New Dual Inhibitors of Neutral Endopeptidase and Angiotensin-Converting Enzyme: Rational Design, Bioavailability, and Pharmacological Responses in Experimental Hypertension

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Received November 16, 1993®

In the treatment of cardiovascular diseases, it could be of therapeutic interest to associate the hypotensive effects resulting from the inhibition of angiotensin II formation, ensured by endothelial angiotensin-converting enzyme (ACE), with the diuretic and natriuretic responses due to the protection of the endogenous atrial natriuretic peptide (ANP) from inactivation by epithelial neutral endopeptidase (NEP). However, an investigation of this hypothesis requires an orally active compound able to jointly inhibit ACE and NEP. Dual inhibitors have therefore been designed by a rational approach, based on the characteristics of the active sites of both enzymes, which belong to the same family of zinc metallopeptidases, and on the structures of their most potent and selective inhibitors. As both NEP and ACE contain a large S'_1-S'_2 domain able to accommodate aromatic residues, the cyclic ACE inhibitor 3-(mercaptomethyl)-3,4,5,6-tetrahydro-2-oxo-1*H*-1-benzazocine-1-acetic acid was selected as a template. Various aliphatic constraints were introduced on the benzyl moiety of the potent NEP inhibitor *N*-[2-(mercaptomethyl)-3-phenylpropanoyl]-L-tyrosine (IC_{50} NEP = 2 nM, IC_{50} ACE = 25 nM) to improve the fit between the computed most stable conformers of these molecules and the ACE template. New dual inhibitors, of general formula, *N*-[2(*R*,*S*)-(mercaptomethyl)-3(*R*,*S*)-phenylbutanoyl]-L-amino acid with IC_{50} values in the nanomolar range for both enzymes were generated by this approach. The separation of the four stereoisomers using chiral amines and the stereoselective synthesis of the 2-(mercaptomethyl)-3-phenylbutanoyl moiety showed that inhibitors with the 2*S*,3*R* configuration are the most potent on both NEP and ACE. The "in vivo" potency of various prodrugs of these inhibitors to inhibit ACE activity in lung and NEP activity in kidney was measured after oral administration in mice. From this pharmacokinetical study the most potent dual inhibitor RB 105 (*N*-[(2*S*,3*R*)-2-(mercaptomethyl)-3-phenylbutanoyl-L-alanine (compound **44c**) (K_I NEP 1.7 nM, K_I ACE 4.5 nM) and its most efficient *in vivo* prodrug mixanpril, [*N*-[(2*S*,3*R*)-2-[(benzoylthio)methyl]-3-phenylbutanoyl]-L-alanine (compound 18) (ED_{50} NEP ~ 1 mg/kg, ED_{50} ACE ~ 7 mg/kg) were selected. Competition experiments with a tritiated inhibitor of ACE or NEP bound to mouse lung and kidney membranes respectively showed that mixanpril has a long duration of action (>8 h). As expected, after iv administration in the spontaneously hypertensive rat (SHR), RB 105 decreased blood pressure and increased diuresis and natriuresis. Both effects were also observed after chronic oral administration of 50 mg/kg mixanpril twice a day in SHR. These results indicate that an efficient and orally active dual inhibitor of NEP and ACE produces beneficial changes in hemodynamics and could represent a therapeutic progress in the treatment of cardiovascular diseases.

Introduction

Angiotensin converting enzyme (EC 3.4.25.1, ACE) and neutral endopeptidase 24.11 (EC 3.4.24.11, NEP, neprilysin) are two ectoenzymes involved in the metabolism of a variety of regulatory peptides (review in ref 1) and particularly those involved in the control of blood pressure and fluid homeostasis (review in ref 2). ACE converts the inactive precursor angiotensin I into angiotensin II, a peptide which promotes vasoconstriction and sodium retention. NEP inactivates the atrial natriuretic peptide (ANP) (review in ref 3), a hormone which induces diuresis and natriuresis, and both ACE and NEP are responsible for the degradation of the vasorelaxant peptide bradykinin at its endothelial and epithelial sites of action respectively.[4,5] Selective inhibitors of ACE (review in ref 6) are clinically useful compounds for the treatment of hypertension[7] and are often used in association with diuretics that increase plasma renin and aldosterone secretion and produce hypokaliema. Selective NEP inhibitors[8-10] have been developed with the aim of protecting ANP and their efficiency in inducing diuretic and natriuretic effects has been demonstrated in various animal models[8-11] and in humans.[12-16] However, these renal effects were not associated with any significant decrease in blood pressure or left ventricular hemodynamic load.[15] This has been suggested to result from the physiological antagonism between the renin-angiotensin system and the facilitation of renal hemodynamics and sodium excretion induced by ANP.[1]

Taken together, these results suggested that a simultaneous inhibition of ACE, to reduce angiotensin II formation, and NEP, to protect ANP from inactivation,

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® Abstract published in *Advance ACS Abstracts*, March 1, 1994.

0022-2623/94/1837-1070$04.50/0

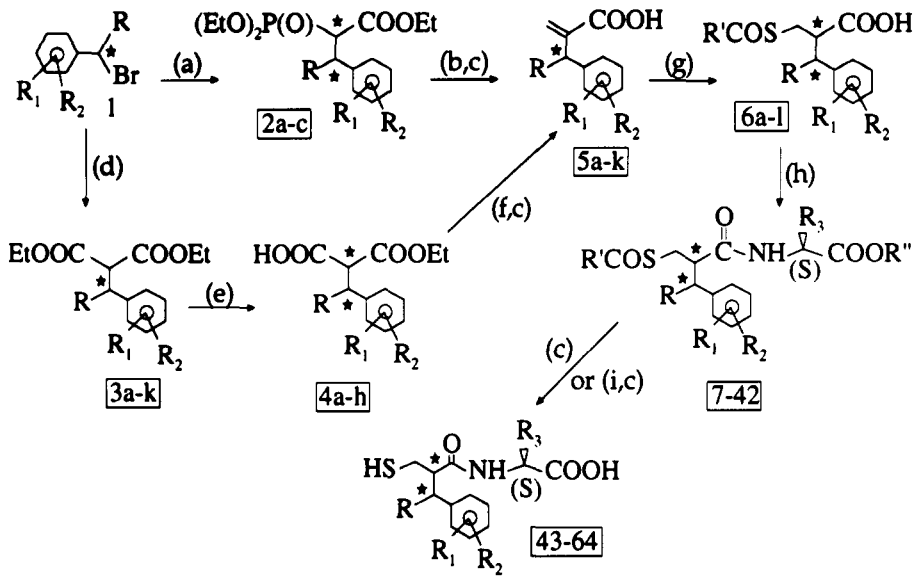


Figure 1. Scheme for the synthesis of the dual NEP/ACE inhibitors as racemic mixtures: (a) NaH, $(EtO)_2P(O)CH_2COOEt$; (b) $H_2C{=}O$, K_2CO_3; (c) OH^- then H_3O^+; (d) $CH_2(COOEt)_2$, EtONa; (e) KOH, EtOH; (f) $H_2C{=}O$, Et_2NH; (g) RCOSH in CH_2Cl_2; (h) (*S*) $H_2NCH(R_3)COOR''$, DCC, HOBt; (i) TFA in CH_2Cl_2.

might have a therapeutic advantage in the treatment of cardiovascular diseases.[17] In agreement with this, the association of selective inhibitors of the two enzymes has been shown to result in a potentiation of their respective effects in various experimental models of hypertension in rats[18,19] and in dogs with pacing-induced heart failure.[20] Nevertheless, for reasons of bioavailability, pharmacokinetics, and toxicity it would be more interesting to use dual inhibitors of NEP and ACE. The first potent mixed inhibitor of NEP and ACE, *N*-[2-(mercaptomethyl)-3-phenylpropanoyl]-L-leucine was described 10 years ago[21-23] and was recently found to produce depressor activity both in DOCA salt rats and spontaneously hypertensive rats (SHR) after iv administration at high doses (100–300 mg/kg).[18] Alatriopril, another closely related analog of thiorphan *N*-[2-(mercaptomethyl)-3-phenylpropanoyl]glycine,[1] was also studied after iv administration in anaesthesized rodents and shown to induce responses associated with ANP protection but without modifying arterial pressure.[24] A highly efficient and constrained mixed inhibitor, able to significantly reduce the blood pressure in SHR and DOCA salt rats after oral administration, has been recently described.[25] However, neither diuretic nor natriuretic effects were reported for this compound. The incomplete responses obtained with all these inhibitors could be related to either a too low affinity for NEP and/or ACE and/or difficulty in reaching the target enzymes.

Independently of these studies, we have designed, with the assistance of molecular modeling, a new series of dual inhibitors of ACE and NEP which possess the critical requirements for possible clinical use, i.e., nanomolar affinity for both enzymes, appropriate bioavailability, allowing inhibition of endothelial ACE and blockade of epithelial NEP, oral activity, and long duration of action. For this purpose, noncyclic and hydrophobic constraints were introduced in the structure of selective inhibitors of NEP, such as thiorphan and analogs[23] by taking into account the similarities and differences in active site recognition of the recently cloned NEP[26] and ACE.[27]

Mercapto- instead of carboxyl-containing molecules, were selected because the thiol group has been shown to yield more potent NEP inhibitors,[28] while the affinities of both types of molecules for ACE are identical. Furthermore, the thiol-containing inhibitors, captopril *N*-[(2*S*)-2-(mercaptomethyl)-3-methylpropanoyl]proline[29] and thiorphan *N*-[2-(mercaptomethyl)-3-phenylpropanoyl]glycine[30] are both on the market and appear to be devoid of major side effects.

Results

(1) Synthesis. The synthesis of the inhibitors **43–64** was easily performed by coupling the various α-amino esters to the appropriate 2-[(acylthio)methyl]-3-(substituted)phenylalkanoic acids **6** by the classical DCC/HOBt method (Figure 1). The deprotection of the functional groups of **7–42** was obtained in a single step by saponification when R″ was a methyl or a benzyl group. When R″ was a *tert*-butyl group, a sequential deprotection was required and partially protected inhibitors could be isolated. Two synthetic pathways were used for the preparation of the acids **6** from their corresponding bromo derivatives **1**. The first, passing by the ethyl 2-substituted (diethylphosphono)acetates **2**, followed by a Wittig–Horner reaction, led to the acids **6** in four steps. Alternatively, going from the 2-substituted diethylmalonates **3** and using a Mannich reaction, the acids **6** were obtained in five steps with similar global yields (Figure 1).

In the two strategies used, compounds **7–42** and **43–64** were obtained as mixtures of four stereoisomers, which are diastereoisomers except when the C-terminal amino acid is a glycine (compounds **7** and **43**). These diastereoisomers could be separated by HPLC under analytical conditions, but not under preparative conditions, because of the closeness of their retention times. Therefore, in order to obtain large quantities of each isomer, chiral amines were used for the separation of the four stereoisomers of the racemic synthon 2-[(acetylthio)methyl]-3-phenylbutanoic acid, **6a**. As shown in Figure 2, the four stereoisomers of **6a** (**6a**$_1$–**6a**$_4$) were obtained using successively, the (*S*)-(−)- and the (*R*)-(+)-α-methylbenzylamines, and the (*S*)-(−)- and (*R*)-(+)-α-naphtylethylamines followed by recrystallization of the precipitated salts in 2-propanol. By this method, an enantiomeric enrichment greater than 95% was observed by HPLC, after coupling of the separate isomers of **6a** with (*S*)-alanine benzyl ester and deprotection. The four stereoisomers

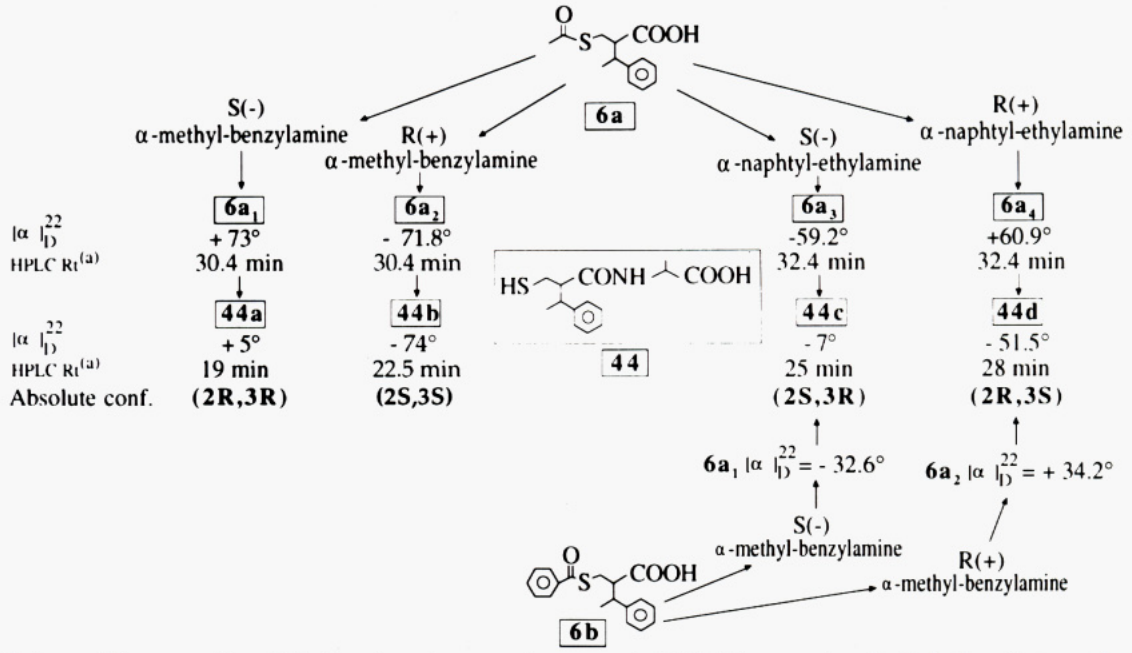


Figure 2. Scheme of the separation of the four stereoisomers of compound **44** [*N*-(2-(mercaptomethyl)-3-phenylbutanoyl)-L-alanine]: (a) C_8 KWI column; TFA 0.05%/CH_3CN, 60/40; (b) C_{18} Kromasil; TFA 0.05%/CH_3CN, 70/30.

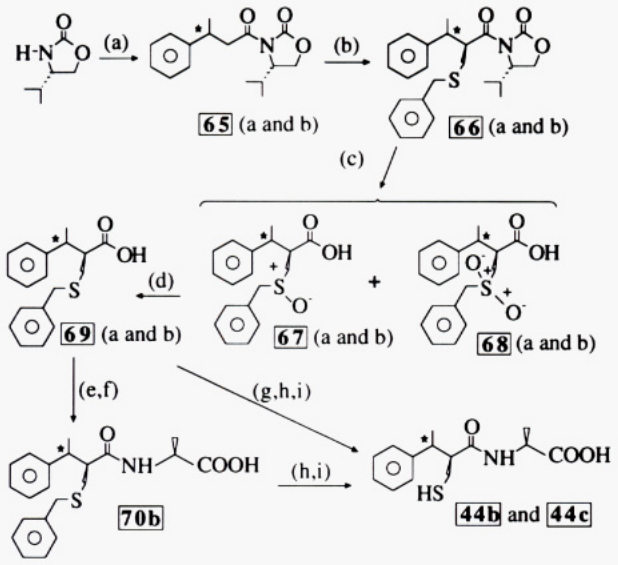


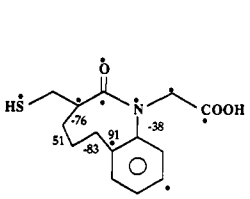
Figure 3. Scheme for the stereoselective synthesis of the 2*S*,3*R* and 2*S*,3*R* isomers of compound **44** [*N*-[2-(mercaptomethyl)-3-phenylbutanoyl)-L-alanine]: (a) $PhCH(CH_3)CH_2COCl$ (*R* and *S*), *n*-BuLi; (b) LDA, $PhCH_2SCH_2Br$; (c) LiOH, H_2O_2; (d) PPh_3, CCl_4; (e) L-Ala OCH_3, DCC, HOBt; (f) OH^- then H_3O^+; (g) L-AlaOCH_2Ph, DCC, HOBt; (h) Na in NH_3(liq); (i) Zn, HCl in methanol.

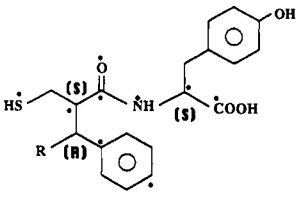
Figure 4. (A, top) ACE pharmacophore defined by the overlap of the biologically active conformation of captopril [(2*S*)-2-(mercaptomethyl)-3-methylpropanoyl]proline (in blue) with the most stable conformer of B [3-(mercaptomethyl)-3,4,5,6-tetrahydro-2-oxo-1*H*-1-benzazocine-1-acetic acid] (in ref). (B, bottom) Superimposition (rms deviation 0.783) of the most stable (F1, Table 1) and populated (41%) conformer of *N*-[(2*S*,3*R*)-2-(mercaptomethyl)-3-phenylbutanoyl]-(*S*)-alanine (**44c**, RB 105) (in yellow) and the ACE template (in red).

obtained, were designated **44a–d** following their elution order in HPLC. At this step, the absolute configurations of the asymmetric carbons of **44a–d** were unknown, but taking in account that **44a** and **44b** issued from $\mathbf{6a_1}$ and $\mathbf{6a_2}$ which were isolated with (*S*)-(−)- and (*R*)-(+)-α-methylbenzylamines respectively, their 2-(mercaptomethyl)-3-phenylbutanoyl moieties were enantiomers. The same argument is valid for **44c** and **44d**, which contain the other set of enantiomers of the (mercaptomethyl)phenylbutanoyl moiety. Interestingly, the same separation was performed on the benzoyl analog **6b**. In this case the salts which crystallized with (*S*)-(−)- and (*R*)-(+)-α-methylbenzylamines led to the inhibitors **44c** and **44d**, respectively.

The absolute configuration of the four stereoisomers of **44** was assigned by means of asymmetric synthesis performed with the chiral synthon, (4*S*)-4-(1-methylethyl)-2-oxazolidinone, developed by Evans,[31] and commercially available (*R*)- and (*S*)-3-methyl-4-phenylbutanoic acids. The 2*S*,3*R* and 2*S*,3*S* isomers of the 2-(mercaptomethyl)-3-phenylbutanoic acid were synthesized by this method using a procedure derived from that developed for the enantioselective synthesis of thiorphan[32] (Figure 3). However, due to the steric hindrance induced by the 3-phenylbutanoyl chain, lithium benzyl oxide was inefficient in releasing the chiral auxiliary, and was therefore replaced by lithium hydroperoxide.[33] Under these conditions, the cleavage was easy and efficient (85%) but the thioether bond was oxidized and a mixture of sulfoxide **67** and sulfone **68** was obtained. After separation, the

Table 1. Percentage of Stable Conformers of Compounds **A**, **48**, and **52** Fitting the Constrained ACE Inhibitor Structure, Determined by a Template-Forcing Minimization Procedure





R	families of conformers	% population	rms dev	E (kcal·mol)$^{-1}$	χ_1	Φ_1	Ψ_1
H	F_1	16	0.747	152	−176	−30	−151
	F_2	14	0.883	150	−137	50	−154
	F_3	19	1.139	153	−106	−19	177
	F_4	51	1.406	143	176	−162	154
CH_3	F_1	41	0.659	145	87	−65	−91
	F_2	48	1.249	147	84	−53	176
	F_3	9	1.388	159	−84	−22	163
C_2H_5	F_1	9	0.662	147	87	−65	−90
	F_2	13	0.981	151	−163	50	150
	F_3	40	1.323	157	−90	44	146
	F_4	38	1.413	162	−43	−1	−100

sulfoxide **67** was reduced by triphenylphosphine and the optically pure (2*S*,3*S*)- and (2*S*,3*R*)-2-[(benzylthio)methyl]-3-phenylbutanoic acids **69a** and **69b** were isolated. The coupling of these compounds with L-alanine methyl ester, followed by deprotection steps, led to the inhibitors [(2*S*,3*S*)-2-(mercaptomethyl)-3-phenylbutanoyl]-L-alanine and [(2*S*,3*R*)-2-(mercaptomethyl)-3-phenylbutanoyl]-L-alanine. The retention time of these two compounds on HPLC indicate that they correspond respectively to compounds **44b** and **44c**. From these results, the 2*S*,3*S* and 2*S*,3*R* configurations were attributed to compounds **44b** and **44c**, respectively. From this, it follows that the configurations of **44a** and **44d** are 2*R*,3*R* and 2*S*,3*S* respectively.

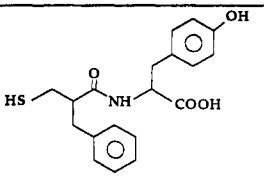
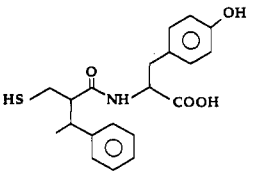
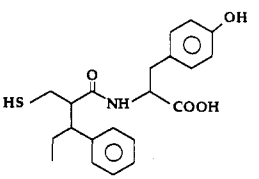
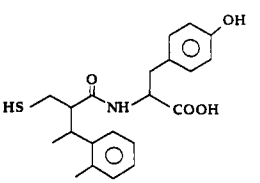
(2) Molecular Modeling. In order to discriminate the relevant conformations for "potent" ACE inhibition, thiol inhibitors bearing linear or cyclic constraints were investigated using 100-ps high temperature molecular dynamics.

The bicyclic and potent lactam inhibitor of ACE, 3-(mercaptomethyl)-3,4,5,6-tetrahydro-2-oxo-1*H*-1-benzazocine-1-acetic acid[34] which contains an aromatic cycle was used as a rigid framework. Three low-energy conformations emerged from the conformational analyses of this inhibitor, one of which (Figure 4A) was very similar to the reported "bioactive" conformation of captopril.[35] This conformation, corresponding to the *R* isomer of the bicyclic lactam, was thus taken as a template for further calculations on our series of inhibitors (Figure 4A).

Conformational analyses of inhibitors ***A***, **48**, and **52** led to three or four low-energy conformers (F1–F4) reported in Table 1. The ability of each form to fit the template structure was then investigated through a template-forcing minimization procedure. The results show that compound **48** is the best candidate for ACE inhibition, as the most populated (41%) conformation, F1, correctly fits the template model (Figure 4B) with a rms deviation of 0.784 Å. The proportion of F1 conformer decreases to 16% in compound ***A***, whereas the conformational constraint induced by the β-ethyl group in compound **52** produced a further decrease in the percentage (9%) of the structure adequately fitting the template.

(3) In Vitro Inhibitory Potencies on NEP and ACE. The inhibitory potencies for NEP and ACE of the thiorphan analog, *N*-[2-(mercaptomethyl)-3-phenylpropanoyl]-L-tyrosine, ***A***,[22] and of derived compounds containing various substituents introduced on the aromatic ring and/or the side chain of the benzyl moiety as noncyclic constraints are reported in Table 2.

Table 2. Effect of the Introduction of Various Constraints in the Phenylpropanoyl Moiety of the Inhibitor *N*-[2-(Mercaptomethyl)-3-phenylpropanoyl]-L-tyrosine on the Inhibitory Potency on NEP and ACE Activities

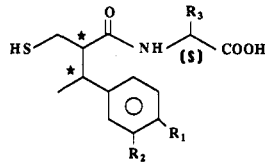
compound	IC_{50} (nM) NEP	IC_{50} (nM) ACE
A 	2.0 ± 0.4	25 ± 6
48 	2.5 ± 0.5	4.3 ± 0.8
52 	2.4 ± 0.3	110 ± 20
53 	11 ± 5	210 ± 40

Compound ***A*** inhibited NEP with a IC_{50} of 2 nM and ACE with a IC_{50} of 25 nM. The introduction of a methyl substituent on the β-methylene group of the benzyl moiety in **48** increased ACE recognition by a factor of 5 without changing NEP inhibition. In contrast, a large increase in IC_{50} values for ACE was observed when an ethyl group in **52** or two methyl groups in **53** were introduced. Compound **48**, being the most potent dual NEP–ACE inhibitor, was selected for further structural investigations.

The inhibitory potencies of the various compounds containing a 2-(mercaptomethyl)-3-phenylbutanoyl moiety, substituted or not on the aromatic ring and coupled to various amino acids, are reported in Table 3. These compounds generally behaved as good NEP inhibitors with IC_{50} values in the nanomolar range. Four exceptions with IC_{50} values of around 10^{-8} M were observed: compound **45** which has a valine in the P'_2 position and compounds **58**, **63**, and **64** which contain hydrophilic groups on the P'_1 benzyl residue. For ACE inhibition, the compounds reported in Table 3 were also efficient, with more than half of them having IC_{50} values in the nanomolar range. Compounds **48**, **55**, **56**, **59**, and **62**, which contain a C-terminal tyrosine, or compounds **44**, **47**, **50**, and **51** with linear side chains were preferred to those having branched amino acids on the P'_2 position (compounds **45** and **46**). Moreover, the presence of hydrophilic groups on the P'_1 benzyl residue slightly increased the IC_{50} values (compare compound **48** to compounds **58**, **59**, and **64**).

In these experiments, the various inhibitors were tested as a mixture of four stereoisomers, which were obtained

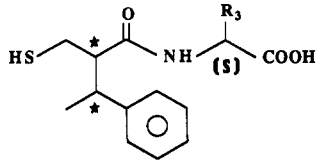
Table 3. Structure-Activity Relationships for Dual Inhibition of NEP and ACE by Various *N*-[2-(Mercaptomethyl)-3-phenylbutanoyl]amino Acids



compd no.	R_1	R_2	R_3	IC_{50} (nM)[a] NEP	ACE
43	H	H	H	1.3 ± 0.3	32 ± 6
44	H	H	CH_3	2.0 ± 0.1	20 ± 2
45	H	H	$CH(CH_3)_2$	15 ± 5	100 ± 10
46	H	H	$CH_2CH(CH_3)_2$	3.5 ± 0.5	19 ± 5
47	H	H	$(CH_2)_3CH_3$	3.1 ± 0.6	7.0 ± 0.8
48	H	H	CH_2(4-OH)Ph	2.5 ± 0.5	4.3 ± 0.8
49	H	H	CH_2OCH_2Ph	4.0 ± 0.8	43 ± 7
50	H	H	CH_2CH_3[b]	6.3 ± 0.5	5.4 ± 0.4
51	H	H	$(CH_2)_2CH_3$[b]	4.0 ± 0.4	4.0 ± 0.5
54	F	H	CH_3	2.0 ± 0.8	10 ± 2
55	F	H	CH_2(4-OH)Ph	5.9 ± 0.3	6.9 ± 0.6
56	OCH_3	H	CH_2(4-OH)Ph	2.5 ± 0.3	3.6 ± 0.6
58	NH_2	H	CH_2(4-OH)Ph	16 ± 8	12 ± 2
59	OH	H	CH_2(4-OH)Ph	9.0 ± 1.0	7.9 ± 0.9
60	$-OCH_2O-$		CH_3	2.0 ± 0.5	9.5 ± 0.6
61	F	F	CH_3	3.5 ± 0.8	5.0 ± 0.8
62	F	F	CH_2(4-OH)Ph	5.2 ± 0.8	4.5 ± 0.5
63	OCH_3	OCH_3	CH_2(4-OH)Ph	16 ± 5	4.0 ± 0.5
64	OH	OCH_3	CH_2(4-OH)Ph	65 ± 10	14 ± 3

[a] The values are the mean ±SEM of three independent experiments. [b] Compounds tested as optically pure 2*S*,3*R* isomers.

Table 4. Inhibitory Potencies of the Separate Stereoisomers of Compounds 44 and 48 on NEP and ACE

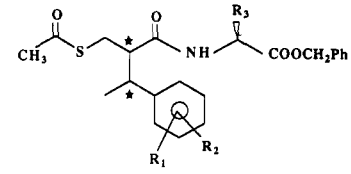


	K_i (nM)			
$R_3 = CH_3$	**44a** (2*R*,3*R*)	**44b** (2*S*,3*S*)	**44c** (2*S*,3*R*)	**44d** (2*R*,3*S*)
NEP	2.3 ± 0.2	2.1 ± 0.2	1.7 ± 0.1	0.7 ± 0.1
ACE	80 ± 6	16 ± 2	4.5 ± 0.5	95 ± 8
$R_3 = CH_2Ph$	**48a** (2*R*,3*R*)	**48b** (2*S*,3*S*)	**48c** (2*S*,3*R*)	**48d** (2*R*,3*S*)
NEP	3.1 ± 0.2	1.6 ± 0.2	3.2 ± 0.3	5.3 ± 0.4
ACE	26 ± 3	15 ± 2	1.9 ± 0.2	10 ± 3

in about equal proportions. It was therefore necessary to compare the relative activities of the separate isomers. This was carried out with compounds **44** and **48**, which were selected due to the favorable pharmacokinetic properties of the former and the good affinities of the latter. For NEP inhibition, no significant modifications were observed whatever the absolute configuration of the C_2 and C_3 carbons (Table 4). As expected, the two isomers having a 2*S* configuration were more active on ACE than those containing a 2*R* residue.[29] In agreement with calculations (not shown here) the most efficient isomers **44c** and **48c** have a 2*S*,3*R* configuration with K_i values of 4.5 and 1.9 nM for ACE and 1.7 and 3.2 nM for NEP, respectively. The former compound was designated **RB 105**.

(4) In Vivo Inhibition of Kidney NEP and Lung ACE in Mouse. The most efficient compounds, selected from their "in vitro" inhibitory potencies or NEP and ACE, were tested to evaluate their "in vivo" potency in mice.

Table 5. Prescreening of Inhibitors (Orally Administered at 2.6 × 10^{-5} mol/kg)[a] for the in Vivo Blockade of Lung ACE



compd no.	R_1	R_2	R_3	% inhibition[b] 20 min	60 min
7	H	H	H	36 ± 5	43 ± 11
8	H	H	CH_3	54 ± 3	44 ± 5
14	H	H	CH_2-(4-OH)Ph	20 ± 4	7 ± 2
15	H	H	CH_2-(4-OCOCH$_3$)Ph	25 ± 6	28 ± 4
13	H	H	$(CH_2)_3CH_3$	34 ± 10	41 ± 6
27	4-F	H	CH_2-(4-OCOCH$_3$)Ph	36 ± 3	11 ± 6
34	4-F	3-F	CH_3	35 ± 6	22 ± 8
29	4-OCH_3	H	CH_2-(4-OCOCH$_3$)Ph	14 ± 6	33 ± 11
37	4-F	3-F	CH_2-(4-OCOCH$_3$)Ph	24 ± 5	19 ± 8

[a] Compounds tested as mixtures of four stereoisomers. [b] The values are the mean ± SEM of three different experiments with three mice per experiment.

For this purpose, the inhibitors were used as prodrugs, protected by thioacetyl and benzyl ester groups, and were orally administered.

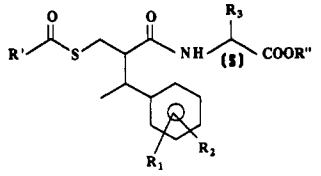
The compounds, tested as mixtures of stereoisomers, were given at a single dose (2.6 × 10^{-5} mol/kg) and the inhibition of binding of a tritiated probe to mouse lung ACE was measured 20 and 60 min after oral administration. The results of this initial inhibitor screening are reported in Table 5. Compounds containing the relatively polar C-terminal tyrosine (compound **14**) were less efficient than those containing more hydrophobic residues, such as CH_3 and $(CH_2)_3CH_3$ (compounds **8** and **13**). The acetylation of the phenol group in **15** did not increase greatly the efficiency of the prodrug in vivo. Various substitutions on the phenyl ring of the 3-phenylbutanoyl moiety were also tested: the presence of fluorine or methoxy group decreased the in vivo activity of the inhibitors as compared to **8**.

The activities of various prodrugs of inhibitor **44** are reported in Table 6. The saponification of the benzyl ester of **8** seemed to have opposing effects; at short times (30 min, 60 min) ACE inhibition by **10** was slightly reduced as compared to **8**, but the inhibition was significantly better at 4 h. The introduction of the adamantoyl moiety as a thiol protecting group in **42** also increased the duration of action, and this effect was more pronounced in the analog **41** which has a free carboxyl group. Using these results, the precursor **6b$_1$**, which contains an hydrophobic benzoylthio ester, was used to obtain the inhibitors **41c**, **18**, **20**, and **22**. A large increase in ACE inhibition was observed at 30 and 60 min with all these compounds.

The four latter compounds were also tested on NEP by measuring the displacement of [^{3}H]HACBOGly binding in mouse kidney (Table 7). A complete blockade of the enzyme was observed until 8 h after the administration of **41c** and **18**. Compounds **20** and **22** were less efficient.

Using the same methodology, the in vivo potency and duration of action of **18** were compared to those of two described dual inhibitors of NEP and ACE (alatriopril and SQ 28133). As shown in parts A and B of Figure 5, compound **18**, designated mixanpril, was distinctly more efficient on both enzymes. Furthermore, dose–response curves for **18** determined for both enzymes gave ED_{50}s ~ 1 mg/kg for NEP and 7 mg/kg for ACE (not shown).

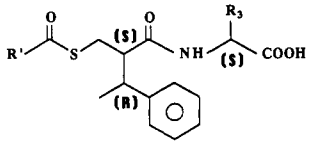
Table 6. Time Course of the in Vivo Inhibition (in Percent of Control) of Lung ACE Activity by Various Inhibitors Orally Administered at 2.6×10^{-5} mol/kg



compd no.	R′	R_1	R_2	R_3	R″	30 min	60 min	120 min	240 min
8	CH_3	H	H	CH_3	CH_2Ph	41 ± 6	46 ± 4	21 ± 6	2 ± 1
10	CH_3	H	H	CH_3	H	31 ± 8	29 ± 7	18 ± 4	16 ± 3
42	Ada[a]	H	H	CH_3	CH_2Ph	41 ± 8	34 ± 7	42 ± 6	16 ± 7
41	Ada[a]	H	H	CH_3	H	40 ± 4	39 ± 5	36 ± 5	35 ± 7
41c	Ada[a,b]	H	H	CH_3	H	72 ± 2	54 ± 5	42 ± 5	37 ± 7
18	Ph[b]	H	H	CH_3	H	75 ± 2	66 ± 5	44 ± 5	32 ± 4
20	Ph[b]	H	H	CH_2CH_3	H	73 ± 2	57 ± 3	39 ± 5	30 ± 2
22	Ph[b]	H	H	$(CH_2)_2CH_3$	H	69 ± 2	52 ± 2	49 ± 3	41 ± 1

[a] Ada = adamantyl. [b] Optically active compounds with a 2*S*,3*R* configuration of the 2-[(alkanoylthio)methyl]-3-phenylbutanoyl moiety.

Table 7. Time Course of the Inhibition (in Percent of Control) of Kidney NEP Activity by Various Inhibitors Orally Administered at 2.6×10^{-5} mol/kg[a]



compd no.	R′	R_3	1 h	2 h	4 h	8 h	16 h
41c	Ada[b]	CH_3	100 ± 2	100 ± 2	100 ± 2	94 ± 4	10 ± 7
18	Ph	CH_3	100 ± 2	100 ± 2	100 ± 1	93 ± 2	12 ± 4
20	Ph	CH_2CH_3	100 ± 2	97 ± 1	73 ± 6		
22	Ph	$CH_2CH_2CH_3$	70 ± 6	55 ± 6	46 ± 2		

[a] The inhibition corresponds to the displacement of the tritiated NEP inhibitor [^{3}H]HACBOGly iv injected. [b] Ada = adamantyl.

(5) Pharmacological Responses. (1) Antihypertensive and Diuretic Responses Induced by iv RB 105 (44c) in SHR. RB 105 [[(2*S*,3*R*)-2-(mercaptomethyl)-3-phenylbutanoyl]-L-alanine, **44c**] was administered iv at 25 mg/kg bolus plus infusion in SHR as previously described.[19] No significant modification of blood pressure and natriuresis was observed after vehicle injection in control animals. RB 105 significantly decreased blood pressure, as compared to control rats, from the 30th min to the end of the experiment (Figure 6A). A maximal effect on natriuresis was also obtained from the 30th min (Figure 6B). Kaliuresis did not change throughout the experiment (not shown).

(2) Effects of Oral Administration of Mixanpril in SHR. SHR were treated with Mixanpril at 50 mg/kg twice a day per os. Blood pressure decreased progressively during the experiment. A maximal decrease was observed after 3 days of gavage (Figure 7). Mixanpril induced an increase in diuresis and natriuresis in SHR during the first days of the experimental protocol (data not shown).

Discussion

With aim of treating and preventing cardiovascular diseases and hypertension, we report here the synthesis and biochemical and pharmacological properties of new compounds able to inhibit jointly NEP, which is responsible for ANP degradation, and ACE, which releases the vasconstrictor peptide angiotensin II. These compounds have been designed by taking into account the characteristics of the active site of both zinc metallopeptidases, deduced from the structures of their most potent and selective inhibitors (reviews in refs 1, 28, and 36).

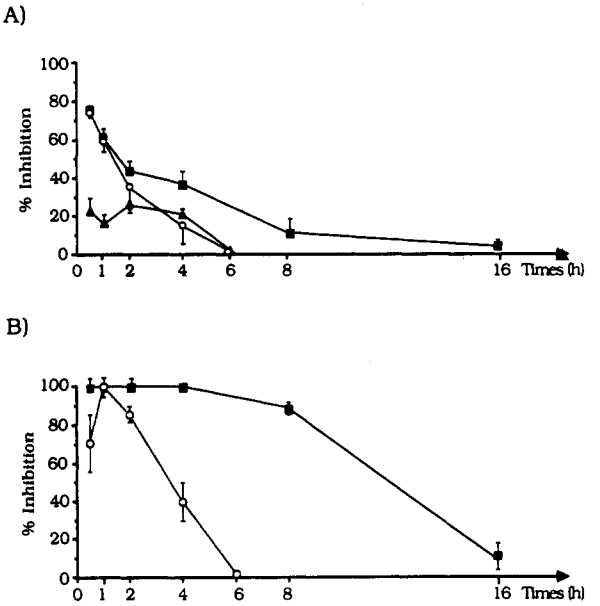


Figure 5. (A) Time course of the in vivo inhibition of lung ACE in mice after oral administration of dual inhibitors of NEP and ACE ($c = 2.6 \times 10^{-5}$ mol/kg). Values are the mean ± SEM of three different experiments; (■) mixanpril, (○) alatriopril [2-[(acetylthio)methyl]-3-[3,4-(methylenedioxy)phenyl]propanoyl]-alanine, (▲) ES 34 = SQ 28133, [2-(mercaptomethyl)-3-phenylpropanoyl]leucine. (B) Time course of the in vivo inhibition of kidney NEP in mice after oral administration of dual inhibitors of NEP and ACE (at concentration = 2.6×10^{-5} mol/kg). Values are the mean ± SEM of three different experiments: (■) mixanpril, (○) alatriopril.

Thus, the ACE active site has been shown to be relatively large, being able to accommodate flexible as well as highly constrained cyclic inhibitors (review in ref 6). In the case of NEP, few constrained inhibitors have been yet reported, but the IC_{50} values of numerous more or less bulky inhibitors suggest that this peptidase also contains a large active site.[1,23] Molecules endowed with noncyclic constraints rather than cyclic compounds were selected because (i) both type of constraints were shown to increase the affinity of various effectors for their target by reducing the thermodynamically unfavorable entropy loss which occurs during the binding process but the residual flexibility associated with linear constraints favourize target adaptation;[37,38] (ii) noncyclic compounds, including pure stereoisomers, are generally easier and less expensive to synthesize than cyclic compounds; (iii) the modulation of the hydrophobic–hydrophilic balance, which was required

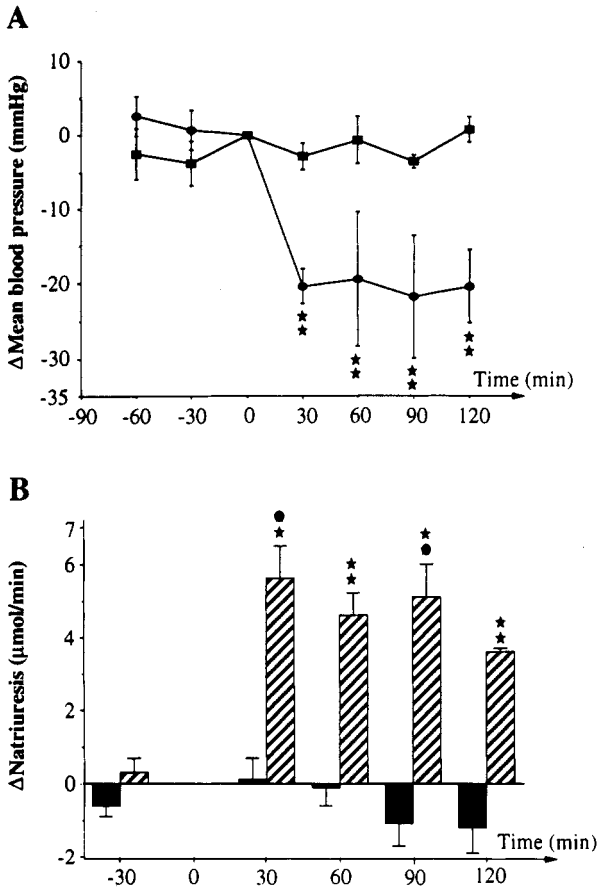


Figure 6. (A) Variation of the mean blood pressure (mmHg) observed in response to RB 105 treatment (iv injection of 25 mg/kg bolus + 25 mg/kg/h infusion) as compared to saline: (■) control, (●) RB 105. (B) Variation in the natriuresis induced in response to RB 105 treatment (iv injection of 25 mg/kg bolus + 25 mg/kg/h infusion) as compared to injection of saline: (■) control, (□) RB 105.

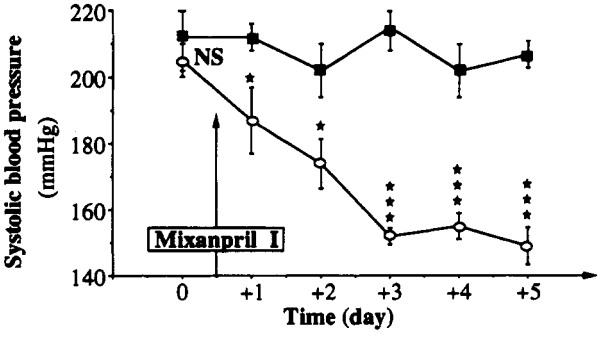


Figure 7. Variation in blood pressure measured during chronic oral administration of 50 mg/kg mixanpril daily to SHR: (■) control, (○) mixanpril.

to optimize the in vivo inhibition of the target enzymes, is more easily manageable with linear constraints.

Mixed NEP/ACE inhibitors must inactivate ACE in the vasculature endothelium (blood compartment) and NEP mainly, but not exclusively,[39] in the brush border membranes of the renal connecting duct (urine compartment). Compounds which are too hydrophobic would be unable to reach the kidney, while those which are too hydrophilic would only transiently inhibit endothelial ACE.

Taking these requirements into account, the potent NEP inhibitor ***A*** (H-SCH$_2$CH(CH$_2$Ph)CONHCH(CH$_2$(*p*-OH)-Ph)COOH containing P′$_1$ and P′$_2$ aromatic components was selected and studied for its ability to fit a template, defined from a molecular dynamics study by the superimposition of the "biologically" active conformation of captopril,[35] with that of the most stable conformer of the highly constrained ACE inhibitor, 3-(mercaptomethyl)-3,4,5,6-tetrahydro-2-oxo-1*H*-1-benzazocine-1-acetic acid ***B***[34] (Figure 4A). In agreement with the relatively weak inhibitory potency of ***A*** on ACE, only a small proportion (16%) of stable conformers was found to fit the template. Various constraints were then introduced in the structure of ***A*** to increase ACE inhibition without loss of potency on NEP. A methyl group in the β-position of the benzyl moiety (compound **48**) improved significantly the recognition of ACE (Table 2) in agreement with a high percentage (41%) of stable conformers fitting the pharmacophore (Table 1). Conversely, the presence of two methyl (compound **53**) or a β-ethyl substituent (compound **52**) decreased the inhibitory potencies (Table 2), in agreement with a reduction in the percentage of the biologically active conformer (Table 1) (Figure 4B). The presence of the β-branched amino acid, valine, in **45** led to a large reduction in ACE affinity (IC_{50} = 100 nM), and the percentage of favorable conformers fell to 2% supporting, the validity of our approach. Furthermore, as shown by the nanomolar IC_{50} values exhibited by the various compounds synthesized for NEP (Table 2), the active site of this enzyme is almost insensitive to the constraints introduced.

From these results, compound **48**, bearing a β-methyl constraint in the benzyl moiety, was selected for the development of a new and potent series of dual inhibitors. It is interesting to note that, to our knowledge, this type of acyclic constraint in the β-position of the P′$_1$ aromatic component has not been reported before in ACE or NEP inhibitors. In the new series of compounds, pure stereoisomers could be obtained by using commercially available chiral synthons developed by Evans et al.[31] Moreover, the use of the nonexpensive chiral amine (*S*)-(−)-α-methylbenzylamine allowed the active precursor **6b$_1$** to be obtained in one step and therefore enabled large quantities of this dual inhibitor to be prepared for pharmacological studies. Very different amino acids can be introduced in the P′$_2$ position without any major reduction in the nanomolar affinity for both enzymes (Table 3). Nevertheless, β-branched amino acids, such as Val in **45** and Leu in **46** led to a reduction in ACE inhibitory potency, which was, as previously described, particularly drastic for the former compound.

Furthermore, none of the modifications of the aromatic ring of the β-methylbenzyl moiety improved the IC_{50} values, showing that the unsubstituted phenyl ring is relatively optimized for the active site of both enzymes (Table 3).

It is well-known that the S′$_1$ subsite of NEP is insensitive to the absolute configuration of the 2-(mercaptomethyl)-3-phenylbutanoyl moiety of thiorphan[40] but that optimal ACE inhibition requires an *S*-configuration for the P′$_1$ residue.[29] This was confirmed with the new series of inhibitors, since the four stereoisomers of **44** were found to be almost equiactive on NEP, whereas only one isomer, **44c**, inhibited ACE with a K_i in the nanomolar range (Table 4).

The in vivo inhibition of NEP and ACE was measured in mice 20 and 60 min after oral administration of a single dose of inhibitor (Table 5). In the first series of experiments the active inhibitors were tested as prodrugs, with

the two polar functions protected by an acetyl group on the sulfydryl and by a benzyl ester on the carboxylate. From the results reported in Tables 3 and 5, it can be seen that the in vivo inhibition of ACE by a prodrug was not directly comparable to the in vitro potency of its precursor. Thus, the most efficient compounds in this prescreening were **8**, the prodrug of compound **44**, while **14**, **29**, and **37**, the prodrugs of **48**, **56**, and **62**, respectively, which have the lowest IC_{50} values for ACE, were significantly less active in vivo. Furthermore, the transient protection of the phenol group by acetylation in **15** and **27**$_1$ did not significantly improve the bioavailability of these compounds. On the other hand, in previous studies with other prodrugs of NEP inhibitors developed as potential analgesics, we have shown that the carboxylate protection is rapidly eliminated in the serum, whereas the sulfhydryl protection was only cleaved in a membraneous environment.[41] Therefore, given that the carboxylate protection was unable to greatly improve the bioavailability of these inhibitors and actually reduced their duration of action, the lipophilic character of the thiol protective group was increased and the carboxylate was kept free. The most active prodrugs, containing the hydrophobic adamantoyl and benzoyl protecting groups in **41c** and **18**, respectively, were tested as pure stereoisomers and were found, as expected, to be very efficient inhibitors of both ACE (Table 6) and NEP (Table 7) in vivo. The benzoyl derivative **18**, being easier to prepare, was designated Mixanpril and was chosen for extensive pharmacological studies. It is interesting to observe that compounds **20** and **22**, which are as potent as **18** on ACE, have a shorter duration of NEP inhibition and were therefore discarded (Table 7). This again emphasizes the necessity of having a good hydrophilic–hydrophobic balance for inhibition of both endothelial ACE and epithelial NEP. However, this also indicates that the duration of action of this class of mixed inhibitors can be modified simply by changing the nature of the C-terminal P'_2 residue.

The pharmacological responses associated with the dual inhibition of NEP and ACE were studied with **44c**, [(2*S*,3*R*)-*N*-[2-(mercaptomethyl)-1-oxo-3-phenylbutyl]-L-alanine, the active form of **18**. This compound, also designated RB 105, was iv injected in SHR. An acute administration of RB 105 at 25 mg/kg bolus + 25 mg/kg/h decreased significantly the blood pressure (Figure 6A) and increased diuresis (Figure 6B) 30 min after the injection and for the duration of the experiment. As previously shown with selective inhibitors of both enzymes, the diuretic effect was specifically related to NEP inhibition and the hypotensive response to ACE inhibition.[19] Consequently, in this work, the dual responses to RB 105 injection can be related to the simultaneous inhibition of both target enzymes. Oral administration of the prodrug Mixanpril (50 mg/kg twice a day) in SHR (Figure 7) also induced hypotensive responses from the first day of the chronic treatment, and this effect was of long duration. The diuretic and the natriuretic effects associated with NEP inhibition were significant only for the first days of the treatment (data not shown). A complete analysis of the properties of Mixanpril, in various models of hypertension and on various biochemical markers of the inhibition of both enzymes, will be published elsewhere.

In conclusion, the new series of potent dual inhibitors of NEP and ACE generated by introducing noncyclic constraints in flexible dipeptide-derived mercaptans fulfill the requirements of potency, bioavailability, and duration of action necessary to reduce blood pressure and to increase sodium excretion in a genetic model of hypertension after oral administration. The co-inhibition of NEP and ACE has previously been shown to increase the mean arterial pressure produced by ACE inhibitors alone in SHR[19] and to diminish blood pressure and left arterial pressure, thus enhancing cardiac output in a canine model of heart failure.[20] Therefore the reduction in the detrimental effects of ACE inhibition on renal function by protecting circulating endogenous ANP with mixed inhibitors such as Mixanpril, could have important therapeutical applications.

Experimental Section

Chemistry. Amino acids were from Bachem (Bubendorf, Switzerland). Reagents were from Aldrich-chemie (Strasbourg, France). (3*R*)- and (3*S*)-3-phenylbutyric acid were from Fluka-chemie (Buchs, Switzerland). The solvents were from SDS (Peypin, France).

The purity of the compounds was checked by thin-layer chromatography on silica gel plates (60F 254, Merck) using the following solvent systems (v/v): (A) hexane/ethyl acetate/acetic acid, 5/5/0.5; (B) hexane/ethyl acetate, 2/1; (C) hexane/ethyl acetate, 6/4; (D) hexane/ethyl acetate/acetic acid, 7/3/0.5; (E) hexane/ethyl acetate, 1/1; (F) CH_2Cl_2/methanol/acetic acid, 9/1/0.5; (G) CH_2Cl_2/methanol, 9/1; (H) hexane/ethyl acetate, 7/3; (I) petroleum ether/diethyl ether, 8/2.

HPLC studies were performed using a reverse-phase C18 kromasil column (i.d. 5 μm, porosity 100 Å) or C_8 KWI column (i.d. 10 μm, porosity 120 Å) (SFCC) with CH_3CN/TFA 0.05% buffer (pH 4.0) as eluent, on a Shimadzu apparatus (detector SPD 6AV, pumps LC9A, recorder CR6A). The eluted peaks were monitored at 210 nm. The structure of all the compounds was confirmed by ^1H NMR spectroscopy (Bruker WH, 270 MHz) in DMSO-d_6 or $CDCl_3$ solutions (5×10^{-3} M). Satisfactory analyses were obtained (C, H, N) for all compounds. Melting points of the crystallized compounds were determined on an Electrothermal apparatus and are reported uncorrected. The following abbreviations were used: AcOH, acetic acid; DCC, dicyclohexylcarbodiimide; DCU, dicyclohexylurea; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; EtOAc, ethyl acetate; EtOH, ethanol; Et_2O, diethyl ether; HOBt, 1-hydroxybenzotriazole; TFA, trifluoroacetic acid.

(I) Synthesis. General Procedure for the Preparation of the Ethyl 2-Substituted (Diethylphosphono)acetates 2. To a solution of ethyl (diethylphosphono)acetate (1 equiv) in dry DMF was added at −5 °C and under inert atmosphere, 1.1 equiv of NaH. After 10 min at the same temperature, the bromide derivative (1.05 equiv) was added slowly. The mixture was stirred overnight at room temperature. The solvent was evaporated in vacuo and the residue taken off with water and extracted with EtOAc. The organic layer was washed with water and brine and dried over Na_2SO_4. After filtration and evaporation of the solvent, compounds **2** were isolated as oils: **2a** (R = CH_3, R_1 = R_2 = H) (99%) R_f(A) 0.54; **2b** (R = CH_3, R_1 = 2-CH_3, R_2 = H) (86%) R_f(A) 0.42; **2c** (R = CH_2CH_3, R_1 = R_2 = H) (99%) R_f(A) 0.49.

General Procedure for the Preparation of the 2-Substituted Malonates 3. To a solution of EtONa in EtOH was added at 0 °C 1 equiv of diethyl malonate. After the mixture was stirred for 15 min at the same temperature, a solution of the bromide derivative (1 equiv) in ethanol was added, and the mixture was stirred overnight at 40 °C. After evaporation of the solvent, the residue was partitioned between water and EtOAc. The organic layer was washed with water and brine and dried over Na_2SO_4. After filtration and evaporation of the solvent, the crude substituted malonates were obtained.

3a (R = CH_3, R_1 = 4-F, R_2 = H): only product (86%); R_f(B) 0.57.

3b (R = CH_3, R_1 = 4-OH, R_2 = H): oily product; purified by chromatography in hexane–EtOAc, 4/1 (43%); R_f(C) 0.52.

3c (R = CH_3, R_1 = 4-OCH_3, R_2 = H). To a solution of **3b** in EtOH were added a solution of KOH in EtOH (1.5 equiv) and

CH_3I (20 equiv). The mixture was stirred for 2 days at room temperature to yield an oily product (98%), R_f(D) 0.52.

3d (R = CH_3, R_1 = 4-OCH_2Ph, R_2 = H). To a solution of **3b** in EtOH were added a solution of KOH in EtOH (1.5 equiv) and $PhCH_2Br$ (1.5 equiv). The mixture was stirred overnight at room temperature to yield a white solid: mp 50 °C (70%); R_f(B) 0.40.

3e (R = CH_3, R_1 = R_2 = H): oily product (86%); R_f(B) 0.51.

3f (R = CH_3, R_1 = 4-NO_2, R_2 = H) was obtained by treatment of **3e** with concentrated H_2SO_4 (1 mL/mmol) and KNO_3 (1 equiv) at −10 °C. After the mixture was stirred for 2 h at −10 °C, water was added and the solution was extracted with Et_2O to yield a white solid: mp 65 °C (84%); R_f(E) 0.73.

3g (R = CH_3, R_1 = 4-F, R_2 = 3-F): oily product (90%); R_f(C) 0.64.

3h (R = CH_3, R_1 = 4-OH, R_2 = 3-OCH_3): oily product (60%); R_f(C) 0.32.

3i (R = CH_3, R_1 = 4-OCH_3, R_2 = 3-OCH_3) was obtained from **3h** as described for **3c**: oily product (60%); R_f(E) 0.43.

3j (R = CH_3, R_1 = 4-OCH_2(4-OCH_3)Ph, R_2 = 3-OCH_3) was obtained by treatment of **3h** with a solution of KOH in EtOH (1.1 equiv) and 4-methoxybenzyl bromide (1.2 equiv). The mixture was stirred for 2 days at room temperature to yield an oily product (85%), R_f(B) = 0.42.

3k (R = CH_3, R_1R_2 = $-OCH_2O-$): oily product (52%); R_f(C) 0.40.

General Procedure for the Preparation of Compounds 4. The 2-substituted diethyl malonates **3** were dissolved in ethanol, and a solution of KOH in ethanol (1.1 equiv) was added at 0 °C. After 1 h at 0 °C, the mixture was stirred overnight at room temperature. The solvent was evaporated in vacuo and the residue dissolved in water. The aqueous layer was washed with Et_2O, acidified to pH 1 by HCl (2 M), and extracted with EtOAc. The organic layer was washed with water and brine and dried over Na_2SO_4. After filtration and evaporation in vacuo, the monoacids **4** were isolated.

4a (R = CH_3, R_1 = 4-F, R_2 = H): oily product (89%); R_f(D) 0.31.

4b (R = CH_3, R_1 = 4-OCH_3, R_2 = H): oily product (88%); R_f(C) 0.70.

4c (R = CH_3, R_1 = 4-OCH_2Phe, R_2 = H): oily product (83%); R_f(B) 0.21.

4d (R = CH_3, R_1 = 4-NO_2, R_2 = H): oily product (93%); R_f(F) 0.58.

4e (R = CH_3, R_1 = 4-F, R_2 = 3-F): oily product (94%); R_f(B) 0.10.

4f (R = CH_3, R_1 = 4-OCH_3, R_2 = 3-OCH_3): oily product (99%); R_f(D) 0.20.

4g (R = CH_3, R_1 = 4-OCH_2(4-OCH_3)Ph, R_2 = 3-OCH_3): oily product (78%); R_f(F) 0.33.

4h (R = CH_3, R_1R_2 = $-OCH_2O-$): oily product (78%); R_f(D) 0.26.

General Procedure for the Preparation of Compounds 5: Procedure A. A mixture of the 2-substituted phosphonoacetates **2** (1 equiv), K_2CO_3 (3 equiv), and a 30% aqueous solution of formol (6.5 equiv) was refluxed for 3 h. After cooling, the mixture was extracted with *n*-hexane. The organic layer was washed with water and brine and dried over Na_2SO_4. After filtration and evaporation in vacuo, an oily residue was obtained. The unsaturated esters (1 equiv) were dissolved in a mixture of acetone and water (7/3), and NaOH (1 M) (1.5 equiv) was added at 0 °C. After the mixture was stirred for 1 h at 0 °C and overnight at room temperature, the solvents were evaporated and the residue taken off with water. The aqueous layer was acidified with (3 M) HCl and extracted with EtOAc. The organic layer was washed with water and brine and dried over Na_2SO_4. After filtration and evaporation of the solvent, the acids **5** were obtained.

Procedure B. A mixture of compound **4** (1 equiv), diethylamine (1 equiv), and 37% aqueous solution of formol (1.5 equiv) was stirred for 2 days at room temperature. The mixture was partitioned between water and Et_2O. The organic layer was washed with water, 10% citric acid solution, water, and brine and then dried over Na_2SO_4. After filtration and evaporation in vacuo, the unsaturated esters were obtained. The saponification of the ester was performed as described in procedure A.

5a (R = CH_3, R_1 = R_2 = H) (procedure A): white solid, mp 100 °C (70%); R_f(D) 0.67.

5b (R = CH_3, R_1 = 2-CH_3, R_2 = H) (procedure A): oily product (63%); R_f(A) 0.65.

5c (R = CH_2CH_3, R_1 = R_2 = H) (procedure A): oily product (68%); R_f(A) 0.69.

5d (R = CH_3, R_1 = 4-F, R_2 = H) (procedure B): white solid; mp 72 °C (72%); R_f(D) 0.69.

5e (R = CH_3, R_1 = 4-OCH_3, R_2 = H) (procedure B): white solid; mp 81 °C (88%); R_f(D) 0.63.

5f (R = CH_3, R_1 = 4-OCH_2Ph, R_2 = H) (procedure B): white solid; mp 87 °C (87%); R_f(B) 0.72.

5g (R = CH_3, R_1 = 4-NO_2, R_2 = H) (procedure B): white solid; mp 102 °C (90%); R_f(D) 0.27.

5h (R = CH_3, R_1 = 4-F, R_2 = 3-F) (procedure B): oily product (98%); R_f(B) 0.48.

5i (R = CH_3, R_1 = 4-OCH_3, R_2 = 4-OCH_3 (procedure B): oily product (66%); R_f(D) 0.27.

5j (R = CH_3, R_1 = 4-OCH_2(4-OCH_3)Ph, R_2 = 3-OCH_3) (procedure B): oily product (71%); R_f(A) 0.53.

5k (R = CH_3, R_1R_2 = $-OCH_2O-$) (procedure B): white solid; mp 102 °C (70%); R_f(D) 0.42.

General Procedure for the Preparation of Compounds 6. The unsaturated acids **5** were dissolved in CH_2Cl_2, and freshly distilled thioacetic acid (3.5 equiv) or thiobenzoic acid (1.2 equiv) was added. The mixtures were stirred at 40–80 °C for 2 days. The solvent was then evaporated in vacuo, and compounds **6** were obtained with a quantitative yield.

6a (R = R′ = CH_3, R_1 = R_2 = H): oily product; R_f(D) 0.68; NMR δ (ppm) 1.20 (CH_3), 2.20–2.25 (CH_3CO), 2.55–3.20 (SCH_2CHCH); 6.70–7.30 (Ar), 12.40 (COOH).

6b (R′ = Ph, R = CH_3, R_1 = R_2 = H): oily product; R_f(D) 0.44; NMR δ (ppm) 1.20–1.35 (CH_3), 2.7–3.2 (SCH_2CHCH), 7.1–7.9 (Ar), 12.50 (COOH).

6c (R = R′ = CH_3, R_1 = 2-CH_3, R_2 = H): oily product; R_f(A) 0.57; NMR δ (ppm) 1.10 (CH_3), 2.20–2.25 (CH_3CO), 2.30 (CH_3-(Ar)), 2.60–3.30 (SCH_2CHCH), 7.10 (Ar), 12.35 (COOH).

6d (R′ = CH_3, R = CH_2CH_3, R_1 = R_2 = H): oily product; R_f(A) 0.60; NMR δ (ppm) 0.60 ($CH_3(CH_2)$), 1.60 ($CH_2(CH_3)$), 2.20–2.25 (CH_3CO), 2.60–3.20 (SCH_2CHCH), 7.20 (Ar), 12.30 (COOH).

6e (R′ = R = CH_3, R_1 = 4-F, R_2 = H): oily product; R_f(D) 0.36; NMR δ (ppm) 1.20 (CH_3), 2.20–2.25 (CH_3CO), 2.55–3.20 (SCH_2CHCH), 7.00–7.40 (Ar), 12.35 (COOH).

6f (R′ = R = CH_3, R_1 = 4-OCH_3, R_2 = H): oily product; R_f(G) 0.48; NMR δ (ppm) 1.20 (CH_3), 2.20–2.25 (CH_3CO), 2.50–3.20 (SCH_2CHCH), 3.70 (OCH_3), 6.80–7.10 (Ar), 12.32 (COOH).

6g (R′ = R = CH_3, R_1 = 4-OCH_2Ph, R_2 = H): white solid; mp 120 °C; NMR δ (ppm) 1.10–1.20 (CH_3), 2.20–2.25 (CH_3CO), 2.50–3.20 (SCH_2CHCH), 5.00 (OCH_2), 6.80–7.45 (Ar), 12.30 (COOH).

6h (R′ = R = CH_3, R_1 = 4-NO_2, R_2 = H): oily product; R_f(D) 0.34; NMR δ (ppm) 1.22 (CH_3), 2.20–2.25 (CH_3CO), 2.60–3.20 (SCH_2CHCH), 7.50–8.10 (Ar), 12.50 (COOH).

6i (R′ = R = CH_3, R_1 = 4-F, R_2 = 3-F): oily product; R_f(E) 0.33; NMR δ (ppm) 1.12–1.30 (CH_3), 2.20–2.25 (CH_3CO), 2.60–3.20 (SCH_2CHCH), 6.95–7.40 (Ar); 12.40 (COOH).

6j (R′ = R = CH_3, R_1 = 4-OCH_2(4-OCH_3)Ph, R_2 = 3-OCH_3): oily product; R_f(A) 0.49 and 0.57; NMR δ (ppm) 1.10–1.30 (CH_3), 2.20–2.30 (CH_3CO), 2.50–3.1 (SCH_2CHCH), 3.70 (2 × OCH_3), 4.90 (OCH_2Ph), 6.60–6.95 (5 H, Ar), 7.45 (2 H, Ar), 12.40 (COOH).

6k (R′ = R = CH_3, R_1 = 4-OCH_3, R_2 = 3-OCH_3): oily product; R_f(G) 0.42; NMR δ (ppm) 1.20 (CH_3), 2.20–2.25 (CH_3CO), 2.50–3.10 (SCH_2CHCH), 3.70–3.75 (OCH_3), 6.60–6.85 (Ar), 12.25 (COOH).

6l (R′ = R = CH_3, R_1R_2 = $-OCH_2O-$): oily product; R_f(D) 0.33; NMR δ (ppm) 1.12 (CH_3), 2.15–2.20 (CH_3CO), 2.50–3.12 (SCH_2CHCH), 5.65 (OCH_2O), 6.50–6.70 (Ar), 12.42 (COOH).

General Procedure for the Synthesis of Compounds 7–40. To a solution of compounds **6** (1 equiv) in dry THF were added successively at 0 °C a solution of the α-amino ester chlorhydrate (1 equiv) and triethylamine (1 equiv) in CH_2Cl_2, a solution of 1-hydroxybenzotriazole (1 equiv) in THF, and a solution of DCC (1.2 equiv) in CH_2Cl_2. The mixtures were stirred overnight at room temperature. After filtration of DCU and evaporation of the solvents in vacuo, the residues were taken off with EtOAc and washed successively with H_2O, a 10% solution of citric acid, H_2O, a saturated solution of $NaHCO_3$, H_2O, and brine. The organic layers were dried over Na_2SO_4, and after filtration and

evaporation of the solvent in vacuo, the crude compounds were purified by flash chromatography on a silica gel column.

7 (R′ = R = CH_3, R_1 = R_2 = R_3 = H, R″ = CH_2Ph): oily product; chromatography in hexane/ethyl acetate, 6/4 (65%); R_f(C) 0.39.

8 (R′ = R = R_3 = CH_3, R_1 = R_2 = H, R″ = CH_2Ph): oily product; chromatography in hexane/ethyl acetate, 75/25; R_f(C) 0.45 (72%).

9 (R′ = R = CH_3, R_1 = R_2 = H, R_3 = CH_3, R″ = tBu): oily product; chromatography in hexane/ethyl acetate, 4/1 (62%); R_f(B) 0.47.

10 (R′ = R = CH_3, R_1 = R_2 = H, R_3 = CH_3, R″ = H): oily product (65%); R_f(D) 0.24.

11 (R′ = R = CH_3, R_1 = R_2 = H, R_3 = iPr, R″ = CH_2Ph): oily product; chromatography in hexane/ethyl acetate, 4/1 (77%); R_f(C) 0.32.

12 (R′ = R = CH_3, R_1 = R_2 = H, R_3 = iBu, R″ = CH_2Ph): oily product; chromatography in hexane/ethyl acetate, 75/25 (72%); R_f(C) 0.45.

13 (R′ = R = CH_3, R_1 = R_2 = H, R_3 = $(CH_2)_3CH_3$, R″ = CH_2Ph): oily product which crystallized slowly; chromatography in hexane/ethyl acetate, 75/25 (77%); R_f(C) 0.48.

14 (R′ = R = CH_3, R_1 = R_2 = H, R_3 = CH_2(4-OH)Ph, R″ = CH_2Ph): oily product; chromatography in hexane/ethyl acetate, 55/45 (64%); R_f(C) 0.32.

15 (R′ = R = CH_3, R_1 = R_2 = H, R_3 = CH_2(4-$OCOCH_3$)Ph, R″ = CH_2Ph): oily product (93%); R_f(A) 0.58.

16 (R′ = R = CH_3, R_1 = R_2 = H, R_3 = CH_2OCH_2Ph, R″ = CH_2Ph): oily product; chromatography in hexane/ethyl acetate, 8/2 (60%); R_f(G) 0.80.

17 (R′ = Ph, R = CH_3, R_1 = R_2 = H, R_3 = CH_3, R″ = tBu): white solid (88%); mp 110 °C; R_f(H) 0.42.

18 (R′ = Ph, R = CH_3, R_1 = R_2 = H, R_3 = CH_3, R″ = H): white solid (83%); mp 80 °C R_f(D) 0.26; NMR δ (ppm) 1.18 (CH_3, Ala, CH_3(CH)), 2.80 (S*CH*$_2$*CHCH*), 4.20 (CHα), 7.10–7.80 (Ar), 8.50 (NH), 12.45 (COOH).

19 (R′ = Ph, R = CH_3, R_1 = R_2 = H, R_3 = CH_2CH_3, R″ = tBu): chromatography in hexane/ethyl acetate, 8/2 (77%); oily product; R_f(C) 0.51.

20 (R′ = Ph, R = CH_3, R_1 = R_2 = H, R_3 = CH_2CH_3, R″ = H): white solid (86%); mp 97 °C; R_f(D) 0.32; NMR δ (ppm) 0.80 ($CH_3(CH_2)$), 1.20 (CH_3(CH)), 1.55–1.70 ($CH_2(CH_3)$), 2.80 (S*CH*$_2$-*CHCH*), 4.10 (CHα) 7.10–7.80 (Ar), 8.35 (NH), 12.55 (COOH).

21 (R′ = Ph, R = CH_3, R_1 = R_2 = H, R_3 = $(CH_2)_2CH_3$, R″ = tBu): chromatography in hexane/ethyl acetate, 7/3 (91%); oily product; R_f(C) 0.53.

22 (R′ = Ph, R = CH_3, R_1 = R_2 = H, R_3 = $(CH_2)_2CH_3$, R″ = H): white solid (72%); mp 98 °C; R_f(D) 0.33; NMR δ (ppm) 0.78 ($CH_3(CH_2)$), 1.19 (CH_3(CH)), 1.26 ($CH_2(CH_3)$), 1.55–1.62 (CH_2-(CH)), 2.80 (S*CH*$_2$*CHCH*), 4.20 (CHα), 7.15–7.80 (Ar), 8.35 (NH), 12.50 (COOH).

23 (R′ = CH_3, R = CH_2CH_3, R_1 = R_2 = H, R = CH_2(4-OH)Ph, R″ = CH_2Ph): chromatography in hexane/ethyl acetate, 7/3 (50%); oily product; R_f(A) 0.49.

24 (R′ = R = CH_3, R_1 = 2-CH_3, R_2 = H, R_3 = CH_2(4-OH)Ph, R″ = CH_2Ph): chromatography in hexane/ethyl acetate, 7/3 (58%); oily product; R_f(C) 0.39.

25 (R′ = R = CH_3, R_1 = 4-F, R_2 = H, R_3 = CH_3, R″ = CH_2Ph): oily compound; chromatography in hexane/ethyl acetate, 7/3 (62%); R_f(B) 0.22.

26 (R′ = R = CH_3, R_1 = 4-F, R_2 = H, R_3 = CH_2-(4-OH)Ph, R″ = CH_2Ph): oily product; chromatography in hexane/ethyl acetate, 7/3 (62%); R_f(B) 0.14. **27_1**: *O*-acetyl derivative (80%); R_f(C) 0.33.

28 (R′ = R = CH_3, R_1 = 4-OCH_3, R_2 = H, R_3 = CH_2-(4-OH)Ph; R″ = CH_2Ph): oily product; chromatography in hexane/ethyl acetate, 65/35 (65%); R_f(C) 0.28. **29_1**: *O*-acetyl derivative (82%); R_f(C) 0.45.

30 (R′ = R = CH_3, R_1 = 4-OCH_2Ph, R_2 = H, R_3 = CH_3, R″ = CH_2Ph): oily product; chromatography in hexane/ethyl acetate, 3/1 (85%); R_f(C) 0.24.

31 (R′ = R = CH_3, R_1 = 4-NO_2, R_2 = H, R_3 = CH_2-(4-OH)Ph, R″ = CH_2Ph): oily product chromatography in hexane/ethyl acetate, 1/1 (59%); R_f(E) 0.39.

32 (R′ = R = CH_3, R_1 = 4-NH_2, R_2 = H, R_3 = CH_2(4-OH)Ph, R″ = CH_2Ph): obtained from **31** by catalytic hydrogenation (Pd/C) in methanol; oily product (85%); R_f(G) 0.46.

33 (R′ = R = CH_3, R_1 = 4-OH, R_2 = H, R_3 = CH_2(4-OH)Ph, R″ = CH_2Ph). **32** was dissolved in a mixture of dioxane/water/H_2SO_4, and a cold solution of $NaNO_2$ (1.6 equiv) was added at 5 °C. After 5 min of stirring, the excess of $NaNO_2$ was destroyed by urea, and the mixture was treated by a boiling aqueous solution of 1% H_2SO_4 for 5 min. The aqueous layer was extracted with EtOAc and, after a standard treatment, an oily product was obtained: chromatography in hexane/ethyl acetate, 6/4 (63%); R_f(E) 0.26.

34 (R′ = R = CH_3, R_1R_2 = OCH_2O, R_3 = CH_3, R″ = CH_2Ph): oily product; chromatography in hexane/ethyl acetate, 75/25 (65%); R_f(B) 0.30.

35 (R′ = R = CH_3, R_1 = 4-F, R_2 = 3-F, R_3 = CH_3, R″ = CH_2Ph): oily product; chromatography in hexane/ethyl acetate, 3/1 (95%); R_f(C) 0.20.

36 (R′ = R = CH_3, R_1 = 4-F, R_2 = 3-F, R_3 = CH_2(4-OH)Ph, R″ = CH_2Ph): oily product; chromatography in hexane/ethyl acetate, 3/1 (85%); R_f(C) 0.24. **37**; *O*-acetyl derivative (81%); R_f(D) 0.20.

38 (R′ = R = CH_3, R_1 = 4-OCH_3, R_2 = 3-OCH_3, R_3 = CH_2(4-OH)Ph, R″ = CH_2Ph): oily product; chromatography in hexane/ethyl acetate, 6/4 (52%); R_f(G) 0.44.

39 (R′ = R = CH_3, R_1 = 4-OCH_2(4-OCH_3)Ph, R_2 = 3-OCH_3, R_3 = CH_2(4-OH)Ph, R″ = CH_2Ph): oily product; chromatography in hexane/ethyl acetate, 6/4 (57%); R_f(C) 0.20.

40 (R′ = R = CH_3, R_1 = 4-OH, R_2 = 3-OCH_3, R_3 = CH_2(4-OH)Ph, R″ = CH_2Ph): obtained from **37** by treatment with TFA in CH_2Cl_2; white solid; mp 130 °C (88%); R_f(C) 0.15.

41 (R′ = adamantyl, R = CH_3, R_1 = R_2 = H, R_3 = CH_3, R″ = H). This compound was prepared by treatment of **42b** with adamantyl chloride in alcalin medium: white solid (77%); mp 105 °C; R_f(A) 0.26; NMR δ (ppm) 1.10 (CH_3(CH)), 1.25 (CH_3-Ala), 1.55, 1.68, 1.90 (adamantyl), 2.50–2.78 (S*CH*$_2$*CHCH*), 4.20 (CHαAla), 7.20 (Ar), 8.35 (NH), 12.40 (COOH).

42 (R′ = adamantyl, R = CH_3, R_1 = R_2 = H, R_3 = CH_3, R″ = CH_2Ph): mixture of stereoisomers; oily product (46%); R_f(D) 0.48; NMR δ (ppm) 1.00–1.30 (CH_3Ala + CH_3(CH)), 1.45–2.00 (adamantyl), 2.5–3.10 (S*CH*$_2$*CHCH*), 3.95–4.40 (CHαAla), 5.04–5.10 (OCH_2Ph), 7.20–7.30 (Ar), 8.08–8.30, 8.52–8.54 (NH).

General Procedure for the Synthesis of Compounds 43–64. Procedure C was used for compounds containing methyl or benzyl esters. These compounds were dissolved in degassed EtOH, followed by the addition at 0 °C and under inert atmosphere of NaOH (1 M) (3 equiv). After being stirred for 1 h at 0 °C and for 2–4 h at room temperature, the mixtures were acidified to pH 1 with HCl (3 N), concentrated in vacuo, taken off with degassed water, and extracted with degassed $CHCl_3$. The organic layers were washed with H_2O and brine and dried over Na_2SO_4. After filtration and evaporation of the solvent, the crude inhibitors were obtained.

Procedure D was used for compounds containing *tert*-butyl esters. They were dissolved in CH_2Cl_2, and TFA (20 equiv) was added at 0 °C. After being stirred for 1 h at 0 °C the mixture was evaporated in vacuo and the residue purified by chromatography. The protection of the thiol group was then eliminated by procedure C.

43 (*R* = CH_3, R_1 = R_2 = R_3 = H): white solid; mp 129 °C (89%); R_f(F) 0.62; NMR δ (ppm) 1.20 (CH_3), 1.40 and 2.40 (*CH*$_2$S + *SH*), 2.50–3.00 (*CHCH*CO), 3.5–3.8 (CH_2Gly), 7.20 (Ar), 8.20–8.50 (NH), 12.43 (COOH). Anal. ($C_{13}H_{17}NO_3S$) C, H, N.

44 (R = R_3 = CH_3, R_1 = R_2 = H): oily compound (75%); R_f(G) 0.27; NMR δ (ppm) 0.80–1.10 (CH_3), 1.20–1.35, (CH_3(Ala)), 1.90–2.40 (*CH*$_2$S + *SH*), 2.5–3.2 (*CHCH*CO), 3.8–4.3 (CHαAla), 7.20 (Ar), 7.9–8.5 (NH), 12.40 (COOH). Anal. ($C_{14}H_{19}NO_3S$) C, H, N.

45 (R = CH_3, R_1 = R_2 = H, R_3 = iPr): oily product (70%); R_f(G) 0.42; NMR δ (ppm) 0.5–0.9 (CH_3(Val)), 1.10 (CH_3), 2.05 (CHβVal), 1.90–2.40 (*CH*$_2$S + *SH*), 2.55–3.10 (*CHCHCO*), 3.9–4.3 (CHαVal), 7.20 (Ar), 7.90–8.20 (NH), 12.40 (COOH). Anal. ($C_{16}H_{23}NO_3S$) C, H, N.

46 (R = CH_3, R_1 = R_2 = H, R_3 = iBu): oily product (72%); R_f(G) 0.33; NMR δ (ppm) 0.80 (CH_3-Leu), 1.12 (CH_3), 1.50 (CH_2β, CHγLeu), 1.90–2.40 (*CH*$_2$S + *SH*), 2.70 (*CHCH*CO), 3.90–4.30

(CHαLeu), 7.20 (Ar), 8.00–8.40 (NH), 12.40 (COOH). Anal. ($C_{17}H_{25}NO_3S$) C, H, N.

47 (R = CH_3, R_1 = R_2 = H, R_3 = $(CH_2)_3$-CH_3): oily product (93%); R_f(G) 0.47; NMR δ (ppm) 0.80 (CH_3Nle), 1.15 (CH_3), centered on 1.20 ($CH_2\gamma$ and δ, Nle), 1.60–1.90 ($CH_2\beta$Nle), 1.90–2.40 (CH_2S + SH), 2.60–3.10 (CHCHCO), 3.70–4.30 (CHαIle), 7.20 (Ar); 7.90–8.35 (NH), 12.45 (COOH). Anal. ($C_{17}H_{25}NO_3S$) C, H, N.

48 (R = CH_3, R_1 = R_2 = H, R_3 = CH_2(4-OH)Ph): white solid; mp 80 °C (81%); R_f(F) 0.43; NMR δ (ppm) 0.90–1.20 (CH_3), 1.40–1.70 (HS), 1.90–2.40 (CH_2S), 2.5–3.0 (CHCHCO + $CH_2\beta$Tyr), 4.20–4.50 (CHαTyr), 6.5–7.4 (Ar), 8.00–8.50 (NH), 9.10 (OH), 12.50 (COOH). Anal. ($C_{20}H_{23}NO_4S$) C, H, N.

49 (R = CH_3, R_1 = R_2 = H, R_3 = CH_2OCH_2Ph): oily product (62%); R_f(G) 0.29; NMR δ (ppm) 1.15 (CH_3), 1.90–2.40 (CH_2S + SH), 2.55–3.00 (CHCHCO), 3.30–3.75 ($CH_2\beta$Ser), 4.20–4.60 (CHαSer + OCH_2(Ph)), 7.20 (Ar), 8.15–8.50 (NH), 12.60 (COOH). Anal. ($C_{21}H_{25}NO_4S$) C, H, N.

50 (R = CH_3, R_1 = R_2 = H, R_3 = CH_2CH_3): white solid; mp 140 °C (80%); R_f(D) 0.19 NMR δ (ppm) 0.90 ($CH_3(CH_2)$), 1.12 ($CH_3(CH)$), 1.60–1.75 ($CH_2(CH_3)$), 1.90 (CH_2S), 2.40 (SH), 2.70 (CHCHCO), 4.20 (CHα), 7.13–7.18 (Ar), 8.30 (NH), 12.50 (COOH). Anal. ($C_{15}H_{21}NO_3S$) C, H, N.

51 (R = CH_3, R_1 = R_2 = H, R_3 = $(CH_2)_2CH_3$): white solid; mp 110 °C (84%); R_f(D) 0.17; NMR δ (ppm) 0.80 ($CH_3(CH_2)$), 1.13 ($CH_3(CH)$), 1.35 ($CH_2(CH_3)$), 1.60 (CH_2CH), 1.90 (CH_2S), 2.40 (SH), 2.65 (CHCHCO) 4.25 (CHαVal), 7.15–7.25 (Ar), 8.30 (NH), 12.50 (COOH). Anal. ($C_{16}H_{23}NO_3S$) C, H, N.

52 (R = CH_2CH_3, R_1 = R_2 = H, R_3 = CH_2(4-OH)Ph): oily product (90%); R_f(F) 0.45; NMR δ (ppm) 0.53 (CH_3), 1.30–1.82 ($CH_2(CH_3)$), 2.20 (HS), 2.40–3.00 ($CH_2\beta$Tyr + SCH_2 + CHCHCO), 4.20–4.50 (CHαTyr), 6.50–7.20 (Ar), 7.9–8.4 (NH), 9.10 (OH), 12.40 (COOH). Anal. ($C_{21}H_{25}NO_4$) C, H, N.

53 (R = CH_3, R_1 = 2-CH_3, R_2 = H, R_3 = CH_2(4-OH)Ph): white solid; mp 70 °C (89%); R_f(F) 0.51; NMR δ (ppm) 1.03 (CH_3CH), 1.50–2.00 (CH_2S), 2.20 (CH_3(Ar)), 2.40–3.20 ($CH_2\beta$Tyr + CHCHCO + SH), 4.12–4.50 (CHαTyr), 6.50–7.20 (Ar), 7.85–8.48 (NH), 9.10 (OH), 12.50 (COOH). Anal. ($C_{21}H_{25}NO_4S$) C, H, N.

54 (R = CH_3, R_1 = 4-F, R_2 = H, R_3 = CH_3): white solid; mp 60 °C (78%); R_f(F) 0.50; NMR δ (ppm) 0.85–1.15 ($CH_3(CH)$), 1.15–1.30 (CH_3Ala), 1.90–2.40 (CH_2S + SH), 2.50–2.80 (CHCHCO)), 3.90–4.35 (CHαAla), 6.90–7.20 (Ar), 8.00–8.50 (NH), 12.40 (COOH). Anal. ($C_{14}H_{18}NFO_3S$) C, H, N.

55 (R = CH_3, R_1 = 4-F, R_2 = H, R_3 = CH_2(4-OH)Ph): oily product (74%); R_f(F) 0.45; NMR δ (ppm) 0.80–1.12 ($CH_3(CH)$), 1.70–2.20 (CH_2S + SH), 2.50–2.80 (CHCHCO), 2.40–3.40 ($CH_2\beta$Tyr), 4.20–4.40 (CHαTyr) 6.50–7.20 (Ar), 8.00–8.40 (NH), 9.20 (OH), 12.60 (COOH). Anal. ($C_{20}H_{22}NFO_4S$) C, H, N.

56 (R = CH_3, R_1 = 4-OCH_3, R_2 = H, R_3 = CH_2(4-OH)Ph): oily product (83%); R_f(F) 0.42; NMR δ (ppm) 0.7–1.1 ($CH_3(CH)$), 1.70–2.30 (HS + CH_2S), 2.50–2.90 (CHCHCO + $CH_2\beta$Tyr), 3.60 (OCH_3), 4.20–4.40 (CHαTyr), 6.50–7.20 (Ar), 8.00–8.40 (NH), 9.10 (OH), 12.50 (COOH). Anal. ($C_{21}H_{25}NO_5S$) C, H, N.

57 (R = CH_3, R_1 = 4-OCH_2Ph, R_2 = H, R_3 = CH_3): colorless paste (82%); R_f(F) 0.15; NMR δ (ppm) 0.90–1.40 (CH_3(CH) + CH_3Ala), 1.90–2.40 (CH_2S + SH), 2.55–3.10 (CHCHCO), 3.90–4.30 (CHαAla), 5.00 (CH_2O), 6.75–7.40 (Ar), 7.90–8.40 (NH), 12.35 (COOH). Anal. ($C_{21}H_{25}NO_4S$) C, H, N.

58 (R = CH_3, R_1 = 4-NH_2, R_2 = H, R_3 = CH_2-(4-OH)Ph): oily product (60%); R_f(F) 0.40; NMR δ (ppm) 0.80–1.20 (CH_3), 1.80–2.40 (HS + CH_2S), 2.60–3.50 (CHCHCO + $CH_2\beta$Tyr), 4.30–4.60 (CHαTyr), 6.50–7.60 (Ar), 8.00–8.50 (NH), 9.00 (OH), 12.45 (COOH). Anal. ($C_{20}H_{24}N_2O_4S$) C, H, N.

59 (R = CH_3, R_1 = 4-OH, R_2 = H, R_3 = CH_2(4-OH)Ph): oily product (95%); R_f(F) 0.24, NMR δ (ppm) 0.85–1.10 (CH_3), 1.80–2.40 (HS + CH_2S), 2.50–3.00 (CHCHCO + $CH_2\beta$Tyr), 4.25–4.50 (CHαTyr), 6.50–7.01 (Ar), 8.00–8.50 (NH), 9.00–9.01 (OH), 12.52 (COOH). Anal. ($C_{20}H_{23}NO_5S$) C, H, N.

60 (R = CH_3, R_1R_2 = OCH_2O, R_3 = CH_3): white solid; mp 80 °C (89%); R_f(F) 0.56; NMR δ (ppm) 0.90–1.30 (CH_3(CH) + CH_3-Ala), 1.95–2.40 (SH + CH_2S), 2.55–3.00 (CHCHCO), 3.95–4.35 (CHαAla), 5.90 (OCH_2O), 6.65–6.85 (Ar), 7.90–8.40 (NH), 12.40 (COOH). Anal. ($C_{15}H_{19}NO_5S$) C, H, N.

61 (R = CH_3, R_1 = 4-F, R_2 = 3-F, R_3 = CH_3): white paste (70%); R_f(G) 0.17; NMR δ (ppm) 0.83–1.30 (CH_3CH + CH_3Ala), 2.00–2.40 (HS + CH_2S), 2.55–2.30 (CHCHCO), 3.90–4.30 (CHαAla), 6.90–7.40 (Ar), 8.00–8.37 (NH), 12.40 (COOH). Anal. ($C_{14}H_{17}NF_2O_3S$) C, H, N.

62 (R = CH_3, R_1 = 4-F, R_2 = 3-F, R_3 = CH_2-(4-OH)Ph): white solid; mp 75 °C (80%); R_f(G) 0.10; NMR δ (ppm) 0.75–1.12 (CH_3-(CH)), 1.85–2.35 (HS + CH_2S), 2.50–3.00 (CHCHCO + $CH_2\beta$Tyr), 4.17–4.55 (CHαTyr), 6.50–7.35 (Ar), 7.97–8.40 (NH), 9.10 (OH), 12.55 (COOH). Anal. ($C_{20}H_{21}NF_2O_4S$) C, H, N.

63 (R = CH_3, R_1 = 4-OCH_3, R_2 = 3-OCH_3, R_3 = CH_2(4-OH)-Ph): oily product (71%); R_f(F) 0.47; NMR δ (ppm) 0.70–1.10 (CH_3), 1.70–2.40 (SH + CH_2S), 2.50–3.00 (CHCHCO + $CH_2\beta$Tyr), 3.70 (OCH_3), 6.50–7.00 (Ar), 8.00–8.40 (NH), 9.10 (OH), 12.40 (COOH). Anal. ($C_{22}H_{27}NO_6S$) C, H, N.

64 (R = CH_3, R_1 = 4-OH, R_2 = 3-OCH_3, R_3 = CH_2(4-OH)Ph): oily product; R_f(F) 0.25; NMR δ (ppm) 0.70–1.10 (CH_3), 1.70–2.30 (SH + CH_2S), 2.50–3.00 (CHCHCO + $CH_2\beta$Tyr), 3.70 (OCH_3), 4.20–4.50 (CHαTyr), 6.50–7.10 (Ar), 8.00–8.40 (NH), 8.60–9.10 (OH), 12.48 (COOH). Anal. ($C_{21}H_{25}NO_6S$) C, H, N.

Separation of the Stereoisomers by Chiral Amines: (1) Separation of the Stereoisomers of 6a. To a solution of 10 g of **6a** in 150 mL of ether was added 5.36 mL of (*S*)-(−)-α-methylbenzylamine. A white precipitate P_1 (4.17 g) was collected. The soluble fraction was treated by 1 N HCl, and 6.85 g of the starting material was recovered. To this fraction was added 3.67 mL of (*R*)-(+)-α-methylbenzylamine, and a second precipitate was filtered (P_2 = 0.7 g). The same deprotection by 1 N HCl was performed, and a third precipitation was obtained from 4.2 g of the initial compound and 2.83 mL of (*S*)-(−)-α-(1-naphthyl)-ethylamine. P_3 (2.3 g) was separated, and after deprotection of the soluble fraction (2.7 g), the last precipitate (P_4 = 0.5 g) was obtained by addition of 1.80 mL of (*R*)-(+)-α-(1-naphthyl)-ethylamine.

The four precipitates P_1–P_4 were recrystallized four times in 2-propanol, and after a final deprotection by HCl (1 N), the four stereoisomers of **6a**, respectively designated **$6a_1$–$6a_4$**, were analyzed by HPLC (C_8 KWI column using TFA 0.05%/CH_3CN, 60/40, as eluent) and their optical rotations measured in $CHCl_3$ (*c* = 0.1).

$6a_1$:$[\alpha]^{22}_D$ +73°; t_R 30.4 min. **$6a_2$**:$[\alpha]^{22}_D$ −71°; t_R 30.4 min. **$6a_3$**: $[\alpha]^{22}_D$ −59°; t_R 32.4 min. **$6a_4$**:$[\alpha]^{22}_D$ = +60°; t_R 32.4 min.

(2) Separation of the Stereoisomers of 6b. The separation of **6b** was performed by the same procedure using only the (*S*-(−)- and (*R*-(+)-α-methylbenzylamine. Two precipitates were obtained and recrystallized from 2-propanol. After deprotection by 1 N HCl, the isomers **$6b_1$** and **$6b_2$** were obtained. **$6b_1$**:$[\alpha]^{22}_D$ −32°. **$6b_2$**:$[\alpha]^{22}_D$ +34.2°.

Preparation of the Four Stereoisomers of the Inhibitor 44 (44a–44d). A coupling step between the four separate isomers of **6a** (**$6a_1$–$6a_4$**), the two separate isomers of **6b** (**$6b_5$** and **$6b_6$**), and alanine benzyl ester was performed by the procedure described for compound **7** followed by the final deprotection (procedure C).

44a: obtained from **$6a_1$**; oily product (60%); R_f(D) 0.32; NMR δ (DMSO) 1.12 (CH_3Ala + CH_3(CH)), 2.00 (SH), 2.25–2.65 (CH_2S), 2.65 (CHCO), 3.05 (CH-Ph), 4.08 (CHαAla), 7.20 (Ph), 8.15 (NH), 12.35 (COOH); $[\alpha]^{22}_D$ (*c* = 0.1, EtOH 95%) + 5 °C.

44b: obtained from **$6a_2$**; oily product (65%); R_f(D) 0.32; NMR δ (DMSO) 0.85 (CH_3Ala), 1.15 ($CH_3(CH)$), 2.00 (SH), 2.55–2.70 (CH_2S), 2.70 (CHCO), 2.85 (CH-Ph), 3.90 (CHαAla), 7.15 (Ph), 7.98 (NH), 12.40 (COOH); $[\alpha]^{22}_D$ (*c* = 0.1, EtOH 95%) −74°.

44c: obtained from **$6a_4$** or **$6b_5$**; oily product (65%); R_f(D) 0.31; NMR δ (DMSO) 1.12 ($CH_3(CH)$), 1.25 (CH_3Ala), 2.00 (SH), 1.92–2.40 (CH_2S), 2.60 (CHCO), 2.70 (CH-Ph), 4.22 (CHαAla), 7.15–7.25 (Ph), 8.40 (NH), 12.38 (COOH); $[\alpha]^{22}_D$ (*C* = 0.1, EtOH 95%) −7°.

44d: obtained from **$6a_3$** or **$6b_6$**; oily product (65%); R_f(D) 0.32; NMR δ (DMSO) 1.08 ($CH_3(CH)$), 1.25 (CH_3Ala), 2.00 (SH), 1.90–2.45 (CH_2S), 2.65 (CHCO), 2.70 (CH-Ph), 4.25 (CHαAla), 7.15–7.25 (Ph), 8.46 (NH), 12.42 (COOH).

Asymmetric Synthesis of (2*S*,3*R*)- and (2*S*,3*S*)-*N*-[2-(Mercaptomethyl)-1-oxo-3-phenylbutyl]-(*S*)-alanine. The same synthetic pathway was used for the two stereoisomers.

(1) (4*S*)-3-[(3*S* or 3*R*)-1-Oxo-3-phenylbutyl]-4-(1-methylethyl)-2-oxazolidinone (65a and 65b). To a solution of (4*S*)-4-(1-methylethyl)-2-oxazolidinone[32] in dry THF (1 eq) cooled to −78 °C were successively added 1.02 eq of *n*-butyllithium in hexane and 1.05 eq of 3(*S*)- or 3(*R*)-phenylbutyl chloride (prepared from

the corresponding acid and $SOCl_2$ in benzene). The temperature was raised to 0 °C, and the mixture was stirred for 30 min at this temperature and then hydrolyzed by addition of 1 M K_2CO_3. After 1 h at room temperature, the mixture was concentrated in vacuo and the resulting aqueous layer was successively washed with water and brine and dried over Na_2SO_4. After evaporation of the solvent, the crude solid was purified by flash chromatography on silica gel column using hexane/ethyl acetate, 8/2, as eluent.

65a: oily product, 9.15 g (96%); R_f(H) 0.57; NMR ($CDCl_3$) δ (ppm) 0.8 ($(CH_3)_2CH$), 1.25 (CH_3CH), 2.25 ($CH(CH_3)_2$), 3.05–3.40 ($COCH_2CH$), 4.05 ($2C_5H$), 4.25 (C_4H), 7.1–7.3 (Ar).

65b: white solid, 4.9 g (80%); mp 54 °C; R_f(H) 0.49; NMR ($CDCl_3$) δ (ppm) 0.6–0.75 ($(CH_3)_2CH$), 1.25 (CH_3-CH), 2.1 ($CH(CH_3)_2$), 2.9–3.45 ($COCH_2$), 3.35 ($CH(CH_3)$), 4.15 ($2C_5H$), 4.3 (C_4H), 7.1–7.3 (Ar).

(2) (4*S*)-3-[(2*S*,3*R* or 3*S*)-1-oxo-2-[[(2-phenylethyl)thio]methyl]-3-phenylbutyl]-4-(1-methylethyl)-2-oxazolidinone (66a and 66b). To a solution of lithium diisopropylamide (prepared from 1 eq of *n*-butyllithium in hexane and 1.1 eq of diisopropylamine in dry THF at 0 °C) cooled to −78 °C was added 1 eq of compounds **66a** or **66b** in dry THF. After 30 min at this temperature, 1.1 eq of freshly distilled benzyl bromomethyl sulfide[42] was added, and the mixture was stirred for 2 h at −20 °C and then hydrolyzed with a 5% solution of NH_4Cl. THF was evaporated in vacuo, and the aqueous layer was extracted with CH_2Cl_2. The organic layer was successively washed with 1 M $NaHSO_4$ (x2), 1 M $KHCO_3$ (x2), and brine and dried over Na_2SO_4. After evaporation of the solvent, the oily product obtained was chromatographed in petroleum ether/diethyl ether, 8/2.

66a: colorless oil, 1.1 g (55%); R_f(I) 0.28; NMR ($CDCl_3$) δ (ppm) 0.75 ($(CH_3)_2CH$), 1.15 (CH_3CH), 2.20 ($CH(CH_3)_2$), 2.65 (CH_2S), 2.90 ($CHCH_3$), 3.30–3.50 (SCH_2Ph), 3.60–3.90 (C_4H + $2C_5H$), 4.50 (CHCO), 7.20 (Ar).

66b: colorless oil, 1.8 g (65%); R_f(I) 0.28; NMR ($CDCl_3$) δ (ppm) 0.90 ($(CH_3)_2CH$), 1.20 (CH_3CH), 2.20–2.54 (CH_2S), 2.41 ($CH(CH_3)_2$), 2.85 ($CHCH_3$), 3.55 (SCH_2Ph), 4.19 ($2C_5H$), 4.50 (C_4H), 4.75 (COCH), 6.20–7.30 (Ar).

(3) (2*S*,3*S* or 3*R*)-2-[(Benzylthio)methyl]-3-phenylbutanoic Acid (69a or 69b). To a solution of **66a** or **66b** in a mixture of THF/H_2O (3/1) was added at 0 °C 8 eq of a H_2O_2 (30%) solution and 4 eq of LiOH. The mixture was stirred for 1 h at 0 °C and overnight at room temperature. After hydrolysis by a solution of Na_2SO_3 (1.5 M), 0.5 M $NaHCO_3$ was added. THF was evaporated and the aqueous layer diluted and extracted with CH_2Cl_2. The aqueous layer was acidified to pH with 2 N HCl and extracted with EtOAc. The oily product obtained was then purified by flash chromatography in *n*-hexane/EtOAc/acetic acid, 4/6/0.5. Three compounds were isolated, the sulfone **68** (R_f = 0.55) and the two diastereoisomeric sulfoxides **67** (R_f = 0.34 and 0.26). The sulfoxides were reduced by a reflux with triethylphosphine (3 eq) in CCl_4 for 2 h. The solvent was evaporated, and the triphenylphosphine oxide was precipitated by addition of *n*-hexane/diethyl ether, 1/1. After filtration and evaporation of the solvents, compound **69a** or **69b** was isolated with a global yield of 35%.

69a: oily product, 0.26 g; R_f(D) 0.48; NMR δ ($CHCl_3$) 1.20 (CH_3), 2.49–2.60 ($SCH_2(CH)$), 2.75 (CHCO), 3.07 (CH-Ph), 3.55 (SCH_2Ph), 7.15 (Ph).

69b: oily product, 0.36 g; R_f(D) 0.47; NMR δ ($CDCl_3$) 1.26 (CH_3), 2.18–2.46 ($SCH_2(CH)$), 2.75 (CHCO), 2.95 (CH-Ph), 3.50 (SCH_2Ph), 7.02–7.18 (Ph).

(4) *N*-[(2*S*,3*S*)-2-(Mercaptomethyl)-1-oxo-3-phenylbutyl]-(*S*)-alanine (44b). Compound **69a** was coupled with alanine benzyl ester following the procedure used for the synthesis of compound **7**. An oily product chromatographied in cyclohexane/ethyl acetate, 8/2, was obtained: 0.22 g (70%); R_f(H) 0.36; NMR ($CDCl_3$) δ (ppm) 1.15 (CH_3-CH), 1.25 (CH_3Ala), 2.15 (CH-CO), 2.60–2.78 ($SCH_2(CH)$), 2.90 (CH-Ph), 3.60 (SCH_2Ph), 4.20 (CHαAla), 5.00 (OCH_2Ph), 5.62 (NH), 7.00–7.40 (Ph). The protected inhibitor (0.48 mmol) was dissolved in THF and dry NH_3 (14 mL), and Na (2.5 mmol) was added. After 15 min at −33 °C, the reaction was quenched by addition of NH_4Cl. The solvents were evaporated, and the residue was dissolved in 1 M KOH, washed with Et_2O, acidified to pH = 1 with 1 N HCl, and extracted with Et_2O. The organic layer was evaporated and the residue dissolved in degassed methanol. Zn (3 eq) and 3 N HCl (6 eq) were added. After 1 h at room temperature, the solvent was evaporated, the residue taken off with degassed water and extracted with degassed $CHCl_3$. The organic layer was dried over Na_2SO_4, filtered, and evaporated in vacuo. An oily product was obtained, 40 mg (30%). All the analytical data obtained for this compound are identical to those described for compound **44b**.

(5) *N*-[(2*S*,3*R*)-2-(Mercaptomethyl)-1-oxo-3-phenylbutyl]-(*S*)-alanine (44c). Compound **69b** (0.15 g) was coupled with alanine methyl ester following the procedure described for the synthesis of compound **7**. An oily product (0.147 g, 77%) was obtained. The deprotection of the carboxylate group was performed according to procedure C, and compound **70b** was obtained as a colorless oil (0.11 g, 85%): R_f(D) 0.25; NMR ($CDCl_3$) δ (ppm) 1.10 (CH_3(CH)), 1.45 (CH_3Ala), 2.15 (CHCO), 2.15–2.60 (CH_2S), 2.80 (CHPh), 3.45 (SCH_2Ph), 4.60 (CHαAla), 5.95 (NH), 6.95–7.30 (Ph).

Compound **70b** was deprotected by Na in liquid NH_3 as described for the preparation of **44b** from **69a**, oily product 0.04 g (53%). All the analytical data obtained for this compound are identical to those found for **44c**.

Molecular Modeling. Conformational analyses were performed through high-temperature molecular dynamics (MD). The calculations were performed with the Discover package (Biosym Technologies Inc., San Diego, CA) using standard forcefield parameters.[43,44] The first-step calculations consisted on equilibrating the system to 600 K during 10 ps. The trajectory was then continued for 100 ps, a structure being extracted every 1 ps. The resulting 100 structures were then submitted to 10 ps of MD at 300 K and further minimized through a procedure consisting on 1000 steps of steepest descent followed by 5000 steps of conjugated gradient method. No conformational constraints were taken into account during the calculations except for the peptide bond for which the trans configuration was assumed. The dielectric constant was kept to 1 during all calculation steps. Each structure was then graphically analyzed using the insight-analysis module, and the various conformers were classified into families. Each family was characterized by a representative conformation and its relative population defined as its percentage of occurrence along the 100-ps simulation. The representative conformers of each family was then compared with the structure of the bicyclic lactam[34] by using the template-forcing procedure (insight II User Guide, version 2.1.0 copyright 1992 Biosym Technologie Inc., San Diego, CA). The nine atoms taken into account for the rms calculations are indicated in Table 1.

Biological Tests. [^{3}H]Tyr-D-Ala^2-Leu-enkephalin (32 Ci/mmol) and [^{3}H]HACBOGly[45] (35 Ci/mmol) was obtained from Dositeck (CEA, France). *N*-Cbz-Phe-His-Leu[46] was from Bachem (Bubendorft, Switzerland). [^{3}H]Trandolaprilate[47] (66 Ci/mmol) was a gift of Roussel Uclaf (France).

Recombinant human angiotensin-converting enzyme obtained as described[27] was a generous gift of Pr. Corvol (College de France, Paris, France).

(1) In Vitro Inhibition of NEP and ACE. Assay for NEP Activity. NEP was purified to homogeneity from rabbit kidney as previously described.[48] NEP (final concentration 1 pmol/100 μL, specific activity on [^{3}H]D-Ala^2-Leu-enkephalin, 0.3 mmol/mg/min) was preincubated for 15 min at 25 °C with or without increasing concentrations of inhibitor in a total volume of 100 μL of 50 mM Tris-HCl buffer pH = 7.4. [^{3}H]D-Ala^2-Leu-enkephalin (K_m = 30 μM) was added to a final concentration of 20 nM, and the reaction was stopped after 30 min by adding 10 μL of 0.5 M HCl. The tritiated metabolite Tyr-D-Ala-Gly was separated on polysterene beads, and the radioactivity was measured by liquid scintillation counting.

Assay for ACE Activity. ACE (final concentration 0.02 pmol/100 μL, specific activity on *N*-Cbz-Phe-His-Leu, 13 mmol/mg/min) was preincubated for 15 min at 37 °C with or without various concentrations of the inhibitors in 0.1 M Tris HCl buffer pH 7.4, and *N*-Cbz-Phe-His-Leu was added to a final concentration of 0.05 mM. The reaction was stopped after 15 min by adding 400 μL of 2 M NaOH. After dilution with 3 mL of water, the concentration of His-Leu was determined following the fluorimetric assay described by Cheung et al.[49] with a MPF 44A

Perkin-Elmer spectrofluorimeter (excitation 365 nm, emission 495 nm). The calibration curve for His-Leu was obtained by addition of increasing concentrations of His-Leu into 0.1 mL of 0.1 M Tris-HCl buffer pH 7.4 containing the denaturated enzyme.

(2) In Vivo Inhibition of Kidney NEP and Lung ACE in Mice. The prodrugs used in these experiments were dissolved in 10% ethanol/10% cremophor/80% water. After oral administration of increasing doses of the various inhibitors, ED_{50} values were determined by competition experiments using iv administered [^{3}H]trandolaprilate (0.5 μCi) for ACE and [^{3}H]HACBOGly (1 μCi) for NEP, which were injected 45 min after inhibitor administration. Fifteen minutes after injection of the tritiated probes, the mice were sacrified and the kidney (for NEP) or the lung (for ACE) was rapidly taken and homogeneized in 50 mM Tris-HCl buffer pH 7.4 (and 1 mM NaCl for ACE) at 4 °C. The homogenate was filtered, and the radioactivity was measured by liquid scintillation counting. Total binding of the radiolabeled probes was determined under the same conditions, without inhibitors. The nonspecific binding was obtained by coinjection of [^{3}H]trandolaprilate with captopril (1000 eq) in the case of lung ACE and by coinjection of [^{3}H]HACBOGly with retrothiorphan[50] (10.000 eq) for kidney NEP.

The time course of the inhibition was obtained by the same procedure using a single dose of inhibitor (2.6×10^{-5} mol/kg) and increasing times (from 30 min to 18 h) before injection of the radiolabeled probes.

Pharmacology. (1) Pharmacological Effects of iv Administration of RB 105 in Spontaneous Hypertensive Rat (SHR). This experiment was performed in male, 12–14 week old SHR ($n = 5$) (Iffa-Credo, St. Germain sur Abresles, France). After anaesthetization with ether, the right carotid artery was cannulated with a polyethylene catheter (Biotrol, Paris, France) for continuous blood pressure monitoring and the right jugular vein with an elastomer catheter for infusions. The bladder was catheterized for urine collection. Saline infusion (1.2 mL/h) was begun immediately, and the rat was allowed to recover for 200 min to stabilize blood pressure and diuresis. The experimental protocol was carried out in unanaesthetized conscious animals. After a base-line period of 1 h, RB 105 or its vehicle were administered by injection plus infusion.

The electrolyte concentrations of fresh urine samples were measured with an ion-selective electrode (Beckman, Brea, CA) and expressed in micromoles per minute.

(2) Pharmacological Effects of Orally Administered Mixanpril in SHR. The effects of the prodrug Mixanpril were assessed after chronic oral administration in SHR. Ten rats were maintained in metabolic cages for 10 days. The animals had free access to tap water and food. Mixanpril or its vehicle was administered by gavage (50 mg/kg) twice a day for 5 days in a cross-over protocol. Blood pressure was measured every day, 2 h after gavage, by the tail cuff method, and diuresis and natriuresis were determined once a day.

Acknowledgment. The authors would like to thank Christine Dupuis for expert manuscript preparation and Dr. Ann Beaumont for stylistic revisions. We are grateful to Dr. B. Molino (Rhône Poulenc Rorer, Collegeville) for helpful discussions. The work was supported by funds from the Institut National de la Santé et de la Recherche Médicale, the Centre National de la Recherche Scientifique, the Université René Descartes and the European economic community (Programme Biomed).

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