14α,14′β-[Dithiobis[(2-oxo-2,1-ethanediyl)imino]]bis(7,8-dihydromorphinone) and 14α,14′β-[Dithiobis[(2-oxo-2,1-ethanediyl)imino]]bis[7,8-dihydro-*N*-(cyclopropylmethyl)normorphinone]: Chemistry and Opioid Binding Properties

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14α,14′β-[Dithiobis[(2-oxo-2,1-ethanediyl)imino]]bis(7,8-dihydromorphinone) (TAMO) (**13**) was synthesized by condensing 14β-amino-7,8-dihydromorphinone (**4**) with acetylthioglycolyl chloride and hydrolyzing the resulting ester with mild base to give a mixture of the thiol **9** and the disulfide **13**. Chromatography of the mixture resulted in conversion of the bulk of the thiol **9** to the disulfide **13** by air oxidation. The disulfide **13** was also prepared by condensing the *tert*-butyldimethylsilyl ether of **4** with the dithiodiglycolyl chloride and treating the resulting product with F^- to give the desired product. The pure thiol **9** free of contamination with the disulfide was prepared by treating **13** with excess *N*-acetyl-L-cysteine and processing the reaction mixture without resorting to chromatography for purification. The corresponding *N*-(cyclopropylmethyl) nor compound **15** was prepared from the silyl ether **6** and acetylthioglycolyl chloride followed by hydrolysis, treatment with F^-, and air oxidation. Incubation of bovine striatal membranes with **13** and **15** resulted in wash-resistant inhibition of the binding of the μ-selective peptide [^{3}H][D-Ala^2,(Me)Phe^4,Gly(ol)^5]-enkephalin (DAMGO). Incubation of membranes with μ but not κ or δ ligands protected the μ binding sites from alkylation by **13** and **15**. The wash-resistant inhibition of μ opioid binding was partially reversed by the addition of the reducing reagent dithiothreitol (DTT). A Scatchard plot of the effect of **13** and **15** on [^{3}H]DAMGO binding showed that these affinity ligands caused a marked decrease in the B_{max} value without affecting the K_d value. The wash-resistant inhibition of binding, the reduction in the number of binding sites, the partial reversal of wash-resistant inhibition of binding by DTT, and previously observed long-term antagonism of μ opioid receptors *in vivo* support the conclusion that **13** and **15** bind covalently to the μ opioid receptor.

Introduction

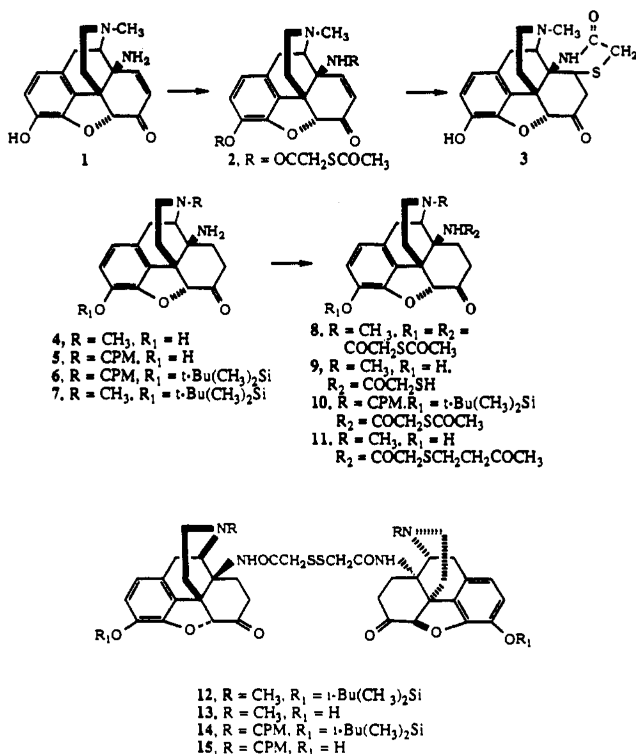
In a preliminary work, we reported the synthesis and some opioid binding properties of a compound to which structure **9** was assigned.[1] The antinociceptive properties of this compound[2] and of the corresponding *N*-(cyclopropylmethyl) nor compound[3] were described recently. The structure originally assigned to **9** was based on the method of synthesis, the NMR spectrum, which contained a signal at δ 3.48 ppm that was assigned to the CH_2SH protons, the formation of an internal Michael adduct **3** upon hydrolysis of the ester amide **2**, and treatment of the compound with methyl vinyl ketone in the presence of base giving the adduct **11**. However, further studies led to the conclusion that TAMO is not the thiol compound **9** but is the corresponding disulfide **13** and that the corresponding *N*-(cyclopropylmethyl) nor analogue is not a thiol but the disulfide **15**.

In this paper, we report the evidence for the revised structural assignments and that **13** forms a covalent bond with the μ opioid receptor.

Chemistry

14β-Aminomorphinone[1] (**1**) was acylated with acetoxythioglycolyl chloride,[4] to give **2**, and the latter was hydrolyzed with dilute base to furnish the Michael adduct **3** (Scheme 1). The NMR spectrum of **3** showed the absence of vinyl protons and the IR spectrum showed carbonyl absorption for the ketonic carbonyl group at 1718 cm^{-1}; thus, the structure of this compound was secured. This result clearly showed that a thiol group must have resulted

Scheme 1



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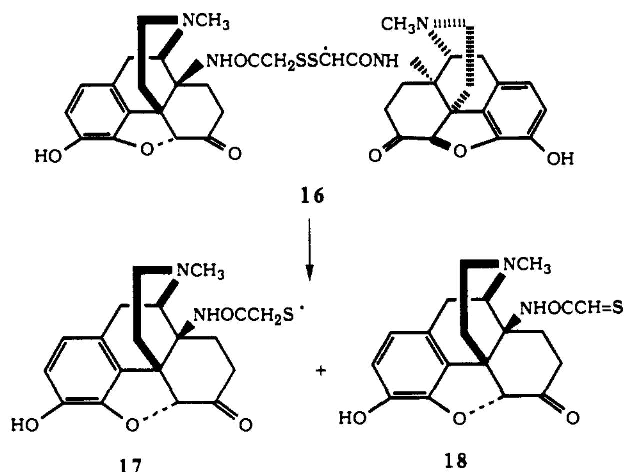
Scheme 2



from the hydrolysis of 2 in order for a Michael reaction to occur. To avoid this reaction 7,8-dihydromorphinones were employed. Treatment of 7,8-dihydromorphinone 4 with acetylthioglycolyl chloride gave the ester amide 8 which was hydrolyzed to give a mixture of products, one of which had an R_f = 0.6 and a more polar material which had an R_f = 0.2 (TLC system, $CHCl_3$/MeOH, 19:1). The less polar substance was the thiol 9 and the more polar material was the disulfide 13.

In order to prepare authentic 14α,14'β-[dithiobis[(2-oxo-2,1-ethanediyl)imino]]bis(7,8-dihydromorphinone) (13, TAMO), dithiodiglycolyl chloride was condensed with the silyl ether 7 to give 12, which in turn was allowed to react with F^- to give 13. The signal for the CH_2S protons in 13 occurred at δ 3.62 ppm, suspiciously close to the previously described signal for the CH_2S^- protons in the NMR spectrum of the product derived by hydrolysis of 8. Owing to the lack of solubility of the compound, the latter spectrum was run as a very dilute solution in $CDCl_3$. The spectrum of the authentic disulfide 13 was run in $CDCl_3$ containing a small amount of CD_3OD. The presence of the small amount of CD_3OD probably was responsible for the difference in the location of the signals for the relevant protons.

When 13 was treated with methyl vinyl ketone in the presence of 1 mol of NaOH, the adduct 11 was formed. Danehy and Kreuz[5] reported that treatment of dithiodiglycolic acid with base at 35 °C resulted in a complex mixture of products, among them being the dianion $^-OOCCH_2S^-$. Presumably, the disulfide 13 furnished the ion 16, which disproportionated to give the mercaptide ion 17 and the thioaldehyde 18 (Scheme 2). The former reacted with methyl vinyl ketone to furnish 11 and the thioaldehyde 18 hydrolyzed to the corresponding aldehyde, which in the presence of strong base underwent a Cannizzaro reaction.[5] In contrast to the disulfide 13, which required strong base to react with methyl vinyl ketone, the thiol 9 reacted smoothly with methyl vinyl ketone in the absence of base to furnish the expected adduct 11.

When 4 was treated with acetylthioglycolyl chloride and the reaction mixture hydrolyzed with dilute base, TLC of the crude reaction mixture revealed the presence of both the thiol 9 and the disulfide 13. When the reaction mixture was worked up and chromatographed on silica gel, none of the desired thiol was obtained. Presumably, the thiol underwent rapid oxidation to the disulfide during the chromatography. Even when purification was tried without chromatography, the thiol 9 was always contaminated with variable amounts of the disulfide 13.

Pure thiol 9 was obtained by treating a solution of the disulfide 13 with excess acetylcysteine and extracting the mixture with $CHCl_3$. Rapid isolation with minimum exposure to air gave the desired thiol, which on the basis of the NMR spectrum was free of the disulfide 13. The signal for the CH_2SH protons occurred at δ 3.29 ppm whereas the signal for the corresponding protons in the disulfide 13 appeared at δ 3.61. Both spectra were run in $CDCl_3$ containing a few drops of CD_3OD. When an aqueous solution of the hydrochloride salt of 9 was allowed to stand exposed to air at room temperature, the $t_{1/2}$ of the oxidation to the disulfide was approximately 90 min. The oxidation was prevented by adding glutathione (GSH) to the solution. Surprisingly, ascorbic acid was not an effective antioxidant.

The *N*-cyclopropylmethyl analogue was prepared using the *tert*-butyldimethylsilyl group as a protecting group for the phenolic hydroxyl group. Compound 5 was converted to 6 and the latter was treated with acetylthioglycolyl chloride to give 10. Hydrolysis followed by exposure to air gave the disulfide 14, which was treated with F^- to give 15. The signal for CH_2SSCH_2 protons in the NMR spectrum appeared at δ 3.64 ppm in agreement with the assigned structure. In view of the sensitivity of the thiol 9 to air oxidation, no attempt was made to prepare the corresponding related to 15.

Table 1. IC_{50} Values for the Inhibition of μ, δ, and κ Opioid Binding to Bovine Striatal Membranes by the Affinity Ligands[a]

radiolabeled opioid	IC_{50} (nM) 9	13	15
[^{3}H]DAMGO, 0.25 nM	0.63 ± 0.12	0.40 ± 0.06	3.29 ± 0.26
[^{3}H]pCl-DPDPE, 0.2 nM	12.1 ± 3.4	10.7 ± 0.59	23.8 ± 2.4
[^{3}H]U69,593, 1 nM	41.1 ± 1.9	40.5 ± 0.29	9.98 ± 0.49

[a] Bovine striatal membranes were incubated with twelve different concentrations of either compound 9, 13, or 15 in the presence of the radiolabeled opioid in 50 mM Tris-HCl, pH 7.5, as described in the Experimental Section. Data are presented as the mean IC_{50} value ± standard error obtained from three experiments.

Biological Results

The IC_{50} values for the inhibition of the binding of the μ-selective peptide [^{3}H][D-Ala^2,(Me)Phe^4,Gly(ol)^5]-enkephalin[6] (DAMGO), the δ-selective peptide [^{3}H][D-Pen^2,*p*-Cl-Phe^4,D-Pen^5]enkephalin[7] (pCl-DPDPE), and the κ-selective ligand [^{3}H]U69,593[8] by compounds 9, 13, and 15 are shown in Table 1. The concentrations of 13 and 15 required to inhibit 50% of [^{3}H]DAMGO binding in a wash-resistant manner were 40 ± 0.1 and 63 ± 7.8 nM, respectively, as shown in Figure 1. At higher concentrations, compounds 13 and 15 completely inhibited [^{3}H]-DAMGO binding. Preincubation of membranes with 100 μM *N*-tosyl-l-phenylalanyl chloromethyl ketone[9] (TPCK), a reagent that reacts with thiol groups and at a concentration of 100 μM, which did not interfere with opioid binding, resulted in a decrease in the concentration of the affinity ligands needed for wash-resistant inhibition of [^{3}H]-DAMGO binding, as shown in Figure 1. The concentrations of compounds 13 and 15 needed to inhibit binding by 50% in a wash-resistant manner in TPCK-treated membranes were 13.3 ± 3.4 and 46 ± 2.1 nM, respectively.

Incubation of membranes with compound 13 at 37 °C resulted in a time-dependent wash-resistant inhibition of [^{3}H]DAMGO binding, as shown in Figure 2. This inhibi-

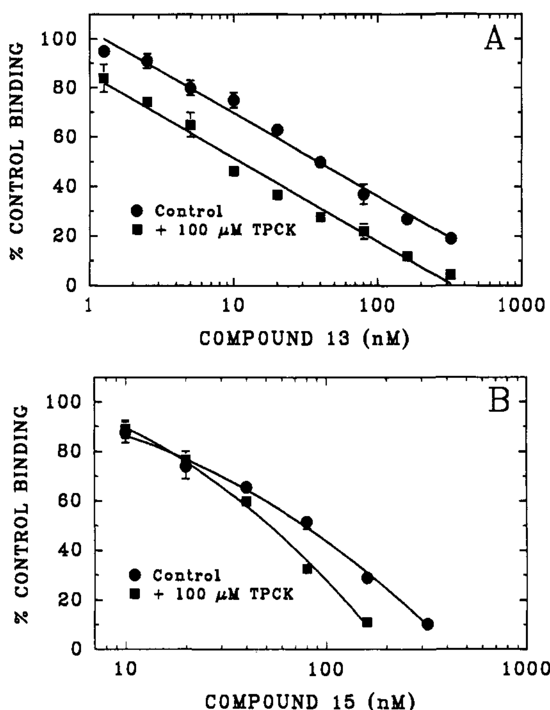


Figure 1. Effect of TPCK on wash-resistant inhibition of opioid binding by the *N*-methyl compound **13** and the *N*-(cyclopropylmethyl) compound **15**. Membranes, 20 mg of protein, were incubated without or with 100 μM TPCK in 16 mL of 50 mM Tris-HCl, pH 7.5, at 37 °C for 30 min. The incubation was terminated by diluting the contents of the tubes to 40 mL with cold buffer, followed by two centrifugal washes. Membranes were resuspended in a final volume of 2 mL of buffer and incubated with different concentrations of **13** (A) or **15** (B) at 37 °C for 5 min, followed by four centrifugal washes. [^{3}H]DAMGO binding to 0.2 mL of membranes was measured as described in the Experimental Section. Data are presented as the mean percentage of control binding ± standard error from three experiments, performed in triplicate.

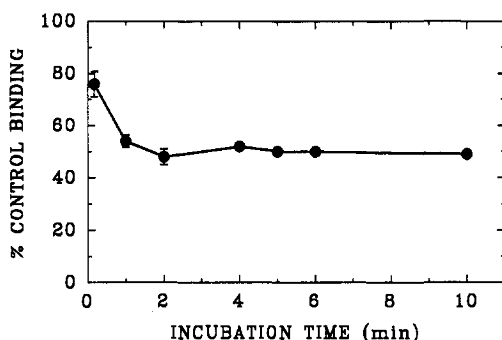


Figure 2. Time course for the wash-resistant inhibition of [^{3}H]-DAMGO binding to membranes by compound **13**. TPCK-treated membranes were incubated with 40 nM of compound **13** in 2 mL of 50 mM Tris-HCl, pH 7.5, at 37 °C for times ranging from 10 s to 10 min. The incubation was terminated by diluting the contents of tubes to 40 mL with cold 50 mM Tris-HCl, pH 7.5, followed by four centrifugal washes. [^{3}H]DAMGO binding to membranes was measured as described in the Experimental Section. Control binding, which was 2040 ± 47 cpm, was to membranes incubated at 37 °C for 10 min without the addition of **13**. Data are presented as the mean percentage of control binding ± standard error from three experiments, performed in triplicate.

tion of binding reached a maximum within 2 min. The *N*-(cyclopropylmethyl) analogue **15** behaved similarly (data not shown). This wash-resistant inhibition was also temperature-dependent as shown in Table 2. Incubating membranes with **13** at 4 or 37 °C for 5 min showed that a 4 °C-incubation resulted in less wash-resistant inhibition than at 37 °C.

Table 2. Effect of Temperature on the Irreversible Inhibition of [^{3}H]DAMGO Binding by Compound **13**[a]

temperature, °C	[^{3}H]DAMGO binding, % of control
4	40 ± 8
37	22 ± 6

[a] Membranes, pretreated with TPCK, were incubated with 80 nM of compound **13** in 2 mL of 50 mM Tris-HCl, pH 7.5, at 4 or 37 °C for 5 min. The incubation was terminated by diluting the contents of the tube to 40 mL with cold 50 mM Tris-HCl, pH 7.5, followed by centrifugation at 39000*g* for 15 min. The membranes were resuspended in 40 mL of buffer, and the washing step was repeated three additional times. Finally, membranes were resuspended in 2 mL of buffer, and 0.25 nM [^{3}H]DAMGO binding to 0.2 mL of membranes was measured as described in the Experimental Section. Control binding, which did not vary with the incubation temperature, was to membranes treated under the same conditions except that compound **13** was not added to the sample. Control [^{3}H]DAMGO binding was 1100 ± 69 cpm. Data are presented as the mean percentage of control binding ± standard error from three experiments, performed in triplicate.

The specificity of the alkylation of opioid binding sites by **13** and **15** was determined by two different methods. Opioid binding to membranes, treated with either **13** or **15** followed by four centrifugal washes, was measured to determine whether the affinity ligands altered the binding of the μ-selective peptide [^{3}H]DAMGO, the δ-selective peptide [^{3}H]pCl-DPDPE, and the κ-selective ligand [^{3}H]-U69,593. [^{3}H]DAMGO binding was irreversibly inhibited by 80% when membranes were treated with 80 nM of compound **13**, a concentration that did not affect the binding of either [^{3}H]pCl-DPDPE or [^{3}H]U69,593 (Figure 3A). The *N*-(cyclopropylmethyl) analogue **15** strongly inhibited [^{3}H]DAMGO binding, moderately inhibited [^{3}H]-U69,593 binding, and weakly inhibited [^{3}H]pCl-DPDPE binding (Figure 3B). Clearly, the *N*-methyl compound **13** was more selective than the *N*-(cyclopropylmethyl) analogue in irreversibly inhibiting μ opioid binding. The IC_{50} values for these affinity ligands showed a similar pattern of selectivity (Table 1).

Protection experiments were performed using the ligands morphine and naloxone for μ binding sites, ICI 174,864[10] and naltrindole[11] for δ sites, and U50,488[12] for κ sites, to determine which, if any, of these opioids could block the wash-resistant inhibition of opioid binding. In these protection experiments, the opioids were incubated with membranes 30 min before the addition of **13** and **15**. After a further 2-min incubation, the membranes were washed and [^{3}H]DAMGO (Table 3) and [^{3}H]U69,593 (Table 4) binding to membranes treated with **13** and **15** was compared to that of untreated membranes and membranes treated only with the corresponding protecting opioids. None of the protecting opioids inhibited either [^{3}H]DAMGO or [^{3}H]U69,593 binding by more than 5% compared with the controls. As shown in Table 3, morphine and naloxone protected the μ opioid binding sites from alkylation by **13** and **15**. The δ opioid ligands ICI 174,84 and naltrindole and the κ opioid U50,488 did not protect the μ binding site from alkylation. The μ-selective peptide DAMGO could not be used because of the difficulty in completely removing the peptide from membranes. As shown in Table 4, U50,488 provided maximal protection of the κ sites from alkylation by compound **15** and naloxone afforded less protection. The μ- and the δ-selective ligands were ineffective in protecting the κ sites.

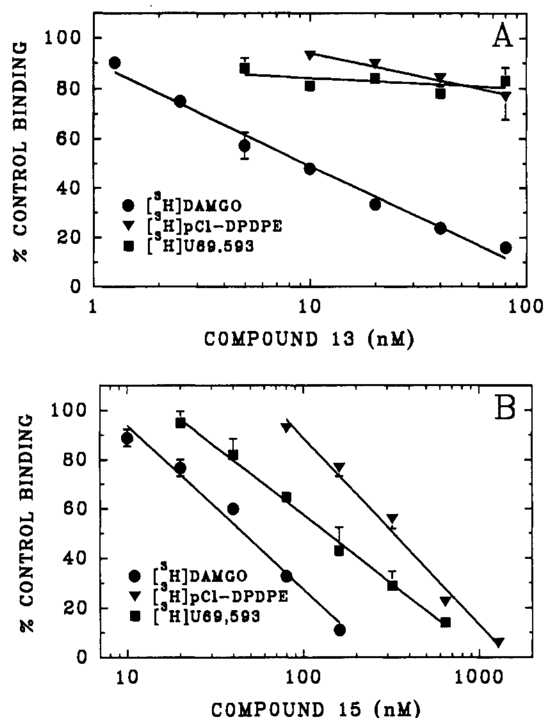


Figure 3. Selectivity of the wash-resistant inhibition of opioid binding by **13** and **15**. TPCK-treated membranes were incubated with different concentrations of **13** (A) or **15** (B) at 37 °C for 5 min, followed by four centrifugal washes. The binding of [3H]-DAMGO, [3H]pCl-DPDPE, or [3H]U69,593 to resuspended membranes was measured as described in the Experimental Section. Control binding was to membranes treated under the identical conditions except that compounds **9** and **11** were not added. Control [3H]DAMGO, [3H]pCl-DPDPE, and [3H]U69,-593 binding was 840 ± 86, 470 ± 88, and 560 ± 41 cpm, respectively. Data are presented as the mean percentage of control binding ± standard error from three experiments, performed in triplicate.

Table 3. Ability of μ-, δ-, and κ-Selective Opioids To Protect the [3H]DAMGO Binding Site from Alkylation by Compounds **13** and **15**[a]

treatment	[3H]DAMGO binding, % of control: 13	15
compound 13	25 ± 2	
compound 15		25 ± 4
naloxone	70 ± 6*	62 ± 5*
morphine	67 ± 8*	62 ± 9*
ICI 174,864	24 ± 2	22 ± 8
naltrindole	25 ± 1	27 ± 5
U50,488	24 ± 1	24 ± 7

[a] TPCK-treated membranes were incubated with 100 nM of the protecting opioids for 30 min at 37 °C, followed by a 2-min incubation with either 40 nM of compound **13** or 80 nM of compound **15**. After washing the membranes, the binding of 0.25 nM [3H]DAMGO to the membranes was measured as described in the Experimental Section. Control binding, which was 1050 ± 112 cpm, was to membranes treated under the same conditions except that the opioid and affinity ligands were not added. Data are presented as the mean percentage of control binding ± standard error from three experiments, performed in triplicate. Inhibition of binding by 100 nM opioids alone was less than 5% of [3H]DAMGO control binding. Significantly different from binding observed with compound **13** or compound **15** alone (*$P \leq 0.05$).

To determine whether compounds **13** and **15** were binding to opioid receptors by forming a mixed disulfide with the binding site, the disulfide-reducing reagent DTT was added to membranes that had been incubated with compounds **13** and **15**. As shown in Table 5, the addition of DTT to the membranes partially reversed the wash-resistant inhibition of [3H]DAMGO binding by **13** and **15**. DTT also partially reversed the wash-resistant inhibition of [3H]U69,593 binding by compound **15** (Table 5). The addition of DTT alone to membranes, followed by four centrifugal washes, did not significantly alter either [3H]DAMGO or [3H]U69,593 binding (Table 5). These results support the view that the *N*-methyl compound **13** forms a mixed disulfide bond with the μ opioid binding site and the *N*-cyclopropylmethyl compound **15** forms mixed disulfide bonds with both μ and κ opioid receptors.

Table 4. Ability of μ-, δ- and κ-Selective Opioids To Protect the [3H]U69,593 Binding Site from Alkylation by the *N*-(Cyclopropylmethyl) Analogue **15**[a]

treatment	[3H]U69,593 binding, % of control
compound 15	30 ± 2
naloxone	51 ± 2*
morphine	31 ± 3
ICI 174,864	26 ± 2
naltrindole	32 ± 4
U50,488	85 ± 2*

[a] TPCK-treated membranes were incubated with 100 nM of the protecting opioids for 30 min at 37 °C, followed by a 2-min incubation with 320 nM of compound **15** in a final volume of 2 mL. After four centrifugal washes, the binding of 1 nM [3H]U69,593 to the membranes was measured as described in the Experimental Section. Control [3H]U69,593 binding was 445 ± 24 cpm. Data are presented as the mean percentage of control binding ± standard error from three experiments, performed in triplicate. Inhibition of binding by 100 nM of the opioids alone was less than 5% [3H]U69,593 control binding. Significantly different from the binding obtained with compound **15** alone (*$P \leq 0.05$).

Table 5. Effect of DTT on Wash-Resistant Inhibition of [3H]DAMGO and [3H]U69,593 Binding by Compounds **13** and **15**[a]

condition	binding, % of control
[3H]DAMGO	
10 nM of compound 13	50 ± 3
10 nM of compound 13, followed by 40 mM DTT	78 ± 3*
80 nM of compound 15	63 ± 5
80 nM of compound 15, followed by 40 mM DTT	87 ± 4*
40 mM DTT alone	104 ± 15
[3H]U69,593	
160 nM of compound 15	35 ± 2
160 nM of compound 15, followed by 40 mM DTT	55 ± 3*
40 mM DTT alone	113 ± 10

[a] TPCK-treated membranes were incubated with either compound **13** or **15** at 37 °C for 5 min, followed by a 10-min incubation with 40 mM DTT at 4 °C in a final volume of 2 mL. After four centrifugal washes, 0.25 nM [3H]DAMGO or 1 nM [3H]U69,593 binding to membranes was measured as described in the Experimental Section. Control binding was to membranes treated in the same manner except for the omission of the affinity ligands and DTT. The addition of DTT to membranes, followed by four centrifugal washes, did not alter the binding of either [3H]DAMGO or [3H]U69,593 to membranes. Control binding was 830 ± 91 and 510 ± 40 cpm for [3H]DAMGO and [3H]U69,593, respectively. Data are presented as the mean percentage of control binding ± standard error from three experiments, performed in triplicate. Significantly different from binding obtained with either **13** or **15** (*$P \leq 0.05$).

[3H]DAMGO and [3H]U69,593 saturation binding to membranes incubated with TPCK alone, TPCK plus **13**, and TPCK plus **15** was investigated to determine whether incubation of membranes with the affinity ligands caused a change in the number of binding sites and/or affinity of the binding sites. Table 6 shows that treating membranes with 100 μM TPCK did not alter either the K_d or B_{max} values for either [3H]DAMGO- or [3H]U69,593-treated membranes compared to untreated membranes. The B_{max} value for [3H]DAMGO binding was significantly decreased in membranes incubated with either **13** or **15**, while the

Table 6. K_d and B_{max} Values for [^{3}H]DAMGO and [^{3}H]U69,593 Binding to Bovine Striatal Membranes Incubated with either TPCK, TPCK, and Compound 13 or TPCK and Compound 15[a]

condition	[^{3}H]DAMGO		[^{3}H]U69,593	
	K_d, nM	B_{max}, fmol/mg of protein	K_d, nM	B_{max}, fmol/mg of protein
control	0.89 ± 0.16	281 ± 59	0.63 ± 0.03	17.2 ± 2.4
TPCK	0.75 ± 0.07	243 ± 24	0.59 ± 0.03	20.1 ± 0.7
TPCK plus 13	0.95 ± 0.26	78 ± 11*	NA	NA
TPCK plus 15	1.27 ± 0.07	46 ± 7*	0.88 ± 0.20	9.3 ± 2.1*

[a] Membranes were incubated without or with 100 μM TPCK in 50 mM Tris-HCl, pH 7.5, at 37 °C for 30 min. After two centrifugal washes, the membranes were resuspended and incubated with or without 40 nM of 13 or 100 nM of 15 at 37 °C for 5 min, followed by four centrifugal washes. [^{3}H]DAMGO and [^{3}H]U69,593 binding to membranes and the protein concentration of membranes were measured as described in the Experimental Section. Control binding was to membranes treated under the same conditions except that TPCK and compounds 13 and 15 were not added to the sample. Data are presented as the mean K_d and B_{max} values ± standard error from three experiments, performed in triplicate. Significantly different from control and TPCK binding (*$P \leq 0.05$).

affinity for the binding sites was unchanged. Similar results were obtained with membranes incubated with compound 15 followed by the measurement of [^{3}H]U69,593 binding. These saturation binding experiments also support the conclusion that the affinity ligands 13 and 15 form covalent bonds with the μ receptor in the case of 13 and with the μ and κ receptors in the case of 15.

Discussion

Opioid affinity ligands have been synthesized for the purpose of labeling opioid receptor(s) for both *in vitro* and *in vivo* experiments. β-Funaltrexamine[13] (β-FNA) has been extensively used to label μ opioid receptors. Other affinity ligands that have been used to affinity label μ opioid receptors include both alkaloids and peptides. Naloxonazine[14] and a series of 14-hydroxydihydromorphinone hydrazones[15-17] (non-peptide ligands) have been used *in vivo* and *in vitro* to block irreversibly μ_1 opioid receptors. When rat brain membranes were incubated with the non-peptide acylating affinity ligand benzimidazolyl isothiocyanate[18] (BIT), binding to μ opioid receptors was selectively inhibited in a wash-resistant manner. The peptide [D-Ala^2,Leu^5,Cys^6]enkephalin (DALCE), which has a sulfhydryl group on the terminal cysteine residue, binds covalently to the δ receptor as demonstrated in antinociceptive[19] and membrane binding[20] studies.

The presence of a sulfhydryl group at or near the opioid binding site was first shown by Pasternak *et al.*[21] and Simon and Groth[22] who demonstrated that reagents such as iodoacetamide and *N*-ethylmaleimide (NEM), which are known to react with thiol groups, inhibited opioid binding to rat brain membranes. The specificity of the reactivity of such agents as NEM with the opioid receptor was demonstrated further by the finding that incubating membranes with opioids before the addition of NEM blocked the inhibitory effect of NEM on opioid binding.[23] These studies suggested that a sulfhydryl group was present at or near the opioid binding site. Many opioid affinity ligands, including β-chlornaltrexamine[24] (β-CNA), β-FNA,[13] DALCE,[20] and the sulfur-containing compounds reported in this paper, have been designed to interact with the thiol group at or near the binding site of the opioid receptor(s). β-CNA reacts with all types of opioid receptors,[25] while β-FNA binds to both κ and μ opioid receptors, but alkylates only the μ receptor.[26] DALCE binds covalently to δ receptors by forming a mixed disulfide bond with the δ opioid binding site.[20]

The *N*-methyl compound 13 and the *N*-(cyclopropylmethyl) analogue 15 bound with high affinity to μ opioid receptors, but the former showed greater selectivity. This pattern of selectivity was also observed in the wash-resistant inhibition of binding. At concentrations which did not inhibit δ or κ opioid binding, both 13 and 15 inhibited μ binding in a wash-resistant manner. At higher concentrations, 15 also inhibited κ binding in a wash-resistant manner and to a lesser degree δ binding. The time course for the wash-resistant inhibition of binding was temperature-dependent and at 37 °C the reaction was complete within 2 min.

Pretreatment of membranes with TPCK decreased the concentration of the affinity ligands needed to inhibit opioid binding in a wash-resistant manner. TPCK binds to sulfhydryl groups and probably reduced but did not eliminate the number of free sulfhydryl groups associated with the striatal membranes. Even with TPCK pretreatment, much larger concentrations of the affinity ligands were needed to inhibit opioid binding in a wash-resistant manner than were needed to inhibit opioid binding in the presence of a radiolabeled opioid. This discrepancy between the concentration of affinity ligand needed for wash-resistant inhibition of binding and competitive inhibition of binding has been observed with other opioid affinity ligands. For example, the IC_{50} value for the inhibition of the binding of 1 nM [^{3}H]dihydromorphine to rat brain membranes by β-FNA was 1.4 nM.[27] However, pretreatment of membranes with 100 nM β-FNA decreased the binding of 1 nM [^{3}H]dihydromorphine, in a wash-resistant manner, by 51%.[27] An IC_{50} value of 0.32 nM was reported for the inhibition of the binding of 2 nM [^{3}H]-DPDPE to rat brain membranes by DALCE.[20] However, when rat brain membranes were incubated with 5 μM DALCE, followed by washing, 39% of the binding of 2 nM [^{3}H]DPDPE remained.[20]

The reason that such large concentrations of affinity ligands are necessary is probably due to nonspecific thiol groups present in membrane homogenates. The total SH content in rat brain membranes is approximately 90 nmol of SH/mg of protein.[28] When membranes were incubated with either 11 or 13, the membrane protein concentration was approximately 5 mg/mL, which corresponds to a total SH concentration of 450 μM. Recently, we have determined that the concentration of endogenous glutathione associated with bovine striatal membranes is 10 nmol/mg of protein.[29] Glutathione, like acetylcysteine will react with compound 13.[29] Since compounds, such as glutathione, which can react with certain affinity ligands, are associated with membrane homogenates, these affinity ligands may react with such thiol-containing compounds but still bind to the opioid receptor in a noncovalent manner. Under such circumstances, higher concentrations of the affinity ligands are required to bind covalently to the opioid receptor(s).

In order to show that the disulfide compounds 13 and 15 were forming mixed disulfide bonds with the μ opioid receptor, membranes were first incubated with the affinity ligands, followed first by the addition of DTT for an additional 10-min incubation at 4 °C and then by four centrifugal washes. As shown in Table 5, the addition of DTT partially restored opioid binding, thus providing evidence that the disulfide-containing affinity ligands were indeed binding covalently to the μ receptor. The reason that partial but not complete reversibility was observed may be due to the rapid reaction of the ligand–DTT complex with the receptor. Even after DTT cleaves the mixed disulfide bond between the ligand and the receptor, thus freeing the sulfhydryl group on the receptor, this newly liberated receptor SH group may react with the mixed disulfide formed between the ligand and DTT. Similarly in the case of DALCE, the addition of DTT only partially restored opioid binding.[23] A 77% wash-resistant inhibition of [^{3}H]DPDPE binding to rat brain membranes was obtained with 10 μM of DALCE. When membranes were incubated with 10 μM DALCE, followed by the addition of 100 mM DTT, a 54% wash-resistant inhibition of binding was observed. This partial reversal of wash-resistant inhibition binding by DALCE, 13, and 15 is evidence that these affinity ligands bind covalently to the opioid receptors.

There are four lines of evidence which lead to the conclusion that the ligands 13 and 15 bind covalently to the μ receptor. First, both ligands produce wash-resistant inhibition of binding to the receptor. Second, DTT partially reverses this wash-resistant binding, and third, the disulfide-containing ligands produce a decrease in the B_{max} values of radiolabeled opioid binding without changing the K_d values. The long-term antagonism of morphine-induced antinociception observed previously in the mouse tail-flick test[2,3] also supports this conclusion.

Experimental Section

Melting points were taken on a laboratory Mel-Temp apparatus and are uncorrected. The ^{1}H NMR spectra were performed in $CDCl_3$, unless otherwise specified. The instrument used was a Varian XL-200 spectrometer using $(CH_3)_4Si$ as the internal standard. IR spectra were run on Perkin-Elmer Model 298 and Perkin-Elmer 1800 Fourier transform infrared spectrometers. Microanalyses were performed by Atlantic Microlab, Norcross, GA, and the results were within ±0.4% of the calculated values.

Internal Michael Adduct of 14β-(Thioglycolamido)morphinone (3). To a stirred mixture of 100 mg of 14β-aminomorphinone[1] and 75 mg of triethylamine in 20 mL of dry CH_2Cl_2 was added a solution of 112 mg of acetylthioglycolyl chloride[4] in 5 mL of dry CH_2Cl_2 dropwise at 15 °C with the exclusion of moisture. The mixture was allowed to warm to room temperature and stirring was continued for another 3 h. The mixture was poured into water, the layers were separated, and the organic layer was washed with water, saturated Na_2CO_3 solution, and again with water. The dried solution was evaporated to dryness and the residue was chromatographed (EtOAc/MeOH, 9:1) to give 2 (100 mg, 56%). The compound was used in the next step without further purification.

To a stirred solution of 100 mg of 2 in 5 mL of MeOH, in an N_2 atmosphere, there was added dropwise 10 mL of 0.1 N NaOH solution at 0 °C. After 1 h, the reaction mixture was neutralized by the addition of 1 N HCl and then extracted with $CHCl_3$. The extract was washed with water and the solvent was removed in vacuo to give 3 (65 mg, 95%). Mp 314–316 °C dec, after crystallization from EtOAc/hexane. IR (KBr): 3380 (OH, NH), 1718 (CO, ketone), 1635 cm^{-1} (CO, amide). NMR: δ 2.40 (s, NCH_3), 3.57 (s, SCH_2), 4.62 (s, 5β-H), 6.68 (q, aromatic H), 7.16 (s, NH). Anal. ($C_{19}H_{20}N_2O_4S$) C, H, N.

14α,14'β-[Dithiobis[(2-oxo-2,1-ethanediyl)imino]]bis(7,8-dihydromorphinone) (13). **(A)** To a stirred suspension of 1.24 g of the dihydrochloride salt of 4[1] in 50 mL of dry CH_2Cl_2 containing 1.5 g of triethylamine cooled to 0 °C was added dropwise a solution of 1.7 g of acetylthioglycolyl chloride[4] in 10 mL of CH_2Cl_2 over a period of 10 min. After stirring for 4 h at 25 °C, the reaction mixture was poured into water and washed with saturated $NaHCO_3$ solution and then again with water. The dried organic layer was evaporated to dryness and the residue was chromatographed on a silica gel column. After elution with $CHCl_3$ to remove impurities, the product was eluted with $CHCl_3$/MeOH (9:1) to give almost pure amido ester 8 (1.26 g, 70.4%). It was suitable for use in the next step. The compound was dissolved in 50 mL of MeOH and a solution of 1.6 g of K_2CO_3 in 20 mL of water was added and the mixture was stirred in a nitrogen atmosphere at room temperature for 30 min. The solution was poured into ice water, neutralized with 2 N HCl, and extracted with $CHCl_3$. The dried extract was evaporated to dryness. An NMR spectrum of the crude residue indicated that the thiol 9 was the major component of the mixture and that 13 was the minor one. The material was chromatographed on silica gel. Elution with $CHCl_3$ removed some impurities and elution with $CHCl_3$/MeOH (9:1) gave pure 13 (for the two steps, 580 mg, 46%). During the chromatography, all of the thiol 9 was oxidized to the disulfide 13. The analytical sample was obtained by crystallization from MeOH/EtOAc. Mp 205–215 °C dec. IR (KBr): 3600–3100 (OH, NH), 1710 (CO, ketone), 1650 cm^{-1} (CO, amide). NMR ($CDCl_3$ + 2 drops of CD_3OD) δ 2.39 (s, NCH_3), 3.61 (s, CH_2SSCH_2), 4.92 (s, 5β-H), 6.68 (q, aromatic H). Anal. ($C_{38}H_{42}N_4O_8S_2 \cdot H_2O$) C, H, N.

(B) To a solution of 100 mg of 14β-amino-7,8-dihydromorphinone 4[1] in dry DMF was added 75 mg of imidazole and 150 mg of *tert*-butyldimethylsilyl chloride. After stirring in a stoppered flask for 3 h at 25 °C, the solution was poured into 100 mL of water and thoroughly extracted with ether. After removal of the solvent, the residue was chromatographed on a column of silica gel to give 117 mg (85%) of the ether 7 suitable for use in the next step.

Forty milligrams of dithiodiglycolic acid was suspended in 1 mL of dry CH_2Cl_2 and the suspension was heated under gentle reflux for 1 h. The resulting clear solution was evaporated to dryness at 40 °C under vacuum. The residue was dissolved in fresh dry CH_2Cl_2 and the process was repeated.

A solution of 19 mg of the above acid chloride in 1 mL of dry CH_2Cl_2 was added over a period of 5 min to a stirred solution of 60 mg of the silyl ether 7 and 18 mg of triethylamine in 8 mL of dry CH_2Cl_2 at −78 °C in an argon atmosphere. After stirring for 10 min, about 10 mL of CH_2Cl_2 was added and the reaction mixture was washed with water, saturated $NaHCO_3$ solution, and again with water. The dried organic layer was evaporated to dryness and the residue was chromatographed on silica gel plates using $CHCl_3$/MeOH (19:1) as the developing solvent. The procedure was repeated twice to furnish 5 mg (8%) of the starting material 7 and 20 mg (28%) of the desired amide 12, which melted at 202–205 °C after crystallization from EtOAc/hexane. IR (KBr): 3304 (NH), 1718 (CO, ketone), 1660 cm^{-1} (CO, amide). NMR: δ 0.151 and 0.216 (2s, 2 CH_3), 0.98 (s, t-Bu), 2.40 (s, NCH_3), 3.59 (d, CH_2SSCH_2), 4.76 (s, 5β-H), 6.63 (q, aromatic H), 7.64 (s, NH). Anal. ($C_{50}H_{70}N_4O_8S_2Si_2 \cdot 0.5H_2O$) C, H, N.

To a stirred solution of 13 mg of the disulfide 12 in 6 mL of dry THF cooled to −78 °C was added 120 μL of a 1 M solution of tetrabutylammonium fluoride in THF. After 30 min, the solution was neutralized with 240 μL of 0.5 N HCl. The solvent was removed and the residue was dissolved in $CHCl_3$. After washing with water, the organic layer was evaporated to dryness to leave 9 mg of crude 13. After chromatography on silica gel plates using $CHCl_3$/MeOH (19:1) as the developing solvent, there was obtained 3.8 mg (38%) of 13 identical in all respects with the sample prepared as described above.

14β-(Thioglycolamido)-7,8-dihydromorphinone (9). A suspension of 100 mg of 13 and 100 mg of *N*-acetyl-L-cysteine in 20 mL of phosphate buffer, pH 7.4, was stirred in an argon atmosphere for 90 min at room temperature. At the end of that time, an additional 100 mg of *N*-acetyl-L-cysteine was added and stirring of the cloudy reaction mixture was continued for an additional 90 min. The almost clear solution was filtered and

the filtrate was treated with 20 mL of phosphate buffer, pH 6.4, and then extracted three times with $CHCl_3$/MeOH (9:1). The combined extracts were washed with saturated salt solution, and the organic layer was evaporated to dryness to leave a solid which was dissolved in 5 mL of MeOH containing 166 μL of 1 N HCl. The solution was evaporated to dryness and the residue was dried in vacuo and triturated with dry ether. The crystalline hydrochloride of **9** was filtered and dried to leave 67 mg of the desired salt. Mp >260 °C dec. The NMR did not show a trace of a signal at δ 3.62, indicating the absence of the disulfide **13**. IR (KBr): 3600–3150, (OH, NH), 2561 (SH, weak), 1717 (CO, ketone), 1653 cm^{-1} (CO, amide). NMR ($CDCl_3$ + 2 drops of CD_3OD): δ 2.40 (s, NCH_3), 3.29 (s, CH_2SH), 4.90 (s, 5β-H), 6.68 (q, aromatic H). Anal. ($C_{19}H_{22}N_2O_4S{\cdot}HCl{\cdot}H_2O$) C, H, N.

14β-Amino-7,8-dihydro-*N*-(cyclopropylmethyl)normorphinonyl *tert*-Butyldimethylsilyl Ether (6). A solution of 480 mg of 14β-amino-7,8-dihydro-*N*-(cyclopropylmethyl)normorphinone dihydrochloride (**5**), 340 mg of imidazole, and 530 mg of *tert*-butyldimethylsilyl chloride were dissolved in 15 mL of dry DMF was allowed to stir in a stoppered flask for 3 h at 25 °C. The solution was poured into 200 mL of H_2O and extracted with three portions of ether. The combined extracts were washed with brine, dried, and evaporated to dryness. Chromatography of the residue on silica gel using $CHCl_3$/MeOH (9:1) as the eluant gave **6** (395 mg, 75%), which melted at 265 °C (dec) after crystallization from ether. The fumarate salt melted at 198–200 °C after crystallization from MeOH and was used for analysis. IR (KBr): 3440 (NH_2), 1723 cm^{-1} (CO, ketone). NMR: δ 0.20 and 0.26 (2s, 2 CH_3), 0.22–0.93 (m, cyclopropyl), 0.99 (s, t-Bu), 5.40 (s, 5β-H), 6.63 (q, aromatic). Anal. ($C_{26}H_{38}N_2O_3Si{\cdot}C_4H_4O_4$) C, H, N.

14β-[Acetyl(thioglycolamido)]-7,8-dihydro-*N*-(cyclopropylmethyl)normorphinonyl *tert*-Butyldimethylsilyl Ether (10). A solution of 146 mg of acetylthioglycolyl chloride in 5 mL of dry CH_2Cl_2 was added dropwise to a solution of 380 mg of **6** and 98 mg of triethylamine in 20 mL of CH_2Cl_2. The reaction mixture was stirred at 0 °C for 30 min and at room temperature for another 30 min. At the end of this time, the mixture was poured into H_2O and the separated organic layer was taken to dryness to give 448 mg (94%) of **10**, which melted at 163–164 °C after crystallization from MeOH. IR (KBr): 3400 and 3300 (NH), 1715 (CO, ketone), 1697 (CO, acetyl), 1640 cm^{-1} (CO, amide). NMR: δ 0.14 and 0.21 (2, s, 2 CH_3), 0.18–0.89 (m, cyclopropyl), 0.97 (s, t-Bu), 2.37 (d, $CH_2C_3H_5$), 2.42 (s, CH_3CO), 3.62 (q, CH_2S), 4.69 (3, 5β-H), 6.60 (q, aromatic H), 7.58 (br, s, NH). Anal. ($C_{30}H_{42}N_2O_5SSi$) C, H, N.

14α,14'β-[Dithiobis[(2-oxo-2,1-ethanediyl)imino]]bis[7,8-dihydro-*N*-(cyclopropylmethyl)normorphinonyl] *tert*-Butyldimethylsilyl Ether (14). A solution of 150 mg of $NaHCO_3$ in 15 mL of water was added to a stirred solution of 410 mg of **10** in 50 mL of MeOH. After stirring overnight, the reaction was poured into water and adjusted to pH 7 with 2 N HCl. The mixture was extracted with $CHCl_3$ and the organic fraction was evaporated to dryness. The residue melted at 127–129 °C after recrystallization from MeOH (360 mg). IR (KBr): 3600–3200 (NH), 1715 (CO, ketone), 1655 cm^{-1} (CO, amide). NMR: δ 0.15 and 0.21 (2s, 4 CH_3), 0.20–0.86 (m, cyclopropyl), 0.97 (s, t-Bu), 3.58 (q, CH_2SSCH_2), 4.73 (s, 5β-H), 6.62 (q, aromatic H). Anal. ($C_{56}H_{78}N_4O_8S_2Si_2{\cdot}1.5H_2O$) C, H, N.

14α,14'β-[Dithiobis[(2-oxo-2,1-ethanediyl)imino]]bis[7,8-dihydro-*N*-(cyclopropylmethyl)normorphinone] (15). To a stirred solution of 350 mg of the disulfide **14** in 75 mL of dry THF cooled to −78 °C was added over a period of 5 min 2.3 mL of a 1 M solution of tetrabutylammonium fluoride in THF. After 30 min of stirring, the reaction mixture was neutralized with 2 N HCl and the solvent was evaporated to dryness in vacuo. The residue was taken up in $CHCl_3$ and the solution was washed with H_2O. The organic layer was evaporated to dryness and the residue was triturated with hexane and filtered, and the solid was washed with hexane to give 263 mg of the crude product. After chromatography on silica gel plates using $CHCl_3$/MeOH (9:1) as the developing solvent, there was obtained a material which after trituration with a small amount of $CDCl_3$ furnished 105 mg of pure free base. Mp >170 °C dec. IR (KBr): 3600–3100 (NH, OH), 1708 (CO, ketone), 1655 cm^{-1} (CO, amide). NMR ($CDCl_3$ + 2 drops of CD_3OD): δ 0.18–0.92 (m, cyclopropyl), 2.39 (dd, $CH_2C_3H_5$), 3.64 (d, CH_2SSCH_2), 4.87 (s, 5β-H), 6.67 (q, aromatic H). The free base was converted to the dihydrochloride and the salt was crystallized from MeOH to furnish the analytical sample. Anal. ($C_{44}H_{50}N_4O_8S_2{\cdot}2HCl{\cdot}3H_2O$) C, H, N.

14β-[(3-Oxobutyl)(thioglycolamido)]-7,8-dihydromorphinone (11). (A) From the thiol 9. To a stirred solution of 35 mg of the thiol **9** in 4 mL of dry THF in an atmosphere of argon was added a solution of 8 μL of methyl vinyl ketone in 1 mL of dry THF. After stirring overnight, the solution was taken to dryness and the residue was chromatographed on a silica gel plate using $CHCl_3$/MeOH (9:1) as the developing solvent. There was obtained 22 mg (53%) of the pure adduct **11**, accompanied by 3 mg of the pure disulfide **13** which was formed by air oxidation during chromatography. The free base was converted to the corresponding hydrochloride by heating to boiling equimolar quantities of the adduct with an aqueous solution of 1 N HCl and evaporating the solution to dryness. Recrystallization of the salt from MeOH/ether gave the analytical sample. Mp 250–260 °C dec. IR (KBr): 3600–3100 (OH, NH), 1710 (CO, ketone), 1650 cm^{-1} (CO, amide). NMR: δ 2.19 (s, $COCH_3$), 2.37 (s, NCH_3), 2.70–2.95 (m, SCH_2CH_2CO), 3.27 (s, $COCH_2S$), 5.02 (s, 5β-H), 6.69 (q, aromatic H). Anal. ($C_{23}H_{28}N_2O_5S{\cdot}1.5H_2O$) C, H, N.

(B) From the disulfide 13. To a stirred solution of 10 mg of **13** in THF in an argon atmosphere was added 2.23 μL of methyl vinyl ketone followed by 26.7 μL of 1 N NaOH. After 30 min, the mixture was evaporated to dryness and the residue dissolved in EtOAc. After washing with water, the organic layer was evaporated to dryness to furnish the crude adduct which was chromatographed as above to give 5 mg (44%) of the pure adduct **11**, identical in all respects with the adduct prepared from the thiol **9**.

Opioid Binding to Bovine Striatal Membranes. Calf striata were homogenized in 10 times their wet weight of cold 50 mM Tris-HCl, pH 7.5, followed by centrifugation at 39000*g* for 20 min at 4 °C. The membranes were resuspended in the original volume of buffer and incubated at 37 °C for 30 min, followed by centrifugation at 39000*g* for 20 min at 4 °C. The membranes were resuspended at a protein concentration of 8–12 mg/mL in 50 mM Tris-HCl, pH 7.5, and stored at −80 °C until use. The protein concentration of membranes was determined by the method of Bradford,[30] using bovine serum albumin as standard.

Membranes were incubated at 25 °C with the radiolabeled ligands in a final volume of 1 mL of 50 mM Tris-HCl, pH 7.5. Incubation times of 60 min were used for the μ-selective peptide [^{3}H]DAMGO and the κ-selective ligand [^{3}H]U69,593, and a 4-h incubation was used with the δ-selective peptide [^{3}H]pCl-DPDPE, in the presence of 5 mM $MgCl_2$ and 0.1 mM phenylmethanesulfonyl fluoride. Nonspecific binding was measured by inclusion of 1 μM naloxone for [^{3}H]DAMGO and [^{3}H]pCl-DPDPE and 1 μM U50,488 for [^{3}H]U69,593. The binding was terminated by filtering the samples through Schleicher & Scheull no. 32 glass-fiber filters using a Brandel 48-well cell harvester. The filters were subsequently washed three times with 3 mL of cold 50 mM Tris-HCl, pH 7.5, and were counted in 2 mL Ecoscint A scintillation fluid. For [^{3}H]U69,593 and [^{3}H]pCl-DPDPE binding, the filters were soaked in 0.25% polyethylenimine for at least 60 min before use.

To determine the IC_{50} values for compounds **9**, **13**, and **15**, membranes were incubated with 12 concentrations of each compound in the presence of the radiolabeled ligand. The thiol **9** was dissolved just before the experiment in a 6 M excess of GSH, which inhibited the oxidation of the thiol **9**.

Incubation of Striatal Membranes with TPCK. In order to reduce nonspecific binding of the affinity ligands, bovine striatal membranes (20 mg of protein) were incubated with 100 μM TPCK in 16 mL of 50 mM Tris-HCl, pH 7.5, at 37 °C for 30 min. TPCK was dissolved in methanol, and the final methanol concentration was 2%. In control experiments, 100 μM TPCK and 2% of methanol did not change the binding of [^{3}H]DAMGO, [^{3}H]U69,593, or [^{3}H]pCl-DPDPE to membranes. After incubation, the membranes were diluted to 40 mL with cold buffer and centrifuged at 39000*g* for 15 min at 4 °C. The washing step was repeated a total of two times. The membranes were then resuspended in 1.6 mL of 50 mM Tris-HCl, pH 7.5, and were incubated immediately with the affinity ligands.

Treating Membranes with the Disulfides 13 and 15. In order to determine the time course of the inhibition of opioid binding to striatal membranes by disulfides 13 and 15, 8–10 mg of TPCK-treated membrane protein was incubated with the affinity ligands at 37 °C for 10 s to 10 min in a final volume of 2 mL of 50 mM Tris-HCl, pH 7.5. The concentrations of 13 and 15 needed to obtain irreversible inhibition of opioid binding were determined by incubating membranes with varying concentrations of the affinity ligands for 5 min at 37 °C. After incubation, the contents of the tubes were diluted to 40 mL with cold buffer and centrifuged at 39000*g* for 15 min at 4 °C. The washing step was repeated a total of four times. Finally, the membranes were resuspended in 2 mL of 50 mM Tris-HCl, pH 7.5, and opioid binding to 0.2 mL of membranes was determined as described above.

Protection of Opioid Binding Sites from Alkylation by Compounds 13 and 15. In order to determine the ability of opioids to protect the opioid binding sites from alkylation, membranes were incubated with a final concentration of 100 nM of either naloxone, morphine, ICI 174,864, naltrindole, or U50,-488 in 1.8 mL of 50 mM Tris-HCl, pH 7.5, at 37 °C for 30 min. After incubation with opioid ligands, the disulfides 13 and 15 were added in a final volume of 2 mL for an additional 2-min incubation at 37 °C. The incubation was terminated by diluting the contents of the tubes to 40 mL with cold buffer, followed by centrifugation at 39000*g* for 15 min. The washing step was repeated four times and opioid binding was determined as described above.

Effect of DTT on the Wash-Resistant Inhibition of Binding by Compounds 13 and 15. Membranes were incubated at 37 °C for 5 min with either compounds 13 or 15, followed by the addition of 40 mM DTT for an additional 10-min incubation at 4 °C in a final volume of 2 mL. The reaction was terminated, the membranes were washed, and binding assays were performed as described above.

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