Design of Orally Active Dual Inhibitors of Neutral Endopeptidase and Angiotensin-Converting Enzyme with Long Duration of Action

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Mercaptoacyl dipeptides, containing a glycine linked to a C-terminal 5-phenylproline, have been synthesized in order to obtain new highly efficient dual inhibitors of the two zinc metallopeptidases, neutral endopeptidase (NEP) and angiotensin-converting enzyme (ACE), which are involved in the control of blood pressure and fluid homeostasis. These compounds have been designed (i) to fit optimally the ACE pharmacophore previously described (Fournié-Zaluski, M. C.; *et al. J. Med. Chem.* **1994**, *37*, 1070−1083), through interaction with the S_1, S_1', and S_2' subsites of this enzyme, (ii) and to interact with the S_1' and S_2' subsites of NEP with the 5-phenylproline moiety outside the catalytic domain (Coric, P.; *et al. J. Med. Chem.* **1996**, *39*, 1210−1219). Replacement of Gly by Ala in these mercaptoacyl dipeptides induced an about 100-fold decrease in ACE inhibition. This shows that, in agreement with molecular modeling studies, a steric constraint as weak as a methyl group hinders optimal ACE active site recognition. Among these compounds, the dual inhibitor **26** (RB 106) (K_i, ACE = 0.35 nM; NEP = 1.6 nM) showed excellent pharmacokinetic properties with an almost complete *in vivo* inhibition of NEP and ACE for more than 4 h after oral administration in mice of a low dose (2.6×10^{-5} mol/kg) of the inhibitor. Moreover, RB 106 remained active 12 h after oral administration. In spontaneous hypertensive rats, a chronic treatment of orally administered RB 106 (25 mg/kg/day) induced a prolonged hypotensive effect (−28 mmHg) still significant 2 days after the end of the treatment. In DOCA salt rats, a hypotensive response and a significant natriuresis were observed after iv administration. RB 106, which is one of the most potent dual inhibitors described to date, could have interesting clinical applications in long term treatment of congestive heart failure and myocardial ischemia.

Introduction

The physiological control of blood pressure and fluid homeostasis is mainly dependent on regulatory peptides: angiotensin II (Ang II) which induces vasoconstriction and indirectly sodium retention through aldosterone secretion[1] and atrial natriuretic peptide (ANP) which increases diuresis and natriuresis and has a slight vasodilatatory action. Furthermore, ANP reduces plasma renin and aldosterone levels.[2] The activity of these two antagonistic systems is regulated essentially by metabolizing processes involving the zinc metallopeptidases, angiotensin-converting enzyme (ACE, EC 3.4.15.1) and neutral endopeptidase (NEP, EC 3.4.24.11, neprilysin). ACE is responsible for the maturation of Ang II from its inactive precursor angiotensin I (Ang I), and NEP inactivates ANP.[3] Moreover, both peptidases are involved in the metabolism of bradykinin,[4,5] a vasodilatatory peptide.

Specific inhibitors of ACE[6,7] are currently used in the treatment of hypertension and congestive heart failure but are generally associated with diuretics which can be responsible for side effects such as activation of pressor system and kaliuresis. Selective inhibitors of NEP[8] have been recently shown to have diuretics and natriuretics properties, without loss of potassium in various experimental models of hypertension,[9−14] but displayed hypotensive effects only in DOCA salt rats.[14−16] Moreover, in patients with heart failure, no significant reduction in arterial blood pressure and left ventricular dimensions was observed in spite of the persistent elevation of plasma ANP levels.[17,18] These limited effects of NEP inhibitors were attributed to the opposite actions of Ang II and ANP on blood pressure and sodium excretion, suggesting that coinhibition of NEP and ACE could be a more efficient approach in the treatment of cardiovascular diseases.[19] Preliminary experiments have shown that the association of selective inhibitors of NEP and ACE leads to synergetic beneficial properties in animal models of hypertension[20,21] and congestive heart failure.[22,23] Therefore numerous dual inhibitors of NEP and ACE have been synthesized, but in most cases their pharmacological properties have not been reported.[24] The first dual inhibitor described, HS-CH_2-CH(CH_2Ph)-CONH-CH[CH_2CH-(CH_3)$_2$]-COOH,[25−27] later designated SQ-28133,[20] elicited hypotensive activity in spontaneous hypertensive rats (SHR) and deoxycorticosterone acetate salt (DOCA salt) hypertensive rats and potentiated ANP and bradykinin hypotensive responses in SHR.[20] Alatrioprilate, a closely related derivative of the selective NEP inhibitor thiorphan, HS-CH_2-CH(CH_2Ph)-CONH-CH_2-COOH,[28] was shown to increase diuresis, natriuresis, and cGMP excretion after iv administration in anesthetized rats, in agreement

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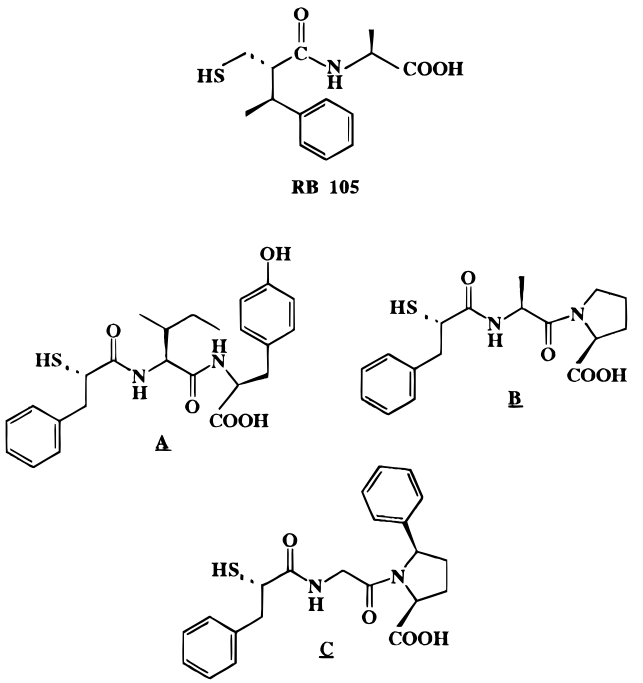


Figure 1. Schematic representation of the dual NEP−ACE inhibitors **A**, [(2*S*)-2-mercapto-3-phenylpropanoyl]-Ile-Tyr, and **B**, [(2*S*)-2-mercapto-3-phenylpropanoyl]-Ala-Pro,[36] which led to the design of the new inhibitor **C**, [2-mercapto-3-phenylpropanoyl]-Gly-(5-Ph)Pro. The structure of the previously designed[31] dual inhibitor RB 105 is also shown.

with its ability to inhibit renal NEP.[29] However, the relatively weak *in vitro* inhibitory potency of SQ-28133 and alatrioprilate for ACE limits their *in vivo* efficiencies.[30]

In contrast, the dual inhibitor RB 105 (or S 21402) (Figure 1) synthesized in our laboratory displays K_i values in the nanomolar range for NEP and ACE.[30] Intravenous infusion of RB 105 decreased blood pressure and increased natriuresis in three animal models of hypertension (renovascular, DOCA salt,[31] and spontaneously hypertensive rats[32]), and strong dose-dependent hypotensive effects were observed after oral administration in spontaneously hypertensive rats.[31] MDL-100,240, another orally active thiol-containing dual inhibitor of NEP and ACE, was reported to have similar properties to RB 105.[33,34]

Given their properties, dual inhibitors could be used in the long term treatment of chronic mild hypertension or myocardial ischemia.[35,64] This requires compounds with a long duration of action as the second generation of dual inhibitors. This could be achieved by increasing the affinities for both NEP and ACE. With this aim, we have recently synthesized mercaptoacyl dipeptides such as **A** and **B**[36] (Figure 1) endowed with nanomolar and subnanomolar inhibitory potencies toward NEP and ACE, respectively. These compounds were shown to interact with the S_1, S_1', and S_2' subsites of ACE, whereas, using both molecular modeling and experiments with mutated Glu[102]NEP, they have been demonstrated to fit the S_1' and S_2' subsites of NEP with the C-terminal amino acid located at the surface of the enzyme.[36] This later result agrees with previously reported data on the localization of various thiol inhibitors within the NEP active site.[37,38] However, the *in vivo* inhibition of lung ACE induced by oral administration of **A** and **B**, in mice, was found to be relatively weak and short.[36] This could result from various factors such as the production of inactive metabolites, the degradation of these modified peptides, or their rapid renal elimination caused by a too high hydrophilicity. It was therefore necessary to improve the bioavailability of these dual inhibitors, for instance, by increasing their hydrophobicity, without decreasing their inhibitory properties toward ACE and NEP. As ACE was reported to contain a large and continuous $S_1'-S_2'$ subsite,[39] a comparison of the structure of **A** and **B** showed that introduction of the phenyl moiety of **A** on the proline ring of **B** could result in potent inhibitors of ACE with enhanced lipophilicity, provided that the amino acid preceding the substituted proline did not hinder the phenyl ring from fitting the enzyme active site. This was achieved by introducing a glycine moiety in the new series of dual inhibitors of type **C** (Figure 1). Substituted proline and thiazolidinecarboxylic acid derivatives have been previously used to obtain selective ACE inhibitors in both the captopril and enalapril series.[6] Given the similarity in their structures, these inhibitors could be assumed to interact with NEP and ACE as their parent compounds **A** and **B**.[36] The synthesis and biochemical and pharmacological properties of these new potent and orally active dual inhibitors are reported in this paper.

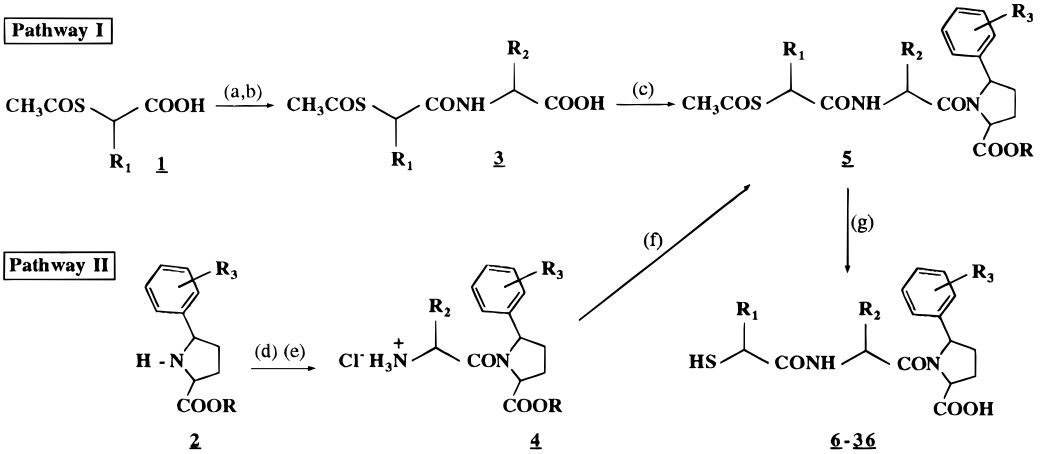
Results

Synthesis. Scheme 1 summarizes the two synthetic pathways used for the preparation of the inhibitors. The key step was the condensation of the highly hindered 5-phenylproline derivatives with either *N*-(acetylthio)-alkanoyl amino acids (pathway I) or N-protected amino acids (pathway II). In both cases, the yields were around 30−50%, whatever the coupling reagents used (DCC/HOBt, BOP, PyBrop...). Nevertheless, pathway II using the acyl chloride method of coupling for the introduction of glycine residue was preferred because the purification steps were found to be easier. The various (acetylthio)alkanoic acids **1** were prepared from the corresponding α-amino acids by the classical two-step synthesis.[40] When optically pure α-amino acids were used, compounds **1** were obtained with a large degree (>90%) of enantiomeric enrichment.

The various 5-phenylproline derivatives **2**, substituted or not on the phenyl ring, have been synthesized (Scheme 2) following the procedures described by Ohta *et al.*[41] for alkylation of pyroglutamate derivatives and by Ezquerra *et al.*[42] for the synthesis of the Δ^1-pyrroline derivatives using pyroglutamic esters as starting materials. These methods have the advantage of giving the 5-phenylprolines with the same absolute configuration at the α-carbon as the initial pyroglutamates. Furthermore, in the last step of the synthesis, a catalytic hydrogenation of the Δ^1-pyrroline yielded a cis proline derivative, while a chemical reduction with sodium borohydride[43] led to a mixture of cis and trans proline isomers which could be easily separated by crystallization[43] allowing the trans isomers to be isolated and studied separately.

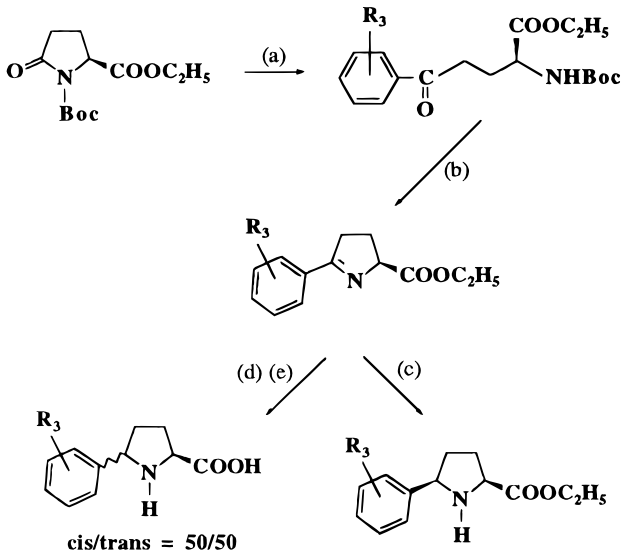
***In Vitro* Inhibitory Potencies**. The inhibitory activities of the newly synthesized compounds were measured on NEP, purified from rabbit kidney,[44] and ACE, purified from rat testes.[45] The IC_{50} values of the initial compound **6** and analogues containing a single

Scheme 1. Scheme for the Synthesis of the Dual NEP−ACE Inhibitors[a]



[a] (a) $H_2NCH(R_2)COOtBu$, DCC/HOBt ; (b) TFA/CH_2Cl_2(50/50) ; (c) H-N(R3)-COOR, DCC/HOBt ; (d) ZNH-CH(R2)-COOH, BOP or ZNH-CH(R2)-COCl, Et_3N ; (e) H_2, Pd/C, HCl ; (f) $CH_3COSCH(R_1)COOH$, DCC/HOBt ; (g) OH^-, then H^+.

Scheme 2. Scheme for the Synthesis of 5-Phenylproline Derivatives[a]



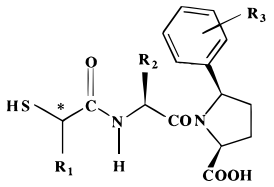
[a] (a) R3-C6H4-MgBr,THF ; (b) TFA/CH_2Cl_2 ; (c) H_2, PdO_2 ; (d) NaOH, then H_3O^+ ; (e) $NaBH_4$.

modification at the level of R_1, R_2, or R_3 are reported in Table 1. As expected from our initial hypothesis, compound **6** was efficient in inhibiting both enzymes with IC_{50} values in the nanomolar range on NEP and subnanomolar range for ACE. As expected from previous studies on selective ACE inhibitors,[6] the inversion of configuration of the mercaptoacyl moiety (compound **7**) was responsible for a large decrease in ACE recognition. The absence of change observed on NEP supports the localization of the benzyl group of the mercaptoacyl moiety within the S_1' subsite of NEP[37,38] since this subsite is well known to be devoid of stereoselective preference.[8,46] The introduction of a substituent in the R_2 position (compound **8**) induced an almost 100-fold loss of potency for ACE and a 6-fold decrease for NEP. On the other hand, the replacement of the benzyl side chain in position R_1 of **6** by aliphatic moieties, either hydrophobic (compound **9**) or positively charged (compound **10**), was unfavorable for NEP and ACE as shown by the large enhancement in the respective IC_{50} values. Conversely, the presence of substituents, such as OH (compound **11**) or NH_2 (compound **12**), on the benzyl moiety of **6** was well accepted by NEP but decreased slightly the recognition of ACE (factor of 8). Substitutions on the phenyl ring of the phenylproline moiety by a hydroxyl group in the meta position (compound **14**) or by hydrophobic substituents (compounds **15** and **16**) were slightly detrimental for ACE recognition. In contrast, **13** (OH group in ortho position) was one of the most potent compounds of this new series of dual inhibitors.

For all the compounds reported in Table 1, the 5-phenylproline moiety has a (2*S*,5*R*) configuration. The influence of this stereochemical parameter on enzyme inhibition was investigated by using inhibitors with a 5-(*ortho*-methylphenyl)proline moiety (Table 2). As compared to **15** (2*S*,5*R*), which has a cis configuration, the inversion of the two asymmetric carbons of the proline ring in **17** (2*R*,5*S*) induces 2- and 10-fold increases in the IC_{50} values for NEP and ACE, respectively. For the (2*S*,5*S*) (trans isomer) **18**, the loss of activity was more important for ACE with IC_{50} values of 48 nM (factor of 16) than for NEP, IC_{50} = 17 nM (factor of 3). The last stereoisomer **19** (2*R*,5*R*) was also less potent than **15** (IC_{50}, NEP = 25 nM; ACE = 112 nM).

Furthermore, the position of the methyl group on the 5-phenylproline substituent had no significant influence on the inhibitory potency (compare **15**, **16**, and **23**), and the presence of a hydroxyl group on the benzyl moiety

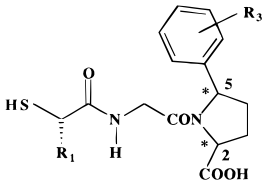
Table 1. *In Vitro* Inhibitory Potencies on NEP and ACE of Various Mercaptoacyl Dipeptides



no.	R_1	abs conf*	R_2	R_3	IC_{50} (nM)[a] NEP	IC_{50} (nM)[a] ACE
6	CH_2Ph	*S*	H	H	1.6 ± 0.3	0.55 ± 0.05
7	CH_2Ph	*R*	H	H	3.2 ± 0.6	25 ± 2
8	CH_2Ph	*S*	CH_3	H	10 ± 2	45 ± 5
9	$CH(CH_3)CH_2CH_3$	*S*	H	H	50 ± 7	3.7 ± 0.8
10	$(CH_2)_4NH_3^+$	*R* + *S*	H	H	500 ± 50	403 ± 57
11	CH_2(*p*-OH)Ph	*S*	H	H	3.4 ± 0.8	4.2 ± 0.4
12	CH_2(*p*-NH_2)Ph	*S*	H	H	1.8 ± 0.4	3.5 ± 0.5
13	CH_2Ph	*S*	H	*o*-OH	1 ± 0.5	0.5 ± 0.1
14	CH_2Ph	*S*	H	*m*-OH	2.4 ± 0.4	1.5 ± 0.5
15	CH_2Ph	*S*	H	*o*-CH_3	5.3 ± 1.0	3.1 ± 0.3
16	CH_2Ph	*S*	H	*m*-CH_3	2.3 ± 0.6	6.2 ± 1.9

[a] IC_{50} values are the mean ± SEM from three independent experiments performed in triplicate.

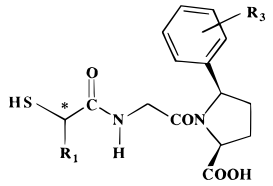
Table 2. *In Vitro* Inhibitory Potencies on NEP and ACE of Various Mercaptoacyl Dipeptides: Influence of 5-Phenylproline Stereochemistry



no.	R_1	abs conf* C_2	abs conf* C_5	R_3	IC_{50} (nM)[a] NEP	IC_{50} (nM)[a] ACE
15	CH_2Ph	*S*	*R*	*o*-CH_3	5.3 ± 1.0	3.1 ± 0.3
17	CH_2Ph	*R*	*S*	*o*-CH_3	8.5 ± 1.6	33 ± 7
18	CH_2Ph	*S*	*S*	*o*-CH_3	17 ± 0.7	48 ± 5
19	CH_2Ph	*R*	*R*	*o*-CH_3	25 ± 3	112 ± 13
20	CH_2(*p*-OH)Ph	*S*	*R*	*o*-CH_3	2.7 ± 0.5	5.0 ± 0.3
21	CH_2(*p*-OH)Ph	*R*	*S*	*o*-CH_3	5.1 ± 1.1	12.5 ± 1.7
16	CH_2Ph	*S*	*R*	*m*-CH_3	2.3 ± 0.6	3.1 ± 0.3
22	CH_2(*p*-OH)Ph	*S*	*R*	*m*-CH_3	1.5 ± 0.3	3.1 ± 1.6
23	CH_2Ph	cis (racemic)		*p*-CH_3	12.5 ± 3.5	6.8 ± 1.4
24	CH_2(*p*-OH)Ph	cis (racemic)		*p*-CH_3	5.5 ± 0.8	2.5 ± 0.5

[a] IC_{50} values are the mean ± SEM from three independent experiments performed in triplicate.

Table 3. Influence of R_1 and R_2 Substituents on the *in Vitro* Inhibitory Potencies on NEP and ACE of Various Mercaptoacyl Dipeptides



no.	R_1	abs conf*	R_3	IC_{50} (nM)[a] NEP	IC_{50} (nM)[a] ACE
25	CH_2(*p*-OH)Ph	*S*	*o*-OH	3.5 ± 0.3	0.6 ± 0.2
26	CH_2(*p*-OH)Ph	*S*	*m*-OH	1.6 ± 0.2	0.7 ± 0.1
27	CH_2(*p*-OH)Ph	*S*	*p*-OH[b]	6.36 ± 0.08	0.46 ± 0.02
28	CH_2(*m*-OH)Ph	*R,S*	*m*-OH	4.3 ± 0.6	3.2 ± 0.8
29	CH_2(*p*-OCH_3)Ph	*S*	*o*-OH	2.2 ± 0.5	0.42 ± 0.05
30	CH_2(*p*-OH)Ph	*S*	*o*-OCH_3	3.4 ± 0.8	4.5 ± 0.1
31	CH_2(*p*-OCH_3)Ph	*S*	*o*-OCH_3	2.5 ± 0.4	2.2 ± 0.8
32	CH_2(*p*-OH)Ph	*S*	*p*-NH_2[b]	26 ± 3	23 ± 3
33	CH_2(*p*-Cl)Ph	*S*	*o*-OH	1.2 ± 0.2	0.9 ± 0.1
34	CH_2(*p*-F)Ph	*S*	*m*-OH	5.3 ± 0.3	3.8 ± 0.4
35	CH_2(*p*-COOH)Ph	*R,S*	*o*-OH	62 ± 1	5.3 ± 0.7
	MDL-100,173[c]			2.2 ± 0.4	2.5 ± 0.4

[a] IC_{50} values are the mean ± SEM from three independent experiments performed in triplicate. [b] These compounds possess a racemic (cis) 5-phenylproline. [c] The K_i values given in ref 50 for MDL-100,173 are 0.11 and 0.08 nM for ACE and NEP, respectively.

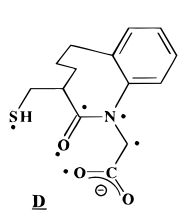
of the inhibitors (**20**−**22** and **24**) is devoid of significant effect on the recognition of both peptidases recognition.

The inhibitory potencies of compounds bearing substituents of different polarities on the aromatic cycles are reported in Table 3. It was observed that, when the benzyl moiety was substituted by a *p*-OH group (**25** to **27**), the compounds had a similar activity on the two enzymes, whatever the position (ortho, meta, or para) of the hydroxyl group on the phenyl ring of the proline moiety. Compounds with a para methoxy (**29**) or a para chlorobenzyl (**33**) group also behaved as efficient inhibitors of both enzymes. The introduction of *o*-OCH_3 as an R_3 substituent decreased activity for ACE by 1 order of magnitude. A large decrease in inhibitory potencies for both enzymes was observed when R_3 = *p*-NH_2 (compound **32**), even taking into account that this molecule has a racemic phenylproline moiety. Finally, the presence of a carboxyl group in **35** is particularly unfavorable for NEP inhibition.

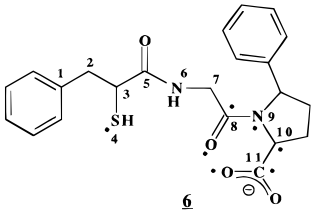
Molecular Modeling. Molecular modeling studies were performed with the aim of defining the biologically active conformations of compound **6** in the active site of ACE. This was done for ACE by using the previously described template **D**[30] developed by using the rigid potent inhibitor (IC_{50} = 4 nM) 3-(mercaptomethyl)-3,4,5,6-tetrahydro-2-oxo-1*H*-1-benzazocine-1-acetic acid (MTBA)[47] and the proposed biologically active conformation of captopril.[48]

Two families of conformers characterized by the presence of the proline peptide bond in cis (16%) and trans (84%) forms were obtained following molecular dynamic studies of **6** (as described in materials and methods), in good agreement with the results of NMR

Table 4. Conformers of Compound **6** Fitting the Constrained ACE Inhibitor Structure **D**[30] Determined by a Template-Forcing Minimization Procedure



D



6

conformers[a]	ψ_2[b]	ϕ_2[b]	ψ_1[b]	χ_1[b]	rmsd dev	E(pot) (kcal mol^{-1})
F_1	174	−89	−43	59	0.326	11.9
F_2	174	−92	−41	178	0.323	12.9
F_3	−175	87	47	−179	0.325	11.1
F_4	−175	84	54	67	0.330	14.1

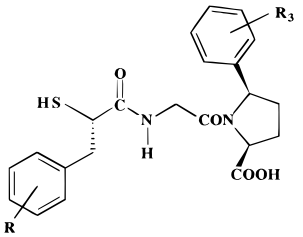
[a] The atoms taken into account for the rms calculations are indicated in the formula of **D** and **6** by dots. [b] The dihedral angles (in degrees) are defined as follows: ψ_2, $N_6-C_7-C_8-N_9$; ϕ_2, $C_5-N_6-C_7-C_8$; ψ_1, $S_4-C_3-C_5-N_6$; χ_1, $C_1-C_2-C_3-C_5$.

experiments. As the proline peptide bond is trans in the bioactive conformation of ACE inhibitors,[48] only the trans conformers were submitted to a template-forcing procedure using the template **D**, leading to two groups of molecules composed of 12 and 5 members, respectively, which differ only by the values of the ϕ_2 angle, −85° ± 10° or 85° ± 10°. All these molecules fit correctly (rmsd < 0.35) the ACE template **D**. The physical characteristics of the four molecules (F_1-F_4) having the lowest potential energies are reported in Table 4.

In the case of **8**, the alanine analogue of **6**, we did not find any low-energy conformers able to overlap the **D** template with satisfactory values of rmsd. This is very likely due to the steric hindrance induced by the 5-phenyl substituent of Pro since the removal of this group led to a compound inhibiting ACE with an IC_{50} value of 0.3 nM.[36] On the other hand, a template-forcing procedure was achieved with **D**, compound **6**, and the two recently described highly constrained cyclic dual inhibitors MDL-100,173[50] and BMS-182,657.[24b] In both cases, a good overlap (rmsd < 0.4) on the pharmacophore and on the conformer (F_2) of **6** was observed (Figure 2). In each case, the orientation of the 2-mercapto-3-phenylpropanoyl side chain corresponds to the proposed biologically active conformation of the enalaprilate side chain ($C_6H_5CH_2CH_2CH(COOH)$)-Ala-Pro.[39] Furthermore, it could be observed that the phenyl rings of the three dual inhibitors partially overlapped. This defines a large hydrophobic domain which could be filled with lipophilic moieties interacting with the continuous $S_1'-S_2'$ subsite of ACE.

***In Vivo* Inhibition of Kidney NEP and Lung ACE.** The most efficient compounds, selected from their *in vitro* inhibitory potencies on NEP and ACE, were tested to evaluate their *in vivo* activities by previously described methods.[30] In an initial screening, a single dose of inhibitor was orally given to mice (2.6×10^{-5} mol/kg) and the inhibition of both enzymes was measured 2 h after administration. The *in vivo* efficiency of the compounds was determined by inhibition of the binding of a tritiated probe ([^{3}H]trandaloprilate) to lung ACE and by inhibition of the degradation of the tritiated substrate [^{3}H]-D-Ala^2-Leu-enkephalin by kidney NEP.

Table 5. *In Vivo* Inhibition of Lung ACE and Kidney NEP 2 h after Oral Administration of a Single Dose of the Dual Inhibitors ($c = 2.6 \times 10^{-5}$ mol/kg)



			inhibition (%)	
no.	R	R_3	lung ACE	kidney NEP
6	H	H	63 ± 3	75 ± 5
15	H	*o*-CH_3	57 ± 5	37 ± 5
23	H	*p*-CH_3	42 ± 10	34 ± 6
11	*p*-OH	H	ND[a]	27 ± 7
20	*p*-OH	*o*-CH_3	60 ± 5	44 ± 3
24	*p*-OH	*p*-CH_3	33 ± 2	3 ± 1
25	*p*-OH	*o*-OH	82 ± 7	69 ± 7
26	*p*-OH	*m*-OH	89 ± 3	72 ± 11
27	*p*-OH	*p*-OH	ND	25 ± 9

[a] ND: not determined.

The results are summarized in Table 5. The modulation of the *in vivo* activity was found more important for NEP than for ACE, and, as previously noticed, with dual inhibitors belonging to the RB 105 series,[30] there is no strict relationship between the *in vitro* IC_{50} values and the *in vivo* efficiencies of these molecules. However, compounds with IC_{50} values >100 nM were found to be inactive (not shown).

The unsubstituted inhibitor **6** exhibited a high *in vivo* efficiency on both enzymes since, 2 h after oral administration, 65% of lung ACE and 75% of kidney NEP were still blocked. The introduction of hydrophobic groups in position R_3 (compounds **15** and **23**) was detrimental for both enzymes but particularly for NEP. The presence of only one hydrophilic R group (compound **11**) decreased significantly the *in vivo* inhibition of NEP, whereas the conjunction of hydrophilic and hydrophobic groups in R and R_3 positions, respectively (compound **20**), restored partially the inhibition of both enzymes except when the methyl group (R_3) was in the para position (compound **24**). Conversely, the introduction of hydroxyl groups on both R_3 and R positions (compounds **25** and **26**) yielded inhibitors endowed with good bioavailabilities, except, once more, when R_3 = *p*-OH (**27**). On the basis of these results, compound **26** which was, along with **6**, the most efficient *in vivo* dual inhibitor of this series, was preferred to the latter due to its better solubility. A time course analysis of the inhibition of NEP and ACE by **26** and the efficient dual inhibitor MDL-100,173[50] was achieved. As shown in Figure 3, the two dual inhibitors, orally administered in mice at the same dose (2.6×10^{-5} mol/kg), have almost identical *in vivo* potencies at short times (60 and 120 min), but compound **26** has a longer duration of action since inhibition of both NEP (Figure 3A) and ACE (Figure 3B) was still significant 12 h after administration.

Pharmacological Responses. The antihypertensive activity of the dual inhibitor **26** was tested after oral administration in SHR. As shown in Figure 4 increasing concentrations of **26**, administered daily for

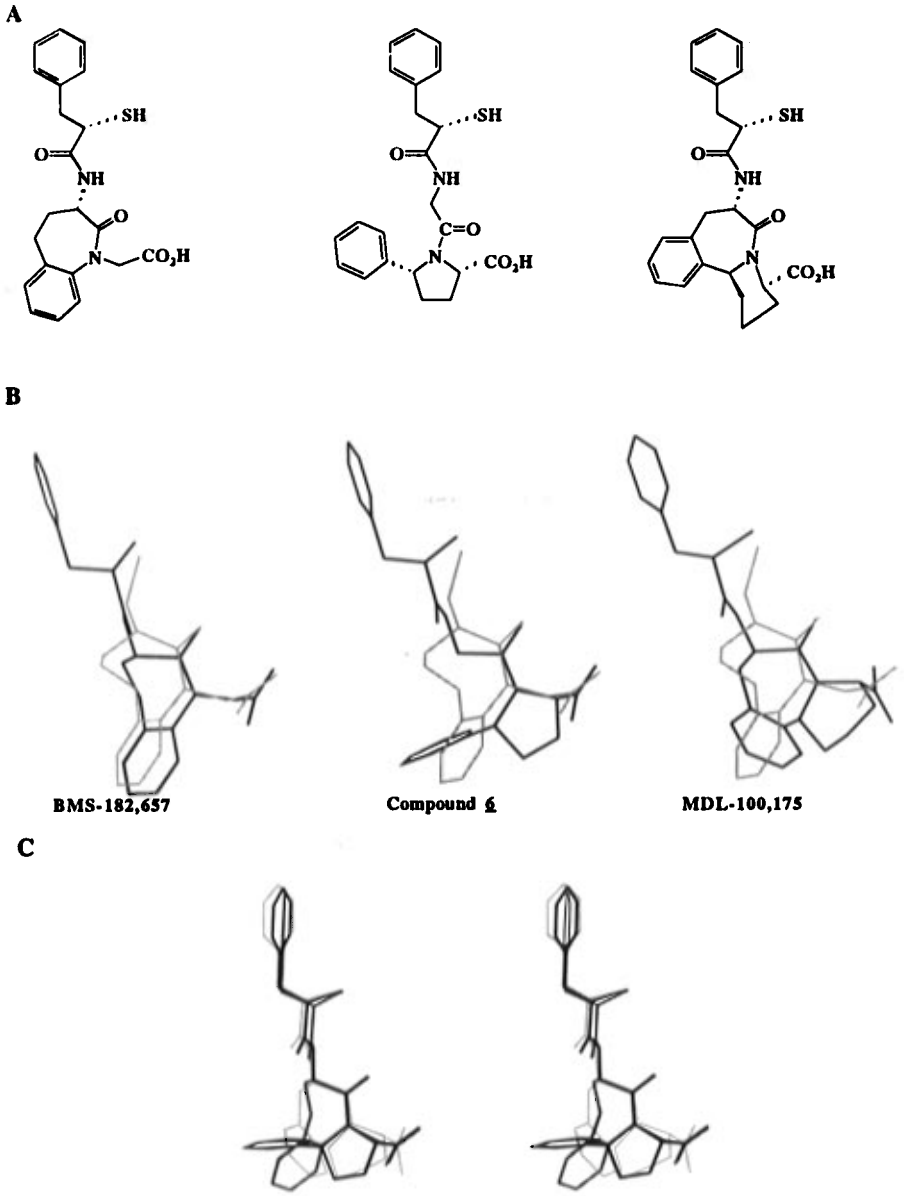


Figure 2. (A) Schematic representation of **6**, MDL-100,173,[50] and BMS-182,657.[24b] (B) Stable conformers of **6**, MDL-100,173, and BMS-182,657 fitting the previously proposed ACE pharmacophore **D** (dotted line). (C) Stereoview of the overlapped assumed biologically active conformation at the ACE active site level of **6** (bold line), MDL-100,173 (dotted line), and BMS-182,657 (medium line). The superimposition shows the similarity in the spatial disposition of the SH, amide, and carboxyl groups in the three inhibitors. Moreover, the large hydrophobic domain evidenced by the partial overlap of the phenyl moiety linked to the cyclic moieties supports the occurrence of a corresponding continuous lipophilic $S_1'-S_2'$ subsite in the ACE active site.

5 days, induced a dose-dependent decrease in blood pressure, statistically significant from 5 mg/kg. A maximum reduction of 28 mmHg was observed for 25 mg/kg, and a plateau was observed for higher doses. Moreover the maximal effect was obtained after 2 days of treatment (Figure 5). At the end of the experiment, the recovery of the initial blood pressure required 3 days, its reduction still remaining significant after 48 h (Figure 5).

Compound **26** was also iv administered to DOCA salt rats at 25 mg/kg^{-1} + 25 mg/kg^{-1} h^{-1} as previously described.[14,32] A decrease in mean blood pressure was observed (Figure 6A) associated with a significant increase in natriuresis (Figure 6B) during the time of the experiment.

Discussion

In this work, we report new orally active dual inhibitors of NEP and ACE endowed with a long duration of action. This property is crucially dependent on the affinity of the inhibitors for both peptidases which must be as high as possible and on their hydrophobic–hydrophilic balance. This latter parameter is assumed to control the renal elimination of the compounds with subsequent inhibition of NEP in the proximal tubule and the life time in the plasma which must be long enough to ensure a prolonged blockade of vascular ACE.[31] With this aim, the recently synthesized mercaptoacyl dipeptides HS-CH(CH_2Ph)CO-Ile-Tyr, **A**, and HS-CH(CH_2Ph)CO-Ala-Pro, **B** (Figure 1), characterized by IC_{50} values in the nanomolar range for NEP and ACE,[36] were used as models. The improvement in pharmacokinetic properties was obtained by introducing a phenyl moiety in the proline ring of molecules of type **B** in which Ala was replaced by Gly. In addition to being endowed with a similar lipophilicity but to be more easily synthesized than the constrained tricyclic

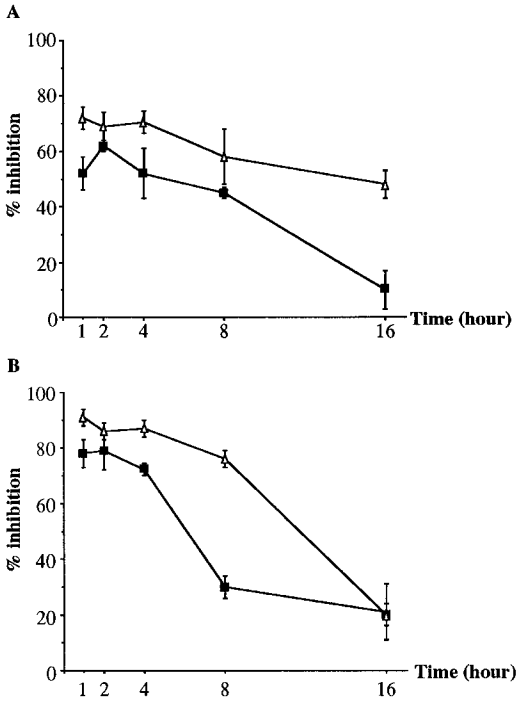


Figure 3. (A) Time course of renal NEP inhibition in mice after oral administration (2.6×10^{-5} mol/kg) of RB 106 (compound **26**) (△) and MDL-100,173[50] (■). (B) Time course of lung ACE inhibition in mice after oral administration (2.6×10^{-5} mol/kg) of RB 106 (compound **26**) (△) and MDL-100,173[50] (■).

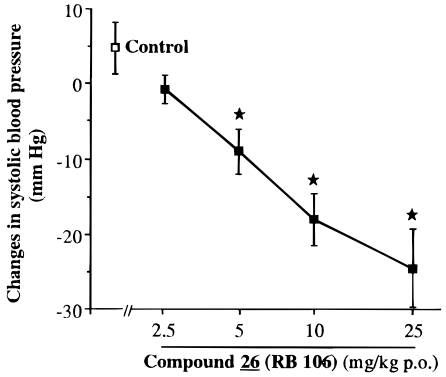


Figure 4. Variation in mean blood pressure observed in response to increasing doses of daily orally administered RB 106 (compound **26**) in SHR; $*p < 0.05$.

inhibitor MDL-100,173[50] or the bicyclic compound BMS-182,657 reported during this work,[24b] the mercaptoacyl dipeptide inhibitors of type **C**, described here, possess a residual flexibility which is expected to facilitate their adaptation in the active site of the two peptidases. The position of the phenyl substituent on the proline ring was selected following molecular modeling studies carried out with the recently designed ACE pharmacophore **D** in which the bicyclic moiety of MTBA is assumed to fit the S_1' and S_2' subsites of ACE, which were proposed to constitute a continuous hydrophobic domain.[30,39] The best overlap with this ACE template was obtained with the cis isomer of the (2*S*)-5-phenylproline. The strong *in vitro* inhibitory potencies ($IC_{50} = 1.6$ nM for NEP and 0.55 nM for ACE) of **6**, which contains the selected 5-phenylproline isomer, confirmed the validity of this strategy. Moreover, **6** has the advantage of possessing two aromatic rings which could be easily substituted by

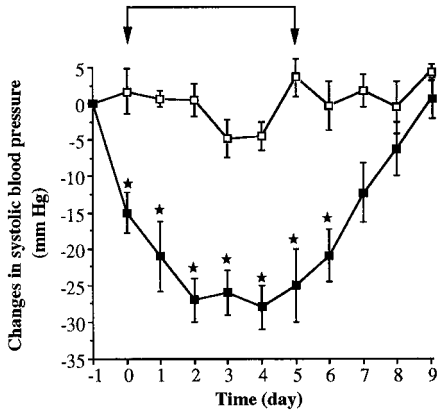


Figure 5. Variation in blood pressure measured during chronic oral administration of 25 mg/kg/day RB 106 (compound **26**) to SHR.

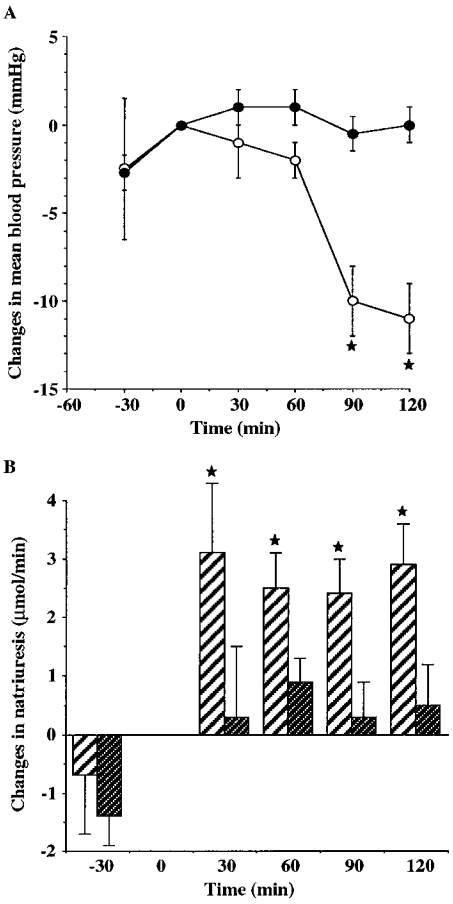


Figure 6. (A) Variation in blood pressure measured after iv administration of 25 mg kg^{-1} bolus + 25 mg kg^{-1} h^{-1} of RB 106 to DOCA salt rats. The arrow represents the begining of the treatment: ●, control animals; ○, treated animals. (B) Variation in natriuresis measured after iv administration of 25 mg kg^{-1} bolus + 25 mg kg^{-1} h^{-1} of RB 106 to DOCA salt rats. The arrow represents the begining of the treatment: white stippled bars, control animals; black stippled bars, treated animals.

hydrophilic or hydrophobic groups in order to modulate the *in vivo* targeting of endothelial ACE and epithelial NEP.[30]

The conformational analysis of **6** showed that the four conformers fitting the **D** template with the lowest energy differ by the relative disposition of the thiol and carboxyl group and the spatial orientation of the mercaptoacyl moiety, defined by $\chi 1$ (Table 4). The con-

former F_2 could correspond to the biologically active form of ACE carboxyl inhibitors such as enalapril and derivatives which have a P_1 benzyl moiety as the dual inhibitors reported here.[6]

Moreover, a template-forcing procedure using **6** and the rigid dual inhibitors MDL-100,173[50] and BMS-182,-657[24b] showed that these two compounds also have an extended conformation of F_2 type (Figure 2) and could bind to the ACE active site in the same manner as **6**. Moreover, the superimposition of these assumed biologically active conformations shows a partial overlap of the phenyl rings linked to the cyclic moieties supporting the occurrence of a large and continuous hydrophobic $S_1'-S_2'$ subsite in ACE.[39] It is important to observe that the mutual orientation of the SH and COOH groups in **6** (conformer F_2) defined by the value of the dihedral angle θ between the noncontinuous atoms $S-C\alpha_{Phe}-C\alpha_{Pro}-C_{COOH}$, $\theta = 24°$, is similar to that found in the biologically active conformation of ACE inhibitors, $\theta = 29°$.[47,48]

The position of **6** inside the NEP active site is very likely identical with that of **B** which has been established recently by using both molecular modeling studies and the mutated Glu^{102}NEP.[36] In these complexes, the C-terminal proline residue is located at the surface of the enzyme active site. The absence of important hydrophobic interactions with the enzyme surface of the C-terminal amino acid of **6** is clearly illustrated by the similarity in the IC_{50} values for NEP of this compound and its precursor HS-CH(CH_2Ph)-CONH-CH_2COOH which are 1.6 and 2.1 nM, respectively. The corresponding IC_{50}'s toward TLN were 1.2 and 2.2 μM, respectively, emphasizing again the great analogies between the active sites of TLN and NEP.[46,49,51] Furthermore, due to the location of the 5-phenylproline moiety outside the active site of NEP, there are no great differences in the inhibitory potencies of the four stereoisomers **15** and **17**−**19**. The slight changes observed could be due to steric contacts with the enzyme's surface.

The computed conformation of **6** inside TLN ($\psi_2 = 163°$, $\phi_2 = -146°$, $\chi_1 = -45°$) and by extension inside NEP, obtained as previously described,[36] is not very different from that (F_2) fitting the ACE template (Table 4). This shows that the molecule is capable of binding to the NEP or ACE active site without major conformational readjustment. Likewise, conformers of similar low energy fitting the ACE and NEP templates were found in MD-100,173. The binding of these two families of conformers to their adapted enzymes seems more convincing than the proposed recognition of ACE and NEP by MD-100,173 with the amide bond of the molecule under trans or cis conformation, respectively.[50] This would require an isomerization process thermodynamically highly unfavorable.

The concept of mixed inhibitors is based on the occurrence of important similarities in the active sites of the targeted peptidases.[27,8] This is the case for NEP, ACE, and TLN which are able to cleave *in vitro* peptides such as enkephalins or bradykinin at the same peptide bond.[8] Nevertheless, due to subtle differences in the NEP and ACE active sites, the various changes introduced in **6** had different effects on each enzyme. For NEP, almost all the compounds synthesized have inhibitory potencies in the nanomolar range, except when the essential characteristics of the active site were not respected. For instance, NEP prefers aromatic residues, uncharged at physiological pH, in the S_1' subsite.[25,27] Accordingly, compounds **9**, **10**, and **35** which contain aliphatic or charged P_1' residues have IC_{50} values in the $10^{-8}-10^{-7}$ M range.

For ACE, the various inhibitors interact with the three S_1, S_1', and S_2' subsites, and consequently their activities were more sensitive to changes affecting the corresponding P_1-P_2' side chains. For the R_1 group, which interacts with the S_1 subsite, the benzyl chain in **6** was preferred to an aliphatic residue in **9** and the (*S*) configuration of the 2-mercaptoacyl moiety was critical (compare **6** and **7**), as previously shown for carboxyalkyl dipeptides belonging to the enalapril series.[6] Moreover, the replacement of the central glycine by an alanine (compound **8** versus **6**) led to a 100-fold decreased activity indicating, as confirmed by molecular modeling, that the simultaneous presence of a 5-phenyl substituent on Pro and a methyl group on the preceding amino acid hinders the molecule from reaching conformations of low energies fitting the ACE template. This is clearly illustrated by the nanomolar IC_{50} values found in corresponding compounds in which the 5-phenyl group has been removed.[36] In accordance with molecular modeling studies done with the ACE template **D**, the optimal interaction with the $S_1'-S_2'$ domains of this peptidase requires a (2*S*,5R) configuration for the 5-phenylproline moiety. This is illustrated by the IC_{50} of compound **15** which was more than 10 times better than those of **17**−**19** (Table 2).

The inhibitory potency measured *ex vivo* after oral administration in mice appeared to be a good reflection of the capacity of a tested compound to reach the target enzymes.[30] For NEP, which has essentially a kidney epithelial localization although the enzyme is also present in the vascular wall[52] and heart,[35] it was striking to observe that the best bioavailabilities were obtained with the initial inhibitor **6** and the two bis-hydroxylated derivatives **25** and **26** which are more hydrophilic. The simplest explanation is that **6** is rapidly metabolized by hydroxylation of the aromatic rings. All the other compounds tested were significantly less efficient (Table 5). For ACE, the results were not very different. Thus, the unsubstituted compound **6** has a relatively good *in vivo* activity, and the introduction of hydrophilic substituents (compounds **25** and **26**) slightly improved the bioavailability. Curiously, this was not the case with compounds **11** and **27**. Compounds **25** and **26** were the most efficient in this prescreening test with more than 80% of lung ACE inhibition and around 70% of kidney NEP inhibition 2 h after a single oral administration of 2.6×10^{-5} mol/kg. Even though these two compounds have the same *in vivo* efficiency, the choice to develop **26** (RB 106) for a complete pharmacological study was influenced by chemical considerations due to the global yield of its synthesis which was always found to be significantly better than that of **25**. Compounds **26** and **6** were shown to behave as competitive inhibitors of NEP and ACE. The K_i values of **26** (RB 106), calculated from the Cheng−Prussof equation, were 1.6 and 0.35 nM for NEP and ACE, respectively.

When orally administered in mice at 2.6×10^{-5} mol/kg, RB 106 led to a prolonged inhibition of renal NEP

and lung ACE (Figure 3). Thus, 12 h after RB 105 administration, the blockade of both peptidases was still higher than 50%. As expected the duration of action of RB 106 was longer than that of a mercaptoacyl dipeptide analogue, HS-CH(CH_2Ph)CO-Ala-Pro, lacking the 5-phenyl substituent.[36] In identical conditions the inhibition of NEP and ACE was around 50% after 3 h with the latter compound, while with RB 106, inhibition of both enzymes was still at maximal levels. The pharmacokinetic properties of RB 106 appear to be significantly better than those of the tricyclic inhibitor MDL-100,173, particularly regarding ACE inhibition (Figure 3B). This could be related to the *in vitro* potencies of both compounds since, in our hands, the IC_{50} values of MDL-100,173 on NEP and ACE were 2.5 ± 0.4 and 2.2 ± 0.4 nM, respectively, *i.e.*, slightly higher than the corresponding values (1.6 ± 0.2 and 0.6 ± 0.2 nM) of RB 106 (Table 3). Nevertheless various factors, including chemical stability, bioavailability, and metabolism, could explain the observed differences in the duration of action of both compounds.

In agreement with these results, RB 106 was found to be very efficient in decreasing the blood pressure of spontaneous hypertensive rats after oral administration at a dose as low as 5 mg/kg (Figure 4). Even more interesting, RB 106 exhibited a strong (−28 mmHg) and prolonged antihypertensive activity in SHR. Thus, at least 2 days were required so that rats, orally treated for 5 days with 25 mg/kg/day RB 106, recovered their initial blood pressure (Figure 5). As expected from its dual inhibitory potency, RB 106 induced also a significant decrease in blood pressure associated with an increase in diuresis (not shown) and natriuresis in the DOCA salt model of hypertension (Figure 6).

In conclusion, the presence of a hydrophobic phenyl group on the 5 position of proline in a series of 2-mercapto-3-phenylpropanoyl-Gly-(5-Ph)-Pro compounds led to very potent orally active dual inhibitors of NEP and ACE endowed with, as expected, a long duration of action. One of these compounds (RB 106) has slightly but significantly better *in vitro* and *in vivo* affinities for NEP and ACE than the dual inhibitors reported to date including the highly constrained polycyclic compounds MDL-100,173 and BMS-182,657. In addition, its pharmacokinetic properties seem also to be more favorable than those of the latter compounds. This could be partly due to a higher flexibility of RB 106 allowing a better adaptation to various targets, including putative intestinal proline transporters, to be obtained. Owing to the strong and persistent reduction of blood pressure observed after chronic oral administration in SHR, RB 106 warrants an extensive pharmacological evaluation now in course in our laboratories.

Experimental Section

Chemistry. The natural amino acid derivatives were purchased from Bachem (Bubbendorf, Switzerland) and the nonnatural amino acids and reagents from Aldrich-Chemie (Strasbourg, France). The synthesis of MDL-100,173 was performed following procedures described,[50] and *para*-carbomethoxyphenylalanine was prepared by alkylation of the diphenylimine of *tert*-butyl glycinate with methyl 4-(bromomethyl)benzoate as described by O'Donnell *et al.*[53] followed by acid hydrolysis of the diphenylimine and *tert*-butyl ester groups: white solid; mp 220 °C dec; R_f (L) 0.31; NMR δ 3.20, $CH_2\beta$; 3.85, OCH_3; 4.30, CHα; 7.42−7.93, Ar; 8.30, NH_3^+. The protection of 2-, 3-, and 4-bromophenols as methoxymethyl derivatives was performed following Winkle *et al.*:[54] *O*-(methoxymethyl)-2-bromophenol, yellow oily product (91%), R_f (B) 0.71; *O*-(methoxymethyl)-3-bromophenol, yellow oily product (92%), R_f (C) 0.60; *O*-(methoxymethyl)-4-bromophenol, yellow oily product (94%), R_f (C) 0.64. The protection of the phenol group of Tyr was performed as described by Koga *et al.*[55]

The purity of the synthesized compounds was checked by thin-layer chromatography on silica gel plates (Merck 60F 254) in the following solvent systems (v/v): A, *n*-hexane/EtOAc = 9/1; B, *n*-hexane/EtOAc = 8/2; C, *n*-hexane/EtOAc = 7/3; D, *n*-hexane/EtOAc = 6/4; E, *n*-hexane/EtOAc = 5/5; F, *n*-hexane/EtOAc/AcOH = 7/3/0.5; G, *n*-hexane/EtOAc/AcOH = 6/4/0.5; H, CH_2Cl_2/MeOH = 9/1; I, CH_2Cl_2/MeOH = 8/2; J, CH_2Cl_2/MeOH = 7/3; K, CH_2Cl_2/MeOH/AcOH = 9/1/0.5; L, CH_2Cl_2/MeOH/AcOH = 8/2/0.5; M, CH_2Cl_2/MeOH/AcOH = 7/3/0.5; N, CH_2Cl_2/MeOH/AcOH = 6/4/0.5; O, *n*-BuOH/AcOH/H_2O = 4/1/1. Plates were revealed with UV, iodine vapor, or ninhydrin. The purity of the final compounds was checked by HPLC on a reverse phase chromasil C_8 (5 μm, 100 Å) column (SFCC) with 0.05% TFA (solvent A)/CH_3CN (solvent B), as the mobile phase, on a Shimadzu apparatus. The eluted peaks were monitored at 210 nm.

The structure of all the compounds was confirmed by ^{1}H NMR spectroscopy (Brüker AC 270 MHz) in DMSO-d_6 using HMDS as an internal reference. Melting points of the crystallized compounds were determined on an Electrothermal apparatus and are reported uncorrected. Satisfactory analyses were obtained (C, H, N) for all final compounds. The optical purity of the various compounds was verified by HPLC.

Abbreviations: Et_2O, ethyl ether; MeOH, methanol; EtOH, ethanol; BuOH, *n*-butanol; EtOAc, ethyl acetate; AcOH, acetic acid; DMF, dimethylformamide; THF, tetrahydrofuran; DCC, dicyclohexylcarbodiimide; DCU, dicyclohexylurea; HOBt, 1-hydroxybenzotriazole; TFA, trifluoroacetic acid; Et_3N, triethylanine; DMSO, dimethyl sulfoxide; HMSD, hexamethyldisiloxane.

General Procedure for the Synthesis of 2-(Acetylthio)-alkanoic Acids $CH_3COSCH(R_1)COOH$ 1. The various 2-(acetylthio)alkanoic acids were prepared in two steps as previously described[40] from the corresponding α-amino acids. Briefly, the α-amino acids (1 equiv) were dissolved in a mixture of HBr 47% (8 equiv) and H_2O (2/3, v/v). At 0 °C, a solution of $NaNO_2$ (6.5 equiv) in H_2O was added. After the classical treatment the 2-bromoalkanoic acids were isolated. The 2-bromoalkanoic acids (1 equiv) were dissolved in DMF, and a solution of CH_3COSK (1 equiv) in DMF was added at 0 °C under inert atmosphere. The crude products were purified by chromatography on silica gel.

1a: R_1 = CH_2Ph; oily product (98%); R_f (O) 0.70; NMR δ 2.25, CH_3CO; 2.88−3.24, $CH_2\beta$; 4.17, CHα; 7.18, Ar; 12.85, COOH. **1b**: R_1 = CH(CH_3)CH_2CH_3; oily product (78%); R_f (F) 0.64; NMR δ 0.82, 2 × CH_3; 1.20−1.30, $CH_2\gamma$; 1.92, CHβ; 2.30, CH_3CO; 4.10, CHα; 12.30, COOH. **1c**: R_1 = CH_2(*p*-OCO_2CH_2-Ph)Ph; oily product (60%); R_f (G) 0.45; NMR δ 2.25, CH_3CO; 2.90−3.12, $CH_2\beta$; 4.16, CHα; 5.20, OCH_2; 7.10, 7.22, 7.38, Ar; 12.85, COOH. **1d**: R_1 = CH_2(*p*-NO_2)Ph; oily product (88%); R_f (A) 0.64; NMR δ 2.30, CH_3CO; 3.10−3.33, $CH_2\beta$; 4.30, CHα; 7.52−8.14, Ar; 12.90, COOH. **1e**: R_1 = CH_2(*p*-Cl)Ph; oily product (90%); R_f (F) 0.31; NMR δ 2.26, CH_3CO; 2.90−3.12, $CH_2\beta$; 4.18, CHα; 7.18−7.30, Ar; 12.84, COOH. **1f**: R_1 = CH_2-(*p*-F)Ph; oily product (90%); R_f (K) 0.60; NMR δ 2.26, CH_3CO; 2.86−3.12, $CH_2\beta$; 4.15, CHα; 7.04−7.18, Ar; 12.92, COOH. **1g**: R_1 = CH_2(*p*-$COOCH_3$)Ph; white solid; mp 220 °C dec (90%); R_f (N) 0.37; NMR δ 2.25, CH_3CO; 2.96−3.24, $CH_2\beta$; 3.78, OCH_3; 4.20, CHα; 7.30−7.80, Ar; 12.88, COOH. **1h**: R_1 = CH_2(*p*-OCH_3)Ph; oily product (40%); R_f (G) 0.42; NMR δ 2.28, CH_3-CO; 2.85−3.04, $CH_2\beta$; 3.68, OCH_3; 4.10, CHα; 6.79−7.08, Ar; 12.80, COOH. **1i**: R_1 = $(CH_2)_4$NH(Boc). **1i** was prepared as a racemic mixture from 6-aminohexanoic acid, and this compound was brominated on the α-carbon by Br_2 in the presence of PBr_3. Thus, after protection of the amino group by a *tert*-butyloxycarbonyl group, the thioacetylation was performed as described: oily product (59%); R_f (F) 0.61; NMR δ 1.35, Boc; 1.10−1.80, $(CH_2)_3$; 2.30, CH_3CO; 2.84, CH_2(NH); 3.90, CHα; 6.75, NH; 12.86, COOH.

General Procedures for the Synthesis of the 5-(Sub-

stituted phenyl)proline Derivatives 2. (a) Synthesis of (*S*)(or *R*)-Ethyl 5-Keto-2-Boc-aminopentanoate. The various (substituted phenyl)magnesium bromides were prepared by addition of a solution of the substituted phenyl bromides (1 equiv) to magnesium (1.1 equiv) in dry THF. The mixtures were kept under reflux until complexion of the reaction (2−3 h) and then were cooled at 0 °C and transferred into a solution of (*S*)- or (*R*)-ethyl *N*-Boc-pyroglutamate (0.85 equiv) in dry THF at −40 °C, under argon atmosphere. After 1 h at −40 °C and 1 h at 0 °C, the reaction was quenched with HOAc/MeOH (1:1) and the mixture diluted with Et_2O. The organic layers were washed with H_2O and brine, dried over Na_2SO_4, filtered, and evaporated leading to crude products, which were purified.

R_3 = H: recrystallization in *n*-hexane/ether = 6/1; white solid; mp 85 °C (71%); R_f (E) 0.65. R_3 = *o*-OCH_2OCH_3: chromatography in *n*-hexane/ethyl acetate = 9/1; oily product (42%); R_f (B) 0.22. R_3 = *m*-OCH_2OCH_3: chromatography in *n*-hexane/ethyl acetate = 9/1; white solid; mp 62 °C (80%); R_f (C) 0.23. R_3 = *p*-OCH_2OCH_3: chromatography in *n*-hexane/ethyl acetate = 9/1; oily product (78%); R_f (C) 0.25. R_3 = *o*-OCH_3: chromatography in *n*-hexane/ethyl acetate = 8.5/1.5; oily product (76%); R_f (D) 0.51. R_3 = *o*-CH_3: chromatography in *n*-hexane/ethyl acetate = 9/1; oily product (30%); R_f (A) 0.27. R_3 = *m*-CH_3: chromatography in *n*-hexane/ethyl acetate = 8/2; white solid; mp 88 °C (40%); R_f (B) 0.20. R_3 = *o*-CH_3 ((2*R*) configuration): chromatography in *n*-hexane/ethyl acetate = 9/1; oily product (35%); R_f (A) 0.27. R_3 = *p*-CH_3: chromatography in *n*-hexane/ethyl acetate = 9/1; oily product (42%); R_f (B) 0.24. R_3 = $NHCOCH_3$: chromatography in *n*-hexane/ethyl acetate = 8/2; oily product (46%); R_f (C) 0.50.

(b) Synthesis of Ethyl 5-(Substituted phenyl)-Δ^1-pyrroline-2-carboxylate. The ethyl 5-keto-2-Boc-aminopentanoates were dissolved in CH_2Cl_2 (1 mL/mmol), and 10 equiv of TFA was added at 0 °C. The mixture was stirred for 2 h at the same temperature. After evaporation in vacuo, the residue was taken off with CH_2Cl_2, washed (×3) with a 10% solution of $NaHCO_3$, H_2O, and brine, and dried over Na_2SO_4. After filtration and evaporation the cyclic compounds were isolated and used without further purification.

R_3 = H: oily product (94%); R_f (E) 0.54. R_3 = *o*-OH: oily product (92%); R_f (C) 0.44. R_3 = *m*-OH: oily product (90%); R_f (C) 0.19. R_3 = *o*-OCH_3: oily product (85%); R_f (E) 0.56. R_3 = *o*-CH_3: oily product (92%); R_f (E) 0.65. R_3 = *m*-CH_3: oily product (93%); R_f (C) 0.37. R_3 = *o*-CH_3 ((2*R*) configuration): oily product (88%); R_f (C) 0.46. R_3 = *p*-OH: oily product (91%); R_f (G) 0.28. R_3 = *p*-NH_2: oily product (92%); R_f (G) 0.50. R_3 = *p*-CH_3: oily product (88%); R_f (K) 0.25.

(c) Synthesis of (2*S*)- or (2*R*)-*cis*-Ethyl 5-(Substituted phenyl)prolinate 2. The various ethyl 5-(substituted phenyl)-Δ^1-pyrroline-2-carboxylates were dissolved in EtOH (3 mL/mmol), and Pt_2O (3 mg/mmol) was added. The mixtures were hydrogenated at room temperature under 30 bars of pressure. After filtration of the catalyst, the organic layers were evaporated in vacuo and the crude products used without further purification.

2a: R_3 = H; oily product (99%); R_f (E) 0.41; NMR δ 1.20, $(OCH_2)CH_3$; 1.50−1.86, $CH_2\beta$Pro; 2.02, $CH_2\gamma$Pro; 3.80−4.08, CHα + ∂Pro; 4.08, OCH_2; 7.20−7.38, Ar. **2b**: R_3 = *p*-NH_2; oily product; R_f (M) 0.52; NMR δ 1.20, $CH_3(CH_2O)$; 1.42−1.90, $CH_2\beta + \gamma$Pro; 3.72−3.85, CHα + ∂Pro; 4.05, OCH_2; 4.84, NH_2; 6.48−7.00, Ar. **2c**: R_3 = *o*-OH; white solid (92%); R_f (C) 0.44; mp 140 °C dec; NMR δ 1.20, $CH_3(CH_2O)$; 1.60−1.85, $CH_2\beta$Pro; 2.00−2.15, $CH_2\gamma$Pro; 3.82−4.22, CHα + ∂Pro; 4.05, OCH_2; 6.60−7.00, Ar; 9.80, OH. **2d**: R_3 = *m*-OH; white solid (98%); R_f (K) 0.24; mp 150 °C dec; NMR δ 1.18, $CH_3(CH_2O)$; 1.45−1.88, $CH_2\beta$Pro; 2.00, $CH_2\gamma$Pro; 3.72−4.00, CHα + ∂Pro; 4.10, OCH_2; 6.50−6.75−7.00, Ar; 9.18, OH. **2e**: R_3 = *p*-OH; oily product; R_f (N) 0.66; NMR δ 1.20, $CH_3(CH_2O)$; 2.10−2.30, $CH_2\beta + \gamma$Pro; 4.10, CH_2O; 4.60, CHα + ∂Pro; 6.80−7.30, Ar; 9.10, OH. **2f**: R_3 = *o*-OCH_3; oily product (99%); R_f (D) 0.28; NMR δ 1.15, $CH_3(CH_2O)$; 1.40−1.80, $CH_2\beta$Pro; 2.02, $CH_2\gamma$Pro; 3.70, OCH_3; 3.75−4.35, CHα + ∂Pro; 4.04, CH_2O; 6.85−7.10−7.55, Ar. **2g**: *o*-CH_3; oily product (97%); R_f (C) 0.41; NMR δ 1.18, $CH_3(CH_2O)$; 1.35−1.85, $CH_2\beta$Pro; 2.05, $CH_2\gamma$Pro; 2.20, CH_3-(Ar); 3.78−4.28, CHα + ∂Pro; 4.05, CH_2O; 7.02−7.60, Ar. **2h**: *m*-CH_3; oily product (96%); R_f (C) 0.34; NMR δ 1.18, CH_3-(CH_2O); 1.50−1.90, $CH_2\beta$Pro; 2.00, $CH_2\gamma$Pro; 2.25, CH_3(Ar); 3.75−4.05, CHα + ∂Pro; 4.05, CH_2O; 6.95−7.13, Ar. **2i**: R_3 = *p*-CH_3; white foam; R_f (K) 0.48; NMR δ 1.20, $CH_3(CH_2O)$; 2.10−2.30, $CH_2\beta + \gamma$Pro; 2.30, CH_3(Ph); 4.08, OCH_2; 4.56, CHα + ∂Pro; 7.20−7.42, Ar.

(d) Synthesis of (2*S*)- and (2*R*)-*trans*-Ethyl 5-(*ortho*-Methylphenyl)prolinate (2j,k). The (*S*) and (*R*) isomers of ethyl 5-(*o*-methylphenyl-Δ^1-pyrroline-2-carboxylate were saponified by alkaline hydrolysis, and the corresponding acids were dissolved in water and treated, at 0 °C, by a solution (1.02 equiv) of $NaBH_4$ in water. The mixtures were stirred overnight at 0 °C, acidified at pH 2, and evaporated in vacuo. A 50/50 mixture of *cis*- and *trans*-(2*S*) or (2*R*)-5-(*o*-methylphenyl)proline was obtained (HPLC C_8 nucleosil CN, CH_3CN/H_2O = 2/98; (t_R = 5.90 and 7.25 min). The trans isomer was precipitated as a tosylate salt (HPLC t_R = 7.25 min) as described[43] (70%). The ethyl ester of the (2*S*) and (2*R*) trans isomers was prepared by treatment with EtOH and $SOCl_2$ (98%): R_f (K) 0.56; NMR δ 1.20, $(OCH_2)CH_3$; 1.40−1.90, $CH_2\beta$; 2.20, $CH_2\gamma$; 2.25, CH_3(Phe); 4.05−4.50, CHα + ∂Pro; 4.10, OCH_2; 7.10−7.50, Ar.

General Procedure for the Synthesis of *N*-2-(Acetylthio)-3-alkanoyl Amino Acids $CH_3COSCH(R_1)CONHCH(R_2)COOH$ 3. To a solution of the 2-(acetylthio)alkanoic acid in $CHCl_3$ were added, at 0 °C, a solution of the chlorhydrate of *tert*-butyl α-amino ester (1 equiv) and Et_3N (1 equiv) in dry THF, a solution of HOBt (1 equiv) in THF, and DCC (1.1 equiv) in $CHCl_3$. After stirring overnight at room temperature, DCU was filtered and the mixture evaporated in vacuo. The residue was taken up in EtOAc and washed as usual. The crude compounds were dissolved in CH_2Cl_2 (0.5 mL/mmol), and 10 equiv of TFA was added at 0 °C. After stirring for 30 min at 0 °C and for 3 h at room temperature, the mixture was evaporated in vacuo and the residue triturated in Et_2O.

3a: R_1 = CH_2Ph, R_2 = H; oily product (90%); R_f (A) 0.57; NMR δ 2.25, CH_3CO; 2.80−3.16, $CH_2\beta$(Ph); 3.63, CH_2Gly; 4.30, CHα(Ph); 7.20, Ar; 8.50, NH; 12.50, COOH. **3b**: R_1 = CH_2-Ph, R_2 = CH_3; white paste (87%); R_f (A) 0.50; NMR δ 1.20, CH_3(Ala); 2.20, CH_3CO; 2.74−3.20, $CH_2\beta$(Ph); 4.10, CHαAla; 4.30, CHα(Ph); 7.20, Ar; 8.50, NH; 12.40, COOH. **3c**: R_1 = $CH(CH_3)CH_2CH_3$, R_2 = H; white paste (75%); R_f (A) 0.37; NMR δ 0.85, 2 × CH_3; 1.20−1.50, $CH_2\gamma$; 1.85, CHβ; 2.30, CH_3CO; 3.68, CH_2Gly; 4.05, CHα; 8.40, NH; 12.40, COOH.

General Procedure for the Synthesis of Ethyl(or methyl) *N*-Glycyl-5-(substituted phenyl)prolinates 4. (1) (*Z*)-Glycine (1 equiv) dissolved in acetone was treated with cyanuric chloride (0.5 equiv) in the presence of Et_3N (1 equiv) at room temperature. After stirring for 3 h, the solvent was evaporated in vacuo and the residue dissolved in CH_2Cl_2. The solution of ethyl(or methyl) 5-(substituted phenyl)prolinate (1 equiv) was added, and the mixture was stirred overnight at room temperature. After evaporation, the residue was taken up with ethyl acetate, washed with 10% $NaHCO_3$ (×3), H_2O, 10% citric acid (×3), H_2O, and brine, and dried over Na_2SO_4. After filtration and evaporation, the crude products was purified by chromatography on silica gel using *n*-hexane/ethyl acetate (5/5) as eluents.

(2) The protected dipeptides were dissolved in ethanol (or methanol) and hydrogenated over 10% Pd/C in the presence of 1.1 equiv of HCl. After completion of the reaction the catalyst was filtered and the mixture evaporated in vacuo. The crude product was used for the following step without further purification.

4a: R_3 = *p*-CH_3, R = CH_3; (1) white foam (65%); R_f (E) 0.29; (2) white solid; mp 115 °C dec (92%); R_f (L) 0.48. **4b**: R_3 = *p*-OH, R = CH_3; (1) white foam (20%); R_f (E) 0.27; (2) white solid; mp 105 °C (75%); R_f (L) 0.20. **4c**: R_3 = H, R = CH_3; (1) white foam (33%); R_f (E) 0.13; (2) oily product (94%); R_f (K) 0.23. **4d**: R_3 = *o*-OH, R = C_2H_5; (1) oily product (42%); R_f (E) 0.20; (2) white wax (95%); R_f (L) 0.58. **4e**: R_3 = *m*-OH, R = CH_3; (1) white solid; mp 173 °C (33%); R_f (E) 0.13; (2) white foam (99%); R_f (L) 0.51. **4f**: R_3 = *o*-OCH_3, R = C_2H_5; (1) colorless oil (45%); R_f (D) 0.19; (2) white foam (85%); R_f (K) 0.42. **4g**: R_3 = *o*-CH_3, R = C_2H_5; (1) oily product (42%); R_f (E) 0.21; (2) white solid; mp 209 °C (94%); R_f (K) 0.37. **4h**: R_3 =

m-CH_3, R = C_2H_5; (1) colorless oil (43%); R_f (E) 0.35; (2) white solid (98 °C dec) (95%); R_f (L) 0.62. **4l**: R_3 = *o*-CH_3, R = C_2H_5 (trans isomer); oil (35%); R_f (E) 0.52; (2) hygroscopic solid (78%); R_f (K) 0.50.

Synthesis of Methyl(or ethyl) *N*-[*N*-[2-(Acetylthio)-alkanoyl]glycyl]-5-(substituted phenyl)prolinates 5. To a solution of 2-(acetylthio)alkanoic acids (1 equiv) in THF were successively added at 0 °C a solution of ethyl(or methyl) *N*-glycyl-5-(substituted phenyl)prolinate, hydrochloride (1 equiv), and Et_3N in $CHCl_3$, a solution of HOBt (1 equiv) in THF, and a solution of DCC (1 equiv) in $CHCl_3$. After stirring for 1 h at 0 °C and overnight at room temperature, DCU was filtered and the mixture evaporated in vacuo. The residue was taken up with EtOAc, washed with H_2O, 10% $NaHCO_3$, H_2O, 10% citric acid solution, H_2O, and brine, and dried over Na_2SO_4. After filtration and evaporation in vacuo, the crude product was purified by chromatography.

5_1: R_1 = CH_2Ph, R_2 = H, R_3 = H ((*S*) configuration); chromatography in *n*-hexane/ethyl acetate = 4/6; oily product (46%); R_f (E) 0.33. **5_2**: R_1 = CH_2Ph, R_2 = H, R_3 = H ((*R*) configuration); chromatography in *n*-hexane/ethyl acetate/acetic acid = 6/4/0.5; oily product (62%); R_f (G) 0.32. **5_3**: R_1 = CH_2Ph, R_2 = CH_3, R_3 = H; chromatography in *n*-hexane/ethyl acetate = 2/1; oily product (40%); R_f (E) 0.39. **5_4**: R_1 = $CH(CH_3)CH_2CH_3$, R_2 = H, R_3 = H; chromatography in *n*-hexane/ethyl acetate = 5/5; white foam (42%); R_f (E) 0.37. **5_5**: R_1 = $(CH_2)_4NHBoc$, R_2 = H, R_3 = H; chromatography in *n*-hexane/ethyl acetate = 2/1; oily product (25%); R_f (C) 0.42. **5_6**: R_1 = CH_2(*p*-$OCOOCH_2Ph$)Ph, R_2 = H, R_3 = H; chromatography in *n*-hexane/ethyl acetate = 5/5; white solid (32%); R_f (E) 0.16. **5_7**: R_1 = CH_2(*p*-NO_2), R_2 = H, R_3 = H; chromatography in *n*-hexane/ethyl acetate = 5/5; oily product (35%); R_f (E) 0.20. **5_8**: R_1 = CH_2(*p*-NH_2), R_2 = H, R_3 = H; obtained by acid hydrolysis of the preceding compound followed by catalytic hydrogenation (Pd/C) in the presence of CH_3COOH (95%); R_f (K) 0.33. **5_9**: R_1 = CH_2Ph, R_2 = H, R_3 = *o*-OH; chromatography in *n*-hexane/ethyl acetate = 4/6; white foam (35%); R_f (E) 0.38. **5_{10}**: R_1 = CH_2Ph, R_2 = H, R_3 = *m*-OH; chromatography in *n*-hexane/ethyl acetate = 4/6; oily product (50%); R_f (E) 0.28. **5_{11}**: R_1 = CH_2Ph, R_2 = H, R_3 = *o*-CH_3; chromatography in *n*-hexane/ethyl acetate = 4/6; white solid; mp 97 °C (58%); R_f (E) 0.40. **5_{12}**: R_1 = CH_2Ph, R_2 = H, R_3 = *m*-CH_3; chromatography in *n*-hexane/ethyl acetate = 6/4; colorless wax (71%); R_f (D) 0.60. **5_{13}**: R_1 = CH_2Ph, R_2 = H, R_3 = *o*-CH_3 (cis (2*R*) configuration); chromatography in *n*-hexane/ethyl acetate = 5/5; oily product (67%); R_f (E) 0.31. **5_{14}**: R_1 = CH_2Ph, R_2 = H, R_3 = *o*-CH_3 (trans (2*S*) configuration); chromatography in *n*-hexane/ethyl acetate = 5/5; oily product (47%); R_f (E) 0.40. **5_{15}**: R_1 = CH_2(*p*-$OCOOCH_2Ph$)Ph, R_2 = H, R_3 = *o*-CH_3; chromatography in *n*-hexane/ethyl acetate = 4/6; oily product (56%); R_f (E) 0.28. **5_{16}**: R_1 = CH_2(*p*-$OCOOCH_2Ph$)Ph, R_2 = H, R_3 = *o*-CH_3 (cis (2*R*) configuration); chromatography in *n*-hexane/ethyl acetate = 5/5; white solid; mp 70 °C (38%); R_f (E) 0.40. **5_{17}**: R_1 = CH_2(*p*-$OCOOCH_2Ph$)-Ph, R_2 = H, R_3 = *m*-CH_3; chromatography in *n*-hexane/ethyl acetate = 5/5; colorless wax (49%); R_f (E) 0.17. **5_{18}**: R_1 = CH_2-Ph, R_2 = H, R_3 = *p*-CH_3; chromatography in *n*-hexane/ethyl acetate = 6/4; white foam (69%); R_f (D) 0.14. **5_{19}**: R_1 = CH_2(*p*-$OCOOCH_2Ph$)Ph, R_2 = H, R_3 = *p*-CH_3; chromatography in *n*-hexane/ethyl acetate = 5/5; white foam (52%); R_f (E) 0.14. **5_{20}**: R_1 = CH_2(*p*-$OCOOCH_2Ph$)Ph, R_2 = H, R_3 = *o*-OH; chromatography in *n*-hexane/ethyl acetate = 4/6; colorless wax (45%); R_f (E) 0.29. **5_{21}**: R_1 = CH_2(*p*-$OCOOCH_2Ph$)Ph, R_2 = H, R_3 = *m*-OH; chromatography in *n*-hexane/ethyl acetate = 4/6; oily product (40%); R_f (E) 0.37. **5_{22}**: R_1 = CH_2(*p*.$OCOOCH_2$-Ph)Ph, R_2 = H, R_3 = *p*-OH; chromatography in *n*-hexane/ethyl acetate = 5/5; oily product (50%); R_f (E) 0.26. **5_{23}**: R_1 = CH_2(*m*-$OCOOCH_2Ph$)Ph, R_2 = H, R_3 = *m*-OH; chromatography in *n*-hexane/ethyl acetate/acetic acid = 7/3/0.25; oily product (19%); R_f (F) 0.19. **5_{24}**: R_1 = CH_2(*p*-OCH_3)Ph, R_2 = H, R_3 = *o*-OH; chromatography in *n*-hexane/ethyl acetate = 4/6; oily product (50%); R_f (E) 0.28. **5_{25}**: R_1 = CH_2(*p*-$OCOOCH_2Ph$)-Ph, R_2 = H, R_3 = *o*-OCH_3; chromatography in *n*-hexane/ethyl acetate = 4/6; oily product (59%); R_f (E) 0.31. **5_{26}**: R_1 = CH_2-(*p*-OCH_2)Ph, R_2 = H, R_3 = *o*-OCH_3; chromatography in *n*-hexane/ethyl acetate = 4/6; white foam (59%); R_f (E) 0.20. **5_{27}**: R_1 = CH_2(*p*-$OCOOCH_2Ph$)Ph, R_2 = H, R_3 = *p*-NH_2; chromatography in CH_2Cl_2/MeOH/AcOH = 9/2/0.1; oily product (25%); R_f (K) 0.35. **5_{28}**: R_1 = CH_2(*p*-Cl)Ph, R_2 = H, R_3 = *o*-OH; chromatography in *n*-hexane/ethyl acetate = 4/6; oily product (53%); R_f (E) 0.38. **5_{29}**: R_1 = CH_2(*p*-F)Ph, R_2 = H, R_3 = *m*-OH; chromatography in *n*-hexane/ethyl acetate = 4/6; white foam (42%); R_f (E) 0.31. **5_{30}**: R_1 = CH_2(*p*-$COOCH_3$)Ph, R_2 = H, R_3 = *o*-OH; chromatography in *n*-hexane/ethyl acetate/acetic acid = 6/4/0.5; white solid mp 105 °C dec (51%); R_f (E) 0.25.

General Procedure for the Preparation of *N*-[*N*-(2-Mercaptoalkanoyl)glycyl]-5-(substituted phenyl)proline. The protected inhibitors (1 equiv) were dissolved in degassed methanol, and 1 N NaOH (5 equiv) was added at 0 °C under inert atmosphere. The mixtures were stirred for 2 h at room temperature. After acidification with 2 N HCl, the methanolic layers were evaporated, diluted in H_2O, and extracted with degassed EtOAc. The organic layers were washed with H_2O and brine, dried over Na_2SO_4, and evaporated in vacuo. All the final compounds were obtained as mixtures of cis (∼20%) and trans (∼80%) rotamers around the Gly−Pro bond. This was easily observed on the NMR spectra by the occurrence of two well-separated peaks for both the CHα and CH∂ protons of proline.

Compound 6: oily product (81%); R_f (A) 0.59; HPLC (45% B) 11.8 min; NMR ∂ (ppm) : 1.78−2.10−2.35, $CH_2\beta$ + γPro; 2.63, SH; 2.70−3.00, $CH_2\beta$Ph; 3.10−3.85, CH_2Gly; 3.68, CHαPh; 4.30(t)−4.69(c), CHαPro; 4.90(c)−5.10(t), CH∂Pro; 7.13−7.35−7.60, Ar; 8.02, NH; 12.40, COOH.

Compound 7: white solid; mp 92 °C (98%); R_f (K) 0.80; HPLC (50% B) 7.94 min; NMR δ 1.80−2.12−2.40, $CH_2\beta$ + γPro; 2.60, SH; 2.68−3.00, $CH_2\beta$Ph; 3.10−3.85, CH_2Gly; 3.65, CHαPh; 4.32(t)−4.70(c), CHαPro; 4.90(c)−5.09(t), CH∂Pro; 7.12−7.35−7.60, Ar; 8.01, NH; 12.50, COOH.

Compound 8: white solid; mp 98 °C (83%); R_f (G) 0.20; HPLC (50% B) 8.7 min; NMR δ 1.10, CH_3Ala; 2.10−2.35, $CH_2\beta$ + γPro; 2.70, SH; 2.80−3.00, $CH_2\beta$Ph; 3.60, CHαPh; 4.28(t)−4.60(c), CHαPro; 4.80, CHαAla; 4.90(c)−5.02(t), CH∂Pro; 7.20−7.60, Ar; 8.40, NH; 12.60, COOH.

Compound 9: white foam (95%); R_f (K) 0.70; HPLC (50% B) 8.7 min; NMR δ 0.80, 2 × CH_3Ile; 1.10−1.35, $CH_2\gamma$Ile; 1.62−1.80−2.16, CHβIle + $CH_2\beta$ + γPro; 2.40, SH; 3.10−3.95, CH_2-Gly; 3.40, CHαIle; 4.35(t)−4.72(c), CHαPro; 4.95(c)−5.12(t), CH∂Pro; 7.20−7.60, Ar; 8.05, NH; 12.65, COOH.

Compound 10: white solid (90%); R_f (K) 0.18; HPLC (28% B) 6.8 min; NMR δ 1.20−1.70, CH2β + γ + ∂, hexanoic chain; 1.80−2.13−2.38, $CH_2\beta$ + γPro; 2.55, SH; 2.68, $CH_2(NH_3^+)$; 3.03−3.85, CH_2Gly; 3.75, CHS; 4.31(t)−4.70(c), CHαPro; 4.90-(c)−5.09(t), CH∂Pro; 7.20−7.31−7.60, Ar; 7.65, NH_3^+; 7.85, NH; 12.60, COOH.

Compound 11: white solid (90%); R_f (M) 0.89; HPLC (28% B) 6.9 min; NMR δ 2.12−2.38, $CH_2\beta$ + γPro; 2.56, SH; 2.70−2.85, $CH_2\beta$Ph; 3.05−3.85, CH_2Gly; 3.52, CHαPh; 4.30(t)−4.70-(c), CHαPro; 4.90(c)−5.08(t), CH∂Pro; 6.55−7.20−7.30, Ar; 7.80, NH; 9.10, OH; 12.55, COOH.

Compound 12: white foam (89%); R_f (K) 0.30; HPLC (25% B) 8.57 min; NMR δ 1.80−2.15−2.38, $CH_2\beta$ + γPro; 2.60, SH; 2.76−3.10, $CH_2\beta$Ph; 3.10−3.88, CH_2Gly; 3.65, CHαPh; 4.32-(t)−4.70(c), CHαPro; 4.89(c)−5.05(t), CH∂Pro; 7.20−7.60, Ar; 8.02, NH; 12.46, COOH.

Compound 13: white foam (93%); R_f (K) 0.53; HPLC (35% B) 14.5 min; NMR δ 1.75−2.10−2.30, $CH_2\beta$ + γPro; 2.62, SH; 2.70−3.04, $CH_2\beta$Ph; 3.15−3.83, CH_2Gly; 3.69, CHαPh; 4.21(t)−4.64(c), CHαPro; 5.15(c)−5.20(t), CH∂Pro; 6.75−7.15−7.80, Ar; 8.03, NH; 9.52, OH; 12.25, COOH.

Compound 14: white wax (85%); R_f (G) 0.29; HPLC (30% B) 30.6 min; NMR δ 1.76−2.10−2.32, $CH_2\beta$ + γPro; 2.62, SH; 2.70−3.05, $CH_2\beta$Ph; 3.15−3.85, CH_2Gly; 3.70, CHαPh; 4.30-(t)−4.60(c), CHαPro; 4.80(c)−4.97(t), CH∂Pro; 6.60−7.10, Ar; 8.00, NH; 9.38, OH; 12.38, COOH.

Compound 15: white solid; mp 110 °C dec (63%); R_f (E) 0.45; HPLC (45% B) 5.70 min; NMR δ 1.70−2.10−2.35, $CH_2\beta$ + γPro; 2.25, CH_3(Ar); 2.55, SH; 2.70−2.90, $CH_2\beta$Phe; 3.08−3.78, CH_2Gly; 3.58, CHαPh; 4.25(t)−4.60(c), CHαPro; 5.02(c)−5.22(t), CH∂Pro; 7.10, Ar; 8.02, NH; 12.50, COOH.

Compound 16: white solid; mp ∼105 °C dec (72%); R_f (G) 0.29; HPLC (40% B) 21.3 min; NMR δ 1.80−2.10−2.35, $CH_2\beta$

+ γPro; 2.26, CH_2(Ar); 2.65, SH; 2.70−3.05, CH_2βPh; 3.10−3.86, CH_2Gly; 3.68, CHαPh; 4.30(t)−4.70(c), CHαPro; 4.89(c)−5.02(t), CH∂Pro; 7.13−7.40, Ar; 8.02, NH; 12.50, COOH.

Compound 17: white solid; mp 102 °C (45%); R_f (K) 0.78; HPLC (45% B) 9.10 min; NMR δ 1.75−2.16−2.40, $CH_2\beta$ + γPro; 2.26, CH_3(Ar); 2.65, SH; 2.70−2.96, CH_2βPh; 3.10−3.89, CH_2Gly; 3.69, CHαPh; 4.30(t)−4.50(c), CHαPro; 5.02(c)−5.24(t), CH∂Pro; 7.00−7.13, Ar; 8.05, NH; 12.50, COOH.

Compound 18: white wax (59%); R_f (K) 0.50; HPLC (45% B) 13.7 min; NMR δ 1.60−1.80−2.02, $CH_2\beta$ + γPro; 2.30, CH_3(Ar); 2.58, SH; 2.68−3.03, CH_2βPh; 3.15−3.92, CH_2Gly; 3.63, CHαPh; 4.58(t)−4.92(c), CHαPro; 5.20(c)−5.35(t), CH∂Pro; 6.94−7.12, Ar; 8.05, NH; 12.54, COOH.

Compound 19: white foam (72%); R_f (K) 0.50; HPLC (45% B) 15.5 min; NMR δ 1.61−1.81−2.0−2.20, $CH_2\beta$ + ∂Pro; 2.30, CH_3(Ar); 2.60, SH; 2.65−3.02, CH_2βPh; 3.20−3.89, Gly; 3.65, CHαPh; 4.60(t)−4.90(c), CHαPro; 5.20(c)−5.37(t), CH∂Pro; 7.00−7.13, Ar; 8.05, NH; 12.50, COOH.

Compound 20: white solid; mp 130 °C dec; R_f (K) 0.74; HPLC (45% B) 6.79 min; NMR δ 1.70−2.10, $CH_2\beta$ + γPro; 2.25, CH_3(Ar); 2.55, SH; 2.70−2.92, CH_2βPh; 3.08−3.80, CH_2Gly; 3.58, CHαPh; 4.28(t)−4.60(c), CHαPro; 5.01(c)−5.20(t), CH∂Pro; 6.55−6.90−7.00−7.10, Ar; 7.95, NH; 9.12, OH; 12.50, COOH.

Compound 21: oily product; R_f (K) 0.74; HPLC (45% B) 5.73 min; NMR δ 1.86−2.18, $CH_2\beta$ + γPro; 2.31, CH_3(Ar); 2.57, SH; 2.64−2.95, CH_2βPh; 3.05−3.95, CH_2Gly; 3.60, CHαPh; 4.35(t)−4.75(c), CHαPro; 5.09(c)−5.70(t), CH∂Pro; 6.60−6.90−7.06−7.20, Ar; 8.06, NH; 9.12, OH; 12.52, COOH.

Compound 22: white solid; mp 105 °C dec (78%); R_f (K) 0.50; HPLC (50% B) 8.52 min; NMR δ 1.75−2.10−2.32, $CH_2\beta$ + γPro; 2.25, CH_3(Ar); 2.52, HS; 2.55−2.90, CH_2βPh; 3.09−3.84, CH_2Gly; 3.58, CHαPh; 4.30(t)−4.70(c), CHαPro; 4.88(c)−5.03(t), CH∂Pro; 6.56−6.90−7.03−7.20−7.40, Ar; 8.00, NH; 9.12, OH; 12.60, COOH.

Compound 23: white foam (89%); R_f (K) 0.59; HPLC (50% B) 9.50 min; NMR δ 1.71−2.10−2.30, $CH_2\beta$ + γPro; 2.20, CH_3(Ar); 2.60, SH; 2.70−3.00, CH_2βPh; 3.10−3.82, CH_2Gly; 3.65, CHαPh; 4.30(t)−4.55(c), CHαPro; 4.84(c)−5.02(t), CH∂Pro; 7.10−7.46, Ar; 8.02, NH; 12.40, COOH.

Compound 24: racemic mixture; white foam (92%); R_f (K) 0.60; HPLC (45% B) 6.17−6.34 min; NMR δ 1.75−2.10−2.32, $CH_2\beta$ + γPro; 2.23, CH_3(Ar); 2.51, SH; 2.58−2.90, CH_2βPh; 3.10−3.90, CH_2Gly; 3.56, CHαPh; 4.30(t)−4.64(c), CHαPro; 4.85(c)−5.00(t), CH∂Pro; 6.50−6.86−7.10−7.45, Ar; 7.98, NH; 9.10, OH; 12.35, COOH.

Compound 25: white foam (80%); R_f (K) 0.18; HPLC (25% B) 19.4 min (C18); NMR δ 1.70−2.10−2.26, $CH_2\beta$ + γPro; 2.52, SH; 2.55−2.60, CH_2βPh; 3.14−3.80, CH_2Gly; 3.58, CHαPh; 4.22(t)−4.62(c), CHαPro; 5.12(c)−5.20(t), CH∂Pro; 6.55−6.75−6.85−7.04−7.79, Ar; 7.98, NH; 9.12−9.67, OH; 12.50, COOH.

Compound 26: white foam (71%); R_f (K) 0.34; HPLC (30% B) 9.94 min (C18); NMR δ 1.76−2.10−2.36, $CH_2\beta$ + γPro; 2.55, SH; 2.60−2.93, CH_2βPh; 3.10−3.89, CH_2Gly; 3.60, CHαPh; 4.30(t)−4.68(c), CHαPro; 4.80(c)−4.96(t), CH∂Pro; 6.58−7.10, Ar; 8.00, NH; 9.14−9.39, OH; 12.40, COOH.

Compound 27: white wax (77%); R_f (K) 0.24; HPLC (25% B) 16.4 min; NMR δ 1.72−2.10−2.30, $CH_2\beta$ + γPro; 2.55, SH; 2.58−2.92, CH_2βPh; 3.10−3.80, CH_2Gly; 3.56, CHαPh; 4.28(t)−4.60(c), CHαPro; 4.82(c)−4.93(t), CH∂Pro; 6.55−6.68−6.88−7.25, Ar; 7.95, NH; 9.10−9.31, OH; 12.60, COOH.

Compound 28: white wax (87%); R_f (K) 0.20; HPLC (25% B) 12.38 and 12.80 min; NMR δ 1.75−2.10−2.32, $CH_2\beta$ + γPro; 2.55, SH; 2.60−2.92, CH_2βPh; 3.13−3.82, CH_2Gly; 3.61, CHαPh; 4.30(t)−4.68(c), CHαPro; 4.80(c)−4.98(t), CH∂Pro; 6.50−6.62−7.00, Ar; 8.01, NH; 9.12−9.40, OH; 12.60, COOH.

Compound 29: white foam (83%); R_f (K) 0.42; HPLC (45% B) 6.48 min; NMR δ1.72−2.12−2.39, $CH_2\beta$ + γPro; 2.55, SH; 2.62−2.95, CH_2βPh; 3.15−3.70, CH_2Gly; 3.53, OCH_3; 3.55, CHαPh; 4.20(t)−4.54(c), CHαPro; 5.12(c)−5.20(t), CH∂Pro; 6.72−7.00−7.80, Ar; 8.00, NH; 9.70, OH; 12.30, COOH.

Compound 30: white foam (91%); R_f (K) 0.50; HPLC (45% B) 5.95 min; NMR δ1.70−2.10−2.30, $CH_2\beta$ + γPro; 2.52, SH; 2.55−2.90, CH_2βPh; 3.16−3.82, CH_2Gly; 3.60, CHαPh; 3.70, OCH_3; 4.23(t)−4.66(c), CHαPro; 5.18(c)−5.23(t), CH∂Pro; 6.60−6.90−7.25−7.90, Ar; 9.13, OH; 12.40, COOH.

Compound 31: white foam (89%); R_f (G) 0.19; HPLC (45% B) 9.90 min; NMR δ 1.70−2.10−2.30, $CH_2\beta$ + γPro; 2.60, SH; 2.64−2.96, CH_2βPh; 3.18−3.70, CH_2Gly; 3.52−3.77, 2 × OCH_3; 3.65, CHαPh; 4.21(t)−4.66(c), CHαPro; 5.20(c)−5.25(t), CH∂Pro; 6.70−6.89−7.00−7.20−7.90, Ar; 8.00, NH; 12.60, COOH.

Compound 32: white foam (62%); R_f (K) 0.30; HPLC (24% B) 13.36 min; NMR δ 1.65−1.80−2.10, $CH_2\beta$ + γPro; 2.62, SH; 2.70−2.90, CH_2βPh; 3.10−3.70, CH_2Gly; 3.60, CHαPh; 4.26(t)−4.60(c), CHαPro; 4.90(c)−5.06(t), CH∂Pro; 6.60−7.70, Ar; 8.10, NH; 9.00, OH; 12.50, COOH.

Compound 33: white foam (88%); R_f (K) 0.36; HPLC (45% B) 9.90 min; NMR δ 1.70−2.10−2.30, $CH_2\beta$ + γPro; 2.66, SH; 2.70−3.00, CH_2βPh; 3.15−3.84, CH_2Gly; 3.67, CHαPh; 4.24-(t)−4.50(c), CHαPro; 4.65(c)−5.20(t), CH∂Pro; 6.72−7.02−7.23−7.79, Ar; 8.03, NH; 9.63, OH; 12.56, COOH.

Compound 34: white foam (97%); R_f (K) 0.28; HPLC (45% B) 7.63 min; NMR δ 1.75−2.10−2.30, $CH_2\beta$ + γPro; 2.64, SH; 2.70−3.00, CH_2βPh; 3.02−3.82, CH_2Gly; 3.54, CHαPh; 4.30(t)−4.70(c), CHαPro; 4.80(c)−4.95(t), CH∂Pro; 6.6−7.10, Ar; 8.00, NH; 9.40, OH; 12.50, COOH.

Compound 35: white foam (67%); R_f (E) 0.22; HPLC (30% B) 10.0 and 11.1 min; NMR δ1.74−2.09−2.32, $CH_2\beta$ + γPro; 2.60, SH; 2.88−3.10, CH_2βPh; 3.03−3.82, CH_2Gly; 3.70, CHαPh; 4.28(t)−4.68(c), CHαPro; 4.80(c)−4.95(t), CH∂Pro; 6.60−7.85, Ar; 8.03, NH; 9.40, OH; 12.60, COOH.

Molecular Modeling. Conformational analyses were performed through high-temperature molecular dynamics (MD) as described.[30,56] The calculations were performed using the Discover Insight software package (Biosym Technologies Inc., San Diego, CA) with the CFF91 force field parameters.[57,58] The first-step calculations consisted of equilibrating the system to 1000 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 simulated annealing at room temperature (300 K) and further minimized through a steepest descent followed by a conjugate gradient algorithm. No conformational constraints were taken into account during the calculations except for the peptide bond which is assumed to be planar. The dielectric constant was kept at 78 during all calculation steps. Each structure was then graphically analyzed using the insight analysis module, and the 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 were then compared with the structure of the pharmacophore by using a template-forcing procedure (Insight II User guide, version 2.1.0., 1992, Biosym Technologies Inc., San Diego, CA). The force constant in this last procedure was set to 50 kcal M^{-1}, and the atoms taken into account for the rms calculation are indicated in Table 1. The relative spatial orientation of the thiol and carboxyl groups in compound **6** was defined by the value of the dihedral angle θ between the noncontinuous groups of atoms S_4-C_3 and $C_{10}-C_{11}$ (Table 4).

Biological Tests. [^{3}H]Tyr-D-Ala^2-Leu-enkephalin (52 Ci/mmol) was obtained from Dositek (CEA Saclay, France); *N*-Cbz-Phe-His-Leu[59] was from Bachem (Buddendorf, Switzerland). Neutral endopeptidase from rabbit kidney[44] and angiotensin converting enzyme from rat testis were purified to homogeneity as previously described.[45] Thermolysin was purchased from SIGMA (France) and used without purification.

***In Vitro* Inhibition of NEP, ACE, and TLN: Assay for Neutral Endopeptidase Activity.** IC_{50} values were determined as previously reported.[60] NEP (final concentration, 1 pmol/100 μL; specific activity for [^{3}H]-D-Ala^2-Leu-enkephalin, 0.3 nmol/mg/min) was preincubated for 15 min at 25 °C with or without increasing concentrations of inhibitor in a total volume of 100 μL in 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 formed, [^{3}H]Tyr-D-Ala-Gly, was separated on polystyrene beads[61] and the radioactivity measured by scintillation counting. Competitive inhibition was demonstrated for **26** from Dickson representation of inhibition curves (not shown here).

Assay for Thermolysin Activity. TLN activity was assayed at 37 °C in a total volume of 100 μL of 50 mM Tris-HCl buffer (pH 7.4) containing 100 mM NaCl and 5 mM $CaCl_2$, with 25 nM [^{3}H][Leu^5]enkephalin ([^{3}H]Tyr-Gly-Gly-Phe-Leu) as a substrate.[62] Reactions were stopped by the addition of 10 μL of 0.5 M HCl, and the metabolite, [^{3}H]Tyr-Gly-Gly, was isolated as previously described for the NEP assay. The radioactivity was quantified by liquid scintillation counting.

Assay for Angiotensin-Converting Enzyme Activity. Enzymatic studies on ACE were performed using N-Cbz-Phe-His-Leu as substrate (K_m = 50 mM). ACE (final concentration, 0.02 pmol/100 μL; specific activity on Cbz-Phe-His-Leu, 13 nmol/mg/min) was preincubated for 15 min at 37 °C with various concentrations of the inhibitors in 50 mM Tris-HCl buffer, pH 8.0. 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 determining following the fluorimetric assay described by Cheung *et al.*[63] with a MPF44A 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 5.0 M Tris-HCl buffer, pH 8.0, containing the denaturated enzyme. Competitive inhibition of **26** and derived K_i value were established by using Dickson representation (not shown).

***In Vivo* Inhibition of Kidney NEP and Lung ACE.** The inhibitors were dissolved in 10% ethanol, 10% cremophor, 80% water. The time course of the inhibition was measured from 1 to 18 h after oral administration in mice of a single dose of inhibitor (2.6×10^{-5} mol/kg). ACE inhibition was determined by competition experiments using [^{3}H]trandaloprilate (0.5 μCi) iv administered. Fifteen minutes after tritiated probe injection, the mice were sacrificed and the lungs were rapidly dissected and homogeneized in 50 mM Tris-HCl buffer (pH 8.0) and 1 mM NaCl at 4 °C. The homogenate was centrifugated, and the radioactivity was measured by liquid scintillation counting. Total binding of the radiolabeled probe was determined under the same conditions, without inhibitors. The nonspecific binding was obtained by coinjection of [^{3}H]trandaloprilate with 1000 equiv of captopril. NEP blockade was determined by inhibition of degradation of [^{3}H]-D-Ala^2-Leu-enkephalin. After sacrifice of mice, the kidneys were rapidly taken and homogeneized in 50 mM Tris-HCl buffer (pH 7.4) at 4 °C. The homogenate was centrifugated for 10 min at 100000*g*, and the membrane fraction was diluted with Tris-HCl buffer. The homogenate (60 μL) was preincubated for 5 min at 25 °C with 10^{-4} M bestatin (10 μL) and 10^{-5} M captopril (10 μL) and then incubated for 10 min with 10 μL of [^{3}H]-D-Ala^2-Leu-enkephalin (2×10^{-8} M). The reaction was stopped by addition of 0.5 M HCl (10 μL). The tritiated metabolite [^{3}H]Tyr-D-Ala-Gly was separated as described,[60] and the radioactivity was measured by liquid scintillation counting. The blanks were obtained by addition of 10^{-5} M retrothiorphan during the preincubation.

Pharmacology: Animals. Age-matched genetically hypertensive rats (SHR) and normotensive Wistar rats were from Iffa Credo (Saint-Germain Abresles, France). DOCA salt hypertension was induced as previously described[14,32] in male Wistar rats (200−220 g) by implanting a deoxycorticosterone acetate (DOCA) pellet (200 mg kg^{-1} of body wt) after unilateral nephrectomy under ether anesthesia. Postoperatively, the rats received standard rat chow and the driking water was supplemented with 1% NaCl and 0.2% KCl. Hypertension occurred 4 weeks later.

Pharmacological Effects. The effects of RB 106 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. In a first series of experiments RB 106, or its vehicle, was administered by gavage twice a day for 6 days at various doses (2.5, 5, 10, and 25 mg/kg) in cross over protocol. Blood pressure was measured every day, 2 h after gavage by the tail cuff method. In a second series of experiments RB 106 was administered by gavage for 6 days, once a day, at 25 mg/kg and blood pressure was measured during 9 days, the 6 days of the experiment and 3 days after.

Pharmacological Effects in DOCA Salt Rats. RB 106 was administered at 25 mg/kg iv + 25 mg/kg/h in conscious animals as previously described.[32] Urine samples were collected via a poly(ethylene) catheter implanted in the bladder. The blood pressure was measured as in the case of SHR.

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References

(1) Ames, R. P.; Borkowski, A. J.; Sicinski, A. M.; Laragh, J. H. Prolonged infusions of angiotensin II and norepinephrine and blood pressure, electrolyte balance and aldosterone and cortisol secretion in man and in cirrhosis with ascites. *J. Clin. Invest.* **1965**, *44*, 1171−1186.

(2) Brenner, B. M.; Ballermann, B. J.; Gunning, M. E.; Zeidel, M. L. Diverse biological actions of atrial natriuretic peptide. *Physiol. Rev.* **1990**, *70*, 665−690.

(3) Kenny, A. J.; Stephenson, S. L. Role of endogenous 24.11 in inactivation of atrial natriuretic peptide. *FEBS Lett.* **1988**, *232*, 507−513.

(4) Erdos, E. G.; Skidgel, R. A. Structure and function of human angiotensin I converting enzyme. *Biochem. Soc. Trans.* **1985**, *13*, 42−44.

(5) Ura, N.; Carretero, O. A.; Erdos, E. G. Role of renal endopeptidase 24.11 in kinin metabolism in vitro and in vivo. *Kidney Int.* **1987**, *32*, 507−513.

(6) Wyvratt, M. J.; Patchett, A. A. Recent developments in the design of angiotensin-converting enzyme inhibitors. *Med. Res. Rev.* **1985**, *5*, 483−531. (This review contains several references to ACE inhibitors containing phenyl-substituted proline or 2-phenyl-4-thiazolidinecarboxylic acid.)

(7) Lawton, G.; Pacioreck, P. M.; Waterfall, J. F. The design and biological profile of ACE inhibitors. *Advances in Drug Research*; Harcourt Brace Jovanovich: London, 1992; Vol. 23, pp 161−220.

(8) Roques, B. P.; Noble, F.; Daugé, V.; Fournié-Zaluski, M. C.; Beaumont, A. Neutral endopeptidase 24.11 : structure, inhibition and experimental and clinical pharmacology. *Pharmacol. Rev.* **1993**, *45*, 87−146.

(9) Seymour, A. A.; Fennell, S. A.; Swerdel, J. N. Potentiation of renal effects of atrial natriuretic factor (99−126) by SQ-29,072. *Hypertension* **1989**, *14*, 87−97.

(10) Sybertz, E. J.; Chiu, P. J. S.; Vemulapalli, S.; Watkins, R.; Haslanger, M. F. Atrial natriuretic factor-potentiating and antihypertensive activity of SCH-34,826. An orally active neutral metalloendopeptidase inhibitor. *Hypertension* **1990**, *15*, 152−161.

(11) Hirata, Y.; Matsuoka, H.; Hayakawa, H.; Sugimoto, T.; Suzuki, E.; Sugimoto, T.; Kasigawa, K.; Matsuo, M. Role of endogenous atrial natriuretic peptide in regulating sodium extraction in spontaneously hypertensive rats. Effects of neutral endopeptidase inhibition. *Hypertension* **1990**, *17*, 1025−1032.

(12) Bralet, J.; Mossiat, C.; Lecomte, J. M.; Charpentier, S.; Gros, C.; Schwartz, J. C. Diuretic and natriuretic responses in rats treated with enkephalinase inhibitors. *Eur. J. Pharmacol.* **1990**, *179*, 57−64.

(13) Seymour, A. A.; Norman, J. A.; Asaad, M. M.; Fennell, S. A.; Little, D. K.; Kratunis, V. J.; Rogers, W. L. Antihypertensive and renal activity of SQ-28,603 on inhibitor of neutral endopeptidase. *J. Cardiovasc. Pharmacol.* **1991**, *17*, 296−304.

(14) Pham, I.; El Amrani, A. I. K.; Fournié-Zaluski, M. C.; Corvol, P.; Roques, B. P.; Michel, J. B. Effects of the selective neutral endopeptidase inhibitor retrothiorphan on renal function and blood pressure in normotensive and in DOCA-salt hypertensive rats. *J. Cardiovasc. Pharmacol.* **1992**, *20*, 847−857.

(15) Sybertz, E. J.; Chiu, P. J. S.; Vemulapalli, S.; Pitts, B.; Foster, C. J.; Watkins, R. W.; Barnett, M.; Haslanger, M. F. SCH-39,-370, a neutral metalloendopeptidase inhibitor potentiates biological responses to atrial natriuretic factor and lowers blood pressure in desoxycorticosterone-acetate-sodium hypertensive rats. *J. Pharmacol. Exp. Ther.* **1989**, *250*, 624−631.

(16) Seymour, A. A.; Norman, J. A.; Asaad, M. M.; Fennell, S. A.; Abboa-Offei, B.; Little, D. K.; Kratunis, V. J.; Delaney, N. G.; Hunt, J. T.; Di Donato, G. Possible regulation of atrial natriuretic factor by neutral endopeptidase 24.11 and clearance receptors. *J. Pharmacol. Exp. Ther.* **1991**, *256*, 1002−1009.

(17) Northridge, D. B.; Jardine, A. G.; Findlay, I. N.; Archibald, M.; Dilly, S. G.; Dargie, H. J. Inhibition of the metabolism of atrial natriuretic factor causes diuresis and natriuresis in chronic heart failure. *Am. J. Hypertens.* **1990**, *3*, 682−687.
(18) Kromer, E. P.; Elsner, D.; Kahles, H. W.; Riegger, C. A. Effects of atriopeptidase inhibitor UK-79,300 on left ventricular hydraulic load in patients with congestive heart failure. *Am. J. Hypertens.* **1991**, *4*, 460−463.
(19) Roques, B. P.; Beaumont, A. Neutral endopeptidase 24.11 inhibitors : from analgesia to antihypertensive? *Trends Pharmacol. Sci.* **1990**, *11*, 245−249.
(20) Seymour, A. A.; Swerdel, J. N.; Abboa-Offei, B. Antihypertensive activity during inhibition of neutral endopeptidase and angiotensin converting enzyme. *J. Cardiovasc. Pharmacol.* **1991**, *17*, 456−465.
(21) Pham, I.; Gonzalez, W.; El Amrani, A. I. K.; Fournié-Zaluski, M. C.; Philippe, M.; Laboulandine, I.; Roques, B. P.; Michel, J. B. Effects of converting enzyme inhibitor and neutral endopeptidase inhibitor on blood pressure and renal function in experimental hypertension. *J. Pharmacol. Exp. Ther.* **1993**, *265*, 1339−1347.
(22) Seymour, A. A.; Asaad, M. M.; Lanoce, V. M.; Langenbacher, K. M.; Fennell, S. A.; Rogers, W. L. Systemic hemodynamics renal function and hormonal levels during inhibition of neutral endopeptidase 3.4.24.11 and angiotensin converting enzyme in conscious dogs with pacing-induced heart failure. *J. Pharmacol. Exp. Ther.* **1993**, *266*, 872−883.
(23) Trippodo, N. C.; Fox, M.; Natarajan, V.; Panchal, B. C.; Dorso, C. R.; Asaad, M. M. Combined inhibition of neutral endopeptidase and angiotensin converting enzyme in cardiomyopathic hamsters with compensated heart failure. *J. Pharmacol. Exp. Ther.* **1993**, *267*, 108−116.
(24) (a) Stanton, J. L.; Sperbeck, D. M.; Trapani, A. J.; Cote, D.; Sakane, Y.; Berry, C. J.; Ghai, R. D. Heterocyclic lactam derivatives as dual angiotensin converting enzyme and neutral endopeptidase 24.11 inhibitors. *J. Med. Chem.* **1993**, *36*, 3829−3833. (b) Robl, J. A.; Simpkins, L. M.; Stevenson, J.; Sun, C.-Q.; Murugesan, N.; Barrish, J. C.; Asaad, M. M.; Bird, J. E.; Schaeffer, T. R.; Trippodo, N. C.; Petrillo, E. W.; Karanewsky, D. S. Dual metalloprotease inhibitors. I. constrained peptidomimetics of mercaptoacyl dipeptides. *Bioorg. Med. Chem. Lett.* **1994**, *4* (15), 1789−1794. (c) Robl, J. A.; Simpkins, L. M.; Sulsky, R.; Sieber-McMaster, E.; Stevenson, J.; Kelly, Y. F.; Sun, C.-Q.; Misra, R. N.; Ryono, D. E.; Asaad, M. M.; Bird, J. E.; Trippodo, N. C.; Karanewsky, D. S. Dual metalloprotease inhibitors. II. Effect of substitution and stereochemistry on benzazepinone based mercaptoacetyls. *Bioorg. Med. Chem. Lett.* **1994**, *4* (15), 1795−1800. (d) Robl, J. A.; Sun, C.-Q.; Simpkins, L. M.; Ryono, D. E.; Barrish, J. C.; Karanewsky, D. S.; Asaad, M. M.; Schaeffer, T. R.; Trippodo, N. C. Dual metalloprotease inhibitors. III. Utilization of bicyclic and monocyclic diazepinone based mercaptoacetyls. *Bioorg. Med. Chem. Lett.* **1994**, *4* (16), 2055−2060. (e) Das, J.; Robl, J. A.; Reid, J. A.; Sun, C.-Q.; Misra, R. N.; Brown, B. R.; Ryono, D. E.; Asaad, M. M.; Bird, J. E.; Trippodo, N. C.; Petrillo, E. W.; Karanewsky, D. S. Dual metalloprotease inhibitors. IV. Utilization of thiazepines and thiazines as constrained peptidomimetic surrogates in mercaptoacyl dipeptides. *Bioorg. Med. Chem. Lett.* **1994**, *4* (18), 2193−2198. (f) Slusarchyk, W. A.; Robl, J. A.; Taunk, P. C.; Asaad, M. M.; Bird, J. E.; DiMarco, J.; Pan, Y. Dual metalloprotease inhibitors. V. Utilization of bicyclic azepinonethiazolidines and azepinonetetrahydrothiazines in constrained peptidomimetics of mercaptoacyl dipeptides. *Bioorg. Med. Chem. Lett.* **1995**, *5* (7), 753−758. (g) Bhagwat, S. S.; Fink, C. A.; Gude, C.; Chan, K.; Qiao, Y.; Sakane, Y.; Berry, C.; Ghai, R. D. 4-substituted proline derivatives that inhibit angiotensin converting enzyme and neutral endopeptidase. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 2673−2676.
(25) Fournié-Zaluski, M. C.; Llorens, C.; Gacel, G.; Malfroy, B.; Swerts, J. P.; Lecomte, J. M.; Schwartz, J. C.; Roques, B. P. Synthesis and biological properties of highly potent enkephalinase inhibitors. *In Peptides 1980, Proc. of the Sixteenth European Symposium*; Brunfeld, K., Ed.; Scriptor: Copenhagen, 1981; pp 476−481.
(26) Gordon, E. M.; Cushman, D. W.; Tung, R.; Cheung, M. S.; Wang, F. L.; Delaney, N. G. Rat brain enkephalinase : characterization of the active site using mercaptopropanoyl amino acid inhibitors and comparison with angiotensin converting enzyme. *Life Sci.* **1983**, *33* (Suppl. 1), 113−116.
(27) Fournié-Zaluski, M. C.; Lucas, E.; Waksman, G.; Roques, B. P. Differences in the structural requirements for selective interaction with neutral metalloendopeptidase (enkephalinase) or angiotensin converting enzyme, molecular investigation by use of new thiol inhibitors. *Eur. J. Biochem.* **1984**, *139*, 267−274.
(28) Roques, B. P.; Fournié-Zaluski, M. C.; Soroca, E.; Lecomte, J. M.; Malfroy, B.; Llorens, C.; Schwartz, J. C. The enkephalinase inhibitor thiorphan shows antinociceptive activity in mice. *Nature* (*London*) **1980**, *288*, 286−288.
(29) Gros, C.; Noel, N.; Souque, A.; Schwartz, J. C.; Danvy, D.; Plaquevent, J. C.; Duhamel, L.; Duhamel, P.; Lecomte, J. M.; Bralet, J. Mixed inhibitors of angiotensin converting enzyme (EC 3.4.15.1) and enkephalinase (EC 3.4.24.11): rational design, properties and potential cardiovascular applications of glycopril and alatriopril. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 4210−4214.
(30) Fournie-Zaluski, M. C.; Coric, P.; Turcaud, S.; Rousselet, N.; Gonzalez, W.; Barbe, B.; Pham, I.; Jullian, N.; Michel, J. B.; Roques, B. P. New dual inhibitors of neutral endopeptidase and angiotensin converting enzyme : rational design, bioavailability and pharmacological responses on experimental hypertension. *J. Med. Chem.* **1994**, *37*, 1070−1083.
(31) Fournié-Zaluski, M. C.; Gonzalez, W.; Turcaud, S.; Pham, I.; Roques, B. P.; Michel, J. B. Dual inhibition of angiotensin converting enzyme and neutral endopeptidase by the orally active inhibitor mixanpril I : a new therapeutic approach in hypertension. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 4072−4076.
(32) Gonzalez-Vera, W.; Fournié-Zaluski, M. C.; Pham, I.; Laboulandine, I.; Beslot, F.; Roques, B. P.; Michel, J. B. Hypotensive and natriuretic effects in hypertensive rats of RB 105, a new dual inhibitor of angiotensin converting enzyme and neutral endopeptidase in hypertensive rats. *J. Pharmacol. Exp. Ther.* **1995**, *272*, 343−351.
(33) French, J. F.; Flynn, G. A.; Giroux, E. L.; Mehdi, S.; Anderson, B.; Beach, D. C.; Koehl, J. R.; Dage, R. C. Characterization of a dual inhibitor of angiotensin I-converting enzyme and neutral endopeptidase. *J. Pharmacol. Exp. Ther.* **1994**, *268*, 180−186.
(34) French, J. F.; Anderson, B. A.; Downs, T. R.; Dage, R. C. Dual inhibition of angiotensin converting enzyme and neutral endopeptidase in rats with hypertension. *J. Cardiovasc. Pharmacol.* **1995**, *26*, 107−113.
(35) Piedimonte, G.; Nadel, J. A.; Long, C. S.; Hoffman, J. I. E. Neutral endopeptidase in the heart : neutral endopeptidase inhibitions prevents isoproterenol-induced myocardial hypoperfusion in rats by reducing bradykinin degradation. *Circ. Res.* **1994**, *75*, 770−779.
(36) Coric, P.; Turcaud, S.; Meudal, H.; Roques, B. P.; Fournié-Zaluski, M. C. Differences in optimal recognition of neutral endopeptidase (NEP) and angiotensin converting enzyme (ACE) actives sites by mercaptoacyldipeptides. A new approach in the design of potent dual inhibitors. *J. Med. Chem.* **1996**, *39*, 1210−1219.
(37) Gomez-Monterrey, I.; Beaumont, A.; Roques, B. P.; Fournié-Zaluski, M. C. New thiol inhibitors of neutral endopeptidase E. C. 24.11 : synthesis and enzyme active-site recognition. *J. Med. Chem.* **1994**, *37*, 1865−1873.
(38) Gomez-Monterrey, I.; Turcaud, S.; Lucas, E.; Bruetschy, L.; Roques, B. P.; Fournié-Zaluski, M. C. Exploration of neutral endopeptidase (EC 3.4.24.11.) active site by a series of new thiol containing inhibitors. *J. Med. Chem.* **1993**, *36*, 87−94.
(39) Pascard, C.; Guilhem, J.; Vincent, M.; Remond, G.; Portevin, B.; Laubie, M. Configuration and preferential solid state conformations of perindoprilate (S-9780). Comparison with the crystal structures of other ACE inhibitors and conclusions related to structure activity relationships. *J. Med. Chem.* **1991**, *34*, 663−669.
(40) Cushman, D. W.; Cheung, H. S.; Sabo, E. F.; Ondetti, M. A. Design of potent competitive inhibitors of angiotensin converting enzyme. Carboxy alkanoyl and mercapto alkanoylamino acids. *Biochemistry* **1977**, *16*, 5484−5491.
(41) Ohta, T.; Hosoi, A.; Kimura, T.; Nozoe, S. Direct chain elongation of N-carbamoylpyroglutamate. An efficient synthesis of (−)-pyrrolidine-2,5-dicarboxylic acid. *Chem. Lett.* **1987**, 2091−2094.
(42) Ezquerra, J.; Pedregal, C.; Rubio, A.; Valenciano, J.; Navio, J. L. G.; Alvarez-Builla, J.; Vaquero, J. J. General method for the synthesis of 5-arylpyrrole-2-carboxylic acids. *Tetrahedron Lett.* **1993**, *34*, 6317−6320.
(43) Overberger, C. G.; David, K. H.; Moore, J. A. Synthesis and resolution of cis and trans 5-methylproline. *Macromolecules* **1972**, *5*, 368−372.
(44) Aubry, M.; Bertheloot, A.; Beaumont, A.; Roques, B. P.; Crine, P. The use of a monoclonal antibody for the high yield purification of kidney enkephalinase solubilized in β octyl glucoside. *Biochem. Cell. Biol.* **1987**, *65*, 398−404.
(45) Pantaliano, M. W.; Holmquist, B.; Riordan, J. F. Affinity chromatographic purification of angiotensin converting enzyme. *Biochemistry* **1984**, *23*, 1037−1042.
(46) Benchetrit, T.; Fournié-Zaluski, M. C.; Roques, B. P. Relationship between the inhibitory potencies of thiorphan and retrothiorphan enantiomers on thermolysin and neutral endopeptidase 24.11 and their interactions with the thermolysin active site by computer modeling. *Biochem. Biophys. Res. Commun.* **1987**, *147*, 1034−1040.
(47) Wattley, J. W. M.; Gavin, T.; Desai, M. Bicyclic lactam inhibitors of angiotensin converting enzyme. *J. Med. Chem.* **1984**, *27*, 816−818.

(48) Andrews, P. R.; Carson, J. M.; Caselli, A.; Spark, M. J.; Woods, R. Conformational analysis and active site modeling of angiotensin converting enzyme inhibitors. *J. Med. Chem.* **1985**, *28*, 393−399.
(49) Roderick, S. L.; Fournié-Zaluski, M. C.; Roques, B. P.; Matthews, B. W. Thiorphan and retrothiorphan display equivalent interactions when bound to crystalline thermolysin. *Biochemistry* **1989**, *28*, 1493−1497.
(50) Flynn, G. A.; Beight, D. W.; Mehdi, S.; Koehl, J. R.; Giroux, E. L.; French, J. F.; Hakes, P. W.; Dage, R. C. Application of a conformationally restricted Phe-Leu dipeptide mimetic to the design of a combined inhibitor of angiotensin I converting enzyme and neutral endopeptidase 24.11. *J. Med. Chem.* **1993**, *36*, 2420−2423.
(51) Benchetrit, T.; Bissery, V.; Mornon, J. P.; Devault, A.; Crine, P.; Roques, B. P. Primary structure homologies between two zinc-metallopeptidases, the neutral endopeptidase 24.11 "enkephalinase" and the thermolysin through clustering. *Biochemistry* **1988**, *27*, 592−596.
(52) Soleilhac, J. M.; Lucas, E.; Beaumont, A.; Turcaud, S.; Michel, J. B.; Ficheux, D.; Fournié-Zaluski, M. C.; Roques, B. P. A 94-kDa protein, identified as neutral endopeptidase-24.11, can inactivate atrial natriuretic peptide in the vascular endothelium. *Mol. Pharmacol.* **1992**, *41*, 609−614.
(53) O'Donnell, M. J.; Eckrich, T. M. The synthesis of amino acid derivatives by catalytic phase transfer alkylations. *Tetrahedron Lett.* **1978**, 4625−4628.
(54) Winkle, M. R.; Ronald, R. C. Regioselective metalation reactions of some substituted (methoxymethoxy) arenes. *J. Org. Chem.* **1982**, *47*, 2101−2108.
(55) Koga, K.; Juang, T. M.; Yamada, S. Stereochemical studies L II Studies on the stereochemical courses in deaminative bromination of 3,5-dichloro-L-tyrosine and in amination of the corresponding α-bromo acid. Existence of strong neighboring phenoxide group participation. *Chem. Pharm. Bull.* **1978**, *26*, 178−184.
(56) Goudreau, N.; Weng, J. H.; Roques, B. P. Conformational analysis of CCK-B agonists using ^{1}H-NMR and restrained molecular dynamics : comparison of biologically active Boc-Trp-(N-Me)Nle-Asp-Phe-NH_2 and inactive Boc-Trp-(N-Me)Phe-Asp-Phe-NH_2. *Biopolymers* **1994**, *34*, 155−169.
(57) Hagler, A. T. Theoretical simulation of conformation, energetics and dynamics of peptides. In *The Peptides: Conformations in Biology and Drug Design*; Hruby, V. J., Meienhofer, J., Eds.; Academic Press: New York, 1985; pp 213−299.
(58) Dauber-Osgerthorpe, P.; Roberts, V. A.; Osgerthorpe, D. J.; Woff, J.; Genest, M.; Hagler, A. T. Structure and energetics of ligand binding to proteins : E-Coli dihydrofolate reductase-trimethoprim, a drug receptor system. *Proteins* **1988**, *4*, 31−47.
(59) Piquilloud, Y.; Reinharz, A.; Roth, M. Studies of the angiotensin converting enzyme with different substrats. *Biochim. Biophys. Acta* **1970**, *206*, 136−142.
(60) Llorens, C.; Malfroy, B.; Schwartz, J. C.; Gacel, G.; Roques, B. P.; Roy, J.; Morgat, J. L.; Javoy-Agid, F. Enkephalin dipeptidyl carboxypeptidase (enkephalinase) : selective radioassay, properties and regional distribution in human brain. *J. Neurochem.* **1982**, *39*, 1081−1089.
(61) Vogel, Z.; Altstein, M. The adsorption of enkephalin to porous polysterene beads : a simple assay for enkephalin hydrolysis. *FEBS Lett.* **1977**, *80*, 332−336.
(62) Benchetrit, T.; Fournié-Zaluski, M. C.; Roques, B. P. Relationship between the inhibitory potencies of thiorphan and retrothiorphan enantiomers on thermolysin and neutral endopeptidase 24.11 and their interactions with the thermolysin active site by computer modeling. *Biochem. Biophys. Res. Commun.* **1987**, *147*, 1034−1040.
(63) Cheung, H. S.; Cushman, D. W. Inhibition of homogenous angiotensin converting enzyme of rabbit lung by synthetic venom peptides of bothrops Jararaca. *Biochim. Biophys. Acta* **1973**, *293*, 451−457.
(64) Seymour, A. A.; Asaad, M. M.; Abboa-Offei, B. E.; Smith, P. L.; Rogers, W. L.; Dorso, C. R. Determinants of *in vivo* activity of neutral endopeptidase 3.4.24.11 and angiotensin converting enzyme inhibitors. *J. Pharmacol. Exp. Ther.* **1996**, *276*, 708−713.

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