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Inhibition of Aminopeptidases by Amastatin and Bestatin Derivatives. Effect of Inhibitor Structure on Slow-Binding Processes[1,2]

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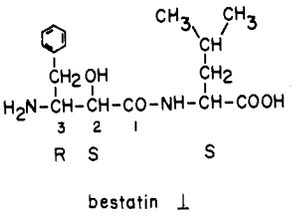
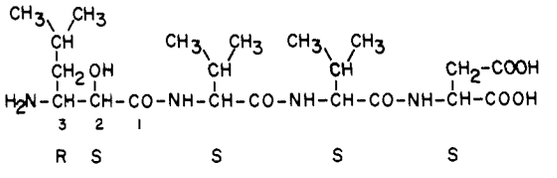
Amastatin [(2*S*,3*R*)-3-amino-2-hydroxy-5-methylhexanoyl-L-valyl-L-valyl-L-aspartic acid] and bestatin [(2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-L-leucine] are slow-binding, competitive inhibitors of aminopeptidase M (AP-M) with net inhibition constants (K_i*) of 1.9×10^{-8} and 4.1×10^{-6} M, respectively. The effect of inhibitor structure on net K_i and on slow-binding inhibition was evaluated for analogues of both inhibitors on AP-M and leucine aminopeptidase (LAP). The (2*S*)-hydroxyl group contributes to the stabilization of a collision complex [EI], which is formed rapidly. In contrast, increasing the peptide chain length of the inhibitor produces more potent inhibitors as a consequence of a slower binding process. A statine analogue of amastatin [(3*S*,4*S*)-Sta-Val-Val-Asp] *stimulated* rather than inhibited LAP. AP-M binds tri- and tetrapeptide inhibitors more strongly than dipeptide inhibitors, whereas LAP binds dipeptide inhibitors more strongly. The difference in binding can be used to distinguish cytosolic from membrane-bound aminopeptidases.

Bestatin (1) and amastatin (2) are two low-molecular-



bestatin 1



amastatin 2

weight peptides isolated by Umezawa and co-workers as part of an effort to discover inhibitors of therapeutically important enzymes.[3] Bestatin (1) is an inhibitor of aminopeptidase B (AP-B) and leucine aminopeptidase (LAP),[4] whereas amastatin (2) inhibits aminopeptidase A (AP-A) and LAP but not AP-B.[5] Bestatin (1) has been reported to enhance cell-mediated immunity in a cell-culture system,[6] produce antitumor effects both in animal systems[7] and in humans,[8] stimulate polysome assembly in T-cell lymphoma (grown in suspension),[9] cause induction of DNA polymerase α,[10] and stimulate DNA and RNA synthesis in T-cells both in vivo and in vitro.[11]

Bestatin and amastatin bind to cell surfaces and inhibit cell-surface aminopeptidases.[12,13] It is believed that their biological properties are an expression of the inhibition of the cell-surface aminopeptidases because analogues that stimulate the immune system are potent inhibitors of aminopeptidases. It is not known how these two properties are linked biochemically.

The fact that leucine aminopeptidase (EC 3.4.11.1) from cytosol of porcine kidney is strongly inhibited by amastatin led us to test amastatin as an inhibitor of aminopeptidase M (EC 3.4.11.2), a particle-bound aminopeptidase of porcine kidney. We found that amastatin is a strong inhibitor of aminopeptidase M and, surprisingly, that it is a slow-binding inhibitor of both aminopeptidase M and leucine aminopeptidase. These results plus a correlation of inhibitor structure with slow binding are reported herein. Comparative data with LAP are reported.

Results

In the absence of inhibitor, the steady-state velocity for hydrolysis of L-Leu-pNA by aminopeptidase M was reached immediately (Figure 1). In the presence of amastatin, however, there is a slow decrease in reaction rate, which varies as a function of the inhibitor concentration. Preincubation of enzyme with inhibitor for an appropriate period of time gave linear reaction velocities, which are defined herein as final steady-state velocities.

(1) Abstracted in part from the Ph.D. Thesis of Byung Jo Moon, 1981, and the M.S. Thesis of Scott Harbeson, 1979, submitted to the University of Wisconsin-Madison Graduate School.
(2) The abbreviations used are as follows: AHMHA, 3-amino-2-hydroxy-5-methylhexanoic acid; AHPBA, 3-amino-2-hydroxy-4-phenylbutanoic acid; L-Leu-pNA, L-leucine-*p*-nitroanilide; Sta, (3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid.
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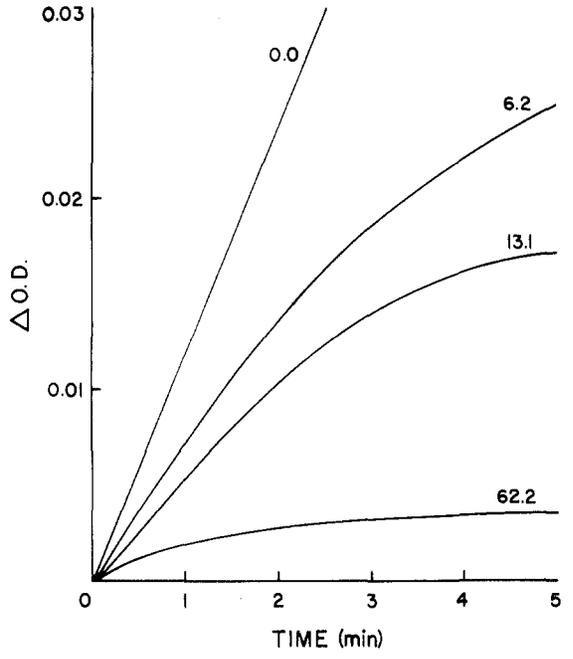

Figure 1. Reaction progress curves for hydrolysis of leucine-*p*-nitroanilide by aminopeptidase M in the presence of increasing concentrations of amastatin. Assays were initiated by adding enzyme at pH 7.2 and 25 °C in 50 mM Tris-HCl to solutions containing 1.5×10^{-4} substrate and 4.9×10^{-9} M enzyme.

A Lineweaver–Burk plot of both initial (before the slow-binding process) and final steady-state velocities established that amastatin is a competitive inhibitor of aminopeptidase M with an initial inhibition constant (K_i) of 2×10^{-5} M from initial velocities and a final inhibition constant (net K_i, K_i^*) of 2×10^{-8} M from final steady-state velocities (Figure 2). In the latter case, it was necessary to lower the enzyme concentration to avoid mutual depletion of enzyme and inhibitor in order to prevent apparent noncompetitive patterns.

Analysis of Time-Dependent Inhibition of Aminopeptidase M by Amastatin and Bestatin. The time-dependent increase in inhibition by amastatin can be interpreted in terms of the mechanisms depicted in Figure 3, where k_3 and k_4 are slow, and $k_3 \gg k_4$ (see Williams and Morrison[14,15]). In mechanism A, the equilibrium between the enzyme and the substrate is rapidly attained, while the equilibrium between the enzyme and an inhibitor is established slowly. In mechanism B, the time-dependent increase in inhibition is caused by a slow conformation change from one free enzyme form to another form of the unbound enzyme prior to formation of the tightened complexes (EI*). In mechanism C, the slow development of inhibition is caused by slow changes in the initially formed collision complex (EI), leading to a new enzyme–inhibitor complex (*EI*) in which the inhibitor is bound more tightly to the enzyme. The latter complex is termed the "Tightened" complex. Duggleby et al. have described how plots of the apparent first-order rate constant for slow binding (k_{obsd}) vs. inhibitor concentration can be used to distinguish between mechanisms A–C,[16] provided the full range of inhibitor concentrations are attainable. Figure 4 shows a plot of k_{obsd} vs. [I]. At lower inhibitor concentrations, the progress curves were too shallow for an accurate determination of k_{obsd}. An increase in AFS could not be used to better define the progress curves due to substrate depletion after an absorbance change of 0.2 ODU or greater. The linear upward plot is consistent with mechanisms A and C under our conditions but excludes mechanism B. Furthermore, mechanism A is not consistent with the half-life for the slow process, which is too long for a diffusion-controlled, bimolecular-associative process. Rate constants for bimolecular collisions are dependent on the size (and shape) of both molecules and on diffusion. For molecules this size, k_1 ranges from 10^7 to 10^8 M^{-1} s^{-1}.[17] Given an amastatin concentration of 2

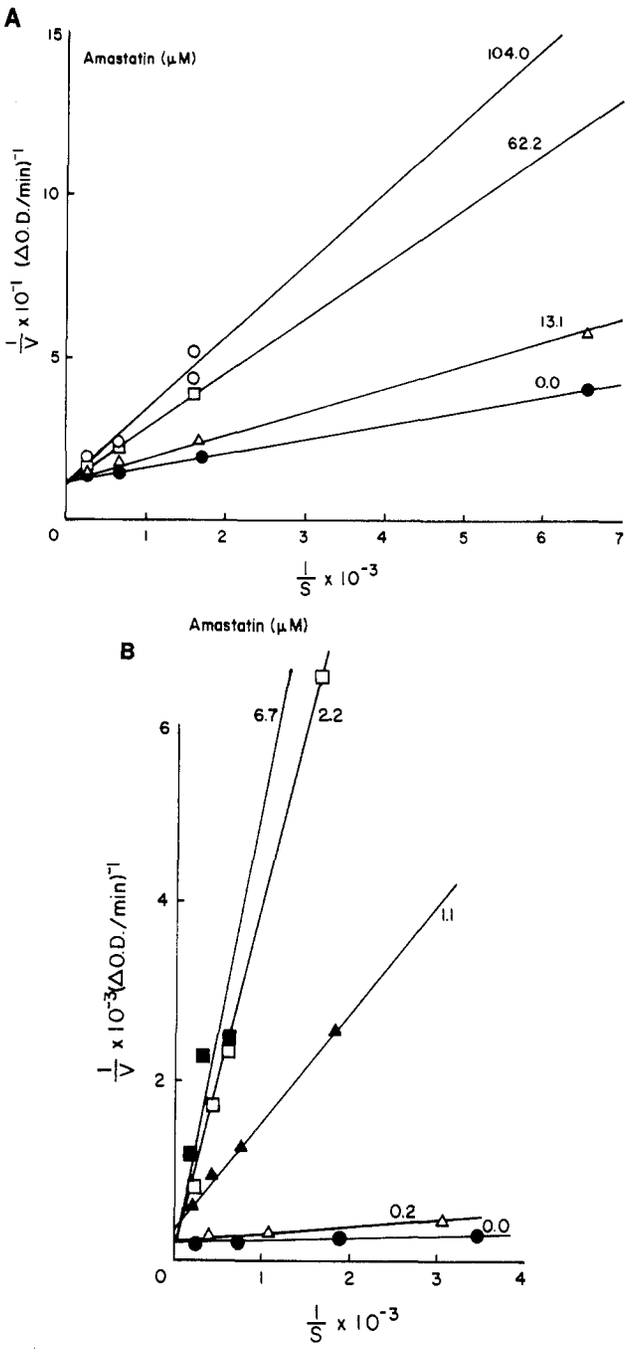

Figure 2. Double-reciprocal plot for inhibition of AP-M by amastatin. (A) Initial velocities were taken at pH 7.2 and 25 °C in 50 mM Tris-HCl buffer. Enzyme concentration was 6.0 nM. (B) Velocities were taken at final steady-state level after 30 min to 2 h preincubation of enzyme and amastatin at pH 7.2 and 25 °C in 50 mM Tris-HCl buffer. Enzyme concentration was 0.83 nM.

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(15) Williams, J. W.; Morrison, J. F.; Duggleby, R. G. *Biochemistry* **1979**, *18*, 2567–2573.
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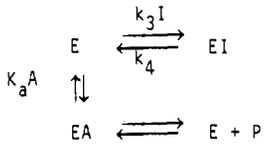
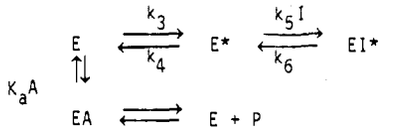
Mechanism A



Mechanism B



Mechanism C

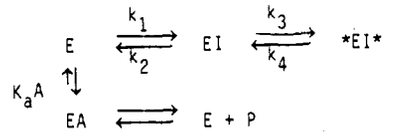


Figure 3. Possible mechanisms for slow-binding inhibition of an enzyme.

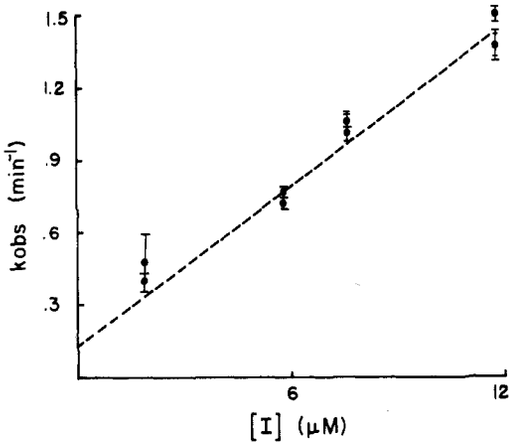


Figure 4. Dependence of k_{obsd} on amastatin concentration for inhibition of AP-M. Progress curves, such as shown in Figure 1, were analyzed as described in the Experimental Section to yield values of the apparent first-order rate constant. These values are plotted against the amastatin concentration.

$\times 10^{-8}$ M and a k_1 of 10^7 M^{-1} s^{-1}, k_{obsd} for the diffusion-controlled bimolecular collision would be about 0.2 s^{-1}, corresponding to a half-life of 3.5. This half-life is too short to account for the slow process observed. Therefore, we have used mechanism C to interpret the slow binding.

The amastatin data were analyzed with the following equations revealed for mechanism C.[18] Because I_t is much larger than E_t in all assays, from initial velocity data,

$$K_i = k_2/k_1 \quad (1)$$

while the net K_i^* value from the final steady-state velocity data is:

$$K_i^* = (k_2/k_1)k_4/(k_3 + k_4) = K_i k_4/(k_3 + k_4) \quad (2)$$

The equilibrium constant for conversion of the collision complex (EI) to the tightened complex (*EI*) is given by eq 3. When these equations were used to analyze the

$$k_3/k_4 = (K_i/K_i^*) - 1 \quad (3)$$

amastatin data with values of $K_i = 2 \times 10^{-5}$ M and $K_i^* = 2 \times 10^{-8}$ M, $k_3/k_4 \simeq 1000$.

In mechanism C, the observed rate constant (k_{obsd}) for a burst or lag in the time course of the assay when the reaction is initiated with enzyme or after preincubation of the enzyme and inhibitor is given by eq 4, where A is

$$k_{obsd} = k_4 + k_3(I/K_i)/(1 + I/K_i + A/K_a) \quad (4)$$

the substrate concentration and K_a is the Michaelis constant. Assay progress curves were fitted to eq 5 by a

$$P = P_0 + (v_i - v_f)/k_{obsd} + v_f t + [(v_f - v_i)e^{-k_{obsd}t}]/k_{obsd} \quad (5)$$

Fortran computer program provided by W. W. Cleland to determine k_{obsd}, where P is the product produced at time t, v_i is the initial velocity, v_f is the final steady-state velocity, k_{obsd} is the first-order rate constant for burst or lag, and P_0 is the product present at time zero. As shown in eq 4, k_{obsd} varies as a function of the amount of inhibitor and substrate in an assay. Under our experimental conditions (generally, the ratios of I/K_i and A/K_a were the same order of magnitude; [E] = 10^{-9} or 10^{-10} M), values for k_{obsd} between 0.14 and 0.23 min^{-1} were obtained, corresponding to a half-life of 3 to 5 min.

In order to determine the dissociation constant for dissociation of the enzyme–amastatin tightened complex, aminopeptidase M (1.75×10^{-7} M, 5 μL) was equilibrated with an excess of amastatin (6.93×10^{-7} M, 10 μL) for about 30 min. The complex was then diluted by the addition of a large volume of buffer that contained substrate (4.0×10^{-4} M, 1.5 mL), so that the final concentration of inhibitor in the assay mixture was well below its inhibition constant (K_i^*). Under these conditions, the second term in eq 4 becomes very small (about 1.6% of the first one) and can be neglected. The value for k_4 obtained was 0.045 min^{-1}, corresponding to a half-life of 15.4 min for the dissociation of the *EI* complex. From $k_3/k_4 \simeq 1000$, k_3 = 45 min^{-1}. The dissociation constant of the "tightened" *EI* complex is slow; therefore, it can be perceived that at enzyme concentrations similar to the inhibitor there will be mutual depletion of enzyme and inhibitor. As described by Williams and Morrison in their paper on slow-binding inhibitors,[14] this depletion would result in the apparent noncompetitive plots.

An identical analysis was carried out for the slow-binding inhibition of leucine aminopeptidase by amastatin and bestatin analogues. Collision and net inhibition constants for both enzymes and inhibitors are presented in Table II.

Net inhibition constants for several other analogues of amastatin and bestatin were determined and are presented in Table III. These compounds serve to delineate the effect that several structural features have on the inhibition of AP-M and supplement those already reported for LAP.[19,20] Deoxyamastatin (**9**), the analogue corresponding to deletion of the 2(*S*)-hydroxyl group, is a much weaker inhibitor of both AP-M and LAP as expected, but it is also a slow-binding inhibitor of both enzymes. The desamino analogue **10**, which corresponds to the deletion of the N-terminal amino group, is a weak inhibitor but not a slow-binding one. Replacement of the C-2 proton on

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Table I. Physical Constants for New Bestatin and Amastatin Analogues[a]

no.	compd	mp, °C	R_f	formula	elemental or amino acid anal.
6	(2*S*,3*R*)-3-amino-2-hydroxy-5-methylhexanoyl-Leu-OH·HCl	162–165[b]	0.35[d]	$C_{13}H_{27}N_4O_4Cl$	C, H, N
9	(3*R*)-3-amino-5-methyl-hexanoyl-Val-Val-Asp(OH)$_2$·HCl	159–162[b]	0.32[d]		Val, 2.0; Asp, 0.98
	(3*R*)-Boc-3-amino-5-methyl-hexanoyl-Val-Val-Asp(OBzl)$_2$	200–202[c]	0.46[e]	$C_{40}H_{58}N_4O_9·CH_3OH$	C, H, N
10	(2*RS*)-2-hydroxy-4-methyl-pentanoyl-Val-Val-Asp(OH)$_2$·HCl	206–208[b]	0.79[f]	$C_{20}H_{35}N_3O_8$	C, H, N
	(2*RS*)-2-hydroxy-4-methyl-pentanoyl-L-Val-L-Val-L-Asp(OBzl)$_2$	156–158[c]	0.60[g]	$C_{34}H_{47}N_3O_8$	C, H, N
11	(3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoyl-Val-Val-Asp(OH)$_2$·HCl	*b*	0.46[d]		Sta, 1.0; Val, 1.6; Asp, 1.0
	(3*S*,4*S*)-Boc-4-amino-3-hydroxy-6-methylheptanoyl-Val-Val-Asp(OBzl)$_2$	162–164[c]	0.56[e]	$C_{46}H_{69}N_5O_{11}$	C, H, N

[a] All amino acids are of the L configuration. [b] Amorphous solid purified by ion-exchange chromatography over Dowex 50W X8 (H^+) with gradient from 0.05 M pyridine formate (pH 2.9) and 0.2 M pyridine–formate (pH 3.1) buffer. [c] Purified by chromatography over silica gel eluting with a gradient from 0.5 to 2% methanol in chloroform. [d] 1-Butanol/acetic acid/water (4:1:1). [e] 5% Methanol in chloroform. [f] 20% EtOAc/benzene. [g] 10% Ethanol/chloroform.

Table II. Effect of Inhibitor Structure on the Slow-Binding Inhibition of Aminopeptidases

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EI^*$$

enzyme	no.	compd	K_i,[a] M	K_i^*, M	IC_{50}, μM	slow binding?
AP-M	2	amastatin	2×10^{-5}	2×10^{-8}		yes
	3	epiamastatin	2×10^{-3}	2×10^{-5}		yes
	1	bestatin	7×10^{-6}	4.1×10^{-6}	3.03[c]	yes
	4	epibestatin	5×10^{-4}	2×10^{-4}		yes
	15	(2*S*,3*R*)AHPBA-Iaa	1.6×10^{-5}	1.6×10^{-5}		no
	7	(2*S*,3*R*)AHMHA-Val-Asp	4×10^{-5}	9×10^{-7}		yes
LAP	2	amastatin	2×10^{-6}	2×10^{-7}	1.1[b]	yes
	3	epiamastatin	2×10^{-4}	2×10^{-5}	>500[b]	yes

[a] $K_i = k_2/k_1$; $K_i^* = K_i[k_4/(k_3 + k_4)]$. [b] Data taken from Tobe et al.[7] [c] Data taken from Leyhausen et al.[13]

bestatin with a methyl group produces analogue 8, which is a 200-fold weaker inhibitor of LAP. Replacement of the 2-hydroxy-3-amino acid, AHMHA, with statine, 3-hydroxy-4-amino-6-methylheptanoic acid, a homologue first identified in pepstatin,[21] gave analogues 11 and 12. The statine-containing inhibitor 11 did not inhibit hydrolysis of substrate even at millimolar inhibitor concentrations. Analysis of the initial velocity data showed that the statine analogue at 1 mM concentration actually stimulated hydrolysis of substrate.

Several amastatin analogues containing two to four amino acids in the peptide chain were prepared (Table III). In general, our results establish that tri- or tetrapeptide derivatives are better inhibitors of AP-M than are dipeptide derivatives and confirm the data of Tobe et al.[20] that shorter peptides are effective inhibitors of LAP. The isoamyl amide derivative of amastatin (14), a slightly weaker inhibitor of both LAP and AP-M, establishes that a free C-terminal carboxyl group is not required in the tri- or tetrapeptides for good inhibition. Removal of the C-terminal carboxyl group from bestatin (15) decreases the inhibition of LAP and AP-M by 25- and 4-fold, respectively.

Discussion

The results described here establish that amastatin is a strong competitive inhibitor of aminopeptidase M and a very slow-binding inhibitor of AP-M and LAP. Thus, maximal inhibition is not reached until the enzyme and inhibitor have equilibrated for over 30 min. This prolonged transient does not appear to be caused by a slow, bimolecular-association reaction, i.e.,

$$E + I \overset{\text{slow}}{\rightleftharpoons} EI$$

because the first-order rate constant (k_{obsd}) is 100–1000 times smaller than expected for a diffusion-controlled bimolecular association and because slow transitions between free enzyme forms are excluded by the data in Figure 2. Thus, the transient appears to be caused by a post-binding process, possibly a conformation change that transforms the initially formed enzyme–inhibitor complex to a new, more tightly bound enzyme–inhibitor complex.

Structure–activity correlations have been obtained for the inhibition of aminopeptidase M by amastatin analogues. Strong inhibition of aminopeptidase M requires a 2(*S*)-hydroxyl group in amastatin, which is identical with the stereochemical requirement found for the inhibition of leucine aminopeptidase and aminopeptidase B by bestatin and amastatin stereoisomers.[12,22] In addition, the size of the inhibitor is very important. Amastatin, a tetrapeptide, is a 100-fold stronger inhibitor of aminopeptidase M than is bestatin, a dipeptide. Another dipeptide analogue (6) in which the (2*S*,3*R*)-AHMHA residue of amastatin replaces the AHPBA residue in bestatin is an even weaker inhibitor and establishes that the differences between amastatin and bestatin are not caused by

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(22) Nishizawa, R.; Saino, T.; Takita, T.; Suda, H.; Aoyagi, T.; Umezawa, H. *J. Med. Chem.* **1977**, *20*, 510–515.

Table III. Inhibition of LAP and AP-M by Amastatin and Bestatin Analogues[a]

no.	compd	LAP K_i*, $\times 10^{-8}$ M	AP-M K_i*, $\times 10^{-8}$ M
	Effect of Amino Acid Residues		
1	(2*S*,3*R*)-AHPBA-Leu (bestatin)	2 (2.0)[b]	410 (3.03)[c]
6	(2*S*,3*R*)-AHMHA-Leu	12	1 176
7	(2*S*,3*R*)-AHMHA-Val-Asp	124 (0.8 μM)[c]	94
2	(2*S*,3*R*)-AHMHA-Val-Val-Asp (amastatin)	22 (1.1)[c]	2
8	(2*S*,3*R*)-2-methylbestatin	378	
	Effect of Hydroxyl Group and Stereochemistry		
1	(2*S*,3*R*)-AHPBA-Leu (bestatin)	2 (2.0)[b]	414
4	(2*R*,3*R*)-AHPBA-Leu (epibestatin)	1 700 (7.5)[e]	
2	(2*S*,3*R*)-AHMHA-Val-Val-Asp (amastatin)	22 (1.1)[c]	2
3	(2*R*,3*R*)-AHMHA-Val-Val-Asp (epiamastatin)	2 300 (<500)[c]	2 600
9	(3*R*)-AMHA-Val-Val-Asp (deoxyamastatin)	68 100	2 500
10	(2*RS*)-HMP-Val-Val-Asp (desaminoamastatin)	74 000	70 000
11	(3*S*,4*S*)-Sta-Val-Val-Asp	570 000 (K_a)[f]	
12	(3*S*,4*S*)-Sta-Val-Val-Iaa	100 000	100 000
13	Boc-Val-Val-Asp	140 000	
14	(2*S*,3*R*)-AHMHA-Val-Val-Iaa	89	18
15	(2*S*,3*R*)-AHPBA-Iaa	50	1 630

[a] All inhibition constants were determined after preincubating the enzyme and inhibitor. [b] Reported by Nishizawa et al.[22] [c] Micromolar IC_{50} value reported by Tobe et al.[7] [d] Data reported by Leyhausen et al.[13] [e] IC_{50} reported by Nishizawa et al.[22] [f] K_a is the apparent K_m in the presence of tripeptide.

the differences in the N-terminal groups, AHMHA and AHPBA. Removal of one valine from amastatin, as in analogue **7**, produces a weaker inhibitor than amastatin but one that is stronger than bestatin. Thus, the Val-Val portion of amastatin enhances the inhibition of aminopeptidase M, and a peptide larger than a di- or tripeptide is required for maximal inhibition. In contrast, inhibition of leucine aminopeptidase or aminopeptidase B[20] is more effective with dipeptide derivatives.

Three analogues that contained modifications of the 2-hydroxy-3-amino acid moiety in amastatin and bestatin were evaluated as aminopeptidase inhibitors. As expected, the analogue lacking the 3-amino group (**10**) was a very poor inhibitor. Addition of a methyl group to bestatin at C-2 (**8**) reduced inhibition 200-fold, although the analogue remains a respectable aminopeptidase inhibitor. The statine analogues **11** and **12** were exceptionally weak inhibitors of both aminopeptidases and, surprisingly, were even weaker inhibitors than the Boc-protected tripeptide **13**, which lacks the vicinal hydroxyamine functionality. Detailed kinetic analysis showed that analogue **11** actually stimulates LAP-catalyzed hydrolysis of Leu-pNA when the inhibitor is added at millimolar concentrations. While we do not have an explanation for this effect, the stimulation suggests that the Sta analogue binds to the aminopeptidases in spite of the extended carbon backbone in statine relative to AHMHA. The binding site is not known.

Effect of Inhibitor Structure on Slow-Binding Inhibition. Inhibition of aminopeptidase M by several analogues of amastatin and bestatin was studied in detail to determine if the functional groups of the inhibitors or the chain length preferentially affected either K_i or K_i*. Surprisingly, we found that the hydroxyl group appears to enhance binding primarily to the collision complex (EI). As shown in Table II, amastatin is a 1000-fold stronger inhibitor of AP-M than is epiamastatin, the analogue that differs only by the configuration of the 2-hydroxyl group. Most of this lost binding is detectable in the collision dissociation constant (K_i), which is 100-fold greater for epiamastatin relative to amastatin. A similar pattern is observed for the two epimers of bestatin (Table II). Epibestatin (**4**), in which the hydroxyl group is 2*R*, is 50 times less effective an inhibitor of AP-M than bestatin, and this loss in potency appears to be due to a 50-fold larger K_i.

These data suggest that the binding of the hydroxyl group to the enzyme first occurs in the initially formed enzyme–inhibitor complex rather than in the tightened complex formed after the slow isomerization.

The number of amino acids in the inhibitor affects the tightness of binding between the inhibitor and AP-M. Our kinetic analysis indicates these added residues contribute most to the slow-binding process (Table II). Bestatin, a dipeptide, is a much weaker inhibitor of aminopeptidase M than is amastatin, a tetrapeptide. As shown in Table II, amastatin and bestatin have similar K_i values, obtained from initial velocity data, but different K_i* values, obtained from the final steady-state velocities. These results indicate that the stronger inhibition of aminopeptidase M by amastatin is caused by the smaller equilibrium constant for the conversion of EI to *EI* between amastatin and aminopeptidase M than that found for bestatin with this enzyme. Tripeptide analogue **7**, which lacks one valine found in amastatin, is a weaker inhibitor. Remarkably, tripeptide **7** has a K_i value quite close to that found for amastatin and bestatin, but its equilibrium constant for the second step (EI $\rightleftharpoons$ *EI*) lies between those of amastatin and bestatin. Modifying the C-terminal substituent in amastatin affected slow-binding inhibition differently than this modification in bestatin. The larger analogue **14**, in which both aspartyl carboxyl groups of amastatin were replaced by alkyl groups (Iaa = isoamyl amide), is a strong, slow-binding inhibitor comparable to amastatin. In contrast, the smaller analogue **15**, derived from bestatin by deletion of the carboxyl group, was not a slow-binding inhibitor. These results suggest that an interaction between the α-hydroxy-β-amino acid residue and the $P_1 \rightarrow P_3'$ residues in the inhibitor is needed to stabilize a tighter complex between aminopeptidase M and amastatin or bestatin.

Mechanism of the Inhibition of Aminopeptidase M by Amastatin and Bestatin. A mechanism for the hydrolysis of peptides by aminopeptidase M has been proposed based on the similarity of AP-M to LAP and the inhibition of the closely related enzymes by bestatin and amastatin (Figure 5).[20] Inhibition of AP-M has been proposed to proceed by chelation of the 2(*S*)-hydroxyl group and the 3-amino group in the AHPBA moiety in bestatin and the AHMHA moiety in amastatin to the zinc ion in the enzyme active site.[22,23] Nishino and Powers[24]

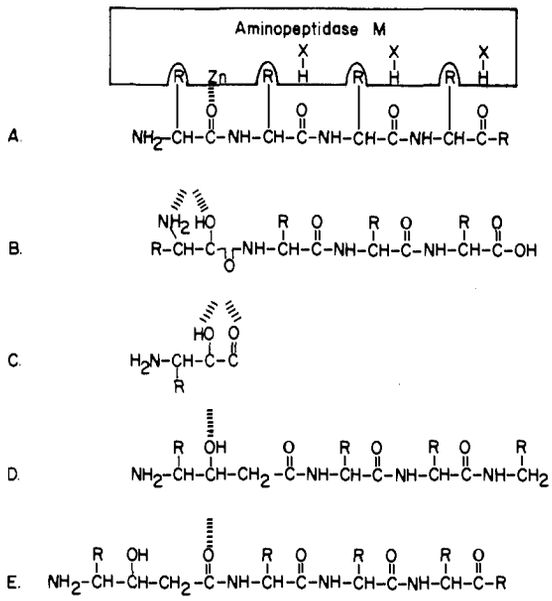

Figure 5. Schematic models for the binding of substrate, amastatin, or bestatin to aminopeptidases: (A) proposed binding of substrate to LAP;[23] (B) model proposed by Nishizawa et al.[22] in which the active-site zinc group is chelated by the amino and hydroxyl groups; (C) model proposed by Nishino and Powers for chelation of the active-site zinc by the hydroxyl and carbonyl groups;[24] (D and E) possible binding of statine-containing analogues to aminopeptidases.

proposed an alternate model for bestatin in which the carbonyl group and the 2(*S*)-hydroxyl group are the zinc ligands (Figure 5). Both models assume that bestatin is an analogue of the transition state or tetrahedral intermediate for amide bond hydrolysis formed via coordination to the active-site zinc atom. The weak inhibition shown by the desamino analogue is consistent with Takita's mechanism, whereas the weak inhibition shown by the Sta analogues **11** and **12** is more consistent with Powers' mechanism.[24] The slow-binding inhibition shown by the most potent analogues further suggests that multiple enzyme–inhibitor complexes are required for maximal inhibition of aminopeptidases.

The structure–inhibition relationships for aminopeptidase M and leucine aminopeptidase closely parallel the substrate specificity of these enzymes. Kim et al.[25] found that brush-border enzymes hydrolyze tripeptides or larger peptides better than substrates that contain one or two amino acids, while cytosolic aminopeptidases hydrolyzed di- or tripeptides but not longer peptides. Thus, the differences between amastatin and bestatin as inhibitors of aminopeptidase M and leucine aminopeptidase parallel the substrate specificities. Recently, Leyhausen et al. found that cell-membrane aminopeptidase activity on L5178Y cells and in mouse liver cell preparations is identical with aminopeptidase M and not AP-B and differs from LAP.[13] They also found that amastatin inhibits AP-M more strongly than does bestatin. Thus, the binding of amastatin and bestatin to aminopeptidases may be helpful for distinguishing between LAP and AP-M in cell preparations in the same way amastatin and bestatin are used to distinguish between AP-A and AP-B. Aminopeptidase M is only the second enzyme found to be more sensitive to amastatin than to bestatin.

Experimental Section

Materials. Amastatin, bestatin, and other inhibitors were prepared from optically pure synthetic *tert*-butyloxycarbonyl-protected-2-hydroxy-3-amino acids by a stepwise solution strategy.[26] Each inhibitor was fully characterized by microanalysis, mass spectrometry, and nuclear magnetic resonance spectroscopy. Details of the amastatin synthesis have been reported.[19,20,26] Physical constants for new analogues are given in Table I. 2-Methylbestatin was a gift from Prof. B. Sharpless, Department of Chemistry, MIT.

Aminopeptidase M (EC 3.4.11.2) was purchased from Sigma Chemical Co. as a suspension in 3.5 M $(NH_4)_2SO_4$ solution, pH ~6, containing 10 mM $MgCl_2$ and was used without further purification. Leucine aminopeptidase was obtained from Sigma Chemical Co. as a chromatographically purified $(NH_4)_2SO_4$ suspension containing 5 mM $MgCl_2$. Unless stated otherwise, this material was used without further purification. Leucine-*p*-nitroanilide hydrochloride was purchased from Sigma Chemical Co. and used without further purification.

Methods. All spectrophotometric assays were carried out with L-leucine-*p*-nitroanilide as substrate for both aminopeptidase M[27] and leucine aminopeptidase,[28,29] with K_m values of 0.4 and 1.8 mM, respectively. Aminopeptidase M assays were performed at pH 7.2 in 50 mM Tris, 5 mM $MgCl_2$. LAP was assayed at pH 8.5 in 0.10 M Tris, 5 mM $MgCl_2$. Initial rates were generally linear for an absorbance change of 0.1 to 0.2 ODU. The appearance of *p*-nitroaniline was monitored at 405 nm with a Gilford 250 spectrophotometer and a Gilford 6051 recorder at 25 °C. The initial hydrolysis rates of leucine-*p*-nitroanilide were determined by placing the substrate solution and inhibitor solution in a cuvette and initiating the reaction by addition of the enzyme.

For the final steady-state velocities, the enzyme solution was preincubated for 15–20 min with a solution of inhibitor prior to initiation of the enzymatic hydrolysis by addition of the substrate. Generally, 0.2 mL of substrate solution, 0.2 mL of inhibitor solution, and 0.02 mL of enzyme solution were used in an assay of aminopeptidase M. In an assay of leucine aminopeptidase, an assay solution contained 0.2 mL of substrate solution, 0.2 mL of inhibitor solution, and 0.1 mL of enzyme solution. All velocities were recorded as the change in optical density with time at 0.04 to 0.20 ODU full scale.

Reaction velocities were first analyzed graphically as Lineweaver–Burk plots. These data were then subjected to computer analysis and fit to the following equations for linear competitive and noncompetitive inhibition according to Cleland's iterative least-squares method:[30]

$$\text{competitive } v = \frac{VS}{K(1 + I/K_{is})} + S$$

$$\text{noncompetitive } v = \frac{VS}{K(1 + I/K_{is}) + S(1 + I/K_{ii})}$$

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Registry No. **1**, 58970-76-6; **2**, 67655-94-1; **3**, 73465-23-3; **4**, 61046-23-9; **6**, 62058-39-3; **7**, 82985-21-5; **8**, 88825-96-1; **9**, 88825-97-2; **10**, 88825-98-3; **11**, 88825-99-4; **12**, 88853-70-7; **13**, 88826-00-0; **14**, 88826-01-1; **15**, 88826-02-2; AP-M, 9054-63-1; LAP, 9001-61-0.

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