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THIOL AND HYDROXAMIC ACID CONTAINING INHIBITORS OF ENDOTHELIN CONVERTING ENZYME

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Abstract

A group of peptido-mimetic molecules containing either a thiol or hydroxamic acid moiety were synthesized and evaluated for *in vitro* inhibition of a putative endothelin converting enzyme (ECE) partially purified from rabbit lung membranes. These compounds were envisioned as analogs of the phosphoramide containing metalloprotease inhibitor phosphoramidon.

Introduction

The potent vasoconstrictor peptide endothelin (1-21), ET-1, was isolated and characterized in 1988 by Yanagisawa and coworkers[1]. A putative endopeptidase, endothelin convering enzyme (ECE), has been proposed to liberate ET via selective cleavge of the Trp^{21}-Val^{22} bond of its precursor big endothelin (1-39), Big-ET-1[1-3]. Abnormal levels of this potent, endogenous vasoconstrictor have been proposed to play a role in hypertension[4-5], acute renal failure[6] and coronary or cerebral vasospasm[7].

It has been postulated that inhibition of ECE would be a viable therapy for the aforementioned disease states[8] since ET-1 is approximately 100 times more potent, as a vasoconstrictor substance, than its precursor Big-ET-1[9]. Therefore, we have initiated a synthetic program aimed at the selective inhibition of the putative ECE. This report describes the initial results of our investigation into thiol and hydroxamic acid containing inhibitors of ECE.

Chemistry

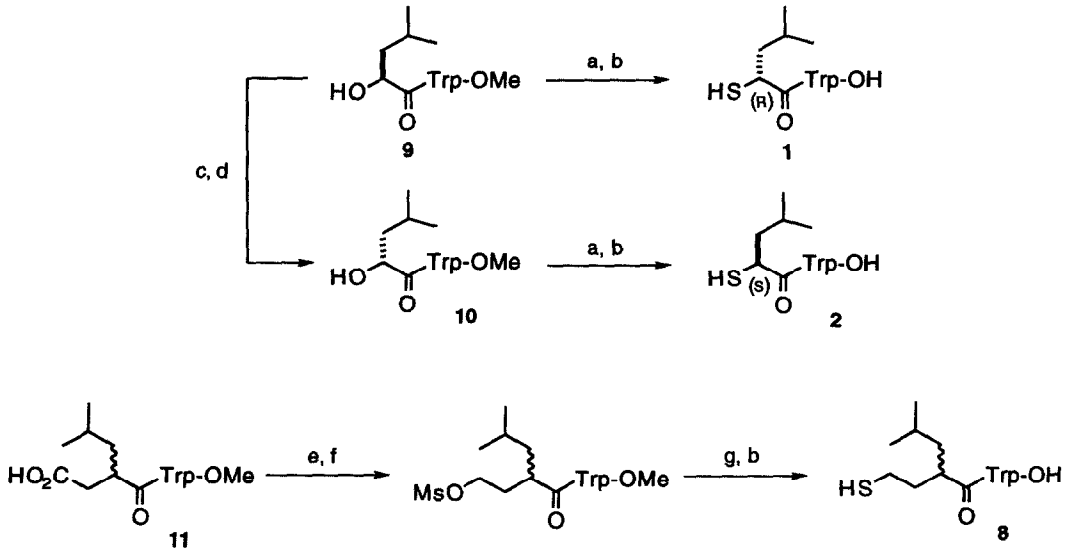
The compounds reported in this communication were prepared by previously described methods for related structures except as outlined in scheme 1. The cysteine and homocysteine containing compounds were synthesized by standard solution phase peptide synthesis employing 1-hydroxybenzotriazole (HOBt) mediated carbodiimide coupling conditions[10]. The thiols were protected during synthesis either by acetylation or symmetrical-disulfide formation.

Compounds **3**, **5** and **7** (Table 1) were prepared by analogy to the syntheses of thiorphan[11], Zincov inhibitor[12] and kelatorphan[13] respectively. Thiols **1**, **2** and **8** derive from displacement of the appropriate activated alcohol with thiol acetic acid[14-15] (Scheme 1). A brief outline of the synthetic protocols for thiols **1**, **2** and **8** is presented below. All compounds synthesized were characterized by ^1H NMR, HPLC (C-18 reverse phase) and mass spectroscopy.

Secondary alcohol **9** (Scheme 1) was prepared by amide bond formation between L-α-hydroxyisocaproic

acid and tryptophan methyl ester[16]. Mitsunobu inversion of **9** with formic acid[17] followed by selective removal of the formate protecting group[18] provided S-alcohol **10** as a single diasteriomer (>95% by ^{1}H NMR). Displacement of **9** or **10** with thiol acetic acid was preformed independently to prepare **1** or **2** respectively. The primary alcohol precursor of **8** was prepared by borane reduction of isobutyl succinate **11**[19] (Scheme 1). Formation of the primary mesylate followed by displacement with thiol acetic acid converted the alcohol to the protected, primary mercaptan[15] precursor of **8** as a mixture of diasteriomers.

Scheme 1



[a] Reaction conditions: (a) THF, AcSH, PPh_3, diisopropyl azodicarboxylate (DIAD), 0 - 25°C; (b) THF/H_2O, 1.2 eq. LiOH, 25°C then acidify with 1N HCl and extract into CH_2Cl_2; (c) THF, HCO_2H, PPh_3, DIAD, 0 - 25°C; (d) THF/H_2O, $NaHCO_3$, 25°C; (e) THF, BH_3·SMe_2, 0 - 25°C then anhydrous MeOH and rotovap; (f) pyridine, 3eq. MsCl; (g) AcSH (neat), 25°C.

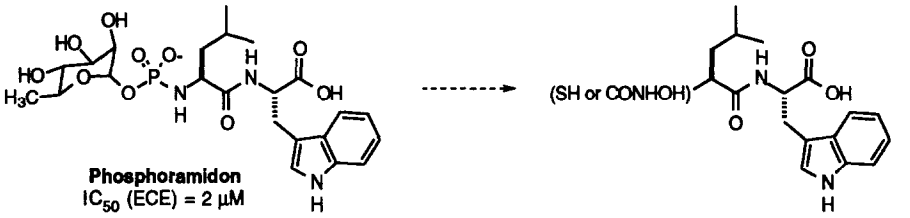
Results and Discussion

It has been demonstrated that several enzymes or classes of enzymes are capable of selectively cleaving the 39 amino acid precursor peptide between residues Trp_{21} and Val_{22} to form ET-1[20]. Human cathepsin E[21] has been implicated as a possible physiologically relevant ECE due to its ability to selectively liberate ET-1 from big ET-1 without further degradation of either peptidic product. Corroboration of these results by *in vivo* experiments with specific inhibitors of these enzymes has not appeared to date. Phosphoramidon, N-(α-L-rhamnopyranosyloxyhydroxyphosphinyl)-L-Leu-L-Trp (Figure 1), has been reported to inhibit the conversion of big-ET-1 to ET-1 *in vitro* as well as *in vivo* suggesting that the physiologically relevant ECE is a metalloprotease[20-22].

A non-homogeneous preparation of ECE isolated from rabbit lung can be inhibited by 1,10

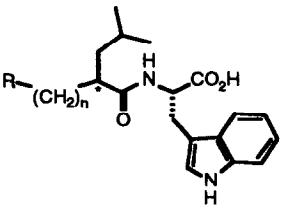
phenanthroline, EDTA and the metalloprotease inhibitor phosphoramidon[23-24]. This is strongly suggestive that ECE is a metalloprotease. We sought to discover new, selective inhibitors of this enzyme by using previously established methods for the design of metalloprotease inhibitors[11-13]. In addition to providing new inhibitors, this approach should furnish additional structural and mechanistic information about ECE. Specifically, our approach to the design of new inhibitors was to combine the metal coordinating properties of either thiol or hydroxamate moieties with the peptide recognition elements found in phosphoramidon (Figure 1).

Figure 1



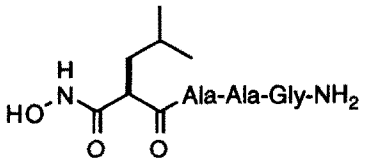
Proper positioning of the putative chelator relative to the recognition elements within an inhibitor molecule is closely tied to inhibitor potency. By varying the distance between these two domains for a group of related compounds (compare **1**, **2**, **5** and **8** or **3** and **7**), we have determined the alignment necessary for inhibition of ECE. For structures **1** - **8**, compounds **3** and **5** were found to be the most potent inhibitors of ECE with IC_{50}'s in the micromolar range, Table 1. With regard to the distance between peptide recognition and chelating substructures, these compounds are analogous to captopril and Zincov inhibitor respectively (Figure 2).

Table 1

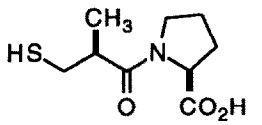


compound #	n	R	*	IC_{50} µM(ECE)
1	0	SH	(R)	100±10
2	0	SH	(S)	100±12
3	0	CONHOH	(R,S)	24±3
4	0	CO_2H	(R,S)	>>100
5	1	SH	(R,S)	12±1
6	1	OH	(R,S)	>>100
7	1	CONHOH	(R,S)	>>100
8	2	SH	(R,S)	>>100
phosphoramidon	-	-	-	4±0.2

Figure 2



Zincov Inhibitor
IC_{50} (Thermolysin) = 64 nM



Captopril
IC_{50} (ACE) = 30 nM

Replacement of the hydroxymate in **3** by carboxylate (compound **4**) or the thiol in **5** by hydroxyl (compound **6**) leads to a greater than 10-fold decrease in potency. This effect is commonly seen when binding / inhibition is due in part to chelation of a metal ion[25]. Although not unexpected, the results of this classical approach indicate that ECE behaves similarly to the more thoroughly studied enzymes ACE[11] (angiotensin converting enzyme) and thermolysin[12].

In an attempt to build SAR around the more potent thiols, our attention was directed towards a source of substituted, preferably chiral, 3-mercapto propionates. It was postulated that N-terminal cysteine containing peptides or peptidomimetic compounds would satisfy the requirement for this functional group. Accordingly, N-terminal cysteine di and tripeptides were prepared and tested for inhibition of ECE (Table 2).

Table 2

compound #	peptide	% @ 100μM	IC_{50} μM(ECE)
12	Cbz-Cys-Leu-Trp-OCH_3	83	31±1
13	Cbz-Cys-Leu-Trp-OH	75	
14	Cbz-Cys-Leu-OCH_3	89	
15	Cbz-Cys-Trp-OCH_3	93	47±3
16	Cbz-Cys-Trp-OH	90	30±4
17	Cbz-(D)Cys-Leu-OCH_3	47	
18	Cbz-homoCys-Leu-OCH_3	94	4±1

Cbz-Cys-Trp-OH (**16**) was similar in potency to the parent thiol inhibitor (**5**). The substitution of a Cbz-amino moiety for the isobutyl group of (**5**) seemed a fairly dramatic change, therefore certain related analogs were also synthesized in order to better understand the activity seen with these new inhibitors. Of the derivatives synthesized, the peptides which contained L-cysteine (**12** through **16**) have fairly similar enzyme activity. Substitution of D-cysteine for L-cysteine (compound **17**) however, significantly decreases inhibitor potency. Evaluating the effect of the position of the putative metal chelating group was accomplished by replacement of cysteine with homocysteine (compound **18**). This substitution led to an approximate ten fold increase in potency. The carboxy-terminal acid derivatives of compounds **14**,**17** and **18** all showed a significant decrease in potency

(< 40% inhibition at 100 μM) when compared with their methyl ester precursors.

Inhibition by compounds **12-14** and **17-18**, the structures of which are no longer close analogs of phosphoramidon, was somewhat unexpected. These data may indicate an extended hydrophobic binding site which tolerates a variety of functional groups (i.e. isobutyl, indole or benzyl) or a completely different mode of binding for these new inhibitors. These initial results do not allow for a distinction between these and other possible explanations. Future investigation of cysteine containing inhibitors will hopefully provide an answer to this question.

Conclusion

Metal chelation appears to be necessary for activity in these compounds. It is not clear, however, whether or not these inhibitors are acting at the active site of the putative metalloprotease. The data for the series of thiols (**1** or **2**, **5** and **8**) demonstrates that inhibition is closely tied to the length of the tether between the mercaptan and peptidic portions of these compounds. This phenomenon may indicate simultaneous interaction of these two moieties with the enzyme or possibly a change in the physical or chemical properties (i.e. metal chelation) of these molecules which affects their ability to inhibit this putative endopeptidase. Synthesis of additional analogs along with more thorough investigation of the kinetics of their inhibition is underway and should provide addition information about this new putative metalloprotease.

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23. The enzyme preparation used in this study was partially purified from the membrane fraction of a rabbit lung homogenate. This enzyme preparation is detergent soluble and was purified approximately 180 fold from the crude lung homogenate. The substrate used for the assay was big-ET-1 and formation of product was monitored by radioimmunoassay for ET-1 (<0.02% crossreactivity for big-ET-1). This preparation was inhibited at neutral pH (optimum between 7.2-7.6) by phosphoramidon ($IC_{50} = 4 \pm 0.2$ μM), 1, 10 phenanthrolene ($IC_{50} = 47 \pm 0.3$ μM), EDTA ($IC_{50} = 0.3 \pm 0.2$ μM) and thiorphan ($IC_{50} = 120 \pm 0.2$ μM) but not by captopril of kelatorphan. Big-ET-1 (Km big-ET-1 (1-39) = 35 μM) is approximately 3-5 fold better as a substrate for this enzyme preparation than either big-ET-2 or big-ET-3. Our preparation is similar to those described in reference twenty-four (24). A detailed description of its purification and assay conditions are found in the supplemental materials section of Bertenshaw, et. al. *J. Med. Chem.* **1993**, 36, 173-176. A full report of the purification and assay procedures is cuurently submitted for publication in a seperate journal.
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