Exploration of Neutral Endopeptidase Active Site by a Series of New Thiol-Containing Inhibitors

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With the aim of characterizing the active site of the neutral endopeptidase [EC 3.4.24.11 (NEP)] and especially its putative S_1 subsite, two series of new thiol inhibitors designed to interact with the S_1, S'_1, and S'_2 subsites of the enzyme have been synthesized. These molecules correspond to the general formula $HSCH(R_1)CH(R_2)CONHCH(R_3)COOH$ (series I) and $HSCH(R_1)CH(R_2)$-$CONHCH(R_3)CONHCH(R_4)COOH$ (series II). Due to the synthetic pathway used, these inhibitors were obtained as mixtures of four stereoisomers. HPLC separation of the stereoisomers of 17 $HSCH[CH_2CH(CH_3)_2]CH(CH_2Ph)CONHCH(CH_3)COOH$ allowed the stereochemical dependence of the inhibitory potency to be determined. The most active isomer **17b** (IC_{50} = 3.6 nM) is assumed to have the *S,S,S* stereochemistry as deduced from both NMR and HPLC data. Although none of the inhibitors obtained were significantly more active than thiorphan, $HSCH_2CH(CH_2Ph)$-$CONHCH_2COOH$ (IC_{50} = 4 nM), which interacts only with the S'_1 and S'_2 subsites of NEP, their enhanced hydrophobicity is expected to improve their pharmacokinetic properties. All these compounds displayed low affinities for ACE (IC_{50}s > 1 μM). The determination of the IC_{50}s of two inhibitors of series II for NEP and for a mutated enzyme in which Arg^{102} was replaced by Glu^{102} allowed their mode of binding to the active site of NEP to be characterized. The R_2 and R_3 chains fit the S'_1–S'_2 subsites, while the R_4 group is probably located outside the active site. Taken together these results indicate that the R_1 chain of these inhibitors creates no additional stabilizing interactions with the active site of NEP. Two hypotheses may account for this: there is no hydrophobic S_1 subsite in NEP or the inhibitors have structures which are too constrained for optimized interactions with the active site.

Introduction

A physiological role for neutral endopeptidase [EC 3.4.24.11 (NEP)] in the central nervous system, where it inactivates the endogenous opioid peptides enkephalins[1] and in the periphery, where it metabolizes the atrial natriuretic peptide mainly in the kidney[2] and in the endothelium of the vasculature,[3] was demonstrated by the antinociceptive responses or the diuresis and natriuresis effects resulting from its inhibition. Owing to the clinical interest of these findings a large number of inhibitors of this enzyme have been synthesized as new analgesics or antihypertensive agents, several of them being now in clinical trials.[4-6]

Given the zinc metallopeptidase nature of NEP, four families of efficient inhibitors have been designed: three of them correspond to dipeptides or pseudodipeptides interacting with the S'_1–S'_2 subsites of the enzyme and bearing thiol,[7] hydroxamate,[8] or phosphorus containing groups[9] as zinc-chelating moieties. Inhibitory potencies in the nanomolar range have been obtained with these molecules. The fourth series of inhibitors contains a

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carboxylate group as zinc chelator initially introduced, as in the preceding series, on dipeptides interacting with the S'_1–S'_2 subsites of the enzyme. These compounds have relatively low NEP affinities with IC_{50}s in the 10^{-6}–10^{-7} range.[10]

In the case of carboxyl inhibitors of angiotensin converting enzyme (ACE), the presence in enalapril [*N*-(1-carboxy-3-phenylpropyl)-L-alanyl-L-proline] of the 1-carboxy-3-phenylpropyl chain increased the inhibitory potency ($K_i = 3.9 \times 10^{-9}$ M) by 3 orders of magnitude as compared to the carboxymethyl analog ($K_i = 2.4 \times 10^{-6}$ M).[11] This large increase in affinity was initially attributed both to the interaction of the hydrophobic 3-phenylpropyl chain with the ACE S_1 subsite (or the enzyme surface) and to the "transition state" nature of this new *N*-carboxyalkyl dipeptide.[12] The complexation mode of the Zn^{2+} cation by the carboxylate was also proposed to account for this improved affinity. Indeed, the X-ray analysis of the enalapril analog, *N*-(1-carboxy-3-phenylpropyl)-L-leucyl-L-tryptophan[13] cocrystallized with thermolysin (TLN), a bacterial enzyme classically used as a model for mechanistic studies on Zn^{2+} metallopeptidases,[1,5] has shown that the carboxylate of the phenylpropyl chain acts as a bidentate chelator for Zn^{2+}, while other carboxylate-containing inhibitors such as, carbobenzoxyphenylalanine[14] or L-benzylsuccinic acid[15] interact as monodentates. This change in the complexation mode may be responsible, in part, for their differences in inhibitory potency towards TLN: 10^{-3} M for the monodendates and 5×10^{-8} M for the bidendate inhibitor.

The same arguments were used to explain the increased affinity of (carboxyalkyl)dipeptide inhibitors of NEP as compared to (carboxymethyl)dipeptides[16-19] although the improvement in inhibitory potencies was not as impressive

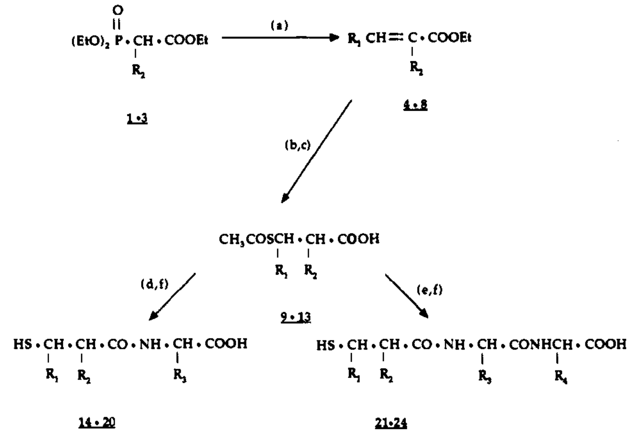
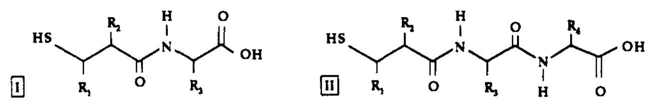


Figure 1. Scheme for the synthesis of inhibitors **14–24**. (a) NaH, 1,2-dimethoxyethane, R_1CHO at reflux temperature; (b) 1 M NaOH/EtOH, then 1 M HCl; (c) CH_3COSH at reflux temperature; (d) $H_2NCH(R_3)COOCH_3$, DCC, HOBt; (e) $H_2NCH(R_3)CONHCH(R_4)COOCH_3$, DCC, HOBt; (f) 1 M NaOH/MeOH, HCl. The different R_1, R_2, R_3, and R_4 groups are reported in Tables I and II.

Scheme I



as with ACE inhibitors. Furthermore, the IC_{50} values of these compounds were shown to be weakly dependent on the nature and the size of the alkyl chain[17] suggesting the lack of a strong interaction of this additional group with the putative S_1 subsite of NEP.

In order to clarify this point, we have extended this approach for the first time to the series of mercapto-containing inhibitors. Thus, various compounds containing a thiol group as a zinc ligand and lateral chains expected to interact with the S_1, S'_1, and S'_2 subsites of NEP and corresponding to the two general formulas I and II, were synthesized (Scheme I).

R_2, R_3 in compounds of series I and R_3, R_4 in compounds of series II were chosen among the residues generally well accepted by the S'_1 and S'_2 subsites of NEP.[7d,10a] Various chains were introduced in position R_1 in series I and R_1 and R_2 in series II, in order to tentatively fit the S_1 subsite of NEP.

As the S_1 subsite of ACE has been relatively well characterized,[5] the compounds synthesized were tested on both NEP and ACE to compare their active sites.

Results

1. Synthesis. The synthesis of inhibitors **14–19** and **20–23** was carried out by coupling the 3-(acetylthio)alkanoic acids **9–13** with the appropriate α-amino esters or dipeptide esters using the DCC/HOBt method, followed by alkaline deprotection of the mercapto and carboxyl group (Figure 1). The various 3-(acetylthio)alkanoic acids

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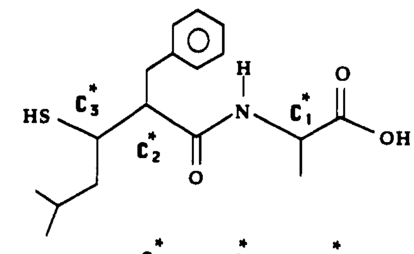
9–13 were prepared using triethyl phosphonoacetate as starting material. The α-alkyl (or aryl) triethyl phosphonoacetates **2** and **3** were obtained by reaction of alkyl (or aryl) halides with triethyl phosphonoacetates (**1**) (R_2 = H) in presence of NaH. The condensation of these phosphonates with various aldehydes according to the Wittig-Horner reaction provided the α,β-unsaturated ethyl esters **4–8** as a (50/50) mixture of *Z* and *E* isomers. After saponification of the ethyl esters, the Michael addition of thiolacetic acid to the α,β-unsaturated acids gave the desired 3-(acetylthio)alkanoic acids **9–13** as a mixture of stereoisomers which were not separated for the following step of the synthesis.

2. **HPLC Separation of the Stereoisomers.** The synthetic pathways used for the preparation of the inhibitors led to a racemization of the asymmetric carbons of the various 3-(acetylthio)alkanoic acids **9–13**. Consequently the inhibitors obtained at the end of the synthesis were a mixture of four stereoisomers, except **23** which contained only two stereoisomers. In order to verify the importance of the stereochemistry of each asymmetric carbon on the activity of these molecules, an HPLC separation was performed on compound **17**. Using a semipreparative C_8 nucleosil column and a mixture of CH_3CN/TFA 0.05% in H_2O as eluent, optically pure forms were obtained. The four stereoisomers were designated **17a** → **17d** following their elution order. In the mixture CH_3CN/TFA 0.05% in H_2O = 35/65 under analytical conditions, the retention time were as follows: **17a**, 28.3; **17b**, 30.1; **17c**, 31.6 and **17d**, 33.5 min, respectively.

3. **Determination of the Absolute Configuration of the Stereoisomers.** The determination of the stereochemistry of each isomer of compound **17** was tentatively carried out using HPLC and NMR data. Firstly, assuming an identical absorption at 210 nm for all the stereoisomers, the areas of the HPLC peaks reflects the proportion of each isomer: these proportions were around 39, 38, 12, and 11% for **17a–d**, respectively. This indicated that one set of enantiomers of the precursor **11** ($CH_3COSC^*H(CH_2CH(CH_3)_2)C^*H(CH_2Ph)COOH$), resulting from addition of thiolacetic acid on $(CH_3)_2CHCH_2CH{=}CH(CH_2Ph)COOH$, was obtained in a greater proportion that the other one. From this, it can be concluded that isomers **17a** and **17b** issued from the major set of enantiomers and have inverted absolute configuration for the two asymmetric carbons C^*_2 and C^*_3 of the 3-mercaptohexanoyl moiety. Conversely **17c** and **17d** were formed from the minor set of enantiomers of **11** (see Figure 2).

Secondly, the ^1H NMR spectra of the separate isomers allows the determination of the relative configuration of the benzyl moiety borne by the C^*_2 asymmetric carbon related to the alanine residue. Indeed as previously demonstrated[20] the chemical shift of the methyl of L-alanine is more shielded in the dipeptide D-Phe-L-Ala than in the L-Phe-L-Ala analog. The chemical shifts for the methyl of Ala were 0.92, 1.12, 1.01, and 1.17 ppm for **17a–d**, respectively. This allows the configurations of the C^*_2 asymmetric carbon of the four stereoisomers to be proposed as summarized in Figure 2.

The configuration of the thiol-bearing asymmetric carbon (C^*_3) was not determined. However, we have



	C^*_3	C^*_2	C^*_1
17a	R (or S)	R	S
17b	S (or R)	S	S
17c	S (or R)	R	S
17d	R (or S)	S	S

Figure 2. Assignment of the absolute configuration of the asymmetric carbons of the four stereoisomers of **17** based on ^1H NMR chemical shifts and elution order on HPLC.

previously shown that a phenylalanine-containing dipeptide of L,L (or D,D) configuration is more rapidly eluted than the L,D (or D,L) analog. In the case of isomer **17a**, the relative absolute configuration of C^*_2 and C^*_1 which are separated by a peptide bond, are inverted, indicating that the proposed rule was not valid. However it may be assumed that owing to the size and the hydrophobic character of the chains borne by C^*_2 and C^*_3 the most important parameter for the retention time was the relative configuration of carbon C^*_3 and C^*_2 rather than that of C^*_2 and C^*_1. If this assumption is correct, C^*_3 and C^*_2 have an identical absolute configuration in **17a** (*R,R* configuration) and **17b** (*S,S* configuration) and an opposite configuration in **17c** (*S,R* configuration) and **17d** (***R,S*** configuration). This led to the assignments proposed in Figure 2. If this assumption is not correct, the inversed configuration for C^*_3, shown in brackets in Figure 2, has to be considered.

4. **Inhibitory Potencies.** The inhibitory potencies of the two series of compounds **14–20** (Table I) and **21–24** (Table II) were measured in both NEP and ACE. As shown in Tables I and II, the IC_{50}s for ACE were in the micromolar range (from 0.3 to 12 μM) indicating a poor recognition of the enzyme's active site by such types of compounds. Conversely, relatively good inhibition was obtained on NEP with a significant modulation of the activity as a function of the chemical structures of the thiol inhibitors.

Five compounds (Table I) were found to exhibit inhibitory potencies towards NEP in the 10^{-8} M range (compounds **14**, **15**, **17–19**), the most efficient being compound **17** with an IC_{50} of 14 nM. This compound was also the most selective since its inhibitory potency on ACE was around 12 μM. The replacement of the C-terminal alanine by a tyrosine in **18** led to a small increase in the IC_{50} for NEP, but a significant decrease in the IC_{50} for ACE (0.7 μM). In contrast, compound **16** which was less potent on NEP (180 nM) was one of the best of this series for ACE (1.6 μM). The four stereoisomers of **17** were tested separately on both enzymes. On NEP the isomer **17b** was the most efficient with a IC_{50} of 3.6 nM, the three others being not significantly different from each other with IC_{50}s from 20 to 40 nM. On ACE, **17a** and **17d** were slightly more active (IC_{50}s of 5 and 4 μM) than **17b** and **17c** (IC_{50}s 16 and 10 μM).

The inhibitors reported in Table II, which correspond to modified tripeptides, were found to be less potent, especially two of them (**22** and **23**) which had IC_{50}s of 380

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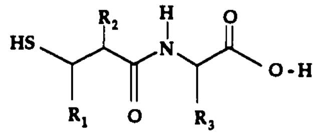
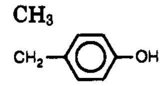
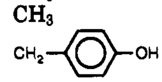
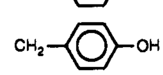
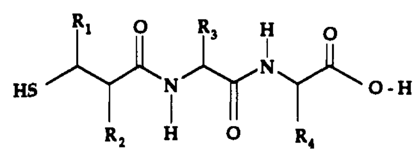
Table I

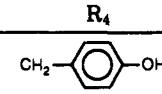
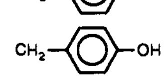
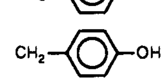
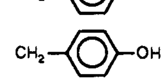


compounds	no. of stereoisomers	R_1	R_2	R_3	IC_{50} (μM)[a] NEP[b]	ACE[c]
thiorphan	2	H	CH_2Ph	H	0.004 ± 0.001	0.14 ± 0.02
14	4	CH_3	CH_2Ph	H	0.050 ± 0.010	11.0 ± 2.0
15	4	$(CH_2)_2Ph$	CH_2Ph	CH_3	0.044 ± 0.012	4.0 ± 0.5
16	4	$(CH_2)_2Ph$	CH_2Ph		0.180 ± 0.005	1.6 ± 0.6
17	4	$CH_2CH(CH_3)_2$	CH_2Ph	CH_3	0.014 ± 0.002	12.0 ± 2.1
17a	1 (*RRS*)	$CH_2CH(CH_3)_2$	CH_2Ph	CH_3	0.020 ± 0.005	5.0 ± 1.2
17b	1 (*SSS*)	$CH_2CH(CH_3)_2$	CH_2Ph	CH_3	0.0036 ± 0.005	16.1 ± 2.5
17c	1 (*SRS*)	$CH_2CH(CH_3)_2$	CH_2Ph	CH_3	0.035 ± 0.005	10.0 ± 3.2
17d	1 (*RSS*)	$CH_2CH(CH_3)_2$	CH_2Ph	CH_3	0.040 ± 0.006	4.0 ± 0.8
18	4	$CH_2CH(CH_3)_2$	CH_2Ph		0.026 ± 0.013	0.71 ± 0.10
19	4	$(CH_2)_2Ph$	$CH(CH_3)_2$		0.053 ± 0.012	4.5 ± 0.5
20	2	$CH_2CH(CH_3)_2$	H	CH_2Ph	0.037 ± 0.006	10.0 ± 2.2

[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 [^{3}H]-D-Ala^2-Leu-enkephalin as substrate. [c] Concentration inhibiting 50% of ACE activity with 50 μM *N*-Cbz-Phe-His-Leu as substrate.

Table II



compounds	no. of stereoisomers	R_1	R_2	R_3	R_4	IC_{50} (μM)[a] NEP[b]	ACE[c]
21	4	CH_3	CH_2Ph	$(CH_2)_3CH_3$		0.050 ± 0.01	6.1 ± 0.5
22	4	CH_3	CH_2Ph	$CH(CH_3)CH_2CH_3$		0.380 ± 0.02	0.3 ± 0.1
23	4	CH_3	CH_2Ph	$CH(CH_3)_2$		0.320 ± 0.08	1.3 ± 0.6
24	2	$CH_2CH(CH_3)_2$	H	CH_2Ph		0.050 ± 0.01	2.8 ± 0.8

[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 [^{3}H]-D-Ala^2-Leu-enkephalin as substrate. [c] Concentration inhibiting 50% of ACE activity with 50 μM *N*-Cbz-Phe-His-Leu as substrate.

and 320 nM, respectively. Nevertheless compounds **21** and **24** displayed inhibitory potencies around 50 nM. On ACE, these compounds were weakly active, but as previously underlined, the most efficient ACE inhibitor of this series, **22** (0.3 μM), was also the least active on NEP (380 nm) emphasizing the already reported structural differences in the active sites of both enzymes.[7b,d,10a]

Discussion

The compounds described in this paper were synthesized with the aim of obtaining thiol inhibitors able to interact with the S_1, S'_1, and S'_2 subsites of neutral endopeptidase. The first series of compounds (Table I) was designed by using the structure of thiorphan, $HSCH_2CH(CH_2Ph)$-$CONHCH_2COOH$,[7a] as template and by assuming that the same type of stabilizing interactions would be preserved after substitution of the thiol-bearing methylene group by various R_1 chains. Indeed, on the basis of the crystallographic data of thiorphan in thermolysin[21] and on the analogies between the active site of thermolysin and NEP, evidenced by several methods[22-24] including site-directed mutagenesis,[25,26] compounds **14–19** were expected to interact with the active site of NEP as depicted in Figure

(21) 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.

(22) Pozsgay, M.; Michaud, C.; Liebman, M.; Orlowski, M. Substrate and inhibitor studies of thermolysin-like neutral metalloendopeptidase from kidney membrane fractions: comparison with bacterial thermolysin. *Biochemistry* **1986**, *25*, 1292–1299.

(23) Benchetrit, T.; Fournié-Zaluski, M. C.; Roques, B. P. Relationships 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 modelling. *Biochem. Biophys. Res. Commun.* **1987**, *147*, 1034–1040.

(24) Benchetrit, J.; 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 thermolysin through clustering analysis. *Biochemistry* **1988**, *27*, 592–596.

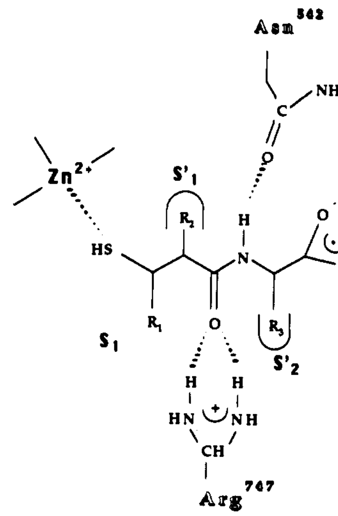


Figure 3. Schematic representation of compounds 14–20 in the active site of NEP, showing the various stabilizing interactions between the inhibitors and the enzyme.

3. The question was therefore to determine if the R_1 chain was able to improve the affinity for the enzyme through efficient interaction with the putative S_1 subsite.

Due to the synthetic pathway used for the preparation of these inhibitors i.e. a Michael addition of thiolacetic on an α,β-unsaturated acid, the final compounds were obtained as mixtures of stereoisomers. These compounds were first studied under their racemic forms to determine the influence of the nature of the R_1, R_2 side chains and that of the coupled amino acid on their inhibitory potencies toward NEP and ACE (Table I). The results reported in Table I show that this influence is relatively weak. It seems that an aliphatic chain in position R_1, a benzyl group in position R_2, and a small hydrophobic residue for the C-terminal amino acid are respectively preferred. Thus, the most efficient compound of this series is **17** (IC_{50}:14 nM), which has only one aromatic residue in position R_2, while the less efficient **16** (IC_{50}:180 nM) contains three aromatic chains. The decreased affinity of **16** could be due to the accumulation of aromatic residues inducing, by steric hindrance, a conformation unfavorable for enzyme recognition.

The role of the stereochemistry of each asymmetric carbon of **17** was studied after separation of the four stereoisomers. As shown in Table I, the most active isomer was **17b** with an IC_{50} of 3.6 nM, the other three being in the 10^{-6} M range. These results agree with the proposed stereochemistry of compound **17b**, since for carboxyl inhibitors of NEP with pseudotripeptide structures, the *S,S,S* configuration has been found to be the most active.[17] These findings also confirm that the stereochemical preference of NEP is not stringent,[7b] since the three other stereoisomers were relatively efficient with IC_{50}s of 20–40 nM.

However, the comparison of the inhibitory potency of **17b** (3.6 nM) with that of thiorphan (4 nM) shows that there is no additional stabilizing factors in NEP active site recognition resulting from introduction of a lipophilic R_1 alkyl chain. Three hypotheses may account for this result: (i) there is no important thermodynamically favorable interaction for the lipophilic chain at the level of the putative NEP S_1 subsite; (ii) the various R_1 hydrophobic chains are not well positioned in the inhibitor backbone for an optimized interaction with the hydrophobic S_1 subsite; (iii) the interaction of the R_1 chain in the hydrophobic S_1 subsite decreases the complexation of Zn^{2+} by the thiol group. These two latter complementary hypotheses seem to be the most convincing, at least when the synthesized compounds were studied as ACE inhibitors. Indeed it has been clearly established that a hydrophobic chain, able to interact with the S_1 subsite, increases the affinity of carboxyalkyl dipeptides for ACE by 3 orders of magnitude.[11] However, in the present series, all the compounds tested had a lower affinity (from 0.7 to 16 μM) for ACE than thiorphan (IC_{50}:0.14 μM) in spite of their additional hydrophobic chains. Likewise none of the four stereoisomers of **17** were more active on thermolysin (IC_{50}s for **17a–d**: $6.7 \pm 0.5 \times 10^{-6}$ M, $4.0 \pm 0.2 \times 10^{-6}$ M, $6.0 \pm 0.3 \times 10^{-6}$ M, and $5.4 \pm 0.5 \times 10^{-6}$ M) than thiorphan ($2.0 \pm 0.8 \times 10^{-6}$ M).[23] Although the new mercapto inhibitors contain an additional R_1 group, their affinity for the zinc metallopeptidases was not improved possibly due to a conformationally induced weakening of the thiol coordination. This is now being investigated in the laboratory by molecular modeling of **17** in the active site of thermolysin.

The second series of compounds (Table II) were pseudopeptides resembling the ACE thiol inhibitors described by Weller et al.[27] to characterize the S_1 subsite of this enzyme. These authors showed that the trans isomer of N-(2-mercapto-1-cyclohexanoyl)-L-Ala-L-Pro is a potent ACE inhibitor (IC_{50}:3 nM). Starting from this model, the *N*-(3-mercaptoalkanoyl dipeptides **21–24** were synthesized, assuming that their R_3 and R_4 side chains interact with the S'_1 and S'_2 subsites of the enzyme, respectively (Figure 4). Under these conditions, the R_1 or R_2 groups were expected to fit the S_1 subsite and to induce a constraint of the backbone favoring the chelation of the Zn^{2+} ion by the thiol group as shown in Figure 4A.

In so far as the C-terminal dipeptide sequences of compounds **21** to **24** were almost identical with a hydrophobic residue (aliphatic or aromatic) as R_3 component and a tyrosine as R_4 moiety, the differences in the potencies between compounds **21** and **24** (IC_{50}s: 50 nM) and compounds **22** and **23** (IC_{50}s: 380 and 320 nM) seem to be due to steric parameters. Indeed the β-branched chains of Ile and Val probably hardly interact with the enzyme subsite when they are wedged between the R_2 and R_4 aromatic moieties. Although unlikely, another possibility is that these compounds interact with different subsites of the active site of NEP. To choose between these two assumptions the NEP subsites occupied by these molecules were experimentally determined using compounds **21** and **22**.

Arg[102] of NEP is located at the edge of the active site of the enzyme and interacts by a salt bridge with the free carboxylate of potent inhibitors such as thiorphan.[26] When site-directed mutagenesis was used to replace Arg[102] by Glu, the K_i of thiorphan for the mutated enzyme increased

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(26) 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.

(27) Weller, H. N.; Gordon, E. M.; Rom, M. B.; Pluscec, J. Design of conformationally constrained angiotensin converting enzyme inhibitors. *Biochem. Biophys. Res. Commun.* **1984**, *125*, 82–89.

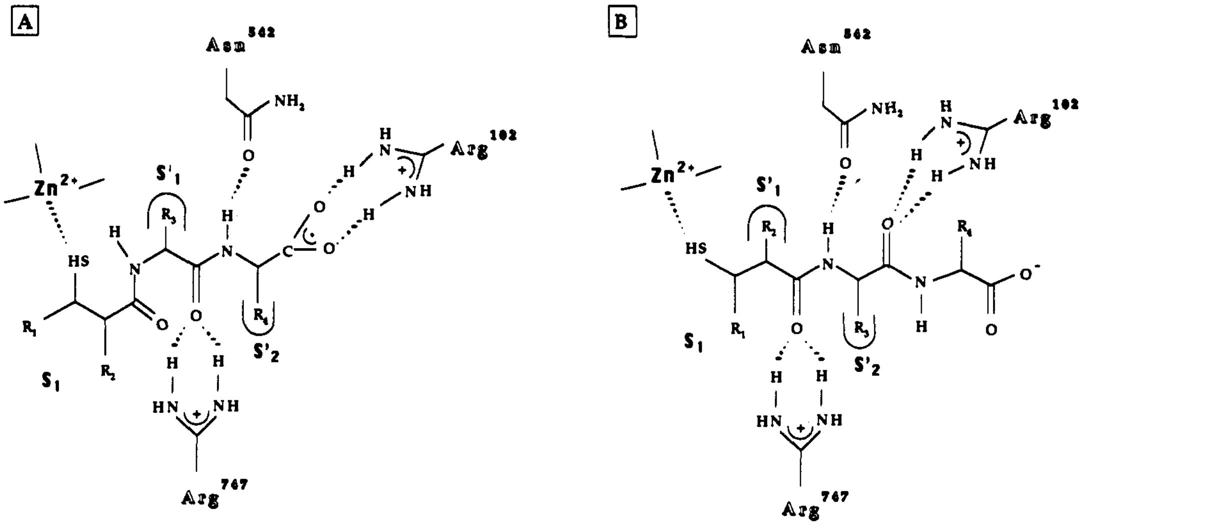

Figure 4. Schematic representation of the two possible models of interaction between compounds **21–24** and the active site of NEP. (A) First hypothesis: the chains R_3 and R_4 interact with the subsites S'_1 and S'_2, respectively, while R_1 or R_2 is able to fit the S_1 subsite. (B) Second hypothesis: Only R_1 is able to fit the S_1 subsite, while R_2 and R_3 interact with the S'_1 and S'_2 subsites, respectively, as proposed in Figure 3. In this case, the C-terminal amino acid $NHCH(R_4)COOH$ is outside the active site.

by over 2 orders of magnitudes.[25] Under the same conditions, the K_i of thiorphan amide which has no free carboxylate was increased only by a factor of 6. Consequently, this mutated enzyme may be an index of inhibitor positioning in the active site of NEP. The IC_{50}s of **21** and **22** on the mutated enzyme were respectively 5×10^{-7} M and 5.6×10^{-7} M, a loss when compared to the natural NEP of only 10-fold for **21** and 2-fold for **22**. Taken together these results indicate that inhibitors **21–24** occupy the active site of NEP in the same manner as inhibitors **14–20**, with R_2 in the S'_1 subsite, R_3 in the S'_2 subsite, and the tyrosine outside the active site (Figure 4B). The differences in potency between compounds of **22** and **23** vs **21** and **24** is therefore very likely due to steric factors as previously discussed. It is interesting to observe that this is not the case for ACE since the best inhibitor for this peptidase, compound **22**, is the weakest for NEP. On the other hand, the loss of an ionic interaction between Arg^{102} of NEP and the free carboxyl group of the compounds **21–24** bound in the active site as proposed in Figure 4B is probably responsible for their lower affinites for NEP as compared to inhibitors such as **17** which interact with the enzyme as shown in Figure 3.

In conclusion, in the series reported in Table I and Table II the R_1 group which was assumed to interact with the S_1 site does not increase the inhibitory potency, showing that no stabilizing interaction has been created. As discussed, this may be interpreted by the absence of a definite hydrophobic subsite in this position or by a constraint of the inhibitor backbone which precludes the interaction of the R_1 chain with the S_1 subsite. Although the inhibitory potencies of the synthesized compounds are not higher than that of thiorphan, these new thiol inhibitors could have improved bioavailability and pharmacological properties thanks to their enhanced lipophilicity (to be published).

Experimental Section

Biological Test. [^{3}H]Tyr-D-Ala^2-Leu-enkephalin (32 Ci/mmole) was obtained from Dositek (CEA, France). *N*-Cbz-Phe-His-Leu was from Bachem (Bubbendorf, Switzerland). Recombinant human angiotensin converting enzyme obtained as described[28] was a generous gift of Pr. Corvol (Collège de France, Paris, France).

Assay for Neutral Endopeptidase. Neutral endopeptidase was purified to homogeneity from rabbit kidney as previously described.[29] IC_{50} values were determined as previously described in detail.[30] NEP (final concentration 1 pmol/100 μL, specific activity on [^{3}H]-D-Ala^2-Leu-enkephalin 0.3 nmol/mg per min) was preincubated for 15 min at 25 °C with or without increasing concentrations of inhibitor in a total volume of 100 μL of 50 mM Tris-HCl buffer pH = 7.4. [^{3}H]-D-Ala^2-Leu-enkephalin (K_m = 30 mM) 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 metabolites formed were separated on polystyrene beads. The mutated enzyme Glu^{102}-NEP was obtained as previously described.[25] The inhibitory potency of the tested inhibitors was determined by the method described for NEP.

Assay for ACE Activity. Enzymatic studies on ACE were performed using *N*-Cbz-Phe-His-Leu[31] as substrate (K_m = 50 mM) as described.[32] ACE (final concentration of 0.02 pm/100 mL; specific activity on Cbz-Phe-His-Leu; 13 mmol/mg per min) was preincubated for 15 min at 37 °C with various concentrations of the inhibitors in 50 mM Tris-HCl buffer (pH = 7.4), and *N*-Cbz-Phe-His-Leu was added to a final concentration of 0.05 mM. The reaction was stopped after 15 min by adding 400 mL 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.[31] with a MPF 44A Perkin-Elmer spectrofluorimeter (excitation 365 nm, emission 495 nm). The calibration curve for His-Leu was obtained by addition of increasing concentrations of His-Leu into 0.1 mL of 0.1 M Tris-HCl buffer pH = 7.4 containing the denatured enzyme.

Synthesis. The protected amino acids were from Bachem (Bubbendorf, Switzerland). Benzyl bromide, 2-iodobutane,

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(32) Cheung, 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.

isovaleraldehyde, hydrocinnamaldehyde, benzaldehyde, and thiolacetic acid were from Aldrich (France). Dicyclohexylcarbodiimide was from Merck (U.S.A.). 1-Hydroxybenzotriazole was from Janssen-Chimica (Belgium) and was used in its hydrated form. 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–AcOH (5/5/0.1); B, EtOAc–hexane (1/4); C, EtOAc–hexane (3/2); D, EtOAc–hexane (1/1); E, CH_2Cl_2–MeOH–AcOH (9/1/0.1) and by HPLC on a reverse-phase Nucleosil C_8 column 250 × 5 mm (SFCC) with CH_3CN/TFA 0.05% in H_2O as mobile phase. The eluted peaks were monitored at 210 nm. The ^1H NMR spectra were taken with a Bruker AC (270 MHz) in $^2H_6$-DMSO using HMDS as internal reference. Melting points of the crystallized products were determined on an Electrothermal apparatus and are reported uncorrected.

The following abbreviations were used: EtOAc, ethyl acetate; AcOH, acetic acid; MeOH, methanol; EtOH, ethanol; DMF, dimethylformamide; THF, tetrahydrofuran; DMSO, dimethyl sulfoxide; HMDS, hexamethyldisiloxane.

General Procedures. Preparation of Triethyl 2-Alkyl(aryl)phosphonoacetates. To a solution of the triethyl phosphonoacetate (1) in dry DMF was added at 0 °C 1 equiv of NaH, and the mixture was stirred at the same temperature for 15 min. After addition of a solution of the alkyl (aryl) halide (1 equiv) in DMF, stirring was continued at room temperature for 24 h. Solvent was removed under reduced pressure. The residue was diluted with EtOAc, washed with H_2O and brine, dried (Na_2SO_4), concentrated in vacuo, and purified by chromatography.

Triethyl 2-Benzylphosphonoacetate (2). Chromatography in EtOAc–hexane, 1/1 colorless oil (70%): ^1H NMR (DMSO) δ 0.9 (3 H, $CH_3(CH_2)$), 1.12–1.18 (6 H, 2 × $CH_3(CH_2)$), 3.00–3.10 (2 H, CH_2Ph), 3.80–3.40 (1 H, CHP), 3.95–4.08 (6 H, 3 × CH_2O), 7.18 (5 H, *Ph*); R_f(A) 0.40.

Triethyl 2-Isopropylphosphonoacetate (3). Chromatography in EtOAc–hexane 1:1 colorless oil (65%): ^1H NMR (DMSO) δ 0.88 and 1.00 ($(CH_3)_2CH$), 1.12–1.20 (9 H, 3 × $CH_3(CH_2)$), 2.15 (1 H, $CH(CH_2)$), 2.7 (1 H, CHP), 3.95–4.05 (6 H, 3 × OCH_2); R_f(A) 0.44.

Preparation of α,β-Unsaturated Ethyl Esters. To a solution of the triethyl 2-alkyl(aryl)phosphonoacetate in dry 1,2-dimethoxyethane [or dioxane–H_2O (2/1)] was added, at 0 °C, 1 equiv of NaH (or 2.5 equiv of K_2CO_3). After stirring for 15 min, 3 equiv of the carbonyl derivative was added, and the mixture was stirred at reflux temperature for 3 h. Removal of the solvent gave a residue which was dissolved in hexane, washed with H_2O and brine, dried (Na_2SO_4), and evaporated. The oily residue was purified by flash chromatography in EtOAc–hexane 1:10.

Ethyl 2-Benzyl-2-butenoate (4). Colorless oil (70%): ^1H NMR (DMSO) δ 1.1 (3 H, $CH_3(CH_2)$), 1.80–1.88 ($CH_3(CH{=})$), 3.52 ($CH_2(Ph)$), 4.02 (OCH_2), 6.1 and 6.9 (CH=), 7.09–7.22 (Ph); R_f(B) 0.77. Anal. ($C_{13}H_{16}O_2$) C, H.

Ethyl 2-Benzyl-5-phenyl-2-pentenoate (5). Colorless oil (68%): ^1H NMR (DMSO) δ 1.08 ($CH_3(CH_2)$), 2.55–2.60 (CH_2CH_2), 3.52 (CH_2Ph), 4.00 (OCH_2), 6.00 and 6.80 (CH=), 7.08–7.20 (Ph); R_f(B) 0.7. Anal. ($C_{20}H_{22}O_2$) C, H.

Ethyl 2-Benzyl-5-methyl-2-hexenoate (6). Colorless oil (68%): ^1H NMR (DMSO) δ 0.80 ($(CH_3)_2CH$), 1.08 ($CH_3(CH_2)$), 1.54 ($CH(CH_3)_2$), 2.10 and 2.28 (2 H, $CH_2CH{=}$), 3.52 (CH_2Ph), 4.00 (OCH_2), 6.00 and 6.8 (CH=), 7.10–7.20 (Ph); R_f(B) 0.8. Anal. ($C_{16}H_{22}O_2$) C, H.

Ethyl 2-Isopropyl-5-phenyl-2-pentenoate (7). Colorless oil (86%): ^1H NMR (DMSO) δ 0.90 ($(CH_3)_2CH$), 1.12 (CH_3CH_2), 2.50 ($CH(CH_3)_2$), 2.62 ($CH_2CH_2(Ph)$), 4.08 (OCH_2), 5.70 and 6.48 (CH=), 7.10–7.20 (Ph); R_f(B) 0.76. Anal. ($C_{16}H_{22}O_2$) C, H.

Ethyl 5-Methyl-2-hexenoate (8). Colorless oil (86%): ^1H NMR (DMSO) δ 0.80 ($(CH_3)_2CH$), 1.15 ($CH_3(CH_2)$), 1.65 ($CH(CH_3)_2$), 2.04 ($CH_2(CH{=})$), 4.02 (OCH_2), 5.8 and 6.8 (CH=); R_f(B) 0.65. Anal. ($C_9H_{16}O_2$) C, H.

Preparation of Substituted 3-(Acetylthio)alkanoic Acids 9–13. A solution of the α,β-unsaturated ethyl ester in EtOH was treated with 2 equiv of 1 N NaOH, and the mixture was stirred at room (or reflux) temperature for 3–24 h. After evaporation of ethanol, the remaining aqueous mixture was diluted with H_2O, acidified with 1 N HCl to pH3, and extracted with EtOAc. The extract was dried (Na_2SO_4) and evaporated to dryness. The α,β-unsaturated acid obtained was dissolved in 10–12 equiv of thiolacetic acid. The reaction mixture was stirred for 24–48 h at reflux temperature and evaporated, and the residue was purified by flash chromatography (EtOAc–hexane 1:1).

3-(Acetylthio)-2-benzylbutanoic Acid (9). Colorless oil (42%): ^1H NMR (DMSO) δ 1.28 ($CH_3(CH)$), 2.30 (CH_3CO), 2.75 (CH_2Ph), 2.90 ($CH(COOH)$), 3.65 (CHS), 7.08–7.20 (Ph), 12.30 (COOH); R_f(C) 0.36. Anal. ($C_{13}H_{16}O_3S$) C, H.

3-(Acetylthio)-2-benzyl-5-phenylpentanoic Acid (10). Colorless oil (50%): ^1H NMR (DMSO) δ 1.80 and 1.95 ($CH_2(CH_2Ph)$), 2.45 (CH_3CO), 2.52–2.70 (CH_2Ph), 2.80–2.92 ($CHCH_2Ph$), 3.69 (CHS), 7.09–7.19 (Ph), 12.42 (COOH); R_f(C) 0.4. Anal. ($C_{20}H_{22}O_3S$) C, H.

3-(Acetylthio)-2-benzyl-5-methylhexanoic Acid (11). Colorless oil (32%): ^1H NMR (DMSO) δ 0.78–0.85 ($(CH_3)_2CH$), 1.69 ($CH(CH_3)_2$), 1.90 (CH_2CH), 2.28–2.35 ($CHCH_2Ph$), 2.50 (CH_3CO), 3.70 (CHS), 7.10–7.20 (Ph), 12.41 (COOH); R_f(C) 0.32. Anal. ($C_{16}H_{22}O_3S$) C, H.

3-(Acetylthio)-2-isopropyl-5-phenylpentanoic Acid (12). Colorless oil (32%): ^1H NMR (DMSO) δ 0.78–0.89 ($(CH_3)_2CH$), 1.70 ($CH(CH_3)_2$), 1.90 (CH_2CH), 2.30 ($CHCOOH$), 2.40 (CH_3COS), 2.6 (CH_2Ph), 3.70 (CHS), 7.10–7.20 (Ph), 12.40 (COOH); R_f(C) 0.32. Anal. ($C_{16}H_{22}O_3S$) C, H.

3-(Acetylthio)-5-methylhexanoic Acid (13). Colorless oil (40%): ^1H NMR (DMSO) δ 0.80 ($(CH_3)_2CH$), 1.40 ($CH_2(CH)$), 1.58 ($(CH_3)_2CH$), 2.29 (CH_3CO), 2.80–3.15 (CH_2COOH), 3.80 (CHS), 12.27 (COOH); R_f(D) 0.22. Anal. ($C_9H_{16}O_3S$) C, H.

General Procedure for the Coupling Step. Procedure IV. To a solution of 3-(acetylthio)propanoic acid derivative in dry THF cooled at 0 °C was added successively 1 equiv of 1-hydroxybenzotriazole, 1.1 equiv of dicyclohexylcarbodiimide, 1 equiv of the corresponding α-amino acid or dipeptide methyl ester hydrochloride, and 1 equiv of triethylamine. After 30 min at 0 °C, the mixture was stirred at room temperature overnight. After filtration of dicyclohexylurea and evaporation of the solvent, the residue was dissolved in EtOAc and washed with H_2O, 10% citric acid, H_2O, 10% $NaHCO_3$, H_2O, and brine, successively. The organic layer was dried (Na_2SO_4) and evaporated. The residue was purified by chromatography.

General Procedure for the Basic Hydrolysis of Esters. Procedure V. To a solution of the protected compound in degassed MeOH at 0 °C under argon was added 3 equiv of 1 N NaOH. After stirring 15 min at 0 °C and 3–9 h at room temperature, the solvent was evaporated and the residue dissolved in H_2O and washed with EtOAc. The aqueous layer was acidified with 1 N HCl to pH 3 and extracted with EtOAc. The organic layer was washed with H_2O and brine, dried over Na_2SO_4, and evaporated.

***N*-(2-Benzyl-3-mercapto-1-oxobutyl)glycine (14).** Colorless oil [63% (ester 78%)]: ^1H NMR (DMSO) δ 1.20 ($CH_3(CH)$), 2.58 (HS), 2.66–3.00 ($CHCH_2Ph$), 3.38–3.75 ($CHCH_3$, CH_2COOH), 7.09 (Ph), 8.11 (NH), 12.31 (COOH). Anal. ($C_{13}H_{17}NO_3S$) C, H, N.

***N*-(2-Benzyl-3-mercapto-5-phenyl-1-oxopentyl)-L-alanine (15).** Colorless solid [51% (ester 87%)]: mp 72 °C; ^1H NMR (DMSO) δ 0.90–1.12 ($CH_3(CH)$), 1.60 and 2.02 (CH_2CHS), 2.52–2.60 (CH_2Ph + HS), 2.70–2.85 ($CHCH_2Ph$), 3.12 (CHS), 4.04 ($CH(CH_3)$), 7.10–7.25 (Ph), 8.02–8.18 (NH), 12.43 (COOH); R_f(E) 0.62; HPLC t_R = 7.3, 7.5, and 7.8 (CH_3CN–TFA 0.05% = 50/50). Anal. ($C_{21}H_{25}NO_3S$) C, H, N.

***N*-(2-Benzyl-3-mercapto-5-phenyl-1-oxopentyl)-L-tyrosine (16).** Colorless solid [57.7% (ester 78%)]: mp 69 °C; ^1H NMR (DMSO) δ 1.60–1.70 (CH_2CHS), 2.35 (HS) centered on 2.75 (CH_2Ph, $CHCH_2Ph$, $CH_2\beta$-Tyr), 3.01 (CHS), 4.26 ($CHCOOH$), 6.55–7.10 (arom), 8.05–8.12 (NH), 9.10 (OH), 12.45 (COOH); R_f(E) 0.71; HPLC t_R = 17.5, 18.7, and 19.8 min (CH_3CN–TFA 0.05% = 50/50). Anal. ($C_{27}H_{29}NO_4S$) C, H, N.

***N*-(2-Benzyl-3-mercapto-5-methyl-1-oxohexyl)-L-alanine (17).** Colorless solid [46% (ester 98%)]: mp 85 °C; ^1H NMR (DMSO) δ 0.72–0.82 ($(CH_3)_2CH$), 0.95–1.15 ($CH_3(CH)$), 1.28–1.48 ($CH_2(CH)$), 1.80 ($CH(CH_2)$), 2.38 (HS), 2.70 ($CH(CH_2Ph)$), 2.88 ($CH_2(Ph)$), 3.05 (CHS), 4.08 ($CH(CH_3)$), 7.10–7.20 (Ph), 8.00–8.12 (NH), 12.38 (COOH); R_f(E) 0.48; HPLC t_R = 10.1 min (CH_3CN–TFA 0.05 = 50/50). Anal. ($C_{17}H_{25}NO_3S$) C, H, N.

N-(2-Benzyl-3-mercapto-5-methyl-1-oxohexyl)-L-tyrosine (18). Colorless solid [50% (ester 52%)]: mp 73 °C; ^{1}H NMR (DMSO) δ 0.60–0.80 ((CH_3)$_2$CH), 1.25 (CH_2CH), 1.75 (CHCH$_2$), 2.31 (SH), 2.56–2.95 (CHCH_2Ph + CH_2β-Tyr), 3.20 (CHS), 4.25 (CH α-Tyr), 6.55–7.10 (arom), 7.99–8.10 (NH), 9.11 (OH), 12.49 (COOH); R_f(E) 0.41; HPLC t_R = 12.6, 13.7, 15.0, 16.0 min (CH_3CN–TFA 0.05% = 50/50). Anal. ($C_{23}H_{29}NO_4S$) C, H, N.

N-(2-Isopropyl-3-mercapto-5-phenyl-1-oxopentyl)-L-tyrosine (19). Colorless solid [58% (ester 85%)]: mp 81 °C; ^{1}H NMR (DMSO) δ 0.50–0.71 ((CH_3)$_2$CH)), 1.60 (CH(CH$_3$)$_2$), 1.91–2.05 (CHCO + CH_2-CH$_2$Ph), 2.45 (SH), 2.50–2.70 (CH_2Ph + CH_2-Tyr), 2.90 (CHS), 4.48 (CH α-Tyr), 6.59 and 7.00 (arom Tyr), 7.12–7.20 (Phe), 8.12 (NH), 9.10 (OH), 12.52 (COOH); R_f(E) 0.31; HPLC t_R = 13.8, 14.3 and 14.8 min (CH_3CN–TFA 0.05% = 50/50). Anal. ($C_{23}H_{29}NO_4S$) C, H, N.

N-(3-Mercapto-5-methyl-1-oxohexyl)-L-phenylalanine (20). Colorless solid [64% (ester 82%)]: mp 145 °C; ^{1}H NMR (DMSO) δ 0.70 ((CH_3)$_2$CH), 1.20 (CH_2), 1.7 (CH(CH$_3$)$_2$), 2.05–2.20 (SH), 2.28 (CH_2CO), 2.75 (CHS), 3.00 (CH_2Phe), 4.40 (CHαPhe), 7.21 (Phe), 8.2 (NH), 12.7 (COOH); R_f(E) 0.59; HPLC t_R = 6.8 min (CH_3CN–TFA 0.05% = 53/47). Anal. ($C_{16}H_{23}NO_3S$) C, H, N.

N-(2-Benzyl-3-mercapto-1-oxobutyl)-L-norleucyl-L-tyrosine (21). Colorless solid [51% (ester 96%)]: mp 132 °C; ^{1}H NMR (DMSO) δ 0.68–0.78 (CH_3Nle), 1.00–1.42 ((CH_2)$_3$Nle), 2.48 (CHCO), 2.52 (SH), 2.71–3.00 (CHS+CH_2Ph + CH_2 β-Tyr), 4.02 (CH αNle), 4.24 (CH αTyr), 6.59 and 6.90 (arom Tyr), 7.02–7.15 (Ph), 7.70–7.90 (NH), 9.12 (OH), 12.50 (COOH); R_f(E) 0.32; HPLC t_R = 9.3, 9.7 min (CH_3CN–TFA 0.05% = 50/50). Anal. ($C_{26}H_{34}N_2O_5S$) C, H, N.

N-(2-Benzyl-3-mercapto-1-oxobutyl)-L-isoleucyl-L-tyrosine (22). Colorless solid [44% (ester 80%)]: mp 128 °C; ^{1}H NMR (DMSO) δ 0.50–0.72 (2 CH_3-Ile), 1.15–1.25 (CH_3(CH)), 1.38 and 1.60 (CH_2-CH(Ile)), 2.40 (SH), 2.60–3.00 (CHCH_2Ph + CH_2 βTyr + CHS), 3.98 (CH αIle), 4.2 (CH αTyr), 6.58 (Tyr), 6.90–7.10 (Ph + Tyr), 7.60–7.72 (NH), 7.98 (NH), 9.12 (OH), 12.4 (COOH); R_f(E) 0.63; HPLC t_R = 8.9 min (CH_3CN–TFA 0.05% = 50/50). Anal. ($C_{23}H_{34}N_2O_5S$) C, H, N.

N-(2-Benzyl-3-mercapto-1-oxobutyl)-L-valinyl-L-tyrosine (23). Colorless solid [44% (ester 86%)]: mp 120 °C; ^{1}H NMR (DMSO) δ 0.38–0.49 ((CH_3)$_2$CH), 0.72–0.81 (CH_3CH), 1.12–1.22 ((CH_3)$_2$CH), 1.60 (CH(CH$_3$)$_2$), 2.40 (SH), 2.60 (CHCO), 2.72–2.90 (CH_2 βPh + CH_2 βTyr), 3.05 (CHS), 3.98 (CH αVal), 4.15–4.28 (CH αTyr), 6.60 (Tyr), 6.95–7.10 (Tyr + Phe), 7.60–7.70 (NH), 7.95 (NH), 9.15 (COOH); 12.45 (1 H, acid); R_f(E) 0.51; HPLC t_R = 6.97 min (CH_3CN–TFA 0.05% = 50/50). Anal. ($C_{25}H_{32}N_2O_5S$) C, H, N.

N-(3-Mercapto-5-methyl-1-oxohexyl)-L-phenylalanyl-L-tyrosine (24). Colorless solid [42% (ester 85%)]: mp 110 °C; ^{1}H NMR (DMSO) δ 0.65–0.75 ((CH_3)$_2$CH), 1.05 and 1.20 (CH_2-(CHS)), 1.62 (CH(CH$_3$)$_2$), 1.85 (HS), 2.10–2.40 (CH_2CO), 2.60–3.00 (CHS + CH_2 βPhe + CH_2 βTyr), 4.30 (CH αPhe), 4.55 (CH αTyr), 6.60 and 6.95 (arom Tyr), 7.10–7.22 (Phe), 8.08–8.18 (2 NH), 9.18 (OH), 12.33 (COOH); R_f(E) 0.52; HPLC t_R = 9.17 min (CH_3CN–TFA 0.05% = 50/50). Anal. ($C_{25}H_{32}N_2O_5S$) C, H, N.

HPLC Separation of the Stereoisomers. The separation of the stereoisomers of 17 was performed using a semipreparative C8 nucleosil column (300 × 7.5 mm) (SFCC) with a Shimatzu LC-9A HPLC apparatus connected to a SPD-6AV UV detector (210 nm). For compound 17, the separation was performed in CH_3CN–TFA 0.005% in H_2O = 30/70. The retention times were 76.9, 81.9, 86.9, and 91.9 min, respectively. The purity was verified in analytical conditions with a C8 nucleosil column (250 × 5 mm) using CH_3CN–TFA 0.05% in H_2O = 35/65. The retention times in these latter conditions were 28.2, 30.1, 31.6, and 33.5 min.

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