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AMINOPHOSPHONATE ENDOTHELIN CONVERTING ENZYME INHIBITORS: POTENCY-ENHANCING AND SELECTIVITY-IMPROVING MODIFICATIONS OF PHOSPHORAMIDON

Takehiro Fukami*, Takashi Hayama, Yuuka Amano, Yuko Nakamura, Yukiko Arai, Kenji Matsuyama, Mitsuo Yano and Kiyofumi Ishikawa

New Drug Discovery Research Laboratories, Tsukuba Research Institute, Banyu Pharmaceutical Co., LTD. Tsukuba Techno-Park Oho, 3 Okubo, Tsukuba, Ibaraki 300-33, Japan

Abstract. A series of α-aminophosphonic acid derivatives and a series of phosphoramidate derivatives have been synthesized and evaluated as inhibitors of a phosphoramidon-sensitive metalloproteinase endothelin converting enzyme (ECE). Some of these compounds exhibit potent ECE inhibitory activity. The most potent inhibitor (**XIIb**) is about 10 times as potent as phosphoramidon.

Introduction

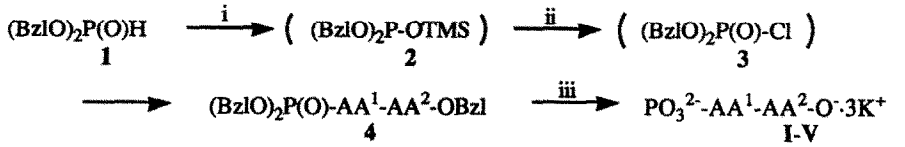
Endothelin (ET)-1, which was first isolated from the culture medium of porcine aortic endothelial cells, is a potent vasoconstrictor consisting of 21 amino acids.[1] Studies including a human genomic analysis have identified two structurally- and functionally-related isopeptides of ET-1 termed ET-2 and ET-3.[2, 3, 4] Since these discoveries, evidence is accumulating that anti-ET agents may provide a novel therapy for the treatment of patients with hypertension,[5,6,7] pulmonary hypertension,[8,9,10] cerebral vasospasm,[11,12] etc.

The biosynthetic pathway of ET-1 has been proposed to be as follows: preproendothelin is initially processed by dibasic-pair-specific endopeptidase(s) to generate an intermediate peptide, big ET-1, which is subsequently cleaved at the Trp^{21}-Val^{22} bond by a putative endothelin converting enzyme (ECE) to yield a mature peptide, ET-1.[1] Since the proposal of this pathway, many efforts have been made to identify an ECE with physiological relevance. Consequently, a phosphoramidon-sensitive neutral metalloproteinase has been identified as the most likely candidate.[13,14,15] This is because 1) a phosphoramidon-sensitive enzyme of vascular endothelial cells hydrolyzes the Trp^{21}-Val^{22} bond specifically without cleaving any other peptide bond of the substrate, big ET-1, and the products, ET-1 and the C-terminal peptide[13] and 2) phosphoramidon inhibits the production of ET-1 in cultured endothelial cells[16] and big ET-1-induced contractions in isolated blood vessels *in vitro*[17] as well as big ET-1-induced hypertension *in vivo*.[18,19] However, phosphoramidon is also known to be a potent inhibitor of other metalloproteinases such as thermolysin and, in particular, enkephalinase (= neutral endopeptidase, NEP, EC 3.4.24.11), which is thought to be an ET-degrading enzyme,[20,21] with an IC_{50} of 4.2 nM. Therefore, it is quite possible that phosphoramidon may potentiate biological responses to ETs, providing a complex explanation of the observed pharmacological data. It was recently reported that higher doses of phosphoramidon suppressed big ET-1-induced bronchoconstriction, while lower doses enhanced the reaction in

guinea pigs.[22] A specific ECE inhibitor is therefore desired for clarifying the pathophysiological role of the ECE.

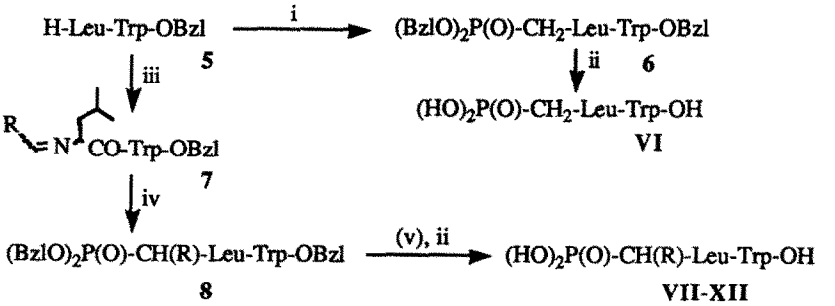
It has been reported that a phosphorous-containing inhibitor produced ECE inhibitory activity similar to that of phosphoramidon with considerable NEP inhibition.[23] However, until now a highly potent and selective ECE inhibitor has not been identified. In this communication, we describe potent and selective ECE inhibitors with aminophosphonate structure derived from phosphoramidon.

Scheme 1.



Reagents: (i) $CH_3C(=NTMS)OTMS$, CH_3CN; (ii) H-AA^1-AA^2-OBzl·HCl, Et_3N, CCl_4; (iii) KOH (3 eq.), H_2/Pd-C, EtOH.

Scheme 2.



Reagents: (i) $(Bzl)_2P(O)CH_2OTf$, Et_3N, THF; (ii) H_2/Pd-C, EtOH; (iii) RCHO, Δ, toluene; (iv) $(BzlO)_2P(O)H$, toluene-CH_3CN; (v) separation on SiO_2

Chemistry

The method for the preparation of phosphoramidate inhibitors (**I-V**) involved phosphorylation of a dipeptide benzyl ester followed by deprotection of the protective groups (Scheme 1). The phosphorylation was carried out in one pot in a manner analogous to the synthesis of phosphonic acid amides.[24] Dibenzyl phosphonate (**1**) was silylated by bis(trimethylsilyl)acetamide to afford dibenzyltrimethylsilyl phosphite (**2**). The phosphite was reacted with CCl_4 to generate the phosphoryl chloride (**3**), which was subsequently reacted with a dipeptide benzyl ester to yield a protected phosphoramidate (**4**). Catalytic hydrogenation of **4** in the presence of three equivalents of KOH[25] gave the desired phosphoramidate inhibitor (**I-V**).

Aminophosphonic acid inhibitors were prepared by the methods described in Scheme 2.[26] Compound **VI** was synthesized by catalytic hydrogenation of the tribenzyl ester **6** prepared from Leu-Trp-OBzl (**5**) and dibenzyl phosphonomethyltriflate in a manner similar to that described by Phillion and Androw.[27] The method for the preparation of compounds **VII-XII** involved the addition of dibenzyl phosphonate to an imine (**7**) prepared from Leu-Trp-OBzl (**5**) and an aldehyde, subsequent separation of two diastereoisomers at the α-position of the

aminophosphonate moiety of the tribenzyl ester (8) by silica gel chromatography, and deprotection by catalytic hydrogenation. Compounds VII and X were prepared as a mixture of diastereoisomers at the α-position of the aminophosphonic acid moiety because the diastereoisomers of 8 could not be separated from each other by silica gel chromatography. All of the final compounds were analyzed for homogeneity and structural integrity by TLC, ^{1}H-NMR, ^{31}P-NMR, and high-resolution FAB-MS.[28]

Biological Results and Discussion

The compounds described herein were tested for their ECE inhibitory activity.[29] Some of the compounds that exhibited potent ECE inhibition were further tested for NEP inhibition.[30]

Initially, we felt that the side-chains of Leu and Trp in phosphoramidon would not fit the S1' and S2' pockets of the ECE enzyme well because in the substrate, big ET-1, the residues that correspond to the S1' and S2' sites are Val and Asn, respectively. On the other hand, it is known that des-rhamnosyl phosphoramidon (phosphoryl-Leu-Trp, I) is more potent than phosphoramidon as an inhibitor of thermolysin and other metalloproteinases.[32] Furthermore, the rhamnose moiety of phosphoramidon is known to be unnecessary for the *in vivo* inhibition of ECE.[33] These findings prompted us to synthesize des-rhamnosyl phosphoramidate derivatives. As shown in Table 1, compound I was a potent ECE inhibitor, as expected (IC_{50} = 0.96 μM). Compound II was a very weak ECE inhibitor (14% inhibition at 100 μM) although it had the same P1' and P2' residues, Val and Asn, as the substrate. All other analogues of I with minor alternations (III-V) were less potent than I. These results suggest that the side-chains of Leu and Trp fit unexpectedly well into the S1' and S2' pockets of the enzyme.

Table 1. ECE Inhibitory Activity of Phosphoramidate Inhibitors

PO_3^{2-}-AA^1-AA^2-O^-·$3K^+$

Compound	AA^1	AA^2	IC_{50}, μM
I	Leu	Trp	0.96
II[a]	Val	Asn	(14%)[b]
III	Leu	Nal[c]	7.4
IV	Ile	Trp	2.8
V	Nle	Trp	1.5

[a] Compound II was prepared as a 3 Et_3N salt. [b] % inhibition at 100 μM.
[c] 3-(1-naphthyl)-L-alanine.

Next, we planned to introduce a putative P1 unit into the inhibitor structures, thus designing α-aminophosphonic acid derivatives; namely, we thought that a substituent at the α-position of the α-aminophosphonic acid moiety might function as P1 (Fig. 1). As shown in Table 2, a reference compound, phosphonomethyl-Leu-Trp (VI), was as potent an ECE inhibitor as phosphoramidon with slightly reduced NEP inhibition. Incorporation of an n-propyl group as a representative alkyl onto the α-position of the aminophosphonic acid moiety (compound VII, a mixture of two diastereoisomers) resulted in a decrease in both ECE and NEP inhibitory activity. In contrast, the introduction of a phenethyl group yielded two diastereoisomers: one isomer (VIIIb) exhibited ECE inhibitory activity 3-fold more potent than that of the reference compound (VI), while the other (VIIIa) was almost inactive. Furthermore, compound VIIIb exhibited about 20-fold

Fig. 1

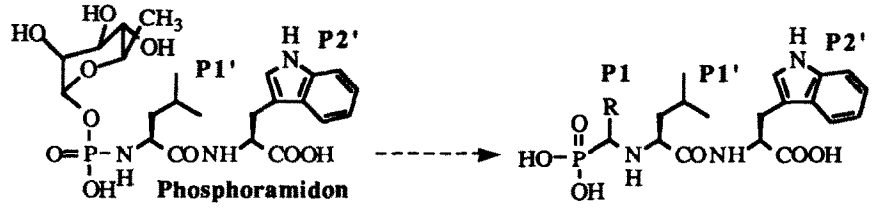
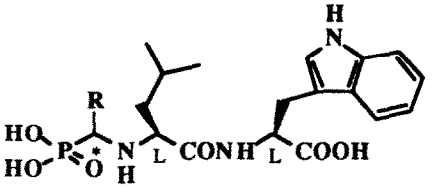


Table 2. ECE and NEP Inhibitory Activity of Aminophosphonic Acid Inhibitors



		IC_{50}, μM		selectivity[a]
Compounds	R	ECE	NEP	ECE/NEP
phosphoramidon		3.1	0.0042	1.4
VI	-H	3.4	0.024	7.1
VII[b]	$-CH_2CH_2CH_3$	15	0.55	37
VIIIa	$-CH_2CH_2C_6H_5$	(33%)[c]	-	-
VIIIb	$-CH_2CH_2C_6H_5$	1.1	0.13	120
IXa	$-CH_2CH_2CH_2C_6H_5$	(38%)[c]	-	-
IXb	$-CH_2CH_2CH_2C_6H_5$	7.1	1.1	150
X[d]	$-CH_2CH_2$-3-Indolyl	1.9	0.56	290
XIa	$-CH_2CH_2-C_6H_4$-4-OH	(42%)[c]	-	-
XIb	$-CH_2CH_2-C_6H_4$-4-OH	3.0	0.13	43
XIIa	$-CH_2CH_2$-1-Naphthyl	31	-	-
XIIb	$-CH_2CH_2$-1-Naphthyl	0.26	0.14	540

[a] (IC_{50} for NEP / IC_{50} for ECE) × 1000. [b] This compound was synthesized as a mixture of diastereoisomers at the α-position of the aminophosphonic acid moiety (55 : 45, estimated by ^{1}H-NMR). [c] % inhibition at 100 μM. [d] This compound was synthesized as a mixture of diastereoisomers at the α-position of the aminophosphonic acid moiety (55 : 45, estimated by ^{1}H-NMR).

greater ECE/NEP selectivity compared with that of the reference compound. These results indicate that ECE strictly discriminates the stereochemistry at this position and that an aryl-containing substituent is preferable for both ECE inhibition and ECE/NEP selectivity. Therefore, we further introduced some (hetero)aryl-alkyl groups onto the α-position to produce compounds **IX-XII**. In each analogue that was synthesized as a single isomer, only one of two diastereoisomers exhibited potent ECE inhibition. Replacement of the phenethyl group of **VIIIb** with a 3-phenylpropyl group resulted in a decrease in both ECE and NEP inhibitory activity (**IXb** vs **VIIIb**). Incorporation of a 2-(3-indolyl)ethyl group onto the α-position yielded compound **X** as a mixture of two diastereoisomers, the ratio of which was estimated as 55/45 by ^{1}H-NMR; therefore, the IC_{50} value of an active isomer of this compound could be estimated at about 1μM. Compound **X** was expected to exhibit highly potent ECE inhibition because the 2-(3-indolyl)ethyl group could mimic the P1 of big ET-1 (the side-chain of Trp^{21}). However, at best this compound exhibited ECE inhibition as potent as that of the phenethyl analogue with slightly improved ECE/NEP selectivity (**X** vs **VIIIb**). Incorporation of a 2-(4-hydroxyphenyl)ethyl group onto the α-position produced reduced ECE inhibition and ECE/NEP selectivity (**XIb** vs **VIIIb**), while incorporation of a 2-(1-naphthyl)ethyl group resulted in a marked increase in ECE inhibitory activity together with significant improvement in ECE/NEP selectivity (**XIIb** vs **VIIIb**). Compound **XIIb** was the most potent ECE inhibitor with the greatest ECE/NEP selectivity among this series of analogues.

Conclusions

The introduction of a P1 unit, which does not exist in the phosphoramidon structure, into inhibitor structures enhances ECE inhibitory activity and ECE/NEP selectivity. The P1 unit appears to be very important for both ECE inhibition and ECE/NEP selectivity. The representative compound, 3-(1-naphthyl)-1-phosphonopropyl-L-leucyl-L-tryptophan (compound **XIIb**), exhibits 10-fold more potent ECE inhibitory activity and 400 times greater ECE/NEP selectivity than does phosphoramidon. This compound is the most potent inhibitor of the phosphoramidon-sensitive metalloproteinase ECE yet known, and will be a useful tool for pharmacological studies of ECE.

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25. For the synthesis of II, Et_3N was used instead of KOH. Therefore, II was obtained as a 3 Et_3N salt.
26. After the final deprotection, these compounds were converted to tripotassium salts by adding three equivalents of KOH.
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28. Representative analytical data is shown below.
I: R_f 0.36 in 2-propanol/concentrated ammonium hydroxide, 1:1; ^{1}H-NMR (300 MHz, D_2O) δ 0.55 (3H, d, J = 6.6 Hz), 0.72 (3H, d, J = 6.6 Hz), 0.90-1.00 (1H, m), 1.10-1.20 (1H, m), 1.24-1.39 (1H, m), 3.14 (1H, dd, J = 9.5, 14.5 Hz), 3.41 (1H, dd, J = 4.7, 14.5 Hz), 3.40-3.50 (1H, m), 4.51 (1H, dd, J = 4.7, 9.5 Hz), 7.15 (1H, t, J = 7.5 Hz), 7.22 (1H, t, J = 7.5 Hz), 7.27 (1H, s), 7.47 (1 H, d, J = 7.5 Hz), 7.73 (1H, d, J = 7.5 Hz); ^{31}P-NMR (121 MHz, D_2O) δ 7.82; HRMS (FAB) calcd for $C_{17}H_{23}N_3O_6PK_2$ (M + 2K - H)$^+$: 474.0599; found: 474.0584.
II: R_f 0.39 in 2-propanol/concentrated ammonium hydroxide, 1:1; ^{1}H-NMR (300 MHz, D_2O) δ 0.84 (3H, d, J = 6.8 Hz), 0.97 (3H, d, J = 6.8 Hz), 1.27 (27H, t, J = 7.2 Hz), 2.03-2.16 (1H, m), 2.65-2.95 (2H, m), 3.19 (18H, q, J = 7.2 Hz), 3.47-3.60 (1H, m), 4.52-4.58 (1H, m); ^{31}P-NMR (121 MHz, D_2O) δ 6.09; HRMS (FAB) calcd for $C_{15}H_{34}N_4O_7P$ (M+ Et_3N + H)$^+$: 413.2165; found: 413.2175.
XIIa: R_f 0.12 in $CHCl_3$/MeOH/AcOH, 10:1:1; ^{1}H-NMR (300 MHz, D_2O) δ 0.71 (3H, d, J = 5.9 Hz), 0.73 (3H, d, J = 5.9 Hz), 1.21-1.50 (3H, m), 1.62-2.06 (2H, m), 2.49-2.61 (1H, m), 2.77-2.95 (2H, m), 3.00-3.35 (3H, m), 4.58-4.68 (1H, m), 6.99-8.01 (12H, m); ^{31}P-NMR (121 MHz, D_2O) δ 19.19; HRMS (FAB) calcd for $C_{30}H_{34}N_3O_6PK_3$ (M + 3K - 2H)$^+$: 680.1096; found: 680.1118.
XIIb: R_f 0.32 in $CHCl_3$/MeOH/AcOH, 10:1:1; ^{1}H-NMR (300 MHz, D_2O) δ 0.78 (3H, d, J = 5.7 Hz), 0.80 (3H, d, J = 5.7 Hz), 1.22-1.53 (3H, m), 1.55-1.84 (1H, m), 1.96-2.15(1H, m), 2.40-2.53 (1H, m), 2.58-2.74 (1H, m), 2.87-3.13 (2H, m), 3.23-3.40 (2H, m), 4.51 (1H, dd, J = 4.9, 8.4 Hz), 6.93-8.00 (12H, m); ^{31}P-NMR (121 MHz, D_2O) δ 20.42; HRMS (FAB) calcd for $C_{30}H_{34}N_3O_6PK_3$ (M + 3K - 2H)$^+$: 680.1096; found: 680.1112.
29. The ECE assay was carried out as previously reported.[13] Briefly, the enzyme fraction was prepared from the microsomal fractions of bovine cultured endothelial cells by solubilization with 0.5% Triton X-100. The substrate used for the assay was 1.0 μM big ET-1 and the product formed was measured by radioimmunoassay for ET-1.
30. The NEP assay was performed according to the method of Orlowski et al.[31] Briefly, microsomal fractions of rat small intestine were used as the NEP preparation. Synthetic substrate (glutaryl-Ala-Ala-Phe-4-methoxy-2-naphthylamide) was used for the assay.
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