

New Thiol Inhibitors of Neutral Endopeptidase EC 3.4.24.11: Synthesis and Enzyme Active-Site Recognition

Isabel Gomez-Monterrey,[†] Ann Beaumont, Patrick Nemecek,[‡] Bernard P. Roques,^{*} and Marie-Claude Fournie-Zaluski

Unité de Pharmacochimie Moléculaire et Structurale, U266 INSERM - URA D1500 CNRS, Université René Descartes, UFR des Sciences Pharmaceutiques et Biologiques, 4, avenue de l'Observatoire, 75270 Paris Cedex 06, France, and Rhône Poulenc Rorer, Centre de Recherche de Vitry-Alforville, 13, quai Jules Guesde, 94403 Vitry/Seine, France

Received May 17, 1993[®]

Selective, as well as mixed, inhibitors of the two zinc metallopeptidases, neutral endopeptidase (NEP) and angiotensin converting enzyme (ACE), are of major clinical interest in the treatment of hypertension and cardiac failure. New thiol inhibitors, corresponding to the general formula HS-CH(R₁)-CH₂-CH(R₂)-CONH-CH(R₃)-COOH, were designed in order to explore the putative S₁ subsite of the active site of NEP. The inhibitors were also tested on ACE and the most representative on thermolysin (TLN) for comparison. The relatively low inhibitory potencies exhibited by these compounds (IC₅₀s in the 10⁻⁷ M range for NEP and in the 10⁻⁶ M range for ACE) as compared to that of thiorphan (IC₅₀s 2.10 × 10⁻⁹ M on NEP and 1.40 × 10⁻⁷ M on ACE) clearly indicate the absence of the expected energetically favorable interactions with the active site of both peptidases. A 100-fold loss of potency for these inhibitors was also observed for thermolysin as compared to thiorphan. Using the mutated Glu¹⁰²-NEP, it was possible to demonstrate that the inhibitors do not fit the S₁ subsite of NEP but interact with the S'₁ and S'₂ subsites through binding of their R₁ and R₂ residues and that the C-terminal amino acid is located outside the active site. These results seem to indicate that these thiol inhibitors are not well adapted for optimal recognition of the S₁ subsite of NEP, and probably ACE, and that other zinc-chelating moieties such as carboxylate or phosphonate groups may be preferred for this purpose.

Introduction

Neutral endopeptidase (EC 3.4.24.11, NEP) is a metallopeptidase involved in the metabolism of a variety of physiologically important peptides and its inhibition offers several interesting new therapeutic possibilities (see review in ref 1). Thus, in the brain, the opioid peptides enkephalins are inactivated by the common action of two zinc metallopeptidases, NEP and aminopeptidase N (APN). Mixed inhibitors of both enzymes have been shown to induce potent analgesic responses without the major side effects of morphine.²⁻⁴ In the intestine, the enkephalins, acting at δ opioid receptors, are mainly involved in the regulation of fluid secretion.⁵ The demonstration that their intestinal actions can be blocked by NEP inhibitors has led to the marketing of thiorphan (HS-CH₂-CH(CH₂Φ)-CONH-CH₂-COOH),⁶ under its prodrug form acetorphan, as an antidiarrheal agent designated tiorfant.^{7,8} In addition, the atrial natriuretic peptide ANP is cleaved by NEP into inactive fragments, and numerous inhibitors have been shown to produce natriuresis and diuresis in animals and humans (see review in ref 9). The association of an NEP inhibitor such as retrothiorphan with an angiotensin converting enzyme inhibitor such as enalapril led to a potentiation of the antihypertensive action of the ACE inhibitor,¹⁰ and mixed inhibitors of the two metallopeptidases could therefore represent a new approach in the treatment of hypertension and cardiac failure.¹¹

The primary sequence of NEP has been deduced from cDNA clones from various species (rabbit,¹² rat,¹³ and

human¹⁴), but its three-dimensional structure remains unknown. However, its mechanism of action and its subsite specificity are well documented, due to its overall analogy with thermolysin (TLN), a bacterial metallopeptidase whose structure was established from X-ray crystallography.¹⁵ These analogies include not only the hydrophobicity of the binding subsites surrounding the catalytic domain¹⁶⁻¹⁹ and the three zinc coordinating ligands²⁰⁻²² but also the amino acids involved in the stabilization of the Michaelis complex.²³⁻²⁶ However, the main difference between NEP and TLN is the presence in the S'₂ subsite of the former of an arginine residue (Arg¹⁰²) which was shown to interact with the free carboxyl group of substrates such as the enkephalins.⁶ In spite of large similarities in the active site of both enzymes, the potency of a large number of inhibitors is about 2 or 3 orders of magnitude lower for TLN than for NEP. This is particularly evident for inhibitors interacting with the S'₁ and S'₂²⁷ subsites of both enzymes. For example, the K_i of (S)-thiorphan (HS-CH₂-CH(CH₂Φ)-CONH-CH₂-COOH) is 1.8 × 10⁻⁶ M for TLN and 1.9 × 10⁻⁹ M for NEP.²⁸ However, the difference is smaller for inhibitors able to interact with the S₁, S'₁, and S'₂ subsites of each enzyme. For example, the K_i of (1-carboxy-3-phenylpropyl)Leu-Trp is 5 × 10⁻⁸ M for TLN,²⁹ and an analogous compound such as SCH 39370 ((1-carboxy-3-phenylpropyl)Phe- β -Ala) has a K_i of 1.1 × 10⁻⁸ M for NEP.³⁰ Taken together, these results seem to indicate that the occupancy of the S₁ subsite is more important in TLN than in NEP for strong inhibition of the enzyme.

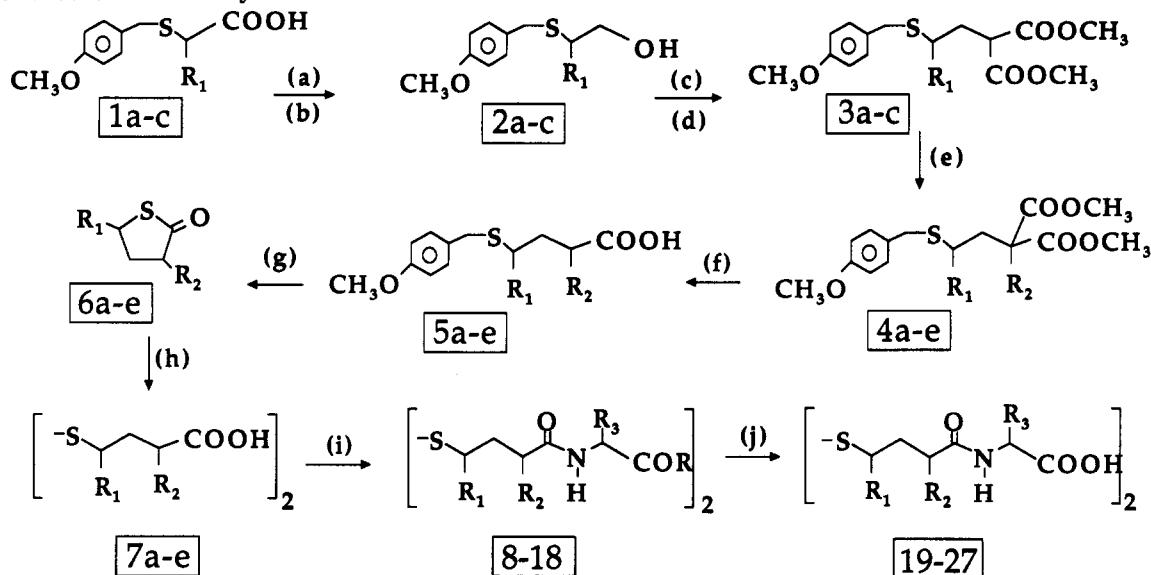
The S₁ subsite of NEP has been investigated with various carboxyalkyl dipeptides³⁰⁻³³ or related compounds, such as UK 69578,³⁴ constructed on the model of enalapril,³⁵ and the results obtained seem to indicate that the S₁ subsite corresponds to a hydrophobic domain. However, the great majority of these compounds have IC₅₀s in the 10⁻⁸ M

* To whom correspondence should be addressed. Tel: (33) 43-25-50-45. FAX: (33) 43-26-69-18.

[†] Present address: Instituto de Química Médica, Juan de la Cierva, 3 Madrid, Spain.

[‡] Centre de Recherche de Vitry-Alforville.

[®] Abstract published in *Advance ACS Abstracts*, May 1, 1994.

Scheme 1. Scheme for the Synthesis of Inhibitors 17-27^a

^a (a) SOCl_2 , MeOH ; (b) NaBH_4 , LiCl ; (c) NaI , $(\text{CH}_3)_3\text{SiCl}$; (d) NaH , $\text{CH}_2(\text{COOCH}_3)_2$; (e) NaH , R_2X ; (f) DMSO-20\% KOH ; (g) CF_3COOH , reflux; (h) NaOH/EtOH , O_2 ; (i) $\text{H}_2\text{N-CH}(\text{R}_3)\text{-COR}$, BOP , DIEA ; (j) OH^- then H_3O^+ .

range, whatever the degree of the hydrophobicity of the P_1 moiety, a result which does not permit a clear characterization of the S_1 subsite.

Recently, we have reported two new series of NEP inhibitors containing a thiol group as the zinc-chelating agent and designed to interact with the S_1 , S'_1 , and S'_2 subsites of the enzyme.³⁶ Some of these compounds were found to be very efficient, essentially those corresponding to the general formula $\text{HS-CH}(\text{R}_1)\text{-CH}(\text{R}_2)\text{-CONH-CH}(\text{R}_3)\text{-COOH}$ with IC_{50} values in the nanomolar range for the most active stereoisomer. Nevertheless, none of these inhibitors is more active than thiorphan ($\text{HS-CH}_2\text{-CH}(\text{CH}_2\Phi)\text{-CONH-CH}_2\text{-COOH}$),⁶ which interacts only with the S'_1 and S'_2 subsites of NEP. Several hypotheses have been proposed to account for this result. The inhibitors might be unable to interact with the S_1 subsite for steric reasons, or the interaction of the R_1 chain in the S_1 subsite might decrease the strength of the zinc complexation. On the other hand, the S_1 subsite of NEP might not be well defined, leaving the inhibitor R_1 side chain to be located in the aqueous medium or at the surface of the enzyme active site.

In order to test the hypothesis that the potency of the previously discussed series is limited by steric hindrance, we have developed new compounds of general formula $\text{HS-CH}(\text{R}_1)\text{-CH}_2\text{-CH}(\text{R}_2)\text{-CONH-CH}(\text{R}_3)\text{-COOH}$, in which a methylene spacer has been introduced between the R_1 and R_2 chains. For R_1 , R_2 , and R_3 , hydrophobic chains were chosen which have previously been found to fit well into the active site of NEP. For comparison, these compounds have also been tested on ACE and some of them on TLN.

Results

(1) **Synthesis.** The compounds 17-27 were prepared following the scheme summarized in Scheme 1. The protected 2-mercaptop alkanoic acids 1a-c were obtained under optically pure forms from the corresponding α -amino acids through desamino halogenation of D -amino acids following the Fischer procedure,³⁷ yielding the corresponding 2-bromo analogs with retention of configuration. The subsequent nucleophilic substitution of the halogen

by the potassium salt of *p*-methoxybenzyl sulfide gave intermediates 1a-c with inversion of configuration. These protected α -mercaptop acids were reduced by lithium borohydride to the corresponding β -mercaptop alcohols 2a-c.

The following synthetic pathway involved three key steps. The first was the formation of the malonate derivatives 3a-c, which necessitated the transformation of the alcohols 2a-c to iodinated derivatives³⁸ before reaction with the sodium salt of dimethyl malonate. The second critical step was the decarboxylation of the hindered intermediates obtained, 4a-e, which was achieved only by heating these compounds in DMSO-20% KOH to give 5a-e. The third difficult step was the deprotection of the thiol group, which could not be performed at the last step of the synthesis either by the classical $\text{Hg}(\text{CF}_3\text{COO})_2$ and H_2S treatment or by reflux in CF_3COOH ³⁹ without major degradation of the final compounds. This problem was bypassed in an intermediate step of the synthesis. Thus, the thiol deprotection was easily obtained from 5a-e by refluxing the compounds in CF_3COOH , owing to the high stability of the thiolactones 6a-c which were spontaneously formed during this step. The synthesis was continued by opening the cyclic structures 6a-e by alkaline treatment and with bubbling O_2 to obtain the disulfides 7a-e. The synthesis was achieved by condensation of α -amino esters or α -amino amides of defined stereochemistry using BOP reagent and final deprotection. The final compounds were isolated as mixtures of diastereoisomers in about equal proportions as shown by NMR and, in some cases, by HPLC. This is due to a racemization of the carbon bearing the protected thiol group, which occurred probably during the drastic treatment with KOH of the intermediates 3a-c.

Compound 32 was obtained by a different pathway (Scheme 2), using the procedure leading to the preparation of α - β -substituted β -mercaptop acids.³⁶ Thus, condensation of triethyl 2-benzyl phosphonoacetate with phenylacetaldehyde led to a mixture of α - β and β - γ unsaturated esters (compounds 28 and 29). Saponification of this mixture yielded only the β - γ unsaturated acid 30 by transposition of the double bond. Addition of thiolacetic acid to 30 gave 31 as a mixture of four stereoisomers in equal

Scheme 2. Scheme for the Synthesis of Inhibitor 32

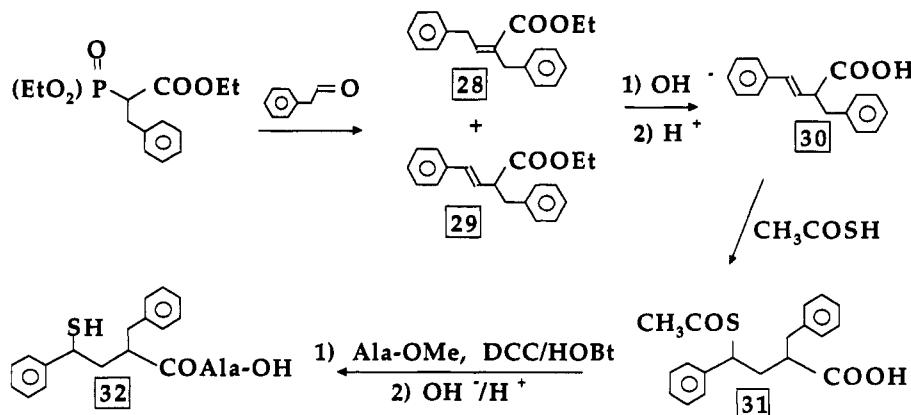


Table 1. Inhibitory Potency of Compounds 17-32 on NEP and ACE Activities

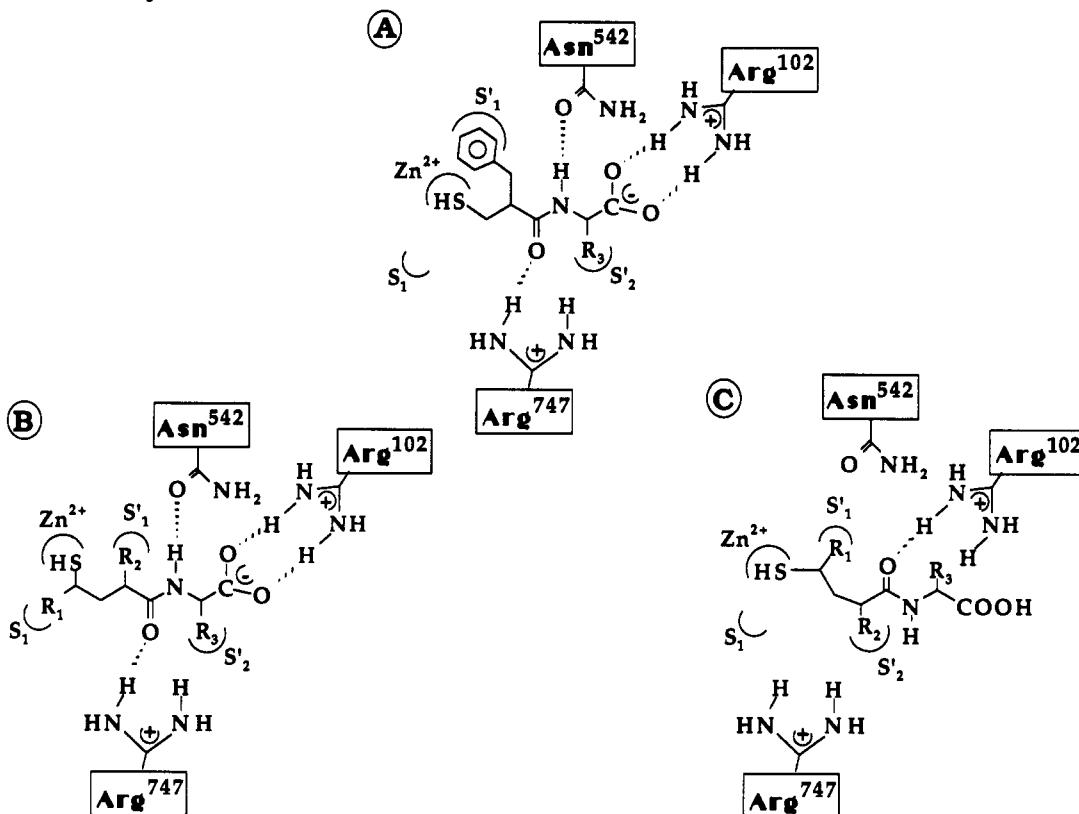
compound	IC ₅₀ (μM) ^a		compound	IC ₅₀ (μM) ^a	
	NEP ^b	ACE ^c		NEP ^b	ACE ^c
A					
	0.044 ± 0.012 ^d	4.0 ± 0.5 ^d			
19	0.19 ± 0.07	3.8 ± 0.8	26	0.38 ± 0.02	3.4 ± 0.6
20	0.29 ± 0.05	3.5 ± 1.5	17	0.36 ± 0.02	4.0 ± 1.0
21	0.32 ± 0.06	3.6 ± 0.5	27S	0.27 ± 0.02	4.4 ± 0.4
22	0.15 ± 0.06	2.4 ± 0.7	27R	0.32 ± 0.05	8.0 ± 0.6
23	0.22 ± 0.03	0.8 ± 0.05	18	0.35 ± 0.05	3.5 ± 0.5
24	0.35 ± 0.05	2.8 ± 0.6	6b	0.22 ± 0.05	2.3 ± 0.6
32	0.13 ± 0.05	3.5 ± 0.5	7b	0.25 ± 0.06	4.0 ± 0.6
			25	0.26 ± 0.04	2.9 ± 0.5

^a Values are the mean ± SEM from three independent experiments computed by log probit of five inhibitor concentrations. ^b Concentration inhibiting 50% of NEP activity using 20 nM [³H]-D-Ala²-Leu-enkephalin as substrate. ^c Concentration inhibiting 50% of ACE activity using 50 μM N-Cbz-Phe-His-Leu as substrate. ^d From ref 36. ^e From ref 40.

proportions, and the final compound 32 was obtained by condensation of alanine methyl ester followed by alkaline hydrolysis of both protective groups.

(2) Inhibitory Activity. The inhibitory potencies of the various compounds synthesized were measured on NEP and ACE as mixtures of stereoisomers and are

summarized in Table 1. The effects of five structural modifications were investigated (i) amidification of the free C-terminal carboxyl group (compound 26 versus 17 or 27S versus 18); (ii) modification of the C-terminal amino acid with successive introduction of an alanine (compound 19), a β-alanine (compound 20), a proline (compound 21),

Chart 1. Schematic Representation of NEP Active Site^a

^a (A) Complex NEP-thiorphan as deduced from X-ray analysis of the complex TLN-thiorphan. (B) Possible model of interaction of inhibitors 17-32 with NEP active site. In this model, the R₁, R₂, and R₃ side chains fit the S₁, S'₁, and S'₂ subsites of the enzyme, respectively. (C) Possible model of interaction of inhibitors 17-32 with NEP active site deduced from experiments with the Glu¹⁰²-mutated enzyme. In this model, the R₁ and R₂ side chains fit the S₁ and S'₂ subsites of NEP and R₃ is outside the active site.

or a tyrosine (compound 22) moiety; (iii) change in the stereochemistry of the C-terminal amino acid with 27S and 27R; (iv) replacement of the benzyl group, assumed to interact with the S'₁ subsite by an isobutyl chain (compounds 22 and 23 or 26 and 27); and finally (v) exploration of the putative S₁ subsite using successively a phenyl (compound 32 versus 19), a benzyl, and phenethyl and isobutyl chains (compounds 23, 25, and 26).

Regarding NEP inhibition, all compounds showed similar potencies, with IC₅₀s in the 1-4 × 10⁻⁷ M range, whatever the chemical modifications introduced in these molecules. For ACE inhibition, the same potency was also obtained for all compounds studied with IC₅₀s in the micromolar range. Furthermore, the synthetic intermediates 6b and 7b were also tested on both enzymes, and their activities were found to be in the 10⁻⁷ M range on NEP and in the micromolar range on ACE. The potencies of the compounds tested on TLN were found in the 10⁻⁴ M range.

Discussion

With the aim of improving NEP active-site recognition, a new series of thiol inhibitors expected to interact with the S₁, S'₁, and S'₂ subsites of the enzyme was synthesized (Table 1). The development of these compounds was based on the structure of a recently described³⁶ thiol inhibitor (compound A, Table 1) and of NEP inhibitors of general formula HOOC-CH(CH₂Ph)-CH₂-CH(CH₂Ph)-CO-AA (compound B, Table 1).⁴⁰ Some of these latter compounds were shown to have IC₅₀s for NEP in the nanomolar range, suggesting that their interaction with the three subsites (S₁, S'₁, and S'₂) of the enzyme are optimized.

In contrast to these results, all the compounds described here exhibited surprisingly similar IC₅₀s for NEP, which were in the 10⁻⁷ M range (Table 1). These data show a significant decreased efficiency of these compounds in binding NEP as compared to that of the thiol inhibitor A³⁶ or the dibenzylglutaryl derivative B. In contrast, the IC₅₀ values for ACE, which were found to be in the 10⁻⁶ M range for all compounds, are not significantly different from those reported for A (Table 1) and derivatives.³⁶ In order to understand in terms of active-site occupancy, the decreased activity of these new compounds as compared to that of other thiol inhibitors such as thiorphan or compound A and its analogs, the activities of 19, 21, 23, 26, 27S, and 27R, chosen as models for this series, were checked on TLN (data not shown). Their IC₅₀s (in the 10⁻⁴ M range) were increased by 3 orders of magnitude as regard to their IC₅₀s for NEP (10⁻⁷ M range). This ratio already found for thiorphan (IC₅₀ on NEP 1.9 × 10⁻⁹ M, IC₅₀ on TLN 1.8 × 10⁻⁶ M) or for constrained thiols such as HS-CH(CH₂CHiPr)-CH(CH₂Ph)-CONHCH(CH₃)-COOH (IC₅₀ on NEP 3.6 × 10⁻⁹ M, IC₅₀ on TLN 4.0 × 10⁻⁶ M) indicates again that the TLN active site can be used as a model to investigate recognition of the active site of NEP. From the crystallographic data of the complex TLN-thiorphan,⁴¹ the assumed interactions of this inhibitor with the active site of NEP are shown in Chart 1A. This model allows two possible types of binding to be proposed. Firstly, the inhibitors bind to the active site of NEP as shown in Chart 1B, i.e., with the three side chains fitting the S₁, S'₁, and S'₂ subsites. In this case, the decreased affinity of these compounds, related to the dibenzylglutaryl derivatives, could be explained by the

interaction of the three side chains with their respective subsites, inhibiting the perfect alignment of the thiol groups with the zinc atom, required for optimal coordination.⁴² In the case of dibenzylglutaryl derivatives, such as **B**, this problem could be overcome by the enhanced degree of freedom of the carboxylate group and its possibility of interacting with the zinc atom, by using one or two of its oxygen atoms, as amply demonstrated by crystallographic analysis of complexes between various carboxyl inhibitors and TLN.¹⁵

However, another explanation may account for the data reported in Table 1. For both compound **A** and its analogs,³⁶ or for compound **B** and its analogs,⁴⁰ the nature of the C-terminal residue is of great importance for the inhibitory activity. In Table 1 it can be seen that IC_{50} s for NEP were the same for compounds **17** and **26** or **18** and **27S**, which differed by the presence of a free carboxylate in **26** and **27S** and an amide group in **17** and **18**. However, it is now well known that, due to the presence of Arg^{102} in the active site of NEP, lower IC_{50} s are obtained for inhibitors containing a free C-terminal carboxylate rather than a carboxamide group.²⁶ More significant was the fact that compounds **21** and **24**, which contain a C-terminal proline, have the same IC_{50} for NEP as **19** or **20** which are terminated by an alanine or a β -alanine residue, as it has also been demonstrated that the S'_2 subsite of NEP poorly recognizes a proline ring.^{16,43} Moreover, the IC_{50} values of **27R** and **27S** on both NEP and ACE were almost identical (Table 1), although the S'_2 subsite of these enzymes has a 100–1000 times better affinity for amino acids of S configuration.^{17,44,45}

Likewise, the comparison of the inhibitory potencies of compounds **20** and **21** on ACE surprisingly showed that they are identical, although it is also well known that ACE does not accept a β -amino acid in its S'_2 subsite, due to its strong carboxy dipeptidase nature.³² Finally, compound **7b** and its cyclic analog **6b**, which is transformed to **7b** during the incubation time at pH 7.4, also have the same inhibitory potencies on NEP and ACE, although they do not contain a C-terminal amino acid. All these arguments seem to favor the mode of interaction with the active site of NEP (and ACE) in which the residues R_1 and R_2 interact with the S'_1 and S'_2 subsites of the enzyme as shown schematically in Chart 1C, with the C-terminal amino acid outside the active site. In this model, the stabilizing hydrogen bonds between the amide group of the inhibitor and the side chains of Arg^{747} and Asn^{542} , which are assumed to be important for the formation of stable complexes with NEP's active site, do not occur.

This proposal seems to be confirmed by experiments performed with **7b** and **25** on the mutated enzyme $Glu^{102-}NEP^{25,26}$ that we have previously used as an index of inhibitor positioning in the enzyme's active site.^{46,36} As shown in Chart 1, Arg^{102} is thought to be positioned to interact with the C-terminal carboxyl of a P'_2 residue. When Arg^{102} was replaced by Glu, the K_i of thiorphan for the mutated enzyme increased by over 2 orders of magnitude, due to the ionic repulsion between the two negatively charged groups. Under the same conditions, the K_i of thiorphan amide which has no free carboxylate was increased only by a factor of 6. The IC_{50} s of **7b** and **25** on the mutated enzyme were respectively 3×10^{-5} and 2×10^{-6} M. These values correspond to a loss of affinity, when compared to the wild-type NEP, of about 120-fold for **7b** and 7-fold for **25**. Taken together, these results

strongly suggest that both compounds interact with S'_1 and S'_2 subsites of NEP and that the C-terminal tyrosine of **25** is outside the active site of the enzyme.

In conclusion, the new series of thiol inhibitors described in this paper was not able to clarify the nature of the S_1 subsite of NEP and ACE. Indeed, it seems that both the hydrophobic character of the S'_1 subsite and the high tendency of the thiol group to optimize the complexation of the Zn^{2+} ion direct the positioning of the inhibitors in such a way that these two interactions are greatly privileged to the prejudice of any other stabilizing interactions, i.e., with Arg^{747} , Asn^{542} , or Arg^{102} . Consequently, it seems more easy to explore the S_1 subsite of NEP with inhibitors which do not contain a thiol group as a zinc ligand. Carboxylates have been intensively used, but hydroxamates or phosphoryl groups may also be useful.

Experimental Section

I. Biological Tests. [³H]Tyr-D-Ala²-Leu-enkephalin (52 Ci/mmol) was obtained from CEA (Saclay, France). N-Cbz-Phe-His-Leu was from Bachem (Bubendorf, Switzerland). Neutral endopeptidase was purified to homogeneity from rabbit kidney as previously described.⁴⁷ Recombinant human angiotensin converting enzyme obtained as described⁴⁸ was a generous gift from Prof. Corvol (Collège de France, Paris, France).

Assay for Neutral Endopeptidase Activity. IC_{50} values were determined as previously described.³⁸ NEP (final concentration 1 pmol/100 μ L, specific activity for [³H]-D-Ala²-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 μ L in 50 mM Tris-HCl buffer, pH 7.4. [³H]-D-Ala²-Leu-enkephalin (K_m = 30 μ M) was added to a final concentration of 20 nM, and the reaction was stopped after 30 min by adding 10 μ L of 0.5 M HCl. The tritiated metabolites formed were separated on polystyrene beads. The mutated enzyme $Glu^{102-}NEP$ was obtained as previously described.²⁶ The inhibitory potency of the inhibitors tested on this enzyme was determined by the method described for NEP.

Assay for Angiotensin Converting Enzyme Activity. Enzymatic studies on ACE were performed using N-Cbz-Phe-His-Leu as substrate (K_m = 50 mM) as previously described.⁴⁹ ACE (final concentration 0.02 pmol/100 μ L, specific activity on Cbz-Phe-His-Leu 13 nmol/mg/min) was preincubated for 15 min at 37 °C with various concentrations of the tested 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 μ L of 2 M NaOH. After dilution with 3 mL of water, the concentration of His-Leu was determined following the fluorimetric assay described by Cheung et al.⁵⁰ with a MPF 44 A 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 denatured enzyme.

As the compounds tested were synthesized under their disulfide forms, 100 equiv of DTT was added to the stock solutions of the inhibitors before dilution.

II. Chemistry. The amino acids were from Bachem (Bubendorf, Switzerland). Chlorotrimethylsilane, dimethyl malonate, benzyl bromide, 2-iodobutane, and (4-methoxyphenyl)methyl mercaptan, were from Aldrich (Strasbourg, France). BOP reagent was from Propeptide (Vert le Petit, France).

The solvents (Normapur label) were from SDS (Peypin, France). 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, EtOAc/hexane = 1/1; B, EtOAc/hexane = 1/4; C, CH_2Cl_2 /MeOH = 14/1; D, EtOAc/hexane = 1/2; E, $CHCl_3$ /MeOH/HOAc = 9/1/0.1; F, $CHCl_3$ /MeOH = 9/1; G, EtOAc/hexane = 2/3. Plates were revealed with UV, iodine vapor, or ninhydrin. The purity of the final compounds was checked by HPLC on a reverse-phase Nucleosil C₈ column (SFCC) with CH_3CN /TFA 0.05% as the mobile phase. The eluted peaks were monitored at 210 nm. The structure of all the

compounds was confirmed by ^1H NMR spectroscopy (Bruker AC 270 MHz) in [$^2\text{H}_6$]DMSO using HMDS as an internal reference. Melting points of the crystallized compounds were determined on an Electrothermal apparatus and are reported uncorrected.

The following abbreviations are used, EtOAc, ethyl acetate; HOAc, acetic acid; MeOH, methanol; EtOH, ethanol; DMF, dimethylformamide; THF, tetrahydrofuran; DMSO, dimethyl sulfoxide; HMDS, hexamethyldisiloxane; Et₃N, triethylamine; HOEt, 1-hydroxybenzotriazole; DCC, dicyclohexylcarbodiimide; DCU, dicyclohexylurea; Et₂O, diethyl ether; TFA, trifluoroacetic acid; BOP, benzotriazol-1-yl-oxytris(dimethylamino)phosphonium hexafluorophosphate; DIEA, diisopropylethylamine.

General Procedure for the Preparation of Compounds 1.

Procedure I. To a solution of (*R*)-2-bromoalkanoic acid (prepared from the corresponding (*R*)- α -amino acid³⁷) in DMF was added at 0 °C a solution of potassium (4-methoxybenzyl)-thiolate (prepared from 4-methoxybenzyl mercaptan and KOH in DMF (5 equiv)). After stirring overnight at room temperature, the mixture was evaporated in vacuo and the residue dissolved in water. The aqueous layer was washed (3X) by EtOAc, acidified to pH 2 by 3 M HCl, and extracted by EtOAc. The organic layer was washed, dried (Na_2SO_4), and evaporated in vacuo.

1a ($\text{R}_1 = \text{CH}_2\text{Ph}$): oily product (98%), R_f (G) 0.55; ^1H NMR δ 2.78 and 3.00 (CH_2Ph), 3.35 ($\text{CH}(\text{COOH})$), 3.70 (CH_3O), 3.80 (CH_2S), 6.8 and 7.10, 7.29 (Ph), 12.50 (COOH).

1b ($\text{R}_1 = (\text{CH}_2)_2\text{Ph}$): oily product (76%), R_f (B) 0.60; ^1H NMR δ 1.77 and 2.00 ($\text{CH}_2\beta$), 2.51 ($\text{CH}_2\gamma$), 3.00 ($\text{CH}(\text{COOH})$), 3.69 (CH_3O), 3.70 (CH_2S), 6.80, 7.00–7.18 (Ph), 12.46 (COOH).

1c ($\text{R}_1 = \text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_3$): oily product (92%), R_f (A) 0.77; ^1H NMR δ 0.72 ($\text{CH}_3(\text{CH}_2)$), 0.85 ($\text{CH}_3(\text{CH})$), 1.10, 1.30, and 1.70 (CH_2CH), 2.9 ($\text{CH}(\text{COOH})$), 3.7 ($\text{CH}_3\text{O} + \text{CH}_2\text{S}$), 6.8 and 7.15 ((*p*-MeO)Ph), 12.43 (COOH).

General Procedure for the Preparation of Compounds 2.

Procedure II. The carboxylic acids 1 were dissolved in MeOH, and SOCl_2 (1.7 equiv) was added dropwise at 0 °C. The mixtures were stirred for 1 h at room temperature and 40 min at 80 °C. The solvents were evaporated. The residues were dissolved in EtOAc, washed with water, 2% NaHCO_3 , and brine, dried (Na_2SO_4), and evaporated in vacuo. The crude esters were dissolved in a mixture of THF and EtOH. NaBH_4 (4 equiv) and LiCl (4 equiv) were added at 0 °C, and the mixtures were stirred overnight at room temperature. After evaporation of the solvents, the residues were taken off in water and extracted by EtOAc. The organic layers were washed, dried (Na_2SO_4), and evaporated in vacuo. The crude alcohols were purified by chromatography on silica gel using EtOAc/hexane = 1/4 as eluents.

2a ($\text{R}_1 = \text{CH}_2\text{Ph}$): colorless solid, 3.9 g (89%), mp 59–60 °C, R_f (A) 0.47; ^1H NMR (DMSO) δ 2.6 and 2.95 (CH_2Ph), 2.70 ($\text{CH}(\text{S})$), 3.30–3.45 (CH_2O), 3.55 (CH_2S), 3.68 (CH_3O), 4.8 (OH), 6.75–7.15 (Ph + ((*p*-MeO)Ph)). Anal. ($\text{C}_{17}\text{H}_{20}\text{O}_2\text{S}$) C, H, S.

2b ($\text{R}_1 = (\text{CH}_2)_2\text{Ph}$): colorless oil, 1.27 g (67%), R_f (A) 0.5; ^1H NMR (DMSO) δ 1.5 and 1.9 ($\text{CH}_2\beta$), 2.49–2.80 ($\text{CH}_2\text{Ph} + \text{CH}(\text{S})$), 3.42–3.52 (CH_2O), 3.68 (CH_2S), 3.72 (CH_3O), 4.75 (OH), 6.80–7.15 (Ph + ((*p*-MeO)Ph)). Anal. ($\text{C}_{18}\text{H}_{22}\text{O}_2\text{S}$) C, H, S.

2c ($\text{R}_1 = \text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_3$): colorless oil, 4.1 g (83%), R_f (A) 0.61; ^1H NMR (DMSO) δ 0.65 (2 CH_3), 1.2 ($\text{CH}_2(\text{CH}_3)$), 1.72 (CHCH_3), 2.52 ($\text{CH}(\text{S})$), 3.35–3.50 (CH_2O), 3.62 (CH_2S), 3.68 (CH_3O), 4.68 (OH), 6.8–7.2 ((*p*-MeO)Ph)). Anal. ($\text{C}_{14}\text{H}_{22}\text{O}_2\text{S}$) C, H, S.

General Procedure for the Preparation of Compounds 3.

Procedure III. To a solution of alcohol 2 in MeCN at 0 °C under argon was added NaI (1 equiv) followed by dropwise addition of chlorotrimethylsilane (1.2 equiv) over 5 min. The mixture was stirred for 90 min, poured into water, and extracted with ether. The organic layer was washed with 5% aqueous $\text{Na}_2\text{S}_2\text{O}_3$ and brine, dried (Na_2SO_4), and evaporated to give the crude derivative. This compound in 1,2-dimethoxyethane at 0 °C was added to a solution of the sodium salt of dimethyl malonate, prepared from dimethyl malonate (1.5 equiv) and NaH (1.5 equiv) in 1,2-dimethoxyethane at 0 °C under argon for 10 min. The mixture was stirred for 40 h at room temperature, evaporated, and partitioned between water and EtOAc. The organic phase was washed with H_2O and brine, dried (Na_2SO_4), evaporated, and purified by flash column chromatography (EtOAc/hexane 1/7).

3a ($\text{R}_1 = \text{CH}_2\text{Ph}$): colorless solid, 4.4 g (83%), mp 64–65 °C, R_f (B) 0.29; ^1H NMR (DMSO) δ 1.7 and 2.0 (CH_2), 2.61 ($\text{CH}(\text{S})$), 2.71–2.82 (CH_2Ph), 3.45–3.65 ($\text{CH}_3\text{O} + \text{CH}_2\text{S} + \text{CH}(\text{malonate})$), 6.8–7.15 (Ph + ((*p*-MeO)Ph)).

3b ($\text{R}_1 = (\text{CH}_2)_2\text{Ph}$): colorless oil, 1.4 g (80%), R_f (B) 0.26; ^1H NMR (DMSO) δ 1.60–1.72 ($\text{CH}_2\beta$), 1.90–2.10 (($\text{CH} + \text{CH}_2\text{CH}$)), 2.38 ($\text{CH}(\text{S})$), 2.50–2.60 (CH_2Ph), 3.49–3.69 ($\text{CH}_3\text{O} + \text{CH}_2\text{S} + \text{CH}(\text{malonate})$), 6.80–7.12 (Ph + ((*p*-MeO)Ph)).

3c ($\text{R}_1 = \text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_3$): colorless oil, 4.0 g (79%), R_f (B) 0.36; ^1H NMR (DMSO) δ 0.65–0.79 (2 CH_3), 1.10–1.35 ($\text{CH}_2(\text{CH}_3)$), 1.55 ($\text{CH}(\text{CH}_3)$), 1.76–2.08 (CH_2), 2.31 ($\text{CH}(\text{S})$), 3.55–3.69 ($\text{CH}_3\text{O} + \text{CH}_2\text{S} + \text{CH}(\text{malonate})$), 6.82 and 7.12 (Ph + ((*p*-MeO)Ph)).

General Procedure for the Preparation of Compounds 4.

Procedure IV. To HNa (1 equiv) in 1,2-dimethoxyethane at 0 °C under argon was added compound 3 (1 equiv). After the mixture was stirred for 15 min at 0 °C, a solution of R_2X (1.3 equiv) in 1,2-dimethoxyethane was added. Stirring was continued at room temperature for 18 h, the solvent was removed, and the residue was diluted with EtOAc and washed with H_2O and brine. After drying (Na_2SO_4), the solvent was removed at reduced pressure and the residue chromatographed (EtOAc/hexane 1/8).

4a ($\text{R}_1 = \text{R}_2 = \text{CH}_2\text{Ph}$): colorless solid, 4.5 g (85%), mp 96–97 °C, R_f (B) 0.35; ^1H NMR (DMSO) δ 1.86 (CH_2), 2.62 ($\text{CH}(\text{S})$), 2.82–3.10 (2 CH_2Ph), 3.48–3.62 ($\text{CH}_3\text{O} + \text{CH}_2\text{S}$), 6.62–7.15 (Ph + ((*p*-OCH₃)Ph)).

4b ($\text{R}_1 = \text{CH}_2\text{Ph}$, $\text{R}_2 = \text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_3$): colorless oil, 2.8 g (56.7%), R_f (B) 0.42; ^1H NMR (DMSO) δ 0.41–0.70 (2 CH_3), 0.75–0.98 ($\text{CH}_2(\text{CH}_3)$), 1.6 ($\text{CH}(\text{CH}_3)$), 1.89 and 2.15 (CH_2), 2.6 (CH_2Ph), 2.80 ($\text{CH}(\text{S})$), 3.45–3.62 ($\text{CH}_3\text{O} + \text{CH}_2\text{S}$), 6.75–7.12 (Ph + ((*p*-MeO)Ph)).

4c ($\text{R}_1 = (\text{CH}_2)_2\text{Ph}$, $\text{R}_2 = \text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_3$): colorless oil, 0.51 g (89%), R_f (B) 0.39; ^1H NMR (DMSO) δ 0.75 (2 CH_3), 0.90–1.40 ($\text{CH}_2(\text{CH}_3) + \text{CH}(\text{CH}_3)$), 1.65 ($\text{CH}(\text{S}) + \text{CH}_2\beta$), 1.92–2.15 (CH_2), 2.60 (CH_2Ph)), 3.50–3.68 ($\text{CH}_3\text{O} + \text{CH}_2\text{S}$), 6.8–7.18 (Ph + ((*p*-MeO)Ph)).

4d ($\text{R}_1 = \text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_3$, $\text{R}_2 = \text{CH}_2\text{Ph}$): colorless oil, 1.75 g (83%), R_f (B) 0.4; ^1H NMR (DMSO) δ 0.65–0.78 (2 CH_3), 1.02–1.40 ($\text{CH}_2(\text{CH}_3) + \text{CH}(\text{CH}_3)$), 1.80 (CH_2), 2.72 ($\text{CH}(\text{S}) + \text{CH}_2\text{Ph}$), 3.54–3.68 ($\text{CH}_3\text{O} + \text{CH}_2\text{S}$), 6.82–7.20 (Ph + ((*p*-OMe)Ph)).

4e ($\text{R}_1 = \text{R}_2 = \text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_3$): colorless oil, 1.2 g (52%), R_f (B) 0.54; ^1H NMR (DMSO) δ 0.60–0.80 (4 CH_3), 0.85–1.80 (2 $\text{CH}_2(\text{CH}_3) + 2\text{CH}(\text{CH}_3)$), 1.8 and 2.15 (CH_2), 2.55 ($\text{CH}(\text{S})$), 3.52–3.65 ($\text{CH}_3\text{O} + \text{CH}_2\text{S}$), 6.78 and 7.1 ((*p*-MeO)Ph).

General Procedure for the Preparation of Compounds 5.

Procedure V. A solution of 4 in DMSO was treated with 20% KOH (3 equiv), and the mixture was stirred for 90 min at 60–70 °C. The mixture was diluted into H_2O and washed with ether. The aqueous layer was acidified with 3 N HCl to pH 3 and extracted with EtOAc. The organic layer was washed with H_2O and brine, dried (Na_2SO_4), evaporated, and purified by flash column chromatography (EtOAc/hexane 3/2).

5a ($\text{R}_1 = \text{R}_2 = \text{CH}_2\text{Ph}$): colorless oil, 2.8 g (77%), R_f (C) 0.53; ^1H NMR (DMSO) δ 1.40–1.72 (CH_2), 2.5–2.82 (2 CH_2CH_2), 3.45 (CH_2S), 3.68 (CH_3O), 6.8–7.12 (2Ph + ((*p*-MeO)Ph)), 12.12 (COOH). Anal. ($\text{C}_{28}\text{H}_{30}\text{O}_3\text{S}$) C, H, S.

5b ($\text{R}_1 = \text{CH}_2\text{Ph}$, $\text{R}_2 = \text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_3$): colorless oil, 0.72 g (56%), R_f (C) 0.5; ^1H NMR (DMSO) δ 0.55–0.68 (2 CH_3), 1.00–1.31 ($\text{CH}(\text{CH}_3) + \text{CH}_2(\text{CH}_3)$), 1.52–1.68 (CH_2), 2.25 ($\text{CH}(\text{COOH})$), 2.60–2.78 (S-CH-CH₂Ph), 3.5 (CH_2S), 3.68 (CH_3O), 6.80–7.10 (Ph + ((*p*-MeO)Ph)), 11.98 (COOH). Anal. ($\text{C}_{28}\text{H}_{30}\text{O}_3\text{S}$) C, H, S.

5c ($\text{R}_1 = (\text{CH}_2)_2\text{Ph}$, $\text{R}_2 = \text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_3$): colorless oil, 0.28 g (70%), R_f (C) 0.51; ^1H NMR (DMSO) δ 0.75–0.78 (2 CH_3), 0.80–1.90 ($\text{CH}_2\beta + \text{CH}_2 + \text{CH}(\text{CH}_3) + \text{CH}_2(\text{CH}_3)$), 2.18 ($\text{CH}(\text{COOH})$), 2.4 ($\text{CH}(\text{S})$), 2.60 (CH_2Ph), 3.50–3.60 ($\text{CH}_3\text{O} + \text{CH}_2\text{S}$), 6.8–7.10 (Ph + ((*p*-MeO)Ph)), 12.5 (COOH). Anal. ($\text{C}_{24}\text{H}_{32}\text{O}_3\text{S}$) C, H, S.

5d ($\text{R}_1 = \text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_3$, $\text{R}_2 = \text{CH}_2\text{Ph}$): colorless oil, 1.05 g (78%), R_f (C) 0.6; ^1H NMR (DMSO) 0.60–0.72 (2 CH_3), 0.80–1.90 ($\text{CH}_2 + \text{CH}(\text{CH}_3) + \text{CH}_2(\text{CH}_3)$), 2.4 (CH-COOH), 2.6–2.8 (S-CH + CH₂Ph), 3.54–3.68 ($\text{CH}_3\text{O} + \text{CH}_2\text{S}$), 6.80–7.15 (Ph + ((*p*-MeO)Ph)), 12.2 (COOH). Anal. ($\text{C}_{20}\text{H}_{30}\text{O}_3\text{S}$) C, H, S.

5e ($\text{R}_1 = \text{R}_2 = \text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_3$): colorless oil, 0.72 g (78.5%), R_f (C) 0.7; ^1H NMR (DMSO) 0.55–0.75 (4 CH_3), 1.08–1.78 (2 $\text{CH}(\text{CH}_3) + 2\text{CH}_2(\text{CH}_3) + \text{CH}_2$), 2.15 ($\text{CH}(\text{COOH})$), 2.35 ($\text{CH}(\text{S})$), 3.48–3.65 ($\text{CH}_3\text{O} + \text{CH}_2\text{S}$), 6.8–7.12 ((*p*-MeO)Ph), 12.15 (COOH). Anal. ($\text{C}_{20}\text{H}_{30}\text{O}_3\text{S}$) C, H, S.

General Procedure for the Preparation of Compounds 6. **Procedure VI.** To the acid 5 was added trifluoroacetic acid (10 equiv). The resulting solution was stirred at 90 °C for 60 min. The solvent was removed at reduced pressure, and the residue was washed with ether, evaporated, and chromatographed (EtOAc/hexane 1/8).

6a ($R_1 = R_2 = \text{CH}_2\text{Ph}$): colorless oil, 1.2 g (92%), R_f (B) 0.7; ^1H NMR (DMSO) δ 1.95–2.18 (CH_2), 2.55–2.95 ($\text{CH}_2(\text{Ph})$), 3.05 (H_2), 4.10 (H_4), 7.00–7.18 (Ph). Anal. ($\text{C}_{18}\text{H}_{18}\text{OS}$) C, H, S.

6b ($R_1 = \text{CH}_2\text{Ph}$, $R_2 = \text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_3$): colorless oil, 0.37 g (83%), R_f (B) 0.74; ^1H NMR (DMSO) δ 0.70–0.85 (2 CH_3), 1.15 (CH_2CH_3), 1.80 (CHCH_3), 2.15–2.22 (CH_2), 2.78 (H_2), 2.91–3.05 ($\text{CH}_2(\text{Ph})$), 4.05 (H_4), 7.2 (Ph). Anal. ($\text{C}_{15}\text{H}_{20}\text{OS}$) C, H, S.

6c ($R_1 = (\text{CH}_2)_2\text{Ph}$, $R_2 = \text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_3$): colorless oil, 90 mg (51%), R_f (B) 0.77; ^1H NMR (DMSO) δ 0.70–0.82 (2 CH_3), 1.15 (CH_2CH_3), 1.58 (CHCH_3), 1.80, 2.00, and 2.55 (CH_2CH_2 –Ph), 2.15–2.30 (CH_2), 2.65 (H_2), 3.70 (H_4), 7.10 (Ph). Anal. ($\text{C}_{16}\text{H}_{22}\text{OS}$) C, H, S.

6d ($R_1 = \text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_3$, $R_2 = \text{CH}_2\text{Ph}$): colorless oil, 0.652 g (94%), R_f (B) 0.74; ^1H NMR (DMSO) 0.78–0.85 (2 CH_3), 1.08–1.48 ($\text{CH}_2(\text{CH}_3)$), 1.58 ($\text{CH}(\text{CH}_3)$), 1.90–2.12 (CH_2), 2.60–2.90 (CH_2 –Ph), 3.10 (H_2), 3.8 (H_4), 7.18 (Ph). Anal. ($\text{C}_{15}\text{H}_{20}\text{OS}$) C, H, S.

6e ($R_1 = R_2 = \text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_3$): colorless oil, 0.31 g (85%), R_f (B) 0.9; ^1H NMR (DMSO) δ 0.7–0.82 (4 CH_3), 1.18–1.45 (2 CH_2 – CH_3), 1.60–1.78 (2 $\text{CH}(\text{CH}_3)$), 2.03–2.25 (CH_2), 2.72 (H_2), 3.75 (H_4). Anal. ($\text{C}_{12}\text{H}_{22}\text{OS}$) C, H, S.

General Procedure for the Preparation of Compounds 7. **Procedure VII.** To a solution of thiobutyrolactone 6 in EtOH was added 4 N NaOH (4 equiv), and the reaction mixture was stirred at room temperature under O₂ for 24 h. The solvents were evaporated, and the residue was diluted in H₂O and washed with hexane. The aqueous phase was acidified with 2 N HCl to pH 3, and the product was extracted with EtOAc. The organic phase was washed with H₂O and brine, dried (Na₂SO₄), and evaporated in vacuo.

7a ($R_1 = R_2 = \text{CH}_2\text{Ph}$): colorless oil, 0.95 g (69%), R_f (D) 0.26; ^1H NMR (DMSO) δ 1.50–1.80 (CH_2), 2.60–2.90 ($\text{CH}_2\text{CH}_2(\text{Ph})$), 6.90–7.10 (Ph), 12.23 (COOH). Anal. ($\text{C}_{23}\text{H}_{38}\text{O}_4\text{S}_2$) C, H, S.

7b ($R_1 = \text{CH}_2\text{Ph}$, $R_2 = \text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_3$): colorless oil, 0.7 g (94%), R_f (D) 0.67; ^1H NMR (DMSO) δ 0.75 (2 CH_3), 0.95–1.25 ($\text{CH}_2(\text{CH}_3)$), 1.4 ($\text{CH}(\text{CH}_3)$), 1.55–1.75 (CH_2), 2.55 ($\text{CH}(\text{COOH})$), 2.6–2.8 ($\text{CHCH}_2(\text{Ph})$), 7.08–7.15 (Ph), 12.11 (COOH). Anal. ($\text{C}_{30}\text{H}_{42}\text{O}_4\text{S}_2$) C, H, S.

7c ($R_1 = (\text{CH}_2)_2\text{Ph}$, $R_2 = \text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_3$): colorless oil, 40 mg (47%), R_f (D) 0.3; ^1H NMR (DMSO) δ 0.68–0.71 (2 CH_3); 1.02–1.30 ($\text{CH}_2(\text{CH})$), 1.51–1.85 ($\text{CH}_2(\text{CH}_3) + \text{CH}(\text{CH}_3) + \text{CH}_2$), 2.20 ($\text{CH}(\text{COOH}) + \text{CH}_2(\text{Ph})$), 2.61 ($\text{CH}(\text{S})$), 7.0–7.10 (Ph), 12.1 (COOH). Anal. ($\text{C}_{32}\text{H}_{46}\text{O}_4\text{S}_2$) C, H, S.

7d ($R_1 = \text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_3$, $R_2 = \text{CH}_2\text{Ph}$): colorless oil, 0.4 g (63%), R_f (D) 0.55; ^1H NMR (DMSO) δ 0.68–0.78 (2 CH_3), 1.00–1.18 ($\text{CH}_2(\text{CH}_3)$), 1.48 ($\text{CH}(\text{CH}_3)$), 1.68–1.82 (CH_2), 2.60–2.78 ($\text{CHCH}_2(\text{Ph}) + \text{CH}(\text{S})$), 7.15 (Ph), 12.20 (COOH). Anal. ($\text{C}_{30}\text{H}_{42}\text{O}_4\text{S}_2$) C, H, S.

7e ($R_1 = R_2 = \text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_3$): colorless oil, 0.21 g (72%), R_f (D) 0.6; ^1H NMR (DMSO) δ 0.70–0.85 (4 CH_3), 1.10–1.75 (2 CH_2 – CH_3 + 2 $\text{CH}(\text{CH}_3)$ + CH_2), 2.20 ($\text{CH}(\text{COOH})$), 2.52 ($\text{CH}(\text{S})$), 12.15 (COOH). Anal. ($\text{C}_{24}\text{H}_{48}\text{O}_4\text{S}_2$) C, H, S.

General Method for Amide Bond Formation. Procedure VIII. To a stirred solution of acids 7a–e (0.1–0.5 mM) in THF (1–5 mL) were added successively BOP reagent (2 equiv), DIEA (4 equiv), and the corresponding α -amino acid methyl ester (or amide) hydrochloride (2 equiv) at 0 °C. After the mixture was stirred at room temperature for 2–3 h, the solvent was removed and the residue was diluted with EtOAc and H₂O. The organic phase was washed with H₂O, saturated citric acid, saturated NaHCO₃, H₂O, and brine. After drying (Na₂SO₄), concentration of the solvent provided an oil, which was purified by chromatography as specified in each case.

General Method for Deprotection. Procedure IX. To a solution of the protected compounds 8 (0.1–0.5 mM) in MeOH (1–5 mL) was added 1 N NaOH (3–4 equiv) at 0 °C, and the mixture was stirred at room temperature for 3–9 h. After evaporation of MeOH, the remaining mixture was diluted with water and washed with ether. The aqueous phase was acidified with 2 N HCl to pH 3 and extracted with CHCl₃. The organic

layer was dried (Na₂SO₄) and evaporated to dryness to afford the final compounds, which were washed with ether and dried under vacuum.

***N,N'*-[4,4'-Dithiobis(1-oxo-2,4-dibenzylbutyl)]bis-L-alanine** (19): white solid, 0.149 g (87%), mp 97–98 °C, R_f (E) 0.4; ^1H NMR (DMSO) δ 1.00–1.18 ($\text{CH}_3(\text{Ala})$), 1.4, 1.8 (CH_2), 2.50–2.90 (2 $\text{CH}(\text{CH}_2\text{Ph})$), 4.10 ($\text{CH}_\alpha(\text{Ala})$), 6.95–7.10 (Ph), 8.0–8.3 (NH), 12.4 (COOH); HPLC t_R 11.3 min, eluent CH₃CN/TFA 0.05% (55/45). Anal. ($\text{C}_{42}\text{H}_{48}\text{N}_2\text{O}_6\text{S}_2$) C, H, N, S.

***N,N'*-[4,4'-Dithiobis(1-oxo-2,4-dibenzylbutyl)]bis- β -alanine** (20): white solid, 0.16 g (83%), mp 60–61 °C, R_f (E) 0.38; ^1H NMR (DMSO) δ 1.40, 1.78 (CH_2), 2.18 (CH_2COOH), 2.55–2.85 (2 $\text{CH}(\text{CH}_2\text{Ph})$), 3.1 ($\text{CH}_2(\text{NH})$), 6.98–7.1 (Ph), 7.78–7.98 (NH), 12.1 (COOH); HPLC t_R 9.7, 10.0, 10.2 min, eluent CH₃CN/TFA 0.05% (55/45). Anal. ($\text{C}_{42}\text{H}_{48}\text{N}_2\text{O}_6\text{S}_2$) C, H, N, S.

***N,N'*-[4,4'-Dithiobis(1-oxo-2,4-dibenzylbutyl)]bis-L-proline** (21): white solid, 0.15 g (77%), mp 99–100 °C, R_f (E) 0.35; ^1H NMR (DMSO) δ 1.50–2.00 ($\text{CH}_2\text{Pro} + \text{CH}_2\gamma\text{Pro} + \text{CH}_2$), 2.55–3.10 (2 $\text{CH}(\text{CH}_2\text{Ph})$), 3.20–3.30 (CH_2Pro), 4.10–4.18 ($\text{H}_\alpha\text{Pro}$), 7.00–7.15 (Ph), 12.2 (COOH); HPLC t_R 12.3, 13.0 min, eluent CH₃CN/TFA 0.05% (55/45). Anal. ($\text{C}_{46}\text{H}_{52}\text{N}_2\text{O}_6\text{S}_2$) C, H, N, S.

***N,N'*-[4,4'-Dithiobis(1-oxo-2,4-dibenzylbutyl)]bis-L-tyrosine** (22): white solid, 0.8 g (87%), mp 100–102 °C, R_f (D) 0.33; ^1H NMR (DMSO) 1.3–1.7 (CH_2), 2.35–2.8 (2 $\text{CH}(\text{CH}_2\text{Ph}) + \text{CH}_2\beta(\text{Tyr})$), 4.3 ($\text{CH}_\alpha(\text{Tyr})$), 6.5–7.1 (Ph + Tyr), 8.0–8.30 (NH), 9.1 (OH), 12.4 (COOH); HPLC t_R 10.5, 10.9 min, eluent CH₃CN/TFA 0.05% (55/45). Anal. ($\text{C}_{54}\text{H}_{56}\text{N}_2\text{O}_8\text{S}_2$) C, H, N, S.

***N,N'*-[4,4'-Dithiobis(1-oxo-2-isobutyl-4-benzylbutyl)]bis-L-tyrosine** (23): white solid, 0.13 g (84%), mp 99–100 °C, R_f (F) 0.22; ^1H NMR (DMSO) δ 0.48–0.70 (2 CH_3), 0.90–1.30 ($\text{CH}_2(\text{CH}_3) + \text{CH}(\text{CH}_3)$), 1.40–1.80 (CH₂), 2.28 (CH-CO), 2.50–2.85 (2 $\text{CH}(\text{CH}_2\text{Ph}) + \text{CH}_2\beta(\text{Tyr})$), 4.4 ($\text{CH}_\alpha(\text{Tyr})$), 6.55–7.18 (Ph + Tyr), 8.0–8.35 (NH), 9.12 (OH), 12.58 (COOH); HPLC t_R 10.2, 10.8 min, eluent CH₃CN/TFA 0.05% (55/45). Anal. ($\text{C}_{48}\text{H}_{60}\text{N}_2\text{O}_8\text{S}_2$) C, H, N, S.

***N,N'*-[4,4'-Dithiobis(1-oxo-2-isobutyl-4-benzylbutyl)]bis-L-proline** (24): white solid, 20 mg (53%), R_f (E) 0.23; ^1H NMR (DMSO) δ 0.71–0.85 (2 CH_3), 1.05, 1.49 ($\text{CH}_2(\text{CH}_3) + \text{CH}(\text{CH}_3)$), 1.50–1.80 (CH₂), 1.85–2.11 ($\text{CH}_2\beta + \text{CH}_2\gamma\text{Pro}$), 2.62–2.88 (2 $\text{CH}(\text{CH}_2\text{Ph}) + \text{CH}_2\text{CO}$), 3.05–3.40 ($\text{CH}_2\delta\text{Pro}$), 4.25 ($\text{CH}_\alpha\text{Pro}$), 7.10–7.20 (Ph), 12.5 (COOH); HPLC t_R 12.9, 13.6 min, eluent CH₃CN/TFA 0.05% (55/45). Anal. ($\text{C}_{40}\text{H}_{56}\text{N}_2\text{O}_6\text{S}_2$) C, H, N, S.

***N,N'*-[4,4'-Dithiobis(1-oxo-2-isobutyl-4-phenethylbutyl)]bis-L-tyrosine** (25): white solid, 20 mg (75%), mp 95–96 °C, R_f (E) 0.23; ^1H NMR (DMSO) δ 0.55–0.75 (2 CH_3), 1.0–1.15 ($\text{CH}_2(\text{CH}_3) + \text{CH}(\text{CH}_3)$), 1.41–1.70 (CH₂ + CH₂(CH)), 1.90–2.30 (2 $\text{CH}(\text{Ph})$), 2.71, 2.90 (CHS + CHCO + CH₂ β -Tyr), 4.41 ($\text{CH}_\alpha(\text{Tyr})$, 6.58–7.18 (Ph + Tyr), 8.00–8.31 (NH), 9.08 (OH), 12.45 (COOH); HPLC t_R 12.2, 12.8 min, eluent CH₃CN/TFA 0.05% (55/45). Anal. ($\text{C}_{52}\text{H}_{64}\text{N}_2\text{O}_8\text{S}_2$) C, H, N, S.

***N,N'*-[4,4'-Dithiobis(1-oxo-2,4-diisobutylbutyl)]bis-L-tyrosine** (26): white solid, 43 mg (91%), mp 105–106 °C, R_f (E) 0.17; ^1H NMR (DMSO) δ 0.62–0.82 (4 CH_3), 1.0 (2 $\text{CH}_2(\text{CH}_3) + 2\text{CH}(\text{CH}_3)$), 1.85–2.25 (CH₂), 2.70–2.90 (CHS + CHCO + CH₂ β -Tyr), 4.30–4.50 ($\text{CH}_\alpha(\text{Tyr})$, 6.6–7.0 (Tyr), 8.0–8.10 (NH), 9.1 (OH), 12.4 (COOH); HPLC t_R 9.7, 10.8 min, eluent CH₃CN/TFA 0.05% (55/45). Anal. ($\text{C}_{42}\text{H}_{48}\text{N}_2\text{O}_8\text{S}_2$) C, H, N, S.

***N,N'*-[4,4'-Dithiobis(1-oxo-2-benzyl-4-isobutylbutyl)]bis-L-tyrosine** (27S): white solid, 0.125 g (85%), mp 103–104 °C, R_f (E) 0.26; ^1H NMR (DMSO) δ 0.51–0.71 (2 CH_3), 0.95 ($\text{CH}_2(\text{CH}_3) + \text{CH}(\text{CH}_3)$), 1.70–1.90 (CH₂), 2.38–2.80 (2 $\text{CH}(\text{CH}_2\text{Ph}) + \text{CHS} + \text{CH}_2\beta(\text{Tyr})$), 4.3 ($\text{CH}_\alpha(\text{Tyr})$, 6.5–7.1 (Ph + Tyr), 8.08–8.22 (NH), 9.1 (OH), 12.4 (COOH); HPLC t_R 9.7, 10.6, 11.6 min, eluent CH₃CN/TFA 0.05% (55/45). Anal. ($\text{C}_{48}\text{H}_{60}\text{N}_2\text{O}_8\text{S}_2$) C, H, N, S.

***N,N'*-[4,4'-Dithiobis(1-oxo-2-benzyl-4-isobutylbutyl)]bis-D-tyrosine** (27R): white solid, 0.153 g (90%), mp 173 °C, R_f (E) 0.25; ^1H NMR (DMSO) δ 0.55–0.81 (2 CH_3), 1.00–1.90 ($\text{CH}_2(\text{CH}_3) + \text{CH}(\text{CH}_3) + \text{CH}_2$), 2.30–2.90 (2 $\text{CH}(\text{CH}_2\text{Ph}) + \text{CHS} + \text{CH}_2\beta(\text{Tyr})$), 4.25–4.45 ($\text{CH}_\alpha(\text{Tyr})$, 6.50–7.20 (Ph + Tyr), 8.00–8.03 (NH), 9.1 (OH), 12.5 (COOH); HPLC t_R 9.8, 10.9, 11.5 min, eluent CH₃CN/TFA 0.05% (55/45). Anal. ($\text{C}_{48}\text{H}_{60}\text{N}_2\text{O}_8\text{S}_2$) C, H, N, S.

***N,N'*-[4,4'-Dithiobis(1-oxo-2,4-diisobutylbutyl)]bis-L-tyrosine amide** (17): white solid, 0.33 g (73%), mp 113–114 °C, R_f (F) 0.22; ^1H NMR (DMSO) δ 0.55–0.72 (2 CH_3), 1.02–1.48 ($\text{CH}_2(\text{CH}_3) + \text{CH}(\text{CH}_3)$), 1.68–1.85 (CH₂), 2.32–2.78 (CHS + 2 $\text{CH}(\text{CH}_2\text{Ph}) + \text{CH}_2\beta(\text{Tyr})$), 4.3 ($\text{CH}_\alpha(\text{Tyr})$, 6.5–7.1 (Ph + Tyr), 8.00–8.03 (NH), 9.1 (OH), 12.5 (COOH); HPLC t_R 9.8, 10.9, 11.5 min, eluent CH₃CN/TFA 0.05% (55/45). Anal. ($\text{C}_{48}\text{H}_{60}\text{N}_2\text{O}_8\text{S}_2$) C, H, N, S.

(Ph) + $CH_2\beta$ -Tyr), 4.31–4.39 ($CH\alpha$ -Tyr), 6.52–6.85 (Tyr), 7.20 (Ph + CONH₂), 8.0–8.08 (NH), 9.08 (OH). Anal. (C₄₈H₆₂N₄O₆S₂) C, H, N, S.

N,N'-[4,4'-Dithiobis(1-oxo-2-benzyl-4-isobutylbutyl)]bis-L-tyrosine amide (18): white solid, 0.33 g (73%), mp 113–114 °C, R_f (F) 0.22; ¹H NMR (DMSO) δ 0.55–0.72 (2CH₃), 1.02–1.48 (CH₂(CH₃) + CH(CH₃)), 1.68–1.85 (CH₂), 2.32–2.78 (CHS + CH-CH₂(Ph) + CH₂ β -Tyr), 4.31–4.39 ($CH\alpha$ -Tyr), 6.52–6.85 (Tyr), 7.20 (Ph + CONH₂), 8.0–8.08 (NH), 9.08 (OH). Anal. (C₄₈H₆₂N₄O₆S₂) C, H, N, S.

Ethyl 2-Benzyl-4-phenyl-2-butenoate (28) and Ethyl 2-Benzyl-4-phenyl-3-butenoate (29): To a solution of triethyl 2-benzyl phosphonoacetate in dry dimethoxyethane was added, at 0 °C, 1 equiv of NaH. After the mixture was stirred for 15 min, 3 equiv of phenylacetaldehyde was added and the mixture was stirred at reflux temperature for 3 h. Removal of the solvent gave a residue dissolved in hexane, washed with H₂O and brine, dried (Na₂SO₄), and evaporated. The oily residue was purified by flash chromatography in EtOAc/hexane 1/10: colorless oil (72%); ¹H NMR 28 isomer *E* δ 1.02 (CH₃), 3.55 (CH₂Ph), 3.70 (CH₂Ph), 3.95 (CH₂O), 6.85 (CH=), 7.12 (Ph), isomer *Z* δ 1.02 (CH₃), 3.50 (CH₂Ph), 3.65 (CH₂Ph), 3.95 (CH₂O), 6.05 (CH=), 7.12 (Ph), 29 δ 1.02 (CH₃), 2.8, 3.0 (CH₂Ph), 3.40 (CH(COOEt)), 3.95 (CH₂O), 6.15, 6.35 (-CH=CH-), 7.12 (Ph).

2-Benzyl-4-phenyl-3-butanoic Acid (30): A solution of the mixture 28 and 29 in EtOH was treated with 2 equiv of 1 N NaOH for 3 h at room temperature. After evaporation of the solvent, the residue was taken off with water, acidified to pH 1, and extracted with EtOAc. A colorless oil (85%) was obtained. ¹H NMR (DMSO): δ 2.90, 3.12 (CH₂Ph), 3.45 (CH(COOH)), 6.39–6.48 (-CH=CH-), 7.16–7.35 (Ph), 12.38 (COOH). R_f (E): 0.6.

4-(Acetylthio)-2-benzylbutanoic Acid (31): The β - γ unsaturated acid was dissolved in 10–12 equiv of thiolacetic acid, and the mixture was stirred for 48 h at reflux temperature and evaporated. The residue was purified by flash chromatography (EtOAc/hexane 1/1). The title compound was obtained as a nearly colorless oil (44%); ¹H NMR (DMSO) δ 2.22 (CH₃(CO)), 2.70–3.00 (CH₂-CH-CH₂), 3.70 and 3.88 (CH(Ph)), 6.90–7.10 (Ph), 12.45 (COOH); R_f (C) 0.35.

N-(2-Benzyl-4-mercaptop-4-phenyl-1-oxobutyl)-L-alanine (32): The title compound was prepared, according to procedures VIII and IX, as a colorless oil (62% (ester), 86%); ¹H NMR (DMSO) δ 0.98–1.22 (CH₃(Ala)), 2.30 (HS), 2.58–2.90 (CH₂CHCH₂), 3.15 (CH(Ph)), 4.10 (CH₂(Ala)), 7.10–7.20 (Ph), 8.12–8.22 (NH), 12.42 (COOH); R_f (E) 0.30; t_R 8.6 min, CH₃CN/TFA 0.05% (50/50). Anal. (C₂₀H₂₃NO₃S) C, H, N, S.

Acknowledgment. We would like to thank Dr. P. Crine for a generous gift of mutated Glu¹⁰²-NEP and C. Dupuis for her assistance in preparing the manuscript. This work was supported by a grant from the Spanish Government (to I. Gomez-Monterrey) and by funds from the Institut National de la Santé et de la Recherche Médicale, the Centre National de la Recherche Scientifique, and the Université René Descartes.

References

- 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, 87–147.
- Maldonado, R.; Féger, J.; Fournié-Zaluski, M. C.; Roques, B. P. Differences in physical dependence induced by selective μ or δ opioid agonists and by endogenous enkephalins protected by peptidase inhibitors. *Brain Res.* 1990, 520, 247–254.
- Noble, F.; Turcaud, S.; Fournié-Zaluski, M. C.; Roques, B. P. Repeated systemic administration of the mixed inhibitor of enkephalin-degrading enzymes, RB 101, does not induce either antinociceptive tolerance or cross-tolerance with morphine. *Eur. J. Pharmacol.* 1992, 223, 83–89.
- Noble, F.; Coric, P.; Fournié-Zaluski, M. C.; Roques, B. P. Lack of physical dependence in mice after repeated systemic administration of the mixed inhibitor prodrug of enkephalin-degrading enzymes RB 101. *Eur. J. Pharmacol.* 1992, 223, 91–96.
- Sheldon, R. J.; Rivière, P. J. M.; Malarichik, M. E.; Mosberg, H. I.; Burks, T. F.; Porreca, F. Opioid regulation of mucosal ion transport in the mouse isolated jejunum. *J. Pharmacol. Exp. Ther.* 1990, 253, 144–153.
- Roques, B. P.; Fournié-Zaluski, M. C.; Soroca, E.; Lecomte, J. M.; Malfroy, B.; Llorens, C.; Schwartz, J. C. The enkephalinase inhibitor thiophorphan shows antinociceptive activity in mice. *Nature (London)* 1980, 288, 286–288.
- Marçais-Collado, H.; Uchida, G.; Costentin, J.; Schwartz, J. C.; Lecomte, J. M. Naloxone reversible antidiarrheal effects of enkephalinase inhibitors. *Eur. J. Pharmacol.* 1987, 144, 125–132.
- Larvol, L.; Baumer, P.; Berard, H.; Cerf, M.; Lecomte, J. M. Rôle des enképhalines endogènes dans la motricité duodénale prost-prandiale. *Gastroenterol. Clin. Biol.* 1992, 15, A142.
- Sybertz, E. J.; Chiu, P. J.; Watkins, R. W.; Vemulapalli, S. Neutral metallopeptidase inhibition: a novel means of circulatory modulation. *J. Hypertens.* 1990, 8, S161–S167.
- Pham, I.; Gonzalez, W.; El Amrani, A. I. K.; Fournié-Zaluski, M. C.; Philippe, M.; Laboulandié, I.; Roques, B. P.; Michel, J. B. Effects of converting enzyme inhibitor and neutral endopeptidase inhibitor in experimental hypertension. *J. Pharmacol. Exp. Ther.* 1993, 265, 1339–1347.
- Roques, B. P.; Beaumont, A. Neutral endopeptidase 24.11 inhibitors: from analgesics to antihypertensives? *Trends Pharmacol. Sci.* 1990, 11, 245–249.
- Devault, A.; Lazure, C.; Nault, C.; Le Moual, M.; Seidah, N. G.; Chretien, M.; Kahn, P.; Powell, J.; Mallet, J.; Beaumont, A.; Roques, B. P.; Crine, P.; Boileau, G. Amino acid sequence of rabbit kidney neutral endopeptidase 24.11 (enkephalinase) deduced from a complementary DNA. *EMBO J.* 1987, 6, 1317–1322.
- Malfroy, B.; Schofield, P. R.; Kuang, W. J.; Seeburg, P. H.; Mason, A. J.; Henzel, W. J. Molecular cloning and amino acid sequence of rat enkephalinase. *Biochem. Biophys. Res. Commun.* 1987, 144, 59–66.
- Malfroy, B.; Kuang, W. J.; Seeburg, P. H.; Mason, A. J.; Schofield, P. R. Molecular cloning and amino acid sequence of human enkephalinase (neutral endopeptidase). *FEBS Lett.* 1988, 229, 206–210.
- Matthews, B. W. Structural basis of the action of thermolysin and related zinc peptidases. *Acc. Chem. Res.* 1988, 21, 333–340.
- 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*; Brunfeld, K., Ed.; Scriptor: Copenhagen, 1981; pp 476–481.
- Llorens, C.; Gacel, G.; Swerts, J. P.; Perdrisot, R.; Fournié-Zaluski, M. C.; Schwartz, J. C.; Roques, B. P. Rational design of enkephalinase inhibitors: substrate specificity of enkephalinase studied from inhibitory potency of various dipeptides. *Biochem. Biophys. Res. Commun.* 1980, 96, 1710–1715.
- Pozsgay, M.; Michaud, C.; Orlowski, M. The active site of endopeptidase 24.11 substrate and inhibitor studies. *Biochem. Soc. Trans.* 1985, 13, 44–47.
- Hersh, L. B.; Morihara, K. Comparison of the subsite specificity of the mammalian neutral endopeptidase 24.11 (enkephalinase) to the bacterial neutral endopeptidase thermolysin. *J. Biol. Chem.* 1986, 261, 6433–6437.
- Benchetrit, T.; Bissey, V.; Mornon, J. P.; Devault, A.; Crine, P.; Roques, B. P. Primary structure homologies between two Zn-metallopeptidases, the neutral endopeptidase 24.11 enkephalinase, and the thermolysin through clustering. *Biochemistry* 1988, 27, 592–596.
- Devault, A.; Sales, V.; Nault, C.; Beaumont, A.; Roques, B. P.; Crine, P.; Boileau, G. Exploration of the catalytic site of endopeptidase 24.11 by site directed mutagenesis. Histidine residues 583 and 587 are essential for catalysis. *FEBS Lett.* 1988, 231, 54–58.
- Le Moual, H.; Devault, A.; Roques, B. P.; Crine, P.; Boileau, G. Identification of glutamic acid 546 as a zinc-coordinating residue in endopeptidase 24.11. *J. Biol. Chem.* 1991, 266, 15670–15674.
- Beaumont, A.; Roques, B. P. Presence of a histidine at the active site of the neutral endopeptidase 24.11. *Biochem. Biophys. Res. Commun.* 1986, 139, 733–739.
- Kim, Y. A.; Shriver, B.; Hersh, L. B. Mutational analysis reveals only one catalytic histidine in neutral endopeptidase (enkephalinase). *Biochem. Biophys. Res. Commun.* 1992, 184, 883–887.
- Bateman, R. C., Jr.; Jackson, D.; Slaughter, C. A.; Unnithan, S.; Chai, Y. G.; Moomaw, C.; Hersh, L. B. Identification of the active site arginine in rat neutral endopeptidase 24.11 (enkephalinase) as arginine 102 and analysis of a glutamate 102 mutant. *J. Biol. Chem.* 1989, 264, 6151–6157.
- Beaumont, A.; Le Moual, H.; Boileau, G.; Crine, P.; Roques, B. P. Evidence that both arginine 102 and arginine 747 are involved in substrate binding to neutral endopeptidase (E.C. 3.4.24.11). *J. Biol. Chem.* 1991, 266, 214–220.
- Schechter, I.; Berger, A. On the size of the active site in proteases I Papain. *Biochem. Biophys. Res. Commun.* 1967, 27, 157–162.
- Benchetrit, T.; Fournié-Zaluski, M. C.; Roques, B. P. Relationships between the inhibitory potencies of thiophorphan and retrothiophorphan enantiomers on thermolysin and neutral endopeptidase 24.11 and their interactions with the thermolysin active site by computer modelling. *Biochem. Biophys. Res. Commun.* 1987, 147, 1034–1040.

(29) Monzingo, A. F.; Matthews, B. W. Binding of N-carboxymethyl dipeptide inhibitors to thermolysin determined by X-ray crystallography: a novel class of transition-state analogues for zinc peptidases. *Biochemistry* 1984, 23, 5724-5729.

(30) Haslanger, M. F.; Sybertz, E. J.; Neustadt, B. R.; Smith, E. M.; Nechuta, T. L.; Berger, J. Carboxyalkyl dipeptides with atrial natriuretic factor potentiating and anti-hypertensive activity. *J. Med. Chem.* 1989, 32, 737-739.

(31) Mumford, R. A.; Zimmerman, M.; TenBroeke, J.; Tank, D.; Joshua, H.; Rothrock, J. W.; Hirshfield, J. M.; Springer, J. P.; Patchett, A. A. Inhibition of porcine kidney enkephalinase by substituted N-carboxymethyl dipeptides. *Biochem. Biophys. Res. Commun.* 1982, 109, 1303-1309.

(32) Berger, J. A. A new approach to analgesia: synthesis and structure activity relationship of some potent and selective inhibitors of enkephalinase A. 19th National Medicinal Chemistry Symposium, Tucson, AZ, 1984.

(33) Chipkin, R. E. Inhibition of enkephalinase: the next generation of analgesics: Correlates in pharmacostucture. *Drugs Future* 1986, 11, 593-604.

(34) Danilewicz, J. C.; Barclay, P. L.; Barnish, I. T.; Brown, D.; Campbell, S. F.; James, K.; Samuels, G. M. R.; Terrett, N. K.; Wythes, M. J. UK-69,578, a novel inhibitor of EC 3.4.24.11 which increases endogenous ANF levels and its natriuretic and diuretic. *Biochem. Biophys. Res. Commun.* 1989, 164, 58-65.

(35) Patchett, A. A.; Harris, E.; Tristram, E. W.; Wyvatt, M. J.; Wu, M. T.; Taub, D.; Peterson, E. R.; Ikeler, T. J.; Ten Broeke, J.; Payne, L. G.; Ondeyka, D. L.; Thorsett, E. D.; Greenlee, W. J.; Lohr, N. S.; Hoffsommer, R. D.; Joshua, H.; Ruyle, W. V.; Rothrock, J. W.; Aster, S. D.; Maycock, A. L.; Robinson, F. M.; Hirschmann, R.; Sweet, C. S.; Ulm, E. H.; Gross, D. M.; Vassil, T. C.; Stone, C. A. A new class of angiotensin converting enzyme inhibitors. *Nature (London)* 1980, 288, 280-283.

(36) Gomez-Monterrey, I.; Turcaud, S.; Lucas, E.; Bruetschy, L.; Roques, B. P.; Fournié-Zaluski, M. C. Exploration of neutral endopeptidase active site by a series of new thiol containing inhibitors. *J. Med. Chem.* 1993, 36, 87-94.

(37) Fischer, E.; Schoeller, W. Derivate des 1-phenylalanin. *Justus Liebigs Ann. Chem.* 1907, 357, 1-24.

(38) Olah, G. A.; Narang, S. C.; Baleram Gupta, B. G.; Malhstra, R. Synthetic methods and reactions. 62. Transformations with chlorotrimethylsilane/sodium iodide, a convenient in situ iodo trimethylsilane reagent. *J. Org. Chem.* 1979, 44, 1247-1251.

(39) Akabori, S.; Sakakibara, S.; Shimonishi, Y.; Nobubara, Y. A new method for the protection of the sulphydryl group during peptide synthesis. *Bull. Chem. Soc. Jpn.* 1964, 37, 433-434.

(40) Ksander, G. M.; Diefenbacher, C. G.; Yuan, A. M.; Clark, F.; Sakane, Y.; Ghai, R. D. Enkephalinase inhibitors I-2,4-dibenzylglutaric acid derivatives. *J. Med. Chem.* 1989, 32, 2519-2526.

(41) Roderick, S. L.; Fournié-Zaluski, M. C.; Roques, B. P.; Matthews, B. W. Thiorphan and retro-thiorphan display equivalent interactions when bound to crystalline thermolysin. *Biochemistry* 1989, 28, 1493-1497.

(42) Fournié-Zaluski, M. C.; Lucas, E.; Waksman, G.; Roques, B. P. Differences in the structural requirements for selective interaction with neutral metallopeptidase (enkephalinase) or angiotensin-converting enzyme. Molecular investigation by use of new thiol inhibitors. *Eur. J. Biochem.* 1984, 139, 267-274.

(43) Fournié-Zaluski, M. C.; Perdrisot, R.; Gacel, G.; Swerts, J. P.; Roques, B. P.; Schwartz, J. C. Inhibitory potency of various peptides on enkephalinase activity from mouse striatum. *Biochem. Biophys. Res. Commun.* 1979, 91, 130-135.

(44) Petrillo, E. W.; Ondetti, M. A. Angiotensin converting enzyme inhibitors: medicinal chemistry and biological actions. *Med. Res. Rev.* 1982, 2, 1-41.

(45) Gordon, E. M.; Cushman, D. W.; Tung, R.; Cheung, H. 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. I), 113-116.

(46) Beaumont, A.; Barbe, B.; Le Moual, H.; Boileau, G.; Crine, P.; Fournié-Zaluski, M. C.; Roques, B. P. Charge polarity reversal inverses the specificity of neutral endopeptidase 24.11. *J. Biol. Chem.* 1992, 267, 2138-2141.

(47) Aubry, M.; Berthelot, A.; Beaumont, A.; Roques, B. P.; Crine, P. The use of a monoclonal antibody for the high yield purification of kidney enkephalinase solubilized in octylglucoside. *Biochem. Cell. Biol.* 1987, 65, 398-404.

(48) Soubrier, F.; Alhenc-Gelas, F.; Hubert, C.; Allegrini, J.; John, M.; Tregear, G.; Corvol, P. Two putative active site centers in human angiotensin I converting enzyme revealed by molecular cloning. *Proc. Natl. Acad. Sci. U.S.A.* 1988, 85, 9386-9390.

(49) Piquilloud, Y.; Reinhartz, A.; Roth, M. Studies of the angiotensin converting enzyme with different substrates. *Biochem. Biophys. Acta* 1970, 206, 136-142.

(50) Cheug, H. S.; Cushman, D. W. Inhibition of homogenous angiotensin converting enzyme of rabbit lung by synthetic venom peptides of bothrops jararaca. *Biochem. Biophys. Acta* 1973, 293, 451-457.