Synthesis of Sulfur-Containing Analogues of Bestatin. Inhibition of Aminopeptidases by α-Thiolbestatin Analogues[1,2]

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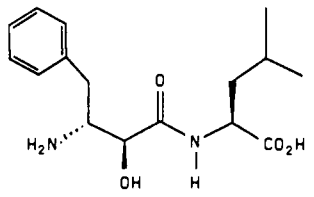
Sulfur-containing amino acid and peptide analogues of bestatin [((2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl)-L-leucine] (1) have been synthesized and evaluated as inhibitors of aminopeptidase M (AP-M), leucine aminopeptidase (LAP), and aminopeptidase B (AP-B). The 2-thiolbestatin analogue (**6**) was found to be a potent inhibitor of all three aminopeptidases (AP-M, K_i = 4.4 μM; LAP, K_i = 0.55 μM; AP-B, K_i = 4.6 nM) but only a slightly better inhibitor of these aminopeptidases than the parent hydroxy-containing compound **1**. Synthetic analogues of L-leucinethiol (**4**), a strong inhibitor of aminopeptidases, were prepared in which the carbon α to the thiol groups was substituted with methyl, methyl carboxylate, and carboxamide derivatives and found to be much weaker inhibitors of all aminopeptidases. A thioamide analogue of bestatin (**49**) is a modest inhibitor of AP-M (K_i = 40 μM), LAP (K_i = 0.33 μM), and AP-B (K_i = 2.4 μM). These results suggest that the sulfur atoms in 2-thiolbestatin and bestatin thioamide do not interact strongly with the active-site zinc atom of these aminopeptidases when the inhibitors are bound to the enzyme. These results are not consistent with proposed models for the inhibition of aminopeptidases by bestatin and related analogues.

Bestatin (**1**), a potent aminopeptidase (APase) inhibitor isolated from culture filtrates of *Streptomyces olivoreticuli* by Umezawa et al.,[3-5] has attracted considerable attention since its discovery in 1976 due to its numerous biological activities, most notably as an immune response modifier[6-12] and a potential analgesic,[13,14] and due to the presence of the novel amino acid, 2(*S*)-hydroxy-3(*R*)-amino-4-phenylbutanoic acid (AHPBA (**2**)) in its structure. Structure-modification studies of bestatin have established that the presence of the 2(*S*)-hydroxyl group in AHPBA is important for tight binding to aminopeptidases.[15] The peptide chain length also is an important contributor to both the slow-binding properties of bestatin and the related inhibitor amastatin **3** and to the preferential binding of **1** and **3** to different aminopeptidases.[15,16]



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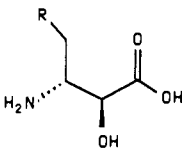
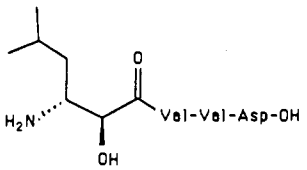


2a, R = C_6H_5

b, R = $^iC_3H_7$



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The structural relationship between bestatin's critical 2(*S*)-hydroxyl group and a probable tetrahedral intermediate for amide bond hydrolysis led to the idea that bestatin might be a transition state analogue inhibitor of aminopeptidases in which the sp^3 geometry and alcohol at the C-2 carbon of the inhibitor mimics the tetrahedral intermediate formed during substrate hydrolysis. Nishizawa et al.[5] suggested, based upon analogy to a proposed catalytic mechanism for peptide cleavage by aminopeptidases,[17] that an essential, active-site zinc ion is chelated by the 2(*S*)-hydroxyl group and the 3-amino group when bestatin is bound to aminopeptidases (Figure 1A). Nishino and Powers[18] (Figure 1B) postulated an alternative mechanism in which the C-2 hydroxyl group and the amide carbonyl bound to the active-site zinc. Both mechanisms suggest an interaction between the 2(*S*)-hydroxyl group of AHPBA (**2**) and the zinc ion presumed to be present in the active site of aminopeptidases.

A logical test of these proposals would be to replace the C-2 hydroxyl group in AHPBA with a thiol group in order

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(1) Abbreviations used: AHPBA, 3-amino-2-hydroxy-4-phenylbutanoic acid; AHMHA, 3-amino-2-hydroxy-5-methylhexanoic acid; EtOAc, ethyl acetate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MOPS, 3-*N*-morpholinopropanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); TFA, trifluoroacetic acid.
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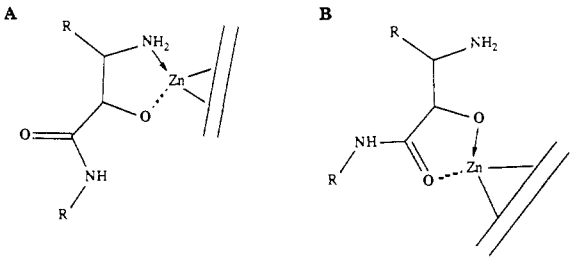
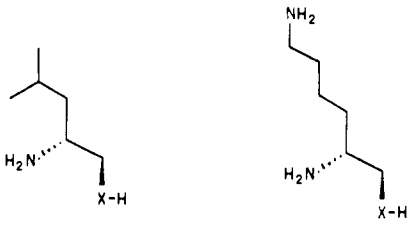


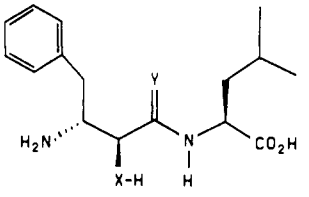
Figure 1. (A) Mechanism of inhibition of aminopeptidases by bestatin as proposed by Nishizawa et al.[3] (B) Mechanism of inhibition of aminopeptidases by bestatin as proposed by Nishino and Powers.[18]

to capitalize on the high affinity of sulfhydryl groups for zinc ions. Two amino thiols, L-leucinethiol (**4**) and L-lysinethiol (**5**) are potent inhibitors of AP-M[19] and AP-B,[20] respectively, and are much more potent inhibitors than the corresponding alcohols (**4a**, **5a**). Thus, if the amino thiols and bestatin bind to APases in the same way, then a thiol bestatin derivative **6** could be a much tighter binding inhibitor of APases than bestatin is. Additionally, replacement of the bestatin amide carbonyl with a thiocarbonyl group, as in compound **7**, might enhance affinity for the active-site zinc atom in the Nishino–Powers model. We report herein the synthesis of two series of sulfur-containing analogues of bestatin exemplified by the C-2 thiol derivative **6** and the thioamide **7**. The inhibition of three aminopeptidases by these compounds is described.



4, X = S
4a, X = O

5, X = S
5a, X = O

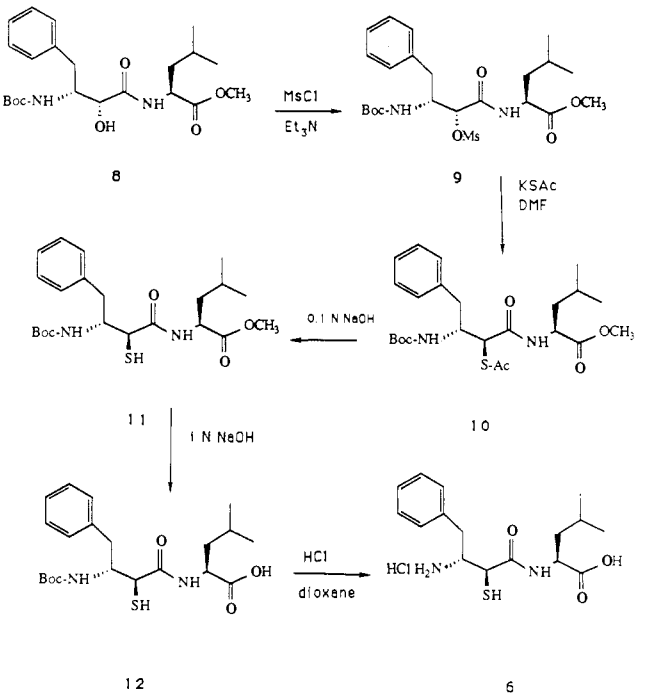


6, X = S; Y = O
7, X = O; Y = S

Results

Synthesis of 2-Thiolbestatin. The synthesis of 2-thiolbestatin is shown in Scheme I. The protected bestatin derivative **8** was converted to the mesylate **9** in a straightforward manner. The C-2 mesylate was displaced with potassium thioacetate to give the protected thiol **10**. Hydrolysis of the thiol ester **10** gave a stable free thiol (**11**), which did not oxidize to the disulfide even after treatment with I_2/MeOH. The C- and N-terminal protecting groups in thiol **11** were removed in two steps to yield the desired 2-thiolbestatin **6**, which was obtained as a hygroscopic solid. A number of peptide chain-length analogues of **6** (Table I) were synthesized by use of similar procedures.

Scheme I



8 **9** **10** **11** **12** **6**

The physical constants for all new intermediates and final products related to compound **6** are given in Table I.

Susceptibility of Thiols to Oxidation. The oxidation of the thio compounds to the corresponding disulfide was surprisingly erratic. The smaller amino thiol analogues (where either a proton or methyl group are α to the thiol group) were easily oxidized to the disulfides by simply passing a stream of air through the reaction mixture during the deacetylation reaction. However, this procedure did not produce a disulfide when a carbonyl group was adjacent to the thiol group. When an isoamylamide group was α to the thiol, oxidation to the disulfide was achieved by reacting the thiol with iodine in methanol, and these disulfides could be reduced back to thiols by reaction with dithiothreitol (DTT). However, thiols that contained a methyl ester group or leucine methyl ester moiety α to the thiol were not oxidized by the same iodine treatment; these compounds were isolated as the free thiols.

Stereochemistry of Thiol Intermediates. The stereochemistry of the 2-thiol derivatives was assigned on the basis of the chemistry utilized to prepare the precursor thiolacetates and from the biological activities of the obtained thiols. It was expected that displacement of the mesylates with potassium thioacetate would proceed with inversion of configuration, so that the 2*R* mesylate was utilized to obtain the desired 2(*S*)-thiol and the 2*S* mesylate used to prepare the 2(*R*)-thiol. Typically, a high degree of inversion was observed, and only a small amount (<5%) of the opposite diastereomer was detected. The one exception was obtained with compound **25**, which inexplicably gave a 1:1 mixture of diastereomers. In several cases (most notably 2(*R*)- and 2(*S*)-thiolbestatin), both diastereomers were synthesized from enantiomerically pure alcohol precursors.[15] The thiolbestatin derivatives assigned the *S* configuration were the more potent aminopeptidase inhibitors.

Thioamide Synthesis. The synthesis of bestatin thioamide **49** is shown in Scheme II. During the reaction with Lawesson's reagent,[33] it was necessary to protect the

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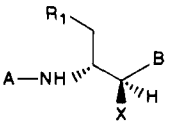
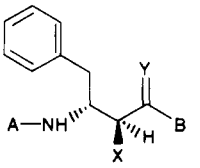
Table I. Physical Constants of Thiolbestatin Analogues and Intermediates



compd	A	R_1	X	B	chirality	TLC[a]	% yield	formula	anal.
13	Boc	$CH(CH_3)_2$	OMs	H	3*S*	0.59 (A)	80	$C_{12}H_{25}NO_5S$	CHN
14	Boc	$CH(CH_3)_2$	SAc	H	3*S*	0.64 (A)	97	$C_{13}H_{25}NO_3S$	CHN
15	Boc	$CH(CH_3)_2$	$S)_2$	H	3*S*	0.66 (A)	91	$C_{22}H_{44}N_2O_4S_2$	CHNS
16	HCl·H	$CH(CH_3)_2$	$S)_2$	H	3*S*	0.22 (C)	91	$C_{12}H_{30}N_2S_2Cl_2$	CHNS
17	Boc	$CH(CH_3)_2$	OMs	H	3*R*	0.60 (A)	94	$C_{12}H_{25}NO_5S$	CHN
18	Boc	$CH(CH_3)_2$	SAc	H	3*R*	0.75 (A)	86	$C_{13}H_{25}NO_3S$	*b*
19	Boc	$CH(CH_3)_2$	$S)_2$	H	3*R*	0.69 (A)	76	$C_{22}H_{44}N_2O_4S_2$	CHNS
20	HCl·H	$CH(CH_3)_2$	$S)_2$	H	3*R*	0.21 (C)	70	$C_{12}H_{30}N_2S_2Cl_2 \cdot {}^1/_4H_2O$	CHNS
21	Boc	$(CH_2)_3NHCbz$	OMs	H	3*S*	0.44 (A)	86	$C_{17}H_{34}N_2O_7S$	CHN
22	Boc	$(CH_2)_3NHCbz$	SAc	H	3*S*	0.58 (A)	92	$C_{18}H_{34}N_2O_5S$	CHNS
23	Boc	$(CH_2)_3NHCbz$	$S)_2$	H	3*S*	0.60 (A)	90	$C_{32}H_{62}N_4O_8S_2$	CHNS
24	HCl·H	$(CH_2)_3NH\cdot HCl$	$S)_2$	H	3*S*	0.03 (K)	63	$C_{12}H_{34}N_4S_2Cl_4 \cdot {}^1/_4CH_3OH$	CHNS
25	Boc	Ph	OMs	CO_2Me	3*R*,3*R*	0.66 (A)	89	$C_{17}H_{25}NO_7S$	CHN
26	Boc	Ph	SAc	CO_2Me	2*R*,3*R*	0.70[c] (A)	50	$C_{18}H_{25}NO_5S$	CHNS
27	Boc	Ph	SH	CO_2Me	2*R*,3*R*	0.68[c] (A)	68	$C_{16}H_{23}NO_4S$	CHNS
28	HCl·H	Ph	SH	CO_2Me	2*R*,3*R*	0.62[c] (C)	73	$C_{11}H_{16}NO_2SCl \cdot {}^1/_4H_2O$	CHNS
29	Boc	$CH(CH_3)_2$	OMs	CH_3	2*RS*,3*S*	0.64[c] (A)	92	$C_{13}H_{27}NO_5S \cdot {}^3/_4H_2O$	*d*
30	Boc	$CH(CH_3)_2$	SAC	CH_3	2*RS*,3*S*	0.72[c] (A)	67	$C_{14}H_{27}NO_3S$	CHNS
31	Boc	$CH(CH_3)_2$	$S)_2$	CH_3	2*RS*,3*S*	0.75[c] (A)	88	$C_{24}H_{48}N_2O_4S_2$	CHNS
32	HCl·H	$CH(CH_3)_2$	$S)_2$	CH_3	2*RS*,3*S*	0.39[c] (C)	85	$C_{14}H_{34}N_2S_2Cl_2$	CHNS
33	Boc	Ph	OMs	CONH-*i*-C_5H_{11}	2*R*,3*R*	0.60 (A)	92	$C_{21}H_{34}N_2O_6S \cdot {}^1/_4H_2O$	CHN
34	Boc	Ph	SAC	CONH-*i*-C_5H_{11}	2*S*,3*R*	0.60 (A)	60	$C_{22}H_{34}N_2O_4S$	CHNS
35	Boc	Ph	$S)_2$	CONH-*i*-C_5H_{11}	2*S*,3*R*	0.62 (A)	85	$C_{40}H_{62}N_4O_6S_2$	*e*
36	TFA·H	Ph	$S)_2$	CONH-*i*-C_5H_{11}	2*S*,3*R*	0.74 (C)	44		
37	HCl·H	Ph	$S)_2$	CONH-*i*-C_5H_{11}	2*S*,3*R*			$C_{30}H_{48}N_4O_2S_2Cl_2 \cdot H_2O$	CHNS
38	Boc	Ph	OMs	CONH-*i*-C_5H_{11}	2*S*,3*R*	0.59 (A)	72	$C_{21}H_{34}N_2O_6S$	CHN
39	Boc	Ph	SAc	CONH-*i*-C_5H_{11}	2*R*,3*R*	0.68 (A)	48	$C_{22}H_{34}N_2O_6S$	CHNS
40	Boc	Ph	$S)_2$	CONH-*i*-C_5H_{11}	2*R*,3*R*	0.65 (A)	53	$C_{40}H_{62}N_4O_6S_2 \cdot H_2O$	CHNS
41	HCl·H	Ph	$S)_2$	CONH-*i*-C_5H_{11}	2*R*,3*R*			$C_{30}H_{48}N_4O_2S_2Cl_2 \cdot H_2O$	CHNS
42	TFA·H	Ph	$S)_2$	CONH-*i*-C_5H_{11}	2*R*,3*R*	0.69 (C)	64		
9	Boc	Ph	OMs	CO-Leu-OMe	2*R*,3*R*	0.60 (A)	97	$C_{23}H_{36}N_2O_8S$	CHN
10	Boc	Ph	SAc	CO-Leu-OMe	2*S*,3*R*	0.63 (A)	58	$C_{24}H_{36}N_2O_6S$	CHNS
11	Boc	Ph	SH	CO-Leu-OMe	2*S*,3*R*	0.60 (A)	73	$C_{22}H_{34}N_2O_5S$	CHNS
6	HCl·H	Ph	SH	CO-Leu-OH	2*S*,3*R*	0.63 (C)	46	$C_{16}H_{25}N_2O_8SCl \cdot 1.2H_2O$	CHNS
43	Boc	Ph	OMs	CO-Leu-OMe	2*RS*,3*R*	0.12, 0.16 (D)	99	$C_{23}H_{36}N_2O_8S$	CHN
44	Boc	Ph	SAc	CO-Leu-OMe	2*RS*,3*R*	0.34[c] (D)	24	$C_{24}H_{36}N_2O_6S$	CHNS
45	Boc	Ph	SH	CO-Leu-OMe	2*RS*,3*R*	0.72, 0.60 (A)	65	$C_{22}H_{34}N_2O_5S$	CHNS
46	HCl·H	Ph	SH	CO-Leu-OH	2*RS*,3*R*	0.57, 0.62 (C)	45	$C_{16}H_{25}N_2O_8SCl \cdot H_2O$	*f*

[a] See the Experimental Section for TLC solvent systems. [b] Calcd: C, 56.69; H, 9.15; N, 5.09; S, 11.64. Found: C, 56.61; H, 9.19; N, 4.44; S, 11.98. [c] No evidence of diastereomers by TLC, but they were seen in the NMR spectra or by HPLC. [d] Calcd: C, 48.35; H, 8.90; N, 4.33. Found: C, 48.10; H, 9.10; N, 4.98. [e] Calcd: C, 63.29; H, 8.23; N, 7.38; S, 8.45. Found: C, 63.02; H, 8.27; N, 7.34; S, 8.92. [f] Calcd: C, 50.72; H, 7.18; N, 7.39; S, 8.46. Found: C, 50.74; H, 6.87; N, 7.46; S, 9.01.

Table II. Physical Constants of Thioamide Analogues



compd	A	X	Y	B	TLC[a]	% yield	formula	anal.
50	Boc	OAc	O	NH-*i*-C_5H_{11}	0.56 (A)	73	$C_{22}H_{34}N_2O_5$	CHN
51	Boc	OAc	S	NH-*i*-C_5H_{11}	0.52 (B)	46	$C_{22}H_{34}N_2O_4S$	CHNS
52	Boc	OH	S	NH-*i*-C_5H_{11}	0.58 (B)	81	$C_{20}H_{32}N_2O_3S$	CHNS
53	HCl-C	OH	S	NH-*i*-C_5H_{11}	0.58 (C)	97	$C_{15}H_{25}N_2ClOS$	CHNS
47	Boc	OAc	O	Leu-OMe	0.62 (A)	87	$C_{24}H_{36}N_2O_7$	CHN
48	Boc	OAc	S	Leu-OMe	0.46 (B)	71	$C_{24}H_{36}N_2O_6S$	*b*
49	TFA·H	OH	S	Leu-OH	0.66 (C)	72	$C_{18}H_{25}N_2O_5SF_3 \cdot H_2O$	CHNS

[a] See the Experimental Section for TLC solvent systems. [b] Calcd: C, 59.98; H, 7.55; N, 5.83; S, 6.67. Found: C, 60.11; H, 7.55; N, 5.77; S, 7.13.

α-hydroxyl group of protected bestatin as the *O*-acetate to prevent its reaction with the thionation reagent. Thus **47** was treated with Lawesson's reagent to give thioamide **48** in 71% yield. Standard deprotections and HPLC pu-

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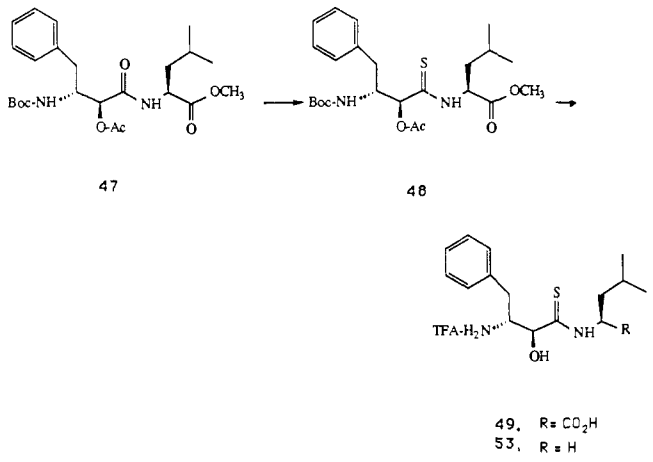
Scheme II



rification gave the desired product, which was isolated as the trifluoroacetate salt **49**. Physical constants for all intermediates and the final bestatin thioamide, as well as those for a simplified isoamylamide analogue **53**, are given in Table II. The presence of the thioamide group in each compound was confirmed by ^{13}C NMR, which showed the appropriate 30 ppm downfield shift in the thiocarbonyl carbon (C=S) relative to the carbonyl carbon (C=O) in the amide analogues.

Biological Results. The sulfur-containing compounds were evaluated as inhibitors of aminopeptidase M (AP-M), leucine aminopeptidase (LAP), and aminopeptidase B (AP-B). The results are summarized in Table III. Surprisingly, 2-thiol bestatin (**6**) was only 2-fold more potent than bestatin (**1**) for inhibition of AP-M and only 13 times more potent than bestatin for inhibition of AP-B. Thiolbestatin **6** was significantly weaker as an inhibitor of LAP.

The effect of replacing the alcohol group by a thiol group is most important in the smaller inhibitors we studied. As already reported,[19,20] it is the amino thiols, leucinethiol (**4**) and lysinethiol (**5**), that show the most pronounced benefit, gaining more than 5 orders of magnitude in potency upon replacement of the hydroxyl group with a thiol group. Again, these results are only valid for AP-M and AP-B; the thiol compounds are actually poorer inhibitors of LAP. Lysinethiol is a slow-binding (time-dependent) inhibitor of AP-B, as are many of the bestatin-derived inhibitors of LAP and AP-M.[15]

All thioamides were significantly poorer inhibitors (5–40 times weaker) than the amide counterpart for all three enzymes tested. The poor inhibition observed for the thioamides neither supports nor disproves an inhibitory chelation mechanism involving the amide carbonyl (Figure 1B) because it is not known how a thiocarbonyl will interact with an active-site metal. The sulfur atom of a thioamide is larger and has reduced hydrogen-bonding capability,[21] compared to the oxygen of an amide, factors that could decrease binding to the enzyme. Except for bond length, the geometrical and conformational properties of the thioamide unit otherwise closely resemble those of an amide.[22]

Table III. Kinetic Constants for Inhibitors

compound	K_i,[a] μM AP-M	LAP	AP-B
L-leucinol (**4a**)	>1000 (-)	>1000 (-)	>1000 (*)
L-leucinethiol (**4**)	0.02 (-)	450 (-)	0.13 (-)
L-lysinol (**5a**)			1000
L-lysinethiol (**5**)	0.11 (-)	>1000 (-)	0.00091 (+)
(2*SR*,3*S*)-α-methylleucinol (**32a**)	355 (+)	507 (+)	>200 (+)
(2*SR*,3*S*)-α-methylleucine-thiol (**32**)	6.0 (-)	>500 (+)	7.0 (+)
(2*S*,3*R*)-AHPBA-OMe (**28a**)	1000 (+)	33 (+)	>1000 (-)
(2*SR*,3*R*) α-thiol of AHPBA-OMe (**28**)	307 (+)	300 (+)	159 (+)
(2*S*,3*R*)-AHPBA-Iaa (**37a**)	20 (*)	0.51 (+)	39 (-)
(2*S*,3*R*) α-thiol of AHPBA-Iaa (**37**)	4.9 (+)	16.7 (+)	4.0 (+)
(2*R*,3*R*) α-thiol of AHPBA-Iaa (**41**)	32 (+)	26 (+)	35.7 (+)
(2*S*,3*R*)-isoamylthioamide (**53**)	703 (*)	4.4 (+)	>1000 (*)
bestatin (**1**)	7.8 (+)	0.02 (+)	0.06 (+)
bestatin thioamide (**49**)	40.3 (-)	0.33 (+)	2.4 (+)
α-thiolbestatin (**6**)	4.4 (+)	0.55 (+)	0.0046 (+)
α-thiolepibestatin (**46**)[b]	9.1 (+)	1.0 (+)	0.0064 (+)

[a] Slow-binding inhibition is indicated in parentheses. An asterisk (*) means that no lag or burst was observed but that increased inhibition was seen after incubation. [b] The 2*RS* diastereomer.

Discussion

The close resemblance of the C-2 hydroxyl group in the N-terminal amino acids **2a,b** in bestatin (**1**) and amastatin (**3**), respectively, to putative tetrahedral intermediates for amide bond hydrolysis led to proposals that these inhibitors bind to the metallo peptidases in a mechanistically related fashion. The Nishizawa et al.[5] model places the critical 2(*S*)-hydroxyl and 3(*R*)-amino groups about the active-site zinc ion (Figure 1A), whereas the Nishino and Powers model pairs the critical 2(*S*)-hydroxyl group with the C-1 carbonyl group for chelation to the active-site zinc ion (Figure 1B). Both modes of binding placed the aromatic side chain of AHPBA in the P_1 position where it would bind to the S_1 enzyme pocket.

If the critical 2(*S*)-hydroxyl group interacts strongly with the active-site zinc in the tightened complexes, then replacement of the hydroxyl group by a sulfhydryl group should lead to tighter binding inhibitors. This strategy has proven successful for the development of inhibitors of other zinc-metallo peptidases, e.g., angiotensin converting enzyme (ACE), which is inhibited by the thiol Captopril,[23] and enkephalinase, which is inhibited by the thiol Thiorphan.[24] Consistent with this postulate is the fact that L-leucinethiol (**4**) strongly inhibits AP-B[20] and AP-M[19] by binding to the target enzyme nearly 10000 times tighter than the corresponding alcohol L-leucinol (**4a**). Subsequently, lysinethiol (**5**) was shown to inhibit AP-B (K_i = 0.1 nM) extremely well,[20] binding about 10000 times more tightly to AP-B than the alcohol **5a**. Thus, replacement of an alcohol with a thiol can greatly enhance the binding of an inhibitor to aminopeptidases in favorable cases.

However, when the corresponding thiolbestatin derivative **6** was prepared by replacing the 2(*S*)-hydroxyl group with the 2(*S*)-thiol group, we were surprised to discover that the inhibitor was only marginally improved relative to the starting bestatin. Thiol bestatin **6** was about 2 times better as an inhibitor of AP-M and 13 times better as an inhibitor of AP-B than **1**; against LAP, **6** was a much weaker inhibitor. In no case did we obtain the 10000

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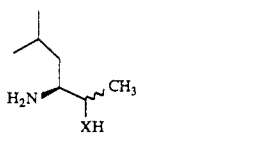
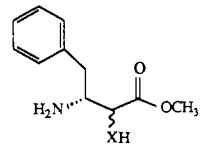
fold enhancements that were observed in the simpler thiol analogues 4, 5.

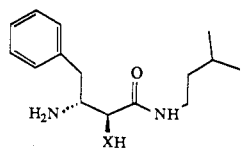
In an attempt to determine why the 2-thiolbestatin derivatives lacked the anticipated enhanced potency, we systematically modified leucinethiol (4) by progressively adding larger functional groups to the inhibitor at the carbon α to the thiol group in order to make each inhibitor more closely resemble the overall structure of bestatin. In this way, the effect of α-methyl, carboxyl, or carboxamide groups on inhibitor potency could be evaluated. Replacing a proton in 4 by a methyl group (32) decreased binding to AP-M and AP-B 300- and 54-fold, respectively. Thus, even the simple modification of adding a methyl group destabilized the interaction between the thiol inhibitors and aminopeptidases, and this accounts for much of the discrepancy between anticipated and observed potency. Incorporation of a carboxyl group α to the thiol, as in 28, gave an even weaker inhibitor. However, addition of the substituted carboxamide functionality α to the thiol, as in 37, favored binding to the enzymes by a factor of about 10 relative to the methyl carboxylate 28, an increase that could be caused by additional stabilizing interactions either to the additional amide proton or to the added isoamyl side chain. Together these results suggest that a substituent α to the thiol group in leucinethiol disfavors binding to the enzymes but that this can be overcome as the size of the inhibitor becomes larger as it is in thiol bestatin 6. Although the idea was not tested, it is possible that longer extensions of the peptide chain might lead to much more potent inhibitors in which the thiol effect is expressed to the same extent it is in L-leucinethiol.



32, X = S
32a, X = O



28, X = S
28a, X = O



37, X = S
37a, X= O
41, X = S; (2R,3R)-diastereomer

In spite of the fact that, in most cases, the α-substituted thiol inhibitor is a weaker inhibitor than L-leucinethiol, replacement of an alcohol hydroxyl group by the thiol group does modestly enhance inhibitor potency. Thus, thiols 32, 28, 37, and 6 are better AP-M and AP-B inhibitors than alcohols 32a, 28a, 37a, and 1. The increased binding varies from a factor of 3 to about 30. These are significant increases although much smaller than observed for L-leucinol to L-leucinethiol.

Our results establish that replacement of the hydroxyl group by the thiol group in bestatin and closely related analogues provides, at best, a modest increase in binding to AP-M and AP-B and weakens binding to LAP. These results suggest that the C-2 hydroxyl in bestatin does not interact strongly with a catalytically active zinc ion presumed to be in the active sites of these three aminopeptidases. In contrast, both L-leucinethiol and L-lysinethiol do bind strongly to AP-B, and L-leucinethiol binds strongly to AP-M, establishing that a zinc–thiol interaction is possible in inhibitors that bind to the S_1 subsite of aminopeptidases. Binding to the S_1 subsite is inferred from the fact that the binding of L-lysinethiol 5 to AP-B is significantly stronger that of L-leucinethiol, bestatin, or thiolbestatin, as would be expected from the presence of a substratelike lysine side chain on lysinethiol that is not present in the other three inhibitors. Since AP-B hydrolyses substrates with N-terminal Lys and Arg residues, a specific interaction between an enzyme anion at the enzyme S_1 subsite and the positively charged ϵ-NH_3 group at P_1 on both substrates and inhibitors is likely, which, in the latter case, would stabilize binding of lysinethiol relative to the nonbasic inhibitors. Because the inhibitor specificity of the thiol inhibitors 4 and 5 parallels the substrate specificity of AP-M and AP-B, we assume that 4 and 5 bind to the S_1 pocket of AP-M and AP-B, respectively. Thus, either the α-substituent in an inhibitor bound at the S_1 subsite hinders the binding of the thiol group to the active-site metal ion or else bestatin does not bind to the S_1 enzyme subsite. Additional experiments will be needed to clarify these questions.

Experimental Section

A. Materials and Methods. 1. Chemical Studies. Melting points were determined with a Fisher-Johns melting point apparatus and are uncorrected. Optical rotations were determined on a Perkin-Elmer 241 polarimeter in a 1.000-dm cell. Proton nuclear magnetic resonance were recorded on a Varian EM390 (90 MHz), a JEOL FX-90Q Fourier transform (90 MHz), or a Bruker W-270 (270 MHz) Fourier transform spectrometer. Chemical shifts are reported in ppm from an internal TMS standard. Carbon-13 nuclear magnetic resonance was recorded on the JEOL FX-90Q (22.5 MHz) spectrometer, and chemical shifts are reported in ppm downfield from internal TMS. Infrared spectra were recorded on a Perkin-Elmer 599B spectrometer. Low-resolution mass spectra were obtained on a Finnegan 1015 GC–mass spectrometer interfaced to a Finnegan M6000 data system. Elemental analyses were determined by combustion analysis by Galbraith Laboratories.

TLC was performed on 0.25-mm thickness silica gel plates (Merck, silica gel 60 F-24). Gravity column chromatography was carried out with Brinkman silica gel 60 (70–270 mesh), while flash chromatography[25] was carried out with Merck silica gel, grade 60, 230–400 mesh. TLC solvent systems used were (v/v) (A) 5% methanol in chloroform; (B) 20% EtOAc/CH_2Cl_2; C) 1-butanol/acetic acid/water, 4:1:1; (D) 20% EtOAc/toluene; (D) 20% EtOAc/$CHCl_3$; (F) 1-butanol/acetic acid/water/pyridine, 15:3:12:10; (G) CH_2Cl_2/CH_3OH/acetic acid, 70:30:1; (H) CH_2Cl_2/CH_3OH/NH_4OH, 70:30:1; (I) 6% EtOAc/$CHCl_3$; (J) 10% CH_3OH/$CHCl_3$; (K) 20% NH_4OH/EtOH. Compounds were visualized on the plates by reaction with (a) ultraviolet light, (b) ninhydrin, (c) chlorox-*o*-tolidine, (d) 5% phosphomolybdic acid in ethanol, (e) iodine, (f) water.

Kinetic Studies. Enzymes. Microsomal aminopeptidase (EC 3.4.11.2), also known as aminopeptidase M, was purchased from Sigma as a suspension in 3.5 M $(NH_4)_2SO_4$ suspension and unless specified was used directly. In order to obtain the (LAP)Zn_6Mg_6 form of the enzyme, the Sigma enzyme was subjected to a 4-h incubation at 37 °C with 10 mM HEPES, pH 8.0, containing 5 mM $MgSO_4$ and 1 M KCl. Aminopeptidase B (EC 3.4.11.6) was obtained from rat liver and purified in this lab by Dr. Scott Harbeson.[26]

Materials. All inhibitors synthesized gave a single spot by TLC and were shown to be analytically pure. Leucine *p*-nitroanilide hydrochloride, dithiothreitol (DTT), and L-leucinethiol were purchased from Sigma Chemical Co. All other chemicals were purchased from Aldrich Chemical Co. except where designated. The following reagents and solvents were used: methanol was distilled from magnesium methoxide; pyridine, benzene, and toluene were distilled from KOH; dimethylformamide was distilled from barium oxide; tetrahydrofuran was distilled from sodium; hexanes were distilled from phosphorus pentoxide; *N*-methylmorpholine and triethylamine were distilled from phthalic anhydride; methylene chloride and ethyl acetate were purchased from Burdick and Jackson Co. (HPLC grade) and used directly. All other reagents and solvents were analytical or reagent grade.

Kinetic Determinations. Spectrophotometric assays were generally carried out as described previously[15] at 25 °C with L-leucine p-nitroanilide as a substrate for aminopeptidase M[27] and cytosolic aminopeptidase,[28,29,15] while L-arginine *p*-nitroanilide was used for aminopeptidase B.[30,26] Hydrolysis of substrate to *p*-nitroaniline was monitored at 405 nm with a Gilford 250 spectrophotometer and a Gilford 6051 recorder. With reference to slow-binding kinetics, initial rates were determined by combining substrate and inhibitor solutions in a cuvette and initiating with enzyme. In the event slow binding was detected, the enzyme solution was incubated for 10–20 min with inhibitor, and then the reaction was initiated by adding substrate. All velocities were recorded as the change in optical density with time at 0.04–0.10 OD full scale.

Typical Assay Conditions. (a) All Enzymes. Substrate concentrations were used that were appropriate to the K_m of the

particular enzyme. For all thiol inhibitors, dithiothreitol was used to keep the inhibitor in the reduced state and was kept at a final concentration of 1.0 mM.

(b) Aminopeptidase M. Typically run in either 0.05 M Tris or MOPS, pH 7.2, containing 5 mM $MgCl_2$. The final enzyme concentration was 5 nM.

(c) Cytosolic Leucine Aminopeptidase. For most experiments, the buffer was 0.1 M Tris, pH 8.5, containing 5 mM $MgCl_2$. In all cases, except for very tight binding inhibitors, the final enzyme concentration was 5 nM. For the very tight binding inhibitors, the enzyme concentration was lowered to 1 nM.

(d) Aminopeptidase B. The buffer was 0.1 M PIPES, pH 7.0, containing 0.2 M NaCl and 1 mM DTT. The final enzyme concentration was estimated to be 4.6 nM[26] for most studies and was diluted 10-fold for use with tight-binding inhibitors.

Data Analysis. The kinetic data were handled in one of two ways.

(1) Computer analysis on a Northstar Horizon computer with the data being fit to one of the following equations for competitive and noncompetitive inhibition with a program written by Professor D. Northrop according to Duggleby's nonlinear regression method:[31]

$$\text{competitive: } V = \frac{VA}{[K_a(1 + I/K_{is})] + A}$$

$$\text{noncompetitive: } V = \frac{VA}{[K_a(1 + I/K_{is}) + A(1 + I/K_{ii})]}$$

(2) The K_i's were determined on the basis of the equation of Cha[32] for tight-binding inhibitors:

$$K_i = \frac{IC_{50} - E_t/2}{1 + S/K_m}$$

[*N*-(*tert*-Butyloxycarbonyl)-*O*-(methylsulfonyl)-3(*R*)-amino-2(*R*)-hydroxy-4-phenylbutanoyl]-L-leucine Methyl Ester (9). Boc-epibestatin methyl ester[15] (**8**) (1.11 mmol) was converted to the mesylate by the following procedure. The free hydroxy compound was dissolved in CH_2Cl_2 (5 mL/mmol) and chilled with an ice bath. To this solution was added TEA (1.5 equiv) and methanesulfonyl chloride (1.3 equiv), and the reaction mixture was stirred for 1 h at 0 °C. The CH_2Cl_2 was evaporated, and the residue was taken up in ethyl acetate and washed with ice H_2O, could 1 N HCl, saturated $NaHCO_3$, and brine. The organic layer was dried (anhydrous $MgSO_4$) and concentrated in vacuo. Precipitation from EtOAc/Skelly B or CH_2Cl_2/Skelly B yielded analytically pure product (1.08 mmol, 97%) as a white solid: $[\alpha]^{23}_D$ +26.8° (*c* 0.37, $CHCl_3$); mp 139–141 °C; ^{1}H NMR (90 MHz, $CDCl_3$) δ 1.00 (d, 6 H, *J* = 5.5 Hz), 1.41 (s, 9 H), 1.72 (br, 3 H), 2.70–3.10 (m, 2 H), 3.10 (s, 3 H), 3.80 (s, 3 H), 4.20–4.82 (m, 3 H), 5.25 (d, 1 H, *J* = 3 Hz), 6.77 (d, 1 H, *J* = 8.8 Hz), 7.30 (s, 5 H).

[*N*-(*tert*-Butyloxycarbonyl)-3(*R*)-amino-2(*S*)-(acetylthio)-4-phenylbutanoyl]-L-leucine Methyl Ester (10). The mesylate **9** (1.0 mmol) was converted to the thioacetate by the following procedure. The mesylate was dissolved in dry DMF (5 mL/mmol) under anhydrous conditions. Solid potassium thioacetate (5 equiv) was then added, and the reaction mixture was stirred at room temperature under a positive N_2 pressure. Primary mesylates generally were completely displaced within 24 h, while the reaction of secondary mesylates often was not complete even after 7 days. Elevated temperatures only served to increase undesirable side products. Workup consisted of adding ethyl acetate (20–30 times by volume) to the dark brown reaction mixture and washing with brine (two times). The organic layer was then dried (anhydrous $MgSO_4$) and concentrated. The residue was taken up in CH_2Cl_2 and treated with activated charcoal (one to two times), which removed much of the brown color. The CH_2Cl_2 was then evaporated, and final purification was achieved with column chromatography. As in the case of the 2*R* isomer, the reaction is very slow and was stopped after 7 days. The crude product was purified via column chromatography (0–4% EtOAc/$CHCl_3$) to yield 0.58 mmol (58%) of the correct 2*S* isomer as a white solid. The reaction did not proceed with complete inversion, however, as 0.05 mmol (5%) of the 2*R* isomer was also isolated: $[\alpha]^{23}_D$ −11.6° (*c* 0.13, $CHCl_3$); mp 157–158 °C; ^{1}H NMR (90 MHz, $CDCl_3$) δ 0.94 (d, 6 H, *J* = 5.5 Hz), 1.32 (s, 9 H), 1.60 (br, 3 H), 2.38 (s, 3 H), 2.92 (br, 2 H), 3.75 (s, 3 H), 4.15–4.93 (m, 4 H), 6.60 (d, 1 H, *J* = 8 Hz), 7.24 (s, 5 H).

[*N*-(*tert*-Butyloxycarbonyl)-3(*R*)-amino-2(*S*)-mercapto-4-phenylbutanoyl]-L-leucine Methyl Ester (11). The thioacetate **10** (0.11 mmol) was dissolved in CH_3OH (10 mL/mmol), and 1.1 equiv of 1 N NaOH was added. The reaction mixture was stirred at room temperature for $^1/_2$ h, diluted with water (10–20 times), and extracted with EtOAc. The organic layer was washed (two times) with brine, dried (anhydrous $MgSO_4$), and concentrated in vacuo to give a stable thiol, which did not oxidize even upon treatment with I_2/MeOH. Flash chromatography (6% EtOAc/$CHCl_3$) of the crude product yielded 0.08 mmol (73%) of the pure product as a white solid: $[\alpha]^{23}_D$ +25 5° (*c* 0.10, $CHCl_3$); mp 160–161 °C; ^{1}H NMR (90 MHz, $CDCl_3$) δ 0.96 (d, 6 H, *J* = 5.5 Hz), 1.34 (s, 9 H), 1.52–1.90 (m, 3 H), 2.20 (d, 1 H, *J* = 9 Hz), 2.95 (d, 2 H, *J* = 6 Hz), 3.52–3.86 (m, 4 H, includes 3.79, s, 3 H), 4.03–4.80 (m, 3 H), 5.08 (d, 1 H, *J* = 10 Hz), 7.27 (s, 5 H). Upon addition of MeOH-d_4 and incubation for 15 min, complete exchange was observed at the 2.20 (SH) resonance and the 5.08 resonance (NH), and less multiplicity was present in the 3.52–3.86 range (α-CH).

(3(*R*)-Amino-2(*S*)-mercapto-4-phenylbutanoyl)-L-leucine Hydrochloride (6). The protected dipeptide 11 (0.80 mmol) was saponified to the acid **12** by dissolving in CH_3OH (2 mL/mmol) and adding 1 N NaOH (1.1 equiv). The reaction was monitored by TLC and upon completion (usually 1–1.5 h) H_2O (2–5 times by volume) was added to the reaction mixture, and then most of the CH_3OH was removed in vacuo. The aqueous layer was washed with ethyl acetate, acidified with solid $KHSO_4$ (pH 1–2), and extracted (two times) with ethyl acetate. The ethyl acetate was then washed with brine (two times), dried (anhydrous $MgSO_4$), and concentrated in vacuo to give a white solid, which was homogeneous by TLC (R_f 0.85; solvent system C) and had an NMR consistent with the free acid. This product (**12**) was used directly and deprotected by reaction with 4 N HCl/dioxane for 30 min to yield a very hygroscopic white solid (0.37 mmol) in 46% yield (two steps). Compound **6** was homogeneous by TLC and HPLC (isocratic, 35% CH_3CN/H_2O with 0.1% TFA) revealed only one isomer in a solvent system that was able to resolve both isomers: $[\alpha]^{23}_D$ −2.0° (*c* 0.10, CH_3OH); ^{1}H NMR (90 MHz, DMSO-d_6) δ 0.88 (d, 6 H, *J* 5 Hz), 1.08–1.87 (m, 3 H), 2.95 (d, 2 H, *J* = 5.5 Hz), 3.04–3.88 (m, includes H_2O peak), 4.19 (br, 2 H), 7.33 (s, 5 H), 8.81 (d, 1 H, *J* = 8 Hz), 9.08 (br, 3 H).

[*N*-(*tert*-Butyloxycarbonyl)-2(*S*)-acetoxy-3(*R*)-amino-4-phenylbutanoyl]-L-leucine Methyl Ester (47). The free hydroxy compound **8** (as previously described)[15] was dissolved in ethyl acetate (5 mL/mmol) in a flask fitted with a drying tube. *N*-methylmorpholine (1.1 equiv) was added, followed by solid DMAP (0.1 equiv). Acetic anhydride (3 equiv) was added, and the reaction mixture was stirred for 1 h at room temperature. After dilution with H_2O, the organic layer was separated and washed with 2 N HCl (two times), saturated $NaHCO_3$ (one time), and brine. The ethyl acetate layer was dried (anhydrous $MgSO_4$), filtered, and concentrated in vacuo. Precipitation from Skelly B/CH_2Cl_2 yielded 0.060 g (0.13 mmol, 87%) of pure product **47** as a white solid: $[\alpha]^{23}_D$ +33.8° (*c* 0.1, $CHCl_3$); mp 136–138 °C; ^{1}H NMR (90 MHz, $CDCl_3$) δ 0.97 (d, 6 H, *J* = 5 Hz), 1.33 (s, 9 H), 1.48–1.74 (m, 3 H), 2.16 (s, 3 H), 2.74–2.92 (m, 2 H), 3.74 (s, 3 H), 4.14–4.81 (m, 2 H), 5.03 (d, 1 H, *J* = 8 Hz), 5.19 (d, 1 H, *J* = 4 Hz), 6.41 (d, 1 H, *J* = 9 Hz), 7.29 (s, 5 H); ^{13}C NMR ($CDCl_3$) δ 20.64, 22.04, 22.75, 24.92, 28.28, 38.34, 41.77, 50.76, 52.40, 53.19, 74.11, 79.74, 126.66, 128.55, 129.32, 137.28, 154.99, 167.72, 169.29, 173.14.

[*N*-(*tert*-Butyloxycarbonyl)-2(*S*)-acetoxy-3(*R*)-amino-4-phenylthiobutanoyl]-L-leucine Methyl Ester (48). To dipeptide **47** (0.49 g, 0.098 mmol) in 1 mL of dry benzene was added 0.20 g (0.049 mmol) of Lawesson's Reagent[33] (Aldrich). The reaction mixture was refluxed 2 h under a slow N_2 flow. The solution was evaporated to dryness in vacuo and then applied directly to a silica gel column, eluting with 2% EtOAc/$CHCl_3$ to yield 0.034 g (0.070 mmol, 71%) of pure **48** as a clear oil: $[\alpha]^{23}_D$ +11.6° (*c* 0.1, $CHCl_3$); ^{1}H NMR (90 MHz, $CDCl_3$) δ 0.96 (d, 6 H, *J* = 3 Hz), 1.30 (s, 9 H), 1.47–2.13 (m, 3 H), 2.21 (s, 3 H), 2.72–3.00 (m, 2 H), 3.71 (s, 3 H), 4.39–5.37 (m, 3 H), 5.61 (s, 1 H, *J* = 3 Hz), 7.27 (s, 5 H), 8.17 (d, 1 H, *J* = 9 Hz); ^{13}C NMR ($CDCl_3$) δ 20.68,

22.34, 22.76, 24.97, 28.19, 38.14, 39.09, 40.82, 41.71, 52.50, 55.71, 79.79, 80.57, 126.63, 128.48, 129.25, 137.12, 154.08, 168.53, 172.40, 197.07.

(2(*S*)-Hydroxy-3(*R*)-amino-4-phenylthiobutanoyl)-L-leucine Trifluoroacetate (49). The protected thiodipeptide **48** (0.17 mmol) was saponified with 0.4 mL of 1 N NaOH, which removed both the acetyl group and the methyl ester as shown by ^{1}H NMR spectroscopy. The reaction was worked up as described for compound **6** to yield a white solid (0.16 mmol) in 94% yield: ^{1}H NMR (90 MHz, $CDCl_3$) δ 0.98 (d, 6 H, J = 6 Hz), 1.26 (s, 9 H), 1.81 (br, 3 H), 2.97 (br, 2 H), 4.30–4.74 (m, 2 H), 5.18 (br, 2 H), 6.61 (br, 1 H), 7.31 (s, 5 H), 9.51 (br, 1 H). The Boc free acid (0.13 mmol) was used directly and deprotected with 4 N HCl/dioxane. Trituration with ether yielded a yellow oil, which was subjected to semipreparative HPLC for purification with a 20–40% gradient of CH_3CN/H_2O, both containing 0.1% TFA. Lyophilization yielded a white solid, which was extremely sensitive to moisture (0.094 mmol, 72%): $[\alpha]^{23}_D$ −22.2° (c 0.12, CH_3OH); ^{1}H NMR (90 MHz, MeOH-d_4 + $CDCl_3$) δ 0.97 (d, 3 H, J = 6 Hz), 1.02 (d, 3 H, J = 6 Hz), 1.02, (d, 3 H, J = 6 Hz), 1.59–2.01 (m, 3 H), 3.02 (d, 2 H, J = 8 Hz), 3.10 (d, 2 H, J = 8 Hz), 3.57–3.84 (m, 1 H), 3.95–4.24 (m, 1 H), 4.87–5.09 (m, 1 H), 7.34 (s, 5 H); ^{13}C NMR (MeOH-d_4) 20.32, 20.75, 24.16, 34.73, 39.11, 56.07, 56.45, 73.41, 126.33, 127.85, 128.18, 134.52, 172.76 (broadened), 200.39.

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Registry No. **1**, 58970-76-6; **4**, 88264-65-7; **4a**, 7533-40-6; **5**, 110675-37-1; **5a**, 110690-36-3; **6**, 114886-46-3; **8**, 77171-42-7; **9**, 114886-47-4; **10**, 114886-48-5; **11**, 114886-49-6; **12**, 114886-50-9; **13**, 109687-65-2; **14**, 112157-31-0; **15**, 112157-32-1; **16**, 112157-33-2; **17**, 114886-51-0; **18**, 114907-39-0; **19**, 114886-52-1; **20**, 114886-53-2; **21**, 114886-54-3; **22**, 114886-55-4; **23**, 114886-56-5; **24**, 114886-57-6; **25**, 114886-58-7; (2*R*,3*R*)-**26**, 114886-59-8; (2*S*,3*R*)-**26**, 114886-86-1; (2*R*,3*R*)-**27**, 114886-60-1; (2*S*,3*R*)-**27**, 114886-87-2; (2*R*,3*R*)-**28**, 114886-61-2; (2*S*,3*R*)-**28**, 114886-88-3; **28a**, 114886-83-8; (2*R*,3*S*)-**29**, 114886-62-3; (2*S*,3*S*)-**29**, 114886-81-6; (2*R*,3*S*)-**30**, 114886-63-4; (2*S*,3*S*)-**30**, 114886-82-7; (2*R*,3*S*)-**31**, 114886-64-5; (2*S*,3*S*)-**31**, 114976-87-3; (2*R*,3*S*)-**32**, 114886-65-6; (2*S*,3*S*)-**32**, 114926-91-9; (2*S*,3*S*)-**32a**, 111061-11-1; (2*R*,3*S*)-**32a**, 111061-12-2; **33**, 114886-66-7; **34**, 114886-67-8; **35**, 114886-68-9; **36**, 114886-70-3; **37**, 114926-84-0; **37a**, 88826-02-2; **38**, 114886-71-4; **39**, 114886-72-5; **40**, 114926-85-1; **41**, 114976-69-1; **42**, 114926-87-3; (2*S*,3*R*)-**43**, 114926-88-4; (2*R*,3*R*)-**44**, 114976-70-4; (2*R*,3*R*)-**45**, 114926-89-5; (2*R*,3*R*)-**46**, 114926-90-8; **47**, 114886-73-6; **48**, 114886-74-7; **48** (saponified), 114886-89-4; **49**, 114886-76-9; **50**, 114886-77-0; **51**, 114886-78-1; **52**, 114886-79-2; **53**, 114886-80-5; AP-M, 9054-63-1; LAP, 9001-61-0; AP-B, 9073-92-1; (2*R*,3*R*)-$PhCH_2CH(NH(BOC))CH(SH)CONH(i\text{-}C_5H_{11})$, 114886-84-9; (2*S*,3*R*)-$PhCH_2CH(NH(BOC))CH(SH)CONH(i\text{-}C_5H_{11})$, 114886-85-0.

Design of Novel Inhibitors of Aminopeptidases. Synthesis of Peptide-Derived Diamino Thiols and Sulfur Replacement Analogues of Bestatin

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Investigations were directed toward inhibition of an aminopeptidase, isolated from rat brain, which has been implicated in the metabolic inactivation of enkephalins. The design rationale and synthesis of novel peptidyl diamino thiol inhibitors of rat brain aminopeptidase are presented, along with accompanying structure–activity analysis. Some of the reported compounds are highly active aminopeptidase inhibitors and possess enzyme inhibitory potency in the nanomolar range (**62**; I_{50} = 1 nM). Analysis of the data permits speculations on possible modes of binding of diamino thiols to aminopeptidase. Other investigations were directed toward understanding the mode of enzyme binding of the naturally occurring aminopeptidase inhibitor bestatin. On the basis of published models of enzyme binding, replacement of the C-2 hydroxyl group of bestatin by a sulfhydryl group was anticipated to lead to enhanced inhibition due to a strengthened interaction of this group with enzymic zinc. Contrary to expectations, "thiobestatin" inhibited rat brain aminopeptidase with only the same degree of effectiveness as the corresponding alcohol. Speculations on the possible mode of enzyme–inhibitor binding of bestatin are offered.

The aminopeptidases (APASE) are a group of exopeptidases that specifically cleave polypeptide chains at the amino terminus. These enzymes are ubiquitous in nature and are of biochemical and medicinal importance due to their key role in the metabolism of numerous biologically active peptides, for example the enkephalins. It is well established that the weak and short-lasting biological activity of the enkephalins can be attributed to their rapid inactivation. Enkephalins are metabolized by several hydrolytic enzymes present in brain: (1) aminopeptidases release Tyr^1, (2) a dipeptidyl aminopeptidase releases the Tyr^1–Gly^2 fragment, and (3) two enzymes cleave the penultimate Gly^3–Phe^4 bond to release the C-terminal dipeptide; angiotensin converting enzyme (ACE) and neutral endopeptidase 24.11, often commonly designated "enkephalinase" (ENKASE)[1,2] (see Figure 1). Cleavage at the Tyr^1–Gly^2 bond may be physiologically important in light of the analgesic effects induced by the aminopeptidase inhibitor bestatin (**1**),[3–6] and because of the complete protection of endogenous enkephalins released from K^+ depolarized brain slices in the presence of **1** together with thiorphan, an enkephalinase inhibitor.[6] A further point bearing on the pharmacological significance of aminopeptidase degradation is that enkephalin analogues fortified against APASE action elicited enhanced analgesic activity.[8] In view of our earlier work directed

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