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N-[2-(Indan-1-yl)-3-mercapto-propionyl] Amino Acids as Highly Potent Inhibitors of the Three Vasopeptidases (NEP, ACE, ECE): In Vitro and In Vivo Activities

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Abstract—We have previously reported the design of a lead compound **1a** for the joint inhibition of neprilysin (NEP, EC 3.4.24.11), angiotensin converting enzyme (ACE, EC 3.4.15.1) and endothelin converting enzyme (ECE-1, EC 3.4.24.71), three metallopeptidases which are implicated in the regulation of fluid homeostasis and vascular tone. We report here the synthesis and biological activities of analogues derived from this lead with inhibitory potencies in the nanomolar range for the three enzymes. Compounds **8b** and **15c** are the most potent triple inhibitors described to date.

Two counteracting peptidergic systems regulate fluid homeostasis and vascular tone,[1] the first one being constituted by endothelin-I (ET-1)[2] and angiotensin II (AII) two potent vasoconstrictive peptides, which are respectively processed by endothelin converting enzyme (ECE-1, EC 3.4.24.71) and angiotensin converting enzyme (ACE, EC 3.4.15.1). The second system considered as physiologically antagonist to the first one, is constituted by bradykinin (Bk) as vasorelaxant peptide, and the atrial natriuretic peptide (ANP) which reduces heart overload by increasing diuresis. The inactivation of the two latter peptides is essentially controled by ACE and neutral endopeptidase (neprilysin, NEP, EC 3.4.24.11) which, like ECE-1, belong to the same family of zinc metallopeptidases.[3] Recent studies have shown that the effects of dual inhibitors of ACE and NEP appear superior to those observed with specific ACE inhibitors like captopril in treatment of severe cardiovascular diseases.[4] However, ET-1 which is the most potent vasoconstrictive factor is inactivated by NEP, thus minimizing the vasorelaxant properties of dual ACE/NEP inhibitors. Therefore, simultaneous inhibition of NEP, ACE, and ECE by a single inhibitor is expected to produce a therapeutic efficacy superior to that observed with dual inhibitors[5] with possible use in several diseases including essential hypertension, chronic heart failure, pulmonary hypertension, vascular remodeling, renal dysfunction, prostatic hypertrophy and ischemia.[6] Up to now, only some compounds acting as inhibitors of the three enzymes have been reported, most of them issued from the few pharmaceutical firms involved in this field.[7,8] We have recently designed a lead compound **1** (Table 2), capable of inhibiting NEP, ACE and ECE with K_i values of 1.8, 20, and 100 nM respectively.[9] However, it was necessary to optimize the compound **1** in order to obtain inhibitors nearly equipotent on the three targeted enzymes. This has been achieved by introducing various substituents on the indanyl moiety of the P'_1 residue, leading to the first inhibitors of NEP, ACE and ECE with nanomolar inhibitory potencies.

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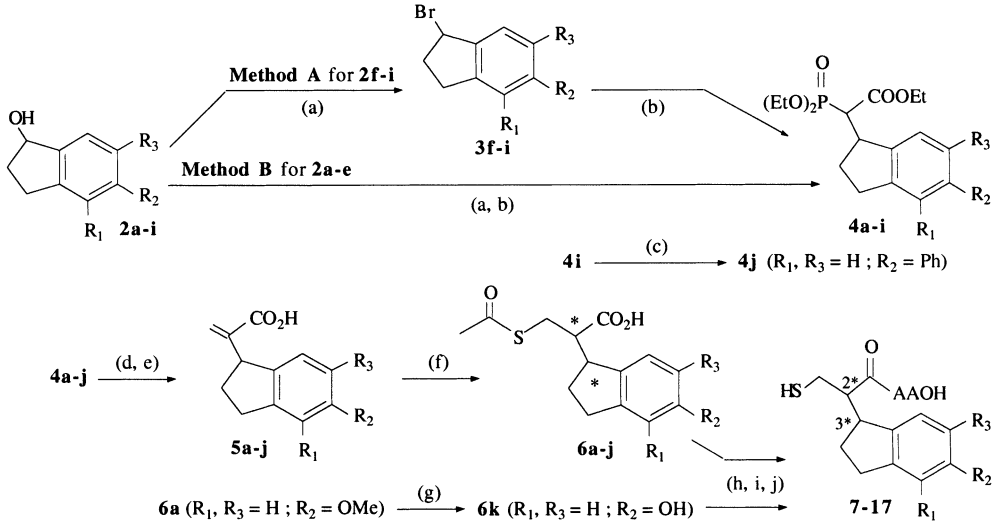


Scheme 1. Synthesis of the inhibitors **7**–**17**. Method A: (a) Me_3SiBr, $CHCl_3$; (b) triethylphosphonoacetate, NaH, DMF; Method B: (a) Me_3SiBr, THF −78 °C ; (b) triethylphosphonoacetate, NaH, THF −78 °C; (c) $PhB(OH)_2$, DME, Na_2CO_3 ; (d) $(H_2CO)_n$, THF, K_2CO_3; (e) NaOH, acetone; (f) thioacetic acid, $CHCl_3$; (g) BBr_3, CH_2Cl_2; (h) EDCI, HOBt, HAAOt-Bu, Et_3N; (i) TFA, CH_2Cl_2; (j) NaOH, then HCl, MeOH.

The synthesis, biochemical and preliminary pharmacological properties of these new vasopeptidase inhibitors (patent Fr No. 00 01937/17 February 2000) are reported in this paper. The target molecules **7**–**17** were prepared (Scheme 1) using the synthetic route previously described[9] starting from various substituted 1-indanol **2a**–**i** as precursors (Table 1). Due to the presence of two unresolved asymmetric carbons in synthon **6**, this method affords the inhibitors as a mixture of four stereoisomers.

Table 1. Various substituted 1-indanol precursors

Compd **2**	**a**[10]	**b**[11]	**c**[12]	**d**[13]	**e**[10]	**f**[10]	**g**[10]	**h**[14]	**i**[10]
R_1	H	H	H	H	H	OMe	H	H	H
R_2	OMe	SMe	NMe_2	OEt	OMe	H	H	CN	Br
R_3	H	H	H	H	OMe	H	OMe	H	H

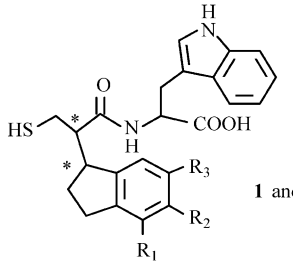
The compounds thus prepared were tested in an initial screening as a mixture of stereoisomers for their ability to inhibit NEP, ACE and ECE activities in vitro according to previously described assays. Table 2 summarizes the results obtained by introduction of various groups in the phenyl ring of the indanyl moiety of **1**. Methoxy, methylthio or bromide group which have about the same dimension were introduced in R_2 position of the indanyl group (**8**, **11**, and **15**) leading to a significant improvement in the recognition of ECE (K_i 22–35 nM) without change on NEP and ACE affinities (Table 2). Conversely, the introduction of one methoxy in R_1 or R_3 position or two methoxy in R_2 and R_3 positions of the indanyl moiety decreases the inhibitory potency on ECE (**7**, **9**, and **10**). The presence of a basic substituent on the P'_1 moiety (**12**) is also unfavourable for ECE recognition. The same result was observed when linear group (**17**) or bulky substituent (**16**) is introduced at the R_2 position of the indanyl group. In the first round of assays, the various inhibitors were tested as a mixture of four stereoisomers (Table 2).

Nevertheless, we have previously shown that the stereochemistry of metallopeptidase inhibitors is important for optimal recognition of the enzymes.

It was therefore critical to compare the relative activities of the separate isomers.

The best compounds **8** and **15** were selected for separation and structure determination. The separation of the four stereoisomers was done by semi-preparative HPLC, as previously described,[9] and the determination

Table 2. In vitro inhibition of NEP,[15] ACE[16] and ECE,[17] activities by compounds **1** and **7**–**17**



1 and **7-17**

				K_i (nM)[a]		
Compd	R_1	R_2	R_3	NEP	ACE	ECE
1	H	H	H	1.8±0.2	20±2	100±10
7	OMe	H	H	16±3	8±1	760±40
8	H	OMe	H	5±0.2	11±1	22±2
9	H	H	OMe	11±1	35±3	79±3
10	H	OMe	OMe	78±7	78±5	560±20
11	H	SMe	H	6±0.1	14±1	35±2
12	H	NMe_2	H	15±1	44±3	370±10
13	H	OEt	H	8±2	18±4	47±3
14	H	OH	H	4±0.1	7±0.2	90±6
15	H	Br	H	5±0.1	12±2	31±3
16	H	Ph	H	14±1	27±7	140±15
17	H	CN	H	11±1	3±0.2	210±10

[a]Values are means of three experiments performed in triplicate. All compounds are mixture of four stereoisomers.

of their absolute configuration was achieved by NMR spectroscopy.[9] The chemical shift of the indanyl CH_2 protons in the vicinity of the substituted asymmetric carbon (C_3, Fig. 1) are dependent on the configuration of the C_2, C_3 carbons as shown in Table 3. This has been clearly established previously with dipeptides[18] or thiol-derived inhibitors.[19] X-ray crystallography was used to confirm the absolute configuration proposed from NMR in the case of compound **15c**. For this purpose, replacement of the tryptophan at the P'_2 position by alanine was achieved in order to facilitate structure assignment of synthon **6i$_1$** obtained in enantiomeric pure form by resolution with *R*-(+)-α-methylbenzylamine (Scheme 2). Coupling of **6i$_1$** with alanine gives compound **18c** (Scheme 2). Suitable crystals were obtained by crystallization in acetonitrile. X-ray crystallography confirms the 2*S*,3*R* absolute configuration of **18c** and thus of **6i$_1$** (Fig. 1).

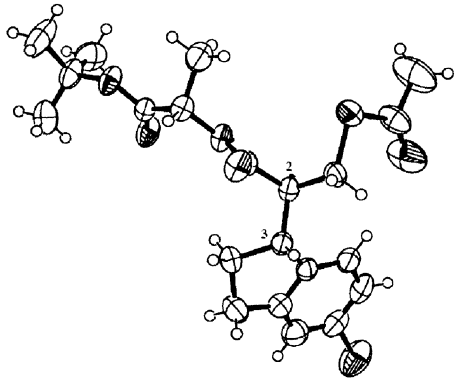
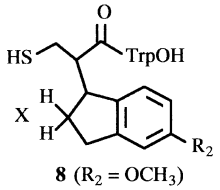


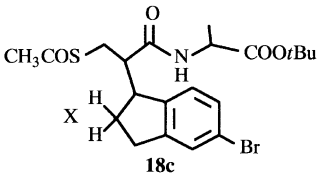
Figure 1. Ortep[20] view of compound **18c**.

As observed in a previous study with compound **1**,[9] the 2*S*,3*R* configuration leads to good NEP/ACE inhibition with K_i values in the nanomolar range for the tested compounds **8** and **15** (Table 4). The 2*R*,3*R* configurations of **8** and **15** are the most efficient regarding ECE

Table 3. ^{1}H NMR data for compounds **8(a–d)**, **15(a–d)** and **18c**



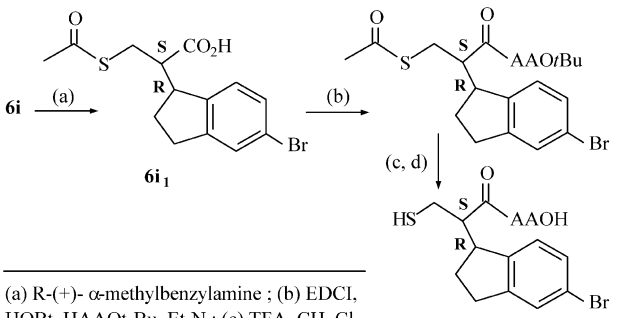
8 (R_2 = OCH_3)
15 (R_2 = Br)



18c

Compd	Stereo	Chemical shifts DMSO-d_6 of X protons (δ ppm)	$[\alpha]_{\Delta}^{23}$
8a	2*S*,3*S*	1.8, 2.0[a]	+36.2
8b	2*R*,3*R*	1.65	−16.1
8c	2*S*,3*R*	1.95	+25.2
8d	2*R*,3*S*	1.5, 1.70[a]	−48.2
15a	2*S*,3*S*	1.8, 2.0[a]	+11.1
15b	2*R*,3*R*	1.65	−5.8
15c	2*S*,3*R*	1.95	+38.0
15d	2*R*,3*S*	1.55, 1.70[a]	−25.9
18c	2*S*,3*R*	2.1	−18.7

Optical rotations were recorded in acetone c = 1.
[a]The values correspond to chemical shifts of non equivalent protons.



(a) R-(+)- α-methylbenzylamine ; (b) EDCI, HOBt, HAAOt-Bu, Et_3N ; (c) TFA, CH_2Cl_2, (d) NaOH, MeOH, then HCl.

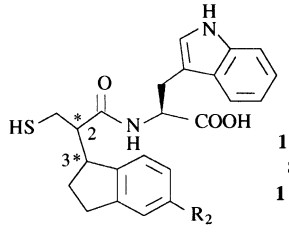
15c : AAOH : Trp
18c : AAOH : Ala

Scheme 2. Synthesis of the inhibitors **15c** and **18c**.

recognition with K_i values in the 10^{-8} molar range (**8b**, **15b**). These values are of the same order than the K_i values obtained for CGS 31447.[21] This inhibitor has been synthesized in our laboratory with the aim to investigate its inhibitory potency towards ACE which was not reported. As shown in Table 4, CGS 31447 has a low affinity for this zinc metallopeptidase. Furthermore, it can be noticed that in this series of inhibitors the stereochemical preferences for ACE and ECE are different. Thus the 2*S*,3*R* stereoisomers are the best ACE inhibitors whereas the most favourable configuration is 2*R*,3*R* for optimal ECE inhibition (Table 4).

NEP displays no clear stereochemical preference although the best inhibitors belong to the 2*R*,3*R* series.[22] This is consistent with the close similarity of NEP and ECE active sites.[23,24] Due to their efficient inhibitory potencies in vitro on NEP, ACE, and ECE compound **15c** was used to measure its in vivo activity. Furthermore,

Table 4. In vitro inhibition of NEP, ACE and ECE, for the pure stereoisomers of compounds **1**, **8** and **15**



1a (R_2 = H),
8 (R_2 = OMe)
15 (R_2 = Br)

Compd	Stereochemistry	K_i (nM)[a] NEP	ACE	ECE
SCH 24470[7]		90[b]	2.5[b]	80[b]
CGS 31447[14]		2.2±0.4	>10000	11±1
1a	2*R*,3*R*	0.7±0.03	43±2	26±3
8a	2*S*,3*S*	30±2	70±2	1460±240
8b	2*R*,3*R*	1.3±0.1	24±3	10±1
8c	2*S*,3*R*	2.7±0.3	4.6±0.5	100±8
8d	2*R*,3*S*	15±2	30±4	180±7
15a	2*S*,3*S*	9.4±0.3	58±6	110±10
15b	2*R*,3*R*	1.8±0.3	55±4	18±3
15c	2*S*,3*R*	3.8±0.3	4.1±0.3	28±2
15d	2*R*,3*S*	13±0.7	66±5	90±10

[a]Values are means of three experiments performed in triplicate.
[b]IC_{50}.

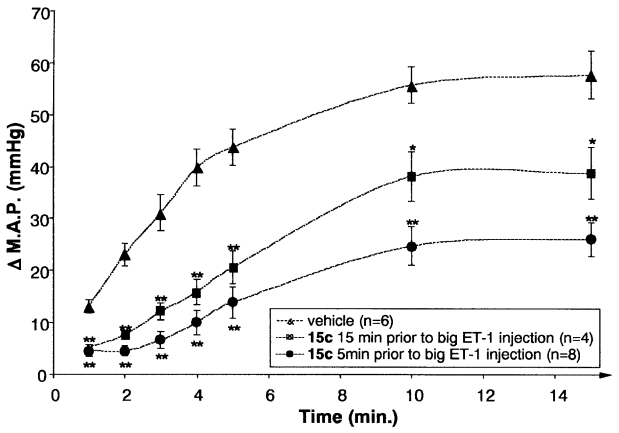


Figure 2. Inhibition of Big ET-1 (1 nmol/kg, iv) pressor response by compound **15c** iv administered in anesthetized rats. $*p<0.05$, $**p<0.01$ versus control group (Dunnett's test).

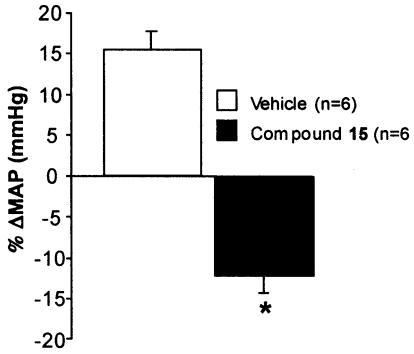


Figure 3. Inhibition of angiotensin I (1 nmol/kg, iv) pressor response by compound **15** iv administered in anaesthetized rats. $*p<0.05$ versus control group (Dunnett's test).

15c was choosen because it can be obtained in larger amounts than **18c** following the synthetic pathway described in Scheme 2.

Compound **15c** was evaluated for its ability to inhibit the pressor response produced by big ET-1 in anesthetized (pentobarbital sodium 60 mg/kg ip), ganglion-blocked (chlorisondamine 2 mg/kg iv) male Wistar rats (Fig. 2).[25] Intravenous injection of 1 nmol/kg big ET-1 in rats treated with vehicle at 1 mL/kg iv (150 μL EtOH, 150 μL Chremophor, 10 μL DMSO, 1350 μL saline) resulted in an 57.2 ± 4.6 mmHg increase in mean arterial pressure (MAP).

In comparison, the sodium salt of **15c** administered iv at 30 mg/kg (59 μmol/kg) 5 or 15 min prior to the big ET-1 challenge, inhibited the big ET-1 pressor response by 54 and 33%, respectively, after 15 min (Fig. 2). It should be noted that injection of the sodium salt of **15c** alone is followed by a -15.2 ± 2.4 mmHg decrease of MAP which is very likely due to ACE inhibition as previously reported.[26]

Furthermore, the in vivo ACE inhibitory effect of compound **15** was evaluated by assessing its effects on MAP responses elicited by angiotensin I (AI 500 ng/kg iv) in anesthetized male Wistar rats (Fig. 3). Intravenous injection of AI in vehicle at 1 nmol/kg iv increased MAP by 15 ± 2 mm Hg. In comparison, the sodium salt of **15** administered iv at 30 mg/kg (59 μmol/kg) 5 min prior to the AI injection completely blocked the pressor response induced by AI.

The in vivo inhibition of lung NEP in mice was carried out as previously described.[27] A 70% blockade of the enzyme is observed for compound **15c** injected at 25mg/kg iv.

In conclusion, the new potent triple inhibitor of NEP, ACE and ECE, **15c** generated after optimization of the lead compound **1** fulfils the requirements of potency for investigations on animal models of cardiovascular diseases. This compound is the first reported inhibitor able to jointly block in vivo the three enzymes involved in the regulation of blood arterial pressure. Further pharmacological studies are necessary to shed more light on the advantages of this first described triple inhibitor over dual NEP/ACE inhibitors in order to validate the concept of triple inhibition.

Supplementary material

Crystal data for **18c**: formula $C_{21}H_{28}BrNO_4S$ monoclinic, $[\alpha]_{\Delta}^{23}=-18.7$ space group $P2_1$; $a=8.532(3)$, $b=9.667(4)$, $c=14.571(8)$ Å, $\beta=105.56(2)$, $V=1158(1)$ Å^3, $Z=2$, 7294 independent reflections, 3920 with $I>2\sigma(I)$, $R1=0.0735$, $wR2=0.2120$. The structure was solved by SHELXS-97[28] and refined using SHELXL-97.[29] Crystallographic data (excluding structure factors) have been deposited to the Cambridge Crystallographic Data Center as supplementary publication no. CCDC 177861. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK (fax: +44-1223-336033; e-mail: deposit@ccdc.cam.ac.uk).

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