PEPTIDES

Structure and Function

Proceedings of the Eighth
American Peptide Symposium

Victor J. Hruby
Daniel H. Rich

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PREFACE

The Eighth American Peptide Symposium was held on the campus of the University of Arizona in Tucson on May 22-27, 1983. About 630 scientists from over 20 countries attended the meeting to exchange ideas and to discuss recent developments in the peptide field. Topics covered in the various sessions included peptide synthesis and biosynthesis, the mechanisms of peptide hormone and neurotransmitter action, and chemical, biological and physical considerations in peptide structure-biological activity relationships. Over 250 oral and poster presentations provided a forum for discussing the latest results.

The meeting was organized to emphasize the growing interdisciplinary nature of peptide research. Virtually all sessions provided examples where the application of various combinations of organic chemistry, analytical chemistry, biochemistry, biophysics, and biology were utilized in an effort to better understand the physical, chemical and biological properties of peptides. For example, in the session organized by Peter Schiller on structural and conformational considerations in the design of biologically active peptides, the latest synthetic methodology was needed to prepare and purify conformationally constrained biologically active peptides containing dehydroamino acids, retroinverso, methylene sulfide and other amide bond replacements, as well as side chain to side chain and side chain to backbone cyclizations. The peptides thus prepared were examined by a variety of biophysical methods including X-ray crystallography and nuclear magnetic resonance spectroscopy. Careful attention was given to a battery of biological studies which provided insights into conformational features associated with biological effects. Enkephalin analogs with high mu and delta selectivity, α-melantropins with high potency, prolonged activity and specific behavior properties, cyclic somatostatin analogs with oral activity and several other peptide hormones were reported. The session on peptide inhibitors of proteases illustrated how careful considerations of the properties of enzyme binding sites based on X-ray crystal structures and on fundamental studies of enzyme mechanisms can lead to the rational design of enzyme inhibitors of high potency. Noteable new examples were presented of inhibitors of pepsin, penicillopepsin, angiotensin-converting enzyme, renin, pyroglutamyl aminopeptidase, and others. Efforts to develop similar approaches and understanding of the structural basis of action of neuropeptides was the major theme of the session on these peptides. Evidence that specific fragments of substance P, ACTH, α-MSH, vasopressin, dynorphin, and enkephalin have different behavior effects was presented, and provided

further evidence for the central importance of peptides as neuromodulators of behavior. The application of these advances to the development of peptides as potential drugs and pharmaceuticals was discussed in a session organized by Wylie Vale. Examples of peptide hormone antagonists, agonists, peptide hormone-toxin and other conjugates, peptide antibodies, enzyme inhibitors and peptide antigens which are being developed for treatment of specific disease states or other biological applications were presented.

In view of these developments, the fourth Alan E. Pierce Award, presented to Ralph Hirschmann for his contributions to peptide and protein synthesis and to the development of orally active peptides, seemed particularly appropriate. In his award address, Dr. Hischmann discussed how development of new knowledge and understanding of peptide synthesis, structural and conformational analysis, and biological function can be utilized for both an understanding of the fundamental properties of peptides and for the development of new drugs.

The importance of understanding biological processes in peptide research was emphasized in sessions on the mechanism of peptide hormone action and on biosynthesis and post-translational processing of peptides. In the former studies the use of receptor specific antagonists, affinity labeled analogs, monoclonal antibodies, and other methods for detecting and examining the structure of receptors, regulatory proteins and other components associated with hormone-receptor structure and function were discussed, as well as other approaches which can be utilized to modulate and control hormone and neurotransmitter function. In a similar manner highly sensitive analytical and genetic techniques are being examined to characterize the biosynthesis of peptide hormones and neurotransmitters. The importance of peptide-membrane interactions and their relation to ion transport was emphasized in a session honoring Erhard Gross, B. Gisin, and W. Veatch all who suffered sudden and untimely deaths since the last symposium. Their contributions to peptide chemistry will be sorely missed.

The session on synthesis was highlighted by Bruce Merrifield's critical assessment of the solid phase method on its 20th anniversary, by Jack Johansen's discussion of the application of proteases to peptide synthesis, and by R. Wenger's report on the total synthesis of cyclosporin A (sandimmune). Continued development of new solution and solid phase methodology including new protecting groups, coupling reagents, *etc.* were presented in many papers, and numerous reports emphasized the application of sensitive analytical and biophysical methods to examine side reactions and mechanism(s) in peptide synthesis.

The continued development of fast atom bombardment mass spectrometry (FAB MS), nuclear magnetic resonance spectroscopy, and increasingly sensitive sequencing methodology for structure determination of minute quantities of materials was emphasized in the session on peptide purification and analysis. Increasingly efficient and selective HPLC methods for proteins and peptides were also critically discussed. A further session emphasized continued development of solution and solid state nmr methods, as well as conformational and dynamic analysis including calculations and molecular dynamics utilizing computer graphics. These methods are providing deeper insight into the physical-chemical properties of peptides and their relation to biological activity.

I thoroughly enjoyed organizing this Symposium. The many useful suggestions for program topics and speakers, the record turnout for the Symposium, and the excellent presentations in both the poster and oral sessions were most gratifying to me. I would like to thank the local committee and especially my students for their critical help with last minute preparations and during the Symposium. I also want to thank Mr. Joe Stanley and his staff of the University of Arizona Conference Center for coordinating the arrangements at the University dormatories and the meals and scientific session at the Memorial Student Union. I especially want to thank Lourdes Gallegos who worked so hard during the past year in the preparation of the abstracts book, the program , and this Proceedings book. Her equanimity and efficient handling of the correspondence, questions, abstract and manuscripts before, during, and after the Symposium are especially appreciated. Finally I thank the bus drivers and a record heat wave for providing some unplanned, but unforgettable experiences.

Once again the publisher of the Proceedings of this Symposium is Pierce Chemical Company of Rockford, Illinois. I am most grateful for the outstanding efforts of Melba Rinaldo in the Editorial Office and of Dr. Robert (Bob) Vigna who served as copy editor of this book. It has been a great pleasure to work with them on the completion of this book.

October 1984 Victor J. Hruby

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University of Arizona

May 22-27, 1983

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ABBREVIATIONS

A

∇AA	cyclopropyl amino acid
Abu	α-aminobutyryl
Abz	aminobenzoyl
Ac	acetyl
ACE	angiotensin-converting enzyme
Acm	acetamidomethyl
Acp	ε-aminocaproyl
AcOH	acetic acid
ACTH	adrenocorticotropic hormone (adrenocorticotropin)
ADH	antidiuretic hormone
Adoc	adamantyloxycarbonyl
AEP	2-aminoethylpyridine
Aib	α-aminoisobutyric acid
AIP	acidic joining peptide
aILE	alloisoleucine
AMC	7-amino-4-methylcoumarin
α-MSH	α-melanotropin, α-melanocyte stimulating hormone
Amp	*p*-(aminomethyl)-phenylalanine
Aoe	2-amino-8-oxo-9,10-epoxydecanoic acid
Arg-H	arginal
Asu	2-aminosuberic acid
Ava	5-aminovaleric acid
AVP	8-arginine vasopressin
AVT	8-arginine vasotocin
AUFS	absorption units full scale

B

BK	bradykinin
β-LPH	β-lipotropin
Boc	*tert*-butyloxycarbonyl
BOP-Cl	N,N-*bis*(2-oxo-3-oxazolidinyl)-phosphorodiamidic chloride
Bpoc	2-(4-biphenyl)propyl(2)oxycarbonyl
BRMAC	bovine renal medulary adenylate cyclase
BSA	bovine serum albumin
tBu or But	*tert*-butyl
Bz	benzoyl
Bzl	benzyl

C

CaM	calmodulin
cAMP	3′,5′-cyclic adenosine monophosphate
CCC	countercurrent chromatography
CCK	cholecystokinin
CD	circular dichroism
CDI	N,N′-carbonyldiimidazole
Cha	β-cyclohexylalanine
CI	chemical ionization
CLIP	corticotropin-like intermediate lobe peptide ($ACTH_{18-39}$)
CNA	β-chlornaltrexamine
CNS	central nervous system
CPY	carboxypeptidase Y
CRF	corticotropin releasing factor
CSA	chemical shift anisotropy
CT	calcitonin

D

Dab	2,4-diaminobutanoic acid
DADLE	[D-Ala^2, D-Leu^5]enkephalin
DCC	dicyclohexylcarbodiimide
DCC-HOBt	dicyclohexylcarbodiimide-1-hydroxybenzotriazole
DCHA	dicyclohexylamine
DCU	*N*-*N*′-dicyclohexylurea
Ddz	2-(3,5-dimethyloxyphenyl)propyl-2-oxycarbonyl
DIEA	diisopropylethylamine
DMA	dimethylacetamide
DMF	dimethylformamide
DMPC	dimyristoylphosphatidylcholine
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
Dnp	2,4-dinitrophenyl
Dns	dansyl, 1-dimethylaminonapthalene-5-sulfonyl
DOPC	dioleylphosphatidylcholine
DPPC	dipalmitoylphosphatidylcholine
DSIP	delta-sleep inducing peptide
dSta	4S-amino-6-methylhepatoic acid
Dts	dithiasuccinoyl
DTT	dithiothreitol
dynA	dynorphin A
dynB	dynorphin B
ΔPhe	α,β-dehydrophenylalanine

E

Eac	epsilon-aminocaproyl
E, EK, Enk	enkephalin
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDT	ethanedithiol
EGF	epidermal growth factor
EI	electron impact
ER	endoplasmic reticulum
Et_3N	triethylamine

F

FAB	fast atom bombardment (mass spectrometry)
FCA	Freunds complete adjuvant
FDMS	field desorption mass spectrometry
Fmoc	9-fluorenylmethoxycarbonyl
For	formyl
FSH	follicle stimulating hormone
FT	Fourier transform

G

GA	gramicidin A
GCMS	gas chromatography mass spectrometry
GH	growth hormone
Gla	γ-carboxyglutamic acid
GLC	gas liquid chromatography
Gn-RH	gonadotropin-releasing hormone (gonadoliberin)
GPI	guinea pig ileum
GRP	gastrin releasing peptide
GSSG	glutathione dimer

H

hArg	homoarginine
hCG	human chorionic gonadotropin
Hcy	homocysteine
HDL	high density lipoprotein
HEPES	*N*-2-hydroxyethylpipirazine-*N'*-2-ethanesulfonic acid
HF	hydrogen fluoride
HG	human gastrin
hGH	human growth hormone
HIF-αA	human leukocyte interferon
HIF-γ	human immune interferon
HMPA	hexamethylphosphoramide

HOBT	N-hydroxybenzotriazole
HOSu	N-hydroxysuccinamide
HPLC	high pressure liquid chromatography
hPTH	human parathyroid hormone
HVE	high voltage electrophoresis

I

Ia	intraortic
Iaa	isoamylamide
IBCF	isobutylchloroformate
icv	intracerebroventricular
IEF	isoelectric focusing
IFG-I	insulin-like growth factor
INF	interferon
IR	infrared
IV	intravenous
IVa	isovaleryl
Ival	isovaline

J

J	spin-spin coupling constant

L

Lac	lactic acid
LDH	lactic dehydrogenase
LH	luteinizing hormone
LH-RH/LHRH	luteinizing hormone releasing hormone (luliberin)
β-LPH	β-lipotropin
γ-LPH	γ-lipotropin
LVP	8-lysine vasopressin
lyso-PC	lysophosphatidylcholine
lyso-PG	lysophosphatidylglycerol

M

MA	mixed anhydride
Mbh	4,4'-dimethyloxybenzhydryl
MCPBA	*m*-chlorperbenzoic acid
MEB	methylbutyryl
MeBMT	N-methyl-(4R)-but-2E-en-1-yl-4-methyl-(L)-threonine
MIF	macrophage inhibiting factor
MNE	magnetic nonequivalence
MS	mass spectrometry
Ms	methanesulfonyl
MSA	methanesulfonic acid

MSH	melanophore (melanocyte) stimulating hormone (melanotropin)
Mts	mesityl-2-sulfonyl
MVD	mouse vas deferens

N

Nal	3-(2-naphthyl)-D-alanine
NAL	naloxone
Napth	β-napthylamine
NMM	N-methylmorpholine
NMR, nmr	nuclear magnetic resonance
NOE	nuclear Overhauser enhancement
NP	neurophysins
Np	nitrophenyl
Nps	2-nitrophenylsulfonyl
Npys	3-nitro-2-piperidinesulfonyl
NT	neurotensin

O

OBzl	benzyl ester (ether)
ODS	octadecasilyl
OMPA	oxymethylphenylacetic
OPA	ortho-phthalaldehyde
ORD	optical rotatory dispersion
OSu	hydroxysuccinimide
OT	oxytocin
O*t*Bu	*tert*-butyl ester (ether)
Ox	oxytocin
Oxy	oxytocin

P

Pac	phenacyl
PAGE	polyacrylamide gel electrophoresis
Pam	phenylacetamidomethyl
Pba	phenylbutyryl
PC	phosphatidylcholine
PEG	polyethyleneglycol
Pen	penicillamine
PET	positron emitting tomography
Pfp	pentafluorophenyl
PG	prostaglandins
ΔPhe	α, β-dehydrophenylalanine
PI	protease inhibitor
Pip	pipecolic acid
PLA	phospholipase A

PMRI	partially modified retro-inverso
PMSF	phenylmethylsulfonylfluoride
pMZ	*p*-methoxybenzyloxycarbonyl
pNA	*p*-nitroanilide
POMC	proopiomelanocortin
Pon	phenylacetoxymethyl-3-nitrobenzamidomethyl
Pop	phenylacetoxypropionyl
PP	pancreatic polypeptide
PPCE	post proline cleaving enzyme
PTH	parathyroid hormone (parathyrine)
PTH	phenylthiohydantoin
Pyr	pyridine
Pyr-MCA	L-pyroglutaminyl-4-methylcoumarinylamide
Pys	2-pyridinesulfenyl
$\psi(CH_2S)$	thiomethyl amide bond replacement

R

RBP	rat blood pressure
REMA	repetitive excess mixed anhydride
RER	rough endoplasmic reticulum
RIA	radioimmunoassay
RNase	ribonuclease
RP-HPLC	reverse phase-high pressure liquid chromatography
RPLC	reverse phase liquid chromatography
RUT	rat uterus

S

SAC	6-aminocaproyl
Sar	sarcosine
Scm	carboxymethylsulfenyl
Scm-Cl	methoxycarbonyl sulfenyl chloride
SDS	sodium dodecylsulfate
SFP	spleen fibrinolytic proteinase
SLP	saturated lower phase
SP	substance P
SPDP	N-succinimidyl-3-(2-pyridyldithio)-propionate
SS	somatostatin
SS-14	somatostatin 14 peptide
SS-28	somatostatin 28 peptide
Sta	statine or 3S-hydroxyl-4S-amino-6-methyl-heptanoic acid
Sto	3-oxo-4S-amino-6-methylheptanoic acid
Suc	succinyl
Sulfmoc	9-(2-sulfo)fluorenylmethyloxycarbonyl

T

TBHP	*tert*-butyl hydroperoxide
tBu	*tert*-butyl
TCA	trichloracetic acid
Tce	trichloroethyl
Tcp	2,4,5-trichlorophenyl
TEA	triethylamine
TEAF	triethylammonium formate
TEAP	triethylammonium phosphate
TEMPO	2,2,6,6-tetramethylpiperidineoxyl
TFA	trifluoroacetic acid
Tfa	trifluoroacetyl
TFE	trifluoroethanol
TFMSA	trifluoromethane sulfonic acid
THF	tetrahydrofuran
Tic	tetrahydroisoquinoline carboxylic acid
Tid	terephthaloyl-*bis*-imidodiacetic acid
TLC	thin layer chromatography
TMG	tetramethylguanidine
TMP·HCl	2,2,6,6-tetramethyl-piperidine hydrochloride
TMS	trimethylsilyl
TNP	trinitrophenyl
Tos	4-toluenesulfonyl
TRH	thyrotropin releasing factor (thyroliberin)
TRIS	tris(hydroxymethyl)aminomethane
Trt	trityl, triphenylmethyl
TSP	3-(trimethylsilyl)propionate

U

UII	urotensin
UUP	unsaturated upper phase
UV	ultraviolet

V

VIP	vasoactive intestinal polypeptide
VP	vasopressin

Z

Z	benzyloxycarbonyl

PEPTIDE RESEARCH IN AN INDUSTRIAL ENVIRONMENT

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Introduction

I am enormously pleased to have been named the recipient of the Alan E. Pierce Award and I should like to express my sincere appreciation for this honor to the Award Committee and to the Pierce Chemical Company.

About twenty years ago, Dr. Robert Denkewalter, then Vice President for Exploratory Research, now retired, had the farsightedness to recommend initiation of peptide research in our laboratories. I have always been grateful that he chose to invite me to head this effort, and I should like to dedicate my remarks this morning to Dr. Denkewalter and also to the memory of Dr. Frederick W. Holly, a long-time friend and associate who had contributed so much to our synthesis of ribonuclease, to our TRH-related research and, until his untimely death in 1980, to our somatostatin programs.

Chemistry of NCA's and NTA's

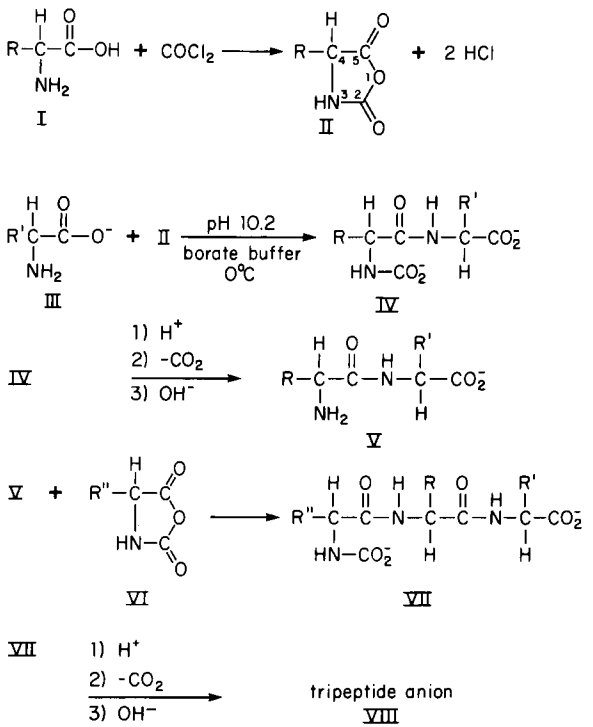
As a result of Dr. Denkewalter's suggestion, Mr. Harvey Schwam, now Dr. Schwam, Mr. Robert Strachan and I set out to explore the potential of N-carboxy α-amino acid anhydrides (NCA's) for the controlled synthesis of peptides. A few months later we were joined by Mr. Victor Garsky, now Dr. Garsky of the Upjohn Company.

None of us had had any prior experience in the synthesis of peptides. We still believe today that the health of peptide chemistry at Merck will continue to be served best through the influx of excellent, broadly trained organic chemists.

When we initiated our program in 1963, NCA's had been used widely for the synthesis of homopolymers, and of random and block heteropolymers. Bailey[1] had reported the synthesis of some oligopeptides *via* the coupling of selected NCA's with amino acid or peptide esters in anhydrous medium at very low temperature, and Bartlett and his associates[2] had published two kinetic studies with NCA's in aqueous medium. The attraction of the NCA's lay in part in the simplicity and low cost of their preparation from an amino acid and phosgene, and in the ease and speed with which the N_{α}-protecting group is removed after a coupling reaction is completed. An idealized reaction sequence for the preparation of a tripeptide by the NCA method is depicted in Scheme I.

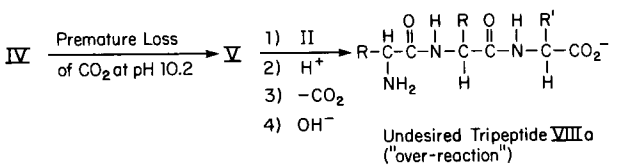
Since the C-5 carbonyl of the NCA II is far more reactive than the urethane carbonyl at C-2, reaction with III should occur only at C-5; at alkaline pH, the resulting carbamate IV would be stable. Acidification leads to the corresponding carbamic acid which spontaneously loses carbon dioxide; the latter can be readily swept out of the reaction mixture with nitrogen. Subsequent readjustment of the pH with base would produce the dipeptide carbanion V and repetition of the cycle would lead to the tripeptide anion VIII, *etc*. A crude hexapeptide can in fact be readily prepared in half a day by this procedure.[3]

We studied the chemistry of these transformations in some detail[4] in order to gain a better understanding of the nature of the side reactions which take place in the hope of thus achieving better control of the reaction and of optimizing



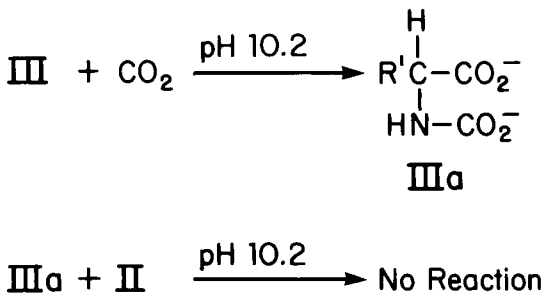
Scheme 1. Idealized scheme for the synthesis of a tripeptide.

the yields. Unfortunately, carbamates such as IV or VII are not completely stable at pH 10 even at 0°. If the carbamate IV loses carbon dioxide prematurely, the resulting dipeptide V can compete with III for any remaining NCA II to give the undesired tripeptide VIIIa (See Scheme 2).



Scheme 2. Acid Catalyzed Side Reaction

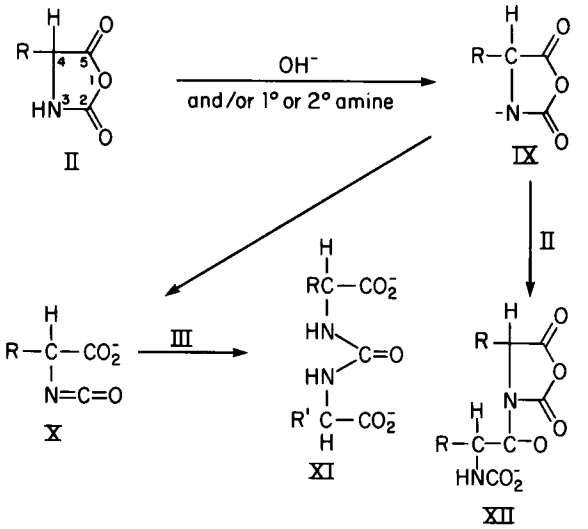
The premature decarboxylation shown in Scheme II is an acid catalyzed side reaction, and the stability of the carbamates therefore increases with pH. Unfortunately, it is not desirable to carry out the reactions at pH 11 where carbamate stability is very good, because of a competing, base-catalyzed side reaction, the hydrolysis of the NCA. In a third side reaction, shown in Scheme 3, any carbon dioxide generated while the desired coupling reaction is in progress can convert a nucleophile such as III into the corresponding carbamate IIIa and thus inactivate it. These three side reactions had already been recognized by Bartlett.



Scheme 3. "Inactivation" of Nucleophile by CO_2

We identified additional base-catalyzed side reactions.[4] One was the formation of a hydantoic acid, typified by structure XI (Scheme 4). A priori one might assume that hydantoic acid formation resulted from the direct attack of an amino acid or peptide on the "wrong" (C2) carbonyl group of the NCA. We have been able to show, however, that this is not the mechanism of hydantoic acid formation, which arises via the isocyanate X (Scheme 4).

In addition, the N-anion IX can react with the NCA II to form XII which retains a reactive anhydride group and which can therefore react with III to generate the unwanted



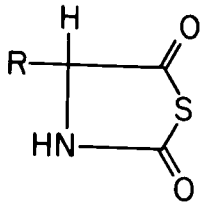
Scheme 4. Mechanism for hydantoic acid formation and for base catalyzed oligomerization.

tripeptide VIIIa, mentioned above. It is formed here by a base-catalyzed mechanism. We found[4] that with relatively soluble NCA's such as the NCA of alanine, highly efficient stirring during the addition of the anhydride as provided by a Waring Blendor will minimize the conversion of II to XII (and thereby to VIIIa) and it will thereby raise the yields.

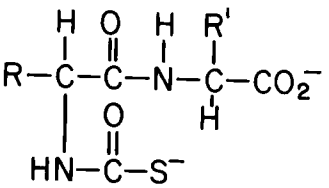
Several years later, Iwakura, Katakai and their associates[5-7] made an important contribution to the problem. They showed that when the coupling reactions are carried out with conventional stirring in the heterogeneous reaction medium acetonitrile-water in the presence of sodium carbonate at low temperatures, side reactions generated via the NCA anion IX such as hydantoic acid formation (XI) or base-catalyzed oligomerization are suppressed. We had found that the NCA of glycine is uniquely prone to undergo conversion to the isocyanate. It is therefore particularly noteworthy that the modifications introduced by the Japanese workers afforded glycyl-L-tryptophan in 96.5% yield.

I should like to mention one other aspect of our work with NCA's. Contrary to our initial expectations, we were able to employ the NCA of aspartic acid[8] and the NCA of glutamic acid without protection of the third functionality to afford the desired peptides uncontaminated by β-aspartyl or γ-glutamyl peptides, respectively. This indicates that an intramolecular rearrangement involving the ω-carboxyl anion of the NCA does not take place under our reaction conditions. Unwanted β-aspartyl peptides are formed, however, when the third functionality of this NCA is protected as an ester.

Because thiocarbamates were expected to show a greater stability at a given pH than the corresponding carbamates, Dr. Ray Dewey, Dr. Erwin Schoenewaldt, Mr. William Palaveda and their associates[9,10] explored the use of 2,5-thiazolidinediones (NTA's) typified by XIII in peptide synthesis. They found, for example, that salts of amino acid thiocarbamates were indeed stable during electrophoresis at pH 11, whereas the carbamate salts decompose under these conditions. The excellent stability of peptide thiocarbamates such as XIV made acid-catalyzed over-reaction less of a problem and permitted the coupling reactions to be carried out at a lower pH than had been the case with the NCA's, thereby suppressing base-catalyzed side reactions. Thus NTA's left less unchanged amino acid and afforded less of the overreaction product than NCA's, generally giving higher yields. The amount of hydantoic acid formed was not changed significantly. We had shown NCA's to afford optically pure products. The 2,5-thiazolidinediones, on the other hand, led to partial epimerization when used under analogous conditions. In spite of this racemization problem, the NTA reaction in aqueous medium can be useful in special situations when conditions can be optimized to minimize racemization and/or to permit facile isolation of the pure products.



XIII



XIV

In spite of the shortcomings of the NCA method, the recent modifications of the NCA method by Iwakura and Katakai and their associates referred to above, and by Drs. Thomas Blacklock and Richard Shuman[11] at Merck, have made possible the practical synthesis of small peptides in kg amounts in high yield and in a cost-effective manner. We were also pleased to learn that Vinick and Jung[12] have succeeded in developing a practical synthesis of aspartame using the NTA of aspartic acid. This process is suitable for the large-scale synthesis of the artificial sweetener.

Synthesis of An Enzyme

Bovine pancreatic ribonuclease (RNase) (Figure 1) was isolated by Rene Dubos and was crystallized by Moses Kunitz in 1940. In 1963, Smyth, Stein and Moore[13] successfully concluded their investigation of the amino acid sequence of this enzyme. The arrangement of the 124 amino acids is shown in Figure 1. It was shown by Richards[14] and his associates in 1958 that treatment of RNase A with the enzyme subtilisin brings about the cleavage of the peptide bond between amino acids 20 and 21. The resulting mixture (RNase S) fully retains the enzymatic activity of RNase A. It was also found by Richards' group that RNase S can be separated into its two components: neither the larger one (S-Protein), which is

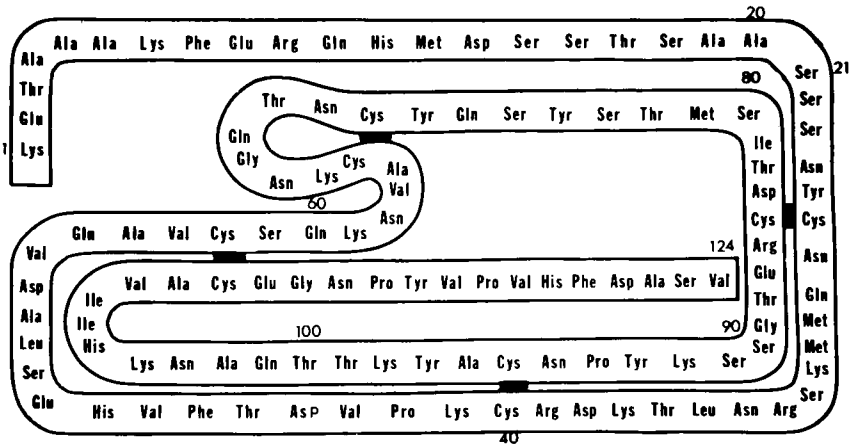


Fig. 1. A two-dimensional representation of the amino acid sequence of bovine pancreatic ribonuclease A based on work of Hirs, Spackman, Smyth, Stein and Moore of the Rockefeller University. The 124-membered chain extends from Lys-1 (amino terminal) to Val-124 (carboxy terminal). The 8 Cys amino acids are joined by disulfide bridges in the positions shown. In ribonuclease S, there is no covalent bond between Ala-20 and Ser-21.

composed of 104 amino acids and which contains the four disulfide bridges, nor the smaller one (S-Peptide), which consists of 20 amino acids, showed any enzymatic activity. On recombination of equimolar portions of S-Protein and S-Peptide, enzymatic activity was fully restored. The recombined enzyme is called RNase S', to distinguish it from RNase S, the original subtilisin cleavage product. The eicosapeptide (S-Peptide) had been synthesized earlier by Hofmann and his associates[15] and subsequently in our laboratory (See Figure 3).

When we undertook the total synthesis of the enzyme RNase S', our experience with NCA's and NTA's encouraged us to keep the use of protecting groups to a minimum.[16] The limitations of the NCA and other solution chemistry methods

convinced us that it would not be possible to undertake the stepwise synthesis of a protein from the C-terminal to the N-terminal amino acid, the strategy chosen by Merrifield and Gutte[16a] in their solid-phase synthesis of RNase A. We elected instead to synthesize the 19 fragments shown in Figure 2 and then to couple the highly purified fragments.[17-21] We employed NCA's and NTA's to form about 40% of the bonds in the peptide fragments of S-protein; the remainder were prepared using the Boc-hydroxysuccinimide esters of Dr. George W. Anderson.

I would like to comment here on only a few aspects of our enzyme synthesis. We had prepared active material in 1968, about five years after we had started peptide chemistry in Rahway. We restricted the use of third functionality protecting groups to our acetamidomethyl blocking group[22,23] for the sulfhydryls of the eight cysteine residues, and to the Cbz-protecting group for the ε-amino groups of the eight lysines. We employed the butyloxycarbonyl group as the acid-labile blocking group of the Anderson active esters. Thus the side chains of aspartic acid, glutamic acid, serine, threonine, tyrosine, arginine, histidine, glutamine and asparagine residues were not protected.[16] The selection of the benzyloxycarbonyl protecting group for the ε-amino function of the eight lysine residues was made at a time when it was widely but incorrectly believed that this protecting group is completely stable under the acidic conditions required for removal of the Boc-group. Although we were able to separate the resulting impurities, we would have been served far better by the isonicotinyloxycarbonyl protecting group[24] which we developed later, not only because it is completely acid stable, but also because it helps to solubilize large peptides in polar solvents such as DMF. To permit the use of the unprotected ω-carboxy groups of aspartic acid and glutamic acids, we relied on the Curtius

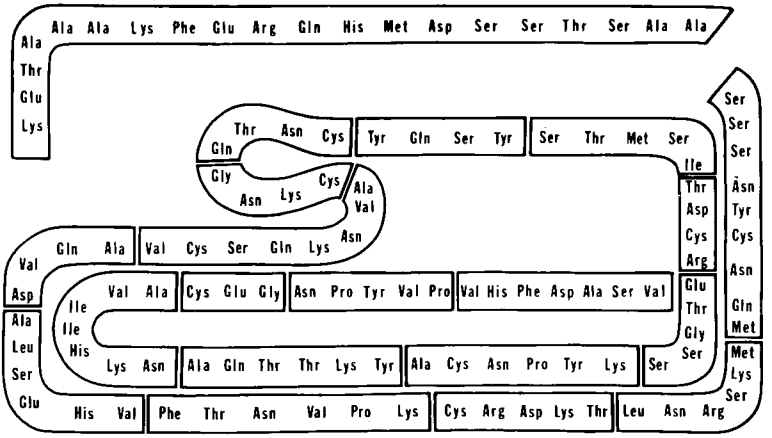


Fig. 2. Fragment coupling method of peptide synthesis. The 19 fragments of S-protein as well as S-peptide are shown.

azide method as modified by Honzl and Rudinger to couple the 19 fragments shown in Figure 2. The coupling methods employed by us, viz. use of NCA's,NTA's, hydroxysuccinimide esters and azides, were consistent with our minimal use of protecting groups.

Using semisynthetic acetamidomethylated octahydro S-protein,[20,23] we were able to optimize the conditions for its conversion to RNase S'. The totally synthetic, protected octahydro S-protein was prepared by Ms. Ruth Nutt, now Dr. Nutt, by coupling 5 mg of the tetratetracontapeptide with 1.6 mg of the hexacontapeptide by the azide method.[21] After removal of the Boc and Cbz protecting groups in the presence of scavengers, the resulting purified acetamidomethylated octahydro S-protein was identical with the natural (semisynthetic) material on disc gel electrophoresis in 30% acrylamide gel at pH 5.5. After removal of the cysteine protecting groups, the resulting octahydro S-protein was oxidized at pH 6.5 in the presence of S-peptide and phosphate ion to afford a protein which had the correct elution volume

from carboxymethyl Sephadex and which catalyzed the hydrolysis of polycytidylic acid and of RNA but not of DNA.[21,25,26] Protein oxidized in the absence of S-peptide was devoid of enzymatic activity.

It seems appropriate to mention that the total synthesis of RNase A or of RNase S' could not have been contemplated without the prior work of Dr. Christian B. Anfinsen and his associates. Referring to his work in his 1972 Nobel Prize lecture,[27] he remarked as follows: "This (thermodynamic hypothesis) states that the three-dimensional structure of a native protein in its normal physiological milieu....is the one in which the Gibbs free energy of the whole system is lowest." When the four cystine bridges of RNase A or of RNase S are reduced, the resulting eight sulfhydryl groups, could--on oxidation--afford as many as 105 possible pairings, only one of which corresponds to the original enzyme. It had been reported by Anfinsen and White[28,29] in the case of RNase A, and by Anfinsen and Haber[30] for RNase S, that the fully reduced enzymes will, under the influence of molecular oxygen alone, form an enzymatically active product in high yield which is indistinguishable from the native enzyme. These observations indicated that the information which determines the secondary and tertiary structure of a protein is contained in the amino acid sequence itself. Without this prior knowledge it would have been senseless to undertake the synthesis of an enzyme containing four disulfide bridges (See Figure 3). Hydrophobic and hydrogen bonding as well as van der Waals interactions, and, to a lesser extent, ionic bonds, are currently thought to contribute significantly to the stabilization of the natural conformation of proteins.

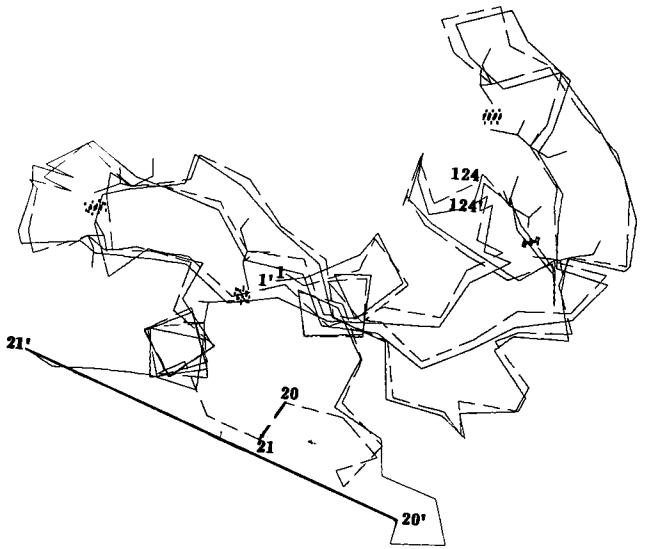


Fig. 3. Superposition of C chains of ribonuclease A (dashed) and ribonuclease S (solid line), showing their amino (1, 1') and carboxy (124, 124') termini. The region containing the subtilisin scissile bond of RNAse A (ala^{20}-ser^{21}) is indicated by a heavy dashed bond. A heavy long line connects the proteolytically cleaved residues 20', 21' of RNAse S. The eight cysteines of RNAse A are indicated with dots showing their pairing: 40:95 (left), 26:84 (left center), 58:110 (right), and 65:72 (upper right, forming a short loop). The positions of the cystine bridges of RNAse S are nearly identical to those of RNAse A. α-Helices are in the middle of the structure (1-12) and to the left (26-33). A less obvious third helix (50-58) is seen on its side in the lower right of this view. The extensive folded anti-parallel β-strand network is also apparent. The structures were obtained from the Brookhaven Protein Data Bank. The bovine ribonuclease A structure of Wlodawer et al. (1982) was refined from 2.0 Å X-ray and neutron diffraction data: A. Wlodawer, R. Bott and L. Sjolin, J. Biol. Chem. **257** 1325-1332 (1982). The older ribonuclease S structure of Wyckoff and Richards (1973) is based on 2.0 Å X-ray data at a lower level of refinement: H. W. Wyckoff, D. Tsernoglou, A. W. Hanson, J. R. Knox, B. Lee, and F. M. Richards, J. Biol. Chem. **245**, 305-328 (1970). Superposition and display was accomplished with the Merck Macromolecular Modeling Graphics Facility version of FRODO by Dr. B. Bush.

Status of Protein Synthesis

The work with NCA's and the enzyme synthesis had taught us a great deal about peptide chemistry, about the behavior of peptides in various solvents, and about the enormous increase in the complexity of the synthetic problems which one encounters as one moves from the synthesis of peptides containing no more than say forty to fifty amino acids, to molecules containing over sixty amino acids. I believe that current methodology is in most cases adequate to permit the synthetic chemist to prepare in a timely fashion the compounds which are today of major interest. This is a result, I believe, largely of advances in separation and spectroscopic techniques made possible by high pressure liquid chromatography and Fourier transform NMR spectroscopy, respectively. I had hoped in the late sixties that the total synthesis of analogs of ribonuclease and of other enzymes might increase our understanding of the importance of particular amino acids in the proper folding and action of enzymes. This hope has not been realized simply because the unambiguous chemical synthesis and characterization of even a comparatively low molecular weight protein (ca. 100-120 amino acids) has remained a formidable undertaking. This is true even when the synthetic target is a natural product where techniques such as affinity chromatography are now often available, and where the natural product can be used as a reference standard for the evaluation of purity. The task of the purification and characterization of a totally synthetic protein becomes even more difficult when one seeks to prepare an analog which does not exist in nature. Recombinant DNA techniques now hold the potential to fill this gap. The objective, it seems to me, remains worthwhile.

Medicinal Chemistry

I think of our study of the chemistry of NCA's as process research, an activity which is very important to us in that it allows us to prepare our therapeutic agents in a practical and cost-effective manner. The enzyme synthesis, on the other hand, exemplified exploratory research which has no disease-related objective. Research of this latter type constitutes a relatively small but still significant portion of our research budget. During 1982, for example, we assigned about 5% of our R&D budget to such exploratory projects.

I should now like to say a few words about two peptide-centered programs which have therapy as their goal and which are thus in the mainstream of our chemical and biological effort in basic research. The first concerns somatostatin, with diabetes as the medical target, and the other, inhibition of angiotensin converting enzyme, which relates to hypertension.

Somatostatin related research: the role of phe^{6} and phe^{11}

It is believed by many diabetologists today that the grave complications of diabetes result from inadequate control by conventional therapy of plasma glucose levels. Although definite evidence is lacking, hyperglycemia is believed to underlie such sequelae as retinopathy, which leads to blindness, nephropathy and neuropathy. A further complication results from the fact that injection of relatively high doses of insulin once or twice a day leads to episodes of hyperinsulinemia, which is believed to lead to the premature development of atherosclerosis. It is also known that growth hormone (GH) levels are abnormally high in

diabetics.[31] This complicates the problem, because GH is known to be an insulin antagonist and to be diabetogenic.[32-34] In addition, GH may play a permissive role in the development of retinopathy.[35,36] Further, Unger has pointed out that plasma levels of glucagon are inappropriately high in diabetics when viewed in the context of the diabetic's hyperglycemia.[37] This tends to increase blood glucose levels and to accentuate the abnormalities of the diabetic state.

Somatostatin, a tetradecapeptide first isolated, characterized and synthesized by Guillemin and his associates at the Salk Institute,[38] owes its name to its ability to inhibit growth hormone release. It was found later by Koerker[39] and by Gerich[40] that somatostatin also suppresses the release of glucagon and of insulin. In the insulin-requiring diabetic, suppression of insulin is not a detriment. Suppression of GH and glucagon release are of potential value. In addition, it has been shown that somatostatin retards gastric emptying and increases gastrointestinal transit time,[41] thus slowing the absorption of oral glucose, another potential benefit. Suppression of GH and glucagon release and delay of glucose absorption made somatostatin of potential interest in the treatment of insulin-requiring diabetics.[42]

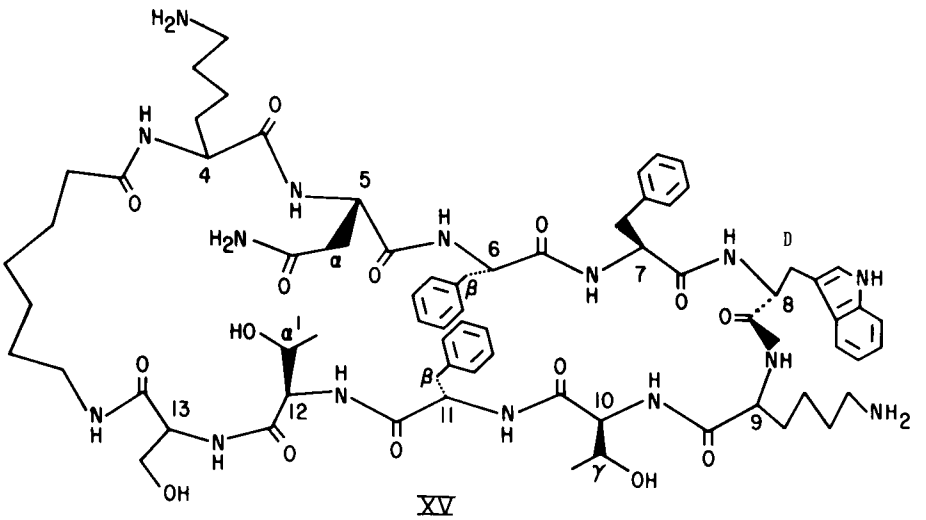
Somatostatin is, however, rapidly degraded by proteolytic enzymes, and it has only a very short biological half life (about one and one-half minutes). This makes it less than satisfactory for clinical studies.

A somatostatin analog suitable for clinical trial should therefore possess a longer duration of action than somatostatin itself. Activity after oral administration is also desirable.

The medicinal chemists at Merck sought therefore to design and synthesize a smaller, more compact analog of

somatostatin in the expectation that it would have a more appropriate biological half life. Under the splendid leadership of Dr. Daniel Veber, the Merck chemists approached the problem by trying to gain some understanding of the conformation of somatostatin at its receptors. We had been able to demonstrate earlier[43] that the cyclic form of somatostatin is biologically active. This observation had provided the first conformational constraint on any model for the bioactive conformation of somatostatin. A second clue was provided by the report by Rivier, Brown and Vale,[44] that the diastereomer of somatostatin having a D-tryptophan in position 8 is more potent than somatostatin. This result suggested[45] the possibility that tryptophan occupies the second position of a β-turn. With this information as a basis, Veber and his associates developed through the synthesis of carefully chosen analogs a conformational model (Figure 4) for the somatostatin analog XV, des (ala^1,gly^2) desamino [cys^3] descarboxy [cys^{14}] $dicarba^{3,14}$ somatostatin.[46]

It is worth pointing out that very significant progress was made possible in these conformational studies through the synthesis and biological testing of analogs. NMR spectroscopic studies, which often form the starting point of conformational investigations, were initiated only later, but eventually made enormous contributions to the problem. I should like to call attention here only to two other aspects of this work. The above model suggested that the β-carbon of phe^6 and phe^{11} might be in close spatial proximity. A bicyclic analog XVI was therefore synthesized in which phe^6 and phe^{11} were replaced by a cystine bridge.[46] In the D-trp^8 series this compound proved to be about equipotent with somatostatin both *in vitro* and *in vivo*. This result demonstrated that phe^6 and phe^{11} are not required for high biological activity and provided strong support for the proposed model. The observed high biological activity was a



XV

Fig. 4. Proposed conformation of des(ala[1],gly[2])desamino[cys[3]]-descarboxy[cys[14]]dicarba[3,14]somatostatin XV. Numbers at each residue refer to the residue number in somatostatin. Compound XVI has a disulfide link between the atoms labeled β. A sense of the relationships of the side chains is obtained by viewing the side chains of Asn[5], Phe[6], Thr[10], Phe[11], and Thr[12] as being axial and the side chains of Trp[8] and Lys[9] as being equatorial.[46]

surprising result in view of the earlier observation by Vale and his associates[47] that replacement of phe[6] by alanine led to an analog having less than 1% of the in vitro GH release suppressing activity of somatostatin, and that the replacement of phe[11] by alanine reduced the activity to 3%. These paradoxical results led directly to the suggestion that the two phenylalanine residues in positions 6 and 11 of somatostatin play an important role in stabilizing the active conformation of this hormone, and that the same conformation is stabilized in the bicyclic analog XVI through the covalent bond of the cystine bridge. Thus the amino acid sequence of a small peptide like somatostatin can be seen to provide

conformational information much as it does in proteins. Elegant NMR spectroscopic studies by Dr. Byron Arison[48] subsequently provided experimental results consistent with the presence of stabilizing hydrophobic interactions between phe^6 and phe^{11}. His results also showed that the stacking of the two aromatic rings is perpendicular rather than parallel. Such a "herringbone pattern" of aromatic rings is seen also in crystalline benzene and in carp parvalbumin. Its energetically favorable implications have been discussed by Dr. Kenneth Thomas and his associates at Merck.[49]

The high activity of XVI, the low activity of (ala^6) and (ala^{11}) somatostatin notwithstanding, shows that a more venturesome approach to medicinal chemistry than is often practiced will at times produce very significant results. Three further examples to illustrate this point will be given later.

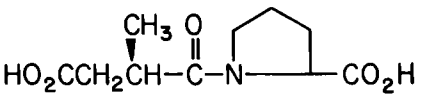
As we reported earlier, Dr. Veber and his associates have succeeded in designing and synthesizing several compounds possessing both high potency and a prolonged duration of action. The simplest molecules prepared to date, suitable for clinical studies, are cyclic hexapeptides.[50] Some of these compounds are active even after oral administration although they are not well absorbed. As we had hoped, Dr. John Gerich of the Mayo Clinic and Drs. Richard Saperstein and Monroe Glitzer in our laboratories showed that these compounds suppress glucagon release in animals and that they possess the desired insulin sparing effect first seen in human subjects by Gerich using somatostatin.[51]. It is noteworthy that one of our cyclic hexapeptides, which was studied in greater detail, was poorly absorbed p.o. even though it was excreted unchanged in the feces. This leads me to the conclusion that making peptides biostable does not ensure good absorption by the oral route.

Angiotensin converting enzyme inhibition

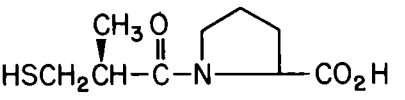
Let me now turn to work in the field of the angiotensin-converting enzyme (ACE) inhibitor design. As you are doubtless aware, the Squibb group headed by Ondetti and Cushman[52] climaxed a series of elegant studies with the design and synthesis of captopril, the first potent, orally active inhibitor of converting enzyme which demonstrated clinically that intervention in the renin angiotensin system provides a novel and attractive approach to the control of hypertension.

The design of captopril grew out of early work with peptides from the venom of a South American pit viper and was built also on the concept developed by Byers and Wolfenden[53] that D-2-benzylsuccinic acid is a potent competitive inhibitor of carboxypeptidase A because it serves as a "biproduct analog."

Ondetti and Cushman reasoned that if the interaction of a carboxyl group in a compound such as XVII with the zinc atom of the enzyme plays an important role in enzyme inhibition, replacement of the carboxyl group by a better zinc ligand should enhance potency. The fact that captopril XVIII is 2000-fold more potent than the prototype amply confirmed this concept. Reports that the use of captopril is accompanied by side effects such as rash and loss of taste, reminiscent of problems encountered with penicillamine, suggested to many workers that the observed toxicity might be due to the sulfhydryl function, which captopril and penicillamine have in common. An intensive effort was therefore launched in many laboratories which sought to replace the sulfhydryl function by another ligand which would bind equally well to the zinc atom of ACE. To my knowledge, no such equipotent mercapto replacement has been found. My colleague,



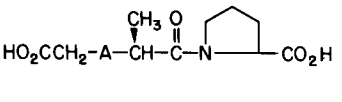
XVII



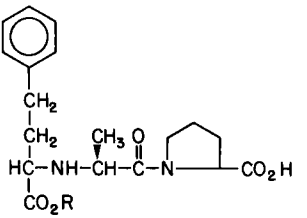
Captopril

XVIII

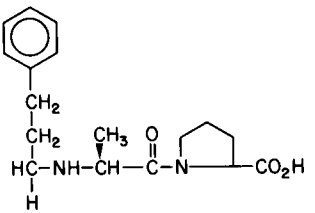
Dr. Arthur Patchett,[54] chose instead to finesse the problem. Having established that XX is an ACE inhibitor of only moderate potency, comparable to Squibb's XIX (both have values of I_{50} in the 2-5 μM range), Dr. Patchett and his associates synthesized XXI (enalaprilic acid) which is more potent than captopril. Esterification afforded XXII, a prodrug possessing good oral activity. Both the carboxyl and the phenethyl residues contribute to the binding to converting enzyme, as evidenced by the fact that enalaprilic acid is more than 3 orders of magnitude more active than either XX or XXIII. Thus, the carboxyl of enalaprilic acid replaces the mercapto group of captopril as a ligand to zinc, but far less effectively. A presumed hydrophobic interaction between the phenethyl residue and some hydrophobic pocket of ACE more than compensates for this loss. Unlike the intramolecular hydrophobic interactions of ribonuclease and somatostatin cited above, the phenethyl group of enalaprilic acid presumably illustrates an intermolecular hydrophobic interaction involving enalaprilic acid and ACE. This interpretation is consistent with the observation by Cushman and his associates that substrates of ACE containing an aromatic amino acid in the third position from the C- terminus are bound much more tightly to the enzyme than those having other residues in this position.[55] Viewed more



XIX A= $-CH_2-$
XX A= $-NH-$



XXI R=H = Enalaprilic Acid
XXII R=Et = Enalapril



XXIII

broadly, the success of my associates illustrates that groups seemingly required for high potency in a biologically active compound can at times be shown not to be required if one can discover an additional or alternate binding site on the receptor. In principle this is possible whenever the group to be deleted does not participate in a unique way in the regulating mechanism. I had cited above the observation that phe^{6} and phe^{11} of somatostatin, seemingly required for high activity,[47] could nevertheless be replaced by a cystine bridge with retention of high potency.[46]

Aspartic Acid Not Required for Gastrin-like Activity

I should like to mention one further example from the literature to illustrate this idea further. As is well known, Tracy and Gregory[56] had shown that the carboxy-

terminal tetrapeptide of gastrin, viz., Trp-Met-Asp-Phe-NH_2 (XXIV), possesses the same biological profile as the parent hormone. Subsequently, extensive studies by Morley[57,58] showed that substitutions are permissible in three of these four amino acids. Morley believed that the aspartic acid residue may have a functional rather than a binding role because it appeared to be indispensable for stimulation of gastric acid secretion. Even the replacement of aspartic acid by glutamic acid led to an inactive compound. Only one analog, in which the β-carboxyl group of aspartic acid was replaced by a tetrazolyl residue, retained activity.

The carboxy-terminal octapeptide of cholecystokinin contains the carboxy-terminal tetrapeptide of gastrin and has gastrin-like activity. It is noteworthy, however, that Trout and Grossman[59] reported in 1971 that the synthetic analog of the C-terminal octapeptide of cholecystokinin in which alanine replaces the penultimate aspartic acid residue (XXV) still retains about 4% of the stimulatory activity of pentagastrin. Whereas XXV produced significant stimulation of acid secretion at doses of 40 μg/kg/hr, Boc-Trp-Met-Ala-Phe-NH_2 had no effect at doses as high as 15 mg/kg/hr.

Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Ala-Phe-NH_2

XXV

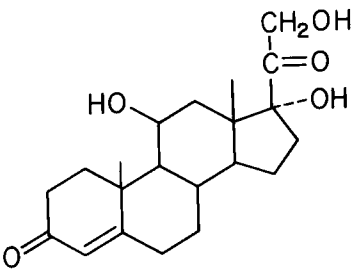
They concluded that the sulfated tyrosine was responsible for the relatively high activity seen with XXV.

It seems likely that the sulfate of tyrosine generates an ionic interaction with the receptor but it is conceivable that the hydrophobic part of tyrosine sulfate is in a hydrophobic environment on the receptor and that it also contributes to binding. In any case I should like to suggest

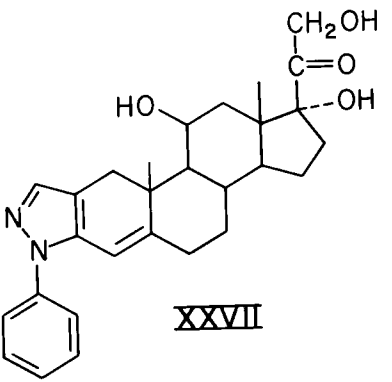
that there is a similarity between Patchett's ACE inhibitor and the alanine analog of the C-terminal octapeptide of cholecystokinin. In one case the seeming requirement for a sulfhydryl, in the other, for aspartic acid was voided by generating an alternate binding site via a phenethyl group or a tyrosine sulfate, respectively.

Δ^4-Pregnene-3-one Not Required for Glucocorticoid Activity

The concept of alternative binding may have a broader application to medicinal chemistry. One illustration from the steroid field serves to illustrate this concept. It had been generally accepted that the α,β-unsaturated ketone of the A-ring of cortisol XXVI is required for anti-inflammatory activity.[60] We were able to show[61] however that phenylpyrazole derivatives such as XXVII, which lack such an unsaturated ketone, are in fact the most powerful activity-enhancing modifiers of glucocorticoids which have been reported. We had proposed earlier[62] that the pyrazole ring may generate an electron distribution resembling that of the A-ring of XXVI. Beyond that, however, I should like to suggest that the powerful effect of the phenyl substitutent may be due to its interaction with an alternate binding site on the intracellular cortisol-binding protein.



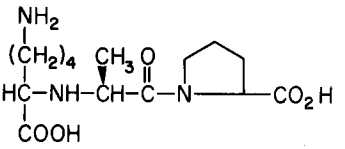
XXVI



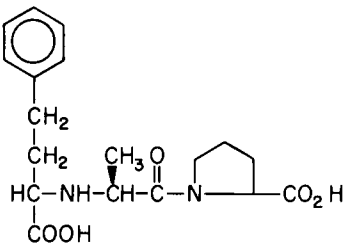
XXVII

Aryl Lysyl Sidechain Interactions

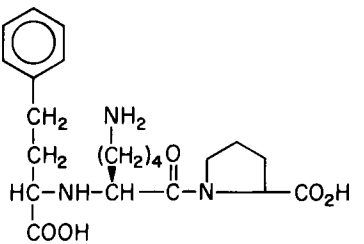
Let me briefly return to another aspect of our somatostatin research. The NMR studies by Dr. Arison with compounds generated by our somatostatin program had revealed in addition to the hydrophobic perpendicular stacking of the aromatic ring of phe^{6} and phe^{11} also a proximity of the side chain of lys^{9} to the aromatic ring of trp^{8} as inferred[63] from the upfield shift of the γ-methylene protons of lysine in the highly active D-trp^{8} analogs. These conclusions were based on prior work by Drs. Henry Joshua and Charles Deber[64] in our laboratories. A more recent study of the NMR spectrum of somatostatin by Van Binst[65] and his associates and by others showed that the γ-methylene of lys^{9} is also shielded, but to a lesser degree than in the D-trp^{8} series. Independently, I had been interested in demonstrating that the side chains of L-trp^{8} and lys^{9} can assume a stacked arrangement resembling that seen with the analogous D-trp diastereomers. Dr. Arison's studies with somatostatin demonstrated a marked temperature dependence of the chemical shift of relevant protons and confirmed that the low energy conformation of somatostatin has the γ-methylene of lys^{9} in the shielding cone of L-trp^{8} and the <u>ortho</u> and <u>meta</u> protons of phe^{6} in the shielding cone of phe^{11}. It is possible that energetically favorable hydrophobic interactions, as are seen intramolecularly between trp^{8} and lys^{9}, may also play a role in intermolecular interactions. In this context it is noteworthy that Patchett and his collaborators[54] showed that the phenethyl side chain of enalaprilic acid (XXI) may be replaced by a lysine side chain as in XXVIII and that the alanyl residue of enalaprilic acid may also be replaced by lysine as in XXIX (MK-521) without loss of inhibitory potency. It is of course possible that in XXVIII and XXIX hydrogen bonds or salt formation make up for the loss of hydrophobic interactions. Nevertheless, intermolecular



XXVIII



XXI Enalaprilic Acid



XXIX (MK-521)

hydrophobic interactions involving the methylenes of the lysine side chain and hydrophobic regions on the receptor might also play a role just as they do intramolecularly for the trp^{8} and lys^{9} of somatostatin. Such interactions would be expected to be weaker than the interactions between two aromatic rings.

Computerized Molecular Modeling

During the early seventies I had an opportunity to support the efforts of Dr. Peter Gund to establish a significant computerized molecular modeling group in our laboratories which would allow a hands-on participation by the synthetic chemists. Fruitful interactions involving Drs. Gund, Graham Smith, Joseph Andose and Bruce Bush of the Merck molecular modeling group, and medicinal chemists such as Drs. Roger Freidinger and Eugene Thorsett eventually materialized and have made important contributions to, for example, our somatostatin and ACE programs. We are very pleased about such success stories, but we also believe that one has to maintain both a healthy skepticism and a lively sense of humor about modeling predictions. This is true in any situation where we might be in danger of believing that we truly "understand" the interaction of a compound with its receptor. We also have to remember that obtaining a pleasing match on the screen is not an end in itself. These considerations do not lessen the impact that computer modeling has on our programs. Any reasonable new idea which occurs to a chemist as a result of computer modeling is worthwhile in the long run. If, as a consequence, a useful compound is synthesized earlier than might otherwise have been the case, something useful has clearly been accomplished whether or not the underlying idea ultimately turns out to have been correct.

Outlook

I should like to conclude by restating a thought which I expressed at the 3rd American Peptide Symposium in Boston in 1972:[66]

"Those of us who have witnessed the rise and decline of adrenocorticoid steroid synthesis from one of the most active fields of hormone research to one which is at present relatively quiescent might well ask whether the same fate is in store for peptide chemistry. I do not think so. There is only so much even nature can do with the cyclopentanoperhydrophenanthrene ring system, and there are only so many alkyl, halo and other substituents which the most imaginative medicinal chemist can attach to this skeleton. On the other hand, by using the 20 coded amino acids, nature has found a way to build molecules of all sizes and shapes which display a high capacity to interact with each other. The future looks bright, not because peptide chemists are so clever but because the building blocks--both natural and unnatural--which are at their disposal permit the synthesis of compounds with seemingly unlimited versatility of chemical, physical and biological properties."

Acknowledgment

I should like to thank Dr. Max Tishler, the former president of our laboratories, for his encouragement and unstinting support of peptide research, especially the enzyme synthesis. Similarly we are indebted to the current head of research, Dr. P. Roy Vagelos, who has strongly encouraged and supported the somatostatin and ACE programs.

It is a great pleasure to acknowledge the contributions of all the scientists, both chemists and biologists, whose work I have described today. I should like particularly to pay tribute to the exceptional dedication, experimental skill

and team spirit of everyone who contributed to the success of the synthesis of ribonuclease. Those who participated in this endeavor will not easily forget it.

Above all I am deeply grateful to all of my associates at Merck who generate an atmosphere for interdisciplinary research which is intellectually exciting, deeply satisfying and, as an added dividend, a great deal of fun.

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AN ASSESSMENT OF SOLID PHASE PEPTIDE SYNTHESIS

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Introduction

The first paper on solid phase peptide synthesis was published just twenty years ago.[1] The purpose of this presentation is to outline briefly the trends in the development of the idea, to pinpoint some of the synthetic achievements, and to predict the future potential of the technique. Current practice is surprisingly close to the general principles initially proposed and it is gratifying to know that the basic idea has held up over this period of time. Essentially all of the details have been changed, however, and much time and effort by many laboratories has been spent in designing and evaluating these changes.

The Solid Support

Foremost among the areas to be considered is the composition and structure of the solid support and the understanding of its chemical and physical behavior during the synthetic process. In principle a wide range of supports is possible, but in practice only two, the original copoly(styrene-divinylbenzene) and the polyacrylamide resins[2] are used extensively. Figure 1 shows a simplified sketch of a resin bead, which can serve as a model for a discussion of its role in the synthesis. The beads consist of a gel-like structure of randomly coiled, lightly crosslinked (1%) polystyrene chains, which become highly solvated in solvents like dichloromethane or dimethylformamide. The peptides are synthesized as covalently anchored pendant chains and may assume random, helical or β-structures

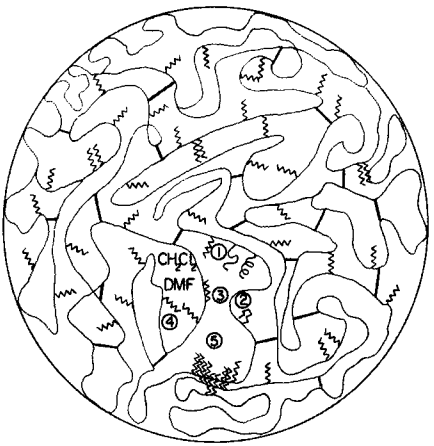

Fig. 1. A sketch of a resin bead containing peptide chains.

depending on their composition and environment. The polymer and peptide chains have been shown both by chemical and physical methods to be highly mobile. This property was examined recently[3] by the synthesis of side-chain protected resin-bound glutathione chains, followed by the acylation of exactly one half of the free α-amino groups with 0.5 mole of succinic anhydride. When the resulting carboxyl groups were activated with DCC and HOBt and allowed to crosslink with the other half of the amino sites, 99.5% of the peptides reacted. The extended lengths of the peptides and their average distance apart on the support were such that extensive movement of the polymer chains was necessary for this to occur. It seems clear now that effective site isolation is a kinetically controlled phenomenon[4] and can only be achieved under special conditions. For peptide synthesis, however, we do not need or want rigid isolation. What we do want is good solvation of the polymer chains and their pendant peptide chains and a polymer gel network that will swell freely so that there is adequate space for the peptide to grow. Recent studies[5] have been enlightening. A series of peptide-resins was synthesized containing high loading of linear peptides up to

6000 MW. Bead diameters showed dramatic increases during the course of the synthesis. When the bead contained 80% peptide and only 20% polystyrene, the dry volume increased 5-fold and the swollen volume in DMF increased from 3.3 ml/g to 28 ml/g, showing that the resin matrix did not fill up with peptide as the chain grew longer, but continued to solvate and swell to accomodate the additional mass. The lightly crosslinked resin and the peptide exert a mutual solubilizing effect on one another and enhance the reactivity. The coupling reactions are observed to be rapid, $k_2 \sim 10$ l/mol·sec, and to proceed nearly to completion. The synthetic efficiency, estimated by analysis of deletions, was unchanged by chain lengths up to at least 60 residues.

The enhancement of solvation and reactivity by the crosslinked resin was demonstrated by studies on synthetic oligoisoleucines. In free solution the homo oligomer becomes insoluble and unreactive at the tetrapeptide stage but when attached to linear non-crosslinked polyethylene glycol insolubility and unreactivity were not reached until 7 or 8 residues.[6] With 1% crosslinked polystyrene, Kent[7] observed no limitation up to 12 residues. I believe that the support is not only of benefit to the physical manipulations of the synthesis but also to the coupling reactions themselves.

Some special peptides have, however, been observed to react slowly and incompletely during synthesis. The coupling of Boc-Tyr(Cl_2Bzl) to the tripeptide H-Ile-Asn-Gly-Resin is a good example.[7] This difficulty at high loading in CH_2Cl_2 was eliminated by reducing the loading and using DMF for the coupling solvent. We have interpreted this as an intermolecular aggregation of peptide chains and have shown that solvents which compete with peptide-peptide interactions are beneficial. A similar effect was observed during the synthesis of cytochrome C (66-104) on a polyacrylamide-resin.[8]

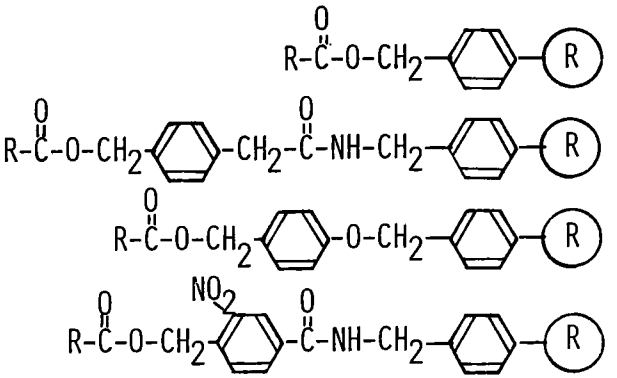

Fig. 2. Handles for attachment of peptide chains.

Attachment to the Solid Support

The original benzyl ester linkage via the chloromethylated resin is still used for small peptides. The main improvements in this area have derived from the introduction of handles (Figure 2), which can be designed to have several advantages: 1, greater stability toward the synthesis conditions, primarily to the acid deprotection step; 2, better characterization of the first attached residue; 3, more flexibility in the design of the synthesis so that the peptide chain can be selectively removed either as a free peptide, a protected peptide, or a protected peptide still containing a handle for reattachment to another resin support. Resins with handles that can be cleaved selectively at two or more sites have been termed multidetachable resins.[9]

The Protection Scheme

The initial scheme was not selective enough and soon evolved into the one now commonly in use, i.e. N^{α}-Boc and benzyl-based side chain groups, which depends on selective acidolysis. Many modifications to enhance the selectivity have been made and those with N^{α}-Bpoc and Ddz are often used. I think the most important new development in the protecting group strategy has been the invention of the base-labile Fmoc group by Carpino,[10] and its introduction in combination with

acid-labile side chain protection into solid phase synthesis by Meienhofer[11] and by Atherton et al.[12] It offers the advantages of mild conditions and high selectivity without serious side reactions, and seems certain to find increased use.

The Coupling Reaction

The DCC coupling method initially employed has served very well, and because of its simplicity continues to be the standard procedure. However, it is gradually being replaced by two newer methods. The most important of these is the symmetric anhydride, introduced by Wieland[13] and applied to solid phase synthesis by Hagenmeier[14] and by Li.[15] Hydroxybenzotriazole esters[16] prepared *in situ* by the DCC reaction have greatly improved the coupling of asparagine and glutamine by eliminating the usual nitrile and amidine by-products. This is the method of choice for these residues.

Monitoring the Coupling and Deprotection Steps

It has been recognized for a long time that analytical control of solid phase synthesis is important. Many discontinuous methods have been developed which require stopping the synthesis and sampling the reaction mixture, e.g. the qualitative fluorescamine[17] and ninhydrin[18] tests for unreacted amino component and a recently developed quantitative ninhydrin analysis.[19] The latter depends on dissociation of the usual resin-bound blue color with a quaternary amine

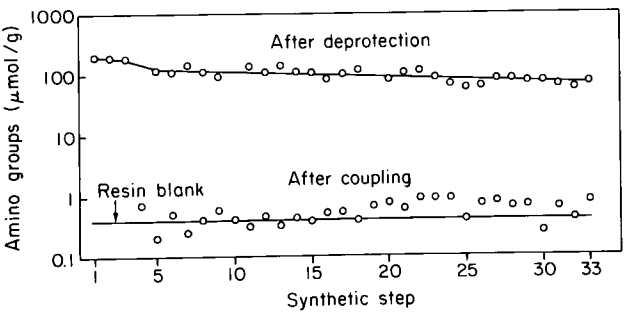

Fig. 3. Monitoring of a cecropin A(1-33) synthesis by a quantitative ninhydrin method.

chloride and spectrophotometric analysis of the solubilized Ruhemann's purple. This can be illustrated by the monitoring of a recent cecropin A(1-33) synthesis[20] (Figure 3). The average deviation from the blank was about 0.2% of total chains. This level of completion of the coupling reactions was in general agreement with the levels found by preview sequencing of the completed peptide-resin and is near the sensitivity of these methods.

Continuous, real time monitoring is more desirable, but in general is less precise near the end of the reaction. Several applications of this approach have been reported for discontinuous synthesis and it is now becoming very effective for monitoring continuous flow syntheses, where spectrophotometric measurement of unreacted reagent can be conveniently measured.

Automation

Early in the development of solid phase peptide synthesis it was suggested that the method would lend itself to automation.[1] An instrument was developed within a few years and many others have followed, including several commercial models. The major improvements have largely been concerned with the electronics and operation controls and several applications of microprocessors and computers have appeared.[21] The most interesting recent developments in automation have been in continuous flow systems first reported by Scott[22] and Erickson. These offer many advantages and may well become the major instruments in the field.

Cleavage and Deprotection

Acidolytic cleavage of peptides by HF is commonly used to liberate the synthetic peptide from the solid support and to deprotect the side chains. Several important side reactions are known, however, and a number of people believe that this is a very harsh treatment and are opposed to subjecting peptides to such a reagent. During the past three years some

important advances in the understanding and use of HF have been made in our laboratory.[24,25] It was recognized that the usual cleavage and deprotection by this reagent proceeds under S_N1 conditions, leading to carbonium and acylium ions, which produce the undesirable side reactions. It was reasoned that conditions which would allow the reactions to proceed by an S_N2 mechanism should avoid these problems. By diluting the HF with a base of proper pKa it was possible to arrive at conditions where the bonds to be cleaved were protonated, while the diluting base was largely unprotonated at the acidity function of the mixture. Dimethylsulfide (DMS), pKa -5.2, was selected and shown to be a suitable nucleophile for the purpose. The initial rate of the reaction

$$\text{Ser(Bzl)} \xrightarrow[0°]{\text{HF/CH}_3\text{SCH}_3} \text{Ser} + \text{Bzl-S(CH}_3)_2$$

and the product analysis for the reaction

$$\text{Tyr(Bzl)} \xrightarrow[0° \text{ 1 hr}]{\text{HF/CH}_3\text{SCH}_3} \text{Tyr} + \text{3-Bzl-Tyr} + \text{Bzl-S(CH}_3)_2$$

were determined for mixtures of HF and DMS from 0 to 100 volume %. The kinetics and by-product analysis both indicated the S_N1 mechanism at high concentrations. Between 40% and 60% HF there was a sharp break in the curves, coinciding with a mechanistic change to an S_N2 reaction. At 25% HF/75% DMS the reactions were still sufficiently rapid to fully deprotect within 1 hr at 0°. Furthermore, the side reactions (e.g. Tyr, Trp, Met and Cys alkylation, and acylation of aromatic rings by Glu) were essentially eliminated. In addition, Met(O) residues were quantitatively reduced to Met and, by the addition of thiocresol to the system, the N^i-formyl group was removed from Trp.

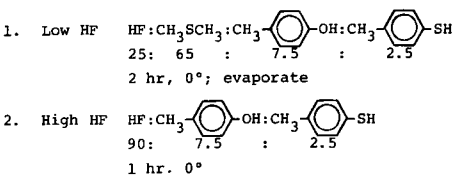

Fig. 4. The recommended low/high HF procedure.

Some groups such as Arg(Tos), Asp(OcHex), Cys(MeBzl) and Pam-resin are not cleaved under these conditions and require a second treatment with 90% HF. The recommended HF procedure is shown in Figure 4.

Synthetic Achievements

The synthesis of small and medium sized peptides (up to 40 or 50 residues) by solid phase methods has gone far beyond what I could imagine in 1963. Peptides are being made in such large numbers they cannot be dealt with individually here. For illustrative purposes two from our own laboratory have been selected. Figure 5 shows the HPLC analysis of cecropin A(1-33)[20] obtained directly after HF cleavage and then after ion exchange chromatography on CM-Sepharose. The crude material after 33 cycles of synthesis was not homogeneous, but was largely (78%) the correct product and could be readily purified in one step to near homogeneity. I think this quality of synthesis is being found in most laboratories for most peptides, although certainly not for all. A more demanding test of the methodology is illustrated by the synthesis of human gastrin I. This 17-residue peptide contains one Tyr, Met, Asp and Arg, two Trp, and a sequence of five Glu residues, all of which can be problems. The HPLC (Figure 6) of the crude cleavage mixture showed a major product, a

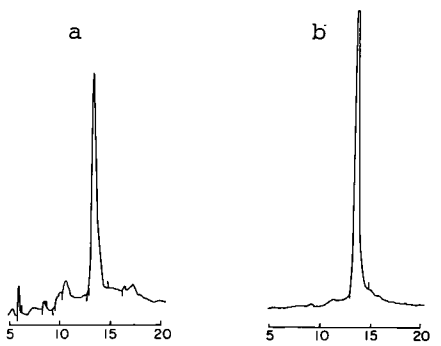

Fig. 5. HPLC analysis of (a) crude and (b) purified synthetic cecropin A(1-33).

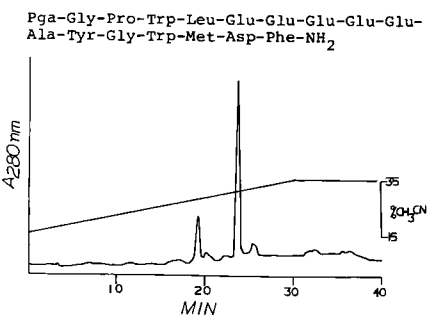

Fig. 6. The sequence and HPLC analysis of crude HF-cleaved human gastrin I.

significant amount of the sulfoxide (formed during workup) and relatively small amounts of other impurities. HGI was isolated in 63% overall yield from the first residue. A comparison with a literature synthesis[28] using similar synthetic procedures, but with cleavage in 90% HF/anisole is quite remarkable.

In contrast, the synthesis of very large peptides and small proteins has not progressed as far as I would have predicted 10 years ago, and relatively few satisfactory syntheses have been reported. We continue to work on this problem, and today I would like to report on our efforts to synthesize interferon. This has been a joint project with Elizabeth Merrifield, Virender Sarin, Richard DiMarchi and James Tam of our laboratory and Matilda Krim, Imre Mecs and Floyd Fox at the Memorial Sloan Kettering Cancer Center. The sequences of human leukocyte interferon α_1[29] and α_2[30] are shown in Figure 7. The α_2 is 10 residues shorter but otherwise there is a high degree of homology. Many samples of smaller peptides were removed at intervals during the synthesis

Fig. 7. The sequences of human leukocyte interferon α_1 and α_2.

α_1 / α_2 1 Cys-Asp-Leu-Pro Glu/Gln Thr-His-Ser-Leu Asp-Asn/Gly-Ser Arg-Arg-Thr-Leu-

16 Met-Leu-Leu-Ala-Gln-Met Ser-Arg/Arg-Lys Ile-Ser Pro-Ser/Leu-Phe Ser-Cys-Leu-

31 Met/Lys Asp-Arg-His-Asp-Phe-Gly-Phe-Pro-Gln-Glu-Glu-Phe Asp/- Gly-

46 Asn-Gln-Phe-Gln-Lys-Ala Pro-Ala/Glu-Thr Ile Ser/Pro Val-Leu-His-Glu Leu/Met

61 Ile-Gln-Gln-Ile-Phe-Asn-Leu-Phe Thr/Ser Thr-Lys-Asp-Ser-Ser-Ala-

76 Ala-Trp-Asp-Glu Asp/Thr Leu-Leu-Asp-Lys-Phe Cys/Tyr Thr-Glu-Leu-Tyr-

91 Gln-Gln-Leu-Asn-Asp-Leu-Glu-Ala-Cys-Val Met/Ile Gln Glu-Glu-Arg/Gly-Val-Gly

106 Val Gly/Thr Glu-Thr-Pro-Leu-Met Asn-Ala/Lys-Glu Asp-Ser-Ile-Leu-Ala-Val-

121 Lys/Arg Lys-Tyr-Phe Arg/Gln Arg-Ile-Thr-Leu-Tyr-Leu Thr/Lys Glu-Lys-Lys-

136 Tyr-Ser-Pro-Cys-Ala-Trp-Glu-Val-Val-Arg-Ala-Glu-Ile-Met-Arg-

166

151 Ser Leu/Phe Ser-Leu-Ser-Thr Asn-Leu-Gln-Glu-Arg-Leu-Arg-Arg-Lys-Glu
- - - - - - - - - -

but none showed any interferon activity. The complete chains of α_1, α_2 and a Ser^1 analog of α_1 were purified as outlined in Figure 8. The initial products contained significant amounts of antiviral activity in human, bovine and mouse cells in a micro CPE inhibition assay using a vesicular stomatitis virus challenge. The preparations were free of cytotoxicity based on growth and morphology of the cells and showed kinetics of development and duration of anti-viral effect comparable to native HuIFα_1. The results suggest that Cys^1 of α_1 may not be involved in an essential disulfide bond. The specific activities have been increased by HPLC on LiChrosorb RP-8 columns.[30] Further purification of very small portions on an antibody column has given synthetic interferon of high specific activity comparable to that of the natural standards.

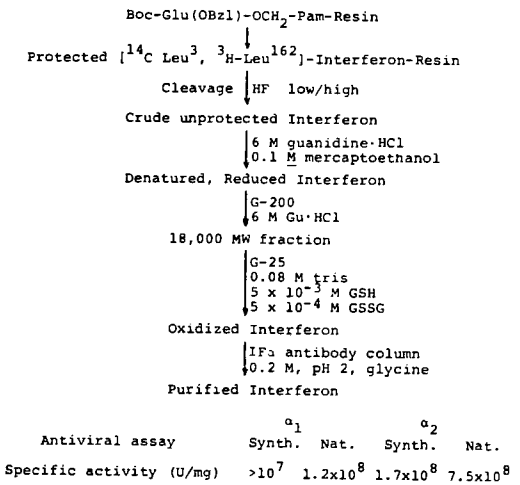

Fig. 8. Summary of interferon syntheses.

We are encouraged by these results and are optimistic that the chemical synthesis of small proteins will continue to improve.

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THE POLYAMIDE METHOD OF SOLID PHASE PEPTIDE SYNTHESIS. SOME RECENT DEVELOPMENTS

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The polyamide method[1,2] represents an attempt to design a solid phase system in which all the chemical operations are carried out under mild and substantially optimised reaction conditions, thus giving maximum opportunity for high yields and freedom from side reactions. It derives, of course, from the basic principles so well laid down by Bruce Merrifield more than twenty years ago,[3] but differs from the established technique in several ways. Notable differences are:

(i) Use of a polar, polyamide gel support[1] in place of the customary polystyrene. This support is freely permeated by polar, aprotic media of the dimethylformamide type which are particularly favourable solvents for many of the reactions involved in peptide synthesis. Solvation differences between the solid support and the attached peptide chains are reduced, minimising aggregation phenomena within the resin matrix.[4]

(ii) Use of fluorenylmethoxycarbonyl (Fmoc) amino-acids[5,6] in place of the more usual Boc derivatives. This is an important feature which avoids the repeated acid treatments associated with the conventional method. The base catalysed deprotection step is exceptionally mild and is favoured by the polar reaction environment. By permitting a new side chain protecting group strategy utilising largely t-butyl (in place of benzyl) derivatives, it also enables the frequently destructive final liquid hydrogen fluoride treatment to be dispensed with.

(iii) Use of a range of reversible peptide-resin linkage agents.[7] These variously substituted benzyl alcohol derivatives confer great flexibility on the method, providing

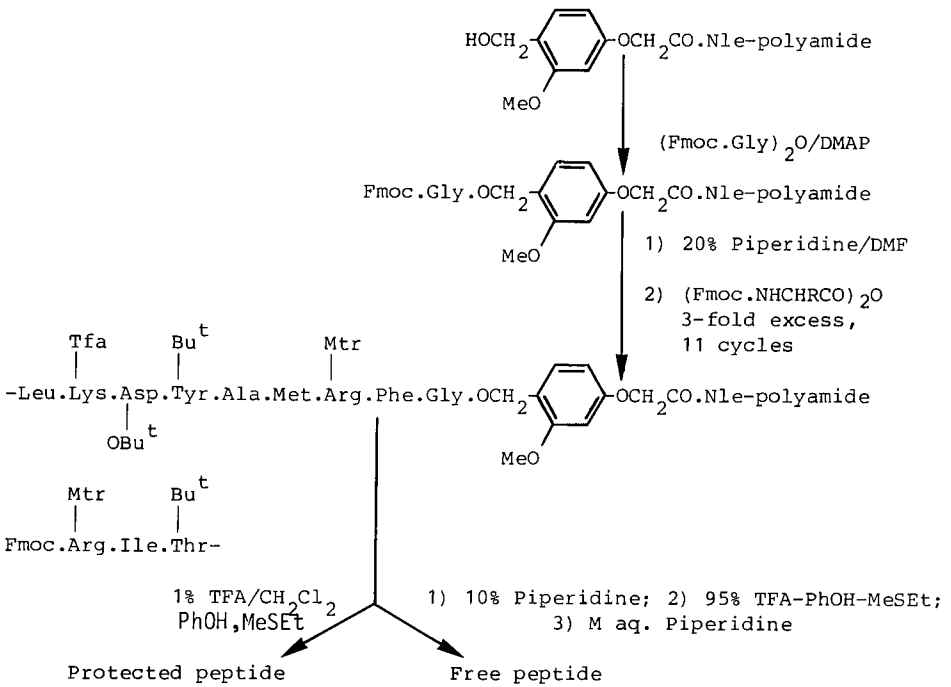
specific strategies for the preparation of, e.g., free peptides, peptide amides, and side chain protected peptides for fragment condensation strategies (see below).

A recent development in the polyamide method has been the introduction of continuous flow methods. Our interest in this area arose early, largely because of the prospect of real time analytical control. Simple polydimethylacrylamide gel resins proved unsatisfactory in pumped column systems. Packing down of the gel occurred with the generation of very high pressures. When the gel is enclosed in a glass column this packing down process is seen to be sometimes quite extreme. Solvent is squeezed from the resin, its volume is reduced to a fraction of the normal expanded state, and the highly solvated internal structure is destroyed. We felt that these observations were of poor augury for efficient synthesis and chose instead to develop a new physically strong support[8] which retained an internal solvated gel structure while permitting free solvent flow.

Flow through a column of gelatinous beads probably occurs largely through the interstitial channels. The beads themselves present a high resistance to forced fluid flow, and solvent and reactant penetration must be largely diffusion controlled. Blockage of the interstitial channels through deformation of the soft swollen beads leads to high pressures and gel compression. In our new resin, the dimethylacrylamide-based monomer mixture is polymerised within the pores of rigid, macroporous inorganic particles.[8] Macroporous kieselguhr has proved suitable for both peptide and oligonucleotide synthesis. Other rigid support-gel combinations have also been prepared but their utility has not yet been fully explored. Diffusion of reactants into and out of the kieselguhr-supported polydimethylacrylamide* is rapid, and negligible pressure is developed under normal pumped flow conditions.

Flow methods based on the new composite resin are now in routine use in our laboratory. The same high chemical

efficiency is obtained as with the unsupported gel.[12] A recent application is outlined in Scheme I. At the last Symposium,[13]



SCHEME I

we reported that short peptides linked to solid supports through the dialkoxy linkage agent illustrated in Scheme I could be detached by very mild acid treatment without the loss of other acid-labile protecting groups, and offered an efficient route to protected peptides for fragment condensation studies. The dodecapeptide sequence comprising residues 4-15 of the cro repressor protein sequence provides a further more substantial example. The tyrosine, threonine, aspartic acid, and arginine (Mtr = 4-methoxy-2,3,6-trimethylbenzene sulphonyl)[9] residues were all protected as trifluoroacetic acid-labile derivatives. Cleavage of the protected peptide from the solid support was achieved efficiently (86-95%) by percolating 1% trifluoroacetic acid-dichloromethane-methyl ethyl sulphide

mixture through the resin bed. The effluent was quenched immediately in dichloromethane containing dimethylformamide, and the Me_2NCHO-CF_3CO_2H complex removed with the aid of Sephadex LH20. The total crude peptide fraction (Found: Asp, 0.98; Thr, 1.05; Gly, 1.00; Ala, 1.04; Met, 1.00; Ile, 0.94; Leu, 1.02; Tyr, 1.05; Phe, 1.05; Lys, 0.95; Arg, 1.86) showed little evidence of side chain deprotection when examined in two widely different hplc systems (Figs. 1a,b). The dodecapeptide was also characterised in its fully deprotected form, confirming the high efficiency of the synthesis (Figs. 2,3).

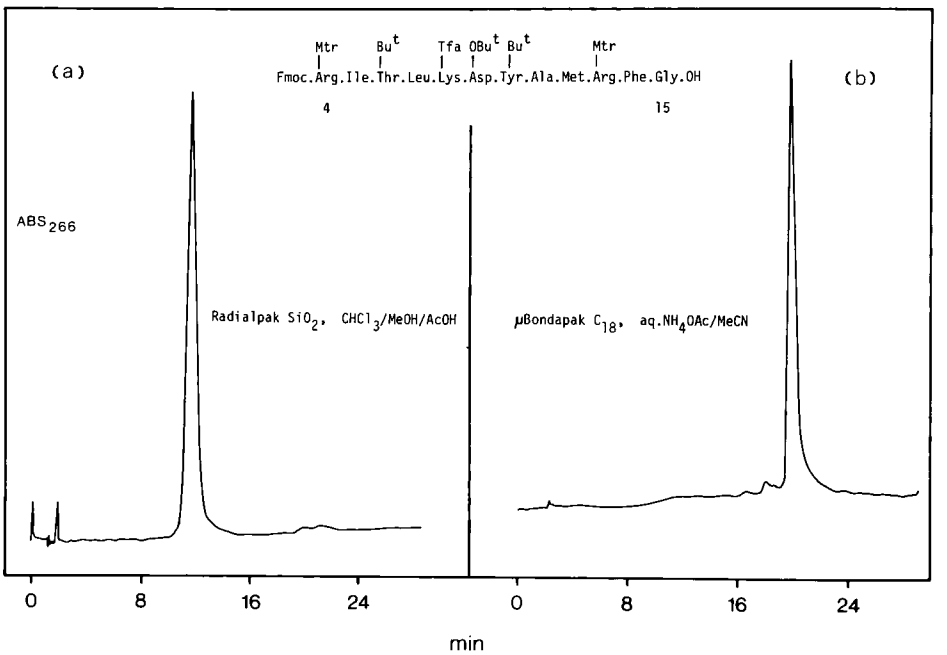


Fig. 1. Hplc of protected cro 4-15 dodecapeptide. Conditions: (a) Radialpak B silica column; A = $CHCl_3$, B = $CHCl_3$/MeOH/AcOH (75:15:5), gradient 20%-80% B over 30 min. (b) μ-Bondapak C_{18} column; A = 0.01 M Mt_4OAc, pH 4.5, B = MeCN, gradient 40%-100% B over 30 min. Flow rates: (a) 3 ml/min; (b) 1.5 ml/min.

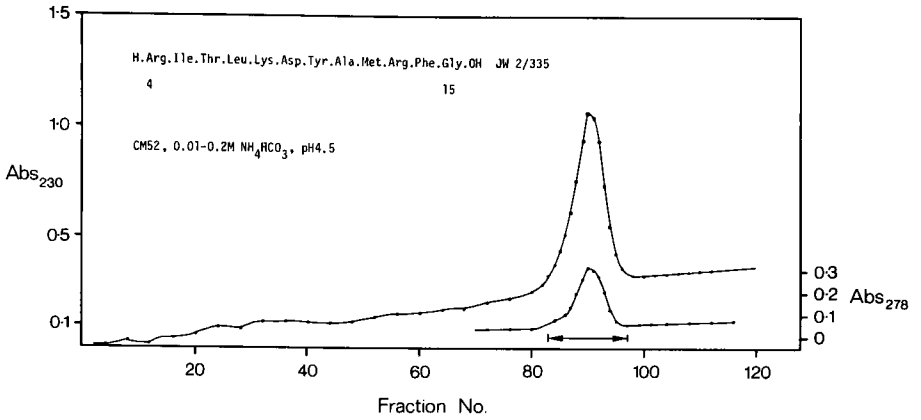


Fig. 2. Ion exchange purification of free cro 4-15 dodecapeptide

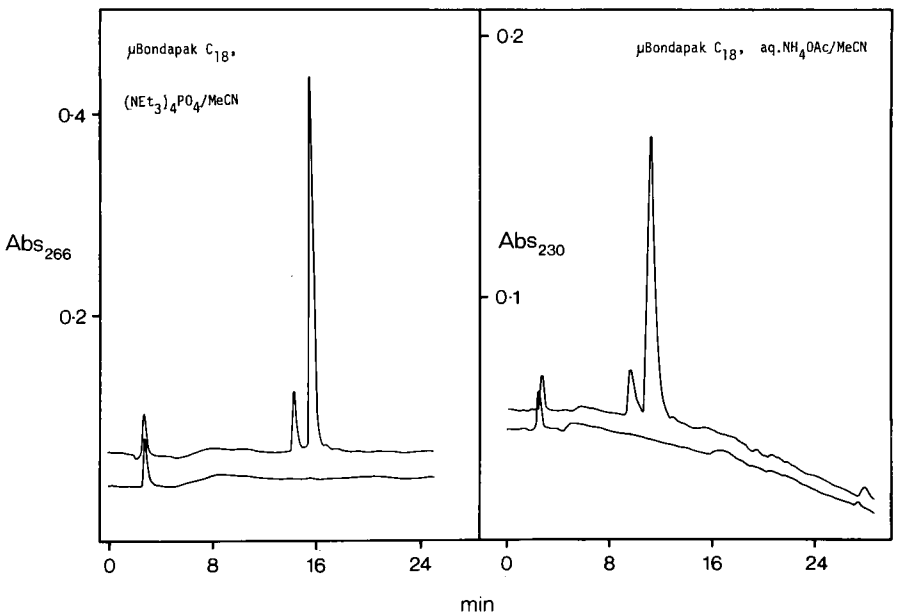


Fig. 3. Hplc of free cro 4-15 dodecapeptide. The smaller, forerunning peak is coincident with sulphoxide produced by hydrogen peroxide oxidation of the main product.

A simple continuous flow synthesiser has been built enabling the composite resin to be used under both manual and computer control.[10] In the latter, besides controlling the various flow and recirculate functions, the microprocessor also receives data from a spectrophotometer which continuously monitors the effluent stream. In principle, the fluorenylmethyl chromophore enables reactant concentration to be measured in the liquid phase in both acylation and deprotection steps, but there are substantial problems. Concentrations are too high for measurement at λ_{max}, even in the narrowest flow cells currently available. Beer's law is not obeyed. In part at least this is due to formation of a fluorescent complex between fluorene derivatives and solvent dimethylformamide. Most of the problems now seem to have been resolved and useful quantitative data obtained, though it will be difficult to achieve the degree of precision which the control of solid phase synthesis really demands. Thus using only a two-fold excess of Fmoc-amino-acid anhydride, the decrease in fluorenylmethyl chromophore would be 25% for complete acylation, whereas an unacceptable 95% reaction would give a decrease of just under 24%. With greater excess of acylating agent the situation worsens. In the acylation step, this difficulty in obtaining precise absolute data may be partly counterbalanced by following the reaction for several passes through the column. Trends in reactant concentration may then be monitored over a period.

A very recent result from step 8 of a current synthesis of avian vasoactive intestinal peptide is shown in Fig. 4. In (a) the absorbance due to recirculating Fmoc.Lys(Tfa) anhydride and acid is measured continuously at 304 nm using a flow cell of path length 0.1 mm. 500 Data points have been collected at 2 sec intervals by a Hewlett Packard HP85 microcomputer, which is simultaneously controlling all other synthesiser functions. The first peak represents total Fmoc absorption before passage through the reaction column; subse-

quent peaks after 1-4 passages. Diffusion in the column and mixing in the reciprocating pump result in progressive broadening and overlap of the peaks, ultimately to a uniform reactant concentration throughout the system. In (b) the data has been corrected and replotted by the computer so that it is now linear in Fmoc concentration rather than absorbance. The solid line is a representation of the areas under successive peaks. Two methods are currently under investigation for evaluating these areas. In the first a simple integration process is applied with verticals dropped from valleys to the

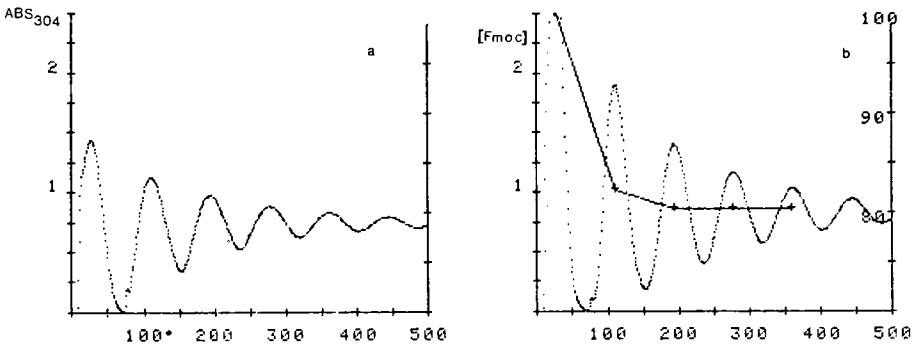


Fig. 4. Photometric record of recirculating Fmoc.Lys(Tfa) anhydride and acid (see text).

baseline. This method becomes progressively less sensitive as the overlap between adjacent peaks increases. In the second method, a curve fitting procedure is applied which includes amongst its variable parameters continuous and incremental diffusion coefficients as well as concentration terms.[11] This procedure is much slower, requiring ten minutes or more computing time after data collection is complete.

The plateauing of successive areas in this and many other similar curves obtained in the VIP synthesis is encouraging and indicates complete reaction after just a few passes through the column. Ninhydrin and trinitrobenzenesulphonic acid colour

tests were negative at the first determination after 25 min. Simple interpretation of the data indicates 91% reaction after a single passage through the column. The preceding step [coupling of Fmoc.Tyr(Bu^t) to leucine] was similarly calculated to be 87% complete after one pass. Much more experience will be necessary in the interpretation of these curves and numerical values to establish their reliability, but if they provide a good indication of the progress of acylation, it will be a simple matter to use the data directly in feedback control.

The deprotection steps in the Fmoc-polyamide procedure provide only simple single peak curves as the piperidine adduct of dibenzofulvene is released into solution. Three such curves are illustrated in Fig. 5. Curve (a) represents

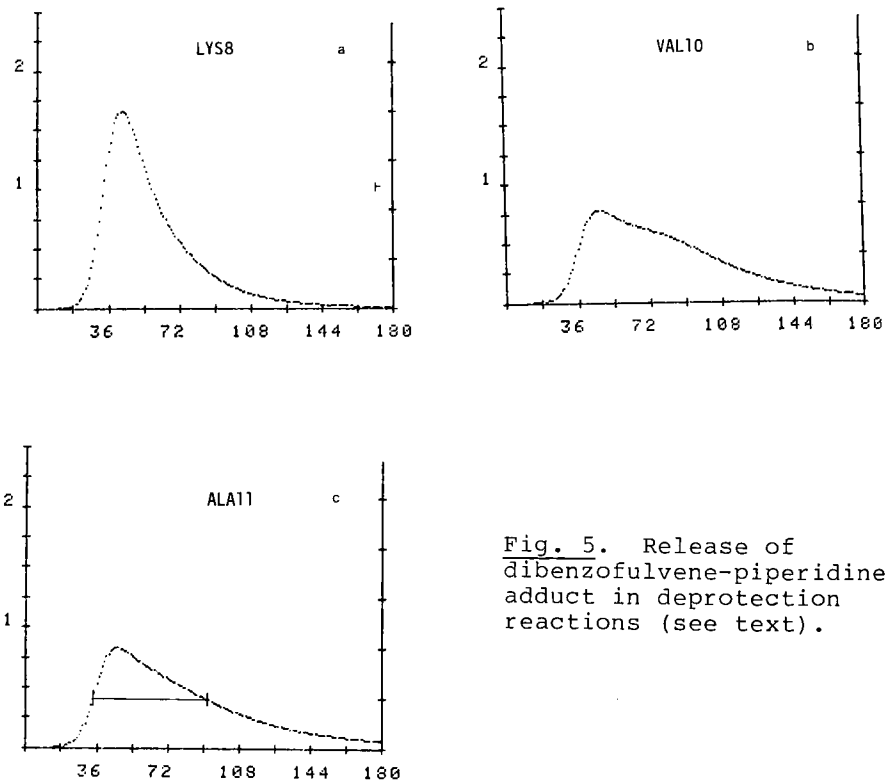


Fig. 5. Release of dibenzofulvene-piperidine adduct in deprotection reactions (see text).

the deprotection of the Fmoc.Lys(Tfa)-peptide resin formed in Fig. 4 above and is a typical result for what we believe to be a normal deprotection step. All the preceding steps in the synthesis and that immediately following gave similar curves with similar areas, although we have not yet established a correction table for linearisation of absorption and concentration. Remarkably, however, an abrupt transition took place at step 10 (deprotection of Fmoc.Val-peptide resin) (Fig. 5b). The following deprotection step is illustrated in Fig. 5c and all subsequent steps were similar. There has evidently been a sharp change in reaction rate possibly associated with a change in peptide-resin structure, though corresponding changes were not found in acylation steps. Only 180 data points were collected during the monitoring of the deprotection reaction so that the curves obtained represent only six minutes of a ten-minute reaction. Fig. 5a indicates complete deprotection (and elution of the reaction product from the column) after this period; Fig. 5b indicates that reaction was incomplete after six minutes. Again much further experience is needed in the interpretation of this data, but at the time we reacted immediately by increasing the deprotection time to 20 minutes. The VIP synthesis is presently incomplete, but assembly of the amino-acid sequence appears to have been satisfactory as judged by preliminary amino-acid analysis results.

In our experience, spectrometric monitoring of continuous flow solid phase synthesis contributes usefully to the conduct of the synthesis. Even in a qualitative sense the information provides a very welcome reassurance that all is proceeding normally. Quantitation is possible and offers the prospect of automatic feedback control. The data is obtained without addition of chemical reagents which might lower the efficiency of the synthesis. It is obtained early in each reaction period when intervention is possible and useful. The ease with which the data may be obtained is certainly associated with use of a resin system which does not generate high pressures in the circulating fluid.

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11. We are grateful to Dr. R. Diamond for developing this procedure.

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* This resin support (Pepsyn K) is available from Cambridge Research Biochemicals, Ltd., Button End Industrial Estate, Harston, Cambridge CB2 5NX, England, and Omnifit, Ltd., 51 Norfolk Street, Cambridge CB1 2LD, England.

NEW METHODS IN PEPTIDE CHEMISTRY

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Introduction

Although a large variety of methods covering almost every aspect in peptide chemistry has been developed over the last years, the synthesis of peptides still remains an undiminished challenge. Besides the unpredictable difficulties inherent in any new peptide sequence, a series of problems related to the known methodology has to be resolved, in order to avoid the formation of undesired side products and to facilitate the final purification

In the following, efforts to improve the existing methodology and to develop new procedures, where urgently required, are discussed on a few examples.

Protection of amino functions

Since the discovery of the benzyloxycarbonyl group[1] benzyloxycarbonyl chloride is used to prepare both in laboratory and industrial scale benzyloxycarbonyl-amino acids as the most widely used starting materials in peptide synthesis. As well known this reagent can hardly be prepared at a high degree of purity; it is generally contamined in varying extents by benzyl chloride as determined by ^{1}H-NMR, and on storage additional decomposition takes place. The contamination lowers the yields in amino acid derivatives and leads to undesired side products particularly at the level of

hydroxy amino acids and arginine. All attempts to replace benzyloxycarbonyl chloride with alternative acylating agents, e.g. mixed carbonates with phenols and N-hydroxysuccinimide[2] or urethane derivatives with oxazoles[3], were unsuccessful partly because of their laborious and costly preparation, partly because of the more difficult workup of the acylation mixture. Our attempts to prepare the dibenzyl-dicarbonate analoguously to the di-tert-butyl-dicarbonate[4] failed, thus prompting us to search for less expensive acylation agents as shown in Fig. 1. These are smoothly prepared via reaction of benzyloxycarbonyl chloride with 2-mercaptobenzoxazole, 2-mercaptobenzothiazole, 2-mercaptobenzimidazole and 2-mercaptopyridine, respectively. The resulting N- and S-acylderivatives are obtained as crystalline, analytically well characterized compounds which are stable on storage. These acyl-donators allow the preparation of benzyloxycarbonyl-amino acids in satisfactory yields (Fig. 2). Their main disadvantage derives from the poisoning effect of traces of these mercapto-compounds on the catalyst used for the subsequent hydrogenolytic removal of the benzyloxycarbonyl group. A promising bypass to this limitation may possibly result from the use of a catalyst newly developed by E. Bayer et al.[5].

For years a serious side reaction accompanying the introduction of amino protecting groups has been overlooked. More efficient chromatographic methods developed recently, allowed us to detect in simple N^{α}-protected amino acid derivatives the presence of related dipeptides to various extents. For example the introduction of the tert-butyloxycarbonyl group particularly into aliphatic amino acids via the di-tert-butyl-dicarbonate was observed to generate related dipeptide derivatives in extents up to 10 % when the reaction is performed at high concentrations in solutions enriched in organic solvents, where formation of mixed anhydrides is favored. Similarly even benzyloxycarbonylation via benzyloxycarbonyl chloride was found to produce as side products dipeptides, e.g. ca. 20 %

Z-Cl + H-Y $\xrightarrow{\text{TEA}}$ Z-Y

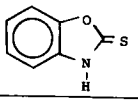
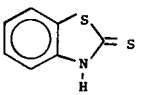
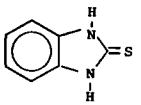
H-Y	yield	m.p.°C	elemental analysis					
	77%	89 - 92	$C_{15}H_{11}NO_3S$ (285.324)	calcd found	C 63.14 63.24	H 3.89 3.86	N 4.91 4.63	S 11.24 11.27
	77%	87	$C_{15}H_{11}NO_2S_2$ (301.391)	calcd found	C 59.78 59.55	H 3.68 3.64	N 4.65 4.64	S 21.28 21.26
	51%	140	$C_{15}H_{12}N_2O_2S$ (284.335)	calcd found	C 63.36 63.27	H 4.25 4.21	N 9.85 9.66	S 11.27 11.39
	71%	37	$C_{13}H_{11}NO_2S$ (245.299)	calcd found	C 63.65 63.59	H 4.52 4.50	N 5.71 5.72	S 13.07 13.44

Fig. 1

Z - Y + H-Val-OH ⟶ Z-Val-OH

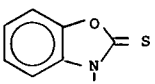
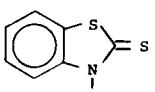
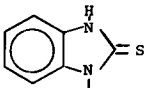
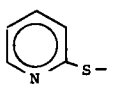
Y	yield	m.p. Lit (66-67 °C)	$[\alpha]_D^{20}$ 15.9 °	$[\alpha]_{546}$ 19.7°	c = 1, $CHCl_3$
	71 %	59 °C	15.7	18.6	
	90 %	60 ° C	15.7	18.5	
	91 %	62 ° C	15.4	18.4	
	82 %	62 ° C	15.4	18.6	

Fig. 2

Z-Thr-Thr-OH is formed during the preparation of Z-Thr-OH at high pH values (above pH 9). Finally the introduction of the fluorenylmethoxycarbonyl group may also be accompanied by a similar side reaction as observed in the case of fluorenylmethoxycarbonyl-glycine (ca. 5 % FMOC-Gly-Gly-OH is formed). Thus it is advisable to carefully test the purity of even simple amino acid derivatives in order to avoid unexpected difficulties in later synthetic steps. Also the chiral analysis of the starting materials should be performed, since derivatization particularly of polyfunctional amino acids involving several synthetic steps, may be accompanied by partial racemization. In this context we have examined as example the preparation of benzyloxycarbonyl-O-tert-butyl-serine and observed that racemization occurs to some extents along the various steps, but in particularly pronounced manner, unexpectedly, in the tert-butylation reaction. Careful crystallizations of the intermediates and of the final derivative leads to the optically pure product.

Protection of thiol functions

Great attention has been paid in the last years to the protection of the thiol function. We have examined in our synthesis of somatostatin-14[6] the usefulness of the tert-butylthio-cysteine derivative[7]. It was found to be perfectly stable under the usual conditions of peptide synthesis, stable in the presence of 2-methylindole to the sulfenyl halides (generated in the haloacids mediated cleavage of sulfenamides) and stable to acid treatments; conversely, it is smoothly removed by reduction with thiols particularly in alkaline media[7] or by phosphines[6]. For the preparation of the S-tert-butylthio-cysteine a new method has recently been elaborated to avoid the use of larger excesses of tert-butylmercaptane as needed in the previous procedure[7], since its elimination creates ecological problems. The reagents developed are the

sulfur-activated sulfenohydrazide derivatives 1-tert-butyl-thio-hydrazine-1,2-dicarboxylic acid diethyl ester, di-tert-butyl ester and dimorpholide, which react smoothly with cysteine, cysteine derivatives or cysteine peptides to produce in practically quantitative yields the corresponding S-tert-butylthio derivatives[8].

Recently the fluorenylmethyl group has been proposed by Bodansky et al.[9] to protect the cysteine thiol function as stable thioether, cleaved by nucleophiles via an elimination reaction. Even if larger experiences with this new cysteine derivative are missing, it may well represent an additional useful alternative to the acetamidomethyl and the trityl group, which are not completely satisfying in regard to their stability and selective cleavage[2].

Protection of other functions

In the last years several new protecting groups have been proposed for particular side chain functions; e.g. for the imidazole function of histidine an interesting group has been introduced by Jones and Ramage[10], i.e. the N(π)-phenacyl-histidine derivative. Unfortunately it has not found wide-spread application probably because of its laborious preparation. For the indole function the formyl group[11] still seems to be the most suited, even if it is unstable to nucleophiles; additionally, it is partially removed by hydrogenolysis and concomitantly saturation of the 2'-3' double bond of the N^{in}-formyl-indole function takes place[12]. The difficulties encountered by its quantitative removal have recently been overcome by adding thiols to the acid deprotection mixtures[13,14]. However, under these conditions its protective effect against indole alkylation in a final acid deprotection step is lost. Of the other N^{in}-protecting groups mainly derivatives of benzenesulfonyl type[15], none has found general

application because of the drastic conditions used in their final cleavage. Although a large number of protecting groups has been developed over the last years, efforts in this context are still welcome not only to refine the orthogonal protection, but also to allow variations in the side chain protecting groups in order to encounter more properly insolubility problems. In fact an interchange of protecting groups was found to considerably increase the solubility of intermediate fragments in the synthesis of somatostatin-14[6], VIP[16] and PHI[17].

Deprotection

A protection scheme which differs from those usually employed and which allows for the final deprotection procedures different from acidolysis, would certainly represent progress. In fact, continously new reports are published about serious side reactions deriving from the acidolytic deprotection procedure. These reports regard i) alkylation reactions[18] at the side chain functions of lysine, tryptophan, tyrosine and methionine, ii) rearrangements of the peptide backbone e.g. to ß-aspartyl-peptides as recently observed in the case of secretin[19], iii) cleavage of acid sensitive peptide bonds, iiii) side reactions of a Friedel-Craft type involving side chain carboxyl functions, etc[18]. A possibly benefical improvement in this context has recently been proposed by Merrifield and coworkers[20]. The use of hydrogen fluoride/dimethylsulfide (1:3) as deprotection medium shall reduce of least some of these side reactions. Particular attention has to be paid to avoid the presence of even small amounts of dimethylsulfoxide which is also generated by oxygen transfer from methionine-S-oxide contaminations; otherwise the Savige-reaction takes place with concomitant cleavage of the peptide chain at the level of tryptophan residues[21].

Additional difficulties deriving from the acidolytic deprotection steps are related to the synthesis of phosphorylated, sulfated and glycosylated peptides. The synthesis of tyrosine-O-sulfate-peptides by direct sulfation was found to produce in all cases a mixture of sulfated and sulfonated derivatives. Significant progress has been achieved by the use of tyrosine-O-sulfate derivatives as successfully exercised by us in the synthesis of CCK-PZ-related peptides[22], whereby the presence of basic amino acids were found to increase the stability of tyrosine-O-sulfate to acids[23]. Concerning glycosyl-peptides first synthetic approaches are going on in various laboratories, but still a lot of research has to be performed in this field, since for these peptide derivatives two difficult chemistries have to be combined: the carbohydrate and the peptide chemistry. On the other hand the synthesis of carbohydrate-containing peptides may become of increasing interest, since the question of whether a greater number of peptide factors is glycosylated in the native state is gaining continuously importance.

Condensation methods

Progress has been achieved in reducing racemization rates by adding various 1,2-dinucleophiles to condensation agents. Nevertheless slow acylation rates, observed to occur frequently in fragment condensation steps, lead to greater degrees of racemization. This phenomenon was encountered in various cases, e.g. in the synthesis of secretin[19] and gastrins[24]. In latter case a comparative investigation of various coupling methods has been performed[23].

In the last coupling step of the little gastrin synthesis attempts to force a quantitative acylation by addition of 1-hydroxybenzotriazole is accompanied by extensive racemization. Condensations with various reagents including the

newly proposed morpholinoethyl-isocyanide[25] indicate the latter reagent as well as dicyclohexylcarbodiimide both in presence of N-hydroxysuccinimide,as preferable. Additionally, the use of 3-hydroxy-4-oxo-3,4-dihydro-1.2.3-benzotriazine as additive should also be more advisable. The fast developments of the chiral analysis by gas-chromatography represents an important improvement in this context since single condensation steps are easily controlled by this fast and sufficiently sensitive method.

Certainly more extended comparative analyses including mixed anhydrides, various organophosphorous reagents as well as a series of newly proposed methods would be necessary to break the strong traditions which in a certain way retard faster improvements.

Unsymmetrical cystine-peptides

For the synthesis of unsymmetrical cystine-peptides mainly three methods are available so far: i) the method of Hiskey[26] based on the activation of one cysteine component as sulfenyl thiocyanate, ii) the method of Harpp[27] via sulfenyl-(diacyl) amide and finally iii) the method of Kamber[28] based on the activation of one cysteine- (or S-protected cysteine-) peptide with sulfenyl halides, e.g. alkoxycarbonylsulfenyl chloride. Latter procedure has successfully been applied for the total synthesis of human insulin[29], which fortunately does not contain any tryptophan residue. In fact, all mentioned procedures are not devoid of side reactions, if tryptophan is present, because of the well known high reactivity of indoles to sulfenyl halides[30].

Consequently, at least for the synthesis of unsymmetrical tryptophan containing cystine-peptides a new method had to be devised. We succeeded in this aim with the sulfenohydrazide procedure[31]. It is based on the activation of one cysteine

component with azodicarboxilic acid derivatives, followed by the reaction of the resulting S-activated sulfenohydrazide with a second cysteine component to yield the desired unsymmetrical cystine-peptide. In both reaction steps the indole function of tryptophan was found to be inert.

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CORRELATION OF COUPLING RATES WITH PHYSICOCHEMICAL PROPERTIES OF RESIN-BOUND PEPTIDES IN SOLID PHASE SYNTHESIS

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In a recent study of solid phase synthesis we have used NMR shifts and relaxation parameters to investigate the molecular physical properties of the resin support[1]. From this a coherent picture of the fundamental aspects of the swollen resin has emerged. In CH_2Cl_2 and DMF the interior of the 1% crosslinked polystyrene (PS) network is a homogeneous environment of well solvated highly mobile chains with properties analogous to a solution of linear PS. The dynamics are characterized by rapid fluctuations of substantial magnitude at frequencies greater than 10^8/s arising from segmental motion in the chains throughout the network. This implies that all sites in the network are readily accessible and there will not be a dispersion in reactivities.

We have examined a number of peptide resins in CH_2Cl_2 and DMF and have found that in general the attached peptide chains also show freedom of motion at rates comparable to those for free molecules in solution, implying good solvation of the peptide. On the basis of these results there is no evidence for fundamental PS resin-caused problems in solid phase synthesis. Some difficulties , formerly attributed to physical inadequacies of the resin, have on more detailed investigations been shown to have their origins in chemical side reactions.[2]

In the solid phase method an occasional problem that does persist is incomplete coupling in the 90-98% range. Perhaps

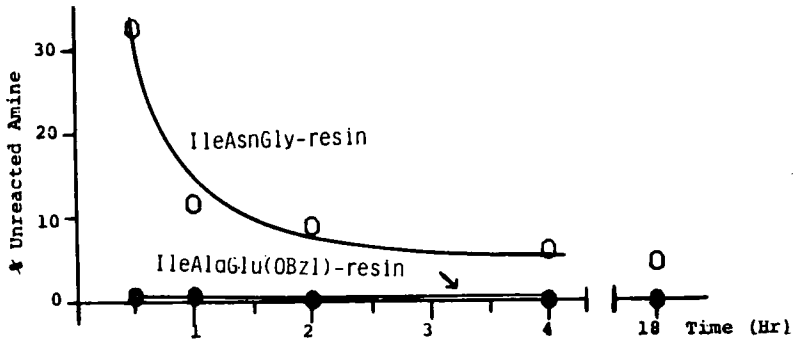

Fig. 1. Unreacted amine vs time for the coupling of $\{Boc\text{-}Tyr\text{-}(Cl_2Bzl)\}_2O$, 0.1M in CH_2Cl_2 to the peptides indicated.

the best documented example is in the synthesis of the decapeptide fragment 65-74 of acyl carrier protein (ACP)[2-4]. In several quantitative studies of the solid phase synthesis of this peptide it was shown that the coupling of the Boc-Tyr to the Ile-Asn-Gly-resin (ACP 74-72-resin) in CH_2Cl_2 was slow and incomplete. The poor yields in chain assembly of this sequence have, in general, been attributed to physicochemical properties of the polymer support[3,4]. We wanted to study the molecular basis of this difficult coupling to understand its origins and test hypotheses advanced to explain it.

$\{Boc\text{-}Tyr\text{-}(Cl_2Bzl)\}_2O$ was coupled to Ile-Asn-Gly-OCH_2-Pam-1% crosslinked PS and Ile-Ala-Glu(OBzl)-OCH_2-Pam-1% crosslinked PS (Figure 1). The tripeptides were assembled by stepwise solid phase synthesis on samples of the same batch of resin (substitution 1.8 meq/g) and with the incorporation of 20% u.l. ^{13}C Ile. Quantitative ninhydrin monitoring[5], amino acid analysis and quantitative Edman degredation confirmed these structures. Each deprotected, neutralized tripeptide resin was allowed to react with $\{Boc\text{-}Tyr\text{-}(Cl_2Bzl)\}_2O$ at a concentration of 0.1M (15 fold excess over peptide). Samples were taken at 0, 0.5,1, 2,4, and 18h, and the amount of unreacted amine determined (Figure 1). Coupling to the Ile-Asn-Gly-resin was slow and incomplete in CH_2Cl_2.

^{13}C NMR measurements were undertaken on the deprotected neutralized resins. For the Ile-Asn-Gly- resin in CH_2Cl_2, ^{13}C

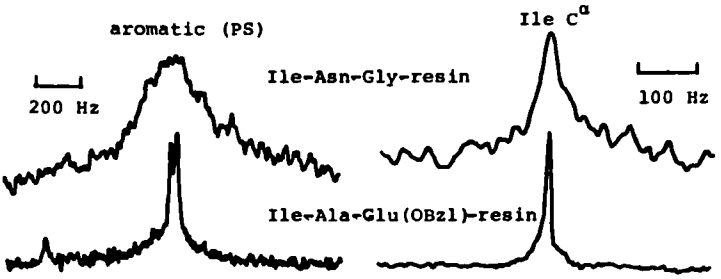

Fig.2. ^{13}C NMR of the aromatic (PS) groups and the Ile C^{α} of the two peptide resins in CH_2Cl_2. Increased width signifies less mobility. There is also a decrease in intensity for the Ile-Asn-Gly-resin due to less motion.

results for the Ile C^{α} and PS carbons (Figure 2) indicate a restriction of motion for both components. In the full spectrum, not shown here, the reduction and distortions in intensity further support a distribution of motional rates where a significant portion are less than 10^6/s. This change indicates from a molecular dynamic level an increase in the effective crosslinking of the resin[6]. This presumably derives from the formation of hydrogen bonded aggregates of some of the peptide chains which can act as non-covalent crosslinks. This change reduces the motion of both peptide and PS and reduces reactivity of the peptides, particularly those in aggregates. Work of Kent and Merrifield[2] on the coupling rates to this peptide as a function of solvent and loading also implicated the aggregation tendency of this peptide in the incomplete coupling in a poor solvent. Hydrogen bonded aggregates of Ile containing peptides attached to linear polymers have also been described.[7] Attachment to crosslinked PS substantially alleviates the chemical reactivity effects of this, however.[2]

This mechanism suggests a straightforward remedy to the difficulty, to employ a solvent such as DMF which can accept hydrogen bonds and displace the peptide-peptide interaction. In this solvent the NMR of the Ile-Asn-Gly-resin shows narrowed lines and longer relaxation times for motions $>10^8$/s for all components. Coupling of the activated Boc-Tyr to this resin was rapid and complete (>99.5% in 0.5 h). As a further test of

the proposed mechanism, Ile-Ala-Glu(OBzl)-resin, containing the Glu(OBzl) which shows a reduced tendency for β aggregation[7], was studied. The NMR did not show motional restriction in either solvent, and couplings in both solvents were complete in 0.5 h.

The phenomenon of poor coupling in the solid phase synthesis of ACP (65-74) is thus not caused by the PS resin support as shown by this controlled comparison in which only the internal sequence of the peptide is changed while all other resin related parameters (batch, loading, chain length, N-terminal and activated amino acid and coupling conditions) were fixed. With NMR it is possible to demonstrate the direct relationship between rapid molecular motion and reactivity and show that DMF is a good solvent for PS and the peptide-resin. In considering the efficacy of other supports, such as polyacrylamides, the potential for restrictive interactions between support and pendant molecules should be born in mind.

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APPLICATION OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY TO THE RAPID SYNTHESIS AND PURIFICATION OF PEPTIDES

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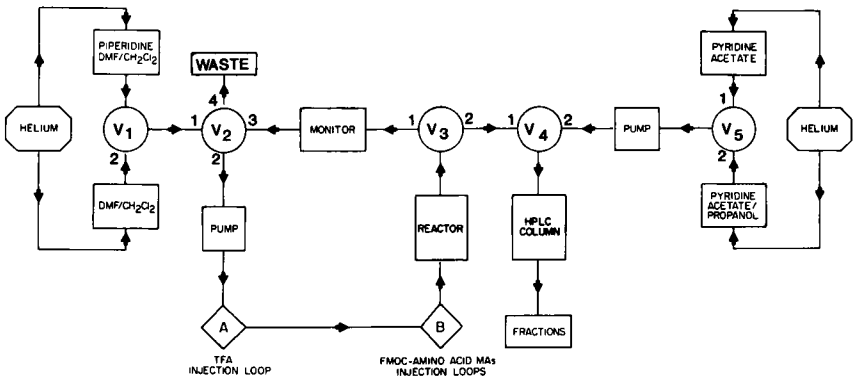
During the last few years the need for biologically active peptides and their analogs has increased tremendously. Their use as antigens in immunological studies, for bioassays, and as standards for high performance liquid chromatography (HPLC) make it necessary to obtain these compounds rapidly and in high purity. Limitations in availability and problems relating to the purity of commercial peptides[1, 2] led us to develop a procedure which allows the rapid preparation and purification of peptides.

Previously described solid-phase techniques in nucleotide[3] and peptide synthesis[4-6] utilize a packed bed in a flow reactor instead of beads in a shaker vessel. Using the former approach it has been possible to assemble an entire continuous flow system from liquid chromatographic equipment in common use in our laboratory[7]. A flow diagram of the solid-phase peptide synthesizer is shown in Figure 1. Microprocessor controlled pumps and valves deliver reagents and solvents. The dimensions of the reactor (4.6x60mm to 22x65mm) permit operation at low back pressure (<50psi) and changes in effluent composition are monitored by UV.



Cycle	Valve position V1	V2	V3	V4	V5	Inject
Wash	2	1,2/3,4	1	-	-	-
Coupling	-	1,4/2,3	1	-	-	B
Deprotection	1	1,2/3,4	1	-	-	-
Detaching	2	1,2/3,4	2	1	-	A
Purification	-	-	-	2	1	-
Elution	-	-	-	2	2	-

Fig. 1. Flow diagram of the solid-phase peptide synthesizer.

The syntheses were performed on a p-benzyloxybenzyl ester polystyrene-1% divinylbenzene resin support. Preformed mixed anhydrides prepared from Fmoc-amino acid derivatives with isobutylchloroformate[8] were injected during the coupling cycles. For Asp, Glu and Tyr, t-butyl sidechain protection was used. The guanidine group of Arg was not blocked. A 15 min coupling was performed with 4 equivalents of amino acid under recycling conditions. The 5 min deprotection cycle was preceeded and followed by wash cycles. After assembling the peptide it was detached from the solid support by injecting TFA and then pumped directly onto a Lichroprep RP-18 column. The column was switched to an HPLC system and washed with 0.5M pyridine acetate pH 4.2 to remove TFA. The crude peptide was eluted with buffer containing 60% 1-propanol and the solvent evaporated.

The enkephalin-containing peptide [Met]enk.-Arg^6-Gly^7-Leu^8 was synthesized both by conventional solid-phase methods

and by utilizing the HPLC instrument. Major identified side products in both syntheses are the truncated peptide sequences 1-4, 1-5 and 1-6 (Fig. 2). The final yields of purified peptide were 23% for the conventional method and 18% for the synthesis without Arg protection.

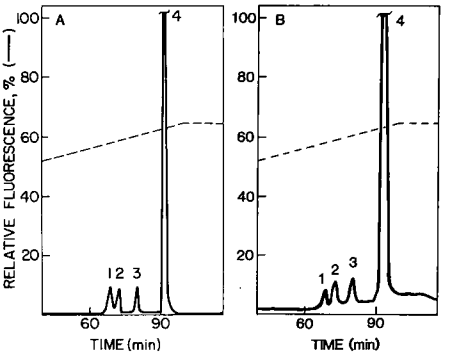


Fig. 2. HPLC of crude [Met]enk.-Arg^{6}-Gly^{7}-Leu^{8} on Ultrasphere ODS using a 1-propanol gradient (10%/h;---) in 0.5M pyridine acetate (pH 4.2) for elution; flow rate 20 ml/h. YGGF (1), YGGFMR (2), YGGFM (3), YGGFMRGL (4). (A) Material derived from conventional synthesis after gel chromatography on Biogel P2; Nα-Boc protection, Tyr(Cl$_2$-Bzl) and Arg(Tos) sidechain protection, hydroxymethyl-polystyrene-1% divinylbenzene resin support. (B) Product from HPLC synthesizer.

Porcine Pronorphin 250-256 produced by synthesis under continuous-flow conditions (see preceeding paper, Jonczyk et al.) is shown in Figure 3. Fluorescamine amino acid analysis (detection limit: 0.05 relative mol% with 100 nmol material) revealed several homologous sequences, which are probably due to impurities in the commercial Fmoc-amino acid derivatives[9].

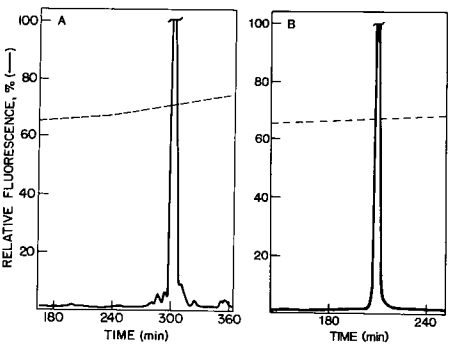


Fig. 3. HPLC of Porcine Pronorphin 250-256. For chromatographic details see Figure 2. (A) HPLC of crude material on Nucleosil 5C18. (B) Rechromatography of the major peak on a diphenyl column.

In conclusion, the high efficiency of liquid and reagent handling under flow conditions together with Fmoc protection, mixed anhydride activation, reverse phase concentration and purification by HPLC permit the rapid preparation of small peptides in less than 48 h. An improvement for Arg-containing peptides would be the development of a suitable sidechain protecting group. The HPLC synthesizer will be used for pilot synthesis in large scale peptide preparations and a fully automated synthesizer is under development.

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AUTOMATED FLOW REACTOR SYNTHESIZER FOR FAST SYNTHESIS OF PEPTIDES USING FMOC PROTECTION

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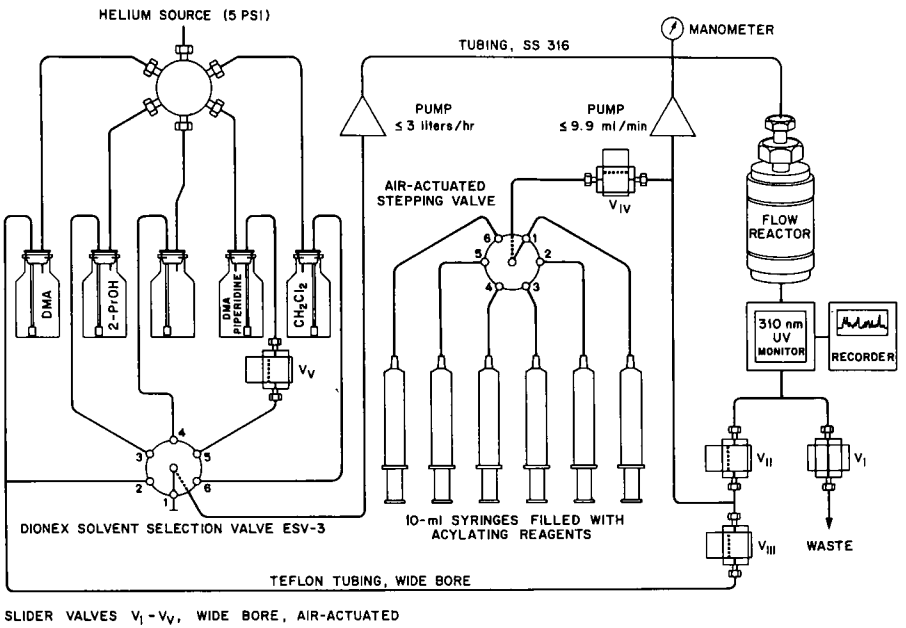
Scott et al.[1] in 1971 undertook to synthesize peptides using a flow reactor. However, the very low degrees of substitution on the polystyrene coated glass beads which were used as a support limited the utility of this approach. In 1980, Erickson et al.[2] used polystyrene bead-packed HPLC columns in conjunction with an analytical HPLC system. In the same year, Verlander, Fuller and Goodman independently developed a large scale, solid phase approach based on a flow reactor.[3] They constructed a prototype synthesizer utilizing a system of nitrogen-pressurized vessels to deliver solvents and reagents to a stainless steel flow reactor.[3] For the synthetic cycles, Fmoc protection and mixed anhydride activation were employed with appropriately derivatized and suitably crosslinked polystyrene as a support.[4] Table I contains a selection of the peptides they synthesized including unpublished information on the scale of the synthesis, purity of the products and yield of the reactions. We constructed a solid phase peptide synthesizer which uses a stainless steel vessel similar to a very short HPLC-column. Solvents for washing steps and reagents for deprotection are pressurized with helium at 5-6 psi to prevent gassing when delivered by an HPLC-pump with a flow of 1-3 L/h. The backpressure is less than 400 psi, usually 30 psi. For couplings of amino acids their

Table I. Peptides Synthesized by the Pressurized Flow Reactor System

Target Peptide	Scale of Synthesis	Overall Yield	Purity of Product
δ-sleep inducing nonapeptide	2.5 mmole	>96%	95%
Fibrino pentadecapeptide	1 mmole	40%	90%
Thymosin α_1	10 mmole	10%	75%

preformed filtered symmetrical anhydrides or their mixed anhydrides with isobutyl chloroformate[4] are used. They are fed into the system via syringes and recycled by a second HPLC-pump. Both reactor heads have the shape of a very flat funnel to assure low dead volumes. A 10 μm frit above the resin bed serves to evenly distribute the solvents, which are then passed through a UV-spectrophotometer to determine the time needed to perform a washing step. The size of the N-(9-fluorenylmethyl)-piperidine peak at 310 nm in a deprotection step can be used to estimate the extent of the previous coupling. A timer controls 5 slider valves, a 6+1 port solvent selection valve with independent actuation of the ports, and a 6+1 port stepping valve with connected syringes for the activated amino acids.

When leaving the UV-unit, solvents go either to waste (V_I) or back (V_{II}) to the recycling pump. V_{III} serves to flush out the tubing and valves of the recycling system, including a back flush in the syringe last used. A slow pump draws the amount of activated amino acid from the syringe which is determined by pump speed and programmed time. The equivalent amount of solvent is displaced from the flow reactor and goes to waste. Hereafter the activated amino acid is recycled for a preset time.



Scheme 1. Flow reactor synthesizer for solid phase peptide synthesis; for Boc-procedure replace DMA/piperidine with TFA/CH_2Cl_2 and fill empty bottle with Et_3N/DMF

The system is flushed out again and ready for the next cycle. The deprotection time in our Fmoc-system with two washings of piperidine/DMA (1:4) is about 3 minutes long. The polystyrene resin with the Fmoc-amino acid[5] coupled via the TFA-labile hydroxymethyl-phenoxymethyl spacer[6], is transferred into a suitable reactor vessel in the most swollen state. Small glass beads may fill up unused space in a larger vessel.

A synthesis of Arg-Ala-Glu-Ile-Met-Arg-Ser [human leukocyte interferon, HIF-αA, (144-150)[7]] was carried out in a 1.5 mmol scale using t-butyl type side-chain protection and the nitro function for arginine. A 62-min cycle included a 15-min double coupling with symmetrical anhydrides. The crude dinitro peptide

was treated with zinc in aqueous TFA[8] to give the desired free peptide which had a correct amino acid analysis.

The peptide Lys-Asn-Trp-Lys-Glu-Glu-Ser, HIF-γ(37-43)[9] was synthesized on a 0.3 mmol scale in the same manner to give two main products with the same amino acid composition. The side product was a t-butylated derivative, as determined by ^{1}H-NMR.

Because symmetrical anhydride coupling may occasionally cause clogging of the syringes by precipitating DCU, coupling with mixed anhydrides of Fmoc-amino acids is preferred. With a 38-min cycle and 15-min single coupling Leu-Ala-Gly-Val was prepared in high purity on a 0.3 mmol scale in nearly quantitative yield.

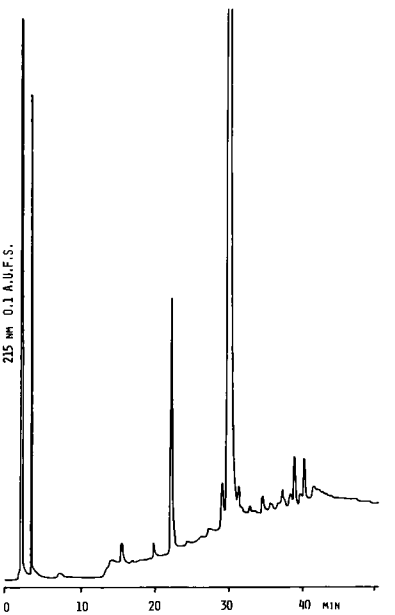


Fig. 1 HPLC of crude pronorphin (250-256) on C-18 in 0.02% TFA, 0-50% CH_3CN in 30 min, 2 mL/min, 20 cm/h.

For the synthesis of Tyr-Glu-Glu-Leu-Phe-Asp-Val (Pronorphin 250-256)[10] on a 0.3 mmol scale, the instrument was readied in 1h. Single coupling steps (15 min) with 2.9 equiv. mixed anhydride were used in a total 39-min cycle. The cleavage (TFA/CH_2Cl_2/mercaptoethanol/anisole, 60:40:1:1) and evaporation were done in 2.5h. A small sample was purified on a C-18 column

(Fig. 1) to obtain the pure peptide in 37% yield which had a correct amino acid analysis. In the following paper, Lahm et al. will describe separation of this peptide in more detail.

Acknowledgment

We wish to thank Dr. M. Goodman, Dr. M. Verlander and collaborators and Dr. B. Erickson and collaborators for discussions and demonstrations of their instruments.

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DETECTION AND PREVENTION OF OLIGOPEPTIDE FORMATION DURING THE SYNTHESIS OF 9-FLUORENYLMETHYLOXYCARBONYL AMINO ACID DERIVATIVES

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Introduction

Since their discovery over 10 years ago[1], 9-fluorenylmethyloxycarbonyl (Fmoc) amino acid derivatives have failed to gain widespread acceptance, despite the possibility of complete selectivity when they are used in conjunction with t-butyl sidechain protection[2,3]. As reported in a preliminary communication, we have recently noted a serious side reaction which occurs during the synthesis of Fmoc-amino acid derivatives[4]. When these compounds are prepared by the standard procedure[1,2], using Fmoc-chloride, the majority of Fmoc-amino acids are contaminated by variable, but often substantial amounts of persistent impurities. We have demonstrated that these are the corresponding Fmoc-oligopeptides, using TLC, HPLC and nmr studies, together with quantitative amino acid analysis and the synthesis of a number of standards[4].

Results

A wide variety of Fmoc-amino acids, both from commercial sources and also prepared in-house, were analyzed by TLC (toluene:acetic acid, 10:1, v/v), HPLC and quantitative amino acid analysis and shown to contain substantial amounts (up to

20%) of the corresponding Fmoc-dipeptides[4]. The contamination was especially noticeable in the case of non-functional amino acids, such as glycine, alanine, phenylalanine, etc. In the case of Fmoc-glycine, a small amount (0.2%) of the corresponding Fmoc-tripeptide was even detected[4].

These results suggested that the oligopeptide contaminants arose through the intermediacy of mixed anhydrides, formed from reaction of the Fmoc-amino acid, as it is formed, with excess Fmoc-chloride. A similar mechanism was proposed when dipeptide formation was noted in the synthesis of benzoylglycine[5]. In order to prove this hypothesis, Fmoc-Leu was treated with an equivalent of Fmoc-chloride in aqueous dioxane containing excess sodium carbonate (typical conditions for the synthesis of an Fmoc-amino acid). After 20 minutes, an equivalent of Leu-OBu^t was added and the reaction allowed to proceed overnight. After the usual work-up, the reaction mixture was treated with TFA to cleave the t-butyl ester, and the crude product analyzed by HPLC. This technique showed the presence of 40% Fmoc-Leu-Leu in the product, while a control sample, to which no Leu-OBu^t had been added, showed no dipeptide. This clearly demonstrates that the oligopeptide contaminants are formed via the intermediacy of the mixed anhydride, which is extremely hydrophobic and therefore sufficiently stable, even in aqueous/organic solvent mixtures, to acylate the amino acid derivative, Leu-OBu^t, competitively with Fmoc-chloride.

The results described above indicate that the use of Fmoc-chloride for the routine synthesis of Fmoc-amino acids is to be avoided. For this reason, alternate acylating reagents, such as mixed carbonates, which are unlikely to lead to the formation of activated amino acid derivatives, were investigated. The unsymmetrical carbonates, Fmoc-O-CO-OR, were prepared by treatment of Fmoc-chloride with the appropriate alcohol, ROH, in the presence of an equivalent of base (triethylamine). The effectiveness of these derivatives as

Table I. Comparison of the Fluorenylmethyl Carbonates, Fmoc-O-CO-OR, for the Synthesis of Fmoc-Ala[a]

R[b]	Reaction Time (hr.)	Yield (%)
Succinimidyl	24	87
Phthalimidyl	79	74[c]
Phenyl	48	--
p-Nitrophenyl	48	--
p-Chlorophenyl	67	4
2,4-Dinitrophenyl	67	13
2,4,5-Trichlorophenyl	75	48
2,4,6-Trichlorophenyl	70	30
Pentachlorophenyl	68	56[c]
Pentafluorophenyl	69	68
1-Benzotriazolyl	24	84[c]

[a]Prepared by treatment of the carbonate with 1.1 equivalents of L-alanine in dioxane/10% aqueous sodium carbonate; reactions were monitored by TLC. [b]Carbonates were prepared by treatment of Fmoc-chloride with the corresponding alcohol in the presence of 1 equivalent of triethylamine. [c]An impure product was obtained.

acylating agents was assessed through treatment of alanine with the reagents in dioxane/10% aqueous sodium carbonate. The results of these comparative studies are summarized in Table I.

From these studies, the optimum reagent for this purpose appeared to be Fmoc-succinimidyl carbonate. (This is in agreement with a recent study[6] concerned with the synthesis of Fmoc-derivatives of hydroxyl-containing amino acids without sidechain protection.) Fmoc-derivatives of all natural amino acids, containing either t-butyl or benzyl-based sidechain protecting groups where necessary, were therefore synthesized using this reagent. The Fmoc-amino acid derivatives prepared in this manner were, without exception, free of the

corresponding dipeptide contaminants and were obtained in high yields (70-95%). In all cases, the properties of the amino acid derivatives corresponded with the literature data[2].

Conclusions

Our studies have shown that a serious side reaction -- the formation of oligopeptides -- can occur during the synthesis of Fmoc-amino acid derivatives under literature conditions, using Fmoc-chloride. We have also shown that the mechanism of this side reaction almost certainly involves the intermediacy of stable mixed anhydrides. However, the side reaction can be avoided completely and the desired Fmoc-amino acid derivatives obtained in pure form and in high yield using Fmoc-succinimidyl carbonate as the acylating reagent. This intermediate is a stable, crystalline compound, obtained in high yield (>90%), by the simple treatment of Fmoc-chloride with N-hydroxysuccinimide in the presence of base. We believe that the use of this reagent to prepare pure, oligopeptide-free Fmoc-amino acids will lead to more widespread use of these versatile, protected amino acid derivatives.

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A NEW APPROACH FOR THE DEPROTECTION OF SYNTHETIC PEPTIDES

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Introduction

A central problem in the chemical synthesis of long and complex peptides is the requirement for a final deprotection method that is efficient and largely free of side reactions. Deprotection by the strong acid is, at present, the best available choice. As a step towards the better understanding of the strong acid deprotection of synthetic peptides, we have studied the S_N2 mechanism in the presence of a weak base such as dimethylsulfide.[1,2] In this paper, we wish to report our studies on the deprotection of synthetic peptides by three strong acid-dimethylsulfide systems: (1) HF (HF-DMS), (2) CF_3SO_3H and CF_3CO_2H (TFMSA-TFA-DMS),[3] and (3) $B(CF_3CO_2)_3$ and CF_3CO_2H (BTFA-TFA-DMS).[4]

Concept of Approach

The inherent problems and side reactions attendant with the use of the strong acid have been mainly associated with its extremely high acidity function leading to the removal of protecting groups by the S_N1 mechanism and to the formation of carbocations that have undesirable effects on synthetic peptides. A different approach to avoid these side reactions is to deprotect peptides in a strong acid by an S_N2 mechanism in which carbocations are not formed.

We have approached the problem of the S_N2 deprotection by seeking a Bronsted weak base, dimethylsulfide, which acts

both as a nucleophile and as a solvent in the dilution of the strong acid so that the acidity function of the resulting strong acid-weak base mixture is optimal for the protonation of the protecting groups without substantial protonation of the nucleophile. In the present study we have investigated three different strong acid systems. TFMSA in TFA is a stronger ionizing acid than HF in DMS. Hence a lower molar concentration of TFMSA in TFA is required to achieve a comparable acidity function to HF in DMS. BTFA is a Lewis acid and in TFA, BTFA-TFA becomes a strong ionizing acid. To further understand these strong acid systems for the purpose of using the S_N2 deprotection mecnanism on synthetic peptides, we have determined the domains of the S_N1 and S_N2 deprotection by measuring the rate profiles and by analyzing products during the deprotection of several benzyl alcohol derived protecting groups over a wide range of acid concentrations.

Table I. The S_N1-S_N2 Deprotection Behavior in Strong Acid-Dimethylsulfide

Deprotection Reactions	Acid	Cosolvent	Method of Analysis	Region of Changeover % Acid
Ser(Bzl)	HF	DMS	Rate[1]	55-60
	CF_3SO_3H	TFA and 10% DMS	Rate	12-15
	$B(TFA)_3$	TFA and 10% DMS	Rate	1.5-2.0M
Tyr(Bzl)	HF	DMS	Product[2] Analysis	50-60
	CF_3SO_3H	TFA and 10% DMS	Product Analysis	12-15
	CF_3SO_3H	TFA and 20% DMS	Product Analysis	30-35
	CF_3SO_3H	DMS	Product Analysis	65-70
	$B(TFA)_3$	TFA and 10% DMS	Product Analysis	1.5-2.0M

[1]Rate changes in the deprotection of Ser(Bzl) to Ser,

[2]Analysis of 3-benzyltyrosine in the deprotection of Tyr(Bzl)

Results and Discussion

A. Rate Profiles. The kinetics of O-benzyl-serine deprotection in all three acid systems and their rates were determined in dilute and concentrate acid concentrations. In the S_N1 deprotection region, the rate changes were rapid (t 1/2 = 5 to 30 sec) with the increase in acid concentration and in the S_N2 deprotection the rate changes were slow (t 1/2 = 10 to 40 min) and relatively independent of the increasing acid concentration.

B. Product Analyses. The products of the S_N1 and S_N2 reactions were studied by the deprotection of Tyr(Bzl). By analyzing the mole percent of tyrosine, 3-benzyltyrosine and dimethylbenzylsulfonium salt, the S_N1 and S_N2 domains were determined. Typically, in the S_N1 region, the 3-benzyltyrosine products accounted for 30 to 45% mole percent of the product in contrast to 0.5 to 5% in the S_N2 regions.

A quantitative distinction between the S_N1 and S_N2 deprotection mechanisms was that the S_N1 deprotection occurs at high acidity function and hence in concentrated strong acid

Table II. Comparison of S_N1 and S_N2 deprotection in HF-dimethylsulfide

Reactions	S_N1 HF vol% >60	S_N2 HF vol% 15 to 50%
Rate of removal of benzyl protecting groups (e.g. Ser(Bzl))	Fast, t 1/2 (5 to 30 sec)	slow, t 1/2 (10-40 min)
Alkylation side reactions e.g. deprotection of Tyr(Bzl) to give 3-Bzl-Tyr	significant 20-33	minimal, 0.5-5%
Reduction of Met(O) to Met	slow and incomplete in 2 h	quantitative in 2 h
Aspartimide formation from a Asp-Gly sequence	significant in 1 h, 0° C. 5-28	minimal 0.5 to 1.5% in 1 h, 0° C.

and S_N2 deprotection occurs at low acidity function and hence in dilute acid concentration. The transition between S_N1 and S_N2 deprotection in all three systems measured by both methods was usually sharp and distinctive. In a binary system such as HF-DMS only one changeover in mechanism was possible and was found to be at 55-60% of HF concentrations. However, in ternary systems such as TFMSA-TFA-DMS or BTFA-TFA-DMS, many changeovers in mechanism were possible depending on the concentrations of TFA and DMS. When the DMS concentration was limited to 10% by volume, the changeover in mechanism with TFMSA-TFA-DMS and BTFA-TFA-DMS was 12-15% of TFMSA and 1.5 to 2.0 M of BTFA respectively. When the DMS concentration was increased to 20% by volume or the binary mixture TFMSA-DMS was used, the changeover in mechanism was found to shift to higher acid concentrations (Table I).

In general, the S_N2 deprotection in these three acid-DMS systems removes most benzyl alcohol derived protecting groups at a slower rate than the S_N1 deprotection, but fulfills our requirement of reduction of alkylation side reactions and aspartimide formation, and in addition reduces Met(O) to Met (Table II).

Acknowledgment

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IMMOBILIZED POLYOXYETHYLENE, A NEW SUPPORT FOR PEPTIDE SYNTHESIS

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Introduction

A plausible and promising approach to solve some of the problems associated with the cross-linked solid resins was the development of liquid-phase peptide synthesis using polyoxyethylene (POE) as a soluble support[1]. It was reasoned that by this modification the unfavorable matrix effect which could cause serious problems during SPPS will be avoided. Moreover, kinetic investigations pointed out that peptide coupling and deprotection should take place homogeneously[2].

Soluble polymers, however, possess certain inherent shortcomings which make them unsuitable for specified manipulations such as washing with different solvents and simple filtration. Consequently, for the removal of reagents and by-products, more time-consuming operations like ultrafiltration or repeated precipitation must be carried out.

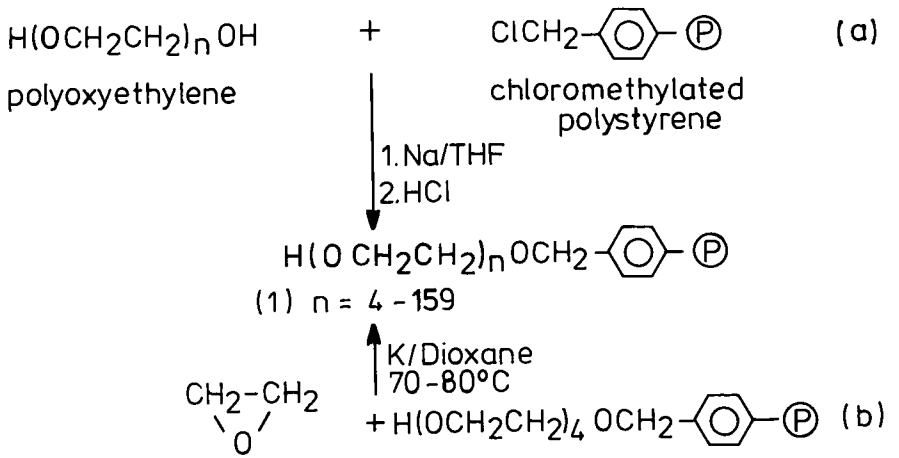
This led to the question as to whether POE can be immobilized on a solid resin, thus combining the advantages of the solid- and liquid-phase techniques.

This graft polymer could be regarded as an ideal carrier having an insoluble core with long polyethylene chains or "hairs" which are extending outwards from the matrix. The results of this investigation are herein presented.

Results and Discussion

Graft polymer (1) was prepared by covalent attachment of POE to cross-linked polystyrene by two different synthetic methods as presented in Scheme 1. Synthesis of immobilized polyoxyethylene via path (a) was carried out by reacting disodium or dipotassium salt of POE with 1 % cross-linked chloromethylated polystyrene in THF or dioxane under nitrogen atmosphere. Under Williamson ether-synthesis conditions, however, two main problems are encountered: First, it seems that cyclization of POE is a major reaction, specially when larger POEs are used. Therefore, this synthesis can successfully be accomplished when monohydroxy POE is employed[3], or when cyclic polymeric ethers are desired[4]. Secondly, only polyoxyethylenes with molecular weight up to 3000 gave relatively good yields but larger polyethers reacted poorly.

Path (b) shows anionic polymerization of ethylene oxide on tetraethylene glycol-polystyrene to obtain (1). The extent of polymerization could be regulated by controlling different parameters such as temperature, reaction time, rate of addition of ethylene oxide and so on.



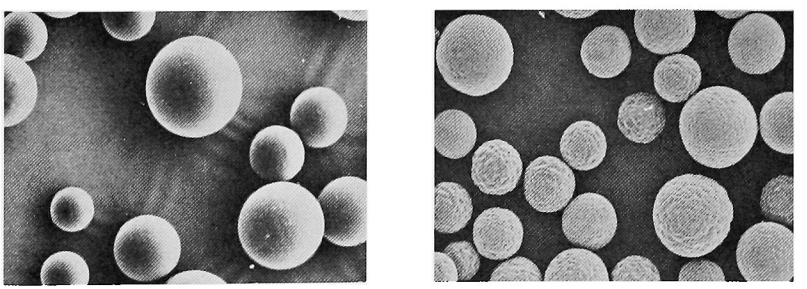
Scheme 1. Synthesis of immobilized polyoxyethylene.

Average molecular weight of the grafted POE is estimated by elemental analysis and measurement of the oxygen content of the copolymer and also by determination of the free terminal hydroxyl group. The approximate number of the hydroxyl group was ascertained by reacting the polymer with isocyanate or its esterification with an amino protected amino acid followed by hydrolysis and quantitative analysis of the amino acid.

Electron micrographs of chloromethylated polystyrene beads and POE-modified resin are shown in Figure 1 which give an impression of the POE structure on and inside the beads.

The mobility of the POE chains of the synthesized graft polymers could be determined by ^{13}C-spin lattice relaxation time measurements which were performed on suspensions of the copolymers in appropriate solvents. These measurements showed that carbon atoms of POE give a sharp signal at 70.5 ppm, whereas the aromatic carbon atoms of polystyrene give very broad signals at ca. 130 ppm, an indication that the mobility of POE chains is greater than that of polystyrene matrix. Addition of ether - a solvent incapable of dissolving POE - to the measuring suspensions lowers the relaxation time of the POE chains profoundly.

These performed studies also pointed out that the relaxation time of the POE side chains increased with the increasing length of the chains, whereas the mobility of soluble POE de-



(a) (b)

Fig. 1. Electron micrographs of polystyrene beads (a) and POE-modified beads (b).

creases as the chains become longer. They also showed that the mobility of the POE side chain of a graft polymer (n=118) was about 60 % of the soluble POE with the same molecular weight.

Initial kinetic studies indicated that coupling of Boc-Gly-ONp with Gly-POE-polystyrene beads proceeds at a faster rate than with the corresponding Gly-POE. The accelerated rate of the coupling in the case of graft polymer can be rationalized that the "hairs" of polystyrene-bound POE assume more ordered arrangements in comparison with randomly oriented chains of the soluble POE which more or less fold together to build a quasi spherical form. Consequently, the terminal groups of the immobilized polyethylenes are more accessible for further reactions, whereas those of soluble POEs could mostly be buried inside the structure.

A sample of the graft polymer (n=118) was employed to synthesize the C-terminal tetra and deca peptides of bovine insulin B-chain by the usual steps. The polymer was first acylated with glycine and then coupled with 4-[N-(tert-butyloxycarbonyl) alanyloxymethyl]-3-nitrobenzoic acid[5] via DCC activation. The protected peptides were cleaved photolytically[6] and purified chromatographically on LH-20 and silica gel.

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ADVANCES IN THE APPLICATION OF N^{α}-Fmoc PROTECTION TO SOLID-PHASE PEPTIDE SYNTHESIS ON ALKOXYBENZYL POLYSTYRENE RESINS

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Introduction

Solid-phase technique has proved to be of value for the chemical synthesis of peptides; nevertheless, apart from the basic principle, it is entirely an open-ended method. Since its inception by R.B. Merrifield in 1962[1], continuous efforts have been made to improve all aspects of the methodology. The present report is concerned with experiences arising from the application of just one of these novel procedures: the use of 9-fluorenylmethyloxycarbonyl (Fmoc) as N^{α} temporary protecting group and tert-alkyl-based side chain protection while the growing peptide is anchored to the solid support through a 4-alkoxybenzyl ester linkage[2,3]. Problems pertinent to the chemistry of anchoring to the resin have been discussed in a previous paper[4]. Here the focus will be primarily on stepwise couplings and final acidolytic deprotection.

Coupling Efficiency

Attainment of a coupling reaction as complete as possible at each cycle of a solid-phase synthesis is essential in order to avoid both the formation of truncated sequences and of deletion sequences if irreversible blockage of unreacted amino groups is not achieved. Efficient couplings improve both yield and homogeneity of products and greatly facilitate the isolation

Table I. Fmoc-amino Acid Incorporation[a] into H-Ile-Ala-Gly-Val-OCH_2-C_6H_4-OCH_2CONH-Resin by Different Procedures

Fmoc-amino acid	symm-Anhydride[b] Coupling 1st	2nd	DCC/HOBt[c] Coupling 1st	2nd	symm-Anhydride[b] 1st	DCC/HOBt[c] Coupling 2nd
Gly	D	C	U	C	D	C
Ala	D	C	D	C	D	C
Val	U	D	U	U	U	C
Leu	U	D	U	U	U	D
Met	D	C	U	C	D	C
Phe	U	C	D	C	U	C
Tyr(Bu^t)	U	C	U	D	U	C
Thr(Bu^t)	U	U	U	U	U	U
Ser(Bu^t)	U	C	D	C	U	C
Asp(OBu^t)	U	C	D	C	U	C
Trp	U	C	U	D	U	C
Arg(Adoc, Adoc)	U	U	U	D	U	D
Lys(Boc)	D	D	U	C	D	C

[a] Determined both by ninhydrin and fluorescamine test: C, complete; U, uncomplete; and D, doubtful incorporation.
[b] Three equiv of preformed symmetrical anhydride, 0.08 M in methylene chloride/DMF 3:1 v/v.
[c] Three equiv of Fmoc-amino acid, HOBt, and DCC, 0.05 M in DMF.

of pure deprotected peptides. In previous syntheses[5] based on N^{α} Fmoc protection, we routinely used the preformed symmetrical anhydride procedure for coupling of protected amino acids (Gln and Asn excepted). Provided that a large excess (three- to four-fold) of anhydride is employed and the protocol includes two coupling steps separated by shrinking and swelling of the resin, this procedure is generally satisfactory. However, the overall process is considerably wasteful and the incorporation of some Fmoc-amino acids required a third coupling. Thus, as a preliminary to the synthesis of glucagon[6], Fmoc-amino acids to be used were coupled separately to a model tetrapeptide-

resin (H-Ile-Ala-Gly-Val-OCH_2-C_6H_4-OCH_2CONH-Resin) bearing a sterically unfavourable isoleucine residue at the amino terminus (no Ile residues are present in glucagon, but this β-branched amino acid is likely to represent a rather stringent test for coupling protocols). Three different double-step protocols were tested: (1) two preformed symmetrical anhydride couplings[7] in CH_2Cl_2/DMF (3:1 v/v); (2) two couplings by the DCC/HOBt[8] procedure in DMF alone; and (3) the first coupling by the symmetrical anhydride and the second one by the DCC/HOBt procedure. The results are shown in Table I. The symmetrical anhydride procedure works well in the majority of the cases, while the DCC/HOBt method alone is less satisfactory. A combination of both methods appears to be attractive because it gave good results (with the notably exception of Fmoc-Thr(Bu^t)) and led to a considerable economy of Fmoc-amino acids. Fmoc-glutamine and Fmoc-asparagine cannot be safely coupled by the anhydride procedure; a comparison was then made by coupling these two protected amino acids by a DCC/HOBt protocol and by their active esters. Although some active esters (p-nitrophenyl, o-nitrophenyl, and 2,4,5-trichlorophenyl) can be easily prepared in good yields, they cannot compete favourably with the DCC/HOBt procedure when coupled to the model peptide-resin.

Final Deprotection

Removal of the target protected peptide from an alkoxybenzyl polystyrene resin can be performed by the action of trifluoroacetic acid, which also cleaves the tert-alkyl-based side chain protecting groups. When methionine and tryptophan residues are present in the peptide-resin, acidolytic cleavage is not without complications and caution must be exerted in order to minimize the danger of both oxidation and alkylation. In order to evaluate the effect of different conditions and scavengers on methionine and tryptophan modification during final cleavage

from the resin, protected glucagon-(23-29)-resin (Boc-Val-Gln-Trp-Leu-Met-Asn-Thr(Bu^t)-OCH_2-C_6H_4-OCH_2CONH-Resin) was synthesized by the Fmoc procedure and submitted to acidolysis. Samples of peptide-resin were treated with trifluoroacetic acid (alone or gradually diluted with CH_2Cl_2) in the presence of a scavenger (thioanisol, methionine, indole, 1,2-dithioethane, and dithiothreitol have been tested) at 25°C or below for various periods of time. The conditions under which minimal side reactions occurred (Trp recovery: 96%; methionine recovery: 98%) but still a satisfactory cleavage yield was obtained (81% based on starting aminoacyl-resin) is as follows: anhydrous CH_2Cl_2, 1,2-ethanedithiol, and trifluoroacetic acid are distilled under argon, one after the other, into a vessel containing the peptide-resin (final concentrations : 5% 1,2-ethanedithiol and 70% trifluoroacetic acid); the suspension is then stirred by a stream of argon for two hours at 20°C and filtered. The combined filtrate and washings are concentrated under high vacuum at 25°C.

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PROPERTIES OF SWOLLEN POLYMER NETWORKS. INTER-SITE REACTIONS: SYNTHESIS OF A CYCLIC ANALOGUE OF OXIDIZED GLUTATHIONE

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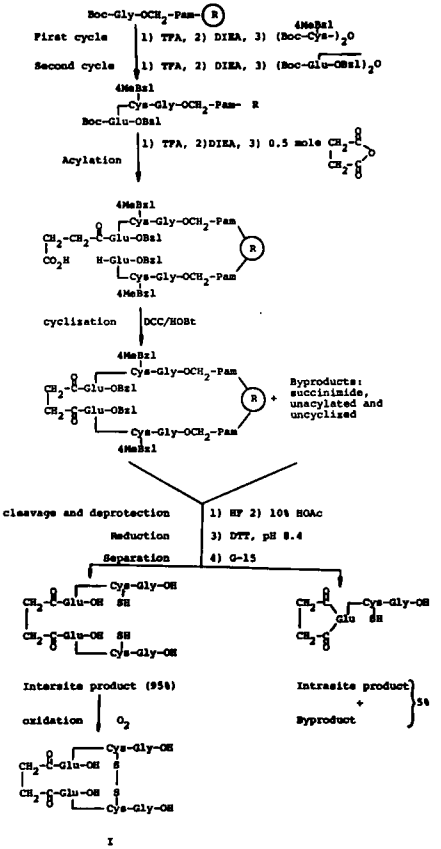
Introduction

In continuation of our studies on the properties of a swollen peptide-resin bead during solid phase peptide synthesis,[1] we have synthesized here a cyclic analogue of glutathione dimer (GSSG), with restricted conformation, to demonstrate the feasibility of carrying out reactions between different reactive sites within the same swollen crosslinked polystyrene bead (Inter-site reaction). There have been contradictory views about the nature of polymer-supported reactions. Some have suggested that cross-linked polystyrene is relatively rigid in nature and that reactive moieties on the polymer support remain isolated from one another.[2-4] This has led to the concept of site isolation where a specific reactive site on a polymeric support remains completely inert toward inter-molecular reactions. In contrast, others have suggested that solvent-swollen cross-linked polystyrene chains have significant molecular mobility.[5,6] This internal flexibility of chains should facilitate the inter-site reaction between peptide chains throughout a swollen polymer gel. Several examples of this type of behavior have been reported under a variety of conditions.[7]

We believe that under the condition normally employed in solid phase peptide synthesis, potential inter-site reactions between different peptide chains in the swollen gel should occur with ease and should be driven to completion if there is no competing reaction. With this view in mind, the cyclic analogue (I) of (GSSG) was synthesized on an amino-



Scheme I

methyl-co-poly(styrene-1%-divinylbenzene) resin support, Scheme I. The desired peptide could be obtained only by inter-peptide chain reaction. The system described here has the advantage that the reaction between peptide chains in the same resin bead can be monitored to completion by using a quantitative ninhydrin test.[8] The quantitation of the desired cyclic peptide formed in this scheme is a good representation of the extent of inter-site reaction possible during solid phase peptide synthesis. As shown in the purification step, Scheme I, side products formed because of intra-site reactions can be distinguished and quantitated.

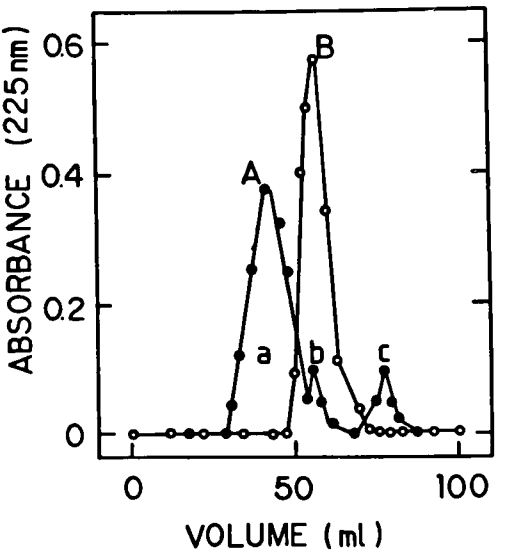
Results and Discussion

The tripeptide-resin Boc-Glu-OBzl (4-MeBzl; Cys-Gly-OCH_2-Pam—R) was synthesized from Boc-glycyl-4-(oxymethyl)phenylacetamidomethyl resin (Boc-Gly-OCH_2-Pam—R) by normal step-wise solid phase peptide synthesis using the preformed symmetric anhydrides of the Boc-amino acids. (Scheme I). An initial substitution of 0.18 mmole/g styrene was selected for this study. The N^{α}-protecting group was removed by treatment with 50% TFA/CH_2Cl_2.

After neutralization with 5% DIEA/CH_2Cl_2, the peptide was reacted with exactly one half molar equivalent of succinic anhydride in DMF. The carboxyl group so generated on one half of the peptide chains in the polymer was coupled to the N^{α}-amino group on the remaining unreacted peptide chains, using a DCC/HOBt coupling protocol in DMF. The coupling was continued until the peptide-resin showed a negative (<0.0003 mmol NH_2/g) ninhydrin test. The peptide liberated after treating the peptide-resin with HF was extracted into 10% aqueous HOAc, then treated at pH 8.5 with dithiothreitol. The reduced peptide mixture was fractionated on Sephadex G-15 (Figure 1, curve A). Peptide under peak a (99.5% of total by amino acid analysis) resulted from inter-site reaction between two tripeptide chains within the same swollen polymer bead and contained the DTT-stable succinyl linkage between two chains. Peak b contained a small amount of disulfide products which reformed after the reduction step. Product under peak c was a mixture of the possible tripeptide derivatives, e.g. unreacted GSH, succinyl-GSH, the acylurea, and the succinimide formed by intramolecular cyclization of the activated succinyl peptide.

The reduced hexapeptide (peak a) was then oxidised by air in 0.1M NH_4OAc buffer, pH 8.2 (negative Ellman's test). The oxidised peptide was lyopholized and again passed through a G-15 column, Figure 1, curve B. A single peak was observed, corresponding to the oxidized cyclic hexapeptide I. Peptide I was also made by classical solution chemistry and they were found to be identical.

The results from this study show that peptide chains in a swollen polymer gel are solvated and possess flexibility and mobility, very similar to those of equivalent peptide chains in solution.[1] These chains move freely and interact with other chains within the same bead to an extent comparable to that found in solution. Under these conditions permanent site isolation does not occur to a measureable extent. Kinetic site isolation was not examined here, but this phenomenon has been demonstrated under special circumstances where rapid competing reactions are present. In the experiments reported now, where there were no rapid competing reactions, it was demonstrated for the first time that reaction between different chains in a low cross-linked polystyrene-divinylbenzene resin bead in a good swelling (solvating) solvent can proceed to completion.



Legend to Figure 1. Chromatographic separation of succinylbisglutathione from synthetic by-products on a Sephadex G-15 column (1.8 x 40 cm). Curve A (•—•) crude reaction product after reduction with dithiothreitol. Curve B (o—o) purified product after air oxidation to the cyclic hexapeptide I. Elution with water at 7.3 ml/h.

Figure 1.

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CHRONIC FORMATION OF ACYLATION-RESISTANT DELETION PEPTIDES IN STEPWISE SOLID PHASE PEPTIDE SYNTHESIS: CHEMICAL MECHANISM, OCCURRENCE, AND PREVENTION.

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The formation of peptide products with one or more internal amino acids missing, compared with the target sequence, is perhaps the most serious practical shortcoming of the stepwise solid phase method for the synthesis of long peptides. Here we show that the chronic formation of acylation-resistant deletion peptides at every step in solid phase synthesis (SPS) is due to a chemical side reaction, and is readily prevented.

Mechanism

The side reaction is caused by aldehyde functionalities on the resin support forming a Schiff's base with the free amino group of the growing peptide chain, which resists acylation under normal conditions (Fig. 1).

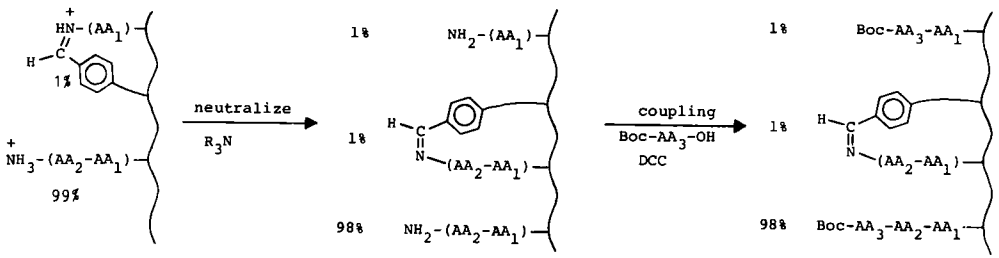

Fig.1. Schiff's base mechanism for formation of deletions.

In the succeeding cycle of the synthesis, the Schiff's base undergoes amine exchange which reveals the previously blocked peptide for further reaction and protects an equal amount of the new, longer chain. Thus, the one set of aldehyde sites will cause deletions at every cycle of the chain assembly.

Evidence

Controlled levels of aldehyde sites were introduced on loaded resins, using a novel procedure based on preparative use of the ninhydrin reaction[1]. These resins were used in stepwise SPS of a model peptide under standard and forcing conditions. The amounts of deletion peptides formed were evaluated by: quantitative ninhydrin monitoring, quantitative Edman degradation, and by direct chromatographic analysis of the crude cleaved products. All three methods gave the same results (Table I).

Table I. Effect of Levels of Aldehyde Sites on Deletions

Concentration of Aldehyde Sites ($x10^6$mol/g)	Product Composition (mole%) LAGV	LAV	LGV	AGV	AV+GV	LV
109*	76.05	3.44	10.64	2.68	4.33	2.36
51	93.51	0.98	2.31	0.93	1.62	0.65
23	97.19	1.18	1.26	0.14	0.02	0.21
1.3	99.46	0.08	0.22	0.14	<0.05	<0.05

Using Boc-Val-OCH_2Pam-Resin, $110x10^{-6}$mol/g Val. Coupling protocol, 2x2hr in situ DCC in CH_2Cl_2; in the case marked (*), a duplicate synthesis in which the couplings were followed by acetic anhydride in pyridine for 1hr gave identical results within experimental error.

The formation of deletion peptides by this mechanism is

insensitive to repeated couplings or acetylation under vigorous conditions. There is a clear correlation between resin-bound aldehyde concentration and levels of deletion peptide formation.

A colorimetric assay for such carbonyl functionalities, based on the quantitative ninhydrin reaction[2], was developed. Ketone functionalities show up to varying extents, correlating with their ability to cause deletion peptides. Results of screening some resins commonly used for SPS are shown in Table II.

Table II. Aldehyde Content of Various Resins

Resin	Loading ($x10^6$mol/g)	Source	Aldehyde Concentration ($x10^6$mol/g)
S-1%DVB	-	Biorad	1.29
$ClCH_2$-Resin	700	Pierce	8.65
	700	Biorad	16.10
	1300	Biorad	22.60
	300	Lab Systems	6.27
NH_2CH_2-Resin	600	ex $ClCH_2$-	25.27
	220	ex (S-DVB)	1.33
	750	ex (S-DVB)	1.90
	1000	ex (S-DVB)	1.00
BHA-Resin	470	Beckman	3.20
	510	Beckman	1.05
	530	Beckman	1.29
p-alkoxybenzyl-	230	Chemalog	8.77
-Resin	260	S. Mojsov	0.85

The aldehyde functionality arises from a variety of side reactions in the derivatization and/or transformation of functionalized resins. The level of aldehydes found in commonly used resins is consistent with the observed chronic levels of deletion using these resins.

Prevention

Aminomethyl-resin prepared using a strong acid Friedel-Crafts catalyst[3] contains minimal amounts of aldehydes (Table II). Using such resins, long peptide chains have been routinely assembled by the stepwise solid phase method with minimal levels of deletions (<0.2% per cycle), as determined by the quantitative Edman degradation (results given in Ref. 4).

Conclusion

These results show that putative physicochemical shortcomings of the resin supports are not responsible for chronic deletion peptide formation in SPS. Rather, this is a chemical side reaction, due to aldehyde functionalities on the resin. Clean resin with very low levels of aldehydes is readily prepared and loaded, and gives chain assembly essentially free of chronic acylation-resistant incomplete reaction.

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SEQUENCE-DEPENDENT COUPLING PROBLEMS IN STEPWISE SOLID PHASE PEPTIDE SYNTHESIS: OCCURENCE, MECHANISM, AND CORRECTION.

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In the course of synthesis of thirty five peptides from hydrophilic sequences of viral coat proteins, encompassing over 700 couplings each monitored by a sensitive quantitative procedure, we have found a number of couplings that did not go to completion (Table I). Here we report detailed analysis of this data which shows that the major incomplete coupling problem is non-random, occurs in about one out of five peptides, and involves four consecutive couplings between 10 and 15 residues from the resin. A mechanism is suggested.

TABLE I. Peptides Synthesized

35 peptides
31 different sequences
723 residues
576 first couplings (w/o Asn, Gln, Arg)
69 incomplete (>0.5%); 48 incomplete (>2%)
10 incomplete second couplings

Stepwise SPPS; Pam-(S-1%DVB); "in situ" DCC activation (0.1M DCC & Boc-amino acid; 2.5-fold excess over peptide) in dichloromethane (DCM) (0-30min), plus 40% DMF (31-60min). Asn, Gln coupled in DMF as HOBt esters. Asn, Gln, Arg couplings slower for activation reasons & excluded from data.

All syntheses were performed under strictly controlled identical conditions using optimized manual stepwise solid phase procedures. Four different peptides were synthesized simultaneously. Thus, the resin, reagents, solvents, and all manipulations were identical except for the different Boc-amino acid added to each vessel in the coupling step. Quantitative ninhydrin monitoring(1) showed that the coupling reactions were normally greater than 99.5% complete. However, from time to time coupling in one of the vessels would be incomplete (0.5-20% unreacted amine). The other syntheses thus served as controls for all the variables except the nature of the incoming Boc-amino acid and the specific sequence of the peptide to which it was being added.

Analysis of the monitoring data was performed in several ways. Figure 1 shows a bimodal distribution of incomplete couplings: most were incomplete at the 3 to 5% level or at the 15 to 20% level. This suggests two distinct types of incomplete coupling, presumably with different origins.

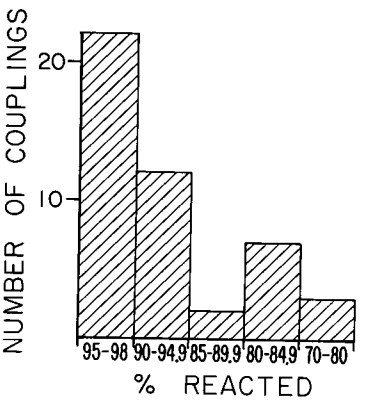

Fig. 1. Number of incomplete first couplings vs. level.

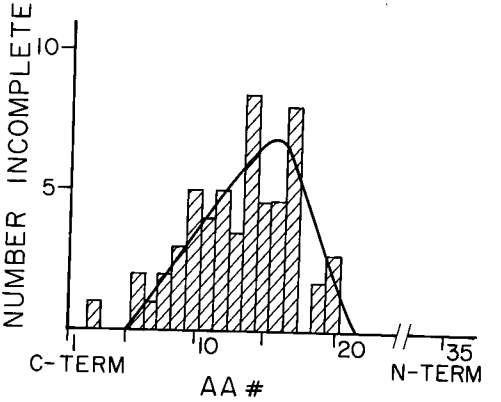

Fig.2 Number of incomplete first couplings vs. position

The data were also examined for correlation with chain length (Figure 2; data normalized to thirty-five first couplings at each chain length). There is a pronounced maximum in the tendency to couple incompletely for protected chains about 15 residues long. This tendency drops off rapidly: no incomplete reactions were observed in all the couplings to chains 21 or more residues in length, even though these represented 30% of the total couplings.

The data in Table II showed that the couplings of the sterically hindered amino acids, Thr(Bzl), Ile, and Val, were more often incomplete. We were unable correlate incomplete coupling with known properties of the N-terminal amino acids involved.

Detailed examination of the data shows that there are clearly two distinct types of incomplete couplings:

i. Random (usually 0.5-2%). These are infrequent: we found 28 randomly spread through 24 peptides (about 400 couplings).

ii. Nonrandom (2-20% level). These occur as strings of 4 residues, usually at positions 11-14 from the resin (Table III). This type of incomplete coupling is responsible for the maxima in the curves shown in Figure 1 & 2. Most of the

non-random incomplete couplings are concentrated in a few specific peptides: 6 of the 31 different sequences showed

TABLE II. Dependence on Activated and N-Terminal Amino Acid

	Total Residues in 35 Peptides	Involvement in Incomplete Reaction			
		Activated Amino Acid	%	N-terminal Amino Acid	%
Asp(OBzl)	27	1	4%	0	0
Asn	28	-	-	0	0
Thr(Bzl)	27	5	19%	0	0
Ser(Bzl)	26	1	4%	0	0
Glu(OBzl)	38	1	3%	3	8%
Gln	36	-	-	12	33%
Gly	35	0	0	2	6%
Ala	45	2	4%	6	13%
Pro	59	5	9%	-	-
Cys(MeBzl)	22	1	5%	0	0
Val	33	7	21%	4	12%
Met	13	0	0	0	0
Ile	26	5	19%	2	8%
Leu	45	6	13%	5	11%
Tyr(BrZ)	33	3	9%	0	0
Phe	13	0	0	0	0
Trp	13	0	0	2	15%
His	23	2	9%	2	7%
Lyz(ClZ)	28	2	7%	2	7%
NH2-Caproic	28	1	4%	2	8%
Arg(Tos)	90	-	-	6	7%

TABLE III. Occurence of Incomplete Coupling

# Res. in Pep.	# Incompl. Couplings	Position <2%	Position >2%	# Res. in Pep.	# Incompl. Couplings	Position <2%	Position >2%
11	2	-	10,11	22	1	12	-
13	1	10	-	23	1	-	16
13	1	-	8	23	1	-	16
13	2	5,9	-	26	1	-	17
14	1	-	10	27	1	-	14
14	1	12	-	27	2	9	14
14	2	7	8	30	1	-	11
15	2	8	7	33#	3	7,15	6
16	0	-	-	35	3	9,17	8
16	2	13	14	12*	4		9-12
19	0	-	-	13*	4		10-13
20#	6	5,12,16	9,14	14*	4	7-10	
20	5	12	3,9, 14,20	19*	4		14-17
				22*	5		16-20
21	0	-	-	34*#	4		12-15

*Incomplete for 4-5 consecutive residues.
Sequence made more than once.

this phenomenon, which accounted for 25 of the 48 couplings more than 2% incomplete. It was found that in DCM the non-random poor coupling was completely reproducible. Furthermore, all 10 incomplete second couplings were found in these sequences, whereas the random incomplete couplings always recoupled successfully.

The major sequence-dependent incomplete coupling is consistent with the ability of some resin-bound peptides to form intermolecular H-bonded aggregates in poor solvents, even when covalently attached to a swollen polymer network(2, 3). This interpretation is supported by the complete coupling of such problem sequences in DMF, a much better, polar, and H-bonding solvent. The maximum tendency at about 15 residues is also consistent with the known dependence of such intermolecular aggregation on chain length(4).

Sequence-dependent coupling problems can be readily overcome with adequate feedback control of the synthesis, by quantitative monitoring and recoupling under more highly solvating conditions where necessary. Peptides synthesized in this way contain negligible levels of deletions (<0.5% per residue). Clearly, it would be desirable to perform all couplings in DMF if a satisfactory and convenient activation method in this solvent were available.

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SOLID PHASE SEGMENT SYNTHESIS AND ASSEMBLING

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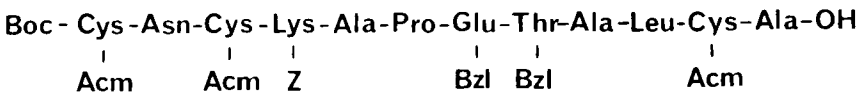
In the course of our studies on the structure-activity relationships of the bee venom toxin apamin, we have been concerned with the synthesis of analogues modified in the C- terminal region, especially at the positions 13 and 14[1]. In order to accelerate the preparation of each analogue we decided to try synthesis of the N- terminal 1-12 segment and subsequent couplings on different 13-18 sequences assembled on a benzhydrylamine resin. This study was also undertaken to see if a segment of twelve amino acids can be extensively purified and characterized as it should be for use in general strategy of solid phase segment synthesis and assembling[2,3] to build larger peptides or proteins.

The work described in this report concerns both synthesis of the 12-membered segment, corresponding the 1-12 sequence of apamin,



on the photosensitive solid support bromomethyl-NBB-resin (α-(4-bromomethyl-3-nitrobenzamido) benzyl copoly (styrene-1 %-divinylbenzene)[3], its intensive purification and characterization and also solid phase couplings of this segment on three different 13-18 apamin sequences to

achieve synthesis of native apamin and of two analogues, p.amino phenylalanine (Pap) 13 and 14 apamin.

Synthesis of apamin 1-12 segment

The bromomethyl-NBB-resin was synthesized as described earlier[2] by coupling of 4-bromomethyl-3-nitrobenzoic acid through its preformed anhydride on a benzhydrylamine resin.

3.39 g of NBB-resin (2.47 meq CH_2Br) were reacted with 1.53 g (4.96 mmol) of cesium Boc-alaninate in DMF at 50°C to yield 2.20 meq of alanine (89 % yield) on the resin. All protected amino-acids were incorporated by standard solid phase procedure, using a double coupling program, except Boc-Leu at position 3 because of high probability to form diketopiperazine. Boc-Leu-OH was coupled by the method of Suzuki[4] and no drop of functionalization was detected by the quantitative ninhydrin test[5] and by amino acid analysis. All other amino acids incorporated also very well.

Photolysis gave excellent yields varying from 82 to 99 % with batches of near one gram of peptide resin (total average yield 89 %) treated for 9 hrs in a mixture of trifluoroethanol in methylene chloride (20:80) after sonication.

The crude peptide was then treated with organic solvents to remove soluble material in ether and chloroform and then precipitated from DMF with water (89 % yield). Column chromatography on LH-60 in DMF gave only one peptidic peak (yield 82 %). Semi-preparative HPLC on C_{18} in DMF/water (82:18) was the final step (yield 80 %).

Amino acid analysis was as expected : Asp 0.99, Thr 1.18, Glu 1.03, Pro 1.01, Ala 3.00, Cys 1.44, Leu 0.99, Lys 0.97. TLC (Rf 0.68, MeOH) and HPTLC (RP_{18}, Rf 0.60,

MeOH-DMF 1-1) gave single spots. $[\alpha]_D^{25}$ = - 35.7 (c, 0.6 DMF). Mp = 150-152°C. Proton NMR spectrum with a 270 MHz (Bruckner) gave all expected signals and no apparent sign of impurities.

Solid phase assembling

On a benzhydrylamine resin, three sequences, analogues of the 13-18 portion of apamin, were first assembled stepwise and then the segment Boc-Apa (1-12)-OH was coupled with DCC and HOBt in DMF. Concentration rather than excess seemed important for high yields. In the first assembling the segment was coupled in a two fold excess in 7 ml DMF (yield 77 %), in the two other assemblings the segment was in 1.3 excess but in 1.5 ml DMF (yields 94 and 96 %) (Fig. 1).

	Sequence on the resin	Coupling yield
	Tos Acm Tos H-Arg-Arg-Cys-Gln-Gln-His-NH-CH- R ø	77%
Boc-Apa(1-12)OH +	Z H-Pap-Arg	94%
	Tos Z H-Arg-Pap	96%

Figure 1.

After HF reaction, cysteine deprotection and disulfide bridging, apamin and the two analogues Pap^{13}-apamin and Pap^{14}-apamin were purified as described earlier (6) but a last HPLC (C_{18} in ethanol and ammonium acetate) purification was added which apparently removed some impurities in very small amounts. Apamin was recovered with excel-

lent analytical data and full toxic activity and binding capacities. The two analogues were much less active (1.4 and 1.6 %).

Conclusion

The results obtained by solid phase segment synthesis and assembling look promising for eventual protein synthesis thanks to rapid synthesis of highly purified segments and to high yields of segment couplings on solid support.

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PEPTIDE SYNTHESIS USING PROPYLPHOSPHONIC ACID ANHYDRIDE AS A COUPLING REAGENT

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Introduction

n-Propylphosphonic acid anhydride is a valuable peptide forming agent. Racemisation is low and comparable with that found with reagents such as DCC/HOBT. The anhydride is soluble in all solvents applied in peptide synthesis and has a practically infinite shelf life at room temperature, if moisture is excluded. A 50 % solution in methylene chloride is usually applied, but 80 % solutions of the reagent in dimethylformamide are also stable (1).

Experiments with aminoacid and peptide derivatives with unprotected side chains (hydroxy, guanido, or imidazolyl groups) showed, that synthesis under the conditions of minimal protection also proceeds well (2).

Results and Discussion

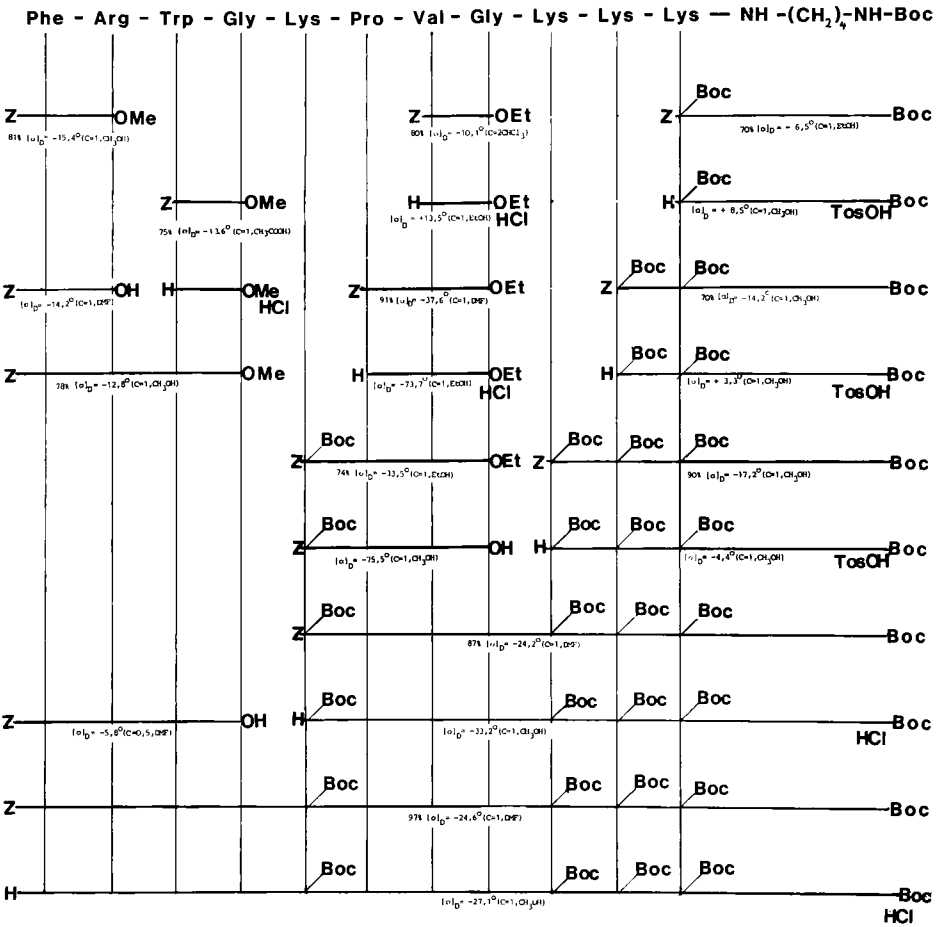
Several peptides of biological interest have been synthesized, e.g.

protected [Lys^{17}]-ACTH-(7-17)undecapeptide-4-amino-n-butylamide (SCHEME 1)

and a dodecapeptide from the C-terminal region of the adenovirus 82 K-protein (SCHEME 2)

SCHEME 1

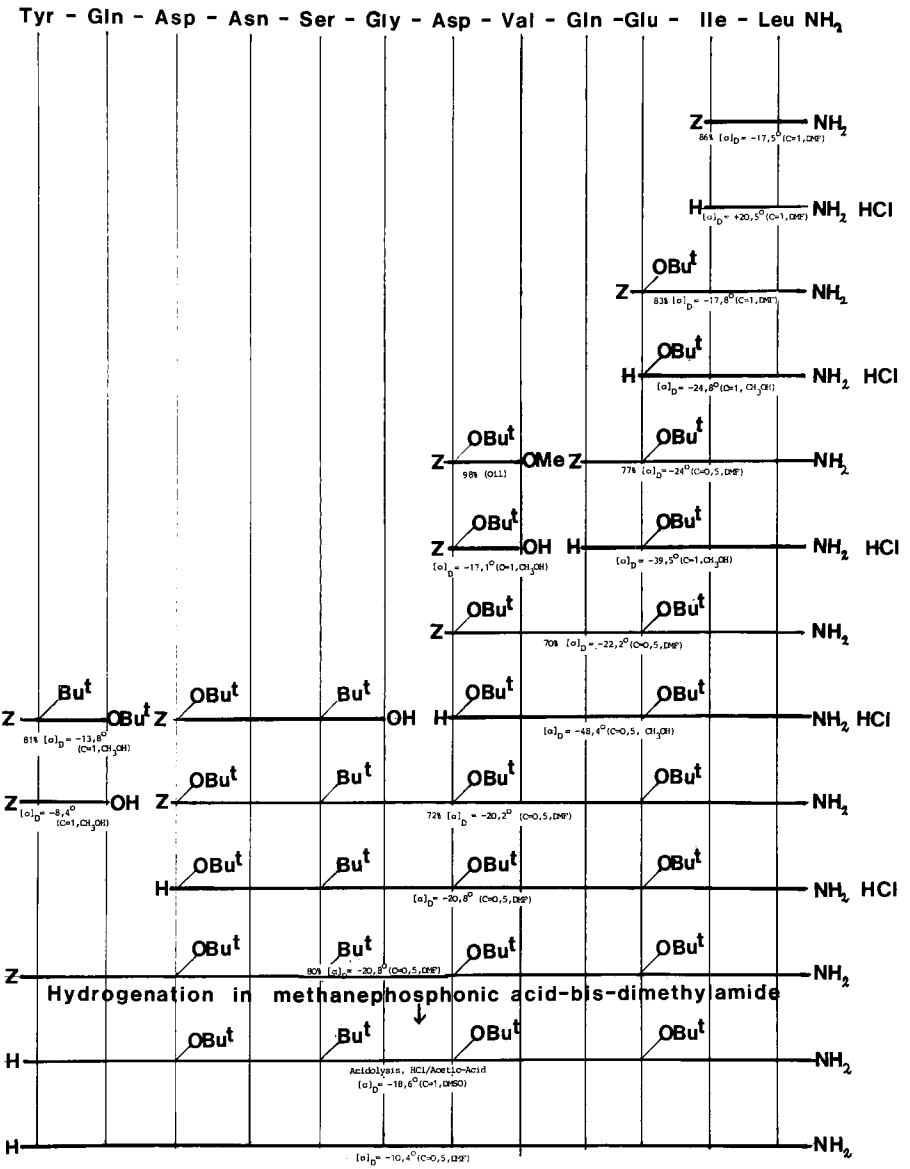
Synthesis of the ACTH partial sequence 11 - 17



SCHEME 2

Synthesis of a dodecapeptide sequence

from the C -terminal region of adenovirus 82 K protein



The procedure of the synthesis is simple:

5 Moles of a tertiary amine are added to the cooled solution of one mole of each of the parts to be coupled in an appropriate solvent. 650 ml of the 50 % solution of propylphosphonic acid anhydride in methylene chloride are added at temperatures below 0°C. After storing the solution overnight at room temperature, solid peptide derivatives were isolated by pouring the solution into saturated sodium-hydrogencarbonate solution, non solidifying peptide derivatives are extracted into ethylacetate, washed with water and 5 % potassium hydrogen sulfate (if basic side chains are absent). After drying over sodium sulfate and evaporation of the ethylacetate solution most of the peptide derivatives solidify upon addition of ether.

Conclusions

The method demonstrated is a low-racemising simple procedure with an easily accessible (3), stable condensing reagent, which avoids filtration of insoluble by-products and is therefore equally well suited for the technical production of small and large peptides, even if they are poorly soluble in the final stages of the synthesis.

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DIMETHYLAMINO-TERT-BUTYLESTER - A NOVEL CARBOXYL PROTECTION

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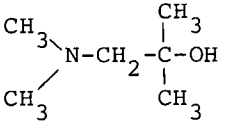
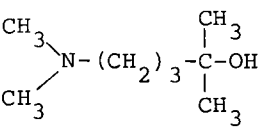
There was a need for us to find carboxy protecting groups, which enhance the solubility of protected peptides, allow sufficient analytics of the peptide and are cleavable. We studied ester functions, where the alcohol component is substituted with ionic groups like phosphoric acid, sulphonic acid or amines. In this communication we would like to describe some features of esters derived from dimethylamino-tert-butanol (I)[1] and attempts with dimethylamino-tert-hexanol (II).[2]

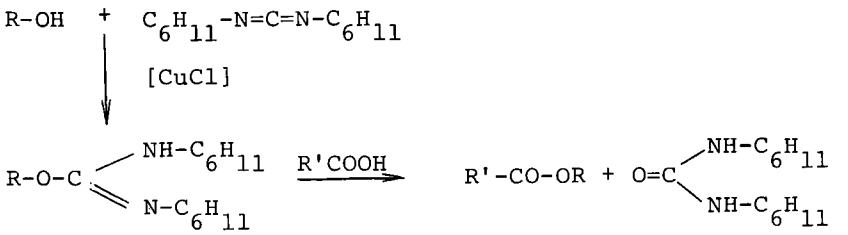


(I)



(II)

To condense carboxy functions with these alcohols we chose their O-alkyl-N,N'-dicyclohexyl-isoureas (Scheme 1). This method was developed by Schmidt and Moosmüller in 1956[3] and later used by Vowinkel[4]. Alcohols can be added to carbodiimides when catalyzed by copper (I)-chloride (or Cu(II) or Zn(II), Däbritz 1966[5]). Because easy available and inexpensive we chose dicyclohexyl carbodiimide.



Scheme 1. Copper (I) catalyzed addition of alcohols to carbodiimides and esterification of carboxy functions with the O-alkyl-N,N'-dicyclohexyl-isourea[3,4].

We prepared the aminoalcohol (I) after Riedel[1] from dimethylamine and iso-butylene oxide with a 70-75% yield and obtained correct analytical data. The aminobutanol was treated with DCC and CuCl (40/20/1, equivalents) at ambient temperature. The reaction was monitored by IR-spectroscopy for a maximum band at 1660 cm^{-1} of the isourea and the disappearing carbodiimide band at 2120 cm^{-1}. With less than 2 equivalents of aminoalcohol the reaction was complete in about 4 weeks, the above conditions allowed workup after 2 weeks. When the temperature was raised to 40°C the batch yielded mostly side products. The isourea was purified on an aluminum oxide column in n-hexane (280 nm), to produce in 95% yield a colorless oil with correct analytical data.

N-protected amino acids can be esterified in high yield (50-80% after silica gel chromatography) in inert solvents like methylene chloride or dimethylformamide with slight excess of the O-dimethylamino-tert-butyl-isourea overnight at ambient temperature. Boc-tyrosine and an excess of the reagent led initially to esterification of the carboxylic function but then the phenol group was also alkylated. With equimolar amounts of the alkylating reagent we could obtain the ester of Boc-tyrosin, and with Boc-tyrosine methylester

the ether derivative. The side chain of asparagine was not affected.

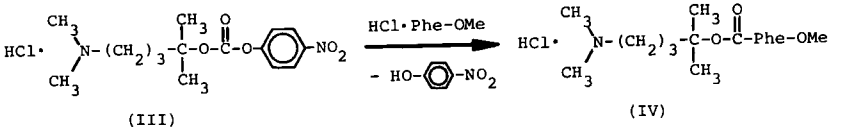
Cleavage of the ester function was studied with Boc-alanine-dimethylamino-tert-butyl ester. It is cleaved with 0.5n NaOH (1.4 equivalents) in 50% aqueous methanol, partly cleaved by 40% HBr in acetic acid, but not affected at all by TFA after 39 hours.

Campbell et al. have shown in 1938[2] that because of the inductive effect of the amino function tertiary alcohols behave more like primary ones in substitution and dehydration reactions the nearer the alcoholic and amino function are located. Because Brady et al.[6] in 1977 proved that 1,4-dimethylpiperidine-4-yl-oxycarbonyl groups need stronger acids for their cleavage than TFA (45% HBr/acetic acid, 1 hour, ambient temperature), we expected TFA-lability of urethanes and esters for the one CH_2-group longer dimethylamino-tert-hexanol (II).

Alcohol (II) was synthesized from the Grignard of 3-dimethylamino propyl chloride[7] and acetone in 40-50% overall yield. The colorless oil gave the expected analytical data. We prepared the N,N'-dicyclohexyl-isourea of this compound in 92% yield as colorless oil with correct analytical data using the same procedure described for alcohol (I). It was not possible to esterify N-protected amino acids to a preparative significant extent. The main reaction was always elimination of dicyclohexylurea which yielded the alkene of the corresponding alcohol.

To prove the likely acid lability of the urethane of this aminoalcohol (II), we prepared its p-nitrophenyl carbonate (III) and treated it with HCl·Phe-OMe (Scheme 2). A

crystalline, analytical correct product (IV) was obtained in 81% yield.



(III) (IV)

Scheme 2. Use of Dimethylamino-tert-hexyl-p-nitrophenyl carbonate for protection of amino groups

The amino protecting group of compound (IV) was cleaved by TFA (10% anisole or thioanisole) to 70% after 1h and quantitatively after 2h, but acetic acid did not affect it at all after 20h. 3.9 equivalents of 0.5m NaOH in 50% aqueous methanol did not cleave the urethane for at least 20h, whereas the methyl ester was cleaved within a few minutes.

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NEW SYNTHETIC APPROACH TO PEPTIDE SULFATE ESTERS. SYNTHESIS AND BIOLOGICAL ACTIVITIES OF CCK ANALOGS

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Introduction

Sulfate ester (SE) formation of hydroxyamino-acids (mostly Tyr) in peptides/proteins seems to be an important regulatory process. Some peptides (cholecystokinin, caerulein) show characteristic biological activities only when in the SE form. In other cases, the cleavage of SE activates the molecule: in proenkephalin one part of the peptide exists as a biological inactive SE[1]. Protein sulfatization on Tyr may have an important role in cell function[2].

The reagents used until now in the synthesis of peptide SE were found to be unsatisfactory. Chlorosulfonic acid in TFA[3] and the pyridine-SO_3 complex[4] were shown to be too reactive and caused several side reactions including sulfonation of Tyr and Trp. The DCCI sulfuric acid method[5,6] can only be used with fully protected peptides.

We describe a new reagent and a general strategy for synthesizing peptides containing hydroxyamino acid SE. Several tertiary ammonium salts of acetylsulfuric acid (ASA) were found to be ideal sulfatizing reagents for N-protected hydroxyamino acids and peptides. Whereas ASA is unstable[7]. Its tertiary ammonium salts (pyridine. 4-dimethylaminopyridine. etc.) are crystalline and stable at + 4°C in the absence of moisture. One of these compounds, the acetyl sulfuric acid pyridinium salt (ASAP) has been reported for introduction of a SE group in Tyr[8] and cholecystokinin-octapeptide[9].

Results and Discussion

Using 50-80% excess of ASAP in pyridine, N-protected Ser, Thr and Hyp are converted to their corresponding SE within 60-90 min at RT. The phenolic hydroxyl of Tyr reacts more slowly (8-12 hours) under the same conditions. Eleven SE derivatives of Boc- or Fmoc-protected hydroxyamino acids were synthesized. All SE's are stable in base (0.1N NaOH.RT), the O-sulfates of Ser, Thr and Hyp are stable in TFA and even in HF (see Table I). Conventional SPPS can therefore be used to generate peptides containing Ser(SE), Thr(SE) and Hyp(SE) when coupling the protected amino acid O-sulfate(Na) with the DCC/HOBT, DCC/PFPOH or DCC/SUOH method. HF treatment, ammonolysis or hydrazinolysis of the peptide do not cause cleavage of SE.

Table I. Stability of Amino Acid Sulfate Esters*

Amino Acid O-Sulfate	100% TFA 20°C, 2 days	50% TFA in CH_2Cl_2 20°C, 2 days	HF 0°C, 45 min	apolar solvents 20°C, 2 hours
H-Ser(SO_3H)-OH	Stable	Stable	5-10% Cleav.	Unstable
H-Thr(SO_3H)-OH	Stable	Stable	5-10% Cleav.	Unstable
H-Hyp(SO_3H)-OH	Stable	Stable	5-10% Cleav.	Unstable
H-Tyr(SO_3H)-OH	10-30% Cleavage (60 min)	10-20% Cleavage (60 min)	80-90% Cleavage	Unstable

*Amino acid SE's are stable in water (pH 1), except for TyrSO_3H. The SE moiety of N protected amino acid SE (alkali salts) show the same stability as that of amino acid SE. Instability in apolar solvents is believed to be due to the inability of an otherwise stable zwitterionic structure to form.

Tyr (SE) is unstable in TFA and HF, therefore in the synthesis of Tyr(SE) containing peptides a slightly different strategy using ASAP had to be designed. It was found that the reagent is unreactive toward free imidazole, indole, guanidino and thioether side chains. Selective and direct sulfatization of Tyr containing N-protected peptides could therefore be achieved in the absence of Ser, Thr and Hyp or in their

presence when protected with an acetyl group. The best examples for this strategy is the first successful solid phase synthesis of CCK-8-SE and caerulein[10]. N-Fmoc-CCK-8-NSE (NSE = nonsulfate ester) was synthesized (SPPS), cleaved by HF, and sulfatized with ASAP. After Fmoc-cleavage (10% piperidine in DMF), the CCK-8-SE was purified with RP-HPLC. In a similar way. the caerulein decapeptide was synthesized using O-acetyl protection for Thr. After HF cleavage, the peptide was sulfatized with ASAP, the O-acetyl group hydrolyzed with 1N NaOH (15 min) and the end product purified with RP-HPLC.

The different reactivities of ASAP towards alcoholic versus phenolic hydroxyl allows for the quasi-selective sulfatization of Ser. Thr or Hyp in the presence of Tyr in a peptide sequence. This is illustrated by the synthesis of a series of analogs of Ac-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH_2 (Ac-CCK(2-8)-NSE) and its SE include substitutions of the Asp residue by SE of Ser. Thr and Hyp. The biological activities of these and other CCK-analogs are summarized in Table II.

Table II. Relative Potencies of CCK-Analogs

Peptide	% CCK-activity	% Gastrinic activity
Pentagastrin	0	100
CCK-8-NSE = [Tyr^2]CCK(1-8)	0.3	100
CCK-8-SE = [$TyrSE^2$]CCK(1-8)	100	0
Ac-[Tyr^2,$SerSE^7$]-CCK(2-8)	0.3	40
Ac-[$TyrSE^2$,$SerSE^7$]-CCK(2-8)	190	0
Ac-[Tyr^2,$ThrSE^7$]-CCK(2-8)	0.2	25
Ac-[$TyrSE^2$,$ThrSE^7$]-CCK(2-8)	166	0
Ac-[Tyr^2,$HypSE^7$]-CCK(2-8)	0.3	0
Ac-[$TyrSE^2$,$HypSE^7$]-CCK(2-8)	300	0
Ac-[$TyrSE^2$,$DAla^4$]-CCK(2-8)	40 (in vivo 200%)	150
Ac-[$TyrSE^2$,$DTrp^5$]-CCK(2-8)	0.7	5
Ac-[$TyrSE^2$,$DMet^6$]-CCK(2-8)	0.6	0

These data show that the presence of the Tyr-O-sulfate in position 2 is necessary for the cholecystokinetic activity. Substitution of Asp-7 with the O-sulfate of Ser, Thr or Hyp gives very active agonists. From these results and 2 dimensional NMR data we propose that the introduction of $TyrSE^2$ causes a significant conformational change. The

nonsulfated CCK-8 and analogs possess a "tetragastrin conformation", and a special receptor population recognizes these peptides resulting in the characteristic "gastrin" activities. Sulfatization of Tyr^2 may induce a preferred conformation which is only recognized by a new set of receptors responsible for CCK like activity.

Introduction of DAla at position 4, even though not particularly favorable, may confer enzymic resistance which results in prolonged activity in vivo. The effect of such substitution on the biological properties of a more potent parent analog such as Ac-[TyrSE2,HypSE7]-CCK(2-8) is presently investigated.

Acknowledgements

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MODIFICATIONS IN THE MIXED ANHYDRIDE METHOD OF COUPLING. N-METHYLPIPERIDINE AS A SUPERIOR BASE.

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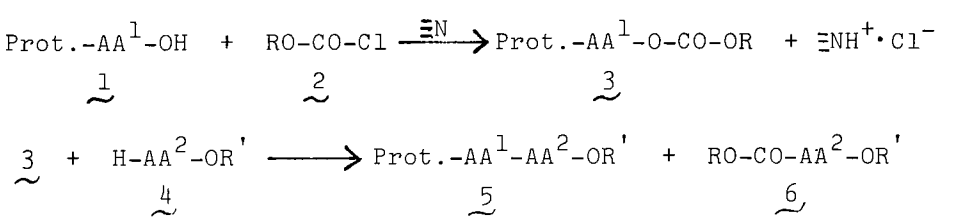
The mixed anhydride method of coupling involves reaction of an N-alkoxycarbonylamino acid or peptide 1 with an alkyl chloroformate 2 in the presence of a tertiary amine base to give the mixed carboxylic-carbonic acid anhydride 3, followed by attack at the activated carbonyl by the primary amine nucleophile 4. Unfortunately, besides the peptide 5, some urethane 6 is also formed because of amination at the wrong carbonyl group [see Meienhofer[1] for review]. More urethane is produced from hindered activated residues.[2] Little else is known about the factors contributing to urethane formation.



We have investigated this side reaction by comparing the yields of peptide and urethane obtained with model systems under various conditions using NMR and/or HPLC for quantitation. Results are expressed in terms of the ratio % urethane/[% urethane + % peptide]. Representative results appear in Table I.

Table I. Urethane/[Urethane + Peptide] Ratios for Couplings

Acid	Base	DCM	THF	DMF
Boc-Ile-OH	Et_3N	65.2	38.6	7.0
	NMM	3.4	5.7	5.5
	NMP	2.8	4.9	5.5
Boc-MeVal-OH	Et_3N	>90	67	24
	NMM	14	24.3	23.5
	NMP	10.5	23.1	25.7
Z-MeVal-OH	NMM		39.5	
	NMP	10	22	
Boc-Leu-OH	Et_3N		0.28	
	NMM		0.26	
	NMP		0.24	

Isobutyl chloroformate was added to base and 5% excess of acid in solvent at -5°. After 90 sec, a solution of H-Lys(Z)-OMe·HCl neutralized with base was added. Products were determined by HPLC on a μBondapak-C_{18} column with CH_3CN-H_2O (1:1) as solvent and absorbance detection at 215 nm. DCM = dichloromethane, NMM = N-methylmorpholine, NMP = N-methylpiperidine.

We conclude that the extent of urethane formation is primarily dictated by the amine base/solvent combination. In THF, Et_3N gives rise to much more urethane than NMM. In DMF, the difference is marginal. In DCM, two extremes obtain. NMM/DCM produces the least urethane, Et_3N/DCM, the most. NMM has been the favored base in peptide synthesis because less racemization accompanies its use.[3] That NMM gives less urethane than Et_3N in mixed anhydride reactions can be suspected from the literature but it has never been reported.

Our findings and other information prompted a search for a better base. We found that N-methylpiperidine gives a higher peptide to urethane ratio and in most cases a higher yield of peptide than NMM or Et_3N. In addition, it lead to less racemization than NMM (15-44% less) during couplings of

Z-Gly-Val-OH and Z-Gly-Phe-OH with H-Lys(Z)-OBzl in DCM and DMF. We recommend NMP/DCM as the best base/solvent combination for mixed anhydride couplings. Both NMP and NMM had been considered in the classical work[3] but NMM was selected for further attention. That DCM is a good solvent for mixed anhydride reactions runs contrary to generally held notions.[1]

During the above study, we realized that mixed anhydrides could be generated comfortably in a non-anhydrous solvent ($DMF-H_2O$, 4:1). This meant that they are not immediately destroyed by water, and therefore might be stable enough to survive a purification step of washing a solution of the anhydride with water. Pure symmetrical anhydrides of N-alkoxycarbonylamino acids have been obtained in this manner[4]. Indeed, when a mixed anhydride prepared in DCM from Boc-Ile-OH, iBuOCOCl and NMP (20 min at -5°) was washed with iced aqueous citric acid and $NaHCO_3$ followed by drying ($MgSO_4$) and removal of solvent under reduced pressure, a clear oil with the appropriate ^{1}H-NMR spectrum for Boc-Ile-O-CO-O-iBu was obtained in 80% yield. Amination with H-Lys(Z)-OMe gave the expected peptide in high yield, thus confirming the integrity of the anhydride. Some urethane was also formed, thus proving for the first time that 'wrong way' acylation indeed is a source of the urethane produced during mixed anhydride coupling. The mixed anhydride of Z-Gly-Val-OH could not be obtained in the same manner; it decomposed during the manipulation.

For racemization assessment in this study, the epimeric peptides were determined, after deprotection, by HPLC on a 10μ μBondapak-C_{18} column [30 x 0.39 cm] with 0.01 M ammonium acetate as solvent and absorbance detection at 208 nm. This system separates the eight H-Gly-Xxx-Lys-OH epimeric peptide pairs which we have examined, as well as many other lysyl-containing peptides. Pertinent data are listed in Table II.

Table II. Epimeric Peptides Well Separated by the System Described.

	Ala	Leu	Val	Ile	Pro	Phe	Tyr	Met	Trp
H-Xxx-Lys-OH		✓	✓	✓	✓	✓	✓		+
H-Gly-Xxx-Lys-OH	✓	✓	✓	✓	✓	✓	✓	✓	
H-Gly-Lys-Xxx-OH	+	✓	✓	✓	o	+			
H-Lys-Xxx-OH	o	+	✓	+✓	o	+	+		

✓0.01 M NH_4OAc; +0.1% H_3PO_4; oNo resolution

Acknowledgements

This work was financially supported by a grant from the Medical Research Council of Canada.

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AN IMPROVED PROCEDURE FOR PEPTIDE CYCLIZATION

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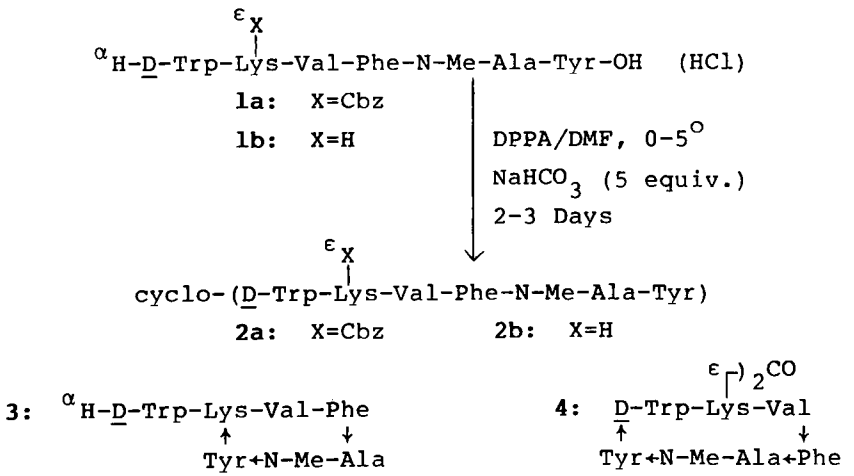
A practical and convenient method for the synthesis of cyclic peptides using the coupling reagent, diphenyl phosphoryl azide (DPPA), has been described.[1] In recent efforts to optimize the cyclization step in the synthesis of a highly potent somatostatin analog (**2b**)[2] (see Scheme I) we explored a number of modifications in this procedure. We were particularly interested in the effect of the type of base on yield, rate of reaction, and racemization. We felt that the insolubility of an inorganic base in DMF should afford two significant advantages over prior art: 1) cyclizations would proceed under conditions of lower solution pH, wherein polymer formation would be suppressed without the need for



Scheme 1. Cyclization Studies

very high dilution; 2) excess of base used at the outset would preclude the need for frequent monitoring and periodic base addition. The results are summarized in Table I.

Our study shows that replacement of triethylamine as base with either sodium bicarbonate or potassium monohydrogen phosphate results in highly efficient cyclization of linear substrate **1a** to product **2a**. The procedure parallels our published technique,[1] except that efficient stirring of the heterogeneous mixture is needed to assure completion of the reaction. Applying the method with bicarbonate to cyclization of **1a** on over a 100-g scale, we were able to isolate >99% pure **2a** in 77% yield.

Less than 0.3% racemization at tyrosine was observed under optimal conditions. The small amount of diastereoisomeric product in the large-scale preparation (the D-Tyr isomer of **2a**) was isolated and characterized. The chirality of D-tyrosine was established by known methods.[3]

Our success with this modified DPPA procedure prompted us

Table I. Cyclization of Protected Linear Substrate **1a**[a]

Base	Temp (°C)	Time (days)	Yield of **2a** (%)[b]	D-Tyr Isomer (%)[b]
Et_3N	5	3	74	1.2
$NaHCO_3$[c]	-30	10	79	1.4
$NaHCO_3$[c]	5	2	88	≤0.3
$NaHCO_3$[c]	20	1	89	≤0.3
Na_2HPO_4[c]	5	7	81	--
K_2HPO_4[c]	5	1[d]	91	<0.3

[a]All reactions were run as described in ref. 1, except for base as shown; total crude product, after precipitation from water, was analyzed by Sephadex gel filtration and RP-HPLC. [b]Determined by RP-HPLC. [c]Five molar equivalents added at start of reaction. [d]Two molar equivalents of DPPA used.

to evaluate an alternative approach to peptide **2b**. We thought selective cyclization of the α-amine (pK_a ~7.7)[4] in substrate **1b** could occur because the concentration of ε-amine (pK_a ~10.3)[4] would be suppressed if insoluble base were used. With $NaHCO_3$ as base we in fact saw a predominance of α-cyclized product **2b** over ε-cyclized product **3**. In contrast, triethylamine strongly favors ε-cyclization (see Table II). Constituents **2b** and **3** were separated by gel filtration on Sephadex G-25 using 2N HOAc. Key evidence supporting the structure of **3** was provided by sequence analysis:[5] (step 1) tryptophan; (step 2) nothing; (step 3) valine.

However, almost half of the total product mixture in the bicarbonate cyclization was dimeric in nature. This fraction consisted largely of ureas, among them urea **4**. This urea was prepared independently from **2b** using DPPA-$NaHCO_3$, and its structure was confirmed by NMR and mass spectral data. Ureas can arise *via* DPPA activation of amine carbonates and thus constitute unique by-products of the $NaHCO_3$ procedure.

When K_2HPO_4 was used in the cyclization of **1b**, dimeric by-products were virtually eliminated (see Table II). How-

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Table II. Selectivity in Cyclization of Substrate **1b**[a]

Base	Temp (°C)	Time (days)	Yield (%)[b] 2b	3	Ratio 2b:3	Dimer (%)
$NaHCO_3$	5	2	26	21	1.2	41
Et_3N	5	3	23	56	0.4	<2
K_2HPO_4	20	1	30	30	1.0	<2

[a]Reaction conducted as in Table I (footnote a), except that treatment with mixed-bed resin was omitted. [b]Based on HPLC analysis of the monomer fraction after gel filtration; peptide **2b** elutes slightly ahead of **3** on a gel column, presumably due to different conformational and adsorptive properties (*cf.* Veber, D.F. *et al.* (1978) *Proc. Nat. Acad. Sci.* **75**, 2636-2640).

ever, the selectivity for α-amine was not as good as that seen in the bicarbonate reaction. Nonetheless, the use of insoluble base allows a limited degree of control over ring closure in a peptide having amino groups with different pK_as.

In summary, the DPPA-bicarbonate procedure constitutes a dependable general technique for peptide cyclization in high yield, with minimal racemization, and is readily adaptable to large-scale synthesis.

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2-(3,5-DI-TERT-BUTYLPHENYL)-PROPYLOXYCARBONYL(t-BUMEOC),3,5-DI-TERT-BUTYLBENZYLOXYCARBONYL(DBZ) AND 2-ADAMANTYL-PROPYL-OXYCARBONYL(ADPOC) AMINO ACIDS AND THEIR USE FOR PEPTIDE SYNTHESES

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Introduction

Considerable progress has been made in recent years in finding groups for peptide synthesis, which can be removed under mild acidolytic conditions. In several cases it was demonstated that the Adpoc group allows selective cleavage in presence of other acid-labile residues[1]. The t-Bumeoc group, used for the first time from our group for peptide synthesis, is cleaved under extremely mild acidolytic conditions. The Dbz group is more stable compared to the Boc group, but both groups show extremely good solubility in organic solvents.

Results and Discussion

The starting material for the t-Bumeoc and Dbz amino acids, 3,5-di-tert.-butylbenzoic acid 4, is synthesized by a Friedel-Crafts reaction from tert.-butylchloride 1 and toluene 2 followed by oxidation of the methyl group with potassium permanganate. Reduction with lithium aluminum hydride or reaction of the methyl ester 5b with methylmagnesium iodide (Grignard

reaction) yields the corresponding alcohols 5a and 6 (Figure 1). The t-Bumeoc residue can easily be incorporated into amino acids using the fluoroformate, for the incorporation of the Dbz group the chloroformate is a suitable reagent. Both protecting groups are stable under alkaline conditions and the t-Bumeoc group is also stable against hydrogenolysis at low proton concentrations. The Dbz residue, however, is cleaved readily by hydrogen in the presence of Pd/C catalyst. The acidolytic cleavage is possible with HCl in ethylacetate at 40°C within 6 hours. The t-Bumeoc group is very mildly removable with 3 % trifluoroacetic acid in methylene chloride, acetic acid/formic acid (83 %)/water (7:1:2 v/v) or 80 % acetic acid. The cleavage in these three solvents can easily be

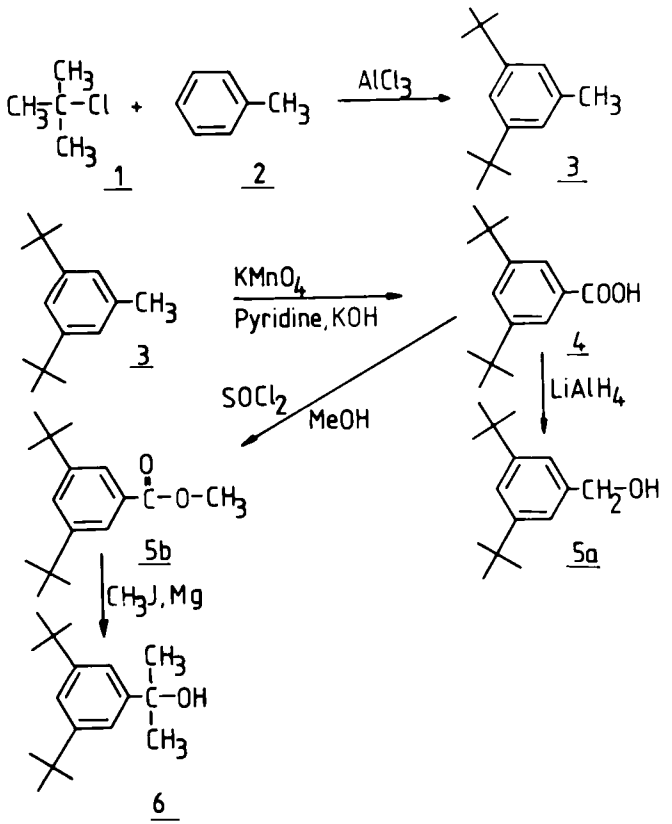


Figure 1. Synthesis of alcohols for the preparation of the reagents for the production of Dbz and t-Bumeoc amino acids.

followed quantitatively by high performance liquid chromatography, and in Table 1 the cleavage rates of t-Bumeoc-Phe-OH in various reagents are collected and compared with values found by Sieber and Iselin for a series of known protective groups[2]. According to our data the cleavage is a reaction of first order and as cleavage products the corresponding alcohols and olefins could be identified. If t-Bumeoc-Lys(Boc)-OH or t-Bumeoc-Ser(Bu^t)-OH are subjected to the solvents mentioned before, no free serine or lysine is found and therefore the t-Bumeoc group is suitable for selective liberation of NH_2 functions in peptide synthesis.

Table. Cleavage Rates of Different Protective Groups.

Protective group	k_1 a) (h^{-1})	k_{rel}	k_1 b) (h^{-1})	k_{rel}
Dbz	$2.1\ 10^{-4}$	0.43	$3.3\ 10^{-4}$	0.16
Boc	$5.0\ 10^{-4}$	1	$2.0\ 10^{-3}$	1
Ppoc	0.35	700	1.5	750
Adpoc	1.2 c)	2400	1.2	600
Bpoc	1.4	2800	3.9	2000
t-Bumeoc d)	2.0	4000	16.0	8000

a) CH_3COOH (80 %); b) CH_3COOH/HCOOH (83 %)/water (7:1:2); c) value determined at 40°C; d) value found for the Phe derivative; for other protected amino acids larger parameters are determined.

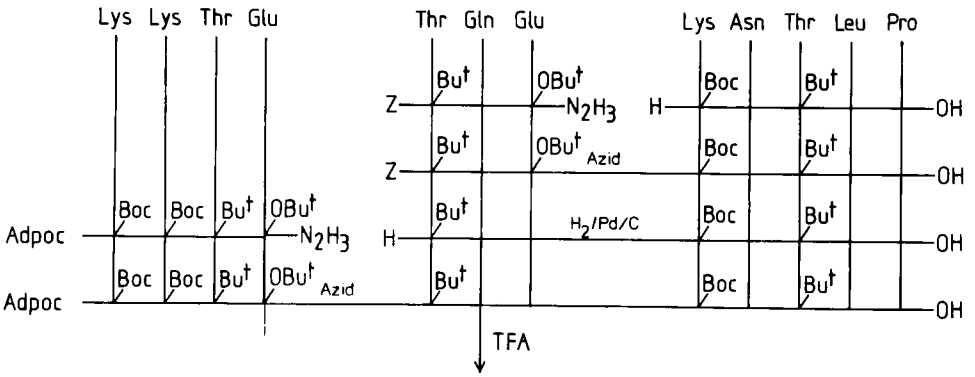
For our synthesis of thymosin ß9 (Ac-Ala-Asp-Lys-Pro-Asp-Leu-Gly-Glu-Ile-Asn-Ser-Phe-Asp-Lys-Ala-Lys-Leu-Lys-Lys-Thr-Glu Thr-Gln-Glu-Lys-Asn-Thr-Leu-Pro-Thr-Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys-Gln-Ala-Lys-OH) the Adpoc group proved to be a valuable residue for selective cleavage from α-amino-protected peptides; to find optimum conditions for removal numerous reagents are tested by us and with the most promising ones the cleavage rates for Adpoc-Trp-Lys(Boc)-OH (somatostatin(8-9)) and Adpoc-Ala-Lys(Boc)-Leu-OMe(thymosin ß9(15-17) are determined(Table 2).

Figure 2 shows the synthetic route to thymosin ß9(18-29).The Adpoc group and the Boc and Bu^t residues in the side chains are removed within one hour in presence of trifluoroacetic

Table 2.Cleavage Rates for Specific Removal of the Adpoc group from Adpoc-Trp-Lys(Boc)-OH(Somatostatin(8-9)) and Adpoc-Ala-Lys (Boc)-Leu-OMe(Thymosin ß9(15-17)) in Various Reagents(TFE =Trifluoroethanol).

Protected peptide	Reagent	$k(min^{-1})$	50 % cleavage	99.9 % (min)
Adpoc-Trp-Lys-(Boc)-OH	50%HCOOH/TFE (1:1)	0.07	10	30
	0.1N HCl/TFE (1:5)	0.02	30	120
	50%HCOOH/TFE / $CHCl_3$ (1:9:1)	0.013	53	300
Adpoc-Ala-Lys-(Boc)Leu-OMe	50%HCOOH/TFE / $CHCl_3$	0.015	46	210
	0.1N HCl/TFE / $CHCl_3$ (1:9:1)	0.09	8	20

acid. The dodecapeptide is received in a yield of 50 % in pure form after separation on a Sephadex G-25 column. From the corresponding methyl ester of the protected dodecapeptide the Adpoc group is selectively removable and thus allows coupling of the fragment thymosin ß9(1-17).



H-L-Lys-L-Lys-L-Thr-L-Glu-L-Thr-L-Gln-L-Glu-L-Lys-L-Asn-L-Thr-L-Leu-L-Pro-OH

Figure 2.Synthetic route to thymosin ß9(18-29).

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TOTAL SYNTHESIS OF THE IMMUNOSUPPRESSIVE DRUG "SANDIMMUNE" (CYCLOSPORIN A)

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Introduction

Cyclosporin A is a metabolite isolated from the fungal species Tolypocladium inflatum Gams.[1] Its structure was established by chemical degradation[2] and X-ray analysis.[3] Its pharmacological and clinical action as a selective immunosuppressive drug has been widely documented.[4] In Cyclosporin A (Figure 1) the amino acid in position 1, N-methyl-(4R)-4-but--2E-en-1-yl-4-methyl-(L)-threonine (MeBmt), was hitherto unknown and was first isolated following the stereospecific synthesis summarized below.

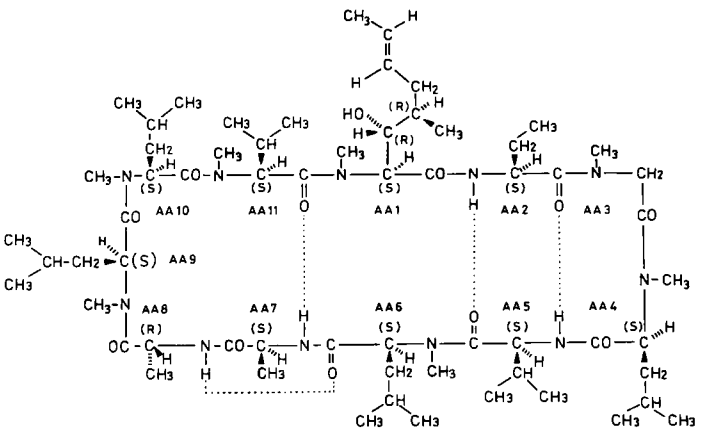

Fig. 1. Cyclosporin A ($C_{62}H_{111}N_{11}O_{12}$; M.W. 1202).

Synthesis of an enantiometrically pure MeBmt-amino acid

(R,R)-(+)-Tartaric acid is used as the basic optically active building block in the synthesis and modified in three major operations as shown in Figure 2.[5]

Synthesis of Cyclosporin A

In the synthesis of Cyclosporin A cyclisation (Figure 1) is effected at the only consecutive pair of non-N-methylated amino acids, between the L-alanine in position 7 and the D-alanine in position 8. A fragment condensation technique introducing the MeBmt-amino acid at the end of the linear undecapeptide synthesis was used. In this way the number of steps after the introduction of the MeBmt unit is minimized. The peptide fragments are built up in the direction shown by the arrows in Figure 3 using the step sequence which is indicated numerically. Using the mixed pivalic anhydride method,[6] bonds 1, 2 and 3 were first made and the tetrapeptide BOC-D-Ala-MeLeu-MeLeu-MeVal-benzylester synthesized. The benzylester of this tetrapeptide could not be made starting

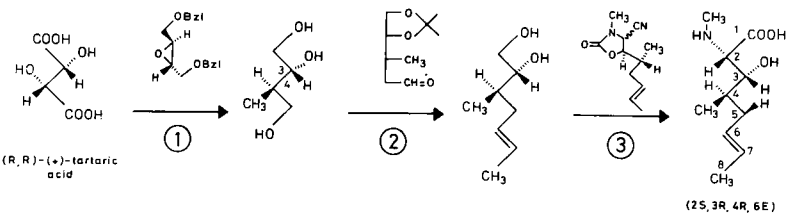

Fig. 2 Strategy used for the synthesis of the amino acid MeBmt.

from the right, by making the bond 3 first, because of instantaneous formation of a diketopiperazine ring when working with the dipeptide H-MeLeu-MeVal-benzylester. Bond 4 was made and the dipeptide BOC-Abu-Sar-benzylester synthesized.

The tetrapeptide BOC-MeLeu-Val-MeLeu-Ala-benzylester was synthesized by forming bonds 5, 6 and 7 in that order. Then by forming the bond 8, the hexapeptide BOC-Abu-Sar-MeLeu-Val--MeLeu-Ala-benzylester was prepared.

The amino acid MeBmt is incorporated as the dimethyloxazolinone derivative to protect the hydroxy and methylamino functions. The peptide bond 9 is made using the DCC method in presence of HOBt to produce, following acidic hydrolysis, the heptapeptide H-MeBmt-Abu-Sar-MeLeu-Val-MeLeu-Ala-benzylester. The BOP-reagent[7] has been used for the formation of bond 10. The use of this reagent has the advantage that either BOC-D-Ala-MeLeu-MeLeu-MeVal-OH (DLLL) or its diastereomer BOC-D-Ala-MeLeu-MeLeu-D-MeVal-OH (DLLD) can serve as starting materials to produce selectively the desired undecapeptide BOC-D-Ala-MeLeu-MeLeu-MeVal-MeBmt-Abu-Sar-MeLeu-Val--MeLeu-Ala-benzylester (DLLL-LLLLLLL). After deprotection of the undecapeptide Cyclosporin A (Figure 1) is obtained in a yield of 35%, if cyclisation is effected using either the BOP-reagent[7] or the mixed phosphonic anhydride method.[8]

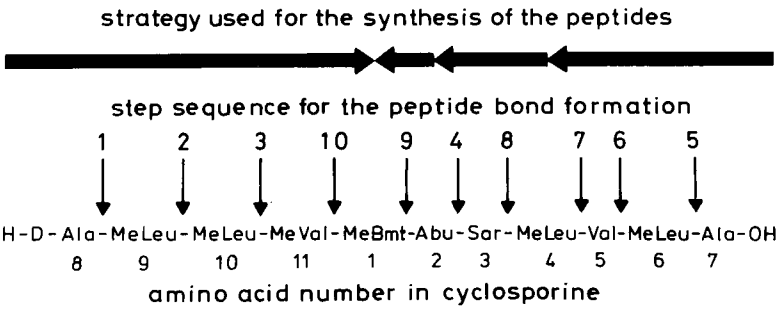

Fig. 3. Strategy used for the synthesis of the peptides.

Conclusion

The successful total synthesis of Cyclosporin A using a fragment condensation technique demonstrates that it is possible to synthesize peptides with N-methylamino acids by this technique under stereochemical control even under conditions recognized as most critical for isomerisation. Moreover it was possible to utilize the isomerisation and to synthesize a peptide containing exclusively the corresponding L-amino acid starting from a D-amino acid.

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SYNTHESIS OF 2-AMINO-8-OXO-9,10-EPOXYDECANOIC ACID DERIVATIVES

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Introduction

The amino acid, 2-amino-8-oxo-9,10-epoxy-decanoic acid, abbreviated Aoe[1] (1), is an epoxy ketone amino acid first found in the plant toxin Cyl-2 (2),[2] and subsequently identified in Chlamydocin (3),[3] a cytostatic cyclic tetrapeptide,[4] and in HC-toxin (4),[5] a host specific plant toxin. The epoxy ketone functional group is critical for the potent cytostatic activities of both chlamydocin (IC_{50} ≃ 2 nM) and HC-toxin (IC ≃ 20 nM) as either the reduced ketone or the opened epoxide derivatives are several thousand fold less potent.[3-5] Aoe derivatives (eg. 5) have been prepared by vinylation of the ω acid chloride of α-amino suberic acid (Asu, 6).[7] However, this route does not directly allow for the synthesis of chiral epoxide. We report here two new routes to Aoe that permit stereoselective synthesis of the chiral epoxy ketone group from the allylic alcohol 7.

Figure 1: Structures of Naturally Occurring Peptides Containing Aoe.

2, Cyl-2 = cyclo(Aoe-D-Tyr(OMe)-L-Ile-L-Pip)

3, Chlamydocin = cyclo(L-Aoe-Aib-L-Phe-D-Pro)

4, HC-Toxin = cyclo(L-Aoe-D-Pro-L-Ala-D-Ala)

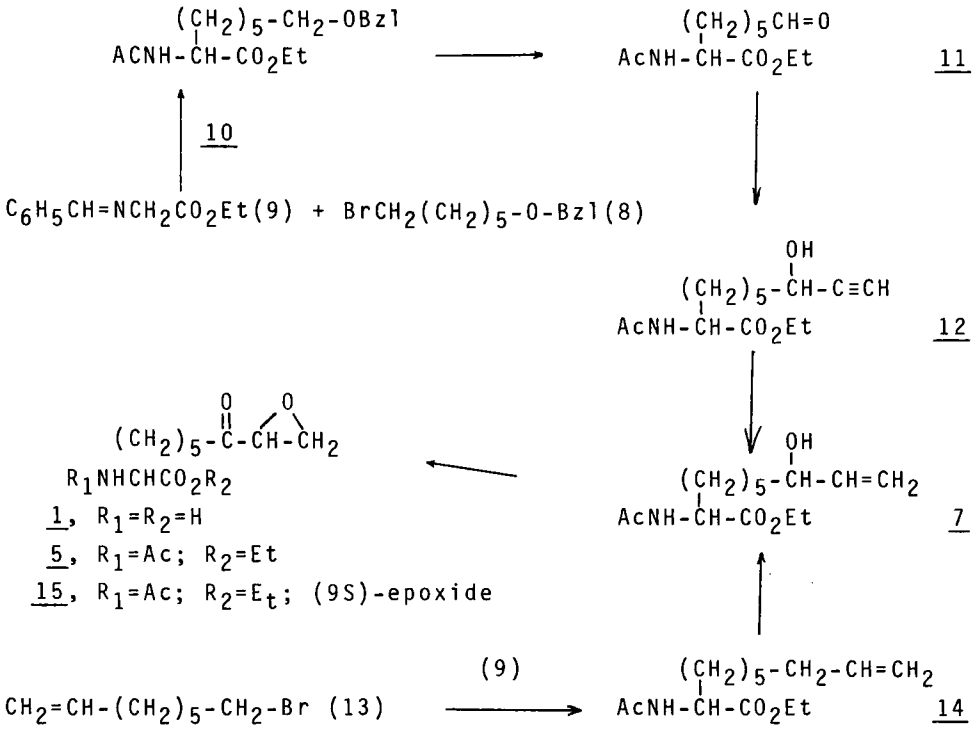
Results

The first approach is based on the addition of acetylene to a preformed aldehyde. Bromo ether 8 was synthesized in one step by treating 1,6-dibromohexane (1 eq.) with NaOBzl (1.0 eq). After distillation (0.2 mmHg), 8 was alkylated with benzylidene glycine ethyl ester 9 according to Stork's procedures[8] (-78°C, KO^tBu, THF). The resulting imine derivative of the amino acid was not purified but cleaved directly (1N HCl) and converted into the N-acetyl protected form, compound 10. The overall yield for the synthesis of this fully protected amino acid was 77% based on benzylidene glycine ethyl ester starting material.

Conversion of benzyl ether 10 into the allylic alcohol moiety by catalytic hydrogenation (60 psi, 2 days) followed by oxidation with SO_3 • pyridine complex in DMSO gave the aldehyde containing amino acid 11. The aldehyde derivative obtained after extractive workup was not purified but alkylated directly with acetylide anion (acetylene, BuLi, -78°C, THF). Acetylenic alcohol 12 was obtained in 40% overall yield based on benzyl ether 10 as starting material. Hydrogenation of 12 over Lindlar catalyst ($Pd/CaCO_3$ with Pb in MeOH) produced the desired allylic alcohol amino acid 7 in 94% yield (29% overall yield).

An alternate approach to the synthesis of 7 also was developed. Alkylation of 8-bromo-1-octene 13 with benzylidene glycine ethyl ester 9 as described previously followed by cleavage of the Schiff base (1.1 eq. $KHSO_4$ in H_2O) gave the free amine of the terminal unsaturated amino acid in 68% yield. Acetylation with acetic anhydride (1.1 eq) using DMAP (0.1 eq) as catalyst gave the N-acetyl derivative 14 in 95% yield. This dehydro-amino acid was converted to allylic alcohol intermediate 7 in 45% yield using a variation on Sharpless' procedures[9] (1 eq. SeO_2, 4 eq. TBHP in CH_2Cl_2, 48 hrs., room temperature; starting material 13 also was recovered in 35% yield). The overall yield to allylic alcohol 7 is 30%.

Figure 2. Synthesis of Derivatives of Aoe (2-Amino-8-oxo-9,10-epoxy-decanoic Acid).[a]



Conversion of 7 into racemic Aoe was carried out with a "one-pot" epoxidation-oxidation procedure described by Cella et al.[10] The allylic alcohol 7 was first treated with 1.2 eq. of MCPBA (0°C, 4 hrs.) followed by an additional 1.5 eq. of MCPBA with 0.04 eq. of TMP · HCl as catalyst (48 hrs., room temperature). After extractive work up and column purification the racemic epoxide (9RS)-Aoe derivative 5 was obtained in 72%

a. All stable intermediates were fully characterized by ^{1}H-NMR, ^{13}C-NMR, and C, H, N, analyses.

yield. Attempts to convert the allylic alcohol 7 into the chiral epoxy-alcohol 15 using the asymetric epoxidation procedure of Sharpless[11] are in progress.[10]

The application of these methods to the synthesis of chlamydocin and HC-toxin with chiral epoxide groups is currently in progress.

Acknowledgements. This work was supported by a grant from the National Cancer Institute, CA 27985.

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1. Abbreviations used: Aoe, 2-amino-8-oxo-9,10-epoxy-decanoic acid; Asu, 2-aminosuberic acid; D-Tyr(OMe), D-O-methyl-tyrosine; Pip, pipecolic acid, Aib, 2-aminoisobutyric acid; $KO^{+}Bu$, potassium tert-butoxide; THF, tetrahydrofuran; DMAP, 4-dimethylamino-pyridine; TBHP, tert-butyl hydroperoxide; MCPBA, m-Chloroperbenzoic acid; TMP • HCl, 2,2,6,6,-tetramethyl-piperidine hydrochloride.

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A GENERAL PROCEDURE FOR THE PREPARATION OF N-tert-BUTYLOXY-CARBONYL, N-ALKYLAMINO ACIDS

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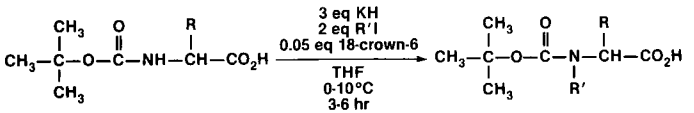
Introduction

Previously, we have used an established procedure[1] to synthesize N-protected, N-methylamino acids. However, this method proved to be unsatisfactory for the synthesis of N-tert-butyloxycarbonyl, N-methylmethionine (Boc-MeMet-OH). We have developed a new method for the efficient synthesis of Boc-MeMet-OH.[2] This N-alkylation procedure has proven to be of general synthetic utility, and with the appropriate alkyl iodide, can be used to synthesize a wide variety of N-protected, N-alkylamino acids (Figure 1).[3]



$R' = -CH_3, -CH_2CH_3, -CH_2-CH_2-CH_3$, or $-CH_2-CH=CH_2$

Fig. 1 Synthesis of Boc-N-alkylamino acids

General Experimental Procedure

In a flask maintained under a N_2 atmosphere, a solution of a Boc-amino acid (50 mmol) in dry THF (30 ml) is added

dropwise to a well stirred, cooled (0°C) suspension of KH (150 mmol) and 18-crown-6 (2.5 mmol, catalyst) in THF (200 mL). An alkyl iodide (100 mmol) dissolved in THF (30 mL) is then added dropwise. The thick suspension is stirred and maintained at 0-10°C and the reaction progress is monitored by TLC. Upon completion of the reaction (3-6 hr), the excess KH is destroyed by careful addition of glacial HOAc (10 mL) followed by H_2O (10 mL). This solution is poured into cold (4°C) H_2O (500 mL) and the pH adjusted to 8.5 with 2N NaOH. The aqueous solution is extracted with Et_2O (2x), adjusted to pH 2.5 with 3N HCl, and washed with EtOAc. The EtOAc layers are combined, dried ($MgSO_4$), and concentrated at reduced pressure to give the Boc-N-alkylamino acid.

Results and Discussion

A number of Boc-N-methylamino acids have been synthesized by the KH/MeI/18-crown-6 procedure (Table I). This N-methylation procedure has proven to be of general synthetic utility and has been used with other alkyl iodides to generate Boc-N-ethyl, propyl, and allyl amino acids (Table II).

Although partial racemization has been observed when the KH procedure is allowed to proceed overnight at room temperature, little or no racemization is evident under the preferred reaction conditions (0-10°C, 3-6 hr) (Table I). In an experiment designed to obtain a relative measure of the racemization potential for the preferred KH procedure, Boc-MeMet-OH was synthesized and crystallized as the dicyclohexylamine salt. The salt was then neutralized and used in a solid phase peptide synthesis of the analgesic enkephalin analog, metkephamid (H-Tyr-D-Ala-Gly-Phe-MeMet-NH_2).[4] After HF cleavage, the crude peptide was analyzed by reversed-phase liquid chromatography with a solvent system designed to separate diastereomeric peptides. This synthetic peptide was

Table I. N-tert-butyloxycarbonyl, N-methylamino acids[a]

Amino Acid	Reaction Time (Temp)	Yield (%)	m.p.(°C)	Optical Rotation (lit)[b] $[\alpha]_D^{25}$ (deg)	Conc.	Solvent
Ala	5 hr (5°C)	70	—	−31.4(−30.4)	1(0.5)	EtOH(EtOH)
			—	−30.9(−31.8)	2(2)	EtOAc(EtOAc)
			169-170[c]	−8.4[c]	0.5	MeOH
Cys (Et)	1.5 hr (0°C)	77	Oil	−61.1	1	EtOH
Cys (Trt)	4 hr (0°C)	80	Oil	−37.3	0.5	$CHCl_3$
			—	−29.5[d]	1	$CHCl_3$
Leu	5 hr (10°C)	83	50-53	−28.4(−24.6)	0.5(0.5)	EtOH(EtOH)
			—	−26.9	1	MeOH
			—	−41.1[d]	1	MeOH
Met	3 hr (0°C)	79	Oil	−51.8	1	MeOH
			120-123[c]	−20.9[c]	1	EtOH
			130-133[d]	−21.0[d]	1	EtOH
D-Met	1.5 hr (5°C)	83	Oil	+50.7	1	MeOH
			132-134[d]	+19.9[d]	1	EtOH
Phe	5 hr (5°C)	72	—	−75.2	1	MeOH
			—	−68.5	1.2	$CHCl_3$
			175-177(176)[b,c]	−25.9(−25.5)[c]	1(0.5)	MeOH(MeOH)
			—	−24.4[d]	1	MeOH
Tyr (Bzl)	6 hr (15°C)	71	131-134	−69.4	1	MeOH
			—	−24.7[d]	1	$CHCl_3$

[a]All data reported for the free acid unless otherwise noted.

[b]Literature Reference: S. T. Cheung and N. L. Benoiton, *Can. J. Chem.*, 55, 906 (1977).

[c]The dicyclohexylamine salt.

[d]The d-(+)-α-methylbenzylamine salt.

Table II. N-tert-butyloxycarbonyl, N-alkylamino acids[a]

Compound	Reaction Time (Temp)	Yield (%)	m.p.(°C)	Optical Rotation[b] $[\alpha]_D^{25}$ (deg)	Conc.
Boc-EtAla-OH	6 hr (5°C)	86	85-86	−46.3	1
Boc-AllylMet-OH	4 hr (0°C)	30	Oil	−77.7	0.93
Boc-EtPhe-OH	6 hr (10°C)	70	137-139	−7.4	1
			124-128[c]	−49.6[c]	1
Boc-PrPhe-OH	4.5 hr (0°C)	30	Oil	−153.3	1
Boc-AllylPhe-OH	4 hr (5°C)	74	Oil	−130.8	0.5
Boc-AllylTyr(tBu)-OH	3 hr (0°C)	54	Oil	−128.9	0.5

[a]All data reported for the free acid unless otherwise noted.

[b]Solvent = MeOH

[c]The dicyclohexylamine salt.

found to contain less than 0.4% of a material that eluted with the same retention time as an authentic sample of the diastereomeric peptide, H-Tyr-D-Ala-Gly-Phe-D-MeMet-NH_2.

In a second study, Cbz-MeLeu-OH (Lit: $[\alpha]_D^{25}$ -26° (C=2, DMF))[5] was synthesized by two independent routes. In one reaction, Cbz-MeLeu-OH was synthesized from Cbz-Leu-OH by the KH procedure (65% yield). In another reaction, Boc-MeLeu-OH was prepared by the KH procedure (75% yield) and then treated sequentially with HCl/HOAc and Cbz-Cl to give Cbz-MeLeu-OH. The optical rotations (C=2, DMF) of the products isolated from these two routes ($[\alpha]_D^{25}$ -27.7° and $[\alpha]_D^{25}$ -27.2°, respectively) indicated that little or no racemization had occurred.

Thus, the KH/MeI/18-crown-6 procedure is capable of generating N-protected, N-alkylamino acids in good yields with excellent optical purity.

Acknowledgments

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ASYMMETRIC SYNTHESIS OF cis-5-ALKYLPROLINE DERIVATIVES

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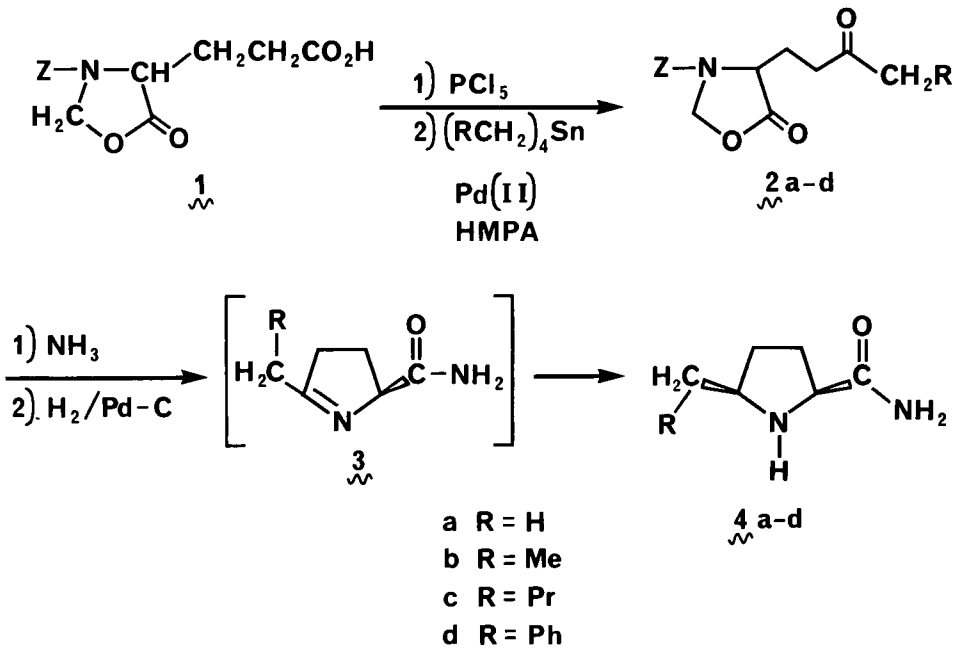
Introduction

During the course of our studies on the asymmetric transformation of amino acids, we developed a versatile and convenient asymmetric synthesis of cis-5-alkylproline amides. Several previous syntheses[1] of 5-methylproline have proceeded through a racemic intermediate (2-methyl-Δ^1-pyrroline-5-carboxylic acid) and generate a mixture of four diastereomers which are separated by multiple recrystallizations of their tartrates.[2] Our procedure uses L-Glu as a chiral synthon for the preparation of an optically pure 2-alkyl-Δ^1-pyrroline-5-carboxamide which is reduced stereoselectively to generate the new asymmetric center at C-5.

Synthesis

Formation of the oxazolidinone[3] (1) from Z-L-Glu-OH served to simultaneously protect selectively the α-carboxyl function and mildly activate it toward subsequent ammonolysis (Scheme 1). The acid chloride was generated in situ and was converted to the required ketones (2) in good yield (Table I) by either of two convenient methods. Reaction of the acid chloride with diazoalkanes ($R\text{-}CHN_2$) formed diazoketones which could be reductively decomposed by shaking with 48% HI to yield 2. The use of tetraalkyltin/$PhCH_2Pd(Ph_3P)_2Cl$[4] (Scheme 1) is more general and does not use hazardous diazoalkanes.



The oxazolidinone ring can be opened to either the acid or the amide, as desired in our studies, in excellent yield. Hydrogenolytic removal of the Z group allows the formation of the chiral 2-alkyl-Δ^1-pyrroline-5-carboxamide which is preferentially reduced by delivery of H_2 from the less hindered side (Scheme 1). The degree of asymmetric induction is catalyst

Table I. Yield of Ketone 2

Ketone 2	R	Yield (%)[1,2]
a	-H	82[1], 74[2]
b	-Me	60[1], 72[2]
c	-Pr	66[2]
d	-Ph	40[1], 33[2]

[1]Diazoalkane route. [2]Tetraalkyltin route.

dependent (Table II), but use of Pd black (from $Pd(OAc)_2$/MeOH) in acidic MeOH gave highly stereoselective reduction (e.g. 5-methylproline amide, 4a; 95% cis). A single recrystallization of 4a from CH_2Cl_2 gave the pure cis isomer (NMR, amino acid analysis).

Table II. Asymmetric Reduction Results

4	R	Yield (%)[1,2,3]	cis/trans[1,2,3]
a	-H	89[1], 83[2], 80[3]	95/5[1], 95/5[2], 70/30[3]
b	-Me	98[1], 75[2]	75/25[1], 80/20[2]
c	-Pr	80[1], 82[2]	90/10[1], 90/10[2]
d	-Ph	85[2]	90/10[2]

[1]Pd(black) catalyst. [2]10% Pd/C catalyst. [3]Adams catalyst.

Conclusions

Proline residues have important conformational properties in polypeptides and proteins, and the study of proline analogs with unusual steric constraints is of interest. We have developed a convenient and general route to optically pure cis-5-alkyl- or -5-aralkyl-proline derivatives which will make such analogs readily available for peptide hormone analog studies.

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THE SYNTHESIS OF AMINO ACIDS USING PHASE-TRANSFER CATALYZED ALKYLATIONS WITH POTASSIUM CARBONATE AS BASE

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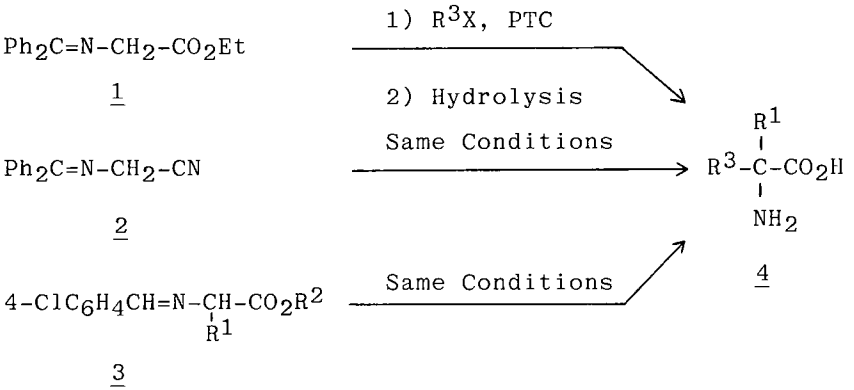
Background

Phase-transfer catalyzed (PTC) alkylation procedures represent a simple and versatile method for the preparation of a wide variety of amino acids. In contrast to the alkylation of suitably protected amino acids under anhydrous conditions using strong base, the PTC method involves: a) a simple reaction procedure, b) mild reaction conditions, c) inexpensive and safe reagents and solvents, d) readily available starting substrates and e) the feasibility of large as well as small scale reactions. We have previously reported practical syntheses of amino acids from Schiff bases derived from glycine esters,[1,2] aminoacetonitrile[3] and higher amino acid esters[4] by PTC alkylation in the presence of sodium or potassium hydroxides. Use of even milder, non-nucleophilic base systems is desirable in order to permit reaction at elevated temperatures, thus allowing for use of less reactive and usually less expensive alkyl halides.

Results

Schiff base derivatives of amino acids are readily alkylated in the presence of solid potassium carbonate in refluxing acetonitrile containing a phase-transfer catalyst.



α-Alkyl amino acids (4, R^1=H) are available from either the ketimines 1 or 2 or aldimine 3a (R^1=H, R^2=Et) while α,α-dialkyl amino acids are prepared from aldimines 3b (R^1=R^2=Me), 3c (R^1=Me, R^2=Et) or 3d (R^1=Ph, R^2=Me) (see Table I).

General Procedure

A heterogeneous mixture of 1 (1.34 g, 5.0 mmol), p-nitrobenzyl chloride (0.86 g, 5 mmol), tetrabutylammonium bromide (0.16 g, 0.5 mmol), finely-ground technical grade potassium carbonate (2.0 g, 15 mmol), and acetonitrile (10 ml) was refluxed with stirring for one hour (the disappearance of starting imine was monitored by HPLC). The mixture was cooled, filtered, the solvent removed in-vacuo, the residue dissolved in ether (15 ml), filtered and then stirred for three hours with 1 N aqueous hydrogen chloride (6.5 mmol). The layers were separated, the aqueous layer was washed with ether and then concentrated hydrogen chloride (6.5 mmol) was added and the solution was refluxed

Table I. Synthesis of Amino Acids 4

	R^3X	Schiff Base	Amino Acid	% Yield[a]
A	$4\text{-}ClC_6H_4CH_2Cl$	1	$4\text{-}ClC_6H_4CH_2\text{-}CH(NH_2)\text{-}CO_2H$	78%
B	$4\text{-}NO_2C_6H_4CH_2Cl$	1	$4\text{-}NO_2C_6H_4CH_2\text{-}CH(NH_2)\text{-}CO_2H$ [b]	80%
C	$HC\equiv C\text{-}CH_2Cl$	1	$HC\equiv CCH_2\text{-}CH(NH_2)\text{-}CO_2H$	68%
D	EtO_2CCH_2Cl	1	$HO_2CCH_2\text{-}CH(NH_2)\text{-}CO_2H$	77%
E	nC_4H_9Br	1	$nC_4H_9\text{-}CH(NH_2)\text{-}CO_2H$	73%
F	$nC_8H_{17}Br$	2	$nC_8H_{17}\text{-}CH(NH_2)\text{-}CO_2H$ [c]	88%
G	$4\text{-}ClC_6H_4CH_2Br$	3a	$4\text{-}ClC_6H_4CH_2\text{-}CH(NH_2)\text{-}CO_2H$	69%
H	$CH_2=CHCH_2Br$	3a	$CH_2=CHCH_2\text{-}CH(NH_2)\text{-}CO_2H$	78%
I	nC_4H_9Br	3a	$nC_4H_9\text{-}CH(NH_2)\text{-}CO_2H$	76%
J	$4\text{-}ClC_6H_4CH_2Cl$	3b	$4\text{-}ClC_6H_4CH_2\text{-}C(CH_3)(NH_2)\text{-}CO_2H$	71%
K	$CH_2=CHCH_2Br$	3c	$CH_2=CHCH_2\text{-}C(CH_3)(NH_2)\text{-}CO_2H$	69%
L	nC_4H_9Br	3c	$nC_4H_9\text{-}C(CH_3)(NH_2)\text{-}CO_2H$	75%
M	EtO_2CCH_2Cl	3d	$HO_2CCH_2\text{-}C(Ph)(NH_2)\text{-}CO_2H$	63%

[a]Isolated yield of pure amino acid on 5-20 mmolar scale. [b]Reaction conducted without phase-transfer catalyst had not consumed starting material after six hours. [c]Prepared in 84% overall yield on 0.5 molar scale.

for six hours. The water was removed in-vacuo, the residue was dissolved in methanol (100 ml) and propylene oxide (5 ml) was added. After stirring overnight two crops of crystals (0.85 g, 80%) of p-nitrophenylalanine were collected.

Acknowledgement

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A CONVENIENT, TWO-STEP SYNTHESIS OF ^{2}H AND ^{13}C SIDE-CHAIN-LABELLED PROLINES

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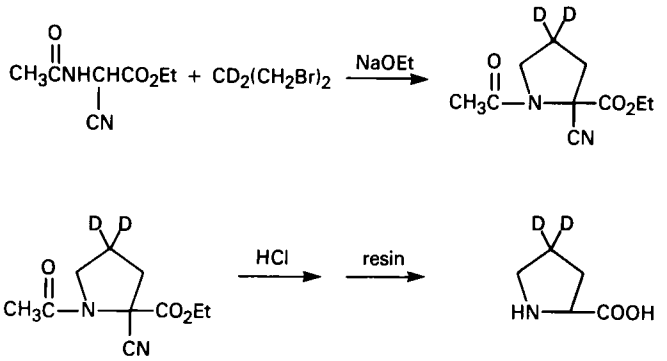
Introduction

The use of isotopically labelled amino acids in nmr studies is well-known[1]. In our solid state nmr studies of collagen, we had need of proline isotopically enriched, in the side-chain only, with deuterium and carbon-13. Such labelled prolines are not available commercially. All literature schemes[2] were multi-step and generally low yield. Since carbon-13 starting materials are expensive, we sought a high yield method, starting with commercially available or easily synthesized labelled material, that allowed ring formation in a single step.

Results

We found the method illustrated in Scheme 1 to proceed smoothly in overall yields of 75-90%. Two equivalents of the inexpensive cyanoacetate were used to assure complete reaction of all labelled dibromo compound. Excess starting material and unwanted dimer were removed by flash chromatography over silica gel. The product of the first reaction may be hydrolyzed quantitatively to proline by treatment with con-



Scheme 1. Synthesis of proline-4,4-d_2.

centrated HCl followed by passage over a weakly basic resin. The final product is a white powder with properties identical to those of commercial D,L-proline. By this method we have now synthesized proline-4,4-d_2, proline-3,3,4,4,5,5-d_6, and proline-4-C^{13}. The proton nmr spectra of the di-deuterated and carbon-13 labelled materials are given in Figure 1.

The use of these compounds in solution and solid state nmr studies of proline, proline-containing peptides, and collagen will be reported elsewhere.

Methods

Dibromopropanes: 1,3-dibromopropane-2-C^{13} was purchased from KOR isotopes. The perdeuterated dibromopropane was purchased from Merck and 1,3-dibromopropane-2,2-d_2 was synthesized in our laboratory from diethyl malonate-d_2 by a method similar to that of Ott[3]. Deuteration was estimated to be greater than 97% on the basis of proton nmr spectra.

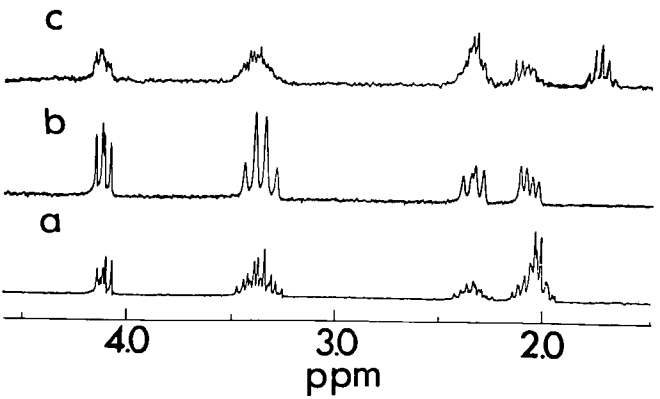


Fig. 1. 220 MHz proton nmr spectra of (a) L-proline, (b) D,L-proline-4,4-d_2, & (c) D,L-proline-4-C^{13} in D_2O.

N-acetamido-2-cyano-4,4-dideuteroproline ethyl ester: Ethyl acetamidocyanoacetate (9.7g, .057 mole) was dissolved in absolute ethanol (150 ml) in a 500 ml round bottom flask equipped with a reflux condenser, dropping funnel, and stirring bar. The mixture was refluxed and a solution of sodium metal (1.31g, .057 mole) dissolved in absolute ethanol (90 ml) was added over a 45 minute period; 1,3-dibromopropane-2,2-d_2 (5.8g, .028 mole) dissolved in absolute ethanol (60 ml) was then added over a 15 minute period. The mixture was allowed to reflux 20 hours. All solvent was removed in vacuo and the residue taken up in methylene chloride and water. The organic layer was separated, washed twice more with water, dried, and stripped. The residue was placed over silica gel (160g) on a flash chromatography column in 98.5% CH_2Cl_2/1.5% EtOH. 125 ml fractions were collected. The product appeared in fractions 3 and 4 (4.63g, 77%). R_f(95% $CHCl_3$/5% MeOH) = 0.5. NMR($CDCl_3$, ppm from TMS): 1.33 (t, 3H), 2.15 (s, 3H), 2.35 (d, 1H), 2.63 (d, 1H), 3.72 (quartet, 2H), 4.28 (quar-

tet, 2H). Parent peak, calculated for $C_{10}H_{12}D_2N_2O_3$ = 212.1130, found = 212.1129. The material is a light yellow oil.

D,L-proline-4,4-d_2: The cyanoacetate (4.39g,.021 mole) was dissolved in concentrated HCl (40 ml) and refluxed for 3 hrs. All solvent was removed in vacuo and the residue dried to constant weight. It was taken up in water and passed over a column of Bio-Rad AG3-X4A resin (60g) which had previously been washed with concentrated NH_4OH. Solvent was collected until no more product was detected by TLC. When stripped and dried, there remained D,L-proline-4,4-d_2 (2.42g, 100%) mp 201-204°C. Its nmr spectrum is shown in Figure 1. The product was recrystallized from 100% ethanol; 1.0g gave .89g, mp 203-206°C. R_f(75% aqueous ethanol) = 0.43.

Acknowledgement

We would like to acknowledge Dr. C.-H. Niu for many helpful discussions and Ms C.E. Sullivan for technical assistance. This work was supported in part by NIH grant 2-SO6-RR08153-07.

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A CONVENIENT GENERAL METHOD FOR SYNTHESIS OF N^{α}-DITHIASUCCINOYL (DTS) AMINO ACIDS : APPLICATION OF POLYETHYLENE GLYCOL AS A CARRIER FOR FUNCTIONAL PURIFICATION[1]

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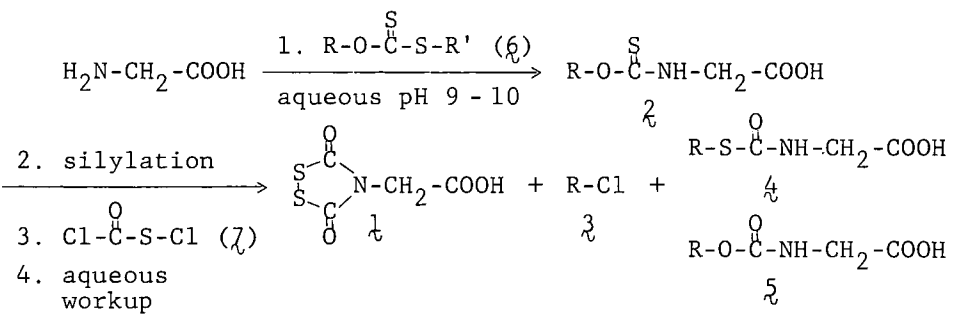
The N^{α}-dithiasuccinoyl (Dts) function (1, drawn for glycine) is being developed for use in mild orthogonal schemes of solid-phase peptide synthesis. Our published methodology for the preparation of 1, shown in Scheme I, utilizes ethoxythiocarbonyl[4] derivatives 2a as starting materials. It has the drawback[5,6] that low levels of urethane by-products (4a and 5a) are formed as well, which may compromise the utility of 1 for peptide synthesis.

SCHEME I



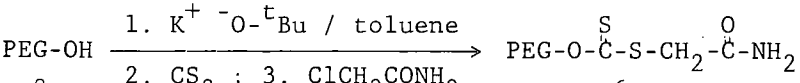
a R = C_2H_5-, R' = -(C=S)-O-R; b R = $CH_3OCH_2CH_2O$-, R' = $-CH_2CONH_2$
c R = $-(CH_2CH_2O)_nCH_2CH_2$-, $\overline{n}$ = 44, PEG-2000, R' = $-CH_2CONH_2$

With the goal to prepare pure 1, we sought functional methods to eliminate unequivocally the urethane impurities. Note that 4 and 5 retain the alkyl group R of starting material 2, whereas desired 1 lacks that alkyl group which has been transformed to R-Cl (3). Therefore, use of an R group which

confers markedly different solubility properties should allow effective and unambiguous resolution of 1 from 2, 3, 4, and 5. We report here that polyethylene glycol (PEG) proves to have an ideal array of properties[7] for these purposes. Bifunctional PEG of average molecular weight 2000 was selected for use, because it is the smallest size for which derivatives are easily obtained in solid "crystalline" form (by precipitation with ethyl ether, or chilling of alcoholic solutions), yet it provides a reasonable capacity of 1 mequiv./gm. Furthermore, the polymer is readily soluble in a variety of organic solvents as well as in water, to extents that ∿ 1 M solutions can be prepared, and PEG-derivatives can be characterized by elemental analyses, IR, UV, 300 MHz H-NMR, 20 MHz ^{13}C-NMR, and amino acid analyses of hydrolysates.

Polymeric xanthate 6c was prepared by an improvement of the protocol of King and Weiner[8], as shown in Scheme II. Thiocarbamate 7c was then prepared by the rapid (complete in 20 min) reaction of the amino acid in aqueous pH 9.5 solution. This polymer was isolated, purified by "recrystallization", and thoroughly dried *in vacuo* over P_2O_5. Recoveries of both polymers were 90%, and functional group transformations were quantitative, as judged by the identity in extinction coefficients between polymeric (series c) and model (series b) compounds.

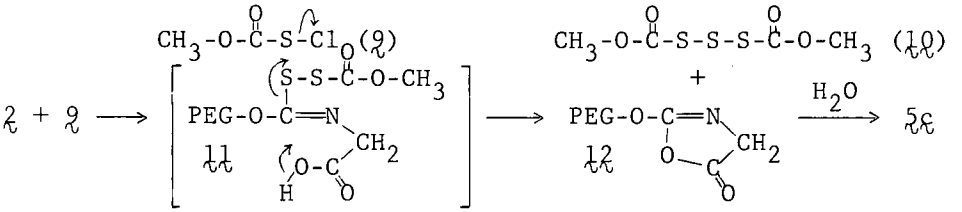
SCHEME II



For subsequent reactions to work, it was necessary to protect the α-carboxyl group; 1,3-bis(trimethylsilyl)urea (BSU) used at 1.1 mol per equiv. amino acid, 25 °C, 30 min, in CH_2Cl_2 - CH_3CN (1:1), proved to be the reagent and conditions of choice. Next, the Dts heterocycle was directly elaborated with chlorocarbonylsulfenyl chloride (7), 25 °C, 10 min, and workup between ether and an aqueous phase partitioned all by-products from the reaction sequence into water. The overall yields of

pure Dts-amino acids were over 85%, and the recovered polymer was primarily 3c but also contained about 7% bound amino acid. When the silylation step was omitted, a 15 to 20% yield of 1 was obtained with about 75% of the amino acid residue retained on the polymer as urethane 5c. Authentic 5c was obtained by reaction of PEG-OH (8) with ethyl isocyanatoacetate, followed by saponification. Remarkably, 5c also formed when methoxycarbonylsulfenyl chloride (9) was substituted for 7 in this experiment; bis(methoxycarbonyl)trisulfane (10)[9] was recovered from the ether phase. These observations may be accounted for as shown in Scheme III, which is not intended to show all intermediates nor to imply concerted reactions. Note that a

SCHEME III



2-alkoxy-5(4H)oxazolone (12) might be involved. Consistent with the proposal of Scheme III is the observation that thiocarbamates derived from γ-aminobutyric and ε-aminocaproic acids were smoothly converted (50 to 70% yields) to their Dts derivatives by direct reaction with 7 and without any need to silylate the free ω-carboxyl group (five-membered transition states, as in 11, not applicable in these cases).

To further evaluate the power of the new technique for the synthesis of pure Dts-amino acids (Scheme I, series c), additional exaggerated conditions were examined. Thus, 6c did react with 7 but the reaction was relatively slow and moreover no product was obtained in the organic phase. A partially derivatized polymer containing both 2c and 6c (in a 7:3 ratio) gave with limiting amounts of 7 the expected Dts-amino acid (1) in high yield and purity selectively, while excess xanthate sites on the polymer remained unaffected. Similarly, the reaction of 2c plus 7 to give 1 proceeded in good yields even

in the presence of additional equivs. of BSU, and any BSU - ζ adducts were water-soluble. These findings were exploited to devise a "one-pot" procedure in which the parent amino acid was limiting, other reagents were used in excess, and intermediate purification of polymer $\zeta\zeta$ was circumvented. Yields of Dts-amino acids were above 80% on both small (0.5 mmol) and large (25 mmol $\cong$ 5 gm product) scales. So far, glycine, alanine, leucine, valine and phenylalanine have been derivatized, but since the only additional protection step is silylation of the α-carboxyl, the method is expected to be general to any side-chain protected amino acid derivative also.

References and Notes

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SOLID PHASE SYNTHESIS OF CECROPIN A

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Introduction

The humoral immune response of certain insects is associated with the cecropins, a newly discovered family of antimicrobial peptides.[1] In particular, cecropin A is known to be remarkably active against both Gram-positive and Gram-negative bacteria. Structural determination of cecropin A showed it to be a basic 37-residue peptide having an unspecified C-terminal blocking group,[2] tentatively postulated to be an amide. In order to establish definitively the nature of this group and to confirm the sequence, a synthes s of cecropin A with a C-terminal carboxamide has been carried out for the first time. Synthesis has provided a fully active peptide that has allowed unambiguous confirmation of the proposed structure.

Results and Discussion

The solid support used for the synthesis (Figure 1) was a modification of a multidetachable benzhydrylamine resin developed in our laboratory.[3] Primarily intended for synthesis of protected peptide amides, it is equally suitable for stepwise work, with the advantage of having a more chemically defined functionalized handle. The synthetic protocols were Boc-Lys(ClZ)-Trp(For)-Lys(ClZ)-Leu-Phe-Lys(ClZ)-Lys(ClZ)-Ile-Glu(OcHex)-Lys(ClZ)-Val-Gly-Gln-Asn-Ile-Arg(Tos)-Asp(OcHex)-Gly Ile-Ile-Lys(ClZ)-Ala-Gly-Pro-Ala-Val-Ala-Val-Val-Gly-Gln-Ala-

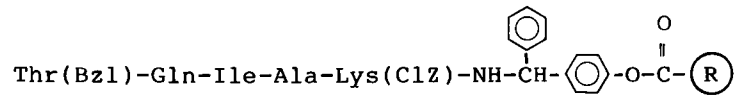

Fig. 1. Synthetic protected cecropin A-resin.

essentially identical to our previous synthesis of cecropin A (1-33).[4] Two radioactive residues were introduced: [^{3}H]Ala^{36} and [^{14}C]Leu^{4}. Quantitative ninhydrin monitoring[5] of substitution indicated a noticeable decrease in the number of growing peptide chains throughout the synthesis (Lys^{37}: 0.125 mmol/g; Lys^{1}: 0.040 mmol/g). Two factors seemed to account for this. First, a 15% drop between Gly^{30} and Gln^{31} due to chain termination by Pca formation[6]. This was confirmed by detection of the corresponding heptapeptide in the final crude. Secondly, a non-specific decrease in substitution was detected at every cycle, amounting to an average 1.4% loss per step. This was later shown to be due to HOBt cleavage of the nucleophilic-sensitive phenyl ester bond between the benzhydrylamine moiety and the polymer matrix. The synthetic protected peptide was cleaved and deprotected by a two-step low/high HF procedure.[7] Low HF was done with HF/Me_2S/p-cresol/p-thiocresol (25:65:8:2), 2h, 0°C. After evaporation, the resin was washed with EtOAc, Et_2O and CH_2Cl_2 to remove residual Me_2S. The deprotection and cleavage were completed with HF/p-cresol (9:1), 1 h, 0°C. The thorough removal of Me_2S increased yields from 38% to 71%. The crude peptide was then purified by CM-Sepharose chromatography using a linear 0.1 M-1.0 M NH_4HCOO gradient at pH 6.6, followed by semi-preparative reverse-phase HPLC on μ-Bondapack C_{18} using a linear acetonitrile gradient on 0.05% aqueous TFA (Figure 2). The purified synthetic cecropin A was thus isolated in a 25% overall yield and with a correct amino acid analysis: Trp 1.00(1), Lys 7.03(7), Arg 1.04(1), Asp 2.08(2), Thr 1.00(1), Glu 3.78(4), Pro 1.17(1), Gly 3.88(4), Ala 4.99(5), Val 4.16(4), Ile 4.68(5), Leu 0.96(1), Phe 0.96(1). It was homogeneous by analytical HPLC and PAGE-SDS and indistinguishable from natural cecropin A by PAGE at pH 4.0.

Final confirmation of the structure of cecropin A was done

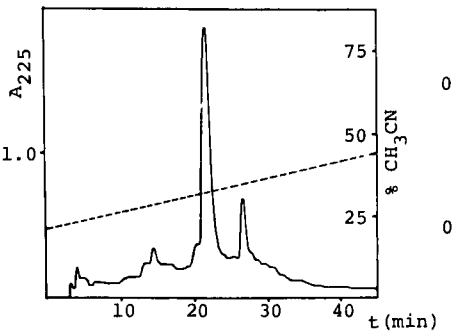

Fig. 2. HPLC purification of synthetic cecropin A.

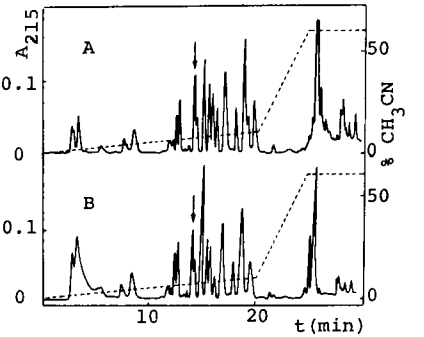

Fig. 3. Fingerprint HPLC of cecropin A digests, A: synthetic; B: natural

by submitting both natural and synthetic peptides to digestion with the InA protease from B. Thuringensis.[8] Analytical finger print HPLC of each digest (Figure 3) showed them to be identical. The ^{3}H-labeled peak eluting at 14.25 min in the synthetic digest chromatogram and its natural homologue (14.19 min) were collected and rechromatographed, both eluting as single peaks differing only in 1 sec over a 30 min program. Amino acid analysis gave Lys:Glu:Thr:Ala:Ile=1, corresponding the the C-terminal pentapeptide of cecropin A. Finally, fission-fragment mass spectroscopy[9] of the natural pentapeptide gave only one major peak of mass 581.48 which agrees very well with the structure [H-Thr-Gln-Ile-Ala-Lys-$NH_2 \cdot Na^+$] (monoisotopic mass, calculated from the most abundant isotopes, is 581.339). These data established the identity of the C-terminal amide of the natural and synthetic peptides and thus conclusively proved that the primary structure originally postulated was correct.

The antibacterial activities of synthetic cecropin A and two analogs[10] were determined by a zone inhibition assay and compared to that of natural cecropin A. Results of Table I show the identity between natural and synthetic materials. They also point out the importance of the two N-terminal

Table I. Lethal Concentrations[11] (μM) of Cecropin A and Analogs.

Bacterium	Strain	Natural	Synthetic		
			(1-37)	(3-37)	[Phe^2](1-37)
E.Coli	D 21	0.32	0.32	2.6	0.34
P.Aeruginosa	OT97	3.5	2.8	90	3.5
B.Megaterium	BmII	0.52	0.51	13	0.78
M.Luteus	MlII	1.9	2.2	> 110	7.4

residues (especially Trp^2) for the complete activity of the molecule.

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STUDIES ON SOLID PHASE FRAGMENT SYNTHESIS: SYNTHESIS OF A 42-RESIDUE PEPTIDE USING A MULTIDETACHABLE RESIN SUPPORT AND IMPROVED DEPROTECTION CONDITIONS

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Introduction

The goal of our research is to prepare a semisynthetic protein that mimics the binding site of the phosphorylcholine binding murine IgA M603.[1] This requires the synthesis of the 120-residue variable region of the M603 heavy chain. We have coupled three peptide fragments to give a 42-residue peptide corresponding to residues 27-68 of the hypervariable regions of the M603 heavy chain. The purpose of this report is to evaluate the preparation of the peptide by fragment synthesis at this stage of the total synthesis.

Results and Discussion

The 42-residue peptide was prepared using three fragments labeled 3(27-42), 4(43-55) and 5(56-68) containing 16, 13 and 13 residues, respectively. The protected peptides 3 and 4 were prepared on a 2-bromopropionyl-resin[2] and were cleaved from the resin by photolysis.[3,4] The protected peptide 5 was prepared using the multidetachable phenylacetoxy-propionyl-resin (Pop-resin)[5] and was cleaved by photolysis to give the protected peptide-oxymethylphenylacetic acid (OMPA) derivative. The OMPA group is a spacer that gives the Pop-resin its multi-detachable properties. We have also synthesized other fragments related to the M603 heavy chain on the Pop-resin and

Table I.[a] Yields for Fragment Coupling and HF Cleavage

	Reaction		Yield
5-OMPA + Br-(R)	KF ⟶	5-OCH_2-Pop-(R)	80%
4 + 5-OCH_2-Pop-(R)	DCC/HOBt ⟶	4-5-OCH_2-Pop-(R)	85%
3 + 4-5-OCH_2-Pop-(R)	DCC/HOBt ⟶	3-4-5-OCH_2-Pop-(R)	75%
3-4-5-OCH_2-Pop-(R)	HF ⟶	Peptide 3-4-5	86%

[a]all yields by amino acid analysis

find it superior due to the suppression of certain side reactions.[3,6]

The fragment assembly began with the reattachment of the purified protected peptide 5-OMPA to 2-bromopropionyl-resin.[7] The protected peptide was added to a suspension of resin (6 fold excess) and anhydrous KF (30 fold excess) in N-methyl-pyrrolidinone (NMP) and was shaken for 42 h. The yield of the reattachment was 80% (Table I). The Pop-resin was chosen as the solid support because of the high yield of the reattachment step and its susceptibility to several methods of cleavage.[5]

Protected peptide 4 was coupled to N^{α}-deprotected peptide 5-OCH_2-Pop-resin using 1 eq. each of DCC and HOBt (preformed HOBt ester for 2.5 h at 0° C). Thee coupling yield was 85% (Table I). The remaining amino sites were terminated using Ac_2O/pyridine. The ratio of peptide:DCC:HOBt was 1:1:1 to insure that there would be no premature termination of amino groups on the resin. We have found that treatment of amino-methyl-resin with DCC/HOBt solutions in DMF leads to termination of the free amino groups as determined by the quantitative ninhydrin reaction.[8] The termination was 10% in a 19 h period. The protected peptide 3 was coupled to the Nαde-protected peptide 4-5-OCH_2-Pop-resin by the same method and the yield was 75% (Table I).

The N^{α}-deprotected peptide 3-4-5-OCH_2-Pop-resin was cleaved and deprotected using our new HF procedure.[9] The peptide-

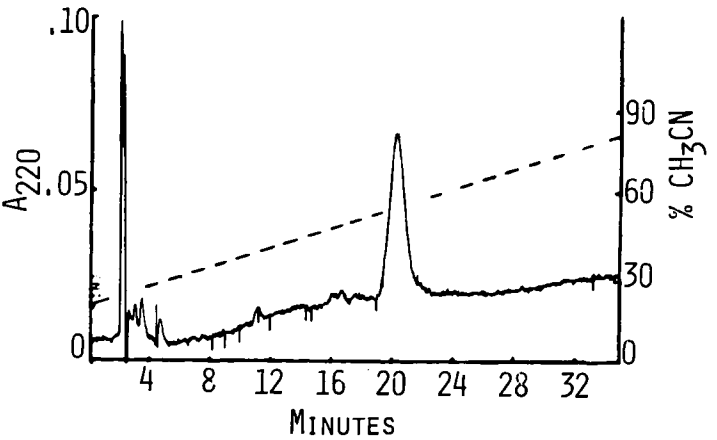

Fig. 1. HPLC on purified peptide 3-4-5.

resin was treated with HF:dimethylsulfide (DMS):p-thiocresol: p-cresol (25:65:5:5) for 2 h at 0°C. The DMS and HF were evaporated, fresh HF was added (95% HF) and the acidolysis was continued for an additional hour at 0°C. The yield of the cleavage was 86% (Table I). The UV spectrum (220→350nm) of the crude peptide indicated a quantitative removal of the formyl protecting group of tryptophan by our new HF method. The full sequence of peptide 3-4-5 (residues 27-68) is as follows:

```
        27
    NH2-Phe-Thr-Phe-Ser-Asp-Phe-Tyr-Met-Glu-Trp-Val-Arg-Gln-
            43
Pro-Pro-Gly-Lys-Arg-Leu-Glu-Trp-Ile-Ala-Ala-Ser-Arg-Asn-Lys-
    56                                                    68
Gly-Asn-Lys-Tyr-Thr-Thr-Glu-Tyr-Ser-Ala-Ser-Val-Lys-Gly-OH
```

The crude peptide was purified by gel filtration on Sephadex G-50 with 10% acetic acid as the eluent. The chromatogram showed three well separated peaks with the major one eluting first. The two minor peaks corresponded to fragment 4-5 and fragment 5 and the major peak to peptide 3-4-5. The major peak appeared as a single band on sodium dodecylsulfate polyacrylamide gel electrophoresis with a molecular weight of

5600 (theory=5680). This result shows that the major peak contains fragments 3, 4 and 5, a conclusion consistent with the amino acid analysis. The major peak from the G-50 column was pooled, rechromatographed on Sephadex G-50, and analyzed by HPLC on a Waters μ-Bondapak C_{18} column (3.9 x 300 mm) using an aqueous acetonitrile solvent containing 0.1% H_3PO_4. A single peak was obtained (Figure 1).

We are currently working on a preparative scale purification of the peptide using reverse-phase C_{18} chromatography and the determination of any binding activity of the 27-68 peptide towards phosphorylcholine.

Acknowledgments

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SYNTHESIS OF HUMAN PARATHYROID HORMONE (1-84) WITH THE STRUCTURE PREDICTED FROM cDNA SEQUENCE ANALYSIS

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Introduction

We recently reported the first synthesis of human parathyroid hormone(1-84),[1] hPTH(1-84), which has the structure determined by Keutmann et al. in 1978, using the solution strategy developed in our laboratory.[2] In 1981, Hendy et al.[3] reported that the Asp residue at position 76 in Keutmann's structure should be Asn, according to sequence analysis of the cDNA cloned for human preproPTH. In the present study, we synthesized hPTH(1-84) with the newly proposed structure applying the same strategy and reported the preliminary data at the 1983 Japanese peptide symposium.[4] Although our final product was isolated by reversed phase HPLC as a single peak, the resolution power may not have been high enough to detect minor impurities from such a high-molecular peptide. Thus, in the present study, we tried to confirm the homogeneity of our product by separating closely related synthetic analogs or fragments of hPTH on our HPLC systems.

Results and Discussion

The hPTH molecule was assembled with the same 13 fully protected segments used for the synthesis of [Asp^{76}]-hPTH, except that the segment(74-78) contained Asn at position

76 instead of the Asp(OBzl) residue. Each segment was protected with the Boc group at the amino terminus, phenacyl ester at the carboxyl terminus, and benzyl type protective groups or the tosyl group at side chain functional groups as has been reported previously.[1,2,4] No particular difficulty was encountered in the segment condensation reactions in solution even when Asp(OBzl) had been replaced with Asn. The fully protected 84-peptide was deprotected with HF and purified similarly, and the desired product was isolated by HPLC.

Amino acid analyses of the isolated material gave reasonable results after acid hydrolysis and Ap-M digestion. The hPTH(1-84) molecule contains two Met residues; if they are partially or fully oxidized to Met(O), three different molecules should be formed. The separability of these molecules was tested on our HPLC system. Of course, the original product gave a single peak, but after treatment with a dilute hydrogen peroxide solution, it gave four peaks including the intact molecule, which were clearly separated

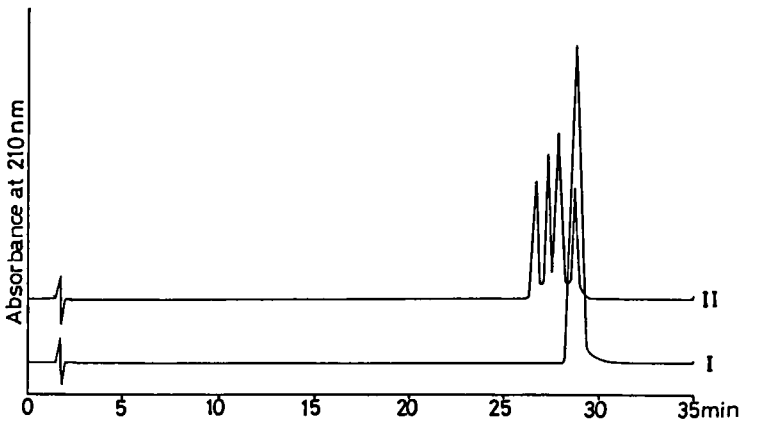

Fig. 1. HPLC of synthetic Asn-hPTH(1-84) and its oxidized products using hydrogen peroxide. I. Asn-hPTH. II. Oxidized products. Column: Nucleosil 5C18 (150 x 4 mm). Eluant: 0.1 M NaCl (pH 2.4) containing MeCN, which was gradiently increased from 10% to 50%.

(Fig. 1). Thus, the present product, which give a single peak on HPLC, was proved to be free of oxidized peptides.

Direct separation of [Asp]- and [Asn]-containing peptides on HPLC was tested using synthetic peptides of various molecular lengths. In the case of the final product (1-84), the difference in retention times was only one minute even under optimum isocratic conditions. When both products were injected into the column as a mixture, they appeared together as a single wide peak even under our optimum conditions (Fig. 2). However, in the cases of fragments (39-84) and (23-84), Asp- or Asn- containing analogs were clearly separated on our HPLC system as shown in Fig. 3. Then, HPLC peptide mapping of tryptic digests were compared with [Asp]- and [Asn]-hPTH (1-84) (Fig. 4). All peaks could be clearly assigned as

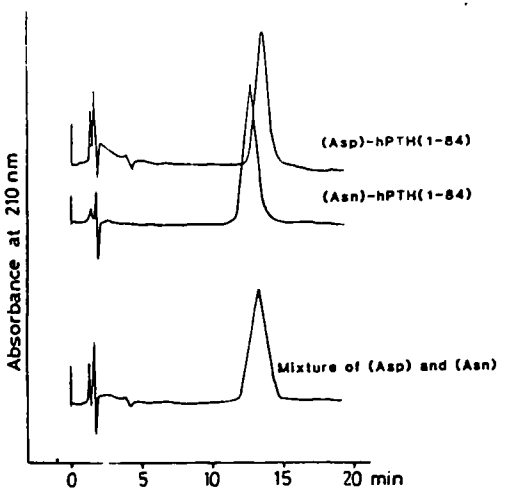

Fig. 2. Separation of Asn- and Asp-hPTH(1-84) on HPLC. Column: Nucleosil 5C18 (150 x 4 mm). Eluant: 0.1 M NaCl(pH 2.0) containing 31% MeCN.

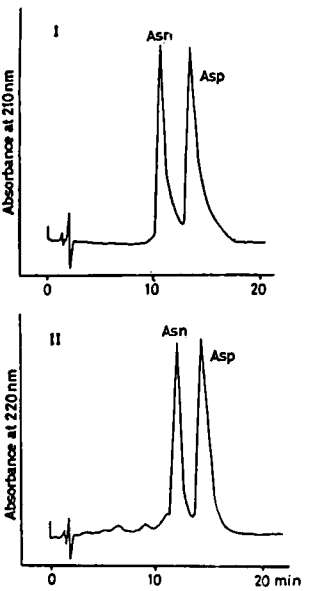

Fig. 3. Separation of Asn- and Asp-containing hPTH(39-84) and (23-84). I. hPTH(39-84). II. hPTH(23-84). Column: Nucleosil 5C18 (150 x 4 mm). Eluants: 10 mM phosphate buffer containing 50 mM Na_2SO_4:MeCN = 81.5: 18.5 for I and 78:22 for II.

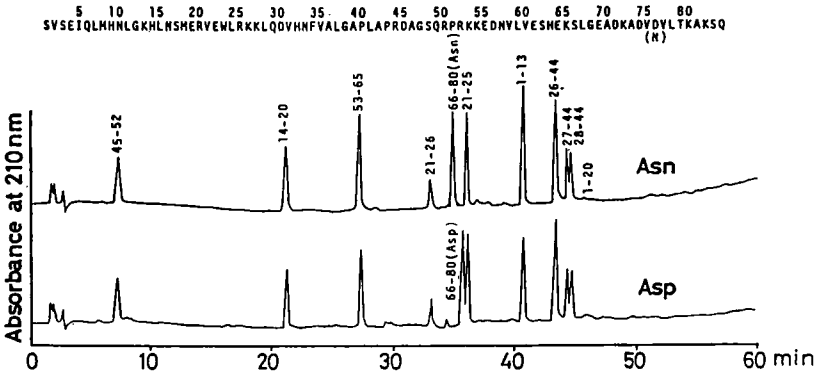

Fig. 4. HPLC mapping of tryptic digests of Asn- and Asp-hPTH(1-84). Column: Nucleosil 5C18 (150 x 4 mm). Eluant: 10 mM phosphate (pH 2.6) containing 50 mM Na_2SO_4 and MeCN, which was increased gradiently from 2% to 50%.

indicated in the figure, and the only difference was observed in the chromatograms at peaks corresponding to fragment (66-80).

From above observations, we concluded that our product was reasonably homogeneous as a peptide with the expected structure. It showed 350 IU/mg in the in vitro assay of rat kidney adenylate cyclase activity and a specific optical rotation value of $[\alpha]_D^{20}$ -89.2° (c 0.2, 1% AcOH).

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ON THE SYNTHESIS OF THE ACTIVE SITE FRAGMENT OF NERVE GROWTH FACTOR

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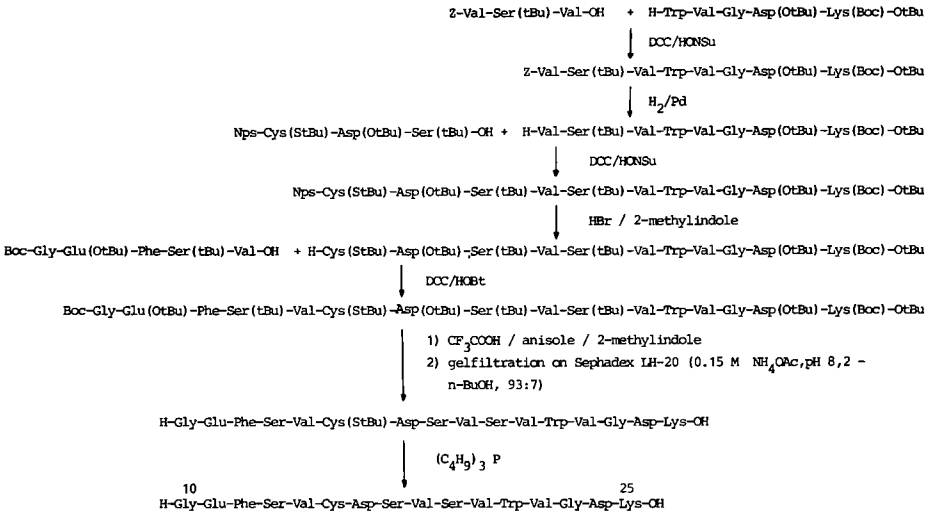
Introduction

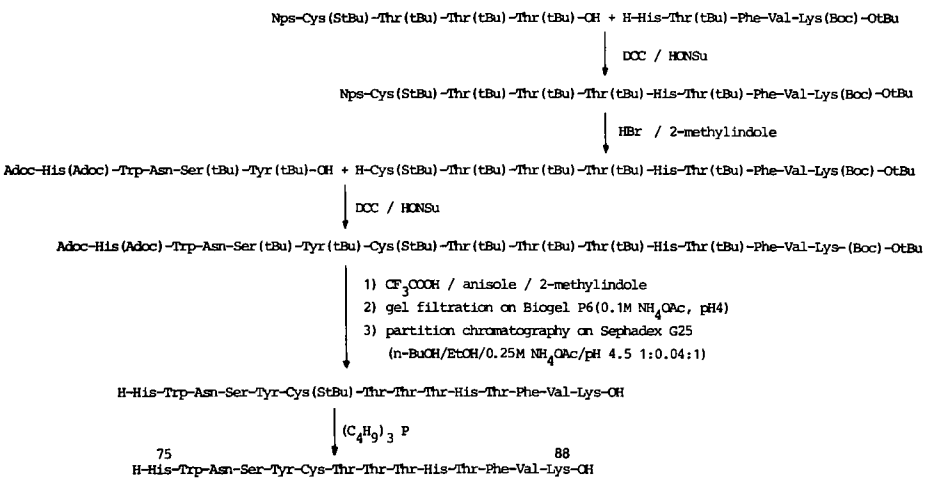
Nerve growth factor (NGF) is a protein, comprising two identical 118-residue peptide chains, that stimulates the outgrowth of neurites from sympathetic and sensory neurons[1,2]. Cyanogen bromide cleavage of NGF followed by tryptic digestion led to the isolation of an unsymmetrical cystine-peptide corresponding to sequences 10-25 and 75-88. This was reported to stimulate nerve fibre outgrowth at 1/100th the molar concentration of the native molecule[3]. As attempts to confirm this potentially important result have so far failed, we decided to resolve this question by synthesizing the corresponding peptide.

Synthesis of the NGF-10-25 / 75-88 fragment

A disulfide bridging of the two peptide chains by air oxidation produces at best the statistically expected proportion of the unsymmetrical cystine-peptide, and this proportion is modified by energy factors. Thus, for a successful synthesis of the title compound a selective cysteine pairing has to be achieved. For this purpose the known procedures[4-6]

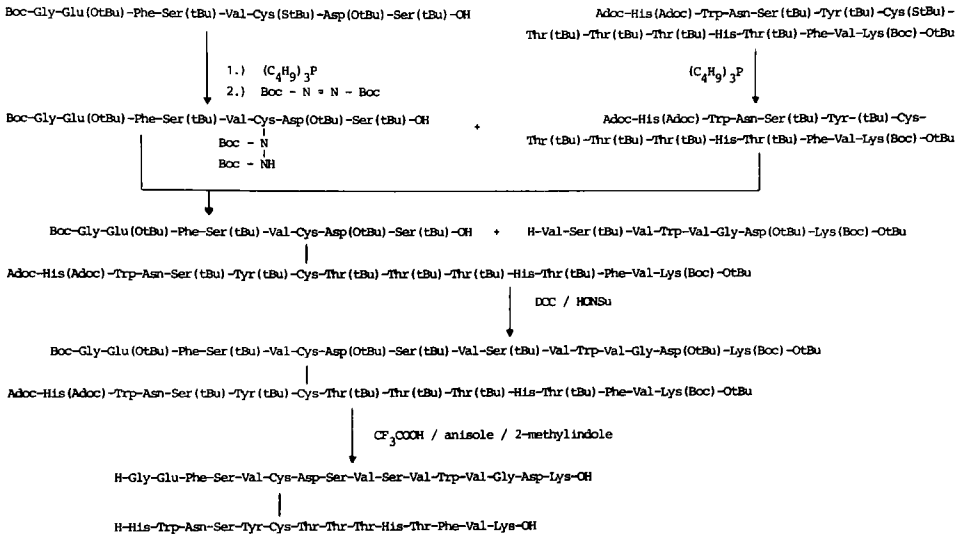


Scheme 1. Synthesis of NGF-sequence 10-25



Scheme 2 Synthesis of NGF-sequence 75-88

cannot be applied in the present case because of the tryptophan residues in both peptide chains. In fact, these methods utilize reagents or sulfur-activated derivatives that react rapidly and irreversibly with the tryptophan side chains to



Scheme 3. Synthesis of the NGF-10-25 / 75-88 fragment

yield the corresponding thio-indole compounds. For this reason a new method for the selective synthesis of unsymmetrical cystine-peptides based on sulfenohydrazide derivatives has recently been developed by us[7]; it can be applied even for tryptophan-containing cysteine-peptides.

The two peptide chains of NGF have been synthesized via the fragment condensation procedure using an acid-labile side chain protection of tert-butanol basis in combination with the S-tert-butylthio group for the selective and reversible masking of the cysteine thiol functions (Scheme 1 and 2). This strategy allowed a selective disulfide bridging of the protected peptide chains upon reductive removal of the S-tert-butylthio group by phosphines followed by activation of the peptide derivative 10-17 as 1-sulfenohydrazine- 1,2-dicarboxylic acid di-tert-butyl ester and its reaction with the cysteine-peptide derivative 75-88 (Scheme 3). The final condensation with fragment 18-25 via DCC/HONSu yielded the fully protected unsymmetrical cystine-peptide 10-25 / 75-88.

Alternatively, upon exposure of the peptide derivatives 10-25 and 75-88 (Scheme 1 and 2) to trifluoroacetic acid in the presence of scavengers, the resulting cysteine-protected peptides 10-25 and 75-88 were purified by gel filtration on LH-20 and Biogel P6, respectively. Then the S-tert-butylthio group was removed by reduction with tri-butylphosphine. The cysteine-peptide 75-88 was reacted with the water-soluble azodicarboxylic acid dimorpholide[8] to yield the sulfur-activated sulfenohydrazide derivative, which in turn was reacted with the cysteine-peptide 10-25 to produce the unsymmetrical disulfide.

Initial attempts to purify the final products so far failed partly because of unexpected solubility problems. Studies of the biological activity are still in progress, but preliminary experiments using partially purified materials indicated that the peptide was devoid of NGF-like activity.

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SYNTHESIS AND BIOLOGICAL ACTIVITIES OF THE MITOGENIC PRINCIPLE OF E. COLI LIPOPROTEIN

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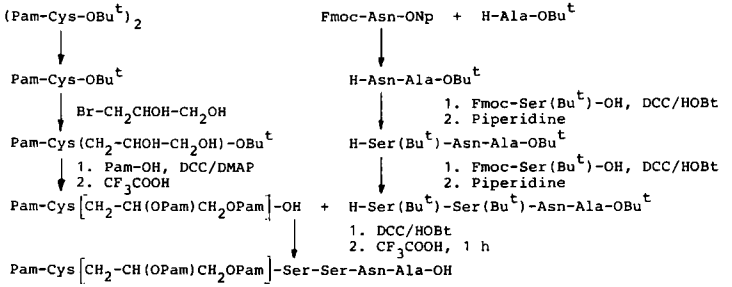
The N-terminal part of the lipoprotein from the outer membrane of Escherichia coli consists of three fatty acids attached to S-glyceryl-L-cysteine[1], followed by a protein consisting of largely α-helical segments[2]. We prepared the N-terminal "tripalmitoyl-pentapeptide": N-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2R and S)-propyl]-(R)-cysteinyl(S)-seryl-seryl-asparaginyl-alanine as outlined in Scheme 1.

$(Pam\text{-}Cys\text{-}OBu^t)_2$ was reduced to the thiol and alkylated with 1-bromopropandiol-1,2. After formation of the diester of the S-glyceryl group with Pam-OH, DCC/DMAP, the cysteine derivative was coupled in 80% yield to the tetrapeptide moiety H-L-Ser-(Bu^t)-Ser(Bu^t)-Asn-Ala-OBu^t, which was obtained via both the Z and Fmoc intermediates. The deprotection required treatment with 100 % trifluoroacetic acid for 1 h to yield the tripalmitoyl-pentapeptide, which is hardly soluble in most solvents. We also separated the diastereomers of N-Pam-Cys[CH_2-CH(OPam)CH_2OPam]-OBu^t on a silica gel column. The pure diastereomers RR and RS were used for the preparation of both diastereomeric tripalmitoyl-pentapeptides. Furthermore the lipopeptide analogue Pam-Ser-Ser-Asn-Ala-OH was prepared. Final products and all intermediates were found to be pure by ^{13}C-NMR (100.1 MHz), FD-MS, elemental and amino acid analysis, GLC on chiral phase and various chromatographic systems[3].



Scheme 1. Synthesis of the tripalmitoyl-pentapeptide.

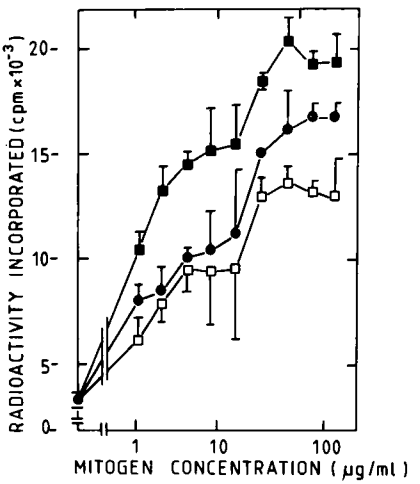


Fig. 1. [^{3}H]Thymidine incorporation (cpm per 3.3 x 10^6 cells) by C3H/HeJ splenocytes after incubation (72 h) with diastereomeric tripalmitoyl-pentapeptide (●), and the diastereomers RR (□) and RS (■). For experimental details see reference 7.

The native lipoprotein is a mitogen towards lymphocytes[4-6], and enzymic fragments carrying three fatty acids have been shown to be mitogenically active[6]. A mixture of the diastereomers induces mitogenesis in splenocytes from different mouse strains, and brings about a marked polyclonal activation of immunoglobulin producing B-lymphocytes in vitro and in vivo[7,8].

Figure 2 shows the induction of DNA synthesis in LPS nonresponder C3H/HeJ mouse splenocytes by the tripalmitoyl-pentapeptides. All three mitogens increased [^{3}H]thymidine incorporation starting at concentrations around 1 mg/ml. Interestingly, the RR diastereomer showed reduced activity, whereas the RS compound exhibited mitogenicity by far superior to the RR and RS/RR samples. Similar results have been found in Balb/c mouse splenocytes, and the specific activation of B-lymphocytes could be demonstrated using C3H/Tif/Bom-nu/nu athymic mice. This stereospecific activation suggests the presence of lipoprotein mitogen receptors on lymphocytes[9]. Pam-Ser-Ser-Asn-Ala-OH also acts as an active mitogen towards C3H/HeJ mouse splenocytes (Table I), whereas palmitic acid or the tetrapeptide alone are

Table I. Mitogenicity of Pam-Ser-Ser-Asn-Ala in splenocytes.

Mitogen Concentration (μg/ml)	[^{3}H]Thymidine Incorporation (cpm x 10^3)
137	419 ± 276
68	5213 ± 232
34	5916 ± 176
17	4999 ± 593
4.3	2707 ± 392
1.1	2053 ± 305
0	1526 ± 242

not active. Thus, a defined tetrapeptide coupled to hydrophobic residue is the minimal structure responsible for the biological activity of a potent bacterial mitogen.

The new synthetic mitogens are valuable tools for continuing the investigation of the molecular mechanism of B-lymphocyte activation; are useful as in vivo stimulants of the immune response, and may non-specifically enhance host-resistance to bacterial infections and tumor growth.

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SYNTHESIS OF A γ-CARBOXYGLUTAMIC ACID CONTAINING HEPTAPEPTIDE CORRESPONDING TO THE 17-23 CYSTINE LOOP OF BOVINE PROTHROMBIN

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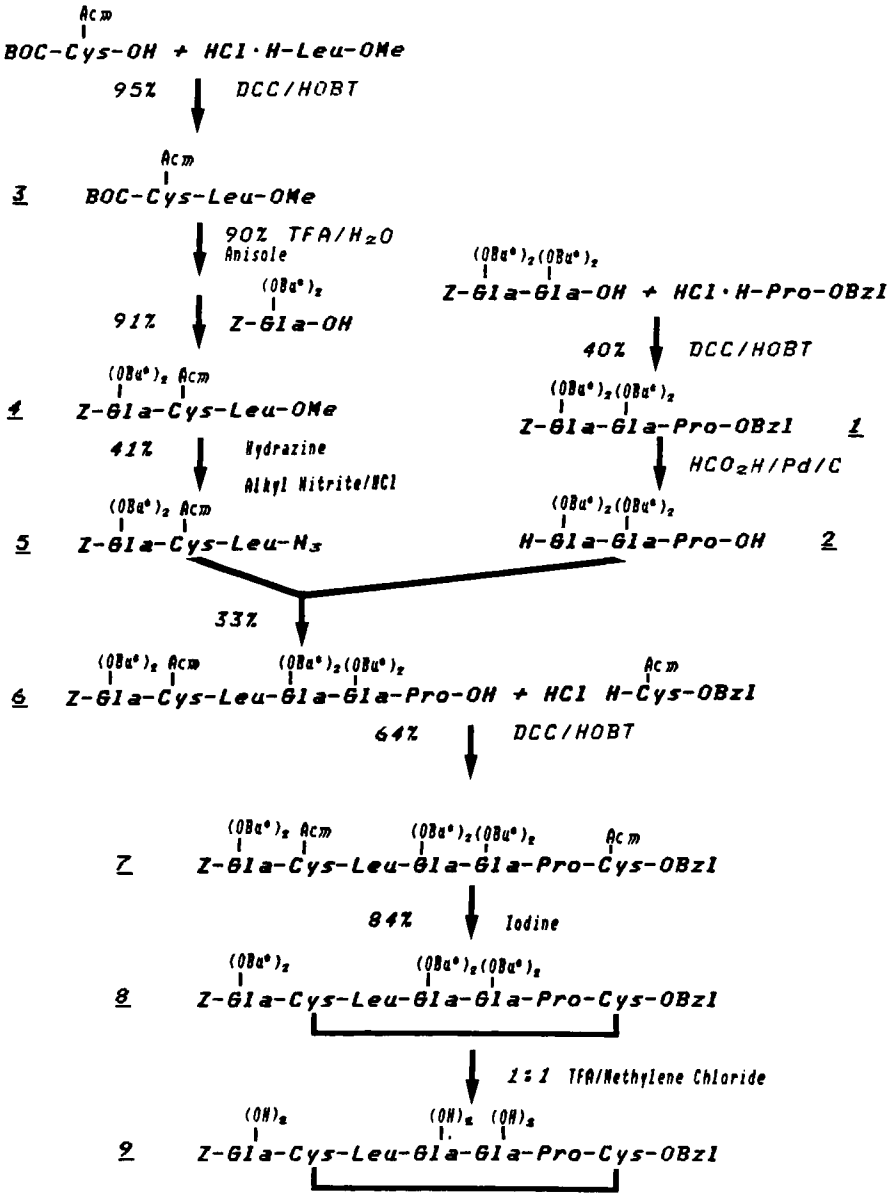
Introduction

The presence and function of γ-carboxyglutamic acid (Gla) residues in vitamin K-dependent plasma proteins has been the subject of several excellent reviews.[1,2] It is known that calcium ion specific binding sites and phospholipid interaction are both dependent on these residues.[3] Bovine prothrombin exhibits an interesting biphasic response on the addition of calcium ions, proposed to arise from a slow trans- to cis-isomerization of proline-22, and a subsequent fast binding of calcium ions.[4] We now wish to report the synthesis of a heptapeptide corresponding to residues 17-23 of bovine prothrombin. The three Gla residues in this peptide are expected to serve as ligands for a calcium ion. Spectroscopic studies should indicate whether the proposed conformational transition can occur in this heptapeptide disulfide loop.

Results and Discussion

The heptapeptide synthetic outline is shown in Scheme 1. Z-Gla(OBu^t)$_2$-Gla(OBu^t)$_2$-OH was synthesized according to Märki et al.,[5] and was coupled with HCl·H-Pro-OBzl using N,N'-dicyclohexylcarbodiimide and 1-hydroxybenzotriazole

Scheme 1. Synthesis of heptapeptide 17-23.



(DCC/HOBt) to give tripeptide 1. This coupling proceeded with racemization, as was evidenced by the separate amide and urethane proton NMR resonances for each diastereomer. A pure diastereomer, presumably the L,L,L-tripeptide could be obtained by crystallization from hexane (plates) or chloroform/hexane (needles) in a 41% yield, mp. 60°C (dec). The proton NMR (250 MHz) of this product was identical to that of a tripeptide prepared via a 2+1 azide coupling, thus confirming the L,L,L assignment. Simultaneous removal of the benzyloxycarbonyl and benzyl ester protecting groups with formic acid catalytic transfer hydrogenation[6] gave the tripeptide 2.

Boc-Cys(Acm)-OH was coupled to HCl·H-Leu-OMe with DCC/HOBT to yield the dipeptide 3 in 95% yield. Subsequent amino deblocking with 90% aqueous trifluoroacetic acid (TFA) and DCC/HOBT coupling to Z-Gla(OBu^t)$_2$-OH afforded the tripeptide 4 in 91% yield as an oil. Hydrazinolysis and crystallization of the product from methanol/ether gave the tripeptide hydrazide in 41% yield, mp. 131°C (dec). Treatment of the hydrazide with isoamyl nitrite/HCl and coupling of the resulting azide 5 with tripeptide 2 gave the hexapeptide 6 in 33% yield after chromatography on silica gel. Loss of tert-butyl esters during azide generation proved to be the source of a major hexapeptide contaminant. A final DCC/HOBT coupling with HCl·H-Cys(Acm)-OBzl, prepared according to Rich et al.,[7] afforded the pure heptapeptide 7 in 64% yield following silica gel chromatography.

Removal of the acetamidomethyl protecting groups and simultaneous disulfide formation was accomplished with iodine[8] in methanol at high peptide dilution to insure intramolecular cyclization. Chromatography on silica gel gave pure cyclic heptapeptide 8 in 85% yield. Subsequent treatment of 8 with 1:1 TFA/methylene chloride and lyophilization from water gave the target heptapeptide 9 as a fluffy white powder. The Ellman reagent test, as extended by Glaser

et al.,[9] gave a positive reaction after sodium borohydride application, confirming the disulfide presence. No free thiols were indicated by this reagent.

Studies on metal ion binding and conformational changes induced by metal ion binding have been undertaken. Magnetic resonance of various nuclei, including ^{43}Ca, ^{25}Mg, ^{13}C and ^{1}H, will be employed. These results will be reported in a future publication.

Acknowledgment

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SYNTHESIS OF A RETROHYDROXAMATE ANALOGUE OF THE IRON-BINDING IONOPHORIC PEPTIDE FERRICHROME

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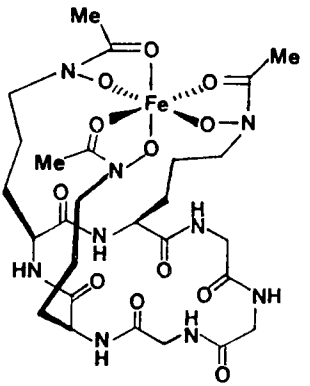
Ferrichrome (1) is a cyclic hexapeptide composed of three units each of glycine and N^{δ}-acetyl-N^{δ}-hydroxy-L-ornithine, and exists as the hexacoordinate ferric ion complex involving ligation by the hydroxamate functions of the ornithine side-chains.[1] Ferrichrome functions as a microbial transport agent responsible for delivery of iron to the cell.[2] Total syntheses of ferrichrome[3] and of enantioferrichrome[4] have been reported. We report in this paper the synthesis of a retrohydroxamate analogue of ferrichrome in which the retro analogue differs from ferrichrome by transposition of the respective N-hydroxy and carbonyl functions in the hydroxamate moiety. This transposition requires replacement of the three N^{δ}-acetyl-N^{δ}-hydroxy-L-ornithine residues in ferrichrome by three N-methyl L-α-aminoadipic acid-δ-hydroxamate units.



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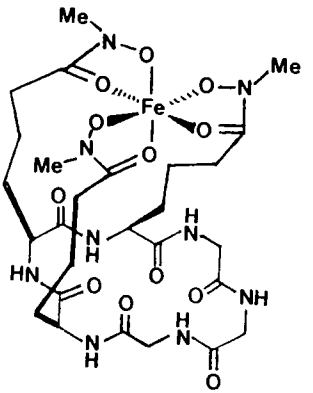

2

N-Methyl-O-benzyl N^{α}-t-Boc-L-α-aminoadipic acid-δ-hydroxamate (6) was prepared from L-α-aminoadipic acid.[5] Protection of the amino group with the t-Boc function was effected by reaction of L-α-aminoadipic acid with di-tert-butyl dicarbonate to give 3. Condensation of 3 with formaldehyde served to protect the α-carboxyl as the corresponding oxazolidinone 4.[6] Carbodiimide-mediated coupling[7] of 4 with N-methyl-O-benzylhydroxylamine[8] furnished hydroxamate 5, which upon saponification (sodium hydroxide in acetone) yielded the required protected α-aminoadipic acid δ-hydroxamate derivative 6. Compound 6 was employed in the peptide synthesis leading to preparation of retrohydroxamate ferrichrome.

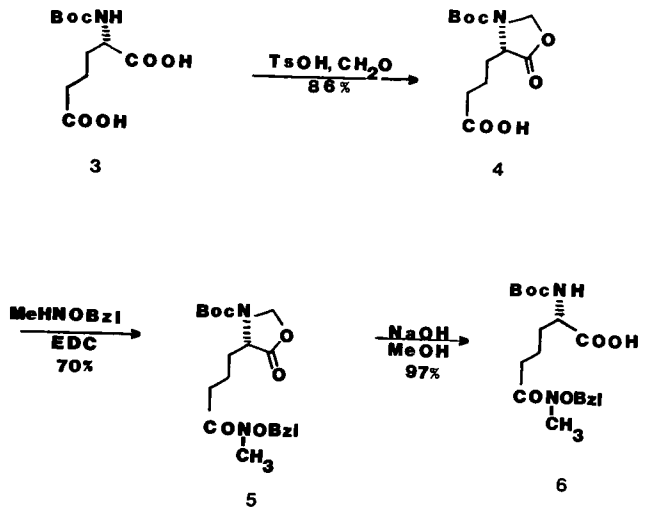

Fig. 1. Synthesis of δ-hydroxamate derivative of N-t-Boc-L-α-aminoadipic acid.

Synthesis of the linear hexapeptide 10 was accomplished by step-wise solution methods. Coupling reactions were effected using the mixed carbonic anhydrides of the individual Boc-amino acids. Removal of the Boc protection at the α-amino groups was accomplished by treatment with 50%

trifluoroacetic acid in dichloromethane. Tripeptide 7 was prepared by reaction of H-Gly-Gly-OMe with 6. A repetition of the sequence of deprotection and coupling effected conversion of 7, via peptides 8 and 9, to hexapeptide 10. Deprotection of the C- and N-termini in 10 and subsequent cyclization using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride:N-hydroxybenzotriazole under conditions of high-dilution in dichloromethane-dimethylformamide gave cyclic product 13 in 51% yield. Removal of the O-benzyl groups on the side-chain hydroxamate functions was effected by hydrogenolysis (H_2, Pd/C) to yield (42%) the deferriretrohydroxamate analogue 14. Complexation of 14 with ferric ion gave retrohydroxamate ferrichrome (2).

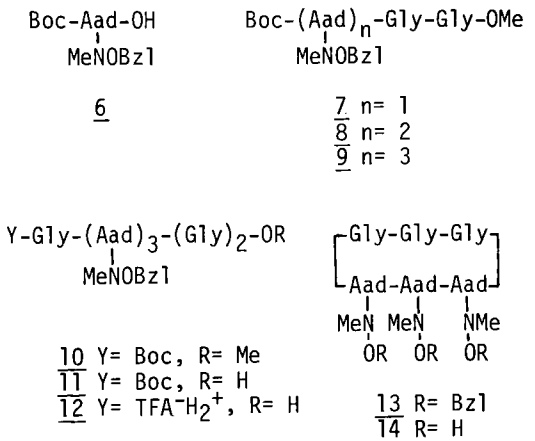

Fig. 2. Synthesis of cyclic hexapeptide.

Preliminary comparison of the retrohydroxamate analogue 2 with the natural ionophore ferrichrome (1) has been made. Significant changes result in the visible spectra of the retro compound upon changing the pH of the solution from 7 to 2, or upon challenging the ferric complex with EDTA. These results indicate that the retro analogue does not bind ferric ion as tightly as ferrichrome, which shows little change in its visi-

ble spectrum under these conditions. Biological assays comparing ferrichrome and the retro analogue show these two ionophores to be indistinguishable. The retro compound was equally effective as ferrichrome in promoting growth of Arthrobacter flavescens, an organism that requires a siderophore such as ferrichrome for growth. In the Bonifas test, both compounds were indistiguishable in their abilities to inhibit the antibacterial activity of the siderophore antibiotic albomycin. Thus, the enzymes involved in iron transport apparently do not distinguish between ferrichrome and its retrohydroxamate analogue.

Acknowledgements

Appreciation is expressed to the National Institutes of Health (General Medical Sciences, Grant GM 26711) for support of this research and to the Colorado State University Regional NMR Center (funded by National Science Foundation Grant No. CHE-78-18581) for 360 MHz NMR spectra.

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SYNTHEIS AND SPECTRAL PROPERTIES OF ($Nle^{56,60}$,34-65) AND (Nle^{209}, 205-231) BACTERIORHODOPSIN

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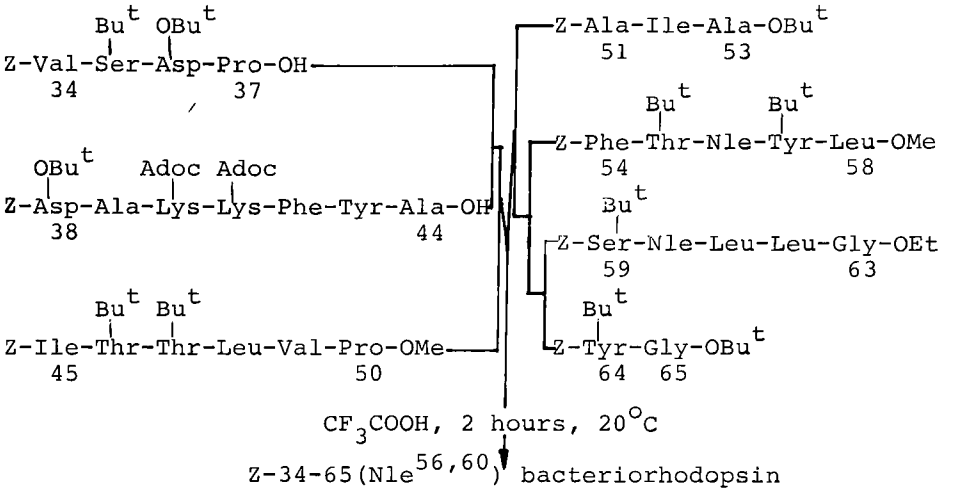
Bacteriorhodopsin, the single protein of the purple membrane of *Halobacterium halobium*, performs photoinduced proton transport through the membrane against the pH gradient[1]. Elucidation of protein conformation in membrane could help in undestanding the mechanism of bacteriorhodopsin functioning. One of the approaches to this rather complex problem is study of model peptides representing various segments of the protein. With this purpose in mind the synthesis of the title peptides, corresponding to the second and seventh α-helical rods of bacteriorhodopsin[2] has been carried out. Met^{56}, Met^{60} and Met^{209} were replaced by isosteric Nle to simplify the synthesis.

As seen from the schemes given below the strategy was employed of solution synthesis with maximum side chain protection with tert-butyl and 1-adamantyl derived protecting groups in combination with Z-group for temporary protection. C-terminal carboxyl was left unprotected or blocked by methyl ester removable by hydrazynolysis with subsequent oxidation according to Meienhofer et al.[3]

Extensive oxidation of the protected tyrosine took place upon oxidation of Z-38-44-N_2H_3 while with Z-54-58-N_2H_3 the same reaction proceeded quite smoothly. Obviously this reaction is sequence dependent. Adoc protection proved much superior to Boc in the solubility of peptides in organic solvents.

All intermediate and end peptides were analyzed by TLC and by HPLC in adsorption, size-exclusion and reverse-phase modes. In a number of cases preparative HPLC were employed for purification of intermediates. Protected peptides 59-65, 54-

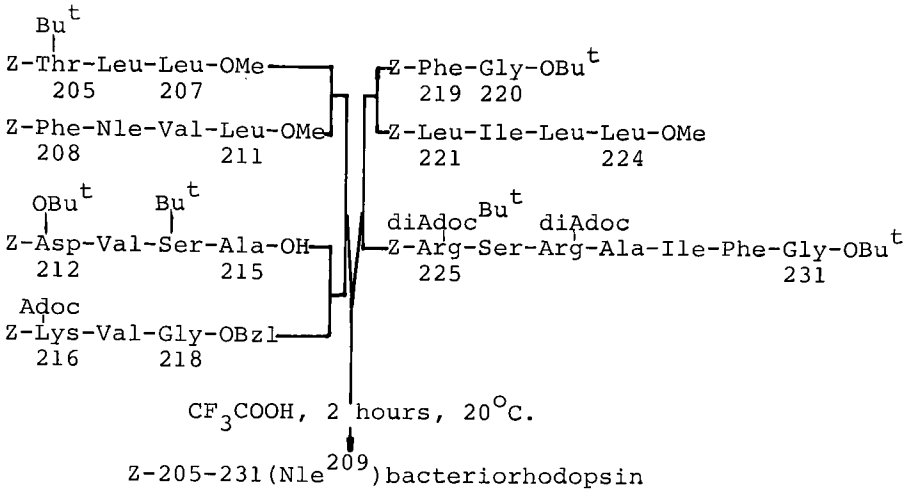


Scheme of synthesis of [Z-Val^{34},Nle^{56,60}]bacteriorhodopsin (34-65)

65, 34-50, 51-65, 34-65, 205-218, 219-231 were purified by high performance preparative size-exclusion chromatography in dimethylformamide, using LH-20 (2,5x45 cm) or Zorbax-Sil (2,5x25 cm) columns and direct pump injection. Analytical size-exclusion chromatography on silicagel Zorbax PSM-60 in dimethylformamide was used to monitor the kinetics of fragment couplings.

Partially protected peptides Z-38-44-OMe, Z-34-44-OMe, Z-34-50-OMe, Z-51-65-OH, Z-34-65-OH, Z-205-218-OH, Z-219-231-OH, Z-205-231-OH were obtained by exposure of respective fully protected segments to trifluoroacetic acid in presence of anisol. Being very hydrophobic these products could not be purified by conventional methods such as ion-exchange or aqueous gel filtration. Reverse phase HPLC on Lichrosorb RP-8 in water-acetonitrile gradient was used for that purpose (Figures 1^{a} and 1^{b}).

Circular dichroism spectra of the partially deblocked peptides 38-44, 34-44, 34-50, 34-65 in trifluoroethanol and trifluoroethanol-water (1:1) mixture were studied (Figure 2). It was rather rewarding to find in the 32-membered product a strong



Scheme of synthesis of [Z-Thr^{205}, Nle^{209}]bacteriorhodopsin (205-231)

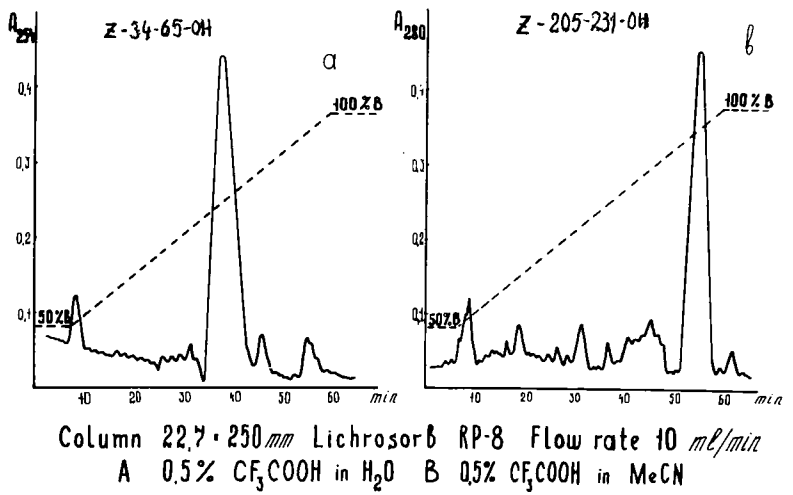


Fig. 1. Purification of the partially deblocked bacteriorhodopsin segments : a. Z-34-65-OH; b. Z-205-231-OH.

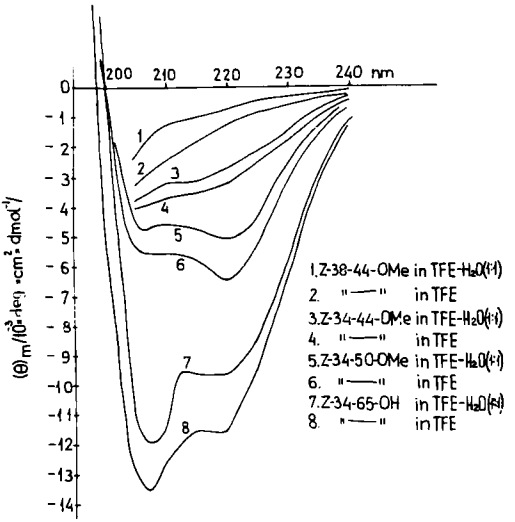


Fig. 2. CD spectra of partially deblocked bacteriorhodopsin segments.

trend to form α-helical structure as follows from the typical intense negative maxima at 220 and 207 nm. This result allows to suggest that α-helical structure of the bacteriorhodopsin transmembrane strands is due to inherent properties of each segment rather than to the packing effect of these strands. Further spectroscopic studies of the peptides obtained are now being carried out at various conditions, including phospholipid vesicles.

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SOLUTION PHASE SYNTHESIS OF CECROPIN A 1-22 AND POTENT ANALOGUES THEREOF USING SEGMENTS PREPARED ON AN OXIME SOLID PHASE SUPPORT

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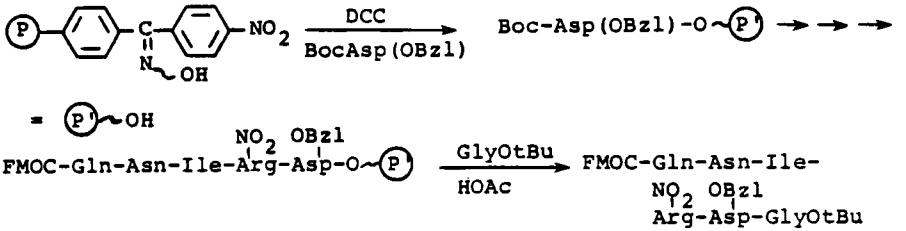
Introduction

Cecropins are a group of antibacterial 37-residue peptides which are synthesized by certain insects as a part of their humoral response[1]. The first 22 residues of these peptides are capable of forming exceptionally basic amphiphilic α-helices[1] suggesting that they may act by mechanisms similar to other amphiphilic cytotoxic peptides such as melittin[2]. To determine whether this secondary structure is essential for the antibacterial properties of the cecropins, cecropin A 1-22 and the idealized amphiphilic α-helical peptides FMOC(Leu-Lys-Lys-Leu-Leu-Lys-Leu)$_n$ (n=1,2) and FMOC(Leu-Glu-Glu-Leu-Leu-Glu-Leu)$_2$ were prepared. They were synthesized by solution phase segment condensation using protected segments prepared exclusively on a p-nitrobenzophenone oxime polymer. Fully protected peptides were removed from the support by a nucleophilic displacement reaction using peptide or amino acid esters[2] (Scheme I). By cleaving α-FMOC blocked peptides from the oxime resin with amino acid t-butyl esters it has been possible to synthesize protected peptides with orthogonal α-carboxy and α-amino protecting groups for bidirectional segment condensation strategies (Scheme II).

Results and Discussion

The use of the oxime ester for the synthesis of a typical protected hexapeptide is illustrated in Scheme I. The remaining peptides were prepared by similar methods. The

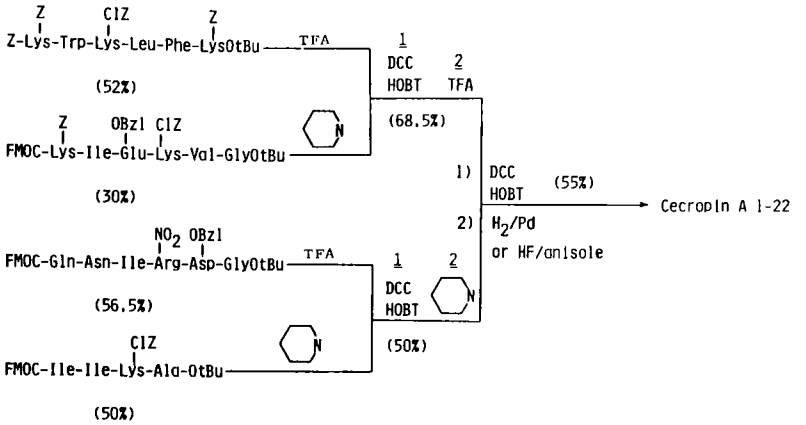
Scheme I



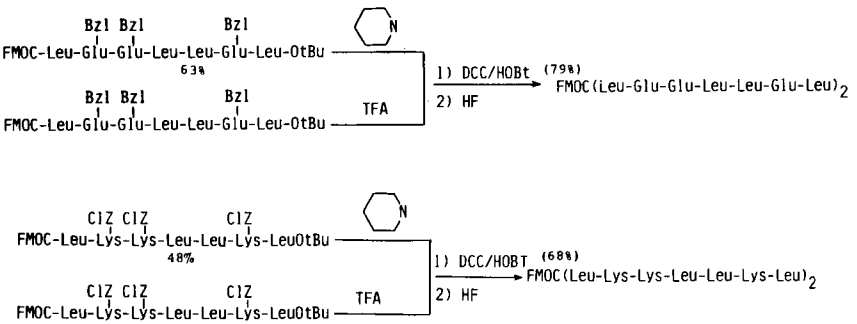
yields in Schemes II and III are those of pure products obtained by crystallization or precipitation. The peptides were homogeneous by criteria of TLC, size exclusion HPLC, and amino acid and elemental analysis.

The segment condensation strategy for the assembly of cecropin A 1-22 is illustrated in Scheme II. Selective deblocking of the FMOC group from segments containing the sequence Asp(OBzl)-Gly at positions 17 and 18 could be accomplished with less than 5% aspartamide formation by treatment with piperidine/DMF/DMSO (1:5:5) at 0° for 15 minutes. The progress of the coupling reactions was conveniently monitored by high performance size exclusion chromatography using a Du Pont Zorbax SIL column (0.9 x 25 cm) and a mobile phase of DMF/TFA (999:1). Intermediates were purified by size exclusion chromatography (Sephadex LH60) and were free of amino or carboxyl components as determined by size exclusion HPLC. After deblocking with HF, cecropin A 1-22 was obtained in approximately 90% purity as determined by isocratic reversed phase HPLC. The final product could readily be purified by size exclusion and ion exchange chromatography.[1] The product obtained by the above

SCHEME II SEGMENT CONDENSATION OF CECROPIN A 1-22



SCHEME III SEGMENT CONDENSATION OF MODEL PEPTIDES



procedure was obtained in comparable yield and purity to cecropin A 1-33 prepared by a stepwise solid phase route.[1] Thus both solution phase and solid phase methods appear to be successful for the preparation of this sequence. The solution phase strategy was preferred for the synthesis of the model peptides in that the fully deprotected peptides were difficult to purify due to their hydrophobicity and aggregation properties. The final purification of these products was aided by purification of the fully protected peptides prior to HF cleavage.

Table I. Antibacterial Activity of Peptides[a]

Peptide	MIC (μM) E. coli	B. subtilis
Cecropin A 1-37[1]	0.3-1[1]	-
Cecropin A 1-22	3-6	1-2
FMOC-(Leu-Lys-Lys-Leu-Leu-Lys-Leu)$_1$	80-40	5-10
FMOC-(Leu-Lys-Lys-Leu-Ley-Lys-Leu)$_2$	0.3-0.6	0.3-0.6
FMOC-(Leu-Glu-Glu-Leu-Leu-Glu-Leu)$_2$	>50	>50

a. Minimal inhibitory concentrations (MIC) were determined by incubating log-phase growing cells (10^5 cells/ml) with increasing concentrations of peptides in Vogel-Bonner minimal medium supplemented with 0.4% glucose. After 16 hours incubation at 37°, the concentration of cells was determined from the absorbance at 565 nm.

Cecropin A 1-22 is approximately one-tenth as active as natural cecropin A 1-37 (Table I). Thus, the active core of cecropin A is localized in a segment which is capable of forming basic amphiphilic helices. As the number of units in FMOC-(Leu-Lys-Lys-Leu-Leu-Lys-Leu)$_n$ is increased from one to two, the helicity (unpublished results) and antibacterial activity increase dramatically (Table I). FMOC-(Leu-Lys-Lys-Leu-Leu-Lys-Leu)$_2$ is as potent as cecropin A 1-33. In contrast, FMOC-(Leu-Glu-Glu-Leu-Leu-Glu-Leu)$_2$ shows no activity. We conclude that the minimal structural requirement for the antibacterial activities of the cecropins is an amphiphilic α-helix with basic residues on one face of the helix and hydrophobic residues on the opposite face.

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ENZYMATIC SEMISYNTHESIS OF HUMAN INSULIN: A PROPOSED PROCEDURE USING IMMOBILIZED ENZYME

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Porcine insulin can be enzymatically converted into human insulin by: 1) coupling Thr-X (X = OR or NHR) with des-alanine(B-30)-porcine insulin (DAI) prepared by digesting porcine insulin with carboxypeptidase A or Achromobacter lyticus protease I (two-step reaction) or 2) subjecting Ala-B-30 in porcine insulin to transpeptidation with Thr-X (one-step reaction). The former is the "coupling" method and the latter, the "transpeptidation" method. Trypsin[1] or Achromobacter lyticus protease I[2] can be used for the former, and trypsin,[3,4] carboxypeptidase Y[5] or Achromobacter lyticus protease I for the latter. In this paper, we propose a model of continuous preparation that can convert porcine insulin into human insulin using immobilized Achromobacter lyticus protease I in the coupling method.

Table I compares the optimal conditions for coupling and transpeptidation in semisynthesis of human insulin. Both are similar except that the transpeptidation requires 50-100 times the amount of enzyme to yield the same degree of human insulin ester (HI-OR). In the coupling process, Achromobacter lyticus protease I shows ten times or higher activity than trypsin at 25°C.

Among five kinds of polymer carrier employed to prepare the immobilized enzyme, the best activity was obtained with the combination of SiO_2-polyglutamic acid and Achromobacter

Figure 1 is a schematic diagram of a tentative procedure for industrial production of human insulin using immobilized enzyme. Immobilized enzyme column may also be used for DAI formation, but the optimal conditions have not yet been determined. In a preliminary experiment, porcine insulin (1 mM in 0.1 M NH_4HCO_3) is hydrolyzed by repeated application to immobilized Achromobacter lyticus protease column (1.2 cm high, flow rate 0.5 cm/hr) and gives 34% (3x appl.) or 69% (6x appl.) of DAI in the effluent. After concentration in vacuo, the effluent is lyophilized and the mixed powder of DAI and porcine insulin thus obtained can be used immediately for the next coupling step.

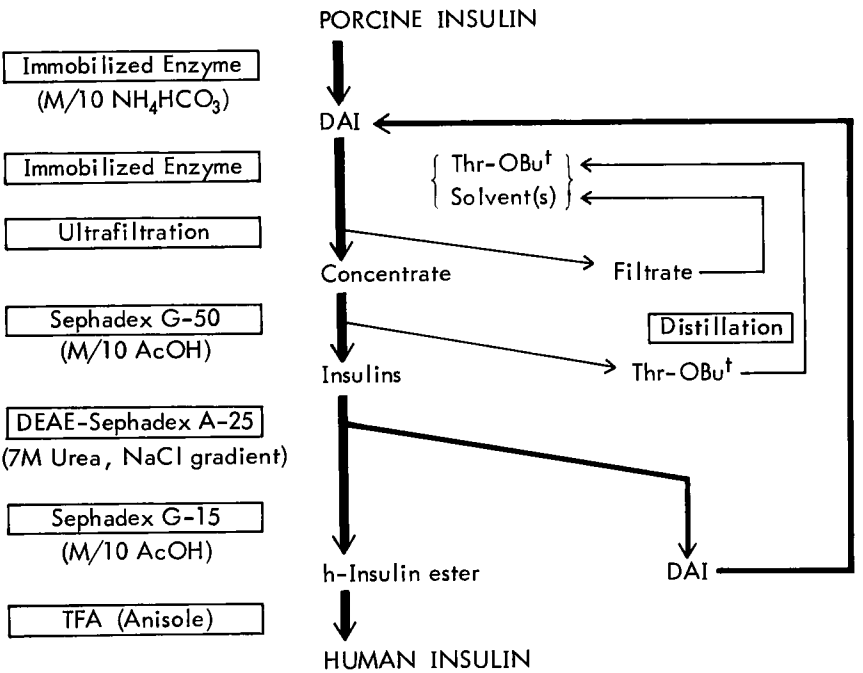

Fig. 1. Semisynthesis of human insulin using immobilized Achromobacter lyticus protease 1.

Table I. Comparison of Optimal Conditions for Coupling and Transpeptidation in Semisynthesis of Human Insulin.

Conditions	Coupling		Transpeptidation	
	Achromo. protease	Trypsin	Achromo. protease	Trypsin*
Reaction mixture	DAI (10 mM) + Thr-OBu^t (0.8 M)		Insulin (10mM) + Thr-OBu^t (0.8M)	Insulin (16mM) + Thr-OBu^t (0.47M)
Opt. pH	6.5-7		5-5.5	ca. 7
Enzyme concn. (Yield, 70%)	1 μM (9 h)	30 μM (4 h)	100 μM (9 h)	350 μM (4 h) Yield, 55%
Water content	<31%	38%	<31%	33% (very strict)
Ability of synthesis (per mg, ratio)	1,000	100	10	(1)
Synthesis by immobilized enzyme	Possible	Difficult	Very difficult	Very difficult

* Jonczyk and Gattner, 1981.

lyticus protease I (37 μM, determined from esterase activity to tosyl-L-lysine methylester). No essential difference was observed between the ratio of the human insulin synthesis to the esterase activity of intact and immobilized enzyme preparations in the coupling. When this immobilized enzyme is used batchwise, repeated application of the same enzyme sample causes almost no change in the enzymatic activity for synthesis.

From the data shown in Table I and the fact that the specific activity of the usable enzyme preparation can not exceed 37 μM, the coupling method using immobilized Achromobacter lyticus protease I seemed to be the best choice for continuous preparation of human insulin semisynthesis. The other methods in the Table were difficult to use because the specific activity of the immobilized enzyme which could be prepared was far from sufficient.

When a column of immobilized enzyme (0.7 cm high, flow rate 0.25 cm/hr) is used for coupling between DAI (5 mM, mixture with porcine insulin) and Thr-OBu^t (0.8 M in 40% of 1,4-butanediol and 2 M of acetate buffer, pH 6.5) to synthesize HI-OR, the yield of the product checked by HPLC increases with repeated application (once: 45%, twice: 63%, thrice: 80%).

By subjecting the reaction mixture to ultrafiltration, insulins are concentrated and the filtrate that contains low-molecular-weight components such as Thr-OBu^t, solvents and buffer can be reused for coupling. The methods of purifying HI-OR and deblocking tertiary butyl ester at B-30 have been described before.[6]

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PYROGLUTAMYL GROUPS AS ENZYME-LABILE PROTECTION OF THE LYSINE SIDE-CHAIN

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N-α-Cbz-lysine[1], stirred in a dimethylformamide solution of pentachlorophenyl pyroglutamate[2] (Cbz-Pc-O-Pcp), dissolved over the course of several hours with the formation of Cbz-Lys(Pc)-OH and pentachlorophenol. The Cbz-Lys(Pc)-OH was either isolated and crystallized or the mixture of Cbz-Lys(Pc)-OH and pentachlorophenol were coupled in situ through the action of dicyclohexylcarbodiimide (Figure 1). Cbz-Lys(Pc)-O-Pcp reacted with glycinamide to yield Cbz-Lys(Pc)-$GlyNH_2$ from which the Cbz group was removed by hydrogenolysis over palladium on charcoal.

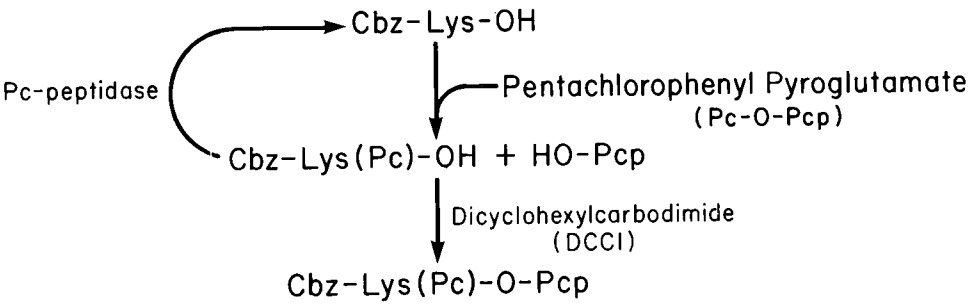

Fig. 1. Synthesis of Cbz-Lys(Pc)-OH and Cbz-Lys(Pc)-O-Pcp.

Treatment of either Cbz-Lys(Pc)-OH or H-Lys(Pc)-$GlyNH_2$ with thiol-activated pyrrolidonecarboxylylpeptidase (calf liver enzyme from Boehringer Mannheim) resulted in specific cleavage of the pyroglutamyl residue from the side-chain of the respective lysine residue. Cleavage of Cbz-Lys(Pc)-OH was monitored by measurement of the Cbz-Lys-OH released (Figure 2,).

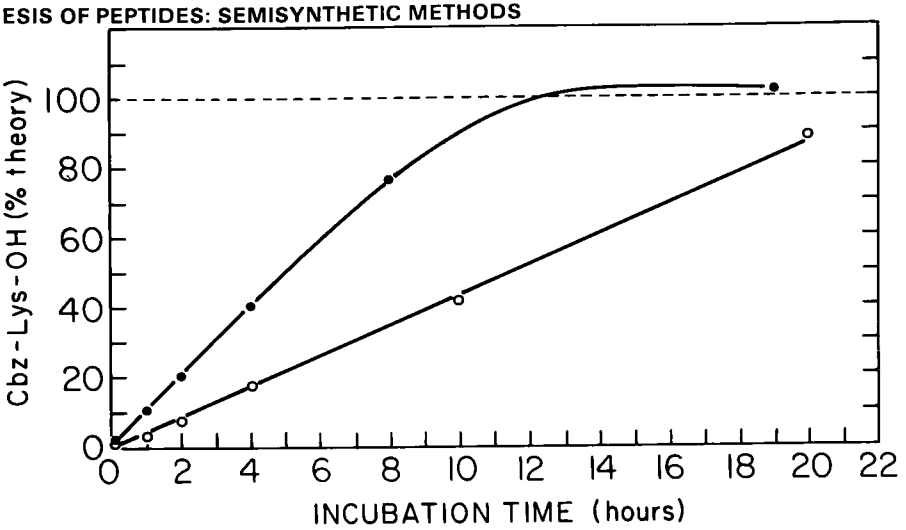

Fig. 2. Enzymatic cleavage of Cbz-Lys(Pc)-OH, Treatment of 2.5 u moles of substrate with 0.025 units (upper curve) or with 0.0125 units of pyrrolidonecarboxylylpeptidase in 0.05 M sodium phosphate buffer at pH 7.1 lead to cleavage of the pyroglutamyl group and release of Cbz-Lys-OH. Quantitative measurement of liberated Cbz-Lys-OH by ion exchange chromatography and ninhydrin colorimetry (short column of Spinco 120C Amino Acid Analyzer) generated the time course of the enzymatic hydrolysis.

The H-Lys-GlyNH$_2$ released by enzymatic hydrolysis of H-Lys(Pc)-GlyNH$_2$ was identified by electrophoretic and chromatographic comparison of the enzymatic cleavage product with authentic lysylglycinamide[3].

Suspensions of bovine pancreatic ribonuclease (RNAse A) and RNAse S-peptide[4] in dimethylformamide / hexamethylphosphoramide mixtures were also treated with Pc-O-Pcp. The suspended polypeptides dissolved in the reaction mixture over periods ranging from a few hours to about one day. The reaction mixtures were diluted with 0.2M acetic acid to give cloudy solutions which clarified upon extraction with ethyl acetate. The aqueous solutions of pyroglutamyl polypeptides were freed of low molecular weight materials by gel filtration on Sephadex G-25. The pyroglutamyl polypeptides prepared in this way were easily soluble in aqueous solutions and amino acid analyses of acid hydrolysates showed a glutamic acid content consistent with quantitative acylation of amino groups with pyroglutamyl groups.

When pyroglutamyl RNAse A was treated with pyrrolidonecarboxylylpeptidase at pH 7.1 in sodium phosphate buffer, a partially deprotected intermediate precipitated from the reaction mixture. This precipitated material corresponded to a product (or mixture of products) in which about half of the pyroglutamyl groups had been removed.

Treatment of 2.5 mg of Pc_3-S-peptide with 0.1 unit of pyrrolidonecarboxylylpeptidase in sodium phosphate buffer at pH 7.5 for 20 hr. followed by gel filtration yielded a product whose acid hydrolysate had an amino acid analysis consistent with complete, or nearly complete, cleavage of the pyroglutamyl groups. This material has not yet been examined thoroughly to prove its purity or its identity with S-peptide.

Discussion

The lysine residue is unique in that it can be reversibly modified and acts as a recognition site for specific endopeptidases which cleave peptide bonds on either the carboxyl side of the residue (trypsin) or on the amino side of the residue (A. mellea protease[5]). Besides the chemical protection required during classical fragment coupling reactions, the reversible modification of lysine residues exerts control over a unique enzymatic site which corresponds, in some ways, to the pallindrome/ restriction nuclease system of double stranded DNA semisynthesis. Both the acetyl group and the phenylacetyl group have been used as enzyme-labile protection for the side-chain of lysine[6,7]. In addition to the attractive properties of these groups and of enzyme-labile protecting groups generally[8-11], the pyroglutamyl group/pyrrolidonecarboxylylpeptidase system has some special properties. Even the large polypeptides RNAse A and RNAse S-peptide gave pyroglutamyl derivatives with good solubility in aqueous and nonaqueous solvent systems. The introduction and removal of blocking groups can be confirmed quantitatively from amino acid analyses of acid hydrolysates. The enzyme used to remove the blocking groups is available commercially in a degree of purity suitable for peptide sequence work. Finally, the pyroglutamic acid released by enzymatic cleavage of the pyroglutamyl group is the substrate of 5-oxo-prolinase[12]. This enzyme cleaves pyroglutamic acid to glutamic acid in an ATP-driven reaction. It should be possible, then, to

develop a coupled enzymatic system in which the cleavage of blocking groups can be monitored by the release of glutamic acid into the reaction mixture.

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KINETICS OF TRYPTIC TRANSPEPTIDATION OF INSULINS

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Substitution of the C-terminal alanine, residue 30 of the B-chain of insulin, with esters of threonine, converts porcine insulin into esters of human insulin. A trypsin catalyzed transpeptidation[1] in a predominately organic medium provides an almost quantitative conversion, and is used in the production of human insulin[2]. The reaction scheme from porcine insulin to human insulin methyl ester is shown in the left side of Fig. 1. An ^{17}O-NMR study using $H_2{}^{17}O$ in the medium provided evidence for that the transpeptidation reaction occurred without the formation of free des-(B30)-insulin as an intermediate[3].

The kinetics of trypsin catalyzed reactions have been studied using a selected set of "dry" conditions. Reaction rates are orders of magnitude slower than hydrolysis in aqueous solution. Fig. 1 shows the various reactions which have been studied. Those within the "broken line box" involve formation and splitting of covalent bonds. Associations between trypsin and substrates are shown outside the box. The models are based on: i) The acyl-enzyme is an intermediate, not only for hydrolysis but also for synthesis of peptide bonds. The acyl-enzyme is des-(B30)-insulinyl-trypsin (Des-(B30)-I-Tr), the carboxyl groups of Lys^{B29} forming an ester bond with the hydroxyl group of Ser^{183} of the active side of trypsin. ii) The non-covalent reactions are fast as compared to the covalent ones, and the model is simplified by assuming rapid or quasi-equilibrium. iii) The release of alanine from porcine insulin is irreversible.

Experimental

Three insulin substrates were used, porcine insulin (PI), des(Ala^{B30}) porcine insulin (Des(B30)-I) and human insulin methyl ester (HI-OMe). Threonine esters, Thr-OMe and Thr(Bu^t)-OBu^t were used, separate and in mixture 1:1, (M/M). Constant reaction conditions were: Concentration of threonine ester 1 M, of acetic acid 2.5 M and of water 20% (w/v). Organic solvent, dimethylacetamide, approximately 60%. Temperature 12°C. The strong acetate puffer formed had, after dilution with water, a pH of 4.5. The substrate concentrations were varied from 2 to 8 mM and trypsin (Tr) concentrations from 0.0125 to 0.2 mM. Table I summarizes the experimental designs. Reactions were followed up to 24 hours. No loss of trypsin activity was found after 24 hours. Samples taken during the reaction progress were precipitated with acetone,and the composition analyzed by HPLC[4]. PI, HI and des-(B30)-I are not separated in this system.

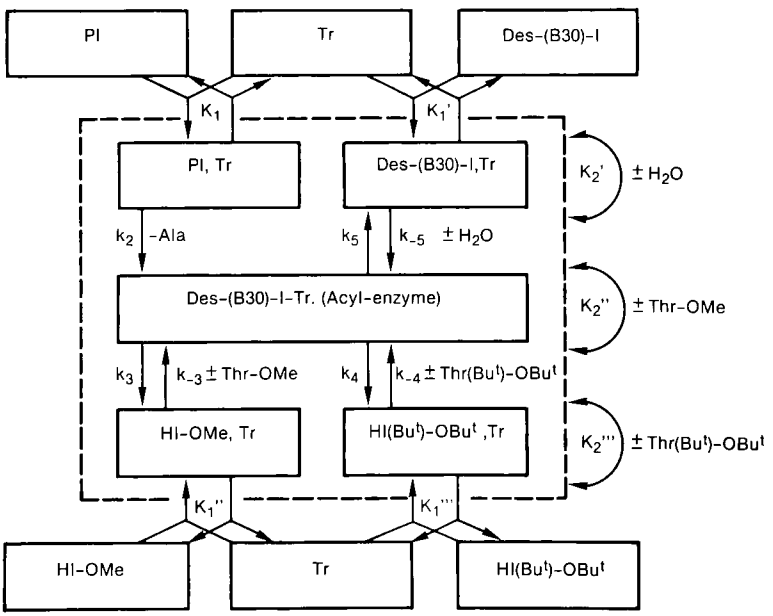

Fig. 1. Reaction scheme. Broken line encloses covalent reactions. K's and k's denote equilibrium and rate constants.

Results and Analysis Based on Simple Plots

The reactions followed 1st order kinetics up to 30-40% conversion. The apparent rate constants are given in Table I, normalized to 1 mM trypsin. Since coupling occurs at a rate 75 times that of transpeptidation (exp. 1 and 4), the release of alanine (k_2) must be the rate limiting step. Transpeptidation from HI-OMe to HI(But)-OBUt is 5 times faster than from PI, suggesting that Thr-OMe is a better leaving group than Ala ($k_{-3}>k_2$). The association of Thr-OMe to trypsin is stronger than association of Thr(But)-OBut ($K_2''>K_2'''$) since synthesis of HI(But)-OBut is markedly suppressed in mixtures of Thr-OMe and Thr(But)-OBut (compare exp. 2 vs. 3 and 5 vs. 6). Transpeptidation proceeds faster with Thr(But)-OBut than with Thr-OMe (exp. 2 vs. exp. 1), and coupling proceeds faster with Thr-OMe (exp. 4 vs. exp. 5). In experiment 7, a transient formation of des-(B30)-I is observed concomittant with the formation of HI(But)-OBut. The Lineweaver-Burk plots did cut the x-axis at points indistinguishable from 0, meaning the K_m's are large, estimated to be $\geq$0.1 M. According to ii) $K_m=1/K_1$ and $K_1 \leq 10\ M^{-1}$. This means that with the highest concentrations of insulin substrate (8 mM) and trypsin (0.2 mM), the amount of insulin-associated trypsin is less than 7.4% of the trypsin.

Insulin substrate	PI			Des-(B30)-I			HI-OMe
Experiment no.	1	2	3	4	5	6	7
Thr-OMe, M	1		0.5	1		0.5	
Thr(But)-OBut, M		1	0.5		1	0.5	1
k_a (h^{-1})							
HI-OMe	0.93		1.4	74		66	
HI(But)-OBut		1.8	0.25		27	6	9
Des-(B30)-I							5.5
Insulin products (%)							
HI-OMe	90		52	96		52	1
HI-(But)-OBut		96	44		96	44	95
PI+Des-(B30)-I	10	4	4	4	4	4	4

Table I. Design of Kinetic Experiments and Apparent 1st Order Rate Constants k_a for Product Formation. The distribution of insulin products are given after 24 hours.

Discussion

Thr-OMe apparently inhibits the release of alanine (k_2), possibly by binding to the imidazol group of His^{46} in trypsin, competing with the alanine to be released.

The much faster rate in coupling than in transpeptidation reactions indicates that the leaving group of the tetrahedral intermediate[5] determines the rate, meaning: $k_{-5} > k_{-3} > k_2$. In an ^{17}O-NMR study[3], it was found that hydrolysis to des-(B30)-I does not occur in the presence of 1 M Thr-OMe (conditions as exp. 1 and 4). In transpeptidation of HI-OMe to HI(Bu^t)-OBu^t, a transient formation of des-(B30)-I was observed (exp. 7). This indicates Thr(Bu^t)-OBu^t less efficiently inhibits hydrolysis than Thr-OMe, meaning $K_2'' > K_2''' \approx K_2'$.

A strong affinity of Thr-OMe for His^{46} in trypsin explains why Thr(Bu^t)-OBu^t couples much slower in competition with Thr-OMe than when present alone (exp. 6 vs.5). Furthermore, k_{-4} must be smaller than k_{-3}, since yields of HI-OMe and HI(Bu^t)-OBu^t after 24 hours are similar despite large differences in apparent rate constants at initial conditions (exp. 3 and 6).

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SEMISYNTHESIS AND PROPERTIES OF SOME CYTOCHROME C ANALOGUES

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Introduction

The strategy for the semisynthesis of horse cytochrome c analogues of Boon[1] can be used for the preparation of any analogue in the domain 66-104 (Fig. 1). The covalently recombined tetrahectapeptides bear homoserine in pos. 65. Exchange of Tyr^{67} → p-fluorophenylalanine and Thr^{78} → Val cause destabilization of the hydrogen bonds of a tightly bonded water molecule in the native electron transferring protein[2]. In the exchange of Phe^{82} → Leu, an (aliphatic) isobutyl residue replaces the (aromatic) benzyl group which might play a role in the process of electron transfer to cytochrome c oxidase[3]. In the exchange Tyr^{97} → Leu the same type of replacement was performed to study the importance of the ever present pair Phe^{10}/Tyr^{97}.

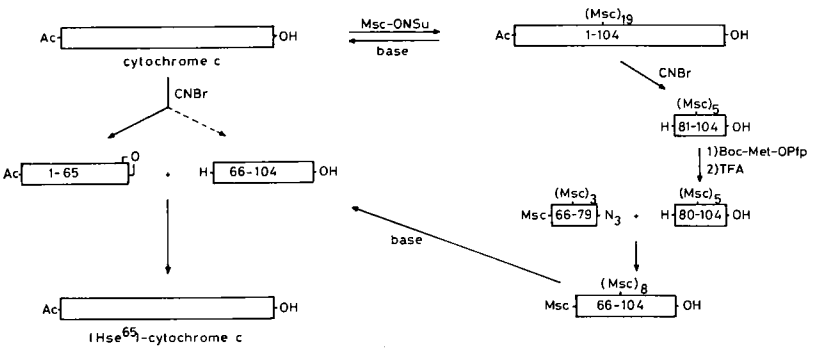

Fig. 1. Semisynthesis of cytochrome c analogues

Results and Discussion

The entropic favourable condensation of the fragments (1-65) and (66-104) was performed[4] at pH 7.2. The yields of the purified products were generally low, presumably due to a less perfect complexation of the two building blocks (Table). With the exception of the Val^{78}-containing analogue, each variant showed a weak absorption at 695 nm, indicative for the proper S-Fe ligation.

Table I Yields and Midpoint Potentials

Analogue	Yield	E_m (mV)
Hse^{65}	35%	263
Hse^{65},$Phe(4F)^{67}$	12%	199
Hse^{65},Val^{78}	3%	178
Hse^{65},Leu^{82}	4%	265
Hse^{65},Leu^{97}	2%	319

In the spectrum of the analogue containing Val^{78}, the Soret peak has shifted to 406 nm and the 695 nm absorption is absent. These facts hint at a transposition of sulfur and nitrogen as the sixth iron ligand (the amino nitrogen of some available lysyl residue can compete, as in the native protein at pH ≥ 11). The redox potentials of the analogues (Table) differ in magnitude. Their values are evidently not directly correlated with the state of the S-Fe ligation, but point to an important functionality of the water molecule, chelated by the side chains of Asn^{52}, Tyr^{67} and Thr^{78}. The periferally located Phe^{82} appears not to cooperate in the assessment of the value of the redox potential but to function in the binding of the entire molecule to its receptor: the oxidase. The unexpectedly great importance of the aromatic pair Phe^{10}/Tyr^{97} as a periferally located junction, essential for attainment of the proper conformation, is a novelty.

The rate of the reduction of oxygen with reduced cytochrome c, Figure 2, depends not only on the redox potential of the

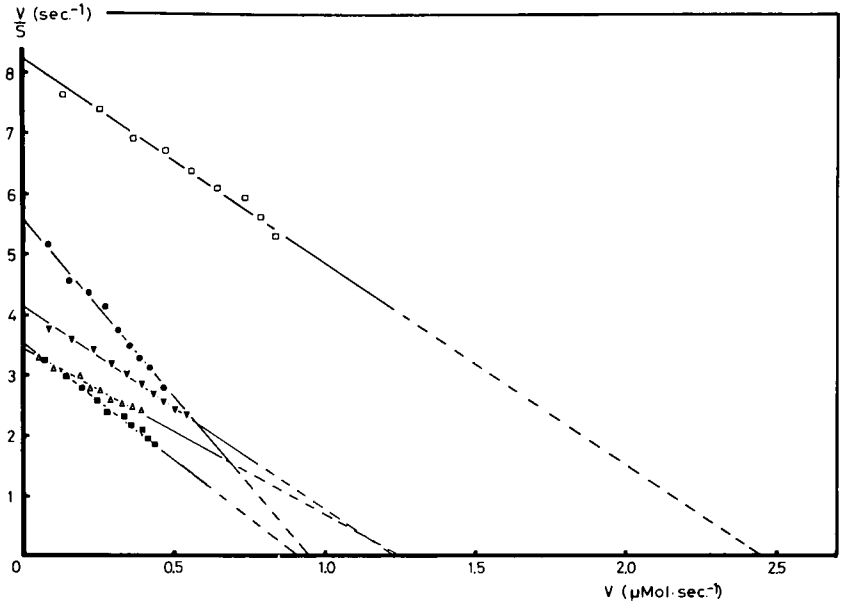

Fig. 2. Electron transfer to cytochrome c oxidase. Cytochrome c and [Hse^{65}]-cytochrome c ●, compared with the analogues additionally bearing Phe(4F)^{67} □, Val^{78} Δ, Leu^{82} ▼, and Leu^{97} ■.

latter, but also on its fixation to the oxidase. We speculate that the analogue lacking the hydroxyl group of Thr^{78} uses one of the lysyl residues designed for binding[5] to the oxidase as the sixth ligand to iron.

Acknowledgement

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IMMOBILIZED N-CARBOXYANHYDRIDES OF ALPHA AMINO ACIDS AS A TOOL IN THE SEMISYNTHESIS OF PEPTIDES

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Introduction

The use of polymer-assisted organic chemical peptide synthesis has increased steadily over the past 20 years. The methods described so far have employed the polymer either as a permanent anchoring agent/protecting group (e.g. Merrifield synthesis[1]), or as a transfer agent for acylation (e.g. polymeric active esters[2]).

In the case of incomplete reactions, both approaches, like the corresponding solution methods, result in mixtures of product and starting peptide.

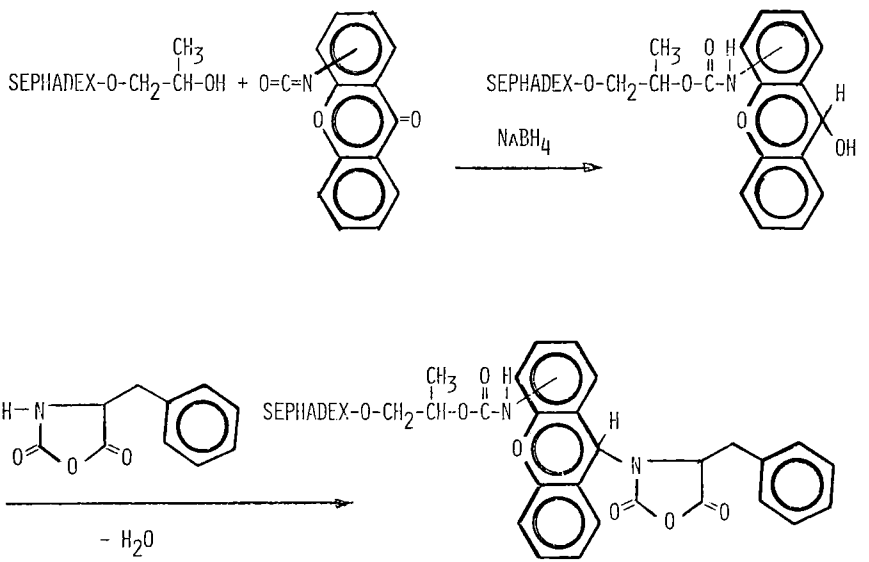
In principle, one way to overcome this problem is to design a system which achieves separation through reaction.

This work describes preliminary experiments using Sephadex as a temporary immobilizing agent for peptides by coupling with pendant alpha amino acid N-carboxyanhydrides (NCAs). The NCA is attached to the Sephadex at the nitrogen atom. Only those peptide molecules which are acylated by the polymeric reagent are retained covalently, i.e. immobilized by the reaction. Since unreacted peptide may be quantitatively recovered from the filtrate and washings, the necessity of applying forcing conditions in order to obtain a quantitative coupling is avoided.

Results and Discussion

Sephadex LH-60 was substituted with xanthydrol groups by reaction with xanthene-2-isocyanate-9-one in dry pyridine, followed by sodium borohydride reduction in isopropyl alcohol /water.

The resulting xanthydrol-Sephadex LH-60 was divided in two portions, of which one was substituted with the NCA of valine, and the other with the NCA of phenylalanine, in boiling toluene/dimethylformamide for 30 to 45 minutes, followed by exhaustive washing with dimethylformamide[3] (cf. Figure).



The level of substitution was ca. 0.1 mmol NCA per gram of NCA-Sephadex, as determined by amino acid analysis. For poly-X-Val NCA, the amino acid content was higher, in accordance with the observation that a fine precipitate of poly-valine was formed as a side reaction during reagent preparation.

The prepared polymeric reagents poly-X-Phe NCA and poly-X-

Val NCA were used to elongate free Thr-Ser-Gly-Pro-Ala first to Phe-Thr-Ser-Gly-Pro-Ala, then to Val-Phe-Thr-Ser-Gly-Pro-Ala, in the following manner:

The peptide and an equivalent of triethylamine were dissolved in a small volume of dimethylformamide, and the dry polymeric reagent was added in deficiency (about 10% less than the equivalent amount). With gentle agitation, a vacuum was applied to remove air and formed carbon dioxide. When the formation of bubbles had ceased, the mixture was gently agitated over night at room temperature.

Unreacted peptide was recovered unchanged from the filtrate by evaporation of the solvent, and after extensive washing with dimethylformamide and water the product peptide was released from the Sephadex carrier by stirring for 2 hrs in acetic acid/ethanol (1/1) at room temperature[3]. After TLC and HPLC-analysis, showing one major peak, the crude product was again immobilized by reaction with the next polymeric reagent.

For reference purposes, Phe-Thr-Ser-Gly-Pro-Ala and Val-Phe-Thr-Ser-Gly-Pro-Ala were also synthesized in aqueous solution at $0^{o}C$ and pH 10, using the free NCAs of phenylalanine and valine in varying amounts in several experiments[4].

The main products of the two types of synthesis were identical by TLC and HPLC, but the following differences were observed. Using even a 100% excess of free NCA, the conversion of starting peptide was far from complete, while the formation of oligomeric products could not be suppressed, even when only equivalent amounts of free NCA were used. The oligomer Phe-Phe-Thr-Ser-Gly-Pro-Ala was isolated by preparative chromatography, and identified by amino acid analysis. In the peptides obtained by reactive immobilization, the formation of oligomers was not observed. The presence of a small amount of unreacted Phe-Thr-Ser-Gly-Pro-Ala in Val-Phe-Thr-Ser-Gly-Pro-Ala is ascribed to the previously mentioned formation of poly-valine during preparation of the polymeric reagent, poly-X-Val NCA. The fine precipitate blocked the filter, making the washing-out process very difficult.

In conclusion, the described procedure of reactive immobilization on polymeric reagents is expected to become useful especially in semisynthetic approaches, where a free peptide is to be elongated at the N-terminus.

Recycling techniques, using columns or cartridges containing the polymeric reagents, may provide the basis for automation, in conjunction with detection systems based on UV-absorption and optical rotation.

The preparation of Sephadex-based polymeric reagents requires further development, in order to obtain higher levels of substitution, with avoidance of side reactions such as polymerization and damage to the Sephadex matrix.

Acknowledgement

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CHARACTERIZATION OF THE ISLET PROHORMONE CONVERTING ACTIVITY OF ISOLATED ISLET MICROSOMES AND SECRETORY GRANULES

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Introduction

There is little doubt that most, if not all, peptide hormones which are destined for secretion from their cells of origin are synthesized initially as preprohormones confirming the "signal hypothesis" originally proposed by Blobel and Sabatini[1]. Several recent reviews have summarized the information available regarding the biosynthesis of ACTH/endorphin[2], parathyroid hormone[3] and the hormones of the pancreatic islets[4,5]. Although a considerable amount of information which indicates the existence of a precursor-product pathway in the formation of many other peptide hormones has been generated, the examples given above have been the most thoroughly studied. After signal cleavage, which probably occurs concomitantly with insertion of the growing polypeptide chain into the cisternae of the endoplasmic reticulum, polypeptide prohormones are subjected to sequential metabolic cleavage during transport to the Golgi apparatus and secretory granules where processing is usually completed. The information available regarding these post-translational processing events was the subject of a recent review by Docherty and Steiner.[6]

We have been investigating the islet prohormone converting

activities present in microsome and secretory granule fractions of anglerfish pancreatic islets. In our initial studies, it was demonstrated that one or more of the converting enzymes involved in processing anglerfish proinsulin, proglucagon(s) and prosomatostatin(s) had unique characteristics which differentiate them from other intracellular proteases. This enzyme(s) is a thiol proteinase, has a pH optimum near 5, cleaves as an endoprotease at basic amino acid residues, may require the presence of segments of the prohormone in addition to the dibasic residues at cleavage sites for substrate recognition and/or binding, and is (secretory granule) membrane associated[7-9]. This enzyme(s) has some of the characteristics of cathepsin B but differs from cathepsin B in that it is not inhibited by TLCK and it has a more restricted substrate specificity. The unique characteristics of the anglerfish islet converting enzyme(s) clearly differentiate it from other enzymes which have been proposed as mediators of proinsulin conversion such as "pancreatic enzyme"[10], cathepsin B[11-13], cathepsin L[11], kallikrein plus kininase[14,15], and plasminogen activator[16]. However, it would not be inconsistent with the data which are presently available to hypothesize that the thiol proteinase(s) found in anglerfish islet performs the initial "trypsin-like" cleavage which was originally proposed as the first processing step necessary in proinsulin conversion[17-22] and is necessary in the processing of most other prohormones. Recently, prohormone converting enzymes with characteristics very similar to the thiol proteinase found in anglerfish islet secretory granules have been identified in secretory granules from rat pituitary neurointermediate lobe[23], bovine posterior pituitary[24] and rat islet[25]. These results provide evidence that enzymes having similar characteristics may be involved in prohormone processing in several different tissue types.

The focus of the study presented here was to examine the differential processing of separate somatostatin precursors

in islet microsomes and secretory granules. Data from studies performed in many laboratories have confirmed the existence of (pre)prohormones for somatostatin(s) which have a similar structure. Precursors having somatostatin (SS-14) at their C-terminus have been identified in anglerfish islets[26-33], catfish islets[34-36], rat islets[37], a rat thyroid medullary carcinoma[38], bovine hypothalamus[39], and a human pancreatic somatostatinoma[40]. In addition, somatostatin-28(SS-28), which comprises the 28 amino acid C-terminus of the mammalian prosomatostatins, has been isolated from ovine[41,42], porcine[43] and rat[44] hypothalami where it presumably represents either a cleavage intermediate in the generation of SS-14 or an alternative cleavage product with SS-like biological activity. In support of the latter possibility, results from numerous studies have demonstrated differential distribution of both SS-28 and SS-14 in various tissues[45-48], and release of both peptides into plasma[47,49,50] as well as from cultured brain cells[51]. Finally, the argument that SS-14 and SS-28 may function as separate hormonal entities is strengthened by reports that the two peptides have differing potencies and/or biologic activity in diverse systems[52-59].

The anglerfish islet presents a model system in which differential processing of somatostatin precursors can be studied. In addition to prosomatostatin I(PSS-I), the precursor of SS-14, a second precursor(PSS-II) which contains at its C-terminus [Tyr^{7},Gly^{10}]SS-14 as a potential cleavage product is also synthesized[60]. However, even though an Arg-Lys pair is located immediately N-terminal to Ala^{1} of the C-terminal tetradecapeptide, [Tyr^{7},Gly^{10}]SS-14 could not be detected in extracts of anglerfish islets[60,61]. Instead, a 28 residue peptide (AF SS-28) having [Tyr^{7},Gly^{10}]SS-14 as its C-terminus was found to be a primary cleavage product of PSS-II (Spiess and Noe, in preparation). As the primary structures of AF PSS-II (determined using recombinant DNA methodology[31]) are similar to those of mammalian prosomatostatins, the differen-

tial processing which is observed provides a situation analogous to the selective cleavage of mammalian prosomatostatins to yield SS-28 and SS-14. By examining processing activity in microsomes and secretory granules from anglerfish islets, it has been possible to demonstrate differential distribution and activity of the enzymes involved in conversion of PSS-I to yield SS-14 and PSS-II to yield AF SS-28.

Results and Discussion

Microsomes (rough endoplasmic reticulum and Golgi) and secretory granules were isolated from anglerfish pancreatic islets by differential and discontinuous density gradient centrifugation[62]. Fraction purity was monitored by assays for RNA and DNA[62], RIA for islet hormones[7,62], and by assays for plasma membrane and lysosomal enzymes[8]. To perform conversion assays, the subcellular fractions were lysed by repeated freezing and thawing then incubated in 100mM sodium acetate-acetic acid, pH 5.2 with ^{3}H-Trp plus ^{14}C-Ile or ^{3}H-Trp plus ^{35}S-Cys labeled islet prohormone mixtures. SS-14, AF SS-28 and their respective precursors become selectively labeled with Trp and Cys whereas insulin and proinsulin are selectively labeled with either Ile or Cys[7-9,26-28]. After termination of conversion assay incubations, extracts of products were subjected to gel filtration and M_r 1,000-2,000 peptides (SS-14 pool), M_r 2,500-4,500 peptides (glucagon and AF SS-28 pool) or M_r 4,500-8,000 peptides (insulin pool) were concentrated for reverse phase HPLC. Accuracy of product cleavage was monitored by comparing the HPLC retention times of radiolabeled *in vitro* conversion products with labeled products from tissue extracts and, in the case of SS-14, with synthetic SS-14. The *in vitro* products which had HPLC retention times identical to those of tissue extract products were considered to be accurately cleaved.

Converting Activity of Secretory Granules

Complete granule lysates, granule lysate supernates and granule membranes were all found to mediate conversion of PSS-I to SS-14. Washing the granule membranes with 1 M KCl prior to incubation resulted in a significant reduction of granule membrane-mediated conversion. These results demonstrate that a portion of the PSS-I converting enzyme(s), is membrane associated. Examination of the cleavage products found in the AF SS-28 and insulin pools revealed that complete granule lysate generated insulin and AF SS-28. However, granule membranes even in the absence of KCl washing, were found to be incapable of producing AF SS-28. This suggests that the PSS-II processing enzyme is not membrane-associated.

Converting Activity of Microsomes

To determine whether the converting activity in microsomes had characteristics similar to that of the enzyme(s) found in secretory granules, assays were performed to monitor the pH optimum and sensitivity to the thiol proteinase inhibitor, PCMB. The pH optimum was found to be near 5. PCMB at 100 μM completely inhibited conversion of all prohormones. Complete microsomal lysates were found to cleave proinsulin to insulin and PSS-II to AF SS-28 accurately, but were incapable of generating SS-14 from PSS-I. Moreover, as was the case for granule membranes, microsomal membranes (not washed with KCl) produced no AF SS-28 from PSS-II, suggesting that this activity is soluble in the microsomes as well.

Considering the results from both secretory granule and microsome-mediated conversion of PSS-I and PSS-II, the clear implication is that two separate enzymes which have a differential distribution are present. First, the PSS-I converting enzyme is active and membrane associated in secretory granules.

This same enzyme is either not present or is inactive in the microsomes. If the converting enzyme(s) is synthesized along with the (pre)prohormones in the rough endoplasmic reticulum and becomes membrane associated after internalization as postulated previously[8], then it is probable that the PSS-I converting enzyme is present, but inactive, in microsomes. The PSS-II converting enzyme is active in both microsomes and secretory granules but is not membrane-associated. This sort of enzyme distribution would allow conversion of PSS-II (and proinsulin) to begin early during the transport process (i.e. in the rough endoplasmic reticulum and/or Golgi complex) whereas the predominant PSS-I processing would occur distally in the secretory granules. An analogous situation has been described in the rat intermediate pituitary by Glembotski[63] who demonstrated that processing of POMC into β-lipotropin occurred in the microsomes, whereas most of the conversion of β-lipotropin into β-endorphin sized peptides occurred in secretory granules.

Purification of Islet Converting Enzymes

Preliminary attempts to isolate the anglerfish islet enzymes which are involved in processing PSS-I and PSS-II have been partially successful. Secretory granules were lysed in 50 mM sodium phosphate buffer, 0.5% deoxycholate, pH 7.4 and subjected to gel filtration in the same buffer. All extract components > M_r 25,000 were then subjected to benzamidine affinity chromatography to remove serine proteinases and then PCMB affinity chromatography to bind polypeptides containing reactive sulfhydryls. After elution from the affinity column, the PCMB bound material was tested for converting activity in the *in vitro* assay. Each of three separate enzyme preparations accurately cleaved PSS-II to yield AF SS-28. Two of the three preparations produced accurately cleaved insulin. It is not surprising that anglerfish insulin could be accurately cleaved from its precursor by a single thiol proteinase. The

B-chain of AF insulin has a C-terminal Lys.[64] Thus, no carboxypeptidase B-like activity is required.

None of affinity purified enzyme preparations generated SS-14 from PSS-I. This latter result is somewhat puzzling since PCMB has been shown to inhibit conversion of PSS-I to SS-14.[7] However, the absence of PSS-I converting activity in an extract in which PSS-II converting activity is found adds support for the contention that separate enzymes are involved in these two processing events. The affinity purification procedure we have developed is a potentially valuable tool in the purification and further characterization of polypeptide prohormone converting enzymes.

The detailed experimental protocols and data which support the results reported herein have been prepared for publication in separate communications.

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HORMONE PRECURSOR PROCESSING IN THE PANCREATIC ISLET

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Introduction

During the 15 years since the discovery of proinsulin, the synthesis of peptide hormones and bioactive effectors by way of cleavage from larger precursor molecules has become recognized as the rule rather than the exception. In fact, more than two dozen peptide effectors are now known to be or are presumed to be formed by limited proteolysis of larger biosynthetic precursors once these molecules have past into the cisternum of the rough endoplasmic reticulum and their signal/leader/pre sequences have been removed[1]. Proteolytic enzymes involved in posttranslational precursor processing may be either endopeptideses or exopeptidases; frequently enzymes of both types are required to yield the net production of the secreted hormone product. Although earlier work on the determination of precursor and hormone structure relied exclusively on protein sequence analysis, advances in the techniques of recombinant DNA research and molecular cloning, together with those of peptide chemistry, have resulted in a recent surge in information on precursor structure and sites for potential precursor cleavage. Still, few really excellent biochemical models have been established for studying cellular and cell-free mechanisms for peptide hormone biosynthesis and precursor processing, and little concrete information is available on the identities and activities of the participating proteases. Study of the biosynthesis of the hormones of the pancreatic islet (insulin, glucagon, somatostatin and pancreatic polypeptide) within the larger framework of information available on other peptide effectors can provide important insights into the complex mechanisms and structural requirements for the posttranslational processing of hormone precursors.

Results and Discussion

Diagrammatic structures for the precursors of mammalian pancreatic polypeptide (PP), insulin, somatostatin and glucagon are illustrated in Figure 1. Structural characteristics of these hormone-containing forms illustrate several general findings on peptide precursors as a whole. First, the bioactive peptide sequence can be N-terminal (as in pro-PP, as well as in prooxytocin and provasopressin), C-terminal (as in prosomatostatin, as well as in proparathormone), both N-terminal and C-terminal (as in proinsulin, as well as in prorelaxin), or "central" (as in proglucagon, proopiomelanocortin, progastrin, procalcitonin and many others). Second, initial cleavage of the bioactive sequence from the precursor occurs by way of endopeptidase action at paired dibasic amino acid residues (whether the pair is Arg-Arg, Lys-Arg, Arg-Lys or Lys-Lys). Third, additional cleavages can occur in nonhormonal sequences of the precursors to liberate cosynthesized and cosecreted peptide products often having as yet ill-defined activities (as in the icosapeptide cleaved from pro-PP[2], and the N-terminal peptide fragment of proglucagon[3], and the N-terminal peptide of somatostatin-28[4], as well as in a many other peptide hormone precursors). Notably, the cosynthesized

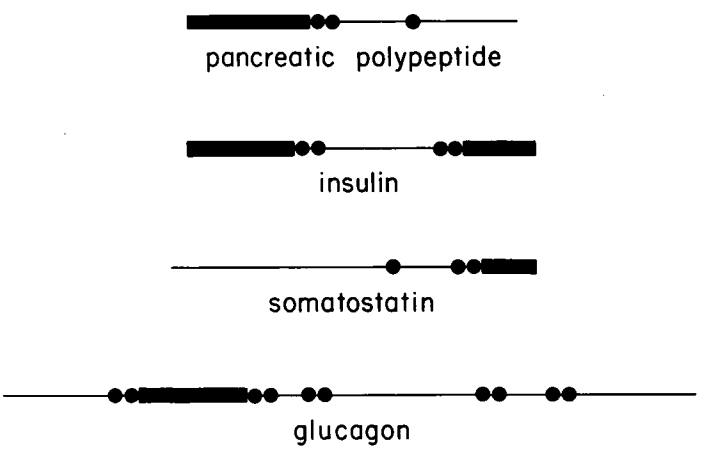

Fig. 1. Digramatic structures of the biosynthetic precursors for islet cell hormones. In each case, the hormone sequence is indicated by a heavy bar and extensions on that sequence, by lines. Dibasic amino acid residues important as precursor conversion sites are indicated by filled circles. The precursors are drawn to approximate scale and do not include N-terminal signal sequences.

and cosecreted product of proinsulin conversion (the C-peptide) is liberated directly during the formation of the hormone itself. Fourth, specific and often tissue-selective processing of the precursor can occur with liberation of larger peptide products which contain the entire hormone sequence (as in the formation of the N-terminally extended somatostatin-28[5] and the N- and C-terminally extended form of glucagon called glicentin[6]. Fifth, additional processing is often required to result in the structure of the final stored and secreted hormone: the simplest case is that of carboxypeptidase action to remove C-terminal basic residues in any hormone sequence which is extended from its C-terminus in its precursor form; a more complex case is that of required C-terminal amidation in the formation of PP as well as in the formation of peptides such as oxytocin, vasopressin, gastrin, calcitonin, secretin, and corticotrophin and growth hormone releasing factors.

Although identification of the dibasic amino acid pair-specific endopeptidases which participate in conversion of the islet cell hormone precursors to their products is a matter of considerable importance, these enzymes have not yet been isolated or characterized: special consideration has been given to enzymes with trypsin-like specificity such as kallikrein and plasminogen activator, but recent evidence strongly favors the participation of cathepsin-related, acidic thiol proteases (see Ref. 1 for a review). Nevertheless, it apears that the structures of the peptide hormones and their precursors may themselves play important roles in directing the converting enzyme to appropriate prohormone cleavage sites. Table 1 documents the distribution frequency of more than 50 dibasic amino acid pairs which occur in a variety of peptide hormones and their precursors. Notably the possible pairs are not equally distributed among those peptides examined, with the frequency decreasing in the order Lys-Arg>Arg-Arg>Lys-Lys=Arg-Lys. Even more intriguing is the finding that the frequency with which each pair occurs as a prohormone converting site (rather than as an uncleaved sequence contained within a hormone product) shows the same order. Among the 27 Lys-Arg pairs included, all were identifiable as probably precursors cleavage sites; among the 8 Arg-Lys pairs tabulated, only two are readily identifiable as sites for prohormone cleavage. On the basis of this simple comparison, the frequencies of Table I suggest that participating converting enzymes greatly favor arginine in the second position of the dibasic pair, whereas they show a somewhat lesser bias against arginine in the first position. The frequent occurrence of uncleaved dibasic pairs emphasizes the need for caution in assigning structures to final hormone products solely by analysis of precursor sequence information.

Table I. Distribution Frequency of Dibasic Amino Acid Pairs in Peptide Hormones and Their Precursors[a]

Dibasic pair	Number of pairs tabulated	Percent of total pairs	Percent of pair cleaved
Arg-Arg	13	24	62
Lys-Arg	27	49	100
Arg-Lys	8	14	25
Lys-Lys	8	14	38

[a]Dibasic amino acid pairs occurring in peptide hormones and their precursors are tabulated for a total of 56 entries derived from 22 hormones and precursors where sequence information is available. The pairs were considered to be prohormone cleavage sites if such cleavage is known to occur (for example in proinsulin) or is likely to occur (for example in the multiple sites occurring in preoenkephalin). Uncleaved pairs occur in hormone sequences themselves; pairs were considered uncleaved when only very low amounts of the cleaved products have been identified and when those products might have arisen from minor processing pathways. Like peptide were included from only a single species. All peptides were of mamalian origin.

Recent biosynthetic studies have further emphasized the importance of dibasic amino acid structure (and sequence) in directing the extent of precursor processing by the converting enzyme: incorportation of the dibasic amino acid analogs canavaline and thialysine into the hormone precursors of anglerfish or rat islets during biosynthetic studies, diminishes the rate of precursor conversion to product[7,8]. The participating endopeptidases thus show significant structural specificity. In addition, two cases of genetic mutations at precursor converting sites in man are known to decrease greatly, or to prevent, precursor processing. In the case of a proalbumin variant (in which the second arginine in the Arg-Arg sequence has been replaced by glutamine), albumin bearing an N-terminal, hexapeptide sequence is secreted from the liver without clinical consequence[9]. In the case of a proinsulin variant (in which arginine in the Lys-Arg pair has been replaced by an unidentified, neutral amino acid) a low activity intermediate of proinsulin conversion with the C-peptide attached to the insulin A chain is secreted from the B-cell with resulting mild diabetes[10]. In another case the sequence of the conversion site for prosomatostatin (Arg-Lys) has been altered in

an additional expressed product of the catfish somatostatin gene family (where the dibasic pair has changed to Lys-Pro)[11]. Not surprisingly, the Lys-Pro sequence is not cleaved, a result emphasizing the potential evolutionary role of mutation in altering peptide structure by the formation or deletion of potential, dibasic pair, prohormone converting sites.

Although the cellular conversion of prohormones to final products is seldom complete (in that small amounts of precursors and conversion intermediates usually remain uncleaved), it is also true that processing of the same gene product can be tissue-specific and in some cases, that processing can seem to be purposefully limited. The clearest example is the processing of proopiomelanocortin to its different products in the anterior and medial lobes of the pituitary[12]. Selective processing of the islet hormone precursors in cells of the pancreas, intestine and hypothalamus results in the ultimate formation of both somatostatin-28 (an extended form of the hormone bearing a 14-residue, N-terminal extension)[13] and glucagon-related peptides bearing both N- and C-terminal extensions on the hormone sequence)[14]. Cleavage of prosomatostatin to somatostatin-28 requires two selective elements of processing: endopeptidase cleavage C-terminal to a single arginine residue at position -29 in the precursor (an unusual example where paired basic residues do not occur) and the absence of cleavage at the Arg-Lys sequence N-terminal to the somatostatin structure. The low tendency for Arg-Lys to be cleaved by processing enzymes (see Table 1) may well contribute to formation of the extended form. On the other hand, processing events resulting in the formation of glicentin (an N- and -terminally extended form of glucagon) and glucagon from the glucagon precursor all occur at Lys-Arg sequences (an easily cleaved pair)[15]. It is thus clear that cleavage selectivity must arise from structural elements beyond the simple dibasic amino acid pair. Residues adjacent to potential conversion sites do not show structural conservation. It is likely that secondary structure plays at least some role in guiding the accuracy of convesion at "correct" sites, while sparing "incorrect" cleavage, e.g. at the Arg-Arg pairs that occur within glucagon and PP themselves.

Exopeptidase processing of precursor conversion intermediates by carboxypeptidase B-like enzymes (to remove C-terminal basic residues) usually occurs as the direct consequences of endopeptidase cleavage at paired basic residues; at least one participating carboxypeptidase B-like enzyme (for the processing of proenkephalin to enkephalin) has been isolated[16]. Other terminal

processing mechanisms for example to produce the pyroglutamyl group from N-terminal glutamine (a spontaneous reaction which may well occur without enzymic catalysis) and to cause N-terminal acetylation (an enzyme-catalyzed reaction requiring acetyl-coenzyme A)[17] play important roles in the biogenesis of a great many bioactive peptides. An equally important mechanism concerns the formation of the peptidyl alpha-carboxamides of potentially active peptides cleaved from larger precursor molecules. The suggestion, now several years old, that carboxamidation requires a glycyl residue C-terminal to the residue destined for amidation[18], has been confirmed by many examples. Considering sequence information on seven precursors of carboxamidated, bioactive peptides, in only one case (the honey bee toxin mellitin), does glycine appear as the C-terminal residue of the precursor; in most cases, paired dibasic amino acid residues occur C-terminal to the glycyl residue, suggesting the participation of specific endopeptidases of the sort described above in the overall processing of the precursors to their final products. The absolute requirement for the carboxamide group in producing active peptides, and the indentification of several new potentially bioactive peptides using chemical methods for the detection of peptidyl carboxamides[19], emphasize the importance of carboxamidation in the posttranslational processing of peptide hormone precursors.

Isolated endocrine cells of the canine duodenal pancreas represent an excellent system for studying both the biosynthesis of pancreatic polypeptide (an alpha-carboxamidated peptide) and cellular mechanisms for carboxamidation. Previous work has documented the synthesis of the hormone by way of a 9,000 molecular weight precursor which bears a C-terminal extension of the PP sequence (see Fig. 1) and the cosynthesis of an icosapeptide which arises from the C-terminal extension itself[2,20]. Conversion of the precursor to PP would thus seem to require the participating of at least three enzyme activities: A dibasic amino acid pair-specific endopeptidase, a dibasic amino acid-specific exopeptidase and the carboxamidating enzyme. A subsequent report showed that the product of biosynthetically labeled pro-PP conversion in cultured endocrine cells of the duodenal canine pancreas (so-calld pseudoislets) is not PP, but is a nonamidated, more acid form of the hormone which presumably contains a C-terminal extension consisting of a single glycine residue[21].

Figure 2 shows the results of a biosynthetic experiment in which the products of pro-PP conversion (PP and the icosapeptide) were examined after incubation of ^{35}S-methionine with newly isolated endocrine cells of the canine duodenal pancreas and with cells subjected to culture for 24 hr and 10 days. Importantly, incorporation of labelled amino acid remained high in all cases. As shown on the left side of the figure, methionine incorporation into PP-related products was always highest for the nonamidated, more acidic form of the peptide. Nevertheless, sizable amounts of fully amidated PP (varying from 15 to 50% of

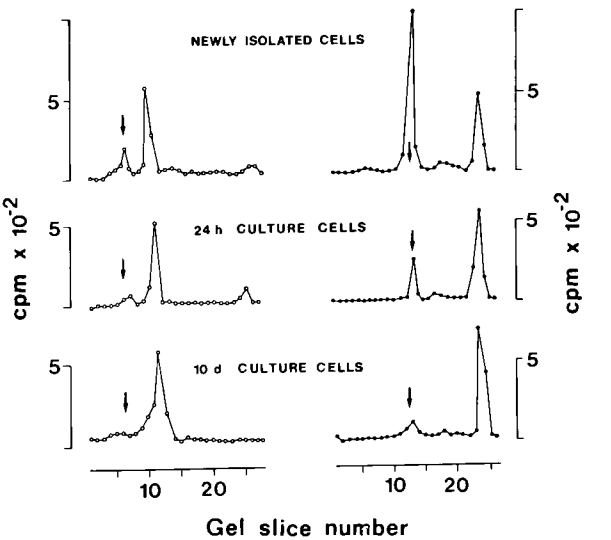

Fig. 2. Analysis of biosynthetic products arising from propancreatic polypeptide conversion in endocrine cells of the canine duodenal pancreas. Isolated cells and cultured islets were prepared and were incubated with ^{35}S-methionine for 90 min as described[2,20]. The washed tissue was then extracted with acetic acid and gel-filtered on Bio-Gel P-30. Separately pooled fractions corresponding to biosynthetically labeled PP and the pro-PP-derived icosapeptide were dried and the peptides subjected to nondenaturing polyacrylamide gel electrophoresis at pH 8.7; labeled peptides present in slices of the tube gels were counted for radioactivity[2,20]. The figure shows results obtained from newly isolated cells and for cells cultured for 24 hr and 10 days. The data shown on the left (open circles) correspond to peptides from the PP region of the gel filtration column, whereas data shown on the right (closed circles) correspond to peptides from the icosapeptide region. The electrophoretic positions taken by native PP (left) and native icosapeptide (right) are indicated by arrows. All labeled peptides shown were precipitable by antibodies directed toward PP (left) or towards the icosapeptide (right).

the total in different experiments) were identified in labelled peptides extracted from newly isolated cells. By even 24 hr of culture, only the nonamidated form of the peptide was identifiable. In addition, pulse chase studies of PP biosynthesis even in fresh cells showed that the nonamidated form of PP was stable and was not converted to PP itself; the nonamidated form identified does not therefore appear to be a precursor for PP biosynthesis. It is not, however, a deamidated peptide, as control experiments showed that carboxyamidated PP remains unaltered when subjected to equivalent purification and analytical procedures.

The right side of Figure 2 shows parallel results examining the formation of the pro-PP-derived icosapeptide in the same cell preparations. In newly isolated cells, the major biosynthetic product corresponds to the isolated peptide, although small amounts of a more acidic form are detected. (It is important to note that the icosapeptide is not itself a carboxamidated peptide). By 24 hr of culture, only small amounts of the usual form of the icosapeptide can be detected; by 10 days of culture, only the more acidic form remains.

The results of Figure 2 document several important aspects of PP biosynthesis and precursor processing: (a) post-translational processing mechanisms resulting in carboxamidation are extremely sensitive to cell culture and presumably to small differences in cellular metabolic state; (b) processing mechanisms resulting in the formation of the icosapeptide are somewhat less sensitive to cell culture, but correct processing is severely altered by prolonged culture conditions; (c) less refined mechanisms involving cleavage of the precursor to smaller peptides resembling the final secreted products (presumably by endopeptidase action at paired basic residues) are not markely sensitive to altered metabolic state and are applied efficiently even in cultured cells; (d) alternative routes of cellular precursor processing may result in the formation of peptides with the character of biosynthetic intermediates even when those peptides escape final processing to normal secreted products. Importantly, both incompletely processed peptides identified in Figure 2 (the acidic forms of PP and the icosapeptide) have been identified chemically in acid-ethanol extracts of canine duodenal pancreas (data not shown). As expected, however, these forms represent less than 10% of the respective, mature peptide products.

Cellular mechanisms for peptide carboxamidation, like those for other aspects of precursor processing remain poorly understood. Nevertheless,

carboxamidating activity has recently been detected in pituitary extracts through the use of model peptide sybstrates bearing C-terminal glycine extensions[22]. The proposed mechanism involving dehydrogenation of the alpha C-N bond of the glycyl residue while it is in amide linkage to the remainder of the peptide resembles likely mechanisms for oxidation of amino acids by the flavine-linked amino acid oxidases. The potential role of basic amino acid residues C-terminal to the amide nitrogen-contributing glycine residue in enhancing reaction rate[23], however, has not yet been examined.

It is clear that the multiplicity of processing steps required for the conversion of peptide hormone precursors to their final products contributes a high degree of complexity to investigations of peptide hormone biosynthesis. Whether considering dibasic amino acid-specific endopeptidases, exopeptidases, carboxyamidation enzymes or cellular mechanisms for translocation and packaging, a great many simultaneous and sequential reactions must be coordinated for final and correct processing to occur. Given the probable low concentrations and lability of participating converting enzymes (some of them perhaps existing as membrane-associated entities), detailed mechanistic and structural analysis of the enzymes has proved difficult. Still, the importance of these enzymes in the biosynthesis of dozens of bioactive peptides underscores the importance of continued investigation on the biochemistry and enzymology of hormone precursor processing. (Supported by grants AM 18347 and AM 20595.)

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STUDIES ON THE NH_2-TERMINAL ACETYLATION OF α-MSH AND β-ENDORPHIN IN THE RAT INTERMEDIATE PITUITARY

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Introduction

In the pituitary pro-ACTH/endorphin is post-translationally processed differently by the anterior and intermediate lobes (1-4). One aspect of this differential processing is the NH_2-terminal acetylation of αMSH- and β-endorphin-related peptides that occurs exclusively in the intermediate pituitary. αMSH, which occurs in des- and α-N-acetylated as well as α-N,O-diacetylated forms in rat, beef and pig intermediate pituitary (5-7), is optimally active as a melanocyte stimulating hormone only when mono- or diacetylated (6), whereas the opiate-like activity of β-endorphin is abolished with α-N-acetylation. Biosynthetic studies with rat intermediate pituitary cell cultures have shown that desacetyl-αMSH is the precursor to α-N-acetyl-αMSH which gives rise to α-N,O-diacetyl-αMSH (8). The post-translational NH_2-terminal acetylation of peptides is a very rare occurrence, being observed for αMSH, β-endorphin, and possibly enkephalins (9), but it is key to determining the biological activities of these peptides. Several recent studies have shown that the acetylation of αMSH and β-endorphin takes place in the secretory granules and that a single enzyme is responsible for the NH_2-terminal acetylation of both αMSH and β-endorphin (8, 10).

To investigate the regulatory mechanism of the acetylation of αMSH and β-endorphin in more detail a study was undertaken to evaluate the possible existence of an enzyme that removes the NH_2-terminal acetyl groups from αMSH and β-

endorphin. This type of enzyme would add a dimension to the mechanism that cells use to control the biological activities of the peptides derived from pro-ACTH/endorphin. To determine whether such an enzyme activity exists, biosynthetic labeling experiments were performed. Rat intermediate pituitary cells were incubated with both (^{3}H)acetate and (^{35}S)methionine such that a steady state level of αMSH and β-endorphin labeling was achieved. Cultures were then chased in non-radioactive medium and the $^{35}S/^{3}H$ ratios of αMSH and β-endorphin before and after the chase incubations were used as measures of the stability of the acetyl groups.

Methods

Primary cultures. Primary cultures of rat intermediate pituitary cells were prepared as previously described (11, 12) and maintained on protamine-coated microwell culture dishes in a complete serum-free medium (13). For biosynthetic labeling of peptides, cultures were incubated in complete serum-free medium containing 250 μCi of (^{35}S)methionine (1265 Ci/mmol; Amersham) and/or 2 mCi of (^{3}H)acetate (2.8 Ci/mmol; New England Nuclear).

Analysis of αMSH- and β-endorphin-related peptides. After the incubations, cultures were extracted with 5N acetic acid plus 0.3 mg/ml phenylmethylsulfonyl fluoride and 1% 2-mercaptoethanol. After freeze-thawing and centrifugation, the supernatants of the extracts were lyophilized. The extracts were then redissolved in 200 μl of 32% acetonitrile in 0.1% trifluoroacetic acid (TFA) and the αMSH- and β-endorphin-sized molecules isolated by high performance liquid chromatography (HPLC) gel filtration using a Bio-Sil TSK-400 column in series with a Bio-Sil TSK-125 column (Bio-Rad); the solvent was 32% acetonitrile/0.1% TFA and the flow rate was 1.0 ml/min (14, 15). The αMSH-sized material was then analyzed by reversed-phase (HPLC) directly or treated first with chymotrypsin followed by RP-HPLC analysis. The chymotrypsin digestion procedure and analysis has been previously described (8, 10). The β-endorphin-sized molecules were submitted to immunoprecipitation with affinity purified antibody Melinda and further analysis by SDS-PAGE using borate tube gels as previously described (11, 12).

Results

Before double-label pulse-chase experiments were performed the labeled αMSH-sized molecules from incubations with (^{3}H)acetate were analyzed to insure that the acetate was not incorporated into the peptide backbone. When cells were incubated for 41h in (^{3}H)acetate-containing medium and the αMSH-sized molecules analyzed by RP-HPLC, the only labeled forms of αMSH were mono- and diacetylated, as expected (Fig. 1). The absence of radioactivity at the elution position of desacetyl-αMSH was preliminary evidence that the acetate was not incorporated into amino acids. Upon analysis of αMSH-sized molecules in a parallel (^{3}H)tryptophan-labeled

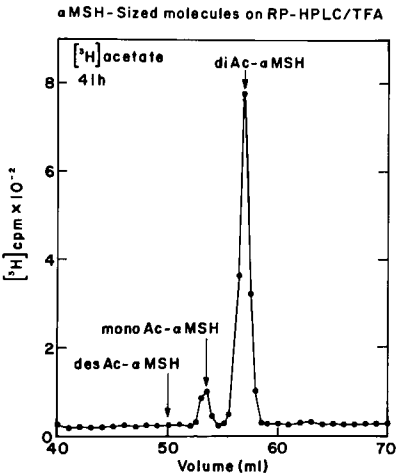

Fig. 1. RP-HPLC analysis of (^{3}H)acetate-labeled αMSH-sized molecules. Intermediate pituitary cells, prepared from two male rats (250 g), were incubated for 41h in medium containing (^{3}H)acetate and analyzed as described in Methods. The HPLC column was a Waters C_{18} μBondapak (4mm x 30cm) and elution was performed using the TFA/acetonitrile solvent system previously described (5,8). Fractions (1 and 0.5 ml) were aliquot counted; the data shown represent the contents of 1/2 the culture.

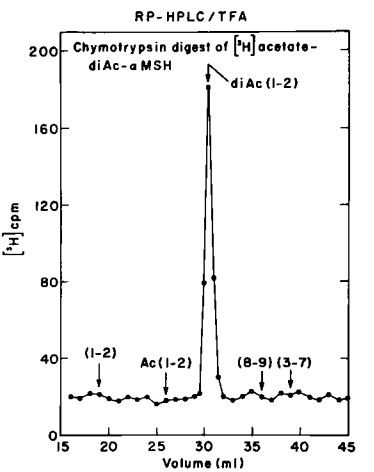

Fig. 2. RP-HPLC analysis of a chymotryptic digest of (^{3}H)acetate-labeled diacetyl-αMSH. One-fourth of the diacetyl-αMSH from the analysis shown in Figure 1 was digested with chymotrypsin and analyzed by RP-HPLC using the TFA/acetonitrile solvent system as previously described (5, 8). A chymotryptic digest of des-, mono-, and diacetyl-αMSH was included in the analysis to provide internal markers for des-, mono-, and diacetyl-ACTH(1-2), ACTH(3-7), and ACTH(8-9). ACTH(10-13)NH_2 elutes at approximately 10 ml (not shown). The elution positions of these peptides were determined by following absorbance at 210 nm.

culture, there were three peaks of radioactivity that correlated with the internal markers for des-, mono-, and diacetyl-αMSH (not shown).

The (^{3}H)acetate-labeled diacetyl-αMSH was characterized further by chymotryptic peptide analysis using RP-HPLC (Fig. 2). The only radioactivity co-migrated with the internal standard for diacetyl-ACTH(1-2), the NH_2-terminal chymotryptic fragment of diacetyl-αMSH. This provided further evidence for the exclusive incorporation of label into the NH_2-terminal acetyl groups.

To verify further the identity of the diacetyl-ACTH(1-2), base treatment was employed to selectively remove the O-acetyl group; this has been previously used in the analysis of αMSH structure (5, 6, 8). There was a nearly quantitative conversion of diacetyl-ACTH(1-2) to mono-acetyl-ACTH(1-2) after treatment with base (Fig. 3). Also, there was a loss of about 50% of the radioactivity from the diacetyl-ACTH(1-2) after base treatment (compare Figures 1 and 2); this suggested that the radioactivity associated with diacetyl-ACTH(1-2) was about equally distributed between the α-N- and O-acetyl groups.

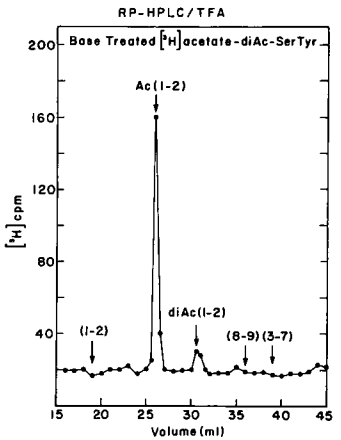

Fig. 3. RP-HPLC analysis of (^{3}H)acetate-labeled diacetyl-ACTH(1-2) after base treatment. Labeled diacetyl-ACTH(1-2) was prepared from one-fourth of the extract described in Figure 1 and treated with 100 mM NaOH for 60 min at 37°. The sample was then diluted with 0.1% TFA and analyzed by RP-HPLC.

The β-endorphin-sized molecules from the (^{3}H)acetate incubation were immunoprecipitated and analyzed by SDS-PAGE (Fig. 4). A single peak of radioactivity migrated to the position for β-endorphin; under these conditions, SDS-PAGE does not distinguish between the various NH_2-terminally acetylated and COOH-terminally shortened forms of β-endorphin (16, 17). Since pro-ACTH/endorphin and βLPH, which are located in slices 10 and 22, respectively, did not contain label it was apparent that the acetate was not being incorporated into the amino acid backbone of any peptides derived from pro-ACTH/endorphin.

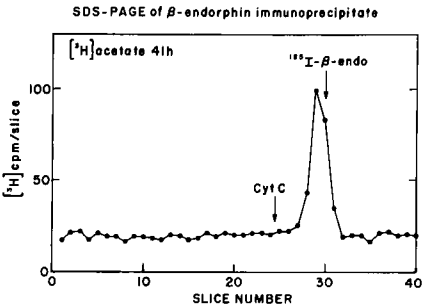

Fig. 4. SDS-PAGE analysis of (^{3}H)acetate-labeled β-endorphin-related peptides. The β-endorphin-related material was purified from cells incubated for 41h in (^{3}H)acetate, as described in Methods. The migration position of (^{125}I)labeled β-endorphin, analyzed on a parallel gel, is shown as well as the location of dansylated cytochrome c, used as an internal marker.

When cells were incubated with both (^{3}H)acetate and (^{35}S)methionine and the αMSH-sized molecules analyzed by RP-HPLC, there was (^{35}S)radioactivity associated with all three forms of αMSH and (^{3}H)radioactivity associated with only the mono- and diacetylated forms (Fig. 5). The ^{35}S/^{3}H ratios for mono- and diacetyl-αMSH were 7.8 and 4.2, respectively. This is consistent with the diacetylated form having about twice the (^{3}H)-acetyl radioactivity per molecule as the monoacetylated form.

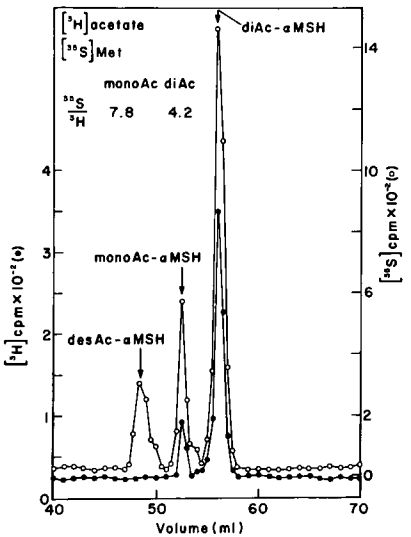

Fig. 5. RP-HPLC analysis of the αMSH-sized molecules from cultures labeled with (^{3}H)acetate and (^{35}S)methionine. The analysis was performed as described in Figure 1 and Methods. Fractions (1 ml and 0.5 ml) were counted for the presence of both (^{3}H)- and (^{35}S) radioactivity on a Tracor Delta 300 LS counter.

The pulse-chase scheme involved labeling three cultures with both (^{3}H)acetate and (^{35}S)methionine for 48h; this incubation was followed by chase incubations of 3, 24, and 48h. As previously shown, a 3h chase incubation is sufficient to allow for the complete conversion of labeled pro-ACTH/endorphin to labeled αMSH- and β-endorphin-sized peptides (11, 12). Diacetyl-αMSH, purified as described in Methods,

was then digested with chymotrypsin and analyzed by RP-HPLC (Fig. 6). The (^{35}S)radioactivity was associated with the chymotryptic peptide, ACTH(3-7), as expected since the methionine is at position 4 of αMSH, while the (^{3}H)radioactivity co-migrated with diacetyl-ACTH(1-2). This clean separation of (^{3}H)- and (^{35}S)radioactivity allowed the simplest, most reliable quantitation of the ^{35}S/^{3}H ratios. The ^{35}S/^{3}H ratios for the 3h, 24h (not shown), and 48h chase incubations did not change significantly. This was also observed when the β-endorphin from these cultures was analyzed by SDS-PAGE (Fig. 7). Here, the SDS-PAGE analysis did not separate the (^{3}H)- and (^{35}S)radioactivity but was employed to insure the proper quantitation of labeled β-endorphin-sized molecules by separation from any non-specifically precipitated material. The ^{35}S/^{3}H ratios for β-endorphin were roughly twice those for αMSH, which is consistent with one acetyl group and one methionine for β-endorphin and two acetyl groups and one methionine for diacetyl-αMSH. It has been shown that a 48h incubation with a labeled amino acid is sufficient to produce mostly acetylated labeled forms of β-endorphin (16, 17).

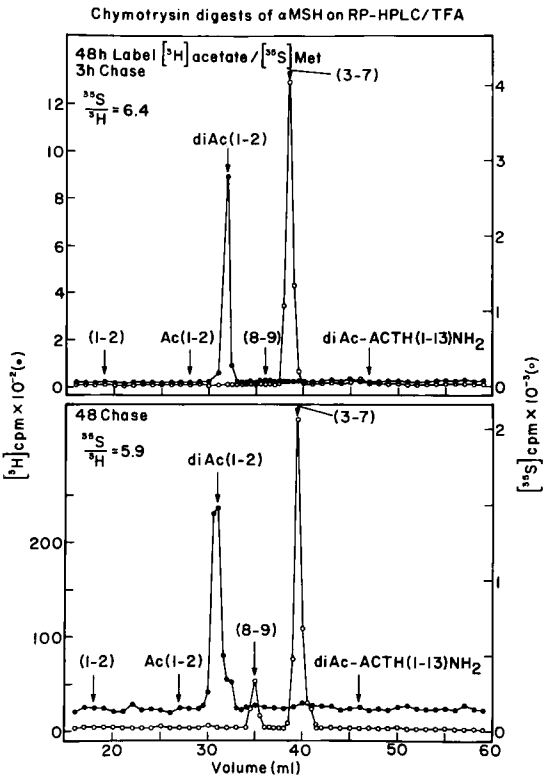

Fig. 6. RP-HPLC analysis of chymotryptic peptides of double-labeled αMSH-sized molecules. Incubations and analyses were performed as described in the text and in Figure 2.

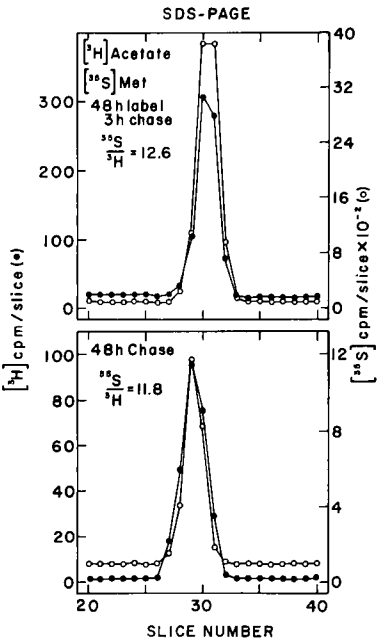

Fig. 7. SDS-PAGE analysis of double-labeled, immunoprecipitated β-endorphin-sized molecules. Incubations and analyses were performed as described in the text and in Figures 1 and 4.

Discussion

This double-label technique has been used previously to demonstrate the stability of the NH_2-terminal acetyl groups on proteins that are co-translationally acetylated as well as the liability of internal acetyl groups on histones (18, 19). Labeling of rat intermediate pituitary cells with (^{3}H) acetate has also been used to observe the secretion of the acetylated forms of β-endorphin (20). In that study pro-ACTH/endorphin and β-endorphin were labeled equally well; the authors attributed this to the incorporation of (^{3}H)acetate into the N-acetyl-glucosamine residues of the carbohydrate groups of pro-ACTH/endorphin. It is apparent from the study presented here that both αMSH and β-endorphin in rat intermediatepituitary cells can be effectively labeled with (^{3}H)acetate such that the radioactivity is associated exclusively with the acetyl groups of these peptides. There was no radioactivity associated with pro-ACTH/endorphin.

Since the $^{35}S/^3H$ ratios of the double labeled αMSH and β-endorphin did not change after 48h of chase incubation, it is clear that rat intermediate pituitary cells

do not deacetylate αMSH and β-endorphin during storage in secretory granules. Undoubtedly, there are deacetylation enzymes in the intermediate pituitary cells that cannot gain access to αMSH and β-endorphin since these peptides are sequestered within secretory granules. Although it is an unsettled issue, the majority of the evidence suggests that αMSH and β-endorphins are acetylated to a lesser extent in brain than in the intermediate pituitary (21, 22). It will be of interest to determine whether acetylation and deacetylation of β-endorphin occur in the brain such as to regulate the opiate-like activity of this peptide.

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AMIDE FORMING ENZYMES IN THYROID AND PITUITARY

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Introduction

The α-amide group is a characteristic structural feature of many peptide hormones, important in most cases for biological activity and conferring a degree of stability against degradation *in vivo*. Recently an enzyme that can catalyse the formation of the α-amide group was shown to be present in porcine and bovine pituitary[1] and its mechanism of action was found to involve dehydrogenation of C-terminal glycine in the biosynthetic precursor of the peptide amide. It was observed that the pituitary enzyme was able to convert substrates based on the C-terminal sequence of α-melanotropin which is elaborated in the pituitary and it was also able to convert substrates based on the C-terminal sequence of gastrin which is elaborated in the gastric antrum. In addition it has been reported that a preparation of the pituitary enzyme can catalyse the conversion of Pyr-His-Pro-Gly to Pyr-His-Pro-$CONH_2$[3]. We present here the results of experiments which demonstrate that an amidating enzyme in rat thyroid has similar specificity to the amidating enzyme in porcine pituitary, suggesting that the same enzyme may be present in the two tissues. The evidence favours a view that the amidating enzyme may be common to a variety of tissues and have similar specificity with a range of substrates.

Materials and Methods

The tripeptide substrates d-Tyr-Val-Gly, d-Tyr-Phe-Gly and d-Tyr-Pro-Gly were prepared by solid phase synthesis and purified by gel filtration on a column (70 x 1cm) of Sephadex G10 in 50% acetic acid, followed by ion exchange chromatography on a column (70 x 1cm) of SP-Sephadex C25 eluted in 0.2M sodium chloride, 20mM sodium phosphate at pH 4.5. Amidating enzyme was extracted from dissected regions of porcine pituitary (1.2g) or from rat thyroid (0.3g) by homogenization in neutral saline at pH 7.0 and by freezing and thawing to disrupt the intracellular particles, as described previously[1]. Further purification of the solubilized enzyme was obtained by salt precipitation in saturated sodium sulphate at pH 5.0. Incubation of the tripeptide substrates was carried out in the presence of 10^{-4}M cupric sulphate with ^{125}I-labelled peptide (10 nmole was labelled with 1mC of iodine and 2 x 10^4cpm were used in each incubation). The amidating enzyme from pituitary or thyroid was stored at -70^{0}C and aliquots (1/40 of the pituitary extract or 1/10 of the thyroid extract) were thawed and added to the peptide substrates in 100µl of 0.2M sodium chloride, 0.02M sodium phosphate at pH 7.0. At intervals, portions of the reaction mixtures were acidified by addition of N-HCl and the formation of dipeptide amide was determined by chromatography on SP-Sephadex C25 (10 x 1cm) in 0.2M sodium chloride 0.05M sodium phosphate at pH 5.5.

Results and Discussion

The rates of conversion of d-Tyr-Val-Gly, d-Tyr-Phe-Gly and d-Tyr-Pro-Gly to the corresponding dipeptide amides by enzyme extracted from porcine anterior pituitary or porcine pars intermedia are indicated in Figure 1a. There was a marked difference in the reaction rates with the different

substrates: the most rapid conversions took place with the phenylalanine containing tripeptide and the slowest reaction with the proline containing substrate. In Figure 1b the rates of conversion of the tripeptides to the respective dipeptide amides catalysed by thyroid enzyme are illustrated. It was notable that the same relative reaction rates were obtained, whether the enzyme was of pituitary or of thyroid origin.

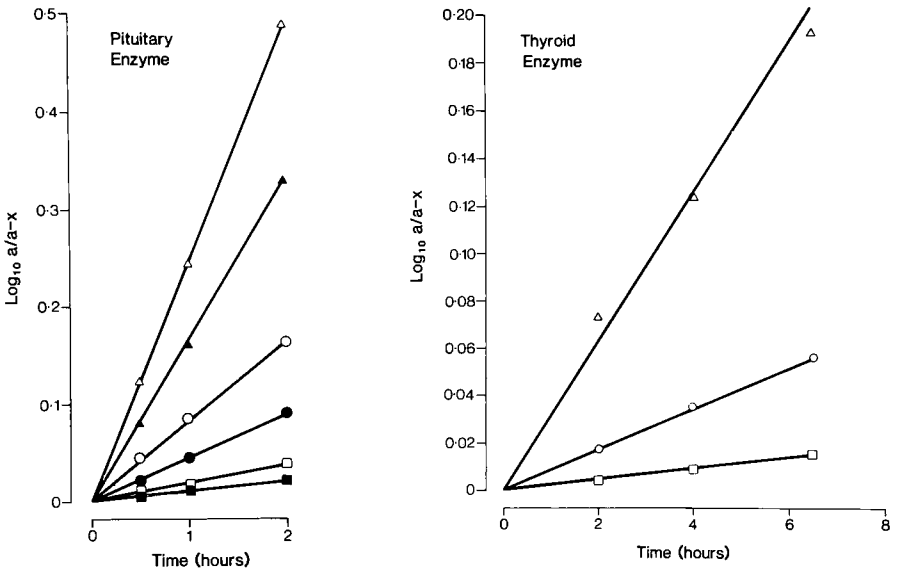

Fig. 1. Relative reaction rates in the conversion of d-Tyr Phe-Gly (Δ-Δ), d-Tyr-Val-Gly (o-o) and d-Tyr-Pro-Gly (□-□) to the corresponding dipeptide amides catalysed by an amidating enzyme extracted from (a) porcine anterior pituitary (open symbols) or porcine pars intermedia (closed symbols), or (b) from rat thyroid.

If the amidating enzyme in the thyroid gland were specific for the thyroid hormone calcitonin, which terminates in proline amide, the thyroid enzyme would be expected to catalyse the amidation of a proline containing substrate more readily than it would a valine containing substrate since the

latter is a model for the terminal amide of a pituitary peptide, α-melanotropin. In contrast, if the pituitary enzyme were specific for valine (or glycine) containing substrates, reflecting the terminal sequence of α-MSH or oxytocin and vasopressin, then it might prove ineffective for catalysing the amidation of d-Tyr-Pro-Gly. The present experiments with d-Tyr-Phe-Gly, d-Tyr-Val-Gly and d-Tyr-Pro-Gly as substrates for amidation show that the amidating enzyme exhibits relative reaction rates that are unrelated to the tissue of origin.

Parallel experiments were performed, under the same conditions, with enzyme extracted from porcine anterior pituitary and porcine pars intermedia (Figure 1a). Surprisingly, the concentration of amidating activity in the anterior pituitary was found to be greater than the concentration of amidating enzyme in the intermediate tissue. Since no anterior pituitary hormone is known that terminates in an α-amide group, our results suggest that the amidating enzyme in this tissue does not normally participate in prohormone processing, either because of sequestration or because it is held under inhibition. The possibility should also be considered that the anterior pituitary may contain one or more hormone amides that have not previously been identified.

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SPECIFIC LABELLING OF PRO-OPIOMELANOCORTIN (POMC)-RELATED PEPTIDES WITH ^{32}P USING CASEIN KINASE.

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A significant proportion of the adrenocorticotropin (ACTH) which is formed in the anterior lobe of the rat and human pituitary is phosphorylated (1,2). Phosphorylated forms of corticotropin-like intermediary lobe peptide (CLIP, $ACTH_{18-39}$) have also been isolated from the rat intermediary pituitary (3). The site of phosphorylation was identified as serine residue 31. Phosphorylation appears to have no effect upon corticotropic activity. In both rat and human ACTH, the phosphorylation site is part of the tripeptide sequence Ser^{31}-Ala^{32}-Glu^{33}. Biosynthetic studies with rat pituitaries have shown that the N-terminal peptides derived from POMC are also phosphorylated, although to a lesser extent than ACTH and CLIP (4). The same tripeptide sequence is present in this portion of the precursor and is thought to be the site of phosphorylation. This sequence conforms to the recognition site for casein kinases: Ser/Thr-X-acidic residue (5). We have prepared extracts of bovine mammary gland and rat pituitary gland containing casein kinases which are able to phosphorylate POMC-related peptides in vitro. The labelling procedure is specific, and by purifying the products with reversed-phase HPLC, active natural ligands having high specific activity can be obtained.

Casein kinase, a cAMP-independent enzyme,is abundant in the lactating mammary gland, where it plays a physiological role in phosphorylating milk proteins (6). An active preparation of mainly casein kinase type I from 100 g of bovine mammary gland was prepared by differential centrifugation and using Triton X-100 to solubilize the membrane-bound enzyme. It was able to phosphorylate rat CLIP (substrate concentration 100 µg/ml) at a maximum rate of 64 pmol/min/mg protein. Using a similar extraction procedure, 500 mg of rat pituitary tissue yielded casein kinase having a similar activity, its maximum phosphorylation rate being 98 pmol/min/mg protein for 10 µg/ml of CLIP (see Table I).

An investigation of the properties of the two preparations suggests that similar enzymes are present in the rat pituitary and bovine mammary gland: their affinity for ATP is similar (8mM) and they are both unable to use GTP as phosphate donor. The Km apparent for rat CLIP was 5.2µM with the mammary kinase and similar for the pituitary enzyme. The mammary kinase was inhibited only by high concentrations of heparin, suggesting that the preparation contains mainly type I casein kinase, with an absolute specificity for serine. This was used for subsequent *in vitro* phosphorylation studies because of its greater concentration.

We found that the mammary kinase is able to phosphorylate synthetic human $ACTH_{1-39}$ *in vitro*, at a rate of the same order as that for CLIP. There is another potential phosphorylation site (Ser^3-Met^4-Glu^5) which appears to be recognized by the enzyme, since ^{32}P is incorporated into $ACTH_{1-24}$ although less efficiently. However, very little phosphorylation of $ACTH_{1-24}$ was observed with the pituitary kinase, and phosphorylation of Ser_3 does not occur *in vivo*. The 49 residue peptide at the N-terminal of POMC, isolated from the rat NIL, can also be phosphorylated by casein kinase, but its

affinity for this substrate is reduced, as expected from the biosynthetic studies (4).

Table I. Properties of Casein Kinase Extracted from the Rat Pituitary and Bovine Mammary Gland.

	Pituitary Kinase	Mammary Kinase
starting material	500 mg (65 whole rat pituitaries)	100 g of lactating bovine mammary gland
Vmax (CLIP)*	98 pmol/min/mg protein	64 pmol/min/mg protein
Km app (CLIP)	4.8 μM	5.2 μM
Vmax (α-casein)**	N.D.	34 pmol/min/mg protein
Km app (α-casein)	N.D.	21 μM
Km app (ATP)	8 mM	8 mM
phosphate donor	only ATP	only ATP
Vmax ($ACTH_{1-39}$)	26 pm/min/mg	20 pm/min/mg
Vmax ($ACTH_{1-24}$)	0.2 pm/m/mg	3.2 pm/min/mg
effect of less than 1 μg/ml heparin	N.D.	no inhibition

N.D: not determined

*: casein kinase activity was determined at pH 7.6 in 100 μl 0.1M Tris-HCl buffer containing 10 mM $MgCl_2$, 0.5-600 μg/ml peptide, 20 μl enzyme preparation and 1 mM [$\gamma^{32}P$] ATP(10-50 Ci/mmol). Incubations were for 20 minutes at 30°C, and were stopped by acidifying the mixture with 1% TFA (100 μl).

**: α-casein was dephosphorylated by incubation with alkaline phosphatase (the extent of dephosphorylation was estimated by amino acid analysis).

Using reversed-phase HPLC to assess the products of the phosphorylation reaction, we showed that the labelling procedure is non-destructive. Non-phosphorylated peptides can be completely resolved from the labelled forms. Thus high specific activities can be achieved.

We have shown that ^{32}P-labelled CLIP is stable during incubations at 37°C with dispersed rat adrenal cells: less than 10% of the label is removed in two hours. Phosphorylated and non-phosphorylated ACTH are equipotent in stimulating steroidogenesis using a dispersed rat adrenal cell assay (2). The ^{32}P-labelled peptides are thus fully-active natural ligands. The method is attractive for the preparation of tracers for receptor binding and in vivo distribution studies. Furthermore, these experiments suggest that the enzyme responsible for phosphorylating POMC-derived peptides in the rat and human pituitary is a Type I casein kinase.

This work was supported by the Medical Research Council of Canada and the Fonds de la Recherche en Santé du Québec.

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AN INVESTIGATION OF THE HETEROGENEITY OF THE ACIDIC JOINING PEPTIDE FROM THE MOUSE PITUITARY.

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The ACTH/β-LPH precursor or pro-opiomelanocortin (POMC) is known to be synthesized within the pituitary in multiple forms. This heterogeneity has been observed both in terms of molecular weight and charge and has been found to be due, in part, to such post-translational modifications as glycosylation and phosphorylation (1,2). It has been speculated that primary sequence heterogeneity may also account for some of the multiple forms. An inspection of the cDNA-derived amino acid sequences for human, rat, mouse and bovine POMC indicates that the least conserved regions are within the γ-LPH and acidic joining peptide (AJP) regions. AJP lies between the 16K or N-terminal fragment and ACTH within POMC and has a large number of acidic residues which cause it to elute early from reversed-phase HPLC columns. Recently Uhler and Herbert have published the cDNA sequence for mouse POMC (3). The sequence for mouse AJP (including the flanking dibasic residues) was predicted as -ArgArgAlaGluGluGluAlaValTrpGlyAspGlySerProGluProSerProArgGluGlyLysArg-. The presence of C-terminal -GlyLysArg- sequence strongly suggests that this peptide actually terminates with a glutamate amide residue. Mouse pituitary tissue was extracted and subjected to reversed-phase HPLC as outlined in the legend to Figure 1.

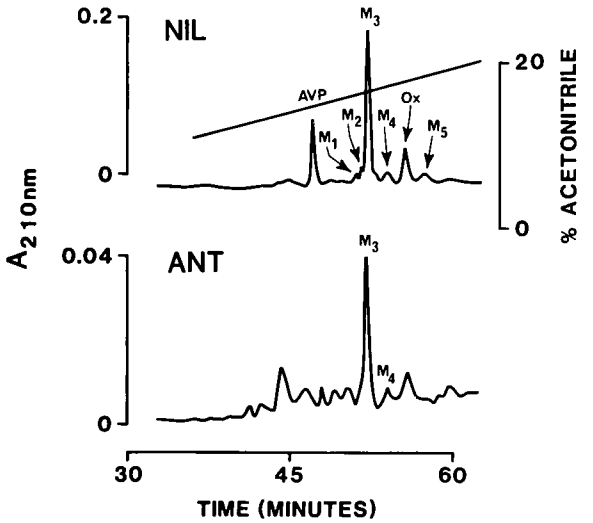

Figure 1. The pituitaries from 50 mice were dissected into anterior (ANT) and neurointermediate (NIL) lobes and extracted using octadecylsilyl-silica cartridges (4). The extracts were subjected to reversed-phase HPLC using a Waters C_{18} μBondapak column as described previously using solvents containing 0.1% trifluoroacetic acid (4). Multiple forms of mouse AJP (M_1 to M_5) were detected in column eluates by observing their strong u.v. absorbance at 280 nm (not shown). Each form was purified using reversed-phase HPLC solvents containing 0.13% heptafluorobutyric acid or 0.01M triethylamine acetate (pH 5.5) as ionic modifiers. These solvents are used to exploit the basic and acidic character of peptides during a purification scheme (4,5). AVP: arginine vasopressin; Ox:oxytocin.

Using the predictive retention coefficients of Browne et al. (6), it was calculated that mouse $AJP_{1-18}NH_2$ should elute just before arginine vasopressin. In fact, various forms of mouse AJP were found to elute slightly later. Four forms of mouse AJP (termed M_1 to M_4) were found in an extract of anterior lobe tissue. The same four forms were found in the NIL extract. In addition an extra form, termed M_5, was found. M_1 to M_5 were subjected to amino acid analysis

following acid hydrolysis. For this purpose M_1 to M_4 from the anterior lobe were combined with the corresponding peaks from the NIL in order to maximize yield.

Table I. Mouse Acidic Joining Peptide: Amino Acid Compositions.

	Asp	Ser	Glu	Pro	Gly	Ala	Val	Arg	Trp	(pmoles/lobe)*	
$mPOMC_{77-95}$	1	2	5	3	3	2	1	1	1	ANT	NIL
M_1	1.2	2.2	5.8	2.9	1.8	1.9	0.9	2.2	ND	1.5	5.4
M_2	1.0	1.7	5.2	2.6	1.5	1.5	1.1	1.0	ND	2.3	6.5
M_3	0.9	2.1	5.2	2.8	1.9	1.9	0.8	1.0	ND	18.0	70.0
M_4	1.0	2.4	5.0	2.4	1.9	1.8	0.8	1.0	ND	3.0	7.5
M_5	0.9	1.9	5.1	3.0	1.7	1.8	0.9	1.0	ND	0	8.0

ND - not determined.
* -estimated from peak heights of purified peptides.

Table 1 shows the amino acid compositions of M_1 to M_5 and their relative yields in the two lobes of the mouse pituitary. All five forms contained only 2 glycine residues suggesting that they were all amidated peptides. In both lobes M_3 predominated and it was assumed that this corresponded to authentic mouse $AJP_{1-18}NH_2$. Interestingly, the elution position of M_5 relative to M_3 is consistant with M_5 being an acetylated form of M_3(6). It is possible that mouse AJP constitutes a weak substrate for the acetyl-transferase that brings about acetylation of both α-MSH and β-endorphin in the intermediate lobe of the pituitary (7). This conclusion is supported by the finding that M_5 is not found in the anterior lobe where it is known that acetylation of these peptides does not take place. M_1 appears to contain an extra arginine residue. The extra arginine is probably located at the amino-terminus of this peptide. M_1 may constitute a short-lived intermediate in the biosynthesis of mouse AJP.

Conclusions

Mouse AJP exists in multiple forms in both anterior and neurointermediate lobes of the pituitary. The difference between these forms can probably be accounted for in terms of post-translational modifications. This study has revealed no positive evidence for multiple gene products. Mouse AJP may be a weak substrate for the actyl-transferase enzyme of the intermediate pituitary.

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STUDY OF PROTEOLYTIC PROCESSING OF NEUROPHYSIN/NEUROHYPOPHYSEAL HORMONE BIOSYNTHETIC PRECURSORS USING A SYNTHETIC PRECURSOR FRAGMENT

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Introduction

Recent recombinant DNA analyses have defined the amino acid sequences of bovine hypothalamic precursors that give rise to neurophysins (NP) and their associated neuropeptide hormones, oxytocin (OT) and arginine vasopressin (AVP).[1,2] In both NPII/AVP and NPI/OT precursors (Fig. 1), the amino terminal hormone and NP are separated by a unique tripeptide, Gly-Lys-Arg, which comprises two typical signals for post-translational processing, X-Gly for formation of C-terminal amides[3] and a dibasic residue sequence for endoproteolytic processing.[4] Based on the disulfide stability of mature neurophysin as well as other considerations, it is likely that the above processing events occur after precursor folding.[5] However, at present, intact precursor is not available in significant amounts. In order to investigate post-translational processing mechanisms of the common precursors, we have studied processing reactions using a synthetic dodecapeptide, Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-Gly-Lys-Arg, containing the oxytocin sequence at positions 1-9 and the OT-NP intervening tripeptide at C-terminal positions 10-12.

Results and Discussion

The dodecapeptide was synthesized on oxymethyl-phenyl-

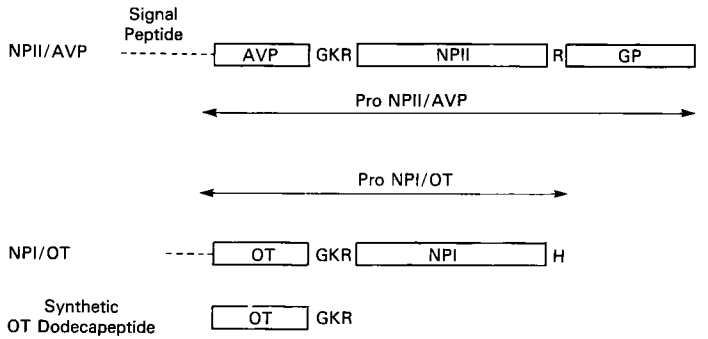

Fig. 1. Schematic diagram of the structures of bovine neurophysin-neurohypophyseal hormone precursors,[1,2] including the in vivo-occurring "pro" form, and the synthetic oxytocin dodecapeptide. Sequences in the N-terminal signal peptide regions contained in pre-pro-NP/H species also are denoted.

acetamidomethyl (PAM) resin by the conventional solid phase method. The protected peptide was deblocked and cleaved from the resin by HF, and the single intramolecular disulfide was formed by oxidation with potassium ferricyanide. Purification of the disulfide containing peptide was achieved by Sephadex G-15 gel permeation chromatography and reverse phase high performance liquid chromatography (HPLC) using an analytical Zorbax-ODS (C-18, Dupont) column. Oxytocin is known to produce a non-covalent complex with NP as a storage form in neurosecretory granules upon completion of precursor processing. The binding affinity of the synthetic peptide and neurophysin was examined by the affinity chromatography method. The dodecapeptide binds tightly to [bovine NP-II]-Sepharose at pH 5.7 and elutes with acid, as does oxytocin. This result is consistent with the hypothesis that dibasic protease cleavage of the folded precursor or precursor intermediate at the arginyl bond amino to NP residue 1 yields a non-covalent complex of hormone- and NP-containing fragments which then is processed further to produce mature hormone-NP complex.

Based upon the view that post-translational processing of

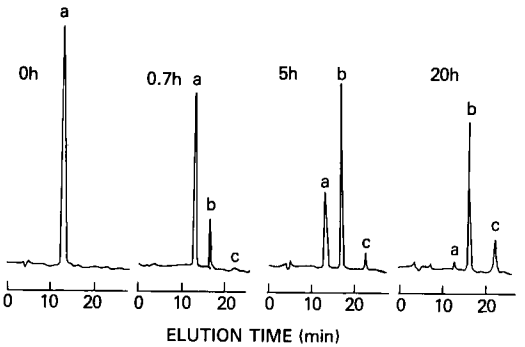

Fig. 2. HPLC profiles of conversion of synthetic oxytocin dodecapeptide by neurosecretory granule lysate. (a) starting dodecapeptide substrate; (b) [des-Arg]-peptide (substrate residues 1-11); (c) [des-Lys-Arg]-peptide (substrate residues 1-10). Column: Zorbax CN (Dupont, 0.46 x 25 cm). Mobile phase: 0.25 M triethylammonium phosphate-acetonitrile linear gradient (5-20% acetonitrile). Elutions were monitored by absorbance at 215 nm.

NP/hormone common precursors takes place in neurosecretory granules during transport from hypothalamus to posterior pituitary,[6] we assayed the processing activity in these granules using oxytocin dodecapeptide as substrate. Neurosecretory granule fraction was prepared by differential centrifugation of bovine posterior pituitary homogenate. The isolated granules were lysed by sonication and subjected to enzyme assay. Incubation of the dodecapeptide with the lysate gave rise to specifically degraded peptides through serial cleavage at the C-terminal arginine and then lysine residue, as judged by amino acid analyses of HPLC fractions (Fig. 2). Release of the arginine residue appeared to be faster than that of lysine. Although prolonged incubation leads to further release of C-terminal amino acid residues, the rates are slow relative to that for the arginine. This result indicates the predominance of carboxypeptidase (CPase) B-like activity in the granule fraction. The granule CPase B activity was maximal at about pH 5.5, in contrast to a pH optimum of 9.0 for pancreatic

CPase B. This can be related to the observation that the internal pH of neurosecretory granules is about 5.8. The granule CPase B was partially purified by gel chromatography on Sephacryl S-300. The major CPase activity (detected using the HPLC assay (Fig. 2)) showed high specificity for the basic residues of the dodecapeptide substrate and was separated from an earlier-eluting non-specific CPase. Although contamination of lysosomal enzyme should be considered, the CPase B-like activity observed here is not due to cathepsin B1 or B2 because the activity is inhibited by both EDTA and p-chloromercuribenzoate.

The data argue that the CPase B-like activity identified here is involved in neuronal processing of NP/hormone precursors. As yet, conversion of the des Lys-Arg product (residues 1-10) to mature OT has not been observed. Since, as described above, the processing likely occurs as part of a noncovalent complex with NP or NP intermediate, it would be helpful to study the effect of complex formation with NP on proteolytic processing of the peptide. This work is in progress.

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NATURAL AND SYNTHETIC PHOSPHORYLATION SITES IN hCG BETA SUBUNIT

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Phosphorylation has been recognized to be an essential step in the function of many metabolic and regulatory enzymes, but phosphate has only recently been found in peptide hormones. None has been reported as yet in the gonadotropins.

With the ultimate aim of defining a possible role for hormone phosphorylation in the action of human chorionic gonadotropin (hCG), we have examined the ability of the hormone and its subunits to act as substrates for *in vitro* phosphorylation by cAMP-dependent protein kinase. The peptides were incubated for varying lengths of time with the kinase from skeletal muscle in presence of (^{32}P)-ATP[1]. The sites of uptake were localized by compositional analysis and Edman degradation of peptide fragments isolated by gel filtration and high pressure liquid chromatography.

Although whole native hCG and alpha subunit did not phosphorylate, the 145-residue beta subunit incorporated one mol/mol phosphate in 24 hr, and after reduction and carboxymethylation (RCXM) the uptake increased to nearly 3 mols/mol. The chemical studies showed two regions to be involved, representing two distinct types of site for cAMP-dependent protein kinase-mediated phosphorylation.

The site in native CGβ was identified as Thr_{97} within the sequence --Cys-Arg-Arg-Ser-Thr-Thr-Asp-Cys-- (residues 93-100) typical of the most common of the phosphate-accepting sequences (--Arg-Arg-X-Ser/Thr--)[2]. In RCXM-hCGβ an additional site was exposed: Ser_{66} within the sequence --Arg-Asp-Val-Arg-Phe--Glu-Ser-Ile-- (residues 60-67). This is a new example of the general sequence --Arg-X-X-Arg-X-X-Ser-- originally found to be an avid acceptor site in rabbit skeletal muscle phosphorylase kinase[3].

There was also evidence for uptake at Ser_{96} in the linearized subunit, suggesting the influence of conformation on sites such as this, involving adjacent susceptible residues. We therefore prepared synthetic peptides comprising the sequence between residues 93 and 101 of hCGβ:

$$H_2N\text{-}Cys_{93}\text{-}Arg\text{-}Arg\text{-}Ser\text{-}Thr\text{-}Thr\text{-}Asp\text{-}Cys_{100}\text{-}Gly\text{-}Tyr\text{-}OH$$

Synthesis was done by the solid-phase procedure on chloromethylated 1% divinylbenzene-styrene resin[4,5]. To permit radiolabeling, a tyrosine was provided at the C-terminus. Initially, the S-ethylthio protecting group[6] was used at Cys_{100}, but proved extremely resistant to subsequent deprotection even after prolonged exposure of the crude peptide to reducing agents such as sodium tetrathionate. Amino acid analysis after total enzymatic digestion showed only one cysteine, and the hydrolysis product of S-ethylthiocysteine (coeluting with alanine) was present after acid hydrolysis.

This peptide was purified for use in experiments requiring the open (linearized) fragment. By carboxymethylcellulose (CMC) chromatography, it eluted early (1.0-1.5 mmho) in a gradient of .01-.3M, pH 6 ammonium acetate buffer, and appeared largely in monomeric form on Biogel P-4 gel filtration.

A second synthesis was carried out using the o-methylbenzyl protecting group at both cysteines, providing the monomeric disulfide form after HF cleavage. The yield of full-length peptide after CMC and P-4 chromatography was quite low. The

major product was a peptide terminating prematurely at threonine, apparently a consequence of steric hindrance by successive O-benzyl protecting groups (used at Ser, Thr and Asp) as well as slow cleavage of BOC-threonine. Some improvement in yield was obtained by use of unprotected threonine and extended cleavage times at the successive BOC-Thr steps.

The two forms of (93--101) peptide were evaluated for phosphate incorporation as done with native hCGβ (Figure 1). Both incorporated one mole of phosphate, but one form labeled predominantly at serine, the other at threonine. By counting the radioactivity at successive Edman cycles, the location of phosphate could be assigned to positions corresponding to

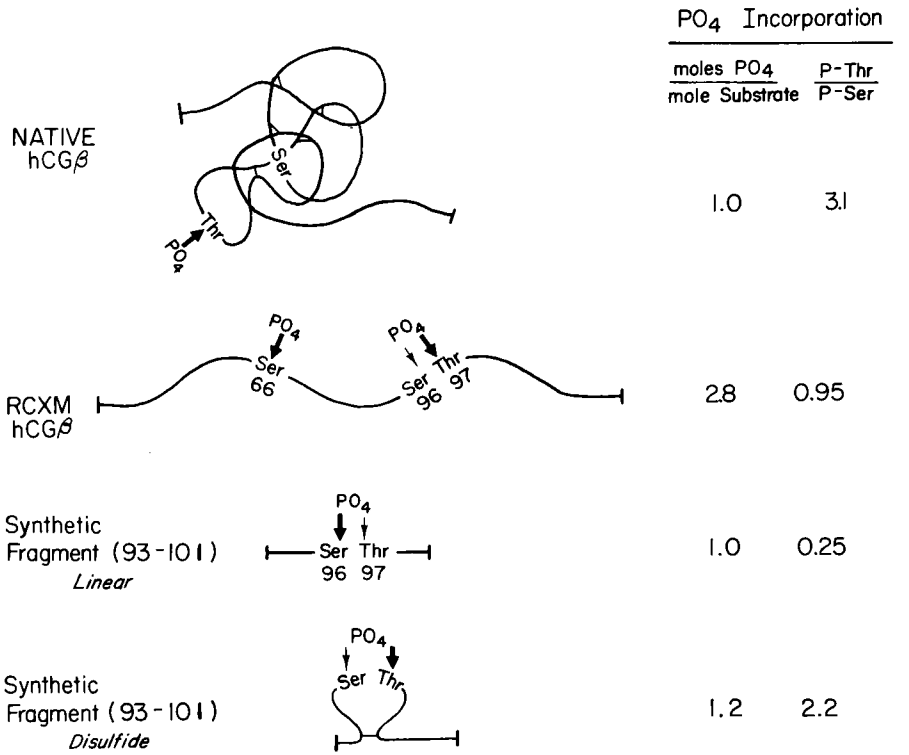

Fig. 1: Summary of in vitro phosphorylation sites in hCG beta subunit. Major sites are denoted by heavy arrows and those with lesser uptake by light arrows.

Ser_{96} in the linear form and Thr_{97} in the "native" closed-disulfide form.

The combined results show the phosphorylation reaction to be a useful conformational probe for hCGβ. While Ser_{66} is an avid acceptor site in the linear sequence, it appears to be "buried" in the native subunit (Figure 1). The (93--100) region is exposed, but the precise site of uptake (Thr_{97} or Ser_{96}) is markedly influenced by the tertiary structure around the site. In whole native hCG, this region is masked, at least in part, by the associated alpha subunit.

There is evidence that the sequence within the (93--100) disulfide loop may be important in determining hormonal potency and specificity[7]. The phosphorylation reaction provides a means for specific modification within this "determinant loop" region for further analysis of structure-function relations in the gonadotropins.

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ASPECTS OF CONFORMATIONAL RESTRICTION IN BIOLOGICALLY ACTIVE PEPTIDES

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Introduction

Whereas several semi-rigid cyclic peptides occur in nature, the majority of small peptide hormones and neurotransmitters are linear and quite flexible. There is increasing evidence that many linear peptides in solution exist in a conformational equilibrium and, furthermore, the notion of conformational heterogeneity and flexibility is quite compatible with the lack of specificity observed with some of these peptides in cases where different subclasses of receptors exist. Conformational restriction represents the most promising tool in efforts to reduce the number of conformational possibilities and not only can provide better insight into the receptor-bound conformation but also may result in enhanced binding selectivity in a situation of receptor heterogeneity. Structural modifications leading to conformational restrictions in peptides have recently been reviewed[1]. Local conformational constraints can be imposed either on the peptide backbone (e.g. through C^{α}- or N^{α}-methylation) or side-chains (e.g. through introduction of α,β-unsaturated amino acids). More drastic conformational restrictions are obtained by synthesis of analogs containing cyclic structures. In particular, side-chain to backbone or side-chain to side-chain cyclizations have produced promising results in recent years. The design of such cyclic analogs demands careful consideration of struc-

ture-activity data and, if possible, of conformational features.

The opioid peptides Met- and Leu-enkephalin (H-Tyr-Gly-Gly-Phe-Met(or Leu)-OH) bind strongly to δ-opiate receptors and show somewhat reduced but still considerable affinity for μ-receptors. Since both bio- and binding assays with reasonable selectivity for μ- and δ-receptors are available to date, the enkephalins and their analogs are uniquely suited for studying the effects of conformational restriction on receptor affinity, "efficacy" and receptor selectivity.

Cyclic Enkephalin Analogs

The observation that introduction of a side-chain in position 2 with D-configuration and replacement of the C-terminal free carboxyl group by N-alkylated carboxamide functions is well tolerated in enkephalin (cf. ref. 2) led to the design of a first type of cyclic analog characterized by substitution of a D-α,ω-diamino acid in position 2 and cyclization of the ω-amino group to the C-terminal carboxyl group (I-IV, Figure 1)[3-5]. Within this series of cyclic homologs subtle variation in conformational restriction is achieved by increasing the number of methylene groups in the side-chain of the 2-position residue from 1 to 4. The rather tight 13- and 14-membered ring structures contained in analogs I and II, respectively, do not permit the formation of various hydrogen-bonded β-bend structures which have been proposed for enkephalin (cf. ref. 6). A particularly attractive feature of this series of cyclic analogs is the possibility to compare the pharmacological profiles of its members with those of the corresponding open-chain analogs (Ia-IVa) which are distinguished from I-IV merely by the opening of a single carbon-nitrogen bond and, therefore, a direct assessment of the effect of the conformational constraint introduced

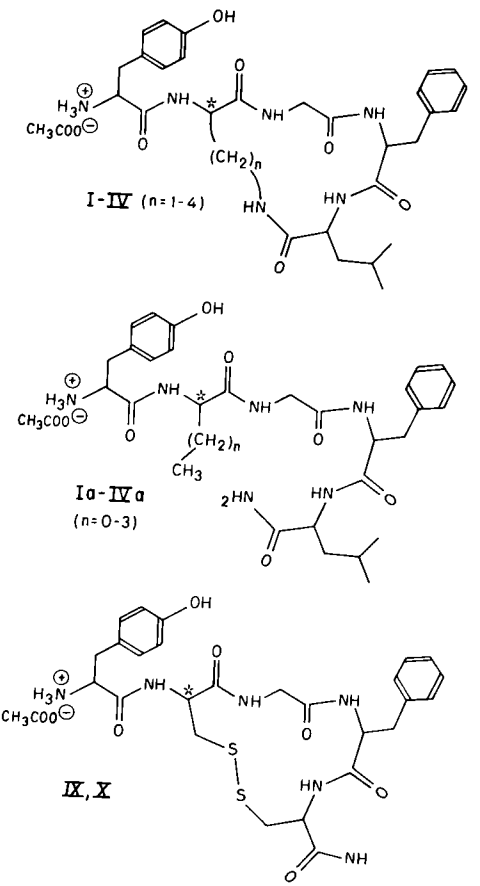

Fig. 1. Structural formulas of cyclic enkephalin analogs I-IV, corresponding open-chain analogs Ia-IVa and cystine-containing analogs IX and X.

through ring closure is possible.

Analogs were tested in bioassays based on inhibition of electrically evoked contractions of the guinea pig ileum (GPI) and the mouse vas deferens (MVD), which are representative for μ- and δ-receptor interactions, respectively. Compared to [Leu^5]enkephalin and the linear correlate IIa, the cyclic prototype II was found to be more potent in the GPI-assay and less potent in the MVD-assay (Table I) and the IC50(MVD)/IC50-(GPI)-ratio of 5.77 resulting for II is indicative of its μ-receptor selectivity. This μ-receptor preference was confirmed through determination of the binding inhibition constants (K_i) by displacement of [^{3}H]naloxone as a μ-receptor selective radio-ligand and [^{3}H][D-Ala^2,D-Leu^5]enkephalin as a δ-receptor selective radio-label from rat brain membrane preparations, which resulted in a K_i^δ/K_i^μ-ratio of II more than ten times higher than that of its corresponding open-chain analog (IIa)[4]. Since this result is mainly due to poor δ-receptor binding of the cyclic analog, it appears that the conformational restriction introduced through ring closure is quite well tolerated at the μ-receptor but less compatible with the topography of the δ-receptor. Furthermore, comparison of the potency patterns of II and IIa permits the conclusion that the μ-receptor selectivity of the cyclic analog is an exclusive consequence of the imposed conformational constraint and permits the unambiguous statement that μ- and δ-receptors differ in their conformational requirements[4].

Within the series of cyclic homologues I-IV all members showed μ-receptor selectivity and the IC50(GPI)/IC50(MVD)-ratio increased gradually with lengthening of the side chain in position 2 (Table I), reaching a value of 29.4 with H-Tyr-c[-N$^\varepsilon$-D-Lys-Gly-Phe-Leu-] (IV). Compared to [Leu^5]enkephalin the latter analog is 50 times more potent on the GPI and 12 times less potent on the MVD. The corresponding open-chain analogs (Ia-IVa) all show IC50-ratios close to unity and therefore, are non-selective. The binding data obtained with

Table I. Potencies of Cyclic and Linear Enkephalin Analogs in the GPI- and MVD-assay.

Compound		GPI IC50 [nM]	MVD IC50 [nM]	MVD/GPI IC50-ratio
I	H-Tyr-c[-N$^{\beta}$-D-A$_2$pr-Gly-Phe-Leu-]	23.4 ± 4.2	73.1 ± 14.5	3.12
II	H-Tyr-c[-N$^{\gamma}$-D-A$_2$bu-Gly-Phe-Leu-]	14.1 ± 2.9	81.4 ± 5.8	5.77
IIa	H-Tyr-D-Abu-Gly-Phe-Leu-NH$_2$	28.7 ± 1.3	45.6 ± 9.1	1.59
III	H-Tyr-c[-N$^{\delta}$-D-Orn-Gly-Phe-Leu-]	48.0 ± 4.3	475 ± 99	9.90
IV	H-Tyr-c[-N$^{\varepsilon}$-D-Lys-Gly-Phe-Leu-]	4.80 ± 1.79	141 ± 28	29.4
IVa	H-Tyr-D-Nle-Gly-Phe-Leu-NH$_2$	24.6 ± 1.6	25.2 ± 7.9	1.02
V	H-Tyr-c[-N$^{\varepsilon}$-D-Lys-Gly-Phe-Met-]	1.20 ± 0.26	34.3 ± 5.5	28.6
VI	H-Tyr-c[-N$^{\varepsilon}$-D-Lys-Gly-Phe(pNO$_2$)-Leu-]	0.504 ± 0.138	13.4 ± 6.3	26.6
VII	H-Tyr-c[-N$^{\varepsilon}$-D-Lys-Gly-Leu-Phe-]	3.36 ± 0.04	41.6 ± 9.4	12.4
VIIa	H-Tyr-D-Nle-Gly-Leu-Phe-NH$_2$	681 ± 55	4'400 ±1'270	6.46
VIII	H-Phe-c[-N$^{\varepsilon}$-D-Lys-Gly-Phe-Leu-]	105 ± 19	4'070 ± 200	38.8
VIIIa	H-Phe-D-Nle-Gly-Phe-Leu-NH$_2$	4'240 ±150	10'900 ±1'400	2.57
IX	H-Tyr-D-Cys-Gly-Phe-D-Cys-NH$_2$	0.780 ± 0.010	0.298 ± 0.037	0.382
X	H-Tyr-D-Cys-Gly-Phe-L-Cys-NH$_2$	1.51 ± 0.03	0.760 ± 0.086	0.503
XI	H-Tyr-L-Cys-Gly-Phe-L-Cys-NH$_2$	210 ± 58	950 ± 160	4.52
XII	H-Tyr-D-Cys-Gly-D-Phe-L-Cys-NH$_2$	135 ± 13	891 ± 139	6.60
XIII	H-Tyr-D-Cys-Gly-Phe(pNO$_2$)-D-Cys-NH$_2$	0.0351± 0.0100	0.0187± 0.0023	0.533
XIV	H-Phe-D-Cys-Gly-Phe-D-Cys-NH$_2$	48.6 ± 6.05	456 ± 159	9.38
XV	H-Phe-D-Cys-Gly-Phe(pNO$_2$)-D-Cys-NH$_2$	13.0 ± 2.1	127 ± 7	9.77
XVI	H-D-Tyr-D-Cys-Gly-Phe(pNO$_2$)-D-Cys-NH$_2$	483 ±112	582 ± 113	1.20
XVII	[Leu^5]enkephalin	246 ± 39	11.4 ± 1.1	0.0463

compounds I-IV are in general agreement with the results of the GPI- and MVD-assay[5]. However, it is interesting to note that throughout this series the cyclic analogs (I-IV) are more potent than their corresponding open-chain analogs (Ia-IVa) in the GPI-assay, but less potent in the μ-receptor selective binding assay. This discrepancy between bio- and binding assay is most pronounced with IV which is 5-times more potent than IVa in the GPI-assay, whereas an exactly reversed potency relationship is observed in the [^{3}H]naloxone binding assay[5]. A divergent effect of ring closure on affinity and "efficacy" at the μ-receptor would be a particularly attractive explanation of this phenomenon. In other words, the more weakly bound cyclic analog (IV) would be much more effective in "activating" the receptor than its bound linear counterpart (IVa).

The important question arises whether the cyclic analogs and their linear counterparts have identical modes of binding or whether one or several moieties in the cyclic and linear peptides bind to different receptor subsites. Studies with linear enkephalin analogs had indicated that L-configuration in positions 1 and 4 and D-configuration in position 2 is required for opiate activity, whereas both L- and D-configuration are tolerated in position 5 (cf. ref. 2). The low potency shown by analogs of II with D-configuration in position 1 (unpublished results) and L-configuration in position 2[3] and the high potency retained upon inversion of the configuration in position 5 of IV[5] indicate that the cyclic analogs have the same configurational requirements as the linear enkephalins. In addition, substitution of a nitro group in para-position of the phenyl ring in position 4 of IV (compound VI) leads to a pronouced increase in potency, as it is the case with linear enkephalin[7]. The ensemble of these results permits the unambiguous conclusion that the cyclic and linear enkephalin analogs share a common mode of binding involving interaction with identical receptor subsites.

Further structure-activity studies with analogs of IV were undertaken. In some cases (e.g. compounds V and VI) amino acid substitutions produce very similar potency shifts in the cyclic analog and in the corresponding linear peptide. On the other hand, transposition of Phe and Leu in cyclic analog IV (compound VII) produces a slight potency enhancement both in the GPI- and MVD-assay, whereas the same modification in the open-chain peptide (VIIa) results in a drastic potency decrease. Perhaps of even greater interest is the observation that substitution of phenylalanine for tyrosine in position 1 of cyclic analog (IV) leads to a compound (VIII) which is more than twice as potent as [Leu^5]enkephalin in the GPI-assay. Again a much more drastic drop in potency is observed with the analogously substituted linear peptide (VIIIa). Since cyclic and linear enkephalins appear to interact with the same binding elements on the receptor surface (see above), these discrepancies in structure-activity relationships between cyclic and linear enkephalin analogs most likely reflect differences in the *binding process*. Because the conformation of the semi-rigid cyclic analogs in solution may closely resemble the receptor-bound conformation, these analogs may interact with the receptor more or less according to Fischer's "lock-and-key" model. On the other hand, the flexible linear analogs are likely to undergo conformational changes in the course of the binding process which might perhaps be best described by the "zipper" model[8]. In the "lock-and-key" interaction certain moieties (e.g. tyrosyl hydroxyl) may no longer be absolutely necessary for efficient binding, whereas in the "zipper-type" interaction certain modifications (e.g. Phe^4-Leu^5 transposition) may be disadvantageous for a stepwise binding process.

A second type of cyclic analog has been obtained through substitution of D-cysteine in position 2 and D- or L-cysteine in position 5 of the enkephalin sequence followed by disulfide bond formation[9] (compounds IX and X, Figure 1). In

comparison to compound II analogs IX and X contain a somewhat more flexible 14-membered ring structure. Conformational studies of cystine-containing enkephalin analogs by fluorescence and NMR-spectroscopy have been initiated. Analogs IX and X are highly potent both in the GPI- and MVD-assay and, therefore, are non-selective. The lack of selectivity of these compounds has also been established in the binding assays[9] and may be due to the relatively high flexibility of their ring structure. Again a mode of binding of the cystine-containing analogs similar to that of linear enkephalins is indicated by the comparable activity of IX and X, the low potencies of XI, XII and XVI and the effect of p-nitro-phenylalanine substitution in position 4 which results in a superactive compound (XIII) showing five times the potency of dynorphin-(1-13) in the GPI-assay and an IC50 of 18.7 picomolar in the MVD-assay. Considerable potency in the GPI-assay is again retained by cystine analogs (XIV and XV) lacking the tyrosyl hydroxyl group. Finally, it is interesting to point out that replacement of the half-cystine in position 2 of IX and X by half-penicillamine (Pen) results in considerable δ-receptor selectivity[10] as a consequence of the additional conformational constraint due to the gem dimethyl substituents in β-position of Pen. Taken together these results demonstrate convincingly how different types and degrees of conformational restriction can produce selective binding to one or the other receptor subclass.

In degradation studies based on incubation with a rat brain membrane suspension at 25°C during up to 1 h both cyclic analog II and cyclic peptides IX and X turned out to be completely stable against enzymolysis[3,9]. The long-lasting analgesic and catatonia-like effect observed following intracerebroventricular administration of compound IV to rats[11] confirms the high resistance of this type of cyclic enkephalin analog to enzymatic degradation.

Conclusions

The results of studies with cyclic enkephalin analogs described above indicate that conformational restriction of peptides can have the following interesting implications:

1) Drastic reduction in conformational possibilities permitting an approximation of the receptor-bound conformation by appropriate physical techniques.
2) Effect on receptor binding (equilibrium dissociation constant and binding kinetics).
3) Enhanced receptor selectivity in a situation of receptor heterogeneity.
4) Effect on "efficacy" (superagonism or antagonism).
5) Different pattern of structure-activity relationships as compared to corresponding open-chain analogs.
6) Increased stability against enzymatic degradation and, thereby, prolonged activity.

Other examples of successful covalent bonding of side-chains in linear peptides include cyclic analogs of LH-RH[12], α-MSH[13], β-endorphin[14] and bradykinin[15]. It remains to be shown whether the high activity of the γ-lactone containing analog of LH-RH and the dramatic potency increase seen with [Cys^4,Cys^{10}]α-MSH are due to enhanced receptor affinity or to increased "efficacy". Both with [Cys^4,Cys^{10}]α-MSH and with the cyclic bradykinin analog a differential effect of the conformational constraint on the biological activity in various assay systems was observed. Undoubtedly, the receptor affinity, selectivity and "efficacy" of cyclic peptides can be further manipulated through modification of peptide bonds or introduction of additional conformational constraints in the peptide backbone or in side-chains, as it is indicated by the antagonist properties of [Pen^1]oxytocin[16,17] and the δ-receptor selectivity of [D-Pen^2,L-Cys^5]enkephalinamide[10]. It can be anticipated that efforts along these lines will have considerable impact on peptide drug development in the future.

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PENICILLAMINE-CONTAINING ENKEPHALIN ANALOGS WITH EXTRAORDINARY DELTA OPIOID RECEPTOR SELECTIVITY

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Introduction

The incorporation of conformational restrictions into analogs of the enkephalins via side-chain to side-chain[1-3] or side-chain to carboxy terminus cyclization[4,5] has resulted in analogs with increased opioid receptor selectivities. A particularly interesting finding is that while the cyclic cystine-containing enkephalinamide analogs; H-Tyr-D-Cys-Gly-Phe-D(or L)-Cys-NH_2, exhibit moderate selectivity for the μ opioid receptor[1], the corresponding 2-D-penicillamine (β,β dimethylcysteine) containing cyclic analogs; H-Tyr-D-Pen-Gly-Phe-D(or L)-Cys-NH_2, display significant δ opioid receptor selectivities.[2] This result was somewhat surprising since enkephalinamide derivatives are generally nonselective or moderately μ receptor selective. Evaluation of the corresponding carboxylic acid terminal peptides, H-Tyr-D-Pen-Gly-Phe-D(or L)-Cys-OH confirmed that these analogs possess increased δ receptor selectivity.[3] It has been shown that substitution of penicillamine for cysteine in the tocin ring portion of oxytocin leads to dramatic reduction in solution conformational flexibility[6,7] and it seems likely that a similar reduction in flexibility underlies the δ receptor selectivities observed with the penicillamine-containing cyclic enkephalin and enkephalinamide analogs. To further examine

the effects of conformational restriction on enkephalin analogs, the penicillamine-containing analogs, [D-Cys^2,D(or L)-Pen^5]enkephalin and the bis-penicillamine analogs, [D-Pen^2,D(or L)-Pen^5]enkephalin were synthesized and evaluated for opioid activity in the rat-brain binding assay and in the mouse vas deferens (MVD) and guinea pig ileum (GPI) bioassays.

Methods

The penicillamine- and bis-penicillamine-containing enkephalins and the reported δ receptor selective analogs [D-Ser^2,Leu^5,Thr^6]enkephalin[8] and [D-Thr^2,Leu^5,Thr^6]enkephalin[9] were synthesized and purified following previously reported protocols.[2,3] GPI and MVD assays, based on the inhibition of electrically induced smooth muscle contractions, and rat brain binding assays were performed as described elsewhere.[2,3]

Table I. Guinea Pig Ileum and Mouse Vas Deferens Assays

Analog	IC_{50}(nM) GPI	MVD	IC_{50}(GPI)/ IC_{50}(MVD)
[D-Cys^2,L-Pen^5]E	39.9 ± 1.5	0.75 ± 0.05	53.2
[D-Cys^2,D-Pen^5]E	66.7 ± 1.3	0.13 ± 0.06	513.
[D-Pen^2,L-Pen^5]E	2720 ± 50.1	2.50 ± 0.03	1090.
[D-Pen^2,D-Pen^5]E	6930 ± 124	2.19 ± 0.30	3160.
DSTLE	234 ± 85.6	0.70 ± 0.08	334.
DTTLE	100 ± 19.6	0.58 ± 0.06	172.
DADLE	24.3 ± 5.3	0.27 ± 0.06	90.

E, Enkephalin

Results and Discussion

As summarized in Table I, all analogs tested were more potent in the MVD assay, in which the δ receptor mediates the effect,[10,11] than in the GPI, in which the μ receptor is the

functional receptor.[10,11] The prototypical δ selective ligand [D-Ala^2,D-Leu^5]enkephalin (DADLE) and [D-Cys^2,L-Pen^5]-enkephalin display only moderate selectivities based on the ratio of IC_{50} values in the two systems, while the previously reported δ specific ligands, [D-Ser^2,Leu^5,Thr^6]enkephalin (DSTLE)[8] and [D-Thr^2,Leu^5,Thr^6]enkephalin (DTTLE)[9] are somewhat more δ selective. [D-Cys^2,D-Pen^5]enkephalin is substantially more δ selective than the preceeding analogs and is the most potent on the MVD of those tested. The bis-penicillamine analogs are the most δ selective of the analogs tested, exhibiting remarkably high IC_{50} (GPI)/IC_{50} (MVD) ratios.

Table II presents results from rat-brain receptor binding assays which show that all the analogs tested are more effective at displacing the δ ligand [^{3}H]DADLE than the μ ligand [^{3}H]naloxone ([^{3}H]NAL). As seen from the ratios IC_{50}^{NAL}/IC_{50}^{DADLE}, however, only the bis-penicillamine enkephalins are highly δ receptor selective in this binding assay. Since elucidation of the physiological role of the δ receptor has been hampered by the lack of ligands highly selective for this receptor, these analogs should greatly aid such studies.

Table II. Rat Brain Binding Assays

Analog	IC_{50} (nM) [^{3}H]NAL	[^{3}H]DADLE	IC_{50}^{NAL}/IC_{50}^{DADLE}
[D-Cys^2,L-Pen^5]E	52.7 ± 2.3	5.4 ± 0.1	9.8
[D-Cys^2,D-Pen^5]E	22.2 ± 2.8	3.5 ± 0.8	6.3
[D-Pen^2,L-Pen^5]E	3710 ± 740	10.0 ± 2.2	371.
[D-Pen^2,D-Pen^5]E	2840 ± 670	16.2 ± 0.9	175.
DSTLE	88 ± 6.0	5.73 ± 0.42	15.4
DTTLE	36.3 ± 3.8	6.40 ± 0.60	5.7
DADLE	16 ± 5.0	3.9 ± 0.7	4.1

E, Enkephalin

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SYNTHESIS AND BIOLOGICAL ACTIVITY OF CYCLIC AND ACYCLIC PARTIAL RETRO-INVERSO ENKEPHALINS

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Introduction

Cyclic and acyclic partially modified retro-inverso enkephalin analogs, in which one or more of the amide bonds of the parent sequence is reversed, have been prepared. The overall effect of the modification is to reverse the direction of the amide bond between selected residues while conserving side chain composition. Reversal of a single amide bond yields two adjacent non-amino acid residues. The amino acid closer to the N-terminal is transformed into the corresponding gem-diaminoalkyl residue, whereas the amino acid closer to the C-terminal is replaced by the corresponding 2-alkylmalonic acid residue. Extension of this transformation to non-adjacent amide bonds necessitates the inversion of chirality of amino acids between the modified residues.

Materials and Methods

Peptides were synthesized by techniques in solution (Figure 1). Monoacyl 2-alkyl gem-diamines were readily generated in good yield from their synthetically accessible peptide

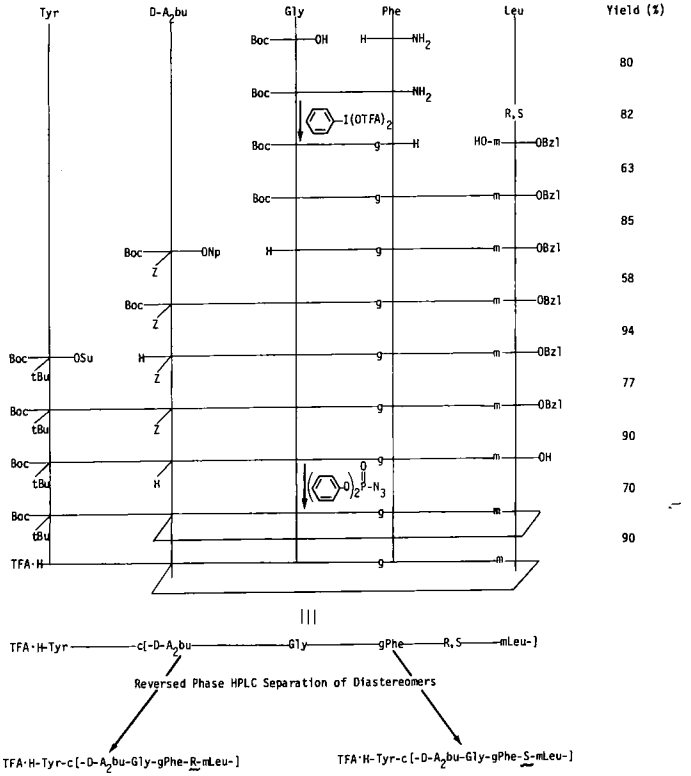

Fig. 1. Representative synthesis of a cyclic partially modified retro-inverso enkephalin.

amide precursors using [bis(trifluoroacetoxy)iodo]benzene.[1,2] Intramolecular cyclization was achieved at high dilution utilizing diphenylphosphoryl azide[3] as the coupling reagent.

The final products were characterized by reverse phase high pressure liquid chromatography (HPLC), thin layer chromatography in three solvent systems, and amino acid analysis. The compounds were tested in bioassays based on inhibition of electrically induced contractions of the quinea pig ileum[4] (GPI) and mouse vas deferens[5] (MVD). The peptides' in vitro enzymatic stabilities towards rat brain membrane suspensions[6] were determined by reverse phase HPLC.

Results and Discussions

Cyclic and acyclic partially modified retro-inverso analogs of H-Tyr-c[-D-A_2bu-Gly-Phe-Leu-][7] and H-Tyr-D-Ala-Gly-Phe-Leu-NH_2[8] are reported in which changes have been made at the amide bonds involving residues 4 and 5. Relative to Leu-enkephalin all the analogs show potent opiate activity and high selectivity towards the putative alkaloid μ-receptor population (Table I).

Table I. Inhibitory Potencies of Enkephalin Analogs in the Guinea Pig Ileum (GPI) and Mouse Vas Deferens (MVD) Assays[a]

	Compound	GPI IC_{50} [nM]	GPI Rel. Potency[b]	MVD IC_{50} [nM]	MVD Rel. Potency[b]	$\frac{IC_{50}(MVD)}{IC_{50}(GPI)}$
	H-Tyr-c[-D-A_2bu-Gly-Phe-Leu-][c]	14.1 ± 2.9	17.5	81.4 ± 5.8	0.140	5.77
	H-Tyr-D-Ala-Gly-Phe-Leu-NH_2[c]	7.63 ± 0.06	32.2	8.26 ± 1.32	1.38	1.08
I	H-Tyr-c[-D-Glu-Gly-gPhe-D-Leu]	19.4 ± 3.4	12.7	313 ± 102.	0.0364	16.1
II	H-Tyr-c[-D-A_2bu-Gly-gPhe-R,S-mLeu-]	3.94 ± 0.57	62.2	11.7 ± 4.0	0.971	2.97
IIf	Faster eluting diastereomer of II	25.5 ± 2.0	9.64	14.9 ± 5.0	0.767	0.584
IIs	Slower eluting diastereomer of II	1.51 ± 0.19	163	7.76 ± 3.17	1.47	5.14
III	H-Tyr-c[-D-Glu-Gly-Phe-gLeu-]	21.6 ± 3.0	11.4	326 ± 28	0.035	15.1
Ia	H-Tyr-D-Ala-Gly-gPhe-D-Leu-For	5.73 ± 2.46	42.9	42.2 ± 13.0	0.270	7.36
IIa	H-Tyr-D-Ala-Gly-gPhe-R,S-mLeu-NH_2	41.2 ± 13.6	5.97	98.3 ± 11.0	0.116	2.39
IIIa	H-Tyr-D-Ala-Gly-Phe-gLeu-For	12.9 ± 1.7	19.1	16.1 ± 2.7	0.710	1.25
	Levorphanol[c]	17.0 ± 3.0	14.5	278 ± 56	0.0041	16.4
	Leu-enkephalin	246 ± 39	1	11.4 ± 1.1	1	0.0463

[a]Mean of three determinations ± SEM.

[b]Potency relative to Leu-enkephalin (= 1).

[c]Data from reference 7.

All the modified enkephalin analogs were found to be highly resistant to proteolytic degradation by rat brain membrane preparations. Under conditions where Leu-enkephalin was completely inactivated in 40 minutes, the modified analogs show no appreciable degradation after 2 hours, and the only partial degradation after days.

The subtle variation in structure achieved through the application of the partial retro-inverso modification represents an attractive approach for the preparation of analogs that resist enzymatic degradation, possess altered selectivity, and maintain substantial or even enhanced biological activity. We have begun to study the conformations of the cyclic partial retro-inverso enkephalins described. Two dimensional shift correlation spectroscopy has allowed unambiguous assignment of ^{1}H resonances. Utilizing an integrated approach combining 2D-J resolved spectroscopy, NOE experiments, molecular dynamics, and energy minimizations we will elucidate the conformational preferences of the cyclic partial retro-inverso enkephalins.

Acknowledgment

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The SYNTHESIS OF D-ALA^2,∇^EPHE^4,LEU^5-ENKEPHALIN

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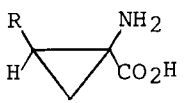
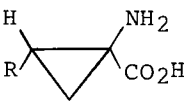
Cyclopropane-containing amino acids in which one side of the cyclopropane ring requires the C_α,C_β bond of the amino acid residue are of interest because of their inherent steric properties. The α-carbon atom is quaternary and, consequently nucleophilic attack at the carbonyl function is sterically hindered. Rigid positioning of the β-substituent and severe constraint of the ϕ and ψ angles of cyclopropyl amino acid (∇AA)[1] residues also follow from their rigid structures. Figure 1 shows cyclopropyl amino acids in their E- and Z-configurations which necessarily have χ_1 angles equal to 0^o and 120^o, respectively. Several of these amino acids have been previously reported in the literature.[2]



(2S,3S) or (2R,3R)∇^Z



(2S,3R) or (2R,3S)∇^E

Figure 1

Incorporation of cyclopropyl amino acids into peptides at appropriate positions should have profound effects on the conformations of those peptides. Models show that the Φ and ψ angles in both the E- and Z-isomers will most probably be small and, consequently, these moieties should promote the formation of β-bends when they can appear at the i+1 and/or i+2 positions of those bends. Equally important, the steric hindrance to nucleophilic attack at the carbonyl functions of these amino acid residues should cause enzymatic hydrolysis to be slow, thus conferring an increased biological lifetime on peptides prepared from these compounds. We have chosen to test these ideas on D-Ala^2, Leu^5-enkephalins because these compounds are relatively easy to synthesize and their <u>in vitro</u> biological assays are readily available.

We have already reported[3] our synthesis of ∇^ZPhe and ∇^EPhe and we now report the incorporation of ∇^EPhe into D-Ala^2 enkephalin. We had previously synthesized dehydro Phe^4 enkephalin (Δ^ZPhe^4-EK) which, of necessity, had the Z-configuration at the dehydro moiety,[4] and it was, therefore, of considerable interest to us to introduce a sterically restricted phenylalanine residue having the E-configuration. The overall synthesis of D-Ala^2, ∇^EPhe^4-enkephalin is outlined in Figure 2. Before proceeding with the synthesis it was, of course, necessary to resolve the ∇^EPhe molecule. The benzyloxycar-

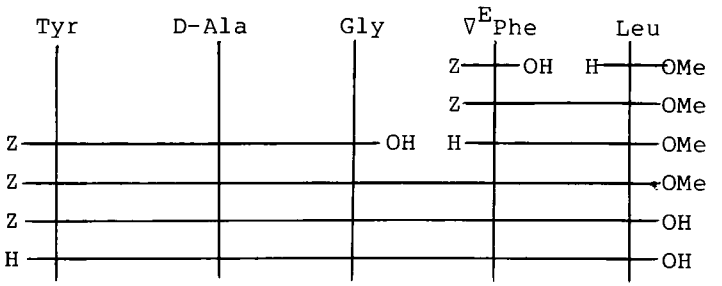


Figure 2

bonyl derivative of ∇^EPhe was resolved by means of its brucine salt and a sample of one of the enantiomers was coupled with

L-leucine methyl ester and its retention time was determined on RPLC. Racemic Z-∇^{E}Phe was then coupled with L-leucine methyl ester by the mixed anhydride procedure and the diastereomeric mixture was separated by RPLC. One of those isomers was identical in RPLC retention time, IR and NMR spectra, to the resolved material. Each diastereomer was then deblocked and coupled with the tripeptide, Z-Tyr-D-Ala-Gly·OH, and converted to the free pentapeptide. Concurrently, the Z-∇^{E}Phe enantiomers were deblocked and their CD spectra were determined under acidic conditions.[5] The (-)-isomer was shown to have the 2S,3R configuration corresponding to the L-form and the (+)-isomer corresponded to the D-form of ∇^{E}Phe.

The bioactivities of the two peptides, as tested against the electrically stimulated mouse vas deferens and guinea pig ileum, are reported in Table 1. These results show that the ∇^{E}Phe moiety has a very deleterious effect on the bioactivity

Table 1

	Mouse Assays	
Enkephalin	Mouse vas Deferens	Guinea Pig Ileum
D-Ala^2,(2R,3S)∇^{E}Phe^4,Leu^5	0.034 mM	0.064 mM
D-Ala^2,(2S,3R)∇^{E}Phe^4,Leu^5	0.096 mM	0.26 mM
D-Ala^2,Leu^5	0.011 μM	--------
D-Ala^2,D-Leu^5	0.0026 μM	--------

of D-Ala^2,Leu^5-enkephalin. Very surprisingly, however, the 2R,3S isomer is the more active of the pair by a factor of three. This is entirely unexpected, since it is known that D-Phe^4 enkephalin is essentially inactive.

Using an amino acid analyzer, we have shown that absolutely no leucine is liberated from either ∇^{E}Phe^4-enkephalin isomer when they are treated with CPase Y for 24 hours at a substrate enzyme of ratio 274:1. The dehydro analog, D-Ala^2, Δ^{Z}Phe^4,Leu^5-EK, was also resistant to CPase Y, but leucine was slowly liberated.[6]

Synthesis of the D-Ala^2,∇^ZPhe^4,Leu^5-enkephalin isomers are underway, and, until the bioactivities of these are determined, we are unable to tell whether the large decrease in bioactivity is due to the E-configuration of the cyclopropyl moiety or the presence of the cyclopropyl ring. Remembering that D-Ala^2, Δ^ZPhe^4-enkephalin was approximately five times as active as the saturated compound,[7] we expect that the configuration is the more important of the two structural modifications.

Acknowledgements

We gratefully acknowledge financial support of NIH-NIDA Grant No. DA02938.

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1. The inverted triangle, ∇, is used to designate "cyclopropyl" with superscripts, ∇^E or ∇^Z, to designate the configurations about the cyclopropane ring. This is not to be confused with the symbol, Δ, meaning "dehydro", as in Δ^ZPhe, etc.

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MULTIPLE CONFORMATIONS OF ENKEPHALIN IN THE CRYSTALLINE STATE

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Introduction

Peptide molecules, especially linear peptides, are extremely flexible and it is possible that a number of different conformations are equally probable. Serendipitously, crystals of Leu^5-enkephalin, Tyr^1-Gly^2-Gly^3-Phe^4-Leu^5, were grown with a large amount of solvent surrounding the peptide molecules, thus partially mimicking a solution environment. By means of x-ray diffraction analysis of a single crystal, sealed in a thin glass capiallary with some of the mother liquor, precise conformational parameters were established for the four distinctly different conformers of enkephalin that had cocrystallized side-by-side in the same unit cell.

The procedure for solving the structure of a crystal containing more than 210 independent C, N and O atoms, without any heavier atoms, the atomic coordinates, bond lengths and angles, and other parameters are being published in a separate paper[1].

Description of Enkephalin Conformers

The four separate enkephalin molecules A-D have been arranged in Figure 1 to show the similarities in the conformations of their extended backbones and the differences in the conformations of the side groups which are shown shaded in the figure. In the backbones the chief differences are the degree of pleating, with B and C being considerably flatter than A and D (*vide infra*, Figures 3-4), and the orientation of the carboxyl terminus which is different in C and D as compared to A and B. The Phe side groups have the gauche$^+$ conformations in all four conformers with only minor differences in the torsional angles about N-C$^{\alpha}$-C$^{\beta}$-C$^{\gamma}$. The Tyr side groups have the trans conformation in A and D and the more unusual gauche$^-$ conformation in B and C. Finally the Leu side chain has the trans conformation in C and the gauche$^+$ conformation in A, B and D.

Aggregation into β-sheets

The enkephalin conformers assemble in an orderly fashion to form a continuous, modified antiparallel pleated β-sheet, shown in Figure 2. In the vertical direction molecules are repeated by translation and there is simple head-to-tail hydrogen bonding between molecules for conformers C and D, while for conformers A and B four water molecules, labelled 1-4 (see middle of diagram), are intimately involved in hydrogen bonding with the amine and carboxyl termini. In the horizontal direction all possible NH$\cdots$O=C hydrogen bonds are formed. The conformers arranged in the order ABCD are repeated in succeeding unit cells. The backbones of conformers A and B are antiparallel with respect to each other and are approximately related by a two-fold rotation. A similar two-fold rotation relationship exists between the backbones of conformers C and D. However, there is no pseudosymmetry between B and C or A and D.

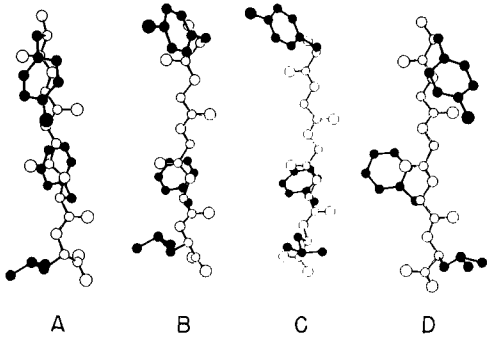


Fig. 1. Four conformers of enkephalin in same crystal.

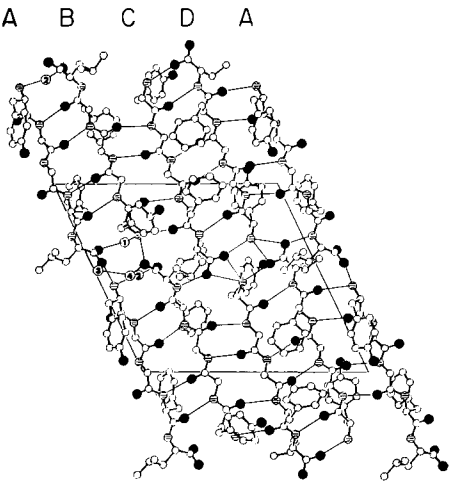
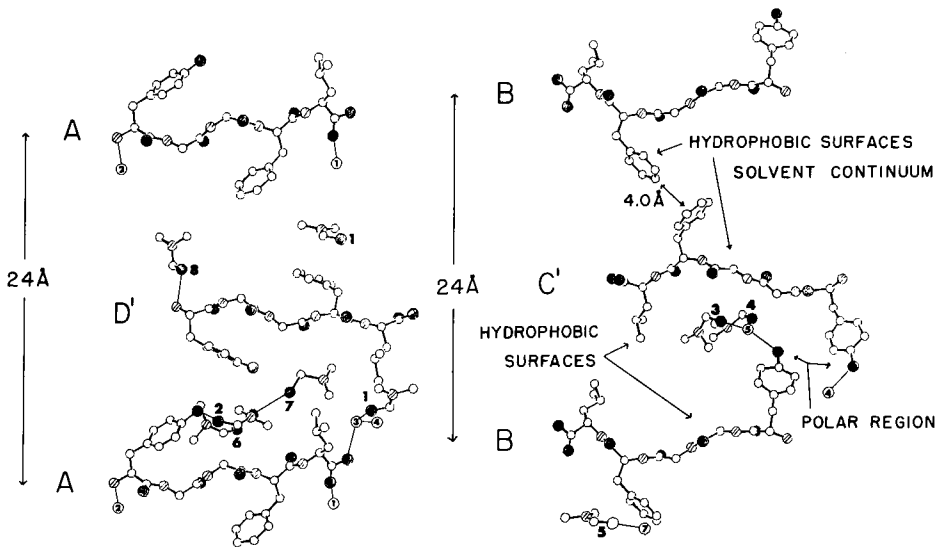


Fig. 2. Beta-sheet



Figs. 3 and 4. Edge-on views of β-sheet. Left: 5 A slice from Z=0 to 1/4. Right: 5 A slice from Z=1/4 to 1/2.

Solvent between β-sheets

The β-sheet is repeated every 24.7 A, the length of the b axis in the unit cell of the crystal, and in between, at 12.3 A spacing, the β-sheet is rotated by 180° to satisfy the two-fold screw symmetry of space group $P2_1$. Figures 3 and 4 show two adjacent slices from an edge-on view of the β-sheets where molecules C' and D' are related to molecules C and D in Figure 2 by the operation of the two-fold screw symmetry.

Side groups protrude above and below the β-sheets into a sea of solvent composed of water and DMFA molecules that are sandwiched between the parallel β-sheets. Side groups are entirely immersed in the solvent layer with almost no contact with side groups from other β-sheets. The forces holding the crystal together appear to be very tenuous.

Only the four OH groups in the Tyr residues, one each in conformers A-D, are available for hydrogen bonding to the solvent. They donate protons to hydrogen bonds with the carbonyl oxygen of DMFA or through water to DMFA, Figures 3 and 4. The hydrogen bonding networks end with DMFA since DMFA is only an acceptor, not a donor. The coordinates for only eight DMFA molecules and eight water molecules have been established in the crystal structure analysis. All of these molecules are held moderately firmly in place by the hydrogen bonds they form with the peptide molecules. The remainder of the solvent molecules exist in a continuum or fluid state with no particular fixed positions.

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CONFORMATIONAL STUDIES OF SOME CYCLIC ENKEPHALIN ANALOGUES BY TWO-DIMENSIONAL NMR SPECTROSCOPY[1]

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Four cyclic enkephalin analogues have been prepared in order to restrict conformational flexibility of the peptide backbone and the side chains in position 4 and 5. They contain a lysyl residue in position 2 which allows cyclization between the side chain N^{ε} group and the terminal carboxyl group in position 5 (Table I). The synthesis of the four compounds is carried out by conventional methods. Tetrahydroisoquinoline carboxylic acid (Tic) has been obtained by condensation of formaldehyde with phenylalanine[2].

The biological activity of the deprotected analogues is presented in Table I. Tests have been performed in the GPI-assay and after intracerebroventricular (i.c.v.) application.

Table I. Cyclic Enkephalin Analogues and Biological Activity of the Deprotected Peptides Related to Morphine-HCl

		GPI	i.c.v.
1	Boc-Tyr-cyclo[-N^{ε}-Lys-Gly-Phe-Leu-]	0	0
2	Boc-Tyr-cyclo[-N^{ε}-D-Lys-Gly-Phe-Leu-][3]	300	30
3	Z-Tyr-cyclo[-N^{ε}-D-Lys-Gly-Phe-Pro-]	3	3
4	Z-Tyr-cyclo[-N^{ε}-D-Lys-Gly-Tic-Pro-]	1.5	< 0.01
	Morphine-HCl	1	1

Peptides 1, 2 and 4 show only one conformation within the NMR time scale, whereas 3 exists in a 40 : 60 mixture of cis/trans Phe-Pro bond isomers.

The ^{1}H NMR signals of 1 and 2 have been assigned by the SECSY technique[4]. For compound 1, the equivalence of the diastereotopic Gly^3-C$^\alpha$H$_2$ resonances, the strong overlap in the C$^\alpha$ and the aliphatic region in addition to the behaviour at variable temperature (Figure 1) and solvent change, suggests a high flexibility of the peptide backbone[5].

The D-lysine peptides appear to be more rigid. The difference to 1 is most obvious in the nonequivalence of the Gly^3-C$^\alpha$H$_2$ protons ($\Delta\delta$ = 0.7 - 0.9 ppm). Internal orientation of D-Lys^2-N$^\varepsilon$H is indicated by the small temperature coefficient (Figure 1). The coupling constants for 2 favor a βI-turn (D-Lys^2-N$^\varepsilon$H → OC-Gly^3). In this case the carbonyl group of the trans peptide bond D-Lys^2-Gly^3 can adopt a position in plane with the Gly^3-C$^\alpha$H$_S$ proton which serves as the explanation of the strong nonequivalence caused by the magnetic anisotropy contribution. The resulting proximity of the Gly^3-NH and D-Lys^2-C$^\alpha$H is demonstrated by a NOE effect between these two protons. Measurements of 2 and the deprotected analogue reveal that the presence of the protecting group does not alter the conformation of the 16 membered ring. The proposed structure for the deprotected 2 derived from the NMR parameters is presented in Figure 2.

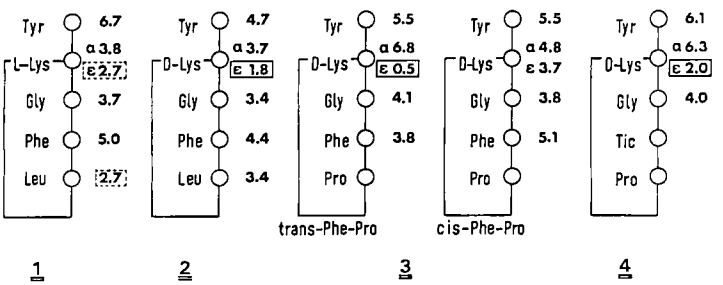

Fig.1. Temperature coefficients (10^{-3} ppm/K) of 1 - 4 in the low temperature range.

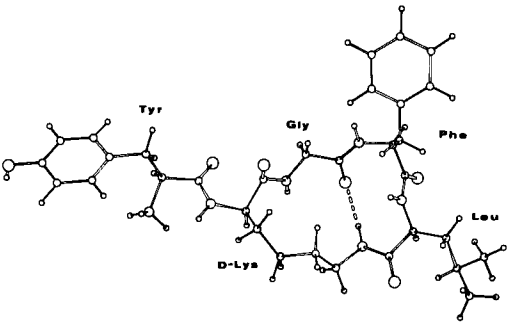

Fig.2. Proposed conformation for deprotected 2.

The presence of a mixture of rotamers in 3 requires the help of several 2D NMR methods to extract the parameters. By 2D-^{1}H-^{13}C shift correlation[6,7] it is possible to correlate the characteristic ^{13}C shifts of the C^{β} and C^{γ} atoms of the cis/trans prolines[8] with the corresponding proton signals. With this information the analysis of the COSY spectrum[6] is feasible. ^{1}H and ^{13}C 2D exchange spectroscopy enables us to follow the chemical exchange process of 3. In the proton case[9] we get support for the assignments made according to the COSY spectrum. The 2D-^{13}C exchange spectrum[10] (Figure 3) allows unambiguous assignments of the ^{13}C signals.

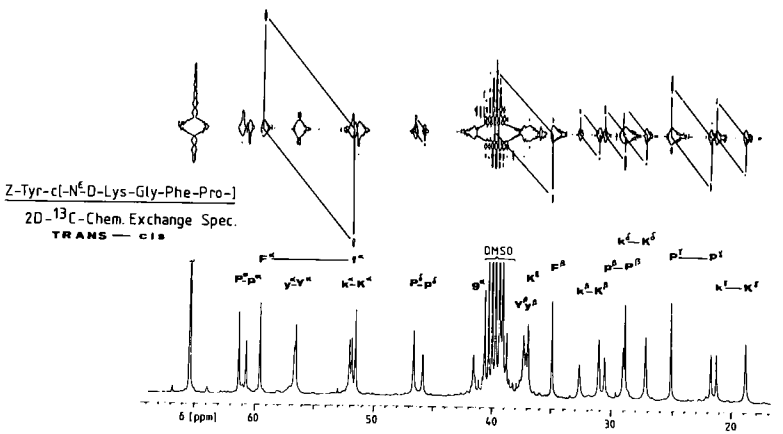

Fig.3. 2D-^{13}C chemical exchange spectrum of 3.

The NMR data (coupling constants, ^{13}C chemical shifts) of the trans isomer of 3 suggest a βI-turn as well. By 2D-^{1}H-^{13}C shift correlation it is shown that 4 contains a trans Tic^{4}-Pro^{5} peptide bond whereas the conformation of the Gly^{3}-Tic^{4} bond cannot be extracted from the data. However, an interpretation of the NMR parameters is only satisfactory if we assume a βI-turn for 4. Evidence for this assumption results e.g. from the identical ^{13}C shifts of the proline residues of 4 and the trans isomer of 3.

In conclusion, if we assume the same backbone structure of 2, 4 and the trans isomer of 3 their difference in biological activity should result from the different orientation of the side chains in position 4 and 5.

Acknowledgements. We thank the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie for financial support.

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CONFORMATIONAL CONSEQUENCES OF INCORPORATING ΔPHE IN AN ENKEPHALIN ANALOGUE AND IN RELATED PEPTIDES

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Despite the existence of natural peptides (e.g. tentoxin[1]) and potent synthetic analogues of biologically active peptides[2] which contain dehydrophenylalanine (Z-α-β-dehydroPhe, ΔPhe), the conformational constraints imposed by the presence of this unusual amino acid in a peptide chain have not been explored in detail. One conformation of this residue (ϕ = -70, $\psi \simeq 0$)[3] can be accommodated in the i+2 position of a β or γ turn, hence it is of particular interest to investigate its participation in reverse turns.

In Table I are gathered ^{1}H NMR data for the NHs of three model peptides containing ΔPhe and three others where L-Phe occurs at the corresponding point in the sequence. In a non-hydrogen-bonding solvent such as chloroform, NHs of a flexible peptide that are exposed to solvent experience little perturbation to their environment as temperature is raised. (Note: solutions must be dilute enough to preclude intermolecular interactions). Those NHs that are intramolecularly hydrogen-bonded will reflect the increasing population of non-hydrogen-bonded states as the temperature is raised, and display a larger $\Delta\delta/\Delta T$.[4] $\Delta\delta/\Delta T$'s of NHs in a rigid peptide, by contrast, are small for intramolecularly hydrogen-bonded residues, since increases in temperature do not alter the conformational

Table I. NH Parameters of Model Peptides [a]

	Boc-Gly-ΔPhe-OMe [b]		Boc-Gly-L-Phe-OMe [b]	
	Gly	ΔPhe	Gly	L-Phe
δ	5.18	7.65	5.02	6.46
Δδ/ΔT [c]	-2.97	-4.80	-2.40	-3.17
Δδ/Δ%solv [d]	43.2	56.1	45.5	42.4
	Boc-Pro-ΔPhe-OMe [e]		Boc-Pro-L-Phe-OMe [e]	
	ΔPhe		L-Phe	
δ	8.43(7.77, *cis*)		7.30(6.50, *cis*) [f]	
Δδ/ΔT [c]	-1.34(-3.23, *cis*)		-1.52(-1.48, *cis*)	
Δδ/Δ%solv [d]	3.1(25.6, *cis*)		- (15.0, *cis*) [f]	
	Boc-Gly-ΔPhe-Leu-OMe [g]		Boc-Gly-L-Phe-Leu-OMe [g]	
	Gly	ΔPhe Leu	Gly	L-Phe Leu
δ	5.23	7.63 6.97	5.01	6.60 6.18
Δδ/ΔT [c]	-3.68	-5.15 -6.74	-2.82	-2.60 -5.17
Δδ/Δ%solv [d]	66.9	63.8 29.8	51.2	35.2 67.6

[a] In $CDCl_3$; δ values, ppm from TMS; Δδ/ΔT, 10^{-3} ppm/deg; Δδ/Δ% solv, 10^{-3} ppm/% acetone. [b] conc 11 mM, RT. [c] Temp range -16 to 20°C. [d] 0-4% acetone V/V. [e] conc 10 mM, -2°C. [f] *trans* Phe NH hidden by aromatic peaks. [g] conc 7 mM, RT.

distribution. In both flexible and rigid peptides, parameters that are usually considered to be indicative of accessibility of NHs (perturbation of δ by addition of a hydrogen-bonding solvent or increase in concentration) will reflect the average accessibility of all states populated by the peptide. Local conformational constraints imposed by sequence will cause decreased average accessibility.

These general strategies led to the following conclusions: 1) Boc-Gly-ΔPhe-OMe and Boc-Gly-L-Phe-OMe both adopt a γ-turn conformation, with a hydrogen bond between the Phe NH and the Boc CO, a large proportion of the time (note large Δδ/ΔT in Phe NH). In neither peptide are the NHs inaccessible to

solvent or other solutes (large concentration dependences (data not shown) and perturbation by acetone. 2) Boc-Pro-ΔPhe-OMe and Boc-Pro-L-Phe-OMe also take up such a γ-turn conformation a large proportion of the time, but their spectral parameters are more typical of rigid peptides: small $\Delta\delta/\Delta T$ (Table I), and small concentration dependences (not shown) and acetone perturbation of NHS. (Note that both peptides exist in two conformations, separated by a high energy barrier, and related by a *cis-trans* isomerization of the Boc-Pro urethane bond.) 3) Boc-Gly-ΔPhe-Leu-OMe and Boc-Gly-L-Phe-Leu-OMe both adopt a β turn conformation, with a hydrogen bond between the Leu NH and the Boc CO, a large proportion of the time (note large $\Delta\delta/\Delta T$ of Leu NH). In the ΔPhe-containing peptide, reduced accessibility of the Leu NH is indicated by the small concentration dependence (not shown) and acetone perturbation of its δ.

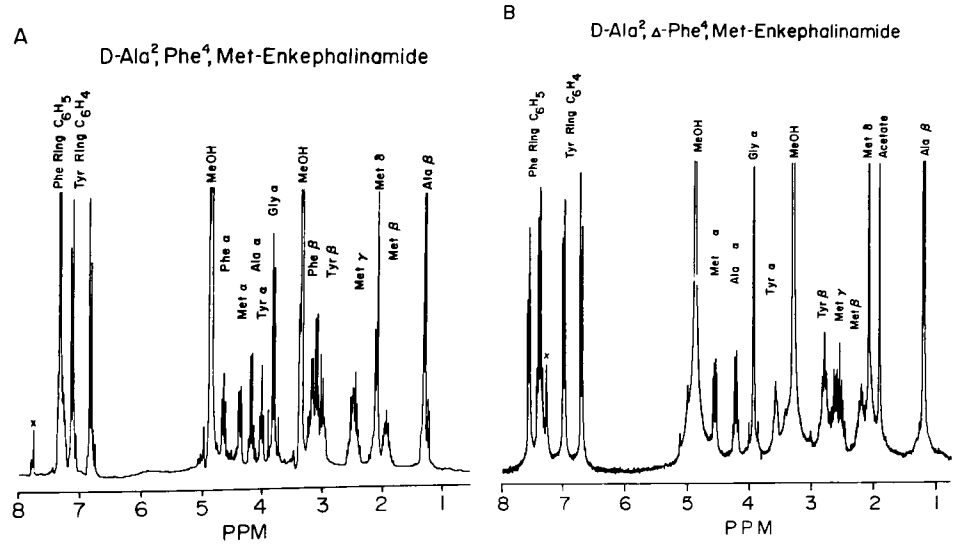

Fig. 1. 250MHz ^{1}H NMR spectrum of: A) D-Ala^2,Phe^4,Met^5-enkephalinamide, 3.83 mg/0.5 ml CD_3OD, 64 scans at room temperature; B) D-Ala^2, ΔPhe^4, Met^5-enkephalinamide, 3.66 mg/0.5 ml CD_3OD, 64 scans at room temperature. X is solvent impurity.

These results illustrate that a ΔPhe can occur in the i+2 position of a β or γ turn without markedly affecting the stability of the turn. Furthermore, an increased rigidity of the peptide is suggested in the region of the ΔPhe residue by the Boc-Pro-ΔPhe-OMe and Boc-Gly-ΔPhe-Leu-OMe accessibility data.

These approaches are currently being applied to D-Ala^2, ΔPhe^4, Met^5-enkephalinamide^2 and its saturated parent peptide; spectra of the ΔPhe analogue and its saturated parent peptide are shown in Fig. 1. Small but significant changes in chemical shifts (Tyr α, Gly α, Met α) between the two molecules suggest some conformational consequence of incorporation of the ΔPhe residue.

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STRUCTURAL, BIOCHEMICAL AND PHARMACOLOGICAL INVESTIGATIONS ON μ AND δ OPIATE RECEPTORS.

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Introduction

Immunocytochemical methods, binding studies, autoradiographic measurements as well as pharmacological assays suggest the occurrence in brain tissue of multiple opioid peptides associated with a multiplicity of binding sites (μ, δ, κ...). This heterogeneity could explain the wide range of pharmacological effects elicited by administration of non-discriminant narcotics. These molecules like the endogenous enkephalins, interact with at least two classes of binding sites (μ and δ). Biochemical and pharmacological properties of these putative μ and δ receptors were investigated using highly selective agonists.

Results and Discussion

Selectivity and binding characteristics of modified enkephalins for μ

and δ receptors subtypes [1-3]. At this time, only few compounds exhibit both a high potency and a large selectivity for μ and δ receptors. The cross-reactivity of the most specific of them is reported in Table 1.

Table 1. Selectivity factor (δ/μ) of modified enkephalins.

	K_I (nM)[a]		K_I DSLET
	[^{3}H] DAGO (μ)	[^{3}H] DSLET (δ)	K_I DAGO
Tyr-D-Ala-Gly-MePhe-Gly-ol (DAGO)	3.9±0.8	700±95	179
Tyr-D-Ala-Gly-NH$(CH_2)_2$-CH$(CH_3)_2$ (TRIMU)	10.0±2.0	1100±120	110
Tyr-D-Ser-Gly-Phe-Leu-Thr (DSLET)	31.0±5.0	4.80±0.80	0.15
Tyr-D-Thr-Gly-Phe-Leu-Thr (DTLET)	25.0±2.5	1.35±0.15	0.05

a) *Mean ± SD of four independent determinations in triplicate performed on crude rat brain membranes.*

Until 20 nM all these compounds bind to only one kind of receptors (μ or δ). Among various differences in the binding characteristics of μ and δ ligands to brain tissue, the most interesting is probably the *selective* loss of μ-binding sites induced by GMP-P(NH)P in the absence of sodium (Fig. 1).

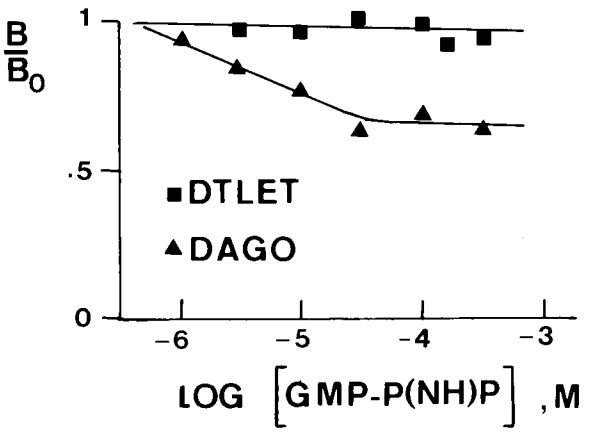

Figure 1.

Autoradiographic measurements of μ and δ binding sites in rat brain.

Till now the distribution of μ and δ receptors in brain tissue has been performed using non-selective ligands [4,5] and results obtained in presence of nucleotides were interpreted by the occurrence of allosteric interconversion between μ and δ binding sites [5].

Figure 2 shows the distribution of μ and δ receptors respectively labelled by 4 nM of [^{3}H] DAGO and 3 nM of [^{3}H] DTLET.

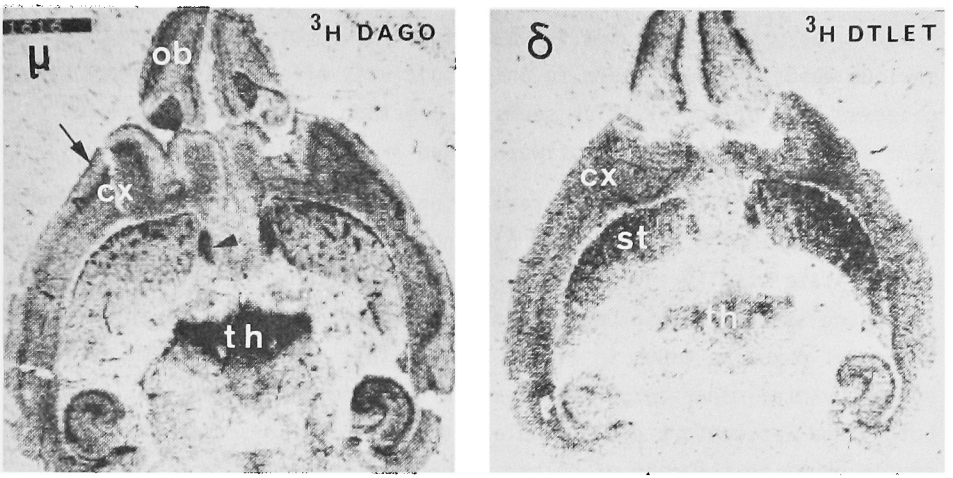

Figure 2. Distribution of μ and δ receptors in horizontal sections of rat brain performed at fig. 58 level of G. Paxinos and C. Watson atlas [6] and processed for autoradiography as described [7]. The images are reconstitutions from data of computerized densitometric analysis of tritium-sensitive films (controls with 1 μM of levorphanol show very low and uniform density). *Left*. μ-receptors are sharply delineated in several areas : cortex (cx), laminea (⟵ = lamina I), olfactory bulbs (ob), thalamus (th), nucleus accumbens (►). In striatum (st), μ- receptors are patchy distributed. *Right*. δ-receptors density clearly decreases from rostral to more caudal levels. As compared to μ-sites, δ-receptors are more homogeneously and diffusely scattered in (cx) and (st) areas. In (nc) and especially (th) the low δ-receptors density strongly contrasts with the dense distribution of μ-sites.

The results of Figure 2 unambiguously confirm the differential distribution of μ and δ receptors in brain. Moreover, from a physiological point of view, it is very interesting to notice that the homogeneous distribution of δ-sites parallels that of dopamine receptors. This result could support the striatal release of dopamine selectively induced by δ-ligands such as DSLET and DTLET [8].

μ-induced analgesia is not increased by δ-receptors-stimulation. Preferential involvement of μ-receptors is clearly shown by the ED_{50} (hot plate

test in mice) of DAGO (∿ 1 nM) and DSLET (130 nM) in good agreement with their K_I on μ-sites. The i.c.v. co-administration of DAGO at its ED_{50} with various doses of DSLET leads to only additivity effects. Therefore, the observed lack of potentiation seems to preclude the recently proposed *in vivo* allosteric interaction between μ and δ-receptors [9].

Conclusion. All the present results unambiguously demonstrate the occurrence of distinct μ and δ opiate binding sites. Antinociceptive responses to hot plate stimili involve a selective stimulation of μ-receptors, whereas δ-binding subtypes could be implicated in behavioural control through modulation of dopamine tonus in limbic system. This hypothesis seems to be supported by preliminary experiments after systemic administration. Finally both μ and δ-receptors could regulate the firing of a single neuron as recently shown in the case of bulbar respiratory neurons [10].

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ROLE OF THE PEPTIDE BACKBONE IN BIOLOGICAL ACTIVITY: SYNTHESIS OF ENKEPHALINS WITH $\psi[CH_2S]$ AND $\psi[CSNH]$ AMIDE BOND REPLACEMENTS

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The endogenous opiate, leucine enkephalin, has proven to be a popular and extremely sensitive host for the introduction of a variety of structural perturbations. Of particular interest has been the examination of more subtle backbone modifications, examined singly and in seriatim, which could lead to dissociated biological activities with respect to the mu (μ) and delta (δ) receptors. We wish to report our results with the amide bond replacements $\psi[CH_2S]$ and $\psi[CSNH]$, which have led to interesting biological consequences.

Using both solution and solid phase methods of peptide synthesis, analogs of leucine enkephalin and [D-Ala^2]leucine-enkephalin containing thioamide and

thiomethylene ether amide bond replacements at each of the four amide bonds (with one exception each as noted below) have been prepared and purified to homogeneity, using various criteria, including analytical reversed phase high performance liquid chromatography. In many cases, the structural assignments were also confirmed by FAB mass spectroscopy.

The pseudopeptides were assayed with respect to their pharmacological activities on the standard guinea pig ileum and mouse vas deferens preparations. These two techniques are believed to offer a useful method for differentiating between affinities of peptides to the μ and δ opiate receptors, respectively, since the mouse preparation consists primarily of the δ receptor population.[1]

It has been previously reported that an enkephalin analog with a ψ[CH_2S] modification and an N-terminal bis-allyl group results in a marked affinity for the δ-receptor when incorporated as a 3-4 amide bond replacement[2] while we demonstrated low activity for Tyr-Gly-Gly ψ[CH_2S]Phe-Leu in the guinea pig ileum assay.[3] Our present results with the additional incorporation of a D-Ala^2 replacement are thus of considerable interest with respect to the question of dissociation of biological responses. As seen in the Table, the incorporation of a ψ[CH_2S] replacement at the C-terminus results in retention of substantial biological activity in both assays. In contrast, activity is virtually abolished when the amide bonds at positions 2-3 and 3-4 are replaced by a thiomethylene ether moiety, even though the activity-enhancing D-Ala^2 modification is present in these analogs. However, it is not yet possible to determine whether the D-Ala^2 moiety either diminishes or potentially enhances the dissociation of biological responses when combined with the ψ[CH_2S] replacement. The reduced activities seen with some of these analogs seems most consistent with the need for a more rigid amide bond replacement in small linear peptides.

The comparison of one of the two possible ψ[CH_2SO] sulfoxide isomers (VI) obtained by peroxide treatment of V reveals it to be approximately 10 times more potent than the sulfide. In contrast, the sulfoxide was no more potent that the sulfide in a binding assay.[3]

The thioamide ψ[CSNH] replacement should be of even greater theoretical interest since it substantially retains all the important structural parameters of the amide group. Although it has not yet been possible to prepare the analogous 3-4 ψ[CSNH] analog, preliminary results from a 2-3 thioamide replacement are even more surprising. In this case, the simple substitution of a carbonyl oxygen by sulfur results in an analog with substantially retained potency on the GPI assay but eliciting one order of magnitude greater potency on the MVD assay. The net result is a peptide possessing a GPI/MVD IC_{50} ratio of greater than 50 to 1.

The other thioamide analogs behave more as their amide counterparts or show decreased potency (Table I). Nevertheless, these results suggest that peptide backbone

Table I. Mouse Vas Deferens (MVD) and Guinea Pig Ileum (GPI) Assay of Enkephalin Analogs

Compound	MVD IC_{50} [nm]	MVD Relative Potency	GPI IC_{50} [nm]	GPI Relative Potency	GPI/MVD IC_{50} Ratio
I. Tyrψ[CSNH]Gly-Gly-Phe-Leu	>57,000	<0.0002	—	--	--
II. Tyr-Glyψ[CSNH]Gly-Phe-Leu	1.20	9.52	61.5	4.00	53.9
III. Tyr-Gly-Gly-Pheψ[CSNH]Leu	5.23	2.18	74.1	3.32±0.09	14.2
IV. Tyr-D-Alaψ[CH_2S]Gly-Phe-Leu	>114,000	<0.0001	inactive	inactive	—
V. Tyr-D-Ala-Glyψ[CH_2S]Phe-Leu	14,400	0.00079	—	--	--
VI. Tyr-D-Ala-Glyψ[CH_2SO]Phe-Leu	1,360	0.0084	—	--	--
VII. Tyr-D-Ala-Gly-Pheψ[CH_2S]Leu	4.37	2.61	34.3±8.5	7.17±1.78	7.85
VIII. Leucine-Enkephalin	11.4	1	246±39	1	21.6

modifications can not only lead to increased potency, or prolonged duration of action, but that they also reveal vital functional roles in a) stabilizing important receptor-active conformational forms and/or b) interacting directly with their receptor counterparts.

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STRUCTURE FUNCTION STUDIES IN DI- AND TRIPEPTIDES RELATED TO DES [GLY]^3 ENKEPHALIN

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Introduction

Recently, dermorphin (HTyralaPheGlyTyrProSerNH_2) isolated from frog skin[1], and morphiceptin (HTyr-Pro-Phe-ProNH_2) a synthetic tetrapeptideamide based on a sequence from β-casein[2], have been found to deviate from typical opioid peptide sequence in that the important tyrosine and phenylalanine residues are linked by a single amino acid. We were interested in investigating the minimum structural requirements[3] in this series with the objective of discovering di- and tripeptides with increased potency in antinociceptive tests.

Methods

Chemistry - The analogues were synthesized by solution methods.[3] The purified peptides were characterized by NMR spectra and amino-acid and elemental analyses and were >98% pure by analytical HPLC.

Biology: The guinea pig ileum preparation was according to the method of Kosterlitz and Watt[4]; the mouse vas deferens preparation according to the method of Hughes, et al.[5] Responses to acetylcholine-induced writhing[6] were measured on

a quantal basis: test drugs were given in saline either by the subcutaneous (s.c.,1 ml/kg) or intercerebroventricular (i.c.v., 5 µl/mouse into lateral ventrical) route.[7] Acetylcholine was administered intraperitoneally 4 mins following i.c.v. and 10 min following s.c. administration. Enzymatic stability was assessed in rat kidney homogenate.[8]

Results and Discussion

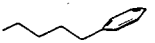
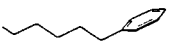
Our initial objective was to determine the minimal structural requirements in the dipeptide series (Table I). It was soon found that the phenylpropyl analogue (2) possessed the optimal chain length for agonist potency.[9] A similar result was obtained in the tripeptide series where the "homophenylalanine" analogue (9) had optimal potency.

A comparison of the effects of methylation in the ala^2 series (Table 1) and the ala^2 enkephalins[10] is of interest: in all series methylation at the tyrosine nitrogen is allowed: methylation at the ala^2 residue is allowed solely in the dipeptide series: methylation at the third residue drastically reduces potency in all series. Methylation at amide nitrogen can have a dramatic effect on enzymatic stability.[10] In the dermorphin series methylation at tyrosine (5, 10) or any internal amide nitrogen (6,8,11,12) resulted in analogues which were essentially stable to rat kidney enzymes. Interestingly, methylation of the C-terminal amide nitrogen (7,13) appeared to destabilize the sequence to rat kidney enzymes.

The dermorphin analogues were also evaluated <u>in vivo</u> using the acetylcholine writhing procedure in the mouse (Table 2).[6] The dipeptides (6,8) and the tripeptide (14) exhibited poor antinociceptive potency by the s.c. route. When given i.c.v.[7] there was a good correlation between the tripeptide (14) and morphine (16) relative to their potency <u>in vitro</u>. The dipeptides were again less potent than their pharmacology

in vitro would suggest. In contrast, the enkephalin derivative (15) was considerably more potent.

Table I

Cmpd	Structure	Guinea Pig Ileum Normorphine= 100	Rat Kidney Homogenate $t^{1/2}$ (min.)
1	HTyr ala NH 	22	
2	HTyr ala NH 	140	
3	HTyr ala NH 	16	
4	HTyr ala NH 	12	
5	MeTyr ala NH Phpr	85	>90
6	HTyr Meala NH Phpr	221	>90
7	HTyr ala NMePhpr	3	24
8	MeTyr Meala NH Phpr	125	>90
9	HTyr ala Hfe NH Ia	165	20
10	MeTyr ala Hfe NH Ia	340	>130
11	HTyr Meala Hfe NH Ia	8	120
12	HTyr ala MeHfe NH Ia	7	>130
13	HTyr ala Hfe NMeIa	44	6

Phpr = 3-phenylpropyl; Hfe = (R)-2-amino-4-phenylbutanoic acid; Ia = 3-methylbutyl

There are two possible explanations for the discrepancy between pharmacological data *in vitro* and *in vivo*: either the receptors in the ileum are dissimilar to the receptors mediating the antiwrithing effects or there are differences in pharmacodynamic profile between the compounds. Studies addressing these issues are in progress.

Table II

Cmpd	Structure	Guinea Pig Ileum NM=100	Ach Writhing Mouse s.c.*	Ach Writhing Mouse i.c.v.+
6	HTyr Meala NHPhpr	220	5.5	N.T.
8.	MeTyr Meala NHPhpr	125	9.4	21
14	HTyr Met (O) Hfe NHIa	1200	4.2	0.1
15	HTyr ala Gly MePhe $NH(CH_2)_2N(O)Me_2$	730	0.2	0.001
16	Morphine	100	0.4	1.0

*s.c. ED_{50} mg kg^{-1}; +i.c.v. ED_{50} nM per mouse

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EFFECT OF THE MODIFIED METHIONINE SIDE CHAIN ON THE BINDING PROPERTIES OF MET^5-ENKEPHALIN

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Introduction

The sulfur atom of the side chain methionine residue in Met^5-enkephalin might be one of the "binding elements". In search of its possible contribution the following analogs have been synthesized:

1. H-Tyr-Gly-Gly-Phe-(S-Et)Cys-$NHCH_3$
2. H-Tyr-Gly-Gly-Phe-(S-Et)Cys-$N(CH_3)_2$
3. H-Tyr-Gly-Gly-Phe-(O-Et)Ser-OH
4. H-Tyr-Gly-Gly-Phe-(O-Me)Hser-OH

Three kinds of modifications were derived: a)The sulfur atom was replaced by the more electronegative oxygen and this was placed either in δ-(O-Me) or γ-(O-Et) position. b)The sulfur was displaced from δ- to γ-position (S-Et). c)The free carboxyl end was converted to monomethyl- and dimethyl-amides (analogs 1 and 2).

Methods

The analogs were synthesized by classical techniques using the N-trityl and the carbobenzoxy groups for the amino protection and mixed anhydride, DCC/HOBt and p-nitro-phenylester couplings in solution. The methodology for the preparation of the protected analogs 3 and 4 has been described elsewhere[1]. Compounds 1 and 2

were synthesized in a similar manner. The deprotected peptides were purified by gel filtration on a Sephadex G-10 column using 2M acetic acid as the eluent, followed by partition chromatography on Sephadex G-15 using the solvent system n-butanol-acetic acid-water (4:1:5). Purified peptides were characterized by amino acid analysis, analytical HPLC and TLC in three solvent systems. Receptor binding assays were performed in homogenates of guinea-pig brain at 0°C using tracers of different selectivity[2] such as [^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5]-enkephalin (μ-site) and [^{3}H]-[D-Ala^2, D-Leu^5]-enkephalin for δ-site (Table I). Binding was studied also in brain synaptosomes[3] and the displacements (μ-site) have been carried out against [^{3}H]-labelled dihydromorphine (Table II).

Results and Discussion

The structural modifications of the methionine side chain in a series of enkephalin analogs indicate: a) Compounds 1 and 4 show higher affinity for the μ receptor in comparison to Leu^5-enkephalin; b) increased hydrophobicity of 1, e.g [(S-Et)Cys-$N(CH_3)_2^5$]enkephalin (2), reduces considerably its binding ability; c) the sulfur containing analogs 1 and 2 have higher affinity for μ-sites while compounds 3 and 4 bind preferentially to δ-sites. Especially, [(O-Et)Ser^5]-enkephalin may be defined as δ-selective antagonist (Table I). It should be noted that all analogs tested against [^{3}H]-dihydromorphine had higher affinity than Leu^5-enkephalin with [(S-Et)Cys-$NHCH_3^5$]-enkephalin being the most potent (Table II). Interesting enough, the same as above modifications of the methionine side chain residue of the C-terminal heptapeptide of Substance P result in analogs with practically no detectable relative affinity and potency[4].

Table I. Inhibitory Effects (K_I, nM) of Enkephalin Analogs on the Binding of the μ-Ligand [^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5]-Enkephalin (1nM) and the δ-Ligand [^{3}H]-[D-Ala^2, D-Leu^5]-Enkephalin (1nM) in Homogenates of Guinea-Pig Brain at 0°C

Analog	μ-site slope	μ-site K_I,nM	δ-site slope	δ-site K_I,nM	μ/δ
[Leu^5]-enkephalin	0.96±0.10	11.5±1.0	0.98±0.06	2.50±0.77	4.6
[(S-Et)Cys-NHCH$_3^5$]-enkephalin	1.05±0.05	3.46±0.32	1.13±0.09	10.7±2.3	0.32
[(S-Et)Cys-N(CH$_3$)$_2^5$]-enkephalin	1.12±0.11	19.2±3.9	1.00±0.09	26.9±6.7	0.71
[(O-Et)Ser^5]-enkephalin	1.04±0.13	13.8±5.2	0.97±0.18	1.67±0.56	8.3
[(O-Me)Hser^5]-enkephalin	1.13±0.09	8.55±1.66	1.33±0.15	3.55±0.36	2.4

The values were the means ± S.E.M and were obtained from 3 estimations.The values for Leu^5--enkephalin obtained from Reckitt and Colman were 18.8±1.18 (μ) and 1.18±0.20 (δ) nM.

Table II. Inhibitory Effects of Enkephalin Analogs on the Binding of the μ-Ligand [^{3}H]-Dihydromorphine in Brain Synaptosomes

Analog	IC_{50}(M)	Activity (%)
[Leu^5]-enkephalin	$2.19.10^{-7}$ [a]	100
[(S-Et)Cys-NHCH$_3^5$]-enkephalin	$2.88.10^{-8}$	760.4
[(S-Et)Cys-N(CH$_3$)$_2^5$]-enkephalin	$4.40.10^{-8}$	497.7
[(O-Et)Ser^5]-enkephalin	$3.39.10^{-8}$	646.2
[(O-Me)Hser^5]-enkephalin	$3.71.10^{-8}$	590.3

Acknowledgments

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SYNTHESIS OF TYROSYL-5-AMINOVALERAMIDE ANALOGS AND THEIR ANALGESIC ACTIVITIES

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Introduction

It was reported that Gly-Gly β-bend of the enkephalin was important to hold its conformation.[1] In the previous report,[2] we synthesized H-Tyr-Ava-Phe-Met-OH(I, Gly-Gly amide bond in methionine-enkephalin was replaced with ethylene bond) to study a role of Gly-Gly amide bond and I showed the analgesic activity by intracisternal administration to mice. To study the minimum structure for the analgesic activity, several tyrosyl-5-aminovaleramide analogs(II,III,IV,V) were synthesized.

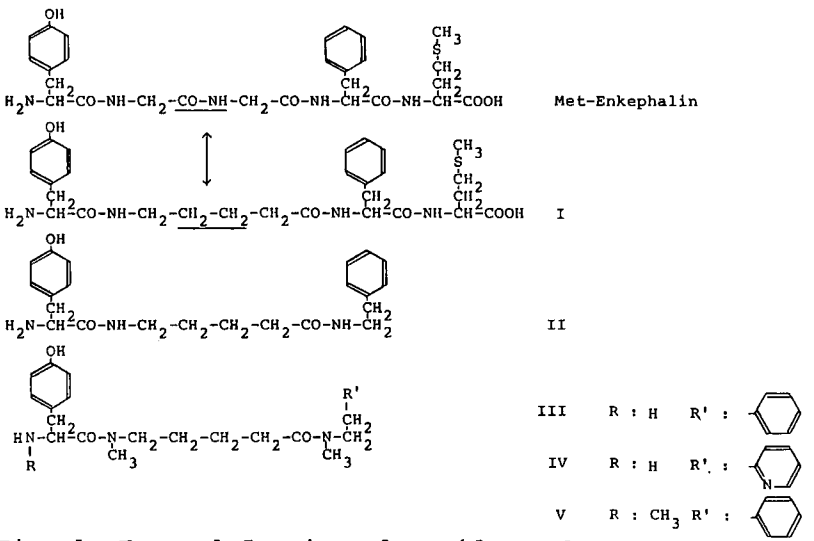

Fig. 1 Tyrosyl-5-aminovaleramide analogs

Methods

The synthetic scheme of II is shown in Fig.2.

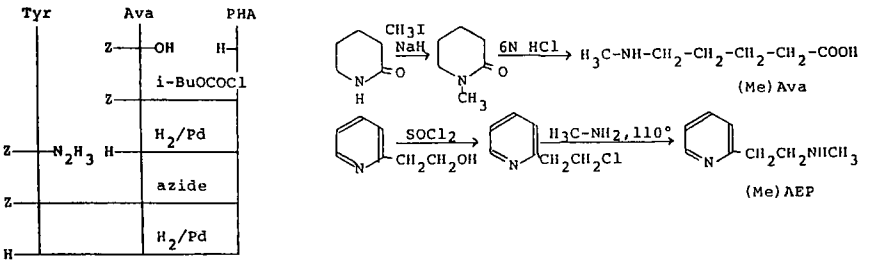

Fig.2. Synthetic scheme of tyrosyl-5-aminovaleramide analogs

III,IV and V were synthesized in the same manner to II. Preparations of (Me)Ava and (Me)AEP are also shown in Fig.2. For synthesis of V, Z-Tyr(Bzl)-OH was methylated in the usual manner and coupled with (Me)Ava-(Me)PHA followed by hydrogenation. The synthetic peptides were tested for the analgesic effect on mice by the tail-pinch method.

Results and Discussion

The analgesic effects of the synthetic peptides are shown in table I.

			5 min	15 min	30 min	
II	s.c.	83mg/Kg	0	0	0	(8)
III	s.c.	40mg/Kg	5	1	0	(10)
	i.v.	40mg/Kg	4	2	1	(8)
IV	s.c.	40mg/Kg	0	0	0	(8)

Table I. Analgesic effects of synthetic peptides. Numbers indicate effected mice. Numbers in parentheses indicate total mice examined.

II was not effective in dose of 40mg/Kg but its N-methylated

compound, III, was effective in the same dose by subcutaneous and intravenous injections. N-Methylations of Ava and PHA might prevent hydrolysis of the peptide by an enzyme and facilitate the peptide to pass the blood-brain barrier. Benzene ring might more effective than pyridine ring comparing the analgesic effects of III and IV. V was inactive in dose of 40mg/kg and the reason was not clear.

To study a adequate distance between Tyr and PHA to the analgesic activity, VI and VII(Ava in II was replaced with 4-aminobutyric acid or 6-aminocaproic acid) were prepared in the same manner to II.

II : Tyr-NH-CH_2-CH_2-CH_2-CH_2-CO-PHA

VI : Tyr-NH-CH_2-CH_2-CH_2-CO-PHA

VII : Tyr-NH-CH_2-CH_2-CH_2-CH_2-CH_2-CO-PHA

The analgesic effects of VI and VII are shown in Table II.

			5 min	15 min	
VI	i.v.	40mg/Kg	2	0	(10)
VII	i.v.	40mg/Kg	1	0	(10)

Table II. The analgesic effects of VI and VII

Both peptides showed weak analgesic effect. A definite distance for the analgesic activity between Tyr and PHA might not be necessary in the primary structure.

Acknowledgements

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DESIGN OF CONFORMATIONALLY-RESTRICTED CYCLIC α-MELANOTROPINS: COMPARISON OF MELANOCYTE-STIMULATING AND BEHAVIORAL ACTIVITIES

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Introduction

α-Melanocyte-stimulating hormone (α-MSH, α-melanotropin) is a linear tridecapeptide (Figure 1) which is biosynthesized and secreted by the intermediate lobe of the pituitary and an extensive system of neurons in the vertebrate central nervous system.[1-4] α-Melanotropin has been implicated in many physiological activities such as integumental melanin pigmentation (stimulation of melanin biosynthesis/melanosome dispersion), behavior (facilitated arousal, attention, motivation, learning, and memory), thermoregulation, fetal development, and somatotropin secretion.[2]

Previous structure-function studies of α-melanotropins have been primarily based on its melanocyte-stimulating activity *in vitro*[5-8] and, more recently, on its behavioral activities *in*

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vivo[4,9]. Although H-Met[4]-Glu[5]-His[6]-Phe[7]Arg[8]-Trp[9]-Gly[10]-OH ($ACTH_{4-10}$) represents an "active site" fragment of α-MSH it is only a weak agonist (about 1/100,000th the potency of α-MSH) in stimulating frog skin melanocytes in vitro. In contrast, in vivo this fragment is apparently equipotent to α-MSH (and ACTH) on delaying the extinction of pole-jumping avoidance behavior in the rat[4]; however, it has been reported to be inactive in effecting excessive grooming behavior (hereafter referred to as grooming behavior) in rats[9].

We have previously examined[10-12] the structural-conformational biological activity determinants of cyclic [Cys[4], Cys[10]]-α-melanotopins on several in vitro melanocyte systems. In this report, we describe the design and comparative bioactivities of several [Cys[4], Cys[10]]-cyclic α-MSH analogues as determined on frog (Rana pipiens) skin melanocytes in vitro and grooming behavior in the rat.

Results and Discussion

Design of [Cys[4], Cys[10]]-Cyclic α-Melanotropins. The [Cys[4], Cys[10]]-cyclic α-MSH analogues investigated in this report are listed in Figure 1. As reported previously[10,11] for [Cys[4], Cys[10]]-α-melanotropins, the design aspects of these conformationally-restricted α-MSH analogues have a similar basis which includes:

I	Ac-Ser(1)-Tyr-Ser-Met(4)-Glu-His-Phe-Arg(7)-Trp-Gly-Lys(10)-Pro-Val(13)-NH_2	α-MSH
II.	Ac-Met-Glu-His-Phe-Arg-Trp-Gly-NH_2	Ac-α-$MSH_{4-10}NH_2$
III.	H-Met-Glu-His-Phe-Arg-Trp-Gly-OH	$ACTH_{4-10}$
IV.	Ac-Cys-Glu-His-Phe-Arg-Trp-Cys-NH_2	Ac-[Cys[4],Cys[10]]-α-$MSH_{4-10}NH_2$
V.	Ac-Cys-Glu-His-Phe-Arg-Trp-Cys-Lys-NH_2	Ac-[Cys[4],Cys[10]]-α-$MSH_{4-11}NH_2$
VI.	Ac-Cys-Glu-His-Phe-Arg-Trp-Cys-Lys-Pro-NH_2	Ac-[Cys[4],Cys[10]]-α-$MSH_{4-12}NH_2$
VII.	Ac-Cys-Glu-His-Phe-Arg-Trp-Cys-Lys-Pro-Val-NH_2	Ac-[Cys[4],Cys[10]]-α-$MSH_{4-13}NH_2$
VIII.	Ac-Ser-Tyr-Ser-Cys-Glu-His-Phe-Arg-Trp-Cys-Lys-Pro-Val-NH_2	[Cys[4],Cys[10]]-α-MSH

Figure 1. Primary Structures of [Cys[4], Cys[10]]-Cyclic α-Melanotropin Peptides Compared to α-MSH and α-MSH Fragment Peptides.

(1) an "isosteric substitution of Cys^{4}~Cys^{10} for Met^{4}~Gly^{10} (Figure 2); (2) incorporation of the "active site" sequence (-Met^{4}-Glu^{5}-His^{6}-Phe^{7}-Arg^{8}-Trp^{9}-Gly^{10}-) into a semirigid 23-membered ring which may mimic a pseudocyclic bioactive conformation (for example, a β-, γ-, or C_7 turn) of the central heptapeptide sequence within α-MSH or other relevant α-MSH_{4-10}-containing analogues/fragments. In addition, we have examined the affect of stepwise elongation of the "active site" (α-MSH_{4-10} sequence) in the C-terminus direction to include the Lys^{11}, Pro^{12}, and Val^{13} residues.

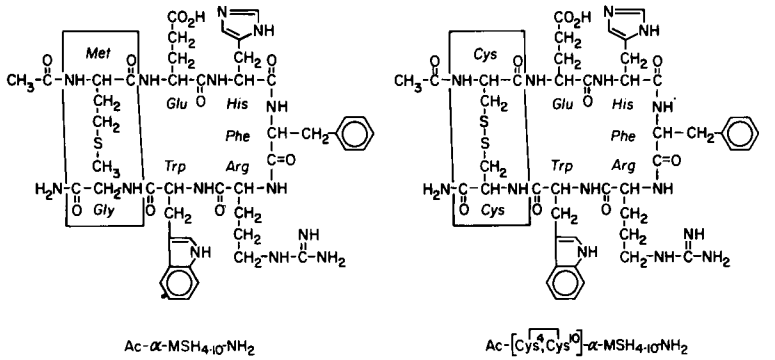

Figure 2. Two-Dimensional Representation of an "Isosteric" Structural Relationship Between Met^{4}~Gly^{10} of Ac-α-MSH_{4-10}-NH_2 (Depicted in a Pseudocyclic Conformation) and Cys^{4}~Cys^{10} of Ac-[Cys^{4}, Cys^{10}]-α-MSH_{4-10}-NH_2 (a cyclic heptapeptide).

It should be emphasized that introduction of the Cys^{4} Cys^{10} disubstitution in α-MSH was based solely upon previous structure-function studies of melanosome-dispersing activity *in vitro*, primarily on frog skin melanocytes. In particular, the unique melanotropic properties of partially racemized α-MSH analogues/fragments and of [Nle^{4}, D-Phe^{7}]-α-MSH[14,15] suggested that secondary structural features were functionally important for the bioactive conformation of α-MSH at its melanocyte receptor. In addition differentiation of melanotropic potency

and prolonged bioactivity was possible using either analogues of [Nle^{4}, D-Phe^{7}]-α-MSH^{16} or of [Cys^{4}, Cys^{10}]-α-MSH^{11,12}

Melanosome-Dispersing Activity In Vitro. The cyclic melanotropin fragments were tested for their relative melanotropic potencies in vitro using the frog (Rana pipiens) skin bioassay (Table 1). Ac-[Cys^{4},Cys^{10}]-α-MSH$_{4-10}$-NH$_2$ was about 100 fold more potent than its linear correlate, Ac-α-MSH$_{4-10}$-NH$_2$ (see Table 1). Successive addition of Lys^{11}, Pro^{12}, and Val^{13} to the C-terminus of the [Cys^{4}, Cys^{10}]-cyclic heptapeptide resulted in greatly increased melanotropic potencies of these

Table I. Relative In Vitro Melanotropic Potencies of [Cys^{4}, Cys^{10}]-Cyclic α-MSH Analogues in the Frog (Rana pipiens) Skin Bioassay.

Peptide	Potency Relative to α-MSH[a]
α-MSH	1.0
ACTH$_{4-10}$(H-α-MSH$_{4-10}$-OH)	0.00001
Ac-α-MSH$_{4-10}$-NH$_2$	0.0006
Ac-[Cys^{4}, Cys^{10}]-α-MSH$_{4-10}$-NH$_2$	0.07
Ac-[Cys^{4}, Cys^{10}]-α-MSH$_{4-11}$-NH$_2$	0.2
Ac-[Cys^{4}, Cys^{10}]-α-MSH$_{4-12}$-NH$_2$	10-100[a,b]
Ac-[Cys^{4}, Cys^{10}]-α-MSH$_{4-13}$-NH$_2$	10-100[a,b]
[Cys^{4}, Cys^{10}]-α-MSH	10-100[a,b]

[a]Relative potency = concentration of α-MSH at 50% response/ concentration of peptide at 50% response. Dose-response curves were generated using assay methods previously reported.[14]

[b]The relative potencies given for these analogues represent the results from assays over the linear portion of the dose-response curves and are quite variable from assay to assay. From studies of the minimal effective dose, however, these cyclic analogs are even more potent than the indicated values. The minimum effective doses are quite variable (ranging from 100 to 10,000 times greater than α-MSH), and appear to be seasonally dependent, and may reflect changes in compartmentalization of ligand and/or receptor sensitivity.[5]

peptides. The cyclic tridecapeptide, [Cys^{4}, Cys^{10}]-α-MSH was equipotent to Ac-[Cys^{4}, Cys^{10}]-α-MSH$_{4-13}$-NH$_2$, and in preliminary studies both were equipotent to Ac-[Cys^{4}, Cys^{10}]-α-MSH$_{4-12}$-NH$_2$. These results suggest a functional role for the C-terminus, particularly the dipeptide sequence Lys-Pro, to "potentiate" the melanotropic activity of the Cys^{4}-Cys^{10} disubstituted "active site" heptapeptide sequence (-Met^{4}-Glu^{5}-His^{6}-Phe^{7}-Arg^{8}-Trp^{9}-Gly^{10}-).

Grooming Behavioral Activity In Vivo. The [Cys^{4}, Cys^{10}]-α-MSH peptides were tested for their relative behavioral activities in vivo using the grooming assay in the rat (Figure 3). ACTH$_{4-10}$ was devoid of grooming activity which confirms previous observations. Acetylation of the N-terminus and carboxamidation of the C-terminus of the linear heptapeptide to give Ac-α-MSH$_{4-10}$-NH$_2$ also did not provide significant enhancement of grooming activity. However the Ac-[Cys^{4}, Cys^{10}]-α-MSH$_{4-10}$-NH$_2$ was very active (about a 6-fold increase relative to its linear counterpart) and produced a greater grooming response (about 20% higher) than α-MSH. Interestingly successive insertion of Lys^{11}, Pro^{12}, and Val^{13} into the C-terminus of Ac-[Cys^{4}, Cys^{10}]-α-MSH$_{4-10}$-NH$_2$ did not augment grooming activity. The Ac-[Cys^{4}, Cys^{10}]-α-MSH$_{4-11}$-NH$_2$ was identical to Ac-[Cys^{4}, Cys^{10}]-α-MSH$_{4-10}$-NH$_2$, and both Ac-[Cys^{4}, Cys^{10}]-α-MSH$_{4-12}$-NH$_2$ and Ac-[Cys^{4}, Cys^{10}]-α-MSH$_{4-13}$-NH$_2$ apparently possess slightly reduced behavioral activity relative to the [Cys^{4}, Cys^{10}]-cyclic heptapeptide. Addition of Ser-Tyr-Ser to the N-terminus of Ac-[Cys^{4}, Cys^{10}]α-MSH$_{4-13}$-NH$_2$, restored grooming activity to that of α-MSH.

Although the molecular basis for the high grooming activities of the [Cys^{4}, Cys^{10}]-cyclic melanotropins may be due to conformational restriction of the cyclic melanotropins compared to the linear melanotropins, the possibility of differential susceptibilities of the peptides to metabolic degradation or differences in biological compartmentalization must also be considered.

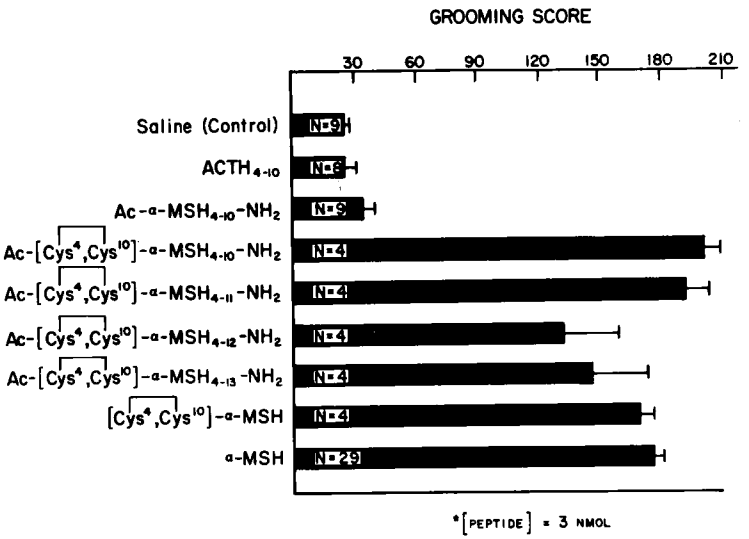

Fig. 3. Relative In Vivo Affects of [Cys^{4}, Cys^{10}]-Cyclic α-MSH Analogues on Grooming Behavior in the Rat. Relative activity = [grooming score of analogue (3 nmol in 5 μL saline)-grooming score of saline (citrate phosphate buffer, pH 7.4)/grooming score of α-MSH (3 nmol in 5 μL saline grooming score of saline] x 100. Grooming behavioral activities were generated using assay methods previously reported[17].

Proposed Reverse-Turn Bioactive Conformation. Integration of both experimental data and theoretical considerations have led us to postulate secondary structural features which may represent the bioactive conformation of α-MSH at the frog skin melanocyte receptor: (1) a peptide chain reversal within the His-Phe-Arg-Trp sequence; (2) a possible β-turn stabilized by intramolecular H-bonding between His^{6} C^{α}-CO and Trp^{9} N^{α}-NH; (3) proximity of Met^{4} and Gly^{10} (as inferred above in Figure 2); (4) lipophilic clustering of Phe^{7} and Trp^{9} side chains; and (5) hydrophilic clustering of Glu^{5}, His^{6}, and Lys^{11}.

Previous conformational studies of oxytocin antagonists and agonists (see reference 18 for a review) indicated that they

also possess topochemical features with a lipophilic surface and a more hydrophilic surface. Similar topographical features have been proposed[19] for other peptide and protein hormones which possess amphiphilic secondary structures (such as α- or π-helics or β-sheets). In the case of α-melanotropin, these same topochemical features also may appear in our proposed conformation. Specifically this would involve intramolecular proximity of the His^6 and Arg^8 side-chains as a positively charged "hydrophilic surface", and a "lipophilic surface" derived from intramolecular proximity of the Phe^7 and Trp^9 lipophilic side-chains (Figure 5).

We have recently described the superagonist melanocyte-stimulating activity of [Nle^4, D-Phe^7]-α-MSH (about 60-fold times more potent than α-MSH)[14,15]. In addition, [Nle^4, D-Phe^7]-α-MSH has been reported to be equipotent to α-MSH in

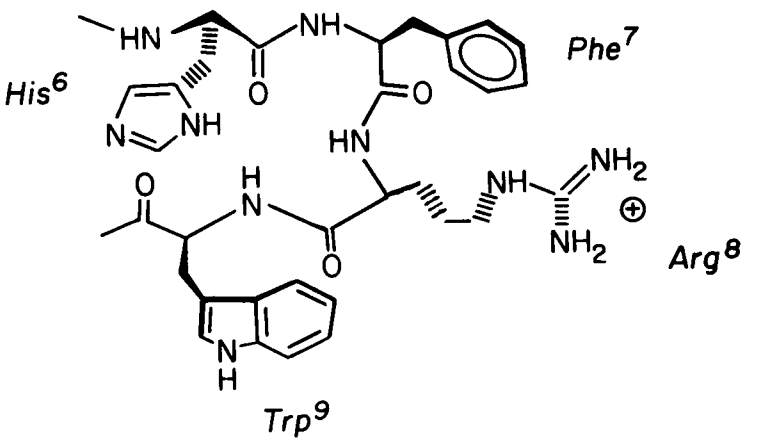

Fig. 4. Pictorial Representation of an "Amphiphilic Reverse Turn" Conformation of α-MSH_{6-9} Sequence (-His-Phe-Arg-Trp-). The peptide backbone of α-MSH_{6-9} is represented approximately within the plane of the page. The amino acid side-chains are subsequently depicted above (Phe^7 and Trp^9) or below (His^6 and Arg^8) the plane of the peptide backbone.

effecting grooming activity.[20] This data suggests that the D-Phe[7] substitution may stabilize or be compatible with a reverse turn-type conformation of the linear molecule which is functionally important to its bioactivity on these two different target tissue systems.

The design of future conformationally-restricted cyclic analogues of α-MSH and analysis of their comparative biological activities and physiochemical properties is expected to provide a more detailed understanding of the structural-conformational determinants at its different proposed physicological sites of action. Such α-melanotropins may find application to the detection or treatment (drug delivery agents) of melanoma (a skin cancer) and to specific neurobehavioral disfunctions in man.

Acknowledgments

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LHRH ANTAGONISTS WITH RESTRICTED CONFORMATION: α-METHYL RESIDUES AND DISULFIDES

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Introduction

Antagonists of LHRH (luteinizing hormone releasing hormone) are interesting because they are potential contraceptive agents. Compounds of the general structure N-acetyl L- or D-$(AA)^1$, D-4-(HAL)Phe^2, D-Trp^3, D-Trp^6 (or D-Nal(2)^6 or D-Arg^6)-LHRH, where $(AA)^1$ can be a variety of residues such as Ala, Phe, Trp, etc. and HAL are halogens, either Cl or F derivatives, are good antagonists and prevent ovulation in rats at subcutaneous doses of a few micrograms per animal (1). Information about the conformation of these antagonists is desirable for the design of even more effective antagonists.

The purpose of this work was to test predictions of conformation based on minimum energy calculations (2) by synthesizing antagonists having conformational restraints in the N-terminal part of the molecule. One preferred structure for the D-Phe^2, D-Trp^3 residues is the α_L, α_L conformational sequence (ϕ_2 = +60 to +80°, ψ_2 = +30 to +50°, ϕ_3 = +80 to +90°, ψ_3 = +60 to +70°). To test the occurrence of the α_L, α_L conformations at the 2 and 3 positions, six analogs having α-methyl residues were

synthesized and tested *in vitro* for inhibition of LH release and *in vivo* for antiovulatory activity.

Results and Discussion

The biological results, shown in Table I, are in agreement with a proposed helical bend type III in the 2 and 3 positions. That is, since α-methyl residues prefer either the α_L or α_R conformations, then analogs having α-methyl residues in these positions should have antagonist activity. Analogs I and II are about as active, both *in vitro* and *in vivo* as their respective parent compounds, VII and VIII.

In position 3, the results are not so clearly defined. Compound IV (D-α-Me-Trp^3) has only 10% as much antagonist activity *in vitro* as the parent compound (VII), while the α-methyl 2,3 analogs V and VI have even lower activity. Probably the very tight helical bend produced by methylating both positions 2 and 3 is unable to achieve a good fit to the receptor. This is not too surprising since the predicted backbone dihedral angles for residue 3 ($\phi \sim 85^\circ$, $\psi \sim 60^\circ$) are somewhat different than those found for α-methyl alanine: ϕ, $50 \pm 5^\circ$, ψ, $45 \pm 7^\circ$ (3,4). The corresponding predicted values for residue 2 are $\phi, \sim 60^\circ$, $\Psi, \sim 40^\circ$, a much better correspondence with the α-methyl values.

A second series of analogs was designed to lock the first four residues into a helical bend structure. These analogs (Table I, compounds IC through IVC) have a disulfide bridge between residues 1 and 4 (Ser^4 becomes Cys^4). The low antagonist activity of these compounds may be a result of the change in position 4, but this is uncertain. Since analogs with Cys^1 linked to a Cys^{10} (VC and VIC) have weak but significant activity (1), probably the Cys^1 modification is not responsible for loss of activity. Conformational energy calculations indicate that residues 2 and 3 are forced into a tight bend structure by the disulfide bond, and this is probably the reason for the low antagonist activity.

Peptide Synthesis

All the peptides were synthesized by the solid phase method using DCC with HOBt in the coupling step and 50% TFA in $CHCl_3$ for removal of the

Table I. Biological Activities of Synthetic LHRH-Analog Antagonists

	Analog	Dose (μg/rat sc)	# Rats	# Rats Ovulated	% Inhibition	$1DR^a_{50}$	Ref.
I	[Acetyl-Ala^1,αMeDPhe^2,DTrp^{3,6}]-LHRH	25	10	6	40	1.7:1	This work
II	[Acetyl-Ala^1,αMeDpClPhe^2,DTrp^{3,6}]-LHRH	6	10	3	70	0.28:1	"
III	[Acetyl-Ala^1,αMeDpFPhe^2,DTrp^{3,6}]-LHRH	6	9	4	56	0.7:1	"
IV	[Acetyl-Ala^1,DPhe^2,αMeDTrp^3,DTrp^6]-LHRH	200	10	8	20	33:1	"
V	[Acetyl-Ala^1,αMeDPhe^2,αMeDTrp^3,DTrp^6]-LHRH	200	9	9	0	73:1	"
VI	[Acetyl-Ala^1,αMePhe^2,αMeTrp^3,DTrp^6]-LHRH	200	9	9	0	110:1	"
VII	[Acetyl-Ala^1,DPhe^2,DTrp^{3,6}]-LHRH	30	10	5	50	2.8:1	b
VIII	[Acetyl-Ala^1,DpClPhe^2,DTrp^{3,6}]-LHRH	12.5	6	0	100	0.37:1	b
		6	11	2	82		
IC	[Acetyl-LCys^1,DpClPhe^2,DTrp^{3,6},LCys^4]-LHRH	200	6	6	0	100:1	"
IIC	[Acetyl-DCys^1,DpClPhe^2,DTrp^{3,6},LCys^4]-LHRH	200	9	7	22		"
		50	5	5	0	73:1	"
IIIC	[Acetyl-LCys^1,DPhe^2,DTrp^{3,6},LCys^4]-LHRH	200	7	6	14	19:1	"
IVC	[Acetyl-DCys^1,DPhe^2,DTrp^{3,6},LCys^4]-LHRH	200	7	6	14	87:1	"
VC	[Acetyl-DCys^1,DpFPhe^2,DTrp^{3,6},Cys^{10}]-LHRH	500	10	9	10	17:1	b
VIC	[Acetyl-DCys^1,DpFPhe^2,DTrp^{3,6},DCys^{10}]-LHRH	500	10	10	0	25:1	b

(a) Pituitary Cell Culture Assay, results expressed as the concentration ratio [Antagonist/LHRH] required to reduce the amount of LHRH-induced LH secreted by 50%.

(b) Analogs described in Ref. 1 - activity data repeated here for comparison purposes.

BOC groups. A benzhydrylamine resin was used for the linear peptides, with deblocking and cleaving from the resin by HF. The α-methyl amino acids were prepared by catalytic phase transfer alkylation (5) and were resolved by the selective action of chymotrypsin at pH 5.0 (6). The usual benzyl ester link to polystyrene was used for the cysteine-containing peptides and they were removed by ammonolysis in methanol. After chromatography on silica gel, the protected (S-ACM) peptides were oxidized with iodine to form the disulfides and then treated with HF to remove the remaining protecting groups. Final purification of all the peptides was by reverse phase preparative HPLC using the procedure described by Gesellchen et al. (7).

Bioassay Methods

The peptides were assayed for their ability to inhibit release of LH by the dispersed pituitary cell culture method of Vale et al. (8). Anti-ovulatory studies were done in rats as described previously (9) using corn oil to dissolve the peptides. They were administered at 12 noon of proestrus.

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ANTAGONISTIC LH-RH ANALOGUES CONTAINING UNNATURAL DIBASIC AMINO ACIDS IN POSITIONS 6 AND 8

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Introduction

Presently, in our laboratory, the most potent antiovulatory leuteinizing hormone - releasing hormone (LH-RH) antagonists contain a D-dibasic amino acid in position 6[1]. For example, the antagonist [Ac-D-p-Cl-Phe^{1,2}, D-Trp^{3} D-Arg^{6}, D-Ala^{10}] LH-RH produced 100% inhibition of ovulation at 5ug in the standard rat antiovulatory assay[2]. Replacement of D-Arg^{6} by D-Phe^{6} resulted in a moderate loss of activity, but analogues with neutral, alkyl amino acids such as D-Ala^{6} and D-Leu^{6} were much less active than the D-Arg^{6} analogue[3]. The aim of the present study was to explore the effect of side chain length and the combination of aromaticity and basicity on the antiovulatory activity of the antagonists by substituting novel amino acids in position 6. Since native porcine LH-RH contains an Arg residue in position 8, substitutions at this position were also studied.

Results and Discussion

To investigate the effect of both aromaticity and basicity

in one residue, the novel amino acid D,L-p-(aminomethyl)-phenylalanine (Amp) was prepared from 4-(bromomethyl)-benzonitrile by hydrogenation and hydrolysis of the diethylacetamidomalonate adduct. The amino acid was incorporated into the standard sequence via conventional solid phase peptide synthesis as its N^{a}Boc-N^{s}Z DCHA salt derivative. The resulting diastereoisomers were separated and purified by two stage preparative reverse-phase liquid chromatography using C-18 silica. The stereoconfiguration of each diastereoisomer was assigned tentatively, by chromatographic data and biological activity. The antiovulatory activity of the analogues is given in Table I.

Table I. Antiovulatory Activity of Position 6 Analogues.

Analogue	Dose /ug.	Inhibition of ovulation /%.
D-Arg^{6}	3.0	100
D-Lys^{6}	3.0	50
D-Amp^{6}	7.5	100
	3.0	67
D-Phe^{6}	7.5	86
Amp^{6}	7.5	17
	15.0	13
Arg^{6}	7.5	11

The D-Amp^{6} analogue had a similar antiovulatory activity to the standard D-Arg^{6} antagonist and was more potent than the D-Phe^{6} analogue. This suggests that the basic nature of the side chain in position 6 is of greater importance than the aromaticity of the residue. As expected, the Amp^{6} analogue and the Arg^{6} control were essentially inactive in the assay.

To study the effect of side chain length on LH-RH antagonism, D and L-2,4-diaminobutanoic acid (Dab) was synthesized stereospecifically, by the Schmidt reaction of

hydrazoic acid with D and L glutamic acid according to the method of van Nispen[4].

Table II. Effect of Side Chain Length on Antagonism.

Analogue	Dose /ug.	Inhibition of ovulation /%.
D-Dab^6	3.0	0
D-Lys^6	3.0	50
D-Arg^6	3.0	100
D-homo-Arg^6	3.0	0
	15.0	100

The results showed that as the apparent side chain length increases from Dab to Lys and from Arg to homo-Arg, the resulting antiovulatory activity increases and declines, with the maximum activity corresponding to the Arg side chain. From these observations, it is apparent that the hydrophilicity of the basic side chain at position 6 is more critical for antagonist activity than the aromaticity of the grouping. However, the length of the side chain is also clearly important since the short side chain D-Dab^6 analogue had very low activity when compared with the standard D-Arg^6, D-Lys^6 or the new D-Amp^6 antagonists.

In native LH-RH and its agonists, the Arg residue in position 8 is essential for gonadotropin release in the rat. We studied therefore, the effect of substitutions at this position. The inclusion of Lys^8 caused very little reduction in antiovulatory activity of the standard antagonist (50% at 3ug) whereas the substitution of Dab^8 caused a much larger loss of activity (18% at 3ug). Substitution of Gln^8 caused a complete loss of activity (0% at 15ug). Thus it is apparent that a basic group is also necessary for activity at position 8 in the standard antagonist sequence. The catastrophic loss of activity in the Gln^8 analogue is interesting since native avian LH-RH differs from porcine

LH-RH only by the substitution of Gln for Arg at position 8^5.

Acknowledgement

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PEPTIDE BACKBONE MODIFICATIONS: THE ψ[CH_2S] MOIETY AS AN AMIDE BOND REPLACEMENT

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Amide bond replacements in peptides have been shown to contribute to prolonged duration of action, altered solubility properties, and a variety of biological consequences including dissociated or selective responses, when contrasted with the parent structures.[1] Carefully chosen modifications have also revealed that the peptide backbone may also perform a function that transcends its historical role as a passive link between and among the active side chain elements.

Since its initial synthesis in 1977,[2] the thiomethylene ether surrogate (CH_2S--designated as ψ[CH_2S] in a generalized nomenclature scheme[3]) has been incorporated within a growing number of biologically active peptides.[4]

Synthetic routes leading to a variety of suitably protected Xxxψ[CH_2S]Yyy dipeptide analogs from appropriate pairs of chiral amino acids have been reported along with evidence of their stereochemical integrity.[5,6]

A recently completed X-ray structural study of Boc-Alaψ[CH_2S]Phe (S,S configuration) has shown the observed bond lengths and angles to be in good agreement with appropriate model compounds.[7] However, due to the somewhat folded nature of the structure, the C_α-C_α carbons of the pseudodipeptide are only 3.45Å apart, as compared to a value

of 3.8Å for a fully extended peptide chain. A model cyclic pseudopentapeptide, cyclo(Pro-Gly-Proψ[CH_2S]Gly-D-Phe) has been examined by proton nmr and been shown to retain two intramolecular (β-turn and γ-turn) hydrogen bonds, suggesting that these structural features are able to coexist with an intercalated amide bond replacement of the CH_2S type.[8] A recently completed synthesis of a biologically active cyclic somatostatin pseudohexapeptide analog (Figure 1), containing a CH_2S at one of the two postulated intramolecular H-bonded β-turns[9] should permit us to evaluate what overall conformational changes, if any, accompany this substitution.

Analogs of LH-RH and leucine enkephalin containing strategically placed ψ[CH_2S] amide bond replacements reveal a wide variation in biological potency ranging from virtual loss of activity to essentially equipotent behavior.[4] There is growing evidence from this and other laboratories that amide bond replacements can lead to a dissociation of biological action among different receptor classes.[10] Whether or not these selectivities are due to different conformational requirements by the hormone receptors is presently under investigation.

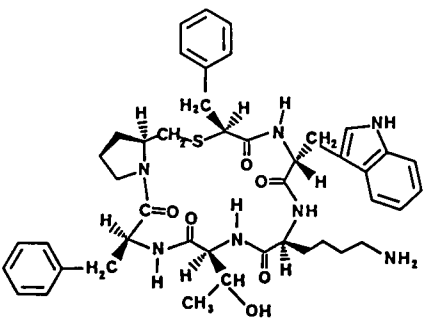

Figure 1 Somatostatin hexapeptide analog (based on the Veber et al. model) containing a CH_2S amide bond replacement between residues proline and phenylalanine (Proψ[CH_2S]Phe).

The stabilities of a number of enkephalin analogs containing $\psi[CH_2S]$ replacements at each of the four amide linkages have been examined with respect to human serum degradation. A typical analog, [D-Ala^2,Glyψ[CH_2S]Phe$^{3-4}$]-Leucine enkephalin, showed minimal degradation in this system (Figure 2). An unexpected observation was that an analog with only a 4-5 amide bond replacement was almost equally resistant, indicating that these backbone modifications can provide some protection to amide linkages at least three positions removed from the modified site.

In summary, the $\psi[CH_2S]$ replacement has been carefully studied in a wide variety of peptides and model compounds and should be considered as part of a useful repertoire of backbone modifications, each having its own unique but ascertainable characteristics.

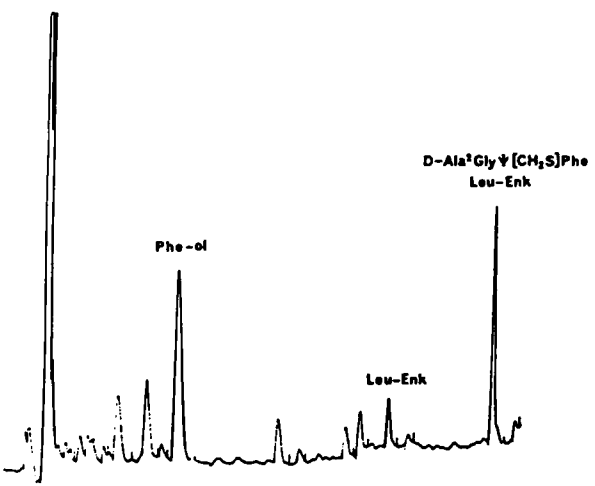

Figure 2. HPLC profile of a minimally degraded pseudo peptide [D-Ala^2,Gly ψ [CH_2S]Phe$^{3-4}$]-Leucine enkephalin analog following 20 min. incubation with human serum; additional peaks represent serum components and degradation fragments from leucine enkephalin as added internal standard. From *Spatola* and *Benovitz,* in preparation.

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STRUCTURAL AND CONFORMATIONAL STUDIES REGARDING TRYPTOPHAN IN A CYCLIC HEXAPEPTIDE SOMATOSTATIN ANALOG

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The tryptophan in somatostatin has been recognized as an important contributor to receptor binding.[1] The highly rigid cyclic hexapeptide analog of somatostatin, cyclo(Pro-Phe-D-Trp-Lys-Thr-Phe)[2] offers a unique framework for structure modification directed at defining the precise nature of the receptor binding of the indole ring.

The cyclic hexapeptides cyclo(Pro-Phe-X-Lys-Thr-Phe), X=D- and L-enantiomers of Trp, 5-F-Trp, 6-F-Trp, 5-MeO-Trp, and 1-Me-Trp were synthesized as examples of analogs containing electronically and sterically modified tryptophan residues. The indole ring substituted tryptophan derivatives were incorporated into the hexapeptides as racemates and the resultant diastereomeric products were separated to >99% isomeric purity by silica gel chromatography. Stereochemical assignments were based on CD and NMR spectral comparisons. The CD spectra showed characteristic differences in the 190-200 nm region for the two series of analogs containing D- and L-Trp.[3]

Biological evaluations (Table I) show that, contrary to prior studies with somatostatin and larger ring somatostatin analogs,[4] the L-Trp containing analog 2 exhibits equal potency to the D-Trp containing analog 1. This unique result indicates that the cyclic hexapeptide structure has stabilized the biologically active conformation in the L-Trp containing analog to the same extent as in the D-Trp analog.

In fact, key solution conformational features described[2] for **1** and measured by NMR chemical shifts, are also present for the L-Trp isomer **2**. These include proximity of the Trp-Lys sidechains, pronounced sidechain stacking in the Phe-Pro-Phe portion, and a cis acyl proline bond. The aqueous CD spectra of **1** and **2** also show similarities above 200 nm.[3]

The analogs **3**, **4**, **5**, and **6**, containing fluoro substituted tryptophans, show potencies equal to the unsubstituted analogs **1** and **2** and also show no difference between the L-and D-Trp isomers. Since fluorine substitutions represent electronic modifications with negligible effects on the steric environment, the high potencies of **3**, **4**, **5**, and **6** are an indication that receptor interaction is not affected by the electron distribution on the indole ring.

Table I. Hormonal Release Inhibition[a] by cyclo(Pro-Phe-X-Lys-Thr-Phe)[b]

Cmpd	X	Insulin	Glucagon	GH (in vitro)
1	D-Trp	5.2(2.4-11)	8.0(1.4-60)	1.7(1.3-2.3)
2	L-Trp	7.9(4.5-16)	9.1(3.8-32)	1.6(1.3-1.9)
3	D-5-F-Trp	8.1(5.3-13)	5.8(2.6-37)	2.4(2.1-2.8)
4	L-5-F-Trp	4.9(3.0-10)	5.0(2.6-22)	2.5(1.9-3.2)
5	D-6-F-Trp	3.7(1.6-14)	1.5(0.05-28)	1.0(0.7-1.4)
6	L-6-F-Trp	2.9(1.4-6.3)	4.2(1.6-3.5)	0.63(0.45-0.91)
7	D-5-MeO-Trp	0.2(0.1-0.4)	0.5(0.2-1.1)	0.02(0.01-0.03)
8	L-5-MeO-Trp	1.9(0.9-4.1)	1.5(0.9-2.8)	0.15(0.11-0.2)
9	D-1-Me-Trp	2.4(1.5-4.0)	5.2(2.4-15)	0.26(0.19-0.37)
10	L-1-Me-Trp	<0.07	<0.07	0.007(0.005-0.012)

[a]Bioassay protocol described in Veber, D.F. *et al*. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2636-2640; activites are relative to somatostatin=1; 95% confidence limits in parentheses. [b]Analogs were synthesized as described in Veber, D.F. *et al*. (1981) *Nature* **292**, 55-58.

Analogs **7**, **8**, **9**, and **10**, unlike the fluoro- and unsubstituted analogs, show significant differences in biological activities which are dependent on the chirality of the substituted tryptophan. The position-5 substituted analogs **7** and **8** represent the first examples of somatostatin analogs where the L-isomer is more active than the corresponding D-isomer. In the analog pair having a substituent in position 1 of the indole rings (**9** and **10**), the opposite activity profile from **7** and **8** is observed, with the D-isomer being the more potent one. These results are indicative of receptor interaction being highly dependent on the steric environment at tryptophan. The high potency of **9** demonstrates that the indole NH is not directly involved in receptor binding. Spectroscopic investigations showed that the variable potencies of **7-10** could not be associated with differences in their solution conformations. The aqueous PMR and CD spectra of the poorly active analogs **10** and **7** show all the characteristic conformational features found in the highly active analogs **2**, **4**, **6**, **8**, and **1**, **3**, **5**, **9**, respectively.

Since the activities of **7-10** could not be explained by differences in their solution conformations, we infer that the low potencies of **7** and **10** are the result of unfavorable receptor interaction. The substitution pattern is supportive evidence that in the D- and L-Trp containing analogs a

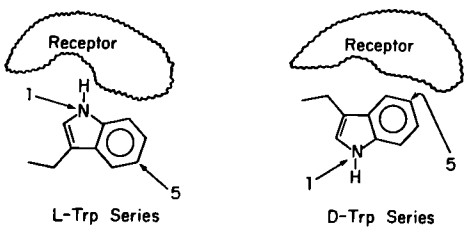

Fig. 1. Indole rings facing in opposite direction when bound to receptor allow substitution of bulky substituents in the 5 position in L- and 1 position in D-isomers.

different edge of the indole ring interacts with the receptor. Molecular modelling studies suggest that the substitution sensitive positions of the indole ring can best be superposed if the NH is facing in opposite directions in the L- and D-isomers (Figure 1). This rotational difference allows an understanding of the SAR observed. These results differ from those observed in larger ring, more flexible analogs.[5] Apparently the conformational rigidity of the cyclic hexapeptides allows less adaptation on receptor binding.

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MODIFIED RETRO ENANTIOMERS ARE POTENT SOMATOSTATIN ANALOGS

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Introduction

Retro enantiomeric peptides present an approach to stabilizing a biologically active peptide to the action of proteases.[1] Strict application of this concept, however, is limited by nonequivalence of side chain topography between the retro enantiomers.[2] Conformational analysis has now led to the design of potent cyclic hexapeptide somatostatin analogs which are retro enantiomers modified to retain the necessary overall molecular shape.

Results and Discussion

The highly active cyclic hexapeptide analog of somatostatin cyclo-(N-Me-Ala-Phe-D-Trp-Lys-Thr-Phe) (1) has been reported.[3] Conformational features in the working model of this structure believed to be important for biological potency include Phe-D-Trp-Lys-Thr and Thr-Phe-N-Me-Ala-Phe β-turns of Types II' and VI respectively, the latter containing a Phe-N-Me-Ala cis peptide bond. In the design of retro enantiomeric analogs of **1**, the overall shape of the molecule resulting from these turns and their attendant side

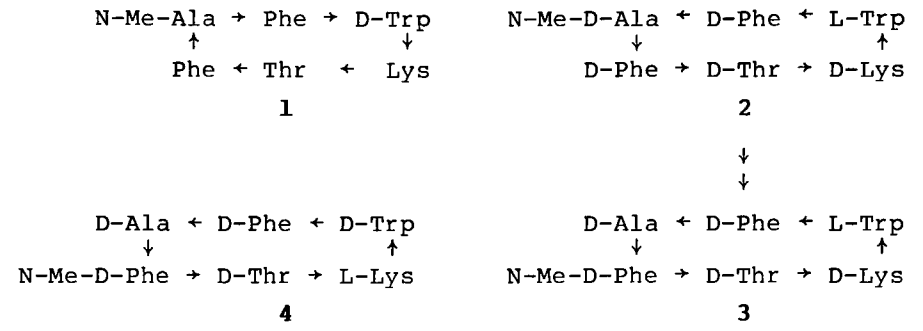

Fig. 1.

chain relationships should be retained. The retro enantiomer of **1** (**2**, Figure 1) would be expected to adopt a different preferred shape due to misplacement of the alanine N-methyl group. Compound **2**[4] was, in fact, only about 0.1% as potent as **1** (Table I).

Examination of molecular models revealed that the desired two β-turn conformation might be restored by moving the N-methyl group from D-Ala[6] to D-Phe[11] in **2**.[5] This change would allow the key cis peptide bond to form in the proper backbone location and should achieve better overall side chain correspondence with **1**. The resultant structure **3** gave a full biological response and showed an increase in potency of two orders of magnitude over **2**. Attention was next turned to improving the side chain correspondence to the postulated Type II' β-turn in **1** by changing D-Lys-L-Trp in **3** to L-Lys-D-Trp. A Type II β-turn should be favored in this new analog (**4**), and since II and II' turns are mirror images, better side chain matching might result. Such is apparently the case since **4** displays 25% of the potency of **1** and is of comparable potency to somatostatin. The overall bioactive conformational correspondence of **1** and **4** must be quite good because these relative potencies reflect a difference of less than 1 Kcal/mole of receptor binding energy. The remaining difference in potency between **1** and **4** could be due to the effect on conformation of small differences in backbone bond

Table I. Potency for Inhibition of Growth Hormone Release In Vitro by Cyclic Hexapeptide Somatostatin Analogs[a]

Somatostatin	1	
1	3.5	(2.6, 4.5)
2	0.003	(0.001, 0.005)
3	0.27	(0.2, 0.36)
4	0.88	(0.7, 1.10)

[a]For method of evaluation, see Veber, D.F. et al. (1981) Nature **292**, 55-58.

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lengths and angles for each pair of corresponding amino acids.[2] Alternatively, a hydrogen bonding contribution from the backbone to the receptor may have been lost in **4**. The results do indicate that the peptide backbone contributes at most a small amount to receptor binding of somatostatin and its analogs.

Evidence for topographic correspondence of **1** and **3** and **1** and **4** in aqueous solution has been obtained from proton nmr spectra (Table II). Upfield shifts of the γ-methylene protons of lysine and the β-methyl protons of alanine are

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Table II. Chemical Shifts of Upfield Shifted Protons in D_2O[a]

	1	2[b]	3[c]	4
Lys^9 γ-CH_2	0.33,0.52	0.48,0.98	0.62,1.05	0.53,0.99
Ala^6 β-CH_3	0.20	1.28	0.20	0.18

[a]Parts per million (ppm) relative to sodium trimethylsilylpropane sulfonate. [b]Major conformer (~75%); a methyl resonance at 0.59 ppm was observed for the minor conformer. [c]One of 2 conformers (1:1); the second conformer does not exhibit shifts of this magnitude.

observed for all three analogs. Two solvent shielded N-H's assigned to Thr^{10} and Phe^{7} in each case are evidence for similar environments for these protons in **1**, **3**, and **4**.

Both biological and solution data indicate that **1** and its modified retro enantiomers **3** and especially **4** have similar preferred shapes. This result was achieved through design based on conformational analysis. The concepts of Shemyakin may be applied, therefore, through conformational modification in spite of the inherent topographic differences between retro enantiomers.

Acknowledgements

We thank Mr. C. Homnick and Mrs. S. Fitzpatrick for hplc and amino acid analyses, Dr. D. Cochran for nmr spectra, Mr. J. Smith for FAB mass spectra, Mrs. M. Banker for typing the manuscript, and Dr. P. Anderson for his support of this work.

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4. Compounds **2-4** were synthesized according to procedures described in Ref. 3 beginning with a protected lysine bound to a solid phase support. Characterization was by amino acid analysis, ^{1}H nmr, and FAB mass spectrometry. HPLC purity was $\geq$92%.
5. Numbering of amino acids is N-Me-Ala^{6}-Phe^{7}-D-Trp^{8}-Lys^{9}-Thr^{10}-Phe^{11} and corresponds to the respective positions in somatostatin.

CONFORMATIONAL CHARACTERIZATION OF CYCLIC SOMATOSTATIN FRAGMENTS

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Structure-activity studies on the tetradecapeptide somatostatin suggested that not all the amino acids are important for eliciting the biological response.[1] The central portion of the molecule is most critical for agonist activity.[1] Veber and his associates synthesized a biologically active cyclic hexapeptide c(Phe^1-Pro^2-Phe^3-D-Trp^4-Lys^5-Thr^6) incorporating only 5 residues of the native molecule.[2] At the same time, we undertook the synthesis of the cyclic pentapeptide c(Phe^1-Phe^2-D-Trp^3-Lys^4-Thr^5), which is inactive.[3] Since the structures are so similar, it is likely that the differences in biological activity arise from conformational effects, which are considered here using preliminary molecular dynamics and nmr results.

Molecular dynamics simulations were carried out[4] to explore conformations for the two cyclic structures. Evolution of selected structures during the approximately 8 picosecond (ps) simulation of the cyclic pentapeptide is shown in Figure 1. A simultaneous change of ψ of tryptophan and ϕ of lysine takes place between 0.3 and 1.8 ps (upper plots). A static view of the structures at 0.8 ps of this transition is shown (left) to be compared to the different backbone conformation of a structure (right) at 3.6 ps. Another coupled motion involves the change of ϕ angle of the threonine and phenyl-

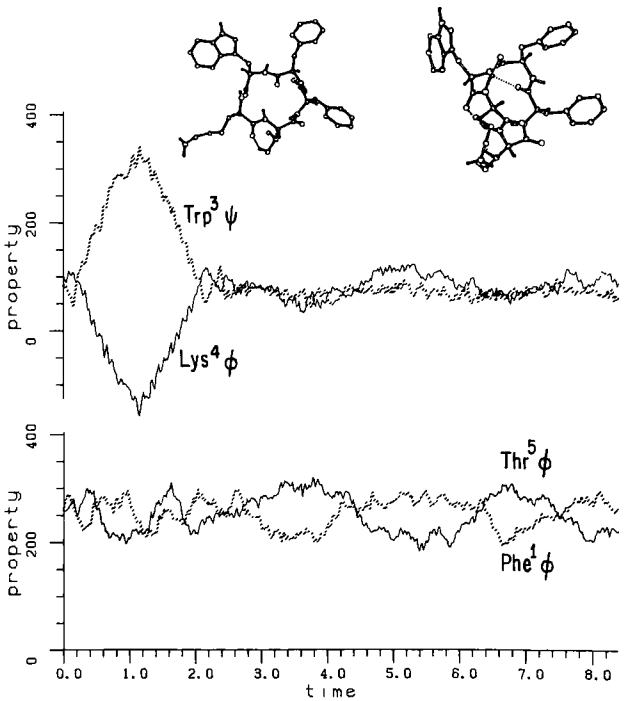

Fig. 1. Histories of selected backbone internal rotation from molecular dynamics simulation of c(Phe^1-Phe^2-D-Trp^3-Lys^4-Thr^5). T = 300°K, time scale in picoseconds. The structures shown occur at 0.8 and 3.6 ps.

alanine (lower plots of Figure 1). There is a wave-like behavior of the two angles which are roughly out of phase with respect to each other from 3 ps until the end of the simulation.

One of the transitions which occurs during a 20 ps molecular dynamics simulation of the cyclic hexapeptide is a coupled motion of the two adjacent backbone torsions, ψ of proline and φ of phenylalanine 3, at around 4.5 ps. Between 8 and 10 ps the side chain of phenylalanine 3 experiences a full rotation.

When the molecular simulations of the cyclic penta- and hexapeptides are viewed, one of the most significant differen-

ces appears to be the spatial relationship of the side chains of the aromatic residues. In the hexapeptide, the phenylalanine 3 and tryptophan side chains are involved in a stacking interaction throughout the simulation. In the pentapeptide the two aromatic rings are on opposite sides of the backbone ring. During these simulations the molecules do not show the interaction of the D-Trp and Lys side chains which was deduced from the upfield shift of γ-methylene protons of the Lys side chain in the nmr spectra of somatostatin analogs in D_2O.[5] The upfield shift was thought to be important for biological activity. However, we have observed the same effect for a number of inactive analogs; most notably the linear and cyclic pentapeptide analogs shown in Table I.

Table I. Chemical Shifts of γ-CH_2 of Lysine in Somatostatin Analogs *

Analog	δ (ppm)
Ac-Gly-Phe-D-Trp-Lys-Thr-NH_2	1.02
c(Gly-Phe-D-Trp-Lys-Thr)	.67
Ac-Phe-Phe-D-Trp-Lys-Thr-NH_2	0.85
c(Phe-Phe-D-Trp-Lys-Thr	0.65

*[in DMSO-d_6/D_2O 45:55]

The backbone conformations are significantly different in the cyclic penta- and hexapeptides. The hydrogen-bonded structures form and dissociate with high frequency during the simulations. In the pentapeptide the C_7 variety dominates at the two phenylalanines and the lysine residue. Characteristic but less frequent are the C_7 structures at the D-Trp and between the threonine OH and lysine CO. The most persistent H-bonded structures in the hexapeptide are a C_{10} around the Phe-Pro dipeptide link and the two C_7 structures around the threonine and phenylalanine 3.

We have examined brief simulations for a biologically inactive cyclic pentapeptide and a very closely related bio-

logically active cyclic hexapeptide. The two simulations do not show the D-Trp-L-Lys side chain interaction. In both series the upfield shift of the γ-methylene protons of the lysine chain is observed. The biological activity may be correlated with the side chain conformations leading to interactions between the aryl and indole rings of the Phe and D-Trp residues[6] present in the cyclic hexapeptide but absent in the cyclic pentapeptide simulations. We are carrying out further studies to assess the relevance of the Lys-Trp interaction to activity and the possible significance of the very different spatial relationship of the Phe and D-Trp side chains found in the simulation in the active and inactive analogs.

Acknowledgment

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SYNTHESIS AND SOME ACTIVITIES OF [1-PENICILLAMINE]DEAMINO-6-CARBA-OXYTOCIN

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Introduction

Analogues of oxytocin having the properties of uterotonic inhibitors of oxytocin could find use in the clinical practice as tocolytics in the cases of menacing miscarriage or preterm delivery[1]. An effective inhibitor must be able to block direct action of oxytocin on myometrial contractions as well as to prevent prostaglandin release by oxytocin stimulated uterus[2]. From the structural features leading to the oxytocin inhibitors in the in vivo uterotonic test we can name the following :

1) Dialkylation of cysteine β-carbon[3] in position 1 (this modification in the case of two methyl groups and molecule without primary amino-group is not sufficient for inhibitory action[4]).

2) Change of configuration of amino-acid in position 2 connected with the lipophilization of tyrosine moiety[5] by substituting the OH group by a more liopophilic one[6].

3) Alkylation of tyrosine hydroxyl, which again as single substitution does not lead to the in vivo acting inhibitor, but which can strikingly increase the inhibitory properties of the analogue, as well as acylation of the amino-group in position 1 (for review see[7]).

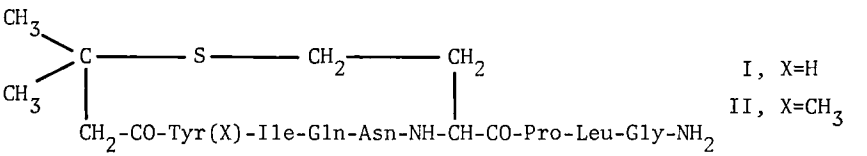
Further modifications leading to the increase of inhibitory activities of analogues are : substitution of threonine for glutamine, dehydroproline for proline and basic amino acid for leucine (for review see[8]).

The modification which we have found[6] extremely useful in the design of inhibitors with D-amino acid in position 2 and which has not been tested up to now in combinations with other structural features given above is carba substitution of disulfide bridge[9]. This substitution has striking

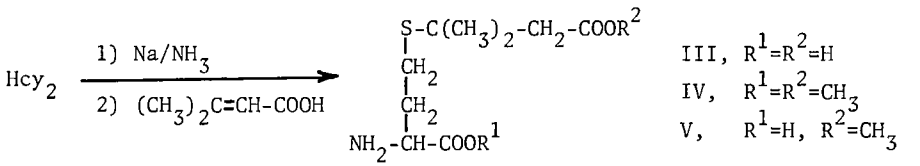
influence on the agonistic activities of prepared analogues[10].

Results and Discussion

We have prepared two analogues (I and II) containing in position 1 the deaminopenicillamine residue and carba-6 modification of the S-S bridge.



The first (I) differs from [1-penicillamine]deamino-oxytocin only by the exchange of methylene for sulfur and the second in addition contains O-methyl-tyrosine in position 2. For their synthesis we have chosen the stepwise elongation of the peptide chain using Nps protecting group and active esters for the coupling reaction. The critical step in the synthesis was the preparation of a suitably modified homocysteine derivative V, which is shown on scheme.



Due to the impossibility of selective esterification of α-carboxyl of compound III, it was necessary to use selective hydrolysis of diester IV. We have performed the cleavage of Nps group either in presence of mercaptoethanol or by the thiosemicarbazide hydrochloride[11] to overcome the splitting of the S-alkyl group. Cyclization was performed by means of active ester according to Krojidlo et al.[12], and the product was purified by reverse -phase HPLC.

The results of biological tests are given in Table I. As can be seen, in comparison with [Pen^1]dOT, analogue I has a very high degree of its own uterotonic activity in vivo and has very weak inhibitory activity in the pressor test. On the other hand analogue II is one of the most potent inhibitors of oxytocin uterotonic activity in vivo, described up to now.

Table I

Uterotonic (UT), galactogogic (GA), antidiuretic (AD) and pressor (BP) activities of prepared analogues determined in rats (I.U./mg). Values in parenthesis mean antagonistic activity given as pA_2.

Compound[a]	UT in vivo	UT in vitro	GA	AD	BP	Ref.
OT	450	450	450	3	3	10
dOT	900	795	536	19	1.4	10
dCOT-6	2792	929	456	118	1.5	10
[Pen^1]dOT	P.A.[b]	(7.14)	Agonist	-	(6.27)	3,15
[Pen^1,Tyr(Me)^2]dOT	(6.86)	(7.76)	(6.94)	0.02	(7.59)	15,16
I	279.8	16.2	6.9	4.81	(6.82)	
II	(7.13)	(8.43)	0[c]	0.52	(7.43)	

a) OT oxytocin, dOT deaminooxytocin, dCOT-6 deamino-6-carba-oxytocin ; b) partial agonist ; c) inactive up to 2×10^{-2} mg.

The high uterotonic activity of compound I can be explained on the basis of Hruby's view on the importance of rigidization of the molecule for the inhibitory activity of analogue[13], as the CH_2-S group has a substantially higher degree of mobility than the S-S grouping. In the case of analogue II either the combination of dimethyl group in position 1 with O-methyltyrosine in position 2 leads to the increased rigidity or the elimination of free OH group (part of the active site of oxytocin[14]) from tyrosine connected with the increased lipophilicity of the analogue is sufficient for the inhibitory activity of the resulting molecule (the reason could provide also an explanation of the inhibitory activity of the D-amino acid in position 2 containing analogues[6]).

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UTEROTONIC OXYTOCIN AND VASOPRESSIN ANTAGONISTS WITH MINIMAL STRUCTURE MODIFICATIONS

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Introduction

Previous studies have shown that [2-Tyr(Et)]-deamino-oxytocin (dE-OXY) is an effective antiuterotonic analogue both in the rat and in the human *in vitro* as well as *in vivo* (1,2,3). In order to develop more effective inhibitors to the actions of vasopressin and oxytocin on the uterus we have now made further modifications at positions 2, 4 and 8 in the deamino-oxytocin structure. At the same time we were interested in the possibilities of achieving high antagonistic activity without the need of gem-disubstitution at the β-carbon atom at position 1. Thus the antagonism will in principle be created by modifications at position 2 (the supposedly "active element") consisting of side chain alterations and/or the change of configuration. Further modifications (positions 4 and 8) will affect factors like affinity to the receptor, distribution and metabolic stability.

Methods

All peptides were synthesized in our laboratories by the solid phase method. 1% Crosslinked polystyrene resin was used with loading in the range of 1 mmole peptide/g resin. The protected peptide was cleaved from the resin by ammonolysis in methanol. The purified protected peptide was deprotected by reduction with sodium in liquid ammonia. The cyclisation was achieved by oxidation with iodine in a mixture of methanol-acetic acid. The final product was purified by gel filtration on Sephadex G-25. The purity of all products was checked by TLC and RP HPLC methods. Final purification was achieved by large scale preparative RP HPLC.
Antiuterotonic activity on rat uterus *in vitro* was assayed according to Holton 1948 as modified by Munsick 1960. Activity *in vivo* was tested on anesthetized rats (1). The inhibitory effect of the synthesized analogues was also evaluated on myometrial strips obtained from pregnant women at term as well as from nonpregnant women (1). The antagonistic potency *in vitro* in rats was expressed in terms of pA_2. *In vivo* in rat as well as *in vitro* on human myometrium it was given as relative potency c f dE-OXY. Oxytocin was used as the agonist with exception only of experiments on nonpregnant human myometrium where AVP was employed. The antidiuretic potency was estimated according to Larsson et al. (4); however, the increase of the urine conductivity was used as a measure of the effect. Blood pressure assays were performed according to Dekanski 1952.

Results and Discussion

Modifications of the original analogue dE-OXY at either position 2, 4 or 8 or combinations of these resulted in analogues with enhanced affinity (higher pA_2 values) for the rat

uterine receptor although their specificity was sometimes lower (Table I). The new analogues were up to 12 times more effective inhibitors than dE-OXY in the rat in vivo (fig. 1, Table I). They were generally more potent inhibitors both on pregnant and nonpregnant human myometrial strips than was dE-OXY. There was a poor correlation between the inhibition found in rats and that found in preparations from the human myometrium. This was particularly evident in the case of the 4-threonine analogues. Furthermore, these peptides were more potent in the nonpregnant than in the pregnant human uterus. Some of the present analogues might be of clinical interest in primary dysmenorrhea and in premature labor, two conditions where vasopressin and oxytocin have been ascribed an ethiological role.

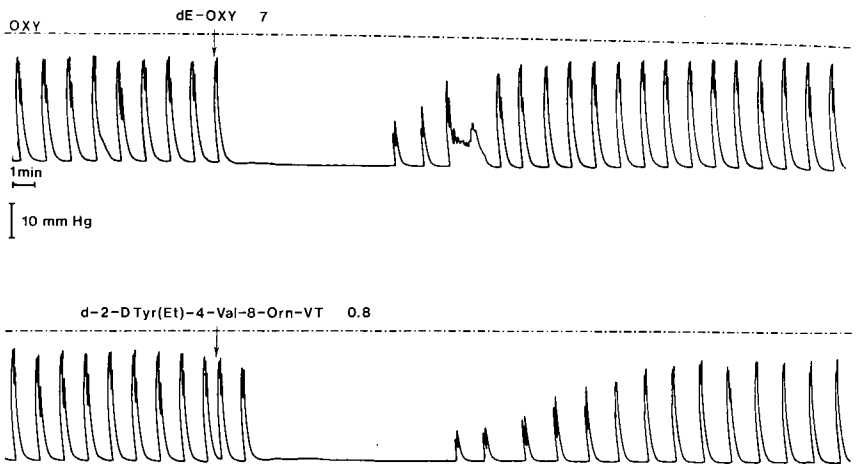

Fig. 1. Inhibitory effects of dE-OXY and d-2-Tyr(Et)-4-Val--8-Orn-VT (µg/100 g body weight, intravenously) on uterine contractions of an anaesthetized rat during continuous OXY-infusion (0.1 µg/min per 100 g body weight).

Table I. Oxytocin Analogues tested for Antagonistic Activity on Rat and Human Myometrium.

Peptide	Anti-diuretic act.	Pressor act.	Antagonistic effects: Rat in vitro pA_2	Rat in vivo	Human in vitro non-pregnant	Human in vitro pregnant
				c f analogue 1		
deamino--oxytocin						
1. $Tyr(Et)^2$	<0.2	<0.1	7.2	1	1	1
deamino--vasotocin						
2. {$Tyr(Et)^2Arg^8$}	37.9	2.5	8.4	2	0.3	
3. {$Tyr(Et)^2Orn^8$}	<1.3	1.4	8.4	5	2.7	0.4
4. {$Tyr(Et)^2Val^4Orn^8$}	0.4	0.9	7.6	6.0	2.9	1.8
5. {$Tyr(Et)^2Thr^4Orn^8$}	11.7	0.1	8.9	6.8	15.5	
6. {$DTyr(Et)^2Arg^8$}	1.0	1.8	8.7	9.4	3.8	4.6
7. {$DTyr(Et)^2Val^4Orn^8$}	0.4	0.2	8.2	12.1	5.2	3.5
8. {$DTyr(Et)^2Thr^4Orn^8$}	2.5	2.4	8.3	4.0	16.6	3.7
9. {$DTrp^2Val^4Arg^8$}	0.1	0.12	8.4	9.8	8.4	

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GLUCAGON RECEPTOR RECOGNITION: STUDIES WITH MODEL AMPHIPHILIC α-HELICAL PEPTIDES

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Introduction

Studies with synthetic models showed that peptide and protein secondary structure plays an important role in determining interactions with amphiphilic surfaces[1]. Examination of the amino acid sequence of the hormone glucagon suggested a potential amphiphilic α-helical region spanning residues 19-29. To determine the contribution of this region to the regulation of glucagon-hepatocyte receptor interaction, model peptides were synthesized which contained the sequence of glucagon (peptide I) from residues 1 through 18 and idealized amphiphilic α-helical structures in the region spanning residues 19-29. One peptide (peptide II) was designed to contain minimal amino acid sequence identity in this region while retaining the proper amphiphilic balance. Another analog (peptide III), designed upon the same principles but containing Phe-22 and Trp-25, was employed in investigating the role of these aromatic residues in receptor interaction and activation.

Results and Discussion

Peptides II and III, prepared by standard solid-phase synthesis, were purified to homogeneity by a combination of gel permeation

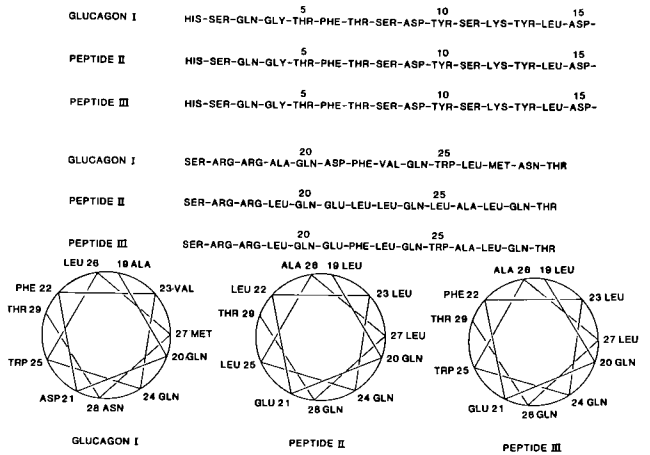

Fig. 1. Amino acid sequences and helical projections of glucagon, peptide II, and peptide III.

chromatography, partition chromatography and preparative HPLC. The sequences and Edmundson helical projections[2] of both peptide analogs are compared with those of glucagon in Figure 1.

Using isolated rat hepatocytes, we have investigated the binding and biological activities of glucagon and these glucagon analogs. Figure 2 illustrates the inhibition of ^{125}I-glucagon binding effected by glucagon and the glucagon analogs. For the binding of glucagon, the curve displays a double sigmoidal behavior which is in agreement with previously reported observations of glucagon binding to canine hepatocytes[3]. In the latter case, the data were analyzed according to a proposed scheme of two non-interacting receptor populations. Similar analysis yielded dissociation constants of $(5.7\pm1.4)\times10^{-11}$ M and $(4.1\pm1.0)\times10^{-8}$ M for the interaction of glucagon with the high and low affinity binding sites, respectively, in rat hepatocytes. On the other hand, the glucagon analogs have significantly altered biological properties. Inhibition of ^{125}I-glucagon binding could not be detected even by 10^{-5} M peptide II. However, peptide III is an inhibitor and displays a single sigmoid binding curve. More importantly, competition

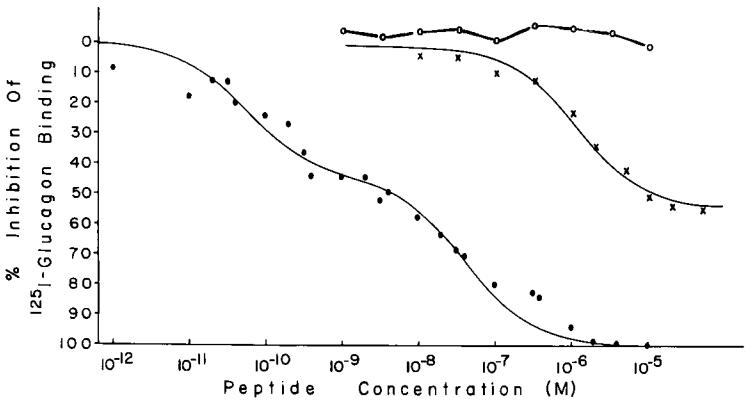

Fig. 2. Binding of glucagon(●), peptide II(O), and peptide III(X) to hepatocyte receptors.

for ^{125}I-glucagon binding by peptide III is circumscribed, a result suggesting interaction with only one of the two hormone binding sites. The data for binding of peptide III were analyzed according to a scheme of a homogeneous hormone-receptor interaction and yielded a K_D = 1.2 x 10^{-6} M.

To determine which receptor class accounts for the binding of peptide III, we studied the inhibition of ^{125}I-glucagon binding by glucagon in the presence of 10^{-5} M peptide III. Under these conditions, glucagon binding displays a homogeneous single sigmoid curve with K_D = 4.6 x 10^{-8} M, a value in close agreement with the dissociation constant of glucagon from the low affinity receptor population. Thus, peptide III binds to the high affinity receptor.

We assayed hormone-stimulated glycogenolysis by measuring the inhibition of radiolabeled carbohydrate flux into hepatocyte glycogen. As shown in Figure 3, peptide II has no measurable inhibitory potency; however, peptide III does exhibit glucagon-like biological activity. Figure 3 also indicates that maximal inhibition of carbohydrate incorporation occurs below saturation of the high affinity binding site

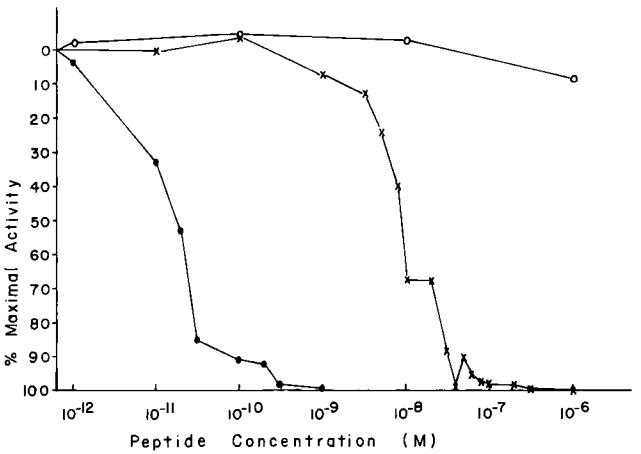

Fig. 3. Inhibition of carbohydrate incorporation in glycogen effected by glucagon(●), peptide II(0), and peptide III(X).

for both glucagon and peptide III. From this, we conclude that ligand interaction with the high affinity receptor population accounts for the physiological hormonal response expressed by glucagon and peptide III.

Our synthetic approach has clarified the analysis of glucagon receptor interaction. Since there is a guaranteed absence of native hormone in our analogs, activity cannot be ascribed to glucagon contamination. Also, our design has allowed the investigation of the function of structural domains of the hormone rather than specific amino acid side chains. Finally, peptide III represents an important and specific probe of the high affinity hepatocyte glucagon receptor.

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STRUCTURE-ACTIVITY STUDIES ON GLUCAGON - A POSITION 4 ANALOG WITH SUPERAGONIST PROPERTIES

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Introduction

Although glucagon is one of the oldest peptide hormones to have been sequenced, investigations into structural features responsible for its potent glucose-stimulating activity have been limited to semi-synthetic analogs derived from natural material and have therefore been quite limited in scope. However, improved solid-phase synthetic techniques and preparative hplc purification methods have made other related peptides such as VIP and secretin[1,2] routinely accessible. Here we report an extension of these methods to glucagon and a series of analogs containing modifications in the important N-terminal region.

Synthesis

Two separate solid-phase syntheses of glucagon and [Arg^{12}]-glucagon were conducted on a Merrifield resin using similar conditions to those reported previously[1,2]. Since glucagon contains a Trp residue, a mixture of TFA(33%):anisole(5%):H_2O(1%) in CH_2Cl_2 was used

for Boc removal. Analogs of the two standard peptides were obtained by splitting the resins at suitable points during the solid-phase syntheses. HF-cleaved peptides were purified by preparative hplc on ODS-silica (15-20 um, Whatman). Yields in the region of 6-10% were obtained. Homogeneity was demonstrated by analytical hplc, tlc, and amino acid analysis of acid and enzyme hydrolysates.

Biological Results and Discussion

In many semi-synthetic studies with glucagon, it has been necessary to protect the ε-amino group of Lys^{12} by conversion to homo-Arg. With this in mind, one batch of resin was prepared with Arg in position 12 in case potential synthetic difficulties forced us to resort to semi-synthesis. Subsequent yields soon made it clear that this was not necessary. The glycogenolytic activities of peptides were determined in the rat and are shown in Table I relative to synthetic glucagon of similar activity to natural material (Lilly). [Arg^{12}]-glucagon retained 50% activity which appears to agree well with moderate levels of activity reported[3] for the homo-Arg analog. A series of [Arg^{12}]-analogs were made with changes to the N-terminal His residue. Neither acetylation, methylation of the 3-imidazole nitrogen, or replacement by Phe resulted in much loss of activity. Even the [des-His]-analog retained 4% activity which is in agreement with activities reported for [des-His]-glucagon[4]. It is probable, then, that the His residue does not form part of an active center in glucagon. Of considerable interest was the Gly residue in position 4 of glucagon since it was possible that there could be a B-bend at this point based on analogous studies with LH-RH and the opiate peptides where replacement of Gly by D-Ala resulted in much improved activities[5,6].

[D-Ala^4,Arg^{12}]-glucagon did indeed exhibit almost full potency relative to [Arg^{12}]-glucagon. In LH-RH, replacement of Gly^6 by a bulkier, aromatic amino acid[7] produces much greater agonist activity. Therefore, [D-Phe^4]-glucagon was made and this was found to be almost 7 times more active than glucagon. The duration of action of this analog was similar to glucagon (unpublished observations) and it thus appears that the increased activity is due to higher receptor affinity rather than decreased enzymatic inactivation. In the whole VIP-secretin-glucagon family of pepides, position 4 is occupied by either Gly or Ala. In view of the present result, the presence or absence of a B-bend configuration could contribute to receptor recogonition and the diverse biological properties of these hormones.

Table I. Glycogenolytic Activities of Glucagon Analogs in the Rat

Analog	% Activity with 95% Confidence Limits
Glucagon	100
Arg^{12}	50(33-76)
desHis,Arg^{12}	4(3-6)
His(3-Me)^1,Arg^{12}	30(20-43)
Phe^1,Arg^{12}	31(22-43)
N-Ac-His^1,Arg^{12}	46(35-60)
D-Ala^4,Arg^{12}	44(32-61)
D-Phe^4	656(509-845)

Acknowledgement

This research was supported by NIH grant AM-30167.

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SYNTHESIS AND BIOLOGICAL ACTIVITY OF (TYR^{22}) GLUCAGON

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Introduction

Glucagon is a 29-residue peptide hormone that elevates blood sugar levels through its action on the liver. We have chosen to study the relationship between the structure of the hormone and its biological action by a total synthesis of glucagon analogs using the stepwise solid phase method.[1] The choice of analogs was based on the Chou-Fasman predictive model for secondary structure of peptides and proteins.[2] Application of these rules to glucagon shows that the C-terminal (19-27) region has a high potential for both α and β structures (<Pα>=1.18 and <Pβ>=1.15) and is, most likely, balanced between the two. This balance can be shifted readily by substituting only one residue. If a phenylalanine residue (<Pα>=1.13; <Pβ>=1.38) in position 22 is replaced by tyrosine (<Pα>=0.70, <Pβ>=1.49) the [Tyr^{22}]glucagon will still have flexible conformation, but one in which the β-pleated sheet will be slightly favored, with an overall potential of <Pα>=1.13 and <Pβ>=1.16.

Results and Discussion

The overall protection scheme for the synthesis of [Tyr^{22}]glucagon is shown in Figure 1.

Boc-His(Tos)-Ser(Bzl)-Gln-{^{14}C}Gly-Thr(Bzl)-Phe-
Thr(Bzl)-Ser(Bzl)-Asp(OcHex)-Tyr(Br-Z)-Ser(Bzl)-
Lys(Cl-Z)-Tyr(Br-Z)-Leu-Asp(OcHex)-Ser(Bzl)-Arg(Tos)-
Arg(Tos)-Ala-Gln-Asp(OcHex)-Tyr(Br-Z)-Val-Gln-
Trp(For)-[^{3}H]Leu-Met(O)-Asn-Thr(Bzl)-OCH_2-C_6H_4-
$CH_2CONHCH_2$-resin

Fig. 1. Protection scheme for the synthesis of [Tyr^{22}]glucagon

At each step of the synthesis the N^{α}-Boc-group was deprotected with 50% TFA/CH_2Cl_2. Each residue was coupled twice, first as a preformed symmetrical anhydride in CH_2Cl_2, followed by HOBt/DCC activation method in DMF. Exceptions were Boc-Asn and Boc-Gln which were coupled both times with HOBt/DCC. The completeness of the coupling reaction was monitored by the quantitative ninhydrin test.[3] At the end of the synthesis the N^{α}-Boc-group was deprotected with 50% TFA/CH_2Cl_2 prior to the final step in which the peptide was cleaved from the resin and all of the protecting groups were removed simultaneously using the improved HF procedure

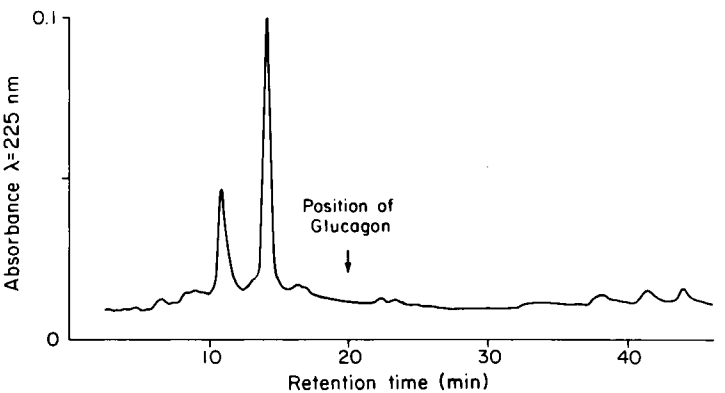

Fig 2. Analytical Bondapak C-18 HPLC of crude [Tyr^{22}]glucagon. Solvent system: Buffer A (10% CH_3CN in 90% 0.1% H_3PO_4), buffer B (50% CH_3CN in 50% 0.1% H_3PO_4). Linear gradient 30% to 80% B in 45 min.

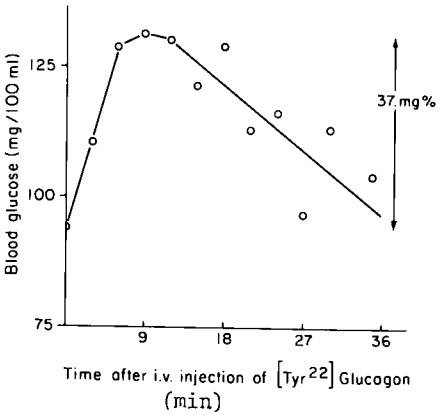

Fig. 3. Hyperglyceмic response in fasted rabbits to i.v. injection of [Tyr^{22}]glucagon at a dosage of 23µg/kg rabbit. Natural glucagon gave a 30-mg% rise at a dose of 5.9 µg/kg.

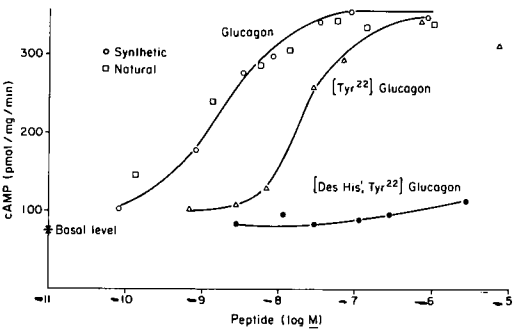

Fig. 4. Activation of adenyl cyclase in rat liver membranes. All peptides were assayed simultaneously. Each point is an average of duplicate sample.

recently developed in our laboratory.[4] The crude material contained 70% of the desired [Tyr^{22}]glucagon as determined by analytical (4 mm x 30 cm) HPLC µ-Bondapak C-18 column (Figure 2). Pure [Tyr^{22}]glucagon was obtained from a semi-preparative HPLC µ-Bondapak column (7.5 mm x 30 cm). Acid hydrolysis as well as enzyme digestion with amino-peptidase M, followed by amino acid analysis showed the expected ratio of amino acids.

The above result clearly shows that, with the use of more acid stable Pam-resin as a solid support, together with improved HF conditions for removal of the side chain protecting groups, medium size peptides of considerable complexity can be synthesized by the stepwise solid phase method in high homogeneity and yield.

Biological activity of [Tyr^{22}]glucagon was tested in vivo by measuring the hyperglycemic response in fasted rabbits. A typical curve is shown on Figure 3.

More quantitative data on the biological action of [Tyr^{22}]-glucagon were obtained by measuring the activation of adenyl cyclase in rat liver membranes[5] (Figure 4). In this assay synthetic glucagon that was obtained from a stepwise solid phase synthesis using the protection scheme shown in Figure 1, activated the hepatic adenyl cyclase to the same extent as natural glucagon (50% activation at 1.4×10^{-9}M). [Tyr^{22}]-glucagon had a specific activity about 10% of that of either natural or synthetic glucagon, but at higher concentration (10^{-6}M) it produced the maximum activation obtained with glucagon. It therefore acts as a full glucagon agonist. In this concentration range [desHis^{1}, Tyr^{22}]glucagon showed only trace activity (<0.1% of [Tyr^{22}]glucagon). The latter result is consistent with the one reported for desHis^{1}-glucagon obtained from the natural hormone.[6]

The reduced biological activity of [Tyr^{22}]glucagon suggests that the C-terminal region of the glucagon molecule plays an important role in the expression of its biological action.

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METALLOCENIC ANGIOTENSIN II ANALOGUES

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Introduction

Angiotensin II (AT), the blood pressure hormone with the sequence Asp-Arg-Val-Tyr-Val-His-Pro-Phe, is one of the longest known and most frequently modified peptide hormones. Structure activity relationship studies have shown the importance of the various amino acid residues, in particular the antagonistic behaviour of selected position 8 modification. Aliphatic replacements of phenylalanine produce competitive inhibitors, e.g. [Leu^8]AT. The potency of these inhibitors was increased by the concomittant replacement of aspartic acid in position 1 with sarcosine (Sar) e.g. [Sar^1,Leu^8]AT or the clinically used saralasin ([Sar^1,Ala^8]AT), and recent studies by some of us with very lipophilic replacements of Phe in position 8 have shown that the duration of action of such an inhibitor prolonges with increasing lipophilicity[1]. [Sar^1,Car^8]AT (Car = o - carboranylalanine, see Fig.) is such an analogue with prolonged activity, but the bulky side chain of Car has also a deleterious influence on the hormone affinity which falls to 0.12% compared to [Sar^1]AT.

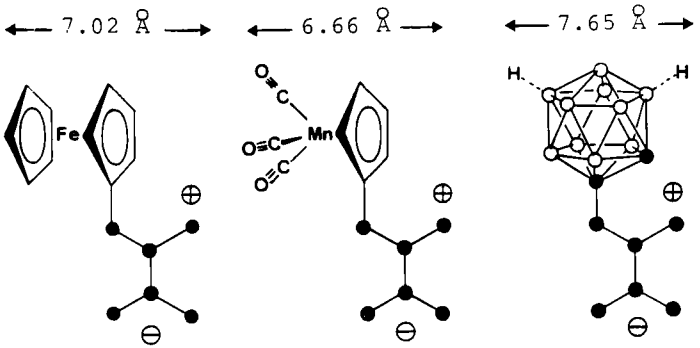
Syntheses

The availability of the interesting metallocenic amino acids ferrocenylalanine (Fer) and cymantrenylalanine (Cym) (see Fig.), which have both lipophilic and aromatic characteristics, made it interesting to investigate more in detail the function of position 8 in AT. Similar studies have already been carried out by some of us with the peptide hormones enkephalin, substance P and bradykinin [2,3]. The syntheses of Fer and Cym containing angiotensins were carried out by the solid phase procedure and the Boc-TFA scheme the enantiomers of D,L-Cym were separated as the brucine salts but their absolute configuration was not determined, D,L-Fer was used as such. These amino-acid residues were protected with the Boc-group and coupled to chloromethylated polystyrene as the cesium salt. Peptide-resin esters were cleaved concomitantely with the side chain protecting groups (Tos for Arg and His, 2,6-Cl_2-bzl for Tyr) by treatment in liquid HF/anisole 10:1, extracted with diluted acetic acid (enough ascorbic acid added to reduce completely any oxidized Fer) and lyophilized. The peptides were purified on LH20, eluted with DMF-ACOH 0.2M 1:3. The identity of the products was assessed by amino acid analysis, atomic absorbtion for Fer, 1.R-spectroscopy for Cym and the purity was verified by HPLC and two TLC systems.

Results and Discussion

The biological activities of the compounds was tested on rabbit aorta strips in vitro, on the binding to purified adrenocortical membranes and the results are presented in the Table. In both bioassays, the Car-containing analogue is the least active compound and retains partial agonistic properties on the rabbit aorta test. Both the D and L Cym diastereomers have in both bioassays almost identical potencies and

Figure: Amino acid structures



Fer Cym Car

TABLE: Biological activities

	R.A. Rabbit aorta	R.A. Membrane binding
[Sar^1]AT	100%	100%
[Sar^1,Car^8]AT	0.12%	4.10%
[Sar^1,D,L-Fer^8]AT	1.0 % A	6.1 %
[Sar^1,(-)Cym^8]	6.7 % A	11.0 %
[Sar^1,(+)Cym^8]	6.7 % A	8.9 %

R.A.$_1$ is the relative affinity of an analogue compared to [Sar^1]AT which has on rabbit aorta strip a half maximal dose (ED_{50}) of $0.74 \cdot 10^{-9}$M and deplaces 50% of bound tritiated [Sar^1]AT on purified bovine adrenocortical membranes at a dose of $9.8 \cdot 10^{-9}$M (= pure antagonist; their relative affinity is expressed by the comparison of the ED_{50} of [Sar^1]AT with the IC_{50} of this compound. IC_{50} is the dose of an antagonist that reduces the response to a double dose of agonist to that of a single dose. The values are the mean of at least six experiments and the standard error is always less than 22% of the indicated value.

properties, a result which is rather surprising. Both compounds are pure antagonists and are 10 to 15 fold less active than [Sar^1]AT, which is still a quite considerable activity for the bulky modification. The diastereomer [Sar^1,Fer^8]AT exhibits a much more important reduction in affinity, especially on the rabbit aorta test (pure antagonist) and is very close to [Sar^1,Car^8]AT (partial agonist).

These results resemble those obtained with the enkephalins, where the replacement of Phe by Cym was much better tolerated than the Fer-substitution[3]. We propose as the most probable explanation that the considerable bulk of the ferrocene-system is too large to be accepted, but that the more nimble carbonyl ligands on Cym are accomodating better with the binding site of the AT-receptor.

The biologically interesting [Sar^1, Cym^8]AT has several promising properties, mainly the photosensitivity of the cymantrene moiety which could be exploited for photoaffinity labeling and the possibility to exchange carbonyls on the Mn atom. The latter technique could become very promising for the visualisation of angiotensin in the living body with the PET-scan technique (positron emitting tomography) by exchanging carbonyls of [Sar^1,Cym^8]AT with positron emitting C-11 monoxide.

The two applications are under investigation.

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SUCCINYL BIS-BRADYKININS: POTENT AGONISTS WITH EXCEPTIONAL RESISTANCE TO ENZYMATIC DEGRADATION

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Introduction

Oligomerization of peptide hormones by attachment to a polyvalent carrier has provided super-potent and long-acting agonists in the MSH and enkephalin field.[1] Dimerization can be viewed as the simplest form of this structure modification, and has provided useful derivatives of several peptides, including LHRH and the enkephalins. Recently, the conversion of a dimeric LHRH antagonist to an agonist by the proper spacing between the LHRH moieties in the molecule has been demonstrated.[2] Bradykinin (BK), almost alone among biologically active peptides, has yet to be modified to produce specific inhibitors. We report here dimerization studies with BK.

Methods

Peptides were synthesized by the solid phase method, and purified by counter current distribution. Dimeric analogs were assembled on the resin from the monomer by treatment of the N-terminal free amino group with a large excess of bis-dinitrophenyl succinate in the presence of 1-hydroxybenzotriazole in DMF. After washing, a 10% excess of monomer was added to the the remaining nitrophenyl ester group on the succinate attached to the BK-resin. After HF cleavage and CCD the dimers were purified additionally on Sephadex G15 in dilute AcOH. Myotropic activity was determined in the isolated rat uterus and guinea pig ileum assays; the lowering of blood pressure in the anesthesized rat after intraaortic (IA) and intravenous (IV) administration was also performed as described.[3]

TABLE 1.

Biological Activities of Bradykinin Monomers and Dimers

BRADYKININ ANALOG	RUT	GPI	RBP-IA	RBP-IV	% BREAKDOWN
BK	100	100	100	100	98
Bz-BK	2	2	9	2	90
Pba-BK	19	15	18	56	90
Oct-BK	13	6	10	26	87
Sta-BK	0.1	0.1	0.7	<5	-
Eac-BK	36	25	13	82	94
Eac-Eac-BK	26	16	17	94	92
Sta-Eac-BK	30	19	9	80	79
Suc-bis(BK)	9	86	75	6521	0
Suc-bis(Eac-BK)	45	42	38	1324	0
Suc-bis(Eac-Eac-BK)	15	13	19	958	0

BK = Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg; Bz = benzoyl (C_6H_5CO); Pba = phenylbutyryl ($C_6H_5(CH_2)_3CO$); Oct = octanoyl ($CH_3(CH_2)_6CO$); Sta = stearoyl ($CH_3(CH_2)_{16}CO$); Eac = epsilon aminocaproyl ($NH_2(CH_2)_5CO$); Suc = succinoyl ($(CH_2CO)_2$). % Breakdown, which represents destruction in the pulmonary circulation, = $100-(ED_{25mm}IA/ED_{25mm}IV)(100)$, where ED_{25mm} is the dose necessary to depress the mean blood pressure 25mm.

Results and Discussion

All of the monomeric and dimeric Eac-containing BK analogs were made from one 1.0 mM batch of BK-resin. The initial batch was split, and each one third produced both the monomeric resin-bound BK analog and the monomer which was added to produce the symmetrical dimer. Calculations of peptide content and purity from amino acid analysis, and elution volumes from Sephadex G15 on columns sized with BK, were consistent with dimeric structures. TABLE 1 lists the BK analogs and their activity in the rat uterus (RUT), guinea pig ileum (GPI) and rat blood pressure (RBP) assays. N-terminal extended BK analogs are known to retain typical BK activities,[4] and the arylalkyl-extended analogs (Bz-BK, Pba-BK) show respectable

potencies and some resistance to pulmonary breakdown. Oct-BK exhibits some dissociation of smooth muscle activity, with a potency on the uterus twice that of the ileum relative to BK. The Eac-monomers of BK, which have a free amino group at their N-terminus, show less breakdown in the lung than BK and good smooth muscle activity.

The dimeric BK analogs show some remarkable activities. The simple BK dimer, Suc-bis(BK), has 9% of the potency of BK on the uterus, an essentially enzyme-free tissue preparation. It is 10 times more potent in the ileum where susceptability to enzymatic breakdown is an essential component of the observed potency. The dimeric Eac-BK and Eac-Eac-BK analogs are equivalent in their activities on the uterus and ileum, a situation normally seen with analogs which have similar resistance to enzymatic breakdown as BK. However, all three dimers are totally resistant to inactivation in the lung. This cannot be attributed solely to their lack of a free N-terminal amino group, nor to the large hydrophobic alkyl group at the N-terminals of the BK sequences within the dimers, as shown by comparison with the other non-dimeric analogs in TABLE 1 which have only a slight resistance to pulmonary breakdown.

The rat blood pressure lowering effect of Suc-bis(BK) after injection IV is second only to that of Lys-Lys-BK, some 65 times the potency of BK. One possible explanation is that in addition to the protection that the succinyl bridge provides in the systemic circulation it may also hold the two BK moieties in the proper spacial arrangement for the dimer to interact at more than one receptor at the same time. Such a cooperative affinity binding of BK sequences to neighboring biological receptors in the systemic circulation, and to a lesser degree in the ileum preparation, could account for the significantly increased activities observed. Increasing the separation of the BK sequences with additional alkyl bridge elements seems to give an optimum range for activity on the uterus with Suc-bis(Eac-BK). Additional bridge lengthening appears to allow too much conformational freedom for the BK moieties to "find" neighboring receptors in the tissues.

If the BK sequences in Suc-bis(BK) are properly spaced to interact with neighboring bio-receptors, then it may be possible to design

inhibitors of BK by incorporating BK analogs which have high affinity but low biological activity into dimers with BK agonist analogs. The combined affinity of the resulting dimer may provide a partial agonist which can then block adjacent receptors.

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SYNTHESIS OF A PEPTIDE WITH GROWTH HORMONE RELEASING ACTIVITY *IN VITRO* AND ITS DEPSIPEPTIDE ANALOG

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Introduction

We have previously described peptides which have specific growth hormone releasing activity in terms of their structure-function.[1-3] The most active pentapeptide described was Tyr-D-Trp-Ala-Trp-D-Phe NH_2 (I), which releases growth hormone in vitro at a dosage of 30 ng/ml medium. Analog studies, as well as conformational energy calculations, have been used to probe for the active conformation.[3] For example, the activity of the analogs Sar^3, Aib^3 and (n-Me) Ala^3 were reported previously and the structural constraints they impose on the backbone conformation lead to a postulated active conformation.

In this report we will continue the analog probe to test for the possible importance of the amide (N-H) at the Ala^3 residue. The depsipeptide Tyr-D-Trp-Lac-Trp-D-Phe-NH_2 (II), was synthesized for this purpose. We describe here the synthesis and biological activity of this analog.

Synthesis

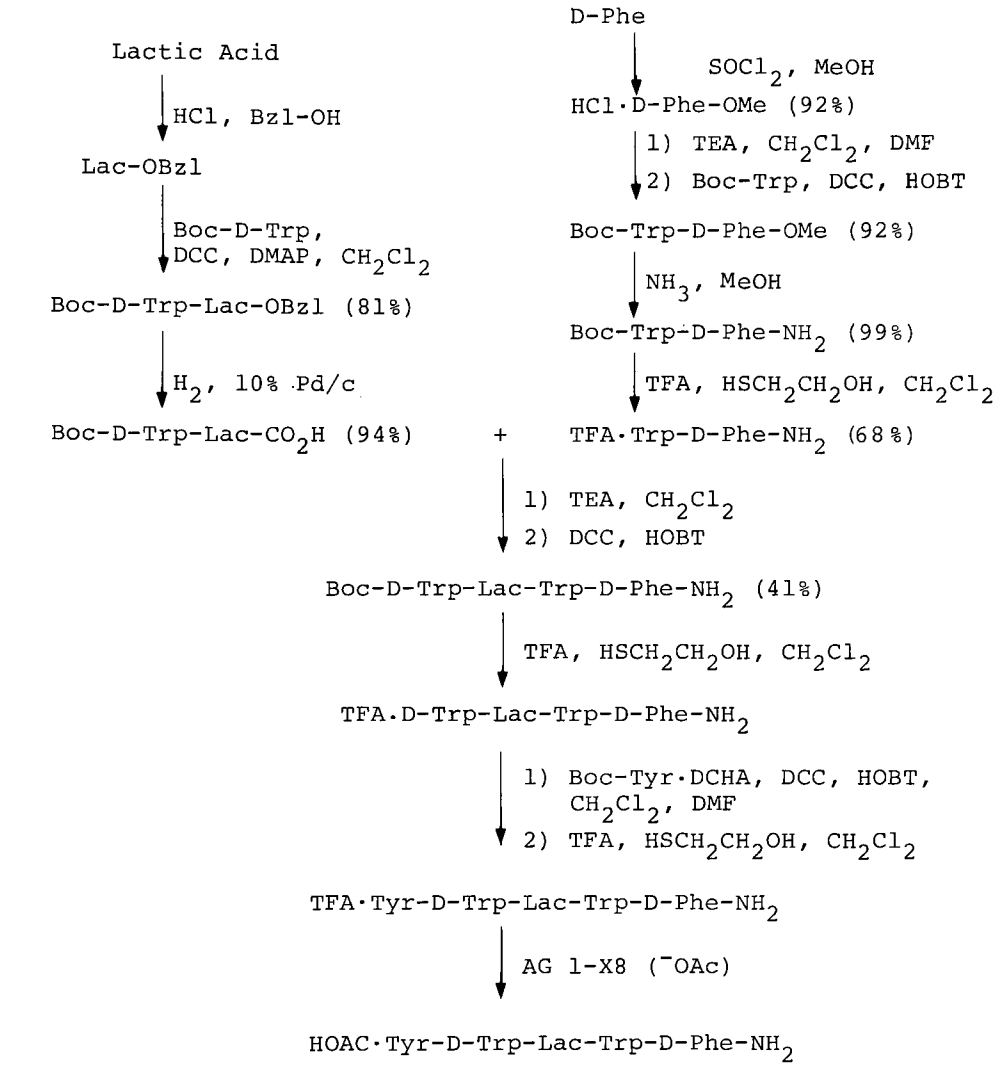
Synthesis of I was carried out by both solid-phase and solution synthesis. Solid-phase synthesis was performed using benzhydrylamine resin. Solution synthesis was performed by stepwise pentachlorophenol active-ester coupling, to form D-Trp-Ala-Trp-D-Phe NH_2. Then Boc Tyr was coupled to the tetrapeptide using DCC-HOBT. Boc-I was deprotected with a solution of trifluoroacetic acid and mercaptoethanol in CH_2Cl_2. The resulting TFA salt of I was purified using partition chromatography with 4:1:5 upper phase as eluent. An LH-20 column using methanol as eluent was used for further purification.

Synthesis of II was carried out by a 1 + 2 + 2 method as shown in Scheme 1. Attempts to synthesize II by a 3 + 2 coupling scheme was not successful. The reason was the failure to remove the C-terminal benzyl group of Boc-Tyr(OBzl)-D-Trp-Ala-OBzl by hydrogenation. Different solvents, e.g., DMF, MeOH and different catalysts, e.g., Pd/black and Pd/C were used in the attempts to remove the C-terminal benzyl group.

Results and Discussion

A comparison of the growth hormone releasing activity of I and II *in vitro*, using pituitaries of 20-day old female rats, is shown in Table I.[2] Peptide I has no LH, FSH or TSH activity at dosages up to 10 μg. Peptide II also gave no LH or FSH response at dosages up to 3 μg, although a slight FSH release was observed at 30 μg ($p < 0.05$).

As previously reported, the ϕ,ψ angle at D-Trp^2 favored a C_7^{eq} conformation. Since N-Me-Ala and Sar were relatively inactive compared to I, we suggested that N-H---O hydrogen bonding may be important for biological activity. The relative inactivity of II may further prove the importance of N-H---O hydrogen bond at the D-Trp^2 residue for the retention of an



Scheme 1. Synthesis of Tyr-D-Trp-Lac-Trp-D-Phe-NH_2

active conformation, since the ester bond in II lacks the NH group yet retains the conformational properties of an amide. Another reason for the relative inactivity of II might be that the NH group contributes to the binding of I to the receptor through direct interaction with a complimentry group on the receptor.

Table 1. In Vitro Growth Hormone Releasing Activity

	Dosage (μg/ml incubation medium)						
Peptide	Control	0.03	0.1	0.3	3	30	100
I*	-287± 187	1073± 74	1495± 199	1788± 155	2463±** 292	6057± 621	4997± 836
II*	-203± 109			-300± 123	463± 90	655± 132	

*Values given are the ΔGH in ng/ml medium (±SEM). P value vs. control was <0.001 except at dosage of 0.3 μg/ml incubation medium of compound II where the P value was not significant.
**Concentration, 1 μg/ml medium.

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SUBSTANCE P AND BEHAVIOR: COMPARISON OF EFFECTS OF N- AND C-TERMINAL FRAGMENTS

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Introduction

The undecapeptide substance P (SP:Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-amide) occurs in a number of central and peripheral neurons. Considerable evidence supports a neurotransmitter role for SP. It is most concentrated in synaptic vesicles and is released under depolarizing conditions; its release is Ca^{++} dependent. Enzymatic degradation probably terminates the action of synaptically-released SP, and two brain enzymes have been shown to cleave the SP molecule at the 4-5 and 7-8 peptide bonds. SP is thought to be a major stimulant of dopamine release in the central nervous system (CNS), and perhaps of serotonin as well. Considerable evidence supports a role for it as a sensory neurotransmitter for pain stimuli in the spinal cord. In spinal cord SP release is stimulated by capsaicin, the "hot" substance in red pepper, and is inhibited by morphine and the opiate peptides. Aside from this proposed role in sensory transmission, little is known about the function of SP in the CNS.[1] Exogenous SP produces a variety of behavioral effects when administered centrally or peripherally to intact animals. In view of the proposed role of SP in pain transmission, it is surprising that low doses of SP produce not an increase, but rather a decrease in responsiveness to painful stimuli.[2] This antinociceptive effect is thought to be due to stimulation of central enkephalin release.[3] In contrast, SP appears to inhi-

bit release of endogenous opiate peptides under certain stress conditions.[4] These and other observations point to complex roles and interactions of SP in the CNS. In the periphery, SP exerts direct effects on smooth muscles, lowers blood pressure, and stimulates secretion of saliva.[5]

Relatively little is known about the receptors for SP. Most of our current understanding of SP structure-activity relationships has come from assays using peripheral tissues. The guinea pig ileum in vitro and rat blood pressure assays have been particularly useful. In these assays, SP(7-11), the C-terminal pentapeptide sequence of SP, is the minimum fragment with activity. This C-terminal region is well conserved within the family of tachykinins, of which SP is a member.[5] The potency increases with chain length up to SP(4-11), which is more potent than SP. N-terminal fragments such as SP(1-7), the N-terminal heptapeptide, have little or no activity. Although studies of the action of SP on single neurons have, for the most part, confirmed the essential role of the C-terminal portion of the peptide molecule in mediating activity[6], this structure-activity relationship is not invariably seen with centrally-mediated actions of SP. Work with different naturally occuring tachykinins has demonstrated that not all responses to these peptides show the same structure-activity relationships and has indicated that the N-terminal part of the peptide molecule may be influencing the biological effects in some specific way. This observation led to the proposal of different SP receptor types.[7] Following our discovery of SP-induced antinociception (analgesia)[2], we demonstrated[8] that this effect is mediated by SP(1-7), an N-terminal fragment produced by action of a brain enzyme and heretofore thought to be a SP degradation product without biological activity. The antinociception is seen following either intraperitoneal or intraventricular injection. Other investigators[9,10] have shown that a C-terminal SP fragment, Pyroglu-Phe-Phe-Gly-Leu-Met-amide (which we designate here as

SP-C), can also produce antinociception following central or peripheral administration. This SP-C fragment is very similar to the one produced by the post-proline cleaving enzyme of brain. These results prompted us to examine the effects of these N- and C-terminal SP fragments in some other behavioral paradigms in which SP has been reported to have effects. We report here that these two peptides have effects on isolation-induced aggression and on some aspects of motor behavior. In general, the N- and C-terminal peptides produce opposite effects on behavior.

Behavioral Experiments

In these experiments SP, SP(1-7) (abbreviated SP-N) and SP-C were administered intraperitoneally in acidified isotonic saline (the solution is 0.01 M in acetic acid). Acidification of the saline increases potency by a factor of 1000.[11] Since SP-C has very low water solubility, a concentrated solution of it in DMSO was diluted with acidified saline for use. After initial dose-response studies showed that these behavioral effects of the peptides, like antinociception, were seen within a limited dose range around 0.6 nmoles/kg body weight, detailed studies were done with that dose of the peptides. As described later, some experiments on motor behavior were also done with intraventricular injection of peptides.

Aggressiveness. Stern and Hadzovic[12] and Uyeno et al.[13] reported that SP can reduce "aggressiveness" in mice. Based on these reports, we studied the effects of SP, SP-N, SP-C and SP(4-11) on fighting behavior in male mice made aggressive by prolonged isolation. Male albino mice, individually housed for two to four weeks prior to testing, were used throughout. Isolation-induced fighting was tested on two consecutive days. On day 1, each of 12 to 15 mice was injected

with either the test peptide or an equivalent volume of acidified saline 30 min before testing. The test consisted of introducing a non-isolated "intruder" male mouse into the cage of the isolated test mouse for a 3 min period. The amount of time, in seconds, that the mice spent in vigorous combat (biting, vocalizing, etc.) was recorded, as was the latency to the first attack. For some experiments, the number of attacks was also counted. On day 2, the injections were reversed: each mouse was injected with the solution not used on day 1, and tested 30 min later with the same intruder as on day 1. For half the subjects, the peptide was injected on day 1, while for the rest, the control injection was administered on day 1. This counterbalanced any bias that would occur if mice were more aggressive, per se, on the first or second day of fighting. Mice which did not fight on both days were eliminated. Results were analyzed using two-tailed paired t-tests, comparing scores on the control injection day with scores on the day of peptide injection.

Dose-response studies showed that SP and SP-N at a dose of 0.60 nmole/kg, I.P., reduced isolation-induced fighting. The C-terminal peptide SP-C, at the same dose, significantly increased the duration of fighting and the number of attacks. The longer C-terminal fragment, SP(4-11), which is extremely potent in peripheral SP assays, was without significant effect on isolation-induced fighting. The ability of SP-N to reduce isolation-induced fighting to an extent comparable to that of SP again demonstrates the significant biological activity of this N-terminal sequence. The enhancement of fighting seen with SP-C indicates that for some SP effects the C-terminal portion of SP may possess biological activity opposite to that of intact SP.

Since some other behavioral effects of SP and related peptides apparently involve endogenous opiate peptides, we

examined the effect of the specific opiate antagonist naloxone on the behavioral response to these peptides in this aggression paradigm. Pretreatment with naloxone potentiated the aggression-inhibiting effect of SP, was without effect on the action of SP-N, and completely blocked the aggression-stimulating effect of SP-C.

Motor Activity. The alteration by exogenous SP of motor behaviors such as locomotion, rearing and grooming has been amply demonstrated in both rats and mice.[14,15] In our experiments, groups of mice were injected with one of the three peptides, while control mice received an equal volume of acidified saline vehicle (10 µl/gm). Thirty minutes later, each mouse was placed in an open field box for 12 minutes. Behavior was recorded continuously during three 2-min periods, once at the beginning of the 12 min session, a second time starting at +5 min, and again during the last 2 min in the box. Behaviors recorded included the number of 6 x 7 cm squares crossed, the number of bouts and total duration of grooming, and the number of rears and leans. (Rearing consists of a hind leg stance with the forepaws not in contact with the walls of the container. A lean, on the other hand, is a hind leg stance with forepaws against the container walls).

To summarize several experiments, we found that SP-N significantly reduced grooming and enhanced rearing/leaning behavior, while SP-C enhanced grooming and reduced rearing/leaning. In some experiments SP significantly increased grooming, but the effects of SP were not as marked as those of the two fragments. In an additional experiment, the effects of daily administration of peptides during three days were examined. Mice were observed each day in the open field box 30 min after receiving a peptide or control injection. Each animal received the same treatment during the three days. All animals

tended to show much rearing/leaning behavior on the first day, with this behavior decreasing each day. In contrast, grooming increased each day. Effects of the SP fragments were consistent with the results of the acute experiments: SP-N enhanced rearing/leaning behavior, most markedly on the first day, and inhibited grooming, most markedly on the third day. SP-C enhanced grooming behavior throughout the experiment.

Intraventricular (ICV) administration of SP in nanogram to microgram doses produces a distinctive behavior pattern in mice characterized by reciprocal hindlimb scratching (RHS)[16]. Work with structurally related tachykinins and C-terminal SP fragments indicates that this effect is largely mediated by the C-terminal portion of SP. We examined the effects of ICV-administered SP and SP fragments on RHS and on the same motor behaviors that were altered following peripheral injections: grooming and rearing/leaning. SP, SP-C and the longer C-terminal fragment <E-SP(6-11) produced a dose-related increase in grooming behavior, including RHS, of short latency and short duration. Enhancement of grooming was seen as a profound increase in both the number and duration of grooming bouts. Both enhanced grooming and RHS were significantly attenuated, but not abolished, by pretreatment with morphine. SP-C also significantly reduced rear/lean behavior. This effect lasted for at least 10 min after ventricular injection, and involved a decrease in both the number and duration of rears and leans. This reduction was not simply a consequence of mice spending more time grooming. When rear/lean behavior was calculated on the basis of time <u>not</u> spent grooming, the reduction in rear/lean behavior was still highly significant. The N-terminal fragments SP(1-4), SP(1-6), SP(1-7), SP(1-7)-amide and SP(1-8) all produced a trend toward reduced grooming and significantly enhanced both the number and the duration of rear/lean episodes.

Discussion

These experiments extend the previous observation that SP-N has specific behavioral activity and show that in several paradigms N-terminal and C-terminal fragments of SP evoke opposite behavioral effects. Intact SP may mimic one or the other of the fragments, or may be without effect. In the latter case, either required processing to active fragments may not taking place or both fragments may be produced and evoke efects which cancel each other. In one paradigm SP-(4-11), which is super-potent in peripheral SP assays, was without effect on behavior. The effects of naloxone suggest that SP and its fragments may interact with the endogenous opioid systems in ways not involving pain responses.

At present, there is little information upon which to base an explanation of how SP fragments operate on the cellular level. However, a few tentative conclusions may be drawn, and a rough hypothesis may at least be stated. The ability of these peptide fragments to alter behavior when peripherally administered in such low doses argues against any major role for non-specific effects such as toxicity, alteration of cellular amino acid uptake, and so on. Interaction with specific high affinity receptors in the CNS seems more likely. CNS receptors with high affinity for SP and for C-terminal fragments such as SP-C have been described.[18] One might suggest that the effects of SP-N are due to simple competitive inhibition of SP binding. Such a mechanism could, at best, only explain an effect of SP-N opposite to that of SP. The similar effects of SP-N and SP on both analgesia and isolation-induced fighting are inconsistent with this hypothesis. One might suggest that N-terminal fragments act via competitive inhibition of an SP degrading enzyme, thus prolonging the action of endogenous SP. Growcott and Tarpey[19] have reported that addition of SP(1-9) to the bathing medium

slows the rate of degradation of SP by guinea pig urinary bladder *in vitro*. Such a mechanism could explain N-terminal effects that are similar to SP effects, but fails for effects of SP-N that are opposite to those of SP, such as the effects on grooming and rear/lean behavior.

SP and related peptides may produce their effects by interaction with monoamine neurotransmitter systems. SP-induced antinociception appears to involve opiate-receptor-rich areas in the central grey area of the brain, and may well act ultimately via serotonin neurons. Central serotonin systems may also be involved in the aggression paradigm, since long-term isolation has been shown to cause a lowering in brain serotonin. The motor effects, on the other hand, may well involve brain dopamine systems, since SP is thought to be a major stimulus for dopamine release in both the nigrostriatal and mesolimbic dopamine systems.[20,21]

Since a peripheral route of administration was used in most of our experiments, a peripheral site of action should be considered, especially since the ability of peptides to cross the blood-brain barrier is uncertain and controversial. However, most of the behavioral effects seen after peripheral administration of these peptides are also seen following intraventricular administration of much lower doses of the same peptides.

The pharmacological effects of SP-N described herein suggest that it, and perhaps other N-terminal fragments as well, may possess physiological properties. Brain has been shown to contain enzymes capable of generating SP-N from SP *in vitro*[17] and some evidence for the presence of SP(1-7) in brain has been presented.[8] While it has not been demonstrated that SP-N is definitely generated *in vivo* and that SP-N so generated can exert any physiological effects, the findings presented here raise the possibility that enzymes thought to

be only SP-degrading enzymes may in fact be SP-processing enzymes. The actions of such enzymes could then give rise to quite different physiological effects, depending on their localization. More research is needed to clarify these issues. Such research may significantly enhance our understanding of the physiological functions of SP.

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ACTH: STRUCTURE ACTIVITY AND NERVOUS SYSTEM EFFECTS

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INTRODUCTION

The classical endocrine view is that the cells of the pituitary are the only source of the peptide hormone corticotropin (ACTH). When it became apparent that ACTH and/or melanotropin (MSH) were capable of influencing the nervous system and behavior in experimental animals and man (see for review De Wied and Jolles, 1982), interest was focused on the presence and possible origin of ACTH and congeners in the nervous system. Immunocytochemical observations are consistent with the notion that opiomelanocortin peptides including ACTH-like peptides, are present in neurons of the arcuate nucleus and in distinct pathways running from this nucleus to the limbic system, midbrain and hindbrain. There is evidence for additional, extra hypothalamic neuronal networks as well. Also developmental studies point to at least two ACTH-containing systems in the body (pituitary and brain). Irrespective of its role as hormone in the periphery or as neuropeptide in the brain, the peptide ACTH is the bearer of multiple information and may activate different receptors involved in the same or different biological response(s). Schwyzer (1980) suggested a linear or sychnologic organization of the information encoded in ACTH. This means that discrete (sometimes overlapping) sequences of adjacent amino acids are responsible for the different components of the total biological action of ACTH. In addition to the active sites (messages) in the molecule, there are auxiliary sites which are described in terms of their function as address and potentiator.

In view of the multiplicity of information-containing sites and the many different effects of ACTH on the central and peripheral nervous systems, it is assumed that the effects of ACTH on nervous tissue are the result of interaction with multiple receptors. In this paper we will review some of the structural requirements for three known effects of ACTH on nervous tissue.

ACTH AND CONDITIONED AVOIDANCE BEHAVIOR IN THE RAT

Conditioned avoidance behavior has been extensively used to assess the role that ACTH plays in the modulation of adaptive behavior in the rat. However, a variety of non-aversively motivated behaviors are influenced by ACTH-like peptides as well (De Wied and Jolles, 1982). Although under certain conditions ACTH has been shown to improve acquisition of shock-motivated active avoidance behavior in rats, in general extinction of the conditioned avoidance response seems to be more sensitive to peptide treatment. It was demonstrated that ACTH-like peptides retard extinction of a pole-jumping, one-way active avoidance response both after systemic and after intracerebroventricular administration in a dose-dependent manner. Initial structure-activity studies revealed that ACTH fragments that lacked classical endocrine activity were able to influence behavior. The behavioral effect was the result of a peptide-brain interaction most likely involving midbrain-limbic structures.

In a long series of experiments, Greven and De Wied (1973) determined what the shortest chain length with full behavioral activity was. Using a fixed test dose of 1 μg peptide s.c., it was found that the fragments (1-10) and (4-10) were as active as ACTH itself, whereas the fragments (11-24) and (25-39) were inactive. Stepwise shortening of the sequence (1-10) from the N- and separately from the C-terminal end resulted in the sequence (4-7) as the smallest fragment containing the essential elements required for full activity (Fig. 1; Greven and De Wied, 1973). In later studies it was found that some fragments, inac-

tive at the fixed screening dose used, induce a complete response when given at a 10-times higher dose (De Wied and Gispen, 1977). The dormant activity in fragments such as (7-10), (11-13) and (25-39) could be expressed by chain elongation. This multiplicity or redundancy of information for a given behavior is reminiscent of other structure activities in ACTH/MSH peptides (Van Nispen and Greven, 1982). Sandman and Kastin (1981) assume that the multiplicity of information in the N-terminal part of the molecule is not so much a reflection of message repetition, but rather that different parts of the molecule code for different behavioral processes. They tested, however, relatively few peptides and therefore the validity of their assumption remains to be proven.

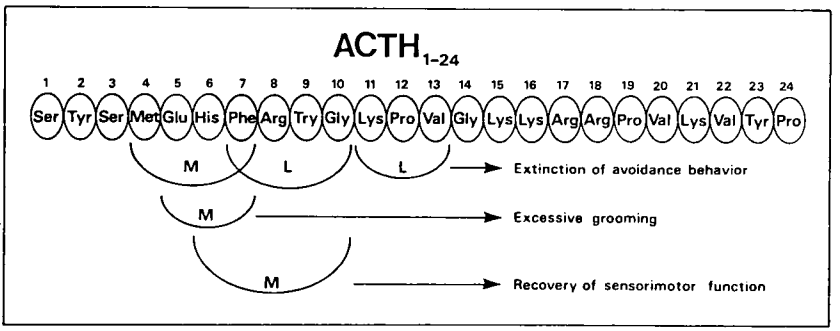

Fig. 1. Primary structure of $ACTH_{1-24}$ with the suggested message sites (M) involved in three different effects of ACTH on the nervous system. L = site with latent activity.

Having established the necessary chain length, Greven and De Wied (1980) extensively studied the effect of amino acid substitutions that are known to improve metabolic stability or to induce residual endocrine activity of the short N-terminal fragments of ACTH. Exchanging L- for D-amino acid residues, which is accompanied by an improved metabolic stability, led to a slight increase in potency of the $ACTH_{4-10}$ or $ACTH_{4-9}$ molecule with two notable exceptions. For, the introduction of a D-Lys

in position 8 in $ACTH_{4-9}$ resulted in a 10-30-fold increase in activity and quite surprisingly, replacement of L-Phe by D-Phe in position 7 completely reversed the direction of the effect of the peptide on behavior: i.e. a facilitation instead of a delay of extinction. Presently it is assumed that the $D\text{-}Phe^7$ peptide may counteract some of the effects of endogenous ACTH-like effects on brain cell firing (De Wied and Jolles, 1982). Applying multiple modification of the parental $ACTH_{4-9}$ molecule, a peptide was synthesized that was a 1000-times more active ($H\text{-}Met(O_2)\text{-}Glu\text{-}His\text{-}Phe\text{-}D\text{-}Lys\text{-}Phe\text{-}OH$, Org.2766). In doses up to 10 µg/kg the peptide was orally active as well. The oxidation of Met, and the substitution of $D\text{-}Lys^8$ and Phe^9 in ACTH-like peptides dramatically reduced corticotropic, melanotropic and lipolytic activity of such peptides (Van Nispen and Greven, 1982). Furthermore, it was suggested that these modifications had improved the selectivity of the peptide as the potentiation was not always expressed when tested in other CNS actions.

Using an one-step passive avoidance behavioral test, it was demonstrated that Org.2766 after subcutaneous injection of low doses (ng quantities) facilitated and after high doses (µg quantities) inhibited retention. Inverted U- or bell-shaped dose response relationships are a common phenomenon but often not well understood in peptide-CNS research. However, in the present case, the differential effect on a given behavior could be ascribed to the involvement of different sites in the molecule. The inhibitory effect was located in the C-terminal part (Phe-D-Lys-Phe). In further studies it was found that the facilitatory influence residing in the N-terminal part was not expressed via a neural substrate containing naloxone-sensitive opiate receptors, whereas the C-terminal inhibitory influence did (Fekete and De Wied, 1982).

From the results of the Free-Wilson analysis carried out on over 50 peptides that delayed extinction of pole-jumping behavior, it was concluded that the behavioral potency of the ACTH-analogues could be considered as the product of independent contributions from individual substitutions (see Van Nispen and

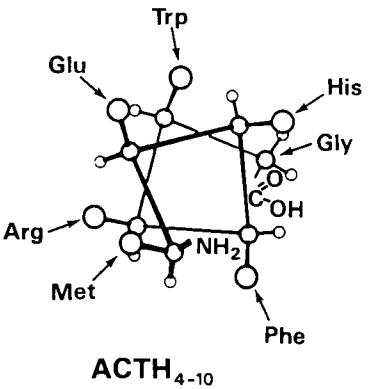

Fig. 2. Suggested stereoconformation of $ACTH_{4-10}$ showing the close proximity of the residues Met^4, Phe^7 and Arg^8 (according to Van Nispen and Greven, 1982).

Greven, 1982). In subsequent research, evidence was obtained to suggest that at the receptor site, $ACTH_{4-10}$ would have an α-helix configuration bringing the residues in positions 4 (Met), 7 (Phe) and 8 (Arg) in close proximity (Fig. 2). The notion that Phe and Met should be in close proximity in order to delay extinction, was substantiated by the behavioral activity of a cyclic peptide containing Phe, Met and a spacer amino acid (Σ amino hexanoic acid; Van Nispen and Greven, 1982). Apparently the amino acids in positions 5 and 6 merely serve as spacers and can indeed be replaced by simple neutral alanine residues without loss of activity.

ACTH AND THE INDUCTION OF EXCESSIVE GROOMING BEHAVIOR IN THE RAT

The first behavioral stereotopy reported to occur after intracranial injection of ACTH into mammals was the stretching and yawning syndrome (Ferrari et al., 1963). In rodents, the onset of this syndrome is preceded by bouts of excessive grooming (Fig. 3). Recently, Spruijt and Gispen (1983) have studied the pattern of ACTH-induced grooming in comparison with the grooming seen after icv saline treatment. In general, grooming is initiated by face washing and follows a cephalocaudal progression. In a dose-dependent manner, ACTH prolonges the bout

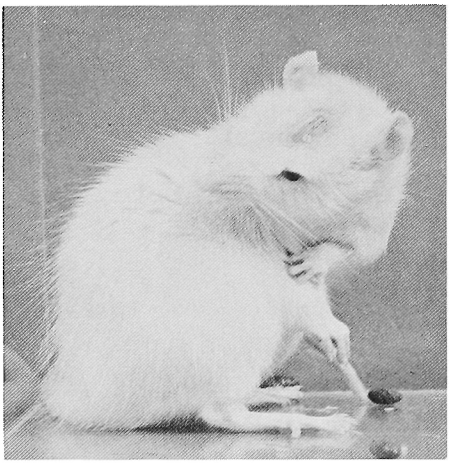

Fig. 3. ACTH-induced excessive grooming in the rat. The figure shows the head-body grooming element, which accounts for 50% of the total displayed grooming elements.

duration by inducing new transitions from scratching to body grooming and vibration, leading via body shakes to face washing.

There is evidence that grooming behavior related to novelty or stress resulting from conflict situations or other stressful stimuli, is initiated by the release of ACTH and congeners from peptidergic pathways (see Gispen and Isaacson, 1981). Although several interpretations seem tenable, at present we prefer to consider ACTH-induced grooming in the context of a behavioral deactivation mechanism operative after the arousing influence of ACTH (Spruijt and Gispen, 1983). As the grooming response to icv ACTH easily can be quantified by means of a time sampling method, it has been very useful in demonstrating the complexity of the structure-activity relationship for ACTH and CNS.

$ACTH_{1-24}$ but neither its composing sequences (1-10) or (11-24) nor their equimolar combination will elicit excessive grooming. The fact that $ACTH_{1-24}$, α-MSH and β-MSH were effective was ascribed to the common core (4-10). However, in contrast to the effects of ACTH on avoidance behavior, administration of the sequence (4-10) per se or Org.2766 was without effect. Yet, gradual shortening at the C-terminal part or at the N-terminal part of the sequence (1-24) revealed that the message site is somewhere in the region (5-13), possibly the sequence (5-7)(Fig. 1). The activity is only expressed when specific C- or N-terminal

elongation is carried out. The sequences (4-7) and (5-14) are active, whereas (4-10) and (5-10) are inactive (Gispen and Isaacson, 1981).

As discussed in the foregoing section, the Phe^7 amino acid residue plays a key role with respect to CNS effects of ACTH. Also with respect to excessive grooming, the $(D\text{-}Phe^7)\text{-}ACTH_{4-10}$ peptide is unique among D-enantiomer substitutions in that it is active in inducing excessive grooming. Other D-enantiomer substitutions in the sequence (4-10) are without effect on the activity of $ACTH_{4-10}$ and make it unlikely that improved metabolic stability can account for the expression of the behavioral effect. A striking example of a single step post-translational activation is the acetylation of the Ser^1 residue of the inactive $ACTH_{1-13}\text{-}NH_2$. The acetylated peptide is α-MSH and that peptide has full grooming activity (Gispen et al., 1975; O'Donohue et al., 1981). Thus, although the structure-activity relationship of the two behavioral activities of ACTH discussed sofar differ, it is clear that the expression of effect follows comparable rules (stereoconformation, proper chain elongation, etc.).

ACTH AND RECOVERY OF SENSORIMOTOR FUNCTION

In studying the neurochemical mechanism of action of ACTH, it became apparent that nervous tissue can be regarded as a target for ACTH in the same way as the adrenal cortex. Indeed, ACTH stimulates the synthesis of RNA and proteins in subcortical brain structures under a variety of experimental conditions (Dunn and Schotman, 1981). This trophic response to ACTH suggests that such peptides may facilitate repair mechanisms that involve the synthesis of (specific) proteins and are operative after brain or nerve damage. Recently Strand and Smith (1980) showed that ACTH accelerates recovery of neuromuscular function following sciatic nerve crush, presumably by a neurotrophic action of the peptide. Bijlsma et al. (see for review Bijlsma et

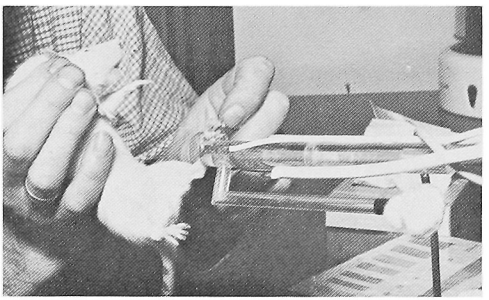

Fig. 4. Foot-flick test as used in the studies on recovery of sensorimotor function after sciatic nerve crush.

al., 1983) confirmed and extended these observations. They studied the recovery of sensorimotor function by means of a foot-flick test after crushing the sciatic nerve in rats (Fig. 4). Subcutaneous treatment (daily or every 48 h) with μg quantities of $ACTH_{1-24}$ facilitated the recovery of function by 10-20%. To investigate what part of the peptide molecule would contain the active site and to exclude steroid mediation, smaller sequences of $ACTH_{1-24}$ devoid of corticotrophic activity were tested. The sequences (1-16), (1-10), (4-10) and (4-9) were active, whereas (11-24) was inactive. Org.2766 had an activity comparable with that of (4-10) and (4-9) and hence the three-fold modification was without potentiating effect in this case. The sequence (4-7) was inactive but (6-10) was as active as (4-10). This observation may be of importance as this sequence (6-10; Fig. 1) still contains melanotropic activity. In fact, treatment with α-MSH was most effective in facilitation of recovery of sensorimotor function after sciatic nerve crush. Hence it was suggested that the return of sensorimotor function was influenced by melanotropic sites in the ACTH molecule.

Light and electron microscopical studies revealed that ACTH treatment resulted in an increased outgrowth of myelinated and unmyelinated neurites (Bijlsma et al., 1983). This process was accompanied by a selective enhancement of the production of cytoskeletal proteins in the grey matter of the lumbar part of the spinal cord (Bijlsma et al., 1983). Further work is in progress to more fully characterize this trophic effect of the peptide on peripheral nerve. Notwithstanding this it is evident

that again a clear structure-activity relationship exists that differs from those discussed above.

CONCLUDING REMARKS

In vivo structure-activity studies of drugs, and of peptides in particular, are extremely difficult to interpret. In general, one must take into account the route of administration, absorption, distribution, metabolism and excretion. Furthermore, one assumes specific binding to some sort of receptor followed by a biological response. With respect to fragments of ACTH chain length and amino acid modifications and/or D-substitutions undoubtedly will affect the afore mentioned processes and the results are often not well characterized. However, it may be that structurally related short peptide fragments may behave in much the same way when the *in vivo* fate of such peptides is considered. Differences in activity under such conditions are much more likely to be determined by peptide-receptor interactions. Even then, in case of ACTH-CNS effects great caution is to be taken as up to date no stereospecific, saturable binding sites for ACTH in the nervous system have been identified.

Despite these drawbacks, it is concluded that the differences in structural requirements for the various effects of ACTH on the nervous system as discussed in this chapter are illustrative of the multiplicity of information encoded in the peptide molecule and of the receptor population recognizing parts of the ACTH molecule. The structure-activity relationships seem at best understood if one assumes: (a) sychnologic information encoding in the ACTH molecule as suggested by Schwyzer; (b) expression of activity by proper terminal end elongation or residu modification; (c) a specific stereoconformation of the peptide crucial to receptor activation.

Identification and characterization of ACTH receptors in the nervous system is of utmost importance to more fully understand the interaction of ACTH with nervous tissue.

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THE DYNORPHIN PEPTIDES

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Introduction: Dynorphin A

The dynorphins comprise the third family of endogenous opioid peptides (Figure 1);[1] the other two are products of the enkephalin gene and the opiomelanocortin gene.[2] The remarkable potency of dynorphin A (dyn A, the 17-residue peptide that was first to be sequenced) is the feature for which it was named.[3,4] In the guinea-pig ileum bioassay it is hundreds of times more potent on a molar basis than morphine or the enkephalins, and 50 times more potent than β-endorphin. Even in the mouse vas deferens, in which the enkephalins are exceptionally potent, dyn A is several times more potent. Evidently some or all of the 12 residues in the COOH-extension of [Leu]enkephalin play key roles in receptor interaction, either with the same or a different receptor from those (δ and μ, respectively) already known to mediate the biologic effects of enkephalins and morphine.

The high potency of dyn A is due to its high affinity for and selective interaction with a specific opioid receptor known as κ, previously characterized as mediating pharmacologic effects of certain benzomorphan alkaloids (e.g., ethylketazocine). By progressive truncation of dyn A from its COOH-terminus, and through the use of analogues, we were able to show[5] that two residues are critically important both for potency and for κ selectivity; these are lysine-11 (in α-neoendorphin and dyn B lysine-10 plays this role) and arginine-7 (lysine-7 in the neo-endorphins). Thus, the biologic potency

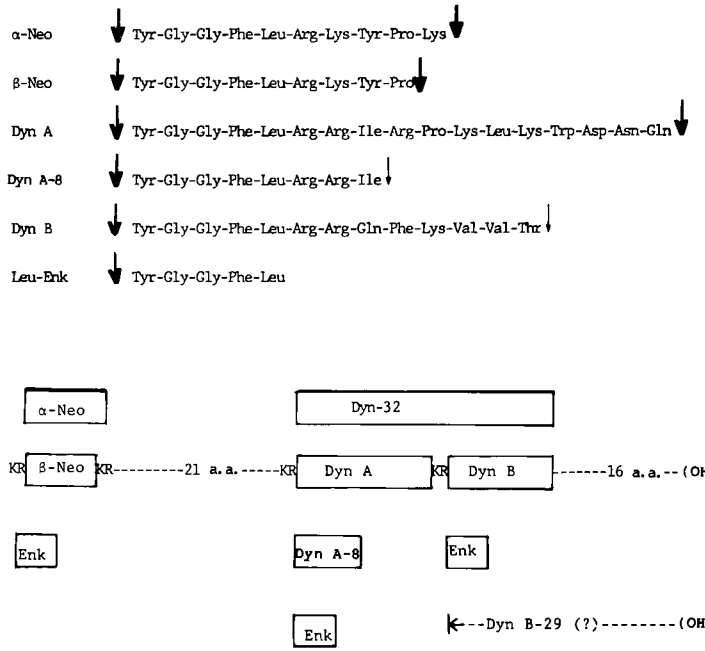

Fig. 1. Opioid Peptides Contained in the Deduced Pro-dynorphin Sequence.[1] Bold arrows show where cleavages occur at Lys-Arg "processing signals", followed by removal of the two basic amino acids. The special case of α-neo is discussed in text. Light arrows indicate cleavages between a neutral aliphatic amino acid and arginine, giving rise to Dyn A-(1-8) and Dyn B. Block diagram indicates various possible patterns of post-translational proteolysis. (From Cone et al.[23]).

and selectivity of the fragment dyn A-(1-13) are not different from those of the full-length dyn A. It is not yet known if κ-opioid selectivity depends upon a particular conformation of the NH_2-terminal enkephalin region,[6] stabilized by intramolecular interactions requiring lysine-11 and arginine-7, or if the κ receptor has specific anionic binding sites complementary to these two residues.

Big Dynorphins, Dynorphin B, and the Neo-Endorphins

In 1982 Fischli et al[7,8] isolated and determined the sequences of two "big dynorphins". These had been purified with the aid of an immunoaffinity resin, using an antiserum[9] highly specific for the sequence of residues 3-12 in dyn A. The most interesting of the two big dynorphins was dynorphin-32 (dyn-32). This proved to contain dyn A followed by Lys^{18}-Arg^{19} and then by an entirely novel 13-residue opioid peptide. The novel peptide was named dynorphin B (Dyn B) (Figure 1), and the original 17-residue dynorphin was accordingly re-named dyn A. Almost simultaneously, Kilpatrick et al[10,11] reported the same 13-residue peptide, isolated as such from bovine pituitary, and called it "rimorphin". The identity across species (dyn-32 had been obtained from porcine pituitary) is noteworthy, suggesting biologic importance of the entire dyn B sequence.

Difficulties in the custom synthesis of dyn-32 delayed proof of identity with the natural peptide; the latter could possibly have been derivatized in some manner. Substituents in the COOH-terminal portion might possibly have been removed during the numerous prior Edman cycles. We have now obtained synthetic dyn-32 (Peninsula Laboratories) and purified it to homogeneity by reverse-phase high-performance liquid chromatography (HPLC), and Dr. M. Hunkapiller (Caltech) has kindly verified its sequence. Figure 2 shows that this synthetic peptide is eluted from HPLC in exactly the same position as "peak 3" of Fischli et al.[8] This HPLC system is capable of separating nearly identical peptides with high resolution; for example, [des-Asn^{17}]dyn-32 is eluted at a distinctly different acetonitrile concentration. The result shown, therefore, makes it highly unlikely that the peptide was derivatized in its natural state, especially by polar residues like sugars, phosphate, or sulfate.

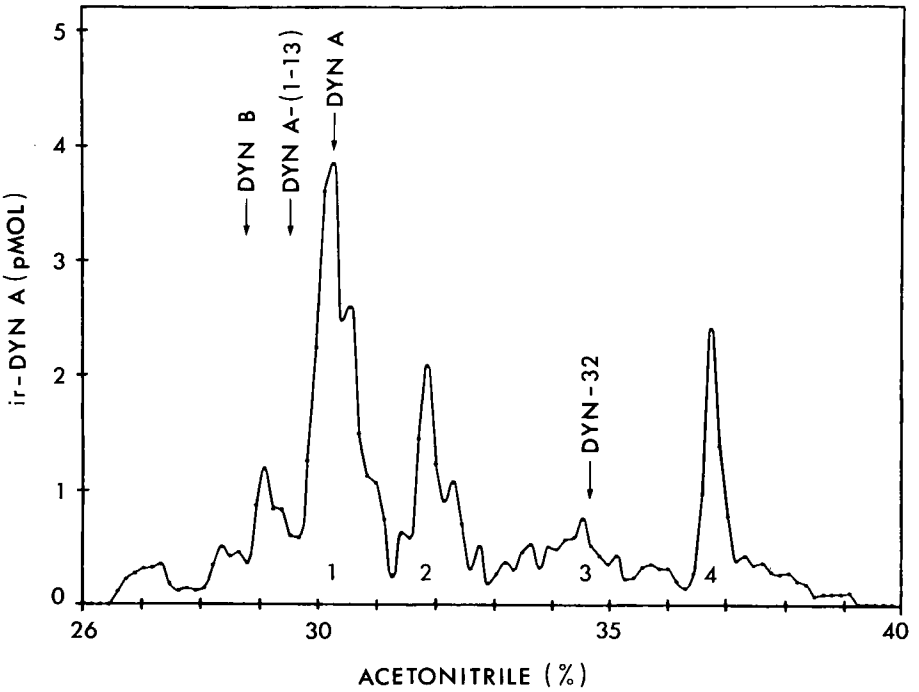

Fig. 2. HPLC Elution Pattern of Porcine Pituitary Extract. Same melanotropin concentrate (1 g) used by Fischli et al.[8] was extracted (100°C, 10 min) with 18 ml of 1 M acetic acid. After centrifuging, supernatant solution was extracted with CM-Sephadex (4°C, 2 hr), then eluted with equal parts of methanol and 0.1 M HCl. After lyophilizing and reconstituting in methanol-HCl, the material was applied to a C_{18} HPLC column as described[8] (linear gradient of CH_3CN in 5 mM trifluoroacetic acid, 20-50% in 30 min, 1.5 ml/min). Total immunoreactive dyn A (ir-dyn A)[9] applied to column was 15 nmol, representing 74% of ir-dyn A in extract. Peaks are numbered according to Fischli et al,[8] who isolated natural dyn-32 in peak 3. Markers indicate elution positions of synthetic peptides in parallel runs; "DYN A-(1-13)" was ^{125}I-labeled internal reference standard.

The sequences of the neo-endorphins (Figure 1) had been reported in 1981,[12,13] correcting an earlier erroneous one.[14] Co-localization studies by radioimmunoassay (RIA) and immunocytochemistry (ICC) suggested that these [Leu]enkephalin-containing peptides and dyn A might be co-synthesized.[15-20]

The second "big dynorphin" isolated by Fischli et al.[7] was dynorphin-24, in which dyn A is followed by Lys^{18}-Arg^{19} and then by the [Leu]enkephalin sequence alone. In other words, this peptide corresponds to dyn-32 in which cleavage has occurred between leucine-24 and arginine-25. It is interesting for two reasons. First, it shows a high degree of homology with BAM peptide E, one of the pro-enkephalin peptides.[21] Peptide E contains [Met]enkephalin at its NH_2- terminus, followed by Arg^6-Arg^7, then by 11 residues, followed by Lys-Arg, and finally by [Leu]enkephalin. Second, dyn-24 is an example -- unique thus far among opioid peptides -- of cleavage at Arg-Arg, which could give rise eventually to free [Leu]enkephalin.

It is satisfying to have the conclusions drawn from peptide chemistry, RIA, and ICC confirmed decisively by the entire pre-pro-dynorphin sequence[1] (we adopt the nomenclature proposed by Rossier[22]); the structures relevant to the three primary [Leu]enkephalin-containing gene products -- α-neo-endorphin, dyn A, and dyn B -- are shown in Figure 1. The expected neo-endorphin product is α-neo-endorphin because the Pro^9-Lys^{10} bond is expected to be resistant to carboxypeptidase B. Indeed, the placement of proline-9 could even be regarded as Nature's clever way of ensuring strong κ-opioid selectivity (see below) by retaining lysine-10 of the Lys^{10}-Arg^{11} pair, which would otherwise be removed. β-Neo-endorphin is present in tissues in very small amounts and has only the partial κ-selectivity conferred by lysine-7.

Very recently, Cone et al.[23] demonstrated that fresh rat brain extracts contain three size classes of dyn A and dyn B

immunoreactivity. The material of approximately 2 kDal was proved, by co-elution with synthetic peptides on HPLC, to be authentic dyn A and dyn B at 2.8 and 12 pmol/g wet weight, respectively. The size class of about 4 kDal was proved in the same manner to be dyn-32. Immunoreactivity detected by both dyn A and dyn B antisera was also present at about 7 kDal; the nature of this material is unknown. In rat pituitary neurointermediate lobe virtually all dyn A and dyn B immunoreactivity was in the 2-kDal size class. In anterior lobe only the 7-kDal immunoreactivity was present, as shown by Seizinger et al.[24] Differences in the relative amounts of immunoreactive α-neo-endorphin, β-neo-endorphin, dyn A-(1-8), dyn A, and dyn B suggested the possibility of differential processing in different brain regions. For example, the concentrations of immunoreactive peptides (pmol/g) were, for these five peptides, respectively: Striatum, 34, 4.3, 32, 9.7, 29; Hippocampus, 18, 4.0, 16, 17, 24. The data for dyn A and dyn B represent the composite contributions of the three size classes; the other three peptides are measured without interference from larger forms.

As shown earlier,[18] dyn A-(1-8) is found in most regions of the central nervous system in amounts larger than those of dyn A. Production of this octapeptide seems to require cleavage at Ile^{8}-Arg^{9} (cf. Figure 1), since cleavage at Arg^{9}-Pro^{10} (followed by carboxypeptidase B removal of arginine-9) is unlikely. Similarly, dyn B is an unexpected product, requiring cleavage at Thr^{13}-Arg^{14} or at Arg^{14}-Ser^{15} (followed by removal of arginine-14). The full COOH-terminal putative opioid peptide dyn B-29 [(H)Y^{1}GGFLRRQFKVVT^{13}RSQEDPNAYYEELFDV^{29}(OH)] has not yet been found in tissues. Whether dyn A-(1-8) and dyn B are indeed products of processing mechanisms, stored in vesicles, and released as neurotransmitters, or whether they are degradation products, the enzymes responsible must have very interesting specificities. They have to recognize Ile-Arg and Thr-Arg, respectively, while ignoring the several other

single and paired arginine and lysine residues in the precursor peptides. Similar examples are now known, in which basic pairs Arg-Arg, Arg-Lys, or Lys-Lys are ignored, while cleavage occurs at Lys-Arg or between an aliphatic neutral amino acid and arginine.

Selectivity of the Dynorphin Peptides for κ-Opioid Receptors

Several lines of evidence from various laboratories indicate that the three primary products of the dynorphin gene -- α-neo-endorphin, dyn A, and dyn B -- are strongly selective for the κ type of opioid receptor, whereas β-neo-endorphin and dyn A-(1-8) have distinctly poorer κ-selectivity. Some of this evidence may be summarized as follows:

(a) The guinea pig ileum preparation (GPI) contains only μ and κ receptors, which can be distinguished by their very different affinities for the opioid antagonist naloxone.[25] The apparent dissociation constant of naloxone (K_e) at μ receptors is about 2 nM, at κ receptors about 30 nM. K_e is about 30 nM for dyn A-(1-13)[3] and for dyn A, dyn B, and α-neo-endorphin (unpublished observations). Dyn A-(1-8) and β-neo-endorphin yield distinctly lower values of naloxone K_e, showing that their effects are mediated in part through μ receptors.

(b) By means of β-chlornaltrexamine (CNA), an opioid site-directed alkylating agent, we were able to inactivate some opioid receptor types while protecting others with a selective ligand. In the GPI, after treatment with CNA in the presence of dyn A, there was a large loss of potency of the μ agonist normorphine but little loss of potency of dyn A.[26] Conversely, if μ sites were protected, there was a differential large loss of potency of dyn A. Ethylketazocine and other putative κ-selective alkaloids behaved like dyn A in these preparations. Similar findings have been obtained in

the mouse vas deferens.[27] We find in GPI that dyn A, dyn B, or α-neo-endorphin protects the same sites against inactivation by CNA, i.e., we find no evidence of κ sub-types.

(c) With the CNA same selective alkylation technique, guinea pig brain membranes can be prepared which are almost homogeneous with respect to opioid receptor type.[28] In such preparations, the equilibrium dissociation constants (K_i) can be determined for a given ligand at μ, δ, and κ sites. A selectivity profile gives the ratios of binding constant at the preferred (highest-affinity) site to the binding constant at each non-preferred site. Dyn-(1-13) amide was studied because it is stable under conditions of the binding assay (most peptides are not); K_i was 21 pM at κ sites, with 30-fold poorer affinity at μ, 78-fold poorer at δ .

(d) In mouse vasa deferentia made tolerant to μ and δ agonists, there was no change in the potency of dynorphin peptides;[29] and after tolerance to the latter, the potencies of the former were unchanged.

(e) In rabbit vas deferens, which is thought to contain only κ receptors,[30] dynorphin and neo-endorphin peptides have very high potency, while μ and δ agonists have low potency.[31] In this preparation, dyn A was several times more potent than dyn A-(1-8).[31,32]

Conclusions

The primary products of the dynorphin gene are α-neo-endorphin, dyn A, and dyn B. All three are highly selective ligands for the κ opioid receptor. This selectivity is conferred upon the [Leu]enkephalin sequence by certain residues in the COOH-terminal extension, namely, lysine-7 in α-neo-endorphin, arginine-7 in the dynorphins; and by lysine-10 in α-neo-endorphin and dyn B, lysine-11 in dyn A. The molecular

interactions through which these two basic residues cause preferential affinity for the κ receptors is unknown.

Dyn A has nearly an order of magnitude higher affinity for κ receptors than have α-neo-endorphin or dyn B. The significance of redundant κ agonists with such widely different affinities is unclear; perhaps a regulated conversion of dyn A to dyn A-(1-8) could be a way of extending the dynamic range of this peptide family.

The dynorphin gene products are widely distributed in neural pathways throughout the central and enteric nervous systems and pituitary. These dynorphinergic systems obviously participate in many and diverse physiologic functions. To establish functional roles of the dynorphin peptides will require better understanding of their post-translational proteolysis and its regulation, of their release from nerve terminals, of the distribution of κ opioid receptors, and of the biochemical and cellular consequences of κ receptor occupancy.[33]

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SYNTHESIS AND BEHAVIOURAL ACTIVITY OF FRAGMENTS OF OXYTOCIN AND ARGININE VASOPRESSIN CONTAINING A CYSTINE RESIDUE IN POSITION 6

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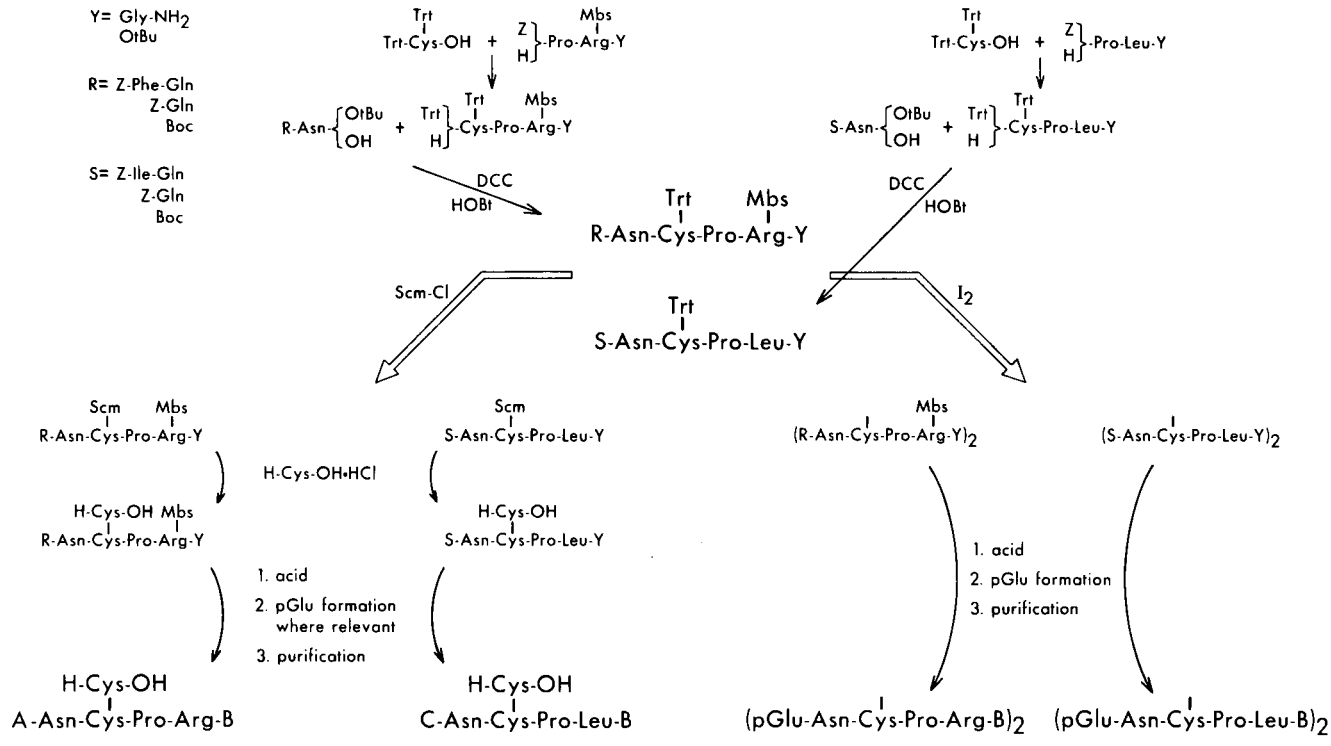
Introduction

Arginine vasopressin (AVP) and oxytocin (OT) have, in addition to their peripheral hormonal activities, effects on behavioural processes[1]. Fragments of these peptides retain central activities[1,2], whereas they are practically devoid of endocrine activity. Incubation of AVP and OT with proteolytic enzymes of a rat brain synaptic membrane preparation results in fragments with an intact disulfide bridge[3]. Of special interest seems the conversion of the fragments starting with Gln into their pyroglutamic acid (pGlu) analogues[3]. We will describe the synthesis of 12 fragments and analogues of AVP and OT, and report on some results of behavioural studies.

Strategy and description of the synthesis

The fragment condensation approach was used to synthesize the key fragments R-Asn-Cys(Trt)-Pro-Arg(Mbs)-Y and S-Asn-Cys(Trt)-Pro-Leu-Y (Fig. 1). The trityl group was chosen for S-protection since on the one hand it can lead to the asymmetrical disulfides by reaction with methoxycarbonylsulfenyl chloride (Scm-Cl), followed by substitution of the introduced Scm group with cysteine via its free thiol group[4], and on the other hand can give rise to symmetrical disulfides (dimers) by treatment with iodine[5]. Treatment of the key peptides with Scm-Cl in CH_2Cl_2 or MeOH/CH_2Cl_2 (1:1, v/v) for 5-60 min at room temperature (RT) gave the corresponding Scm-



I	A = H-Phe-Gln ; B = $Gly-NH_2$	VII	C = H-Ile-Gln ; B = $Gly-NH_2$	V	B = $Gly-NH_2$
II	A = pGlu ; B = $Gly-NH_2$	VIII	C = pGlu ; B = $Gly-NH_2$	VI	B = OH
III	A = pGlu ; B = OH	IX	C = pGlu ; B = OH		
IV	A = H ; B = $Gly-NH_2$	X	C = H ; B = $Gly-NH_2$		

XI	B = $Gly-NH_2$
XII	B = OH

Fig. 1 Synthesis of asymmetrical and symmetrical disulfide fragments and analogues of AVP and OT.

-peptides. Conversion into the mixed disulfide with a slight excess of cysteine.HCl was performed in MeOH, MeOH/CH_2Cl_2 or DMF at RT for 10-60 min. For the preparation of some symmetrical disulfides, the corresponding Cys(Trt) monomer was treated with I_2 (0.005 M in acetic acid) for 1 h at RT. After removal of the protecting groups with methanesulfonic acid in TFA in the presence of thioanisole and conversion into the acetate form, the peptides were purified by chromatography on SiO_2 or by counter current distribution. The H-Gln peptides convert spontaneously (slowly) into the pGlu form; a good and more rapid conversion takes place in 50% aqueous acetic acid at 50°C for approx. 5 h (prior to purification). The peptides were analyzed by TLC in several solvent systems, HPLC, electrophoresis, amino acid analysis and isotachophoresis[6].

Results and Discussion

In the case of insufficient solubility of the Cys(Scm) containing peptides in MeOH or MeOH/CH_2Cl_2 to form the asymmetrical disulfide, DMF can be used as a solvent and Cys.HCl is added to the peptide solution. Removal of excess Cys.HCl by precipitation, extraction or Sephadex LH-20 chromatography is necessary. Purification of the partially protected cystine (Cyt) peptides prior to removal of protecting groups and pGlu formation results in a clearly higher yield (20-25% higher) of the end-product. Conversion of the Cys(Scm) fragments into their Cyt peptides is in general a rapid reaction (10-15 min) but does not go to completion (~90%); longer reaction times are not beneficial.

The behavioural results obtained with AVP fragments II and III (Table 1) may be interpreted as an improvement of consolidation; those obtained with the analogues VIII and IX, like OT, indicate an opposite effect. II and VIII have a small effective dose range, whereas III and IX have a more extended range. These four peptides are clearly more potent in this behavioural assay than the respective parent nonapeptides.

Table 1. Indications for purity and behavioural activity

Peptide		HPLC[a]	Potency ratio[b]	Dose range tested (pg)
II	[pGlu^4,Cyt^6]AVP-(4-9)-NH_2	95.5%	3000[c]	0.01-1000
III	[pGlu^4,Cyt^6]AVP-(4-8)-OH	98.3%	$\geqslant$ 1000[c]	0.1 - 10
VIII	[pGlu^4,Cyt^6]OT-(4-9)-NH_2	98.3%	100[d]	0.1 -1000
IX	[pGlu^4,Cyt^6]OT-(4-8)-OH	98.5%	1 - 10[d]	1 -1000
X	[Cyt^6]OT-(5-9)-NH_2	96.0%	inactive[d]	10 - 100

a Nucleosil 10 C-18. Gradient elution with tetramethylammonium phosphate (pH 2.8)/MeOH systems at 20°C; detection : 210 nm[6].

b Studied in a one-trial learning, step-through passive avoidance test[7]. Immediately after the learning trial the animals were injected intracerebroventricularly with peptide solution (in saline) or placebo. The latency (in sec) to re-enter the dark compartment was measured after 24 h.

c Increased latency; AVP as reference[8].

d Decreased latency; OT as reference[8].

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CROSS-TOLERANCE BETWEEN A VASOPRESSIN ANALOG (DESAMINO LYSINE^8 N_ϵ-$(Gly)_3$-VASOPRESSIN)(DLGVP) AND MORPHINE IN THE MOUSE WRITHING TEST

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Introduction

Lysine-vasopressin has previously been reported to produce antinociceptive effects following intracerebroventricular (icv) or subcutaneous (sc) administration to rats.[1] Although the analgesic effects of this peptide were not blocked by naloxone, it facilitated the development of tolerance to morphine.[2] Similarly, arginine-vasopressin (AVP) was found to have antinociceptive effects in the mouse which were also not blocked by naloxone.[3] Recently, vasopressin was found to be co-localized with dynorphin in neurons of the hypothalamus and pituitary[4] and to potentiate morphine analgesia while reversing dynorphin antagonism of morphine analgesia in the mouse.[5] These findings suggest a physiological role for vasopressin in the modulation of endorphinergic control of pain. We now report the synthesis and characterization of a novel vasopressin analog and present further evidence supporting an interaction between the opioid system and vasopressin.

Synthesis and Methods

Desamino lysine^8-N_ϵ-$(Gly)_3$-vasopressin (DLGVP) was prepared by treating desamino lysine^8-vasopressin (0.76 gm, 0.73 mmol) with Boc-triglycine (0.61 gm, 2.1 mmol) in the presence of triethylamine, 1-hydroxybenzotriazole (0.63 gm, 3.9 mmol) and dicyclohexylcarboiimide (0.23 gm, 1.11 mmol) in DMF at 4°C for 0.5 hr and then at 25°C for an additional 3 hr. This product was then isolated by precipitation and lyophilization. It was then treated with trifluroacetic acid in the presence of anisole at 0°C and the deprotected product isolated by lyophilization and purified by gel filtration to yield 0.7 gm (0.53 mmol) of the triglycyl analog in 73% yield and 99% purity by analytical HPLC. The desamino lysine^8-vasopressin was synthesized by the Merrifield solid-phase procedure on a benzhydrylamine support. The disulfide bridge was formed by the potassium ferricyanide method. Purification was accomplished by gel filtration and partition chromatography on Sephadex G-25.

Analgesic effects were assessed in mice by the acetic acid writhing test with percent analgesia calculated as: 100 - (control writhes - treatment writhes)/control writhes. Male, ICR mice (20-30 gm) received graded sc doses of DLGVP, AVP or morphine sulfate 20 min prior to an intraperitoneal injection of acetic acid (0.2 N, 1 ml/kg). After 15 min, writhing was recorded for one 5 min interval. Analgesia was also evaluated in the hot-plate test with latency to hind-paw lick taken as the endpoint. Mice were made tolerant to morphine by twice-daily (0900 and 1900 hr) sc injections of increasing doses of morphine (10, 30, 100 and 100 mg/kg) over 4 days.

Results and Discussion

Administration of DLGVP (0.01 - 3 mg/kg, sc) produced a maximum analgesia of 62% (n = 10) in the writhing test (A_{50} = 0.03 mg/kg) while AVP (0.01 - 3 mg/kg, sc) also inhibited writhing with a maximum effect of 70% (n = 10)(A_{50} = 0.16 mg/kg). Administration of naloxone (2 mg/kg, sc) 10 min prior to DLGVP or AVP only partially attenuated the analgesic effect of these peptides. Morphine (0.01 - 3 mg/kg, sc) produced a dose-related, naloxone-reversible inhibition of writhing. DLGVP (1, 3 or 10 ug) given icv was also effective in this test. DLGVP was not effective in the hot-plate test at 55°C when given at 3 mg/kg; this dose was effective, however, when the surface temperature was lowered to 48°C and peak effects were seen at 10 min. Pretreatment of mice for 4 days with morphine displaced the morphine dose-response curve (writhing test) to the right of that seen in naive animals by a factor of 4.6, indicating the development of tolerance to this morphine effect. This pretreatment also flattened the DLGVP curve demonstrating cross-tolerance between this peptide and morphine (Figure 1). Application of DLGVP to the electrically stimulated guinea-pig ileum (longitudinal muscle-myenteric plexus) preparation (an opioid sensitive system) had no effect. These results indicate an interaction of DLGVP with the opioid system. Since DLGVP did not inhibit contractions of the isolated ileum, it is concluded that this peptide does not interact with an opioid receptor in that tissue. Conversely, the consistent naloxone attenuation of the DLGVP analgesic response taken together with the demonstrated cross-tolerance between this peptide and morphine suggests that (a) the peptide interacts with an opioid receptor in vivo, but not in vitro, or (b) the compound is affecting levels of endogenous analgesic (endorphinergic) peptides. On the basis of the work in vitro, the latter seems more likely. The demonstration of cross-tolerance between morphine and DLGVP suggests a final

common pathway in the analgesic effects of vasopressin and the opioid peptide system and supports further the concept of physiological control of opioid analgesia.

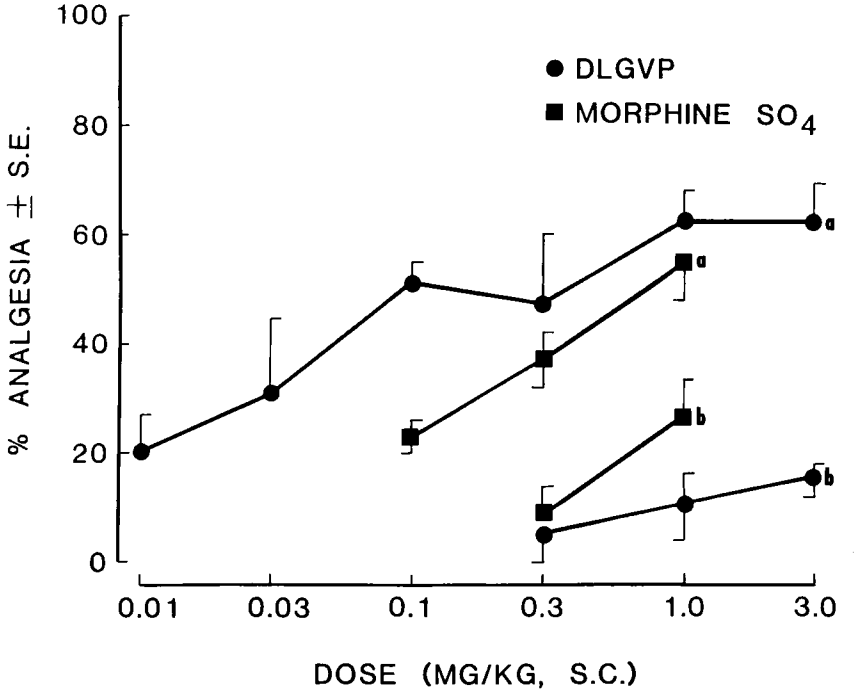

Figure 1. Dose-response curves of DLGVP and morphine before (a) and after (b) pretreatment with morphine for 4 days.

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BINDING OF α-MSH AND STRUCTURALLY-RELATED PEPTIDES TO RAT-BRAIN OPIATE RECEPTORS

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Introduction

Observations of the effects of ACTH and α-MSH on behavior, learning, and analgesia,[1-3] coupled with recent information on α-MSH biosynthesis[4] and its localization in brain prompted us to study directly the binding of α-MSH and structurally related peptides to the rat-brain opiate receptor utilizing radioactive ligands selective for classes of opiate receptors.

Using a rat-brain cortical preparation and biologically active radioligands, ^{3}H-dihydromorphine and ^{3}H[D-Ala^{2},D-Leu^{5}]-enkephalin, we studied the binding of ACTH, α-MSH, [Nle^{4},D-Phe^{7}]α-MSH, and parathyroid hormone (PTH 1-34), to the rat-brain opiate receptor. The analogue of α-MSH was selected because of its previously demonstrated enhanced stimulation of adenylate cyclase, tyrosinase activity and melanosomal dispersion.[5] Parathyroid hormone (1—34) was studied because it contains, in the region PTH-(17—24), a sequence of marked homology to ACTH-(4—10).

For study of α-MSH receptors, we chose an oxidation-stable synthetic analogue of α-MSH of enhanced biopotency, [Nle^{4},D-Phe^{7}]α-MSH, for attachment of both iodine and a photolabile moiety and α-MSH responsive Cloudman melanoma cells, as a source for α-MSH receptors.

Results and Discussion

α-MSH, [Nle^4,D-Phe^7]α-MSH, ACTH (1—39), and PTH-(1—34) each bound to the opiate receptor at concentrations ranging from 1 x 10^{-6} M to 1 x 10^{-4} M. The apparent K_B for α-MSH is 1.5 x 10^{-5} M for the delta class of cortical opiate receptors, and 5 x 10^{-5} M for the mu class of cortical opiate receptors, defined respectively by displacement of class-specific radioligands ^{3}H-(D-Ala^2,D-Leu^5)enkephalin and ^{3}H-dihydromorphine. The α-MSH, ACTH and PTH-(1—34) peptides bound to brain membranes less avidly than did β-endorphin by a factor of approximately 10^4. PTH-(53—84), which contains no structural homology to the ACTH-(4—10) sequence, demonstrated no inhibition of radioligand-specific binding. HPLC analysis of iodinated α-MSH following enzymic digestion with papain, prolidase and amino peptidase indicated that only the monoiodin-

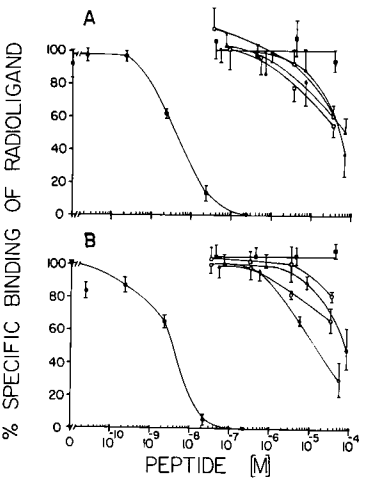

Fig. 1. Plot of competitive inhibition of radioligand binding to rat brain cortical opiate receptors. Radioligands are: ^{3}H-dihydromorphine in panel A and ^{3}H-[D-Ala^2,D-Leu^5]enkephalin in panel B. The peptides are represented by (●) β-endorphin; (▲) [Nle^4,D-Phe^7]α-MSH; (○) ACTH-(1—39); (△)α-MSH; and (□) PTH-(1-34). The peptide hPTH-(53-84) (■) was used as a control.

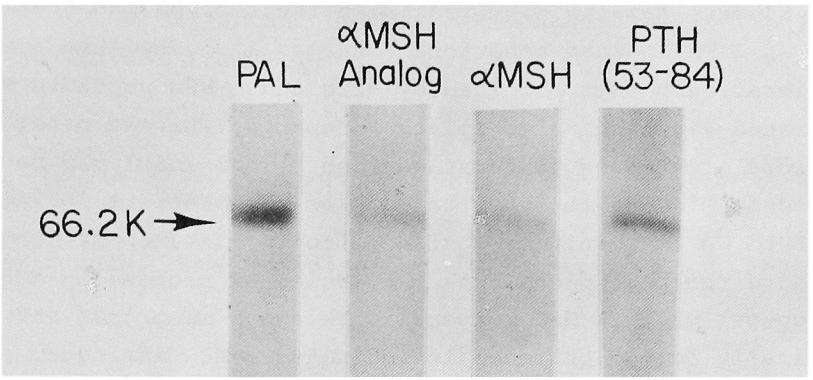

Fig. 2. Radioautograph of FNPA-reacted ^{125}I[Nle^4,D-Phe^7]α-MSH photoaffinity-labeled melanoma plasma membranes after solubilization, reduction and SDS-polyacrylamide gel electrophoresis. The presence of α-MSH and [Nle^4,D-Phe^7]α-MSH, which compete for membrane binding sites, markedly reduces the intensity of the band that labels.

ated tyrosine [Nle^4,D-PHE^7]α-MSH was biologically active. Although many protein bands are seen after Coomassie blue staining of SDS-polyacrylamide gels of solubilized Cloudman melanoma membranes, only one distinct band is visualized by radioautography. This band, migrates on SDS-polyacrylamide gel electrophoresis at approximate M_r=70,000.

Our studies, with a preparation of rat brain cortex and biologically active opiate radioligands, demonstrate that α-MSH, [Nle^4,D-Phe^7]α-MSH, ACTH-(1—39) and PTH-(1—34) can all bind to the cortical opiate receptor. There is little selectivity of the binding of the α-MSH structurally related peptides to the mu or delta subclass of opiate receptor. Furthermore, our findings suggest that structural determinants lying within the "active core" sequence of ACTH, and, in addition, present in a homologous sequence of PTH are responsible for specific interaction with brain opiate receptors.

[Nle^4,D-Phe^7]α-MSH, did not possess enhanced affinity for the opiate receptor compared to unsubstituted α-MSH.

Studies showing differential effects of naloxone antagonism of ACTH-induced behavioral changes indicate that some behavioral responses elicited by ACTH and α-MSH peptides may be mediated via binding to opiate receptors, whereas other behavioral responses (not antagonized by naloxone) may be mediated by binding to other categories of receptors for ACTH and α-MSH. We have initiated studies to obtain information regarding the physicochemical properties of proposed α-MSH receptors using α-MSH responsive melanoma membranes and a biologically active photolabile iodinated α-MSH analogue, and have demonstrated an α-MSH receptor on Cloudman melanoma cells. We now plan to identify α-MSH receptors in the central nervous system.

High local concentrations of α-MSH and ACTH in regions of the central nervous system may permit a neurotransmitter or neuromodulator role for these peptides through their interaction with the opiate receptor and may explain some of their effects on the complex events associated with behavior and learning, whereas other behavioral effects may occur by direct binding to specific α-MSH receptors in brain.

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EVIDENCE FOR AN ENDOGENOUS CENTRAL NERVOUS SYSTEM LIGAND FOR THE PHENCYCLIDINE RECEPTOR

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Introduction

Phencyclidine (1-(phenylcyclohexyl piperidine), PCP or angel dust) is a major drug of abuse. Its use in man often results in clinical manifestations which include euphoria, aggressive behavior and psychotic disturbance. The symptoms are difficult to distinguish from schizophrenia and treatment of PCP abuse often requires prolonged psychiatric management.

The dramatic psychopathological actions of PCP appear to be mediated by its interaction with highly specific and selective PCP receptors in the central nervous system. These receptors have a selective regional distribution that is distinct from the receptor distribution of any known endogenous ligand and their localization in cortical regions is consistent with the observed psychotic actions of PCP administration[1]. The identification of an endogenous ligand which can interact with the angel dust receptor is of obvious clinical and biological interest. In this study, we report the identification of angel dustin, an endogenous agonist for the PCP receptor.

Methods and Results

Porcine brains were obtained from Eskay Co. and homogenized at 4°C in five volumes of a mixture of 50 ml formic acid, 150 ml trifluoracetic acid (TFA), 10 g NaCl and 90 ml HCl (37%) per liter. The homogenate was then centrifuged at 15,000 x g for 20 min and the pellet was discarded. Lipids were removed from the supernatant by extracting twice with two volumes of diethyl ether.

The supernatant was then chromatographed on a preparative liquid chromatograph (System 500A, Waters Assoc.) containing two reverse phase C_{18} cartridges (each 5.7 x 30 cm) in series. The sample was injected at 100 ml/min and the columns were eluted using a step gradient of 10, 20, 30, 40, 50 and 100% acetonitrile in 0.1% trifluorocetic acid at 250 ml/min. Aliquots were removed, dried by vacuum centrifugation and assayed in two dilutions for the ability to inhibit receptor binding of ^{3}H-PCP as described previously[1]. These fractions containing angel dustin activity were concentrated and chromatographed by gel filtration or reversed phase analytical high pressure liquid chromatography (HPLC).

For gel filtration experiments, the supernatants were resuspended in 0.1 M acetic acid and chromatographed on columns of Sephadex G-10 (1.4 x 19 cm, 6ml/hr), G-25 fine or G-50 fine (1.6 x 70cm, 10 ml/hr) eluted with 0.1 M acetic acid. Angel dustin activity eluted in the void of the G-10 column, near the included volume of the G-50 and in the fractionated range of the G-25 column suggesting a molecular weight of 1500-2000.

For analytical HPLC experiments, samples were resuspended in 0.25 M triethylammonium formate buffer (TEAF) and chromatographed on a 4mm x 30 cm μBondapak C_{18} column using a gradient of acetonitrile and TEAF, as illustrated in Figure 1, run at 1.0 ml/min. Samples from the analytical column which contained PCP activity were subjected to peptidase digestion or further tests of PCP bioactivity.

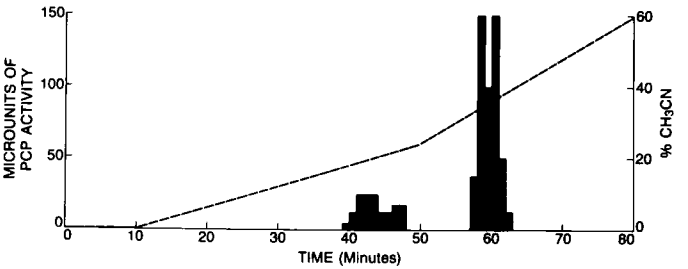

Figure 1. Elution of angel dustin activity from analytical HPLC. Angel dustin activity is expressed in terms of equivalents of PCP required to displace ^{3}H-PCP receptor binding (1 unit of activity = 1 mole of PCP).

Fractions containing angel dustin were incubated with chymotrypsin (0.3 mg/ml), and trypsin (3.0 mg/ml) for 1 hour at 36°C; with leucine aminopeptidase (0.5 mg/ml) and pronase (0.5 mg/ml) for 20 min at 37°C and with carboxypeptidase A (0.1 unit/ml) for 10 min at 37°C. Controls were obtained by incubating bioactive fractions with denatured enzymes (80°C for 10 min prior to incubation). PCP bioactivity was significantly decreased by incubating with all enzymes except chymotrypsin.

The active angel dustin fraction was tested on behavioral and electrophysiological tests for PCP activity. An injection of 25 nmol of PCP in 2 ul of saline caused a dramatic increase in contralateral rotation (63 $\pm$ 8 contralateral turns compared to 25 $\pm$ 13 contralateral for saline or an HPLC fraction of porcine brain extract that does not contain angel dustin activity). An injection of angel dustin bioactivity in 2 ml saline (HPLC fraction 60 - see Fig 1), like PCP, resulted in a dramatic significant increase in contraversive turning (65 $\pm$ 9 contraversive turns). The results of this study demonstrate

that angel dustin has the same effect as PCP after intranigral injection. The active fraction was also tested for electrophysiological actions after pressure injection onto cortical or hippocampal pyramidal cells. Micropippettes were filled with 10 mM PCP solution of the active angel dustin fragments immediately before experiments and single unit extracellular recordings were made. Iontophoresis of PCP (10 nA for 30s) onto cortical or hippocampal cells inhibits spontaneous activity. Similarly, pressure injection of a fraction containing angel dustin activity (HPLC fraction 60) dramatically inhibits spontaneous activity. Pressure injection of a fraction that does not contain angel dustin activity (fraction 24) fails to alter spontaneous electrical activity.

Discussion

The results of these studies indicate that there is an endogenous ligand, angel dustin, for the PCP receptor. Angel dustin appears to be a peptide with a molecular weight of 1500-2000 daltons. Thus PCP appears to be an agonist for endogenous angel dustin systems as PCP and angel dustin have the same behavioral and electrophysiological activities. If, in fact, angel dustin is an endogenous ligand for the phencylidine receptor, disorders of this neuronal system could lead to psychopathologies and development of antagonists to this compound could lead to the discovery of a new class of antipsychotic agents.

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STRUCTURE AND ANTAGONIST ACTIVITY IN A SERIES OF HEXAPEPTIDE SUBSTANCE P ANALOGS

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Introduction

The localization of Substance P (SP) in primary afferent neurons of the class which transmit nociceptive information[1] has led to the hypothesis that SP antagonists should, in principle, provide a route to novel analgesics.

The seminal publication by Piercey, et al[2] of the structure of an antagonist of SP, [DPro^2, DPhe^7, DTrp^9]SP, led us to incorporate features of this and subsequently reported antagonists[3] into our ongoing program of Pro^6 SP(6-11) agonist analogs. Here we report the antagonist profiles of Pro^{6}SP(6-11) peptides having one or more residues replaced by D-aromatic aminoacids.

Methods

Chemistry - Peptides were synthesized by solution techniques, purified by chromatography on silica gel and/or C_{18} reverse phase and tested as the lyophilized phosphate salts.

Biology - SP receptor binding was determined using a modification of the method of Hanley, et al.[4] Isolated Tissue: Guinea pig ileum longitudinal muscle strips were prepared according to Lee et al.[5] and pA_2 calculated according to Bjorkroth et al.[6] Acetylcholine writhing was

determined in male Swiss-Webster mice.[7] The appropriate dose of the test compound was given intrathecally and the animals were challenged after 5 minutes with acetylcholine. In the rotarod test[8] the test compounds were given intrathecally and performance scored, blind, at 5, 15, 30, 45 and 60 minutes.

Results and Discussion

In light of the theory of "address" and "message" portions of peptides,[9] and in consequence of a concern that the N-terminal portion of SP might be important for receptor interactions,[10] the first question was whether an acylated C-terminal pentapeptide of Substance P would have sufficient affinity for the SP receptor to function as an antagonist. Our methodology for discovering and measuring the potency of SP antagonists was based on a two-step in vitro program: firstly, affinity for the SP receptor was assessed in a rat brain binding assay; then the ability of the test compound to antagonize the effect of SP in the unstimulated guinea pig ileum was measured and expressed as a pA_2.

Shown in Table I are three Pro^6 C-terminal hexapeptide analogs which satisfied our preliminary screening requirements. They bind to the SP receptor in rat brain and show antagonist potency in g.p.i., as shown by pA_2 measurements of 5.4 to 5.7. These pA_2 values compare well to those of the undecapeptides reported by Piercey and Folkers. Additionally, they appear to exhibit a reduced agonist component. We were satisfied, therefore, that a hexapeptide could provide the structural requirements for the elaboration of SP antagonists.

The acetylcholine writhing test is an accepted means of assessing antinociceptive activity.[7] In this test 6 produced a dose-related inhibition of the writhing response. The ED_{50} of 6 (2.7 μg/mouse, i.t.) demonstrates comparable potency to that of the undecapeptide 3 (4.6 μg/mouse).

Table I Substance P Antagonists

No.	Structure	Binding (IC_{50}) μM	Rel. Agonist Potency	pA_2
1.	ArgProLysProGlnGlnPhePheGlyLeuMetNH$_2$	.002	100	-
2.	ArgDProLysProGlnGlnDPhePheDTrpLeuMetNH$_2$	1.4	.005	4.6*
3.	ArgDProLysProGlnGlnDTrpPheDTrpLeuMetNH$_2$	3.7	.02	5.5^{+}
4.	ProDPhePheDTrpLeuMetNH$_2$	26.8	.0001	5.6
5.	ProDPhePheDPheLeuMetNH$_2$	30.7	<.001	5.4^{++}
6.	ProDTrpPheDTrpLeuMetNH$_2$	16.1	.00001	5.7

*A value of 4.61 is reported for this compound[6]

$^{+}$A value of 5.43 is reported for this compound[6]

$^{++}$The slope of the Schild plot is -0.49

Recently, Rodriguez, et al.[11] have suggested that the apparent antinociceptive activity of peptide 3, [DPro^2, DTrp^{7,9}]-SP, is secondary to its profound, long-lasting motor impairments. The effects 3 and 6 were compared in the rotarod test, a standard test for motor performance.[10] Mice receiving the hexapeptide 6 at a dose 2-3 times its analgesic ED_{50} showed motor function which is indistinguishable from vehicle controls (Figure 1); in contrast mice receiving the undecapeptide 3, as reported, showed significant motor impairment at this dose. There is more than an 11-fold separation between analgesic (ACh) ED_{50} and motor impairment (rotarod) ED_{50} for 6 (2.7 mg/mouse vs. >30 μg/mouse) as contrasted with the two-fold separation of the same parameters in the case of 3 (4.6 vs. 8.4 μg/mouse).

Conclusions

We have shown that hexapeptides modeled on the C-terminus of Substance P can provide SP antagonists of comparable potency to known undecapeptide antagonists, and, further, that antinociception can, in these hexapeptides, be separated from motor function impairment.

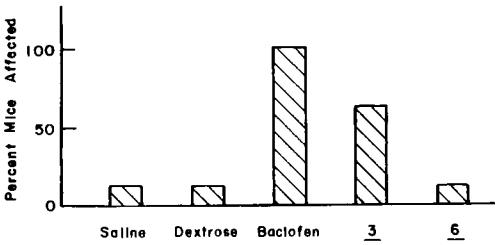

Fig. 1. Impairment of motor function in mice as measured by rotarod performance. Peptides 3 and 6 were administered at 10 μg i.t.; Baclofen was administered at 1.75 mg/kg s.c. as positive control.

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CHOLECYSTOKININ OCTAPEPTIDE FRAGMENTS: SYNTHESIS AND STRUCTURE -ACTIVITY RELATIONSHIP

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Introduction

Cholecystokinin (CCK) acts on gastrointestinal, pancreatic and hepatobiliary systems. This peptide also produces a variety of effects in the central nervous system. In order to characterize more precisely the types of receptors that mediate peripheral and central effects of CCK, we have studied the ability of all the C-terminal fragments of CCK 26-33 (H-Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2) to elicit contractions of isolated guinea pig gallbladder and ileum and to inhibit [^{3}H]pentagastrin binding on rat striatum slide-mounted sections.

Experimental Procedures

CCK 26-33 and its C-terminal fragments were synthesized by solid-phase method. N^{α}-Boc-amino acids were esterified to the Merrifield support by the cesium salt method. N^{α}-Boc-amino a-

cids were used throughout with protection of β-carboxyl, phenolic hydroxyl and β-indole functions by β-phenacyl, O-2,4-dinitrophenyl and N^{in}-formyl groups, respectively. Coupling was achieved by preformed symmetrical anhydrides. N^{α}-Boc groups were removed by 0.1N HCl in formic acid. The last Boc was removed after ammonolysis (non-sulfated peptides) with a solution of TFA in 10 and 100 fold excess, over the peptide, of ethanedithiol and dimethylsulfide respectively or after sulfation with a solution of TFA in 10 fold excess, over the peptide, of 2-methylindole. The 2,4-dinitrophenyl ether was removed by reaction with 1M thiophenol in DMF (2h, 23°C) whereas the β-phenacyl ester was removed with 1M selenophenol in DMF (72h, 23°C) Cleavage of peptides from the solid support and removal of the N^{in}-formyl group were performed in a 30% solution of ammonia in methanol (48h, 4°C). Sulfation of the phenolic hydroxyl group was brought about by reacting the N^{α}-Boc-peptides with a solution of sulfur trioxide pyridine complex in anhydrous pyridine (≃12h, 23°C). All the peptides were purified by countercurrent distribution and high performance liquid chromatography. Homogenous peptides gave correct amino acid analyses after acidic and enzymatic hydrolysis.

Gallbladder and ileum were prepared according to Rubin et al.[1] Concentrations of peptides ranging from 10^{-10} to 10^{-4}M of all peptides were assayed on both preparations. Inactive peptides and weak agonists were tested as putative antagonists. Binding assays on rat striatum slide-mounted sections were performed as described by Gaudreau et al.[2]

Results and Discussion

Table I shows the potencies of various C-terminal fragments of CCK 26-33, CCK 1-33, gastrin I and pentagastrin in gallbladder and ileum bioassays and in rat striatum slide-mounted binding assay. The order of potency of all the peptides studied is very similar in gallbladder and ileum. CCK 26-33 > CCK 1-33

Table I. Potencies of Various Cholecystokinin/Gastrin-Related Peptides in Rat Brain Section Binding Assay and in Guinea Pig Gallbladder and Ileum Bioassays

Compound	Rat Brain Binding Assay IC_{50} (nM)[1]	RP (%)	Guinea Pig Gallbladder EC_{50} (nM)[2]	RP (%)	Guinea Pig Ileum EC_{50} (nM)[2]	RP (%)
CCK 26-33	0.55 ± 0.03	100	2.5 ± 0.4	100	3.9 ± 0.5	100
CCK 26-33 NS*	0.55 ± 0.09	91	6840 ± 200	0.04	6400 ± 100	0.06
CCK 27-33	0.65 ± 0.05	77	6.2 ± 0.6	40	9.7 ± 0.4	40
CCK 27-33 NS*	1.35 ± 0.06	37	11200 ± 900	0.02	10000 ± 1300	0.04
CCK 28-33	2.50 ± 0.05	20	28000 ± 2200	< 0.01	13800 ± 2700	0.03
CCK 29-33	2.80 ± 0.05	18	-	< 0.01	27500 ± 6500	0.01
CCK 30-33	3.70 ± 0.06	13	-	< 0.01	28500 ± 2500	0.01
CCK 31-33	Inactive[3]	0	Inactive[3]	0	Inactive[3]	0
CCK 32-33	Inactive[3]	0	Inactive[3]	0	Inactive[3]	0
CCK 1-33	4.0 ± 0.9	13	4.8 ± 0.8	52	6.9 ± 0.2	56
Gastrin I	13 ± 3	4	-	0.01	6400 ± 430	0.06
Pentagastrin			40000 ± 1500	0.01	10800 ± 2100	0.04

[1] IC_{50}, concentration displacing 50% of specifically bound [^{3}H]pentagastrin. Values represent mean ± SEM of three experiments, each in triplicate. [2] EC_{50}, concentration producing 50% of the maximal effect. [3] No effect at 10^{-4}M. * NS = non-sulfated.

> CCK 27-33 with other fragments becoming much less active as a function of the chain length. Gastrin I and pentagastrin are weaker agonists in the gallbladder assay than in the ileum. In both preparations, the minimal structure able to induce a biological response is CCK 30-33. Neither the inactive fragments nor the weak agonists have antagonistic properties. In the rat striatum binding assay, the order of potency is different from the one observed in the bioassays. CCK 26-33 ≃ CCK 26-33NS > CCK 27-33 > CCK 27-33NS with smaller fragments, CCK 1-33 and gastrin I having less affinity for the CCK receptors. However, CCK 28-33, 29-33 and 30-33 still have a high affinity. CCK 31-33 and 32-33 are inactive. These findings show that in all preparations the minimal structure able to bind and/or to induce a biological response is the C-terminal tetrapeptide. In peripheral tissues, the sulfate ester moiety is critical for the maximal activity of CCKs to their receptors. In the brain, the sulfate ester seems to be less important since the non-sulfated octapeptide is almost as potent as its sulfated parent peptide. The order of potency of a series of related peptides being a first criterium in receptor classification, our results confirm earlier findings of Innis and Snyder[3] in that brain CCK receptors appear to be different from the peripheral ones. Alternatively, our data could be explained by the different kinetics of enzymatic inactivation in central vs peripheral tissues. However, the fact that pentagastrin (a CCK/gastrin analog resistant to aminopeptidase degradation) is very weak in both bioassays does not support this hypothesis.

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BINDING OF METHIONINE-ENKEPHALIN TO MICELLES: EFFECT ON PEPTIDE SIDE CHAIN CONFORMATION

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Introduction

Transfer of peptide hormones from an aqueous phase to the lipid-rich environment of their protein receptors may be mediated by interactions with the surrounding membrane phospholipids. Peptides involved in such processes are typified by the neurotransmitters methionine-enkephalin (Tyr-Gly-Gly-Phe-Met) (Met-Enk) and leucine-enkephalin (Tyr-Gly-Gly-Phe-Leu) (Leu-Enk), endogeneous brain peptides which operate at the same receptor as natural opiates and their antagonists (1). Enkephalins are known to bind negatively-charged lipids, such as phosphatidylserine via ionic attractions (2). Using high-resolution proton (360 MHz) and carbon-13 (90 MHz) NMR, we have shown (a) that hydrophobic interactions are sufficient to bind an opioid peptide effectively to a model membrane regardless of whether or not formal electrostatic attractions are present, as in the case of Met-Enk or Leu-Enk and the zwitterionic (net neutral) lipid, lysophosphatidylcholine (lyso-PC); and (b) that these peptides bind to lipids *via* a combination of electrostatic and hydrophobic interactions when the lipid is negatively charged (e.g., lysophosphatidylglycerol (lyso-PG) (3). Lysophospholipids and others which form relatively lower molecular weight micellar particles (vs. lipid vesicles) are preferred in these studies because of the clarity of NMR spectra obtainable (4-6).

One possible role lipid might play in hormone/receptor events is that of converting (or at least facilitating the conversion of) the peptide into the "biologically active" conformation required for receptor binding. In the present work, we have examined this question by monitoring the behavior of β-proton resonances from the two enkephalin aromatic residues as a function of lipid/peptide mole ratios.

Results and Discussion

Proton-NMR (360 MHz) spectra of the aliphatic side-chain β-methylene protons of Met-Enk Tyr and Phe residues in the presence of increasing concentrations of lyso-PG micelles are shown in Figure 1. Linewidth, chemical shift, and coupling constant data for Phe and Tyr β-CH_2 protons, obtained from Figure 1 and related spectra, are summarized in Figure 2. Resonance line broadening -- arising from reduction in rates of molecular motion upon binding the (much larger) lipid particle -- is evident; for example, the Δ(linewidth) for Tyr β-CH_2 resonances = 5.5 Hz for lipid-bound peptide (Fig. 1E) vs. free Met-Enk (Fig. 1A). Selective chemical shift changes upon lipid binding are also noted throughout the spectra; in the region examined, the higher-field (β-H^A) components of the Phe signals shift ~30 Hz, while the downfield (β-H^B) components shift ~4 Hz (Fig. 2).

Peptide or protein binding to membrane preparations is frequently reported to be accompanied by alterations in gross conformation [e.g., (7)]. However, significant variations in the vicinal coupling constants -- which might be expected to accompany major redistribution of side chain rotamer populations on lipid binding -- did not occur. This was confirmed for ^3J values of all enkephalin resonances not obscured by lipid resonances (i.e., Phe β-CH_2, Tyr β-CH_2 (Fig. 1), and also Phe α-CH, Met β-CH_2, and Met γ-CH_2). These data thus

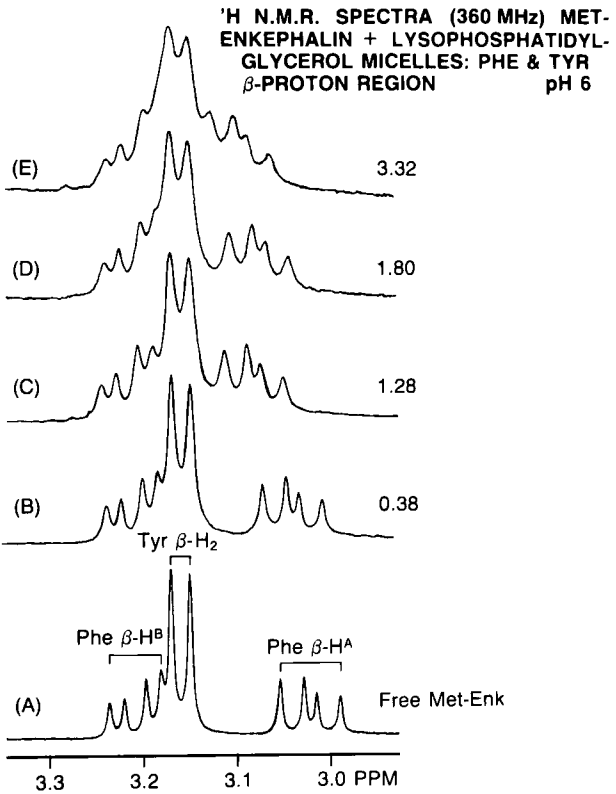

Fig. 1. ^{1}H-NMR spectrum (360 MHz) of [Met^{5}]-enkephalin (2.5 mg in 0.5 ml D_2O, pH = 6, 23°C) in the region of the aliphatic side-chain β-methylene protons of the Phe and Tyr residues. (A) Free peptide (concentration = 8.72 x 10^{-3}M); (B)-(E), sample (A) to which successive weighed amounts of lipid have been introduced (0.8, 2.7, 3.8 and 7.0 mg of egg L-α-lysophosphatidyl-DL-glycerol, respectively). Mole ratios of lipid/peptide are shown in the diagram. Spectra were recorded in the Fourier transform mode with 16K data points and typically 250 accumulations per spectrum. Chemical shifts are referenced to external tetramethylsilane.

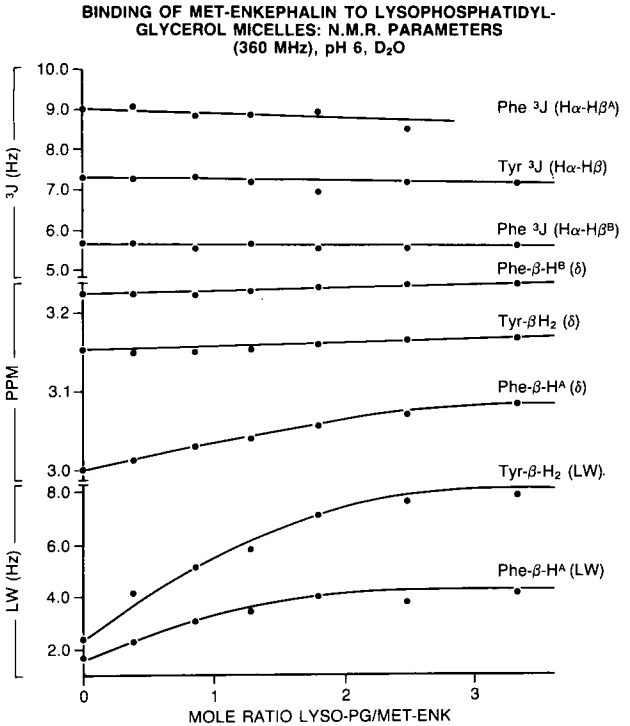

Fig. 2. Effect on [Met^5]-enkephalin ^{1}H-NMR parameters as a function of L-α-lysophosphatidyl-DL-glycerol/peptide ratios. LW = linewidth (uncorrected); δ = chemical shift; ^{3}J = vicinal coupling constant. Data are obtained from spectra such as presented in Fig. 1. Values given for Phe parameters are the average of two determinations, i.e., both branches of the ABX octet of each proton. Resonance broadening and overlap precluded accurate determination of some ^{3}J values at higher lipid/peptide ratios.

demonstrate that ^3J-values remain unchanged during the transition from free to bound enkephalin. The ^3J-values obtained are in good agreement with those reported by Zetta et al. (8) and Kobayashi et al. (9). (Since NH protons have been exchanged, no direct information concerning enkephalin backbone conformation(s) was obtained.)

Several solution NMR studies on Met-Enk have led to proposals for folded (β-turn) conformations in organic solution (8,10,11), but for largely extended conformations in D_2O solution (12). Met-Enk should be able to bind to micelles in either of these conformational categories, although the binding should generally favor peptide structures which place enkephalin hydrophobic substituents on a non-polar "face" suitable for lipid interaction. Indeed, specificity or "sidedness" for the peptide-lipid complex is suggested by both (a) the differential extents of line broadening for Tyr and Phe β-CH_2 resonances and (b) the selective chemical shift change of the Phe H_β^A vs. the H_β^B resonance (Fig. 2)*. However, binding of free Met-Enk to the lipid particle appears to occur without alteration of the key peptide side chain conformational parameters, the vicinal coupling constants.

The results of these and our previous studies (3) lead us to suggest that the major influences of lipid upon enkephalin peptides reside in (i) binding them in a residue-specific manner via a combination of electrostatic attractions and hydrophobic interactions (the "attraction-interaction" model); (ii) reducing their rates of conformational fluctuations; and (iii) facilitating their entry into, and thereby increasing their effective local concentration in the membrane microenvironment.

*Another example of the apparent specific binding of enkephalin to lipid was observed in ^{13}C spectra (90 MHz) of the additions of lyso-PG to Leu-Enk, where one Leu δ-CH_3 carbon resonance shifted 38 Hz and the other 17 Hz (both downfield) in bound (lyso-PG/Leu-Enk mole ratio = 5) vs. free peptide.

Acknowledgements

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SPECTRAL STUDIES OF GRAMICIDIN A AND ITS DERIVATIVES IN MEMBRANES. ENSUING MODELS OF ION CHANNELLING.

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Introduction

In a number of preceeding publications[1-7] we have considered conformational states of gramicidin A (GA, 1) and its covalently crosslinked bis-derivatives (2-10) in solution. Experimental IR spectra in the amide I region were decomposed into component spectra calculated by the method of Chirgadze and Nevskaya[8] for various helical structures of GA. Fig.1 represents some of the computed spectra. Complementary information was obtained from the CD spectra (the helical sence and intermolecular association) and from fluorescence spectra (association).

L L D L D L D L D L D L D L
Form-ValGlyAlaLeuAlaValValValTrpLeuTrpLeuTrpLeuTrp-EA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Gramicidin A (Form-formyl, EA-ethanol amine) (GA) (1)

EA-(15-1)-CO(CH_2)$_n$CO-(1-15)-EA (2-4)
Head-to-head, n=2,4,6

Form-(1-15)-EA-CO-(1-15) (5-8)
Head-to-tail, n=1,2,6,7

Form-(1-15)-EA-(CH_2)$_n$CO-EA-(15-1)-Form (9-10)
Tail-to-tail, n=2,3

It was found that none of the four slowly interconverting GA dimers[12] is conformationally homogeneous. In dioxane

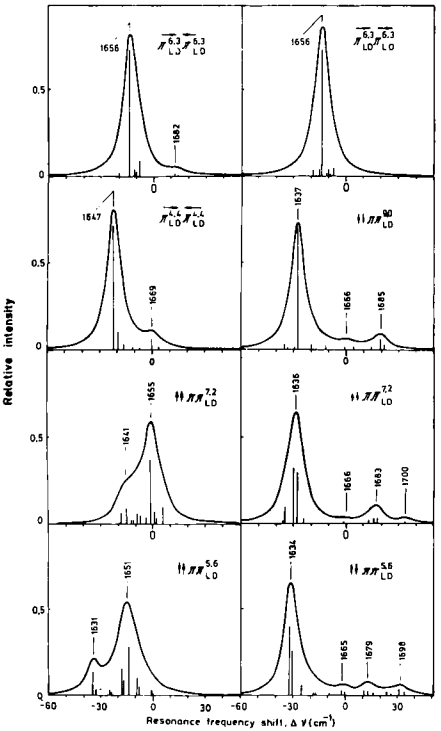


Fig. 1. Calculated IR spectra of GA dimers. $\pi_{LD}\pi_{LD}$ and $\pi\pi_{LD}$ denote the single and double stranded helices. The former were originally proposed by Urry[9,10] and by Ramachandran and Chandrasekaran[11], the latter - by Veatch et al.[12]. Subscript (4.4, 5.6, 6.3, 7.2 or 9.0) indicates the number of amino acid residues per helical turn. A pair of arrows, → ←, or → → shows the association mode in the single stranded helix: N-terminus to the N-terminus (head-to-head) or N-terminus to the C-terminus (head-to-tail). A pair of arrows, ↑↑ or ↑↓ shows the mode of peptide chain alignment in the double helix: parallel or antiparallel.

each of these species is an equilibrium of a number of helical conformers of which the antiparallel double helix predominates

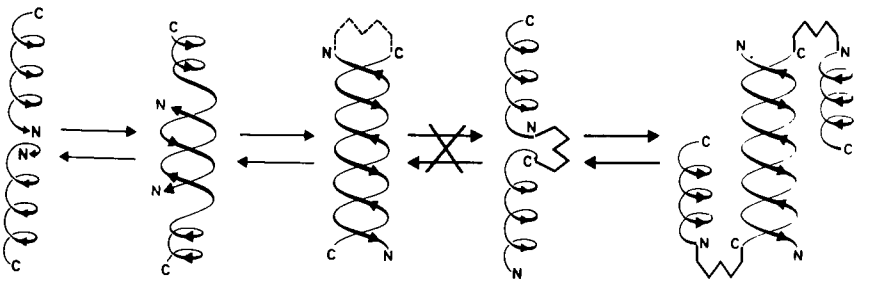


Fig. 2. "Zipper" mechanism of antiparallel double helix formation by GA and its head-to-tail bis-derivatives.

and the single stranded helices are the minor forms. Bis-derivatives (2-10) show in dioxane a strong tendency to form $\downarrow\uparrow\pi\pi_{LD}$-helical aggregates. At higher dilution, i.e. in the monomeric state they predominantly form single stranded helices ($\overrightarrow{\pi_{LD}}\overleftarrow{\pi_{LD}}$, $\overrightarrow{\pi_{LD}}\overrightarrow{\pi_{LD}}$ or $\overleftarrow{\pi_{LD}}\overrightarrow{\pi_{LD}}$ depending upon the junction site)[4-6].

Behavior of the "head-to-tail" analogs (5-8) for which there was no *a priori* reason to expect destabilization of the $\downarrow\uparrow\pi\pi_{LD}$-helix in the monomer supports the "sliding" or "zipper" mechanism of formation of these helices[5,6]. Such mechanism was hypothetically considered in 1975 by Urry et al.[13]. According to that mechanism formation of $\downarrow\uparrow\pi\pi_{LD}$-helices is preceeded by head-to-head or tail-to-tail association of single stranded π_{LD}-helices (i.e. by formation of $\overrightarrow{\pi_{LD}}\overrightarrow{\pi_{LD}}$ or $\overleftarrow{\pi_{LD}}\overrightarrow{\pi_{LD}}$-structures) with subsequent "screwing in" of one peptide chain into the other (Fig. 2). Either the first or the second part of this prosess is no longer possible in the presence of a cross-link and the $\downarrow\uparrow\pi\pi_{LD}$ helix is not formed.

The $\overrightarrow{\pi_{LD}}\overleftarrow{\pi_{LD}} \rightarrow \downarrow\uparrow\pi\pi_{LD}$ transition includes replacement of a number of intramolecular hydrogen bonds with intermolecular ones as well as concomitant synchronous, stepwise redistribution of many intramolecular hydrogen bonds. Energy computations showed that redistribution of hydrogen bonds in GA helices does

not imply passage of high energy barriers, since the non-hydrogen-bonded π^{5}_{LD}-helix, intermediate between the $\pi^{4.4}_{LD}$ and the $\pi^{6.3}_{LD}$ helices, proved to be one of the most favourable structures[14].

Interpretation of spectral data on GA in membranes is more restricted that in solution due to heterogeneity of the system, interference with IR and UV absorbtion bands of the lipid, light scatter, etc. E.g. the lipid:GA $\geqslant$ 1000:1 concentration range (where GA is a dimer as shown in fluorescence and single channel

Table 1. IR amide I Frequences (ν, cm^{-1}) and Conformations of GA in Phospholipid Liposomes.

Lipid	GA:lipid, M:M	After sonication		After sonication + incubation		
		ν	Conformation	Conditions of incubation	ν	Conformation
DPPC	1:10	1634[a]	$\downarrow\uparrow\pi\pi_{LD}$-helix + + $\uparrow\downarrow\beta$-sheet	15 hr, 70°	1630	$\downarrow\uparrow\beta$-sheet
	1:20	1634[a]	$\downarrow\uparrow\pi\pi_{LD}$-helix + + $\uparrow\downarrow\beta$-sheet	10 hr, 70°	1630	$\downarrow\uparrow\beta$-sheet
	1:75	1632	$\uparrow\downarrow\beta$-sheet	3 hr, 70°	1629	$\downarrow\uparrow\beta$-sheet
	1:150	1632	$\uparrow\downarrow\beta$-sheet	10 hr, 70°	1630	$\downarrow\uparrow\beta$-sheet
	1:310	1634	$\downarrow\uparrow\pi\pi_{LD}$-helix	3 hr, 70°	1630	$\downarrow\uparrow\beta$-sheet
DMPC	1:10	1633[a]	$\downarrow\uparrow\pi\pi_{LD}$-helix + + $\downarrow\uparrow\beta$-sheet	2 hr, 50°	1632	$\uparrow\downarrow\pi\pi_{LD}$-helix + + $\downarrow\uparrow\beta$-sheet
	1:35	1632[b]	$\downarrow\uparrow\beta$-sheet	2 hr, 50°	1631	$\downarrow\uparrow\beta$-sheet
	1:150--350	1630--1631	$\downarrow\uparrow\beta$-sheet	2 hr, 50°	1630--1631	
DOPC	1:100	1629	$\uparrow\downarrow\beta$-sheet + $\downarrow\uparrow\pi\pi_{LD}$ + $\pi^{\overrightarrow{6.3}\ \overleftarrow{6.3}}_{LD}$-helix	2 hr, 50°	1628	$\uparrow\downarrow\beta$-sheet + + $\downarrow\uparrow\pi\pi_{LD}$-helix
	1:10	1632[a]	$\uparrow\downarrow\pi\pi_{LD}$-helix + + $\downarrow\uparrow\beta$-sheet	2 hr, 50°	1628	$\uparrow\downarrow\beta$-sheet + $\uparrow\downarrow\pi\pi_{LD}$-helix

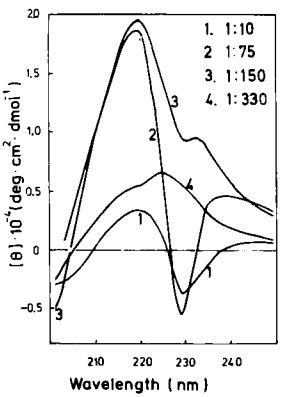
[a] For these conditions fluorescence maximum shifts from 330 to 334 nm, pointing to non-complete incorporation of GA into the lipid.

[b] The spectrum taken at 52°.

experiments[15]) is out of reach of modern IR and CD instruments. Nonetheless attempts are continuously being made to reach progress in that area[16-21] and the present work is one such attempt.

Results and Discussion

IR, CD and fluorescence spectra of GA were studied in various lipids and lipid:GA ratios; the incubation time and temperature were also varied. The results obtained are shown in Table 1 and Figs 3-5. The IR frequency, ν=1634 cm^{-1} obtained at 20° with sonicated dipalmitoyl phosphatidylcholine (DPPC) liposomes con-



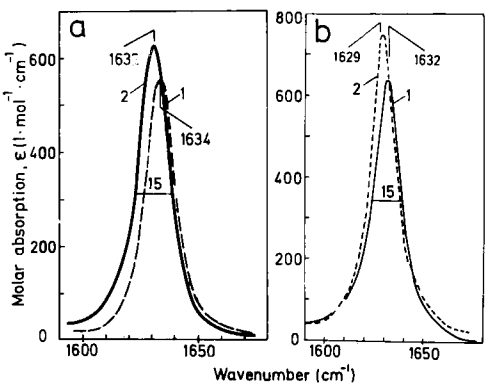


Fig. 3. CD spectra of GA in DPPC liposomes at various DPPC:GA ratio. The overall concentration of GA is (1-9)x10^{-4}M, T=20°. Spectra measured directly after sonication. 1,310:1, 2,150:1; 3,75:1, 4,10:1.

Fig. 4. Effect of increased concentration (a, spectra measured directly after sonication) and incubation (b, DPPC:GA=75:1) on the IR spectra of GA in DPPC liposomes.

(a) 1, DPPC:GA=310:1, 2, DPPC:GA=75:1

(b) 1, spectrum measured directly after sonication; 2, incubation 3 hr at 70°.

taining the smallest proportion of GA (lipid:GA = 310:1 which approaches the above mentioned ratio characteristic for the dimeric state) agrees with the ↓↑ double helical structure ($\pi\pi^{5.6}_{LD}$ or $\pi\pi^{7.2}_{LD}$) and excludes from consideration the single stranded helix as well as the ↑↑ double helix. Positive chiroptical effects in the CD spectrum (Fig. 3-1) point to higer than in dioxane content of right handed forms under these conditions. Increase in the GA concentrations is accompanied by notable changes in the CD and IR spectra (Figs 3 and 4a) due to further than dimer aggregation. The amide I band becomes more intensive and shifted to 1630 cm^{-1} at (75-100):1 ratio which is best explained by formation of a pleated β-sheet.[3] At further concentration increase the fluorescence maximum shifts from 330 nm to 334 nm indicating that the Trp chromophores are no longer merged into

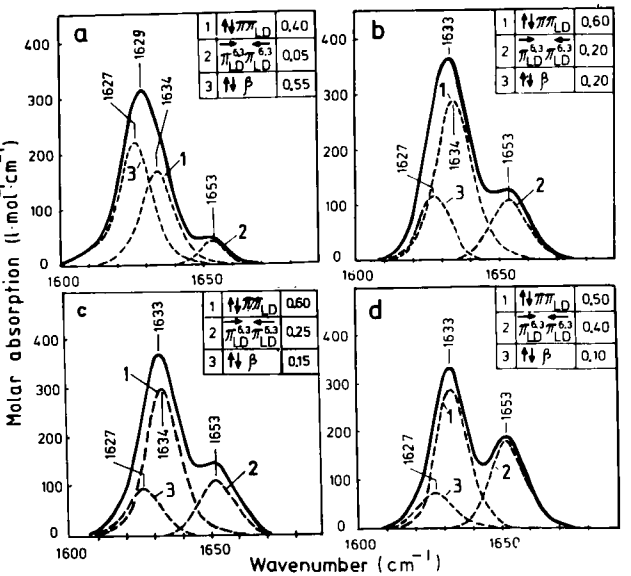


Fig. 5. IR spectra of GA and its N-carboxylic derivatives (11-13) (b-d) in DOPC liposomes (20°). Decomposition of the experimental profile into individual components is shown. (a) DOPC:GA=100:1; (b) DOPC:(11)=50:1; (c) DOPC:(12)=100:1 (d) DOPC:(13)=(170-340):1.

Table 2. Conformations of Bis-GA Derivatives in Liposomes.

Analog	Lipid	Lipid:peptide	Molar fraction $\uparrow\downarrow\pi\pi_{LD}$	$\pi_{LD}\pi_{LD}$	$\uparrow\downarrow\beta$
3,	DPPC	200:1	0.60	-	0.40
head-to-head	DOPC	30:1[a]	0.50	0.20	0.30
n=4		250:1	0.25	0.30	0.45
7,	DPPC	(190-340):1	0.60	-	0.40
head-to-tail	DOPC	40:1	0.30	0.15	0.55
n=6		170:1	0.25	0.40	0.35
10	DPPC	40:1	0.35	0.15	0.50
		200:1	0.70	0.20	0.10
tail-to-tail	DOPC	40:1	0.25	-	0.75
n=3		200:1	0.25	0.25	0.50

[a] Peptide is not completely incorporated into the lipid matrix, as judged from fluorescence spectra.

the hydrophobic core of the membrane. Incubation of the liposomes at 70° and repeated IR measurements at 20° show that under these conditions a well incorporated $\uparrow\uparrow\beta$sheet (ν=1629-1630 cm^{-1}) is always formed (Fig.4b , Table 1). Very similar result was obtained in the dimiristoyl phosphatidylcholine (DMPC) liposomes even with 350:1 ratio without incubation (Table 1). A β-hairpin structure with DL-turn at the 5-8 site was proposed for GA in the aggregate. 5,6,22 From this follows that the results of earlier studies of GA containing liposomes where the antibiotic was *a priori* assumed dimeric even at (1.5-45):1 ratios[16-21] require reinterpretation.

In dioleyl phosphatidylcholine (DOPC) dimeric form is relatively more stable than in DPPC or DMPC. It is noteworthy that here a band at 1653 cm^{-1} appeared, reflecting the presence of ca 5% of the $\pi_{LD}^{\overrightarrow{6.3}}\pi_{LD}^{\overleftarrow{6.3}}$-helix (Fig. 5a). The carboxylic derivatives (11-13) obtained as described in[4] showed even higher content of single stranded helices (Figs 7b-7d).

$HOOC(CH_2)_nCO$-(1-15)-EA

n=2, N-succinyldesformylgramicidin A (11)

n=3, N-glutaryldesformylgramicidin A (12)

n=6, N-suberyldesformylgramicidin A (13)

Bis-derivatives (3,7 and 10) representing all three junction modes were also studied in the DPPC and DOPC liposomes (Table 2), Aggregates are clearly present in DPPC with head-to-head and head-to-tail analogs(3 and 7). Their principal structures reflecting the stable 3:2 $\uparrow\downarrow\pi\pi_{LD}$:$\downarrow\uparrow\beta$ ratio are suggested in Fig. 6. In DOPC, as with GA itself aggregates are less stable and the IR intensities are concentration dependent. Single stranded π_{LD}-helices appear in the equilibrium and their fraction increases on diluting the peptide with the lipid. In part these π_{LD}-structures might be formed by "halfes" of bis-derivatives and located at the edges of the aggregates shown in Fig. 6. However, in a number of cases (Table 2) this structure is absent in the aggregate which means that the major part of π_{LD}-helices, especially at high lipid:peptide ratio are due to non-associated $\pi_{LD}\pi_{LD}$-helical structures. As in solution, the non-associated head-to-tail derivative does not form antiparallel double helices. Therefore one might assume that the "zipper" mechanism discussed above for dioxane solution can be applied to membrane environment as well.

The results obtained in this work show that both GA, and its bis-derivatives, form in membrane a variety of helical structures which are in equilibrium with each other. Since the $\downarrow\uparrow\pi\pi_{LD}$ and the $\pi_{LD}\pi_{LD}$-helices are known to have similar dimensions of the axial cavity[12] we assume that all these structures are able to function as ion channels and to contribute to the observed conductivity according to their molar fractions. Such proposal is in contrast with the current view which strongly favors the $\pi^{\overrightarrow{6.3}}_{LD}\pi^{\overleftarrow{6.3}}_{LD}$-helix as the major ion conducting species (see e.g. [16,18,23,24] and the references cited therein).

According to that model appearence and disappearence of the channel conductivity reflect formation and decay of the dimer which are controlled by lateral diffusion of GA (Fig. 7a,b). We suggest an alternative model based on a double helical

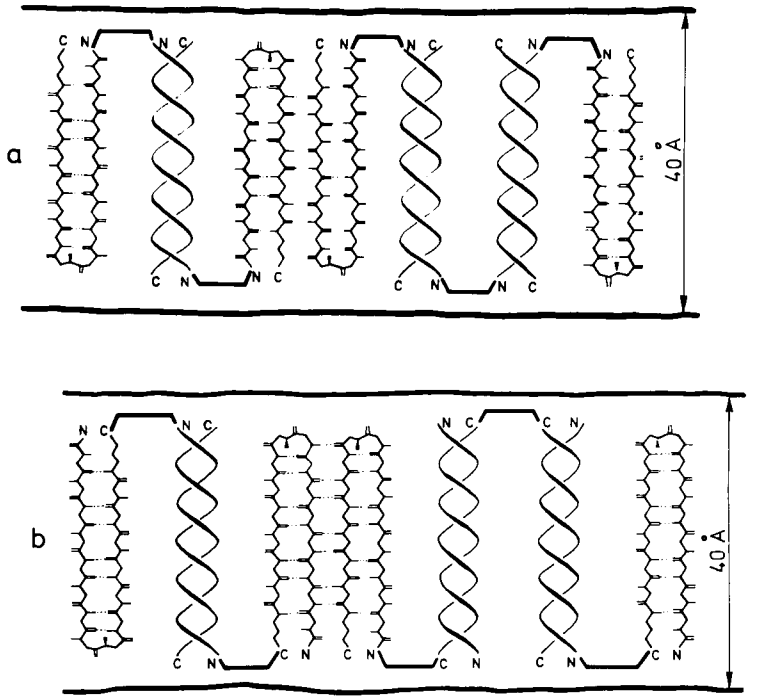


Fig. 6. Intermolecular association of head-to-head (a) and head-to-tail (b) bis-gramicidins in phospholipid membranes.

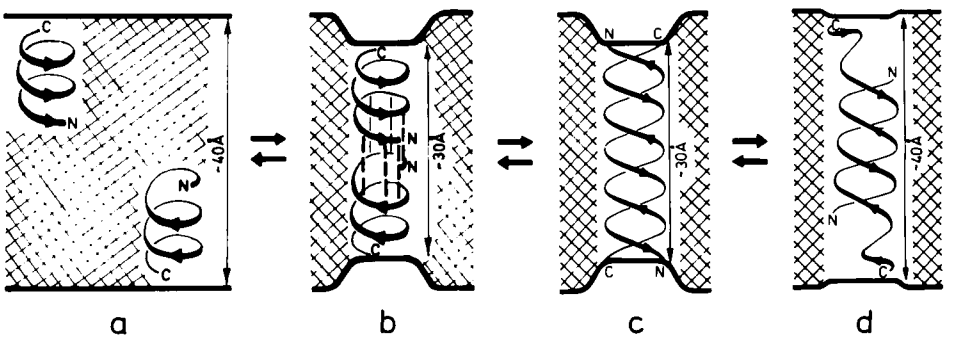


Fig. 7. Switching on and off the single stranded (a,b) and double stranded (c,d) helical GA channels.

structure of the conducting channel. According to that model (Fig. 7c,d) onset and decay of the channel is coupled to fluctuation of the membrane width and the ensuing partial unwinging of the helix with concomitant formation of terminal non-conducting $\pi_{LD}^{4.4}$-helical segments. Kinetic properties of the ionic channels induced by GA and its carboxylic analogs correlate with the increasing $\overrightarrow{\pi_{LD}}\overleftarrow{\pi_{LD}}/\uparrow\uparrow\pi\pi$ ratio (Fig. 5) in the (1)-(11)-(13) series (for details see[6,7,25]).

As shown above, monomeric bis-gramicidins form in the membrane single stranded $\overrightarrow{\pi_{LD}}\overleftarrow{\pi_{LD}}$, $\overrightarrow{\pi_{LD}}\overrightarrow{\pi_{LD}}$ or $\overleftarrow{\pi_{LD}}\overrightarrow{\pi_{LD}}$-helices with the same dimensions of the axial cavity as in the GA dimers. It is not surprizing therefore that all of them form ion channels closely resembling in conductivity and ion selectivity the GA channels. On the other hand, channels made of bis-gramicidin cannot follow the mechanisms depicted in Fig 7. Accrdingly, all these channels have very long life times, 8-13 sec which is 40-60 times longer than the typical life time of the GA channel (~ 0,2 sec)[4-6].

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PROPERTIES OF IONIC CHANNELS FORMED BY ANALOGS AND DERIVATIVES OF GRAMICIDIN A

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Introduction

Channel-forming peptides such as gramicidin A have been used in the past years as models for biological ion-transport systems. Valine-gramicidin A is a linear pentadecapeptide with the structure HCO-NH-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val--L-Val-D-Val-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-CO-NH CH_2CH_2OH[1]. Characteristic features of this structure are the alternating sequence of D- and L- amino acids and the fact that all residues, with the exception of glycine in position 2, are hydrophobic. Gramicidin A has been shown to increase the alkali ion permeability of biological membranes[2] and of artificial lipid bilayer membranes[3] (for a recent survey of the literature, see Ref. 4). Structural models of the gramicidin channel have been proposed by Urry[5] and by Ramachandran and Chandrasekaran[6] on the basis of conformational energy considerations. The model of Urry consists of a helical dimer that is formed by head-to-head (formyl end to formyl end) association of two gramicidin monomers, and that is stabilized by intra- and intermolecular hydrogen bonds. The central hole along the axis of the π^6(L,D)-helix has a diameter of about

0.4 nm and is lined with the peptide C-O moieties, whereas the hydrophobic residues are located on the exterior surface of the helix. The total length of the dimer is about 3 nm which is the lower limit of the hydrophobic thickness of a lipid bilayer. The length of the dimer is thus sufficient for bridging the membrane if a local thinning of the lipid structure is assumed. Although in organic solvent different dimeric structures of gramicidin A may exist[7], virtually all experimental results obtained with gramicidin A incorporated into lipid bilayers are consistent with Urry's model of a head-to-head associated dimer.

Analogs of Gramicidin A

The synthetic work of Erhard Gross and his associates[8] on gramicidin A analogs opened up the possibility of studying the relationship between chemical structure and transport properties of ionic channels[9]. After incorporation into artificial lipid bilayer membranes the ionic permeability of single gramicidin channels can be studied using sensitive elctrical measuring techniques[10]. The single-channel method, as well as electrical relaxation experiments[11] and noise analysis[12,13] have been used for the characterisation of ion transporting properties of gramicidin analogs[9,14-18]. Such studies have shown that substitution of a single amino acid in the pentadecapeptide (e.g., replacement of L-tryptophan in position 11 by L-phenylalanine) results in significant changes of single-channel conductance[9]. Furthermore, peptides with much simpler amino-acid sequence, such as HCO-NH-(L-Trp-D-Leu)$_7$-L-Trp-CO-NHCH$_2$CH$_2$OH have been shown to form ionic channels with similar properties as gramicidin A[15].

Derivatives with Charged Residues

The permeability of ionic channels in biological membranes is likely to be modulated by the presence of ionized groups near the channel mouth. Evidence for the influence of free charges on the entry rate of ions exists for the sodium channel in nerve[19]. For this reason studies with model channels bearing charged residues at the entrance are of considerable interest[16,20,21]

O-Pyromellitylgramicidin

O-Pyromellitylgramicidin (Figure 1) is a derivative with three carboxyl groups at the hydroxyl end of the peptide which, according to the model of Urry, is located at the mouth of the channel. If this compound is added to one side of a lipid membrane, only a small membrane conductance develops, whereas addition to both sides results in a drastic increase of conductance[20]. This finding is in contrast to the

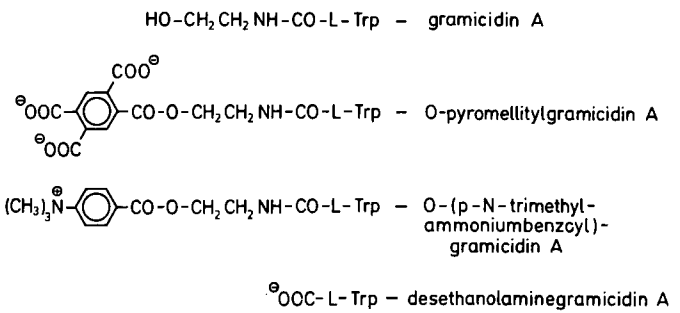

Fig. 1. Electrically charged derivatives of gramicidin A

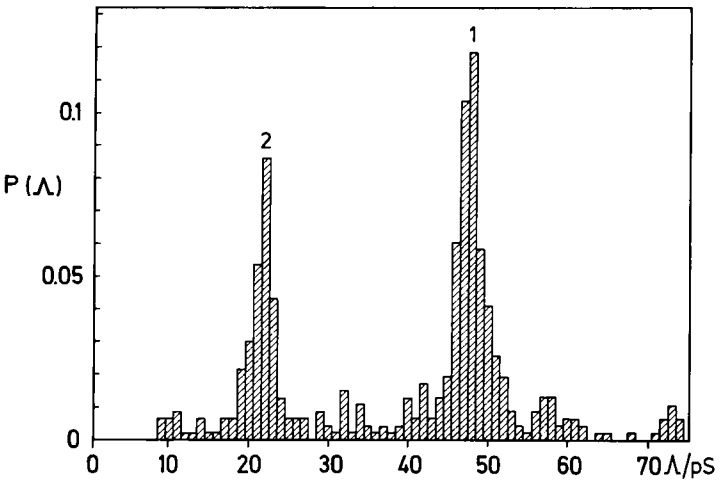

Fig. 2. Probability distribution of single-channel conductance values. Desethanolaminegramicidin A in a glycerolmonooleate/n-hexadeane membrane, 1 M CsCl, pH 4, T=25°C. A voltage of 100 mV was applied to the membrane. The total number of events was N=465.

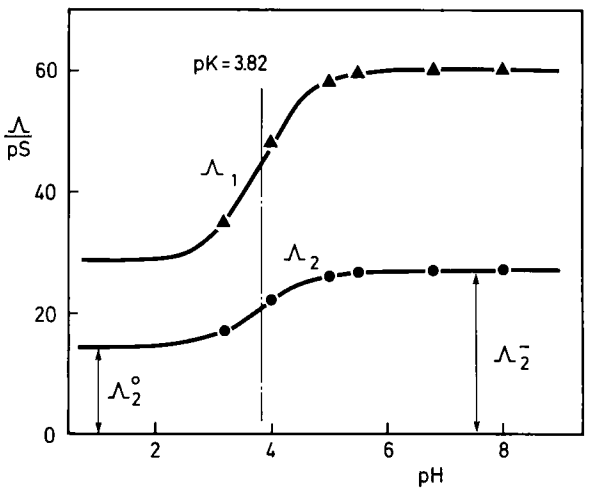

Fig. 3. pH dependence of the two conductance states of the desethanolaminegramicidin channel. Λ_1 and Λ_2 are the conductance values at the peaks of the probability distribution (Fig. 2). At the lowest experimental pH value (pH = 3.2) Λ_1 and Λ_2 have been corrected for the H^+ conductance of the channel. The curves represent theoretical fits using Eqns. 1 and 2.

behaviour of unmodified gramicidin which yields nearly the same membrane conductance, irrespective whether the peptide is added to only one or to both aqueous compartments. These observations can be explained on the basis of the dimer model assuming that the two halves of the channel can combine only if they are on opposite sides of the bilayer. Unmodified gramicidin which is strongly hydrophobic apparently can easily cross the membrane, whereas the negatively-charged derivative presumably stays on the side of the bilayer where it has been added.

O-Pyromellitylgramicidin exhibits a peculiar dependence of single-channel conductance on ion concentration. At high concentration the conductance is smaller than that of gramicidin. This difference may arise from a partial obstruction of the channel entrance by the bulky pyromellityl residue. At low ionic concentrations (0.01 M) the negatively charged channel has a five to sixfold higher conductance than the neutral channel. This finding is consistent with the assumption that the three negative charges of the pyromellityl residue are located near the mouth of the channel. The electrical potential created by the fixed negative charges leads to an accumulation of permeable cations near the entrance of the channel; according to the Debye-Hückel theory of interionic attraction, the accumulation effect becomes stronger at low ionic strength.

O-(p-N-Trimethylammoniumbenzoyl)-gramicidin

A gramicidin derivative with a positive charge at the channel mouth is obtained by introducing a p-N-trimethylammonium residue at the hydroxyl end of the peptide[16] (Figure 1). Experiments with black lipid membranes show that this compound is able to form ionic channels, but in this case the conductance of the channel is considerably smaller than the conduct-

ance of the neutral gramicidin channel. An interesting experiment consists in adding the positively charged derivative to one aqueous phase and the negatively charged derivative to the other. Under this condition hybride channels may be expected to form that possess an intrinsic structural asymmetry. Indeed the current-voltage characteristic of a lipid membrane modified in this way is found to exhibit a pronounced rectifying behaviour. At 200 mV the conductance is about 3.5 times higher in the forward direction (cations moving from the negative to the positive end of the channel) than in the reverse direction.

Desethanolaminegramicidin

Cleaving the ethanolamine residue from gramicidin A yields a derivative with a free carboxyl terminus[21]. A surprising property of this compound is the occurence of two peaks in the probability distribution of single-channel sizes (Figure 2). Under the same conditions gramicidin A yields only a single peak in the histogram. Since the reaction product has been extensively purified, a chemical heterogeneity seems unlikely. The possibility that the two conductance states correspond to the protonated and unprotonated form of the terminal carboxyl group can be excluded, as both conductance values vary in the same way with pH (see below).

A possible explanation consists in the assumption that the peptide can assume two different conformations in the region near the carboxyl terminus, where the bulky tryptophane residues are located[22,23]. If one conformation (H) has a higher conductance than the other (L), then peak 1 in Figure 2 would result from state HH of the dimer and peak 2 from state HL. (State L may interact more strongly than H with the polar head groups of the lipids, so that the HL form of the dimer is located symmetrically with respect to the central plane of the membrane; this could explain why a still lower conductance

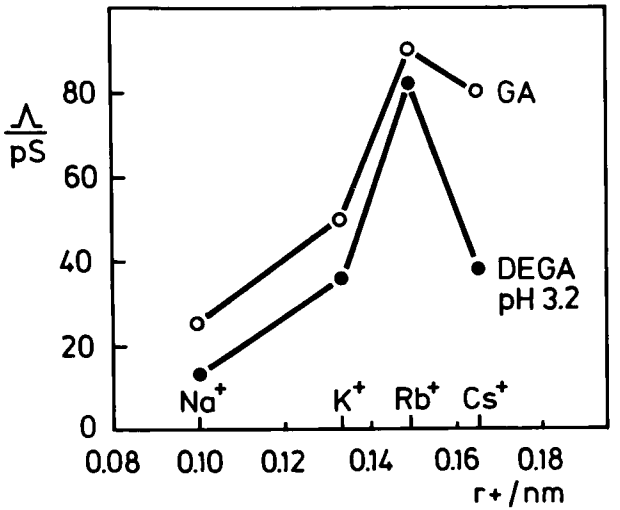

Fig. 4. Single-channel conductance Λ of gramicidin A (GA) and desethanolaminegramicidin A (DEGA) in the presence of different alkali ions (ion concentration 1 M). The values of DEGA correspond to peak 1 in the histogram (Fig. 2). DEGA is present mainly in the protonated form at pH = 3.2.

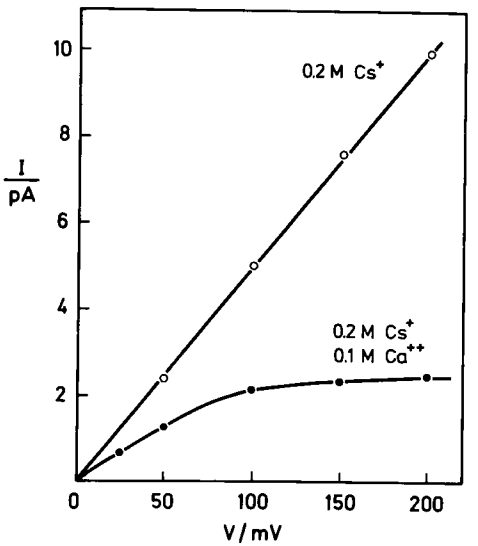

Fig. 5. Single-channel current I as a function of voltage V in the presence and absence of Ca^{++}. The values of I correspond to peak 1 in the histogram (Fig. 2).

state LL is not observed).

The pH dependence of both conductance states (represented by the peak values Λ_1 and Λ_2) could be fitted by a simple titration curve (Figure 3). Since protonation - deprotonation reactions are fast within the time scale of single-channel conductance fluctuations, a given conductance state is likely to represent a time-average of the conductance of the protonated and the deprotonated form of the channel. If α is the fraction of time the channel spends in the deprotonated state, Λ_i (i=1,2) can be expressed by

$$\Lambda_i = \alpha\Lambda_i^- + (1-\alpha)\Lambda_i^o \qquad (1)$$

Λ_i^- and Λ_i^o are the conductance values in the deprotonated and protonated state, respectively. Introducing the pK-value of the terminal carboxyl group, the quantity α may be obtained from the Henderson-Hasselbalch equation:

$$pH = pK + \log \frac{\alpha}{1-\alpha} \qquad (2)$$

As shown by Figure 3, Λ_1 as well as Λ_2 could be fitted with Eqns. 1 and 2 using the same pK of 3.82. A pK of this magnitude is not unreasonable for a terminal carboxyl group of a peptide; for instance, N-acetylalanine has a pK value of 3.72[24].

Desethanolaminegramicidin A and gramicidin A differ in their selectivity towards alkali ions (Figure 4). There is a general tendency for increasing single-channel conductance with increasing size of the ion. The largest ion, cesium, however, has a conductance comparable to rubidium in the gramicidin A channel; with desethanolaminegramicidin A the conductance of the smaller Rb^+ ion is much higher than the conductance of Cs^+. This indicates that the chemical structure at the channel entrance is important for ion selectivity.

In the presence of calcium ions the conductance of the desethanolaminegramicidin channel is strongly reduced (Figure 5).

This effect which has already been observed with gramicidin A at higher calcium concentrations[25] becomes visible at concentrations above 10 mM. As shown by Figure 5 calcium ions not only diminish the ohmic conductance of the channel,but also markedly affect the current-voltage characteristic. Whereas in the absence of Ca^{++} the current-voltage curve is nearly linear, a strongly saturating I (V)-behaviour is observed in the presence of Ca^{++}. Calcium ions alone do not induce any appreciable conductance. Furthermore, the amplitudes of the two channel types (Figure 2) monotonously decrease with increasing Ca^{++} concentration without appearance of additional conductance states. A tentative explanation of these findings consistsin the assumption that Ca^{++} binds to a site near the channel entrance in a fast association - dissociation reaction and thereby creates a virtually voltage-independent energy barrier that limits the entry rate of permeable cations[25].

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CHANNEL-FORMING MOLECULES: CONFORMATION OF PEPTIDES WITH ALTERNATING L AND D RESIDUES AS MODELS OF GRAMICIDIN A.

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Introduction

Linear L,D sequential polypeptides can be considered as models of gramicidin A, a natural linear pentadecapeptide, which allows diffusion of alkaline ions across natural or synthetic lipid bilayer membranes by a channel mechanism. Experimental and theoretical studies on peptide systems containing sequences of regularly alternating L and D residues carried out in our laboratories[1-2], have indicated a propensity to give rise to structures of the β-type. Semiempirical energy calculations performed in the regions of conformational space "allowed", are in good agreement with the solid-state results obtained by x-ray crystallographic analyses on single crystals of model peptides.

X-ray data and Conformational calculations

Conformational parameters, ω , ϕ , ψ , obtained by single crystals structure determination, for two alternated dipeptides containing the sequences D-Leu-L-Leu and L-Ile-D-aIle, are reported in table I. The two dipeptides adopt an U-shape structure, whereas an hexapeptide containing the sequence (D-aIle--L-Ile)$_3$, present an hairpin-shaped structure stabilized by a novel intramolecular hydrogen bonding scheme and backbone bulky side-chain interactions. The experimental conformational parameters have been already published in a brief communication[3].

Conformational calculations have been performed on selected conformations of LL and LD dipeptides of some hydrophobic aminoacid residues, using ECEPP[4] (Q.C.P.E. 286) adapted to an Univac 1100/80. The dipeptides used in the calculation had the N-terminus blocked with an N-acetyl group, different from the t-Boc group of the dipeptides, studied by x-ray. The most stable conformation of the leucine dipeptide is very close to that observed experimentally in the solid-state. Different choices for the torsional parameters, found in the case of DL valine sequences[5], are higher in energy (table I). For the L-Ile-D--aIle dipeptide, the conformation calculated for the LD helix is lower in energy with respect to the experimental one, but the values of the conformational parameters are quite similar. The theoretical conformations of the side chain are close to those found experimentally. As far as the stabilization of the more favourable conformations is concerned, the ECEPP potential shows they are destabilized by electrostatic interactions and stabilized by non-bonded interactions.

Conformation calculations on Ac-(D-Ala-L-Ala)$_2$-D-Ala-NHMe

have been performed in order to point out key interactions which allow the formation of a type II β-turn fused with an α-turn, both included in a intermolecularly hydrogen bonded C_{17} ring structure, as found in the crystal of the linear hexapeptide[3]. While the β-turn is achieved with accessible low energy, the α-turn requires a ψ value for L-Ala[4] residue at about -50°, which is almost 2 Kcal/mol higher than the corresponding conformation with positive ψ value. The formation of the C_{17} ring is not sufficient to reverse the stability of the two minima for the ψ angle of the L-Ala[4] residue. The conformation observed experimentally could be explained by the strong dependence of the energy on the χ^1 side chain torsion angle of the L-Ala[4] residue. Bulkier side chains (Ile or Val) stabilize the formation of the C_{13} α-turn and then the hydrogen bonded C_{17} ring structure.

Table I: Geometry and Energy Parameters for Some Selected Conformations of the peptides reported in the Text.

a. Ac-(D-Leu-L-Leu)-OMe

ϕ_2	ψ_2	ω_2	ϕ_3	ψ_3	ω_3^*	E_T (Kcal/mol)
88	-135	177	-138	-61	175	4.12 (Calc from Obs Model)
-46	60	180	-52	-54	180	71.62 (α_R-Poly-DL-Val)
139	-125	180	-112	125	180	143.18 (LD-Helix Poly-DL-Val)

b. Ac-(L-Ile-D-aIle)-OMe

ϕ_2	ψ_2	ω_2	ϕ_3	ψ_3	ω_3^*	E_T (Kcal/mol)
-122	115	-176	129	-93	177	19.30 (Calc from Obs Model)
139	-125	-176	-112	125	180	16.27 (LD-Helix

*Referred to the torsion angle C_3^{α}-C'_3-O_3-Me.

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MEMBRANE ACTIVE PEPTIDES CONTAINING α-AMINOISOBUTYRIC ACID

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Introduction

The formation of voltage-gated transmembrane channels by several naturally occurring peptides rich in α-aminoisobutyric acid (Aib) has provided the rationale for extensive investigations on the conformations and membrane modifying activity of Aib containing peptides[1,2]. Figure 1 provides a representative set of sequences of membrane active Aib peptides of microbial origin, of which, alamethicin is the most extensively studied member. This report summarizes studies carried out at Bangalore, which have focussed on the structural chemistry of Aib peptides in the solid state and in solution, their association in organic and aqueous phases and the membrane modifying activity of synthetic fragments of the natural ionophores.

Conformation of Aib Peptides

The presence of an additional methyl group at C^{α} in Aib as compared to Ala results in a dramatic reduction in the degree of conformational freedom for the Aib residue[3]. The theoretically computed conformational energy map for Ac-Aib-NHMe illustrates these constraints (Figure 2a). Only limited regions of ϕ, ψ space are energetically favourable, with the minimum falling in the right and left handed helical ($3_{10}/\alpha$) region of the Ramachandran map. The predictions of theoretical calculations are amply borne out by the cystallographic studies on over three dozen Aib

Alamethicin I(II) : Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro
(Aib)
-Val-Aib-Aib-Glu-Gln-Phol

Suzukacillin A(B): Ac-Aib-Ala-Aib-Ala-Aib-Ala-Gln-Aib-Aib-Aib-Gly-Leu-Aib-Pro-Val
(Iva) (Aib) (Leu)
-Aib-Iva-Glu-Gln-Phol
(Aib)(Gln)

Trichotoxin A 40/50 : Ac-Aib-Gly-Aib-Leu-Aib-Gln-Aib-Aib-Aib-Ala-Aib-Aib-Pro-Leu
(Ala) (Ala)
-Aib-Iva-Glu-Valol
(Aib)(Gln)

Emerimicin III(IV): Ac-Phe-Aib-Aib-Aib-Val-Gly-Leu-Aib-Aib-Hyp-Gln-Iva-Hyp
-Ala-Phol
(Aib)

Zervamicin IIA : Ac-Trp-Ile-Gln-Aib-Ile-Thr-Aib-Leu-Aib-Hyp-Gln-Aib-Hyp
-Aib-Pro-Phol

Fig. 1. Representative sequences of Aib containing membrane active peptides.

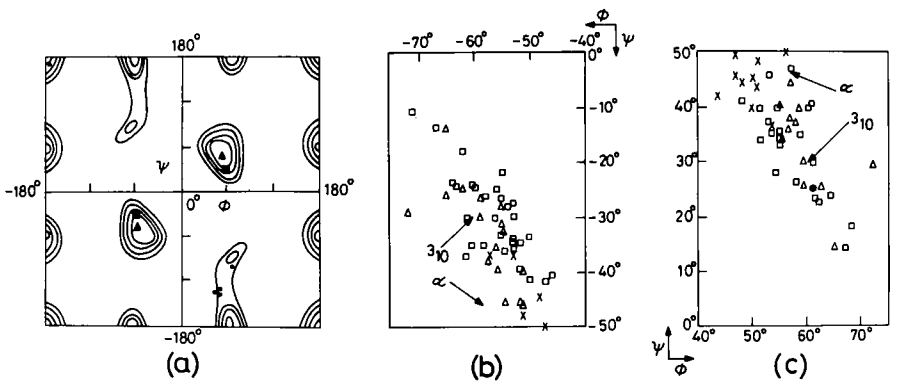


Fig. 2.(a) Potential energy map for Ac-Aib-NHMe. Contours at 1 kcal mol^{-1} intervals. Ideal 3_{10} (□) and α (Δ) - helical conformations are shown. • represent crystal structure observations of Aib in non-helical conformations.

(b,c) Crystal structure observations for Aib in helical regions ΔN-terminal residues □ non-terminal residue ×C-terminal residue as ester or acid.

peptides, summarized in Fig. 2b,c. In protected dipeptide amides and tripeptides β-turn structures are observed while in longer sequences (4 to 5 residues) consecutive β-turn or 3_{10}-helical structures are formed. α-Helices have been found in a model 11-residue peptide Boc-(Ala-Aib)$_2$-Ala-Glu-(OBzl)-(Ala-Aib)$_2$-Ala-OMe[4] and the natural product, alamethicin[5]. However, a 10-residue peptide Boc-Aib-Pro-Val-Aib-Val-Ala-Aib-Ala-Aib-Aib-OMe adopts a 3_{10}-helical structure in the solid state[6], while fibre diffraction studies favour a 3_{10} helix for poly(Aib)[7]. ^{1}H NMR studies of Aib peptides in organic solvents have been interpreted in terms of 3_{10} helical conformations for sequences ranging from 5-15 residues in length[8-11]. CD spectra of Aib rich oligopeptides resemble patterns obtained for α-helical polypeptides[2]; however, it is uncertain whether chiroptical distinctions can be made between 3_{10} and α-helices[12]. In general there has been good agreement between conformational studies in solution, by NMR, and X-ray diffraction results, emphasizing the role of Aib residues in limiting the structural flexibility of acyclic sequences in solution, resulting in a substantial degree of conformational homogeneity. 3_{10} and α-helices differ very slightly in ϕ, ψ values[3], and can be differentiated by their hydrogen bonding patterns (3_{10}, 4 → 1; α, (5 → 1). More elaborate studies are necessary to determine the conditions which favour a specific type of helical folding. It is clear, however, that sequences of the type listed in Figure 1 are constrained to adopt largely helical conformations (3_{10} or α), characterized by the absence of an internal channel. This is in contrast to the π_{LD} or double helical structures suggested for gramicidin A, which have large internal diameters[13]. Aib rich peptide channels must therefore be necessarily formed by aggregates of helical peptides.

Peptide Association

Aggregation of a model peptide (Boc-Gln-Aib-Leu-Aib-Gly-

Leu-Aib-Pro-Val-Aib-Aib-OMe), corresponding to residues 7-17 of suzukacillin has been studied by ^{1}H NMR in organic solvents. Comparison with a peptide analog, in which Gln(1) is replaced by Ala established that peptide association in apolar media is promoted by intermolecular hydrogen bonding involving the side chain carboxamide group[14]. Synthetic peptides incorporating a fluorescent dansyl group have been employed to monitor aqueous phase aggregation, which is accompanied by transfer of the fluorophore to a less polar environment, resulting in a blue shift and increase in the quantum yield of emission (Fig. 3). Critical micelle concentrations have been determined for a series of alamethicin fragments. Aggregation is facilitated by increasing chain length but inhibited by the presence of a C-terminal

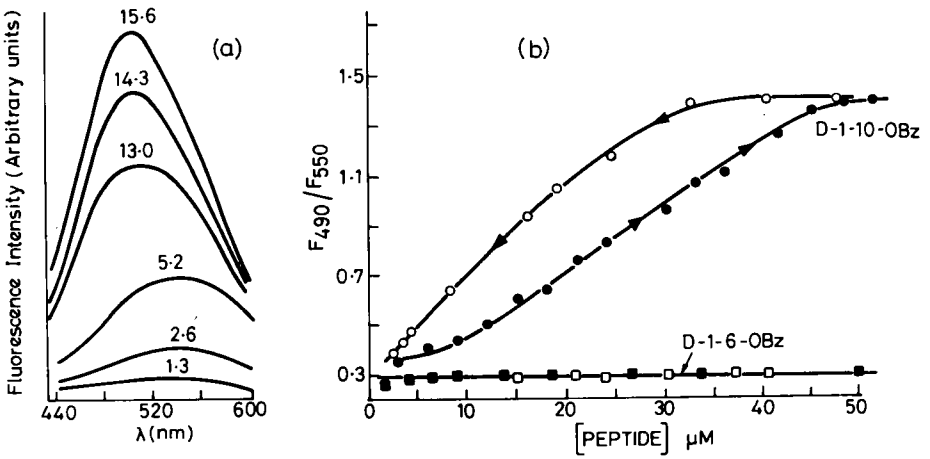


Fig. 3.(a) Fluorescence spectra of a dansylated alamethicin fragment (Dans-Gly-1-17-OBzl) as a function of peptide concentration (λ_{ex} = 330 nm). Concentrations in μM indicated. The ratio of intensities F_{490}/F_{550} is an index of aggregation.

(b) • Aggregation titration o curve generated by rapid sequential dilution from 50 μM in distilled water.

carboxylate[15]. Figure 3 illustrates the possibility of hysteresis in the aggregation-disaggregation curves for these hydrophobic peptides, suggesting a "memory effect" as noted in early CD studies of alamethicin[16].

Membrane Modifying Activity

The ability of synthetic fragments of alamethicin and suzukacillin to form channels across membranes has been monitored using two assay systems[15]. 1) A fluorescence method for monitoring Ca^{2+} transport across liposomal membranes, using chlorotetracycline as a probe, and 2) peptide induced uncoupling of oxidative phosphorylation in rat liver mitochondria.

The effectiveness of peptides as membrane modifying agents measured in these assays, correlates well with their ease of aggregation in aqueous media as shown in Table I.

Table I. Membrane modifying and aggregation properties of alamethicin and fragments

Peptide[a]	Ca^{2+}flux[b]	Uncoupling[c]	cmc[d]
Alamethicin	3	100	2.5[e]
D-1-17-OBzl	1.9	90	10
D-1-17-OH	0.5	44	50
D-1-13-OBzl	0.7	90	3
D-1-13-OH	0.6[f]	30	50
D-1-10-OBzl	0[g]	0[g]	30
D-1- 6-OBzl	0[g]	0g	50

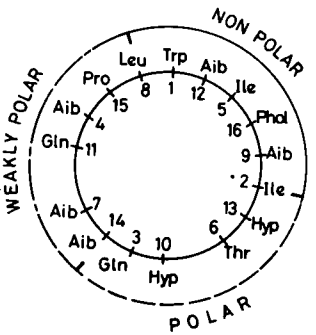
a) D = dansylglycyl. Residue numbers are for the alamethicin I sequence b) Measured as initial slope of fluorescence increase for 5 μM peptide c) % reduction in respiratory control index of rat liver mitochondria by 2.8 μM peptide d) cmc in μM e) From ref 16 f) At 15 μM g) At 20 μM.

Peptide association at the membrane-water interface may, therefore, be implicated in the formation of peptide channels although conclusive evidence for insertion into the membrane of an aqueous phase preaggregate remains to be obtained[1]. The structure activity studies suggest that peptides as short as 13 residues can form pores, while the C-terminal polar tripeptide is not essential for activity. Studies on a 16-residue (2-17) suzukacillin fragment in which the central Gln residue is replaced by Ala further establish that completely apolar peptides are also active. This finding is in agreement with the recent report that peptides of the type $Boc(Ala-Aib-Ala-Aib-Ala)_n-OMe$ are capable of altering lipid bilayer conductance, with activity detectable even for a pentapeptide[17].

Helix Dipole Model

A feature of the helical structures suggested for Aib rich peptides is the presence of a large "macrodipole moment" along the helix axis[1]. For peptide helices of length 10-20 residues, dipole-dipole interactions can be significant in low dielectric constant media like lipid bilayers. It is estimated that for two α-helix dipoles of 9 residues in length separated by 8 Å, the antiparallel orientation is 18 kcal $mole^{-1}$ more stable than the parallel arrangement[18]. Such interactions may contribute towards holding apolar peptide aggregates together in lipid bilayers. The presence of residues with polar, hydrogen bonding side chains can result in amphipathic helical structures as illustrated for an α-helical arrangement of the zervamicin IIA sequence (Figure 4). In the case of the remaining sequences listed in Figure 1 the lone central Gln residue, provides a site for hydrogen bonding[14]. Cylindrical peptide helices can associate in the lipid phase by specific interactions, schematically indicated for a hexameric aggregate in Figure 4. Close packed aqueous phase aggregates with polar faces pointing outwards are converted, by rotation about the helix axis,

into lipid phase structures linked by intermolecular hydrogen bonds. In the closed form of the channel a central "core" peptide helix, which is not linked by hydrogen bonds to its neighbours, blocks the pore. Application of a transmembrane voltage can induce ejection of this helix dipole, generating an open channel (Figure 4). Conductance state fluctuations can then be envisaged as involving peptide monomer exchange, by lateral diffusion in the membrane plane. Changes in aggregate size and hence channel diameter or net dipole moment can lead to altered pore state characteristics (Figure 5). Transmembrane flip-flop[19], a high energy process



ZERVAMICIN IIA

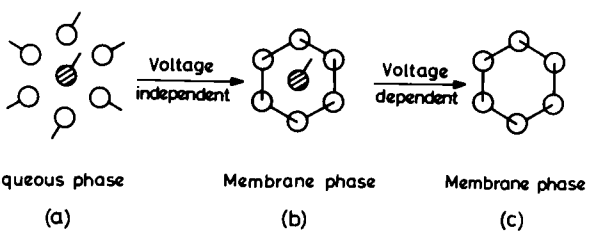


Fig. 4. (top) Helical wheel representation of the zervamicin IIA sequence.
bottom) Model for generation of an open channel a) aqueous phase aggregate; projection represents hydrogen bonding helix face b) lipid phase aggregate c) open channel.

need not necessarily be involved, since lateral exchange processes can be considered as formally equivalent. Pore state lifetimes should be modulated by the precise nature of the interpeptide interactions, while pore state transitions should be influenced by "fluidity" of the lipid bilayer. The model proposed here differs in several important respects from that based on the alamethicin crystal structure[5]. In the case of short synthetic peptides, channel formation must also require head to tail association of helices[3,17]. While precise sequence effects may be relatively unimportant in determining the membrane activity of these peptides, the presence of Aib residues provides a sure means of generating helical conformations, which can readily aggregate in lipid bilayers.

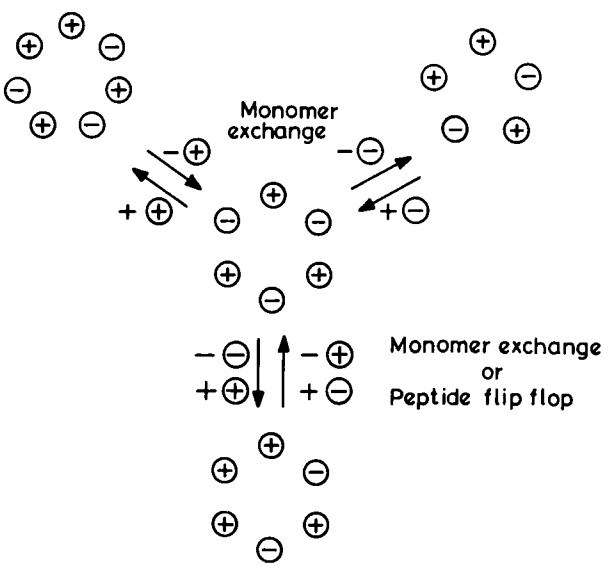


Fig. 5. Schematic representation of pore state fluctuations. + and - refer to oppositely oriented helical peptides.

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PROPOSED MOLECULAR ARCHITECTURE FOR THE ALAMETHICIN PORE

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Introduction

Alamethicin induces a voltage-dependent, fluctuating conductance in lipid bilayers due to the formation of a voltage-gated ion pore. Fox and Richards[1] have recently proposed a model for the alamethicin channel based on the crystal structure of alamethicin which is essentially alpha-helical. While many features of this model are attractive, conductance properties of some analogs are not easily reconciled.

NMR studies have led to a conformation in solution[2] characterized by a parallel beta-sheet dimer of the C-terminal residues 15-20 with the N-terminal residues 3-9 in a near alpha-helical conformation. We propose a model in which the off-state is composed of a parallel beta barrel with the N-terminal helical segments folded back against the barrel which does not completely penetrate the lipid bilayer. Under an imposed electrical gradient, the helical segments would swing down to complete the ion channel with a hybrid parallel N-terminal helical barrel and C-terminal beta barrel.

Requirements from Bilayer Studies

Various properties of the alamethicin channel can be deduced from detailed studies of alamethicin and its analogs on artificial bilayers.[3] When alamethicin is added to one side of the bilayer, a positive voltage

elevates the conductance more than a negative voltage[4] implying that the gating event must be the net movement of a positive charge. As alamethicin and other analogs exhibiting this behavior do not have a formal positive charge, a change in the dipole orientation of an alpha helix is suggested. Alpha helices have applicable dipole moments[5] as does alamethicin in solution. In accord with this reasoning, the negative charge associated with residue-18, Glu, fixes the C-terminal and prevents gating with negative voltage.

The number of monomers per channel and the charge per monomer which crosses the membrane are important constraints for model building. The apparent power dependence (N) of conductance at a fixed voltage on aqueous peptide concentration for a set of alamethicin analogs varies for alamethicin from 10.5 to 5.6 for des-Ac-Aib on membranes prepared from bacterial phosphatidyl ethanolamine. The product (alpha) of the charge on one monomer and the fraction of the distance across the membrane which this charge moves during the gating event remains constant at 0.5 regardless of analog or lipid bilayer. Any model must reconcile the variability of N with membrane composition and analog as well as the relatively constant value of alpha at 0.5. If the charge on each monomer moves all the way across the membrane, then the value would be approximately 0.5 electronic charge equal to the effective charge estimated at each end of an alpha helix[6]. The major conclusions are a variable number of monomers per pore with the same gating charge per monomer consistent with a helical dipole model. An additional constraint comes from the observation that a shortened analog, alamethicin 1-17, shows nearly identical gating properties while requiring higher concentrations for similar conductance. This implies that the C-terminal region is a determinant of association while the N-terminal region contains the common gating structure.

Molecular Model of Gating Mechanism

Starting with the suggestion of a parallel beta structure at the C-terminus from NMR and strong evidence for the first 10 residues as alpha helical from both NMR and x-ray, a hybrid pore was constructed

based on the observation of beta barrels due to the natural curvature of beta sheets. A hinge region corresponding to Gly^{11}-Leu^{12} would allow the N-terminal alpha helical segment to fold back over the beta barrel to help stabilize dipolar repulsion both between the parallel beta sheets and the alpha helices. Under an appropriate potential across the membrane, the N-terminal helices would swing down through the bilayer moving the half-charge associated with the dipole across the membrane and complete the channel. The C-terminal would determine the association prior to activation. Due to the increased length of the beta sheet, residues 18-20 are not necessary for channel formation in normal width membranes. Approximately half the channel (16 angstroms) is contributed by the helix with the other half arising from residues 11-17 as beta sheet.

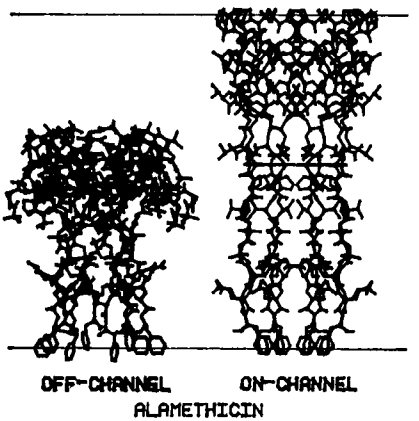

Fig. Hybrid pore model for alamethicin octomer channel.

Strong experimental support for the off-channel comes from the observation of an identical tertiary structure of helices flanking parallel beta sheet in the crystal structure of TIM, triose phosphate isomerase[7]. We modeled the off-channel by replacing the residues of TIM 110-130 with those of alamethicin followed by energy minimization. The resulting structure was almost identical implying a local minimum for alamethicin for a conformer of the correct off-channel architecture. Symmetry operators generated the off-channel structure shown in the Figure, and

the on-channel was simply constructed by torsional rotations of the hinge region, Gly^{11}-Leu^{12}. The off-channel architecture is one in which the minimization of dipolar repulsion has clearly been accomplished. Proteins with similar structures become possible candidates for natural voltage-gated channels. The central pore would be sensitive to the external field with increased strength making both the beta sheet and helices more parallel. This would increase the cross-channel repulsion and expand the radius of the beta barrel.

In contrast to the model of Fox and Richards, the hybrid pore model does not require opposition of parallel alpha helices with strong dipolar repulsion in the absence of an imposed electrical field, and gating is determined by reorientation of the N-terminal alpha helical segments in accord with most measurements. Further characterization of alamethicin analogs will be necessary to distinguish between these models of the alamethicin pore. In this regard, a dimer of alamethicin in which cross-linking between Glu-18 residues with ethylene diamine has occurred shows both increased activity and stability as would be predicted by the hybrid pore model. The presence of an almost identical molecular architecture in triose phosphate isomerase[7] lends credence not only to the hybrid pore model for the alamethicin pore, but suggests a similar mechanism for voltage-gating in natural membrane channels.

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VOLTAGE-GATED MEMBRANE PORES ARE FORMED BY A FLIP-FLOP OF α-HELICAL POLYPEPTIDES

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We have shown that voltage-dependent pores are formed in lipid bilayer membranes by a variety of α-helical polypeptides: alamethicin F30/F50, suzukacillin A, trichotoxin A40/A50 and its chemically modified analogues, a synthetic nonadecapeptide, melittin, acetylated melittin, the tryptic melittin fragment 1-21 and its acetylated derivative[1]. Simple helical peptide oligomers, Boc-(L-Ala-Aib-L-Ala-Aib-L-Ala)$_n$-OMe (n = 1-4)[2] also induce voltage-dependent, ion conducting pores, although at higher concentrations than with alamethicin[3]. Furthermore an eicosapeptide conjugate with a partially α-helical interferon segment was found to induce a voltage-dependent conductivity: Boc-L-Asn-Arg(NO_2)-Arg(NO_2)-Ala-Leu-Ile-Leu-Leu-Ala-Gln-(Ala-Aib-Ala-Aib-Ala)$_2$-OMe. We conclude that a lipophilic stable α-helical segment[4] is the sufficient presequisite for the formation of voltage-gated pores. The side chains of Gln-, Glu-, Aib-residues, particular C- or N-termini or helix/coil transitions are of no fundamental importance with respect to the voltage-dependence of the gating mechanism[1,5]. Consequently we strictly contradict the mechanism proposed by Fox and Richards[6].

According to our 'dipole flip-flop gating model' the helical polypeptide enters the lipid phase via its more lipophilic terminus. Additional evidence comes from the opposing asymmetry behaviour in the current-voltage characteristic of alamethicin and the interferon segment containing analogue, respectively. Within the membrane polypeptide molecules spontaneously change the orientation and after some time stable antiparallel

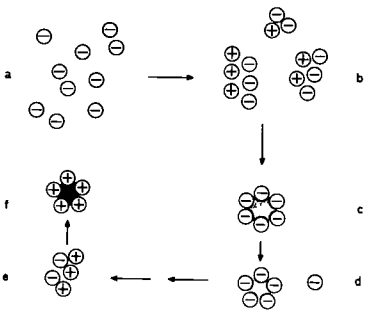

Fig. 1. Voltage-gated pore formation by α-helical dipoles according to the 'dipole flip-flop gating model' (schematic).

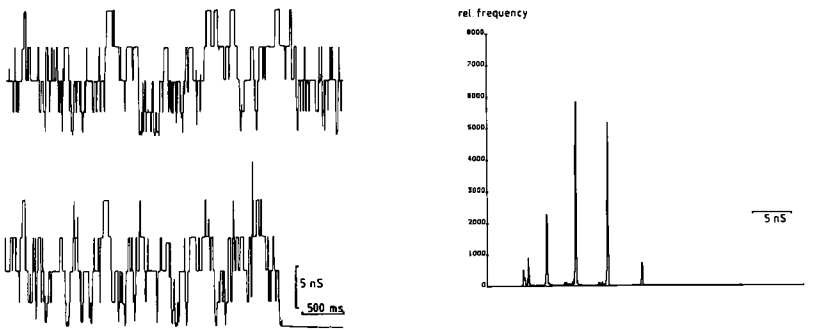

Fig. 2. Current fluctuation trace and distribution of conductance states of a single pore formed by the highly pure synthetic alamethicin. Experimental conditions: 1-palmitoyl-2-oleoyl-phosphorylcholin (1,2-POPC)/bacterial cephalin (9/1) using the glass pipette technique[5]; 3 M KCl + 10 mM Tris·HCl pH 7.0; 5 nM alamethicin in bath; +130 mV; +20°C.

dipole aggregates are formed (Figure 1 a,b). After application of a sufficiently high voltage the flipping of these α-helices within an oligomeric aggregate which are oriented antiparallely to the electric field leads to a parallel arrangement (c). Immediately the helix dipoles repel each other thereby opening a pore of distinct diameter which depends on the number of oligomers involved. The repulsive forces between the parallel dipoles are reduced by dielectric and ionic screening as a consequence of inflowed water and ions. After voltage sign reversal the ion pathway closes in a stepwise manner and after some time a new pore opens with reversed alignment of the α-helix dipoles (d,e,f). Experimental data supporting this view of channel formation are summarized elsewhere[5].

We recently synthesized alamethicin F30 which was obtained in very high purity. Figure 2 demonstrates the extreme homogeneity in the conductance levels which is less pronounced with the purified native alamethicin F30 (from T. viride) due to the presence of closely related natural analogues. Surprisingly, the fluctuation pattern of single pore events (Figure 3) induced by the highly pure synthetic precursor of alamethicin F30, Boc-L-Pro-Aib-Ala-Aib-Ala-Gln-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu(OBzl)-Gln-Pheol (protected alamethicin 2-20), is very similar to that of the helical peptide oligomers[3]. The large values of the only one or two conductance levels observed correspond to those of the 8th, 9th level of alamethicin F30.

We conclude that voltage-dependent pore formation is caused by the interaction of helix dipoles. The sequential occurrence and stability of pore states, however, are determined by the particular N- and C-termini[1].

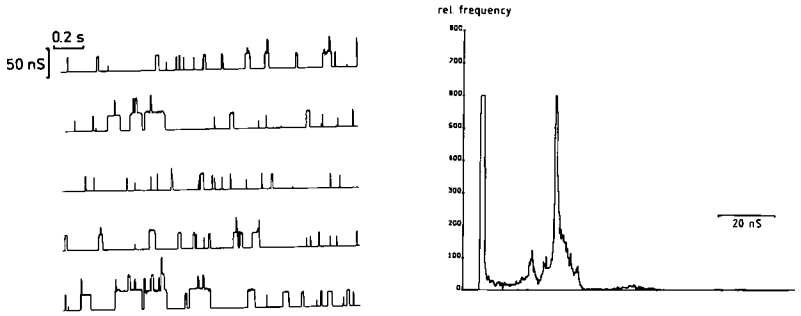

Fig. 3. Current fluctuation trace and conductance distribution of a series of pores formed by the highly pure protected synthetic alamethicin 2-20 (100 nM). Up to three pores are seen simultaneously. Experimental conditions same as in Figure 1 except for only 1,2-POPC; +330mV.

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MEMBRANE-ACTIVE PEPTAIBOL ANTIBIOTICS: CONFORMATIONAL PREFERENCES OF THE 2-9 SEGMENT OF EMERIMICINS III AND IV AND ALL RELATED SHORT SEQUENCES

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Introduction

In this communication we present the results of a conformational investigation in solution of the fully protected 2-9 segment of emerimicins III and IV[1] (two components of the family of membrane-active peptaibol antibiotics produced by Emericellopsis Microspora),[2-4] Z-(Aib)$_3$-L-Val-Gly-L-Leu-(Aib)$_2$-OMe, and all related short sequences using IR absorption and CD. For Z-(Aib)$_3$-OtBu, Z-(Aib)$_3$-L-Val-OMe, and Z-(Aib)$_3$-L-Val-Gly-OMe the solid-state conformations have been determined by X-ray diffraction.[5,6] The selection of the emerimicin family for this investigation was based on the fact that, among all the known peptaibol antibiotics,[2] emerimicins III and IV each incorporate an octapeptide segment (residues 2-9) which contains the longest sequence (the triplet -Val-Gly-Leu-) of C^{α}-monoalkylated α-amino acid residues within two blocks of $C^{\alpha,\alpha}$-dialkylated residues. It was expected that this would provide some information relating the effect of main-chain length and specific amino acid sequences on the type of helical structure (either 3_{10}- or α-helix)[7] adopted by natural antibiotics containing a high proportion of $C^{\alpha,\alpha}$-dialkylated residues.

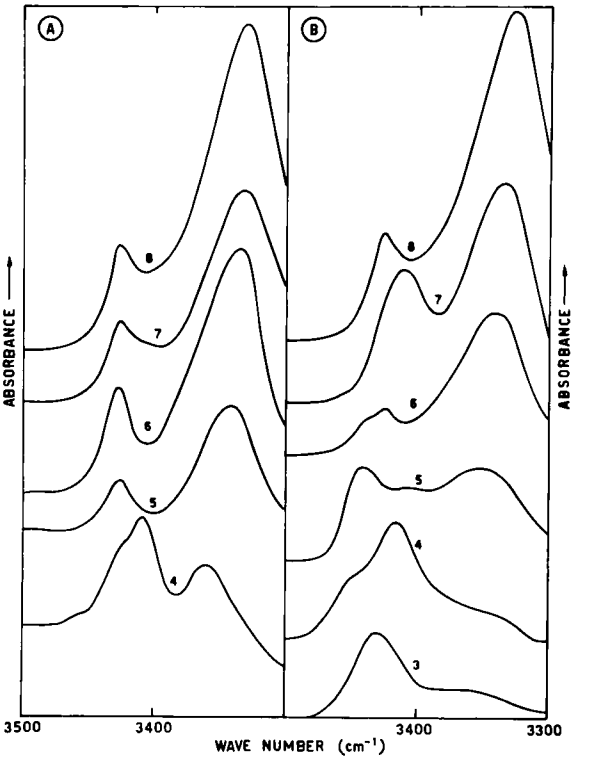

Fig.1. IR absorption in $CDCl_3$ at high dilution (conc.$5x10^{-5}$M) of the fully protected 2-9 segment of emerimicins III and IV and related short sequences starting from: (A) the N-terminus and (B) the C-terminus.

Results and Discussion

In $CDCl_3$ at high dilution, where self-association is minimized, the ratios of the integrated intensity of the IR absorption bands of intramolecularly hydrogen-bonded N-H groups (3350 cm^{-1})[5] to free N-H groups (3430 cm^{-1})[5] of the protected emerimicin fragments increase with increasing main-chain length and Aib content (Figure 1). This technique did not allow us to discriminate between the types of intramolecularly hydrogen-bonded species (either incipient 3_{10}-or α-helices)

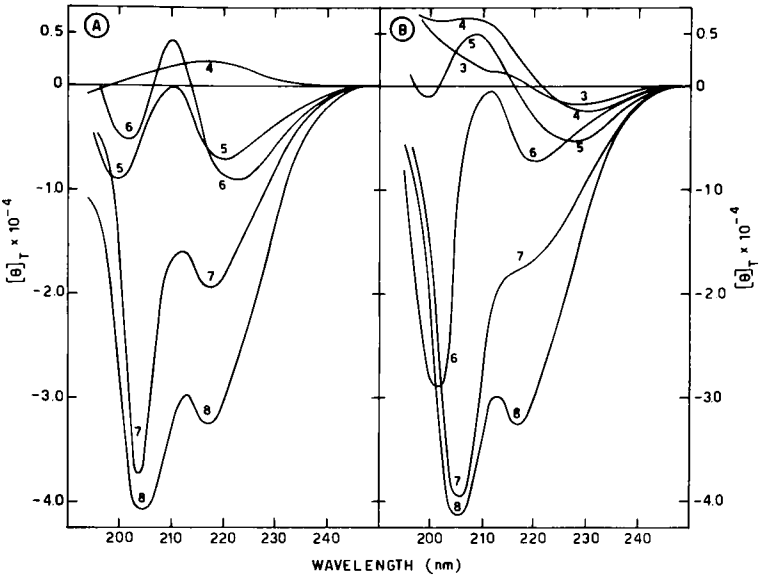

Fig.2. CD spectra in TFE of the fully protected 2-9 segment of emerimicins III and IV and related short sequences starting from: (A) the N-terminus and (B) the C-terminus.

which are formed.

In TFE the CD spectra of the 7-peptides and the 8-peptide are typical of a right-handed α-helix[8] (Figure 2). The shapes of the curves of the shorter peptide sequences are reminiscent of some of those calculated by Woody[9] for β-turn conformations. Thus, the CD investigation has provided information on the conformational preferences of these peptides in solution that is complementary to that given by IR absorption.

The X-ray diffraction investigation of the protected emerimicin fragments Z-(Aib)$_3$-OtBu, Z-(Aib)$_3$-L-Val-OMe, and Z-(Aib)$_3$-L-Val-Gly-OMe already indicated that these three peptides form incipient 3_{10}-helices (one β-turn in the 3-peptide, two β-turns in the 4-peptide, three β-turns in the 5-peptide).[5,6] Helices of both screw senses are present in the tripeptide; in contrast,

in the structures of the 5-and 4-peptides the single chiral L-Val residue imposes only one screw sense (right-handed for the 5-peptide, while left-handed for the 4-peptide) to the helical molecules. The standard 3_{10}-helix is somewhat distorted in the 5-peptide by the presence in an internal position of the Val residue.

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COMPARISON OF CATION BINDING BY THREE CYCLIC PENTAPEPTIDES

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The conformational adjustments upon cation complexation of three cyclic pentapeptides, *cyclo*-(Gly^1-Pro-Gly^2-D-Ala-Pro), I, *cyclo*-(D-Phe-Pro-Gly-D-Ala-Pro), II, and *cyclo*-(Ala-Pro-Gly-D-Phe-Pro), III, have been determined by ^{13}C nuclear magnetic resonance (NMR) and by circular dichroism (CD). Peptide I, as previously reported,[1] forms two distinct 1:1 complexes with perchlorates of Li^+, Mg^{++}, Mn^{++}, Ca^{++}, or Ba^{++} in acetonitrile. One of these 1:1 species (PC_{cis}) has one *cis* X-Pro bond, and the other (PC_{trans}) is all *trans*. At high concentrations of salt, a species that has all-*trans* bonds and a stoichiometry of 1:2, peptide:cation, is formed, and the proportion of the PC_{cis} and PC_{trans} species diminishes accordingly. The complexation equilibria for this peptide are summarized below, and Figure 1b shows ^{13}C NMR spectra in the region of the Pro C^β and C^γ signals as Mg^{++} is added to the peptide in acetonitrile. Note the shifting of the resonances attributable to the all-*trans* species, and the appearance of an additional set of resonances in positions typical of a species with one *cis* X-Pro bond. These latter signals decrease in relative intensity at high salt.

$$\begin{array}{ccccccc} P_{trans} & + & C & \overset{K_1}{\rightleftharpoons} & PC_{trans} & + & C \overset{K_2}{\rightleftharpoons} PC_{2\,trans} \\ \uparrow\downarrow \text{ slow} & & & & \uparrow\downarrow \text{ slow} & & \\ (P_{cis}) & + & C & \overset{K_1'}{\rightleftharpoons} & PC_{cis} & & \end{array}$$

Model building led to the hypothesis that the bond undergoing *cis-trans* isomerization was the Gly^1-Pro bond. It was

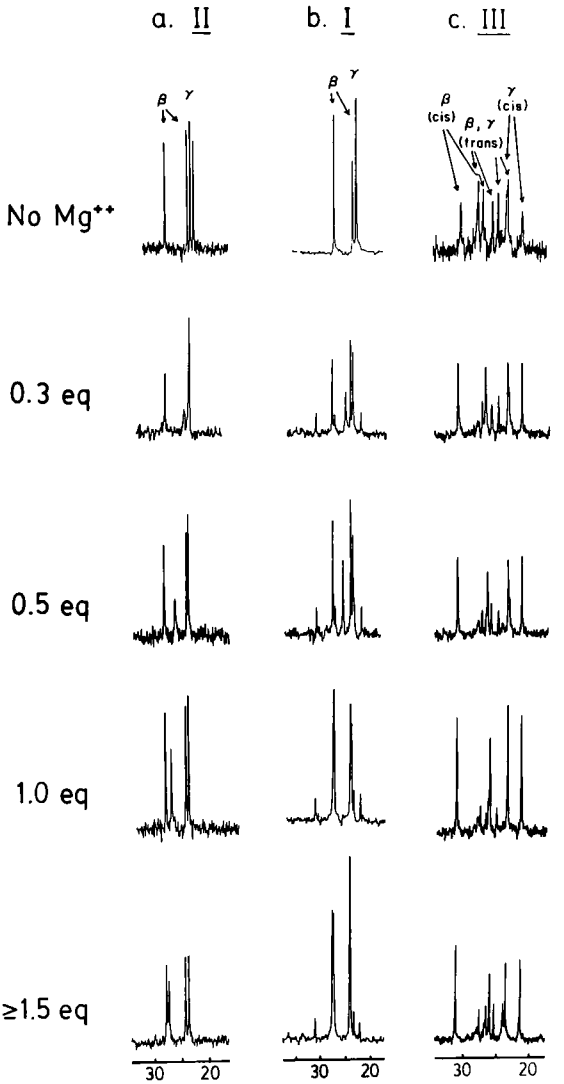

Fig. 1. Proline C^{β} and C^{γ} region of 62.9 MHz ^{13}C NMR spectra of cyclic pentapeptides upon titration with $Mg(ClO_4)_2$: a. *cyclo*-(D-Phe-Pro-Gly-D-Ala-Pro), II, conc 5 mg/ml in 1:1, CD_3CN:$CDCl_3$; b. *cyclo*-(Gly-Pro-Gly-D-Ala-Pro), I, conc 19 mg/ml in CD_3CN; c. *cyclo*-(Ala-Pro-Gly-D-Phe-Pro), III, conc 16 mg/ml in CD_3CN. In c., the resonances labeled *cis* or *trans* arise from the one-*cis* or all-*trans* species, respectively.

predicted that an analogous peptide with a D residue replacing Gly^1 would not adopt the PC_{cis} conformation, while insertion of an L residue at this position would strongly favor this complex. Peptides II and III were designed and synthesized to test these predictions.

Peptide II, with a D-Phe in place of Gly^1, occurs in a free conformation that is very similar to that of peptide I in a variety of solvents[2] and in crystals.[3] In Figure 1a are shown ^{13}C NMR spectra in the region of the Pro C^{β} and C^{γ} resonances upon addition of $Mg(ClO_4)_2$ to II in acetonitrile/chloroform (1:1). Note that no new resonances are seen as salt is added, but that shifts do occur in the signals associated with the all-*trans* species. The most pronounced shift is that of the Pro C^{β}. Comparison with the titration data for I (Figure 1b) reveals that the shifts of the all-*trans* form of the peptides are similar. As in the case of I, the CD spectral changes (not shown) indicate that at least two binding equilibria are involved in the salt-peptide interaction, one leading to a 1:1 complex, and the other to a 1:2, PC_2 complex. A crystal structure of a 1:1 complex of peptide II and $Mg(SCN)_2$ has been determined.[4]

Peptide III, with an L-Ala in place of Gly^1, adopts two conformations in the absence of cations; one conformation is all-*trans*, and the other has a *cis* Ala-Pro bond. In solvents of medium polarity such as acetonitrile, these forms are of comparable stability (see Figure 1c). In more polar solvents, the one-*cis* form is preferred. Addition of $Mg(ClO_4)_2$ leads to a marked shift in the distribution of conformers, with the one-*cis* species increasing in population. At 1:1, Mg^{++}:peptide, the all-*trans* form is nearly absent. Addition of higher concentrations of salt eventually leads to a somewhat greater proportion of the all-*trans* form.

Spectral data and inspection of molecular models show that a rearrangement of the intramolecularly hydrogen-bonded all-*trans* free conformation of peptides I and II leads to a site

with three carbonyls oriented appropriately for cation binding. In peptide I this site can be maintained without significant change while an isomerization of the Gly^1-Pro bond takes place to the one-*cis* form. Because of steric hindrance involving the D-Phe side chain, peptide II cannot take up the one-*cis* form. It adopts only the PC_{trans} complex, whose stability is approximately equal to that of I. While the all-*trans* complex has the remaining two carbonyl oxygens oriented such that a second ion could be bound, the one-*cis* conformer cannot take up an additional cation since one carbonyl oxygen is pointed away from the ring. Both I and II favor all-*trans* PC_2 complexes at high salt. In peptide III steric hindrance between the Ala methyl and the δ-CH_2 of the following Pro leads to a competitive stability of the all-*trans* and one-*cis* conformers in the absence of salt. Complexation strongly favors the one-*cis*, 1:1 form in which a comparable binding site to that of the all-*trans* species can be arranged *and* the steric interaction is relieved. Nonetheless, at high concentrations of Mg^{++}, the formation of an all-*trans* PC_2 species is indicated by an increased relative intensity of the *trans* signals in ^{13}C NMR (Figure 1c) and by an additional inflection in CD titrations as a f[salt] (not shown).

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IRON-BINDING CYCLIC HEXAPEPTIDES OF ASPERGILLUS OCHRACEOUS

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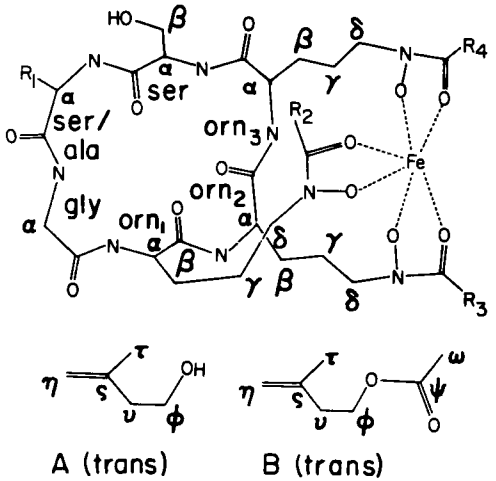
Introduction

Aerobic micro-organisms growing under iron stress conditions, produce various types of Fe^{3+} ion chelating compounds (siderophores) to acquire iron from the environment. Aspergillus ochraceous strain gold, cultured in this laboratory, produces a large number of siderophores, most of which belong to the ferrichrome family. The structure of a novel ferrichrome, asperchrome C, isolated from this fungus has been determined using high resolution ^{1}H and ^{13}C NMR spectroscopy.

Results and Discussion

Structures of eight out of eleven compounds isolated from A. ochraceous have been determined by X-ray crystallography and NMR spectroscopy. Seven of these are closely related members of the ferrichrome family and the eighth compound, fusarinine C (or fusigen) is a cyclic triester of N-acyl ornithine. Ferrirubin (I), Ferrichrysin (II) and fusarinine C have been previously reported from other fungal cultures.[1 2 3 4] Asperchrome A (III), B_1 (IV), B_2 (V), C (VI) and D(VII) are novel compounds; the last four are unique in having dissimilar ornithine N-acyl groups. Crystal structures of ferrirubin, asperchrome A and B_1 have been determined.[5 6] Asperchrome C crystallized from water with the following unit cell

parameters: a = 13.701(8) Å, b = 19.310(2) Å, c = 11.157(10) Å, α = 95.30°(6), β = 88.65°(7), γ = 97.09°(6) space group P_1 (Z = 2). The X-ray diffraction study shows the compound to be similar to ferrirubin, but a complete structure has not been determined because of the poor quality of the crystals.



	R_1	R_2	R_3	R_4
I	CH_2OH	A	A	A
II	CH_2OH	CH_3	CH_3	CH_3
III	CH_3	A	A	A
IV	CH_2OH	CH_3	A	A
V	CH_2OH	A	1 CH_3 & 1 A	
VI	CH_2OH	——2 A & 1 B——		
VII	CH_2OH	——2 CH_3 & 1 A—		

A (trans) B (trans)

Deferriferrirubin and deferriasperchrome C were prepared for NMR studies and the results show that asperchrome C is a monoacetyl ferrirubin formed by acetylation of one of the N-acyl OH functions. It was found that ferrirubin can be produced from asperchrome C by acid and alkaline hydrolysis. Assignment of the NMR signals (Figure 1) has been carried out by deuterium exchange studies, coupling constants correlation and by comparison with the published results on deferriferrichrysin[7] and our own results on deferriferrirubin. The peptide ring conformation of deferriasperchrome C appears to be identical with that of deferriferrirubin as both of these show similar chemical shift values for all ring protons and similar coupling constants ($J_{N\alpha}$ for the NH protons and J_{gem} for the glycine α protons). The chemical shifts for the protons in the acyl side chains in deferriferrirubin and deferriasper-

chrome C (Figure 1) shows the dissimilarity in their N-acyl side chain constitution.

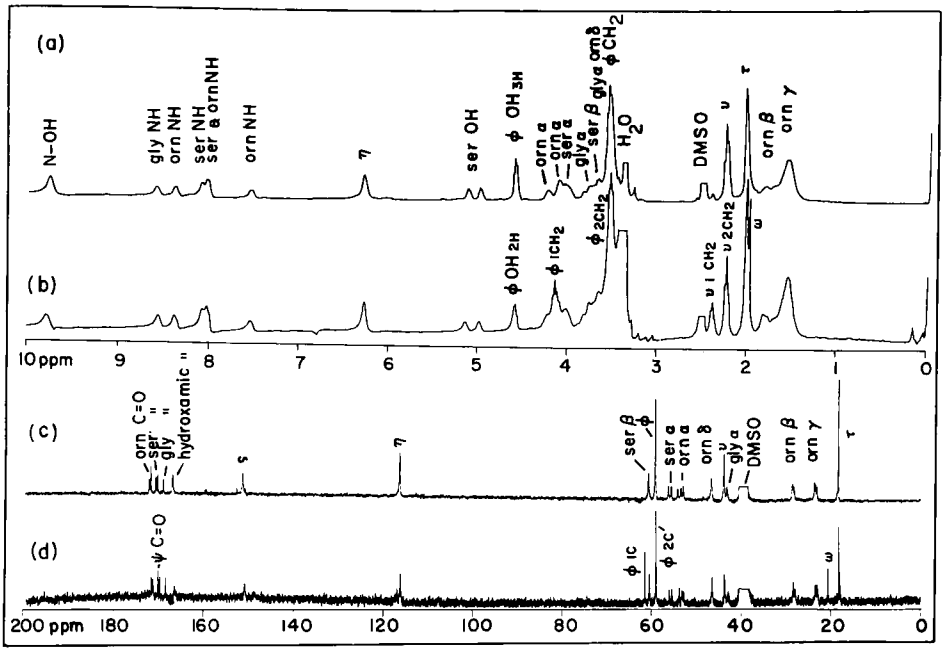


Fig. 1. ^{1}H (300 MHz) and ^{13}C (75.4 MHz) NMR spectra of deferriferrirubin (A and C) and deferriasperchrome C (B and D) in DMSO.

Acetylation of one of these N-acyl chains, as present in deferriasperchrome C, causes the following changes: (i) the 3 proton OH triplet at 4.62 δ ppm in DMSO reduces to a 2 proton triplet, (ii) triplet for one CH_2 group at ν shifts downfield by 0.14 δ ppm compared to that of the other two CH_2 groups at the same position, (iii) one of the φ CH_2 resonates 0.60 δ ppm downfield from the other two φ CH_2 groups and the acetyl CH_3 protons produce a distinct singlet at 2.01 δ ppm slightly upfield from the 9 proton methyl singlet. Further evidence for the structure of deferriasperchrome C is provided

by the ^{13}C NMR spectra which show the resonances for the acetyl CH_3 and C=O carbon atoms in addition to the complete ferrirubin backbone (Figure 1). Moreover, the methylene carbon atom bearing the acetyl group shows a downfield shift in resonance by 2.6 δ ppm compared to the other two CH_2 carbon atoms at position ϕ. Characterization of the individual N-acyl group resonances cannot be done in the deferri compound because of the superimposition of the signals of homologous protons. Further studies on their metal chelates (Ga^{3+}) in which the conformation of the side chains are somewhat restricted may offer additional information so that it will be possible to identify which of the three N-acyl groups is esterified.

Acknowledgement: This research is supported by DHHS grant GM 21822.

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INTERACTION OF CALCIUM IONS WITH PEPTIDE HORMONES OF THE GASTRIN FAMILY. A CD STUDY ON Nle^{11}-HG-13 AND Nle^{15}-HG-17

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Introduction

In recent years it has been observed that some of the physiological actions of gastrins seem to be related to the presence of calcium ions[1]. Preliminary investigations carried out on Nle^{11}-HG-13 (Des-Trp^{1}-Nle^{12}-minigastrin) revealed that the hormone strongly interact with calcium in trifluoroethanol (TFE) while no interaction has been observed in water[2]. In TFE the binding process induces an order-disorder conformational change of the peptide backbone, and seems to involve two metal ions per mole of hormone. In the present work, taking advantage of the presence of aromatic probes in the C-terminal tetra-peptide Trp-Nle-Asp-Phe-NH_2 , CD studies have been carried out in the near UV in order to determine if this biologically important sequence is directly involved in the interaction with ions. Investigations have been extended to Nle^{15}-HG-17.

Results and Discussion

The results of CD measurements on Nle^{11}-HG-13 in the aromatic absorption region are shown in Figure 1. The band envelop dominated by L_a and L_b Trp transitions[3] changes substantially upon addition of calcium. At saturation levels of ions a negative band envelop centered at about 280 nm becomes evident. The difference between the initial and the final CD spectra of Figure 1 corresponds to the CD pattern of c(L-Val-L-Trp) (Figure 2) with reversed sign[3]. We therefore conclude that the variation of the CD properties in this spectral region largely reflects a change of the environment of the Trp residue in the C-terminal

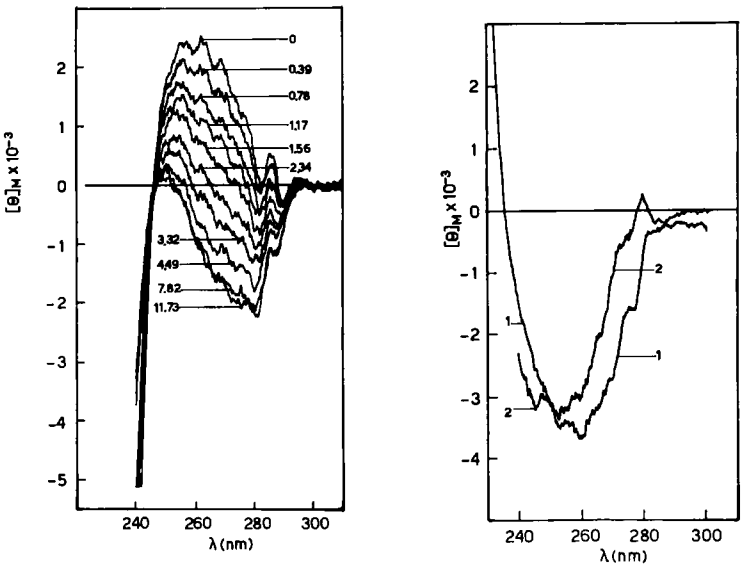

Fig.1 CD spectra of Nle^{11}-HG-13 in the aromatic region, in TFE and in the presence of Ca^{++}. The Ca^{++}/hormone molar ratios are indicated on the spectra. Ellipticity values $[\theta]_M$ are espressed per mole of hormone.

Fig.2 Computed differential CD spectra of Nle^{11}-HG-13 (curve 1) and of Nle^{15}-HG-17 (curve 2) obtained by subtraction of the CD spectra of the pure hormones from the CD spectra recorded at saturation levels of calcium.

sequence. Most important the effect of calcium ions on the CD spectra in the near UV does not parallel that previously observed in the far UV[2]. In fact the binding curve (Figure 3) shows that three, and not two, metal ions are bound per mole of hormone. These findings seem to indicate that the environment of the biologically important sequence is modified not only because of the calcium-induced conformational change of the peptide backbone (monitored by the CD spectra in the far UV), but also because of a direct interaction with the metal ions.

Elongation of the peptide chain from Nle^{11}-HG-13 to Nle^{15}-HG-17 does not provide any additional binding site for calcium. In this case however there is a parallel response from the near and far UV CD properties which indicate that three metal ions are always bound to a gastrin molecule.

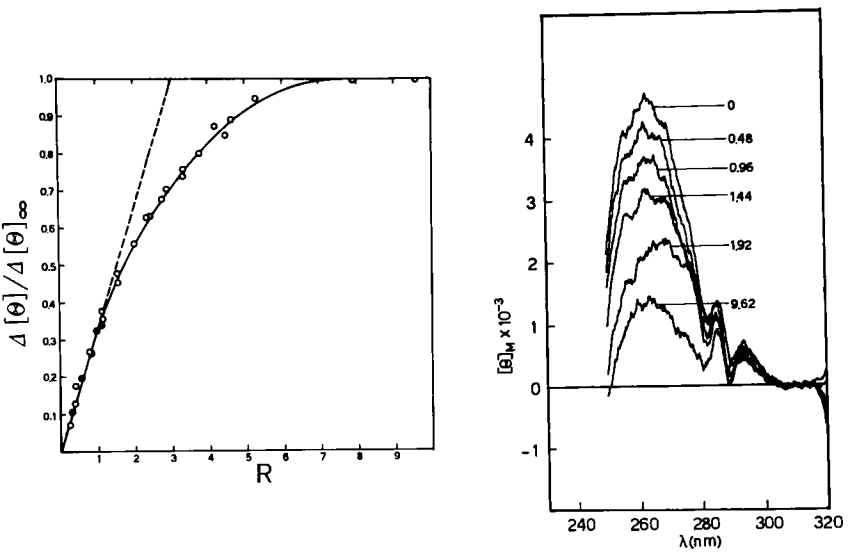

Fig.3 Relative variation of molar ellipticity,measured at 270 nm, of Nle^{11}-HG-13 in TFE, as a function of the calcium/hormone molar ratio .

Fig.4 CD spectra on Nle^{15}-HG-17 in the aromatic region in TFE at various Ca^{++}/hormone molar ratios (indicated on the spectra).

Binding curves very similar to that reported in figure 3 have in fact be obtained. The CD spectra reported in Figure 4 seem to indicate that only one of the two Trp residues of the hormone (at positions 4 and 14) is sensitive to the presence of calcium. In fact the difference between the final andinitial CD spectra of Figure 4 is very similar to that obtained for Nle^{11}-HG-13 (Figure 2). Keeping in mind our previous conclusion on Nle^{11}-HG-13 we suggest that also in the case of Nle^{15}-HG-17 the Trp residue involved in the interaction with the metal ions is the one located in the C-terminal sequence.

From saturation curves such as that shown in Figure 3 and using the general equation for a system containing three independent binding sites per molecule a set of binding constants have been estimated using a "best fitting" procedure. Two very similar sets of binding constants have been obtained for the

two hormones with K_3 of the order of $1x10^5$ M^{-1} and K_1 higher by at least one order of magnitude.

In conclusion the biologically active analogs of minigastrin and little gastrin bind strongly three calcium ions per mole with binding constants of the order of 10^5 or higher. On the other hand it is known that the capability of stimulating pancreatic enzyme secretion of the gastrins appear reduced or abolished in the absence of calcium. A correlation between these two facts might be possible especially if we take into account that also divalent cation ionophores increase pancreatic enzyme secretion, and their action is reduced by removing calcium.

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DESIGN OF PROTEASE INHIBITORS

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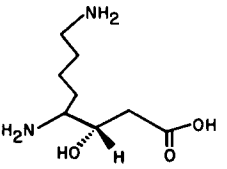
Novel protease inhibitors are needed in increasing numbers for medical and biochemical applications. The successful treatment of hypertension with angiotensin converting enzyme inhibitors (e.g., Captopril)[1] and the use of protease inhibitors to elucidate mechanisms of enzyme action[2] and peptide biosynthesis provide three examples where these compounds have proven most helpful. New strategies for designing protease inhibitors are needed in addition to a greater understanding of how known inhibitors bind to enzymes. Together, these efforts can lead to significant advances in enzymology and medicinal chemistry. At the present time, most efforts aimed at designing protease inhibitors start by synthesizing analogs of enzymatic reaction intermediates, e.g., tetrahedral intermediates, collected substrates or collected products. Several potent inhibitors of proteases have been prepared in this way.[3,4]

An alternative approach for designing inhibitors begins with naturally occurring inhibitors as the parent structures. The objective is to modify the inhibitor so as to achieve selectivity for a particular target enzyme without sacrificing the potency of the parent structure. This approach has not been applied often because there are only a few naturally occurring inhibitors of proteases (e.g., pepstatin, bestatin and amastatin, phosphoramidon and the peptide aldehydes related to leupeptin)[5] and because the relationships between inhibitor structure and the structure of either substrates or



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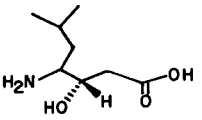

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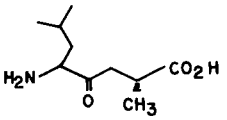

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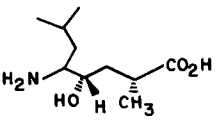

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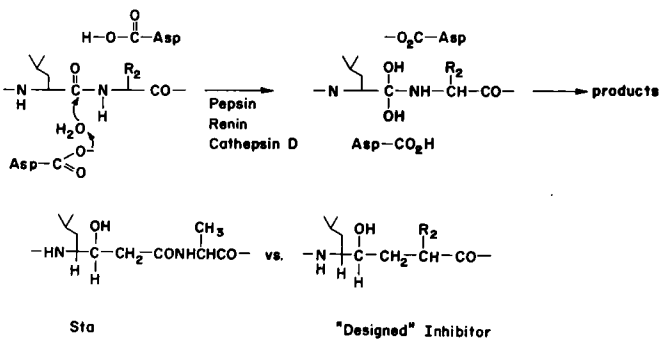


Fig. 1. Top. Schematic representation for formation of a tetrahedral intermediate during the hydrolysis of amide bonds by aspartyl proteases. Bottom. Comparison of statine and "isosteric" structures with the tetrahedral intermediate.

reaction pathway intermediates are obscure. For example, pepstatin (Iva-Val-Val-Sta-Ala-Sta) (1) inhibits most aspartyl proteases with dissociation constants generally in the range of 10^{-9} to 10^{-10}M except for renin. The central residue of statine, (3S,4S)-4-amino-3-hydroxy-6-methylheptanoic acid (2), is essential for tight-binding inhibition.[6] It has been postulated that the 3S-hydroxyl group of statine in pepstatin mimics the tetrahedral intermediate for amide bond hydrolysis (Figure 1).[7,8] However, pepstatin is not completely isosteric with a tetrahedral intermediate since statine contributes two extra atoms at C-1 and C-2.

We describe herein the synthesis and properties of three new inhibitors of aspartyl proteases. These compounds are variations of pepstatin modified so that the essential statine residue more closely approximates a substrate for the target enzyme. One inhibitor incorporates a new analog of statine, DAHOA (3). The other inhibitors incorporate the ketomethylene (4) and hydroxyethylene (5) amide bond isosteres and are analogs that more closely resemble a substrate or a tetrahedral intermediate.

The rationale underlying the synthesis of the lysine side chain analog 3 comes from the substrate specificity of fungal proteases. One major difference between mammalian aspartyl proteases (e.g., pepsin) and those isolated from fungi (e.g., penicillopepsin) is that the fungal proteases have the ability to activate trypsinogen, an activation process which requires specificity for lysine. None of the mammalian aspartyl proteases show this specificity as they prefer to hydrolyze amide bonds between hydrophobic residues. The best penicillopepsin substrate, N-Ac-Ala-Ala-Lys-Phe(NO_2)-Ala-Ala-NH_2 (6), is cleaved by penicillopepsin at the Lys-Phe(NO_2) bond.[9] A specific interaction between the positively charged ε-amino group of lysine and some negatively charged group on the enzyme is indicated because penicillopepsin cleaves Lys-Phe bonds faster than amide bonds between hydro-

phobic residues and because the kinetic parameters Km and k_{cat} for cleavage of this substrate show strong pH dependence. Using the refined crystal structure of the complex between penicillopepsin and Iva-Val-Val-Sta-OEt (7)[10] and molecular graphic simulations, James and Hofmann examined a possible tetrahedral intermediate that would be formed during penicillopepsin catalyzed hydrolysis of a substrate that contains a Lys-Phe segment. These studies suggested it is possible to form a solvated ion pair between the bis-carboxylate pair, Glu16 and Asp115, and the ε-amino group of substrate lysine.[11] If the model proposed by James and Hofmann is correct, then the pepstatin analog, Iva-Val-Val-DAHOA-OEt (8) in which the side chain of the statine residue in 7 has been changed from an isobutyl group to a 4-aminobutyl side chain should be a stronger inhibitor of penicillopepsin than the corresponding statine-containing inhibitor 7 owing to the additional ionic attraction. A schematic representation of the penicillopepsin-tripeptide inhibitor 8 complex is shown in Figure 2. The coordinates are based on the X-ray structure of tripeptide 7 but with the lysine side-chain in the DAHOA analog 8. The enzyme has been omitted here to permit visualization of the appropriate interactions between the side chain of inhibitor and the enzyme carboxyl groups.

(3S,4S)-4,8-Diamino-3-hydroxyoctanoic acid (DAHOA) (3) was synthesized by the route shown in Scheme 1. Boc-DAHOA(Z)-OEt (11) was obtained as a mixture of 3S and 3R diastereomers which were converted to the trichloroethoxycarbonyl derivatives 12ab and separated by chromatography over silica gel. The higher Rf material 12a was assigned the 3S configuration based on the H' NMR chemical shifts of the C-2 protons which resonate slightly downfield from the C-2 protons of the 3R diastereomer. The trichloroethoxycarbonyl groups in 12a and 12b were cleaved by reaction with cadmium in DMF/acetic acid[12] to yield the diastereomers 11a (3S,4S) and 11b (3R,4S). Compounds 11a and 11b were used to synthe-

size the Cbz-protected peptides 13a and 13b via standard stepwise coupling methods.[13] The free amine 8 was prepared by catalytic hydrogenation over palladium in DMF/acetic acid. Tetrapeptide 15 was prepared in a similar fashion.

Inhibition of penicillopepsin by all statine and DAHOA pepstatin analogs was measured using substrate 6. Assays were performed at 25°C in pH 5.5, .02M sodium acetate buffer.[9] The results of these determinations are shown in Table I.

Replacement of (3S,4S)-statine in 7 with (3S,4S)-DAHOA leads to tripeptide 8 in which Ki has been decreased by a factor of about 100. Tetrapeptide 15 is 8 times more potent than the corresponding statine analog. The decrease in Ki for 8 relative to 7 corresponds to an increased binding interaction of 2-3 Kcal, a value close to the interaction expected for a solvated ion pair.[14] An ionic interaction also is suggested by the fact that Cbz protected DAHOA tripeptide 13, which cannot form an ion pair at the terminal nitrogen, is a much poorer inhibitor of penicillopepsin (Ki = 10^{-7}M). There is evidence that the ionic interaction is with the acidic amino acid in position 115 because tighter binding is seen with penicillopepsin, a fungal protease, but not with pepsin, a mammalian aspartyl protease, in which the amino acid 115 is tyrosine rather than aspartic acid.[11,15] The Ki of DAHOA tripeptide 8 is greater than 10^{-6}M on porcine pepsin whereas the corresponding statine analog 7 has a Ki of 10^{-8}M on porcine pepsin. Interestingly, the very weak binding of 8 to porcine pepsin relative to 7 suggests the positively charged ion actually may be repelled from the active site of pepsin.

These data suggest a carboxylate group in penicillopepsin attracts the free epsilon amino group of the lysine side chain derivative of statine. If the binding mode of DAHOA inhibitor 8 is close to that found for the bound inhibitor,

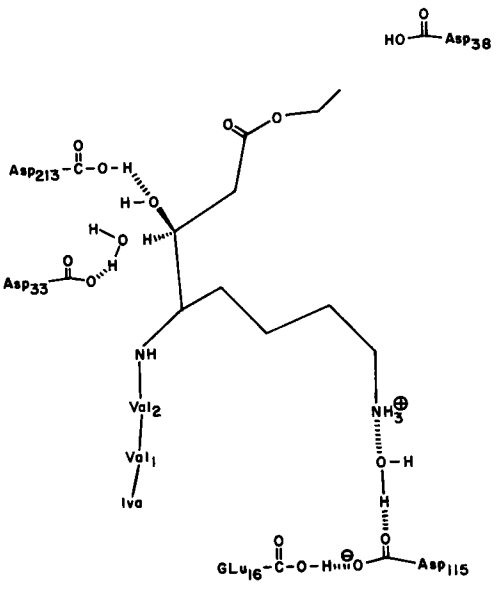


Fig. 2. Schematic representation of the binding of the lysine side-chain analog of statine, Iva-Val-Val-DAHOA-OEt to penicillopepsin.

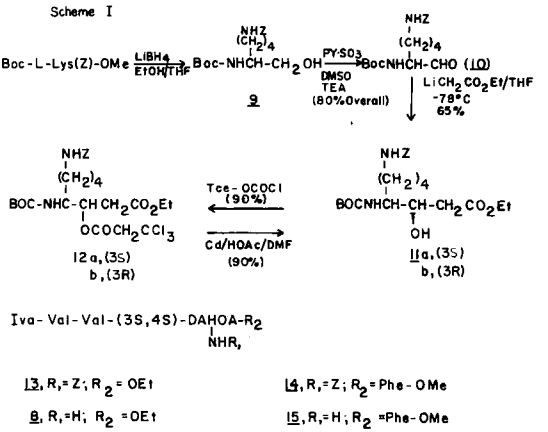


Table I. Inhibition of Aspartyl Proteases by Pepstatin Analogs

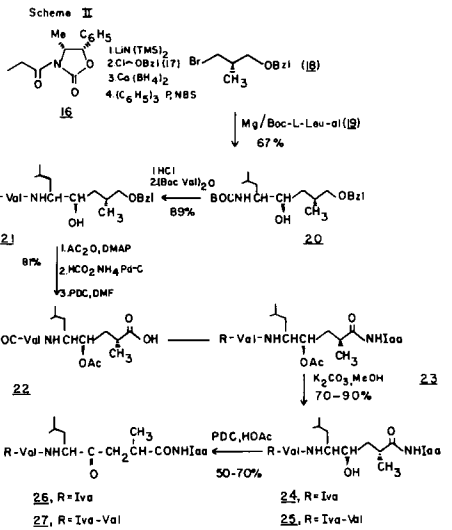
Inhibition of Aspartyl Proteases by Pepstatin Analogs

#	Compound[a]	Ki, M ($\times 10^9$) porcine pepsin	penicillopepsin
1	Iva-Val-Val-Sta-Ala-Sta-OH	.056	.15
7	Iva-Val-Val-Sta-OEt	10	47
8	Iva-Val-Val-DAHOA-OEt	>1000	0.4
	Iva-Val-Val-Sta-Phe-OMe	--	1.0
15	Iva-Val-Val-DAHOA-Phe-OMe	--	0.08
28	Iva -Val-Sto-Ala-Iaa[b]	56	--
26	Iva-Val-Leu($COCH_2$)Ala-Iaa	970	--
29	Iva-Val-Val-Sto-Ala-Iaa[b]	10	85
27	Iva-Val-Val-Leu($COCH_2$)Ala-Iaa	25	--
30	Iva-Val-Sta-Ala-Iaa	3	7600
24	Iva-Val-Leu($\underset{OH}{CHCH_2}$)Ala-Iaa (4S?)	27	--
	(4R?)	750	--
31	Iva-Val-Val-Sta-Ala-Iaa	0.1	6.5
25	Iva-Val-Val-Leu($\underset{OH}{CHCH_2}$)Ala-Iaa (4S?)	9.2	--
	(4R?)	500	--

a. All statine analogs have the (3S,4S) configuration.

b. Sto, 4-Amino-3oxo-6-methylheptanoic acid.

Scheme II



Iva-Val-Val-Sta-OEt (7), it is likely that this interaction is with the carboxylate groups in the Glu16 and Asp115 residues.

Ketomethylene and Hydroxyethylene Analogs

The ketomethylene (4) and hydroxyethylene (5) isosteres of a Leu-Ala dipeptide sequence were synthesized in order to establish the importance of the two extra atoms in statine relative either to substrate or to the tetrahedral intermediate (Figure 1). Alkylation of the chiral oxazolidinone 16 with benzyl chloromethyl ether 17 followed by borohydride reduction gave the chiral alcohol (Scheme II).[16a] After conversion to the bromide 18,[16b] the corresponding Grignard reagent was allowed to react with Boc-L-leucinal 19[17] to give the hydroxy ether 20 as a mixture of diastereomers in 67% yield. This compound could be deprotected at nitrogen and acylated as shown to provide the extended analog 21.

The ether group in 21 was converted to acid 22 as shown in Scheme II. Acid 22 was converted to the isoamylamide by reaction with DCC/HOBt and isoamylamine, the peptide chain was extended by the symmetrical anhydride method and the diastereomers were separated by silica gel chromatography. Alcohols 24, 25 were obtained by methanolysis and oxidized to ketones 26, 27 by reaction with pyridinium dichromate in acetic acid.

Inhibition of porcine pepsin by the ketomethylene and hydroxyethylene derivatives was determined using the reported spectrophotometric assay.[13] Ketones 26, 27 which correspond to analogs of substrates that contain a Leu-Ala sequence, are weaker inhibitors of pepsin than the corresponding ketostatine analogs 28, 29. One diastereomer of each of the hydroxyethylene isosteres 24, 25 was found to be a stronger inhibitor of pepsin than the corresponding ketones, as would be

expected from the similarity of the alcohol derivatives to the tetrahedral intermediate (Figure 1). However, the hydroxyethylene analogs were significantly weaker than the corresponding statine derivatives 30, 31. It must be noted that none of the side chain substituents in analogs 25 and 26ab correspond to substituents known to favor rapid hydrolysis of substrate by pepsin. It is likely that better inhibitors of aspartyl proteases derived from the ketomethylene and hydroxyethylene isosteres will be found when the isobutyl and methyl side chains in 25 and 26 are replaced by the hydrophobic substitutents known to favor binding to pepsin.[18]

These results indicate that in otherwise identical peptide sequences incorporation of the Sta-Ala segment in place of the Leu-Ala dipeptide isosteres produces a stronger inhibitor of pepsin. The effectiveness of the statine containing inhibitors is consistent with statine atoms C-3 to C-7 mimicking a tetrahedral intermediate whereas the atoms at C-2 and C-1 may serve some other function such as to allow the inhibitor to bind to product binding sites. In this mode pepstatin would be both a transition-state analog and a collected-product analog. Alternatively, pepstatin could mimic a dipeptide unit of the tetrahedral intermediate as proposed by Boger.[19]

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CRYSTALLOGRAPHIC ANALYSIS OF A PEPSTATIN ANALOGUE BINDING TO THE ASPARTYL PROTEINASE PENICILLOPEPSIN AT 1.8 Å RESOLUTION

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Introduction

X-ray crystallographic analysis of inhibitor or substrate analogue binding to the active sites of enzymes can be used to deduce productive binding modes of substrates and the enzymes' catalytic pathways. The binding sites on the aspartyl proteinase penicillopepsin[1-3] have been probed by the pepstatin analogue Iva-Val-Val-Sta-OEt (Iva = isovaleryl; StaOEt = ethylester of statine [(4S,3S)-4-amino-3-hydroxyl-6-methylheptanoic acid]). The high resolution refined structure of penicillopepsin[1] was used to provide phasing information to compute a 1.8 Å resolution difference electron density map of the bound inhibitor Iva-Val-Val-Sta-OEt.[4] This map was of high quality and showed not only the binding mode of the inhibitor but also revealed a large conformational change in the enzyme that had resulted from the inhibitor binding. However, difference electron density maps suffer from two sources of error: (1) the phases used for the computation are those of the native enzyme, thus, are only approximations to the correct phases for the complexed crystal and (2) the inhibitor displaces solvent molecules from the native enzyme structure thereby introducing distortions in the difference electron density map. In order to obtain a more accurate description of the complex, we have refined the structure of the pepstatin analogue bound to penicillopepsin at 1.8 Å resolution.

Methods

X-ray intensity data were collected from a single crystal of penicillo-

pepsin with the bound inhibitor Iva-Val-Val-StaOEt to 1.8 Å resolution.[1,4] A molecular model of the inhibitor peptide was derived from the difference map[4] as well as an approximation of the conformational change of the β-loop region of the enzyme, from Trp71 to Ser82. These atomic coordinates plus the coordinates of the remainder of the penicillopepsin molecule were subjected to 21 cycles of restrained-parameter least-squares refinement.[5] At several stages during the refinement, difference maps with coefficients $|F_o|-|F_c|$ (where $|F_o|$ and $|F_c|$ are the measured and calculated structure factor amplitudes, respectively) and calculated phases, α_c, were used in conjunction with $2|F_o|-|F_c|$, α_c maps to make corrections to the atomic coordinates and to find additional ordered solvent molecules.

Results and Discussion

Table 1 presents some of the refinement statistics obtained after 21 cycles of least-squares on the data from the crystal of the enzyme-inhibitor complex. In this process, not only is it important to attain a low crystallographic R-factor (0.131) but also the molecular geometry should remain close to that expected from highly refined atomic resolution structures of the individual amino acids. It can be seen from the data in Table 1 that the refined structures of the native enzyme and the inhibitor bound enzyme are of comparable high quality so that deductions regarding

Table 1. Summary of refinement statistics from restrained-parameter least-squares

Data	Native penicillopepsin[§]	Penicillopepsin + pepstatin analogue	Stereochemistry of resulting model	Native penicillopepsin[§]	Penicillopepsin + pepstatin analogue
Resolution Range	8.0 - 1.8 Å	8.0 - 1.8 Å	rms Δ bond distances[†]	0.014 Å	0.015 Å
Total Reflections	23,881	23,920	rms Δ angle distances	0.038 Å	0.038 Å
Reflections used ($I>\sigma(I)$)	21,962	21,197	rms Δ planarity	0.015 Å	0.017 Å
Total No. Atoms	2363	2401	rms Δ ω peptide bonds	3.0°	3.3°
No. Solvent Atoms	319	322			
No. Cycles	86	21			
R-factor*	0.136	0.131			

* Defined as $R = \Sigma||F_o|-|F_c||/\Sigma|F_o|$.

† These values are the root mean square deviation of the refined stereochemical parameters from those in the small molecule dictionary of "ideal" values (Sielecki et al., 1979).

§ Data from James and Sielecki (1983).

binding modes and conformational changes are on a firm basis.

At cycle 12 of the refinement, a steric problem between the CH_2COOEt group of the statine residue and the carbonyl oxygen of Gly35 was observed. Also, the N-terminal isovaleryl group had an excessively large isotropic temperature factor. These two groups were removed from the calculations for 4 of the subsequent refinement cycles and a second difference map after cycle 17, omitting their contribution, was computed (coefficients, $|F_o|-|F_c|$; phases, α_c). A portion of this map is shown in Figure 1 along with

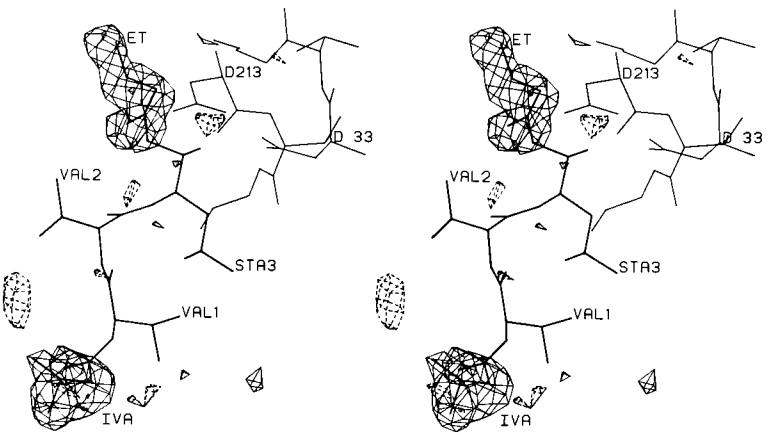

Fig. 1. Difference electron density map at cycle 17. The ethyl ester, $CH_2COOCH_2CH_3$, and the isovaleryl group had been omitted from the structure factor calculations. The pepstatin analogue model is the final refined model at cycle 21. A small portion of the active site of penicillopepsin is included as reference. All the figures were prepared using program M3, designed and implemented by C. Broughton for the MMS-X interactive graphics system.

the molecular model of Iva-Val-Val-StaOEt that has resulted from the 21 cycles of refinement. The positive contour regions show the electron density associated with the isovaleryl group and the ethyl ester. The conformation of the isovaleryl group was quite clear on this map and the high atomic temperature factors are probably a result of the relatively few contacts it makes with penicillopepsin. The electron density for the ethyl ester was not so easily interpretable. We have chosen one possible conformation of the ester group that requires a non-planar conformation, i.e. the atoms of the ethyl group are not in the plane determined by the atoms of the carboxyl group, CH_2COO. The refined torsional angle,

$CH_2-\overset{O}{\overset{\|}{C}}-O-CH_2$ is -82.7°. This conformation was not evident on the original difference map.[4] The distortion of that density was probably the result of the proximity of three strongly bound solvent molecules in the native enzyme structure. If one tries to maintain the planar conformation of the ester in the refined orientation of the carboxyl group, it is not possible to fit the electron density shown in Figure 1 satisfactorily and a too close contact of the ethyl group with the carbonyl oxygen atom of Gly35 in penicillopepsin results.

In addition to the altered conformation of the ethyl ester, the whole inhibitor molecule has shifted from the position deduced from the original difference map.[4] The two positions are compared in Figure 2. The root

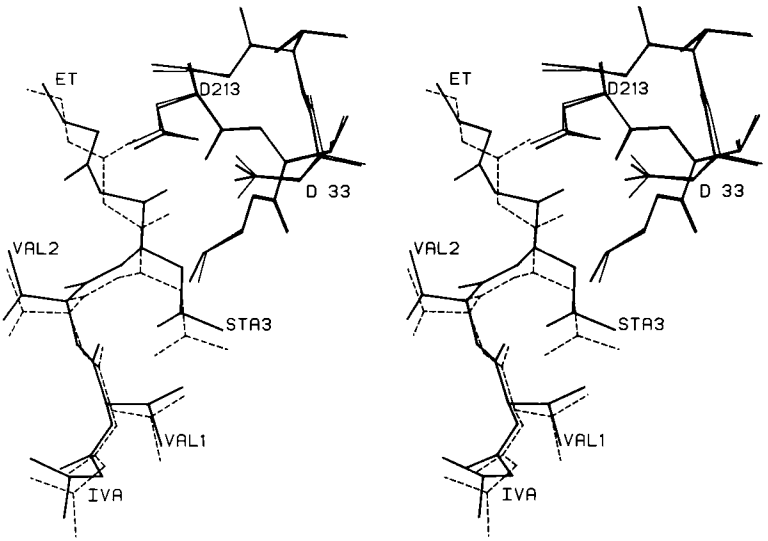

Fig. 2. Comparison of the positions of Iva-Val-Val-StaOEt as deduced from the initial difference map (dashed line) and after the 21 cycles of least-squares refinement (full thick line). The conformational change of the active site residues is also indicated (full thin line, native penicillopepsin; full thick line, penicillopepsin-pepstatin complex).

mean square distance between corresponding atoms of the molecule in these two positions is 0.90 Å. The largest differences are associated with the ester group (the carbonyl oxygen atoms differed by 3.36 Å). The whole statine residue, especially the 3-OH group close to the catalytic aspartyl groups, has shifted by ∿0.85 Å. Even though the initial coordinates of

the inhibitor were derived from a good quality 1.8 Å resolution difference electron density map, large movements of this inhibitor relative to the catalytic groups on the enzyme have resulted from refinement. It is clear that reliable deductions regarding substrate binding and the enzymes catalytic mechanism should only be made from the refined coordinates.

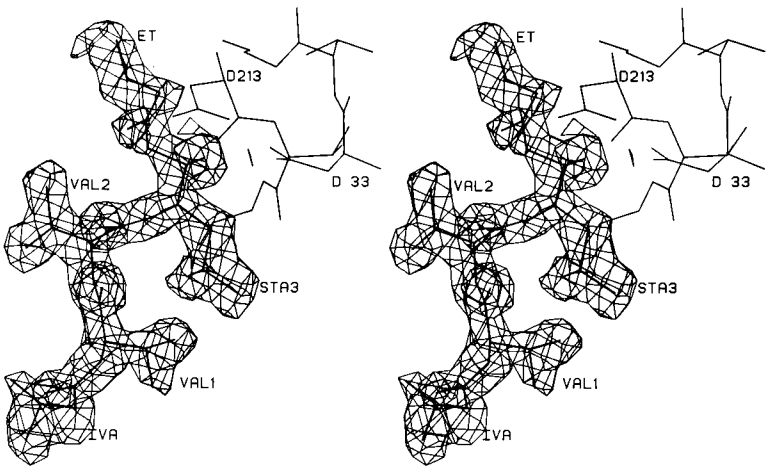

Fig. 3. Electron density map $2|F_o|-|F_c|$, α_c showing the density for Iva-Val-Val-StaOEt at the conclusion of the refinement.

The proposed non-planar ester group represents a high energy conformation for this system. At present it is not possible to reconcile the relatively large destabilization energy ($\sim$ 10 kcal/mole) necessary to maintain the non-planar conformation of the ester and still have the inhibitor bound to penicillopepsin. A computer search indicates that there are no reported non-planar esters of this type contained within the Cambridge Crystallographic Data Bank of small molecule structures. Nevertheless, the interpretation of the electron density of the $2|F_o|-|F_c|$, α_c map after 21 cycles of refinement seems well satisfied by the refined atomic coordinates (Figure 3). Moreover, these same coordinates also adequately fit the model independent difference electron density of Figure 1. Still, the weaker electron density and higher temperature factors associated with atoms of the ester group indicate that an alternative conformation of this group should not be ruled out.

The overall binding mode of the pepstatin analogue to penicillopepsin is shown in Figure 4. The hydrogen-bonding interactions of the peptide to

the enzyme are listed in Table 2; the number of intermolecular non-bonded contacts less than or equal to 4.0 Å are given in Table 3.

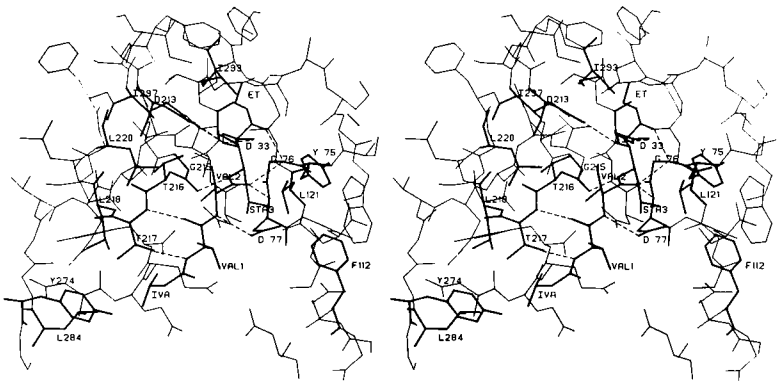

Fig. 4. Details of binding interactions of Iva-Val-Val-StaOEt to the active site of penicillopepsin. Hydrogen bonds between enzyme and inhibitor are shown as dashed lines; some of those residues of penicillopepsin implicated in binding are shown in thicker full lines.

Table 2. Hydrogen-bonding involving P_3 P_2 P_1 and P_1' residues

Pepstatin Fragment		Penicillopepsin	Dist (Å)	Pepstatin Fragment		Penicillopepsin	Dist (Å)
P_3	Val NH	Thr217 $O^{\gamma 1}$	2.83	P_1	Sta NH	Gly215 C=O	3.07
	Val C=O	Thr217 NH	3.03		Sta-OH	Asp33 $O^{\delta 2}$	2.50
		Wat540 O	2.80			Asp33 $O^{\delta 1}$	3.31
P_2	Val NH	Asp77 $O^{\delta 1}$	2.71			Asp213 $O^{\delta 1}$	2.89
	Val C=O	Gly76 NH	3.21			Asp213 $O^{\delta 2}$	2.60
		Asp77 NH	3.24	P_1'	Ester C=O	Gly76 NH	3.25
					Ester C=O	Wat606 O	2.86

Relatively few contacts with atoms of penicillopepsin are made by the P_4 Iva atoms. A small hydrophobic niche formed by Leu218, Leu284 and the aromatic plane of Tyr274 can be discerned (Figure 4). In addition to the two strong hydrogen bonding interactions between the main chain of the inhibitor at P_3 Val and Thr217 of the enzyme, the valyl side chain makes hydrophobic contact to the methylene carbons of Glu15. All the hydrogen bonding interactions involving the polypeptide chain atoms of P_2 Val are to residues of the flap (Table 2). Hydrophobic interactions for the P_2 Val side chain atoms involve residues Thr216 $C^{\gamma 2}$, Leu220 $C^{\delta 1}$ and Ile297 $C^{\delta 1}$. A leucyl residue in this P_2 position could be easily accommodated

and should enhance the binding interaction with penicillopepsin.

Table 3. Summary of non-bonded contacts (≤4.0Å) between IvaValValStaOEt and penicillopepsin

Pepstatin analogue \ Penicillopepsin	Glu 15	Asn 31	Asp 33	Gly 35	Tyr 75	Gly 76	Asp 77	Ser 79	Leu 121	Phe 190	Ile 211	Asp 213	Gly 215	Thr 216	Thr 217	Leu 218	Leu 220	Leu 284	Ile 297	Total
P_4 Iva	1														3	1		1		6
P_3 Val	1						3*						2	3	7(2)					16
P_2 Val					1	4(1)	11(2)							3			1		1	21
P_1 Sta		1	8(2)		5		1	1	2			4(2)	9(1)	2						33
P_1' ester				1	2	1(1)				2	2	2		1						11
Total	2	1	8	1	8	5	15	1	2	2	2	6	11	9	10	1	1	1	1	87

* Contacts within this boxed region are to atoms of residues that comprise the flap of penicillopepsin.

There are a large number of contacts involving the atoms of the P_1 statyl residue. A hydrogen bond from the main chain NH of P_1 Sta to the carbonyl oxygen of Gly215 in penicillopepsin helps to position the scissile bond correctly at the active site. The importance of this hydrogen bond to substrate binding in the aspartyl proteinases is appreciated from the subsite specificity data for porcine pepsin.[6] A proline in the P_1 position has almost no cleavage probability with porcine pepsin. Hydrophobic interactions involving the statyl side chain atoms and the aromatic plane of the strongly conserved Tyr75 residue of the flap seem most important. The C^{δ} atoms of Leu121 undergo a conformational change and are also involved in binding the statyl side chain.

The 3-OH group of P_1 statine figures prominently in the inhibitory mechanism of pepstatin and the present analogue towards the aspartyl proteinases. The hydroxyl oxygen atom displaces a strongly bound solvent molecule (039) from the native enzyme.[1] The refined positions of the 3-OH oxygen and this solvent molecule, 039, differ by only 0.25 Å and make essentially identical non-bonded contacts with the two carboxyl groups, Asp33 and Asp213, at the active site of penicillopepsin. The carboxyl group of Asp33 undergoes a slight rotation upon inhibitor binding [35° in χ^2 (C^{β}-C^{γ} bond)], whereas that of Asp213 remains essentially fixed. The hydrogen bonding interaction from Thr216 $O^{\gamma 1}$ to $O^{\delta 2}$ of Asp213 may account for the added rigidity of this residue.[1] The approximately equal distances from the 3-OH to the $O^{\delta 1}$ and $O^{\delta 2}$ atoms of Asp213 suggest a bifurcated hydrogen bond involving the 3-hydroxyl proton to the carboxyl group oxygen atoms.

Similarly, hydrogen bonding distances from Asp33 $O^{\delta 2}$ to Asp213 $O^{\delta 1}$ and to the 3-OH of P_1 statine (2.83 Å and 2.50 Å, respectively) imply a bifurcated hydrogen bond as well with the proton on $O^{\delta 2}$ of Asp33. The exact hydrogen bonding interactions cannot be determined unequivocally with X-ray diffraction at this resolution, since the H atoms are not locatable experimentally. The contact distances are relatively accurate however, and show the asymmetric interaction that the 3-OH group makes with these two aspartic acid residues (Table 2, Figure 3).

The largest conformational changes of residues of penicillopepsin that can be attributed to peptide inhibitor binding are associated with the flap. Atoms comprising Gly76 move on average 4.0 Å from their position in the native enzyme. The hinge like motion of this hairpin β loop region of penicillopepsin is shown in Fig. 5. Residues Trp71 and Gly83 do not move

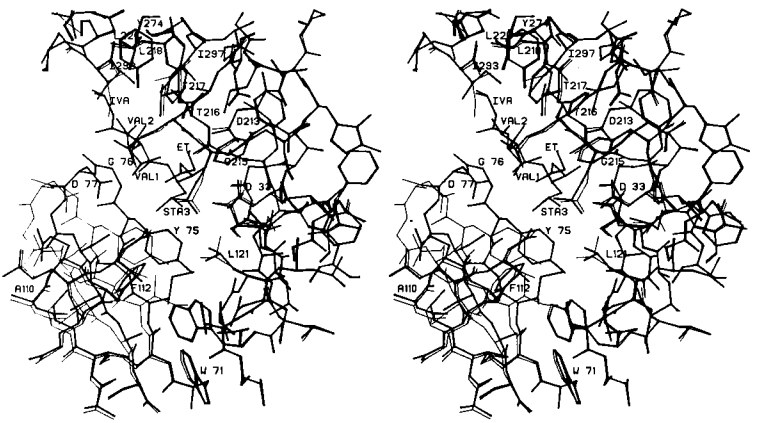

Fig. 5. View of the active site of penicillopepsin with the bound inhibitor showing the details of the conformational changes induced on binding. The native enzyme conformation is shown in thin lines; the complex conformation by thick lines. Ninety percent of the enzyme atoms move by less than 0.3 Å; residues Gly76, Asp77 move by ∿ 4.0 Å towards the inhibitor molecule thus closing the cleft.

from their position in the native enzyme. Relatively small changes in the ϕ and ψ conformational angles along the main chain result in the large movement of the atoms comprising the β-bend Tyr75, Gly76, Asp77, Gly78. The movement of these residues of the flap results in the displacement of three of the ordered water molecules in the penicillopepsin native structure and a re-ordering of the other bound solvent in the region. A total

of 11 ordered water molecules in the native enzyme structure are displaced by the inhibitor binding and concommitant conformational change of the flap. Other residues that move in a concerted fashion following the movement of the flap are Gln107 to Gln111, with the C^{α} atoms of Ser109 and Ala110 moving 0.58 and 0.50 Å respectively (Figure 5). These residues form a third antiparallel strand of a small β-sheet region of the molecule.[1] Individual side chains that move by more than 0.7 Å as a result of the inhibitor binding include Glu16, Asp33, Phe112, Leu121, Leu284, Ile293 and Ile297. The overall rms shift for all atoms of penicillopepsin is 0.47 Å with 2118 atoms out of 2363 atoms total moving less than 0.3 Å.

Several possible binding modes for substrates with aspartyl proteinases have been proposed.[2,3,7-9] None of these proposals were based on refined crystal structures. The study described here has allowed for more reliable model building at the active site of penicillopepsin. Towards this end we have built a P_1 Lys and a P_1 Phe residue at the P_1 Sta position in order to analyze the contacts. A lysyl side chain would make favorable interactions with a carboxyl-carboxylate pair Glu16, Asp115, the possible anionic site in penicillopepsin. Such an interaction is well known for the microbial aspartic proteinases.[10] A P_1 phenylalanine could also be accommodated in the S_1 binding site of penicillopepsin (Figure 6). Nevertheless, due to the very different shape of its side chain, the interactions would be much

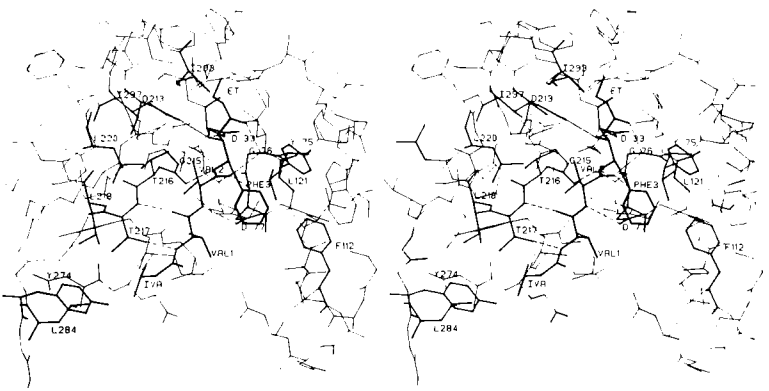

Fig. 6. Model building of an analogue of Iva-Val-Val-StaOEt with a phenylalanyl side chain replacing the leucyl side chain of P_1 Sta. No serious too-short non-bonded contacts are made with residues of penicillopepsin.

less favorable than those of an homologous P_1 leucine residue.

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PEPSTATIN BINDING TO RHIZOPUS CHINENSIS ASPARTYL PROTEINASE

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Introduction

X-ray diffraction investigations of enzyme-inhibitor complexes can provide accurate information about the molecular basis of inhibitor binding. Although this is necessarily static information, it can be related to kinetic properties and can provide a three-dimensional basis for substrate binding and for the mechanism of action of the enzyme. Pepstatin,[1] a hexapeptide (Figure 1) isolated from streptomyces is a powerful inhibitor of aspartyl proteases (Ki = 5×10^{-11}M for pepsin.[2] We have previously reported the three-dimensional structure of pepstatin complexed to the aspartyl protease from Rhizopus chinensis,[3] and have concluded that the statine-4 residue approaches a transition state analog in agreement with the suggestion of Marciniszyn et. al.[4] Similar results have been reported for a fragment of pepstatin bound to penicillopepsin.[5] The binding of pepstatin suggests a model of substrate binding and of the tetrahedral intermediate. This has led us to a proposed mechanism of action involving general acid-base catalysis with no covalent intermediates.

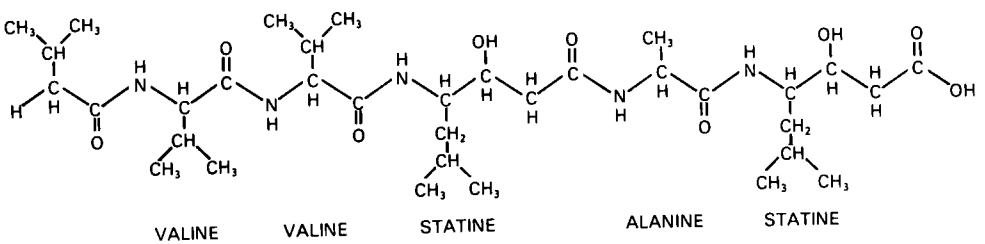

Fig. 1 Structure of pepstatin.

Results and Discussion

The preparation and properties of the pepstatin-Rhizopus pepsin crystals have been previously described.[3] The Rhizopus pepsin crystal structure is being refined at 1.8 Å resolution. The phases from the current structure have been combined with the 2.5 Å amplitudes [|F pepstatin| - |F native|] to yield a 2.5 Å difference electron density map.

This map contained one prominent extended feature that clearly corresponded to the pepstatin bound in the major groove of the enzyme. Apart from some peaks and troughs in the vicinity of the pepstatin (discussed below) the overall appearance of the difference map was nearly featureless, indicating that the protease does not undergo any large conformational change upon binding the inhibitor. The improved phases used in calculating this map resulted in a much clearer map with less ambiguity. Refitting pepstatin to the map led to readjustment of ival-val2 and of the conformation of the peptide bond between sta4 and ala4. The region around the sta-ala peptide bond had been ambiguous in the earlier difference map, but could now be resolved (Figure 2).

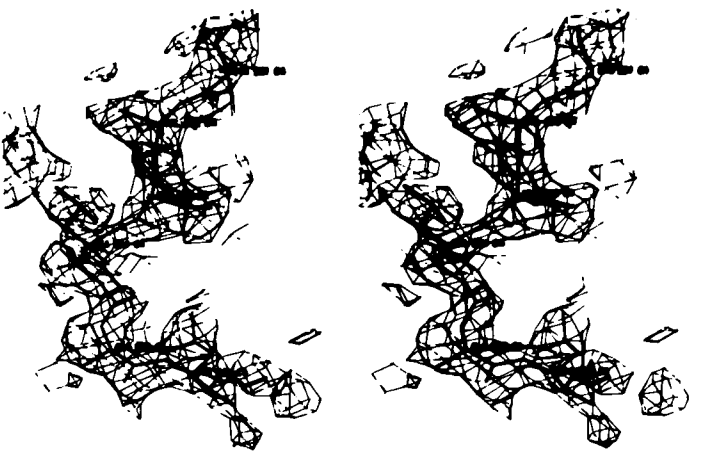

Fig. 2 Model of pepstatin on difference electron density map.

The other features of the difference map indicate that there is a conformational change of one part of the enzyme after binding pepstatin. There is a β-hairpin loop, residues 70-85, the end of which moves toward the pepstatin (Figure 3) with an rms movement of 1.2 Å for the residues from Trp71 to Asp77. The remaining significant features of positive and negative density were due to the exclusion or movement of water molecules or ions after binding the pepstatin.

The pepstatin is bound in the major cleft between the two domains. The first four residues adopt an extended conformation with side chains alternating in opposite directions. The main chain makes an abrupt turn after the 3-hydroxyl of statine 4. This is probably the result of the two additional main chain carbon atoms of the statine 4 which displace the carboxyl terminal two residues of the pepstatin about 3 Å along the cleft relative to a normal peptide substrate.

Chemical studies of the binding of pepstatin to pepsin have shown that it is dependent on the 3-OH of statine[6] and is highly stereospecific.[7,8] Recent studies using ^{13}C NMR

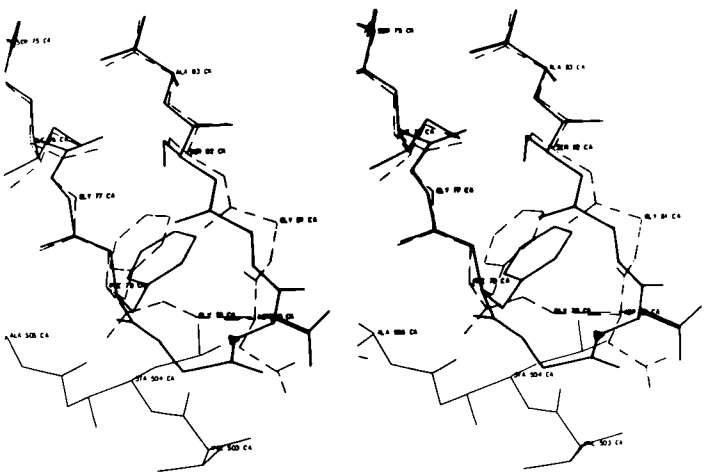

Fig. 3 Movement of the flap residues 75-83 (dashed = native, solid, with pepstatin.

provide evidence for a tetrahedral intermediate binding of a ketone analog of pepstatin.[9]

The interactions between the pepstatin and the enzyme observed in the crystal involve hydrogen bonds as well as numerous van der Waals contacts. The hydrogen bonds are listed in Table I and illustrated in Figure 4. The leucyl-like side chain of statine 4 fits in a mainly hydrophobic pocket formed by residues Phe75, Gly76, Asp77, Phe111 and Leu120. The 3-hydroxyl oxygen is positioned straddling the carboxyl groups of Asp32 and Asp215.

We have concluded that the first four residues of pepstatin mimic the binding of substrate, and we have accordingingly constructed a hypothetical model of valyl-valyl-leucyl-phenylalanyl-leucine bound to the cleft in an extended configuration. In this model, the first three residues of the substrate follow the positions of the second, third and fourth

Table I. Inhibitor - Enzyme Interactions Between Pepstatin and Rhizopus chinensis Aspartyl Proteinase

Hydrogen bonds						
pepstatin			protease			distance
Valine	2	N	Serine	219	O_γ	3.3 Å
Valine	2	O	Serine	219	N	3.0 Å
Valine	3	N	Aspartic acid	77	OD_2	3.1 Å
Valine	3	O	Aspartic acid	77	N	3.1 Å
Statine	4	N	Glycine	217	O	3.0 Å
Statine	4	OH	Aspartic acid	32	OD_2	2.5 Å
Statine	4	OH	Aspartic acid	215	OD_2	2.8 Å
Statine	4	O	Glycine	76	N	3.2 Å
Alanine	5	N	Glycine	34	O	2.8 Å
Alanine	5	O	Tryosine	189	OH	2.7 Å

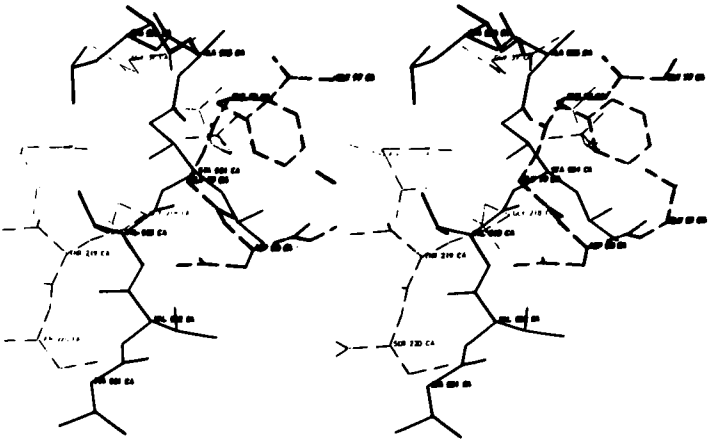

Fig. 4 Pepstatin (solid) and hydrogen bonding residues of Rhizopus chinensis aspartyl protese (dashed, consecutive numbering).

residues of pepstatin with the leucyl side chain bound in the P1 hydrophobic pocket. Of the remaining side chains, the phenylalanine is directed towards another hydrophobic pocket (P1') using convention pf Berger et. al.[10] formed by Ile213, Ile301 and Tyr189, and the carboxyterminal leucine is in contact with Ile73 and Phe75 of the flap and with Ser35 (P2'). The cleft is sufficiently long to accommodate several additional residues.

The substrate modeled in this way has the carboxyl oxygen of the scissile peptide bond directed toward the carboxyl of Asp215 which is believed from chemical evidence to be protonated. This oxygen is situated in roughly the same position as the hydroxyl of statine 4. The nitrogen of the scissile bond is situated in roughly the same position as the main chain CH2 of pepstatin.

The tetrahedral intermediate can be constructed as an extension of the substrate model by placing a second oxygen at the position indicated by the hydrogen on the C3 atom of statine 4, and by carrying out the minimum adjustments necessary

to create a tetrahedral carbon atom. We have constructed such a model with a leucyl side chain preceding and phenylalanyl side chain following the scissile bond. In this model, which still approximates the modeled substrate, the second oxygen can be accommodated in the active site of the enzyme without requiring any rearrangement of the protein (Figure 5).

The model tetrahedral intermediate makes chemically reasonable contacts with the enzyme, and examination of this complex has led to a proposed mechanism for peptide cleavage by the enzyme (Figure 6). In this mechanism, the carbonyl carbon undergoes nucleophilic attack by a water molecule with Asp32 serving as a general base catalyst. Simultaneously, the protonated carboxyl of Asp215 polarizes the carbonyl bond thus enhancing the attraction of the carbonyl carbon for the nucleophile.

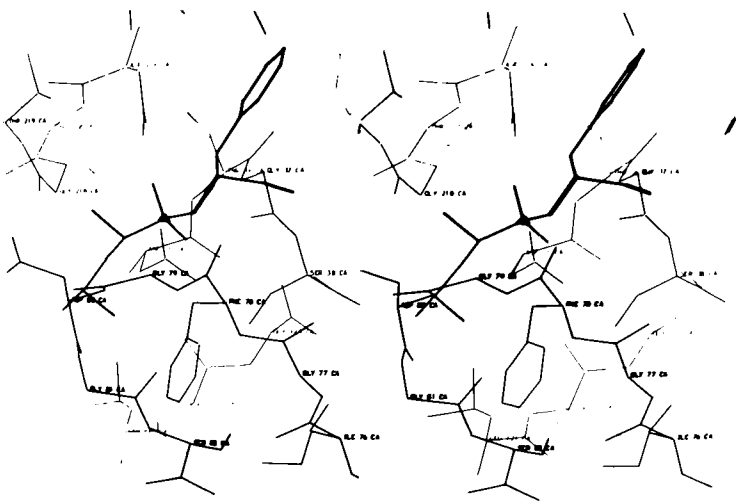

Fig. 5 Model of tetrahedral intermeidate and Rhizopus chinensis aspartly protease active site (consecutive numbering).

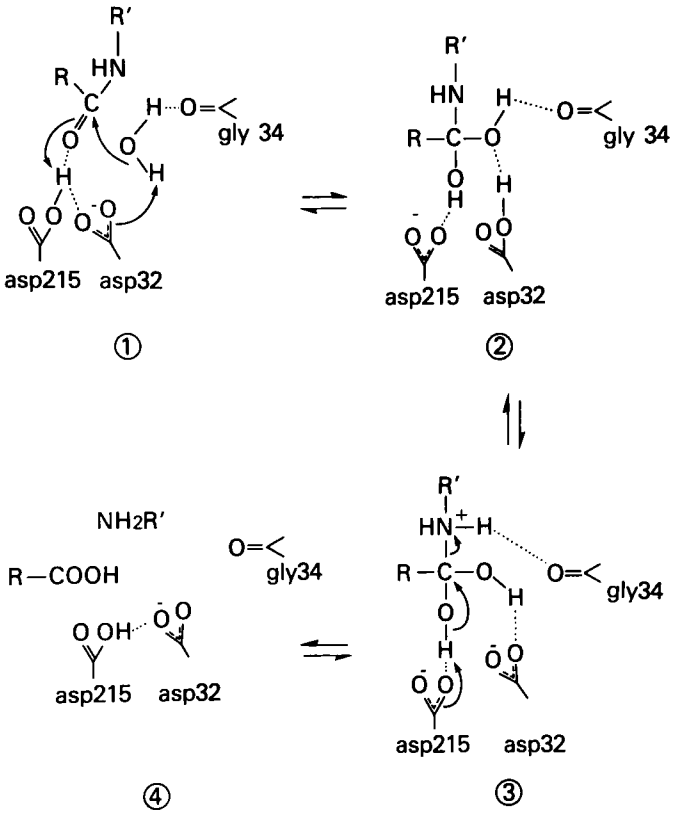

Fig. 6 Proposed mechanism of action.

In this mechanism the side chains of aspartyl 215 and 32 serve as an acid and base catalysts respectively to catalyze the nucleophilic attack by an hydroxide ion. The amide nitrogen may then be protonated by another water molecule or by a proton from the tetrahedral intermediate itself, mediated by the presence of the carboxyl of gly 34 (37 in the consecutive numbering shown in Figure 5). However the amide nitrogen becomes protonated, the products are then formed by an elimination reaction.

PEPTIDE INHIBITORS OF PROTEASES

The aspartyl proteases share a number of functional and structural properties that include similar specificities for the peptide bond bracketed by large hydrophobic residues; inhibition by pepstatin, 1,2epoxy 3-nitrophenoxypropane (EPNP),[12,13] and diazo compounds such as diazo acetyl DL norleucine (DAN); a common molecular weight for the active enzyme; sequence homology[14] and very similar three-dimensional structures. The structure of penicillopepsin at 1.8 Å has been reported,[15-17] and a comparison with the Rhizopus pepsin shows them to have remarkably similar three-dimensional structures. The currently refined model of the Rhizopus pepsin can be aligned with penicillopepsin so that the main chain atoms of 260 residues (out of 320 in the Rhizopus pepsin) are displaced by an rms distance of only 1.2 Å. This structural homology extends to the conformations of the side chains as well as conformations of the active site residues around the S1 and S1' subsites in the cleft (Figure 7). Similarly, the binding of a fragment of pepstain to penicillopepsin seems to resemble closely the binding of pepstatin to the Rhizopus pepsin. It is therefore very likely that these two enzymes cleave the peptide bond through identical overall mechanisms, although there may be fine tuning through the differences in amino acid sequence. The mechanism we propose is consistent

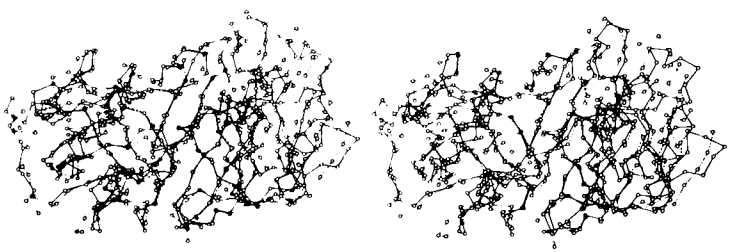

Fig. 7 Backbone folding of Rhizopus aspartyl protease (solid) with penicillopepsin (dashed, superimposed).

with O^{18} exchange studies reported for pepsin.[19-21]. It is therefore likely that this mechanism can in the same way be generalized to account for the other aspartic proteases, both microbial and mammalian.

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ANGIOTENSIN-CONVERTING ENZYME INHIBITORS: PHOSPHINIC ACID ANALOGS OF ACYL TRIPEPTIDES.

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Introduction

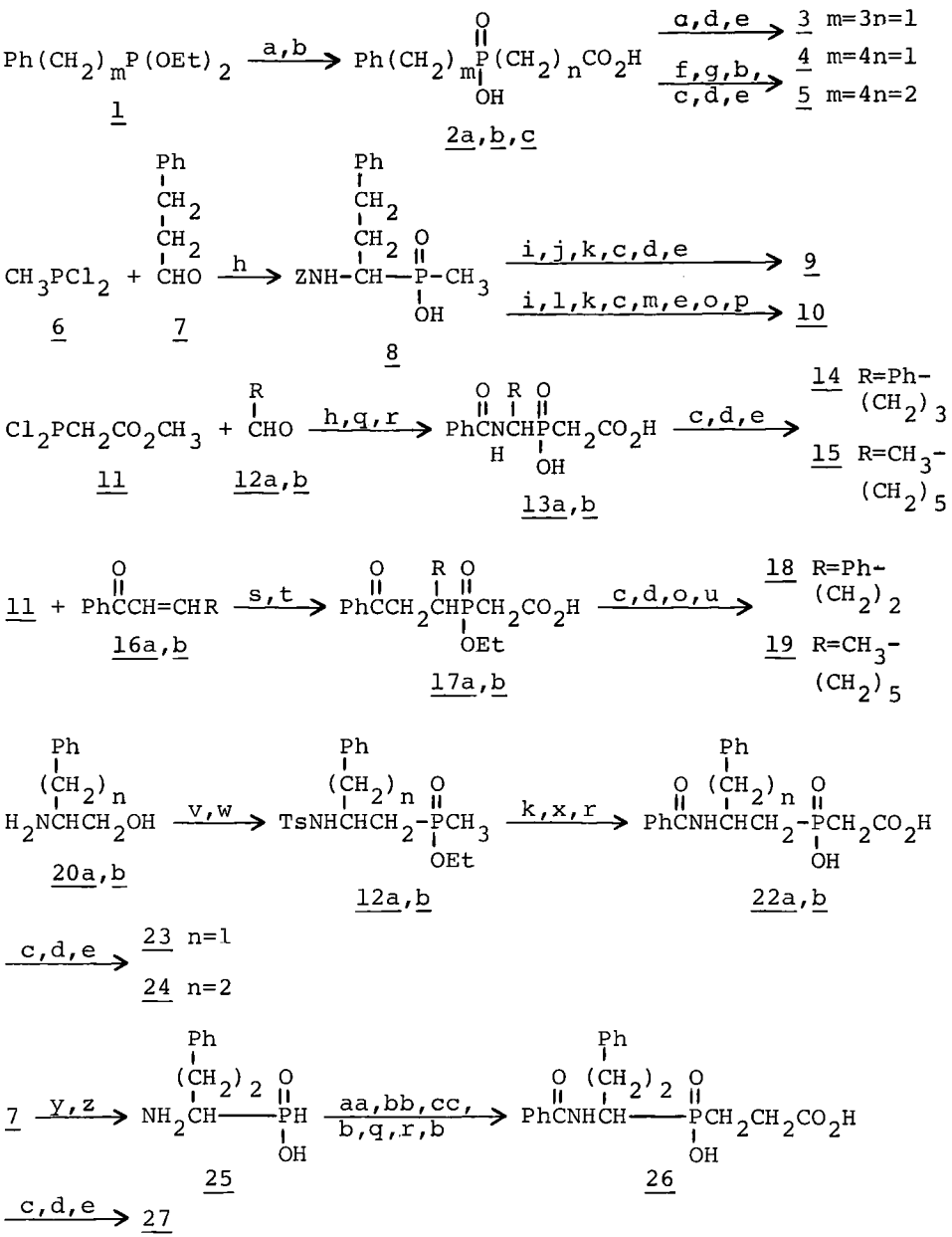
We have developed a novel series of phosphinic acids as potent inhibitors of angiotensin-converting enzyme (ACE).[1] These compounds achieve tight binding to ACE due to the presence of a strong ligand for the active site zinc ion as well as features which mimic the binding of acyl tripeptides. In addition, we will report the extension of this concept to phosphonate and phosphonamidate ACE inhibitors.

Results

Synthesis of Phosphinic Acid Acyl Tripeptide Analogs

Scheme 1 outlines the synthesis of the compounds listed in Table I. Except in the case of 10, the phosphinic acid side chains were fully elaborated before coupling to proline esters. Compounds 2, 13, 22 and 26 were coupled with the phosphinic acid groups unprotected. The precursors of 9, 10, 14 and 15 were prepared by a new method involving reaction of phosphines such as 6 or 11 with aldehydes and benzyl carbamate.[2] Ketophosphinic acids 17a,b were prepared by conjugate addition of 11 to enones 16a,b. The optically active β-aminoalkyl phosphinic acids 21a,b were prepared by a new

Scheme 1.



Notes: See Scheme 2.

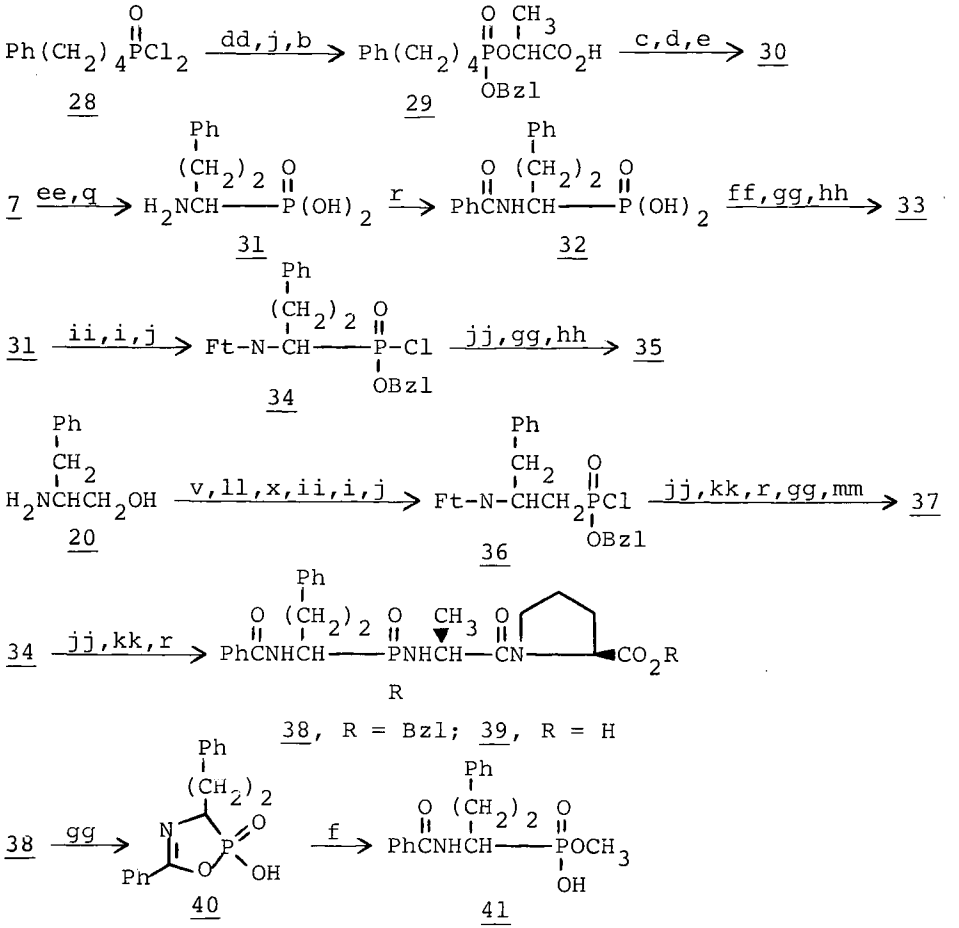
reaction of phosphinates with aminoalcohol N,O-ditosylates derived from amino acids.[3] The side chain for 27 was constructed by conjugate addition of 25 to methyl acrylate. All compounds were fully characterized by spectral and elemental analyses and were generally tested as dilithium salts. Compounds 9, 10, 14, 18, 15, 19 and 27 were tested as 1:1 mixtures of epimers at the side chain asymmetric center; however, a single diastereomer of 10 could be isolated due to fortuitous crystallization of an intermediate in its synthesis. The diastereomeric mixtures of compounds 23 and 24 were also prepared and tested for comparison with the (S) diastereomers shown.

Scheme 2 shows the synthesis of phosphonate and phosphonamidate analogs of acyl tripeptides. With the exception of 30, these compounds were prepared by phosphonylation of a dipeptide[4] or hydroxyacyl amino acid. Phosphonic acid 32 reacts with DCC to give a reactive intermediate, probably 40, which rapidly phosphonylates alcohols; this reaction fails with the phthaloyl derivative of 31. Compound 35 is stable at neutral pH, but 37 suffers P-N bond cleavage at pH <9 and must be isolated in the presence of 1 mole LiOH. Its half-life at the assay pH (8.3) is 1 hour. When 38 was hydrogenated under conditions which allowed isolation of 37, α-benzamido derivative 39 could not be isolated. In methanol, hydrogenation of 38 produces 41. This result implicates oxaphosphazole 40, formed by intramolecular participation of the benzamido group, as an intermediate in the decomposition. This interpretation is consistent with the observed order of stability, 35>>37>> 39, since the phthalimido group of 35 cannot participate in assisted decomposition and the 6-membered ring intermediate analogous to 40 would be expected to form more slowly in the breakdown of 37.

Angiotensin-converting Enzyme Inhibition

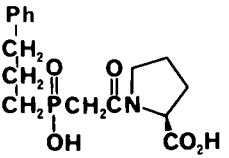
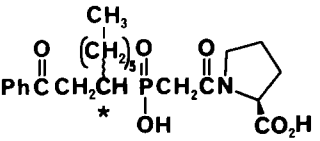
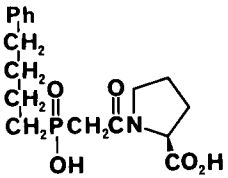
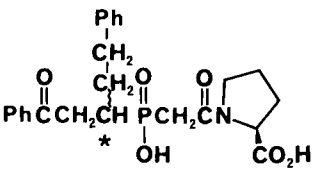
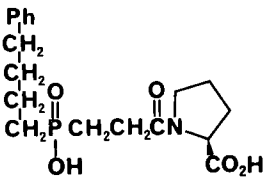
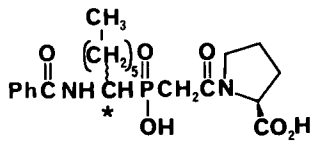
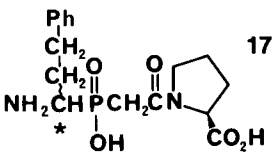
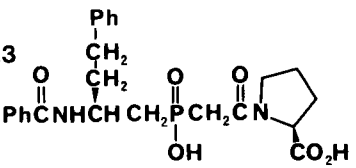
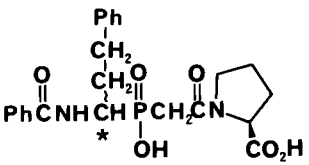
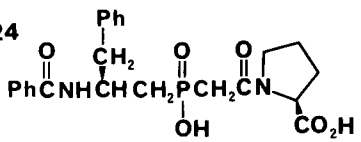
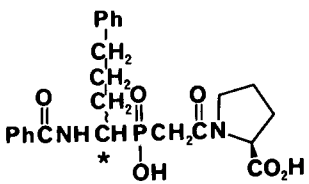
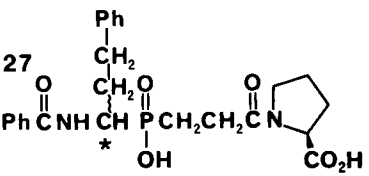
Table I gives the concentrations of test compounds which produce 50% inhibition of the hydrolysis of hippuryl histidyl

Scheme 2.



Notes to Schemes 1 and 2. Ft=phthaloyl; Bzl=$PhCH_2$-; Z=$PhCH_2$-OCO; a-$Br(CH_2)_nCO_2Et$; b-NaOH; c-carbonyldiimidazole; d-Pro-OBzl; e-H_2,Pd/C; f-CH_3OH; g-$PhCH_2N{=}NNHTol$; h-ZNH_2, $tBuCO_2H$; i-BSA, PCl_5; j-BzlOH, Et_3N; k-LDA, CO_2; l-EtOH, Et_3N; m-Pro-OtBu; n-$PhCO_2H$, DCC, HOBT; o-Me_3SiBr; p-TFA; q-6N HCl; r-PhCOCl, base; s-Ac_2O, EtOH; t-HCl, AcOH; u-HBr, AcOH; v-TsCl, py; w-$NaPO(OEt)CH_3$; x-9N HBr, phenol; y-Ph_2CHNH_2, H_3PO_2; z-17N HBr; aa-Z-Cl, NaOH; bb-EtOCOCl, NEt_3; cc-$NaOCH_3$,$CH_2{=}CH-CO_2Me$; dd-$HOCH(CH_3)CO_2CH_3$; ee-PCl_3, Z-NH_2, $tBuCO_2H$; ff-DCC, L-Lac-ProOBzl; gg-H_2,Pd/C, Et_3N; hh-Ag-50 (Li^+); ii-Ft-anhyd.; jj-Ala-ProOBzl, Et_3N; kk-H_2NNH_2; ll-$NaPO(OEt)_2$; mm-LiOH.

Table 1. Inhibition of ACE by Phosphinic Acids.

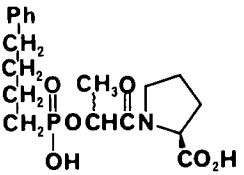
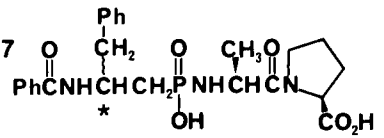
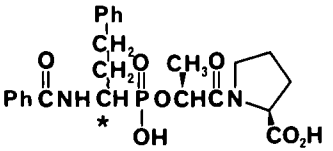
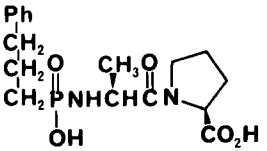
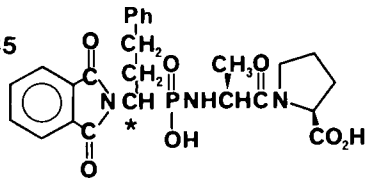
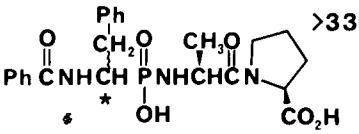
No.		I_{50}(nM)	No.		I_{50}(nM)
3		530	15		33
4		180	18		910
5		1700	19		76
9		170000	23		12[b]
10		73[a]	24		10[c]
14		31	27		50

Notes: * indicates 1:1 epimer mixture; a - I_{50} = 770 for single epimer of unknown configuration; b - I_{50} = 27 for R,S-mixture; c - I_{50} = 20 for R,S-mixture.

leucine by ACE.[5] As we reported previously,[1] the activities of simple phosphinic acids 3, 4 and 5 show that the phosphinyl acetyl compound 4 is about 10-fold more potent than the propanoyl analog 5, and that the phenylpropyl moiety of 3 binds less well than the phenylbutyl group of 4. The poor activity of compound 9 shows that an amino function α to the phosphinic acid moiety is not tolerated. However, the α-benzamido compounds 10 and 14 show increased activity relative to their parent compounds 3 and 4. The diminished activity of a single diastereomer 10 (not shown) indicates the importance of the proper configuration at the side chain asymmetric center. The substitution of an aliphatic side chain (15) for the phenylalkyl group leads to potent inhibition. Comparison of the ketone 18 with its amide isostere 10 shows a 12-fold loss of activity resulting from CH_2 for NH substitution, but the corresponding change in 19 *vs.* 15 shows only a 2-fold loss of activity. Compound 23 is an isomer of 10 in which the benzamido group is displaced β to the phosphinic acid; inhibitory potency increases, and the active diastereomer is found to have the S-configuration corresponding to the natural peptide stereochemistry. Similarly, compound 24 is more active than its isomer 14. Phosphinyl propanoyl derivative 27 is an isomer of 24 in which the phosphinic acid group and the adjacent methylene have been transposed, but inhibitory activity is diminished only slightly.

In Table II the effect of adding benzamido groups to phosphonate and phosphonamidate inhibitors is shown. The simple phosphonate ester 30 inhibits ACE with about twice the potency of its phosphinic acid isostere, but not as well as the corresponding phosphonamidate.[6] The increased activity of compound 33 relative to 30 can be attributed to the presence of the α-benzamido group, but the phenylalkyl groups of 30 and 33 differ in length, so the magnitude of the effect is hard to judge. Phosphonamidate 42 is a potent inhibitor of ACE, and its β-benzamido derivative 37 appears to be slightly less

Table II. Phosphonate and Phosphonamidate ACE Inhibitors.

No.		I_{50} (nM)	No.		I_{50} (nM)
30		94	37		36
33		12	42		15[a]
35		61	43		>33000[b]

Notes: a-10 nM (Ref. 4); b-25% inhibition at 33 μM (Ref. 4).

active. However, the true affinity of 37 for ACE may be masked by the moderate instability of the compound as well as its diastereomeric composition. The α-phthalimido derivative 35 is about 4 times less potent than 42, indicating that the presence of the phthalimido group does not have a marked effect on binding to ACE. We were unable to measure the inhibitory activity of α-benzamido derivative 39 due to its rapid decomposition. Thorsett *et al.*[4] report very poor ACE inhibition by 43, but this result may reflect decomposition before or during the assay procedure rather than poor binding to the enzyme.

Discussion.

Our results show that the phosphinic acid and phosphonate inhibitors with benzamido substituents bind more tightly to ACE than the corresponding compounds without these substituents. The figure schematically depicts the binding of an idealized peptide and inhibitors 23 and 33 to the ACE active site. Part of the binding affinity of both compounds is due to previously studied interactions involving the acyl proline moiety.[1] In addition, each inhibitor benefits from the hydrophobic interaction of the phenylalkyl group with the S_1 subsite and the binding of the zinc ion to the phosphinate or phosphonate ligand. The phosphonate ester oxygen of 33 may

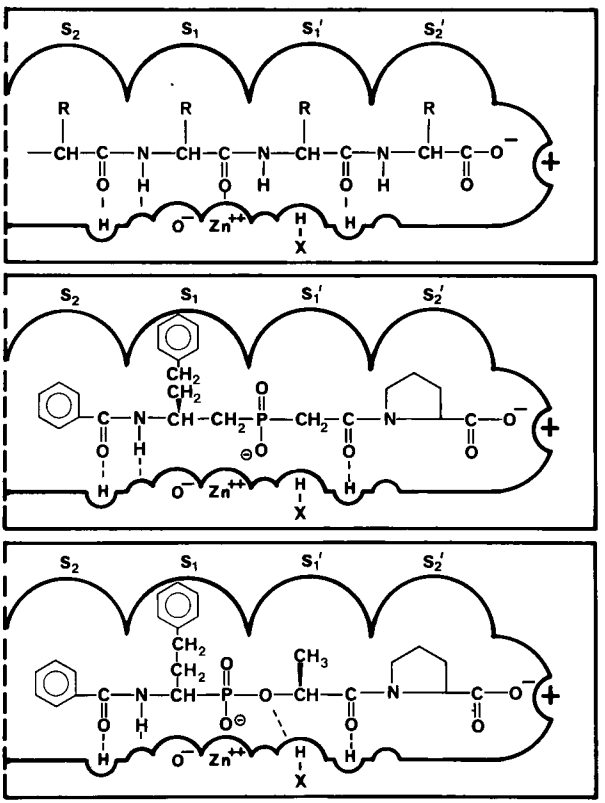


Figure. Schematic of ACE inhibitor binding.

also contribute to binding. Phosphonate 30 is more active than its CH_2 isostere[1] but less active than its N-H isostere,[6] which indicates that the oxygen can partly mimic the phosphonamidate NH. This situation is consistent with the NH acting as a hydrogen bond acceptor. However, others have attributed the role of H-bond donor to this group.[4,7]

ACE will not hydrolyze a tripeptide lacking a terminal N-acyl group.[8] Also, a terminal benzamido group is essential for optimal ACE inhibition by a ketone tripeptide analog.[9] We suggest that the benzamido group of our inhibitors mimics the terminal acyl group of an acyl tripeptide. The observation that the side chain configuration of the β-benzamido series must match the peptide L-configuration strengthens this conclusion. We picture the benzamido N-H as being involved in a hydrogen bond to an acceptor site on the enzyme, but this interaction may be affected by the nature of the adjacent side chain. The enzyme tolerates some variation in the placement of the phosphinic acid and benzamido functions along the backbone. The structure of 23 and 24 appears to be optimum, but the alternatives represented by 14 and 27 bind nearly as well. Recent reports[4,7] suggest that incorporation of benzamido groups into phosphonyl dipeptides severely disrupts the tight binding of these compounds to ACE. Our results show that the benzamido group of 33 contributes to tighter binding to ACE. The benzamido group of 37 and the phthalimido group of 35 do not appear to enhance binding relative to the parent 42, but their presence is clearly tolerated by the enzyme. Our observation of the instability of 37 and 39 is readily explained by a mechanism for facile hydrolysis assisted by the benzamido group. Facile hydrolysis of a phosphonamide assisted by a neighboring carboxylate was recently observed in a related system.[10] We conclude that the poor activity reported for 43[4] is due to hydrolysis before or during assay, rather than to poor binding to the enzmye. A similar explanation probably applies to the poor activity of the phosphonyl analog of hippuryl-Ala-Pro reported by Galardy *et al.*[7]

We have demonstrated that incorporation of the benzamido group into simple phosphinic acids leads to potent inhibitors of ACE which are similar in potency to other classes of inhibitors such as mercaptoacyl amino acids, carboxylalkyl dipeptides and peptide ketone analogs. This strategy is ineffective when applied to phosphonyl dipeptides due to instability of the target compound, but leads to a potent inhibitor in the phosphonate series.

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BICYCLIC INHIBITORS OF ANGIOTENSIN-CONVERTING ENZYME

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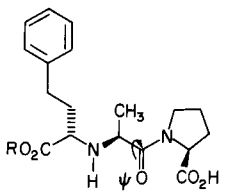
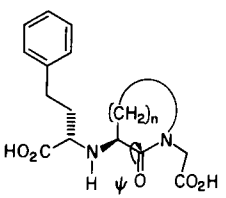
During the last decade much effort has been directed toward the discovery of inhibitors of the zinc metalloenzyme, angiotensin-converting enzyme (ACE).[1] MK-422 (1b) is an extremely potent inhibitor of this enzyme which has good oral activity as its monoethyl ester 1a (MK-421, enalapril).[2] Recently, Thorsett et al. have established that 7-, 8-, and 9-membered monocyclic lactams (2) are excellent mimics of the L-alanyl-L-proline unit in inhibitors of ACE (Figure 1).[3] In addition to establishing that MK-422 binds to ACE in the trans peptide rotamer, this work illustrated the importance of the ψ angle (Figure 1) in inhibitors of this enzyme. This work evolved from the concept that bicyclic lactams would be conformationally rigid mimics of L-alanyl-L-proline.



1 a) R = C_2H_5, MK-421
b) R = H, MK-422



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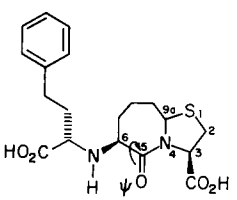

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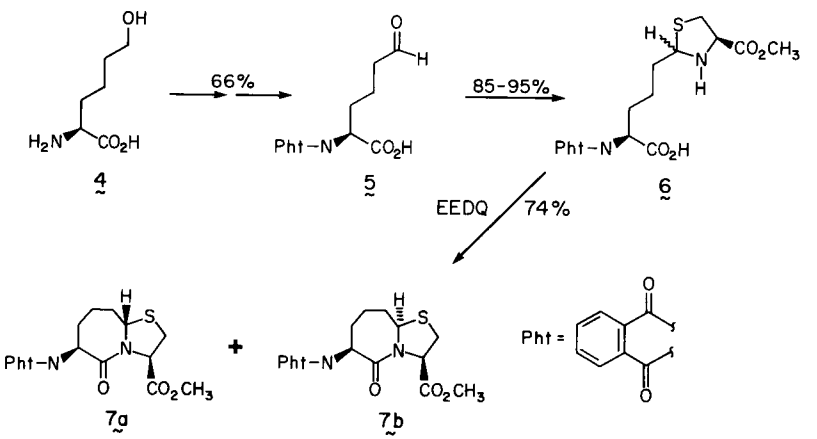
Fig. 1

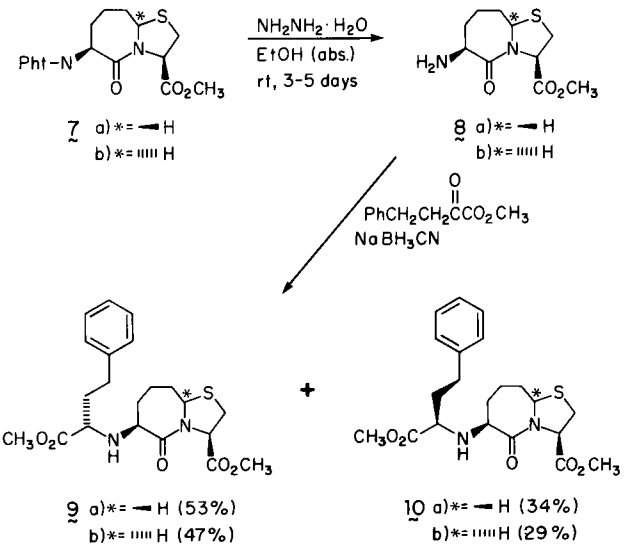
To test this proposal, bicyclo[5.3.0]decane derivative 3 (Figure 1) appeared synthetically attractive. The near equivalency of L-4-thiaproline and L-proline in inhibitors of ACE has previously been established.[1,2] The most challenging aspect of preparing molecules of this type is the control of stereochemistry. In accordance with our design, (R) and (S) configurations are required at $C_{(3)}$ and $C_{(6)}$, respectively. This is accomplished by incorporating (R)-cysteine and (S)-2-amino-6-hydroxyhexanoic acid (4)[4] into our synthesis as depicted in Scheme 1.

(S)-2-amino-6-hydroxyhexanoic acid (4) is protected as its N-phthaloyl benzyl ester derivative. Oxidation of this intermediate with pyridinium chlorochromate followed by hydrogenolysis of the benzyl ester gave the requisite aldehydic acid 5. Condensation of 5 with (R)-cysteine, methyl ester afforded a 2:1 mixture of diastereomeric thiazolidines 6. Cyclization is effected with EEDQ to give a 1:1 mixture of bicyclic derivatives 7a and 7b. The diastereomers are separated on a reverse phase column and the stereochemistry for 7b established by x-ray crystallography.

The phthaloyl group in 7 is removed with hydrazine under mild conditions to give 8 (Scheme 2). Aminoesters 8a and 8b



Scheme 1

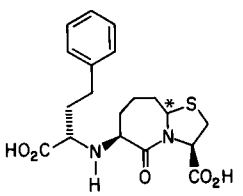


Scheme 2

are then reductively alkylated with excess methyl 2-oxo-4-phenylbutyrate in the presence of sodium cyanoborohydride. The resulting diastereomers 9 and 10 are separated by silica gel chromatography. The four diastereomeric diesters are individually hydrolyzed to the corresponding diacids and then tested *in vitro* for their ACE inhibitory activity. The stereochemical assignment of the phenethyl side chain in 9 and 10 are made (vide infra) on the basis that greater inhibitory activity resides in the (S) isomer in inhibitors of this type.[2] This assignment has been confirmed for 11b by x-ray crystal structure analysis.

The *in vitro* ACE inhibitory results (I_{50}'s) for the four diastereomeric diacids are illustrated in Table I. Potent inhibitors (11a and 11b) are obtained with both bicyclic ring systems. However, bicyclic inhibitor 11b with I_{50} of 6.0×10^{-10}M is particularly noteworthy when compared to MK-422 ($I_{50} = 1.2 \times 10^{-9}$M). The binding affinity of 11b for rabbit angiotensin-converting enzyme has been determined by competitive equilibrium dialysis against MK-422 ($K_i = 2.1 \times 10^{-10}$M). The measured K_i value of 7.6×10^{-11}M at pH 7.5 makes 11b the

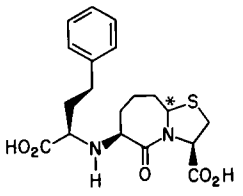
Table I



11

a) * = ◄ H 4.0×10⁻⁹ M

b) * = ····· H 6.0×10⁻¹⁰ M



12

a) * = ◄ H 1.6×10⁻⁷ M

b) * = ····· H 1.8×10⁻⁸ M

most potent inhibitor of ACE yet reported.

This high level of potency for 11b encourages us to suggest that the angles and molecular volume defined by 11b (ψ = 168°) are a good approximation of the S_1' and S_2' subsite geometries in angiotensin-converting enzyme. The promising *in vivo* activities of some of these compounds will be separately reported.

Acknowledgments: The authors thank Dr. E. D. Thorsett for his encouragement, Dr. E. H. Ulm and B. L. LaMont for I_{50} determinations, and Dr. H. G. Bull for the enzyme kinetics.

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CONFORMATIONALLY RESTRICTED INHIBITORS OF ANGIOTENSIN CONVERTING ENZYME

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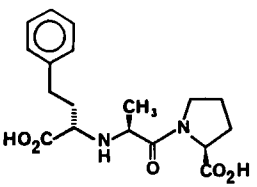
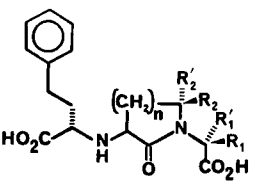
The design of non-peptide drugs derived from bioactive peptides is one of the great challenges of medicinal chemistry. Our approach to this problem involved the use of conformational analysis to explore the structural properties of substrates and inhibitors of angiotensin converting enzyme (ACE). Initial calculations were based on the inhibitor Phe-Ala-Pro and later the X-ray structure of MK-422(1) as well as known structure activity data for ACE inhibition.[1] The results of these studies suggested that we could take advantage of the inherent conformational restraint of the Ala-Pro subunit (2a) to design new inhibitor structures having the potential to provide additional information about the ACE active site.



1



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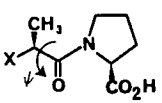
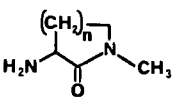


2 a. X = NH_2
b. X = CH_2SH



4

The new class of inhibitors arising from our studies is characterized by a medium sized lactam ring acting as a trans-dipeptide mimic (3). The lactam ring is formed by insertion of a variable length polymethylene bridge between the methyl group and the proline C-5 position. The conformational result is to restrict the distribution of available low energy ψ-values. For synthetic expediency we chose simple lactams (3, all R=H) for our initial studies. The lactam approach to peptide conformational restraint has been independently developed by Freidinger.[2] The general validity of this approach for ACE inhibitors modeled after captopril(2b) was reported by us in a preliminary communication.[3] The work on derivatives of 2b confirmed our prediction that inhibitor potency would be greatest for 7 and larger membered lactams.

Since 1 is so potent ($K_i=2x10^{-10}$M) we assumed it was bound to ACE in or near its low energy conformation. MM2 calculations gave $\psi=140^{o}$ for 1 in its lowest energy form while X-ray data gave $\psi=143^{o}$. To more fully evaluate the structure-activity relationships for 3 with respect to our assumed binding conformation for 1 we explored the conformational characteristics of the lactam inhibitors. We used simplified lactam models 4 for our conformational calculations within the MM2 framework. Table I summarizes the computational results for 4 and gives a partial distribution of available low energy ψ values above the minimum energy for each ring size. Though two or more conformations are likely for each ring system, the population distribution indicates that the ψ angle is constrained to a narrow region (e.g. 8a,b; 9a,c).

A correlation of inhibitor potencies (I_{50}) for lactam inhibitors 3 with the lowest energy ψ values of the model lactams 4 is suggested from inspection of Table I. However, the range of low energy ψ values for the 7-9 member lactam inhibitors, the potencies of which are quite close, leads to the reasonable conclusion that a window of ψ angles from ca 130-170^{o} is consistant with tight binding of the inhibitor to ACE.

Table I. Low Energy ψ-Angles for Lactams 4 and ACE-Inhibition by Lactams 3 (R=H)

Ring size	n	ψ(deg)	Erel[a]	%[b]	I_{50}[c](nM)
5a	1	-132	0.0	52.0	5,300
5b	1	-106	0.04	48.0	
6a	2	-138	0.0	88.4	430
6b	2	-111	1.2	11.6	
7a	3	166	0.0	75.0	19
7b	3	-72	0.7	23.0	
8a	4	145	0.0	69.4	4.8
8b	4	144	0.5	29.8	
9a	5	135	0.0	42.5	8.1
9b	5	-64	0.6	15.4	
9c	5	143	0.8	11.0	
9d	5	116	0.9	9.3	
1	-	140	-	-	1.2[d]

a Comparison of Erel is valid only within a given ring size

b Populations were calculated from the descrete Boltzmann distribution function: Pi=1/Σexp[(Ei-Ej)/RT]as the mole ratio.

c All values are for racemic lactam inhibitors 3.

d Value for all-S isomer

The introduction of R_1 and R_2 substituents into 3 was found to lead to changes in potencies. These substituent effects were explored most thoroughly for the 7-membered lactams using methyl groups (Table II). Inhibitor potency is quite sensitive to R_1 stereochemistry. The S-isomer enhances potency while its epimer decreases activity relative to the parent lactam. This is consistent with stereochemical requirements of ACE for the terminal carboxyl group of substrates and inhibitors.[1] Substitution at the R_2 and R_2' positions enhances potency regardless of stereochemistry. Furthermore, there is only a modest I_{50} difference between the epimers. The lack of stereochemical preference at the R_2 position is indicative of spatial tolerance in the ACE active site. Our computations

Table II. R_1 and R_2 Substituent Effects on 3 (n=3)

R_1	R_1'	R_2	R_2'	I_{50}(nM)
H	H	H	H	19 (ψ=166°)
CH_3	H	H	H	8
H	CH_3	H	H	57
H	H	CH_3	H	3 (ψ=174°)
H	H	H	CH_3	7 (ψ=165°)

show that the orientation of the methyl group does not substantially affect the ψ-angle in the lowest energy conformation (Table II).

Our results confirm that a distinct conformational element exists in the binding of inhibitors to ACE which we can relate to the ψ-angle of 2. In addition, these results along with those of Freidinger and coworkers[2] suggest that lactams can play an important role in the design of conformationally restrained peptides and peptide mimics.

Acknowledgements: We thank Dr. Edgar H. Ulm and his staff for providing I_{50} determinations and Dr. Roger Freidinger for helpful discussions during the early course of this work.

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THE DESIGN OF SUBSTRATE ANALOG RENIN INHIBITORS

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Introduction

Renin (EC 3.4.99.19) is an aspartic proteinase elaborated by the juxtaglomerular cells of the kidney and released into the venous circulation in response to a variety of stimuli[1]. The enzyme cleaves circulating angiotensinogen to generate angiotensin I which is transformed into biologically active angiotensin II. This peptide maintains central blood pressure both by constriction of vascular smooth muscle and regulation of fluid balance (Figure 1). Both direct and indirect effects are involved in these processes.

Like other highly specific proteases,[2] renin binds synthetic peptides which are homologous with the amino acid sequence of the naturally occurring substrate around the site of cleavage[3]. These substrate analogs are capable of inhibiting the enzyme both in vitro and in vivo[4].

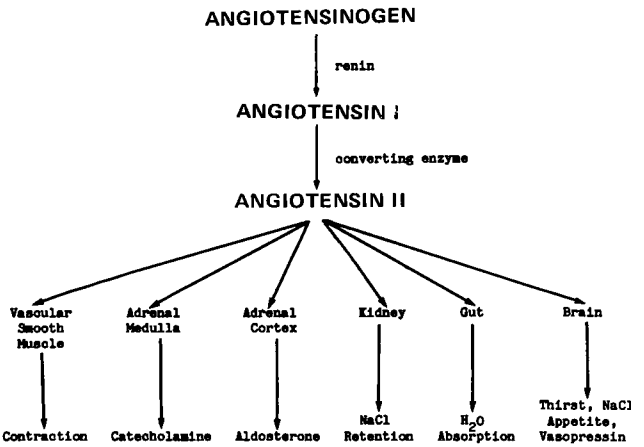

Fig. 1. Functions of angiotensin II.

The octapeptide sequence His-Pro-Phe-His-Leu-Leu-Val-Tyr, found between positions 6-13 of equine angiotensinogen, was shown by L. Skeggs and co-workers to be the minimal sequence efficiently cleaved by renin. Deletion of either the N-terminal histidyl or C-terminal tyrosyl residue from this peptide markedly decreased enzyme specificity (V_{max}/K_M). The octapeptide (RI-5) is relatively insoluble at physiologic pH and binds renin at least an order of magnitude more poorly than angiotensinogen. Conversion of this sequence into a useful renin inhibitor requires that solubility of the peptide be increased, affinity for renin improved, and circulating half-life extended. A program of specific modifications aimed at imparting these properties to the octapeptide was undertaken.

Development of an effective renin inhibitor involved the synthesis of numerous analogs of the octapeptide. Several of these are listed in Figure 2.

		K_I (μM)	Solubility (μM)	Half-life (sec)	Specificity (Renin/ACE)
RI-5:	His-Pro-Phe-His-Leu-Leu-Val-Tyr	40*	160	---	---
RI-19:	Pro ———————————————	39	320	---	---
RI-56:	Pro ———————— Phe-Phe ————	1	100	<15	---
RI-68:	Pro ———————— Phe-Phe ———— Lys	2	840	225	15
RI-71:	Pro ———————— Phe-Phe ———— D-Lys	<1	330	300	300

*pH 5.5

Fig. 2. Key peptides prepared during development of substrate analog renin inhibitors.

Addition of prolyl residues to the N-terminus increases solubility of the octapeptide (RI-19). Other modifications which increase solubility were developed but proline addition is the only change synergistic with substitutions which increase affinity for renin. Other solubility enhancing substitutions, such as incorporation of hydrophilic amino acid residues, decrease inhibition of the enzyme by the peptide.

Affinity for renin was increased by replacing the leucyl residues of the enzyme with more lipophilic aromatic analogs such as phenylalanine (RI-56). A quantitative relationship between lipophilicity and inhibitory properties was shown to exist in the center of the octapeptide. Replacement of the tyrosyl residue at position 3 with either more hydrophobic or hydrophilic residues however, decreases inhibitory properties of the peptide. Similar substitutions at the C-terminal tyrosyl residue do not change binding of the peptide to renin. A quantitative relationship between lipophilicity and inhibitory properties exists only at the putative cleavage site of the substrate analog[6].

Addition of a lysyl residue to the C-terminus of the peptide increases both circulating half-life and solubility of the peptide. The resulting decapeptide Pro-His-Pro-Phe-His-Phe-Phe-Val-Tyr-Lys (RIP, RI-68) has been used for both

in vitro and in vivo inhibiton of renin[5] .

Current Research

For therapeutic relevance, the renin inhibitors should be orally active, have a high affinity, and be specific for renin. Research discussed here is focused on development of an orally active analog of RIP. This, rather than affinity or specificity was chosen as the first objective of the design program. Little is known about properties which enhance absorption of peptides; until these are defined, modifications designed to increase affinity or improve specificty may be incompatible with oral activity.

Research from several groups[7] demonstrated that the passage of small neutral molecules across living membranes from a variety of tissues is inversely proportional to the molecular weight (Mol. Wt.) and directly proportional to the lipophilicity (P) of the material. The equation:

Eq. 1. $\text{Transport} = \text{Transport}_{std} \, (P^a/\text{Mol. Wt.}^b)$

relates these properties to the rate of passsage and provides objective goals for the design of renin inhibitors.

Improving the rate at which RIP analogs cross mucosal surfaces requires that the size of the peptide be decreased and lipophilicity increased.

Size Reduction:

Two advantages arise from the size reduction of the inhibitors. First smaller peptides should pass through mucosal surfaces more rapidly. Second, the shorter peptides contain reduced number of sites at which they may be inactivated by proteolytic enzymes. Fewer modifications are needed to make the peptides resistant to proteolytic degradation. Both Kokubo and colleagues[8] and Johnson[9] synthesized tetrapeptides based on the sequence Leu-Leu-Val-Tyr and showed that deletion of the N-terminal four residues reduced affinity for renin between two to three orders of magnitude. The C-terminal pentapeptide amide from RIP (Phe-Phe-Val-Tyr-Lys-NH_2, RI-77) has an IC_{50} value equal to about 2% that of the parent decapeptide. This peptide should diffuse across mucosal surfaces about three times as rapidly as RIP.

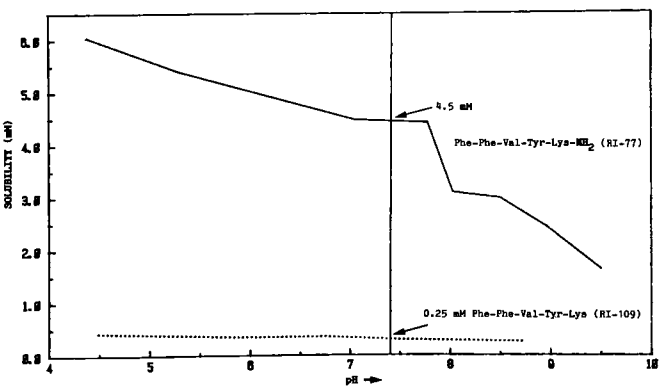

Fig. 3. Solubility of the C-terminal pentapeptide from RIP (RI-109) and the pentapeptide amide (RI-77).

One problem with size reduction is that the short substrate analogs are frequently insoluble and thus difficult to purify and test. Replacement of the C-terminal carboxyl with a carboxamide residue leads to an eighteen fold increase in solubility of the peptide at physiologic pH with little change in inhibitory properties.

Variations in Lipophilicity

The rate of passage across biologic membranes is directly proportional to the lipophilicity of a compound (log P) raised to the 1.3 power. Variations in lipophilicity probably offer the best means of controlling the rate at which peptides cross mucosal surfaces. A series of analogs in which the side chains of the first four residues were systematically modified to contain either a more hydrophilic (-OH) or more hydrophobic (-Cl) substituent were prepared. The effect of these substitutions on lipophilicity of the pentapeptides is shown in Figure 4.

OH:	-0.13		-0.04		-0.31			
H:	Phe	-	Phe	-	Val	-	Tyr	- Lys - NH_2
Cl:	+0.50		+0.48		-0.16		+0.36 (H)	
							+0.89 (Cl)	

Fig. 4. Effect of various substitutions on the lipophilicity of the pentapeptide amides.

Difference in log P between the most hydrophilic analog (Phe-Phe-Thr-Tyr-Lys-NH_2) and the most hydrophobic analog (Phe-Phe-Val-Phe(4Cl)-NH_2) is 1.20 (+0.89 - (-0.31)). The most hydrophobic analog should cross mucosal surfaces about fifty times more rapidly than the most hydrophilic analog.

Identical replacements may not change lipophilicity of the peptide to the same degree. Replacement of the -OH with -H in the p-position of the aromatic residue at position 4 increases log P by +0.36. The same substitution at position 1 changes log P of the peptide by only +0.13 units and at position 2 a smaller change of +0.04 is observed. The discrepancy between predicted and observed changes in lipophilicity may be due to changes in the conformation of the peptide. A position dependent difference is not observed when p-chlorophenylalanine is used. In this case log P at positions 1, 2, and 4 changes by +0.50, +0.48, and +0.53 units respectively.

Effect of the various substitutions on inhibitory properties of the substrate analogs is shown in Figure 5.

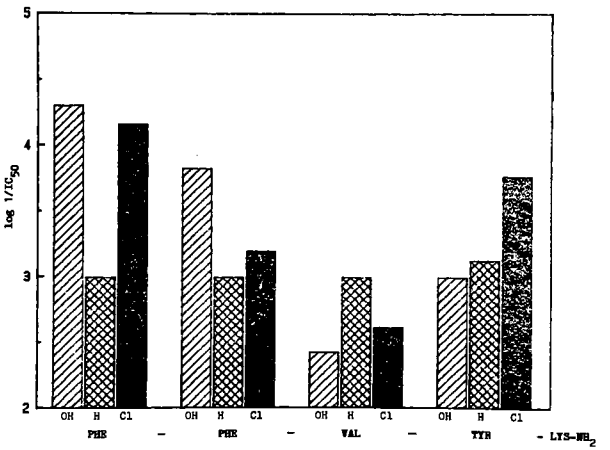

Fig. 5. Effect of various substitutions on log $1/IC_{50}$.

Lipophilicity is directly related to renin inhibition only at position 4 of the pentapeptides. At the first two residues, replacement of H with either OH or Cl improves binding to the enzyme. Both substitutions at position 3 decrease inhibitory properties of the peptides.

Figure 6 shows the effect of inhibitor concentration on enzyme inhibition. One new analog Phe-Phe(4I)-Val-Tyr-Lys-NH_2 (RI-103) inhibits human renin about as well as RIP. The peptide is also a potent hypotensive agent in vivo.

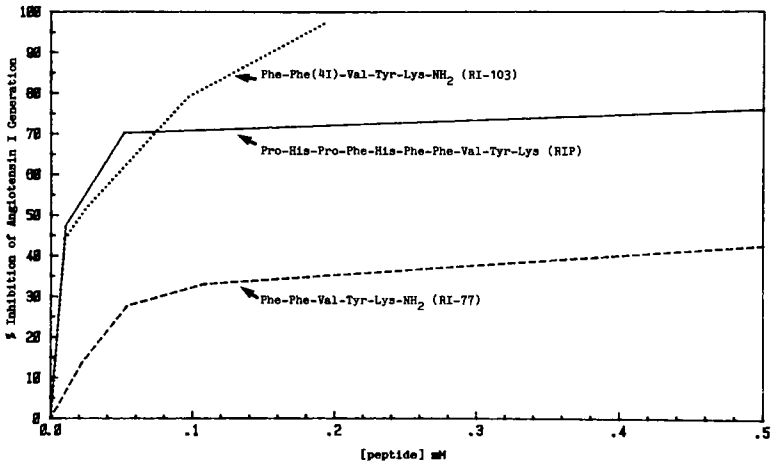

Fig. 6. Inhibition of human renin by various substrate analogs.

In summary, early research led to the design and synthesis of a renin inhibitor which is effective both in a monkey model of renovascular hypertension, and more recently, in man. Conversion of this peptide into a therapeutically relevant renin inhibitor requires that it be made orally active. To accomplish this, size of the substrate analog has been reduced to about one-half, and lipophilicity increased by two orders of magnitude. Affinity of one inhibitor for renin is equal to that of RIP.

Supported by a grant from the Upjohn Company.

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RENIN INHIBITORS. DESIGN OF ANGIOTENSINOGEN TRANSITION-STATE ANALOGS CONTAINING STATINE

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Introduction

The proteolytic enzyme renin cleaves the protein substrate angiotensinogen to yield angiotensin I, the decapeptide substrate transformed by converting enzyme into the octapeptide pressor substance angiotensin II.[1] Interruption of this proteolytic cascade by inhibition of renin could provide a novel approach to the treatment of hypertension.[2]

Species specific inhibitors of human and canine renins have been reported by Burton et al.[3] and by Szelke et al.,[4,5] in which modifications around the cleavage site in the minimum substrate,[6] octapeptide (6-13) **1** (Figure 1), have given competitive inhibitors of reasonable potency (IC_{50} = 10^{-6}-10^{-8}M). The general aspartyl-protease inhibitor pepstatin, isovaleryl-Val-Val-Sta-Ala-Sta **7**, a naturally-occurring pentapeptide containing two units of statine (Sta, see Figure 1), is a strong competitive inhibitor of pepsin (K_I = 4.6 x 10^{-11}M),[7] but a much weaker inhibitor of renins (K_I = 3.9-130 x 10^{-7}M), Table II). It has been proposed that the 3S-hydroxyl of the central Sta residue is an analog for the transition-state or tetrahedral-intermediate of the scissile peptide bond,[8] an hypothesis supported by ^{13}C-NMR studies on pepstatin analogs bound to pepsin[9] and by an x-ray crystallographic structure of pepstatin bound to a bacterial aspartyl protease from Rhizopus chinensis.[10]

PEPTIDE INHIBITORS OF PROTEASES: RENIN

Considering the high substrate specificity exhibited by renin, we postulated that incorporation of statine into a peptide sequence more closely resembling that of angiotensinogen would yield improved inhibitors of renin and have recently reported[11] the development of highly potent (K_I = 10^{-8}-10^{-9}M) inhibitors of human, monkey, dog, hog, cat, and rabbit renins. We describe here computer modeling studies which played a key role in the design of these inhibitors and report additional results in which computer assisted design has led to the syntheses of potent conformationally restricted inhibitors.

Results and Discussion

Our initial hypothesis was that Sta could replace Leu-10 of the substrate sequence (Figure 1). With such a substitution, the analogy between inhibitor and substrate residues can be exact on the N-terminal side of the Sta side chain, but on the C-terminal side the analogy is problematical. For a highly specific endopeptidase like renin, the Sta substitution for Leu-10 would seem to throw subsequent important

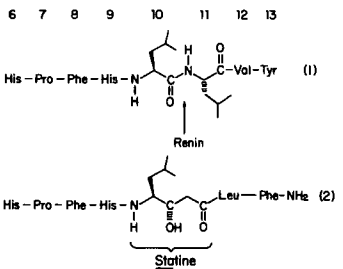

Fig. 1. Analogy between minimum renin substrate, angiotensinogen (6-13) octapeptide **1**, and statine-containing heptapeptide inhibitor **2**.

residues "out of phase". Using the Merck Molecular Modeling System,[12] we investigated how residues appended onto the C-terminus of Sta might model onto important substrate residues. We found that, for some possible conformations of substrate positions 10-13, Sta could function as an approximate dipeptide analog, allowing full restoration of the "phase shift" within two subsequent amino acid residues. The availability of x-ray coordinates for pepstatin 7 in Rhizopus chinensis aspartyl protease, from Bott and colleagues,[10] presented the opportunity to model inversely. We recognized that the details of substrate recognition would differ between renin and the Rhizopus relative, but assumed that the fundamental architectures of the two enzymes and their modes of binding substrates would be similar.

We matched Leu*Leu-Val-Phe onto the pepstatin 7 structure, where the scissile carbonyl of the substrate fragment was converted to a tetrahedral hydroxyl, and the amide bond was lengthened appropriately (= *). Matching of the Leu*10 side chain (α and β) and the once-carbonyl hydroxyl onto the analogous Sta positions can be exact. Of the eight backbone

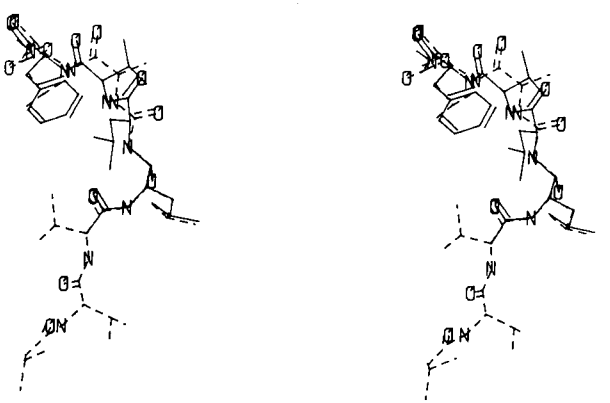

Fig. 2. Ac-Leu*Leu-Val-Phe-NH_2 (solid), where * denotes a tetrahedral carbonyl (-CHOH-NH-), matched onto isovaleryl-Val-Val-Sta-Ala-Sta 7 (dashed, pepstatin) as in the complex with Rhizopus chinensis aspartyl protease.

dihedral angles from Leu*10 to the Phe-13 Ø angle, we allowed six to rotate, freezing only the remaining two amide bonds, and matched the Phe-13 side chain (α and β) onto the C-5 and C-6 carbons of the terminal Sta in pepstatin 7. The resulting fit, with an average deviation of <0.04Å for the six fitted positions, is shown in Figure 2. The fitted substrate-intermediate structure satisfies two stringent and independent tests: 1) it is energetically reasonable; and 2) it can be placed into the Rhizopus enzyme active site without significant unfavorable substrate-enzyme interactions.

This modeling emboldened us to prepare inhibitors in which Sta-Xxx-Phe would be analogous to Leu-Leu-Val-Tyr, substrate positions 10-13. Heptapeptide inhibitors 2 and 3 (Table I) inhibit hog kidney renin with an IC_{50} more than

Table I. Inhibition of Hog Kidney Renin

Number	Structure 6 7 8 9 10 11 12 13	IC_{50}, M
1	His-Pro-Phe-His-Leu-Leu-Val-Tyr	(5.5×10^{-5})*
2	His-Pro-Phe-His-Sta---Leu---Phe-NH_2	2.0×10^{-8}
3	Iva-His-Pro-Phe-His-Sta---Leu---Phe-NH_2	3.1×10^{-8}
4	Ibu-His-Pro-Phe-His-Sta-NH_2	2.9×10^{-5}
5	Boc-Phe-His-Sta---Leu---Phe-NH_2	3.6×10^{-8}
6	Boc-Phe-His-Sta---Leu---Sta	3.6×10^{-8}
7	Iva-Val-Val-Sta---Ala---Sta	1.0×10^{-6}
8	Iva-His-Pro-Phe-His-Sta^{+}--Leu---Phe-NH_2 (3R)	1.4×10^{-5}

Hog kidney renin assay (pH 7.3, 30°C) as described previously.[11] Compounds here and in Tables II-IV characterized by TLC, HPLC, ^{1}H NMR, amino acid analyses, and elemental analyses. Details of syntheses will be reported elsewhere. Iva=isovaleryl; Ibu=iso-butyryl; Sta=statine, (3S,4S)-4-amino-3-hydroxy-6-methyl heptanoyl. *K_M value, ref. 3. $^{+}$3R isomer, Sta isomer with epimeric 3-hydroxyl.

1000-fold lower than the K_M value for the comparable octapeptide substrate **1**. The C-terminal-truncated inhibitor **4** demonstrates the importance of the two residues following Sta. For hog renin, Leu is the optimal "spacer" between Sta-10 and Phe-13, although Ala is tolerated without significant loss in inhibition. For the 13-position in the inhibitor, Phe or Tyr are optimal, and extending further at the C-terminus has no effect on potency (data not shown).

No loss of potency occurs where Boc-Phe replaces His-Pro-Phe in pentapeptide inhibitors such as **5** (Table I). A comparison between pentapeptides **6** and **7** suggests that for pepstatin **7**, the misfit to renin leading to modest inhibition may be due primarily to inappropriate residues at the N-terminus, with the C-terminal ...Ala-Sta of pepstatin being an adequate substitution for Leu-Phe.

Despite the significant species differences in renin enzymes and substrates[13,14] and the strong species specificity exhibited by other reported renin inhibitors,[3-5] we based our inhibitor design on the hog substrate sequence. Since hog angiotensinogen is a good substrate for human and dog

Table II. Species Specificity of Renin Inhibitors

Number	Structure (6 7 8 9 10,11 12 13)	Renin, IC_{50}, 10^{-9}M: Human Kidney*	Human Plasma	Dog Plasma
3	Iva-His-Pro-Phe-His-Sta--Leu-Phe-NH_2	19	16	42
9	Iva-His-Pro-Phe-His-Sta--Ile-Phe-NH_2	1.9	1.9	38
10	Iva-His-Pro-Phe-His-Sta--Ile-Phe-OCH_3	2.5	0.63	13
7	Iva-Val-Val-Sta--Ala-Sta	13000	22000	1300

Human and dog plasma renin assays (pH 7.4, 37°C) and human kidney renin assay (pH 7.2, 37°C) as described in ref. 11. Iva=isovaleryl. *K_I values, 10^{-9}M, consistent with competitive inhibition.

renins (though human substrate is cleaved only by human renin),[14] an inhibitor which closely matched hog substrate structure should be broadly inhibitory for renins. Inhibitor **3** is equipotent against human, dog, and hog renins (Table II and Table I). Enhanced potency versus human renin is gained by an Ile for Leu substitution, as in **9** and **10** (Table II), the most potent reported inhibitors of human renin. Unlike pepstatin **7**, the inhibitors reported here are relatively specific for renins versus other aspartyl proteases such as pepsin and cathepsin D.[11]

Further molecular modeling, based upon inhibitor **2** fit to the pepstatin **7** structure in Rhizopus aspartyl protease (Figure 2), suggested that a cycle could be constructed from the His-9 side chain position toward the inhibitor C-terminus (see Figure 1 and Table III). Modeling of disulphide containing bridges of varying lengths showed allowed cycles of the type Cys-(or Hcy)-Sta-Leu-Phe-NH-CH_2-CH_2-S, while maintaining the analogy established with pepstatin **7**. One such cycle is shown in Figure 3. Preparation of **13** and **14**

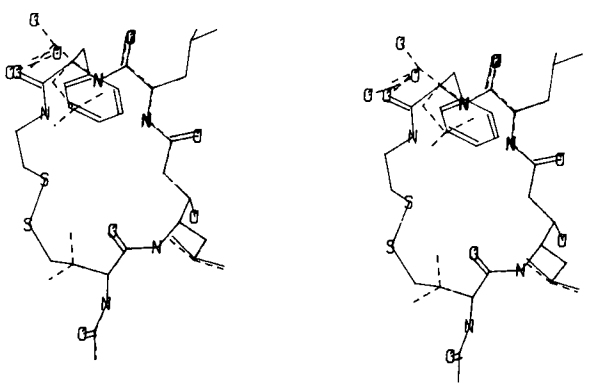

Fig. 3. Ac-Hcy-Sta-Leu-Phe-NH-CH_2-CH_2-S (solid) matched onto Val-Sta-Ala-Sta portion of pepstatin **7** (dashed).

Table III. C-Terminal Cyclic Renin Inhibitors

Number	Structure (6 7 8 9 10,11 12 13)	Inhibition, 10^{-9}M Hog	Human
11	Ibu-His-Pro-Phe-His-Sta---Leu-Phe-NH_2	46	110
12	Ibu-His-Pro-Phe-Abu-Sta---Leu-Phe-NH_2	280	34
13	Ibu-His-Pro-Phe-Cys-Sta---Leu-Phe-N(H)-S (cyclic)	130	380
14	Ibu-His-Pro-Phe-Hcy-Sta---Leu-Phe-N(H)-S (cyclic)	190	200

Hog kidney renin assay (IC_{50}, pH 7.3, 30°C) and human kidney renin assay (K_I, pH 7.2, 37°C) as described in ref. 11. Cyclic inhibitors were characterized additionally by fast atom bombardment mass spectrometry. Abu=L-α-aminobutyryl; Hcy=L-homocysteinyl.

(Table III) confirmed the predicted inhibitory potency, comparable to the linear analog **12**. Testing *in vivo*, in hog-renin-infused anesthetized rats,[11] showed the expected potency for **13** and **14** as blood-pressure lowering agents. Unfortunately duration of action was comparable to **11**.

Previous work from Paiva and colleagues[15] on conformationally restricted renin substrate analogs (for example, **20**, Table IV) has implied a turn structure for the Pro-Phe

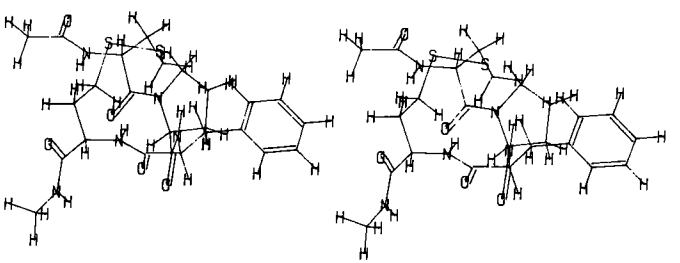

Fig. 4. Ac-Hcy-Pro-Phe-Hcy-NHMe, type I β-turn.

Table IV. N-Terminal Cyclic Renin Inhibitors

Number	Structure (6 7 8 9 10,11 12 13)	Hog Renin IC_{50}, 10^{-9} M
2	His-Pro-Phe-His-Sta-----Leu-Phe-NH_2	20
15	Abu-Pro-Phe-Abu-Sta-----Leu-Phe-OCH_3	24
16	Cys-Pro-Phe-Cys-Sta-----Leu-Phe-OCH_3	2200
17	Cys-Pro-Phe-Hcy-Sta-----Leu-Phe-OCH_3	3100
18	Hcy-Pro-Phe-Cys-Sta-----Leu-Phe-OCH_3	280
19	Hcy-Pro-Phe-Hcy-Sta-----Leu-Phe-OCH_3	49
20*	Cys-His-Pro-Phe-His-Cys-Leu-Val-Tyr-Lys	6900*

Hog kidney renin assay (pH 7.3, 30°C) as described in ref. 11. Cyclic inhibitors were characterized additionally by fast atom bombardment mass spectrometry. *Data from ref. 15; hog kidney renin pH 6.0, 37°C. Abu = L-α-aminobutyryl; Hcy = L-homocysteinyl.

segment in the octapeptide minimum substrate **1** (Figure 1). Modeling suggested that an alternative to a Paiva-type cycle would be a tighter cycle from positions 6 to 9 (see Figure 1 and Table IV). The optimal size for such a cycle was found by modeling to be dependent on turn type and details of the turn structure. General conclusions are that Type I or III turns[16] are best fit with a Cys-Pro-Phe-Cys (abbreviated: Cys-6, Cys-9) or Hcy-6, Hcy-9 bridges (Hcy = L-homocysteine). Type II turns[16] are best fit with Hcy-6, Cys-9 or Cys-6, Hcy-9 bridges. Additionally, the Cys-6, Cys-9 structure is highly restrictive, with significant sulfur to main-chain non-bonded repulsions difficult to avoid. Syntheses of cyclic inhibitors **15-19** (Table IV) confirmed an optimal turn structure for hog and human renins as Hcy-Pro-Phe-Hcy, consistent with Types I or III turns. An example is shown for a Type I turn in Figure 4. The optimal inhibitor **19** was

tested in the hog-renin-infused anesthetized rat model,[11] and found to have no significant increase in duration over **2**.

Matching cycle **19** onto the pepstatin **7** structure in Rhizopus protease reveals a possible match between Phe-8 of the inhibitor and the isovaleryl group of pepstatin, raising the possibility that the isovaleryl group of pepstatin might fulfill the important Phe-8 binding role in the pepstatin inhibition of renin.

The molecular modeling studies described here catalyzed the design of novel, potent inhibitors of renin, and led to conformationally restricted inhibitors helping to define the bound inhibitor (and substrate) conformations. Although starting from a relatively poor inhibitor of renin (pepstatin **7**) in a distantly related enzyme (Rhizopus chinensis aspartyl protease), this approach, in which modeling ideas are tested rapidly by synthesis and biological evaluation, demonstrates considerable power for the design of enzyme inhibitors.

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NOVEL TRANSITION-STATE ANALOGUE INHIBITORS OF RENIN

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Renin is an aspartic proteinase which catalyses the first and rate-limiting step in the conversion of angiotensinogen into the pressor octapeptide angiotensin-II. There is extensive sequence homology and structural similarity between renin and the other aspartic proteinases whose structure

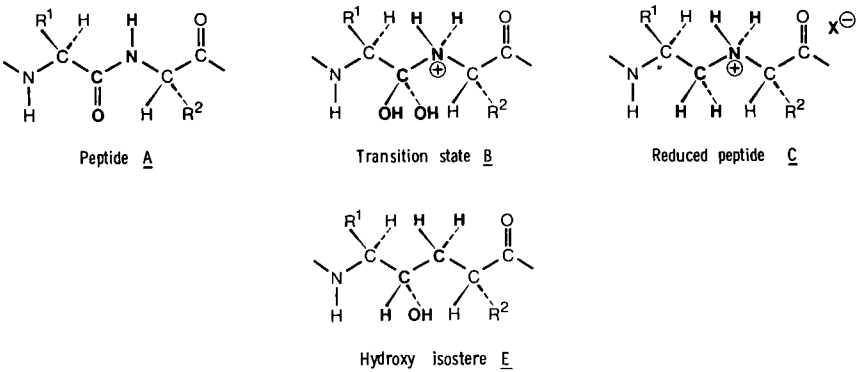
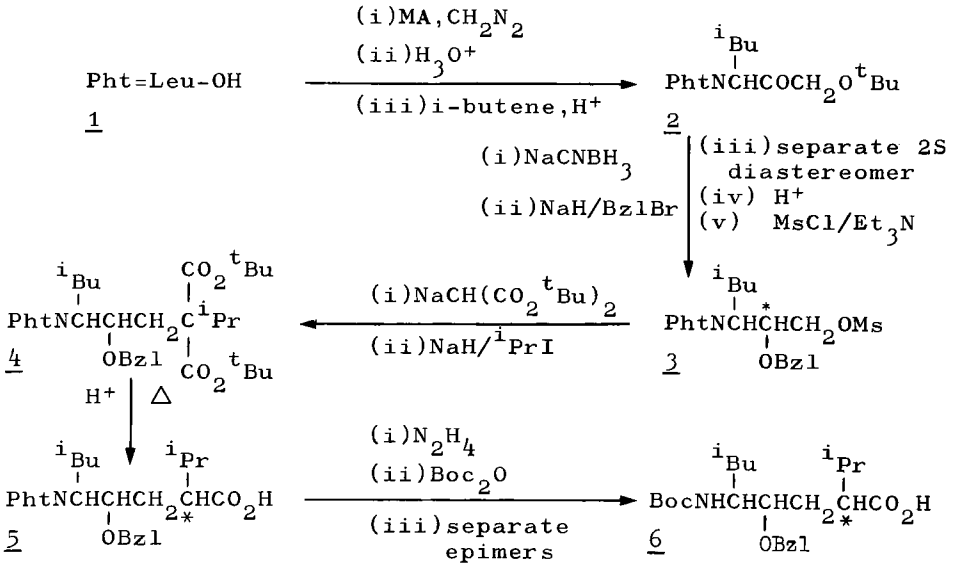


Fig. Structures of transition-state B, reduced isostere C, hydroxy isostere E and statine analogue D of peptide bond A.

and catalytic mechanism has been investigated in detail [1,2]. These studies indicate that the transition-state formed during hydrolysis of the scissile bond has structure B (see Figure), in which the trigonal carbon of the amide carbonyl has been transformed into a tetrahedral one, and the amide -NH- into an immonium ion. Because the enzyme has a much greater binding affinity for the transition-state B than for substrate A, non-hydrolysable analogues of the transition-state are expected to be potent inhibitors of the enzyme [3,4].

Previous work from these laboratories has shown[5] that chemical reduction of the scissile peptide bond to the secondary amine C (Figure) in the minimum substrate sequence 11 (Table) of human angiotensinogen yields highly active inhibitors of human renin (e.g. compound 12 in the Table).

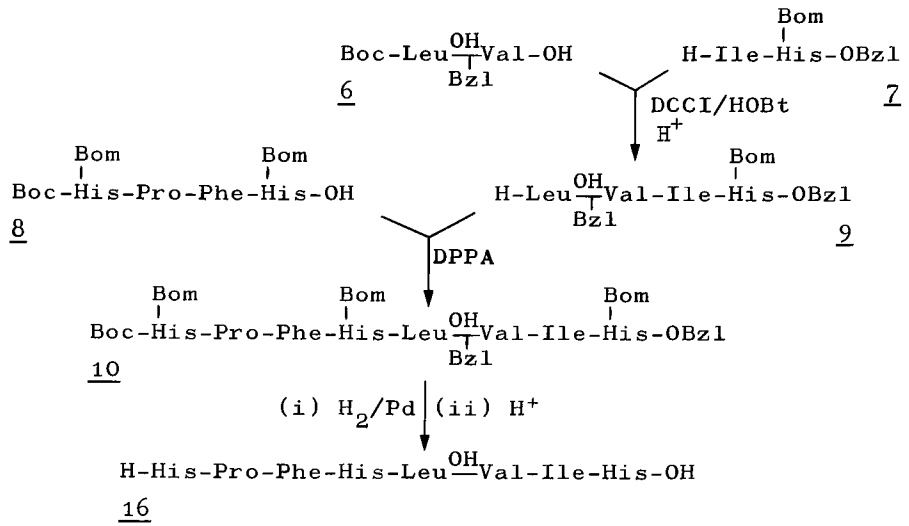
We now describe new transition-state analogues 16 and 17 (Table) in which the scissile bond of the substrate is replaced with the non-basic hydroxy-ethylene group ("hydroxy



Pht=phthaloyl MA=mixed anhydride

*configuration at asymmetric centres in the pure epimers of 3 and 6 was determined by X-ray crystallography[6].

Scheme 1. Synthesis of the protected hydroxy isostere 6



Scheme 2. Synthesis of the transition-state analogue 16

isostere") of structure E (Figure) containing two tetrahedral carbon atoms. Scheme 1 shows the synthesis of the protected Leu—Val hydroxy isostere 6 which was incorporated in place of Leu-Val into the (6-13)octapeptide sequence of human angiotensinogen according to Scheme 2.

The *in vitro* inhibitory potencies against human and canine renin of 16 and 17 are compared with those of the known[5] reduced isostere 12, the novel keto isostere[7] 13 and the statine analogue 14. Clearly, the hydroxy isostere is superior to the other modifications and analogue 17 with $IC_{50}=7\times10^{-10}M$ is the most potent inhibitor of human renin yet reported. We believe that the high potency of hydroxy isosteres reflects their ability to mimic the transition-state very closely. Replacement of Leu in 11 with statine yields a less potent inhibitor 14, presumably because the side-chains (R^1 and R^2 in structure D) are misaligned with the hydrophobic pockets[1] that normally bind the side-chains of the substrate scissile dipeptide sequence A and its transition state B.

Table. Inhibition of Plasma Renin[8] by Analogues 12 - 17

No.	Human angiotensinogen Renin 6 7 8 9 10 | 11 12 13	IC_{50} (nM) human renin	IC_{50} (nM) dog renin
11	H His Pro Phe His Leu ▼ Val Ile His OH	313000	1000000
12	Leu-R-Val	190	155000
13	Leu-K-Val	500	-
14	Sta	17	220
15	Leu-OH-DVal	22000	-
16	Leu-OH-Val	2.7	30
17	Boc Leu-OH-Val	0.7	20

R= $-CH_2-NH-$ K= $-CO-CH_2-$ OH= $-CH(OH)-CH_2-$

In compounds 12 - 17, only variations from the human substrate octapeptide 11 are shown.

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NEW POTENT INHIBITORS OF HUMAN RENIN.

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Renin is a special aspartyl protease, active at neutral pH and specific for a unique substrate, angiotensinogen. It releases angiotensin I which is converted into the pressor hormone angiotensin II by converting enzyme. The recent development of converting enzyme inhibitors has shown that blockade of renin-angiotensin system is an extremely effective treatment for hypertension[1,2]. An alternative to block the renin system is the inhibition of renin. Renin can be inhibited either specifically by modified renin substrates[3,4] or by the non specific aspartyl protease inhibitor, pepstatin[5,6] and its derivatives[7]. Renin substrate analogs are peptides of eight or more residues and are very difficult to protect against proteolysis. Pepstatin is a relatively poor inhibitor of human renin with a K_i in the 10^{-5} molar range when tested on human angiotensinogen at physiological pH[8]. In order to get short, highly potent and specific inhibitors for human renin, we designed to combine the favorable structural features of both pepstatin and substrate analogs.

Pepstatin is a natural peptide having the structure : Iva-Val-Val-Sta-Ala-Sta, where statine (Sta) is (3S,4S)-4-amino-3-hydroxy-6-methylheptanoic acid. Statine 3 has been found to be essential for inhibitory potency of pepstatin on pep-

sin[9]. It has been proposed that this statyl residue is a transition-state analog and mimics the tetrahedral intermediate involved in peptide bond hydrolysis[10]. In view of the strong homology that pepsin and mouse submaxillary renin share in their primary structures, particulary in the regions of their active sites[11], we thought that information about pepstatin inhibiting pepsin could be applicable to renin. Thus we decided to save statin in position 3. Modifications were aimed at the N-terminus of pepstatin by substituting the valines in positions 1 and 2. No attempt was made to modify the C-terminal part, Ala_4-Sta_5. Statine was produced in high yield and large quantities following a procedure which will be reported elsewhere[12,13]. These pepstatin analogs have been tested as inhibitors of human plasma renin activity using a pool of renin-rich human plasmas[8]. Inhibition of porcine pepsin was measured using bovine haemoglobin as substrate[14]. IC_{50} values are given below.

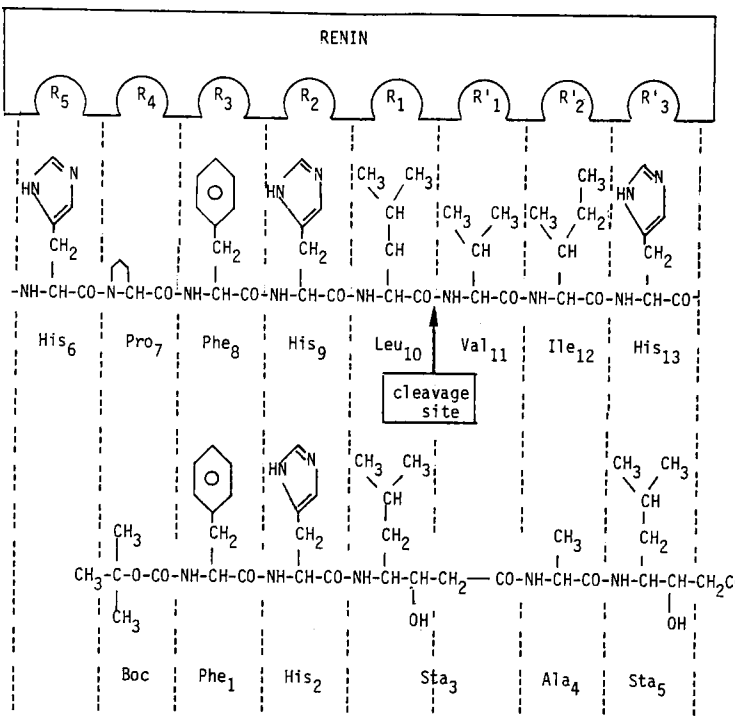
Table. Inhibition of renin and pepsin by pepstatin analogs A-X-Y-Sta-Ala-Sta-OR.

	Pepstatin Analog				$IC_{50}(\mu M)$		
n°	A	X	Y	R	Renin pH6	Renin pH7.4	Pepsin pH2.0
1**	Iva	Val	Val	H	1.4	13.0	0.02
2	Z	Phe	Val	Me	0.01	0.18	0.06
3	Z	Trp	Val	Me	0.0067	0.15	0.029
4	Z	Tyr	Val	Me	0.17	2.2	0.024
5	Boc	dPhe	Val	Me	0.3	10.	0.047
6	Z	Phe	dVal	Me	100	100	1.9
7	Z	Phe	Phe	Me	0.017	3.5	0.03
8	Z	Phe	Phe	H	0.07	1.	0.09
9	Boc	Phe	Phe	Me	0.0035	0.19	0.014
10	Adoc	Phe	Phe	Me	0.045	3.	0.08
11	Z	Tyr	Tyr	Me	2.2	100	0.07
12	Boc	Trp	Trp	Me	0.017	0.15	0.12
13	Z	Phe	Trp	Me	0.08	1.4	0.065
14	Boc	Phe	His	Me	0.0018	0.027	1.3
15	Boc	Phe	His	H	0.0040	0.045	0.55
16	Boc	Trp	His	Me	0.0055	0.14	0.85

** pepstatin

It is clear that the presence of an aromatic residue (Phe or Trp) in position 1 of pepstatin has a key role in potent renin inhibitors. This modification decreased IC_{50} by two orders of magnitude (items 2, 3, 7 to 10, 12 to 16). The most

effective modification to position 2 was replacement of valine by histidine. (items 14 to 16). This substitution produced the most potent inhibitors of the series at physiological pH and also decreased affinity for pepsin. The introduction of D-ami-



Structural comparison of human angiotensinogen (I) and pepstatin analog - Item 14

no acids in position 1 or 2 considerably diminished inhibitory potency. Thus it seems that the residues in positions 1 and 2 occupy special sites in the enzyme active center. Furthermore, the most potent and most specific inhibitor, Boc-Phe-His-Sta-Ala-Sta-OMe, (item 14), shares structural homology with natural renin substrate (Figure). A correspondence between the statine 3 of pepstatin andthe cleavage site of the substrate of aspartyl proteases has already been proposed. From the present study it appears that Phe_1 and His_2 of item 14 occupy the same renin subsites (R_3 and R_2 respectively) as Phe_8 and His_9 of angiotensinogen. At the same time statine 3 iso-

butyl chain which is homologous to substrate Leu_{10} side chain should occupy subsite R_1. Boger et al [15] have recently reported the synthesis of heptapeptide analogs of the pig renin substrate in which the scissile Leu_{10}- Leu_{11} bond was replaced by a statyl residue. These analogs were highly potent renin inhibitors when His_6 and Pro_7 are saved in the structure. From our data, His_6 could be omitted and Pro_7 replaced by a Boc protecting group without significantly loosing inhibitory potency. With IC_{50} in the $10^{-8}M$ range at physiological pH, the new pepstatin analogs reported here constitue the shortest efficient inhibitors for human renin.

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SYNTHESIS OF EPOXYPOLYPEPTIDES AS INHIBITORS OF RENIN

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Introduction

Because of the potential value that inhibitors of renin have as pharmacological tools and as therapeutic agents, there has been an ongoing effort to develop inhibitors of this enzyme. Epoxides such as 1,2-epoxy-3-(p-nitrophenoxy)propane have been shown to inactivate the aspartyl proteases pepsin[1] and renin[2] by specifically labelling one of the aspartic acid residues present within the active site of these enzymes. In an attempt to develop an irreversible epoxide inhibitor that would be specific for renin, epoxypolypeptides 10-12 were synthesized. It was envisioned that by placing an epoxide moiety in a position within peptide fragments of angiotensinogen which corresponds to the amide bond cleaved by renin, the epoxide would be attacked by the carboxyl group of one of the aspartic acid residues involved in renin's catalytic process. This proposed process, which is depicted in Figure 1, would lead to irreversible inhibition of the enzyme.

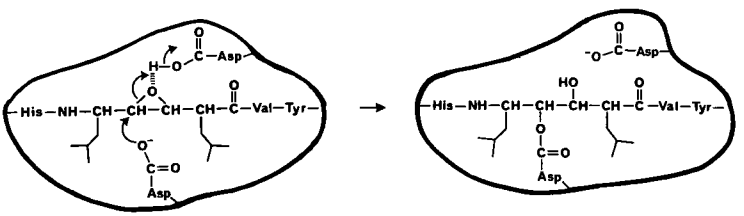
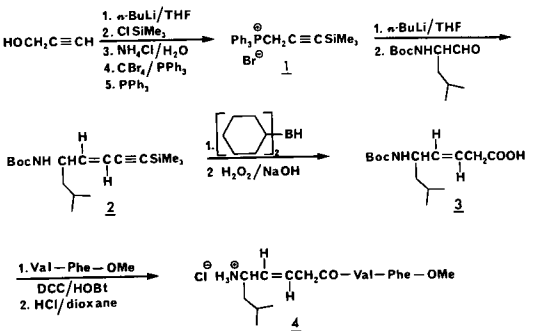


Fig. 1. Hypothetical model of the renin-epoxypolypeptide reaction

Results and Discussion

The key intermediate in the synthesis of epoxypolypeptides 10-12 was the trans-olefinic tetrapeptide 4. This material was synthesized as outlined in Scheme 1. Wittig reagent 1, which was obtained from propargyl alcohol, was condensed with Boc-L-leucinal to give the trans-enyne 2 in 94% yield. The treatment of 2 with dicyclohexylborane followed by alkaline hydrogen peroxide as described by Hann et al.[3] afforded the protected trans-olefinic dipeptide 3. This material was coupled to Val-Phe-OMe using DCC/HOBt. Removal of the tert-butoxycarbonyl group from the resulting tetrapeptide analogue yielded 4. Olefinic tetrapeptide 4 was coupled to Z-His, Z-Gly, $PhCH_2CH_2CO$-Gly, and Z-Pro-Phe-Gly to give the trans-olefinic peptides 6-9. Compounds 7-9 were treated with m-chloroperoxybenzoic acid to give diastereoisomeric mixtures of epoxypolypeptides 10-12, respectively.

Epoxypolypeptides 10-12, as well as the olefinic-polypeptides 6-9 and the angiotensinogen peptide fragment 5, were tested for their ability to inhibit both hog kidney renin and human amniotic renin[4] using an assay system that has been described previously[5]. The IC_{50} values for these analogues are shown in Table I.



Scheme 1

Table I. Renin Inhibitory Activity of Olefinic- and Epoxypolypeptides

No.	Compound	IC_{50} (10^{-5}M)[a] Human	Hog
5	Phe-His-Leu-Leu-Val-Phe-OMe	40	23
6	Z-His-NHCHCH=CHCH$_2$CO-Val-Phe-OMe	48	55
7	Z-Gly-NHCHCH=CHCH$_2$CO-Val-Phe-OMe	200	64
8	PhCH$_2$CH$_2$CO-Gly-NHCHCH=CHCH$_2$CO-Val-Phe-OMe	43	32
9	Z-Pro-Phe-Gly-NHCHCH=CHCH$_2$CO-Val-Phe-OMe	>10	b
10	Z-Gly-NHCHCH-CHCH$_2$CO-Val-Phe-OMe (O)	>300	180
11	PhCH$_2$CH$_2$CO-Gly-NHCHCH-CHCH$_2$CO-Val-Phe-OMe (O)	10	32
12	Z-Pro-Phe-Gly-NHCHCH-CHCH$_2$CO-Val-Phe-OMe (O)	7	b

[a] Average of two determinations. [b] No inhibition was observed with concentrations up to 1 x 10^{-4}M.

In general, the trans-olefinic and trans-epoxypolypeptides synthesized in this study showed renin inhibitory activities comparable to the angiotensinogen peptide fragment after which they were modeled, polypeptide 5. The most active compound in this series was epoxypolypeptide 12. This analogue possessed an IC_{50} of 7 x 10^{-5}M against human amniotic renin. These results, thus, demonstrate that the dipeptide segment which corresponds to the Leu 10-Leu 11 scissile amide bond of angiotensinogen can be replaced with either the trans-olefinic or

trans-epoxide analogues of the dipeptide Leu-Gly without seriously affecting the binding of the resulting polypeptide analogues to renin.

In order to determine whether or not epoxypolypeptides 10-12 would irreversibly inactivate renin, varying concentrations of these epoxides were preincubated with renin at 25°C for various time periods (0-60 min.) before the enzymatic assay was carried out. None of the epoxypolypeptides demonstrated any ability to inactivate renin in a time-dependent manner under the conditions employed in this study. There are several possible reasons why the epoxypolypeptides 10-12 fail to inactivate renin. It may be that the epoxide moiety in these molecules is not reactive enough to react with the aspartyl carboxyl group within renin's active site. Another possible reason may be that although the epoxide moiety is in the position corresponding to the scissile amide bond in analogues 10-12, these analogues may interact with renin's active site in such a manner that the epoxide moiety is not in the proper position for it to be attacked by renin's aspartyl carboxyl group.

Acknowledgements

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SYNTHESIS OF PEPTIDES RELATED TO THE PROSEGMENT OF RENIN PRECURSOR : A NEW WAY IN THE SEARCH FOR RENIN INHIBITORS

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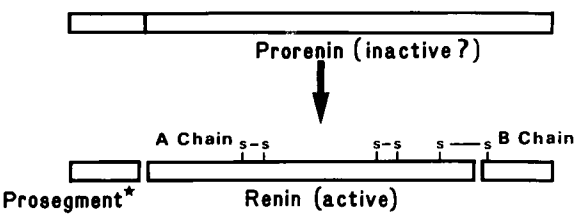
Introduction

Renin, a specific aspartyl protease, cleaves a substrate angiotensinogen to release angiotensin I at the starting point of the renin-angiotensin-aldosterone system which controls the regulation of blood pressure[1]. The complete structure of the gene coding for mouse submaxillary renin has recently been elucidated and has provided evidence for the existence of a prosegment[2] (Fig. 1).



10 20 30 40

*TFSLPTGTTFERIPLKKMPSVREILEERGVDMTRLSAEWDVFTKR

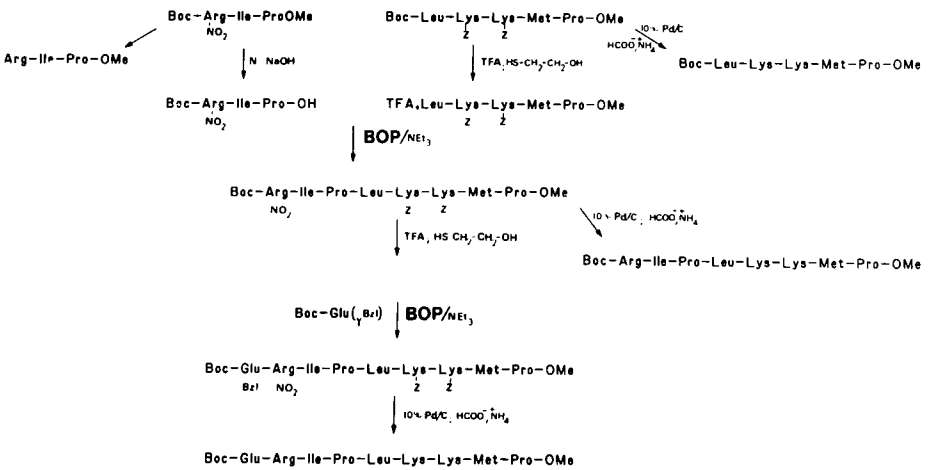
Fig. 1 : A model for renin processing and the sequence of renin prosegment.

Several other aspartyl proteases such as pepsin and chymosin are also synthesized as inactive precursors and enzyme activation occurs with the release of a N-terminal peptidic fragment - the prosequence - of the precursor[3,4,5]. Furthermore, fragments from the prosequence of pepsinogen inhibit pepsin activity with high affinity[6,7,8]. In the case of porcine pepsin, maximum inhibition was observed with the 1-16 and 1-12 peptides of pepsinogen. By analogy with pepsin, we hypothesized that the renin prosegment, particularly peptidic fragments located at its N-terminus, might inhibit renin activity.

We report the synthesis of four peptides related to the renin prosegment and their effects on the enzymatic activity of mouse submaxillary renin.

We designed to prepare the nonapeptide Glu-Arg-Ile-Pro-Leu Lys-Lys-Met-Pro(11-19 peptide of renin prosegment) by the classical method via fragment condensation. The strategy for synthesis is summarized in scheme 1.

Scheme 1



All peptidic bonds were formed using BOP (benzotriazolyl-oxy-tris dimethyl-amino-phosphonium hexafluorophosphate)[9] as the activating agent. To prevent methionine from sulfur alkylation during the removal of the Boc protecting group, mercaptoethanol(1%) was added to the trifluoroacetic acid solution. At the end of the synthesis, the side-chain functions were deblocked by catalytic transfer hydrogenolysis using ammonium formate as a hydrogen donor[10]. The presence of methionine decreased the rate of hydrogenolysis. Complete deprotection occured within 48 to 72 hours after several further additions of catalyst.

Enzymatic Studies

The nonapeptide and three synthetic intermediates, Boc-Leu-Lys-Lys-Met-Pro-OMe, Arg-Ile-Pro-OMe and Boc-Arg-Ile-Pro-Leu-Lys-Lys-Met-Pro-OMe were tested as inhibitors of the in vitro reaction of mouse submaxillary renin on the porcine angiotensinogen tetradecapeptide (pH 6.0). Enzyme activity was determined as the release of angiotensin I which was measured by radioimmunoassay[11]. The concentrations inhibiting 50% of renin activity (IC_{50}) were determined for each peptide (Table I). Pepstatin, a potent aspartyl protease inhibitors[12], was tested in the same conditions. All four synthetic peptides related to renin prosegment were found to inhibit the activity of renin. The most potent inhibitors of the series were the entire nonapeptide and the pentapeptide Boc-Leu-Lys-Lys-Met-Pro-OMe with IC_{50} estimated at 2 and 3 μM respectively. The octapeptide lacking the N-terminal glutamic residue was one order of magnitude less potent than the nonapeptide, indicating that this residue is important for the affinity with renin.

Table I

Peptide	IC_{50} (μM)
Boc-Glu-Arg-Ile-Pro-Leu-Lys-Lys-Met-Pro-OMe	3
Boc-Arg-Ile-Pro-Leu-Lys-Lys-Met-Pro-OMe	20
Arg-Ile-Pro-OMe	200
Boc-Leu-Lys-Lys-Met-Pro-OMe	2
Pepstatin	0.4

Conclusion

Our study suggests the existence of an inactive renin precursor analogous to inactive precursors of other aspartyl proteases (pepsinogen, prochymosin, cathepsinogen). It opens a new approach in the search for renin inhibitors which are of considerable pharmacological interest for the treatment of renin-dependent hypertension.

Acknowlegement

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RENIN INHIBITION BY LINEAR AND CYCLIC ANALOGS OF THE ANGIOTENSINOGEN-(6-11) SEQUENCE

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Introduction

The rate-limiting step in the series of reactions leading to the formation of the octapeptide hormone angiotensin II is the release of the decapeptide angiotensin I from the N-terminal portion of a protein substrate (angiotensinogen), catalyzed by renin. This enzyme is structurally similar to the pepsin-like acid proteases,[1] but differs from them by not acting at low pH, and, most characteristically, by its strict specificity for a Leu-Leu or Leu-Val bond and by the requirement of a minimum octapeptide size.[2] This property, and the proposal of a β-turn involving residues 6-9 (His-Pro-Phe-His) of the angiotensinogen-(1-14)-tetradecapeptide sequence, led to the suggestion that the substrate conformation may be important for renin activity.[3] This hypothesis was supported by the finding that conformationally restricted analogs of the angiotensinogen-(5-14) sequence, in which positions 5 and 10 were bridged by a cystine residue, competitively inhibited renin with K_i values of the same order as the K_m for the hydrolysis of the tetradecapeptide substrate.[4]

To further characterize the possible role of the proposed β-turn for the binding of the substrate to the enzyme, we have now synthesized linear and cyclic analogs of the angiotensinogen-(6-11)-hexapeptide containing the His-Pro-Phe-His sequence, and assayed them as potential renin inhibitors.

Methods

Peptides were synthesized by the solid phase method on the classical Merrifield resin, using the tosyl protection for histidine. The linear peptides were obtained by cleavage with anhydrous HF. The cyclic peptide was obtained by hydrazinolysis followed by cyclization by the azide method. All peptides were purified by ion exchange and gel filtration chromatography.

Human renin (0.05 Goldblatt units/mg protein) was kindly supplied by Dr. E. Haas, Mount Sinai Hospital, Cleveland. Renin assays were done at pH 7.5 in 0.1 M phosphate buffer, with angiotensinogen-(1-14)-tetradecapeptide as substrate, and the release of angiotensin I was determined by radioimunoassay.[5] K_i values were estimated from Lineweaver-Burk plots in the absence and in the presence of the analogs, and the data were treated with a weighted non-linear regression program.

Results and Discussion

The cyclic peptide 8 (Table I), in which the β-turn is present,[6] inhibited human renin with a K_i that was close to the K_m (2.5×10^{-5}M) found for the tetradecapeptide substrate. On the other hand, similar inhibition was obtained with the linear analogs 2 (with L-Phe) and 3 (with D-Phe), suggesting that the more rigid cyclic structure is not necessary for binding to the enzyme. However, the lack of inhibition by compound 7, in which the proline residues hinder the β-turn, suggests that the ability to assume this conformation may be important for binding. It is interesting to note that the analog in which the amino group was acetylated (4) had a three-fold decrease in affinity for the enzyme, while blocking of the carboxyl as the azide (6) resulted in a two-fold decrease, and no inhibition ocurred with the ξ-aminocaproyl derivative (5). These findings suggest that electrostatic interactions between the ends of the hexapetide analogs may affect their ability to

assume the β-turn conformation. In order to better understand these interactions we have determined the pK values for the titratable groups of the compounds shown in Table I.

Table I. Inhibitory Constants and pK Values

Compound*		K_i (M)†	pK values				
Nº	Sequence	x 10^5	COOH	N_2H_3	Imidazoles		Amino
1	-His-Pro-Phe	n.i.	3.65	-	5.78	-	7.61
2	His-Pro-Phe-His-Pro-Phe	4.93	3.50	-	5.60	6.95	8.05
3	His-Pro-Phe-His-Pro-D-Phe	3.85	3.47	-	5.68	7.04	8.22
4	Ac-His-Pro-Phe-His-Pro-Phe	17.10	3.28	-	6.45	7.35	-
5	Acp-His-Pro-Phe-His-Pro-Phe	n.i.	3.14	-	6.03	7.00	10.86
6	His-Pro-Phe-His-Pro-Phe-N_2H_3	9.14	-	3.65	5.20	6.43	7.69
7	His-Pro-Pro-His-Pro-Phe	n.i.	3.18	-	5.38	6.79	8.30
8	His-Pro-Phe-His-Pro-D-Phe	5.31	-	-	5.85	6.83	-

*Ac, Acetyl; Acp, ξ-aminocaproyl †n.i., no inhibition

The carboxyl pK values for compounds 1, 2 and 3 are very similar to those previously obtained for angiotensin II, which has the same C-terminal His-Pro-Phe sequence.[7] The acetylation of the amino group (compound 4), or the addition of an ξ-aminocaproyl residue (compound 5), decreases significantly the carboxyl pK, indicating an interaction between the terminal amino and carboxyl groups of compound 2.

The lower imidazole pK values in compounds 2, 3, 6 and 7 can be attributed to the N-terminal histidine, since they are similar to the pK found for compound 1. The influence of the α-carboxyl on the imidazole group titration can be observed by comparing the N^{α}-acylated compound 4 with the cyclic peptide 8. The imidazole pK values in the latter compound are 0.60 and 0.52 units lower than in the former. The blocking of the carboxyl group in compound 6, in comparison to 2 or 3, also decreases the imidazole pK values by 0.40 and 0.50 units. These

similar decreases indicate that the carboxyl group may be equidistant from both imidazole groups.

The pK values for the amino groups of compounds 2 and 3 are significantly higher than that for the tripeptide 1. Furthermore, blocking of the C-terminal carboxyl shifts the amino pK to almost the same value as that in compound 1. These data suggest a proximity between the amino and the carboxyl terminal groups, as would be expected if the sequence -His-Pro-Phe-His- were in a β-turn.

Substitution of Pro for Phe in compound 3 to give 7, significantly decreased the pK of the carboxyl and imidazole groups and raised the amino group pK. This indicates some structure in the sequence His-Pro-Pro-His, so that the ends of compound 7 are closer than in the cases of 2 or 3.

Our results support the hypothesis that the ability to form a β-turn involving the sequence His-Pro-Phe-His may be important for binding of the substrate to renin, and that this sequence may be a recognition site for the enzyme.

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X-RAY CONFORMATIONS OF RENIN SUBSTRATES.LEU-LEU-VAL-TYR(OMe) AND Ø-O-CH_2-CO-LEU-VAL-PHE(OMe).

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Introduction

Renin plays an important role in the regulation of blood pressure. It cleaves angiotensinogen to give angiotensin I which is subsequently converted in angiotensin II, the potent vasoconstrictor. The inhibition of the renin at the level of the renin-angiotensinogen reaction is a possible way to lead to new antihypertensive drugs.

Carboxyl (acid) proteases are widely studied[1 2 3 4] but there is no precise information concerning the sequence and the conformation of human renin. As all carboxyl proteases, renin has a specificity for peptide bonds located between hydrophobic residues and is inhibited by compounds like pepstatin.

The present investigation was undertaken to develop new types of renin inhibitors. Our approach involves conformational studies of natural or synthetic substrates and the synthesis of compounds which might mimic the endogenic substrate or the postulated transition state of the renin-angiotensinogen reaction. Renin specifically cleaves the Leu^{10}-Leu^{11} peptide bond of angiotensinogen. So consequently,we were at first concerned with the conformations of peptides containing the Leu-Leu sequence.

In the present paper,we report the X-Ray conformation of the 10-13 fragment of angiotensinogen: Leu- Leu- Val- Tyr (OMe) (1) and the conformation of a derivative with a modified Leu-Leu peptide bond: Ø-O-CH_2-CO-Leu- Val- Phe (OMe) (2).

Results and Discussion

Colorless crystals of compounds 1 and 2 were grown from aqueous dimethylsulfoxyde solutions. The unit cell parameters were evaluated on Weissemberg photographs and refined after careful centering of at least 20 reflexions for each crystal. The crystal data are listed in Table I.

	Leu-Leu-Val-Tyr(OMe)	Ø-O-CH_2-CO-Leu-Val-Phe(OMe)
Chemical formula	$C_{27}H_{44}N_4O_6$	$C_{29}H_{39}N_3O_6$
Space group	$P2_12_12_1$	$P2_12_12_1$
Z	4	4
Cell parameters	a= 4.979(1)Å b=22.482(4)Å c=26.559(5)Å	a= 4.866(1)Å b=22.311(4)Å c=27.203(5)Å
V	2973A^3	2953A^3
Dc	1.16g cm^{-3}	1.18g cm^{-3}
R	0.07	0.10

Table I. Crystal data of Leu-Leu-Val-Tyr(OMe) and Ø-O-CH_2-CO-Leu-Val-Phe-(OMe).

The structures were solved by direct methods using the Multan 78 system of computer programs for molecule 1 and Multan 80 for molecule 2. The missing atoms on the E-maps were located from difference Fourier maps after few cycles of refinement (minimization of $(Fc-Fo)^2$). When possible, hydrogen atoms were calculated in theoretical position. Due to the high thermal parameters and the statistical positions of a methyl group(Leu 2, compound 1) some hydrogen atoms were missing on difference Fourier maps. The last cycle of least square refinements with anisotropic thermal parameters for all non hydrogen atoms converged to values of 0.07 for compound 1 and 0.10 for compound 2.

As seen on the drawings of the two peptides molecules (Figure I.),the

main chains are rather extended with the side chains alternatively on the right and left.

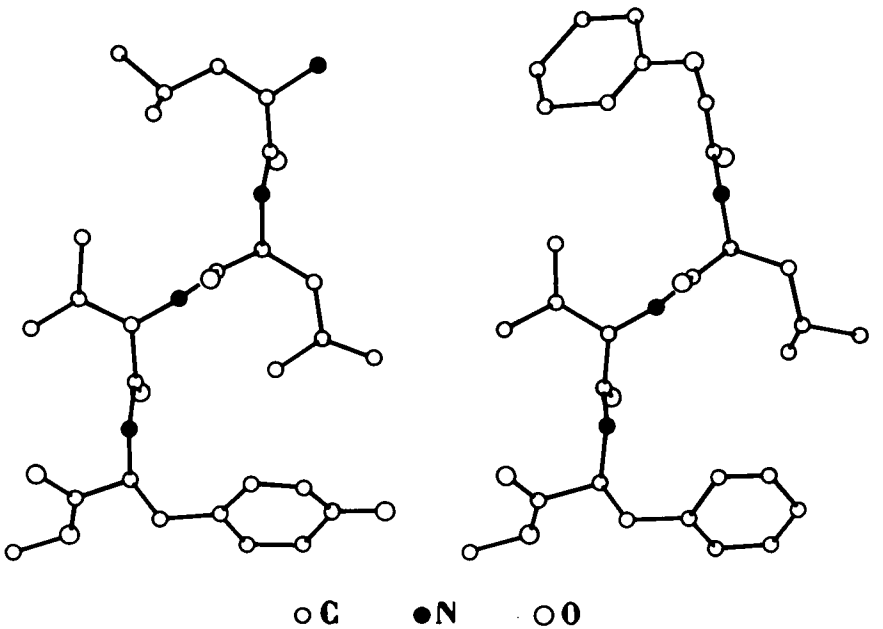

Fig. I. Conformations of Leu-Leu-Val-Tyr(OMe) and Ø-O-CH_2-CO-Leu-Val-Phe(OMe).

	Leu-Leu-Val-Tyr (OMe)				Ø-O-CH_2-CO-Leu-Val-Phe (OMe)			
	Leu.	Leu.	Val.	Tyr.	Ø- -CO	Leu.	Val.	Phe.
φ		-129	-124	- 84		-125	-124	- 93
ψ	125	124	120	154	-171*	112	120	153
ω	179	178	176		180	172	174	

Table II. The main chains torsion angles.*The first ψ value for Ø-O-CH_2-CO-Leu-Val-Phe(OMe) is defined by Ø-CH_2-C_α-NH. The torsion angles are given in accordance with the IUPAC-IUB Commission on Biochemical Nomenclature[5].

The conformation angles are shown in Table II. The angles ω are within

$\pm 8°$ of the trans conformation. For each molecule, the Leu(2) and Val(3) have the usual ϕ and ψ values for the βsheet[6]. The conformations of the side chains are described χ^i. The rotation angles around $C_\alpha - C_\beta$ bonds have classical values[7]. For instance, in the Leu-Leu-Val-Tyr(OMe) structure, χ_1 has a value of $-73°$ close to the $-60°$ corresponding to the most stable conformation. The phenyl ring is gauche to the nitrogen atom and trans to the terminal carboxyl group. In the crystallographic 'a' direction, molecules are linked by hydrogen bonds and form parallel βsheet-type structures. Every hydrogen bond is built with the oxygen of one peptide bond and the nitrogen of the same peptide bond of the next molecule. These fully extended conformations observed in the crystals are in accordance with the extended binding site observed for the acid proteases[1,8] and with the extended conformations observed for the inhibitors in the complexes proteases-pepstatin (or fragment of pepstatin)[9,10].

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NEW SUBSTRATES AND INHIBITORS FOR THE ASPARTYL PROTEASES

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Introduction

Studies of the Aspartyl Proteases have been stimulated by recent crystallographic results[1,2]. Additional progress in understanding the action of this class of proteases has been limited by the available assays. We have recently developed a new spectrophotometric substrate[3] and have demonstrated[4] its utility for the assay of enzymes in this family. In this report we present preliminary data on the characterization of six new substrate analogs. Additionally, we have extended our studies[5] of the pepsinogen activation peptide based inhibitors by preparing four new analogs.

Experimental

Peptides were synthesized by standard solid phase methods on chloromethylated polystyrene using a Vega Model 50 synthesizer. Cleavage was done with anhydrous HF and the peptides were extracted with 50% acetic acid/water. Purification was done by Dowex or CM-Sepharose chromatography.

Kinetics were carried out on a Gilford Model 250 equipped with a Lauda water bath. Data was plotted in double reciprocal format and values of V_{max} and K_m were calculated from intercepts and slopes. For inhibition studies the enzyme was pre-incubated with inhibitor and the assay initiated by the addition of the substrate Lys-Pro-Ala-Glu-Phe-(NO_2)Phe-Arg-Leu. The data was plotted according to Dixon[6].

Results and Discussion

We have synthesized a series of substrates represented by:

Lys-Pro-X-Glu-Phe-(NO_2)Phe-Arg-Leu

where X is a variable residue in the P3 position of the substrate. These substrates are cleaved between Phe and (NO_2)Phe yielding a shift in the absorbance spectra of the (NO_2)Phe residue sufficient to measure the rate of hydrolysis. Such data has given the results presented in Table I for the current series.

Table I. Kinetic Parameters for Pig Pepsin Catalyzed Hydrolysis of Substrates, pH 3.5, 37°, 0.1M sodium formate.

P3 residue	k_{cat}(sec^{-1})	Km(mM)	k_{cat}/Km (sec^{-1})mM^{-1})
Val	19.2	0.060	320
Pro	218	0.187	1166
His	9.5	0.415	23
Lys	15.4	0.622	25
Ala	96.7	0.035	2770
Ile	106.4	0.049	2175

For comparison, the values for the parent compound, Pro-Thr-Glu-Phe-(NO_2)Phe-Arg-Leu were k_{cat} of 90 sec^{-1}, Km of 0.090 mM and k_{cat}/K_m of 1000 sec^{-1} mM^{-1}. It can be seen in Table I that three new peptides have greater values of k_{cat}/K_m for P3 equal to Pro, Ala, and Ile. Also, the extension of the substrate by an extra Lys residue has improved the solubility sufficiently that they can be dissolved directly in the kinetic buffer. Finally, it can be seen that a positively charged residue in position P3 is not well accommodated, with lower k_{cat} values and higher K_m values.

Using the best of these new substrates for the assay, we have studied the inhibition of this activity by:

Leu-Val-X-Val-Pro-Leu-Val-Arg

Where again X is a variable residue. In this series we have retained the Val-Pro-Leu-Val sequence in positions 4 - 7 that we have previously shown to be essential for tight binding to pepsin[5].

Table II. K_I Values for Inhibition of Porcine Pepsin Catalyzed Hydrolysis, pH 5.5, 37°, 0.1 M Sodium Acetate.

P3 residue	K_I μM
Glu	>200
Val	4
Ser	0.8
Lys	6

For comparison, the porcine pepsinogen (1 - 12) peptide has given a K_I value of 0.2 μM in similar assays.

It can be seen from Table II that a negatively charged residue is not well accommodated in the P3 position. Even more striking was our observation that these new, shorter inhibitors give almost instantaneous inhibition whereas we have reported that (1 - 12) or (1 - 16) inhibitors of related sequence exhibit a two-step mechanism of inhibition involving a slow second step[7].

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STUDIES ON THE ACTIVE SITE STRUCTURE OF LEUCINE AMINOPEPTIDASE (BOVINE LENS): EVIDENCE FOR MULTIPLE BINDING SUBSITES AND CATALYSIS OF TRANSPEPTIDATION

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Introduction

Leucine aminopeptidase (LAP) is a well known metalloprotease[1,2] whose mechanism of action is poorly understood. LAP requires two metal ions per subunit for activity. Its substrates must contain a free, unprotonated amino group. There is a preference for hydrophobic side chains, though most N-terminal amino acid residues will eventually be cleaved. One of LAP's most unusual features is its size. It is a hexamer composed of six identical subunits of 54,000 daltons each. This seems large for an enzyme that is apparently a simple protease. These studies were undertaken in an effort to see whether LAP's large size might be involved in its mechanism of action. One possibility was an unusual catalytic process, requiring a large active site. Such a process could be a processional mechanism, where the substrate is sequentially hydrolyzed without release by the enzyme after each bond is broken. This would result in early appearance of the second amino acid of a peptide substrate. Two series of peptides of the form Leu-Ala_n and Gly-Ala_n were used to test this hypothesis. The kinetics of hydrolysis of these peptide substrates were compared to determine what effect, if any, length of the peptide chain had on the kinetic parameters K_m and k_{cat}. The products of hydrolysis, the Ala_n peptides, were also assayed

*Deceased December 5, 1982

to determine what effect product competition would have on the kinetics of the leucyl and glycyl peptides.

Experimental

Preparation of peptide substrates. The leucyl and glycyl peptides used were synthesized using standard solution techniques. The final protected peptides (Z-leu-Ala$_n$-OBzl or Z-Gly-Ala$_n$-OBzl) were purified by crystallization and characterized by melting point, TLC, and elemental analysis. The peptides were deprotected and further characterized with TLC, titration, ion exchange chromatography, amino acid analysis, elemental analysis, and optical rotation. All substrates were >98% pure as judged by these methods.

Determination of kinetic parameters. LAP was isolated from bovine lenses according to the procedure of Hanson et al.[3] as modified by Melbye.[4] The same preparation of LAP was used for all kinetic experiments. Prior to each assay, the LAP was activated with Mg^{++}.[5] Varying substrate concentrations (usually a 10- to 20-fold range spanning the substrate's K_m) were incubated in 0.05 to 0.10 M Tris·HCl, pH 8.5, 0.01 M $CO_3^=$ at 30°. The reactions were initiated by the addition of the enzyme. At three time intervals following initiation, aliquots of the enzyme mixture were placed into pH 2.2 citrate buffer to inactivate the enzyme and prepare the sample for analysis. Product formation was quantitated by ion exchange chromatography in citrate buffer 0.2 N in sodium, pH 3.25 to 4.25 (depending on substrate), using a Beckman 120B amino acid analyzer equipped with a Spectra-Physics System AA integrator. At no time did hydrolysis exceed 5% of the initial substrate concentration. Initial velocities (in μmoles substrate hydrolyzed per minute per mg LAP) were calculated from three time points using linear regression. The kinetic parameters K_m and k_{cat} (V_{max}/E) were calculated using the computer program hyperb.[6]

K_m values were corrected for substrate pK_a to reflect the amount of unprotonated amino groups present at pH 8.5.

Results and Discussion

The results of the kinetic studies of the leucyl and glycyl peptides are summarized in Table I. For all substrates, hydrolysis of the N-terminal peptide bond followed Michaelis-Menten kinetics. With the exception of Gly-Ala_3, the only products observed were Ala_n and either Leu or Gly. In the case of Gly-Ala_3, Ala and Ala_2 were also produced in small amounts. Subsequent analysis showed that one of the initial products, Ala_3, was a much better substrate than the starting material and was able to compete effectively for the LAP even at a 1% level. There was no evidence for a processional mechanism of action for LAP. The data do support an extended active site for LAP, however. This is seen by the decreasing K_m values, particularly in the glycyl series, with increasing peptide chain length. There are apparently binding sites for at least the first three residues of a polypeptide substrate.

Table I. LAP Kinetic Constants for Leucyl and Glycyl Peptides

Substrate	k_m mM	k_{cat} µmole/min/mg
Leu-NH_2	5.5±0.8	680±40
Leu-Ala	0.44±0.7	610±70
Leu-$AlaNH_2$	1.3±0.1	680±60
Leu-Ala_2	0.40±0.05	720±120
Leu-Ala_3	1.1±0.3	680±110
Gly-NH_2	630±20	6.8±1.5
Gly-Ala	99±48	38±12
Gly-Ala_2	32±2	1830±130
Gly-Ala_3	32±2	570±40

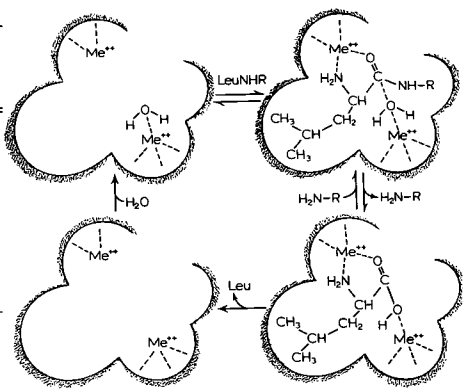

Fig. 1. Proposed Mechanism of Action for LAP

Suprisingly, close inspection of the Ala_3 reaction with LAP showed the formation of Ala_4 as well as Ala and Ala_2. The Ala_4 apparently resulted from a transpeptidation reaction competing with the hydrolysis reaction. Reaction of Ala_2 and Ala_4 with LAP also yielded transpeptidation as well as hydrolysis products. The kinetics of product appearance were consistent with the following scheme:

$$Ala_n + LAP \rightleftharpoons Ala_n \cdot LAP \rightarrow Ala_n \cdot LAP^* \xrightarrow{H_2O} \begin{cases} Ala + Ala_{n-1} + LAP \\ Ala_n \end{cases} \quad Ala_{n+1} + Ala_{n-1} + LAP$$

Transpeptidation catalysis by LAP also supports an extended active site for the enzyme. Furthermore, transpeptidation is more consistent with a stepwise rather than concerted mechanism. Since our many attempts to isolate an acyl enzyme intermediate have failed, we propose an intermediate in which the attacking nucleophile is a water molecule coordinated to one of the metal ions. This proposal is illustrated in Figure 1. This mechanism is consistent with the observed reactions of LAP.

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THE SELECTIVE INHIBITION OF ENTEROKINASE BY SYNTHETIC PEPTIDE ALDEHYDES BASED ON THE ACTIVATION PEPTIDE OF TRYPSINOGEN

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Introduction

Enterokinase and trypsin share trypsinogen as a common substrate. Both hydrolyse the susceptible Lys-Ile bond on the C-terminal side of the characteristic tetraaspartyl sequence in the activation peptide of all mammalian trypsinogens. However, the rate of trypsinogen activation by the two enzymes varies by several orders of magnitude due to their different affinities for this polyanionic sequence[1]. This communication reports the synthesis of the peptide aldehydes Boc-$(Asp)_n$-argininal where n = 2 to 4 and demonstrates the selective inhibition of enterokinase, but not trypsin-catalysed hydrolysis of the common substrates, Gly-$(Asp)_n$-Lys-2-Napthylamide[2] and α-N-[^{3}H]-acetyltrypsinogen[3]. Mechanism of inhibition is presumed to be formation of an enzyme-aldehyde adduct resembling the tetrahedral intermediate of proteolysis .

Experimental

The peptide aldehydes Boc-$(Asp)_n$-argininal, where n = 2, 3 or 4 were prepared by a method similar to that described by Shimizu et al.[5]. Z-(N-ω-nitro)-arginine was converted to the aldehyde by controlled reduction with diisobutyl aluminium hydride in THF at -42°C. The crude product was extracted in CH_2Cl_2 and immediately protected by conversion to its semicarbazone which was crystallised from

70% ethanol, m.p. 109-111°C (reported m.p. 107-109°C). The α-amino group was deprotected with HBr in acetic acid and the product coupled to Boc(Bzl)-$(Asp)_n$ by the DCC/HOBt preactivation method. The protected peptide semicarbazones were crystallised from ethyl acetate and gave single ninhydrin fluorescent or 2,4-DNP positive spots on TLC. The aldehydes were regenerated overnight at room temperature in 10 equivalents HCHO:20 equivalents HAc in MeOH[6]. The remaining groups were removed by hydrogenation over Pd/charcoal. The peptide aldehydes all gave two phenanthrene-quinone positive spots for arginine on TLC which may have reflected either tautomeric forms of the aldehyde or partial deprotection of the α-NH_2 group. The products were all 90% or more peptide by weight and were used for inhibition studies without further purification. The inhibition of human enterokinase or bovine trypsin by these aldehydes was compared with leupeptin (acetyl-leu-leu-argininal) using either Gly-$(Asp)_4$-Lys-2-Nap or α-N-[^{3}H]-acetyl trypsinogen as substrate. K_i values for enterokinase were estimated by linear regression analysis of plots of 1/v against I at three concentrations of substrate. Hydrolysis of Gly-$(Asp)_4$-Lys-2-Nap (0.625mM, 1.25mM, 1.875mM) was linear with time whereas the assay using α-N-[^{3}H]-acetyl trypsinogen was modified so that in the presence of an excess of enterokinase (>50ng) the initial rate of release of α-N-[^{3}H]-acetyl activation peptide was directly proportional to substrate concentration (0.4 - 0.8μCi = 60 - 120μg trypsinogen). Trypsinolysis of the substrates was complex and inhibition was expressed as the IC_{50}, the concentration of aldehyde causing 50% inhibition of activity.

Results and Discussion

Enterokinase, like trypsin, has an absolute requirement for lysine or arginine on the carbonyl side of the susceptible peptide bond. To date, no naturally occurring

anti-serine proteinase peptide aldehyde has been shown to contain lysinal nor have any been successfully synthesised presumably due to its chemical reactivity. Other considerations for preparing argininal-derivatised activation peptide analogues are the reported stability of the cyclic carbanolamine towards racemisation and possible non-specific nucleophilic attack .

K_i values for human enterokinase of 0.2μM, 0.1μM and 0.06μM for Boc$(Asp)_n$-Argininal where n = 2 to 4 respectively, were obtained with Gly$(Asp)_4$-Lys-2-Nap as substrate (Table 1). These were four orders of magnitude or more lower than the corresponding K_i values for two synthetic activation peptide analogues confirming the reactivity of the aldehyde compared to the free acid . The corresponding K values for enterokinase with α-N-[^{3}H]-acetyl-trypsinogen as substrate were approximately 1 log unit lower still. By contrast the IC_{50} values for trypsin inhibition by these aldehydes were between 2 and 4 orders of magnitude higher against both substrates reflecting the lower affinity of trypsin for the polyanionic sequences. More importantly the inhibitory constants for leupeptin inhibition of trypsin and enterokinase-catalysed hydrolysis of (a) Gly-$(Asp)_4$-Lys-2-Nap, were 1.0μM and 0.175μM respectively and (b) α-N-[^{3}H]-acetyl trypsinogen, were 1.0μM and 0.35μM respectively. This would suggest that the two leucyl residues were non-specifically bound to the sub-sites adjacent to the active sites of both enzymes.

This study has shown that the unique specificity of enterokinase for the tetraaspartyl sequence of trypsinogen activation peptide can be exploited to discriminate it from trypsin, an enzyme with which it shares many catalytic properties. The potency of peptide aldehydes as anti-proteinases together with selectivity of action conferred by judicious choice of amino-acids would suggest that they may be exploited in vivo for the management of diseases where

inappropriate proteinase activity is suspected to contribute to the pathgenesis.

TABLE I

INHIBITION CONSTANTS FOR HUMAN ENTEROKINASE (K_i) AND TRYPSIN (IC_{50})

Substrate / Inhibitor	Gly-(Asp)$_4$-Lys-2-Nap			α-N-[^{3}H]-acetyltrypsinogen		
	Enterokinase(K_i)		Trypsin(IC_{50})	Enterokinase(K_i)		Trypsin(IC_{50})
Boc(Asp)$_2$Argal	0.2	μM	>20μM	0.01	μM	>30μM
Boc(Asp)$_3$Argal	0.1	μM	>50μM	0.02	μM	>100μM
Boc(Asp)$_4$Argal	0.055	μM	>50μM	0.01	μM	>100μM
Leupeptin	1.0	μM	0.175μM	1.0	μM	0.35μM
(Asp)$_4$Lys	100	μM	-	-		-
Ala-Pro-Phe-(Asp)$_4$-Lys	750	μM	-	-		-

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INHIBITORS OF PYROGLUTAMYL AMINOPEPTIDASE

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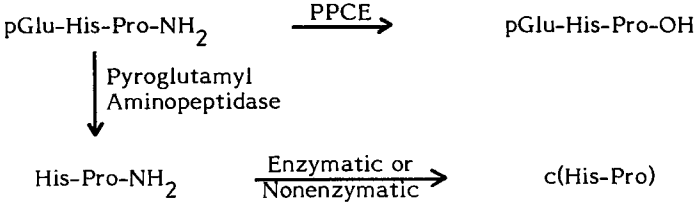
Introduction

Thyrotropin releasing hormone (TRH), a tripeptide amide with the structure L-Pyroglutamyl-L-Histidyl-L-Proline amide, has recently been found to have a role in the central nervous system (CNS) in addition to a well defined role in the endocrine system. TRH is distributed throughout the extrahypothalamic nervous system,[1] the spinal cord, the retina,[2] as well as other tissues, and has been found to have a variety of effects in the CNS.[3-5] These CNS effects are independent of the integrity of the endocrine system. The CNS actions of TRH, together with the presence of TRH in nerve terminals and synaptosome fractions of the brain, support the hypothesis that the tripeptide plays a role in the modulation of neuronal activity, possibly as a neurotransmitter.[1,6]



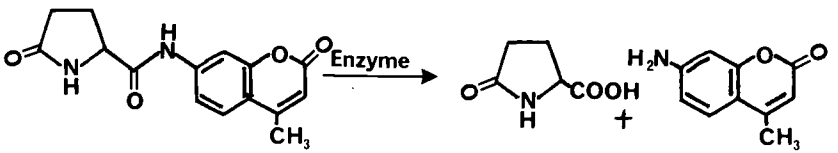
Scheme 1. TRH degradative pathways

Rapid enzymatic degradation of TRH occurs in tissue and serum[7] (Scheme 1). The deamidation of TRH in tissue is catalyzed by an enzyme with a molecular weight of 76,000 and is called postproline cleaving enzyme (PPCE).[8,9] Cleavage of the L-Pyroglutamyl-L-Histidyl peptide bond of TRH occurs in serum and tissue. In serum, this cleavage is catalyzed by an enzyme with a molecular weight of 260,000 which exhibits characteristics distinctly different from the pyroglutamyl aminopeptidases from various tissues known to catalyze the same reaction.[10] In tissue, cleavage of the L-Pyroglutamyl-L-Histidyl peptide bond is catalyzed by a pyro-

glutamyl aminopeptidase with a molecular weight of 28,000.[11] Unlike the serum enzyme which appears to be specific for TRH, the tissue enzyme can catalyze the hydrolysis of various pyroglutamyl-containing peptides such as pGlu-Ala and pGlu-His.[12] Inhibition of this tissue enzyme could provide an indirect means of elevation of TRH levels in the CNS. A series of chloroacetyl-substituted TRH analogs previously synthesized in this laboratory[13,14] were found to possess TRH-like CNS activity in antagonizing some behavioral effects of morphine in mice.[15] These TRH analogs, however, did not have significant TRH-like effects on the pituitary. Therefore, the inhibition of TRH degradation was considered a possibility.

Experimental

The inhibition of pyroglutamyl aminopeptidase by these potentially irreversible inhibitors has been investigatd using calf liver enzyme preparations and the fluorogenic substrate, L-pyroglutamyl-4-methylcoumarinylamide (Pyr-MCA), (Scheme 2). The enzyme activity is monitored by observing the fluorescence intensity of the cleavage product, 7-amino-4-methylcoumarin (AMC).



Pyr-MCA **AMC**

Scheme 2. Fluorescencent enzyme assay reaction

The potential inhibitors were assayed at 37°C in the presence of Pyr-MCA, enzyme, and phenylmethylsulfonylfluoride (PMSF) in TRIS buffer, pH=7.4, containing 2mM EDTA and 2mM DTT. Relative fluorescence was determined at approximately 3, 25, and 45 minutes. These readings were then compared to control samples run simultaneously, which contained enzyme, Pyr-MCA, and PMSF. For each compound, assays were done at varying inhibitor concentrations with Pyr-MCA concentration held constant in order to determine the optimum inhibitor concentration for approximately 50% inhibition. These were then followed by assays in which the compound concentration remained constant and Pyr-MCA concentration varied in order to generate Lineweaver-Burk Plots (Figure 3 - 6).

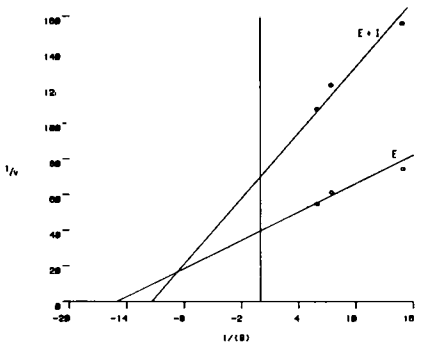


Fig. 3. Effect of pGlu-Phe-Pyrr on enzyme activity

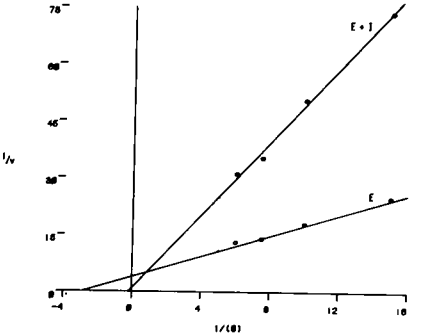


Fig. 4. Effect of TRH on enzyme activity

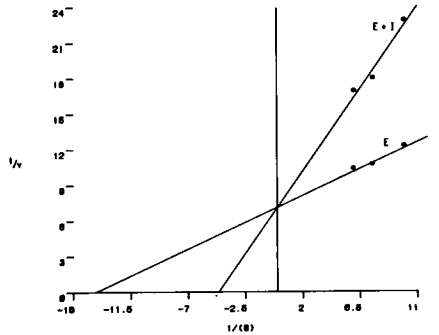


Fig. 5. Effect of mClAcBz-Phe-Pyrr on enzyme activity

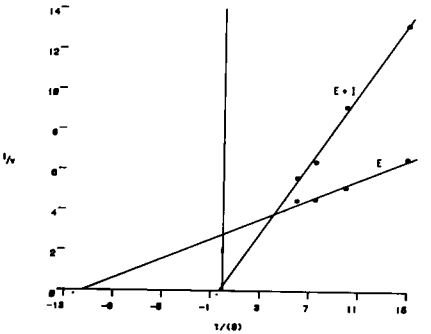


Fig. 6. Effect of pClAcBz-Ala-Phe-Pyrr on enzyme activity

Results and Discussion

Of six chloroacetyl-substituted TRH analogs investigated, four showed substantial enzyme inhibition (Table I). It was interesting to note that while pClAcBz-Ala-Phe-Pyrr led to appreciable enzyme inhibition, no inhibition was observed with mClAcBz-Ala-Phe-Pyrr. TRH and the substrate analog, pGlu-Phe-Pyrr, were also found to inhibit the generation of AMC apparently because they are better substrates for the enzyme.

A very interesting parallel was observed which indicated that those analogs with CNS activity also inhibited the enzyme, pyroglutamyl aminopeptidase. It is

thus suggested that the TRH-like CNS activity of these analogs was derived from an inhibition of the breakdown of endogenous TRH and not from a direct action on the CNS receptor.

Table I. Enzyme inhibition and CNS activities of TRH analogs

Compound	CNS Act	Enz Inhib
pGlu-Phe-Pyrr	NO	YES
mClAcBz-Phe-Pyrr	YES	YES
mClAcBz-Ala-Phe-Pyrr	NO	NO
pClAcBz-Ala-Phe-Pyrr	YES	YES
ClAcAla-Phe-Pro-NH_2	YES	YES
mClAcBz-Phe-Pro-NH_2	NO	NO
cyclo(Phe-Pro)	YES	NO
TRH	YES	YES

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SYNTHESIS OF INHIBITORS OF A BACTERIAL PEPTIDOGLYCAN SYNTHETASE

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Introduction

The cytoplasmic steps of biosynthesis of bacterial peptidoglycan involve uridine diphospho-N-acetylmuramyl-peptide precursors. Each step is catalyzed by a particular synthetase, which is highly specific for both the nucleotide and the amino acid (or dipeptide) substrates[1]. In order to find specific inhibitors of these enzymes, which might act as antibacterial agents, we have undertaken the synthesis of analogues of the glycopeptide part of the precursors. In particular, we have recently reported[2] that propionyl- and N-acetylmuramyl-L-Ala-D-Glu-OH inhibit the UDP-MurNac-L-Ala-γ-D-Glu-meso-DAP synthetase of Escherichia coli. In this communication, we describe the synthesis of new N^{α}-acyl - dipeptides, and their effect on this meso-DAP-adding enzyme.

Synthesis of the peptides

Peptides with the general formula R-L-Ala-D-Xxx-OH [R = D-lactyl, L-lactyl, DL-2-methylbutyryl (DL-2-Meb) ; Xxx = Glu, Asp] were prepared by coupling Nps-L-Ala-OSu to H-D-Xxx(OBzl)-OBzl. After removal of the Nps group by pyridine hydrobromide/indole[3,4], the N^{α}-amino function was acylated by R-OSu, and the benzyl groups were removed by catalytic hydrogenolysis.

D-Methionine sulfoxide [D-Met(O)] and D-methionine sulfoximine [D-Met (O)(NH)], synthesized according to the literature[5], were coupled with

Z-Ala-ONp in DMF-H_2O in the presence of $NaHCO_3$. After removal of the Z group by HBr/AcOH, the N^{α}-amino function was acylated by Pr-ONp. Due to the chiral sulfur atom, D-Met(O), D-Met(O)(NH) and their N^{α}-Pr-L-Ala derivatives were each obtained as a mixture of two diastereoisomers.

When necessary, final N^{α}-acyl-dipeptides were purified by HPLC. Their purity was checked by TLC, HPLC and amino acid analysis.

MDP derivatives were synthesized as already described[6,7].

Enzymatic results

The preparation of the E. coli enzyme used and the enzymatic test, which was carried out by measuring the addition of meso-[^{14}C]DAP to UDP-MurNac-L-Ala-D-Glu in the presence of ATP and Mg^{2+}, were previously described[8]. The results obtained with the different synthetic compounds are reported in Table I.

Discussion

In a previous communication[2], we showed that acylation of the L-Ala-D-Glu moiety is necessary for an inhibitory effect. Among the acylated dipeptides, MurNac-L-Ala-D-Glu-OH, which mostly resembles the structure of the nucleotide substrate, gives the best result. Some substitutions, which partly mimic the MurNac group (Pr, D-Lac), lead to comparable inhibitory effect. With more important modifications (L-Lac or D-Asp) no significant inhibition is observed.

It should be pointed out that the lack of a uridine diphospho group in MurNac-L-Ala-D-Glu-OH strongly reduces the affinity of the compound for the enzyme. Indeed, whereas the Km of the nucleotide substrate[8] is 35 µM, a good inhibitory effect by the MurNac-dipeptide is only observed at 10mM.

If we consider MurNac-L-Ala-D-Glu-NH_2 (MDP) as a basic structure, we can see that modifications of the peptide moiety do not improve the inhibitory capacity. However, substitutions of the 1-OH of MurNac residue

Table I. Inhibitory Effect of the Synthetic Compounds

Compounds	Residual enzymatic activity (%) at 10 mM	at 1 mM
H-L-Ala-D-Glu-OH	95[a]	113
Pr-L-Ala-D-Glu-OH	59[a]	88[a]
MurNac-L-Ala-D-Glu-OH	48	99
D-Lac-L-Ala-D-Glu-OH	58	103
L-Lac-L-Ala-D-Glu-OH	96	99
DL-2-Meb-L-Ala-D-Glu-OH	82	-
D-Lac-L-Ala-D-Asp-OH	82	-
D-Met(O)	100	-
Pr-L-Ala-D-Met(O)-OH	88	-
MurNac-L-Ala-D-Glu-NH_2 (MDP)	79[a]	-
MurNac-D-Ala-D-Glu-NH_2	82	-
MurNac-NMe-L-Ala-D-Glu-NH_2	97	-
MurNac-L-Ala-D-Glu-OMe	89	-
MurNac-L-Ala-D-Gln-OH	74	-
1-O-α-methyl-MDP	38	-
1-O-α-benzyl-MDP	73	-
1-O-β-p-aminophenyl-MDP	38	-
1-O-β-p-nitrophenyl-MDP	55	-
D-Met(O)(NH)[b]	105	102
Pr-L-Ala-D-Met(O)(NH)-OH[b,c]	20	57

[a]Ref.2; [b]after pre-incubation for 15 min with the enzyme, ATP and Mg^{2+}; [c]79% at 100 μM, 97% at 10 μM

by α-methyl, β-p-aminophenyl and β-p-nitrophenyl yield good inhibitors. This observation suggests that the prolongation of the structure beyond the MurNac residue is necessary to obtain a higher affinity for the enzyme.

L-Met(O) and L-Met(O)(NH) were found to inhibit glutamine synthetase[9,10], which catalyzes the same kind of reaction as meso-DAP-adding enzyme. Furthermore, L-Met(O)(NH) is known to be a "suicide" substrate

of glutamine synthetase[11] and γ-glutamylcysteine synthetase[12]. When used in the direct inhibition assay, D-Met(O), D-Met(O)(NH) and their N^{α}-Pr-L-Ala derivatives have no significant effect even at 10mM. However, when pre-incubated with the enzymatic preparation in the presence of ATP and Mg^{2+}, Pr-L-Ala-D-Met(O)(NH)-OH inhibits the enzymatic activity. This result suggests that this propionyl-dipeptide acts according to a suicide type mechanism. The fact that D-Met(O)(NH) has no effect shows the importance of the Pr-L-Ala moiety, which probably increases the affinity of the inhibitor for the enzyme.

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SPECIFIC PEPTIDE INHIBITORS FOR NON-PLASMIN FIBRINOLYTIC PROTEINASES: HUMAN LEUKOCYTE ELASTASE (LE) AND HUMAN SPLEEN FIBRINOLYTIC PROTEINASE (SFP)

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Previously, we reported that Suc-Tyr-Leu-Val-pNA and Suc-Ala-Tyr-Leu-Val-pNA were specific substrates for human spleen fibrinolytic proteinase (SFP)[1] (k_{cat}/K_m=22600 and 84000, respectively) as well as for human leukocyte elastase (k_{cat}/K_m= 17600 and 48500, respectively), which is responsible for the tissue destruction that occurs in emphysema and bronchitis and we indicated that the substrate specificity of the both enzymes was identical so far examined.[2-4] In order to study their physiological roles, specific inhibitors were required. This report deals with selective peptide inhibitors for SFP and LE as well as their application to affinity chromatography to purify the both enzymes. Based on the structure of the substrates described above, two types of peptide inhibitors were designed and synthesized.

Reversible Peptide Inhibitors

First of all, eight kinds of stereoisomers of Suc-Tyr-Leu-Val-pNA were synthesized. Two isomers, Suc-L-L-L-pNA and Suc-D-L-L-pNA were hydrolyzed by SFP and LE to release p-nitroaniline, whereas other isomers were not hydrolyzed by SFP and LE and exhibited inhibitory activity against SFP and LE. Out of those inhibitors, Suc-L-D-D-pNA showed the most effective inhibitory activity.[5,6]

Next, the C-terminal or N-terminal of the peptide was modified and their inhibitory activity against SFP and LE was assessed. The results are summarized in Table I.

Table I. Inhibitory Effect on Amidolysis by SFP and LE

Compound	Dioxane (%)	Inhibition (%) SFP	LE
Suc-L-Tyr-L-Leu-L-Val-Pip	0.3	<5	0
Suc-L-Tyr-D-Leu-D-Val-Pip	0.3	<10	0
Dan-L-Tyr-L-Leu-L-Val-pNA	9	36	47
Dan-L-Tyr-D-Leu-D-Val-pNA	9	40	56

Substrate: Suc-Ala-Tyr-Leu-Val-pNA (0.3 mM); Inhibitors: 0.3 mM; Pip: 4-methyl piperidine; Dan: dansyl

Although Suc-L-Tyr-D-Leu-D-Val-pNA was an effective inhibitor, Suc-L-Tyr-D-Leu-D-Val-Pip exhibited weak inhibitory activity only against SFP, suggesting that pNA group could bind with some part of those enzymes to act as an effective inhibitor. It is also interesting that substitution of dansyl for succinyl of Suc-L-Tyr-L-Leu-L-Val-pNA changed the specific substrate for SFP and LE to the specific inhibitor against SFP and LE although the reason still remain to be solved.

In order to obtain a useful tool for rapid and simple purification of SFP and LE, an effective inhibitor, Suc-L-Tyr-D-Leu-D-Val-pNA was selected as ligand and coupled with AH-Sepharose 4B by the method described previously[7] to afford a selective and durable affinity sepharose. In the case of small scale purification, one step purification of LE and SFP by this affinity chromatography gave a 59-fold purification and a 116-fold purification, respectively. However, for large scale purification, performance of this affinity chromatography in combination with other conventional purification methods is preferable.

Irreversible Peptide Inhibitors: Peptide Chloromethyl Ketones

Peptide chloromethyl ketones were designed based on the

Table II. Kinetic Constants of SFP and LE for $-CH_2Cl$ Type Inhibitors

Inhibitor	Enzyme	I ($Mx10^4$)	$10^4 k_{obsd}$ (s^{-1})	Half Life (sec)	k_{obsd}/I ($M^{-1}s^{-1}$)
Suc-L-Tyr-L-Leu-L-Val-CH_2Cl	SFP	0.2	<23	>300	<115
	LE		<23	>300	<115
Boc-L-Tyr-L-Leu-L-Val-CH_2Cl	SFP	0.1	224	31	2240
	LE		117	59	1170
Boc-D-Tyr-L-Leu-L-Val-CH_2Cl	SFP	0.1	267	26	2670
	LE		112	62	1120
Boc-L-Ala-L-Tyr-L-Leu-L-Val-CH_2Cl	SFP	0.1	495	14	4950
	LE		330	21	3300
Dan-L-Ala-L-Tyr-L-Leu-L-Val-CH_2Cl	SFP	0.1	301	23	3010
	LE		267	26	2670
Ac-L-Ala-L-Tyr-L-Leu-L-Val-CH_2Cl	SFP	0.1	248	28	2480
	LE		151	46	1510
Suc-L-Tyr-D-Leu-D-Val-CH_2Cl	SFP	2.5	-	-	-
	LE		-	-	-
Boc-L-Tyr-D-Leu-D-Val-CH_2Cl	SFP	2.5	-	-	-
	LE		-	-	-
Boc-L-Tyr-L-Leu-L-Val-CH_2Cl	SFP	2.5	175	40	69.2

Amidolysis was performed for 5 min. Substrate: Suc-L-Ala-L-Tyr-L-Leu-L-Val-pNA (0.5 mM); Enzyme: about 0.35 μM in Tris/HCl buffer (0.1 M, pH 8.0) containing 2 M $NaClO_4$; Dioxane concentration: 1.5% except for I=2.5 (7.5%); Suc or Boc-L-Tyr-D-Leu-D-Val-CH_2Cl did not inhibit the both enzymes at the concentration (I=0.25 mM).

structure of the substrates, Tyr-Leu-Val and Ala-Tyr-Leu-Val, and synthesized. Their inhibitory activity against SFP and LE is summarized in Table II. Suc-L-Tyr-L-Leu-L-Val-CH_2Cl exhibited inhibitory effect expectedly. Boc-L-Tyr-L-Leu-L-Val-CH_2Cl, which had no minus charge at N-terminus, exhibited much more effective inhibitory activity against SFP and LE specifically. Chloromethyl ketones derived from Ala-Tyr-Leu-Val exhibited an inhibitory effect more strongly and selectively.

It is reasonable that Suc- or Boc-L-Tyr-D-Leu-D-Val-CH_2Cl did not inhibit those enzymes because chloromethyl ketone functional group might be placed in the opposite direction against active site histidine residue. However, it is interesting that Suc-L-Tyr-D-Leu-D-Val-pNA inhibits and Suc- or Boc-L-Tyr-D-Leu-D-Val-CH_2Cl does not inhibit those enzymes at the same concentration. The existence of some binding site in the enzymes suitable for pNA moiety might be again hypothesized. These relationship was further proved by preparing eight kinds of stereoisomer of Boc-Tyr-Leu-Val-CH_2Cl and assessing their inhibitory effect. The potency of inhibitory activity of those chloromethyl ketone derivatives was in inverse proportion to that of pNA derivatives.

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SUBSTRATE ANALOG INHIBITORS OF IGA_1 PROTEASE

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Introduction

Highly specific proteolytic enzymes cleave either a single or restricted number of substrates in vivo to effect a biologic function. Examples of these proteases are: IgA_1 protease, renin, kallikrein, and rec A[1]. These enzymes may be contrasted with less specific proteases such as those isolated from the gastrointestinal tract which cleave many types of peptide bond. In vitro, highly specific proteases often bind and may cleave peptides which are homologous with the amino acid sequence of the naturally occurring substrate at the cleavage site[2]. These substrate analogs are capable of blocking the in vivo reaction of a highly specific protease[3].

IgA_1 proteases are a class of enzymes isolated from pathogenic bacteria which inactivate both serum and secretory IgA_1 by cleavage of a single peptide bond in the hinge region of the human antibody. Three sites of cleavage have been identified in the hinge region as shown in Figure 1. Commensal members of the bacterial species do not produce the protease which is assumed to assist in the invasion of the host by the pathogen[4].

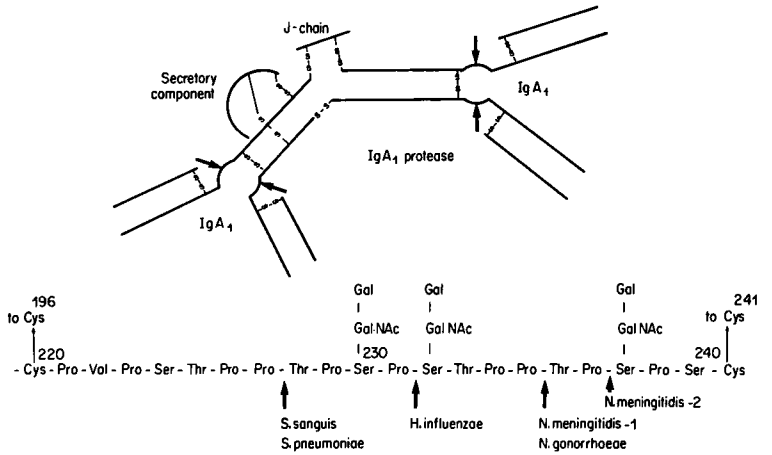

Fig. 1. Model of secretory IgA_1 with cleavage sites.

Experimental

Peptides were prepared by solid phase synthetic techniques[5]. Both the standard chloromethylated polymer and the p-methylbenzhydrylamine polymer were used as supports. After synthesis, cleavage and purification by chromatography on Sephadex G-25 peptides were characterized by a rapid microtechnique. Approximately 200 μg of the peptide was subjected to hplc on a semipreparative reverse phase column (1 x 25 cm). The column effluent passed through a flow cell mounted in an HP-8450A spectrophotometer which measured the effluent spectrum from 200-400 nM every three seconds. U-v spectra taken during the ascending and decending limbs of a peak were stored in the spectrophotometer memory, normalized, and superimposed to check homogeneity of the eluting material. Each peak was caught in a computer controlled x-y fraction collector and evaporated to dryness with N2. The residue was diluted to a standard volume and characterized by amino acid analysis, [M] , u-v spectrum, specific activity, tlc, and hplc.

Results and Discussion:

The octapeptide sequence Thr-Pro-Pro-Thr-Pro-Ser-Pro-Ser which is found between position 225-233 of human IgA_1, is an inhibitor of the IgA_1 protease from N. gonorrhoeae. Blockage of N- and C-terminal functional groups by acetylation and amidation improves the IC_{50} about seven-fold. The peptide is also an inhibitor of both IgA_1 proteases from N. meningitidis, but has little effect on other IgA_1 proteases (Figure 2).

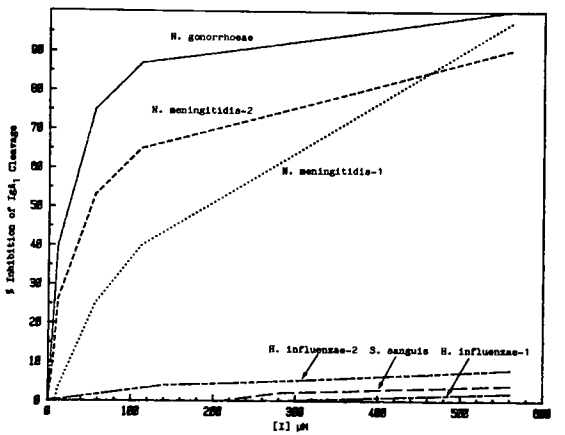

Fig. 2. Inhibition of various IgA_1 proteases by Ac-Thr-Pro-Pro-Thr-Pro-Ser-Pro-Ser-NH_2

Other investigators have proposed that the hinge region of IgG exists as a polyproline helix with the γ-chains of the immunoglobulin held in close proximity [6]. If this model applies to the hinge region of IgA_1, elements of both chains could be in contact with the enzyme active site. A substrate analog inhibitor consisting of two peptide chains might have increased inhibitory effect. To prepare these inhibitors, each hydroxy amino acid was sequentially replaced with cysteine and the resulting peptide dimerized. None of the disulfide linked peptides is a better inhibitor of IgA_1 protease than the blocked octapeptide (Figure 3).

Inhibition of IgA_1 Protease by Immunoglobulin Hinge-Region Substrate Analogs

	Enzyme Source		
	N. gonorrhoeae	S. sanguis	H. influenzae
	IC_{50} (μM)	IC_{50} (μM)	IC_{50} (μM)
Thr-Pro-Pro-Thr-Pro-Ser-Pro-Ser	180	---	---
Ac—————NH_2	28	28,000 (29%)	---
Ac-Cys(S)—————NH_2	800	---	7600
Ac-Cys(S)—————NH_2			
Ac———Cys(S)—————NH_2	3000	---	---
Ac———Cys(S)—————NH_2			
Ac———Cys(S)—————NH_2	6000	---	---
Ac———Cys(S)—————NH_2			
Ac———Cys—NH_2(S)	220	---	---
Ac———Cys—NH_2(S)			

Fig. 3. Effect of dimerization on IC_{50} for the IgA_1 protease substrate analog inhibitors.

IC_{50} values for the IgA_1 protease from N. gonorrhoeae by the peptide crosslinked at the C-terminus and the free sulfhydryl (not shown) are 220 μM and 160 μM respectively. Diminuation appears to be due to replacement of the seryl residue with cysteine and not dimerization.

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STUDIES OF PARATHYROID HORMONE-RECEPTOR INTERACTIONS: DESIGN OF AN ANTAGONIST EFFECTIVE IN VIVO AND PHOTOAFFINITY LABELING OF PARATHYROID HORMONE

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Introduction

Parathyroid hormone (PTH) plays a critical role in calcium homeostasis through its direct action on kidney and bone and indirect effects on gut to prevent hypocalcemia. The rapid effects of PTH on target tissues make it responsible for minute-to-minute regulation of calcium levels in the extracellular fluid, a function which is essential for the normal activity of muscle, nerve, enzymes, blood clotting factors and other hormones.

For the last decade, efforts have been made in our laboratory to characterize PTH receptors and elucidate the nature of the PTH-receptor interaction leading to expression of hormonal activity. Two general approaches have been used. The first is indirect characterization of the PTH receptor and hormone-receptor interactions through extensive structure-activity studies in multiple bioassay systems.[1] The structural elements within the PTH molecule necessary for receptor-binding and for expression of hormone action have been identified as well-delineated and separable domains. From these studies, the nature of complementary structural features within receptors was inferred, and directions were established leading to the design and synthesis of a PTH inhibitor effective in vivo.[2,3]

The second approach, photoaffinity labeling, has led to direct characterization of PTH receptors[4] and comparison of receptors present in different species and target tissues.[5]

Development of a PTH Antagonist Effective *In Vivo*

PTH is an 84-amino acid single-chain polypeptide. All of the structural determinants necessary for full biological activity of PTH reside within the N-terminal one-third (residues 1—34) of the native hormone.[1,6] Within the molecule, there is a domain responsible for receptor binding, the region 3—34, and a small but critical region (positions 1 and 2) necessary for hormone action. Based on these findings, potent *in vitro* antagonists were prepared. An analog of the 3—34 region, [Nle-8,Nle-18,Tyr-34]bPTH-(3—34)amide, proved to be the most potent *in vitro* antagonist of PTH yet designed. *In vitro*, this compound is a true competitive inhibitor which has avidity for PTH receptors comparable to that of PTH (its inhibitory constant (K_i) is approximately equal to the affinity constant (K_m) for PTH). *In vitro*, this analog is devoid of PTH-like agonist activity.[1,7,8] However, in two *in vivo* studies,[9,10] the analog failed to display inhibitory properties; in other *in vivo* investigations, it demonstrated weak, but definite, PTH-like agonist properties in the dog,[11] the rat,[12] and in a canine isolated perfused bone system.[13] Taken together, these studies revealed the 3—34 analog to be an agonist for several parameters of PTH action, including stimulation of hypercalcemia, urinary phosphate and cAMP excretion, and production of 1α,25-dihydroxyvitamin D_3. The potency of this analog was less than 1% that of PTH on a molar basis.

Our approach to generating an *in vivo* PTH inhibitor was to modify the N-terminus through further truncation to eliminate the last traces of agonist activity from the 3—34 sequence

without losing completely avidity for PTH receptors.

A series of fragments of PTH containing N-terminal deletions were synthesized and evaluated in a renal radioreceptor assay for PTH.[14] Stepwise deletions at the N-terminus produce a progressive decline in receptor avidity, and identify the region 25—34, most distal from the N-terminus, as the principal binding domain of the hormone.[15] The intermediate sequence 7—34 binds well to PTH receptors, although avidity was diminished to 1/10-1/100th of the 3—34 sequence. In addition, substitution of norleucine for methionine is poorly tolerated *in vivo* leading to a 15-fold decline in potency of analogs containing this substitution.[16]

Using these directions established *in vitro* and *in vivo*, we selected for synthesis the analog [Tyr-34]bPTH-(7—34)-amide (Figure 1). The peptide was prepared by the solid-phase method of Merrifield[17,18] and purified by gel-filtration followed by semi-preparative high pressure liquid chromatography.[19]

The biological properties of this analog *in vivo* were evaluated for effects on PTH-stimulated urinary phosphate and cAMP excretion. Highly purified native bPTH-(1—84) (kindly

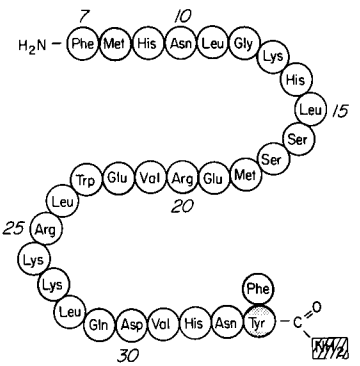

Fig. 1. Structure of [Tyr-34]bPTH-(7—34)amide. Tyrosine at position 34 replaces phenylalanine; the C-terminus is modified from a carboxylic acid to a carboxyamide.

provided by Dr. Henry T. Keutmann) or the antagonist, or a combination of both peptides was infused intravenously into vitamin D-deficient thyroparathyroidectomized rats and urine volume, phosphate, and cAMP levels determined every 30 min for 2.5-3.5 h. When inhibitor was administered, infusion began 1 h prior to beginning PTH infusion.[2] The inhibitor was infused at a molar dose-rate 200-fold greater than native PTH. Figure 2 depicts a compilation of data obtained from 40 test animals. The analog displayed no PTH-like agonist properties for phosphaturia. The analog inhibited greater than 70% of the maximal PTH-stimulated phosphaturic response. When phosphate is normalized for creatinine excretion,

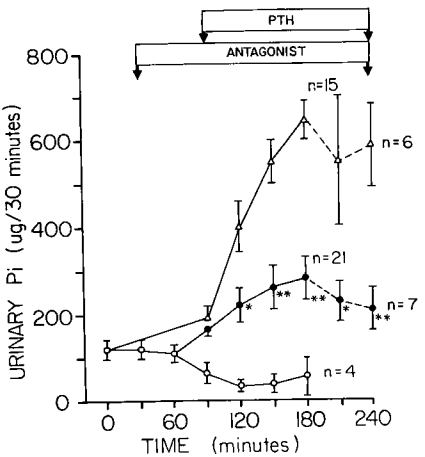

Fig. 2. Evaluation of the effects of [Tyr-34]bPTH-(7—34)-amide on urinary phosphate excretion in vivo in rats. Data shown are compiled from a total of 40 animals. Fifteen rats (Δ) comprised the control group and received native bPTH-(1—84) alone intravenously at a molar dose rate of 0.27 nmol/h. Twenty-one rats received the inhibitor at a dose rate of 54 nmol/h and PTH (0.27 nmol/h) (●). Analog infusion was begun one hour prior to beginning the PTH infusion. Four animals (o) received antagonist alone. Dotted line shows continuation of study for 6 animals receiving PTH alone and 7 receiving PTH and antagonist. Determinations in animals receiving antagonist and PTH differ significantly from animals receiving PTH alone, *$p < 0.025$ and **$p < 0.01$ by Student's t-test.

complete inhibition of phosphaturia is obtained. Comparable antagonist properties and lack of agonist activity were observed for the PTH-stimulated cAMP response (not shown). The analog can antagonize PTH action for at least 2.5 hours.

Further studies have recently been undertaken to assess effects of the 7—34 analog on the PTH-mediated calcemic response. A newly developed *in vivo* assay, based on the serum calcium-elevating response to PTH which occurs in acutely thyroparathyroidectomized rats, was used.[3] Given in intravenous boluses before, simultaneous with, and after a single bolus of PTH (antagonist:agonist=300:1 for each bolus), the antagonist completely blocked the calcemic response to PTH (Figure 3).

Hence, structure-activity studies have led to the design and synthesis of the first inhibitory analog of PTH active *in vivo*. This antagonist exerts its effects through competing with PTH for occupancy of receptor sites. When administered

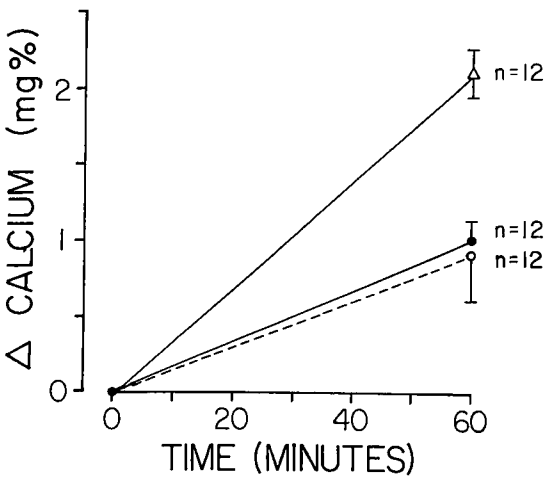

Fig. 3. Inhibition of PTH-stimulated calcemic response in acutely thyroparathyroidectomized rats. Data shown are for animals receiving 5 M.R.C. Units hPTH-(1—34) (Δ), [Tyr-34]-bPTH-(7—34)amide + hPTH-(1—34), molar ratio 300:1, (●), and controls (no peptide)(o).

in sufficiently high dosage, this analog inhibits completely the principal actions of PTH *in vivo*, namely stimulation of phosphaturia and urinary cAMP excretion, and elevation of serum calcium levels.

Photoaffinity-Labeling of PTH Receptors

To identify the PTH binding component in target tissues we developed a biologically active, photolabile, radioiodinated analog of PTH.[4] We used this photoaffinity radioligand to covalently label PTH binding elements in membranes derived from canine renal cortical tissue. A single membrane component, M_r = 70,000, labeled specifically and is presumed to represent the PTH receptor or a binding subunit of the receptor. Employing a different photolabile radioligand, Draper *et al.* have identified a PTH binding element with similar characteristics in canine renal membranes.[20]

The biological properties of PTH fragments and analogs are consistent across different bioassay systems, suggesting that the binding requirements of receptors may be conserved. To examine physicochemically PTH receptors and to compare receptors from different target tissues and species, we used photoaffinity methods to label the PTH binding component in human tissues of skeletal and dermal origin and in canine renal tissue.

Monolayer cell cultures derived from human giant cell tumors of bone respond to PTH by increasing intracellular and extracellular levels of cAMP. Although skin is not conventionally regarded as a PTH-target tissue, some monolayer cultures of fibroblasts from human foreskin and adult dermis are also PTH-responsive.

We have also employed the photoaffinity labeling technique to examine the mechanism of desensitization to PTH. Preincu-

bation with PTH of cells cultured from human giant-cell tumors or human fibroblasts results in subsequent loss of the cAMP response to PTH.[8] Desensitization in these cells must result ultimately from either decreased receptor number or availability, or uncoupling of receptor from its intracellular effector system(s). In order to address directly the role of receptor availability during desensitization to PTH, we used the photoaffinity radioligand as a probe of receptor binding capacity before and after desensitization.

A radioligand analog of PTH was reacted with cells. After photolysis, cells were extracted and the proteins denatured, reduced, and separated by electrophoresis (SDS-polyacrylamide gels) followed by autoradiography. A single membrane component, M_r = 70,000; was labeled specifically in cells cultured from skeletal and dermal tissue (Figure 4). To compare directly the physicochemical properties of the photoaffinity-labeled binding components detected in the canine renal membrane and intact human cells, we mixed labeled preparations from membranes or cells in pairs and ran each combination simultaneously on adjacent lanes of an SDS-polyacrylamide electorphoresis slab gel (not shown). Components derived from each system appeared identical in terms of molecular size.

Pre-incubation of cells derived from giant cell tumors with PTH results in desensitization to this hormone. Photoaffinity labeling of the PTH binding component was markedly diminished by pre-incubation with this hormone, indicating that a decrease in receptor number or availability is responsible for desensitization to PTH. Pre-incubation with a PTH antagonist which fails to produce desensitization does not result in diminished photoaffinity labeling of the binding component. This result suggests that simple occupancy of receptors does not result in loss of the PTH binding component and that post-receptor events related to hormonal action are required for desensitization.

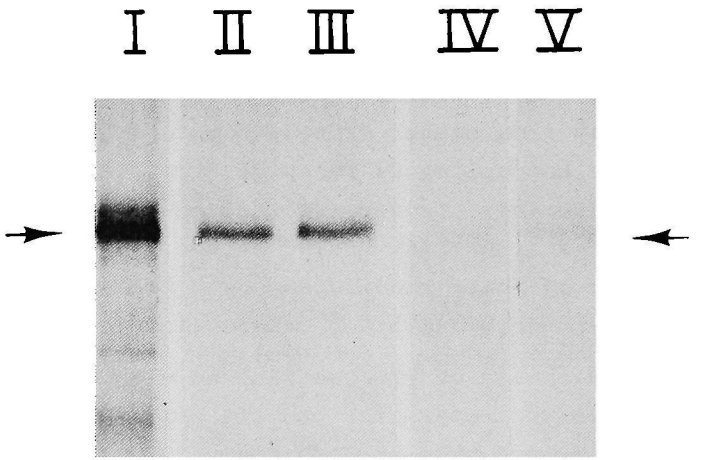

Fig. 4. Identification of the PTH binding component in canine renal membranes and cells derived from human giant cell tumors of bone and human dermis. Renal membranes and intact cells were incubated with the photolabile PTH radioligand in the presence or absence of competing hormone. Radioautograph displays component of comparable mobility (arrow) in renal tissue (lane I) and human cells of skeletal (lane II) and dermal (lane III) origin. Addition of excess PTH prior to light exposure results in almost complete disappearance of the labeled component in the human cells, lanes IV (skeletal cells) and V (dermal cells).

Conclusions

PTH receptors and the nature of hormone-receptor interaction have been investigated by structure-activity studies and by direct covalent labeling of receptors. These studies have led to the development of the first PTH antagonist effective *in vivo*.[2] These studies establish feasibility and future directions for design of PTH inhibitors.[2] Attempts are now underway to design more potent antagonists. Provided agonist properties are not restored to inhibitory molecules by the process, further N-terminal extension may generate more

effective antagonists: analogs of sequence length 6—34 or 5—34 may have enhanced inhibitory potency.

Finally, direct characterization of PTH receptors demonstrates that receptors are conserved across species and target tissue. The photoaffinity labeling approach also provides direct evidence supporting a decrease in receptor number or availability as the mechanism responsible for desensitization of human cells to PTH. The identification of a specific PTH binding component in human cells (which can be grown in monolayer culture) should facilitate harvesting, further characterization, and ultimately isolation of the human receptor for PTH.

Acknowledgements

This review describes studies performed in collaboration with several investigators. The contribution of Drs. Samuel H. Doppelt, Steven R. Goldring, Noboru Horiuchi, Stephen M. Krane, Robert M. Neer, and John T. Potts, Jr. is gratefully acknowledged. George A. Tyler, Gary L. Shepard, Merrilee Roelke, Carolyn Rourke, and Panfilo Federico provided expert technical assistance. This work was supported in part by Grant No. AM11794 from the N.I.A.D.D.K.

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REGULATION OF CELL GROWTH BY POLYPEPTIDE FACTORS: AGENTS OF THE NERVOUS SYSTEM

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Cell growth, both *in vitro* and *in vivo*, is a complex phenomenon involving a variety of nutritive and regulatory substances as well as environmental factors that include contact with other cells and extracellular substances. In terms of response, there are three main categories: (a) cells which are constantly dividing, (b) cells which have the capacity to divide but exist in an arrested state (G_0) until properly stimulated and (c) cells which have lost the capacity to divide and are therefore in their terminally differentiated state. It is the cells of the second category that have been most useful in understanding features of growth regulation but all three categories have contributed useful information.

Among the various stages that appear to be important for the control of growth are a subset of hormones known collectively as growth factors. These are primarily, although not exclusively, polypeptides with most known entities possessing molecular weights in the range of 5-30 kDa.[1] Not unexpectedly they have a broad spectrum of target tissues that can vary in their responsiveness and can be delivered by systemic transport and/or diffusion. In this regard, Sporn and Todaro[2] recognized three basic types of hormones and growth factors, which they termed autocrine,

paracrine and endocrine. Both autocrine and paracrine substances reach their target tissue by diffusion, which in the former case is the cell of origin and in the latter a different cell type. Endocrine systems, as classically defined, depend upon systemic transport. At our present level of understanding, these differences are not further manifested by differences in mechanism of action. The majority of polypeptide growth factors appear to operate in a paracrine fashion.

One particular set of growth factors that has received considerable attention in the last several years are those found in the nervous system. They can be subdivided into four principal classes formed by substances synthesized in neuronal cells which act on either neuronal or non-neuronal targets and substances elaborated in non-neuronal cells which can in turn act on neuronal or non-neuronal cells. Further subdivisions are possible if one takes into account whether the neurons are derived from the peripheral or central nervous systems and whether the non-neuronal cells are located inside or outside either system. Potential examples of all of these have been identified,[3] albeit that in a number of cases the identification of the cells of origin has not been made. This is particularly true of substances that are derived from neuronal tissue; the factors that are elaborated in non-neuronal cells are much better defined. By far the best studied example is nerve growth factor (NGF)[4,5] but there are a considerable number of other neurotrophic factors that have now been reported.[3,6] In a like fashion, a number of polypeptide growth factors that can affect glial cells[1,6,7] represent the latter grouping.

NGF has been particularly useful in serving as a prototype of polypeptide growth factors synthesized in non-neuronal tissues that act

on target neurons. This group, which is a specialized subset of paracrine agents designated retroneurocrine, apparently act by interacting with presynaptic membrane receptors followed by internalization and retrograde axonal transport to the cell body.[8] In this fashion they provide trophic stimulation of the neuron before, during and after synapse formation. It follows from such a model that the internalization process plays a crucial, but not necessarily exclusive, role in the transmission of the hormonal signal. However, definitive evidence is still lacking and thus mechanism(s) by which the internalized hormone modulates response is only speculatory.[1,4,5,9]

Although detail regarding the functural properties of NGF (as well as other neurotrophic hormones) is still sparse, there is considerable new information regarding the biosynthesis of the molecule.

Biosynthesis of Nerve Growth Factor

Murine submandibular gland NGF occurs as a complex (denoted 7S) of three types of polypeptide chains.[10] Each is elaborated by a separate gene, with the mature form produced by limited proteolysis from a precursor molecule.[11-13] The β subunit, which possesses all of the hormonal activity of the complex,[4,5] is composed of two identical polypeptide chains of approximately 13,200 molecular weight.[14] In the absence of α and γ subunits, it occurs as a tightly bound noncovalent dimer that is considered to be the active form.[15] As judged by sequence analysis, the γ subunit is an arginine-specific peptidase of the serine family.[16] Based on the arginine specificity, the presence of this residue at the C-terminal position of the β chain and a proposed

relationship to insulin that involved gene duplication, Angeletti and Bradshaw[14] suggested a possible precursor processing role for this subunit. Berger and Shooter[17] identified a precursor to β NGF of 22 kDa that could be processed by the γ subunit, among other proteases, to the mature form. However, elucidation of the complete structure of the precursor molecule, as deduced from the nucleic acid sequence of a cDNA clone, revealed that the mature protein segment was situated at the carboxyl terminus.[11,12] Only two residues (Arg-Gly) extended past the previously identified C-terminal arginine residue. The remainder of the precursor structure, containing either 121 or 187 amino acids depending on correct identification of the initiator methionine position,[11,12] is removed sequentially or en bloc by enzymes[15] yet to be identified. It is entirely possible as has been suggested,[11] that the γ subunit has this function in addition to removing the C-terminal dipeptide. However, some doubt concerning this role for the γ subunit has been engendered by the observation that guinea pig prostate NGF is completely lacking in γ subunit as judged both by enzymatic assays and Northern blot analysis.[18,19] A similar finding has been recently reported for NGF isolated from human fibroblast.[20] If the precursor to the β subunit in the guinea pig prostate is similar to that identified from the murine submandibular gland, it would suggest a processing mechanism that does not involve the γ subunit.

The α subunit of the 7S complex, which has no known biological function, has also been identified as a serine protease by amino acid sequence analysis.[13] However, it lacks enzymatic activity against several potential substrates and the putative active site serine is not normally reactive with diisopropylfluorophosphate. The as yet

unidentified structure blocking the amino terminal residue probably accounts for this lack of catalytic activity. Partial sequence data indicates that the α subunit is closely related to the γ subunit but does not explain its role in the 7S complex.[13] Like the γ subunit, its existence may be limited to the submandibular gland and be unrelated to the retroneurocrine function of β NGF.

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A MODEL FOR THE ACTIVATION AND DESENSITIZATION OF VASOPRESSIN-SENSITIVE ADENYLATE CYCLASE IN A CULTURED PIG KIDNEY CELL LINE ($LLC\text{-}PK_1$)

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Introduction

The antidiuretic action of the peptide hormone vasopressin is mediated by the stimulation of adenylate cyclase and the production of cAMP (1). Adenylate cyclase is an ubiquitous enzyme that mediates the production of cAMP in response to a large number of hormones and neuro-transmitters in different cells. During the last decade it has become clear that adenylate cyclase is part of a complex regulatory system consisting of at least three components structured within the lipid framework of the cell membrane (2). Located at the outer membrane surface is the receptor (R) containing a specific site for the binding of hormone. At the inner surface of the membrane are the catalytic unit (C) and the guanine nucleotide regulatory component (G). The latter contains a GTP binding site(s) and is responsible for mediating the effects of GTP, hormones, sodium fluoride (NaF), and cholera toxin on the activity of C (3). A general scheme of hormone-receptor adenylate cyclase interactions has been proposed (4,5). While quantitative differences occur in the kinetics of the interactions in various cell types, the following sequence of events appears to be qualitatively similar in all systems studied.

$$H + R \underset{}{\overset{(a)}{\rightleftharpoons}} HR + G_{GDP} \overset{(b)}{\rightleftharpoons} HRG_{GDP} + GTP \overset{(c)}{\rightleftharpoons} HRG^*_{GTP} \quad \text{(eq 1)}$$

$$HRG^*_{GTP} \overset{(d)}{\rightleftharpoons} HR + G^*_{GTP} + C \overset{(e)}{\rightleftharpoons} G^*_{GTP}C^* \overset{(f)}{\rightleftharpoons} G_{GDP} + C + Pi$$

Upon binding of H to R, an HR complex is formed (reaction a). HR then interacts with inactive G which contains a bound GDP (reaction b). This results in the hormone-stimulated release of bound GDP and the binding of GTP (reaction c). The activation of G appears to be the rate limiting step in the activation of the enzyme (4). The complex, HRG^*_{GTP}, may activate C directly or the HR complex may dissociate from the now activated G (reaction d) allowing G^*_{GTP} to combine with inactive C (reaction e) to form an active enzyme complex responsible for the generation of cAMP from MgATP. G can also be activated independently of hormone by the addition of GTP or its analogs, NaF or cholera toxin. Those agents proposed to directly activate C are less well defined but have been claimed to include MnATP (3), Ca^{++}-calmodulin (6), and forskolin (7). Inactivation of the enzyme (reaction f) results from the hydrolysis of GTP to GDP in the G unit, presumably the result of the endogenous GTPase activity of G itself (5). Furthermore, hormone-induced alterations in the state of coupling of the receptor to the other components of the enzyme may be associated with a shift in the affinity of the receptor to the hormone (7). Recently, it has also been suggested that R, G or C units may exist as inactive oligomers in the basal state and that enzyme activation is associated with disaggregation of oligomers into active monomers (2). This "disaggregation-coupling" model of adenylate cyclase activation was based upon experimental evidence obtained using the radiation inactivation of glucagon-sensitive adenylate cyclase in hepatic cell membranes (9).

The above interactions have been explored in studies utilizing a cultured pig kidney epithelial cell, $LLC\text{-}PK_1$, which responds to vasopressin by increasing adenylate cyclase activity twenty-fold (10). The availability of this cell line has

allowed study of hormone binding and cyclic-AMP production in intact cells under various conditions as well as adenylate-cyclase activation in particulate fractions derived from these cells. Based upon the results of such studies which have been previously reported by Roy et al. (11), we now propose a model for the activation and desensitization of adenylate cyclase in this hormone-receptor system. This model is consistent with many of the features of the "disaggregation-coupling" model originally proposed by Rodbell and coworkers, and has the additional strength of being derived from studies in intact cell. Also, in preliminary experiments using target analysis of radiated intact cells we have obtained further supportive evidence for this model.

Binding Studies in Intact Cells

The techniques used for establishment of LLC-PK_1 monolayer cell cultures, and assay of vasopressin binding and cAMP accumulation have been published previously (12). The basic characteristics of the vasopressin receptor reported in these studies were similar to those found in rat and bovine renal membranes. However, it was possible to observe the regulation of receptor properties by hormone and other effectors in these intact cells. Association and dissociation rate constants ranged from $1.0 - 1.4 \times 10^7$ M^{-1} litre min^{-1} and 0.125 - 0.343 min^{-1} respectively yielding a derived K_D of 11-24 nM. Data obtained using a 10,000 g particulate fraction gave similar values indicating that there was no access problem for the ligand in monolayers cells. Scatchard analysis of equilibrium binding data yielded a curvilinear plot consistent with a non-homogeneous population of binding sites. Such data have been interpreted as consistent with negative cooperativity. The results of reversibility studies however, make this possibility unlikely since similar values were obtained for the initial dissociation rate following removal of radioligand both

in the absence and presence of excess unlabelled hormone. Studies using various analogues of vasopressin gave competition curves which were all monophasic and parallel making it unlikely that there were two independent populations of binding sites. Furthermore, when dose-dependency was studied over a wide range of Mg^{++} concentrations the ratio of affinities for the two sites was constant, making the possibility of two independent sites unlikely. Additional evidence against the presence of two or more independent binding sites was obtained from studies in EDTA suspended cells. Despite an association time curve comparable to that seen on monolayer cells, Scatchard analysis of the dose-dependency revealed a homogeneous population of binding sites without loss of total binding capacity. An alternative explanation consistent with all of the binding data was the possibility of hormone induced "receptor transition". According to this hypothesis the receptor exists as an oligomer with identical subunits (R) of high affinity (K_1) prior to occupancy. With the binding of hormone to one site of the oligomer the affinity of the other sites are lowered to a new value, K_2. For a dimer this is summarized as:

$$\underset{}{H + RR} \overset{K_1}{\rightleftharpoons} \underset{(a)}{HRR} \rightleftharpoons \underset{(b)}{HRR'} \overset{H\ K_2}{\rightleftharpoons} \underset{(c)}{HRR'H} \qquad \text{(Eq 2)}$$

The hypothesis that the receptor is a dimer is the simplest assumption for the receptor transition model that imposes maximum statistical contraints by limiting the number of parameters. It is also possible to allow the receptors to exits as higher order aggregates without changing the statistical evaluation of the data.

STUDIES OF CAMP PRODUCTION AND ADENYLATE CYCLASE ACTIVITY

Roy and coworkers subsequently examined the physiologic relevance of receptor transition to hormone induced adenylate

cyclase activation and desensitization (10). Exposure of cells to maximal doses of vasopressin for several minutes resulted in greatly reduced adenylate cyclase activity. This uncoupling or desensitization of the hormone response was specific for vasopressin since NaF and GTP activation of the enzyme were unaffected. The time course for desensitization was slower than hormonal binding consistent with the conclusion that receptor transition to R' is not the rate limiting step in desensitization. In addition the concentration range for the development of desensitization paralleled the concentration range for occupancy of the low affinity site (10).

The kinetics of hormone induced cAMP accumulation in intact monolayer cells at various concentrations of hormone were also consistent with an activation and desensitization process of the enzyme linked to hormone induced receptor transition. Initial exposure to a maximal concentration of hormone which rapidly occupied both high and low affinity binding sites resulted in a decreased rate of cAMP production by 6 minutes (11), consistent with the development of a desensitization process. The maximun cAMP response of the intact cells is observed at vasopressin concentrations leading to less than 50% of hormonal occupancy. A significant decrease in cAMP production is observed with uncoupling of greater than 50% of the receptors.

In subsequent studies intact cell conditions were manipulated to further explore the causal relationship between activation and subsequent desensitization of the enzyme. Cells incubated in the presence of hypertonic NaCl demonstrated a homogeneous population of low affinity (R') receptors presumably because receptor transition occurred before hormone addition (13). This receptor transition was dissociated from desensitization which did not occur. Despite the apparent R' form of the receptor, the initial rate of maximal hormone stimulation of adenylate cyclase was at least as great in these cells as in control cells, and prolonged hormone stimulation

(several minutes) was more effective in generating cAMP due to the prevention of desensitization. This supports the notion that both the R and R' form of the receptor are active in coupling to adenylate cyclase. Thus it appears that receptor transition is not a sufficient condition for desensitization to occur, but is likely an early step in the sequential activation of the enzyme and subsequent desensitization following occupancy by hormone.

The Model

From the foregoing considerations, it is clear that a model for adenylate cyclase activation and desensitization by vasopressin in LLC-PK_1 cells must take into account the following features:

1. There is a large discrepancy between the circulating low levels of the hormone (pM) and the affinity of the hormone to its receptor (nM) indicating the presence of functional "spare" receptors. Correspondingly, a nonlinear coupling relationship is observed between receptor occupation by hormone and stimulation of adenylate cyclase.

2. Occupancy of one receptor (R) is followed by transition of nearby receptors to a lower affinity (R') state (site-site interaction, possibly within an oligomer).

3. Both the R and R' form of the receptor appear to be initially active in stimulating adenylate cyclase.

4. Vasopressin stimulation of adenylate cyclase is reduced after prior exposure of intact cells to the hormone (desensitization).

5. The desensitization process follows the concentration range for occupancy of the low affinity site but is much slower

than enzyme activation and is only slowly reversible (72h).

The model we propose is similar to the "disaggregation coupling" model of Rodbell (2) with some differences for the system we are studying. According to this model the receptor and enzyme exist in an oligomeric configuration prior to hormonal stimulation. Upon binding of H to one unit of the oligomer with affinity K_1 (R form) the remaining R sites undergo receptor transition to the R' form. In this state G_{GDP} is capable of interacting with GTP and the catalytic unit is activated. The exact configuration of the activated catalytic unit with respect to the other components of the receptor enzyme complex is not specified. Activation of the enzyme is associated with disaggregation of the oligomer which in turn is followed by the establishment of the desensitizated state. The exact constituents of the monomers (R,G,C) are not specified and may vary, providing a source for the recycling of individual receptor-enzyme proteins. GTP hydrolysis is responsible for the turn-off of enzyme activity.

Schlegel et al. used the technique of radiation inactivation to probe the state of assembly of the glucagon responsive adenylate cyclase of hepatic cell membranes (10). Based on this approach, it was proposed that the unstimulated state of the receptor-enzyme complex involved a tetrameric aggregate of R and G units in an inactive state. According to this formulation hormone induced disaggregation is associated with coupling to or activation of the catalytic unit. Our modified model emanates from considerations raised by the binding, activation and desensitization data for the vasopressin-responsive adenylate cyclase system in LLC-PK_1 cells. The presence of an aggregate of receptors in the unstimulated state provides the best model to explain these data but does not provide proof of disaggregation with hormonal stimulation and also does not probe the components of the oligomer. In order to explore the possibility of either disaggregation of the oligomer with acti-

vation or desensitization of the enzyme we have also used the technique of radiation inactivation and applied it to intact LLC-PK_1 cells in monolayer. In preliminary experiments, cells which have not been exposed to vasopressin or other activators of adenylate cyclase were irradiated and particulate fractions prepared for assay of basal and vasopressin-stimulated adenylate cyclase following irradiation. The activity dose-relation was compared with that obtained for cells which had been stimulated with vasopressin or other activators of adenylate cyclase. We found a marked difference in the shape of the activity dose relations. Unstimulated cells gave complex curves for the radiation dose-basal cyclase activity relation with prominent upward convexity. Membranes prepared from vasopressin stimulated cells and assayed in the presence of hormone gave a simple exponential decay of enzyme activity with radiation dose. With the help of Dr. Alan Verkman of the Biophysical Laboratories at Harvard Medical School, we derived the theoretically predicted activity-dose relation for oligomeric enzyme systems and tested the predicted effect of a variety of physical assumptions on this relation. According to this formulation, the predicted activity-dose relation for an enzyme existing in two states, that of an oligomer with limited activity and that of a monomer with greater activity, corresponds to the convex curve we observed for the unstimulated cells. This formulation assumes that only the monomeric unit hit within the oligomer is inactivated with the release of the remaining active monomers, and also assumes complete equilibration between oligomers and monomers following irradiation. The simple exponential decay observed for the hormone activated cells corresponds to the disaggregated monomeric form of the enzyme. It should be noted that concave upward activity-dose relations such as those observed by Schlegel et al. (9) in unstimulated hepatic cell membranes, correspond best to a model assuming the presence of two or more non-interacting target enzymes. We are currently determining the vasopressin concentration and time dependence of the change from a complex to a

simple exponential activity dose relation in order to discern the precise relationship of disaggregation to enzyme activation and desensitization. Evidence for an oligomer in the unstimulated state as assessed by assay of enzyme activity suggests that the catalytic unit also must be involved in the oligomer. Definition of the precise coupling relationship of R, G and C units within the oligomer and their interaction with enzyme activation will require careful studies of the kinetics of enzyme activation following manipulation of R, G and C.

Consequences of the Model and Conclusions

The presence of a highly ordered oligomeric structure suggests that the receptor-enzyme complex resides within selected lipid domains of the plasma membrane (13). In this sense, the activation of adenylate cyclase by fatty acids (14), microtubule disruptors (15), and methylation of phospholipids (16) might result from the release of constraints on receptor-enzyme components within localized submicrometer domains. An oligomeric structure of the hormone enzyme complex allows for homotropic subunit interactions such that minimal occupation by hormone may cause near-maximal formation of the functionally active monomeric unit. Desensitization which follows receptor affinity transition and initial enzyme activation most likely also requires disaggregation into monomers. While desensitization is only observable at pharmacological concentrations of hormone, it is possible that its physiological signficance reflects a need to "recycle" the active state of individual components of the receptor-enzyme complex which is facilitated by disaggregation of the complex. Since so few receptors are ever occupied by hormone at physiological concentrations, such a desensitization or recycling of less than 1% of receptors would not impede the cells ability to respond to hormone.

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SPECIFIC INTERACTIONS BETWEEN PEPTIDE HORMONES AND ARTIFICIAL LIPID MEMBRANES

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Introduction

Peptide hormones and neuropeptides elicit their biological action by associating saturably and reversibly with specific receptors in the outer membranes of their target cells. The lipid phase of the membrane acts as a matrix for the receptors and is essential for the functionality and the topological arrangement of the receptor proteins. Is it also responsible for the proper function of peptide agonists?

We wondered whether the lipid phase of the target cell membranes might possibly interact as an antenna with peptides to capture them from the surrounding fluid as suggested by Adam and Delbrück,[1] and Berg and Purcell,[2] and to induce secondary structures and topological arrangements that would facilitate rapid and correct receptor contacts.[3,4]

Conformational aspects of this problem have been intensively investigated.[5] Unfortunately, most spectroscopic methods fail with membraneous particles larger than micelles. However, Pitner and Urry[6] introduced trifluoroethanol as a solvent promoting conformational transitions in peptides and proteins akin to those caused by membrane surroundings. With

this tool, valuable information about conformational changes in the isotropic and symmetrical surroundings of water and trifluoroethanol has accrued, e.g.[7 8 9] The observed secondary structures were usually assigned to particular segments of the peptide primary structures by parallel investigations of the corresponding synthetic fragments[7 8 9] and by application of the Chou and Fasman rules.[10]

Quite different secondary structures, specific orientations, and topological arrangements of the peptides might be induced by the anisotropic and unsymmetrical liquid crystalline ultrastructure of lipid bilayer membranes, provided that the peptides are actually adsorbed. We addressed this problem using three methods:

Capacitance minimization.- This method[11] detects the adsorption of charged peptides to planar bilayers composed of neutral lipids (e.g. lecithin). Amounts of adsorbed charges and thermodynamic parameters can be estimated.[12]

Vesicle-mediated hydrophobic photolabelling.- The extremely hydrophobic photolabel, [^{125}I]3-trifluoromethyl-3-(m-iodophenyl)diazirine, [^{125}I]TID, was introduced by Brunner and Semenza for the study of proteins in biological membranes.[13] By carefully observing certain limitations of the method, it is possible to characterize hydrophobic contacts between peptides and lipid vesicles (liposomes), and to distinguish them from the labeling caused by hydrophobic peptide-[^{125}I]TID aggregates in solution and on the vesicle surface.[14 15] Hydrophilic peptides or segments that are adsorbed to the vesicles by electrostatic interactions, only, are very weakly labeled.

Infrared attenuated total reflection spectroscopy (IR-ATR).- This method[16] can be used to study lipid bilayer membranes and peptides associated with them in the dry and in the hydrated states.[17 18] Position and shape of the IR bands allow the detection of peptide secondary structures; their orientation with respect to the membrane plane can be determined with polarized radiation. Adsorption from and escape into the

aqueous surroundings, penetration through the membranes, and ^{1}H-^{2}H exchange kinetics can easily be studied.[19 20]

Results and Discussion

Adrenocorticotropin-(1-24)-tetrakosipeptide, ACTH(1-24), +SYSME(-)HF-R(+)WGK(+)PVGK(+)K(+)R(+)R(+)PVK(+)VYP- (6+), and its component peptides, ACTH(1-10) (0), and ACTH(11-24) (6+) were investigated by all three methods. ACTH(5-24), ACTH(7-24), ACTH(1-13)amide, acetyl ACTH(1-13)amide (α-MSH), dynorphin(1-13), +YGGFLR(+)R(+)IR(+)PK(+)LK(+)- (5+), leuenkephalin, +YGGFL-, and a number of synthetic enkephalin analogs were studied by hydrophobic labeling and are awaiting IR-ATR.

ACTH(1-24), ACTH(1-10), ACTH(11-24).- Capacitance minimization shows that ACTH(1-24) is readily adsorbed to lecithin (PC) and to dioleoylphosphatidylcholine (DOPC) bilayers from 0.1 mM solutions. With natural membranes carrying 10% negative charges, the Boltzman distribution predicts a similar adsorption from sub-micromolar concentrations.[21] Thus, an antennae-like capture with reduction of dimensionality for the search of receptors[1 2] is a likely process in both types of membrane. ACTH (11-24) was not adsorbed and ACTH(1-10) (zero net charge) could not be observed.

IR-ATR and vesicle-mediated hydrophobic photolabeling gave consistent results that led to the following model:[15 20 22] ACTH(1-24) is adsorbed to membranes by electrostatic and hydrophobic forces (this was supported by the results of equilibrium dialysis). Its C-terminal, pharmacological 'address' segment (residues 11-24) remains on the membrane surface in an irregular extended conformation in which the planes of the peptide bonds are oriented perpendicularly to the plane of the membrane. The N-terminal, pharmacological 'message' segment (residues 1-9) assumes a helical conformation (in agreement with trifluoroethanol studies[7 8 9]) that penetrates into the hydrophobic layers of the membranes (Gly^{10} might be

part of a 'hinge'[4]). This specific type of interaction is favored by low ACTH(1-24)/lipid molar ratios and occurs by adsorption of the peptide from aqueous solutions in which it has a random secondary structure. ACTH(1-10), despite its hydrophobicity, does not interact with membranes and is not labeled. If forced into dry membranes, it assumes a β-pleated sheet structure oriented perpendicularly to the membrane planes, but escapes immediately on contact with water. ACTH(11-24) interacts electrostatically with anionic membranes, but is not labeled. It does not interact with neutral membranes, but if it is sandwiched in between hydrated layers, it shows the same conformational characteristics as the corresponding segment (residues 11-24) in ACTH(1-24).[23]

It thus appears that the amphiphilicity provided by the combination of the more hydrophobic message with the strongly hydrophilic address is responsible for the adsorption of ACTH(1-24) to membranes and also for the penetration by the message in its helical structure. This agrees with the pharmacological observations: inactive peptides ACTH(1-10) and ACTH(11-24) in assays for steroidogenesis, highly potent combination of message and address in ACTH(1-24). The perpendicular helix imbedded in the membrane might prefer receptors with their recognition sites exposed 'laterally' to the hydrophobic layer of the outer membrane leaflet of the target cells. This is consistent with a helical message model derived from pharmacological observations.[4] ACTH(1-10) is as active as ACTH(1-24) in behavioural assays.[4] This could mean that, on its brain target cells, the receptors expose their recognition sites towards the aqueous phase to trap the ACTH(1-10) that cannot interact with the lipid phase of the membrane. Perhaps these receptors have a requirement for the β-pleated sheet structure that is quite obviously preferred by ACTH(1-10).[20]

ACTH(5-24, ACTH(7-24), ACTH(1-13)amide, acetyl ACTH(1-13)amide.- The peptides containing the address, residues 11-24, are strongly labeled in the presence of lipid vesicles. Chymotryptic hydrolysis and Edman degradation revealed a hydrophobic interaction of the residual

message of the antagonist, ACTH(7-24). The partial agonist, ACTH(5-24), was not investigated in this respect. The intensity of vesicle-mediated hydrophobic labeling of the investigated ACTH peptides correlated excellently with their EC(50) and IC(50) values for in vitro steroidogenesis (Figure 1). The correlation was equally excellent for the inhibition of a synaptosomal protein kinase.[24]

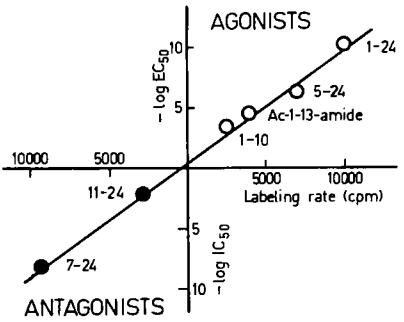

Fig. 1. Correlation between in vitro steroidogenic potency and vesicle-mediated labeling rate of corticotropin peptides (lipid:phosphatidic acid-lecithin 1:9 mixture).

These results indicate that model membrane studies have a good chance of being biologically significant.

Dynorphin(1-13) and enkephalin.- Dynorphin(1-13) has been subdivided into a pharmacological message and an address,[25] analogously to ACTH and ACTH(1-24). Vesicle-mediated hydrophobic photolabeling revealed that the message (YGGF) enters the hydrophobic layers, but that the address (LRRIRPKLK) remains in aqueous surroundings.[14,15] Thus the pharmacological behaviour and the vesicle interaction agree, much the same as for ACTH(1-24). IR-ATR studies are in progress.

Enkephalin peptides and their amides have very weak hydrophobic interactions with lecithin-phosphatidic acid and phosphatidylserine vesicles.

The free acids, e.g. leuenkephalin, interact more weakly still with brain cerebroside sulfate vesicles. However, the amides react very strongly with this type of vesicle. The enkephalin free acids are zwitterionic and the amides carry one positive charge. This cannot be the reason for their different behaviour towards phosphatidylserine and cerebroside sulfate vesicles, because both vesicles are strongly negatively charged. Furthermore, dynorphin(1-13) interacts with equal strength with all three types of vesicle. The free acids, however, are preferential δ-opiate receptor agonists, whereas the amides are preferential μ-agonists. Another agonist that is known to be adsorbed more strongly to cerebroside sulfate than to phosphatidylserine[5] is morphine. Thus, our results[14,15] suggest a strong lipid head group specificity of opioid peptides that might become of practical value for distinguishing κ, δ, and μ agonists from one another by their interaction with artificial membranes.

Conclusions

Capacitance minimization and, especially, vesicle-mediated hydrophobic photolabeling and infrared attenuated total reflection spectroscopy are excellent tools for studying molecular interactions between peptides and model lipid membranes.

The hydrophobic interaction of peptides with model membranes depends on their amphiphilic character (in our case, caused by a message-address combination, in others, perhaps by the induction of amphiphilic helices[26]).

Hydrophobic interactions and head group specificity correlated well with known pharmacological properties of the investigated peptides, suggesting the biological significance of such model studies.

Solution and crystal conformations of peptide agonists need not

necessarily reflect the secondary structure requirements of the receptors.[3] These may correspond more closely to the structures present in trifluoroethanol and, especially, in model lipid membranes.

A new requirement of receptors, open for future studies, emerges from our work: the topological deployment of peptides on and in the target cell membrane.

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Synthetic Epidermal Growth Factor Fragments Induce Phosphorylation of Endogenous Membrane Proteins

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Introduction

Epidermal growth factor (EGF) is a potent stimulator of cellular proliferation and inhibitor of gastric acid secretion. EGF is a single polypeptide of 53 residues with three disulfide bonds, defining three looped regions from residues 1 to 20, 14 to 31 and 32 to 53. To date, only the importance of the C-terminal sequence of EGF 47 to 53 has been studied in some detail. Derivatives of EGF and urogastrone lacking five or six residues exhibit a marked reduction in both receptor affinity and mitogenic activity in vitro. Despite the reduced potency, EGF(1-47) is capable of inducing a full biological response. Therefore, the residues critical to the intrinsic activity of the molecule are contained within the 1 to 47 sequence (for a recent review on EGF see Ref. 1). Recent studies on the mechanisms of mitogenic action of EGF indicate that upon binding to its diffusely distributed receptor, rapid clustering of the EGF receptor occurs. The EGF-receptor complexes are then internalized, and the complexes form vesicles inside the cell. Concurrently, EGF induces

rapid responses such as the uptake of ions[2], enhanced phosphorylation of various membrane proteins including the autophosphorylation of the EGF-receptor[3], changes in cell morphology[4], and the reorganization of cytoskeletal proteins[5]. How these early cellular responses to EGF relate to the mitogenic process, and the way in which EGF activates its receptors to initiate these responses are unknown . We have prepared various sequence overlap synthetic EGF fragments in order to define the mechanism of EGF stimulation for the early responses mediated by EGF-receptors.

Results and Discussion

Using the Merrifield solid phase method, the following fragments were prepared:[Ala-14](1-20), [(ACM)Cys-20,31](20-31), [Ala-20](14-31),[(ACM) Cys-31](25-42), (32-48), and (43-51). The peptides were purified by ion exchange chromatography on DEAE-cellulose or CM-cellulose. Purity of the synthetic peptides was monitored by HPLC using Waters Associates μBondapak C18 analytical columns with water/acetonitrile gradients containing 0.1% CF_3COOH. Where further purification was necessary, chromatography using semipreparative μBondapak C18 columns was performed. The levels of EGF-receptor autophosphorylation enhancement by the synthetic peptides were determined as follows: shed membrane proteins(30 μg) from human epidermoid carcinoma A431 cells prepared as described by Cohen et. al. were preincubated with synthetic peptides at 25°C for 25 minutes and for 5 minutes at 0°C. Then, an aliquot of [γ-^{32}P]ATP was added and incubated an additional 10 minutes at 0°C. The samples were then electrophoresed on 5-15% SDS/PAGE gradient gels, and the autoradiograms of the gels were taken. Figure 1 illustrates the level of EGF-receptor autophosphorylation by EGF (lane 1), and by the synthetic peptides. Note that only the peptides containing the residues 20 to 31 enhanced the levels of EGF-receptor phosphorylation (a main band of lane 1 corresponds to 170,000 dalton EGF-receptor marked with an arrow). In a separate experiment, relative receptor affinities of the synthetic fragments as compared with native EGF were determined using human foreskin fibroblasts. Again only the peptides containing the residues 20 to 31 showed any ability to compete for ^{125}I-EGF

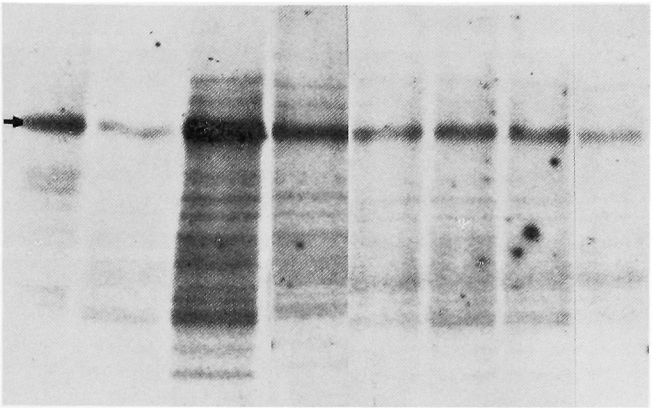

Fig. 1. Enhancement of EGF-receptor autophosphorylation induced by 33nM EGF, 350 μM [Ala-14](1-20), 350 μM [Ala-20](14-31), 125 μM [(ACM)Cys-20,31](20-31), blank, 100 μM [(ACM)Cys-31](25-42), 210 μM (32-48), 210 μM (43-51) respectively for lane 1 to 8. Arrow in lane 1 indicate the position for 170,000 dalton EGF-receptor.

in binding to EGF-receptors. The concentration for [Ala-20](14-31) at which the peptide exhibited a half maximal binding competition with ^{125}I-EGF was about 75 μM. The same synthetic peptides also induced the delayed response of EGF(induction of DNA synthesis) in human foreskin fibroblasts as judged by the incorporation of [^{3}H]thymidine into DNA. In order to confirm that synthetic peptide-induced early and delayed responses are indeed receptor mediated cellular responses, we turned to yet another early receptor response to EGF binding: receptor clustering(see reference 6 for detailed experimental procedures). Again only the peptide with 20 to 31 residues initiated the receptor-clustering (see Fig. 2). Therefore, the induced cellular responses by the peptides containing the residues 20-31 are receptor mediated events. Further, the activation of EGF-receptors by EGF fragments leads to induction of early and delayed responses. The levels of an early cellular response to EGF, phosphorylation of membrane proteins, is related to receptor occupancy, not the size of ligand. It appears that there is no segregation of the residues responsible for receptor binding and receptor activation. Further,the residues 20 to 31 seem

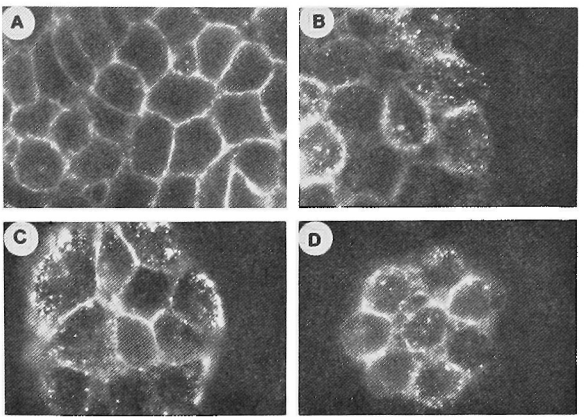

Fig. 2. Fluorescently labelled EGF-receptors : patches of fluorescence indicate the clustering of EGF-receptors induced by (B) 10nM EGF,(C) 430 μM [Ala-20](14-31),(D) 190 μM [(ACM)Cys-20,31](20-31) and (A) blank with only fluorecently labelled monoclonal anti EGF-receptor antibody added.

to play a key role in specificity of receptor binding, activation of phosphorylation and induction of DNA synthesis.

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REACTIVE ANALOGS OF ARGININE VASOPRESSIN AS TOOLS FOR RECEPTOR LOCALIZATION, ISOLATION AND ANALYSIS OF HORMONE-RECEPTOR INTERACTIONS: STUDIES IN THE TOAD BLADDER

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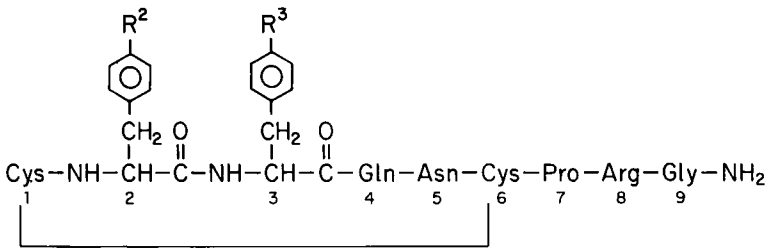
Introduction

The attachment of a hormone to its target tissue is generally too unstable to permit the hormone to serve as a marker for the isolation of the hormone-receptor complex --- because the specific binding properties of the receptor are altered or lost entirely during the solubilization steps of even mild purification procedures. A promising approach to this problem evolved in the sixties with the introduction of photogenerated and other reagents for labeling of hormone receptors and other biologically significant molecules.[1] More recently stable (irreversibly bioactive) hormone-receptor complexes have been formed in which hormone analogs, serving as affinity probes, make possible the isolation of receptors for several hormones.

Our particular interest in this problem centers on the neurohypophyseal hormonal (NH) peptides. The studies reported below are concerned with arginine vasopressin, AVP, and the hydroosmotic receptor system in the urinary bladder of the toad, Bufo Marinus. We have evaluated the level and persistence of the activity of four analogs of AVP (Figure 1) in the intact isolated toad bladder system using Bentley's gravimetric

method.[2] Each of these analogs differs from AVP either at position 2 or at position 3 where a photoreactive para-azido-phenylalanyl, or a chemically-reactive para-bromacetylamino-phenylalanyl, residue replaces the naturally-occurring tyrosyl and phenylalanyl residues at these positions.



R^2=	R^3=	
OH	H	AVP
N_3	H	[Phe(p-N_3)^2] AVP (2a)
$NHCOCH_2Br$	H	[Phe(p-$NHCOCH_2Br$)^2] AVP (2c)
H	N_3	[Phe^2, Phe(p-N_3)^3] AVP (3a)
H	$NHCOCH_2Br$	[Phe^2, Phe(p-$NHCOCH_2Br$)^3] AVP (3b)

Fig. 1. Formulas for AVP and four synthetic AVP analogs.

Results and Discussion

The 3-substituted azido analog of AVP, [Phe^2,Phe(p-N_3)^3]AVP, when activated by ultraviolet light of relatively long wave length (280-360 nanometers) in the presence of the toad bladder, is converted to reactive intermediates which bind at or near the functional sites of the NH hydroosmotic receptor and thereby initiates irreversibly the train of events that leads to increase in water flow along the osmotic gradient across the bladder wall. This hydroosmotic response has been found to persist for as long as 24 hours after resuspending the bladders in an agonist-free serosal bath of fresh amphibian Ringers solution. This is in sharp contrast to the behavior of bladders in control experiments in which

[Phe^2,Phe(p-N)^3]AVP was used without ultraviolet irradiation. In these control experiments the analog also proved to be an agonist (with an ED_{50} of 4.5×10^{-7}M and with an intrinsic hydroosmotic activity of 74% of that of the parent hormone, AVP), but in this case, when the [Phe^2,Phe(p-N_3)^3]AVP-containing serosal bath was replaced with fresh, agonist-free Ringer's solution, the hydroosmotic response declined immediately and was terminated within minutes. The immediate reversal and rapid termination of the hydroosmotic response noted in these control experiments is identical to the reversal and termination of response that occurs when a bladder, challenged in a serosal bath containing AVP (or other functionally similar NH agonists), is "washed" by resuspension in a fresh, agonist-free serosal bath. The prolonged hydroosmotic response of the toad bladder following exposure to photoactivated [Phe^2,Phe(p-N_3)^3]AVP suggests that this analog of AVP can serve as a covalently-bonded agonistic affinity probe for studies of receptor function (signal generation, turnover, recycling, inactivation) and ultimately for receptor isolation (when ^3H and/or other appropriate labels are incorporated into the analog).

The 2-substituted azido analog of AVP, [Phe(p-N_3)^2]AVP did not have significant hydroosmotic activity in the concentration range 8×10^{-8} to 8×10^{-7}M; but this analog was found to be a potent reversible inhibitor of the hydroosmotic action of AVP. However the inhibitory effect of [Phe(p-N_3)^2]AVP was not preserved by photolysis in this system. [Phe^2,Phe(p-$NHCOCH_2Br$)^3]AVP was also found to be a potent but reversible inhibitor of the hydroosmotic action of AVP whereas [Phe(p-$NHCOCH_2Br$)^2]AVP proved to be inactive as agonist or antagonist. The irreversible hydroosmotic response to [Phe^2,Phe(p-N_3)^3]AVP was markedly decreased when photolysis of this analog was carried out with AVP or [Phe(p-N_3)^2]AVP or [Phe^2,Phe(p-$NHCOCH_2Br$)^3]AVP also included in the serosal bath of the toad bladder assay system. Thus the parent hormone, its

2-substituted azido analog and its 3-substituted bromoacetylamino analog all compete with the photoactivated 3-substituted azido analog at the hydroosmotic receptor sites.

The relative hydroosmotic potencies of the 2-substituted and 3-substituted analogs in the toad bladder system differ from the relative abilities of these analogs to stimulate membrane preparations of bovine renal medullary adenylate cyclase (BRMAC). 2-azido-AVP and 2-bromoacetyl-AVP have higher apparent affinity for activation of BRMAC and stimulate this enzyme to much greater maximal velocities than 3-azido-AVP and 3-bromoacetyl-AVP[3].

In summary, the irreversible hydroosmotic response of the toad bladder to photoactivated [Phe^2,$Phe(p\text{-}N_3)^3$]AVP indicates that this analog binds covalently and functionally to the NH receptor and therefore that, with appropriate labeling, it can be used as a tool for NH receptor isolation and for studies of receptor action and turnover.

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SYNTHESIS AND BIOLOGICAL PROPERTIES OF CHOLECYSTOKININ 27-32 AMIDE, CCK-27-32-NH_2 : A NEW CLASS OF CHOLECYSTOKININ RECEPTOR ANTAGONIST.

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Introduction

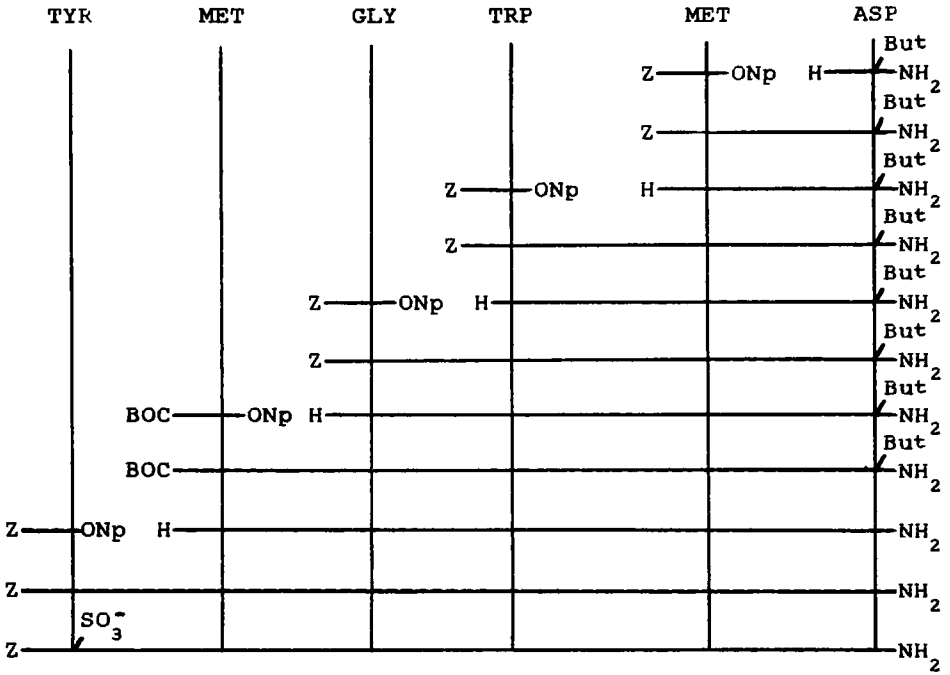
Previous studies in our laboratories concerning studies on structure relationships of N-protected CCK-27-33, showed that replacement of aspartic acid residue in position 32 could produce analogues for which stimulation of enzyme secretion from pancreas (pancreozymin-like activity) and stimulation of gall bladder contraction (cholecystokinin-like activity) are diferentiated[1,2]. In fact, it appears from our investigations 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 those residues located at the N-terminus of the CCK molecule. To further explore the structural requirements of these residues, we undertook the study of the role of the phenylalanine in position 33. For this purpose, CCK-27-32-NH_2, Z-Tyr(SO_3-)-Met-Gly-Trp-Met-Asp-NH_2 was synthesized.

Synthesis

Ammonia was passed over a solution in ethyl acetate of the active ester, BOC-L-aspartic acid-β-benzyl-α-p-nitrophenyl ester[3] to yield BOC-L-aspartic acid-β-benzyl-α-amide. This compound was partially deprotected by TFA. The resulting salt was allowed to react with Benzyloxycarbonyl-L-methionine p-nitrophenyl ester[4] in the presence of 1-hydroxybenzotriazole[5]. Partial deprotection of the N-terminal amino group was performed by hydrogenolysis in a mixture of DMF, DIEA, water, in the presence of $Pd/BaSO_4$ as described previously[6]. The chain was lengthened by stepwise acylations[7] with active esters of Benzyloxycarbonyl amino acids in the presence of 1-hydroxybenzotriazole, except in the preparation of the pentapeptide intermediate were BOC-L-methionine p-nitrophenyl ester[8] was applied. Simultaneous removal of the N-terminal BOC group and tert-butyl ester group from the α-carboxyl of the aspartyl residue afforded the amphoteric pentapeptide amide, Met-Gly-Trp-Met-Asp-NH_2. Benzyloxycarbonyl-tyrosine was incorporated as its p-nitrophenyl ester. The partially protected hexapeptide derivative Z-Tyr-Met-Gly-Trp-Met-Asp-NH_2 was treated with SO_3-pyridine complex[6] to produce the peptide Z-Tyr(SO_3^-)-Met-Gly-Trp-Met-Asp-NH_2, CCK-27-32-NH_2. The α-amino protecting group was not removed because earlier studies[9] showed that acyl derivatives of the C-terminal heptapeptide of CCK were more potent then the heptapeptide segment with the free α-amino group. In fact, the potency in the release of amylase from pancreatic acini or in the stimulation of the gall bladder contraction of the N-protected hexapeptide was equal to that of the C-terminal octapeptide segment of the molecule, CCK-26-33. The peptide CCK-27-32-NH_2 as a di-sodium salt, was purified by chromatography on a column of silica gel. The purified compound was homogeneous by TLC and HPLC and gave the expected amino acid composition and elemental analysis. Its U.V. spectrum was essentially that of tryptophane. In the I.R. spectrum, the characteristic band of a sulfate ester (1040 cm^{-1}) could be observed. The synthesis of this peptide is summarized in the chart.

Biological activities

Amylase secretion was determined using the techniques previously described[10]. In dispersed acini from guinea pig pancreas, cholecystokinin 27-32 amide did not alter amylase secre-



Synthesis of CCK-27-32 amide.

tion, at concentration as high as 10μM[11]. However, this peptide was able to antagonize the action of CCK-26-33 and causes a parallel rightward shift in the dose-response curve for the stimulation of enzyme secretion induced by CCK-26-33. Cholecystokinin 27-32 amide also inhibited binding of ^{125}I labeled CCK to pancreatic acini. CCK-27-32-NH_2 caused half maximal inhibition of both CCK-26-33 stimulated amylase secretion and binding of ^{125}I labeled CCK at a concentration approximatively 3μM. Cholecystokinin 27-32 amide did not show activity on stimulation of gall bladder contraction, even at very high doses.

Conclusions

It is clear that the C-terminal phenylalanine residue of cholecystokinin is not essential for the binding of CCK or struc-

turally related peptides to the CCK receptors, but is essential for intrinsic CCK-like biological activities. The present results suggest that CCK-27-32-NH_2 is a fully competitive cholecystokinin antagonist[11]. Compared to other cholecystokinin receptor antagonists, CCK-27-32-NH_2 is the most potent antagonist described to date. The present findings constitute the first report of a peptide that can function as a fully competitive CCK-receptor antagonist. Relations existing between structures and activities of these peptides are under investigations.

Acknowledgements

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N^{ε}-ACYL ANALOGS OF THE α-FACTOR FROM *SACCHAROMYCES CEREVISIAE*

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Introduction

Sexual conjugation of *a* and α-haploids of *Saccharomyces cerevisiae* is mediated by a tridecapeptide, Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr, known as the α-factor (αF).[1] Treatment of *a* cells with this pheromone results in cell cycle arrest, cessation of DNA synthesis, and the formation of aberrant morphologies known as Shmoos.[1] We recently synthesized analogs of the des-Trp^1-αF in which Trp^3 was replaced by β-cyclohexylalanine (Cha)[2]. Many of these analogs retained high biological activity although the morphology of treated *a*-cells was significantly altered from those resulting from incubation with natural α-factor. The α-factor is thought to bind to a receptor located in the yeast cell membrane. We reasoned that increasing the lipophilicity of the pheromone might raise its solubility in the membrane and thereby increase its activity. Since the des-Trp^1,Cha^3,Lys^7(Ac)-analog retained its biological activity we felt that the N^{ε}-amine of the Lys^7 residue would be an excellent position to add hydrophobic groups to the α-factor. In this communication we report the synthesis and biological properties of a number of N^{ε}-acyl analogs of the α-factor which contain saturated fatty acids of increasing chain length.

Experimental

The synthesis of mating factor segments was carried out using solution phase techniques with either mixed anhydrides or HOBt accelerated p-nitrophenyl active esters as the coupling agents. Dodecapeptides were assembled by coupling the des-Trp[1] amine terminal heptapeptide to the carboxyl terminal pentapeptide. For the synthesis of the N^{ε}-Lys[7]-acyl analogs, the acyl group [$H(CH_2)_nCOOH$, n = 1,3,7,11,17] was attached to lysine using p-nitrophenyl active esters. Best results were obtained when the amine terminal segment was first acylated and then incorporated into the dodecapeptide. Except for the case of the N^{ε}-Lys[7](Ac) analog, attempts to directly acylate the dodecapeptide resulted in very low yields. After deprotection, all α-factor analogs were purified to greater than 98% homogeneity using reversed-phase HPLC on a μBondapak C_{18} column with $CH_3OH/H_2O/CF_3COOH$ as the mobile phase. Details of the synthesis will be published elsewhere.[3] Biological activity of α-factor analogs was assessed by determining the minimum concentration of peptide which resulted in the formation of aberrant morphologies after incubation with a-cells (Shmoo assay).[2]

Results and Discussion

The biological activity of N^{ε}-acyl-α-factor analogs and some physical properties are given in Table I. As previously noted for the N^{ε}-Lys[7](Ac)-derivative both the butanoyl and octanoyl derivatives retain high biological activity. Attachment of lauric acid (C_{12}) to the N^{ε}-amine caused six fold reduction in activity, and the analogous octadecanoyl (stearyl) derivative was inactive at 20,000 ng/ml. Since this α-factor analog is quite insoluble in both buffer and growth medium it was not possible to measure activity at higher concentrations. These results confirm our conclusion that a free N^{ε}-Lys[7] amino group is not necessary for activity and show that conjugates containing up to a 12-carbon atom chain at this position have access to the receptor. Thus large modifications are possible at the lysine side chain. Since none of the peptides had increased activity as compared to the des-Trp[1],Cha[3]-αF the

hydrophobicity of this analog appears to be sufficient for it to efficiently partition into the membrane. However, we recently determined that the des-Trp^{1},Cha^{3},Lys(Ac)^{7}-αF is cleaved by whole cells twice as fast as the unacetylated homologue. We are currently studying the degradation rates of the other N$^{\varepsilon}$acyl analogs to see whether pheromone degradation influences their activity.

Table I. Biological and Chemical Properties of α-Factor Analogs

Peptide	$[\alpha]_D^{25}$	R_f [b]	Biological Activity[c]
1) Natural Sequence[a]	-34.7° (c 0.11,AcOH)	0.18	50
2) des-Trp^{1},Cha^{3}-αF	-37.7° (c 0.28,AcOH)	0.13	250
3) des-Trp^{1},Cha^{3},Lys^{7}(Ac)[e]-αF	-40.0° (c 0.20,AcOH)	0.19	1000
4) des-Trp^{1},Cha^{3},Lys^{7}(Bu)[e]-αF	-65.0° (c 0.14,AcOH)	0.23	800
5) des-Trp^{1},Cha^{3},Lys^{7}(Oc)[e]-αF	-36.2° (c 0.28,AcOH)	0.29	800
6) des-Trp^{1},Cha^{3},Lys^{7}(La)[e]-αF	-71.8° (c 0.11,AcOH)	0.26	6000
7) des-Trp^{1},Cha^{3},Lys^{7}(St)[e]-αF	-39.3° (c 0.09,AcOH)	0.30	NA

[a]The natural sequence is: Trp^{1}His^{2}Trp^{3}Leu^{4}Gln^{5}Leu^{6}Lys^{7}Pro^{8}Gly^{9}Gln^{10}Pro^{11}Met^{12}Tyr^{13}. [b]Mobile phase 1-BuOH-AcOH-H_2O (4:1:5, upper phase). [c]The minimum concentration (ng/ml) that results in shmoo formation (morphogenesis). [d]NA is not active at concentration up to 20 μg/ml. [e]Ac = acetyl; Bu = butanoyl; Oc = octanoyl; La = Lauryl; St = stearyl.

The N$^{\varepsilon}$-Lys^{7}-acyl analogs cause a-cells to form severely distorted morphologies. Rather than inducing the formation of pear or peanut shaped shmoos, treatment with all of the above dodecapeptides resulted in irregular, amoeba-like shapes for the yeast cells. It is clear that analogs of the tridecapeptide α-factor must be investigated before definitive conclusions can be made. Results to date, however, demonstrate that the tridecapeptide α-factor induces pear-shaped shmoos whereas dodecapeptides lead to amoeba-like morphologies.

A recent investigation reported the isolation and characterization of peptidal sex hormones with hydrocarbon side chains.[4] Few reports in the literature have discussed the synthesis of such compounds and only recently has attention been given to the effect of hydrocarbonaceous side chains on peptide conformation and activity.[5] In this context application of Chou-Fasman probabilities to the α-factor suggests that this peptide has a statistical tendency to form either helical or β-sheet structures. We are currently assessing the conformation of the des-Trp^1,Cha^3-αF using circular dichroism and ^1H nmr spectroscopy and will investigate the influence of the fatty acyl substitutions on the conformational equilibria in this peptide.

Acknowledgements

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UPTAKE OF EPIDERMAL GROWTH FACTOR INTO A LYSOSOMAL ENZYME-DEFICIENT ORGANELLE CORRELATES WITH THE MITOGEN'S ABILITY TO STIMULATE CELLULAR DNA SYNTHESIS

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Introduction

Epidermal growth factor (EGF) is internalized by cultured fibroblasts through endocytic pathways which results in association of the mitogen with several subcellular compartments. The compartments include coated pits on the cell surface, [1,2] endocytic vacuoles, [1,3] lysosomes and multivesicular bodies, [1-4] and the Golgi apparatus.[3,4] In addition we have recently shown that EGF is taken up into a dense subcellular fraction which does not have the properties of any of these subcellular organelles.[5,6] This dense subcellular organelle lacks significant lysosomal enzyme activity, is different in density from the major lysosomal fraction and contains a different protein content than the lysosomal fraction.[5,6] The results described here, together with other recent work, [6] demonstrate that uptake of the mitogen into this dense subcellular organelle is correlated with the ability of EGF to stimulate DNA synthesis.

Results

Human fibroblasts (HF) were preincubated for 1 day with either DMEM containing 0.1% bovine serum albumin (DMEM-BSA) or 10% fetal calf serum (DMEM-FCS). They were then treated with ^{125}I-EGF for 3 h at 37 C by adding the growth factor directly to the culture medium. The cells were then lysed and fractionated on a 41% Percoll gradient.[6] As shown in Figure 1,

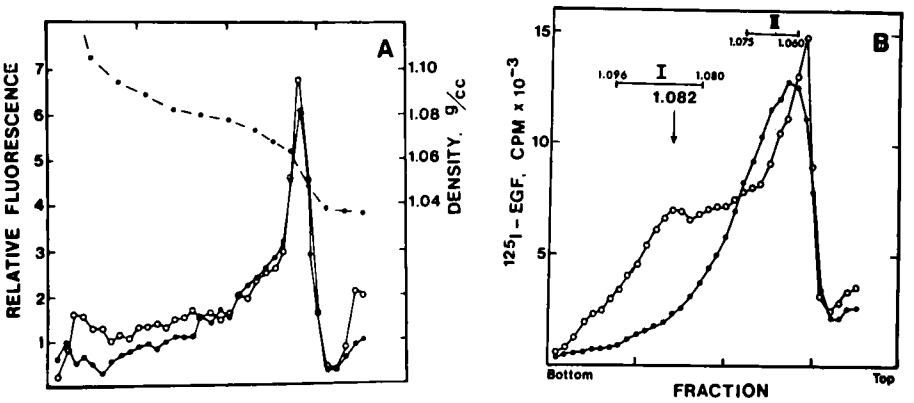

Fig.1. Distribution of ^{125}I-EGF on 41% Percoll gradients. Confluent HF cells were preincubated for 1 d in either DMEM-FCS (o) or DMEM-BSA (o). A) -galactosidase activity and density (o-o). B) ^{125}I-EGF. Arrow indicates a density of 1.082.

in the presence of 10% FCS, EGF is taken up into a dense, lysosomal enzyme-deficient fraction with a peak density of 1.082. In contrast, in the absence of serum very little EGF is taken up into this fraction. In both the presence and absence of serum a lighter peak of activity is observed. This peak is coincident with the major peak of lysosomal enzyme activity (Figure 1A).

The ability of EGF to induce DNA synthesis in HF cells in either the presence or absence of serum was tested (Figure 2). Under serum containing conditions (DMEM-FCS) the HF cells were quite responsive with maximum DNA synthesis 17-20 h after EGF addition while very little induction of DNA synthesis was observed in serum-free conditions. Thus, DNA synthesis is induced under the same conditions in which EGF uptake into a dense lysosomal enzyme-deficient component is observed. The possible interrelationship between these two phenomena was therefore studied in greater detail.

Human fibroblasts or 208F rat fibroblasts were incubated

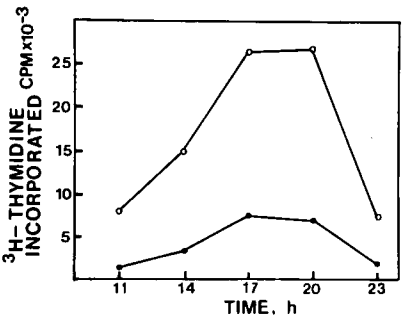

Fig.2. EGF (10 ng/ml) stimulation of thymidine incorporation in HF cells which had been incubated for 1 d in DMEM-FCS (o) or DMEM-BSA (o). Thymidine incorporated in a control, lacking EGF, was subtracted from each time point. This baseline level was constant throughout the experiment at 6500 cpm for cells in DMEM-FCS and 4700 cpm for cells in DMEM-BSA.

with varying concentrations of serum, drugs or effectors as indicated in Table I. The cells were then assayed for their ability to take up EGF into the dense lysosomal enzyme-deficient organelle and their ability to respond to EGF under each condition. In every case, a decrease in the ability of the cells to take up EGF into the dense fraction was accompanied by a decrease in responsiveness to the mitogen. In contrast there was no correlation between uptake of EGF into the major lysosome fraction and cellular responsiveness. In addition the results suggest the involvement of calcium related processes and actin filaments in the delivery of EGF into the dense, lysosomal enzyme-deficient organelle.

Discussion

The results described here demonstrate that EFG is taken up into a dense subcellular organelle. This organelle is different in density and enzyme content than the major lysosome fraction. These data are in agreement with our previous observations[5,6] which also demonstrate that the protein content of the dense fraction is different from the major lysosome fraction. These results also show a strong correlation

Table I. Effect of Serum Concentation, Drugs and Effectors on EGF Uptake and Mitogenic Activity

Treatment	EGF uptake Fraction I % of Control	EGF uptake Fraction II % of Control	DNA Synthesis % of Control
10% serum	100	100	100
5% serum	60.6	116	75.5
2.5% serum	47.3	118	48.9
1% serum	38.7	142	39.4
0% serum	33.7	136	28.3
15mM methylamine	45.8	417	23
50uM cytochalasin B	62.7	150.5	9
20mM $CaCl_2$	108.4	113.3	117
2mM EGTA[#2]	42.2	123.1	88

Human fibroblasts or 208F rat fibroblasts (#) were treated as indicated. Fractions I and II are as indicated in Figure 1.

between uptake of EGF into this fraction and the ability of the cells to respond to the mitogen. No correlation is observed between responsiveness and uptake of EGF into the lysosome fraction. Although our data show a simple correlation between uptake of EGF into a dense subcellular organelle and EGF stimulation of cellular DNA synthesis we have not yet demonstrated a direct cause and effect relationship between these two events. However, a strong possibility exists that uptake of EGF into this fraction or the endocytic pathway leading to this association is involved in producing a mitogenic signal.

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THE "CAGY" PEPTIDE: A PROBE FOR GLYCOPROTEIN-HORMONE-SUBUNIT INTERACTION

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The hormone-specific beta subunits of the pituitary and placental glycoprotein hormones (lutropin, LH; follitropin, FSH; thyrotropin, TSH; choriogonadotropin, CG) differ markedly in amino acid sequence, in contrast to the virtual identity in primary structure among the common alpha subunits. Limited regions of homology can be found among beta subunits, however, including a segment toward the amino-terminus centered about the sequence --Cys-Ala-Gly-Tyr-- (C-A-G-Y; residues 34--37 in human LH and CG). This sequence also occurs in the beta subunit of choleratoxin and in several serine proteases[1,2]. The CAGY sequence is immediately preceded by a hydrophobic residue and in the hormones it is also flanked by sets of homologous threonine residues (Figure 1).

It has been suggested[3] that this homologous sequence could represent a site of interaction with alpha subunit during association into biologically active hormone. We therefore prepared synthetic peptides representing the CAGY region, and tested their ability to inhibit recombination of human choriogonadotropin subunits as measured by immunoassay, and by receptor binding and adenylate cyclase activation in rat ovarian luteal cells.

Two peptides were synthesized by the solid phase procedure

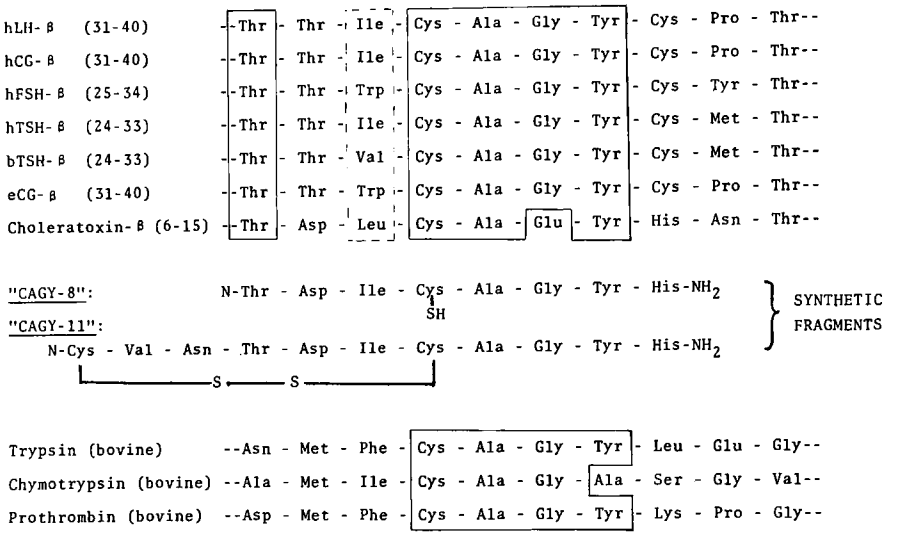

Fig. 1. Comparison of "CAGY" region sequences among glycoprotein hormone and choleratoxin beta subunits, serine proteases, and the synthetic peptides prepared for this investigation.

using benzhydrylamine resin[4,5] (Figure 2). Acetamidomethyl protecting groups were removed from cysteines with mercuric acetate and the peptides purified by Biogel P-2 gel filtration and high performance liquid chromatography. An eight-residue fragment ("CAGY-8", Figure 1) included a free sulfhydryl corresponding to residue 34 of LH and CG, and an extended eleven-residue fragment ("CAGY-11") incorporated a disulfide bridge involving Cys-34, as found in the native subunit.

Alpha and beta subunits of human CG were co-incubated at pH 8.5, 25° for 24 hr in presence and absence of added CAGY

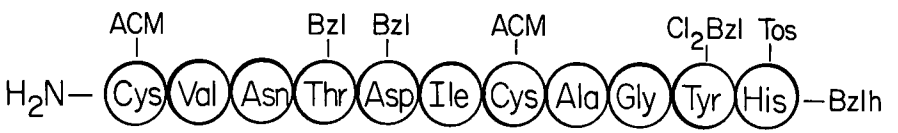

Fig. 2. Solid-phase synthesis of CAGY peptides.

TABLE I. Effect of CAGY Fragments on Subunit Recombination

Hormone preparation	Receptor binding		Adenylate cyclase	
	ID_{50}*	RP†	ID_{50}	RP
Native hCG	5.5	1.0	.105	1.0
α + β	8.5	.65	.175	.60
α + β + CAGY-11	11.2	.49	.208	.51
α + β + CAGY-8	10.2	.54	.204	.52

*Hormone dose providing half-maximal response (receptor assay = ng/ml; cyclase assay = μg/ml).

†Potency relative to native human CG standard (CR-123).

peptide in 50M excess. As summarized in Table I, recombination in the presence of CAGY peptides was comparable to the control incubation; activities of the recombination products by receptor and adenylate cyclase assays were not significantly different. Superimposed response curves were also found by radioimmunoassay using antisera specific for whole, recombined hCG. CAGY-8 and CAGY-11 alone showed no inherent activity in any of these assay systems.

Immunological cross-reactivity among antigens involving this sequence was evident, however, from studies by Jagiello and Mesa-Tejada[6] showing that the CAGY fragments could inhibit immunoperoxidase staining of protease components in oocytes by an anti-human LH-beta antiserum.

The potential involvement of this sequence in enzyme-substrate interaction was determined by inhibition of protease action on synthetic and natural substrates. We observed a small but significant effect of CAGY-11, in 100-fold excess, on chymotrypsin cleavage of benzyl tyrosine ethyl ester. Chymotryptic cleavage of a larger protein substrate, casein, as measured by generation of TCA-soluble peptides, was not inhibited. The peptides did not affect cleavage by trypsin of the synthetic substrate p-toluenesulfonyl-L-arginine methyl ester.

The results of these experiments indicate that peptides containing the CAGY sequence may fit partially into an enzyme binding site, and share certain antigenic determinants with proteases and glycoprotein hormone beta subunits. However, they do not associate sufficiently with the surface of interaction between alpha and beta subunits to modify or interfere with subunit recombination.

A broader surface, including multiple points of contact between subunits, is probably involved in the process of association. Alternatively, conformational changes may occur during recombination that produce a hydrophobic "pocket" involving the CAGY sequence region.

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STABLE ISOTOPE CONTAINING PEPTIDES AS PROBES OF LIGAND/ RECEPTOR INTERACTIONS: DEUTERO-FORMYL-METHIONYL-LEUCYL-PHENYLALANINE

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Introduction

N^{α}-Formyl-Methionyl-Leucyl-Phenylalanine (CHO-Met-Leu-Phe-OH) is the prototype of a series of small molecular weight oligopeptides which stimulate chemotaxis, lysosomal enzyme secretion and a variety of other cellular responses in mammalian phagocytes[1,2]. These effects are mediated by interaction of the peptide with a specific membrane receptor[3,4] the structural requirements of which have recently been summarized[5]. This summary, expressed as a hypothetical model of the receptor of rabbit neutrophils, included speculation about several specific interactions between the tripeptide and its receptor. In light of the almost absolute requirement for the presence of the formyl group it was postulated that the marked enhancement produced by formylation was the result of hydrogen bonding of the formyl proton with a critical area on the receptor. As a test of this hypothesis we have attempted to determine if the well known stable isotope enhancement of hydrogen bonding seen with some enzyme/substrate interactions[6] would also be evident with this

ligand/receptor interaction. Accordingly, we have synthesized Deutero-Formyl-Methionyl-Leucyl-Phenylalanine (C^2DO-Met-Leu-Phe-OH). This analaog has been evaluated analytically, relative to CHO-Met-Leu-Phe-OH, and for its ability to induce lysosomal enzyme secretion from rabbit neutrophils[1].

Results and Discussion

The tripeptide benzyl ester, H-Met-Leu-Phe-OBzl was prepared by a rapid mixed anhydride method using isobutylchloroformate[7]. Deuteroformic acid (Merck) was coupled via a conventional anhydride procedure using isovalerylchloride as reported perviously[8]. Final deprotection was with anhydrous hydrogen fluoride/anisole (45 min, 4°C) and the compound was purified by crystallization from acetone/H_2O. Side by side comparison of the deuterated analog and authentic CHO-Met-Leu-Phe-OH showed that the two were indistinguishable with respect to chromatographic mobility and melting point. Furtheremore, a NMR spectra of CDO-Met-Leu-Phe-OH was obtained in DMSO and compared to that previously reported for CHO-Met-Leu-Phe-OH[9]. With the exception of the total absence of the formyl proton resonance, the position and relative intensity of the other resonances were identical for both peptides.

The ability of each of induce lysosomal enzyme secretion from rabbit neutrophils[1] is shown in Figure 1 and clearly indicate a significantly enhanced activity for the deuterated analog. The apparent affinities (i.e. the concentration of produce 50% of the maximum effect) were 1.5×10^{-10}M (CHO-Met-Leu-Phe-OH) and 3.0×10^{-11}M (CDO-Met-Leu-Phe-OH) respectively. There was a similar decrease in the threshold concentration for CDO-Met-Leu-Phe-OH but no change in the maximum effect. Overall, this isotopic substitution results in a 4-5 fold enhancement in the biological activity of the deuterated analog versus CHO-Met-Leu-Phe-OH.

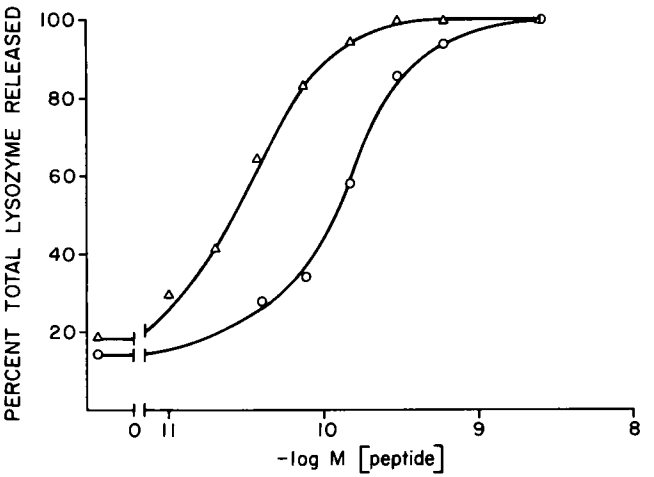

Fig. 1 Lysosomal enzyme secretion from cytochalasin-B treated rabbit neutrophils. Concentration dependency for CDO-Met-Leu-Phe-OH (Δ) and CHO-Met-Leu-Phe-OH (o).

Obviously biological activity of any complex ligand is a function both of the three dimensional structure of the ligand and the effectiveness of the interaction with its receptor. Any chemical alteration may therefore be expected to change either or both of these determinants. The unequivocal answer to the question posed here then requires that no change in the conformation result from the deuterium substitution. That this is the case is strongly supported by the finding that the NMR spectra, less the formyl proton resonance, of the deuterated analog is identical to that of authentic CHO-Met-Leu-Phe-OH[9]. It should be noted, however, that the spectra was taken in DMSO and therefore does not take into account peptide/solvent interactions in physiological buffers.

In summary, we have prepared CDO-Met-Leu-Phe-OH and found it to be indistinguishable from CHO-Met-Leu-Phe-OH in all ways except for its enhanced biological activity. This we believe is good evidence in support of the hypothesis that the formyl proton hydrogen bonds with the receptor of rabbit neutrophils. In addition to its specific use here we also submit that stable isotope substitutions may be useful probes to evaluate ligand/receptor interactions in general.

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IMMUNOGENICITY OF IMMUNOREACTIVE CALMODULIN PEPTIDES

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Introduction

Vertebrate calmodulin (CaM) is a 148-residue calcium-binding protein that may regulate the action of many enzymes in response to the intracellular concentration of calcium ion. The use of site-directed antisera in immunochemical mapping procedures has been important for correlation of calmodulin structural domains with calmodulin functional regions.[1]

Results and Discussion

We have identified a 7-residue segment of vertebrate CaM as an immunoreactive site.[2,3] As illustrated in Table I, the 137-143 segment (Asn-Tyr-Glu-Glu-Phe-Val-Gln-NH_2) is as immunoreactive as intact CaM towards an antiserum[2] against performic acid-oxidized vertebrate CaM. It is the only 7-residue segment with full immunoreactivity. Also, all 7 of its side chains are important for its full immunoreactivity.[3]

Lerner[4] postulated that essentially any segment at least 7 residues long and exposed on the protein surface should be immunogenic when bound to a carrier and should elicit antibody molecules that react with the intact protein. We have tested this hypothesis for CaM by binding the synthetic peptide amide

Table I. Immunoreactivity of Calmodulin Peptide Amides: Specificity of a Rabbit Antiserum Against Performic Acid-Oxidized Vertebrate Calmodulin

Region	Structure of calmodulin peptide amides[a] 134 136 137 138 142 143 144	Relative activity
1-148	Chicken gizzard calmodulin	100
134-143	Gly-Glu-Val-Asn-Tyr-Glu-Glu-Phe-Val-Gln-a	100
136-142	Val-Asn-Tyr-Glu-Glu-Phe-Val-a	4
136-143	Val-Asn-Tyr-Glu-Glu-Phe-Val-Gln-a	100
137-143	Asn-Tyr-Glu-Glu-Phe-Val-Gln-a	100
137-144	Asn-Tyr-Glu-Glu-Phe-Val-Gln-Met(O)-a	100
138-144	Tyr-Glu-Glu-Phe-Val-Gln-Met(O)-a	1

[a] a = NH_2 = amide.

Table II. Immunoreactivity of Calmodulin Peptide Amides: Specificity of a Rabbit Antiserum Against Keyhole Limpet Hemocyanin-Cys-CaM-(137-143)-NH_2

Region	Structure of calmodulin peptide amides[a] 134 137 143 144	Relative activity
1-148	Chicken gizzard calmodulin	< 0.1
134-143	Gly-Glu-Val-Asn-Tyr-Glu-Glu-Phe-Val-Gln-a	80
137-143	Asn-Tyr-Glu-Glu-Phe-Val-Gln-a	100
137-144	Asn-Tyr-Glu-Glu-Phe-Val-Gln-Met(O)-a	0.2

[a] a = NH_2 = amide.

Cys-CaM-(137-143)-NH_2 through its thiol group to keyhole limpet hemocyanin using iminothiolane. As shown in Table II, this conjugate elicited antisera in rabbits that recognized the immunoreactive-site peptide CaM-(137-143)-NH_2 as well as longer peptides ending with Gln-143 amide. But addition of just one residue after Gln-143 decreased reactivity 500-fold. No immunoreactivity was seen towards intact vertebrate CaM. This antiserum is evidently directed more towards the $CONH_2$

Table III. Immunoreactivity of Calmodulin Peptide Amides: Specificity of a Rabbit Antiserum Against Chicken Ovalbumin-Cys-CaM-(134-148)-NH_2

Region	Structure of calmodulin peptide amides[a] 136 137 138 142 143 144 145	Relative activity
1-148	Chicken gizzard calmodulin	100
136-143	Val-Asn-Tyr-Glu-Glu-Phe-Val-Gln-a	11
136-144	Val-Asn-Tyr-Glu-Glu-Phe-Val-Gln-Met(O)-a	33
136-145	Val-Asn-Tyr-Glu-Glu-Phe-Val-Gln-Met(O)-Met(O)-a	110
137-143	Asn-Tyr-Glu-Glu-Phe-Val-Gln-a	9
137-144	Asn-Tyr-Glu-Glu-Phe-Val-Gln-Met(O)-a	11
137-145	Asn-Tyr-Glu-Glu-Phe-Val-Gln-Met(O)-Met(O)-a	29
138-142	Tyr-Glu-Glu-Phe-Val-a	< 0.02
138-144	Tyr-Glu-Glu-Phe-Val-Gln-Met(O)-a	0.2

[a] a = NH_2 = amide.

terminus of the immunogenic peptide than towards side chains.

We next modeled the molecular enviroment of the calmodulin 7-residue immunoreactive site by adding residues to each end of the site. The 16-residue peptide Cys-CaM-(134-148), Cys-Gly-Gln-Val-Asn-Tyr-Glu-Glu-Phe-Val-Gln-Met-Met-Thr-Ala-Lys, has 3 CaM residues before and 5 CaM residues after the site. This peptide was synthesized by the solid-phase method and was attached through its thiol group to chicken ovalbumin using N-succinimidyl 3-(2-pyridyldithio)propionate. This conjugate elicited antisera in rabbits that showed high titers towards CaM. In a competition radioimmunoassay that used vertebrate CaM as tracer, one antiserum was nearly as reactive with intact vertebrate CaM as with the immunogenic peptide Cys-CaM-(134-148). As shown in Table III, it also reacted well with the 7-residue immunoreactive-site peptide CaM-(137-143)-NH_2. The presence of Val-136 or Met(O)-144 increased activity modestly. But the absence of Asn-137 or Gln-143 decreased immunoreactivity substantially. Thus the specificity of this anti-peptide serum was similar to that of the anti-CaM serum.

In addition, this anti-peptide serum was 100 times more reactive with vertebrate CaM than with spinach CaM, which has lysine in place of glutamine at position 143.

These results show that a synthetic segment of vertebrate CaM containing the 7-residue immunoreactive site can be immunogenic when bound to a carrier and can elicit antibodies that react with intact vertebrate CaM. More important, they show that successful engineering of a site-directed antiserum against an intact protein using a synthetic peptide-carrier as the immunogen is not generally as simple as choosing a peptide with minimal length and surface exposure.[4] As in the present case, success may require the presence of additional features that allow the potentially immunogenic peptide to adopt a conformation and orientation similar to those displayed by the corresponding segment of the intact protein. This approach offers a rational alternative to "shotgun" methods and modern hybridoma technology for the development of site-directed antisera for use in specific immunochemical assays.

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THE ABILITY OF CALCITONIN TO FORM LIPOPROTEIN COMPLEXES: RELATIONSHIP TO OTHER HORMONES AND TO RECEPTOR BINDING.

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Introduction

In an amphipathic helix one face is hydrophobic while the opposite face is hydrophilic. In order to fold into an amphipathic helix, a peptide must contain sequences in which hydrophobic residues are regularly spaced at every third or fourth residue along the chain. Several peptides and proteins, containing sequences which would allow them to fold into an amphipathic helix, can solubilize phospholipids in the form of disc-shaped lipoprotein particles. Among the peptides which have this property are a number of hormones, including calcitonin. Although there is considerable variation in the amino acid sequence of calcitonins from different species, they all maintain the capability of segregating hydrophobic and hydrophilic residues through the formation of an amphipathic helical segment.

Results

A more detailed description of the interaction of calcitonins with phospholipids is presented elsewhere[1]. The present work will discuss the essential features of these findings and their possible relationship to the structural properties of other hormones.

Salmon, porcine and human calcitonins (sCT, pCT and hCT) have little or no helical content in aqueous solution as revealed by circular dichroism. Addition of the zwitterionic phospholipid, dimyristoyl phosphatidylcholine (DMPC), has no effect on the conformational properties of these calcitonins nor do these peptides alter the phase transition properties of this phospholipid. In contrast, a variety of negatively charged, acidic phospholipids promote an increase in the helix content of sCT. The sCT is capable of disrupting the multilamellar structure of these acidic phospholipids, forming structures of much smaller size which appear visually transparent. In the case of dimyristoyl phosphatidylglycerol (DMPG), the complexes formed with sCT appear, when visualized under the electron microscope with negative staining, to have a morphology similar to that of the discoidal lipoprotein particles formed between DMPC and glucagon or between DMPC and the apolipoprotein A-1. The phase transition characteristics of DMPG are also markedly affected by sCT, the transition becoming broader and having a lower enthalpy with increasing peptide to lipid ratios. Similar effects of disrupting the multilamellar structure of DMPG and of broadening the phospholipid phase transition and lowering the transition enthalpy are observed in the presence of hCT. However, in the case of hCT the interaction with DMPG is not accompanied by a large increase in the helix content of the peptide. In addition, sCT can solubilize mixtures of 75% DMPC and 25% DMPG to produce particles of similar morphology to those formed from sCT and DMPG alone but without a large increment in helical structure.

Discussion

The ability of hCT to solubilize DMPG or of sCT to solubilize mixtures of DMPC and DMPG without a large increase in the helix content of the peptide brings into question the role of the amphipathic helix in the solubilization of phospholipids. However, even if the conformation of the lipid-bound peptide is not identical to that of an α-helix it is almost certainly amphipathic. Several of the hydrophobic amino acid residues of the peptide must spacially group together so as to allow them to bind strongly to the phospholipid matrix, while leaving the hydrophilic residues exposed to the aqueous environment to aid in the solubilization of the lipoprotein particle. Integral membrane proteins and hydrophobic peptides can bind to phospholipids because their hydrophobic residues are spacially segregated as a result of the primary structure. However, most peptides and proteins do not form complexes with phospholipids nor can they solubilize phospholipids. There are, however, a number of peptides and proteins including several apolipoproteins[2], amyloid A[3], glucagon[4], calcitonin[1] and mellitin[5] which have segments with hydrophobic amino acids regularly spaced at every third or fourth residue along the chain and which can solubilize phospholipids. Since these peptides almost certainly form an amphipathic structure in solubilizing phospholipids and since these peptides all have segments which would become amphipathic if the peptide folded into an α-helix, it seems likely that a structure similar, but not necessarily identical to an amphipathic helix is formed by the peptide in the lipoprotein complex. Most of the evidence concerning the conformation of the lipid-bound peptide comes from circular dichroism. In the case of the hCT-DMPC complex or the sCT complex with DMPG and DMPC, the conformation of the peptide is clearly not highly helical. However, because of the many uncertainties in the interpretation of circular dichroism spectra it is not

possible to give a very quantitative estimate of the extent of helix formation or about how far removed from an α-helical conformation the actual structure is. In the case of the calcitonins, electrostatic interactions appear to be important in promoting an increase in the helical content. Thus, hCT which has an overall charge of +1 at pH 7.4 forms less helix with DMPG than sCT which has an overall charge of +3. In addition, the negatively charged DMPG induces more helix in sCT than do mixtures of DMPG and the zwitterionic DMPC.

Calcitonin is one of several non-homologous, polypeptide hormones which have hydrophobic residues regularly spaced along the peptide chain[6]. These hormones include glucagon β-endorphin, parathyroid hormone as well as calcitonin. The presence of the segments of the polypeptide chain which can form an amphipathic helix adds to the receptor-binding affinity of these hormones. It is possible that the ability to form an amphipathic structure is a common feature of many membrane-active peptides allowing them to interact with a hydrophobic region in the membrane.

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STRUCTURAL STUDIES ON UNUSUAL PEPTIDES

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Introduction

Despite the obvious power of X-ray crystallography, it is still true today that the majority of challenging structural problems in the field of bio-organic chemistry are not solved by this method. The reasons are numerous, and include situations where (i) insufficient material is available for crystallization (ii) even though sufficient material may be available for crystallization, suitable crystals cannot be obtained and (iii) even though crystals are obtained, the structure cannot be solved by X-ray methods. In these circumstances, structures may be solved by less direct methods. When >1 mg of a substance is available, a combination of mass spectrometric and nmr methods is powerful, with the latter often playing the major rôle. When the quantities available are <1 mg, then the rôle played by mass spectrometry is often necessarily greater. However, it is usually only in the case of standard peptides that a structure may be determined by mass spectrometry alone. In most cases, and even in the case of unusual peptides, mass spectrometry must be used in conjunction with microchemical reactions.

Until the mid-1970s, a severe limitation of the mass spectrometric approach lay in the lack of volatility of many organic molecules. Even with appropriate derivatization (e.g. acetylation, permethylation), the mass range for successful molecular weight determination was frequently <1,000 Daltons.

Although the advent of field desorption had slightly alleviated the problem, it had only been with the advent of particle induced desorption mass spectrometry that the situation has changed dramatically. An important early break-through occurred with the application of californium plasma desorption mass spectrometry.[1] For the first time, molecular weights of highly polar molecules, such as tetrodotoxin, could be directly determined (without derivatization) by mass spectrometry. A second break-through occurred with the introduction of fast atom bombardment (FAB) mass spectrometry by Barber and coworkers.[2] In this technique, a few μg of the substrate, dissolved in a matrix of glycerol (or thioglycerol, or aminoglycerol) is bombarded with fast Xe atoms (prepared from Xe^+ ions of several keV energy). Under these conditions, both positive and negative ions of the substrate (usually a polar molecule) are expelled from the matrix. Both positive and negative ion spectra may be obtained, often (but not always) with comparable sensitivities. This paper is concerned with structural studies on polar molecules using this technique in conjunction with micro-chemical manipulations.

Discussion

Our approach will be illustrated by two examples. Both are concerned with the structure elucidation of unusual peptides. While the structure elucidation of peptides obtained by enzymic digestion of proteins (and hence normally unblocked and containing only L-amino acids) can be carried out by conventional solution methods, peptides which are blocked, cyclic and/or contain D-amino acids are advantageously examined by FAB in conjunction with micro-chemical manipulations.

The poisoning of cattle by ingestion of larvae of an Australian species of sawfly (*Lophyrotoma interrupta*) is a serious problem in several of the grazing areas of Queensland.[3]

The first report of the isolation of a toxic factor is due to Leonard.[4] An improved method for isolation of an essentially pure toxin with an $L.D._{100}$ of 2 mg/kg was reported by Oelrichs *et al.*[5] Amino-acid analysis showed the presence of the following amino acids (molar ratios given in parentheses): Asp (1.89), Glu(1.93), Ala(1.00), Val(1.16), Ile(0.82), and Phe (0.92) (relative to Ala = 1.00). Electron impact (EI) mass spectrometry established benzoyl as an N-terminal blocking group, and determined[5] the partial sequence PhCO-Ala-Phe-Val-Ile.

The sequence of amino acids in the toxin was determined by FAB mass spectrometry. The presence of an abundant MH^+ ion at *m/z* 1040, in conjunction with the amino acid analysis, is in accord with the presence of four carboxy-groups and one primary amide in the toxin. Four kinds of sequence ions are observed in the spectrum. Those sequence ions which contain the N-terminal portion of the peptide correspond to acylium ions (1), or protonated amide ions (2).[6] Ions corresponding to (1) occur at *m/z* 105, 176, 323, 422, 535, 650, 765 and 894 (and MH^+ at 1040) and indicate the sequence PhCOAlaPheValIle-AspAspGluGln; or the C-terminal Gln could equally be replaced by the isomeric amino acid carrying the amide on the backbone carbonyl, and the carboxy-function in the sidechain (iso-Gln). Ions corresponding to (2) at *m/z* 667, 782 and 911 (in conjunction with MH^+) confirm the nature of the three amino acids constituting the C-terminal portion.

$$R\text{-}\overset{+}{C}{=}O \qquad (1)$$

$$(RCONH_2)H^+ \qquad (2)$$

Those sequence ions which contain the C-terminal portion of the peptide correspond to protonated amine fragments (3),[6] or the ion-radical fragments (4). Ions corresponding to (3) at *m/z* 865, 718, 619, and 506 indicate the sequence XPheValIleY, where the masses of X and Y are in accord with the proposed

sequence. Very low abundance ions, corresponding to (4), at *m/z* 849, 702, 603, 490 support this sequence.

$(H_2NR)H^+$ (3) $(\dot{R}CHCO-)H^+$ (4) $RCONH^-$ (5)

A number of the structural conclusions derived from the positive ion FAB spectrum could be confirmed from the negative ion FAB spectrum. This shows an extremely abundant $(M - H)^-$ ion at *m/z* 1038. A series of sequence ions, most generally expressed in terms of the anion (5), occur at *m/z* 338, 437, 550, 665, 780, and 909. These, together with the $(M - H)^-$ ion at *m/z* 1038, indicate the sequence XValIleAspAspGluGln (or, as before, the C-terminal residue may be iso-Gln).

The ambiguity of Gln or iso-Gln at the C-terminus of the toxin was removed by carrying out a Hofmann degradation on the toxin with [bis(trifluoroacetoxy)iodo]benzene.[7] The resulting peptide was totally hydrolysed and the products subjected to amino-acid analysis. The analysis established the production of (molar ratios in parentheses, relative to Ala = 1.00) Ala (1.00), Phe(0.96), Val(0.94), Ile(1.01), Asp(1.93), Glu(1.19), and a product with the same retention time as 2,4-diaminobutyric acid (DAB , 0.89). These experiments establish that the C-terminal residue is Gln and not iso-Gln.

The absolute configurations of the amino-acids in the toxin were determined by gas chromatography on a chiral column. A sample of toxin was subjected to total acid hydrolysis, and the resulting amino acids were converted into their N-trifluoroacetyl isopropyl esters. Gas chromatography of these derivatives on a 'Chirasil-Val' column, and a comparison of their retention times with those of authentic samples, established that Ala, Phe, one Asp, and Glu or Gln, have the D-configuration; and that Val, Ile, one Asp, and Glu or Gln have the L-configuration. Peak assignments were confirmed by EI and chemical ionisation (CI) mass spectrometry.

The ambiguity of absolute configurations at Glu^7 and Gln^8 was resolved by carrying out a total acid hydrolysis on the previously mentioned crude product of Hofmann degradation, and analysing the N-trifluoroacetyl isopropyl ester derivatives of the resulting amino acids on the chiral g.c. column. Co-chromatography with authentic derivatives of D and L Glu, and D and L DAB, establish the hydrolysate to contain D and L Glu in the approximate ratio 93:7, and D and L DAB in the approximate ratio 5:95. Thus, the C-terminal residue of the toxin is L-Gln, and the penultimate residue Glu^7, has the D-configuration.

The ambiguity of absolute configurations at Asp^5 and Asp^6 was resolved by analysing products of partial acid hydrolysis of the toxin. Cleavage by dilute acid hydrolysis of a peptide residue involving an aspartate residue is known to be a facile process compared with other cleavages.[8] Partial acid hydrolysis of the toxin was effected by 10% aqueous HCl at 100°C for 1.5 h. Fractions were separated by reversed-phase preparative h.p.l.c., and the fractions analysed by FAB mass spectrometry. One fraction (fraction 28) was observed to give very abundant ions in its positive ion FAB mass spectrum at *m/z* 493 and 247. These ions correspond to MH^+ signals from the peptides PheVal-IleAsp and IleAsp. Support for the assignment of the peak at *m/z* 493 came from the fragmentation pattern which established loss of C-terminal Asp [loss of 116 to give ion type (2), and loss of 133 to give ion type (1)]. Additionally, esterification of the peptide with MeOH-HCl gave, by FAB analysis, a diester (MH^+ at *m/z* 521); and acetylation with Ac_2O-H_2O gave a mono-acetyl derivative (MH^+ at *m/z* 535), as required by the proposed sequence. The *m/z* 247 ions also showed the anticipated increments in these experiments.

Total hydrolysis of a sample of the above fraction, and analysis of the N-trifluoroacetyl isopropyl ester derivatives of the resulting amino-acids on the chiral g.c. column established that the D:L Asp ratio was approximately 5:1. Allowing

for traces of other peptides in fraction 28, these data indicate that the absolute configuration at Asp^5 is D; and, in the light of data obtained previously, the absolute configuration at Asp^6 must be L.

We have therefore determined that the structure of the toxin, for which the name lophyrotomin has been suggested previously,[3] is PhCO-D-Ala-D-Phe-L-Val-L-Ile-D-Asp-L-Asp-D-Glu-L-Gln.[9]

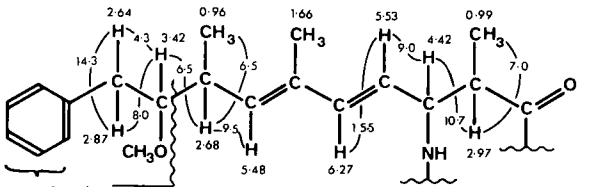
The presence of four D-amino acids in a peptide isolated from an animal is without precedent. However, at this stage we cannot be certain that the peptide, or some or all of its D-amino acid components, is biosynthesised by one or more simpler organisms with which the larvae may interact.

The second example of structure elucidation is concerned with a more complex peptide toxin isolated from *Microcystis aeruginosa*. Several instances of animal poisoning in many parts of the world have been attributed to the sporadic blooms of toxic strains of this blue-green algae.[10] Four toxin variants were isolated from a laboratory clone of *M. aeruginosa* forms *aeruginosa* cultured from a natural bloom in Witbank Dam.[11] Details of the absolute configurations of common amino acids contained in the four toxin variants have been reported.[12] We have recently established the structure of a novel β-amino acid and an amino acid sequence of the toxin variant BE-4.[13]

The reported[12] amino acid composition of BE-4 is (molar ratios in parentheses): erythro-β-methyl-D-Asp(1); D-Glu(1); D-Ala(1); L-Ala(1); L-Leu(1); and N-methyldehydroalanine (N-MeDha)(1). The peptide has a blocked N-terminus (no acetylation with Ac_2O/H_2O), and is resistant to enzymic digestion. The molecular weight is determined as 909 Daltons by fast atom bombardment (FAB) mass spectrometry; but neither this spectrum, nor the EI spectrum of permethylated BE-4, showed evidence of the sequence ions normally observed in the spectra of linear peptides.[14,15] On the basis of the above evidence, we conclude

that BE-4 is likely to be a cyclic peptide. If the peptide is monocyclic, then the component(s) to be added to the already identified units has a mass of 313 Daltons. The identification of this component as a novel β-amino acid is first presented.

In addition to the presence of the above-mentioned amino acids, the 400 MHz ^1H nmr spectrum of BE-4 in D_2O showed carbon-bound protons corresponding to the β-amino acid residue (6). The connectivity was established by spin decoupling experiments (J values indicated in (6)) and negative nOes [{5.53} → 1.66(-12%), {6.27} → 5.48(-15%), and {5.48} → 6.27 (-15%)], the nOes being determined in $(CD_3)_2SO$ solution at 303K.



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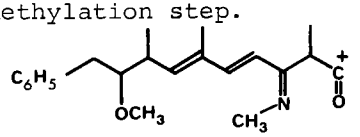
The nature of the functional groups in (6) was established by EI mass spectrometry. An abundant fragment ion at *m/z* 135 in the mass spectrum of BE-4 (or permethylated BE-4) is in accord with a methoxy substituent β to the phenyl group. This conclusion is consistent with the chemical shift of the adjacent proton (3.42 ppm), and the observation of a methyl resonance at 3.35 ppm (D_2O solution). Fragment ions in EI mass spectra of permethylated BE-4 before, and after, pulse hydrolysis (6N HCl, 5 min., 100°C, followed by acetylation) correspond to structures (7) and (8), respectively. These assignments are in accord with high resolution mass measurements, and the shifts to *m/z* 329 and 275 when CD_3I replaces CH_3I in the permethylation step.



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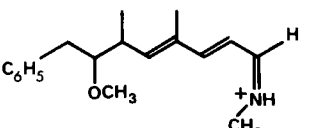


8

Reduction of the N-MeDha residue in BE-4 with $NaBH_4$ (or $NaBD_4$) gives a product with a molecular weight increased by two (or three) Daltons, as established by FAB-MS. The reduced toxin was subjected to pulse hydrolysis (6N HCl, 5 min., 100°C) and two major products, BE-4I and BE-4II, were isolated by reversed phase HPLC; their molecular weights (FAB-MS) were 812 and 794 Daltons, and both showed loss of C-terminal "Glu" residue[15] (where "Glu" represents either Glu or β-MeAsp, since these amino acids have the same molecular weights). These molecular weights were unchanged when reduction of BE-4 was carried out with $NaBD_4$. Thus the residue which has been lost is reduced N-MeDha. FAB-MS analysis of the esterified (MeOH/HCl) or acetylated (Ac_2O/H_2O) products revealed that both have a free amino group; while BE-4I has three, and BE-4II has two, carboxylic acid groups. These results suggest that BE-4I is a linear peptide produced from hydrolysis (+ 18 Daltons) of monocyclic BE-4, with losses of the reduced N-MeDha unit (- 85 Daltons) and methanol (- 32 Daltons). The last loss is readily accommodated from (6). Product BE-4II is concluded to differ from BE-4I by dehydration involving one carboxyl group.

BE-4I was acetylated (Ac_2O/d_6-Ac_2O, 1:1 in H_2O) and then deutero-permethylated. The EI mass spectrum of the product shows pairs of acylium sequence ions at *m/z* 131/134, 261/264, 424/427, 512/515, 810/813, and 973/976. These, in conjunction with the above FAB data, define the sequence: Ac-Ala-Leu-"Glu"-Ala-βaa'-"Glu" where βaa' is the β-amino acid (6) minus methanol. A similar experiment on BE-4II gave the partial sequence Ac-Ala-Leu-"Glu"-Ala... .

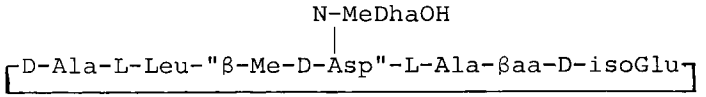
Analysis of the N-trifluoroacetyl isopropyl esters of the amino acids derived from acid hydrolysis of BE-4II, by a GC-MS system fitted with a chiral capillary column, showed D-Ala: L-Ala in the ratio 1:1.5. After one Edman degradation cycle on BE-4II, the FAB mass spectrum of the remaining peptide[16] showed that N-terminal Ala was cleanly removed. Analysis of the remaining amino acid components of this peptide as above

showed L-Ala as before, but no D-Ala was detected. Thus, D-Ala is the N-terminal residue and L-Ala the fourth residue from the N-terminus. The second Edman cycle on BE-4I or BE-4II removed L-Leu, but the third Edman cycle failed as evidenced by no change in the molecular weight as determined by FAB-MS. Dansylation of this tetrapeptide, followed by hydrolysis, gave DNS-β-MeAsp, establishing β-MeAsp as residue 3. Since it is not removed by the Edman procedure, it must be β-linked to the next residue. Thus, the structure of BE-4I (except the stereochemistry of βaa') is (9), where Glu is now restricted to Glu or isoGlu in the parent peptide.

D-Ala-L-Leu-erythro-β-methyl-D-isoAsp-L-Ala-βaa'-D-Glu

(9)

To determine the positions of free carboxyl groups in BE-4, its ^{1}H nmr spectrum was recorded as a function of 'pH' (NaOD added to a solution in D_2O). Only the resonance of the C_α-proton of the Glu residue (4.14 ppm) shifted significantly upfield (to 3.86 ppm) with increasing 'pH'. We conclude that the α-carboxyl of Glu is free, whereas the γ-carboxyl, and both the α- and β-carboxyls of β-methylAsp (or β-methylisoAsp) are blocked. Since the molecular weight of the toxin requires two free carboxyl groups in the structure, the nmr data can be satisfied if the second carboxyl group is attached to the α-carbon of N-MeDha (lacking an α-CH). This conclusion is additionally supported by the observation that $NaBH_4$-reduced BE-4 forms a dimethyl ester (0.02M HCl/MeOH, followed by FAB-MS), whereas BE-4 itself forms a monomethyl ester; evidently under these conditions, the conjugate carboxyl group is not esterified. Thus, (10), where "β-Me-D-Asp" is either erythro-β-methyl-D-Asp or erythro-β-methyl-D-isoAsp, satisfies all structural information we have for BE-4.



(10)

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UTILIZATION OF ORTHOPHTHALALDEHYDE IN THE EDMAN DEGRADATION OF RAT HYPOTHALAMIC CORTICOTROPIN RELEASING FACTOR (CRF)

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Introduction

In 1981, Bhown et al[1] described their experience with fluorescamine, which was found to be useful in the suppression of peptide background presumably built up during Edman degradation by nonspecific peptide bond cleavage and as a result of incomplete coupling or cleavage or both (carry over problem). Fluorescamine was applied to the spinning cup of a modified Beckman 890C sequencer in cycles in which proline was N-terminus of the main peptide. By this application, peptide background was significantly reduced facilitating further sequence analysis of the main peptide. This effect was explained by the blocking of contaminating peptides (carrying N-terminal primary amines) with fluorescamine which did not affect the N-terminal secondary amine proline of the main peptide. However, the peptide background was found to reappear 8-10 Edman cycles after blocking with fluorescamine.

When Machleidt and Hofner[2] employed this approach of background suppression to solid phase sequence analysis, they were not successful with fluorescamine, but they could achieve background suppression with orthophthalaldehyde (OPA).

In view of this (latter) finding, we have been especially interested in OPA as a selective blocking agent. However, our interest was not based on the need to suppress peptide background, since carryover and nonspecific cleavage were not observed to play a major role under the conditions of Edman degradation[3] worked out by us for peptides containing up to 40-50 residues. Instead, we found it attractive to establish a sequence analytical strategy allowing at least partial

sequence analysis of proline containing peptides under quasi-homogeneous conditions. We therefore employed this OPA strategy of Edman degradation for the first time to an unknown polypeptide, rat hypothalamic corticotropin-releasing factor (CRF). Purification of this 41-residue polypeptide[4] and its sequence analysis[5] have been described elsewhere. We here want to provide additional information about the OPA strategy which was applied to a CRF preparation of a purity of approximately 50%[4,5].

Results and Discussion

To date, OPA has been mainly used in peptide chemistry to generate fluorescent derivatives of amino acids and peptides in the presence of thiols[6-9]. Based on the investigation of the reaction of n-propylamine with OPA and β-mercaptoethanol, Simons and Johnson[8] proposed that the fluorescent products of primary amines with OPA and thiols were 1-alkylthio-2-alkyl-substituted isoindoles. In view of the finding that the nitrogen in the isoindole structure was contributed by the primary amine, it was understandable that secondary amines would not yield a fluorescent isoindole derivative[8]. However, the observation that secondary amines such as proline did not form fluorescent products with OPA and thiol would not exclude the possibility of the formation of non-fluorescent adducts. That secondary amines can react with OPA to form stable adducts has been demonstrated[10].

When proline was treated with OPA as described under Figure 1 for the blocking of peptides, amino acid analysis did not provide any evidence for the formation of reaction products with OPA. Other amino acids representing primary amines did react under the same conditions. Sequence analysis of synthetic peptides after incubation with OPA showed that peptides with N-terminal methionine, lysine, tyrosine, phenylalanine, serine and glycine were successfully blocked with OPA, whereas peptides with N-terminal proline were not affected. The stability of the OPA block was revealed by PTH-amino acid analysis of every cycle in the Edman degradation of native rat hypothalamic CRF of a purity of 50% (Fig. 1). After blocking of the contaminating peptide in cycle 4, only

one major PTH-amino acid was detected per cycle (Fig. 1). The identified PTH-amino acids could be assigned to one peptide corresponding to rat hypothalamic CRF (4-39). The N- and C-terminal sequences were either determined by Edman degradation utilizing OPA to block CRF, or by peptide mapping on reverse-phase HPLC as described in detail elsewhere[5].

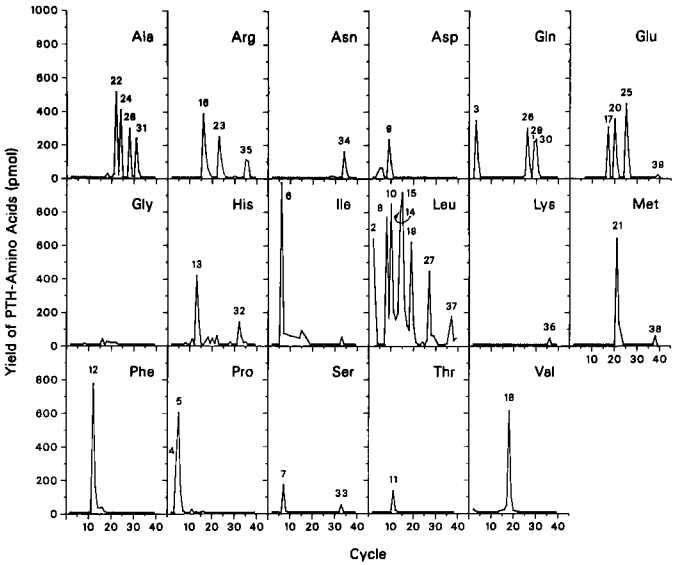

Fig. 1. Edman degradation of approximately 1.4 nmol of native rat hypothalamic CRF pretreated with 3-sulfo-phenylisothiocyanate[5]. Sequence analysis was performed in a Wittmann-Liebold[11] modified (Beckman 890C) spinning cup sequencer as described[3,12]. With this sequencer and reverse-phase HPLC for the identification of PTH-amino acids, complete Edman degradation of unknown 40-50 residue polypeptides has been achieved after application of 1 nmol of peptide or less to the cup[12,13]. OPA (1 mM) was added in aqueous Quadrol to the dry Polybrene peptide film in cycle 4 through the delivery line and reacted with the peptide as described[5]. Total PTH-amino acid yields are presented.

After the establishing of the OPA strategy by the application to an unknown peptide (rat CRF), we expect that this strategy may advantageously be used, especially in the partial characterization of precious partially purified peptides containing proline residues.

Acknowledgements

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MEASUREMENT WITH OPTIMAL MOLECULAR SPECIFICITY OF ENDOGENOUS PEPTIDES IN NERVE TISSUE BY MEANS OF FAB-CAD-B/E-B'/E'-SIM METHODOLOGY

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Introduction

The objective of this research program is twofold: to develop the capability of measuring subnanogram amounts of endogenous neuropeptides g^{-1} of wet weight tissue and significantly, to attach unambiguous molecular specificity to that measurement. The aims of this research program are manifold: calibrate an immunoassay antibody; describe molecular processes involved in nociception[1], extend the mass range of endogenous peptides to be measured, synthesis of individual stable isotope-labeled peptide internal standards[2], measure[3-9] endogenous neuropeptides in several peripheral and central nervous system tissues to compare those peptide amounts in unstimulated control tissue versus electrostimulated tissue, and utilize a radioimmunoassay to "screen" antibody-responsive RP-HPLC peaks.

Materials and Methods

A Finnigan MAT 731 mass spectrometer outfitted with a fast atom bombardment (FAB) source (Ion Tech) and

operating in the collision activated dissociation (CAD) mode[10] is utilized. Individual ion currents are analyzed by two alternating linked field scans, B/E for the peptide of interest and B'/E' for the ^{18}O-labeled peptide four mass units higher. Tissue extracts are purified by utilizing a combination of commercially available C18 Sep-Pak (Waters) cartridge and reverse phase C18 high performance liquid chromatography utilizing a volatile triethylamine formate buffer[11,12]. The organic modifier is acetonitrile and peptide UV absorption is monitored at 200 nm. The C-terminal tripeptide fragment (GFL or GFM) is selected for selected ion monitoring. This analytic measurement mode provides the optimum specificity available.

Results and Discussion

Table I collects preliminary analytical data obtained for FAB-CAD-B/E-B'/E'-SIM measurement of the two endogenous pentapeptides methionine enkephalin (ME = YGGFM) and leucine enkephalin (LE = YGGFL) in tooth pulp tissue for unstimulated controls versus electrostimulated tissue.

TABLE I. Measurement of Neuropeptides in Tooth Pulp Tissue (ng of enkephalin g^{-1} wet weight tissue)

	Unstimulated	Stimulated
ME	355 (n=2)	240 (n=2)
LE	20 (n=3)	8 (n=2)

This data indicates a decrease of 20% of the ME and 40% of the LE following electrostimulation of canine tooth pulp. While these peptide decreases also may be due to an increased metabolic breakdown of those enkephalin

pentapeptides, it is difficult to assess the extent of pre-enkephalin breakdown in the metabolic cascade

precursor → pentapeptide → metabolite(s).

Table II collects data from FAB-CAD-B/E-B'/E'-SIM measurement of endogenous enkephalin pentapeptides in the canine anterior and posterior pituitary.

TABLE II. Measurement of Neuropeptides in Pituitary Tissue (ng pentapeptide g^{-1} wet weight tissue)

	Anterior Pituitary	Posterior Anterior
LE	70	2
ME	2,950	3,760

These data demonstrate that there is clearly more methionine enkephalin in both lobes, more methionine enkephalin in the posterior pituitary versus the anterior pituitary, and the reverse distribution for leucine enkephalin. This type of analytic data obtained with high molecular specificity in the FAB-CAD-B/E-B'/E'-SIM mode indicates the possibility that differential peptide processing mechanisms may be involved in the canine anterior and posterior sections of the pituitary reflecting different metabolic profiles and post-translational events.[13] The opioid peptide precursors may be processed in several ways because different metabolism pathways are available in different tissue and different peptide metabolic products may be found in those different tissues. A variety of post-translational modifications (tyrosyl O-sulfation, phosphorylation, oxidation of the methionine enkephalin thioether to the sulfoxide, amidation, and acetylation) may alter the peptide hydrophobicity and shift the HPLC enkephalin peak.

It is important to understand and accurately

delineate the metabolic profile of opioid neuropeptides represented by these two enkephalin pentapeptides because a current hypothesis indicates that these neuropeptides may decrease neuronal firing via neuromodulatory pathways.

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AMINO ACID SEQUENCE OF POLYPEPTIDES BY ENZYMATIC HYDROLYSIS AND DIRECT DETECTION USING A THERMOSPRAY LC/MS

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Introduction

Rapid determination for the C-terminal sequence of peptide and protein samples has been carried out with on-line enzymatic hydrolysis using immobilized Carboxypeptidase Y (CPY) and thermospray liquid chromatography/mass spectrometry (LC/MS).

Since thermospray LC/MS provides stable vaporization and ionization for non-volatile samples with very high sensitivity[1], this system is particularly well suited to analysis of biological samples. This system can easily handle flow rates up to 2 ml/min of aqueous mobile phase producing mainly quasi-molecular ions $(M+H)^+$ along with a few fragments. In this work, peptide or protein samples were injected through a narrow bore column containing immobilized CPY, and released C-terminal amino acids were carried directly into the thermospray ion source by the continuous flow of an aqueous buffer. Since this system allows very reproducible response for the protonated molecular ions and the response is linear over the working range, sequence information could be obtained by the recovery yield of $(M+H)^+$ for each amino acid.

Correct sequence of the first several residues from the C-terminus has been elucidated for various angiotensins and RCM RNase at picomolar levels in less than 2 minutes.

Material and Methods

Ten mg of CPY were immobilized on 2 g of long chain alkylamine controlled pore glass beads (purchased from Electro-Nucleonics, Inc.) via glutaraldehyde bridge[2] in phosphate buffer at pH 6. Unreacted functional groups were covered with 0.5 M glycylmethyl ester. A twenty cm long, 2.1 mm ID stainless steel column was packed by a slurry method under high pressure. RCM RNase was prepared by reducing the cystine bonds of bovine pancreatic ribonuclease followed by carboxymethylation. Sample solutions were delivered into the thermospray LC/MS by 0.1 M ammonium acetate buffer at pH 5.5 with a Spectra-Physics model SP 8700 pump system. The recovery yields were determined by injecting an amino acid standard mixture before or after the sample injection.

Results and Discussion

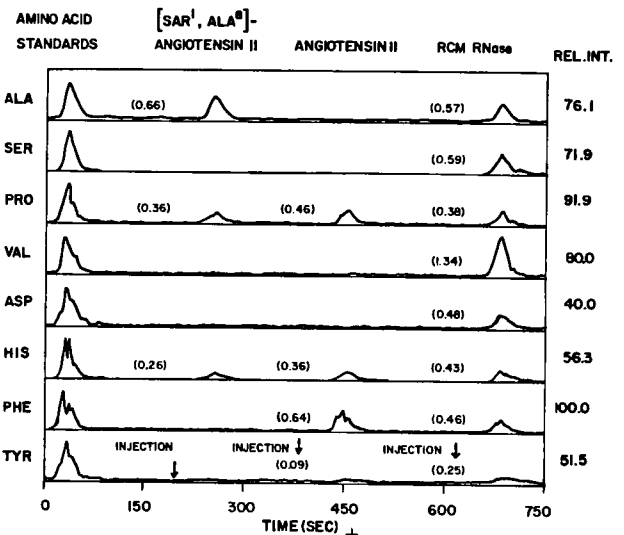

Fig. 1. Mass chromatograms for MH^+ ions from amino acid standard mixture and from CPY hydrolysis of the indicated substrates at a column temperature of 42°C. Figures in parenthesis represent the recoveries.

Figure 1 shows some of the C-terminal amino acids cleaved from 200 pmoles of [Sar^1, Ala^8] angiotensin II, angiotensin II and RCM RNase by injecting the substrates through the CPY column. Figure 2 illustrates the mass spectrum produced from injection of RCM RNase in Figure 1 with background subtraction. Except for mass 150, only the MH^+ ions for the amino acids expected are seen as prominent peaks in the mass spectrum. Mass 120 is a fragment of phenylalanine. From the recovery yields calculated from the amino acid standard mixture, the first four, five and ten C-terminal amino acids could be easily determined from [Sar^1, Ala^8] angiotensin II (Sar-Arg-Val-Tyr-Ile-His-Pro-Ala), angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) and RCM RNase (Gly-Asn-Pro-Tyr-Val-Pro-Val-His-Phe-Asp-Ala-Ser-Val) respectively. Even though a single injection of substrate along with the standard injection could allow correct sequence determination, control of the extent of hydrolysis by either changing column temperature or flow rate could provide more reliable sequence information, especially for the substrates which contain some of the same amino acids repeat-

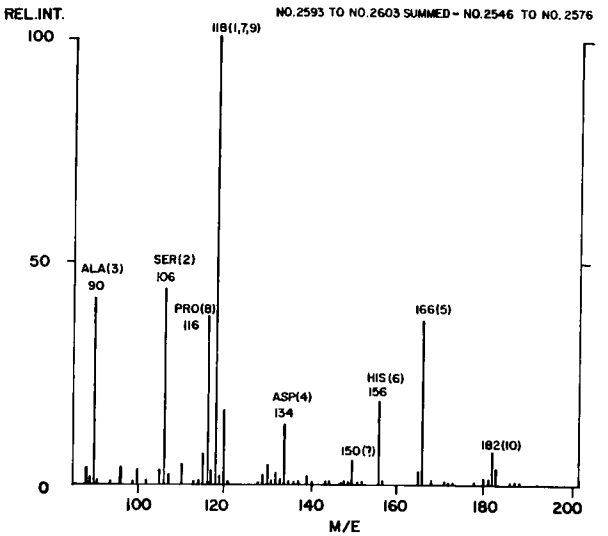

Fig. 2. Summed and background subtracted mass spectrum of amino acids recovered from 200 pmoles of RCM RNase from one of the results shown in Figure 1.

ed in the chain. As shown in Table I, more residues and higher yields were obtained for [Sar^1, Ala^8] angiotensin II by increasing the column temperature.

Table I. Recovery Yields of Amino Acids from [Sar^1, Ala^8]-Angiotensin II at Various Temperatures

	22°C	32°C	42°C
Ala	0.21	0.29	0.66
Pro	0.08	0.09	0.36
His	*	0.07	0.26
Ile	*	*	0.16
Tyr	*	*	*
Val	*	*	*

* Less than 0.03

Conclusions

With the thermospray LC/MS and immobilized CPY the correct C-terminus sequence can be determined very rapidly. The advantages of this technique are the following:

1) no need to derivatize;
2) sufficiently sensitive to provide sequence information at low picomolar level;
3) total analysis time is less than 2 minutes;
4) substrates are not limited to small peptides;
5) not limited to the C-terminus sequence determination only; can be extended to the N-terminus by using aminopeptidase.

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DETERMINATION OF THE ENANTIOMERIC COMPOSITION OF AMINO ACID CONSTITUENTS OF PEPTIDES WITH HIGH PERFORMANCE LIQUID CHROMATOGRAPHY: A METHOD FOR EVALUATING THE EFFECTS OF PEPTIDE SEQUENCE ON THE RACEMIZATION RATES OF BASIC AMINO ACIDS

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Introduction

Since the initial findings of Hare and Gil-Av[1], research has continued on the development of aqueous mobile phases in which ion pairing, incorporating chiral complexes, can be used in HPLC to resolve the D- and L-enantiomers of amino acids. Previous analyses of the extent of racemization of acidic and neutral amino acid constituents of heat-alkali treated melanotropins led to the design and synthesis of a highly potent melanotropin with ultralong biological activity.[2] The basic amino acid constituents of the melanotropins that were investigated, however, were not amenable to enantiomeric analyses with the gas chromatographic procedures employed.[3] In the present study, α-MSH, peptide fragments of α-MSH and (Nle^4)-α-MSH, and dipeptides were subjected to heat-alkali treatment (0.1 N NaOH, 10 min, 100°C). Next, the peptides were hydrolyzed (6 N HCl, 24 hr, 100°C). The extent

of racemization of Lys, His and Arg in the peptide hydrolyzates was determined by HPLC using an LC-18 reversed-phase column and an aqueous mobile phase containing L-proline coordinated to Cu^{++} as the chiral additive. Portions of the peptides were hydrolyzed directly (6 N HCl) to determine if partial racemization occurred during synthesis or acid hydrolysis. The effects of heat-alkali treatment on the stereochemistry of free L-Lys, L-His and L-Arg was also investigated.

Results and Discussion

The D/L values for the Lys, His and Arg constituents of the peptide hydrolyzates and for free Lys, His and Arg are listed in table I. Heat-alkali treatment did not result in partial racemization of the free amino acids. Lysine did not racemize in internal positions or as the C-terminal or N-terminal substituent, although the extent of racemization of Lys in the N-terminal position must be studied further since the N-terminal positions of the peptides studied were, with the exceptions of Arg-Lys and His-Phe, blocked with acetyl groups. Histidine racemized rapidly in internal peptide positions. In addition to stabilization of the incipient α-carbanion of His by the imidazole group, adjacent amino acids may be contributing to the formation of the α-carbanion of His. In α-MSH, and in the peptide Ac-Nle-Glu-His-Phe-Arg-Trp-Gly-NH_2, it is possible that the CO_2^- group of the R substituent of Glu, which is adjacent to His, may enhance the rate of α-proton extraction from His, thus increasing the rate of racemization.

Histidine was only slightly racemized in the N-terminal position of the dipeptide His-Phe. Histidine, however, racemized rapidly in the peptide Ac-Ser-Tyr-Ser-Nle-Glu-His-NH_2, although this peptide terminated with an NH_2 group, therefore

Table I. D/L Values* of Basic Amino Acids

Sample	0.1 N NaOH 100°C, 10 min	D/L Lys	D/L His	D/L Arg
Ac-α-MSH-NH_2	No	0.019 ±0.013	0.013 ±0.003	0.013 ±0.008
	Yes	0.014 ±0.006	0.439 ±0.021	0.389 ±0.023
Ac-α-MSH-NH_2 11-13	No	0.026 ±0.006	np†	np
	Yes	0.035 ±0.001	np	np
Ac-α-MSH-NH_2 7-10	No	np	np	...§
	Yes	np	np	0.683 ±0.012
Ac-(Nle^4)-α-MSH-NH_2 4-10	No	np	0.034 ±0.002	0.020 ±0.002
	Yes	np	0.497 ±0.019	0.450 ±0.011
Ac-(Nle^4)-α-MSH-NH_2 1-6	No	np	0.051# ±0.008	np
	Yes	np	0.502 ±0.004	np
Arg-Lys	No	...	np	...
	Yes	...	np	...
His-Phe	No	np	0.035 ±0.009	np
	Yes	np	0.064 ±0.032	np
Arg	No	np	np	...
	Yes	np	np	...
Lys	No	...	np	np
	Yes	...	np	np
His	No	np	...	np
	Yes	np	...	np

*Average of at least three chromatographic determinations.

†Not present.

§D-enantiomer below detection.

#Acid hydrolysis of this sample was conducted at 110°C for 24 hr, resulting in this slightly higher value.

eliminating the possibility of destabilizing the incipient α-carbanion of His by CO_2^-. Histidine racemized at a slower rate in α-MSH than in the peptide fragments of α-MSH and (Nle^4)-α-MSH. The rate of racemization of His may therefore be affected by factors in addition to its R substituent and the steric or electrostatic effects of its adjacent neighbors Glu and Phe. Arginine, like His, racemized at a slightly different rate in peptides that differed in overall chain length, although the amino acids adjacent to Arg (Phe-Arg-Trp) remained the same. In the dipeptide Arg-Lys, however, Arg did not racemize. Additional analyses of this peptide by HPLC prior and subsequent to heat-alkali treatment revealed that heat-alkali treatment did not result in the hydrolysis of this peptide to free Arg and Lys.

In summary, the racemization observed for Arg and His varied as a function of peptide position. The mechanisms that control this variance are no doubt complex and are dependent on a variety of parameters that include the structure of adjacent amino acids and perhaps overall peptide chain length, composition and conformation. The HPLC techniques used to perform the above analyses afforded a rapid, sensitive (picomole) method for monitoring the enantiomeric composition of amino acid constituents of naturally occurring and synthetic peptides. We thank V. J. Hruby and T. K. Sawyer for providing the peptide fragments for this study.

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SOME VARIABLES IN REVERSED PHASE CHROMATOGRAPHY OF PROTEINS AND PEPTIDES

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It may be said without exaggeration that high performance reversed-phase liquid chromatography (RPC) has had a major impact on protein chemistry. The degree of purification achieved today with synthetic peptides, proteolytic digests of large proteins and cyanogen bromide cleavage products was impossible before the advent of RPC. A problem is that it is so widely used and accepted that many users do not realize it is still in an evolving state. Concepts on mobile phase selection, column selection and retention mechanism have changed substantially in the past few years; all of which provide a more rational basis for optimization of resolution.

It is the objective of this paper to provide a limited examination of some variables involved in column selection and retention in RPC of polypeptides. Reversed phase HPLC has been used for almost two decades in the separation of small molecules where it was thought that the inorganic support was simply a passive carrier of the alkyl silane bonded phase. Although there were some selectivity differences between various manufacturers columns, this was attributed to differences in bonding chemistry and RPC was hailed as a universal technique by manufacturers and academic scientists alike. This concept of universality that emerged in analytical chemistry in the mid-seventies had the effect of directing attention away from the column in the case of some very disturbing problems that protein chemists were observing. Recovery and resolution of higher molecular weight, hydropho-

bic polypeptides was very difficult and not reproducible between laboratories. Even when the problem was traced to columns, it was again attributed to bonded phase chemistry.

The surprise was that the nature of the silica itself has a far greater impact on resolution and recovery of polypeptides than either the type of alkyl silane (C_1, C_8 or C_{18}) or the bonding chemistry used in its application. The first hint of the support contribution to resolution came when Lewis (1) showed that supports of 300 Å or larger pore diameter gave superior resolution of collagens over 200 kilodaltons (KD). This increase in resolution was attributed to the more extensive penetration and enhanced mass transfer of polypeptides within the macroporous matrix of the chromatography support. Studies by both Bennett (2) and Pearson (3) showed that the macroporous support used by Lewis also gave superior resolution and recovery of peptides under 10 KD. This was puzzling because these peptides were sufficiently small to gain ready access to pores of even 100 Å. A support with 300 Å pores should not have provided any substantial gain in resolution and recovery. The anomally was solved when Pearson (4) examined a variety of RPC supports that he had prepared with the same silane bonding chemistry but different silicas. Results of resolution and recovery studies with several of these supports on cyanogen bromide cleavage fragments of fetal hemoglobin and the bovine serum albumin-ovalbumin pair are shown in Table I. It is seen that the actual source of the silica is far more important than porosity. It may be concluded from these studies that the support matrix plays a major role in RPC of polypeptides. The exact mechanism by which this occurs has not presently been determined.

A second factor examined by Pearson (4) was the value of theoretical plate measurements in predicting the peptide resolving power of columns. A comparison of theoretical plate measurements and resolution is shown in Table II. Other than

Table I. Effects of Silica Matrix on Protein Separations

Silica type	Pore diameter (Å)	R_s[a]	R_{ec}[b]	R_s[c]	R_{ec}[d]
Nucleosil 100-5	100	1.39	1.97	0.75	5.02
Hypersil	120	0.43	0.70	0.99	2.62
Spherosil XOBO75	300	Poor	Poor	Poor	Poor
Spherisorb SG30F	300	1.80	4.33	1.81	6.65
LiChrospher Si 300	300	1.72	3.80	1.00	6.25
Vydac TP	330	2.38	5.70	2.37	6.59
LiChrospher Si 1000	1000	1.54	4.17	0.79	-

[a]Resolution of the bovine serum albumin-oalbumin pair.
[b]Recovery of ovalbumin relative to bovine serum albumin.
[c]Resolution of the alpha-CB_2 and -CB_3 peptides from CNBr cleavage of fetal hemoglobin.
[d]Relative recovery of the CB_2/CB_3 pair.

Table II. Protein Resolution vs. Theoretical Plates

Column BSA/OVA	N[a]	h[b]	R_s[c]
Partisil ODS-3	4050	6.17	2.03
LiChrosorb-RPC	3918	6.38	Poor
Partisil C8	2397	10.43	1.52
Bio-Rad ODS-5S	2330	21.46	1.74
Vydac-test-C_8	830	60.24	2.90

[a]Theoretical plate values were determined with $NaNO_3$ in H_2O at 0.5 ml/min.
[b]The symbol h designates reduced plate height.
[c]Resolution of the bovine serum albumin-ovalbumin pair.

the column prepared by Pearson, the columns chosen for examination were not specifically designed for polypeptide separations and it is expected that they should be inferior. It is seen that there is little relationship between the theoretical plate measurements and resolution. The interpretation of these results is that the contribution of the various silicas in resolving polypeptides dominates the smaller differences in theoretical plates. When the support variable is removed and comparisons are made between columns packed with the same support, correlations between resolution and theoretical plate height will then be found. An extremely important observation made during these studies was that column length made little contribution to the resolution of peptides over 5 KD. Columns 5 cm in length gave good resolution.

The data in Tables I and II illustrate that the most suitable technique for evaluating RPC columns is with a peptide mixture; preferably a higher molecular weight, hydrophobic mixture. Hopefully, a "bench mark" will be developed for commercial columns that evaluates them on their ability to resolve polypeptides.

The second issue this paper will address is the differences between retention of small molecules and polypeptides. Isocratic elution is common in the resolution of molecules under one kilodalton. In contrast, numerous investigators have noted that isocratic elution of polypeptides becomes increasingly unsatisfactory as molecular weight increases (1,5,6). Retention curves, i.e. plots of retention time versus displacing agent concentration, become increasingly concave in proportion to molecular size. A change of several percent in displacing agent concentration can cause a transition from retention to elution with large proteins. Clearly there is some major difference in the retention of large molecules.

When one visualizes adsorption onto the hydrophobic surface of a reversed phase support of a molecule 1000 times the size of benzene with a hundred or more hydrophobic moieties, it is intuitive that 1) multiple hydrophobic groups on both the support and polypeptide must participate in the adsorption process and 2) multiple solvent molecules must participate in solute desorption. In addition to these concepts of the adsorption process, Geng and Regnier (7) proposed that a stoichiometric number (Z) of solvent molecules would be required for desorption based on the nature of the stationary phase, the specific protein, organic displacing agent and pairing agents. Protein retention in RPC may be related to at least three variables as shown in the equation below; affinity of the protein for the support, number of solvent molecules (Z) required to displace the protein and displacing agent concentration $[D_o]$.

$$k' = I/[D_o]^Z = (T_r - T_o)/T_o$$

The k' term is a capacity factor which is related to both the chromatographic retention time (T_r) of the particular protein in question and that of a non-retained protein (T_o). The equation predicts that as Z increases retention curves will become more concave. This has been observed experimentally as noted above.

This retention equation has been used in treating the retention data of several proteins chromatographed on 300 Å pore diameter octyl silane columns (SynChrom RPP) eluted with either phosphoric, trifluoracetic or formic acid in isopropanol. Table III shows that the number of solvent molecules (Z) required for displacement is a function of both the protein and acid component of the mobile phase. Dependence of Z on the particular protein is easily explained. As the number of hydrophobic residues in a polypeptide increase either by increasing molecular size or by changing the hydrophobic to hydrophilic amino acid ratio in the molecule, it is to be expected that Z will increase. In contrast, the observed dependence of Z on acid composition was

Table III. The Influence of Acid on Z

	IPA-H_3PO_4 (0.05 M) pH 2.50		IPA-0.1% TFA		IPA-Formic acid (60%)	
	Z	log I	Z	log I	Z	log I
Insulin	15	6.52	16	8.1	4	-0.83
Cytochrome c	30	15.2	32	17.7	8	-1.9
Lysozyme			34	19.6	8	-0.6
Bovine Serum Albumin	117	63.8	96	57.3	33	-0.80
Carbonic Anhydrase	45	28.9	36	23.3	17	2.2
-Lacto-globulin	49	30.7			17	2.3

unexpected. The function of the acid in RPC has been thought to be two-fold; 1) it suppresses the ionization of surface silanols on the support and ionic interactions of solutes with these groups and 2) it forms ion pairs with cationic species in the polypeptide. Ion pairs with either formic or phosphoric acid should be hydrophilic and decrease both the interaction of a polypeptide with the reversed phase column and concomitantly, retention. Ion pairs with trifluoroacetic acid are said to have the reverse behavior because of its more hydrophobic character. Table III does not support this concept of how ion-pairing agents function. There is little difference between phosphoric acid and TFA. Although used a much higher concentration and lower pH, Z values with formic acid are 60 to 80% less than with the other acids. Clearly, another explanation of how these pairing agents work is required.

Solvent stoichiometry (Z) is also dependent on the type of displacing agent as shown in Table IV. As expected, fewer molecules of isopropanol and acetonitrile are required to displace a protein because of their more hydrophobic

Table IV. The Influence of Organic Solvent on Z

Protein	IPA-H_2O 0.1% TFA		CH_3OH-H_2O 0.1% TFA		ACN-H_2O 0.1% TFA	
	Z	log I	Z	log I	Z	log I
Insulin	16	8.1	24	27.9	19	14.4
Cytochrome c	32	17.7	51	59.8	38	29.0
Lysozyme	34	19.6	47	56.3	35	28.4
Bovine Serum Albumin	96	57.3	208	-	135	112.8

character. Probably the most surprising data in this table is the large Z value of bovine serum albumin with methanol.

From the data presented in this paper it may be concluded that; 1) the silica matrix makes a major contribution to the resolution of polypeptides on alkyl silane bond phase supports, 2) there is little correlation between the number of theoretical plates produced by a column with small molecules and its ability to resolve polypeptides, 3) column length makes little contribution in the resolution of peptides and proteins in RPC, i.e. short columns of 5 cm or less in length may be used, 4) multiple solvent molecules are required to desorb polypeptides from RPC columns and 5) there is an exact stoichiometry between the number of solvent molecules required for desorption and the particular protein.

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HIGH-PERFORMANCE PARTITION CHROMATOGRAPHY (HPPC) OF PEPTIDES ON CROSS-LINKED AGAROSE AND TSK GELS

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Introduction

The purification of peptides synthesized by chemical methods requires the separation of molecular species that do not differ in charge. Two useful separation methods are (a) partitioning in two-phase solvent systems [countercurrent distribution and partition chromatography (PC)] and (b) adsorption chromatography (HPLC). For a quantitative approach to PC, distribution constants of acetyl-amino acid amides, along with acetyl-Gly-Gly-NH_2 and acetyl-Gly-Gly-Gly-NH_2, were measured. By applying the Martin hypothesis[1-3] we can calculate ΔR_M (residue) values which are measures of the effect amino acid residues have on the mobility of a peptide in PC. Values for the rather typical solvent system A, 1-butanol: pyridine:0.6 M NH_4OAc (5:3:10, v/v), are: Trp, -3.03; Phe, -2.34; Tyr, -2.13; Leu, -1.91; Ile, -1.64; Met, -1.26; Val, -1.10; Ala, -0.08; His, -0.04; Pro, 0.09; Thr, 0.13; Gly, 0.30; Ser, 0.40; Gln, 0.56; Arg, 0.58; Asn, 0.73; Lys, 1.43; Glu, 1.86; Asp, 2.12. The ΔR_M (residue) values in the 31-peptide camel β-endorphin (β_C-EP) in solvent system A are reduced by a factor of two.[3] This was interpreted to mean that in β-endorphin the solvent accessibility of amino acid residues is no less than one-half of full accessibility. This finding was very encouraging since solvent accessibility governs the ability of PC to effect separations. The relationship of ΔR_M to the separation process is given by the

equation $R_S = (\sqrt{n}/4)\ (\Delta R_M)(1-R_f)$ where R_S is degree of resolution and n is the number of theoretical plates.[4] Current partition columns on Sephadex and agarose exhibit at most 500 theoretical plates and are, therefore, capable of effecting separations (R_S = 1) only when ΔR_M is 0.25 or greater. It is evident that when ΔR_M is about 0.10 a column of 3000 theoretical plates is required.

Results and Discussion

The compressibility of conventional gels limits the flow rates attainable and, therefore, attempts to decrease particle size and gain efficiency are defeated by the consequent slow flow rates. A new cross-linked 6% agarose (CL-agarose) with wet particle diameters of 20-40 μm (Pharmacia) can be operated in the PC mode under moderate pressure (5 p.s.i.) to yield practical flow rates (4-7 ml/hr/cm^2) without substantial shrinkage. Since pressure requirements are relatively low, conventional open columns are easily and inexpensively adaptable to these new conditions. The column can also be regenerated with 30% aqueous pyridine. An example of a separation in a 3000-plate column is shown in Figure 1, with a run on a 500-plate Sephadex G-50 column shown for comparison.

The TSK-gel column appears to offer greater promise for rapid PC. A commercially available column (7.5 x 30 mm TSK-G 4000 SW, 13 μm particle diameter, Alltech) was run at 500 p.s.i. in 50% aqueous methanol and then opened at the top and the dead volume filled with more gel. The steel frit at the top was reinserted and not removed subsequently. The most critical factor in preventing efficient PC in high-pressure systems appears to be the accumulation of aqueous droplets in various obstructions in the system while the organic phase is running. Vast improvement was achieved by use of an "undersaturated" organic phase. As a specific

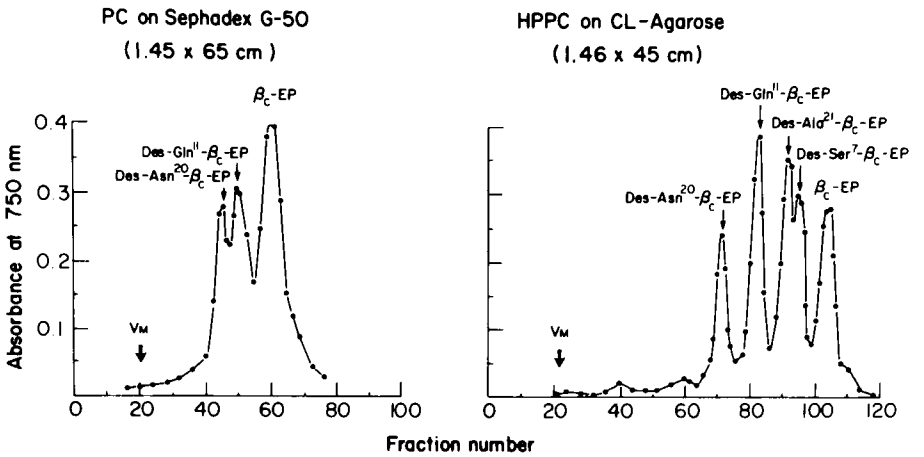

Fig. 1. Partition chromatography of peptide mixtures on Sephadex G-50 and CL-agarose in solvent system A.

example, a mixture of 98 ml of saturated upper phase (SUP) of solvent system A and 2 ml of 1-butanol:pyridine (5:3, v/v) gives a clear fluid capable of dissolving ca. 0.5 ml of saturated lower phase (SLP). This is defined as a 0.5% undersaturated upper phase (0.5% UUP). A complete cycle consisted of (1) SLP, (2) SUP, (3) flush of pre-column system with 50% aqueous methanol and 0.5% UUP, (4) 0.5% UUP, (5) chromatography in 0.5% UUP, and (6) regeneration with 50% aqueous methanol. When steps (1) and (6) were run at 0.4 ml/min and steps (2), (4), and (5) at 0.2 ml/min, pressures ranged 150-250 p.s.i. The TSK-gel showed 1000 plates as compared to the 3000-plate performance of CL-agarose for the same mixture (Figure 2). Although the TSK-gel appears unfavorable in this comparison, its speed of operation and the potential for further improvement makes it a very promising support for rapid and efficient PC.

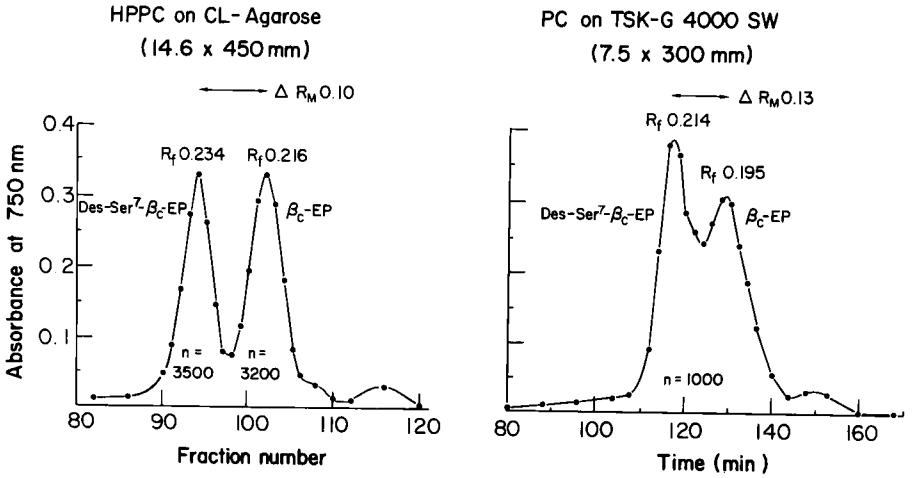

Fig. 2. Partition chromatography of a peptide mixture on CL-agarose and TSK-gel in solvent system A.

The cross-linked 6% agarose was a generous gift from Dr. Jan-Christer Janson (Pharmacia Fine Chemicals). This work was supported in part by a grant from the National Institute of Health (GM-30072).

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MULTIDIMENSIONAL REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF PEPTIDES: A SYSTEMATIC APPROACH FOR SELECTIVITY OPTIMISATION

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INTRODUCTION

Reversed-phase high performance liquid chromatography (RP-HPLC) based on microparticulate porous silicas chemically bonded with hydrocarbonaceous ligands has become the dominant technique for peptide analysis and purification during the past several years (1,2). With a specified non-polar stationary phase a single elution protocol will often not exhibit sufficient resolving power to accomodate the vagaries of complex peptide mixtures. Rechromatography under different elution conditions of unresolved components in subsequent discrete experiments or via sequential column switching procedures is thus mandatory. The present investigation was addressed towards a systematic approach for selectivity optimisation with peptides where such multidimensional RP-HPLC is required.

RESULTS AND DISCUSSION

Peptide retention in RP-HPLC with alkylsilica supports occurs by two discrete processes based on size exclusion effects and surface adsorption effects. In isocratic systems, retention can be defined in terms of the capacity factor (k') whilst under gradient elution retention can be discussed in terms of the median capacity factor (k') which is the instantenous k'-value as the peptide passes the midpoint of the column (3). At a fixed pH, buffer

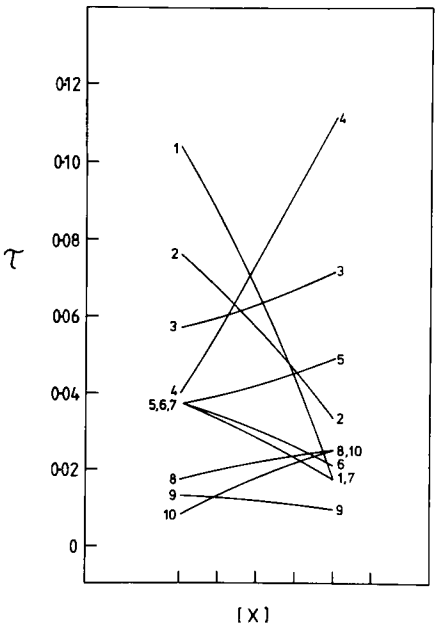

Figure. Plot of τ versus pairing ion concentration over range 1.5 m M to 65 m M for hGH tryptic peptides. Peptide code, 1 = T-11/T-1; 2 = T-2/T-8; 3 = T-18/T-20; 4 = T-12/T-14; 5 = T-1/T-18; 6 = T-4/T-11; 7 = T-6/T-10; 8 = T-10/T-4; 9 = T-18/T-2; 10 = T-20/T-15. Sequence assignment as in ref. 5.

composition and ionic strength, the general form of the dependency between peptide retention and solvent content follows (3,4) the relationship

$$k'_1 = k'_{w,1} \exp (F_{1,1}(\psi)) + (k'_{o,1} F_{1,2}(\psi))^{-1} + k'_{E,1} F_{1,3}(\psi) \qquad (1)$$

Systematic variation of bulk parameters, such as the ligand surface density, or a conditional parameter such as the pH, which influence a primary or secondary equilibrium process will give rise to a N-dimensional selectivity matrix in terms of k'_1, k'_2, k'_3, etc., due to conditions 1,2,3 etc. This selectivity matrix can be visualised from plots of ln k' as a function of one or more experimental variables. Individual values of the parameters k_w, k_o, etc. can be derived experimentally or calculated by standard methods of numerical analysis including Gaussian

elimination statistical procedures. For low pH, water rich eluents where 0.5, selectivity of polypeptides is dominated by the magnitude of the solubility parameter (k'_w), solvation parameter (k'_o) terms and the number of non-polar and polar sites (y and z) on the alkylsilica stationary phase to which the polypeptide binds. Relative evaluation of the y- and z- values can be achieved from the slopes of the decending and ascending aspects of the lnk' versus ψ plots over the range of eluent compositions where adequate solute solubility exists. Further, the linearised forms of these expressions for regular reversed phase elution behaviour of peptides in which little uniquely developed secondary structure is evident, under isocratic or gradient elution conditions are respectively given by equations (2) and (3).

$$\ln k' = \ln k'_w - S\psi \qquad (2) \qquad \ln k' = \ln k'_w - b(t/t_o) \qquad (3)$$

where

$b = S.V_m . d\psi/dt$, F^{-1}; $t_o = V_o.F^{-1}$; F = volumetric flow rate, and

S = solvent desorption parameter of a specified peptide.

Since selectivity (α) is defined under these regular reversed phase conditions as

$$\ln \alpha_{i,j} = \tau = \ln k'_{w,i}/\ln k'_{w,j} - \Delta S \psi \qquad (4)$$

$$\text{where } \tau_{i,j} = \frac{\Delta (\Delta G_{vdw})_{i,j} + \gamma N (\Delta A_j - \Delta A_i)}{2.3\ RT} \qquad (5)$$

and $\Delta(\Delta G_{vdw})_{i,j}$ is the difference in van der Waals interaction energies for solutes, i and j, γ is the instantaneous bulk surface tension of the mobile phase, and ($\Delta A_j - \Delta A_i$) is the difference in respective molecular contact areas, then selectivity optimisation for two peptide components which may have coincidental retention behaviour, i.e $\tau = 0$, under a particular chromatographic condition requires evaluation of τ in terms of solubility parameter and s-value differences or alternatively evaluation of τ in terms of $\Delta A_{i,j}$ as the mobile phase composition is varied. As no two peptides will have identical $\ln k'_w$ and S-values under several different RP-HPLC conditions, unique solutions to eqtn. 5 can be obtained from the selectivity matrix. Alternatively, with small peptides of known sequence, relative retention can be predicted with reasonable correlation from topographic indices such as retention coefficients obtained under one set of chromatographic

conditions and intersystem conversion factors (5). For any value of the experimental variable represented by the x-axis in these τ-plots, the lowest pair of intersecting lines on the plot correspond to the two peptide pairs having the poorest resolution. Illustrative of this approach is the τ-plot for several peptide pairs derived from the tryptic map of human growth hormone (22K-hGH) shown in the Figure. Although the two pairs of tryptic peptides T-2 (LFDNAMLR)/T-18 (DMDKVETFLR) and T-15 (FDTNSHNDDALLK)/T-20 (IVQCRSVECSCGF) of native 22K-hGH are the four peptides from the complete digest which are least readily resolvable on μBondapak C_{18} columns under low pH phosphate or TFA-aqueous acetonitrile elution conditions, optimal resolution for semi-preparative RP-HPLC separations could be readily achieved from only several exploratory analytical experiments. Similar selectivity optimisation methods have been utilised in this laboratory for the resolution of closely related microheterogeneous forms of polypeptide hormones including relaxins and growth hormones.

In summary, selectivity optimisation based on an analysis of the τ-plots dependencies greatly expands the versatility of RP-HPLC methods for peptide separation and experimentally provides guidelines for the selection of alternative chromatographic conditions to permit optimal resolution of peptide mixtures.

ACKNOWLEDGEMENTS

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CHARACTERIZATION OF β-ENDORPHIN PROCESSING IN BIOLOGICAL MATERIALS BY REVERSED-PHASE LIQUID CHROMATOGRAPHY

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Introduction

The endorphins are a class of neuropeptides that have created a great deal of research interest in the neurosciences. These naturally occurring β-lipotropin (β-LPH) fragments have biological actions similar to opiates such as morphine. Recent evidence from our laboratory and others has shown that the metabolism (processing) of one of these peptides derived from the C-terminus of β-LPH, β-endorphin (βE; βE 1-31), not only results in the breakdown of this peptide but leads to the formation of fragments that may be involved in various behavioral and physiological processes.[1-3]

β-endorphin is also released into the systemic circulation in response to a number of physiological stimuli. Increases in endorphin levels have been shown to change cardiovascular, respiratory and metabolic function but no specific target for circulating βE has been identified. Recently, we reported that perfusion of the dog small intestine with βE results in an increase in phasic and tonic motility and the production of several α- (βE 1-16) and γ- (βE 1-17) type endorphins which were active.[4] These observations led to the hypothesis that a balance exists between α-, γ- and β-type endorphins in specific tissues that is necessary for physiological homeostasis and an alteration in this balance may be responsible for pathological states. To address this hypothesis, it was necessary to develop selective, precise and quantitative high performance liquid chromatography (HPLC) procedures capable of separating the many βE related fragments that could be produced by in vitro incubations with membrane homogenates from tissues of interest.

Materials and Methods

The *in vitro* metabolism of human βE was studied using membrane preparations initially homogenized in 1mM Na/K phosphate buffer, washed twice by centrifugation at 50,000 x g for 10 min and resuspended in 50 mM phosphate buffer (pH 7.4). Tissues were obtained from adult mongrel dogs as described previously.[4] After 5 to 90 min incubations with βE (20 μM) at 37°C, tissues were boiled for 15 min and centrifuged for 60 min at 15,000 x g. The supernatant was assayed for βE and related peptide fragments by HPLC.

Peptides were separated on Beckman Ultrasphere ODS-5μ columns using a series of gradients of acetonitrile against 0.1M sodium phosphate buffer (pH.2.2). Peptides were detected at 210 nM with a Perkin-Elmer Model LC-65T Detector. Variation in background absorbance drift due to solvents was subtracted using a Cole Scientific Axxiom Model 301-99 Data Saver. Recording and quantification was accomplished using a Hewlett-Packard Model 3390A Integrator. The flow rate was maintained at 2.0 ml/min and the column temperature was 40°C.

Endorphin fragments were generously donated by Dr. H.M. Greven and J.W. van Nispen (Organon International, OSS, The Netherlands).

Results and Discussion

Figure 1 illustrates the high resolution HPLC separation and specific retention times for βE and 27 related peptide standards. β-endorphin has one of the longest retention times (70 min), whereas more polar fragments such as βE 2-9, βE 18-31, and βE 1-5 (methionine enkephalin) elute much earlier. Since peptide separations on hydrocarbonaceous columns (C-18: ODS) are highly dependent on the degree of hydrophobicity, those peptide fragments containing more of the hydrophobic amino acids leucine, phenylalanine and isoleucine elute later in the chromatographic profile shown.

Time-course processing of βE in membrane homogenates of canine whole brain minus cerebellum and the nerve/muscle region of the small intestine are demonstrated in Figures 2 and 3. Note the similarity in the ratio and

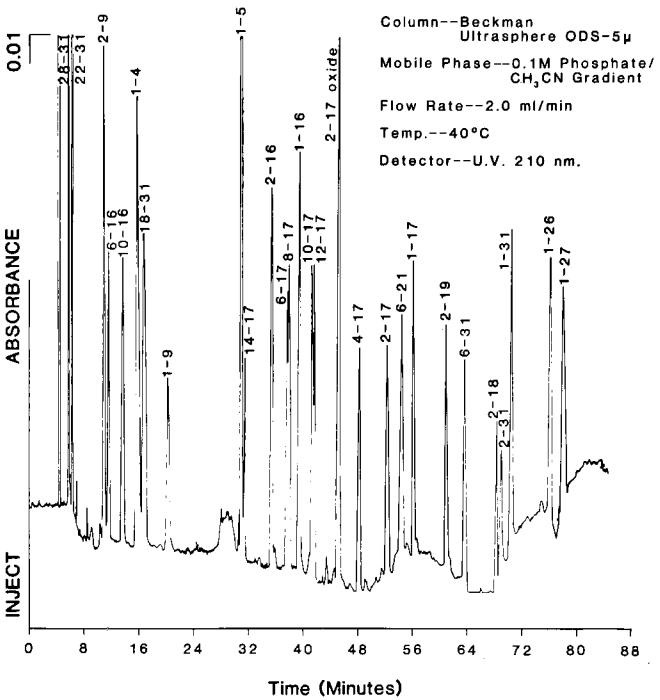


Fig. 1. Reversed phase HPLC separation of 28 β-endorphin related standards. Each peak corresponds to 70-200 ng injected.

pattern of α- and γ-related peptides formed. Extrapolation of the βE concentration by HPLC analysis over the time-course of 5 to 90 min resulted in an experimental zero time concentration which was only 10% lower than the absolute amount (Figure 2 and 3, inset). Analysis of half-life kinetics resulted in similar rates between brain and the nerve/muscle region ($T\frac{1}{2}$=141 vs 82 min), whereas the mucosa of the small intestine and whole kidney had βE half-lives of 22 and 19 min, respectively (data not shown). The mucosa and kidney also exhibited a very random rate of fragment formation which differed qualitatively and quantitatively from the nerve/muscle region and the brain. Although the nerve/muscle region had a 10-fold higher concentration of fragments produced per mg of protein, the ratio and pattern of α- to γ-type endorphins was very similar. Therefore, these data suggest that βE processing in the nerve/muscle region of the small intestine may be occurring at the membrane of the nerve.

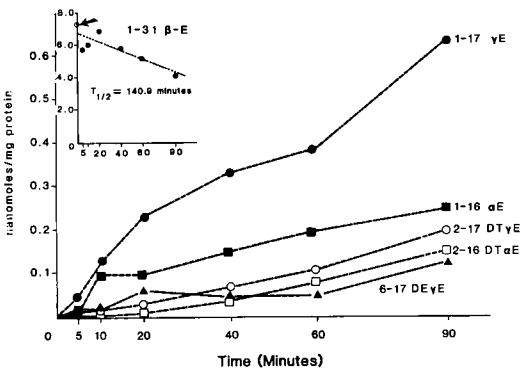
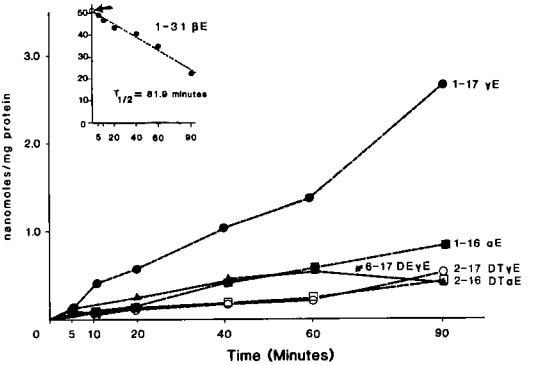


Fig. 2. Time-course processing of βE in membrane homogenates of brain. Inset describes kinetics of βE degradation. The arrow points to absolute concentration of βE added per mg protein (5.35 mg protein/ml). Standard deviations of duplicate analyses were within 10%.

Fig. 3. Time-course processing of βE in membrane homogenates of small intestine nerve/muscle region (0.64 mg protein/ml). Inset is as described above. Standard deviations of duplicate analyses were within 10%.



Conclusions

The data presented offers evidence that a specific balance exists between the formation of α- and γ-type endorphins in different tissues. The question of whether this balance in the gastrointestinal system and kidney is altered in pathological states, as we have previously shown centrally in post-mortem brains from schizophrenics,[3] remains to be established.

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SIDE REACTION ON A SYNTHETIC PEPTIDE GENERATED BY FORMATE BUFFERS DURING PURIFICATION BY PREPARATIVE H.P.L.C.

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Introduction

Preparative reversed phase high pressure liquid chromatography becomes more and more used in the purification of synthetic peptides as a complementary technique to the usual procedures (such as ion-exchange chromatography, adsorption chromatography or gel filtration). [1,2,3] In this type of chromatography buffers containing the triethylammonium counter-ion have proven to be eluents giving good separation of peptides on octadecyl silica gel. This paper reports the purification of des Gly-NH_2, (Arg^8)-vasopressin (dGAVP) for which a preparative HPLC was required. The use of a triethylammonium formate buffer gives rise to a chemical modification of the peptide. This reaction and the recovery of dGAVP from the side-product are described.

Results and Discussion

Solid phase peptide synthesis - N-α-Boc-Arg (ω-Tos) was esterified on a 1% crosslinked chloromethyl-polystyrene by the caesium salt procedure[4] to an extent of 0.32 mmoles/g. Protected N-α-Boc amino acids used during the solid phase synthesis were: Arg (ω-Tos), Pro, Cys (p-OMe-Bzl), Asn-ONp, Gln-ONp, Phe, Tyr (2-Br-Z).

2.5 equiv. of each Boc amino acids were coupled with DCC (except Asn and Gln coupled as their p-nitrophenyl esters). The Boc group was removed with 50 % TFA in CH_2Cl_2 (30 min). The crude peptide was cleaved from the resin by anhydrous HF containing 10 % anisole during 45 min. at 0°C. Oxidation with potassium ferricyanide has permitted cyclisation of dGAVP through disulfide bridge formation.

Purification - After gel filtration on Sephadex G15 in 2N AcOH the monomeric peptide was chromatographed on silica gel (Lobar® Merck) with n-BuOH-Pyr-AcOH-H_2O (4:1:1:2) as eluent. The recovered product was homogeneous on TLC in the solvent systems : A : n-BuOH-Pyr-AcOH-H_2O (4:1:1:2) and B : AcOEt-Pyr-AcOH-H_2O (5:5:1:3). The overall yield was 5 % based on the substitution of the polystyrene with N-α-Boc-Arg (ω-Tos). Nevertheless two minor impurities were detected by HPLC analysis. They are eluted after the main peak of dGAVP on a Hibar prepacked column RT 250-4 (4 mm x 25 cm) of octadecyl silica gel (10 µ, Merck) using a linear gradient of 10 % to 20 % CH_3CN in triethylammonium formate (1 % formic acid adjusted to pH 3.5 with TEA) over 20 min. (Figure 1 a). One g of the peptide was submitted to a preparative reversed phase chromatography in the same buffer system (1 L) on a column (2,6 cm x 40 cm) filled with 50 g of octadecyl silica gel (Nucleosil, 25-40 µ, Macherey-Nagel). The flow rate was 50 ml/h. Fractions characterized by one single peak by HPLC analysis were pooled and concentrated by evaporation. The residue was diluted with water, lyophilized twice and desalted on Sephadex G10 (in 2N AcOH). TLC and HPLC analyses indicated that the product was transformed into a less polar compound, (Figures 1 b and 2). This product was visualized by the Pauly, Sakaguchi and chlorine/tolidine reagents but not with ninhydrin. As the modification was shown to occur after the evaporation and lyophilization, we postulated that the presence of large excess of concentrated formic acid at this stage could lead to N-formylation of the peptide. In order to cleave this N-formyl group we dissolved 170 mg of the modified peptide in 10 ml of 1 N H Cl. After 16 hours of reaction time the solution was passed through a Sephadex G10 column (2N AcOH).

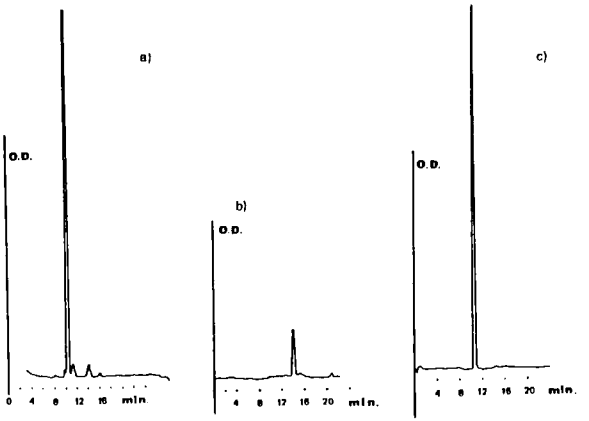

Fig. 1. HPLC analysis of dGAVP : a) dGAVP after silica gel chromatography (200 µg, O.D. full scale = 0.1 at 274 nm); b) dGAVP after preparative HPLC and desalting on Sephadex G10. (20 µg, O.D. full scale = 0.2); c) pure dGAVP obtained through purification on CM-cellulose and silica gel chromatography (200 µg, O.D. full scale = 0.2 at 274 nm).

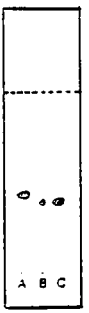

Fig. 2. Thin layer chromatography analysis (solvent system A) of : A - 50 µg of peptide obtained after preparative HPLC and desalting; B - 50 µg of pure dGAVP; C - 50 µg of product A after 1N HCl treatment (16 h).

A main fraction of 77 mg of homogeneous dGAVP was recovered. The product is eluted as a single peak in HPLC and reacts with ninhydrin (see Fig. 2 for TLC analysis).

To avoid the reversed phase chromatography step we developed another purification scheme involving ion-exchange chromatography. 4.7 g of peptide issued from the first gel filtration on Sephadex G15 were purified on a carboxymethylcellulose (with gradient elution of AcOH concentration varying from 0.05 M to 1.75 M) followed by a silica gel chromatography (in solvent system A). 1.1 g of pure dGAVP was obtained after a last gel filtration on Sephadex G10. The HPLC profile of this product is shown in Figure 1 c. Its amino acid composition is : Asx : 0.97; Glx : 1.02; Pro : 1.00; Cys : 1.73; Tyr : 0.98; Phe : 1.01; Arg : 1.00.

Conclusion

The resolution capacity and the volatility of triethylammonium formate buffer seems to make it a suitable solvent for the preparative purification of synthetic peptides. However, we have been able to show that the concentration of the eluted fraction leads to extensive N-formylation of the peptide. This side reaction limits thus the usefulness of this buffer for preparative HPLC.

Acknowledgement

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USE OF SYNTHETIC PEPTIDES FROM RHODOPSIN'S CARBOXYL-TERMINUS TO ELUCIDATE SPECIFICITY OF ANTI-RHODOPSIN MONOCLONAL ANTIBODIES

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We have raised monoclonal antibodies against rod cell outer segment membranes which contain the visual pigment rhodopsin as their principal protein component.[1] Among those antibodies produced we identified several with specificity for the carboxyl-terminal region of rhodopsin.[2] We then synthesized a family of peptides containing sequences from rhodopsin's carboxyl-terminal region. These peptides were used in a radioimmunoassay to compete for binding of antibody to rhodopsin in order to determine the binding specificity for each monoclonal antibody.

Monoclonal antibodies were produced and screened for specificity as previously described.[1,2] Three of these antibodies (1D4, 1C5, and 3A6) were chosen for further characterization of their binding specificity.

Peptides from the carboxyl-terminal region of rhodopsin were synthesized manually using the modified solid-phase procedure of Merrifield.[3] The amino acid sequence of the carboxyl-terminal 18 amino acids of bovine rhodopsin is[4]: $Asp^{18'}$-Glu-Ala-$Ser^{15'}$-Thr-Thr-Val-Ser-$Lys^{10'}$-Thr-Glu-Thr-Ser-

$Gln^{5'}$-Val-Ala-Pro-$Ala^{1'}$-COOH. Synthesis of the family of peptides 1'-4', 1'-6', 1'-8'... to 1'-18' was started using 1 mmol of Boc-Ala-PAM-resin (0.59 meq Ala per gram resin). Boc amino acids were used, and prior to coupling, the Boc group was removed by treatment with 50% TFA in CH_2Cl_2. Neutralization was carried out with 10% DIEA in CH_2Cl_2. The side chain functional groups of Ser, Thr, Glu, and Asp were protected by the benzyl group, and the ϵ-amino group of lysine was blocked with the 2-chlorocarbobenzoxy group. [^{3}H]Ala was incorporated in position 3' (1 mCi/mmol) to facilitate peptide purification and concentration determination. All couplings were performed by the DCC/HOBt method and double coupling was employed at every cycle. The peptides were cleaved from the resin by treatment with HF containing 10% anisole for 45 min at 0°C. The crude peptides were desalted on a Bio-Gel P-2 column (2.5 x 100 cm) in 50% HAc and submitted to preparative HPLC as shown in Figure 1A. Peptides 3'-18' and 3'-18'(Gln 8') were prepared by the same procedure. All synthetic peptides gave the expected molar ratios on amino acid analysis and were >90% pure by analytical HPLC as illustrated in Figure 1B.

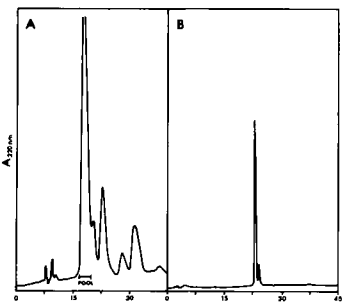

Fig. 1(A) Preparative HPLC of peptide 1'-12' (14.7 mg) on a Whatman ODS-3 (Magnum 20) column using 75% A (0.1% TFA in water) and 25% B (methanol) at 10 ml/min with 2.0 AUFS. (B) Analytical HPLC of purified peptide 1'-12' on a Whatman ODS-3 0.5 x 25 cm Column using 0 to 30% B over 40 min where A = 0.1% H_3PO_4 in H_2O and B = 0.05% H_3PO_4 in CH_3CN.

When monoclonal antibody 1D4 was tested in the solid phase radioimmunoassay (Figure 2A), competition was observed with peptide 1'-8' and improved with increasing peptide length (1'-10' and 1'-12'). Longer peptides (1'-12' to 1'-18') bound to antibody nearly as well as rhodopsin itself. The carboxyl-terminal residues 1' and 2' must be part of the antigenic site since peptide 3'-18' did not compete (Figure 2A). We conclude that the antigenic site bound by monoclonal antibody 1D4 is from residues 1'-2' to 11'-12'. Monoclonal antibody 1C5 (Figure 2B) binds to the amino-terminal region of peptide 1'-18': peptide 1'-18' competes, and changes at positions 1', 2', and 8' have no effect. Monoclonal antibody 3A6 (Figure 2C) binds to the middle of peptide 1'-18': changes at positions 1' and 2' have no effect, but substitution of Gln for Glu at position 8' abolishes binding activity.

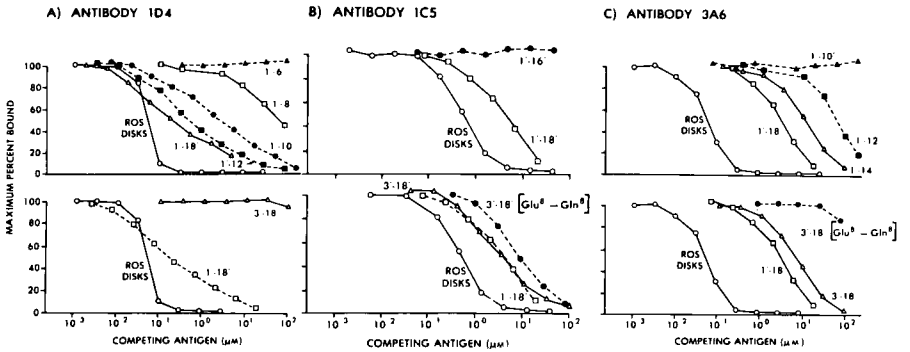

Figure 2. Competition of peptides with rhodopsin in a solid phase radioimmunoassay using anti-rhodopsin monoclonal antibodies.

In conclusion we have synthesized a family of peptides representing portions of the amino acid sequence from the carboxyl-terminus of bovine rhodopsin. These peptides have been evaluated as competitors for the binding of rhodopsin

to anti-rhodopsin monoclonal antibodies. Each monoclonal antibody tested showed different regions of binding within the carboxyl-terminal sequence. Monoclonal antibodies with such well-defined specificities will be of use in study of the structure and function of rhodopsin's carboxyl-terminus, its cellular location and protein molecular topography.

Acknowledgements

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PTH-AMINO ACID IDENTIFICATION BY MULTICOMPONENT ANALYSIS OF FIRST AND SECOND DERIVATIVE ULTRAVIOLET SPECTRA

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Introduction

PTH-amino acids, the end products of protein sequence analysis, are typically analyzed by HPLC, a sensitive technique with a turnaround time of 20-30 minutes per cycle.[1] Although nondestructive and highly compatible with HPLC analysis, ultraviolet spectroscopy has not been employed because of the extreme spectral similarities of these compounds. It has been previously shown that multi-component analysis of first and second derivative UV spectra can be used to identify PTH-amino acids (Levine & Lehrman, in preparation). We now report preliminary findings which indicate that these methods can be applied to protein sequence analysis.

Materials and Methods

PTH-amino acids (Pierce) were purified by HPLC, using a modification of a known procedure, and diluted to 20-50μM with 20% CH_3CN. The purity of each derivative was then checked, and repurified if greater than 2% impurity was observed. CH_3CN and water were HPLC grade.

[19-24]-Calcitonin was synthesized by standard solid phase techniques, and sequenced on a microprocessor-controlled

Beckman 890C protein sequenator.

For these studies, a Hewlett-Packard 8450A UV Spectrophotometer was used. This instrument was able to collect 20 spectra from 200-800 nm, subtract background, and average the results within a period of 10 seconds. After calculation of the first and second derivatives, a simple procedure enabled muticomponent analysis of unknown compounds vs. stored spectra. Statistical parameters evaluating the validity of the data were automatically determined.

Results and Discussion

Primary UV spectra of 19 PTH-amino acids were recorded and the derivative spectra calculated. In general, the derivative spectra were more distinctive than absorption spectra and therefore exploited for identification purposes. For example, absorption spectra of Pth-tyrosine and -phenylalanine are quite similar (Figure 1, panel A), but dramatic differences in the second derivative spectra of these compounds are observed from 265-285 nm (panel C). Second derivative spectra have been previously shown to be useful in quantitative determinations of protein-bound tyrosine, phenylalanine and tryptophan.[2]

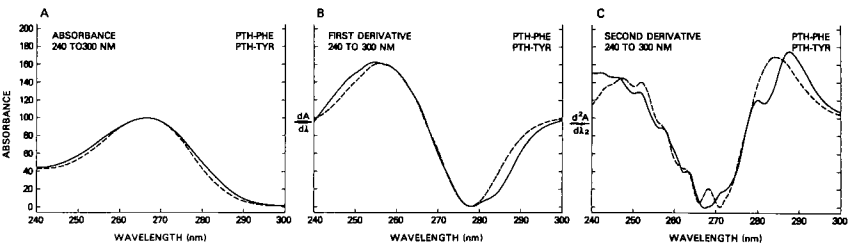

Fig. 1. Normalized absorption, first and second derivative spectra of PTH-tyrosine and -phenylalanine.

The first four residues of [19-24]-calcitonin were sequenced, and the resultant PTH-amino acids were analyzed by modification of a published HPLC procedure. Multicomponent analysis on the HP 8450A, after HPLC purification and dilution with 20% acetonitrile, confirmed the identity of each of these cycles (see Figure 2). Cycle 3 was identified as PTH-threonine by multicomponent analysis vs. PTH-threonine, serine, glutamine and asparagine despite the fact that most of the material underwent dehydration and other side reactions. As shown in Table I, cycle 4 was properly identified as PTH-tyrosine when analyzed against standard spectra of PTH-tyrosine and alanine. When the analysis was expanded to include PTH-valine, methionine and proline, however, incorrect assignments were made. This indicates that further development of the method is required. Sequence analysis of the last 2 amino acids was not possible, presumably due to washout of the peptide.

Simultaneous determination of retention time and full-scan UV spectra is possible with currently available HPLC detectors. Our findings indicate that multicomponent analysis of such data may permit development of an on-line system for routine confirmation of PTH-amino acid assignments.

Table I. Multicomponent Analysis of Cycles 1,2, and 4 From [19-24]-Calcitonin Sequence Analysis

Cycle 1		
Amino Acid	Concentration (μM)	Relative standard deviation
Leu	18.2	0.13
Ile	4.2	0.82
Phe	N.D.	—

Cycle 2		
Amino Acid	Concentration (μM)	Relative standard deviation
Gln	23.5	0.09
Ser	0.43	2.08
Thr	N.D.	—
Asn	N.D.	—

Cycle 4		
Amino Acid	Concentration (μM)	Relative standard deviation
Tyr	16.4	0.34
Ala	N.D.	—

[19-24]-Calcitonin: H-Leu-Gln-Thr-Tyr-Pro-Arg-OH. N.D.= not detected.

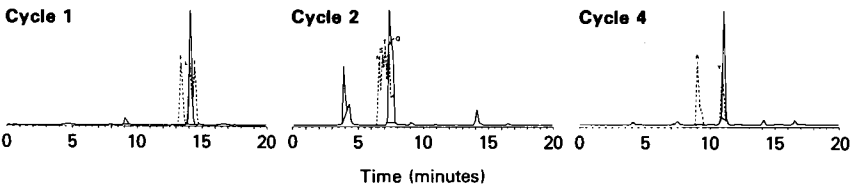

Fig. 2. HPLC profiles of the cycles 1,2 and 4 from [19-24]-calcitonin sequence analysis. Solid lines (——) indicate elution profiles. Dashed lines (---) indicate the elution positions of other PTH-amino acids used in multicomponent analyses (see Table I).

Summary

We have demonstrated that multicomponent analysis of derivative spectra can be used for PTH-amino acid identfication and have applied these methods to confirm HPLC analysis of four PTH-amino acids derived from sequence analysis of [19-24]-calcitonin.

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SYNTHESIS OF AMINO-TERMINAL FRAGMENTS OF CHOLECYSTOKININ 26-33

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Cholecystokinin (CCK) has been identified in brain and found to coexist with dopamine in certain dopamine-containing neurons. The role of CCK remains to be elucidated. We have reported that CCK produces changes in animal behaviors similar to that induced by haloperidol.[1] To establish the minimal structural requirements of CCK 26-33 for this activity, we have prepared peptides comprising the amino-terminal sequence including the sulfated Tyr residue. Solid-phase synthesized and sulfated peptides were purified by countercurrent chromatography (CCC) on the flow-through coil planet centrifuge (Figure 1).

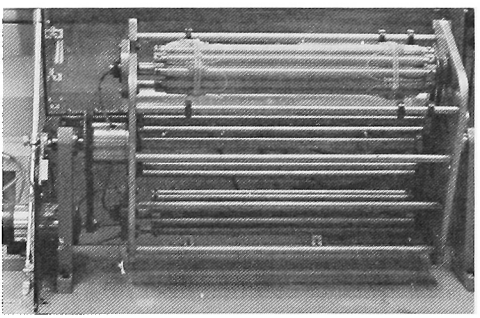

Fig. 1. Kontes prototype. On gear side, top, is mounted the preparative chromatography coil. Lower coil is 0.55 mm i.d. tubing for analytical scale chromatography. Accessory equipment are the Electro-Craft Motomatic drive, Milton Roy minipump, MTS 4-way sample loading slide valve, UV detector and fraction collector.

The instrument is comprised of a coil of 1.5 mm i.d. PTFE tubing wound on rods mounted on the gear side of the apparatus. The total volume is 130 ml or 260 ml when 2.5 mm i.d. tubing is used. Samples of 100 to 400 mg are loaded in a small volume of a two-phase solvent system onto the coil already filled with stationary phase. During the chromatography the coil is rotated 400 rpm in the counterclockwise direction and mobile phase pumped at 24 ml/hr. Under these conditions, 50% of the stationary phase is retained. Fractions of 15 min are collected. The UV absorbance of the effluent is monitored and fractions containing peptide are concentrated and analyzed. Details of the methodology are described elsewhere[2].

Table I Cholecystokinin Fragment Peptides

	Sequence	Solvent System	K
1.	Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp NH_2	BAW	.27
2.	Ac Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp NH_2	NH_4OAc	.03
3.	Ac Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp	BAW	.88
4.	Ac Asp-Tyr(SO_3H)-Met-Gly-Trp-Met NH_2	NH_4OAc	.90
5.	Ac Asp-Tyr(SO_3H)-Met-Gly-Trp-Met	NH_4OAc	.03
6.	Ac Asp-Tyr(SO_3H)-Met-Gly-Trp NH_2	HH_4OAc	.16
7.	Ac Asp-Tyr(SO_3H)-Met-Gly	BAW	.12

Upper phase of solvent systems was used as mobile phase.
BAW = n-butanol, acetic acid, water (4:1:5 by volume)
NH_4OAc = 0.2 to 0.4 M ammonium acetate, n-butanol (1:1)
K = partition coefficient = solute conc. in mobile phase/ solute conc. in stationary phase[2].

The peptides prepared are listed above. Unsulfated N-acetylated peptides were assembled on the chloromethyl resin (peptides 3, 5, and 7), benzhydrylamine resin (1 and 2) or p-methylbenzhydrylamine resin (4 and 6). Boc amino acids including β-Bzl-Asp and 2Br-CBZ-Tyr were coupled in 2.5 molar excess. Peptide was cleaved by HF/anisole including ethyl methyl sulfide for 45 min at 4°C. The product was submitted

to CCC. The results of the purification of unsulfated Ac CCK 26-29 are shown in Figure 2. The K was 0.48.

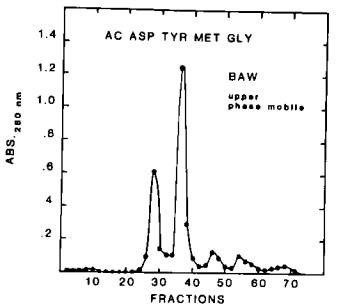

Fig. 2. Chromatography on the coil planet centrifuge of 291 mg synthetic product. Mobile phase solvent front emerged at fraction 22. Fractions 35-40 contained 176 mg of purified peptide.

The other unsulfated peptides are relatively hydrophobic and were eluted very close to the solvent front when the upper phase was mobile. Conditions of lower K's (0.5 to 0.03) resulting in better separations were effected by using the BAW system with the lower phase mobile. Peptides were eluted later and distributed in a larger volume but well separated from impurities. The CCC of unsulfated Ac 26-32 NH_2 is such an example presented in Figure 3.

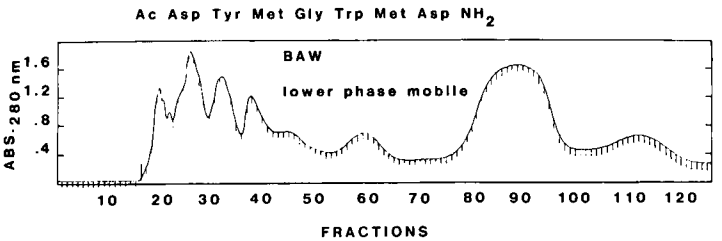

Fig. 3. Chromatography of the heptapeptide. Solvent front occurred at fraction 17. Fractions 76-98 were 60 mg which was subsequently sulfated.

The purified peptides were sulfated by pyridine—SO_3, and purified by CCC in the conditions listed in Table I. Being more hydrophilic, the sulfated peptides were eluted well after the solvent front and before, or sometimes with, the excess salt of the sulfation reaction. Ac CCK 26-32 (peptide 3),

Ac CCK 26-31 NH_2 (4) and Ac CCK 26-30 NH_2 (6) required no further purification. However, Ac CCK 26-32 NH_2 (2) as shown in Figure 4 was contained in the last peak, fractions 98-106 of the CCC, and had to be separated from salt by HPLC (C_{18}, .8 x 30 cm column in 0.02 M NH_4OAc and an acetonitrile gradient of 10% to 25%). The yield after this step was 52 mg or 80% yield from the sulfation reaction. For the synthesis of unacetylated peptides, trifluoroacetyl peptides were prepared and after sulfation the Tfa group was removed. The purity of the peptides was determined by TLC, HPLC and amino acid analysis. Spectral analyses established the presence of the sulfate group. CCC on the coil planet centrifuge proved to be useful in purifying these peptides.

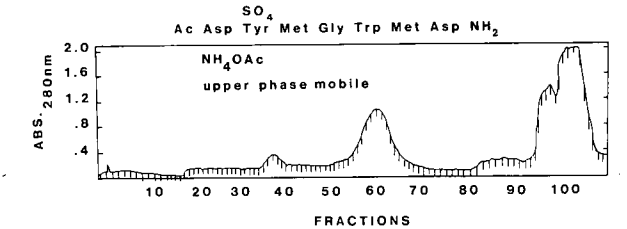

Fig. 4. CCC of the heptapeptide sulfation reaction. Solvent front emerged at fraction 18; at fraction 81 rotation was stopped and contents were pumped out. Sulfated peptide was included in fractions 98-106.

The interactions of Ac CCK 26-32 NH_2, Ac CCK 26-31 NH_2, Ac CCK 26-30 NH_2 and Ac CCK 26-29 were assessed with CCK receptors of guinea pig brain. Only Ac CCK 26-32 NH_2 displayed specific binding to the central nervous system receptors and did so more potently than dibutyryl cyclic GMP, a known CCK antagonist.

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SOLID STATE NMR OF PEPTIDES IN MEMBRANE BILAYERS

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Introduction

Many peptides and proteins are associated with membranes. There are a wide variety of plausible mechanisms for interactions between amphiphilic lipids and amphiphilic peptides. The most obvious limiting cases are for charged interactions between functionalized sidechains of amino acids and the polar headgroups of lipids, and for hydrophobic interactions between both backbone and sidechain parts of the peptide and the hydrocarbon chains of the lipids. There are few well characterized examples of these limiting cases, much less for multiple and more subtle means of molecular interactions. These complex biological systems, whether isolated intact or reconstituted from pure components, are very difficult to study by physical methods. In particular, NMR studies of peptides (and proteins) in membrane environments, with a few exceptions[1-3], have been limited to describing the effects of peptides on the lipids.[4]

Solid state NMR offers several promising simplifications for these studies, as compared to conventional, high resolution, solution NMR. First of all, peptides associated with

extended multilamellar bilayers can be studied, so the research is not limited to sonicated vesicle or micelle model systems. The lack of overall reorientation of the peptide-lipid complex means that internal motions can be separated from overall reorientation of the molecules themselves. The use of specific isotopic labels (here ^{15}N and ^{2}H) means that the peptide portions can be characterized independent of the lipid portions.

Synthetic hydrophobic peptides with one or a few labelled sites were prepared and found to be associated with lipids in the presence of excess water. These provide an excellent model system for characterizing the basic dynamical properties. They also provide background for yet more complex biological problems, such as membrane associated proteins. The coat proteins of the filamentous bacteriophages are small hydrophobic proteins that reside in the cell membrane prior to virus assembly. Important features of peptides and proteins in membrane bilayers are shown to be similar by the comparative study of hydrophobic synthetic peptides and viral coat proteins.

The motions of membranes are as crucial to their function as other chemical and physical properties. The great deal of background on the dynamics of lipids stands in contrast to the little information on molecules imbedded in bilayers. The preliminary results described here are designed to begin a full elucidation of the dynamics of peptides and proteins in membrane environments.

Peptide Backbone Dynamics by NMR

In general, molecular motions influence NMR spectral results by their averaging effects on lineshapes and by inducing spin relaxation. The largest, and qualitatively simplest, effects are seen in the averaging of powder pattern

lineshapes by motions that are rapid relative to the timescale pertinent to the nuclear spin interaction. Roughly speaking, this timescale is the breadth in frequency units of the static powder pattern.

^{15}N chemical shift anisotropy (CSA) has proven useful in characterizing peptide backbone dynamics.[5] Figure 1 compares the effects of various degrees of motional averaging on the static CSA powder pattern. Isotropic reorientation results in complete averaging of the CSA to the single line of Figure 1A, as seen in liquid samples. Small amplitude motions, here modeled as librations about the CSA principal axis system, that occur rapidly on the 10^3 Hz timescale alter the lineshape.

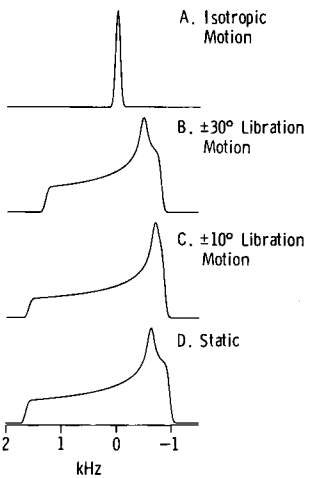

Fig. 1. Motional averaging of amide ^{15}N CSA.

Experimental ^{15}N peptide bond CSA powder patterns from peptides in bilayers are shown in Figure 2 and compared to a calculated static pattern (Figure 2E). A polycrystalline peptide has a rigid backbone by these measures as seen by comparing the breadth and shape of Figure 2D to that of Figure 2E. The three hydrophobic synthetic peptides with ^{15}N labeled backbone sites give similar, static patterns

in Figures 2A-2D. This demonstrates that these three peptides, including a dipeptide (Figure 2A), have backbone amide linkages immobilized by the lipid-peptide interaction.

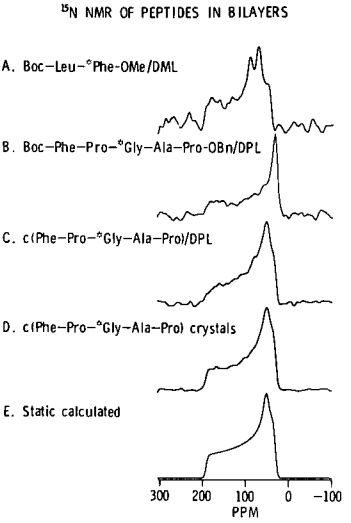

Fig. 2. ^{15}N Spectra of amide sites of peptides in bilayers as labeled in the Figure.

Figure 3C shows that the entire backbone of the coat protein in the filamentous bacteriophage fd is rigid.[5] Figure 3B shows that the coat protein from fd and the similar bacteriophage Pfl when reconstituted in a membrane protein complex have partially flexible peptide backbones. Most of the peptide linkages are immobile, as seen for the intact virus and the synthetic peptides in bilayers. However, a significant percentage give a narrow, effectively isotropic resonance superimposed on the broad powder pattern of the rigid sites, indicative of substantial motional averaging. The presence of two distinct motional regimes for the coat protein backbones in bilayers mirrors the theoretical comparison of Figure 1.

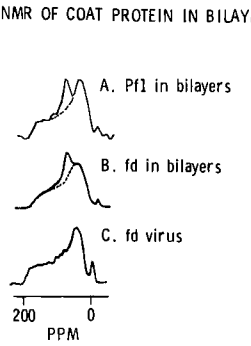

Fig. 3. ^{15}N NMR spectra of uniformly labeled filamentous bacteriophage coat proteins. The main band is from the amide sites. The upfield resonance is from lysine sidechains.

Sidechain Dynamics by NMR

Large amplitude, rapid motions substantially alter the powder patterns from nuclear spin interactions. The ^{2}H quadrupole powder pattern from specific C-^{2}H sites has been widely used to characterize molecular dynamics in the solid state. Figure 4 shows calculated powder patterns for the ε (or δ) C-^{2}H bonds of phenyl sidechains. The static pattern is in Figure 4D; its breadth is substantially greater than that of the ^{15}N CSA pattern shown in Figure 1A. This shifts the timescale for motions that influence the powder patters from 10^{3} Hz to 10^{6} Hz. Figure 4A is the fully averaged single line spectrum from complete, isotropic motional averaging. Figures 4B and 4C demonstrate the effect of rapid motion about the C_{β}—$C\gamma$ bond axis. The difference lies in the type of motion, where free diffusion gives the axially symmetric pattern of Figure 4B while 180° flips give the asymmetric pattern of Figure 4C.

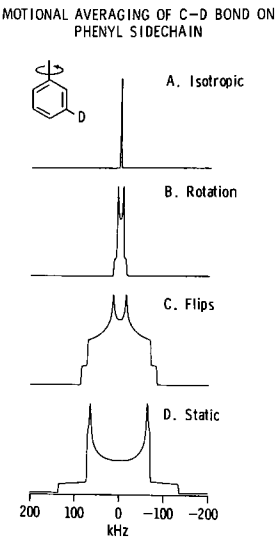

Fig. 4. Calculated ^2H NMR spectra for ring sites.

Tyrosine and phenylalanine rings in amino acids, peptides, and proteins have been shown to be either rigid or undergoing rapid 180° flips between equivalent sites by solid state NMR.[2,5-8] Figure 5A is the experimental spectrum from d_5Phe labeled Boc-Leu-Phe-OMe in DML bilayers. Figures 5B-5D are calculated patterns for the five deuterons of a phenylalanine undergoing rotational, flip, or no motion about the C_β-C_γ bond axis, where the four δ and ε deuterons are averaged, but the ξ deuteron is unaffected. The phenylalanine of the dipeptide in bilayers clearly has unhindered diffusion of the ring about the C_β—C_γ bond axis. Figure 2A shows the peptide bond to be rigid, while Figure 5A shows the ring to be rotating.

Contrasting dynamics are observed for the phenylalanine and tyrosine rings of fd coat protein in bilayers. The two tyrosine residues in the central hydrophobic portion of the sequence undergo only 180° flip motions, since the lineshape of Figure 6B is closely similar to that in Figure 4C. By contrast, some of the phenylalanine rings show substantial

motional averaging, giving an essentially isotropic resonance in Figure 6A.

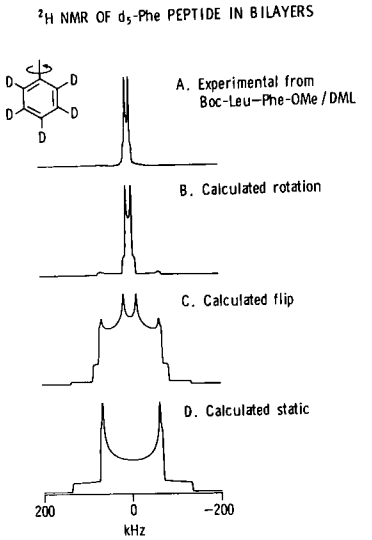

Fig. 5. A. ^{2}H NMR spectrum of d_5Phe labeled Boc-Leu-Phe-OMe in DML bilayers. B-D. Calculated ^{2}H NMR spectra for d_5Phe.

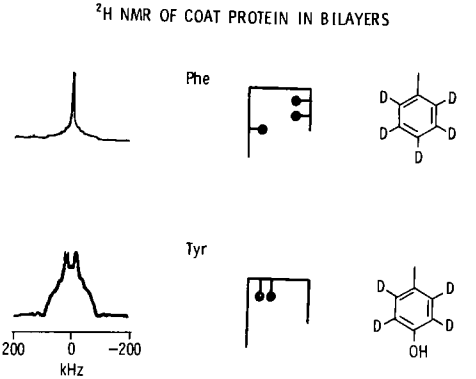

Fig. 6. ^{2}H NMR spectra of d_5Phe and d_4Tyr labeled fd coat protein in DPL bilayers.

Discussion

The relatively small synthetic peptides give readily interpreted results, in that the peptide backbones are immobilized by the lipid bilayer environment. The phenylalanine ring of the dipeptide undergo rotation about the C_β—C_γ bond axis, but no other motions of substantial amplitude. The results from fd coat protein are more complex, presenting a partially flexible backbone. If that portion of the backbone with many degrees of motional freedom included at least two of the phenylalanine residues this would explain the extensive motional averaging seen in Figure 6A. However, specific ^{15}N site labeling experiments show that all three phenylalanine residue have immobile peptide bonds. Therefore, complex combination of C_α-C_β and C_β-C_γ motions, but not of the peptide bond, must be present in the phenylalanine residues.

The dynamics of peptides and proteins in membrane bilayers are considerably more complex than for globular protein in solution or native structural proteins, where only rigid backbones and rigid or flipping rings have been observed. These preliminary studies in membrane bilayers show that rigid and partially flexible backbones exist and that a wide range of phenyl sidechain motions, including rotational diffusion and effectively isotropic averaging are present.

Acknowledgements

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SOME ELEMENTS IN CONFORMATIONAL DESIGN OF PEPTIDES

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Introduction

Peptides are flexible molecules. Each amino acid residue in the chain has a number of sterically reasonable conformations which differ slightly in stability. Further, the conformational states of successive residues are quite independent. Thus the number of conformational states becomes very large even for oligopeptides.

Polypeptides and proteins utilize long-range cooperative effects and the hydrophobic effect to stabilize particular conformations. The thermodynamic driving force for protein folding is the hydrophobic effect[1]. That is, amino acids with lipid-like side chains are preferentially placed in the interior of the protein, sequestered from the solvent. Regular structures, α helix and β sheet, amplify subtle differences in conformational energy to stabilize a single overall conformation and give the structure rigidity.

Oligopeptides have no interior that is completely shielded from solvent and are too short to fully utilize cooperative effects. Therefore, in order to stabilize particular oligopeptide conformations, more covalent restrictions must be employed than in proteins. Examples are numerous among both naturally-occurring and synthetic peptides. Some of the restricting elements employed are cyclization, D-amino acids, and steric bulk.

Four important conformational determinants for oligopeptides are covalent structure, excluded steric volume, hydrogen bonds and solvation. Because of the strength of chemical bonds, conformations determined by the covalent structure are the most rigid. Atoms are only slightly compressible, so that steric volume excludes many conformations. Peptide hydrogen bonds and solvation affect the population of the sterically allowed regions.

Since oligopeptides have a large fraction of their surface exposed, solvation strongly influences which conformations are observed. In this paper, current efforts to elucidate peptide hydration will be briefly summarized. Some aspects of peptide conformation in aqueous solution can be explained by hydration-shell models[2] and by consideration of explicit water molecules in the first two hydration layers[3]. The solvent has been more thoroughly treated via molecular dynamics[4] and Monte Carlo methods[5,6]. These latter studies have shown that the potential functions employed describe molecular interactions in aqueous peptide systems quite well. Additional studies are needed to predict conformations in dilute aqueous solution for testing against experimental observations. It is hoped that empirical methods can be parameterized to reproduce many of the conformational predictions of molecular dynamics and Monte Carlo simulations.

In the following sections steric control of peptide conformation and some factors which influence the strength of peptide hydrogen bonds will be discussed. This discussion will be based on isomeric methylproline peptides. Methylprolines can give a specific conformation to a region of a peptide chain.

Excluded Steric Volume

Cis-trans isomerism of the peptide bond preceding proline is well known. We have shown that adding a methyl group to either the C^{α} or C^{δ} of proline affects the rate or the position of equilibrium for this isomerism[7,8]. Observed percentages cis based on ^{13}C NMR spectra are given in Table I for AcProNHMe and Ac-2-MeProNHMe. The 15-25% cis observed for AcProNHMe is typical of many proline peptides. The absence of cis isomer for Ac-2-MeProNHMe can be qualitatively understood from the increased steric bulk at C^{α}. In the trans isomer the smaller carbonyl oxygen is adjacent to C^{α}, but in the cis isomer the larger methyl group is in this position. The experimentally observed percentages cis are well matched by predictions based on the consistent force field (CFF) method of Lifson and co-workers[9]. The increase in percentage cis in going from chloroform to water is overestimated by the in vacuo energy computed with an infinite dielectric constant. The fact that this crude model does not correspond well with the behavior in aqueous solution is no surprise. The CFF calculations predict a majority of the cis conformation for Ac-5,5-Me_2ProNHMe. While we have no

experimental data on this peptide, the related peptide N-acetyl-2,2-dimethyl-thiazolidine-4-carboxylic acid has been observed to have only the cis isomer in dimethylfulfoxide[10].

TABLE I. Percentage Cis Peptide Bond Isomer for Ac-X-NHMe

X	Percentage Cis			
	Theory		Experimental[a]	
	$\epsilon = 1$	$\epsilon = \infty$	$CHCl_3$	H_2O
Pro	15	45	15	25
2-MePro	0	5	0	0
5,5-Me_2Pro	50	95	--	--

a. From reference 8. Based on ^{13}C NMR spectra.

TABLE II. Percentage C_7 Conformer for Ac-X-NHMe[a]

X	Percentage C_7	
	CCl_4	$CHCl_3$
Pro	95	85
anti-3-MePro	90	75
syn-3-MePro	60	15

a. From reference 8. Based on infrared spectra.

For many peptides containing the trans peptide bond isomer, the intramolecularly hydrogen-bonded C_7 conformer (ϕ , $\psi \cong -100^o, 80^o$) is preferred in non-polar solvents. The experimental percentage of this conformation for three proline peptides is given in Table II. For AcProNHMe, all of the population which has the trans peptide bond isomer also has the C_7 conformation. Since the proline carbonyl oxygen eclipses C^β in the C_7 conformation, steric bulk at C^β should decrease the population of C_7. Indeed, Ac-syn-3-MeProNHMe has only 15% C_7 in chloroform solution, and 60% C_7 in CCl_4. Even chloroform, a relatively inert solvent, interacts strongly with peptides[11]. For this peptide the enthalpy of transfer from carbon tetrachloride to chloroform is -7.0 kcal/mol compared to -2.9 kcal/mol for transfer from chloroform to water (Table III).

TABLE III. Enthalpies of Transfer for Ac-X-NHMe[a]

X	Enthalpy (kcal/mol)	
	CCl_4 to $CHCl_3$	$CHCl_3$ to H_2O
Pro	-6.0	-2.5
anti-3-MePro	-4.0	-2.4
syn-3-MePro	-7.0	-2.9

a. Calorimetric enthalpies from reference 11.

For AcProNHMe, CFF calculations predict 85% C_7 and an expectation value of 80° for ψ. For Ac-syn-3-MeProNHMe, 70% C_7 and $\langle\psi\rangle = 98^{\circ}$ are predicted. This prediction is consistent with observations in carbon tetrachloride solution. Predictions for Ac-syn-(3,4)-methyleneProNHMe are similar to those for Ac-syn-3-MeProNHMe. Even though the methyl and methylene groups are separated in space, both are expected to be effective in disrupting the C_7 conformation. In contrast, Ac-(3,4)-dehydroProNHMe and AcProNHMe which have similar covalent structure and steric bulk, are predicted to have similar conformational behavior. Infrared spectra indicate 60% C_7 in $CHCl_3$ for Ac-(3,4)-dehydroProNH$_2$. In crystals this peptide has the cis peptide isomer[12]. Perhaps increased rigidity of the five-membered ring relative to AcProNHMe stabilizes the cis isomer at the expense of the C_7 conformer.

Hydrogen Bonding

The crystals and molecular structures of the seven isomeric AcMeProNHMe[13] and AcProNHMe[14] have been determined by X-ray diffraction. The strengths of the hydrogen bonds in the crystals have been gauged from the amide A (NH stretch) frequency in their infrared spectra.

The relationship between the hydrogen bond donors and acceptors in crystals of the eight proline peptides is shown in Figures 1 and 2. All of the amide hydrogens are on the 2.0 A sphere around the oxygen (O—H distances range from 1.93 to 2.08 A). The distances of the amide hydrogen to the plane of the acceptor amide show considerable variation (Figure 2).

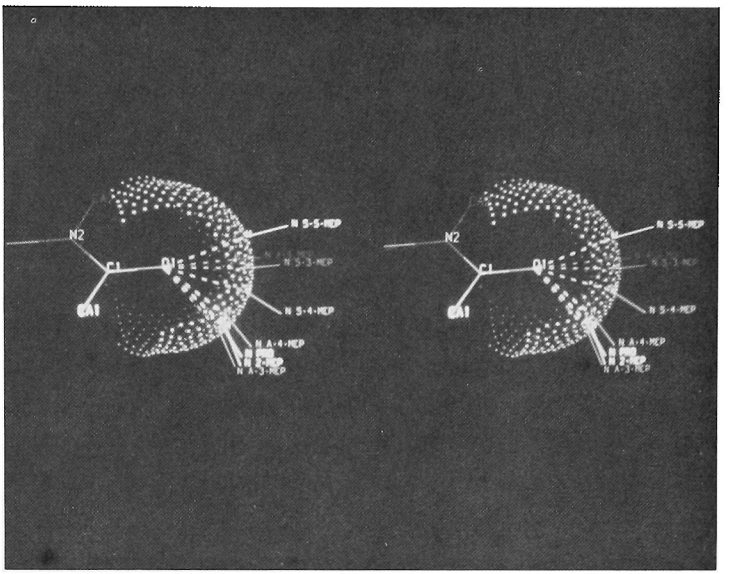

Fig. 1. Stereoview of positions of the amide NH relative to the hydrogen bond acceptor in crystals of eight methyl proline peptides, Ac-X-NHMe, X is indicated in the figure. A sphere of 2.0 A radius about the oxygen atom is shown.

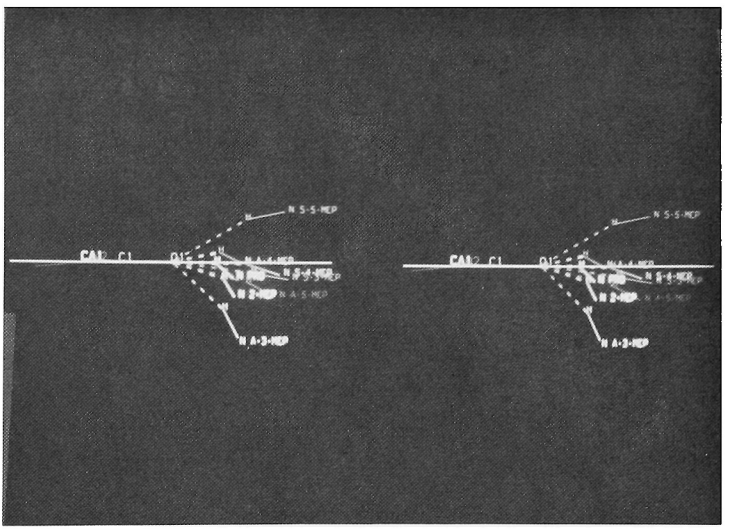

Fig. 2. Stereoview as in Figure 1 showing positions of the amide NH's relative to the plane of the hydrogen bond acceptor.

Hydrogen bond lengths and angles are plotted versus amide A frequency in Figure 3. The hydrogen bond length shows little variation and no clear relationship to amide A frequency. The NH—O angle is not far from linear (180^{o}) throughout the series. The CO—N and CN—O angles fall in two groups. The CO—N angle is near 120^{o} for the hydrogen bonds with lower frequencies and near 160^{o} for those with higher frequencies. The CN—O angle is ca. 130^{o} or 110^{o} for the hydrogen bonds with lower and higher frequencies, respectively.

The distances of the hydrogen bond donor (H) to the plane of the acceptor amide and of the acceptor (O) to the plane of the donor are plotted versus amide A frequency in Figure 4. The oxygen distances fall roughly in two groups near 0.0 and 0.4 A. The hydrogen distances tend to increase with increasing frequency. A regression line can be drawn with only the point at 3287 cm^{-1}, 1.1 A (Ac-anti-3-MeProNHMe) showing a large deviation.

The data presented here supports other theoretical and experimental studies which indicate that the strongest hydrogen bonds have the CO—N and CN—O angles near 120^{o}, values expected for sp^{2} hybridization, and NH—O nearly linear. This study raises the possibility that the out-of-plane distance of the amide H is also an important parameter in determining hydrogen bond strength. Ab initio quantum chemical calculations are in progress to test this possibility.

The amide A infrared band of Ac-syn-5-MeProNHMe in the solid state matches that of the C_7 conformation for the peptides in solution. The hydrogen bond parameters, especially the out-of-plane distance of the amide hydrogen, are also well matched in the two cases. The strength of the C_7 hydrogen bond is, therefore, expected to be similar to the weakest intermolecular hydrogen bond in the solid state series.

Conclusions

The studies summarized here illustrate that methyl substituents can control cis-trans peptide bond isomerism and the population of the C_7 conformation. The methylprolines are conformationally defined building blocks for peptide synthesis. The study of hydrogen bonding indicates that the relationship between hydrogen bond strength and out-of-plane distance of the donor should be more thoroughly investigated.

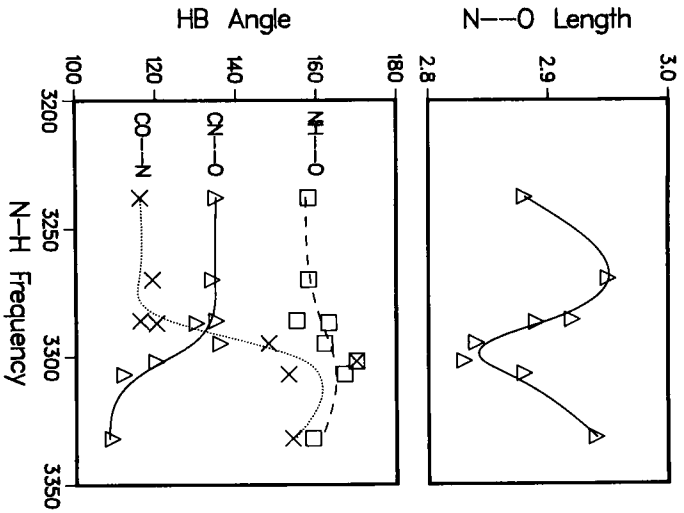

Fig. 3. Hydrogen bond parameters versus amide A frequency in crystals of eight proline peptides, Ac-X-NHMe. In order of increasing frequency the X's are Pro, anti-4-MePro, 2-MePro, anti-3-MePro, syn-4-MePro, syn-3-MePro, anti-5-MePro, and syn-5-MePro, respectively. The distance is in Angstroms, angles in degrees, and frequency in cm^{-1}. Data are from reference 13.

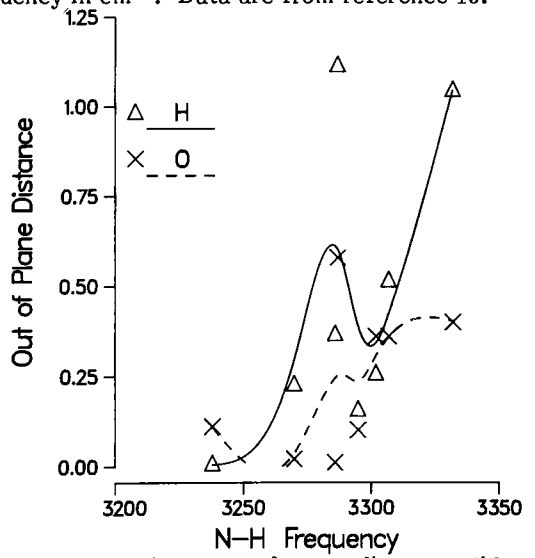

Fig. 4. Out-of-plane distances for proline peptides versus amide A frequency. Peptides as in Figure 3. The distances are from the amide hydrogen (H) to the plane of the acceptor and from carbonyl oxygen (O) to the plane of the donor. Data from reference 13.

Acknowledgments

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REVERSE TURNS IN HYDROPHOBIC ENVIRONMENTS

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The effect of a hydrophobic environment on the tendency of a polypeptide chain to adopt reverse turn conformations has been examined in two complementary ways. 1) A computer search of globular proteins of known structure has yielded several examples of "buried" reverse turns that occur in the hydrophobic interior region of the proteins. 2) Conformational studies of synthetic peptides designed to model hydrophobic turn sequences have been carried out by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) and by circular dichroism (CD).

Nine interior β turns were found in a search of 22 proteins whose X-ray structures have been determined and refined, and for which coordinates of solvent molecules in the crystal are also available.[1] A striking finding is that each interior turn interacts with at least one crystallographically located interior water molecule (Figure 1). Visualization of these interior turns revealed that all of their polar groups were hydrogen bonded either to waters or to other groups on the protein. Furthermore, the turn-associated waters were found to be well-buried in the proteins, and to participate in at least three hydrogen bonds.

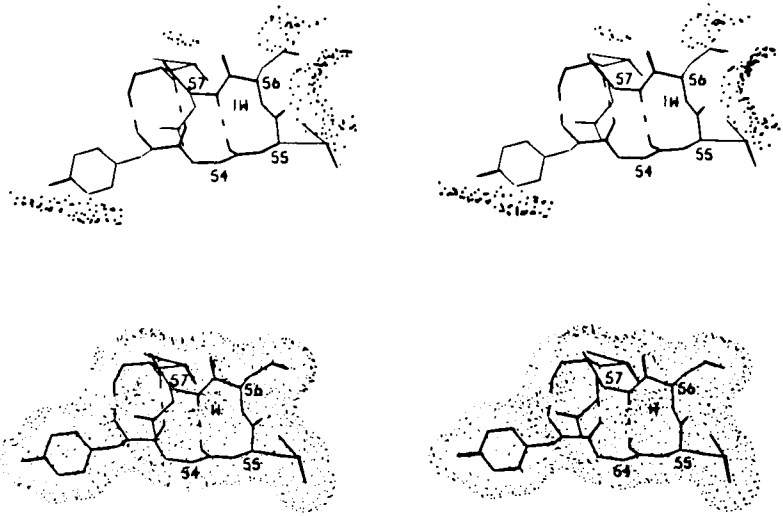

Fig. 1. Two stereoviews of the "buried" β turn in lysozyme. In the top view, the solvent-accessible surface of the turn in the protein is shown (stippled). In the bottom view, the solvent-accessible surface of the isolated turn is shown. The turn-associated water is labeled "W" in these views.

Reverse turns are intrinsically polar moieties, since they necessarily expose hydrogen bonding groups to the medium around them. A large proportion of turns in globular proteins occurs at the solvent-accessible surface. These examples of interior turns show that water molecules incorporated into the protein structure can help to provide a mechanism to stabilize buried turns. A similar strategy may be invoked in the folding of membrane proteins into turns within the interior of a bilayer.

To explore the conformational preferences of the synthetic model peptides in hydrophobic microenvironments, NMR and CD studies were carried out on peptides solubilized in sodium dodecyl sulfate (SDS) detergent micelles. The peptides' spectral properties indicate: 1) preferred interactions with the micelle (versus the bulk solvent), 2) oriented interactions

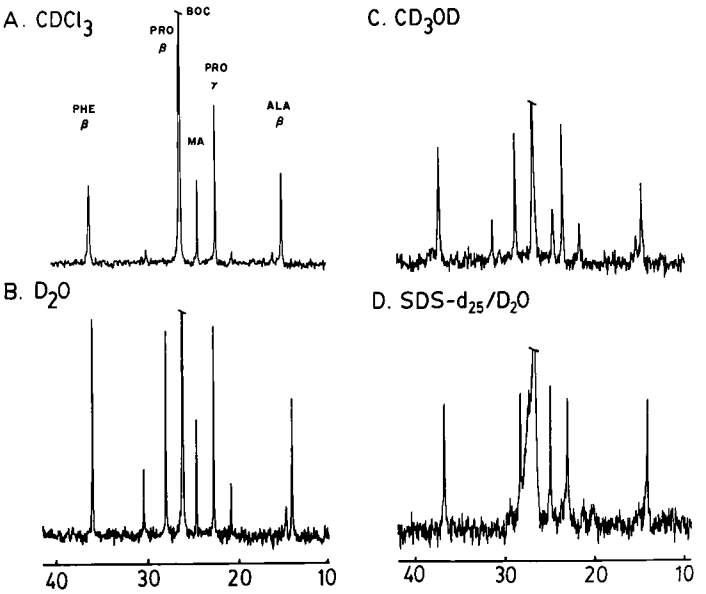

Fig. 2. 62.9 MHz ^{13}C NMR spectra (upfield region) of *t*Boc-D-Phe-D-Ala-Pro-$NHCH_3$. A. Conc 0.02 M in $CDCl_3$. B. Conc 0.01 M in D_2O; C. Conc 0.02 M in CD_3OD; D. Conc 0.01 M in 0.050 M SDS in D_2O. Note the small proportion of *cis* X-Pro conformer in SDS. "MA" signifies methyl amide.

with respect to the micelle surface, and 3) conformational distributions distinct from those observed to occur in bulk solvents, including reverse turn structures.

The tripeptide *t*Boc-D-Phe-D-Ala-Pro-$NHCH_3$ is soluble in water, but undergoes changes in its CD spectrum as SDS is added to an aqueous solution. ^{13}C NMR data reveal that the interaction with SDS micelles shifts the equilibrium between *cis* and *trans* conformers (around the D-Ala-Pro bond) towards that distribution seen in solvents less polar than water (i.e. chloroform) (Figure 1). Yet the chemical shift difference between the Pro C^{β} and C^{α} resonances ($\Delta\delta_{\beta\gamma}$), which is indicative of the average local conformation of the prolines, reflects an effective environment similar to methanol or water. No evidence

of a turn conformation is obtained in this short peptide. The related tetrapeptide, Cbz-Pro-D-Phe-D-Ala-Pro-$NHCH_3$, manifests a preferred orientation with respect to the micelle surface. Not only do the two prolines yield spectral characteristics indicative of a more polar effective microenvironment around the N-terminal portion of the molecule (higher *cis/trans* ratio) than around the C-terminal portion, but also differential shifts for different parts of the molecule are seen in ^1H NMR spectra upon addition of a paramagnetic ion (Eu^{+3}) to the aqueous phase. The $\Delta\delta_{\beta\gamma}$ data indicate that the C-terminal part of the peptide adopts a γ turn conformation a large portion of the time.[2]

The sequence of the buried β turn in lysozyme, Tyr-Gly-Ile-Leu-Gln (where Ile-Leu are in positions i+1 and i+2 of the turn), has been incorporated into a synthetic model peptide. Studies of the linear hexapeptide, *t*Boc-D-Tyr-Gly-Ile-Leu-Gln-Pro-OH indicate a very favorable interaction with SDS micelles. Preliminary NMR data (temperature dependences of NH resonances and NH exchange kinetics) suggest that this peptide takes up a folded conformation in methanol, dimethylsulfoxide, and aqueous SDS solutions. Some initial indications of nuclear Overhauser enhancements between remote parts of the peptide in SDS solution have been observed.

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NMR STUDIES OF NATURAL DERMORPHIN AND ITS SYNTHETIC ANALOGUES

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Introduction

The natural opioid heptapeptide dermorphin isolated from the skin of frogs (1) has an analgesic activity many times higher than natural enkephalins. Its sequence was shown to be $Tyr^{1}DAla^{2}Phe^{3}Gly^{4}Tyr^{5}Pro^{6}Ser^{7}NH_{2}$. (2) Here we report (i) proton assignments of cationic dermorphin, the L-Ala isomer and the N-terminal fragment, (ii) determination of the relative amide proton solvent exposures, (iii) a preliminary conformational analysis of dermorphin using $^{3}J_{\phi}$ coupling constants and ϕ and ψ NOEs.

Results and Discussion

All resonances in Table 1 were assigned by one dimensional double resonance methods (3) and/or 2D ^{1}H:^{1}H shift correlation (4) at 200,

TABLE I

		tri*	tetra	penta	hexa	hepta	L-hepta
		CHEMICAL SHIFTS (δ) IN PPM					
Y^1	H_α	4.050	-	4.050	4.020	4.060	4.030
	H_β	2.960	2.930	2.940	2.950	2.960	2.920
	$H_{\beta'}$						3.020
a^2 (A^2)	H_α	4.387	4.382	4.400	4.395	4.409	4.459
	H_β	0.863	0.905	0.822	0.859	0.838	1.303
	H_N	8.532	8.480	8.416	8.361	8.508	8.670
F^3	H_α	4.532	4.637	4.658	4.700	4.695	4.717
	H_β	2.842	2.836	2.832	2.839	2.818	2.814
	$H_{\beta'}$	3.128	3.172	3.128	3.125	3.117	3.128
	H_N	8.448	8.490	8.556	8.521	8.492	8.387
G^4	H_α		3.700	3.632	3.680	3.670	3.700
	$H_{\alpha'}$		3.800	3.924	3.890	3.900	3.860
	H_N		8.380	8.405	8.415	8.400	8.301
Y^5	H_α			4.390	4.641	4.656	4.644
	H_β			2.753	2.763	2.751	2.754
	$H_{\beta'}$			2.956	3.045	3.066	3.021
	H_N			8.510	8.505	8.430	8.277
		COUPLING CONSTANTS (J) IN Hz					
a^2 (A^2)	$J_{\alpha\beta}$	7.0	6.9	6.8	7.0	7.0	7.0
	$J_{\alpha N}$	7.7	8.7	8.4	8.1	8.0	8.2
F^3	$J_{\beta\beta'}$	-13.7	-13.8	13.6	-13.8	-13.7	-14.2
	$J_{\alpha\beta}$	10.5	10.4	11.2	9.6	10.1	8.5
	$J_{\alpha\beta'}$	4.2	4.0	3.7	4.4	4.3	4.7
	$J_{\alpha N}$	8.9	8.7	9.0	8.6	8.2	8.0
G^4	$J_{\alpha\alpha'}$		-16.7	-16.6	-16.7	-16.8	-16.8
	$J_{\alpha N}$		5.8	5.8	5.9	5.8	5.7
Y^5	$J_{\beta\beta'}$			-13.8	-14.0	-14.1	-14.3
	$J_{\alpha\beta}$			9.4	9.9	10.1	8.8
	$J_{\alpha\beta'}$			4.6	4.5	4.2	4.7
	$J_{\alpha N}$			7.9	8.8	8.8	8.4

*This refers to the aminoterminal fragments from HCl·Y-a-F-NH_2 to HCl·Y-a-F-G-Y-P-S-NH_2-L-hepta and the letters in parenthesis refer To the L-Ala^2 analogue.

270, 400, 500 and 600 megahertz. Only the chemical shift of the D-Ala methyl group is significantly anomalous (5) in all fragments but this anomalous shift is reduced when L-Ala is substituted. Detection of an NOE between the D-Ala methyl group and the aromatic rings of both Tyr^1 and Phe^3 indicated that the 0.5 ppm upfield shift was due to ring currents from both rings. The principal conformational determinants from our experiments are: (a) relatively high $^3J_\phi$ values for all NH-CH fragments (Table 1) (b) small ϕNOEs and large ψNOEs for all NH-CH-CONH fragments, (c) $\Delta\delta/\Delta T$ coefficients for amide protons that lie between those values for a fully solvent shielded and fully solvent exposed proton (6). The lack of a significant dependence of the pmr parameters over a concentration range of 10^{-2} to 10^{-4} ppm indicated a monomeric heptapeptide. All of these parameters plus the side chain-side chain NOEs and anomalous shifts of the D-Ala methyl groups are accounted for by a linear extended structure. The latter is one of several closely related conformations that are consistent with the NMR data. Our data needs to be further quantitated before we can distinguish between a unique linear conformation or a set of conformations in which extended conformations predominate.

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CONFORMATIONAL STUDIES OF ACTINOMYCIN-RELATED PEPTIDE LACTONES

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Introduction

NMR studies of the actinomycin-related pentapeptide lactone I (R = CH_3) were reported by Lackner[1]. This compound exists in two different solution conformations designated A and C. The former, observed in acetone, resembles that of the two peptide moieties in actinomycin D. The latter, observed in chloroform, was postulated to have all peptide bonds trans and an intramolecular hydrogen bond between the Val NH and Sar C=O. The A conformation is believed to be similar to that found for the peptide units in the crystal structure of the deoxyguanosine-actinomycin D complex[2], featuring cis Val-Pro and Pro-Sar peptide bonds. The present study extends investigation to the analogues of I depicted in Figure 1.

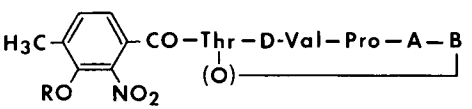

Fig. 1. Peptide lactones. I: A = Sar, B = MeVal; II: A = Sar, B = MeAla; III: A = Sar, B = Val; IV: A = Gly, B = MeVal.

Results and Discussion

Syntheses of peptide lactones I-IV were achieved via cyclization of the O-peptides at the A-B peptide bond with N,N-bis(2-oxo-3-oxazolidinyl)phosphorodiamidic chloride[3] (BOP-Cl), which gave better yields (>50%) than the use of dicyclohexylcarbodiimide.

Proton NMR spectra of I and II (R = Bzl) in $CDCl_3$ presented a mixture of A and C conformers; the A/C ratio increased with concentration reflecting self-association of A. In this conformer the Val NH may be involved in an intermolecular hydrogen bond in view of the concentration-dependence of its chemical shift. With R = H, only C conformers were observed in chloroform.

For III and IV, which each lack an N-methyl group of I, only C conformers were seen in both chloroform and acetone, in contrast to I and II. This reluctance to adopt the A conformation extends to the corresponding actinomycin analogues, which consequently fail to bind to DNA, unlike those related to I and II.

FT NMR spectra were obtained at 360 MHz and assignments were based upon two-dimensional homonuclear correlated spectra (COSY). This technique[4] employs a pair of 90° pulses separated by an interval which is systematically varied and where the free induction decay is recorded after the second pulse. Fourier transformation yields a 2D COSY spectrum in which crosspeaks at the intersections of the relevant perpendiculars indicate couplings between proton pairs. An example of a 2D contour plot and 1D spectrum is shown in Figure 2 for IV. Peptide backbone conformationally sensitive NMR parameters for the C conformers did not vary significantly among these analogues, and the D-valyl NH protons generally displayed the slowest deuterium exchange rate in accord with Lackner's findings for I.

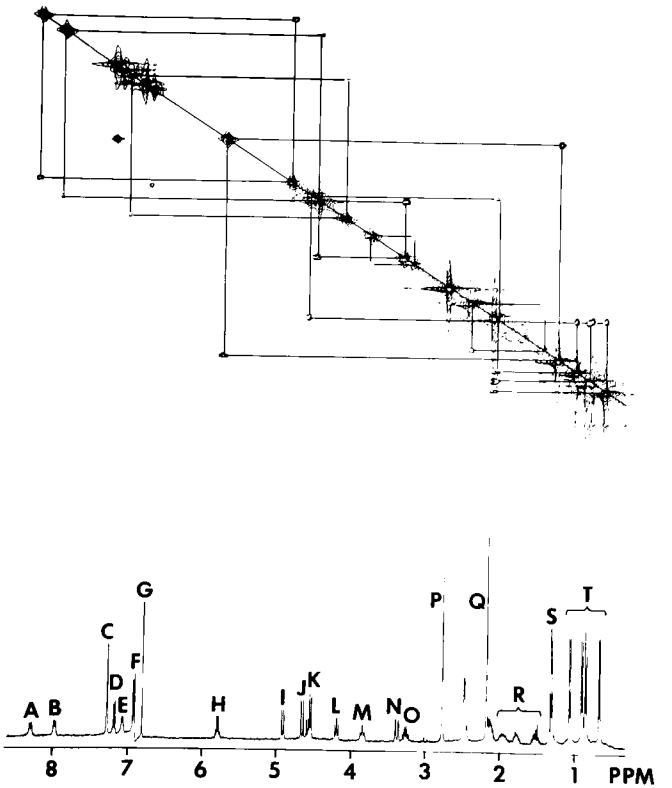

Fig. 2. 2D Contour plot and 1D NMR spectrum of IV in $CDCl_3$/C_6D_6 (2:1). A = Thr NH; B = Gly NH; C = C_6H_6; D & F = Ar-H; E = Val NH; G = $CHCl_3$; H = Thr β-H; I = Thr α-H; J = MeVal α-H; K = Pro & Gly α-H; L = Val α-H; M & O = Pro δ-H; N = Gly α-H; P = N-CH_3; Q = Ar-CH_3; R = MeVal & Val β-H & Pro β- & α-H; S = Thr CH_3; T = Val & MeVal C-CH_3.

Peptide II (R = H) separated from ethyl acetate solution in three crystal forms, one of which is orthorhombic with the space group $P2_12_12_1$ (a = 12.892, b = 21.740, c = 24.909 Å, Z = 8). An X-ray crystallographic study of this crystal is in progress. Figure 3 depicts the peptide geometry which features cis Val-Pro and Pro-Sar peptide bonds and resembles the

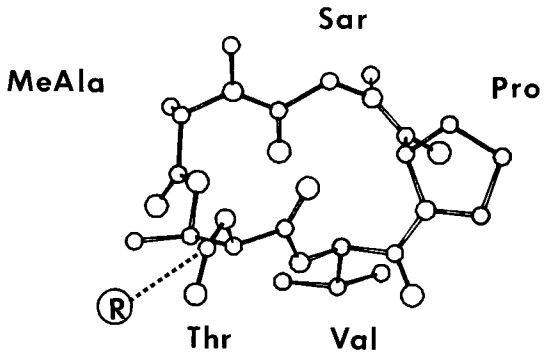

Fig. 3. X-ray structure of the peptide part of II. Ⓡ here denotes the aromatic ring.

A conformation present in actinomycin[2]. Two molecules are associated in this crystal structure by intermolecular hydrogen bonds from a Val NH to a Val C=O and vice-versa. This situation recalls that existing within the actinomycin molecule[2] and the above NMR studies indicate that related association may occur in solution. In conclusion, this peptide displays a tendency to actinomycin-related dimerization even in the absence of the bridging phenoxazinone chromophore. This tendency may play a role in the biosynthesis of the actinomycins.

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CONFORMATIONS OF DIASTEREOMERIC CYCLIC OCTAPEPTIDES

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Synthetic cyclic peptides of defined backbone conformation are of interest as analogs for determining the biologically active conformations of naturally occurring peptides. We report here studies directed at identifying sequences that produce conformationally stable cyclic octapeptides.

In the present work we desired to produce cyclic octapeptide backbones of C_2 symmetry fixed by two beta turns, using proline residues to determine the turns. To minimize the population of *cis* Xxx-Pro peptide bond isomers that could result from interference between the Xxx β and Pro γ groups in the *trans* isomers, we chose to incorporate Gly-Pro. To keep the NMR spectra simple, we used Ala and Phe as the remaining residues.

The 4 diastereomeric cyclo-(Ala-Gly-L-Pro-Phe)$_2$ peptides were prepared for examination by standard solution methods, using active ester or mixed anhydride coupling to form the tetrapeptides, Z-Ala-Gly-L-Pro-Phe-OMe. These were converted to octapeptides *via* the azide method and cyclized at 5 mM in dimethylformamide using the same method. Cyclization yields were between 45 and 70 %. All of the peptides were obtained in crystalline, analytically pure form and were proved to be cyclic octapeptides by the mass of the M+1 ion produced by fast atom bombardment.

Proton and ^{13}C NMR spectra of the peptides in dimethyl sulfoxide solution were examined. In each of the 4 cases there was one major component of C_2 symmetry in the NMR average. Symmetrical and unsymmetrical minor forms were visible. ^{13}C measurements indicated that the major form had trans Gly-Pro peptide bonds in all cases.[1] The relative stability of the dominant form is indicated below:

I.	cyclo-(D-Ala-Gly-L-Pro-D-Phe)$_2$	(< 2% minor forms)
II.	cyclo-(D-Ala-Gly-L-Pro-L-Phe)$_2$	(10% minor forms)
III.	cyclo-(L-Ala-Gly-L-Pro-L-Phe)$_2$	(25% minor forms)
IV.	cyclo-(L-Ala-Gly-L-Pro-D-Phe)$_2$	(50% minor forms)

The table on the next page gives some of the NMR observations for I, II and III.

In the major form of III the particular values of the Gly vicinal HNCH coupling constants and the high value of the Gly geminal coupling[2] indicate little mobility at that residue, and a large chemical shift non-equivalence for the glycine α-protons (0.64 ppm) is in agreement. The values for HNCH couplings in Ala and Phe suggest that averaging in those residues is also likely to be over a narrow range of dihedral angles. It thus seems legitimate to match the observed couplings to dihedral angles in search of a single conformational model for III. The data in the Table give the following constraints:[1-3]

$$\phi_{Ala} \cong -110^\circ \text{ or } -130^\circ,\ \phi_{Gly} = \pm 150^\circ,\ \psi_{Gly} \cong 180^\circ,$$

$$\psi_{Pro} \cong -40^\circ \text{ or } 160^\circ,\ \phi_{Phe} \cong -100^\circ \text{ or } -140^\circ.$$

A single model with C_2 symmetry consistent with these constraints can readily be found. It has the following approximate backbone angles.

	L-Ala	Gly	L-Pro	L-Phe
ϕ	-110°	-150°	-60°	-100°
ψ	-60°	180°	-40°	-10°

Backbone NMR Observations of cyclo-(Ala-Gly-Pro-Phe)$_2$ Isomers

I.(D-Ala, D-Phe)	Ala	Gly	Phe
(NH)	7.68	6.84	8.77
dδ/dT (ppm/deg)	-.0012	.0003	-.0043
$k_{nitroxyl}$ ($M^{-1}s^{-1}$)	180	250	1200
J_{HNCH}	8.4	7.7, 6.7	7.8
other J		17.0 (HCH)	2.5, 12.0(α-β)

II.(D-Ala, L-Phe)	Ala	Gly	Phe
(NH)	7.42	7.51	8.07
dδ/dT (ppm/deg)	-.0015	-.0015	-.0046
$k_{nitroxyl}$ ($M^{-1}s^{-1}$)	290	660	680
J_{HNCH}	7.6	6.5, 5.1	7.7
other J		16.5 (HCH)	3.8, 11.6(α-β)

III.(L-Ala, L-Phe)	Ala	Gly	Phe
(NH)	7.62	8.10	8.09
dδ/dT (ppm/deg)	-.0026	-.0030	-.0010
$k_{nitroxyl}$ ($M^{-1}s^{-1}$)	150	180	330
J_{HNCH}	8.8	7.8, <2	8.1
other J		17.8 (HCH)	ca. 3, 12(α-β)

All have Pro C^{β} - C^{γ} shift differences of 4.0-4.5 ppm.
All have one Pro H^{β} 0.5-0.6 ppm upfield at ca. 1.4 ppm.

In this conformation the Ala and Gly N-H are directed inwards, and the Phe N-H, central in an approximately Type I beta turn, is somewhat shielded by the Pro and Phe side chains. The two Ala methyls are quite close to each other across the ring. The conformation is similar to that half of the cyclic octapeptide β-amanitin crystal structure[4] which contains the Hyp-dihydroxy-Ile Type I turn,

	L-Cys	L-Asp	L-Hyp	L-diOH-Ile	L-Trp
ϕ	-121^{o}	-172^{o}	-60^{o}	-79^{o}	-109^{o}
ψ	-85^{o}	179^{o}	-37^{o}	-23^{o}	-40^{o}

Overall the peptide backbone differs from that of β-amanitin in that the latter has a type II Ile-Gly turn in the Gly-Ile-Gly-Cys half of its ring. In β-amanitin the Trp and Cys side chains are oxidatively coupled to form a bicyclic structure, but it would appear from this result with cyclo-(L-Ala-Gly-L-Pro-L-Phe)$_2$ that the β-amanitin backbone conformation is determined by the sequence and ring size, not by the bridge between side chains.

A conformation with Type II turns at L-Pro-D-Phe is consistent with the data for I. It is analogous in a number of respects to that for III.

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PEPTIDE PROTON EXCHANGE IN A CYCLIC DODECAPEPTIDE

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Because of the interest accorded to proton exchange as a measure of conformation, we thought it valuable to make a careful study of exchange rates in a peptide of stable conformation large enough to possess solvent-exposed and solvent-shielded backbone N-H protons. We report here a study of **cyclo-(Met^1-Val^2-Gly^3-Pro^4-Asn^5-Gly^6)$_2$**, which NMR studies have indicated to be suitable. Its proton spectrum shows C_2 symmetry on the NMR time average but, in distinction to the spectra of many linear oligopeptides, the ranges of the NMR observables - coupling constants, chemical shifts, and indications of solvent exposures - are large, indicating that averaging does not occur over a wide range of conformations. Further, the observables are consistent with a single (average) conformation in a variety of solvents.[1] A search through conformation space for low energy conformations has also indicated that the conformation proposed on NMR grounds is a stable one.[2] The proposed model has beta turns of approximately Type III (3_{10}) at Pro-Asn. In it, the backbone N-H protons of the Met^1, Gly^3 and Gly^6 residues are sequestered, and the C=O bonds of Met^1 and Gly^3 are also directed into the peptide ring.

First order rates of peptide and side chain amide proton exchange were measured in H_2O from pH 1.2 to 9, at 21°, using linewidths between 2 and 120 sec^{-1}, saturation transfer between 0.2 and 15 sec^{-1} and direct observation of peak growth between 10^{-4} and 10^{-3} sec-^{1}. Observation was by the Redfield

2-1-4 pulse sequence at 300 MHz. Second order rate constants for hydroxide ion and hydrogen ion catalyzed reactions were obtained from the rate *vs* pH data. Over the 10^{-4} to 10^{2} s^{-1} range of first order rates there was no departure from linear dependence on $[H_3O^+]$ and $[OH^-]$ and thus no evidence of a rate determining conformation change. To determine the steric influence of backbone conformation, the second order constants were compared with constants calculated using the correlation proposed by Molday, *et al.*,[3] which estimates primarily the polar effects of nearest neighbor side chains. Side chain rates were compared with experimental results for N-acetylasparagine N'-methylamide.[4] Base catalyzed exchange is compared below with two other measures of solvent exposure, nitroxyl-induced T_1 relaxation rate constants (k_{nit}) and chemical shift temperature coefficients, both for H_2O solutions.

Proton	k_{OH} $M^{-1}s^{-1}$	k_{obs}/k_{pred}	k_{nit} $M^{-1}s^{-1}$	$d\delta/dT$ ppm/deg
Met	$.73 \times 10^7$	.049	210	.0045
Val	3.4×10^7	.45	410	.0091
Gly^3	$.12 \times 10^7$	.0057	160	.0029
Asn	$47. \times 10^7$	2.5	overlap	.003
Gly^6	$.84 \times 10^7$	.014	overlap	.003
NH_2(E)	4.1×10^7	.48	550	.0055
NH_2(Z)	1.3×10^7	.65	730	.0053

Base catalyzed exchange rates of the sequestered Met and Gly protons are 20-170 times slower than predicted from the model substances. The nitroxyl relaxation data are in accord with the exchange data in that the values of k_{nit} decrease in the order Val >> Met > Gly^3. Unfortunately, the Asn and Gly^6 N-H resonances coincide, and although significant differences in exchange rate were observed and measured, the nitroxyl rates

were not obtained. The temperature coefficients for Met, Val and Gly^3 also parallel the observed/predicted ratios.

Full parallelism among the three measures of solvent exposure is not to be expected, since the underlying phenomena differ. Although the Asn backbone proton exchanges 400 times faster than the Gly^6 proton, the two have the same temperature coefficients - their overlapping resonances shift together between 10° and 60°. Exchange of the Asn backbone proton may be intramolecularly assisted by the side chain carbonyl, which is associated with it in the proposed conformation. The side chain amide proton <u>trans</u> to oxygen exchanges faster than the <u>cis</u>, because of polar effects, but the temperature coefficients of the two protons are the same, and the nitroxyl rates, presumably strongly dependent on steric factors, differ in the opposite sense, <u>cis</u> to the carbonyl more exposed, consistent with the model.

Philson and Bothner-By measured exchange rates for gramicidin S.[5] We estimate from their data that the internal Val and Leu N-H protons exchange 1500 and 250 times slower than predicted, while the exposed Orn and D-Phe protons go at 0.1 and 1.0 times respectively, consistent with the known conformation. In both peptides the oberved/predicted ratio for base catalyzed exchange thus appears to measure solvent exposure, at least in the absence of intramolecular catalysis.

The results for proton catalyzed exchange are as follows:

Proton	Met	Val	Gly^3	Gly^6	$NH_2(E)$	$NH_2(Z)$
k_H, $M^{-1}s^{-1}$	.039	.020	.034	.017	320	360
k_{obs}/k_{pred}	.07	.05	.10	.06	.5	.6

In the acid-catalyzed process, there seems to be no conformation dependence for the backbone protons of cyclo-(Met-Val-

Gly-Pro-Asn-Gly)$_2$. In gramicidin S, (rates from Philson and Bothner-By[5]) the distinction between inside Val and Leu N-H (k_{obs}/k_{pred} = .0025. and .0031) and exposed Orn and Phe (.10 and .084) is present but less than for the base catalyzed reaction. In both peptides the exposed backbone protons exchange at least 10 times slower than predicted.

We suggest that reduced sensitivity of the acid catalyzed rates to proton exposure agrees with N-protonation as the dominant mechanism for exchange, and that the inhibition of acid catalyzed exchange is a consequence of the rigidity of the cyclic peptide structures. N-Protonation requires nitrogen bond angles to become tetrahedral. In a sufficiently stable initial conformation this change would introduce a barrier or reduce the equilibrium fraction of protonated nitrogen and thereby hinder exchange.

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PRIMARY AMIDE EXCHANGE RATES OF NEUROHYPOPHYSEAL HORMONES

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Introduction

Hydrogen exchange rates of neurohypophyseal hormones and their analogs have been employed to determine the extent of solvent exposure of peptide hydrogens.[1] Here we have extended these measurements to the primary amide hydrogens of oxytocin (OT), 8-lysine vasopressin (LVP), α-desamino LVP (dLVP).

Materials and Methods

Oxytocin was purchased from Vega Biochemicals (Tucson, AZ) and LVP from Bachem (Torrence, CA). Desamino LVP was synthesized by the solution method.[2] The preparation of N-α-acetylglutamine-N'-methylamide (Gln') has been previously described.[3]

Proton FT-NMR spectra were measured by the Redfield 2-1-4-1-2 pulse sequence[4] on a Bruker WH-400 spectrometer.

Peptides were studied as 5 mM solutions in 25 mM sodium acetate buffer in 90% H_2O-10% D_2O (v/v). Exchange rates were measured by analysis of resonance linewidths (see footnote to Table I in reference 1a).

Results and Discussion

The energy of activation for exchange of the primary amide hydrogens of Gln' had to be determined in order to correct for the difference in the temperature employed in this study and in the previous analysis of this model compound.[3] Exchange rates were measured separately for acid (pH 2.02) and base catalysis (pH 8.02) at 299-319° K. Activation energies, determined by linear least squares fitting of rate constants to the Arrhenius equation are presented in Table I. These energies of activation are similar to values reported for N-methylacetamide (17 and 23 kcal/mole for acid and base catalysis, respectively) and for poly-D,L-alanine (17 and 22 kcal/mole, respectively).[5]

The pH dependence of the primary amide hydrogen exchange rate of OT and LVP is presented in Table II. Exchange rates calculated for these hydrogens in the fully solvent exposed state on the basis of model compound data[3] are shown in parenthesis. Within a factor of about 2-3 (the expected

Table I. Activation Energies and Rate Constants at 25°C of Gln'[a]

	Activation Energy in kcal/mole		k_H	k_{OH}
	E_H	E_{OH}	$(M^{-1}S^{-1})$	$(M^{-1}S^{-1})$
trans	14.65 ± 1.31	19.08 ± 0.99	1729	7.6×10^7
cis	15.37 ± 1.36	21.59 ± 0.73	1705	2.0×10^7

[a]The subscripts H and OH refer to acid and base catalysis, respectively

Table II. Hydrogen-Exchange Rates (S^{-1}) of Primary Amide Protons[a]

pH	*trans* proton			*cis* proton		
	Asn	Gln	Gly	Asn	Gln	Gly
1. Oxytocin						
6.59	2.1(3.7)	2.1(3.0)	3.1(5.3)	2.1(0.9)	b	5.2(3.1)
6.95	9.4(8.4)	3.1(6.8)	5.2(12.0)	15.7(2.0)	b	14.7(7.0)
7.22	11.5(15.6)	5.2(12.0)	7.3(22.4)	17.8(3.7)	b	16.8(13.1)
7.48	17.8(28.4)	10.5(23.0)	14.7(40.8)	27.2(6.6)	b	26.2(23.9)
7.70	36.7(47.1)	22.0(38.1)	28.3(67.7)	b	b	34.6(39.6)
7.91	53.4(76.4)	30.4(61.8)	41.9(109.7)	b	b	44.0(64.2)
2. 8-lysine vasopressin						
6.58	2.6(3.6)	4.2(2.9)	4.2(5.1)	1.0(0.8)	2.1(0.8)	b
6.76	3.7(5.4)	5.3(4.4)	5.3(7.8)	1.6(1.3)	3.1(1.2)	b
7.01	7.9(9.6)	11.6(7.8)	14.7(13.8)	2.1(2.3)	4.2(2.1)	b
7.27	15.2(17.5)	20.0(14.2)	b	3.1(4.1)	5.3(3.8)	b
7.52	35.2(31.1)	44.1(25.3)	b	15.8(7.3)	10.5(6.7)	b
7.65	48.9(42.0)	51.5(34.1)	b	18.9(9.8)	13.7(9.1)	b
7.84	61.5(65.0)	82.0(52.8)	b	26.3(15.2)	23.1(14.1)	b

[a]Calculated values are given in parenthesis, [b]resonance overlap

accuracy of the predicted rates) the observed rates are not diminished from values expected for fully solvated protons. It appears therefore that significant internal hydrogen bonding does not occur for primary amide hydrogens whose rates could be monitored. These data are not consistent with the model of Von Dreele et al.[6] for LVP in which the trans carboxamide hydrogen of Gly_9 is hydrogen bonded. Note, however, that because of resonance overlap, the exchange rates of the cis primary amide hydrogens of Gln_4 of OT and Gly_9 of LVP could not be measured. The only primary amide exchange rate of dLVP that could be determined was that of the cis proton of Gly_9 and the ratios of the calculated to the observed rates for this proton were of the order of 2 and less for seven pH values studied in the range 6.5-7.8.

The exchange rates of the cis primary amide hydrogens of Asn_5 of OT are more rapid than rates predicted for the fully solvated hydrogens. This anomaly may result from

partial overlap of this resonance with the Tyr o-CH peak or from differences between the conformations of the solvated sidechain of the Asn_5 residue of OT and the Asn sidechain of the reference model compound.[3]

Acknowledgements

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PMR STUDIES OF Cu(II) AND Zn(II) INTERACTION WITH GLYCYL-L-HISTIDYL-L-LYSINE AND RELATED PEPTIDES

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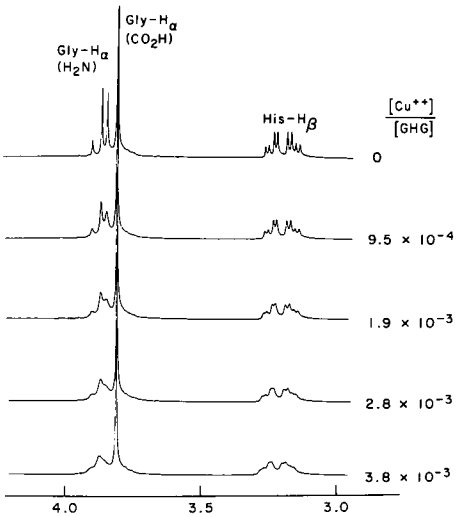
Introduction

The binding of metal ions to glycyl-L-histidyl-L-lysine (GHL) is of interest in view of the bioactivity of both the free tripeptide and its Cu(II) complex.[1] Previously magnetic resonance studies have been performed 1) on the Cu(II)-GHL system with emphasis on the determination of chemical shifts, T_{1p} and T_{2p} for $^{13}C^{2}$, and 2) on the Zn(II)-GHL system where the Zn(II)-GHL ratio is one.[3]

Results and Discussion

The PMR spectra of GH, GHG and GHL were recorded in the presence of a 1:1 DPO_4^{2-}:$D_2PO_4^-$ buffer as a function of added Cu(II) (Figure 1). All proton resonances of GH, GHG and GHL are broadened significantly with added Cu(II) except those of the C-terminal glycine of GHG and those of the lysine of GHL. (All protons with observable resonances which are broadened are marked with a ▲ on the structures.) For the Cu(II)-GHL system a $1/T_1$ plot vs. added Cu(II) was generated (Figure 2). The imidazole proton resonances and the N-terminal CH_2 reso-

nances of a Cu(II)-GHL system (where $CuCl_2$:GHL = $1.8x10^{-3}$) significantly broaden on changing the temperature from 5 to 30°C whereas the His-H_β and Lys-H_δ sharpen significantly.



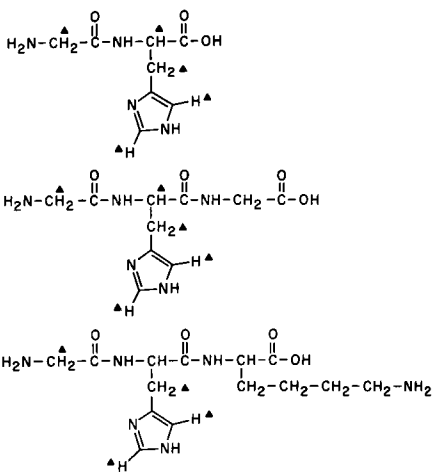


Fig. 1. Selected portion of GHG PMR spectra as a function of added Cu(II).

We conclude that the glycyl and histidyl residues of GH, GHG and GHL interact with Cu(II) all in a very similar manner. By contrast there is no evidence of interaction of the C-terminal glycine of GHG or of the ε-NH_2 of GHL. The selective broadening of the imidazole and N-terminal CH_2 proton resonances in the Cu(II)-GHL system is consistent with a proposal that their $1/T_{2p}$ values are dominated by the complex lifetime term at ~30°C (Laussac, et al.)[2].

The imidazole proton resonances of a Zn(II)-GHL system (Figure 3) reveal that there are at least three unique imidazole environments at the lowest temperature. One of these is undoubtedly uncomplexed GHL (Rabenstein)[3]. Upon increasing the temperature the upfield peaks (*) are reduced in relative intensity and the sets of two downfield peaks (the "doublets")

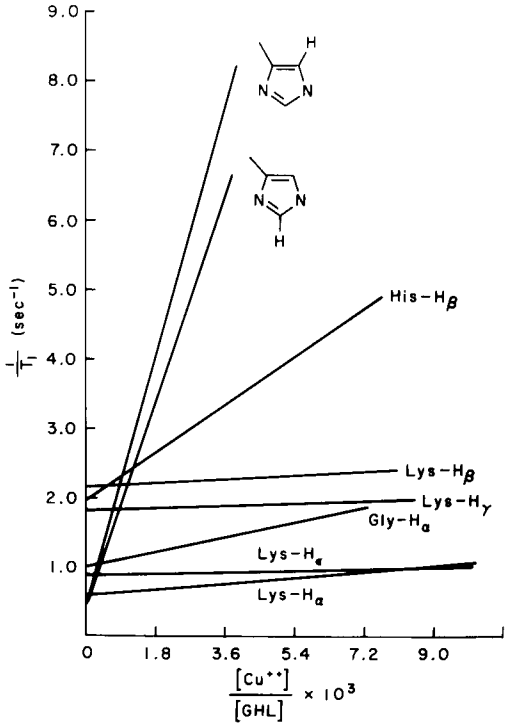


Fig. 2. Plot of $1/T_1$ vs. added Cu(II) for GHL proton resonances.

appear to coalesce to singlets. Increasing the Zn(II)-GHL ratio by adding zinc acetate results in reduction of the upfield peaks (*) and in the "doublets" being replaced by sharp singlets. It is proposed that the upfield peaks (*) represent uncomplexed GHL in slow exchange with the complexed GHL and that the "doublets" represent two different Zn(II) complexes in relatively rapid exchange at or above ~40°C.

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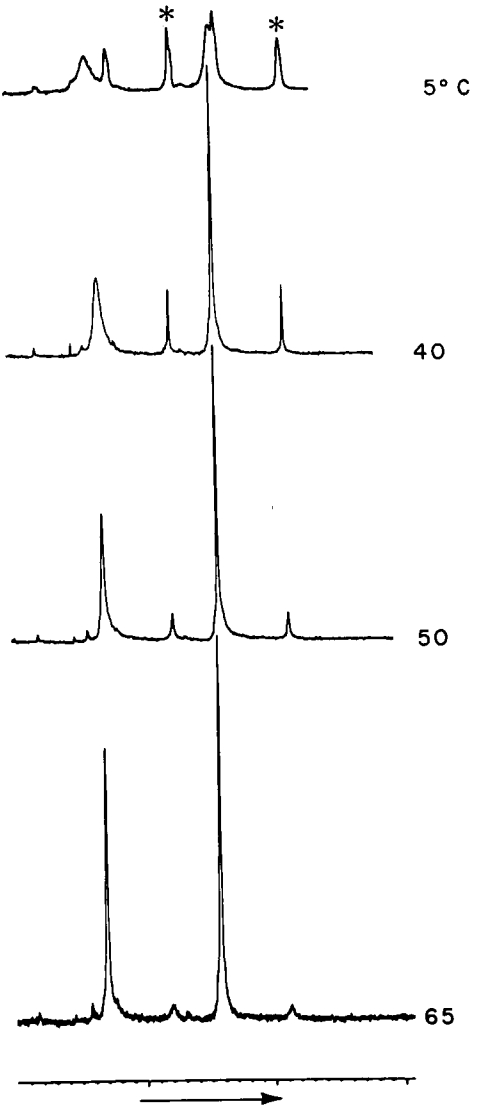

Fig. 3. PMR of the imidazole proton region of a 1.6$Zn(OAc)_2$·$2H_2O$:1GHL·HOAc system.

STRUCTURAL PROPERTIES OF TRH ANALOGS: PROBING STRUCTURE-CNS ACTIVITY RELATIONSHIPS AT THE MOLECULAR LEVEL

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Introduction

TRH, thyrotropin releasing hormone, (L-pGlu-L-His-L-Pro-NH_2) is as well known for its CNS activity as it is for its endocrinological properties. There has been considerable interest in the possible application of TRH analogs with extended biological half lives as psychotherapeutic agents. Consequently a number of peptide analogs with dramatically different chemical structure have been prepared and tested for TRH like CNS activity.

We have been carrying out an extensive program of crystal structure analyses for TRH analogs in an effort to contribute to an understanding of the interrelationships between chemical structure, conformation and CNS activity. To date we have determined crystal structures for seven analogs, each of which displays a degree of CNS activity. This report presents some current thoughts on the meaning of the crystallographic study.

The chemical structures of the TRH analogs for which crystal structures are known are presented in Figure 1. They are grouped into two main classes R^1 and R^2 according to which residue was modified. R^1 analogs are subdivided as R^1_S and R^1_U depending on the hybridization of their "α"-carbon atoms.

Results and Discussion

Crystal structures for I, IV, V, VIII, & IX contain two mole-

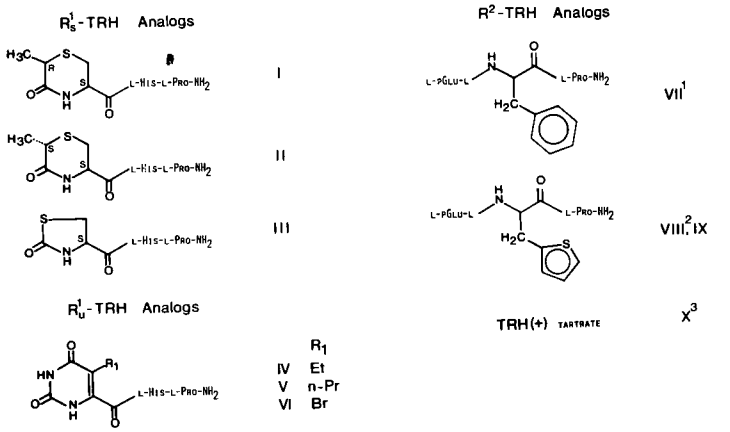

Fig. 1. Chemical structures for TRH analogs for which crystal structures have been determined in our (I-VII) and other (VIII-V) laboratories.

cules of TRH analog per asymmetric unit, which provides for a total of 15 examples of conformation. They may be summarized as follows. All peptide bonds are *trans*. The heterocycles of residue 1 adopt four orientations, two for R^1_S analogs (including pGlu), $\langle\psi_1\rangle$ = 8 and 154°, and two for R^1_U analogs, $\langle\psi_1\rangle$ = -113 and 111°. Conformations for the remainder of the peptide backbone are very similar, except for the proline amide, which adopts two orientations differing by rotation of ∿ 180° about the C_α-C' bond. There is an apparent preferred conformation for the histidine free base side chain: χ_1 ∿ 70° and χ_2 ∿ -65°. Examples of the observed conformations are presented as a superposition diagram in Figure 2, which also presents a composite hydrogen bonding environment.

We prepared the composite hydrogen bonding environment in an effort to gain insight into the kind of intermolecular interactions that might allow such chemically different species as the R^1_S and R^1_U analogs to bind effectively to the same receptor. As indicated in the diagram there are at least two areas in space where a receptor hydrogen bond donor could in-

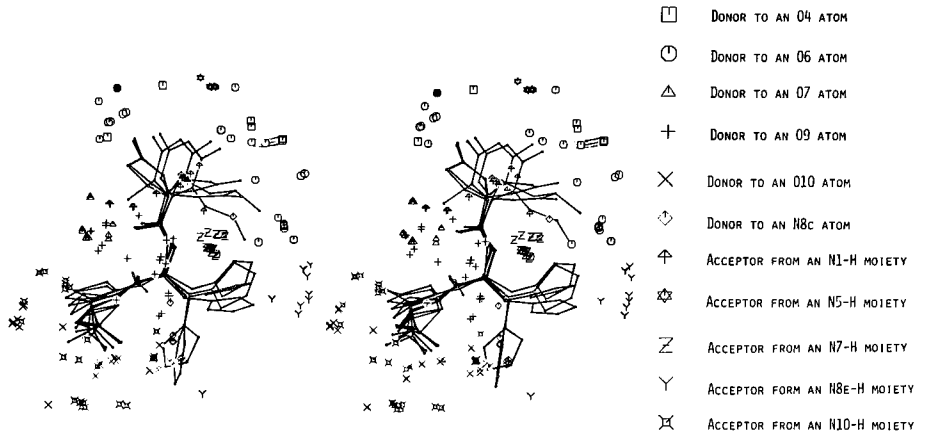

Fig. 2. A stereoscopic superposition diagram of the conformations of one molecule each of I, V, VI, VII, IX, & X. The observed hydrogen bonding interactions (to 3.2 A) from all crystal structure determinations are presented with the indicated symbols. The darkened symbol in the upper right of each figure and the band of three symbols in the upper left represent possible common receptor hydrogen bond donor cites for binding R^1_S and R^1_U analogs.

teract with a heterocycle carbonyl moiety from either class.

It has been recognized for sometime that CNS active compounds often interact either competitively or complementarily. There is reason to believe that TRH may interact with opiate receptors. Consequently we have looked for conformational similarities between the TRH analogs and Leu^5-enkephalin for which there are two known crystal structures.[4,5] Figure 3 contains superposition diagrams with four examples of Leu^5-enkephalin molecules fit to the backbone of I. In each stereo pair, the best and worst fit, as determined by least squares methods, is presented.

We find the similarities in conformation of the last three residues of Leu^5-enkephalin molecules with the TRH analogs to be striking enough to suggest that appropriate biological tests be carried out to determine if there are direct TRH-enkephalin interactions under physiological conditions.

Fig. 3. Stereoscopic superposition plots with Leu^5-enkephalins.

Acknowledgement

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THE APPLICATION OF ^{13}C-^{1}H NMR COUPLINGS TO THE DETERMINATION OF PEPTIDE CONFORMATIONS IN SOLUTION

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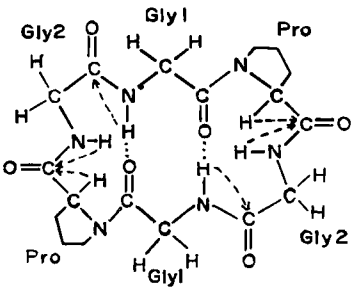
Introduction

Various nuclei such as ^{1}H, ^{13}C, and ^{15}N in a peptide are coupled to each other by two dominant mechanisms; i) spin-spin, which occurs between the nuclei that are covalently bonded and ii) dipole-dipole, which is mainly responsible for relaxation phenomena between the nuclei. These two coupling mechanisms between the ^{13}C and ^{1}H nuclei have been utilized in this study, first to assign both the ^{13}C and ^{1}H resonances and then to detect an intramolecular hydrogen bonded pair (NH...O=C) in a peptide. The technique is demonstrated by using a cyclic hexapeptide, cyclo-(Gly-L.Pro-Gly)$_2$, as a model system. The conformation of this peptide has been well studied, both in the solution[1-3] and crystalline states[4]. A common conformational feature of this molecule is the type II β-turn stabilized by a ten-membered intramolecular hydrogen bond as shown by the dotted line in the scheme below:



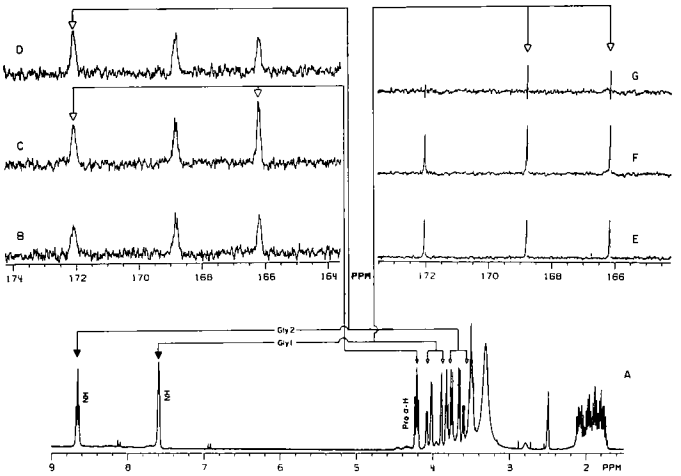


Fig. 1. A) 300 MHz ^{1}H NMR spectrum of cyclo-(Gly-Pro-Gly)$_2$ in DMSO-d_6 (10mM), B) 75 MHz ^{13}C carbonyl NMR resonances of the same sample as in A coupled without NOE - 512 scans with a pulse delay of 30 sec, C) same as B but proline α-H proton at 4.21 ppm selectively irradiated, D) same as B while the most down field glycine NH proton at 8.66 ppm was irradiated, E) same as B but ^{1}H broadbanded decoupled without NOE, F) same as E while Gly^{1} NH at 7.95 ppm was selectively irradiated and G) the E and F difference spectrum showing the absolute NOE enhancement factors. Note that the irradiated nuclei are indicated by (▼) while the observed resonances are shown by (∇).

Results and Discussion

The 300 MHz ^{1}H NMR spectrum of cyclo-(Gly^{1}-L-Pro-Gly^{2})$_2$ is depicted in figure 1A. The initial step is to establish the connectivities between the NH proton and the α-proton resonances of the peptide. This was achieved by homonuclear

^{1}H double resonance experiments and indicated by the arrows in the figure. The second step is to assign each set of glycine resonances to a given glycine residue of the molecule (Gly^{1} or Gly^{2}). It should be noted in the scheme that the proline carbonyl carbon is simultaneously ^{2}J coupled to its own α-H proton and to the NH proton of the succeeding glycine residue, (Gly^{2}).

The 75 MHz coupled ^{13}C NMR spectrum of the carbonyl region of the peptide is presented in figure 1B. Selective irradiation of the proline α-H proton at 4.21 ppm shows the reduction in linewidth of the most downfield C=O carbon at 172.0 ppm and the most upfield C=O carbon resonance at 166.1 ppm as indicated by the arrows in figure 1C. Similar ^{1}H irradiation of the most downfield glycine NH proton signal at 8.66 ppm, (see figure 1A), however, reduces the linewidth of the most downfield carbonyl ^{13}C resonance at 172.0 ppm, as shown in figure 1D. This carbonyl ^{13}C resonance at 172.0 ppm, according to the scheme above, therefore, belongs to the proline residue, and the most downfield NH signal at 8.66 ppm is assigned to the glycine residue, designated as Gly^{2}. The remaining set of glycine resonances belongs to Gly^{1}. Selective irradiation of the known α-H proton resonances connects them with their respective ^{13}C carbonyl resonances as reported[5] previously. Thus, the complete assignment of both the ^{13}C and ^{1}H NMR resonances has been obtained by using the selective ^{1}H irradiation technique, and is in agreement with results obtained by using the selective isotopic enrichment of the peptide[1].

Figure 1E shows the ^{13}C carbonyl resonances, now assigned, obtained by broadband decoupling of ^{1}H, but without NOE. Figure 1F shows the same carbonyl groups with NOE derived selectively from the Gly^{1} NH proton at 7.59 ppm. The absolute enhancement factors are noted in the difference spectrum in figure 1G. The Gly^{2} carbonyl carbon derives part of its NOE from interaction with the adjacent Gly^{1} NH proton as shown by

the broken line in the scheme. Therefore, the observed NOE (0.47) on the Gly^2 C=O carbon at 168.8 ppm from the Gly^1 NH proton is self-explanatory. However, the observation of the NOE (0.34) on the Gly^1 carbonyl carbon at 166.1 ppm, figure 1G, resulting from the ^1H irradiation of its own NH proton, indicates that the Gly^1 C=O and NH groups are linked by an intramolecular hydrogen bond as shown in the scheme (dotted lines). Such an effect has been observed in valinomycin[6] and in some small organic molecules[7].

The selective NOE method described here should find general application in the assignment of ^{13}C and ^1H NMR resonances and, most importantly, in the simultaneous detection of N-H...O=C hydrogen bond pairs.

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HISTIDINE AND β-FOLDING

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The His residue known to exist in both basic and protonated forms at the physiological pH is rather commonly found in the so-called $(i+2)^{th}$ position of β-turns in proteins[1]. A conformational analysis by ir and ^{1}H-nmr spectroscopies and X-ray diffraction studies was carried out on the model dipeptide Bu^tCO-L-Pro-L-His-NHMe(1) in both basic (a) and protonated (b) states,with reference to the homologous L-Pro-L-Phe derivative (2). PF_6^- anion was associated to the protonated form because of the easy dissociation of N^+-H...PF_6^- ion pairs in organic solvents[2].

Solid state

Stereoviews of the molecular conformations of 1a and 1b are reproduced in Figure 1.

Peptide 1a is βI-folded (Pro: ϕ = -63°, ψ = -22°; His: ϕ = -70°, ψ = -20°, χ^1 = 60°, χ^2 = -86°; i+3 → i interaction, N...O = 2.95 Å) with a short intramolecular contact between His NH bond and N^π atom of the imidazole ring (N...N^π = 3.22 Å). The His N^π atom is also intermolecularly bonded to the N^τH bond (N^τ...N^π = 2.87 Å).

The protonated form 1b accommodates a quite different conformation (Pro: ϕ = -64°, ψ = 147°; His^+: ϕ = -133°, ψ = 159°, χ^1 = -74°, χ^2 = 66°) with an intramolecular (His^+)N H...OC(Bu^t)

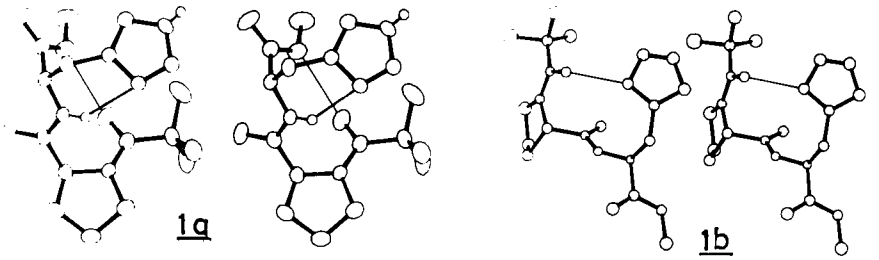

Fig. 1. Stereoviews of the molecular conformations.

interaction ($N^{\pi}...O$ = 2.72 Å) and the His^{+} residue in the C_5 disposition. Both NH amide groups are weakly bound to PF_6^- anions (N...F = 2.9-3.5 Å) and $N^{\tau}H$ is the donor site to the His^{+} CO group($N^{\tau}...O$ = 2.67 Å) of a neighboring molecule.

The βII-folded form of 2 (Pro: ϕ = -64°, ψ = 139°; Phe: ϕ = 62°, ψ = 23°, χ^1 = -42°, χ^2 = -68°; i+3 → i interaction, N...O = 3.00 Å) is the second example of such a bent homochiral sequence in oligopeptides[3] and stacking of aromatic rings prevents them from intramolecular interactions.

Solute state

Ir spectra of 1a and 1b in the NH and CO stretching absorption domains (Figure 2) have been assigned on the basis of ir studies on possible interactions of the imidazole ring in both basic and protonated states.

Ir spectrum of 1a exhibits two absorption bands at 1610 and 3350 cm^{-1} characteristic of β-folding mode[4] the ratio of which estimated from the intensity of the residual free NH(Me) absorption exceeds 80% against only 35% for 1b. The major conformer of 1b is an open-form with the His residue in the C_5 disposition and the imidazolium ring hydrogen bonded to the CO(Bu^t)carbonyl group. Because of its very low frequency,the

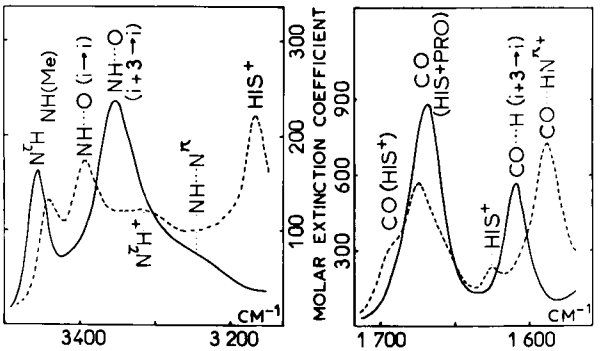

Fig. 2. Ir absorption spectra of 1a and 1b (CH_2Cl_2/MeCN 1%, 0,005 mol/l).

wide absorption band of the bonded $N^{\pi}H$ vibrator interferes with the complex CH absorption below 3100 cm^{-1}.

The above experiments are corroborated by the variation of NH proton nmr signals against the solvent composition in $CHCl_3$/DMSO mixtures (Figure 3) . It can be pointed out that peptides 1b and 2 give variations characteristic for solvent exposed NH protons. In the opposite, both 1a amide protons with low variation are solvent protected, in good agreement with the existence of two intramolecular hydrogen bonding types in βI-folded conformers.

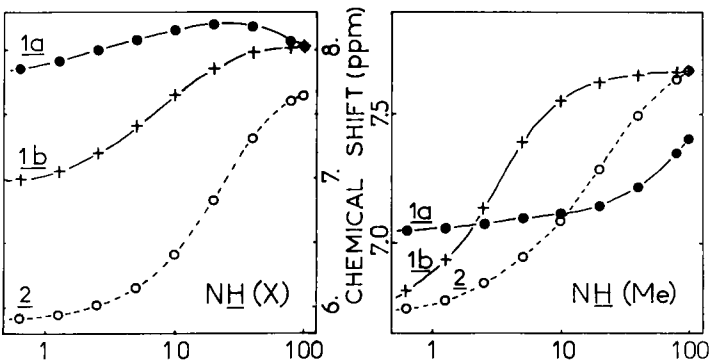

Fig. 3. Variation of the NH proton nmr signals against % DMSO in $CHCl_3$.

The $^{3}J(NH-C^{\alpha}H)$ coupling constant of 7.8 Hz for 1a in MeCN is consistent with the βI-folding mode[5] and the low $^{3}J(C^{\alpha}H-C^{\beta}H_2)$ coupling constants (4.0-4.5 Hz) for both 1a and 1b indicate a large dominance, up to 80%, of the His $C^{\alpha}-C^{\beta}$ rotamer III ($\chi^1 = 60°$) in both βI-folded and open conformers[6].

Conclusion

Pro-His peptide gives an example of a conformational transition induced by protonation of His side-chain. Due to the $NH...N^{\pi}$ interaction in basic His residue, the βI-folded conformation with the disposition of His $C^{\alpha}-C^{\beta}$ bond corresponding to rotamer III, is favored in both solute and solid states of 1a. β-folded conformers are seriously challenged upon protonation by open forms in which the imidazolium ring is the proton donor site to the N-terminal carbonyl group. This interaction is retained in the solid state but the dominant His $C^{\alpha}-C^{\beta}$ rotamer III in solution turns out to be of the type I in the crystal, probably because of the molecular packing forces.

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A MODEL FOR THE THREE-DIMENSIONAL STRUCTURE OF RENIN

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Introduction

Renin, like other acid proteases,[1,2] has two aspartic acid residues at the active site. Unlike its congeners, however, it displays a very high degree of substrate specificity.[3] To understand this specificity and evaluate possible inhibitors of pharmacological interest, we propose a model for the three-dimensional structure of renin.

X-ray structures of three acid proteases are available at 2.8 Å resolution or better.[1,2] Comparison of these structures demonstrates high sequence and structural homology, particularly for the protein interior and the region around the active site. Since the sequence of renin isolated from the mouse submaxillary gland[4,5] has considerable homology with the other acid proteases, their structures can be employed to predict that of renin. The method used and the structure obtained are described briefly in this report.

Methods

The initial alignment of renin with the other acid proteases was based on sequence and structural homology, including the location of disulfide bonds. The renin model was then constructed from the coordinates of the acid protease from Rhizopus chinensis. Where the sequences matched, the coordinates for the backbone of R. chinensis were used. Where additions and deletions were needed, the backbone was constructed using a three-dimensional graphics system.[6] Side chains were built following the coordinates of R. chinensis as closely as possible. The structure generated in this way was rationalized by energy minimization with the program CHARMM[7]; for comparison, corresponding energy calculations were done for the R. chinensis.

To analyze the interactions between the protein and substrate, models were built in the active site of renin of the natural substrate and of inhibitors containing phenylalanine on either side of the scissile bond. The models were based on the crystal structure for pepstatin bound to R. chinensis.[8]

Results

The total energy of the crystal structure of R. chinensis and the model for renin were calculated with CHARMM; the energy of both structures was high, with that of the renin model considerably higher due to a few close van der Waals contacts. After energy refinement, satisfactory energies including attractive van der Waals interactions, were obtained. The results for the various energy contributions are given in Table I, which also lists the RMS deviations from the initial structure. It is clear from the table that both structures have appropriate stereochemistry and nonbonded interactions; however, it can not be concluded from this result that the renin structure is correct. Fig. 1 shows the main chain of renin obtained

from the model building and energy refinement procedure.

Table I. Energy Contributions and RMS for the Refined Structures of Renin and Rhizopus

Energy (kcal/mole)[a]	Rhizopus	Renin
Bonds	61	72
Bond Angles	1247	1411
Dihedral Angles	794	878
Improper Torsion	118	143
Van der Waals	-2220	-2459
Electrostatic	-604	-674
Hydrogen Bond	-600	-686
Total	-1204	-1315
RMS (Å)[b]	0.673	0.935

[a]The energy refinement involved 6 steps of 90 cycles each and utilized the adopted base Newton Raphson algorithm; see ref. 7 for a definition of the energy contributions.
[b]Root-mean-square deviation from the initial structure.

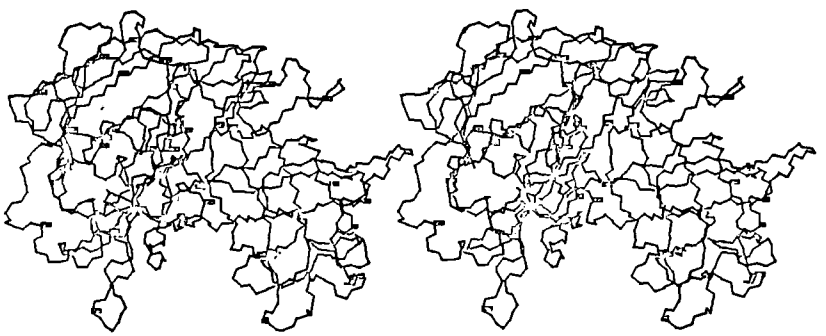

Fig. 1. Stereoscopic drawing of the peptide backbone of the renin model.

Although the renin structure is similar to that of R. chinensis, as would be expected from the modeling procedure, there is a clear difference in the active site region. Immediately adjacent to the two aspartic acid residues is the proposed "P1" binding site for the side chain of the substrate on the N-terminal side of the scissile bond. In R. chinensis, a deep pocket is present which does not appear in renin. The active site model for renin is such that the natural substrate

can be accommodated with the leucine on the amino side of the scissile bond making close contact with the pocket residues. When the leucine is replaced with phenylalanine, steric repulsion occurs that may force the scissile bond of the substrate away from the aspartic acid residues and explain the inhibition by such substrate analogs.

The present model for renin is highly speculative. It is most likely to be valid for the core of the enzyme; however, the proposed substrate-enzyme interaction awaits validation by a high resolution electron density map. It is hoped that the model will serve as a reference point for experimental tests and aid in the analysis of renin's mechanism of action and its inhibition.

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ECEPP83: AN IMPROVED EMPIRICAL POTENTIAL ENERGY PROGRAM

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In 1975 the program ECEPP (Empirical Conformational Energy Program for Peptides)[1] was introduced to study polypeptide structure. Several deficiences in ECEPP have since been found.[2-3] This paper describes some corrections to these problems. Studies using the 4-21G set of Gaussian orbitals, with total geometry relaxed to achieve optimized structures of the molecule N-acetyl-N'-methyl-alanyl amide, have recently been completed.[4-5] The results were used as guides in the refinement of the ECEPP potentials. The energies between atoms were earlier parameterized by molecular orbital and crystal packing studies.[6-7] The change in potentials presented here involves only the non-bonded interactions between atoms separated by three bonds (i.e. denoted 1-4 interactions). The special nature of these interactions was discussed previously.[1] Unfortunately, in the original work, there was little rationale for choosing a scale factor for the 1-4 interactions. To better help define this problem, 4-21G *ab initio* geometry optimized structures of a model dipeptide (Ala) were compared directly with the empirically calculated conformations. Table I compares ECEPP83 to old ECEPP and to the *ab initio* results (see IV→VI for improved agreement). Further, access to the bridge region ($\psi \sim 0$) using ECEPP is restricted by a barrier of $\sim$ 3 Kcal/mol.

Softening the 1-4 potentials lowers this barrier to ∿ 0.8 Kcal/mol. Equation 1 was found by successively reducing the coefficients and calculating the dipeptide energy positions.

$$E_{n-b}^{1-4}(r_{ij}) = 0.3\epsilon[0.3(r_{ij}/r_o)^{-12} - 2(r_{ij}/r_o)^{-6}] \qquad (1)$$

Table I. Conformations of Low Energy by 4-21G, ECEPP, and ECEPP83 for N-Acetyl-N'-Methyl-L-Alanylamide.

	Low Energy Conformations			Relative Energy (Kcal/mol)		
	4-21G	ECEPP	ECEPP83	4-21G	ECEPP	ECEPP83
I	ϕ -85	-84	-84	0.0	0.0	0.0
	ψ 73	79	78			
II	ϕ-166	-154	-156	1.4	0.4	0.3
	ψ 167	153	157			
III	ϕ 75	78	78	2.8	8.8	7.9
	ψ -62	-64	-63			
IV	ϕ-134	-150	-151	3.9	0.7	0.4
	ψ 38	72	44			
V	ϕ -92	-74	-75	4.9	1.1	0.5
	ψ -6	-45	-33			
VI	ϕ 61	54	55	6.7	2.3	1.8
	ψ 41	57	50			
VII	ϕ-162	-158	-160	7.9	1.6	1.4
	ψ -55	-58	-57			

These in turn were compared to the *ab initio* results, and to the Hartree-Fock (HF) and Thomas-Fermi-Dirac (TFD) repulsive force constants (See Fig. 1). Because no geometry changes were included in ECEPP83, the *ab initio* results could only be used to establish trends, but the agreement in dihedral angles between ECEPP83 and *ab initio* data improves. No correlation with *ab initio* energies was attempted.[5] We also changed the peptide bond length from 1.325 Å (ECEPP)[7] to 1.330Å (ECEPP83),[8] without changing the partial atomic charges.

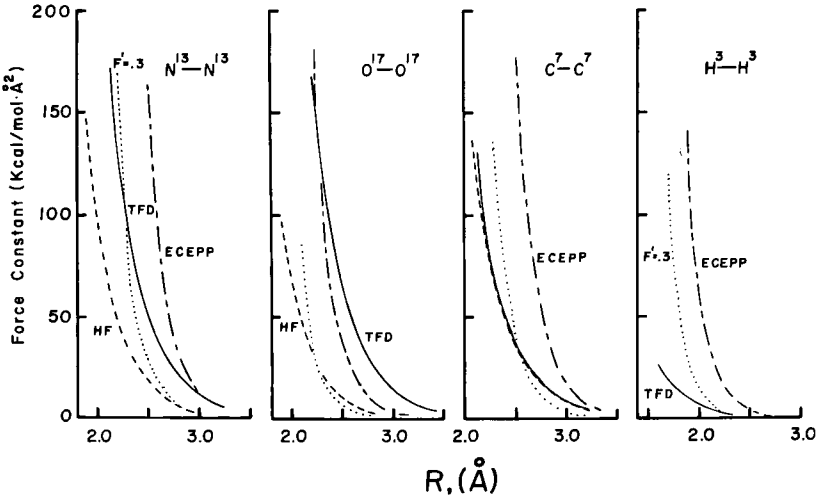

Fig. 1. Force constant, f_r versus interactomic distance for N---N, O---O, C---C and H---H 1-4 interactions. Solid lines, TFD; dash, HF; dash-dot, ECEPP; dot, ECEPP83.

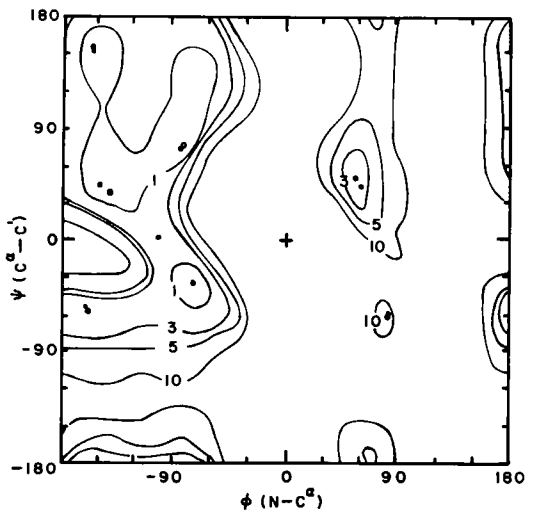

Fig. 2. Isoenergetic contour ϕ-ψ map of N-acetyl-N'-methyl alanylamide by ECEPP83. Circles are ECEPP83 minima, dots are 4-21G geometry optimized minima. Energy is in kcal/mol.

The conformational isoenergetic contour map calculated using ECEPP83, is shown in Fig. 2 for N-acetyl-N'-methyl alanyl amide. Comparing this map to that obtained using ECEPP[3], one sees the major difference in the bridge region (i.e. $\psi \sim 0°$). No barrier in this region is found from the ab initio calculations, only a low-energy trough. Further changes in side-chain potentials made in ECEPP83 will be reported elsewhere[9].

Acknowledgement

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MONTE CARLO SIMULATION OF WATER STRUCTURE AROUND THE TETRAPEPTIDE COMMON TO ENKEPHALINS, TYR-GLY-GLY-PHE

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Introduction

The interest in the structure-activity relationships of enkephalins, the endogenous opioid pentapeptides has induced a considerable number of conformational studies under various experimental conditions[1]. Many of these studies are concerned with the conformation in aqueous solution. Even if evidences for the existence of a conformational equilibrium in aqueous solution have been accumulated[1] it is still not clear what is the state of the enkephalins in water. Considering that theoretical calculations using molecular dynamics or Monte Carlo (MC) methods are potentially powerful methods for solving this problem, we have undertaken a MC study of water structure around the N-terminal tetrapeptide Tyr-Gly-Gly-Phe (YGGF) which is the smallest potent structure common to enkephalins[2]. It is therefore a convenient model for computing the relative stabilities of various conformers in aqueous solution.

One of the conformers examined (I) is that found in the crystal of the zwitterionic form of YGGF[3] and the two others were selected from theoretical studies : a conformer (II) representing a spatial structure compatible with the requirements for binding at the μ receptor[2] and a low energy more extended conformer (III) obtained from a model for hydrated peptides described previously[4].

Results and discussion

Our MC simulation was performed on the three point charge model[5] (SPC) for water molecules packed in cubic cells containing the peptide (density 1.000 ce^{-1}), using the Metropolis algorithm. At least two layers of water molecules surrounded the peptides. Geometry and potential functions for the peptide were that derived by the same authors from those determined by Hagler et al.[6]. The terminal groups of the zwitterionic YGGF bear a complete one electron charge distributed on all the atoms of the group ($NH_3^{(+)}$ and $COO^{(-)}$). The usual periodic boundary conditions (cut-off distance of 8 Å for all interactions) were used. From the total of 310^6 configurations, the initial 210^6 were rejected. Problems which arise from boundary conditions when long range electrostatic forces between charges (ions) and dipolar solvant molecules are present and the influence of boundary conditions on the results will be examined in a further study.

The three conformers are shown on Figure 1.

In the absence of solvent the conformational energy is 12 kcal higher for I relative to II and III which are of comparable energy. The water-peptide interaction energy, equal, in Kcal, to -298.(I), -272.(II) and -356. (III) ± 15 kcal, is also the lowest for III. Using the Born model[7] it is possible to estimate the energy of interaction (E_{R_C}) between a charge located at the center of a spherical eluster of radius R_C with a dipolar medium outside the eluster treated as a continuum. Each of the charges, if alone contributes 21 kcal to that interaction. Even if the neglected energy of long range interaction is of that order, the differences between the values of E_{R_C} for individual conformers should be considerably smaller.

We have examined the extent of hydration of various chemical groups by computing the pair distribution function $g_{OP}(r)$ of oxygens of water around a given solute atom P, which is related to the probability of finding a water molecule at a distance r from that atom. For the three conformers, inclusively for the more folded one (I), the terminal charged group show a well-defined hydration shell, whereas for the dipolar group of the peptide bonds the hydration shell is small. When computing the number of water molecules in the first hydration shell, it is found that the stabilization

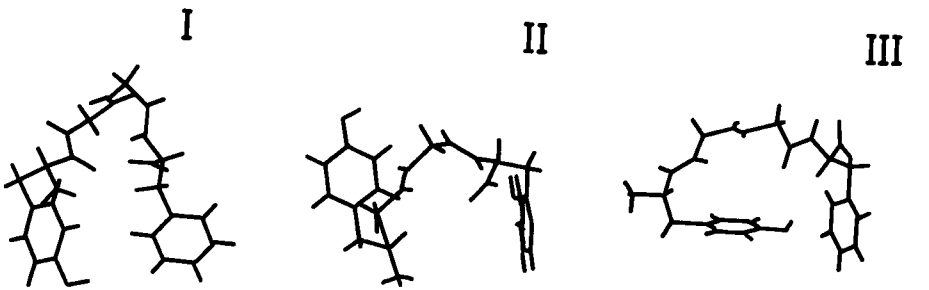

Fig. 1. Models of conformer I, II and III.

of the "extended" conformer III by hydration arises from a good exposure to solvant of various polar groups and of the terminal charged group. For example, the number of water molecules in the first hydration shell is 3,8 (I), 2.5 (II) and 4.4 (III) for the NH_3^+ group and 7.2 (I), 3.1 (II) and 8.2 (III) for the two oxygens of the COO^- group. The better hydration of conformer III is due in addition to a larger distance between the charges.

In spite of a greater unfavourable water-water interaction, conformer III remains more stable in water than the two other conformers examined.

In conclusion, the results obtained suggest that the state of YGGF in water may be represented by an equilibrium between various conformers, but that the extended structures are favoured over the folded ones.

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EVALUATION OF S-PEPTIDE SEQUENCE MODELING BY CRYSTALLOGRAPHIC CHARACTERIZATION OF A SIMPLIFIED SEMISYNTHETIC RIBONUCLEASE S

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INTRODUCTION

Ribonuclease S-peptide has been the subject of numerous synthetic analogue studies[1]. Most often, the S-peptide amino acid sequence has been subjected to step-by-step residue replacement or truncation. In an effort to define a more comprehensive view of how S-peptide sequence contributes to conformation and function in bovine pancreatic ribonuclease, 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 and to demonstrate the validity of such basic factors through design and synthesis of a modeled S-peptide. We have reported earlier computer-assisted modeling of ribonuclease S-peptide based on structural and local interaction analysis[2,3]. The analyses provided the following insights: 1) nonbonding interactions involving Phe-8 and Met-13 contribute major complex stabilizing free energy(Figure 1) and 2) the α-helical framework of S-peptide is essential to orient these two side chains optimally toward hydrophobic residues Val-47,54, and 108 and Leu-51 of S-protein. In addition, previous studies have showed

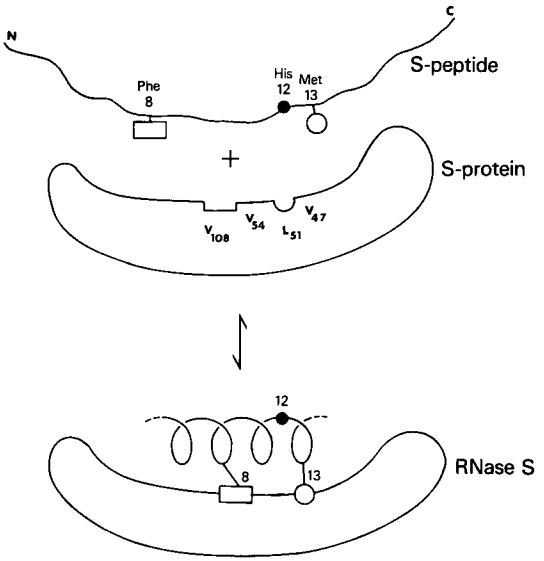

Fig.1. Schematic view of RNase S-peptide and S-protein complexation. The scheme illustrates the key α-helical conformation for S-peptide in order to effect critical nonbonding interactions among Phe-8 and Met-13 of S-peptide and Val-47,54, and 108 and Leu-51 of S-protein. The nonbonding interactions among these residues are predicted to provide specific recognition and resultant stabilization for RNase S complexation.

the critical catalytic role of His-12. These insights led to the design of a model peptide, AEAAAAKFARAHMAA, where alanine residues were predicted to provide the α-helical framework to orient the side chains of Phe-8 and Met-13. Biochemical and physical properties of the model (1-15) S-peptide have been reported[4]. This modeled S-peptide formed a catalytically active complex with S-protein consistent with its design to mimic the biological function of native S-peptide.

In order to verify that the designed structure actually was achieved, crystal structure determination of the model semi-synthetic complex was undertaken.

RESULTS AND DISCUSSION

Circular dichroism spectra and estimated free energies of α -helical stabilization of model peptide as compared to those of native S-peptide suggested a similar if not increased tendency for the model S-peptide to form α-helical structure[3,4]. The preliminary 3 Å resolution X-ray structure map of the model peptide:S-protein complex indicates that, as intended, the model peptide assumes an α-helical structure to the same extent as does native S-peptide in RNase S(Figure 2).

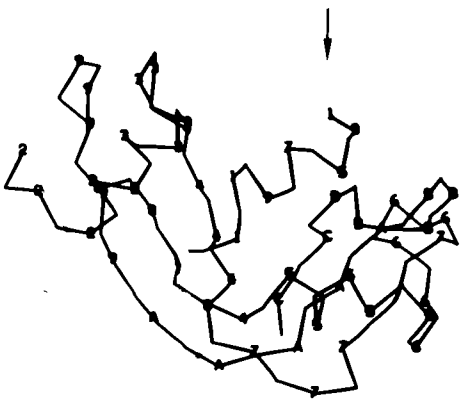

Fig.2. Preliminary 3 Å resolution structure of model semisynthetic RNase S determined by x-ray diffraction analysis. The arrow indicates the N-terminal residue of model S-peptide; the α -helical conformation within the modeled peptide is seen.

Furthermore, the critical complex-stabilizing nonbonding interactions (see Figure 1) arising from the side chains of Phe-8 and Met-13 are retained in the crystal structure of the complex.

Hence, the crystal structure of the S-peptide in model semisynthetic RNase S complex indeed has the structure intended from sequence modeling. The results support the notion that the functional role of a given residue in a sequence can be simplified as either a local structure determinant or a structure stabilizer through long range interaction. Lastly, the results emphasize the usefulness of conformation-based sequence consideration for designing simplified synthetic peptides and proteins.

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PEPTIDE-TOXIN COMPLEXES AND PITUITARY PROLACTIN SECRETION

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This paper deals with the toxin of Corynebacterium diphtheriae, its structural requirements for cell binding and cell entry, and the synthesis and action of a hybrid protein formed from a portion of the diphtheria toxin and the hypophysiotropic hormone thyrotropin releasing hormone (TRH).

Diphtheria toxin is extremely potent; as little as 50 to 100 ng/kg is lethal for sensitive mammalian species. A single molecule of the toxin that enters the cell is sufficient to produce irreversible cell damage. Native toxin has a molecular weight of 62 K; under denaturing and reducing conditions it can be separated into two peptides: an amino terminal fragment designated A (MW 24 K), and a carboxyl-terminal fragment designed B (MW 38 K) (Fig. 1). The A and B fragments define

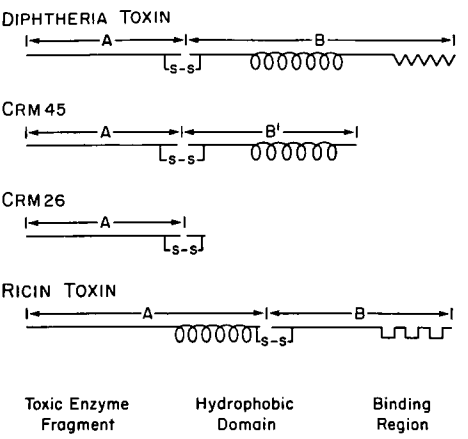


Figure 1
Diagramatic representation of diphtheria toxin (MW 62 K), and two related toxins, the product of mutant strains of Corynebacterium diphtheriae. These are Cross Reacting Material 45 (CRM45) and CRM26. For comparison the structure of ricin, another toxin commonly used for studies of toxin-protein hybrids is shown. CRM45 differs from the native toxin in lacking the binding domain, while CRM26 lacks both this region and the hydrophobic domain binding domains.

functionally distinct regions with enzyme activity (A chain) and receptor binding capacity (B chain). Upon entry into cell cytosol, fragment A inactivates elongation factor-2 (EF-2), an enzyme responsible for the translocation of activated amino acids from the amino site on the ribosome to the peptidyl site. Fragment A catalyzes the following reaction:

$$NAD + EF\text{-}2 \rightleftharpoons ADPR\text{-}EF\text{-}2 + \text{nicotinamide} + H^+$$

Adenylated EF-2 is inactive and as a result, protein synthesis is inhibited.

Although fragment A is highly active as an enzyme in broken cell preparations, it is not toxic for eukaryotic cells because alone it is unable to penetrate through the cytoplasmic membrane. The B fragment of diphtheria toxin is essential for toxicity. It has two domains, a cell binding determinant that interacts with a specific mammalian cell receptor, and a hydrophobic region postulated by Boquet and Pappenheimer[1] to be essential for translocation of the toxin complex into the cell.

Diphtheria-cross reacting material 45 (CRM45) consists of the A chain linked to the hydrophobic region but lacking the binding domain. It is ineffective as a toxin in whole cell preparations but can catalyse the adenylation of EF-2 in broken cell preparations with a potency equivalent to native toxin. Cross reacting material 26 (CRM26) consist only of the A chain and lacks the hydrophobic domain of CRM45 and of native toxin. For comparison, Fig. 1 also shows the structure of ricin, a toxin that recently has been widely used to prepared immunotoxins.

The molecular basis of the interaction which allows for the translocation of fragment A into the cytosol is the subject of considerable speculation.[1] Recent work suggest that in addition to cell binding, the toxin must be endocytosed by an active process, and that certain specific conditions be met to allow the toxin to pass through the endocytic membrane into the cytosol.[2-4] This hypothesis is outlined in Figure 2.

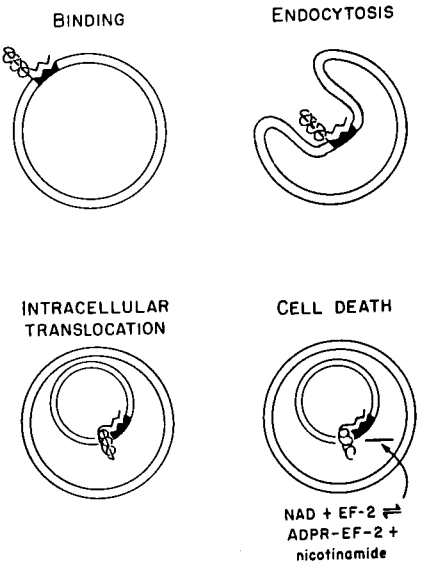


Figure 2
Schematic outline of mechanism of cell intoxication by diphtheria toxin. The binding fragment of the toxin interacts with a specific receptor on the cell membrane. The receptor with the bound toxin is internalized. Next the hydrophobic domain in some way facilitates the translocation of the toxic fragment (shown as the straight bar) into the cytosol where the toxic enzyme interferes with the function of elongation factor-2.

Our interest in developing peptide-diphtheria toxin hybrid proteins arose from efforts to elucidate the molecular mechanism of toxin entry into cells. We also wished to determine whether such hybrids could selectively destroy classes of cells containing specific receptors both in the elucidation of normal functions and possibly as specific therapeutic agents for cancer therapy. The development of targeted toxin hybrids using monoclonal antibodies directed against specific cell membrane determinants[5-7], hormone-toxin hybrids[8-9], and other ligand-toxin hybrids such as asialofetuin[10] is an extremely active area of current reseach (Table 1).

As our first approach to testing the modified Boquet-Pappenheimer model of diphtheria toxin entry into cells we attempted to prepare complexes of CRM45 with human chorionic gonadotropin (hCG). This hormone was chosen because it has a high bonding affinity to receptors on gonadal cells and the kinetics of binding and mechanisms of action have been well worked out.[11] Furthermore, hCG B chain-ricin A chain complexes have been shown[12] to have limited but definite toxicity to a Leydig tumor line. Unfortunately, we found that all standard

Table 1

Examples of Reported Chimeric Toxins

LECTINS

Concanavalin A * **
Misteria Lectin **

ANTIBODIES

Dinitrophenyl Group **
SV40 **
Mumps **
Colorectal Carcinoma * **
Transferrin Receptor *
Iodiotypic Immunoglobulins on B Cells *
Thy 1,2 Antigen on T Cells *

CARBOHYDRATE MOIETIES

Monophosphopentamannose * **
Asialofetuin * **

HORMONES

Human Placental Lactogen **
Insulin **
Epidermal Growth Factor * **
Thyrotropin-Releasing Hormone **

* Ricin ** Diphtheria

protein linking methods used to combine hCG or hLH to CRM45 led to loss of specific binding to dispersed testicular cells. This finding underlines the crucial importance of the link between the toxin and the ligand for binding and entry into cells.

We chose to construct a peptide hormone complex with CRM45 utilizing TRH. TRH is a tripeptide (pGlu-His-Phe-NH_2) which binds to specific receptors on the pituitary gland.[13-18] TRH stimulates the secretion of TSH and of prolactin from normal pituitary cells, and under some circumstances of growth hormone. It is also active in stimulating prolactin secretion in several pituitary tumor cell lines, including the well studied GH lines first isolated by Tashjian et al.[19] Table II summarizes the mode of binding and action of this peptide on GH cells. The availability of several *in vivo* and *in vitro* model systems that permit the measurement of both receptor binding and biological responsiveness of pituitary cells make TRH an especially useful molecule for the study that we had proposed.

Table 2
Mode of Action of TRH on GH Cells

Binds to receptor on cell membranes
Receptor complex initially dissociable, later not
Approximately 125,000 receptors per cell
Apparent Kd of 10 nM
Is internalized, but internalization not essential
Endocytosis not essential for effect
Stimulates adenylcyclase, but an epiphenomenon
Mobilizes intracellular pool of calcium

To determine the importance of the hydrophobic domain of diphtheria toxin for intoxication of cells we also studied a hybrid prepared by linking TRH with a product of another diphtheria mutant strain designated CRM26 (Fig. 1). CRM26 is structurally and functionally similar to fragment A in that it contains the enzymatically active domain of the toxin molecule.

For these studies diphtheria toxin-related products CRM26 and CRM45 were purified from culture supernatant fluids of *C. diphtheriae* strains C7 (β-*tox*-2) and C7 (β-*tox*-45) respectively by ammonium sulfate precipitation and DEAE ion-exchange chromatography.[20] CRM concentrations were determined using the BioRad Protein Assay Kit, and confirmed in the case of CRM45 by rocket immunoelectrophoresis.[21] TRH immunoreactivity was measured as previously described.[22] GH3 cells, a clonal strain of rat pituitary tumor cells[19] were obtained from the American Type Culture Collection. They were maintained as monolayers in Ham's F10 medium supplemented with 15% horse serum, 2.5% fetal calf serum and 2mM glutamine. A control line of cells that do not have TRH receptors (3T3 mouse fibroblasts) were also studied.

CRM45 was thiolated with N-succimidyl-3-(2-pyridyldithio)-propionate(SPDP, Pharmacia), and was coupled to TRH modified by the addition of acetylcystamine (See Fig. 3).[23] CRM26 was coupled through its terminal sulfhydyl, but also was studied as a complex through the SPDP linking procedure.

In our first paper dealing with this project we characterized the two TRH-diphtheria toxin-related protein hybrids

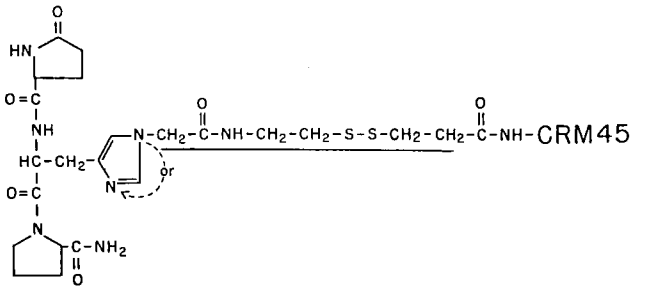


Figure 3
Diagram of the structure of the TRH-CRM45 hybrid molecule.

with respect to their ability to bind specifically to anti-TRH antibody, and to GH_3 rat pituitary tumor cells.[24] Although the TRH-CRM26 conjugate bound with a slightly higher affinity than did TRH-CRM45, TRH-CRM45 caused a 50% inhibition of protein synthesis at 3 x 10^{-9} M, while the TRH-CRM26 conjugate was non-toxic even at 10^{-7}M (Fig. 4). TRH-CRM45 did not inhibit protein synthesis in the 3T3 cell line which lacks TRH receptors. These studies demonstrated that it was feasible to construct a neuropeptide-toxin complex capable of selectively binding to and destroying certain types of cells. These experiments also demonstated for the first time in isogenic hybrid toxin studies that the hydophobic domain of diphtheria toxin is essential for the translocation of a polypeptide fragment containing ADPR-transferase activity into the cell. The findings lend strong support to the hypothesis of Boquet and Pappenheimer.[1] Their work has shown that the hydrophobic domain of fragment B is buried within the three dimensional structure of the diphtheria toxin molecule, but is exposed on CRM45.[25]

Several other studies have provided additional physical and chemical evidence of the unique structure of CRM45 and of the potential importance of the hydrophobic region. Lambotte et al.[26] showed that the primary structure of the fragment B hydrophobic region is very similar to the lipid-binding domain of integral membrane proteins. The lipid-binding properties of

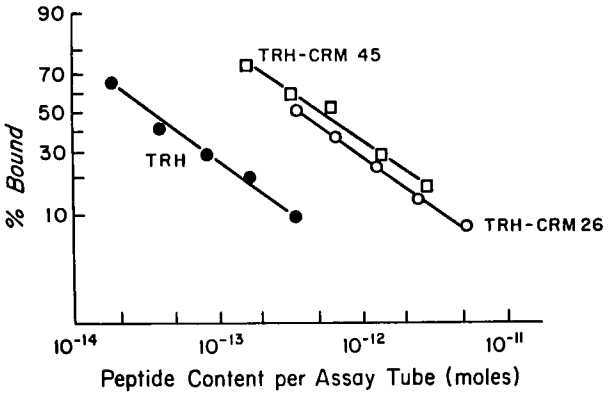


Figure 4
A typical dose-response toxicity curve for TRH-CRM45 compared with the effects of TRH-CRM26, and the unconjugated respective toxins. Inhibition of protein synthesis is measured by [14C]-leucine incorporated into acid precipitable protein. In this experiment 24 hr exposure to TRH-CRM45 at a concentration of 3 x 10^{-9} M caused a 50% inhibition of protein synthesis in GH3 cells. Unconjugated CRM45 was found to be 200 to 500 times less toxic in this system. In contrast, neither CRM26 nor the TRH-CRM26 conjugate, though enzymatically active (in broken cell assay), inhibited protein synthesis even at 10^{-7} M. Native diphtheria toxin is virtually non-toxic at this concentration because GH3 cells are derived from the rat, a relatively resistant species.

this domain have been demonstrated by studies which showed that CRM45 is capable of inserting itself directly into both liposomes[27] and artificial phospholipid bilayers.[4] In fact, the latter investigators have shown that the fragment B portion of CRM45 is able to form a pore in artifical phospholipid bilayers large enough to allow the passage of an extended fragment A molecule. These studies also demonstrated that it was feasible to construct a neuropeptide-toxin complex capable of selective binding and destruction of certain types of cells.

Next we sought to determine whether TRH-CRM45 complex binds selectively to organs known to possess TRH receptors when administered by intravenous injection into intact animals. To do this, the regional localization of specifically and non-specifically bound TRH-CRM45 complex was studied in the rat.[24] For comparison the fate and binding of CRM45 alone was also

studied. CRM45 and TRH-CRM45 were radioiodinated[28] and purified by Sephadex chromatography in 0.15 M NaCl, 0.01 M sodium phosphate (pH 7.4). The specific activity of these preparations was between 2 and 3 μCi/pmole.

Approximately 107 dpm of either TRH-[^{125}I]-CRM45 or [^{125}I]-CRM45 in 0.1 ml normal saline was injected intravenously into 5 normal male rats, 150-200 gm b.w. At various times the animals were killed and tissue and blood samples removed. Organ concentrations of radioiodinated material were corrected by Sephadex G 50 chromatography of tissue extracts to exclude fractions corresponding to degraded CRM, i.e. less than 30,000 daltons. The extent of degradation in different tissues varied widely; at 15 ml 85% of radioactivity present in anterior pituitary was as intact CRM, although only 64% of the material in blood and 24% in kidney was undegraded at the same time interval. Radioactive TRH-CRM45 disappeared rapidly from blood: at 5 min 2.9% of the injected dose/ml was present; at 15 min 2.9% and at 30 min 1.2%.

Distribution of radioactive TRH-CRM45 conjugate in various organs given as tissue:plasma (T:P) ratios at 5, 15, and 30 min after intravenous injection is shown in Fig. 5.

Concentrations of intact peptide greater than those attributable to tissue bovine serum albumin (BSA) space were found (in descending order) in kidney, anterior pituitary, liver, testis, and hypothalamus. T:P ratios increased with the passage of time in most tissues. These time-related differences were significant statistically only for anterior pituitary, hypothalamus and testis. Mean kidney T:P ratios reached peak values at 15 min, and had fallen by 30 min, but none of the values were statistically different from one another.

If tissue uptake of TRH-CRM45 is mediated via the TRH receptor, one would predict that receptor-containing tissues would have higher T:P ratios for the conjugate alone and that TRH should compete for those tissue binding sites. T:P ratios at

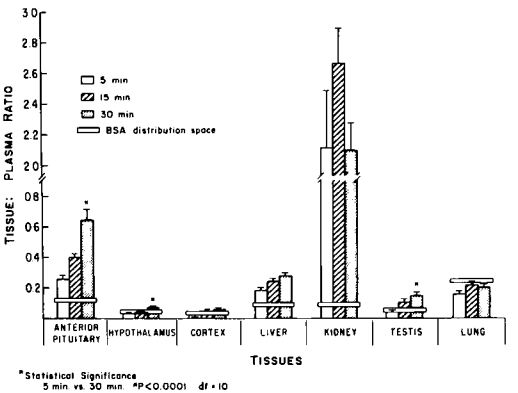


Figure 5
Tissue:plasma ratios of TRH-[^{125}I]-CRM45 in various tissues at 5, 15 and 30 min after intravenous injection. The values have been corrected for the undegraded fraction. The ratio of labeled bovine serum albumin (BSA) which represents the blood vascular compartment is shown for comparison.

15 min for animals given radioiodinated TRH-CRM45 with and without a large excess of unlabeled TRH and iodinated CRM45 are shown in Table III. TRH-CRM45 was more highly concentrated than CRM45 in 5 tissues: anterior pituitary, hypothalamus, testis, ovary, and breast (of lactating rats). The latter studies were carried out in a separate experiment.[24] The most marked difference in concentration of labeled complex was in the anterior pituitary. In three tissues (anterior pituitary, hypothalamus, and testis), TRH-CRM45 T:P ration were reduced significantly by concomitant administration of excess unlabeled TRH.

High concentrations of radioactivity were found in the kidney of animals given either CRM45 of TRH-CRM45 with and without TRH. We concluded therefore that the enhanced uptake of this organ was a property of the CRM45 and not of the TRH moiety. In fact T:P ratios for CRM45 for all other organs examined were virtually identical at 15 min to those obtained using radioiodinated BSA except that for the kidney where the ratio was much higher for CRM45 (1.935 $\pm$ 0.158 vs. 0.085 $\pm$ 0.019; $p < 0.005$; Table 3). Therefore the kidney must handle CRM45 differently that it does BSA, a finding possibly attributable to the smaller molecular size of CRM45 and its greater negative ionic charge at physiological pH.

TABLE 3

Tissue:plasma ratio of TRH-(^{125}I)-CRM45, (^{125}I)-CRM45, and (^{125}I)-bovine serum albumin (BSA) 15 min after i.v. injection corrected for undegraded fraction.

Tissue	TRH-CRM45	CRM45	*TRH + TRH-CRM45	BSA
Kidney	1.752±0.116	1.935±0.158	1.545±0.153	0.085±0.019
Pituitary	0.305±0.016	0.088±0.003	0.229±0.018	0.113±0.017
Lung	0.234±0.011	0.239±0.020	0.221±0.017	0.230±0.024
Ovary	0.183±0.022	0.136±0.008	0.175±0.023	0.133±0.033
Liver	0.152±0.004	0.150±0.011	0.150±0.012	0.088±0.005
Breast	0.119±0.017	0.058±0.006	0.098±0.022	0.064±0.005
Pancreas	0.100±0.009	0.092±0.004	0.095±0.003	1.010±0.002
Testis	0.053±0.007	0.030±0.003	0.037±0.002	0.031±0.002
Hypothalamus	0.038±0.003	0.024±0.002	0.023±0.002	0.031±0.004
Cortex	0.026±0.002	0.021±0.003	0.020±0.002	0.021±0.002

Statistical significance versus TRH-CRM45

$P < 0.05$ ——

$P < 0.01$ ═══

$P < 0.005$ ≡≡≡

*TRH + TRH-CRM45 injected simultaneously with 0.5 mg unlabeled TRH.

These studies showed that the TRH-CRM45 complex is selectively concentrated in normal anterior pituitary and that its uptake in this organ is reduced by concomitant administration of cold TRH. We conclude therefore that selective uptake of TRH-CRM45 is indicative of binding to specific TRH receptors. Other sites in which displaceable binding could be demonstrated were the hypothalamus and testis. Non-displaceable uptake relative to CRM45 alone was demonstrated in ovary and the breast parenchyma. Studies of *in vitro* of TRH binding have previously shown high affinity TRH receptors in anterior pituitary,[14,25] and various brain regions including the hypothalamus[30,31] and low affinity binding sites in the liver.[30] Binding in the testis has been reported at slightly above that of background.[30]

One of the long range goals of our work with TRH-CRM45 hybrids was to develop molecules which can be targeted to specific populations of cells bearing TRH receptors and produce selective destruction of these cells. In the next series of experiments we sought to determine whether TRH-CRM45 administered to intact animals could specifically damage lactotropes. The lactating rat was chosen as the test system for study because prolactin levels in such animals are very high and a physiologically meaningful response could be gauged by observing lactational performance. These studies will be reported in detail separately. A brief summary is provided here.

Pregnant rats were obtained from Charles River Breeding Laboratories. On the day of delivery, mothers (5 or 6 per group) were injected with 10 or 50 µg/100 g bw TRH-CRM45 or CRM45 alone. Controls were injected with saline, and litter size adjusted to 10 pups for each mother. The weights of pups and mothers were measured daily. On day 7 the mothers were decapitated approximately 15 min after nursing began when hormone levels associated with suckling are expected to be high. Blood was collected directly into the heparinized tube on ice followed by rapid separation of plasma from cells. Rat prolactin concentrations were determined by double antibody RIA using anti-PRL-7 antibody and reference preparation rat PRL-RP2 from the National Hormone Distribution Program of the NIAMDD. As compared to controls, treatment of lactating rats with TRH-CRM45 but not CRM45 alone caused a significant decrease in PRL levels measured during a suckling episode. PRL levels in the TRH-CRM45 rats, though low, are greater than those measured in nonlactating female rats (<5ng/ml) (Table 4).

Table 4

Effect of Treatment with TRH-CRM45 and CRM45 on Plasma Hormone Levels in Lactating Rats.

Treatment	Prolactin (µg/ml)	Oxytocin (µU/ml)	Corticosterone (µg%)
Saline	255 ± 69	3.42 ± 1.05	10.36 ± 2.28
TRH-CRM45 (10 µg/100g BW)	85 ± 40*	-	-
TRH-CRM45 (50 µg/100g BW)	35 ± 8*	5.95 ± 2.26	11.00 ± 3.21
CRM45 (50 µg/100 BW)	201 ± 27*	-	-

*P < 0.05
**P < 0.005

Pups from TRH-CRM45 treated mothers grew less well than controls (Fig. 6) and had a significant mortality rate (Table 5). Since several pups from the TRH-treated mothers died during the experiment, average weight of those surviving was used as a measure of growth. Therefore changes in growth rate as pictured in Fig. 6 are less striking than if the entire group of animals had been included. In order to demonstrate that the decreased weight gain in the pups from conjugate treated mothers was not due to direct poisoning of the animals, litters from treated and untreated mothers were cross-fostered. The growth pattern of the pups followed that of their foster

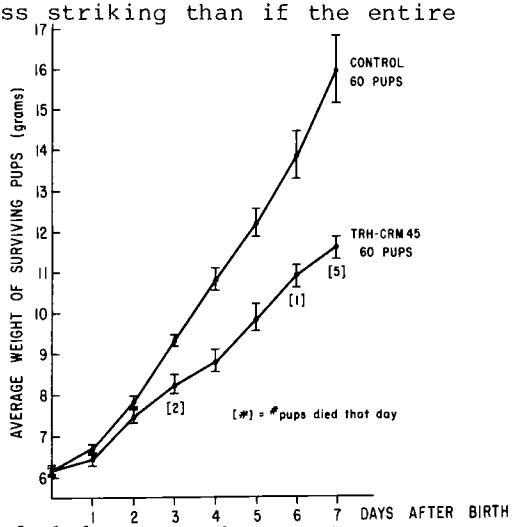


Figure 6
Growth patterns in pups from litters of mothers treated on the day of delivery with either 50 µg/100 g bw TRH-CRM45 or saline as a control.

Table 5

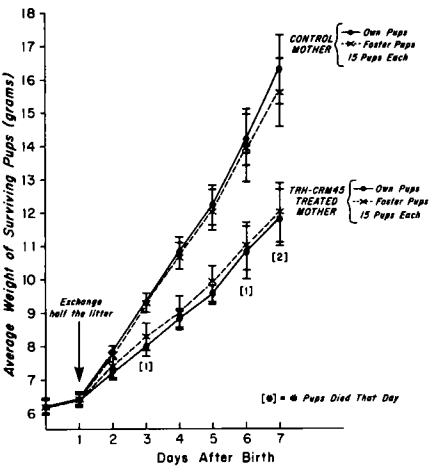
Effect on Growth and Survival of Litters of TRH-CRM45 and CRM45 Treated Rats

Treatment	Average 1 wk Weight Gain of Surviving Pups (g)	# Pups Died ÷ # Pups Studied
Saline	9.7 ± 0.8	0/60
TRH-CRM45 (10 µg/100g BW)	8.4 ± 0.4	0/50
TRH-CRM45 (50 µg/100g BW)	5.6 ± 0.4*	8/60
CRM45 (50 µg/100g BW)	7.9 ± 0.7	0/50

*P < 0.005

siblings rather than that of their litter mates (Fig. 7).

Figure 7
Growth patterns of pups from control and TRH-CRM45 treated mothers which were cross-fostered 24 hr post delivery. Cross fostering was carried out to determine if the impaired growth of the pups was due to the toxin being transmitted in the milk during the first hours of life. Only the pups that were nursed by the TRH-CRM45 treated mothers showed impaired growth.



Analysis of milk for a large molecular weight radioactive compound from mothers given TRH-[^{125}I]-CRM45 demonstrated that the toxin was not secreted by the breast.

Decreased lactation following the fall of maternal prolactin secretion is the most likely explanation for the poor growth rate of the pups. Levels of two other hormones related to lactation, oxytocin and corticosterone were normal (Table 4) and direct analysis showed that the toxin was not secreted in the milk. Since the pups of mothers treated with CRM45 alone as

controls grew normally, it is unlikely that systemic toxicity of CRM45 was an important factor in causing poor growth in their young. We cannot eliminate the possibility that, in addition to reduction of prolactin levels, some damage to breast parenchyma is incurred by the toxin since TRH-CRM45 does localize to this tissue, though not in a displacable manner.

A potential drawback to the systemic use of toxin-TRH hybrids is the possibility that tissues like liver or kidney may clear the complex as part of a normal detoxification mechanism. In this study, the kidney was found to take up CRM45 much more avidly than did the pituitary, and the effect was unrelated to the presence of TRH in the hybrid. The functional and pathological significance of this localization is under study. Nevertheless, the demonstration of specific binding of TRH-CRM45 to specific receptors, both in vivo and in vitro, the evidence of damage to specific cells in tissue culture, and the impairment of function of prolactinotropes after systemic injection, indicate that it is feasible to construct toxin-peptide hybrids that are capable of selective destruction of cells, which can be used both for physiological studies, and conceivably for the destruction of neoplastic cells.

Acknowledgement

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HUMAN AND RAT HYPOTHALAMIC GROWTH HORMONE RELEASING FACTORS (GRF)

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Introduction

Several GRF's originating from 2 different human pancreatic tumors have recently been characterized independently in 2 laboratories.[1-3] Whereas Guillemin et al.[3] reported on the isolation, sequence analysis and synthesis of 3 different GRF species: hpGRF-44NH_2, hpGRF-40 and hpGRF-37, the tumor made available to us contained hpGRF-40 predominantly.

The question of whether these peptides belonging to the glucacon/secretin family and which showed most sequence homology with PHI were indeed the long sought hypothalamic GRF, remained unanswered. In order to obtain direct evidence of the nature of hypothalamic GRF, we isolated it from both rat hypothalami made available to us by Dr. A. Parlow, and human infundibulum provided to us by Dr. S. Reichlin.

Experimental

HPLC systems have been described earlier.[1,4,5] Synthetic (syn.) solid phase approach was that reported earlier.[1] A preparative system using Waters Associates Prep LC-500 and handpacked cartridges using Vydac C_{18}, C_4 or phenyl 300Å, 15-20μ silica was used for the purification of syn. peptides. Increasing concentration of CH_3CN was generated by an Eldex low pressure gradient maker. Rat hypothalami were extracted and first chromatographed on a gel permeation column as described by Vale et al.[6] We followed GRF activities throughout our purification schemes with an in vitro method for assaying the ability of GRF to stimulate the secretion of GH by primary culture of rat pituitary cells.

Results and Discussion

HPLC purification of rat hypothalamic GRF (rhGRF) yielded after 6 reverse phase steps (Table I) a highly purified material (3.4 nmoles) which was subjected to sequence analysis in an automatic spinning cup sequencer after derivatization with 3-sulphophenylisothiocyanate.[7] The complete sequence was confirmed by comparative peptide mapping of both natural and syn. peptides.

TABLE I. HPLC Purification of rhGRF

HPLC Step	Column[a)]	Solvent Composition[b)]	Gradient Shape (%)[c)]	Retention Volumes[d)]
1	C_{18}	TEAP	$7 \xrightarrow{40'} 60$	1600-1800
2	C_4	TEAP	$25 \xrightarrow{25'} 95$	41.1-45.6
3	diphenyl	.1%TFA	$25 \xrightarrow{15'} 45$	20.4-22.5
4	C_{18}	.1%TFA	$35 \xrightarrow{15'} 60$	14.0-16.2
5	diphenyl	.1%TFA	$20 \xrightarrow{40'} 50$	24.0-25.4
6	C_{18}	.1%TFA	$35 \xrightarrow{40'} 60$	35.6-36.5

a) All columns were from Vydac. Step 1 was preparative (5x30 cm cartridge). Step 2 was semipreparative (1x25 cm). All other steps were run on analytical columns (0.46x25 cm). b) TEAP (pH 2.25) was made as described earlier.[4] B buffer was 60% CH_3CN diluted in A. c) $7 \xrightarrow{40'} 60\%$ means that a gradient going from 7%B to 60%B was generated in 40 min. Flow rates were 75 ml/min for step 1;3 ml/min for step 2;1.2 ml/min for steps 3 to 6. d) Retention volume of the active zone was measured in ml from the time of start of the gradient (does not include loading volume which varied). (See ref 5 for further details).

The 43-residue GRF molecule which sequence was determined to be H-His-Ala-Asp-Ala-Ile-Phe-Thr-Ser-Ser-Tyr-Arg-Arg-Ile-Leu-Gly-Gln-Leu-Tyr-Ala-Arg-Lys-Leu-Leu-His-Glu-Ile-Met-Asn-Arg-Gln-Gln-Gly-Glu-Arg-Asn-Gln-Glu-Gln-Arg-Ser-Arg-Phe-Asn-OH was twice as potent as hpGRF-40, but showed only 67% sequence homology with the known hpGRF. The question of whether this relatively low level of homology was the result of tissue rather than species specificity was resolved by our purifying and characterizing human hypothalamic GRF (hhGRF). After a first extraction and gel permeation separation similar to that reported for hpGRF[1] and rhGRF[7], HPLC was used for the isolation (Table II) of 2 distinct molecular species which were separated at Step 5. In addition to sharing similar

immunologic properties syn. (oxidized) hpGRF-44NH_2 and (oxidized) hhGRF-44NH_2 coelute under HPLC conditions illustrated in Fig 1A. Similarly, syn. (oxidized) hpGRF-40 coeluted with the 2nd (oxidized) hhGRF entity under chromatographic conditions that separated both hpGRF-40 from hpGRF-44NH_2 and from their respective Met(O)^{27} analogs as illustrated in Fig. IB. Comparison of the clostripain digest maps by HPLC and amino acid composition of the fragments generated from both natural and syn. preparations suggest that both hp and hhGRF share a common sequence.

Table II. HPLC Purification of hhGRF-44NH_2

HPLC Step	Column[a]	Solvent Composition[b]	Gradient Shape[c]	Retention Volume[d]
1	C_4	.1%TFA	20 45' 80	38.5-40.8
2	diphenyl	TEAP	20 30' 50	36.8-42.0
3	diphenyl	.1%TFA	25 18.5' 53	30.6-32.4
4	diphenyl	.1%TFA	25 20' 53	33.0-33.6
5	C_{18}	.1%TFA	35 20' 54	29.8-30.8
6[e]	diphenyl	.1%TFA	35 20' 55	27.4-29.2
7	diphenyl	.1%TFA	35 20' 55	24.8-25.7
6'[f]	C_4	.1%TFA	35 15' 52	26.4-27.6
7'[f]	diphenyl	.1%TFA	35 20' 55	24.4-25.0

a) All steps were carried out on Vydac analytical columns. b),c) and d) see Table I. e) Active zones from Step 5 were treated with H_2O_2 (see ref 5). f) hhGRF-40 was separated from hhGRF-44NH_2 at step 5 and further processed in Steps 6' and 7'.

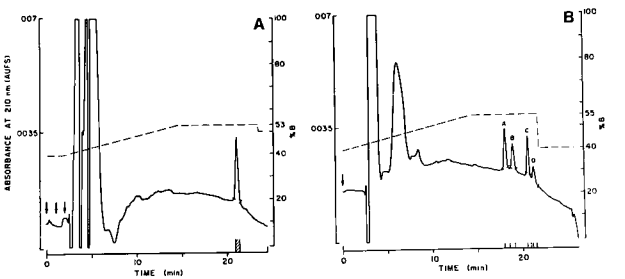

Fig. I.
A: Coelution of syn. [Met(O)^{27}]-hpGRF-44 and oxidized hhGRF from Step 7 (Table II).
B: Separation of syn. [Met(O)^{27}]-hpGRF-40(A) and -44(B) and hpGRF-40(C) and -44(D) under conditions reported in Table II Step 7; gradients as shown by dotted lines; loads ~40ng.

Structure activity relationships.

In order to develop an effective GRF analog we have investigated the effect of chain shortening and replacements by L and D amino acids (AA) on biological potency as measured in our in vitro bioassay where hpGRF-40 has a potency of 1. We found that the active core encompassed residues 1-27 and that C-terminal amidation of these short analogs [GRF(1-29);GRF(1-32)] lead to peptides which are up to 3 fold more potent than their corresponding free acids. Extension by 1 amino acid at the N-terminal or deletion of Tyr^1 resulted in analogs with low potency [$DAla^0$]-hpGRF(1-32)-NH_2 (0.076); des-Tyr^1-hpGRF(1-32)-NH_2 (0.0008). Position 1 replacements in hpGRF(1-32)-NH_2 show the following potencies: [$DTyr^1$]- (0.74);[Phe^1]- (0.13);[$N\alpha CH_3Phe^1$]- (0.12);[His^1]- (2.15);[$4ClPhe^1$]- (0.02);[$C\alpha CH_3 4Cl$-Phe^1]- (0.047);[Met^1]- (0.36);[Arg^1]- (0.018); [Glu^1]- (0.0024). Replacement of Met^{27} by Nle^{27} increased potency by a factor of 2, while biopotency is retained by replacement of some amino acids by their D-isomers: [$DAla^{15}$]-hpGRF(1-32)-NH_2 (0.43);[$DMet^{27}$]-hpGRF(1-32)-NH_2 (0.22). Potent and enzymatically resistant GRF analogs may be useful for growth promotion both in humans and domestic animals.

Acknowledgements

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OXYTOCIN AND PROSTAGLANDINS IN UTERINE CONTRACTIONS AT TERM AND THE POTENTIAL OF OXYTOCIN ANTAGONISTS IN THE PREVENTION OF PRETERM LABOR

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Introduction

Oxytocin (OT) and prostaglandins (PGs) are believed to be the major mediators in uterine contractions. The sensitivity of the pregnant uterus to OT increases abruptly at term. The term pregnant uterus also exhibits a markedly enhanced production of PGs. We have shown that OT has dual actions on the isolated pregnant rat uterus, a direct myometrial stimulating action and a PG-releasing action.[1] OT, therefore, may play a pivotal role in labor. Inhibition of OT actions by specific OT antagonists may be an effective method for the treatment of preterm labor or threatened abortion. In this presentation, we describe the relationship between the development of OT sensitivity and uterine PG production in the pregnant rat uterus and the effects of OT antagonists on the uterotonic action and PG-releasing action of OT in the pregnant rats.

Results and Discussion

OT sensitivity was determined in pregnant rats on Day 19, 20, 21 and 22 of the gestation period. The rats were divided into two groups. One group served as controls and the other group as experimentals. The experimental group was treated with diclofenac sodium, a water soluble potent PG synthesis inhibitor, 1.0 mg/rat orally twice a day for two and a half days to suppress endogenous synthesis of PGs. The control group was given water under the same protocol.

Uterine contractile responses to 40 mU and 80 mU of OT i.v. were measured in each rat on either Day 19, 20, 21 or 22 of pregnancy. Day 19 and 20 pregnant rats showed only minimal responses to these two doses of OT. In the control rats, there was an abrupt increase in OT sensitivity on Day 21 and 22 of pregnancy. In the experimental group, in which endogenous PG synthesis had been suppressed, the increase in OT sensitivity on Day 21 and 22 was greatly attenuated. Figure 1 shows the uterine responses to OT in two control rats and two experimental rats.

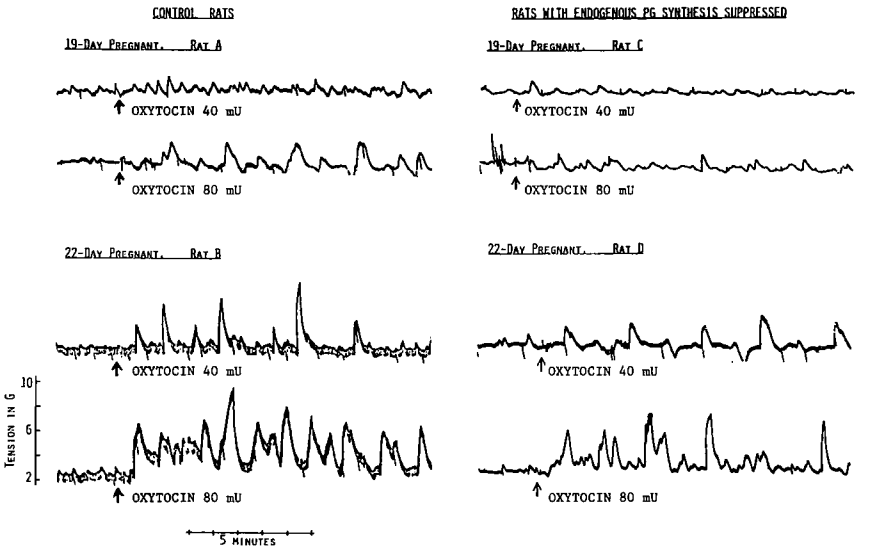

Fig. 1. Development of OT sensitivity in the term pregnant rats and the effects of inhibition of PG synthesis on OT sensitivity. Uterine contractions were recorded in vivo with an isometric force-displacement transducer. Each rat was given two doses of OT, 40 and 80 mU i.v. Note the marked increase in OT sensitivity in the Day 22 pregnant control rat (Rat B vs Rat A). In rats in which endogenous PG synthesis had been suppressed (Rats C and D), OT sensitivity was reduced.

In a separate series of experiments, uterine and placental tissues were removed for the measurement of tissue contents of $PGF_{2\alpha}$ and PGE_2. Tissue samples were homogenized in isopropyl alcohol with a Polytron homogenizer. An equal volume of phosphate buffer, pH 7.4, was added to the homogenate. After centrifugation, the alcoholic supernatant was

washed with hexanes, acidified to pH 4.0 and extracted for PGs with chloroform. The chloroform extract was dried to a residue and its $PGF_{2\alpha}$ and PGE_2 contents determined by radioimmunoassays.

In control rats, there was a 3 to 4-fold increase in uterine tissue concentration of $PGF_{2\alpha}$ on Day 21 and 22. Uterine concentrations of PGE_2 did not change significantly over Day 19 to Day 22. In the experimental rats in which endogenous PG synthesis had been suppressed, there was no increase in $PGF_{2\alpha}$ on Day 21 and 22.

Placental tissue concentrations of $PGF_{2\alpha}$ and PGE_2 did not vary significantly over the four days measured. Therefore, only $PGF_{2\alpha}$ levels were found to relate to the development of OT sensitivity.

The effects of OT antagonists on OT-induced uterine contractions were studied in intact term pregnant rats. Two highly potent OT antagonists, [Pen^1, Phe^2, Thr^4]OT and [Pen^1, Phe^2, Thr^4, $\Delta^{3,4}$-Pro^7, Orn^8]OT with *in vitro* pA_2 values of 7.35 and 7.45 respectively, were found to be effective *in vivo* OT inhibitors. These two OT antagonists produced a dose-dependent inhibition of the OT-induced uterine contractions in the Day 22 and 23 pregnant rats. The uterotonic response to 80 mU of OT was nearly completely inhibited by 10 to 20 µg of the antagonists. In our previous work, we have shown that [Pen^1, Phe^2, Thr^4]OT also inhibited the PG-releasing action of OT.[2] Our work-in-progress suggests that this may be a common effect shared by OT antagonists.

The duration of inhibitory action of [Pen^1, Phe^2, Thr^4]OT and [Pen^1,-Phe^2, Thr^4, $\Delta^{3,4}$-Pro^7, Orn^8]OT was determined in the term pregnant rat. The response to 50 mU of OT was first determined. Inhibition of the OT response was then produced by 5 µg of the antagonists. After the initial inhibition of OT response, the rat was injected 50 mU OT every 30 min until the uterine contractile response had recovered to greater than 50% of the control value. Figure 2 shows the recovery time for these two OT antagonists. All four rats receiving [Pen^1, Phe^2, Thr^4]OT recovered nearly 100% of the control response at the next injection interval, that is 30 minutes post injection of the antagonist. For the five rats receiving [Pen^1, Phe^2, Thr^4, $\Delta^{3,4}$-Pro^7, Orn^8]OT, similar recovery was not attained even after 120 minutes. The estimated time for 50% recovery was 70 minutes.

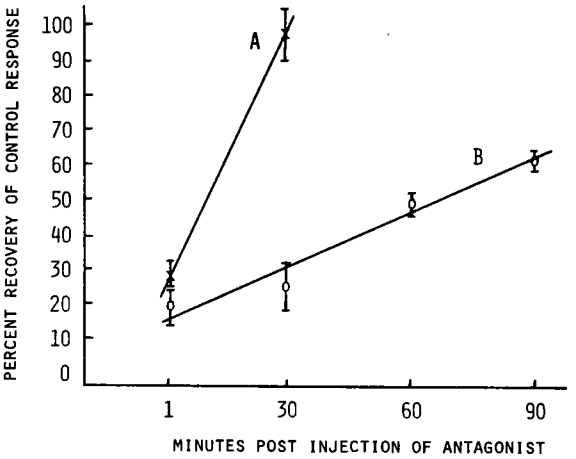

Fig. 2. Recovery of OT uterotonic response following a single injection of OT antagonists. A: [Pen^1, Phe^2, Thr^4]OT, average values for 4 rats. B: [Pen^1, Phe^2, Thr^4, $\Delta^{3,4}$-Pro^7, Orn^8]OT, average values for 5 rats.

The parturient uterus is characterized by increases in OT sensitivity and OT receptor concentration.[3,4] Our study here shows that the development of OT sensitivity is dependent on the capacity of the uterus to generate PG, principally $PGF_{2\alpha}$. Both the uterotonic action and the PG-releasing action of OT could be blocked by OT antagonists. Structural modifications of OT analogs could yield antagonists with greater potency as well as longer duration of action. Such OT antagonists may have potential as tocolytic agents for the treatment of preterm labor.

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NEW LUTEINIZING HORMONE-RELEASING FACTOR ANTAGONISTS

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Introduction

In our studies of LRF agonists and antagonists we focused on the effects of substitution by very hydrophobic, unnatural amino acids on the potency and pharmacokinetics of these compounds.[1] The 3-(2-naphthyl)-D-alanine residue [D-Nal(2)] provided particularly potent agonistic ([D-Nal(2)^6]LRF; 200x LRF)[2] and antagonistic (1) analogs.[1] High potency and prolonged duration were reported[3] for an analog containing D-Arg (2). The higher potency of 2 in corn oil vehicle indicated to us that increased hydrophobicity could be beneficial, and we have prepared analogs containing N^G,$N^{G'}$-dialkyl-homo-Arg, residues capable of electrostatic and hydrophobic interaction.

Synthesis

The D-hArg(R_2) derivatives can be prepared from Z-D-Orn-OBzl or Z-D-Lys-OBzl by reaction with the corresponding N,N'-dialkylcarbodiimide (toxic) in t-BuOH. Most analogs contained D-hArg(R_2) analogs because of the lower cost of D-Lys, but some D-Arg(R_2) derivatives were made. Boc-D-hArg(R_2)-OH

derivatives were incorporated without side chain protection; R = Me, Et, Pr, hexyl, i-Pr, c-hexyl. Cleavage and deprotection from BHA-resin by liq. HF (0°, 1 hr) resulted in complete dealkylation of the D-hArg(c-hexyl$_2$) side chain (only D-hArg products obtained), while D-hArg(i-Pr$_2$) yielded D-hArg(i-Pr$_2$) and D-hArg(i-Pr) products. Alkyl chains with a primary carbon attached to the guanido function (R = Me, Et, Pr, hexyl) gave good yields of the desired dialkyl products (Table I).

Results and Discussion

HF deprotection of a D-hArg(c-hexyl$_2$) analog unexpectedly gave the D-hArg analog 4, which was essentially equipotent to the standard 2 (Table I), showing that side chain length is not critical in position 6. The D-hArg(Me$_2$) analogs (5,6) were as potent as their parent structures (2,3) in the antiovulatory assay, but 5 and 6 exhibited substantially prolonged activity in the castrated adult male rat LH suppression assay (Figure 1; 5 not shown). The D-hArg(Pr$_2$) analogs (12-14) are slightly less potent (Table I), both acutely (PE) and by duration of action (D_{II} dosing). The D-hArg(Et$_2$) analogs (7-11) are all very potent antagonists and 7, 9-11 exhibit prolonged activity, reflected by their D_{II} data. All are substantially longer acting than the standards (2,3) in the LH suppression assay in castrated adult male rats (Figure 1). Compound 11 is the most potent analog that we have tested, both acutely and by D_{II} dosing. This duration is especially dramatic in the LH suppression assay (Figure 1), where this analog causes full suppression at 24 hr at the dose shown. At a 25 µg/kg dose of 11 the degree of suppression at 24 hr is not significantly different (LH level 36%) from that shown at 50 µg/kg. It is estimated from these data that at least a 5-fold higher dose of the standard (2) is needed to achieve the same degree of suppression at 24 hr in this assay. Compound 11 is clearly

Table I. Potency of LRF Antagonists -- Rat Antiovulatory Assay

	Analog	Time (Vehicle)	ED_{50} (μg) PE[a] (p.g.)[b]	PE[a] (c.o.)[b]	D_{II}[a] (c.o.)[b]
1	[N-Ac-Pro^1,D-pF-Phe^2,D-Nal(2)^{3,6}]LRF[c]		2.2	3.3	140
2	[N-Ac-D-pCl-Phe^1,D-pCl-Phe^2,D-Trp^3,D-Arg^6,D-Ala^{10}]LRF[d]		1.7	1.2	5
3	[N-Ac-D-Nal(2)^1,D-pF-Phe^2,D-Trp^3,D-Arg^6]LRF[c,e]		2.4	1.1	9
4	[N-Ac-D-pCl-Phe^1,D-pCl-Phe^2,D-Trp^3,D-hArg^6,D-Ala^{10}]LRF		1.3	2.0	<8
5	[N-Ac-D-pCl-Phe^1,D-pCl-Phe^2,D-Trp^3,D-hArg(Me$_2$)^6,D-Ala^{10}]LRF		1.6	1.0	5.6
6	[N-Ac-D-Nal(2)^1,D-pF-Phe^2,D-Trp^3,D-hArg(Me$_2$)^6]LRF		1.7	0.8	5.0
7	[N-Ac-D-pCl-Phe^1,D-pCl-Phe^2,D-Trp^3,D-hArg(Et$_2$)^6,D-Ala^{10}]LRF		1.7	1.6	3.3
8	[N-Ac-D-pCl-Phe^1,D-pCl-Phe^2,D-Trp^3,D-hArg(Et$_2$)^6]LRF		2.0	2.4	16
9	[N-Ac-D-Nal(2)^1,D-pF-Phe^2,D-Trp^3,D-hArg(Et$_2$)^6]LRF		1.6	1.4	3.5
10	[N-Ac-D-Nal(2)^1,D-pCl-Phe^2,D-Trp^3,D-hArg(Et$_2$)^6]LRF		2.0	1.0	2.5
11	[N-Ac-D-Nal(2)^1,D-pCl-Phe^2,D-Trp^3,D-hArg(Et$_2$)^6,D-Ala^{10}]LRF		0.7	0.5	2.5
12	[N-Ac-D-pCl-Phe^1,D-pCl-Phe^2,D-Trp^3,D-hArg(Pr$_2$)^6,D-Ala^{10}]LRF		2.6	2.0	14
13	[N-Ac-D-pCl-Phe^1,D-pCl-Phe^2,D-Trp^3,D-hArg(Pr$_2$)^6]LRF		2.6	2.3	
14	[N-Ac-D-Nal(2)^1,D-pF-Phe^2,D-Trp^3,D-hArg(Pr$_2$)^6]LRF		2.4	4.7	12

[a]Time of dosing: noon on proestrus (PE) or 24 hr earlier at noon on diestrus II (D_{II}). [b]Vehicle was 40% propylene glycol/saline (p.g.) or corn oil (c.o.). [c]Nestor, et al., 1983.[1] [d]Coy, et al., 1982.[3] [e]Rivier, et al., 1983.[4]

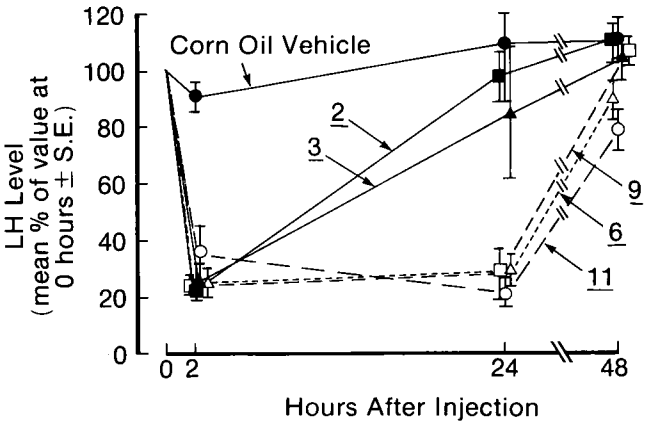

Figure 1. LH levels in castrated male rats following injection of LRF antagonists at 50μg/Kg. (n = 11 in vehicle group and 5 or 6 in antagonist groups)

one of the most potent LRF antagonists yet reported, and the clinical pharmacology of this compound will be explored.

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PRINCIPLES OF ACTIVE SITE FORMATION IN PEPTIDES AND PROTEINS

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An analysis of interaction between peptide/protein ligands and receptor molecules in the cell has been conducted by us with recourse to three principles of information theory - those of signature, ambiguity and equivocation, respectively[1-2]. According to the signature principle, molecular interaction is determined by the sets of properties (signatures) possessed by the active sites (signature carriers) in the electronic structures of molecules. What properties of the ligand are exactly responsible for interaction is determined by the structure of the second component in the reaction, e.g. the cell receptor. This implies the possibility of finding selectively acting analogues of natural ligands. A molecule has signatures corresponding to each of its functions. As any given molecule may carry a multitude of signatures, this leads to the uncertainty of elicited effects (principle of ambiguity). Under certain conditions, molecules varying as to their structures may carry similar signatures and fulfill similar functions (principle of equivocation). The signature and equivocation principles add to the understanding of equifunctionality of structurally different amino acid residues. For instance, Arg, Lys, Gln and Asn are equifunctional with respect to their propensity to form hydrogen bonds.[1]

The structure-function analysis of peptide and protein bioregulators carried out on the basis of these principles has led to the identification of equifunctional "common fragments" in the structures of various peptide and protein ligands.[1] A characteristic feature of such fragments is the presence of a basic amino acid or aminodicarboxylic acid amide (Arg, Lys,

Gln, Asn) located next to one or several Pro, Val or Gly residues. The structure of these fragments may be depicted as Gly/a↔b↔Pro/Val↔Pro/Val (type I) and b↔Pro/Val↔b↔Pro/Val (type II), where a is an amino acid with a free COOH group, and b is a basic amino acid or aminodicarboxylic acid amide. The most typical structure element of the common fragments is the dipeptide unit b↔Pro/Val. Given below are several examples of common fragments (numbers of terminal amino acids are indicated):

vasopressin 9-7	GKP	ß-MSH 18-15	DKPP	substance P 1-4	RPKP
α-MSH 10-13	GKPV	angiotensin 1-3	DRV	fibrinopeptide B 13-16	RPKP
wasp kinin 9-12	GRPP	insulin 30-28	EKP	BPP-B 9-6	KPRP
ACTH 18-20	RPV	ranatensin 4-2	QPV	BPP-V-c_2^e 6-3	QPRP
BPP-A 3-5	RPP	evolidin 1-6	NPV	Naja Naja oxiana toxin 46-43	KVKP

In 1979, Galaktionov obtained preliminary data on the space structure of bradykinin using the method of total semiempirical conformation analysis in vacuo.[3,4] Later, steric structure calculations were performed for angiotensin, bradykin potentiating peptide, tuftsin and other peptides containing common fragments or structural elements[3,5], and it was found that stable conformations of these compounds are quasicyclic, and that (see Figure) stabilization of the quasicycles is attained by interaction between a basic amino acid (or aminocarboxylic acid amide) and a carboxylic or amide group of the C-terminal. Quasicyclization results in unique three-dimensional structure, and its profile may correspond to the "pocket" of the active site on the receptor molecule. This provides a high selectivity of recognition and binding and drastically decreases conformational entropy. The above calculations led to the synthesis of biologically active cyclic analogues of linear peptides.[6] Examples of this type of structures are given below for different series of analogues: bradykinin (I, IV, VI), kallidin (II, III, V), angiotensin (VII - IX), corticotropin and melanotropin (X, XI), tuftsin (XII - XVI), neurotensin (XVII - XXI), substance P (XXII - XXIII),

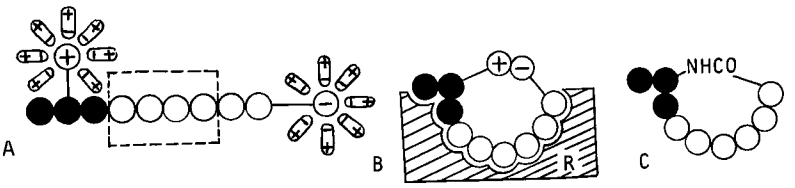

Fig. 1 Models of peptide structures. A - primary peptide structure. Common fragment location is indicated in black, location of the specific active site is framed. Hydration of the charged groups is shown. B - quasicyclic structure of ligand bound to the cell receptor. C - cycloanalogue of the linear peptide, where the ionic bond is replaced by the peptide one.

enkephalin (XXIV, XXV) and wasp kinin (XXVI, XXVII):

KPPGFGFFR	(I)	KVYVHPF	(VII)	TKPR	(XIV)	KPYILG	(XX)
RKPPGFGPFR	(II)	OrnVYVHPG	(VIII)	TKPRG	(XV)	KRPYILG	(XXI)
KRPPGFSPFR	(III)	KRVYIHPF	(IX)	TOrnPRG	(XVI)	KFFGLM	(XXII)
RPPGFSPFR	(IV)	KHFRWG	(X)	KTKPR	(XVII)	KQFFGLM	(XXIII)
KRPPGFSPFR	(V)	KHFRWGG	(XI)	KPYIL	(XVIII)	YKGFM	(XXIV)
RPPGFSPFR—$CO(CH_2)_{11}NH$	(VI)	KPR (XII) TKPR	(XIII)	RKPYIL	(XIX)	YKGFL	(XXV)
		KKKLRGKPPGFGPFR	(XXVI)	KKKKPPGFGPFR			(XXVI)

The biological studies carried out with the cycloanalogues have found that a sharp differentiation of biological properties often occurs following cyclization. For example, a characteristic, although not general, feature is the prolongation of the biological action of cyclopeptides as compared to their linear precursors. Some cycloanalogues (I-IV, IX, etc.) were active in vivo for several hours, whereas their linear counterparts were destroyed during some minutes.

Many quasicyclic structures of peptides include about 6-8 amino acid residues.[7] For example, carboxypepidase A, papain, α-chymotrypsin, and protease B are known to interact with 5, 7, 6 and 7 amino acids of the substrate, respectively. The active sites of inhibitors of the trypsin-type enzyme comprise about 6-8 amino acid residues regardless of the molecule size.[7] The same is true of antigen-antibody interaction. For instance, the antigenic determinants of myoglobin correspond to the peptide chain fragments with amino acid sequence 15-22 (8 amino

acid residues), 56-62 (7), 94-99 (6), 113-119 (7) and 145-151 (7), those of ferredoxin 1-7 (7) and 51-55 (5).[8] Hence, it appears that the specific interaction with receptor molecules is provided by the "active" site on the ligand comprising, on the average, 6-8 amino acid residues. Characteristically, the functionally active disulphide cycles are comparable, with respect to size, with quasicycles.[1]

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STRUCTURAL REQUIREMENTS FOR LH-RH MURAMYL DIPEPTIDE (MDP) CONJUGATES IN INDUCING IMMUNOLOGICAL CASTRATION.

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Introduction

Immunization against LH-RH (luteinizing hormone releasing hormone) obtained by administration of this decapeptide coupled to a carrier and given with Freunds complete adjuvant (FCA) can inhibit gonadal function in male and female (1). Recently, we demonstrated that immunological castration of male mice can be obtained without carrier and FCA (2) by immunization with LH-RH directly coupled to synthetic N-acetylmuramyl-L-Alanyl-D-Isoglutamine-L-Lysine (MDP-Lys). MDP is the minimal adjuvant active structure of mycobacteria in FCA (3), and has been shown an effective adjuvant in several models of conventional or synthetic vaccines (4,5). In the present report we compare the activities of conjugates obtained by coupling MDP or MDP-Lys to LH-RH-COOH or (2-10)LH-RH. Our results demonstrate that both conjugates are devoid of hormonal activity when tested on

pituitary glands *in vitro*. In mice, both were capable of inducing a marked decrease in testicular testosterone levels. However, LH-RH-MDP-Lys was far more active than MDP-(2-10)LH-RH in effecting castration.

Results

LH-RH-COOH was coupled to MDP-Lys and (2-10)LH-RH to MDP using a mixed anhydride method as described by Naithani (6). The resulting compounds were : pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-CO-NH-Lys-DisoGln-Ala-MurNac and MurNac-Ala-DisoGln-CO-NH-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2. These conjugates were tested *in vitro* for their capacity to induce the release of LH in a superfusion system according to a method previously described (7).

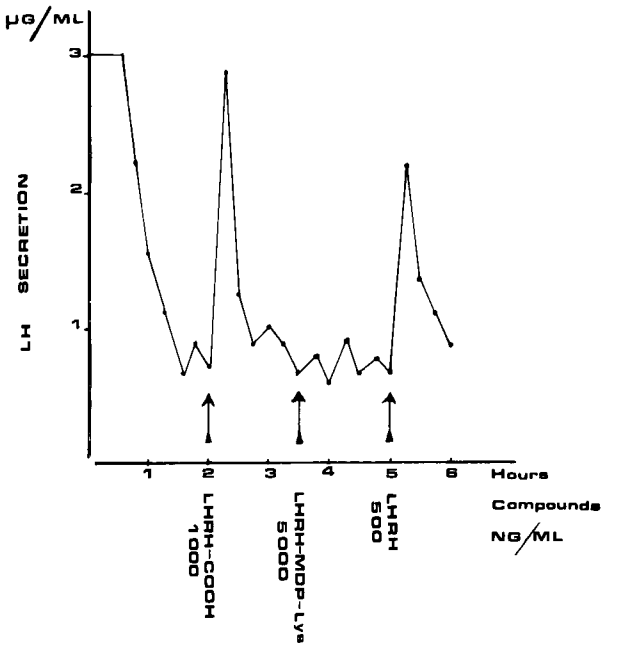


Figure 1. Pituitary response to LH-RH-MDP-Lys.

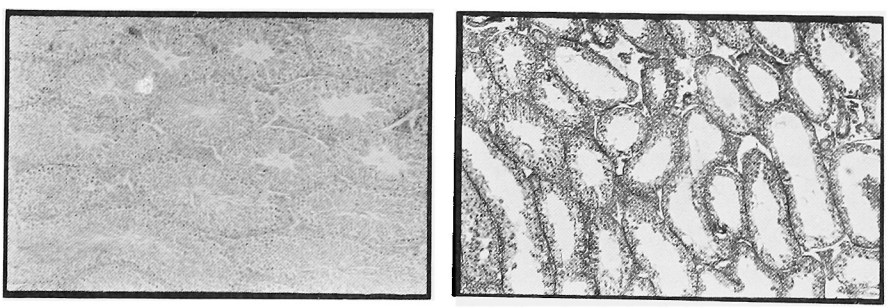
The anterior hemipituitaries of rats were perfused with medium containing control or experimental compounds and LH was measured in the effluent by a radioimmunoassay. The results obtained with LH-RH-MDP-Lys compared to LH-RH-COOH and LH-RH are schematically represented on Figure 1. The same loss of hormonal activity was obtained with MDP-(2-10)LH-RH.

To test the immunogenic activity of these compounds *in vivo*, male swiss mice were immunized subcutaneously at two day intervals with 50 µg or 10 µg of conjugates in saline. After one month, they received a boost of LH-RH or (2-10)LH-RH at the same dosages. Two months after primary immunization mice were sacrificed and the level of testicular testosterone was determined by radioimmunoassay (8). Results are shown in Table 1.

Table 1. Testosterone level in ng per testis

Groups	PBS	LH-RH-MDP-Lys		MDP-(2-10)LH-RH	
Dose	-	50µg	10µg	50µg	10µg
Testosterone Level	160	40	90	72	10

A significant decrease in all conjugate treated groups as compared with controls could be observed. The most impressive result was obtained with the low dose of MDP-(2-10)LH-RH.



a. b.

Figure 2. a. Testis from control mouse. b. Testis from mouse immunized with LH-RH-MDP-Lys.

Histological examination confirmed these findings since, unlike control animals (Fig. 2.a.), the two groups treated with LH-RH-MDP-Lys exhibited marked atrophy of the seminiferous tubules and absence of spermatozoides in the lumen (Fig. 2.b.).

The effect was less marked in groups treated with MDP-(2-10) LH-RH since spermatozoides were still present in many tubules. Nevertheless, even in these animals, spermatogenesis was affected as indicated by the delay in or complete lack of spermatocyte maturation.

Conclusion

These results demonstrate the critical importance of the structure of the adjuvant LH-RH conjugate in obtaining an efficient synthetic vaccine.

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SYNTHETIC FIBRIN-LIKE PEPTIDES USED AS ANTIGENS YIELD FIBRIN-SPECIFIC ANTIBODIES

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Thrombus detection remains an important clinical problem. The use of antibodies as agents for the in vivo localization of fibrin deposits has been hampered by antifibrin cross-reactivity with fibrinogen, the circulating fibrin-precursor. Blood clots when thrombin cleaves two pairs of small peptides from fibrinogen to yield fibrin monomers. Spontaneously, fibrin monomers aggregate to form an insoluble gel which is then covalently stabilized by Factor XIIIa. Despite the dramatic gelation, fibrin retains 98% of the original covalent structure of fibrinogen. Thus it is understandable that antifibrin sera cross-react strongly with fibrinogen, and that there has been only one isolated report of a fibrin-specific serum.[1]

Results and Discussion

Rather than examining antibodies elicited by the entire fibrin molecule with all the epitopes it shares with fibrinogen, we chose to focus the immune response on synthetic fibrin-unique peptides. We anticipated that antibodies which recognized a synthetic fibrin epitope might bind to fibrin exclusive of fibrinogen.

Synthesis of cross-linked fibrin-like peptide. Rationaliz-

ing that the Factor XIIIa cross-link site of fibrin γ-chains would represent a fibrin-unique epitope, two peptides containing ε-(γ-Glu)Lys were synthesized. The first cross-link peptide was assembled by solid-phase segment condensation of two 7-peptides; the second by cyclization of a linear 12-peptide with diphenylphosphorylazide.[2] Fast atom bombardment mass spectrometry was used advantageously to characterize the cyclic fibrin-like peptide and to verify the amino acid sequence of its linear precursor. Unfortunately, mice immunized with the cross-linked 14-peptide produced only low levels of antipeptide antibodies which were completely inhibited by human fibrinogen.

Synthesis of linear fibrin-like peptides. Rationalizing that the amino termini exposed by thrombin cleavage would also be unique to fibrin, two linear 10-peptides from the amino termini of human fibrin α- and β-chains were synthesized.[3] The fibrin-like peptides were purified by carboxymethylcellulose chromatography, conjugated to keyhole limpet hemocyanin and used for the immunization of rabbits. The antisera derived from both the α- and β-fibrin peptides could be rendered specific for fibrin by immunoadsorption with human fibrinogen-Sepharose. The resulting specifically purified anti-α and anti-β peptide sera were devoid of antihuman fibrinogen activity as expected, but retained their original antihuman fibrin immunoreactivity.[4]

Based upon these results a related linear fibrin-like peptide was synthesized for preparation of monoclonal antibodies. Using the Merrifield solid-phase method,[3] the first seven amino acids of the fibrin β-chain were assembled on Cys(Dmb)-OCH_2-resin in a stepwise fashion. A sample of the completed peptidyl-resin, Gly-His(Tos)-Arg(Tos)-Pro-Leu-Asp(Bzl)-Lys(2-Clz)-Cys(3,4-Dmb)-OCH_2-resin, was evaluated by quantitative solid-phase Edman degradation. The resulting sequencing data: (1) confirmed that the desired amino acid sequence was pres-

ent, and (2) indicated that the average level of amino acid deletions, expressed as percent preview per cycle[5] was less than 1%. Following anhydrous hydrogen fluoride treatment, the fully deprotected peptide, which gave an acceptable amino acid composition (± 5%), was coupled to maleimidobenzoyl-KLH[6] and used to immunize BALB/c mice. Six weeks after primary immunization, immune spleen cells were harvested and fused with Sp2/0 myeloma cells to yield eventually three independent hybridoma cell lines.[7] In the screening immunoassay, human fibrin monomer was used as solid-phase bound antigen. Before testing culture supernatants were mixed with fibrinogen as inhibitor. Thus, only those hybridomas which produced fibrin-specific monoclonal antibodies were selected.

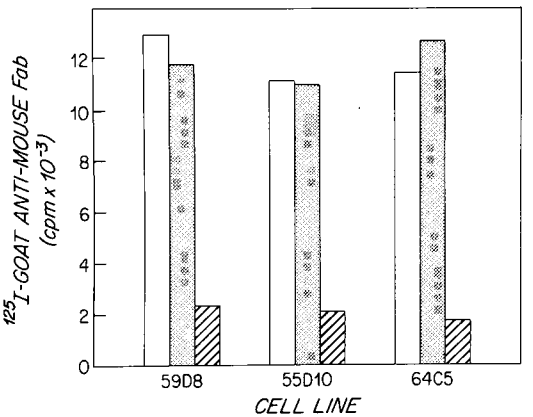

Fig. 1. Binding of monoclonal antibodies to clotted fibrin.

Because fibrin clots are polymers of fibrin monomers, it was important to determine whether clots would be also recognized by the monoclonal antibodies. Thus, an in vitro fibrin disc assay was devised. On 9.5-mm discs of filter paper, human fibrinogen (40 µg, Kabi L grade) was clotted by adding human thrombin (Sigma, 1.25 U) in the presence of $CaCl_2$ and Trasylol. When such fibrin discs were used to test each monoclonal antibody, fibrin clot recognition could be confirmed as shown in Figure 1. Each monoclonal antibody as detected by a radio-

iodinated second antibody bound well to clotted fibrin discs (open bars) as compared to buffer controls (hatched bars). Importantly, this binding was unaffected by human fibrinogen at a concentration of 4 mg/ml (stippled bars).

In summary, immunization with a fibrin-like peptide which contains the γ-chain cross-link site has yielded antisera which are fully cross-reactive and therefore not useful. On the other hand, linear fibrin-like peptides which corresponded to the amino termini of the α- and β-chains of fibrin have provided both polyclonal and monoclonal fibrin-specific antibodies. It is significant that the monoclonal antibodies were capable of binding to fibrin polymer as well as fibrin monomers. The potential for applying these monoclonal antibodies for thrombis detection in a clinical setting is of considerable interest. (Supported by NIH Grant HL-28015).

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APPROACH TO A SYNTHETIC VACCINATION AGAINST CHOLERA

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Introduction

Vibrio cholerae secretes a toxin (CT) which causes severe diarrhea in human (1). CT is composed of two subunits A and B. Subunit A possesses the adenylate cyclase-stimulating activity, whereas subunit B, composed of five β chains, contains the membrane binding sites to the ganglioside GM_1 of intestinal cells (2). In order to prevent the action of CT, we assumed that a short specific sequence of β chain including residues involved in the toxin binding process could: a) elicit antibodies able to inhibit CT binding, b) stimulate local immune response, and c) compete with CT on cell membrane receptors.

Fragments corresponding to sequence 30-50 β (I) and 50-75 β (II) were synthesized:

A*-S-L-A-G-K-R-E-M-A-I-I-T-F-K-N-G-A-T-F-E-V-COOH (I),

A*-V-E-V-P-G-S-Q-H-I-D-S-Q-K-K-A-I-E-R-M-K-N-T-L-R-I-A-COOH (II)

14C Ala was added in the N-terminal position in order to facilitate the monitoring of the purification process. Both peptides are located in a random structure area, as predicted by the method of Chou and Fasman; one contains 35 Arg, essential in the binding process according to Duffy and Lai (3), and both are hydrophilic enough to represent good immunogenes (4).

Results

Synthesis: Both sequences were synthesized by the solid phase procedure of Merrifield with a semi-automatic peptide synthesizer SAP IV (Sempa-Chimie, France). After chromatographic purification, the free peptides were pure by TLC, HPLC, polyacrylamide gel electrophoresis and amino-acid analysis.

Biological properties: Only 30-50 β containing 35 Arg involved in the cell membrane binding process (3) bound on human erythrocytes (Table I).

Ala*30-50 β	Ala*50-75 β	Ala*44-68 hPTH	Ala*Ala 186-201 Diphteria toxin
13%	5%	2%	1%

Table I : Fixation of different 14C labeled peptides on human erythrocytes, results are expressed in remaining radioactivity on erythrocytes after overnight incubation.

Furthermore in excess quantities in inhibited the overproduction by CT of mice adrenal cell tumors (Y_1) secreted steroids consecutive to activation of adenylate cyclase (TableII) (5).

Table II.

CT M	30-50 M	50-75 M	20α Dihydroprogesterone	%Inhibition
0	0		0.5 µg./ml.	
10^{-8}	0		2.2 "	
10^{-8}	$4 10^{-6}$		1.7 "	30%
10^{-8}	10^{-6}		1.30 "	53%
0		0	0.4 "	
10^{-8}		0	1.2 "	
10^{-8}		$4 10^{-6}$	1.3 "	0%
10^{-8}		10^{-6}	1.3 "	0%

Effect of 30-50 & 50-75 fragments on steroids synthesis by CT stimulated adrenal Cells(Y1) Incubation: 24 h. 37°
Steroids production was mesured fluorimetrically as µg. of 20α-dihydro-progesterone

Immunological properties :

1) Both IP, OP administration of 30-50 β or 50-75 β, without carrier or adjuvant, induced serum antibodies in mice;

2) Antibodies against 30-50 β or 50-75 β recognized CT, B and A subunits and LT enterotoxin of E. Coli, whereas anti CT antibodies poorly recognize these fragments, the A subunit and LT (6, 7);

3) Mice orally immunized with 50-75 β were protected against a CT challenge in the intestinal ligated loop test (Table III). This protection was not correlated with the amount of antibodies present in the serum or intestinal fluid.

CT	.191	.234	.210	.185	.213	.199
50-75 β	.079	.158	.050	.114	.050	

Table III : Intestinal loop challenge of immunized mice. C57 black mice were orally immunized with CT (5µg) or 50-75 β (5µg). Each animal was operated under slight ether anesthesia; around 10 cm of ileum was ligated 5 cm from the pylorus and CT (1µg./100µl buffer) was injected. 4 hours later, animals were sacrificed, the loop was dissected. Results are expressed in ratio of weight (g) on length (cm) of ligated loop. Control:buffer .060, TC .200

4) Some 50-75 β antisera presented vibriocidal properties against the Inaba strain, contrary to antisera raised against CT, 30-50 β.

Conclusion

Data reported here demonstate that short synthetic fragments of CT B subunit are able to compete with the whole toxin in its fixation process, to elicit antibodies in mice which recognize the whole toxin and to protect the animal against CT after several OP administrations.

Acknowledgments

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SYNTHETIC ANTIGENIC DETERMINANTS OF ROUS SARCOMA VIRUS pp60^{src} PROTEIN.

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Introduction

The calculatory assumption of Hopp and Woods[1] for the selection of antigenic determinants in a given protein structure is based upon the search for maximal hydrophilicity values. In these terms trifunctional amino acids like Arg, Lys, Asp, Glu contribute to the best. Previous synthetic experiences for peptides rich in those trifunctional residues induced the utilization of the same strategy for the construction of antigenic peptides potentially determining the antigenicity of the transforming phosphoprotein pp60^{src} from Rous Sarcoma Virus. Peptides identified to represent epitopic regions of the viral protein allow further insight into structure-function relations for the phosphorylation mechanism of pp60^{src} [2].

Materials and Methods

For the rapid synthesis of as many as possible potentially antigenic determinants we have used our carbonyldiimidazole/1-hydroxybenzotriazole method of activation of N^{α}-2-(3,5-dimethoxyphenyl)-propyl-2-oxycarbonyl-(Ddz) amino

Table I. Physico-Chemical Characteristics of Synthetic Peptides Comprising Structural Elements of the RSV pp60^{src} Phosphoprotein.

Sequence Position	Amino Acid Sequence	Hydrophilicity Value	Yields %	HPLC (min.)	$[\alpha]_{589}^{20}$ g)	
103-108	KKGERL	1.7	23	19,7 d)	-11°	(1)
155-160	RRESER a)	2.6	23	21,1 d)	-15°	(2)
175-178	RKSE a)	2.3	50	-	-01°	(2)
315-321	KKLRHEK	1.8	24	15,1 e)	-28°	(1,5)
316-321	KLRHEK	1.6	36	13,8 e)	-21°	(1,5)
318-321	RHEK	2.1	41	-	-36°	(1)
326-333	YAVVSEEP a)	0	70	26,5 f)	-96°	(0,6)
409-415	RLIEDNE	1.2	30	18,0	-22°	(2,5)
412-415	EDNE a)	2.3	60	-	-01°	(0,7)
419-427	RQGAKFPIK a)	0.5	27	25,0 e)	-09°	(0,5)
458-463	KGRVPY	0.4	44	13,1 b)	-13°	(2)
500-505	RKDPEE	3.0	40	14,0 c)	-60°	(3)
500-506	RKDPEER a)	2.1	61	-	-56°	(1)
501-506	KDPEER	2.5	42	-	-90°	(1,8)

a) Conventional synthesis in solution; HPLC gradient buffer A: 0.1 M KH_2PO_2, pH 2.1; B: acetonitrile, b)%B 0-60 (0-22 min.); c)%B 0-60 (0-38 min.); d)%B 0-80 (0-32 min.); e)%B 0-60 (0-27 min.); f)%B 0-45 (5-35 min.); detection 210 nm, coulmn RP 18 SERVACHROM 4 x 250 mm.g) optical rotation, (c) in H_2O.

acid tert.butyl derivates in the gel phase synthesis[3]. As presented in Table I, another portion of the selected structures has been successfully prepared in solution by excess Ddz-amino acid anhydrides[4]. The purified peptides have been bonded both to BSA and KLH by the aid of N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide with pH control for the direction of N-, respectively C-terminal anchorage of the peptides[2].

Table II. Immunogenicity of Synthetic Antigenic Determinants of RSV pp60^{src} Protein.

Sequence Position	Peptide Antigen	Hydrophilicity Value, Ø	Antiserum titer in ELISA	Peptide specific IgG isolated by affinity chromat. (µg/30mg IgG)	
				AH-Seph.	CH-Seph.
103-108	KKGERL	1.7	1 : 10000	1476	1408
155-160	RRESER	2.6	1 : 5000	2470	2120
315-321	KKLRHEK	1.8	1 : 10000	1796	1644
326-333	YAVVSEEP	0	1 : 100	170	0
409-415	RLIEDNE	1.2	1 : 100	500	370
419-427	RQGAKFPIK	0.5	1 : 500	939	85
458-463	KGRVPY	0.36	1 : 5000	1700	0
500-506	RKDPEER	2.5	1 : 5000	520	253

Results and Discussion

As presented in Table I, 14 peptides of RSV pp60^{src} structure have been obtained pure in 23-60% yield from polymer-supported operations and 23-70%, respectively, from synthesis in solution. N- and C-terminal conjugates of 8 peptides with KLH on simultaneous injection into rabbits resulted in antibody titers as presented in Table II. On isolation of the antibodies superior binding was observed when the antigenic peptides were C-terminally linked to the affinity gel. Cationic charges in the determinants, which are freely accessible in the solvating phase, obviously are more essential than lipophilic and anionic elements in triggering B lymphocytes for antibody production and for immune precipitation. Though identical residues terminate the structures of KKLRHEK (315-321) and RKDPEER (500-506), superior antibody binding by affinity gels as obtained from C-terminally anchored antigens, which exhibited cationic clusters to the solvating phase. Structures comprising good average hydro-

philicity most probably are of enhanced antigenicity, however, there is no linear correlation. The combination of calculatory guide lines from Chou and Fasman[5] together with those of Hopp and Woods[1] allowed us to select and synthesize short determinants, which were found to represent epitops of RSV $pp60^{src}$. Antibodies produced by the aid of KKLRHEK, RLIEDNE and RKDPEER, which do present cationic clusters to the solvating medium and are rich in helix inducing residues, did precipitate $pp60^{src}$ protein[2]. In addition, peptides KKGERL and RRESER though not inducing $pp60^{src}$ reactive antibodies, were recognized by immunoglobulins from RSV tumor bearing rabbits, suggesting that these two peptides as well are related (or identical) to epitopes of $pp60^{src}$. In conclusion, antigenic synthetic determinants do not necessarily need to be of extended length and of stabilized conformation to represent epitopes for antibody recognition[6].

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SMALL PEPTIDES OF THYMIC ORIGIN STIMULATE T-LYMPHOCYTE SUBSETS.

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Introduction

Previous studies in our groups on immunomodulatory activities of synthetic intermediates of thymosin-α_1 have shown that the N-terminal portion of the parent molecule exhibits stimulatory activities in the α-amanitine inhibited E-rosette assay and much less in the mixed lymphocyte culture, whereas C-terminal sections behave inversely[1-3]. These findings were judged as qualitative indications for a possibly structure dependent regulation of the immune balance expressed in T-lymphocyte subset proportions.We are searching for further support of our molecular signal hypothesis on the T-lymphocyte subset regulation[4].

Materials and Methods

C-Terminal sections of thymosin-α_1 are rich in charged side functions, whereas the serine and threonine residues are mainly located in a middle portion of the polypeptide.The region 17-28 of the parent molecule therefore was subdivided

into 11 overlapping peptide segments and investigated for structure-related immunomodulatory activities. 2 fragments from which we had synthesized thymosin-α_1, together with 1 fragment analog were included in this investigation. Details of the preparations are published elsewhere[1-3,5]. The samples were tested in three systems[3,5], the results are presented in Table I.

Table I. Comparison of Immunological In Vitro Activities of Small Synthetic Peptides Comprising Structural Elements of Thymosin-α_1 in Three Assay Systems.

Position	Sequence	$ER^{a)}_{Az}$	$MLC^{b)}_{\alpha\text{-Ama}}$	$ER^{c)}_{\alpha\text{-Ama}}$
17-24	KEKKEVVE	10^{-7}	58	37
18-24	EKKEVVE	10^{-5}	60	53
19-24	KKEVVE	IA	64	15
20-24	KEVVE	10^{-5}	56	31
21-24	EVVE	IA	49	35
20-25	KEVVEE	10^{-6}	28	42
20-26	KEVVEEA	IA	90	12
20-27	KEVVEEAE	10^{-6}	34	-
20-28	KEVVEEAEN	10^{-5}	51	15
25-27	EAE	IA	94	35
25-28	EAEN	IA	88	42
1'-6	KDAAVD	IA	28	66
Thy-α_1 [d)]	AcSDAAVDTSSEITTKDL--KEKKEVVEEAEN	10^{-6}	100	100

a) Azathioprine E-rosette inhibition assay;
b) α-amanitine inhibited mixed lymphocyte culture;
c) α-amanitine E-rosette inhibition assay;
d) synthetic thymosin-α_1.

Results and Discussion

The search for differentiated responses in the assays mentioned was indicated because of our finding[2] that the N-terminal hexapeptide of thymosin-α_1 modified by lysine instead of acetyl serine showed strong stimulatory activity in the α-amanitine inhibited E-rosette assay, but much less in the α-amanitine inhibited mixed lymphocyte culture and no response in the azathioprine E-rosette inhibition assay. This particular analog, Lys-Asp-Ala-Ala-Val-Asp (next to the last entry in Table I), already represented the basic-acidic-lipophilic sequence character extensively inspected in lit. 5. Six of 12 thymosin-α_1 derived peptides are inactive in the azathioprine E-rosette inhibition assay. This test is performed with spleen cells, which are known to have mainly cytotoxic characteristics. The same peptides are significantly (49%, compared to thymosin-α_1) or even very active (94%,compared to thymosin-α_1) in the α-amanitine inhibited mixed lymphocyte culture with human peripheral T cell populations. Because α-amanitine inhibits the protein biosynthesis in general and not selectively for specific cells, we can assume that all populations in this assay are maintained in an immature state of differentiation. Addition of those peptides which were found unable to restore spleen cell sensitivity to azathioprine, does sensibilize T cell populations for stimulation by foreign lymphocytes in the MLC. On the other hand, the same peptides by themselves and in absence of cellular antigens are scarcely active in the α-amanitine inhibited E-rosette assay (see entry 3,5,7,10,11 in Table I). In conclusion this means, in the presence of those peptides, only one specific subgroup can respond in the α-amanitine inhibited MCL triggered by allogeneic lymphocytes, most probably non-lytic T helper cells[6,7]. The opposite arguments can be used to explain the action of peptides in the $MLC_{\alpha\text{-Ama}}$ and

in the $ER_{\alpha\text{-Ama}}$, which were found as active as thymosin-α_1 in the azathioprine E-rosette inhibition assay, ER_{Az}. Obviously from this latter data, peptides containing the basic-acidic-lipophilic sequence character function as a soluble growth factor for cytolytic lymphocytes in the $MLC_{\alpha\text{-Ama}}$ in addition to stimulation by cellular antigens. This results in general proliferation (see $ER_{\alpha\text{-Ama}}$ data) paralleled by cytolytic action of CTL, which causes less than 50% increase of the counts as measured in the MLC. Investigations performed in our laboratory with an Ortho Cytofluorograph™, subset specific Orthomune™ antibodies and cell sorting as yet are not conclusive and therefore are not considered in this discussion.

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LARGE SCALE SYNTHESIS OF THYMOSIN α_1 BY FRAGMENT CONDENSATION USING TERT-BUTYL SIDE CHAIN PROTECTION

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Several thymic peptides have been shown to play roles in T-cell maturation.[1] Thymosin α_1 is a highly acidic N^{α}-acetyl 28-peptide, isolated from calf thymus gland,[2] chemically characterized and reported to exhibit biological activities involved in the development of thymus-dependent lymphocytes.[2] Syntheses of thymosin α_1 have been reported by solution[3,4] and solid phase[5-7] methods.

Synthesis of a 100 gram amount for clinical studies was carried out in solution for ready scale up using t-butyl side chain protection. Provisions were made to minimize racemization, optimize purification and allow analog preparation.

Seven segments (Figure 1, I-VII) were prepared by stepwise chain elongation using either the mixed anhydride (iBuOCOCl) or HOSu method. In each case N^{α}-Z and global side chain t-butyl protecting groups were used to permit selective deprotection of the former under catalytic hydrogenolysis conditions.

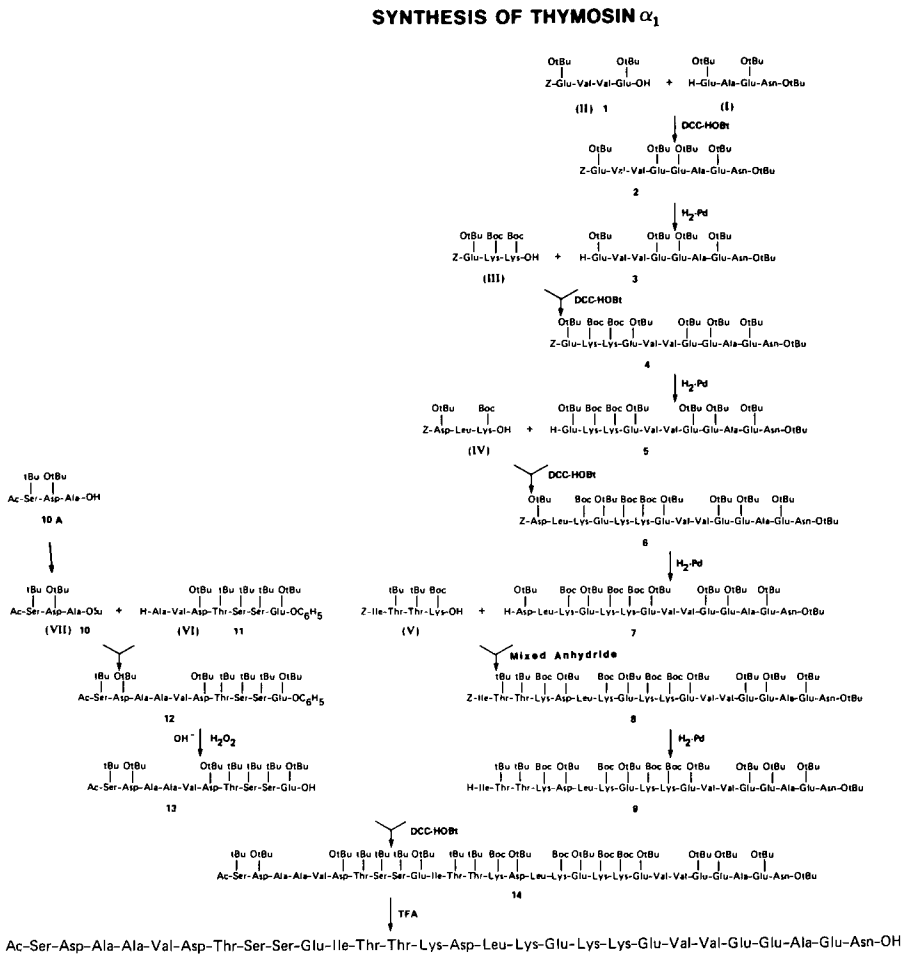

Fig. 1. Overall synthetic outline to thymosin α_1 via fragment condensation using tert-butyl side chain protection

Assemblage of the intermediate fragments led to the preparation of two key protected intermediates (9, 13), Figure 1.

Tetrapeptides I and II (Figure 1) were coupled by the DCC-HOBt procedure and the ensuing protected octapeptide (2) hydrogenated to produce 3. Coupling of 3 with Fragment III by the DCC-HOBt procedure afforded the protected 11-peptide, 4 (71.5%) Catalytic hydrogenolysis of 4 produced 5. DCC-HOBt coupling

of 5 with tripeptide IV gave the protected 14-peptide, 6 (78.6%). Hydrogenolysis of 6 in trifluoroethanol (TFE) gave 7 (97.8%) which was coupled with tetrapeptide V by the mixed anhydride procedure in DMF to produce the protected 18-peptide, 8 (93.2%). Final hydrogenation in TFE provided the key COOH-terminal octadecapeptide intermediate 9.

Coupling of fragments VI and VII provided the decapeptide phenyl ester 12 (91.7%). The phenyl ester group was cleaved by the procedure of Kenner and Seeley[8] in aqueous TFE to the NH_2-terminal decapeptide key intermediate 13 (98.8%).

Alternate coupling conditions of 9 and 13 were investigated to optimize the yield and purity of the fully protected 28-peptide, 14. The coupling was best achieved in TFE with 1.8 equiv. of 13 using DCC (5.6 equiv.) and HOBt (10.1 equiv.). The protected 28-peptide, 14, was readily purified by chromatography on silica gel and the product shown to be homogeneous (tlc) and in full agreement with the expected amino acid analysis, elemental analysis and nmr spectrum.

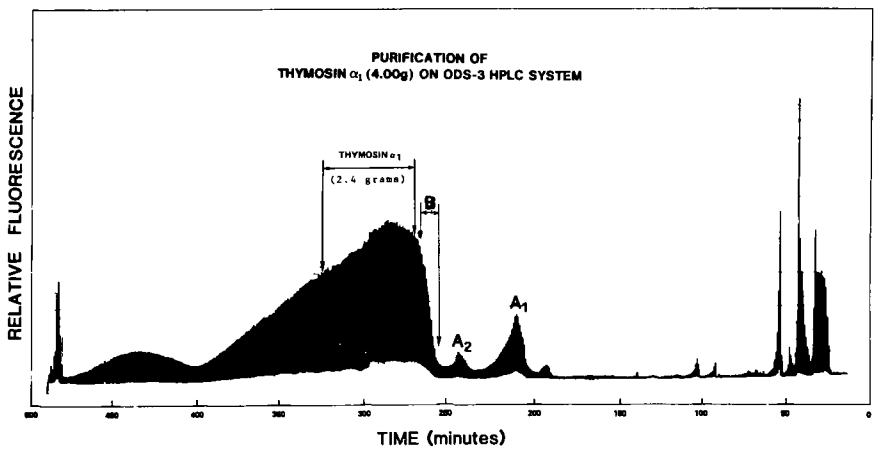

Fig. 2. Preparative HPLC of semi-purified thymosin α_1 on a (2x100 cm) C_{18} reversed phase column. Eluant: Pyr (3%): AcOH (3%) with a gradient of CH_3CN (0 to 13.25%).

Deprotection of the crude protected 28-peptide, 14, was carried out with 1:1 TFA-CH_2Cl_2. Preparative HPLC of the resultant thymosin α_1 was performed in 2 stages: (1) partial purification on a Jobin-Yvon Chromatospac Prep. 100 (8x100 cm) containing C_8 reverse phase [Eluant: pyridine (6%): acetic acid (2.25%) with a stepwise gradient of CH_3CN (0 to 1%)] and (2) final purification (~2.5g product per run) on the Whatman ODS-3 system (2x100 cm) with simultaneous discontinuous monitoring of each fraction with fluoropa which permitted pooling of appropriate fractions (Figure 2).

Three minor stereoisomer contaminants from the thymosin α_1 synthesis were also isolated during the preparative HPLC [Figure 2: products A_1, A_2 and B]. The stereoisomers were identified as D-Lys^{17}-thymosin α_1 (A_1); D-Lys^{14}-thymosin α_1 (A_2) and D-Ala^{3}-thymosin α_1 (B) resulting from trace racemization during the thymosin α_1 synthesis.

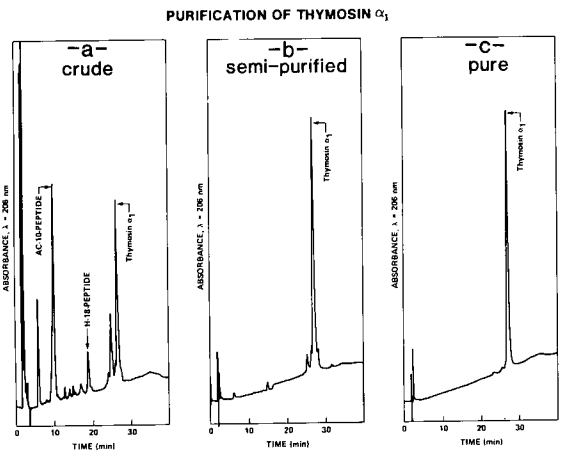

Fig. 3. Analytical HPLC on Bondapak C_{18}. Eluant: (0.022% TFA)—CH_3CN; Gradient: 4 to 24% (32 min); (a) crude thymosin α_1 (b) after preliminary purification (c) thymosin α_1 after final purification.

Analytical HPLC of thymosin α_1 at each stage of purification is shown in Figure 3. HPLC of the crude thymosin α_1 (Figure 3a) resulted in complete separation of all the contaminants [excess Ac-10-peptide (10 min); unreacted H-18-peptide (18.8 min); D-Lys^{14}-thymosin α_1 (24.8 min)] from thymosin α_1 (26.5 min).

The overall yield for the final 4 stages of thymosin α_1 synthesis was 30% and the product was shown to be homogeneous and in agreement with expected values (TLC, HVE, IEF, amino acid analysis, peptide mapping, ^{13}C-NMR and ^{1}H-NMR). Thymosin α_1 prepared by this procedure (nearly 100 grams) was obtained in microcrystalline form and released for clinical studies.

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A SYSTEM FOR THE STUDY OF PEPTIDE TRANSPORT ACROSS THE GUT

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Intestinal transport of di- and tri-peptides occurs,[1] but intact intestinal transport of larger peptides in adult animals is not established. We describe a system to investigate the intestinal transport of large peptides. An Ussing chamber, which allows separation of intestinal mucosal and serosal surfaces, was used to measure the rate at which a highly-labeled radioactive peptide was transported transmurally.[2] Transported material was characterized by HPLC. The nonapeptide studied (RI-52) has the amino acid sequence Pro-His-Pro-Tyr-His-Leu-Phe-Val-Tyr and is a known inhibitor of renin.[3]

Materials and Methods. Peptides were synthesized by solid phase techniques,[4] deprotected with HF, and purified by chromatography on Sephadex G-15 and Biogel P-2. Iodinated RI-52 was converted to the highly labeled peptide by catalytic reduction with tritium.[5] Homogeneity of products was determined by HPLC using a Beckman ODS C_{18}-semi-preparative column.

Transport of peptide was determined from the mucosal to serosal side of a rabbit jejunal epithelial sheet from which the serosa and muscularis propria had been removed. Tissue was mounted between two modified Ussing chambers and transmural movement of peptide was examined with and without 1,000 kIU Trasylol/ml added to the mucosal and serosal

surfaces. Approximately 100 nmol RI-52 (740 Ci/Mol) was added to the mucosal side and one ml samples removed at 20, 40, 60, and 80 minutes from the serosal and at 10 and 90 minutes from the mucosal side and stored at -80°. Identity of the radioactive material was inferred from a comparison of the radioactivity elution profile of the sample and authentic RI-52 and its potential catabolites, Tyr, Val-Tyr, and Leu-Phe-Val-Tyr.

Results and Discussion. Transported material had an HPLC retention time similar to that of RI-52 (Fig. 1) which suggests that intact nonapeptide was transported across rabbit jejunum. By 80 min approximately 0.1% of the nonapeptide crossed the jejunum.

Radioactive material was transported linearly over time, 20 minutes was required to reach a constant rate of flux (Fig. 2). Material transported after 20 minutes was quite homogeneous with a retention time similar to intact RI-52 (Fig. 1a,c). After 80 minutes the transported material was less homogeneous (Fig. 1b,d). The constant rate of transport probably represents multiple processes: a decreasing rate of nonapeptide transport due to its decreasing mucosal concentration, and increasing rates of transport of breakdown products.

Trasylol inhibited the degradation of nonapeptide (Fig. 1). The rate of radioactive material transported was decreased in the presence of Trasylol (Fig. 2). If transport of intact nonapeptide is slower than the transport of amino acids and di- and tri-peptides, the total amount of radioactivity transported by 80 minutes in the presence of Trasylol would be less, since a smaller concentration of more rapidly transported materials would be available for transport.

The mechanisms by which a nonapeptide may be transported intact are unknown. A carrier-mediated process similar to

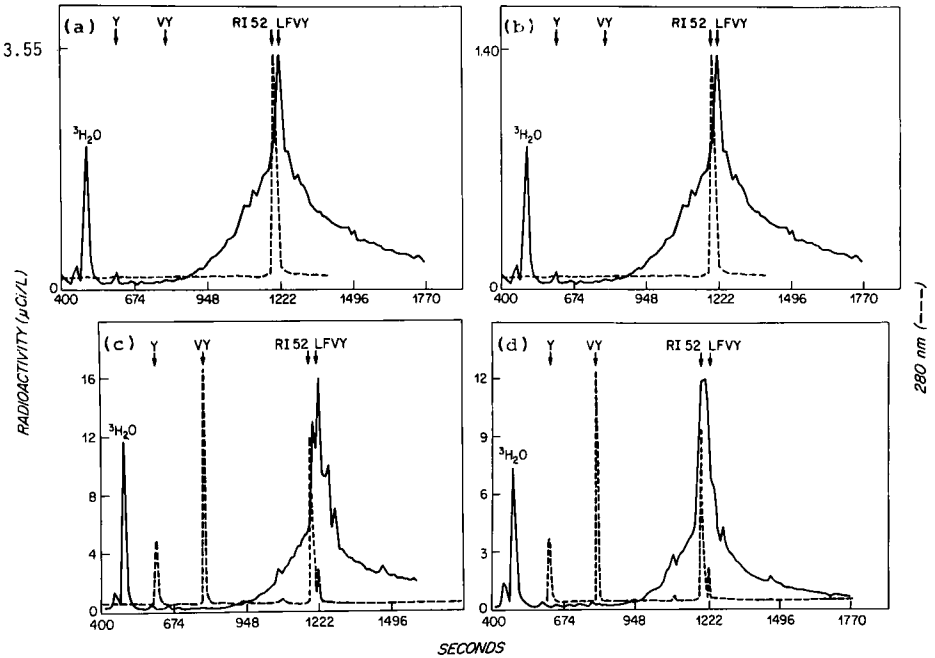

Fig. 1. HPLC's of radioactive material (——) transported from mucosal to serosal side: by 20 minutes in absence of Trasylol (a) and in presence of 1,000 kIU Trasylol/ml (b); by 80 minutes in absence of Trasylol (c); and in presence of 1,000 kIU Trasylol/ml (d). Co-chomatography (-----) of nonapeptide (RI52), Tyr (Y), Val-Tyr (VY), and Leu-Phe-Val-Tyr (LFVY) is superimposed. Retention time of ^3H_2O is indicated.

but separate from that used by amino acids and small peptides[1,6] would be unlikely. Endo- and exo-cytotic processes may play a role as they do in the intestinal uptake of proteins. A systematic investigation of the structural and chemical features of peptides which affect their intestinal transport should help suggest how biologically active peptide drugs might be developed which could be effective in oral preparations.

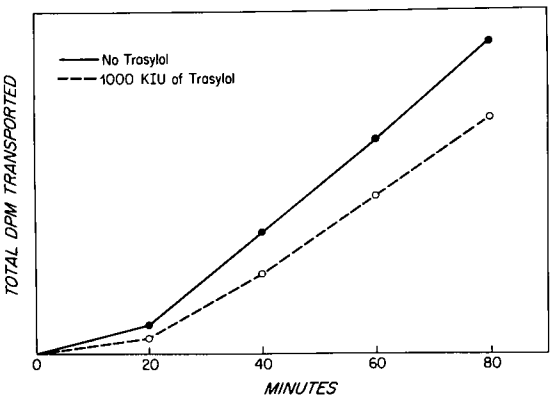

Fig. 2. Transport of radioactive material from mucosal to serosal side.

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