Inhibition of Peptidylglycine α-Amidating Monooxygenase by N-Substituted Homocysteine Analogs

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C-terminal amidation is a posttranslational modification found in many neuropeptides. Peptidylglycine α-amidating monooxygenase (PAM) catalyzes the synthesis of the biologically essential C-terminal amide from a glycine-extended precursor peptide. Reported herein are the first potent inhibitors of PAM. Dipeptides containing a C-terminal homocysteine and an N-acylated hydrophobic amino acid were found to inhibit PAM with IC_{50}s in the low nanomolar range. Inhibition potency was dependent on both the carboxylate and the thiolate functionalities of the homocysteine and on the hydrophobic groups of the second amino acid. The thiolate was postulated to produce high binding affinities through coordination with the active-site copper. The compound series also exhibited potent inhibition of PAM in rat dorsal root ganglion cells as demonstrated by a dose-dependent increase in the substance P-Gly/substance P ratio. These results indicate that the compounds have sufficient potency and intracellular bioavailability to aid future studies focused on neuropeptide function and the contributions of neuropeptides to various disease processes.

Introduction

C-terminal amidation is a posttranslational modification present in nearly half of all endocrine and neuroendocrine peptide hormones.[1] In most cases, the C-terminal amide is a prerequisite for full biological activity. Biosynthesis of the amidated peptide begins with proteolytic processing of a preprohormone peptide to generate a glycine-extended precursor peptide. Conversion of the glycine to the corresponding carboxamide[2] requires two sequential enzymatic steps (Scheme 1a).[3] The first step is catalyzed by the enzyme peptidylglycine 2-hydroxylase (PGH, EC 1.14.17.3) and entails the oxidation of the glycine to the α-hydroxyglycine intermediate. The second step is catalyzed by the enzyme peptidylamidoglycolate lyase (PGL, EC 4.3.2.5) and results in the cleavage of the C–N bond to yield the amidated peptide and glyoxylate.[4] Through alternative splicing, a single gene encodes for a monofunctional protein containing monooxygenase activity and for a membrane-associated bifunctional enzyme containing both the monooxygenase and lyase activities.[5] The bifunctional enzyme (peptidylglycine α-amidating monooxygenase, PAM) has two catalytic domains that act in a sequential manner to generate the C-terminal amide.

The monooxygenase activity exhibited by both PAM and PGH is dependent on copper, molecular oxygen, and ascorbate[6] and involves the stereospecific abstraction of the *pro-S* hydrogen of glycine[7] by a hydroxy radical formed from a copper–oxo species.[8] The resulting α-carbon-centered glycyl radical is then rapidly trapped by another hydroxy radical to form the α-hydroxyglycine peptide (Scheme 1b). This mechanism is analogous to that proposed for dopamine β-hydroxylase.[9] SAR studies on PAM indicate that the monooxygenase activity is very specific for peptides with a C-terminal glycine.[10] In contrast, a wide variety of amino acids are tolerated in the penultimate position,[11] suggesting either that the monooxygenase has very broad specificity or that there are isoforms of PAM expressed in a tissue-specific manner. As shown in Table 1, PAM activity has been found in all tissues expressing amidated peptides,[12] including pituitary, thyroid, hypothalamus, serum, antrum, cerebrospinal fluid, and the submandibular gland.

One amidated peptide that has received considerable attention over the past decade is substance P (SP). Interest in SP is in part due to the finding that SP levels are elevated during inflammation and correlate with the degree of inflammation.[13] SP may also contribute to the pathogenesis of rheumatoid arthritis as evidenced by its stimulation of prostaglandin E_2 and collagenase release in synoviocytes[14] and its enhanced release during passive movements of inflamed ankle joints relative to the control joint.[15] Furthermore, infusion of SP into the rat knee increases the severity of experimental arthritis.[16] Hence, an SP antagonist[17] or an inhibitor of the amidating enzyme responsible for production of SP could be useful in the treatment of rheumatoid arthritis.

Inhibition of peptide amidation was expected to yield higher levels of the biologically inactive glycine-extended precursor, which in turn would be released and degraded by other enzymes. Several studies have shown that amidated peptide levels can be modulated. In one study, vitamin C deficient guinea pigs were shown to have elevated glycine-extended gastrin levels (30-fold) and a 2-fold lower gastrin level relative to the control.[18] Since glycine-extended gastrin is a good PAM substrate and since ascorbate serves as the natural cofactor responsible for supplying two-electron reducing equivalents per turnover, these effects were attributed to inhibition of PAM. A second study showed that the copper chelator *N,N*-diethyldithiocarbamate (DDC) or its disulfide dimer disulfiram (Antabuse) were able to inhibit PAM *in vitro* and were able to decrease levels of the amidated pro-ACTH/endorphin-derived peptides and α-melanotropin (αMSH) in mouse pituitary corticotropic tumor cells. Furthermore, disulfiram-treated

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Scheme 1

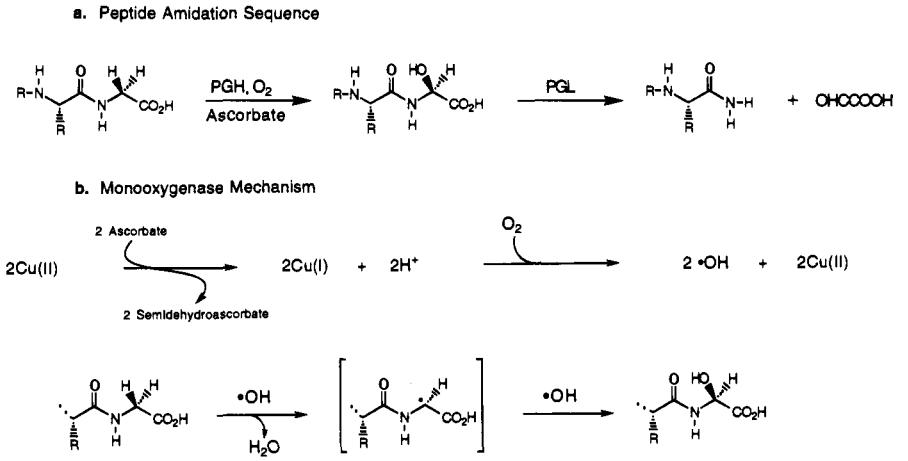


Table 1. Amidated Peptide[a] Characteristics

amidated peptide	penultimate amino acid	synthesis site[b]	possible function
substance P	Met	EH, GI, HT	inflammation mediator
neuropeptide Y	Tyr	AM, Brain, GI	vasoconstrictor
CGRP	Phe	Brain, SN, P	glycogen synthesis inhibitor
amylin	Tyr	P	glycogen synthesis inhibitor
gastrin	Phe	EH, GI, HT	stomach acid secretion
cholecystokinin	Phe	EH, GI, HT	stomach acid secretion
vasopressin	Gly	AM, GO, GI, HT	antidiuresis regulation
oxytocin	Gly	HT, GO	uterus contraction
αMSH	Val	EH, IL	melanocyte regulation
calcitonin	Pro	thyroid	calcium homeostasis

[a] Only a fraction of known amidated peptide hormones. [b] Abbreviations: CGRP, calcitonin gene-related peptide; αMSH, melanocyte stimulating hormone; AM, adrenal medulla; EH, extrahypothalamic brain areas; GI, gastrointestinal tract; GO, gonads; HT, hypothalamus; IL, intermediate lobe of pituitary; P, pancreas; SN, sensory neurons.

rats (11 days at 4 mg/kg) showed a dose-related increase in glycine-extended αMSH relative to the control (38% vs 5%).[19] Both studies indicate that inhibition of peptide amidation will affect levels of amidated hormones. Unfortunately, the compounds used in these studies are inhibitors of cofactor binding and therefore are subject to potential specificity problems since a wide variety of enzymes use the same cofactors. Consequently, we initiated a program to find competitive inhibitors of PAM with the hope that these compounds could ultimately be tailored to inhibit specific PAM isoforms responsible for the synthesis of specific amidated peptides.

Only a few competitive inhibitors of PAM have been described in the literature. For example, α-keto acids such as acetopyruvate[20] and [(4-methoxybenzoyl)oxy]-acetic acid[21] are competitive inhibitors of PAM with K_is of 0.25 and 0.48 mM, respectively. Other competitive inhibitors of PAM include 4-phenyl-3-butenoic acid[21] and peptides with a C-terminal vinylglycine,[22] both of which are reported to produce time-dependent inactivation. Considering the K_m of the amidated peptides (e.g., substance P; K_m = 2.2 μM), these compounds are relatively weak inhibitors and would not be expected to have an effect *in vivo*. Reported herein are the first potent competitive inhibitors of PAM and their effect on substance P synthesis in cell culture.

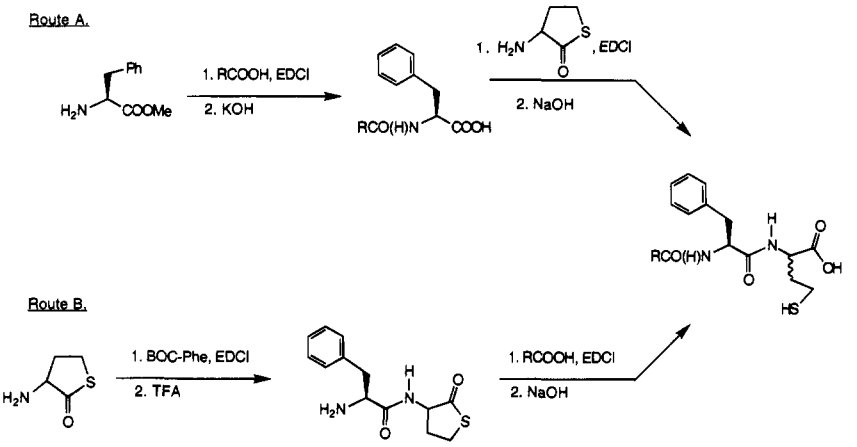
Chemistry

The dipeptides shown in Table 2 were readily prepared (Scheme 2) by either (i) acylating the N-terminal amino acid methyl ester with the corresponding carboxylic acid followed by ester hydrolysis, coupling of the carboxylate-protected C-terminal amino acid and ester hydrolysis (route A) or (ii) coupling the carboxylate-protected C-terminal amino acid with the corresponding BOC-protected amino acid followed by BOC deprotection, acylation of the free amino, and carboxylate deprotection (route B). Tripeptide **1** was prepared by route A and the N-terminal BOC cleaved at the end of the synthesis with TFA. Compounds containing homocysteine were prepared by hydrolysis of the corresponding peptide containing D,L-homocysteine thiolactone to give an inseparable 1:1 mixture of diastereomers. Attempts to hydrolyze peptides containing L-homocysteine thiolactone under either acidic or basic conditions led to significant racemization (15–30%) as did procedures involving mercuric ion-assisted hydrolysis.[23] Since the extent of racemization was variable, compounds were prepared as a 1:1 mixture of diastereomers and evaluated in the inhibition assay. Isolation of the single diastereomer was accomplished by preparative HPLC (compounds **22** and **23**). C-terminal cysteine analogs (**7** and **24**) were prepared from L-cystine diethyl ester by following route A with the addition of a disulfide reduction step using zinc in acetic acid prior to the final ester hydrolysis. The presence of the thiol was confirmed in all cases by either NMR or by titration[24] (see the Experimental Section).

Results and Discussion

Previous substrate SAR studies indicate that PAM activity requires a C-terminal glycine residue.[10,11] Remarkably, replacement of the glycine with close analogs of glycine, e.g., β-alanine, α-aminobutyric acid, sarcosine, and L-alanine, leads to peptides that are neither

Scheme 2. General Synthesis of Homocysteine-Containing PAM Inhibitors



substrates nor inhibitors of PAM. Only substitution with D-alanine[10] yields a weakly binding substrate. Previous substrate SAR studies also showed PAM to have a strong preference for tripeptides with hydrophobic amino acids in the penultimate position and at the P-2 site. Further enhancement of the tripeptide affinity for PAM was achieved by blocking the N-terminal amine with various hydrophobic groups (Table 2, compounds **2**–**4**). Although these compounds showed a 10-fold increase in affinity for PAM, they were considered still too weak to be effective *in vivo*. Hence, we focused our attention on the C-terminal glycine in the hope that a replacement could be found that would transform these tripeptide substrates into potent and specific inhibitors.

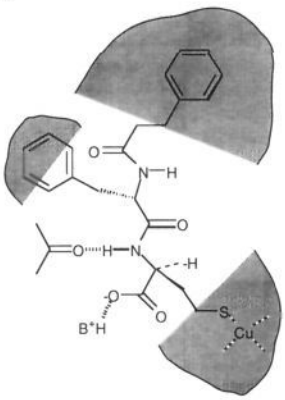
The general mechanism of copper monooxygenases[25] (e.g., dopamine β-hydroxylase[9]) guided our efforts to find a glycine replacement. As shown in Scheme 1, the PAM catalytic mechanism begins with abstraction of the glycine *pro-S* hydrogen[7] by a hydroxy radical formed from a copper-oxo species. The resulting carbon radical is then rapidly trapped by another hydroxy radical to form the α-hydroxyglycine peptide. Since hydroxy radicals have a very short diffusion limit,[26] we speculated that the active-site copper must be in the vicinity of the glycine methylene. Furthermore, the high stereospecificity of the hydrogen abstraction coupled with the requirement of the glycine carboxylate and amido groups for activity[11a] (Table 2) strongly suggested that the PAM active site binds and rigidly holds the C-terminal glycine in a single conformation (Scheme 3). Consequently, our strategy was to design bisubstrate inhibitors (peptide substrate and molecular oxygen) containing a peptidic backbone and a group that could coordinate to the active-site copper. We reasoned that replacement of the *pro-S* hydrogen with a group capable of copper coordination should lead to enhanced affinity

Table 2. Compound [R-AA1-AA2] Data Summary

compd	R	AA1	AA2	route	% yield[a]	formula[b]	IC_{50} (nM)
1	D-Tyr	Phe	Gly	A	60	$C_{20}H_{23}N_3O_5 \cdot 0.5H_2O$	>10000
2	Dan-D-Tyr	Phe	Gly	B	76	$C_{32}H_{34}N_4O_7S \cdot 1.5H_2O$	4200
3	CBZ-D-Tyr	Phe	Gly	B	80	$C_{28}H_{29}N_3O_7 \cdot 1.75H_2O$	3500
4	BOC-D-Tyr	Phe	Gly	B	75	$C_{25}H_{31}N_3O_7 \cdot H_2O$	4000
5	BOC-D-Tyr	Phe	Ala	A	80	$C_{26}H_{33}N_3O_7 \cdot 0.5H_2O$	>10000
6	BOC-D-Tyr	Phe	Ser	A	50	$C_{26}H_{33}N_3O_8$	>10000
7	BOC-D-Tyr	Phe	Cys	A	69	$C_{26}H_{33}N_3O_7S \cdot 0.5H_2O$	340 ($n = 1$)
8	BOC-Phe	Phe	HC	B	100	$C_{27}H_{35}N_3O_6S$	39(±8)
9	BOC	Phe	HC	A	40	$C_{18}H_{26}N_2O_5S$	53(±12)
10	$(CH_3)_2CH_2CO$-	Phe	HC	B	80	$C_{19}H_{26}N_2O_4S$	29(±4)
11	CBZ	Phe	HC	A	77	$C_{21}H_{24}N_2O_5S$	18(±3)
12	indole-2-CO-	Phe	HC	B	40	$C_{22}H_{23}N_3O_4S \cdot 0.5H_2O$	8(±1)
13	2-naphthoic-	Phe	HC	B	77	$C_{24}H_{24}N_2O_4S$	10(±3)
14	$PhOCH_2CO$-	Phe	HC	B	60	$C_{21}H_{24}N_2O_5S$	14(±3)
15	3,4-DCC	Phe	HC	B	80	$C_{22}H_{24}Cl_2N_2O_4S$	55(±5)
16	$PhCH_2CH_2CO$-	Phe	HC	A	77	$C_{22}H_{26}N_2O_4S \cdot 0.5H_2O$	15(±3)
17	$PhCH_2CH_2CO$-	Met	HC	A	73	$C_{18}H_{26}N_2O_4S_2$	18(±2)
18	$PhCH_2CH_2CO$-	Val	HC	A	92	$C_{18}H_{26}N_2O_4S$	160(±30)
19	$PhCH_2CH_2CO$-	Leu	HC	A	35	$C_{19}H_{28}N_2O_4S \cdot 0.5H_2O$	300(±17)
20	$PhCH_2CH_2CO$-	NL	HC	A	63	$C_{19}H_{26}N_2O_4S$	23(±2)
21	$PhCH_2CH_2CO$-		HC	A	83	$C_{13}H_{17}NO_3S$	7500(±1700)
22	$PhCH_2CH_2CO$-	Phe	*l*-HC	A		$C_{22}H_{26}N_2O_4S \cdot 0.5H_2O$	10(±1)
23	$PhCH_2CH_2CO$-	Phe	*d*-HC	A		$C_{22}H_{26}N_2O_4S \cdot 0.5H_2O$	610(±80)
24	$PhCH_2CH_2CO$-	Phe	Cys	A	50	$C_{21}H_{24}N_2O_4S$	330(±70)
25	$PhCH_2CH_2CO$-	Phe	dCHC	A	80	$C_{21}H_{26}N_2O_2S$	1600
26	$PhCH_2CH_2CO$-	Phe	Asp	A	85	$C_{22}H_{24}N_2O_6$	>10000
27	$PhCH_2CH_2CO$-	Phe	Asn	A	66	$C_{22}H_{25}N_3O_5 \cdot H_2O$	>10000
28	$PhCH_2CH_2CO$-	Phe	Gln	A	91	$C_{23}H_{26}N_2O_6$	>10000
29	$PhCH_2CH_2CO$-	Phe	HC-OBn	A	40	$C_{29}H_{32}N_2O_4S$	>10000
30	$PhCH_2CH_2CO$-	Phe	*l*-HCTL	A	80	$C_{22}H_{24}N_2O_3S$	>10000

Abbreviations: AA1, N-terminal amino acid; AA2, C-terminal amino acid; 3,4-DCC, 3,4-dichlorohydrocinnamoyl; NL, norleucine; Dan, dansyl; HC, D,L-homocysteine; dCHC, descarboxyhomocysteine, i.e -N(H)$(CH_2)_3$SH; HCTL, D,L-homocysteine thiolactone. [a] Denotes yield of final step. [b] Formula indicates C, H, N analysis was within ±0.4% of the theoretical value.

Scheme 3. Hypothetical Active-Site Structure of PAM



via both the increased number of favorable interactions and the chelation effect.[27]

Imidazoles, thioimidizoles, and thiolates are known to coordinate to cooper. Given the likelihood that the site surrounding the active-site copper was sterically encumbered, we chose to synthesize tripeptides containing C-terminal alkylthiolates. Replacement of the C-terminal glycine with L-cysteine gave tripeptide **7**, which was >10-fold more potent than the corresponding glycine containing peptide **4**. Evidence that the thiolate was responsible for the increased potency is apparent from comparing the IC_{50}s for tripeptides **5**–**7**. Replacement of the glycine *pro-S* hydrogen in tripeptide **4** with a methyl or hydroxymethyl (compounds **5** and **6**, respectively) resulted in a complete loss in inhibitory activity (>90% remaining activity at 10 μM concentration) whereas replacement with the larger, more sterically-demanding thiomethyl group led to a 10-fold enhancement in potency. This result coupled with the known propensity of thiolates to coordinate with copper strongly suggests that tripeptide **7** interacts with the active-site copper.[32]

The SAR of **7** was further explored by synthesis of a series of analogs (Table 2). First, the tripeptide **8** was synthesized to determine the optimal length of the alkylthiolate sidechain. The finding that a C-terminal D,L-homocysteine residue gave another 10-fold increase in potency suggested that the distance between the glycine methylene and the copper ion was not adequately transversed by the thiomethyl group. Next, the SAR for the amino acid residues attached to the C-terminal homocysteine residue in tripeptide **8** was explored. Removal of one amino acid gave the N-blocked dipeptide **9**, which resulted in no loss in potency whereas removal of both amino acids gave the N-blocked homocysteine analog **21**, which resulted in a 500-fold loss in potency. Synthesis of a series of N-blocked dipeptides containing a C-terminal homocysteine indicated that aliphatic groups attached to the N-terminal end of the dipeptide were slightly worse than aryl groups (compounds **9**–**16**). Furthermore, the aryl group could be directly attached to the amide (e.g., **12** and **13**) or via a spacer (e.g., **14**–**16**). SAR studies of the N-terminal amino acid side chain indicated a strong preference for hydrophobic residues.[11] A comparison of the hydrophobic residues showed that analogs with Phe (**16**) amd Met (**17**) were significantly more potent than analogs with branched aliphatic amino acids (e.g., Val (**18**) and Leu (**19**)). The final area of SAR explored was the homocysteine residue. Similar to the SAR found for the tripeptide analogs, homocysteine was strongly preferred over cysteine (**22** vs **24**). Furthermore, consistent with the predicted position of the active-site copper relative to the glycine methylene, the dipeptide containing the L-isomer of homocysteine (**22**) was shown to be 60-fold more potent than the corresponding peptide containing the D-homocysteine isomer (**23**). The last piece of SAR uncovered in this study was that the carboxylate of the homocysteine residue is critical for inhibition potency as evidenced by the lack of activity found for the descarboxy analog **25**, the benzyl ester **29**, and the thiolactone **30**.

Dipeptides containing a C-terminal homocysteine represent very potent inhibitors of the rat medulla PAM and a considerable improvement over the previous best known PAM inhibitors [[(4-methoxybenzoyl)oxy]acetic acid; K_i = 480 μM[21]]. The magnitude of the inhibition constants were found to depend on whether the inhibitor was preincubated with the enzyme prior to initiation of the reaction. For example, without preincubation, compound **16** had an IC_{50} of 33 nM, whereas with a 20 min preincubation at room temperature the IC_{50} dropped to 15 nM. Importantly, preincubation of PAM with 0.3 μM **16** for 20 min followed by a 200-fold dilution led to total recovery of the starting activity over the following hour. Hence, the compounds bind reversibly with the highest affinity binding state apparently attained only through a kinetically slow process.[28]

Inhibition of Substance P Biosynthesis in Cell Culture

Substance P is a C-terminal amidated undecapeptide that is biosynthesized in the central and peripheral nervous systems. The possible role of substance P in neurogenic inflammation and potentially even in the pathogenesis of rheumatoid arthritis makes SP antagonists and/or inhibitors of SP biosynthesis/release an attractive target. Since the dorsal root ganglion (DRG) cell represents the cell responsible for release of SP in neurogenic inflammation and also the site where SP is biosynthesized in the peripheral nervous system, we established a rat primary DRG cell culture in order to monitor the intracellular bioavailability of our PAM inhibitors.[29]

The effect of PAM inhibitors on SP biosynthesis in the DRG cell culture assay is shown in Table 3. All of the PAM inhibitors tested showed some inhibition of SP biosynthesis at 10 μM concentration. The apparent potency, however, was significantly less than the IC_{50} generated with the isolated enzyme, suggesting that the compounds had limited intracellular bioavailability. Since a free carboxylate can often limit cell penetration,

Table 3. PAM Inhibition in DRG Cells

compd	% *I* in DRG[a]	compd	% *I* in DRG[a]
8	34 ± 7	**15**	37 ± 15
10	10 ± 4	**16**	28 ± 7
11	40 ± 7	**20**	7 ± 4
12	42 ± 8	**22**	25 ± 4
13	67 ± 5	**30**	37 ± 9
14	22 ± 12		

[a] Denotes percent inhibition in DRG cells at 10 μM concentration of the inhibitor.

we are preparing several proesters of the most active PAM inhibitors to optimize their intracellular bioavailability.

Conclusions

Dipeptides containing a C-terminal homocysteine and an N-acylated hydrophobic amino acid were found to inhibit PAM with IC_{50}s in the low nanomolar range. Inhibition potency was dependent on both the carboxylate and the thiolate functionalities of the homocysteine and on the hydrophobic groups of the second amino acid. The thiolate was postulated to produce high binding affinities through coordination with the active-site copper. The compound series also exhibited inhibition of PAM in rat dorsal root ganglion cells as demonstrated by a dose-dependent increase in the substance P-Gly/substance P ratio. These results indicate that the compounds have sufficient potency and intracellular bioavailability to aid future studies focused on neuropeptide function and on the role neuropeptides play in various disease processes.

Experimental Section

Melting points were determined in open capillary tubes with a Thomas-Hoover Uni-Melt apparatus and are uncorrected. Proton NMR spectra were recorded on either a Brucker AC-250, a Varian XL400, or a Varian XL300 spectrometer. Chemical shifts are reported in parts per million (ppm) downfield from tetramethylsilane. Coupling constants are given in hertz. Infrared (IR) spectra and MS spectra were measured on a Nicolet 5SXB FTIR spectrometer and a Hewlett-Packard GC/MS 5985B spectrometer, respectively. Microanalyses were carried out at Robertson Laboratory, Inc., Madison, NJ. Optical rotations were recorded on a JASCO DIP-370 polarimeter. Thiol determinations were recorded on a Perkin-Elmer UV–vis spectrophotometer according to the known procedure.[24]

Thin-layer chromatography was performed on Analtech silica gel GF plates. Visualization was accomplished by UV illumination or by staining with a $KMnO_4$ solution. Column chromatography was performed with silica gel Kieselgel 60 (0.040–0.063 mm) from EM Scientific. High-performance liquid chromatography (HPLC) was accomplished with a Waters Delta prep 3000 and a 254 absorbance detector. Peak separations were achieved using a 250 mm × 30 cm YMC pack D.SIL-10-b s-10 120a silica gel column with a mobile phase of 1% acetic acid in ethyl acetate/hexane (1:1) and a flow rate of 5.00 mL/min.

General Amino Acid Coupling Procedure. To a solution containing an N-protected amino acid (1 equiv, 0.1 M) in CH_2Cl_2 was added an amino acid ester (1 equiv, usually as an HCl salt) and 1-hydroxybenzotriazole (HOBT; 1–1.5 equiv) followed by triethylamine (TEA; 1 equiv) and *N*-[3-(dimethylamino)propyl]-*N'*-ethylcarbodiimide hydrochloride (EDCl; 1.5-2 equiv). The mixture was stirred for 4 h at room temperature or until TLC analysis indicated that the reaction was complete. The reaction mixture was then diluted with ethyl acetate and washed with 1 N HCl (2×), saturated sodium bicarbonate (2×), and brine. The organic layer was dried over $MgSO_4$ and concentrated to dryness in vacuo. The resulting product was either directly used in the next step or purified further by flash chromatography.

General Deprotection Procedures. BOC-amino acids were deprotected by stirring a CH_2Cl_2:trifluoroacetic acid (1:1) solution at room temperature for 4 h. The solvent was removed in vacuo and the last traces of trifluoroacetic acid removed by repeated evaporations from dichloromethane. Methyl esters were deprotected by dissolving the compound in methanol at a concentration of about 0.1 M and slowly adding 1.2 equiv of 1 N NaOH. After completion of the reaction (1–4 h), the solvents were removed in vacuo and the residue was dissolved in ethyl acetate. The organic layer was washed with 1 N HCl and then water and dried over Na_2SO_4.

Hydrocinnamoyl-L-phenylalanyl-L-homocysteine Thiolactone (30). Route A. A solution of hydrocinnamic acid (15 g, 0.099 mol), L-phenylalanine methyl ester hydrochloride salt (20 g, 0.102 mol), 1-hydroxybenzotriazole (13.5 g, 0.099 mol), triethylamine (10.4 g, 0.102 mol), and *N*-[3-(dimethylamino)-propyl]-*N'*-ethylcarbodiimide hydrochloride (38.3 g, 0.2 mol) in 100 mL of methylene chloride was stirred at room temperature for 4 h. The reaction mixture was diluted with 150 mL of ethyl acetate and washed with 1 N HCl (2 × 50 mL) and saturated $NaHCO_3$ (2 × 50 mL). The organic layer was dried ($MgSO_4$) and concentrated to give the desired amide as a white solid (25.17 g, 81%): mp 60–62 °C; IR (KBr) 1754, 1712, 1647, 1535 cm^{-1}; ^1H NMR ($CDCl_3$) 2.42–2.47 (m, 2H), 2.78–2.83 (t, J = 7.1 Hz, 2H), 2.90 (dd, J = 8.5, 13.8 Hz, 1 H), 3.09 (dd, J = 5.8, 13.8 Hz, 1 H), 3.68 (s, 3H), 4.65 (dd, J = 5.7, 8.7 Hz, 1H), 7.1–7.3 (m, 10H); MS (DCl/CH_4) 312 (M + 1). Anal. ($C_{19}H_{21}NO_3$) C, H, N.

The ester (25 g, 0.08 mol) was then treated with 250 mL of 2 N NaOH in MeOH for 1 h at room temperature. The reaction mixture was concentrated to half of the original volume and then diluted with 250 mL of water. The aqueous solution was washed with ether (2 × 60 mL) and acidified with 12 N HCl at 0 °C. The resulting suspension was extracted with ethyl acetate (3 × 100 mL), and the combined extracts were dried (Na_2SO_4) and evapoarated to give hydrocinnamoyl-L-phenylalanine as a white solid (24 g, 100%): mp 158–159 °C; IR (KBr) 1730, 1700, 1633, 1522 cm^{-1}; ^1H NMR (CD_3OD) 2.44 (dd, J = 6.5, 8.6 Hz, 2H), 2.77–2.82 (m, 2H), 2.91 (dd, J = 8.9, 13.9 Hz, 1H), 3.15 (dd, J = 5.1, 13.9 Hz, 1H), 4.65 (dd, J = 5.1, 8.9 Hz, 1H), 7.1–7.3 (m, 10H); MS (DCl/CH_4) 298 (M + 1). Anal. ($C_{18}H_{19}NO_3$) C, H, N.

The synthesis was completed by stirring a solution containing the acid (10 g, 0.034 mol), L-homocysteine thiolactone hydrochloride (5.2 g, 0.034 mol), 1-hydroxybenzotriazole (4.5 g, 0.034 mol), triethylamine (3.47 g, 0.034 mol), *N*-[3-(dimethylamino)propyl]-*N'*-ethylcarbodiimide hydrochloride (9.8 g, 0.051 mol), and CH_2Cl_2 at room temperature. After 4 h, the reaction mixture was poured into ether (500 mL) and washed with 1N HCl (2 × 200 mL), sat. $NaHCO_3$ (2 × 200 mL) and brine (1 × 100 mL). The organic layer was then dried (Na_2SO_4) and concentrated in vacuo. The resulting residue was chromatographed on silica gel (60% ethyl acetate in hexane) to give thiolactone **30** as a white solid (12.5 g, 93%): mp 168–169 °C; IR (KBr) 1705, 1639, 1540 cm^{-1}; ^1H NMR (CD_3OD) 1.86–2.00 (m, 1H), 2.37–2.49 (m, 3H), 2.77–2.88 (m, 3H) 3.04 (dd, J = 6.7, 13.7 Hz, 1H), 3.19–3.25 (m, 1H), 3.29–3.40 (m, 1H) 4.54 (dd, J = 6.9, 13.6 Hz, 1H), 4.63 (dd, J = 6.8, 9.2 Hz, 1H), 7.12–7.28 (m, 10H); MS (DCl/CH_4) 397 (M + 1); $[\alpha]^{25}_D$ = −11.42 (6.8 mg/mL in MeOH). Anal. ($C_{22}H_{24}N_2O_3S$) C, H, N.

2-Naphthoyl-L-phenylalanyl-D,L-homocysteine Thiolactone. Route B. A solution of BOC-L-phenylalanine (2.0 g, 7.5 mmol), D,L-homocysteine thiolactone hydrochloride (1.16 g, 7.5 mmol), TEA (762 mg, 7.5 mmol), HOBT (1.01 g, 7.5 mol), EDCl (2.88 g, 15 mmol), and CH_2Cl_2 (100 mL) was stirred at room temperature. After 4 h, the reaction mixture was poured into 200 mL of ethyl acetate and washed with 1 N HCl (2 × 50 mL), saturated $NaHCO_3$ (2 × 50 mL), and brine (1 × 25 mL). The organic layer was dried (Na_2SO_4) and evaporated to dryness to give the dipeptide as a white solid (2.5 g, 100%): mp 89–90 °C; IR (KBr) 3326, 1700, 1655, 1538, 1520, 1250, 1166 cm^{-1}; NMR ($CDCl_3$) 1.34 (s, 9H), 2.06–2.17 (m, 1H), 2.53–2.61 (m, 1H), 2.82 (dd, J = 9.3, 13.8 Hz, 1H), 3.12 (dd, J = 4.9, 13.9 Hz, 1H) 3.39 (dd, J = 5.4, 11.7 Hz, 1H), 3.43 (dd, J = 5.3, 11.6 Hz, 1H), 7.14–7.26 (m, 5H); MS (CDl/CH_4) 365 (M + 1). Anal. ($C_{18}H_{24}N_2O_4S$) C, H, N.

The BOC-protected dipeptide (546 mg, 1.5 mmol) was then dissolved in 50% TFA/CH_2Cl_2 and stirred at room temperature. After 4 h, the reaction mixture was concentrated to dryness and redissolved in 10 mL of CH_2Cl_2. To this solution was added 2-naphthoic acid (275 mg, 1.6 mmol), TEA (163 mg, 1.6 mmol), HOBT (216 mg, 1.6 mmol), and EDCl (613 mg, 3.3 mmol). After the solution was stirred for 4 h at room temperature, it was poured into 60 mL of ethyl acetate and

washed with 1 N HCl (2 × 20 mL), saturated $NaHCO_3$ (2 × 30 mL), and brine. The organic layer was then dried (Na_2SO_4) and concentrated in vacuo. The resulting residue was chromatographed on silica gel (50% ethyl acetate in hexane) to give the desired compound as a white solid (400 mg, 63.8%): mp 94–95 °C; IR (KBr) 3281, 1806, 1641, 1531 cm^{-1}; NMR ($CDCl_3$) 1.90–2.27 (m, 1H), 2.43–2.69 (m, 1H), 3.08–3.15 (m, 1H), 3.20–3.46 (m, 3H), 4.59–4.69 (m, 1H), 4.90–4.96 (m, 1H), 7.16–7.37 (m, 5H), 7.51–7.60 (m, 2H), 7.74–7.81 (m, 1H), 7.81–7.95 (m, 3H), 8.26 (d, J = 10.9 Hz, 1H); MS (DCl/CH_4) 419 (M + 1). Anal. ($C_{24}H_{22}N_2O_3S$) C, H, N.

Hydrocinnamoyl-L-phenylalanyl-L-homocysteine (22). General Procedure for Thiolactone Hydrolysis. A 0 °C solution of hydrocinnamoyl-L-phenylalanyl-D,L-homocysteine thiolactone (2.0 g, 0.005 mol) and methanol (10 mL) was degassed by bubbling nitrogen through the solution for 5 min and then treated with 2 N NaOH (3 mL, 0.006 mol). After being stirred for 2 h at room temperature, the reaction mixture was acidified with 0 °C 12 N HCl and poured over EtOAc. The organic layer was separated and washed with 1 N HCl, saturated $NaHCO_3$, and brine. The mixture of diastereoisomers was separated by preparative HPLC using a Waters Delta prep 3000 and a 250 mm × 30 cm YMC pack D.SIL-10-b s-10 120 a silica gel column with a mobile phase of 1% acetic acid in ethyl acetate/hexane (1:1) and a flow rate of 5.00 mL/min. The L,L isomer (**21**) and the D,D isomer (**22**) had retention times of 25 and 38 min, respectively.

Compound **22** (129 mg): mp 117–119 °C; IR (KBr) 1720, 1632, 1453 cm^{-1}; NMR (CD_3OD) 1.76–2.02 (m, 3H), 2.41–2.56 (m, 3H), 2.75–2.89 (m, 3H), 3.11 (dd, J = 5.4, 14 Hz, 1H), 4.56 (dd, J = 4.7, 9.3 Hz, 1H), 4.61–5.69 (m, 1H), 7.1–7.52 (m, 10H); MS (DCl/CH_4) 415 (M + 1). Anal. ($C_{22}H_{26}N_2O_4S$·0.5H_2O) C, H, N.

Compound **23** (99 mg): mp 152–154 °C; NMR (CD_3OD) 2.06–2.18 (m, 3H), 2.21–2.89 (m, 3H), 2.75–2.89 (m, 3H), 3.0 (dd, J = 8.5, 13.6 Hz, 1H), 4.47 (dd, J = 4.7, 9.3 Hz, 1H), 4.61–4.69 (m, 1H), 7.10–7.52 (m, 10H); MS (DCl/CH_4) 415 (M + 1). Anal. ($C_{22}H_{26}N_2O_4S$·0.5H_2O) C, H, N.

D-Tyrosyl-L-phenylalanylglycine (1): mp 185–187 °C; IR (KBr) 2555, 1743, 1682, 1653 cm^{-1}; ^1H NMR (D_2O) δ 2.81–2.90 (m, 3H), 3.14 (dd, J = 4.3, 14.4 Hz, 1H), 3.74 (q, 2H), 4.17 (t, J = 5.8 Hz, 1H), 4.63–4.68 (m, 1H), 6.80 (s, 4H), 7.21–7.23 (m, 2H), 7.31–7.39 (m, 3H); MS (DCl/CH_4) 546 (M + 1); $[\alpha]^{25}_D$ = −25.6 (8.1 mg/mL in MeOH). Anal. ($C_{20}H_{23}N_3O_5$·0.5H_2O) C, H, N.

Dansyl-D-tyrosyl-L-phenylalanylglycine (2): mp 174–177 °C; NMR (CD_3OD) δ 2.30–2.46 (m, 2H), 2.72 (dd, J = 9, 14 Hz, 1H), 2.83 (s, 6H), 3.09 (dd, J = 5.2, 13.9 Hz, 1H), 3.70 (dd, J = 5.3, 9 Hz, 1H), 3.86 (dd, J = 7.8, 5.2 Hz, 1 H), 4.56 (dd, 2H), 6.33 (dd, 4H), 7.10–7.21 (m, 6H), 7.38–7.44 (m, 2H), 8.05 (dd, J = 8.5, 16.5 Hz, 2H), 8.46 (d, 8.5 Hz, 1H); MS (FAB) (M + 1) 619. Anal. ($C_{32}H_{34}N_4O_7S$·1.5H_2O) C, H, N.

(Benzyloxycarbonyl)-D-tyrosyl-L-phenylalanylglycine (3): mp 124–126 °C; IR (KBr) 1694, 1649, 1535, 1515 cm^{-1}; ^1H NMR (CD_3OD) δ 2.48–2.60 (m, 1H), 2.61–2.83 (m, 2H), 3.05–3.18 (m, 1H), 3.60–3.91 (m, 2H), 4.06–4.20 (m, 1H), 4.59–4.65 (m, 1H), 4.90–5.00 (m, 2H), 6.72 (dd, 4H), 7.12–7.24 (m, 10H). Anal. ($C_{28}H_{29}N_3O_7$·1.75H_2O) C, H, N.

(*tert*-Butoxycarbonyl)-D-tyrosyl-L-phenylalanylglycine (4): mp 150–151 °C; IR (KBr) 1738, 1633, 1646, 1614 cm^{-1}; ^1H NMR (CD_3OD) δ 1.35 (s, 9H), 2.51–2.58 (m, 1H), 2.72 (dd, J = 6.8, 13 Hz, 1H), 2.86 (dd, J = 9, 14 Hz, 1H), 3.88 (dd, 2H), 4.11 (t, J = 7.0 Hz, 1H), 4.60–4.63 (m, 1H), 6.65 (dd, 4H), 7.12–7.23 (m, 5H); MS (DCl/CH_4) 486 (M + 1). Anal. ($C_{25}H_{31}N_3O_7H_2O$) C, H, N.

(*tert*-Butoxycarbonyl)-D-tyrosyl-L-phenylalanylalanine (5): mp 117–119 °C; IR (Nujol) 1717, 1652, 1641, 1516 cm^{-1}; ^1H NMR (CD_3OD) δ 1.29–1.50 (m, 12H), 2.51–2.60 (m, 1H), 2.71–2.81 (m, 1H), 2.82–2.93 (m, 1H), 3.10–3.20 (m, 1H), 4.13 (dd, J = 5.6, 8.6 Hz, 1H), 4.39 (dd, J = 7.3, 14.5 Hz, 1H), 4.56–4.80 (m, 1H), 6.90 (dd, 4H), 7.13–7.25 (m, 5H); MS (DCl/CH_4) 500 (M + 1). Anal. ($C_{26}H_{33}N_3O_7$·0.5H_2O) C, H, N.

(*tert*-Butoxycarbonyl)-D-tyrosyl-L-phenylalanylserine (6): mp 123-124 °C; IR (KBr) 1720, 1650, 1514 cm^{-1}; ^1H NMR (CD_3OD) δ 1.35 (s, 9H), 2.57–2.60 (m, 1H), 2.74–2.80 (m, 1H), 2.89 (dd, J = 8.5, 13.8 Hz, 1H), 3.76–3.89 (m, 2H), 4.12–4.18 (m, 1H), 4.46 (dd, J = 4.8, 10 Hz, 1H), 4.58–4.73 (m, 1H), 6.64–6.67 (m, 2H), 6.87–6.91 (m, 2H), 7.18–7.33 (m, 5H); MS (DCl/CH_4) 516 (M + 1). Anal. ($C_{26}H_{33}N_3O_8$) C, H, N.

(*tert*-Butoxycarbonyl)-D-tyrosyl-L-phenylalanylcysteine (7): mp 97–99 °C; IR (KBr) 2556, 1743, 1682, 1653 cm^{-1}; ^1H NMR (CD_3OD) δ 1.35 (s, 9H), 2.03–2.08 (m, 1H), 2.46–2.63 (m, 2H), 2.73–2.91 (m, 2H), 3.06 (dd, 1H), 3.30–3.45 (m, 1H), 4.12 (t, 1H), 4.39–4.51 (m, 1H), 4.58–4.64 (m, 2H), 6.66 (d, 2H), 6.90 (d, 2H), 7.15–7.25 (m, 5H). Anal. ($C_{26}H_{33}N_3O_7S$·0.5H_2O) C, H, N.

(*tert*-Butoxycarbonyl)-D-tyrosyl-L-phenylalanyl-D,L-homocysteine (8): mp 92–94 °C; IR (KBr) 2554, 1716, 1690, 1650, 1516 cm^{-1}; ^1H NMR (CD_3OD) δ 1.34 (s, 9H), 1.98–2.48 (m, 4H), 2.50–2.54 (m, 1H), 2.60–2.78 (m, 1H), 2.87–3.10 (m, 3H), 4.20–4.23 (m, 1H), 4.47 (dd, 0.5H), 4.55 (dd, 0.5H), 4.65–4.72 (m, 1H), 7.01–7.31 (m, 10H); MS (DCl/CH_4) 530 (M + 1). Anal. ($C_{27}H_{35}N_3O_6S$) C, H, N.

(*tert*-Butoxycarbonyl)-L-phenylalanyl-D,L-homocysteine (9): mp 60–62 °C; IR (KBr) 2556, 1717, 1698, 1658, 1525 cm^{-1}; ^1H NMR (CD_3OD) δ 1.367 (s, 4.5H), 1.37 (s, 4.5H), 1.85–2.11 (m, 2H), 2.23–2.35 (m, 1H), 2.53 (t, J = 7.3 Hz, 1H), 2.78–2.89 (m, 1H), 3.05 (dd, 0.5H), 3.12 (dd, 0.5H), 4.28–4.33 (m, 1H), 4.51 (dd, 0.5H), 4.59 (dd, 0.5H), 7.20–7.27 (m, 5H); MS (DCl/CH_4) 383 (M + 1). Anal. ($C_{18}H_{26}N_2O_5S$) C, H, N.

Isobutyryl-L-phenylalanyl-D,L-homocysteine (10): mp 67–68 °C; IR (KBr) 1640, 1548, 1719 cm^{-1}; ^1H NMR (CD_3OD) δ 0.84–0.85 (m, 6H), 1.27–1.40 (m, 3H), 1.70–2.35 (m, 5H), 2.46–2.53 (m, 1H), 2.82–2.93 (m, 1H), 3.04–3.15 (m, 1H), 4.51 (dd, 0.5H), 4.62 (dd, 0.5H), 4.67–4.74 (m, 1H), 7.18–7.29 (m, 5H); MS (DCl/CH_4) 381 (M + 1). Anal. ($C_{19}H_{28}N_2O_4S$) C, H, N.

(Benzyloxycarbonyl)-L-phenylalanyl-D,L-homocysteine (11): mp 52–53 °C; IR (KBr) 2556, 1708, 1661, 1527 cm^{-1}; ^1H NMR (CD_3OD) δ 1.82–2.31 (m, 3H), 2.42–2.59 (m, 1H), 2.79–2.93 (m, 1H), 3.04–3.17 (m, 1H), 4.36–4.41 (m, 1H), 4.44–4.60 (m, 1H), 5.00–5.04 (m, 2H), 7.20–7.33 (m, 10H); MS (DCl/CH_4) 417 (M + 1). Anal. ($C_{21}H_{24}N_2O_5S$) C, H, N.

(Indol-2-ylcarbonyl)-D-tyrosyl-L-phenylalanyl-D,L-homocysteine (12): mp 176–179 °C; IR (KBr) 3297, 1719, 1624, 1565, 1539, 745 cm^{-1}; ^1H NMR (CD_3OD) δ 1.98–2.21 (m, 2H), 2.55 (t, J = 7 Hz, 2H), 3.07 (dd, J = 9.1, 13.9 Hz, 1H), 3.25–3.31 (m, 1H), 4.60 (dd, J = 4.5, 9 Hz, 1H), 4.80–4.9 (m, 1H), 7.01–7.32 (m, 8H), 7.39 (d, J = 8.4 Hz, 1H), 7.58 (d, J = 8 Hz, 1H); MS (DCl/CH_4) 408 (−H_2O) (M + 1). Anal. ($C_{22}H_{23}N_3O_4S$·0.5H_2O) C, H, N.

2-Naphthoyl-L-phenylalanyl-D,L-homocysteine (13): mp 102–103 °C; IR (KBr) 3295, 1719, 1638, 1533, 1539, 1233 cm^{-1}; ^1H NMR (CD_3OD) δ 1.85–2.40 (m, 4H), 2.58 (t, J = 7 Hz, 1H), 3.06–3.16 (m, 0.5H), 3.22–3.33 (m, 0.5H), 4.56–4.63 (m, 1H), 4.89–4.99 (m, 1H), 7.16–7.36 (m, 5H), 7.51–7.59 (m, 2H), 7.73–7.96 (m, 3H), 8.27 (d, J = 15.7 Hz, 1H); MS (DCl/CH_4) 419 (−H_2O) (M + 1). Anal. ($C_{24}H_{24}N_2O_4S$) C, H, N.

(Phenoxyacetoyl)-L-phenylalanyl-D,L-homocysteine (14): mp 68–71 °C; IR (KBr) 1722, 1651, 1538, 1237 cm^{-1}; ^1H NMR (CD_3OD) δ 1.88–2.34 (m, 4H), 2.5–2.52 (m, 1H), 2.94–3.21 (m, 2H), 4.39–4.59 (m, 4H), 6.85–7.0 (m, 4H), 7.21–7.30 (m, 6H); MS (DCl/CH_4) 417 (M + 1). Anal. ($C_{21}H_{24}N_2O_5S$) C, H, N.

(3,4-Dichlorobenzoyl)-L-phenylalanyl-D,L-homocysteine (15): mp 76–78 °C; IR (KBr) 1718, 1644, 1550 cm^{-1}; ^1H NMR (CD_3OD) δ 1.8–2.4 (m, 3H), 2.4–2.6 (m, 3H), 2.7–3.0 (m, 3H), 3.0–3.2 (m, 1H), 4.49 (dd, J = 4.5, 9.3 Hz, 1H), 4.56–4.70 (m, 1H), 7.1–7.4 (m, 8H); MS (DCl/CH_4) 484 (weak), 466 (−H_2O) (M + 1). Anal. ($C_{22}H_{24}Cl_2N_2O_4S$) C, H, N.

Hydrocinnamoyl-L-phenylalanyl-D,L-homocysteine (16): mp 65–67 °C; IR (KBr) 1720, 1632, 1453 cm^{-1}; ^1H NMR (CD_3OD) δ 1.76–2.32 (m, 2H), 2.39–2.50 (m, 3H), 2.75–2.89 (m, 3H), 3.01 (dd, 0.5H), 3.11 (dd, 0.5H), 4.47 (dd, 0.5H), 4.56 (dd, 0.5H), 4.59–4.69 (m, 1H), 7.10–7.29 (m, 10H); MS (DCl/CH_4) 415 (M + 1). Anal. ($C_{22}H_{26}N_2O_4S$·0.5H_2O) C, H, N.

Hydrocinnamoyl-L-methionine-D,L-homocysteine (17): mp 44–45 °C; ^1H NMR (CD_3OD) δ 1.79–1.89 (m, 1H), 1.95–2.13 (m, 4H), 2.04 (s, 3H), 2.32–2.38 (m, 2H), 2.39–2.58 (m, 4H), 2.88–2.93 (t, J = 6.8 Hz, 2H), 4.39–4.49 (m, 1H), 4.52–

4.60 (m, 1H), 7.13–7.28 (m, 5H); MS (DCl/CH_4) 399 (M + 1). Anal. ($C_{18}H_{26}N_2O_4S_2$) C, H, N.

Hydrocinnamoyl-L-valine-D,L-homocysteine (18): mp 60–62 °C; IR (KBr) 1721, 1638, 1546 cm^{-1}; ^1H NMR (CD_3OD) δ 0.84–0.99 (m, 6H), 1.92–2.15 (m, 3H), 2.44–2.59 (m, 4H), 2.91 (t, J = 7.6 Hz, 2H), 4.09–4.23 (m, 1H), 4.53–4.64 (m, 1H), 7.12–7.27 (m, 5H); MS (DCl/CH_4) 367 (M + 1). Anal. ($C_{18}H_{26}N_2O_4S$) C, H, N.

Hydrocinnamoyl-L-leucine-D,L-homocysteine (19): mp 68–70 °C; IR (KBr) 1719, 1638, 1549 cm^{-1}; ^1H NMR (CD_3OD) δ 0.875 (dd, J = 5.4 Hz, 6H), 1.45–1.51 (m, 3H), 1.96–2.14 (m, 2H), 2.44–2.58 (m, 4H), 2.91 (t, J = 7.0 Hz, 2H), 4.33–4.39 (m, 1H), 4.46–4.55 (m, 1H), 7.13–7.27 (m, 5H); MS (DCl/CH_4) 381 (M + 1). Anal. ($C_{19}H_{28}N_2O_4S{\cdot}0.5H_2O$) C, H, N.

Hydrocinnamoyl-L-norleucine-D,L-homocysteine (20): mp 146–148 °C; IR (KBr) 1763, 1672, 1510, 1276 cm^{-1}; ^1H NMR (CD_3OD) δ 0.89 (s, J = 3.9 Hz, 3H), 1.17–1.49 (m, 4H), 1.51–1.62 (m, 1H), 1.66–1.77 (m, 1H), 1.91–2.15 (m, 2H), 2.42–2.57 (m, 4H), 2.85–3.0 (m, 2H), 4.23–4.33 (m, 1H), 4.53–4.60 (m, 1H), 7.12–7.27 (m, 5H); MS (DCl/CH_4) 381 (M + 1). Anal. ($C_{19}H_{28}N_2O_4S$) C, H, N.

Hydrocinnamoyl-D,L-homocysteine (21): mp 96–97 °C; IR (KBr) 1731, 1646, 1628, 1541 cm^{-1}; ^1H NMR (CD_3OD) δ 1.28–2.07 (m, 2H), 2.24–2.43 (m, 2H), 2.54 (t, J = 7.3 Hz, 2H), 2.91 (t, J = 7.5 Hz, 2H), 4.54 (dd, J = 4.5, 9.5 Hz, 1H), 7.13–7.28 (m, 5H); MS (DCl/CH_4) 268 (M + 1). Anal. ($C_{13}H_{17}NO_3S$) C, H, N.

Hydrocinnamoyl-L-phenylalanyl-L-cysteine (24). Hydrocinnamoyl-L-phenylalanine (200 mg, 0.67 mmol) and cystine dimethyl ester (90 mg, 0.37 mmol) were coupled under normal conditions to yield after workup a yellow solid (230 mg, 80%): mp 73–75 °C; ^1H NMR ($CDCl_3$) 2.39–2.4 (m, 2H), 2.72–2.98 (m, 5H), 3.05–3.13 (m, 1H), 3.69 (s, 3H), 4.57–4.71 (m, 2H) 7.02–7.3 (m, 10H). Zinc dust (50 mg) was added to a solution of the disulfide (220 mg, 0.27 mmol) in acetic acid (5 mL) and the reaction stirred overnight. After filtration and removal of the solvent, the residue was purified by flash chromatography on silica gel with 50% ethyl acetate as the eluent to afford the desired compound (200 mg, 91%): ^1H NMR ($CDCl_3$) 2.4–2.5 (m, 2H), 2.7–3.2 (m, 6H), 3.39 (s, 3H), 4.7–4.82 (m, 2H), 7.02–7.3 (m, 10H). Hydrolysis of the methyl ester was accomplished by deoxygenating a methanol (5 mL) solution of the methyl ester (200 mg, 0.48 mmol), cooling to 0 °C, and adding 2 N KOH (0.36 mL, 0.73 mmol). After 1 h, the reaction was acidified with 1 N HCl and extracted with ethyl acetate. The combined organic layers were washed with water and dried (Na_2SO_4) to yield 97 mg of a white solid (50%): mp 73–75 °C; IR (KBr) 1724, 1639, 1532 cm^{-1}; ^1H NMR (CD_3OD) δ 2.42–2.49 (m, 2H), 2.76–3.06 (m, 5H), 3.04–3.16 (m, 1H), 4.55–4.70 (m, 2H), 7.10–7.24 (m, 10H); MS (CDl/CH_4) 401 (M + 1); $[\alpha]^{25}_D = -8.27°$ (4.0 mg/mL in MeOH). Anal. ($C_{21}H_{24}N_2O_4S$) C, H, N.

Hydrocinnamoyl-L-phenylalanyldecarboxyhomocysteine (25): mp 127–129 °C; IR (KBr) 3309, 3285, 1643, 1537 cm^{-1}; ^1H NMR (CD_3OD) δ 1.60–1.67 (m, 2H), 2.28–2.33 (t, J = 7.1 Hz, 2H), 2.44–2.49 (m, 2H), 2.78–2.86 (m, 3H), 2.96–3.03 (dd, J = 7.3, 13.5 Hz, 1H), 3.15–3.27 (m, 2H), 4.51 (t, J = 7.5 Hz, 1H), 7.13–7.29 (m, 10H); MS (DCl/CH_4) 371 (M + 1). Anal. ($C_{21}H_{26}N_2O_2S$) C, H, N.

Hydrocinnamoyl-L-phenylalanyl-L-aspartic acid (26): mp 84–86 °C; IR (KBr) 1724, 1669, 1529 cm^{-1}; ^1H NMR (CD_3OD) δ 2.39–2.47 (m, 2H), 2.70–2.93 (m, 5H), 3.06–3.18 (m, 1H), 4.64–4.70 (m, 2H), 7.11–7.24 (m, 10H); MS (DCl/CH_4) 413 (M + 1). Anal. ($C_{22}H_{24}N_2O_6$) C, H, N.

Hydrocinnamoyl-L-phenylalanyl-L-asparagine (27): mp 113–114 °C; IR (KBr) 1729, 1650, 1538 cm^{-1}; ^1H NMR (CD_3OD) δ 2.40–2.47 (m, 2H), 2.68 (dd, J = 5.5, 10.8 Hz, 1H), 2.73–2.87 (m, 4H), 3.08–3.18 (m, 1H), 4.63–4.72 (m, 2H), 7.10–7.27 (m, 10H); MS (DCl/CH_4) 412 (M + 1). Anal. ($C_{22}H_{25}N_3O_5{\cdot}H_2O$) C, H, N.

Hydrocinnamoyl-L-phenylalanyl-L-glutamine (28): mp 78–79 °C; IR (KBr) 1718, 1638, 1544 cm^{-1}; ^1H NMR (CD_3OD) δ 1.75–2.23 (m, 3H), 2.34–2.49 (m, 3H), 2.74–2.89 (m, 3H), 3.03 (dd, J = 7.0, 13.6 Hz, 0.5H), 3.12 (dd, J = 5.3, 14 Hz, 0.5H), 4.36 (dd, J = 4.3, 8.8 Hz, 1H), 4.41 (dd, J = 4.8, 9.1 Hz, 1H), 7.1–7.28 (m, 10H); MS (DCl/CH_4) 427 (M + 1). Anal. ($C_{23}H_{26}N_2O_6$) C, H, N.

Hydrocinnamoyl-L-phenylalanyl-D,L-homocysteine Benzyl Ester (29). Benzyl alcohol (436 mg, 4.03 mmol) in anhydrous THF (5 mL) was treated with sodium metal (49 mg, 2.13 mmol) at room temperature. After 2 h hydrocinnamoyl-L-phenylalanyl-D,L-homocysteine thiolactone (800 mg, 2.02 mmol) in anhydrous THF (10 mL) was added and the reaction stirred at room temperature. After 1 h, the reaction was quenched with 0.1 M HCl and extracted with ethyl acetate. The combined organic layer were washed with saturated $NaHCO_3$ and brine and dried ($MgSO_4$). After removal of the solvents, the crude product was chromatographed on silica gel (EtOAc/hexane, 3:1) to obtain a white solid (210 mg, 40%): mp 129–131 °C; IR (KBr) 1743, 1622 cm^{-1}; ^1H NMR (CD_3OD) δ 1.72–2.19 (m, 3H), 2.34–2.48 (m, 3H), 2.85–3.02 (m, 4H), 4.60–4.73 (m, 2H), 5.05–5.16 (m, 2H), 6.08–6.13 (m, 0.5H), 6.52–6.63 (m, 0.5H), 7.05–7.38 (m, 15H); MS (CDl/CH_4) 505 (M + 1). Anal. ($C_{29}H_{32}N_2O_4S$) C, H, N.

PAM Enzyme Assay. PAM from conditioned medium derived from cultured rat medullary thyroid carcinoma CA-77 cells (Unigene Laboratories; Fairfield, NJ) was partially purified using a DEAE cartridge followed by Sephacryl 300 SF column chromatography.[11] Reaction mixtures (100 μL) containing PAM (13.5 μU) and the inhibitor at the desired concentration were preincubated for 20 min at room temperature in 150 mM TES buffer (pH = 7.0) containing 0.001% Triton-100. The substrate *N*-dansyl-D-Tyr-Phe-Gly (2.0 μM) and ascorbate (3 mM) were added to initiate the reaction. At the designated time, 10 μL of 0.1 M EDTA solution was added to quench the reaction. Separation and quantification (peak integration) of the product (Dan-D-Tyr-Phe-NH_2) and starting tripeptide were performed on a C_{18}-reverse phase column with fluorometric detection.[30] Each inhibitor was dissolved in DMSO (10 mM) and then diluted in 150 mM TES, pH = 7.0. A minimum of seven different inhibitor concentrations were tested in duplicate with each inhibitor tested three times. The percent inhibition for each compound was determined by comparing the reaction rates to the control and then constructing a log dose–response curve. Inhibitor IC_{50} determinations were obtained by least squares analyses of the linear portions of the log dose–response curves.

DRG Cell Assay. Primary cultures of dorsal root ganglion (DRG) neurons were prepared as previously described.[29] In the absence of ascorbate, the ratio of substance P-Gly/substance P (SP-Gly/SP) changes from 0.05 on day one to 3.3 on day six. On the sixth day, the inhibitor was added followed by the addition of ascorbate (500 μM) 12 h later. After incubation overnight, the conversion of substance P-Gly to substance P was monitored by an RIA using an antibody that recognizes SP with an affinity 10 000-fold higher than SP-Gly.[31] The SP-Gly level was determined by treating a duplicate sample with 3.5 μg of PAM followed by the RIA measurement of SP. The difference in SP measured between the two samples gave the level of SP-Gly. Percent inhibition was determined at a 10 μM inhibitor concentration.

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References

(1) (a) Eipper, B. A.; Mains, R. E. Peptide α-Amidation. *Annu. Rev. Physiol.* **1988**, *50*, 333–344. (b) Judd, A. K.; Schoolnik, G. K. Peptides: Chemistry, Biology, and Pharmacology. *Adv. Pharmacol.* **1990**, *21*, 221–285.

(2) (a) Bradbury, A. F.; Finnie, M. D. A.; Smyth, D. G. Mechanism of C-Terminal Amide Formation by Pituitary Enzymes. *Nature* **1982**, *298*, 686–688; (b) Bradbury, A. F.; Smyth, D. G. Peptide Amidation. *Trends Biol. Sci.* **1991**, *16*, 112–115.

(3) Eipper, B. A.; Stoffers, D. A.; Mains, R. E. The *Biosynthesis of Neuropeptides*: Peptide α-Amidation. *Annu. Rev. Neurosci.* **1992**, *15*, 57–85.

(4) (a) Katopodis, A. G.; Ping, D.; May, S. W. A Novel Enzyme from Bovine Neurointermediate Pituitary Catalyzes Dealkylation of α-Hydroxyglycine Derivatives, Thereby Functioning Sequentially with Peptidylglycine α-Amidating Monooxygenase in Peptide

Amidation. *Biochemistry* **1990**, *29*, 6115–6120. (b) Tajima, M.; Iida, T.; Yoshida, S.; Komatsu, K.; Namba, R.; Yanagi, M.; Noguchi, M.; Okamoto, H. The Reaction Product of Peptidylglycine α-Amidating Enzyme is a Hydroxyl Derivative at α-Carbon of the Carboxyl-terminal Glycine. *J. Biol. Chem.* **1990**, *265*, 9602–9605.

(5) Yun, H.-Y.; Keutmann, H. T.; Eipper, B. A. Alternative Splicing Governs Sulfation of Tyrosine or Oligosaccharide on Peptidylglycine α-Amidation Monooxygenase. *J. Biol. Chem.* **1994**, *269*, 10946–10955.

(6) Eipper, B. A.; Mains, R. E.; Glembotski, C. C. Identification in Pituitary Tissue of a Peptide α-Amidation Activity that acts on Glycine-Extended Peptides and Requires Molecular Oxygen, Copper and Ascorbic Acid. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 5144–5148.

(7) (a) Young, S. D.; Tamburini, P. P. Enzymatic Peptidyl α-Amidation Proceeds through Formation of an α-Hydroxyglycine Intermediate. *J. Am. Chem. Soc.* **1989**, *111*, 1933–1934. (b) Ramer, S. E.; Cheng, H.; Palcic, M. M.; Vederas, J. C. Formation of Peptide Amides by Peptidylglycine α-Amidating Monooxygenase: A New Assay and Stereochemistry of Hydrogen Loss. *J. Am. Chem. Soc.* **1988**, *110*, 8526–8532. (c) Ping, D.; Katopodis, A.; May, S. W. Tandem Stereochemistry of Peptidylglycine α-Monooxygenase and Peptidylamidoglycolate Lyase, the Two Enzymes Involved in Peptide Amidation. *J. Am. Chem. Soc.* **1992**, *114*, 3998–4000.

(8) (a) Freeman, J. C.; Villafranca, J. J.; Merkler, D. J. Redox Cycling of Enzyme-Bound Copper during Peptide Amidation. *J. Am. Chem. Soc.* **1993**, *115*, 4923–4924. (b) Kulathila, R.; Consalvo, A. P.; Fitzpatrick, P. F.; Freeman, J. C.; Snyder, L. M.; Villafranca, J. J.; Merkler, D. J. Bifunctional Peptidylglycine α-Amidating Enzyme Requires Two Copper Atoms for Maximum Activity. *Arch. Biochem. Biophys.* **1994**, *311*, 191–194. (c) Merkler, D. J.; Kulathila, R.; Consalvo, A. P.; Young, S. D.; Ash, D. E. ^{18}O Isotopic ^{13}C NMR Shift as Proof that Bifunctional Peptidylglycine α-Amidation Enzyme is a Monooxygenase. *Biochemistry* **1992**, *31*, 7282–7288.

(9) (a) Fitzpatrick, P. F.; Villafranca, J. J. Mechanism-Based Inhibitors of Dopamine β-Hydroxylase. *Arch. Biochem. Biophys.* **1987**, *257*, 231–250. (b) Kent, U. M.; Fleming, P. J. Purified Cytochrome b_{561} Catalyzes Transmembrane Electron Transfer for Dopamine β-Hydroxylase and Peptidyl Glycine α-Amidating Monooxygenase Activities in Reconstituted Systems. *J. Biol. Chem.* **1987**, *262*, 8174–8178.

(10) Landymore-Lim, A. E. N.; Bradbury, A. F.; Smyth, D. G. The Amidating Enzyme in Pituitary Will Accept a Peptide with C-Terminal D-Alanine as Substrate. *Biochem. Biophys. Res. Commun.* **1983**, *117*, 289–293.

(11) (a) Tamburini, P. P.; Jones, B. N.; Consalvo, A. P.; Young, S. D.; Lovato, S. J.; Gilligan, J. P.; Wennogle, L. P.; Erion, M.; Jeng, A. Y. Structure-Activity Relationships for Glycine-Extended Peptides and the α-Amidating Enzyme Derived from Medullary Thyroid CA-77 Cells. *Arch. Biochem. Biophys.* **1988**, *267*, 623–631. (b) Mehta, N. M.; Gilligan, J. P.; Jones, B. N.; Bertelsen, A. H.; Roos, B. A.; Birnbaum, R. S. Purification of a Peptidylglycine α-Amidating Enzyme from Transplantable Rat Medullary Thyroid Carcinomas. *Arch. Biochem. Biophys.* **1988**, *261*, 44–54. (c) Dickinson, C. J.; Daugherty, D.; Guo, Y. J.; Stadler, B.; Finniss, S.; Yamada, T. Substrate Specificity of the Gastrin-amidating Enzyme. *J. Biol. Chem.* **1993**, *268*, 15929–15934.

(12) (a) Eipper, B. A.; Myers, A. C.; Mains, R. E. Peptidyl-Glycine α-Amidation Activity in Tissues and Serum of the Adult Rat. *Endocrinology* **1985**, *116*, 2497–2504. (b) Vaeroy, H.; Nyberg, F.; Franzen, H.; Terenius, L. Characterization of a Substance P-Gly Amidating Enzyme in Human Cerebrospinal Fluid. *Biochem. Biophys. Res. Commun.* **1987**, *148*, 24–30. (c) Wand, G. S.; Ney, R. L.; Baylin, S.; Eipper, B.; Mains, R. E. Characterization of a Peptide α-Amidation Activity in Human Plasma and Tissues. *Metabolism* **1985**, *34*, 1044–1052.

(13) (a) Gilligan, J. P.; Lovato, S. J.; Erion, M. D.; Jeng, A. Y. Modulation of Carrageenan-Induced Hind Paw Edema by Substance P. *Inflammation* **1994**, *18*, 285–292. (b) Tissot, M.; Pradelles, P.; Giroud, J. P. Substance P-Like Levels in Inflammatory Exudates. *Inflammation* **1988**, *12*, 25–35. (c) Colpaert, F. C.; Donnerer, J.; Lembeck, F. Effects of Capsaicin on Inflammation and on the Substance P Content of Nervous Tissues in Rats with Adjuvant Arthritis. *Life Sci.* **1983**, *32*, 1827–1834. (d) Helme, R. D.; Andrews, P. V.; Watson, B. A. Neurogenic Inflammation Caused by Wool Fabric in the Rat: Possible Mediation by Substance P. *Neurosci. Lett.* **1986**, *66*, 333–337. (e) Goetzl, E. J.; Chernov, T.; Renold, F. Neuropeptide Regulation of the Expression of Immediate Hypersensitivity. *J. Immunol.* **1985**, *135*, 802s–805s. (f) Perianin, A.; Snyderman, R.; Malfroy, B. Substance P Primes Human Neutrophil Activation: A Mechanism for Neurological Regulation of Inflammation. *Biochem. Biophys. Res. Commun.* **1989**, *161*, 520–524.

(14) Lotz, M.; Vaughan, J. H.; Carson, D. A. Effect of Neuropeptides on Production of Inflammatory Cytokines by Human Monocytes. *Science* **1988**, *241*, 1218–1221.

(15) Oku, B.; Satoh, M.; Takagi, H. Release of substance P from the spinal dorsal horn is enhanced in polyarthritic rats. *Neurosci. Lett.* **1987**, *74*, 315–319.

(16) Levine, J. D.; Clark, R.; Devor, M.; Helms, C.; Moskowitz, M. A.; Basbaum, A. I. Intraneuronal Substance P Contributes to the Severity of Experimental Arthritis. *Science* **1984**, *226*, 547–549.

(17) Lembeck, F.; Donnerer, J.; Tsuchiya, M.; Nagahisa, A. The non-peptide tachykinin antagonist, CP-96,345, is a potent inhibitor of neurogenic inflammation. *Br. J. Pharmacol.* **1992**, *105*, 527–530.

(18) Hilsted, L.; Rehfeld, J. F.; Schwartz, T. W. Impaired α-Carboxyamidation of gastrin in vitamin C-deficient Guinea Pigs. *FEBS Lett.* **1986**, *196*, 151–154.

(19) (a) Mains, R. E.; Park, L. P.; Eipper, B. A. Inhibition of Peptide Amidation by Disulfiram and Diethyldithiocarbamate. *J. Biol. Chem.* **1986**, *261*, 11938–11941. (b) Marchand, J. E.; Hershman, K.; Kumar, M. S. A.; Thompson, M. L.; Kream, R. M. Disulfiram Administration Affects Substance P-like Immunoreactive and Monoaminergic Neural Systems in Rodent Brain. *J. Biol. Chem.* **1990**, *265*, 264–273.

(20) Kizer, J. S.; Bateman, R. C., Jr.; Miller, C. R.; Humm, J.; Busby, W. H., Jr.; Youngblood, W. W. Purification and Characterization of a Peptidyl Glycine Monooxygenase from Porcine Pituitary. *Endocrinology* **1986**, *118*, 2262–2267.

(21) Katopodis, A. G.; May, S. W. Novel Substrates and Inhibitors of Peptidylglycine α-Amidating Monooxygenase. *Biochemistry* **1990**, *29*, 4541–4548.

(22) Zabriskie, T. M.; Cheng, H.; Vederas, J. C. Mechanism-Based Inactivation of Peptidylglycine α-Hydroxylating Monooxygenase (PHM) by a Substrate Analogue, D-Phenylalanyl-L-Phenylalanyl-D-Vinylglycine: Inhibition of Formation of Peptide C-Terminal Amides. *J. Am. Chem. Soc.* **1992**, *114*, 2270–2272.

(23) Bos, L. B.; Arens, J. F. Chemistry of acetylenic ethers. LXIII. The synthesis of α,β-unsaturated thiolo esters from carbonyl compounds, acetylenic ethers and boron trifluoride. *Recl. Trav. Chim. Pays-Bas* **1963**, *82*, 157–171.

(24) Ellman, G. L. A colorimetric method for determining low concentrations of mercaptans. *Arch. Biochem. Biophys.* **1958**, *74*, 443–450.

(25) Walsh, C. *Enzymatic Reaction Mechanisms*; Freeman: San Francisco, CA, 1979; pp 458–463.

(26) Hertzberg, R. P.; Dervan, P. B. Cleavage of DNA with Methidiumpropyl-EDTA-Iron(II): Reaction Condition and Product Analyses. *Biochemistry* **1984**, *23*, 3934–3945.

(27) Byers, L. D. Binding of Reactive Intermediate Analogs to Enzymes. *J. Theor. Biol.* **1978**, *74*, 501–512.

(28) Erion, M. D.; Walsh, C. T. 1-Aminocyclopropanephosphonate: Time-Dependent Inactivation of 1-Aminocyclopropanecarboxylate Deaminase and Bacillus stearothermophilus Alanine Racemase by Slow Dissociation Behavior. *Biochemistry* **1987**, *26*, 3417–3425.

(29) Wong, M.; Jeng, A. Y. Posttranslational Modification of Glycine-Extended Substance P by an α-Amidating Enzyme in Cultured Sensory Neurons of Dorsal Root Ganglia. *J. Neurosci. Res.* **1994**, *37*, 97–102.

(30) Jones, B. N.; Tamburini, P. P.; Consalvo, A. P.; Young, S. D.; Lovato, S. J.; Gilligan, J. P.; Jeng, A. Y.; Wennogle, L. P. A Fluorometric Assay for Peptidyl α-Amidation Activity Using High-Performance Liquid Chromatography. *Anal. Biochem.* **1988**, *168*, 272–279.

(31) Jeng, A. Y.; Wong, M. Lovato, S. J.; Erion, M. D.; Gilligan, J. P. A Radioimmunoassay for Measuring α-Amidating Enzyme Activity. *Anal. Biochem.* **1990**, *185*, 213–219.

(32) Inhibition of peptide amidation was determined using the bifunctional enzyme isolated from CA-77 cells.[30] Since the bifunctional enzyme contains the catalytic domains for both the monooxygenase and the lyase, these results alone do not prove unambiguously that inhibition occurs through inhibition of the monooxygenase activity. The fact that the intermediate hydroxyglycine[7] was not detected, however, strongly suggests that the observed inhibition is not through inhibition of the lyase activity. Furthermore, the magnitude of the enhanced potency exhibited by the thiol-containing inhibitors is consistent with a thiolate–metal interaction and not a relatively weak thiolate hydrogen bond or hydrophobic interaction.