ysis), G. D. Roberts and S. Rottschaefer (mass spectra), P. J. Hieble (biological data), and C. Kaiser for helpful discussions.

**Registry No.** 1, 57987-77-6; 1 (free base), 61563-24-4; 2, 78104-37-7; 3, 78104-35-5; 5, 83903-85-9; 5 (free base), 78104-49-1; 6, 78104-36-6; 7, 78104-31-1; 8, 78104-32-2; 9, 78104-33-3; 9a, 78104-34-4; 10, 23707-37-1; 11, 120546-57-8; 12, 78104-40-2; 13, 78234-22-7; 13 (free base), 78104-50-4; 14, 55766-82-0; 15, 120546-58-9; 16, 78104-43-5; 17, 120546-59-0; 17 (free base), 120546-72-7; 18, 120546-60-3; 19, 109909-64-0; 20, 120546-61-4; 20 (free base), 109544-44-7; 21, 120546-62-5; 22, 120546-63-6; 22 (free base), 120546-73-8; 23, 120546-64-7; 24, 120546-65-8; 24 (free base), 120546-74-9; 25, 56623-96-2; 26, 56623-97-3; 26 (free base), 56623-99-5; 27, 35317-80-7; 28, 35317-81-8; 29, 120546-66-9; 30, 120546-67-0; 31, 120546-68-1; 32, 56623-93-9; 33, 120546-69-2; 34, 120546-70-5; 35, 120546-71-6; 36, 109220-61-3; PNMT, 9037-68-7; benzyl mercaptan, 100-53-8; 7-amino-8-nitroisoquinoline, 56623-94-0; dimethylthiocarbamoyl chloride, 16420-13-6.

**Supplementary Material Available:** Tables of positional and thermal parameters, general temperature factor expressions, $B$'s, bond distances, bond angles, and torsional angles (5 pages). Ordering information is given on any current masthead page.

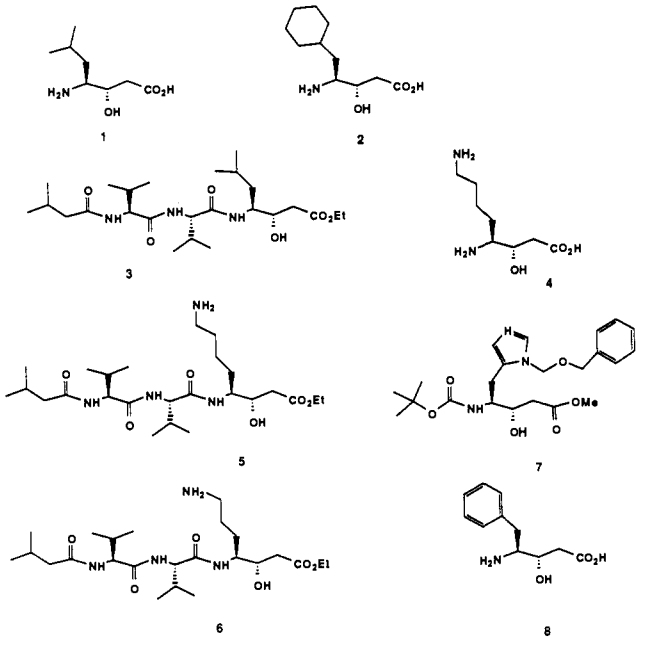
# Synthesis of the Novel π-(Benzyloxymethyl)-Protected Histidine Analogue of Statine. Inhibition of Penicillopepsin by Pepstatin-Derived Peptides Containing Different Statine Side-Chain Derivatives[1]

Juergen Maibaum and Daniel H. Rich*

*School of Pharmacy, University of Wisconsin—Madison, 425 North Charter Street, Madison, Wisconsin 53706.*
*Received August 8, 1988*

The synthesis of aspartic proteinase inhibitors derived from a new histidine side-chain analogue of statine (Sta), (3*S*,4*S*)-4-amino-3-hydroxy-5-(imidazol-4-yl)pentanoic acid (HiSta, **20**), is reported. Boc-HiSta(BOM)-OMe (**7**) was prepared in 16% overall yield from Boc-His(π-BOM)-OH via formation of the tetramic acid derivative **11** and stereoselective cis reduction with $NaBH_4$ to the 4-hydroxy lactam **12**. Removal of the Boc group from ester **7** (enantiomeric purity ee = 88–90%) and coupling to the tripeptide segment Iva-Val-Val-OH (**13**) by the DCC/HOBt preactivation method followed by hydrogenolytic removal of the π-BOM group over $Pd(OH)_2$ on carbon gave Iva-Val-Val-HiSta-OMe (**16**). This new peptide **16** is a very potent inhibitor of the fungal aspartic proteinase penicillopepsin ($K_i = 4.5 \times 10^{-9}$ M) that is 10 times more active than the comparable Sta-containing inhibitor **3** and 2–3 times more potent than the new (3*S*,4*S*)-4-amino-3-hydroxy-5-phenylpentanoic acid (AHPPA) analogue **17** ($K_i = 1.5 \times 10^{-8}$ M). However, compound **16**, which has an imidazole residue at the $P_1$ position, is a significantly weaker inhibitor of the enzyme than the corresponding analogues with the lysine (**5**) and ornithine (**6**) side chains at $P_1$. Considerations that led to the synthesis of **16** and the results of the enzyme kinetics are discussed in detail.

In recent years, numerous analogues of the natural product inhibitor pepstatin[2] and substrate-derived peptides containing statine [(3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid (Sta, **1**)] have been developed as



potent inhibitors of pepsin,[3–7] penicillopepsin,[6,8] renin,[9,10] and other fungal and mammalian aspartic proteinases.[8,11,12] X-ray crystal structures of statine-containing inhibitors bound to penicillopepsin,[13,14] *Rhizopus chinensis* pepsin,[15]

* Address correspondence to this author.

(1) Abbreviations used follow the IUPAC–IUB commission on Biochemical Nomenclature recommendations. Additional abbreviations are as follows: Boc, *tert*-butyloxycarbonyl; π-BOM, π-(benzyloxymethyl); DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; Iva, isovaleryl; Nph, 4′-nitrophenylalanine; ACHPA, (3*S*,4*S*)-4-amino-3-hydroxy-5-cyclohexylpentanoic acid; AHPPA, (3*S*,4*S*)-4-amino-3-hydroxy-5-phenylpentanoic acid; HiSta, (3*S*,4*S*)-4-amino-3-hydroxy-5-(imidazol-4-yl)pentanoic acid; LySta, (3*S*,4*S*)-4,8-diamino-3-hydroxyoctanoic acid; OrnSta, (3*S*,4*S*)-4,7-diamino-3-hydroxyheptanoic acid; Sta, (3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid.

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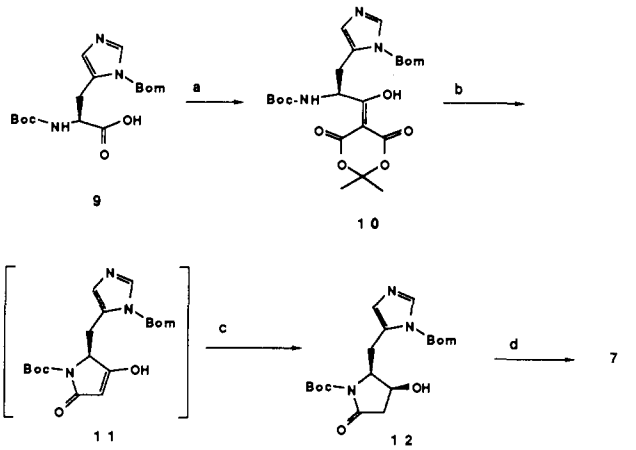
and endothiapepsin[16] have been used to identify the binding interactions of several peptide inhibitors within the catalytic cleft of the enzyme, from which it has been proposed that statine functions as the transition-state bioisostere of a dipeptide unit.[17] Furthermore, detailed proposals for the catalytic mechanism of aspartic proteinases have been based upon these and other[16,18] crystal structures and on $^{13}C$ NMR studies.[6b,19]

Molecular modeling based on the X-ray crystal data has led to the design of new side-chain analogues of statine with enhanced potency and higher selectivity for the target enzyme. Some of the most potent inhibitors of human renin so far reported have been obtained by replacing the statine isobutyl residue at the $P_1$-binding site[21] with the more bulky and hydrophobic cyclohexylmethyl side chain,[10] ACHPA (2). Molecular modeling of the crystal structure of Iva-Val-Val-Sta-OEt (3) complexed to penicillopepsin[21] and considerations of the substrate specificity of penicillopepsin[8] had suggested that peptides containing the lysine side-chain derivative of statine, LySta (4), would show enhanced binding due to an electrostatic interaction of the $\epsilon$-nitrogen of LySta with the anionic Glu-16/Asp-115 residues of the enzyme. The LySta tripeptide 5 and the OrnSta analogue 6 were synthesized and were, in fact, found to be much better inhibitors with high selectivity for penicillopepsin.[8] However, the X-ray structure of the inhibitor–enzyme complex[14] revealed that peptide 5 was bound to the active site in a new conformation stabilized by an unexpected electrostatic interaction to Asp-77 rather than the Glu-16/Asp-115 pair suggested by the modeling studies.

Thus, although a few side-chain analogues of statine have been incorporated into peptide inhibitor sequences,[22] only limited information is available about the structure–activity relationships and the influence on enzyme selectivity with regard to structural variations at the $P_1$ position.

Scheme I[a]




[a] (a) Meldrum's acid, DCC, DMAP, 24 h in $CH_2Cl_2$; (b) MeCN, 30-min reflux; (c) $NaBH_4$, $CH_2Cl_2$–acetic acid (9:1), 0 °C; (d) $NaOCH_3$, MeOH.

In our search for potent, subnanomolar inhibitors of penicillopepsin,[8,19] we became interested in whether the $S_1$ pocket of this enzyme could accommodate hydrophobic residues of steric bulk, and whether basic side-chain derivatives of peptide 5 with increased hydrophobicity at $P_1$ could lead to improved enzyme inhibition. We report herein the synthesis of the basic statine analogue Boc-4-(*S*)-amino-3(*S*)-hydroxy-5-($\pi$-BOM-imidazol-4-yl)pentanoic acid methyl ester [Boc-HiSta(BOM)-OMe, 7]. This new amino acid derivative and the benzyl side-chain analogue 4(*S*)-amino-3(*S*)-hydroxy-5-phenylpentanoic acid (AHPPA, 8)[3] were used to prepare two new inhibitors of penicillopepsin.

## Chemistry

The new side-chain analogue of statine Boc-HiSta-(BOM)-OMe (7) was synthesized from $\pi$-BOM-protected histidine via the 4-hydroxy-substituted lactam intermediate 12 according to a modified method reported recently[23] (Scheme I). DCC-mediated condensation[24] of $N^{\alpha}$-Boc-His($\pi$-BOM)-OH[25] (9) with Meldrum's acid in the presence of 4-(*N*,*N*-dimethylamino)pyridine (DMAP) afforded the crude compound 10 in good yield (70%). Refluxing of 10 in acetonitrile gave, besides a considerable amount of unidentified side products, the tetramic acid derivative 11 which without purification was reduced with sodium borohydride under acidic conditions to the N-protected lactam 12 (26% yield for two steps).

Attempts to improve the yield of this reaction sequence under varied conditions by using different solvents for the cyclization to 11 as the key reaction, or to purify compound 11 from major side products prior to reduction,[23] were unsatisfactory. The cyclization of compound 10 to give the tetramic derivative 11 was attempted in acetonitrile, ethyl acetate, methanol, and 2-propanol under varying reaction temperatures. No reaction at all was observed in ethyl acetate, in which the completely recovered starting material is insoluble. Only traces of the desired product, and some side products as well, were formed in methanol

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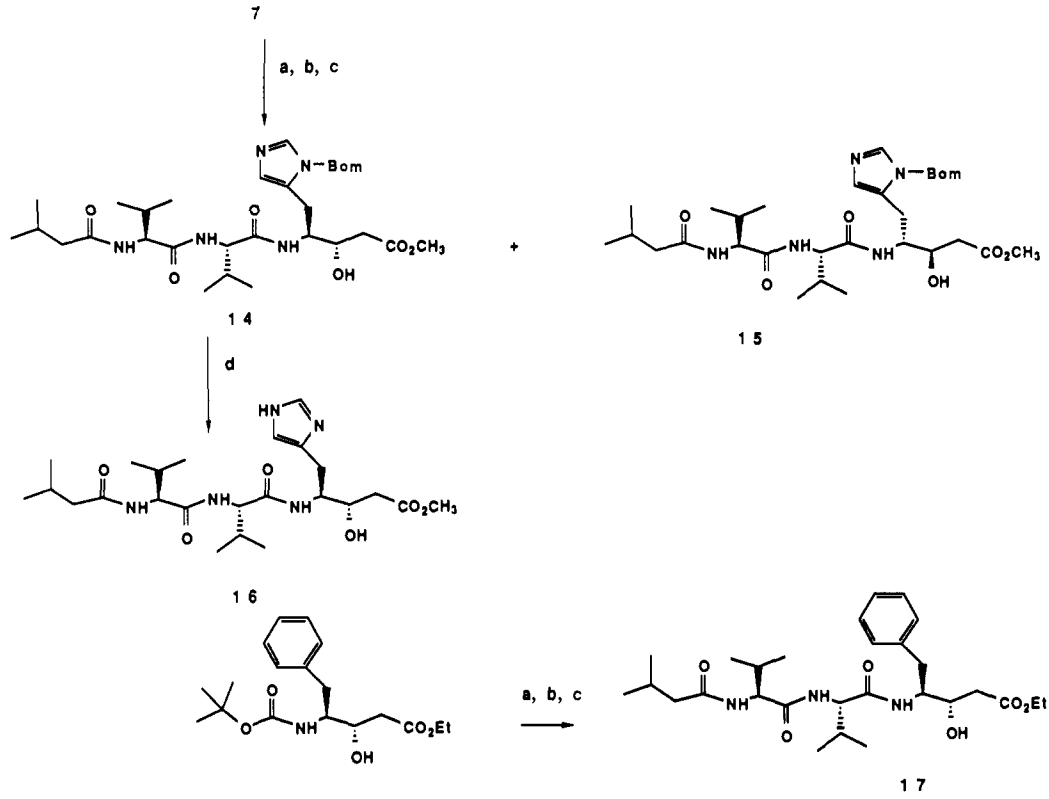
**Scheme II**[a]




[a] (a) 4 N HCl in dioxane; (b) Iva-Val-Val-OH (13), DCC, HOBt (preactivation at 0 °C, 2 h); (c) DIEA; (d) $H_2/Pd(OH)_2$, MeOH.

(reaction temperature >75 °C) and in refluxing 2-propanol. This is in contrast to the "quantitative" reactions in each of these solvents (except 2-propanol) that have been obtained by Jouin et al.[23] TLC monitoring of the reaction in acetonitrile at 110–120 °C revealed complete formation of product after 30–45 min; shorter reaction times or lower temperatures did not improve the ratio of compound 11 versus side products.

Purification of crude compound 11 according to the procedure of Jouin et al.[23] by extraction from an ethyl acetate solution with 5% aqueous $NaHCO_3$ in the first step, followed by acidifying the water phase to pH 3 with citric acid and reextraction with ethyl acetate, led to the recovery of only a small percentage of 11 because of its water solubility at mild acidic and even neutral pH. The noticeable water solubility of compound 11 could be due to a zwitterionic structure at neutral conditions.

In the final step, Boc-HiSta(BOM)-OMe (7) was obtained from 12 by methanolysis with sodium methoxide in 16% overall yield, starting from the protected amino acid. The proton NMR spectra (500 MHz) of $N^{\alpha}$-Boc lactam 12 (coupling constant $J_{cis\text{-}4,5}$ = 6.6 Hz) and of 7 confirmed the high stereoselectivity by which 11 is reduced to the cis isomer 12, thus providing 7 with high diastereomeric purity. The enantiomeric purity (ee) of 7 was established by Boc deprotection and derivatization of the free amine with optically pure (*R*)-(+)- and (*S*)-(−)-1-phenylethyl isocyanate.[26,27] Analysis of the proton NMR spectra of the resulting diastereomer products revealed the presence of small amounts of a second urea diastereomer, respectively, indicating ee = 88–90% for Boc-HiSta-(BOM)-OMe (7).

Synthesis of peptide 14 was achieved by coupling the dipeptide segment Iva-Val-Val-OH (13) as its active ester, which was formed by preactivation with DCC/HOBt,[19] to the corresponding Boc-deprotected HiSta ester derivative 7 in 85–95% yield (cf. Scheme II). Coupling of $N^{\alpha}$-deprotected 7 to the dipeptide gave, in addition, small amounts (5–10%) of a peptide side product which is most likely the 3*R*,4*R* diastereomer, compound 15, and which could be removed by silica gel column chromatography. Minor contamination of peptide 14 with its D-Val[3] epimerization side product (see below) which showed similar $R_f$ values on TLC could not be chromatographically removed. The BOM group was removed from compound 14 by hydrogenolysis over $Pd(OH)_2$ on carbon to give peptide 16 in 87% yield. In a similar fashion, the AHPPA tripeptide 17 and the D-Val-Sta peptide 18 were prepared from compounds 13 and 19 respectively.[19] Epimerization at the C-terminal valine residue in 13 during preactivation coupling was in the range of 2–5% as determined by reversed-phase HPLC.

## Enzyme Kinetic Analysis

Inhibition constants ($K_i$) for the inhibition of penicillopepsin are summarized in Table I. Kinetic data for compound 3 and the new peptides 16–18 were determined by the methods described previously,[8] with Ac-Ala-Ala-Lys-Nph-Ala-Ala-$NH_2$ as the substrate. In the case of slow-binding kinetics, the enzyme and inhibitor were preincubated for 10 min before substrate was added. $IC_{50}$ values obtained from plots of the initial steady-state velocities of uninhibited vs inhibited enzyme were converted

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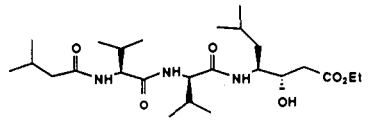

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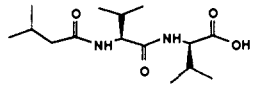

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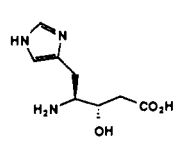

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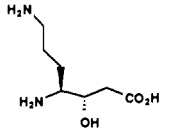

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to $K_i$ inhibition constants by using the correlation of Cha et al.:[29]

$$K_i = (IC_{50} - 0.5E_t)/(1 + S/K_M)$$

where $E_t$ and $S$ are the concentration of the enzyme and substrate in the assay and $K_M$ is the Michaelis constant for the substrate. Similar $K_i$ values for compounds **16–18** were calculated by computer fit of the enzyme reaction velocities to the equation of Morrison[30] for tight-binding, competitive inhibition.

The new compounds **16** and **17** both were potent, low-nanomolar level inhibitors of penicillopepsin, peptide **16** being 2–3-fold more active than the AHPPA analogue **17**, which contains a benzyl side chain at $P_1$. Peptide **17** was about 5 times more active than the reference Sta-containing inhibitor **3**, whereas the basic HiSta peptide **16** showed a 2–5-fold higher $K_i$ value than that reported for the inhibitors **5** and **6** containing the basic lysine and ornithine side chain, respectively, at $P_1$. The D-Val diastereomeric peptide **18** showed no enzyme inhibition at a concentration of 18 μM and thus is more than 250-fold less active than the corresponding L-Val diastereomer **3**. Accordingly, the inhibition constant for the HiSta derivative **16** should not be influenced significantly by one minor contamination (<5%; vide supra) with the corresponding D-epimer.

## Discussion

Incorporation of the LySta and OrnSta analogues into pepstatin-derived peptides as replacement for the statine residue has led to inhibitors of penicillopepsin with enhanced potency and higher selectivity for the fungal enzyme.[8] The $K_i$ values of peptides **5** and **6**, for example, were 10–100 times smaller than the $K_i$ of the reference peptide Iva-Val-Val-Sta-OEt (**3**) when tested against penicillopepsin (Table I), but both **5** and **6** were, in contrast, exceptionally weak inhibitors of porcine pepsin.[8] The X-ray crystal structure of the peptide **5**–penicillopepsin complex[8,14] revealed that the tighter binding of **5** can be attributed to the formation of a strong ion pair between the ε-nitrogen of the LySta side chain and the Asp-77 carboxylate of the "mobile flap" region of the enzyme. This electrostatic interaction is additionally stabilized by hydrogen bonds of the amino group to the Asp-77 carboxylate, the hydroxyl of Ser-79, and an enzyme-bound water molecule. Moreover, the lipophilic portion of the LySta side chain in its bound conformation induces considerable van der Waals interactions to several amino acid residues of the binding cleft, such as Tyr-75, Leu-121, and Phe-112,[8,14] which generate a hydrophobic environment at the $S_1$-binding pocket. The X-ray crystal data for the bound peptide **5** and the equipotency of the OrnSta analogue **6** as an inhibitor of penicillopepsin suggest[8] that the $N^{\delta}$-amino group of the shorter side chain inhibitor also forms a tight ion pair with Asp-77. From these data we considered that increasing the lipophilicity of the basic peptide residue at $P_1$, without weakening the binding energy from ion-pair formation, should lead to inhibitors with improved potency against penicillopepsin. We therefore synthesized the new AHPPA-containing peptide **17** and the basic peptide **16** which contained the novel histidine side-chain analogue of statine HiSta (**20**).

**Table I.** Inhibition of Penicillopepsin by Peptides Containing Statine and Side-Chain Analogues[1]

| no. | compound | $K_i$, nM | slow binding |
|---|---|---|---|
| **3** | Iva-Val-Val-Sta-OEt | 72[a] | – |
| **18** | Iva-Val-D-Val-Sta-OEt | >18 000[b] | – |
| **17** | Iva-Val-Val-AHPPA-OEt | 15 | – |
| **5** | Iva-Val-Val-LySta-OEt | 2.1[c] | + |
| **6** | Iva-Val-Val-[(3*RS*,4*S*)-OrnSta]-OEt | 1.1[c,d] | + |
| **16** | Iva-Val-Val-HiSta-OMe | 4.5 | + |

[a] Lit.[6,8] $K_i$ = 47 nM for compound **3** synthesized by stepwise coupling methodology. [b] No inhibition of the enzyme at an inhibitor concentration of μM. The inhibitor precipitated from solution at higher concentrations before addition of enzyme. [c] Data taken from ref 8. [d] Reported $K_i$ had been determined on a 1:1 mixture of 3*R*/3*S*. Number reported is half the number obtained for the mixture.

As shown in Table I, peptide **17** is a better inhibitor of penicillopepsin than the statine analogue **3**, indicating that the enzyme has a preference for the hydrophobic aromatic residue. The importance of a hydrophobic residue at $P_1$ of Sta-containing inhibitors for maximum binding has also been demonstrated for other aspartic proteinases.[10] Thus, increased binding to human renin and cathepsin D was obtained when statine with its isobutyl side chain was replaced by the more hydrophobic AHPPA analogue **8** (benzyl side chain) or, more efficiently, by the cyclohexylalanyl analogue **2** (a 55–70-fold better inhibitor). In contrast, no significant increase in binding to porcine pepsin[3,10] and *R. chinensis* pepsin[10] was observed for peptides containing more bulky and lipophilic side chain analogues of Sta.

The HiSta compound **16** with the basic imidazole side chain is a better inhibitor than **17**, but **16** is also a significantly weaker inhibitor than the LySta and OrnSta derivatives **5** and **6**. This result indicates that the favorable effects of the van der Waals interaction (suggested by the preference of penicillopepsin for the aromatic phenyl residue) and the electrostatic interactions from ion-pair formation are not additive when peptide **16** binds to penicillopepsin. There may be several reasons for this: although HiSta (**20**) is a conformationally restricted structural isostere of OrnSta (**21**), the bond lengths and angles in the aromatic system differ from those of the corresponding portions of the aliphatic side chain, and this may significantly alter the interactions between the enzyme peptide backbone and the imidazole τ-nitrogen. Steric or electronic interactions of the imidazole with the amino acid

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residues in the $S_1$ enzyme binding site may favor a conformation of the inhibitor side chain (as well as the conformation of the enzyme mobile flap region) in which a potential electrostatic ion-pair interaction is weakened. Alternatively, the dipolar electron density of the heteroaromatic system may disfavor the van der Waals interactions with the enzyme amino acid residues in the binding site. Finally, the HiSta side chain in **16** in solution will by hydrated, and these hydrogen-bonded waters must be stripped before **16** can bind to the hydrophobic environment of the $S_1$ site, a process that would disfavor binding. It is notable that peptide **16**, in contrast to the hydrophobic analogue **17**, binds slowly to penicillopepsin, a process that may result from slow or improbable desolvation of either the imidazole side chain or the $S_1$ pocket.[31,32] It is not possible to differentiate between these possibilities from our data.

In summary, the incorporation of the phenylalanyl analogue of statine AHPPA (**8**) and the histidine side-chain analogue HiSta **20** into a pepstatin-derived peptide sequence gave very potent inhibitors of penicillopepsin. The present results further indicate that the fungal enzyme prefers more bulky, hydrophobic residues at the $S_1$-binding site than the isobutyl side chain in statine and confirm that suitable basic residues at the $P_1$ position, including basic aromatic groups, further improve the inhibitor binding. However, peptide **16** with the imidazole residue at $P_1$ is not a better inhibitor of the enzyme than the LySta and OrnSta analogues **5** and **6**. The structural requirements for maximum binding of peptide inhibitors that contain statine side-chain analogues at the $P_1$–$P_1'$ binding site, to the $S_1$ pocket of the enzyme, as well as the specificity of the enzyme–inhibitor interaction at the $P_1$ position with regard to different aspartic proteinases are not yet completely understood.

## 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 polarimeter. Proton nuclear magnetic resonance spectra were recorded with a Bruker EM-500 instrument at 500 MHz. Chemical shifts are reported as units (ppm) relative to tetramethylsilane. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN, and given data were within the range of ±0.4% of calculated values. TLC was performed on 0.25-mm thickness silica gel plates (Merck, silica gel 60 $F_{254}$). The following TLC solvent systems were used: (A) 10% methanol in methylene chloride; (B) 5% methanol in methylene chloride: (C) chloroform–methanol–acetic acid 85:15:3 (v/v/v). Compounds were visualized on plates by UV light, and by reaction with ninhydrin and with 5% phosphomolybdic acid in ethanol. For column chromatography, Merck silica gel, grade 60, 230–400 mesh, was used. HPLC analyses were carried out on a Waters system consisting of two Model 510 HPLC pumps in combination with an automated gradient controller, a UV detector Model 441 (detection at 214 nm), and a data module Model 730. A Waters Bondapac C-18 reversed-phase column and acetonitrile–water solvent mixtures were used. Kinetic parameters for inhibition of penicillopepsin were determined with Ac-Ala-Ala-Lys-Nph-Ala-Ala-$NH_2$ as substrate, according to the methods described previously.[8] Total enzyme concentration of the inhibition assays was $[E]_t = 1.4 \times 10^{-8}$ M, and the substrate concentration was $[S] = 1.5 \times 10^{-4}$ M. Inhibition constants were calculated from IC values, by using Cha's equation[29] and Morrison's equation for tight-binding inhibitors.[30]

**General Procedure A. Removal of the *N*-Boc Protecting Group.** A solution of the *N*-Boc-protected compound (0.1–0.3 mmol) in 4 N HCl in dioxane (3 mL) was stirred at room temperature for 30–45 min. The reaction was monitored by TLC. Excess of reagent was removed under reduced pressure, and the obtained oily hydrochloride salt was dried for 2 h in vacuo over KOH.

**General Procedure B. Preactivation Coupling with DCC/HOBt.** To a solution of the tripeptide Iva-Val-Val-OH[19] or Iva-Val-D-Val-OH[19] in $CH_2Cl_2$–DMF (6:1), cooled to 0 °C, were added dicyclohexylcarbodiimide (1 equiv) and 1-hydroxybenzotriazole (1 equiv). The mixture was stirred for 2 h at 0 °C. Precipitated dicyclohexylurea (DCU) was removed by filtration, and the filtrate was immediately added to the amino acid hydrochloride (1 equiv). The solution thus obtained was then neutralized by adding diisopropylethylamine (1 equiv) at 0 °C, and stirring was continued for 3 h at 0 °C (a gel-like precipitate appeared after a short reaction time) and overnight at room temperature. The mixture was concentrated under reduced pressure, and the residue was purified by silica gel chromatography.

**5-[1-Hydroxy-2(*S*)-[(*tert*-butyloxycarbonyl)amino]-3-[3-(benzyloxymethyl)imidazol-4-yl]propylidene]-2,2-dimethyl-1,3-dioxane-4,6-dione (10).** To a solution of 1.88 g (5.0 mmol) of Boc-His($\pi$-BOM)-OH (**9**) in methylene chloride (20 mL) were added 2,2-dimethyl-1,3-dioxane-4,6-dione, Meldrum's acid, (0.75 g, 5.2 mmol), DMAP (0.915 g, 7.5 mmol), and DCC (1.18 g, 5.73 mmol), and stirring was continued for 24 h at room temperature. The mixture was filtered, and the organic phase was washed twice with 5% aqueous $KHSO_4$ and saturated NaCl solution and evaporated. The residue was taken up in absolute ethanol (100 mL), filtered through glass wool, and reevaporated under reduced pressure to remove traces of water. The crude oily product was triturated with ether, filtered, and dried in high vacuo. The pale yellow solid (1.75 g, 70%), which was not further purified, contained traces of the starting amino acid and other impurities: $R_f = 0.40$ (C); $^1$H NMR (DMSO-$d_6$) $\delta$ 1.27 (s, 9 H), 1.48 (s, 6 H), 2.67 and 3.13 (2 m, 2 H), 4.58 (s, 2 H), 5.39 (m, 1 H), 5.81 (m; AB, $J$ = 10 Hz; 2H), 6.24 (d, $J$ = 8 Hz 1 H), 7.2–7.4 (m, 7 H), 9.15 (s, 1 H).

**(4*S*,5*S*)-1-(*tert*-Butyloxycarbonyl)-4-hydroxy-5-[[3-(benzyloxymethyl)imidazol-4-yl]methyl]pyrrolidin-2-one (12).** A solution of 500 mg (1.0 mmol) of the crude product **10** in acetonitrile (20 mL) was refluxed for 0.5 h (bath temperature 110 °C). After cooling, the solution was concentrated under reduced pressure and dried in vacuo. The oily residue was dissolved in a mixture of methylene chloride–acetic acid (6 mL, 9:1) and cooled to 0 °C, and 95 mg (2.51 mmol) of $NaBH_4$ was added in one portion. Stirring was continued for 4 h at 0 °C before the reaction was quenched by addition of water (1 mL). The aqueous phase was neutralized with solid $NaHCO_3$ and then extracted with methylene chloride and the combined organic phase dried over $MgSO_4$ and evaporated. Purification by silica gel chromatography (eluent, $CH_2Cl_2$–MeOH 96:4) afforded 105 mg (26%) of a colorless oil: $R_f = 0.32$ (A); $[\alpha]^{22}_D = +8.4$; $[\alpha]^{22}_{436} = +21.5$ ($c$ = 1, $CH_2Cl_2$); $^1$H NMR ($CDCl_3$) $\delta$ 1.49 (s, 9 H), 2.46 (dd, $J$ = 8, 17 Hz; 1 H), 2.64 (dd, $J$ = 7, 17 Hz; 1 H), 3.10 (dd, $J$ = 4, 15 Hz; 1 H), 3.16 (dd, $J$ = 9.5, 15 Hz; 1 H), 4.35 (ddd, $J$ = 4, 6.6, 9.5 Hz; 1 H), 4.44 (s, 3 H), 4.47 (m, 1 H), 5.35 (m; AB, $J$ = 11 Hz; 2 H), 6.80 (s, 1 H), 7.2–7.35 (m, 5 H), 7.39 (s, 1 H). Anal. ($C_{21}H_{27}N_3O_5 \cdot 0.5H_2O$) C, H, N.

**(3*S*,4*S*)-5-[3-(Benzyloxymethyl)imidazol-4-yl]-4-[(*tert*-butyloxycarbonyl)amino]-3-hydroxypentanoic Acid Methyl Ester (7).** To a solution of 110 mg (0.274 mmol) of compound **12** in methanol (2 mL) was added 720 $\mu$L (0.288 mmol) of a 25 wt % solution of sodium methoxide in methanol. The mixture was stirred for 15 min at room temperature and then immediately flash chromatographed on silica gel with 10% MeOH–$CH_2Cl_2$ as eluent. The product thus obtained was purified by silica gel chromatography ($CH_2Cl_2$–MeOH 97:3) to give 105 mg (88%) of a colorless oil: $R_f = 0.35$ (A); $[\alpha]^{22}_D = -19.0$; $[\alpha]^{22}_{436} = -38.9$ ($c$ = 0.6, MeOH); $^1$H NMR ($CDCl_3$) $\delta$ 1.39 (s, 9 H), 2.32 (dd, $J$ = 2, 17 Hz; 1 H), 2.55 (dd, $J$ = 10, 17 Hz; 1 H), 2.93 (m, 2 H), 3.62

(31) The slow extrusion of a water molecule bound to the carboxyl–carboxylate pair Asp-33/Asp-213 (aspartyl sequence numbering refers to penicillopepsin) in the active site of the enzyme has been discussed in detail as a cause of the slow-binding process of tight-binding pepstatin analogues.[32]

(32) Rich, D. H.; Northrop, D. B. In *Computer-Aided Drug Design*; Perun, T., Probst, C., Eds.; Marcel-Dekker: New York, pp 185–250. See also: Rich, D. H. *J. Med. Chem.* **1985**, *28*, 263–273.

(m, 1 H), 3.66 (s, 3 H), 3.74 (m, 1 H), 3.97 (d, $J$ = 10 Hz; 1 H), 4.45 (m; AB, $J$ = 12 Hz; 2 H), 4.96 (d, $J$ = 9 Hz; 1 H), 5.32 (m; AB, $J$ = 11 Hz; 2 H), 6.91 (s, 1 H), 7.25–7.35 (m, 5 H), 7.46 (s, 1 H). Anal. ($C_{22}H_{31}N_3O_6 \cdot 0.5H_2O$) C, H, N.

**Derivatization with Optically Active 1-Phenylethyl Isocyanate (PEI).** Compound **7** (15 mg, 0.035 mmol) was treated with 1 mL of 50% trifluoroacetic acid in methylene chloride at 0 °C. After 45 min, the mixture was concentrated under reduced pressure and the residue taken up in $CH_2Cl_2$ (1 mL) and reevaporated. The crude TFA salt was dissolved in $CH_2Cl_2$ (2 mL) and washed with saturated aqueous $NaHCO_3$ solution (1 mL). The organic phase was dried over $Na_2SO_4$ for 5 min and decanted. To one part of the solution of the free base (about 1 mL) was added 15 μL (0.154 mmol) of (*R*)-(+)-PEI at room temperature. After 30 min (only one product spot was detected by TLC), the resulting mixture was chromatographed on a silica gel column with $CH_2Cl_2$–MeOH (95:5) as eluent to isolate the pure diastereomeric urea product. In the same manner, the urea derivative from **7** and (*S*)-(−)-PEI was prepared. Analysis of the $^1$H NMR spectra of each purified urea product, both showing identical $R_f$ values on TLC, indicated the presence of 5.5 and 5.7%, respectively, of the other diastereomer, corresponding to an enantiomeric purity ee = 88–89% for compound **7**.

**Diastereomer from (*R*)-(+)-PEI:** $R_f$ = 0.33 (A); $^1$H NMR ($CDCl_3$) δ 1.42 (d, $J$ = 6 Hz; 3 H), 2.29 (dd, $J$ = 3, 16 Hz; 1 H), 2.52 (dd, $J$ = 10, 16 Hz; 1 H), 2.79 (dd, $J$ = 8, 14.5 Hz; 1 H), 2.89 (dd, $J$ = 6.5, 14.5 Hz; 1 H), 3.64 (s, 3 H), 3.80 (m, 1 H), 3.98 (m, 1 H), 4.43 (m; AB, $J$ = 12 Hz; 2 H), 4.75 (m, 1 H), 4.85 (m, 1 H), 4.95 (m, 1 H), 5.25 (m; AB, $J$ = 11 Hz; 2 H), 6.78 (s, 1 H), 7.2–7.4 (m, 10 H), 7.44 (s, 1 H).

**Diastereomer from (*S*)-(−)-PEI:** $R_f$ = 0.33 (A); $^1$H NMR ($CDCl_3$) δ 1.41 (d, $J$ = 6 Hz; 3 H), 2.1–2.2 (m, 2 H), 2.88 (dd, $J$ = 8, 15 Hz; 1 H), 2.93 (dd, $J$ = 6.5, 15 Hz; 1 H), 3.63 (s, 3 H), 3.79 (m, 1 H), 3.92 (m, 1 H), 4.42 (m; AB, $J$ = 12 Hz; 2 H), 4.69 (m, 1 H), 4.9–5.0 (m, 2 H), 5.31 (m; AB, $J$ = 10 Hz; 2 H), 6.83 (s, 1 H), 7.2–7.35 (m, 10 H), 7.45 (s, 1 H).

***N*-Isovaleryl-L-valyl-L-valyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoic Acid Ethyl Ester (3).** The title compound was prepared by general procedure B from HCl·$H_2$N-(*S*,*S*)-Sta-OEt [obtained from 91 mg (0.30 mmol) of Boc-(*S*,*S*)-Sta-OEt,[28] enantiomeric purity ee = 97.5%, by general procedure A] and Iva-Val-Val-OH (90 mg, 0.30 mmol) in $CH_2Cl_2$–DMF (7 mL). Silica gel chromatography (2% MeOH–$CH_2Cl_2$), followed by recrystallization from methanol–ether, gave 125 mg (86%) of the pure diastereomer as a white solid: mp 237–239 °C; $[\alpha]^{22}_D$ = −82 ($c$ = 0.065, MeOH) (lit.[5] mp 235–236 °C; $[\alpha]^{22}_D$ = −80 ($c$ = 0.06, MeOH)); $R_f$ = 0.16 (B); $^1$H NMR (DMSO-$d_6$) δ 0.7–0.95 (m, 24 H), 1.14 (t, $J$ = 7 Hz; 3 H), 1.15–1.6 (m, 3 H), 1.75–2.45 (m, 7 H), 3.7–4.25 (m, 6 H; q at 4.01 ppm, $J$ = 7 Hz), 4.96 (d, $J$ = 5 Hz; 1 H), 7.4–7.9 (m, 3 H). Anal. ($C_{25}H_{47}N_3O_6$) C, H, N.

***N*-Isovaleryl-L-valyl-D-valyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoic Acid Ethyl Ester (18).** The title compound was prepared as described above from HCl·$H_2$N-(*S*,*S*)-Sta-OEt [from 45 mg (0.15 mmol) of the Boc-protected compound] and Iva-Val-D-Val-OH (45 mg, 0.15 mmol) in $CH_2Cl_2$–DMF (3 mL). The product was purified by silica gel chromatography (1% MeOH–$CH_2Cl_2$, then 2% MeOH–$CH_2Cl_2$) and recrystallization from methanol–ether to give 60 mg (82%) of the pure diastereomer as a white solid: mp 191–193 °C; $[\alpha]^{22}_D$ = −15.9 ($c$ = 0.063, MeOH); $R_f$ = 0.23 (B); $^1$H NMR (DMSO-$d_6$) δ 0.75–0.95 (m, 24 H), 1.1–1.65 (m, 6 H; t at 1.15 ppm, $J$ = 7 Hz), 1.8–2.45 (m, 7 H), 3.75–3.95 (m, 2 H), 4.01 (q, $J$ = 7 Hz; 2 H), 4.05–4.25 (m, 2 H), 4.92 (d, $J$ = 5 Hz; 1 H), 7.35–7.55 (m, 1 H), 7.75–7.95 (m, 2 H). Anal. ($C_{25}H_{47}N_3O_6$) C, H, N.

***N*-Isovaleryl-L-valyl-L-valyl-4(*S*)-amino-5-[3-(benzyloxymethyl)imidazol-4-yl]-3(*S*)-hydroxypentanoic Acid Methyl Ester (14).** The title compound was prepared according to general procedure B from HCl·$H_2$N-HiSta(π-BOM)-OMe [obtained from 40 mg (0.092 mmol) of compound **7** by general procedure A], Iva-Val-Val-OH (**13**; 30 mg, 0.10 mmol), HOBt (15.5 mg, 0.10 mmol), DCC (21 mg, 0.10 mmol), and DIEA (18 μL, 0.10 mmol) in 2.5 mL of solvent. The mixture was concentrated under reduced pressure, followed by purification of the oily residue by silica gel chromatography with $CH_2Cl_2$–MeOH (gradient from 99:1 to 96:4), to afford a fraction containing the pure compound (20 mg, 35%) as a white solid (one spot on TLC) and a second fraction (25 mg, 44%) containing minor amounts of a second diastereomer with lower $R_f$ value: mp 211–214 °C; $R_f$ = 0.28 (A); $[\alpha]^{22}_D$ = −70.1 ($c$ = 0.1, MeOH); $^1$H NMR (DMSO-$d_6$) δ = 0.7–0.95 (m, 18 H), 1.85–2.05 (m, 5 H), 2.30 (m, 2 H), 2.63 (dd, $J$ = 8, 16 Hz; 1 H), 2.89 (dd, $J$ = 6, 16 Hz; 1 H), 3.52 (s, 3 H), 3.9–4.2 (m, 4 H), 4.41 (m, 2 H), 5.26 (d, $J$ = 6 Hz; 1 H), 5.44 (m; AB, $J$ = 11 Hz; 2 H), 6.76 (s, 1 H), 7.2–7.35 (m, 5 H), 7.7–7.9 (m, 4 H). Anal. ($C_{32}H_{49}N_5O_7$) C, H, N.

***N*-Isovaleryl-L-valyl-L-valyl-4(*S*)-amino-3(*S*)-hydroxy-5-(imidazol-4-yl)pentanoic Acid Methyl Ester (16).** To a solution of 20 mg (32.5 mol) of π-BOM-protected peptide **14** in methanol (10 mL) was added 20 mg of 20% palladium hydroxide on carbon. A slow stream of hydrogen was bubbled through the mixture for 7 h at room temperature, followed by filtration over Celite 545, washing with methanol, and concentration under reduced pressure. The oily residue was purified by silica gel chromatography (eluent, 10% MeOH–$CH_2Cl_2$) to give 14 mg (87%) of a white solid (one spot on TLC): mp 228 °C dec; $R_f$ = 0.11 (A); $[\alpha]^{22}_D$ = −89.1 ($c$ = 0.064, MeOH); $^1$H NMR (DMSO-$d_6$) δ 0.75–0.85 (m, 18 H), 1.85–2.05 (m, 5 H), 2.26 (dd, $J$ = 9.5, 16 Hz; 1 H), 2.35 (dd, $J$ = 4, 16 Hz; 1 H), 2.57 (dd, $J$ = 8, 15 Hz; 1 H), 2.79 (dd, $J$ = 6, 15 Hz; 1 H), 3.53 (s, 3 H), 3.95 (m, 1 H), 4.00 (m, 1 H), 4.10 (q, $J$ = 7, 9 Hz; 1 H), 4.16 (q, $J$ = 7, 8 Hz; 1 H), 5.2 (br, 1 H), 6.83 (s, 1 H), 7.64 (d, $J$ = 8 Hz; 1 H), 7.75–7.8 (m, 2 H), 7.84 (d, $J$ = 9 Hz; 1 H). Anal. ($C_{24}H_{41}N_5O_6$) C, H, N.

***N*-Isovaleryl-L-valyl-L-valyl-4(*S*)-amino-3(*S*)-hydroxy-5-phenylpentanoic Acid Ethyl Ester (17).** The title compound was prepared according to general procedure B from HCl·$H_2$N-AHPPA-OEt [obtained from Boc-(*S*,*S*)-AHPPA-OEt[28] (34 mg, 0.10 mmol), enantiomeric purity ee = 95%], HOBt (15.5 mg, 0.10 mmol), DCC (21 mg, 0.10 mmol), and DIEA (18 μL, 0.1 mmol) in $CH_2Cl_2$–DMF (2.5 mL, 6:1). The reaction mixture was concentrated under reduced pressure, and the crude product obtained was purified by chromatography on silica gel ($CH_2Cl_2$–MeOH 98.5:1.5). A white solid (50 mg, 96%) was obtained which showed a single spot on TLC: mp 238–240 °C; $R_f$ = 0.38 (A); $[\alpha]^{22}_D$ = −83.6 ($c$ = 0.07, MeOH); $^1$H NMR (DMSO-$d_6$) δ 0.7–0.95 (m, 18 H), 1.10 (t, $J$ = 7 Hz; 3 H), 1.8–2.05 (m, 5 H), 2.24 (dd, $J$ = 9, 16 Hz; 1 H), 2.34 (dd, $J$ = 3, 16 Hz; 1 H), 2.56 (dd, $J$ = 8, 14 Hz; 1 H), 2.84 (dd, $J$ = 6, 14 Hz; 1 H), 3.9–4.05 (m, 4 H), 4.09 (m, 1 H), 4.13 (m, 1 H), 5.20 (d, $J$ = 8 Hz; 1 H), 7.1–7.25 (m, 5 H), 7.66 (d, $J$ = 9 Hz; 1 H), 7.72 (d, $J$ = 9 Hz; 1 H), 7.84 (d, $J$ = 9 Hz; 1 H). Anal. ($C_{28}H_{45}N_3O_6$) C, H, N.

**Acknowledgment.** This work was supported by research funds provided by the National Institutes of Health (DK10200). We thank Prof. Theo Hofmann for a generous sample of penicillopepsin and Dr. Jim Blodgett for his kindly assistance with the kinetic assay.

**Registry No.** **3**, 84062-22-6; **7**, 120742-06-5; **7**·TFA (BOC-deblocked), 120742-14-5; **9**, 79950-65-5; **10**, 120742-07-6; **11**, 120742-08-7; **12**, 120742-09-8; **13**, 59452-57-2; **14**, 120742-10-1; **15**, 120849-35-6; **16**, 120742-11-2; **17**, 120742-12-3; **18**, 120849-36-7; (*R*)-(+)-PEI, 33375-06-3; (*S*)-(−)-PEI, 14649-03-7; (*R*)-$H_3$CCH(Ph)NHCO-HiSta(π-BOM)-OMe, 120742-15-6; (*S*)-$H_3$CCH(Ph)NHCO-HiSta(π-BOM)-OMe, 120849-37-8; BOC-Sta-OEt, 67010-43-9; H-Sta-OEt·HCl, 84851-46-7; Iva-Val-D-Val-OH, 63986-08-3; H-HiSta(π-BOM)-OMe·HCl, 120742-16-7; BOC-AHPPA-OEt, 72155-46-5; H-AHPPA-OEt·HCl, 120742-17-8; 2,2-dimethyl-1,3-dioxane-4,6-dione, 2033-24-1; penicillopepsin, 9074-08-2.