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

Daniel H. Rich* and Francesco G. Salituro

School of Pharmacy, University of Wisconsin, Madison, Wisconsin 53706. *Received September 16, 1982*

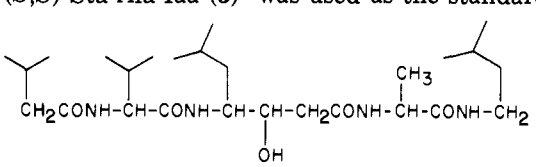
A series of pepstatin analogues having structural variations in the $P_{2'}$, $P_{1'}$, and P_2 positions have been synthesized and tested for inhibition of porcine pepsin. The standard peptide for this study was Iva-Val-Sta-Ala-Iaa. Structural variations in the $P_{2'}$ and $P_{1'}$ positions have relatively little effect on K_i; however, small variations in the P_2 position have a more dramatic effect on K_i and time-dependent inhibition. A series of pepstatin fragments were also synthesized and tested for inhibition of porcine pepsin.

Pepstatin (1) is a naturally occurring pentapeptide, which was isolated by Umezawa[1,2] from various species of actinomycetes. Since its discovery, pepstatin has been shown to be an inhibitor of carboxyl proteases[3-5] characterized by low toxicity and low dissociation constants (4.57×10^{-11} M for pepsin).[6]

Inhibition studies on pepsin with various structural analogues of pepstatin have shown that the central amino acid, 3(*S*)-hydroxy-4(*S*)-amino-6-methylheptanoic acid (statine, Sta, **2**) is essential for tight binding inhibition.[7,8] Further studies have illustrated the importance of the P_3 subsite (see Figure 1; P_1, P_2, etc. are defined by Schecter and Berger[9]) on inhibition of porcine pepsin.[10] Dramatic changes in K_i are produced by small structural changes in this position. We report here the results of a study to further characterize the structural factors affecting inhibition. Our results show the importance of various structural features contained in pepstatin for binding at the $S_{1'}$, $S_{2'}$, and S_2 sites of pepsin.

Results

Chemistry. A variety of pepstatin analogues have been synthesized and studied kinetically. The peptide Iva-Val-(*S*,*S*)-Sta-Ala-Iaa (**3**)[8] was used as the standard for comparison between analogues because it has been shown to have the minimal structural requirements that produce the slow binding, time-dependent inhibition characteristic of pepstatin.



Iva-Val-Sta-Ala-Iaa (3)

Most of the peptides shown in Tables I and II were synthesized by standard methods that have been previously described.[7] Active esters were employed when propylamine was coupled to Boc amino acids **16** and **25**. Initial attempts to prepare (*S*,*S*)-Boc-Sta-Ipa[1] via standard DCC/HOBT or EEDQ coupling methods gave poor yields of product. This was apparently due to the formation of a stable salt between amine and carboxylate, which was insoluble in methylene chloride. The pentafluorophenyl active ester method proved to be suitable for the preparation of these compounds. Reaction between this active ester is rapid and proceeds with a low degree of racemization.[11] Preparation of the active ester by the DCC-PFP "complex" method[12] also is rapid (~20 min). Since pentafluorophenol has a low pK (~6), it was necessary to add extra equivalents of base to prevent isopropylamine-PFP salt formation. Thus, for the preparation of **25**, 3 equiv of *N*-methylmorpholine was used (DCC-PFP complex is in a 1:3 ratio) because the crude reaction mixture was used without any purification after the filtering of DCU. For the preparation of compound **16**, *N*-methylmorpholine was not used and 2 equiv of nucleophilic base were slowly added to the solution of purified active ester. Compound **14** was prepared by means of a similar procedure.

Physical constants and crystallization solvents for synthetic intermediates are reported in Table II, and those for final products are reported in Table I. NMR data are reported only for final products, since the assignments did not vary significantly from intermediates.

Kinetics. Inhibition constants (K_i) for inhibition of pepsin by pepstatin analogues (Table I) were determined as previously described[10] from IC_{50} values taken from plots of V_i/V_0 vs. inhibitor concentration, where V_i is the inhibited velocity and V_0 is the velocity in the absence of inhibitor. IC_{50} values were converted to K_i by the equation of Cha:[13]

$$K_i = \left(IC_{50} - \frac{E_t}{2}\right)\left(1 + \frac{S}{K_m}\right)^{-1}$$

where E_t 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 min, and the reaction was initiated by

(1) Abbreviations used follow IUPAC-IUB tentative rules as described in *J. Biol. Chem.* **1972**, *247*, 977. Additional abbreviations used are: DCC, dicyclohexylurea; DCU, dicyclohexylcarbodiimide; Boc, *tert*-butyloxycarbonyl; Ipoc, isopropyloxycarbonyl; Iva, isovaleryl; Sta, 4-amino-3-hydroxy-6-methylheptanoic acid; Tempo, 2,2,6,6-tetramethylpiperidinyl-1-oxy; Iaa, isoamylamide; PFP, pentafluorophenyl; Ipa, isopropylamide; NMM, *N*-methylmorpholine; DMF, dimethylformamide; THF, tetrahydrofuran; EtOAc, ethyl acetate; MeOH, methanol; TDI, time-dependent inhibition.

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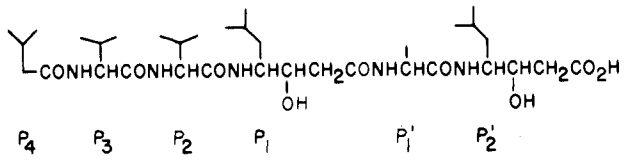

Figure 1. Structure of pepstatin (1) showing positions designated P_1, P_2, P_3, P_4, $P_{1'}$, and $P_{2'}$.

addition of substrate. Velocities were taken at apparent steady state. Inhibitors that 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, v/v. Some inhibitors (**45** and **48**) required 10% methanol because of their poor solubility in the buffer. Only inhibitor **37** was insufficiently soluble to prevent precise quantitation of its true K_i.

The data in Table I show how structural differences in the $P_{2'}$, $P_{1'}$, and P_2 subsites affect K_i. Inhibition constants for analogues with $P_{2'}$ variations or deletions exhibit K_i's from $\sim 10^{-9}$ to 10^{-7} M; however, this range encompasses relatively large structural variations. Compounds **3**, **6**, and **9** 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 highly selective and that the carboxyl of the $P_{2'}$ site is not required. Removal of the hydrophobic side chain (**12**), however, increases K_i by about 40-fold and eliminates time-dependent inhibition, indicating a need for a hydrophobic or chiral side chain. Compounds **15** and **19** further illustrate the effect of branching at the α carbon of the $P_{2'}$ site. The isopropylamide compound **19** has about a 10-fold lower K_i than the methylamide **15** and exhibits time-dependent inhibition.

The role of the $P_{2'}$ NH group can be illustrated by comparing compounds **15** and **23**. 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 $P_{2'}$ carbon skeleton (**21** and **27**) further increases K_i.

Similarly, relatively large structural changes in the $P_{1'}$ subsite produce small changes in K_i (10^{-9} to 10^{-8} M). Compounds **3** and **31** are comparable inhibitors, while complete removal of the side chain (**35**) 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 $P_{2'}$ site (10^{-9} to 10^{-7} M). However, relatively subtle changes in structure are represented in this range. Replacement of the isopropyl side chain (**3**) with an isobutyl (**41**) or benzyl group (**43**) in the P_2 subsite increases K_i 5- to 10-fold and eliminates time-dependent inhibition. Interestingly, a methyl-substituted analogue (**39**) remains time dependent, although a 10-fold weaker inhibitor. Removal of the P_2 side chain (**37**) increases K_i dramatically.

Discussion

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 $P_{3'}$ of pepstatin. The naturally occurring pepstatins, which differ only in the size of the *N*-acyl chain in the P_4 position, inhibit pepsin to a similar extent,[14] although inhibition of

Table I

no.	compd	K_i, M	TDI[a]	mp, °C	TLC: R_f (solvent)[b]	$[\alpha]^{24}_D$, deg (c, solv)	yield, %	formula	anal.
3	Iva-Val-(*S,S*)-Sta-Ala-Iaa[c]	3.0×10^{-9}	+	237–238	0.41 (A)	−61	77		C, H, N
6	Iva-Val-(*S,S*)-Sta-Ala-Phe-OMe	1.5×10^{-9}	+	224–227	0.34 (A)	−52	75	$C_{31}H_{50}N_4O_7$	C, H, N
9	Iva-Val-(*S,S*)-Sta-Ala-Leu-OMe	1.1×10^{-9}	+	204–207	0.45 (A)	−72	80	$C_{28}H_{52}N_4O_7$	C, H, N
12	Iva-Val-(*S,S*)-Sta-Ala-Gly-OMe	6.8×10^{-8}	−	211–213	0.20 (A)	−50	66	$C_{24}H_{44}N_4O_7$	C, H, N
19	Iva-Val-(*S,S*)-Sta-Ala-Ipa	2.0×10^{-8}	+	>250	0.41 (A)	−48	80	$C_{24}H_{46}N_4O_5$	C, H, N
15	Iva-Val-(*S,S*)-Sta-Ala-NHCH$_3$	1.4×10^{-7}	−	233–235	0.17 (B)	−33	15	$C_{22}H_{42}N_4O_5 \cdot H_2O$	C, H, N
23	Iva-Val-(*S,S*)-Sta-Ala-OCH$_3$	6.0×10^{-7}	−	187–188	0.37 (B)	−84	60	$C_{22}H_{41}N_3O_6$	C, H, N
21	Iva-Val-(*S,S*)-Sta-OCH$_2$CH$_3$	8.0×10^{-7}	−	181–182	0.50 (A)	−58	90	$C_{20}H_{38}N_2O_5$	C, H, N
27	Iva-Val-(*S,S*)-Sta-Ipa	3.0×10^{-7}	−	222–223	0.47 (A)	−67	87	$C_{21}H_{41}N_3O_4$	C, H, N
3	Iva-Val-(*S,S*)-Sta-Ala-Iaa	3.0×10^{-9}	+						
31	Iva-Val-(*S,S*)-Sta-Leu-Iaa	4.6×10^{-9}	+	188–200	0.51 (A)	−50	82	$C_{29}H_{56}N_4O_5$	C, H, N
35	Iva-Val-(*S,S*)-Sta-Gly-Iaa	5.6×10^{-8}	−	204–205	0.30 (A)	−60	40	$C_{25}H_{48}N_4O_5$	C, H, N
3	Iva-Val-(*S,S*)-Sta-Ala-Iaa	3.0×10^{-9}	+						
37	Iva-Gly-(*S,S*)-Sta-Ala-Iaa[d]	$\sim 1.0 \times 10^{-7}$	−	91–92	0.14 (A)	−25	70	$C_{23}H_{44}N_4O_5 \cdot H_2O$	C, H, N
39	Iva-Ala-(*S,S*)-Sta-Ala-Iaa	3.0×10^{-8}	+	157–158	0.26 (A)	−57	70	$C_{24}H_{46}N_4O_5$	C, H, N
41	Iva-Leu-(*S,S*)-Sta-Ala-Iaa	1.3×10^{-8}	−	149–150	0.35 (A)	−51	77	$C_{27}H_{52}N_4O_5$	C, H, N
43	Iva-Phe-(*S,S*)-Sta-Ala-Iaa	2.1×10^{-8}	−	205–206	0.42 (A)	−31	86	$C_{30}H_{50}N_4O_5$	C, H, N
45	Iva-Val-Val-(*S,S*)-Sta-OCH$_2$CH$_3$	1.0×10^{-8}	−	235–236	0.52 (A)	−80	70	$C_{25}H_{47}N_3O_6$	C, H, N
48	Iva-Val-Val-(*S,S*)-Sta-Ala-OCH$_3$	3.5×10^{-9}	+	>250	0.23 (B)	−88	85	$C_{27}H_{50}N_4O_7 \cdot H_2O$	C, H, N

[a] TDI = time-dependent inhibition. [b] A = 10% MeOH in $CHCl_3$ (v/v); B = 5% MeOH in $CHCl_3$. [c] Previously reported by Rich et al.[7] [d] Low solubility of this inhibitor presented difficulties in precise quantitation of K_i.

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Table II

no.	compd	mp,[a] °C	TLC: R_f (solv)[c]		$[\alpha]^{24}_D$, deg (c, solv)	yield, %	formula	anal.
4	Boc-(*S,S*)-Sta-Ala-Phe-OMe	103–131 (A)	0.59 (A)	−36	(0.67, MeOH)	81	$C_{26}H_{41}N_3O_7$	C, H, N
5	Boc-Val-(*S,S*)-Sta-Ala-Phe-OMe	176–177 (A)	0.52 (B)	−50	(0.13, MeOH)	80	$C_{31}H_{50}N_4O_8$	C, H, N
7	Boc-(*S,S*)-Sta-Ala-Leu-OMe	*b*	0.28 (B)	−31	(0.64, MeOH)	90	$C_{23}H_{43}N_3O_7$	C, H, N
8	Boc-Val-(*S,S*)-Sta-Ala-Leu-OMe	*b*	0.53 (A)	−64	(0.18, MeOH)	85	$C_{28}H_{52}N_4O_8$	C, H, N
10	Boc-(*S,S*)-Sta-Ala-Gly-OMe	116–118 (A)	0.15 (B)	−20	(0.17, MeOH)	60	$C_{19}H_{35}N_3O_7$	C, H, N
11	Boc-Val-(*S,S*)-Sta-Ala-Gly-OMe	155–158 (A)	0.31 (A)	−40	(0.83, MeOH)	60	$C_{24}H_{44}N_4O_8$	C, H, N
13	Boc-Ala-pFp							
14	Boc-Ala-$NHCH_3$	112–113 (A)	0.35 (B)	−13	(0.71, MeOH)	40	$C_9H_{18}N_2O_3$	C, H, N
16	Boc-Ala-Ipa	111–112 (B)	0.26 (B)	−18	(0.53, MeOH)	50	$C_{11}H_{22}N_2O_3$	C, H, N
17	Boc-(*S,S*)-Sta-Ala-Ipa	151–152 (A)	0.33 (B)	−40	(0.34, MeOH)	75	$C_{19}H_{37}N_3O_5$	C, H, N
18	Boc-Val-(*S,S*)-Sta-Ala-Ipa	218–220 (C)	0.53 (A)	−44	(0.51, MeOH)	40	$C_9H_{18}N_2O_3$	C, H, N
20	Boc-Val-(*S,S*)-Sta-OEt	121 (A)	0.65 (A)	−53	(0.1, MeOH)	70	$C_{20}H_{38}N_2O_6$	C, H, N
22	Iva-Val-(*S,S*)-Sta-OH							
24	Boc-(*S,S*)-Sta-PFP							
25	Boc-(*S,S*)-Sta-Ipa	129–131 (B)	0.34 (B)	−38	(0.13, MeOH)	80	$C_{16}H_{32}N_2O_4$	C, H, N
26	Boc-Val-(*S,S*)-Sta-Ipa	148–149 (A)	0.38 (A)	−46	(0.32, MeOH)	70	$C_{21}H_{41}N_3O_5$	C, H, N
28	Boc-Leu-Iaa	89–90 (D)	0.62 (B)	−25	(0.80, MeOH)	87	$C_{16}H_{32}N_2O_3$	C, H, N
29	Boc-(*S,S*)-Sta-Leu-Iaa	oil	0.36 (B)	−39	(0.20, MeOH)	78	$C_{24}H_{17}N_3O_5$	C, H, N
30	Boc-Val-(*S,S*)-Sta-Leu-Iaa	*b*	0.19 (B)	−40	(0.17, MeOH)	65	$C_{29}H_{56}N_4O_6$	C, H, N
32	Boc-Gly-Iaa	oil	0.51 (B)			90	$C_{12}H_{24}N_2O_3$	C, H, N
33	Boc-(*S,S*)-Sta-Gly-Iaa	127–129 (A)	0.18 (B)	−37	(0.43, MeOH)	88	$C_{20}H_{39}N_3O_5$	C, H, N
34	Boc-Val-(*S,S*)-Sta-Gly-Iaa	131–132 (B)	0.12 (B)	−49	(0.17, MeOH)	75	$C_{25}H_{48}N_4O_6$	C, H, N
36	Boc-Gly-(*S,S*)-Sta-Ala-Iaa	*b*	0.16 (A)	−22	(0.20, MeOH)	52	$C_{23}H_{43}N_4O_6$	C, H, N
38	Boc-Ala-(*S,S*)-Sta-Ala-Iaa	75–80 (A)	0.18 (B)	−43	(0.14, MeOH)	84	$C_{24}H_{46}N_4O_6$	C, H, N
40	Boc-Leu-(*S,S*)-Sta-Ala-Iaa	88–92 (E)	0.15 (B)	−41	(0.53, MeOH)	93	$C_{27}H_{52}N_4O_6$	C, H, N
42	Boc-Phe-(*S,S*)-Sta-Ala-Iaa	164–166 (A)	0.41 (B)	−26	(0.20, MeOH)	50	$C_{30}H_{50}N_4O_6$	C, H, N
44	Boc-Val-Val-(*S,S*)-Sta-OEt	172–173 (A)	0.50 (A)	−70	(0.1, MeOH)	85	$C_{25}H_{47}N_3O_7$	C, H, N
46	Boc-Val-Val-(*S,S*)-Sta-OH							
47	Boc-Val-Val-(*S,S*)-Sta-Ala-OMe	199–201 (A)	0.48 (B)	−79	(0.25, MeOH)	82	$C_{27}H_{50}N_4O_8 \cdot 0.5H_2O$	C, H, N

[a] Crystallization solvents: A = EtOAc/hexane; B = ether/hexane; C = ether; D = hexane; E = ether/petroleum ether. [b] Compound recovered as an amorphous solid. [c] A = 10% MeOH in CHCl (v/v); B = 5% MeOH in $CHCl_3$ (v/v).

renin is dependent upon the size of the chain. Modification of the P_3 position[10] has shown the importance of geometry in this position on both inhibition constant and, more interestingly, on time-dependent inhibition. Urethane derivatives (Boc and Ipoc replacing Iva in P_3) are weaker inhibitors than compound **3** and are not slow-binding inhibitors. This may reflect differences between the preferred geometry for urethanes vs. the required geometry for maximum binding of inhibitor to enzyme at the P_3 side chain. The importance of the P_3 site was also illustrated by Umezawa,[15] who compared several N-acylated semisynthetic tetrapeptide analogues of pepstatin. The tetrapeptide Val-Sta-Ala-Sta was prepared by cleavage of pepstatin by pepstatin hydrolase and subsequently N-acylated to give a number of P_3 analogues. Although the tetrapeptide itself is a poor inhibitor, most of the N-acylated compounds were found to be nearly as potent as pepstatin as pepsin inhibitors. Substitutions in the $P_{3'}$ position, prepared by coupling high-molecular-weight synthetic polymers to the C-terminal carboxyl group of pepstatin, produce little effect on the K_i for inhibition of renin or pepsin.[16] These pepstatin–dextran conjugates are more soluble than pepstatin; however, they retain most of pepstatin's inhibitory potency in spite of the increased steric bulk at the C terminus. Similarly, addition of amino acids with charged side chains[17] to the $P_{3'}$ position gave more soluble compounds that inhibited renin with K_i's similar to that of pepstatin. The present study provides the first data on the effect of structure in the P_2, $P_{1'}$ and $P_{2'}$ positions of pepstatin on inhibition of pepsin.

Inhibition constants for analogues with $P_{1'}$ and $P_{2'}$ 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 inhibition is much less sensitive to the nature of the side chain beyond the need for α-amino acids. The $P_{2'}$ residue is required, since trunkated analogues are poor inhibitors; however, the only requirement of this residue is that it should be a hydrophobic one. The insensitivity of the $P_{3'}$ position to modification of structure also is consistent with the finding that adding amino acids or polymers to the $P_{3'}$ site does not affect K_i markedly. Furthermore, recent ESR data, utilizing spin-labeled pepstatin analogues,[22] indicate that the mobility of the C-terminal region of pepstatin is only partially restricted 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 analogue, Tempo-Val-(*S,S*)-Sta-Ala-Iaa, is almost completely immobilized. This is consistent with much tighter binding at the P_3 subsite and with the much stricter structural requirements at P_3 for tight binding inhibition.

Inhibition of pepsin by a variety of pepstatin analogues[10,18–20] can be time dependent. The kinetics for this process are consistent with the formation of at least two enzyme inhibitory complexes. These results have led to a proposal for the minimum kinetic mechanism (eq 1) involving three distinct enzyme–inhibitor complexes for

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$$E + I \underset{k_2}{\overset{k_1}{\rightleftarrows}} EI \overset{\text{fast}}{\underset{k_4}{\overset{k_3}{\rightleftarrows}}} EI^* \overset{\text{slow}}{\underset{k_6}{\overset{k_5}{\rightleftarrows}}} EI^{**} \quad (1)$$

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.[8] Inhibitors that exhibit time-dependent inhibition then undergo a slow conversion to a tightened EI** complex. It is also possible the tightened complex is formed directly from free enzyme via a branched pathway and not directly from the intermediate complex. Our present kinetic data cannot distinguish between these possibilities.

The kinetic data reported here for the P_2 analogues suggest that this site may be involved in the conformational changes associated with the slow binding process. Analogues having side chains larger than an isopropyl group (**41** and **43**) do not bind slowly, possibly because steric bulk prohibits conformational changes required to achieve the tightened EI** complex. The analogue with the smaller side chain, such as the methyl group in the alanine analogue **39**, may allow this conformational change to occur due to its small size, although it lacks the hydrophobicity of large groups and has a larger dissociation constant for EI*.

An X-ray structure for the complex of pepstatin analogue **34** bound to penicillopepsin has recently been obtained.[21] Analogue **45** adopts a β-sheet conformation when bound in the active site of the enzyme with 15 nonbonded 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 Asp-77 residue after a major conformational change has occurred (flap closing) may be involved.

Experimental Section

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 with Varian Model EM-390, JEOL FX-900 Fourier transform, and Bruker HX-90E Fourier transform spectrometers. Chemical shifts were reported as δ units (parts per million) 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. The following TLC solvent systems were used: A, 10% methanol in chloroform (v/v); B, 5% methanol in chloroform (v/v). Compounds were visualized on the plates by reaction with ninhydrin, chlorox–*o*-tolidine, 5% phosphomolybdic acid in ethanol, and ultraviolet light. All compounds used in kinetic studies appeared as a single spot on TLC. Kinetic constants were measured with synthetic heptapeptide Phe-Gly-His-Phe(NO_2)-Phe-Ala-Phe-OMe as described and a Gilford Model 250 spectrophotometer connected to a Gilford 6051 recorder.[8]

General Procedure A. Removal of the *tert*-Butyloxycarbonyl Group. Boc amino acids were deprotected with 4 N HCl in dioxane as previously described.[10]

General Procedure B. Coupling Reactions with Dicyclohexylcarbodiimide/1-Hydroxybenzotriazole.[23] Peptides were prepared with DCC/HOBT in methylene chloride as previously described,[7] except that *N*-methylmorpholine was used in place of triethylamine. The peptide was purified by silica gel chromatography, when necessary, followed by crystallization from a suitable solvent or solvent mixture.

General Procedure C. Preparation of Symmetrical Anhydrides.[24] The Boc amino acid or carboxylic acid symmetrical anhydride was prepared with DCC in methylene chloride as previously described.[10]

General Procedure D. Coupling Reactions and N-Acylation Reactions via Symmetrical Anhydrides. The symmetrical anhydrides were reacted with free amines in either methylene chloride or DMF as previously described,[10] except that *N*-methylmorpholine was used in place of triethylamine. The peptide was purified by silica gel chromatography when necessary and crystallized.

General Procedure E. Coupling Reactions with EEDQ.[25] To a chilled solution of Boc amino acid (1 equiv) and free amine (1 equiv) was added *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (1 equiv, 97% EEDQ). The reaction was stirred at 0 °C for 2–4 h and at room temperature overnight. The reaction was worked up as described in procedure B.

General Procedure F. Saponification of Peptide Esters. The peptide ester (1 mmol) was dissolved in methanol (~9 mL). To this solution was added 1 N NaOH (1.1 mmol). When reaction was complete by TLC, the methanol was removed in vacuo, and water 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 peptide acid.

General Procedure G. Preparation of Pentafluorophenyl Active Esters via the DCC–PFP "Complex" Method.[12] The Boc amino acid (1 equiv) was dissolved in ethyl acetate (50 mg/mL) and chilled to 0 °C. DCC–PFP complex (1 equiv) was added, the mixture was stirred, and the progress of the reaction was followed by TLC. After ~0.5 h, the reaction mixture was chilled over dry ice for 20 min and then filtered. The organic layer was worked up as in general procedure B.

***N*-(*tert*-Butyloxycarbonyl)-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl-L-alanyl-L-phenylalanine Methyl Ester (4).** The title compound was prepared by general procedure B from HCl·Ala-Phe-OMe and (3*S*,4*S*)-Boc-Sta-OH. Silica gel chromatography (3% MeOH/$CHCl_3$), followed by crystallization, yielded pure product. See Table II for physical and microanalytical data.

***N*-(*tert*-Butyloxycarbonyl)-L-valyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl-L-alanyl-L-phenylalanine Methyl Ester (5).** The title compound was prepared by general procedure D from HCl·(*S*,*S*)-Sta-Ala-Phe-OMe and Boc-L-Val anhydride with methylene chloride as solvent. Chromatography (5% MeOH/$CHCl_3$), followed by crystallization, yielded pure product. See Table II for physical and microanalytical data.

***N*-Isovaleryl-L-valyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl-L-alanyl-L-phenylalanine Methyl Ester (6).** The title compound was prepared by general procedure D with HCl·Val-(*S*,*S*)-Sta-Ala-Phe-OMe, isovaleric anhydride, and DMF as solvent. Crystallization yielded pure product: NMR (methanol-d_4) δ 0.67–1.11 (m, 18 H), 1.15–1.78 (m, 8 H, includes doublet, $J \approx 7$ Hz), 1.9–2.44 (m, 6 H, includes doublet, $J = 7$ Hz), 2.89–3.2 (m, 2 H), 3.67 (s, 3 H), 3.78–4.89 (m, 3 H), 7.1–7.3 (m, 5 H). See Table I for other physical and microanalytical data.

***N*-(*tert*-Butyloxycarbonyl)-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl-L-alanyl-L-leucine Methyl Ester (7).** The title compound was prepared by general procedure B from HCl·Ala-Leu-OMe and (3*S*,4*S*) Boc-Sta-OH. The product was chromatographed (3% MeOH/$CHCl_3$) and recovered as an amorphous solid. See Table II for physical and microanalytical data.

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***N*-(*tert*-Butyloxycarbonyl)-L-valyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl-L-alanyl-L-leucine Methyl Ester (8).** The title compound was prepared by general procedure D from HCl·(*S,S*)-Sta-Ala-Leu-OMe and Boc-L-Val anhydride with methylene chloride as solvent. Chromatography (3% MeOH/$CHCl_3$) yielded pure product as an amorphous solid. See Table II for physical and microanalytical data.

***N*-Isovaleryl-L-valyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl-L-alanyl-L-leucine Methyl Ester (9).** The title compound was prepared by general procedure D from HCl·Val-(*S,S*)-Sta-Ala-Leu-OMe and isovaleric anhydride with DMF as solvent. Crystallization yielded pure product: NMR ($CDCl_3$) δ 0.78–1.2 (m, 24 H), 1.33–1.83 (m, 8 H, includes doublet, J = 7 Hz), 1.89–2.22 (m, 7 H), 2.44–2.76 (1 H), 3.38 (m, 1 H), 3.73 (s, 3 H), 3.78–4.22 (m, 2 H), 4.33–4.89 (m, 2 H), 6.02 (d, $J \approx 8$ Hz, 1 H), 6.78 (d, J = 8 Hz, 1 H), 7.44 (d, J = 8 Hz, 1 H), 7.82 (d, J = 8 Hz, 1 H). See Table I for other physical and microanalytical data.

***N*-(*tert*-Butyloxycarbonyl)-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl-L-alanylglycine Methyl Ester (10).** The compound was prepared by general procedure B from HCl·Ala-Gly-OMe and (*S,S*)-Boc-Sta-OH. Chromatography (5% MeOH/$CHCl_3$) yielded pure product as an amorphous solid. See Table II for physical and microanalytical data.

***N*-(*tert*-Butyloxycarbonyl)-L-valyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl-L-alanylglycine Methyl Ester (11).** The title compound was prepared by general procedure D from HCl·(*S,S*)-Sta-Ala-Gly-OMe and Boc-Val anhydride with methylene chloride as solvent. Chromatography (7% MeOH/$CHCl_3$), followed by crystallization, yielded pure product. See Table II for physical and microanalytical data.

***N*-Isovaleryl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl-L-alanylglycine Methyl Ester (12).** The title compound was prepared by general procedure D from HCl·Val-(*S,S*-Sta-Ala-Gly-OMe and isovalerly anhydride with DMF as solvent. Crystallization yielded pure product: NMR ($CDCl_3$) δ 0.78–1.11 (m, 18 H), 1.33–1.55 (m, 7 H), 1.89–2.67 (m, 5 H), 3.33–3.72 (m, 4 H, includes singlet at δ 3.71), 3.89–4.22 (m, 4 H), 4.44 (m, 1 H), 4.89 (m, 1 H), 5.78 (d, J = 8 Hz, 1 H), 6.89 (m, 1 H), 7.33–7.78 (m, 2 H). See Table I for other physical and microanalytical data.

***N*-(*tert*-Butyloxycarbonyl)-L-leucyl Isoamylamide (28).** The title compound was prepared by general procedure E from isoamylamine and Boc-Leu. Precipitation gave the product as an amorphous solid. See Table II for physical and microanalytical data.

***N*-(*tert*-Butyloxycarbonyl)-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl-L-leucyl Isoamylamide (29).** The title compound was prepared by general procedure B from HCl·Leu-Iaa and (3*S*,4*S*)-Boc-Sta-OH. Chromatography (5% MeOH/$CHCl_3$) gave pure compound as a clear oil. See Table II for physical and microanalytical data.

***N*-(*tert*-Butyloxycarbonyl)-L-valyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl-L-leucyl Isoamylamide (30).** The title compound was prepared by general procedure D from HCl·(*S,S*)-Sta-Leu-Iaa and Boc-Val anhydride with methylene chloride as solvent. Chromatography (5% MeOH/$CHCl_3$) gave product as an amorphous solid. See Table II for physical and microanalytical data.

***N*-Isovaleryl-L-valyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl-L-leucyl Isoamylamide (31).** The title compound was prepared by general procedure D from HCl·Val-(*S,S*)-Sta-Leu-Iaa and isovaleric anhydride with DMF as solvent. Crystallization yielded pure product: NMR ($CDCl_3$) δ 0.67–1.11 (m, 30 H), 1.22–1.89 (m, 11 H), 1.90–2.22 (m, 4 H), 3.00–3.44 (m, 3 H), 3.76–4.44 (m, 3 H), 5.07 (d, $J \approx 8$ Hz, 1 H), 6.00–6.22 (m, 2 H), 7.78–8.0 (m, 2 H). See Table I for other physical and microanalytical data.

***N*-(*tert*-Butyloxycarbonyl)glycyl Isoamylamide (32).** The title compound was prepared by general procedure E from isoamylamine and Boc-Gly-OH. The product was obtained as a clear oil. See Table II for physical and microanalytical data.

***N*-(*tert*-Butyloxycarbonyl)-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoylglycyl Isoamylamide (33).** The title compound was prepared by general procedure B from HCl·Gly-Iaa and (*S,S*)-Boc-Sta-OH. Chromatography (5% MeOH/$CHCl_3$), followed by crystallization, yielded pure product. See Table II for physical and microanalytical data.

***N*-(*tert*-Butyloxycarbonyl)-L-valyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoylglycyl Isoamylamide (34).** The title compound was prepared by general procedure D from HCl·(*S,S*)-Sta-Gly-Iaa and Boc-Val anhydride with methylene chloride as solvent. Chromatography (5% MeOH/$CHCl_3$), followed by crystallization, yielded pure product. See Table II for physical and microanalytical data.

***N*-Isovaleryl-L-valyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoylglycyl Isoamylamide (35).** The title compound was prepared by general procedure D from HCl·Val-(*S,S*)-Sta-Gly-Iaa and isovaleric anhydride with DMF as solvent. Crystallization yielded pure product: NMR ($CDCl_3$), δ 0.79–1.11 (m, 24 H), 1.20–1.78 (m, 8 H), 1.89–2.55 (m, 4 H), 3.27 (q, J = 6 Hz, 2 H), 3.44–4.22 (m, 5 H), 4.67 (d, $J \approx 7$ Hz, 1 H), 5.83 (m, 2 H), 7.5 (m, 2 H). See Table I for other physical and microanalytical data.

***N*-(*tert*-Butyloxycarbonyl)glycyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl-L-alanyl Isoamylamide (36).** The title compound was prepared by general procedure B from HCl·(*S,S*)-Sta-Ala-Iaa[7] and Boc-Gly-OH. Chromatography (5% MeOH/$CHCl_3$) gave the product as an amorphous solid. See Table II for physical and microanalytical data.

***N*-Isovalerylglycyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl-L-alanyl Isoamylamide (37).** The title compound was prepared by general procedure D from HCl·Gly-(*S,S*)-Sta-Ala-Iaa and isovaleric anhydride with DMF as solvent. Crystallization yielded pure product: NMR ($CDCl_3$) δ 0.78–1.11 (m, 18 H), 1.22–1.78 (m, 10 H, includes doublet at δ 1.38), 2.00–2.55 (m, 4 H), 3.27 (q, $J \approx 7$ Hz, 2 H), 3.67–4.22 (m, 4 H), 4.25–4.55 (m, 2 H), 6.33–6.67 (m, 2 H), 7.06–7.33 (m, 2 H). See Table I for other physical and microanalytical data.

***N*-(*tert*-Butyloxycarbonyl)-L-alanyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl-L-alanyl Isoamylamide (38).** The title compound was prepared by general procedure D from HCl·(*S,S*)-Sta-Ala-Iaa and Boc-Ala anhydride with methylene chloride as solvent. Chromatography (5% MeOH/$CHCl_3$) yielded pure product as an amorphous solid. See Table II for physical and microanalytical data.

***N*-Isovaleryl-L-alanyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl-L-alanyl Isoamylamide (39).** The title compound was prepared by general procedure D from HCl·Ala-(*S,S*)-Sta-Ala-Iaa and isovaleric anhydride with DMF as solvent. Crystallization yielded pure product: NMR ($CDCl_3$) δ 0.89–1.11 (m, 18 H), 1.33–1.78 (m, 13 H, includes two doublets at δ 1.42 and 1.38), 2.00–2.67 (m, 4 H), 3.11–3.56 (m, 3 H, includes quartet at δ 3.27), 3.78–4.44 (m, 3 H), 4.85 (d, J = 8 Hz, 1 H), 5.89–6.28 (m, 2 H), 7.55–7.89 (m, 2 H). See Table I for other physical and microanalytical data.

***N*-(*tert*-Butyloxycarbonyl)-L-leucyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl-L-alanyl Isoamylamide (40).** The title compound was prepared by general procedure D from HCl·(*S,S*)-Sta-Ala-Iaa and Boc-Leu anhydride with methylene chloride as solvent. Chromatography (5% MeOH/$CHCl_3$), followed by crystallization, yielded pure product. See Table II for physical and microanalytical data.

***N*-Isovaleryl-L-leucyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl-L-alanyl Isoamylamide (41).** The title compound was prepared by general procedure D from HCl·Leu-(*S,S*)-Sta-Ala-Iaa and isovaleric anhydride with DMF as solvent. Crystallization yielded pure product: NMR ($CDCl_3$) δ 0.78–1.11 (m, 24 H), 1.22–1.78 (m, 13 H, includes doublet at δ 1.38), 2.0–2.67 (m, 4 H), 3.22 (q, $J \approx 7$ Hz, 2 H), 3.78–4.44 (m, 4 H), 4.89 (d, J = 8 Hz, 1 H), 5.89–6.11 (m, 2 H), 7.78–8.11 (m, 2 H). See Table I for other physical and microanalytical data.

***N*-(*tert*-Butyloxycarbonyl)-L-phenylalanyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl-L-alanyl Isoamylamide (42).** The title compound was prepared by general procedure D from HCl·(*S,S*)-Sta-Ala-Iaa and Boc-Phe anhydride with methylene chloride as solvent. Chromatography (5% MeOH/$CHCl_3$), followed by crystallization, yielde pure product. See Table II for physical and microanalytical data.

***N*-Isovaleryl-L-phenylalanyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl-L-alanyl Isoamylamide (43).** The title compound was prepared by general procedure D from HCl·

Phe-(*S,S*)-Sta-Ala-Iaa and isovaleric anhydride with DMF as solvent. Crystallization yielded pure product: NMR ($CDCl_3$) δ 0.67–1.11 (m, 18 H), 1.22–1.78 (m, 10 H), 1.89–2.27 (m, 4 H), 2.89–3.33 (m, 4 H), 3.44–4.00 (m, 2 H), 4.22–4.83 (m, 3 H), 6.44 (m, 1 H), 6.71 (m, 1 H), 6.89–7.44 (m, 6 H), 7.71 (m, 1 H). See Table I for other physical and microanalytical data.

Ethyl *N*-(*tert*-Butyloxycarbonyl)-L-valyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoate (20). The title compound was prepared by general procedure D from HCl·(*S,S*)-Sta-OEt and Boc-Val anhydride with methylene chloride as solvent. Crystallization yielded pure product. See Table II for physical and microanalytical data.

Ethyl *N*-Isovaleryl-L-valyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoate (21). The title compound was prepared by general procedure D from HCl·Val-(*S,S*)-Sta-OEt and isovaleric anhydride with DMF as solvent. Crystallization yielded pure product: NMR ($CDCl_3$) δ 0.75–1.11 (m, 18 H), 1.15–1.75 (m, 8 H, includes triplet at δ 1.25), 2.15 (m, 2 H), 2.44 (d, $J \approx 7$ Hz, 2 H), 3.65 (m, 1 H), 3.85–4.35 (m, 5 H, includes quartet at δ 4.07), 6.15 (d, $J = 9$ Hz, 1 H), 6.47 (d, $J = 9$ Hz, 1 H). See Table I for other physical and microanalytical data.

***N*-Isovaleryl-L-valyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoic Acid (22).** The title compound was prepared by general procedure F from Iva-Val-(*S,S*)-Sta-OEt in about 95% yield. The compound was used in the next step without further purification.

***N*-Isovaleryl-L-valyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl-L-alanine Methyl Ester (23).** The title compound was prepared by general procedure B from Iva-Val-(*S,S*)-Sta-OH and HCl·Ala-OMe. Chromatography (3% MeOH/$CHCl_3$), followed by crystallization, yielded pure product: NMR ($CDCl_3$) δ 0.67–1.11 (m, 18 H), 1.15–1.67 (m, 8 H, includes doublet at δ 1.42), 1.78–2.55 (m, 4 H), 3.42 (m, 1 H), 3.73 (s, 3 H), 3.78–4.11 (m, 2 H, includes quartet at δ 3.91), 4.57 (t, $J = 8$ Hz, 1 H), 4.89 (d, $J = 8$ Hz, 1 H), 5.84 (d, $J \approx 7$ Hz, 1 H), 7.29 (m, 1 H), 7.6 (d, $J \approx 8$ Hz, 1 H). See Table I for other physical and microanalytical data.

Ethyl *N*-(*tert*-Butyloxycarbonyl)-L-valyl-L-valyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoate (44). The title compound was prepared by general procedure D from HCl·Val-(*S,S*)-Sta-OEt and Boc-Val anhydride with methylene chloride as solvent. Crystallization yielded pure product. See Table II for physical and microanalytical data.

Ethyl *N*-Isovaleryl-L-valyl-L-valyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoate (45). The title compound was prepared by general procedure D from HCl·Val-Val-(*S,S*)-Sta-OEt and isovaleric anhydride with DMF as solvent. Crystallization yielded pure product: NMR ($CDCl_3$) δ 0.90–1.11 (m, 24 H), 1.24 (t, $J = 7$ Hz, 3 H), 1.38–1.78 (m, 4 H), 1.89–2.33 (m, 4 H), 2.41 (d, $J \approx 7$ Hz, 2 H), 3.46 (m, 1 H), 3.78–4.44 (m, 6 H, includes quartet at δ 4.1), 6.22–6.67 (m, 2 H), 6.67–7.0 (m, 1 H). See Table I for other physical and microanalytical data.

***N*-(*tert*-Butyloxycarbonyl)-L-valyl-L-valyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoic Acid (46).** 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*)-hydroxy-6-methylheptanoyl-L-alanine Methyl Ester (47).** The title compound was prepared by general procedure B from HCl·Ala-OMe and Boc-Val-Val-(*S,S*)-Sta-OH. Chromatography (3% MeOH/$CHCl_3$), followed by crystallization, yielded pure product. See Table II for physical and analytical data.

***N*-Isovaleryl-L-valyl-L-valyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl-L-alanine Methyl Ester (48).** The title compound was prepared by general procedure D from HCl·Val-Val-(*S,S*)-Sta-Ala-OMe and isovaleric anhydride with DMF as solvent. Crystallization yielded pure product: NMR ($CDCl_3$) δ 0.89–1.11 (m, 24 H), 1.22–1.67 (m, 9 H), 2.0–2.33 (m, 4 H), 3.73 (s, 3 H), 4.0–4.67 (m, 6 H), 7.0 (m, 1 H), 7.11–7.42 (m, 3 H). See Table I for other physical and microanalytical data.

Pentafluorophenyl *N*-(*tert*-Butyloxycarbonyl)-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoate (24). 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 further purification.

***N*-(*tert*-Butyloxycarbonyl)-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl Isopropylamide (25).** To a chilled ethyl acetate solution of Boc-(*S,S*)-Sta-PFP was added NMM (3 equiv), followed by isopropylamine (1.1 equiv). The reaction was stirred at 0 °C and closely followed by TLC. After 1 h 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 yielded pure product. See Table II for physical and microanalytical data.

***N*-(*tert*-Butyloxycarbonyl)-L-valyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl Isopropylamide (26).** The title compound was prepared by general procedure D from HCl·(*S,S*)-Sta-Ipa and Boc-Val anhydride with methylene chloride as solvent. Crystallization yielded pure product. See Table II for physical and microanalytical data.

***N*-Isovaleryl-L-valyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl Isopropylamide (27).** The title compound was prepared by general procedure D from HCl·Val-(*S,S*)-Sta-Ipa and isovaleric anhydride with DMF as solvent. Crystallization yielded pure product: NMR ($CDCl_3$) δ 0.76–1.11 (m, 18 H), 1.11–1.22 (m, 8 H, includes doublet at δ 1.16), 1.33–1.67 (m, 3 H), 1.89–2.33 (m, 4 H), 3.78–4.33 (m, 5 H), 5.95 (m, 2 H), 6.22 (d, $J \approx 9$ Hz, 1 H). See Table I for other physical and microanalytical data.

***N*-(*tert*-Butyloxycarbonyl)-L-alanine Pentafluorophenyl Ester (13).** 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 Methylamide (14).** 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 min. The reaction was monitored closely by TLC and appeared to be complete after about 0.5 h. 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, and the solvent was removed in vacuo. Crystallization yielded pure product. See Table II for physical and microanalytical data.

***N*-Isovaleryl-L-valyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl-L-alanyl Methylamide (15).** The title compound was prepared by general procedure B from HCl·Ala-NH-CH_3 and Iva-Val-(*S,S*)-Sta-OH. Chromatography (5% MeOH/$CHCl_3$), followed by crystallization, yielded pure product: NMR ($CDCl_3$) δ 0.78–1.11 (m, 18 H), 1.22–1.79 (m, 8 H, includes doublet at δ 1.39), 1.89–2.44 (m, 4 H), 5.0 (d, $J \approx 5$ Hz, 3 H), 3.33–3.56 (m, 1 H), 3.78–4.11 (m, 2 H), 4.35 (t, $J = 7$ Hz, 1 H), 4.73 (d, $J = 8$ Hz, 1 H), 6.11 (d, $J = 8$ Hz, 1 H), 6.39 (m, 1 H), 7.38–7.78 (m, 2 H). See Table I for other physical and microanalytical data.

***N*-(*tert*-Butyloxycarbonyl)-L-alanyl Isopropylamide (16).** Boc-Ala-PFP (13; 1 equiv) was dissolved in EtOAc (100 mg/mL) and chilled to 0 °C. Isopropylamine (2 equiv) was slowly added to the solution over a period of 20 min. The reaction mixture was allowed to stir for about 2 h. 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 yielded pure product. See Table II for physical and microanalytical data.

***N*-(*tert*-Butyloxycarbonyl)-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoylalanyl Isopropylamide (17).** The compound was prepared by general procedure B from HCl·Ala-Ipa and Boc-(*S,S*)-Sta-OH. Chromatography (3% MeOH/$CHCl_3$), followed by crystallization, yielded pure product. See Table II for physical and microanalytical data.

***N*-(*tert*-Butyloxycarbonyl)-L-valyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl-L-alanyl Isopropylamide (18).** The title compound was prepared by general procedure D from HCl·(*S,S*)-Sta-Ala-Ipa and Boc-Val anhydride with methylene chloride as solvent. Chromatography (5% MeOH/$CHCl_3$), followed by crystallization, yielded pure product. See Table II for physical and microanalytical data.

***N*-Isovaleryl-L-valyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl-L-alanyl Isopropylamide (19).** The title

compound was prepared by general procedure D from HCl·Val-(*S,S*)-Sta-Ala-Ipa and isovaleric anhydride with DMF as solvent. Crystallization yielded pure product: NMR (Me_2SO-d_6) δ 0.83–1.17 (m, 24 H), 1.20–1.50 (m, 8 H), 2.0–2.33 (m, 4 H), 3.55–4.44 (m, 5 H), 4.84 (d, J = 6 Hz, 1 H), 7.22 (m, 1 H), 7.60–7.67 (m, 3 H). See Table I for other physical and microanalytical data.

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Registry No. **3**, 72155-64-7; **4**, 84850-91-9; **5**, 84850-92-0; **6**, 84850-93-1; **7**, 84850-94-2; **8**, 84850-95-3; **9**, 84850-96-4; **10**, 84850-97-5; **11**, 84850-98-6; **12**, 84850-99-7; **13**, 50903-48-5; **14**, 84851-00-3; **15**, 84851-01-4; **16**, 84851-02-5; **17**, 84851-03-6; **18**, 84851-04-7; **19**, 84851-05-8; **20**, 84851-06-9; **21**, 84851-07-0; **22**, 72155-75-0; **23**, 84851-08-1; **24**, 84851-09-2; **25**, 84851-10-5; **26**, 84851-11-6; **27**, 84851-12-7; **28**, 84863-67-2; **29**, 84851-13-8; **30**, 84851-14-9; **31**, 84851-15-0; **32**, 84851-16-1; **33**, 84851-17-2; **34**, 84851-18-3; **35**, 84851-19-4; **36**, 84851-20-7; **37**, 84851-21-8; **38**, 84851-22-9; **39**, 84863-68-3; **40**, 84851-23-0; **41**, 84851-24-1; **42**, 84851-25-2; **43**, 84851-26-3; **44**, 84851-27-4; **45**, 84062-22-6; **46**, 84851-28-5; **47**, 84851-29-6; **48**, 84851-30-9; Ala-Phe-OMe·HCl, 2280-75-3; (3*S*,4*S*)-Boc-Sta-OH, 58521-49-6; (*S,S*)-Sta-Ala-Phe-OMe·HCl, 84851-31-0; Boc-L-Val anhydride, 33294-55-2; Val-(*S,S*)-Sta-Ala-Phe-OMe·HCl, 84851-32-1; Ala-Leu-OMe·HCl, 59515-79-6; (*S,S*)-Sta-Ala-Leu-OMe·HCl, 84851-33-2; Val-(*S,S*)-Sta-Ala-Leu-OMe·HCl, 84851-34-3; Ala-Gly-OMe·HCl, 23404-09-3; (*S,S*)-Sta-Ala-Gly-OMe·HCl, 84851-35-4; Val-(*S,S*)-Sta-Ala-Gly-OMe·HCl, 84851-36-5; Boc-Leu-OH, 13139-15-6; Leu-Iaa·HCl, 84851-37-6; (*S,S*)-Sta-Leu-Iaa·HCl, 84851-38-7; Val-(*S,S*)-Sta-Leu-Iaa·HCl, 84863-69-4; Boc-Gly-OH, 4530-20-5; Gly-Ias·HCl, 84851-39-8; (*S,S*)-Sta-Gly-Iaa·HCl, 84851-40-1; Val-(*S,S*)-Sta-Gly-Iaa·HCl, 84851-41-2; (*S,S*)-Sta-Ala-Iaa·HCl, 84894-08-6; Gly-(*S,S*)-Sta-Ala-Iaa·HCl, 84851-42-3; Boc-Ala anhydride, 33294-53-0; Ala-(*S,S*)-Sta-Ala-Iaa·HCl, 84851-43-4; Boc-Leu anhydride, 51499-91-3; Leu-(*S,S*)-Sta-Ala-Iaa·HCl, 84851-44-5; Boc-Phe anhydride, 33294-54-1; Phe-(*S,S*)-Sta-OEt·HCl, 84851-45-6; (*S,S*)-Sta-OEt·HCl, 84851-46-7; Val-(*S,S*)-Sta-OEt·HCl, 84851-47-8; Ala-OMe·HCl, 2491-20-5; Val-Val-(*S,S*)-Sta-OEt·HCl, 84851-48-9; Val-Val-(*S,S*)-Ala-OMe·HCl, 84851-49-0; (*S,S*)-Sta-Ipa·HCl, 84851-50-3; Val-(*S,S*)-Sta-Ipa·HCl, 84851-51-4; Iva-Val-(*S,S*)-Sta-OH, 84851-52-5; Ala-NH-CH_3·HCl, 61275-22-7; Ala-Ipa·HCl, 84851-53-6; (*S,S*)-Sta-Ala-Ipa·HCl, 84851-54-7; Val-(*S,S*)-Sta-Ala-Ipa·HCl, 84851-55-8; isovaleric anhydride, 1468-39-9; isoamylamine, 107-85-7; isopropylamine, 75-31-0; methylamine, 74-89-5; pepsin, 9001-75-6.

Synthesis and Biological Properties of New Hexapeptide Substrates for Vitamin K Dependent Carboxylase. Evidence for X-Pro Cis/Trans Amide Bond Interconversions in Prothrombin Precursor Fragment 18–23

Daniel H. Rich,*[†] Megumi Kawai,[†] Hedda L. Goodman,[‡] and John W. Suttie[‡]

School of Pharmacy and Department of Biochemistry, College of Agriculture and Life Science, University of Wisconsin-Madison, Madison, Wisconsin 53706. *Received August 23, 1982*

Three hexapeptide analogues, corresponding to sequence 18–23 of bovine prothrombin precursor [-Cys-Leu-Glu-Glu-Pro-Cys-] have been synthesized and evaluated as substrates for vitamin K dependent carboxylase. These new hexapeptides are moderately good substrates for the carboxylase but do not significantly inhibit carboxylation of Phe-Leu-Glu-Glu-Leu, a good substrate for the enzyme. Based on proton and carbon-13 NMR experiments, it is established that the conformation of sequence 18–23, which contains proline at position 22, has a trans amide bond for the Glu-Pro[22] sequence in chloroform-*d*. This amide bond is converted to the cis amide geometry in Me_2SO-d_6. It is proposed that good substrates for the carboxylase require a trans amide bond between residues 21 and 22.

Vitamin K is required for the posttranslational carboxylation of L-glutamyl (Glu) residues to γ-carboxyglutamyl residues (Gla)[1] in microsomal precursors of prothrombin,[2–4] clotting factors VII, IX, and X,[5] plasma proteins C, Z, and S,[5,6] and a limited number of other proteins.[6,7] This transformation from Glu to Gla converts these inactive prothrombin precursors to their biologically active forms.[7,8] The enzyme catalyzing this reaction, vitamin K dependent carboxylase,[9] requires oxygen, reduced vitamin K, and carbon dioxide in order to carboxylate the γ-carbon of Glu.[6,8] Location of Gla residues in three clotting proteins is shown in Figure 1.

We have reported that the synthetic pentapeptides H-Phe-Leu-Glu-Glu-Leu-OH (**1**), a pentapeptide corresponding to prothrombin precursor sequence 5–9 (see Figure 1), and the shorter Boc-Glu-Glu-Leu-OMe (**2**) are substrates for the carboxylase and have been utilized to characterize the substrate requirements of the enzyme.[10,11] The carboxylase appears to act selectively at L-glutamyl residues because peptides containing L-aspartyl, L-homoglutamyl, L-glutaminyl, and D-glutamyl residues were not measurably carboxylated in our assay system.[12a] (Recent data suggest L-aspartyl may be carboxylated in certain systems.)[12b] The peptide sequence adjacent to the site of

[†] School of Pharmacy.
[‡] Department of Biochemistry.

(1) Abbreviations used follow IUPAC–IUB tentative nomenclature described in *J. Biol. Chem.*, **247**, 977 (1972). Additional abbreviations used are: Gla, γ-carboxyglutamic acid; DCC, dicyclohexylcarbodiimide; DCU, dicyclohexylurea; Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; Acm, acetamidomethyl.
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