Optimal Recognition of Neutral Endopeptidase and Angiotensin-Converting Enzyme Active Sites by Mercaptoacyldipeptides as a Means To Design Potent Dual Inhibitors

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An interesting approach for the treatment of congestive heart failure and chronic hypertension could be to avoid the formation of angiotensin II by inhibiting angiotensin converting enzyme (ACE) and to protect atrial natriuretic factor by blocking neutral endopeptidase 24.11 (NEP). This is supported by recent results obtained with potent dual inhibitors of the two zinc metallopeptidases, such as RB 105, $HSCH_2CH(CH_3)PhCONHCH(CH_3)COOH$ *(Fournié-Zaluski et al. Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91, 4072*−4076), which reduces blood pressure in experimental models of hypertension, independently of the salt and renin angiotensin system status. In order to develop new dual inhibitors with improved affinities, long duration of action, and/or better bioavailabilities, various series of mercaptoacyldipeptides corresponding to the general formula $HSCH(R_1)CONHCH(R_1')CON(R)CH(R_2')COOH$ have been synthesized. The introduction of well-selected β-branched chains in positions R_1 and R_1', associated with a tyrosine or a cyclic amino acid in the C-terminal position, led to potent dual inhibitors of NEP and ACE such as **21** [*N*-[(2*S*)-2-mercapto-3-methylbutanoyl]-Ile-Tyr] and **22** [*N*-[(2*S*)-2-mercapto-3-phenylpropanoyl]Ala-Pro] which have IC_{50} values in the nanomolar range for NEP and subnanomolar range for ACE. These compounds could have different modes of binding to the two peptidases. In NEP, the dual inhibitors seem to interact only with the S_1' and S_2' subsites, whereas additional interactions with the S_1 binding subsite of ACE probably account for their subnanomolar inhibitory potencies for this enzyme. The localization of the Pro residue of **22** outside the NEP active site is supported by biochemical data using (Arg^{102},Glu)NEP and molecular modeling studies with thermolysin used as model of NEP. One hour after oral administration in mice of a single dose (2.7×10^{-5} mol/kg), **21** inhibited 80% and 36% of kidney NEP and lung ACE, respectively, while **22** inhibited 40% of kidney NEP and 56% of lung ACE.

Introduction

The design of dual inhibitors of neutral endopeptidase (NEP, EC 3.4.24.11 or neprilysin) and angiotensin-converting enzyme (ACE, EC 3.4.25.1) has become an important therapeutic challenge in the last few years.[1] ACE belongs to the enzymatic cascade of the renin−angiotensin system and releases the vasoconstrictor peptide angiotensin II from its inactive precursor, angiotensin I. NEP inactivates the atrial natriuretic peptide (ANP)[2] which induces diuresis, natriuresis, and a slight vasodilatation. Furthermore, NEP and ACE are both involved in the inactivation of bradykinin (Bk), a vasodilatatory peptide, at their epithelial and endothelial sites, respectively.[3,4] However, as shown in various pharmacological experiments, there is a physiological antagonism between the renin−angiotensin and ANP systems, reducing the optimal action expected from the inhibition of one of these enzymes.[5,6] This suggests that a simultaneous inhibition of ACE and NEP, reducing angiotensin II formation and protecting ANP and Bk from inactivation, might have, as proposed some years ago,[1] therapeutic advantages in the treatment of cardiovascular diseases, as compared to selective inhibition of a single enzyme.[7−9]

Cloning and sequencing of NEP and ACE have shown that both enzymes belong to the thermolysin group of Zn^{2+} metallopeptidases,[10,11] and have significant analogies in their active sites, as shown by their capacity to cleave *in vitro* identical peptides at the same bond (for example bradykinin and Leu- or Met-enkephalin[12]). Consequently, the same types of molecules are able to block the active sites of these enzymes, as illustrated by mercaptoacyl amino acids and carboxyalkyl dipeptides (thiorphan, $HSCH_2CH(CH_2Ph)CONHCH_2COOH$,[13] and SCH-32,615, $PhCH_2CH(COOH)NHCH(CH_2Ph)-CONH(CH_2)_2COOH$,[14] for NEP, captopril, $HSCH_2CH-(CH_3)CO$-Pro,[15] and enalapril, $PhCH_2CH_2CH(COOH)-NHCH(CH_3)CO$-Pro,[16] for ACE). The selectivity of these molecules results from subtle differences in the active sites of both peptidases.[17−19] Efficient NEP inhibitors preferentially contain aromatic residues in the P_1' position,[20] and most ACE inhibitors are characterized by a small residue (Ala) in the P_1' position and a cyclic amino acid (Pro) in the P_2' position.[21] However, it has been possible to design dual inhibitors of these peptidases by introducing more hydrophobic residues in the selective inhibitor of NEP, thiorphan,[13] and compounds such as *N*-[2-(mercaptomethyl)-3-phenylpropanoyl]leucine[17,18] or alatrioprilate[22] exhibit relatively good affinities for both enzymes.

Recently, more potent dual inhibitors containing hindered substituents have been designed giving new information about the structures of the binding domains of each enzyme. Thus, the development of RB 105 [(2*S*,3*R*)-$HSCH_2CH(CH(CH_3)Phe)CO$-L-Ala][23,24] has

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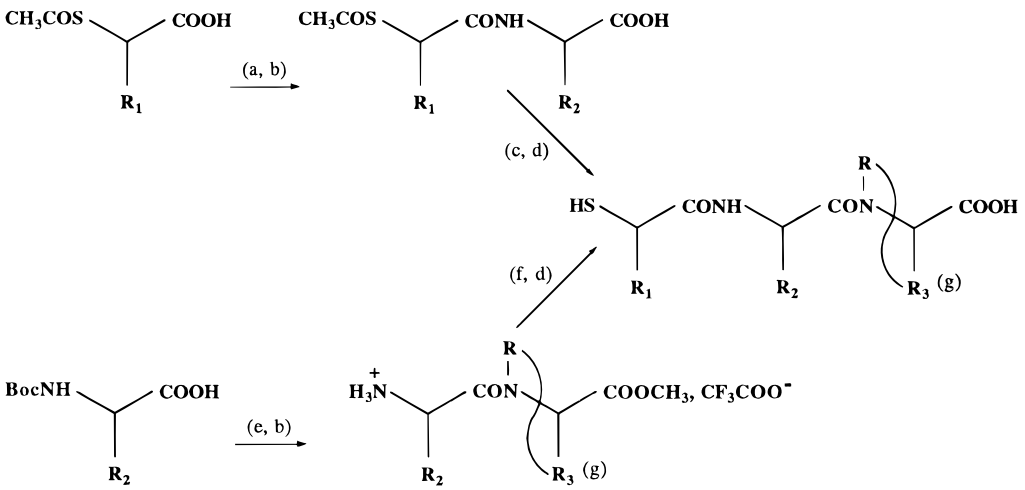
Scheme 1[a]



[a] (a) $H_2NCH(R_2)COOtBu$, DCC, HOBt in $CH_2Cl_2/THF = 1/1$; (b) TFA, CH_2Cl_2; (c) $HN(R)CH(R_3)COOCH_3$, DCC, HOBt in $CH_2Cl_2/THF = 1/1$; (d) OH^- then H^+ in $H_2O/MeOH = 2/1$; (e) $HN(R)CH(R_3)COOCH_3$, DCC, HOBt in $CH_2Cl_2/THF = 1/1$; (f) $CH_3COSCH(R_1)COOH$, DCC, HOBt, Et_3N in $CH_2Cl_2/THF = 1/1$; (g) R, R_3 may constitute the side chain of a cyclic amino acid.

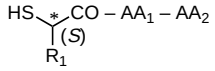
shown that the S_1' subsite of both peptidases is able to accommodate constrained moieties such as a β-methylbenzyl group. However, conformational analysis of RB 105 and its derivatives, to determine thermodynamically acceptable overlaps between stable conformers of these inhibitors and the ACE pharmacophore, designed from rigid selective inhibitors, showed that the presence of the β-methylbenzyl group in the P_1' position, precludes the presence of bulky residues in the P_2' position.[23] Recently, a great number of molecules containing polycyclic residues at the P_2' position have been described, showing that NEP seems to possess, like ACE, a large S_2' domain,[25] in agreement with earlier observations.[19] Taken together, these data support the idea that the S_1' and S_2' subsites of NEP and ACE constitute hydrophobic domains which could be efficiently occupied by cyclic or linear structures.[20,21] The introduction of cyclic moieties in the structure of the inhibitors is expected to reduce the unfavorable entropic factor in enzyme binding. However, this advantage is often outweighed by (i) difficulties in synthesizing these compounds, particularly if they have to be obtained in optically pure forms; (ii) the problems of manipulating the hydrophilic−hydrophobic balance to ensure the inhibition of vascular ACE and kidney NEP, with the same efficiency.[24] In order to improve our knowledge of the active site of both peptidases and to try to obtain new potent dual inhibitors of NEP and ACE, we have designed compounds corresponding to mercaptoacyldipeptides of general formula $HSCH(R_1)COAA_1AA_2$, in which linear or cyclic constraints have been introduced. The synthesis and inhibitory potencies of these compounds on both NEP and ACE are reported in this paper. The blockade of lung ACE and renal NEP by the two best inhibitors have been studied after oral administration. Finally, structure−activity studies including the use of a mutated NEP and molecular modeling studies with thermolysin (TLN) have allowed a model to be proposed for the recognition of the NEP and ACE subsites by these new inhibitors.

Results

1. Synthesis. The various 2-(acetylthio)alkanoic acids were synthesized by the classical two-step procedure which allowed these compounds to be obtained with a high degree of optical purity from the corresponding α-amino acids of defined absolute configuration. The α-amino acids led to the corresponding 2-bromoalkanoic acids with retention of configuration by the Fischer procedure.[26] Subsequent S_N2 thioacetylation of these bromo derivatives[15] yielded the expected (acetylthio)alkanoic acids. The coupling steps were performed by the DCC/HOBt method, and the deprotection of the sulfhydryl and carboxyl groups was achieved by alkaline hydrolysis (Scheme 1).

2. Inhibitory Activities on NEP and ACE. A comparison of the inhibitory potencies of compound **1** on NEP and ACE activities (Table 1), with those of the corresponding precursors **A** and **B**, shows that compound **1** is significantly better than **A**, which does not possess a substituted mercaptoalkyl chain ($R_1 = H$), and is also more potent than **B**, which does not contain the C-terminal alanine ($AA_2 = OH$), for both enzymes. Therefore, the inhibitory potencies of compounds containing a *N*-[2-mercapto-3-phenyl propanoyl]-L-phenylalanyl moiety, and differing only by the nature of the C-terminal amino acid (compounds **2**−**7**), were compared to those of compound **1**. For NEP inhibition, only one derivative, which contains a C-terminal tyrosine (compound **7**), was as efficient as **1**. All of the other compounds, which have more hydrophobic moieties, were significantly less active by approximately 1 order of magnitude. For ACE inhibition, compounds **6** and **7**, which contain respectively a tryptophan and a tyrosine residue, had activities in the same range as compound **1**. For this latter enzyme, slight increases in IC_{50} values were also observed with compounds **2**−**5**. Taking into account that compound **7** is a relatively good dual NEP/ACE inhibitor, some modifications were introduced on the mercaptoalkanyol moiety of this molecule in order to optimize the recognition of both enzymes. The lengthening of the alkyl chain (compound **8** versus **7** or **10** versus **9**) led to a slight reduction in the affinity for NEP (factors 7 and 3, respectively) and a small improvement in ACE recognition. Furthermore, the shortening of the thiol-containing chain (compound **11** versus **12**) had no significant effect on the inhibitory potencies for both peptidases. The nature of the amino

Table 1. Structure−Activity Relationships for Dual Inhibition of NEP and ACE by Various Mercaptoacyl Dipeptides



compd	R_1	AA_1	AA_2	IC_{50} (nM)[a] NEP	IC_{50} (nM)[a] ACE
A	H	Phe	Ala	14 ± 2	220 ± 20
B	CH_2Ph	Phe	OH	23 ± 3	44 ± 4
C	CH_2Ph	Ala	OH	4 ± 1	40 ± 4
1	CH_2Ph	Phe	Ala	2.3 ± 0.2	7.1 ± 0.5
2	CH_2Ph	Phe	1-ACP[b]	45 ± 6	30 ± 4
3	CH_2Ph	Phe	O(Bzl)Ser	50 ± 5	12 ± 2
4	CH_2Ph	Phe	S(Bzl)Cys	40 ± 3	14 ± 4
5	CH_2Ph	Phe	1-Nal[c]	120 ± 10	50 ± 5
6	CH_2Ph	Phe	Trp	50 ± 4	7.9 ± 0.6
7	CH_2Ph	Phe	Tyr	2.9 ± 0.6	5.6 ± 0.6
8	$(CH_2)_2Ph$	Phe	Tyr	20 ± 5	1.5 ± 0.3
9	CH_2Ph	Val	Tyr	3.6 ± 0.5	4.0 ± 1.0
10	$(CH_2)_2Ph$	Val	Tyr	10 ± 2	1.6 ± 0.3
11	Ph	Ile	Tyr	4.5 ± 0.3	3.5 ± 0.3
12	CH_2Ph	Ile	Tyr	3.0 ± 0.3	2.5 ± 0.2
13	CH_2Ph	Nle	Tyr	2.8 ± 0.6	4.0 ± 0.5
14	CH_2Ph	Leu	Tyr	2.2 ± 0.4	3.0 ± 0.3
15	CH_2Ph	(O-Et)Thr	Tyr	5.5 ± 0.6	6.3 ± 0.8
16	$CH(CH_3)CH_2CH_3$	Val	Tyr	5.3 ± 0.6	1.9 ± 0.4
17	$CH(CH_3)CH_2CH_3$	Phe	Tyr	3.5 ± 0.5	10 ± 2
18	$CH(CH_3)CH_2CH_3$	β(Me)Phe[d]	Tyr	32 ± 2	33 ± 4
19	$CH(CH_3)CH_2CH_3$	(OBzl)Thr	Tyr	100 ± 30	34 ± 4
20	$CH(CH_3)OCH_2Ph$	Ile	Tyr	4.1 ± 0.6	7.1 ± 0.6
21	$CH(CH_3)_2$	Ile	Tyr	1.4 ± 0.1	0.26 ± 0.02

[a] IC_{50} values are the means ± SEM of three independent determinations performed in triplicate. [b] 1-ACP, 1-aminocyclopentanoic acid. [c] 1-Nal, 1-naphthylalanine. [d] β(Me)Phe is a mixture of the four stereoisomers.

acid "AA_1" is not of major importance since the replacement of Phe by amino acids with alkylated side chains such as Val (compound **9**), Ile (compounds **11** and **12**), Nle (compound **13**), Leu (compound **14**), or (O-Et)Thr (compound **15**) led to nanomolar IC_{50} values on both enzymes. In addition, given the selective beneficial role of a C-terminal β-branched amino acid in the recognition of the active site of ACE, previously demonstrated in RB 105 series,[23] a similar modification was introduced in the mercaptoalkanoyl moiety of the inhibitors: three models of β-branched residues, corresponding respectively to the lateral chains of Ile (compounds **16**−**19**) (*O*-benzyl)Thr (compound **20**) and Val (compound **21**), were chosen. It is interesting to observe that the best dual inhibitor of this series contains two short β-branched chains (compound **21**) with IC_{50} values of 1.4 and 0.26 nM for NEP and ACE, respectively. The presence of more hindered substituents induced a significant decrease in the inhibitory potencies for both peptidases (compounds **18** and **19**).

Finally, a series of pseudotripeptide inhibitors containing a C-terminal cyclic amino acid was synthesized. Concerning NEP inhibition, the main observation was that the introduction of C-terminal cyclic amino acids such as a proline (compounds **22**, **23**, and **26**), a pipecolinic acid (**32**), or a perhydroindole carboxylic acid (**33**) did not modify the IC_{50} values significantly, as compared to their mercaptoalkanoyl amino acid precursors **B** or **C**. Conversely, the majority of the inhibitors containing these cyclic amino acids have IC_{50} values in the nanomolar or subnanomolar range for ACE. Changing the absolute configuration of the residue R_1 did not modify the activity on NEP but was unfavorable for ACE. This is illustrated by compound **22** (*S* configuration) which is 75 times more potent than its *R* isomer **23** on ACE. Furthermore, in this series of compounds, the lengthening of R_1 (compound **24** versus **22**) decreased the affinity for NEP, while the shorthening of this chain (compound **29** versus **28**) reduced the activity on both enzymes. In this series, the replacement of the benzyl group by a branched chain (compounds **34**−**37**) in position R_1 is highly unfavorable for NEP inhibition. For interaction with the ACE active site, a β-branched aliphatic residue is well accepted in the R_1 position (compound **34**) if the C-terminal residue is not a bicyclic amino acid (compounds **35** and **36**), and the presence of an α-methyl amino acid in position AA_1 (compound **25**), the modification of the proline residue in position AA_2 by decarboxylation (compound **30**), or hydroxylation (compound **31**) led to a decreased activity on both enzymes.

Compounds **C** and **1** were also tested on the mutated (Arg^{102},Glu) NEP.[27] The IC_{50} values of **C** and **1** on this enzyme were respectively 150 and 18 nM, i.e. 50-fold and 6-fold less efficient than for the wild-type NEP.

3. Molecular Modeling. Molecular modeling studies were performed with the aim of defining the position of compound **22** in the active site of NEP. This was done by using the coordinates of the NEP inhibitor thiorphan co-crystallized inside the active site of TLN as framework.[28]

The biologically active conformation of **22** in the NEP active site was investigated using the template-forcing procedure. The resulting conformation was first docked inside the active site of TLN and then refined through a minimization procedure (see the Experimental Section). As compared to the thiorphan−TLN complex, the distance between the thiol group of **22** and the zinc atom was found to be almost identical. Due to the presence in thiorphan of an additional CH_2 group between the SH and the α-carbon of the mercaptoacyl moiety, this resulted in a slight shift of the benzyl group of **22** in

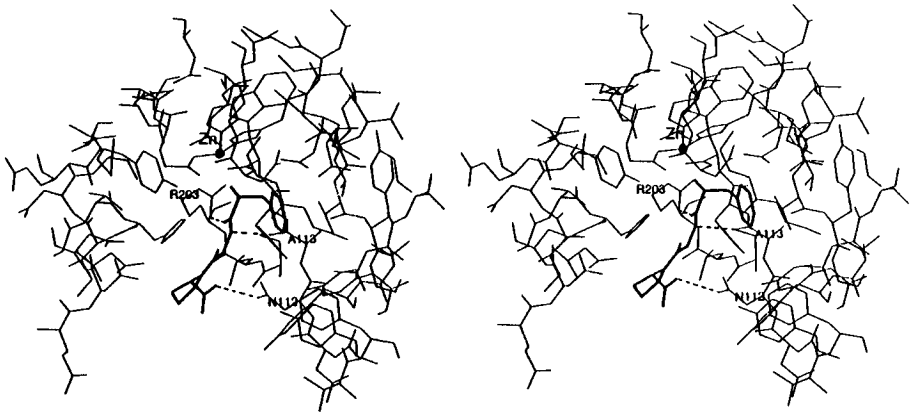
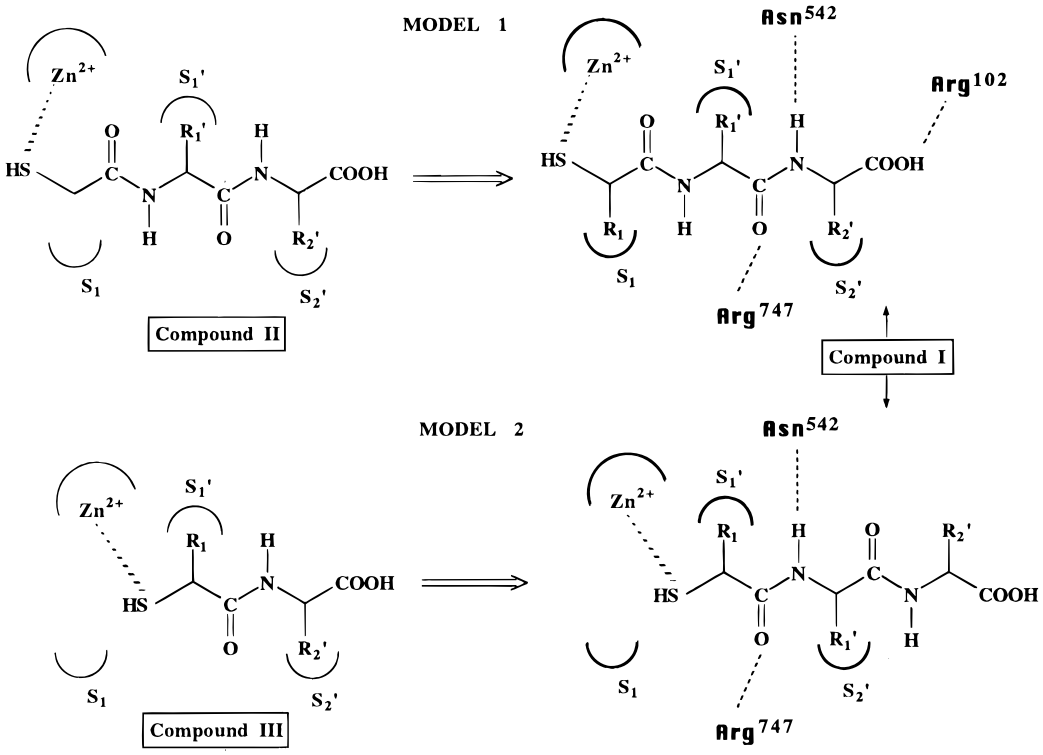


Figure 1. Stereoview of the complex between thermolysin and the dual inhibitor **22** [HSCH(CH_2Ph)CO-Ala-Pro]. Dotted lines show the hydrogen bonds between **22** and TLN active site amino acids. In this complex the C-terminal proline moiety is outside the S_2' subsite.

Scheme 2



the S_1' subsite (0.9 Å calculated from the barycenter of the phenyl rings in **22** and in thiorphan). The main hydrogen bonds found in the thiorphan−TLN complex, such as the interaction between the carbonyl of the mercaptoalkanoyl moiety and Arg^{203}, were also observed in the complex **22**−TLN. However, in this complex, the NH of the amide bond of **22** was hydrogen bonded to the CO of Ala^{113} and the free carboxylate of the proline moiety formed a hydrogen bond with the NH_2 group of Asn^{112} driving the proline moiety at the edge of the active site cleft (Figure 1).

Discussion

In order to develop a new series of dual inhibitors of NEP and ACE, we have synthesized molecules corresponding to the general formula of compound **I** (Scheme 2) which have two possible binding modes to the peptidases depending on the position of the R_1 substituent in the S_1 or S_1' subsites. In each case, the sulfhydryl group is coordinated to the zinc atom present in the catalytic site.[29] Mercaptoacetyl dipeptides of type II (Scheme 2) previously described as NEP inhibitors[30,31] have been proposed to interact by the R_1' and R_2' groups with the S_1' and S_2' subsites of the enzyme, respectively.[30] On the basis of these results the alkyl group R_1 introduced on the mercaptoacetyl moiety of compound **II** (Scheme 2) would be expected to bind the S_1 subsite (model 1). Alternatively the new inhibitors of type I could be considered to derive from the coupling of an amino acid, $H_2NCH(R_2')COOH$, with the free carboxylate group of a mercaptoacyl amino acid **III** (Scheme 2), which interacts with the S_1' and S_2' subsites of the enzyme.[19] In this case, the additional residue, R_2', would be outside this part of the active site (model 2).

In order to choose between these models, the inhibitory potencies of compounds **1**−**7** were compared with those of the corresponding precursors **A** and **B**.

Compound **1**, resulting from the introduction of a benzyl group in **A**, has an inhibitory potency for NEP 6

times better than its precursor. This could be interpreted as resulting from an additional favorable interaction of the benzyl side chain with the putative S_1 subsite of NEP (model 1). This is not contradicted by the IC_{50} values of compounds **2**−**6**, which are slightly greater than that of **1**. However, the decreased activities of compound **8** versus **7** and **10** versus **9** are difficult to explain with this model of interaction. Indeed it has been shown with carboxyalkyl dipeptide inhibitors of NEP that the S_1 subsite of this enzyme is more efficiently fitted by a phenethyl group than by a benzyl group. Thus, the compound, [$PhCH_2CH(COOH)$]-Phe-Ala, $IC_{50} = 300$ nM, is less active than [$PhCH_2CH_2CH(COOH)$]-Phe-Ala, $IC_{50} = 90$ nM.[32] This is the reverse in the S_1' subsite where the phenethyl group in HomoPhe has been shown to be detrimental for NEP recognition: $PhCH_2CH(COOH)$-HomoPhe-βAla, $IC_{50} = 8500$ nM; $PhCH_2CH(COOH)$-Phe-βAla, $IC_{50} = 21$ nM.[32] Consequently, the relative IC_{50} values of compounds **7**/**8** and **9**/**10** for NEP are more satisfactorily explained by the second model of enzyme recognition.

In order to confirm this hypothesis, the IC_{50} values of **C** and **1** were measured using recombinant rabbit NEP and its mutated analog (Arg^{102},Glu)NEP, both expressed in Cos cells.[27] As previously shown, this mutated NEP can be used as an index of inhibitor positioning in the enzyme active site.[33,34] Indeed Arg^{102} is thought to be located at the edge of the S_2' subsite where it probably interacts with the C-terminal carboxyl group of a P_2' residue, accounting for the dipeptidyl carboxypeptidase activity of NEP.[35−37] When Arg^{102} is replaced by Glu, the IC_{50} of thiorphan for the mutated enzyme is increased by more than 2 orders of magnitude, due to the ionic repulsion between the two negatively charged groups. Under, the same conditions, the IC_{50} of thiorphan amide which has no free carboxylate was increased only by a factor 6. The IC_{50} values of **C** and **1** on the mutated enzyme are respectively 150 and 18 nM, while they are 3.2 and 2.8 nM respectively, on the recombinant wild-type enzyme. The IC_{50} ratios of 50 for **C** and 6 for **1** support the model 2 of NEP recognition for these compounds in agreement with previous results obtained with other thiol inhibitors of NEP.[33,34] Additional evidence, for this model, comes from the tridimensional structure of the complex formed between thermolysin and the sulfhyldryl-containing inhibitor BAG [$HSCH_2CH(CH_2Ph)CO$-Ala-Gly-NH_2].[38] The crystallographic data show that the benzyl moiety and alanyl residue interact respectively with the S_1' and S_2' subsites of the enzyme whereas the C-terminal glycinamide emerges at the surface of the enzyme (model 2).[38] This mode of interaction was also observed with larger C-terminal amino acids.[29] The increased activity of compound **1** as compared to **B** remains to be explained. Delaney et al. has proposed the existence of an S_3' subsite in NEP.[39] Although this hypothesis cannot be excluded, it is difficult to reconcile it with the existence of an Arg^{102} residue at the surface of the enzyme. The increased potency of **1** versus **B** could therefore be due to favorable nonspecific interactions with the enzyme surface. An increase in the size of the C-terminal amino acid (compounds **2**−**6**) led to a reduction in NEP affinity probably for steric and/or conformational reasons.

For ACE inhibition an increased potency (30-fold) was obtained for compound **1** as compared to **A**, in agreement with the binding of the benzyl group of **1** in the well-characterized hydrophobic S_1 subsite of this peptidase[16,21] (model 1). The S_1' and S_2' domains in ACE could not be optimally fitted by two concomitant voluminous residues as illustrated by the decreased activities of **2** and **5** as compared to **1** or **7**. Furthermore the slight increase in IC_{50} values for **8** and **10** as compared to **7** and **9** is also consistent with model 1, since a phenethyl chain is preferred to a benzyl chain for S_1 binding.[21]

The presence of a valine as the central residue is favorable for NEP and ACE inhibition (compound **16**), but the introduction of more bulky groups in **18** and **19** decreased the interaction with both peptidases, probably due to steric hindrance. It is interesting to note that **21**, in which the β-branched chains are reversed as compared to those in **16**, is the most active compound of this series, on both enzymes, with a subnanomolar activity ($IC_{50} = 0.26$ nM) on ACE. With this compound all the hydrophobic interactions with S_1, S_1', and S_2' subsites of ACE (model 1, Scheme 2) are probably satisfied, and therefore β-methyl-substituted alkyl chains represent an interesting "working model" to further improve dual inhibition.

The proposed mode of interaction of these novel dual inhibitors is reinforced by the results obtained with the derivatives containing cyclic amino acids with the ACE active site such as proline or analogues in the C-terminal position (Table 2). It is known that a proline residue is well recognized by the S_2' subsite of ACE.[15,21] Thus, the introduction of a proline residue in **C** (compound **22** in Table 2) induced a large increase in affinity leading to a subnanomolar IC_{50} value (0.3 nM). This compound has been previously described by Ondetti *et al.*[40] as an ACE inhibitor and was recently reevaluated by Delaney *et al.*[39] on both NEP and ACE, the IC_{50} values reported (NEP = 400 nM and ACE = 30 nM) were about 100 times greater than those found in the present work (IC_{50} on NEP, 3.8 nM; IC_{50} on ACE = 0.3 nM). This discrepancy might be due to differences in the experimental conditions used for the tests. In all our measurements, thiol inhibitors were preincubated in the presence of β-mercaptoethanol, in order to prevent oxidation of the mercaptans. Subnanomolar potencies were also obtained with compounds **27** and **33** (Table 2) and probably also for **32**, taking into account that racemic pipecolinic acid was used.

In the case of NEP, it has been shown that a proline ring does not favorably fit the S_2' subsite.[18,20] If we compare compounds **22**, **32**, and **33** with the precursor **C**, or compound **26** with **B**, there are no significant differences in IC_{50} values. This strongly supports the second model of NEP recognition in which the C-terminal amino acid is outside the specific binding domains of the enzyme.[29,33,34] Compound **22** has been docked in TLN, taken as a model of the NEP active site (Figure 1). This was carried out, as described in the Experimental Section, by using the coordinates of the NEP inhibitor thiorphan, which has been cocrystallized with TLN.[28] This showed that the benzyl moiety fits the S_1' subsite and that the proline residue of **22** emerged outside the active site cleft of this enzyme as the glycinamide moiety in the TLN-BAG complex.[38] The conformation of **22** inside the TLN active site is not crucially different from that of thiorphan except for a

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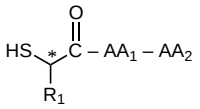

compd	R_1	stereo (*)	AA_1	AA_2	IC_{50} (nM)[a] NEP	IC_{50} (nM)[a] ACE
B	CH_2Ph	*S*	Phe	OH	23 ± 3	44 ± 4
C	CH_2Ph	*S*	Ala	OH	4 ± 1	40 ± 4
22	CH_2Ph	*S*	Ala	Pro	3.8 ± 0.6	0.3 ± 0.1
23	CH_2Ph	*R*	Ala	Pro	1.3 ± 0.2	25 ± 5
24	$(CH_2)_2Ph$	*S*	Ala	Pro	20 ± 2	0.35 ± 0.05
25	CH_2Ph	*S*	Aib[b]	Pro	46 ± 10	1.6 ± 0.6
26	CH_2Ph	*S*	Phe	Pro	30 ± 8	1.1 ± 0.2
27	CH_2Ph	*S*	Val	Pro	24 ± 9	0.85 ± 0.08
28	CH_2Ph	*S*	Ile	Pro	24 ± 5	1.7 ± 0.6
29	Ph	*S*	Ile	Pro	130 ± 15	28 ± 5
30	CH_2Ph	*S*	Ala	Pyr[c]	22 ± 5	150 ± 30
31	CH_2Ph	*S*	Val	Hyp[d]	63 ± 8	100 ± 20
32	CH_2Ph	*S*	Ala	(R,S)Pip[e]	9.5 ± 0.8	1.6 ± 0.6
33	CH_2Ph	*S*	Ala	Phi[f]	5.3 ± 0.2	0.40 ± 0.03
34	$CH(CH_3)CH_2CH_3$	*S*	Val	Pro	190 ± 30	0.58 ± 0.04
35	$CH(CH_3)CH_2CH_3$	*S*	Ala	Ind[g]	180 ± 15	3.3 ± 0.2
36	$CH(CH_3)_2$	*S*	Ile	Phi[f]	175 ± 30	28 ± 2
37	$CH(CH_3)Ph$	*S*	Val	(*R*,*S*)Pip[e]	180 ± 30	110 ± 20

[a] IC_{50} values are the means ± SEM of three independent determinations performed in triplicate. [b] Aib, 2-amino isobutyric acid. [c] Pyr, pyrrolidine. [d] Hyp, 4-hydroxyproline. [e] Pip, pipecolinic acid. [f] Phi, 1-perhydroindole-2-carboxylic acid. [g] Ind, indoline-2-carboxylic acid.

slight displacement of the benzyl moiety in the S_1' subsite, which does not seem to modify the van der Waals interactions responsible for the preference of TLN and NEP for aromatic moieties as the P_1' component. The absence of important hydrophobic interactions between the C-terminal amino acid of **22** and the enzyme surface is clearly illustrated by the similarity in the IC_{50} values for NEP of this compound and its precursor **C** which are 4 and 3.8 nM, respectively. Likewise, the inhibitory potencies of **22** and its precursor for TLN were 3.5×10^{-7} and 2×10^{-7} M respectively, emphasizing again the great analogies between the active sites of TLN and NEP.[28,42,43] Nevertheless, compounds **16** and **21** (Table 1) which have two branched chains followed by a tyrosine residue, and which have nanomolar IC_{50} values for both peptidases, exhibit a 30-fold decrease in NEP affinity when a cyclic amino acid replaces the tyrosine (compounds **34**, **36**, and **37**, in Table 2). As discussed before for compounds **18** and **19** compared with **16** and **17**, one of the simplest explanations could be that the C-terminal cyclic moieties induce a conformational change unfavorable for NEP recognition.

In conclusion, highly potent dual inhibitors can be obtained in the series of mercaptoacyldipeptides by introduction of linear constraints, such as β-branched chains, in favorable positions (compound **21**). In this series, the introduction of a C-terminal cyclic amino acid does not increase the recognition of either enzyme. Structure–activity relationships and molecular-modeling studies indicate that nanomolar inhibition of NEP can be obtained with compounds optimized for the interaction with the S_1' and S_2' domains of the enzyme, reinforcing the assumption that no true S_1 subsite exists in NEP.[41] For ACE inhibition, subnanomolar inhibitory potencies were obtained by improving interactions with the S_1, S_1', and S_2' subsites of this peptidase. This might be obtained using C-terminal cyclic amino acids such as Pro and analogues, but also with Tyr as ultimate amino acid, if residues in the P_1 and P_1' positions are judiciously chosen. The oral activity of **21** and **22** has been measured in mice. The inhibition of kidney NEP and lung ACE was measured 1 h after oral administration of a single dose (2.7×10^{-5} mol/kg) of these compounds. In these conditions, **21** inhibits 80% and 36% of kidney NEP and lung ACE, respectively, while **22** inhibits 40% of kidney NEP and 56% of lung ACE. At the same dose, the potencies of orally administered dual inhibitors belonging to the mixanpril series were better on pulmonary ACE and renal NEP. However, using the approach developed in the present work, new dual inhibitors, with both high inhibitory potency and increased oral activity and duration of action, could be designed by appropiate modifications of the hydrophilic balance of the constituting P_1, P_1', and P_2' residues. This work is now in progress in our laboratory.

Experimental Section

I. Biological Tests. [^{3}H]Tyr-D-Ala^2-Leu-enkephalin (52 Ci/mmol) was obtained from Dositek (CEA Saclay, France), *N*-Cbz-Phe-His-Leu[44] was obtained from Bachem (Buddendorf, Switzerland). Neutral endopeptidase from rabbit kidney[45] and angiotensin converting enzyme from rat testis[403] were purified to homogeneity as previously described. (Arg^{102},Glu)NEP was cloned and expressed as described.[27] Thermolysin was purchased from Sigma (France) and used without further purification.

Assay for Neutral Endopeptidase Activity. IC_{50} values were determined as previously reported.[47] NEP (final concentration 1 pmol/100 μL, specific activity for [^{3}H]Tyr-D-Ala^2-Leu-enkephalin, 0.3 nmol/mg/min) was preincubated for 15 min at 25 °C with or without increasing concentrations of inhibitor in a total volume of 100 μL in 50 mM Tris-HCl buffer, pH 7.4. [^{3}H]Tyr-D-Ala^2-Leu-enkephalin (K_m = 30 μM) was added to a final concentration of 20 nM, and the reaction was stopped after 30 min by adding 10 μl of 0.5 M HCl. The tritiated metabolites formed were separated on polystyrene beads.[48] The assay for the mutated-Glu^{102} NEP was identical to that described for wild-type 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). ACE (final concentration 0.02 pmol/100 μL, specific activity on Cbz-Phe-His-Leu (13 nmol/mg per min) was preincubated for 15 min at 37 °C with various concentrations of the inhibitors in 50 mM Tris-HCl buffer, pH 8.0. *N*-Cbz-Phe-His-Leu was added to a final concentration of 0.05 mM. The reaction was stopped after 15 min by adding 400 μL of 2 M NaOH. After dilution with 3 mL of water, the concentration of His-Leu was determined following the fluorimetric assay described by Cheung *et al.*[49] with a MPF44A Perkin-Elmer spectrofluorimeter (excitation 365 nm, emission 495 nm). The calibration curve for His-Leu was obtained by addition of increasing concentrations of His-Leu into 0.1 mL of 5.0 M Tris-HCl buffer, pH 8.0, containing the denaturated enzyme.

Assay for Thermolysin. IC_{50} values were determined as previously reported[42] using [^{3}H]Leu-enkephalin as substrate.

II. Molecular Modeling. The docking of **22** in the TLN active site was done by a template-forcing procedure (Insight II User guide, Version 2.1.0. copyright 1992, Biosym Technologies Inc., San Diego, CA) using the coordinates of thiorphan cocrystallized with TLN[28] followed by minimization. For this purpose, the heavy atoms of the (2-mercapto-3-phenylpropanoyl)glycyl moiety of **22** and all the corresponding atoms of thiorphan were used. The Gly-Pro bond of **22** was fixed in a trans conformation.

Two minimization steps were performed: first, compound **22** was minimized inside the TLN active site, whose structure was maintained fixed, and second, minimization of the complex TLN/**22** without any constraints was carried out. A zinc force field derived from *ab initio* calculations, parametrized in the Amber framework, was used in this procedure to take into account metal−protein interactions.

III. Chemistry. The natural amino acid derivatives and β-naphthyl-L-alanine were purchased from Bachem (Bubbendorf, Switzerland). The non-natural amino acids, pipecolinic acid, homophenylalanine, indoline-2-carboxylic acid, and the reagents were purchased from Aldrich-Chemie (Strasbourg, France). The synthesis of β-methylphenylalanine, (βMe)Phe,[50] and perhydroindolecarboxylic acid,[51] (Phi) were performed following procedures previously described.

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, CH_2Cl_2/MeOH = 9/1; B, CH_2Cl_2/MeOH/AcOH = 9/1/0.5; C, CH_2Cl_2/MeOH/AcOH = 9/0.5/0.5; D, CH_2Cl_2/MeOH/AcOH = 9/0.3/0.1; E, BuOH/AcOH/H_2O = 4/1/1; F, c-hexane/EtOAc/AcOH = 7/3/0.5; G, CH_2Cl_2/MeOH/AcOH/H_2O = 5/5/0.5/1. Plates were analyzed with UV, iodine vapor, or ninhydrin. The purity of the final compounds was checked by HPLC on a reverse phase kromasil C_8 (5 μm, 100 Å) column (SFCC) with TFA 0.05% (solvent A)/CH_3CN (solvent B) as the mobile phase on a Shimadzu apparatus. The eluted peaks were monitored at 210 nm.

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

The following abbreviations were used: Et_2O, ethyl ether; MeOH, methanol; BuOH, 1-butanol; EtOAc, ethyl acetate; AcOH, acetic acid; DMF, dimethylformamide; THF, tetrahydrofuran; DCC, dicyclohexylcarbodiimide; DCU, dicyclohexylurea; HOBt, 1-hydroxybenzotriazole; TFA, trifluoroacetic acid; DMSO, dimethyl sulfoxide; HMSD, hexamethyldisiloxane.

General Procedure for the Synthesis of 2-Bromoalkanoic Acid, $R_1CH(Br)COOH$. The α-amino acid (1 equiv) was dissolved in a mixture of HBr 48% (8 equiv) and water (2/3, v/v) at 0 °C. An aqueous solution of $NaNO_2$ (3.2 equiv) was added slowly, and the mixture was stirred for 2 h at the same temperature. The mixture was degassed *in vacuo* and then extracted by Et_2O. The organic layer was washed with water and saturated NaCl solution, dried over Na_2SO_4, filtered, and evaporated *in vacuo*. The 2-bromoalkanoic acids were obtained as oily products which crystallized slowly: R_1 = Ph (96%), R_f (B) 0.63; $R_1 = CH_2Ph$ (88%), R_f (A)0.60; $R_1 = (CH_2)_2$-Ph (98%), R_f (B) 0.64; $R_1 = CH(CH_3)Ph$ (84%), R_f (E) 0.90; $R_1 = CH(CH_3)_2$ (70%), R_f (C) 0.52; $R_1 = CH(CH_3)CH_2CH_3$ (87%), R_f (*C*) 0.70; $R_1 = CH(CH_3)OCH_2Ph$ (98%), R_f (A) 0.56.

General Procedure for the Synthesis of 2-(Acetylthio)-alkanoic Acid, $R_1CH(SCOCH_3)COOH$. A solution of sodium thioacetate (1.5 equiv) in DMF was added at 0 °C to a solution of 2-bromoalkanoic acid (1 equiv) in DMF. After stirring overnight at room temperature, the solvent was evaporated *in vacuo*. The residue was taken up with water, acidified with 1 N HCl, and extracted with EtOAc. The organic layer was washed with water and saturated solution of NaCl, dried over Na_2SO_4, filtered, and evaporated *in vacuo*. An oily product was obtained.

R_1 = Ph (62%): R_f (F) 0.37; ^{1}H NMR ∂ (ppm) 2.28 (CH_3CO), 5.14 (CHα), 7.30 (Ar), 12.60 (COOH). $R_1 = CH_2Ph$ (98%): R_f (E) 0.70; ^{1}H NMR ∂ (ppm) 2.25 (CH_3CO), 2.88−3.24 ($CH_2\beta$); 4.17 (CHα), 7.18 (Ar), 12.85 (COOH). $R_1 = (CH_2)_2Ph$ (79%): R_f (D) 0.29; ^{1}H NMR ∂ (ppm) 1.85−2.05 ($CH_2\beta$), 2.28 (CH_3-CO), 2.55 ($CH_2\gamma$), 3.90 (CHα), 7.15 (Ar), 12.80 (COOH). $R_1 = CH(CH_3)Ph$ (96%): R_f (A) 0.60; ^{1}H NMR ∂ (ppm) 1.18−1.23 ($CH_3\beta$), 2.15−2.30 (CH_3CO), 3.14 (CHβ), 4.18 (CHα), 7.20 (Ar), 12.70 (COOH). $R_1 = CH(CH_3)_2$ (94%): R_f (C) 0.40; ^{1}H NMR ∂ (ppm) 0.90 ($2CH_3$), 2.10 (CHβ), 2.30 (CH_3CO), 3.85 (CHα), 12.40 (COOH). $R_1 = CH(CH_3)CH_2CH_3$ (78%): R_f (A) 0.44; ^{1}H NMR ∂ (ppm) 0.82 ($2CH_3$), 1.20−1.30 ($CH_2\gamma$), 1.92 (CHβ), 2.30 (CH_3CO), 4.10 (CHα), 12.30 (COOH). $R_1 = CH(CH_3)OCH_2Ph$ (64%): R_f (A) 0.46; ^{1}H NMR ∂ (ppm) 1.10 ($CH_3\beta$), 2.34 (CH_3-CO), 3.90 (CHβ), 4.20 (CHα), 4.45 (OCH_2), 7.25 (Ar), 12.90 (COOH).

General Procedure for the Coupling Steps. A solution of the α-amino ester or dipeptide ester (1 equiv) and triethylamine (1.1 equiv) in $CHCl_3$, a solution of HOBt (1 equiv) in THF, and a solution of DCC (1.5 equiv) in $CHCl_3$ were successively added to a solution of the compound containing a free carboxylic group (Boc-amino acid or 2-(acetylthio)-alkanoic acid) in THF at 0 °C. After stirring overnight, the mixture was filtered and evaporated *in vacuo*. The residue was dissolved in EtOAc, washed, and dried as classically reported.

General Procedures for the Deprotection of the Functional Groups. The N-Boc protection was cleaved by treatment with 10 equiv of TFA in CH_2Cl_2 (50/50, v/v). The ester and the acetyl protection of thiol group were cleaved by alkaline hydrolysis with degassed reagents in order to prevent the oxidation of the free thiol group.

Compound 1: white solid; mp 145 °C; R_f (F) 0.22; HPLC t_R (40% B) 13.5 min; ^{1}H NMR ∂ (ppm) 1.22, (CH_3(Ala)), 2.40 (SH), 2.50 and 3.08 ($2CH_2\beta$), 3.60 (*CH*SH), 4.12 (CHα(Ala)), 4.48 (CHα(Phe)), 7.17 (Ar), 8.12 and 8.27 (NH), 12.50 (COOH).

Compound 2: white solid; mp 88−91°C dec; R_f (C) 0.60; HPLC t_R (55% B) 7.9 min; ^{1}H NMR ∂ (ppm) 1.52 (4H), 1.76 (2H), 1.95 (2H, cyclopentyl), 2.55 (HS), 2.70, 2.90, and 3.02 ($2CH_2\beta$), 3.65 (*CH*SH), 4.50 (CHα(Phe)), 7.20 (Ar), 8.15 (NH), 12.12 (COOH).

Compound 3: white solid; mp 125 °C; R_f (C) 0.43; HPLC t_R (55% B) 11.2 min; ^{1}H NMR ∂ (ppm) 2.30 (SH), 2.70−3.00 ($2CH_2\beta$), 3.45−3.70 (CH_2O + *CH*SH), 4.49 (CHα(Phe) + O*CH*$_2$-Ph), 4.60 (CHα(Ser)), 7.10−7.20 (Ar), 8.18−8.30 (NH), 12.10 (COOH).

Compound 4: white solid; mp 135 °C; R_f (C) 0.51; HPLC t_R (55% B) 12.45 min; ^{1}H NMR ∂ (ppm) 2.30 (HS), 2.75−3.75 (3 × $CH_2\beta$), 3.4 (*CH*SH), 3.68 (S*CH*$_2$Ph), 4.36 (CHα(Phe)), 4.57 (CHα(Cys)), 7.12 (Ar), 8.20−8.32 (NH), 12.60 (COOH).

Compound 5: white solid; mp 145 °C; R_f (A) 0.28; HPLC t_R (37% B) 6.2 min; ^{1}H NMR ∂ (ppm) 2.40 (SH), 2.55−3.05 (3 × $CH_2\beta$), 3.50 (*CH*SH), 4.50 (CHα(Phe) + CHα(Nal)), 7.1 (Ar-(Phe)), 7.3−8.05 (Ar(Nal)), 8.05−8.45 (NH), 12.40 (COOH).

Compound 6: white solid; mp 156 °C; R_f (C) 0.42; HPLC t_R (55% B) 9.85 min; ^{1}H NMR ∂ (ppm) 2.30 (SH), 2.70−3.05 (3 × $CH_2\beta$), 3.60 (*CH*SH), 4.44 (CHα(Trp)), 4.51 (CHα(Phe)), 6.80−7.40 (Ar), 8.10−8.13 (NH), 10.8 (NH(Ind)), 12.60 (COOH).

Compound 7: white solid; mp 189 °C; R_f (B) 0.56; HPLC t_R (55% B) 6.45 min; ^{1}H NMR ∂ (ppm) 2.38 (HS), 2.60−3.10 (3 × $CH_2\beta$), 3.60 (*CH*SH), 4.30 (CHα(Tyr)), 4.50 (CHα(Phe)), 6.40−6.98 (Ar(Tyr)), 7.14 (Ar(Phe)), 8.15−8.20 (NH), 9.18 (OH-(Tyr)), 12.60 (COOH).

Compound 8: white solid; mp 138 °C; R_f (B) 0.42; HPLC t_R (55% B) 7.50 min; ^{1}H NMR ∂ (ppm) 1.70−1.90 (*CH*$_2$(CHSH)), 2.50 (HS), 2.65−3.00 (2 × CH$_2\beta$ + CH$_2\gamma$), 3.70 (*CH*SH), 4.30 (CHα(Tyr)), 4.50 (CHα(Phe)), 6.60−6.95 (Ar(Tyr)), 7.20 (Ar-(Phe)), 8.20 (2 × NH), 9.20 (OH(Tyr)), 12.70 (COOH).

Compound 9: white solid; mp 182 °C; R_f (C) 0.24; HPLC t_R (45% B) 8.00 min; ^{1}H NMR ∂ (ppm) 1.30 (CH$_3$(Val)), 1.86 (CHβ(Val)), 2.64 (SH), 2.70−3.10 (2 × CH$_2\beta$), 3.70 (*CH*SH), 4.20 (2 × CHα), 6.60−6.95 (Ar(Tyr)), 7.12 (Ar(Phe)), 7.96−8.18 (NH), 9.12 (OH(Tyr)), 12.45 (COOH).

Compound 10: white solid; mp 190 °C; R_f (B) 0.44; HPLC t_R (45% B) 9.80−10.35 min; ^{1}H NMR ∂ (ppm) 0.80 (CH$_3$(Val)), 1.75−1.90 (*CH*$_2$-CH-SH), 1.90 (CHβ(Val)), 2.50 (HS), 2.65−2.95 (CH$_2\beta$ + CH$_2\gamma$), 3.45 (*CH*SH), 4.20−4.28 (2 × CHα), 6.00−6.95 (Ar(Tyr)), 7.15 (Ar(Phe)), 8.00−8.15 (NH), 9.14 (OH(Tyr)), 12.50 (COOH).

Compound 11: white solid; mp >250 °C dec; R_f (B) 0.42; HPLC t_R (50% B) 6.88 min; ^{1}H NMR ∂ (ppm) 0.80 (2 × CH$_3$-(Ile)), 1.08−1.40−1.68 (CHβ + CH$_2\gamma$(Ile)), 2.58 (SH), 2.60−2.90 (CH$_2\beta$(Tyr)), 4.20−4.25 (CHα(Ile + Tyr)), 4.85 (CH(Ph)), 6.55−6.95 (Ar(Tyr)), 7.30 (Ar(Ph)), 8.15 (2NH), 9.10 (OH(Tyr)), 12.45 (COOH).

Compound 12: white solid; mp 185 °C; R_f (B) 0.28; HPLC t_R (45% B) 9.23 min; ^{1}H NMR ∂ (ppm) 0.80 (2CH$_3$(Ile)), 1.03−1.35−1.68 (CHβ + CH$_2\gamma$(Ile)), 2.65 (SH), 2.75−3.20 (CH$_2\beta$(Ph + Tyr)), 3.68 (*CH*SH), 4.10 (CHα(Ile)), 4.30 (CHα(Tyr)), 6.65−7.00 (Ar(Tyr)), 7.18 (Ar(Ph)), 8.05−8.20 (NH), 9.10 (OH(Tyr)), 12.55 (COOH).

Compound 13: white solid; mp 130−140 °C dec; R_f (D) 0.38; HPLC t_R (46% B) 9.4 min; ^{1}H NMR ∂ (ppm) 0.80 (ϵCH$_3$-(Nle)), 1.25 (CH2γ,∂(Nle)), 1.55 (CH2β(Nle)), 2.60 (SH), 2.80−3.20 (CH2β(Ph + Tyr)), 3.65 (*CH*SH), 4.00−4.12 (CHα(Nle + Tyr)), 6.60−6.95 (Ar(Tyr)), 7.24 (Ar(Ph)), 8.05−8.20 (NH), 9.55 (OH(Tyr)), 12.48 (COOH).

Compound 14: white solid; mp 134 °C; R_f (B) 0.45; HPLC t_R (50% B) 7.25 min; ^{1}H NMR ∂ (ppm) 0.75 (CH$_3$(Leu)), 1.30 (CH$_2\beta$(Leu)), 1.48 (CHγ(Leu)), 2.60 (SH); 2.70−3.10 (CH$_2\beta$(Ph + Tyr)), 3.54 (*CH*SH), 4.22 (CHα(Leu + Tyr)), 6.60−6.95 (Ar-(Tyr)), 7.15 (Ar(Ph)), 7.92−8.10 (NH), 9.15 (OH(Tyr)), 12.50 (COOH).

Compound 15: Oily compound; R_f (B) 0.44; HPLC t_R (45% B) 9.00 min; ^{1}H NMR ∂ (ppm) 1.00 (2 × CH$_3$), 2.60 (SH), 2.70−3.15 (CH$_2\beta$(Ph + Tyr)), 3.40 (OCH$_2$), 3.70 (CHβ(Thr)), 3.85 (*CH*SH), 4.34 (CHα(Tyr + Thr)), 6.60−6.95 (Ar(Tyr)), 7.18 (Ar-(Ph)), 7.80−8.00 (NH), 9.20 (OH(Tyr)), 12.4 (COOH).

Compound 16: white product; mp 150 °C; R_f (D) 0.20; HPLC t_R (50% B) 5.76 min; ^{1}H NMR ∂ (ppm) 0.75 (CH$_3$(Val + Ile)), 1.00−1.25 (CH$_2\gamma$(Ile)), 1.52 (CHβ(Ile)), 1.90 (CHβ(Val)), 2.40 (SH), 2.75−2.88 (CH$_2\beta$(Tyr)), 3.28 (*CH*SH), 4.16−4.30 (CHα(Val + Tyr)), 6.58−6.95 (Ar(Tyr)), 7.90−8.12 (NH), 9.12 (OH(Tyr)), 12.56 (COOH).

Compound 17: white product; mp 120 °C; R_f (B) 0.40; HPLC t_R (50% B) 8.10 min; ^{1}H NMR ∂ (ppm) 0.72 (2 × CH$_3$-(Ile)), 0.95−1.20 (CH$_2\gamma$(Ile)), 1.60 (CHβ(Ile)), 2.15 (SH), 2.60−3.00 (CH$_2\beta$(Phe + Tyr)), 3.20 (*CH*SH), 4.32 (CHα(Tyr)), 4.52 (CHα(Phe)), 6.60−6.95 (Ar(Tyr)), 7.20 (Ar(Phe)), 9.12 (OH-(Tyr)), 12.62 (COOH).

Compound 18 (mixture of diastereoisomers): white product; mp 125 °C; R_f (B) 0.50−0.60; HPLC t_R (50% B) 9.13 min; ^{1}H NMR ∂ (ppm) 0.50−0.90 (3 × CH$_3$), 0.95−1.20−1.50 (CHβ + CH$_2\gamma$(Ile)), 2.12 (SH), 2.65−3.20 (CH$_2\beta$(Tyr) + CHβ(Phe)), 3.30 (*CH*SH), 4.30−4.60 (CHα(Phe + Tyr)), 6.60−6.95 (Ar-(Tyr)), 7.18 (Ar(Phe)), 7.82−8.50 (NH), 9.10 (OH(Tyr)), 12.52 (COOH).

Compound 19: Oily product; R_f (C) 0.30; HPLC t_R (45% B) 8.72 min; ^{1}H NMR ∂ (ppm) 0.75−1.05 (2 × CH$_3$(Ile) + CH$_3$-(Thr)), 1.35−1.60−1.70 (CHβ + CH$_2\gamma$(Ile)), 2.45 (SH), 2.80−2.90 (CH$_2\beta$(Tyr)), 3.45 (*CH*SH), 3.85 (CHα(Tyr)), 4.40 (OCH$_2$ + CHα + CHβ(Thr)), 6.60−6.85 (Ar(Tyr)), 7.25 (Ar(Ph)), 7.92−8.08 (NH), 9.15 (OH(Tyr)), 12.70 (COOH).

Compound 20: white solid; mp 82 °C; R_f (B) 0.45; HPLC t_R (50% B) 8.00 min; ^{1}H NMR ∂ (ppm) 0.75 (2 × CH$_3$(Ile)), 1.15 (CH$_3$(Thr)), 1.00−1.35−1.75 (CHβ + CH$_2\gamma$(Ile)), 2.55 (SH), 2.68−2.80 (CH$_2\beta$(Tyr)), 3.62 (*CH*SH), 3.68 (CHβ(Thr)), 4.28 (CHα(Ile + Tyr)), 4.40 (CH$_2$O), 6.55−6.90 (Ar(Tyr)), 7.18 (Ar-(Ph)), 8.00−8.20 (2 × NH), 9.12 (OH(Tyr)), 12.43 (COOH).

Compound 21: white solid; mp 199 °C; R_f (B) 0.42; HPLC t_R (50% B) 5.84 min; ^{1}H NMR ∂ (ppm) 0.72−0.85 (2 × CH$_3$-(Val) + 2 × CH$_3$(Ile)), 1.00 (CHβ(Ile)), 1.38−1.65 (CH$_2\gamma$(Ile)), 1.80 (CHβ(Val)), 2.30 (SH), 2.75−2.98 (CH$_2\beta$(Tyr)), 3.15 (*CH*SH), 4.18−4.32 (CHα(Ile + Tyr)), 6.55−6.90 (Ar(Tyr)), 7.90−8.08 (2 × NH), 9.15 (OH(Tyr)), 12.50 (COOH).

Compound 22: white solid; mp 74 °C; R_f (B) 0.59; HPLC t_R (37% B) 6.96 min; ^{1}H NMR ∂ (ppm) 1.12 (CH$_3$(Ala)), 1.78 (CH$_2\gamma$(Pro)), 1.81−2.00 (CH$_2\beta$(Pro)), 2.70 (SH), 2.75−3.08 (CH$_2\beta$(Ph)), 3.25−3.33 (CH$_2$∂(Pro)), 3.62 (*CH*SH), 4.10 (CHα-(Pro)), 4.45 (CHα(Ala)), 7.15 (Ar(Ph)), 8.25 (NH), 12.35 (COOH).

Compound 23: white solid; mp 157 °C; R_f (B) 0.61; HPLC t_R (37% B) 7.05 min; ^{1}H NMR ∂ (ppm) 0.92 (CH$_3$(Ala)), 1.78 (CH$_2\gamma$(Pro)), 1.75−2.08 (CH$_2\beta$(Pro)), 2.69 (SH), 2.88−3.02 (CH$_2$-(Ph)), 3.45−3.55 (CH$_2$∂(Pro)), 3.65 (*CH*SH), 4.15 (CHα(Pro)), 4.45 (CHα(Ala)), 7.12 (Ar(Ph)), 8.18 (NH), 12.40 (COOH).

Compound 24: white paste; R_f (C) 0.60; HPLC t_R (45% B) 6.42 min; ^{1}H NMR ∂ (ppm) 1.14 (CH$_3$(Ala)), from 1.80 to 2.10 (CH$_2\beta$,γ(Pro) + CH$_2\beta$(Ph)), 2.52 (CH$_2\gamma$(Ph)), 2.65 (SH), 3.37 (*CH*SH), 3.46−3.60 (CH$_2$∂(Pro)), 4.18 (CHα(Pro)), 4.67 (CHα-(Ala)), 7.15 (Ar(Ph)), 8.25 (NH), 12.35 (COOH).

Compound 25: Oily product; R_f (F) 0.37; HPLC t_R (40% B) 6.6−6.8 min; ^{1}H NMR ∂ (ppm) 1.25 (2 × CH$_3$), 1.70−2.2 (CH$_2\beta$ + γ(Pro)), 2.6 (SH), 2.7−3.1 (CH$_2\beta$(Phe)), 3.2−3.55 (*CH*SH + CH$_2$∂(Pro)), 4.1 (CHα(Pro)), 7.18 (Ar(Ph)), 8.12 (NH), 12.1 (COOH).

Compound 26: white solid; mp 69 °C; R_f (C) 0.60; HPLC (50% B) 8.10 min; ^{1}H NMR ∂ (ppm) 1.85 (CH$_2\gamma$(Pro)), 1.85−2.05 (CH$_2\beta$(Pro)), 2.50 (SH), 2.72−3.00 (CH$_2\beta$(Phe + Ph)), 3.61 (*CH*SH), 4.10 (CHα(Pro)), 4.60 (CHα(Phe)), 7.12 (Ar(Ph + Phe)), 8.35 (NH), 12.40 (COOH)).

Compound 27: white solid; mp 155 °C; R_f (A) 0.26; HPLC t_R (50% B) 6.25 min; ^{1}H NMR ∂ (ppm) 0.80 (2 × CH$_3$(Val)), 1.78−2.04 (CHβ(Val) + (CH$_2\beta$, CH$_2\gamma$(Pro)), 2.65 (SH), 2.78−3.05 (CH$_2\beta$(Phe)), 3.48 (CH$_2$∂(Pro)), 3.70 (*CH*SH), 4.10 (Cα-(Pro)), 4.25 (CHα(Val)), 7.14 (Ar(Ph)), 8.15 (NH), 12.33 (COOH).

Compound 28: white solid; mp 158−162 °C dec; R_f (D) 0.22; HPLC t_R (45% B) 9.22 min; ^{1}H NMR ∂ (ppm) 0.80 (2 × CH$_3$), 1.04 (CHβ(Ile)), 1.50−1.78 (CH$_2\gamma$(Ile)), 1.78−2.05 (CH$_2\beta$ + γ(Pro)), 2.70 (SH), 2.80−3.05 (CH$_2\beta$(Ph)), 3.50−3.70 (CH$_2$∂-(Pro) + *CH*SH), 4.08 (CHα(Pro)), 4.38 (CHα(Ile)), 7.12 (Ar-(Ph)), 8.20 (NH), 12.35 (COOH).

Compound 29: Oily compound; R_f (C) 0.33; HPLC t_R (50% B) 7.94 min; ^{1}H NMR ∂ (ppm) 0.80 (2 × CH$_3$), 1.15 (CHβ(Ile)), 1.50−1.80 (CH$_2\beta$(Ile)), 1.80−2.10 (CH$_2\beta$ + γ(Pro)), 2.45 (SH), 3.25−3.78 (CH$_2$∂(Pro)), 4.10 (CHα(Pro)), 4.32 (CHα(Ile)), 4.80 (CH(Ph)), 7.20−7.40 (Ar(Ph)), 8.40 (NH), 12.36 (COOH).

Compound 30: Oily product; R_f (A) 0.40; HPLC t_R (45% B) 6.80 min; ^{1}H NMR ∂ (ppm) 1.10 (CH$_3$), 1.79 (CH$_2\beta$,β'(Pyr)), 2.68 (SH), 2.80−3.08 (CH$_2\beta$(Ph)), 3.2 (CH$_2\alpha$,α'(Pyr)), 3.64 (*CH*SH), 4.45 (CHα(Ala)), 7.15 (Ar(Ph)), 8.20 (NH), 12.36 (COOH).

Compound 31: white solid; mp 100 °C dec; R_f (C) 0.30; HPLC t_R (50% B) 7.6 min; ^{1}H NMR ∂ (ppm) 0.50−0.85 (2 × CH$_3$(Ile)), 1.05−1.50−1.65 (CHβ, CH$_2\gamma$(Ile)), 1.90−2.05 (CH$_2\beta$-(Hyp)), 2.65 (SH), 2.70−3.10 (CH$_2\beta$(Ph)), 3.60 (CHα(Ph) + CH$_2$∂(Hyp)), 4.25 (CHα(Ile) + (CHα + CHγ(Hyp)), 5.10 (OH-(Hyp)), 7.20 (Ph)), 8.15 (NH), 12.37 (COOH).

Compound 32 (mixture of stereoisomers): oily product; R_f (C) 0.48; HPLC t_R (50% B) 5.75 min; ^{1}H NMR ∂ (ppm) 1.10 (CH$_3$(Ala)), 1.25−1.55−2.04 (CH$_2\beta$,γ,∂(Pip)), 2.70 (HS), 2.75−3.08 (CH$_2\beta$(Ph)), 3.10−3.55 (CH$_2\epsilon$(Pip)), 3.65 (CHα(Ph)), 4.70 (CHα(Ala)), 4.95 (CHα(Pip)), 7.18 (Ar), 8.20 (NH), 12.05 (COOH).

Compound 33: white solid; mp 148 °C; R_f (G) 0.79; HPLC t_R (50% B) 7.67 min; ^{1}H NMR ∂ (ppm) 1.10 (CH$_3$), 1.00−2.30 (CH$_2$(Phi)), 2.65 (SH), 2.75−3.10 (CH$_2\beta$(Ph)), 3.65 (*CH*SH), 4.04 (CHα(Phi)), 4.46 (CHα(Ala)), 7.12 (Ar(Ph)), 8.30 (NH), 12.28 (COOH).

Compound 34: oily product; R_f (B) 0.59; HPLC t_R (50% B) 6.86 min; ^{1}H NMR ∂ (ppm) 0.90 (4 × CH$_3$), 1.00−2.12 (CHβ-(Ile) + CH$_2\gamma$(Ile) + CHβ(Val) + (CH$_2\beta$ + γ(Pro)), 2.40 (SH), 3.54 (*CH*SH), 3.30−3.76 (CH$_2$∂(Pro)), 4.17 (CHα(Pro)), 4.30 (CHα(Val)), 8.16 (NH), 12.36 (COOH).

Compound 35: white solid; mp >200 °C dec; R_f (A) 0.61; HPLC t_R (50% B) 9.32 min; ^{1}H NMR ∂ (ppm) 0.80 (2 × CH$_3$-

(Ile)), 1.04 (CHβ(Ile)), 1.25 (CH_3(Ala)), 1.30−1.50 ($CH_2\gamma$(Ile)), 1.65 ($CH_2\beta$(Ind)), 2.38 (SH), 3.60 (*CH*SH), 4.50 (CHα(Ala)), 5.10 (CHα(Ind)), 7.00−7.18 (Ar(Ind)), 8.10 (NH), 12.36 (COOH).

Compound 36: white solid mp >250 °C dec; R_f (C) 0.54; HPLC t_R (50% B) 9.03 min; ^{1}H NMR ∂ (ppm) 0.80−0.90 (CH_3-(Val + Ile)), 1.00−2.25 (CHβ(Ile) + $CH_2\gamma$(Ile) + CHβ(Val) + CH_2(Phi)), 2.40 (SH), 3.58 (*CH*SH), 4.12 (CHα(Phi)), 4.25 (CHα(Ile)), 8.30 (NH), 12.36 (COOH).

Compound 37 (mixture of stereoisomers): oily product; R_f (B) 0.48; HPLC t_R (50% B) 9.3, 9.8, 10.3, and 10.6 min; ^{1}H NMR ∂ (ppm) from 0.70 to 2.20 ($CH_2\beta,\gamma,\partial$(Pip) + CH_3(Val) + $CH_3\beta$-(Ph) + CHβ(Val)), 2.60 (SH), 2.95 (CHβ(Ph)), 3.60 (*CH*SH), 3.70−3.95 ($CH_2\partial$(Pip)), 4.30 (CHα(Val)), 5.05 (CHα(Pip)), 7.20 (CH(Ar)), 8.20 (NH), 12.60 (COOH).

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