Toward an Optimal Joint Recognition of the S_1' Subsites of Endothelin Converting Enzyme-1 (ECE-1), Angiotensin Converting Enzyme (ACE), and Neutral Endopeptidase (NEP)

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The formation of vasoconstrictors (e.g., angiotensin II and endothelin) and the inactivation of vasodilators (e.g., bradykinin and atrial natriuretic) by membrane-bound zinc metallopeptidases are key mechanisms in the control of blood pressure and fluid homeostasis. The way in which these peptides modulate physiological functions has been intensively studied. With the aim to develop compounds that can jointly block the three metallopeptidases—neutral endopeptidase (NEP, neprilysin), angiotensin-converting enzyme (ACE), and endothelin-converting enzyme (ECE-1)—we studied the common structural specificity of the S_1' subsites of these peptidases. Various mercaptoacyl amino acids of the general formula HS-CH_2-CH(R_1')CO-Trp-OH, possessing more or less constrained R_1' side chains, were designed. The mercapto-acyl synthons contain one or two asymmetrical centers. The K_i values of the separated stereoisomers of the most efficient inhibitors were used to determine the stereochemical preference of each enzyme. A guideline for the joint inhibition of the three peptidases was obtained with the (2*R*,3*R*) isomer of compound **13b**. Its K_i values on NEP, ACE, and ECE were 0.7, 43, and 26 nM, respectively.

Introduction

Four peptidergic systems are involved in the regulation of blood pressure, fluid volume, and electrolyte homeostasis. Two of them, angiotensin II (AII) and endothelin I (ET-1), are derived from inactive precursors and are strong vasoconstrictors. Conversely, bradykinin (Bk) is a potent vasodilatator, and atrial natriuretic peptide (ANP) has natriuretic and diuretic activities.[1] Consequently, modulating these physiologically counteracting systems could be of great interest for the treatment of various cardiovascular diseases. One way to obtain such a result is to decrease the formation of AII and ET-1 and to increase the amount of Bk and ANP. Theoretically, this could be achieved by simultaneously inhibiting the peptidases involved in the generation of vasoconstrictor peptides, i.e., angiotensin-converting enzyme (ACE, EC 3.4.15.1) for AII and endothelin converting enzyme (ECE-1, EC 3.4.24.71) for ET-1, and in the inactivation of the diuretic and vasorelaxant peptides, i.e., neutral endopeptidase (NEP, EC 3.4.24.11, neprilysin) for ANP and NEP and ACE for Bk (reviews in refs 2−5).

These three peptidases belong to the same family of membrane-bound zinc metallopeptidases and have the same mechanism of action.[4] Selective inhibitors have been reported for each enzyme.[6−8] ACE inhibitors are the most commonly used drugs for the treatment of hypertension and congestive heart failure.[9,10] The concept of dual NEP/ACE inhibition[11] led to the generation of a large number of compounds with interesting therapeutic profiles compared to selective inhibitors.[5] However, one possible limitation of this approach could be an increase in plasma levels of ET-1 since the degradation of this peptide is achieved by NEP.[12] This problem can be overcome by inhibiting ECE with compounds that block the enzymatic activity of the three targeted metallopeptidases. This can be achieved by synthesizing small molecules containing efficient zinc ligands.

Therefore, we tried to design such compounds so that we could optimize the control of the various cardiovascular functions associated with the four peptidergic systems. We developed compounds that interact with the S_1' and S_2' subsites of the three enzymes and that bear a thiol group as a zinc ligand. All of the molecules designed have the general formula HS-CH_2-CH(R_1')-CO-TrpOH. The tryptophan residue was selected as P_2' component for all of these molecules because the indole moiety is well recognized by the S_2' subsite of ECE.[13,14] Various R_1' side chains containing aromatic rings were tested for interaction with the S_1' subsite of the three targeted enzymes. Mercaptoacyl synthons contain one or two asymmetrical centers, thus the corresponding inhibitors are mixtures of two or four diastereoisomers. We separated each of these isomers and used physicochemical methods and X-ray crystallography to determine and to confirm the absolute configuration of each isomer. Their K_i values on the three peptidases were measured, and their stereochemical preferences were determined so that triple inhibitors with about the same nanomolar inhibitory potency for the three peptidases could be obtained.

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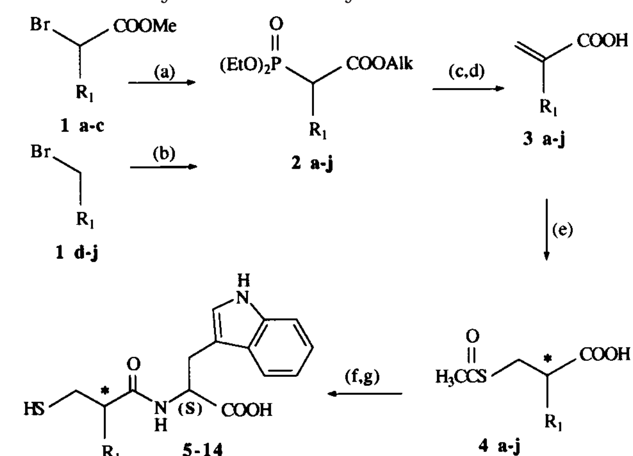
Scheme 1. Synthetic Pathway of Inhibitors **5**−**14**[a]



[a] (a) $(EtO)_3P$; (b) triethylphosphonoacetate, NaH, THF; (c) $(H_2CO)_n$, K_2CO_3, THF; (d) NaOH, acetone; (e) thioacetic acid, $CHCl_3$; (f) EDCl, HOBT, Et_3N, HCl, Trp-OR; (g) TFA, NaOH, MeOH.

Results

I. Chemistry. The various inhibitors were prepared as outlined in Scheme 1. In brief, compounds **2** were condensed with formaldehyde by a Wittig−Horner olefination. This was followed by alkaline hydrolysis, which yielded substituted acrylic acids (**3**). Racemic mixtures (**4**) were obtained by Michäel addition of thiolacetic acid to compounds **3**. The inhibitors (**5**−**14**) were obtained by coupling compounds **4** with tryptophan methyl or *tert*-butyl ester by use of the EDCI/HOBt method, followed by alkaline or acidic hydrolysis of the thioester and ester groups. The 2-substituted phosphonoacetates **2** were obtained by two methods depending on the nature of the substituent R_1' (Scheme 1). The first method was an Arbusov reaction between triethyl phosphite and methyl-1-bromo-1-aryl acetates (**1a**−**c**). The second method involved the reaction between the triethylphosphonoacetate anion with various bromoalkyl derivatives (**1d**−**j**). Due to the presence of two unresolved asymmetrical carbons in synthons **4e**−**j**, the inhibitors **9**−**14** were obtained as a mixture of four stereoisomers. To obtain optically pure species, the most interesting compounds (**9**, **10**, **13**, and **14**) were separated by a two-step process (Scheme 2). The corresponding synthons (**4e**, **4f**, **4i**, and **4j**) were separated by preparative HPLC into two diastereoisomeric sets of enantiomers, designated $\mathbf{4_1}$ and $\mathbf{4_2}$ according to their elution order. The fractions $\mathbf{4_1}$ and $\mathbf{4_2}$ were present in 30% and 70% of the separated synthons, respectively.

Each pair of enantiomers ($\mathbf{4_1}$ and $\mathbf{4_2}$) was coupled with the optically pure *S*-tryptophan *tert*-butyl ester, leading to a new mixture of diastereoisomers which were also separated by preparative HPLC and deprotected (Scheme 2). The different stereoisomers obtained in enantiomeric pure form (≥98% based on analytical HPLC) were designated "a" and "b" when they were derived from $\mathbf{4_1}$ and "c" and "d" when they were derived from $\mathbf{4_2}$.

As the HPLC separation was poorly efficient for compounds **9a**−**d**, the four optically pure stereoisomers were obtained from the four chiral synthons of **4e**, resolved by chiral amines, as previously described.[15]

II. Assignment of the Absolute Configuration of the Asymmetrical Carbons in the Separated Isomers of Inhibitors 9, 10, 13, and 14. A convergent approach was used to assign the absolute configuration of the asymmetrical carbons in the separated isomers of inhibitors **9**, **10**, **13**, and **14**.

In a previous report[15] the four stereoisomers of **4e** were separated by chiral amines and their respective absolute configurations determined by using stereoselective syntheses. These results show that, in our experimental conditions, the same assignment based on the HPLC retention times could be proposed: the first set of enantiomers ($\mathbf{4e_1}$) is a mixture of 2*R*,3*R* and 2*S*,3*S* isomers, the other set ($\mathbf{4e_2}$) contained the other two stereoisomers 2*R*,3*S* and 2*S*,3*R*. The first set of enantiomers ($\mathbf{4e_1}$) only represented 30% of the initial mixture, whereas the second set ($\mathbf{4e_2}$) was the most abundant (70%).

As the relative proportion of each pair $\mathbf{4f_1}/\mathbf{4f_2}$, $\mathbf{4i_1}/\mathbf{4i_2}$, and $\mathbf{4j_1}/\mathbf{4j_2}$ was identical to the relative proportion of $\mathbf{4e_1}/\mathbf{4e_2}$, the same stereochemical assignment was postulated for the four synthons.

NMR has been used to assign the stereochemistry of a dipeptide or a pseudo dipeptide.[16] The general rule is that, in a dipeptide unit containing one aromatic side chain, the relative absolute configuration of each residue can be deduced from the chemical shifts of the protons of the nonaromatic side chain: the protons were shielded when the two residues had opposite absolute configuration (*R*,*S* or *S*,*R*) as compared to those having identical configurations (*R*,*R* or *S*,*S*).

All of the inhibitors studied have an *S*-tryptophan in the C terminal position. Thus, the relative chemical shifts of the γ methyl side chain in **9** and **10**, or of the γ methylene in **13** and **14**, allow the absolute configurations of C2 to be determined in the four stereoisomers (Table 1).

The γ methyl in **9** and **10** and the γ methylene in **13** and **14** are more shielded in the "b" and "d" isomers than in the "a" and "c" isomers indicating that in the former C2 has an *R* configuration and that in the latter it has an *S* configuration (Table 1).

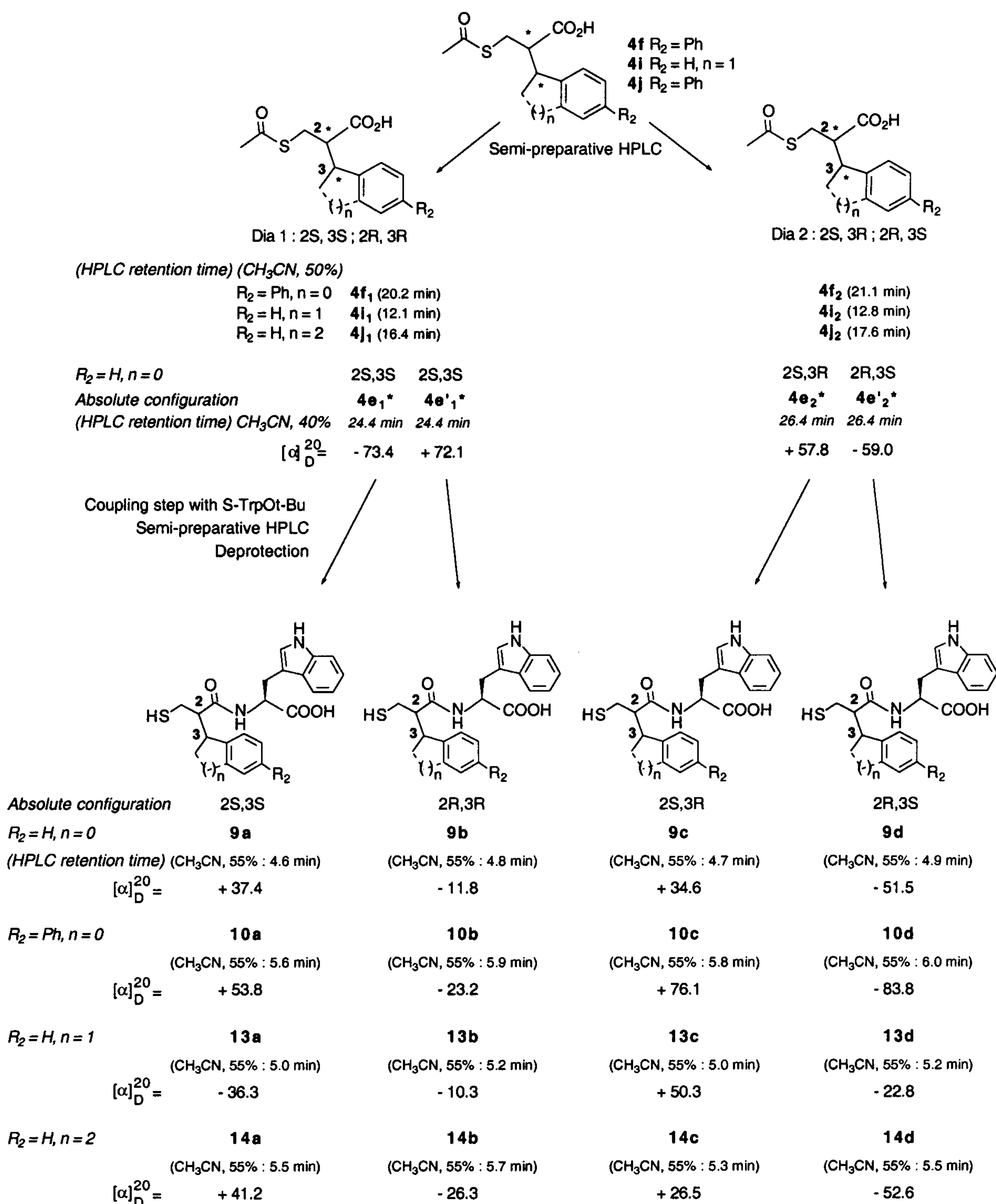
Taken together these data allow a complete stereochemical assignment of the four stereoisomers of **9**, **10**, **13**, and **14**. The absolute configurations of the carbon C2 and C3 in the mercaptoacyl synthons are 2*S*,3*S* in "a", 2*R*,3*R* in "b", 2*S*,3*R* in "c", and 2*R*,3*S* in "d" (Scheme 2).

Furthermore, the empiric rule used to determine the absolute configuration of the stereoisomers **9**, **10**, **13**, and **14** was confirmed by X-ray crystallography on the protected form of compound **13c**. As expected, compound **13c** is in the 2*S*,3*R* absolute configuration (Figure 1).

It is noteworthy that when HPLC was carried out in identical conditions the elution order was also related to the stereochemistry of the compounds, the 2*S* isomers "a" and "c" being slightly less retained than the 2*R* analogues "b" and "d". This result further confirms our hypothesis.

III. Inhibitory Potencies of the Various Inhibitors. In a preliminary screening, the synthesized compounds were tested as a mixture of stereoisomers for their ability to inhibit ECE-1, NEP, and ACE in vitro (Table 2). The R_1' residues of the first series of compounds (**5**−**7**) are bicyclic structures, the spatial orientations of which are constrained by the absence of a

Scheme 2. Separation of the Four Stereoisomers of Compounds **9**, **10**, **13**, and **14**[a]



[a] Retention time on a C18 Kromasil column using TFA 0.05%/CH_3CN as mobile phase. *Stereochemistry of compound **4e** was determined as previously reported.[15]

methylene spacer. Compound **5**, which has a *p*-biphenyl moiety, was the least efficient inhibitor with K_i values between 10^{-7} and 10^{-6} M for the three enzymes. In contrast, the *m*-biphenyl residue in **6** significantly improves ECE recognition, with a K_i value of 0.9 μM and inhibitory potencies between 10^{-8} and 10^{-7} M for NEP and ACE, respectively. The naphth-1-yl residue found in **7** is well recognized by NEP (K_i = 22 nM) but is less efficient for ECE and ACE.

In the second series of compounds (**8** to **12**), a methylene spacer was introduced and was replaced by a phenyl (compound **8**) or methyl group (**9** to **12**). The biphenyl methyl moiety of **8** is unfavorable for the three enzymes with K_i values of about 10^{-7} M for NEP and ACE and greater than 10^{-5} M for ECE. The replacement of one of the phenyl groups by a methyl in **9** allowed the recovery of high affinity for NEP and ACE (K_i values 3.0 and 40 nM, respectively) and a significant increase

Table 1. Chemical Shift of Some Characteristic Groups of Protons for the Separated Isomers of Compounds **9**, **10**, **13**, and **14**

compd	n	R_2	chemical shift (δ ppm) A	C	D	SH	proportion	config
9a	0	H	2.6−2.65	2.9	1.1	1.75	15	2*S*3*S*
9b			2.4−2.65	2.85	0.9	1.95	15	2*R*3*R*
9c			1.85−2.56	2.65	1.1	1.5	35	2*S*3*R*
9d			1.85−2.40	2.7	0.8	1.85	35	2*R*3*S*
10a	0	Ph	2.3−2.70	3.2	1.20	1.75	15	2*S*3*S*
10b			2.4−2.65	3.0	1.05	1.90	15	2*R*3*R*
10c			1.86−2.45	2.8	1.15	1.50	35	2*S*3*R*
10d			1.9−2.4	2.8	0.85	1.90	35	2*R*3*S*
13a	1	H	2.15−2.55	3.35	1.75−1.95	1.65	15	2*S*3*S*
13b			2.15−2.65	3.2	1.6	2.0	15	2*R*3*R*
13c			2.45−2.65	3.05	1.9−2.0	1.60	35	2*S*3*R*
13d			2.45−2.70	3.0	1.50−1.70	2.0	35	2*R*3*S*
14a	2	H	1.90−2.70	3.15	1.5−1.60	1.75	10	2*S*3*S*
14b			1.9−2.65	2.9	1.05−1.25	1.90	10	2*R*3*R*
14c			2.2−2.6	2.75	1.45−1.75	1.60	40	2*S*3*R*
14d			2.2−2.70	2.70	1.1−1.25	2.05	40	2*R*3*S*

Figure 1. Molecular structure of the protected form of **13c**, determined with single-crystal X-ray crystallography.

in ECE recognition (0.3 μM). An additional phenyl group in **10** was slightly detrimental for NEP and ACE (4.8 and 62 nM, respectively) but increased ECE recognition. The fusion of the two cycles in a naphth-2-yl (compound **11**) and naphth-1-yl (compound **12**) moiety was unfavorable for ECE in the first case and for the three enzymes in the latter case.

In the last series of inhibitors (**13** and **14**), the methyl group found in **9** was introduced into a cyclic structure, creating an indanyl group in **13** and a tetrahydronaphthyl residue in **14**, at the P_1' position. The K_i value obtained with **14** was not significantly different from that obtained with **9** but the indanyl moiety in **13** resulted in the most efficient ECE inhibitor described in this paper with a K_i value of 100 nM.

On the basis of these preliminary results, we decided to study compounds **9**, **10**, **13**, and **14** in more detail. The different stereoisomers were separated so that the inhibitory potency of each compound could be determined (Table 3).

Table 2. In Vitro Activity (K_i, nM) of the Thiol Inhibitors on NEP, ACE, and ECE

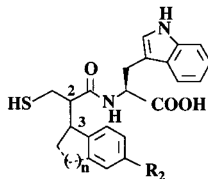
Compounds	R_1'	NEP [a]	ACE [a]	ECE [a]
5		135 ± 10	77 ± 7	4,150 ± 900
6		35 ± 3	150 ± 10	900 ± 30
7		22 ± 1	150 ± 30	1,560 ± 50
8		140 ± 5	250 ± 30	> 10,000
9		3.0 ± -0.2	40 ± -4	300 ± -20
10		4.8 ± 0.1	62 ± 4	220 ± 10
11		5.4 ± 0.1	47 ± 3	980 ± 60
12		51 ± 3	79 ± 2	600 ± 100
13		1.8 ± 0.2	20 ± 2	100 ± 10
14		4.5 ± 0.9	18 ± 3	300 ± 40
Thiorphan		4.0 ± 0.1	> 100	> 10,000
Captopril		> 1000	2 ± 0.1	> 10,000
CGS-31,447		2.2 ± 0.5	> 10,000	10.8 ± 0.2

[a] All compounds were tested under mixtures of two or four stereoisomers.

The four stereoisomers of compound **9** all had the same effect on NEP activity. However, lengthening the S_1' side chain of **10** induced a slight stereodependence, which was attributed to steric hindrance in the "2*S*" isomers. Indeed, the affinity of **10a** and **10c** was decreased by about 1 order of magnitude, whatever the configuration at the C3 carbon. For **13** and **14**, which contain a bicyclic moiety in S_1' position, another stereopreference was induced. This seemed to be related to the absolute configuration at the C3 carbon: isomers **13a**/**13d** and **14a**/**14d** were significantly less active than **13b**/**13c** and **14b**/**14c**.

In general, the inhibition of ACE was greatly dependent on the backbone stereochemistry: an *S* configuration is required at C2 atom. This was verified for the four inhibitors. The isomers **9c**, **10c**, **13c**, and **14c**, which have the 2*S*,3*R* configuration, were the most active with K_i values of about 10^{-8} M. The second most active were the "a" isomers, which have the 2*S*,3*S* configuration. In

Table 3. In Vitro Activity (K_i, nM) for the Separated Isomers of Compounds **9**, **10**, **13**, and **14** on NEP, ACE, and ECE



compd	*n*	R_2	config	NEP	ACE	ECE
9	0	H	racemic	3 ± 0.2	40 ± 4	300 ± 20
9a			2*S*3*S*	2.2 ± 0.3	29 ± 6	100 ± 10
9b			2*R*3*R*	2.4 ± 0.8	33 ± 1	200 ± 30
9c			2*S*3*R*	2.5 ± 0.2	15 ± 2	780 ± 40
9d			2*R*3*S*	5.0 ± 0.4	290 ± 10	580 ± 40
10	0	Ph	racemic	4.8 ± 0.1	62 ± 4	220 ± 10
10a			2*S*3*S*	30 ± 1	65 ± 1	110 ± 5
10b			2*R*3*R*	1.5 ± 0.3	130 ± 6	135 ± 10
10c			2*S*3*R*	35 ± 5	38 ± 1	920 ± 10
10c			2*R*3*S*	5.0 ± 0.4	330 ± 3	190 ± 10
13	1	H	racemic	1.8 ± 0.2	20 ± 2	100 ± 8
13a			2*S*3*S*	10 ± 1	41 ± 3	1140 ± 60
13b			2*R*3*R*	0.7 ± 0.03	43 ± 2	26 ± 3
13c			2*S*3*R*	2.1 ± 0.1	10 ± 1	290 ± 20
13d			2*R*3*S*	27 ± 1	170 ± 1	680 ± 9
14	2	H	racemic	4.5 ± 0.9	18 ± 3	300 ± 40
14a			2*S*3*S*	8.0 ± 0.6	26 ± 2	670 ± 50
14b			2*R*3*R*	3.0 ± 0.4	76 ± 6	250 ± 20
14c			2*S*3*R*	3.0 ± 0.6	10 ± 0.6	190 ± 8
14d			2*R*3*S*	43 ± 5	134 ± 20	1240 ± 20

all cases, the least efficient compound had the 2*R*,3*S* configuration, with K_i values of about 10^{-7} M.

Finally, for ECE inhibitors a different stereopreference appeared between compounds **9** and **10**, containing the branched synthons, and compounds **13** and **14**, containing a fused bicyclic structure. For compounds **9** and **10**, the "a" (K_i 100 and 110 nM, respectively) and "b" (K_i 200 and 135 nM, respectively) isomers were the most efficient. For compound **13**, the 2*R*,3*R* isomer (**13b)** had a K_i value of 26 nM. For compound **14**, the 2*S*,3*R* isomer (**14c**) (K_i 190 nM) and the 2*R*,3*R* isomer (**14b**) (K_i 250 nM) were virtually equipotent.

Discussion

The three enzymes involved in the maturation or degradation of the major vasoactive peptides (AII, ET-1, Bk, and ANP) belong to the gluzincin subfamily of zinc metallopeptidases. Thus, all three enzymes have virtually identical mechanisms of action and their active sites are similarly organized[4,17−20] as shown by the study of various dual NEP/ACE inhibitors.[15,20] As a result, a common pharmacophore has been proposed for NEP and ACE. ECE-1 presents significant primary sequence homology with NEP (51% in the active site), including consensus sequences and essential amino acids in the catalytic site, suggesting a close similarity between NEP and ECE.[21] This was recently confirmed by a three-dimensional model of ECE based on the X-ray structure of NEP.[18,19] These studies suggest that there are differences in the topological organization of the S_2' subsites and that ECE has a deeper S_1' pocket than NEP. This is consistent with the relative activities of CGS-26,303 and CGS-34,043, the P_1' residues of which are of different sizes and which behave, respectively, as a selective NEP inhibitor (K_i(NEP) 0.9 nM; K_i(ECE) 410 nM) and a selective ECE inhibitor (K_i(NEP) 2300 nM; K_i(ECE) 22 nM).[8] In this work, our main objective was to compare the S_1' subsites of ECE-1, NEP, and ACE. We maintained the P_2' moiety so that we could determine the most favorable spatial disposition of S_1' and design compounds to inhibit the three peptidases with equipotent activities.

Compounds **5**, **6**, and **7**, which have P_1' side chains derived from phenyl glycine moieties, retained a relatively good affinity for NEP and ACE, but their lack of mobility seemed to preclude an efficient recognition of the ECE S_1' subsite (Table 2). We also tested *β*-branched residues derived from phenylalanine. The second *β*-branched aromatic group in **8** was shown to be highly detrimental compared to the parent compound $HSCH_2$-(CH_2Ph)CO-TrpOH (K_i(ECE) 340 nM),[14,22] essentially for ECE inhibition. However, a *β*-methyl chain (**9**) is well accepted by the active sites of NEP and ACE as previously demonstrated[15] and also by the ECE active site.

The effect of an additional aromatic ring in the P_1' component of the inhibitor (compounds **10**−**12**) was highly dependent on its position: the biphenyl moiety of **10** slightly increased the recognition of ECE, reflecting a better occupancy of this S_1' pocket. Conversely, the naphthyl moieties of **11** and **12** were significantly less efficient for both NEP and ECE recognition.

Finally, the best result was obtained with **13**, which can be considered to be a constrained analogue of **10** and which had the best affinity of this series of compounds for the three enzymes, suggesting that the three enzymes share strong similarities for the occupancy of the S_1' subsite.

These results were analyzed in more detail after separating the various stereoisomers obtained during this nondiastereoselective synthesis. The first parameter that should be highlighted is the asymmetric induction observed during the Michael addition of thioacetic acid on the racemic derivatives **3a**−**j**. The 30/70 ratio reflects a diastereotopic transition state for this reaction, with differences in the accessibility of the intermediate enolate double bond.

However, whatever the mechanism responsible for this diastereoselectivity, the stereoisomers **c** and **d** were always obtained in greater proportion than **a** and **b**, and this has to be taken into account for the synthesis of such inhibitors.

The second interesting point concerns the stereochemistry of the most efficient isomers (Table 3), which are directed both by the different targeted enzymes and by the structure of a given inhibitor.

Thus, in the case of ACE, the most active inhibitors were derived from the 2*S*3*R* synthons. This has been previously observed in the mixanpril series,[15] in which the presence of a small C-terminal residue, such as an alanine, led to K_i values in the nanomolar range. In the new inhibitors reported here, the bulky C-terminal tryptophan slightly decreases the affinity for ACE, leading to K_i values of about 10^{-8} M.

The S_1' subsite of NEP is relatively large and can accommodate various side chains without stereochemical preference.[18,23] This has been previously shown with inhibitors such as thiorphan[24] and mixanpril[15] and was also the case with compound **9**: the four stereoisomers of which have K_i values between 2 and 5 nM. However, the lengthening of the P_1' chain in **10** and the constraint

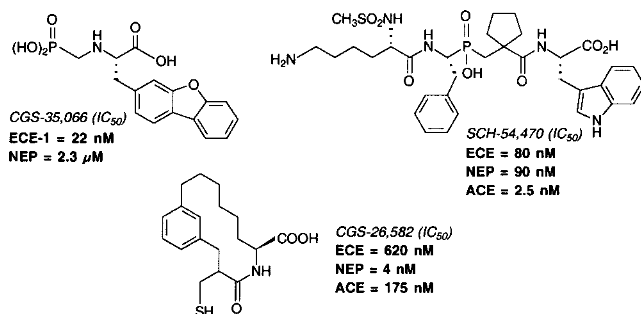


Figure 2. Example of triple NEP/ACE/ECE inhibitors.

introduced by the bicyclic moiety in **13** and **14** led to some restrictions in the optimal recognition of the S_1' NEP subsite. Thus, the 2*R*3*R* isomers of **10b**, **13b**, and **14b** were the most efficient compounds, although the 2*S*3*R* isomers of **13** and **14** (**13c** and **14c**) also exhibit good affinities for NEP. Compounds **13c** and **14c** were relatively good dual NEP/ACE inhibitors. This is in contrast with **10**, which had opposite stereopreference for NEP and ACE (Table 3).

Finally, the proposed molecules are not optimized for ECE inhibition, and their inhibitory potencies have to be improved. However, given the molecular modeling study of the active site of ECE,[19] this could be achieved by modifying the P_2' residue. Our results are more difficult to interpret in terms of stereochemical recognition. Thus, for the bicyclic structures **13** and **14**, only one stereoisomer (**13b**) emerges significantly, with a K_i value of 26 nM on ECE, indicating that, in this spatial orientation, the residue fits into the S_1' subsite of ECE relatively well. The indanyl moiety of **13b** is smaller than the P_1' side chain of CGS-35,066 (K_i(ECE) 22 nM (Figure 2)), which could be an unfavorable parameter, but has reduced degrees of freedom, probably resulting in a more favorable entropic contribution during the recognition of the active site of the enzyme.

In conclusion, a compound must be nearly equipotent on the three targeted enzymes if it is to be considered as a triple inhibitor. None of the separated isomers completely fulfilled this requirement. Compound **10a**, with K_i values of 30, 65, and 110 nM on NEP, ACE, and ECE, respectively, could be considered as a triple inhibitor, because the ratios between these K_i values are only 2 and 4. However, their affinities are relatively weak for pharmacological studies. Compound **13b**, with K_i values of 0.7 nM, 40 nM, and 26 nM on NEP, ACE, and ECE, respectively, could be an interesting candidate for the triple inhibition of the targeted enzymes. This compound is comparable with two other triple inhibitors, SCH-54,470 and CGS-26,582[25,26] (Figure 2). SCH-54,470 is a phosphinic inhibitor, which interacts with the S_2−S_2' subsites of the three vasopeptidases (NEP, ACE, and ECE) with K_i values of 90 nM, 2.5 nM, and 80 nM, respectively. CGS-26,582 is a macrocyclic inhibitor bearing a thiol as a zinc-chelating group with K_i values of 4 nM, 175 nM, and 600 nM on NEP, ACE, and ECE, respectively. Thus, CGS-26,582 can be considered as a selective inhibitor of NEP. Furthermore, a previous study[27] ruled out the possibility of using a thiol group in ECE inhibitors. However, our work and recent results[28] demonstrate that this efficient zinc coordinating agent could be an alternative to the phosphonate group used to optimize CGS-31,447.[29] Our promising results should allow triple NEP/ACE/ECE inhibitors with nanomolar affinities for each enzyme to be designed. Moreover, by comparing the activities of **9** and **10** on ECE, it can be concluded that substituting various groups in suitable positions on the aromatic rings of **13** should improve the recognition of ECE.

Experimental Section

Chemistry. ^{1}H NMR spectra were measured on a Brüker AC 270 MHz or AC 400 MHz spectrometer using tetramethylsilane as internal standard. Electrospray mass spectra (MS-ES) were recorded on a Esquier-Brüker spectrometer. Flash columm chromatography was performed using 40−63 μm silica gel. Reaction progress was determined by either TLC analysis or monitored using analytical reverse-phase HPLC (Shimadzu, LC10 AD-vp with a Class-VP5.03 software or Shimadzu, SCL 6B) using a Kromasil C_{18} column (Touzart-Matignon, France), with mobile phase consisting of water containing 0.05% TFA and acetonitrile. HPLC separations were performed using a reverse-phase C18 Kromasil column (5 μM, porosity 100 Å) with CH_3CN/TFA 0.05% buffer (pH 4.0) as eluent, on a Waters apparatus (detector 2487, pump 600 controler). The eluted peaks were monitored at 210 nm. Reagents were obtained from commercial sources and are used without further purification. Optical rotation were determined on a Jasco P-1030 polarimeter.

General Procedure for Synthesis of Compounds 1a−c. A mixture constituted of the appropriate phenylacetic methyl ester (65 mmol), *N*-bromosuccinimide (130 mmol), and a catalytic amount of benzoyl peroxyde (0.65 mmol) in dried CCl_4 (50 mL) was refluxed 24 h. After cooling, the precipitate was removed by filtration, and the reaction adduct was evaporated in vacuo, yielding the titled compounds as yellow oils which were used without further purification.

1-Bromo-biphen-4-yl-acetic acid methyl ester (1a): 18.2 g (92%). TLC (cyclohexane:ethyl acetate, 80:20): R_f = 0.36. HPLC (CH_3CN:H_2O, 60:40): R_t = 18.2 min. ^{1}H NMR ($CDCl_3$) δ 3.7 (s, 3H), 5.45 (s, 1H), 7.3 (m, 4H), 7.5 (m, 5H).

1-Bromo-biphen-3-yl-acetic acid methyl ester (1b):[30] 17.1 g (86%). TLC (cyclohexane:ethyl acetate, 80:20): R_f = 0.38. HPLC (CH_3CN:H_2O, 70:30): R_t = 9.1 min. ^{1}H NMR ($CDCl_3$) δ 3.7 (s, 3H), 5.45 (s, 1H), 7.25 (m, 4H), 7.5 (m, 4H).

1-Bromo-naphth-1-yl-acetic acid methyl ester (1c): 18 g (99%). TLC (cyclohexane:ethyl acetate, 80:20): R_f = 0.43. HPLC (CH_3CN:H_2O, 70:30): R_t = 8.7 min. ^{1}H NMR (CDCl3) δ 3.7 (s, 3H), 6.1 (s, 1H), 7.4 (m, 3H), 7.65 (d, *J* = 7.9 Hz, 1H), 7.8 (dd, *J* = 7.9 Hz, 2H), 8.05 (d, *J* = 7.9 Hz, 1H).

General Procedure for Synthesis of Compounds 2a−c. A mixture constituted of the appropriate 1-bromophenylacetic methyl ester **1** (25 mmol) and triethyl phosphite (37.5 mmol) was heated at 120 °C for 24 h. After cooling, evaporation in vacuo gave a residue which was purified by column chromatography on silica gel with 30% of ethyl acetate in cyclohexane, yielding compound **2** as an oil.

Diethoxyphosphoryl-2-(biphen-4-yl)-acetic acid methyl ester (2a): 7.8 g (87%). TLC (cyclohexane:ethyl acetate, 70:30): R_f = 0.17. HPLC (CH_3CN:H_2O, 70:30): R_t = 5.3 min. ^{1}H NMR ($CDCl_3$) δ 1.25 (m, 6H), 3.65 (s, 3H), 4.05 (m, 4H), 4.30 (d, *J* = 24 Hz, 1H), 7.25 (m, 3H), 7.5 (m, 6H).

Diethoxylphosphoryl-2-(biphen-3-yl)-acetic acid methyl ester (2b): 6.0 g (67%). TLC (cyclohexane:ethyl acetate, 70:30): R_f = 0.15. HPLC (CH_3CN:H_2O, 70:30): R_t = 5.0 min. ^{1}H NMR ($CDCl_3$) δ 1.2 (m, 6H), 3.65 (s, 3H), 4.0 (m, 4H), 4.35 (d, *J* = 24 Hz, 1H), 7.4 (m, 8H), 7.7 (s, 1H).

Diethoxyphosphoryl-2-(naphth-1-yl)-acetic acid methyl ester (2c): 7.8 g (93%). TLC (cyclohexane:ethyl acetate, 70:30): R_f = 0.14. HPLC (CH_3CN:H_2O, 70:30): R_t = 4.2 min. ^{1}H NMR ($CDCl_3$) δ 0.9 (t, *J* = 7 Hz, 3H), 1.1 (t, *J* = 7 Hz, 3H), 3.65 (s, 3H), 3.9 (m, 4H), 5.05 (d, *J* = 24 Hz, 1H), 7.35 (m, 3H), 7.75 (dd, 7 Hz, 2H), 8 (m, 2H).

General Procedure for Synthesis of Compounds 2d−j. To a solution of triethylphosphonoacetate (40 mmol) in dry DMF (50 mL) was added by portions (44 mmol) sodium hydride

at 0 °C, and the mixture was stirred at this temperature for 0.5 h. Compound **1** (40 mmol) in dry DMF (20 mL) was added to this mixture, and the solution was stirred at room temperature overnight. The solvent was removed in vacuo, and the residue was diluted with ethyl acetate, washed with H_2O, and dried over Na_2SO_4. After removal of the solvent, the residue was purified by column chromatography (4:6 ethyl acetate: cyclohexane) to give **2**.

2-(Diethoxyphosphoryl)-3-diphenyl-propionic acid ethyl ester (2d):[31] 7.8 g (87%). TLC (cyclohexane:ethyl acetate, 70:30): R_f = 0.17. HPLC (CH_3CN:H_2O, 70:30): R_t = 5.3 min. ^{1}H NMR ($CDCl_3$) δ 1.25 (m, 6H), 3.65 (s, 3H), 4.05 (m, 4H), 4.30 (d, J = 24 Hz, 1H), 7.25 (m, 3H), 7.5 (m, 6H).

2-(Diethoxyphosphoryl)-3-methyl-3-phenyl-propionic acid ethyl ester (2e):[15] 11.9 g (91%). TLC (cyclohexane: ethyl acetate:acetic acid, 5:5:0.5): R_f = 0.54. ^{1}H NMR (DMSO-d_6) δ 0.75 (dd, 3H), 0.95 (td, 3H), 1.1 (m, 6H), 3.3 (m, 1H), 3.65 (m, 2H), 4.0 (m, 4H), 7.25 (m, 5H).

2-(Diethoxyphosphoryl)-3-methyl-3-(biphen-4-yl)-propionic acid ethyl ester (2f): 14.2 g (88%). TLC (cyclohexane: ethyl acetate:acetic acid, 5:5:0.5): R_f = 0.54. HPLC (CH_3CN: H_2O, 70:30): R_t = 8.4−8.7 min. ^{1}H NMR (DMSO-d_6) δ 0.8 (td, 3H), 1.2 (m, 6H), 3.4 (m, 2H), 3.7 (s, 2H), 4.05 (m, 4H), 7.25−7.6 (m, 9H).

2-(Diethoxyphosphoryl)-3-methyl-3-(naphthalen-2-yl)-propionic acid ethyl ester (2g): 9.3 g (63%). TLC (cyclohexane:ethyl acetate:acetic acid, 5:5:0.1): R_f = 0.89. ^{1}H NMR (DMSO-d_6) δ δ 0.7 (td, 3H), 1.25 (m, 6H), 3.4 (m, 2H), 3.7 (s, 2H), 4.1 (m, 4H), 7.4 (m, 3H), 7.8 (m, 4H).

2-(Diethoxyphosphoryl)-3-methyl-3-(naphthalen-1-yl)-propionic acid ethyl ester (2h): 12.6 g (83%). TLC (cyclohexane:ethyl acetate, 50:50): R_f = 0.17. ^{1}H NMR ($CDCl_3$) δ 0.5−1.5 (m, 12H), 3.4−3.8 (m, 4H), 4.1 (m, 4H), 4.4 (m, 1H), 7.3 (m, 4H), 7.6 (m, 1H), 7.7 (t, J = 7 Hz, 1H), 8.1 (t, J = 7 Hz, 1H).

Diethoxyphosphoryl-(indan-1-yl)-acetic acid methyl ester (2i): 13.2 g (97%). TLC (cyclohexane:ethyl acetate, 60: 40): R_f = 0.14. ^{1}H RMN ($CDCl_3$): 1.3 (m, 9H), 2.3 (m, 2H), 2.9 (m, 2H), 3.3 (m, 1H), 3.9 (m, 1H), 4.2 (m, 6H), 7.30 (m, 4H).

Diethoxyphosphoryl-(1,2,3,4,tetrahydro-naphth-1-yl)-acetic acid methyl ester (2j): 13 g (95%). HPLC (CH_3CN: H_2O, 60:40): R_t = 9.2−9.5 min. ^{1}H NMR ($CDCl_3$) δ 1.2 (m, 9H), 2.0 (m, 4H), 2.7 (m, 2H), 3.3 (m, 1H), 3.55 (m, 1H), 4.15 (m, 6H), 6.9 (m, 4H).

General Procedure for Synthesis of Compounds 3a−j. To a solution of a compound **2** (15 mmol) in dry THF (50 mL) were added paraformaldehyde (30 mmol) and potassium carbonate (30 mmol). The mixture was stirred for 24 h under reflux. After warm filtration on Celite, THF was removed under reduced pressure. The residue was disolved in acetone (180 mL), and 90 mL of a 1 M sodium hydroxide solution was added to the solution. The mixture was stirred for 12 h at room temperature. The solvent was removed in vacuo. The residue was diluted with H_2O (100 mL) and washed with ether (2 × 70 mL). The aqueous layer was acidified with 1 N HCl (120 mL) and extracted with ether (3 × 100 mL). The combined organic layers were dried over Na_2SO_4 and evaporated to dryness to yield the pure acid **3**.

2-(Biphenyl-4-yl)-acrylic acid (3a):[32] 3.15 g (95%). TLC (cyclohexane:ethyl acetate:acetic acid, 7:3:0.5): R_f = 0.37. HPLC (CH_3CN:H_2O, 65:35): R_t = 5.5 min. ^{1}H NMR ($CDCl_3$) δ 6.1 (s, 1H), 6.6 (s, 1H), 7.3 (m, 5H), 7.6 (m, 4H).

2-(Biphenyl-3-yl)-acrylic acid (3b): 1.6 g (47%). HPLC (CH_3CN:H_2O, 65:35): R_t = 5.5 min. ^{1}H NMR ($CDCl_3$) δ 6.0 (s, 1H), 6.5 (s, 1H), 7.3 (m, 5H), 7.5 (m, 4H).

2-(Naphthalen-1-yl)-acrylic acid (3c):[33] 2.0 g (60%). (CH_3CN:H_2O, 65:35): R_t = 4.4 min. ^{1}H NMR ($CDCl_3$) δ 5.9 (s, 1H), 6.8 (s, 1H), 7.2−7.4 (m, 4H), 7.7−7.9 (m, 3H).

2-(1,1-Diphenyl-methyl)-acrylic acid (3d):[34] 3.2 g (89%). TLC (cyclohexane:ethyl acetate:acetic acid, 6:4:0.5): R_f = 0.55. ^{1}H NMR ($CDCl_3$) δ 5.2 (s, 1H), 5.3 (s, 1H), 6.5 (s, 1H), 7.0 (m, 4H), 7.2 (m, 6H).

2-(1-Phenyl-ethyl)-acrylic acid (3e):[15] 2.2 g (82%). TLC (cyclohexane:ethyl acetate:acetic acid, 7:3:0.5): R_f = 0.67. ^{1}H NMR ($CDCl_3$) δ 1.2 (d, 7 Hz, 3H), 4.0 (q, 7 Hz, 1H), 5.6 (s, 1H), 6.1 (s, 1H), 7.2 (m, 3H), 7.3 (m, 2H).

2-[1-(Biphenyl-4-yl)-ethyl]-acrylic acid (3f): 2.6 g (70%). TLC (cyclohexane:ethyl acetate:acetic acid, 6:4:0.5): R_f = 0.61. ^{1}H NMR (DMSO-d_6) δ 1.3 (d, 7 Hz, 3H), 3.9 (q, 7 Hz, 1H), 5.6 (s, 1H), 6.1 (s, 1H), 7.2 (m, 3H), 7.3 (m, 2H), 7.5 (m, 4H).

2-[1-(Naphthalen-2-yl)-ethyl]-acrylic acid (3g): 2.9 g (85%). TLC (cyclohexane: ethyl acetate:acetic acid, 5:5:0.1): R_f = 0.6. ^{1}H NMR (DMSO-d_6) δ 1.4 (d, 7 Hz, 3H), 4.1 (q, 7 Hz, 1H), 5.7 (s, 1H), 6.2 (s, 1H), 7.4 (m, 3H), 7.6 (s, 1H), 7.8 (m, 3H).

2-[1-(Naphthalen-1-yl)-ethyl]-acrylic acid (3h): 1.2 g (35%). TLC (cyclohexane:ethyl acetate, 5:5): R_f = 0.5. ^{1}H NMR ($CDCl_3$) δ 1.5 (d, 7 Hz, 3H), 4.7 (q, 7 Hz, 1H), 5.5 (s, 1H), 6.4 (s, 1H), 7.4 (m, 4H), 7.7 (d, J = 7 Hz, 1H), 7.8 (d, J = 7 Hz, 1H), 8.0 (d, J = 7 Hz, 1H).

2-(Indan-1-yl)-acrylic acid (3i): 1.9 g (69%). HPLC (CH_3CN:H_2O, 50:50): R_t = 8.9 min. ^{1}H NMR ($CDCl_3$) δ 1.9 (m, 1H), 2.4 (m, 2H), 2.9 (m, 2H), 4.2 (t, J = 7 Hz, 1H), 5.4 (s, 1H), 6.4 (s, 1H), 7.1 (m, 4H), 7.2 (m, 1H).

2-(1,2,3,4,Tetrahydro-naphth-1-yl)-acrylic acid (3j): 1.6 g (52%). HPLC (CH_3CN:H_2O, 60:40): R_t = 6.7 min. ^{1}H NMR ($CDCl_3$) δ 1.7 (t, J = 7 Hz, 2H), 1.9 (m, 2H), 2.7 (m, 2H), 4.1 (t, J = 7 Hz, 1H), 5.1 (s, 1H), 6.4 (s, 1H), 6.9 (m, 1H), 7.1 (m, 3H).

General Procedure for Synthesis of Compounds 4a−j. Thiolacetic acid (12 mmol) was added to a solution of compound **3** (10 mmol) in $CHCl_3$ (10 mL). The mixture was refluxed for 12 h. The solvent was removed under reduced pressure. The residue was purified by column chromatography on silica gel (ethyl acetate:cyclohexane 4:6) to give **4** as an oil.

3-Acetylsulfanyl-2-(biphenyl-4-yl)-propanoic acid (4a): 1.5 g (48%). TLC (cyclohexane:ethyl acetate:acetic acid, 7:3: 0.5): R_f = 0.28. HPLC (CH_3CN:H_2O, 65:35): R_t = 5.9 min. ^{1}H NMR ($CDCl_3$) δ 2.2 (s, 3H), 3.3 (m, 2H), 3.8 (td, J = 7 Hz, 1H), 7.2 (m, 5H), 7.6 (m, 4H). ES-MS $[M+Na]^+$ 323.

3-Acetylsulfanyl-2-(biphenyl-3-yl)-propanoic acid (4b): 2.0 g (67%). TLC (cyclohexane:ethyl acetate, 6:4): R_f = 0.1. HPLC (CH_3CN:H_2O, 65:35): R_t = 5.8 min. ^{1}H NMR ($CDCl_3$) δ 2.2 (s, 3H), 3.3 (m, 2H), 3.8 (td, J = 7 Hz, 1H), 7.2 (m, 5H), 7.5 (m, 4H). ES-MS $[M+Na]^+$ 323.

3-Acetylsulfanyl-2-(naphthalen-1-yl)-propanoic acid (4c): 0.9 g (35%). TLC (cyclohexane:ethyl acetate, 5:5): R_f = 0.15. HPLC (CH_3CN:H_2O, 65:35): R_t = 4.8 min. ^{1}H NMR ($CDCl_3$) δ 2.2 (s, 3H), 3.4 (m, 2H), 4.6 (td, J = 7 Hz, 1H), 7.4 (m, 4H), 7.7 (m, 2H), 8.2 (d, J = 7 Hz, 1H). ES-MS $[M+H]^+$ 275.

2-Acetylsulfanylmethyl-3,3-diphenyl-propanoic acid (4d): 1.8 g (56%). TLC (cyclohexane:ethyl acetate, 6:4): R_f = 0.12. ^{1}H NMR ($CDCl_3$) δ 2.2 (s, 3H), 2.7 (m, 1H), 3.1 (m, 1H), 3.5 (m, 1H), 4.1 (m, 1H), 7.2 (m, 10H). ES-MS $[M+Na]^+$ 337.

2-Acetylsulfanylmethyl-3-phenyl-butanoic acid (4e):[15] 2.4 g (95%). TLC (cyclohexane:ethyl acetate:acetic acid, 7:3: 0.5): R_f = 0.5. ^{1}H NMR ($CDCl_3$) δ 1.2 (s, 3H), 2.2 (s, 3H), 2.5−3.2 (m, 4H), 6.7−7.3 (m, 5H). ES-MS $[M+H]^+$ 253.

Compound **4e** was resolved by chiral amine as previously described, leading to the four separate stereoisomers: **4e₁**, **4e′₁**, **4e₂**, **4e′₂**.[15]

2-Acetylsulfanylmethyl-3-(biphenyl-4-yl)-butanoic acid (4f): 3.2 g (96%). TLC (cyclohexane:ethyl acetate:acetic acid, 5:5:0.5): R_f = 0.42. HPLC (CH_3CN:H_2O, 65:35): R_t = 7.9 min. ^{1}H NMR (DMSO-d_6) δ 1.2 (s, 3H), 2.2 (s, 3H), 2.6−3.1 (m, 4H), 7.1−7.6 (m, 9H). ES-MS $[M+NH_4]^+$ 346.

The two diastereoisomers of **4f** were separated using preparative HPLC, leading to **4f₁** and **4f₂**. HPLC (CH_3CN:H_2O, 50:50): R_t (**4f₁**) 20.2 min, (**4f₂**) 21.1 min.

2-Acetylsulfanylmethyl-3-(naphthalen-2-yl)-butanoic acid (4g): 2.2 g (74%). TLC (cyclohexane:ethyl acetate:acetic acid, 5:5:0.1): R_f = 0.56. ^{1}H NMR (DMSO-d_6) δ 1.3 (d, 7 Hz, 3H), 2.2 (s, 3H), 2.7 (m, 2H), 2.9−3.2 (m, 2H), 7.2 (m, 3H), 7.6 (s, 1H), 7.8 (m, 3H).

2-Acetylsulfanylmethyl-3-(naphthalen-1-yl)-butanoic acid (4h): 2.5 g (83%). TLC (cyclohexane:ethyl acetate, 5:5): R_f = 0.24. HPLC (CH_3CN:H_2O, 65:35): R_t = 5.7 min. ^{1}H NMR

($CDCl_3$) δ 1.3 (m, 3H), 2.1 (s, 3H), 2.7−3.1 (m, 3H), 4 (m, 1H), 7.3 (m, 4H), 7.7 (m, 2H), 8.1 (m, 1H). ES-MS [M+Na]$^+$ 325.

3-Acetylsulfanyl-2-(indan-1-yl)-propanoic acid (4i): 2.1 g (85%). TLC (cyclohexane:ethyl acetate, 6:4): R_f = 0.53. HPLC (CH_3CN:H_2O, 40:60): R_t = 18.9−20.3 min. ^{1}H NMR ($CDCl_3$) δ 2−2.3 (m, 5H), 2.8−3.1 (m, 5H), 3.6 (m, 1H), 7.1 (m, 4H).

The two diastereoisomers of **4i** were separated using preparative HPLC, leading to **4i$_1$** and **4i$_2$**. HPLC (CH_3CN:H_2O, 50:50): R_t (**4i$_1$**) 12.1 min, (**4i$_2$**) 12.8 min.

3-Acetylsulfanyl-2-(1,2,3,4,tetrahydro-naphthalen-1-yl)-propanoic acid (4j): 2.3 g (84%). HPLC (CH_3CN:H_2O, 60:40): R_t = 6.0−6.3 min. ^{1}H NMR ($CDCl_3$) δ 1.8 (m, 4H), 2.3 (s, 3H), 2.8 (m, 2H), 3.1 (m, 4H), 7.1 (m, 4H). ES-MS [M+H]$^+$ 279

The two diastereoisomers of **4j** were separated using preparative HPLC, leading to **4j$_1$** and **4j$_2$**. HPLC (CH_3CN:H_2O, 50:50): R_t (**4j$_1$**) 20.2 min, (**4j$_2$**) 21.1 min.

General Procedure for Synthesis of Compounds 5−14. Compound **4** (2.5 mmol), was dissolved in a mixture of THF and $CHCl_3$ (2.2 mL) containing tryptophan methyl ester (3 mmol), triethylamine (3 mmol), EDC (3 mmol), and HOBt (3 mmol). The reaction mixture was stirred for 14 h at room temperature, then the solvents were removed in vacuo, and the residue partitioned between EtOAc and satured $NaHCO_3$. The EtOAc layer was extracted two times with satured $NaHCO_3$, three times with 10% aqueous citric acid, and finally with brine. The organic layer was dried with Na_2SO_4, the solvent removed in vacuo, and the residue dissolved in MeOH (5 mL) under argon with stirring. Degassed 2 M NaOH solution (6 equiv) was added, and the mixture was stirred for 3 h. The organic solvent was evaporated in vacuo, and the remaining aqueous solution was acidified with 1 M HCl under stirring until pH 1. The aqueous solution was dried in vacuo, and the residue was purified by preparative reverse-phase HPLC on a C_{18} column, using TFA 0.05% in H_2O and CH_3CN as mobile phases. The eluate was lyophilized to afford the desired compound as a white solid.

(2*S*)-[2-(4-Biphen-4-yl)-3-mercapto-propanoylamino]-3-(1(*H*)-indol-3-yl)-propanoic acid (5): 0.88 g (80%). HPLC (CH_3CN:H_2O, 65:35): R_t = 5.9 min. ^{1}H NMR ($CDCl_3$) δ 1.6 (m, 1H, SH), 2.7 (m, 2H, CH_2 Trp), 3.1−3.3 (m, 2H, CH_2S), 3.5 (m, 1H, CHCO), 4.8 (m, 1H, Hα Trp), 6.0 (m, 1H, NH), 6.7−7.6 (m, 14H, Ar), 7.9 (NH, indoyl). ES-MS [M+H]$^+$ 445. Anal. ($C_{26}H_{24}N_2O_3S$) C, H, N.

(2*S*)-2-[2-(3-Biphen-4-yl)-3-mercapto-propanoylamino]-3-(1(*H*)-indol-3-yl)-propanoic acid (6): 0.49 g (44%). HPLC (CH_3CN:H_2O, 65:35): R_t = 5.7 min. ^{1}H NMR ($CDCl_3$) δ 1.4 (m, 1H, SH), 2.7 (m, 2H, CH_2 Trp), 3.1−3.3 (m, 2H, CH_2S), 3.5 (m, 1H, CHCO), 4.8 (m, 1H, Hα Trp), 6.1 (m, 1H, NH), 6.7−7.5 (m, 14H, Ar), 7.6−7.8 (NH, indoyl). ES-MS [M+H]$^+$ 445. Anal. ($C_{26}H_{24}N_2O_3S$) C, H, N.

(2*S*)-2-[2-(Naphthalen-1-yl)-3-mercapto-propanoylamino]- 3-(1(*H*)-indol-3-yl)-propanoic acid (7): 0.32 g (31%). HPLC (CH_3CN:H_2O, 50:50): R_t = 11.2 min. ^{1}H NMR ($CDCl_3$−TFA) δ 3.1 (m, 2H, CH_2 Trp), 3.3−3.5 (m, 3H, CH−CH_2S), 4.5−4.9 (m, 1H, Hα Trp), 6.5 (m, 1H, NH), 6.8−8 (m, 12H, Ar). ES-MS [M+H]$^+$ 419. Anal. ($C_{24}H_{22}N_2O_3S$) C, H, N.

(2*S*)-2-[2-Mercaptomethyl-3-diphenyl-propanoylamino]-3-(1(*H*)-indol-3-yl)-propanoic acid (8): 0.58 g (51%). HPLC (CH_3CN:H_2O, 65:35): R_t = 4.8−5.0 min. ^{1}H NMR ($CDCl_3$) δ 1.2 (m, 1H, SH), 2.5 (m, 2H, CH_2 Trp), 2.6 (m, 1H, CHCO), 4.0 (m, 1H, PhCHPh), 4.5(m, 1H, Hα Trp), 6.2 (m, 1H, NH), 7.0−7.3 (m, 15H, Ar), 8.1 (m, 1H, NH, indoyl). ES-MS [M+H]$^+$ 459. Anal. ($C_{27}H_{26}N_2O_3S$) C, H, N.

(2*S*)-2-[2-Mercaptomethyl-3-phenyl-butanoylamino]-3-(1(*H*)-indol-3-yl)-propanoic acid (9): 0.26 g (26%). HPLC (CH_3CN:H_2O, 70:30): R_t = 4.1−4.3 min. ^{1}H NMR (DMSO-d_6) δ 0.8−1.1 (m, 3H, CH_3), 1.9 (m, 1H, SH), 2.6 (m, 3H, CH_2S, CHCO), 2.9 (m, 2H, CH_2 Trp), 3.1 (m, 1H, CH), 4.4 (m, 1H, Hα Trp), 6.9−7.4 (m, 10H, Ar), 8.1−8.3 (m, 1H, NH), 10.8 (m, 1H, NH, indoyl). ES-MS [M+H]$^+$ 397. Anal. ($C_{22}H_{24}N_2O_3S$) C, H, N.

(2*S*)-2-[2-Mercaptomethyl-3-(biphen-4-yl)-butanoylamino]-3-(1(*H*)-indol-3-yl)-propanoic acid (10): 0.86 g (73%). HPLC (CH_3CN:H_2O, 50:50): R_t = 20.6−21.1−24.5−25.6 min. ^{1}H NMR (DMSO-d_6) δ 0.8−1.3 (m, 3H, CH_3), 1.9 (m, 1H, SH), 2.6 (m, 3H, CH_2S, CHCO), 2.9 (m, 2H, CH_2 Trp), 3.1 (m, 1H CH), 4.2−4.6 (m, 1H, Hα Trp), 6.8−7.6 (m, 14H, Ar), 8.1−8.4 (m, 1H, NH), 10.7 (m, 1H, NH, indoyl). ES-MS [M+H]$^+$ 473. Anal. ($C_{28}H_{28}N_2O_3S$) C, H, N.

(2*S*)-2-[2-Mercaptomethyl-3-(naphthalen-2-yl)-butanoylamino]-3-(1(*H*)-indol-3-yl)-propanoic acid (11): 0.72 g (65%). HPLC (CH_3CN:H_2O, 65:35): R_t = 5.4−5.6−5.8 min. ^{1}H NMR (DMSO-d_6) δ 0.9−1.2 (m, 3H, CH_3), 1.7 (m, 1H, SH), 2.7 (m, 3H, CH_2S, CHCO), 3.1 (m, 3H, CH, CH_2 Trp), 4.2−4.6 (m, 1H, Hα Trp), 6.7−7.7 (m, 12H, Ar), 8.1−8.3 (m, 1H, NH), 10.7 (m, 1H, NH, indoyl). ES-MS [M+H]$^+$ 447. Anal. ($C_{26}H_{26}N_2O_3S$) C, H, N.

(2*S*)-2-[2-Mercaptomethyl-3-(naphthalen-1-yl)-butanoylamino]-3-(1(*H*)-indol-3-yl)-propanoic acid (12): 0.55 g (50%). HPLC (CH_3CN:H_2O, 65:35): R_t = 5.2−5.4−5.5−5.7 min. ^{1}H NMR (DMSO-d_6) δ 0.9−1.2 (m, 3H, CH_3), 1.6−1.7 (m, 1H, SH), 2.7−3.5 (m, 6H, CH_2S, CHCO, CH, CH_2 Trp), 4.2−4.5 (m, 1H, Hα Trp), 6.5−7.7 (m, 12H, Ar), 8.2 (m, 1H, NH), 10.7 (m, 1H, NH, indoyl). ES-MS [M+Na]$^+$ 447. Anal. ($C_{26}H_{26}N_2O_3S$) C, H, N.

(2*S*)-2-[2-(Indan-1-yl)-3-mercapto-propionylamino]-3-(1(*H*)-indol-3-yl)-propanoic acid (13): 0.25 g (25%). HPLC (CH_3CN:H_2O, 60:40): R_t = 5.1−5.3 min. ^{1}H NMR (DMSO-d_6) δ 1.5 (m, 1H, SH), 1.8−2.2 (m, 2H, CH_2), 2.4−3.1 (m, 8H), 4.4−4.6 (m, 1H, Hα Trp), 6.8−7.5 (m, 9H, Ar), 8.2 (m, 1H, NH), 10.8 (m, 1H, NH, indoyl). ES-MS [M+H]$^+$ 409. Anal. ($C_{23}H_{24}N_2O_3S$) C, H, N.

(2*S*)-2-[3-Mercapto-2-(1,2,3,4,tetrahydro-naphthalen-1-yl)-propionylamino]-3-(1(*H*)-indol-3-yl)-propanoic acid (14): 0.73 g (69%). HPLC (CH_3CN:H_2O, 60:40): R_t = 5.7−6.0−6.2 min. ^{1}H NMR ($CDCl_3$) δ 1.3 (m, 1H, SH), 1.4−1.7 (m, 4H, CH_2CH_2), 2.2 (m, 1H, CHCO), 2.5 (m, CH_2 Trp), 2.7−3.3 (m, 5H), 4.8 (m, 1H, Hα Trp), 5.8 (m, 1H, NH), 6.8−7.4 (m, 8H, Ar), 8.1 (m, 1H, NH, indoyl). ES-MS [M+H]$^+$ 423. Anal. ($C_{24}H_{26}N_2O_3S$) C, H, N.

Preparation of the Four Stereoisomers of Inhibitors 9, 10, 13, and 14. A coupling step between the two separate diastereoisomers of **4e−f**, **4i−j** (**4$_1$** and **4$_2$**), and tryptophan *tert*-butyl ester was performed by the procedure described for compound **5−14** followed by a separation of the diastereoisomers by preparative HPLC. After final deprotections, the diastereoisomers were found to have a purity of ≥98% by analytical HPLC. They were characterized by ^{1}H NMR using two-dimensional Cosy and HMQC experiments.

(2*S*)-2-[2-(2*S*)-Mercaptomethyl-3-(3*S*)-phenyl-butanoylamino]-3-(1*H*-indol-3-yl)-propanoic Acid (9a). Obtained from **4e$_1$**: HPLC CH_3CN, 50%, 10.23 min. ^{1}H NMR δ (DMSO) 1.12 (d, 8.5 Hz, CH_3), 1.75 (s, SH), 2.3 (t, 7H, 1H), 2.6 (m, 2H), 2.9 (m, 2H), 3.15 (m, 1H), 4.4 (q, CHαTrp), 6.8 (t, 1H), 6.9 (t, 1H), 7 (m, 6H), 7.25 (d, 1H), 7.5 (d, 1H), 8.15 (d, 7Hg, NH), 10.8 (s, 1H, NH indoyl). $[\alpha]^{20}_D$ (c = 0.288, acetone) +37.4.

(2*S*)-2-[2-(2*S*)-Mercaptomethyl-3-(3*R*)-phenyl-butanoylamino]-3-(1*H*-indol-3-yl)-propanoic Acid (9b). Obtained from **4e′$_1$**: HPLC CH_3CN, 50%, 11.00 min. ^{1}H NMR δ (DMSO) 0.9 (d, 8.5 Hz, CH_3), 1.95 (t, SH), 2.4 (m, 1H), 2.65 (m, 3H), 2.9 (m, 2H), 4.3 (m, CHαTrp), 6.8 (s, 1H), 6.9 (t, 1H), 7.0 (t, 1H), 7.1 (m, 5H), 7.25 (d, 2H), 7.4 (d, 2H), 8.15 (d, NH), 10.7 (s, 1H). $[\alpha]^{20}_D$ (c = 0.246, acetone) −11.8.

(2*S*)-2-[2-(2*S*)-Mercaptomethyl-3-(3*R*)-phenyl-butanoylamino]-3-(1*H*-indol-3-yl)-propanoic Acid (9c). Obtained from **4e$_2$**: HPLC CH_3CN, 50%, 11.65 min. ^{1}H NMR δ (DMSO) 1.1 (d, CH_3), 1.5 (t, SH), 1.75 (m, 1H), 2.5 (m, 1H), 2.65 (m, 2H), 3.05 (m, 1H), 3.2 (m, 1H), 4.6 (m, CHαTrp), 6.9 (t, 1H), 7.0 (t, 1H), 7.1 (m, 7H), 7.5 (d, 1H), 8.4 (d, 1H), 10.8 (s, 1H). $[\alpha]^{20}_D$ (c = 0.274, acetone) +34.6.

(2*S*)-2-[2-(2*S*)-Mercaptomethyl-3-(3*S*)-phenyl-butanoylamino]-3-(1*H*-indol-3-yl)-propanoic Acid (9d). Obtained from **4e′$_2$**: HPLC CH_3CN, 50%, 12.20 min. ^{1}H NMR δ (DMSO) 0.8 (d, CH_3), 1.8 (t, SH), 1.9 (m, 1H), 2.4 (m, 1H), 2.6 (m, 2H), 3.0 (s, 1H), 3.2 (s, 1H), 4.5 (m, CHαTrp, 1H), 6.9−7.0 (m, 2H), 7.1−7.3 (m, 7H), 7.5 (d, 1H), 8.4 (d, NH), 10.8 (s, NH indoyl). $[\alpha]^{20}_D$ (c = 0.200, acetone) −51.5.

(2*S*)-2-[2-(2*S*)-Mercaptomethyl-3-(3*S*)-biphen-4-ylbutanoylamino]-3-(1*H*-indol-3-yl)-propanoic Acid (10a). Obtained from **4f₁**: HPLC CH_3CN, 60%, 7.63 min. ^1H NMR δ (DMSO) 1.2 (d, 8.5 Hz, CH_3), 1.7 (t, 7 Hz, SH), 2.3 (m, 1H, CH_2S), 2.7 (m, 1H, CH_2S), 2.8 (m, 1H, CHCl), 2.95−3.1 (m, CH_2Trp), 3.2 (m, 1H, $CHCH_3$), 4.4 (m, 1H, CHTrp), 6.9 (m, 1H), 7.0 (m, 1H), 7.1 (m, 1H), 7.3 (m, 1H), 7.4 (m, 4H), 7.45 (m, 1H), 7.5 (m, 1H), 7.6 (m, 2H), 7.7 (m, 2H), 8.2 (d, NH), 10.8 (s, NH indoyl). $[\alpha]^{20}_D$ (c = 0.218, acetone) +53.8.

(2*S*)-2-[2-(2*S*)-Mercaptomethyl-3-(3*R*)-biphen-4-ylbutanoylamino]-3-(1*H*-indol-3-yl)-propanoic Acid (10b). Obtained from **4f₁**: HPLC CH_3CN, 60%, 7.85 min. ^1H NMR δ (DMSO) 1.05 (d, 8.5 Hz, CH_3), 1.9 (t, 7 Hz, SH), 2.4 (m, 1H), 2.55−2.7 (m, 2H), 2.8 (m, 2H), 3.0 (m, 1H), 4.35 (q, CH-Trp), 6.7−7.0 (m, 4H), 7.2 (m, 2H), 7.4 (m, 4H), 7.6 (s, 1H), 7.7 (m, 3H), 8.2 (d, NH), 10.65 (s, NH indoyl). $[\alpha]^{20}_D$ (c = 0.2, acetone) −23.2.

(2*S*)-2-[2-(2*S*)-Mercaptomethyl-3-(3*R*)-biphen-4-ylbutanoylamino]-3-(1*H*-indol-3-yl)-propanoic Acid (10c). Obtained from **4f₂**: HPLC CH_3CN, 60%, 5.03 min. ^1H NMR δ (DMSO) 1.15 (d, 7 Hz, CH_3), 1.5 (t, SH), 1.9 (t, 1H), 2.4 (m, 1H), 2.75 (m, 1H), 2.8 (m, 1H), 3.1 (m, 1H), 3.2 (m, 1H), 4.6 (m, 1H, CHαTrp), 6.9−7.0 (m, 2H), 7.2 (s, 1H), 7.3 (d, 1H), 7.35−7.45 (m, 4H), 7.55 (d, 1H), 7.65 (s, 1H), 7.8 (m, 4H), 8.5 (d, NH), 10.8 (s, NH indoyl). $[\alpha]^{20}_D$ (c = 0.280, acetone) +76.1.

(2*S*)-2-[2-(2*S*)-Mercaptomethyl-3-(3*S*)-biphen-4-ylbutanoylamino]-3-(1*H*-indol-3-yl)-propanoic Acid (10d). Obtained from **4f₂**: HPLC CH_3CN, 60%, 8.27 min. ^1H NMR δ (DMSO) 0.85 (d, 7 Hz, CH_3), 1.85 (m, 1H), 1.9 (m, SH), 2.45 (m, 1H), 2.7 (m, 2H), 3.05 (m, 1H), 3.2 (m, 1H), 4.6 (m, CHαTrp), 6.9−7.0 (m, 2H), 7.2 (s, 2H), 7.25 (m, 2H), 7.40 (m, 2H), 7.5 (m, 2H), 7.8 (m, 4H), 8.5 (d, NH), 10.8 (s, NH indoyl). $[\alpha]^{20}_D$ (c = 0.202, acetone) −83.8.

(2*S*)-2-[(2*S*)-2-(1*S*)-Indan-1-yl-3-mercapto-propionylamino]-3-(1*H*-indol-3-yl)-propanoic Acid (13a). Obtained from **4i₁**: HPLC CH_3CN, 60%, 6.44 min. ^1H NMR δ (DMSO) 1.65 (t, 7 Hz, SH), 1.75−1.95 (m, 2H, CH_2-$\mathbf{CH_2}$-CH), 2.15−2.65 (m, CH_2S), 2.65−2.75 (3H, m), 3.0−3.15 (m, CH_2Trp), 3.45 (m, 1H), 4.6 (CH-Trp, m), 6.85−7.0 (m, 4H), 7.1−7.2 (m, 3H), 7.3 (d, 1H), 7.5 (d, 1H), 8.35 (d, 1H), 10.8 (s, 1H). $[\alpha]^{20}_D$ (c = 0.235, acetone) +36.3.

(2*S*)-2-[(2*R*)-2-(1*R*)-Indan-1-yl-3-mercapto-propionylamino]-3-(1*H*-indol-3-yl)-propanoic Acid (13b). Obtained from **4i₁**: HPLC CH_3CN, 60%, 6.72 min. ^1H NMR δ (DMSO) 1.6 (m, 2H, CH-$\mathbf{CH_2}$−CH_2), 2.0 (t, SH), 2.15 (t, 1H), 2.5−2.65 (m, 3H), 2.75 (m, 1H), 2.9 (m, 1H), 3.15 (m, 2H), 4.6 (m, CH-Trp), 6.7−7.1 (m, 7H), 7.3 (d, 1H), 7.5 (d, 1H), 8.3 (d, NH), 10.8 (s, NH indoyl). $[\alpha]^{20}_D$ (c = 0.233, acetone) −10.3.

(2*S*)-2-[(2*S*)-2-(1*R*)-Indan-1-yl-3-mercapto-propionylamino]-3-(1*H*-indol-3-yl)-propanoic Acid (13c). Obtained from **4i₂**: HPLC CH_3CN, 60%, 6.46 min. ^1H NMR δ (DMSO) 1.55 (m, SH), 1.9−2.0 (m, 2H, CH-$\mathbf{CH_2}$-CH_2), 2.4−2.5 (m, 2H), 2.6 (m, 2H), 2.85−3.0 (m, 2H), 3.15 (m, 2H), 4.5 (m, CHαTrp), 6.9 (t, 1H), 7.0−7.3 (m, 7H), 7.5 (d, 1H), 8.4 (d, NH), 10.8 (s, NH indoyl). $[\alpha]^{20}_D$ (c = 0.264, acetone) +50.31.

(2*S*)-2-[(2*R*)-2-(1*S*)-Indan-1-yl-3-mercapto-propionylamino]-3-(1*H*-indol-3-yl)-propanoic Acid (13d). Obtained from **4i₂**: HPLC CH_3CN, 60%, 6.71 min. ^1H NMR δ (DMSO) 1.5−1.7 (m, 2H, CH-$\mathbf{CH_2}$-CH_2), 2.0 (m, 5H), 2.45 (m, 1H), 2.55 (m, 2H), 2.7−2.9 (m, 3H), 3.05 (m, 1H), 3.1 (m, 1H), 4.5 (m, CHαTrp), 6.8−7.1 (m, 7H), 7.2 (d, 1H), 7.7 (d, 1H), 8.4 (d, NH), 10.7 (d, NH indoyl). $[\alpha]^{20}_D$ (c = 0.222, acetone) −22.8.

(2*S*)-2-[3-Mercapto-2-(2*S*)-((1*S*),1,2,3,4-tetrahydro-naphthalen-1-yl)-propionyl-amino]-3-(1*H*-indol-3-yl)-propanoic Acid (14a). Obtained from **4j₁**: HPLC CH_3CN, 60%, 7.15 min. ^1H NMR δ (DMSO) 1.4−1.6 (m, 3H), 1.6 (m, 1H), 1.65 (t, 1H, SH), 1.9 (m, 1H), 2.5−2.7 (m, 3H), 3.0−3.2 (m, 4H), 4.55 (q, 1H, CH-Trp), 6.9−7.1 (m, 5H), 7.15 (s, 1H), 7.2 (m, 2H), 7.5 (m, 1H), 8.4 (d, 1H, NH), 10.8 (s, 1H, NH indoyl). $[\alpha]^{20}_D$ (c = 0.254, acetone) +41.2.

(2*S*)-2-[3-Mercapto-2-(2*R*)-((1*R*),1,2,3,4-tetrahydro-naphthalen-1-yl)-propionyl-amino]-3-(1*H*-indol-3-yl)-propanoic Acid (14b). Obtained from 4j₁: HPLC CH_3CN, 60%, 7.43 min. ^1H NMR δ (DMSO) 1.1 (m, 1H), 1.3 (m, 2H), 1.6 (m, 1H), 1.9 (t, SH), 1.95 (m, 1H), 2.6 (m, 2H), 2.65 (m, 1H), 2.9 (m, 2H), 3.1 (m, 2H), 4.55 (q, CH-Trp), 6.9−7.0 (m, 6H), 7.2 (d, 2H), 7.45 (d, 1H), 8.3 (d, NH), 10.7 (s, NH indoyl). $[\alpha]^{20}_D$ (c = 0.202, acetone) −26.3.

(2*S*)-2-[3-Mercapto-2-(2*S*)-((1*R*),1,2,3,4-tetrahydro-naphthalen-1-yl)-propionyl-amino]-3-(1*H*-indol-3-yl)-propanoic Acid (14c). Obtained from **4j₂**: HPLC CH_3CN, 60%, 6.82 min. ^1H NMR δ (DMSO) 1.4 (m, 1H), 1.45 (m, 1H), 1.6 (m, SH), 1.7 (m, 1H), 2.0 (m, 1H), 2.2 (t, 1H), 2.6 (m, 3H), 2.7−2.85 (m, 2H), 3.0 (m, 1H), 3.15 (m, 1H), 4.6 (q, 1H), 6.9 (t, 1H), 7.0 (m, 5H), 7.1 (s, 1H), 7.25 (d, 1H), 7.5 (d, 1H), 8.3 (d, NH), 10.8 (s, NH indoyl). $[\alpha]^{20}_D$ (c = 0.266, acetone) +26.5.

(2*S*)-2-[3-Mercapto-2-(2*R*)-((1*S*),1,2,3,4-tetrahydro-naphthalen-1-yl)-propionyl-amino]-3-(1*H*-indol-3-yl)-propanoic Acid (14d). Obtained from **4j₂**: HPLC CH_3CN, 60%, 7.13 min. ^1H NMR δ (DMSO) 1.25 (m, 2H), 1.4 (m, 1H), 1.8 (m, 1H), 2.0 (t, SH), 2.2 (t, 1H), 2.55 (m, 2H), 2.7 (m, 3H), 2.95 (m, 1H), 3.15 (m, 1H), 4.5 (m, CHαTrp), 7.0 (m, 6H), 7.1 (s, 1H), 7.3 (d, 1H), 7.5 (d, 1H), 8.4 (d, NH), 10.7 (s, NH indoyl). $[\alpha]^{20}_D$ (c = 0.285, acetone) −52.6.

In Vitro Inhibition of NEP, ACE, and ECE Activities. 1. Enzymes. NEP was purified to homogeneity from rabbit kidney as previously described.[35] ACE was purified from rat testis.[36] Recombinant human ECE-1c (hECE-1c) was expressed in Cos-7 cells.[37]

2. Substrates. Inhibitory potencies were determined by using DGPA (Dansyl-Gly-(p-NO_2)Phe-β-ala)[38] (K_m = 37 μM) for NEP, N-Cbz-Phe-His-Leu (K_m = 50 μM)[39] for ACE, and the new substrate (Pya[21]-(p-NO_2)Phe[22])bigET(19−35) (K_m = 20 μM) for ECE-1c.[37]

3. Assay for NEP. NEP (final concentration 250 ng/mL, specific activity on DGPA, 345 mmol/mg/min) was preincubated for 10 min at 37 °C in black 96-well microplates with or without increasing concentrations of inhibitors in a total volume of 100 μL of 50 mM Tris-HCl buffer pH = 7.4. DGPA was added to a final concentration of 50 μM, and the reaction was stopped after 1 h by adding 150 μL of DMSO. The fluorescence was measured at λ_{ex} = 340 nm, λ_{em} = 530 nm.

4. Assay for ACE. ACE (final concentration 0.02 pmol/100 μL, specific activity on N-Cbz-Phe-His-Leu, 13 nmol/mg/min) was preincubated for 10 min at 37 °C in black 96-well microplates with or without various concentrations of inhibitors in 50 mM Tris/HCl/NaCl 1% buffer pH 7.4. N-Cbz-Phe-His-Leu was added to a final concentration of 50 μM. The reaction was stopped after 30 min by adding 50 μL of 2 N NaOH. After dilution with 50 μL of water, the concentration of His-Leu was determined following the fluorometric assay previously described.[39]

5. Assay for ECE. ECE activity was performed in black 96-well microplates in a final volume of 100 μL with recombinant human ECE-1c (hECE-1c). hECE-1c at 4 μg total protein by well was preincubated for 10 min at 37 °C in 50 mM tris-maleate pH 6−8 with or without increasing concentrations of inhibitors. The reaction was initiated by addition of 20 μM of the substrate (Pya[21]-(p-NO_2)Phe[22])bigET(19−35), and the mixture was incubated 1 h at 37 °C. The reaction was stopped by cooling, and the fluorescence was measured (λ_{ex} = 340 nm, λ_{em} = 400 nm).

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