New α-Thiol Dipeptide Dual Inhibitors of Angiotensin-I Converting Enzyme and Neutral Endopeptidase EC 3.4.24.11

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Dual inhibitors of the two zinc metallopeptidases, neutral endopeptidase (NEP, EC 3.4.24.11) and angiotensin-I converting enzyme, have been the focus of much clinical interest for the treatment of hypertension and congestive heart failure. A novel series of α-thio dipeptides containing central cyclic non-natural amino acids were prepared and were evaluated for their ability to inhibit these two metallopeptidases *in vitro* and *in vivo*. Most of these compounds were found to be excellent dual inhibitors of ACE and NEP *in vitro* and several were also found to inhibit angiotensin-I (AI) pressor response in conscious rats when given by intravenous administration. Compound **6n**, one of our most potent dual inhibitors *in vitro*, was found to be more efficacious than captopril in the AI pressor experiment when administered orally to conscious rats. This compound was also found to inhibit plasma NEP activity following oral administration to conscious rats and was more efficacious than acetorphan. The structure–activity relationships and biological activity of these dual inhibitors will be discussed.

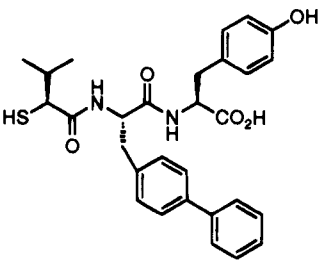
Introduction

Recently considerable interest has been devoted to the development of dual-acting inhibitors of the membrane-bound zinc metalloproteases, angiotensin-I converting enzyme and neutral endopeptidase EC 3.4.24.11.[1-12] These two ectoenzymes are involved in the metabolism of a variety of regulatory peptides which play an important role in blood pressure control and fluid homeostasis. Angiotensin-I converting enzyme (ACE) is part of the enzymatic cascade of the renin–angiotensin–aldosterone system (RAAS) and catalyzes the conversion of the decapeptide angiotensin-I to the octapeptide angiotensin-II. Angiotensin-II is a potent vasoconstrictor and promotes the release of aldosterone, which leads to sodium and fluid retention.

Neutral endopeptidase (NEP) is involved in the metabolic degradation of atrial natriuretic factor (ANF).[13-15] ANF is a 28-amino acid polypeptide which is synthesized primarily in the atrial myocytes and is secreted into circulation in response to atrial distention. The interaction of ANF with its receptors leads to the generation of c-GMP and elicits a number of biological responses including diuresis, natriuresis, vasodilatation, and the reduction of renin and aldosterone plasma levels.[16,17]

ACE inhibitors have gained wide acceptance clinically and are commonly prescribed for the treatment of hypertension and congestive heart failure (CHF).[18] To further increase the efficacy of this class of therapeutic agents, they can be co-administered with a diuretic.[19] Diuretics, however, can evoke a number of undesired side effects such as activation of pressor systems, hypokalemia, hyperglycemia, and elevation of plasma lipids.[20] Selective inhibitors of NEP have been studied in animal models and in humans and have been found to produce significant diuretic and natriuretic effects without kaliuresis.[21] Moreover, the co-administration of a selective ACE and NEP inhibitor in models of hypertension and CHF has shown a potentiation of its respective effects over administration of the single agents separately.[22,23]

In a recent communication, we disclosed a series of α-mercaptodipeptides that were found to be dual-acting ACE and NEP inhibitors.[24] One of the most potent dual inhibitors disclosed was compound **1** ((ACE)IC_{50} = 62



1

nM, (NEP)IC_{50} = 28 nM). This compound was found to inhibit angiotensin-I (AI) induced pressor response in normotensive conscious rats after intravenous dosing, indicating that it was efficacious *in vivo* as an ACE inhibitor. Unfortunately, its duration of effect was short; 50% of the AI pressor response was inhibited for only 60 min at 10 mg/kg after intravenous administration. We decided to use this compound as a lead structure and strived to improve its duration of action. We were hopeful that by replacing the amino acids in this molecule with non-natural amino acids that would not impair the *in vitro* potency of these compounds we might improve the metabolic stability and thus the efficacy of compound **1**. In our earlier work we investigated the preparation of a number of α-mercaptoacyl dipeptides. None of these compounds contained spiroalkyl unnatural amino acids. We believed that a spiroalkyl amino acid would provide more bulk about the amide bond and thus may protect it from hydrolysis. Herein we report our results on a series of spiroalkyl α-mercaptodipeptide dual ACE and NEP inhibitors.

Chemistry

The target α-mercaptodipeptides were prepared as outlined in Scheme 1. Synthesis of these dual inhibitors

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Scheme 1

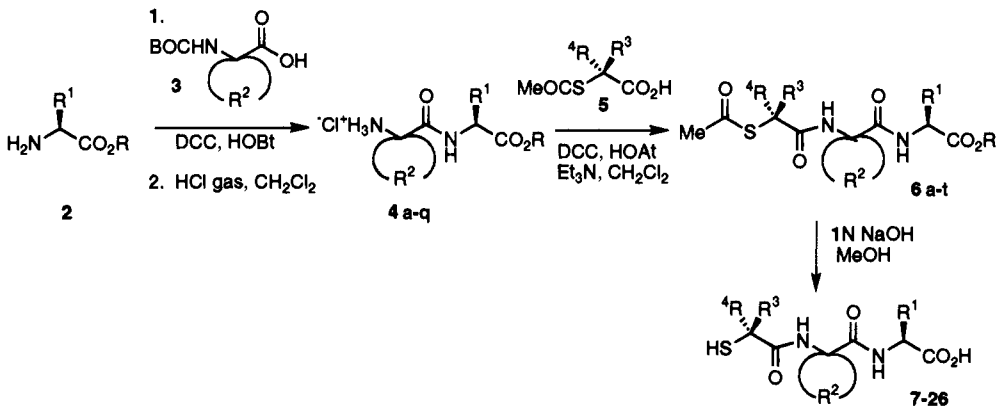
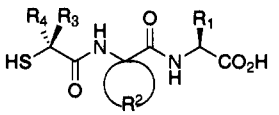


Table 1. Physiochemical Properties of α-Mercaptodipeptides



compd	R^1	R^2	R^3	R^4	mp (°C)	formula	analysis
7	CH_2(*p*-OHPh)	$(CH_2)_2$	$CH(CH_3)_2$	H	91–92	$C_{18}H_{24}N_2O_5S$	C, H, N
8	CH_2(*p*-OHPh)	$(CH_2)_4$	$CH(CH_3)_2$	H	209–210	$C_{20}H_{28}N_2O_5S$	C, H, N
9	CH_2(*p*-OHPh)	$(CH_2)_5$	$CH(CH_3)_2$	H	112–114	$C_{21}H_{30}N_2O_5S$	C, H, N
10	CH_2(*p*-OHPh)	$((CH_2)_2O(CH_2)_2)$	$CH(CH_3)_2$	H	221	$C_{20}H_{28}N_2O_5S$	C, H, N
11	CH_2(*p*-OHPh)	2-norbornyl[a]	$CH(CH_3)_2$	H	105	$C_{22}H_{30}N_2O_5S$	C, H, N
12	CH_2(*p*-OHPh)	1,2-$(CH_2)_2$Ph	$CH(CH_3)_2$	H	186–187	$C_{24}H_{28}N_2O_5S$	C, H, N
13	CH_2(*p*-OHPh)	$(CH_3)_2$	$CH(CH_3)_2$	H	180–181	$C_{18}H_{26}N_2O_5S$	C, H, N
14	CH_2(*p*-MeOPh)	$(CH_2)_4$	$CH(CH_3)_2$	H	182–183	$C_{21}H_{30}N_2O_5S$	C, H, N
15	CH_2(*p*-FPh)	$(CH_2)_4$	$CH(CH_3)_2$	H	168–169	$C_{20}H_{27}FN_2O_4S$	C, H, N
16	CH_2(2-thienyl)	$(CH_2)_4$	$CH(CH_3)_2$	H	175–176	$C_{18}H_{26}N_2O_4S_2$	C, H, N
17	CH_2(3-thienyl)	$(CH_2)_4$	$CH(CH_3)_2$	H	182–184	$C_{18}H_{26}N_2O_5S_2$	C, H, N
18	CH_2(2-pyridyl)	$(CH_2)_4$	$CH(CH_3)_2$	H	175–176	$C_{19}H_{27}N_3O_4S$	C, H, N
19	CH_2(3-pyridyl)	$(CH_2)_4$	$CH(CH_3)_2$	H	218–219	$C_{19}H_{27}N_3O_4S$	C, H, N
20	CH_2(3-indolyl)	$(CH_2)_4$	$CH(CH_3)_2$	H	145–146	$C_{22}H_{29}N_3O_4S$	C, H, N
21	CH_2(5-OH-3-indolyl)	$(CH_2)_4$	$CH(CH_3)_2$	H	105	$C_{22}H_{29}N_3O_5S$	C, H, N
22	CH_2(*p*-PPh)	$(CH_2)_4$	$CH(CH_3)_2$	H	180–181	$C_{26}H_{32}N_2O_4S$	C, H, N
23	CH_2CH_2Ph	$(CH_2)_4$	$CH(CH_3)_2$	H	203–204	$C_{21}H_{30}N_2O_4S$	C, H, N
24	CH_2(*p*-OHPh)	$(CH_2)_4$	H	H	118–119	$C_{17}H_{22}N_2O_5S$	C, H, N
25	CH_2(*p*-OHPh)	$(CH_2)_4$	CH_2Ph	H	187–189	$C_{24}H_{28}N_2O_5S$	C, H, N
26	CH_2(*p*-OHPh)	$(CH_2)_4$	$-(CH_2)_4-$		90	$C_{21}H_{28}N_2O_5S$	C, H, N

[a] Compound is racemic at this center.

was initiated by coupling various α-amino acid esters (**2**) to cyclic Boc-protected amino acids by the classical DCC/HOBt method.[25,26] Deprotection of the Boc-protected dipeptides with anhydrous HCl gas in methylene chloride afforded the amine hydrochloride salts (**4**). Coupling of the α-mercaptoacyl acids[27] (**5**) to the dipeptide amine hydrochlorides (**4**) under the standard DCC/HOBt method gave poor yields (<15%) and led to significant racemization of the α-thiol center. A number of alternative peptide coupling procedures were investigated, and we found that the new coupling additive, 1-hydroxy-7-azabenzotriazole (HOAt) recently described by Carpino gave superior results.[28] We were able to obtain reasonable yields of the α-mercaptoacyl dipeptides (**6**). The reaction times were shorter, and no or only trace amounts of the undesired diasteromers were detected by ^1H NMR. Double deprotection of the α-mercaptoacyl dipeptides (**6**) with degassed 1 N sodium hydroxide in methanol yielded the desired α-thiols. The α-mercaptodipeptides (compounds **7**–**26**) that were prepared are shown in Table 1.

Results and Discussion

***In Vitro* Inhibitory Potencies on ACE and NEP.** The *in vitro* inhibitory potencies for ACE and NEP of the α-mercaptodipeptides prepared are reported in Table 2. The ACE assay was carried out as described in the literature.[29] The NEP assay was carried out with a slight modification to the literature procedure as described in the Experimental Section.[30] For each round of IC_{50} determinations, enalaprilat and captopril served as controls for the ACE assay and thiorphan and phosphoramidon served as controls for the NEP assay. In our initial structure–activity relationship studies of our dipeptide **1**, the C-terminal and α-thiol portions of the molecule were held constant while the central amino acid was varied. Replacement of the (*S*)-biphenylylalanine residue with a variety of non-natural amino acids yielded compounds **7**–**13**. To simplify the structure of **1**, with the exception of compound **11**, achiral cyclic amino acids were placed at this center. These compounds all exhibited good *in vitro* inhibitory activity toward ACE and NEP. The best compound in this series was compound **8**, the cycloleucine derivative. This compound was more potent with respect to its *in vitro* ACE and NEP inhibitory potencies than our lead biphenylylalanine dipeptide **1**, and in comparision to thiorphan and phosphoramidon, it was a more potent

Table 2. *In Vitro* ACE and NEP-Inhibitory Activity of α-Mercaptodipeptides[a]

compd	IC_{50}(ACE) (nM)	IC_{50}(NEP) (nM)
1	62	28
7	15	5.0
8	7.0	1.5
9	33	8.7
10	11	6.4
11	65	15
12	26	25
13	15	9.2
14	19	2.2
15	24	1.8
16	18	1.5
17	11	2.6
18	35	3.0
19	37	3.1
20	10	2.1
21	13	2.0
22	139	16
23	30	1.2
24	58	15
25	22	3.0
26	17	5.2
thiorphan		5.4 ± 0.18[b]
phosphoramidon		26
captopril	10 ± 0.8[c]	
enalaprilat	8.8	

[a] Details of the enzyme assays are described in the Experimental Section, number of determinations for each IC_{50} value was 2 ($n = 2$). [b] $n = 6$. [c] $n = 14$.

Table 3. Plasma Inhibition of ACE Activity in the Conscious Rat[a]

compd	dose (mg/kg)	% inhibition of AI pressor response 1 h	2 h	4 h	6 h
7	10 (iv)	91 ± 4	43 ± 11	30 ± 16	17 ± 7
8	10 (iv)	90 ± 1	91 ± 4	83 ± 1	68 ± 9
8	10 (po)	48 ± 1	59 ± 7	36 ± 13	15 ± 12
9	10 (iv)	22 ± 3	10 ± 5	6 ± 6	
10	10 (iv)	43 ± 3	27 ± 4	4 ± 3	
12	10 (iv)	86 ± 3	59 ± 3	24 ± 8	9 ± 0
13	10 (iv)	88 ± 4	81 ± 3	69 ± 3	67 ± 5
15	10 (iv)	85 ± 8	80 ± 6	38 ± 6	28 ± 6
16	10 (iv)	79 ± 2	68 ± 7	44 ± 15	29 ± 12
17	10 (iv)	97 ± 3	100 ± 0	87 ± 0	71 ± 3
18	10 (iv)	73 ± 6	52 ± 10	28 ± 8	17 ± 3
20	10 (iv)	98 ± 2	92 ± 1	86 ± 3	82 ± 3
20	10 (po)	45 ± 14	50 ± 16	29 ± 12	26 ± 16
23	10 (iv)	72 ± 8	69 ± 2	79 ± 6	75 ± 4
24	10 (iv)	44 ± 13	15 ± 4	7 ± 2	
25	10 (iv)	86 ± 5	75 ± 14	43 ± 5	17 ± 3
26	10 (iv)	3 ± 7			
6n	12.3 (po)[b]	83 ± 3	72 ± 4	71 ± 9	64 ± 8
captopril	10 (ia)[c]	96 ± 4	60 ± 3	26 ± 11	3 ± 3
	10 (po)	100 ± 0	78 ± 1	36 ± 12	13 ± 12
benazeprilat	10 (ia)[c]	100 ± 0	74 ± 4	27 ± 10	7 ± 5

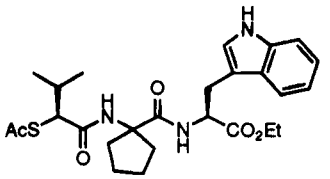
[a] Tests were carried out as described in the Experimental Section. [b] Administered po in 3% cornstarch (1 mg/mL), molar equivalent 10 mg/kg of **20**. [c] No experimental differences could be detected between ia or iv administration.

NEP inhibitor. Compound **8** was also found to be equipotent to enalaprilat and captopril as an ACE inhibitor.

Compounds that contained the cycloleucine central amino acid and the same terminal α-thiol portion as compound **1**, but where modifications were made to the C-terminal amino acid residue, were also investigated. Analogs which contained a variety of natural and unnatural aryl and heteroarylamino acids residues at this position were prepared. Compounds **14**–**23**, with the exception of compound **22**, were all found to be potent dual inhibitors of ACE and NEP. None of these molecules were more potent than the tyrosine-derived analog, compound **8**, although compounds **17**, **20**, and **21** were similar in potency. Interestingly, we found it was possible to replace the tyrosine hydroxyl functionality in compound **8** with either a fluorine (compound **15**) or a methoxy group (compound **14**) and retain NEP activity. The ACE inhibitory activity was about 2–3 times less. Also, we found that it was possible to replace the tyrosine with heterocyclic amino acids such as thienylalanine (compounds **16** and **17**), pyridylalanine (compounds **18** and **19**), or tryptophan (compound **20**) and retain most of the compounds NEP activity compared to compound **8**. The ACE activity of these compounds was compromised slightly.

A few modifications were made to the α-thiol terminal portion of the molecule (compounds **24**–**26**). No improvement in ACE or NEP *in vitro* inhibitory activity was seen.

***In Vivo* ACE and NEP Inhibition.** Most of the compounds that were found to be potent dual acting ACE and NEP inhibitors *in vitro* (IC_{50}'s < 50 nM) were evaluated for their *in vivo* ACE potency in conscious rats (Table 3).[31] Many of these compounds were found to be longer acting inhibitors of AI pressor response in the conscious rat than either captopril or benazeprilat. In particular compounds **8**, **13**, **17**, **20**, and **23** were found to inhibit 50% of the AI pressor response for more than 6 h. Interestingly, it was found that the spirocyclopentyl derivative compound **8** was longer acting *in vivo* than the spirocyclopropyl or spirocyclohexyl derivatives, compounds **7** and **9**, despite their nearly equal ACE and NEP *in vitro* activities. Replacing the tyrosine hydroxyl group with a fluorine (compound **15**) decreased the compound's duration of effect compared to compound **8** *in vivo*. Compounds **24**, **25**, and **26**, where modifications were made to the isopropyl group of compound **8**, were also found to be shorter acting than compound **8**. Perhaps the α-thiol is more accessible to deactivation in these compounds. Compound **8** was also administered to a conscious rat at 10 mg/kg po and was found to be orally active with a similar duration of effect as captopril. We also evaluated the plasma inhibition of ACE activity of the simple ethyl ester *S*-acetyl analog of **20** (compound **6n**), which was one of our most efficacious inhibitors after iv administration. Compound **6n**, at 12.3 mg/kg po, was found to have improved oral activity over compound **20**. It inhibited AI pressor response by greater than 50% for more than 6 h, making it more efficacious than captopril (Figure 1).



6n

We also investigated the ability of **6n** to inhibit plasma NEP activity following oral administration to conscious rats. Compound **6n** at 12.3 mg/kg po was found to significantly increase exogenous plasma ANF concentrations, approximately 100% over controls at 4

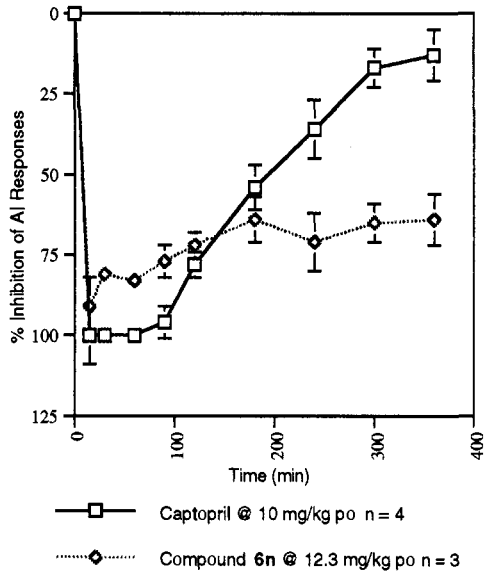


Figure 1. Inhibition of angiotensin-I pressor response in conscious rats treated with captopril and compound **6n** at 12.3 mg/kg po.

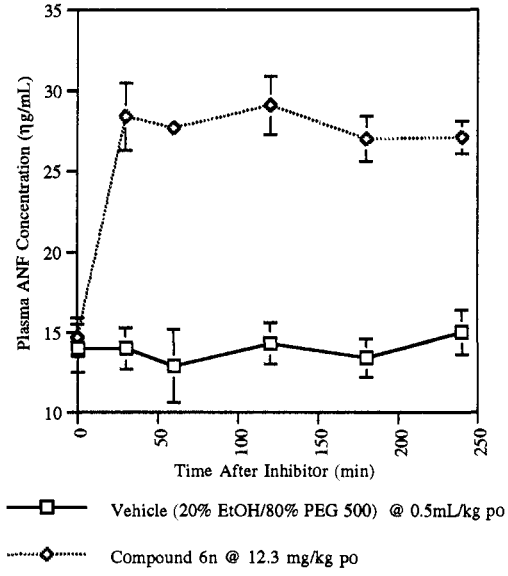
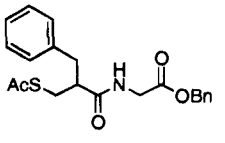


Figure 2. Potentiation of infused ANF in conscious rats due to compound **6n** at 12.3 mg/kg po.

h (Figure 2). In this assay, compound **6n** was found to be more efficacious than acetorphan (Figure 3), the thioacetate analog of thiorphan.



Acetorphan

In summary a number of potent dual acting *in vitro* α-mercaptodipeptide inhibitors of ACE and NEP activity have been described. Several of these compounds were found to have better *in vitro* potency and also displayed greatly improved *in vivo* ACE inhibitory activity over our lead structure **1** and over literature standards. Compound **6n**, one of our most potent *in vivo* ACE inhibitors, was found to be an orally active inhibitor of plasma ACE and NEP activity in conscious normotensive rats. We are in the process of preparing additional

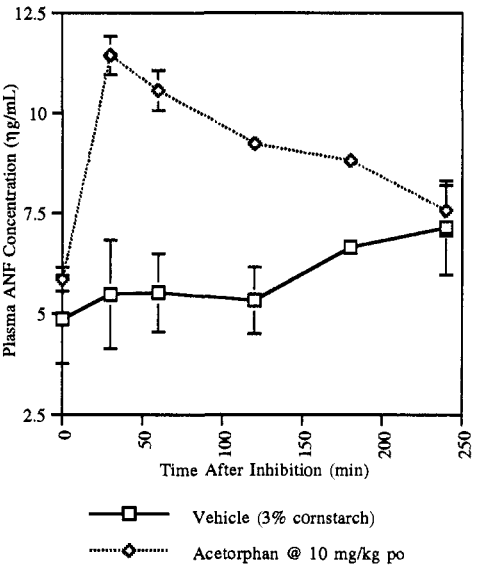


Figure 3. Potentiation of infused ANF in conscious rats due to acetorphan at 10 mg/kg po.

analogs of the compounds discussed and are evaluating their *in vivo* ACE and NEP inhibitory activity upon oral administration and their effect on blood pressure and renal function.

Experimental Section

I. Chemistry. Melting points (mp) were determined on a Thomas-Hoover or Melt-Temp melting point apparatus and are uncorrected. Infrared (IR) spectra were recorded on a Nicolet 5SXB FTIR spectrometer and are reported in cm^{-1}. Proton NMR spectra were recorded on a Bruker AC-250, Varian XL-300, or Varian XL-400 spectrometer with tetramethylsilane as the internal standard. The chemical shifts (δ) are reported in parts per million (ppm). Mass spectra were recorded on a Hewlett-Packard GC/MS 5985 spectrometer using chemical ionization (DCI) or on a Vestec 201 using thermospray technology (TSP). Microanalyses were carried out at Robertson Laboratory Inc., Madison, NJ. Flash chromatography under a nitrogen atmosphere on silica gel 60 (0.04–0.06 mm) (Baker) was used for compound purification. The amino acids used were commercially available, and all organic solvents used were of anhydrous grade.

General Procedure for the Preparation of Compounds 4. To a stirred solution of the amino acid ester **2** (1 equiv) and the Boc-protected amino acid **3** (1 equiv) in dichloromethane were added 1,3-dicyclohexylcarbodiimide (1 equiv) and 1-hydroxybenzotriazole (1 equiv). The mixture was stirred for 16–24 h, and then the solid precipitate was removed by filtration. The organic phase was washed with a saturated solution of sodium bicarbonate, and brine and dried over magnesium sulfate. The mixture was then filtered and concentrated under reduced pressure to give a white foam. The foam was purified by flash chromatography on silica gel, eluting with hexane–ethyl acetate. The pure fractions were combined and concentrated. The purified Boc-protected dipeptide was then dissolved in dichloromethane, and dry HCl gas was bubbled through the solution for 15 min. The solvent was removed under reduced pressure to yield a white foam. The compound was used without further purification.

4a (R = Et, $R^1 = CH_2(p\text{-HOPh})$, $R^2 = (CH_2)_2$): white foam (85%); ^1H NMR (CD_3OD) δ 1.27 (t, 3H), 1.35 (m, 4H), 2.88 (dd, 1H), 3.12 (dd, 1H), 4.12 (q, 2H), 4.59 (dd, 1H), 6.67 (d, 2H), 7.01 (d, 2H).

4b (R = Me , $R^1 = CH_2(p\text{-HOPh})$, $R^2 = (CH_2)_4$): white foam (62%); ^1H NMR (CD_3OD) δ 2.24–1.80 (m, 8H), 2.89 (dd, 1H), 3.16 (dd, 1H), 3.70 (s, 3H), 4.70 (dd, 1H), 6.72 (d, 2H), 7.08 (d, 2H).

4c (R = Me, $R^1 = CH_2$(*p*-HOPh), $R^2 = (CH_2)_5$): white foam (81%), ^{1}H NMR (CD_3OD) δ 2.05–1.39 (m, 10H), 2.88 (dd, 1H), 3.20 (dd, 1H), 3.72 (s, 3H), 4.67 (dd, 1H), 6.70 (d, 2H), 7.03 (d, 2H).

4d (R = Et, $R^1 = CH_2$(*p*-OHPh), $R^2 = (CH_2)_2O(CH_2)_2$): white foam (85%); ^{1}H NMR (CD_3OD) δ 1.23 (m, 7H), 3.12 (m, 2H), 4.10 (m, 6H), 4.71 (dd, 1H), 6.69 (d, 2H), 7.06 (d, 2H).

4e (R = Me, $R^1 = CH_2$(*p*-HOPh), R^2 = 2-norbornyl): white foam (73%); ^{1}H NMR (CD_3OD) δ 1.65–1.41 (m, 6H), 2.35 (m, 4H), 2.95 (m, 1H), 3.21 (m, 1H), 3.71 (s, 3H), 4.70 (m, 1H), 6.69 (d, 2H), 7.05 (d, 2H).

4f (R = Et, $R^1 = CH_2$(*p*-HOPh), $R^2 = CH_2$(*o*-CH_2Ph)): white foam (65%); ^{1}H NMR (CD_3OD) δ 1.23 (t, 3H), 2.85 (dd, 1H), 3.19 (dd, 1H), 3.42 (d, 1H), 3.75 (d, 1H), 4.15 (m, 2H), 4.69 (dd, 1H), 6.69 (d, 2H), 6.95 (d, 2H), 7.31 (m, 4H).

4g (R = Et, $R^1 = CH_2$(*p*-HOPh), $R^2 = (CH_3)_2$): white foam (90%); ^{1}H NMR (CD_3OD) δ 1.25 (t, 3H), 1.40 (s, 3H), 1.52 (s, 3H), 2.90 (dd, 1H), 3.20 (dd, 1H), 4.15 (q, 2H), 4.61 (dd, 1H), 6.67 (d, 2H), 7.07 (d, 2H).

4h (R = Me, $R^1 = CH_2$(*p*-MeOPh), $R^2 = (CH_2)_4$): white foam (66%); ^{1}H NMR (CD_3OD) δ 1.90 (m, 8H), 2.91 (dd, 1H), 3.22 (dd, 1H), 3.75 (s, 3H), 3.78 (s, 3H), 4.71 (dd, 1H), 6.80 (d, 2H), 7.11 (d, 2H).

4i (R = Me, $R^1 = CH_2$(*p*-FPh), $R^2 = (CH_2)_4$): white foam (78%); ^{1}H NMR (CD_3OD) δ 2.23–1.89 (m, 8H), 3.15 (m, 2H), 3.73 (s, 3H), 4.71 (m, 1H), 7.00 (m, 2H), 7.22 (d, 2H).

4j (R = Me, $R^1 = CH_2$(2-thienyl), $R^2 = (CH_2)_4$): white foam (78%); ^{1}H NMR (CD_3OD) δ 2.10 (m, 8H), 3.21 (dd, 1H), 3.47 (dd, 1H), 3.79 (s, 3H), 4.71 (dd, 1H), 6.87 (m, 2H), 7.21 (d, 1H).

4k (R = Me, $R^1 = CH_2$(3-thienyl), $R^2 = (CH_2)_4$): white foam (75%); ^{1}H NMR (CD_3OD) δ 1.90 (m, 8H), 3.09 (dd, 1H), 3.27 (dd, 1H), 3.75 (s, 3H), 4.79 (dd, 1H), 6.95 (d, 1H), 7.12 (bs, 1H), 7.31 (m, 1H).

4l (R = Me, $R^1 = CH_2$(2-pyridyl), $R^2 = (CH_2)_4$): white foam (76%); ^{1}H NMR (CD_3OD) δ 1.98 (m, 8H), 3.59 (dd, 1H), 3.75 (s, 3H), 3.79 (dd, 1H), 4.95 (dd, 1H), 8.05 (m, 2H), 8.55 (dd, 1H) 8.71 (d, 1H).

4m (R = Me, $R^1 = CH_2$(3-pyridyl), $R^2 = (CH_2)_4$): white foam (75%); ^{1}H NMR (CD_3OD) δ 1.98 (m, 8H), 3.41 (dd, 1H), 3.67 (dd, 1H), 3.80 (s, 3H), 4.90 (m, 1H), 8.07 (dd, 1H), 8.61 (d, 1H), 8.80 (d, 1H), 8.91(s, 1H).

4n (R = Et, $R^1 = CH_2$(3-indolyl), $R^2 = (CH_2)_4$): white foam (48%); ^{1}H NMR (CD_3OD) δ 1.29 (t, 3H), 1.82 (m, 8H), 3.80 (m, 2H), 4.17 (q, 2H), 4.81 (dd, 1H), 7.07 (m, 3H), 7.25 (d, 1H), 7.53 (d, 1H).

4o (R = Et, $R^1 = CH_2$(5-HO-3-indolyl), $R^2 = (CH_2)_4$): white foam (83%); ^{1}H NMR (CD_3OD) δ 1.15 (t, 3H), 1.78 (m, 8H), 3.24 (m, 2H), 4.13 (q, 2H), 4.73 (dd, 1H), 6.64 (d, 1H), 6.91 (d, 1H), 7.02 (s, 1H), 7.13 (d, 1H).

4p (R = Me, $R^1 = CH_2$(*p*-PhPh), $R^2 = (CH_2)_4$): white foam (90%); ^{1}H NMR (CD_3OD) δ 1.98 (m, 8H), 3.05 (dd, 1H), 3.35 (dd, 1H), 3.75 (s, 3H), 4.85 (dd, 1H), 7.60–7.26 (m, 9H).

4q (R = Et, $R^1 = (CH_2)_2$Ph, $R^2 = (CH_2)_4$): white foam (80%); ^{1}H NMR (CD_3OD) δ 1.25 (t, 3H), 2.40–1.91 (m, 10H), 2.69 (m, 2H), 4.18 (q, 2H), 4.67 (dd, 1H), 7.20 (m, 5H).

General Procedure for the Preparation of Compounds 6. To a solution of the dipeptide hydrochloride **4** (1 equiv) in dichloromethane was added triethylamine (1 equiv). The mixture was stirred for 5 min and then the α-mercaptoacyl acid **5** (1 equiv), 1,3-dicyclohexylcarbodiimide (1 equiv), and 1-hydroxy-7-azabenzotriazole (1 equiv) were added. The mixture was stirred for 16 h, and then the solid precipitate was removed by filtration. The organic phase was washed with a saturated solution of sodium bicarbonate and brine, dried over magnesium sulfate, filtered, and concentrated under reduced pressure to give a white foam. The foam was purified by flash chromatography on silica gel, eluting with hexane–ethyl acetate. The pure fractions were combined and concentrated under reduced pressure.

6a (R = Et, $R^1 = CH_2$(*p*-HOPh), $R^2 = (CH_2)_2$, R^3 = H, $R^4 = CH(CH_3)_2$, $R^5 = C(CH_3)_3$): white solid (44%); mp 132–133 °C. Anal. ($C_{25}H_{36}N_2SO_6$) C, H, N.

6b (R = Me, $R^1 = CH_2$(*p*-HOPh), $R^2 = (CH_2)_4$, R^3 = H, $R^4 = CH(CH_3)_2$, $R^5 = CH_3$): white solid (55%); mp 157–158 °C. Anal. ($C_{23}H_{32}N_2SO_6$) C, H, N.

6c (R = Me, $R^1 = CH_2$(*p*-HOPh), $R^2 = (CH_2)_5$, R^3 = H, $R^4 = CH(CH_3)_2$, $R^5 = CH_3$): white solid (38%); mp 172–173 °C. Anal. ($C_{24}H_{34}N_2SO_6$) C, H, N.

6d (R = Et, $R^1 = CH_2$(*p*-HOPh), $R^2 = (CH_2)_2O(CH_2)_2$), R^3 = H, $R^4 = CH(CH_3)_2$, $R^5 = CH_3$): white solid (35%); mp 74–77 °C. Anal. ($C_{24}H_{34}N_2SO_7$) C, H, N.

6e (R = Me $R^1 = CH_2$(*p*-HOPh), R^2 = (2-norbornyl), R^3 = H, $R^4 = CH(CH_3)_2$, $R^5 = CH_3$): white solid (52%); mp 74–77 °C. Anal. ($C_{25}H_{34}N_2SO_6$) C, H, N.

6f (R = Et, $R^1 = CH_2$(*p*-HOPh), $R^2 = CH_2$(*o*-CH_2Ph), R^3 = H, $R^4 = CH(CH_3)_2$, $R^5 = CH_3$): white solid (56%); mp 145–146 °C. Anal. ($C_{28}H_{34}N_2SO_6$) C, H, N.

6g (R = Et, $R^1 = CH_2$(*p*-HOPh), $R^2 = (CH_3)_2$, R^3 = H, $R^4 = CH(CH_3)_2$, $R^5 = CH_3$): white solid (44%); mp 137–138 °C. Anal. ($C_{22}H_{32}N_2SO_6$) C, H, N.

6h (R = Me, $R^1 = CH_2$(*p*-MeOPh), $R^2 = (CH_2)_4$, R^3 = H, $R^4 = CH(CH_3)_2$, $R^5 = CH_3$): white solid (58%); mp 104–105 °C. Anal. ($C_{24}H_{34}N_2SO_6$) C, H, N.

6i (R = Me, $R^1 = CH_2$(*p*-FPh), $R^2 = (CH_2)_4$, R^3 = H, $R^4 = CH(CH_3)_2$, $R^5 = CH_3$): white solid (50%); mp 123–124 °C. Anal. ($C_{23}H_{31}FN_2SO_5$) C, H, N.

6j (R = Me, $R^1 = CH_2$(2-thienyl), $R^2 = (CH_2)_4$, R^3 = H, $R^4 = CH(CH_3)_2$, $R^5 = CH_3$): white solid (43%); mp 123–124 °C. Anal. ($C_{21}H_{30}N_2S_2O_5$) C, H, N.

6k (R = Me, $R^1 = CH_2$(3-thienyl), $R^2 = (CH_2)_4$, R^3 = H, $R^4 = CH(CH_3)_2$, $R^5 = CH_3$): white solid (38%); mp 110–112 °C. Anal. ($C_{21}H_{30}N_2S_2O_5$) C, H, N.

6l (R = Me, $R^1 = CH_2$(2-pyridyl), $R^2 = (CH_2)_4$, R^3 = H, $R^4 = CH(CH_3)_2$, $R^5 = CH_3$): white foam (48%).

6m (R = Me, $R^1 = CH_2$(3-pyridyl), $R^2 = (CH_2)_4$, R^3 = H, $R^4 = CH(CH_3)_2$, $R^5 = CH_3$): white solid (37%); mp 122–124 °C. Anal. ($C_{22}H_{31}N_3SO_5$) C, H, N.

6n (R = Et, $R^1 = CH_2$(3-indolyl), $R^2 = (CH_2)_4$, R^3 = H, $R^4 = CH(CH_3)_2$, $R^5 = CH_3$): white solid (39%); mp 112–113 °C. Anal. ($C_{26}H_{35}N_3SO_5$) C, H, N.

6o (R = Et, $R^1 = CH_2$(5-HO-3-indolyl), $R^2 = (CH_2)_4$, R^3 = H, $R^4 = CH(CH_3)_2$, $R^5 = CH_3$): white solid (41%); mp 189–190 °C. Anal. ($C_{26}H_{35}N_3SO_6$) C, H, N.

6p (R = Me, $R^1 = CH_2$(*p*-PhPh), $R^2 = (CH_2)_4$, R^3 = H, $R^4 = CH(CH_3)_2$, $R^5 = CH_3$): white solid (72%); mp 128–129 °C. Anal. ($C_{29}H_{36}N_2SO_5$) C, H, N.

6q (R = Et, $R^1 = ((CH_2)_2$Ph), $R^2 = (CH_2)_4$, R^3 = H, $R^4 = CH(CH_3)_2$, $R^5 = CH_3$): white solid (52%); mp 101–103 °C. Anal. ($C_{25}H_{36}N_2SO_5$) C, H, N.

6r (R = Me, $R^1 = CH_2$(*p*-HOPh), $R^2 = (CH_2)_4$, R^3 = H, R^4 = H, $R^5 = CH_3$): white solid (60%); mp 91–92 °C. Anal. ($C_{20}H_{26}N_2SO_6$) C, H, N.

6s (R = Et, $R^1 = CH_2$(*p*-HOPh), $R^2 = (CH_2)_4$, R^3 = H, $R^4 = CH_2$Ph $R^5 = CH_3$): white solid (30%); mp 137–138 °C. Anal. ($C_{28}H_{34}N_2SO_6$) C, H, N.

6t (R = Bu, $R^1 = CH_2$(*p*-HOPh), $R^2 = (CH_2)_4$, R^3, $R^4 = (CH_2)_3$ R^5 = (no acetyl group, SH)): white solid (57%); mp 106–107 °C. Anal. ($C_{25}H_{36}N_2SO_5$) C, H, N.

General Procedure for the Preparation of Compounds 7–26. To a solution of the α-mercaptoacetyl dipeptide **6** (0.1–0.5 mM) in degassed methanol (5–10 mL) under an atmosphere of nitrogen was added a degassed solution of 1 N NaOH (3 equiv). The mixtures were stirred at room temperature for 2–4 h and then acidified to pH 1 with 1 N HCl. The solution was concentrated *in vacuo*. Ethyl acetate and a saturated solution of sodium chloride were added. The layers were separated, and the organic phase was washed with brine and dried over magnesium sulfate. After filtration and concentration, the α-thiol was obtained in quantitative yield after dryng under vacuum.

***N*-[[1-[(2(*S*)-Mercapto-3-methyl-1-oxobutyl)amino]-1-cyclopropyl]carbonyl]-L-tyrosine (7):** white solid; mp 91–92 °C; IR (KBr) 3281, 2962, 1652, 1516, 1205 cm^{-1}; ^{1}H NMR(DMSO-d_6): δ 0.89 (d, 3H), 0.96 (d, 3H), 1.22 (m, 4H), 1.85 (m, 1H), 2.51 (d, 1H), 2.85 (m, 2H), 2.96 (t, 1H) 4.37 (q, 1H), 6.62 (d, 2H), 6.91 (d, 2H), 7.05 (d, 1H), 8.62 (bs, 1H), 9.21 (bs, 1H), 12.81 (bs, 1H). MS (CDI, CH_4) *m*/*z* 381 (MH^+). Anal. ($C_{18}H_{24}N_2O_5S$) C, H, N.

***N*-[[1-[(2(*S*)-Mercapto-3-methyl-1-oxobutyl)amino]-1-cyclopentyl]carbonyl]-L-tyrosine (8):** white solid; mp 209–210 °C; IR (KBr) 3301, 2963, 1722, 1650, 1517, 1200 cm^{-1}; ^{1}H

NMR (CD_3OD) δ 0.91 (d, 3H), 1.06 (d, 3H), 1.67 (m, 3H), 1.95 (m, 3H), 2.15 (m, 3H), 3.01 (m, 3H), 4.69 (t, 1H), 6.67 (d, 2H), 7.05 (d, 2H); MS (CDI, CH_4) m/z 409 (MH^+). Anal. ($C_{20}H_{28}N_2O_5S$) C, H, N.

***N*-[[1-[(2(*S*)-Mercapto-3-methyl-1-oxobutyl)amino]-1-cyclohexyl]carbonyl]-L-tyrosine (9):** white solid; mp 112–114 °C; IR (KBr) 3325, 2562, 1734, 1653, 1616, 1515, 1223 cm^{-1}; ^1H NMR (CD_3OD) δ 0.90 (d, 3H), 1.05 (d, 3H), 1.30–2.10 (m, 9H), 3.00 (m, 3H), 4.55 (m, 1H), 6.67 (d, 2H), 7.01 (d, 2H), 7.21 (d, 1H), 7.94 (s, 1H); MS (TSP) m/z 422 ($M - 1H^+$). Anal. ($C_{21}H_{30}N_2O_5S$) C, H, N.

***N*-[[4-[(2(*S*)-Mercapto-3-methyl-1-oxobutyl)amino]-4-tetrahydropyranyl]carbonyl]-L-tyrosine (10):** white solid; mp 221 °C; IR (KBr) 3420, 2556, 1737, 1674, 1656, 1515, 1223 cm^{-1}; ^1H NMR (CD_3OD) δ 0.91 (d, 3H), 1.06 (d, 3H), 2.00 (m, 5H), 3.00 (m, 3H), 3.50–3.80 (m, 4H), 4.61 (d, 1H), 6.67 (d, 2H), 7.01 (d, 2H), 7.83 (d, 1H), 8.24 (s, 1H); MS (DCI, CH_4) m/z 425 (MH^+). Anal. ($C_{20}H_{28}N_2O_5S$) C, H, N.

***N*-[[2-[(2(*S*)-Mercapto-3-methyl-1-oxobutyl)amino]-2-norbornyl]carbonyl]-L-tyrosine (11):** white solid; mp 105 °C; IR (KBr) 3315, 2562, 1727, 1654, 1515, 1224 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.78–0.95 (m, 6H) 1.09–1.60 (m, 7H), 1.75–1.96 (m, 1H), 2.10 (m, 1H), 2.30 (m, 1H), 2.67–2.93 (m, 2H), 3.09 (m, 1H), 3.44 (m, 1H), 4.31 (m, 1H), 6.61 (d, 2H), 6.92 (d, 2H), 7.39 (d, 1H), 7.49 (d, 1H), 8.24 (d, 1H), 9.20 (s, 1H), 12.69 (bs, 1H); MS (DCI, CH_4) m/z 435 (MH^+). Anal. ($C_{22}H_{30}N_2O_5S$) C, H, N.

***N*-[[2-[(2(*S*)-Mercapto-3-methyl-1-oxobutyl)amino]-1,3-dihydro-2*H*-inden-2-yl]carbonyl]-L-tyrosine (12):** white solid; mp 186–187 °C; IR (KBr) 3330, 2555, 1739, 1658, 1615, 1515, 1226, 822, 738 cm^{-1}; ^1H NMR (CD_3OD) δ 0.84 (d, 3H), 1.00 (d, 3H), 1.85 (m, 1H), 2.91 (d, 1H), 3.05 (m, 2H), 3.19–3.68 (m, 4H), 4.62 (t, 1H), 6.68 (d, 2H), 7.03 (d, 2H), 7.16 (m, 4H); MS (DCI, CH_4) m/z 457 (MH^+). Anal. ($C_{24}H_{28}N_2O_5S$) C, H, N.

***N*-[[1-[(2(*S*)-Mercapto-3-methyl-1-oxobutyl)amino]-1-methylethyl]carbonyl]-L-tyrosine (13):** white solid; mp 180–180 °C; IR (KBr) 3416, 2562, 1720, 1673, 1615, 1515, 1220 cm^{-1}; ^1H NMR (CD_3OD) δ 0.94 (d, 3H), 1.05 (d, 3H), 1.41 (s, 3H), 1.43 (s, 3H), 1.92 (m, 1H), 2.95 (d, 1H), 3.03 (m, 2H), 4.59 (t, 1H), 6.68 (d, 2H), 7.03 (d, 2H); MS (DCI, CH_4) m/z 383 (MH^+). Anal. ($C_{18}H_{26}N_2O_5S$) C, H, N.

***N*-[[1-[(2(*S*)-Mercapto-3-methyl-1-oxobutyl)amino]-1-cyclopentyl]carbonyl]-*O*-methyl-L-tyrosine (14):** white solid; mp 182–183 °C; IR (KBr) 3401, 3278, 2542 1732, 1653, 1513, 1249, 1178, 827 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.85 (d, 3H), 0.94 (d, 3H), 1.59–1.83 (m, 7H), 2.00 (m, 2H), 2.49 (d, 1H), 2.90 (m, 2H), 3.08 (t, 1H), 3.69 (s, 3H), 4.39 (q, 1H), 6.81 (d, 2H), 7.07 (d, 2H), 8.12 (bs, 1H), 10.20 (s, 1H), 12.78 (s, 1H); MS (DCI, CH_4) m/z 423 (MH^+). Anal. ($C_{21}H_{30}N_2O_5S$) C, H, N.

***N*-[[1-[(2(*S*)-Mercapto-3-methyl-1-oxobutyl)amino]-1-cyclopentyl]carbonyl]-4-fluoro-L-phenylalanine (15):** white solid; mp 168–169 °C; IR (KBr) 3407, 2549, 1734, 1650,1510, 1223 cm^{-1}; ^1H NMR (CD_3OD) δ 0.93 (d, 3H), 1.04 (d, 3H), 1.76 (m, 4H), 1.96 (m, 3H), 2.17 (m, 2H), 2.95 (d, 1H), 3.10 (m, 3H), 4.62 (q, 1H), 6.69 (d, 2H), 7.20 (d, 2H), 7.34 (d, 1H), 8.30 (s, 1H); MS (DCI, CH_4) m/z 411 (MH^+). Anal. ($C_{20}H_{27}FN_2O_4S$) C, H, N.

***N*-[[1-[(2(*S*)-Mercapto-3-methyl-1-oxobutyl)amino]-1-cyclopentyl]carbonyl]-L-2-thienylalanine (16):** white solid; mp 175–176 °C; IR (KBr) 3271, 2555, 1742, 1653, 1514, 1191 cm^{-1}; ^1H NMR (CD_3OD) δ 0.93 (d, 3H), 1.05 (d, 3H), 1.72 (m, 4H), 1.94 (m, 3H), 2.20 (m, 2H), 2.94 (d, 1H), 3.34 (m, 3H), 4.64 (m, 1H), 6.90 (d, 2H), 7.20 (d, 1H), 7.34 (d, 1H), 8.32 (s, 1H); MS (DCI, CH_4) m/z 399 (MH^+). Anal. ($C_{18}H_{26}N_2O_4S_2$) C, H, N.

***N*-[[1-[(2(*S*)-Mercapto-3-methyl-1-oxobutyl)amino]-1-cyclopentyl]carbonyl]-L-3-thienylalanine (17):** white solid; mp 182–184 °C; IR (KBr) 3274, 2549, 1730, 1704, 1668, 1523 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.88 (d, 3H), 0.98 (d, 3H), 1.55 (m, 4H), 1.80–2.10 (m, 5H), 2.45 (m, 1H), 3.00 (m, 3H), 4.39 (q, 1H), 6.94 (d, 1H), 7.17 (d, 1H), 7.23 (d, 1H), 7.39 (m, 1H), 8.12 (s, 1H), 12.75 (s, 1H); MS (DCI, CH_4) m/z 399 (MH^+). Anal. ($C_{18}H_{26}N_2O_4S_2$) C, H, N.

***N*-[[1-[(2(*S*)-Mercapto-3-methyl-1-oxobutyl)amino]-1-cyclopentyl]carbonyl]-L-2-pyridylalanine (18):** white solid; mp 175–176 °C; IR (KBr) 3303, 2549, 1730, 1668, 1653, 1515, 1199 cm^{-1}; ^1H NMR (CD_3OD) δ 0.92 (d, 3H), 1.05 (d, 3H), 1.73 (m, 4H), 1.98 (m, 3H), 2.15 (m, 2H), 3.00 (d, 1H), 3.30 (m, 2H), 4.75 (t, 1H), 7.33 (m, 1H), 7.48 (m, 1H), 7.84 (m, 1H), 8.48 (m, 1H); MS (TSP) m/z 392 ($M - H^+$). Anal. ($C_{19}H_{27}N_3O_4S$) C, H, N.

***N*-[[1-[(2(*S*)-Mercapto-3-methyl-1-oxobutyl)amino]-1-cyclopentyl]carbonyl]-L-3-pyridylalanine (19):** white solid; mp 218–219 °C; IR (KBr) 3382, 3294, 2550, 1720, 1658, 1521, 1190 cm^{-1}; ^1H NMR (CD_3OD) δ 0.92 (d, 3H), 1.06 (d, 3H), 1.73 (m, 4H), 1.94 (m, 3H), 2.14 (m, 2H), 2.98 (d, 1H), 3.22 (m, 2H), 4.67 (m, 1H), 7.48 (m, 1H), 7.90 (m, 1H), 8.46 (m, 2H); MS (DCI, CH_4) m/z 394 (MH^+). Anal. ($C_{19}H_{27}N_3O_4S$) C, H, N.

***N*-[[1-[(2(*S*)-Mercapto-3-methyl-1-oxobutyl)amino]-1-cyclopentyl]carbonyl]-L-tryptophan (20):** white solid; mp 145–146 °C; IR (KBr) 3400, 2356, 1731,1655, 1519, 1230, 1201 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.82 (d, 3H), 0.95 (d, 3H), 1.61 (m, 4H), 1.80–2.00 (m, 5H), 2.49 (d, 1H), 3.10 (m, 3H), 4.47 (q, 1H), 6.96 (t, 1H), 7.08 (t, 1H), 7.15 (s, 1H), 7.22 (d, 1H), 7.30 (d, 1H), 7.49 (d, 1H), 8.14 (s, 1H), 10.83 (s, 1H), 12.70 (s, 1H); MS (DCI, CH_4) m/z 432 (MH^+). Anal. ($C_{22}H_{29}N_3O_4S$) C, H, N.

***N*-[[1-[(2(*S*)-Mercapto-3-methyl-1-oxobutyl)amino]-1-cyclopentyl]carbonyl]-L-5-hydroxytryptophan (21):** white solid; mp 105 °C; IR (KBr) 3338, 2556, 1726, 1655, 1514, 1205 cm^{-1}; ^1H NMR (CD_3OD) δ 0.86 (d, 3H), 0.98 (d, 3H), 1.67 (m, 4H), 1.88 (m, 3H), 2.10 (m, 2H), 2.90 (d, 1H), 3.19 (m, 2H), 4.65 (t, 1H), 6.60 (dd, 1H), 6.90 (s, 1H), 7.05 (s, 1H), 7.10 (d, 1H); MS (DCI, CH_4) m/z 448 (MH^+). Anal. ($C_{22}H_{29}N_3O_5S$) C, H, N.

***N*-[[1-[(2(*S*)-Mercapto-3-methyl-1-oxobutyl)amino]-1-cyclopentyl]carbonyl]-L-4-phenylphenylalanine (22):** white solid; mp 180–181 °C; IR (KBr) 3325, 3270, 2556, 1739, 1669, 1628, 1520, 1200 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.90 (d, 3H), 1.00 (d, 3H), 1.71 (m, 4H), 1.89 (m, 3H), 2.15 (m, 2H), 2.93 (d, 1H), 3.14 (m, 2H), 4.68 (t, 1H), 7.31 (m, 3H), 7.40 (m, 2H), 7.54 (m, 4H); MS (DCI, CH_4) m/z 469 (MH^+). Anal. ($C_{26}H_{32}N_2O_4S$) C, H, N.

***N*-[[1-[(2(*S*)-Mercapto-3-methyl-1-oxobutyl)amino]-1-cyclopentyl]carbonyl]-L-homophenylalanine (23):** white solid; mp 203–204 °C; IR (KBr) 3347, 3279, 2558, 1727, 1692, 1669, 1532, 1518 cm^{-1}; ^1H NMR (CD_3OD) δ 1.00 (d, 3H), 1.10 (d, 3H), 1.75 (m, 4H), 2.19 (m, 6H), 2.44 (m, 1H), 2.70 (t, 2H), 3.04 (d, 1H), 4.39 (m, 1H), 7.20 (m, 5H), 7.48 (d, 1H), 8.38 (s, 1H); MS (DCI, CH_4) m/z 407 (MH^+). Anal. ($C_{21}H_{30}N_2O_4S$) C, H, N.

***N*-[[1-[(2-Mercaptoacetyl)amino]-1-cyclopentyl]carbonyl]-L-tyrosine (24):** white solid; mp 118–119 °C; IR (KBr) 3356, 3276, 2562, 1701, 1657, 1515, 1226 cm^{-1}; ^1H NMR (CD_3OD) δ 1.70 (m, 4H), 1.89 (m, 2H), 2.12 (m, 2H), 3.00 (m, 2H), 3.09 (s, 2H), 4.57 (m, 1H), 6.69 (d, 2H), 7.00 (d, 2H), 7.30 (d, 1H), 8.26 (s, 1H); MS (DCI, CH_4) m/z 367 (MH^+). Anal. ($C_{17}H_{22}N_2O_5S$) C, H, N.

***N*-[[1-[(2(*S*)-Mercapto-1-oxo-3-phenylpropyl)amino]-1-cyclopentyl]carbonyl]-L-tyrosine (25):** white solid; mp 187–189 °C; IR (KBr) 3394, 2966, 1653, 1515, 1223 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.39 (m, 4H), 1.75 (m, 4H), 2.75 (m, 4H), 3.09 (d, 1H), 3.59 (m, 1H), 3.59 (m, 1H), 4.34 (q, 1H), 6.61 (d, 2H), 6.90 (d, 2H), 7.21 (m, 5H), 8.09 (bs, 1H), 9.20 (s, 1H), 12.18 (bs, 1H); MS (DCI, CH_4) m/z 457 (MH^+). Anal. ($C_{24}H_{28}N_2O_5S$) C, H, N.

***N*-[[1-[[(1-Mercapto-1-cyclopentyl)carbonyl]amino]-1-cyclopentyl]carbonyl]-L-tyrosine (26):** white solid; mp 90 °C; IR (KBr) 3342, 2541, 1730, 1650, 1515, 1226 cm^{-1}; ^1H NMR (CD_3OD) δ 1.70–2.20 (m, 16H), 3.05 (m, 2H), 4.55 (t, 1H), 6.63 (d, 2H), 6.95 (d, 2H); MS (DCI, CH_4) m/z 421 (MH^+). Anal. ($C_{21}H_{26}N_2O_5S$) C, H, N.

II. Biological Tests. *In Vitro* Inhibition of Angiotensin-I-Converting Enzyme (ACE). The *in vitro* inhibition of the angiotensin-I- converting enzyme (ACE) was performed by a method analogous to that given in the literature.[29] The assay was adapted to a 96-well plate format for use with a Beckman Biomek workstation and performed at 25 °C. Rabbit lung ACE was used for this assay. The buffer for the ACE

assay consists of 300 mM NaCl, 100 mM KH_2PO_4 (pH 8.3). The reaction is initiated by the addition of 50 μL of hippuryl-histidylleucine (2 mg/mL) to wells containing enzyme and drug in a volume of 125 μL, and the plates are incubated for 75 min at room temperature. The reaction is terminated by the addition of 40 μL of 1 N NaOH. A freshly prepared *o*-phthalaldehyde solution, 50 μL (2 mg/mL in methanol), is added to the wells, and the contents are mixed and allowed to stand at 25 °C. After 10 min, 50 μL of 1 N HCl is added, the well contents are mixed, and the optical density is read at 360 nm.

The results are plotted against drug concentration to determine the IC_{50}.

***In Vitro* Inhibition of Neutral Endopeptidase EC 3.4.24.11.** Neutral endopeptidase EC 3.4.24.11 activity is determined by the hydrolysis of the substrate glutaryl-Ala-Ala-Phe-2-naphthyl amide (GAAP) using a modified procedure of Orlowski and Wilk.[30] The incubation mixture (total volume 125 μL) contains 4.2 μg of protein (rat kidney cortex membranes homogenates[13]), 50 mM Tris buffer (pH 7.4 at 25 °C), 500 μM GAAP (final concentration), and leucine aminopeptidase M (2.5 μg). The mixture was incubated for 25 min at 25 °C, and 100 μL of fast garnet (250 μg fast garnet/mL of 10% Tween 20 in 1 M sodium acetate, pH 4.2) was added. Enzyme activity is measured spectrophotometrically at 540 nm. One unit of NEP 3.4.24.11 activity is defined as 1 nmol of 2-naphthylamine released per minute at 25 °C and pH 7.4. To determine IC_{50} values of the inhibitors, increasing concentrations of each compound were preincubated for 10 min at 25 °C with the membranes. Experiments were carried out in duplicate, and inhibition curves were constructed on the basis of seven data points near the IC_{50}.

***In Vivo* Angiotensin-I-Converting Enzyme Inhibition.** The *in vivo* tests for intravenously administered compounds are performed with male, conscious normotensive Sprague–Dawley rats (275–390 g). The rats were anesthetized with methohexital sodium (75 mg/kg, ip) and instrumented with femoral arterial and venous catheters for direct blood pressure measurement and iv administration of compounds, respectively. On the following day, the pressor responses to three challenges of angiotensin-I (300 ng/kg, iv) were obtained. Test compounds were then administered iv or po, and the rats were rechallenged with angiotensin-I at scheduled times thereafter. All responses obtained after the administration of test compound were compared to the average of the initial three responses. Any observed decrease of said pressor response is an indication of angiotensin-I-converting enzyme inhibition.

***In Vivo* Neutral Endopeptidase EC 3.4.24.11 Inhibition.** Male Sprague–Dawley rats (275–390 g) were anesthetized with ketamine (150 mg/kg)/acepromazine (10%) and instrumented with catheters in the femoral artery and vein to obtain blood samples and infuse ratANF(99-126), respectively. The rats were tethered with a swivel system and were allowed to recover for 24 h before being studied in the conscious, unrestrained state.

In the assay, plasma ANF levels were determined in the presence and absence of NEP inhibition. On the day of study, all rats were infused continuously with ANF at 450 ng/kg/min iv for the entire 5 h of the experiment. Sixty minutes after beginning the infusion, blood samples for baseline ANF measurements were obtained (time 0), and the rats were then randomly divided into groups to be treated with the test compounds or vehicle. Additional blood samples are taken at 30, 60, 120, 180, and 240 min after administration of the test compound.[32]

Plasma ANF concentrations were determined by a specific radioimmunoassay. The plasma was diluted (×50, ×100, and ×200) in buffer containing 19 mM sodium phosphate monobasic and 81 mM sodium phosphate dibasic (pH 7.4), 50 mM NaCl, 0.1% BSA, 0.1% Triton X-100, and 0.1% NaN_3. One hundred microliters of standards [rANF(99-126)] or samples were added to 100 μL of rabbit anti-rANF serum and incubated at 4 °C for 16 h. Ten thousand cpm of [^{125}I]rANF were then added to the reaction mixture which is incubated at 4 °C for an additional 24 h. Goat anti-rabbit IgG serum coupled to paramagnetic particles was added to the reaction mixture and bound [^{125}I]rANF was pelleted by exposing the mixture to an attracting magnetic rack. The supernatant was decanted and the pellets counted in a gamma counter. All determinations are performed in duplicate.

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(31) Compounds **14**, **19**, and **21** could not be solubilized for *in vivo* experimentation; compounds **11** and **22** had ACE $IC_{50} > 50$ nM and thus were not considered to be potent enough for *in vivo* evaluation.
(32) In this assay there is some variability in the steady state concentrations of ANF obtained. We attribute this variability to two sources: differences in clearance rates of ANF from one group of animals to the next and the standard curve for the ANF radioimmunoassay can vary from one assay to another.

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