Dr. B. Whitehouse, Queen Elizabeth College, London.

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FAST ATOM BOMBARDMENT MASS SPECTROMETRY APPLIED TO PEPTIDES

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Two years ago we outlined the General Procedure below for assigning structures to peptide antibiotics and other peptides which relies heavily on high resolution mass spectrometry (HRMS), including field desorption (HRFDMS), gas chromatography/electron ionization and chemical ionization (GC/HREIMS and GC/CIMS).[1]

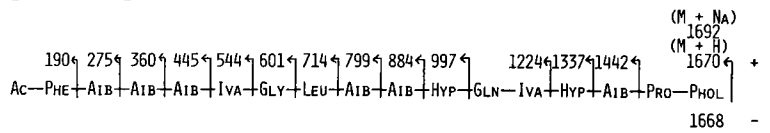
General Procedure

1. Total hydrolysis of the antibiotic to amino acids, identified by: a) HRFDMS; b) derivatization and GC/HREIMS; and c) derivatization and GC retention times on a chiral column.
2. Quantitation of the amino acids by GC and amino acid analyzer.
3. Assignment of a molecular formula to the antibiotic by molar ratios of amino acids and by HRFDMS on the molecular ion.
4. Assignment of a partial sequence to the antibiotic from fragment peaks in its HREI mass spectrum.
5. Partial hydrolysis of the antibiotic to oligopeptides, identified by: a) derivatization and GC/HREIMS and GC/FIMS; and b) HRFDMS.
6. Overlapping of the oligopeptides, combined with HREIMS on the intact antibiotic, to assign the structure.

Quite recently a new mass spectrometric technique has been developed, called fast atom bombardment mass spectrometry (FABMS) by its inventors at the University of Manchester Institute of Science and Technology (UMIST).[2] The FAB technique employs an ion gun which accelerates argon ions to 2-10 KeV, then passes the argon ion beam through an atmosphere of argon gas where the ions undergo charge exchange, yielding a beam of accelerated argon atoms, which is directed onto a ribbon of metal or plastic which has been coated with a solution or suspension of the sample to be studied, in a matrix of glycerol. The argon atoms ionize and vaporize the sample molecules (along with glycerol)

and the pseudomolecular ions—$(M + H)^+$, $(M + Na)^+$, $(M - H)^-$, etc.—are either detected as such or cleave to give fragmentation ions. The technique is an outgrowth of secondary ion mass spectrometry (SIMS) but differs from it in the use of an atom beam (rather than an ion beam) and in the use of a glycerol matrix. The neutral atom beam is a particular advantage in a magnetic sector mass spectrometer where the potential in the ion source may be as much as 10,000 volts above ground.

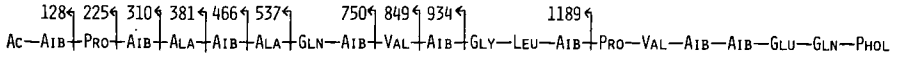
The FABMS technique has been applied to problems of peptide structure, including two peptides with which we were familiar from our earlier efforts on peptaibophol antibiotics.[3,4] As seen in Scheme 1, antiamoebin I gave an $(M + Na)^+$ ion at m/z 1692 by FABMS, accompanied by an $(M + H)^+$ ion at m/z 1670. In addition, the FAB mass spectrum gave fragment ions up to m/z 1442, indicative of clean fragmentation between adjacent amino acids in the peptide chain, as well as ions at m/z 242 and 341 indicative of the sequences Hyp-Gln and Hyp-Gln-Iva. The single FAB step actually replaces two from our previous General Procedure (Steps 3 and 4), involving FDMS to assign the molecular weight of the antibiotic plus EIMS to assign fragmentations. It also reduces greatly the necessity for identification of oligopeptides following partial hydrolysis (Steps 5 and 6). It should be noted, too, that fragmentation ions by EIMS ceased at m/z 884,[3] while FDMS gave only $(M + Na)^+$ and no $(M + H)^+$. In addition, FABMS has the advantage that in the negative ion mode it gave a strong $(M - H)^-$ ion at m/z 1668, confirming the molecular weight. Finally, at low masses the positive ion FAB mass spectrum contained ions characteristic of the individual amino acids Aib, Pro, Iva, Leu-Hyp, and Phe at m/z 58, 70, 72, 86, 91, and 120, thus confirming the presence of those amino acids assigned by HRFDMS; FABMS might thus replace Step 1a of the General Procedure.



Scheme 1. Antiamoebin I fragmentation.

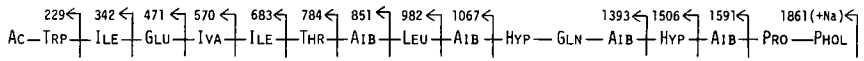
Preliminary studies on FABMS as applied to alamethicin also indicated its utility in assigning fragment ion peaks, as shown in Scheme 2. Here again, the fragmentations are considerably cleaner than by EIMS and extend to higher masses (EIMS fragmentation peaks in this sequence ceasing at m/z 537 and no sequence peaks appearing above m/z 1002).[4]

Concurrent with our work on the antiamoebins and the alamethi cins, we have been investigating the structures of the zervamicins, peptai-



Scheme 2. Alamethicin I fragmentation.

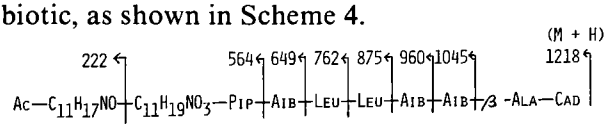
bophol antibiotics which generally have lower antibacterial activity but higher pore-forming activity than the antiamoebins and the alamethicins. Zervamicins, however, are more refractory to mass spectrometric investigation due to the presence of the more polar and more fragile amino acids threonine and tryptophan, and the General Procedure had little success in dealing with these compounds. On the other hand, FABMS gave the same sort of information with zervamicin IC that it did with antiamoebin I, as shown in Scheme 3. Additional fragmentation peaks at m/z 242 and 327 assigned the sequence Hyp-Gln-Iva, the low mass peaks at m/z 58, 70, 72, 86, and 130 confirmed the amino acids Aib, Pro, Iva, Ile-Leu-Hyp and Trp and a negative ion FABMS peak at m/z 1837 confirmed the molecular weight.



Scheme 3. Zervamicin IC fragmentation.

Recent improvements in high pressure liquid chromatography (HPLC) have separated a total of seven neutral compounds of the zervamicin II family and four acidic compounds of the zervamicin I family (Figure 1). All of these structures have now been assigned by FABMS. FABMS combined with HPLC has also indicated that emerimicins IIA and IIB are identical to zervamicins IIA and IIB.

We have also tested the FABMS technique with a number of other peptides, with generally excellent success. For example, the antitumor antibiotic CC 1014[1] yields not only molecular ion information but also sequence information beyond that supplied in the EI mass spectrum of the antibiotic, as shown in Scheme 4.



Scheme 4. CC 1014 fragmentations.

Other peptides successfully investigated by FABMS include the marine-derived didemnins A and B (MW's 942, 1111), gramicidin S (1140), berninamycin A (1146), polymyxins B (1183, 1197), zorbamycin and its copper chelate (1411, 1474), zorbonomycin B and its copper chelate (1413, 1476), and saramycetin (1450). Of these, berninamycin A and saramycetin yielded little or no information by procedures employing FDMS.

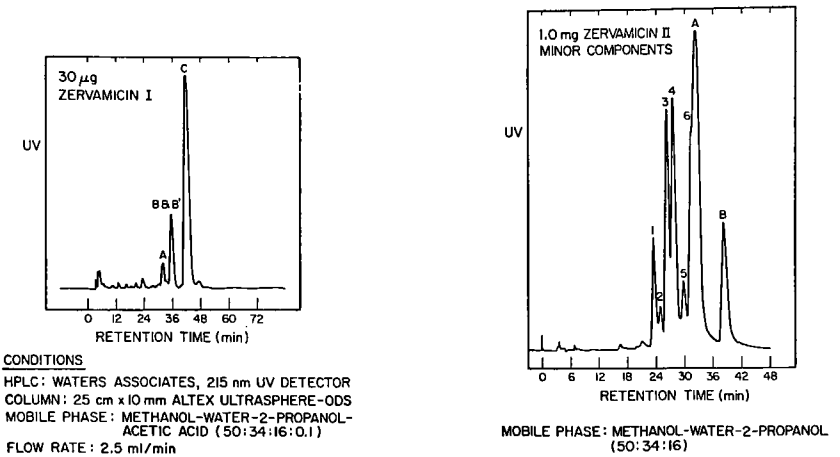


Fig. 1. HPLC separations of zervamicins I (acidic) and II (neutral).

FABMS offers a superior alternative to a number of other techniques of mass spectrometry, combining a number of the best features of FDMS, CIMS, and EIMS. Precise mass measurement is not yet routine by FABMS, but we have carried out some high resolution measurements by peak matching FABMS peaks against various standards. For example, accurate mass measurement of the m/z 1067 peak in the FAB spectrum of zervamicin IC determined (9 ppm accuracy) that the two Hyp residues were to the right of the fragmentation giving the peak, while the accurate mass of the $(M + H)^+$ peak for zorbamycin at m/z 1412 was shown to agree within 7 ppm with the value expected by Argoudelis and Baczynskyj (personal communication). The recently developed multichannel signal analyzer methodology,[6] which thus far proved valuable for precise mass measurement with FDMS, CIMS, and EIMS, will probably be of considerable use with the FAB technique as well.

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HPLC ISOLATION AND FD-MS QUANTIFICATION OF PICOMOLE AMOUNTS OF MET-ENKEPHALIN IN CANINE TOOTH PULP

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Introduction

The tooth is considered to be a singular sensory site in the body from which the only sensation experienced is pain.[1] Thus, a nociceptor system may exist in teeth as part of the body's neurophysiological control of pain perception. The recent discovery of endogenous molecules (enkephalins, endorphins, etc.) that are opiate-like substances and may play a role in pain perception suggests a physiological system key to nociceptive information processing. These endogenous opiate peptides and their receptors hold promise for understanding pain mechanisms as a neuronal system mediating the organ's capacity for pain suppression. The specific aims of this research were to extract from dental tissue (tooth pulp) biologically active neuropeptides which putatively participate in the pain process, to purify, and to quantify endogenous levels of these oligopeptides.

A unique combination of chromatographic separation and mass spectrometric techniques has been developed for a novel method for measurement of picomole amounts of endogenous oligopeptides in biological tissue.[2,3] Reverse phase high performance (pressure) liquid chromatography (RP-HPLC) is utilized for rapid high resolution separation of peptides. A new buffer system, dilute triethylamine-formic acid (TEAF), is utilized. Field desorption mass spectrometry (FD-MS) data provide unambiguous measurement and identification of the intact molecular structure of the isolated neuropeptides.[3]

Materials and Methods

Dogs are anesthetized and exsanguinated through a femoral artery. During the terminal phase of this procedure, canine tooth pulps are removed, immediately frozen in liquid nitrogen, and stored at -70° C until used. Pulps are thawed and weighed. Six ppm of ^{2}Ala-Leucine-enkephalin (^{2}Ala-LE) is added as internal standard, and the pulps are homogenized with a cell disrupter in 5 volumes of 1N acetic acid. The

homogenate is centrifuged at 15,000 rpm. The supernatant is extracted with ether (2x), lyophilized, residue resuspended in pH 7.5 tris buffer, and centrifuged. The supernatant is placed on a previously washed Bio-Beads SM2 column (polystyrene-divinylbenzene), eluted with 2 ml methanol, and evaporated with nitrogen. The sample is dissolved in 100 μl TEAF and chromatographed on a Waters RP-HPLC. Standard synthetic peptides [Methionine-enkephalin (ME), Leucine-enkephalin (LE), ^{2}Ala-LE] are chromatographed on RP-HPLC. ^{3}H-LE was added to the homogenate to assess extraction recovery efficiency. A Finnigan (Varian) MAT 731 (Bremen, W. Germany) mass spectrometer of Mattauch-Herzog double-focusing geometry equipped with a field desorption/field ionization/ electron ionization combination source was used to produce protonated molecular ions. Lyophilized pulp samples collected from RP-HPLC are dissolved in 100 μl MeOH. Volume is reduced to less than 10 μl with nitrogen, and the entire sample placed on the FD emitter. MS scans for sample and internal standard $(M+H)^{+}$ ions verify molecular weight which relates to the intact molecular structure of the neuropeptide of interest.

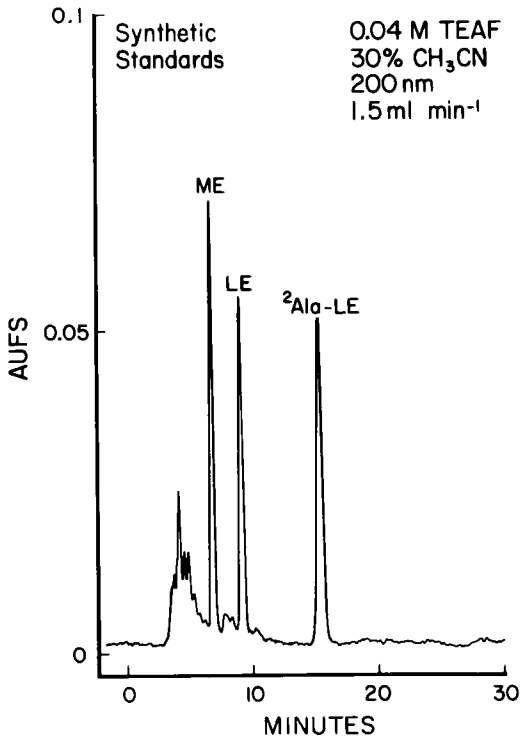

Fig. 1. RP-HPLC chromatogram of a standard mixture of ME (6.5 min), LE (8.6 min), and ^{2}Ala-LE (14.5 min). Experimental parameters were 0.1 AUFS; 200 nm; 1.5 ml min^{-1}; 2 μBondapak C18 columns in series; 1.0 μg of each peptide injected; 0.04 M TEAF (70%); 30% CH_3CH(30%).

Results

Figure 1 contains the RP-HPLC chromatogram of standard synthetic peptides. Figure 2 contains the RP-HPLC chromatogram of an extract from 306 mg of dog tooth pulp. This sample was compared to the standards in Figure 1. The pulp tissue contains a large number of peaks eluted from the Bio-Beads SM2 column which allows separation of peptides in the 600-14,000 molecular weight range. Labeled are peaks of ME and LE and the internal standard ^{2}Ala-LE. The extraction recovery of ^{3}H-LE was 25% for this sample. Figure 3 contains a photograph of the oscillographic paper recording illustrating the alternation between $(M+H)^{+}$ of internal standard (IS) and ME. Individual peak heights for both IS and ME are integrated. The endogenous amount of ME is calculated as the ratio of integrated ME/IS ion current x amount of IS originally added. The value obtained for pulp sample was 3 μg/g tissue.

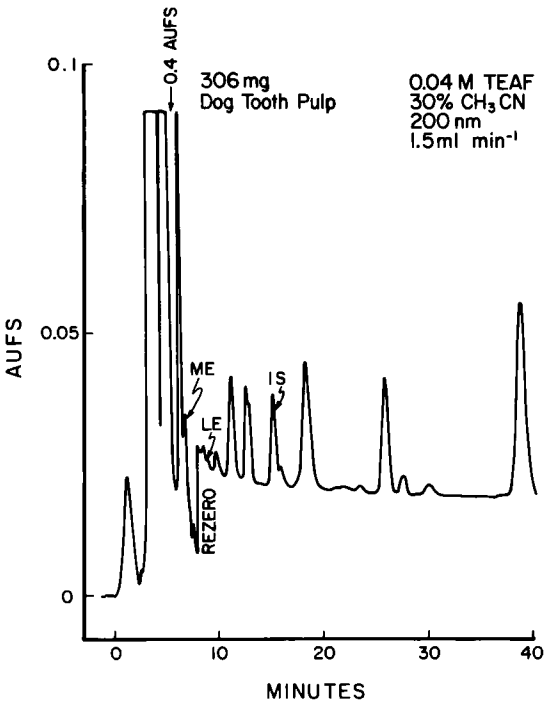

Fig. 2. RP-HPLC chromatogram of an extract from 306 mg tooth pulp. Experimental parameters listed in legend of Figure 1.

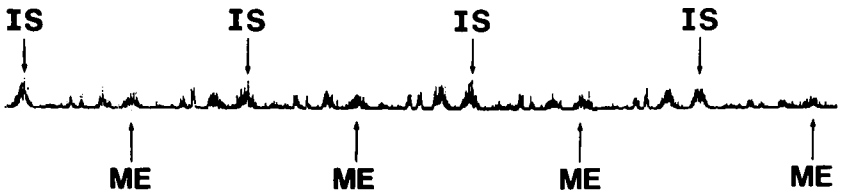

Fig. 3. Photograph of oscillographic paper record of $(M+H)^+$ ions of internal standard (IS) and ME.

Conclusions

Several conclusions result from this research:

1. For the first time an opiate-like peptide (ME) was isolated and quantified by FD—MS in a tooth pulp extract.
2. LE was previously quantified by RP-HPLC technique only.[3]
3. Optimal HPLC resolution has been combined with maximal molecular specificity to quantify specific dental neuropeptides.
4. The overall recovery is 25% and the internal standard allows quantification of neuropeptides by FD-MS-selected ion monitoring techniques.
5. The data presented in this paper opens up an intriguing question -what is the role of ME in tooth pulp?

Acknowledgements

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RAPID PARTIAL SEQUENCING OF PROTEINS BY GAS CHROMATOGRAPHIC MASS SPECTROMETRY

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Introduction

A gas chromatographic mass spectrometric (GCMS) technique has been developed to rapidly obtain a large amount of sequence information from an intact protein. Briefly, the GCMS strategy[1,2] consists of partial hydrolysis of the protein with 6N HCl or non-specific enzymes to a complex mixture of di- to pentapeptides. The mixture is then derivatized as follows[3]:

1) Methylation with 3 N HCl/MeOH or CH_2N_2
2) Trifluoroacetylation with $CF_3CO_2CH_3/(CH_3CH_2)_3N$
3) Reduction with B_2D_6/THF
4) O-Trimethylsilylation with $(CH_3)_3SiN(CH_2CH_3)_2$/pyridine

The resulting mixture of O-TMS polyamino alcohols is separated by gas chromatography, and the mass spectrum of each component is recorded. Interpretation of these data allows for the identification of 50-100 di- to pentapeptides. The essential feature of this technique is that partial sequence information can be obtained rapidly since no primary degradation fragments need to be separated and isolated, a task that is particularly time-consuming for large proteins. Three applications of the GCMS technique are presented to illustrate how it effectively complements both DNA-based protein sequencing and the Edman degradation.

Results and Discussion

Phase Checking of Protein Sequences Derived from DNA Sequences — Rapid methods for DNA sequencing have made it possible to determine the primary structure of proteins too large or intractable to be easily sequenced by the Edman degradation. Although DNA sequencing may largely replace protein sequencing as the method of choice for determining the sequence of large proteins, there is still a need for data derived from the protein itself. Positive identification of initiation and termination coding sequences requires comparison with amino and carboxyl terminal sequences. The former is most easily obtained by the Edman degradation (if the protein does not contain a blocked N-terminus), and the latter is best determined by carboxypeptidase diges-

tion. In addition, sequence data from throughout the protein is useful particularly when long nucleotide sequences are being translated into protein sequences. An error leading to the insertion or deletion of a single nucleotide will result in a reading frame shift which, when translated, will produce a grossly incorrect protein sequence even though 99% of the DNA sequence is correct. GCMS protein sequencing is an ideal way to obtain a large number of peptide sequences from throughout the molecule thereby eliminating the possibility of reading frame errors.[4] These peptide sequences can be obtained in the same time frame as the DNA sequence and require only nanomole quantities of protein since the GCMS strategy obviates the time and material consuming steps of peptide isolation.

The complete amino acid sequence of *E. coli* alanine tRNA synthetase, an 875 amino acid long protein, has been determined by this combined DNA/GCMS approach.[5] GCMS peptides derived from throughout the protein were matched to each of the three amino acid sequences obtained from translation of each of the possible reading frames. Peptide matches delineate blocks of properly phased DNA, and any phasing errors are readily apparent. When these are corrected and all the peptides are aligned in a single reading frame, the possibility of a remaining phasing error is greatly reduced. Figure 1 demonstrates that all the tri-, tetra- and pentapeptides identified by GCMS fit only into reading frame 1 and none match any amino acid sequence corresponding to reading frame 2 or 3.

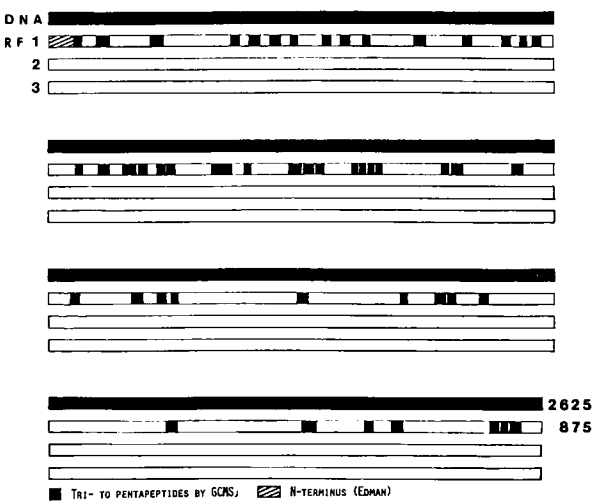

Fig. 1. Schematic representation of alanine tRNA synthetase. The structural gene is 2625 nucleotides long and encodes an 875 amino acid long protein. The three possible reading frames are labeled "RFI", "2", and "3". The shaded areas in reading frame 1 represent GCMS peptides. The hatched box at the beginning of RFI indicates Edman degradation data from the intact protein.

Location of Post Translationally Modified Amino Acids — The GCMS peptide sequencing strategy has also been used to determine sites of post translational conversion of specific glutamic acid residues to γ-carboxyglutamic acid (Gla) in osteocalcin, a small protein derived from bone. The presence of this amino acid cannot be inferred from a DNA sequence, and since it readily decarboxylates to glutamic acid, it is difficult to determine by conventional methods. The GCMS strategy[6] consists of decarboxylation of the protein with DCl with converts Gla into γ, γ dideuteroglutamic acid. After decarboxylation the protein is analyzed by GCMS as described above. Figure 2 summarizes the data resulting from a single experiment on chicken osteocalcin, a 50 amino acid long protein which contains three Gla residues. Fourteen peptides were identified which contained the Gla derivative. If the sequence of this protein had been previously established, the data from this single experiment would suffice to locate all three of the γ-carboxyglutamic acid residues as well as confirm 92% of the sequence. The full sequence was completed by mass spectrometric analysis of several enzymatic hydrolyzates of osteocalcin.[7]

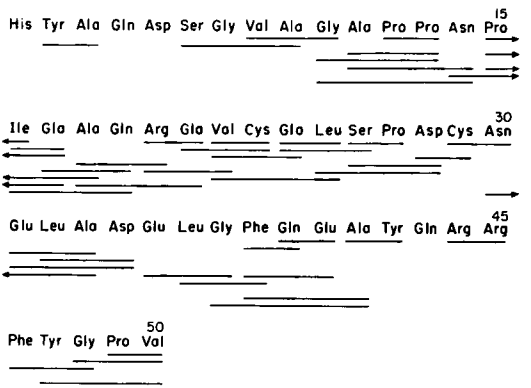

Fig. 2. Amino acid sequence of chicken osteocalcin. Underlinings represent the peptides identified in a single partial acid hydrolyzate.

Sequencing Proteins by Combined GCMS/Edman Degradation — Macromomycin, a protein with antitumor activity related to neocarcinostatin is being sequenced by a combination of GCMS and Edman degradation. The sequencing strategy involves tryptic cleavage at lysine followed by Edman and GCMS sequencing experiments on both the four isolated tryptic fragments and the intact protein. To place the tryptic peptides in the proper order the partial acid hydrolyzate of the intact macromomycin was derivatized and the GCMS data searched for the three required lysine overlap peptides. Thus, the protein can be sequenced from a single set of primary degradation fragments.

Another example of the efficiency of the combined sequencing strategy is the determination of the sequence of the C-terminal tryptic fragment of macromomycin. An Edman degradation experiment yielded the N-terminal 18 residues and residue 20 as shown in Figure 3. Carboxypeptidase digestion indicated that phenylalanine and alanine were the C-terminal residues. A GCMS experiment on a partial acid hydrolyzate of intact macromomycin is shown in Figure 4. Among the peptides identified were Ala-Ala-Glx-Ala-xLeu, Ala-xLeu-Thr-Phe and xLeu-Thr-Phe-Ala (xLeu = Leu or Ile). These peptides established that residue 19 was alanine and completed the sequence of this fragment as shown in Figure 3 without resorting to additional fragmentation and peptide isolation. The GCMS peptides also confirm a large part of the N-terminal sequence established by Edman degradation. Finally, it should be noted that the 115 peptides identified in the experiment shown in Figure 3 represent approximately 85% of the sequence of this protein, which is about 112 amino acids long.

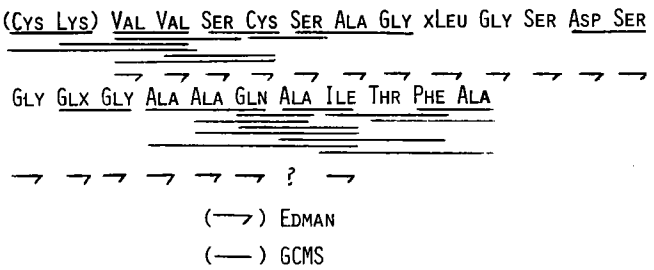

Fig. 3. C-terminal tryptic peptide of macromomycin.

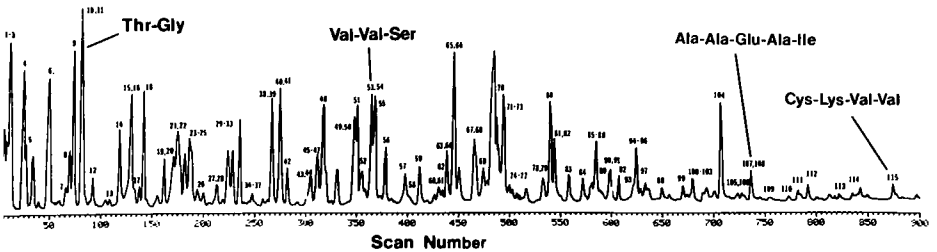

Fig. 4. Total ionization plot derived from a partial acid hydrolysis experiment on macromomycin. The y axis is Relative Intensity.

Conclusion

These three examples illustrate how the GCMS peptide sequencing methodology can be used to obtain partial sequence data more easily than conventional techniques. In all cases the advantage of using GCMS is that an analysis can be carried out on an intact protein without the isolation of specific peptides.

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HPLC PURIFICATION OF OVINE CRF, RAT EXTRA HYPOTHALAMIC BRAIN SOMATOSTATIN AND FROG BRAIN GnRH

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Introduction

HPLC has become an indispensable tool to the peptide chemist. Indeed, numerous are its uses as an analytical as well as preparative instrument that can be applied to naturally occurring or synthetic peptide/protein mixtures. Depending on the detection system, analyzed samples can be totally or partially (in the case of post column derivatization) collected and further purified when taking advantage of solvent or column selectivity, temperature effects or different gradient shapes. The purification of ovine hypothalamic corticotropin releasing factor (CRF), rat extra hypothalamic brain somatostatin and frog brain gonadotropin releasing hormone (GnRH) are examples used to illustrate those very points.

Experimental

Both HPLC systems and buffer composition (triethylammonium phosphate: TEAP, or formate: TEAF) have been described earlier.[1] Columns, solvent, flow rate, temperature and load, are described in the Figure legends illustrating each RP-HPLC purification step. Buffer A is the aqueous buffer, buffer B is 60% CH_3CN in A. Chart speed was 1 cm/min. Column cuts are shown above the abscissa and the active zone is hatched. Gradient shape is shown by a dotted line. An *in vitro* assay based on the ability of a putative CRF to stimulate the secretion of corticotropin by primary cultures of rat anterior pituitary cells[2] was used to follow CRF-like activity. Starting material for the purification of CRF was 490,000 sheep hypothalami which were acid extracted, defatted and partitioned in a 1-But-OH: Pyr: 0.1% AcOH system as reported by Burgus *et al.*[3] The lower phase of this last step (2 kg) contained a fraction of the total CRF-like activity exhibited by the defatted material. Ultrafiltration or dialysis yielded 15 g of desalted peptide/protein concentrate which was applied, batch-wise, to gel permeation on G-50 at 4° C. Two zones of activity were obtained; that eluting at 1.3 Ve/Vo had a significantly larger secretory V_{max}[2] than the retarded material (2.0 Ve/Vo) which, upon further purification, was found to contain AVP. Treatment

of the larger molecular weight active fraction with GnHCl/AcOH (pH 2.5) and chromatography on Bio-Gel P-10 using 4M Gn.HCl/AcOH (pH 2.5) as eluant gave one single peak of activity which was only partially retarded, thus excluding the possibility that the activity had been associated with larger molecular weight carriers. The pool of this zone from several columns was the starting material for the RP-HPLC purification of ovine CRF.

Four hundred rat brains (minus hypothalamus and median eminence) were lyophilized. An acetone powder was obtained and extracted with hot 3N HOAc. The supernatant after centrifugation was the starting material for the RP-HPLC purification steps. Somatostatin like immunoactivity (SS-LI) was followed by radioimmunoassay (RIA).[4]

Similarly 100 frog brains *(Rana catesbeiana)* were lyophilized. An acetone powder was obtained and extracted with 3N AcOH. The retarded material which contained the GnRH-like immunoactivity (GnRH-LI)[5] after gel permeation on Sephadex G-50 was the starting material for the RP-HPLC purification. GnRH-LI was followed by RIA.

Results and Discussion

HPLC purification of ovine CRF — Early attempts to purify CRF by RP-HPLC using μBondapak C_{18} or even μBondapak CN and NH_4OAc/CH_3CN buffer resulted in complete loss of CRF biological activity. This disappointing observation was the main incentive to search for a buffer system that would be UV transparent, non-toxic or volatile so that bioassays could be carried out without any further manipulation of the samples. The trialkylammonium buffers and TEAP[1] in particular, were found to indeed increase recovery as well as overall column performance. The role of the added alkylamine to the mobile phase was to competitively inhibit the participation of the sample in the solute, in the ion exchange or adsorption reactions with the non-bonded silanols on the stationary phase. This early observation was subsequently documented by Sokolowsky and Wahlund.[6]

Figure 1 shows the scheme of purification used to obtain the CRF fraction subjected to sequence analysis. The first step is a rough separation where recovery was emphasized rather than resolution. The active zones from more than a hundred such runs were pooled to generate 28-125-00 (150,000 fragments in 8.75 ml). This step achieved two purposes: a) it freed the active fraction from Gn.HCl and b) resulted in an approximately hundred-fold purification.

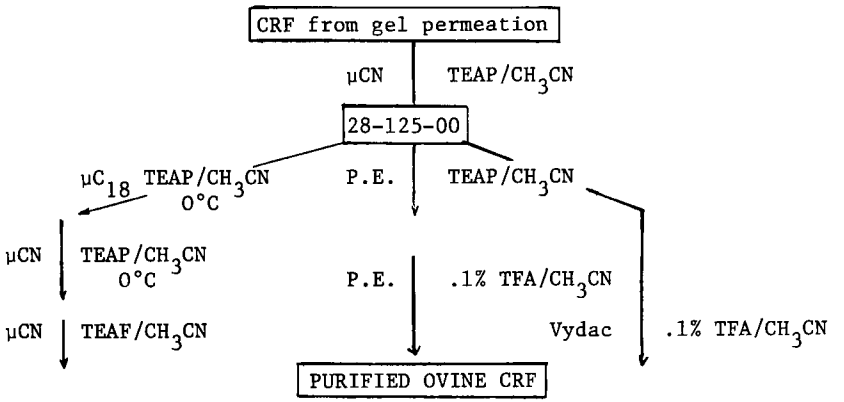

Fig. 1. RP-HPLC purification of ovine CRF.

At this point a great number of HPLC systems were tried and tested in an effort to resolve what semed to be an untractable mixture. In most cases one would either lose biological activity when using C_{18} columns (μBondapak or SupelcoSil) or not achieve any separation with the μBondapak CN. Several solvent systems made of different organic modifiers (CH_3CN, 1-propanol, 1-butanol, 2-propanol or mixes thereof) and TEAP or NH_4OAc at different concentrations (.1 to .5 N) or pH (2.25 to 7.2) were tested without success.

It was only when we used a μBondapak C_{18} (but not SupelcoSil C_{18}) immersed in ice-water that the TEAP/CH_3CN buffer system would yield acceptable resolution and recovery. At that stage, gradient conditions were optimized on the C_{18} column (Figure 1, left column and Figure 2), isocratic conditions were found on the μCN columns and desalting could be achieved with the TEAF/CH_3CN buffer at room temperature. The main drawbacks of this original isolation scheme was a relatively low overall yield due to adsorption on the column and in the case of the final desalting step with TEAF, of the inability to monitor the column effluent since CRF did not seem to adsorb at 255-280 nm and TEAF would not be UV transparent at the concentration used. We therefore had to resort to following CRF by biological testing or amino acid analysis while arbitrarily fractionating the column effluent. About 10 nanomoles of CRF were purified in that manner.

Most of these difficulties were later resolved by using large pore size silica (300-330 Å) that had been properly derivatized (C_{18}) and end capped [i.e., Perkin-Elmer (P.E.) (10μ) Figure 1 center column and Figure 3 or Vydac (5μ) Figure 1 right column and Figure 4]. Whereas

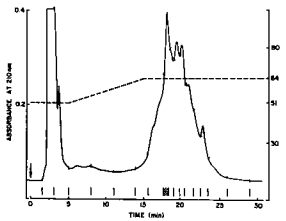

Fig. 2.: μBondapak C_{18} (0.46 x 30 cm); Load: CRF 28-125-00 (125 μl = 2150 f); A = TEAP pH 3.0; Flow rate: 1.2 ml; Temperature 0°C.

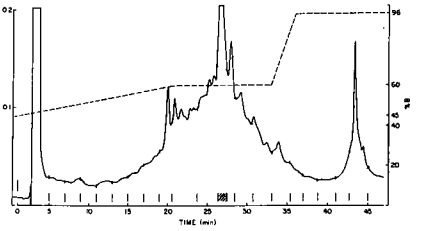

Fig. 3.: P.E. ODS-HC SIL-X-1 (0.26 x 25 cm); Load: CRF 28-125-00 (80 μl = 1400 f); A = TEAP pH 2.25; Flow rate: 0.7 ml/min; Room temperature.

acceptable recovery of CRF-like activities could be obtained in the 100 Å support by cooling the column, thus driving the equilibrum of mass transfer from the solid support toward the solute, one could do the reverse and increase resolution (as illustrated in Figure 3 and 4) by using monolayered end capped C_{18}, 330 Å silica at room temperature. Higher resolution was obtained on the 5μ particles (Vydac) (Figure 4) than on the 10μ particles (P.E.) (Figure 3). The use of solvent systems having a relatively high concentration of alkyl ammonium salt also became obsolete due to the absence of residual silanols, thus allowing for purification and desalting of 28-125-00 in one step using 0.1% TFA/CH_3CN as eluant. Rechromatography of purified ovine CRF is shown in Figure 5. Amino acid and sequence analyses of this material were consistent with a structure having 41 residues.[7,8]

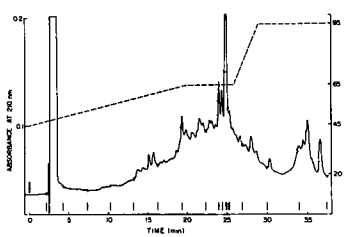

Fig. 4: Vydac C_{18} (5μ: 0.45 x 25 cm); Load: CRF 28-125-00 (80 μl = 1400 f); A = .1% TFA B = 60% CH_3CN in 0.24% TFA; Flow rate ; 1.2 ml/min; RT

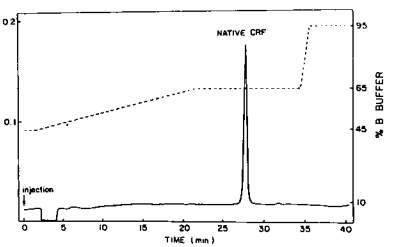

Fig. 5: Vydac C_{18} (5μ: 0.45 x 25 cm); Load: purified CRF (ca 4 μg); A,B, flow rate and temperature as in Fig. 4.

HPLC purification of rat extra hypothalamic brain SS. - The presence of SS-LI in extra hypothalamic rat brain tissues was shown by Vale *et al.*[4] at concentrations close to 1/6 that found in the hypothalamus. The

chemical nature and possible role of SS in the brain were however unknown. The SS-containing starting material (described in the experimental section) was run through seven successive HPLC steps which led to a material that behaved like SS-14 in several chromatographic systems and radioimmunoassays. Furthermore, it was found to have the amino acid composition of SS-14. We cannot at this stage exclude the presence in brain or larger forms of SS such as SS-28 first purified from porcine gut and characterized by Pradayrol *et al.*[9], since the early steps of purification using C_{18} cartridges in the Prep LC-500 of Waters Associates may have selectively adsorbed such large molecular weight substance.

HPLC purification of a frog brain GnRH — Whereas SS-like molecules have now been characterized from numerous tissues in several species, the decapeptide GnRH has been fully characterized from ovine and porcine hypothalami only. A report by Eiden and Eskay[10] would indicate that GnRH-LI immunoreactivity in the frog sympathetic ganglia is different from the known decapeptide. We have made similar observations (based on chromatographic behavior of the activity) and extended the work to the purification of frog brain GnRH-LI (see Figure 6). This isolated material was found to behave chromatographically and in several radioimmunoassays identically to GnRH and had the amino acid composition of the decapeptide GnRH. It is therefore probable that frog brain GnRH-LI is identical to mammalian GnRH.

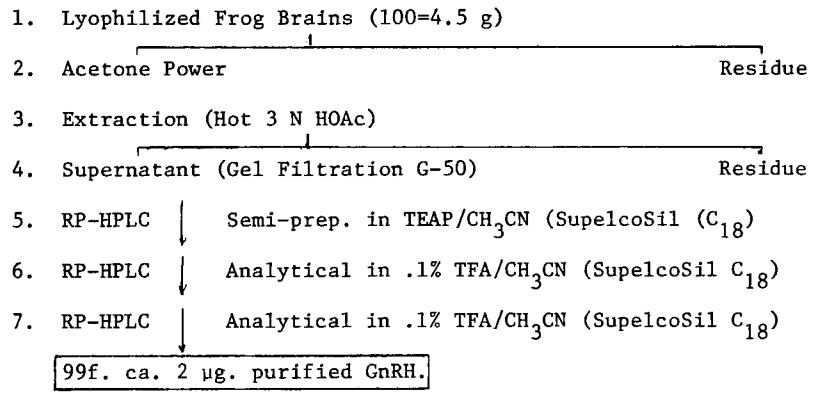

Fig. 6. Purification scheme for frog brain GnRH

Conclusion

The ability to purify the above mentioned peptides is the result of constant improvement in HPLC technology (optimization of column supports, solvent systems, flow rate, gradient shape, temperature). Whereas isolation programs have focused so far on purifying enough of the material of interest for identification (RIA's, amino acid analysis and sequencing) the, as yet, unmet challenge is to ascertain 100% recovery of a biological activity throughout its purification scheme, thus allowing for absolute quantitation of a given substance in a given tissue.

Acknowledgements

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A GENERAL APPROACH TO THE MICROISOLATION OF PEPTIDES

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Introduction

In view of the rapidly increasing knowledge concerning the biological significance of endogenous peptides it is important that efficient methodology should be available for the isolation and chemical characterization of novel peptides. Peptide isolation has, however, been notoriously difficult, due to the very low tissue concentrations of most peptides and the considerable inefficiency of classical isolation methodology, which usually leads to large losses.

In this report we describe a methodology for the isolation of peptides which is generally applicable, rapid, simple and, due to the elimination of major losses, efficient. Furthermore, the methodology is applicable to large and small scale isolation of peptides from tissue extracts, culture and physiological fluids. This approach is based on previous experiments where octadecasilyl-silica (ODS) was used for peptide extraction, followed directly by reverse-phase high performance liquid chromatography (HPLC) for peptide isolation.[1-3] We have used these procedures extensively for the isolation of novel peptides from various tissues, *e.g.* the isolation of luteinizing hormone releasing factor (LRF) from 3000 rat hypothalami.

Experimental Procedures

Tissue was homogenized in 10-20 volumes of a mixture of aqueous formic acid (5% v/v), trifluoroacetic acid (TFA: 1% v/v), NaCl (1% w/v) and 1 M HCl[2]. After centrifugation the supernatant was adjusted to pH 2.5 with 10 M NaOH and defatted with methylene chloride or petroleum ether. The aqueous phase was separated by centrifugation and residual organic solvent removed in vacuo. The solution was then passed through a column of ODS (LRP-2, Whatman) as previously described.[3,4] The ODS column was washed free of salt with 0.2 M acetic acid and the adsorbed peptides eluted with a minimal volume of 0.36 M pyridine formate, pH 3, in 60% (v/v) *n*-propanol. For peptide isolation, two reverse-phase HPLC systems were used, consisting of C18 and C8

columns in conjunction with either 0.36 M pyridine formate, pH 3/*n*-propanol or 0.5% (v/v) TFA/acetonitrile as the mobile phases. For amino acid analysis[5] and bioassay[6] (release of luteinizing hormone [LH] from pituitary monolayer culture) published procedures were employed.

Results and Discussion

Peptide isolation is achieved by means of four procedures. Tissue extraction with Bennett's solution[2] will remove most of the large proteins; however, medium-sized and small peptides are recovered in good yield.[2] Lipids are removed by extraction with organic solvent. A key step is reverse-phase peptide extraction, which is suitable for the rapid preparation of a crude but highly enriched peptide fraction. It facilitates transition from large volumes/masses of original tissue extracts to amounts which can be subjected directly to HPLC. The capacity of ODS for peptides is high; *i.e.* at least 10 g for a 7.5 x 25 cm column. HPLC is used immediately for the isolation of peptides from crude peptide extracts. Depending on the amount of material, initial chromatography is performed with an analytical (0.46 x 25 cm, 10 mg), semi-preparative (0.9 x 2.5 cm; 50-100 mg), or preparative (C18) (2.2 x 50 cm, 1 g) column, with pyridine formate/propanol as the mobile phase. The desired peptide fraction is then rechromatographed on a smaller column in the same solvent system under isocratic conditions. Although such two-step HPLC purification can yield a pure peptide, rechromatography in another HPLC system with different solute selectivity, such as a C8 column in conjunction with the TFA/acetonitrile mobile phase, is often required. Gel filtration may also be used for the preliminary fractionation of peptide extracts, particularly when quantities exceeding the capacity of even preparative HPLC columns are processed.

In peptide purification on a microscale, successful isolation of peptides in sufficient amounts for structural characterization largely depends on the effective control of losses. The proposed isolation approach is designed for minimization of losses. Procedures known to be associated with loss (*e.g.* conventional ion-exchange chromatography and thin-layer separations) are avoided. The techniques used here afford good peptide recovery (94 ± 10% [S.D.] for ODS peptide extraction; >90% with HPLC). However, extensive loss of peptide usually occurred when column fractions were lyophilized prior to further purification. In our isolations of rat hypothalamic somatostatin-14[7] and somatostatin-28[8] losses in excess of 90% occurred when gel filtration fractions were lyophilized prior to HPLC. Subsequently we determined that such loss was entirely eliminated if lyophilization of column fractions was avoided and

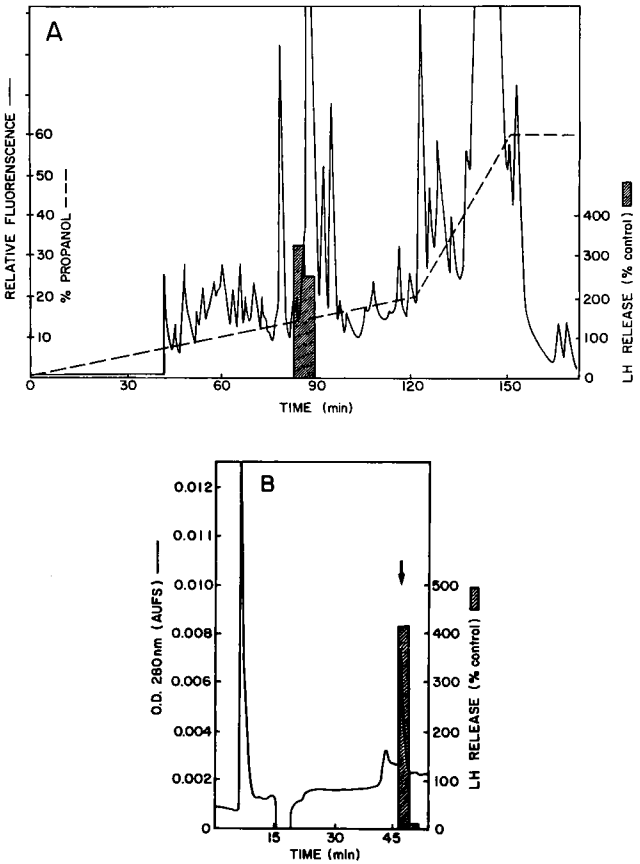

Fig. 1. Isolation of LRF from rat hypothalamus. An acetic acid extract of 97,000 hypothalami was passed through an anti-somatostatin-immunoaffinity column.[8] The non-retained material (in 12 l phosphate buffer) was subjected to ODS reverse-phase peptide extraction (7.5 x 25 cm ODS column). Yield: 1.4 g. A) HPLC of 50 mg peptide extract on an RP-18 Ultrasphere column (0.9 x 25 cm) in the pyridine formate/propanol mobile phase. Fractions containing LH releasing activity were pooled and further purified by HPLC on an RP-18 column using the TFA/acetonitrile mobile phase (data not shown). B) Final purification (after 2 steps of rechromatography) of the active fraction on RP-8 (0.46 x 25 cm) under isocratic conditions using 0.5% TFA in 23.2% acetonitrile. The arrow indicates the retention time of synthetic LRF.

instead the sample (after appropriate dilution) was directly loaded onto an HPLC column by pumping it through the column.

An example of the capability of the isolation methodology is shown in Figure 1. LRF was isolated from a side fraction of an extract of 3000 rat hypothalami used for the isolation of somatostatin 28.[8] 400 pmol of pure peptide were obtained, the amino acid composition of which was

determined as $Asx_{0.18}$, $Ser_{1.06}$, $Glx_{1.09}$, $Pro_{0.94}$, $Gly_{2.14}$, $Ala_{0.14}$, $Val_{0.13}$, $Leu_{1.01}$, $Tyr_{0.95}$, $His_{0.86}$, $Trp_{0.99}$, $Arg_{1.13}$. From this composition, and the chromatographic behavior of the peptide, we conclude that rat hypothalamic LRF is identical in structure to ovine and porcine LRF. Other examples of applications of this technique are the isolation and complete structural characterization of γ_1-melanotropin from the neurointermediate lobes of 12 bovine pituitaries [9] and of somatostatin-28(1-12).[10]

Acknowledgements

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PREPARATIVE REVERSE PHASE HPLC: AN EFFICIENT PROCEDURE FOR THE RAPID PURIFICATION OF LARGE AMOUNTS OF BIOLOGICALLY ACTIVE PROTEINS

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Introduction

Reverse phase liquid chromatography (RPLC) combined with fluorometric detection has been proven to be a powerful tool for the separation of closely related proteins, such as insulin derivatives or hemoglobin subunits and their genetic variants[1,2] as well as for the isolation of small quantities of biologically active proteins, such as interferon.[3] We have therefore investigated the usefulness of this method for the purification of epidermal growth factor (EGF), a protein hormone with a molecular weight of 6000 Daltons. EGF is produced by the submaxillary glands of male mice and stimulates the proliferation of a variety of target cells. This protein should be available in large quantities for biological studies and also be free of contaminants which, even if present in small quantities, might interfere with biological assays because of structural similarities or high intrinsic activity.

Usually, EGF is prepared by homogenization of salivary glands, followed by centrifugation and gel filtration of the resulting supernatant: the fraction containing EGF activity is then directly used for biological studies. The purity of this material is evaluated by SDS-polyacrylamide gel electrophoresis and/or isoelectric focusing. One disadvantage of these methods is the fact that only limited amounts of material can be applied to the gels so that contaminants present at less than 1% are difficult to detect.

We demonstrate that by the use of one additional reverse phase HPLC step (separation based mainly on hydrophobicity) one obtains a further purification which also reveals the existence of molecules present in small quantities. A closely related species not detectable with classical methods is also present.

In addition, the procedure can be scaled up for the rapid purification of this protein from more complex protein mixtures.

Materials and Methods

RPLC, fluorometric detection system and solvent system are those described previously.[2,3,4,5] Columns were obtained from Altex-Beckman (Mountain View, CA): RP-8, 5μ particle size, 100A pore size 0.46 X 25 cm; RP-8, 5 μ particle size, 100A pore size 1.0 X 25 cm.

Mouse epidermal growth factor was prepared either by gel filtration on Bio-Gel P10 in the presence of a NaCl/ HCl buffer system (pH 1.5)[6] or in the presence of a Tris-HCl buffer system (pH 8.5). In the latter case the factor is first isolated as a complex with its binding protein and then this complex dissociated to obtain EGF (unpublished). The presence of EGF was determined by radioimmunoassay.

Results

Mouse EGF, prepared as described in Materials and Methods, was purified on an analytical RP-8 column. Usually 4-5 mg of material were injected. Shallow gradients (0.14% n-propanol increase/ minute) and low flow rates (220 μl/ minute) gave best separations. The chromatography revealed the presence of a variety of additional polypeptides present in these preparations usually used for biological studies (Figure 1). Interestingly, all of the EGF samples (prepared by either of the two methods described above) contain two major components in approximately the same quantities. Both of these proteins possess EGF immunoreactivity and are indistinguishable on SDS-polyacrylamide or on IEF gels. The presence of these components on HPLC did not depend on the age of the growth factor preparation. Chymotryptic mapping of these two molecules reveals differences in their peptide fragments whose chemical characterization is under way.

Up to 20 mg of a mouse EGF preparation can be loaded on an analytical column without exceeding its capacity. Less than 0.001% of the EGF immunoreactivity is eluted from the column within 30 minutes after injection in the absence of organic modifier from the mobile phase. Nearly 100% of the immunoreactivity is recovered after the run. The presence of peptides with similar retention times leads to broadening of the peaks so that for quantities larger than 4-5 mg the use of semipreparative columns (25 X 1.0 cm) is recommended.

Conclusions

Reverse phase HPLC is a useful tool for the final purification of natural proteins like the epidermal growth factor. Quantities up to 4-5 mg can be loaded to analytical columns without loss of resolution or

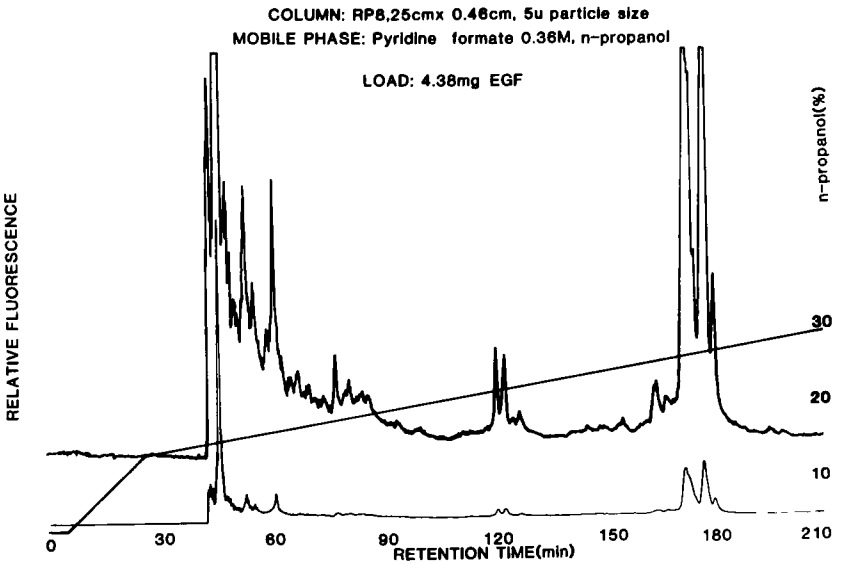

Fig. 1. Chromatography of mouse epidermal growth factor (flow rate 220 μl/minute, pH 3.0).

exceeding the capacity. This allows the detection of "contaminations" present in small quantities. In addition, the high resolution achieved reveals the presence of structurally related molecules, which are not detectable with other methods (SDS-PAGE and IEF). For larger quantities semipreparative columns are recommended. Best results were obtained with stationary phases of 5μ particle size and octyl group coating at low flow rates (10-15 ml/h).

Acknowledgements

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COMPLETE PURIFICATION OF PITUITARY PEPTIDES USING REVERSED-PHASE HPLC ALONE

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Previous studies from this laboratory have demonstrated how the combination of an octadecylsilyl-silica (ODS—silica) extraction procedure[1,2,3] and reversed-phase high performance liquid chromatography (RP-HPLC) can provide a means of rapidly purifying tissue peptides without the need for conventional ion-exchange or gel-filtration chromatography.[4] However, the use of only one principle of chromatography invites the criticism that the purity of isolated peptides is not ensured. Furthermore the choice of solvent composition in the final isocratic RP-HPLC step was frequently difficult and the peptides of interest were eluted in relatively large volumes. To circumvert these problems, we undertook to develop means of altering the selectivity of the reversed-phase column and thereby introduce a different chromatographic principle into the purification scheme. Final purification would then be achieved by gradient RP-HPLC and peptides would be eluted in small solvent volumes.

We have recently compared the properties of perfluorinated carboxylic acids as hydrophobic ion-pairing reagents.[5] Of the acids examined heptafluorobutyric (HFBA) had the most useful properties. Like its homolog trifluoroacetic acid (TFA), HFBA is a strong acid and completely volatile. HFBA was observed to be a stronger hydrophobic ion-pairing reagent than TFA. With all other variables constant (i.e. reversed-phase column, organic solvent, elution gradient), it could be seen that all test peptides had greater retention times in a system containing 0.1 M HFBA throughout than one containing 0.1 M TFA.[5] This effect was related to the number of basic amino acids within each peptide. Thus it is possible to alter the retention times of peptides simply by exchanging HFBA for TFA in the RP-HPLC solvent system. In this fashion, the elements of cation exchange chromatography can be introduced into a RP-HPLC purification scheme.

The usefulness of this technique is illustrated by the purification of the major form of immunoreactive α-melanotropin (α-MSH) from the neurointermediary lobe (NIL) of the rat pituitary. 190 rat NILs were extracted with an acidic medium designed to maximize peptide solubili-

zation and minimize peptidase activity.[1,6] This initial extract was in turn extracted with ODS—silica (C_{18} Sep-Pak, Waters) in a batch procedure. ODS—silica has a low capacity for salts and high molecular weight proteins and a relatively high affinity for peptides. The ODS-silica eluate, now largely salt and protein free, was loaded onto the reversed-phase column as described previously.[4,6] The column was eluted as described in the legend to Figure 1. The main peak of immunoreactive α-MSH (Figure 1) was loaded back onto the same column which was eluted as described in the legend to Figure 2. The original peak resolved into seven

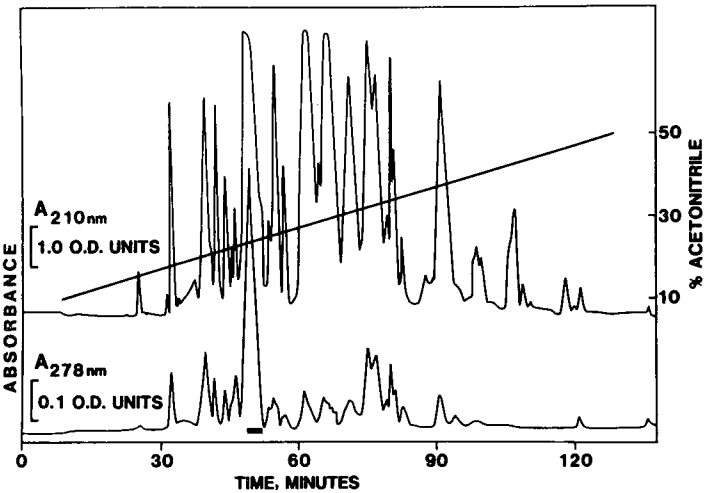

Fig. 1. RP-HPLC of an extract of 190 rat NILs. The column (C_{18} μBondapak, Waters) was eluted over two hours with a linear gradient of 10-50% acetonitrile containing 0.1% TFA. The solvent was delivered at 1.5 ml per minute using a Waters HPLC system and UV absorbance monitored continuously at 278 nm and 210 nm. The main peak of immunoreactive α-MSH eluted between 50 and 52 minutes (marked with a bar) and this material was subjected to further RP-HPLC on the same column.

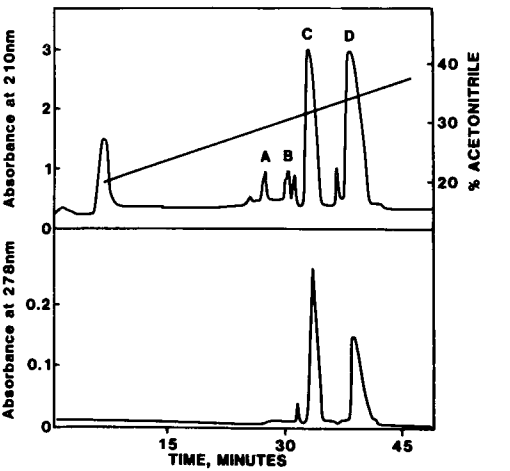

Fig. 2. RP-HPLC of the material eluting between 50 and 52 minutes in Figure 1. The column was eluted over one hour with a linear gradient 20 to 45% acetonitrile containing 0.13% HFBA. Other conditions as for Figure 1.

components in the new solvent system (Figure 2). The materials contained in Peaks A, B, C and D were identified by amino acid analysis and appeared to be homogeneous. Immunoreactive α-MSH was found to correspond to peak C. Structural elucidation showed this peptide to be N,O-diacetyl-Ser_1-α-MSH.[6] The other major component (peak D) was found to correspond in amino acid composition to rat γ-LPH.[7] While these two peptides co-elute in the TFA solvent system, γ-LPH elutes considerably later in the HFBA system. This behavior is due largely to the presence of seven charged amino groups in rat γ-LPH compared to only three in the α-MSH. Peaks A and B were found to contain phosphorylated-glycosylated $ACTH_{18-39}$ and glycosylated $ACTH_{18-39}$ respectively.[8] The resolution of these two peptides is due mainly to the polar phosphate group which causes peak A to emerge first. In this instance there are an equal number of charged amino groups in the two peptides available for ion-pairing (i.e. three). However, we have found that HFBA also enhances separation due to simple polarity differences when compared with TFA.

The combination of the two solvent systems permits the purification of every component in a complex mixture such as a pituitary extract even when, as in this case, the column has been overloaded. From the extract of 190 rat NILs, we have identified the following peptides by amino acid analysis and peptide mapping: oxytocin, vasopressin, several neurophysins, des-acetyl, mono-acetyl- and di-acetyl-α-MSH, γ_3-MSH,[9] γ-LPH, eight forms of corticotropin-like itermediary lobe peptide,[8] corticotropin (phosphorylated and non-phosphorylated) and α-, β-, γ- and δ-endorphin.

The availability of HPLC protein analysis columns has provided a means of confirming the purity of isolated peptides. They also permit the estimation of the molecular weight of peptides of unknown structure. We have used the Waters I-125 column for this purpose (eluted with 32% aqueous acetonitrile containing 0.1% TFA at a flow rate of 1 ml per minute). We have observed an excellent correlation between the elution position and the log molecular weight of a range of synthetic and natural peptides (effective range 1,000 to 15,000 Daltons). Using UV monitoring of the column eluate at 210 nm it is possible to obtain a molecular weight estimate on less than 200 ng. This technique has confirmed the high degree of purity of our isolated peptides and in many instances provided valuable molecular weight information.

Acknowledgements

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MECHANISM OF ACTION OF PARATHYROID HORMONE

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Introduction

Parathyroid hormone (PTH) has a critical role in the physiology of calcium homeostasis: through its principal actions on kidney and bone (and indirect effects on intestine), PTH is responsible for the minute-to-minute regulation of calcium levels in blood and extracellular fluid. PTH interacts with receptors on the plasma membrane of target tissue cells. This interaction initiates a cascade of intracellular events: generation of cAMP, phosphorylation of intracellular proteins by PTH-activated kinases, intracellular entry of calcium, and activation of enzymes which ultimately contribute to or ellicit a full spectrum of ensuing metabolic consequences.

PTH-Receptor Interactions

Structure-activity studies of PTH have revealed a great deal concerning the initial step in hormonal action, interaction with receptors. The principal secreted form of PTH contains 84 amino acids. The NH_2-terminal one-third (positions 1-34) contains all the structural requirements necessary for full bioactivity in multiple assay systems and binding of bPTH-(1-34) to renal membranes is equal, on a molar basis, to binding of bPTH-(1-84).[1] No function has yet been established for the remaining (COOH-terminal) portion of the molecule (positions 35-84).

The active region of PTH can be separated into functional domains: a region primarily responsible for receptor-binding and a small but distinct region responsible for hormone action once receptor binding has occurred. Deletion of positions 1 and 2 causes complete loss of biopotency, yet receptor binding properties are retained.[1] This delineation of function has permitted design of potent *in vitro* inhibitors of PTH.[2,3]

Studies directed at determining the principal binding domain for PTH have identified at least two relatively small (10 amino acids or smaller) regions of the molecule. The region 25-34 is, in relative terms, the sequence containing the principal structural determinants of receptor interaction.[4]

Photoaffinity Radiolabeling of the PTH-Receptor

Much has been learned about the PTH-receptor from structure-activity studies. However, this knowledge is limited to presumptions regarding complementary groups in the receptor whose presence is inferred. Recently, we[5] and others[6] have covalently labeled the PTH receptor using a biologically active, radiolabeled, photolabile PTH analog. ^{125}I-labeled [Nle-8, Nle-18, Tyr-34]-bPTH(1-34)amide was conjugated in darkness through lysine side-chains to photolabile aromatic azides: 4-fluoro-3-nitrophenyl azide (FNPA) or N-succinimidyl-6 (4′-azido-2′-nitrophenyl amino) hexanoate (SMHA). Incubation in darkness of the analog with canine renal membranes, followed by exposure to light, revealed specific labeling of a membrane component (M.W. = 70,000) representing either the PTH—receptor or a binding subunit (Figure 1). These studies should facilitae harvesting and eventual further characterization of the PTH receptor.

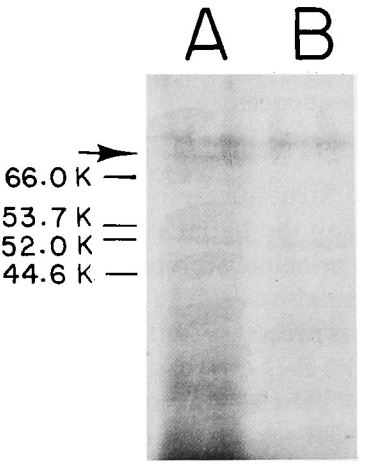

Fig. 1. Radioautograph of SMHA-reacted-^{125}I-[Nle-8, Nle-18, Tyr-34]bPTH-(1-34)amide photoaffinity-labeled canine renal membranes after solubilization, reduction, and SDS-polyacrylamide gel electrophoresis. In lane A, the photolabile radioligand was added alone; several membrane components were visualized. Lane B depicts marked reduction in labeling of a single band (arrow) when photolysis was performed after pre-incubation of membranes with 10 μg of PTH agonist, [Nle-8, Nle-18, Tyr-34] bPTH-(1-34)amide. Indicated at left are migration positions of proteins of known molecular weight.

Post Receptor Events

PTH action in kidney has long been associated with the concept of cAMP as a second messenger.[7] Generation of cAMP is followed by activation of cAMP-dependent kinases. Ausiello *et al.*[9] have demonstrated increased activity of cAMP-dependent kinase activity in monolayer cell cultures of human giant cell tumors of bone exposed to PTH. Phosphokinase activity is completely abolished when PTH is added in the presence of a 3-fold molar excess of a PTH inhibitor. Three endogenous substrates were found for the phosphokinase: phosphoproteins of M.W. = 55,000, 43,000 and 38,000. Dephosphorylation of proteins of M.W. = 200,000 and 120,000 also occurred. These studies indicate that

PTH action may be mediated by the phosphorylation and dephosphorylation of specific substrates.

Calcium may also serve as an intracellular messenger for PTH action. Levels of intracellular calcium in bone cells rise rapidly after PTH-stimulation. The rise follows a time course similar to that observed for cAMP, but appears independent of cAMP. Increasing intracellular calcium levels results in RNA synthesis and release of lysozomal enzymes associated with bone resorption. Calmodulin may be activated by calcium and might then regulate activity of a number of intracellular enzymes. Calmodulin may also act to stimulate adenylate cyclase independent of the guanyl nucleotide regulatory subunit.

Finally, target tissue may modulate its responsiveness to PTH. Prolonged or repeated exposure to hormone may produce desensitization. Monolayer cell cultures derived from human giant cell tumors and human fibroblasts respond to PTH by increasing intra- and extracellular levels of cAMP. However, after a few hours, subsequent exposure to PTH produces a diminished cAMP response. By 12-36 hours, the cells are completely refractory.[9]

This phenomenon does not result from simple or irreversible occupancy of receptors by PTH since cells washed after exposure to PTH are fully responsive to the hormone if challenged within minutes to one hour of initial exposure. Secondly, receptor occupancy alone is not sufficient to cause desensitization. The PTH inhibitor, [Nle-8, Nle-18, Tyr-34]bPTH-(3-34)amide, when added in the presence of bPTH-(1-84), can inhibit completely the cAMP response to hormone, yet it fails to elicit the desensitization response. It appears likely that generation of cAMP or other post-receptor events are required for desensitization.

Numerous PTH analogs have been used in studies of receptor interaction, intercellular metabolism, and regulation of mineral-ion transport. Extension of these investigations will yield a more sophisticated understanding of the mechanisms of peptide hormone action. In such studies lies the promise of generating highly refined and perhaps clinically useful analogs of PTH.

Acknowledgements

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HORMONE-REGULATED PHOSPHORYLATION OF ATP-CITRATE LYASE

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Introduction

By the early 1970's, it was possible to describe the glucagon activation of hepatic glycogenolysis as a cascade of reactions that start with the glucagon-receptor interaction, and conclude with the hydrolysis of glycogen to glucose-1-PO_4 mediated by phosphorylase a. The identity of the key intermediate steps were provided by the discovery of cAMP and the adenylate cyclase reaction by Sutherland and his colleagues,[1] and the subsequent discovery of the cAMP-dependent protein kinase by Walsh, Perkins and Krebs.[2]

It quickly became clear that the significance of these two discoveries extended far beyond glucagon-mediated glycogenolysis. The realization that numerous different hormone receptors were coupled to adenylate cyclase indicated that cAMP mediated actions of many hormones.[3] In addition, as the list of physiologic substrates for the cAMP-dependent protein kinase enlarged, it became clear that the broad substrate specificity of this enzyme permitted the simultaneous covalent modification of a large number of regulatory enzymes and other protein substrates of unrelated function; thus, this single protein kinase reaction underlies the so-called "pleiotypic" cellular response.[4]

cAMP Independent Hormones

A variety of peptide hormones alter cell function without elevation of cellular cAMP levels. In attempting to understand the mechanisms underlying these hormonal actions, several features of the "cAMP" pathway have greatly influenced the investigative approach:

1) hormone action initiated by a cell surface receptor without a requirement for internalization;
2) pronounced amplification of the extracellular signal via the generation of an intracellular messenger molecule at the cell surface;
3) a series of altered ligand-protein and protein-protein interactions

initiated by the increased intracellular level of the messenger molecule; 4) the ubiquitous role of covalent protein modification (especially phosphorylation).

In recent years, substantial insight has been gained into the actions of hormones which utilize calcium as the messenger molecule. Calcium, recruited from the extracellular space or some intracellular site of sequestration, binds to calmodulin, and the calcium-calmodulin complex modifies cell function by interacting with a hierarchy of intracellular proteins.[5] While many of the general features of the cAMP pathway are applicable to the calcium pathway, interesting differences have emerged. For example, in contrast to cAMP, where altered protein phosphorylation appears to be an obligatory intermediate step in all actions of the nucleotide in eucaryotic cells, altered phosphorylation appears to mediate only a subset of calcium-calmodulin actions (*e.g.* via the activation of myosin light chain kinase). Overall, however, the conceptual framework provided by the cAMP pathway has served quite well in extending our understanding of certain cAMP-independent, calcium-mediated actions of vasopressin, angiotensin II and the alpha adrenergic agents.

An Approach to Insulin Action

By contrast, although much is known concerning the interaction of insulin with its cell surface receptor, progress in detailing the subsequent signalling events has proceeded inchmeal. While insulin can alter cAMP metabolism and transcellular calcium fluxes, there is no compelling evidence for either of these molecules as a primary intracellular signal (*i.e.* second messenger) for insulin.[6] Insulin action at the cellular level, however, is clearly analogous to actions of hormones which recruit cAMP and/or calcium in at least one respect: several of the metabolic actions of insulin, *e.g.*, stimulation of glycogen synthase and pyruvate dehydrogenase, are due to alterations in protein phosphorylation. Moreover, when insulin is added as the sole hormone to ^{32}P-labelled target cells, the hormone rapidly induces a unique pattern of altered protein phosphorylation, with examples of both increased and decreased ^{32}P incorporation into specific ^{32}P-peptides as separated on detergent gel electrophoresis.[7, 8] Given the central role of stimulated protein phosphorylation in the ultimate expression of glucagon and vasopressin action, we have pursued an experimental strategy aimed at the identification of major insulin-stimulated phosphopeptides in target cells, and the identification and characterization of the protein kinases/phosphatases mediating these alterations, with the goal of understanding the insulin regulation of these interconverting enzymes.

ATP-Citrate Lyase: A Reporter Molecule for Hormone-Stimulated Phosphorylation

Such a program has been developed most completely in the case of a cytosolic phosphoprotein of subunit molecular weight 123,000 daltons, which undergoes enhanced phosphorylation when ^{32}P-labelled hepatocytes or adipocytes are exposed to either insulin, glucagon or vasopressin. This protein was subsequently purified from hepatocytes and identified as the subunit of ATP-citrate lyase, the first cytosolic enzyme of lipid biosynthesis in non-ruminants.[9] Incubation of isolated hepatocytes with insulin or insulin infusion into intact rats results in an increase in the net content of alkali-labile (serine) phosphate in the hepatic enzyme.[10,11] Only three general mechanisms could explain this effect of insulin, *i.e.* stimulation of a protein kinase, inhibition of a protein phosphatase or a hormonally directed but ligand-mediating modification of lyase which alters its properties as a substrate for kinase or phosphatase action. To date, our efforts have been directed toward the identification of the protein kinase mediating the insulin-stimulated phosphorylation of lyase so as to determine whether this kinase is regulated by insulin.

In order for a lyase kinase to be implicated in a hormone signalling cascade, it must fulfill at least two criteria: 1) its activity must increase with hormone addition, and 2) it must phosphorylate the same site on purified lyase *in vitro* as is phosphorylated *in vivo* after hormone addition. In the case of glucagon-directed lyase phosphorylation in intact hepatocytes, glucagon activates a lyase kinase in hepatocytes which is indistinguishable from the cAMP-dependent protein kinase.[7] Moreover, the site on lyase phosphorylated in isolated hepatocytes following glucagon addition is on the same tryptic peptide (Thr-Ala-Ser(P)-Phe-Ser-Glu-Ser-Arg) as that phosphorylated by purified cAMP-dependent protein kinase *in vitro*.[13]

Clearly, the putative insulin-directed kinase cannot be the cAMP-dependent protein kinase, since insulin action proceeds without increased cAMP levels or cAMP-dependent protein kinase activation.[8] In order to identify the specific cAMP-independent lyase kinase which mediates the insulin-stimulated phosphorylation, we have simultaneously undertaken the identification of the site(s) on lyase phosphorylated under insulin's influence *in vivo*, and the isolation of the lyase kinase from rat liver capable of phosphorylating these sites.

Our approach to the determination of the site of insulin-stimulated phosphorylation of lyase is as follows: ^{32}P-labelled hepatocytes, treated with and without insulin (7 nM x 10 min) were prepared. ^{32}P-lyase was

purified from both pools of cells, in parallel, under conditions previously shown to maintain the phosphorylation state. ^{32}P-lyase purified from insulin-treated cells contained 1.7 fold more ^{32}P radioactivity than control ^{32}P-lyase. Both preparations were briefly digested with trypsin, the phosphopeptides containing the phosphorylation site quantitatively released and separated from the core enzyme by precipitation of the latter with perchloric acid. Analytic HPLC peptide maps of the perchloric acid supernatant of digests of the control and insulin ^{32}P-lyase each revealed two main peaks eluting 29 and 34 mins after sample injection, respectively (Figure 1, panels A and B). These peaks have identical retention times to a pair of ^{32}P-labelled tryptic peptides (termed peptides A and B) derived from parallel tryptic digestion of lyase phosphorylated *in vitro* by cAMP-dependent protein kinase (Figure 1, panel C). Peptide B is a pentadecapeptide whose amino-terminal octapeptide is peptide A; peptide A contains three serines (vide supra and reference 13). Direct comparison of the elution pattern of the tryptic ^{32}P-phosphopeptides from the three sources shown in Figure 1 was carried out in mixing experiments: control, insulin and cAMP kinase-phosphorylated lyase were co-injected and ^{32}P-phosphopeptide separated under shallow gradient conditions, to magnify the resolution (Figure 2). Under circumstances where minor differences in elution would be easily detectable, the radioactive peptides derived from ^{32}P-lyase isolated from intact ^{32}P-labelled hepatocytes co-eluted with ^{32}P-peptides A and B derived from lyase phosphorylate *in vitro* with the cAMP-dependent protein kinase. This preliminary evidence of the close similarity, if not identity, between the ^{32}P-phosphopeptide from these various forms of lyase indicates that both glucagon (via cAMP-dependent protein kinase) and insulin may direct the phosphorylation of the same octapeptide domain on lyase. Precise comparison of the sites must await the unambiguous assignment of PO_4 to one or more of the three serines present in the peptide sequence (threonine is excluded as a phosphorylation site since no phosphothreonine is detected after acid hydrolysis of peptide).

Concomitantly, we have undertaken the isolation of the lyase kinases of rat liver, based on the premise that a knowledge of the site specificity and regulatory properties of these enzymes will permit the identification of the kinase mediating the insulin-stimulated phosphorylation of lyase. When rat liver cytosol is fractionated by salt gradient elution from a phosphocellulose column, five peaks of ATP-citrate lyase kinase activity are resolved. Two peaks have been identified as the cAMP-dependent protein kinase and its free catalytic subunit; a third

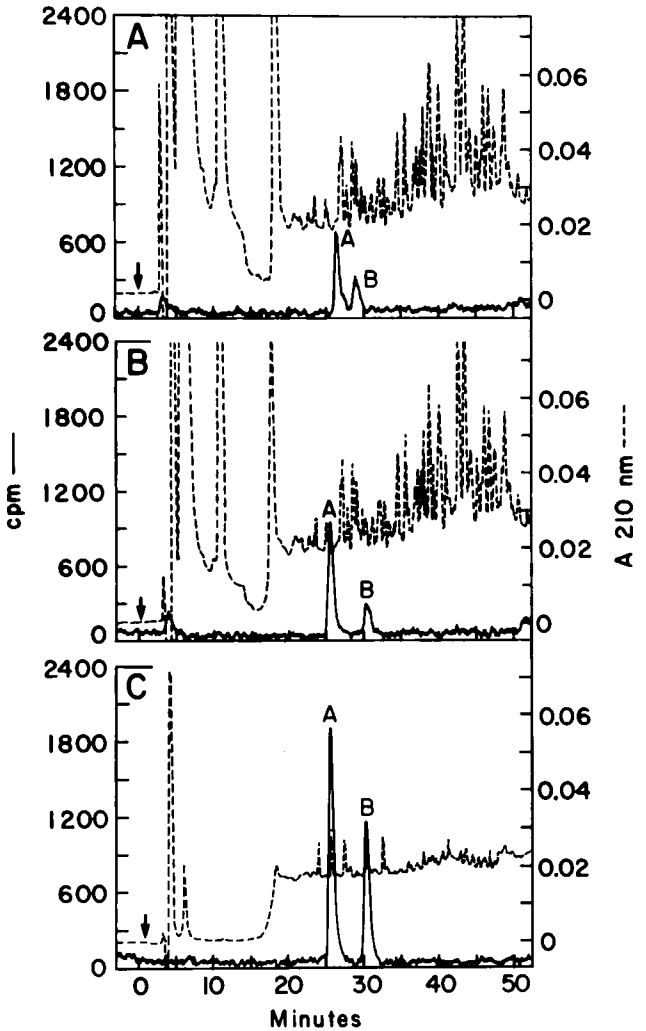

Fig. 1. HPLC tryptic map of ^{32}P-ATP-citrate lyase phosphorylated *in vivo* in ^{32}P-labelled hepatocytes incubated with or without insulin, or phosphorylated *in vitro* by the cAMP-dependent protein kinase.

^{32}P-ATP-citrate lyase, purified from control and insulin-treated ^{32}P-labelled hepatocytes, was digested by TPCK-treated trypsin 1% (w/w) in parallel with a sample of ^{32}P-lyase phosphorylated *in vitro* by the cAMP-dependent protein kinase. The ^{32}P-ATP-citrate lyase samples were digested in buffer consisting of 10 mM NaH_2PO_4, 1 mM DTT and 1 mM KEDTA, pH 7.5. Protein concentrations, specific radioactivity and length of tryptic digestion at 37°C in each case were as follows: control 0.57 mg/ml, 18,420 cpm/mg, 40 min; insulin .58 mg/ml, 31,534 cpm/mg, 40 min; cAMP kinase 10.0 mg/ml, 8,000 cpm/mg, 5 min. After precipitation of the indigested core enzyme with perchloric acid (3% w/w), over 90% of the ^{32}P was recovered in the supernatant in each case. Samples of this supernatant were injected into the chromatograph at time zero (arrow) after the column had

been equilibrated in 0.1% TFA and 5% (v/v) acetonitrile. The column (Altex Ultraphase ODS C_{18}, 0.466 x 25 cm) was perfused with the same solution for 5 min and then in all cases, the peptides were eluted in a linear gradient from 5 to 60% acetonitrile (1.0% acetonitrile/min) in 0.1% TFA. The sample and volume injected with each run are as follows: 0.15 ml control (panel A), 0.15 ml insulin (panel B) and 0.1 ml cAMP kinase (panel C). cpm = solid line; A_{210} nm = dotted line.

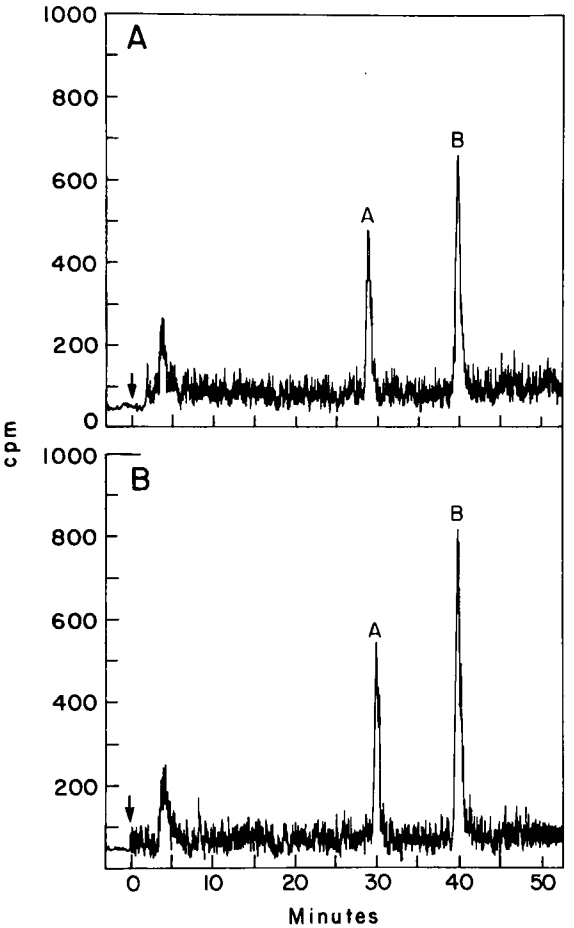

Fig. 2. HPLC tryptic maps of ^{32}P-ATP-citrate lyase: mixing experiments.

The samples come from the same tryptic digests described in the legend to Figure 1, except that control and insulin samples were obtained after only 10 min of tryptic digestion. After equilibration of the volume in 0.1% TFA and 5% acetonitrile (v/v), the samples were injected. Peptides were then eluted in a linear gradient from 5 to 20% acetonitrile (0.5% acetonitrile/min) in 0.1% trifluoroacetic acid. The sample and volume injected with each run are as follows: Panel A = 0.1 ml control lyase digest plus 0.1 ml insulin lyase digest; Panel B = 0.1 ml control plus 0.1 ml insulin plus 0.1 ml cAMP. cpm = solid line.

peak of lyase kinase is active only in the presence of calcium/calmodulin. As indicated above, these kinases are likely to mediate the glucagon- and vasopressin-induced phosphorylation of lyase observed in isolated hepatocytes, respectively. The remaining two peaks of lyase kinase activity are unaffected by calcium and cAMP, and each is capable of further stoichiometric phosphorylation of ATP-citrate lyase beyond the maximal extent catalyzed by the cAMP-dependent protein kinase. Thus, these kinases are our candidates for mediating the insulin-induced, cAMP-independent lyase phosphorylation. Of interest, both of these calcium and cAMP-independent protein kinases also phosphorylate acetyl-CoA carboxylase, another protein whose phosphorylation in intact liver cells is enhanced after insulin stimulation.[14] Thus, it will be of considerable interest to compare the site(s) on lyase phosphorylated by these kinases, with the site(s) on lyase phosphorylated *in vivo* after insulin stimulation. These studies are currently in progress.

Acknowledgements

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OXYTOCIN-DEPENDENT PHOSPHORYLATION OF MYOSIN LIGHT CHAIN IN MAMMARY MYOEPITHELIAL CELLS

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Introduction

The myoepithelial cells of the mammary gland contract in response to oxytocin and a limited number of other muscle stimulants. This is a potentially useful model for studying the mode of action of oxytocin and the phenomenon of excitation-contraction coupling. Information obtained from studies with oxytocin may also be useful in understanding the actions of other peptide hormones with amino acid sequences containing oxytocin-like loop structures.

Although little is known about the myoepithelial contractile system, it appears to resemble that of smooth muscle.[1] There is increasing evidence that Ca^{2+}-dependent phosphorylation of the 20,000-M_r light chain of smooth muscle myosin may be important in the regulation of contraction: this has been reviewed recently by Small and Sobieszek.[2]

We wish to report a method for monitoring the phosphorylation of the myosin light chain of isolated mammary myoepithelial cells in response to stimulation by oxytocin.

Methods

The litters were weaned from lactating rats, and on the 7th day of involution, the mammary glands were removed. Myoeptihelial cells were obtained by collagenase dispersion of the involuted mammary tissue. The isolated cells (1-2 x 10^6) were incubated with 25μCi [^{32}P]orthophosphate ($^{32}P_i$) in a preoxygenated physiological salt solution at 37° C for 60 min to label the intracellular nucleotide pools. The cells were then challenged with oxytocin, and the incubation was terminated by the addition of ice-cold trichloroacetic acid. Phosphorylated proteins were separated by electrophoresis on 15% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS-PAGE) by the method of Laemmli.[3] Two-dimensional isoelectric focusing/SDS-PAGE was performed according to O'Farrell.[4] The polyacrylamide gels were fixed, stained, and destained.[5] After drying, the gels were autoradiographed for 1-5 days and analyzed for ^{32}P incorporation into proteins.

Results and Discussion

The lactating mammary gland contains secretory cells and myoepithelial cells. After weaning, the mammary tissue involutes, resulting in the regression of the secretory cells and retention of the myoepithelial cells. The myoepithelial cells used in the following experiments were obtained from involuted mammary tissue. The cell membranes had binding sites for oxytocin with an apparent K_d of 4.6 nM, which is similar to the value reported for myoepithelial cells isolated from lactating mammary tissue.[6]

When the cells were incubated for 60 min with $^{32}P_i$, using conditions similar to those of the hormone binding assay, a number of myoepithelial cell proteins were labeled. Subsequent addition of oxytocin to the cells caused a marked increase in the level of phosphorylation of a 20,000-M_r protein (Figure 1a and b). This protein was identified as the myosin light chain after co-electrophoresis with ^{32}P-labeled rat mammary actomyosin[1] (Figure 1c and d). Two-dimensional PAGE confirmed that no other ^{32}P-labeled proteins co-migrated with the phosphorylated light chain and that oxytocin had no detectable effect on the level of phosphorylation of other ^{32}P-labeled proteins.

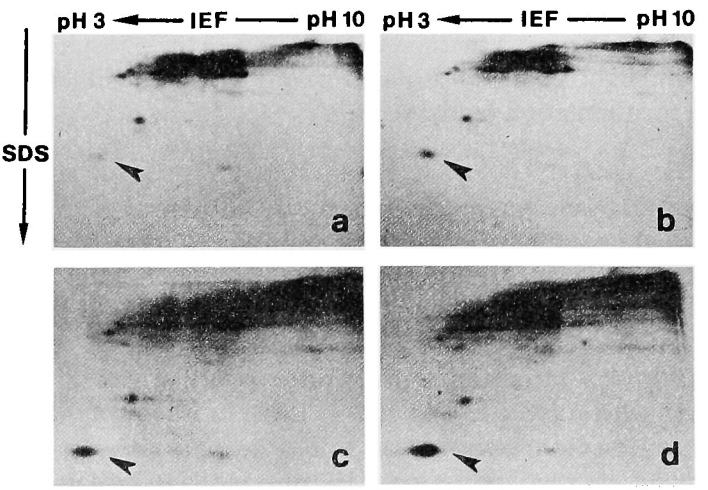

Fig. 1. Autoradiographs of ^{32}P-labeled proteins, separated by two-dimensional gel electrophoresis, from myoepithelial cells incubated in the absence (a) and presence (b) of 100 nM oxytocin for 3 min. Samples (c) and (d) were from cells incubated with 100 nM oxytoin for 3 min, but (d) was electrophoresed with ^{32}P-labeled rat mammary actomyosin. Arrows indicate the position of the 20,000-M_r light chain of myosin.

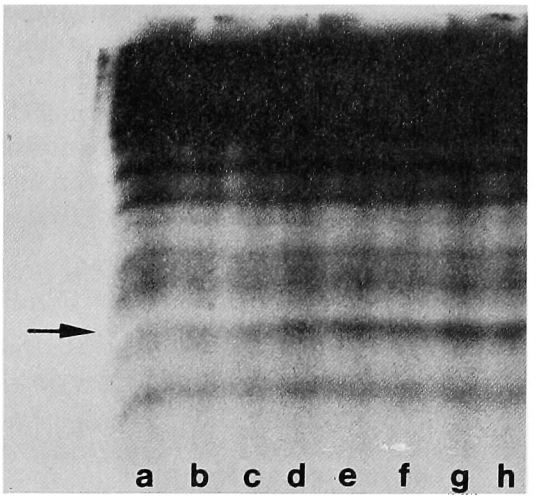

Fig. 2. Autoradiograph of ^{32}P-labeled proteins from myoepithelial cells incubated for 0.5 min with (a) 0 nM; (b) 1 nM; (c) 2 nM; (d) 4 nM; (e) 6 n M; (f) 8 nM; (g) 10 nM and (h) 100 nM oxytocin. Samples were electrophoresed on a 15% polyacrylamide gel containing 0.1% SDS. The arrow indicates the position of the 20,000-M_r light chain of myosin.

The concentration of oxytocin in the incubation medium affected both the rate and extent of myosin light chain phosphorylation. Figure 2 shows the electrophoretic pattern of ^{32}P-labeled proteins from myoepithelial cells stimulated with oxytocin. Oxytocin concentrations of 4 nM or more caused a dramatic increase in the incorporation of ^{32}P into the myosin light chain within 0.5 min. There was a similar increase in light chain phosphorylation when the cells were incubated for 3 min with 2 nM oxytocin. After the initial rapid phosphorylation of myosin, there was always a slower dephosphorylation to a basal level over a period of about 20 min.

Calcium is known to have an important role in smooth muscle contraction.[2] Chelation of Ca^{2+} in the incubation medium with ethylene glycol *bis*(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA) inhibited the usual response to oxytocin. There was, however, a transient phosphorylation of the light chain in response to 100 nM oxytocin within 0.5 min, which decayed to a basal level in 1.5 min. Re-addition of Ca^{2+} to the medium after 10 min resulted in an oxytocin-dependent phosphorylation of the myosin light chain. A similar transient response has been reported for the tension development of smooth muscle strips stimulated to contract in a Ca^{2+}-free medium.[7] Thus, the results show that the myosin light chain could undergo cyclic phosphorylation-dephosphoryla-

tion-rephosphorylation, and suggest that Ca^{2+} plays an important role in the intracellular events which follow stimulation by oxytocin.

In summary, we have developed a method for monitoring the cellular response of mammary myoepithelial cells to physiological concentrations of oxytocin by observing the level of phosphorylation of the 20,000-M_r myosin light chain. This system should prove useful in studying the mode of action of oxytocin and the cellular events involved in excitation-contraction coupling.

Acknowledgements

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STUDIES ON THE INTERNALIZATION OF PEPTIDE HORMONES *IN VIVO*

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Introduction

The view that peptide hormone receptors are almost exclusively cell surface structures has been discarded with the appreciation that peptide hormone receptors are highly concentrated in intracellular vesicles. We have used a combination of morphological and biochemical approaches to show that: (1) polypeptide hormone receptors are highly concentrated in rodent liver Golgi fractions; (2) Golgi receptors are derived from both intracellular production and internalization from the cell surface; (3) intracellular receptors exist in unique vesicles of intermediate density between Golgi and lysosomes; (4) peptide hormones are internalized and concentrated, in substantially intact form, in Golgi elements; and (5) internalization to Golgi elements is followed by the apparent transfer of radioactivity to unique vesicles - a sequence which might relate to the intracellular processing of peptide hormones.

The proof that polypeptide hormone receptors exist in Golgi elements and not in contaminating plasma membrane components is based on the observations summarized in Table I.

Table I. Evidence for Receptors in Golgi Elements[a]

1. Purified Golgi fractions bound hormones in a different pattern from purified plasma membrane.
2. Freeze-thawing enhanced hormone binding to Golgi but not plasma membrane.
3. Electron microscope radioautography demonstrated ^{125}I-labeled hormone bound to morphologically defined Golgi vesicles.
4. Golgi receptors were more stable than plasma membrane receptors on extended incubation at 30° C.
5. The pattern of regulation of Golgi and plasma membrane receptors has been found to differ for both insulin and prolactin.

[a] The observations upon which this summary is based can be found in references 1 to 7.

Unique Vesicles — The existence of polypeptide hormone receptors in other subcellular structures has been sought. The reports of insulin receptors in cell nuclei[9-11] have been unconfirmed in our own laboratories.[1,12] In view of many studies claiming internalization of peptide hormones to lysosomes[13] we searched for receptors in secondary lysosomes. The L fraction of rat liver was subfractioned by centrifugation on a discontinuous metrizamide gradient as described by Wattiaux *et al.*[14] The highest binding of peptide hormones was not found in the typical secondary lysosome subfractions as judged by both enzymatic analysis and electron microscopy. Rather, binding was maximal in a subfraction of lower density containing many VLDL-filled vesicles. Since this fraction contained low levels of the Golgi marker enzyme, galactosyl transferase, the vesicles could not be regarded as characteristic Golgi vesicles.

To test the contention that receptors are most enriched in structures distinct from classical secondary lysosomes the L fraction of rat liver was subfractionated on a continuous isoosmotic Percoll gradient (Figure 1).

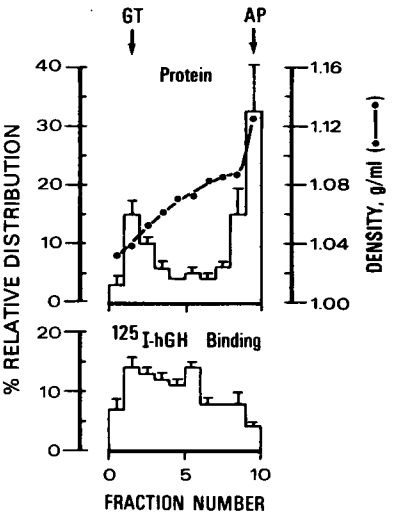

Fig. 1. The distribution of protein and ^{125}I-hGH binding after Percoll gradient centrifugation of the L fraction from female rat liver. The top panel depicts protein in each fraction as a per cent of the total protein recovered. The bottom panel depicts the distribution of ^{125}I-hGH binding sites calculated in the same way. The data are adapted from Khan *et al.*[20] wherein experimental details are described.

Galactosyl transferase activity (GT), the Golgi marker enzyme, and acid phosphatase activity (AP), the lysosomal marker enzyme, sedimented in the low (fraction 2) and high (fraction 10), regions of the gradient respectively. However, lactogen receptors (^{125}I-hGH binding sites) were recovered over a range of densities with a substantial amount found in the region of intermediate density (fractions 2 to 6 of the gradient). These data indicate that receptors are located not only in classical Golgi vesicles but also in unique vesicles which cosediment, at least in part, in the L

fraction. Current studies indicate that these vesicles contain VLDL, possess no galactosyl transferase, and have reduced levels (about 1/3) of acid phosphatase compared to secondary lysosomes. Unique vesicles also constitute a portion of the Golgi intermediate and heavy fractions.

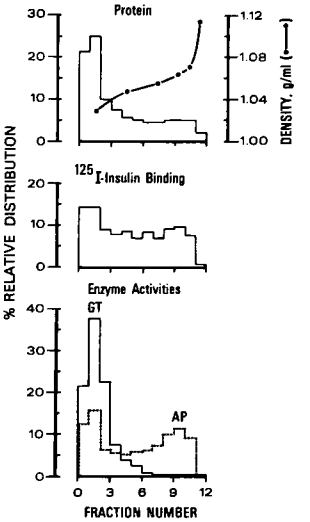

Fig. 2. The distribution of protein, ^{125}I-insulin binding, and enzyme activities after Percoll gradient centrifugation of the Golgi intermediate fraction from female rat liver. The top panel depicts protein in each fraction as a percent of total protein recovered. The middle panel depicts the distribution of ^{125}I-insulin binding sites, and the lower panel the distribution of galactosyl transferase (GT, ———) and acid phosphatase (AP, · · · ·) calculated as a percent of total recovered. Note the different gradient employed here as compared to the study illustrated in Figure 1.

Figure 2 depicts the distribution of protein, insulin binding sites, and enzyme activities obtained when the Golgi intermediate fraction was subjected to Percoll gradient centrifugation. The bulk of protein was in the low density, galactosyl transferase rich region of the gradient (top panel). ^{125}I-insulin binding sites were distributed across the gradient with a substantial fraction of the sites found in the higher density regions (middle panel). Galactosyl transferase (GT) activity was in the low density region of the gradient whereas acid phosphatase (AP) activity was found co-sedimenting with GT in the light region as well as separately in the high density region of the gradient. The latter peak corresponds to structures devoid of GT but containing insulin as well as lactogen (data not shown) receptors. These structures have been examined under electron microscopy and consist of VLDL-containing vesicles and not secondary lysosomes. In addition the specific activity of AP is about 1/3 of that seen in characteristic secondary lysosomes (M.N. Khan, B.I. Posner, R.J. Khan, and J.J.M. Bergeron, manuscript in preparation). They are thus comparable to the structures found cosedimenting in the L fraction and we have used the term 'unique vesicle'[20] to describe these entities for the time being.

Internalization of Peptide Hormones — We have combined morphological and biochemical techniques to study the problem of internalization of peptide hormones. Using *in vivo* radioautography we have mapped peptide hormone receptor distribution in various tissues,[15-17] and have demonstrated its powerful applicability in localizing hormone receptor-bearing cells within complex multicellular tissues such as brain.[18-19] With this approach it has been possible to study the time course of uptake and subcellular distribution of ^{125}I-insulin in rat liver.[20] In parallel the uptake of ^{125}I-labeled peptide hormones into various subcellular fractions of rat liver has been examined.[22,23] The two approaches give a consistent picture of internalization which is summarized in Table II.

Table II. Internalization of Peptide Hormones into Rat Liver *in Vivo*.

1. *Injected ^{125}I-insulin or ^{125}I-prolactin is rapidly concentrated in Golgi elements* as observed with *in vivo* radioautography and subcellular fractionation procedures. Radioautography of isolated Golgi fractions showed radiolabeled material intimately associated with morphologically characteristic Golgi vesicles.
2. *Uptake of ^{125}I-hormone is a receptor-mediated process.* Uptake was inhibited by coinjected unlabeled hormone in parallel with its biological activity. Uptake of ^{125}I-prolactin was greater in female than male rat liver in parallel with greater receptor content in the former.[24]
3. *Internalized radioactivity is relatively intact.* Radiolabel extracted from isolated Golgi fractions was chromatographed and bound to fresh hormone receptors.
4. *Uptake of ^{125}I-hormone into secondary lysosomes is low.* Radioactivity concentration in secondary lysosomes was $\leqslant 10\%$ that in Golgi fractions. This proportion was not affected by chloroquine treatment.[8]

Sequence of Hormone Uptake into Vacuolar Components — We have studied the time course of uptake into the different components of the Golgi fractions. As noted above the Golgi intermediate fraction contains both Golgi and unique vesicles. ^{125}I-insulin was injected and the Golgi intermediate fraction isolated and further fractionated on a Percoll gradient at the times noted in Figure 3. Radiolabel first appeared in the light and later in the heavier regions of the gradient. This suggests that hormone was first internalized to the lighter, GT-enriched, Golgi vesicles

and was later transferred to the heavier unique vesicles. A similar sequence has been defined for prolactin (R.J. Khan, M.N. Khan, B.I. Posner, and J.J.M. Bergeron, manuscript in preparation). The sequential transfer of internalized hormone first to Golgi and then to unique vesicles probably reflects a central pathway of intracellular processing. A scheme depicting the flow of hormone-receptor complexes in relation to vesicle 'traffic' is depicted in Figure 4.

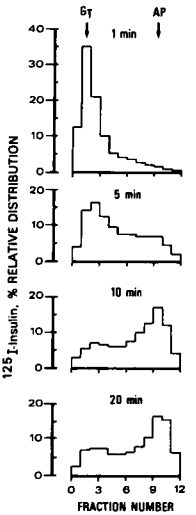

Fig. 3. The distribution of radioactivity as a function of time after ^{125}I-insulin in a Golgi intermediate fraction subfractionated on an isopycnic Percoll gradient. The gradient was identical to that in Figure 2. The positions of the two marker enzyme peaks, acid phosphatase (AP) and a galactosyl transferase (GT) are indicated in the top panel. The radioactivity per fraction is represented as a percent of the total radioactivity recovered. Data are from Khan *et al.*[27]

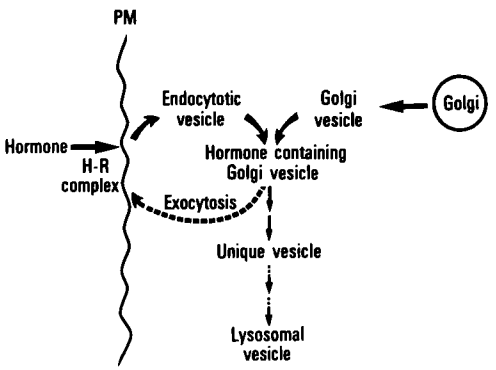

Fig. 4. A probable scheme of vesicle and hormone-receptor (H-R) complex flow. The interrupted lines refer to less well-established routes. PM denotes plasma membrane.

Significance of Intracellular Receptors and Internalization — Intracellular Golgi receptors appear to arise from two sources. They are produced within the cell as suggested by our studies on lactogen receptor induction.[5] In addition, they probably derive in part from the internalization of surface receptors. These observations are compatible with a receptor cycle in which new receptors appear in the Golgi and are transferred to the plasma membrane, perhaps during the process of exocytosis, and surface receptors are brought back to the Golgi during internalization. This sequence may involve reutilization of receptors, though not necessarily hormone. Down regulation of receptors is likely, in part, a consequence of the internalization of hormone-receptor complexes. The existence of receptors within Golgi vesicles is compatible with a biological function therein not dissimilar in principle from what has been observed in respect to the plasma membrane.

It is likely that internalization of hormones is linked to their degradation as suggested by Terris and Steiner.[25,26] We suggest however, that the rapid concentrative internalization of substantially intact polypeptide hormone, probably as hormone-receptor complexes, into characteristic Golgi vesicles, implies a role for these complexes in respect to Golgi function. The hormone-receptor complexes could couple to unique Golgi membrane effectors (*viz.* enzymes) and thus modulate their activity. Alternatively the internalization of hormone-receptor complexes may be a way of re-introducing membrane structures to the Golgi where they may be covalently modified (*viz.* by glycosylation) and subsequently redistributed elsewhere in the cell. This redistribution process need not be restricted to the ligand's receptor but could involve other membrane proteins which may interact with the hormone-receptor complex (*viz.* various effectors).

Acknowledgements

This work was supported by grants MT-4182 (BI. Posner) and MT-5605 (J.J.M. Bergeron) from the Medical Research Council of Canada, and by grant 1R01 AM 19573 from the U.S. Public Health Service. Drs. Posner and Bergeron are Chercheurs boursiers du Que'bec

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IN VIVO AND *IN VITRO* STUDIES WITH GLUCAGON INHIBITORS

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Introduction

Glucagon is a 29 amino acid peptide hormone which is important in glucose homeostasis, stimulating glycogenolysis in the liver and gluconeogenesis in the liver and other tissues. Its role in diabetes is controversial, [1,2] but in view of the critical importance of glucose in maintaining life, it is difficult to imagine that only a single hormone, insulin, would be important. In an effort to better understand the role of glucagon in normal and diabetic states, for sometime we have been investigating appraoches to the development of an *in vivo* glucagon inhibitor (antagonist).[3,4] During that time we obtained several partial agonists[3-6] and investigated their conformational properties. These results led us to postulate that a change in the spatial relationship of the N-terminal and central regions of glucagon might lead to antagonist analogs. During the past two years we have utilized this approach to develop four glucagon inhibitors,[5,6] at least three of which have no intrinsic agonist activity in the *in vitro* hepatic adenylate cyclase system. In this paper, we report some of the *in vitro* and *in vivo* activities of these antagonists. Of particular importance is our finding that the most potent inhibitor, [1-N^{α}-trinitrophenylhistidine, 12-homoarginine]-glucagon lowers blood glucose levels in diabetic animals 40-60% *in vivo*. These results indicate that glucagon antagonists may be useful agents in the treatment of diabetes.

Results and Discussion

All of the glucagon inhibitors and partial agonists reported in this paper were prepared by semisynthetic chemical methods. As with most chemical reactions, quantitative yields were not obtained. Thus, it was important to develop highly effective purification methods. Indeed this turned out to be the most difficult and critical aspect of this research, and it was only after these methods had been developed[5,6] that the partial agonist or antagonist activities of several of these analogs were recognized.

The standard assay which we have used to evaluate the biological activities of our glucagon analogs has been the liver plasma membrane adenylate cyclase system.[7] In this assay system [1-N^{α}-carbamoylhistidine, 12-N^{ϵ}-trinitrophenyllysine]-glucagon (1), [1-des-histidine][12-N^{ϵ}-phenylthiocarbamoyllysine]-glucagon (2), [1-des-histidine] [2-N^{α}-trinitrophenylserine, 12-homoarginine]-glucagon (3), and [1-N^{α}-trinitrophenylhistidine, 12-homoarginine]-glucagon (4) had no agonist activity. However, they competitively blocked the action of glucagon. The pA_2 values for these glucagon inhibitors are given in Table I. These results indicate that effective antagonists are obtained by modification at the N-terminal region of glucagon (removal of His^1 and/or addition of a bulky lipophilic group) *and* at the ϵ-amino group of Lys^{12} by increased steric size utilizing either bulky lipophilic groups (**1,2**) or a guanidyl group (**3,4**).

Table I. pA_2 Values of Glucagon Antagonists in Hepatic Adenylate Cyclase Assay.

Compound	pA_2[a]
1. [N^{α}-CAR-His^1, N^{ϵ}-TNP-Lys^{12}]-Glucagon	6.42
2. [des-His^1][N^{ϵ}-PTC-Lys^{12}]-Glucagon	7.15
3. [des-His^1][N^{α}-TNP-Ser^2, $HArg^{12}$]-Glucagon	7.41
4. [N^{α}-TNP-His^1, $HArg^{12}$]-Glucagon	8.16

[a] pA_2 = Negative log to the base 10 of the average molar concentration of an antagonist that will reduce the response of 2X units of agonist to X units of agonist.

We next investigated the *in vitro* binding of the inhibitors to hepatic membrane receptors using either displacement of radiolabeled glucagon by antagonist or radiolabeled antagonist as a measure of binding activity. GTP has been known for sometime to decrease glucagon binding to hepatic receptors. However, we have found that GTP does not significantly affect the binding of the inhibitor [N^{α}-TNP-His^1, $HArg^{12}$]-(**4**) to this glucagon receptor. On the other hand, Mn^{+2} and Mg^{+2}, both of which have *little effect* on glucagon binding, substantially *increase* binding of the antagonists to the liver plasma membrane receptors for glucagon. Moreover, pyrophosphate which *increases* glucagon agonist binding, has very little effect on glucagon antagonist binding. These results suggest that glucagon agonist and antagonists have different binding modes to liver membrane receptors.

In view of the very high *in vitro* antagonist and binding activities of [N^{α}-TNP-His^{1}, $HArg^{12}$]-glucagon (**4**) , we decided to investigate the ability of this inhibitor to lower blood glucose levels *in vivo*. We first examined its ability to lower blood glucose levels in streptozotocin-treated diabetic rats. A 0.5 mg/kg bolus of the inhibitor in saline has a substantial effect on blood glucose levels, decreasing them by about 25-30% within five minutes. Blood glucose levels returned to baseline levels within 20-25 minutes. In subsequent experiments we examined the effects of a continuous but low level infusion of the glucagon antagonist **4** into the animal. The results of a 60 minute infusion are shown in Figure 1. As can be seen, the results are dramatic. Blood glucose levels were reduced 40-60% below the basal glucose levels in the diabetic animals, and the effect persisted for sometime after infusion was stopped. These results indicate that glucagon plays a significant role in the hyperglycemia of streptozotocin-induced diabetic rats. It further suggests that the glucagon antagonists may be useful agents in the treatment of uncontrolled diabetes.

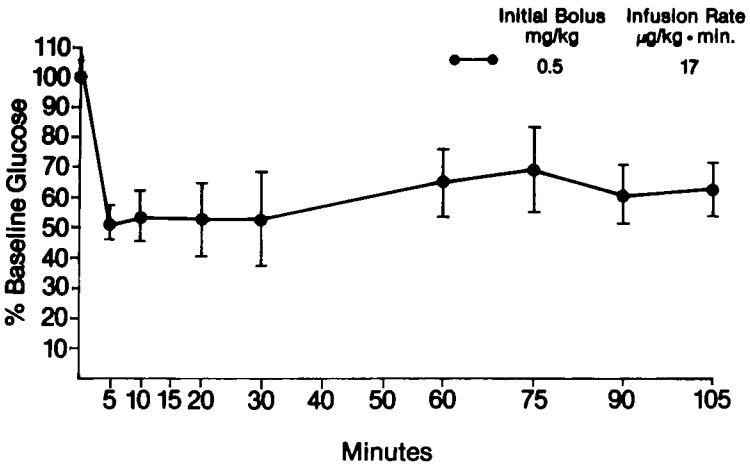

Fig. 1. The effect of glucagon antagonist [N^{α}-TNP-His^{1}, $HArg^{12}$]-glucagon on blood glucose of streptozotocin-diabetic rats. Each animal received 0.5 mg/kg of antagonist followed by continuous infusion of 0.017 mg/kg ·min antagonist for 60 min. Glucose is expressed as % time zero values. Results are means ± SEM for three rats.

Acknowledgements

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BIOLOGICALLY ACTIVE CONJUGATES OF ACTH AND CYTOTOXIC DRUGS: PROPERTIES OF ACTH ANALOGS CONTAINING DAUNORUBICIN

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Introduction

Successful site-mediated chemotherapy, which may increase drug effectiveness while decreasing toxicity, requires a carrier with selective affinity for receptor sites in tumor tissue and a means for attaching the carrier to a cytotoxic drug without loss of biological activity. The anthracycline daunorubicin has been successfully coupled to high molecular weight carriers.[1]

$ACTH_{1-24}$ has been a diffucult hormone to modify with retention of receptor affinity. The N-terminal amino acids are important in eliciting a steroidogenic response, while the basic residues in positions 11-18 are essential for receptor binding. Modifications near the C-terminus are less critical.[2] We have synthesized derivatives of $ACTH_{1-24}$, replacing tyrosine^{23} with cysteine, in order to obtain analogs containing a selectively modifiable thiol. These analogs may prove useful for attachment of various ligands of interest. In this study, we have conjugated two (Cys^{23}) analogs to the amino sugar of daunorubicin[3] via a bifunctional cross-linking agent.

Methods

ACTH derivatives were synthesized by standard solid phase procedures. The acetamidomethyl (Acm) function was used for thiol protection. Cleavage from the resin and side chain deblocking exclusive of Acm, were carried out in anhydrous HF. The products were stored as the S-Acm derivatives and were deblocked as needed by treatment with mercuric acetate, followed by β-mercaptoethanol to generate the free thiol.

The procedure for the cross-linking of daunorubicin to the (ACTH peptides is illustrated in Figure 1.

Daunorubicin-HCl was reacted with 1.1-1.2 equivalents of *m*-maleimidobenzoyl N-hydroxysuccinimide ester (*m*-MBS) in the presence of 1.2 equivalents of triethylamine in CH_3CN 15-20 hours in the dark. The solvent was evaporated and the product was purified by

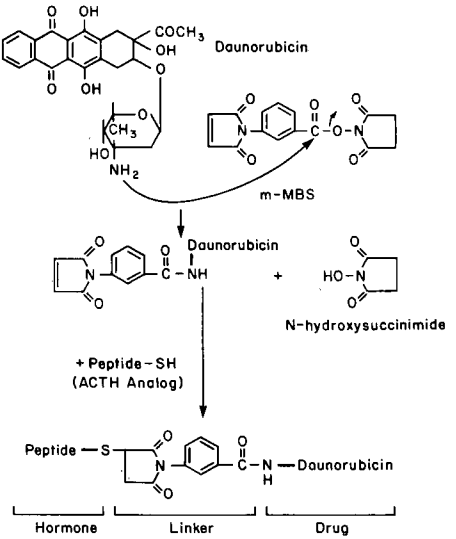


Fig. 1. Synthesis of Daunorubicin-Peptide conjugates using *m*-MBS

partition chromatography on Sephadex G-10 using butanol:acetic acid: water (4:1:5), upper and lower layers, as mobile and stationary phases. For conjugation to (Cys^{23}) ACTH$_{6-24}$ and (Nle^4, Cys^{23}) ACTH$_{1-24}$, the *m*-MBS—Daunorubicin was dissolved to 4mM in DMF and added in 10λ aliquots to an equimolar solution of the peptide in 0.5M phosphate buffer, pH 6.1. After the reaction was complete, 1 or 2 drops of glacial acetic acid were added to clarify the mixture, which was then diluted and lyophilized. The conjugated peptides were isolated by chromatography on Bio-Gel P-6 in 1N acetic acid.

Adenylate cyclase activation was measured by the conversion of ^{32}P-ATP to ^{32}P-cAMP by aliquots of rat adrenocortical membrane suspensions containing approximately 50 μg of protein.

Results

The (Cys^{23})ACTH peptides and their daunorubicin derivatives appeared to be homogenous by thin-layer chromatography and amino acid analysis. Performic acid oxidation and subsequent acid hydrolysis of the daunorubicin derivates yielded no cysteic acid, indicating that conjugation to *m*-MBS-daunorubicin was complete.

Figure 2 shows the ability of the derivatives to activate adenylate cyclase. (Nle^4, Cys^{23})ACTH$_{1-24}$ and Dauno-(Nle^4, Cys^{23})-ACTH$_{1-24}$ are partial agonists in this assay. Their ED$_{50}$ values are almost identical to that of ACTH$_{1-24}$, indicating that even in the analog substituted with a

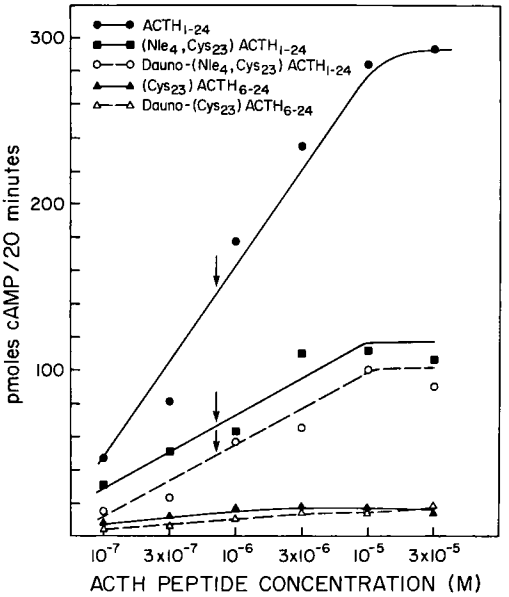


Fig. 2. Activation of adenylate cyclase by ACTH derivatives. Arrows indicate ED_{50} values.

bulky daunorubicin derivative at position 23, receptor affinity is retained. $(Cys^{23})ACTH_{6-24}$ and Dauno-$(Cys^{23})ACTH_{6-24}$, which lack amino acid residues believed to be important in eliciting a steroidogenic response, fail to activate the enzyme.

Figures 3 and 4 indicate that the daunorubicin-analogs can competitively antagonize $ACTH_{1-24}$.

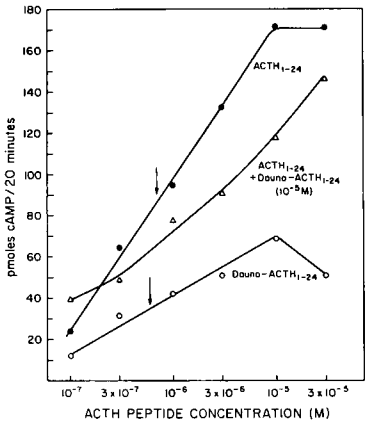
Experiments to study effects of the daunorubicin analogs on cultured Y-1 adrenal tumor cells are in progress. Early observations have indicated a slight, but significant, inhibition of cell growth by Dauno-$(Nle^4, Cys^{23})ACTH_{1-24}$ at 10^{-6}M.

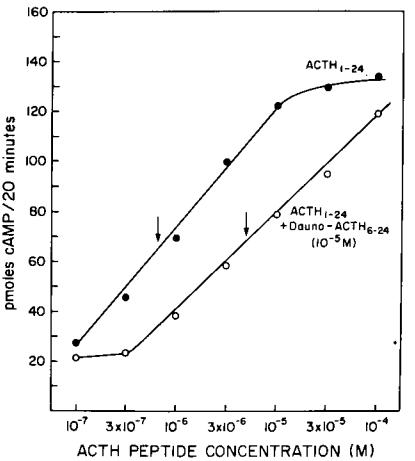
Conclusions

ACTH analogs containing cysteine in position 23 retain affinity for the receptor. The bifunctional reagent, *m*-MBS, can be used to couple daunorubicin to Cys^{23} in these peptides without loss of receptor binding. Specific cytotoxic effects of the daunorubicin-peptide conjugates remain to be determined.

Acknowledgements

We are indebted to Dr. George Babcock of Ives Laboratories for the gift of daunorubicin hydrochloride.





Figs. 3 & 4. Inhibition of $ACTH_{1-24}$ by Dauno-(Nle^4, Cys^{23})$ACTH_{1-24}$ and Dauno-(Cys^{23})-$ACTH_{6-24}$. Dauno-(Cys^{23})$ACTH_{6-24}$ has no agonist activity, but contains the basic amino acids essential for receptor binding. The *m*-MBS-daunorubicin derivative of glutathione has no effect on the dose-response curve of $ACTH_{1-24}$, implying that the antagonism seen with the ACTH derivatives is receptor-mediated.

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ON THE METABOLIC BREAKDOWN OF LHRH BY RENAL TISSUE

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Introduction

Previous studies indicate that the kidney is involved in the clearance and metabolism of Luteinizing Hormone-Releasing Hormone (LHRH) and therefore may participate in the regulation of plasma levels of this hormone.[1-3] We have demonstrated[4] that rat renal homogenates hydrolyze (pGlu-3,4-^{3}H) LHRH into pGlu (**1**) pGlu-His (**2**) pGlu-His-Trp (**3**) pGlu-His-Trp-Ser (**4**) pGlu-His-Trp-Ser-Tyr (**5**) and pGlu-His-Trp-Ser-Tyr-Gly (**6**).

However, attempts to establish metabolization of a peptide hormone in an organ by using whole organ homogenates is physiologically meaningless, since the enzymes which would normally be found in discrete cellular compartments are scrambled, dissolved or even destroyed during homogenization. We have combined *in vivo* and *in vitro* studies on the metabolization of (pGlu-3,4,-^{3}H) LHRH in order to attempt to elucidate the fate of this hormone in renal tissue. The analysis of breakdown products was performed by high performance liquid chromatography (HPLC) by comparison to known standards.[5]

***In vitro* Studies** — Rate studies on ^{3}H-LHRH metabolization by rat renal homogenate supernatants showed that incubation for a few minutes resulted in the formation of metabolites **1-6,** whereas incubation for prolonged time resulted in the formation of metabolites **1-4.** Hence, extensive incubation results in destruction of peptides **5** and **6,** and underscores the pitfalls in doing a metabolic study at a single enyzme concentration for an arbitrarily selected time period.

Microperfusion of isolated segments of rabbit proximal tubule with ^{3}H-LHRH showed that the collection fluid contained label corresponding to peptides **2-4** and to intact hormone, whereas the bathing medium contained metabolites **1-4**. There was very little sequestration of label in the tubule cells during the perfusion interval so that most of the reabsorbed labeled peptide moved across the epithelium.

Rate studies on tritiated hormone incubated with isolated rabbit renal microvilli membranes show that after 5 min metabolite **4** was the

product detectable in largest amount. After 10 min, peptides **3** and then **2** gradually increased in concentration over a 60 min period, during which the concentration of hormone and peptide **4** fell steadily. Hence, proximal tubular handling of LHRH appears to involve hydrolysis by luminal brush border enzymes, resulting in initial cleavage at position **4**, followed by carboxypeptidase type cleavage yielding peptides **3** and then **2**. In a subsequent step there would be a partial reabsorption of hormone and/or peptides **2, 3** and **4** followed by further intracellular hydrolysis which would yield metabolite **1** detected in the bathing medium. It is noteworthy that no intact hormone was found in the bathing medium even though the tritiated hormone was perfused in amounts exceeding physiological levels. This finding leads us to predict that once LHRH undergoes glomerular filtration it will not be reabsorbed intact, and thus will be irreversibly removed from the circulation. Furthermore, peptides **2, 3** and **4** and perhaps hormone should be the products of LHRH renal metabolic clearance and should be found in urine, their relative percentages depending largely on the length of the interaction with brush border enzymes.

The tubular processing of LHRH is unusual in that peptide metabolites are reabsorbed. Previous microperfusions of proximal straight renal tubule segments with small linear peptides resulted in the recovery of intact labeled hormone and the labeled amino acid in the collection fluid, and labeled amino acid but not peptides in the bathing medium. LHRH, with its unusual N-terminus is immune to attack by some proteases, which may account for the inability of the proximal tubule brush border to degrade it down to pGlu. However, our results clearly demonstrate that one can discriminate between the qualitative cleavage of the peptide hormone taking place at the luminal membrane and that taking place inside the cells of the epithelial lining of the tubule, thus revealing differences in enzymatic content at these histological sites.

***In vivo* Studies** — The microinfusion of ^{3}H-LHRH into rat proximal tubules *in vivo,* by the technique of Gottschalk *et al.*[6] resulted in the recovery of intact hormone and peptides **2, 3** and **4** in the urine, as predicted in our *in vitro* studies, whereas microinfusion into distal tubules resulted in the recovery of only intact hormone. These results agree with those obtained by microperfusion of isolated segments of rabbit proximal tubules or incubation of isolated rabbit renal brush border microvilli membranes. Collectively, these data suggest that partial hydrolysis of ^{3}H-LHRH to these peptide fragments takes place *in vivo* in the proximal tubule through contact digestion by brush border enzymes.

When (^{3}H) LHRH was presented to the renal artery of the rat kidney *in vivo,* peptides **2, 3** and **4** were found in the urine, but mainly metabolites **1** and **4** and trace amounts of peptides **2** and **3** were found in the renal venous blood. Arterial perfusion of ^{3}H-LHRH *in vivo* through the ureteral ligated, nonfiltering kidney of the rat, however, yielded only intact hormone in renal venous blood leading to the conclusion that the renal vasculature and interstitium do not detectably degrade LHRH. These findings provide strong support for the view that LHRH is filtered by the glomerulus and hydrolyzed by the proximal tubule, with the resulting metabolites undergoing either reabsorption or urinary loss. The findings of metabolites **1** and **4** in venous blood and traces of peptides **2** and **3** suggests that peptides **2-4** and possibly intact hormone are reabsorbed from the proximal tubular lumen and undergo further intracellular hydrolysis, perhaps by a pryoglutamase or by carboxypeptidase(s) in successive steps, to fragment **1**. The breakdown products, **1-4**, diffuse through the basalateral cell membranes and enter the venous circulation (Figure 1).

In view of the high rates for renal blood flow and glomerular filtration, and the great capacity of the proximal tubule for hydrolyzing LHRH, the kidney probably plays a dominant role in the clearance and metabolic degradation of this hormone, preventing its accumulation in the general circulation. Thus, patients with renal failure have slower metabolic clearance rates and prolonged disappearance half-times for LHRH compared to normal controls.[2] The renal clearance and degradation of LHRH would prevent the inappropriate LH and FSH release from the pituitary as well as extrapituitary actions of LHRH.

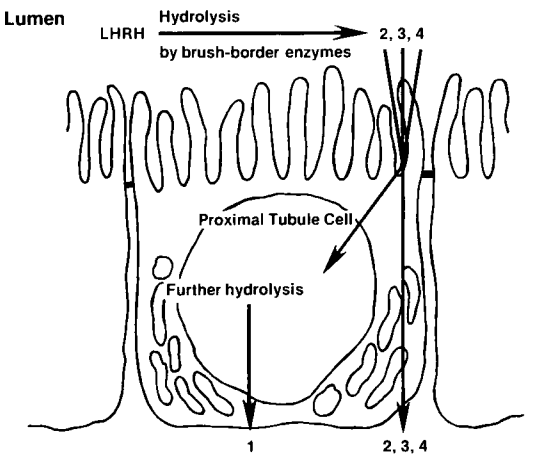

Fig. 1. Proposed breakdown products of LHRH in renal tubular cells.

Our findings are unique in that: a) The brush border degrades the hormone to pyroglutamyl peptide metabolites; b) The pyroglutamyl peptides are reabsorbed; c) Degradation of peptides appears to occur also within the cell. Previous studies have reported proximal tubular degradation of small linear peptides to amino acids and the reabsorption of the latter. The reabsorption of small peptides by the proximal tubule followed by further intracellular degradation has not been previously reported. As these reabsorbed peptides are not completely degraded to pGlu intracellularly (as evidenced by appearance of luminal peptides **2, 3** and **4** in the contraluminal compartment *in vivo* and *in vitro*), this finding raises the expectation that biologically active peptides that escape brush border degradation may be reabsorbed intact, possibly allowing the expression of a protracted biological action.

The concept of contact digestion, first described in the small intestine, postulates that small peptides are degraded by hydrolases in the luminal glycocalyx and luminal cell membrane. Two mechanisms have been described: 1) Membrane hydrolysis followed by absorption of amino acids and 2) Carrier-mediated absorption of di- and tri-peptides with subsequent intracellular hydrolysis. Our studies suggest that the proximal tubular cell and the enterocyte of the small intestine might hydrolyze and transport small linear peptides by similar mechanisms. Additionally, our studies suggest that LHRH agonists or antagonists should be prepared, featuring the substitution of L-serine, and perhaps L-pyroglutamic acid, with unnatural amino acids which would render the analogs less susceptible to breakdown by kidney enzymes possibly leading to analogs which would be longer acting *in vivo*.

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