Inhibition of Aspartic Proteinases by Peptides Containing Lysine and Ornithine Side-Chain Analogues of Statine[1]

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The synthesis of two new analogues of statine are reported corresponding to analogues with the lysine side chain and the ornithine side chain. These analogues were designed on the basis of substrate specificity and molecular modeling of three-dimensional structures of the penicillopepsin: Iva-Val-Val-Sta-OEt crystal structure. 4,8-Diamino-3-hydroxyoctanoic acid [LySta] and 4,7-diamino-3-hydroxyheptanoic acid [OrnSta] were synthesized respectively from Boc-Lys(Z)-al and Boc-Orn(Bzl,Z)-al by addition of lithio ethyl acetate to the aldehyde group. The [LySta] derivative was converted to the trichloroethoxycarbonyl derivative and separated into the corresponding 3*S*,4*S* and 3*R*,4*R* diastereomers. The [OrnSta] derivative was used as a mixture of 3-position diastereomers. These new amino acids were used to prepare the following inhibitors: Iva-Val-Val-[LySta]-OEt and Iva-Val-Val-[OrnSta]-OEt as well as the corresponding synthetic intermediates. Inhibition constants (K_i values) were measured for inhibition of porcine pepsin and penicillopepsin. Both compounds were potent inhibitors of penicillopepsin with K_i values 10–100 times smaller (2.1 and 1.1 nM, respectively) than the K_i of Iva-Val-Val-Sta-OEt (47 nM). In contrast both inhibitors are exceptionally weak inhibitors of porcine pepsin with K_i values greater than 1 μM. These results are correlated with the ability of the basic group in the new inhibitors to bind to aspartic acid-77 in penicillopepsin.

Molecular modeling of small molecule–protein complexes is being used increasingly to visualize binding sites on proteins,[2] to rationalize enzyme catalytic mechanisms,[3] and to design novel inhibitors of therapeutically important enzymes. Use of known X-ray crystal structures of enzyme–inhibitor complexes has made it possible to obtain tighter binding inhibitors of a few enzymes. Kuyper et al.[4] converted trimethoprim (**1a**) to superactive inhibitors (e.g., **1b**) of dihydrofolate reductase by incorporating a carboxyl group into the inhibitor that could bind to the guanidine group of Arg-57 in DHFR. Difference density electron maps of the *Escherichia coli*–**1b** crystal complex were in agreement with the postulated binding mechanism.

However, predictions based on the crystal structures of enzyme–inhibitor complexes are not always straightforward. A particularly interesting example of the uncertainties associated with the design of inhibitors by detailed consideration of an X-ray crystal structure is found in the work of Boger et al.,[5,6] who designed novel potent human renin inhibitors, in part, by consideration of the X-ray crystal structure of the complex between pepstatin and the fungal aspartic proteinase *Rhizopus chinensis* pepsin. Pepstatin [Iva-Val-Val-Sta-Ala-Sta (**2**), where Sta is 4-(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoic acid[1]] is the first general inhibitor[7] of the class of enzymes called aspartic proteinases, defined as such because of the presence of two catalytically essential aspartic acids in the active site of the enzyme.[8,9] Numerous analogues of pepstatin have been synthesized and tested as inhibitors of pepsin,[10-17] renin,[18,19] penicillopepsin,[15,16] and other aspartic proteinases.[20-22] X-ray crystal structures of pepstatin bound to *R. chinensis* pepsin[23,24] and of the shortened tripeptide analogue Iva-Val-Val-Sta-OEt (**3**) bound to penicillopepsin[25,26] have served to identify binding interactions between these inhibitors and the active site of the aspartic proteinases. Several detailed proposals for the catalytic mechanism of aspartic proteinases are based upon these crystal structures.[23,24,27] Computer graphic analysis of the binding interactions in the *R. chinensis* pepsin–pepstatin complex suggested that additional binding interactions might be obtained by adding alkyl groups at C-2 of statine (as in **4**) or by extending the isobutyl side chain of Sta to a cyclohexylmethyl side chain (ACHPA as in **5**). Compounds **4** and **5** were synthesized and found to inhibit human renin (K_i = 1.7 and 0.16 nM, respectively)[5,6] much better than the corresponding statine-derived inhibitors. But surprisingly these compounds were not improved in-

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(1) (a) Abstracted in part from the Ph.D. dissertation of Francesco G. Salituro submitted to University of Wisconsin—Madison in Aug 1984. (b) Abbreviations used: Statine (Sta), 3-hydroxy-4-amino-6-methylheptanoic acid; [LySta], 4,8-diamino-3-hydroxyoctanoic acid; [OrnSta], 4,7-diamino-3-hydroxyheptanoic acid; DMAP, 4-(*N*,*N*-dimethylamino)pyridine; TEA, triethylamine.

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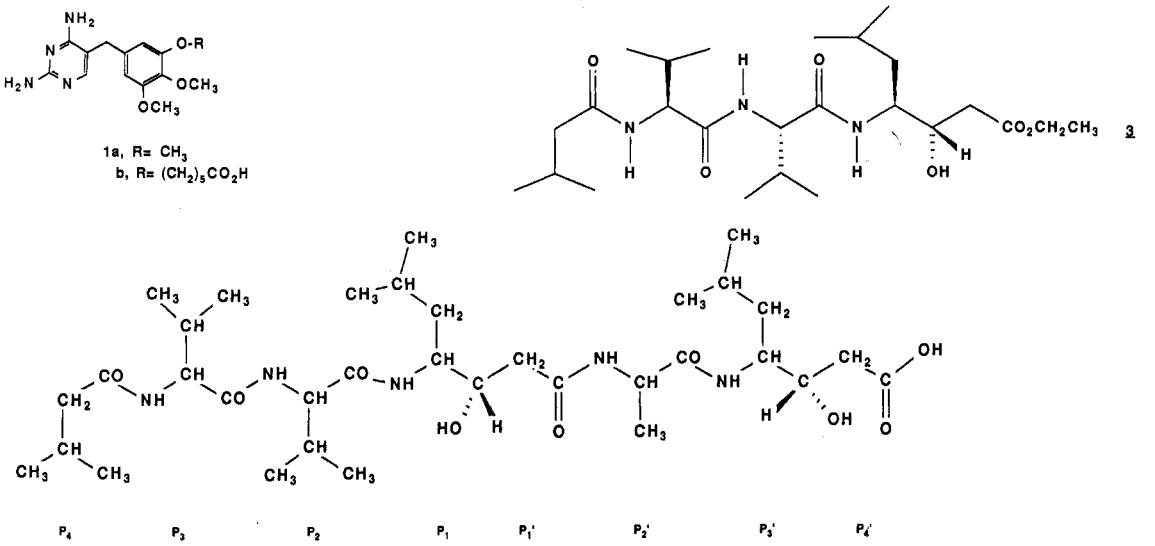
Chart I



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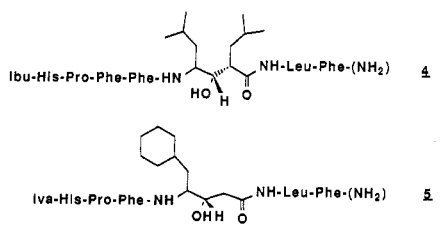
hibitors relative to the statine-containing peptides of *R. chinensis* pepsin, the enzyme on which the computer modeling was based.



We initiated the present study to see if selective, potent inhibitors of penicillopepsin could be designed from considerations of substrate specificity[28,29] and from the 1.8 Å resolution crystal structure of the complex between penicillopepsin and the tripeptide inhibitor Iva-Val-Val-Sta-OEt (**3**).[25] The idea was to determine if substitutions that improved the interactions between substrate and penicillopepsin would enhance interactions between an inhibitor and this enzyme. At the same time differences between aspartic proteinases isolated from fungal sources and mammalian sources (e.g., porcine pepsin) could be examined.

One major difference between these enzymes is that the fungal aspartic proteinases can activate trypsinogen, an activation process that requires the enzyme to recognize a lysine residue and to cleave a Lys-Ile bond.[29] None of the mammalian aspartic proteinases are known to activate trypsinogen and these enzymes usually do not cleave peptides at Lys-Xxx bonds. Instead, the mammalian enzymes prefer cleavage sites flanked by hydrophobic residues such as Leu-Leu, Phe-Phe, or related combinations.[8,9] In contrast, one of the best substrates for penicillopepsin is the synthetic peptide, Ac-Ala-Ala-Lys-Phe(NO_2)-Ala-Ala-NH_2 (**6**), which is cleaved by penicillopepsin between the lysine and *p*-nitrophenylalanine residues. Lysine was placed in the P_1 position (P_1 and P_1' refer to amino acid substitutions in substrates and inhibitors located to the left and right, respectively, of the bond cleaved by the enzyme[30]) to exploit the fact that penicillopepsin hydrolyzes Lys-Xxx bonds. Lysine placed in this position does enhance binding of the peptide to the enzyme.

We reasoned that replacement of the isobutyl side chain of statine in the inhibitor Iva-Val-Val-Sta-OEt (**3**) with the 4-aminobutyl side chain found in lysine could lead to a new inhibitor that would bind more tightly to penicillopepsin than the statine-containing analogue **3**, if **3** and substrate **6** bind similarly to the enzyme at S_1.[15,16] We report herein the synthesis of two new basic statine analogues, 4,8-diamino-3-hydroxyoctanoic acid (**7**, [LySta]) and 4,7-diamino-3-hydroxyheptanoic acid (**8**, [OrnSta]). (The bracket nomenclature is intended to emphasize the relationship of the statine analogue to the amino acid side chain. The former amino acid was abbreviated DAHOA in our preliminary reports of this work.[15,16]) These new amino acids have been converted into new inhibitors of pepsin, penicillopepsin, and *Rhizopus* pepsin and used to differentiate binding between these enzymes.

Methods

Chemistry. Synthesis of Lysine-Statine [LySta] Derivatives. The synthesis of the protected lysine derivative of statine [LySta], (4,8-diamino-3-hydroxyoctanoic

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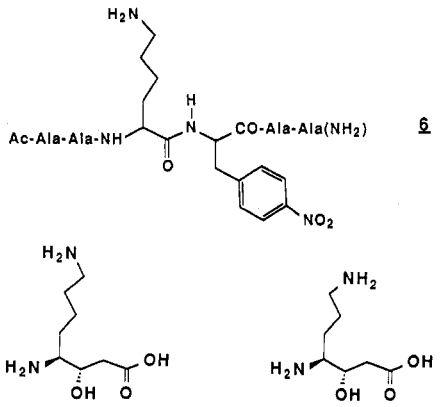
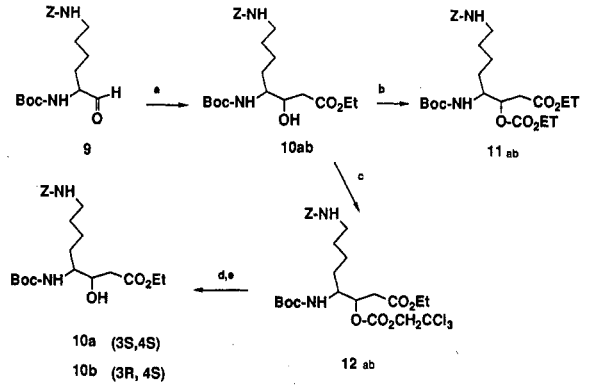


acid) began with the preparation of α-Boc-ε-Cbz-lysinal (**9**) by the method of Hamada and Shiori[31] (Scheme I). Reaction of **9** with ethyl lithioacetate at −78 °C for 30 min gave the desired fully protected amino acid **10ab** in about 70% yield as a mixture of diastereomers. The diastereomers of this compound were not separable by TLC in over 15–20 systems examined. However, the O-protected compounds were separable by TLC and column chromatography especially when run in ethyl ether systems such as ethyl ether/hexane. Initially compound **11ab** 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. In light of this, the ethoxycarbonyl protecting group was abandoned for the (trichloroethoxy)carbonyl protection scheme. Initial attempts to prepare compound **12ab** by using methods employed for the preparation of **11ab** failed. However, compound **12ab** was easily formed in high yield by reaction with trichloroethyl chloroformate in pyridine with a trace of DMAP as catalyst. Diastereomers **12ab** were easily separable by chromatography. The higher R_f of **12a** and relative NMR data were used to assign this compound the 3*S*,4*S* configuration. This assignment was confirmed by the relative potencies of the analogues (compare **16a** and **16b** in Table I) and from the X-ray of **16a** bound to penicillopepsin (see Figure 2).[32] Deprotection by reaction of compounds **12a** and **12b** with zinc in a 1:1 mixture of DMF/acetic acid was slow (~12 h). However, the use of 20 equiv of activated powdered cadmium in place of zinc[33] in a 1:1 mixture of DMF/acetic acid converted **12a** to **10a** in about 90% yield after about 4 h. Interestingly, compound **12b** was deprotected less rapidly (~57%) after the same amount of time. Fortunately, no side products were formed in these reactions, and unreacted **12b** was easily recovered.

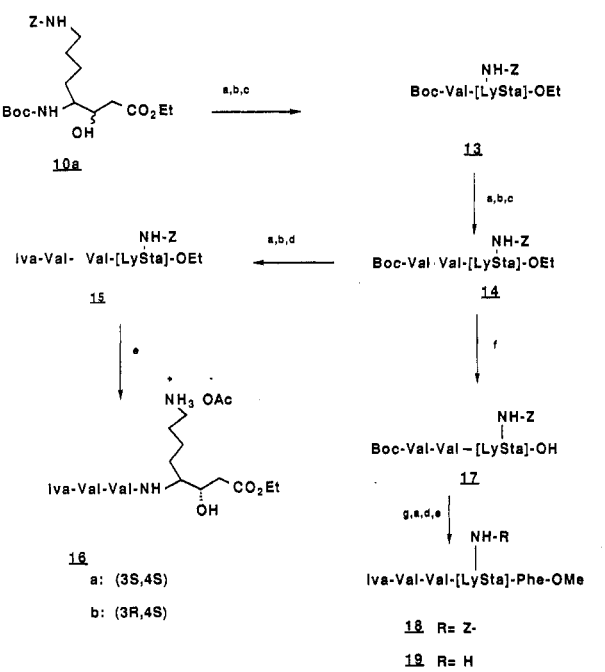
Compounds **10a** and **10b** were used separately to prepare tripeptide esters **16ab** and **19ab** by the routes outlined in Scheme II. Tripeptides **16a** and **16b** were prepared by sequentially coupling Boc-valine anhydride two times to the starting compounds **10a** and **10b** followed by acylation with isovaleryl anhydride to obtain **15a** and **15b**. The Cbz group was then removed by using standard catalytic hydrogenation procedures to give **16a** and **16b**. Compound **18a** was prepared by saponification of intermediate **14a** followed by coupling of **17** to phenylalanine methyl ester

Scheme I[a]



[a] a, $LiCH_2CO_2Et$, THF, −78 °C; b, EtOCOCl, DMAP, TEA, CH_2Cl_2; c, CCl_3CH_2OCOCl, pyridine, DMAP; d, chromatography, silica gel; e, Cd (30 equiv), DMF/HOAc, 25 °C 3–4 h).

Scheme II[a]



[a] 3*S*,4*S* = **a**, 3*R*,4*S* = **b**. Reagents used: a, HCl/dioxane; b, TEA; c, $(Boc\text{-}Val)_2O$; d, $(Iva)_2O$; e, H_2/Pd/C, DMF, HOAc; f, 1.1 equiv of NaOH/MeOH; g, DCC, HOBt, HPhe-OMe.

hydrochloride and acylation. Hydrogenation of **18** as described for compound **15** gave **19a**. In all cases the amines were obtained as the acetate salts.

Synthesis of Ornithine-Statine [OrnSta] Derivatives. The successful synthesis of Boc-[OrnSta]-OEt (**27**) is shown in Scheme III. Boc-Orn(Z)-OMe (**20**) was reduced to the alcohol **21**. The δ-Cbz group was cleaved from alcohol **21** by using standard catalytic transfer hydrogenolysis[34] to form the amino alcohol **22**. (It should be noted that Cbz removal must be carried out on alcohol **21** rather than the methyl ester **20** since removal of the Cbz group of **20** formed the lactam.) Compound **22** was then treated with benzaldehyde to form **23**, which was immediately reacted with sodium borohydride to form the *N*-benzyl derivative **24** in 65% yield. Reaction of **24** with benzyl chloroformate gave the bis-protected amine **25**, which was converted to aldehyde **26** via the method of Hamada and Shiori.[31] Aldehyde **26** was subsequently treated with ethyl lithioacetate in tetrahydrofuran to yield **27** as a mixture

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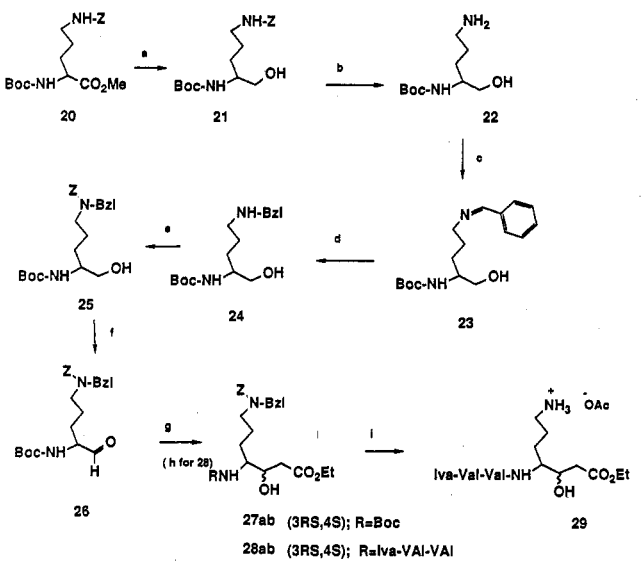
Table I. Inhibition of Penicillopepsin by [LySta] and [OrnSta] Analogues

compound	K_i, nM penicill	K_i, nM pepsin	K_i, nM *R. chinensis*
3 Iva-Val-Val-Sta-OEt	47	10	42
16a Iva-Val-Val-[LySta]-OEt	2.1[a]	>1000	33
15a Iva-Val-Val-[LySta]-OEt	100	ND[e]	ND[e]
33 Iva-Val-Val-[LySta]-OEt	100	1900	29
16b Iva-Val-Val-[(3*R*, 4*S*)LySta]-OEt	50	ND	ND
34 Iva-Val-Val-Sta-Phe-OMe	1.3[b,c]	ND	11
19 Iva-Val-Val-[LySta]-Phe-OMe	0.08[b]	ND	ND
29 Iva-Val-Val-[(3*RS*, 4*S*)OrnSta]-OEt	1.1[b,d]	>1000	47

[a] This value is an average of three independent determinations of this K_i (1.66, 0.4, 4.4 nM). [b] Time-dependent inhibition TDI) (slow binding, $T_{1/2}$ > 30 s) was observed for this inhibitor. [c] Data taken from ref 13. [d] K_i was determined on a 1:1 mixture of 3*R*/3*S*. Number reported is one-half the number obtained for the mixture. [e] ND, not assayed.

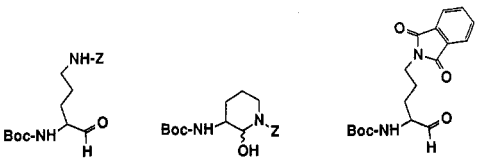
Scheme III[a]



[a] a, $LiBH_4$, EtOH, THF; b, Pd–C, NH_4O_2CH; c, $MgSO_4$, C_6H_5C-HO; d, $NaBH_4$, MeOH; e, Cbz-Cl, TEA, dioxane, water; f, pyridine, SO_3, Me_2SO, TEA; g, $LiCH_2CO_2Et$, −78 °C, 30 min; h, same sequence of reactions as for compounds **10** → **15**; i, 20% $Pd(OH)_2$, DMF, HOAc.

of diastereomers. This mixture was used throughout the remainder of the synthesis. Twice coupling with Boc-valine anhydride followed by acylation with isovaleryl anhydride gave protected tripeptide **28**. The benzyl and Cbz groups were then simultaneously removed by hydrogenation over 20% palladium hydroxide on carbon at 30 psi in DMF with a trace of acetic acid for 3 h. The acetate salt **29** was isolated by the procedure developed for the lysine-statine peptides.

Two other routes to the [OrnSta] derivative **27ab** were investigated. Attempts to form **27ab** via the aldehyde of γ-Z Orn **30** were unsuccessful as only the cyclic aminal **31** was isolated. Ethyl lithioacetate does not add to the latent aldehyde in this compound. The use of δ-phthaloyl protection on ornithine also proved unproductive as ethyl lithioacetate reacted rapidly at the imide carbonyl of **32**.[35] The major product contained two units of ethyl acetate.



30 **31** **32**

Kinetic Results. Inhibition of Penicillopepsin by [LySta] and [OrnSta] Analogues. The kinetic data in Table I were obtained by the methods described for inhibition of pepsin[11,12] and penicillopepsin and *R. chinensis* pepsin.[28] All compounds listed have the 3*S*,4*S* configurations unless stated otherwise, and all amines are the acetate salts. Compound **29** was tested as a 1:1 mixture of 3*S*,3*R* diastereomers. The K_i value obtained for the mixture (2.3 nM) was divided in half to determine the K_i value of the 3*S* compound. NMR data (not shown) indicated both diastereomers were equally present.

Table I clearly shows a major difference between penicillopepsin and porcine pepsin. Replacement of Sta with [LySta] in compound **3** produces compound **16a**, which is a 10–100-fold better inhibitor of penicillopepsin than compound **3** but at least 100 times worse as an inhibitor of porcine pepsin. Protection of the 8-amino group in this peptide, e.g., analogues **15a** and the *N*-acetyl derivative **33**, produces substantially worse inhibitors of penicillopepsin. Extension of the peptide chain of **16a** to incorporate a P_2' phenylalanine as in **19** produces a remarkably potent inhibitor of penicillopepsin, one that binds about 2 times tighter to penicillopepsin than pepstatin (K_i pepstatin = 0.15 nM for penicillopepsin[15]).

When the side chain of [LySta] is shortened by one carbon to the [OrnSta] derivative **29**, inhibition is only slightly affected. Compound **29** is essentially equipotent to **16a** as an inhibitor of penicillopepsin or pepsin.

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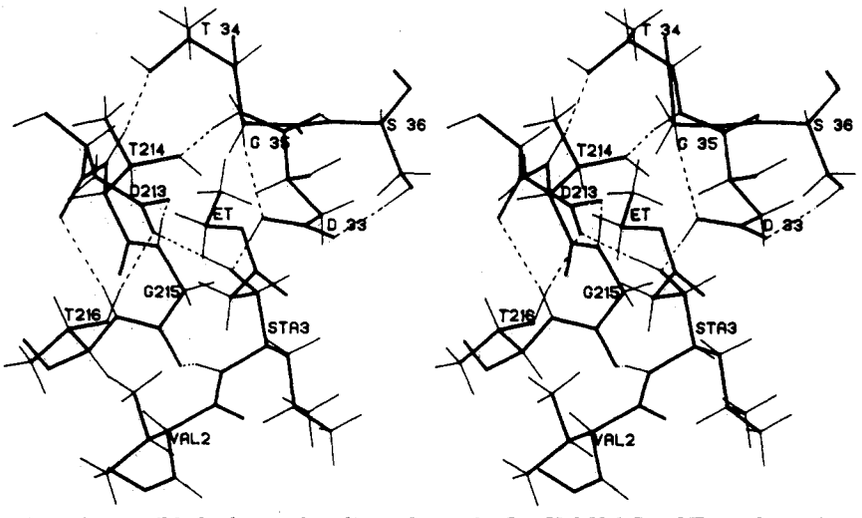

Figure 1. Stereoscopic view of a possible hydrogen-bonding scheme for Iva-Val-Val-Sta-OEt at the active site of penicillopepsin.[29] Only the P_1 (Sta) and P_2 (Val) residues are shown.

The 3*S* diastereomer of the [LySta] peptides is at least 100 times stronger inhibitor of penicillopepsin than the 3*R* diastereomer **16b**.

Discussion

The fact that penicillopepsin rapidly hydrolyzes peptide and protein substrates at Lys-Xxx bonds implies a specific interaction between this amino acid and an acidic enzyme residue. This idea was supported by extensive kinetic studies that showed that the kinetic parameters K_m and K_{cat} for the synthetic substrate **6** are strongly pH dependent with the maximum binding consistent with a specific interaction between the ϵ amine of substrate lysine and a carboxyl group on the enzyme.[29] Initially, it was thought that the acidic residue might be Asp-38.[36] This proposal was based on early computer graphic modeling of the native penicillopepsin without peptide-like inhibitors bound in the active site of the enzyme. However, when the crystal structure of Iva-Val-Val-Sta-OEt (**3**) was obtained, a major conformational change in the enzyme was discovered in which the "flap" region of the enzyme closed on the tripeptide inhibitor **3**.[25] This conformational change prevented access of the P_1 side chain to Asp-38 so that this initially postulated electrostatic interaction was not possible.

The availability of the crystal structure for the Iva-Val-Val-Sta-OEt–penicillopepsin complex led to a second search for anionic groups that could interact via an electrostatic bond with the ϵ amine group of substrate lysine. Computerized molecular modeling was based on the X-ray crystal structure with the assumption that the torsion angles of substrate lysine would adopt the same side-chain torsion angles found for the isobutyl side chain of statine in the X-ray crystal structure of Iva-Val-Val-Sta-OEt (**3**) bound to penicillopepsin (Figure 1). Hofmann et al. proposed that the carboxyl–carboxylate pair of Glu-16 and Asp-115 with a net negative charge of −1 could interact with the ϵ amino group of substrate lysine via a solvent-separated electrostatic interaction.[29] A model of this interaction is shown in Figure 2A. Additional phylogenetic considerations suggested that this interaction would not occur in pepsin or other mammalian aspartic proteases that do not activate trypsinogen.

The [LySta]-containing inhibitor **16a** was synthesized to test this hypothesis. If lysine substrates are stabilized by the solvent-separated electrostatic interaction and if substrate **6** and inhibitor **3** bind to penicillopepsin in the same way with respect to the enzyme interaction at P_1, then inhibitors with a lysine side chain in P_1 should bind more tightly to penicillopepsin than the reference tripeptide inhibitor **3** by a factor of about 2–3 kcal.[37]

The kinetic results in Table I clearly show that the [LySta] analogues bind more tightly to penicillopepsin than the reference inhibitor **3**. Compound **16a** is about 10–100 times more potent an inhibitor of penicillopepsin than **3**. This enhanced binding is consistent with about a 1–3-kcal increase in binding energy between enzyme and inhibitor and is in the range expected for the addition of an electrostatic interaction onto an otherwise hydrophobic interaction between the lysine side chain and the enyme S pocket. Compound **19a** is about 20-fold better than the corresponding statine-containing compound **34**. The N^{ϵ}-protected compounds **15a** and **33** indicate that the enhanced binding is dependent upon the free amino group.

The data in Table I also show that enhanced binding occurs only with penicillopepsin. The binding of Sta and [LySta] inhibitors to *Rhizopus* pepsin is little changed. However with porcine pepsin, the binding is much weaker; the K_i value of **16a** is greater than 1 μM whereas the parent Sta compound **3** has a K_i value of 10 nM. This remarkable loss in binding indicates that the free amine is actually excluded from the active site in porcine pepsin.

In order to probe the structural requirements for the postulated electrostatic interaction between the 8-amino group on [LySta] and the Glu-16 and Asp-115 carboxylate pair, we synthesized the lower analogue of [LySta] that contains the amino propyl side chain found in ornithine. It was expected that retraction of the positive charge on the amino group into a hydrophobic S_1 pocket would severely destabilize the enzyme–inhibitor interaction. While separation of electrostatic bonds is not strongly distance dependent, immersion of a positive charge into the hydrophobic pocket should be disfavored.

We were therefore surprised to find that the [OrnSta] analogue **29** is not significantly weaker as an inhibitor of penicillopepsin than the [LySta] analogue **16a**. Thus, the kinetic data suggest that either the Glu-16 and Asp-15 pair did not stabilize the [LySta] peptide **16a** binding or that some other enzyme group had emerged to stabilize the positive charge on the 7-amino group of [OrnSta] in **29**.

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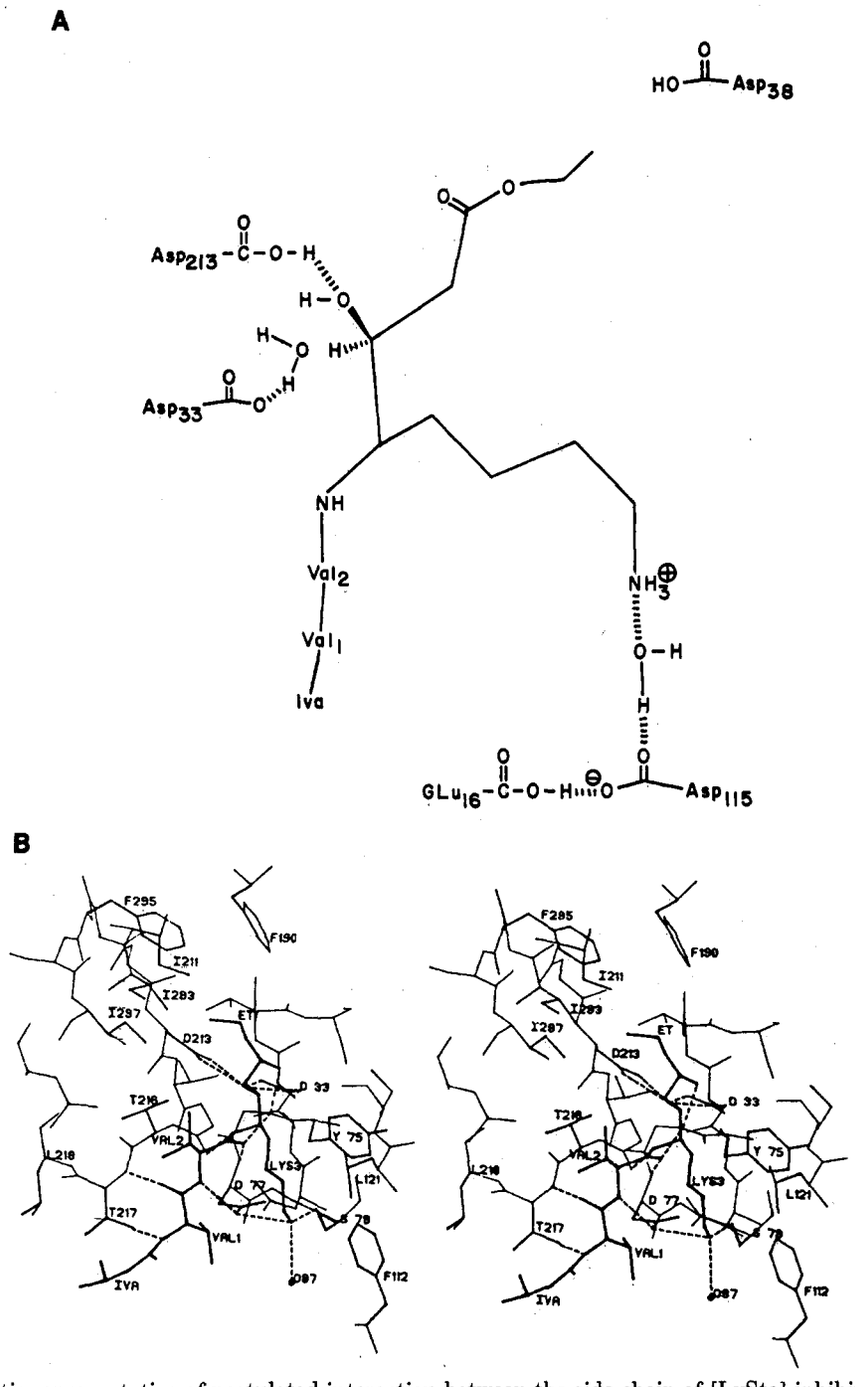

Figure 2. (A) Schematic representative of postulated interaction between the side chain of [LySta] inhibitor **16a** with Glu-16 and Asp-115 in the active site of penicillopepsin.[29] This model assumed side-chain torsion angles in the [LySta] inhibitor **16a** were the same as for the Sta side chain in Figure 1. (B) Observed bound conformation of Iva-Val-Val-[LySta]OEt in the binding cleft of penicillopepsin shown in stereo.[32] The 8-amino group of the P_1 lysyl side chain forms hydrogen bonds to $O^{\delta 1}$ of Asp-77, O^{γ} of Ser-79, and to water O87 and not to Glu-16 and Asp-115. van der Waals interactions are from this side chain to Tyr-75, Leu-121, and Phe-112. Reprinted with permission from ref 32. Copyright deGruyter, 1985.

Recently, James et al. have prepared crystals of Iva-Val-Val-[LySta]-OEt (**16a**) complexed with penicillopepsin suitable for X-ray crystallography.[32] The X-ray structure shows (Figure 2B) that the electrostatic interaction between enzyme and inhibitor arises from a tight ion pair between the 8-amino group of the [LySta] side chain and aspartic acid-77 (not Glu-16, Asp-115) on the enzyme. The new interaction with Asp-77 is made possible by small rotations about the [LySta] side chain torsion angles away from the torsion angles of the statine isobutyl side chain in Figure 1. These results illustrate the hazards of designing enzyme inhibitors from static representations of X-ray crystal structures in that only slight modifications of either enzyme or inhibitor conformation can provide favorable alternate binding modes. A further consequence of the structure in Figure 2B is that the shorter side-chain analogues such as the [OrnSta] derivative **29** would not be expected to be significantly weaker inhibitors of penicillopepsin since the short distance between the inhibitor backbone and Asp-77 of the enzyme can accommodate an ornithine side chain as well as the lysine side chain. Instead, one might predict that longer side chain analogues, such as found in homolysine side chains, might be the weaker penicillopepsin inhibitors.

The structural basis for the weak binding of the [LySta] peptide to porcine pepsin has not been satisfactorily ex-

plained. The binding of **16a** to pepsin is not enhanced by incorporation of the lysine side chain, which is consistent with substrate specificity data. What is surprising is that the binding of **16a** to porcine pepsin is weakened over 100 times relative to the tripeptide **3**. Porcine pepsin contains threonine, a β-branched amino acid, at position 77, whereas both penicillopepsin and *R. chinensis* pepsin have aspartic acid at the corresponding position.[6] Aspartic acid is not β-branched so that steric factors may prevent the positively charged and hydrated amines in **16a** and **29** from penetrating the S_1 pocket. However the energetics of binding may be very subtle because neither basic inhibitor **16a** nor **29** is an improved inhibitor (relative to **3** and **34**, respectively) of *Rhizopus* pepsin, which contains Asp-79 (homologous to Asp-77 in penicillopepsin). These subtle differences between aspartic proteinases are exciting and may ultimately lead to a better understanding of the structural basis of enzyme specificity. It is likely that a detailed understanding of enzyme–inhibitor interactions will emerge only when many more crystal structures have been solved and when the free energies of the enzyme–inhibitor interactions inclusive of solvent have been characterized.

Experimental Section

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 with 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). The following TLC solvent systems were used: (A) 10% methanol in chloroform (v/v), (B) 5% methanol in chloroform (v/v), (C) 50% ethyl acetate in hexane (v/v), (D) 20% ethyl acetate in toluene (v/v), (E) 75% ethyl ether in hexane (v/v), (F) 66% ethyl ether in hexane (v/v), (G) 40% ethyl acetate in methylene chloride (v/v), (H) 50% ethyl ether in hexane (v/v), (I) 4:1:1 1-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 by using synthetic heptapeptide Phe-Gly-His-Phe(NO_2)-Phe-Ala-Phe-OMe for porcine pepsin assays as described[12] and Ac-Ala-Ala-Lys-Phe(NO_2)-Ala-Ala-NH_2[28] for the penicillopepsin and *R. chinensis* pepsin assays, which were carried out at pH 5.5 in 0.02 M sodium acetate buffer and pH 4.0 in 0.02 M formate buffer, respectively, as described by Hofmann and Hodges.[28] Kinetics were carried out on a Gilford Model 250 spectrophotometer connected to a Gilford 6051 recorder. Inhibition constants (K_i) were calculated from IC_{50} values determined from duplicate sets of 8–10 data points as described previously for porcine pepsin.[13]

General Synthetic Procedures. General Procedure A. Removal of the *tert*-Butoxycarbonyl Group. The Boc peptide (1 equiv) in a solution of 4 N HCl in dioxane (10 equiv of HCl) was stirred at room temperature and the reaction monitored by TLC. Complete reaction was generally achieved in about 30 min. Excess reagent was removed under reduced pressure to give a solid residue. The residue was reevaporated 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.[38] The amino hydrochloride (1.0 mmol) was dissolved in methylene chloride (5 mL) and neutralized at 0 °C 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 °C for 2–4 h 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 $NaHCO_3$, and 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.[39,40] Boc-amino acid (2 equiv) and DCC (1 equiv) 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 of DMF or methylene chloride) was cooled on an ice bath and neutralized with *N*-methylmorpholine (1 equiv). After addition of the symmetrical anhydride (2 equiv), stirring was continued at 0 °C for 2–4 h 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 $NaHCO_3$, and saturated NaCl, and dried ($MgSO_4$). The peptide was purified by silica gel chromatography when necessary and crystallized.

General Procedure E. 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) and the reaction was monitored by TLC. When the 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 F. Removal of Benzyloxycarbonyl Group via Catalytic Hydrogenation. The *N*-Cbz-protected compound was dissolved in a minimum amount of dry DMF that contained 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 h. After this time the mixture was filtered over Celite and washed with DMF. The filtrate was then concentrated in vacuo. Pure product was precipitated by using ethyl ether and collected by filtration.

***N*^{4}-(*tert*-Butyloxycarbonyl)-*N*^{8}-(benzyloxycarbonyl)-4-(*S*),8-diamino-3(*R*,*S*)-hydroxyoctanoic Acid Ethyl Ester ([LySta], 10ab).** To 6 mL of dry tetrahydrofuran cooled by dry ice/CCl_4 was added diisopropylamine (20.4 mmol) under a nitrogen atmosphere, followed by a 1.6 N solution of *n*-butyllithium in hexane (20.4 mmol). After 1 h the bath temperature was lowered to −78 °C and dry ethyl acetate (20.4 mmol) was added via syringe and stirred for 15 min. α-Boc-ε-Cbz-lysinal[31] (4.96 g, 13.6 mmol) 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 with 20% ethyl acetate in toluene. Pure compound was obtained as a mixture of 3*R* and 3*S* diastereomers in 62% yield: TLC R_f (D) 0.08, R_f (B) 0.41. Anal. ($C_{23}H_{36}N_2O_7$) C, H, N.

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N^4-(*tert*-Butyloxycarbonyl)-N^8-(benzyloxycarbonyl)-*O*-[(2,2,2-trichloroethoxy)carbonyl]-4(*S*),8-diamino-3(*R*,*S*)-hydroxyoctanoic Acid Ethyl Ester (12ab). Compound **10ab** (2.6 g, 5.74 mmol) was dissolved in pyridine (40 mL). (Dimethylamino)pyridine (0.57 mmol) and trichloroethyl chloroformate (11.48 mmol) 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 3*R* and 3*S* isomers. The 3*S* isomer **12a** was obtained as an oil in about 43% yield: TLC R_f (H) 0.36; NMR ($CDCl_3$) δ 1.1–1.65 (m, 18 H, includes triplet δ 1.23, J = 7.5 Hz), 2.71 (d, J = 7 Hz, 2 H), 3.05–3.20 (m, 2 H), 3.7–4.0 (m, 1 H), 4.15 (q, J = 7.5 Hz, 2 H), 4.50–4.95 (m, 4 H), 5.0–5.40 (m, 3 H, includes a singlet δ 5.09), 7.01 (s, 5 H). The 3*R* isomer **12b** was isolated as an oil in about 42% yield: TLC R_f (H) 0.32; NMR ($CDCl_3$) δ 1.15–1.65 (m, 18 H, includes triplet δ 1.27, J = 7.5 Hz), 2.67 (d, J = 7.0 Hz, 2 H), 3.0–3.30 (m, 2 H), 3.70–4.35 (m, 4 H, includes quartet δ 4.22, J = 7.5 Hz), 4.55 (br, J = 9 Hz, 1 H), 4.81 (s, 2 H), 5.0–5.30 (m, 3 H, includes singlet δ 5.13), 7.40 (s, 5 H).

N^4-(*tert*-Butyloxycarbonyl)-N^8-(benzyloxycarbonyl)-4(*S*),8-diamino-3(*S*)-hydroxyoctanoic Acid Ethyl Ester ((3*S*,4*S*)-[LySta], 10a). Compound **12a** (1.4 g, 2.23 mmol) was dissolved in a 1:1 mixture of DMF/acetic acid (5 mL). Metallic cadmium powder (67 mmol) was added to the solution and the mixture was stirred for 7 h 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) 0.54, R_f (C) 0.17; $[\alpha]^{24}_D$ –7.8° (*c* 0.60, EtOH); NMR ($CDCl_3$) δ 1.17–1.65 (m, 18 H, includes triplet δ 1.27, J = 7.5 Hz), 2.51 (dd, J = 7 Hz, 3 Hz, 2 H), 3.0–3.30 (m, 2 H), 3.30–3.75 (m, 2 H), 3.90–4.35 (m, 3 H, includes quartet δ 4.20, J = 7.5 Hz), 4.50–5.15 (m, 4 H, includes singlet δ 5.13), 7.39 (s, 5 H). Anal. ($C_{23}H_{36}N_2O_7$) C, H, N.

N^4-(*tert*-Butyloxycarbonyl)-N^8-(benzyloxycarbonyl)-4(*S*),8-diamino-3(*R*)-hydroxyoctanoic Acid Ethyl Ester (10b). The title compound was prepared from compound **12b** (1.5 g, 2.39 mmol) by using a procedure analogous to that for the preparation of **10a**. 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 (C) 0.16; $[\alpha]^{24}_D$ –16.9° (*c* 0.60, EtOH); NMR ($CDCl_3$) δ 1.15–1.70 (m, 18 H, includes triplet δ 1.29, J = 7.5 Hz), 2.49 (dd, J = 2 Hz, 6 Hz, 2 H), 3.10–3.35 (m, 2 H), 3.35–3.80 (br m, 2 H), 3.85–4.35 (m, 3 H, includes quartet δ 4.21, J = 7.5 Hz), 4.45–5.05 (m, 2 H), 5.13 (s, 2 H), 7.40 (s, 5 H). Anal. ($C_{23}H_{36}N_2O_7$) C, H, N.

***N*-(*tert*-Butyloxycarbonyl)-L-valyl-N^8-(benzyloxycarbonyl)-4(*S*),8-diamino-3(*S*)-hydroxyoctanoic Acid Ethyl Ester (13a).** Compound **10a** (200 mg, 44 mmol) was deprotected according to general procedure A. The resulting hydrochloride was coupled with Boc-valine anhydride (0.88 mmol) according to general procedure D with methylene chloride as solvent. Pure product was obtained by crystallization in about 70% yield (two crops): mp 116–117 °C (ethyl acetate/hexane); TLF R_f (B) 0.37; $[\alpha]^{24}_D$ –35.8° (*c* 0.067, MeOH); NMR ($CDCl_3$) δ 0.75–1.0 (m, 6 H), 1.10–1.6 (m, 18 H, includes triplet δ 1.25, J = 7 Hz), 2.0–2.30 (m, 1 H), 2.42–2.55 (m, 2 H), 3.0–3.30 (m, 2 H), 3.50 (m, 1 H), 3.60–4.30 (m, 5 H, includes quartet δ 4.30, J = 7 Hz), 4.80–5.20 (m, 3 H, includes singlet δ 5.15, 2 H), 6.4 (d, J = 10.5 Hz, 1 H), 7.25–7.45 (m, 5 H). Anal. ($C_{28}H_{45}N_3O_8$) C, H, N.

***N*-(*tert*-Butyloxycarbonyl)-L-valyl-N^8-(benzyloxycarbonyl)-4(*S*),8-diamino-3(*R*)-hydroxyoctanoyl Ethyl Ester (13b).** Compound **10b** (160 mg, 0.353 mmol) was deprotected according to general procedure A. The resulting hydrochloride was coupled with Boc-valine anhydride (0.70 mmol) according to general procedure D with methylene chloride as solvent. Crystallization yielded pure product in about 65% yield: mp 131–132 °C (ethyl acetate/hexane); TLC R_f (B) 0.33; $[\alpha]^{24}_D$ –27.5° (*c* 0.12, MeOH); NMR ($CDCl_3$) δ 0.75–1.05 (m, 6 H), 1.15–1.70 (m, 18 H, includes triplet δ 1.27, J = 7 Hz), 1.90–2.25 (m, 1 H), 2.47 (d, J = 6 Hz, 2 H), 3.02–3.30 (m, 2 H), 3.42–3.58 (m, 1 H), 3.65–4.32 (m, 5 H, includes quartet δ 4.18, J = 7 Hz), 4.90–5.25 (m, 4 H, includes singlet δ 5.11), 6.33 (br d, J = 8 Hz, 1 H), 7.46 (s, 5 H). Anal. ($C_{28}H_{45}N_3O_8$) C, H, N.

***N*-(*tert*-Butyloxycarbonyl)-L-valyl-L-valyl-N^8-(benzyloxycarbonyl)-4(*S*),8-diamino-3(*S*)-hydroxyoctanoic Acid Ethyl Ester (14a).** Boc compound **13a** (125 mg, 0.23 mmol) was deprotected according to general procedure A. The resulting hydrochloride was then coupled to Boc-valine anhydride (0.46 mmol) according to general procedure D with methylene chloride as solvent. Crystallization afforded product in about 92% yield: mp 169–171 °C (ethyl acetate/hexane); TLC R_f (A) 0.60, R_f (B) 0.33; $[\alpha]^{24}_D$ –42.8° (*c* 0.105, MeOH); NMR ($CDCl_3$) δ 0.80–1.10 (m, 12 H), 1.15–1.75 (m, 18 H), includes triplet δ 1.25, J = 7 Hz), 1.85–2.32 (m, 2 H), 2.47 (d, J = 6 Hz, 2 H), 3.0–3.30 (m, 2 H), 3.42–3.70 (m, 1 H), 3.75–4.39 (m, 6 H), 5.05–5.38 (m, 4 H, includes singlet δ 5.12), 6.48–6.72 (m, 2 H), 7.23–7.45 (m, 5 H). Anal. ($C_{33}H_{54}N_4O_9$) C, H, N.

***N*-(*tert*-Butloxycarbonyl)-L-valyl-L-valyl-N^8-(benzyloxycarbonyl)-4(*S*),8-diamino-3(*R*)-hydroxyoctanoic Acid Ethyl Ester (14b).** Boc compound **13b** (30 mg, 0.054 mmol) was deprotected according to general procedure A. The resulting hydrochloride was coupled to Boc-valine anhydride (0.10 mmol) according to general procedure D with methylene chloride as solvent. After workup 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) 0.27; $[\alpha]^{24}_D$ –34.5° (*c* 0.087, MeOH); NMR ($CDCl_3$) δ 0.75–1.10 (m, 12 H), 1.10–2.10 (m, 20 H), 2.44 (d, J = 6 Hz, 2 H), 3.05–3.30 (m, 2 H), 3.35–3.70 (m, 2 H), 3.80–4.30 (m, 5 H), 4.90–5.30 (m, 4 H, includes singlet δ 5.12), 6.41–6.65 (m, 2 H), 7.23–7.40 (m, 5 H). Anal. ($C_{33}H_{54}N_4O_9$) C, H, N.

***N*-(*tert*-Butyloxycarbonyl)-L-valyl-L-valyl-N^8-(benzyloxycarbonyl)-4(*S*),8-diamino-3(*S*)-hydroxyoctanoic Acid (17a).** Compound **14a** (70 mg, 0.108 mmol) was saponified to the free acid according to general procedure E. The product was obtained in about 89% yield and was used in the next reaction without further purification.

***N*-(*tert*-Butyloxycarbonyl)-L-valyl-L-valyl-N^8-(benzyloxycarbonyl)-4(*S*),8-diamino-3(*S*)-hydroxyoctanoyl-L-phenylalanine Methyl Ester (18a).** Compound **17** (60 mg, 0.096 mmol) was coupled to phenylalanine methyl ester (0.12 mmol) according to general procedure B. The resulting material was chromatographed on a 10-g silica gel column eluted 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) 0.40; $[\alpha]^{24}_D$ –37.2° (*c* 0.105, MeOH); NMR ($CDCl_3$) δ 0.75–1.10 (m, 2 H), 1.10–1.85 (m, 18 H), 1.80–2.25 (m, 2 H), 2.92–3.30 (m, 4 H), 3.35–4.45 (m, 8 H, includes singlet δ 3.73), 4.60–5.20 (m, 4 H, includes singlet δ 5.1), 6.55 (m, 1 H), 6.82–7.20 (m, 12 H). Anal. ($C_{41}H_{61}N_5O_{10}\cdot H_2O$) C, H, N.

***N*-Isovaleryl-L-valyl-L-valyl-N^8-(benzyloxycarbonyl)-4(*S*),8-diamino-3(*S*)-hydroxyoctanoic Acid Ethyl Ester (15).** Boc compound 14 (30 mg, 0.046 mmol) was deprotected according to general procedure A. The resulting hydrochloride was coupled to isovaleric anhydride (0.10 mmol) as described in general procedure D with DMF as the solvent. Precipitation from ethyl ether gave the title compound in about 90% yield: mp 216–218 °C (ethyl ether); TLC R_f (B) 0.31; $[\alpha]^{24}_D$ –69.7° (*c* 0.076, MeOH); NMR (MeOH-d_4) δ 0.78–1.15 (m, 18 H), 1.17–1.78 (m, 9 H, includes triplet δ 1.23, J = 7 Hz), 1.87–2.27 (m, 5 H), 2.40 (d, J = 7 Hz, 2 H), 3.08 (m, 2 H), 3.51–4.27 (m, 6 H), 5.04 (s, 2 H) 7.28 (s, 5 H). Anal. ($C_{33}H_{54}N_4O_8\cdot H_2O$) C, H, N.

***N*-Isovaleryl-L-valyl-L-valyl-N^8-(benzyloxycarbonyl)-4(*S*),8-diamino-3(*R*)-hydroxyoctanoic Acid Ethyl Ester (15b).** Boc compound **14b** (33 mg, 0.50 mmol) was deprotected according to general procedure A. The hydrochloride was coupled to isovaleric anhydride (0.10 mmol) as described for general procedure D with DMF as solvent. Precipitation from ethyl ether gave product in about 70% yield: mp 217–219 °C (ethyl ether); TLC R_f (B) 0.25; $[\alpha]^{24}_D$ –42.5° (*c* 0.073, MeOH); NMR (MeOH-d_4) δ 0.79–1.17 (m, 18 H), 1.19–1.77 (m, 9 H), 1.88–2.25 (m, 5 H), 2.38

(d, J = 7 Hz, 2 H), 3.10 (m, 2 H), 3.50 4.27 (m, 6 H), 5.05 (s, 2 H), 7.29 (s, 5 H). Anal. ($C_{33}H_{54}N_4O_8 \cdot H_2O$) C, H, N.

N-Isovaleryl-L-valyl-L-valyl-N^8-(benzyloxycarbonyl)-4(S),8-diamino-3(S)-hydroxyoctanoyl-L-phenylalanine Methyl Ester (18). Boc compound **17** (30 mg, 0.0403 mmol) was deprotected by using general procedure A. The hydrochloride was reacted with isovaleric anhydride (0.08 mmol) according to general procedure D with DMF as solvent. Precipitation from ethyl ether gave product in 90% yield: mp >250 °C; TLC R_f (B) 0.18; $[\alpha]^{24}_D$ −250° (c 0.12, MeOH); NMR MeOH-d_4/acetic acid-d_4, 1/1, v/v) δ 0.67–1.10 (m, 18 H), 1.20–1.78 (m, 6 H), 1.80–2.22 (m, 5 H), 2.33 (d, J = 6 Hz, 2 H), 2.93–3.33 (m, 4 H), 3.55–3.78 (m, 4 H, includes singlet δ 3.67), 3.78–4.33 (m, 3 H) , 4.71 (t, J = 8 Hz, 1 H), 5.07 (s, 2 H), 7.0–7.44 (m, 10 H). Anal. ($C_{41}H_{61}N_5O_9 \cdot H_2O$) C, H, N.

N-Isovaleryl-L-valyl-L-valyl-4(S),8-diamino-3(S)-hydroxyoctanoic Acid Ethyl Ester Acetate (16a). The Cbz group of compound **15a** (19 mg, 0.03 mmol) was removed as described in general procedure F. Precipitation from ethyl ether gave product in about 77% yield: mp 228–230 °C; TLC R_f (F) 0.55; $[\alpha]^{24}_D$ −75° (c 0.08, MeOH); NMR (Me_2SO-d_6) δ 0.77–1.15 (m, 18 H), 1.34–1.91 (m, 9 H), 2.10–2.33 (m, 5 H), 2.48 (m, 2 H), 3.0–3.31 (m, 2 H), 3.67–3.85 (m, 4 H), 4.05–4.45 (m, 3 H). Anal. ($C_{27}H_{52}N_4O_8 \cdot H_2O$) C, H, N.

N-Isovaleryl-L-valyl-L-valyl-4(S),8-diamino-3(R)-hydroxyoctanoic Acid Ethyl Ester Acetate (16b). Compound **15b** (19 mg, 0.03 mmol) was subjected to conditions described in general procedure H. Precipitation from ethyl ether gave product in 80% yield: mp 220 °C; TLC R_f (I) 0.53; $[\alpha]^{24}_D$ −48° (c 0.10, MeOH); NMR (Me_2SO-d_6) δ 0.78–1.13 (m, 18 H), 1.35–1.89 (m, 9 H), 1.92–2.26 (m, 5 H), 2.38 (m, 2 H), 3.11 (m, 2 H), 3.52–4.33 (m, 6 H). Anal. ($C_{27}H_{52}N_4O_8 \cdot 1.5H_2O$) C, H, N.

N-Isovaleryl-L-valyl-L-valyl-4(S),8-diamino-3(S)-hydroxyoctanoyl-L-phenylalanine Methyl Ester Acetate (19). Compound **18** (20 mg, 0.027 mmol) was treated as described in general procedure H. Ethyl ether precipitation afforded product in 83% yield: mp 200 °C; TLC R_f (I) 0.61; $[\alpha]^{24}_D$ −270° (c 0.10, MeOH); NMR (CD_3CO_2D) δ 0.70–1.10 (m, 18 H), 1.33–1.89 (m, 6 H), 2.0–2.33 (m, 5 H), 2.49 (m, 2 H), 2.98–3.33 (m, 4 H), 3.67–3.82 (m, 4 H, includes singlet at δ 3.73), 4.05 (m, 1 H), 4.39 (m, 2 H, includes triplet, J = 8 Hz), 4.84 (m, 1 H), 7.22 (s, 5 H). Anal. ($C_{35}H_{59}N_5O_9 \cdot H_2O$) C, H, N.

N-Isovaleryl-L-valyl-L-valyl-N^8-acetyl-4(S),8-diamino-3-(S)-hydroxyoctanoic Acid Ethyl Ester (33). Compound **15a** (30 mg, 0.083 mmol) was deprotected according to general procedure A. The resulting hydrochloride was acylated with acetic anhydride by using general procedure D. After workup 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) 0.42; $[\alpha]^{24}_D$ −71.0° (c 0.10, MeOH); NMR (MeOH-d_4) δ 0.78–1.11 (m, 18 H), 1.30 (t, J = 7 Hz, 3 H), 1.44 (m, 9 H), 2.00–2.23 (m, 5 H, includes singlet δ 2.18), 2.53 (d, J = 7.5 Hz, 2 H), 3.11 (m, 2 H), 4.00–4.27 (m, 6 H). Anal. ($C_{27}H_{50}N_4O_7$) C, H, N.

N^2-(*tert*-Butyloxycarbonyl)-N^5-(benzyloxycarbonyl)-2-(S),5-diaminopentanol (21). α-Boc-δ-Cbz-ornithine methyl ester (**20**; 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 °C and a 2 N solution of lithium borohydride in tetrahydrofuran (9 mL, 18 mmol) was slowly added dropwise. The reaction was monitored by TLC. After 2 h 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 and dried ($MgSO_4$), and the solvent removed in vacuo to yield 3.1 g of a clear oil (95%): TLC R_f (A) 0.49. The product was used in the next step without further purification.

N^2-(*tert*-Butyloxycarbonyl)-2(S),5-diaminopentanol (22). Compound **21** (3.1 g, 8.8 mmol) 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 mmol) was added, followed by 10% palladium on carbon (300 mg). The reaction progress was monitored by TLC. After 3 h the mixtures 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) 0.08; NMR ($CDCl_3$) δ 1.33–1.78 (m, 13 H), 2.74 (m, 2 H), 3.38–3.71 (m, 3 H), 4.55–4.86 (m, 3 H), 5.55 (br m, 1 H).

N^2-(*tert*-Butyloxycarbonyl)-N^5-benzyl-2(S),5-diaminopentanol (24). Free amine **22** (530 mg, 2.41 mmol) was dissolved in methylene chloride (10 mL). Triethylamine (242 μL, 2.41 mmol) and benzaldehyde (245 μL, 2.41 mmol) followed by $MgSO_4$ (1 g) were added. The mixture was stirred at room temperature for 4 h 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 mmol) was added to the solution in small portions over a period of 0.5 h. The mixture was stirred for 3 h 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) 0.08; NMR ($CDCl_3$) δ 1.33–1.70 (m, 13 H, includes singlet δ 1.40), 2.62 (m, 2 H), 3.40–3.67 (m, 3 H), 3.70–4.00 (m, 4 H), 5.43 (br m, 1 H), 7.29 (s, 5 H).

N^2-(*tert*-Butyloxycarbonyl)-N^5-benzyl-N^5-(benzyloxycarbonyl)-2(S),5-diaminopentanol (25). Compound **24** (500 mg, 1.62 mmol) was dissolved in a 1:1 mixture of dioxane/H_2O (10 mL) and chilled to 0 °C. NaOH (1 N, 1.62 mL) was added followed by a dropwise addition of benzyl chloroformate (3.24 mmol) over a period of 3 h. The pH was carefully maintained at pH 10 with 1 N NaOH. The mixture was stirred at room temperature for 3 h 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 (C) 0.21; NMR ($CDCl_3$) δ 1.14–1.89 (m, 13 H), 3.25 (m, 2 H), 3.30–3.73 (m, 3 H), 4.03 (m, 1 H), 4.43 (s, 2 H), 4.82–5.21 (m, 3 H, includes singlet δ 5.14), 7.0–7.40 (m, 10 H). Anal. ($C_{25}H_{34}N_2O_5 \cdot 0.75H_2O$) C, H, N.

N^2-(*tert*-Butyloxycarbonyl)-N^5-benzyl-N^5-(benzyloxycarbonyl)-2(S),5-diaminopentanol (26). Alcohol **25** (470 mg, 1.06 mmol) was dissolved in dry Me_2SO (1.5 mL). Trimethylamine (445 μL, 3.2 mmol) was added and the mixture was cooled to 0 °C. Pyridine–sulfur trioxide complex[31] (510 mg, 3.2 mmol) in Me_2SO (1.5 mL) was added and the mixture was stirred at room temperature for 0.5–0.75 h. 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) 0.39, R_f (C) 0.50.

N^4-(*tert*-Butyloxycarbonyl)-N^7-benzyl-N^7-(benzyloxycarbonyl)-4(S),7-diamino-3(R,S)-hydroxyheptanoic Acid Ethyl Ester (27ab). To 1 mL of dry tetrahydrofuran cooled by dry ice/CCl_4 was added diisopropylamine (1.6 mmol) under a nitrogen atmosphere, followed by a 1.6 N solution of n-butyllithium in hexane (1.6 mmol). After 1 h, the bath temperature was lowered to −78 °C and dry ethyl acetate (1.6 mmol) was added via syringe. The solution was stirred for 15 min. Aldehyde **26** (1.06 mmol) 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 and 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 **25**): TLC R_f (B) 0.53, R_f (C) 0.20; NMR ($CDCl_3$) δ 1.11–1.74 (m, 16 H, includes triplet δ 1.27, J = 7.5 Hz), 2.44 (m, 2 H), 3.12–3.80 (m, 4 H), 3.81–4.32 (m, 3 H), includes quartet δ 4.17, J = 7.5 Hz), 4.47 (s, 2 H), 4.90 (br s, 1 H), 5.15 (s, 2 H), 7.12–7.50 (m, 10 H).

Anal. ($C_{29}H_{40}N_2O_7$) C, H, N.

***N*-(*tert*-Butyloxycarbonyl-L-valyl-L-valyl-N^7-benzyl-N^7-(benzyloxycarbonyl)-4(*S*),7-diamino-3(*R*,*S*)-hydroxyheptanoic Acid Ethyl Ester.** Compound **27ab** (100 mg, 0.19 mmol) was deprotected according to general procedure A. The resulting hydrochloride was coupled with Boc-valine anhydride (0.4 mmol) according to general procedure D with methylene chloride as solvent. The crude product obtained was chromatographed over 10 g of silica gel eluting with 40% ethyl acetate in methylene chloride. Product was isolated as an oil in about 80% yield: TLC R_f (B) 0.50, R_f (G) 0.25; NMR ($CDCl_3$) δ 0.79–1.18 (m, 6 H), 1.23 (t, J = 7.5 Hz, 3 H), 1.30–1.71 (m, 13 H), 2.05 (m, 1 H), 2.39 (m, 2 H), 3.11–3.56 (m, 3 H), 3.62–4.30 (m, 5 H, includes quartet δ 4.13, J = 7.5 Hz), 4.45 (s, 2 H), 5.0 (m, 1 H), 5.14 (s, 2 H), 6.39 (br m, 1 H), 7.08–7.45 (m, 10 H).

***N*-(*tert*-Butyloxycarbonyl)-L-valyl-L-valyl-N^7-benzyl-N^7-(benzyloxycarbonyl)-4(*S*),7-diamino-3(*R*,*S*)-hydroxyheptanoic Acid Ethyl Ester.** Boc-Val-[OrnSta]-OEt (66 mg, 0.105 mmol) was deprotected according to general procedure A. The resulting hydrochloride was coupled with Boc-valine anhydride (0.2 mmol) according to general procedure D with 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 (G) 0.13; NMR ($CDCl_3$) δ 0.75–1.11 (m, 12 H), 1.15–1.62 (m, 16 H, includes triplet δ 1.26, J = 7 Hz), 1.83–2.25 (m, 2 H), 2.43 (m, 2 H), 3.22 (m, 2 H), 3.60–4.40 (m, 7 H, includes quartet δ 4.18, J = 7 Hz), 4.45 (s, 2 H), 5.18 (s, 2 H), 5.38 (d, J = 8 Hz, 1 H), 6.65–7.10 (m, 2 H), 7.10–7.52 (m, 10 H).

***N*-Isovaleryl-L-valyl-L-valyl-N^7-benzyl-N^7-(benzyloxycarbonyl)-4(*S*),7-diamino-3(*R*,*S*)-hydroxyheptanoic Acid Ethyl Ester (28ab).** Boc-Val-Val-[OrnSta]-OEt (37 mg, 0.051 mmol) was deprotected according to general procedure A. The resulting hydrochloride was coupled to isovaleric anhydride according to general procedure D with 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) 0.61; NMR (MeOH-d_4) δ 0.77–1.13 (m, 18 H), 1.16–1.64 (m, 7 H, includes triplet δ 1.28, J = 7 Hz), 1.82–2.26 (m, 5 H), 2.45 (m, 2 H), 3.10 (m, 2 H), 3.61–4.60 (m, 8 H), 5.28 (s, 2 H), 7.30 (m, 10 H). Anal. ($C_{39}H_{58}N_4O_8$) C, H, N.

***N*-Isovaleryl-L-valyl-L-valyl-4(*S*),7-diamino-3(*R*,*S*)-hydroxyheptanoic Acid Ethyl Ester Acetate (27a).** Compound **28ab** (28 mg, 0.04 mmol) 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 psi for 3 h. 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 about 88% yield: mp 228–230 °C; TLC R_f (I) 0.52; NMR (MeOH-d_4) δ 0.91–1.10 (m, 18 H), 1.23 (t, J = 7 Hz, 3 H), 1.65 (m, 4 H), 1.80–2.21 (m, 8 H), 2.46 (m, 2 H), 2.90 (m, 2 H), 3.63–4.41 (m, 6 H). Anal. ($C_{26}H_{50}N_4O_8$) C, H, N.

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Registry No. **9**, 82689-16-5; **10a**, 98045-10-4; **10b**, 98063-00-4; **12a**, 105562-69-4; **12b**, 105562-70-7; **13a**, 105562-71-8; **13b**, 105617-26-3; **14a**, 105562-72-9; **14b**, 105617-27-4; **15a**, 91416-63-6; **15b**, 91464-93-6; **16a**, 105617-28-5; **16a** (free base), 91416-61-4; **16b**, 105660-65-9; **16b** (free base), 105617-32-1; **17**, 105562-73-0; **18**, 105562-74-1; **19**, 105617-29-6; **19** (free base), 91416-62-5; **20**, 2480-95-7; **21**, 105562-75-2; **22**, 105562-76-3; **23**, 105562-77-4; **24**, 105562-78-5; **25**, 105562-79-6; **26**, 105562-80-9; **27a**, 105562-81-0; **27b**, 105562-82-1; **28a**, 105562-83-2; **28b**, 105617-30-9; **29a**, 105562-85-4; **29a** (free base), 105562-84-3; **29b**, 105660-66-0; **29b** (free base), 105617-33-2; **33**, 105562-86-5; $ClCO_2CH_2CCl_3$, 17341-93-4; Boc Val anhydride, 33294-55-2; H-Phe-OMe, 2577-90-4; Boc-L-Val-L-Val-N^8-Cbz-(3*S*,4*S*)-[LySta]-L-Phe-OMe, 105562-87-6; ZCl, 501-53-1; AcOEt, 141-78-6; Boc-L-Val-N^7-Bzl-N^7-Cbz-(3*S*,4*S*)-[OrnSta]-OEt, 105660-72-8; Boc-L-Val-N^7-Bal-N^7-Cbz-(3*R*,4*S*)-[OrnSta]-OEt, 105562-88-7; Boc-L-Val-L-Val-N^7-Bzl-N^7-Cbz-(3*S*,4*S*)-[OrnSta]-OEt, 105562-89-8; Boc-L-Val-L-Val-N^7-Bzl-N^7-Cbz-(3*R*,4*S*)-[OrnSta]-OEt, 105617-31-0; isovaleric anhydride, 1468-39-9; benzaldehyde, 100-52-7; penicillopepsin, 9074-08-2; aspartic proteinase, 78169-47-8; pepsin, 9001-75-6.

Inhibitors of Cyclic AMP Phosphodiesterase. 1. Analogues of Cilostamide and Anagrelide[1]

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Evaluation of a series of lactam heterocyclic analogues of cilostamide (**2**) as inhibitors of cyclic AMP phosphodiesterase derived from both human platelets and rat heart in comparison with their corresponding methoxy-substituted heterocycles has revealed that the *N*-cyclohexyl-*N*-methyl-4-oxybutyramide side chain of **2** is an important lipophilic and/or steric pharmacophore. Attachment of this side chain to the parent heterocycle of the potent cyclic AMP phosphodiesterase inhibitor anagrelide (**3**) afforded the hybrid structure RS-82856 (**1**), shown to be more potent than either of its progenitors as an inhibitor of cyclic AMP phosphodiesterase or of ADP-induced platelet aggregation. The available in vitro data suggest that **1** possesses potentially useful antithrombotic and cardiotonic properties.

Current therapeutic approaches to the treatment of heart failure rely on the stimulation of cardiac contractility with the administration of cardiac glycosides or sympathomimetic agents. The absence of a safe, orally active, positive inotropic agent has prompted the search for such drugs. Recently, considerable interest has focused on the properties of some inhibitors of cyclic AMP phosphodiesterase (PDE).[2,3] Phosphodiesterase inhibitors have been described with cardiotonic and vasodilatory properties

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(2) For an excellent review of the current status of selective PDE inhibition, see: Weishaar, R. E.; Cain, M. H.; Bristol, J. A. *J. Med. Chem.* **1985**, *28*, 537.
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