experiments, and Warnell Hall, who prepared the manuscript.

Registry No. (*E*)-**2**, 104575-08-8; (*Z*)-**2**, 104575-09-9; (*E*)-**5**, 76579-59-4; (*Z*)-**5**, 76579-58-3; (*E*)-**6**, 104575-10-2; (*Z*)-**6**, 104575-11-3; (*E*)-**7**, 104575-21-5; (*Z*)-**7**, 104575-12-4; (*E*)-**8**, 104575-22-6; (*Z*)-**8**, 104575-13-5; (*E*)-**9**, 104575-23-7; (*Z*)-**9**, 104575-14-6; **10**, 104575-15-7; **11**, 70354-02-8; **13**, 67106-71-2; **14**, 104575-16-8; **15**, 104575-17-9; **16**, 104575-18-0; **17**, 104575-19-1; **18**, 104575-20-4; MEM chloride, 3970-21-6; 4-$BrC_6H_4OCH_2CH_2NEt_2$, 1823-62-7; benzyl 4-hydroxyphenyl ketone, 2491-32-9; 4-cyanophenol, 767-00-0; 4-bromophenol, 106-41-2; 2-(diethylamino)ethyl chloride hydrochloride, 869-24-9; benzyl chloride, 100-44-7; benzyl cyanide, 140-29-4; ethyl iodide, 75-03-6.

Inhibition of Cathepsin D by Substrate Analogues Containing Statine and by Analogues of Pepstatin

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Five new cathepsin D inhibitors were synthesized and tested as inhibitors of bovine cathepsin D. The compounds were derived by replacing a Phe-Phe dipeptidyl unit of a good cathepsin D substrate, Boc-Phe-Leu-Ala-Phe-Phe-Val-Leu-OR, with statine ((3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid, Sta) or with Sta-Phe. The best inhibitor, Boc-Phe-Leu-Ala-(*S*,*S*)-Sta-Val-Leu-OMe, inhibited cathepsin D with a K_i value of 1.1 nM. In general, the more effective inhibitors were consistent with the proposal that statine functions as a replacement for a dipeptidyl unit. Thirty-five known pepstatin analogues also were evaluated as cathepsin D inhibitors. Substituents in the P_4 and P_3' positions are important for maximal inhibition of this aspartic proteinase, and the P_4 substituent appears more important for inhibition of cathepsin D than for inhibition of other aspartic proteinases.

Cathepsin D (EC 3.4.23.5), an aspartic proteinase first isolated from bovine spleen by Press et al.,[1] is thought to be involved in intracellular tissue degradation.[2] As a result, several attempts to develop synthetic substrates[3] and inhibitors[4] of cathespin D have been reported. At present, the most potent reversible inhibitor of cathepsin D reported (K_i = 0.5 nM)[5] is the pentapeptide inhibitor pepstatin [isovaleryl-L-valyl-L-valyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl-L-alanyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoic acid; Iva-Val-Val-Sta-Ala-Sta (**1**, Figure 1)] first isolated by Umezawa et al.[6]

The importance of the central statine [4(*S*)-amino-3-(*S*)-hydroxy-6-methyl-heptanoyl (sta, **2**)] residue for maximum inhibition of pepsin[5,7a-d] and other aspartic proteinases[8] by pepstatin analogues has focused attention on the possible interactions between this novel amino acid and the active site of the inhibited enzyme. Powers, noting similarities between the structures of pepstatin and good substrates of aspartic proteinases, suggested that statine might replace a dipeptide unit when the inhibitor was bound at the active site of the enzyme.[9] Marciniszyn et al.[10] and Marshall,[11] noting similarities between the structures of statine and possible reaction pathway intermediates, suggested that statine might be a transition-state analogue inhibitor of aspartic proteinases. Boger extended these ideas via molecular modeling methods, describing the close structural similarities between the conformation of pepstatin in the crystal structure of the pepstatin–*Rhizopus chinensis* pepsin complex[12] and a realistic tetrahedral intermediate for hydrolysis of substrate.[13] This new concept that statine could replace a dipeptide tetrahedral intermediate portion of the substrate was used by Boger et al. to synthesize potent renin inhibitors in which the Leu-Val or Leu-Leu units in peptides derived from various angiotensinogen sequences were replaced with statine analogues.[14a,b] Independently, Szelke et al. developed remarkably potent renin inhibitors by incorporating a hydroxyethylene isostere of Leu-Val (**3**) in place of the scissile dipeptide moiety,[15] and the close agreement in potency of the statine-derived and hydroxyethylene-derived inhibitors is consistent with the statine $\simeq$ dipeptide tetrahedral intermediate hypothesis. Rich

(1) Press, E. M.; Porter, R. R.; Cebra, J. *Biochem. J.* **1960**, *74*, 501–514.
(2) Barrett, A. J. In *Proteinases in Mammalian Cells & Tissues*; Barrett, A. J. Ed.; Elsevier/North Holland: Amsterdam/New York, 1977; pp 209–248. Dingle, J. T.; Poole, A. R.; Lazarus, G. S.; Barrett, A. J. *J. Exp. Med.* **1973**, *137*, 1124–1141.
(3) Agarwal, N.; Rich, D. H. *Anal. Biochem.* **1983**, *130*, 158–165.
(4) Lin, T. Y.; Williams, H. R. *J. Biol. Chem.* **1979**, *254*, 11875–11883.
(5) Knight, C. G.; Barrett, A. J. *Biochem. J.* **1976**, *155*, 117–125.
(6) Umezawa, H.; Aoyagi, T.; Morishima, H.; Matusaki, M.; Hamada, H.; Takeuchi, T. *J. Antibiot.* **1970**, *23*, 259–262.
(7) (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.
(8) Rich, D. H. In *Proteinase Inhibitors*; Barrett, A. J., Salverson, G. S., Eds.; Elsevier Science, in press.
(9) 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.
(10) Marciniszyn, J., Jr.; Hartsuck, J. A.; Tang, J. *J. Biol. Chem.* **1976**, *251*, 7088–7094.
(11) Marshall, G. R. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1976**, *35*, 2494–2501.
(12) 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.
(13) Bott, R., Subramanian, E.; Davies, D. R. *Biochemistry* **1982**, *21*, 6956–6962.
(14) (a) 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. (b) 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.
(15) 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.

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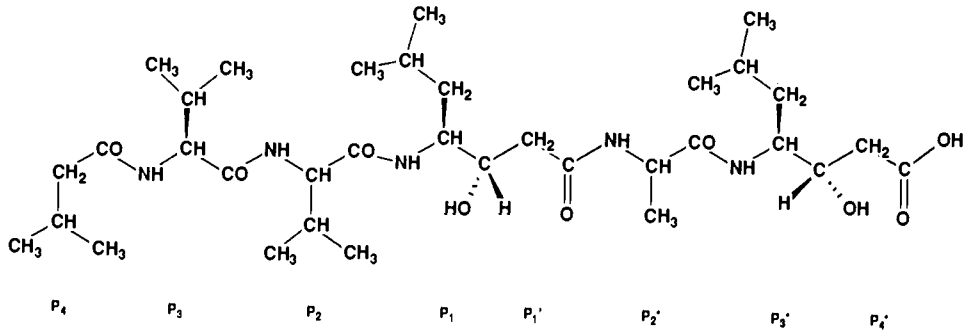

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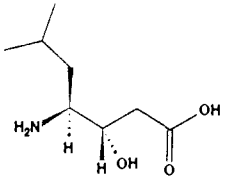
Figure 1. Structure of pepstatin showing the amino acid positions $P_4 \rightarrow P_4'$.

Table I. Inhibition of Three Aspartic Proteinases by Statine-Containing Analogues of Substrates

		K_i, nM							
no.	compound	cathepsin D	pepsin	*R. chinensis* pepsin	mp, °C	yield, %	TLC,[a] R_f	molecular formula	anal.
5	Boc-Phe-Ala-Ala-(*S,S*)-Sta-Phe-Val-Leu-OMe	60	1.7	51	217–219	60	0.30	$C_{49}H_{75}N_7O_{11}$	C, H, N
6	Boc-Phe-Ala-Ala-(*S,S*)-Sta-Val-Leu-OMe	12	2.8	78	207–208	63	0.27	$C_{40}H_{66}N_6O_{10}$	C, H, N
7	Boc-Phe-Leu-Ala-(*S,S*)-Sta-Phe-Val-Leu-OMe	5.8	0.64	9.2	235–236	66	0.41	$C_{52}H_{81}N_7O_{11}\cdot H_2O$	C, H, N
8	Boc-Phe-Leu-Ala-(*S,S*)-Sta-Val-Leu-OMe	1.1	6.1	7.2	184–186	73	0.35	$C_{43}H_{72}N_6O_{10}\cdot 0.5H_2O$	C, H, N
9	Boc-Phe-Phe-Ala-(*S,S*)-Sta-Phe-Val-Leu-OMe	12	24	14	218–220	56	0.48	$C_{55}H_{79}N_7O_{11}\cdot 1.5H_2O$	C, H, N
10	Boc-Phe-Ala-Ala-(*S,S*)-Sta-Ala-Iaa	100	3.3	210	196–197	76	0.27	$C_{36}H_{60}N_6O_8\cdot 0.5H_2O$	C, H, N

[a] $CHCl_3$–MeOH (9:1).

and co-workers have also found that replacement of statine by the hydroxyethylene isostere of [Leu-Ala] produces essentially equipotent inhibitors of pepsin.[16-18] More recently, Powell et al. have reported a potent inhibitor of chymosin devised by replacing the Phe-Met cleavage dipeptide with statine,[19] demonstrating further the utility of this strategy.



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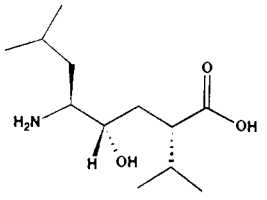
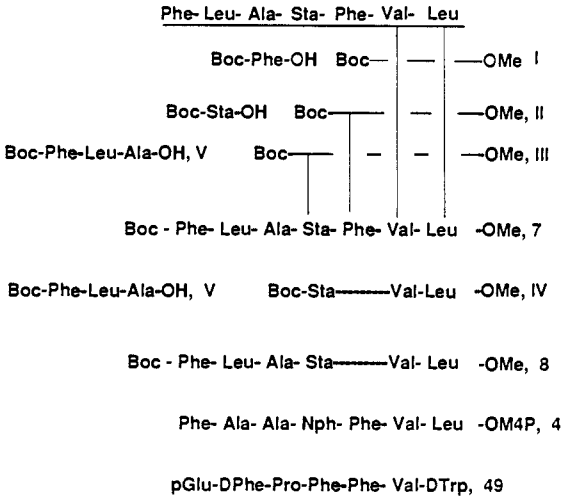


3

In order to evaluate further the generality of replacing dipeptides in a substrate sequence with statine as a strategy for preparing inhibitors, we have synthesized five new substrate-derived inhibitors of cathepsin D in which the scissile dipeptide unit of a low-K_m cathepsin D substrate was replaced by a statine residue or by a Sta-Phe unit. These new cathepsin D inhibitors are compared with reported pepstatin-derived pepsin inhibitors[7-10,16-18,20] for inhibiton of cathepsin D and *R. chinensis* pepsin. All five new compounds are highly potent cathepsin D inhibitors, with the best inhibitors formed by replacing the dipeptidyl unit with statine.

Scheme I



Methods

Chemistry. The new substrate-derived inhibitors incorporating a statine residue were derived from the reported[3] peptide substrate Phe-Ala-Ala-Nph-Phe-Val-Leu-OM4P (4) in which OM4P is the ester derived from

(16) Rich, D. H.; Salituro, Fl. G.; Holladay, M. W. 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 511–520.
(17) Rich, D. H.; Salituro, F. G.; Holladay, M. W.; Schmidt, P. G. In *Conformationally Directed Drug Design*; Vida, J. A., Gordon, M., Eds.; ACS Symposium Series 251; American Chemnical Society: Washington, DC, 1984; pp 211–237.
(18) Rich, D. H. *J. Med. Chem.* **1985**, *28*, 263–273.
(19) 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 Proteinases and Their Inhibitors*; Kosta, V., Ed.; Walter de Gruyter: Berlin, in press.
(20) Rich, D. H.; Bernatowicz, M. S.; Agarwal, N. S.; Kawai, M.; Salituro, F. G.; Schmidt, P. G. *Biochemistry* **1985**, *24*, 3165–3173.

Table II. Inhibition of Cathepsin D by Analogues of Pepstatin

no.	compound	K_i, nM
1	Iva-Val-Val-(3*S*,4*S*)-Sta-Ala-Sta-OH	0.5
11	Iva-Val-Val-(3*S*,4*S*)-Sta-Ala-Iaa[8]	3.7
12	Iva-Val-Val-(3*R*,4*S*)-Sta-Ala-Iaa[18]	>100
13	Boc-Val-Val-(3*S*,4*S*)-Sta-Ala-Iaa[8]	1.1
14	Boc-Val-Val-d-Sta-Ala-Iaa	445
15	Iva-Val-Val-Sto-Ala-Iaa[9]	25
16	Iva-Val-Val-DMS-Sto-Ala-Iaa[9]	107
17	Iva-Val-Val-(3*S*,4*S*)-AHPPA-Ala-Iaa[17,20]	1.0
18	Iva-Val-Val-(3*R*,4*S*)-AHPPA-Ala-Iaa[17,20]	100
19	Boc-Val-Val-(3*S*,4*S*)-AHPPA-Ala-Iaa[17,20]	2.1
20	Boc-Val-Val-(3*R*,4*S*)-AHPPA-Ala-Iaa[17,20]	95
21	Iva-Val-Val-(3*S*,4*S*)-Me[3]Sta-Ala-Iaa[17,20]	400
22	Iva-Val-Val-(3*R*,4*S*)-Me[3]Sta-Ala-Iaa[17,20]	214
23	Boc-Val-Val-(3*S*,4*S*)-Me[3]Sta-Ala-Iaa[17,20]	620
24	Boc-Val-Val-(3*R*,4*S*)-Me[3]Sta-Ala-Iaa[17,20]	31
25	Iva-Val-Val-(3*S*,4*S*)-Me[3]AHPPA-Ala-Iaa[17,20]	2000
26	Iva-Val-Val-(3*R*,4*S*)-Me[3]AHPPA-Ala-Iaa[17,20]	47
27	Boc-Val-(3*S*,4*S*)-Me[3]AHPPA-Ala-Iaa[17,20]	930
28	Boc-Val-Val-(3*R*,4*S*)-Me[3]AHPPA-Ala-Iaa[17,20]	12
29	Iva-Val-Val-(3*S*,4*S*)-Sta-Ala-OMe[10]	90
30	Iva-Val-Val-(3*S*,4*S*)-Sta-OEt[10]	180
31	Iva-Val-(3*S*,4*S*)-Sta-Ala-Iaa[7,8]	220
32	Iva-Val-(3*R*,4*S*)-Sta-Ala-Iaa[7]	2100
33	Iva-Val-d-Sta-Ala-Iaa[7]	1400
34	Boc-Val-(3*S*,4*S*)-Sta-Ala-Iaa[7,8]	2700
35	Boc-Val-(3*S*,4*S*)-AHPPA-Ala-Iaa[7]	370
36	Boc-Val-(3*R*,4*S*)-AHPPA-Ala-Iaa	4860
37	Iva-Val-(3*S*,4*S*)-AHPPA-Ala-Iaa[7]	52
38	Iva-Val-(3*R*,4*S*)-AHPPA-Ala-Iaa	1070
39	*i*-bu-Val-(3*S*,4*S*)-Sta-Ala-Iaa[8]	590
40	2-MeBu-Val-(3*S*,4*S*)-Sta-Ala-Iaa[8]	230
41	*i*-POC-Val-(3*S*,4*S*)-Sta-Ala-Iaa[8]	620
42	*t*-BuAc-Val-(3*S*,4*S*)-Sta-Ala-Iaa[8]	110
43	DiEtAc-Val-(3*S*,4*S*)-Sta-Ala-Iaa[8]	120
44	Iva-Val-(3*S*,4*S*)-Sta-Ala-OMe[10]	1800
45	Iva-Val-(3*S*,4*S*)-Sta-Ala-Leu-OMe[10]	43
46	Iva-Val-(3*S*,4*S*)-Sta-Ala-Phe-OMe[10]	23

4-(hydroxymethyl)pyridine and Nph = (NO_2Phe).[21] Compounds **5**–**10** (Table I) were prepared by condensation of the N-terminal tripeptide with the appropriate C-terminal fragment using the standard DCC/HOBt coupling method[22] (Scheme I). Boc-Sta-Phe-Val-Leu-OMe (III), prepared stepwise from Leu-OMe, was coupled with Boc-Phe-Leu-Ala-OH (V)[3] by using DCC/HOBt.[22] The shorter peptide chain analogue Boc-Phe-Leu-Ala-Sta-Val-Leu-OMe (**8**) was prepared similarly by coupling tripeptide V with Sta-Val-Leu-OMe (IV). Compounds **5** and **6** were synthesized by coupling III and IV, respectively, with Boc-Phe-Ala-Ala-OH.[3] Compound **9** was prepared by coupling Boc-Phe-Phe-Ala-OH[3] with III. Compound **10** resulted from the coupling of Boc-Phe-Ala-Ala-OH[3] with H-Sta-Ala-Iaa.[7] Details of the syntheses of several representative compounds are described in the Experimental Section, and physical constants are listed in Table I.

(21) Abbreviations used follow IUPAC–IUB tentative rules as described in *J. Biol. Chem.* **1972**, *247*, 977. Additional abbreviations used are the following: DCC, dicyclohexylcarbodiimide; DCU, dicyclohexylurea; DMF, dimethylformamide; Boc, *tert*-butyloxycarbonyl; Iva, isovaleryl; Sta, 4-amino-3-hydroxy-6-methylheptanoic acid (statine); Iaa, isoamylamide; *t*-BuAc, *tert*-butylacetyl; *i*-POC, isopropyloxycarbonyl; *i*-bu, isobutyryl; 2-MeBu, 2-methylbutyryl; DiEtAc, diethylacetyl; AHPPA, 4-amino-3-hydroxy-5-phenylpentanoic acid; HOBt, 1-hydroxybenzotriazole; d-Sta, 4-amino-6-methylheptanoic acid (deoxystatine); Nph, 4′-nitrophenylalanine; NMM, *N*-methylmorpholine; TLC, thin-layer chromatography; Sto, 4-amino-3-oxo-6-methylheptanoic acid; DMS-Sto, 4-amino-2-(dimethylsulfenyl)-3-oxo-6-methylheptanoic acid; -OM4P, 4-(hydroxymethyl)pyridine.

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

Most of the pepstatin-derived inhibitors listed in Table II have been reported previously.[7-10] The new pepstatin analogues **14**, **36**, and **38** were prepared by application of the previously described synthetic methods. Physical constants for these compounds are given in the Experimental Section.

Kinetic Assays

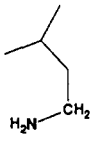
Inhibition constants (K_i) for inhibition of cathepsin D by analogues of pepstatin (Tables I and II) were determined in a manner similar to that described for pepsin.[7,8] IC_{50} values were taken from plots of V_i/V_0 vs. inhibitor concentration, where V_i is the inhibited velocity and V_0 is the velocity in the absence of inhibitor. The substrate used was Phe-Ala-Nph-Phe-Val-Leu-OM4P (**4**).[3] To convert IC_{50} values to K_i values, the equation of Cha et al.[23] was utilized:

$$K_i = [IC_{50} - (E_t/2)][1 + (S/K_m)]^{-1}$$

where E_t is the total enzyme concentration, K_m is the Michaelis constant for the substrate[3] (7.1×10^{-6} M), and S is the substrate concentration. Initial steady-state velocities were taken. No time-dependent or slow-binding inhibition was seen in this system for periods up to 30 min. Inhibitor solutions generally contained ≤1% methanol, v/v. Compound **12** was insufficiently soluble in this solvent system to yield a good estimate for the IC_{50} value. This K_i value is denoted by a > sign in the table.

Results and Discussion

Inhibitors Derived from Pepstatin. A substantial number of pepstatin analogues have been synthesized in recent years and evaluated as inhibitors of pepsin and renin.[7] These compounds were used to help delineate the overall peptide chain length needed to efficiently inhibit cathepsin D. The data in Table II show the effects on K_i as different pepstatin subsites are altered. The most notable outcome of screening these analogues against cathepsin D is that both the P_3' and P_4 subsites contribute substantially to maximum inhibition of cathepsin D (where the convention $P_4 \rightarrow P_4'$ designates amino acid positions to the left (P) and right (P′) of the scissile amide bond in the substrate;[24] Figure 1). For the numbering used here, statine is assumed to span both the P_1 and P_1' positions.[12] At the C-terminus of the inhibitor, deleting the propionic acid portion of the P_3' statine moiety by replacing Sta (**2**) with the C-terminal amide formed from isoamylamine (isoamylamide group, **47**) weakens inhibition seven-fold



47

(**11**, Table II). Further reduction in the size of the P_3' substituent from the isoamylamide group (**47**) in **11** to the methyl ester in **29** decreases binding 180-fold. The importance of this apparent lipophilic binding interaction between the P_3' site and cathepsin D is further demonstrated by tripeptide analogues **44** and **46**, which differ in the substituent in the P_3' position. Replacement of the methyl ester in **44** with phenylalanine (P_3' substituent

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

(24) Schechter, I.; Berger, A. *Biochem. Biophys. Res. Commun.* **1967**, *27*, 157–162.

Phe-OMe) decreases K_i about 100-fold.

The P_4 substituent of pepstatin-derived inhibitors also is important for maximum inhibition of cathepsin D. Removal of a valine from the pepstatin-derived inhibitors leads to the tripeptide inhibitors with the general formula **48** where X–Y = N-terminal blocking group. Compounds

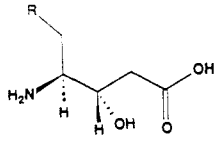
$$\underset{X}{P_4}\ \underset{Y}{P_3}\ \underset{Val}{P_2}\ \underset{Sta}{P_1}\ P_1'\ \underset{Ala}{P_2'}\ \underset{Iaa}{P_3'}$$

48

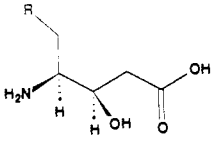
lacking P_4 substituents are ca. 2 orders of magnitude weaker inhibitors (see **31**, **34**, **35**, **39**, **43**). Thus, the P_4 substituent is much more important for inhibiting cathepsin D than it is for inhibiting pepsin.[7-10] It is interesting that addition of bulky substituents to other positions in the inhibitor can compensate for some of the binding interactions between inhibitor and cathepsin D lost by removal of the P_4 substituent. Thus, the inhibitor **37**, which contains the larger 4-amino-3-hydroxy-5-phenylpentanoic acid (AHPPA) residue at P_1, binds 5 times more tightly than the corresponding statine-containing analogue **31**. Similary, compounds **45** and **46**, which contain Leu or Phe at P_3', are 40–80 times improved inhibitors relative to **44** in spite of the lost P_4 substituent. While the P_1 and P_3' substitutions evaluated in this study restore part of the binding lost upon deletion of the P_4 residue, these modifications did not compensate completely for this loss. The best inhibitors of cathepsin D appear to require a peptide chain length spanning P_4 to P_3'. It is interesting to note that the potent substrate-derived cathepsin D inhibitor (K_i = 3 nM) *p*-Glu-DPhe-Pro-Phe-Phe-Val-DTrp (**49**) also spans the $P_4 \rightarrow P_3'$ positions.[4]

The data in Table II also illustrate that favorable interactions between functional groups and enzyme may not be additive when grouped together in one inhibitor structure. In general, we observed that (3*S*)-statine-derived inhibitors containing the *tert*-butyloxycarbonyl group on nitrogen at P_4 are slightly better inhibitors than ones containing an N-terminal isovaleryl group (cf. **13** and **11**). However, the Boc group does not appear to enhance binding relative to the Iva group in inhibitors containing the more bulky AHPPA residue (cf. **19** and **17**) or in the (3*R*)-statine or Me^{3}Sta series of compounds.

The configuration of the hydroxyl group in statine, important for tight-binding inhibition of pepsin[7-9] and human renin,[14] also is important for maximum inhibition of cathepsin D. In both the statine-containing (**50**, **51**) and AHPPA-containing (**52**, **53**) derivatives, the 3(*S*)-hydroxyl

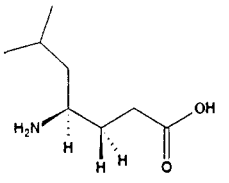


50, R = $CH(CH_3)_2$
52, R = C_6H_5



51, R = $CH(CH_3)_2$
53, R = C_6H_5

compounds **50** and **52** are 10–100 times better inhibitors of cathepsin D than the diastereomeric 3(*R*)-hydroxyl derivatives **51** and **53**, as is evident from a comparison of the K_i data for **11** vs. **12**, **17** vs. **18**, **19** vs. **20**, **31** vs. **32**, **35** vs. **36**, and **37** vs. **38**. Removal of the 3-hydroxy group from statine gives deoxystatine (**54**),[30] which produces a much



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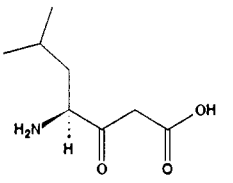

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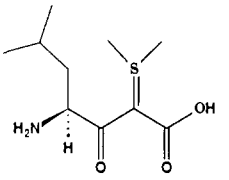
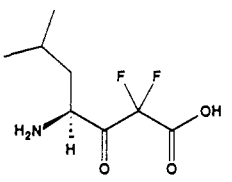


56

poorer inhibitor (compare **13** vs. **14**, **33** vs. **31**), again emphasizing the importance of the hydroxyl group. Oxidation of the 3-hydroxyl group in statine to the corresponding ketone group (Sto, **55**) leads to a sixfold decrease in inhibition (**11** vs. **15**), but even a much more sterically hindered ketone compound, e.g., **16**, which contains a (dimethylsulfenyl)statone (**56**), is a better inhibitor than the deoxystatine derivative **14**. This latter result may suggest a substantial degree of hydration of the ketone in **56**, leading to improved binding to the enzyme in spite of the presence of the dimethylsulfonium group. However, these ketone analogues are clearly much less potent inhibitors than the difluorostatone (**57**) containing analogues of pepstatin and renin substrates, which are excellent inhibitors of pepsin[25] and renin.[26]



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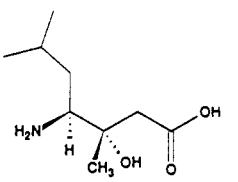
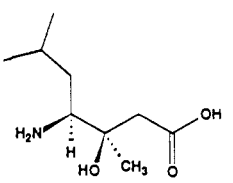


58



59

When a second substituent is added to C-3 of statine-derived inhibitors, the more active diastereomer has the 3*R* configuration (**59**) rather than the 3*S* configuration (**58**). In the 3-methyl statine series, the 3(*R*)-hydroxyl derivatives **22** and **24** are more potent than the 3(*S*)-hydroxyl compounds **21** and **23**. The Me^{3}AHPPA derivatives **25–28** also are stronger inhibitors when C-3 has the *R* configuration. Several reasons for the change in relative potency for the different 3-position diastereomers have been reviewed.[20]

(25) Gelb, M. H.; Svaren, J. P.; Ables, R. M. *Biochemistry* **1985**, *24*, 1813.
(26) Thaisrivongs, S.; Pals, D. T.; Kati, W. M.; Turner, S. R.; Thomasco, L. M. *J. Med. Chem.* **1985**, *28*, 1555–1558.
(27) Evin, G.; Castro, B.; Devin, J.; Ménard, J.; Corool, P.; Guegan, R.; Diaz, J.; Demarne, H.; Cazaubon, C.; Gagnol, J. P. In *Peptides: Structure and Function*; Hruby, V. J., Rich, D. H., Pierce Chemical Co.: Rockford, IL, 1983; pp 583–586.
(28) Lübke, K.; Schröder, E. *Ann. Chem.* **1963**, *665*, 205–212.
(29) Rich, D. H.; Sun, E. T.; Boparai, A. S. *J. Org. Chem.* **1978**, *43*, 3624–3626.
(30) Rich, D. H.; Sun, E. T. O.; Singh, J. *Biochem. Biophys. Res. Commun.* **1977**, *74*, 762–767.

Inhibitors Derived from Substrate Sequences. The major objective of our work with cathepsin D was to determine if the strategy of replacing dipeptide units in substrate sequences with (3*S*,4*S*)-statine would lead to good cathepsin D inhibitors. The methyl ester analogue of Phe-Ala-Ala-Nph-Phe-Val-Leu-OM4P, an efficient substrate for cathepsin D cleaved at the Nph–Phe bond,[3] was modified by replacing the scissile Nph-Phe unit with statine and with a Sta-Phe unit. The corresponding

Boc-Phe-Ala-Ala-Sta-Phe-Val-Leu-OMe, 5

Boc-Phe·Ala-Ala-Sta-------Val·Leu-OMe, 6

Boc-Phe-Leu-Ala-Sta-Phe-Val-Leu-OMe, 7

Boc-Phe-Leu·Ala-Sta-------Val-Leu-OMe, 8

Boc-Phe-Phe-Ala-Sta-Phe-Val-Leu-OMe, 9

Boc-Phe-Ala-Ala-Sta-Ala-Iaa, 10

methyl ester of a related cathepsin D substrate, Phe-Leu-Ala-Nph-Phe-Val-Leu-OM4P,[3] also was modified to produce inhibitors **7** and **8**. The use of leucine rather than alanine in the P_3 position leads to a substrate with an unusually low K_m (1 μm) for this series of compounds. Compound **9** is derived from another cathepsin D substrate sequence, while compound **10**, a substrate–pepstatin hybrid, combines the C-terminal pepstatin sequence with the substrate N-terminal sequence. All of these compounds are capable of spanning the binding region $S_4 \rightarrow S_3'$ required for maximum inhibition of cathepsin D.

Kinetic analysis showed that dipeptide isosteres **6** and **8** are remarkably potent inhibitors of cathepsin D (Table I) in spite of the fact that the isobutyl side chain of statine is smaller than the benzyl side chain present in Phe (or Nph) at the P_1 position of substrate. Consistent with predictions based on the Boger hypothesis that statine replaces a dipeptidyl unit,[12] both compounds **6** and **8** are about 5 times stronger inhibitors of cathepsin D than the longer peptides **5** and **7**. The best inhibitor synthesized in this study (**8**) was obtained by modifying the cathepsin D substrate with the lowest K_m.

The C-terminal peptide sequence of the inhibitors contributes to enzyme–inhibitor binding. The substrate–pepstatin hybrid peptide **10** inhibits cathepsin D about 10 times less strongly than the substrate-derived inhibitor **6**. Whether this is due to the absence of the Val side chain or the carboxy group is not known. The contribution to binding of specific residues in the C-terminal dipeptide fragment appears to be very similar to that found with substrate–pepstatin hybrid inhibitors of human renin.[27] The results in Table I suggest that Val at P_2' is comparable to Ala for inhibition of cathepsin D. A benzyl side chain at P_2' may be too bulky since a fivefold decrease in potency is observed going from **5** to **6** and **7** to **8**. However, it is also possible that the inhibitor molecules **5**, **7**, and **9**, extending from P_5 to P_4', simply are too long to adequately fit the binding pocket. At the other end of the molecule, replacement of Ala by Leu (**5** vs. **7** and **6** vs. **8**) leads to an order of magnitude improvement in potency, indicating the availability of additional hydrophobic binding. But the introduction of a more bulky benzyl side chain at P_3 (compound **9**) results in a twofold weaker inhibitor. It should be noted that these observations about the effects of side-chain substituents in the P_3 and P_2' positions on inhibitor binding are only preliminary and that no attempt has been made to vary the C-terminal or N-terminal peptide side chains to produce highly selective cathepsin D inhibitors.

To our knowledge, compound **8** is the most potent substrate-derived inhibitor of cathepsin D reported so far. It is equipotent with the best pepstatin-derived inhibitors **13** and **17** (Table II) synthesized in this laboratory and half as active as pepstatin itself, and our results suggest that in this enzyme system statine is again acting as a dipeptide isostere. However, while the substrate-derived compounds are potent inhibitors of cathepsin D, they are not nearly as selective (Table I) as the substrate-derived renin inhibitors.[14] Compounds **6** and **8** effectively inhibit pepsin and the fungal protease derived from *R. chinensis* nearly as well as cathepsin D. The potent inhibition of these other aspartic proteinases is not surprising since the starting cathepsin D substrates also are excellent substrates for pepsin and, to a lesser extent, for *R. chinensis* pepsin.

Experimental Section

Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN. Amino acid analyses were determined on a Durrum 500 amino acid analyzer. Melting points were determined on a Fisher-Johns apparatus and are uncorrected. Proton nuclear magnetic resonance spectra were recorded with Bruker 90– or 270-MHz instruments. All synthesized compounds gave satisfactory NMR spectra. TLC was performed on 0.25-mm thickness silica gel plates (Merck, silica gel 60 F-254). For column chromatography, Brinkman silica gel 60, 70–270 mesh, was used. The following TLC solvent systems were used: A, 10% methanol in chloroform (v/v); B, 5% methanol in chloroform (v/v). Compounds were visualized on the plates by UV light, by reaction with ninhydrin and chlorox–*o*-tolidine. Kinetic parameters for inhibition of cathepsin D were measured with synthetic heptapeptide Phe-Ala-Ala-Nph-Phe-Val-Leu-OM4P as described[3] with use of a Gilford Model 250 spectrophotometer connected to a Gilford 6051 recorder. Pepsin inhibition was measured as described previously.[7-10] *R. chinensis* pepsin was assayed by following the hydrolysis of Ac-Ala-Ala-Lys-Nph-Ala-Ala-NH_2 at 296 nm.[31]

***N*-(*tert*-Butyloxycarbonyl)-valyl-leucine Methyl Ester (Boc-Leu-OMe) (I).** A suspension of 5.46 g (30 mmol) of leucine methyl ester hydrochloride in 60 mL of CH_2Cl_2 was neutralized with 3.3 mL (30 mmol) of *N*-methylmorpholine with stirring and cooling. A solution of Boc-Val-OH (6.51 g, 30 mmol) and *N*-hydroxybenzotriazole (4.6 g, 30 mmol) in DMF–CH_2Cl_2 (1:9) was added at 0 °C. Dicyclohexylcarbodiimide (6.2 g, 30 mmol) was then added, and the mixture was stirred at 0 °C for 6 h and at room temperature for 18 h. The separated DCU was removed by filtration, the filtrate was concentrated in vacuo, and the residue was dissolved in ethyl acetate. The organic layer was washed with water, cold 0.1 N NaOH, water, 1 N citric acid, water, 1 N $NaHCO_3$, and water and dried (anhydrous $MgSO_4$) to give 8.51 g (82% of theory) of I after crystallization from Skelly B: mp 135–36.5 °C (lit.[28] mp 125–126 °C); TLC, R_f 0.62 (A).

Boc-Phe-Val-Leu-OMe (II). Boc-Val-Leu-OMe (I) (7.23 g, 21 mmol) was deprotected with 4 N HCl–dioxane for 60 min at room temperature. The solvent was removed under reduced pressure, and the residue was triturated three times with CH_2Cl_2 and dried. Following the procedure described for the preparation of I, the HCl salt of the peptide was neutralized with NMM (2.3 mL, 21 mmol). The reaction of the free dipeptide with Boc-Phe-OH (5.57 g, 21 mmol), using DCC/HOBt as the coupling reagent, gave 8.3 g (80%) of II: mp 153.5–154.5 °C; TLC, R_f 0.5 (A). Anal. Calcd for $C_{26}H_{41}N_3O_6$: C, H, N.

Boc-(*S*,*S*)-Sta-Phe-Val-Leu-OMe (III). Following the procedure described for the preparation of II, the reaction of II (122.75 mg, 0.25 mmol) with Boc-statine[29] (68.75 mg, 0.25 mmol)

(31) Hofmann, T.; Hodges, R. S. *Biochem. J.* **1982**, *203*, 603.

using DCC/HOBt as coupling reagent gave the crude tetrapeptide, which was purified by silica gel chromatography using 2% MeOH in $CHCl_3$ as the eluant. Yield 130.0 mg (80%); mp 118–119 °C; TLC, R_f 0.60 (A). Anal. Calcd for $C_{34}H_{56}N_4O_8$: C, H, N.

Boc-(*S*,*S*)-Sta-Val-Leu-OMe (IV). Following the procedure described for the preparation of II, the reaction of I (120.4 mg, 0.35 mmol) with Boc-statine[29] (96.25 mg, 0.35 mmol) using DCC/HOBt as the coupling reagent gave the crude tripeptide, which was purified by silica gel chromatography (eluant: 1% MeOH in $CHCl_3$). Yield 138 mg (79%); mp 165–166 °C; TLC, R_f 0.47 (A). Anal. Calcd for $C_{25}H_{47}N_3O_7 \cdot 0.5H_2O$: C, H, N.

Boc-Phe-Leu-Ala-(*S*,*S*)-Sta-Phe-Val-Leu-OMe (7). Tetrapeptide III (25 mg, 0.0386 mmol) was deprotected by using 4 N HCl–dioxane as described for the synthesis of II. The hydrochloride salt was dried, dissolved in 3 mL of DMF–CH_2Cl_2 (1:3), and neutralized with 4.25 μL (0.0386 mmol) of NMM. Boc-Phe-Leu-Ala-OH (V)[3] (17.3 mg, 0.0386 mmol), obtained by saponification of the corresponding methyl ester with 0.1 NaOH, and 5.9 mg (0.0386 mmol) of HOBt were added. The mixture was cooled and stirred in an ice bath, 8.0 mg (0.0386 mmol) of DCC was added, and stirring continued for 5 hr at 0 °C and 43 h at room temperature. After filtration and concentration, excess ethyl acetate (30 mL) was added, and the jelly-like material was collected by filtration, washed with water, 1 N $NaHCO_3$, H_2O, saturated $KHSO_4$ solution, and H_2O, and dried in vacuo to give 25.0 mg (66%) of the title compound.

Other peptides reported here were synthesized by closely related procedures employing conventional methods and were purified by recrystallization or silica gel chromatography. Physical constants for the final products are listed in Table I.

***N*-(*tert*-Butyloxycarbonyl)-L-valyl-L-valyl-4(*S*)-amino-6-methylheptanoyl-L-alanine Isoamylamide (14).** This compound was prepared from Boc-Val-OH and Boc-Val-d-Sta-Ala-Iaa[7] in a manner similar to that for the preparation of II. Yield 62%; mp 234–236 °C; TLC, R_f 0.45 (A). Anal. Calcd for $C_{31}H_{59}N_5O_6$: C, H, N.

***N*-(*tert*-Butyloxycarbonyl)-L-valyl-((*S*)-amino-3(*R*)-hydroxy-5-phenylpentanoyl-L-alanine Isoamylamide (36).** The title compound was prepared in an analogous manner to the synthesis of Boc-Val-(3*S*,4*S*)-AHPPA-Ala-Iaa described by Rich et al.[7] Yield 73%; mp 210–212 °C; TLC, R_f 0.19 (B). Anal. Calcd for $C_{29}H_{48}N_4O_6 \cdot 1.5H_2O$: C, H, N.

Isovaleryl-L-valyl-4(*S*)-amino-3(*R*)-hydroxy-5-phenylpentanoyl-L-alanine Isoamylamide (38). This compound was prepared from the hydrochloride salt of **27** and isovaleryl anhydride. Yield 70%; mp >250 °C; TLC, R_f 0.34 (A). Anal. Calcd for $C_{29}H_{48}N_4O_5 \cdot H_2O$: C, H, N.

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Registry No. 1, 26305-03-3; 5, 104487-47-0; 6, 104487-48-1; 7, 104465-35-2; 8, 104465-36-3; 9, 104465-37-4; 10, 104465-38-5; 11, 81921-69-9; 12, 96479-22-0; 13, 81921-68-8; 14, 104487-49-2; 15, 85702-32-5; 16, 85702-30-3; 17, 96411-86-8; 18, 96479-28-6; 19, 96394-75-1; 20, 96443-84-4; 21, 104465-40-9; 22, 104528-70-3; 23, 104465-41-0; 24, 104528-71-4; 25, 104465-42-1; 26, 104528-72-5; 27, 104465-43-2; 28, 104528-73-6; 29, 84851-30-9; 30, 84062-22-6; 31, 72155-64-7; 32, 72174-07-3; 33, 72155-74-9; 34, 72155-63-6; 35, 72155-67-0; 36, 104528-74-7; 37, 75846-13-8; 38, 104528-75-8; 39, 81921-65-5; 40, 104528-76-9; 41, 81921-64-4; 42, 81921-63-3; 43, 81921-67-7; 44, 84851-08-1; 45, 84850-96-4; 46, 84850-93-1; I, 15215-73-3; II, 104465-44-3; III, 104465-45-4; IV, 104465-46-5; V, 61533-27-5; Boc-Val-OH, 13734-41-3; H-Val-Leu-OMe·HCl, 23365-04-0; Boc-Phe-OH, 13734-34-4; H-(*S*,*S*)-Sta-Phe-Val-Leu-OMe·HCl, 104465-47-6; Boc-Val-*d*-Sta-Ala-Iaa, 72155-72-7; cathepsin D, 9025-26-7; leucine methyl ester hydrochloride, 7517-19-3; Boc-statine, 58521-49-6; isovaleryl anhydride, 1468-39-9; pepsin, 9001-75-6.

Synthesis and Calcium Channel Antagonist Activity of Dialkyl 1,4-Dihydro-2,6-dimethyl-4-(pyridinyl)-3,5-pyridinedicarboxylates

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The Hantzsch condensation of alkyl acetoacetates **3** with methyl 3-aminocrotonate (4) and pyridinecarboxaldehydes **5** afforded the unsymmetrical alkyl methyl 1,4-dihydro-2,6-dimethyl-4-(pyridinyl)-3,5-pyridinedicarboxylates **6**, whereas condensation of **3** with **5** and ammonium hydroxide gave the symmetrical dialkyl 1,4-dihydro-2,6-dimethyl-4-(pyridinyl)-3,5-pyridinedicarboxylates **7**. The calcium channel antagonist activities of disubstituted 1,4-dihydro-3,5-pyridinedicarboxylates **6**, **7**, and **9** were determined with use of the muscarinic-receptor-mediated Ca^{2+}-dependent contraction of guinea pig ileal longitudinal smooth muscle. The relative potency order for isomeric pyridinyl analogues **6** and **7** was 2-pyridinyl > 3-pyridinyl > 4-pyridinyl. Increasing the size of the alkyl ester substituents enhanced activity. Compounds having nonidentical ester substituents were more potent than those having identical ester substituents. Replacement of the C-3 and/or C-5 ester substituent(s) by a cyano substituent(s) decreased activity significantly. An approximate 1:1 correlation between the IC_{50} value for inhibition of [^{3}H]nitrendipine binding and inhibition of the tonic component of the muscarinic-induced contractile response was observed. The test results suggest that a 4-(pyridinyl) substituent is bioisosteric with a 4-(nitrophenyl) substituent on a 1,4-dihydropyridine ring system where *o*-, *m*-, and *p*-nitrophenyl are bioisosteric with 2-pyridinyl, 3-pyridinyl, and 4-pyridinyl, respectively.

The 1,4-dihydropyridine calcium channel antagonists nifedipine (**1a**) and its *o*-CF_3 analogue (**1b**) exhibit negative inotropic and marked muscle relaxant properties.[1,2] Nifedipine is used clinically as an antianginal agent in the treatment of ischemic heart disease.

Structure–activity correlations for dialkyl 1,4-dihydro-2,6-dimethyl-4-aryl-3,5-pyridinedicarboxylates indicate that the nature and position of substitution in the aryl ring

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(1) Loev, B.; Goodman, M. M.; Snader, K. M.; Tedeschi, R.; Macko, E. *J. Med. Chem.* **1974**, *17*, 956.
(2) Rodenkirchen, R.; Bayer, R.; Steiner, R.; Bossert, F.; Meyer, H.; Moller, E. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1979**, *310*, 69.

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