Potent and Selective Inhibitors of an Aspartyl Protease-like Endothelin Converting Enzyme Identified in Rat Lung

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Two structurally distinct series of potent and selective inhibitors of an aspartyl protease-like endothelin converting enzyme (ECE) activity identified in the rat lung have been developed. Pepstatin A, which potently inhibits the rat lung ECE, served as the basis for the first series. Alternatively, selected renin inhibitors containing the dihydroxyethylene moiety were shown to be inhibitors of rat lung activity. Subsequent modifications improved inhibition of the rat lung ECE while eliminating renin activity. Both series of ECE inhibitors demonstrated a range of selectivity over Cathepsin D. Water-solubilizing moieties were appended onto selected compounds to facilitate in vivo testing. Partial reduction of the pressor response to exogenously administered Big ET-1 was observed with selected rat lung ECE inhibitors.

In recent years, the vascular endothelium has been investigated extensively for its ability to secrete both relaxing and constricting factors that operate in concert with other vasoactive substances to regulate smooth muscle tone. Endothelium-derived relaxing factor (EDRF) is an example of the former that is released by endothelial cells in response to various physical and chemical stimuli to affect vasodilation.[1] Endothelin (ET), a 21-amino acid bicyclic peptide isolated and characterized from cultured porcine endothelial cells by Yanagisawa and colleagues, is being studied as a possible endothelium-derived constricting factor (EDCF).[2] Endothelin produces a potent and long-lasting arterial constriction in vitro and elevation of arterial pressure in vivo. Three isoforms of endothelin, ET-1, ET-2, and ET-3, each a distinct gene product encoded in the human genome, have been identified.[3] ET-1 (2) appears to be the prevalent isoform circulating in man and has been the primary focus of pharmacological investigations reported to date.

Yanagisawa and co-workers also proposed a biosynthetic pathway for the production of ET based on c-DNA sequencing of the cloned gene.[2] Preproendothelin, the 203-amino acid precursor, is initially cleaved adjacent to pairs of basic residues by conventional hormone processing enzymes to form a 38-amino acid peptide prohormone, Big ET (1). In the final step, Big ET is cleaved between the Trp^{21}-Val^{22} bond to release the mature peptide 2 (Figure 1). The unusual cleavage site in Big ET suggests that a novel endothelin converting enzyme (ECE) is involved in the synthesis of the vasoconstricting peptide. The physiological relevance of this cleavage is suggested by the greater in vitro potency (>100-fold) of ET over Big ET. In addition, the substrate, Big ET, and its cleavage products, ET and the C-terminal fragment, have all been detected in the supernatant of cultured endothelial cells to further support the validity of the proposed biosynthetic pathway.[4]

A number of studies have implicated ET in various pathophysiological conditions. Elevated ET levels were detected in animal models of acute renal failure,[5] acute pulmonary hypoxia,[6] and cyclosporin-induced nephrotoxicity.[7] In man, increased plasma levels of ET were measured following myocardial infarction[8] and in patients with congestive heart failure[9] and asthma.[10] Endothelin

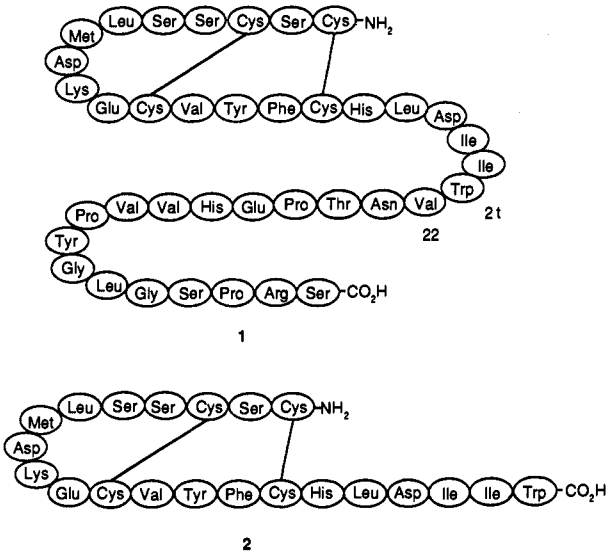

Figure 1. Big Endothelin-1 (Big ET-1) (1) and Endothelin-1 (ET-1) (2).

has been shown to possess mitogenic properties[11] and its levels were significantly elevated in human breast cancer tissue.[12] These findings have sparked considerable speculation surrounding the possible involvement of ET in these as well as other disease states. Preventing the potential pathological actions of ET through obstruction of its biosynthesis, i.e. ECE inhibition, has emerged as an attractive new area for the potential development of novel therapeutics.

A number of enzyme activities have been reported that generate ET from Big ET. Several laboratories[13] have reported similarly on a membrane-bound, neutral pH-active enzyme activity derived from various tissues and endothelial cells that is inhibited, albeit with modest potency (IC_{50} ~1-10 μM), by phosphoramidon (*N*-[(α-L-rhamnopyranosyloxy)hydroxyphosphinyl]-L-leucyl-L-tryptophan), a potent inhibitor of neutral endopeptidase (NEP; EC 3.4.24.11). The activity also is inhibited by metal-chelating agents such as EDTA and o-phenanthroline, suggesting this ECE to be a metal-dependent enzyme. Other metalloprotease inhibitors such as thiorphan, another potent NEP inhibitor, and the angiotensin converting enzyme inhibitors, e.g. Captopril, are inactive or very weak

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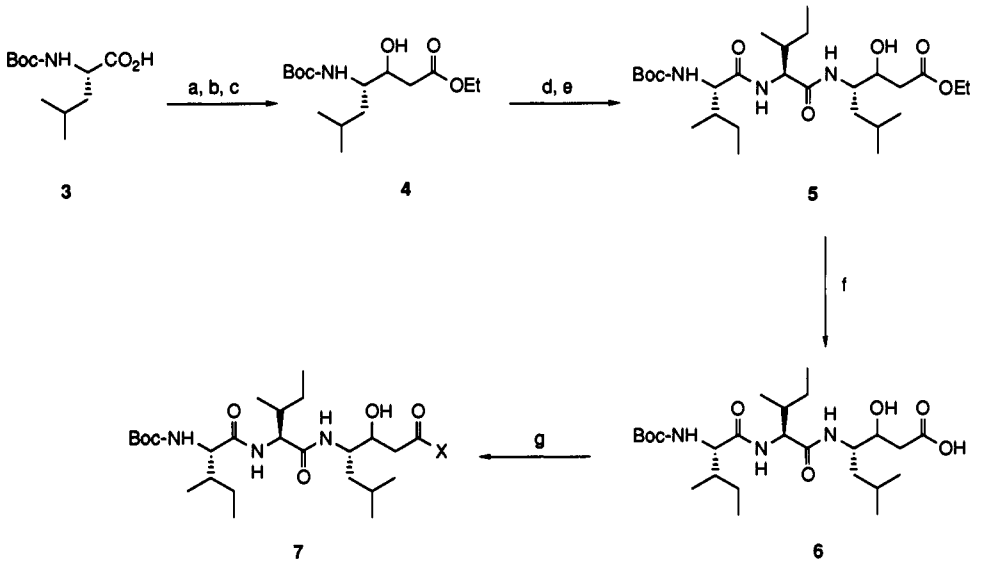
Scheme I [a]



[a] Reagents: (a) 3,5-dimethylpyrazole, HOBt, EDCI, CH_2Cl_2; (b) $LiAlH_4$, THF, −78 °C; (c) $LiCH_2CO_2Et$, THF, −78 °C; (d) HCl(g)-dioxane; (e) Boc-Ile-Ile-OH, IBCF, NMM, CH_2Cl_2, −20 °C; (f) NaOH, MeOH, H_2O; (g) IBCF, NMM, HNR_2.

inhibitors of this ECE activity. To support its role as physiologically relevant ECE, phosphoramidon, but not thiorphan or Captopril, was shown to abolish the systemic and regional hemodynamic effects of exogenously administered Big ET in rats.[14] In addition, phosphoramidon inhibits the release of ET from cultured endothelial cells and affects the relative ratios of ET to Big ET in a manner consistent with the inhibition of its synthesis.[15] Curiously, no reports have emerged to date describing compounds that are more potent or selective inhibitors of this ECE activity than phosphoramidon.

A number of ECE's that possess maximal activity at lower pH and sensitivity to Pepstatin-A have also been reported. Several groups[16] have reported on ECE activities found in cultured endothelial cells and in bovine adrenal medulla that resemble the aspartyl protease Cathepsin D, based on similarity in size and reactivity with a Cathepsin D antibody. However, the likelihood of Cathepsin D as a physiologically relevant ECE is diminished by its rapid degradation of ET-1. Another aspartyl protease, Cathepsin E, has also been proposed as an ECE based on its ability to specifically cleave Big ET-1 to yield ET-1 without subsequent degradation of the product.[17]

We have identified an enzyme activity from plasma membrane fractions prepared from rat lung that specifically cleaves Big ET-1 at the Trp^{21}-Val^{22} bond.[18] This activity appears to be similar to yet distinct from the other aspartyl-like activities that have been reported. The activity of this rat lung enzyme is optimal at pH 4.0 and is potently inhibited by Pepstatin-A with an IC_{50} of 20 nM. Inhibitors of the other enzyme classes, e.g. phosphoramidon, thiorphan, E-64, PMSF, aprotinin, and TLCK, did not inhibit the conversion of Big ET in this assay. This rat lung activity preferentially cleaves Big ET-1 over Big ET-3. Partial purification of this rat lung enzyme has been achieved and the activity appears to reside in a single band of apparent molecular weight of 90 kDa by SDS-PAGE.

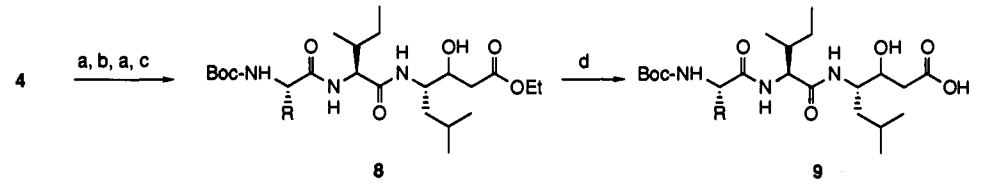
In order to determine whether this converting activity identified in the rat lung is indeed a biologically relevant enzyme responsible for the generation of endogenous ET-1, potent and selective inhibitors are required along with their subsequent evaluation in appropriate pharmacological models. We have developed two structurally distinct series of inhibitors that potently and selectively inhibit this rat lung activity in vitro. Pepstatin-A served as the basis for the first series, utilizing statine, the key inhibitory moiety, as the structural core onto which various residues were appended to impart potency and selectivity for the rat lung enzyme. A second, structurally distinct lead structure emerged as a result of screening existing in-house aspartyl protease inhibitors. Several renin inhibitors possessing a common dihydroxyethylene fragment were identified that possessed modest inhibition of the rat lung enzyme and were subsequently modified to improve potency for ECE while eliminating renin activity. In this paper, we describe the development of these two lead structures into potent inhibitors of the rat lung activity that are selective over other biologically relevant aspartyl proteases such as Cathepsin D and renin. In addition, water solubility was introduced into selected compounds in order to facilitate their testing in various pharmacological models.

Methods

Chemistry. The protected (3*RS*)-statine **4** was synthesized from Boc-Leu (**3**) by modification of a procedure described by Rich[19] and initially elaborated into the final compounds as a mixture (~1:2, *R*/*S*) of diastereomers. Removal of the Boc-protecting group of **4** and addition of the two isoleucine residues under standard conditions yielded tripeptide **5** as shown in Scheme I. Amino acid couplings were conducted routinely using either 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDCI) or isobutyl chloroformate (IBCF) and *N*-methylmorpholine (NMM). Removal of the Boc-protecting group was accomplished with anhydrous HCl in dioxane. Base hydrolysis of **5** to the acid **6** and its subsequent conversion to various amides via the mixed anhydride (IBCF-NMM) yielded the series of statine-based inhibitors **7** modified at the C-terminus. A series of statine-based tripeptide esters **8** and acids **9** containing amino acid modifications at the P_3 residue were prepared from **4** using the standard methodology outlined in Scheme II.

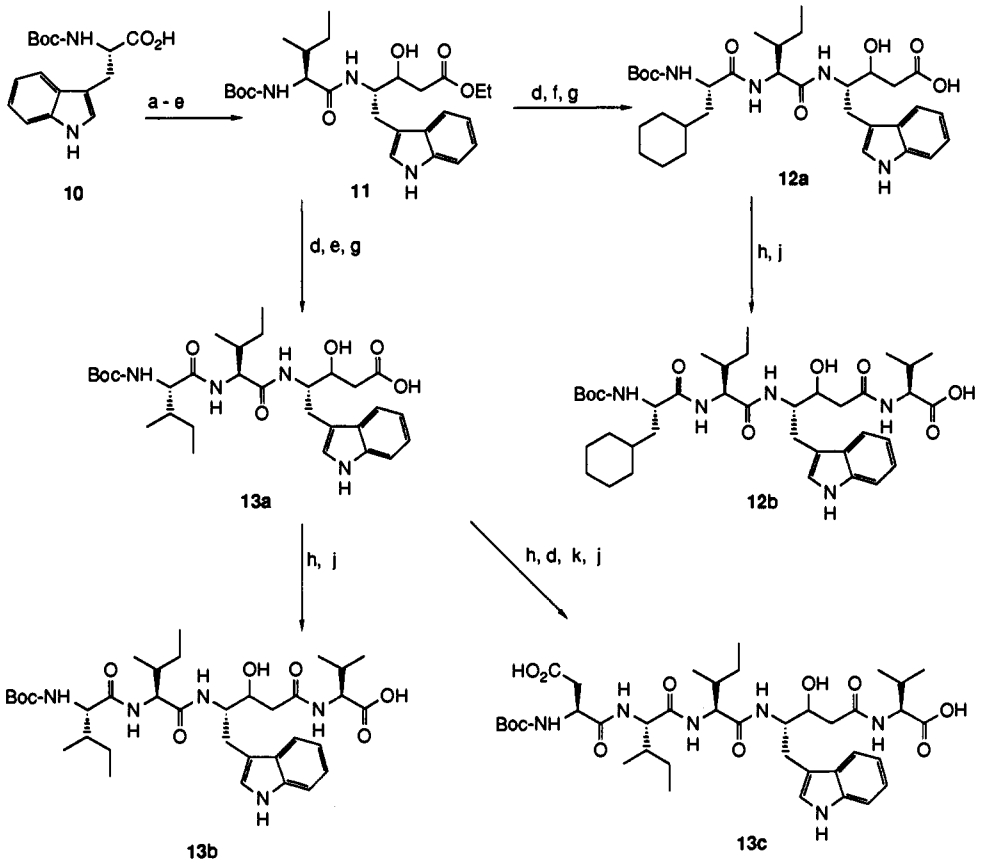
The statine derivative containing the indol-3-yl group in the side chain was synthesized from Boc-Trp **10** in an

Scheme II [a]



[a] Reagents: (a) HCl(g), dioxane; (b) Boc-Ile-OH, IBCF, NMM, CH_2Cl_2, −20 °C; (c) Boc-NH-CH(R)CO_2H, EDCI, HOBt, DMF; (d) NaOH, MeOH.

Scheme III [a]



[a] Reagents: (a) 3,5-dimethylpyrazole, HOBt, EDCI, CH_2Cl_2; (b) $LiAlH_4$, THF, −78 °C; (c) $LiCH_2CO_2Et$, THF, −78 °C; (d) HCl(g)-dioxane; (e) Boc-Ile-OH, IBCF, NMM, CH_2Cl_2, −20 °C; (f) Boc-Cha-OH, IBCF, NMM, −20 °C; (g) NaOH, MeOH, H_2O; (h) Val-OBn, EDCI, HOBt, DMF; (j) H_2/Pd(C), AcOH; (k) Boc-Asp(β-OBn)-OH, IBCF, NMM, −20 °C.

analogous manner as **4**. The indolylstatine derivative was also prepared as an epimeric mixture (~1:2, *R*/*S*) at the 3-hydroxyl position. The N-terminus was extended in a step-wise manner using standard methods as shown in Scheme III to yield tripeptides **12a** and **13a**. Coupling valine to the C-terminus produced the tetrapeptides **12b** and **13b** and further extension of **13a** to the pentapeptide **13c** was achieved using standard deprotection and coupling reactions.

The (3*S*)- and (3*R*)-statines were chromatographically separated from mixture **4** and elaborated into the diastereomerically pure tripeptides **15** and **16** according to the standard methods described in Scheme IV.

The dihydroxyethylene derivatives **18** were prepared from the corresponding Boc-protected amino acid **17** according to the procedure described by Luly.[20] Elaboration into the pseudotripeptides **19–22** was achieved using the standard methods described in Scheme V.

ECE Inhibition Assay. The compounds were tested for their ability to inhibit the conversion of Big ET-1 to ET-1 in a preparation of rat lung membrane as previously described.[18]

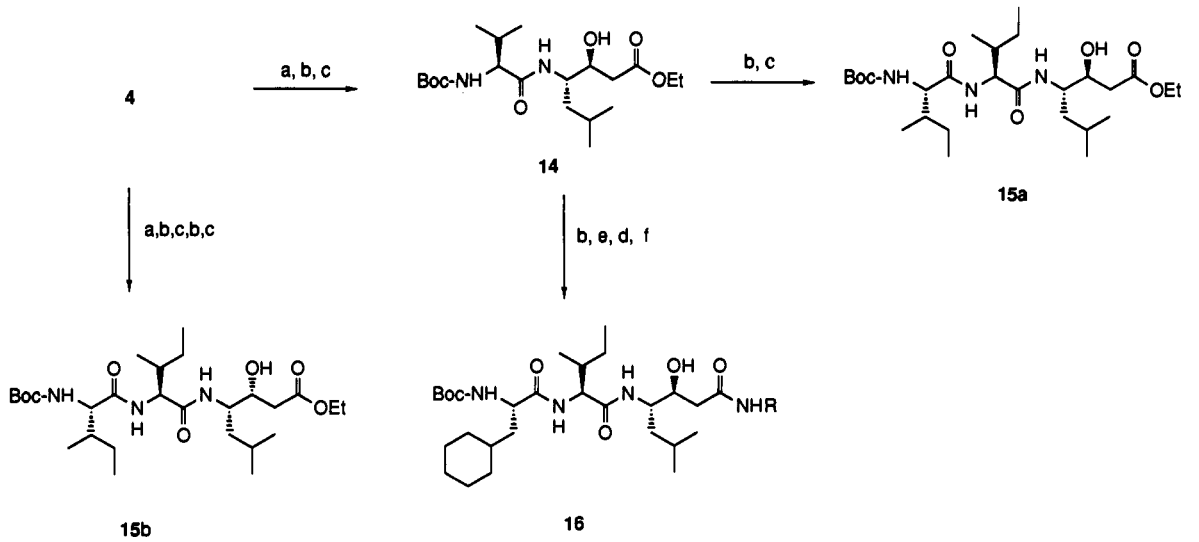
Cathepsin D Inhibition Assay. The compounds were tested for their ability to inhibit the conversion of hemoglobin by commercially purchased bovine spleen Cathepsin D as previously described.[21]

Renin Inhibition Assay. The compounds were tested for their ability to inhibit partially purified human renin at pH 6.0 as previously described.[22]

Results and Discussion

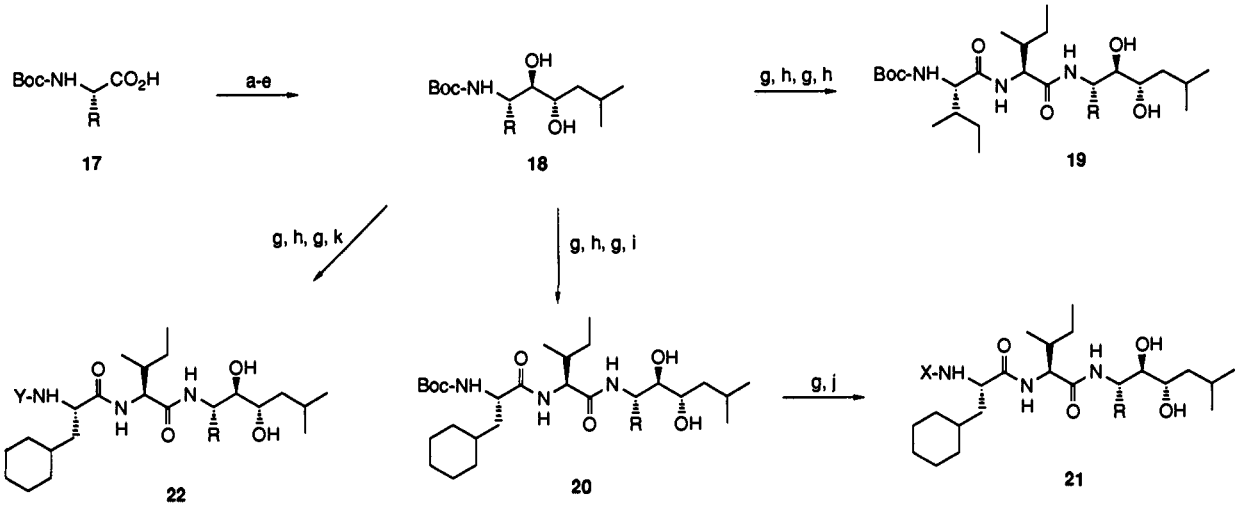
Statine-Based Inhibitors. The initial series of compounds designed and developed to inhibit the endothelin converting enzyme activity found in the rat lung utilized statine ((3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid), a unique amino acid contained in Pepstatin A that serves a critical role for potent inhibition of aspartyl proteases. Statine itself did not inhibit the rat lung activity (data not shown), suggesting that additional residues were required for recognition by the enzyme. On the basis of analogy to the well-established mechanism by which

Scheme IV [a]



[a] Reagents: (a) separate diastereomers; (b) HCl(g), dioxane; (c) Boc-Ile-OH, IBCF, NMM, THF, −20 °C; (d) NaOH, H_2O, MeOH; (e) Boc-Cha-OH, IBCF, NMM, THF, −20 °C; (f) RNH_2, EDCI, HOBt, CH_2Cl_2.

Scheme V [a]



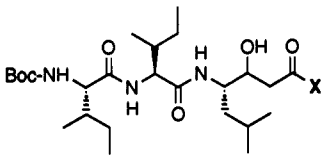
[a] Reagents: (a) BH_3, THF; (b) DMSO, $(COCl)_2$, then Et_3N; (c) TMSCN, ZnI_2; (d) ^iBuMgCl, Et_2O; (e) H_3O^+; (f) $NaBH_4$, THF; (g) HCl(g), dioxane; (h) Boc-Ile-OH, IBCF, NMM; (i) Boc-Cha-OH, IBCF, NMM; (j) X-Cl, amine base; (k) Y-Cha-OH, EDCI, HOBt, NMM.

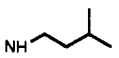
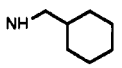
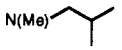
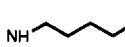
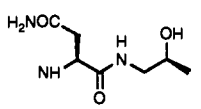
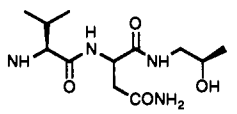
statine-based compounds inhibit a number of known aspartyl proteases,[23] the 3-hydroxyl group of statine was expected to interact with two aspartic acids in the active site of the rat lung enzyme. The isobutyl side chain of statine was assumed to occupy the S_1 binding site in the ECE enzyme. Additional binding energy was therefore anticipated upon appending two isoleucine residues, reflecting the amino acids in the P_2 and P_3 positions of the substrate, Big ET-1 (1), onto the N-terminus of **4**. The resulting tripeptide **5** did demonstrate inhibition of the rat lung activity with an IC_{50} of 65 nM (Table I). Hydrolysis of the C-terminal ester to the acid **6** was detrimental for ECE inhibition as was conversion to the primary amide **7a**. Compounds **5**, **6**, and **7a** were weak inhibitors of Cathepsin D and the free acid **6** was inactive when tested in the renin assay.

Extension of the C-terminus of amide **7a** by various branched alkyl groups provided the first significant improvement in inhibitor potency. The secondary isopropyl amide **7b** was slightly more potent than both primary amide **7a** and ester **5** while the equipotent isobutyl **7c** and isovaleryl **7d** derivatives further improved activity, achieving nearly 8- and 5-fold greater potency than the primary amide **7a** and ester **5**, respectively. Extending the alkyl group into the cyclohexylmethyl resulted in a weaker inhibitor (**7e**) than the isobutyl or isovaleryl examples. Interestingly, conversion of the potent isobutylamide **7c** to the tertiary *N*-methylisobutylamide **7f** did not significantly affect inhibition, suggesting that the hydrogen in the secondary amide is not participating in interactions important for recognition or affinity and that a degree of steric bulk can be accommodated in that region of the molecule by the enzyme. The branched alkyl amides **7b–f** exhibited roughly 4- to 9-fold selectivity for the rat lung enzyme over Cathepsin D. Selected compounds when tested for their ability to inhibit renin were found to be inactive.

Various functional groups could be extended out from the C-terminal amide of the tripeptide **7a** with varying results. Incorporating an acid or ester, exemplified by the β-alanine **7g** and β-alanine ethyl ester **7h**, yielded derivatives with improved ECE potency over the primary amide. Straight-chain alkyl alcohols of varying lengths were extended off the C-terminus to yield amides **7i–k**, all active in inhibiting the rat lung enzyme. The acid **7g**,

Table I. Biological Data of Statine-Based Inhibitors 5–7



no.	X	formula	anal.[a]	IC$_{50}$ (nM) ECE[b]	IC$_{50}$ (nM) Cathepsin D[b]	IC$_{50}$ (nM) renin[c]
5	OEt	$C_{27}H_{51}N_3O_7$	C,H,N	65 ± 8.9 (3)	160 ± 24 (3)	nd[d]
6	OH	$C_{25}H_{47}N_3O_7$·0.25TFA	C,H,N	370 ± 62 (3)	2600 ± 1200 (3)	>10 000
7a	NH_2	$C_{25}H_{48}N_4O_6$	e	100 ± 17 (3)	410 ± 93 (3)	nd
7b		$C_{28}H_{54}N_4O_6$·0.25H_2O	C,H,N	32 (2)	120 (2)	nd
7c		$C_{29}H_{56}N_4O_7$·H_2O	C,H,N	13 ± 1.4 (3)	65 ± 15 (3)	>10 000
7d		$C_{30}H_{58}N_4O_6$·0.4H_2O	C,H,N	11 (2)	82 (2)	nd
7e		$C_{32}H_{60}N_4O_6$·0.25H_2O	C,H,N	94 (2)	540 (2)	nd
7f		$C_{30}H_{58}N_4O_6$·0.5H_2O	f	6.5 (2)	58 (2)	>10 000
7g		$C_{28}H_{52}N_4O_8$	C,H,N	58 (1)	190 (1)	>10 000
7h		$C_{30}H_{56}N_4O_8$	C,H,N	13 (2)	60 (2)	nd
7i		$C_{29}H_{56}N_4O_7$·H_2O	g	13 (2)	82 (2)	nd
7j		$C_{30}H_{58}N_4O_7$	C,H,N	21 (1)	51 (1)	nd
7k		$C_{31}H_{60}N_4O_7$	C,H,N	11 (1)	68 (1)	nd
7l		$C_{28}H_{54}N_4O_7$	C,H,N	15 (1)	120 (1)	nd
7m		$C_{32}H_{60}N_6O_8Na$	h	270 ± 34 (3)	1700 ± 180 (3)	nd
7n		$C_{37}H_{69}N_7O_{10}Na$	i	17 ± 3.5 (3)	200 ± 15 (3)	nd

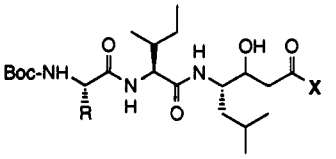
[a] Compounds gave satisfactory analyses within ±0.4% of theoretical calculations. [b] Number of determinations is indicated in parentheses. Means and ±SE are indicated for those compounds with three or more determinations. [c] Single point determination. [d] Not determined. [e] Calcd C, 59.97; H, 9.66; N, 11.19; found C, 59.77; H, 9.46; N, 10.77. [f] Calcd C, 62.15; H, 10.26; N, 9.66; found C 61.95; H, 9.79; N, 9.35. [g] Calcd C, 58.96; H, 9.90; N, 9.42; found C, 58.67; H, 9.43; N, 9.26. [h] HRMS Calcd for $C_{32}H_{60}N_6O_9Na$: 695.4319, found: 695.4315. [i] HRMS Calcd for $C_{37}H_{69}N_7O_{10}Na$: 794.5004, found: 794.5007.

ester **7h**, and alcohol derivatives **7i–k** all maintained selectivity over Cathepsin D; **7g** did not exhibit any renin inhibition.

Threonine is the P_3' residue in the substrate, Big ET-1; incorporating 2-hydroxypropylamine, a threonine surrogate lacking the α-carboxyl group, yielded **7l**, which possessed similar potency to the structurally similar isobutyl amide **7c**. However, additional incorporation of asparagine, which is the P_2' residue in the substrate, into **7l** yielded a compound **7m** of much weaker activity. Surprisingly, further incorporation of valine, the P_1' residue in Big ET-1, resulted in **7n**, a very potent ECE inhibitor (IC_{50} = 17 nM) containing the P_1' through P_3' residues found in Big ET-1. The comparative biological results of **7l**, **7m**, and **7n** suggest that the statine in this particular series of inhibitors may be serving as a single amino acid replacement. This is in direct contrast to statine-based inhibitors of other aspartyl proteases such as renin and Cathepsin D where similar structure–activity studies indicated that statine was serving as a dipeptide replacement.[23] These compounds maintained varying degrees of selectivity over Cathepsin D inhibition.

Replacement of the P_3 Ile residue in **5** and **6** with cyclohexylalanine (Cha) resulted in compounds **8a** and **9a**, respectively, that possessed significantly improved ECE potencies. Other aliphatic groups substituted into the P_3 position had varying effects on the ability of the compound to inhibit the rat lung enzyme. Incorporating *tert*-butylalanine yielded inhibitors **8b** and **9b** that were comparable to the corresponding Cha derivatives **8a** and **9a**. Compounds **8c** and **9c**, which omitted the methylene carbon between the amino acid α-center and the cyclohexyl group, were weaker inhibitors than the respective Cha-containing inhibitors. Likewise, *tert*-butylglycine replacement at the P_3 position yielded compounds **8d** and **9d** that were weaker than the homologous *tert*-butylalanine derivatives. P_3 substitution by *O-tert*-butylthreonine also produced weak inhibitors **8e** and **9e** of the rat lung activity. Apparently, additional branching from the 4-position, but not the 3-position, of the P_3-amino acid is tolerated by the

Table II. Biological Data of P_3-Modified Statine-Based Inhibitors 8 and 9



no.	R	X	formula	anal.[a]	IC_{50} (nM) ECE[b]	Cathepsin D[b]	renin[c]
8a		OEt	$C_{30}H_{55}N_3O_7$	C,H,N	16 ± 0.97 (3)	58 ± 2.9 (3)	>10 000
9a		OH	$C_{28}H_{51}N_3O_7 \cdot 0.5H_2O$	C,H,N	28 (1)	130 (1)	>10 000
8b		OEt	$C_{28}H_{53}N_3O_7$	C,H,N	16 ± 3.7 (3)	72 ± 1.2 (3)	nd[d]
9b		OH	$C_{26}H_{49}N_3O_7 \cdot H_2O$	C,H,N	58 (1)	150 (1)	nd
8c		OEt	$C_{29}H_{53}N_3O_7$	C,H,N	48 ± 6.5 (3)	93 ± 26 (3)	nd
9c		OH	$C_{27}H_{49}N_3O_7 \cdot H_2O$	C,H,N	200 (1)	350 (1)	nd
8d		OEt	$C_{27}H_{51}N_3O_7$	C,H,N	100 ± 18 (3)	280 ± 29 (3)	>10 000
9d		OH	$C_{25}H_{47}N_3O_7 \cdot 0.5H_2O$	C,H,N	750 (1)	1100 (1)	>10 000
8e		OEt	$C_{29}H_{55}N_3O_8$	C,H,N	130 ± 15 (3)	470 ± 23 (3)	nd
9e		OH	$C_{27}H_{51}N_3O_8$	C,H,N	1100 (1)	1300 (1)	nd

[a] Compounds gave satisfactory analyses within ±0.4% of theoretical calculations. [b] Number of determinations is indicated in parentheses. Means and ±SE are indicated for those compounds with three or more determinations. [c] Single point determination. [d] Not determined.

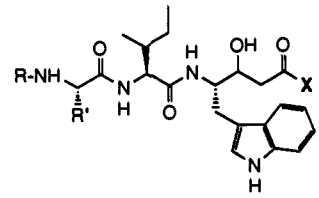
enzyme. In all cases, the free acids were 2- to 8-fold less potent than the corresponding esters. The selectivity of these various P_3-substituted inhibitors of the rat lung ECE over Cathepsin D varied from essentially nonselective, e.g. **8c**, to those that were 5-fold selective, e.g. **8b**. Selected compounds in this series were tested for their ability to inhibit renin and were shown to be inactive at a concentration of 10 μM.

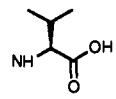
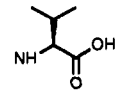
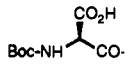
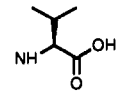
In order to reflect the tryptophan that resides in the P_1 position of the substrate, the isopropyl side chain of statine was replaced with the indol-3-yl group (Table III). Surprisingly, the indolylstatine derivative **12a** was a significantly weaker inhibitor than the parent compound **9a**. Introducing the P_1 valine onto the C-terminus of **12a** did yield a potent inhibitor **12b**, suggesting again that the statine derivative is serving as a single amino acid replacement. As was observed in the statine series, isoleucine at the P_3 position (**13a,b**) dramatically decreased potency from the cyclohexylalanine examples. Extending **13b** at the N-terminus by addition of aspartic acid, the P_4 residue in the substrate, yielded pseudopentapeptide **13c** that exhibited even weaker potency. The indolylstatine derivatives maintained or improved selectivity for ECE over Cathepsin D, achieving up to 25-fold selectivity with **12b**. Several of the inhibitors in this series were tested in the renin assay and shown to be inactive.

The inhibitors discussed in Tables I–III have been isolated and tested as a roughly 1:2 (*R*/*S*) mixture of diastereomers, epimeric at the 3-position of the statine and the indolylstatine residues. To establish that the (3*S*)-isomer is preferred for potent inhibition of the rat lung enzyme as had been established for other aspartyl proteases such renin and Cathepsin D, the diastereomerically pure statine isomers were separated from the epimeric mixture **4** and elaborated into a select series of inhibitors (Table IV). The diastereomerically pure tripeptide **15a** bearing the (3*S*)-hydroxyl was 35-fold more potent than the (3*R*)-isomer **15b** and 5-fold more potent than the 1:2 (*R*/*S*) mixture **5**, thus suggesting a similarity in the active site requirements for the rat lung ECE as the other established aspartyl proteases. Incorporating several of the key structural elements that are important for potency, i.e. (3*S*)-statine, cyclohexylalanine at the P_3 position, and extension of the C-terminus into the isobutyl amide, into a single compound produced **16a**, a potent ECE inhibitor in this series. Selectivity over Cathepsin D by these diastereomerically pure inhibitors ranged from 2- to 4-fold; renin inhibition by these compounds remained weak.

In order to impart water solubility into these diastereomerically pure statine-based inhibitors, various water-solubilizing groups were extended out from the C-terminus. Tripeptide **16b**, possessing the morpholinylethyl amide,

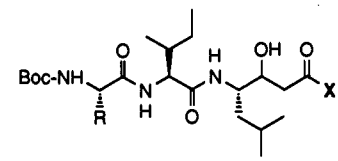
Table III. Biological Data of Indolylstatine-Based Inhibitors 12 and 13

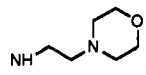
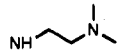
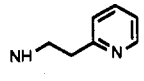


no.	R	R′	X	formula	anal.[a]	IC_{50} (nM) ECE[b]	IC_{50} (nM) Cathepsin D[b]	IC_{50} (nM) renin[c]
12a	Boc		OH	$C_{33}H_{50}N_4O_7 \cdot 0.65TFA$	C,H,N	890 ± 49 (3)	12 000 ± 1600 (3)	nd[d]
12b	Boc			$C_{38}H_{59}N_5O_8 \cdot 0.6TFA$	C,H,N	24 ± 8.6 (3)	620 ± 68 (3)	>10 000
13a	Boc		OH	$C_{30}H_{46}N_4O_7 \cdot 0.7TFA$	C,H,N	>10 000 (1)	>10 000 (1)	>10 000
13b	Boc			$C_{35}H_{55}N_5O_8 \cdot 0.5TFA$	C,H,N	960 (2)	>10 000 (2)	nd
13c				$C_{39}H_{60}H_6O_{11} \cdot TFA$	C,H,N	7 500 (2)	≥10 000 (2)	nd

[a] Compounds gave satisfactory analyses within ±0.4% of theoretical calculations. [b] Number of determinations is indicated in parentheses. Means and ±SE are indicated for those compounds with three or more determinations. [c] Single point determination. [d] Not determined.

Table IV. Biological Data of Statine-Based Inhibitors 15 and 16



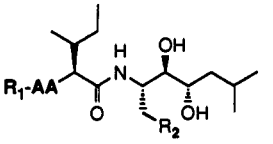
no.	R	X	config[a]	formula	anal.[b]	IC_{50} (nM) ECE[c]	IC_{50} (nM) Cathepsin D[c]	IC_{50} (nM) renin[d]
15a		OEt	*S*	$C_{27}H_{51}N_3O_7$	C,H,N	12 ± 2.5 (3)	21 ± 1.8 (3)	nd[e]
15b		OEt	*R*	$C_{27}H_{51}N_3O_7$	C,H,N	440 ± 84 (3)	4600 ± 840 (3)	nd
16a			*S*	$C_{32}H_{60}N_4O_6$	C,H,N	5.7 (2)	16 (2)	1400
16b			*S*	$C_{34}H_{61}N_5O_7 \cdot 1.3TFA$	C,H,N	7.5 ± 1.4 (3)	480 ± 11 (3)	nd
16c			*S*	$C_{32}H_{61}N_5O_6 \cdot 1.1TFA$	C,H,N	7.8 (2)	300 (2)	nd
16d			*S*	$C_{35}H_{59}N_5O_6 \cdot 1.25TFA$	C,H,N	5.2 (2)	37 (2)	>1000

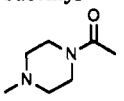
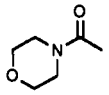
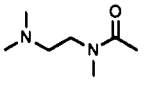
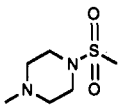
[a] Absolute configuration of 3-hydroxyl group in statine. [b] Compounds gave satisfactory analyses within ±0.4% of theoretical calculations. [c] Number of determinations is indicated in parentheses. Means and ±SE are indicated for those compounds with three or more determinations. [d] Single point determination. [e] Not determined.

maintained potent inhibition of the rat lung ECE (IC_{50} = 7.5 nM) with respect to the isobutyl amide **16a** while significantly improved selectivity (64-fold) over Cathepsin D was achieved. Water solubility in the range of 3 mg/mL was attained by **16b**. Likewise, the dimethylethylamino **16c** and the 2-pyridylethyl **16d** derivatives were potent and water-soluble ECE inhibitors although Cathepsin D selectivity of **16d** was significantly reduced relative to **16b**.

Dihydroxyethylene-Based Inhibitors. In addition to developing the statine-containing inhibitors that derived

Table V. Biological Data of Dihydroxyethylene Inhibitors **19–22**



no.	R_1	AA	R_2	formula	anal.[a]	IC_{50} (nM) ECE[b]	IC_{50} (nM) Cathepsin D[b]	IC_{50} (nM) renin[c]
19a	Boc	Ile	cyclohexyl	$C_{31}H_{59}N_3O_6$	C,H,N	6.3 ± 2.0 (3)	61 ± 16 (3)	34
19b	Boc	Ile	1-naphthyl	$C_{35}H_{55}N_3O_6$	C,H,N	660 (1)	>10,000 (2)	>10,000
19c	Boc	Ile	2-naphthyl	$C_{35}H_{55}N_3O_6$	C,H,N	550 (2)	>10,000 (2)	>10,000
20a	Boc	Cha	cyclohexyl	$C_{34}H_{63}N_3O_6$	C,H,N	11 ± 0.87 (3)	55 ± 5.2 (3)	<10
20b	Boc	Cha	1-naphthyl	$C_{38}H_{59}N_3O_6 \cdot 0.5H_2O$	C,H,N	23 ± 7.5 (3)	>10,000 (3)	nd[d]
20c	Boc	Cha	2-naphthyl	$C_{38}N_{59}N_3O_6$	C,H,N	3.1 ± 0.40 (3)	63 ± 9.6 (3)	>10,000
20d	Boc	Cha	3-indolyl	$C_{36}H_{58}N_4O_6 \cdot 0.25H_2O$	C,H,N	5.9 ± 0.63 (3)	210 ± 14 (3)	>10,000
21a	H (HCl)	Cha	2-naphthyl	$C_{33}H_{52}ClN_3O_4$	C,H,N	68 (2)	2500 (2)	nd
21b	Ac	Cha	2-naphthyl	$C_{35}N_{53}N_3O_5$	*e*	4.2 (2)	180 (2)	nd
21c	succinyl	Cha	2-naphthyl	$C_{37}N_{55}N_3O_7 \cdot H_2O$	C,H,N	3.1 ± 0.77 (3)	60 ± 9.7 (3)	nd
22a		Cha	2-naphthyl	$C_{39}H_{62}N_5O_5 \cdot HCl \cdot 2.5H_2O$	C,H,N	6.0 (2)	1100 (2)	>1000
22b		Cha	2-naphthyl	$C_{38}H_{58}N_4O_6 \cdot H_2O$	C,H,N	2.1 (2)	68 (2)	350
22c		Cha	2-naphthyl	$C_{39}H_{63}N_5O_5 \cdot 1.7TFA$	C,H,N	5.9 ± 0.50 (3)	280 ± 14 (3)	500
22d		Cha	2-naphthyl	$C_{38}H_{61}N_5O_6S \cdot 1.5TFA$	C,H,N	3.5 (2)	110 (2)	660

[a] Compounds gave satifactory analyses within ±0.4% of theoretical calculations. [b] Number of determinations is indicated in parentheses. Means and ±SE are indicated for those compounds with three or more determinations. [c] Single point determination. [d] Not determined. [e] Calcd C, 70.56; H, 8.97; N, 7.05; found C, 71.34; H, 8.81; N, 6.53.

from Pepstatin-A, a structurally distinct series of compounds that also possessed potent and selective ECE inhibition was desired as a means of verifying any pharmacological findings that might be obtained from the first set of inhibitors. Therefore, a number of in-house compounds generated from other aspartyl protease-based projects, e.g. renin and HIV, were screened for their ability to inhibit the rat lung activity. A number of renin inhibitors possessing a common dihydroxyethylene "hook" 18 (where R = cyclohexylmethyl) was demonstrated to inhibit the ECE activity with IC_{50}'s in the range of 50 to 100 nM. Since these compounds were very potent renin inhibitors (IC_{50} < 1 nM), attempts to reverse the order of selectivity between renin and the rat lung ECE were pursued.

The first inhibitor synthesized in this series contained two isoleucines (P_2 and P_3 residues of Big ET-1) that were attached onto the N-terminus of the cyclohexyl renin hook. Compound **19a** was a potent inhibitor of the rat lung activity with an IC_{50} of 6 nM, however significant renin activity was maintained (Table V). Assuming that the P_1-cyclohexyl group of the dihydroxyethylene moiety fits into the S_1 site of the rat lung converting enzyme, a study of the renin model suggested that greater selectivity between the two activities might be achieved by substituting the cyclohexyl group with a more sterically extended residue such as an indole, which is the P_1 residue (tryptophan) in Big ET-1. A bulkier residue would prevent binding into the S_1 site of renin and thereby diminish or eliminate renin activity. Thus, the 1- and 2-naphthalene derivatives **19b** and **19c**, respectively, were prepared and tested. Although significantly weaker ECE inhibition was observed, both compounds were inactive against renin. The ability of the compounds to inhibit Cathepsin D was also abolished upon introduction of a larger group at P_1.

As previously discussed, substituting P_3 isoleucine with cyclohexylalanine significantly improved potency in the statine-based inhibitors. Applying this same strategy to the dihydroxyethylene series resulted in a similar improvement in ECE potency in some compounds. Replacing the P_3 isoleucine in **19a** with cyclohexylalanine yielded **20a**; this substitution did not affect potency for ECE and Cathepsin D inhibition and possibly diminished selectivity over renin. Subsequent replacement of the P_1 cyclohexane residue with the isomeric naphthalenes provided the potent ECE inhibitors **20b** and **20c** with IC_{50}'s of 23 and 3 nM, respectively. The 2-naphthyl derivative is a 7-fold more potent inhibitor of the rat lung activity than the 1-naphthyl isomer, suggesting that a discrete lipophilic pocket exists in the ECE into which the aromatic group is fitting. More importantly, the more potent ECE inhibitor **20c** is now a very weak or possibly inactive inhibitor of renin and 20-fold selective over Cathepsin D. In addition, the 1-naphthyl compound **20b** represents one of the most selective (>1000-fold) inhibitors of ECE over Cathepsin D. Replacing the naphthalene with the 3-indolyl group, thereby reflecting the P_1 residue found in the substrate, yielded a derivative **20b** that possesses a similar inhibitory profile to **20c**.

Removal of the Boc-protecting group in **20c** resulted in a significantly weaker inhibitor **21a**. However, subtitution of the Boc with the acetyl group produced a compound

21b that was equipotent for ECE as **20c**. Likewise, the succinyl derivative **21c** was a potent inhibitor of the rat lung enzyme exhibiting an IC_{50} of 3 nM and possessing a similar 20-fold selectivity over Cathepsin D as **20c**.

Lastly, water solubility was introduced into the dihydroxyethylene series of ECE inhibitors as had been accomplished with the statine-based compounds. Water-solubilizing residues were appended onto the N-terminus of selected analogues with the hopes of improving solubility while maintaining ECE potency and selectivity. The solubilizing groups were linked to the inhibitor via the urea or sulfonyl; the derivatives in this series, **22a–d**, maintained potent ECE inhibition (IC_{50}'s < 10 nM) relative to the parent compound **21b**. These compounds were soluble in water at levels of approximately 3 mg/mL. Their selectivity for ECE over Cathepsin D varied from 30- to 180-fold. Surprisingly, introduction of the water-solubilizing groups significantly improved the compounds' ability to inhibit renin although modest selectivity (80- to 190-fold) for ECE was maintained.

In general, parallel trends between ECE and Cathepsin D inhibition were observed in both the statine- and dihydroxyethylene-based series, suggesting that the two enzymes possess similar properties with regard to inhibitor binding requirements. In most cases, selectivity ranged from 3- to 20-fold. However, there are compounds within certain series that demontrate significant selectivity (>50-fold), e.g. **16b**, **20b**, **22a**, over structurally related compounds that only modestly discriminate, suggesting that the two enzymes are indeed distinct. Establishing whether the rat lung enzyme is truly a unique activity cannot be accomplished at this time; further purification and characterization along with possible cloning of the enzyme will be required.

Several of the potent, selective and water-soluble ECE inhibitors such as **16b**, **16c**, and **22a** were tested in rats (iv administration) for their ability to inhibit the pressor response of exogenously administered Big ET-1 according to a previously described protocol.[14] Although some inhibition of the pressor response was observed, consistent results could not be obtained (data not shown) and further investigations with these compounds are continuing. Since the conversion of exogenously administered Big ET-1 may be occurring via a nonphysiologically relevant ECE, testing these inhibitors in an animal model in which endogenous levels of ET can be elevated is desired. Efforts to develop such models are currently underway.

In summary, we have developed two structurally distinct series of potent and selective inhibitors of an endothelin converting enzyme activity identified in the rat lung. Determining the biological significance of this enzyme activity and whether the compounds will be of therapeutic utility awaits the development of appropriate pharmacological models in which to evaluate these compounds.

Experimental Section

Solvents and other reagents were reagent grade and used without further purification unless otherwise noted. Amino acids and *tert*-butyloxycarbonyl (Boc) protected amino acids were purchased from Bachem, Inc., Torrance, CA or Sigma Chemical Co., St. Louis, MO. ^{1}H-NMR spectra were recorded at 300 MHz and expressed as ppm downfield from tetramethylsilane (TMS) as an internal standard. Column chromatography was performed on silica gel 230–400 mesh (E. Merck). Preparative reverse-phase HPLC chromatography was performed on a Gilson 303 dual pump delivery system using a Gilson Holochrom variable wavelength UV detector at 225 nm. The column was a Dynamax 30A column (2.5 × 30 cm) and the solvents used were A, 0.1% trifluoroacetic acid in water and B, acetonitrile. A linear gradient beginning with 5% B and ending with 50% B over 30 min was employed. Elemental analyses were performed by Abbott Laboratories Analytical Department, North Chicago, IL, or by Robertson Laboratory, Madison, NJ and are within ±0.4% of calculated unless otherwise stated. The following abbreviations have been used: Boc, *tert*-butyloxycarbonyl; EDCI, 1-[3-(dimethylamino)-propyl]-3-ethylcarbodiimide hydrochloride; HOBt, 1-hydroxybenzotriazole. Other peptide and amino acid abbreviations and conventions are those recommended by the IUPAC-IUB Commission (*Biochem. J.* 1984, 219, 345–373).

Boc-Isoleucylisoleucyl-(3*R*,*S*)-statine Ethyl Ester (5). A solution of **4** (425 mg, 1.4 mmol) in anhydrous HCl in dioxane (20 mL) was stirred at room temperature for 2 h. Water was added to the mixture and the solution frozen and lyophilized to give the amine hydrochloride salt as an oil (302 mg, 90%). To a 0 °C solution of the resulting salt (150 mg, 0.63 mmol), Boc-Ile-Ile-OH (150 mg, 0.62 mmol), and *N*-methylmorpholine (70 mg, 0.70 mmol) in methylene chloride (3 mL) was added EDCI (134 mg, 0.70 mmol) and HOBt (94 mg, 0.70 mmol). The solution was allowed to warm to ambient temperature and stirred overnight. The reaction was diluted with ethyl acetate, washed with 1 M H_3PO_4 (3×), saturated $NaHCO_3$ solution (3×), and brine. After drying over Na_2SO_4, the solvent was removed in vacuo and the residue was chromatographed (ethyl acetate/hexane) to yield **5** (130 mg, 40%) as a white solid, MS (DCI/NH_3) *m/e* 530 $(M + H)^+$, 547 $(M + NH_4)^+$.

Boc-Isoleucylisoleucylstatine (6). To a solution of ester **5** (33 mg, 0.064 mmol) in MeOH (7 mL) was added 1 N NaOH (0.45 mL, 0.45 mmol). The reaction mixture was stirred overnight at room temperature. The MeOH was removed in vacuo and the residue was diluted with EtOAc and washed with 1 M H_3PO_4 and brine. After drying ($MgSO_4$), evaporation of the solvent in vacuo yielded a white solid which was purified by preparative HPLC. The desired fractions were frozen and lyophilized to yield **6** as a white solid (7 mg, 22%): ^{1}H NMR (DMSO-d_6, 300 MHz) δ 0.80 (m, 18 H), 1.00–1.75 (m, 9 H), 1.37 (s, 9 H), 2.05–2.40 (m, 2 H), 3.60–3.90 (m, 3 H), 4.15 (m, 1 H), 6.77 (d, 1 H), 7.65 (m, 2 H); MS (FAB) *m/e* 502 $(M + H)^+$, 524 $(M + Na)^+$. Anal. ($C_{25}H_{47}N_3O_7 \cdot 0.25CF_3COOH$ C, H, N.

General Procedure for the Preparation of Tripeptide Amides 7. Boc-Isoleucylisoleucylstatyl-NH_2 (7a). To a −5 °C solution of the acid **6** (40 mg, 0.08 mmol) in THF (4 mL) were added successively *N*-methylmorpholine (8 mg, 0.08 mmol) and isobutyl chloroformate (11 mg, 0.08 mmol) and the reaction mixture was stirred for 20 min. Concentrated NH_4OH solution (0.025 mL, 0.36 mmol) was added and the reaction was allowed to stir an additional 2.5 h. The solvent was removed in vacuo and the residue was dissolved in MeOH, diluted with $CHCl_3$, and washed with 1 M H_3PO_4 (2×), saturated $NaHCO_3$ (2×), and brine. Drying (Na_2SO_4) and concentration in vacuo followed by flash chromatography eluting with 93.5:6.5 $CHCl_3$/EtOH afforded **7a** (7 mg, 18%) as a white solid: ^{1}H NMR (DMSO-d_6, 300 MHz) δ 0.8 (m, 18 H), 1.4 (s, 9 H), 1.0–1.8 (m, 9 H), 2.0–2.2 (m, 2 H), 3.6–3.9 (m, 3 H), 4.35 (d of d, 1 H), 6.8–7.7 (m, 4 H); MS (FAB) *m/e* 501 $(M + H)^+$. Anal. Calcd for $C_{25}H_{48}N_4O_6$: C, 59.97; H, 9.66; N, 11.19; Found: C, 59.77; H, 9.46; N, 10.77.

General Procedure for the Preparation of P_3-Modified Tripeptide Esters 8. Boc-Cyclohexylalanylisoleucylstatine Ethyl Ester (8a). Boc-Cyclohexylalanylisoleucyl-OH (172 mg, 0.45 mmol) and statine ethyl ester hydrochloride (107 mg, 0.45 mmol) were coupled employing HOBt and EDCI in an analogous manner as described in the preparation of **5**. The final compound was purified by flash chromatography on silica gel eluting with 3:1 hexane/ethyl acetate to afford **8a** (146 mg, 57%) as a white solid: ^{1}H NMR (DMSO-d_6, 300 MHz) δ 0.8 (m, 15 H), 1.00–1.73 (m, 19 H), 1.40 (s, 9 H), 2.15–2.43 (m, 2 H), 3.65–4.20 (m, 6 H), 4.90 (d of d, 1 H), 6.93 (d of d, 1 H), 7.60 (d of t, 2 H); MS (FAB) *m/e* 570 $(MH)^+$. Anal. Calcd for $C_{30}H_{55}N_3O_7$: C, 63.27; H, 9.70; N, 7.38; Found: C, 63.14; H, 9.71; N, 7.34.

General Procedure for the Preparation of P_3-Modified Tripeptide Acids 9. Boc-Cyclohexylalanylisoleucylstatyl-OH (9a). The ester **8a** (85 mg, 0.15 mmol) was hydrolyzed and purified by the method described for **6** to give **9a** as a white solid: ^{1}H NMR (DMSO-d_6, 300 MHz) δ 0.80 (m, 12 H), 1.35 (s, 9 H),

1.00–1.73 (m, 19 H), 2.10–2.30 (m, 2 H), 3.67 (m, 1 H), 3.83 (m, 1 H), 3.95 (m, 1 H), 4.13 (m, 1 H), 6.92 (m, 1 H), 7.43–7.65 (m, 2 H); MS (FAB) *m/e* 542 $(M + H)^+$, 564 $(M + Na)^+$. Anal. Calcd for $C_{28}H_{51}N_3O_7 \cdot 0.5H_2O$: C, 61.07; H, 9.52; N, 7.63; Found: C, 61.47; H, 9.49; N, 7.63.

(3*RS*,4*S*)-4-(Boc-Cha-Ile-Amino)-5-(3-indolyl)-3-hydroxypentanoic Acid (12a). a. (4*S*)-4-Boc-Amino-5-(3-indolyl)-3-hydroxypentanoic Acid Ethyl Ester. To a 0 °C solution of Boc-Trp-OH (6.00 g, 24.3 mmol), 3,5-dimethylpyrazole (2.33 g, 24.3 mmol), and HOBt (3.6 g, 26 mmol) in methylene chloride (150 mL) was added EDCI (5.12 g, 26.6 mmol). The reaction mixture was allowed to warm to room temperature, stirred overnight, and concentrated in vacuo. The residue was diluted with ethyl acetate and washed with 1 M H_3PO_4. The aqueous washes were back-extracted with ethyl acetate, and the combined organic extracts were washed with 1 M H_3PO_4 (2×), saturated $NaHCO_3$ solution (3×), and brine. After drying over Na_2SO_4, the solvent was removed in vacuo and the resulting crude product was flash-chromatographed on silica gel eluting with 2:1 hexane/ethyl acetate to yield the pyrazolide (6.95 g, 75%). The pyrazolide (4.00 g, 10.47 mmol) was dissolved in anhydrous tetrahydrofuran (50 mL) and cooled to −78 °C. Lithium aluminum hydride (0.64 g, 16.8 mmol) was added to the solution in 5 portions over 70 min. Following the addition, the reaction mixture was maintained at −78 °C for an additional 1 h and then carefully poured with vigorous stirring into 10% citric acid solution (500 mL). This mixture was extracted with Et_2O (3×), and the combined organic extracts were washed with brine, dried over $MgSO_4$, and concentrated in vacuo to give the aldehyde as a tacky residue. To a −78 °C solution of ethyl acetate (5 mL) in THF (50 mL) was added a solution of 1 M lithium hexamethyldisilazide (120 mL) over 30 min. After stirring an additional 1 h at −78 °C, a solution of the aldehyde in THF (20 mL) was added. The reaction mixture was stirred at −78 °C for 70 min and then quenched by addition to 1 N HCl (100 mL). This mixture was extracted twice with Et_2O, and the organic extracts were washed with saturated $NaHCO_3$ solution (2×) and brine. After drying over $MgSO_4$, the solution was concentrated in vacuo and the residue purified by flash chromatography eluting with 15% ethyl acetate in hexanes to afford the ethyl ester (1.91 gm, 60%) as a white solid. **b. (3*RS*,4*S*)-4-(Boc-Cha-Ile-Amino)-5-(3-indolyl)-3-hydroxypentanoic Acid Ethyl Ester.** The tripeptide ester was prepared according to the method described for the preparation of **5c**. **c. (3*RS*,4*S*)-4-(Boc-Cha-Ile-Amino)-5-(3-indolyl)-3-hydroxypentanoic Acid (12a).** The acid **12a** was prepared according to the method described for the preparation of **6**: MS (FAB) *m/e* 615 $(M + H)^+$, 637 $(M + Na)^+$; ^1H NMR ($CDCl_3$, 300 MHz) δ 0.70–1.05 (m, 6 H), 1.40 (2s, 9 H), 1.05–2.60 (m, 13 H), 3.1 (m, 2 H), 4.00–4.45 (m, 3 H), 4.90 (m, 1 H), 6.60–7.20 (m, 4 H), 7.33 (d, 1 H), 7.60 (2d, 2 H), 8.20 (m, 1 H). Anal. ($C_{33}H_{50}N_4O_7 \cdot 0.65CF_3COOH$) C, H, N.

(3*RS*,4*S*)-4-(Boc-Ile-Ile-Amino)-5-(3-indolyl)-3-hydroxypentanoic Acid (13a). The compound was prepared in a similar manner to that described for **12a**: MS (DCI/NH_3) *m/e* 575 $(M + H)^+$, 592 $(M + H + NH_3)^+$; ^1H NMR ($CDCl_3 + CD_3OD$, 300 MHz) δ 0.70–1.00 (m, 12 H), 1.00–1.90 (m, 6 H), 1.45 (s, 9 H), 1.70 (m, 2 H), 2.30 (m, 2 H), 2.90–3.15 (m, 2 H), 3.97 (m, 3 H), 4.32 (bd, 1 H), 7.10 (m, 3 H), 7.33 (d, 1 H), 7.70 (bt, 1 H). Anal. ($C_{30}H_{46}N_4O_7 \cdot 0.7CF_3COOH$) C, H, N.

General Procedure for the Preparation of Dihydroxyethylene Inhibitors 19 and 20. *N*-[(2*S*,3*R*,4*S*)-1-(2-naphthyl)-3,4-dihydroxy-6-methyl-2-heptyl]-Boc-Cha-Ile-NH_2 (20c). A solution of **18** (R = 2-naphthyl; prepared by the method of Luly;[20] 60 mg, 0.15 mmol) in 4 N HCl in dioxane (3 mL) was stirred at ambient temperature for 1 h and then concentrated in vacuo. The resulting hydrochloride salt was dissolved in THF (2 mL) and adjusted with 1 N NaOH to pH 12. The mixture was extracted with CH_2Cl_2 (5×). The combined organic extracts were dried over K_2CO_3 and concentrated in vacuo to afford the free amine (45 mg, 99%). To a −20 °C solution of Boc-Ile-OH hemihydrate (37 mg, 0.15 mmol) in THF (10 mL) were added *N*-methylmorpholine (151 mg, 1.49 mmol) and isobutyl chloroformate (203 mg, 1.48 mmol). After stirring an additional 15 min, the free amine of **18** (45 mg, 0.15 mmol) was added to a solution of the mixed anhydride. The reaction was allowed to warm to room temperature then stirred an additional 4 h. Ethyl acetate was added and the solution was washed with 1 M H_3PO_4 (3×), saturated $NaHCO_3$ solution (3×), and brine. After drying over $MgSO_4$, the solvent was removed in vacuo to yield the *N*-[(2*S*,3*R*,4*S*)-1-(2-naphthyl)-3,4-dihydroxy-6-methyl-2-heptyl]-Boc-Ile-NH_2 (77 mg, 99%) as a white solid. The product was deprotected with HCl in dioxane and coupled with Boc-Cha-OH as described above to yield **20c** after recrystallization from ethyl acetate/hexane as a white solid: mp 173–174 °C; MS (FAB) *m/e* 654 $(M + H)^+$; ^1H NMR ($CDCl_3$, 300 MHz) δ 0.70–1.05 (m, 12 H), 1.10–1.90 (m, 19 H), 1.47 (s, 9 H), 3.20 (m, 1 H), 3.25 (d of d, 1 H), 3.48 (m, 2 H), 4.00–4.20 (m, 2 H), 4.55 (m, 1 H), 5.78 (bd, 1 H), 7.22–7.50 (m, 4 H), 7.70–7.83 (m, 3 H). Anal. ($C_{38}H_{59}N_3O_6$) C, H, N.

General Procedure for the Preparation of Dihydroxyethylene Inhibitors 22. (2*S*,3*R*,4*S*)-2-{*N*-[[(4-Methylpiperazin-1-yl)carbonyl]cyclohexylalanylisoleucyl]amino}-1-(2-naphthyl)-3,4-dihydroxy-6-methylheptane Hydrochloride (22a). To cyclohexylalanine methyl ester hydrochloride salt (1.00 g, 4.51 mmol) suspended in toluene (25 mL) was added bis(trichloromethyl) carbonate (669.2 mg, 2.25 mmol). The reaction mixture was heated at 100 °C for 3 h and then concentrated in vacuo to afford a semisolid residue. To this residue dissolved in methylene chloride (15 mL) and cooled to 0 °C was added *N*-methylpiperazine (0.23 g, 4.5 mmol). After stirring for 1 h, the reaction mixture was concentrated at reduced pressure and the residue purified on silica gel eluting with 4% methanol in chloroform to afford *N*-[(4-methylpiperazin-1-yl)carbonyl]cyclohexylalanine methyl ester (629 mg, 45%). To a solution of the methyl ester (629 mg, 2.02 mmol) in THF (16 mL) and water (2 mL) cooled to 0 °C was added lithium hydroxide monohydrate (169 mg, 4.03 mmol) in water (2 mL). After 90 min, the THF was removed under reduced pressure and the aqueous solution acidified to pH 3 by the careful addition of 1 N HCl. This solution was placed on a column of Dowex 1-X8 and 50W-X8 made acidic by packing and equilibrating with 1 N HCl. The column was eluted with 0.1 N HCl and 1 N HCl. *N*-[(4-Methylpiperazin-1-yl)carbonyl]cyclohexylalanine was obtained as an amorphous solid. *N*-[(2*S*,3*R*,4*S*)-1-(2-naphthyl)-3,4-dihydroxy-6-methyl-2-heptyl]Ile-NH_2 (18.1 mg, 0.06 mmol, described in preparation of **20c**) was coupled with *N*-[(4-Methylpiperazin-1-yl)carbonyl]-cyclohexylalanine (22.2 mg, 0.055 mmol) in an analogous manner as described for the preparation of **5**. Flash chromatography on silica gel eluting with 20% methanol in methylene chloride afforded the product (31 mg, 76%) which was taken up in 4 N HCl in dioxane (1.5 mL) and then concentrated at reduced pressure to afford the hydrochloride salt **22a**: MS (FAB) *m/e* 680 $(M + H)^+$; ^1H NMR (DMSO-d_6, 300 MHz) δ 0.62–0.89 (m, 12 H), 0.90–1.80 (m, 19 H), 2.23 (m, 3 H), 2.90–3.20 (m, 4 H), 3.30 (s, 3 H), 3.40–3.74 (m, 3 H), 4.18 (m, 1 H), 4.32 (m, 1 H), 5.00 (d, 0.5 H), 6.55 (d, 0.5 H), 7.40–7.57 (m, 4 H), 7.70–7.88 (m, 4 H). Anal. ($C_{39}H_{62}ClN_5O_5 \cdot 2.5H_2O$) C, H, N.

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