Inhibition of Porcine Pepsin by Two Substrate Analogues Containing Statine. The Effect of Histidine at the P_2 Subsite on the Inhibition of Aspartic Proteinases

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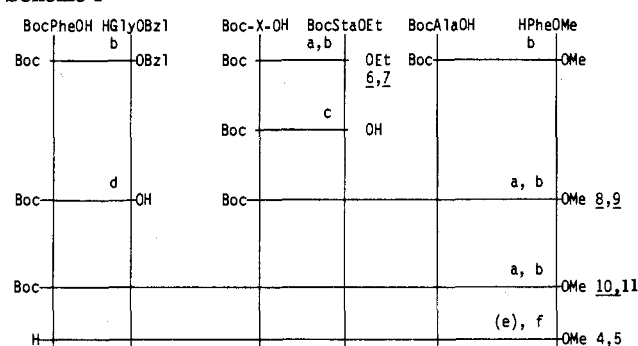
Two new inhibitors, 4 and 5, of the aspartic proteinase porcine pepsin were synthesized. These compounds, which span the P_4-P'_3 binding subsites of the enzyme, were derived by replacing the Nph-Phe dipeptidyl unit of a good pepsin substrate, H_2N-Phe-Gly-His-Nph-Phe-Ala-Phe-OMe (**3**), with statine [(3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid, Sta]. Hexapeptide **5**, H_2N-Phe-Gly-Val-(*S*,*S*)-Sta-Ala-Phe-OMe, is an extremely potent inhibitor of pepsin with a K_i value < 1 nM. This result is consistent with the proposal that statine functions as a bioisosteric replacement for a substrate dipeptidyl unit. Compound 4, which contains His at P_2, is 2 orders of magnitude less active than the valine analogue **5** (K_i = 150 nM). The factor for the decrease in binding to pepsin effected by replacement of Val by His at P_2 parallels the ratio of protonated vs unprotonated imidazole group in peptide 4 at pH 4, according to the Henderson–Hasselbach equation. This result suggests that a positively charged side chain at P_2 is undesirable for maximum pepsin inhibition. Kinetic constants for several known inhibitors of pepsin and renin are presented that demonstrate that the effect of His incorporation at P_2 on pepsin inhibition depends upon the peptide sequence and that the effect is considerably different for renin inhibitors. We further suggest that the high selectivity of potent renin inhibitors known to be only weak pepsin and cathepsin D inhibitors is due in part to the extent of histidine protonation at P_2 arising from pH differences in the inhibition kinetics assay of renin (neutral conditions) compared to other aspartic proteinases (acid pH 2–4).

The aspartic proteinase pepsin is strongly and reversibly inhibited by the naturally occurring pentapeptide pepstatin [Iva-Val-Val-Sta-Ala-Sta-OH, (1)],[1,2] the first general inhibitor[3-7] of this class of enzymes, which includes renin, penicillopepsin, cathepsin D, and others. Pepstatin, which contains the unusual γ-amino acid (3*S*,4*S*)-statine (**2**), is one of the most potent inhibitors of porcine pepsin (K_i = 0.056 nM)[3,7] reported. Several studies with analogues of 1 have demonstrated the importance of the central statine residue for tight-binding inhibition of pepsin[6,8] and other aspartic proteinases.[9] The proposal that statine might be a transition-state analogue of the scissile dipeptide unit of a substrate, which was based on the structural similarities between pepstatin and enzyme substrate sequences[10] and the possible reaction pathway intermediate,[11,12] respectively, was supported by ^{13}C NMR studies[13] and by the crystal structures of pepstatin bound to *Rhizopus chinensis* pepsin[14] and penicillopepsin.[15] Boger et al.[16] extended this hypothesis by computer-assisted molecular modeling based on conformational data of the pepstatin–*R. chinensis* pepsin complex, suggesting that statine could function as a dipeptide isostere of a tetrahedral intermediate for the hydrolysis of a substrate occupying the S_1-S'_1 binding site (definition by Schechter and Berger[17]) of the enzyme.[18] This concept of functional isosterism or bio isosterism was successfully used to syn-

Scheme I[a]



4; X = His

6,8,10; X = His(π-BOM)

5,7,9,11; X = Val

[a] (a) 4 N HCl/dioxane, (b) DCC/HOBt, (c) NaOH, (d) H_2, Pd/C, (e) H_2, Pd/C, 80% AcOH, (f) HCl/EtAc, -20 °C.

(1) Abbreviations used follow the IUPAC–UIB Commission on Biochemical Nomenclature recommendation. Additional abbreviations are as follows: ACHPA, (3*S*,4*S*)-4-amino-3-hydroxy-5-cyclohexylpentanoic acid; AHPPA, (3*S*,4*S*)-4-amino-3-hydroxy-5-phenylpentanoic acid; Boc, *tert*-butyloxycarbonyl; DCC, dicyclohexylcarbodiimide; DCU, dicyclohexylurea; DIEA, diisopropylethylamine; DMF, dimethylformamide; HOBt, 1-hydroxybenzotriazole; Iaa, isoamylamide; Iva, isovaleryl; Nph, 4'-nitrophenylalanine; π-BOM, 3'-(benzyloxy)methyl; Sta, 4-amino-3-hydroxy-6-methylheptanoic acid (statine).

(2) Umezawa, H.; Aoyagi, T.; Morishima, H.; Matusaki, M.; Hamada, H.; Takeuchi, T. *J. Antibiot.* **1970**, *23*, 259–262.
(3) Kunimoto, S.; Aoyagi, T.; Nishizawa, R.; Komai, T.; Takeuchi, T.; Umezawa, H. *J. Antibiot.* **1974**, *27*, 413–418.
(4) Aoyagi, T. In *Bioactive Peptides Produced by Microorganisms*; Umezawa, H., Takita, T., Shiba, T., Eds.; Halsted: New York, 1978; pp 129–151.
(5) Barrett, A. J.; Dingle, J. T. *Biochem. J.* **1972**, *127*, 439–441.
(6) Knight, C. G.; Barrett, A. J. *Biochem. J.* **1976**, *155*, 117–125.
(7) Workman, R. J.; Burkitt, D. W. *Arch. Biochem. Biophys.* **1979**, *194*, 157–164.
(8) (a) Rich, D. H.; Sun, E. T. O.; Ulm, E. *J. Med. Chem.* **1980**, *23*, 27–33. (b) Rich, D. H.; Bernatowicz, M. S. *J. Med. Chem.* **1982**, *25*, 791–795. (c) Rich, D. H.; Bernatowicz, M. S. *J. Org. Chem.* **1983**, *48*, 1999–2001. (d) Rich, D. H.; Salituro, R. G. *J. Med. Chem.* **1983**, *26*, 904–910.
(9) Rich, D. H. In *Proteinase Inhibitors*; Barrett, A. J., Salverson, G., Eds.; Elsevier: New York, 1986; pp 179–217.
(10) Powers, J. C.; Harley, A. D.; Myers, D. V. In *Acid Proteases-Structure, Function and Biology*; Tang, J., Ed.; Plenum: New York, 1977; pp 141–157.
(11) Marciniszyn, J., Jr.; Hartsuck, J. A.; Tang, J. *J. Biol. Chem.* **1976**, *251*, 7088–7094.
(12) Marshall, G. R. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1976**, *35*, 2494–2501.
(13) Rich, D. H.; Bernatowicz, M. S.; Schmidt, P. G. *J. Am. Chem. Soc.* **1982**, *104*, 3535–3536.
(14) Bott, R.; Subramanian, E.; Davies, D. R. *Biochemistry* **1982**, *21*, 6956–6962.
(15) James, M. N. G.; Sielecki, A.; Salituro, F.; Rich, D. H.; Hofmann, T. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 6137–6141.
(16) Boger, J. In *Peptides Structure and Function*; Proceedings of the Eighth American Peptide Symposium; Hruby, V. J., Rich. D. H., Eds.; Pierce Chemical Co.: Rockford, IL, 1983; pp 569–578.
(17) Schechter, I.; Berger, A. *Biochem. Biophys. Res. Commun.* **1967**, *27*, 157.
(18) Rich, D. H. *J. Med. Chem.* **1985**, *28*, 263–273.

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Table I. Physical Constants and Enzyme Inhibition of Porcine Pepsin by Statine-Containing Analogues of Substrate **3**

no.	compound	mp, °C	yield,[a] %	TLC,[b] R_f	molecular formula	anal.	K_i,[c] nM
3	H_2N-Phe-Gly-His-Nph-Phe-Ala-Phe-OMe						40000[d]
4	H_2N-Phe-Gly-His-(*S*,*S*)-Sta-Ala-Phe-OMe	170–173	27	0.51 (C), 0.63 (E)	$C_{38}H_{54}Cl_2N_8O_8 \cdot 4H_2O$	C, H, N	150
5	H_2N-Phe-Gly-Val-(*S*,*S*)-Sta-Ala-Phe-OMe	130–133	42	0.17 (A), 0.31 (B)	$C_{37}H_{55}ClN_6O_8 \cdot 3H_2O$	C, H, N	<1.0[e,f]

[a] From Boc-(*S*,*S*)-Sta-OEt. [b] Single spot; for solvent systems, see the Experimental Section. [c] Inhibition constants, determined as described[28,30] in 0.02 M sodium acetate buffer (pH 4, 25 °C). [d] Apparent K_m of pepsin substrate at pH 3.5–4.0, 37 °C.[23] [e] Upper limit estimated from IC_{50} value close to total enzyme concentration [E_t]. [f] Time-dependent inhibition, TDI (slow binding $T_{1/2}$ > 30 s).

thesize potent renin inhibitors by incorporating statine (**2**) in place of the scissile dipeptide unit of sequence analogues of the renin substrate angiotensinogen.[19,20] More recently, potent statine-containing inhibitors of the aspartic proteinases chymosin[21] and cathepsin D[22] have been reported that derived from good substrates of these enzymes. Not surprisingly, cathepsin D inhibitors derived from substrates that are also rapidly cleaved by other enzymes like pepsin revealed no inhibition specificity between these enzymes.

We report herein the synthesis of two new inhibitors of porcine pepsin, hexapeptides **4** and **5**, in which the scissile Nph-Phe dipeptide unit of the parent pepsin substrate, H_2N-Phe-Gly-His-Nph-Phe-Ala-Phe-OMe (**3**)[23] was replaced by a statine residue. The synthetic substrate **3**, which is efficiently cleaved by pepsin ($k_{cat.}/K_m$ = 500 mM^{-1} s^{-1}) and which shows some selectivity for the pepsin-catalyzed hydrolysis compared to cathepsin D and, to a lesser extent, to *R. chinensis* pepsin,[23] contains a histidine residue at the P_2 position, which is often found in good pepsin substrates.[23,24] In contrast, several very potent renin inhibitors with His at P_2 have been reported that showed high specificity for renin.[19,25b,d,f,33,34] The considerable difference in pepsin inhibition observed for the new compounds **4** and **5**, differing in the amino acid at P_2 (His vs Val), is compared to reported data for the inhibition of pepsin and renin by related pairs of statine-containing inhibitor sequences, in which His at P_2 was replaced with other amino acids. On the basis of the kinetic data of the new compounds **4** and **5** and on comparative data for the inhibition of pepsin, cathepsin D, and renin by inhibitors containing His at P_2, we suggest that the protonated state of the imidazole group at P_2 is a contributor to the enzymes' specificity.

Results

Chemistry. The new substrate-derived inhibitors **4** and **5** were synthesized in a straightforward manner by stepwise coupling of dipeptide fragments to give the protected precursors **10** and **11**, by using conventional deprotection procedures and the standard DCC/HOBt coupling method[26] (Scheme I). Synthesis of the histidine containing peptide intermediates **6**, **8**, and **10**, starting with the condensation of Boc-His(π-BOM)-OH with H_2N-(*S*,*S*)-Sta-OEt to form the dipeptide **6** (yield 74%), was accomplished by using the $N^{\pi\text{-imid}}$-benzyloxymethyl (π-BOM) group[27] as a semipermanent imidazole protecting group. Reasonable yields were obtained at each coupling step, allowing convenient purification of the products. Unfortunately, selective His deprotection of hexapeptide **10** by hydrogenolysis (10% Pd/C in 80% acetic acid)[27] proved to be very sluggish, as has been observed in other cases depending on the peptide sequence,[27] with 40–50% of compound **10** being recovered after 12 h at room temperature. Complete removal of the π-BOM group was finally achieved by repeating the hydrogenolysis reaction after replacing the Pd catalyst with fresh catalyst to obtain *N*-Boc-protected **10** in only 69% yield. Removal of the π-BOM group of **10** by acidolysis with HBr–trifluoroacetic acid, which has been reported as the method of choice,[27] was avoided because of expected side reactions at the statine moiety. Finally, Boc deprotection with saturated HCl in ethyl acetate at −20 °C gave the hexapeptide hydrochlorides **4** and **5** in

(19) Boger, J.; Lohr, N. S.; Ulm, E. H.; Poe, M.; Blaine, E. H.; Fanelli, G. M.; Lin, T.-Y.; Payne, L. S.; Schorn, T. W.; LaMont, B. I.; Vassil, T. C.; Sabilito, I. I.; Veber, D. F.; Rich, D. H.; Boparai, A. S. *Nature (London)* **1983**, *303*, 81–84.

(20) Boger, J.; Payne, L. S.; Perlow, D. S.; Lohr, N. S.; Poe, M.; Blaine, E. H.; Ulm, E. H.; Schorn, T. W.; LaMont, B. I.; Lin, T.-Y.; Kawai, M.; Rich, D. H.; Veber, D. F. *J. Med. Chem.* **1985**, *28*, 1779–1790.

(21) Powell, M. J.; Holdsworth, R. J.; Baker, T. S.; Titmas, R. C.; Bose, C. C.; Phipps, A.; Eaton, M.; Rolph, C. E.; Vallner, M. J.; Kay, J. In *Aspartic Proteinase and Their Inhibitors*; Kostka, V., Ed.; Walter de Gruyter: Berlin, 1985; pp 479–483.

(22) Agarwal, N. S.; Rich, D. H. *J. Med. Chem.* **1986**, *29*, 2519–2524.

(23) Fruton, J. S. *Adv. Enzymol. Relat. Areas, Mol. Biol.* **1976**, *44*, 1–36.

(24) Medzihradszky, K.; Voynick, I. M.; Medzihradszky-Schweiger, H.; Fruton, J. S. *Biochemistry* **1970**, *9*, 1154–1162.

(25) (a) Evin, G.; Castro, B.; Devin, J.; Ménard, J.; Corvol, P.; Guegan, R.; Diaz, J.; Demarne, H.; Cazaubon, C.; Gagnol, J.-P. In *Peptides Structure and Function*; Proceedings of the Eighth American Peptide Symposium; Hruby, V. J., Rich, D. H., Eds.; Pierce Chemical Co.: Rockford, IL, 1983; pp 583–586. (b) Thaisrivongs, S.; Pals, D. T.; Kati, W. M.; Turner, S. R.; Thomasco, L. M. *J. Med. Chem.* **1985**, *28*, 1555–1558. (c) Boger, J. In *Aspartic Proteinases and Their Inhibitors*; Kostka, V., Ed.; Walter de Gruyter: Berlin, 1985; pp 401–420. (d) Thaisrivongs, S.; Pals, D. T.; Kati, W. M.; Turner, S. R. *J. Med. Chem.* **1986**, *29*, 2088–2093. (e) Thaisrivongs, S.; Pals, D. T.; Lawson, J. A.; Turner, S. R.; Harris, D. W. *J. Med. Chem.* **1987**, *30*, 536–541. (f) Luly, J. R.; Plattner, J. J.; Stein, H.; Yi, N.; Soderquist, J.; Marcotte, P. A.; Kleinert, H. D.; Perum, T. J. *Biochem. Biophys. Res. Commun.* **1987**, *143*, 44–51.

(26) Koenig, W.; Geiger, R. *Chem. Ber.* **1970**, *103*, 788–798.

(27) (a) Brown, T.; Jones, J. H.; Richards, J. D. *J. Chem. Soc., Perkin Trans. 1* **1982**, 1553–1561. (b) Bish, C. J.; Brown, T.; Jones, J. H.; Rajasekharan, K. N. In *Peptides 1984*; Proceedings of the Eighteenth European Peptide Symposium; Ragnarsson, U., Ed.; Almqvist & Wiksell International: Stockholm, Sweden; pp 97–100.

(28) Rich, D. H.; Sun, E. T. O. *Biochem. Pharmacol.* **1980**, *29*, 2205–2212.

(29) (a) Dunn, B. M.; Parten, B.; Jimenez, M.; Rolph, C. E.; Valler, M. J.; Kay, J. In *Aspartic Proteinases and Their Inhibitors*; Kostka, V., Ed.; Walter de Gruyter: Berlin, 1985; pp 221–243. (b) Dunn, B. M.; Kay, J. *Biochem. Soc. Trans.* **1985**, *13*, 1041–1043. (c) Pohl, J.; Dunn, B. M. Tenth American Peptide Symposium, Abstracts, Washington University, St. Louis, MS; May 23–28, 1987.

(30) Cha, S.; Agarwal, R. P.; Parks, R. E., Jr. *Biochem. Pharmacol.* **1975**, *24*, 2187–2197.

(31) Szelke, M.; Jones, D. M.; Atrash, B.; Hallett, A.; Leckie, B. In *Peptides Structure and Function*; Proceedings of the Eighth American Peptide Symposium; Hruby, V. J., Rich, D. H.; Eds.; Pierce Chemical Co.; Rockford, IL, 1983; pp 579–582.

(32) Hollands, T. R.; Voynick, I. M.; Fruton, J. S. *Biochemistry* **1969**, *8*, 575–585.

(33) Szelke, M.; Leckie, B., Hallett, A.; Jones, D. M.; Sueiras, J.; Atrash, B.; Lever, A. F. *Nature (London)* **1982**, *299*, 555–557.

(34) Hallett, A.; Jones, D. M.; Atrash, B.; Szelke, M.; Leckie, B. J.; Beattie, S.; Dunn, B. M.; Valler, M. J.; Rolph, C. E.; Kay, J.; Foundling, S. I.; Wood, S. P.; Pearl, L. H.; Watson, F. E.; Blundell, T. L. In *Aspartic Proteinases and Their Inhibitors*; Kostka, V., Ed.; Walter de Gruyter: Berlin, 1985; pp 467–478.

almost quantitative yield (Table I).

Enzyme Kinetics. Enzyme assays for the inhibitors of porcine pepsin (EC 3.4.23.1) were carried out as described previously,[28] with Lys-Pro-Ala-Glu-Phe-Nph-Arg-Leu[29] as the substrate. The reaction rate was followed by monitoring the decrease in absorbance at 290 nm. In the case of **5** (slow binding), the enzyme and inhibitor were incubated for 10 min prior to addition of substrate. The reaction rates were followed for the first 5–10% of reaction, and the data were plotted as percent activity versus inhibitor concentration. Inhibition constants (K_i) were calculated from IC_{50} values by using the equation of Cha et al.[30]

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

where E_t and S are the concentration of the enzyme and substrate in the assay, respectively, and K_m is the Michaelis constant for the substrate (40 μM).[29] Kinetic analysis showed that the hexapeptides **4** and **5** are effective inhibitors of porcine pepsin but differ considerably in their kinetic behavior (Table I). Compound **4** is over 100 times weaker as an inhibitor of porcine pepsin than **5**. Furthermore, compound **4** is not a slow-binding inhibitor[18] of pepsin. In contrast, **5** binds in a time-dependent manner to the enzyme and is one of the most potent pepsin inhibitors yet described.

Discussion

We have found that the statine-containing inhibitor **5**, which is derived from the pepsin substrate **3** by replacing the His-Phe-Phe unit with a Val-Sta unit, is a remarkably potent inhibitor of the aspartic proteinase pepsin. This result is consistent with the postulate of Boger et al. that the novel γ-amino acid statine mimics a dipeptidyl tetrahedral intermediate in the enzyme-catalyzed cleavage of substrate to products.[16] Thus, replacement of the dipeptide cleavage site in an aspartic proteinase substrate by statine gives an excellent inhibitor of porcine pepsin and complements the results obtained previously with renin, cathepsin D, and chymosin for which very good inhibitors were obtained by the same strategy. Even more remarkable is the fact that the statine-containing inhibitor **5** is a subnanomolar inhibitor in spite of the fact that statine contains a single isobutyl side chain and not the two benzyl side chains found at P_1-P_1' in the substrate. It may well be that even more potent pepsin inhibitors would be found when statine is replaced by either the corresponding benzyl side chain analogue of statine (AHPPA) or the cyclohexylmethyl derivative (ACHPA), either of which could bind more strongly by hydrophobic interactions suggested by the preference of the enzyme for aromatic residues at P_1-P_1' in substrates.

In previous tests of the statine–dipeptidyl isostere hypothesis,[19,22] replacement of the substrate P_1-P_1' sites by statine was all that was needed to obtain very good inhibitors. This pattern was not retained in the case of pepsin; replacement of the Nph-Phe P_1-P_1' unit in **3** with statine produced an inhibitor (**4**), which has a K_i (0.15 μm) much weaker than anticipated from literature precedents. However, when His at P_2 is replaced by Val (**5**), the affinity of the inhibitor ($K_i \leq 1$ nm) is increased more than 150-fold relative to **4**, indicating an unfavorable effect on pepsin inhibition when the inhibitor contains an imidazole side chain in P_2.

The unfavorable effect of histidine at P_2 in pepsin inhibitor **4** cannot be ascribed to steric effects between the His side chain and S_2 of pepsin. For one thing, histidine is often found at P_2 in rapidly hydrolyzed synthetic pepsin substrates such as **3**.[23,32] Furthermore, larger amino acids, such as phenylalanine, which has a benzyl side chain rather than the imidazole methyl side chain, are tolerated at P_2 both in statine-containing inhibitors[8d] and in pepsin inhibitors containing novel Leu-Val isosteric replacements.[38] More likely, the difference between **4** and **5** (and between P_2-Phe derived inhibitors[8d,38]) is due to the basic nature of the histidine imidazole side chain. At the pH of the assay medium, which is maintained at 4 for our pepsin assays, the imidazole ring ($pK_a = 6.8$) of His will be over 99% protonated in the unbound form of peptide **4**. Assuming the Henderson–Hasselbach equation holds at these dilute conditions, at pH 4 the concentration of the unprotonated species available for binding to pepsin is calculated to be about 1% of the total inhibitor concentration. Thus, the corrected K_i for the unprotonated form of **4** is calculated to be about 1 nM, which is very close to the observed K_i of inhibitor **5**, which contains Val in P_2 rather than His and is comparable to inhibitors with Phe at P_2.[8d,38] These results thus suggest that it is the positively charged imidazole side chain at P_2 in inhibitors that destabilizes binding to pepsin.

Many statine-containing inhibitors and closely related analogues (e.g., hydroxyethylene analogues)[9,31] have been synthesized as potential inhibitors of human renin.[16,19,20,25,31,38] One objective of these synthetic studies has been the desire to attain high enzyme selectivity for renin over other mammalian aspartic proteinases, e.g., pepsin or cathepsin D. Several examples of very potent renin inhibitors that are at the same time quite weak inhibitors of pepsin, cathepsin D, and other enzymes are known. No attempt has been reported to rationalize this preference.

Our data suggest that at least part of the selectivity of these inhibitors for renin over pepsin is due to the presence of His in the P_2 position of most renin inhibitors, which destablizes binding to pepsin but not to renin. A review of related pairs of statine-containing inhibitors, one with His, and one without (Table II), demonstrates that His in P_2 invariably decreases binding to pepsin. The effect ranges from a factor of about 5 (for example, **20** vs **21**) to 100 (**14** vs **15**). It is obvious from this data that the effect of His at P_2 depends upon different peptide sequences. These differences probably derive from subtle differences in ionic interactions at several positions along the active-site cleft of pepsin, including the S_2 position, as has been suggested to occur with lysine-P_2-containing substrates.[29c] Recent X-ray analyses of renin inhibitors complexed to the aspartic proteinase endothiapepsin[37] show that the side-chain imidazole of His in P_2 of two *endothia* pepsin inhibitors is oriented in significantly different ways despite the presence of the Asp-77 residue at the P_2 binding site. Such effects, if these occur in pepsin as well, could moderate the unfavorable interaction between His-P_2 of pepsin inhibitors and pepsin to give the range of K_i values observed.

In the case of renin, the effect of His at P_2 is very different. The examples in Table II where the peptides differ by only a single side chain substituent formed by replacing

(35) Rich, D. H.; Sun, E. T. O.; Boparai, A. S. *J. Org. Chem.* **1978**, *43*, 3624–3626.
(36) Salvadori, S.; Sarto, G.; Tomatis, R. *Int. J. Pept. Protein Res.* **1982**, *19*, 536–542.
(37) Foundling, S. I.; Cooper, J.; Watson, F. E.; Cleasby, A.; Pearl, L. H.; Sibande, B. L.; Hemmings, A.; Wood, S. P.; Blundell, T. L.; Valler, M. J.; Norey, C. G.; Kay, J.; Boger, J.; Dunn, B. M.; Leckie, B. J.; Jones, D. J.; Atrash, B.; Hallett, A.; Szelke, M. *Nature (London)* **1987**, *327*, 349–352.
(38) Luly, J. R.; Yi, N.; Soderquist, J.; Stein, H.; Cohen, J.; Perun, T. J.; Plattner, J. J. *J. Med. Chem.* **1987**, *30*, 1609–1616.

Table II. Inhibition of Pepsin and Renin. Comparison of Inhibition Constants[a] of His-P_2-Containing Peptides with Their Corresponding Analogues Modified at the P_2 Subsite

no.	sequence	human renin (pH)	porcine pepsin (pH)	lit.
	P_6 P_5 P_4 P_3 P_2 P_1P_1' P_2' P_3'			
12	Boc-Trp-Trp-Sta-Ala-StaOMe	17 (6.0)	120 (2.0)	25a
13	-------------His------------------------	5.5	850	
14	Boc-Phe-Phe-Sta-Ala-StaOMe	3.5 (6.0)	14 (2.0)	25a
15	-------------His------------------------	1.5	1300	
16	Z-Phe-Val-Sta-Ala-StaOMe	10 (6.0)	60 (2.0)	25a
17	Boc-------His-------------------------	1.8	1300	
18	H_2N-Abu-Pro-Phe-Abu-Sta-Leu-PheOMe	9 (7.4)	<1 (4.0)	25c
19	H_2N-His------------- His------------------NH_2	8	34	
20	Ibu-His-Pro-Phe-Ala-Sta-Leu-PheNH_2	99 (7.4)	4 (4.0)	25c
21	-----------------------His-------------------------	16	23	
22	POA-Leu-Sta-Leu-PheOMe	1700 (7.4)	12 (4.0)	19
23	------- His--------------------------	1100	67	
22	for cathepsin D[b]		35 (4.0)	19
23			3500	

[a] Reported data at given pH of the enzyme kinetic assays. IC_{50} values (nM) are given for compounds 12-17, and K_I values (nM) for compounds 18-23. [b] Inhibition constants for rabbit liver cathepsin.

Table III. Aspartic Proteinase Specificity of Renin Inhibitors

no.	compound	IC_{50}, nM (pH)[a] human renin	porcine pepsin	bovine cathepsin D	lit.
24	ProHisProPheHis-NH / CH_2NH / CO-IleHisLysOH	1.0×10^{-8} (7.0)	-	at 7.0×10^{-4} no inhibition (3.3)	33
		1.0×10^{-8} (7.0)	6.0×10^{-5} [b] (3.1)	4.0×10^{-5} [c] (3.1)	34
25	BocPheHis-NH / CF_2CO-IleAmp / O	1.4×10^{-9} (6.0)	4.2×10^{-5} (2.0)	1.7×10^{-6} (3.2)	25b
26	BocProPhe-N-Me-His-NH / CO-IleAmp / OH	2.6×10^{-10} (6.0)	6.8×10^{-6} (2.0)	6.3×10^{-6} (3.2)	25d
27	BocPheHis-NH / S / $NHCO(CH_2)_2C_6H_5$ / OH	7.0×10^{-9} (6.0)	>1.0×10^{-4} (1.9)	>1.0×10^{-4} (3.1)	25f

[a] Reported compatative data for different aspartic proteinases at given pH of the enzyme kinetic assays. [b] Inhibition constant for human pepsin. [c] Inhibition constant for human cathepsin D.

the P_2 residue with His (e.g., **14** vs **15**, **22** vs **23**), plus other reported data for renin inhibitors,[25d-f] suggest that His at P_2 *increases* binding to renin slightly by a factor of 1-10. Because the pK_a of the His imidazole group is near the pH at which most renin assays are carried out (generally 6.0-7.4), both the protonated and unprotonated imidazole groups are present in comparable concentrations. It is not possible to conclude from these data whether renin prefers the protonated or unprotonated form of the His imidazole group, but if the analogy to pepsin holds, then renin would bind the unprotonated species. The fact that several renin inhibitors have been reported in which Phe successfully replaces His at P_2 is consistent with this observation. NMR studies could be carried out with inhibitors ^{13}C labeled at the imidazole ring to identify the protonated state when these inhibitors bind to human renin to determine this definitely.

Comparative inhibition constants (IC_{50}) of renin inhibitors with His at P_2 (e.g., compounds **24-27**[25b,d,f,33,34]) illustrate that inhibitor selectivity between renin and pepsin or cathepsin D, respectively, can be as high as a factor of 10^4. As shown in Table III, the renin selectivity for inhibitors **24-27** (a factor of 10^3-10^4 in K_i) remarkably parallels the difference in pH (3-4 pH units) of the inhibition kinetics assay for renin and the two other aspartic proteinases. Thus, the extent to which the His residue is protonated under different assay conditions (acidic vs almost neutral pH) appears to be a major factor contributing to the inhibitor selectivity between renin and pepsin, as well as between renin and cathepsin D. It will be interesting to see if X-ray crystal structures of pepsin-inhibitor complexes will provide an explanation of the unfavorable effect of histidine at P_2 in inhibitors of pepsin.

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 200-MHz instrument and COSY NMR spectra were recorded on a Bruker EM-500 instrument. All synthesized compounds gave satisfactory NMR spectra. Chemical shifts are reported as δ units (ppm) relative to tetramethylsilane as internal standard. 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, methylene chloride-methanol-concentrated ammonia,

85:10:1 (v/v/v); C, methylene chloride–methanol–concentrated ammonia, 80:20:2 (v/v/v); D, butanol–acetic acid–water, 4:1:1 (v/v/v); E, butanol–acetic acid–pyridine–water, 15:3:12:10 (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. Kinetic parameters for inhibition of porcine pepsin (Sigma, 3200 units/mg of solid, Lot. 64F-8080) were determined by use of the methods described previously,[28] with synthetic Lys-Pro-Ala-Glu-Phe-Nph-Arg-Leu[29] as substrate. Total enzyme concentration of the inhibition assays was $[E_t]$ = 3.5 nM, and the substrate concentration was [S] = 0.12 mM. Hydrolysis reactions were followed at 290 nm with the use of a Gilford Model 250 spectrometer connected to a Gilford 6051 recorder. Inhibition constants (K_i) were calculated from IC_{50} values.[30]

***N*-(*tert*-Butyloxycarbonyl)-$N^{\pi\text{-imid}}$-(benzyloxycarbonyl)-L-histidyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoic Acid Ethyl Ester (6).** Boc-(*S,S*)-Sta-OEt[35] (230 mg, 0.76 mmol) was deprotected with saturated hydrochloride solution in ethyl acetate (1 mL) at -20 °C for 1 h. Most of the HCl was removed by a stream of dry nitrogen at 0 °C, and the residual solvent was evaporated at reduced pressure. After being dried in vacuo over P_2O_5, the obtained oil was dissolved in CH_2Cl_2–DMF (7 mL, 1:1), and Boc-His(π-BOM)-OH (285 mg, 0.76 mmol), *N*-hydroxybenzotriazole (116 mg, 0.76 mmol), and diisopropylethylamine (132 μL, 0.76 mmol) were added at 0 °C. Dicyclohexylcarbodiimide (172 mg, 0.84 mmol) was then added, and the mixture was stirred at 0 °C for 3 h and at room temperature for 15 h. DCU was removed by filtration, the filtrate was concentrated, and the residue was dissolved in ethyl acetate. The organic phase was washed with saturated $NaHCO_3$ and water, dried over Na_2SO_4, and evaporated. The crude product was purified by silica gel chromatography with CH_2Cl_2–MeOH (95:5) as eluant to give a colorless oil. Precipitation from ether–petroleum ether, filtration, and drying in vacuo gave 315 mg (74%) of the title compound as a white solid: mp 65–69 °C; $[\alpha]^{25}_D$ -34.9 (c 2, MeOH); TLC, R_f 0.58 (B). Anal. Calcd for $C_{29}H_{44}N_4O_7$: C, H, N.

***N*-(*tert*-Butyloxycarbonyl)-$N^{\pi\text{-imid}}$-[(benzyloxy)methyl]-L-histidyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl-L-alanyl-L-phenylalanine Methyl Ester (8).** To a solution of Boc-His(π-BOM)-(*S,S*)-Sta-OEt (6) (280 mg, 0.50 mmol) in dioxane–water (2 mL, 2:1) was added 1 N NaOH (1 mL), and the mixture was stirred at room temperature for 1 h. The mixture then was neutralized with 1 N HCl and concentrated in vacuo to remove dioxane. The residue was dissolved in water (10 mL), and the aqueous phase was extracted repeatedly with butanol. The free acid was precipitated from the concentrated butanol solution (5 mL) by adding petroleum ether. The gelatinous precipitate was filtered, washed with petroleum ether, and dried to give 225 mg (85%) of the dipeptide free acid as white solid: mp 225–228 °C dec; TLC, R_f (D) 0.34. Boc-Ala-Phe-OMe [124 mg, 0.35 mmol; with mp 85–86 °C; $[\alpha]^{25}_D$ -20.9 (c 1, MeOH)] was deprotected with saturated HCl in ethyl acetate as described above. The hydrochloride salt of the dipeptide, dissolved in CH_2Cl_2 (7 mL), was added to the suspension of Boc-His(π-BOM)-(*S,S*)-Sta-OH (186 mg, 0.35 mmol) in DMF (7 mL) at 0 °C, followed by neutralization with DIEA (60 μL, 0.35 mmol). Coupling reaction with DCC–HOBt, as described for the preparation of 6, followed by silica gel chromatography (eluant: CH_2Cl_2–MeOH, 97:3) and precipitation from ethyl acetate–*n*-hexane, gave 200 mg (75%) of the title compound as a white solid: mp 79–82 °C; $[\alpha]^{25}_D$ -33.4 (c 0.5, MeOH); TLC, R_f 0.48 (B). Anal. Calcd for $C_{40}H_{56}N_6O_9 \cdot H_2O$: C, H, N.

***N*-(*tert*-Butyloxycarbonyl)-$N^{\pi\text{-imid}}$-[(benzyloxy)methyl]-L-phenylalanylglycyl-L-histidyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl-L-alanyl-L-phenylalanine Methyl Ester (10).** Tetrapeptide 8 (55 mg, 0.072 mmol) was deprotected with 4 N HCl–dioxane for 1 h at room temperature. The solvent was removed under reduced pressure, and the residue was dried in vacuo. The hydrochloride was dissolved in CH_2Cl_2-DMF (3 mL, 1:1) and neutralized with DIEA (12.5 μL, 0.072 mmol) at 0 °C. Boc-Phe-Gly-OH (23 mg, 0.072 mmol), obtained by palladium-catalyzed hydrogenolysis of the corresponding benzyl ester [mp 134–136 °C; $[\alpha]^{25}_D$ -6.1 (c 1.2, MeOH) (lit.[36] mp 136–137 °C; $[\alpha]^{25}_D$ -5.1 (c 1, MeOH)], was added to the solution, followed by HOBt (11 mg, 0.072 mmol) and DCC (18 mg, 0.087 mmol). Stirring was continued for 3 h at 0 °C and overnight at room temperature. Workup and purification by silica gel chromatography, as described for compound 8, afforded 60 mg (86%) of the title compound: mp 103–108 °C; TLC, R_f 0.27 (A). Anal. Calcd for $C_{51}H_{68}N_8O_{11} \cdot 1.5H_2O$: C, H, N.

***N*-(*tert*-Butyloxycarbonyl)-L-phenylalanylglycyl-L-valyl-4(*S*)amino-3(*S*)-hydroxy-6-methylheptanoyl-L-alanyl-L-phenylalanine Methyl Ester (11).** Via the procedure described for the preparation of 10, the reaction of Boc-Val-(*S,S*)-Sta-Ala-Phe-OMe[8d] (9) (91 mg, 0.15 mmol), after deprotection, with Boc-Phe-Gly-OH (49 mg, 0.15 mmol) in CH_2Cl_2-DMF (7 mL, 2:1), using DCC/HOBt as the coupling reagent, gave the crude hexapeptide, which was purified by silica gel chromatography (eluant: CH_2Cl_2–MeOH, 95:5). After precipitation from methanol–ether, trituration with ether, and drying, 95 mg (78%) of a white solid was obtained: mp 175–177 °C; $[\alpha]^{25}_D$ -41.1 (c 1.2, MeOH); TLC, R_f 0.38 (A). Anal. Calcd for $C_{42}H_{62}N_6O_{10} \cdot H_2O$: C, H, N.

2-Amino-L-phenylalanylglycyl-L-histidyl-4(*S*)-amino-3-(*S*)-hydroxy-6-methylheptanoyl-L-alanyl-L-phenylalanine Methyl Ester Dihydrochloride (4). To the solution of hexapeptide 10 (40 mg, 0.041 mmol) in 80% acetic acid (5 mL) was added 8 mg of 10% palladium on charcoal. Hydrogenolysis was carried out for 12 h at room temperature and under normal pressure (incomplete reaction). The mixture was filtered over Celite, washed with 80% acetic acid, and the filtrate was concentrated under reduced pressure. The hydrogenolysis reaction was then repeated under similar conditions. Purification of the crude product by silica gel chromatography (CH_2Cl_2–MeOH–concentrated NH_3, 90:10:1) and precipitation from ethyl acetate–ether afforded 24 mg (69%) of *N*-Boc-protected 4 [mp 132 °C (begin of melting); $[\alpha]^{25}_D$ -30.1 (c 0.5, MeOH); TLC, R_f 0.58 (C)]. The compound (16 mg, 0.019 mmol) was Boc-deprotected with saturated HCl in ethyl acetate at -20 °C for 1 h. Most of the excess of HCl was removed by a stream of dry nitrogen, thereby concentrating the mixture. A small amount of ether was added, and the obtained precipitate was filtered and repeatedly washed with ether, while the white solid was kept under a solvent blanket. After the solid was dried vigorously in vacuo over P_2O_5, 14 mg (95%) of the title compound was obtained: ^1H NMR (DMSO-d_6, 500 MHz) δ 0.80 and 0.83 (d, *J* = 7 Hz, 6 H), 1.14 (d, *J* = 7 Hz, 3 H), 1.1–1.4 (m, 2 H), 1.51 (m, 1 H), 2.0–2.15 (m, 2 H), 2.8–3.51 (m, 6 H), 3.57 (s, 3 H), 3.7–3.85 (m, 4 H), 4.09 (m, 1 H), 4.28 (m, 1 H), 4.43 (m, 1 H), 4.64 (m, 1 H), 4.95 (m, 1 H), 7.15–7.25 (m, 10 H), 7.65–7.75 (m, 1 H), 7.97 (m, 1 H), 8.3–8.4 (m, 2 H), 8.8–8.9 (m, 2 H).

2-Amino-L-phenylalanylglycyl-L-valyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl-L-alanyl-L-phenylalanine Methyl Ester Hydrochloride (5). Via the procedure described for the preparation of 4, hexapeptide 11 (20 mg, 0.024 mmol) was deprotected with saturated HCl in ethyl acetate, and the title compound was obtained as a white solid: yield 17 mg (95%); ^1H NMR (DMSO-d_6, 500 MHz) δ 0.75–0.85 (m, 12 H), 1.13 (d, *J* = 7 Hz, 3 H), 1.29 (m, 2 H), 1.52 (m, 1 H), 2.00 (m, 1 H), 2.05–2.15 (m, 2 H), 2.9–3.15 (m, 4 H), 3.55 (s, 3 H), 31.75–3.9 (m, 4 H), 4.06 (m, 1 H), 4.23 (m, 1 H), 4.26 (m, 1 H), 4.29 (m, 1 H), 4.85 (m, 1 H), 7.15–7.3 (m, 10 H), 7.57 (m, 1 H), 7.88 (m, 1 H), 8.00 (m, 1 H), 8.05–8.15 (m, 1 H), 8.29 (m, 1 H).

Acknowledgment. This work was supported by a grant from NIH (DK20100). J.M. thanks the Deutsche Forschungs gemeinschaft (F.R.G.) for financial support. We thank Prof. Ben Dunn for a generous sample of pepsin substrate[29] and Karen Rebholz for assistance with the kinetic assay.

Registry No. 3, 50572-79-7; **4**·2HCl, 112151-83-4; **4** (free base), 112317-45-0; **5**·HCl, 112151-84-5; **5** (free base), 112317-46-1; **6**, 112151-85-6; **8**, 112151-86-7; **9**, 84850-92-0; **10**, 112151-87-8; **11**, 112151-88-9; BOC-(*S,S*)-Sta-OEt, 67010-43-9; H-(*S,S*)-Sta-OEt·HCl, 84851-46-7; BOC-His(π-BOM)-OH, 79950-65-5; BOC-His(π-BOM)-(*S,S*)-Sta-OH, 112151-89-0; BOC-Ala-Ph-OMe, 2280-66-2; H-Ala-Phe-OMe·HCl, 2280-75-3; H-His(π-BOM)-(*S,S*)-Sta-Ala-Phe-OMe·HCl, 112151-90-3; BOC-Phe-Gly-OBzl, 42280-29-5; BOC-Phe-Gly-OH, 25616-33-5; BOC-Phe-Gly-His-(*S,S*)-Sta-Ala-Phe-OMe, 112151-91-4; H-His-OH, 71-00-1; pepsin, 9001-75-6; aspartic proteinase, 78169-47-8.