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Inhibition of Aspartic Proteases by Pepstatin and 3-Methylstatine Derivatives of Pepstatin. Evidence for Collected-Substrate Enzyme Inhibition†

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ABSTRACT: The synthesis of 10 analogues of pepstatin modified so that statine is replaced by 4-amino-3-hydroxy-3,6-dimethylheptanoic acid (Me^3Sta) or 4-amino-3-hydroxy-3-methyl-5-phenylpentanoic acid (Me^3AHPPA) residues is reported. Both the 3*S*,4*S* and 3*R*,4*S* diastereomers of each analogue were tested as inhibitors of the aspartic proteases, porcine pepsin, cathepsin D, and penicillopepsin. In all cases the 3*R*,4*S* diastereomer (rather than the 3*S*,4*S* diastereomer) of the Me^3Sta and Me^3AHPPA derivatives was found to be the more potent inhibitor of the aspartic protease (K_i = 1.5–10 nM for the best inhibitors), in contrast to the results obtained with statine (Sta) or AHPPA derivatives, where the 3*S*,4*S* diastereomer is the more potent inhibitor for each diastereomeric pair of analogues. The Me^3Sta- and Me^3AHPPA-containing analogues are only about 10-fold less potent than the corresponding statine and AHPPA analogues and 100–1000-fold more potent than the corresponding inhibitors lacking the C-3 hydroxyl group. Difference NMR spectroscopy indicates that the (3*R*,4*S*)-Me^3Sta derivative induces conformational changes in porcine pepsin comparable to those induced by the binding of pepstatin and that the (3*S*,4*S*)-Me^3Sta derivatives do not induce the difference NMR spectrum. These results require that the C-3 methylated analogues of statine-containing peptides must inhibit enzymes by a different mechanism than the corresponding statine peptides. It is proposed that pepstatin and (3*S*)-statine-containing peptides inhibit aspartic proteases by a collected-substrate inhibition mechanism. The enzyme–inhibitor complex is stabilized, relative to pepstatin analogues lacking the C-3 hydroxyl groups, by the favorable entropy derived when enzyme-bound water is returned to bulk solvent. The water is displaced from the enzyme active site by the (3*S*)-statine hydroxyl group. The relative potencies of (3*R*)-statine, (3*S*)-Me^3Sta, and (3*R*)-Me^3Sta are rationalized in terms of the mechanism proposed for pepstatin. The relationship of this type of inhibition mechanism to transition-state-analogue inhibitor mechanisms is discussed.

Enzyme inhibitors can be used effectively to elucidate the nature of enzyme active sites when the geometry of the inhibitor closely approximates the pathway geometry of a reaction intermediate for a particular enzymic reaction. Pepstatin [isovalerylvalylvalyl-(3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoylalanyl-(3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid (Iva-Val-Val-Sta-Ala-Sta) (**1**) (Figure 1)], a tight-binding inhibitor of aspartic proteases discovered by Umezawa et al. (1970), has been shown to bind to the active sites of most aspartic proteases with unusually small dissociation constants (4.57×10^{-11} M in the case of pepsin) (Workman & Burkitt, 1979). The extraordinarily tight binding to this class of proteases led to the hypothesis that pepstatin inhibits by approximating some reaction pathway intermediate, possibly the tetrahedral intermediate for amide bond hydrolysis shown in Figure 2 (Marciniszyn et al., 1976; Marshall, 1976). The relationship between the tetrahedral intermediate and the structure of pepstatin is most clearly evident at the statine residue in the third position of the peptide chain. Pepstatin is postulated to mimic the tetrahedral intermediate by placing the *pro-S* hydroxyl group[1] of statine in a position normally occupied by one of the oxygens of the tetrahedral intermediate produced during amide bond hydrolysis. Experiments with *O*-acetylpepstatin (**2**) and dideoxypepstatin (**3**), both of which are very much weaker inhibitors of pepsin, confirmed the importance of this *pro-S* hydroxyl group, and the latter compound, which is an over 4000-fold weaker inhibitor than pepstatin, established that the

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[1] Abbreviations: Sta, 4-amino-3-hydroxy-6-methylheptanoic acid; Sto, 4-amino-6-methyl-3-oxoheptanoic acid; AHPPA, 4-amino-3-hydroxy-5-phenylpentanoic acid; dSta, 4(*S*)-amino-6-methylheptanoic acid; Iva, isovaleric acid; dideoxypepstatin, Iva-Val-Val-dSta-Ala-dSta; Iaa, isoamylamide; O4MP, 4-(hydroxymethyl)pyridine; Boc, *tert*-butoxycarbonyl; NOE, nuclear Overhauser effect; TLC, thin-layer chromatography. Sta^3 refers to Sta in the third position of the peptide chain in pepstatin; C-3 refers to the third carbon in the backbone of statine or AHPPA. *pro-S* designates a substituent (hydroxyl or methyl) replacing the hydroxyl group in (3*S*,4*S*)-statine; *pro-R* designates a substituent replacing the C-3 proton in (3*S*,4*S*)-statine.

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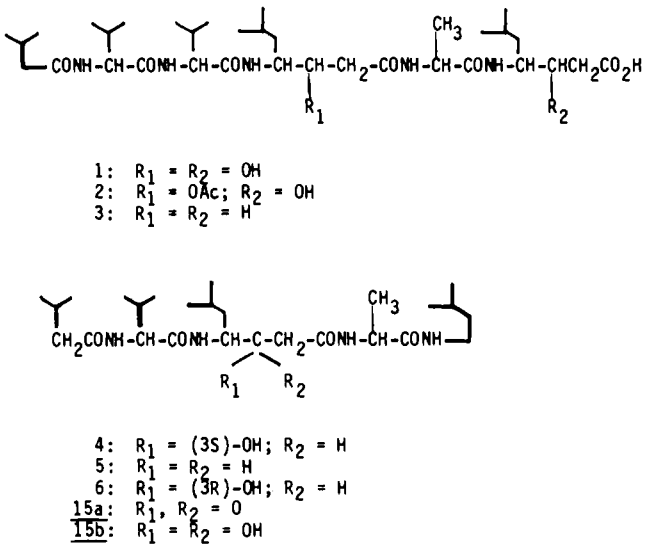
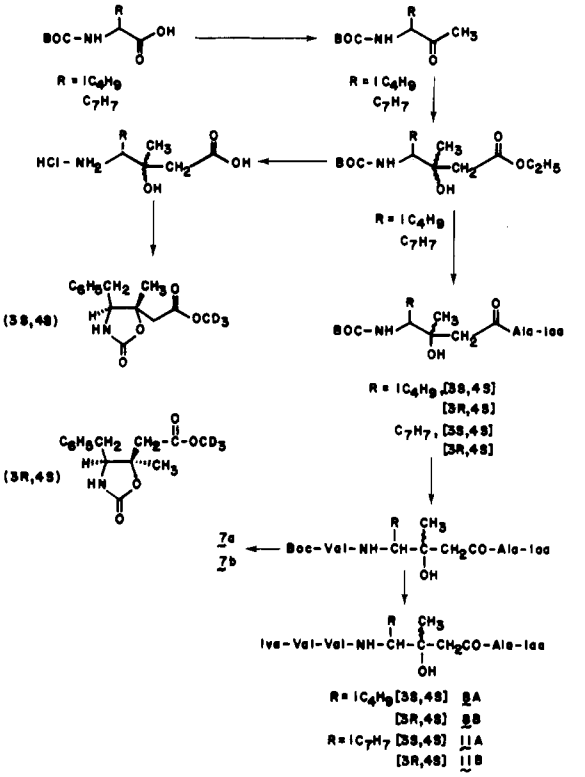


FIGURE 1: Structure of pepstatin and some related analogues.

poor activity of the *O*-acetyl derivative need not be caused by steric hindrance between the inhibitor and the enzyme (Rich et al., 1977). The C-3 *pro-S* hydroxyl group[1] also is essential for binding of shortened analogues to pepsin, e.g., **4**, where removal (**5**) or epimerization (**6**) gave correspondingly weaker inhibitors (Figure 1). Renin inhibitors that contain (3*R*)-statine also are weaker than the corresponding analogues that contain (3*S*)-statine (Boger et al., 1983).

The discovery that the C-3 *pro-R* hydroxyl[1] inhibitors [(3*R*,4*S*)-Sta] are so much weaker than the C-3 *pro-S* analogues has not been satisfactorily explained because a tetrahedral intermediate for amide hydrolysis (Figure 2A) formed by addition of water to the amide carboxyl group possesses two hydroxyl groups and, a priori, the C-3 *pro-R* hydroxyl group of (3*R*)-Sta could replace the *pro-R* hydroxyl group in the tetrahedral intermediate. Subsequent X-ray crystallographic studies have shown that the C-3 *pro-S* hydroxyl group in pepstatin or in the shortened analogue, Iva-Val-Val-(3*S*,4*S*)-Sta-OEt (Rich & Salituro, 1983), is hydrogen bonded to the carboxyl groups of Asp-213 and Asp-33 in penicillopepsin (James et al., 1982) and Asp-220 and Asp-35 in *Rhizopus chinensis* pepsin (Bott et al., 1982), the catalytically active side chain carboxyl groups in these enzymes, but the crystal structures do not identify whether the C-3 *pro-S* hydroxyl group mimics the tetrahedral oxygen that is derived from the substrate carbonyl group (Hofmann et al., 1984) or the enzyme-bound water that adds to the carboxyl group. Bott et al. (1982) proposed a related model of the tetrahedral intermediate for amide bond hydrolysis based on preliminary coordinates for the pepstatin–*Rhizopus* pepsin complex in which the *pro-R* oxygen is hydrogen bonded to Asp-35 but not to Asp-220.

Scheme I



With these models in mind we synthesized two statine analogues in which the proton on carbon 3 was replaced with a methyl group. These new amino acids, 4-amino-3-hydroxy-3,6-dimethylheptanoic acid (Me^{3}Sta) and 4-amino-3-hydroxy-3-methyl-5-phenylpentanoic acid (Me^{3}AHPPA) (Scheme I), were incorporated into the Me^{3}Sta and Me^{3}AHPPA derivatives **7**–**11** of pepstatin. The idea was that the *pro-R* methyl group[1] on C-3 would not interfere with binding to the enzyme through steric interactions between the added methyl group and the protein if the X-ray structure for the pepstatin–*R. chinensis* pepsin was correct and representative of the pepstatin–enzyme interaction with other aspartic proteases. Surprisingly, we found that the (3*R*)-Me^{3}Sta derivatives (which contain *pro-R* hydroxyl and *pro-S* methyl groups) are bound by the enzyme more tightly than the (3*S*)-Me^{3}Sta derivatives. We report herein the synthesis and

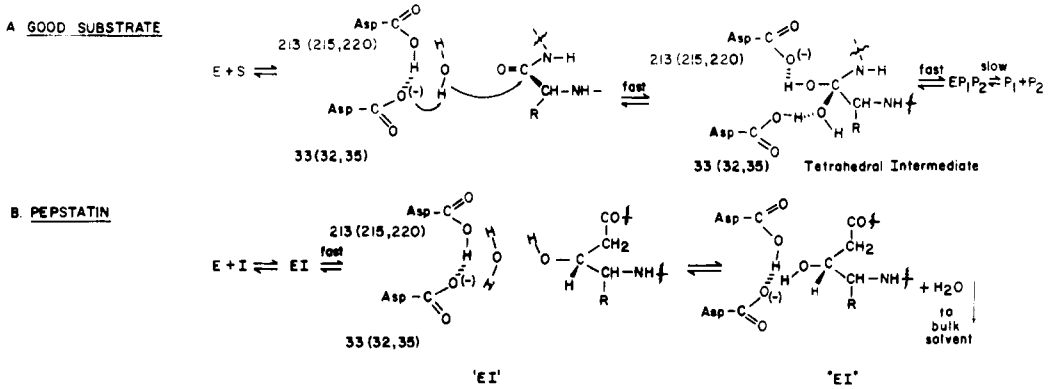


FIGURE 2: Schematic representation of the relationships between proposed catalytic and inhibitory mechanisms: (A) catalytic chemical and kinetic events associated with substrate hydrolysis by pepsin; (B) displacement of enzyme-bound water by statine hydroxyl group during inhibition of pepsin by pepstatin. Variable aspartyl sequence numbers refer to penicillopepsin (pepsin, *Rhizopus* pepsin), respectively.

Table I: 270-MHz NMR Data for Me^3Sta and Me^3AHPPA Peptide Derivatives in Me_2SO-d_6[a]

compd	ppm C-2 H_2	C-3 CH_3	C-3 OH
Boc-Me^3Sta-Ala-Iaa			
3*S*,4*S*	2.20 (14)[b]	1.16	4.90
3*R*,4*S*	2.23 (14)	0.98	5.22
Boc-Val-Me^3Sta-Ala-Iaa			
3*S*,4*S*	2.19 (13)	1.09	5.07
3*R*,4*S*	2.23 (13.5)	1.03	5.20
Boc-Val-Me^3AHPPA-Ala-Iaa			
3*S*,4*S*	2.26 (13.5)	1.22	5.32
3*R*,4*S*	2.32 (14)	1.09	5.47
Boc-Val-Val-Me^3AHPPA-Ala-Iaa			
3*S*,4*S*	2.28 (13.5)	1.16	5.22
3*R*,4*S*	2.28 (14)	1.11	5.50
Iva-Val-Me^3Sta-Ala-Iaa			
3*S*,4*S*	–	–	–
3*R*,4*S*	2.22 (14)	1.03	5.16
Iva-Val-Val-Me^3Sta-Ala-Iaa			
3*S*,4*S*	–	–	–
3*R*,4*S*	2.22 (13.5)	1.02	5.25
Boc-Val-Val-Me^3Sta-Ala-Iaa			
3*S*,4*S*	2.21 (14)	1.07	4.97
3*R*,4*S*	2.22 (13)	1.03	5.20
Iva-Val-Me^3AHPPA-Ala-Iaa			
3*S*,4*S*	2.28 (13)	1.17	5.20
3*R*,4*S*	2.30 (13)	1.12	5.37
Iva-Val-Val-Me^3AHPPA-Ala-Iaa			
3*S*,4*S*	2.27 (13.5)	1.17	5.22
3*R*,4*S*	2.27 (14)	1.10	5.57

[a] A dash indicates that the spectra were not taken because of the insolubility in Me_2SO-d_6 (as well as MeOH-d_4). [b] Numbers in parentheses are *J* values given in hertz.

inhibition constants against pepsin, penicillopepsin, and cathepsin D of a series of pepstatin analogues incorporating Me^3Sta and Me^3AHPPA. Difference NMR spectra establish that the (3*R*)-Me^3Sta analogues induce conformational changes in the enzyme similar to those induced by the binding of pepstatin. However, the different configuration of the hydroxyl group in this new class of aspartic protease inhibitor requires a new set of interactions between enzyme and inhibitor to stabilize binding to the active site of aspartic proteases and is interpreted in terms that pepstatin is a collected-substrate inhibitor of aspartic proteases.

Materials and Methods

The synthesis of Boc-Me^3Sta and Boc-Me^3AHPPA has been described previously (Kawai et al., 1983). The chirality of both diastereomers was determined by converting the free amino acid as a mixture of diastereomers into the oxazolidones, as shown in Scheme I, which were then analyzed by ^{13}C NMR and difference NOE experiments (Kawai et al., 1983). These experiments established the 3*S*,4*S* diastereomer as the major product in both analogues. Each amino acid derivative and each peptide derivative were analyzed further by 270-MHz NMR to determine chemical shift patterns to aid assignment of chirality in peptide products. The partial data shown in Table I established that the 3*S*,4*S* diastereomer consistently gave upfield chemical shifts for the C-2 protons but downfield resonances for the C-3 methyl. The mixture of diastereomeric amino acids was coupled to L-Ala-Iaa. The diastereomeric dipeptides were separated by chromatography and utilized to synthesize the corresponding pepstatin analogues by a reported stepwise approach [Rich & Bernatowicz (1982) and references cited therein]. All compounds were characterized by microanalysis and NMR and were shown to be homogeneous on TLC. Physical constants are reported in Table II.

Kinetic constants were measured by using synthetic peptide substrates incorporating the *p*-nitrophenylalanine group in a spectrophotometric assay (Medzihradszky et al., 1970). For pepsin the synthetic heptapeptide substrate Phe-Gly-His-Phe(NO_2)-Phe-Ala-Phe-OMe was utilized as previously described (Rich & Sun, 1980) by monitoring the increased absorbance at 310 nm with a Gilford Model 250 spectrophotometer and recorder. Cathepsin D activity was monitored by using the synthetic peptide Phe-Ala-Ala-Phe(NO_2)-Phe-Val-Leu-O4MP and assay conditions (25 °C, 0.01 M sodium formate at pH 3.5, 1-cm semimicrocuvettes) as described (Agarwal & Rich, 1983). All solutions were prepared in polypropylene tubes. Penicillopepsin activity was monitored by following the decreased ultraviolet absorbance at 296 nM as the synthetic substrate *N*-Ac-Ala-Ala-Lys-Phe(NO_2)-Ala-Ala(NH_2) is hydrolyzed at Lys-Phe(NO_2) (Hofmann & Hodges, 1982).

Table II: Properties of Synthetic Me^3Sta- and Me^3AHPPA-Containing Peptides

no.	compd	mp (°C, uncor)	TLC R_f[a]	crystn solvent	formula	calcd C	calcd H	calcd N	found C	found H	found N
7A	Iva-Val-(3*S*,4*S*)-Me^3Sta-Ala-Iaa	262–263	0.56	MeOH/Et_2O	$C_{27}H_{52}N_4O_5$	63.25	10.22	10.93	63.48	10.47	10.97
7B	Iva-Val-(3*R*,4*S*)-Me^3Sta-Ala-Iaa	240–242	0.53	MeOH/Et_2O					63.49	10.36	10.92
8A	Iva-Val-Val-(3*S*,4*S*)-Me^3Sta-Ala-Iaa	>280	0.53	insol in Me_2SO or MeOH	$C_{32}H_{61}N_5O_6$	62.81	10.05	11.45	62.95	10.18	11.3
8B	Iva-Val-Val-(3*R*,4*S*)-Me^3Sta-Ala-Iaa		0.51	EtOAc/MeOH/Et_2O (10:2:20)					62.60	10.13	11.26
9A	Boc-Val-Val-(3*S*,4*S*)-Me^3Sta-Ala-Iaa	247–249	0.61	MeOH	$C_{32}H_{61}N_5O_7$	61.21	9.79	11.15	61.36	9.98	11.21
9B	Boc-Val-Val-(3*R*,4*S*)-Me^3Sta-Ala-Iaa	216–219	0.59	chrom[b]					61.09	9.97	11.08
10A	Iva-Val-(3*S*,4*S*)-Me^3AHPPA-Ala-Iaa	256–257		chrom[b]	$C_{30}H_{50}N_4O_5$	65.90	9.22	10.25	65.83	9.27	10.11
10B	Iva-Val-(3*R*,4*S*)-Me^3AHPPA-Ala-Iaa	255–256	0.53	EtOAc/MeOH/Et_2O (10:1:20)							
11A	Iva-Val-Val-(3*S*,4*S*)-Me^3AHPPA-Ala-Iaa	271–272 dec	0.55	chrom[b]	$C_{35}H_{59}N_5O_6$	65.09	9.21	10.84	64.53	9.48	10.73
11B	Iva-Val-Val-(3*R*,4*S*)-Me^3AHPPA-Ala-Iaa	271–272	0.41	MeOH/Et_2O (12:20)					65.13	9.39	10.75
12A	Iva-Val-Val-(3*S*,4*S*)-AHPPA-Ala-Iaa	>250 dec	0.31	MeOH	$C_{34}H_{57}N_5O_6 \cdot {}^1/_2H_2O$	63.75	9.06	10.94	63.60	9.27	10.75
12B	Iva-Val-Val-(3*R*,4*S*)-AHPPA-Ala-Iaa	257–259	0.22	MeOH	$C_{34}H_{57}N_5O_6 \cdot {}^1/_2H_2O$				63.56	8.89	10.88

[a] The solvent system used was 10% methanol in chloroform. [b] Chromatographed, not crystallized.

Table III: Inhibition of Pepsin, Penicillopepsin, and Cathepsin D by Me^3Sta and Me^3AHPPA Analogues

no.	compd	K_i pepsin ($\times 10^9$ M)	K_i cathepsin D ($\times 10^8$ M)	K_i penicillopepsin ($\times 10^9$ M)
7A	Iva-Val-(3*S*,4*S*)-Me^3Sta-Ala-Iaa	10000		
7B	Iva-Val-(3*R*,4*S*)-Me^3Sta-Ala-Iaa	100		
8A	Iva-Val-Val-(3*S*,4*S*)-Me^3Sta-Ala-Iaa	1200	>40[a]	>5000
8B	Iva-Val-Val-(3*R*,4*S*)-Me^3Sta-Ala-Iaa	1.5	21.4	80
9A	Boc-Val-Val-(3*S*,4*S*)-Me^3Sta-Ala-Iaa	>100	>62	
9B	Boc-Val-Val-(3*R*,4*S*)-Me^3Sta-Ala-Iaa	12	3.1	
10A	Boc-Val-Val-(3*S*,4*S*)-Me^3AHPPA-Ala-Iaa	20000	ND[c]	
10B	Boc-Val-Val-(3*R*,4*S*)-Me^3AHPPA-Ala-Iaa	72	ND	
11A	Iva-Val-Val-(3*S*,4*S*)-Me^3AHPPA-Ala-Iaa	2000	93	
11B	Iva-Val-Val-(3*R*,4*S*)-Me^3AHPPA-Ala-Iaa	2.1	1.2	
12A	Iva-Val-Val-(3*S*,4*S*)-AHPPA-Ala-Iaa	0.12	0.096	
12B	Iva-Val-Val-(3*R*,4*S*)-AHPPA-Ala-Iaa	>200	>10	
13A	Iva-Val-Val-(3*S*,4*S*)-Sta-Ala-Iaa	0.1	0.37[b]	6.5
13B	Iva-Val-Val-(3*R*,4*S*)-Sta-Ala-Iaa	100	>100	
4	Iva-Val-(3*S*,4*S*)-Sta-Ala-Iaa	2.9	22	
5	Iva-Val-(4*S*)-dSta-Ala-Iaa	>1000	>500	
6	Iva-Val-(3*R*,4*S*)-Sta-Ala-Iaa	2400	>500	
15	Iva-Val-(4*S*)-Sto-Ala-Iaa	60		
14A	Iva-Val-Val-(3*S*,4*S*)-Sta-Ala-OMe		ND	5
14B	Iva-Val-Val-(3*R*,4*S*)-Sta-Ala-OMe		ND	1000

[a] Inhibition not observed at this concentration. [b] Value for N-terminal Boc group is 0.11. [c] ND, not assayed.

Inhibition constants (K_i) for all analogues (Table III) were determined from IC_{50} values taken from the 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 IC_{50} values were converted to K_i by the equation of Cha (1975): $K_i = (IC_{50} - E_t/2)(1 + S/K_m)^{-1}$, where E_t is the total enzyme concentration, K_m is the Michaelis constant for the substrate, and S is the substrate concentration. In cases where inhibition was time dependent, the inhibitor was incubated with the enzyme for 10–30 min depending on the time needed for equilibration, and the substrate was added to start the reaction. Inhibitors that did not display time-dependent inhibition gave linear, steady-state initial velocities when the reactions were initiated by addition of enzyme. These initial velocities were the same as those produced following preincubation of enzyme with inhibitor and initiation of the reaction by addition of substrate.

Enzymes. Porcine pepsin (lot no. 83C-8080) was purchased from the Sigma Chemical Co., St. Louis, MO, and used without further purification. Cathepsin D (specific activity, 64.3 units/mg) from porcine spleen was a gift from J. Tang. Penicillopepsin was purified as previously described (Hofmann, 1976).

NMR spectra were run at 300 MHz with a CMS 70/50 magnet and 5-mm probe (Cryomagnet Systems, Indianapolis, IN). A Nicolet 1180 computer and an in-house quadrature detection console completed the system. Samples were field frequency locked by using the deuterated solvent's resonances.

Difference spectra, resulting from peptide binding to pepsin, were generated as described previously (Schmidt et al., 1982). Care was taken to match solution conditions (except for the presence of peptide in one of them) for each sample and its control. For NMR experiments porcine pepsin was prepared from pepsinogen as described previously (Schmidt et al., 1982). ^2H_2O (99.96 atom %) and methanol-d_4 (99.5 atom %) were obtained from Aldrich Chemical Co. (Milwaukee, WI).

Results

Synthesis of Peptides. Assignment of Chirality. The Me^3Sta- and Me^3AHPPA-containing analogues were synthesized and their configurations assigned by the routes outlined in Scheme I. Synthetic details for the preparation of amino acid derivatives have been reported (Kawai et al., 1983). The Me^3AHPPA analogues were obtained as a mixture of the 3*R* and 3*S* diastereomers in a ratio of 3:7 (assigned via the oxazolidones) whereas the corresponding Me^3Sta derivatives were obtained in ratios of 1:9 (*R*:*S*). Because we were unable to separate the 3*S*,4*S* diastereomer from the 3*R*,4*S* diastereomer at the protected amino acid stage for either amino acid, each mixture of diastereomers was coupled with L-alanylisoamylamide, to form a mixture of two diastereomers, 3*S*,4*S*-L and 3*R*,4*S*-L, which were separated by column chromatography over silica gel and recovered in 90–95% yield. The ratios of the diastereomers in the Boc-protected starting materials permitted assignment of the chirality in the dipeptide products. Thus the major dipeptide product isolated (63% yield for Me^3AHPPA and 80% yield for Me^3Sta) is the 3*S*,4*S* diastereomer, and the minor product isolated (27% for Me^3AHPPA and 10% for Me^3Sta) is the 3*R*,4*S* diastereomer. The separated peptides were then carried through the remaining steps in Scheme I to afford the final products in optically pure form. Assignments of Me^3Sta and Me^3AHPPA chirality were corroborated throughout the syntheses of the analogues by the observation that the C-2 protons resonated upfield and the C-3 methyl group resonated downfield in the 3*S*,4*S* diastereomers relative to the 3*R*,4*S* diastereomer, both in the amino acid derivatives and in the peptide products (Table I).

Inhibition constants (K_i) for each of the analogues shown in Table III were obtained from the IC_{50} values as described under Materials and Methods. The data show that, for each diastereomeric pair, one peptide is consistently a substantially stronger inhibitor of the aspartic protease, whether it is pepsin, penicillopepsin, or cathepsin D. In the case of the methylated analogues **7A,B–11A,B**, the minor (3*R*,4*S*) diastereomer is the better inhibitor of the pair by a factor of 100–1000. As the peptide chain is lengthened, the inhibitors become stronger and the difference between the inhibition constants for the respective diastereomers becomes greater. For example, extension of the peptide chain by one residue (**7B** vs. **8B**) decreased K_i for pepsin by a factor of about 100. At the same time the difference in K_i between diastereomers increased from 100 to nearly 1000. This same pattern is followed with all the diastereomeric pairs on each enzyme in Table III.

In contrast to the pattern obtained with Me^3Sta derivatives, statine or AHPPA analogues lacking the methyl group at

carbon 3 are better inhibitors when the hydroxyl group has the *pro-S* configuration (Rich & Sun, 1980; Boger et al., 1983). Several additional synthetic Sta and AHPPA analogues extend this observation. (3*S*)-AHPPA peptide **12A** is about a 1000-fold better inhibitor than the corresponding (3*R*)-AHPPA diastereomer **12B**. Similarly, the (3*S*)-statine derivative **13A** is a 1000-fold better inhibitor than the corresponding (3*R*)-statine diastereomer **13B** (Rich & Bernatowicz, 1982; Rich & Sun, 1980). Shorter statine peptides retain the pattern, and the deoxy analogue **5** shows that the poor inhibition of the (3*R*)-statine [or (3*R*)-AHPPA] analogue is not due to steric hindrance between the *pro-R* hydroxyl group and the enzyme.

All analogues inhibited pepsin more efficiently than cathepsin D. Replacement of the isobutyl side chain in Sta with a benzyl group to form AHPPA analogues greatly increases the effectiveness against cathepsin D in contrast to pepsin, where little improvement is gained. Thus AHPPA analogue **12A** is a 4-fold stronger inhibitor than the corresponding Sta derivative **13A**. When the methyl group is added to carbon 3 of the amino acid, the difference between the inhibitors is even greater. The Me^{3}AHPPA derivative **11B** is nearly 20 times more potent than the corresponding Me^{3}Sta derivative **8B**. Other side chain replacements distinguish the two enzymes. The N-terminal Boc derivative **9B** is a better inhibitor of cathepsin D relative to the isovaleryl derivative **8B**. This situation is reversed for pepsin, where the isovaleryl **8B** is the better inhibitor. The results with the N-terminal Boc substituents and the benzyl vs. isobutyl side chain replacements serve to highlight the differences that must exist within the active sites of these aspartic proteases.

Inhibition of penicillopepsin by pepstatin analogues has been shown to require substituents in the P_4–P_3' positions of the inhibitor [F. Salituro, T. Hofmann, and D. H. Rich, unpublished results; designation of residues by P_1, etc., follows the convention introduced by Schechter & Berger (1967)]. Statine can be considered to occupy the P_1–P_1' positions (Boger, 1983; Rich et al., 1984). Thus only a few inhibitors in Table I were expected to be tight-binding inhibitors of penicillopepsin. The results obtained with analogues **8A**, **8B**, **13A**, **14A**, and **14B** showed that inhibition of penicillopepsin follows the pattern found for pepsin and cathepsin D.

The tight-binding Me^{3}Sta and Me^{3}AHPPA pepsin inhibitors were also found to be unusually slow binding inhibitors at saturating inhibitor concentrations. While detailed experiments to determine the half-life for onset of inhibition were not carried out, preincubation for 10–30 min, consistent with half-lives for onset of inhibition of about 2–5 min, was required for maximum inhibition of pepsin, cathepsin D, and penicillopepsin by the (3*R*)-Me^{3}Sta analogues. These times are significantly longer than the 31-s half-life found for binding of pepstatin to pepsin (Rich & Sun, 1980). Thus, the methyl group added to carbon 3 of the statine derivative affects both the rate of binding to the enzyme and the dissociation constant.

NMR Difference Spectra. Iva-Val-(3*S*,4*S*)-Me^{3}Sta-Ala-Iaa added to ^2H_2O (containing 5% methanol-d_4) gives only a hint of resonances in ^{1}H NMR spectra (Figure 3B) because of its low solubility. It is soluble, however, in a ^2H_2O solution containing equimolar porcine pepsin. Figure 3C shows a difference spectrum generated by subtracting a spectrum of 0.5 mM pepsin (plus 5% methanol-d_4) from one for 0.5 mM pepsin plus 0.5 mM 3*S*,4*S* peptide and 5% methanol-d_4. Methyl peaks from the peptide appear clearly in this spectrum near 0.8 ppm. They are broadened and shifted somewhat compared to the compound in methanol (Figure 3A) because

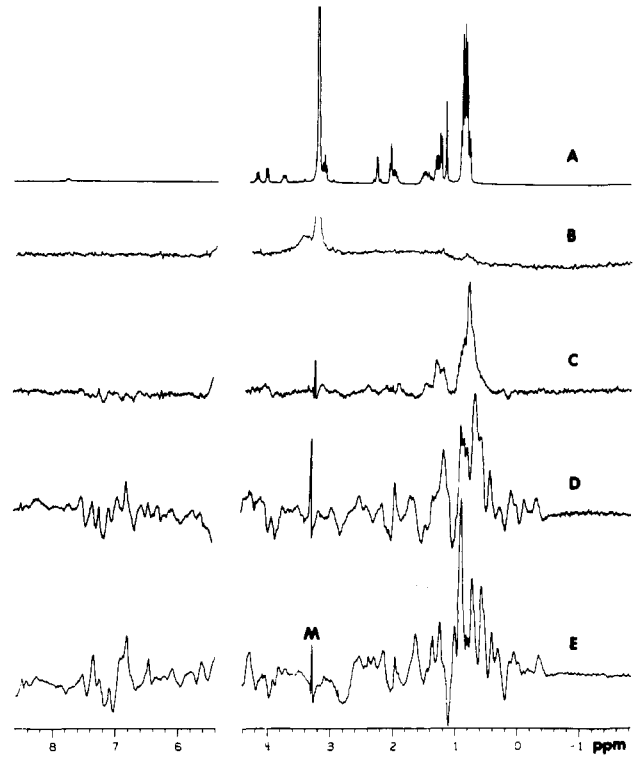


FIGURE 3: 300-MHz ^{1}H NMR spectra of pepsin–pepstatin analogue complexes. (A) Iva-Val-(3*S*,4*S*)-Me^{3}Sta-Ala-Iaa in MeOH-d_4. (B) (3*S*,4*S*)-Me^{3}Sta peptide in ^2H_2O. A 25-μL aliquot of 10 mM peptide stock in MeOH-d_4 was added to 500 μL of ^2H_2O. A total of 400 scans were collected. Spectral conditions: 90° pulses, 1.5-s pulse delay, 2-Hz line broadening, 300 MHz, and 23 °C. (C) Difference spectrum. A sample of 0.5 mM (3*S*,4*S*)-Me^{3}Sta peptide (25 μL of 10 mM stock in MeOH-d_4) was added to 0.5 mM pepsin in ^2H_2O and run under the same NMR spectral conditions as spectrum B. From this was subtracted the spectrum of 0.5 mM pepsin plus 25 μL of MeOH-d_4. (D) Difference spectrum. Conditions were the same as in spectrum C, but the (3*R*,4*S*)-Me^{3}Sta diastereomer was used. (E) Difference spectrum. Conditions were the same as in spectra B and C, but pepstatin A with (3*S*,4*S*)-statine was used. M indicates the residual methanol methyl peak, present in all spectra shown here. Its chemical shift (3.28 ppm) provided an internal shift standard.

the peptide is bound to the enzyme. Other peptide peaks are not so easily identified because of their multiplicities and low amplitudes. When the 3*R*,4*S* isomer is used, a much more complex difference spectrum results. Peaks above and below the base line are indicative of perturbations induced by peptide binding, which generate changes in chemical shifts of protein resonances (Schmidt et al., 1982). Peptide resonances are in this spectrum also, but they are difficult to assign because of changes in the methyl region of the protein manifested in the difference spectrum. The difference spectrum of pepstatin produced by binding to porcine pepsin is given in Figure 3E.

Discussion

Previous studies of pepstatin inhibition of pepsin and other aspartic proteases have established that the C-3 *pro-S* hydroxyl[1] group of statine in pepstatin or pepstatin analogues is essential for tight-binding inhibition of aspartic proteases and that analogues lacking the hydroxyl group (deoxy-Sta analogues), or those with a *pro-R* hydroxyl group, are about 1000-fold weaker inhibitors (Rich et al., 1980; Boger et al., 1983). It was therefore surprising that when a proton at C-3 is replaced by a methyl group in Sta or AHPPA to form Me^{3}Sta- and Me^{3}AHPPA-containing analogues of pepstatin, very potent inhibitors of pepsin, penicillopepsin, and cathepsin D are produced when the hydroxyl group has the *pro-R* configuration. These are remarkably good inhibitors in spite of

the changed hydroxyl configuration. With inhibition constants near 10^{-9} M for pepsin and penicillopepsin, and 10^{-8} M for cathepsin D, (3*R*)-Me^{3}Sta and (3*R*)-Me^{3}AHPPA inhibitors **8B** and **11B** are only about 20-fold weaker inhibitors than the corresponding (3*S*)-Sta and (3*S*)-AHPPA analogues **13A** and **12A** and 2–3 orders of magnitude stronger inhibitors than either the corresponding (3*S*)-Me^{3}Sta diastereomers **8A** and **11A**, the 3*R* diastereomers of Sta or AHPPA (**12B, 13B, 6**), or the deoxy analogues **3** and **5**. Relative to the deoxy-Sta derivatives, the hydroxyl group in the (3*R*)-Me^{3}Sta and (3*R*)-Me^{3}AHPPA inhibitors must stabilize the binding of inhibitor to enzyme. Because the C-3 configuration is reversed, the orientation of the hydroxyl group toward the catalytic aspartic carboxyl groups must be reversed, relative to that between pepstatin and enzyme, or if the hydroxyl group orientation remains the same, the binding of inhibitor positions P_1'–P_3' to enzyme positions S_1'–S_3' must be dramatically different. Either way, some new interaction between enzyme and inhibitor is needed to rationalize the potent inhibition observed.

One possible explanation was that the hydrogen bonding within the active sites of pepsin and cathepsin D differed from that found in the fungal aspartic proteases characterized by X-ray crystallography. However, this was shown not to be the case because the (3*R*)-Me^{3}Sta peptide **8B** is several hundred times more potent an inhibitor of penicillopepsin than the corresponding 3*S* diastereomer **8A**. Furthermore, the (3*S*)-Sta analogue Iva-Val-Val-(3*S*,4*S*)-Sta-Ala-OMe (**14A**) inhibits penicillopepsin several hundred times more strongly than the corresponding 3*R* diastereomer (Table III). Thus the effect of hydroxyl group configuration on inhibition constant in both types of inhibitors is the same for penicillopepsin as it is for pepsin and cathepsin D.

We therefore conclude that (3*R*)-Me^{3}Sta- and (3*R*)-Me^{3}AHPPA-containing analogues of pepstatin inhibit aspartic proteases by a *mechanism in which both the pro-R hydroxyl group and the pro-S methyl group* contribute to binding since the (3*R*)-Sta derivatives that lack the additional methyl group on C-3 are much poorer inhibitors of these enzymes (Table III). To interpret our data, it is first necessary to examine the nature of the interaction between pepstatin and the active site of aspartic proteases.

Is Pepstatin a Collected-Substrate Inhibitor? The relationship between pepstatin's structure and that of the tetrahedral intermediate for amide bond hydrolysis has been noted on several occasions (Marcinizyn et al., 1976; Marshall et al., 1976; Rich et al., 1982a,b, 1984; James et al., 1982; Bott et al., 1982), but the remarkable binding of the inhibitor to aspartic proteases has not been satisfactorily explained. One aspect of transition-state-analogue theory holds that enzymes expend less energy to bind inhibitors that contain structures resembling the geometry of transition states or tetrahedral intermediates (Wolfenden, 1972; Lienhard, 1973). Whereas this effect is no doubt important to the binding of pepstatin, it is insufficient to completely explain the potent binding of pepstatin to aspartic proteases, because dideoxypepstatin **3** (or **5**), which also has tetrahedral geometry at C-3, is a 4000-fold weaker inhibitor of pepsin than pepstatin. Clearly the *pro-S* hydroxyl group of statine contributes up to 4000-fold to the binding of inhibitor to enzyme over and above the peptide–protein interactions. The *pro-S* hydroxyl group multiply hydrogen bonds to the two aspartic acid carboxyl groups in the enzyme active site, but this process is more subtle than it first appears. According to James & Sielecki (1983), the native enzyme also forms multiple hydrogen bonds between the two aspartic acid carboxyl groups and a water molecule. Thus the binding of pepstatin to the enzyme leads to the formation of about the same number of hydrogen bonds to the *pro-S* C-3 hydroxyl group as are broken when the water is expelled from the active site. Hydrogen bonding to the statine *pro-S* hydroxyl group by itself appears insufficient to explain the 4000-fold increase in binding relative to the deoxystatine compounds.

A major contribution to binding must come from the effect of inhibitor binding to the aspartic protease upon the number of bound water molecules (Figure 2B). James & Sielecki (1983) have noted that multiple water molecules are bound in the active site of native penicillopepsin and that these are displaced by inhibitor binding. Return of "bound" water to bulk solvent can be assumed to be the same for both pepstatin and dideoxypepstatin *except* for the water molecule bound between Asp-213 and Asp-33. When the *pro-S* hydroxyl group of statine derivatives is inserted into the space between the two aspartic acid carboxyl groups, a bound water molecule is displaced from the active site, and this "desolvation" increases the interaction between enzyme and statine-containing inhibitor relative to deoxystatine-containing inhibitor by increasing the entropy of water. Return of this single bound water to bulk solvent can affect the free energy of binding enough to account for the much stronger binding of pepstatin relative to the dideoxypepstatin analogue. The increase in entropy derived from the release of bound water to bulk solvent has been estimated to involve between 10 and 16 eu (Jencks, 1976) for 3–5 kcal of energy favorable to inhibitor binding. This amount is consistent with the 4–5-kcal increased binding energy calculated by comparing the dissociation constants of pepstatin with dideoxypepstatin (Table III).[2] This analysis predicts that pepstatin is a stable analogue of the two substrates for amide bond hydrolysis, bound water and the peptide chain. Pepstatin thus is a collected-substrate (bisubstrate; Wolfenden, 1972) inhibitor in addition to an analogue of the tetrahedral intermediate.

The high selectivity between the (3*R*)- and (3*S*)-Me^{3}Sta and -Me^{3}AHPPA diastereomers for inhibition of pepsin, penicillopepsin, and cathepsin D and the surprising finding that analogues with the *pro-R* hydroxyl are the potent inhibitors require a different explanation for the tight binding of these inhibitors to the enzyme active site.[3] The problem is complicated by the fact that any explanation must rationalize both the good inhibition of the (3*R*)-Me^{3}Sta derivatives and the poor inhibition of the (3*R*)-Sta derivatives lacking the C-3 methyl group. Thus, while one can propose rotations about the single bonds connecting C-3 of Sta to the α-carbon of Ala (–CH(OH)–CH$_2$–CONH–CH–) that permit the *pro-R* hydroxyl group on (3*R*)-Me^{3}Sta to form hydrogen bonds to Asp-33 and Asp-213, an examination of molecular models indicates that these rotations markedly displace (1–2 Å) the inhibitor peptide chain atoms toward the -Tyr^{75}-Gly^{76}- residues of the enzyme, two residues that are part of the mobile flap region of these enzymes. The effects of placing a *pro-R* hydroxyl at the point to which the *pro-S* hydroxyl of pepstatin binds are substantial and alter the inhibitor–enzyme interactions far from the corresponding statine positions. Even the

[2] It is possible that steric hindrance between the C-3 proton in pepstatin and the bound water molecule weakens the binding interaction further. Thus the 4–5-kcal difference between **1** and **3** may be the sum of both displacement of bound water and a steric interaction.

[3] Asp-33(35), Asp-213(220): Designation of the position of active site aspartic acid residues in aspartic proteases lists the position in penicillopepsin first; the position in *R. chinensis* pepsin is shown in parentheses.

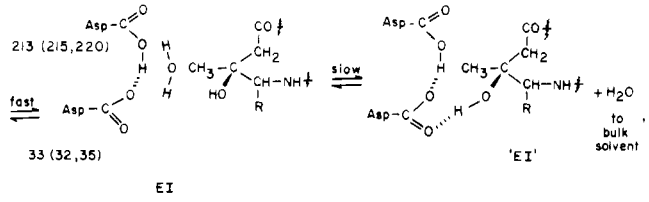


FIGURE 4: Possible collected-substrate mechanism for inhibition of aspartic proteases by (3*R*)-Me^{3}Sta and (3*R*)-Me^{3}AHPPA peptides. This model differs from the mechanism proposed in Figure 2 by the displacement of water by the *pro-S* methyl group and the formation of a new hydrogen bond between the *pro-R* hydroxyl and Asp-33(32) groups.

S_3' NH group is displaced over 1 Å with respect to the position of the same group in pepstatin when bound to *R. chinensis* pepsin. Presumably, these displaced interactions would diminish the number of favorable interactions with the enzyme in P_1'–P_3' region with a substantial loss of binding energy. Molecular models suggest that other binding modes are conceivable that would permit the P_3' NH group to bind to the normal S_3' site, but these conformations force the *pro-R* hydroxyl group to move away from Asp-213 about 0.5–1.0 Å while simultaneously displacing the statine $-CH_2CONH-$ atoms toward the mobile flap. While it may be possible that these displacements of the statine atoms toward the mobile flap are not sterically prohibited by the resulting enzyme conformation and that new, favorable enzyme–inhibitor interactions are formed, none of these binding modes provides an explanation for the 100–1000-fold poorer binding of the (3*R*)-Sta analogues relative to the (3*R*)-Me^{3}Sta analogues. The (3*R*)-Sta derivatives should be able to bind to the enzyme in the same ways as the methylated analogues.

The alternative possibility is that the (3*R*)-Me^{3}Sta inhibitor binds to the enzyme in essentially the same orientation as does pepstatin or the statine tripeptide, with the result that placement of the hydroxyl group is on the *pro-R* side and placement of the methyl group on the *pro-S* side of the inhibitor. That is, in this binding mode, all interactions between enzyme and inhibitor for P_3–P_3' except those at the C-3 position of statine near the Asp-213(220) and Asp-33(32) carboxyl groups would be preserved.

This hypothesis is reasonable only if one of the two major hydrogen bonds between Asp-213(220) or Asp-33(35) and water is replaced by another favorable interaction such as a hydrogen bond. One reasonable replacement would be a hydrogen bond between the *pro-R* hydroxyl group and Asp-33(35) (Figure 4). This hydrogen bond would resemble the probable hydrogen bond between Asp-33(35) and the *pro-R* hydroxyl group in the tetrahedral intermediate for amide bond hydrolysis (Bott et al., 1982). Because the (3*R*)-Me^{3}Sta derivatives are 15–30-fold weaker inhibitors of aspartic proteases than the (3*S*)-statine derivatives, it is not necessary nor likely that the number of hydrogen bonds to the aspartyl carboxyl groups will be the same in the complexes to the methylated inhibitors. We have noted that the (3*R*)-Sta peptides do not bind as efficiently to the enzyme as do the (3*R*)-Me^{3}Sta derivatives. Because the *pro-R* hydroxyl group in the (3*R*)-Sta peptides would be expected to perform the same function as the *pro-R* hydroxyl group in the (3*R*)-Me^{3}Sta peptides in the enzyme–inhibitor complex, then the *pro-S* methyl group must make some *positive* contribution to stabilize the enzyme–inhibitor complex. One possibility is that the methyl group replaces the steric bulk of the deleted *pro-S* hydroxyl group and, at the same time, displaces the bound water molecule. Thus the methyl group prevents water molecules from occupying the void between Asp-213(220) and Asp-33(35) created by the replacement of the *pro-S* hydroxyl group by a proton. This role for the methyl group is not without its problems as it requires the insertion of a hydrophobic group into what is a hydrophilic environment in the reported crystal structures.

It is not yet possible to differentiate experimentally between these two binding modes. If substantial changes in binding of P_1'–P_3' groups to enzyme have occurred, they are not detectable by the difference NMR spectra (vide infra) obtained to date. It is likely that the exact nature of the novel interaction between 3(*R*)-methylstatine (and Me^{3}AHPPA) derivatives will be found only from X-ray crystal structures of the inhibitor–enzyme complexes.

The poor inhibition of the (3*S*)-Me^{3}Sta and (3*S*)-Me^{3}AHPPA analogues also is difficult to explain. We propose it may be caused by either of the following: either there are steric interactions between the *pro-R* C-3 methyl group and enzyme atoms (i.e., computer graphic projections of crystal data may overestimate the available space) or the hydrophobic methyl group that would be close to the Asp-33(32) carboxylate group may desolvate or perturb hydrogen bonds to this negatively charged group. Either of these possibilities would destabilize the complex.

The binding of a ketone analogue of pepstatin to pepsin is consistent with the binding of both *pro-R* and *pro-S* hydroxyl groups formed in the active site of aspartic proteases by hydration of the carbonyl group. Ketone analogue **15A** (Sto, 4-amino-6-methyl-3-oxoheptanoyl; Figure 1) has been shown by ^{13}C NMR studies to bind to porcine pepsin as a tetrahedral species, later shown to be the *gem*-diol **15B** (Rich et al., 1982a, 1984). The dissociation constant (6×10^{-8} M; Rich et al., 1982b) is in the range expected for a *gem*-diol species that can form a hydrogen bond between the *pro-S* hydroxyl group and the Asp-215 and Asp-32 carboxyl groups (Table III). The other, *pro-R* hydroxyl group would occupy the *pro-R* position of a tetrahedral intermediate, but in this case, because the bound water is added to the carbonyl and not released to bulk solvent, the entropic contribution to binding energy discussed previously is not gained. The 20-fold difference between the ketone's inhibition constant (60×10^{-9} M) and that of the corresponding statine analogue (3×10^{-9} M) may indicate the hydrogen bond from Asp-33(32) to the *pro-R* hydroxyl group does not compensate for the loss of the favorable entropy factor, or it may be a reflection of the unfavorable equilibrium constant (0.05) for hydration of a statone derivative. ^{13}C-Labeled statone **15** is more than 95–99% trigonal (**15A**) in buffer; small amounts of the *gem*-diol species (**15B**) were not detectable under the experimental conditions employed so that the equilibrium constant is less than 0.05–0.01. The equilibrium constant for hydration of a carbonyl group in aqueous buffer depends greatly on the structure of the ketone. Acetamidoacetone is hydrated less than 1% in water whereas the equilibrium constant for chloroacetone hydrate formation is 0.08 (Lewis & Wolfenden, 1977). The inductive effect of a carboxamide group is very similar to that of a chlorine atom (pK_a or chloroacetic acid, 2.86; pK_a of malonic acid, 2.83) so that the equilibrium constant for the statone derivative **15** in water in the absence of enzyme would be near 0.08. Thus the K_i observed for the ketone analogue **15** is comparable to that expected if formation of the enzyme–inhibitor complex is destabilized only by the unfavorable equilibrium for hydration of the ketone; the slight difference between estimated and observed values may not be significant or may reflect other equilibria, such as enol formation, that lower hydration.

For the reasons cited previously, our analysis of the binding of the (3*R*)-Me^{3}Sta and (3*R*)-Me^{3}AHPPA analogues to

pepsin, penicillopepsin, and cathepsin D has suggested the possibility that these inhibitors bind to the active site of these enzymes in the same way that pepstatin binds, with the exception of the effects on water and the two catalytically active Asp carboxyl groups. In the absence of X-ray data for complexes of these inhibitors to an aspartic protease, we carried out difference proton NMR binding studies to pepsin for both the (3*R*)- and (3*S*)-Me^{3}Sta analogues **8B** and **8A**, relative to pepstatin, in order to determine if this assumption is reasonable. Pepstatin and several of its analogues, upon binding to pepsin, have been shown to induce protein conformational changes leading to ^{1}H NMR difference spectra (Schmidt et al., 1982). The effect of pepstatin is seen in Figure 3E. Corresponding difference spectra were obtained for analogues **8A** and **8B** and are shown in Figure 4B–D. While some features of spectra D and E of Figure 3 match fairly closely, others do not. In the aromatic region a small shift in the His-53 ϵ-C gives rise to difference peaks near 8.5 ppm in both spectrum D and spectrum E, but the bulk of the difference peaks do not match. We conclude that the (3*R*,4*S*)-Me^{3}Sta peptide induces changes in protein structure when bound but that these changes are not identical with those produced by pepstatin binding, although the differences may be subtle.

Most interesting is the contrast in difference spectra produced by the (3*S*,4*S*)- and (3*R*,4*S*)-Me^{3}Sta diastereomers **8A** and **8B**. The former induces almost no change in the protein while the latter causes substantial shifts. This behavior is opposite to that for the related pair of diastereomers containing Sta, Boc-Val-(3*S*,4*S*)-Sta-Ala-Iaa and Boc-Val-(3*R*,4*S*)-Sta-Ala-Iaa, where the largest effect on the protein difference NMR spectrum is induced by the 3*S*,4*S* species (Schmidt et al., 1982).

The origin of the difference spectra appears to be caused by reorientation of the protein diamagnetic anisotropic groups such as aromatic rings and carbonyls (Schmidt et al., 1982). X-ray crystallographic data show that binding of the inhibitor Iva-Val-Val-Sta-OEt to penicillopepsin induces a conformational change in the enzyme for a portion of the structure called the "β-flap" that includes residues 70–80 in pepsin (James et al., 1982). This movement is associated with a closing of the flap onto the inhibitor. Tyr-75 is on this flap in pepsin, and its ring is part of the hydrophobic pocket for the aliphatic side chain of the Sta^3 residue in pepstatin (Bott et al., 1982; James et al., 1982). Tyr-75 lies very close to Phe-111 and to Trp-39 (the ring centers are 5–7 Å apart). These three residues are conserved in at least four aspartic protease sequences including pepsin and penicillopepsin (Tang et al., 1973; Foltmann & Pederson, 1977). Aromatic rings in such close proximity will mutually affect each other's chemical shifts through the ring current anisotropy effect. With 14 protons between them, movement of the Tyr-75 side chain upon pepstatin binding could provide difference spectra wherein about 10 net protons are shifted. Because the added methyl group in the Me^{3}Sta analogues would perturb the final resting point of the inhibitor in the "closed" complex, exact identity between the statine analogue–pepsin difference spectra and the Me^{3}Sta analogue–pepsin spectra would not be expected. Instead, one would expect that the difference spectra in the aromatic region of the NMR spectra would be indicative of a partially closed flap. In view of the tight binding of these inhibitors (Table III) it is likely that the difference NMR spectra indicate that the (3*R*)-Me^{3}Sta derivative binds to pepsin in a closed complex closely analogous to that observed with the (3*S*)-Sta analogues. In contrast, the (3*S*)-Me^{3}Sta diastereomers are clearly bound but in a complex that does not produce the difference in the aromatic region of the NMR; hence, it can be inferred that the β-flap is not closed.

The discovery that the (3*R*)-Me^{3}Sta and (3*R*)-Me^{3}AHPPA analogues of pepstatin are potent inhibitors of aspartic proteases has at least three important implications. First is the fact that these inhibitors must inhibit the enzyme by a novel mechanism. We propose that pepstatin derives a substantial portion of its binding via a collected-substrate–inhibitor mechanism (Figure 2B) and that the (3*R*)-Me^{3}Sta and (3*R*)-Me^{3}AHPPA derivatives inhibit the enzymes via a modified collected-substrate mechanism in which either the *pro-R* hydroxyl group hydrogen bonds to Asp-33 and the *pro-S* methyl group displaces bound water (Figure 4) or else the 3(*R*)-hydroxyl binds at the solvent site and the P_1'–P_3' residues form new interactions with the enzyme. In either case, the primary driving force for tight binding relative to the corresponding deoxy-Sta derivatives is the entropy generated by the return of bound water to bulk solvent. Presumably, the hydroxyethylene dipeptide isosteres (Szelke et al., 1983; Holladay & Rich, 1983; Rich et al., 1984) also inhibit aspartic proteases in part by this mechanism. This is a novel inhibition mechanism that apparently has not been described for another enzyme–inhibitor system although it is possible other putative transition-state-analogue inhibitors that mimic tetrahedral hydration intermediates, e.g., coformycin (Cha et al., 1975) or tetrahydrouridine (Wentworth & Wolfenden, 1975), could function by this mechanism and derive considerable binding energy by displacing bound water from catalytic sites.

The collected-substrate–water mechanism also could serve as a prototype for the development of inhibitors of other enzymes. One application to the development of inhibitors of aspartic proteases would be the synthesis of stable *gem*-diol species (i.e., stable hydrates of statone derivative **15B**). Such an analogue could have a smaller dissociation constant for aspartic proteases if hydrated completely in solution prior to binding and would be a more potent inhibitor ($\ll 10^{-10}$ M) due to the added hydrogen bond between the *pro-R* hydroxyl group and Asp-33(32), or to an "oxyanion hole", supplementing the hydrogen bonding and entropic displacement of water. Thus stable hydrates of **15B** would provide an experimental test for the inhibition mechanisms proposed here. For the same reasons, the report that phosphinate analogues of statine bind much more tightly ($K_i < 40$ pM) to aspartic proteases (Bartlett & Kezer, 1984) is consistent with the collected-substrate interaction being further stabilized by an additional hydrogen bound to the second oxygen on the phosphinate.

Second, the fact that the potent inhibitors of Me^{3}Sta (and Me^{3}AHPPA) have the 3*R* configuration requires that these inhibitors bind differently to aspartic proteinases than do the (3*S*)-Sta [or (3*S*)-AHPPA] inhibitors. The discovery of new binding sites between aspartic proteinases and (3*R*)-Me^{3}Sta-derived inhibitors has important consequences since it may be possible to develop new renin inhibitors that maximize interactions to these new sites.

Finally, the assignment of the more potent inhibitor to the unnatural 3*R* chirality for the necessary hydroxyl group is unusual. It is a common practice in biochemical transformations, especially in cases where proteases are utilized to carry out transformations on highly substituted amino acids and peptides of unknown chirality, to assume that the diastereomer that retains the most activity has the same chirality as a natural product model of established chirality. Our results, in which the unnatural diastereomer of the methyl analogue is consistently the more potent compound, establish that this assumption does not always hold.

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