Communications to the Editor

Phosphorus-Containing Inhibitors of Endothelin Converting Enzyme: Effects of the Electronic Nature of Phosphorus on Inhibitor Potency

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Introduction. Since the discovery of endothelin (ET-1) by Yanigasawa[1,2] and co-workers, an enormous effort throughout the scientific community has been directed toward understanding the biological significance of this potent vasoconstrictor peptide.[3] Numerous studies aimed at determination of the physiology and pathophysiology of endothelins,[3b,c] as well as synthetic programs probing antagonism[4] of the endothelin receptors and inhibition of a putative endothelin converting enzyme (ECE),[5] have been undertaken. This report describes the initial findings of our program concerned with the design and synthesis of inhibitors of a putative metalloprotease, ECE, partially purified from rabbit lung.[6,7]

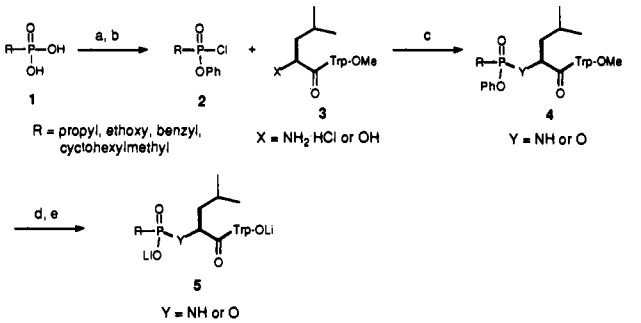
ET-1 is approximately 100 times more potent than its precursor, big-ET-1; consequently it has been proposed that inhibition of a physiologically relevant ECE may be of therapeutic advantage in disease states characterized by overproduction of endothelin.[3] These diseases may include hypertension,[8] acute renal failure,[9] and coronary or cerebral vasospasm.[10]

Chemistry. Complete synthetic procedures and analytical data for the compounds presented in this communication are contained within the supplemental materials section. All compounds synthesized were characterized by ^1H and ^{31}P NMR and mass spectrometry. A brief outline of the synthetic protocols is presented below.

Compounds **15–22** were prepared by condensation of either leucyltryptophan methyl ester[11] or its hydroxy derivative **3** (X = OH) with an appropriate phosphorus acid chloride[12] **2** followed by hydrolysis of both phosphorus and carboxylic esters (Scheme I). Alkaline hydrolysis of alkyl phosphonamide esters can be quite sluggish. Therefore, it was decided that a more labile ester intermediate would be used. Accordingly, phosphorus acid dichlorides (either commercially available or prepared from phosphorus diacids, oxalyl chloride, and a catalytic amount of DMF[12]) were first treated with 1 equiv of phenolate followed by addition of the peptide or its oxygen analog **3** (Scheme I).

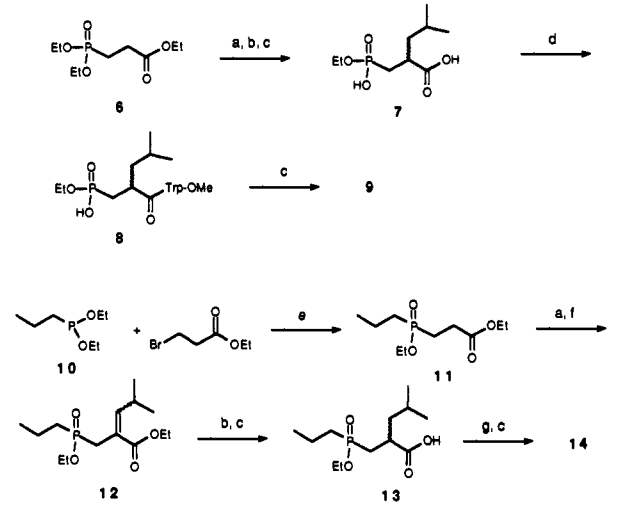
The protected phosphorus amides **4** (Scheme II) were

Scheme I. Synthesis of Phosphonamide- and Phosphoramide-Containing Compounds[a]



[a] Reaction conditions: (a) CH_2Cl_2, $(COCl_2)$ 1.1 equiv, DMF 0.1 equiv, reflux; (b) CH_2Cl_2, phenol 1.0 equiv and Et_3N 1.0 equiv in CH_2Cl_2 added dropwise; (c) THF, 4-DMAP; (d) THF/H_2O, LiOH 3.1 equiv, reflux; (e) to reaction mixture in (d) add 1.1 equiv of HCl and H_2O and then lyophilize.

Scheme II. Synthesis of 3-Phosphonopropionate-Containing Compounds[a]



[a] Reaction conditions: (a) THF, LDA, isobutyraldehyde, −78 °C to room temperature; (b) EtOH, 10% Pd/C (10 weight %), H_2 40 psi; (c) THF/H_2O, LiOH 2.2 equiv and then HCl; (d) THF/DMF, 2 equiv of 4-methylmorpholine and then 1 equiv of isobutyl chloroformate (stir 10 min) and then Trp-OMe 1 equiv; (e) toluene, reflux 16 h; (f) CCl_4, $SOCl_2$, and then EtOH; (g) THF/DMF, EDC, HOBt.

stable to flash chromatography. After mild alkaline hydrolysis (<80 °C for 2–10 h) of both the phosphorus and carboxylic esters, HCl was added to the reaction mixture to neutralize the phenolate present. The phenol byproduct is readily removed by lyophilization of an aqueous solution of phenol and the dilithium salt of the desired product **5** (Scheme I).

The syntheses of phosphonate **9** and phosphinate **14** began with Stobbe condensation[13] of isobutyraldehyde and either triethyl phosphono-3-propionate (**6**) or diethyl (propylphosphinyl)-3-propionate[14] (**11**), respectively (Scheme II). Appropriate protecting group manipulation and olefin reduction followed by either carbodiimide or mixed anhydride amide bond formation to tryptophan methyl ester provided the precursors to **9** and **14**, respectively. Hydrolysis and reverse-phase (C-18) HPLC yielded **9** and **14**, both as a mixture of two diastereomers.

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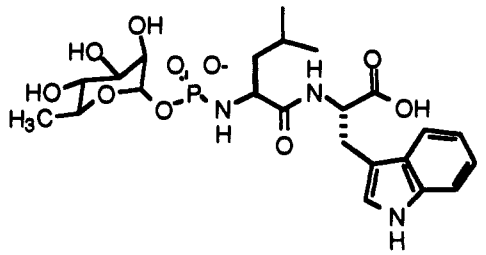


Figure 1. Phosphoramidon IC_{50} (rabbit lung ECE) = 2 ± 0.1 μM (value is mean ± SEM for three separate experiments).

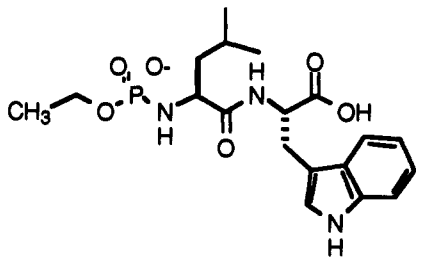


Figure 2. Ethyl phosphoramidate 15. IC_{50} (rabbit lung ECE) = 109 ± 32 μM (value is mean ± SEM for three separate experiments).

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.[15] Human cathepsin E[16] 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-rhamnopyranosyloxy)hydroxyphosphinyl]-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.[15-17]

The systematic design and synthesis of analogues of phosphoramidon would seem to be a promising path toward more potent and specific inhibitors of ECE. A significant obstacle to this approach lies in the synthetic challenge associated with the synthesis of molecules containing a variety of chemically sensitive functional groups. The incompatibility of the synthetic and protection protocols for this phosphorus linked glycopeptide would hinder an effort to synthesize numerous analogues in a timely fashion.

It was our contention that if certain of these groups could be removed without a significant loss in potency, a systematic investigation would be synthetically tractable. The initial results of our studies indicate that this is possible through replacement of the rhamnose moiety by simple alkyl groups. For example, replacement of the rhamnose ring in phosphoramidon by an ethoxy group provides inhibitor 15 (Figure 2) with an IC_{50} of 109 μM,[6] about 60-fold less potent than phosphoramidon, but a synthetically more attractive target. As shown in Figure 3, phosphoramidate 15 also inhibits the pressor response to big-ET-1 in the ganglion-blocked rat in a dose-dependent manner.[18]

It has been demonstrated that inhibition of metalloproteases by phosphorus-based inhibitors is sensitive to the electronic nature of the phosphorus atom.[19,20] Consequently, we have synthesized analogues of 15 (Table I) containing various phosphorus acids and amides. Propyl phosphonamide 17 represents a novel inhibitor with potency similar to phosphoramidon[21] and a structure significantly more amenable to rapid and thorough investigation of the structural requirements for inhibition of ECE. Although the IC_{50} for phosphonamide 17 is identical to phosphoramidon in vitro, this compound was 5 times more potent in vivo[18,22] (Figure 3).

The relative potency for compounds 9 and 14–18 parallels, with one exception, that described for a related series of thermolysin inhibitors.[19,20] On the basis of the results for thermolysin, one would expect the phosphinic acid analogue 14 to be of similar potency to phosphonamide 17. This is clearly not the case for ECE. Failing to establish a close correlation between ECE and the more thoroughly studied metalloprotease thermolysin, the above series was expanded to better understand the enzyme/inhibitor system under consideration.

Additional pairs of inhibitors which vary only in the oxidation state of phosphorus (phosphoramide versus phosphonamide) were synthesized and tested for inhibition of ECE. In vitro inhibition by structures 19–22 (Figure 4) illustrates that the phosphonamide oxidation state does not provide a consistent improvement in potency over the phosphoramide. This result suggests that the electronic nature of the putative transition-state analog is not solely responsible for relative inhibitor potency even in such structurally similar molecules. As Bartlett proposes,[19] differences in binding between closely related inhibitors cannot be accounted for by the comparison of a single

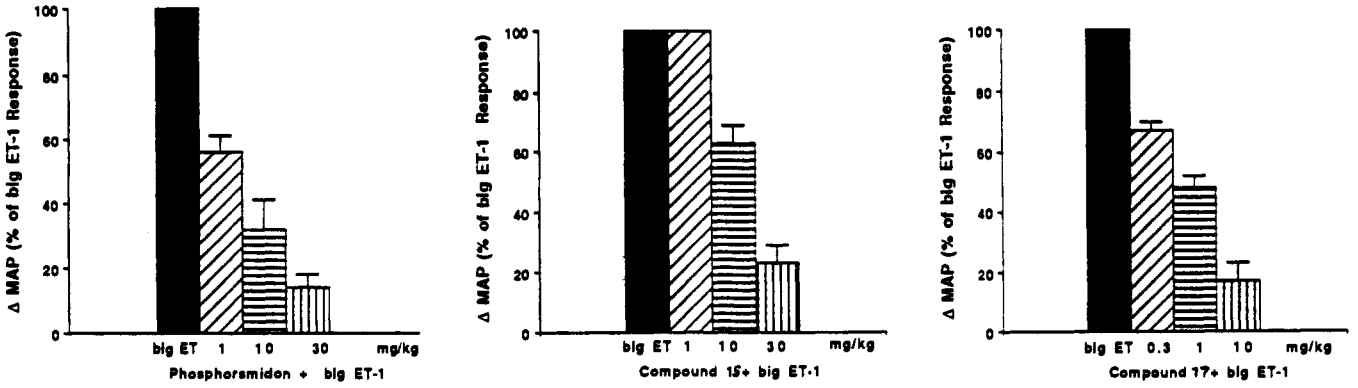
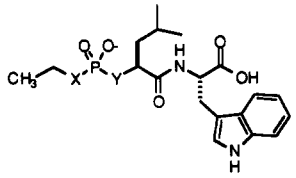


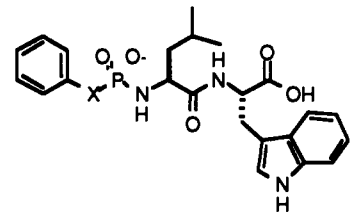
Figure 3. Effect of phosphoramidon and compounds 15 and 17 on the pressor response to big ET-1 in the ganglion-blocked anesthetized rat. For in vivo inhibition determinations the compounds were given intravenously 10–15 s prior to the administration of big ET-1 at 1 nmol/kg (n = 3 or 4 for each dose). Neither phosphoramidon nor compound 17 antagonize the pressor response to ET-1 in a rat pressor assay. For methodological details, see reference 17.

Table I. ECE Inhibition by Structural Analogs of 15

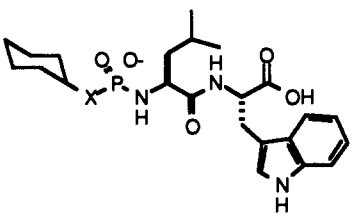


no.	X	Y	IC_{50} (μM)
15	O	NH	109 ± 32[a]
16	O	O	≫100[b]
9[c]	O	CH_2	≫100[b]
17	CH_2	NH	2 ± 0.1[a]
18	CH_2	O	≫100[b]
14	CH_2	CH_2	≫100[b]

[a] IC_{50} data were determined in an RIA assay using big ET-1 as substrate (values are means ± SEM for three separate experiments). [b] This IC_{50} value corresponds to a compound which gave <25% inhibition when assayed at a 100 μM screening concentration (n = 2). [c] This compound was synthesized as a mixture of diastereomers at the point of attachment of the isobutyl side chain.



19: X = O, IC_{50} = 37 ± 2 μM
20: X = CH_2, IC_{50} = 61 ± 2 μM



21: X = O, IC_{50} = 48 ± 5 μM
22: X = CH_2, IC_{50} = 4.8 ± 0.7 μM

Figure 4. Phosphonamidate and phosphoramidate inhibitors of ECE. Analogs of phosphoramidon.

factor such as phosphorus oxy anion basicity, minor structural changes, or variation in solvation energy.

Presumably, a variety of factors influenced the relative potency between a given phosphonamide/phosphoramide pair in this study. The difference in potency between phosphoramide and phosphonamide oxidation states for the three sets compared (15 vs 17, 19 vs 20, and 21 vs 22) was greatest for the smallest group attached to phosphorus (propyl vs ethoxy, 15 vs 17). This may suggest that the relative potency in the more substituted cases (benzyl or cyclohexylmethyl) was dominated by increased hydrophobic or steric interactions with the enzyme which masked the effects of changing the electronic nature of phosphorus.

Our future work in this area will include studies directed at gaining a better understanding of the intriguing trends noted above. In addition, we hope to develop SAR for ECE through the synthesis of phosphorus-containing inhibitors related to those presented in this communication. These studies should provide a more complete description of the structural requirements for inhibition of ECE.

Supplementary Material Available: Detailed synthetic procedures and analytical data as well as a description of the partial purification of ECE from rabbit lung and assay conditions (19 pages). Ordering information is given on any current masthead page.

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(6) The enzyme preparation used in this study was partially purified from the membrane fraction of a rabbit lung homogenate. It was inhibited at neutral pH (optimum between 7.2 and 7.6) by phosphoramidon (IC_{50} = 2 ± 0.1 μM), 1,10-phenanthrolene (IC_{50} = 47 ± 0.3 μM), EDTA (IC_{50} = 0.3 ± 0.2 μM), and thiorphan (IC_{50} = 120 ± 0.2 μM) but not by captopril or 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. This preparation is similar to those described in ref 7. A detailed description of its purification and assay conditions are found in the supplemental materials section of this manuscript.

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(18) *In vivo* data for the compounds described in this manuscript were obtained according to the procedure of McMahon et al. See ref 17 above. The pressor assay monitors blood pressure in normotensive rats (male Sprague-Dawley, 300–350 g) after injection of big ET-1 with or without prior administration of an inhibitor. ED_{50}s are based on a percent of the control response (blood pressure increase) to big ET-1 injection.

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(21) Both phosphoramidon and propyl phosphonamide–leucyltryptophan 17 demonstrated competitive kinetics with K_i values of 4 and 8 μM, respectively.

(22) Compound 17 inhibits ACE (IC_{50} = 4 μM) (IC_{50} = 30 nM for captopril in this assay) and NEP (EC 3.4.24.11) (IC_{50} = 10 ± 0.8 nM) (IC_{50} = 17 nM for kelatorphan in this assay). This inhibition, however, does not affect the *in vivo* results as it has been demonstrated that ACE and NEP inhibitors do not block the conversion of big ET-1 to ET-1 either *in vitro* or *in vivo* (see ref 17).