Arylacetamide-Derived Fluorescent Probes: Synthesis, Biological Evaluation, and Direct Fluorescent Labeling of κ Opioid Receptors in Mouse Microglial Cells

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Received November 2, 1995[⊗]

Fluorescein isothiocyanate isomer I (FITC-I) conjugates of 2-(3,4-dichlorophenyl)-*N*-methyl-*N*-[1-(3- or 4-aminophenyl)-2-(1-pyrrolidinyl)ethyl]acetamide (**10** and **14**) were prepared either without or with an intervening mono-, di-, or tetraglycyl linker. The 3-substituted fluorescent probes (**2**−**5**) were found to retain potent agonist activity in smooth muscle preparations as well as high κ receptor affinity and selectivity in receptor binding assays. The 4-substituted series (**6**−**9**) were substantially less potent than the corresponding 3-substituted compounds. Flow cytometric analysis demonstrated high levels of direct κ-specific staining of mouse microglial cells by the fluorescent probe **5** containing a tetraglycyl linker, as indicated by a 41% decrease in percent cells positively labeled and a 61% decrease in mean fluorescence intensity in the presence of the κ-selective antagonist, norbinaltorphimine (norBNI). In similar studies, the probe **2** without a linker exhibited only nonspecific binding. This is the first report of direct, selective staining of κ opioid receptors by a fluorescent nonpeptide opioid ligand. The results of the present study illustrate the importance of introducing hydrophilic linkers to reduce nonspecific binding of fluorescent probes for opioid receptors.

Among the three major types of opioid receptors (μ, κ, and δ),[1] κ receptors have attracted special attention because they mediate analgesia with minimal dependence and respiratory depression.[2] In addition to analgesia, other potential applications of κ-selective agonists have been reported.[3] Because κ receptors represent important therapeutic targets, selective ligands can serve as important pharmacologic tools in order to gain a better understanding of their mechanisms of action. Moreover, the need for additional selective ligands has been heightened by the recent cloning of κ opioid receptors.[4−9]

Fluorescent ligands can be used to label receptors in cell cultures or tissue preparations. The fluorescent labeling can be studied by fluorescence microscopy,[10,11] confocal laser microscopy,[12] or flow cytometry.[13] Furthermore, the high sensitivity of fluorescent labeling may also allow the detection of receptors in heterogeneous populations of immune cells where radioligand binding studies have failed to do so.[13] Although immunofluorescent methods have been useful in identifying opioid receptors,[14] pharmacologically active fluorescent opioid ligands would have a potential advantage over biologically inactive immunofluorescent probes because they can in principle be used to correlate the staining of opioid receptors with function. In spite of reports on the synthesis of fluorescent opioid ligands,[15−19] no successful applications of such ligands for the *direct* visualization of opioid receptors have been published. Recently, a fluorescein-conjugated arylacetamide ligand was successfully employed in the *indirect* immunofluorescent detection of κ opioid receptors in the mouse immune system,[13] but direct staining with this resulted only in nonspecific binding. Here we report on the development of κ-selective fluorescent probes **2**−**9** that are derived from the potent κ-selective agonist, (*S*)-2-(3,4-dichlorophenyl)-*N*-methyl-*N*-[1-phenyl-2-(1-pyrrolidinyl)ethyl]acetamide (**1**, ICI199,441).[20] Members of this series retain κ agonist activity and selectivity, and one of its members (**5**) has been found to label κ opioid receptors. This is the first report of *direct* κ receptor selective staining by a nonpeptide fluorescent opioid ligand.

Design Rationale and Chemistry

Because structure−activity relationship studies have indicated that the central phenyl group of **1** can tolerate substitutions with minimal effect on affinity and agonist potency,[20] we chose to attach a fluorescein moiety to the meta and para positions. The required *m*- and *p*-NH_2 intermediates, **10** and **14**, were prepared as previously reported.[21] Intermediate **10** was obtained in enantiomerically pure form, while **14** was prepared as the racemate.

Since high levels of nonspecific staining were observed in our laboratory for δ-selective fluorescent opioid receptor ligands,[22] it was postulated that the lipophilicity of the fluorescent probes may lead to high levels of nonspecific binding as a result of receptor internalization and solubility in membranes. In order to minimize this possibility, the polarity of the ligands was modulated through the insertion of a glycyl or oligoglycyl linker between the fluorophore and the pharmacophore. In addition, we thought that longer linkers might also minimize unfavorable interactions of the fluorophore with the receptor.

Because fluorescein is unstable in light and difficult to purify, conjugation to fluorescein isothiocyanate (FITC-I) was performed in the last step of the prepara-

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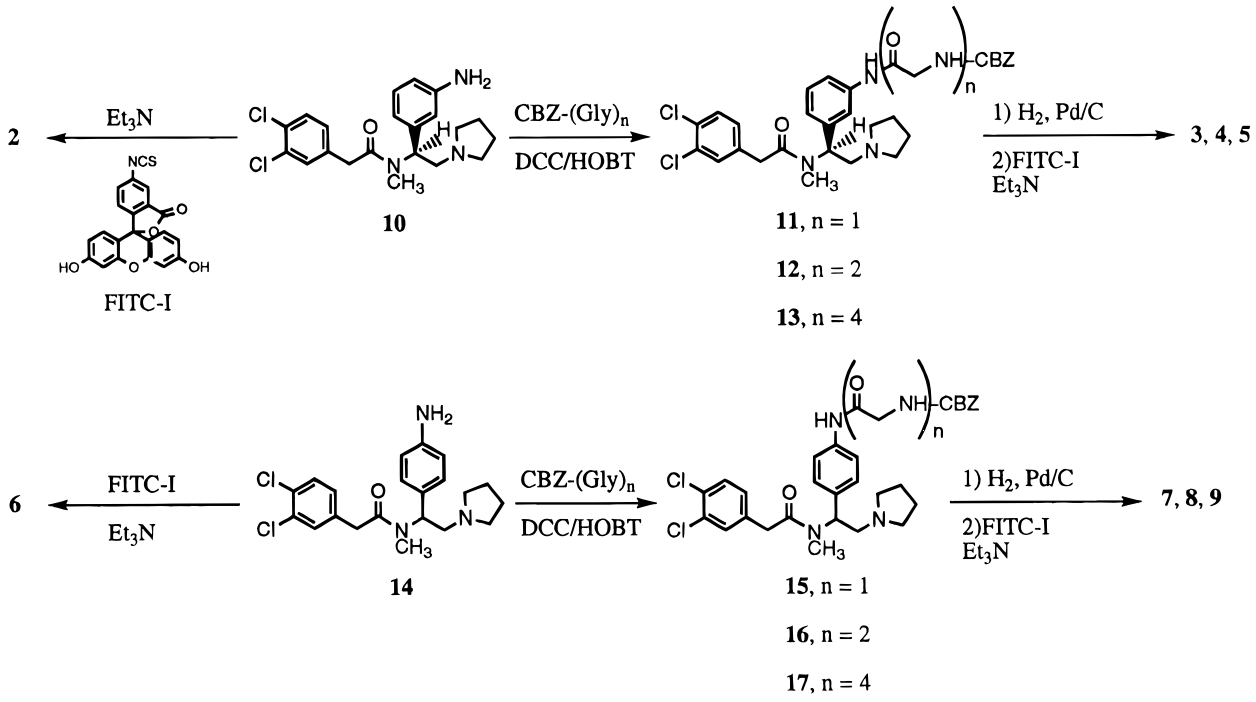
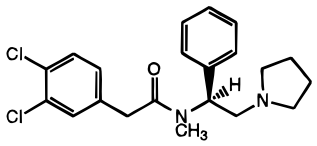
[⊗] Abstract published in *Advance ACS Abstracts,* March 1, 1996.

0022-2623/96/1839-1729$12.00/0

Scheme 1



tion of the fluorescent probes (Scheme 1). The glycyl and oligoglycyl linkers were first attached by DCC/HOBT coupling of *N*-CBZ-$(Gly)_n$[23,24] (n = 1, 2, and 4) to **10** and **14**. The resulting conjugates **11**−**13** and **15**−**17** and the parent amines **10** and **14** were deprotected by catalytic hydrogenolysis and coupled to FITC-I in the presence of triethylamine to afford the meta- or para-substituted fluorescent probes **2**−**9**.



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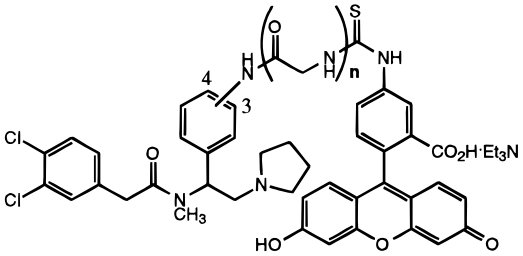

compd	isomer	position	n
2	S	3	0
3	S	3	1
4	S	3	2
5	S	3	4
6	R,S	4	0
7	R,S	4	1
8	R,S	4	2
9	R,S	4	4

Biological Results

Smooth Muscle Assays. Target compounds were tested on the electrically stimulated guinea pig longitudinal ileal muscle[25] (GPI) and mouse vas deferens[26] (MVD) preparations as described previously.[27] In wash-resistant activity studies, the agonist-treated smooth muscle preparation was incubated with naltrexone (500 nM) for 10 min before the tissue was washed with buffer to remove the naltrexone (NTX).

In GPI the meta series of fluorescent probes (**2**−**5**) generally were highly potent opioid agonists and comparable to the parent amine **10** and the unsubstituted ligand **1** (Table 1). The ligand with the diglycyl linker (**4**) exhibited the lowest potency in this series. On the other hand, the para-substituted series **6**−**9** were substantially less potent by factors ranging from 14 to 1100. Both the meta and para series were generally less potent agonists in the MVD relative to the GPI.

Fluorescent probes **2**−**5** were tested for wash-resistant agonist activity by washing the treated preparation with buffer or naltrexone (Table 2). The agonist activity of unsubstituted compounds **1** and fluorescent probes **2** and **4** in the GPI preparation was only partially reversed by extensive washing with buffer, but the activity of **3** and **5** could be fully reversed by washing with naltrexone, followed by washing with buffer to remove the NTX. In the MVD preparation, the agonist activities of **2**−**5** could be removed by treatment with buffer alone.

Binding Studies. The opioid receptor binding affinities and selectivities of **3** and **5** were determined by competition with radioligands in guinea pig brain membranes by employing a modification of the method of Werling et al.[28] Binding to κ receptors was evaluated using 1 nM [^{3}H]-(5α,7α,8β)-(−)-*N*-methyl-*N*-1-pyrrolidinyl-1-oxaspiro[4.5]dec-8-ylbenzeneacetamide[29] ([^{3}H]U69,593), to μ receptors with 2 nM [^{3}H][D-Ala^2,MePhe^4,Gly-ol^6]enkephalin[30] ([^{3}H]DAMGO), to δ_1 receptors with 5 nM [^{3}H][D-Pen^2,D-Pen^5]enkephalin[31] ([^{3}H]DPDPE), and to δ_2 receptors with 2 nM [^{3}H]Tyr-

Table 1. Agonist Potencies in Smooth Muscle Preparations

compd	IC_{50} (nM) ± SEM[a]	
	GPI	MVD
1	0.27 ± 0.09[b]	2.5 ± 1.7[b]
2	0.37 ± 0.18	1.2 ± 0.19
3	0.17 ± 0.03	1.2 ± 0.34
4	1.2 ± 0.3	1.5 ± 0.12
5	0.15 ± 0.06	6.5 ± 1.3
6	5.2 ± 0.88	12.6 ± 2.1
7	62 ± 19	102 ± 69
8	48 ± 14	170 ± 15
9	166 ± 45	421 ± 110
10	0.17 ± 0.08	0.12 ± 0.01
14	0.65 ± 0.20	4.5 ± 1.4

[a] Values are arithmetic means of at least three experiments. [b] Reference 21.

Table 2. Percent Recovery of Twitch Height in the GPI and MVD after Wash Treatment[a]

compd	GPI		MVD
	buffer[b]	NTX[c]	buffer[b]
1	20	32	
2	21	61	100
3	39	97	79
4	34	37	100
5	44	131	100

[a] $n = 1$. [b] Percent recovery of twitch height after 30−40 washes with buffer alone. [c] Percent recovery after treatment with 500 nM of NTX and removal of NTX by 40 washes with buffer.

D-Ser-Gly-Phe-Leu-Thr ([^{3}H]DSLET).[32] As shown in Table 3, both **3** and **5** bind the κ opioid receptor with high affinity and selectivity.

Fluorescent Labeling Studies

Direct fluorescent labeling of mouse microglial cells by the fluorescent probes **2** and **5** was analyzed by flow cytometry using a Coulter XL100 instrument (Coulter, Miami, FL) with an excitation wavelength of 488 nm and an emission wavelength of 525 nm. Microglial cells have been shown to constitutively express the κ opioid receptor[33] and were prepared as previously reported.[34] Because the microglial cells are believed to be functionally equivalent to monocytes or tissue macrophages of the somatic immune system, the conditions of the fluorescence studies are as previously reported in studies with mouse immune cells.[13] Specificity of the fluorescent labeling was determined by including the κ-selective antagonist, norbinaltorphimine (norBNI),[35] or the μ-selective antagonist, β-funaltrexamine (β-FNA),[36] in the fluorescent labeling studies. Mean fluorescence intensity was determined from the percent positively labeled cells. The background fluorescence intensity (1.53) was determined from cells not treated with any fluorescent probe. NorBNI was used at a concentration of 1 mM because the 500 μM to 2 mM range was previously shown to be optimal in similar studies with mouse immune cells.[13] In each sample, about 10 000 cells were analyzed. Fluorescent probes **2** and **5** were studied to determine whether the tetraglycyl linker in **5** had increased the polarity sufficiently to allow observation of κ-selective fluorescent labeling.

First, the concentration-dependence studies indicated that both **2** and **5** are nearly equipotent in their ability to label the microglial cells (Figure 1). However, when norBNI (1 mM) was included, the percent of cells that were positively labeled by **5** (50 μM) was significantly decreased by 41% (Table 4). Mean fluorescence intensity was also reduced by 61%, as determined by the following formula: [(total fluorescence − background fluorescence) − (fluorescence in the presence of norBNI − background fluorescence)]/(total fluorescence − background fluorescence). The addition of β-FNA (500 μM) did not significantly affect the percent of cells positively labeled or mean fluorescence intensity by **2** (50 μM) and **5** (50 μM) (Table 4).

Table 3. Opioid Receptor Binding Selectivities in Guinea Pig Brain

compd	K_i (nM)[a]				K_i selectivity ratio		
	κ	μ	δ_1	δ_2	μ/κ	δ_1/κ	δ_2/κ
3	0.31	286	18.0	43.8	922	58	141
5	0.91	631	96.2	264	693	105	290

[a] Values are geometric means of three experiments.

Discussion

The attachment of fluoroscein, through a glycyl or oligoglycyl linker, to a κ-selective pharmacophore has afforded a series of fluorescent ligands. Members of the meta-substituted series (**2**−**5**) generally retained κ agonist potency in smooth muscle preparations, whereas the corresponding members of the para series (**6**−**9**) were considerably less potent.

Since a high degree of nonspecific binding to cellular components would render the ligands ineffective as fluorescent probes, we investigated the facility with which the agonist activity of **2**−**5** could be removed from the GPI. This was evaluated by measuring the residual agonist effect after repeated washing of the preparation with buffer alone or with naltrexone. The finding that the agonist effect of **3** and **5** on the GPI could be removed upon washing, while the remaining members of the meta series exhibited wash-resistant agonism, led us to investigate these compounds further in an effort to develop them as fluorescent probes. This was based on the assumption that wash-resistant agonism may in part be due to the partitioning of the ligand into the cell membrane and to internalization.

After the κ selectivity of **3** and **5** was verified through binding studies using guinea pig membranes, the most hydrophilic (**5**) and the most hydrobic (**2**) members of the meta series were evaluated by flow cytometry on microglial cells which have been reported[34] to contain κ receptors. The fluorescent labeling of microglial cells by **5** was found to be κ-selective, as indicated by significant decreases in the fraction of cells stained and in the staining intensity upon exposure to the κ antagonist, norBNI. In contrast to **5**, the staining of microglial cells by compound **2** was not changed by norBNI treatment. This is consistent with a previous report[13] on the staining of mouse immune cells by the racemic form of **2**.

The results of the flow cytometry studies clearly demonstrate the advantage of the tetraglycyl linker in **5**. This hydrophilic linker most likely reduces nonspecific staining by limiting internalization and membrane partitioning of the fluorescent probe. In contrast, the considerably more hydrophobic ligand **2** may more easily penetrate membranes and the cell so that the total binding far exceeds specific binding.

In conclusion, this study presents the first report of *direct* selective staining of κ receptors by a fluorescent nonpeptide opioid ligand (**5**). The reduction of non-

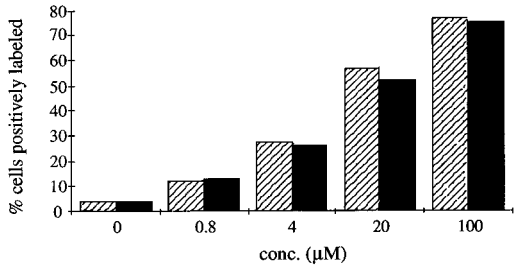

Figure 1. Concentration dependence of the percent of cells that are positively labeled by **2** or **5**. Microglial cells expressing high autofluorescence (about 4−8% of total cell population) were eliminated by gating. Data are mean values of approximately 10 000 cells/group and are representative of two separate experiments.

Table 4. Microglial Cell κ Opioid Receptors Demonstrated by Immunofluorescence[a]

compd	treatment	% cells positively labeled	mean fluorescence intensity
5	medium	57.5	2.63
	+ NorBNI	33	1.04
	+ β-FNA	62	3.03
2	medium	69.6	3.53
	+ NorBNI	68.9	3.52
	+ β-FNA	71.4	3.88

[a] Microglia cells (2×10^4) were treated with 50 μM of **5** or **2** in the absence (medium) or presence of norBNI (1 mM) or β-FNA (500 μM) and analyzed by flow cytometry. Values are expressed both as the percent of cells positively labeled and mean fluorescence intensity. The mean background fluorescence intensity (1.53), determined from cells not treated with any fluorescent probe, was subtracted. Microglial cells having high autofluorescence (about 4−8% of total cell population) were eliminated by gating.

specific staining by an oligoglycyl linker that joins the pharmacophore to the fluorophore suggests this may be used as a general strategy to diminish internalization and nonspecific binding of nonpeptide ligands. Fluorescent probe **5** may prove to be a useful tool in pharmacologic and biochemical studies.

Experimental Section

All NMR spectra were recorded on a GE 300 MHz spectrometer or a Varian-500 MHz spectrometer at room temperature. Optical rotations were measured using a Rudolph Research Autopol III automatic polarimeter. Melting points were determined using a Thomas Hoover capillary melting point apparatus and are uncorrected. Elemental analyses were conducted by M-H-W Laboratories in Phoenix, AZ. Mass spectra were recorded by the Chemistry Mass Spec Labs at the University of Minnesota's Chemistry Department. Fluorescein isothiocyanate isomer I was purchased from Aldrich Chemical Co. *N*-CBZ-Gly and *N*-CBZ-GlyGly were purchased from Sigma Chemical Co.

2-(3,4-Dichlorophenyl)-*N*-methyl-*N*-{(1*S*)-1-[*N*-(*N*-CBZ-glycinamido)-3-aminophenyl]-2-(1-pyrrolidinyl)ethyl}acetamide (11). To an ice-cold solution of *N*-CBZ-Gly (0.2449 g, 1.17 mmol) in dry CH_2Cl_2 under N_2 was added HOBT (0.1599 g, 1.18 mmol), followed by a CH_2Cl_2 solution of DCC (0.2467 g, 1.20 mmol). After 1 h at 0 °C, a CH_2Cl_2 solution of **10**[21] (0.3174 g, 0.781 mmol) was added, and the reaction mixture was stirred at 25 °C under N_2 for 4 days before it was filtered through Celite. The residue after evaporation of the filtrate was dissolved in EtOAc and washed successively with saturated $NaHCO_3$ and water, and the organic fraction was dried ($MgSO_4$), filtered, and evaporated. Flash-column chromatography with $CHCl_3$:2% NH_3:5% MeOH and crystallization afforded 0.3178 g (68%) of **11**·HCl: mp 220 °C; $[\alpha]^{25}_D$ +113.6° (c = 0.28, MeOH); ^{1}H NMR (DMSO-d_6) δ 1.90−1.95 (m, 4H, CH_2CH_2), 2.76 (s, 3H, NCH_3), 3−4.1 (complex, 10H, 5 CH_2), 5.01 (s, 2H, $ArCH_2O$), 6.07 (m, 1H, CH), 6.94 (m, 1H, amide H), 7.25−7.56 (complex, 12H, aromatic H), 10.1 (s, 1H, amide H); MS (FAB) m/z 597.2. Anal. ($C_{31}H_{34}N_4O_4Cl_2$·HCl) C, H, N, Cl.

2-(3,4-Dichlorophenyl)-*N*-methyl-*N*-{(1*S*)-1-[*N*-(*N*-CBZ-glycylglycinamido)-3-aminophenyl]-2-(1-pyrrolidinyl)ethyl}acetamide (12). Compound **12** was prepared from *N*-CBZ-GlyGly (0.3056 g, 1.15 mmol), HOBT (0.1565 g, 1.16 mmol), DCC (0.2416 g, 1.17 mmol), and **10**[21] (0.3108 g, 0.76 mmol) in dry CH_2Cl_2 (28 mL). The procedure and reaction conditions are similar to those employed for the preparation of **11**. After 18 h, the mixture was filtered through Celite, and the filtrate was washed with saturated $NaHCO_3$ before it was dried (Na_2SO_4), filtered, and evaporated. Column chromatography with $CHCl_3$:2% NH_3:5% MeOH followed by evaporation of a 2-propanol solution of the HCl salt yielded **12**·HCl (0.3308 g, 63%): mp 224 °C; $[\alpha]^{25}_D$ +105.9° (c = 0.31, MeOH); ^{1}H NMR (DMSO-d_6) δ 1.94 (br s, 4H, CH_2CH_2), 2.77 (s, 3H, NCH_3), 3.06−4.07 (complex, 12H, 6 CH_2), 5.0 (s, 2H, $ArCH_2O$), 6.07 (m, 1H, CH), 6.95 (d, J = 7.8 Hz, 1H, amide H), 7.26−7.58 (complex, 12H, aromatic), 8.23 (m, 1H, amide H), 10.02 (s, 1H, amide H); MS (FAB) m/z 654.2. Anal. ($C_{33}H_{37}N_5O_5Cl_2$·HCl) C, H, N, Cl.

2-(3,4-Dichlorophenyl)-*N*-methyl-*N*-{(1*S*)-1-[*N*-(*N*-CBZ-glycylglycylglycylglycinamido)-3-aminophenyl]-2-(1-pyrrolidinyl)ethyl}acetamide (13). A solution of DCC (0.1273 g, 0.62 mmol) in dry EtOAc (12 mL) was added to an ice-cold solution of *N*-CBZ-GlyGlyGlyGly (0.2204 g, 0.58 mmol) and HOBT (0.0800 g, 0.59 mmol) in dry DMF (50 mL), and the mixture was stirred in ice bath under N_2 for 1 h. A solution of **10**[21] in dry EtOAc (10 mL) was added, and the mixture was stirred at 25 °C under N_2 for 21 h before it was evaporated to dryness. The residue was then suspended in hot EtOAc and filtered, and the filtrate was washed with saturated $NaHCO_3$ before it was dried (Na_2SO_4), filtered, and evaporated. Some remaining dicyclohexylurea (DCU) was removed by flash-column chromatography with $CHCl_3$:2% NH_3:10% MeOH, and evaporation of an iPrOH solution of the HCl salt yielded **13**·HCl (91.7 mg, 29%): mp 210 °C; $[\alpha]^{25}_D$ +88.42° (c = 0.19, MeOH); ^{1}H NMR (DMSO-d_6) δ 1.95 (br s, 4H, CH_2CH_2), 2.77 (br s, 3H, NCH_3), 3.03−4.04 (broad, 16H, 8 CH_2), 5.0 (br s, 2H, $ArCH_2O$), 6.07 (m, 1H, CH), 6.95 (m, 1H, amide H), 7.31−7.52 (broad, 12H, aromatic), 8.24 (broad, 3H, amide H), 9.94 (br s, 1H, amide H); MS (FAB) m/z 768.2. Anal. ($C_{37}H_{43}N_7O_7Cl_2$·HCl·H_2O).

2-(3,4-Dichlorophenyl)-*N*-methyl-*N*-{(1*R*,*S*)-1-[*N*-(*N*-CBZ-glycinamido)-4-aminophenyl]-2-(1-pyrrolidinyl)ethyl}acetamide (15). Compound **15** was prepared from *N*-CBZ-Gly (0.3167 g, 1.514 mmol), HOBT (0.2087 g, 1.544 mmol), DCC (0.3222 g, 1.562 mmol), and **14**[21] (0.3996 g, 0.9834 mmol) in dry CH_2Cl_2 (26 mL). The procedure and reaction conditions are similar to those employed for the preparation of **11**. After 15 h, H_2O was added to destroy excess active esters. The suspension was then filtered through Celite, and the DCU was washed with a little CH_2Cl_2. The filtrate was then washed with saturated $NaHCO_3$ before it was dried (Na_2SO_4), filtered through Celite, and evaporated. Upon concentration, more DCU precipitated and was removed by filtration through a cotton-plugged pipet. The oil remaining was purified by flash-column chromatography using $CHCl_3$:2% NH_3:5% MeOH before it was converted to the HCl salt with Et_2O·HCl and crystallized from MeOH:Et_2O to yield **15**·HCl (0.4636 g, 72%): mp 145 °C dec; ^{1}H NMR (DMSO-d_6) δ 1.87−1.95 (m, 4H, CH_2CH_2), 2.74 (s, 3H, NCH_3), 3.0−4.1 (complex, 10 H, 5 CH_2), 5.01 (s, 2H, benzylic), 6.04 (m, 1H, CH), 7.15−7.58 (complex, 12 H, aromatic); MS (FAB) m/z 597.1. Anal. ($C_{31}H_{34}N_4O_4Cl_2$·HCl) C, H, N, Cl.

2-(3,4-Dichlorophenyl)-*N*-methyl-*N*-{(1*R*,*S*)-1-[*N*-(*N*-CBZ-glycylglycinamido)-4-aminophenyl]-2-(1-pyrrolidinyl)ethyl}acetamide (16). To an ice-cold solution of **14**[21] (0.3043 g, 0.7489 mmol) in dry CH_2Cl_2 (20 mL) were sequentially added *N*-CBZ-GlyGly (0.2993 g, 1.124 mmol), HOBT (0.1546 g, 1.144 mmol), DCC (0.2389 g, 1.158 mmol), and dry CH_2Cl_2 (4 mL) with stirring under N_2. The mixture was allowed to

slowly warm to 25 °C overnight. After 64 h at 25 °C, H_2O was added to destroy excess active esters before the suspension was filtered through Celite, and the DCU was washed with a little CH_2Cl_2. The filtrate was diluted with $CHCl_3$ to 200 mL and washed with saturated $NaHCO_3$ before it was dried (Na_2SO_4), filtered through Celite, and evaporated. More DCU precipitated and was removed by filtration through a cotton-plugged pipet to yield an oil which was flash-column chromatographed using $CHCl_3$:2% NH_3:5% MeOH and then purified on 2 mm chromatotron plates using $CHCl_3$:2% NH_3:3% MeOH. The product was converted to the HCl salt with Et_2O·HCl and solidified by addition and evaporation of iPrOH to yield **16**·HCl (0.1590 g, 31%): mp 137 °C dec; ^{1}H NMR (DMSO-d_6) δ 1.94 (m, 4H, CH_2CH_2), 2.74 (s, 3H, NCH_3), 3.1−4.0 (complex, 12H, 6 CH_2), 5.01 (s, 2H, benzylic), 6.05 (m, 1H, CH), 7.17−7.56 (complex, 12H, aromatic); MS (FAB) *m/z* 654.3. Anal. ($C_{33}H_{37}N_5O_5Cl_2$·HCl) C, H, N, Cl.

2-(3,4-Dichlorophenyl)-*N*-methyl-*N*-{(1*R*,*S*)-1-[*N*-(*N*-CBZ-glycylglycylglycylglycinamido)-4-aminophenyl]-2-(1-pyrrolidinyl)ethyl}acetamide (17). To an ice-cold mixture of **14**[21] (0.3013 g, 0.7415 mmol) and *N*-CBZ-GlyGlyGlyGly (0.5612 g, 1.475 mmol) in anhydrous DMF (100 mL) were added HOBT (0.2040 g, 1.510 mmol) and DCC (0.3139 g, 1.521 mmol) with stirring under N_2. The mixture was allowed to slowly warm to 25 °C overnight. After 21 h, the reaction was incomplete, and *N*-CBZ-GlyGlyGlyGly (0.2808 g, 0.7383 mmol), HOBT (0.1020 g, 0.7548 mmol), and DCC (0.1570 g, 0.7609 mmol) were added with cooling in ice−H_2O. The mixture was stirred at 0 °C for 1 h and at 25 °C for 15 h before H_2O was added to destroy excess active esters. After evaporation of DMF, the residue was partitioned between 0.5 N HCl and $CHCl_3$ (200 mL) before the aqueous fraction was basified with NaOH(s) and extracted with EtOAc (400 mL), which was dried (Na_2SO_4), filtered through Celite, and evaporated. The residue was purified by flash-column chromatography using $CHCl_3$: 2% NH_3:10% MeOH and converted to the HCl salt with Et_2O·HCl to yield **17**·HCl (0.0699 g, 12%): mp 144 °C dec; ^{1}H NMR (DMSO-d_6) δ 1.94 (br s, 4H, CH_2CH_2), 2.74 (s, 3H, -NCH_3), 3.0−4.0 (complex, 16H, 8 CH_2), 5.00 (s, 2H, benzylic), 6.05 (m, 1H, CH), 7.15−7.61 (complex, 12H, aromatic); MS (FAB) *m/z* 768.3. Anal. ($C_{37}H_{43}N_7O_7Cl_2$·HCl) C, H, N.

2-(3,4-Dichlorophenyl)-*N*-methyl-*N*-{(1*S*)-1-[*N*-(fluoresceinyl-5-thioureido)-3-aminophenyl]-2-(1-pyrrolidinyl)-ethyl}acetamide (2). With stirring at 25 °C under N_2, a solution of **10**[21] (0.1992 g, 0.4902 mmol) in absolute ethanol (6 mL) and Et_3N (2 mL) was added to a solution of fluorescein isothiocyanate isomer I (FITC-I, 0.1526 g, 0.3919 mmol) in freshly distilled THF (3 mL) and absolute ethanol (1 mL). After stirring for 16 h in the dark, the solvent was evaporated, and Et_3N was removed azeotropically with dry CH_3CN. The solid was then washed thoroughly with absolute EtOH and MeOH before it was purified by 0.25 mm reverse-phase-18 TLC plates using 100% MeOH as the solvent to yield **2**·Et_3N (80.1 mg, 23%): mp 185 °C dec; $[\alpha]^{25}_D = +118°$ (c = 0.1, MeOH with 1 drop Et_3N); ^{1}H NMR (DMSO-d_6) δ 1.6 (br s, 4H, CH_2CH_2), 5.79−5.82 (m, 1H, CH); MS (FAB) *m/z* 795.1 (MH^+). Anal. ($C_{42}H_{36}N_4O_6SCl_2$·($C_2H_5)_3N$) C, H, N, Cl.

2-(3,4-Dichlorophenyl)-*N*-methyl-*N*-{(1*S*)-1-[*N*-(*N*-fluoresceinyl-5-thioureidoglycinamido)-3-aminophenyl]-2-(1-pyrrolidinyl)ethyl}acetamide (3). Intermediate **11**·HCl (0.3050 g, 0.511 mmol) was deprotected by hydrogenation with 48 mg of 10% Pd/C in MeOH (10 mL) under 10 psi for 65 h. To a solution of FITC-I (0.0808 g, 0.208 mmol) in dry THF (1 mL) and absolute ethanol (1 mL) was added a solution of the free base of deprotected **11** (0.0928 g, 0.200 mmol) in absolute ethanol (2 mL) and Et_3N (1 mL) with stirring at 25 °C under N_2. After stirring for 23 h in the dark, the reaction mixture was diluted with absolute ethanol and concentrated under reduced pressure. Once concentrated, dry CH_3CN was added to remove Et_3N azeotropically. The solution was then evaporated to dryness, and the residue was suspended in absolute ethanol and separated by filtration. The solid was washed thoroughly with absolute ethanol and dried to yield **3**·Et_3N (0.1228 g, 64%): mp 200 °C dec; $[\alpha]^{25}_D$ +71.6° (c = 0.11, MeOH with 2 drops Et_3N); ^{1}H NMR (DMSO-d_6) δ 1.655−1.977 (br m, 4H, CH_2CH_2), 2.720 (s, 3H, NCH_3), 5.785−6.176 (br m, 1H, CH); MS (FAB) *m/z* 852.1 (MH^+). Anal. ($C_{44}H_{39}N_5O_7SCl_2$·($C_2H_5)_3N$) C, H, N, Cl.

2-(3,4-Dichlorophenyl)-*N*-methyl-*N*-{(1*S*)-1-[*N*-(*N*-fluoresceinyl-5-thioureidoglycylglycinamido)-3-aminophenyl]-2-(1-pyrrolidinyl)ethyl}acetamide (4). Intermediate **12**·HCl (0.3170 g, 0.485 mmol) was deprotected by hydrogenation with 49 mg of 10% Pd/C in MeOH (14 mL) under 10 psi for 69 h. To a solution of FITC-I (0.0841g, 0.216 mmol) in dry THF (1 mL) was added a solution of the free base of deprotected **12** (0.1080 g, 0.2075 mmol) in absolute ethanol (2 mL) and Et_3N (1 mL) with stirring at 25 °C under N_2. After stirring for 24 h in the dark, the reaction mixture was filtered through a cotton plug in a pipet, diluted with absolute ethanol, and concentrated under reduced pressure. Additional absolute ethanol and dry CH_3CN were added, and the mixture was concentrated again. This was repeated twice before the solution was evaporated to dryness, and the residue was suspended in absolute ethanol and separated by filtration. The solid was washed thoroughly with absolute ethanol and dried to yield **4**·Et_3N (0.1540 g, 73%): mp 205 °C dec; $[\alpha]^{25}_D$ +66.7° (c = 0.15, MeOH with 1 drop Et_3N); ^{1}H NMR (DMSO-d_6) δ 1.62 (br s, 4H, CH_2CH_2), 2.68 (s, 3H, NCH_3), 5.81 (br m, 1H, CH); MS (FAB) *m/z* 909.3 (MH^+). Anal. ($C_{46}H_{42}N_6O_8SCl_2$·($C_2H_5)_3N$) C, H, N, Cl.

2-(3,4-Dichlorophenyl)-*N*-methyl-*N*-{(1*S*)-1-[*N*-(*N*-fluoresceinyl-5-thioureidoglycylglycylglycylglycinamido)-3-aminophenyl]-2-(1-pyrrolidinyl)ethyl}acetamide (5). Intermediate **13**·HCl (0.2037 g, 0.265 mmol) was deprotected by hydrogenation with 30 mg of 10% Pd/C in MeOH (16 mL) under 10 psi for 3 days. With stirring at 25 °C under Ar, FITC-I (0.0735 g, 0.189 mmol) was added to a mixture of the monohydrochloride salt of deprotected **13** (0.1209 g, 0.1802 mmol) and Et_3N (4 mL) in freshly distilled THF (6 mL) and absolute ethanol (4 mL). After stirring for 63 h in the dark, more fluorescein isothiocyanate isomer I (0.0699 g, 0.180 mmol) was added, and the reaction was complete (by reverse-phase TLC) after 28 h more of stirring. After evaporation of solvent and azeotropic removal of Et_3N with dry CH_3CN, the product was dissolved in MeOH:Et_3N (99:1) and purified on 0.25 mm reverse-phase-18 TLC plates using 100% MeOH as the solvent to yield **5**·Et_3N (112.9 mg, 55.7%): mp 205 °C dec; $[\alpha]^{25}_D = +66.7°$ (c = 0.12, MeOH with 1 drop Et_3N); ^{1}H NMR (DMSO-d_6) δ 1.5−1.7 (m, 4H, CH_2CH_2), 5.78−5.82 (m, 1H, CH); MS (FAB) *m/z* 1123.3 (MH^+). Anal. ($C_{50}H_{48}N_8O_{10}SCl_2$·($C_2H_5)_3N$) C, H, N, Cl.

2-(3,4-Dichlorophenyl)-*N*-methyl-*N*-{(1*R*,*S*)-1-[*N*-(fluoresceinyl-5-thioureido)-4-aminophenyl]-2-(1-pyrrolidinyl)-ethyl}acetamide (6). A mixture of **14**[21] (0.1088 g, 0.2678 mmol), FITC-I (0.1085 g, 0.2786 mmol), and Et_3N (2 mL) in anhydrous EtOH:THF (5 mL, 3:2) was stirred at 25 °C under N_2 for 17 h before it was evaporated in the dark, and Et_3N was azeotropically removed with dry CH_3CN to yield a solid that was suspended in absolute EtOH (4 mL) and collected by filtration. Washing successively with absolute EtOH (8 mL) and anhydrous ethyl ether yielded **6**·Et_3N (0.1441 g, 60%): mp 194 °C dec; ^{1}H NMR (DMSO-d_6) δ 1.63 (br s, 4H, CH_2CH_2), 2.72 (s, 3H, NCH_3), 2.40−4.0 (complex, 8H, 4 CH_2), 5.79 (m, 1H, CH); MS (FAB) *m/z* 795.3 (MH^+). Anal. ($C_{42}H_{36}N_4O_6SCl_2$·($C_2H_5)_3N$) C, H, N, Cl.

2-(3,4-Dichlorophenyl)-*N*-methyl-*N*-{(1*R*,*S*)-1-[*N*-(*N*-fluoresceinyl-5-thioureidoglycinamido)-4-aminophenyl]-2-(1-pyrrolidinyl)ethyl}acetamide (7). Intermediate **15**·HCl (0.2024 g, 0.3193 mmol) and 30 mg of 10% Pd/C were mixed in MeOH (12 mL) and hydrogenated at 25 °C under 10 psi for 70 h before the mixture was filtered through Celite, and the Pd/C was washed thoroughly with hot MeOH (100 mL). After evaporation of the filtrate, the residue was partitioned between 2 N NaOH (200 mL) and EtOAc (300 mL), and the organic fraction was dried (Na_2SO_4), filtered through Celite, and evaporated. To a solution of the deprotected intermediate (0.1229 g, 0.2652 mmol) in absolute EtOH (3 mL) and Et_3N (1 mL) were added FITC-I (0.1072 g, 0.2753 mmol) and freshly distilled THF (1 mL) with stirring under N_2. The mixture was evaporated after 48 h in the dark, and Et_3N was removed azeotropically with dry CH_3CN to yield a solid which was suspended in 95% EtOH and collected by filtration. The solid

was washed successively with 95% EtOH and anhydrous ethyl ether to yield **7**·Et_3N (0.1538 g, 60.8%): mp 203 °C dec; ^1H NMR (DMSO-d_6) δ 1.62 (br s, 4H, CH_2CH_2), 5.79 (m, 1H, CH); MS (FAB) *m/z* 852.3 (MH^+). Anal. ($C_{44}H_{39}N_5O_7SCl_2$·$(C_2H_5)_3N$) C, H, N, Cl.

2-(3,4-Dichlorophenyl)-*N*-methyl-*N*-{(1*R*,*S*)-1-[*N*-(*N*-fluoresceinyl-5-thioureidoglycylglycinamido)-4-aminophenyl]-2-(1-pyrrolidinyl)ethyl}acetamide (8). Intermediate **16**·HCl (0.1195 g, 0.1729 mmol) and 24 mg of 10% Pd/C were mixed in MeOH (6 mL) and hydrogenated at 25 °C under 10 psi for 138 h before the mixture was filtered through Celite, and the Pd/C was washed thoroughly with hot MeOH. After evaporation of the filtrate, the residue was partitioned between 1 N NaOH (150 mL) and EtOAc (300 mL), and the organic fraction was dried (Na_2SO_4), filtered through Celite, and evaporated. To a solution of the deprotected intermediate (0.0615 g, 0.1182 mmol) in absolute EtOH (3 mL) and Et_3N (2 mL) were added FITC-I (0.0479 g, 0.1229 mmol) and freshly distilled THF (1 mL) with stirring at 25 °C under N_2. After 7 days in the dark, the mixture was evaporated, and Et_3N was removed azeotropically with dry CH_3CN (16 mL) to yield a solid which was suspended in absolute EtOH (8 mL) and collected by filtration. Washing with anhydrous ethyl ether and drying yielded **8**·Et_3N (0.0887 g, 74%): mp 208 °C dec; ^1H NMR (DMSO-d_6) δ 1.61 (m, 4H, CH_2CH_2), 5.80 (m, 1H, CH); MS (FAB) *m/z* 909.3 (MH^+). Anal. ($C_{46}H_{42}N_6O_8SCl_2$·$(C_2H_5)_3N$) C, H, N, Cl.

2-(3,4-Dichlorophenyl)-*N*-methyl-*N*-{(1*R*,*S*)-1-[*N*-(*N*-fluoresceinyl-5-thioureidoglycylglycylglycylglycinamido)-4-aminophenyl]-2-(1-pyrrolidinyl)ethyl}acetamide (9). Intermediate **17**·HCl (0.0729 g, 0.0905 mmol) and 16 mg of 10% Pd/C were mixed in MeOH (20 mL) and hydrogenated at 25 °C under 10 psi. After 72 h, the mixture was filtered through Celite. The Pd/C was washed thoroughly with hot MeOH (100 mL), and the combined filtrate was evaporated. To a solution of the 1 HCl salt of the deprotected intermediate (0.0500 g, 0.0745 mmol) in absolute EtOH (5 mL) and Et_3N (4 mL) was added FITC-I (0.0309 g, 0.0794 mmol) in dry THF (2 mL) with stirring at 25 °C under N_2 in the dark. After 3 days, more fluorescein isothiocyanate isomer I (0.0298 g, 0.0765 mmol) and 100% EtOH (4 mL) were added, and the stirring continued for 2 days. The mixture was then evaporated, and Et_3N was azeotropically removed with dry CH_3CN (8 mL). The solid was purified on 0.25 mm reverse-phase-18 TLC plates eluting with 100% MeOH. The residue solidified upon addition of dry CH_3CN, and the solid was collected by filtration to yield **9**·Et_3N (0.0343 g, 41%): mp 198 °C dec; ^1H NMR (DMSO-d_6) δ 5.80 (m, 1H, CH); MS (FAB) *m/z* 1023.2 (MH^+). Anal. ($C_{50}H_{48}N_8O_{10}SCl_2$·$(C_2H_5)_3N$) C, H, N, Cl.

Acknowledgment. We thank Veronika Phillips, Joan Naeseth, and Michael Powers for in vitro testing of the compounds. We also acknowledge funding from U.S. Public Health Service Grants DA-08131, DA-04381, and DA-09924.

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JM950813B