Apstatin Analogue Inhibitors of Aminopeptidase P, a Bradykinin-Degrading Enzyme

Linda L. Maggiora,*,† Arthur T. Orawski,‡ and William H. Simmons‡

Pharmacia and Upjohn, Inc., Kalamazoo, Michigan 49001, and Department of Molecular and Cellular Biochemistry, Stritch School of Medicine, Loyola University Chicago, Maywood, Illinois 60153

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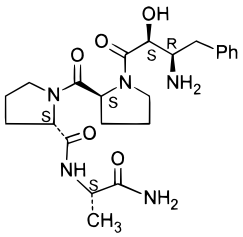
Membrane-bound aminopeptidase P (AP-P) participates in the degradation of bradykinin in several vascular beds. We have developed an inhibitor of AP-P called apstatin (**1**) (*N*-[(2*S*,3*R*)-3-amino-2-hydroxy-4-phenyl-butanoyl]-L-prolyl-L-prolyl-L-alaninamide); $IC_{50,human} = 2.9\ \mu M$. In the rat, apstatin can potentiate the vasodilatory effect of bradykinin, reduce blood pressure in an aortic-coarctation model of hypertension, and reduce cardiac damage and arrhythmias induced by ischemia/reperfusion. In this study, we have determined structure−activity relationships for apstatin analogues as well as for other chemical classes of inhibitors using AP-P isozymes from different sources. The most potent inhibitor was one in which the N-terminal residue of apstatin was replaced with a (2*S*,3*R*)-3-amino-2-hydroxy-5-methyl-hexanoyl residue (**6**, $IC_{50,human} = 0.23\ \mu M$). The (2*R*,3*S*)-analogue of **6** was equipotent with **6** while the (2*S*,3*S*)- and (2*R*,3*R*)-analogues were considerably less potent. Apstatin analogues lacking the L-alanine or having hydroxyproline in place of the proline in the second position had reduced affinity. Certain thiol-, carboxylalkyl-, and hydroxamate-containing compounds were inhibitory in the low micromolar range. Human cytosolic AP-P isozymes and *Escherichia coli* AP-P exhibited different inhibitor profiles than mammalian membrane-bound AP-P isozymes. The effects of the compounds on X-Pro dipeptidase (prolidase) and leucyl aminopeptidase are also presented.

Introduction

Aminopeptidase P can inactivate bradykinin by cleaving the Arg[1]−Pro[2] bond.[1] Aminopeptidase P and angiotensin converting enzyme (ACE) (which cleaves the Pro[7]−Phe[8] bond of bradykinin) are totally responsible for the extensive degradation of bradykinin which occurs in the pulmonary and coronary circulations of the rat.[2−4] Since bradykinin exhibits potent vasodilatory and cardioprotective effects,[5−7] there may be a therapeutic benefit to inhibiting these two enzymes and thereby increasing endogenous levels of bradykinin. Indeed, inhibitors of ACE are widely prescribed for a variety of cardiovascular disorders. Many of the beneficial effects of ACE inhibitors, particularly cardioprotective effects, have been shown to be due to inhibition of bradykinin degradation rather than inhibition of angiotensin II formation which is the other function of this enzyme.[6−7] By analogy, we have predicted that inhibitors of aminopeptidase P will also exhibit cardioprotective effects by potentiating endogenously released bradykinin, possibly acting synergistically with ACE inhibitors in this regard.

Aminopeptidase P (X-Pro aminopeptidase; EC 3.4.11.9) removes the N-terminal amino acid from peptides which have a proline residue in the second position.[8−11] There are different isoforms of aminopeptidase P including a membrane-bound form and a cytosolic form which appear to differ in their substrate specificities.[11−12] It has been assumed that the membrane-bound form is responsible for the extracellular degradation of bradykinin in the pulmonary and coronary circulations. This form has been shown to be located on the plasma membrane of vascular endothelial cells.[13]

Membrane-bound aminopeptidase P has been purified from various mammalian tissues.[1,14−18] The enzyme is a zinc-containing metallopeptidase.[19] Substrate specificity studies[12] have indicated that this enzyme has a broad specificity for the first amino acid of a peptide substrate but requires proline (or dehydroproline or homoproline) in the second position. The enzyme requires at least a third amino acid (dipeptides are not cleaved), and this third residue must have a small side chain (e.g., Ala, Pro, Gly, and sometimes Val). On the basis of this information, a lead inhibitor compound referred to as apstatin (**1**) was prepared.[3]



Apstatin **1**

(2*S*,3*R*)-3-Amino-2-hydroxy-4-phenyl-butanoic acid (AHPB) was used as the N-terminal residue of apstatin because of its metal-chelating properties. AHPB is present in bestatin, a bacterial product which inhibits broad-specificity aminopeptidases.[20] This residue in

* To whom correspondence should be addressed. Phone: 616-833-9345. Fax: 616-833-2500. E-mail: Linda.L.Maggiora@am.pnu.com.
† Pharmacia and Upjohn, Inc.
‡ Loyola University Chicago.

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bestatin has been shown by X-ray crystallography to coordinate an active site Zn^{2+} in leucyl aminopeptidase.[21] The second and third amino acids of apstatin are proline residues to accommodate the specificities of the corresponding binding subsites in aminopeptidase P.[12] A fourth amino acid amide (Ala−NH_2) was added since tetrapeptide substrates show higher binding affinities than tripeptides.[1,12,17] Apstatin has K_i values of 2.6 μM and 0.64 μM for rat and human membrane-bound aminopeptidase P, respectively.[12]

Apstatin can block the cleavage of the Arg^1−Pro^2 bond of bradykinin in the rat pulmonary and coronary circulations.[3−4] Apstatin can potentiate the blood pressure lowering effects of bradykinin given by i.v. injection.[22] When combined with an ACE inhibitor, apastatin can substantially reduce blood pressure in rats made hypertensive by aortic coarctation.[23] Apstatin also exhibits cardioprotective effects. Using the isolated perfused rat heart model of ischemia/reperfusion injury, apstatin was shown to reduce the duration of reperfusion-induced ventricular fibrillation and to reduce cardiac damage as evidenced by a decrease in the outflow of the cytosolic enzymes, creatine kinase, and lactate dehydrogenase, during reperfusion.[24]

The present study was designed to investigate the structure−activity relationships of apstatin analogues for various aminopeptidase P isoforms and to test whether aminopeptidase P-specific modifications of known metallopeptidase inhibitors (e.g., thiols and hydroxamates) can also inhibit these enzymes.

Chemistry

All amino acids shown in the tables are of the L-configuration unless noted otherwise. The compounds shown in Table 1 (**1**−**5**) were synthesized by standard solid phase peptide chemistry methods using commercially available Boc protected amino acids. Compounds **6**, **8**, and **10** shown in Table 2 were synthesized by solution phase methods using commercially available starting materials having the configurations shown in the table. The (2*RS*,3*S*)-3-amino-2-hydroxy-5-methylhexanoic acids needed for compounds **7** and **9** were synthesized by literature methods via a cyanohydrin intermediate starting with L-leucinal.[25] The stereoisomers were separated by reverse phase chromatography of the final product peptides. The carboxy alkyl inhibitor, compound **11**, was synthesized by a coupling reaction of Et-2-oxo-4-phenylbutyrate to Pro-Pro-Ala-NH_2 tripeptide in a standard reductive amination procedure using $NaCNBH_4$, followed by saponification of the ethyl ester.

The inhibitors shown in Table 3 were synthesized by coupling the appropriate cyclic carboxylic acids to the dipeptide, Pro-Ala-NH_2. For compounds **12** and **13** *cis*- and *trans*-2-(4-methoxy-benzylthio)-cyclopentanecarboxylic acid was synthesized by base-catalyzed addition of 4-methoxybenzyl mercaptan to cyclopentene-1-carboxylic acid under phase transfer conditions.[26] NMR analysis of the mixture of isomers indicated an approximate 2 to 1 ratio of isomers, although the identity of each of the isomers could not definitively be assigned by NMR. An aliquot of the mixture of isomers was separated by reverse phase chromatography. The faster moving, higher melting isomer was tentatively assigned the cis configuration by analogy to the melting point of the *S*-benzyl analogue.[26] Correspondingly, the lower melting, slower moving isomer was assigned the trans configuration. The mixture of cis and trans isomers was coupled to the Pro-Ala-NH_2 dipeptide, and the 4-methoxybenzyl protecting group was removed from the thiol by treatment with HF. The cis and trans peptide stereoisomers were separated by reverse phase chromatography. It is presumed that the faster moving isomer has the cis configuration, while the slower moving isomer is the trans. Compounds **14**, **15**, and **16** were synthesized starting from *cis*- or (±)*trans*-1,2-cyclohexanedicarboxylic anhydride using a solid phase procedure. Each anhydride was reacted with 1 equiv of Pro-Ala-NH_2. For the *trans*-cyclohexane starting material, the stereoisomers were separated by reverse phase chromatography. Each peptide isomer was then treated with *O*-benzyl hydroxylamine followed by catalytic hydrogenation to generate the hydroxamate final product. The *cis*-hydroxamate was synthesized in an analogous fashion.

Results and Discussion

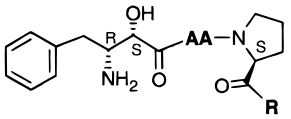
Table 1 shows the effects of various analogues of apstatin on the activity of membrane-bound aminopeptidase P from different species. All of the compounds in Table 1 contain an N-terminal (2*S*,3*R*)-3-amino-2-hydroxy-4-phenyl-butanoyl (AHPB) residue which serves as a putative chelator of an active site Zn^{2+} ion.

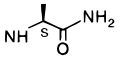
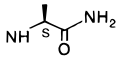
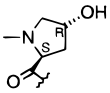
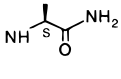
Replacement of the L-proline residue in the second position of apstatin (**1**) with L-thiaproline (**2**) led to a 2−4-fold decrease in affinity. This is in contrast to results with another proline-specific peptidase, prolyl oligopeptidase, in which a similar replacement greatly increased the affinity of inhibitors for this enzyme.[27] Replacement of L-proline in the second position with *trans*-4-hydroxy-L-proline (**3**) reduced affinity by 2 orders of magnitude.

Compound **4**, which is missing the C-terminal alanyl residue of apstatin, exhibited a 15−140-fold reduction in affinity. In previous studies, tripeptide substrates were also shown to have reduced affinity compared with tetrapeptide and larger substrates,[1,12,17] suggesting that occupation of a fourth binding subsite in the enzyme is required for maximal binding. Compound **5**, the longest compound in the series of inhibitors which contain an N-terminal AHPB residue, also had the highest affinity in this series for each of the membrane-bound aminopeptidase P preparations tested.

Table 2 shows the effect of analogues of apstatin having N-terminal modifications on membrane-bound aminopeptidase P from various species. In compounds **6**−**9**, the N-terminal AHPB residue of apstatin is replaced with a 3-amino-2-hydroxy-5-methyl-hexanoyl (AHMH) residue. Compound **6,** which has an AHMH residue with 2*S*,3*R* stereochemistry, exhibited a 2−46-fold higher affinity than apstatin and was the most potent inhibitor of the human enzyme ($IC_{50} = 0.23\ \mu M$). The data indicate that the leucine-like isobutyl side chain of AHMH interacts more favorably with membrane-bound aminopeptidase P than the phenylalanine-like benzyl side chain of the AHPB residue. A bacterial product called amastatin which inhibits broad-specific-

Table 1. Modifications of the Penultimate Proline or the C-Terminal Residue of Apstatin

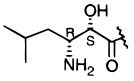
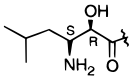
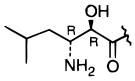
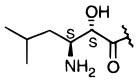
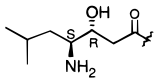
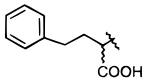


			IC$_{50}$ (μM) for membrane-bound aminopeptidase P[a]			
cmpd #	AA	R	human	monkey	rat	bovine
1[c]			2.9[b] (K_i = 0.64)	6.1	4.1[b] (K_i = 2.6)	9.4[b] (K_i = 7.8)
2			11.	18.	9.2	34.
3			600.	470.	1700.	>2000
4		—NH_2	79.	89.	390.	1300.
5		-ala-β-ala- -cys-NH_2 (L-amino acids)	2.8	3.4	0.66	4.0

[a] IC$_{50}$s determined in triplicate by linear regression analysis of the linear portion of the rate vs log inhibitor concentration plot. Average correlation coefficients for all determinations equal to 0.96. IC$_{50}$s determined with 0.5 mM Arg-Pro-Pro in 0.1 M Hepes, pH 8.0. [b] Reference 3. [c] Cmpd **1** = apstatin, available from Sigma Chemical Company.

Table 2. N-Terminal Modifications of Apstatin

R-Pro-Ala-NH_2

		IC$_{50}$ (μM) for membrane-bound aminopeptidase P[a]			
Cmpd #	R	human	monkey	rat	bovine
6		0.23	0.13	0.56	4.5
7		0.43	0.23	0.31	2.1
8		31.	30.	19.	50.
9		88.	58.	100.	470
10		>1000	>2000	1200.	>2000
11		2.6	8.4	3.8 (1.5)[b]	4.6 (1.1)[b]

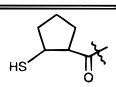
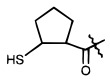
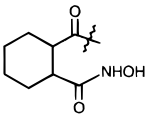
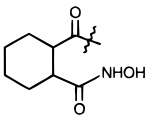
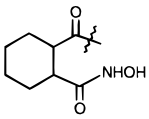
[a] See footnote *a*, Table 1. [b] In the presense of 4 mM $MnCl_2$.

ity aminopeptidases also has an N-terminal (2*S*,3*R*)-AHMH residue.[28] Compound **7**, the 2*R*,3*S* stereoisomer of **6**, had similar affinities compared to **6** for the membrane-bound aminopeptidase P enzymes. However, the 2*R*,3*R* and 2*S*,3*S* stereoisomers (**8** and **9**, respectively) had notably lower affinities than **6** and **7**. Compound **10**, which has a methylene group inserted between the carbinol and carbonyl functions of **7**, had no inhibitory activity.

The data for compounds **1**−**9** indicate that there are species differences with respect to the degree of inhibition of membrane-bound aminopeptidase P by apastatin analogues. The human and cynomolgus monkey enzymes exhibited very similar IC$_{50}$ values. The bovine enzyme gave consistently higher IC$_{50}$ values than the other enzymes. Bovine aminopeptidase P has also been shown to exhibit higher K_m values for bradykinin homologue substrates compared to the rat enzyme.[1,17]

Table 3. Substitutions for the AHPB-Pro Residues of Apstatin

R-Pro-Ala-NH_2

Cmpd #	R	stereoisomer	IC_{50} (μM) for membrane-bound aminopeptidase P[a] human	monkey	rat	bovine
12		*trans* (?) fast isomer[b]	29	13.	3.4	10.
13		*cis* (?) slow isomer[b]	390.	300.	150.	300.
14		*trans* fast isomer[b]	48.	21.	37.	50.
15		*trans* slow isomer[b]	>1300	9000	>500	>500
16		*cis*	>5000	>5000	>5000	>5000

[a] See footnote *a*, Table 1. [b] Fast and slow moving isomers from reverse phase HPLC.

The inhibitor IC_{50} values were higher for the bovine enzyme regardless of where in the apstatin molecule the structural modifications were made. These results suggest that bovine aminopeptidase P may have an altered primary-specificity proline binding subsite (S_1')[29] which consistently results in reduced affinity for an inhibitor or substrate.

In compound **11**, the AHPB residue of apstatin is replaced with an *N*-[-1-(*R*,*S*)-carboxy-3-phenylpropyl]-group. This carboxyalkyl-type compound was inhibitory in the low micromolar range, suggesting that a carboxyl group may serve as a Zn^{2+}-chelating function. The presence of 4 mM $MnCl_2$ in the assay increased the affinity for **11** by 2.4−4.2-fold. This effect of Mn^{2+} was much less than that seen for enalaprilat and ramiprilat, carboxyalkyl inhibitors of ACE which can also inhibit aminopeptidase P.[17,19] Mn^{2+} was shown to enhance the inhibitory potency of enalaprilat toward rat aminopeptidase P by 150-fold.[17] The Mn^{2+}-potentiation of enalaprilat inhibition may relate to the presence of a free C-terminal carboxyl group not present in **11**.[30]

Table 3 shows the inhibitory potencies of thiol and hydroxamate compounds for the various membrane-bound aminopeptidase P preparations. In compounds **12** and **13,** a (2-thiolcyclopentyl)carbonyl group replaces the terminal AHPB-Pro of apstatin. In these analogues, the thiol group is designed to coordinate the active site Zn^{2+}. The putative trans isomer (**12**) exhibited inhibitory activity in the low micromolar range while the putative cis isomer (**13**) was considerably less potent. It is not known whether the cyclopentane ring of these compounds occupies the first binding subsite of the enzyme (S_1)[29] or the second primary-specificity proline-binding subsite (S_1'). If it occupies the S_1 subsite, it can be predicted that extending the compound by one more amino acid so that the fourth (S_3') subsite is also occupied should increase affinity.

Compounds **14**−**16** are apstatin analogues in which the AHPB-Pro has been replaced with a [(*cis*- or *trans*-2-hydroxyaminocarbonyl)cyclohexyl]carbonyl group. In these compounds, the hydroxamate is designed to coordinate the active site Zn^{2+}. Compound **14** was inhibitory while compounds **15** and **16** were not. While the S_1' subsite of aminopeptidase P has been shown to accommodate a six-membered ring (homoproline),[17] it is not known whether the cyclohexyl group of **14** occupies this site or the S_1 subsite.

Table 4 shows the effect of the compounds on cytosolic aminopeptidase P preparations from human heart, human platelets, and *Escherichia coli* as well as on prolidase (X-Pro dipeptidase) (EC 3.4.13.9) and leucyl aminopeptidase (EC 3.4.11.1). The latter two enzymes were included in the screening since they had been shown previously to be inhibited by apstatin.[3] In addition, prolidase is structurally, and presumably mechanistically, related to aminopeptidase P but is specific for X-Pro dipeptides.[31,32]

The human cytosolic aminopeptidase P had a significantly different inhibitor profile than the human membrane-bound isozyme (Table 4). The majority of the compounds were considerably less potent as inhibitors of the cytosolic enzyme than of the membrane-bound form. For example, **7**, **8**, **11**, and **14** were 53-, >48-, 280-, and >63-fold, respectively, poorer inhibitors of the cytosolic than of the membrane-bound enzyme. On the other hand, **4**, a pseudo tripeptidamide, was a somewhat better inhibitor of the cytosolic enzyme. This may be related to the fact that the cytosolic enzyme appears to be better at hydrolyzing di- and tripeptide substrates than the membrane-bound form.[1,12,17,33] The thiol-

Table 4. Specificity of Inhibitors

	IC_{50} (μM)				
	aminopeptidase P isozymes[a]				
cmpd	human heart cytosolic	human platelet	*E. coli*	prolidase[b]	leucyl aminopeptidase[c]
1	21[d]	18	9.0	4.9[d]	0.3[d]
2	82		41.	29.	0.18
3	>2000	>2000	160	110	1.6
4	45.	250.	16.	2.2	2.5
5	2.7	6.8	7.4	130	0.29
6	6.3	1.2	2.2	2.2	12.
7	23.	1.1	1.7	0.66	7.8
8	>1500	>1500	1300	410	>1000
9	92	>800	390	59.	450
10	>2000		>2000	>2000	~800
11	720	5.7	2.1	>2000	>1000
12	6.3			>2000	>1000
13	53.		>3000	>2000	>1000
14	>3000		130	1700	220
15	>2000		>2000	>2000	>1000
16	>5000		2000	>50000	~5000

[a] See footnote *a*, Table 1. [b] From pig kidney; assayed using 0.5 mM Arg-Pro in 0.1 M Hepes, pH 8.0. [c] From pig kidney; assayed using 0.5 mM leucyl-*p*-nitroanilide in 0.1 M Hepes, pH 8.0, containing 0.2 mM $MnCl_2$ and 3.5% DMSO. [d] Reference 3.

containing compounds **12** and **13** were also better inhibitors of the cytosolic enzyme. Size may also be a factor in these cases if the cyclopentane ring is actually binding to the first (S_1) subsite of each of these isozymes instead of the second (S_1') subsite. If this is the case, the fourth (S_3') subsite remains unoccupied. This orientation of the inhibitor may favor binding to the cytosolic enzyme. Overall, the results provide additional evidence that the cytosolic and membrane-bound forms of aminopeptidase P are distinct gene products.

Human platelet aminopeptidase P activity was found in the 43000*g* supernatant of platelet extracts. However, the sensitivity of this activity to the various compounds tested as inhibitors (Table 4) was different from that of either the human heart cytosolic enzyme or the human lung membrane-bound enzyme. For example, **11** was a 130-fold better inhibitor of the platelet enzyme than of the heart cytosolic enzyme. On the other hand, compounds **8** and **9** revealed clear differences between the platelet and membrane-bound enzymes. The platelet enzyme is like the membrane-bound enzyme, however, in showing a strong Mn^{2+}-dependence for inhibition by the pseudodipeptide AHMH-Pro, a characteristic not found for the cytosolic form.[30] In addition, the inhibitory potency of the nonspecific chelating agent, 1,10-phenanthroline, for the platelet enzyme (IC_{50} = 97 μM) is similar to that for the membrane-bound enzyme (IC_{50} = 93 μM) but considerably different than that for the cytosolic enzyme (IC_{50} = 9.2 μM) (unpublished data).

Recombinant *E. coli* aminopeptidase P was inhibited by several of the compounds (Table 4). The most potent inhibitor was **7** (IC_{50} = 1.7 μM).

Prolidase (X-Pro dipeptidase) was inhibited to some extent by all compounds which had an α-hydroxy-*β*-amino acid in the first position (**1**−**9**) (Table 4). None of the thiol, carboxyalkyl, or hydroxamate compounds (**11**−**16**) inhibited prolidase. For the compounds which had a terminal AHPB-Pro- sequence, the affinity decreased with increasing length (IC_{50}s: **4** < **1** ≪ **5**), which is opposite of the results seen with the aminopeptidase P isozymes. This is consistent with substrate specificity data showing that prolidase is specific for dipeptides while aminopeptidase P can hydrolyze oligopeptides.[12,34] In fact, the pseudodipeptide, AHMH-Pro, has been found to be an extremely potent inhibitor of prolidase (K_i = 16 nM).[30] Prolidase, like the aminopeptidase P preparations, preferred the isobutyl group of AHMH (e.g., **6** and **7**) over the benzyl group of AHPB (**1**). Prolidase showed a preference for the 2*S*,3*R* and 2*R*,3*S* stereoisomers of AHMH (**6** and **7**) over the 2*S*,3*S* and 2*R*,3*R* isomers (**8** and **9**), similar to aminopeptidase P.

Leucyl aminopeptidase, a cytosolic broad-specificity aminopeptidase, was inhibited in the micromolar range by some of the compounds investigated despite the fact that this enzyme cannot hydrolyze X-Pro bonds[35] (Table 4). Of the compounds containing an α-hydroxy-*β*-amino acid, those with an AHPB residue were better inhibitors than those with an AHMH residue (e.g., **1** vs **6**). This indicates that, in contrast to aminopeptidase P and prolidase, leucyl aminopeptidase prefers the benzyl group over the isobutyl group in the first position of these pseudopeptides. Similar conclusions were reached previously using analogues of amastatin[36] and bestatin.[37] Replacement of the proline residue in the second position of apstatin (**1**) with hydroxyproline (**3**) did not greatly affect the affinity for leucyl aminopeptidase in keeping with the broader specificity of the S_1' subsite of this enzyme compared with that of aminopeptidase P. Of the stereoisomers of **6**, the 2*S*,3*R* (**6**) and 2*R*,3*S* (**7**) isomers were much better inhibitors than the 2*R*,3*R* (**8**) and 2*S*,3*S* (**9**) isomers. Previously, the 2*S*,3*R* isomers of amastatin[36,37] and bestatin[37] were shown to be more potent inhibitors of leucyl aminopeptidase than the corresponding 2*R*,3*R* isomers. Neither the carboxyalkyl (**11**) nor the thiol compounds (**12** and **13**) inhibited leucyl aminopeptidase. Of the hydroxamates (**14**−**16**), only **14** showed some inhibitory activity.

Conclusions

This study examined the inhibitory effects of different analogues of apstatin on the activity of aminopeptidase P isozymes. Particular emphasis was placed on the membrane-bound form of the enzyme which appears to be important in the metabolism of bradykinin by vascular endothelial cells. The most potent inhibitor of human membrane-bound aminopeptidase P was **6**. The

structural features of **6** contributing to its potency include the N-terminal α-hydroxy-β-amino acid with 2*S*,3*R* stereochemistry and an isobutyl side chain, an unmodified proline in the second position, and the tetrapeptide length. The substitution of the N-terminal benzyl side chain of apstatin with an isobutyl side chain to give **6** increased potency by 10-fold. This suggests that other side chain modifications in this residue, as well as in the third and fourth residues, could further increase potency. This study also revealed that the terminal amino group is not required as a putative metal-chelating function but can be replaced by a thiol, carboxyl, or even hydroxamate group.

The testing of the apstatin analogues on different preparations of aminopeptidase P activity indicated that humans have at least three different isozymes of aminopeptidase P. These include the membrane-bound form and two cytosolic forms, one found in heart extracts and the other in platelets. Some of the analogues showed selectivity in their inhibition of these different forms.

Apstatin has recently been shown to (1) potentiate the hypotensive effects of bradykinin, (2) significantly reduce blood pressure in a severe hypertensive rat model when combined with an ACE inhibitor, and (3) greatly reduce necrosis and ventricular arrhythmias in the isolated perfused rat heart subjected to ischemia and reperfusion.[22−24] These results indicate that aminopeptidase P inhibitors may be efficacious in the treatment of various cardiovascular disorders by increasing levels of endogenously formed bradykinin. This paper identifies compounds with submicromolar affinity for aminopeptidase P which could serve as leads for the development of more potent inhibitors. Although the apstatin analogues tested are peptidic in nature, they were designed to be protease resistant.[3] Studies in rats showed that the bradykinin-potentiating activity of apstatin is undiminished 5 h after intravenous administration,[23] confirming its metabolic stability. The oral bioavailability of apstatin analogues has yet to be investigated.

The unusual specificity of membrane-bound aminopeptidase P and its ability to inactivate bradykinin, a potent endogenous vasodilator and cardioprotective peptide, make this enzyme an attractive target for the development of new cardiovascular drugs.

Experimental Section

General Procedures. Commercially available reagents were purchased from Sigma, Aldrich, Lancaster, Advanced Chem Tech, and Cal Biochem and used without further purification. ^{1}H NMR were obtained on a Brucker 300 MHz spectrometer, and mass spectral data were obtained on a VG70SE instrument. Silica gel chromatography refers to flash chromatography using silica gel 60 (230−400 mesh). Analytical reverse phase HPLC was performed on a 5 μm Vydac C18 25 × 0.3 cm column using a Hewlitt Packard Series 1100 instrument equipped with a diode array detector and column heater. The following two solvent systems were used to validate the purity of each of the final peptide products: (1) linear gradient of 100% H_2O (0.1% TFA) to 30% H_2O (0.1% TFA)/70% CH_3CN (0.06% TFA) in 20 min, (2) linear gradient of 95% H_2O (25 mM KH_2PO_4)/5% CH_3CN to 28% H_2O (25 mM buffer)/72% CH_3CN in 22 min. The UV spectrum of each product peak was monitored across the entire peak from 200 to 500 nm using the diode array detector. The spectra from the slopes and apex of the peak were overlayed for confirmation of purity. In some cases, as for compound **4**, the column was heated to 50 °C to sharpen the peaks. Preparative reverse phase chromatography was performed on a Vydac C18 (15−20 μm particle size) column (2.2 cm i.d. × 29.0 cm length) using a linear gradient system.

General Procedure for Solid Phase Couplings. *p*-Methylbenzhydrylamine resin was used as solid support, and standard solid phase techniques were used for Boc-amino acid couplings.[38] The peptides were removed from the resin by treatment with HF/5% anisole at 0 °C for 1 h and purified by reverse phase chromatography followed by lyophilization from H_2O.

Boc-Pro-Ala-NH_2. Boc-Pro and Ala-NH_2·HCl were coupled using diethyl cyanophosphonate (DEPC) as the activating agent in the presence of diisopropylethylamine (DIPEA) to yield the known dipeptide. The Boc group was removed immediately prior to use by treatment with 4 N HCl in dioxane.

(2*S*,3*R*)-(3-Amino-2-hydroxy-4-phenyl-butanoyl)-L-prolyl-L-prolyl-L-alaninamide (1). Compound **1**, apstatin, was synthesized by the general solid phase coupling procedure: purity >99% by analytical HPLC, Rt = 8.42 min; ^{1}H NMR (D_2O, 300 MHz) δ 1.26−1.31 (d, 3 H), 1.84−1.97 (m, 6 H), 2.14−2.31 (m, 2 H), 2.92−3.0 (m, 1 H), 3.02−3.07 (m, 1 H), 3.34−3.55 (3, 3 H), 3.71−3.73 (m, 2 H), 4.12−4.19 (q, 1 H), 4.30−4.34 (m, 1 H), 4.42−4.43 (d, 1 H), 4.63−4.68 (m, 1 H), 7.25−7.35 (m, 5 H); FAB-MS *m*/*z* (M + H$^+$) 460; M_r = 459.2 calcd for $C_{23}H_{33}N_5O_5$; HRMS (FAB) calcd for $C_{23}H_{33}N_5O_5 + H_1$ 460.2560, found 460.2581.

(2*S*,3*R*)-(3-Amino-2-hydroxy-4-phenyl-butanoyl)-L-thioprolyl-L-prolyl-L-alaninamide (2). Compound **2** was synthesized by the general solid phase coupling procedure: purity 95% by analytical HPLC, Rt = 9.43 min; NMR ^{1}H NMR (D_2O, 300 MHz) δ 1.3−1.4 (d, 3 H), 1.8−2.08 (m, 3 H), 2.13−2.37 (m, 1 H), 2.89−3.13 (m, 2 H), 3.17−3.38 (m, 1 H), 3.43−3.58 (m, 1 H), 3.61−3.73 (m, 1 H), 3.78−3.93 (m, 2 H), 4.20−4.27 (m, 1 H), 4.37−4.42 (m, 1 H), 4.42−4.46 (d, 1 H) 7.30−7.48 (m, 5H), 3H under HOD peak; FAB-MS for *m*/*z* (M + H)$^+$ 478; M_r = 477.02 calcd for $C_{22}H_{31}N_5O_5S$; HRMS (FAB) calcd for $C_{22}H_{31}N_5O_5S + H_1$ 478.2124, found 478.2143.

(2*S*,3*R*)-(3-Amino-2-hydroxy-4-phenyl-butanoyl)-L-4-hydroxyprolyl-L-prolyl-L-alaninamide (3). Compound **3** was synthesized by the general solid phase coupling procedure using Boc-*O*-benzyl-L-4-hydroxyproline: purity 95% by analytical HPLC, Rt = 7.86; ^{1}H NMR (D_2O, 300 MHz) δ 1.25−1.32 (m, 3 H), 1.85−1.97 (m, 4 H), 2.22−2.32 (m, 2 H), 2.90−3.06 (m, 2 H), 3.47−3.62 (m, 3 H), 3.68−3.75 (m, 2 H), 4.11−4.17 (m, 1 H), 4.31−4.46 (m, 3 H), 4.76−4.79 (m, 1 H), 7.24−7.36 (m, 5 H); FAB-MS for *m*/*z* (M + H$^+$) 476; M_r = 475.24 calcd for $C_{23}H_{33}N_5O_6$; HRMS (FAB) calcd for $C_{23}H_{33}N_5O_6 + H_1$ 476.205, found 476.2491.

(2*S*,3*R*)-(3-Amino-2-hydroxy-4-phenyl-butanoyl)-L-prolyl-L-prolinamide (4). Compound **4** was synthesized by the general solid phase coupling procedure: purity 90% by analytical HPLC, Rt = 8.53 min; ^{1}H NMR (D_2O, 300 MHz) δ 1.84−1.98 (m, 6 H), 2.13−2.46 (m, 2 H), 2.87−3.10 (m, 2 H), 3.28−3.53 (m, 4 H), 3.69−3.81 (m, 1 H), 4.25−4.37 (m, 1 H), 4.42−4.44 (d, 1 H), 4.61−4.68 (m, 1 H), 7.28−7.35 (m, 5 H); FAB-MS for *m*/*z* (M + H$^+$) 389; M_r = 388.21 calcd for $C_{20}H_{28}N_4O_4$; HRMS (FAB) calcd for $C_{20}H_{28}N_4O_4 + H_1$ 389.2188, found 389.2203.

(2*S*,3*R*)-(3-Amino-2-hydroxy-4-phenyl-butanoyl)-L-prolyl-L-prolyl-L-alanyl-β-alanyl-L-cysteinamide-NH_2 (5). Compound **5** was synthesized by the general solid phase coupling procedure: purity 95% by analytical HPLC, Rt = 8.75 min; ^{1}H NMR (D_2O, 300 MHz) δ 1.24−1.27 (d, 3 H), 1.86−2.01 (m, 6 H), 2.19−2.32 (m, 2 H), 2.42−2.53 (bt, 2 H), 2.81−2.84 (m, 2 H), 2.88−3.13 (m, 2 H), 3.38−3.62 (m, 5 H), 3.69−3.82 (m, 2 H), 4.10−4.18 (q, 1 H), 4.30−4.42 (m, 2 H), 4.43−4.50 (d, 1 H), 7.28−7.37 (m, 5 H), 1H under HOD peak; FAB-MS for *m*/*z* (M + H$^+$) 634, and (2M + H$^+$) for dimer; M_r = 633.294 calcd for $C_{29}H_{43}N_7O_7S_1$; HRMS (FAB) calcd for $C_{29}H_{43}N_7O_7S + H_1$ 634.3022, found 634.3032.

(2*S*,3*R*)-(3-Amino-2-hydroxy-5-methyl-hexanoyl)-L-prolyl-L-prolyl-alaninamide (6). Compound **6** was synthesized

by the general solid phase coupling procedure: purity 100% by analytical HPLC, Rt = 7.54 min; ^{1}H NMR (D_2O, 300 MHz) δ 0.82−0.86 (dd, 6 H), 1.24−1.32 (d, 3 H), 1.44−1.49 (t, 2 H), 1.58−1.65 (m, 1 H), 1.84−1.97 (m, 6 H), 2.15−2.33 (m, 2 H), 3.48−3.77 (m, 5 H), 4.12−4.21 (q, 1 H), 4.27−4.33 (t, 1 H), 4.47−4.52 (m, 1 H), 1H under HOD peak; FAB-MS for *m/z* (M + H$^+$) 426; M_r = 425.26 calcd for $C_{20}H_{35}N_5O_5$; HRMS (FAB) calcd for $C_{20}H_{35}N_5O_5$ + H_1 426.2716, found 426.2702.

(2*R*,3*S*)-(3-Amino-2-hydroxy-5-methyl-hexanoyl)-L-prolyl-L-prolyl-alaninamide (7). The mixture of Boc-(2*RS*,3*S*)-3-amino-2-hydroxy-5-methylhexanoic acids, synthesized essentially by the procedure in ref 25 was coupled to the Pro-Pro-Ala-BHA resin and treated with HF by the standard procedure. The two isomeric product peptides were separated and purified by preparative reverse phase chromatography: purity 95% by analytical HPLC, Rt = 7.74 min, the 5% impurity detected by HPLC is compound **9**; ^{1}H NMR (D_2O, 300 MHz) δ 0.8−0.88 (dd, 6 H), 1.28−1.30 (d, 3 H), 1.40−1.48 (t, 2 H), 1.52−1.68 (m, 1 H), 1.78−2.0 (m, 6 H), 2.13−2.38 (m, 2 H), 3.39−3.78 (m, 5 H), 4.10−4.23 (q, 1 H), 4.30−4.38 (t, 1 H), 4.42−4.5 (d, 1 H), 1H under HOD peak; FAB-MS for *m/z* (M + H$^+$) 426; M_r = 425.26 calcd for $C_{20}H_{35}N_5O_5$; HRMS (FAB) calcd for $C_{20}H_{35}N_5O_5$ + H_1 426.2716, found 426.2729.

(2*R*,3*R*)-(3-Amino-2-hydroxy-5-methyl-hexanoyl)-L-prolyl-L-prolyl-alaninamide (8). Compound **8** was synthesized by the general solid-phase coupling procedure: purity 100% by analytical HPLC, Rt = 8.20 min; ^{1}H NMR (D_2O, 300 MHz) δ 0.79−0.85 (dd, 6 H), 1.25−1.60 (m, 6 H), 1.78−2.02 (m, 6 H), 2.14−2.30 (m, 2 H), 3.48−3.78 (m, 5 H), 4.15−4.37 (m, 3 H), 1H under HOD peak; FAB-MS for *m/z* (M + H$^+$) 426; M_r = 425.26 calcd for $C_{20}H_{35}N_5O_5$; HRMS (FAB) calcd for $C_{20}H_{35}N_5O_5$ + H_1 426.2716, found 426.2737.

(2*S*,3*S*)-(3-Amino-2-hydroxy-5-methyl-hexanoyl)-L-prolyl-L-prolyl-alaninamide (9). Compound **9** was obtained from the preparative chromatography performed for compound **7**: purity 98% by analytical HPLC, Rt = 8.16 min; ^{1}H NMR (D_2O, 300 MHz) δ 0.78−0.86 (dd, 6 H), 1.25−1.48 (m, 5 H), 1.55−1.68 (m, 1 H), 1.78−2.02 (m, 6 H), 2.15−2.34 (m, 2 H), 3.3−3.41 (m, 1 H), 3.48−3.78 (m, 4 H), 4.12−4.22 (q, 1 H), 4.30−4.42 (m, 2 H), 1 H under HOD peak; FAB-MS for *m/z* (M + H$^+$) 426; M_r = 425.26 calcd for $C_{20}H_{35}N_5O_5$; HRMS (FAB) calcd for $C_{20}H_{35}N_5O_5$ + H_1 426.2716, found 426.2702.

(3*R*,4*S*)-(4-Amino-3-hydroxy-6-methyl-heptanoyl)-L-prolyl-L-prolyl-alaninamide (10). Commercially available Boc-statine was coupled to the tripeptide by the solid phase method and purified by reverse phase chromatography to yield compound **10**: purity 95% by analytical HPLC, Rt = 8.52 min; ^{1}H NMR (D_2O, 300 MHz) δ 7.8−0.91 (dd, 6 H), 1.28−1.31 (d, 3 H), 1.32−1.62 (m, 3 H), 1.80−1.97 (m, 6 H), 2.15−2.29 (m, 2 H), 2.48−2.65 (m, 2 H), 3.32−3.43 (m, 1 H), 3.54−3.58 (m, 3 H), 3.69−3.74 (m, 1 H), 4.12−4.19 (q, 1 H), 4.23−4.35 (m, 2 H), 4.58−4.69 (m, 1 H); FAB-MS for *m/z* (M + H$^+$) 440; M_r = 439.28 calcd for $C_{21}H_{37}N_5O_5$; HRMS (FAB) calcd for $C_{21}H_{37}N_5O_5$ + H_1 440.2873, found 440.2861.

***N*-(1-(*R*,*S*)-carboxy-3-phenylpropyl)-L-prolyl-L-prolyl-alaninamide (11).** The TFA salt of Pro-Pro-Ala-NH_2 was synthesized by the solid phase method. The tripeptide (100 mg, 0.252 mmol) and Et-2-oxo-4-phenyl butyrate (72 mg, 0.376 mmol) were dissolved in 10 mL of absolute EtOH and stirred for 20 min at room temperature under a nitrogen atmosphere. $NaCNBH_4$ (5 mg, 0.08 mmol) was added. After 4 h, 20 mg (0.104 mmol) more of keto ester and 5 mg more of $NaCNBH_4$ were added, and the mixture was stirred overnight. The crude reaction mixture was placed on the preparative reverse phase column and eluted with a gradient from 100% H_2O to 60% H_2O/40% CH_3CN (1% TFA) over 180 min to yield 54 mg of the ethyl ester of compound **11** that was 80% pure according to analytical HPLC: FAB-MS for *m/z* (M + H$^+$) 473; M_r = 472.27 calcd for $C_{25}H_{36}N_4O_5$.

Without further purification, the ethyl ester was saponified by treatment with a mixture of 5 mL of MeOH and 10 mL of 1 N NaOH for 2 h. The final product was purified by reverse phase chromatography to yield 16 mg of the (*R*,*S*)-carboxyalkyl peptide **11**: purity 100% by analytical HPLC, Rt = 9.51 min; ^{1}H NMR (D_2O, 300 MHz) δ 1.30−1.35 (dd, 3 H), 1.78−2.18 (m, 8 H), 2.19−2.32 (m, 1 H), 2.36−2.52 (m, 1 H), 2.56−2.82 (m, 2 H), 3.12−2.23 (m, 1 H), 3.38−3.48 (m, 2 H), 3.58−3.69 (m, 1 H), 3.71−3.79 (m, 1 H), 4.1−4.28 (m, 1 H), 4.31−4.40 (m, 1 H), 4.43−4.51 (m, 1 H), 7.19−7.36 (m, 5 H); FAB-MS for *m/z* (M + H$^+$) 445; M_r = 444.24 calcd for $C_{23}H_{32}N_4O_5$; HRMS (FAB) calcd for $C_{23}H_{32}N_4O_5$ + H_1 445.2451, found 445.2454.

***N*-[(2-Thiocyclopentyl)carbonyl]-L-prolyl-alaninamide (12). Faster Moving HPLC Isomer.** *cis/trans*-2-[[(4-Methoxyphenyl)methyl]thio]-cyclopentanecarboxylic acid was synthesized by the method described in ref 26. As described in the reference, the synthesis resulted in an approximate 2:1 mixture of isomers as determined by ^{1}H NMR.

The mixture of isomers was resolved by preparative reverse phase HPLC with a gradient from 80/20 to 50/50 H_2O/CH_3CN. The slower moving isomer, recrystallized from hexanes, had a melting point of 67−69 °C: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.55−1.98 (m, 4 H), 2.02−2.14 (m, 2 H), 2.74−2.82 (m, 1 H), 3.30−3.37 (q, 1 H), 3.75 (s, 2 H), 3.79 (s, 3 H), 6.82−6.86 (m, 2 H), 7.23−7.26 (m, 2 H); MS (EI) for *m/z* 266; M_r = 266.10 calcd for $C_{14}H_{18}O_3S_1$. This is presumed to be the trans isomer.

The faster moving isomer had a melting point of 97−98 °C: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.48−1.59 (m, 1 H), 1.69−1.98 (m, 4 H), 1.99−2.09 (m, 1 H), 2.98−3.08 (m, 2 H), 3.64−3.70 (d, 2 H), 3.72 (s, 3 H), 6.74−5.78 (d, 2 H), 7.17−7.19 (d, 2 H); MS same as for slower isomer. This is presumed to be the cis isomer.

The mixture of isomers was coupled to the dipeptide by the following procedure. The cis/trans mixture of the cyclopentanecarboxylic acid derivative (105 mg, 0.395 mmol), the HCl salt of Pro-Ala-NH_2 dipeptide (88 mg, 0.395 mmol), and DEPC (71 μL, 0.474 mmol) were mixed with 30 mL of CH_2Cl_2. Upon the addition of 202 μL (1.16 mmol) of DIPEA everything dissolved. The reaction was stirred under argon at room temperature for 2 h. The solvent was removed, and the residual oil was purified by silica gel chromatography using 8% MeOH/2% HOAc, 90% EtOAc as eluant to give 104 mg (61% yield) of cis/trans product. ^{1}H NMR was taken of the fractions enriched in one of the isomers: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.26−1.36 (m, 3 H), 1.53−2.30 (m, 10 H), 2.72−2.78 (m, 1 H), 2.23−3.29 (m, 1 H), 3.42−3.55 (m, 2 H), 3.70−3.78 (m, 2 H), 3.80 (s, 3 H), 4.38−4.55 (m, 2 H), 5.84 (bs, 2 H), 6.55 (bs, 1 H), 6.81−6.84 (m, 2 H), 6.97 and 7.05 (bs, 1 H), 7.17−7.27 (m, 2 H).

The 4-methoxybenzyl protecting group was removed by treating the 104 mg from above with 5 mL of HF plus 300 μL of *p*-cresol at 0 °C for 1 h. The solvents were removed, and the residue was extensively washed with hexanes and then applied to a reverse phase column with a linear gradient from 100% H_2O to 80% H_2O/20% CH_3CN. Two products were collected. The faster moving isomer was 90% pure by analytical HPLC with 10% impurity of the slower moving isomer: Rt = 9.56 min; ^{1}H NMR (CD_3OD, 300 MHz) δ 1.36−1.41 (m, 3 H), 1.52−1.67 (m, 1 H), 1.7−2.28 (m, 9 H), 2.88−3.0 (q, 1 H), 3.62−3.9 (m, 2 H), 4.28−4.49 (m, 2 H); MS (ESI+) *m/z* (M + H$^+$) 314; M_r = 313.15 calcd for $C_{14}H_{23}O_3N_3S_1$; HRMS (FAB) calcd for $C_{14}H_{23}O_3N_3S_1$ + H_1 314.1538, found 314.1536.

***N*-[(2-Thiocyclopentyl)carbonyl]-L-prolyl-L-alaninamide (13). Slower Moving HPLC Isomer.** Compound **13** was obtained from the procedure reported above for compound **12**. The slower moving isomer was 100% pure by analytical HPLC: Rt = 9.93; ^{1}H NMR (CD_3OD, 300 MHz) δ 1.35−1.39 (m, 3 H), 1,50−2.38 (m, 10 H), 2.93−2.96 (q, 1 H), 3.53−3.8 (m, 2 H), 3.85−3.97 (m, 1 H), 4.24−4.45 (m, 2 H); MS (ESI+) *m/z* (M + H$^+$) 314; M_r = 313.15 calcd for $C_{14}H_{23}O_3N_3S_1$; HRMS (FAB) calcd for $C_{14}H_{23}O_3N_3S_1$−H_1 312.1382, found 312.1387.

***N*-[[((±)-*trans*-2-Hydroxyaminocarbonyl)cyclohexyl]-carbonyl]-L-prolyl-L-alaninamide. Faster Moving HPLC Isomer (14) and Slower Moving Isomer (15).** Coupling of the anhydride to the dipeptide was performed with the following procedure: 162 mg (1.0 mM) of (±)-*trans*-1,2-cyclohexanedicarboxylic acid anhydride and 221 mg (1.0 mmol) of the HCl salt of Pro-Ala-NH_2 were mixed with 20 mL of 4 Å sieve-dried DMF. DIPEA (348 μL, 2.0 mmol) was added, and the mixture was stirred overnight under argon. The solvent

was removed under reduced pressure. The sample was purified by preparative reverse phase chromatography using a gradient from 100% H_2O to 70% H_2O/30% CH_3CN with 1% TFA as counterion. Both 177 mg of the faster moving isomer and 151 mg of the slower moving isomer were recovered: yield = 96%.

Faster isomer: analytical HPLC, Rt = 8.91 min; ^{1}H NMR (CD_3OD, 300 MHz) δ 1.28−1.41 (m, 7 H), 1.73−2.25 (m, 8 H), 2.59−2.80 (m, 2H), 3.66−3.74 (m, 1 H), 3.79−3.89 (m, 1 H), 4.27−4.42 (m, 2 H); FAB-MS for *m*/*z* (M + H^+) 340; M_r = 339.18 calcd for $C_{16}H_{25}N_3O_5$.

Slower isomer: analytical HPLC, Rt = 10.23 min; ^{1}H NMR (CD_3OD, 300 MHz) δ 1.3−1.48 (m, 7 H), 1.75−1.98 (m, 3 H), 1.99−2.1 (m, 3 H), 2.2−2.34 (m, 2 H), 2.67−2.78 (m, 2 H), 3.65−3.78 (m, 1 H), 3.90−4.0 (m, 1 H), 4.21−4.39 (m, 2 H); FAB-MS same as for faster isomer.

Formation of the benzyl protected hydroxamate derivative was performed with the following procedure: 32 mg (0.094 mmol) of the slow moving isomer from above, 15 mg (0.094 mmol) of *O*-benzyl hydroxylamine HCl, 42 mg (0.094 mmol) of BOP, and 82 μL (0.188 mmol) of DIPEA were dissolved in 20 mL of CH_2Cl_2. The reaction was stirred overnight at room temperature under argon. The solvent was removed and the residue was dissolved in EtOAc. The organic layer was washed with 0.1 N HCl and dried over $MgSO_4$. The solvent was removed, and the product was purified by preparative HPLC using a gradient from 100% H_2O to 60% H_2O/40% CH_3CN to yield 24 mg of *O*-benzylhydroxamate product: yield = 58%; analytical HPLC 13.85 min; ^{1}H NMR (CD_3OD, 300 MHz) δ 1.3−1.49 (m, 7 H), 1.72−2.1 (m, 7 H), 2.19−2.35 (m, 1 H), 2.48−2.61 (m, 1 H), 2.74−2.89 (m, 1 H), 3.63−3.77 (m, 1 H), 3.94−4.08 (m, 1 H), 4.19−4.42 (m, 2 H), 4.78−4.80 (s, 2 H), 7.29−7.41 (m, 5 H); FAB-MS for *m*/*z* (M + H^+) 445; M_r = 444.24 calcd for $C_{23}H_{32}N_4O_5$.

The *O*-benzylhydroxamate derivative of the faster isomer was formed by the same procedure as above: analytical HPLC, Rt = 11.5 min; ^{1}H NMR (CD_3OD, 300 MHz) δ 1.28−1.55 (m, 7 H), 1.72−1.87 (m, 3 H), 1.9−2.09 (m, 4 H), 2.1−2.22 (m, 1 H), 2.3−2.43 (m, 1 H), 2.78−2.88 (m, 1 H), 3.62−3.72 (m, 1 H), 3.78−3.89 (m, 1 H), 4.22−4.39 (m, 2 H), 4.72−4.78 (s, 2 H), 7.3−7.45 (m, 5 H); FAB-MS same as for slower isomer.

Removal of the *O*-Benzyl Protecting Group. The slower moving isomer (52 mg) of the *O*-benzylhydroxamate derivative was hydrogenated with 20 mg of 10% Pd/C in EtOH in a Parr bottle for 4 h at 40 psi H_2. The catalyst and solvent were removed, and the sample was purified by reverse phase HPLC with a gradient from 100% H_2O to 90% H_2O/10% CH_3CN to yield 12 mg of pure product (**14**): purity > 99% by analytical HPLC, Rt = 8.64 min; ^{1}H NMR (CD_3OD, 300 MHz) δ 1.30−1.51 (m, 7 H), 1.72−2.09 (m, 7 H), 2.18−2.31 (m, 1 H), 2.45−2.58 (m, 1 H), 2.78−2.9 (m, 1 H), 3.6−3.7 (m, 1 H), 3.94−4.03 (m, 1 H), 4.24−4.40 (m, 2 H); FAB-MS for *m*/*z* (M + H^+) 355; M_r = 354.19 calcd for $C_{16}H_{26}N_4O_5$; HRMS (FAB) calcd for $C_{16}H_{26}N_4O_5 + H_1$ 355.1981, found 355.1993.

The same conditions were used for the removal of the *O*-benzyl protecting group for the slower moving HPLC isomer. A major side product identified by FAB-MS from the reaction was over-reduction of the hydroxamic moiety to the primary amide. The desired product (**15**) had a purity of 95% by analytical HPLC, Rt = 7.59 min: ^{1}H NMR (CD_3OD, 300 MHz) δ 1.29−1.47 (m, 7 H), 1.79−1.92 (m, 3 H), 1.95−2.1 (m, 4 H), 2.12−2.25 (m, 1 H), 2.33−2.48 (m, 1 H), 2.78−2.92 (m, 1 H), 3.61−3.72 (m, 1 H), 3.78−3.89 (m, 1 H), 4.23−4.39 (m, 2 H); FAB-MS same as for compound **14**; HRMS (FAB) found 355.1993.

***N*-[[((*cis*)-2-Hydroxyaminocarbonyl)cyclohexyl]carbonyl]-L-prolyl-L-alaninamide (16).** Compound **16** was synthesized with the same procedures as for the trans isomers **14** and **15**: 90% purity by analytical HPLC, Rt = 8.4 min; ^{1}H NMR (CD_3OD, 300 MHz) δ 1.38−1.56 (m, 5 H), 1.58−2.31 (m, 10 H), 2.38−2.58 (m, 1 H), 2.91−3.0 (m, 1 H), 3.30−3.45 (m, 1 H), 3.59−3.78 (m, 1 H), 4.2−4.48 (m, 2 H); FAB-MS for *m*/*z* (M + H^+) 355; M_r = 354.19 calcd for $C_{16}H_{26}N_4O_5$; HRMS (FAB) calcd for $C_{16}H_{26}N_4O_5 + Na_1$ 377.1801, found 377.1795.

Membrane-Bound Aminopeptidase P. Membrane-bound aminopeptidase P was purified to homogeneity from bovine lung[1] and rat lung.[17] Partially purified membrane-bound aminopeptidase P was obtained from both human lung and monkey (cynomolgus) lung by treatment of lung microsomes with phosphatidylinositol-specific phospholipase C (*Bacillus thurnigiensis*) (ICN, Costa Mesa, CA) followed by centrifugation as described previously.[1]

Human Heart Cytosolic Aminopeptidase P. Human cytosolic aminopeptidase P was assayed using the 40000*g* supernatant of a human heart homogenate prepared in 0.1 M potassium phosphate, pH 6.8 (1:3 w/v). In some cases, a partially purified form of the enzyme was used: The 40000*g* supernatant was brought to 1 M NaCl, centrifuged to remove precipitate, and applied (9 mL) to a TNB-thiol agarose column (1.5 mL) (Pierce, Rockford, IL). Following washing of the column, the enzyme was eluted with 10 mM dithiothreitol in 0.1 M potassium phosphate, 1 M NaCl, pH 6.8. Active fractions were pooled, and the dithiothreitol was removed by repeated concentration and dilution with 0.1 M Hepes, pH 8.0, using Centricon-30 ultrafiltration units.

Human Platelet Aminopeptidase P. Aminopeptidase P was extracted from 1 day expired human platelets (Rh positive, O, 422 mL containing 78 mL of ACD-A anticoagulant) prepared by the plateletpheresis method (Central Blood Bank, Pittsburgh, PA, and supplied courtesy of Alan Hoffstadter, Loyola Blood Bank). The platelet suspension was stored for several days at 25 °C with agitation on a Nutrator rotator mixer. A portion (40 mL) of the suspension was centrifuged at 250*g* for 7 min at 25 °C. The supernatant was decanted and recentrifuged at the same speed for 5 min. The resulting supernatant was centrifuged at 2790*g* for 15 min at 25 °C. The 2790*g* pellet was carefully resuspended in 40 mL of phosphate-buffered saline (PBS) containing 1 μg/mL prostacyclin (Schering AG, Berlin/Berkamen) at 25 °C. After centrifugation at 2790*g* for 15 min, the pellet was washed again with 40 mL of PBS−prostacyclin and recentrifuged under the same conditions. The final pellet was resuspended in 4 mL of PBS−prostacyclin. The suspension was placed in a Corex tube, rapidly frozen in a dry ice/acetone bath, thawed at 37 °C, sonicated for 60 s in a Branson B-12 ultrasonic cleaner containing ice water, and then centrifuged at 43000*g* for 2 h in a Sorvall RC-2B refrigerated superspeed centrifuge. The resulting supernatant, containing over 90% of the aminopeptidase P activity, was used for enzyme assays.

Other Enzymes. Recombinant *E. coli* aminopeptidase P[39] was generously supplied by Dr. Tadashi Yoshimoto (Nagasaki University). Purified prolidase (X-Pro dipeptidase) (EC 3.4.13.9) and purified cytosolic leucyl aminopeptidase (EC 3.4.11.1), both from porcine kidney, were obtained from Sigma Chemical Co. (St. Louis, MO).

Enzyme Assays. All forms of aminopeptidase P were assayed using 0.5 mM Arg-Pro-Pro (Bachem Biosciences, Philadelphia, PA) in 0.1 M Hepes, pH 8.0. The enzyme reaction was followed by measuring the increase in production of free arginine by a fluorescence assay described previously.[1] *E. coli* aminopeptidase P was incubated with 0.73 mM $MnCl_2$ prior to assay (residual $MnCl_2$ in the assay was 0.02 mM). Prolidase activity was determined with the same fluorescence assay[1] using 0.5 mM Arg-Pro (Sigma Chemical Co.) as substrate in 0.1 M Hepes, pH 8.0. Leucyl aminopeptidase activity was determined using 0.5 mM leucyl-*p*-nitroanilide (Sigma Chemical Co.) in 0.1 M Hepes, pH 8.0, containing 0.2 mM $MnCl_2$ and 3.5% DMSO and measuring the rate of leucine formation by the fluorescence assay described previously.[1] Each IC_{50} value, the inhibitor concentration required to inhibit 50% of the enzyme activity under the conditions of the assay, was obtained in the following way: (1) enzyme rates were measured in the presence of a wide range of inhibitor concentrations to yield an approximate IC_{50} value; (2) the experiment was then repeated using 10 different concentrations of inhibitor in the vicinity of this value, each in triplicate, followed by linear regression analysis of the linear portion of the rate vs log inhibitor concentration plot to determine the IC_{50} value.

The average correlation coefficient for all regression analyses in this study was 0.96. K_i values were determined as described previously.[3]

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