J. Enzyme Inhibition, 1997, Vol. 12, pp. 71–77
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Amsterdam B.V. Published in The Netherlands
by Harwood Academic Publishers
Printed in Malaysia

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/17/11
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PHOSPHONAMIDE INHIBITORS OF NEUTRAL ENDOPEPTIDASE (EC 3.4.24.11)

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(Received 29 October 1996)

Keywords: 3.4.24.11 neutral endopeptidase; NEP; phosphonoamide; inhibitors; structure-activity relationship.

INTRODUCTION

Atrial Natriuretic Factor (ANF) is a peptide hormone secreted primarily from granules in atrial myocytes. ANF is produced in response to atrial expansion and has potent diuretic, and systemic and coronary vasodilatory properties. Endocrine effects include inhibition of renin release and reduction of aldosterone and noradrenaline levels.[1,2] These properties suggest that ANF may be of therapeutic value in cardiovascular disorders. It has been reported that prolonged infusion of ANF in hypertensive animals and man reduced blood pressure.[3,5] Moreover, intravenous infusion of the peptide in patients with severe Congestive Heart Failure (CHF) may have beneficial effects on cardiac function by reducing preload and afterload.[6,7] However, the therapeutic usefulness of exogenous ANF is limited both by its lack of oral bioavailability and by a rapid inactivation.[8,9] ANF is inactivated both by enzymatic degradation, by the neutral endopeptidase EC 3.4.24.11 (NEP), and by the binding with clearance receptors.

One approach to this problem has been to delay the inactivation of endogenous ANF by inhibiting the NEP activity. Endopeptidase inhibitors showed hypotensive effect in several animal models of hypertension[10–12], whereas in hypertensive patients they increased plasma ANF and diuresis and suppressed the renin-angiotensin-aldosterone system even though its effect in lowering blood pressure

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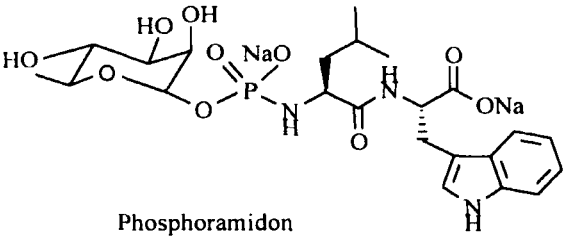
is questionable. In patients with CHF, the NEP inhibitor Candoxatrilat produced nearly as much natriuresis as furosemide, but had the advantage of causing greater falls in pulmonary wedge pressure and having a more favourable neuroendocrine profile.[13]

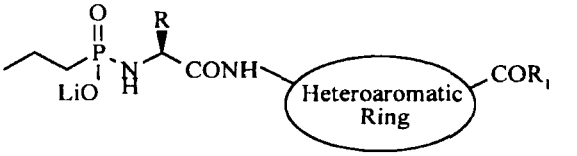
Recently, it has been reported that Candoxatril increased exercise tolerance and reduced symptoms in patients with mild and moderate CHF.[14] These results suggest that NEP inhibitors could be used as first-line therapy in mild CHF. Many centres are involved in the research and development of potent NEP inhibitors and some compounds have been identified.[15] Among these, Phosphoramidon has been reported to be a potent NEP inhibitor.[16] On the other hand to our knowledge, only phosphonamide derivatives of natural dipeptide as dual ACE and NEP inhibitors[17], or as Endothelin Converting Enzyme inhibitors[18], were investigated.

Thus, the preparation of phosphonamide derivatives with a non-peptide structure has not reported even though recently, De Lombaert *et al.*[19] have discovered a series of phosphonomethyl non-peptide inhibitors of NEP.

Furthermore with carboxylic acid inhibitors it has been already reported [20,21] that the P_2' residue could be replaced by anthranilic acid but the structure-activity relationships of the stereoelectronic requirements of the S_2' site of the enzyme have not yet been completely clarified. Therefore we investigated the following general formula (see Figure 1) and the structure-activity relationship is presented in this paper.



Phosphoramidon



R = alkyl, arylalkyl; R_1=OLi, OC_2H_5, $N(CH_3)_2$
Heteroaromatic Ring: pyridine, pyrrole, thiazole

FIGURE 1 Phosphoramidon and proposed analogues.

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METHODS

Neutral Endopeptidase Activity Assay

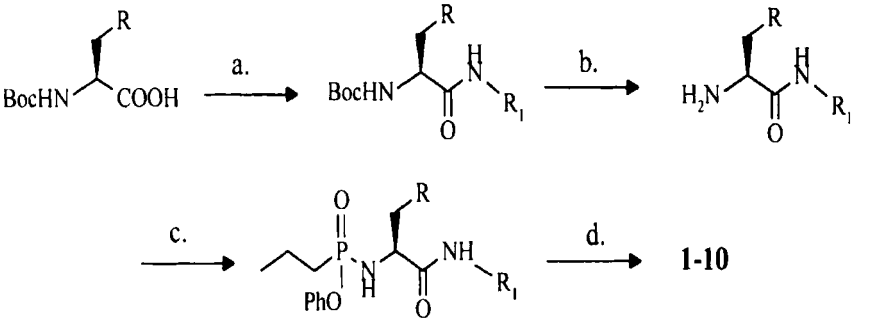
Membranes from rat kidney cortex were prepared as described by Maeda *et al.*[22] The cortex was dissected from rat kidney and suspended in phosphate buffer pH 7.4. The tissue was homogenized, layered over a solution of 41% (w/v) sucrose and centrifuged. The membranes were collected from the buffer/sucrose interface, washed twice and stored at −70° C. The NEP assay was performed according to the method of Llorens *et al.*[23] [^{3}H][Leu^5] Enkephalin was added as substrate and the metabolite [^{3}H]-Tyr-Gly-Gly was quantified, after chromatography on polystyrene bed columns, by liquid scintillation spectrometry.

Angiotensin-Converting Enzyme Activity Assay

The ACE activity was evaluated in purified rabbit lung preparation by a spectrophotometric technique using furylacryloyl-phenyl-alanyl-glycyl-glycine (FAPGG) as substrate in accordance with Holmquist.[24]

Synthesis

The general method of synthesis for the compounds described in Table I is reported in Scheme 1. Compounds **1–10** were synthesized using standard methods



SCHEME 1 General scheme of synthesis.
R = iPr; C_6H_5; 3,4-$Cl_2C_6H_3$; 4-F-C_6H_4; 4-C_6H_5-C_6H_4
R_1 = 4-CH_3OOC-2-pyridyl; 1-C_2H_5OOC-4-pyrrolyl;4-$C_2H_5OOCCH_2$-2-thiazolyl, 4-$(CH_3)_2NCOCH_2$-2-thiazolyl.
a. (1) *N*-Hydroxysuccinimide DCC, 3h, 20°C, (2) R_1-NH_2, dioxane, 16h, 20°C;
b. HCl, AcOEt, 2h, 20°C;
c. n-PrPO$(Cl)_2$, phenol, TEA, CH_2Cl_2, 16h, 20°C;
d. base, THF/water.

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by condensing the appropriate *N*-t-(Butyloxycarbonyl)-L-amino-acid with the heterocycloamines (R_1-NH_2)[25] followed by the removal of the protecting group (Boc). A solution of propylphosphonic dichloride, phenol and triethylamine[26] in methylene chloride was stirred at room temperature for 2 h then added to a solution of the dipeptide dissolved in methylene chloride. Finally, the phenoxy ester and the protection of the terminal carboxylic acid were simultaneously removed with lithium hydroxide in THF/water affording **1–10**, except **8** and **9**. These two compounds were obtained by mild basic selective hydrolysis of the phenoxy ester with $NaHCO_3$ in THF/water.

Compounds **1–10** were isolated and tested as the lithium or sodium salt; they showed a complete stability in aqueous solution at pH ≥ 4 but at lower pH the hydrolysis of the phosphonoamido bond was observed.

RESULTS AND DISCUSSION

Phosphoramidon is a natural compound possessing a potent NEP inhibition action but the presence of a sugar moiety and a natural dipeptide backbone suggests a rapid clearance due to metabolism. S. Bertenshaw *et al.*[18] have demonstrated that the replacement of the rhamnose ring by an n-propyl group provided **1** with an $IC_{50} = 30$ nM on NEP (the report IC_{50} was 10 nM) substantially maintaining the phosphoramidon potency.

Starting from this structure, we investigated the substitution of tryptophan with non-natural aminoacids characterized by an heteroaromatic ring (Table I). Compounds **1–10** were tested as NEP inhibitors and the results are summarized in Table I. The first compound of this class was the pyridine derivative **2** which was as active as **1**. Since it has been reported that NEP inhibitors accept an aromatic substitute at P_1' position[26], we introduced a phenyl ring i.e. **3** vs **2**, which led to a 5–6 fold increase in activity. Other heteroaromatic rings such as the pyrrole **4** and thiazole **7**, with opposite electronic features, were investigated. It is worth noting that both maintain the same activity, demonstrating the high steric and electronic tolerance of the S_2' site of the enzyme. Moreover, although the substitution of the carboxylic acid group with either ester **8** or amide **9**, decreased the activity 4-fold, it is interesting that both compounds are still potent inhibitors. On the other hand, previous SAR studies with NEP inhibitors have already demostrated that the carboxylic acid is not essential for this activity.[27] It was also found that the introduction of one methylene group between the carboxylic acid and the aromatic ring was allowed i.e. **7** vs **3** and **4**.

Other structural requirements at P_1' position were investigated. The introduction of halogens such as chlorine **5** and fluorine **10** seemed to be well accepted. In the case

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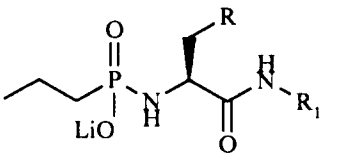
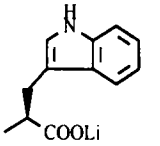
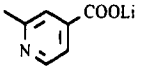
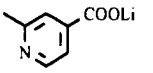
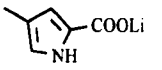
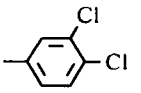
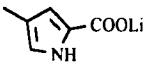
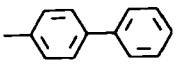
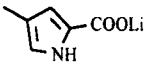
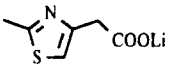
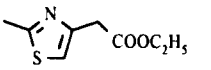
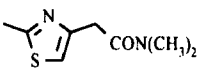
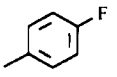
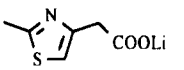
TABLE I Effect of structural modification on NEP inhibition.



	R	R_1	*NEP-IC50 (nM)*
	Phosphoramidon	–	20
1	-$CH(CH_3)_2$		30[a]
2	-$CH(CH_3)_2$		35
3			6
4			7
5			6
6			11
7			10
8[b]			42
9[b]			43
10			13

[a]Literature value IC_{50} = 10 nM[18], [b]Sodium salt. The IC_{50} value of the compounds tested was determined using increasing concentrations in triplicate. Each value represents the mean of two experiments.

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of the N-phosphonomethyl class, De Lombaert *et al.*[28] identified that the presence of a 4-phenylphenyl moiety at the P'_1 position increased the activity by no less than three orders of magnitude compared with its simpler phenyl analogue. However **6**, which is characterized by the same substitution, did not show any improvement in activity but confirmed the high volume tolerance of the S'_1 site of the enzyme.

All compounds were also tested in order to evaluate Angiotensin Converting Enzyme inhibition but none of our compounds elicited inhibitory effects up to 1000 nM concentration.

To summarize,

- Structural flexibility and opposite electronic effects exist in the selection of the amino acid interacting with the S'_2 site (**2–10**).
- The carboxylic acid modulates, but is not essential for the activity (**8** and **9**)
- An aromatic ring at the P'_1 position is more readily accepted than an alkyl group (**3** vs **2**).
- The S'_1 site of the enzyme accepts bulky lipophilic substitutes (**5** and **6**).

In conclusion, potent NEP inhibitors can be obtained with a single phosphorylated α-amino-acid coupled with heteroarylamines and our results contribute to the general understanding of the structural requirement for potent inhibitory binding with NEP.

Acknowledgements

We thank Dr. P. Castelnovo, R. Fattori and Zambon's Analytical Chemistry Staff for providing the analytical data on compounds described in this study.

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