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SYNTHETIC AND MECHANISTIC STUDIES OF ASPARTIC PROTEINASE INHIBITORS. PEPSTATIN ANALOGS BASED ON SUBSTRATE SPECIFICITY.

BY

FRANCESCO G. SALITURO

A thesis submitted in partial fulfillment of the

requirements for the degree of

PHARMACY LIBRARY
SCHOOL OF PHARMACY

DOCTOR OF PHILOSOPHY

(Pharmacy)

at the

UNIVERSITY OF WISCONSIN-MADISON

1984

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PEPSTATIN ANALOGS BASED ON SUBSTRATE SPECIFICITY.

A thesis submitted to the Graduate School of the University of Wisconsin-Madison in partial fulfillment of the requirements for the degree of Doctor of Philosophy

by

PHARMACY LIBRARY
SCHOOL OF PHARMACY

FRANCESCO G. SALITURO

Degree to be awarded: December 19 84 May 19____ August 19____

Approved by Thesis Reading Committee:

Daniel H. Rich

Major Professor

7/28/84

Date of Examination

Phillip A. Hart

CR Hutchinson

Dean, Graduate School

SYNTHETIC AND MECHANISTIC STUDIES OF ASPARTIC PROTEINASE INHIBITORS.
PEPSTATIN ANALOGS BASED ON SUBSTRATE SPECIFICITY.

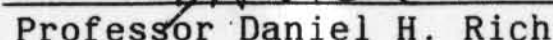
FRANCESCO G. SALITURO

(Under the supervision of Professor Daniel H. Rich)

The synthesis of a variety of structurally varied pepstatin analogs were described. The effects of structural variations in the P_2, $P_{2'}$ and $P_{3'}$ inhibitor subsites on inhibition of porcine pepsin were determined and the same done for the P_4, P_3, P_2, $P_{2'}$ and $P_{3'}$ on inhibition of penicillopepsin. P_1 analogs which resemble good penicillopepsin substrates were also synthesized and studied kinetically. These studies provided useful information concerning the difference in specificity between these two enzymes.

The x-ray crystal structure of a pepstatin fragment bound to penicillopepsin and kinetic studies on ketomethylene and hydroxyethylene pepstatin analogs which resemble good porcine pepsin substrates, have suggested that pepstatin may act as a collected substrate inhibitor.

Based on these kinetic and x-ray results, in addition to data available through research collaborators, a general acid-base mechanism for aspartic proteinases is proposed.

APPROVED: 
Professor Daniel H. Rich

DATE: 9/28/84

To my parents, wife and son.

ACKNOWLEDGMENTS

I express my deep gratitude and sincere appreciation to my advisor Professor Daniel H. Rich for his guidance, encouragement and critical advice throughout the course of my thesis research.

I also thank Professor Dexter B. Northrop and Professor Phillip A. Hart for their helpful discussions.

I must also thank the numerous members of Dr. Rich's group with whom I had the pleasure of collaborating, especially Dr. Megumi Kawai, Dr. Mark Holladay and Tim Ocain. Their helpful discussions, advice and friendship have been and will always be very valuable to me.

A very special thanks goes to my undergraduate advisor Professor Bruce R. Branchini who initiated my interest in bioorganic research. His contribution to my education and his friendship will not be forgotten.

Thanks are also due to Linda Frei for an excellent typing job.

Deepest appreciation I reserve for the members of my family. To my parents who have loved and encouraged me not only throughout my education, but my entire life. To my brothers and sister for their love. To my wife Vicky, who has been an inspiration to me and has always stood behind me especially when times were hard. And finally to my son Joseph who has made me the proudest father in the world.

LIST OF FIGURES

Figure		Page
1.	Schematic illustration of the renin-angiotensin system. . . .	7
2.	Mechanism A. General acid-base mechanism of aspartic proteinases, and comparison of the central Sta of pepstatin with tetrahedral intermediate	15
3.	Mechanism B. Nucleophilic mechanism of aspartic proteinases, and comparison of Sta with the tetrahedral intermediate.	16
4.	Synthesis of statine.	24
5.	Comparisons between statine, statone, dipeptide and dipeptide isosteres	32
6.	Synthesis of Leu-Ala ketomethylene and hydroxyethylene analogs	33
7.	Synthesis of Leu-Phe ketomethylene and hydroxyethylene analogs	36
8.	Synthesis of Leu-D-Ala ketomethylene and hydroxyethylene analogs	39
9.	Synthesis of Leu-D-Phe isosteres.	41
10.	Boc-Leucinal chelated to magnesium ion.	42
11.	Synthesis of diamino-hydroxy-octanoic acid.	45
12.	A,B. Synthesis of LySta peptides	47
13.	Attempted preparations of alpha-Boc-epsilon-Cbz ornithinal. .	49
14.	Attempted preparation of N^4-(Boc)-N^7-(phthaloyl)-4(S),7-diamino-3(R,S)-hydroxy-heptanoic acid ethyl ester	50
15.	Synthesis of N^4-(Boc)-N^7-(benzyl)-N^7-(Cbz)-4(S),7-diamino-3(R,S)-hydroxy-heptanoic acid ethyl ester (OrnSta) and OrnSta peptides.	52
16.	Stereo view of the active site of penicillopepsin with bound inhibitor 56	55

Figure | Page

17. Comparison of Leu-Leu-Val-Phe tetrahedral intermediate with Sta-Ala-Sta 66

18. EI complexes mimicking various ES complexes 70

19. Required binding sites for porcine pepsin and penicillopepsin 82

20. Penicillopepsin substrate 86

21. Schematic representation of inhibitor 56 bound to penicillopepsin. 87

22. Schematic representation of LySta inhibitor 115a bound to penicillopepsin 88

23. Two proposed mechanisms for peptide bond hydrolysis by aspartic proteinases. 92

24. Two possibilities of ketone hydration 95

25. Schematic representation of the addition of water to the C-3 carbonyl of Sto peptide 11, catalyzed by porcine pepsin 96

26. ^{13}C spectra of ketone 148 bound to porcine pepsin in H_2O and ^2H_2O. 98

27. Isotope effect of ^{18}O on ^{13}C NMR spectra of 148 bound to pepsin. 100

28. (A) Stereo view of the active site of penicillopepsin showing water molecule 039 positioned between Asp-33 and Asp-213 103

28. (B) Stereo view of the active site of penicillopepsin with bound inhibitor 56. C-3 hydroxyl replaces water molecule 039 103

29. Schematic representation of the relationships between proposed catalytic and inhibitory mechanisms. 104

30. Hypothetical reaction coordinate for pepsin catalyzed hydrolysis of peptide bonds 110

31. Hypothetical ΔG binding coordinate for intermediates in peptide bond cleavage by pepsin. 111

LIST OF TABLES

Table — Page

I. Nomenclature of several known pepstatins. 5

II. Inhibition of porcine pepsin by several P_3 analogs. 11

III. Final P_2, $P_{2'}$ and $P_{3'}$ analogs 26

IV. Intermediates in the synthesis of P_2, $P_{2'}$ and $P_{3'}$ analogs . 27

V. Various dipeptide isosteres prepared. 34

VI. Ketomethylene and hydroxyethylene final products. 35

VII. Inhibition constants for pepstatin analogs on porcine pepsin 59

VIII. Inhibition constants for ketomethylene and hydroxyethylene compounds on porcine pepsin 64

IX. Kinetic constants for several porcine pepsin substrates with $S_{1'}$ variations. 69

X. Potential substrates and inhibitors for porcine pepsin isosteric to ketomethylene analogs 76

XI. Inhibition of penicillopepsin by pepstatin analogs. 77

XII. Inhibition of penicillopepsin by LySta and OrnSta analogs. 83

XIII. Inhibition of porcine pepsin by pepstatin analogs 102

ABBREVIATIONS

Nomenclature and symbols of amino acids and peptides generally follow the recommendation of the IUPAC-IUB Commission on Biological Nomenclature [1. Biochemistry 5, 2485 (1966); 2. Ibid., 6, 362 (1967); 3. Ibid., 11, 1726 (1972)]. Unless otherwise specified, amino acids are of the L configuration.

The following abbreviations are used:

Ac	acetyl
Boc	*tert*-butoxycarbonyl
Bu	butyl
t-Bu	*tert*-butyl
Cbz	benzyloxycarbonyl
$CDCl_3$	deuterated chloroform
d	deuterium
DCC	dicyclohexylcarbodiimide
DCU	dicyclohexylurea
DIBAL-H	diisobutylaluminum hydride
dm	decimeter
DMAP	dimethylaminopyridine
DMSO	dimethylsulfoxide
dSta	deoxystatine or 4(S)-amino-6-methylheptanoic acid
ESR	electron spin resonance
Et	ethyl
EtOAc	ethyl acetate
FT	Fourier transform

HOAc	acetic acid
HOBT	1-hydroxybenzotriazole
hr	hours
Iaa	isoamylamide
Ipoc	isopropyloxycarbonyl
Iva	isovaleryl
K_{diss}	dissociation constant
K_i	inhibitory binding constant
Km	Michaelis constant
LD	lethal dose
ln	natural logarithm
LySta	4,8-diamino-3-hydroxyl-octanoic acid
M	molar
mM	millimolar
Me	methyl
Me^3Sta	3-methylstatine
mp	melting point
NBS	N-bromosuccinimide
NMR	nuclear magnetic resonance
OrnSta	4,7-diamino-3-hydroxyl-heptanoic acid
PDC	pyridinium dichromate
PLC	preparative thin layer chromatography
$Py \cdot SO_3$	pyridine sulfurtrioxide complex
Sta	4-amino-3-hydroxyl-6-methylheptanoic acid or statine, (3S,4S) unless otherwise specified
Sto	3-oxo-4(S)-amino-6-methylheptanoic acid or statone

TDI	time-dependent inhibition
TEA	triethylamine
THF	tetrahydrofuran
TLC	thin layer chromatography
TMS	tetramethylsilane
UV	ultraviolet
v/v	volume to volume
δ	chemical shift

TABLE OF CONTENTS

Page

Acknowledgments. iv

List of Figures. v

List of Tables vii

Abbreviations. viii

I. Introduction 1

A. Historical 1

1. The biological significance of proteinases 1

2. Isolation and structure elucidation of pepstatin . . 3

3. Biological properties of pepstatin 4

4. Structure-activity relationships 9

B. Mechanism of Action of Aspartic Proteinases. 12

1. Crystal structures of aspartic proteinases and aspartic proteinase complexes. 12

2. Mechanism of pepstatin inhibition. 17

II. Research Plan. 21

III. Results and Discussion 23

A. Synthetic Studies. 23

1. P_2, $P_{2'}$, $P_{3'}$ Analogs of pepstatin. 23

a. Synthesis of $P_{3'}$ analogs 25

b. Synthesis of $P_{2'}$ analogs 29

c. Synthesis of P_2 analogs. 29

d. Synthesis of pepstatin fragments 30

Page

2. Synthesis of Ketomethylene and Hydroxyethylene Analogs. 31

a. Synthesis of Leu-Phe isosteres 34

b. Synthesis of Leu-D-Ala isosteres 38

c. Synthesis of Leu-D-Phe isosteres 40

3. Synthesis of Lysine-Statine (LySta) Derivatives. . . 43

4. Synthesis of Ornithine-Statine (OrnSta) Derivatives. 48

B. X-Ray Crystal Structure of Iva-Val-Val-Sta-OEt (56) Bound to Penicillopepsin 53

C. Kinetic Studies. 57

1. Effect of structure in subsites P_2, $P_{2'}$ and $P_{3'}$ on inhibition of porcine pepsin. 57

a. Results. 57

b. Discussion 60

2. Inhibition of porcine pepsin by ketomethylene and hydroxyethylene dipeptide isosteres. 62

a. Results. 62

b. Discussion 65

3. Effect of structure in subsites P_4, P_3, P_2, $P_{2'}$ and $P_{3'}$ on the inhibition of penicillopepsin 76

a. Results. 76

b. Discussion 78

4. Inhibition of penicillopepsin by lysine-statine and ornithine-statine analogs. 82

a. Results. 82

b. Discussion 84

D. Catalytic Mechanism of Aspartic Proteinases. 91

Page

E. Mechanism of Pepstatin Inhibition. 100

F. Conclusions. 108

IV. Experimental 114

A. Materials and Methods. 114

B. General Synthetic Procedures 115

C. Specific Procedures. 118

D. $H_2{}^{18}O$ Experiments. 180

V. References 182

I. INTRODUCTION

A. Historical

1. The Biological Significance of Proteinases

It is well known that the general class of enzymes called proteinases are very important in the control and maintenance of a healthy physiological state. This has prompted extensive investigation of these enzymes since alteration of some enzyme activity may possibly counteract the symptoms of particular diseases.

As a general class, proteinases are subgrouped into more specific classes which reflect the various catalytic groups involved in amide bond hydrolysis.

The serine proteinases are a group of enzymes so termed because of a catalytically required serine residue in the active site of these enzymes. The serine hydroxyl group is deprotonated, presumably via a proton transfer mechanism[1], and attacks the carbonyl of the scissle amide bond. These enzymes, which are involved in such diverse biological functions as digestion, fertilization, and blood clotting, have been extensively studied because of their involvement in several diseases. For example, unusually high elastase activity has been implicated in the lung disease emphysema. It is thought that an imbalance between elastase and its naturally occurring inhibitors such as $alpha_1$-PI may result in increased proteolytic activity in the lung, thereby causing destruction of alveolar sacs.[2] Also, deficiencies in various serine proteinases of the blood clotting cascade are involved in the congenital bleeding disorder hemophilia. Factor IX is a plasma

glycoprotein involved in blood coagulation. It is a zymogen of the catalytically active serine proteinase Factor IXa, and is activated by the action of Factor XIa.[3] Without this enzyme the further steps involved in the cascade, which result in a fibrin clot, are not possible.

Metalloproteases are a group of enzymes which have in their active site a required metal ion. Aminopeptidases and carboxypeptidases, for example, have a required zinc atom in their active sites. This metal ion is thought to be involved in the mechanism of these enzymes by polarizing the carbonyl of the scissle amide bond, thereby rendering it more susceptible to nucleophilic attack of a nucleophile which occurs with the aid of two other catalytically required glutamic acid residues. Enzymes of this type are involved in the processing and degradation of peptide hormones such as angiotensin II (Fig. 1) and the enkephalins.

The class of enzymes that this thesis will address are those called aspartic proteinases. These enzymes have two aspartic acid residues in their active site which catalyze the cleavage of amide bonds. As of yet, it is not clear whether the mechanism of these enzymes involves a nucleophilic attack of one of the aspartic acid carboxyl groups or a general acid-base mechanism where water acts as the nucleophile, however later sections of this thesis will present data which clarifies this problem. These enzymes are involved in various biological functions such as digestion and blood pressure regulation (pepsin and renin, respectively). Other aspartic proteinases such as cathepsin D have been implicated in disease states associated with

muscular atrophy or inflammation.[4]

From these examples on the biological significance of proteinases, it is obvious that the study of enzyme specificity and mechanisms are well warranted. It is well known that specific inhibitors of enzymes can be useful tools for the study of enzyme mechanisms and their role in physiological and biochemical processes, and they may also be useful for analysis and treatment of various diseases.

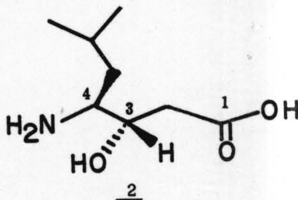
2. Isolation and Structure Elucidation of Pepstatin

Many naturally occurring enzyme inhibitors are known today. A significant number have been discovered by Hamao Umezawa and his coworkers, who in 1965 initiated the screening of various microbial cultural filtrates in search of inhibitors. His work has led to the finding of leupeptins[5], antipain[6] and numerous other inhibitors[7-10], the most recent of which are the arphamenines[11]. In 1970 they also reported the isolation, from various actinomycetes species, of an aspartic proteinase inhibitor called pepstatin[12]. Pepstatin 1 was found to be a pentapeptide inhibitor with a free carboxyl group and an N-terminus acylated with isovaleric acid (Iva). It was found to contain 2 residues of L-valine, one residue of isovaleric acid, one of alanine and two residues of a novel amino acid, 4-amino-3-hydroxyl-6-methylheptanoic acid (AHMHA)[13,14], known today as statine (Sta)[15]. High resolution mass spectroscopy studies of permethylated pepstatin[13] established the sequence as isovaleryl-L-valyl-L-valyl-statyl-L-alanyl-statine:

Iva-Val-Val-Sta-Ala-Sta-OH

(1) Pepstatin

This structure was confirmed by its synthesis in 1972.[16] The absolute stereochemistry of statine was later established by the work of Kinoshita and coworkers[17,18], who in 1973 synthesized the 4 possible diastereomers of statine. Its absolute configuration was shown to be 4(S)-amino-3(S)-hydroxyl-6-methylheptanoic acid (2) and this was



confirmed by x-ray structure determination.[19] Since then this unusual amino acid has also been found in the didemnins, which are depsipeptides isolated from Caribbean tunicates[20]. However, the stereochemistry of the Sta moiety in these peptides has not been established.

Numerous other pepstatins, which differ only in the size of the N-acyl moiety (Table 1), have also been isolated. Pepstatin (1) was named pepstatin A and, unless otherwise stated, the name pepstatin when used in this thesis refers to pepstatin A.

3. Biological Properties of Pepstatin

All of the pepstatins have been shown to be potent, specific inhibitors of aspartic proteinases including porcine pepsin,

TABLE I. Nomenclature of several known pepstatins

R-Val-Val-(S,S)-Sta-Ala-(S,S)-Sta-OH

Compound	R
Pepstatin Ac	Acetyl
Pepstatin Pr	Propionyl
Pepstatin Bu	n-Butyryl
Pepstatin A	Iso-valeryl
Pepstatin B	n-Caproyl
Pepstatin C	Iso-caproyl
Pepstatin D	n-Heptanoyl
Pepstatin E	Iso-heptanoyl
Pepstatin F	Anteiso-heptanoyl
Pepstatin G	n-Caprylyl
Pepstatin H	Iso-caprylyl

cathepsin D, various fungal aspartic proteinases and renin[14,21], although renin is inhibited to a lesser extent. The specificity of pepstatin toward inhibition of aspartic proteinases is indicated by the fact that it does not inhibit serine proteinases or thiol proteinases. Some of the biological properties which prompted an initial interest in pepstatin were the effective prevention of stomach ulceration in ligated rats, presumably through an inhibition of pepsin or gastricsin and its low toxicity towards several lab mammals.[7] Some clinical studies also demonstrated a potential utility for the treatment of human gastric ulcer.[22]

Cathepsin D, which has been implicated in diseases associated with muscular atrophy, is also inhibited by pepstatin and it may, therefore, have potential therapeutic value for the treatment of disorders of this type in the future.

Although pepstatin is less effective at inhibiting renin than pepsin or cathepsin D, it is still the most potent naturally occurring inhibitor of this enzyme. Renin is an important enzyme involved in the biosynthesis of angiotensin II (Fig. 1), the most potent hypertensive substance formed naturally in man. Circulating angiotensin II directly constricts arterioles and produces immediate elevation of blood pressure. Either angiotensin II or III[23] also stimulates the release of the sodium-retaining steroid aldosterone to cause an increase in body fluid and an increase in blood pressure. It has been shown, through extensive study over the past 15 years, that inhibition of the carboxydipeptidase, angiotensin converting enzyme (ACE), which produces angiotensin II from I, can lower blood pressure in hypertensive subjects.

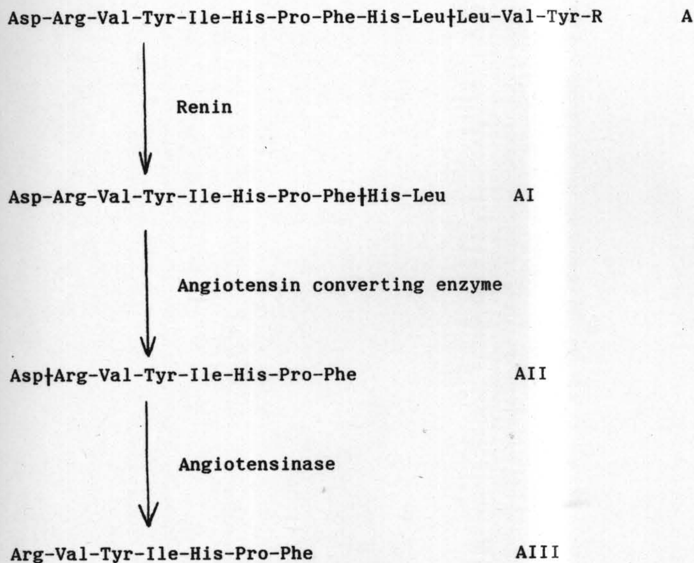


Fig. 1. Schematic illustration of the renin-angiotensin system. Markers (|) indicate points of amide bond cleavage.

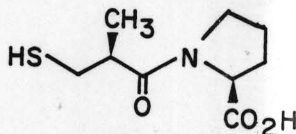
A = Angiotensinogen

AI = Angiotensin I

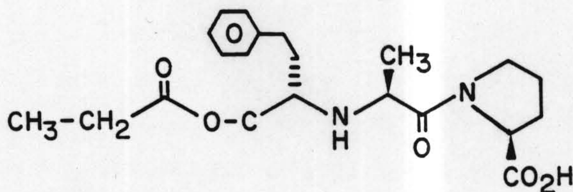
AII = Angiotensin II

AIII = Angiotensin III

The compound SQ14225 (D-3-mercapto-2-methylpropanoyl-L-proline, registered as captopril) (3), which was developed by Ondetti et al.[24,25], was shown to be a potent, orally active inhibitor of ACE. This drug is currently marketed under the trade name Captoten. Merck has also recently developed an inhibitor of ACE. MK-421 (4) is also a potent, orally active inhibitor and has been selected for clinical trials as an antihypertensive drug.[26]



3 Captopril



4 MK-421

These studies have prompted numerous pharmaceutical companies to pursue other lines of exploitation of the renin-angiotensin system, specifically, the inhibition of renin. Pepstatin is a good inhibitor of renin although a relatively weak one compared with other aspartic proteinases. However, it has recently been shown that through structural

modifications of pepstatin, more potent inhibitors of renin can be prepared.[119] If it can be shown that these analogs of pepstatin are effective in lowering blood pressure, in vivo, without increasing the already low toxicity of pepstatin, a significant breakthrough will have been achieved in the regulation of hypertension, since many antihypertensive drugs currently on the market have significant side effects and toxicity. (A major drawback in the treatment of hypertension has been patient compliance.)

One of the most striking properties of pepstatin is the extraordinarily small dissociation constant of the pepstatin-enzyme complex ($K_i \sim 10^{-10}$ M for pepsin and cathepsin D).[27,28] This makes pepstatin for all intensive purposes an irreversible inhibitor and this inhibition is stoichiometric. It has been shown by Umezawa and coworkers that pepstatin does in fact bind to the active site of pepsin[22,27], and more recent x-ray crystallographic data have confirmed this. Studies of pepstatin complexed to the aspartic proteinase from _Rhizopus chinensis_[29,30] and of a pepstatin fragment complexed to the fungal aspartic proteinase penicillopepsin[31a,b] have indicated an extended conformation for both inhibitors in a region of the enzyme which contains the catalytically required aspartic acid residues. These x-ray data will be discussed in more detail in later sections of this thesis.

4. Structure-Activity Relationships

This section briefly reviews the structure-activity relationships of pepstatin analogs on pepsin inhibition known at the onset of this work. The nomenclature of Schechter and Berger[39] is used

where enzyme subsites S_3, S_2, S_1, $S_{1'}$ and $S_{2'}$ are occupied by inhibitor residues P_3, P_2, P_1, $P_{1'}$ and $P_{2'}$.

Data obtained in this lab by Dr. E. T. O. Sun under the supervision of Dr. D. H. Rich have conclusively shown that the hydroxyl group of the central Sta in pepstatin is required for complete inhibitory activity.[32] Dideoxypepstatin (in which the 3(S)hydroxyl group is replaced by a proton in both statine residues) was shown to be an approximately 4,000-fold less effective inhibitor of pepsin. Subsequent studies revealed that the hydroxyl group of the C-terminal Sta was not necessary for tight binding inhibition of pepsin[33,34], and that this residue could be replaced with an isoamylamide moiety while retaining full potency. In addition, these studies showed that the 3(S) stereochemistry of statine was critical and that a minimum peptide length was required for tight binding inhibition. When the stereochemistry of the 3(S) hydroxyl group in the analog Iva-Val-(3S,4S)Sta-Ala-Iaa, 5, was changed to 3(R), an inhibitor approximately 3,000-fold less active was produced. The observation was also made that longer peptides which contain a moiety in the P_4 position are more potent than those which lack a P_4 residue. These data are in agreement with earlier findings.[21,35]

Numerous other structure-activity relationships have been carried out to define the effects of structural changes on subsites P_4, P_3 and $P_{4'}$. The naturally occurring pepstatins, which differ only in the size of the N-acyl chain the P_4 position (Table I), inhibit pepsin to a similar extent[36], although inhibition of renin is dependent upon the size of the chain. Modification of the P_3 position[37] has shown the

importance of geometry in this position on both the inhibition constant and more interestingly, on time-dependent inhibition. The kinetic properties of derivatives 6 and 8 may provide evidence for preferred geometry of the P_3 group when bound to the enzyme. These Ipoc (6) and Boc (8) urethane derivatives are weaker inhibitors than compounds 5 and 7 and are not slow binding inhibitors even though the pairs 5 and 6, and 7 and 8 are essentially isosteric with each other. This may reflect differences between preferred geometry of urethanes vs. the required geometry for maximum binding of inhibitor to the enzyme at the P_3 site.

The importance of the P_3 site was also illustrated by Umezawa[35], who compared several N-acylated semisynthetic tetrapeptide analogs of pepstatin. The tetrapeptide Val-Sta-Ala-Sta was prepared by cleavage of pepstatin by pepstatin hydrolase, and subsequently N-acylated to give several P_3 analogs. Although the tetrapeptide itself is a poor inhibitor, most of the other N-acylated compounds were nearly as potent as pepstatin for pepsin inhibition.

TABLE II. Inhibition of porcine pepsin by several P_3 analogs.[37]

	Compound	K_i (nM)	TDI
5	Iva-Val-(S,S)Sta-Ala-Iaa	3	+
6	Ipoc-Val-(S,S)Sta-Ala-Iaa	96	-
7	t-BuAc-Val-(S,S)Sta-Ala-Iaa	7.5	-
8	Boc-Val-(S,S)Sta-Ala-Iaa	170	-

B. Mechanism of Action of Aspartic Proteinases

1. Crystal Structures of Aspartic Proteinases and Aspartic Proteinase Complexes

X-ray crystallographic studies have yielded the molecular structures of several microbial aspartic proteinases at resolutions of 3 Å or less.[38,40,50] The 3-dimensional structure of porcine pepsin has also been partially resolved.[41] All of these aspartic proteinases which include those from *Rhizopus chinensis*, *Endothia parasitica* and *Penicillium janthinellum* show a great deal of tertiary structure similarity. The structure of the *Penicillium janthinellum* proteinase penicillopepsin has since been refined to 1.8 Å.[42] This molecule has, approximate dimensions of 65 x 49 x 39 Å. The others have dimensions similar to this. The molecule is bilobal with an extended hydrophobic interior. The two lobes are separated by a deep cleft that is approximately perpendicular to the 65 Å length. The catalytically important aspartates Asp-33 and Asp-213 (penicillopepsin numbering) are found buried in this groove but accessible to solvent. The importance of these residues in the mechanism of penicillopepsin was initially confirmed when it was found that incubation of enzyme with 1,2-epoxy-3-(p-nitrophenoxy)propane resulted in inactivation of the enzyme via covalent attachment of the reagent to these aspartic acids.[43] This study also implicated the Tyr-75 hydroxyl group as a proton donor to the product free amine in the mechanism of peptide bond hydrolysis, however, more recent studies have raised some questions about this possibility.[31a,b] Although it was unclear in an earlier 2.8 Å resolution crystal structure[38], a refined 1.8 Å picture has clearly shown the

existence of a water molecule bound to the catalytic aspartyl residues.[42] This region of the enzyme is extensively hydrogen bonded and is the least mobile area of the enzyme. Conformational changes in this vicinity so that either Asp-33 or Asp-213 would be more accessible to the substrate are, therefore, unlikely. However, conformational flexibility of a segment of the molecule, from residues Trp-71 to Ser-82, often called the mobile "flap" region has been implicated in substrate induced conformational changes in the enzyme.[44] The importance of these conformational changes will be discussed in other sections of this thesis.

Other points of agreement between groups working on different enzymes are that: 1) the two aspartic acid groups have different pK_a's, one at about 4.5 and the other at 1.5. It has been the general consensus that Asp-213 has the higher pK_a, thereby acting as a proton donor to the substrate carbonyl oxygen which is attacked by a nucleophile. Recent modeling studies using the 1.8 Å penicillopepsin structure supported this hypothesis.[42] 2) Asp-33 would be about 5 Å away from a substrate carbonyl and appears to be too far away to be involved in a direct nucleophilic attack, unless extensive conformational changes occur. This was determined through enzyme-substrate modeling studies by Blundell and coworkers.[50] 3) The binding of substrate with induced conformational changes need not displace water hydrogen bonded to the active site.[38]

Recently the x-ray crystal structure of the Rhizopus chinensis aspartic proteinase complexed to pepstatin has been reported.[45] This study has confirmed that pepstatin binds in an extended conformation in

the cleft region of the enzyme which contains the catalytic aspartates. The critical 3(S) hydroxyl group of the central statine is strategically placed between these two aspartates indicating that pepstatin may act as a transition state analog[46] inhibitor by mimicking the transition state or tetrahedral intermediate for peptide bond hydrolysis (see Figures 2 and 3).

Another complex between a pepstatin fragment, synthesized in this lab, and penicillopepsin has also been resolved.[31a,b] This complex will be described in more detail in later sections.

All of the x-ray crystallographic studies mentioned and numerous other studies[47-49] have suggested two possible mechanisms for aspartic proteinases (Fig. 2, 3). Although it is unclear whether the mechanism proceeds via a general acid-base mechanism A (Fig. 2) or by a nucleophilic mechanism B (Fig. 3), many of these studies have pointed to mechanism A as the most likely.

However, extrapolations from Rhizopus chinensis-pepstatin complexes must be done with caution since the resolution of 2.8 Å is not sufficient to clarify the orientation of the 3(S) hydroxyl group with respect to the catalytic aspartates or to establish the existence or non-existence of an enzyme bound water molecule. Other problems are that the x-ray picture may not correlate with the dynamic enzymatic events in solution, and that pepstatin is not isosteric with normal substrates.

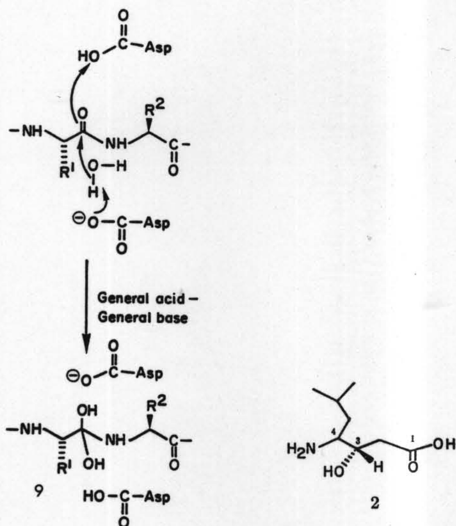


Fig. 2: Mechanism A. General acid-base mechanism of aspartic proteinases, and comparison of the central Sta of pepstatin 1 with the tetrahedral intermediate 9.

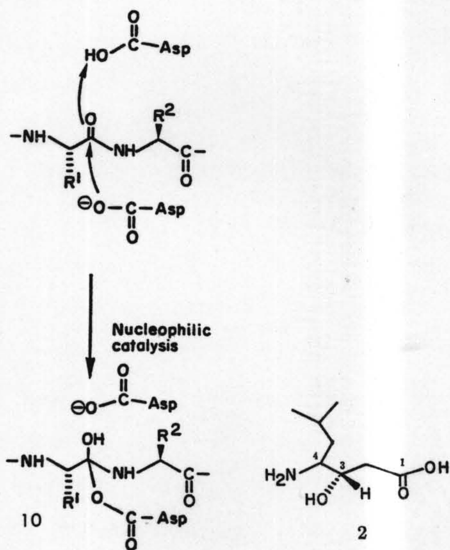


Fig. 3: Mechanism B. Nucleophilic mechanism of aspartic proteinases, and comparison of Sta with tetrahedral intermediate 10.

2. Mechanism of Pepstatin Inhibition

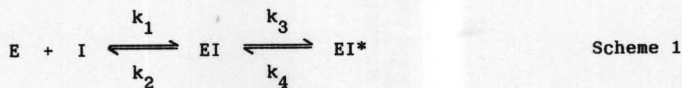
The unusually low dissociation constant of pepstatin on porcine pepsin (4.5×10^{-11} M)[51] has led some people to conclude that pepstatin is a transition state analog inhibitor.[46,52] The best known substrates for pepsin have dissociation constants on the order of 10^{-5} M[53], making pepstatin a $\simeq 10^{6}$ fold better binder; this difference is typical of transition state analogs.

The concept that an enzyme's affinity for the transition state is higher than that for substrates was first pointed out by Linus Pauling[54,55] and was later recognized and developed for its potential application to the study of enzymes by Wolfenden and Leinwald.[56-59,114]

Two mechanisms for peptide bond hydrolysis by aspartic proteinases have previously been mentioned. Mechanism B involves the nucleophilic attack of one of the aspartic acids to the carbonyl to generate the tetrahedral intermediate 10 and subsequent cleavage of the peptide bond to form products. Mechanism A involves the attack of a water molecule, aided by the abstraction of a proton by one of the aspartates, to form tetrahedral intermediate 9 followed again by product formation. The structure of pepstatin shows considerable similarity to each of these transition states especially from C-3 to C-7 of statine. This portion is directly isosteric with the S_1 site in the substrate, although statine atoms C_1 and C_2 are not isosteric with the $S_{1'}$ site. Because of these atoms, statine can be considered 2 atoms too long to be isosteric with an amino acid or 1 atom too short to be a dipeptide isostere. This has led to some confusion concerning the mechanism of pepstatin inhibition. This subject will be dealt with in more detail in later

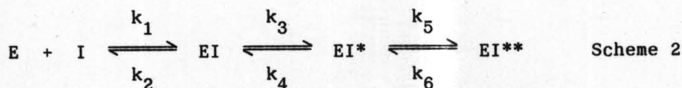
sections.

The kinetic mechanism of pepstatin has also been extensively studied by previous workers in this lab.[34] Besides its structure and dissociation constant, another unusual feature of pepstatin is the phenomena of time-dependent inhibition or slow-binding, which this molecule and numerous analogs exhibit. Because of its non-steady state behavior, time was required to achieve maximum inhibition when porcine pepsin was added to a solution of substrate and inhibitor. This time-dependent decrease in absorbance was analyzed by the mechanism of Kitz and Wilson[60] and found to be consistent with the following Scheme 1.



$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EI^* \qquad \text{Scheme 1}$$

This mechanism indicates that the initial collision complex EI proceeds via a slow process (k_3) to a more tightened EI* complex. It should be noted that this represents a minimum mechanism since other intermediates could exist along the reaction pathway. From this study[34] it was concluded that $k_2/k_1 = 1.3 \times 10^{-8}$ M, $k_3 = 0.0022\ sec^{-1}$ and $k_4 = 4.6 \times 10^{-5}$ M. Therefore a dissociation constant on the order of 10^{-8} was attributable to the initial EI complex which could proceed through a slow process to an EI* complex. This is consistent with kinetic data which point to k_{diss} of $\sim 10^{-8}$ M as the cut-off for observation of slow binding. As a result of kinetic studies reported by Kitagishi et al.[61], the mechanism was further modified as shown in Scheme 2. This scheme indicates the presence of another intermediate in the reaction



pathway. These stopped flow kinetic studies indicated the presence of an intermediate formed much faster than could be observed using conventional kinetic methods. The value of k_2/k_1 reported from this study was 8 x 10^{-4} M and k_3 was 600 sec^{-1}. Computer simulations performed by Dr. Michael Bernatowicz[62] were consistent with this proposed minimum mechanism.

Further studies aimed at the elucidation of the mechanism of pepstatin inhibition were carried out by Rich et al.[64] utilizing an analog of compound 5 in which the C_3 hydroxyl of Sta was oxidized to a ketone (Sto).

Iva-Val-Sto-Ala-Iaa (11)

Compound 11 inhibits porcine pepsin with a K_i of 6 x 10^{-8} M (~20X less than parent compound 5) and does not exhibit time-dependent inhibition, suggesting its inability to form a tightened EI** complex.[63] It was later shown[64] that this inhibitor bound to the enzyme as a C_3 tetrahedral adduct. ^{13}C NMR studies clearly showed the ^{13}C resonance of C_3 in buffer at ~200 ppm, whereas in the presence of enzyme the resonance was shifted to ~99 ppm indicating a tetrahedral geometry and that the added atom was oxygen. The study, however, was unable to determine the nature of the added oxygen (water or aspartate) and it was not rigorously proven whether the gem diol formation was catalyzed by the enzyme

as opposed to a mechanism involving gem diol formation in solution followed by binding to the enzyme.

II. RESEARCH PLAN

At the onset of this work a great deal was known about the structural requirements for pepstatin inhibition of porcine pepsin. A crystal structure of pepstatin bound to the *Rhizopus chinensis* aspartic proteinase resolved to 2.8 Å had also been obtained, which suggested that statine was acting like a tetrahedral intermediate in peptide bond hydrolysis. However, a more detailed account of the mechanism of pepstatin inhibition was still lacking.

To address this issue, several approaches were taken. It was assumed that pepstatin and rationally designed analogs, could be used to clarify the mechanism of catalysis of aspartic proteinases. Therefore, several series of pepstatin analogs were synthesized for kinetic evaluation on two aspartic proteinase systems: porcine pepsin and penicillopepsin. The major approaches taken were as follows:

1. Analogs which had structural variations in the P_2, P_2' and P_3' positions were synthesized. Compounds of this type were tested on porcine pepsin since little was known about the effects of structural variation in these positions. Several of these and other previously synthesized analogs were also tested on penicillopepsin for which no pepstatin structure-activity relationships were known.

2. Syntheses of compounds were also carried out which more closely approximated good penicillopepsin substrates (LySta compounds). It should be mentioned that this aspect of the project came about during the early stages of this work, during which several pepstatin analogs were checked by collaborators for possible co-crystallization with

the enzyme. Fortuitously, one analog Iva-Val-Val-Sta-OEt (56) was co-crystallized and a 1.8 Å x-ray crystal structure was obtained. The analogs which resembled substrate could then be tested in solution and, along with the crystal structure, possibly provide information regarding the mechanism of aspartic proteinases.

3. Peptides containing ketomethylene and hydroxyethylene dipeptide isosteres which more closely resemble porcine pepsin substrates were synthesized. Analogs which contained bulky substituents in $P_{1'}$ were prepared since pepstatin lacks a substituent in this position.

Experiments were also carried out to determine if the formation of the tetrahedral species of the Sto analog 11 was an enzyme catalyzed process. This was done by incubating the inhibitor and enzyme in the presence of $H_2{}^{18}O$. Analysis of the recovered ketone by mass spectrometry could then determine if formation of the tetrahedral species was an enzyme catalyzed process or a non-specific hydration in solution. This experiment was also carried out on a ketomethylene analog (148).

In summary, the objective of this research was to elucidate in more detail the mechanism of inhibition by pepstatin, which ultimately could provide information concerning the mechanism of aspartic proteinases. The general approach was to synthesize and kinetically test various analogs of pepstatin which more closely resemble good substrates for various aspartic proteinases.

III. RESULTS AND DISCUSSION

A. Synthetic Studies

1. P_2, P_2', P_3' Analogs of Pepstatin

To date there have been several syntheses of statine reported in the literature.[17,65,115-118] The procedure that was utilized for the work described herein was a modification of the procedure of D. H. Rich et al.[65] This procedure involved the preparation of Boc-leucinal 13 via reaction of Boc-leucine methyl ester with diisobutyl aluminum hydride in toluene (pathway A, Fig. 4) and subsequent reaction with lithio ethyl acetate to form Boc-Sta-OEt, 14, as a mixture of 3R,3S diastereomers. This method for aldehyde preparation suffers, however, from several drawbacks. One is that production of a mixture of methyl ester, aldehyde and the overreduction product alcohol is unavoidable. In addition, the aldehyde at this point cannot be purified due to its tendency to racemize on silica gel.[65] Therefore, alkylation must be performed on an impure mixture. Also, because of the methanol quench involved in the reaction[65], some hemiacetal is formed which must be hydrolyzed, prior to alkylation, in order to form aldehyde. Incomplete formation of aldehyde could result in poor yields in the alkylation step. For these reasons, Boc-leucinal was prepared by the method of Hamada and Sioriri.[66] This method, which can be utilized for the preparation of various Boc or Cbz protected amino aldehydes, involves the reduction of the methyl ester (Fig. 4, path B) to alcohol 12 using lithium borohydride in a mixture of ethanol and tetrahydrofuran, followed by oxidation to aldehyde 13 with pyridine sulfur

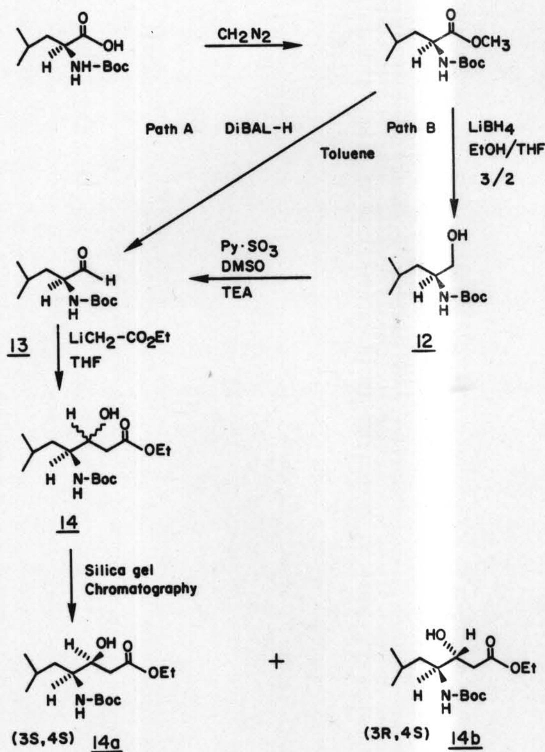


Fig. 4: Synthesis of statine.

trioxide complex, DMSO and triethylamine (a modification of the Moffet oxidation). Although path B involved two steps vs. path A's one step, there were advantages to path B. The preparation of Boc-leucinol was very clean, fast (~3 hrs) and proceeded in high yield (~90%). Also chromatography could be utilized at this stage to obtain 100% pure alcohol. The oxidation step also proceeded relatively cleanly, rapidly (~10 min) and in good yields (~80%). Some side products[67] may be observed occasionally, however they are rarely over ~10-15% under the conditions of the reaction. Reaction with lithio ethyl acetate can then be performed on a much cleaner aldehyde. Silica gel chromatography then afforded the two diastereomers 14a and 14b. Statine prepared in this manner was utilized to prepare all the compounds shown in Table III. These compounds represent a systematic variation of the $P_{3'}$, $P_{2'}$ and P_2 positions of the standard peptide 5. Kinetic analysis of these compounds provided the first data on the effect of structural variations in these positions on the inhibition of porcine pepsin. The intermediates in the synthesis of these compounds are in Table IV.

1a. Synthesis of $P_{3'}$ analogs

The first set of compounds in Table III represents variations in the $P_{3'}$ position of the standard peptide 5 (compounds 17, 20, 23, 30, 26, 34, 32, 38). Most of these peptides were synthesized by a standard stepwise coupling procedure which involved systematic addition of a new residue working from C-terminal to N-terminal. The preparation of compounds 17 and 20 was initially attempted via a 2 + 2 coupling of Iva-Val-Sta-OH 33 and alanyl-phenylalanine-methyl ester

TABLE III. Final P_2, $P_{2'}$ and $P_{3'}$ Analogs

	Compound
5)	Iva-Val-(S,S)Sta-Ala-Iaa
17)	Iva-Val-(S,S)Sta-Ala-Phe-OMe
20)	Iva-Val-(S,S)Sta-Ala-Leu-OMe
23)	Iva-Val-(S,S)Sta-Ala-Gly-OMe
30)	Iva-Val-(S,S)Sta-Ala-Ipa
26)	Iva-Val-(S,S)Sta-Ala-$NHCH_3$
34)	Iva-Val-(S,S)Sta-Ala-OCH_3
32)	Iva-Val-(S,S)Sta-OCH_2-CH_3
38)	Iva-Val-(S,S)Sta-Ipa
5)	Iva-Val-(S,S)Sta-Ala-Iaa
42)	Iva-Val-(S,S)Sta-Leu-Iaa
46)	Iva-Val-(S,S)Sta-Gly-Iaa
5)	Iva-Val-(S,S)Sta-Ala-Iaa
48)	Iva-Gly-(S,S)Sta-Ala-Iaa
50)	Iva-Ala-(S,S)Sta-Ala-Iaa
52)	Iva-Leu-(S,S)Sta-Ala-Iaa
54)	Iva-Phe(S,S)-Sta-Ala-Iaa
56)	Iva-Val-Val-(S,S)Sta-OCH_2CH_3
59)	Iva-Val-Val-(S,S)Sta-Ala-OCH_3

Iva = Iso-valeryl;
Iaa = Isoamyamide;
Ipa = Isopropylamide

TABLE IV. Intermediates in the Synthesis of P_2, $P_{2'}$ and $P_{3'}$ Analogs

Compound	Compound
15) Boc-(S,S)Sta-Ala-Phe-OMe	37) Boc-(S,S)Sta-Ipa
16) Boc-Val-(S,S)Sta-Ala-Phe-OMe	39) Boc-Leu-Iaa
18) Boc-(S,S)Sta-Ala-Leu-OMe	40) Boc-(S,S)Sta-Leu-Iaa
19) Boc-Val-(S,S)Sta-Ala-Leu-OMe	41) Boc-Val-(S,S)Sta-Leu-Iaa
21) Boc-(S,S)Sta-Ala-Gly-OMe	43) Boc-Gly-Iaa
22) Boc--Val(S,S)Sta-Ala-Gly-OMe	44) Boc-(S,S)Sta-Gly-Iaa
24) Boc-Ala-pFp	45) Boc-Val-(S,S)Sta-Gly-Iaa
25) Boc-Ala-$NHCH_3$	47) Boc-Gly-(S,S)Sta-Ala-Iaa
27) Boc-Ala-Ipa	49) Boc-Ala-(S,S)Sta-Ala-Iaa
28) Boc-(S,S)Sta-Ala-Ipa	51) Boc-Leu-(S,S)Sta-Ala-Iaa
29) Boc-Val-(S,S)Sta-Ala-Ipa	53) Boc-Phe-(S,S)Sta-Ala-Iaa
31) Boc-Val-(S,S)Sta-Oet	55) Boc-Val-Val(S,S)Sta-OEt
33) Iva-Val-(S,S)Sta-OH	57) Boc-Val-Val-(S,S)Sta-OH
35) Boc-(S,S)Sta-pFp	58) Boc-Val-Val-(S,S)Sta-Ala-OMe
36) Boc-(S,S)Sta-Ipa	

pFp = pentafluorophenol

hydrochloride and alanyl-leucine methyl ester hydrochloride, respectively. However, the yields of these reactions were poor to modest (10-50%). Problems with this reaction were: 1) poor solubility of Iva-Val-Sta-OH; 2) poor solubility of the product which made aqueous work-up and necessary chromatography very difficult; 3) the poor yields also represented a significant waste of valuable statine. For these reasons it was decided to proceed with a stepwise synthesis. In this series only compounds 26 and 34 were prepared by coupling Iva-Val-Sta-OH to the respective amine components. This was done only for the sake of convenience. Other compounds which initially posed some difficulty were those which contained the isopropylamine (Ipa) moiety. Initial attempts at the preparation of Boc-Sta-Ipa 36 via standard DCC/HOBT or EEDQ coupling methods (see experimental section) gave poor yields of product. This was apparently due to the formation of a stable salt between the amine and the carboxylate, which was insoluble in the reaction solvent, methylene chloride. This salt formed very rapidly (~10 sec) after addition of the amine to the solution of Boc-Sta-OH and coupling agent. This problem was solved by utilization of an active ester type coupling whereby the active ester could be preformed or even isolated prior to reaction with isopropylamine. The pentafluorophenyl active ester method proved to be suitable for the preparation of these compounds. Reaction between active ester and amines is rapid and proceeds with a low degree of racemization.[68] Furthermore, preparation of the esters by the DCC-pFp "complex" method[69] is also rapid (~20 min). Some other aspects of this reaction, however, had to be taken into consideration. Because of the low pK_a of pentafluorophenol (~6), it was

necessary to add extra equivalents of base to prevent formation of an isopropylamine-pFp salt. The number of extra equivalents added depended upon the method used. For example, if the active ester was formed in situ via the DCC-pFp complex method[69] (36), three extra equivalents of base were added since the ratio of DCC to pFp is 1:3. If the active ester was isolated prior to reaction with free amine (25, 27), then only one extra equivalent of base was required. In the case of compounds 25 and 27, two equivalents of the nucleophile were used rather than using a different tertiary amine base.

1b. Synthesis of $P_{2'}$ analogs

Only two new compounds with $P_{2'}$ variations were synthesized. Again, compounds 42 and 46 were prepared using a standard stepwise approach. Isoamylamide intermediates 39 and 43 were synthesized from the corresponding Boc compounds and isoamylamine using the EEDQ method rather than the DCC/HOBT method which was used to prepare Boc-Ala-Iaa in previous studies.[33] The only reason for this was to facilitate the synthesis by eliminating chromatography which would almost inevitably be involved for the removal of DCU. These compounds were obtained analytically pure after aqueous work-up in high yields (80-90%). The remaining steps toward 42 and 46 were straight-forward (see experimental section).

1c. Synthesis of P_2 analogs

The syntheses of compounds 50, 52 and 54 were analogous to that of the standard peptide 5 whereby Boc-Val-anhydride was

coupled to Sta-Ala-Iaa hydrochloride, followed by deprotection and N-acylation. This procedure was used due to the failure of the DCC/HOBT coupling of Boc-Val-OH to the dipeptide in the preparation of Boc-Val-Sta-Ala-Iaa.[70] The anhydride method was therefore utilized for the preparation of compounds 49, 51 and 53. Yields for these compounds were good (80-90%) except for compound 53 which could only be obtained in 50% yield. Whether this was due to an unfavorable steric interaction with the Sta isobutyl side chain is, however, unclear since the Gly compound 47 could also only be obtained in about 50% yield. This compound was prepared by both the anhydride method and the DCC/HOBT method. Yields for both reactions were similar (~50%) but only the DCC/HOBT procedure is reported in the experimental section.

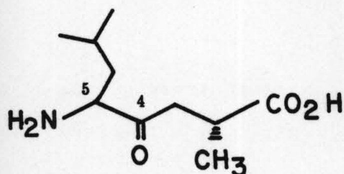
1d. Synthesis of pepstatin fragments

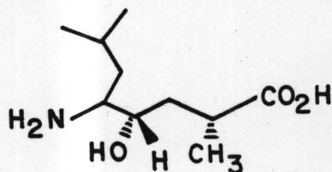
Some pepstatin fragments were also synthesized (56 and 59). Compound 56 was obtained by sequential coupling of Boc-Val-anhydride, Boc-Val-anhydride and isovaleryl anhydride. Compound 59 was prepared from an intermediate of the above. Intermediate 55 was saponified to compound 57 and subsequently coupled to alanine methyl ester hydrochloride. The resulting material was then used to prepare the final product 59. The high yield (82%) of this coupling reaction suggests that the problem of solubility was the reason for failure of the 2 + 2 fragment couplings described earlier, since Boc-Val-Val-Sta-OH (57) was much more soluble in the reaction solvent than Iva-Val-Sta-OH (33).

2. Synthesis of Ketomethylene and Hydroxyethylene Analogs

The unusual structure of statine in comparison to substrates has raised many questions concerning its mechanism of action: 1) Is statine an analog of an amino acid or of a dipeptide? Statine can be considered as being 2 atoms too long to be an amino acid analog or 1 atom shorter than a dipeptide (Fig. 5). 2) If statine is a dipeptide analog, what would be the effect of varying substituents placed in the $P_{1'}$ position?

To answer the first question, Holladay and Rich set out to prepare several ketomethylene and hydroxyethylene dipeptide isosteres (60, 61).[71] These isosteres were incorporated into various peptides and tested for inhibition of porcine pepsin. Kinetic results obtained were consistent with the proposal that statine was more like a dipeptide (this will be discussed in more detail in later sections).[72,73] The synthesis of these compounds is outlined in Figure 6.[71]





60 61

The question concerning the effect of variation in the $P_{1'}$ position still remained. Several analogs were prepared in order to investigate the effect of size and chirality of substituents in this position on inhibition of porcine pepsin. The structures for the isostere

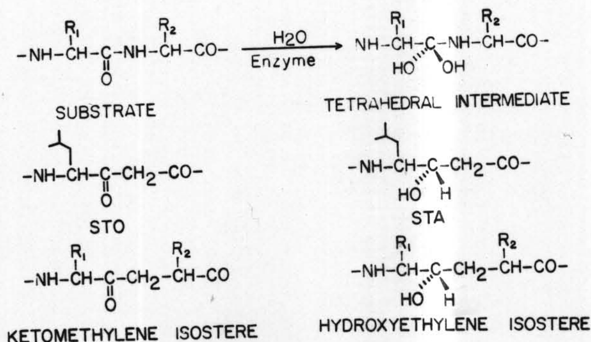


Fig. 5: Comparisons between statine, statone, dipeptide and dipeptide isosteres.

portions of these compounds are shown in Table V, along with their abbreviations. Unless designated otherwise, by appropriate letters under the OH of the hydroxyethylene isosteres compounds should be assumed to be of the 4(S) chirality, they should also be assumed to be isosteric with an L-amino acid in the $P_{1'}$ position unless designated by a D. These isosteres were incorporated into the peptides shown in Table VI.

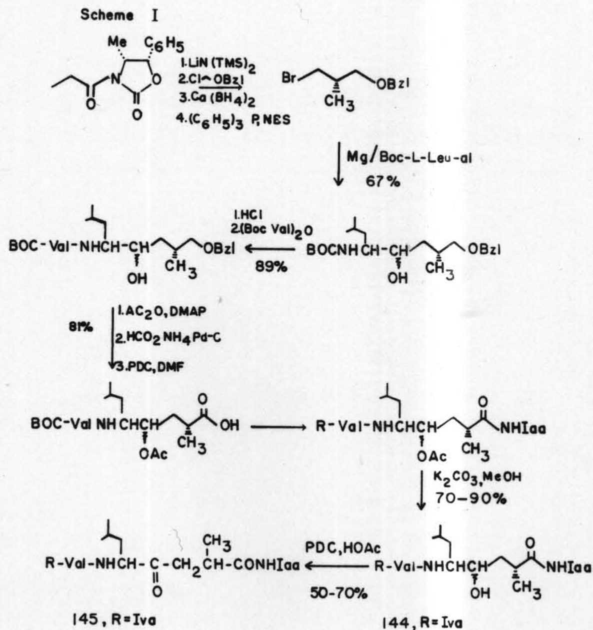


Fig. 6: Synthesis of Leu-Ala ketomethylene and hydroxyethylene analogs.

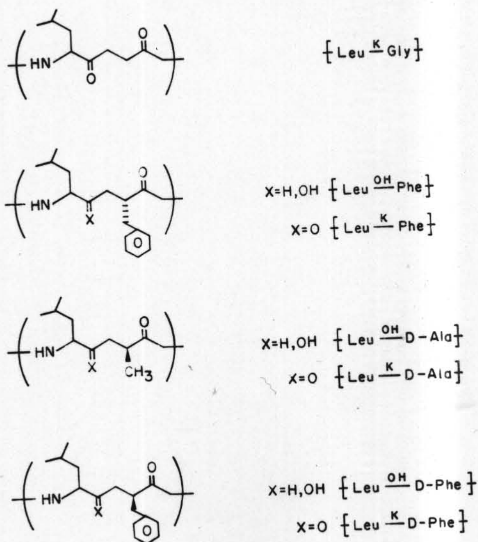


TABLE V. Various dipeptide isosteres prepared.

2a. Synthesis of Leu-Phe isosteres

The synthesis of analogs 75 and 76 is outlined in Figure 7, and is analogous to the synthesis developed by Holladay and Rich.[71] The first step involved acylation of the known 2-oxazolidone 63[74] with dihydrocinnamoyl chloride, using a literature method[74], to form compound 64, which could be stereospecifically alkylated with benzyl bromomethyl ether[75] to form compound 65. This particular

TABLE VI. Ketomethylene and hydroxyethylene final products.

81) Iva-Val-[Leu $\overset{OH}{\text{—}}$ Phe]-Iaa

82) Iva-Val-[Leu $\overset{K}{\text{—}}$ Phe]-Iaa

75) Iva-Val-[Leu $\overset{OH}{\text{—}}$ Phe]-Ala-Iaa

76) Iva-Val-[Leu $\overset{K}{\text{—}}$ Phe]-Ala-Iaa

92a) Iva-Val-[Leu $\overset{OH}{\text{—}}$ D-Ala]-Ala-Iaa

92b) Iva-Val-[Leu $\overset{OH}{\underset{R}{\text{—}}}$ D-Ala]-Ala-Iaa

93) Iva-Val-[Leu $\overset{K}{\text{—}}$ D-Ala]-Ala-Iaa

106) Iva-Val-[Leu $\overset{OH}{\text{—}}$ D-Phe]-Ala-Iaa

107) Iva-Val-[Leu $\overset{K}{\text{—}}$ D-Phe]-Ala-Iaa

137) Iva-Val-[Leu $\overset{K}{\text{—}}$ Gly]-Ala-Iaa

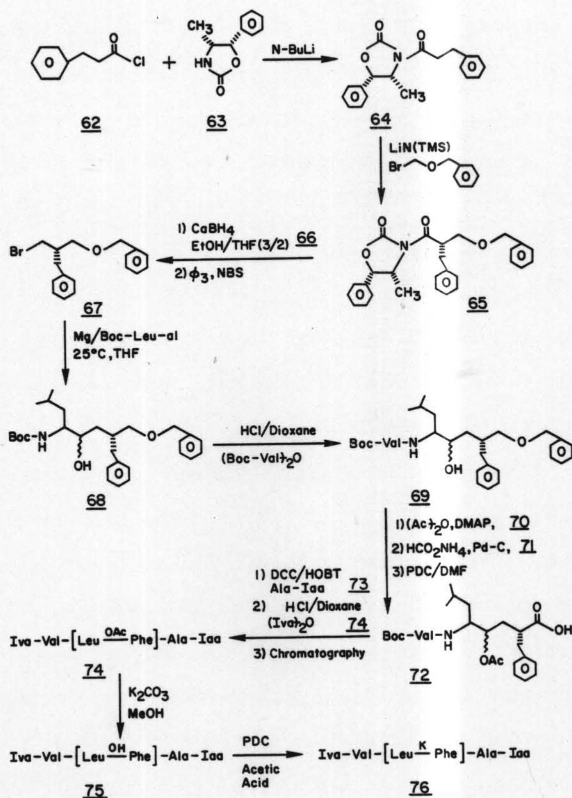


Fig. 7: Synthesis of Leu-Phe ketomethylene and hydroxyethylene analogs.

alkylation proved to be quite sensitive to the nature of the base and the halomethyl benzyl ether. The best yields (~67%) were obtained when lithium hexamethylsilamide was used as base and when bromine was the leaving group. When LDA was used as base or when chloromethyl benzyl ether was used as the electrophile, the yields dropped to the 30% range. Compound 65 was then recrystallized to constant rotation before reduction to the free alcohol. This reduction was carried out in a mixture of THF/EtOH (2/3, v/v). The amount of ethanol in the reaction was critical as a 1/1 mixture resulted in a very slow reaction (<20% conversion in 6 hrs). When calcium borohydride was used as the reducing agent, better yields (~80% vs. 60%) were obtained than when lithium aluminum hydride was used. This finding is consistent with previous observations.[71] Bromide 67, which was prepared from alcohol 66 via a literature procedure[76], was then converted to the Grignard reagent and reacted with Boc-leucinal at 25°C. The product 68 was obtained in a very low yield (~25%) as a mixture of C_4 diastereomers. These diastereomers were easily separable on TLC and it was found that the ratio of 4S:4R was about 3:2. The reason for the low yield is not known but similar reactions (preparation of the Leu $\overset{OH}{\text{—}}$ D-Phe isostere) also gave consistently lower yields (20-30%) than the Leu-Ala isosteres. All of the proceeding steps leading to compounds 75 and 76 were carried out as previously reported.[71] These reactions were performed on a mixture of C_4 diastereomers which were later separated prior to the final deacylation step. The upper major isomer was assumed to be the 4S isomer by analogy to the Leu-Ala isosteres where it was rigorously established that the major, faster R_f isomer formed from the Grignard reaction was

in fact 4S.[71] Compounds 81 and 82 were prepared by a similar procedure as shown in Figure 7 by reacting 72 with the isoamylamine rather than with Ala-Iaa.

2b. Synthesis of Leu-D-Ala-isosteres

Compounds 92a, 92b and 93 were synthesized using essentially the same procedure as developed for the Leu-L-Phe isosteres.

Alcohol 83 (Fig. 8) was prepared according to the procedure reported by Evans et al.[74] which utilizes the oxazolidone derived from L-valine to establish chirality at C-2. This alcohol was converted to bromide 84 using the same procedure as described for the preparation of 67.

In this synthesis the temperature of the Grignard reaction was varied. This reaction was carried out at 0°C rather than at 25°. The yield and the ratio of 4S to 4R, however, were not greatly affected. In the case of the preparation of the Leu-Ala isostere, the 25° Grignard reactions proceeded in ~50-65% yields with 4S:4R ratios of about 4:1.[71] At 0°C the reaction product 85 was obtained in 55% yield with a ratio of 4S:4R of about 2:1. The two diastereomers were, however, not separable by TLC at this stage. Several TLC systems were used in an attempt to resolve the diastereomers of compounds 85 and 86 but these attempts were unsuccessful. Reverse phase HPLC using various gradients of acetonitrile and water was also ineffective towards the separation of the compound 86 diastereomers. Upon acetylation, however, (compound 87) the diastereomers were easily distinguishable on

Fig. 8: Synthesis of Leu-D-Ala ketomethylene and hydroxyethylene analogs.

TLC and by NMR. The acetyl CH_3 peak of the major component was shifted downfield by about 4 Hz and the amide NH of the major isomer was shifted about 13 Hz upfield. Estimations of the ratio of 4S:4R isomers were therefore made at this stage.

The remainder of the synthesis was straightforward as described in Fig. 8 and in the experimental section. As shown in Fig. 8, the 4S:4R diastereomeric mixture was carried through the reaction sequence up to the deacylation step. At this stage compounds 91a and 91b were separated and individually deacylated to compounds 92a and 92b.

2c. Synthesis of Leu-D-Phe isosteres

Compounds 106 and 107 (Fig. 9) were synthesized by a method similar to that of the Leu-L-Phe isosteres. Compound 94 was used as the oxazolidone chiral auxiliary as was the case for the preparation of alcohol 83. The synthesis of compound 95 was straightforward. Alkylation with benzylbromomethyl ether using lithium hexamethylsilamide as the base afforded 96 in high yield (~90%). This compound was recrystallized to constant rotation prior to reduction with lithium borohydride using ethanol/THF (2/1) as solvent to form alcohol 97. Again, this reduction was sensitive to the amount of ethanol. The reaction was initially carried out using a 1/1 mixture of the solvents, but after 4 hrs no product was observed by TLC. Upon addition of more ethanol, however (to make solution 2/1 ethanol/THF), the reaction proceeded to form alcohol 97 in 90% yield after an additional 4 hrs. The optical rotation of the alcohol (-26.5°) was almost exactly the opposite of alcohol 66 (+23.6°). Bromide 98 was prepared as described for

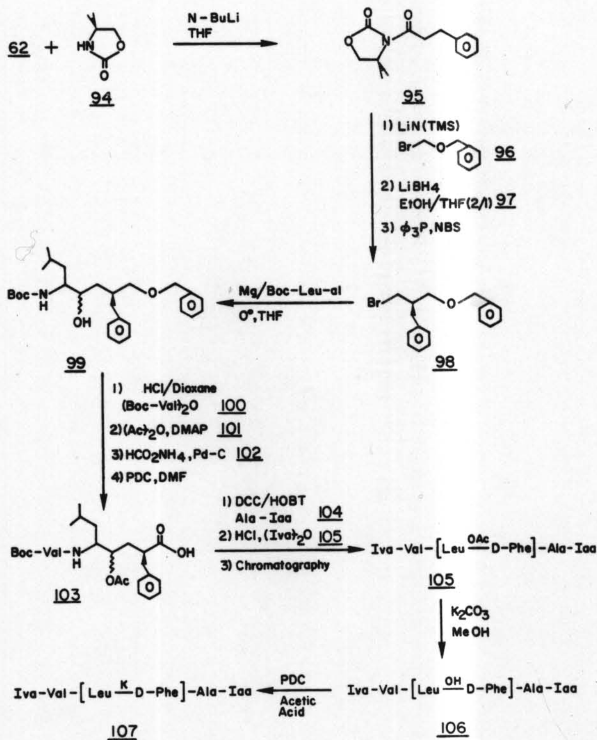


Fig. 9: Synthesis of Leu-D-Phe isosteres.

the other bromides. The rotation of this compound was also almost exactly opposite its enantiomer 67.

The Grignard reaction to form product 99 was carried out at 0° as opposed to 25° for the Leu-L-Phe isosteres. This temperature difference seemed to have a significant effect on the ratio of 4S:4R isomers. The ratio in this case was about 10-20:1 as opposed to about 2:1 in the case of the preparation of 68 at 25°C. It seems unlikely that this difference is due to the altered stereochemistry at C_2 since the attack of the Grignard reagent is supposedly controlled by the stereochemistry of the aldehyde.[77] Attack of a Grignard reagent on the magnesium chelated Boc-L-leucinal (Fig. 10) should be from the back, less hindered face, thereby giving rise to more 4S than 4R compound. If such a species as in Fig. 10 is stabilized at 0°, one would expect a greater amount of the 4S derivative.

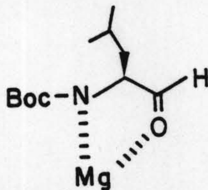


Fig. 10: Boc-L-Leucinal chelated to Mg^{++}.

The yield of this reaction (~30%) is consistent with the yields obtained for the preparation of 68. The fact that the Grignard reaction consistently gave poor yields with the 2-benzyl-3-benzyloxy-1-bromopropanes (67, 98) and the fact that the reactions for preparing 85 and 99 were done side by side, where 85 was obtained in approximately 2-fold better yield, indicates the effect of the size of the 2

substituent in the bromide on the yield of these reactions. Larger side chains have consistently given poorer yields.

As was the case for the Leu-D-Ala isosteres, diastereomers were not observable for the Leu-D-Phe isosteres until the O-acetyl stage (compound 101). At this point the mixture could be estimated by NMR and TLC to contain about a 20:1 ratio of 4S to 4R diastereomer. The mixture was used for the synthesis without separation as with the other isosteres, however, after the chromatography step (105 ⟶ 105a + 105b) none of the lower minor isomer was detected. The small amount that was present at earlier stages had probably been separated during the numerous columns that were run prior to this stage. With an approximate 20:1 4S:4R ratio, the amount of Grignard product (99) that was used would have had about 25 mg of the 4R isomer in with 475 mg of the 4S isomer. In the final column purification of compound 105, about 50 mg were applied. The amount of 4R material here could have been no higher than 2 or 3 mg based on these ratios. It is therefore not surprising that no 4R compound was detected in the final stages of the synthesis.

3. Synthesis of Lysine-Statine (LySta) Derivatives

The synthesis of the lysine derivative of statine 109 (LySta or DAHOA, 4,8-diamino-3-hydroxy octanoic acid) began with the preparation of α-Boc-ε-Cbz-lysinal 108, by the method of Hamada and Shiriri.[66] Reaction of 108 with lithio ethyl acetate at -78°C for 30 minutes gave the desired fully protected amino acid 109a,b in about 70% yield as a mixture of diastereomers. The diastereomers of this compound, however, were not separable in 15-20 TLC systems. It was found,

however, that O-protected compounds were easily separable by TLC or column chromatography especially when run in ethyl ether systems such as ethyl ether/hexane. Initially compound 110a,b was synthesized in order to develop a separation procedure, but the method was not applied to the actual separation because deprotection of the carbonate derivatives was too slow. After 24 hrs at room temperature with 10 equivalents of K_2CO_3, neither compounds 110a nor 110b underwent significant reaction. When heated to 50°C, compound 110a gave product 109a but only after several hours reaction. Compound 110b, however, still showed no significant transformation to 109b. In light of this, the ethoxy carbonyl mode of protection was abandoned for the trichloroethoxycarbonyl protection scheme. Initial attempts to prepare compound 111a,b using methods similar to the preparation of 110a,b failed. However, compound 111a,b was easily formed in high yield in 20 min by reaction with trichloroethoxy chloroformate in pyridine with a trace of DMAP. These diastereomers were also easily separable by chromatography. Deprotection by reaction of compounds 111a and 111b with zinc in a 1:1 mixture of DMF/acetic acid was slower than desirable (~12 hrs). This problem was circumvented by the use of cadmium powder in place of zinc.[78] The cadmium used was a cadmium 200 mesh powder purchased from Alfa. It was activated by successively washing with 1N HCl, methanol and ethyl ether followed by vacuum drying. Therefore, reaction of 111a with 20 equivalents of cadmium in a 1:1 mixture of DMF/acetic acid afforded compound 109a in about 90% yield after about 4 hrs. However, compound 111b was deprotected to a lesser extent (~57%) after the same amount of time. Fortunately, no side products were

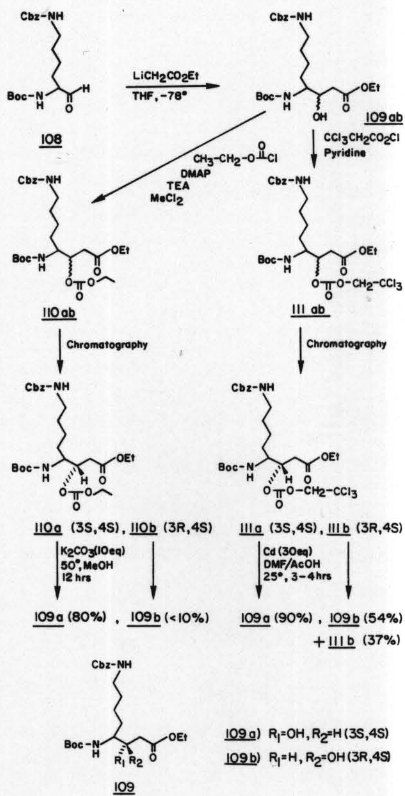


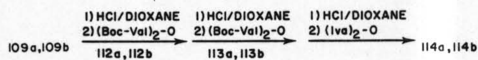
Fig. 11: Synthesis of diamino-hydroxy-octanoic acid (LySta or DAHOA).

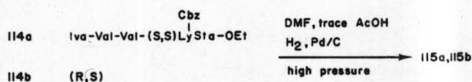
formed in these reactions and unreacted 111b was easily recovered.

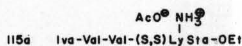
It is uncertain why in both cases (compounds 110a,b and 111a,b) the 3R O-protected compounds were deprotected slower than the 3S compounds, but one explanation may involve the relative orientation of the C_3 and C_4 substituents. If compounds 110a,b and 111a,b are held in a rigid conformation (the extraordinary differences between the 3S and 3R isomers on TLC may indicate that this is a possibility), then the protected hydroxyl group of the 3S derivative is trans to the 4S side chain and the 3R derivative is cis to the 4S side chain. The size of the 4S side chain may, therefore, be involved in reagent access problems in the 3R isomer since the side chains are closer together than in the 3S isomers.

Compounds 109a and 109b were separately used to prepare peptides 115a, 115b and 119a by the routes described in Figure 12. 115a and 115b were prepared by sequentially coupling Boc-valine anhydride two times to the starting compounds 109a and 109b followed by an isovaleryl anhydride coupling to obtain 114a and 114b. The Cbz group was then removed using standard catalytic hydrogenation procedures. Compound 119a was prepared by saponification of intermediate 113a followed by coupling to phenylalanine methyl ester hydrochloride using DCC and HOBT. 117a was subsequently treated as described for compound 114a to yield 119a. In all cases the final product amines were assumed to be the acetate salts, since hydrogenation was carried out in DMF in the presence of acetic acid.

A

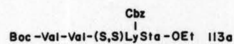


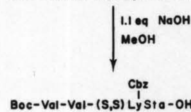


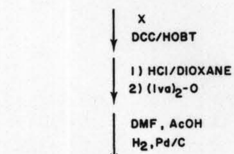


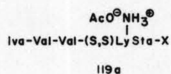
115b (R,S)

B









119b X = H_2N-Phe-OMe

Fig. 12A,B: Synthesis of LySta peptides.

4. Synthesis of Ornithine-Statine (OrnSta) Derivatives

The synthesis of the ornithine-statine (OrnSta) analog 132 is described in Figure 15. Initial attempts to prepare compound 123 by path A or path B (Fig. 13) failed. When compound 120 was subjected to conditions analogous to that for the preparation of 108, the cyclic aminal 122 was obtained rather than the aldehyde. The structure of 122 was consistent with the NMR data of the observed products (2 diastereomers). The products showed no aldehyde peaks and no Cbz NH protons. Both NMR spectra also contained a signal which integrated for one proton at approximately 5.7 ppm (5.7 ppm for the upper isomer, 5.6 ppm for the lower). This is consistent with the calculated chemical shift of a proton on a carbon which is alpha to a carbamate and a hydroxyl group. Elemental analysis was also consistent with the structure.

122 was isolated from the reaction mixture (Fig. 13, path A) in about 60% yield from 121 as a 1:1 mixture of diastereomers, which were very easily separable on silica gel. Only one attempt at the conversion of 122 to 123 was performed. Compound 122 was treated with 2.5 equivalents of lithio ethyl acetate at -78°C for 30 minutes, however, only starting material was recovered.

The synthesis of a derivative of 123 was therefore achieved by a different route. The solution to the problem was to protect both delta NH's of ornithine. The phthaloyl group is one protection group which will mask both NH's so this was investigated as a possible solution (Fig. 14) even though there were several obvious drawbacks. One is that the phthaloyl group is very sensitive to borohydride and other

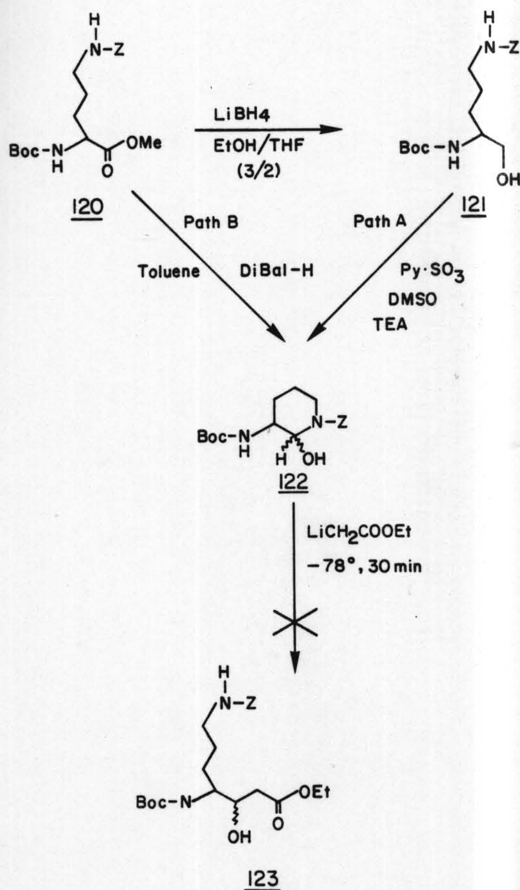


Fig. 13: Attempted preparations of α-Boc-δ-Cbz ornithinal.

124 → (MeOH, 4hrs.) → 133 → (Py·SO$_3$, DMSO, TEA) → 134 → ($LiCH_2COOEt$, THF, -78°, 20 min) → 135 (<10%) + 136 (50-60%)

Fig. 14: Attempted preparation of N^4-(Boc)-N^7-(phthaloyl)-4(S),7-diamino-3(R,S)-hydroxy-heptanoic acid ethyl ester.

hydride reductions[79], which are reactions required to form the needed amino alcohols. It is thought that these reagents lead to reduction of the phthaloyl carbonyls to alcohols.[80] This problem, however, could be circumvented by preparing the phthaloyl derivative of amino alcohol 121. This was easily done by reaction of compound 121 with carbethoxy phthalimide[81] in methanol for 4 hrs to form compound 133 in about 78% yield. Another anticipated problem was the enolate addition. It was observed by E. T. O. Sun that the addition of phthaloyl leucinal to lithio ethyl acetate gave poor yields of phthaloyl statine ethyl ester, although inverse additions gave yields in the 50% range.[70] The attempted preparation of 135 was therefore carried out in such a manner where lithio ethyl acetate was added to the aldehyde 134. Several products, however, were isolated from this reaction. The desired product 135 was obtained in less than 10% yield as estimated by NMR. The major product obtained (~50-60%) was assumed to be compound 136. The NMR of this compound contained an aromatic signal at 7.55 ppm as opposed to 7.75 ppm for a typical phthaloyl aromatic signal. This upfield shift is consistent with the observed shift for the reduced phthaloyl ring product described previously.[80] Also the NMR indicated a doubling of the signals associated with the ethyl ester moiety. These data are consistent with the assumption that an extra ethyl acetate had indeed attacked the phthaloyl carbonyls to form 136. About 20-30% of the starting aldehyde was also recovered indicating that a shorter reaction time to decrease formation of 136 would be impractical. The phthaloyl procedure was therefore abandoned for the procedure described in Figure 15.

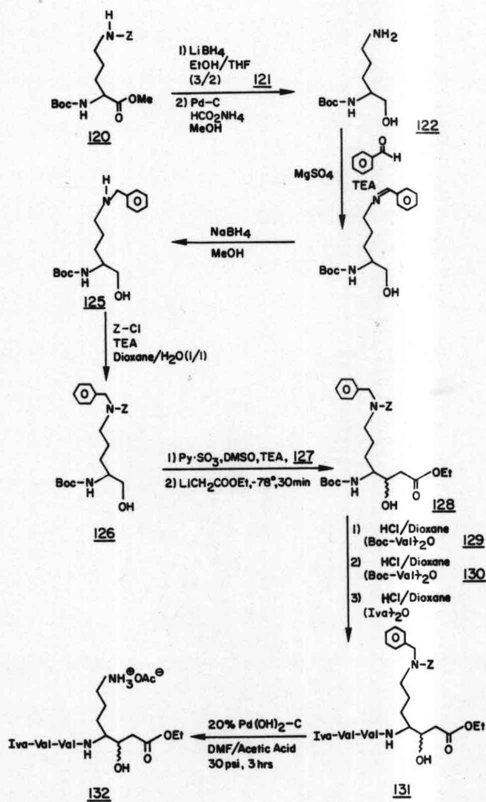


Fig. 15: Synthesis of N^4-(Boc)-N^7-(benzyl)-N^7-(Cbz)-4(S),7-diamino-3-(R,S)-hydroxy-heptanoic acid ethyl ester (OrnSta) and OrnSta containing peptides.

Compound 121 was deprotected using standard catalytic transfer hydrogenolysis[82] for the removal of Cbz groups to form amino alcohol 124. It should be noted that this procedure must be carried out on alcohol 121 rather than the methyl ester 120 since removal of the Cbz group of 120 resulted in lactam formation. Compound 124 was then treated with benzaldehyde, $MgSO_4$ and triethylamine in methylene chloride to form the Schiff base. The Schiff base was isolated by filtering magnesium sulfate and removing the solvent in vacuo. The crude material was immediately dissolved in dry methanol and reacted with sodium borohydride to form 125 in 65% yield from 124. Treatment with benzyl chloroformate at pH 10 in a dioxane/water mixture then afforded alcohol 124, which was converted to aldehyde 127 via the method of Hamada and Shioriri.[66] Compound 127 was subsequently treated with lithio ethyl acetate in tetrahydrofuran to yield 128 as a mixture of C_3 diastereomers. This mixture was used throughout the remainder of the synthesis which involved sequential couplings to Boc-valine anhydride twice and isovaleryl anhydride to afford compound 131. The benzyl and Cbz groups were then simultaneously removed by treatment with 20% palladium hydroxide on carbon at 30 psi in DMF with a trace of acetic acid for 3 hrs, to form the acetate salt of the free amine 132. This compound was isolated in a manner similar to the lysine-statine peptides.

B. X-Ray Crystal Structure of Penicillopepsin Bound to Iva-Val-Val-Sta-OEt (56)

X-ray crystallographic analysis of inhibitor binding to the active sites of enzymes can provide significant information concerning

the binding modes of substrates and the enzymes' catalytic pathway. Recently James et al. have reported the x-ray crystal structure of the complex between the aspartic proteinase penicillopepsin and the tripeptide statine containing inhibitor Iva-Val-Val-Sta-OEt (56).[31a,b] The high resolution refined crystal structure of penicillopepsin[42] was used to compute a 1.8 Å electron density map of the bound inhibitor.

Penicillopepsin is an aspartic proteinase isolated from the mold Penicillium janthinillum. This enzyme shows significant sequence homology to other known aspartic proteinases. Penicillopepsin, as well as other aspartic proteinases, is characterized by a long binding cleft which can accommodate 7-8 amino acid residues of an oligopeptide substrate in an extended conformation. Asp-33 and Asp-213, the two catalytically important residues for penicillopepsin are located centrally in this cleft.

The electron density map of this enzyme bound to inhibitor 56 has clearly demonstrated the binding mode of the inhibitor on the enzyme and has also revealed a large conformational change in the enzyme, produced as a result of inhibitor binding.

The inhibitor (56) is bound to penicillopepsin in an extended, approximate ß-sheet conformation with the 3S hydroxyl of Sta situated between the catalytic aspartate carboxyl groups. The P_4 isovaleryl group of 56, which makes relatively few non-bonded contacts with the enzyme, lies in a small hydrophobic pocket defined by Leu-218, Leu-284 and Tyr-274. The P_3 valine makes two strong hydrogen bonding interactions to Thr-217 as well as several other non-bonded contacts of less than 4.0 Å. A surface depression on the enzyme formed by the side

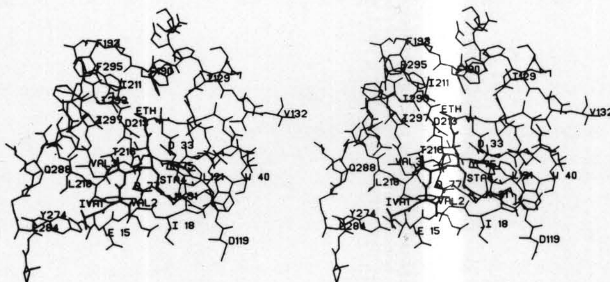


Fig. 16: Stereo view of the active site of penicillopepsin with bound inhibitor 56.

chains of Glu-15 and Glu-16 discerns this binding site which appears to be able to accommodate larger groups. The P_2 valine makes 21 non-bonded interactions with the enzyme, 15 of which are with the residues of the movable "flap" (residues 71-83). Eleven of these 15 interactions are with Asp-77.

The P_1 statyl residue has the largest number of contacts to the enzyme. A hydrogen bond between the Sta NH and Gly-215 helps position what would be the scissle amide bond in a substrate correctly at the active site. The formation of this hydrogen bond in substrates is

likely to be of great importance because, in the case of porcine pepsin, proline in the S_1 position of a substrate dramatically reduces cleavage possibility.[87] The 3S hydroxyl group of Sta displaces a strongly bound water molecule from the active site of native enzyme. The position of the Sta oxygen and the water oxygen in the native enzyme[42] differ by only 0.25 Å.

The largest conformational changes of the enzyme that can be attributed to inhibitor binding are associated with the residues of the mobile flap.[31a,b] Ninety percent of the enzyme's remaining atoms move by less than 0.3 Å. The carbonyl of Asp-33 undergoes a slight rotation but Asp-213 is fixed. Gly-76 and Asp-77 of the flap move by about 4 Å towards the inhibitor thus closing the binding cleft and permitting the formation of hydrogen bonds with the inhibitor backbone. Since these contacts are exclusively to the peptide backbone and are not associated with side chain specificity, similar contacts could also form with the backbone of a good substrate. These interactions could therefore be involved in positioning the scissle bond in the proper orientation for cleavage. The movement of the flap has also eliminated the possible role of the hydroxyl group of Tyr-75 as a proton donor, as was thought from earlier studies.[43] This study suggested that the Tyr-75 hydroxyl was involved in the catalytic mechanism by donating a proton to the leaving group nitrogen, however, this more recent crystal structure has suggested that Tyr-75 is involved only in enhancement of specific binding interactions to the S_1 side chain which often exhibits an aromatic preference.[87]

Computer graphic modeling studies, performed by Hofmann et al.[91], have been carried out to study the possible binding modes of good penicillopepsin substrates. Their proposals were based on the refined crystal structure described. A more detailed discussion of this will be treated in later sections concerning the lysine-statine (LySta) analog inhibitors.

C. Kinetic Studies

1. Effect of Structure in Subsites P_2, $P_{2'}$ and $P_{3'}$ on Inhibition of Porcine Pepsin

a. Results

Inhibition constants (K_i) for inhibition of pepsin by pepstatin analogs (Table VII) were determined as previously described[37] from IC_{50} values taken from plots of Vi/Vo vs. inhibitor concentration, where Vi is the inhibited velocity and Vo is the velocity in the absence of inhibitor. IC_{50} values were converted to K_i by the equation of Cha[83]:

$$K_i = (IC_{50} - \frac{Et}{2})(1 + \frac{S}{k_m})^{-1}$$

where Et is the total enzyme concentration, K_m is the Michaelis constant for the substrate (4.0×10^{-5} M), and S is the substrate concentration. When inhibitors exhibited time-dependent inhibition, the inhibitor was incubated with enzyme for 10 minutes and the reaction was initiated by addition of substrate. Velocities were taken at apparent steady state. Inhibitors which did not display time-dependent

inhibition gave linear steady-state initial velocities when reactions were initiated with enzyme. Inhibitor solutions were generally made to contain 1% methanol by volume. Some inhibitors (56, 59) required 10% methanol because of their poor solubility in the buffer. Only inhibitor 48 was insufficiently soluble to prevent precise quantitation of its true K_i.

The data in Table VII show how structural differences in the $P_{3'}$, $P_{2'}$ and P_2 subsites affect K_i. Inhibition constants for analogs with $P_{3'}$ variations or deletions exhibit K_i's from ~10^{-9} M to 10^{-7} M; however, this range encompasses relatively large structural variations. Compounds 5, 17 and 20 all have K_i's near 10^{-9} M and exhibit time-dependent inhibition, indicating that the size or nature of the hydrophobic group is not important, and that the carboxyl of the $P_{3'}$ site is not required. Removal of the hydrophobic side chain (23), however, increases K_i by about 40 fold and eliminates time-dependent inhibition, indicating a need for a hydrophobic or chiral side chain. Compounds 26 and 30 further illustrate the effect of branching at the carbon of the $P_{3'}$ site. Isopropylamide compound 30 has about a 10 fold lower K_i than the methyl amide 26 and does not exhibit time-dependent inhibition.

The role of the $P_{3'}$ NH can be demonstrated by comparing compounds 26 and 34. Our results indicate that the amide NH is relatively unimportant for inhibitor binding and is probably not involved in any critical hydrogen bonds. Complete removal of the $P_{3'}$ carbon skeleton (32, 38) further increases K_i.

TABLE VII. Inhibition Constants for Pepstatin Analogs on Porcine Pepsin

	Compound	K_i (nm)	TDI
5)	Iva-Val-(S,S)Sta-Ala-Iaa	3	+
17)	Iva-Val-(S,S)Sta-Ala-Phe-OMe	1.5	+
20)	Iva-Val-(S,S)Sta-Ala-Leu-OMe	1.1	+
23)	Iva-Val-(S,S)Sta-Ala-Gly-OMe	68	-
30)	Iva-Val-(S,S)Sta-Ala-Ipa	20	-
26)	Iva-Val-(S,S)Sta-Ala-$NHCH_3$	140	-
34)	Iva-Val-(S,S)Sta-Ala-OCH_3	600	-
32)	Iva-Val-(S,S)Sta-OCH_2-CH_3	800	-
38)	Iva-Val-(S,S)Sta-Ipa	300	-
5)	Iva-Val-(S,S)Sta-Ala-Iaa	3	+
42)	Iva-Val-(S,S)Sta-Leu-Iaa	4.6	+
46)	Iva-Val-(S,S)Sta-Gly-Iaa	56	-
5)	Iva-Val-(S,S)Sta-Ala-Iaa	3	+
48)	Iva-Gly-(S,S)Sta-Ala-Iaa	100	-
50)	Iva-Ala-(S,S)Sta-Ala-Iaa	30	+
52)	Iva-Leu-(S,S)Sta-Ala-Iaa	13	-
54)	Iva-Phe(S,S)-Sta-Ala-Iaa	21	-
56)	Iva-Val-Val-(S,S)Sta-OCH_2CH_3	10	-
59)	Iva-Val-Val-(S,S)Sta-Ala-OCH_3	3.5	+

Similarly, relatively large structural changes in the $P_{2'}$ subsite produce small changes in K_i (10^{-9} to 10^{-8} M). Compounds 5 and 42 are comparable inhibitors, while complete removal of the side chain (46) increases K_i about 20 fold, suggesting preference for a hydrophobic or chiral residue.

The P_2 subsite on the other hand, is relatively more sensitive to structural modification. The compounds tested in this series exhibit a range of K_i's similar to those in the $P_{3'}$ site (10^{-9} to 10^{-7} M). However, relatively subtle changes in structure are represented in this range. Replacement of the isopropyl side chain (5) by isobutyl (52) or benzyl (54) in the P_2 subsite increases K_i 5-10 fold and eliminates time-dependent inhibition. Interestingly, a methyl substituted analog (50) remains time-dependent although it is a 10-fold weaker inhibitor. Removal of the P_2 side chain (48) increases the K_i dramatically.

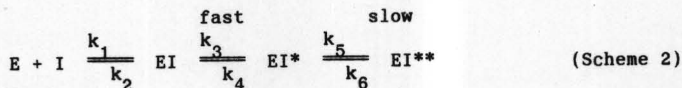
b. Discussion

As described in previous sections, a number of structure-activity relationship studies have been carried out on the pepstatin-pepsin system to define the effect of structural changes on subsites P_4, P_3 and $P_{4'}$ of pepstatin. The present study provides the first data on the effect of structure in the P_2, $P_{2'}$ and $P_{3'}$ positions of pepstatin on inhibition of pepsin.

Inhibition constants for analogs with $P_{2'}$ and $P_{3'}$ variations indicate that there is probably a less specific hydrophobic interaction between enzyme and inhibitor at these sites. Our data show that the C-terminus is needed but is much less sensitive to the nature of the side

chain beyond the need for α-amino acids. The $P_{3'}$ residue is required as truncated analogs are poor inhibitors, however, the only requirement of this residue is that it should be a hydrophobic one. Recent ESR data, utilizing spin labeled pepstatin analogs[84] indicate that the C-terminal region of pepstatin has only partial restriction of mobility when bound to pepsin, which is consistent with loose interactions between pepsin and the $P_{3'}$ site. In contrast, the N-terminal substituted spin label analog is almost completely immobilized and is consistent with much tighter binding at the P_3 subsite.

Recent studies on pepsin using various structural analogs of pepstatin[37,63,61,85] have led to the proposal of a minimum kinetic mechanism (Scheme 2)



$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_4}{\overset{k_3}{\underset{\text{fast}}{\rightleftharpoons}}} EI^* \underset{k_6}{\overset{k_5}{\underset{\text{slow}}{\rightleftharpoons}}} EI^{**} \qquad \text{(Scheme 2)}$$

involving 3 distinct enzyme inhibitor complexes for inhibition of this enzyme. The initial collision complex (EI) isomerizes via a fast first order process to an intermediate (EI*) complex. The dissociation constant for EI* has been determined to be about 1×10^{-8} M for pepstatin.[34] Inhibitors which exhibit time-dependent inhibition then undergo a slow conversion to a tightened EI** complex. It is possible that the tightened complex is formed directly from free enzyme <u>via</u> a branched pathway. Our present kinetic data cannot distinguish between these possibilities.

The data for P_2 analogs suggests that this site may be involved in the conformational changes required for the slow binding process. Analogs having side chains larger than isopropyl (52, 54) do not bind slowly, possibly because steric bulk prohibits conformational changes required to achieve the tightened EI** complex. The analog with the smaller side chain (50) may allow this conformational change to occur due to its small size although it lacks the hydrophobicity of larger groups and has a larger dissociation constant for EI*.

An x-ray structure for the complex of pepstatin analog 56 bound to penicillopepsin has recently been obtained.[31] This x-ray structure has been discussed in more detail in the previous section. Analog 56 adopts a ß-sheet conformation when bound in the active site of the enzyme with 15 non-bonded interactions between atoms in the P_2 site and sites on the enzyme. Twelve of these interactions involve Asp-77 which is found in the movable "flap" of penicillopepsin. Although the nature of the slow-binding process is not yet known, it is reasonable to speculate that a further tightening between the P_2 position and the residue in porcine pepsin homologous to penicillopepsin Asp-77 after a major conformational change has occurred (flap closing) may be involved.

2. Inhibition of Porcine Pepsin by Ketomethylene and Hydroxyethylene Dipeptide Isosteres

a. Results

Inhibition constants for the inhibition of porcine pepsin by various ketomethylene and hydroxyethylene isosteres are shown in Table VIII. Several compounds (144-148) were previously

synthesized[71] and tested kinetically[72,73] by Dr. Mark Holladay in the lab of Dr. D. H. Rich and are used for comparison of other $P_{1'}$ derivatives. The configuration of the substituent in the $P_{1'}$ position corresponds to that of an L-amino acid unless specified by a D. The configuration of the 4-hydroxyl group is S in all analogues unless specified by an R under the OH sign of the isostere. All compounds were tested on porcine pepsin as previously described[37] using synthetic heptapeptide substrate.

The compounds in Table VIII show how variations in structure and chirality in the $P_{1'}$ position of the dipeptide isosteres affect K_i. Replacement of the 2R methyl group in isostere 144 by a 2R benzyl group (81) has no effect on K_i. Comparison of the ketomethylene analogs 145 and 82 unfortunately is difficult since the true K_i of 82 was not determined due to poor solubility.

For analogs extended in the C-terminal direction, replacement of the pro-R hydrogen in the 2 position of 146 by a methyl group results in a 16 fold more potent inhibitor. However a bulkier substituent (75) does not have any further effect on K_i. The analogous ketomethylene compounds 76, 137 and 148 show an interesting trend. The Leu$\overset{K}{—}$Gly compound has a poor K_i which is decreased almost 100 fold by replacing the $P_{1'}$ hydrogen with a 2R methyl group. However, further increase of steric bulk of the $P_{1'}$ site (76) raises K_i by about a factor of 3. None of these ketones are slow binders.

Inhibitors which contain a 2S substituent (Leu-D-amino acid isosteres) follow the same trend found for the 2R compounds; however they are significantly weaker inhibitors. Compounds 92a and 106

TABLE VIII. Inhibition Constants for Ketomethylene and Hydroxyethylene Compounds on Porcine Pepsin

	Compound	K_i (nm)	TDI
5)	Iva-Val-Sta-Ala-Iaa[a]	3	+
144)	Iva-Val-[Leu $\overline{OH}$ Ala]-Iaa[a]	27	-
81)	Iva-Val-[Leu $\overline{OH}$ Phe]-Iaa	27	-
145)	Iva-Val-[Leu $\overline{K}$ Ala]-Iaa[a]	970	-
82)	Iva-Val-[Leu $\overline{K}$ Phe]-Iaa*	~500	-
146)	Iva-Val-[Leu $\overline{OH}$ Gly]-Ala-Iaa[a]	50	-
147)	Iva-Val-[Leu $\overline{OH}$ Ala]-Ala-Iaa[a]	3	+
75)	Iva-Val-[Leu $\overline{OH}$ Phe]-Ala-Iaa	2.5	+
137)	Iva-Val-[Leu $\overline{K}$ Gly]-Ala-Iaa	2140	-
148)	Iva-Val-[Leu $\overline{K}$ Ala]-Ala-Iaa[a]	27	-
76)	Iva-Val-[Leu $\overline{K}$ Phe]-Ala-Iaa	72	-
92a)	Iva-Val-[Leu $\overline{OH}$ D-Ala]-Ala-Iaa	160	-
92b)	Iva-Val-[Leu $\frac{OH}{R}$ D-Ala]-Ala-Iaa	>1000	-
106)	Iva-Val-[Leu $\overline{OH}$ D-Phe]-Ala-Iaa	140	-
93)	Iva-Val-[Leu $\overline{K}$ D-Ala]-Ala-Iaa	480	-
107)	Iva-Val-[Leu $\overline{K}$ D-Phe]-Ala-Iaa	850	-

[a]Compounds previously reported ref. 72, 73. [b]Compounds previously reported ref. 33. *True K_i unavailable due to poor solubility. K = ketomethylene. TDI = time dependent inhibition. OH = hydroxyethylene derivative. Unless mentioned otherwise by lettering under the OH, the isosteres have the 4(S) configuration. P_1` positions correspond to L-amino acid groups unless mentioned otherwise.

inhibit porcine pepsin to a similar extent, but they are about 50 fold weaker inhibitors than the corresponding Leu-L-amino acid isosteres. Ketone compounds 93 and 107 also show a trend similar to the ketomethylenes which contain a 2R group 148, 76. Compound 107 which contains a 2S benzyl group is about a 2 fold worse inhibitor than compound 93.

b. Discussion

The unusual structure of statine has raised several questions concerning its mechanism of action as an inhibitor of aspartic proteinases. The first question concerns statine's analogy to either an amino acid or a dipeptide. Because of carbons 1 and 2 of statine it can be considered as being two atoms too long to be an amino acid analog or one atom too short to be a dipeptide analog. Powers suggested on the basis of extensive comparison of pepsin substrate sequences that statine may be closer to a dipeptide.[97] More recently, Boger proposed a more specific model, using x-ray data for pepstatin bound to Rhizopus chinensis aspartic proteinase. He postulated that statine is an analog of an enzyme-bound dipeptide substrate in the tetrahedral intermediate form. A comparison between the C-terminal segment of pepstatin and the tetrahedral form of -Leu-Leu-Val-Phe- is shown in Figure 17.[88] From these models, it was clear that the isobutyl and hydroxyl groups of the central statine of pepstatin and the first leucine of the substrate can bind to the same enzyme site (S_1), while at the same time the isobutyl side chain of the second statine and the benzyl group of the substrate can bind to the $S_{3'}$ site. This steric match could only be possible if statine serves as a dipeptide

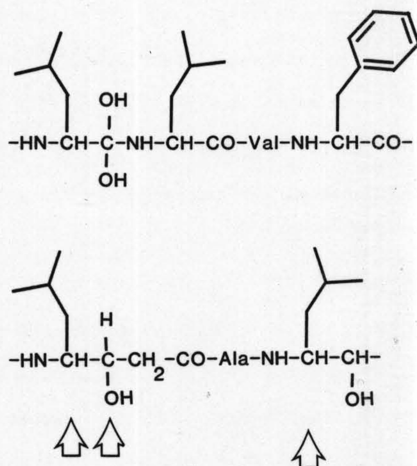


Fig. 17: Comparison of Leu-Leu-Val-Phe tetrahedral intermediate with Sta-Ala-Sta.

replacement. Szelke et al.[89] and Boger et al.[119] have recently reported the synthesis of various renin inhibitors which are based on renin substrates. When the Leu-Leu or Leu-Val sequence of a substrate was replaced by statine, potent inhibitors of renin were obtained.[119] When statine was then replaced by a hydroxyethylene dipeptide isostere, an inhibitor of similar potency was obtained.[89] These data are again consistent with the hypothesis that statine is acting as a dipeptide.

Dr. Mark Holladay, working in the laboratory of Dr. D. H. Rich, has also studied this problem and has synthesized various ketomethylene

and hydroxyethylene dipeptide isosteres (60, 61).[71-73] These isosteres were prepared and incorporated into peptides (144-148, Table VIII) to test for inhibition of the porcine pepsin aspartic proteinase system.

Comparison of hydroxyethylene inhibitor 144 and 147 with the Sta containing inhibitor 5 reveals that the K_i of 147, in which the Leu-Ala isostere replaces Sta is remarkably similar to the K_i of 5. However, the K_i of 144, in which the Leu-Ala isostere replaces Sta-Ala of 5, is about 10 fold higher than the K_i of 5.

These effects on pepsin inhibition along with those of renin reported by Szelke and Boger support the postulate that statine is an analog of a dipeptide tetrahedral intermediate. The isobutyl group of statine corresponds to the P_1 substituent that binds to the S_1 subsite on the enzyme. The C-1 and C-2 atoms serve to span the distance between the P_1 and $P_{2'}$ sites. Therefore, the alanine of pepstatin binds approximately to the $S_{2'}$ site and not the $S_{1'}$ site.

This proposal then leads nicely into the second question concerning statine's structure. If statine is a dipeptide analog and serves to span the distance between the P_1 to $P_{2'}$ binding sites, what would be the result of adding substituents which could bind to the $P_{1'}$ position? Since statine lacks a side chain which could bind to this site on the enzyme, one could suggest that replacement of statine with a dipeptide isostere, which contained a $P_{1'}$ side chain that mimics the $S_{1'}$ side chain of a good substrate, may result in inhibitors which are more potent than Sta containing inhibitors due to the added interaction of the $P_{1'}$ site in the dipeptide isostere. I therefore set out to prepare several $P_{1'}$ analogs (Table VIII) which would more closely

approximate the $S_{1'}$ site of good pepsin substrates. Compounds 75, 76, 81, 82 were synthesized and tested as porcine pepsin inhibitors. These inhibitors contain a benzyl group in the $P_{1'}$ position mimicking an L-phenylalanine residue in the $S_{1'}$ site of a substrate. It is clear, however, from Table VIII that a benzyl group in this position does not decrease K_i for the hydroxyethylene analogs compared to the Leu-Ala isosteres. Compounds 81 and 144 have virtually identical Ki values. Likewise, compounds 75 and 147 have similar K_i's. There is a favorable interaction by replacing the pro-R hydrogen of C-2 in the Leu-Gly analog (146) by a methyl group, but increasing steric bulk has no further effects. In the case of the ketomethylene analogs (137, 148, 76), a similar trend is observed where replacing a proton with a methyl (137 vs. 148) results in a better inhibitor, however, in this case, compound 76, which more closely approximates a good substrate is about a three fold weaker inhibitor than 148.

In light of these results, a literature review on porcine pepsin-substrate specificity seemed in order. This topic has been the subject of many studies, mainly by Dr. Joseph Fruton.[47-49,53] His studies have addressed numerous questions on this topic. One interesting observation that he has made concerns the relative variance of kinetic parameters such as K_m and K_{cat} with substrate structure when comparing large numbers of substrates. He has found that K_m varies little over markedly different substrates, whereas the K_{cat} parameters vary widely. For example, compounds of the type Z-His-X-Y-OMe (a sample of which are shown in Table IX) where X and Y are Gly, Leu, Tyr or Phe have been prepared and tested as substrates of porcine pepsin. The K_m's for

TABLE IX. Kinetic Constants for Several Porcine Pepsin Substrates with $S_{1'}$ Variations

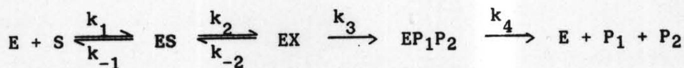
Substrates	$V/K \times 10^4$	$K_m \times 10^3$	$K_{cat} \times 10^4$
S_2 $S_{1'}$ $S_{1'}$			
Z-His-Phe-Gly-OMe	13	1.6	21
-Ala-OMe	21	1.8	37
-Leu-OMe	100	.5	52
-Tyr-OMe	5900	.29	1700

these substrates range from .18-1.8 mM (about one order of magnitude), however, the K_{cat} values vary more than 1000 fold. It has been proposed that for this system the K_m values approximately equal the K_s dissociation constant for enzyme and substrate. The rate limiting step is then involved in catalysis.[53] In view of these results, the side chain specificity of pepsin is an expression of kinetic, rather than binding, specificity. It can be seen from Table IX that K_{cat} as well as V/K increase markedly for larger groups in the $S_{1'}$ position.

In light of this, it may be postulated that steps involved in K_{cat} (amide cleavage or product release for example), in an enzyme substrate reaction, results in a conformational change which effects this position such that products with larger groups may be released faster from the enzyme surface following catalysis. A conformational change of this type is a reasonable hypothesis since some changes in the enzyme must occur to deal with the opposing forces of substrate binding and

product release. If such a process is occurring, substrates which have variations in the $S_{1'}$ position may associate and dissociate with enzyme to a similar extent if the pocket is not a specific or required one for binding. However, differences will be seen in the rates of catalysis if the pocket is somehow forced closed at this site, with the faster rates associated with larger groups.

In terms of enzyme-inhibitor interactions, however, the problem becomes more subtle and more difficult because it may not be clear whether the enzyme-inhibitor complex is mimicking a ground state collected-substrate complex, a tetrahedral intermediate, a collected product, or any other intermediate in an enzyme-substrate reaction (this is, of course, assuming that there is in fact an analogy between substrate binding and inhibitor binding). This is important because



a) $E + I \rightleftharpoons EI$

b) $E + I \rightleftharpoons EI \rightleftharpoons EI'$

c) $E + I \rightleftharpoons EI \rightleftharpoons EI' \rightleftharpoons EI''$

Fig. 18: EI complexes mimicking various ES complexes.

if the enzymatic steps or intermediates which are sensitive to structural variation in a substrate are farther to the right, in a reaction coordinate, from the type of intermediate that the inhibitor is mimicking (Fig. 18) then one might not expect that these same structural

changes in an inhibitor would have an effect similar to that observed for the substrate (i.e., what's good for a substrate may have no effect on an inhibitor). For example, in the case of a ground state collected substrate inhibitor (Fig. 18 a), if the sensitive steps in the substrate reaction are K_{cat} or product release steps then preparing the inhibitors which look more like substrate may not result in better inhibitors since these enzymatic steps or intermediates cannot be achieved in a ground state complex. It should be noted that Fig. 18 is not meant to suggest that only one step is involved in the formation of an EI complex (a) or 2 steps for an EI' complex (b), etc., since there may be multiple steps unique to the enzyme inhibitor interaction which lead to the particular species. The main point of Fig. 18 is: what is the inhibitor mimicking and are the enzymatic steps which are sensitive to structural variations in substrates involved in achieving a particular enzyme inhibitor complex.

It appears that this line of reasoning may explain the lack of variation between compounds 147 and 75. From the data obtained by Fruton for various pepsin substrates, it appears that enzyme-product (EP_1P_2) complexes are stabilized and destabilized by substrate structure variations more than the enzyme-substrate (ES) complexes. The invariability of K_m for pepsin substrates and the large variability of K_{cat} seem to support this. The hydroxyethylene compounds may therefore mimic a particular enzyme-substrate intermediate, achieved prior to K_{cat} steps in an enzyme-substrate reaction, that is not significantly altered in stability by structural changes. The increase in the K_i of 76 vs. 148 may also be explained using this treatment. It is known

that ketones of this type are converted by the enzyme to a tetrahedral species (evidence will be presented in later sections). If this catalysis is similar to the catalysis in substrates and conformational changes (such as those described earlier) occur on the enzyme to restrict the $P_{1'}$ or $S_{1'}$ position to favor product release, then a bulkier substituent in $P_{1'}$ may result in a weaker inhibitor since a bulkier group in this site is now unfavorable (enzyme is set up for product release). Therefore the ketomethylenes may be mimicking an enzyme substrate complex that is further to the right (Fig. 18) than the hydroxyethylenes where structurally sensitive enzymatic steps or portions of these steps may be realized.

Bartlett has recently synthesized several phosphonamidate compounds, which are approximately isosteric with the proposed tetrahedral intermediate of several substrates for thermolysin.[90] His study indicated that the K_i values of the inhibitors correlated well with K_m/K_{cat} of the substrates but not K_m. This supposedly provided evidence that the phosphonamidates are transition state analogs and not just multisubstrate ground state analogs which would require correlation to K_m. This type of treatment for our inhibitors, at this stage, would, however, not be useful for several reasons. One is that there are too few ketomethylenes and hydroxyethylenes analogs to make a good plot. Also, the similar K_i values of <u>147</u> and <u>75</u> and the dramatically different K_i value for <u>146</u> do not plot with known substrates in Table IX and will not correlate with the kinetic parameters of substrates isosteric with the inhibitors (i.e., Iva-Val-Leu-Phe-Ala-Iaa). Because of the similar K_i values of Ala and Phe compounds all plots will result in "L" shapes

and inverted "L" shapes which are constricted or expanded on the horizontal axis (plots are K_i vs. K_m or K_i vs. K_m/K_{cat}). However, later sections will present x-ray and kinetic data which are consistent with the proposal that statine is a collected substrate analog.

The compounds which have altered chirality in the $P_{1'}$ position (92a,b, 93, 106, 107) were prepared in light of data on pepsin substrates reported by Fruton some 15 years ago.[47] He reported that when the $S_{1'}$ position of substrates such as those in Table IX were changed to D-amino acids, inhibitors were obtained. The K_i of these inhibitors, however, equaled the K_m value of the analogous substrate, indicating that these inhibitors worked by somehow disallowing the K_{cat} steps in the enzyme mechanism. We therefore set out to prepare these analogs to determine the affect of altered chirality on inhibitors.

Table VIII shows that the K_i's for the D hydroxyethylene compounds are about 50 times weaker than the analogous L inhibitors. This seems to indicate that there is a fundamental difference between these inhibitors and the substrates tested by Fruton. If these inhibitors are bound to the enzyme in purely an ES fashion (Fig. 18), one would expect that the K_i's between the 2S and 2R inhibitors would be the same based on the results obtained by Fruton. The fact that they are not the same may suggest that there are some enzymatic processes available to the L-amino acid isostere that are not available to the D-amino acid isostere. This process would also have to be one which is independent or not involved with the K_s for the substrates and inhibitors prepared by Fruton.

One possibility may be "flap" closing. It has been shown from x-ray data that some residues of the movable flap of penicillopepsin move by about 4.0 Å, relative to native enzyme, to close in on the bound inhibitor 56.[31a,b] Using enzyme-substrate molecular modeling studies and the coordinates of the enzyme-inhibitor complex, Hofmann et al. has proposed the binding mode of good penicillopepsin substrate.[91] Analysis of this complex, however, indicates the possibility for a serious steric interaction between the side chain of a D-amino acid in the $S_{1'}$ position and some residues of the flap (in particular Tyr-75). This may suggest that steps involved in flap closing are responsible for the results observed. If flap closing and subsequent tightening of the enzyme inhibitor complex are inhibited by the $P_{1'}$ side chains in compounds 92a and 106, it would be expected that they would be weaker inhibitors. Further evidence for this involves comparison to the Leu$\frac{OH}{}$Gly compound 146. Compounds 92a and 106 are weaker inhibitors than 146 indicating that loss of the binding site is not solely responsible for the weaker K_i values. Therefore K_m (which approximately equals K_s in Fruton's study) would be independent of flap closing for the Fruton substrates. The question that now remains is why is flap closing available to these inhibitors? The answer may involve the tetrahedral geometry of the C_4 carbon of the isosteres (and the C_3 of Sta) vs. the trigonal geometry of substrates. The tetrahedral geometry may be involved in lowering the activation energies in steps leading to the transition state since the enzyme need not apply force for the distortion of various bond angles.[92] The expulsion of bound water in the active site of the enzyme by the 3S hydroxyl of Sta

or the 4S hydroxyl of the hydroxyethylenes may also be involved.[31a,b] Therefore it may be easier for the inhibitors with tetrahedral geometries to achieve enzyme inhibitor complexes which are further to the right in a reaction coordinate than an EX collision complex. Statine and the hyroxyethylene analogs may therefore not simply mimic ES complexes (Fig. 18) but a tightened ES complex somewhere between the ES complex and the EX transition state.

Ketone analogs 93 and 107 are weaker inhibitors than 148 and 76 by 10-20 fold. This decrease in inhibition may be explained by the proposals above. Ketone 148, and probably 76, are converted by porcine pepsin to tetrahedral species[93], therefore further tightening of the inhibitor associated with these catalytic steps may be available to these inhibitors. However, if steps analogous to K_{cat} for substrates are inhibited by a 2S substituent in isosteres in the same way as a D-amino acid in substrates and if the potential inhibition of flap closing by a 2S side chain in the $P_{1'}$ position of inhibitors is involved, certainly the result would be higher K_i values for compounds like 93 and 107. It would be interesting to see how the K_i's of 93 and 107 compared to the value of k_{-1}/k_1 or k_s (assuming analogy to substrate binding) for 148 and 76. Based on this hypothesis the values may be equal. Also it would be of interest to compare the K_m's and/or K_i's of compounds in Table X with the K_i's of 93 and 107. This may provide data that would further clarify the mechanism of statine and hydroxyethylene inhibitors.

TABLE X. Potential Substrates and Inhibitors for Porcine Pepsin Isosteric to Ketomethylene Analogs

Iva-Val-Leu-Ala-Ala-Iaa
Iva-Val-Leu-Phe-Ala-Iaa
Iva-Val-Leu-D-Ala-Ala-Iaa
Iva-Val-Leu-D-Phe-Ala-Iaa

3. Effect of Structure in Subsites P_4, P_3, P_2, $P_{2'}$ and $P_{3'}$ on the Inhibition of Penicillopepsin

a. Results

Inhibition constants (K_i) for inhibition of penicillopepsin by pepstatin analogs (Table XI) were determined using the synthetic hexapeptide substrate Ac-Ala-Ala-Lys-Phe(NO_2)-Ala-Ala-NH_2.[86] Assays were carried out at 25°C in pH 5.5, .02 M sodium acetate buffer as described.[86] The appearance of H_2N-Phe-(NO_2)-Ala-Ala-NH_2 was monitored by following a decrease in absorbance at 296 nm.[86] Both enzyme and substrate were generous gifts from Dr. Theo Hofmann. Inhibition constants were determined from IC_{50} values as described for pepsin kinetics.

The data in Table XI illustrate how structural variations in the P_4, P_3, P_2, $P_{2'}$ and $P_{3'}$ positions affect K_i for penicillopepsin. Although this is a small sampling of compounds the data also yield some information about the differences and similarities between

TABLE XI. Inhibition of Penicillopepsin by Pepstatin Analogs

	Compound	K_i (nm)	TDI[a]
142)	Iva-Val-Val-Sta-Ala-Iaa[b]	6.5	+
141)	Boc-Val-Val-Sta-Ala-Iaa[b]	4.0	+
5)	Iva-Val-Sta-Ala-Iaa	7600	-
8)	Boc-Val-Sta-Ala-Iaa	6100	-
6)	Ipoc-Val-Sta-Ala-Iaa	5700	-
142)	Iva-Val-Val-Sta-Ala-Iaa	6.5	+
140)	Iva-Val-Phe-Sta-Ala-Iaa	50	-
142)	Iva-Val-Val-Sta-Ala-Iaa	6.5	+
59)	Iva-Val-Val-Sta-Ala-OMe	4.9	+
139)	Iva-Val-Val-Sta-Phe-OMe	1.3	+
56)	Iva-Val-Val-Sta-OEt	47	-
143)	Iva-Val-Val-(R,S)Sta-Ala-OMe	1000	-

[a]TDI = time dependent inhibition.

[b]Compounds previously synthesized and tested.[85] All peptides contain (3S,4S)Sta unless mentioned otherwise.

penicillopepsin and porcine pepsin.

Compounds with P_4 variations show little variation in K_i as illustrated by compounds 141 and 142. Both compounds are potent inhibitors and are time dependent. Deletion of the P_4 site, however (compounds 5, 6, 8), dramatically increases the K_i, although structural variations of these P_3 analogs is of little significance to penicillopepsin. Slight variations in the P_2 position do, however, affect K_i. Compound 140 is about 10 fold less potent than the parent 142, and is not a slow binding inhibitor. Bulkier substituents in the $P_{2'}$ position seem to be more favorable in the case of penicillopepsin as 139 is about a four fold better inhibitor than 59. Deletion of the $P_{2'}$ position (56) further increases K_i by about 10 fold (vs. 59). Deletion of the $P_{3'}$ position (59) has little effect on K_i as 59 and 142 are comparable inhibitors.

b. Discussion

Previously, there have been no structure-activity relationships carried out on the pepstatin-penicillopepsin system. This study (Table XI) provides the first data on the effect of the structural variations on several inhibitor positions and provides some insight into the differences between the aspartic proteinases porcine pepsin and penicillopepsin.

Inhibition constants for compounds which have P_4 changes or deletions indicate that there is a distinct but nonselective interaction between enzyme and inhibitor at this position. Compounds 141 and 142 are essentially equipotent with each other and both bind slowly. This

is in contrast to porcine pepsin where compound 142 is about a 60 fold better inhibitor although both are also time dependent on porcine pepsin. Porcine pepsin seems to be more selective, at this position, towards groups which will provide optimal binding, whereas penicillopepsin seems to make little distinction between urethane and amide moieties at this site. The x-ray crystal structure of 56 bound to penicillopepsin has shown that there are few non-bonded contacts made by the enzyme to the Iva moiety of 56, although a small hydrophobic pocket can be discerned.[31a,b] Unfortunately, there are no enzyme-inhibitor complexes of porcine pepsin for which x-ray data exist. Deletion of this site results in dramatic changes in K_i as seen in the comparisons of compounds 5, 6 and 8 with 141 and 142. This comparison clearly points to the requirement for a substituent in the P_4 position to occupy this hydrophobic pocket in the enzyme. This is again in contrast to porcine pepsin where deletion of the P_4 position need not coincide with large increases in K_i. Compound 5 is a very potent inhibitor of porcine pepsin and is comparable to 141 and 142. Various other analogs which lack the P_4 position and have P_3 variations have been prepared and some have been found to be almost equipotent with pepstatin on porcine pepsin.[37] Penicillopepsin is also quite insensitive to P_3 variations. Compounds 5, 6 and 8 have similar K_i's on penicillopepsin but differ widely on porcine pepsin (Table II). The porcine pepsin data (Table II) have pointed to possible differences between the required geometry for inhibitors at the P_3 position and the preferred geometry for urethanes[37], however this selectivity does not seem to come into play or is not as important in the case of

penicillopepsin. This is consistent with the x-ray crystal structure which indicates that there is a surface depression on the enzyme which could be involved in the binding of a group larger than the isopropyl group in the P_3 position of 56.[31a,b] Again, however, there are no comparative x-ray studies for porcine pepsin.

The P_2 position appears to be a point of similarity between penicillopepsin and porcine pepsin. Replacing an isopropyl side chain with a benzyl side chain in this position results in a 10 fold weaker inhibitor which does not exhibit time dependent inhibition (142 vs. 140). Similar results were obtained with compounds 5 and 54 (Table VII) on porcine pepsin inhibition. As previously mentioned, this P_2 position is the site of 15 non-bonded interactions involving the movable "flap" of penicillopepsin and the bound inhibitor 56. This was extrapolated to the porcine pepsin case and interpreted as being part of the inhibition mechanism associated with slow binding. The speculation can, therefore, certainly be made in this case that the larger benzyl group interferes with flap closing thereby lowering potency and eliminating the slow binding step. The x-ray crystal structure also indicates a possible steric interaction between groups larger than isopropyl in this position with leucine 220 and threonine 216. These groups make 1 and 3 non-bonded contacts of ≤ 4.0 Å with the P_2 valine of 56, respectively.[31a,b]

The $P_{2'}$ position of penicillopepsin appears to differ somewhat from the $P_{2'}$ of porcine pepsin. Addition of a bulkier substituent at this site (139) decreases K_i by about a factor of four or five on penicillopepsin but has little effect or slightly increases the K_i for

porcine pepsin (compound 42, Table II). Unfortunately, a binding pocket for the $P_{2'}$ position cannot be clearly discerned from the crystal structure since 56 lacks this position. However a hydrophobic area between Phe-190, Ile-211 and Phe-295 could engulf a bulky $P_{2'}$ residue, such as a benzyl group. Removal of the $P_{2'}$ substituent (56) increases K_i by about 10 fold (compared to 59) and indicates a requirement for a hydrophobic substituent in this position. This is consistent with the data for porcine pepsin.

The $P_{3'}$ position of penicillopepsin appears to be of little importance to binding as compounds 142 and 59 have similar dissociation constants. This position, therefore, represents a major difference between penicillopepsin and porcine pepsin. As previously mentioned, on porcine pepsin the $P_{3'}$ position was non-selective towards various substituents but was required, whereas on penicillopepsin this position is not required.

A 3R hydroxyl Sta compound was also tested on penicillopepsin and found to be much weaker than the 3S compounds. This is consistent with all previous data, which indicates a strict requirement for a 3S hydroxyl, and with the crystal structures of enzyme-inhibitor complexes which show the 3S hydroxyl positioned between the two catalytic aspartates.

In summary, this study along with the x-ray crystal structure of 56 bound to penicillopepsin has helped to define the required binding sites on penicillopepsin. As opposed to porcine pepsin, which requires an inhibitor with residues in the $P_3 \longrightarrow P_{3'}$ positions for tight binding, penicillopepsin requires a span of residues from $P_4 \longrightarrow P_{2'}$ (Fig.

19) for tight binding. This indicates that there is a difference in the active sites of these closely related aspartic proteinases and represents a point of possible selectivity between the two enzymes. Because of this extrapolations of the penicillopepsin system to porcine pepsin must be made with caution.

P_3-P_2-P_1-P_1'-P_2'-P_3' Porcine pepsin

P_4-P_3-P_2-P_1-P_1'-P_2' Penicillopepsin

Fig. 19: Required binding sites for porcine pepsin and penicillopepsin.

4. Inhibition of Penicillopepsin by Lysine-Statine and Ornithine-Statine Analogs

a. Results

The kinetic results in Table XII were obtained by the methods described in previous sections for penicillopepsin and pepsin. All compounds are 3S,4S unless stated otherwise, and all free amines are assumed to be the acetate salts. Compound 132 was tested as a 1:1 mixture of 3S:3R diastereomers. The K_i obtained for the mixture, 2.3 nm, was divided in half to determine the K_i of the 3S compound.

Table XII clearly shows a major difference between penicillopepsin and porcine pepsin. Replacement of Sta with LySta free amine to compound 56 results in compound 115a which is a 100 fold better inhibitor than 56 on penicillopepsin but at least 100 times worse on porcine pepsin. Protection of the 8 amino group of LySta (114a, 134), however,

TABLE XII. Inhibition of Penicillopepsin by LySta and OrnSta Analogs

Compound	K_i (nm) penicill.	TDI	K_i (nm) pepsin	TDI
56) Iva-Val-Val-Sta-OEt	47	-	10	-
115a) Iva-Val-Val-LySta-OEt (LySta bearing NH_2)	0.4	+	>1000	-
114a) Iva-Val-Val-LySta-OEt (LySta bearing HN-Z)	100	-		
134) Iva-Val-Val-LySta-OEt (LySta bearing HNAc)	100	-	1900	
115b) Iva-Val-Val-(R,S)LySta-OEt (LySta bearing NH_2)	50	-		
139) Iva-Val-Val-Sta-Phe-OMe	1.3	+		
119) Iva-Val-Val-LySta-Phe-OMe (LySta bearing NH_2)	.08	+		
132) Iva-Val-Val-(3R,S,4S)OrnSta-OEt (OrnSta bearing NH_2)	1.1*	+	>1000	-

LySta = 4,8-diamino-3-hydroxy-octanoic acid, compounds are 3S,4S unless stated otherwise.

OrnSta = 4,7-diamino-3-hydroxy-heptanoic acid.

*K_i was determined on a 1:1 mixture of 3R:3S. Number reported is one-half the number obtained for the mixture.

results in inhibitors that are worse than 115a on penicillopepsin. Extension of the peptide chain of 115a to incorporate a $P_{2'}$ phenylalanine (119) results in an even more potent inhibitor of penicillopepsin, which is about 2 times more effective at inhibition than pepstatin (K_i pepstatin = .15 nm on penicillopepsin[72]).

When the side chain of LySta is shortened by one carbon (132), inhibition is only slightly affected as 132 is about three times worse than 115a.

Compound 115b indicates the preference for the 3S diastereomer. The 3R isomer is still a rather potent inhibitor but is still about 100 times weaker than the 3S isomer.

b. Discussion

The study of lysine-statine analogs stemmed from the results obtained from studies of 56[31a,b] bound to penicillopepsin and subsequent substrate modeling studies[91]. The modeling studies were designed to help explain the differences observed between fungal and mammalian aspartic proteinases.

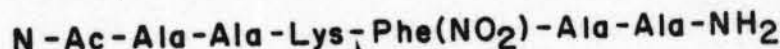
One major difference between these enzymes is that the fungal enzymes have the ability to activate trypsinogen, an activation process which requires specificity for lysine (involves cleavage of Lys-Ill bond). None of the mammalian aspartic proteinases show this type of specificity as they prefer to cleave between hydrophobic residues.

One of the best substrates for penicillopepsin is Ac-Ala-Ala-Lys-Phe(NO_2)-Ala-Ala-NH_2 (Fig. 20).[86] This substrate, designed and synthesized by Hofmann and Hodges, is cleaved by penicillopepsin between the

lysine and the para-nitro-phenylalanine bond. The lysine in S_1 was chosen to exploit the fact that, like other fungal aspartic proteinases, penicillopepsin is lysine specific. Hydrolysis of this bond can be followed by a decrease in absorbance at 296 nm upon release of the free amine H-Phe-(NO_2)-Ala-Ala-NH_2.

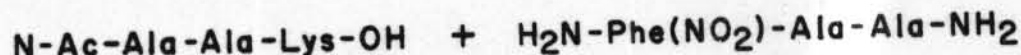
The fact that penicillopepsin shows preference for lysine over hydrophobic amino acids in the S_1 position and that the kinetic parameters K_m and K_{cat} for this substrate show strong pH dependence[91] implies that there may be a specific interaction between the epsilon amine of lysine and some group on the enzyme. To rationalize this, Hofmann et al. have proposed an interaction between the lysine epsilon amine and the carboxyl-carboxylate pair of Glu 16, Asp 115 on the enzyme.[91] This interaction was proposed to be via a hydrogen bonded water molecule rather than a close ion pair due to the distance between the groups. The hypothesis was based on computer modeling studies in which the penicillopepsin substrate was modeled into the enzyme using the binding coordinates for the bound inhibitor 56. It should be noted that in all the mammalian aspartic proteinases that have been sequenced, the

Penicillopepsin Substrate



penicillopepsin cleaves here

K_m	K_{cat}
0.078 mM	36 sec^{-1}



$\Delta\varepsilon = -1800\ M^{-1}cm^{-1}$ at 296 nm

pH 5.5, 0.02M NaAcetate buffer, 25°C

T. Hofmann and R.S. Hodges

Fig. 20: Penicillopepsin substrate.

position homologous to Asp-115 in penicillopepsin is replaced by a hydrophobic amino acid. In fungal aspartic proteinases, this acidic residue is generally conserved.[91]

A schematic representation of the enzyme-inhibitor (56) complex is shown in Fig. 21 in which the bulk of the enzyme residues have been deleted to show the approximate orientation of the groups in question. Figure 22 shows a new inhibitor which more closely mimics the substrate aminobutyl side chain.

If lysine substrates are stabilized by this type of electrostatic interaction and if inhibitor 56 binds in the same fashion as do

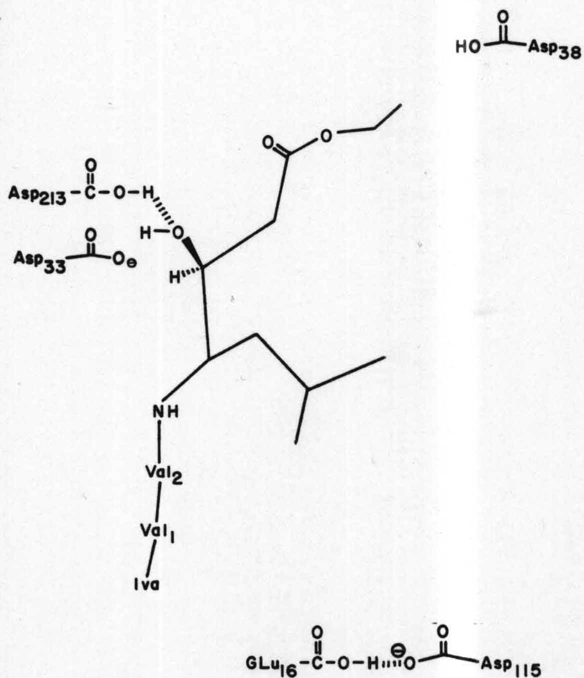


Fig. 21: Schematic representation of inhibitor 56 bound to the active site of penicillopepsin.

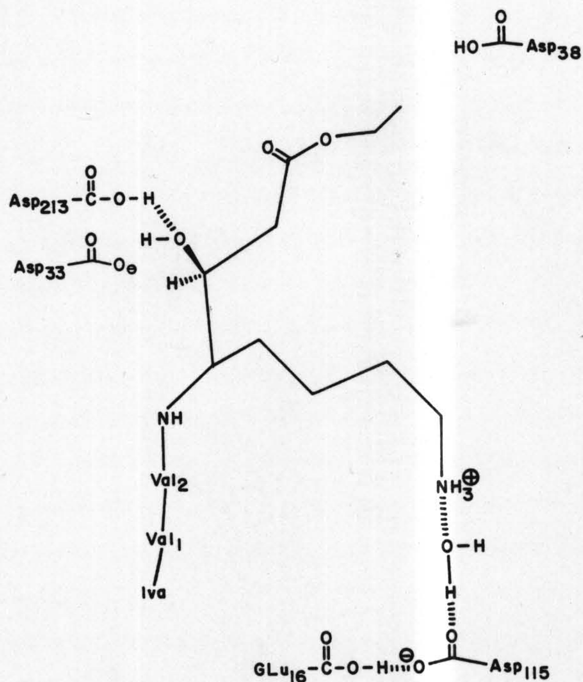


Fig. 22: Schematic representation of LySta inhibitor 115a bound to active site of penicillopepsin. Drawing indicates proposed interaction with Glu-16 and Asp-115.

substrates, then this new inhibitor with a lysine side chain should be a better inhibitor than the parent Sta compound 56. One would expect that it should be better by approximately 2-3 Kcal.[94] This study would therefore help to identify the nature of the specificity that this enzyme shows, and also represents a good test to show that inhibitor binding is analogous to substrate binding. X-ray analysis of enzyme inhibitor complexes can be very useful for determining the mechanism of action of enzymes. However, in order for mechanistic conclusions to be valid, it should be demonstrated that the inhibitor is analogous to substrate. One way to show this is to study inhibitors that closely resemble substrate. These experiments represent such a study.

The kinetic results in Table XII clearly show an enhancement of binding for LySta analogs. Compound 115a is about 100 times more potent than 56 for inhibition of penicillopepsin. This is consistent with about a 2-3 Kcal increase in binding. Compound 119 is about 20 fold better than its parent Sta compound 139 and is also consistent with this proposal. Compounds 114a and 134 indicate a requirement for the free amine.

Table XII also shows that the enhancement of binding is specific for penicillopepsin. The K_i of 115a is greater than 1 μM on porcine pepsin whereas the parent Sta compound 56 has a K_i of 10 nM. This may indicate that the free amine is actually repelled from the active site of porcine pepsin.

These data are consistent with the proposal that there is a group on the enzyme that can interact with the free amine of a lysine side chain. However, a recent x-ray crystal structure of the complex

between inhibitor 115a and penicillopepsin has indicated that the interaction is not with the carboxyl-carboxylate pair Glu-16 and Asp-115[120], but rather with Asp-77 of the mobile "flap." It appears that the interaction is a close ion pair and that the side chain is not in an extended conformation. The fact that 115a is a better inhibitor than 56, however, still supports the idea that binding of 56 is a good model for substrate binding.

Ornithine analog 132 was prepared to determine the effects of shortening the side chain which contains the free amine. It was thought that by so doing the electrostatic interaction seen between the lysine side chain and the enzyme may be eliminated or reduced drastically. This, however, was not the case. Analog 132 is only three times weaker than the LySta analog 115a and is significantly stronger than the statine analog 56. The slight decrease may be an indication of increased distance between the amine and the carboxyl groups, however the results seem to suggest that the enzyme can make certain conformational changes, within limits, if these changes result in a favorable stabilizing interaction.

These results are not surprising in light of the new x-ray data which suggest that the P_1 side chain is not extended. Therefore, shorter analogs need not be significantly weaker since the distance between the inhibitor backbone and Asp-77 of the enzyme is shorter than the distance between the inhibitor and Asp-115.

D. Catalytic Mechanism of Aspartic Proteinases

The presence of two catalytically active aspartic acid groups in the active sites of aspartic proteinases was deduced from pH-dependence and alkylation experiments.[53] Subsequent x-ray crystallographic data on various aspartic proteinases have confirmed these conclusions.[38-43] The residues on penicillopepsin have been assigned as Asp-33 and Asp-213.[95] Numerous proposals for a catalytic mechanism for aspartic proteinases have been proposed as the result of transpeptidation and ^{18}O-exchange experiments. These mechanisms usually featured direct nucleophilic attack by an enzyme carboxylate on a peptide substrate carbonyl to form either an "amino enzyme" carboxamide intermediate or a mixed carbonic anhydride "acyl enzyme" intermediate[96-101] (Fig. 23). However, amino-transfer transpeptidation is observable only for specific substrates, suggesting that proposed mechanisms involving amino-enzyme intermediates are inconsistent with a general mechanism for peptide bond hydrolysis.[102] Furthermore, ^{18}O-exchange studies during the transpeptidation reactions have discounted a carboxamide derivative from an enzyme carboxyl group and an amine product as a viable intermediate.[103] The failure to trap an activated enzyme species with nucleophiles[105] and x-ray data which argue against a covalent mechanism on spacial grounds[43,45] are further evidence against an acyl enzyme intermediate. The possibility that a general acid-base mechanism could be operable, that is, one in which water attacks the carbonyl of the scissle peptide bond with the active site carboxylates mediating the appropriate proton transfers, gained acceptance after it was realized[53] that the resynthesis of peptide bonds from final

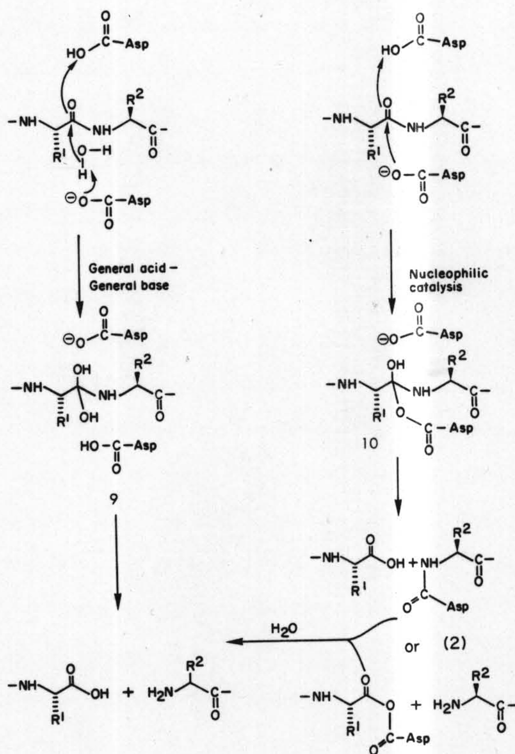


Fig. 23: Two proposed catalytic mechanisms for peptide bond hydrolysis by aspartic proteinases.

hydrolysis products was an energetically reasonable process under the appropriate conditions.[104] Thus, most of the results of transpeptidation experiments could be rationalized by invoking the microscopic reversibility of a general acid-base catalyzed forward reaction, which includes a structure-dependent ordered release of hydrolysis products.[43,53] Results from ^{18}O-exchange studies[103,106,107] are also most consistent with a general acid-base mechanism. However, all possibilities consistent with a covalent mechanism have not been rigorously excluded and more conclusive evidence is required to support the general acid-base mechanism.

One approach to this problem, which has been pursued in the laboratory of Dr. D. H. Rich, is to study the interactions between pepsin and ketones whose structures are based on pepstatin. The strategy involved the design of ketones which could serve as pseudo-substrates, that is, be subject to the catalytic action of the enzyme but only to the point of formation of a tetrahedral intermediate, which because of its stability would not be susceptible to cleavage. Such a stable complex could then be amenable to study by appropriate physical methods.

The statone (4-amino-3-oxo-6-methylheptanoic acid) containing peptide, Iva-Val-Sto-Ala-Iaa 11, was used in initial studies. This peptide was synthesized in the laboratory of Dr. D. H. Rich and found to be a good inhibitor of pepsin (K_i = 56 nm[63]). In a study utilizing ^{13}C-NMR spectroscopy, where the C-3 of Sto in compound 11 was labeled with ^{13}C, this center was shown to be >95% trigonal in aqueous buffer. However, upon addition of pepsin, the ^{13}C signal shifts from 199 ppm to 99 ppm, indicating that the geometry of C-3 is tetrahedral and that the

added atom is oxygen.[64]

It was, however, necessary to establish unambiguously that the transformation from trigonal to tetrahedral geometry was an enzyme catalyzed process as opposed to one in which the ketone was hydrated in solution followed by binding to the enzyme (Fig. 24). Thus when statone analog 11 was incubated with pepsin in 99% $H_2{}^{18}O$ for three hours, recovered ketone contained less than 10% ^{18}O at C-3 as determined by mass spectral analysis (see experimental section for details). In a mechanism involving ketone hydration prior to binding, ^{18}O incorporation in recovered inhibitor should be at least 50%, corresponding to the expected value for a single cycle of nonstereospecific addition/ non-stereospecific elimination of water to the ketone carbonyl (Fig. 24). The actual results then indicate that addition-elimination is a highly stereospecific process and thus enzyme catalyzed.

The origin of the oxygen nucleophile that adds to the Sto C-3 carbonyl was established by NMR experiments performed by Dr. P. G. Schmidt whereby the ^{13}C chemical shift for the C-3 of compound 11 was measured in both H_2O and ^2H_2O in the presence of porcine pepsin.[108] In ^2H_2O the C-3 carbon resonance is shifted upfield 0.36 ppm relative to the carbon resonance obtained in H_2O. Previous studies have established that carbon resonances are shifted upfield by 0.15 ppm per hydroxyl upon changing the solvent from H_2O to ^2H_2O due to the addition of one deuterium.[109-111] Therefore the 0.36 ppm shift is consistent with two hydroxyl groups attached to C-3 carbon of 11 in the tetrahedral species, which is in turn consistent with the general acid-base mechanism which utilizes water as the nucleophile, rather than aspartate.

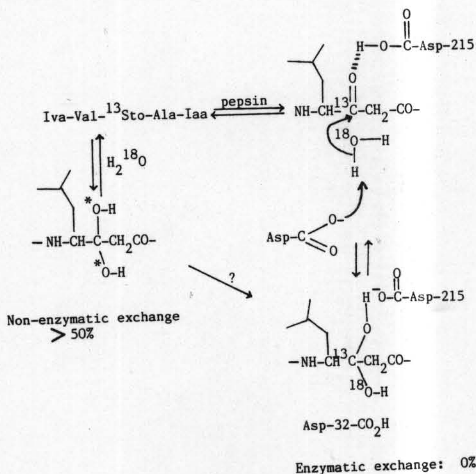


Fig. 24: Two possibilities of ketone hydration. Non-specific hydration (left) yields at least 50% incorporation of ^{18}O. Enzyme catalyzed hydration results in no incorporation.

A similar experiment in which the ^{13}C NMR of <u>11</u> was carried out in $^{2}H_2O$ and $^{2}H_2{}^{18}O$ verified the conclusions of the previous experiment by showing a 0.05 ppm upfield shift in $^{2}H_2{}^{18}O$ relative to $^{2}H^{16}O$. Isotope effects of ^{18}O on ^{13}C carbon resonances produce chemical shifts typically in the range of 0.03-0.05 ppm and are expected to be upfield

relative to ^{16}O.[111] The upfield shift in the carbon resonance establishes that the oxygen nucleophile that adds to C-3 of 11 must come from water (Fig. 25). These labeling studies are, therefore, not consistent with the addition of an aspartate in the catalytic mechanism of aspartic proteinases because exchange of ^{18}O into enzyme carboxyl groups is slow (Fig. 23).[103]

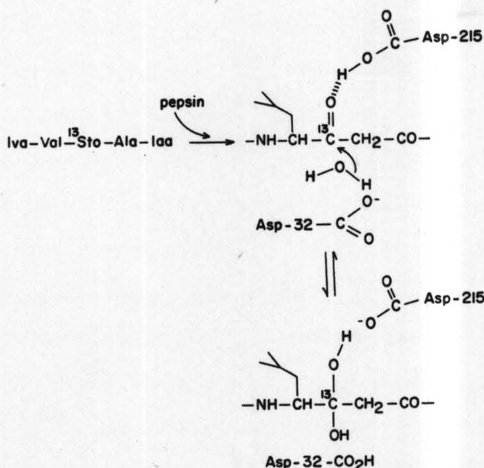


Fig. 25: Schematic representation of the addition of water to the C-3 carbonyl of Sto peptide 11, catalyzed by porcine pepsin.

In order to establish that the addition process observed for 11 in the active site of pepsin is analogous to that which occurs for substrate, the same experiments were carried out on a ketomethylene dipeptide isostere which was 99% enriched in ^{13}C at C-4. These experiments were necessary to establish that the results observed for Sto peptide 11 were not due to a mechanism different to what occurs in normal substrates. Since Sto containing peptides are not isosteric with substrates, the previous conclusions are subject to the valid criticism that a different mechanism could be involved due to possible different spacial requirements for interaction with the enzyme. Therefore the experiments were repeated using isostere 148.

Addition of ketomethylene peptide 148 to a solution of porcine pepsin in the manner described for the corresponding Sto analog[108], produced a 112 ppm upfield shift of the C-4 carbonyl ^{13}C resonance.[112]

Again, experiments were performed in order to confirm that the addition of the nucleophile is an enzyme catalyzed process, as opposed to non-enzymatic hydration followed by binding. Therefore, one equivalent of ketone peptide 148 was incubated with pepsin in 99% $H_2{}^{18}O$ for three hours, then isolated and analyzed by mass spectrometry. The recovered ketone contained less than 12% ^{18}O at C-4, establishing by the arguments described previously that hydration-dehydration is a highly stereoselective process and thus enzyme catalyzed. Prolonged exposure (3 weeks) of 148 to labelled water leads to only 30% incorporation of labelled oxygen at C-4. Thus, exchange is slow and nonstereospecific when 148 is free in solution. The half-life, estimated by NMR, for ketone hydration by the enzyme is, on the other hand, highly

stereoselective and fast (t½ < 2 min). This value represents only the upper limit of the half-life; kinetic data[72,73] establish that 148 binds to pepsin without a lag transient so that a half-life for formation of the tetrahedral species is on the order of t½ < 20 sec. In experiments analogous to those performed on the Sto peptide, the C-4 carbon resonance of 148 was shifted upfield by 0.30 ppm, relative to a sample run in H_2O, when the spectrum was obtained in ^2H_2O (Fig. 26).

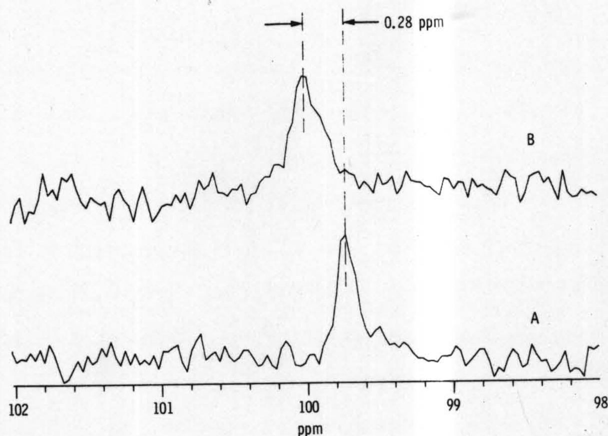


Fig. 26: ^{13}C NMR spectra of ketone 148 bound to porcine pepsin.

A) ^2H_2O solution of .5 mM pepsin and .5 mM 148.

B) 90% H_2O, 10% ^2H_2O for solvent.

When the spectrum was obtained in $^2H_2{}^{18}O$, the resonance for isotopically enriched C-4 in 148 was shifted upfield 0.04 ppm relative to the chemical shift in $^2H_2{}^{16}O$ (Fig. 27). Deuterated water was used because narrower ^{13}C line widths are obtained permitting more accurate measurements of the ^{18}O isotope shift. The line widths (~10 Hz) are consistent with line broadening for an inhibitor bound to a protein of 35,000 molecular weight. As described previously[108], these upfield shifts are consistent with the presence of two hydroxyl groups on C-4 of 148. The additional upfield shift in $^2H_2{}^{18}O$ provides direct evidence that the nucleophile is water, since exchange of ^{18}O into the enzyme carboxyl groups is negligible under the conditions of the experiment.[103]

In summary, these data establish unambiguously that pepsin catalyzes the stereospecific addition of water to a Sto containing peptide and a ketone isostere of a peptide substrate. In the key experiments, isotope effects on ^{13}C chemical shifts were used to directly observe the gem-diol tetrahedral intermediate and to exclude the possibility that the observed tetrahedral adduct might have resulted from the direct attack of an enzyme carboxylate on the ketone carbonyl. Thus, given the reasonable assumption that pepsin-catalyzed hydration of a peptide substrate to a tetrahedral intermediate 9 (Fig. 23) occurs in a process parallel to the observed pepsin-catalyzed hydration of pseudosubstrate ketone 148 to a gem-diol species, then pepsin catalyzes the hydrolysis of peptides by a general acid-general base mechanism and not by a nucleophilic mechanism. In view of this conclusion, it follows that a slow, structure dependent, ordered release of products more

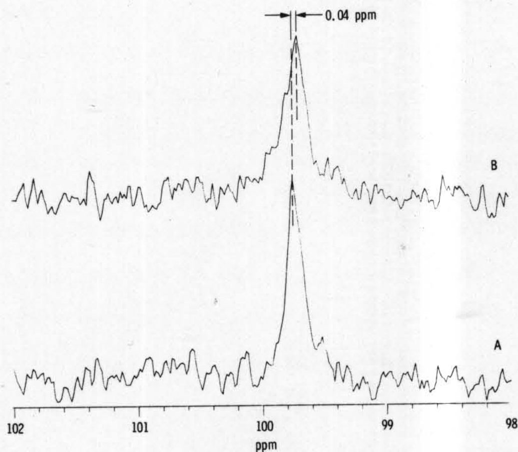


Fig. 27: Isotope effect of ^{18}O on ^{13}C NMR spectra of 148 bound to pepsin. A) $^{2}H_2{}^{16}O$ solution of 148 and pepsin as in Fig. 26. B) $^{2}H_2{}^{18}O$ as solvent.

reasonably accounts for data which earlier had been interpreted as evidence for covalent intermediates.

E. Mechanism of Pepstatin Inhibition

In the past, pepstatin has been regarded as a transition state analog based on the tetrahedral geometry of Sta C-3 and its

similarity to a possible transition state during peptide bond hydrolysis. However, kinetic data[32,33], recent x-ray data[31a,b] and the proposed general acid-base mechanism of aspartic proteinases[108,112] has led to a more detailed explanation of pepstatin's action.

Previous studies have revealed that pepstatin analogs which have altered chirality at C-3 (3R, compound 149) or analogs that lack a C-3 hydroxyl group (dideoxy compound 150) have dissociation constants 20,000 and 4,000 fold higher than their appropriate (3S,4S)-Sta analogs, respectively (Table XIII).[32,33] The fact that these compounds, which have a tetrahedral geometry about C-3, are significantly weaker inhibitors than (3S,4S)-Sta peptide 142 implies a more specific and important role for the 3S hydroxyl group. The importance of this group has been reinforced by the x-ray crystal structure of the complex between pepstatin and R. chinensis aspartic proteinase which shows that the hydroxyl group is within hydrogen bonding distance of the catalytically essential aspartates.[45] A remarkably similar structure has been observed for the complex of the tripeptide Iva-Val-Val-Sta-OEt (56)[31a] as discussed in previous sections. Moreover, recent high resolution refinements of these data have revealed that the position of the 3S hydroxyl group of 56 is nearly identical to the site occupied by a water molecule hydrogen bonded to Asp-33 and Asp-213 in the native enzyme (Fig. 28A,B).[31b,120] Therefore, inhibitor binding must be accompanied by displacement of a water molecule (Fig. 29).

TABLE XIII.

	Compound	K_i (nm) porcine pepsin	TDI
1)	Iva-Val-Val-(3S,4S)-Sta-Ala-(3S,4S)-Sta-OH	.056	
142)	Iva-Val-Val-(3S,4S)-Sta-Ala-Iaa	.1	+
149)	Iva-Val-Val-(3R,4S)-Sta-Ala-Iaa	2000	-
150)	Iva-Val-Val-dSta-Ala-dSta[a]	210	-
151)	Iva-Val-Val-(3R,4S)-Me^3Sta-Ala-Iaa[b]	1.5	+

[a]dSta = 4(S)-amino-6-methylheptanoic acid

[b]Me^3Sta = 4-amino-3-hydroxyl-3,6-dimethylheptanoic acid.

The significance of this result becomes apparent when one considers the extent to which the positive entropy change associated with water displacement will contribute to the strength of inhibitor binding. Jencks[92] has estimated that the return of a bound water molecule to bulk solvent increases entropy from 10-16 eu to produce 3-5 Kcal of energy favorable to binding at room temperature. Since the hydrogen bonds between the enzyme aspartyl groups and the statine 3-S hydroxyl may be considered only as replacements for hydrogen bonds between the bound water and the native enzyme, the net enthalpic change for water displacement is probably small. The increase in entropy, however, does result in a significant increase in binding.

Such a process may also be responsible for the slow binding or time dependent step observed for pepstatin and various analogs. One could hypothesize that this particular displacement of water is slow

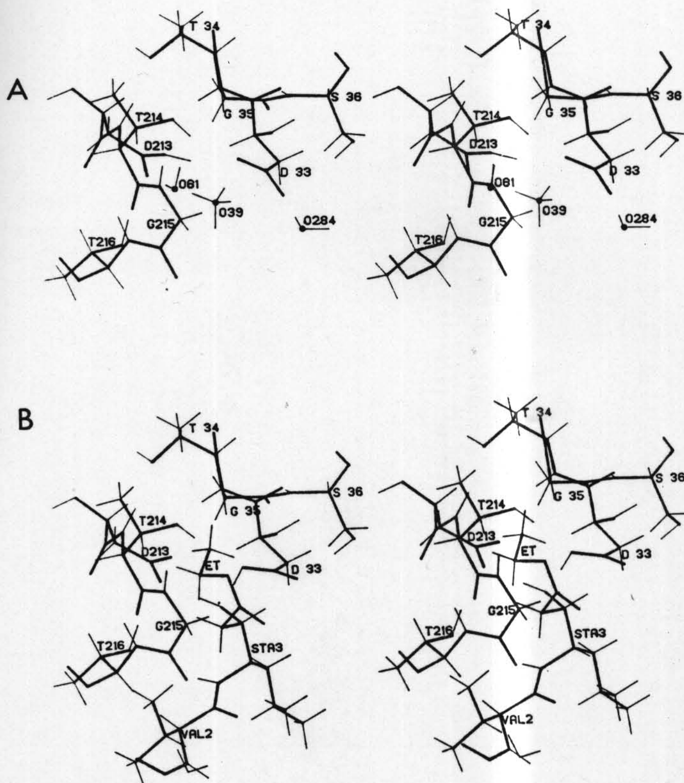


Fig. 28: (A) Stereo view of the active site of penicillopepsin showing water molecule 039 positioned between Asp-33 and Asp-213.

(B) Stereo view of the active site of penicillopepsin with bound inhibitor <u>56</u>. C-3 hydroxyl replaces water molecule 039.

A. GOOD SUBSTRATE

$E + S \rightleftharpoons$ 213 (215,220) Asp–C(=O)–O–H ··· H–O–H ··· $O^{(-)}$–C(=O)–Asp 33 (32,35) + O=C(N–H)–CH(R)–NH– $\overset{fast}{\rightleftharpoons}$ Tetrahedral Intermediate $\overset{fast}{\rightleftharpoons} EP_1P_2 \overset{slow}{\rightleftharpoons} P_1 + P_2$

B. PEPSTATIN

$E + I \rightleftharpoons EI \overset{fast}{\rightleftharpoons}$ 'EI' $\rightleftharpoons$ *EI* $+ H_2O$ (to bulk solvent)

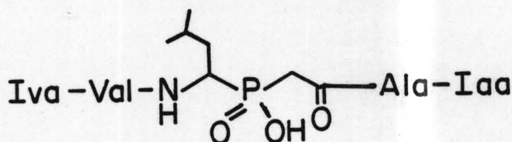
Fig. 28: Schematic representation of the relationships between proposed catalytic and inhibitory mechanisms. A) General acid-base mechanism of substrate hydrolysis. B) Inhibitory mechanism of pepstatin. The pro-S hydroxyl displaces a bound water molecule. Variable aspartic sequence numbers refer to penicillopepsin (pepsin, Rhizopus pepsin) respectively .

because strong hydrogen bonds between the native enzyme and water must be broken in order for their reformation with the statine hydroxyl. Furthermore, the water molecule would have to be squeezed past the bulky inhibitor in order to return to bulk solvent.

This mechanism is reinforced by the fact that when the pro-S hydrogen in the weak 3R statine inhibitor 149 is replaced by a methyl group (151), a potent inhibitor is obtained (Table XIII) which exhibits very slow binding ($t_{1/2}$ ~2-5 min as compared to $t_{1/2}$ ~30 sec for pepstatin.)[113] It should be noted that no 3R hydroxyl statine peptide has ever exhibited tight binding inhibition on any aspartic proteinases that have been studied. Furthermore, the (3R,4S)-Me^3Sta compounds are potent inhibitors on all the aspartic proteinases that have been studied.[113] Such a radical 1000 fold increase in binding may, again, be due to the increase in entropy by displacing the bound water molecule in the active site of aspartic proteinases. Such a process would not be possible for (3R,4S)-Sta since the pro-S proton lacks the bulk to displace the water molecule. The very slow binding observed for the Me^3Sta compounds may be explained as the result of several features. One is that the hydrogen bonds broken between the enzyme and the water molecule are not replaced by other hydrogen bonds due to the presence of the methyl group, and there may be an unfavorable interaction between the enzyme carboxyl groups and the pro-S methyl group (the 10-20 fold increase in K_i for 151 vs. 142 may reflect this). Also there may be more steric interactions near the active site, due to the presence of both a methyl and a hydroxyl group, which would result in greater difficulty for water displacement. However, this favorable

displacement of water to bulk solvent, would override the other unfavorable interactions mentioned.

Recent data from other groups have reinforced the proposals presented here. Bartlett and Kezer have recently reported the synthesis and kinetic analysis of phosphinic acid dipeptide analogs on porcine pepsin.[121]



152

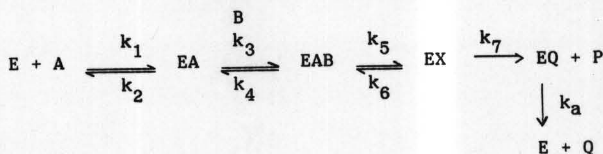
Since the equilibrium Sto ═ hydrate lies far to the left when free in solution, the binding energy available to the hydrate is in principle greater than that represented by the K_i of the ketone. Such a tetrahedral phosphorous analog 152 could mimic the tetrahedral hydrate to take advantage of the additional binding energy. Compound 152 was found to be a potent inhibitor of porcine pepsin (K_i ~ .07 nM); about 20 times more potent than the parent Sta compound 5. It was also found to be an extraordinarily slow binding inhibitor ($t_{1/2}$ ~ 115 min). Based on the proposals of pepstatin inhibition, compound 152 would displace water from the active site of pepsin but it would also form an additional, new hydrogen bond due to the presence of two oxygens. Therefore 152 would have about 1-2 Kcal stabilization over 5. The numbers observed are consistent with this. The matter of the extremely slow binding could again be explained by the steric argument used for Me^3Sta

compound 151. The presence of a phosphorous atom and two oxygen atoms would make the active site region even more crowded than in the Me^3Sta compounds and would result in an even slower displacement of water.

Thus, although pepstatin still may be considered a transition state analog inhibitor owing to the tetrahedral geometry at C-3 of statine, pepstatin may also be considered a collected substrate inhibitor because the statine pro-S hydroxyl group mimics the enzyme-bound substrate water molecule. This is consistent with the observed hydroxyethylene data which seem to point to an enzyme inhibitor complex which more resembles an ES complex (although tetrahedral) rather than an EP complex owing to the invariability between various P_1' analog inhibitors. The dissociation constant for dideoxypepstatin analog 150 is about 10-100 fold smaller than the K_s for comparable substrates, and addition of a pro-S hydroxyl group to C-3 (142) contributes an additional 1000-4000 fold to inhibition binding. It is reasonable to attribute much of the tighter binding of 150, compared with substrates, to the tetrahedral geometry of C-3, in accord with transition state analog theory. To the extent that steric interactions between a proton on C-3 of the central deoxySta residue and the bound water might interfere with optimum binding of 150, the contribution of tetrahedral geometry to the binding of inhibitors which do displace water (and thus do not encounter steric interactions) could be greater. As a result it is difficult to assign precisely the degree to which entropic considerations are responsible for the tighter binding of 1 compared to 150, but, as discussed above, this contribution is likely to be substantial.

F. Conclusions

Previous kinetic data has led to a proposed minimum kinetic mechanism (Scheme 2) for inhibition of porcine pepsin by pepstatin. This mechanism suggests the fast formation of an EI collision complex and a fast EI* complex followed by the slow formation of an EI** complex. The crystal structure of 56 bound to penicillopepsin and other kinetic data presented in this thesis may serve to define the slow process of EI** formation as the displacement of a water molecule in the active site of the enzyme by the 3S hydroxyl group of statine. It has also been proposed that this final complex mimics an enzyme substrate complex which is tetrahedral but still resembles a collected substrate complex. It has also been demonstrated that the hydroxyethylene inhibitors described in this thesis do not correlate with the K_m values or K_m/K_{cat} values for known substrates, although the equations of Bartlett predict a possible correlation. These results may be explained from the analysis of a minimum mechanism for pepsin hydrolysis (Scheme 3).



Scheme 3

This minimum mechanism, which involves two substrates (A is water, B is peptide), indicates the formation of an enzyme-collected substrate complex EAB followed by a catalytic step to the tetrahedral intermediate EX and an ordered release of products P and Q (2 peptide fragments).

Northrop[122] has recently analyzed this mechanism and has indicated that the mechanism can only be broken down into two segments. One segment involves steps from EAB to EQ + P and the other from EQ to E. In light of this it is not surprising that hydroxyethylene compounds do not correlate with K_m or K_m/K_{cat}. Because the first segment of this mechanism involves both binding and catalysis steps, these two processes cannot be separated and analyzed individually and therefore any correlation with one parameter and not the other becomes coincidental. It is, on the other hand, surprising that a correlation was seen in Bartlett's study. It should be mentioned that the plot of K_i vs. K_m/K_{cat} in Bartlett's study showed a correlation coefficient equal to .94, while the r = .70 for the plot of K_i vs. K_m. However, if only one point from the latter plot was excluded then r = .89.

These results obtained in this thesis may be further clarified by examining a hypothetical reaction coordinate based on the mechanism in Scheme 3 (Fig. 30). In this diagram the ΔG_{act} (1) is suggested to be attack of the substrate water molecule on the peptide substrate to form the tetrahedral intermediate EX. ΔG_{act} (2) designates the energy required for the first irreversible step in the mechanism and would involve processes necessary to permit release of products such as enzyme and/or substrate conformational changes. Two transition states associated with these steps would occur (ETS and ETS*).

Now, Fig. 30 describes the hypothetical situation in which all the energy is available for binding (i.e., the geometries of the compounds binding are such that the enzyme need not apply strain to distort bonds so all available energy can be utilized for binding). It should be

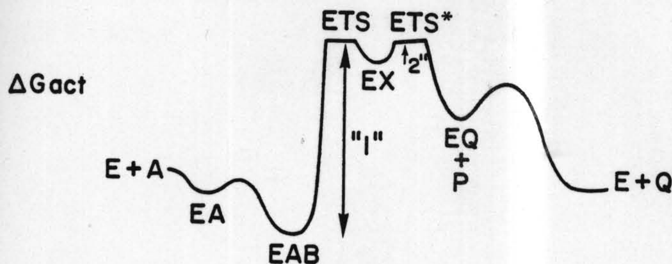


Fig. 30: Hypothetical reaction coordinate for pepsin catalyzed hydrolysis of peptide bonds.

mentioned here that Figures 30 and 31 are not suggesting that the energy levels of the intermediates are known to be as shown, as this is a purely qualitative analysis of the coordinates. Figure 31 then suggests two different tightly bound transition state analogs ETS and ETS*. The former may resemble the collected substrate complex while the latter may be closer to an enzyme-products complex following the previously suggested conformational changes. Therefore, pepstatin

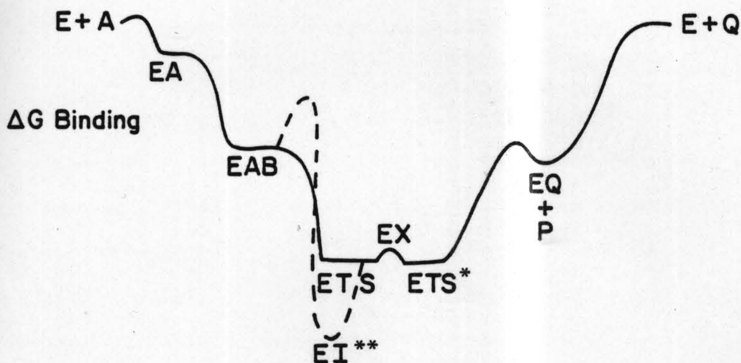


Fig. 31: Hypothetical ΔG binding coordinate for intermediates in peptide bond cleavage by pepsin.

as well as hydroxyethylene analogs <u>may</u> mimic an ETS complex (dotted line, Fig. 31) or something close to it. There are, however, factors that must be taken into consideration. The energy well for the EI** complex should theoretically be lower than the ETS complex because of more favorable binding due to entropic factors not available to the enzyme substrate reaction (i.e., displacement of bound water). The inhibitory mechanism of these compounds probably does not parallel

an enzyme substrate reaction because of these differences. It becomes very difficult then to say that pepstatin exactly or nearly exactly mimics an ETS complex. I am, therefore, saying that pepstatin and hydroxyethylenes may mimic this complex or something close to it. This is also another reason why a correlation (if any correlation is possible) as suggested by Bartlett was not seen (the mechanism of inhibition and catalysis are not necessarily parallel).

The ketomethylenes and Sto compounds, however, probably closely parallel the substrate reaction up to the formation of ETS* where the complex becomes "stuck" because of the increased stability of the carbonyl-carbon bonds of these compounds versus amide bonds in substrates. It would be expected that this complex would be in an energy well higher than the EI** of pepstatin, since no enzyme bound water molecule is displaced. It is also being suggested that this complex which is mimicking ETS* is more to the right of the EI** complex of pepstatin. The fact that ketomethylenes do show more variation in K_i than hydroxyethylenes suggests that the activation energy (2) (Fig. 30) for the formation of products would be different for different substrates. Therefore the inhibitors would lie in ETS* wells of varying stability (Fig. 31). Better substrates (and lower activation energies (2)) would mean more weakly bound ETS* complexes. This is consistent with what was observed. This is also consistent with the observation that product release steps are sensitive to structural variation in substrates (segment 2 of Scheme 3).

In summary, the work presented in this thesis has led to a more detailed proposal for the inhibition of aspartic proteinases by pepstatin, hydroxyethylene peptides and ketomethylene peptides. Along with the aid of numerous collaborators, a more detailed account of peptide hydrolysis has also been proposed.

IV. EXPERIMENTAL SECTION

A. Materials and Methods

Melting points were determined with a Fisher-Johns melting point apparatus and are uncorrected. Optical rotations were determined on a Perkin-Elmer Model 241 automatic polarimeter (0.9999 dm cell). Proton nuclear magnetic resonance spectra were recorded at 90 MHz using Varian Model EM-390, Jeol Fx-90Q Fourier transform and Bruker HX-90E Fourier transform spectrometers. Chemical shifts were reported as δ units (ppm) relative to tetramethylsilane as internal standard.

TLC was performed on 0.25 mm thickness silica gel plates (Merck, silica gel 60 F-254). For column chromatography, Brinkman silica gel 60, 70-270 mesh was used for gravity columns while medium pressure liquid chromatography (mplc) grade Merck silica gel, grade 60, 230-400 mesh was used for flash columns (columns run under positive pressure). TLC solvent systems used were: A) 10% methanol in chloroform (v/v); B) 5% methanol in chloroform (v/v); C) 3% methanol in chloroform (v/v); D) 100% ethyl ether; E) 75% ethyl ether in hexane (v/v); F) 66% ethyl ether in hexane (v/v); G) 60% ethyl ether in hexane (v/v); H) 50% ethyl ether in hexane (v/v); I) 30% ethyl ether in hexane (v/v); J) 5% ethyl ether in hexane (v/v); K) 20% ethyl acetate in hexane (v/v); L) 40% ethyl acetate in hexane (v/v); M) 50% ethyl acetate in hexane (v/v); N) 66% ethyl acetate in hexane (v/v); O) 20% ethyl acetate in toluene (v/v); P) 40% ethyl acetate in methylene chloride (v/v); Q) 80% methylene chloride in hexane (v/v); R) 4:1:1 n-butanol/acetic acid/water (v/v/v). Compounds were visualized on the plates by reaction with:

ninhydrin, chlorox-o-tolidine, 5% phosphomolibdic acid in ethanol, ultraviolet light and water. All compounds used in kinetic studies appeared as a single spot on TLC and were analytically pure. Kinetic constants were measured using synthetic heptapeptide Phe-Gly-His-Phe(NO_2)-Phe-Ala-Phe-OMe for porcine pepsin assays as described[8] and Ac-Ala-Ala-Lys-Phe(NO_2)-Ala-Ala-NH_2[86] for penicillopepsin assays, which were carried out at pH 5.5 in .02 M sodium acetate buffer as described by Hofmann and Hodges.[86] Kinetics were carried out on a Gilford Model 250 spectrophotometer connected to a Gilford 6051 recorder.

B. General Synthetic Procedures

General Procedure A. Removal of the Tert-Butyloxycarbonyl Group

Boc peptide (1 eq) in a solution of 4 N HCl in dioxane (10 eq HCl) was stirred at room temperature and the reaction monitored by TLC. Complete reaction was generally achieved in about 30 minutes. Excess reagent was removed under reduced pressure to give a solid residue. The residue was re-evaporated from ether several times and dried in vacuo over KOH and P_2O_5 for several hours. The resulting hydrochlorides were used without further purification.

General Procedure B. Coupling Reactions Using Dicyclohexylcarbodiimide/1-Hydroxybenzotriazole[123]

The amino hydrochloride (1.0 mmol) was dissolved in methylene chloride (5 ml) and neutralized at 0° with N-methylmorpholine (1.0 mmol). Boc amino acid (1 mmol) and HOBT (1.5 mmol) were added

followed by a solution of DCC (1 mmol) in methylene chloride (5 ml). The reaction mixture was allowed to stir at 0° for 2-4 hours and at room temperature overnight. DCU was filtered, and the filtrate was evaporated under reduced pressure and elevated temperature. The residue was dissolved in ethyl acetate or ethyl ether, washed successively with cold 1 N HCl, saturated $NaHCO_3$, saturated NaCl and dried ($MgSO_4$). The peptide was purified by silica gel chromatography when necessary and crystallized from a suitable solvent or solvent mixture.

General Procedure C. Preparation of Symmetrical Anhydrides[124]

Boc amino acid (2 eq) and DCC (1 eq) in methylene chloride were stirred at 0° C for 40 min. The reaction mixture was cooled for 20-30 min on dry ice and filtered to remove DCU. The filtrate was used immediately without further purification. Carboxylic acid anhydrides were prepared in a similar manner but were purified by vacuum distillation.

General Procedure D. Coupling Reactions and N-Acylation Reactions via Symmetrical Anhydrides

A solution of peptide hydrochloride (~100 mg/ml DMF or methylene chloride) was cooled on an ice bath and neutralized with N-methylmorpholine (1 eq). After addition of the symmetrical anhydride (2 eq), stirring was continued at 0° for 2-4 hours and at room temperature overnight. The solvent was removed under reduced pressure and elevated temperature. The residue was dissolved in ethyl acetate,

washed with cold 1 N HCl, saturated $NaHCO_3$, saturated NaCl and dried ($MgSO_4$). The peptide was purified by silica gel chromatography when necessary and crystallized.

General Procedure E. Coupling Reactions Using EEDQ[125]

To a chilled solution of Boc amino acid (1 eq) and free amine (1 eq), N-ethoxycarbonyl-2-ethoxy-1,2-dihydro-quinoline (1 eq, 97% EEDQ) was added. The reaction was stirred at 0° C for 2-4 hours and at room temperature overnight. The reaction was worked up as described in procedure B.

General Procedure F. Saponification of Peptide Esters

Peptide ester (1 mmol) was dissolved in methanol (~9 ml). To this 1 N NaOH (1.1 mmol) was added and the reaction was monitored by TLC. When reaction was complete the methanol was removed in vacuo and H_2O was added to the mixture. This solution was washed with ether twice and the aqueous layer was acidified to pH 2-3 with 1 N citric acid. The aqueous layer was extracted with ethyl acetate. The organic layer was dried ($MgSO_4$) and evaporated to yield product.

General Procedure G. Preparation of Pentafluorophenyl Active Esters via DCC-Pentafluorophenol "Complex" Method[69]

Boc amino acid (1 eq) was dissolved in EtOAc (50 mg/ml) and chilled to 0° . DCC-pFp "complex" (1 eq) was added and the mixture was stirred and followed by TLC. After ~1/2 hour the reaction mixture was chilled on dry ice for 20 min then filtered. The organic layer

was worked up as in General Procedure B.

General Procedure H. Removal of Benzyloxycarbonyl Group via Catalytic Hydrogenation

N-Cbz protected compound was dissolved in a minimum amount of dry DMF which contains about 1 drop of acetic acid/ml of solvent. To this solution was added 10% Palladium on carbon and the mixture was shaken on a Parr hydrogenation apparatus at 20-30 psi for 3-4 hrs. After this time the mixture was filtered over celite and washed with DMF. The filtrate was then concentrated in vacuo. Pure product was precipitated using ethyl ether and collected by filtration.

C. Specific Procedures

N-(tert-Butyloxycarbonyl)-4(S)-amino-3(S)-hydroxyl-6-methylheptanoyl-L-alanyl-L-phenylalanine Methyl Ester (15). The title compound was prepared by general procedure B from HCl·Ala-Phe-OMe[126] (.545 mmoles) and (3S,4S)-Boc-Sta-OH (.36 mmoles). Silica gel chromatography (3% MeOH/$CHCl_3$) followed by crystallization yielded pure product in about 81% yield: mp 130-131°C (EtOAc/hexane); TLC R_f (A) 0.59; $[\alpha]_D^{24}$ -36° (c = .675, MeOH); NMR ($CDCl_3$) δ .95 (d, J = 6 Hz, 6H), 1.25-1.80 (m, 15H, includes singlet 1.41), 2.32 (m, 2H), 3.10 (d, J = 6.5 Hz, 2H), 3.70 (s, 3H), 3.80-4.10 (m, 2H), 4.10-4.95 (m, 4H), 6.30-6.80 (m, 2H), 7.0-7.40 (m, 5H). Anal. calcd. for $C_{26}H_{41}N_3O_7$: C, 61.52; H, 8.14; N, 8.28. Found: C, 61.82; H, 8.25; N, 8.22.

N-(tert-Butyloxycarbonyl)-L-valyl-4S-amino-3(S)-hydroxyl-6-methylheptanoyl-L-alanyl-L-phenylalanine Methyl Ester (16). The title

compound was prepared by general procedure D from HCl-(S,S)Sta-Ala-Phe-OMe[126] (.2 mmoles) and Boc-L-valine anhydride (.4 mmoles) using methylene chloride as solvent. Chromatography (5% MeOH/$CHCl_3$) followed by crystallization yielded pure product in about 80% yield: mp 176-177°C (EtOAc/hexane); TLC R_f (B) .52; $[\alpha]_D^{24}$ -50° (c = .125, MeOH); NMR ($CDCl_3$) δ .67-1.11 (m, 12H), 1.2-1.5 (m, 14H), 1.56-2.67 (m, 4H), 3.08 (d, J = 6.7 Hz, 2H), 3.22-4.22 (m, 6H, includes singlet δ 3.64), 4.35 (t, J = 6.7 Hz, 1H), 4.67-5.39 (m, 3H), 6.47 (d, J = 8 Hz, 1H), 6.90-7.78 (m, 7H). Anal. calcd. for $C_{31}H_{50}N_4O_8$: C, 61.36; H, 8.31; N, 9.23. Found: C, 61.09; H, 8.37; N, 9.00.

N-Isovaleryl-L-valyl-4(S)-amino-3(S)-hydroxyl-6-methylheptanoyl-L-alanyl-L-phenylalanine Methyl Ester (17). The title compound was prepared by general procedure D using HCl·Val-(S,S)Sta-Ala-Phe-OMe[126] (.3 mmoles), isovaleric anhydride (.6 mmoles) and DMF as solvent. Crystallization gave product in about 75% yield: mp 224-227°C (MeOH: ethyl ether); TLC R_f (A) .34; $[\alpha]_D^{24}$ -52° (c = .062, MeOH); NMR (methanol-d_4) δ .67-1.11 (m, 18H), 1.15-1.78 (m, 8H, includes doublet J ≅ 7 Hz), 1.9-2.44 (m, 6H, includes doublet J = 7 Hz), 2.89-3.2 (m, 2H), 3.67 (s, 3H), 3.78-4.89 (m, 3H), 7.1-7.3 (m, 5H). Anal. calcd. for $C_{31}H_{50}N_4O_7$: C, 62.98; H, 8.53; N, 9.53. Found: C, 62.73; H, 8.66; N, 9.61.

N-(tert-Butyloxycarbonyl)-4(S)-amino-3(S)-hydroxyl-6-methylheptanoyl-L-alanyl-L-leucine Methyl Ester (18). The title compound was prepared by general procedure B from HCl·Ala-Leu-OMe[126] and

(3S,4S)Boc-Sta-OH. The product was chromatographed (3% MeOH/$CHCl_3$) and recovered as an amorphous solid in about 90% yield: TLC R_f (B) .28; $[\alpha]_D^{24}$ -31° (c = .64, MeOH); NMR ($CDCl_3$) δ .95-1.15 (m, 12H), 1.45-1.95 (m, 18H), 2.47 (m, 2H), 3.82 (s, 3H), 4.0-4.15 (m, 2H), 4.40-4.90 (m, 4H), 6.60-6.82 (m, 2H). Anal. calcd. for $C_{23}H_{43}N_3O_7$: C, 58.33; H, 9.15; N, 8.87. Found: C, 58.07; H, 9.24; N, 8.62.

N-(tert-Butyloxycarbonyl)-L-valyl-4(S)-amino-3(S)-hydroxyl-6-methylheptanoyl-L-alanyl-L-leucine Methyl Ester (19). The title compound was prepared by general procedure D from HCl·(S,S)Sta-Ala-Leu-OMe[126] (.32 mmoles) and Boc-L-valine anhydride (.64 mmoles) using methylene chloride as solvent. Chromatography (3% MeOH/$CHCl_3$) yielded pure product as an amorphous solid in about 85% yield: TLC R_f (A) .53; $[\alpha]_D^{24}$ -64° (c = .18; MeOH); NMR ($CDCl_3$) δ .8-1.1 (m, 18H), 1.4-1.8 (m, 17H), 1.85-2.2 (m, 2H), 2.4-2.6 (m, 2H), 3.4 (m, 1H), 3.6-3.8 (m, 4H), 4.1 (m, 1H), 4.5 (m, 1H), 4.7 (m, 1H), 5.0 (d, J = 7.2 Hz, 1H), 5.2 (d, J ≅ 7 Hz, 1H), 6.6 (d, J = 10.8 Hz, 1H), 7.4 (m, 1H), 7.75 (m, 1H). Anal. calcd. for $C_{28}H_{52}N_4O_8$: C, 58.72; H, 9.15; N, 9.78. Found: C, 58.59; H, 9.10; N, 9.73.

N-Isovaleryl-L-valyl-4(S)-amino-3(S)-hydroxyl-6-methylheptanoyl-L-alanyl-L-leucine Methyl Ester (20). The title compound was prepared by general procedure D from HCl·Val-(S,S)Sta-Ala-Leu-OMe[126] (.25 mmoles) and isovaleric anhydride (.5 mmoles) using DMF as solvent. Precipitation from ethyl ether gave product in about 80% yield: mp 204-207°C; TLC R_f (A) .45; $[\alpha]_D^{24}$ -72° (c = .1, MeOH); NMR ($CDCl_3$) δ .78-

1.2 (m, 24H), 1.33-1.83 (m, 8H, includes doublet J = 7 Hz), 1.89-2.22 (m, 7H), 2.44-2.76 (m, 1H), 3.38 (m, 1H), 3.73 (s, 3H), 3.78-4.22 (m, 2H), 4.33-4.89 (m, 2H), 6.02 (d, J ≅ 8 Hz, 1H), 6.78 (d, J = 8 Hz, 1H), 7.44 (d, J = 8 Hz, 1H), 7.82 (d, J = 8 Hz, 1H). Anal. calcd. for $C_{28}H_{52}N_4O_7$: C, 60.36; H, 9.41; N, 10.10. Found: C, 60.14; H, 9.51; N, 9.91.

N-(tert-Butyloxycarbonyl)-4(S)-amino-3(S)-hydroxyl-6-methylheptanoyl-L-alanyl-glycine Methyl Ester (21). The compound was prepared by general procedure B from HCl·Ala-Gly-OMe[126] (.2 mmoles) and (S,S)Boc-Sta-OH (.2 mmoles). Chromatography (5% MeOH/$CHCl_3$) followed by crystallization gave product as an amorphous solid in about 60% yield: mp 116-118°C (EtOAc/hexane); TLC R_f (B) .15; $[\alpha]_D^{24}$ -29° (c = .17, MeOH), NMR ($CDCl_3$) δ .79-1.10 (m, 6H); 1.25-1.75 (m, 15H), 2.40 (m, 2H), 3.55-3.75 (m, 4H, includes singlet δ 3.68), 3.80-4.20 (m, 4H), 4.52 (m, 1H), 4.90 (m, 1H), 6.90-7.40 (m, 2H). Anal. calcd. for $C_{19}H_{35}N_3O_7$: C, 54.65; H, 8.45; N, 10.06. Found: C, 54.83; H, 8.46; N, 9.89.

N-(tert-Butyloxycarbonyl)-L-valyl-4(S)-amino-3(S)-hydroxyl-6-methylheptanoyl-L-alanyl-glycine Methyl Ester (22). The title compound was prepared by general procedure D from HCl·(S,S)Sta-Ala-Gly-OMe[126] (.10 mmoles) and Boc-valine anhydride (.2 mmoles) using methylene chloride as solvent. Chromatography (7% MeOH/$CHCl_3$) followed by crystallization gave product in about 60% yield: mp 155-158°C (EtOAc/hexane); TLC R_f (A) .31; $[\alpha]_D^{24}$ -40° (c = .83, MeOH); NMR ($CDCl_3$) δ .75-

1.15 (m, 12H), 1.25-1.70 (m, 15H), 1.80-2.60 (m, 3H), 3.31-3.85 (m, 4H), includes singlet δ 3.79), 3.90-4.20 (m, 3H), 4.55 (m, 1H), 4.81-5.16 (m, 2H), 7.0-7.70 (m, 3H). Anal. calcd. for $C_{24}H_{44}N_4O_8$: C, 55.80; H, 8.59; N, 10.85. Found: C, 55.64; H, 8.54; N, 10.75.

N-Isovaleryl-4(S)-amino-3(S)-hydroxyl-6-methylheptanoyl-L-alanyl-glycine Methyl Ester (23). The title compound was prepared by general procedure D from HCl·Val-(S,S)Sta-Ala-Gly-OMe[126] (.06 mmoles) and isovaleryl anhydride (.12 mmoles) using DMF as solvent. Precipitation from ethyl ether gave product in about 66% yield: mp 211-213°C (ethyl ether); TLC R_f (A) .20; $[\alpha]_D^{24}$ -50° (c = .05, MeOH); NMR ($CDCl_3$) δ .78-1.11 (m, 18H), 1.33-1.55 (m, 7H), 1.89-2.67 (m, 5H), 3.33-3.72 (m, 4H, includes singlet δ 3.71), 3.89-4.22 (m, 4H), 4.44 (m, 1H), 4.89 (m, 1H), 5.78 (d, J = 8 Hz, 1H), 6.89 (m, 1H), 7.33-7.78 (m, 2H). Anal. calcd. for $C_{24}H_{44}N_4O_7$: C, 57.58; H, 8.86; N, 11.19. Found: C, 57.30; H, 8.93; N, 11.01.

N-(tert-Butyloxycarbonyl)-L-leucyl Isoamylamide (39). The title compound was prepared by general procedure E from isoamylamine (2 mmoles) and Boc-leucine (2 mmoles). Crystallization gave product as an amorphous solid in about 87% yield. mp 89-90°C (hexane); TLC R_f (B) .62; $[\alpha]_D^{24}$ -25° (c = .80, MeOH); NMR ($CDCl_3$) δ .75-1.10 (m, 12H), 1.2-1.8 (m, 15H, includes singlet δ 1.42), 3.2 (q, J $\cong$ 6 Hz, 2H), 4.1 (m, 1H), 5.3 (m, 1H), 6.6 (m, 1H). Anal. calcd. for $C_{16}H_{32}N_2O_3$: C, 63.97; H, 10.74; N, 9.32. Found: C, 63.86; H, 10.62; N, 9.08.

N-(tert-Butyloxycarbonyl)-4(S)-amino-3(S)-hydroxyl-6-methylheptanoyl-L-leucyl Isoamylamide (40). The title compound was prepared by general procedure B from HCl·Leu-Iaa[126] (.54 mmoles) and (3S,4S)Boc-Sta-OH (.36 mmoles). Chromatography (5% MeOH/$CHCl_3$) gave pure compound as a clear oil in about 78% yield: TLC R_f (B) .36; $[\alpha]_D^{24}$ -39° (c = .2, MeOH), NMR ($CDCl_3$) δ .70-1.10 (m, 18H), 1.20-1.80 (m, 18H), includes singlet at δ 1.42), 2.35 (m, 2H), 3.2 (q, J ≅ 6 Hz, 2H), 3.55 (m, 1H), 3.80-4.10 (m, 2H), 4.39 (m, 1H), 4.80 (m, 1H), 6.40-6.80 (m, 2H). Anal. calcd. for $C_{24}H_{47}N_3O_5$: C, 62.99; H, 10.35; N, 9.18. Found: C, 62.77, H, 10.39; N, 9.04.

N-(tert-Butyloxycarbonyl)-L-valyl-4(S)-amino-3(S)-hydroxyl-6-methylheptanoyl-L-leucyl Isoamylamide (41). The title compound was prepared by general procedure D from HCl·(S,S)Sta-Leu-Iaa[126] (.3 mmoles) and Boc-valine anhydride (.6 mmoles) using methylene chloride as solvent. Chromatography (5% MeOH/$CHCl_3$) gave product as an amorphous solid in about 65% yield: TLC R_f (B) .19; $[\alpha]_D^{24}$ -40° (c = .17, MeOH); NMR ($CDCl_3$) δ .75-1.10 (m, 24H), 1.20-1.80 (m, 19H), 2.36 (m, 2H), 3.20 (q, J = 6 Hz, 2H), 3.5-4.5 (m, 5H), 4.9 (m, 1H), 6.30-6.80 (m, 2H), 7.85 (m, 1H). Anal. calcd. for $C_{29}H_{56}N_4O_6$: C, 62.56; H, 10.14; N, 10.06. Found: C, 62.56; H, 10.24; N, 9.94.

N-Isovaleryl-L-valyl-4(S)-amino-3(S)-hydroxyl-6-methylheptanoyl-L-leucyl Isoamylamide (42). The title compound was prepared by general procedure D from HCl·Val-(S,S)Sta-Leu-Iaa[126] (.05 mmoles) and isovaleric anhydride (.1 mmole) using DMF as solvent.

Precipitation yielded product in about 82% yield: mp 188-200°C (ethyl ether); TLC R_f (A) .51; $[\alpha]_D^{24}$ -50° (c = .117, MeOH); NMR ($CDCl_3$) .67-1.11 (m, 30H), 1.22-1.89 (m, 11H), 1.90-2.22 (m, 4H), 3.00-3.44 (m, 3H), 3.76-4.44 (m, 3H), 5.07 (d, J ≅ 8 Hz, 1H), 6.00-6.22 (m, 2H), 7.78-8.0 (m, 2H). Anal. calcd. for $C_{29}H_{56}N_4O_5$: C, 64.41; H, 10.44; N, 10.36. Found: C, 64.11; H, 10.24; N, 10.31.

N-(tert-Butyloxycarbonyl)-glycyl Isoamylamide (43). The title compound was prepared by general procedure E from isoamylamine (2 mmoles) and Boc-Gly-OH (2 mmoles). Product was obtained as a clear oil in about 90% yield: TLC R_f (B) .51; NMR ($CDCl_3$) δ .81 (d, J = 6 Hz, 6H), 1.1-1.7 (m, 13H, includes singlet δ 1.41), 3.20 (q, J ≅ 6 Hz, 2H), 3.70 (d, J = 6 Hz, 2H), 5.45 (m, 1H), 6.45 (m, 1H). Anal. calcd. for $C_{12}H_{24}N_2O_3$: C, 58.99; H, 9.90; N, 11.47. Found: C, 58.79; H, 9.71; N, 11.64.

N-(tert-Butyloxycarbonyl)-4(S)-amino-3(S)-hydroxyl-6-methyl-heptanoyl-glycyl Isoamylamide (44). The title compound was prepared by general procedure B from HCl·Gly-Iaa[126] (.94 mmoles) and (S,S)Boc-Sta-OH (.73 mmoles). Chromatography (5% MeOH/$CHCl_3$) followed by crystallization gave product in about 88% yield: mp 127-129°C (EtOAc/hexane); TLC R_f (B) .18; $[\alpha]_D^{24}$ -37° (c = .43, MeOH); NMR ($CDCl_3$) δ .70-.95 (m, 12H), 1.2-1.6 (m, 15H, includes singlet at δ 1.4), 2.4 (d, J = 7.5 Hz, 2H), 3.2 (q, J = 6 Hz, 2H), 3.4-4.2 (m, 5H), 4.85 (m, 1H), 6.55 (m, 1H), 7.0 (m, 1H). Anal. calcd. for $C_{20}H_{39}N_3O_5$: C, 59.83; H, 9.79; N, 10.47. Found: C, 59.63; H, 9.87; N, 10.26.

N-(tert-Butyloxycarbonyl)-L-valyl-4(S)-amino-3(S)-hydroxyl-6-methylheptanoyl-glycyl Isoamylamide (45). The title compound was prepared by general procedure D from HCl·(S,S)Sta-Gly-Iaa[126] (.3 mmoles) and Boc-valine anhydride (.6 mmoles) using methylene chloride as solvent. Chromatography (5% MeOH/$CHCl_3$) followed by crystallization gave product in about 75% yield: mp 131-132°C (EtOAc/pet. ether). TLC R_f (B) .12; $[\alpha]_D^{24}$ -49° (c = .165, MeOH); NMR ($CDCl_3$) δ .95-1.15 (m, 18H), 1.30-1.60 (m, 16H, includes singlet at δ 1.4), 2.42 (m, 2H), 3.25 (q, J ≅ 7 Hz, 2H), 3.50-4.30 (m, 5H), 4.45 (m, 1H), 5.02 (m, 1H), 6.45 (m, 1H), 7.15-7.4 (m, 2H). Anal. calcd. for $C_{25}H_{48}N_4O_6$: C, 59.98; H, 9.67; N, 11.19. Found: C, 59.87; H, 9.64; N, 11.03.

N-Isovaleryl-L-valyl-4(S)-amino-3(S)-hydroxyl-6-methylheptanoyl-glycyl Isoamylamide (46). The title compound was prepared by general procedure D from HCl·Val-(S,S)Sta-Gly-Iaa[126] (.08 mmoles) and isovaleric anhydride (.16 mmoles) using DMF as solvent. Crystallization gave product in about 40% yield: mp 204-205°C (ethyl ether); TLC R_f (A) .30; $[\alpha]_D^{24}$ -60° (c = .143, MeOH); NMR ($CDCl_3$) δ .79-1.11 (m, 24H), 1.20-1.78 (m, 8H), 1.89-2.55 (m, 4H), 3.27 (q, J = 6 Hz, 2H), 3.44-4.22 (m, 5H), 4.67 (d, J ≅ 7 Hz, 1H), 5.83 (m, 2H), 7.5 (m, 2H). Anal. calcd. for $C_{25}H_{48}N_4O_5$: C, 61.96; H, 9.98; N, 11.56. Found: C, 61.60; H, 9.97; N, 11.31.

N-(tert-Butyloxycarbonyl)-glycyl-4(S)-amino-3(S)-hydroxyl-6-methylheptanoyl-L-alanyl Isoamylamide (47). The title compound was prepared by general procedure B from HCl·(S,S)Sta-Ala-Iaa (.24 mmoles)

and Boc-Gly-OH (.24 mmoles). Chromatography (5% $MeOH/CHCl_3$) gave product as an amorphous solid in about 52% yield: TLC R_f (A) .16; $[\alpha]_D^{24}$ -22° (c = .19, MeOH); NMR ($CDCl_3$) δ .78-1.00 (m, 12H), 1.11-1.67 (m, 18H), 2.44 (d, J = 7.5 Hz, 2H), 3.42 (m, 2H), 3.55-3.89 (m, 3H), 4.00-4.55 (m, 2H), 4.89-5.55 (m, 2H), 6.0-6.44 (m, 2H), 6.89 (m, 1H). Anal. calcd. for $C_{23}H_{43}N_4O_6$: C, 57.84; H, 9.08; N, 11.73. Found: C, 57.79; H, 9.20; N, 11.61.

N-Isovaleryl-glycyl-4(S)-amino-3(S)-hydroxyl-6-methylheptanoyl-L-alanyl Isoamylamide (48). The title compound was prepared by general procedure D from HCl·Gly-(S,S)Sta-Ala-Iaa[126] (.06 mmoles) and isovaleric anhydride (.12 mmoles) using DMF as solvent. Precipitation gave product in about 70% yield: mp 91-92°C (ethyl ether); TLC R_f (A) .14; $[\alpha]_D^{24}$ -25° (c = .10, MeOH); NMR ($CDCl_3$)δ.78-1.11 (m, 18H), 1.22-1.78 (m, 10H, includes doublet δ 1.38), 2.00-2.55 (m, 4H), 3.27 (q, J = 7 Hz, 2H), 3.67-4.22 (m, 4H), 4.25-4.55 (m, 2H), 6.33-6.67 (m, 2H), 7.067.33 (m, 2H). Anal. calcd. for $C_{23}H_{44}N_4O_5 \cdot H_2O$: C, 58.20; H, 9.35; N, 11.80. Found: C, 57.90; H, 9.52; N, 11.48.

N-(tert-Butyloxycarbonyl)-L-alanyl-4(S)-amino-3(S)-hydroxyl-6-methylheptanoyl-L-alanyl Isoamylamide (49). The title compound was prepared by general procedure D from HCl·(S,S)Sta-Ala-Iaa (.21 mmoles) and Boc-alanine anhydride (.42 mmoles) using methylene chloride as solvent. Chromatography (5% $MeOH/CHCl_3$) and crystallization gave product as an amorphous solid in about 84% yield: mp 75-80°C (EtOAc/hexane); TLC R_f (B) .18; $[\alpha]_D^{24}$ -43° (c = .142, MeOH); NMR ($CDCl_3$) δ .78-1.11 (m,

12H), 1.22-1.78 (m, 21H), 2.0-2.67 (m, 2H), 3.11-3.56 (m, 3H), 3.89-4.44 (m, 3H), 4.67 (d, J = 8 Hz, 1H), 4.98 (d, J = 6 Hz, 1H), 6.22 (m, 1H), 7.33-7.55 (m, 2H). Anal. calcd. for $C_{24}H_{46}N_4O_6$: C, 59.36; H, 9.53; N, 11.54. Found: C, 59.36; H, 9.82; N, 11.37.

N-Isovaleryl-L-alanyl-4(S)-amino-3(S)-hydroxyl-6-methylheptanoyl-L-alanyl Isoamylamide (50). The title compound was prepared by general procedure D from HCl·Ala-(S,S)Sta-Ala-Iaa[126] (.07 mmoles) and isovaleric anhydride (.14 mmoles) using DMF as solvent. Precipitation gave product in about 70% yield: mp 157-158°C (ethyl ether); TLC R_f (A) .26; $[\alpha]_D^{24}$ -57° (c = .137, MeOH); NMR ($CDCl_3$) δ .89-1.11 (m, 18H), 1.33-1.78 (m, 13H, includes two doublets δ 1.42 and δ 1.38), 2.00-2.67 (m, 4H), 3.11-3.56 (m, 3H, includes quartet δ 3.27), 3.78-4.44 (m, 3H), 4.85 (d, J = 8 Hz, 1H), 5.89-6.28 (m, 2H), 7.55-7.89 (m, 2H). Anal. calcd. for $C_{24}H_{46}N_4O_5$: C, 61.25; H, 9.85; N, 11.90. Found: C, 61.33; H, 9.89; N, 11.67.

N-(tert-Butyloxycarbonyl)-L-leucyl-4(S)-amino-3(S)-hydroxyl-6-methylheptanoyl-L-alanyl Isoamylamide (51). The title compound was prepared by general procedure D from HCl·(S,S)Sta-Ala-Iaa (.21 mmoles) and Boc-leucine anhydride (.42 mmoles) using methylene chloride as solvent. Chromatography (5% MeOH/$CHCl_3$) followed by crytallization gave product in about 93% yield: mp 88-92°C (ethyl ether/pet. ether); TLC R_f (B) .15; $[\alpha]_D^{24}$ -41° (c = .53, MeOH); NMR ($CDCl_3$) δ .75-1.15 (m, 18H), 1.2-1.83 (m, 21H), 2.0-2.55 (m, 2H), 2.95-3.70 (m, 3H), 3.75-4.55 (m, 3H), 4.85 (m, 1H), 5.10 (m, 1H), 6.65 (m, 1H), 7.35-7.75 (m, 2H).

Anal. calcd. for $C_{27}H_{52}N_4O_6 \cdot \frac{1}{2} H_2O$: C, 60.41; H. 9.94; N, 10.42. Found: C, 60.75; H, 9.59; N, 10.42.

N-Isovaleryl-L-leucyl-4(S)-amino-3(S)-hydroxyl-6-methylheptanoyl-L-alanyl Isoamylamide (52). The title compound was prepared by general procedure D from HCl·Leu-(S,S)Sta-Ala-Iaa[126] (.075 mmoles) and isovaleric anhydride (.15 mmoles) using DMF as solvent. Precipitation gave product in about 77% yield: mp 149-150°C (ethyl ether); TLC R_f (A) .35; $[\alpha]_D^{24}$ -51° (c = .14, MeOH); NMR ($CDCl_3$) δ .78-1.11 (m, 24H), 1.22-1.78 (m, 13H, includes doublet δ 1.38), 2.0-2.67 (m, 4H), 3.22 (q, J ≅ 7 Hz, 2H), 3.78-4.44 (m, 4H), 4.89 (d, J = 8 Hz, 1H), 5.89-6.11 (m, 2H), 7.78-8.11 (m, 2H). Anal. calcd. for $C_{27}H_{52}N_4O_5$: C, 62.52; H, 10.10; N, 10.80. Found: C, 62.43; H, 10.07; N, 10.63.

N-(tert-Butyloxycarbonyl)-L-phenylalanyl-4(S)-amino-3(S)-hydroxyl-6-methylheptanoyl-L-alanyl Isoamylamide (53). The title compound was prepared by general procedure D from HCL·(S,S)Sta-Ala-Iaa (.21 mmoles) and Boc-phenylalanine anhydride (.42 mmoles) using methylene chloride as solvent. Chromatography (5% MeOH/$CHCl_3$) followed by crystallization gave product in about 50% yield: mp 164-166°C (EtOAc/hexane); TLC R_f (B) .41; $[\alpha]_D^{24}$ -26° (c = .19, MeOH); NMR ($CDCl_3$) δ .67-1.00 (m, 12H), 1.11-1.55 (m, 18H, includes singlet δ 1.41), 1.78-2.55 (m, 2H), 3.02 (d, J = 7 Hz, 2H), 3.24 (q, J = 7 Hz, 2H), 3.78-4.44 (m, 5H), 5.0 (d, J = 6 Hz, 1H), 6.00-6.33 (m, 2H), 6.89-7.33 (m, 6H). Anal. calcd. for $C_{30}H_{50}N_4O_6$: C, 64.15; H, 8.79; N, 9.97. Found: C, 64.05; H, 8.83; N, 9.88.

N-Isovaleryl-L-phenylalanyl-4(S)-amino-3(S)-hydroxyl-6-methylheptanoyl-L-alanyl Isoamylamide (54). The title compound was prepared by general procedure D from HCl·Phe-(S,S)Sta-Ala-Iaa[126] (.07 mmoles) and isovaleric anhydride (.14 mmoles) using DMF as solvent. Precipitation gave product in about 86% yield: mp 205-206°C (ethyl ether); TLC R_f (A) .42; $[\alpha]_D^{24}$ -31° (c = .08, MeOH); NMR ($CDCl_3$) δ .67-1.11 (m, 18H), 1.22-1.78 (m, 10H), 1.89-2.27 (m, 4H), 2.89-3.33 (m, 4H), 3.44-4.00 (m, 2H), 4.22-4.83 (m, 3H), 6.44 (m, 1H), 6.71 (m, 1H), 6.89-7.44 (m, 6H), 7.71 (m, 1H). Anal. calcd. for $C_{30}H_{50}N_4O_5$: C, 66.03; H, 9.05; N, 10.27. Found: C, 66.27; H, 9.39; N, 10.08.

N-(tert-Butyloxycarbonyl)-L-valyl-4(S)-amino-3(S)-hydroxyl-6-methylheptanoic Acid Ethyl Ester (31). The title compound was prepared by general procedure D from HCl·(S,S)-Sta-OEt[126] (1 mmole) and Boc-valine anhydride (2 mmoles) using methylene chloride as solvent. Crystallization yielded product in about 70% yield: mp 121-121.5°C (EtOAc/hexane); TLC R_f (A) .65; $[\alpha]_D^{24}$ -53° (c = .1, MeOH); NMR ($CDCl_3$) δ .75-1.10 (m, 12H), 1.15-1.90 (m, 15H, includes triplet δ 1.25, J = 7 Hz), 2.10 (m, 1H), 2.44 (d, J ≅ 6 Hz, 2H), 3.51 (m, 1H), 3.70-4.30 (m, 5H, includes quartet δ 4.15, J = 7 Hz), 5.2 (m, 1H), 6.2 (m, 1H). Anal. calcd. for $C_{20}H_{38}N_2O_6$: C, 59.67; H, 9.52; N, 6.96. Found: C, 59.61; H, 9.60; N, 6.93.

N-Isovaleryl-L-valyl-4(S)-amino-3(S)-hydroxyl-6-methylheptanoic Acid Ethyl Ester (32). The title compound was prepared by general procedure D from HCl·Val-(S,S)Sta-OEt[126] (.25 mmoles) and isovaleric

anhydride (.5 mmoles) using DMF as solvent. Crystallization gave product in about 90% yield: mp 181-182°C (EtOAc/hexane); TLC R_f (A) .50; $[\alpha]_D^{24}$ -58° (c = .063, MeOH); NMR ($CDCl_3$) δ .75-1.11 (m, 18H), 1.15-1.75 (m, 8H, includes triplet δ 1.25), 2.15 (m, 2H), 2.44 (d, J = 7 Hz, 2H), 3.65 (m, 1H), 3.85-4.35 (m, 5H, includes quartet δ 4.07), 6.15 (d, J = 9 Hz, 1H), 6.47 (d, J = 9 Hz, 1H). Anal. calcd. for $C_{20}H_{38}N_2O_5$: C, 62.15; H, 9.91; N, 7.25. Found: C, 61.95; H, 10.16; N, 7.35.

N-Isovaleryl-L-valyl-4(S)-amino-3(S)-hydroxyl-6-methylheptanoic Acid (33). The title compound was prepared by general procedure F from Iva-Val-(S,S)Sta-OEt (.16 mmoles) in about 95% yield. The compound was used in the next step without further purification.

N-Isovaleryl-L-valyl-4(S)-amino-3(S)-hydroxyl-6-methylheptanoyl-L-alanine Methyl Ester (34). The title compound was prepared by general procedure B from Iva-Val-(S,S)Sta-OH (.308 mmoles) and HCl·Ala-OMe (.46 mmoles). Chromatography (3% MeOH/$CHCl_3$) followed by crystallization gave product in about 60% yield: mp 187-188°C (ethyl ether); TLC R_f (B) .37; $[\alpha]_D^{24}$ -84° (c = .11, MeOH); NMR ($CDCl_3$) δ .67-1.11 (m, 18H), 1.15-1.67 (m, 8H, includes doublet δ 1.42), 1.78-2.55 (m, 4H), 3.42 (m, 1H), 3.73 (s, 3H), 3.78-4.11 (m, 2H, includes quartet δ 3.91), 4.57 (t, J = 8 Hz, 1H), 4.89 (d, J = 8 Hz, 1H), 5.84 (d, J ≅ 7 Hz, 1H), 7.29 (m, 1H), 7.6 (d, J ≅ 8 Hz, 1H). Anal. calcd. for $C_{22}H_{41}N_3O_6$: C, 59.57; H, 9.32; N, 9.47. Found: C, 59.40; H, 9.54; N, 9.21.

N-(tert-Butyloxycarbonyl)-L-valyl-L-valyl-4(S)-amino-3(S)-hydroxyl-6-methylheptanoic Acid Ethyl Ester (55). The title compound was prepared by general procedure D from HCl·Val-(S,S)Sta-OEt[126] (.19 mmoles) and Boc-valine anhydride (.38 mmoles) using methylene chloride as solvent. Crystallization gave product in about 85% yield: mp 172-173°C (EtOAc/hexane); TLC R_f (A) .50; $[\alpha]_D^{24}$ -70° (c = .11, MeOH); NMR ($CDCl_3$) δ .70-1.10 (m, 18H), 1.12-1.65 (m, 15H, includes triplet δ 1.25, J ≅ 7 Hz), 1.80-2.20 (m, 2H), 2.38 (m, 2H), 3.75-4.55 (m, 7H, includes quartet δ 4.12, J ≅ 7 Hz), 6.05 (m, 1H), 7.10-7.60 (m, 2H). Anal. calcd. for $C_{25}H_{47}N_3O_7$: C, 59.85; H, 9.44; N, 8.38. Found: C, 59.92; H, 9.38; N, 8.32.

N-Isovaleryl-L-valyl-L-valyl-4(S)-amino-3(S)-hydroxyl-6-methylheptanoic Acid Ethyl Ester (56). The title compound was prepared by general procedure D from HCl·Val-Val-(S,S)Sta-OEt[126] (.12 mmoles) and isovaleric anhydride (.24 mmoles) using DMF as solvent. Crystallization gave product in about 70% yield: mp 235-236°C (MeOH/ethyl ether); TLC R_f (A) .52; $[\alpha]_D^{24}$ -80° (c = .059, MeOH); NMR ($CDCl_3$) δ .90-1.11 (m, 24H), 1.24 (t, J = 7 Hz, 3H), 1.38-1.78 (m, 4H), 1.89-2.33 (m, 4H), 2.41 (d, J ≅ 7 Hz, 2H), 3.46 (m, 1H), 3.78-4.44 (m, 6H, includes quartet δ 4.1), 6.22-6.67 (m, 2H), 6.67-7.0 (m, 1H). Anal. calcd. for $C_{25}H_{47}N_3O_6$: C, 61.82; H, 9.76; N, 8.65. Found: C, 61.79; H, 9.77; N, 8.68.

N-(tert-Butyloxycarbonyl)-L-valyl-L-valyl-4(S)-amino-3(S)-hydroxyl-6-methylheptanoic Acid (57). The title compound was prepared

by general procedure F from Boc-Val-Val-(S,S)Sta-OEt. Crystals of the product precipitated from the acidified aqueous solution. The material was filtered and dried to give product in about 91% yield. The compound was used without further purification.

N-(tert-Butyloxycarbonyl)-L-valyl-L-valyl-4(S)-amino-3(S)-hydroxyl-6-methylheptanoyl-L-alanine Methyl Ester (58). The title compound was prepared by general procedure B from HCl·Ala-OMe (.09 mmoles) and Boc-Val-Val-(S,S)Sta-OH (.64 mmoles). Chromatography (3% MeOH/$CHCl_3$) followed by crystallization gave product in about 82% yield: mp 199-201°C (EtOAc/hexane); TLC R_f (B) .48; $[\alpha]_D^{24}$ -79° (c = .25, MeOH); NMR ($CDCl_3$) δ .83-1.11 (m, 18H), 1.33-1.78 (m, 15H), 2.0-2.55 (m, 4H), 3.61-4.23 (m, 8H, includes singlet δ 3.75), 4.53 (t, J = 7 Hz, 1H), 4.93 (m, 1H), 6.44 (m, 1H), 6.78 (m, 1H), 7.33 (m, 1H). Anal. calcd. for $C_{27}H_{50}N_4O_8 \cdot \frac{1}{2} H_2O$: C, 57.12; H, 9.23; N, 9.87. Found: C, 56.90; H, 9.15; N, 9.68.

N-Isovaleryl-L-valyl-L-valyl-4(S)-amino-3(S)-hydroxyl-6-methylheptanoyl-L-alanine Methyl Ester (59). The title compound was prepared by general procedure D from HCl·Val-Val-(S,S)Sta-Ala-OMe[126] (.06 mmoles) and isovaleric anhydride (.12 mmoles) using DMF as solvent. Crystallization gave product in about 85% yield: mp > 250°C (MeOH/ethyl ether); TLC R_f (B) .23; $[\alpha]_D^{24}$ -88° (c = .11, MeOH); NMR ($CDCl_3$) δ .89-1.11 (m, 24H), 1.22-1.67 (m, 9H), 2.0-2.33 (m, 4H), 3.73 (s, 3H), 4.0-4.67 (m, 6H), 7.0 (m, 1H), 7.11-7.42 (m, 3H). Anal. calcd. for $C_{27}H_{50}N_4O_7 \cdot H_2O$: C, 57.90; H, 9.35; N, 9.99. Found: C,

58.22; H, 9.29; N, 9.85.

N-(tert-Butyloxycarbonyl)-4(S)-amino-3(S)-hydroxyl-6-methyl-heptanoic Acid Pentafluorophenyl Ester (35). The title compound was prepared by general procedure G. The DCU from the reaction was filtered and the filtrate was used in the next step without any further purification.

N-(tert-Butyloxycarbonyl)-4(S)-amino-3(S)-hydroxyl-6-methyl-heptanoyl Isopropylamide (36). To a chilled ethyl acetate solution of Boc-(S,S)Sta-pFp, NMM (3 eq) was added followed by isopropylamine (1.1 eq). The reaction was stirred at 0° and closely followed by TLC. After 1 hr the solution was washed with cold 1 N HCl, saturated $NaHCO_3$ and saturated NaCl. The organic layer was dried ($MgSO_4$) and removed in vacuo. Crystallization gave product in about 80% yield: mp 129-131°C (ethyl ether/hexane); TLC R_f (B) .34; $[\alpha]_D^{24}$ -38° (c = .13, MeOH); NMR ($CDCl_3$) δ .90 (d, J = 6 Hz, 6H), 1.18 (d, J = 7 Hz, 6H), 1.30-1.75 (m, 12H), 2.25 (m, 2H), 3.52 (m, 1H), 3.75-4.20 (m, 2H), 4.32 (m, 1H), 4.78 (d, J ≅ 10 Hz, 1H), 6.0 (m, 1H). Anal. calcd. for $C_{16}H_{32}N_2O_4$: C, 60.73; H, 10.20; N, 8.85. Found: C, 60.56; H, 10.46; N, 8.91.

N-(tert-Butyloxycarbonyl)-L-valyl-4(S)-amino-3(S)-hydroxyl-6-methylheptanoyl Isopropylamide (37). The title compound was prepared by general procedure D from HCl·(S,S)Sta-Ipa[126] (.44 mmoles) and Boc-valine anhydride (.88 mmoles) using methylene chloride as solvent. Crystallization gave product in about 70% yield: mp 148-149°C (EtOAc/hexane); TLC R_f (A) .38; $[\alpha]_D^{24}$ -46° (c = .32, MeOH); NMR ($CDCl_3$)

δ .65-1.30 (m, 18H), 1.35-1.88 (m, 12H), 2.0-2.5 (m, 3H), 3.75-4.25 (m, 5H), 4.90 (m, 1H), 5.90 (m, 1H), 6.35 (m, 1H). Anal. calcd. for $C_{21}H_{41}N_3O_5$: C, 60.70; H, 9.95; N, 10.11. Found: C, 60.73, H, 9.78; N, 10.09.

N-Isovaleryl-L-valyl-4(S)-amino-3(S)-hydroxyl-6-methylheptanoyl Isopropylamide (38). The title compound was prepared by general procedure D from HCl-Val-(S,S)Sta-Ipa[126] (.07 mmoles) and isovaleric anhydride (.14 mmoles) using DMF as solvent. Precipitation gave product in about 87% yield: mp 222-223°C (ethyl ether); TLC R_f (A) .47; $[\alpha]_D^{24}$ -67° (c = .16, MeOH); NMR ($CDCl_3$) δ .76-1.11 (m, 18H), 1.11-1.22 (m, 8H, includes doublet δ 1.16), 1.33-1.67 (m, 3H), 1.89-2.33 (m, 4H), 3.78-4.33 (m, 5H), 5.95 (m, 2H), 6.22 (d, J ≅ 9 Hz, 1H). Anal. calcd. for $C_{21}H_{41}N_3O_4$: C, 63.13; H, 10.34; N, 10.52. Found: C, 62.90; H, 10.42; N, 10.32.

N-(tert-Butyloxycarbonyl)-L-alanine Pentafluorophenyl Ester (24). The title compound was prepared by general procedure G in about 70% yield. The product was crystallized from hexane and used without purification in the next step.

N-(tert-Butyloxycarbonyl)-L-alanyl Methyl Amide (25). Boc-Ala-pFp (600 mg, 1.7 mmol) was dissolved in freshly distilled THF (5 ml) and chilled to 0°C. A 1.4 M solution of methylamine in THF (2.42 ml, 3.4 mmol) was added dropwise over a period of about 20 minutes. The reaction was monitored closely by TLC and appeared to be complete

after about ½ hour. The THF was removed in vacuo and EtOAc was added to the residue. The solution was then washed with cold 1 N HCl, saturated $NaHCO_3$ and saturated NaCl. The organic layer was dried ($MgSO_4$) and the solvent was removed in vacuo. Crystallization gave product in about 40% yield: mp 112-113°C (EtOAc/hexane); TLC R_f (B) .35; $[\alpha]_D^{24}$ -13° (c = .71, MeOH); NMR ($CDCl_3$) δ 1.25-1.65 (m, 12H, includes singlet δ 1.41), 2.85 (d, J = 5 Hz, 3H), 4.0-4.40 (m, 1H), 5.15 (m, 1H), 6.37 (m, 1H). Anal. calcd. for $C_9H_{18}N_2O_3$: C, 53.45; H, 8.97; N, 13.85. Found: C, 53.28; H, 9.08; N, 13.69.

N-Isovaleryl-L-valyl-4(S)-amino-3(S)-hydroxyl-6-methylheptanoyl-L-alanyl Methyl Amide (26). The title compound was prepared by general procedure B from HCl·Ala-NH-CH_3[126] and Iva-Val-(S,S)Sta-OH. Chromatography (5% MeOH/$CHCl_3$) followed by precipitation gave product in about 15% yield: mp 233-235°C (ethyl ether); TLC R_f (B) .17; $[\alpha]_D^{24}$ -33° (c = .10, MeOH); NMR ($CDCl_3$) δ .78-1.11 (m, 18H), 1.22-1.79 (m, 8H, includes doublet δ 1.39), 1.89-2.44 (m, 4H), 5.0 (d, J ≅ 5 Hz, 3H), 3.33-3.56 (m, 1H), 3.78-4.11 (m, 2H), 4.35 (t, J = 7 Hz, 1H), 4.73 (d, J = 8 Hz, 1H), 6.11 (d, J = 8 Hz, 1H), 6.39 (m, 1H), 7.38-7.78 (m, 2H). Anal. calcd. for $C_{22}H_{42}N_4O_5 \cdot H_2O$: C, 57.40; H, 9.63; N, 12.23. Found: C, 57.80; H, 9.23; N, 11.91.

N-(tert-Butyloxycarbonyl)-L-alanyl Isopropylamide (27). Boc-Ala-pFp (13) (1.55 mmoles) was dissolved in EtOAc (100 mg/ml) and chilled to 0°. Isopropylamine (3.1 mmoles) was slowly added to the solution over a period of 20 min. The reaction mixture was allowed to

stir for about 2 hrs. After this the mixture was washed with cold 1 N HCl, saturated $NaHCO_3$ and saturated NaCl. The organic layer was dried and the solvent was removed in vacuo. Crystallization gave product in about 50% yield: mp 111-112°C (ethyl ether/hexane); TLC R_f (B) .26; $[\alpha]_D^{24}$ -18° (c = .53, MeOH); NMR ($CDCl_3$) δ 1.13 (d, J = 6 Hz, 6H), 1.25-1.85 (m, 12H), 3.80-4.25 (m, 2H), 4.95 (m, 1H), 5.90 (m, 1H). Anal. calcd. for $C_{11}H_{22}N_2O_3$: C, 57.37; H, 9.63; N, 12.16. Found: C, 57.46; H, 9.70; N, 12.11.

<u>N-(tert-Butyloxycarbonyl)-4(S)-amino-3(S)-hydroxyl-6-methylheptanoyl-alanyl Isopropylamide (28)</u>. The compound was prepared by general procedure B from HCl·Ala-Ipa[126] (.37 mmoles) and Boc-(S,S)Sta-OH (.37 mmoles). Chromatography (3% MeOH/$CHCl_3$) followed by crystallization gave product in about 75% yield: mp 151-152°C (EtOAc/hexane); TLC R_f (B) .33; $[\alpha]_D^{24}$ -40° (c = .34, MeOH); NMR ($CDCl_3$) δ .79-1.30 (m, 12H), 1.35-1.80 (m, 15H), 2.20-2.60 (m, 3H), 3.61 (m, 1H), 3.81-4.23 (m, 3H), 4.42 (m, 1H), 4.81 (m, 1H), 6.35 (m, 1H), 6.82 (m, 1H). Anal. calcd. for $C_{19}H_{37}N_3O_5$: C, 58.89; H, 9.63; N, 10.84. Found: C, 58.71; H, 9.68; N, 10.60.

<u>N-(tert-Butyloxycarbonyl)-L-valyl-4(S)-amino-3(S)-hydroxyl-6-methylheptanoyl-L-alanyl Isopropylamide (29)</u>. The title compound was prepared by general procedure D from HCl·(S,S)Sta-Ala-Ipa[126] (.2 mmoles) and Boc-valine anhydride (.4 mmoles) using methylene chloride as solvent. Chromatography (5% MeOH/$CHCl_3$) followed by precipitation gave product in about 75% yield: mp 218-220°C (ethyl ether); TLC R_f (A)

.53; $[\alpha]_D^{24}$ -44° (c = .51, MeOH); NMR ($CDCl_3$) δ .72-1.25 (m, 18H), 1.28-1.75 (m, 14H), 1.80-2.70 (m, 4H), 3.40-4.40 (m, 6H), 5.0 (m, 1H), 6.28 (m, 1H), 7.40-7.70 (m, 2H). Anal. calcd. for $C_{24}H_{46}N_4O_6$: C, 59.24; H, 9.53; N, 11.51. Found: C, 59.17; H, 9.49; N, 11.35.

N-Isovaleryl-L-valyl-4(S)-amino-3(S)-hydroxyl-6-methylheptanoyl-L-alanyl Isopropylamide (30). The title compound was prepared by general procedure D from HCl·Val-(S,S)Sta-Ala-Ipa[126] (.1 mmole) and isovaleric anhydride (.2 mmole) using DMF as solvent. Crystallization gave product in about 80% yield: mp > 250°C (MeOH/ethyl ether); TLC R_f (A) .41; $[\alpha]_D^{24}$ -48° (c = .10, MeOH); NMR (DMSO-d_6) δ .83-1.17 (m, 24H), 1.20-1.50 (m, 8H), 2.0-2.33 (m, 4H), 3.55-4.44 (m, 5H), 4.84 (d, J = 6 Hz, 1H), 7.22 (m, 1H), 7.60-7.67 (m, 3H). Anal. calcd. for $C_{24}H_{46}N_4O_5$: C, 61.25; H, 9.85; N, 11.90. Found: C, 61.10; H, 10.04; N, 11.74.

(4R,5S)-3-[3-Phenyl-propionyl]-4-methyl-5-phenyloxazolidinone (64). (4R,5S)-4-Methyl-5-phenyloxazolidinone[74] (16.7 g, .09 moles) was dissolved in dry THF (90 ml) in a 2-necked, 500 ml round bottom flask under an N_2 atmosphere. The solution was then chilled to -78°C and to this a 1.6 N solution of n-butyllithium in hexane (.09 moles) was added dropwise. The solution was stirred at -78°C for 15 min. After this a solution of dihydrocinnamoyl chloride (.09 moles) in dry THF (40 ml) was added over a period of about .5 hr. The mixture was allowed to stir at -78° for an additional 15 min and then allowed to slowly warm to room temperature. The mixture was poured over brine and extracted with ethyl acetate (2 x 100 ml). The organic phase was washed with 1 N

HCl, saturated $NaHCO_3$ and brine. The organic layer was dried ($MgSO_4$) and removed in vacuo. The resulting residue was then recrystallized from hexane to yield pure product in about 96% yield (2 crops): mp 83-85°C; TLC R_f (D) .64, R_f (Q) .25; $[\alpha]_D^{24}$+32.7° (c = .97, $MeCl_2$); NMR ($CDCl_3$) δ .82-.95 (d, J = 7 Hz, 3H), 2.87-3.34 (m, 4H), 4.71 (m, 1H), 5.60 (d, J = 7 Hz, 1H), 7.13-7.48 (m, 10H). Anal. calcd. for $C_{19}H_{19}NO_3$: C, 73.77; H, 6.19; N, 4.53. Found: C, 73.95; H, 6.17; N, 4.41.

<u>(4S,5S)-3-[(2S)-2-Benzyl-3-benzyloxypropionyl]-4-methyl-S-phenyloxazolidinone (65)</u>. Compound <u>64</u> (25 g, 81 mmoles) was dissolved in dry THF (30 ml) in a 2-necked 500 ml round bottom flask under an N_2 atmosphere and chilled to -78°C. To this solution was added a 1 M solution of lithium bis(trimethysilyl)amide in THF (84 mmoles) dropwise via syringe over a period of .5 hr. The mixture was stirred for an additional .5 hr before benzyl bromomethyl ether[74] (240 mmoles) in THF (20 ml) was added over a period of .5 hr via a pressure equalized addition funnel. The mixture was stirred at -78°C for .5 hr, warmed to -45°C (acetonitrile-dry ice) for 4 hr then warmed to 0° for 1 hr. To the stirred mixture was added dry pyridine (18 ml, 228 mmol) and acetic anhydride (14.4 ml, 152 mmol). The mixture was stirred at room temperature for 3 hr and then diluted with 190 ml of 2 M aqueous $KHCO_3$. The mixture was stirred until gas evolution ceased. The mixture was concentrated in vacuo to about 200 ml. The residue was extracted with methylene chloride (2 x 150). The organic phase was washed with 1 N HCl, saturated $NaHCO_3$, and saturated brine, dried ($MgSO_4$) and removed

in vacuo. The residue was recrystallized from hexane to yield pure product in about 65% yield (2 crops): mp 85-86°; TLC R_f (Q) .20; $[\alpha]_D^{24}$ -23.0° (c = 1.02, $MeCl_2$), NMR ($CDCl_3$ δ .81 (d, J = 7 Hz, 3H), 2.93 (d, J = 7.5 Hz, 2H), 3.55-3.97 (m, 2H), 4.38-4.85 (m, 4H includes singlet δ 4.52), 5.27 (d, J = 7.5 Hz, 1H), 7.10-7.50 (m, 15H). Anal. calcd. for $C_{27}H_{27}N_1O_4$: C, 75.51; H, 6.33; N, 3.26. Found: C, 75.69; H, 6.46; N, 3.19.

(2R)-2-Benzyl-3-benzyloxypropanol (66). Calcium chloride (2.88 g, 25.9 mmoles) was dissolved in dry absolute ethanol (42 ml) and chilled to -20°C. Sodium borohydride (1.96 g, 51.8 mmoles) was added, and the mixture was stirred for 1 hr. Compound 65 (7 g, 17.3 mmoles) in THF (28 ml) was added slowly via syringe. The mixture was stirred at -10°C for 3 hr before the reaction was quenched with 1 N $KHSO_4$. The aqueous layer was extracted with ethyl acetate (2 x 50 ml) and the organic layer was subsequently washed with 1 N $KHSO_4$, saturated $NaHCO_3$ and brine. The organic layer was dried ($MgSO_4$) and removed in vacuo. Ethyl ether was added to the residue to precipitate the recyclable oxazolidinone. This was filtered and the filtrate was again concentrated in vacuo to a clear oil. This residue was applied to a 400 g MPLC column and eluted with 40% ethyl ether/hexane at a flow rate of about 6 ml/min. Product was obtained as a clear oil in about 90% yield: TLC R_f (H) .40; $[\alpha]_D^{24}$ +23.6° (c = .72, EtOH); NMR ($CDCl_3$) δ 2.12 (m, 1H), 2.40 (m, 1H), 3.65 (d, J = 7.5 Hz, 2H), 3.40-3.75 (m, 4H), 4.48 (s, 2H), 7.05-7.44 (m, 10H). Anal. calcd. for $C_{17}H_{20}O_2$: C, 79.66; H, 7.86. Found: C, 79.48; H, 7.67.

(2S)-2-Benzyl-3-benzyloxy-1-bromopropane (67). Alcohol 66 (3.7 g, 14.4 mmoles) was dissolved in methylene chloride (25 ml) and the solution was chilled to 0°C. Triphenylphosphine (3.9 g, 14.8 mmoles) was added followed by N-bromosuccinimide (2.6 g, 14.8 mmoles) which was added in small portions over a period of .5 hr. The reaction was stirred at room temperature overnight. The mixture was concentrated in vacuo and 50% ethyl ether/hexane (70 ml) was added to precipitate triphenylphosphine oxide. The filtrate was evaporated in vacuo and the residue dissolved in ethyl acetate. The organic phase was washed with 1 N HCl, saturated $NaHCO_3$ and brine, dried ($MgSO_4$) and evaporated in vacuo. The resulting residue was chromatographed over 200 g of silica gel (MPLC grade silica gel) eluting with 5% ethyl ether/hexane under positive pressure (~5 ml/min). Product was obtained as a clear liquid in about 80% yield: TLC R_f (H) .57; $[\alpha]_D^{24}$ -2.75 °(c = .8, EtOH); NMR ($CDCl_3$) δ 2.24 (m, 1H), 3.68 (d, J = 7.5 Hz, 2H), 3.30-3.55 (m, 4H), 4.42 (s, 2H), 7.05-7.40 (m, 10H). Anal. calcd. for $C_{17}H_{19}BrO$: C, 63.96; H, 6.00. Found: C, 63.74; H, 6.16.

(2R,4RS,5S)-2-Benzyl-1-benzyloxy-5-tert-butyloxycarbonylamino-4-hydroxyl-7-methyl octane (68). Into a dry, 2-necked 25 ml round bottom flask under a stream of dry nitrogen were added magnesium powder (170 mg, 7.1 mmoles) and a few crystals of iodine. The flask was heated with a heat gun until I_2 vapors were visible, then allowed to cool to room temperature. Dry THF (2 ml) was added via syringe followed by 1 drop of (2S)-2-benzyl-3-benzyloxy-1-bromopropane (67). The mixture was warmed until reaction began, then the remainder of the

bromoether (total 1.75 g, 5.48 mmol) in THF (1 ml) was added over a period of 1.5 hr. The mixture was then stirred for 4 hr before Boc-leucinal (591 mg, 2.75 mmoles) in THF was added over a period of 2 hr. After stirring an additional 4 hr at room temperature, the mixture was poured into saturated aqueous NH_4Cl. Ethyl acetate was added and the mixture was acidified with 2 N HCl. The organic layer was then washed with saturated $NaHCO_3$ and brine, dried ($MgSO_4$) and removed in vacuo. The residue was chromatographed over 50 g of silica gel (MPLC grade silica gel) and eluted with 15% ethyl acetate/hexane under positive pressure (~5 ml/min). Product was obtained as a mixture of 4R,4S diastereomers in about 30% yield (4S:4R ~ 3:2) as an oil: TLC R_f (K) .15; NMR ($CDCl_3$) δ .84 (d, J = 6 Hz, 6H), 1.15-1.68 (m, 14H), 2.38 (m, 1H), 2.70 (dd, J = 7 Hz, J = 3 Hz, 2H), 3.04 (m, 1H), 3.37-3.88 (m, 4H), 4.51 (s, 2H), 4.71 (m, 1H), 7.10-7.40 (m, 5H), 7.42 (s, 5H). Anal. calcd. for $C_{28}H_{41}NO_4$: C, 73.81; H, 9.07; N, 3.07. Found: C, 73.63; H, 8.80; N, 3.30.

N-(tert-Butyloxycarbonyl)-L-valyl-5(S)-amino-2(R)-benzyl-1-benzyloxy-4(R,S)-hydroxyl-7-methyl octane (69). Compound 68 (110 mg, .24 mmoles) was deprotected according to general procedure A. The resulting hydrochloride was coupled with Boc-valine anhydride (.48 mmoles) according to general procedure D using methylene chloride as solvent. The crude compound was chromatographed on 10 g of silica gel (MPLC grade) and eluted with 1% $MeOH/CHCl_3$ under positive pressure (~5 ml/min). Pure compound was obtained as an oil in about 75% yield: TLC R_f (B) .66; NMR ($CDCl_3$) δ .85-1.0 (m, 12H), 1.15-1.75 (m, 14H), 1.95-

2.33 (m, 2H), 2.61 (m, 2H), 3.22-3.91 (m, 6H), 4.46 (s, 2H), 5.00 (m, 1H), 5.97 (m, 1H), 6.90-7.39 (m, 10H). Anal. calcd. for $C_{33}H_{50}N_2O_5$: C, 71.45; H, 9.08; N, 5.05. Found: C, 71.31; H, 9.29; N, 5.24.

N-(tert-Butyloxycarbonyl)-L-valyl-4(R,S)-acetoxy-5(S)-amino-2(R)-benzyl-1-benzyloxy-7-methyl octane (70). Compound 69 (70 mg, .125 mmoles) was dissolved in ethyl acetate (1.5 ml), and triethylamine (34 µl, .126 mmoles), acetic anhydride (23 µl, .252 mmoles) and 4-dimethylaminopyridine (~1.5 mg, .0126 mmoles) were added. After stirring 5 hr at room temperature, the mixture was diluted with ethyl acetate and washed with 1 N HCl, saturated $NaHCO_3$ and brine. The organic layer was dried and removed in vacuo. The residue was chromatographed over 10 g of silica gel eluting with 50% ethyl ether/hexane. Pure product was obtained as an oil in quantitative yield: TLC R_f (H) .28 (4S isomer), .22 (4R isomer); NMR ($CDCl_3$) δ .75-1.10 (m, 12H), 1.12-1.75 (m, 14H), 1.90-2.18 (m, 5H includes singlet δ 2.08), 2.40-3.0 (m, 2H), 3.37 (d, J = 5 Hz, 2H), 3.70-3.95 (m, 1H), 4.19 (m, 1H), 4.48 (s, 2H), 4.92-5.21 (m, 2H), 5.90 (d, J = 10 Hz, 1H), 7.05-7.30 (m, 5H), 7.32 (s, 5H). Anal. calcd. for $C_{35}H_{52}N_2O_6$: C, 70.44; H, 8.78; N, 4.69. Found: C, 70.32; H, 8.93; N, 4.80.

N-(tert-Butyloxycarbonyl)-L-valyl-4(R,S)-acetoxy-5(S)-amino-2(R)-benzyl-1-hydroxyl-7-methyl octane (71). The benzyl ether 70 (76 mg, .127 mmoles) was dissolved in a mixture of 50% isopropanol/acetic acid (2 ml) and the solution was flushed with N_2 gas. Powdered ammonium formate (31 mg, .51 mmoles) followed by 10% palladium on carbon

(30 mg) were added and the mixture was stirred overnight. The mixture was filtered through celite rinsing with ethyl acetate (20 ml). The organic layer was washed with 1 N HCl, saturated $NaHCO_3$ and brine, dried ($MgSO_4$) and removed in vacuo. The residue was chromatographed on 10 g of silica gel (MPLC grade) eluting with 60% ethyl ether/hexane under positive pressure (~5 ml/min). Pure product was obtained as an oil in about 95% yield: TLC R_f (G) .10; NMR ($CDCl_3$) δ .75-1.10 (m, 12H), 1.20-1.75 (m, 14H), 1.90-2.25 (m, 5H includes singlet δ 2.04), 2.51-2.79 (m, 3H), 3.54 (m, 1H), 3.82 (m, 1H), 4.20 (m, 1H), 5.03 (m, 1H), 5.20-5.65 (m, 2H), 6.16 (d, J = 10 Hz, 1H), 7.24 (s, 5H). Anal. calcd. for $C_{28}H_{46}N_2O_6 \cdot \frac{1}{2} H_2O$: C, 65.35; H, 9.20; N, 5.44. Found: C, 65.39; H, 8.97; N, 5.46.

N-(tert-Butyloxycarbonyl)-L-valyl-4(R,S)-acetoxy-5(S)-amino-2(R)-benzyl-7-methyl octanoic acid (72). Compound 71 (61 mg, .12 mmoles) was dissolved in dry DMF (1 ml). Pyridinium dichromate (342 mg, .91 mmoles) was added and the mixture was stirred under an atmosphere of N_2 for 30 hrs. The mixture was diluted with H_2O (2 ml) and ethyl acetate (20 ml). While cooling and stirring the reaction, the H_2O layer was acidified with solid $KHSO_4$. The layers were separated and the H_2O layer was re-extracted with ethyl acetate (2 x 10 ml). The combined organic extracts were dried ($MgSO_4$) and evaporated in vacuo. The crude product, obtained in about 95% yield was used without further purification: TLC R_f (A + 10 drops acetic acid) .42; NMR ($CDCl_3$) δ .75-1.10 (m, 12H), 1.21-1.62 (m, 13H), 1.80-2.25 (m, 5H), 2.55-3.14 (m, 3H), 3.72 (m, 1H), 4.10 (m, 1H), 5.00 (m, 1H), 5.29 (m, 1H), 7.20 (s,

5H), 9.00 (m, 1H).

N-(tert-Butyloxycarbonyl)-L-valyl-4(R,S)-acetoxy-5(S)-amino-2(R)-benzyl-7-methyloctanoyl-isoamylamide (79). Compound 72 (30 mg, .058 mmoles), was coupled to isoamylamine (.063 mmoles), according to general procedure B. After work-up the crude peptide was chromatographed over 10 g of silica gel (MPLC grade) eluting with 1% MeOH/$CHCl_3$ under positive pressure (~3 ml/min). Product was obtained as an oil in about 85% yield: TLC R_f (C) .47; NMR ($CDCl_3$) δ .75-1.07 (m, 18H), 1.12-2.2 (m, 21H), 2.30-2.85 (m, 3H), 3.15 (m, 2H), 3.78 (m, 1H), 4.11 (m, 1H), 4.78-5.04 (m, 2H), 5.72-5.98 (m, 2H), 7.19 (s, 5H).

N-Isovaleryl-L-valyl-4(R,S)-acetoxy-5(S)-amino-2(R)-benzyl-7-methyl-octanoyl-isoamylamide (80). Compound 79 (30 mg, .05 mmoles) was deprotected according to general procedure A. The resulting hydrochloride was coupled to isovaleryl anhydride (.10 mmoles) according to general procedure D in DMF. Following aqueous work-up the crude compound was chromatographed over 10 g of silica gel (MPLC grade) eluting with 40% ethyl acetate/hexane under positive pressure (~3-5 ml/min) to afford pure major component (2R,4S,5S) in 77% yield as a white solid and pure minor component (2R,4R,5S) in 12% yield as a white solid. Major component 80a (2R,4S,5S): mp 156-157°C (ethyl ether/hexane), TLC R_f (M) .25; NMR ($CDCl_3$) δ .79-1.12 (m, 24H), 1.15-1.70 (m, 8H), 1.70-2.43 (m, 8H), 2.71-3.38 (m, 4H), 4.21 (m, 2H), 4.96 (m, 1H), 5.81 (m, 1H), 6.07 (d, J = 10 Hz, 1H), 6.25 (d, J = 8.5 Hz, 1H), 7.22 (m, 5H).

N-Isovaleryl-L-valyl-5(S)-amino-2(R)-benzyl-4(S)-hydroxyl-7-methyl-octanoyl-isoamylamide (81). Compound 80a (26 mg, .046 mmoles) was dissolved in dry MeOH (1 ml). Powdered K_2CO_3 (30 mg, .22 mmoles) was added and the mixture was stirred at room temperature for 3-4 hrs. After this time, it appeared that the product had precipitated from the methanol. H_2O was added to the mixture to dissolve K_2CO_3 and the insoluble peptide was collected by filtration and dried to yield pure compound as a white solid in 75% yield: mp 223-225°C; TLC R_f (A) .61; $[\alpha]_D^{24}$ -34° (c = .14, MeOH); NMR (d_4-MeOH) δ .66-1.01 (m, 24H), 1.11-1.76 (m, 8H), 1.83-2.22 (m, 5H), 2.56-3.56 (m, 4H), 3.76-4.72 (m, 3H), 7.22 (s, 5H). Anal. calcd. for $C_{31}H_{53}N_3O_4 \cdot \frac{1}{2} H_2O$: C, 68.85; H, 10.06; N, 7.77. Found: C, 68.79; H, 9.89; N, 7.62.

N-Isovaleryl-L-valyl-5(S)-amino-2(R)-benzyl-7-methyl-4-oxo-octanoyl-isoamylamide (82). Compound 81 (.01 mmoles) was dissolved in glacial acetic acid (1 ml). To this, pyridinium dichromate (.03 mmoles) was added and the mixture was stirred for 8 hrs. Then, the mixture was diluted with $CHCl_3$ (10 ml) and H_2O (10 ml). The layers were separated and the organic layer was washed with 1 N HCl, saturated $NaHCO_3$ and brine. The organic layer was dried ($MgSO_4$) and removed in vacuo. Recrystallization from methanol/ethyl ether afforded pure product in 90% yield: mp 167-168°C, TLC R_f (A) .72; $[\alpha]_D^{24}$ -56° (c = .17, MeOH); NMR (d_4-MeOH) δ .89-1.02 (m, 24H), 1.35-1.68 (m, 4H), 1.78-2.16 (m, 8H), 2.42 (m, 1H), 3.22-3.41 (m, 4H), 4.03 (m, 1H), 4.21-4.41 (m, 1H), 7.14-7.33 (m, 5H). Anal. calcd. for $C_{31}H_{51}N_3O_4 \cdot \frac{1}{2} H_2O$: C, 69.11; H, 9.73; N, 7.80. Found: C, 69.00; H, 9.62; N, 7.55.

N-(tert-Butyloxycarbonyl)-L-valyl-4(R,S)-acetoxy-5(S)-amino-2(R)-benzyl-7-methyl-octanoyl-L-alanyl-isoamylamide (73). Compound 72 (20 mg, .038 mmoles) was coupled with HCl·Ala-Iaa (.042 mmoles), according to general procedure B. After work-up the crude peptide was chromatographed over 10 g of silica gel (MPLC grade) eluting with .75% MeOH/$CHCl_3$ under positive pressure (~3 ml/min). Product was obtained as an oil in about 80% yield: TLC R_f (A) .73, R_f (B) .41; NMR ($CDCl_3$) δ .75-1.10 (m, 18H), 1.15-2.20 (m, 21H), 2.25-2.84 (m, 3H), 3.03 (m, 2H), 3.75 (m, 1H), 4.05-4.57 (m, 3H), 4.65-5.12 (m, 2H), 5.79-6.20 (m, 2H), 6.50 (m, 1H), 7.23 (m, 5H). Anal. calcd. for $C_{36}H_{60}N_4O_7$: C, 65.43; H, 9.15; N, 8.48. Found: C, 65.44; H, 9.20; N, 8.42.

N-Isovaleryl-L-valyl-4(R,S)-acetoxy-5(S)-amino-2(R)-benzyl-7-methyl octanoyl-L-alanyl-isoamylamide (74). Compound 73 (26 mg, .039 mmoles) was deprotected according to general procedure A. The resulting hydrochloride was coupled to isovaleryl anhydride (.08 mmoles) according to general procedure D in DMF. Following aqueous work-up the crude compound was chromatographed over 10 g of silica gel eluting with 66% ethyl acetate/hexane to afford pure major component 74a (2R,4S,5S) in 70% yield as a white solid and pure minor component 74b (2R,4R,5S) as a white solid in 10% yield. Major component 74a (2R,4S,5S): mp 142-143°C (ethyl ether/hexane); TLC R_f (A) .77, R_f (N) .13; $[\alpha]_D^{24}$ -44° (c = .10, MeOH); NMR ($CDCl_3$) δ .71-1.12 (m, 24H), 1.21-2.25 (m, 19H, includes singlet δ 2.08), 2.33-3.24 (m, 4H), 4.04-4.52 (m, 3H), 4.94 (m, 1H), 5.90-6.44 (m, 3H), 6.58 (d, J = 7 Hz, 1H), 7.04-7.34 (m, 5H). Anal. calcd. for $C_{36}H_{60}N_4O_6 \cdot H_2O$: C, 65.23; H, 9.42; N, 8.45. Found:

C, 65.35; H, 9.14; N, 7.97.

N-Isovaleryl-L-valyl-5(S)-amino-2(R)-benzyl-4(S)-hydroxyl-7-methyl-octanoyl-L-alanyl Isoamylamide (75). Compound 74a (15 mg, .023 mmoles) was dissolved in dry MeOH (1 ml). Powdered K_2CO_3 (9 mg, .07 mmoles) was added and the mixture was stirred at room temperature for 3-4 hrs. After this time it appeared that the product has precipitated from the methanol. H_2O was added to the mixture to dissolve K_2CO_3 and the insoluble peptide was collected by filtration and dried to yield pure compound as a white solid in 87% yield: mp >250°C; TLC R_f (A) .47; $[\alpha]_D^{24}$ -33° (c = .21, MeOH); NMR (d_4-MeOH) δ .66-1.05 (m, 24H), 1.11-1.78 (m, 11H), 1.80-2.22 (m, 5H), 2.56-3.56 (m, 4H), 3.60-4.33 (m, 4H), 7.18 (s, 5H). Anal. calcd. for $C_{34}H_{58}N_4O_5 \cdot H_2O$: C, 65.78; H, 9.74; N, 9.02. Found: C, 65.50; H, 9.42; N, 8.78.

N-Isovaleryl-L-valyl-5(S)-amino-2(R)-benzyl-7-methyl-4-oxo-octanoyl-L-alanyl Isoamylamide (76). Compound 75 (8 mg, .011 mmoles) was dissolved in glacial acetic acid (1 ml). To this pyridinium dichromate (.033 mmoles) was added and the mixture was stirred for 7-8 hrs. The mixture was diluted with $CHCl_3$ (10 ml) and H_2O (10 ml). The layers were separated and the organic layer was washed with 1 N HCl, saturated $NaHCO_3$ and brine. The organic layer was then dried ($MgSO_4$) and removed in vacuo. Recrystallization from methanol/ethyl ether afforded pure product in 83% yield: mp 238-239°C; TLC R_f (A) .63, R_f (B) .57; $[\alpha]_D^{24}$ -21.5° (c = .2, MeOH); NMR (MeOH-d_4) δ .81-1.02 (m, 24H), 1.23-1.76 (m, 11H), 1.91-2.15 (m, 4H), 2.68 (m, 1H), 2.91 (m,

2H), 3.12 (m, 2H), 4.11-4.24 (m, 2H), 4.39 (m, 1H), 7.24 (m, 5H). Anal. calcd. for $C_{34}H_{56}N_4O_5 \cdot 1.5$ MeOH: C, 65.71; H, 9.52; N, 8.63. Found: C, 65.73; H, 9.14; N, 8.68.

(2R)-3-Benzyloxy-1-bromo-2-methylpropane (84). Alcohol 83[74] (6 g, 33.3 mmoles) in $MeCl_2$ (50 ml) chilled to 0°C, triphenylphosphine (8.7 g, 33.3 mmoles) and N-bromosuccinimide (5.72 g, 33.3 mmoles) were converted to the title compound following the procedure described for enantiomer 67. Product was obtained as a clear liquid in about 70% yield: TLC R_f (J) .23; $[\alpha]_D^{24}$ -12.2° (c = 5.5, EtOH); NMR ($CDCl_3$) δ 1.0 (d, J = 7 Hz, 3H), 2.07 (m, 1H), 3.28-3.52 (m, 4H), 4.43 (s, 2H), 7.27 (s, 5H). Anal. calcd. for $C_{11}H_{15}BrO \cdot \frac{1}{4}$ hexane: C, 56.71; H, 7.04. Found: C, 56.94; H, 7.03.

(2S,4RS,5S)-1-Benzyloxy-5-tert-butyloxycarbonyl-amino-4-hydroxyl-2,7-dimethyloctane (85). Into a dry, 3-neck 100 ml round bottom flask under a stream of dry nitrogen were added magnesium powder (.575 g, 18.53 mmoles) and a few crystals of iodine. The flask was heated with a heat gun until I_2 vapors were visible, then allowed to cool to room temperature. Dry THF (7 ml) was added via syringe followed by 1 drop of (2R)-3-benzyloxy-1-bromo-2-methylpropane. The mixture was warmed until reaction began, then the remainder of the bromoether (total 4.5 g, 18.53 mmol) in THF (5 ml) was added over a period of 1.5 hr. The mixture was then stirred for 4 hrs, then cooled to 0° before Boc-leucinal (2.0 g, 9.3 mmoles) in THF was added over a period of 2 hr. After stirring an additional 4 hr at 0°C, the mixture was

poured into saturated aqueous NH_4Cl. Ethyl acetate was added and the mixture was acidified with 2 N HCl. The organic layer was then washed with saturated $NaHCO_3$ and brine, dried ($MgSO_4$) and removed in vacuo. The residue was then chromatographed on a 200 g silica gel column (MPLC grade silica gel) and eluted with 15% ethyl acetate/hexane under positive pressure (~5 ml/min). Product was obtained as a mixture of 4R,4S diastereomers in about 50% yield (4S:4R ~ 2:1[127]) as an oil: TLC R_f (K) .29; NMR ($CDCl_3$) δ .75-1.04 (m, 9H), 1.10-1.60 (m, 14H), 2.05 (m, 1H), 3.20-3.80 (m, 5H), 4.52 (s, 2H), 4.79 (m, 1H), 7.33 (s, 5H). Anal. calcd. for $C_{22}H_{37}NO_4$: C, 69.62; H, 9.83; N, 3.69. Found: C, 69.38; H, 9.91; N, 3.72.

N-(tert-Butyloxycarbonyl)-L-valyl-5(S)-amino-1-benzyloxy-4(R,S)-hydroxyl-2(S),7-dimethyloctane (86). Compound 85 (820 mg, 2.16 mmoles) was deprotected according to general procedure A. The resulting hydrochloride was then coupled with Boc-valine anhydride (4.3 mmoles) according to general procedure D using methylene chloride as solvent. The crude compound was chromatographed on 50 g of silica gel (MPLC grade) and eluted with 66% ethyl ether/hexane under positive pressure (~5 ml/min). Pure compound was obtained as an oil in about 65% yield: TLC R_f (F) .20; NMR ($CDCl_3$) δ .75-1.05 (m, 15H), 1.12-1.65 (m, 14H), 1.79-2.20 (m, 2H), 3.10-3.48 (m, 2H), 3.50-4.15 (m, 4H), 4.55 (s, 2H), 5.08 (d, J = 8 Hz, 1H), 6.16 (m, 1H), 7.37 (s, 5H). Anal. calcd. for $C_{27}H_{46}N_2O_5$: C, 67.75; H, 9.69; N, 5.85. Found: C, 67.51; H, 9.66; N, 5.89.

N-(tert-Butyloxycarbonyl)-L-valyl-4(R,S)-acetoxy-5(S)-amino-1-benzyloxy-2(S),7-dimethyloctane (87). Compound 86 (400 mg, .83 mmoles) was dissolved in ethyl acetate (10 ml). To this, triethylamine (227 µl, .83 mmoles), acetic anhydride (146 µl, 1.6 mmoles) and 4-dimethylaminopyridine (10 mg, .083 mmoles) were added. The reaction was carried out as for compound 70. The organic layer was then dried. The crude product was chromatographed on a 20 g silica gel column (MPLC grade) eluting with 50% ethyl ether/hexane under positive pressure (5 ml/min). Product was obtained as an oil in 95% yield: TLC R_f (H) .29 (4S isomer), .21 (4R isomer); NMR ($CDCl_3$) δ .75-1.12 (m, 15H), 1.15-1.90 (m, 14H), 1.94-2.30 (m, 5H), 3.29 (m, 2H), 3.83 (m, 1H), 4.25 (m, 1H), 4.45 (s, 2H), 4.82-5.39 (m, 2H), 5.95-6.25 (m, 1H), 7.29 (s, 5H). Anal. calcd. for $C_{29}H_{48}N_2O_6$: C, 66.89; H, 9.29; N, 5.38. Found: C, 66.53; H, 9.29; N, 5.36.

N-(tert-Butyloxycarbonyl)-L-valyl-4(R,S)-acetoxy-5(S)-amino-1-hydroxyl-2(S),7-dimethyloctane (88). The title compound was prepared from benzyl ether 87 (340 mg, .653 mmoles), dissolved in 8 ml of 50% isopropanol/acetic acid, powdered ammonium formate (199 mg, 2.9 mmoles) and 10% Pd-C (180 mg) as described for compound 71. The crude product was chromatographed on a 20 g silica gel column (MPLC grade) and eluted with 75% ethyl ether/hexane under positive pressure (~5 ml/min). Pure product was obtained as an oil in about 75% yield: TLC R_f (E) .12; NMR ($CDCl_3$) δ .80-1.15 (m, 15H), 1.20-1.95 (m, 14H), 2.0-2.25 (m, 5H), 2.81 (m, 1H), 3.44 (m, 2H), 3.81 (m, 1H), 4.22 (m, 1H), 4.84-5.31 (m, 2H), 6.15 (m, 1H). Anal. calcd. for $C_{22}H_{42}N_2O_6 \cdot H_2O$: C, 58.90; H,

9.87; N, 6.24. Found: C, 59.04; H, 9.69; N, 6.14.

N-(tert-Butyloxycarbonyl)-L-valyl-4(R,S)-acetoxy-5(S)-amino-2(S),7-dimethyloctanoic acid (89). The title compound was prepared from alcohol 88 (150 mg, .35 mmoles) and pyridinium dichromate (1.05 g, 2.8 mmoles) as described for compound 73. The crude product, obtained in about 75% yield, was used without further purification: TLC R_f (A) .23 (major isomer), .12 (minor isomer); NMR ($CDCl_3$) δ .76-1.10 (m, 12H), 1.15-1.77 (m, 16H), 2.92-2.19 (m, 5H), 2.51 (m, 1H), 3.81 (m, 1H), 4.22 (m, 1H), 5.03 (m, 1H), 5.48 (m, 1H), 6.20-6.75 (m, 1H), 10.0 (m, 1H).

N-(tert-Butyloxycarbonyl)-L-valyl-4(R,S)-acetoxy-5(S)-amino-2(S),7-dimethyloctanoyl-L-alanyl-isoamylamide (90). Compound 89 (100 mg, .22 mmoles), was coupled to HCl·Ala-Iaa (.33 mmoles), according to general procedure B. After work-up the crude peptide was chromatographed on a 15 g silica gel column (MPLC grade) eluting with .75% MeOH/$CHCl_3$ under positive pressure (~3 ml/min). Product was obtained as an amorphous solid, upon precipitation from ethyl ether, in about 85% yield: TLC R_f (A) .54 (major), .42 (minor); NMR ($CDCl_3$) δ .90 (m, 18H), 1.05-1.80 (m, 21H), 1.90-2.34 (m, 7H), 3.22 (m, 2H), 3.82 (m, 1H), 4.18 (m, 1H), 4.49 (m, 1H), 4.92 (m, 1H), 5.19 (d, J = 8 Hz, 1H), 6.21 (d, J = 9 Hz, 1H), 6.63 (d, J = 8 Hz, 1H), 6.88 (m, 1H). Anal. calcd. for $C_{30}H_{50}N_4O_7 \cdot 1(Et)_2O$: C, 61.98; H, 10.09; N, 8.50. Found: C, 61.86; H, 9.90; N, 8.82.

N-Isovaleryl-L-valyl-4(R,S)-acetoxy-5(S)-amino-2(S),7-dimethyloctanoyl-L-alanyl-isoamylamide (91). Compound 90 (79 mg, .135 mmoles) was deprotected according to general procedure A. The resulting hydrochloride was coupled to isovaleryl anhydride (.27 mmoles) according to general procedure D in DMF. Following aqueous work-up the crude compound was chromatographed on a 10 g silica gel column (MPLC grade) eluting with .75% MeOH/$CHCl_3$ under positive pressure (2-3 ml/min) to afford pure major component (2S,4S,5S) 91a in 57% yield as a white solid and pure minor component (2S,4R,5S) 91b in 23% yield as a white solid. Major component 91a (2S,4S,5S): mp 199-203°C (ethyl ether/hexane); TLC R_f (B) .21; $[\alpha]_D^{24}$ -59° (c = .11, MeOH); NMR ($CDCl_3$) δ .92 (m, 27H), 1.10-1.80 (m, 13H), 1.82-2.35 (m, 6H), 3.22 (m, 2H), 4.21 (m, 2H), 4.52 (m, 1H), 5.00 (m, 1H), 6.18-6.65 (m, 3H), 6.78 (m, 1H). Anal. calcd. for $C_{30}H_{56}N_4O_6$: C, 63.35; H, 9.92; N, 9.85. Found: C, 63.06; H, 9.90; N, 9.63. Minor component 91b (2S,4R,5S): mp 211--214°C (ethyl ether/hexane); TLC R_f (A) .15; $[\alpha]_D^{24}$-27° (c = .10, MeOH); NMR ($CDCl_3$) δ .67-1.07 (m, 27H), 1.29 (m, 11H), 1.82 (m, 2H), 2.09 (m, 6H), 3.29 (m, 2H), 4.0-4.56 (m, 3H), 4.93 (m, 1H), 6.11 (m, 1H), 6.14-6.67 (m, 3H). Anal. calcd. for $C_{30}H_{56}N_4O_6 \cdot \frac{1}{4} H_2O$: C, 62.85; H, 9.93; N, 9.77. Found: C, 62.54; H, 9.86; N, 9.41.

N-Isovaleryl-L-valyl-5(S)-amino-4(S)-hydroxyl-2(S),7-dimethyloctanoyl-L-alanyl-isoamylamide (92a). The title compound was prepared from 91a (26 mg, .046 mmoles) and K_2CO_3 (57 mg, .41 mmoles) as described for compound 81. Pure product was obtained as a white solid in 83% yield: mp 246-249°; TLC R_f (A) .37; $[\alpha]_D^{24}$ -40° (c = .37, MeOH);

NMR (d_4-MeOH) δ .85 (m, 24H), .99 (d, J = 7.2 Hz, 3H), 1.11-1.61 (m, 15 H), 1.79-1.94 (m, 3H), 2.21 (q, J = 7.1 Hz, 1H), 2.84 (t, J = 7.2 Hz, 2H), 3.21 (m, 1H), 3.52 (m, 1H), 3.67 (d, J = 8.0 Hz, 1H), 3.79 (q, J = 7.8 Hz, 1H). Anal. calcd. for $C_{28}H_{54}N_4O_5 \cdot H_2O$: C, 61.72; H, 10.36; N, 10.28. Found: C, 61.50; H, 10.18; N, 10.00.

N-Isovaleryl-L-valyl-5(S)-amino-4(R)-hydroxyl-2(S),7-dimethyloctanoyl-L-alanyl-isoamylamide (92b). The title compound was obtained from 91b (13 mg, .023 mmoles) and powdered K_2CO_3 (28 mg, .20 mmoles) as described for compound 81. Pure product was obtained as a white solid in 81% yield: mp 233-235°C; TLC R_f (A) .37; $[\alpha]_D^{24}$ -36° (c = .11, MeOH); NMR (d_4-MeOH) δ .81-1.02 (m, 24H), 1.10 (d, J = 7.0 Hz, 3H), 1.22-1.73 (m, 15H), 1.94-2.18 (m, 3H), 2.64 (m, 1H), 3.10-3.40 (m, 6H), 3.85 (m, 1H), 4.14 (m, 1H), 4.27 (m, 1H). Anal. calcd. for $C_{28}H_{54}N_4O_5 \cdot H_2O$: C, 61.72; H, 10.36; N, 10.28. Found: C, 61.36; H, 10.20; N, 9.96.

N-Isovaleryl-L-valyl-5(S)-amino-4-oxo-2(S),7-dimethyloctanoyl-L-alanyl Isoamylamide (93). Compound 92b (15 mg, .028 mmoles) was dissolved in glacial acetic acid (2 ml). To this, pyridinium dichromate (.084 mmoles) was added and the mixture was stirred for 7-8 hrs. The mixture was then diluted with $CHCl_3$ (20 ml) and H_2O (10 ml). The layers were separated and the organic layer was washed with 1 N HCl, saturated $NaHCO_3$ and brine. The organic layer was then dried ($MgSO_4$) and removed in vacuo. The resulting solid was chromatographed on a 5 g silica gel column, eluting with 1% MeOH/$CHCl_3$. Pure product was

obtained in about 84% yield: mp >250°C; TLC R_f (A) .46; $[\alpha]_D^{24}$ -45° (c = .11, MeOH); NMR ($CDCl_3/d_4$-MeOH) δ .67-.98 (m, 24H), 1.09 (d, J = 7.3 Hz), 1.13-1.32 (m, 7H), 1.41-1.72 (m, 4H), 2.0 (m, 3H), 2.40-2.70 (m, 2H), 3.12 (m, 2H), 4.11 (m, 1H), 4.25 (m, 1H), 4.51 (m, 1H). Anal. calcd. for $C_{28}H_{52}N_4O_5 \cdot 1.25$ acetic acid: C, 61.01; H, 9.57; N, 9.37. Found: C, 60.64; H, 9.25; N, 9.75.

N-Isovaleryl-L-valyl-5(S)-amino-4-oxo-7-methyl-octanoyl-L-alanyl Isoamylamide (137). N-Isovaleryl-L-valyl-5(S)-amino-4(R,S)-hydroxyl-7-methyl-octanoyl-L-alanyl-isoamylamide (40 mg, .078 mmoles) was dissolved in acetic acid (2 ml). Pyridinium dichromate (.23 mmoles) was added and the mixture was stirred for 12 hr. The mixture was diluted with $CHCl_3$ (20 ml) and H_2O (10 ml) and the layers separated. The organic phase was washed with 1 N HCl, saturated $NaHCO_3$ and brine, dried ($MgSO_4$) and removed in vacuo. Recrystallization from MeOH/ethyl ether afforded product in 70% yield: mp 227-228°C; TLC R_f (A) .38; $[\alpha]_D^{24}$ -93° (c = .76, MeOH); NMR (MeOH-d_4) δ .80-1.08 (m, 24H), 1.25-1.82 (m, 8H), 1.91-2.30 (m, 5H), 2.47 (m, 2H), 2.84 (m, 2H), 3.18 (m, 2H), 4.17-4.55 (m, 3H). Anal. calcd. for $C_{27}H_{50}N_4O_5$: C, 63.49; H, 9.87; N, 10.97. Found: C, 63.45; H, 9.77; N, 10.84.

(4S)-3-[3-Phenyl-propionyl]-4-(2-propyl)-2-oxazolidinone (95). (4S)-4-(2-Propyl)-2-oxazolidinone[74] (15.0 g, .116 moles) was dissolved in dry THF (90 ml) in a 2-necked, 500 ml round bottom flask under an N_2 atmosphere. The solution was then chilled to -78°C and to this a 1.6 N solution of n-butyllithium in hexane (.116 moles) was

added dropwise. The solution was then stirred at -78°C for 15 min. After this a solution of dihydrocinnamoyl chloride (.116 moles) in dry THF (40 ml) was added over a period of about .5 hr. The mixture was then allowed to stir at -78° for an additional 15 min and then allowed to slowly warm to room temperature. The mixture was then poured over brine and extracted with ethyl acetate (2 x 100 ml). The organic phase was then washed with 1 N HCl, saturated $NaHCO_3$ and brine. The organic layer was then dried ($MgSO_4$) and removed in vacuo. The resulting residue was then recrystallized from ethyl acetate/hexane to yield pure product in about 82% yield (2 crops): mp 62°C; TLC R_f (L) .56, R_f (I) .12; $[\alpha]_D^{24}$ +70.90° (c = 1.5, $MeCl_2$); NMR ($CDCl_3$) δ .80-.92 (m, 6H), 2.18-2.48 (m, 1H), 2.85-3.40 (m, 4H), 4.12-4.50 (m, 3H), 7.26 (s, 5H). Anal. calcd. for $C_{15}H_{19}NO_3 \cdot \frac{1}{4} H_2O$: C, 67.78; H, 7.39; N, 5.27. Found: C, 67.53; H, 7.23; N, 4.82.

<u>(4S)-3-[(2R)-2-Benzyl-3-benzyloxypropionyl]-4-(2-propyl)-2-oxazolidinone (96)</u>. Compound <u>95</u> (22 g, 84 mmoles) was dissolved in dry THF (30 ml) in a 2-necked, 500 ml round bottom flask under an N_2 atmosphere and chilled to -78°C. To this a 1 M solution of lithium bis(trimethylsilyl)amide in THF (84 mmoles) was added dropwise via syringe over a period of .5 hr. The mixture was stirred for an additional .5 hr before benzyl bromomethyl ether (240 mmoles) in THF (20 ml) was added over a period of .5 hr via a pressure equalized addition funnel. The mixture was stirred at -78°C for .5 hr, warmed to -45°C (acetonitrile-dry ice) for 4 hr, then warmed to 0° for 1 hr. To the stirred mixture was added dry pyridine (18 ml, 228 mmol) and acetic anhydride

(14.4 ml, .52 mmol). The mixture was stirred at room temperature for 3 hr and then diluted with 190 ml of 2 M aqueous $KHCO_3$. The mixture was stirred until gas evolution ceased. The mixture was concentrated in vacuo to about 200 ml. The residue was extracted with methylene chloride (2 x 150 ml). The organic phase was then washed with 1 N HCl, saturated $NaHCO_3$ and saturated brine, dried ($MgSO_4$) and removed in vacuo. The residue was recrystallized from ethyl ether/hexane to yield pure product in about 90% yield (2 crops): mp 60°C; TLC R_f (L) .59; $[\alpha]_D^{24}$ +59.1° (c = 2.0, $MeCl_2$); NMR ($CDCl_3$) δ .80-.95 (m, 6H), 2.32 (m, 1H), 2.94 (dd, J = 4 Hz, 8Hz, 2H), 3.55-4.18 (m, 5H), 4.22-4.64 (m, 4H, includes singlet δ 4.46), 7.13-7.40 (m, 10H). Anal. calcd. for $C_{23}H_{27}NO_7 \cdot ½ H_2O$: C, 70.75; H, 7.23; N, 3.58. Found: C, 70.91; H, 7.18; N, 3.27.

<u>(2S)-2-Benzyl-3-benzyloxypropanol (97)</u>. Oxazolidinone <u>96</u> (17.16 g, 45 mmoles) was dissolved in absolute ethanol (134 ml) and chilled to -20°C under an N_2 atmosphere. To this a 2 N solution of $LiBH_4$ in THF (67 ml, 134 mmoles) was slowly added. After 4 hr the reaction was worked up as for compound <u>66</u>. The residue was then applied to a 400 g MPLC column and eluted with 20% ethyl ether/hexane at a flow rate of 6 ml/min. Product was obtained as a clear oil in about 95% yield: TLC R_f (H) .19; $[\alpha]_D^{24}$ -26.50° (c = 7.4, EtOH); NMR ($CDCl_3$) δ 2.04 (m, 1H), 3.59 (d, J = 7 Hz, 2H), 3.72 (m, 1H), 3.34-3.70 (m, 4H), 4.38 (s, 2H), 7.0-7.40 (m, 10H). Anal. calcd. for $C_{17}H_{20}O_2 \cdot ½ H_2O$: C, 76.95; H, 7.97. Found: C, 77.12; H, 7.69.

<u>(2R)-2-Benzyl-3-benzyloxy-1-bromopropane (98)</u>. Alcohol <u>97</u> (8.45 g, 33 mmoles) was dissolved in $MeCl_2$ (50 ml) and chilled to 0°C. To this triphenylphosphine (8.7 g, 33.3 mmoles) was then added in small portions over a period of .5 hr. The reaction and work-up conditions were the same as described for compound <u>67</u>. The crude product was then chromatographed on a 300 g silica gel column (MPLC grade silica gel) eluting with 5% ethyl ether/hexane under positive pressure (~5 ml/min). Product was obtained as a clear liquid in about 77% yield: TLC R_f (H) .60; $[\alpha]^{24}_{D}$ +2.1° (c = 8.0, EtOH); NMR ($CDCl_3$) δ 2.20 (m, 1H), 3.67 (d, J = 7.5 Hz, 2H), 3.31-3.57 (m, 4H), 4.45 (s, 2H), 7.04-7.45 (m, 10H). Anal. calcd. for $C_{17}H_{19}BrO \cdot 1/8\ H_2O$: C, 63.51; H, 6.04. Found: C, 63.47; H, 6.21.

<u>(2S,4RS,5S)-2-Benzyl-1-benzyloxy-5-tert-butyloxycarbonyl-amino-4-hydroxyl-7-methyloctane (99)</u>. Into a dry, 3-neck 100 ml round bottom flask under a stream of dry nitrogen were added magnesium powder (.575 g, 18.53 mmoles) and a few crystals of iodine. The flask was heated with a heat gun until I_2 vapors were visible, then allowed to cool to room temperature. Dry THF (7 ml) was added via syringe followed by 1 drop of (2R)-2-benzyl-3-benzyloxy-1-bromopropane. The mixture was warmed until reaction began, then the remainder of the bromoether (total 5.9 g, 18.53 mmol) in THF (5 ml) was added over a period of 1.5 hr. The mixture was then stirred for 4 hr. The mixture was then cooled to 0° and Boc-leucinal (2.0 g, 9.3 mmoles) in THF was added over a period of 2 hr. After stirring an additional 4 hr at 0°, the mixture was poured into saturated aqueous NH_4Cl. Ethyl acetate was

added and the mixture was acidified with 2 N HCl. The organic layer was then washed with saturated $NaHCO_3$ and brine, dried ($MgSO_4$) and removed in vacuo. The residue was then chromatographed on a 200 g silica gel column (MPLC grade silica gel) and eluted with 15% ethyl acetate/hexane under positive pressure (~5 ml/min). Product was obtained as a mixture of 4R,4S diastereomers in about 35% yield (4S:4R ~ 10-20:1[127]) as an oil: TLC R_f (O) .40; NMR ($CDCl_3$) δ .90 (d, J = 5.5 Hz, 6H), 1.20-1.65 (m, 14H), 2.12 (m, 1H), 2.57 (d, J = 6 Hz, 2H), 3.10-3.70 (m, 5H), 4.45 (s, 2H), 4.62 (m, 1H), 7.15 (m, 5H), 7.30 (s, 5H). Anal. calcd. for $C_{28}H_{41}NO_4$: C, 73.81; H, 9.07; N, 3.07. Found: C, 73.55; H, 8.97; N, 3.15.

N-(tert-Butyloxycarbonyl)-L-valyl-5(S)-amino-2(S)-benzyl-1-benzyloxy-4(R,S)-hydroxyl-7-methyloctane (100). Compound 99 (500 mg, 1.1 mmoles) was deprotected according to general procedure A. The resulting hydrochloride was then coupled with Boc-valine anhydride (2.2 mmoles) according to general procedure D using methylene chloride as solvent. The crude compound was chromatographed on 50 g of silica gel (MPLC grade) and eluted with 66% ethyl ether/hexane under positive pressure (~5 ml/min). Pure compound was obtained as an oil in about 82% yield: TLC R_f (F) .24; NMR ($CDCl_3$) δ .75-1.05 (m, 12H), 1.15-1.68 (m, 14H), 1.84-2.31 (m, 2H), 2.56 (d, J = 7 Hz, 2H), 3.17-4.04 (m, 6H), 4.43 (s, 2H), 5.07 (d, J = 9 Hz, 1H), 6.12 (d, J = 9 Hz, 1H), 6.95-7.37 (m, 10H). Anal. calcd. for $C_{33}H_{50}N_2O_5$: C, 71.45; H, 9.08; N, 5.05. Found: C, 71.52; H, 9.13; N, 5.05.

N-(tert-Butyloxycarbonyl-L-valyl-4(R,S)-acetoxy-5(S)-amino-2(S)-benzyl-1-benzyloxy-7-methyloctane (101). Compound 100 (300 mg, 54 mmoles) was dissolved in ethyl acetate (10 ml). To this, triethylamine (148 ml, 54 mmoles), acetic anhydride (100 ml, 1.1 mmoles) and 4-dimethylaminopyridine (6.5 mg, .054 mmoles) were added. The reaction was then carried out as described for compound 70. The crude product was chromatographed on a 20 g silica gel column (MPLC grade) and eluted with 50% ethyl ether/hexane under positive pressure (~5 ml/min). Product was obtained as an oil in about 95% yield: TLC R_f (H) .31 (4S isomer), .25 (4R isomer); NMR ($CDCl_3$) δ .72-1.08 (m, 12H), 1.15-1.70 (m, 14H), 1.85-2.20 (m, 5H, includes singlet δ 2.08), 2.46-2.79 (m, 2H), 3.28 (m, 2H), 3.79 (m, 1H), 4.24 (m, 1H), 4.44 (s, 2H), 4.92-5.21 (m, 2H), 5.97 (broad d, J = 10 Hz, 1H), 7.15 (m, 5H), 7.34 (s, 5H). Anal. calcd. for $C_{35}H_{52}N_2O_6$: C, 70.44; H, 8.78; N, 4.69. Found: C, 70.65; H, 9.00; N, 4.44.

N-(tert-Butyloxycarbonyl)-L-valyl-4(R,S)-acetoxy-5(S)-amino-2(S)-benzyl-1-hydroxyl-7-methyloctane (102). Benzyl ether 101 (240 mg, .40 mmoles) dissolved in a mixture of 50% isopropanol/acetic acid (6 ml), powdered ammonium formate (122 mg, 1.8 mmoles) and Pd-C (150 mg) were reacted as described for compound 71. The crude product was chromatographed on a 20 g silica gel column (MPLC grade) and eluted with 75% ethyl ether/hexane under positive pressure (~5 ml/min). Pure product was obtained as an oil in about 94% yield: TLC R_f (E) .22; NMR ($CDCl_3$) δ .74-1.05 (m, 12H), 1.10-1.80 (m, 14H), 1.90-2.20 (m, 5H, includes singlet δ 2.07), 2.42 (m, 3H), 3.43 (m, 2H), 3.78 (m, 1H),

4.21 (m, 1H), 4.95-5.25 (m, 2H), 6.12 (m, 1H), 7.14 (s, 5H). Anal. calcd. for $C_{28}H_{46}N_2O_6$: C, 66.38; H, 9.15; N, 5.53. Found: C, 66.44; H, 9.31; N, 5.66.

N-(tert-Butyloxycarbonyl)-L-valyl-4(R,S)-acetoxy-5(S)-amino-2(S)-benzyl-7-methyl-octanoic acid (103). Compound 102 (150 mg, .3 mmoles) was dissolved in dry DMF (1 ml) and reacted with pyridinium dichromate (885 mg, 2.36 mmoles) as described for compound 72. The crude product, obtained in about 80% yield, was used without further purification: TLC R_f (A) .27, R_f (N) .29; NMR ($CDCl_3$) δ .81-1.1 (m, 12H), 1.15-1.65 (m, 13H), 1.82-2.24 (m, 5H), 2.53-3.00 (m, 3H), 3.54-4.31 (m, 2H), 4.98 (m, 1H), 5.22 (m, 1H), 6.21 (m, 1H), 7.14 (s, 5H), 7.91 (m, 1H).

N-(tert-Butyloxycarbonyl)-L-valyl-4(R,S)-acetoxy-5(S)-amino-2(S)-benzyl-7-methyl-octanoyl-L-alanyl Isoamylamide (104). Compound 103 (80 mg, .152 mmoles), was coupled to HCl·Ala-Iaa (.23 mmoles), according to general procedure B. After work-up the crude peptide was chromatographed on a 15 g silica gel column (MPLC grade) eluting with .75% MeOH/$CHCl_3$ under positive pressure (~3 ml/min). Product was obtained as an oil in about 90% yield: TLC R_f (A) .59 (major), .50 (minor), R_f (N) .33; NMR ($CDCl_3$) δ .85 (m, 18H), 1.10-2.20 (m, 23H, includes singlets at δ 1.42 and 2.06), 2.30-2.84 (m, 3H), 3.18 (m, 2H), 3.81 (m, 1H), 4.08-4.36 (m, 3H), 4.83-5.10 (m, 2H), 5.86 (d, J = 7.5 Hz, 1H), 6.13 (d, J = 9.5 Hz, 1H), 6.52 (m, 1H), 7.15 (m, 5H). Anal. calcd. for $C_{36}H_{60}N_4O_7 \cdot 1.5$ MeOH: C, 63.53; H, 9.38; N, 7.90. Found:

C, 63.52; H, 9.11; N, 7.62.

N-Isovaleryl-L-valyl-4(R,S)-acetoxy-5(S)-amino-2(S)-benzyl-7-methyl-octanoyl-L-alanyl Isoamylamide (105). Compound 104 (49 mg, .074 mmoles) was deprotected according to general procedure A. The resulting hydrochloride was coupled to isovaleryl anhydride (.14 mmoles) according to general procedure D in DMF. Following aqueous work-up the crude compound was chromatographed on a 10 g silica gel column (MPLC grade) eluting with .75% MeOH/$CHCl_3$ under positive pressure (~2 ml/min) and recrystallized to afford pure major component (2S,4S,5S) 105a in 90% yield as a white solid and minor component (2S,4R,5S) 105b in 5% yield. Major component (2S,4S,5S): mp 138-139°C (ethyl ether/hexane); TLC R_f (B) .24; $[\alpha]_D^{24}$ -60° (c = .10, MeOH); NMR ($CDCl_3$) δ .94 (m, 24H), 1.16-1.74 (m, 9H), 1.81-2.59 (m, 10H), 2.85 (m, 2H), 3.21 (m, 2H), 4.11-4.54 (m, 3H), 5.08 (m, 1H), 5.98 (m, 1H), 6.30 (d, J = 9 Hz, 1H), 6.44-6.85 (m, 2H), 7.05-7.45 (m, 5H). Anal. calcd. for $C_{36}H_{60}N_4O_6 \cdot \frac{1}{2} H_2O$: C, 66.13; H, 9.40; N, 8.57. Found: C, 65.91; H, 9.31; N, 8.39.

N-Isovaleryl-L-valyl-5(S)-amino-2(S)-benzyl-4(S)-hydroxyl-7-methyl-octanoyl-L-alanyl Isoamylamide (106). Compound 105a (37 mg, .056 mmoles) was reacted with powdered K_2CO_3 (40 mg, .29 mmoles) as described for compound 81. Pure product was obtained as a white solid in 95% yield: mp 220-223°C; TLC R_f (A) .50; $[\alpha]_D^{24}$ -64° (c = .24, MeOH); NMR (d_4-MeOH) δ .67-1.10 (m, 24H), 1.14-1.78 (m, 9H), 1.89-2.22 (m, 7H), 2.69 (m, 2H), 3.11 (m, 2H), 3.56-4.21 (m, 4H), 7.17 (s, 5H).

Anal. calcd. for $C_{34}H_{58}N_4O_5 \cdot MeOH$: C, 66.22; H, 9.80; N, 8.82. Found: C, 66.17; H, 9.42; N, 8.53.

N-Isovaleryl-L-valyl-5(S)-amino-2(S)-benzyl-7-methyl-4-oxo-octanoyl-L-alanyl Isoamylamide (107). Compound 106 (17 mg, .028 mmoles) was dissolved in glacial acetic acid (1 ml). To this, pyridinium dichromate (.084 mmoles) was added and the mixture was stirred for 7-8 hrs. The mixture was then diluted with $CHCl_3$ (20 ml) and H_2O (10 ml). The layers were separated and the organic layer was washed with 1 N HCl, saturated $NaHCO_3$ and brine. The organic layer was then dried ($MgSO_4$) and removed in vacuo. The resulting crude solid was chromatographed on a 5 g silica gel column, eluting with 1% MeOH/$CHCl_3$. Pure product was obtained by precipitation from hexane, in 81% yield: mp 222-223°C; TLC (R_f (A) .57; $[\alpha]_D^{24}$ -83° (c = .54, MeOH); NMR ($CDCl_3$/d_4-MeOH) δ .75-1.10 (m, 24H), 1.11-1.65 (m, 11H), 2.0 (m, 4H), 2.43-3.21 (m, 5H), 4.01-4.52 (m, 3H), 7.18 (m, 5H). Anal. calcd. for $C_{34}H_{56}N_4O_5$: C, 65.73; H, 9.14; N, 8.68. Found: C, 65.96; H, 9.16; N, 8.62.

N^4-(tert-Butyloxycarbonyl)-N^8-(benzyloxycarbonyl)-4(S),8-diamino-3(R,S)-hydroxyl-octanoyl Ethyl Ester (109a,b). To 6 ml of dry tetrahydrofuran cooled by dry ice-CCl_4 was added diisopropylamine (20.4 mmoles) under a nitrogen atmosphere, followed by a 1.6 N solution of n-butyllithium in hexane (20.4 mmoles). After 1 hr the bath temperature was lowered to -78°C and dry ethyl acetate (20.4 mmoles) was added via syringe and stirred for 15 min. α-Boc-ε-Cbz-lysinal[66] (4.96 g, 13.6 mmoles) in 15 ml of dry tetrahydrofuran was added via syringe over

about 5 min. The reaction was stirred for 20-25 min before 1 N HCl was added. The mixture was warmed to room temperature, acidified to pH 2-3, and extracted with ethyl acetate two times. The organic layer was washed with saturated NaCl, dried, and evaporated in vacuo. The resulting oil was chromatographed on 400 g of silica gel using 20% ethyl acetate in toluene. Pure compound was obtained as a mixture of 3R and 3S diastereomers in 62% yield: TLC R_f (O) .08, R_f (B) .41. Anal. calcd. for $C_{23}H_{36}N_2O_7$: C, 61.05; H, 8.20; N, 6.19. Found: C, 60.98; H, 8.11; N, 6.12.

N^4-(tert-Butyloxycarbonyl)-N^8-(benzyloxycarbonyl)-O-(ethoxycarbonyl)-4(S),8-diamino-3(R,S)-hydroxyloctanoyl Ethyl Ester (110a,b). Compound 109a,b (80 mg, .18 mmoles) was dissolved in dry methylene chloride (2 ml). Triethylamine (.36 mmoles) was added followed by ethyl chloroformate (1.08 mmoles), dimethylaminopyridine (.02 mmoles) and the reaction was stirred at room temperature overnight. Ethyl acetate (15 ml) was added to the mixture which was then washed with 1 N HCl, saturated $NaHCO_3$ and saturated NaCl. The organic phase was dried ($MgSO_4$) and removed in vacuo. The residue was chromatographed over 8 g silica gel eluting with 50% diethyl ether/hexane which separated the 3(R) and 3(S) diastereomers. The 3(S) isomer of compound 110a,b (110a) was isolated in about 54% yield: TLC R_f (E) .56; NMR ($CDCl_3$) δ 1.10-1.40 (m, 6H), 1.40-1.60 (m, 15H), 2.65 (d, J = 7 Hz, 2H), 3.0-3.30 (m, 2H), 3.50-4.0 (m, 1H), 4.0-4.30 (m, 6H), 4.50-4.90 (m, 2H), 5.05-5.25 (m, 3H includes singlet δ 5.11, 2H), 7.36 (s, 5H). The 3(R) isomer (110b) was isolated in about 38% yield: TLC R_f (E) .46; NMR $CDCl_3$ δ

1.10-1.60 (m, 21H), 2.61 (d, J = 7 Hz, 2H), 3.0-3.30 (m, 2H), 3.60-4.0 (m, 6H), 4.40-4.75 (m, 2H), 5.00-5.20 (m, 3H includes singlet δ 5.11, 2H), 7.37 (m, 5H).

N^4-(tert-Butyloxycarbonyl)-N^8-(benzyloxycarbonyl)-O-(2,2,2-trichloroethoxycarbonyl)-4(S),8-diamino-3(R,S)-hydroxyl octanoyl Ethyl Ester (111a,b). Compound 109a,b (2.6 g, 5.74 mmoles) was dissolved in pyridine (40 ml). Dimethylaminopyridine (.57 mmoles) and trichloroethyl chloroformate (11.48 mmoles) were added sequentially to this solution. After 20 min of stirring at room temperature, ethyl acetate (100 ml) was added and the mixture was washed with 1 N HCl, saturated $NaHCO_3$ and saturated NaCl. The organic phase was dried and reduced in vacuo. The residue was applied to a 200 g MPLC column and eluted with 30% ethyl ether/hexane at a flow rate of about 2-5 ml/min, which separated the 3R and 3S isomers. The 3(S) isomer 111a was obtained as an oil in about 43% yield: TLC R_f (H) .36; NMR ($CDCl_3$) δ 1.1-1.65 (m, 18H, includes triplet δ 1.23, J = 7.5 Hz), 2.71 (d, J = 7 Hz, 2H), 3.05-3.20 (m, 2H), 3.7-4.0 (m, 1H), 4.15 (q, J = 7.5 Hz, 2H), 4.50-4.95 (m, 4H), 5.0-5.40 (m, 3H, includes a singlet δ 5.09), 7.01 (s, 5H). The 3(R) isomer 111b was isolated as an oil in about 42% yield: TLC R_f (H) .32; NMR ($CDCl_3$) δ 1.15-1.65 (m, 18H, includes triplet δ 1.27, J = 7.5 Hz), 2.67 (d, J = 7.0 Hz, 2H), 3.0-3.30 (m, 2H), 3.70-4.35 (m, 4H, includes quartet δ 4.22, J = 7.5 Hz), 4.55 (broad doublet, J = 9 Hz, 1H), 4.81 (s, 2H), 5.0-5.30 (m, 3H, includes singlet δ 5.13), 7.40 (s, 5H).

N[4]-(tert-Butyloxycarbonyl)-N[8]-(benzyloxycarbonyl)-4(S),8-diamino-3(S)-hydroxyl-octanyl Ethyl Ester (109a). Compound 111a (1.4 g, 2.23 mmoles) was dissolved in a 1:1 mixture of DMF/acetic acid (5 ml). Metallic cadmium powder (67 mmoles) was added to the solution and the mixture was stirred for 7 hrs at room temperature. The suspension was filtered and washed with DMF/acetic acid (5 ml) and ethyl acetate (30-40 ml). The solution was neutralized with solid K_2CO_3, washed with saturated NaCl, dried ($MgSO_4$) and evaporated in vacuo. The residue was chromatographed on a 100 g silica gel column (MPLC grade silica gel) and eluted with 50% ethyl acetate/hexane under positive pressure (flow ~ 5-7 ml/min). The product was isolated as an oil in about 90% yield: TLC R_f (B) .54, R_f (M) .17; $[\alpha]_D^{24}$ -7.8° (c = .60, EtOH); NMR ($CDCl_3$) δ 1.17-1.65 (m, 18H, includes triplet δ 1.27, J = 7.5 Hz), 2.51 (dd, J = 7 Hz, 3Hz, 2H), 3.0-3.30 (m, 2H), 3.30-3.75 (m, 2H), 3.90-4.35 (m, 3H, includes quartet δ 4.20, J = 7.5 Hz), 4.50-5.15 (m, 4H, includes singlet δ 5.13), 7.39 (s, 5H). Anal. calcd. for $C_{23}H_{36}N_2O_7$: C, 61.05, H, 8.20; N, 6.19. Found: C, 61.14; H, 8.09; N, 6.21.

N[4]-(tert-Butyloxycarbonyl)-N[8]-(benzyloxycarbonyl)-4(S),8-diamino-3(R)-hydroxyl-octanoyl Ethyl Ester (109b). The title compound was prepared from compound 111b (1.5 g, 2.39 mmoles) using a procedure analogous to that for the preparation of 111a. Starting material was recovered by chromatography as an oil in about 37% yield and product was obtained as an oil in about 54% yield: TLC R_f (M) .16; $[\alpha]_D^{24}$ -16.9° (c = .60, EtOH); NMR ($CDCl_3$) δ 1.15-1.70 (m, 18H, includes triplet δ 1.29, J = 7.5 Hz), 2.49 (dd, J = 2 Hz, 6 Hz, 2H), 3.10-3.35 (m, 2H),

3.35-3.80 (broad multiplet, 2H), 3.85-4.35 (m, 3H, includes quartet δ 4.21, J = 7.5 Hz), 4.45-5.05 (m, 2H), 5.13 (s, 2H), 7.40 (s, 5H). Anal. calcd. for $C_{23}H_{36}N_2O_7$: C, 61.05; H, 8.20; N, 6.19. Found: C, 61.02; H, 8.11; N, 6.19.

N-(tert-Butyloxycarbonyl)-L-valyl-[N^8-(benzyloxycarbonyl)]-4(S),8-diamino-3(S)-hydroxyl-octanoyl Ethyl Ester (112a). Compound 109a (200 mg, 44 mmoles) was deprotected according to general procedure A. The resulting hydrochloride was coupled with Boc-valine anhydride (.88 mmoles) according to general procedure D using methylene chloride as solvent. Pure product was obtained by crystallization in about 70% yield (2 crops): mp 116-117°C (ethyl acetate/hexane); TLF R_f (B) .37; $[\alpha]_D^{24}$ -35.8° (c = .067, MeOH); NMR ($CDCl_3$) δ .75-1.0 (m, 6H), 1.10-1.6 (m, 18H, includes triplet δ 1.25, J = 7 Hz), 2.0-2.30 (m, 1H), 2.42-2.55 (m, 2H), 3.0-3.30 (m, 2H), 3.50 (m, 1H), 3.60-4.30 (m, 5H, includes quartet δ 4.30, J = 7 Hz), 4.80-5.20 (m, 3H, includes singlet δ 5.15, 2H), 6.4 (d, J = 10.5 Hz, 1H), 7.25-7.45 (m, 5H). Anal. calcd. for $C_{28}H_{45}N_3O_8$: C, 60.97; H, 8.22; N, 7.62. Found: C, 61.07; H, 8.21; N, 7.44.

N-(tert-Butyloxycarbonyl)-L-valyl-[N^8-(benzyloxycarbonyl)]-4(S),8-diamino-3(R)-hydroxyl-octanoyl Ethyl Ester (112b). Compound 109b (160 mg, .353 mmoles) was deprotected according to general procedure A. The resulting hydrochloride was coupled with Boc-valine anhydride (.70 mmoles) according to general procedure D using methylene chloride as solvent. Crystallization yielded pure product in about 65%

yield: mp 131-132°C (ethyl acetate/hexane); TLC R_f (B) .33; $[\alpha]_D^{24}$ -27.5°(c = .12, MeOH); NMR ($CDCl_3$) δ .75-1.05 (m, 6H), 1.15-1.70 (m, 18H, includes triplet δ 1.27, J = 7 Hz), 1.90-2.25 (m, 1H), 2.47 (d, J = 6 Hz, 2H), 3.02-3.30 (m, 2H), 3.42-3.58 (m, 1H), 3.65-4.32 (m, 5H, includes quartet δ 4.18, J = 7 Hz), 4.90-5.25 (m, 4H, includes singlet δ 5.11), 6.33 (broad doublet, J = 8 Hz, 1H), 7.46 (s, 5H). Anal. calcd. for $C_{28}H_{45}N_3O_8$: C, 60.97; H, 8.22; N, 7.62. Found: C, 60.83; H, 8.06; N, 7.45.

N-(tert-Butyloxycarbonyl)-L-valyl-L-valyl-[N^8-(benzyloxycarbonyl)]-4(S),8-diamino-3(S)-hydroxyl-octanoyl Ethyl Ester (113a). Boc compound 112a (125 mg, .23 mmoles) was deprotected according to general procedure A. The resulting hydrochloride was then coupled to Boc-valine anhydride (.46 mmoles) according to general procedure D using methylene chloride as solvent. Crystallization afforded product in about 92% yield: mp 169-171°C (ethyl acetate/hexane); TLC R_f (A) .60, R_f (B) .33; $[\alpha]_D^{24}$ -42.8° (c = .105, MeOH); NMR ($CDCl_3$) δ .80-1.10 (m, 12H), 1.15-1.75 (m, 18H), includes triplet δ 1.25, J = 7 Hz), 1.85-2.32 (m, 2H), 2.47 (d, J = 6 Hz, 2H), 3.0-3.30 (m, 2H), 3.42-3.70 (m, 1H), 3.75-4.39 (m, 6H), 5.05-5.38 (m, 4H, includes singlet δ 5.12), 6.48-6.72 (m, 2H), 7.23-7.45 (m, 5H). Anal. calcd. for $C_{33}H_{54}N_4O_9$: C, 60.91; H, 8.36; N, 8.61. Found: C, 60.88; H, 8.34; N, 8.54.

N-(tert-Butyloxycarbonyl)-L-valyl-L-valyl-[N^8-(benzyloxycarbonyl)]-4(S),8-diamino-3(R)-hydroxyl-octanoyl Ethyl Ester (113b). Boc compound 112b (30 mg, .054 mmoles) was deprotected according to general

procedure A. The resulting hydrochloride was coupled to Boc-valine anhydride (.10 mmoles) according to general procedure D using methylene chloride as solvent. After work-up the resulting material was chromatographed on a 10 g silica gel column eluting with 5% methanol in chloroform. Appropriate fractions were collected and evaporated. Precipitation from ethyl ether gave product in about 85% yield: mp 193-194°C; TLC R_f (B) .27; $[\alpha]_D^{24}$ -34.5° (c = .087, MeOH); NMR ($CDCl_3$) δ .75-1.10 (m, 12H), 1.10-2.10 (m, 20H), 2.44 (d, J = 6 Hz, 2H), 3.05-3.30 (m, 2H), 3.35-3.70 (m, 2H), 3.80-4.30 (m, 5H), 4.90-5.30 (m, 4H, includes singlet δ 5.12), 6.41-6.65 (m, 2H), 7.23-7.40 (m, 5H). Anal. calcd. for $C_{33}H_{54}N_4O_9$: C, 60.91; H, 8.36; N, 8.61. Found: C, 61.05; H, 8.57; N, 8.65.

<u>N-(tert-Butyloxycarbonyl)-L-valyl-L-valyl-[N^8-(benzyloxycarbonyl)]-4(S),8-diamino-3(S)-hydroxyl-octanoic acid (116a)</u>. Compound <u>113a</u> (70 mg, .108 mmoles) was saponified to the free acid according to general procedure F. The product was obtained in about 89% yield and was used in the next reaction without further purification.

<u>N-(tert-Butyloxycarbonyl)-L-valyl-L-valyl-[N^8-(benzyloxycarbonyl)]-4(S),8-diamino-3(S)-hydroxyl-octanoyl-L-phenylalanine Methyl Ester (117a)</u>. Compound <u>116a</u> (60 mg, .096 mmoles) was coupled to phenylalanine methyl ester (.12 mmoles) according to general procedure B. The resulting material was chromatographed on a 10 g silica gel column eluting with 5% methanol in chloroform. Fractions were then collected and evaporated in vacuo. Precipitation of material afforded product in

70% yield: mp 163-165°C (ethyl ether/hexane); TLC R_f (A) .40; $[\alpha]_D^{24}$ -37.2° (c = .105, MeOH); NMR ($CDCl_3$) δ .75-1.10 (m, 2H), 1.10-1.85 (m, 18H), 1.80-2.25 (m, 2H), 2.92-3.30 (m, 4H), 3.35-4.45 (m, 8H, includes singlet δ 3.73), 4.60-5.20 (m, 4H, includes singlet δ 5.1), 6.55 (m, 1H), 6.82-7.20 (m, 12H). Anal. calcd. for $C_{41}H_{61}N_5O_{10} \cdot H_2O$: C, 61.41; H, 7.92; N, 8.73. Found: C, 61.41; H, 8.02; N, 8.60.

<u>N-Isovaleryl-L-valyl-L-valyl-[N^8-(benzyloxycarbonyl)]-4(S),8-diamino-3(S)-hydroxyl-octanoyl Ethyl Ester (114a)</u>. Boc compound <u>113a</u> (30 mg, .046 mmoles) was deprotected according to general procedure A. The resulting hydrochloride was coupled to isovaleric anhydride (.10 mmoles) as described in general procedure D using DMF as the solvent. Precipitation from ethyl ether gave the title compound in about 90% yield: mp 216-218° (ethyl ether); TLC R_f (B) .31; $[\alpha]_D^{24}$-69.7° (c = .076, MeOH); NMR (d_4-MeOH) δ .78-1.15 (m, 18H), 1.17-1.78 (m, 9H, includes triplet δ 1.23, J = 7 Hz), 1.87-2.27 (m, 5H), 2.40 (d, J = 7 Hz, 2H), 3.08 (m, 2H), 3.51-4.27 (m, 6H), 5.04 (s, 2H), 7.28 (s, 5H). Anal. calcd. for $C_{33}H_{54}N_4O_8 \cdot H_2O$: C, 57.55; H, 8.48; N, 8.14. Found: C, 57.78; H, 8.07; N, 7.92.

<u>N-Isovaleryl-L-valyl-L-valyl-[N^8-(benzyloxycarbonyl)]-4(S),8-diamino-3(R)-hydroxyl-octanoyl Ethyl Ester (114b)</u>. Boc compound <u>113b</u> (33 mg, .50 mmoles) was deprotected according to general procedure A. The hydrochloride was coupled to isovaleric anhydride (.10 mmoles) as described for general procedure D using DMF as solvent. Precipitation from ethyl ether gave product in about 70% yield: mp 217-219°C (ethyl

ether); TLC R_f (B) .25; $[\alpha]_D^{24}$ -42.5° (c = .073, MeOH); NMR (d_4-MeOH) δ .79-1.17 (m, 18H), 1.19-1.77 (m, 9H), 1.88-2.25 (m, 5H), 2.38 (d, J = 7 Hz, 2H), 3.10 (m, 2H), 3.50-4.27 (m, 6H), 5.05 (s, 2H), 7.29 (s, 5H). Anal. calcd. for $C_{33}H_{54}N_4O_8 \cdot H_2O$: C, 57.55; H, 8.48; N, 8.14. Found: C, 57.81; H, 8.07; N, 8.01.

N-Isovaleryl-L-valyl-L-valyl-[N^8-(benzyloxycarbonyl)]-4(S),8-diamino-3(S)-hydroxyl-octanoyl-L-phenylalanine-methyl Ester (118a). Boc compound 117a (30 mg, .0403 mmoles) was deprotected using general procedure A. The hydrochloride was reacted with isovaleric anhydride (.08 mmoles) according to general procedure D using DMF as solvent. Precipitation from ethyl ether gave product in 90% yield: mp >250°C; TLC R_f (B) .18; $[\alpha]_D^{24}$ -250° (c = .12, MeOH); NMR (d_4-MeOH/d_4-acetic acid, 1/1, v/v) δ .67-1.10 (m, 18H), 1.20-1.78 (m, 6H), 1.80-2.22 (m, 5H), 2.33 (d, J = 6 Hz, 2H), 2.93-3.33 (m, 4H), 3.55-3.78 (m, 4H, includes singlet δ 3.67), 3.78-4.33 (m, 3H), 4.71 (t, J = 8 Hz, 1H), 5.07 (s, 2H), 7.0-7.44 (m, 10H). Anal. calcd. for $C_{41}H_{61}N_5O_9 \cdot H_2O$: C, 62.66; H. 8.08; N, 8.91. Found: C, 62.63; H, 7.99; N. 8.71.

N-Isovaleryl-L-valyl-L-valyl-4(S),8-diamino-3(S)-hydroxyl-octanoyl Ethyl Ester Acetate (115a). The Cbz group of compound 114a (19 mg, .03 mmoles) was removed as described in general procedure H. Precipitation from ethyl ether gave product in about 77% yield: mp 228-230°C; TLC R_f (F) .55; $[\alpha]_D^{24}$ -75° (c = .08, MeOH); NMR (dmso-d_6) δ .77-1.15 (m, 18H), 1.34-1.91 (m, 9H), 2.10-2.33 (m, 5H), 2.48 (m, 2H), 3.0-3.31 (m, 2H), 3.67-3.85 (m, 4H), 4.05-4.45 (m, 3H). Anal. calcd. for

$C_{27}H_{52}N_4O_8 \cdot H_2O$: C, 56.03; H, 9.39; N, 9.68. Found: C, 56.27; H, 9.02; N, 9.39.

N-Isovaleryl-L-valyl-L-valyl-4(S),8-diamino-3(R)-hydroxyl-octanoyl Ethyl Ester Acetate (115b). Compound 114b (19 mg, .03 mmoles) was subjected to conditions described in general procedure H. Precipitation from ethyl ether gave product in 80% yield: mp 220°C; TLC R_f (R) .53; $[\alpha]_D^{24}$ -48° (c = .10, MeOH); NMR (dmso-d_6) δ .78-1.13 (m, 18H), 1.35-1.89 (m, 9H), 1.92-2.26 (m, 5H), 2.38 (m, 2H), 3.11 (m, 2H), 3.52-4.33 (m, 6H). Anal. calcd. for $C_{27}H_{52}N_4O_8 \cdot 1.5\ H_2O$: C, 55.18; H, 9.43; N, 9.53. Found: C, 55.20; H, 9.05; N, 9.44

N-Isovaleryl-L-valyl-L-valyl-4(S),8-diamino-3(S)-hydroxyloctanoyl-L-phenylalanine-methyl Ester Acetate (119a). Compound 118a (20 mg, .027 mmoles) was treated as described in general procedure H. Ethyl ether precipitation afforded product in 83% yield: mp 200°C; TLC R_f (R) .61; $[\alpha]_D^{24}$ -270° (c = .10, MeOH); NMR (CD_3CO_2D) δ .70-1.10 (m, 18H), 1.33-1.89 (m, 6H), 2.0-2.33 (m, 5H), 2.49 (m, 2H), 2.98-3.33 (m, 4H), 3.67-3.82 (m, 4H, includes singlet at δ 3.73), 4.05 (m, 1H), 4.39 (m, 2H, includes triplet, J = 8 Hz), 4.84 (m, 1H), 7.22 (s, 5H). Anal. calcd. for $C_{35}H_{59}N_5O_9 \cdot H_2O$: C, 57.60; H, 8.69; N, 9.60. Found: C, 57.33; H, 8.29; N, 9.49.

N^4-(tert-Butyloxycarbonyl)-N^8-(acetyl)-4(S),8-diamino-3(S)-hydroxyl-octanoyl Ethyl Ester (133). Compound 109a (90 mg, .20 mmoles) was deprotected as described in general procedure H, except methanol

was used in place of DMF. Evaporation of solvent following filtration of catalyst afforded a clear oil. This resulting material was dissolved in methylene chloride (5 ml) and neutralized with N-methylmorpholine (.20 mmoles). Acetic anhydride (.30 mmoles) was added and the mixture stirred at room temperature for 4 hr, after which the mixture was diluted with ethyl acetate. The mixture was washed with 1 N HCl, saturated $NaHCO_3$, saturated NaCl, dried ($MgSO_4$) and evaporated in vacuo. Pure compound was obtained as an oil in about 72% yield: TLC R_f (A) .30; $[\alpha]_D^{24}$ -8.2° (c = .20, MeOH); NMR ($CDCl_3$) δ 1.22 (t, J = 7 Hz, 3H), 1.35-1.92 (m, 15H), 2.16 (s, 3H), 2.66 (m, 2H), 3.25-3.85 (m, 4H), 3.98-4.50 (m, 3H, includes quartet δ 4.35, J = 7 Hz), 4.93 (m, 1H), 5.91 (m, 1H).

N-Isovaleryl-L-valyl-L-valyl-[N^8-(acetyl)]-4(S),8-diamino-3(S)-hydroxyl-octanoyl Ethyl Ester (134). Compound 133 (30 mg, .083 mmoles) was deprotected according to general procedure A. The resulting hydrochloride was coupled to Iva-Val-Val-OH using general procedure B. After work-up the resulting material was recrystallized from methanol/ethyl ether to afford pure product in about 30% yield: mp 256-257°C; TLC R_f (A) .42; $[\alpha]_D^{24}$ -71.0° (c = .10, MeOH); NMR (d_4-MeOH) δ .78-1.11 (m, 18H), 1.30 (t, J = 7 Hz, 3H), 1.44 (m, 9H), 2.00-2.23 (m, 5H, includes singlet δ 2.18), 2.53 (d, J = 7.5 Hz, 2H), 3.11 (m, 2H), 4.00-4.27 (m, 6H). Anal. calcd. for $C_{27}H_{50}N_4O_7$: C, 59.76; H, 9.29; N, 10.32. Found: C, 59.63; H, 9.23; N, 10.27.

N^2-(tert-Butyloxycarbonyl)-N^5-(benzyloxycarbonyl)-2(S),5-diamino-pentanol (121). α-Boc-δ-Cbz-ornithine methyl ester (3.5 g, 9.2 mmol) was dissolved in absolute ethanol (14 ml). The mixture, which was kept under an N_2 atmosphere, was chilled to 0° and a 2 N solution of lithium borohydride in tetrahydrofuran (9 ml, 18 mmoles) was slowly added dropwise. The reaction was monitored by TLC. After 2 hr the reaction was quenched by pouring the reaction solution into citric acid (1 N), and extracting the product with ethyl acetate. The organic layer was then washed with saturated NaCl, dried ($MgSO_4$) and the solvent removed in vacuo to yield 3.1 g of a clear oil (95%): TLC R_f (A) .49. The product was used in the next step without further purification.

N^2-(tert-Butyloxycarbonyl)-2(S),5-diamino-pentanol (124). Compound 121 (3.1 g, 8.8 mmoles) was dissolved in dry methanol (20 ml) and the solution was purged of oxygen by vigorous bubbling with nitrogen. Ammonium formate powder (1.6 g, 26 mmoles) was added, followed by 10% palladium on carbon (300 mg). The reaction progress was monitored by TLC. After 3 hr the mixture was filtered through celite and the filtrate washed with methanol (20 ml). The methanol was removed in vacuo and ethyl acetate (20 ml) was added to dissolve the residue. The free amine was extracted into saturated $KHSO_4$ (the success of this procedure was followed by TLC). The aqueous layer was basified to pH 10 with solid NaOH and washed with ethyl acetate (100 ml). The organic layer was then dried ($MgSO_4$) and evaporated in vacuo to yield a clear oil (1.94 g, 98%) which was used without further purification: TLC R_f (A) .08; NMR ($CDCl_3$) δ 1.33-1.78 (m, 13H), 2.74 (m, 2H), 3.38-3.71 (m,

3H), 4.55-4.86 (m, 3H), 5.55 (broad m, 1H).

N^2-(tert-Butyloxycarbonyl)-N^5-(phthaloyl)-2(S),5-diaminopentanol (133). Free amine 124 (900 mg, 4.08 mmoles) was dissolved in methanol (5 ml). To this, N-carboethoxy-phthalimide[81a,b] (1.3 g, 4.1 mmoles) was added and the mixture was stirred at room temperature for 3-4 hr. The solution was diluted with ethyl acetate (30 ml) and was washed with 1 N HCl, saturated $NaHCO_3$ and saturated NaCl. The organic layer was dried ($MgSO_4$) and evaporated in vacuo. The residue was recrystallized from hexane/ethyl acetate to give a white powder (1.1 g in 2 crops) in about 78% yield: mp 88-90°C; TLC R_f (A) .50, R_f (M) .14; NMR ($CDCl_3$) δ 1.33-1.97 (m, 13H, includes singlet 1.36), 2.23 (m, 1H), 3.33-3.86 (m, 5H), 4.73 (m, 1H), 7.77 (m, 4H). Anal. calcd. for $C_{18}H_{24}N_2O_5$: C, 62.06; H, 6.94; N, 8.04. Found: C, 61.97; H, 6.91; N, 7.89.

N^2-(tert-Butyloxycarbonyl)-N^5-(benzyl)-2(S),5-diaminopentanol (125). Free amine 124 (530 mg, 2.41 mmoles) was dissolved in methylene chloride (10 ml). Triethylamine (242 µl, 2.41 mmoles) and benzaldehyde (245 µl, 2.41 mmoles) followed by $MgSO_4$ (1 g) were added. The mixture was stirred at room temperature for 4 hr after which time the $MgSO_4$ was filtered and the solvent evaporated in vacuo. The residue was dissolved in methanol (10 ml) and solid sodium borohydride (7.24 mmoles) was added to the solution in small portions over a period of 0.5 hr. The mixture was stirred for 3 hr and then quenched by pouring onto iced water. The water layer was acidified with saturated $KHSO_4$

and extracted with ethyl acetate. The aqueous layer was basified to pH 10 with solid NaOH and extracted with ethyl acetate (100 ml). The organic layer was dried ($MgSO_4$) and removed in vacuo to give a clear oil (500 mg, 70%) which was used in the next step without further purification. TLC R_f (A) .08; NMR ($CDCl_3$) δ 1.33-1.70 (m, 13H, includes singlet δ 1.40), 2.62 (m, 2H), 3.40-3.67 (m, 3H), 3.70-4.00 (m, 4H), 5.43 (broad m, 1H), 7.29 (s, 5H).

<u>N^2-(tert-Butyloxycarbonyl)-N^5-(benzyl)-N^5-(benzyloxycarbonyl)-2(S),5-diamino-pentanol (126)</u>. Compound <u>125</u> (500 mg, 1.62 mmoles) was dissolved in a 1:1 mixture of dioxane/H_2O (10 ml) and chilled to 0°C. 1 N NaOH (1.62 ml) was added followed by a dropwise addition of benzyl chloroformate (3.24 mmoles) over a period of 3 hr. The pH was carefully maintained at pH 10 with 1 N NaOH. The mixture was stirred at room temperature for 3 hr at pH 10. After this the reaction mixture was diluted with H_2O (10 ml) and extracted with ethyl acetate. The organic layer was dried ($MgSO_4$) and removed in vacuo. The residue was chromatographed on a 10 g silica gel column (MPLC grade silica gel) eluting with 50% ethyl acetate in hexane under positive pressure (flow rate ~ 5 ml/min). Appropriate fractions were combined and evaporated in vacuo to yield a clear oil (508 mg, 71%): TLC R_f (M) .21; NMR ($CDCl_3$) δ 1.14-1.89 (m, 13H), 3.25 (m, 2H), 3.30-3.73 (m, 3H), 4.03 (m, 1H), 4.43 (s, 2H), 4.82-5.21 (m, 3H, includes singlet δ 5.14), 7.0-7.40 (m, 10H). Anal. calcd. for $C_{25}H_{34}N_2O_5 \cdot \frac{3}{4} H_2O$: C, 65.84; H, 7.85; N, 6.14. Found: C, 65.75; H, 7.84; N, 6.01.

N[2]-(tert-Butyloxycarbonyl)-N[5]-(benzyl)-N[5]-(benzyloxycarbonyl)-2(S),5-diamino-pentanal (127). Alcohol 126 (470 mg, 1.06 mmoles) was dissolved in dry DMSO (1.5 ml). Triethylamine (445 µl, 3.2 mmoles) was added and the mixture was cooled to 0°C. Pyridine-sulfurtrioxide complex[66] (510 mg, 3.2 mmoles) in DMSO (1.5 ml) was added and the mixture was stirred at room temperature for .5 hr-.75 hr. The solution was poured onto iced water to stop the reaction, and the product was extracted with ethyl acetate. The organic layer was dried ($MgSO_4$), evaporated in vacuo, and vacuum dried overnight. The resulting oil was used without further purification: TLC R_f (B) .39, R_f (M) .50.

N[4]-(tert-Butyloxycarbonyl)-N[7]-(benzyl)-N[7]-(benzyloxycarbonyl)-4(S),7-diamino-3(R,S)-hydroxyl-heptanoyl Ethyl Ester (128). To 1 ml of dry tetrahydrofuran cooled by dry ice-CCl_4 was added diisopropylamine (1.6 mmoles) under a nitrogen atmosphere, followed by a 1.6 N solution of n-butyllithium in hexane (1.6 mmoles). After 1 hr the bath temperature was lowered to -78°C and dry ethyl acetate (1.6 mmoles) was added via syringe. The solution was stirred for 15 min. Aldehyde 127 (1.06 mmoles) dissolved in 1 ml of dry tetrahydrofuran was added via syringe over about 5 min. The reaction was stirred for 20-25 min before 1 N HCl was added. The mixture was warmed to room temperature and acidified to pH 2-3, then extracted with ethyl acetate twice (40 ml). The organic layer was washed with saturated NaCl, dried ($MgSO_4$), and evaporated in vacuo. The resulting oil was chromatographed on 20 g silica gel (MPLC grade silica gel) eluting with 50% ethyl acetate in hexane under positive pressure (flow rate ~ 5 ml/min). The pure 3(R,S)

diastereomeric mixture was obtained as an oil in 53% yield (from alcohol 126): TLC R_f (B) .53, R_f (M) .20; NMR ($CDCl_3$) δ 1.11-1.74 (m, 16H, includes triplet δ 1.27, J = 7.5 Hz), 2.44 (m, 2H), 3.12-3.80 (m, 4H), 3.81-4.32 (m, 3H, includes quartet δ 4.17, J = 7.5 Hz), 4.47 (s, 2H), 4.90 (broad s, 1H), 5.15 (s, 2H), 7.12-7.50 (m, 10H). Anal. calcd. for $C_{29}H_{40}N_2O_7$: C, 65.89; H, 7.67; N, 5.30. Found: C, 65.67; H, 7.63; N, 5.26.

N-(tert-Butyloxycarbonyl-L-valyl-[N^7-(benzyl)-N^7-(benzyloxycarbonyl)]-4(S),7-diamino-3(R,S)-hydroxyl-heptanoyl Ethyl Ester (129). Compound 128 (100 mg, .19 mmoles) was deprotected according to general procedure A. The resulting hydrochloride was coupled with Boc-valine anhydride (.4 mmoles) according to general procedure D using methylene chloride as solvent. The crude product obtained was chromatographed over 10 g silica gel eluting with 40% ethyl acetate in methylene chloride. Product was isolated as an oil in about 80% yield: TLC R_f (B) .50, R_f (P) .25; NMR ($CDCl_3$) δ .79-1.18 (m, 6H), 1.23 (t, J = 7.5 Hz, 3H), 1.30-1.71 (m, 13H), 2.05 (m, 1H), 2.39 (m, 2H), 3.11-3.56 (m, 3H), 3.62-4.30 (m, 5H, includes quartet δ 4.13, J = 7.5 Hz), 4.45 (s, 2H), 5.0 (m, 1H), 5.14 (s, 2H), 6.39 (broad multiplet, 1H), 7.08-7.45 (m, 10H).

N-(tert-Butyloxycarbonyl)-L-valyl-L-valyl-[N^7-(benzyl-N^7-(benzyloxycarbonyl)]-4(S),7-diamino-3(R,S)-hydroxyl-heptanoyl Ethyl Ester (130). Compound 129 (66 mg, .105 mmoles) was deprotected according to general procedure A. The resulting hydrochloride was coupled

with Boc-valine anhydride (.2 mmoles) according to general procedure D using methylene chloride as solvent. Silica gel column purification (10 g) eluting with 40% ethyl acetate in methylene chloride afforded pure compound as an oil in 75% yield: TLC R_f (P) .13; NMR ($CDCl_3$) δ .75-1.11 (m, 12H), 1.15-1.62 (m, 16H, includes triplet δ 1.26, J = 7 Hz), 1.83-2.25 (m, 2H), 2.43 (m, 2H), 3.22 (m, 2H), 3.60-4.40 (m, 7H, includes quartet δ 4.18, J = 7 Hz), 4.45 (s, 2H), 5.18 (s, 2H), 5.38 (d, J = 8 Hz, 1H), 6.65-7.10 (m, 2H), 7.10-7.52 (m, 10H).

N-Isovaleryl-L-valyl-L-valyl-[N^7-(benzyl)-N^7-(benzyloxycarbonyl)]-4(S),7-diamino-3(R,S)-hydroxyl-heptanoyl Ethyl Ester (131). Compound 130 (37 mg, .051 mmoles) was deprotected according to general procedure A. The resulting hydrochloride was coupled to isovaleric anhydride according to general procedure D using DMF as solvent. Precipitation from ethyl ether gave product as a white powder in about 96% yield: mp 148-152°C; TLC R_f (A) .61; NMR (MeOH-d_4) δ .77-1.13 (m, 18H), 1.16-1.64 (m, 7H, includes triplet δ 1.28, J = 7 Hz), 1.82-2.26 (m, 5h), 2.45 (m, 2H), 3.10 (m, 2H), 3.61-4.60 (m, 8H), 5.28 (s, 2H), 7.30 (m, 10H). Anal. calcd. for $C_{39}H_{58}N_4O_8$: C, 65.90; H, 8.22; N, 7.89. Found: C, 65.72; H, 8.12; N, 7.71.

N-Isovaleryl-L-valyl-L-valyl-4(S),7-diamino-3(R,S)-hydroxyl-heptanoyl Ethyl Ester Acetate (132). Compound 131 (28 mg, .04 mmoles) was dissolved in methanol (3 ml) and 3-4 drops of acetic acid were added. The solution was then purged of oxygen with nitrogen before 20% palladium hydroxide on carbon (10 mg) was added. The mixture was put

on a parr hydrogenation apparatus at 30 p.s.i. for 3 hr. After this the catalyst was removed by filtration over celite and washed with methanol. The solvent was concentrated in vacuo (~1 ml) and product was precipitated with ethyl ether and collected as a white powder in about 88% yield: mp 228-230°C; TLC R_f (R) .52; NMR (d_4-MeOH) δ .91-1.10 (m, 18H), 1.23 (t, J = 7 Hz, 3H), 1.65 (m, 4H), 1.80-2.21 (m, 8H), 2.46 (m, 2H), 2.90 (m, 2H), 3.63-4.41 (m, 6H). Anal. calcd. for $C_{26}H_{50}N_4O_8$: C, 57.13; H, 9.22; N, 10.25. Found: C, 57.31; H, 9.14; N, 10.32.

N-(tert-Butyloxycarbonyl)-L-valyl-L-valyl-4(S)-amino-3(S)-hydroxyl-6-methylheptanoyl-L-phenylalanine Methyl Ester (138). The title compound was prepared by general procedure B from Boc-Val-Val-Sta-OH (.15 mmoles) and HCl·Phe-OMe (.18 mmoles). Chromatography (3% MeOH/$CHCl_3$) followed by crystallization gave product in about 66% yield: mp 138-140°C (ethyl acetate/hexane); TLC R_f (B) .42; $[\alpha]_D^{24}$ -50° (c = .30, MeOH); NMR (MeOH-d_4) δ .83-1.05 (m, 18H), 1.15-1.63 (m, 11H, contains singlet δ 1.42), 1.80-2.24 (m, 3H), 2.43 (d, J = 7 Hz, 2H), 2.83 (m, 2H), 3.72 (s, 3H), 3.81-4.42 (m, 4H), 4.82 (m, 1H), 7.36 (s, 5H). Anal. calcd. for $C_{33}H_{54}N_4O_8$: C, 62.44; H, 8.58; N, 8.83. Found: C, 62.19; H, 8.57; N, 8.84.

N-Isovaleryl-L-valyl-L-valyl-4(S)-amino-3(S)-hydroxyl-6-methylheptanoyl-L-phenylalanine Methyl Ester (139). Compound 138 (30 mg, .048 mmoles) was deprotected according to general procedure A. The resulting hydrochloride was then coupled with isovaleric anhydride

according to general procedure D. Precipitation from ethyl ether afforded pure compound as a white powder in 80% yield: mp 224-225°C; TLC R_f (B) .29; $[\alpha]_D^{24}$ -62° (c = .15, MeOH); NMR (d_4-MeOH) δ .89-1.02 (m, 24H), 1.36-1.76 (m, 4H), 1.90-2.32 (m, 4H), 2.43 (d, J = 7 Hz, 2H), 2.91 (m, 2H), 3.44 (m, 1H), 3.76-4.43 (m, 6H, includes singlet δ 3.78), 4.81 (m, 1H), 7.42 (s, 5H). Anal. calcd. for $C_{33}H_{54}N_4O_7$: C, 64.06; H, 8.79; N, 9.07. Found: C, 63.99; H, 8.69; N, 8.85.

N-Isovaleryl-L-valyl-L-phenylalanyl-4(S)-amino-3(S)-hydroxyl-6-methylheptanoyl-L-alanyl Isoamylamide (140). Compound 53 (.05 mmoles) was deprotected according to general procedure A. The resulting hydrochloride was reacted with Boc-valine anhydride (.1 mmoles) according to general procedure D. The resulting compound was without further purification deprotected by general procedure A, and reacted with isovaleryl anhydride (.10 mmoles) using general procedure D. The resulting material was chromatographed on a 10 g silica gel column eluting with 5% methanol in chloroform. Product was obtained in an overall yield of 72%: mp 247-250°C; TLC R_f (A) .31; $[\alpha]_D^{24}$ -48° (c = .1, MeOH); NMR (MeOH-d_4) δ .68-1.09 (m, 24H), 1.22-1.78 (m, 10H), 1.88-2.28 (m, 5H), 2.89-3.34 (m, 4H), 3.43-4.05 (m, 2H), 4.32-4.82 (m, 2H), 7.43 (s, 5H). Anal. calcd. for $C_{35}H_{58}N_5O_6$: C, 65.19; H, 9.07; N, 10.86. Found: C, 65.28; H, 9.12; N, 10.95.

D. $^{18}OH_2$ Experiments

Statone compound 11 (.125 µmoles in methanol) was added to a solution of porcine pepsin, .125 µmoles in 50 mM, pH 5.0 oxalate buffer

made up with 99% enriched $H_2{}^{18}O$. In control experiments, pepstatin (.125 μmoles) was added prior to addition of Sto peptide. Assays were incubated for 3 hr (or the desired amount of time) and freeze dried. To the powder, chloroform (1 ml) was added and the insoluble material was filtered. The organic layer was applied to a .25 mm thickness 20 x 10 cm TLC plate and chromatographed with 10% methanol in chloroform. The band corresponding to starting Sto peptide was scraped from the plate and extracted with 1% methanol in chloroform. The residue was analyzed by mass spectrometry. Mass peak (M) and M+2 peak heights were analyzed to determine the extent of ^{18}O incorporation. The same experiment was carried out on compound <u>148</u>.

REFERENCES

1. R. M. Stroud, M. Kneger, R. E. Koeppe, A.A. Kossiakoff and J. L. Chambers. 1975. In Proteases and Biological Control, ed. E. Reich, D. Rifkin and E. Shaw (Long Island, N.Y.: Cold Spring Harbor Press), p. 13.
2. J. C. Powers, A. Yasutake, N. Nishino, B. F. Gupton and C.-M. Kam. 1981. In Peptides: Synthesis-Structure-Function, ed. D. H. Rich and E. Gross (Rockford, IL: Pierce Chemical Co.), p. 391.
3. K. Fujikawa, M. E. Legas, H. Kato and E. W. Davie, Biochemistry, 13, 4508 (1974).
4. C. G. Knight and A. J. Barrett, Biochem. J., 155, 117 (1976) and references therein. A. A. Iodice, Life Sciences, 19, 135 (1976).
5. T. Aoyagi, T. Takeuchi, A. Matsuzaki, K. Kawamura, S. Kondo, M. Hamada, M. Maeda and H. Umezawa, J. Antibiot., 22, 283 (1969).
6. H. Suda, T. Aoyagi, M. Hamada, T. Takeuchi and H. Umezawa, J. Antibiot., 25, 263 (1972).
7. H. Umezawa. 1972. Enzyme Inhibitors of Microbial Origin. Tokyo: University of Tokyo Press.
8. H. Umezawa. 1976. In Methods in Enzymology, Vol. XLV, p. 678.
9. T. Aoyagi and H. Umezawa. 1975. In Structures and Activities of Protease Inhibitors of Microbial Origin, Cold Spring Harbor Conf. Cell Proliferation to Proteases Biol. Control, pp. 429-454.
10. H. Umezawa, Jpn. J. Antibiot., XXX Suppl. S-138-162 (1977).

11. H. Umezawa, T. Aoyagi, S. Ohuchi, A. Okuyama, H. Suda, T. Takita and T. Takeuchi, J. Antibiotics, 36, 1572-1575 (1983).

12. H. Umezawa, T. Aoyagi, H. Morishima, M. Matsuzaki, M. Hamada and T. Takeuchi, J. Antibiotics, 23, 259 (1970).

13. H. Morishima, T. Takita, T. Aoyagi, T. Takeuchi and H. Umezawa, J. Antibiot., 23, 263 (1970).

14. T. Aoyagi, S. Kunimoto, H. Morishima, T. Takeuchi and H. Umezawa, J. Antibiot., 24, 687 (1971).

15. J. Marciniszyn, Jr., J. A. Hartsuck and J. Tang, J. Biol. Chem., 251, 7088 (1976).

16. H. Morishima, T. Takita and H. Umezawa, J. Antibiot., 25, 551 (1972).

17. H. Morishima, T. Takita and H. Umezawa, J. Antibiot., 26, 115 (1973).

18. M. Kinoshita, S. Aburake, A. Hagiwara and J. Imai, J. Antibiot., 26, 249 (1973).

19. H. Nakamura, H. Morishima, T. Takita, H. Umezawa and Y. Iitaka, J. Antibiot., 26, 255 (1973).

20. K. L. Rinehart Jr., J. B. Gwer, R. G. Hughes, H. E. Renis, J. P. McGovern, E. B. Swynenberg, D. A. Springfellow, S. L. Kuentzel and L. H. Li, Science, 212, 933-935 (1981).

21. T. Aoyagi, H. Morishima, R. Nishizawa, S. Kunimoto, T. Takeuchi, H. Umezawa and H. Ikezawa, J. Antibiot., 25, 689 (1972).

22. S. Kunimoto, T. Aoyagi, H. Morishima, T. Takeuchi and H. Umezawa, J. Antibiot., 25, 251 (1972).

23. M. A. Ondetti and D. W. Cushman. 1979. Annual Reports in Medicinal Chemistry (New York: Academic Press), pp. 82-91.

24. M. A. Ondetti, B. Rubin and D. W. Cushman, Science, 196, 441 (1977).

25. D. W. Cushman, H. S. Cheung, E. F. Sabo and M. A. Ondetti, Biochem., 16(25), 5484 (1977).

26. S. F. Campbell and J. C. Danilewicz. 1981. Annual Reports in Medicinal Chemistry (New York: Academic Press), pp. 73-82.

27. S. Kunimoto, T. Aoyagi, R. Nishizawa, T. Komai, T. Takeuchi and H. Umezawa, J. Antibiot., 27, 413 (1974).

28. C. G. Knight and A. J. Barrett, Biochem. J., 155, 117 (1976).

29. E. Subramanian, I. D. A. Swan, M. Liu, D. R. Davies, J. A. Jenkins, I. J. Tickle and T. L. Blundell, Proc. Natl. Acad. Sci. USA, 74, 556 (1977).

30. R. B. Bott and D. R. Davies. 1983. In Peptides: Structure and Function. Proceedings of Eighth American Peptide Symposium, ed. V. J. Hruby and D. H. Rich (Rockford, IL: Pierce Chemical Co.), p. 531.

31. a) M. N. G. James, A. Sielecki, F. G. Salituro, D. H. Rich and T. Hofmann, Proc. Natl. Acad. Sci. USA, 79, 6137 (1982).

    b) M. N. G. James, A. R. Sielecki and J. Moult. 1983. In Peptides: Structure and Function. Proceedings of Eighth American Peptide Symposium, ed. V. J. Hruby and D. H. Rich (Rockford, IL: Pierce Chemical Co.), p. 521.

32. D. H. Rich, E. Sun and J. Sing, Biochem. Biophys. Res. Comm., 74, 762 (1977).

33. D. H. Rich and E. T. O. Sun, J. Med. Chem., 23, 27 (1980).

34. D. H. Rich and E. T. O. Sun, Biochem. Pharm., 29, 205 (1980).

35. Y. Matsushita, H. Tone, S. Hori, Y. Yagi, A. Takamatsu, M. Morishima, T. Aoyagi, T. Takeuchi and H. Umezawa, J. Antibiot., 28, 1016 (1975).

36. T. Aoyagi, Y. Yagisawa, M. Kumagai, M. Hamada, H. Morishima, T. Takeuchi and H. Umezawa, J. Antibiot., 26, 539 (1973).

37. D. H. Rich and M. S. Bernatowicz, J. Med. Chem., 25, 791 (1982).

38. I. N. Hsu, L. T. J. Delbaere and M. N. G. James, Nature, 266, 140 (1977).

39. I. Schechter and A. Berger, Biochem. Biophys. Res. Comm., 27, 157 (1957).

40. E. Subramanian, I. D. A. Swan, M. Liu, D. R. Davies, J. A. Jenkins, I. J. Tickle and T. L. Blundell, Proc. Natl. Acad. Sci. USA, 74, 556 (1977).

41. N. S. Andreeva, A. E. Gustchina, A. A. Fedorov, N. E. Shutzkever and T. V. Volnova, Adv. in Exptl. Med. and Biol., 95, 23 (1977).

42. M. N. G. James and A. R. Sielecki, J. Mol. Biol., 163, 299 (1983).

43. M. N. G. James, I-Nan Hsu and L. T. J. Delbaere, Nature, 267, 808 (1977).

44. J. S. Fruton, Mol. Cell. Biochem., 32, 105 (1980).

45. R. Bott, E. Subramanian and D. R. Davies, Biochem., 21, 6956 (1982).

46. J. Marciniszyn Jr., J. A. Hartsuck and J. Tang, J. Biol. Chem., 251, 7088 (1976).

47. J. S. Fruton, Adv. in Enzymology, 33, 401-443 (1970).

48. J. S. Fruton. 1971. In The Enzymes, Vol. III, P. D. Boyer, ed. (New York, NY: Academic Press), pp. 119-164.

49. J. S. Fruton, Accounts Chem. Res., 7, 241 (1974).

50. T. L. Blundell, H. B. Jones, G. Khan, G. Taylor, B. T. Sewell, L. H. Pearl and S. P. Wood, Proc. FEBS Meet., 60, 281 (1980).

51. R. J. Workman and D. W. Burkitt, Arch. Biochem. Biophys., 194, 157 (1979).

52. P. Sepulveda, J. Marciniszyn, D. Liu and J. Tang, J. Biol. Chem., 250, 5082-5088 (1975).

53. J. S. Fruton, Adv. in Enzymology, 44, 1-36 (1976).

54. L. Pauling, Chem. Eng. News, 24, 1375 (1946).

55. L. Pauling, Amer. Sci., 36, 51 (1948).

56. R. Wolfenden, Nature, 223, 704 (1969).

57. R. Wolfenden, Accounts Chem. Res., 5, 10 (1972).

58. R. Wolfenden, Ann. Rev. Biophys. Bioeng., 5, 271 (1976).

59. R. Wolfenden. 1978. In Transition States of Biochemical Processes, R. D. Gandour and R. L. Schowen, ed. (New York: Plenum Press), pp. 555-578.

60. R. Kitz and I. B. Wilson, J. Biol. Chem., 237, 3245 (1962).

61. K. Kitagishi, H. Nakatni and K. Hiromi, J. Biochem., 573 (1980).

62. M. S. Bernatowicz, Ph.D. Thesis.

63. M. S. Bernatowicz, A. S. Boparai and D. H. Rich. 1981. In Peptides: Synthesis-Structure-Function. Proceedings of the Seventh American Peptide Symposium, D. H. Rich and E. Gross, eds. (Pierce Chem. Co.), p. 439.

64. D. H. Rich, M. S. Bernatowicz and P. G. Schmidt, J. Am. Chem. Soc., 104, 3535 (1982).

65. D. H. Rich, E. T. Sun and A. S. Boparai, J. Org. Chem., 43, 3624 (1978).

66. Y. Hamada and T. Shiori, Chem. Pharm. Bull., 30, 1921 (1982).

67. K. Omura and D. Swern, Tetrahedron, 34, 1651 (1978).

68. J. Kovacs, E. J. Davis, H. Johnson, H. Cortegiano and J. Roberts. 1972. In Chemistry and Biology of Peptides (Ann Arbor, MI: Ann Arbor Science Publishers), p. 359.

69. J. Kovacs, L. Kisfaludy and M. Q. Ceprini, J. Am. Chem. Soc., 89, 183 (1967).

70. Eric T. O. Sun, Ph.D. Thesis.

71. M. W. Holladay and D. H. Rich, Tetrahedron Lett., 24, 4401 (1983).

72. D. H. Rich, F. G. Salituro and M. W. Holladay. 1983. "Design of Protease Inhibitors," in Peptides: Structure and Function. Proc. Eighth American Peptide Symposium, V. Hruby and D. H. Rich, eds. (Rockford, IL: Pierce Chemical Co.), p. 511.

73. D. H. Rich, F. G. Salituro and M. W. Holladay. 1983. "Design and Discovery of Aspartyl Protease Inhibitors. Mechanism and Clinical Implications," in Drug Design Based on Peptide and Nucleic Acid Conformational Structure, J. Vida, ed. (New York, NY: Academic Press), p. 211.

74. D. A. Evans, M. D. Ennis and D. J. Mathre, J. Am. Chem. Soc., 104, 1737 (1982).

75. Benzyl-bromomethyl ether was prepared by stirring 1 eq of benzyl-chloromethyl ether (Fluka) with 1 eq of LiBr in THF (~2 mM solution).

76. Q. Branca and A. Fischli, Helv. Chim. Acta, 60, 925 (1977).

77. W. C. Still, J. H. McDonald, Tetrahedron Lett., 21, 1031-1038 (1980) and references therein.

78. G. Hancock, I. J. Galpin and B. A. Morgan, Tetrahedron Lett., 23, 249 (1982).

79. T. W. Green. 1981. Protecting Groups in Organic Synthesis, (New York: John Wiley and Sons), p. 329.

80. Paul D. Gesellchen, Ph.D. Thesis.

81. a) P. M. Worster, C. C. Leznoff and C. R. McArthur, J. Org. Chem., 45, 174 (1980).

    b) C. R. McArthur, P. M. Worster, J. L. Jiang and C. C. Leznoff, Can. J. Chem., 60, 1836 (1982).

82. M. K. Anser and A. F. Spatola, Synthesis, 929 (1980).

83. S. Cha, R. P. Agarwal and R. E. Parks Jr., Biochem. Pharmacol., 24, 2187 (1975).

84. P. G. Schmidt, M. S. Bernatowicz and D. H. Rich, Biochemistry, 21, 1830 (1982).

85. P. G. Schmidt, M. S. Bernatowicz and D. H. Rich, Biochemistry, 21, 6710 (1982).

86. T. Hofmann and R. S. Hodges, Biochem. J., 203, 603 (1982).

87. J. C. Powers, A. D. Harley and D. V. Myers. 1977. In Acid Proteases-Structure, Function and Biology, J. Tang, ed. (New York: Plenum), pp. 141-157.

88. J. Boger. 1983. In Peptides Structure and Function. Proceedings of the Eighth American Peptide Symposium, V. J. Hruby and D. H. Rich, eds. (Rockford, IL: Pierce Chem. Co.), p. 569.

89. M. Szelke, D. M. Jones, B. Atrash, A. Hallett and B. Leckie, ibid., p. 579.

90. P. A. Bartlett and C. K. Marlowe, Biochemistry, 22, 4613 (1983).

91. T. Hofmann, R. S. Hodges and M. N. G. James, Biochemistry, 23, 635 and references therein (1984).

92. W. P. Jencks, Adv. in Enzymology, 43, 219 (1975).

93. M. W. Holladay, F. G. Salituro, P. G. Schmidt and D. H. Rich, J. Amer. Chem. Soc., submitted for publication (1984).

94. A. R. Fersht, J. Mol. Biol., 64, 497 (1972).

95. A. Cunningham, H. M. Wang, S. R. Jones, A. Kurosky, L. Rao, C. I. Harris, S. H. Rhee and T. Hofmann, Can. J. Biochem., 54, 902 (1976).

96. M. L. Bender and F. J. Kezdy, Ann. Rev. Biochem., 34, 49-76 (1965).

97. G. R. Delpierre and J. S. Fruton, Proc. Nat. Acad. Sci. U.S.A., 54, 1161-1167 (1965).

98. T. C. Bruice and S. Benkovic. 1966. Bioorganic Mechanisms, Vol. I (New York: W. A. Benjamin, Inc.), pp. 2-4.

99. J. Knowles, Phil. Trans. Roy. Soc. Lond., B257, 135-146 (1970).

100. G. E. Clement, Prog. Bioorganic Chem., 2, 177-238 (1973).

101. M. Takahashi, T. T. Wang and T. Hofmann, Biochem. Biophys. Res. Comm., 57, 39-46 (1974).

102. M. S. Silver and M. Stoddard, Biochemistry, 11, 191 (1972).

103. V. K. Antonov, in ref. 87, pp. 179-198.

104. L. V. Kozlov, L. M. Ginodman, V. N. Orekhovich and T. A. Valueva, Biokhimiya, 31, 315-321 (1966).

105. A. J. Cornish-Bowden, P. Greenwell and J. R. Knowles, Biochem. J., 113, 369-375 (1969).

106. V. K. Antonov, L. M. Ginodman, Y. V. Kapitannikov, T. N. Barshevskaya, A. G. Gurova and L. D. Rumsh, FEBS Lett., 88, 87-90 (1978).

107. V. K. Antonov, L. M. Ginodman, L. D. Rumsh, Y. V. Kapitannikov, L. P. Barshevskaya, A. G. Gurova and L. I. Volkova, Eur. J. Biochem., 117, 195-200 (1981).

108. P. G. Schmidt, M. W. Holladay, F. G. Salituro and D. H. Rich, submitted for publication.

109. P. E. Pfeffer, K. M. Valentine and F. W. Parrish, J. Am. Chem. Soc., 101, 1265 (1978). S. C. Ho, H. J. Koch and R. S. Stuart, Carbohydr. Res., 64, 251 (1978).

110. J. Reuben, J. Am. Chem. Soc., 105, 3711 (1983).

111. H. Batiz-Hernandez and R. A. Bernheim, Prog. Nucl. Magn. Reson. Spectrosc., 3, 63 (1967). C. J. Jameson, J. Chem. Phys., 66, 4983 (1977).

112. M. W. Holladay, F. G. Salituro, P. G. Schmidt and D. H. Rich, submitted for publication.

113. D. H. Rich, M. S. Bernatowicz, N. S. Agarwal, M. Kawai, F. G. Salituro and P. G. Schmidt, submitted for publication.

114. G. E. Lienhard, Ann. Rep. Med. Chem., 7, 249 (1972). G. E. Lienhard, Science, 180, 149 (1973).

115. M. Kinoshita, A. Hagiwara and S. Aburaki, Bull. Chem. Soc. Jap., 48, 570 (1975).

116. R. Steulmann and H. Klostermeyer, Justus Liebigs Ann. Chem., 2245 (1975).

117. T. Katsuki and M. Yamaguchi, Bull. Chem. Soc. Jap., 49, 3287 (1976).

118. W. S. Liu and G. I. Glover, J. Org. Chem., 43, 754 (1978).

119. J. Boger, N. S. Lohr, E. H. Ulm, M. Poe, E. H. Blaine, G. M. Fanelli, T.-Y. Lin, L. S. Payne, T. Schorn, B. I. Lamont, T. C. Vassil, I. I. Sabilito, D. F. Veber, D. H. Rich and A. S. Boparai, Nature, 303, 81 (1983).

120. Personal communication, M. N. G. James.

121. P. A. Bartlett and W. B. Kezer, J. Amer. Chem. Soc., 106, 4282 (1984).

122. K. Radika and D. B. Northrop, J. Biol. Chem., in press.

123. W. Konig and R. Greiger, Chem. Ber., 103, 788 (1970).

124. a) H. Schussler and H. Zahn, Chem. Ber., 95, 1076-1080 (1962).
b) T. Wieland, W. Kern and R. Sehring, Justus Liebigs Ann. Chem., 569, 117 (1950).

125. a) M. Muhlemann, M. I. Titov, R. Schwyzer and J. Rudinger, Helv. Chim. Acta, 55, 2854 (1972).
b) D. Belleau and G. Malek, J. Amer. Chem. Soc., 90, 1651 (1968).

126. Prepared from corresponding Boc protected compound by general procedure A.

127. Ratios estimated from NMRs of O-acetylated compounds.