J. Med. Chem. 1995, 38, 2119–2129

Hydroxamic Acids as Potent Inhibitors of Endothelin-Converting Enzyme from Human Bronchiolar Smooth Muscle

Ron Bihovsky,*,[†] Barry L. Levinson, Rivka C. Loewi, Paul W. Erhardt,[‡] and Mark A. Polokoff[§]

Berlex Laboratories, Inc., 110 East Hanover Avenue, Cedar Knolls, New Jersey 07927

Received August 29, 1994[⊗]

Hydroxamic acids **6a–h**, derived from malonyl amino acids, and **25a–d**, derived from succinyl amino acids, were synthesized as inhibitors of human bronchiolar smooth muscle endothelin-converting enzyme (HBSM ECE). Several unexpected side reactions were discovered, particularly in the synthesis of hydroxamates derived from succinates. *In vitro* evaluation against human bronchiolar ECE revealed that in all cases hydroxamates derived from malonate were more potent than hydroxamates derived from succinate. Isopropyl and isobutyl P_1' side chains were suitable; omission of the P_1' side chain seriously diminished potency. In the P_2' position, several amino acids gave potent malonate-derived hydroxamate inhibitors (**6b,d–h**, IC_{50} = 0.2–6.8 nM), and β-Ala provided an extremely potent inhibitor (**6c**, IC_{50} = 0.01 nM). C-terminus carboxylates are much more potent ECE inhibitors than the corresponding amides. Most of the hydroxamates were also potent inhibitors of thermolysin and neutral endopeptidase (NEP); however, the P_2' β-Ala derivative **6c** uniquely inhibited HBSM ECE much more potently than NEP.

Introduction

Human endothelin-converting enzyme (ECE) is a novel enzyme which cleaves the $Trp^{21}-Val^{22}$ bond of big endothelin-1 (big ET-1), an essentially inactive 38-amino acid precursor, to produce the 21-amino acid peptide endothelin-1 (ET-1), a potent vasoconstrictive hormone thought to play a pathophysiological role in congestive heart failure, renal failure, hypertension, vasospasm, atherosclerosis, subarachnoid hemorrhage, and other diseases.[1,2] Big ET-1 appears to be enzymatically processed to ET-1 within endothelial cells and also released intact extracellularly.[3] ET-1 produced by endothelial cells may feed back to these cells and diffuse to adjacent smooth muscle, initiating vasoconstriction. Alternatively, big ET-1 itself may diffuse to the smooth muscle cells, where it could be converted to ET-1 by ECE to produce local vasoconstriction. Thus endothelial cells or smooth muscle cells are probable sources of ECE.

A single authentic physiologically relevant human ECE, the enzyme which converts big ET-1 to ET-1 under physiological conditions, has not yet been positively identified. Various enzymes convert big ET-1 to ET-1 but are of doubtful physiological relevance because they would not normally be exposed to big ET-1 or because they also cleave ET-1, precluding efficient conversion. Initially, various cysteine, aspartyl, and serine proteases which convert big ET-1 into ET-1 were proposed to be ECE.[4] Currently, however, the weight of evidence suggests that the physiologically relevant ECE is a metalloprotease.[4] Metalloprotease ECE preparations have been obtained from various porcine, bovine, rat, rabbit, guinea pig, and human cells and tissues,[4–6] and several related but distinct ECE sequences from rat, bovine, and human lung and aortic endothelial cells were recently cloned and expressed.[7] These preparations clearly differ from each other. For example, IC_{50}s reported for the natural product phosphoramidon range 4 orders of magnitude, from 0.3 nM to >10 μM, and IC_{50}s for the neutral endopeptidase (NEP) thiol inhibitor thiorphan range from 30 nM to ≫ 100 μM.[5–7] Selectivity toward substrates big ET-1, -2, and -3 differs drastically, and reported molecular weights range from 120 to 500 kDa.

We have isolated ECE from human bronchiolar smooth muscle (HBSM) cells.[8] This enzyme fulfilled the important criterion of cleaving big ET-1 to ET-1, without further degrading ET-1. HBSM ECE was potently inhibited (see Table 1) by known inhibitors of metalloproteases, including the natural product phosphoramidon (IC_{50} = 0.8 nM), thiorphan (8 nM), and two commercially available hydroxamates, kelatorphan (8 nM) and zincov (11 nM). (See the results section.) Concurrent with our research, several hydroxamates were found to be weak inhibitors of a partially purified ECE from rabbit lung homogenate.[9]

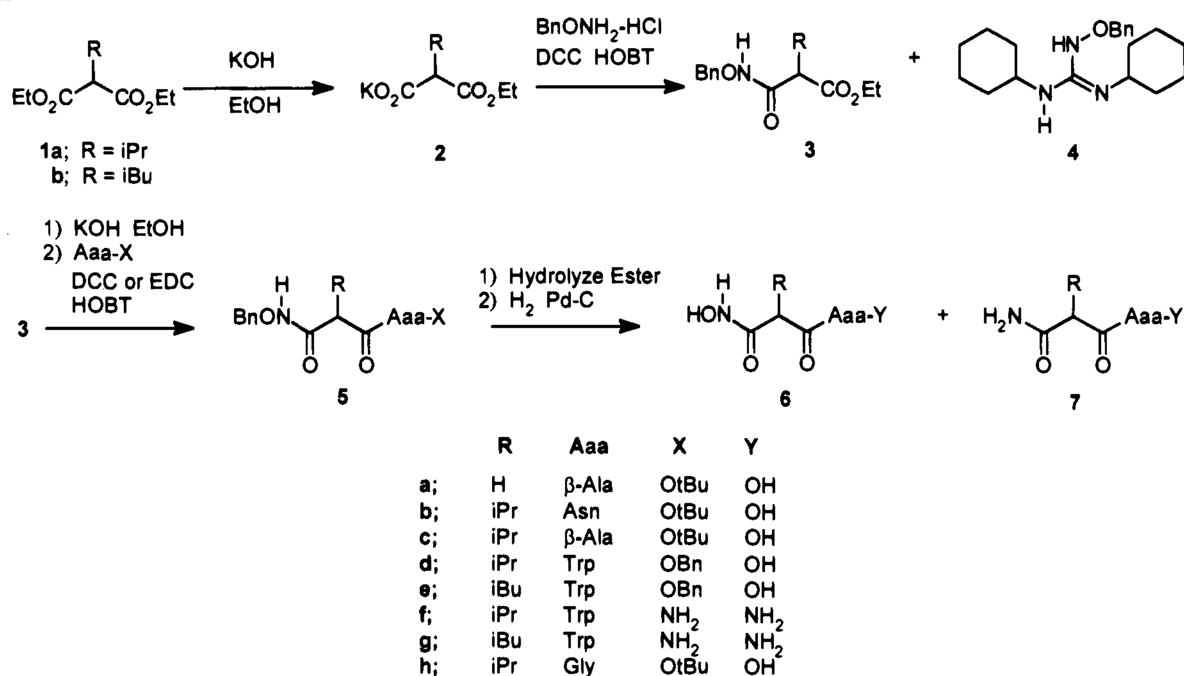
Hydroxamic acids are known to be potent inhibitors[10] of several other metalloproteases, including thermolysin, neutral endopeptidase (NEP, enkephalinase), collagenase, angiotensin converting enzyme, stromelysin, and thermolysin. Historically, the first hydroxamic acid found to be a metalloprotease inhibitor was the naturally occurring antibiotic actinonin.[11] Related natural products were discovered more recently.[12] Potent hydroxamic acid inhibitors of other zinc proteases, with IC_{50}s in the low nanomolar concentration range, were also prepared synthetically.[13] Subsequently, foroxymithine, a naturally occurring hydroxamic acid (of very different structure), was found to be a weak zinc protease inhibitor.[14] The hydroxyl and carbonyl groups of hydroxamic acids inhibit by forming a bidentate chelate with the zinc atom of the enzyme's active site.[10]

Encouraged by the known potency of hydroxamates toward other metalloproteases, we set out to prepare a series of hydroxamic acids as inhibitors of HBSM ECE.[15]

* Author to whom correspondence should be addressed.
[†] Current address: Cephalon, Inc., 145 Brandywine Parkway, West Chester, PA 19380-4245.
[‡] Current address: College of Pharmacy, University of Toledo, Toledo, OH 43606-3390.
[§] Current address: Berlex Biosciences, 15049 San Pablo Ave., Richmond, CA 94804.
[⊗] Abstract published in *Advance ACS Abstracts*, May 15, 1995.

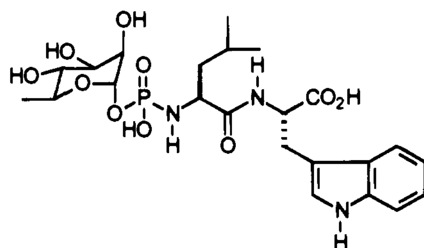
0022-2623/95/1838-2119$09.00/0 © 1995 American Chemical Society

Scheme 1



	R	Aaa	X	Y
a;	H	β-Ala	OtBu	OH
b;	iPr	Asn	OtBu	OH
c;	iPr	β-Ala	OtBu	OH
d;	iPr	Trp	OBn	OH
e;	iBu	Trp	OBn	OH
f;	iPr	Trp	NH_2	NH_2
g;	iBu	Trp	NH_2	NH_2
h;	iPr	Gly	OtBu	OH

Hydroxamic acids derived from malonyl amino acids and succinyl amino acids were of interest since both are known to be potent inhibitors of other metalloproteases. Our initial hydroxamate inhibitor targets contained side chains corresponding to Val-Asn, the P_1' and P_2' amino acids of big ET-1 (the two amino acids which follow the cleavage site). Other inhibitors were based upon the Leu-Trp sequence found in the natural product phosphoramidon (see figure), which inhibits HBSM ECE with $IC_{50} = 0.8$ nM. β-Alanine was incorporated at P_2' because it had afforded potent carboxylate inhibitors of NEP.[16]



Phosphoramidon

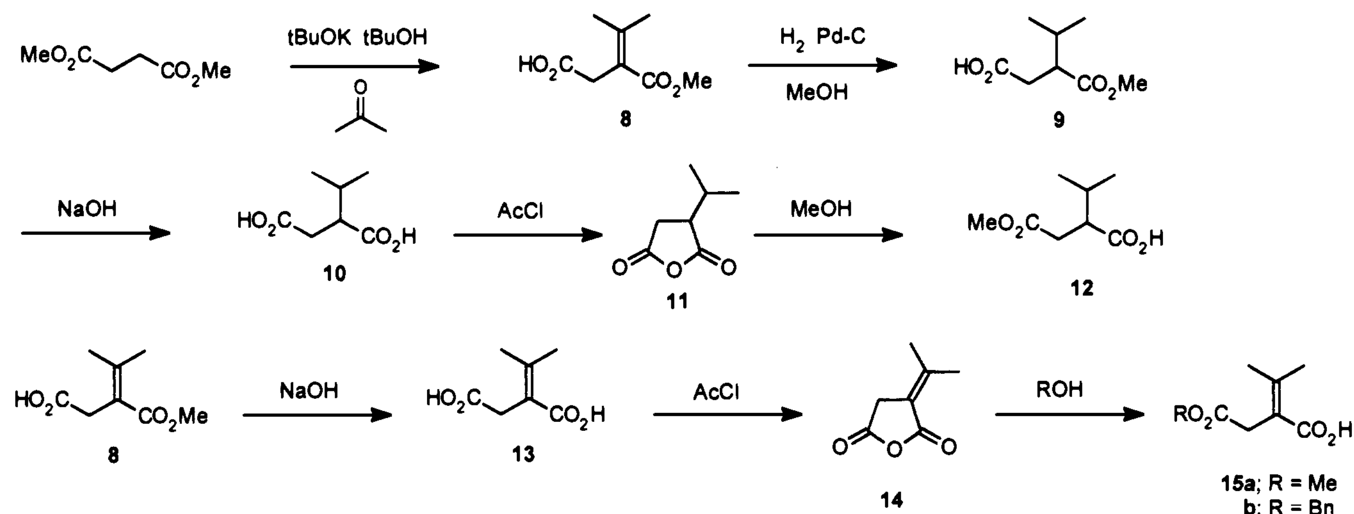
Synthesis

Synthesis of the malonate-derived hydroxamate series (Scheme 1), following literature precedent,[17] was relatively straightforward. Malonate monoethyl esters **2** and (benzyloxy)amine were condensed using DCC and HOBT in dichloromethane to afford benzyl hydroxamates **3**. An unexpected guanidine side product, **4**, was also formed.[18] Interestingly, (benzyloxy)amine does not react with DCC at 20 °C. However, in the presence of HOBT, (benzyloxy)amine does react with DCC to produce **4**. In this respect, (benzyloxy)amine behaves differently from amino acid esters, which do not form guanidine adducts with DCC under these conditions.[18]

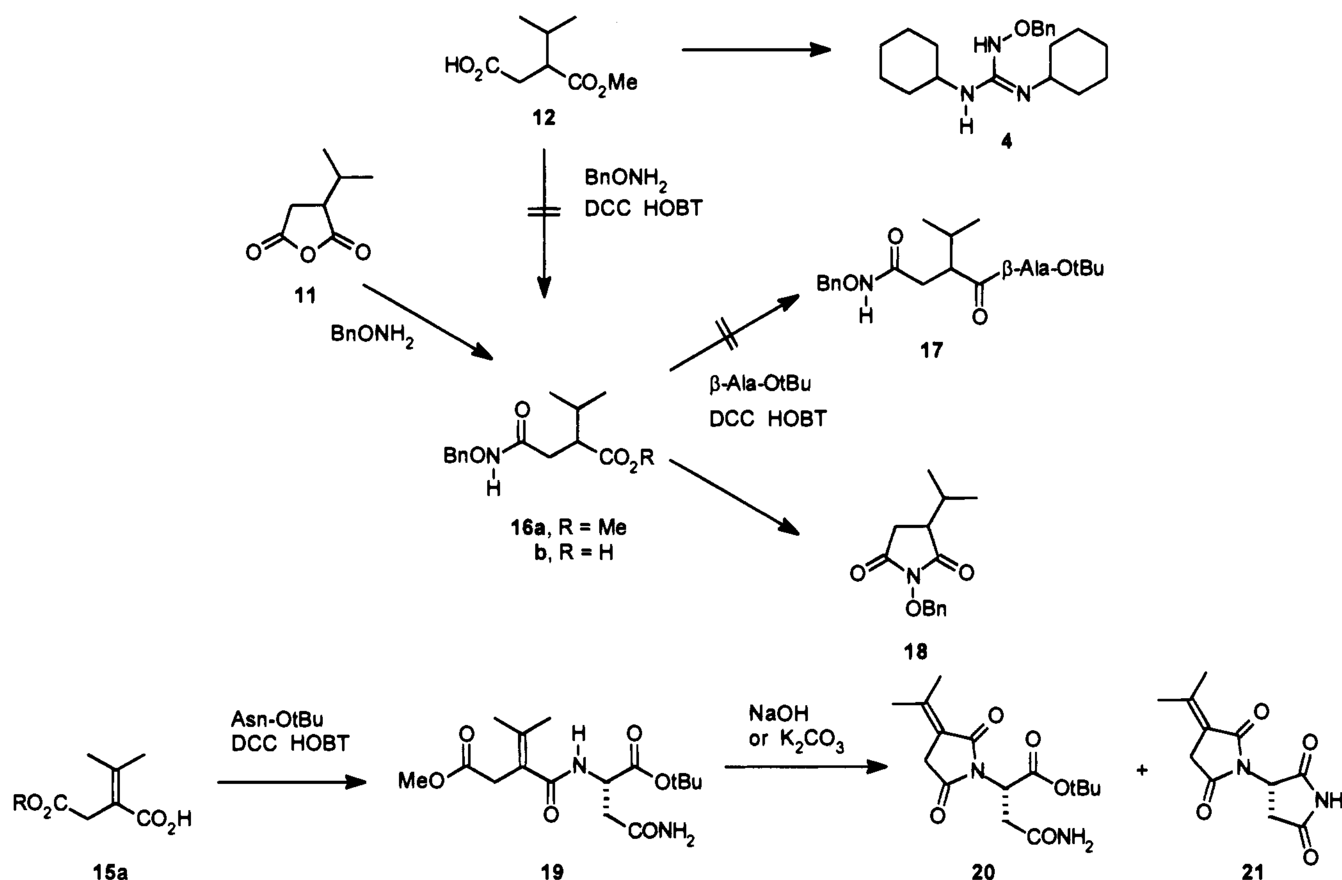
Hydrolysis of **3** required 2 equiv of hydroxide, since 1 equiv was consumed abstracting the hydroxamate N-H proton. Subsequent condensation with Trp-NH_2, Asn-OtBu, Trp-OBn, β-Ala-OtBu, and Gly-OtBu afforded **5**. The esters (where present) were hydrolyzed or hydrogenolyzed, and hydrogenolysis of the hydroxamate benzyl protecting group gave the target hydroxamic acids **6**. Hydrogenolysis over Pd/C catalyst, under standard catalytic or transfer hydrogenation conditions, was accompanied by significant reduction of the hydroxamate to the corresponding amide **7**. Although precedent does exist,[19] this side reaction has rarely been noted in the synthesis of related hydroxamate metalloprotease inhibitors. We have found that amide formation during Pd/C hydrogenolysis can be suppressed by addition of sodium bicarbonate to neutralize acidic impurities or by hydrogenolysis over $Pd(OH)_2/C$ and careful monitoring of hydrogen uptake.

Despite precedent,[20] synthesis of the succinate-derived hydroxamate series proved more troublesome and less efficient. The isopropyl(idene)succinic acid starting materials **11**, **12**, and **15** were prepared by Stobbe condensation and regioselective esterification as shown (Scheme 2).[21,22] Attempted condensation of succinate monomethyl ester **12** with (benzyloxy)amine in the presence of DCC and HOBT, analogous to the above malonate synthesis, gave a complex mixture containing guanidine side product **4** but little of the desired benzyl hydroxamate, **16a** (Scheme 3). Reaction of (benzyloxy)amine with α-isopropylsuccinic anhydride (**11**) gave the desired benzyl hydroxamate **16b** in modest yield. However, attempted condensation of **16b** with β-Ala-OtBu in the presence of DCC and HOBT gave none of the desired product **17** but only effected cyclization to *N*-(benzyloxy)succinimide **18**.[22,23] Monomethyl ester **15a** was successfully condensed with Asn-OtBu; however, attempted hydrolysis of the methyl ester in **19** with sodium hydroxide or potassium carbonate resulted in succinimide formation by cyclization with displacement of the methyl ester and, partially, the *tert*-butyl ester, affording a mixture of **20** and **21**. Cleavage of the *tert*-butyl ester under mild alkaline conditions appears remarkable. However the analogous succin-

Scheme 2



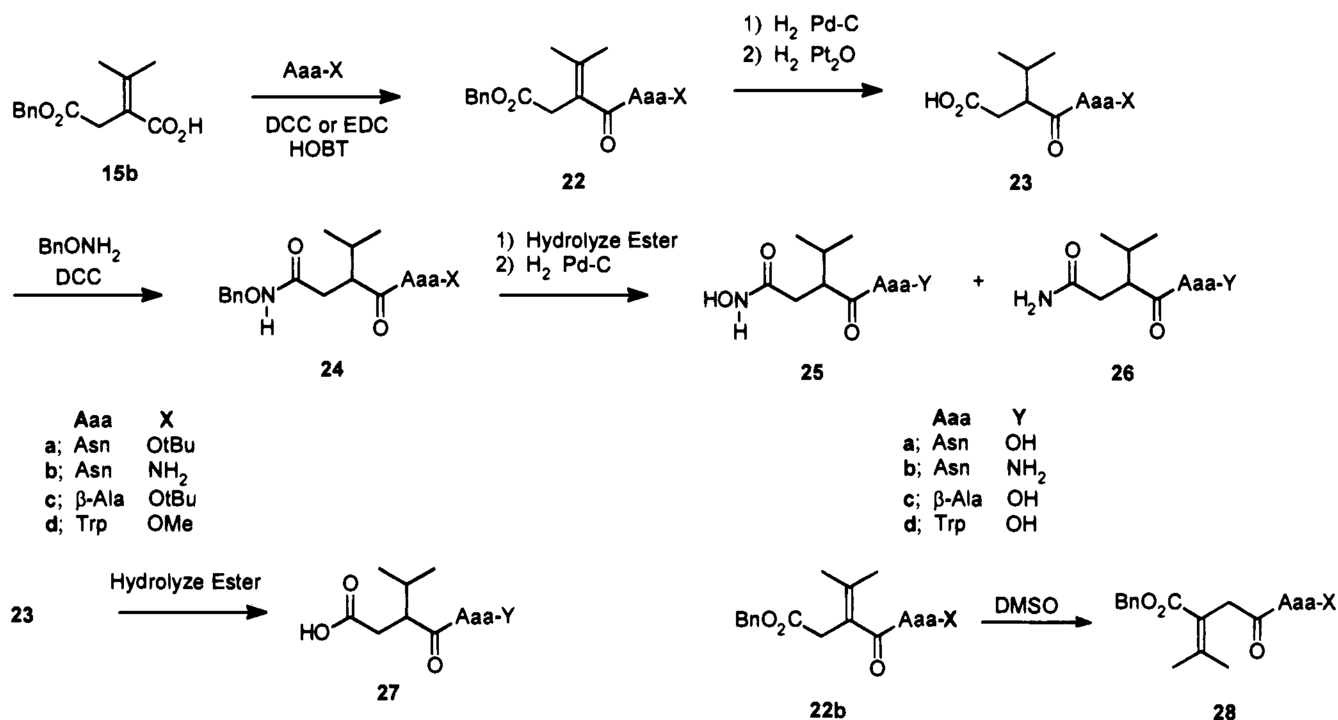
Scheme 3



imide formation occurs when Z-Asn-OtBu is treated with hydroxide.[24]

In light of these complications, we were forced to utilize a route less efficient for succinate analog preparation (Scheme 4), which avoided subjecting Asn *tert*-butyl esters or succinamide monoester derivatives (i.e., **19**) to alkaline conditions. Thus condensation of succinate monobenzyl ester **15b** with Asn-OtBu, Asn-NH_2, β-Ala-OtBu, or Trp-OMe in the presence of HOBT and DCC or EDC afforded benzyl esters **22**. A DMSO solution of benzyl ester **22b** slowly isomerized to **28**, which was also formed as a side product in the condensation of **15b** with Asn-NH_2. A plausible mechanism for formation of **28** involves cyclization of **22** to a succinimide and subsequent ring opening by benzyl alcohol. Hydrogenolysis of **22** with Pd/C followed by catalytic hydrogenation over Pt afforded **23**. This was coupled with (benzyloxy)amine in the presence of DCC or EDC to give **24**, again accompanied by guanidine **4**. Exclusion of HOBT from this reaction appeared to improve the yield of **24** and suppress formation of guanidine **4**. The *tert*-butyl esters of **24a,c** were cleaved with trifluoroacetic acid, while the methyl ester of **25d** was hydrolyzed with sodium hydroxide, without cyclization to a succinimide. Subsequent hydrogenolysis of the benzyl group gave the desired target hydroxamates **25**. Prolonged hydrogenation of **24c** in the presence of residual trifluoroacetic acid again gave partial reduction

Scheme 4



to the corresponding amide **26c**.[19] The corresponding carboxylic acids **27**, also of interest as inhibitors, were obtained by hydrolysis of esters **23**.

Inhibition of ECE, NEP, and Thermolysin

ECE isolated and purified from human bronchiolar smooth muscle (HBSM) cells has optimum activity at pH 7.2 and K_m for big ET-1 of 12 μM, with k_{cat} = 83 min^{-1}.[8] This HBSM ECE does not process big ET-3 efficiently. It requires calcium cation (>10 nM) and is completely dependent on chloride, having no activity in the absence of NaCl and maximal activity at 150–200 mM NaCl. ECE from human bronchiolar smooth muscle cells[8] has properties distinct from those reported for ECE isolated from endothelial cells. Notably, human bronchiolar smooth muscle ECE is much more sensitive to phosphoramidon (IC_{50} = 0.8 nM; see Table 1) than most endothelial cell ECE preparations (reported IC_{50}s range from 0.3 nM to >10 μM, but generally 0.5–5 μM),[5-7] including human umbilical vein endothelial cell (HUVEC) ECE which we partially purified in preliminary experiments. Human bronchiolar smooth muscle ECE is also much more potently inhibited by thiorphan (IC_{50} = 8 nM) than most other ECE preparations. These differences in inhibitor sensitivity likely reflect the presence of different isozymes of ECE (see the Discussion section).

Human bronchiolar smooth muscle ECE activity was measured by following the hydrolysis of human big ET-1 to ET-1. Because of the ability of crude preparations to generate products other than ET-1, some of which could cross-react with anti-ET-1 antibodies, hydrolysis of human big ET-1 to ET-1 was instead quantitatively measured by HPLC, with comparison to standards.

Evaluation of the hydroxamic acid targets (see Table 1) as HBSM ECE inhibitors reveals that in all cases hydroxamates derived from malonate are more potent than the homologous hydroxamates derived from succinate. Analogously, malonate-derived hydroxamates are known to be more potent than succinate hydroxamates as inhibitors of NEP, while succinate hydroxamates are more potent inhibitors of collagenase.[10]

In the malonate series, isopropyl appears to be slightly preferred over isobutyl at P_1' (**6d–g**); omission of this side chain, as in **6a**, seriously diminishes potency toward ECE. Isopropyl at P_1' is also satisfactory in the succinate series (**25a– 25d**), while kelatorphan, which bears a benzyl P_1' side chain, is the most potent succinate tested. The stereochemistry of the isopropyl P_1' side chain causes a relatively minor difference in potency of the diastereomers of **25d**. In the potent malonate series, Asn is only slightly preferred over Gly in the P_2' position, while Trp gives a potent inhibitor, and β-Ala gives the *extremely* potent inhibitor **6c**, with an IC_{50} of 0.01 nM. It is unexpected but noteworthy that the Asn residue present at P_2' in the ECE substrate big ET-1 is not required or optimal for inhibitor potency. In the less potent succinate series, however, Trp is preferred over Asn, which is essentially equipotent to β-Ala at P_2'.

Concurrent with our research, **6e**, envisioned as a hydroxamate analog of phosphoramidon, was also prepared in another laboratory and found to be a weak inhibitor of a partially purified ECE from rabbit lung.[9] In contrast, we find that **6e** is one of our more potent inhibitors and more potent than phosphoramidon as an inhibitor of ECE isolated from human bronchiolar smooth muscle cells.

As expected, the hydroxamic acid functionality is essential for potent inhibition of ECE. Thus *O*-benzyl hydroxamate precursors **5g** and **24b**, unable to form bidentate ligands with zinc, are very weak inhibitors of ECE. Amide **26c**, obtained by reduction of the hydroxamate **25c**, is also a weak ECE inhibitor. The carboxylates **27b,c** do not inhibit ECE, while **27d** and the carboxylate analog of **6c** (IC_{50} ~ 1000 nM) are modest inhibitors of ECE, though much weaker than the corresponding hydroxamates. Enalaprilat, a potent

Table 1. Inhibition of ECE, NEP, and Thermolysin by Hydroxamic Acids and Related Compounds

			IC_{50} (nM)[a]			
			HBSM ECE	NEP		thermolysin
inhibitor	R	Aaa-Y	big ET-1	big ET-1	Leu-Enkeph	Leu-Enkeph
		Hydroxamates Derived from Malonates				
6a[c]	H	β-Ala-OH	3000[b]			
6b	iPr	Asn-OH	3.0	20	1	
6c	iPr	β-Ala-OH	0.01	1	2	1900
6d	iPr	Trp-OH	0.20	0.03	0.4	5
6e	iBu	Trp-OH	0.25	0.07	0.03	5
6f	iPr	Trp-NH_2	1.2	3	2	9
6g	iBu	Trp-NH_2	4.8	7	2	2
6h	iPr	Gly-OH	6.8	8	3	
zincov	iBu	Ala-Gly-NH_2	11	40	2	175
		Hydroxamates Derived from Succinates				
25a	iPr	Asn-OH	150[b]	750	170[b]	
25b	iPr	Asn-NH_2	3000[b]			
25c	iPr	β-Ala-OH	190	270	30	
25d[c]	iPr	Trp-OH	17	30	5	2000
25d[c]	iPr	Trp-OH	40	40	18	
actinonin[c]	pentyl	Val-prolinol	4000	8200		
kelatorphan[c]	benzyl	Ala-OH	8	8	1	3
		Miscellaneous Compounds				
5g	iBu	Trp-NH_2	~10 000[b]			
24b	iPr	Asn-NH_2	≫10 000			
26c	iPr	β-Ala-OH	6500			
27b	iPr	Asn-NH_2	>10 000			
27c	iPr	β-Ala-OH	>10 000			
27d	iPr	Trp-OH	500[b]	1000	950	
Z-Pro-Leu-Gly-NHOH[c]			33 000	2300		
phosphoramidon[c]			0.8	2	6	33
thiorphan			8	20	5	

[a] Except where noted otherwise, IC_{50}s were determined in at least two independent experiments with duplicate measurements at each inhibitor concentration. Typical standard errors were 17 ± 6%. [b] IC_{50} determined in only one experiment. [c] Single diastereomer. Two diastereomers of **25d** were separated. Other compounds are mixtures of diastereomers or (in the case of **6c**,**h**, **25c**, **26c**, **27c**, and thiorphan) enantiomers.

inhibitor of angiotensin-converting enzyme which bears a carboxylate-chelating moiety, does not inhibit ECE (>10 000 nM, data not shown) since it bears inappropriate side chains.

The hydroxamic acids were also tested as inhibitors of two other metalloproteases, thermolysin and a soluble form of rabbit kidney neutral endopeptidase (NEP, EC 3.4.24.11),[25] in order to evaluate specificity. [Leu^5]-enkephalin was utilized as a substrate for NEP and thermolysin. Big ET-1 was also utilized as a substrate for NEP, under conditions identical to the ECE assay to allow direct comparison for selectivity against ECE. The IC_{50}s of well-known NEP inhibitor standards, thiorphan, phosphoramidon, and kelatorphan, are consistent with literature values.[16]

The IC_{50}s observed for inhibition of NEP by hydroxamates are somewhat substrate dependent; however, potencies of the inhibitors against NEP (with big ET-1 as substrate) correlate strongly with their potencies against HBSM ECE. This is consistent with the recent finding that NEP and ECE are closely related but distinct proteases[7] with similar structural requirements for substrates (i.e., big ET-1) and for potent inhibitors. While most of the inhibitors inhibit NEP as well as or better than ECE, the P_2' asparagine residue in **6b** and **25a**, which corresponds to P_2' of big ET-1, produces a 5–7-fold selectivity toward ECE. More significantly, P_2' β-Ala derivative **6c** exhibits 100-fold selectivity toward ECE. Thus, while P_2' β-Ala affords potent hydroxamate and carboxylate[16] inhibitors of NEP, it facilitates particularly potent binding to ECE, affording the observed selectivity. Compounds **6d**,**e**, prepared as hydroxamate analogs of phosphoramidon, are much more potent inhibitors of NEP than is phosphoramidon. Interestingly, **6d**,**e** inhibit NEP more potently than ECE and rank as some of the most potent NEP inhibitors yet reported.[16,17,25] Thermolysin is potently inhibited by most of the potent ECE inhibitors. Notable exceptions are **6c** and **25d**, which are good inhibitors of ECE and NEP but much weaker inhibitors of thermolysin.

Discussion

At the outset of this research, we screened many different tissues and cultured cells for enzymes capable of converting big ET-1 to ET-1. Tissue sources tended to be heavily contaminated with other enzymes. In addition, we desired a source of human ECE. We found that several cultured human cells, including human umbilical vein endothelial cells (HUVEC) and human bronchiolar smooth muscle (HBSM) cells produced ECE-like activity. The ECE of HBSM cells was pursued[8] on the basis of the concept that big ET-1 released from adjacent endothelial cells is hydrolyzed by the underlying smooth muscle cells, causing vasoconstriction, and that this process could be a target for pharmacological intervention. This view is supported by the observation that the vasoconstrictor activity of big ET-1 *in vivo*, and on porcine coronary arteries *lacking* endothelial cells, is inhibited by phosphoramidon.[26]

We found that ECE from HBSM cells was more sensitive to inhibition by phosphoramidon and thiorphan than other ECE preparations. These differences suggest the existence of different ECE isozymes. The existence of ECE isoforms has also been proposed recently to explain the lack of endothelial-type ECE-1 expression in neurons and other cells which are known

to produce ET-1.[7] Likewise, existence of distinct ECE subtypes has been postulated to explain differences in substrate specificity between isolated and expressed rat lung ECE[7] and could also explain the broad range of sensitivities reported for inhibitors of various other ECE preparations. It is very likely that additional ECE isozymes, with properties somewhat different from those of either the endothelial cell or smooth muscle cell forms, will be found. The difference in sensitivity to inhibitors is exciting, as it implies that selective pharmacological intervention should be possible.

The sensitivity of HBSM ECE to phosphoramidon and thiorphan raised the possibility that it might actually be neutral metalloendopeptidase (NEP, EC 3.4.24.11). However this possibility was eliminated since the inhibitor profiles were distinct (see Table 1), the specific activity of ECE on big ET-1 is more than 100 times greater than that of NEP, and NEP rapidly cleaves ET-1, while human bronchiolar smooth muscle ECE does not. Furthermore, human bronchiolar smooth muscle ECE was not immunoprecipitated by anti-NEP monoclonal antibody. Inhibition of ECE by low concentrations of phosphoramidon and thiorphan is consistent with the inhibitory effect which these inhibitors display *in vivo* against big ET-1.[5,6,26]

Hydroxamates derived from succinates (**25**) and particularly malonates (**6**) proved to be potent inhibitors of HBSM ECE. In the malonate series, Val is the preferred P_1' amino acid and β-Ala is the preferred P_2' amino acid. C-terminus carboxylates **6d,e** and **25a** are much more potent ECE inhibitors than the corresponding amides **6f,g** and **25b**, respectively. This suggests that the carboxylate in these inhibitors, a moiety absent in the analogous region of big ET-1, binds to a positively charged residue such as Arg in ECE. Indeed, recently reported sequences reveal the presence of an Arg residue near the active site of ECE,[7] analogous to NEP.[27] It is possible that the P_2' carboxylate in the potent carboxylate inhibitors **6d,e** occupies the site normally taken by the carboxylate in the side chain of P_5' Glu in big ET-1, which is brought into proximity by a turn involving the P_4' Pro. The outstanding potency of β-Ala derivative **6c** (IC_{50} = 0.01 nM) may similarly be caused by favorable binding to a carboxylate-binding site in ECE.

Malonate-derived hydroxamate **6c** is an exquisitely potent inhibitor of HBSM ECE which displays unique selectivity toward ECE over NEP inhibition. We anticipate that these hydroxamates, particularly **6c**, will be useful tools for defining roles of ECE *in vitro* and *in vivo*, possibly leading to therapeutic agents.

Experimental Section

Unless otherwise stated, crude reaction mixtures were normally processed as follows: The organic solution was rinsed with water, 1 M HCl, saturated sodium carbonate, and saturated NaCl and then dried over anhydrous magnesium sulfate. The organic solvent was then evaporated *in vacuo* to afford the crude product. Column chromatography was performed on silica gel 60 (400−230 mesh). Preparative reverse-phase HLPC was performed on C_{18} silica gel eluted with a gradient of water−acetonitrile containing trifluoroacetic acid (0.1%). Hydroxamic acids **6** and **25** gave a deep burgundy-colored reaction with 2.5% aqueous $FeCl_3$. TLC plates were visualized with 4% phosphomolybdic acid in ethanol or with UV light. Hydroxamates were visualized after TLC with 2.5% aqueous $FeCl_3$ diluted with acetone (2:3). NMR spectra were recorded at 300 MHz. Z-Pro-Leu-Gly-NHOH was obtained from Sigma.

Potassium Monoethyl (1-Methylethyl)malonate (2a). A solution of KOH (5.19 g, 92.7 mmol) in ethanol (60 mL) was added to diethyl (1-methylethyl)malonate (15 g, 74.17 mmol) over 50 min. After 20 h, the ethanol was evaporated and the precipitate was rinsed with ether to afford the desired potassium salt (16.4 g, 104% crude yield) as a white solid: mp 110−125 °C; NMR (D_2O) δ 0.93 (6 H, 2 d), 1.24 (3 H, t), 2.24 (1 H, m), 2.98 (1 H, d), 4.19 (2 H, q).

Potassium Monoethyl (2-Methylpropyl)malonate (2b). This compound was prepared as above, affording a very hygroscopic solid: NMR (D_2O) δ 0.91 (6 H, d), 1.19 (3 H, t), 1.45 (1 H, m), 1.60 (2 H, dd), 3.32 (1 H, t), 4.12 (2 H, q).

Ethyl 2-[[(Phenylmethoxy)amino]carbonyl]-3-methylbutanoate (3a). Potassium salt **2a** (4.79 g, 22.6 mmol) was dissolved in dichloromethane (100 mL). HOBT (3.45g, 22.5 mmol) and (benzyloxy)amine hydrochloride (3.60 g, 22.6 mmol) were added. This heterogeneous mixture was cooled to 5 °C, DCC (4.89 g, 23.7 mmol) was added, and the mixture was stirred for 1 h at 5 °C and for 68 h at 20 °C. Water (1 mL) was added. After stirring for 30 min, the mixture was filtered, and the filtrate was worked up as usual to afford the desired product as a pale yellow wax (6.96 g, 108% crude yield): NMR ($CDCl_3$) δ 0.90 (6 H, 2 d), 1.23 (2 H, t), 2.30 (1 H, m), 2.90 (1 H, d), 4.18 (3 H, q), 4.90 (2 H, s), 7.30 (5 H, m).

Ethyl 2-[[(Phenylmethoxy)amino]carbonyl]-4-methylpentanoate (3b). This compound was prepared as above: NMR ($CDCl_3$) δ 0.89 (6 H, m), 1.5−2.0 (3 H, m), 3.22 (1 H, t), 4.13 (2 H, s), 7.40 (5 H, s), 9.16 (1 H, br s).

***N,N'*-Dicyclohexyl-*N''*-(phenylmethoxy)guanidine (4).** The crude material was conveniently obtained from the aqueous base-insoluble precipitate deposited after hydrolysis of **3**. Chromatography (ether−hexanes, 50:50) afforded a white solid, mp 69−70 °C.

Alternatively, DCC (233 mg, 1.13 mmol), HOBT (175 mg, 1.14 mmol), and (benzyloxy)amine (138 mg, 1.12 mmol) in CH_2Cl_2 (2 mL) were stirred for 23 h. After normal processing, the crude product was dissolved in CH_2Cl_2 and washed with 1 M NaOH and the solvent evaporated to afford crude product (340 mg, 91% crude yield). Chromatography (ether) afforded pure product (57% yield): mp 69−72 °C; NMR ($CDCl_3$) δ 1.0−1.4 (10 H, m), 1.5−1.8 (6 H, m), 1.82 (2 H, m), 1.99 (2 H, m), 2.94 (1 H, br s, exchanges), 2.99 (1 H, br s), 3.30 (1 H, br s), 4.76 (1 H, br d, exchanges), 4.81 (2 H, s), 7.25−7.40 (5 H, m); FAB MS *m/e* 330 (M + H). Anal. ($C_{20}H_{31}N_3O$) C, H, N.

***N*-[2-[[(Phenylmethoxy)amino]carbonyl]-3-methylbutanoyl]-L-asparagine 1,1-Dimethylethyl Ester (5b).** KOH (6.03 g, 107 mmol, 221 mol %) dissolved in ethanol (54 mL) and water (27 mL) was added over 20 min to a stirred solution of ethyl ester **3a** (13.61 g, 48.7 mmol) dissolved in ethanol (54 mL). After 18 h, the solvent was evaporated and water (130 mL) was added. The mixture was filtered, and the filtrate was acidified with 1 M HCl. 2-[[(Phenylmethoxy)amino]carbonyl]-3-methylbutanoic acid (3.54 g, 29% yield) was collected by filtration. The filtrate was extracted four times with ethyl acetate to give more crude acid (5.39 g, 44%) which could be recrystallized from water to give white crystals: mp 121−123 °C; NMR ($CDCl_3$) δ 0.93 (3 H, d), 0.95 (3 H, d), 2.24 (1 H, m), 2.81 (1 H, d), 4.89 (2 H, d), 7.34 (5 H, m). Anal. ($C_{13}H_{17}NO_4$) C, H, N.

The carboxylic acid from hydrolysis of **3a** (250 mg, 0.995 mmol) was treated with L-asparagine *tert*-butyl ester hydrochloride (224 mg, 0.995 mmol), DCC (215 mg, 1.05 mmol), HOBT−H_2O (153 mg, 0.995 mmol) and $EtNiPr_2$ (0.18 mL, 1.04 mmol), in DMF (2.5 mL) for 1 h at 0 °C and then for 48 h at 20 °C. The mixture was filtered, and water was added to the filtrate. The resulting precipitate was dissolved in EtOAc (500 mL) and processed as usual to afford the product (402 mg, 96% yield) as a wax: NMR (CD_3OD) δ 0.94 (6 H, m), 1.46 (9 H, s), 2.54 (1 H, d), 2.74 (2 H, m), 4.61 (1 H, m), 7.37 (5 H, m).

***N*-[2-[[(Phenylmethoxy)amino]carbonyl]-3-methylbutanoyl]-β-alanine 1,1-Dimethylethyl Ester (5c).** The carboxylic acid from hydrolysis of **3a** (8.93 g) was coupled with β-alanine *tert*-butyl ester hydrochloride as above. The mixture was filtered, and the DMF was evaporated *in vacuo* (60−70

°C). The residue was triturated with ether to afford the product (9.15 g, 67% yield) as an off-white solid: NMR ($CDCl_3$) δ 0.94 (6 H, 2 d), 2.48 (2 H, m), 3.46 (2 H, m), 4.91 (2 H, s), 6.84 (1 H, br), 7.36 (5 H, s), 9.37 (1 H, s).

***N*-[2-[[(Phenylmethoxy)amino]carbonyl]-3-methylbutanoyl]-L-tryptophan Phenylmethyl Ester (5d).** The carboxylic acid from hydrolysis of **3a** was coupled with L-tryptophan phenylmethyl ester hydrochloride as above. The residue was recrystallized from methanol to afford the product as a white solid: mp 178−182 °C (69% yield); NMR (DMSO-d_6) δ 0.69 (3 H, d), 0.76 (3 H, d), 2.10 (1 H, d), 3.18 (1 H, m), 4.60 (1 H, q), 4.72 (2 H, s), 5.04 (2 H, d), 6.98 (1 H, t), 7.07 (1 H, t), 7.14 (1 H, s), 7.24 (1 H, m), 7.34 (10 H, m), 7.49 (1 H, d), 8.07 (1 H, d), 10.90 (1 H, s), 11.04 (1 H, s). Anal. ($C_{31}H_{33}N_3O_5$·$0.5H_2O$) C, H, N.

***N*-[2-[[(Phenylmethoxy)amino]carbonyl]-4-methylpentanoyl]-L-tryptophan Phenylmethyl Ester (5e).** KOH (3.22 g, 57 mmol, 252 mol %) dissolved in ethanol (26 mL) and water (12 mL) was added over 20 min to a stirred solution of ethyl ester **3b** (6.69 g, 22.8 mmol) dissolved in ethanol (54 mL). After 68 h, the solvent was evaporated and water (130 mL) was added. The mixture was filtered, and the filtrate was acidified with 1 M HCl. 2-[[(Phenylmethoxy)amino]carbonyl]-4-methylpentanoic acid (4.46 g, 74% yield) was collected by filtration as a white solid: mp 81−84 °C; NMR ($CDCl_3$) δ 0.89 (6 H, m), 1.60 (1 H, m), 1.76 (2 H, m), 3.18 (1 H, t), 4.92 (2 H, s), 7.38 (5 H, s), 8.92 (1 H, br s). Anal. ($C_{14}H_{19}NO_4$·$1.05H_2O$) C, H, N.

The carboxylic acid from hydrolysis of **3b** was coupled with L-tryptophan phenylmethyl ester hydrochloride as above. The crude product (65% yield) was recrystallized from methanol to afford the product as a white solid: mp 177−181 °C; NMR (DMSO-d_6) δ 0.74 (6 H, m), 1.3 (1 H, m), 1.5 (2 H, m), 3.00 (1 H, t), 3.18 (2 H, m), 4.60 (1 H, m), 4.71 (2 H, s), 5.08 (2 H, s), 6.9−7.5 (16 H, m), 8.04 (1 H, d), 10.91 (1 H, s), 11.02 (1 H, s). Anal. ($C_{32}H_{35}N_3O_5$·$0.23H_2O$) C, H, N.

***N*-[2-[[(Phenylmethoxy)amino]carbonyl]-3-methylbutanoyl]-L-tryptophanamide (5f).** The carboxylic acid from hydrolysis of **3a** was coupled with L-tryptophanamide hydrochloride as above, except that DCC was replaced with 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC). The crude product (130% crude yield) precipitated from the reaction mixture residue upon addition of water and was not further purified.

***N*-[2-[[(Phenylmethoxy)amino]carbonyl]-4-methylpentanoyl]-L-tryptophanamide (5g).** The carboxylic acid from hydrolysis of **3b** was coupled with L-tryptophanamide hydrochloride as described for **5f**. The product precipitated from water as a white solid and was rinsed with 1 M HCl, dilute $NaHCO_3$, and water: mp 194−200 °C; NMR (DMSO-d_6) δ 0.74 (6 H, m), 1.22 (1 H, m), 1.50 (2 H, m), 3.0 (2 H, m), 3.18 (1 H, m), 4.71 (2 H, d), 6.9−8.0 (10 H, m), 10.82 (1 H, s), 10.05 (0.6 H, s), 11.14 (0.4 H, s). Anal. ($C_{25}H_{32}N_4O_4$·0.21 H_2O) C, H, N.

***N*-[2-[[(Phenylmethoxy)amino]carbonyl]-3-methylbutanoyl]glycine 1,1-Dimethylethyl Ester (5h).** The carboxylic acid from hydrolysis of **3a** was coupled with glycine *tert*-butyl ester hydrochloride as described for **5b**. The residue was triturated with ether to give the product (67% yield) as a white solid: NMR (CD_3OD) δ 0.96 (3 H, d), 1.03 (3 H, d), 1.51 (9 H, s), 2.34 (1 H, m), 2.60 (1 H, d), 3.88 (1 H, s), 4.89 (2 H, s), 7.43 (5 H, m).

***N*-[3-(Hydroxyamino)-1,3-dioxopropyl]-β-alanine (6a).** HCl gas was bubbled intermittently into a suspension of *tert*-butyl ester **5a** (140 mg) suspended in ether (2 mL). The precipitate dissolved, and after 1 h, the solvent was evaporated and the residue was triturated three times with refluxing ether to give the carboxylic acid as a white solid (88 mg, 75% yield), mp 130−131 °C. This solid was dissolved in methanol (1.5 mL) and hydrogenated for 3.5 h at 1 atm over 10% Pd/C (9 mg) in the presence of $NaHCO_3$ (9 mg). Removal of the catalyst and evaporation of the solvent afforded the desired product (60 mg, 98% yield) as a hygroscopic off-white wax: $FeCl_3$ positive; NMR (CD_3OD) δ 2.56 (2 H, t), 3.20 (2 H, s, exchanges), 3.46 (2 H, t); FAB MS *m/e* 191 (M + H). Anal. ($C_6H_{10}N_2O_5$·0.2HOAc) C, H, N.

***N*-[2-[(Hydroxyamino)carbonyl]-3-methylbutanoyl]-L-asparagine (6b).** *tert*-Butyl ester **5b** (402 mg) was dissolved in trifluoroacetic acid at 5 °C. After 1 h at 20 °C, the solvent was evaporated and the residue was triturated with ether. The resulting carboxylic acid (244 mg, 70% yield, mp 147−151 °C) was dissolved in methanol (5 mL) and hydrogenated over 5% Pd/C (33 mg) for 4 h at 1 atm. Removal of the catalyst and evaporation of the solvent afforded the crude product (154 mg, 84% crude yield) which was purified on reverse-phase HPLC to give the product as a white solid: mp 138−142 °C; NMR (CD_3OD) δ 1.00 (6 H, m), 2.30 (1 H, d), 2.60 (1 H, d, exchanges), 2.78 (2 H, m), 4.70 (1 H, m); FAB MS *m/e* 276 (M + H). Anal. ($C_{10}H_{17}N_3O_6$·0.1TFA) C, H, N, F.

***N*-[2-[(Hydroxyamino)carbonyl]-3-methyl-1-oxobutyl]-β-alanine (6c).** HCl gas was bubbled intermittently into a suspension of *tert*-butyl ester **5c** (9.15 g, 24.2 mmol) suspended in ether (90 mL), affording a solution. After 1 h, the solvent was evaporated. The residue was dissolved in water and lyophilized to afford the carboxylic acid as a yellow solid (8.32 g, 107% crude yield), mp 108−115 °C. Anal. ($C_{16}H_{12}N_2O_5$) C, H, N. A portion (5.02 g) was dissolved in methanol (60 mL) and hydrogenated at 1 atm over 10% Pd/C (500 mg) and $NaHCO_3$ (500 mg) for 90 min. The mixture was filtered; the filtrate was concentrated and suspended in water (35 mL). The minor precipitate was removed, and the supernatant was lyophilized to give the product as a white solid (3.28 g, 91% yield): mp 127−132 °C; NMR (CD_3OD) δ 0.90 (6 H, d), 2.22 (1 H, m), 2.58 (2 H, t), 2.65 (1 H, d), 3.46 (2 H, m); FAB MS *m/e* 233.1132 (M + H), calcd 233.1138. Anal. ($C_9H_{16}N_2O_5$·$0.7H_2O$) C, H, N.

***N*-[2-[(Hydroxyamino)carbonyl]-3-methylbutanoyl]-L-tryptophan (6d).** A solution of benzyl ester **5d** (200 mg) in methanol (8 mL, dissolved by brief heating) was hydrogenated at 1 atm over 10% Pd/C (30 mg) and $NaHCO_3$ (500 mg) for 3.5 h. The mixture was filtered; the filtrate was concentrated, suspended in water, and dried *in vacuo* to give the product as a white solid (119 mg, 90% yield): mp 100−110 °C; NMR (CD_3OD) δ 0.74 (1.4 H, d), 0.84 (2 H, d), 0.91 (2.6 H, d), 2.20 (1 H, m), 2.50 (0.6 H, d), 2.70 (0.4 H, d), 3.21 (2 H, m), 4.70 (1 h, m), 7.08 (3 H, m), 7.30 (1 H, d), 7.58 (1 H, d); FAB MS *m/e* 348 (M + H). Anal. ($C_{17}H_{212}N_3O_5$·$1.1H_2O$) C, H, N.

***N*-[2-[(Hydroxyamino)carbonyl]-4-methylpentanoyl]-L-tryptophan (6e).** This compound was prepared from **5e** by the method used for **6d**. The crude product was chromatographed on silica gel (propanol−water−NH_4OH, 74:19:7), suspended in water, and filtered. The filtrate was lyophilized to give the product as a white solid: mp 173−181 °C; NMR (CD_3OD) δ 0.76 (6 H, m), 1.29 (1 H, m), 1.52 (1 H, m), 1.65 (1 H, m), 3.01 (1 H, m), 3.17 (1 H, m), 3.39 (1 H, m), 4.61 (1 H, m), 7.00 (4 H, m), 7.28 (1 H, d), 7.59 (1 H, d); FAB MS *m/e* 362 (M + H). Anal. ($C_{18}H_{23}N_3O_5$·$1.3H_2O$·$0.51NH_3$) C, H, N.

***N*-[2-[(Hydroxyamino)carbonyl]-3-methylbutanoyl]-L-tryptophanamide (6f).** A suspension of benzyl ester **5f** (273 mg) in methanol (6 mL) was hydrogenated at 1 atm over 5% Pd/C (33 mg) at 20 °C for 1.5 h and at 50 °C for 2 h. After filtration, the filtrate was concentrated and chromatographed on silica gel (propanol−water−NH_4OH, 80:10:10) to give the product as a white solid (96 mg, 44% yield): mp 176−180 °C; NMR (CD_3OD) δ 0.90 (6 H, m), 2.25 (1 H, m), 2.50 (1 H, 2 d), 3.11 (1 H, m), 4.68 (1 H, t), 6.97−7.14 (3 H, m), 7.30 (1 H, m), 7.63 (1 H, m); FAB MS *m/e* 347 (M + H). Anal. ($C_{17}H_{22}N_4O_4$·$0.67H_2O$) C, H, N.

***N*-[2-[(Hydroxyamino)carbonyl]-4-methylpentanoyl]-L-tryptophanamide (6g).** This compound was prepared from **5g** by the method used for **6d** and then chromatographed on silica gel (propanol−water−NH_4OH, 80:10:10) to give the product as a white solid: mp 190−198 °C; NMR (CD_3OD) δ 0.76 (6 H, dd), 1.23−1.60 (3 H, m), 2.95 (1 H, m), 3.60 (1 H, m), 4.45 (1 H, m), 7.03 (4 H, m), 7.31 (1 H, d), 7.45 (2 H, m), 7.71 (0.5 H, d), 7.85 (0.5 H, d), 10.50−10.82 (1 H, br s); FAB MS *m/e* 361 (M + H). Anal. ($C_{18}H_{24}N_4O_4$·$0.83H_2O$·0.22PrOH) C, H, N.

***N*-[2-[(Hydroxyamino)carbonyl]-3-methylbutanoyl]glycine (6h).** This compound was prepared from **5h** by the method used for **6a**. The product (59% yield) was collected as white crystals after being rinsed with EtOAc: mp 102−110

°C; NMR (CD_3OD) δ 0.98 (6 H, 2 d), 2.30 (1 H, m), 2.60 (1 H, d), 3.89 (2 H, d); FAB MS *m/e* 219 (M + H). Anal. ($C_8H_{14}N_2O_5$·0.15EtOAc) C, H, N.

N-[2-(Aminocarbonyl)-3-methyl-1-oxobutyl]-β-alanine (7c). This amide (36% yield) was separated from hydroxamate **6c** by preparative reverse-phase HPLC: no $FeCl_3$ reaction; NMR (CD_3OD) δ 0.98 (6 H, 2 d), 2.20 (1 H, m), 2.51 (2 H, t), 2.71 (1 H, d), 3.46 (2 H, t); FAB MS *m/e* 217 (M + H).

4-Methyl 2-(1-Methylethyl)butanedioate (12). Dihydro-3-(methylethyl)-2,5-furandione[22] (**11**, 108 mg) was refluxed with methanol (1.1 mL) for 17 h. The solvent was evaporated to give **12** (133 mg, 100% yield) as a colorless oil: NMR ($CDCl_3$) δ 0.95 (3 H, d), 0.98 (3 H, d), 2.07 (1 H, m), 2.45 (1 H, dt), 2.74 (2 H, m), 3.69 (3 H, s).

(1-Methylethylidene)butanedioic Acid (13). Monomethyl ester **8**[21] (5.12 g, 29.8 mmol) was stirred with NaOH (3.27 g, 81.8 mmol) in water (50 mL) for 12 h. Concentrated HCl (7.5 mL, 90 mmol) was added, the mixture was cooled to 0 °C, and the white solid product (2.90 g, 62%) was collected by filtration, mp 166–167 °C (lit.[21] mp 164 °C). Additional product (650 mg, 14%) was obtained by concentrating the filtrate to 20 mL: NMR (DMSO-d_6) δ 1.79 (3 H, s), 2.06 (3 H, s), 3.23 (2 H, s).

Dihydro-3-(methylethylidene)-2,5-furandione (14). Diacid **13** (14.32 g, 90.6 mmol) was stirred with acetyl chloride (30 mL) for 5 min at 20 °C and for 20 min at reflux. The solvent was evaporated, and the residue was Kugelrohr distilled (140–155 °C, 1.5 mmHg) to give the product as a pale yellow solid (12.36 g, 97% yield): mp 38–42 °C (lit.[21] mp 44 °C); NMR ($CDCl_3$) δ 1.99 (3 H, s), 2.38 (3 H, s), 3.50 (2 H, br s).

4-Methyl (1-Methylethylidene)butanedioate (15a). Acetyl chloride (27 mg, 0.35 mmol) was cautiously added to a suspension of **13** (1.00 g, 6.33 mmol) in methanol (1 mL). After 20 min of refluxing, the solvent was evaporated to give crude product (1.08 g) which was recrystallized (hexanes–EtOAc, 10:1) to give **15a** (842 mg, 77% yield) as a white solid, mp 91–95 °C. Alternatively, anhydride **14** (810 mg) dissolved in methanol (10 mL) was refluxed for 15 h. The solvent was evaporated to give **15a** (989 mg, 98% yield) as a white solid: mp 96–98 °C; NMR ($CDCl_3$) δ 1.92 (3 H, s), 2.24 (3 H, s), 3.41 (2 H, br s), 3.71 (3 H, s).

4-Phenylmethyl (1-Methylethylidene)butanedioate (15b). Anhydride **14** (12.36 g, 88.3 mmol), benzyl alcohol (14.36 g, 133 mmol), and ethanol-free chloroform (110 mL) were refluxed for 3 days. Evaporation of the solvent followed by recrystallization from ethyl acetate and hexanes gave the product (15.1 g, 69% yield) as a white solid: mp 120–124 °C; NMR ($CDCl_3$) δ 1.90 (3 H, s), 2.23 (3 H, s), 3.46 (2 H, s), 5.14 (2 H, s), 7.34 (5 H, m); ^{13}C NMR ($CDCl_3$) δ 23.65, 24.10, 35.17, 66.50, 119.82, 127.98, 128.46, 135.90, 153.79, 171.20, 173.15. Anal. ($C_{14}H_{16}O_4$) C, H.

N-[2-(Methylethylidene)-1,4-dioxo-4-(phenylmethoxy)-butyl]-L-asparagine 1,1-Dimethylethyl Ester (22a). A mixture of L-asparagine *tert*-butyl ester hydrochloride (670 mg, 2.98 mmol) and **15b** (730 mg, 2.94 mmol) in CH_2Cl_2 (10 mL) was stirred with iPr_2NEt (0.52 mL, 2.98 mmol), HOBT–H_2O (450 mg, 2.94 mmol), and DCC (640 mg, 3.10 mmol) at 0 °C for 1 h and at 20 °C for 46 h. Water (0.1 mL) was added, the mixture was filtered, and the filtrate was worked up as usual to afford the desired product as an off-white solid (1.16 g, 94% yield): mp 81–85 °C; NMR ($CDCl_3$) δ 1.46 (9 H, s), 1.72 (3 H, s), 1.94 (3 H, s), 3.78 (2 H, d of AB-q), 3.36 (2 H, AB-q), 4.74 (1 H, m), 5.14 (2 H, s), 5.42 (1 H, br s), 6.00 (1 H, br s), 7.14 (1 H, d), 7.37 (5 H, s).

N-[2-(Methylethylidene)-1,4-dioxo-4-(phenylmethoxy)-butyl]-L-asparaginamide (22b). A mixture of L-asparaginamide hydrochloride (500 mg, 2.99 mmol) and **15b** (740 mg, 2.98 mmol) in DMF (7 mL) was stirred with iPr_2NEt (0.52 mL, 2.98 mmol), HOBT–H_2O (460 mg, 3.00 mmol), and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) (640 mg, 3.33 mmol) at 20 °C for 47 h. The mixture was filtered. The precipitate was rinsed with water, saturated $NaHCO_3$, and water and then dried *in vacuo* to afford **28** (500 mg, 46% yield). The DMF was evaporated from the filtrate *in vacuo* ($T < 33$ °C). Water (15 mL) was added, and the resulting precipitate was collected by vacuum filtration, rinsed with dilute $NaHCO_3$ and water, then dried *in vacuo* to afford the desired product (300 mg, 28% yield) as a white solid: mp 180–104 °C; NMR (DMSO-d_6) δ 1.69 (3 H, s), 1.80 (3 H, s), 2.50 (2 H, m), 3.33 (2 H, s), 4.56 (1 H, m), 5.08 (2 H, s), 6.88 (1 H, s), 7.03 (1 H, s), 7.10 (1 H, s), 7.33 (5 H, m), 7.92 (1 H, d); ^{13}C NMR (DMSO-d_6) δ 20.58 (q), 22.10 (q), 35.32 (t), 36.63 (t), 49.75(d), 65.73 (t), 124.83 (s), 127.81 (d), 127.96 (d), 128.35 (d), 135.99 (s), 136.28 (s), 168.95 (s), 171.02 (s), 171.75 (s), 172.94 (s); FAB MS *m/e* 362 (M + H). Anal. ($C_{18}H_{23}N_3O_5$) C, H, N.

N-[2-(Methylethylidene)-1,4-dioxo-4-(phenylmethoxy)-butyl]-β-alanine 1,1-Dimethylethyl Ester (22c). β-Alanine *tert*-butyl ester hydrochloride and **15b** were coupled with DCC as described for **22a** to give **22c** (100% yield) as an off-white solid: mp 67– 70 °C; NMR ($CDCl_3$) δ 1.45 (9 H, s), 1.69 (3 H, s), 1.84 (3 H, s), 2.46 (2 H, t), 3.32 (2 H, s), 3.50 (2 H, q), 5.12 (2 H, s), 7.35 (5 H, br s).

N-[2-(Methylethylidene)-1,4-dioxo-4-(phenylmethoxy)-butyl]-L-tryptophan Methyl Ester (22d). L-Tryptophan methyl ester hydrochloride and **15b** were coupled with EDC as described for **22b** to give **22d** (100% yield) as a yellow wax: NMR ($CDCl_3$) δ 1.65 (3 H, s), 1.28 (3 H, s), 3.25 (4 H, m), 3.67 (3 H, s), 4.97 (1 H, dd), 6.79 (1 H, d), 7.00 (1 H, d), 7.09 (1 H, t), 7.19 (1 H, t), 7.32 (7 H, m), 7.54 (1 H, d), 7.99 (1 H, br s).

N-[4-Hydroxy-2-(methylethyl)-1,4-dioxobutyl]-L-asparagine 1,1-Dimethylethyl Ester (23a). Compound **22a** (1.16 g) dissolved in methanol (12 mL) was hydrogenated over 10% Pd/C (150 mg) at 40 psi for 13 h (affording hydrogenolysis of the benzyl group and partial reduction of the double bond) and then over PtO_2 (115 mg) for 23 h. Removal of the catalyst gave the product (896 mg, 98%) as a white solid: mp 130–135 °C; NMR ($CDCl_3$) δ 0.99 (6 H, m), 1.47 (4.5 H, s), 1.48 (4.5 H, s), 1.90 (1 H, m), 2.45 (2 H, m), 2.80 (3 H, m), 4.41 (0.5 H, m), 4.72 (0.5 H, m).

N-[4-Hydroxy-2-(methylethyl)-1,4-dioxobutyl]-L-asparaginamide (23b). Compound **22b** was hydrogenated as above to give **23b** (97% yield) as a white powder: mp > 280 °C; NMR (D_2O) δ 0.93 (6 H, m), 2.65 (1 H, m), 2.68 (2 H, m), 2.89 (1 H, m), 4.86 (0.5 H, m), 4.81 (0.5 H, m); FAB MS *m/e* 274 (M + H), 257 (M–NH_2). Anal. ($C_{11}H_{19}N_3O_5$·0.3H_2O) C, H, N.

N-[4-Hydroxy-2-(methylethyl)-1,4-dioxobutyl]-β-alanine 1,1-Dimethylethyl Ester (23c). Compound **22c** (1.03 g) was hydrogenated as above to give **23c** (92% yield) as a colorless oil: NMR ($CDCl_3$) δ 0.93 (6 H, m), 1.43 (9 H, s), 1.90 (1 H, m), 2.15 (1 H, m), 2.45 (2 H, m), 2.75 (2 H, m), 3.46 (2 H, m), 6.6 (1 H, t); FAB MS *m/e* 288 (M + H), 232 (M + H – C_4H_8). Anal. ($C_{14}H_{25}NO_5$) C, H, N.

N-[4-Hydroxy-2-(methylethyl)-1,4-dioxobutyl]-L-tryptophan Methyl Ester (23d). Compound **22d** was hydrogenated as above to give **23d** (79% yield) as a pink solid: mp 80–86 °C; NMR ($CDCl_3$) δ 0.78–0.96 (6 H, m), 1.86 (1 H, m), 2.42 (2 H, m), 2.86 (1 H, m), 3.26 (2 H, m), 3.59 (1 H, s), 3.67 (2 H, s), 4.91 (1 H, m), 6.42 (0.65 H, d), 6.56 (0.35 H, d), 7.08 (3 H, m), 7.33–7.58 (2 H, m), 8.68 (0.35 H, s), 9.00 (0.65 H, s). Anal. ($C_{19}H_{24}N_2O_5$·0.25H_2O) C, H, N.

N-[2-(Methylethyl)-1,4-dioxo-4-[(phenylmethoxy)amino]butyl]-L-asparagine 1,1-Dimethylethyl Ester (24a). Compound **23a** (665 mg, 2.015 mmol) was dissolved in DMF (6.5 mL). DCC (450 mg, 2.18 mmol) and (benzyloxy)amine (270 mg, 2.20 mmol) were added, and the mixture was stirred for 41 h at 20 °C. After filtration, the solvent was evaporated from the filtrate *in vacuo* at 30 °C. The residue was dissolved in chloroform and worked up as usual. Chromatography on silica gel (chloroform–methanol, 94:6) gave the desired product (510 mg, 58% yield) as a white solid: mp 182–185 °C; NMR ($CDCl_3$) δ 0.94 (3 H, m), 0.96 (3 H, m), 1.42 (4.5 H, s), 1.46 (4.5 H, s), 1.84 (1 H, m), 2.28 (1 H, m), 2.45 (1 H, m), 4.30 (0.5 H, m), 4.76 (0.5 H, s), 6.46 (0.5 H, s), 6.59 (0.5 H, s), 7.35 (5 H, m), 8.19 (0.5 H, d), 9.68 (1 H, d). Anal. ($C_{22}H_{33}N_3O_6$·0.25H_2O) C, H, N.

N-[2-(Methylethyl)-1,4-dioxo-4-[(phenylmethoxy)amino]butyl]-L-asparaginamide (24b). The crude product, prepared as above, was triturated twice with refluxing EtOAc to give **24b** (56% yield) as a white solid: mp 189–190 °C; NMR (CD_3OD) δ 0.93 (6 H, m), 1.74 (1 H, m), 2.2–2.9 (5 H, m), 4.63

(1 H, t), 4.81 (2 H, AB-q), 7.37 (5 H, m); FAB MS *m/e* 379 (M + H). Anal. ($C_{18}H_{26}N_4O_5{\cdot}0.5H_2O$) C, H, N.

***N*-[2-(Methylethyl)-1,4-dioxo-4-[(phenylmethoxy)amino]butyl]-*β*-alanine 1,1-Dimethylethyl Ester (24c).** Compound **23c** was coupled with (benzyloxy)amine hydrochloride as described for **3a** to give **24c** (43%) as a white solid after chromatography on silica gel (ether–ethyl acetate, 90:10): mp 93–96 °C; NMR ($CDCl_3$) δ 0.93 (6 H, m), 1.40 (9 H, s), 1.79 (1 H, m), 2.39 (5 H, m), 3.28 (1 H, m), 3.44 (1 H, m), 4.83 (2 H, s), 6.8 (1 H, t), 7.30 (5 H, m), 10.1 (1 H, br s).

***N*-[2-(Methylethyl)-1,4-dioxo-4-[(phenylmethoxy)amino]butyl]-L-tryptophan Methyl Ester (24d).** The crude product, prepared like **23a** but in CH_2Cl_2, was purified by chromatography (EtOAc–hexanes, 50:50–100:0) to afford the following two diastereomers of **24d** as light orange solids (45% and 30% yields): mp 168–170 °C; NMR ($CDCl_3$) δ 0.91 (6 H, m), 1.8–2.5 (4 H, m), 3.26 (2 H, m), 3.68 (3H, s), 4.86 (2H, br s), 4.92 (1 H, m), 6.31 (1 H, d), 7.14 (3 H, m), 7.37 (5 H, s), 7.52 (1 H, d), 8.24 (1 H, br s), 8.74 (1 H, br s). Anal. ($C_{26}H_{31}N_3O_5{\cdot}0.3H_2O$) C, H, N. Second diastereomer: mp 142–144 °C; NMR ($CDCl_3$) δ 0.75 (6 H, m), 1.8–2.5 (4 H, m), 3.28 (2 H, d), 3.69 (3H, s), 4.80 (3 H, br s), 6.52 (1 H, d), 7.14 (3 H, m), 7.37 (5 H, s), 7.53 (1 H, d), 8.57 (1 H, br s), 9.20 (1 H, br s). Anal. ($C_{26}H_{31}N_3O_5$) C, H, N.

***N*-[4-(Hydroxyamino)-2-(methylethyl)-1,4-dioxobutyl]-L-asparagine (25a).** Compound **24a** (470 mg) was hydrolyzed with TFA (4.5 mL) at 0 °C for 1 h and at 20 °C for 1 h. The TFA was evaporated, and the residue was triturated with ether and then lyophilized from water to give the benzyl hydroxamate as a white solid (328 mg, 97% yield): mp 164–167 °C; NMR (CD_3OD) δ 0.93 (6 H, m), 1.82 (1 H, m), 2.28 (1 H, dd), 2.36 (1 H, dd), 2.55 (1 H, m), 2.74 (2 H, m), 4.80 (3 H, m), 7.35 (5 H, m), 8.18 (1 H, t). Anal. ($C_{18}H_{25}N_3O_6{\cdot}0.4H_2O$) C, H, N.

The benzyl hydroxamate (114 mg) in methanol (3 mL) was hydrogenated at 1 atm over 5% Pd/C for 1.25 h. The precipitate was removed, and the solvent was evaporated from the filtrate to give **25a** (85 mg, 98%) as a white solid: mp 102–106 °C; NMR (CD_3OD) δ 0.96 (6 H, m), 1.84 (1 H, m), 2.32 (2 H, m), 2.55 (1 H, m), 2.74 (2 H, m), 4.66 (0.5 H, t), 4.79 (0.5 H, t); FAB MS *m/e* 290 (M + H). Anal. ($C_{11}H_{19}N_3O_6{\cdot}0.3H_2O{\cdot}0.2MeOH$) C, H, N.

***N*-[4-(Hydroxyamino)-2-(methylethyl)-1,4-dioxobutyl]-L-asparaginamide (25b).** A suspension of **24b** (280 mg) and $NaHCO_3$ (30 mg) in methanol (5 mL) and EtOAc (2.5 mL) was hydrogenated at 1 atm over 5% Pd/C for 2 h. The precipitate was removed, and the solvent was evaporated from the filtrate. Chromatography (chloroform–methanol–acetic acid, 80:20:10) gave the product as a hygroscopic white solid: mp 170 °C dec; NMR (D_2O) δ 0.89 (3 H, d), 0.95 (3 H, d), 1.79 (1 H, m), 12.47 (3 H, m), 2.71 (1 H, m), 2.89 (1 H, m); FAB MS *m/e* 289.1500 (M + H), calcd 289.1512. Anal. ($C_{11}H_{20}N_4O_5{\cdot}1.5NaOAc$) C, H, N.

***N*-[4-(Hydroxyamino)-2-(methylethyl)-1,4-dioxobutyl]-*β*-alanine (25c).** Compound **24c** (192 mg) was hydrolyzed with TFA (1.8 mL) at 0 °C for 1 h and at 20 °C for 1 h. The TFA was evaporated, and the residue was triturated with ether to give the benzyl hydroxamate as a white solid (127 mg, 77%): NMR ($CDCl_3$) δ 0.96 (6 H, m), 1.78 (1 H, m), 2.3–2.6 (5 H, m), 3.55 (2 H, m), 4.84 (2 H, s), 7.28 (5 H, s), 7.5 (1 H, br s), 10.8 (1 H, br s).

The benzyl hydroxamate (127 mg) in methanol (1.5 mL) was hydrogenated over 5% Pd/C for 10.5 h to give a 1:1 mixture of **25c** and amide **26c**. Reverse-phase HPLC gave **25c** as a hygroscopic wax: NMR (CD_3OD) δ 0.92 (3 H, d), 0.94 (3 H, d), 1.80 (1 H, m), 2.28 (2 H, m), 2.46 (3 H, m), 3.40 (2 H, m); FAB MS *m/e* 247 (M + H). Anal. ($C_{10}H_{18}N_2O_5{\cdot}0.3TFA$) C, H, N, F.

***N*-[4-(Hydroxyamino)-2-(methylethyl)-1,4-dioxobutyl]-L-tryptophan (25d).** A mixture of diastereomers **24d** (100 mg, 0.215 mmol) was hydrolyzed with NaOH (0.50 mL of 1.0 M) in methanol (0.6 mL) for 20 min and then filtered. The filtrate was acidified with 1 M HCl and extracted into EtOAc to give the benzyl hydroxamate (94 mg, 97% yield). This was hydrogenated as described for **24b**. Chromatography (iPrOH–NH_4OH–H_2O, 90:5:5–80:10:10) gave two diastereomers (26% and 15% yields) as light pink solids: mp 122–126 °C; NMR (CD_3OD) δ 0.87 (3 H, d), 0.91 (3 H, d), 1.80 (1 H, m), 2.23 (2 H, m), 2.51 (1 H, m), 3.18 (1 H, dd), 3.31 (1 H, dd), 4.86 (1 H, dd), 7.00 (2 H, m), 7.14 (1 H, s), 7.29 (1 H, d). Anal. ($C_{18}H_{23}N_3O_5{\cdot}0.75H_2O{\cdot}0.13NH_3$) C, H, N. Second diastereomer: mp 126–129 °C; NMR (CD_3OD) δ 0.62 (3 H, d), 0.70 (3 H, d), 1.64 (1 H, m), 2.24 (2 H, m), 2.42 (1 H, m), 3.10 (1 H, dd), 3.36 (1 H, dd), 4.70 (1 H, dd), 7.01 (2 H, m), 7.10 (1 H, s), 7.28 (1 H, d), 7.62 (1 H, d). Anal. ($C_{18}H_{23}N_3O_5{\cdot}H_2O{\cdot}0.37NH_3$) C, H, N.

***N*-[4-Amino-2-(methylethyl)-1,4-dioxobutyl]-*β*-alanine (26c).** This amide was separated from **25c** by HPLC to give a pink solid: no reaction with $FeCl_3$; mp 160–164 °C; NMR (CD_3OD) δ 0.92 (3 H, d), 0.95 (3 H, d), 1.81 (1 H, m), 2.3–2.6 (5 H, m), 3.41 (2 H, m); FAB MS *m/e* 231.1350 (M + H), calcd 231.1345. Anal. ($C_{10}H_{18}N_2O_4{\cdot}0.43H_2O$) C, H, N.

***N*-[4-Hydroxy-2-(methylethyl)-1,4-dioxobutyl]-L-asparaginamide (27b).** See **23b**.

***N*-[4-Hydroxy-2-(methylethyl)-1,4-dioxobutyl]-*β*-alanine (27c).** Compound **23c** was hydrolyzed according to the procedure used to prepare **6c**. The product **27c** was obtained as a viscous, colorless syrup: NMR (D_2O) δ 0.96 (6 H, 2d), 1.81 (1 H, m), 2.46 (1 H, q), 2.62 (4 H, m), 3.50 (2 H, m). Anal. ($C_{10}H_{17}NO_5{\cdot}0.2HCl$) C, H, N.

***N*-[4-Hydroxy-2-(methylethyl)-1,4-dioxobutyl]-L-tryptophan (27d).** Compound **23d** was hydrolyzed according to the procedure used to prepare **25c**. The product **27d** was obtained as a pink solid: mp 90–105 °C; NMR (CD_3OD) δ 0.68 (2 H, d), 0.92 (4 H, d), 1.84 (1 H, m), 2.40 (1 H, m), 2.54 (2 H, m), 3.24 (2 H, m), 4.79 (1 H, m), 7.03 (3 H, m), 7.30 (1 H, d), 7.59 (1 H, m). Anal. ($C_{18}H_{22}N_2O_5{\cdot}0.8H2O{\text -}0.2MeOH$) C, H, N.

***N*-[4-Methyl-3-[(phenylmethoxy)carbonyl]-3-pentenoyl]-L-asparaginamide (28).** This side product was obtained in the synthesis of **22b**, mp 187–188 °C. A solution of **22b** in DMSO-d_6 at 20 °C slowly rearranged to a mixture of **22b** and **28**, but **22b** was stable under these conditions: NMR (DMSO-d_6) δ 1.83 (3 H, s), 2.25 (3 H, s), 2.58 (1 H, dd), 2.99 (1 H, dd), 3.21 (2 H, br s), 4.49 (2 H, d), 4.92 (1 H, t), 5.14 (1 H, t), 6.74 (1 H, br s), 7.07 (1 H, br s), 7.26 (1 H, m), 7.31 (6 H, m); ^{13}C NMR (DMSO-d_6) δ 20.03 (q), 23.54 (q), 33.86 (t), 33.94 (t), 49.18 (d), 62.83 (t), 119.71 (s), 126.35 (d), 126.54 (d), 127.96 (d), 142.5 (s), 147.29 (s), 168.74 (s), 169.71 (s), 171.46 (s), 173.04 (s); FAB MS *m/e* 362 (M + H). Anal. ($C_{18}H_{23}N_3O_5$) C, H, N.

Isolation and Purification of Human Bronchiolar Smooth Muscle Endothelin-Converting Enzyme (HBSM ECE). Human bronchiolar smooth muscle (HBSM) cells, cultured as described,[28] were utilized as the source of ECE.[8] The harvested centrifuged cells (1.5 g), suspended in physiological saline solution containing 10% sucrose and 0.1% NaN_3, were disrupted with a Parr cell disruption bomb and centrifuged at 370*g*. This process was repeated, and the combined supernatants were centrifuged at 20000*g* for 45 min. The resulting supernatant was centrifuged at 150000*g* for 60 min to afford the cell membrane fraction pellet. The membrane fractions (170 μL, 1.4 mg of protein) were solubilized with 10 mM Tris-HCl (pH 8) buffer containing 1% *n*-octyl *β*-glucopyranoside and 1 mM PMSF (330 μL) and then centrifuged at 19500*g* for 10 min. The supernatant (1.2 mg of protein) was chromatographed on a Pharmacia Mono Q HR 5/5 column eluted with 0–1 M NaCl in 50 mM Tris-HCl and 25 mM *n*-octyl *β*-glucopyranoside. The fractions containing ECE activity (0.044 mg of protein) were chromatographed on a Pharmacia Superose 12 HR 10/30 column eluted with 50 mM (pH 7.2) Na-MOPS (3-(*N*-morpholino)propanesulfonic acid) containing 250 mM NaCl and 25 mM *n*-octyl *β*-glucopyranoside. For assay purposes, MonoQ fractions were usually sufficiently free of contaminating proteases to yield linear production of ET-1 from big ET-1, with minimal degradation of product ET-1.

For characterization, HBSM ECE was further purified using Superose 12, upon which ECE chromatographed in the 400 kDa range. In other experiments, larger quantities of ECE were purified, following Q-Sepharose, at pH 8, using blue-Sepharose (NaCl gradient elution) and heparin-Sepharose (NaCl gradient elution), or ConA-agarose (NaCl and α-methylmannoside elution), followed by the Superose 12 step, yielding 100 ng of enzyme from 10 mg of membrane fraction.

SDS–PAGE under reducing conditions showed bands at 205, 140, and 100 kDa, which, upon electroelution and microsequencing, appeared to be N-terminal blocked.

Under the assay conditions used, HBSM ECE was found to have a K_m for big ET-1 of 12 μM, with k_{cat} = 83 min^{-1} (assumed MW = 100 kDa; V_{max} = 50 000 units/mg; 1 unit = 1 nmol/h of conversion) and optimum activity at pH 7.2. The specific activity was estimated to be ~10 000 units/mg. HBSM ECE does not cleave big ET-3 (<10% conversion under conditions which cleave big ET-1). It requires calcium cation (>10 nM) and is completely dependent on chloride, having no activity in the absence of NaCl and maximal activity at 150–200 mM NaCl. Activation was not achieved by NaOAc. Less than 20% of the activity could be immunoprecipitated by mouse anti-human CALLA (NEP) monoclonal antibody (A. J. Turner, personal communication).

Inhibition of Human Bronchiolar Smooth Muscle Endothelin-Converting Enzyme (HBSM ECE), Neutral Endopeptidase (NEP), and Thermolysin. ECE assays were conducted at 37 °C for 4–5 h with 3 μM hBET-1 (human big ET-1) in 50 mM (pH 7.2) Na-MOPS, 150 mM NaCl, 2.5 mM *n*-octyl β-glucopyranoside, 30 μM $CaCl_2$, 1 μM amastatin, and 1 mM phenylmethanesulfonyl fluoride. In 250 μL total volume, HBSM ECE (sufficient to produce 2–5 of pmol ET-1/h) was preincubated with the inhibitor (dissolved in 1 μL of DMSO) for 30 min at 37 °C, prior to the start of the assay. hBET-1 was added to a final concentration of 3 μM to begin the reaction. The reaction was terminated by addition of Na-EDTA, pH 7.0, to 3 mM and placed on ice. PCMS (*p*-(chloromercuri)benzenesulfonic acid), used by others to inactivate contaminating thiol proteases, was not utilized as it inhibits HBSM ECE 88% at 1 μM and 25% at 0.1 μM.

Samples of 200 μL were analyzed by reverse-phase HPLC on a Vydac 218TP5405 column (4.6 × 50 mm, 5 μm, 300 Å pore size, C_{18}). Elution was performed at 30 °C and 1.5 mL/min with a gradient of 50 mM (pH 6.5) sodium acetate: acetonitrile (90:10–75:25 over 1.5 min, 75:25 for 2 min, 75:25–50:50 over 2.5 min). ET-1 and hBET-1 were quantified by fluorescence (excitation = 225 nm, emission = 340 nm), with reference to authentic ET-1 and hBET-1. IC_{50} calculations were made by least-squares curve fit. Except where noted otherwise, each IC_{50} in Table 1 was determined in at least two independent experiments with duplicate measurements at each of five inhibitor concentrations bracketing the IC_{50}.

The hydroxamate targets were also tested as inhibitors of a soluble form of rabbit kidney neutral endopeptidase (NEP, EC 3.4.24.11; obtained from Dr. Philippe Crine[25]) under the same conditions employed for ECE. They were also tested as inhibitors of thermolysin (TLN, EC 3.4.24.4; Sigma) with 0.5 μM [3,5-^{3}H-Tyr^1][Leu^5]enkephalin (NEN) as substrate. These assays were conducted at 20 °C for 10 min in 50 mM Tris-Cl (pH 7.5) buffer. The enzyme was preincubated with inhibitor for 15 min, prior to the start of the assay.

Acknowledgment. We are grateful to Victor Ilozue, Lori Bolognese, and Ray Myers for skillful assistance in enzyme assays and to Lynne H. Parker-Botelho for stimulating discussions. Human bronchiolar smooth muscle cells were a kind gift of Charles Twort.

References

(1) Yanagisawa, M.; Kurihara, H.; Kimura, S.; Tomobe, Y.; Kobayashi, M.; Mitsui, Y.; Yazaki, Y.; Goto, K.; Masaki, T. A Novel Potent Vasoconstrictor Peptide Produced by Vascular Endothelial Cells. *Nature* **1988**, *332*, 411–415.

(2) Doherty, A. M. Endothelin: A New Challenge. *J. Med. Chem.* **1992**, *35*, 1495–1508. Underwood, R. D.; Chan, D. P.; Burnett, J. C. Endothelin: An Endothelium-Derived Vasoconstrictor Peptide and Its Role in Congestive Heart Failure. *Heart Failure* **1991**, *April/May, 50–58*. Yanagisawa, M.; Masaki, T. Molecular Biology and Biochemistry of the Endothelins. *TIPS* **1989**, *10*, 374–378. Leppaluoto, J.; Ruskoaho, H. Endothelin Peptides: Biological Activities, Cellular Signalling and Clinical Significance. *Ann. Med.* **1992**, *24*, 153–161. Masaki, T.; Kimura, S.; Yanagisawa, M.; Goto, K. Molecular and Cellular Mechanism of Endothelin Regulation. *Circulation* **1991**, *84*, 1457–1468. Shiosaki, K.; Opgenorth, T. J. Potential Therapeutic Utility of ECE Inhibitors and ET Receptor Antagonists. *Drug News Perspect.* **1994**, *10*, 593–602.

(3) Emori, T.; Hirata, Y.; Ohta, K.; Shichiri, M.; Shimokado, K.; Marumo, F. Concomitant Secretion of Big ET and its C-Terminal Fragment from Human and Bovine Endothelial Cells. *Biochem. Biophys. Res. Commun.* **1989**, *162*, 217–223. Sawamura, T.; Kimura, S.; Shinmi, O.; Sugita, Y.; Yanagisawa, M.; Masaki, T. Analysis of Endothelin Related Peptides in Culture Supernatant of Porcine Aortic Endothelial Cells: Evidence for Biosynthetic Pathway of Endothelin-1. *Biochem. Biophys. Res. Commun.* **1989**, *162*, 1287–1294. Hexum, T. D.; Hoeger, C.; Rivier, J. E.; Baird, A.; Brown, M. R. Characterization of Endothelin Secretion by Vascular Endothelial Cells. *Biochem. Biophys. Res. Commun.* **1990**, *167*, 294–300. Saijonmaa, O.; Nyman, T.; Hohenthal, U.; Fyhrquist, F. Endothelin-1 is Expressed and Released by a Human Endothelial Hybrid Cell Line (EA.hy 926). *Biochem. Biophys. Res. Commun.* **1991**, *181*, 529–536. Pacher, R.; Berger-Klein, J.; Globits, S.; Teufelsbauer, H.; Schuller, M.; Krauter, A.; Ogris, E.; Rodler, S.; Wutte, M.; Hartter, E. Plasma Big Endothelin-1 Concentrations in Congestive Heart Failure Patients. *Am. J. Cardiol.* **1993**, *71*, 1293–1299.

(4) For reviews, see: Opgenorth, T. J.; Wu-Wong, J. R.; Shiosaki, K. Endothelin-Converting Enzymes *FASEB J.* **1992**, *6*, 2653–2659. Turner, A. J. Endothelin-Converting Enzymes and Other Families of Metallo-Endopeptidases. *Biochem. Soc. Trans.* **1993**, *21*, 697–701.

(5) Ohnaka, K.; Takayanagi, R.; Yamauchi, T.; Okazaki, H.; Ohashi, M.; Umeda, F.; Nawata, H. Identification and Characterization of Endothelin Converting Activity in Cultured Bovine Endothelial Cells. *Biochem. Biophys. Res. Commun.* **1990**, *168*, 1128–1136. Okada, K.; Miyazaki, Y.; Takada, J.; Matsuyama, K.; Yamaki, T.; Yano, M. Conversion of Big Endothelin-1 by Membrane-Bound Metallopeptidase in Cultured Bovine Endothelial Cells. *Biochem. Biophys. Res. Commun.* **1990**, *171*, 1192–1198. Matsumura, Y.; Ikegawa, R.; Tsukahara, Y.; Takaoka, M.; Morimoto, S. Conversion of Big Endothelin-1 to Endothelin-1 by Two Types of Metalloproteinases of Cultured Porcine Vascular Smooth Muscle Cells. *Biochem. Biophys. Res. Commun.* **1991**, *178*, 899–905. Sessa, W. C.; Kaw, S.; Hecker, M.; Vane, J. R. *Biochem. Biophys. Res. Commun.* **1991**, *174*, 613–618. Devine, E. M.; Marselle, C. A.; Pederson, T. M.; Wu-Wong, J. R.; Budzik, G. P.; Dillon, T. P.; Opgenorth, T. J. Identification of a Neutral Metalloendopeptidase Endothelin Converting Enzyme from Various Cultured Endothelial Cell Lines. *FASEB J.* **1991**, *5*, A1417. Shields, P. P.; Gonzales, T. A.; Charles, D.; Gilligan, J. P.; Stern, W. Accumulation Of Pepstatin In Cultured Endothelial Cells And Its Effect On Endothelin Processing. *Biochem. Biophys. Res. Commun.* **1991**, *177*, 1006–1012. Ahn, K.; Beningo, K.; Olds, G.; Hupe, D. The Endothelin-Converting Enzyme from Human Umbilical Vein is a Membrane-Bound Metalloprotease Similar to that from Bovine Aortic Endothelial Cells. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 8606–8610. Sawamura, T.; Shinmi, O.; Kishi, N.; Sugita, Y.; Yanagisawa, M.; Goto, K.; Masaki, T.; Kimura, S. Characterization of Phosphoramidon-sensitive Metalloproteinases with Endothelin-Converting Enzyme Activity in Porcine Lung Membrane. *Biochim. Biophys. Acta* **1993**, *1161*, 295–302. Bertenshaw, S. R.; Rogers, R. S.; Stern, M. K.; Norman, B. H.; Moore, W. M.; Jerome, G. M.; Branson, L. M.; McDonald, J. F.; McMahon, E. G.; Palomo, M. A. Phosphorus-Containing Inhibitors of Endothelin Converting Enzyme: Effects of the Electronic Nature of Phosphorus on Inhibitor Potency. *J. Med. Chem.* **1993**, *36*, 173–176. Takahashi, M.; Matsushita, Y.; Iijima, Y.; Tanzawa, K. Purification and Characterization of Endothelin-Converting Enzyme from Rat Lung. *J. Biol. Chem.* **1993**, *268*, 21394–21398. Fukami, T.; Hayama, T.; Amano, Y.; Nakamura, Y.; Arai, Y.; Matsuyama, K.; Yano, M.; Ishikawa, K. Aminophosphonate Endothelin Converting Enzyme Inhibitors: Potency-Enhancing and Selectivity-Improving Modifications of Phosphoramidon. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 1257–1262. Shima, H.; Kawashima, Y.; Ohmori, K.; Sugiura, M.; Kawashima, K. Endothelin Converting Enzymes in Guinea-Pig Lung Membrane Fractions: Purifications and Characterizations. *Biochem. Mol. Biol. Int.* **1994**, *34*, 1227–1234. Balwierczak, J. L.; Kukkola, P. J.; Savage, P.; Jeng, A. Y. Effects of Metalloprotease Inhibitors on Smooth Muscle Endothelin-Converting Enzyme Activity. *Biochem. Pharmacol.* **1995**, *49*, 291–296.

(6) Ikegawa, R.; Matsumura, Y.; Tsukahara, Y.; Takaoka, M.; Morimoto, S. Phosphoramidon, a Metalloproteinase Inhibitor, Suppresses the Secretion of Endothelin-1 from Cultured Endothelin Cells by Inhibiting Big-Endothelin-1 Converting Enzyme. *Biochem. Biophys. Res. Commun.* **1990**, *171*, 669–675. Okada, K.; Miyazaki, Y.; Takada, J.; Matsuyama, K.; Yamaki, T.; Yano, M. Conversion of Big-Endothelin 1 by Membrane-Bound Metalloendopeptidase in Cultured Bovine Endothelial Cells. *Biochem. Biophys. Res. Commun.* **1990**, *171*, 1192–1198. McMahon, E. G.; Palomo, M. A.; Moore, W. M.; McDonald, J. F.; Stern, M. K. Phosphoramidon Blocks The Pressor Activity of Porcine Big

Endothelin-1-(1–39) In Vivo and Conversion of Big Endothelin-1-(1–39) to Endothelin-1-(1–21) In Vitro. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 703–707. Hioki, Y.; Okada, K.; Ito, H.; Matsuyama, K.; Yano, M. Endothelin Converting Enzyme of Bovine Carotid Artery Smooth Muscles. *Biochem. Biophys. Res. Commun.* **1991**, *174*, 446–451.

(7) Shimada, K.; Takahashi, M.; Tanzawa, K. Cloning and Functional Expression of Endothelin-converting Enzyme from Rat Endothelial Cells. *J. Biol. Chem.* **1994**, *269*, 18275–18278. Xu, D.; Emoto, N.; Giaid, A.; Slaughter, C.; Kaw, S.; deWit, D.; Yanagisawa, M. ECE-1: A Membrane-Bound Metalloprotease that Catalyzes the Proteolytic Activation of Big Endothelin-1. *Cell* **1994**, *78*, 473–485. Ikura, T.; Sawamura, T.; Shiraki, T.; Hosokawa, H.; Kido, T.; Hoshikawa, H.; Shimada, K.; Tanzawa, K.; Kobayashi, S. cDNA Cloning and Expression of Bovine Endothelin Converting Enzyme. *Biochem. Biophys. Res. Commun.* **1994**, *203*, 1417–1422. Schmidt, M.; Kröger, B.; Jacob, E.; Seulberger, H.; Subkowski, T.; Otter, R.; Meyer, T.; Schmalzing, G.; Hillen, H. Molecular Characterization of Human and Bovine Endothelin Converting Enzyme (ECE-1). *FEBS Lett.* **1994**, *356*, 238. Shimada, K.; Matsushita, Y.; Wakabayashi, K.; Takahashi, M.; Matsubara, A.; Iijima, Y.; Tanzawa, K. Cloning and Functional Expression of Human Endothelin-Converting Enzyme cDNA. *Biochem. Biophys. Res. Commun.* **1995**, *207*, 807–812. Yorimitsu, K.; Moroi, K.; Inagaki, N.; Saito, T.; Masuda, Y.; Masaki, T.; Seino, S.; Kimura, S. Cloning And Sequencing of a Human Endothelin Converting Enzyme in Renal Adenocarcinoma (ACHN) Cells Producing Endothelin-2. *Biochem. Biophys. Res. Commun.* **1995**, *208*, 721–727.

(8) Parker Botelho, L. H.; Garrigan, M. C.; Johns, A.; Levinson, B. L.; Patterson, K. C.; Polokoff, M. A. Endothelin Converting Enzyme. World Patent Application 92/13944, 1992.

(9) Bertenshaw, S. R.; Talley, J. R.; Rogers, R. S.; Carter, J. S.; Moore, W. M.; Branson, L. M.; Koboldt, C. M. Thiol and Hydroxamic Acid Containing Inhibitors of Endothelin Converting Enzyme. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 1953–1958.

(10) For a review of hydroxamic acid proteinase inhibitors, see: Powers, J. C.; Harper, J. W. Inhibitors of Metalloproteases. In *Proteinase Inhibitors*; Barrett, A. J., Salvesen, G., Eds.; Elsevier: New York, 1986; pp 244–253.

(11) Gordon, J. J.; Devlin, J. P.; East, A. J.; Ollis, W. D.; Sutherland, I. O.; Wright, D. E.; Ninet, L. Studies Concerning the Antibiotic Actonin. Part I. Constitution of Actinonin. A Natural Hydroxamic Acid with Antibiotic Activity. *J. Chem. Soc. Perkin Trans. I* **1975**, 819–825. Umezawa, H.; Aoyagi, T.; Tanaka, T.; Suda, H.; Okuyama, A.; Naganawa, H.; Hamada, M.; Takeuchi, T. Production of Actinonin, an Inhibitor of Aminopeptidase M, by Actinomycetes. *J. Antibiot.* **1985**, *38*, 1629–1630.

(12) Lelievre, Y.; Bouboutou, R.; Boiziau, J.; Cartwright, T. Inhibition of Synovial Collagenase by Actinonin. Study of Structure/Activity Relationship. *Pathol. Biol.* **1989**, *37*, 43–46. Inaoka, Y.; Takahashi, S.; Kinoshita, T. Propioxatins A and B, New Enkephalinase B Inhibitors II. Structural Elucidation. *J. Antibiot.* **1986**, *39*, 1378–1381. Sato, T.; Shibazaki, M.; Morioka, M.; Suzuki, K.; Takebayashi, Y. YL-01869P Substance and Production Thereof. Jap. Patent Application 3157, 372, 1989. Tamaki, K.; Ogita, T.; Tanzawa, K.; Sugimura, Y. Synthesis and Determination of the Absolute Configuration of Matlystatin B. *Tetrahedron Lett.* **1993**, *34*, 683–686.

(13) Devlin, J. P.; Ollis, W. D.; Thorpe, J. E.; Wright, D. E. Studies Concerning the Antibiotic Actinonin. Part VI. Synthesis of Structural Analogues of Actinonin by DCC Coupling Reactions. *J. Chem. Soc. Perkin Trans. I* **1975**, 848–851, and preceeding papers in series. Nishino, N.; Powers, J. C. Peptide Hydroxamic Acids as Inhibitors of Thermolysin. *Biochemistry* **1978**, *17*, 2846. Ondetti, M. A.; Rubin, B.; Cushman, D. W. Design of Specific Inhibitors of Angiotensin-Converting Enzyme: New Class of Orally Active Antihypertensive Agents. *Science* **1977**, *196*, 441–444. Nishino, N.; Powers, J. C. Pseudomonas aeruginosa Elastase. Development of a New Substrate, Inhibitors, and an Affinity Ligand. *J. Biol. Chem.* **1980**, *255*, 3482–3486.

(14) Umezawa, H.; Aoyagi, T.; Ogawa, K.; Obata, T.; Iinuma, H.; Naganawa, H.; Hamada, M.; Takeuchi, T. Foroxymithine, a New Inhibitor of Angiotensin-Converting Enzyme Produced by Actinomycetes. *J. Antibiot.* **1985**, *38*, 1813–1815.

(15) Bihovsky, R. H.; Erhardt, P. W.; Lampe, J. W.; Mohan, R.; Shaw, K. J. Inhibitors of the Conversion of Big Endothelin to Endothelin. World Patent Application 93/11154, 1993.

(16) Gomez-Monterrey, I.; Turcaud, S.; Lucas, E.; Bruetschy, L.; Roques, B. P.; Fournie-Zaluski, M.-C. Exploration of Neutral Endopeptidase Active Site by a Series of New Thiol-Containing Inhibitors. *J. Med. Chem.* **1993**, *36*, 87–94. Sybertz, E. J. Drugs Inhibiting the Metabolism and Inactivation of ANF: Pharmacological Actions and Therapeutic Implications. *Cardiovasc. Drug Rev.* **1990**, *8*, 71–82. Roques, B. P.; Beumont, A. Neutral Endopeptidase-24.11 Inhibitors: From Analgesics to Antihypertensives. *TIPS* **1990**, *11*, 245–249.

(17) Cherot, P.; Devin, J.; Fournie-Zaluski, M. C.; Roques, B. P. Enkephalin-Degrading Dipeptidylaminopeptidase: Characterization of the Active Site and Selective Inhibition. *Mol. Pharmacol.* **1986**, *30*, 338–344. Fournie-Zaluski, M.-C.; Coulaud, A.; Bouboutou, R.; Chaillet, P.; Devin, J.; Waksman, G.; Costentin, J.; Roques, B. P. New Bidentates as Full Inhibitors of Enkephalin-Degrading Enzymes: Synthesis and Analgesic Properties. *J. Med. Chem.* **1985**, *28*, 1158–1168. Nishino, N.; Powers, J. C. Peptide Hydroxamic Acids as Inhibitors of Thermolysin. *Biochemistry* **1978**, *17*, 2846–2850.

(18) Khorana, H. G. The Chemistry of Carbodiimides. *Chem. Rev.* **1953**, *53*, 145–165. Merrifield, R. B.; Gisin, B. F.; Bach, A. N. The Limits of Reaction of Radioactive DCC with Amino Groups During Solid-Phase Peptide Synthesis. *J. Org. Chem.* **1977**, *42*, 1291–1295. Lerch, U.; Moffatt, J. G. Carbodiimide-Sulfoxide Reactions XIII. Reactions of Amines and Hydrazine Derivatives. *J. Org. Chem.* **1971**, *36*, 3861–3869. Schumann, E. L. Aralkoxyguanidines, Aryloxyalkoxyguanidines, and Salts Thereof in Anorectic Methods and Compositions. U.S. Patent 3, 456, 058, 1969.

(19) Gipson, R. M.; Pettit, F. H.; Skinner, C. G.; Shive, W. Catalytic Hydrogenolysis of Hydroxamic Acids to Amides *J. Org. Chem.* **1963**, *28*, 1425–1426. Masaki, M.; Ohtake, J.; Sugiyama, M.; Ohta, M. The Selective Debenzylation of *N*-Acyl-*O*-Benzylhydroxylamines. *Bull. Chem. Soc. Jpn.* **1965**, *38*, 1802. Fuji, T.; Hatanaka, Y. A Synthesis of Rhodotorulic Acid. *Tetrahedron* **1973**, *29*, 3825–3831. Nikam, S. S.; Kornberg, B. E.; Johnson, D. R.; Doherty, A. M. Synthesis of Hydroxamic Acids: Pd/$BaSO_4$ as a New Catalyst for the Deprotection of O-Benzyl Hydroxamates. *Tetrahedron Lett.* **1995**, *36*, 197–200. Hydrogenation in the presence of pyridine or transfer hydrogenation did not suppress amide formation in our hands.

(20) Handa, B. K.; Johnson, W. H.; Machin, P. J. Hydroxylamine Derivatives. European Patent Application 236, 872, 1987. Xie, J.; Soleilhac, J.-M.; Schmidt, C.; Peyroux, J.; Roques, B. P.; Fournie-Zaluski, M.-C. New Kelatorphan-Related Inhibitors of Enkephalin Metabolism: Improved Antinociceptive Properties. *J. Med. Chem.* **1989**, *32*, 1497–1503. Cartwright, T.; Bouboutou-Tello, R.; Lelievre, Y.; Fournie-Zaluski, M.-C. Novel Compounds with Collagenase-Inhibiting Activity U.S. Patent 4, 918, 105, 1990.

(21) Stobbe, H. Bemerkung zu die Darstellung der Teraconsäure. (Comments about the Preparation of Teraconic Acid.) *Chem. Ber.* **1903**, *36*, 197–199. Overberger, C. G.; Roberts, C. W. The Preparation of 2-Alkyl-1,4-butanediols. *J. Am. Chem. Soc.* **1949**, *71*, 3618–3621. Kofron, W. G.; Wideman, L. G. Specific Synthesis And Selective Alkylation And Condensation of Monoesters of Substituted Succinic Acids. *J. Org. Chem.* **1972**, *37*, 555–559. Almquist, R. G.; De Graw, J. I. Oxoalkanoic Acid Derivatives as Inhibitors of Angiotensin Converting Enzyme. U.S. Patent 4, 329, 473, 1982. Hancock, J. E. H.; Linstead, R. P. Positional Isomerides Derived From Methylsuccinic Acid; A Note on the Rearrangement of Unsymmetrical Compounds of This Type. *J. Chem. Soc.* **1953**, 3490–3496.

(22) Groutas, W. C.; Brubaker, M. J.; Stanga, M. A.; Castrisos, J. C.; Crowley, J. P.; Schatz, E. J. Inhibition of Human Leukocyte Elastase by Derivatives of N-Hydroxysuccinimide. *J. Med. Chem.* **1989**, *32*, 1607–1611.

(23) Successful application of this approach had previously been reported; see Shaw, A.; Wolanin, D. J. Hydroxamic Acids. Eur. Patent Application 231, 081, 1987. Inaoka, Y.; Takahashi, S.; Sato, S.; Propioxatins A and B, New Enkephalinase Inhibitors III. Total Synthesis of Propioxatin A. *J. Antibiot.* **1986**, *39*, 1382–1385.

(24) Roeske, R. Preparation of *tert*-butyl Esters of Free Amino Acids *J. Org. Chem.* **1963**, *28*, 1251–1253.

(25) Lemay, G.; Waksman, G.; Roques, B. P.; Crine, P.; Boileau, G. Fusion of a Cleavable Signal Peptide to the Ectodomain of Neutral Endopeptidase (E.C. 3.4.24.11) Results in the Secretion of an Active Enzyme in COS-1 Cells. *J. Biol. Chem.* **1989**, *264*, 15620–15623.

(26) Fukuroda, T.; Noguchi, K.; Tsuchida, S.; Nishikibe, M.; Ikemoto, F.; Okada, K.; Yano, M. Inhibition of Biological Actions of Big Endothelin-1 by Phosphoramidon. *Biochem. Biophys. Res. Commun.* **1990**, *172*, 390–395.

(27) Jackson, D. G.; Hersh, L. B. Reaction of Neutral Endopeptidase 24.11 (Enkephalinase) with Arginine Reagents. *J. Biol. Chem.* **1986**, *261*, 8649–8654.

(28) Twort, C.; Van Breemen, C. Human Airway Smooth Muscle in Culture. *Tissue Cell* **1988**, *20*, 339–344.

JM940567O