14β-[(p-Nitrocinnamoyl)amino]morphinones, 14β-[(p-Nitrocinnamoyl)amino]-7,8-dihydromorphinones, and Their Codeinone Analogues: Synthesis and Receptor Activity

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A series of 14β-[(nitrocinnamoyl)amino]codeinones and morphinones, some of which contain a 5β-methyl group, were prepared from 14β-aminocodeinones and 14β-[N-(cyclopropylmethyl)-amino]norcodeinones. The affinities of the target compounds for the μ, δ, and κ opioid receptors were determined by radiolabeled binding experiments using bovine brain membranes. An analogous series of 7,8-dihydrocodeinones and morphinones was prepared and assayed in the same systems. The 3-methoxy derivatives **3** and **4** were more selective than the corresponding morphinones for the μ receptor. The 5β-methylcodeinones **25** and **27** had lower affinity at all receptors than the corresponding morphinones, but the 5β-methylmorphinones had affinities similar to the morphinones **5** and **6**. A similar pattern was observed in the 7,8-dihydro series. Two compounds, 5β-methyl-14β-[(p-nitrocinnamoyl)amino]-7,8-dihydromorphinone, **20** (MET-CAMO), and N-(cyclopropylmethyl)-14β-[(p-nitrocinnamoyl)amino]-7,8-dihydronormorphinone, **22** (N-CPM-MET-CAMO), acted as nonequilibrium ligands in antinociception and membrane binding studies. In mice after icv administration, neither ligand showed any agonist activity but 8–24 h after administration both compounds acted as potent μ antagonists. A Scatchard plot of the effect of N-CPM-MET-CAMO on [^{3}H]DAMGO ([^{3}H]D-Ala^2, (Me)-Phe^4, Gly(ol)^5]enkephalin) binding to bovine striatal membranes showed that there was a significant decrease in the B_{max} value and a marginal effect on the K_d value suggesting that the number of binding sites was reduced. When taken together, these results support the view that **20** and **22** bind covalently to the μ receptor. On the other hand, when N-acetylcysteine and **22** were allowed to react in a buffered solution, **22** was recovered unchanged. Under these conditions no Michael reaction was observed.

Introduction

Recently, papers have appeared describing the biological activities of some 14β-[(p-halocinnamoyl)amino]- and [(p-methylcinnamoyl)amino]-7,8-dihydromorphinones and codeinones.[1,2] The dihydromorphinones showed long-acting narcotic antagonist properties, and the dihydrocodeinones were partial agonists in the mouse tail-flick assay.[1] One member of this series, 14β-[(p-chlorocinnamoyl)amino]-7,8-dihydro-N-(cyclopropylmethyl)normorphinone (clocinnamox, C-CAM), was studied more extensively.[2] The authors concluded that C-CAM was producing its antagonistic effect via a nonequilibrium mechanism and that the "C-14 substituent probably confers a weak Michael acceptor function to the compound".[2] In support of this suggestion Burke[3] reported that, in a Scatchard analysis, C-CAM caused a reduction in the number of binding sites without affecting the K_d value.

Since a nitro group is more electron-withdrawing than either a halogen or a methyl group, it was anticipated that replacing the p-chloro and p-methyl substituents on the cinnamoylamino moiety with a p-nitro group would make the cinnamoylamino function as a better Michael acceptor. The synthesis and some biological properties of such compounds are the subject of this paper.

Chemistry

The synthesis of the 14β-[(p-nitrocinnamoyl)amino]-morphinones and 7,8-dihydromorphinones is shown in Scheme I. 14β-Aminocodeinone (**1**) prepared from thebaine by the method of Kirby and McLean[4] was condensed with p-nitrocinnamoyl chloride to give **3**. Demethylation of **3** with the aid of BBr_3 furnished the morphinone **5**. N-(Cyclopropylmethyl)northebaine[5] was converted to **2** by the Kirby–McLean procedure[4] and was acylated to give **4**. Demethylation of **4** furnished the morphinone **6**. Catalytic hydrogenation of **1** and **2** gave **7** and **8**, respectively, and these 7,8-dihydro intermediates were converted to their respective (p-nitrocinnamoyl)amino derivatives **9**, **10**, **11**, and **12** by acylation with p-nitrocinnamoyl chloride to give **9** and **10** followed by BBr_3 demethylation to give **11** and **12**.

Several years ago it was reported that 5β-methyl-7,8-dihydromorphinone (metopon) was a more effective oral analgesic than morphine.[6] Widespread clinical use was hindered by the difficulty in the original synthesis of the compound, particularly with respect to the introduction of the 5β-methyl group.[7] Since that time greatly improved procedures have been developed for the direct one-step introduction of the 5β-methyl group into thebaine.[8,9] A number of 5β-methyl-14-[(p-nitrocinnamoyl)amino]morphinones and 7,8-dihydromorphinones were prepared by

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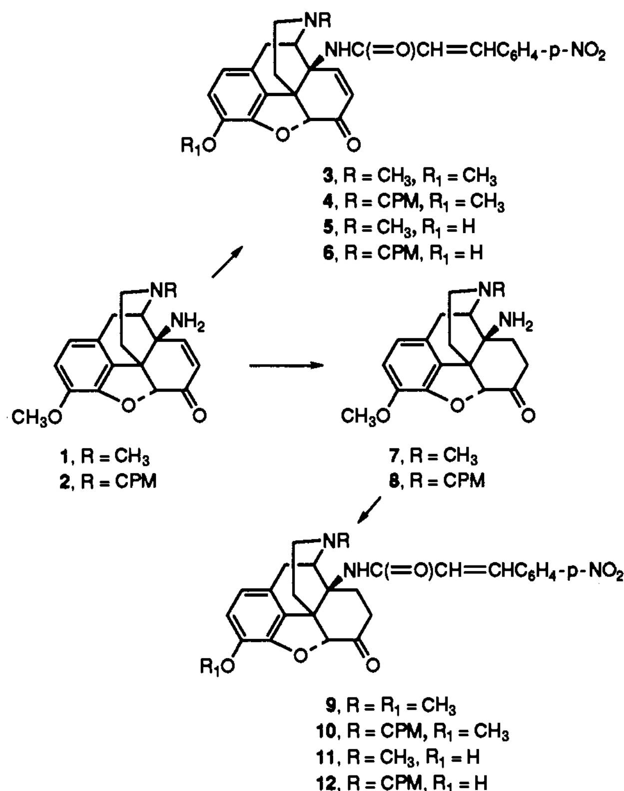
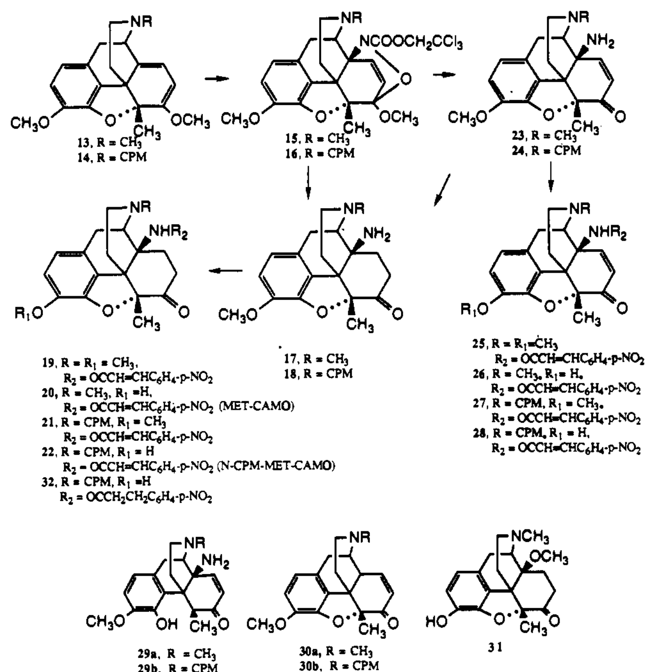
Scheme I



Scheme II



the methods shown in Scheme II. 5β-Methylthebaine[8,9] (13) and *N*-(cyclopropylmethyl)-5β-methylnorthebaine (14) were converted to their respective adducts 15 and 16 by the Kirby–McLean procedure.[4] Reductive cleavage of these adducts using HCl in ethylene glycol followed by zinc reduction in the presence of NH_4Cl and finally hydrolysis gave not only the desired 14β-amino-5β-methylcodeinone derivatives **23** and **24** but also the dihydrothebainones **29a** and **29b** as well as the 5β-methylcodeinones **30a** and **30b**. The structures of the dihydrothebainones, formed by cleavage of the C–O bond, were secured by elemental analyses, IR spectra, and NMR

Table I. Physical Data and Opioid Binding Values of the 14β-[(p-Nitrocinnamoyl)amino]morphinones and Codeinones and Their 5β-Methyl Analogues

compd	R	R_1	R_2	IC_{50} (nM) μ	δ	κ
3	CH_3	CH_3	H	1.01	45.5	68
4	CPM	CH_3	H	1.45	54.0	5.01
5	CH_3	H	H	1.45	4.35	4.5
6	CPM	H	H	7.8	106.0	10.8
25	CH_3	CH_3	CH_3	36.2	374	171
26	CH_3	H	CH_3	0.80	25.9	184
27	CPM	CH_3	CH_3	36.8	542.0	274.0
28	CPM	H	CH_3	1.88	24.7	25.5

spectra. In the latter, the signal for the 5β-methyl group appeared as a doublet rather than as a singlet as in the case of **30a** and **30b** showing that ring cleavage occurred in the formation of **29a** and **29b**. Compounds **30a** and **30b** resulted from cleavage of the C_{14}–N bond.

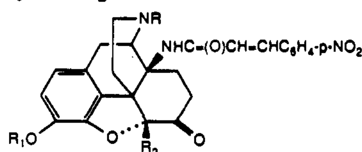
Schmidhammer and his colleagues[9] converted 5β-methylthebaine to 5β-methyl-7,8-dihydrothebainone by catalytic hydrogenation in acetic acid solution using a Pd/C catalyst. If the reduction is carried out in MeOH in the presence of a NaOAc/HOAc buffer, **23** and **24** or preferably the adducts **15** and **16** furnish the codeinone derivatives **17** and **18** without cleavage of the C–O bond. The [(p-nitrocinnamoyl)amino]codeinones **25** and **27** and the corresponding morphinone derivatives **26** and **28** were prepared by the same methods employed to prepare the corresponding analogues shown in Scheme I. The 5β-methyl-7,8-dihydrocodeinones **19** and **21** and the corresponding 5β-methyl-7,8-dihydromorphinone **20** and **22** were prepared the same way.

Biological Results and Discussion

The IC_{50} values at the μ, δ, and κ receptors for the 14β-(cinnamoylamino)morphinones and codeinones are reported in Table I. The 3-methoxy derivatives **3** and **4** had the same affinities as the morphinone **5** at the μ receptor. Surprisingly, the affinity of **6**, a morphinone for all three receptors, was relatively poor. In contrast, the 5β-methylcodeinones **25** and **27** had far lower affinity for all three receptors than the corresponding morphinones **26** and **28**. The latter were about as active as the 5β-desmethyl compound **5**.

A similar SAR pattern was observed in the 7,8-dihydro series (Table II). The dihydrocodeinones **9** and **10** showed about the same binding affinity for the μ receptor as the dihydromorphinones **11** and **12**, but there was a large difference in affinities between the 5β-methyldihydrocodeinones **19** and **21** and the morphinones **20** and **22**. The last two compounds were bioassayed in bovine striatal membranes. The binding affinities of **20** and **22** at the μ receptor compared favorably with the morphinone **5** and with the dihydromorphinones **10** and **12**, which lack a methyl group at the 5-position of the 4,5-epoxymorphinan ring system. Schmidhammer[11] reported that 14β-methoxy-5β-methyl-7,8-dihydromorphinone **31** (14β-methoxymetopon) was 150 times as potent as oxymorphone in the mouse writhing assay and that 14β-methoxynaloxone

Table II. Physical Data and Opioid Binding Values of the 14β-[(p-Nitrocinnamoyl)amino]-7,8-dihydromorphinones and Their 5β-Methyl Analogues



compd	R	R_1	R_2	IC50 (nM) μ	δ	κ
9	CH_3	CH_3	H	0.44	19.7	27.2
11	CH_3	H	H	0.28	3.5	6.6
10	CPM	CH_3	H	3.77	248	39.1
12	CPM	H	H	0.87	21.2	12.1
19	CH_3	CH_3	CH_3	104.4	>1 μM	>1 μM
20[a]	CH_3	H	CH_3	0.33 ± 0	50.9 ± 5.2	116 ± 21
21	CPM	CH_3	CH_3	32.0	672	>1 μM
22[a]	CPM	H	CH_3	0.52 ± 0.07	10.7 ± 0.8	126 ± 2.9
32[b]	CPM	H	CH_3	24.0	22.6	162

[a] Details of the bioassay methods using bovine striatal membranes are described in ref 10. [b] The 14β-[(p-nitrodihydrocinnamoyl)amino] derivative.

Table III. [³H]DAMGO Saturation Binding to Bovine Striatal Membrane Homogenates Treated in the Absence and Presence of 20 nM of 22

treatment	K_D (nM)	B_{max} (fmol bound)/mg of protein
none	0.66 ± 0.04	195 ± 3
N-CPM-MET-CAMO	0.82 ± 13	100 ± 18

[a] Values are the mean ± SEM of three experiments performed in triplicate.

and 14β-methoxynaltrexone were substantially less active as narcotic antagonists in the mouse writhing test.

Both **20**[12] and **22**[13] behaved as nonequilibrium ligands in bovine striatal membranes. The binding affinities of these ligands were in the same range as that of **31**, 14β-methoxymetopon.[11] The latter displayed agonist activity in the mouse writhing assay. In contrast, neither **20** nor **22** produced antinociception in the mouse tail-flick test when administered icv in doses up to 100 nmol, nor did either act as morphine antagonists when given simultaneously with the administration of morphine. However, a 1 nmol dose given by the icv route 8–24 h prior to the administration of 3 nmol of morphine resulted in the complete antagonism of the morphine-induced antinociception.[12] Neither **20** nor **22** antagonized the antinociceptive effects of either δ or κ-mediated antinociception.

A Scatchard plot (Table III) of the effect of **22** on the binding of [³H]DAMGO in bovine striatal membranes resulted in a decrease in the B_{max} without affecting the K_D (Table III). These data are compatible with the hypothesis that **22** binds covalently to the μ receptor.

5β-Methyl-14β-[(dihydrocinnammoyl)amino]-7,8-dihydro-*N*-(cyclopropylmethyl)normorphinone, **32**, was synthesized from p-nitrodihydrocinnamoyl chloride and **18** followed by BBr_3 demethylation. The binding affinities are reported in Table II. This compound behaved as a normal reversible ligand since it washed out of the receptor preparation by normal washing procedures. Since the relevant double bond in **32** was missing, this compound could not behave as a Michael acceptor.

When **22** and *N*-acetylcysteine were allowed to react in an aqueous THF medium at pH 8.0, the ligand **22** was recovered unchanged. In another experiment, the course of the reaction was followed spectrophotometrically by monitoring the reduction in the λ_{max} of (p-nitrocinnamoyl)-amino UV absorption peak. No change in absorption was observed during the course of the reaction.

Although the biochemical and pharmacological evidence supports the hypothesis that **20** and **22** bind covalently to the μ receptor, the chemical evidence does not. We have reported that 14β-[(5-azido-2-nitrophenacetyl)amino]-7,8-dihydromorphinone and its N-CPM analogue behave as nonequilibrium ligands at the μ receptor prior to UV irradiation.[15] It is possible that ligands which show very slow dissociation kinetics but do not necessarily bind covalently to the receptor may cause a reduction in the B_{max} value without affecting the K_d values in a Scatchard analysis. In order to determine whether **20** and **22** bind covalently or pseudoirreversibly to the μ receptor we plan to radiolabel **22** and allow it to bind to the receptor in bovine membrane preparations. If the radioactivity persists after extensive washing the membranes will be separated on SDS polyacrylamide gels using DTT to cleave S–S bonds. If the ligand binds covalently to the now denatured receptor it should be visualized easily.

Experimental Section

Melting points were taken on a laboratory Melt-Temp apparatus and are uncorrected. The ¹H-NMR spectra were run on Varian XL-200 MHz and Varian Unity 500 MHz spectrometers. The IR spectra were run on a Perkin-Elmer Model 298 infrared spectrometer. Microanalyses were performed by Atlantic Microlab, Norcross, GA, and are within ±0.4% of the calculated values.

14β-[(p-Nitrocinnamoyl)amino]codeinone (3). A solution of 100 mg of 14β-aminocodeinone (1)[5] in 25 mL of CH_2Cl_2 containing 75 μL of triethylamine was cooled to −30 °C. A solution of p-nitrocinnamoyl chloride, freshly prepared from 100 mg of p-nitrocinnamic acid and $SOCl_2$, in 25 mL of CH_2Cl_2 was added dropwise with stirring. The reaction mixture was allowed to warm to room temperature and was stirred for an additional 3 h. At the end of this time, a solution of saturated $NaHCO_3$ was added carefully, and the organic layer was separated, washed with $NaHCO_3$ solution and brine, and dried (Na_2SO_4). The solution was taken to dryness, and the residue was chromatographed on a silica gel column using EtOAc/hexane (3:1) as the eluant. There was obtained 110 mg of the desired product, mp 314–316 °C dec. IR (KBr): 3310, 1670, 1620, 1590 cm⁻¹. ¹H NMR ($CDCl_3$): δ 2.45 (3H, s, NCH_3), 3.08 (1H, d, H_9), 3.20–3.29 (1H, d, H_{10}), 3.83 (3H, s, OCH_3), 4.45 (1H, s, H_5), 6.18–6.23 (2H, q, H_8 and NHCOC*H*=), 6.57–6.70 (3H, m, H_1, H_2 and H_7), 7.60–7.68 (3H, m, =C*H*Ar and ArH), 8.19–8.23 (2H, d, ArH). Anal. ($C_{27}H_{25}N_3O_6 \cdot 0.5H_2O$) C, H.

14β-[(p-Nitrocinnamoyl)amino]-*N*-(cyclopropylmethyl)-norcodeinone (4). A solution of 60 mg of 14β-amino-*N*-(cyclopropylmethyl)norcodeinone (2)[10] in 15 mL of CH_2Cl_2 containing 40 μL of triethylamine was cooled to −20 °C with stirring while a solution of p-nitrocinnamoyl chloride, prepared from 70 mg of p-nitrocinnamic acid and $SOCl_2$, in CH_2Cl_2 was added dropwise. The reaction mixture was allowed to warm to room temperature, and after being stirred for 1 h, it was treated with a saturated $NaHCO_3$ solution. The layers were separated, and the organic layer was worked up as described for **3**. After chromatography on silica gel using EtOAc/hexane as the eluant there was obtained 35 mg of pure **4**, mp 165–167 °C. IR (KBr): 3290, 1670, 1630, 1590 cm⁻¹. ¹H NMR ($CDCl_3$): δ 3.09–3.18 (1H, d, H_{10}), 3.37–3.39 (1H, d, H_9), 3.82 (3H, s, OCH_3), 4.96 (1H, s, H_5), 6.15–6.28 (2H, q, H_8 and NHCOC*H*=), 6.51–6.70 (3H, m, H_1, H_2 and H_7), 7.61–7.68 (3H, m, =C*H*Ar and ArH), 8.19–8.23 (2H, d, ArH). Anal. ($C_{30}H_{29}N_3O_6 \cdot 0.5H_2O$) C, H.

14β-[(p-Nitrocinnamoyl)amino]morphinone (5). To a cooled (−20 °C) solution of **3** (76 mg, 0.15 mmol) in CH_2Cl_2 was added BBr_3 (63.6 μL) in CH_2Cl_2 dropwise with stirring. The reaction mixture was slowly brought to room temperature and stirred at this temperature for 4 h. Then 2 mL of MeOH and saturated $NaHCO_3$ solution were added, and the organic layer

was separated. The aqueous layer was extracted two times with a $CHCl_3$/MeOH (4:1) mixture. The combined extracts were washed with brine (20 mL) and dried over anhydrous Na_2SO_4, and the solvent was removed under reduced pressure. The residue was chromatographed over silica gel using EtOAc as the eluant to furnish pure **6** (50 mg) as a pale yellow solid, mp 290 °C dec. ^{1}H NMR ($CDCl_3$): δ 2.43 (3H, s, NCH_3), 3.1–3.2 (1H, d, H_9), 3.25–3.32 (1H, d, H_{10}), 5.05 (1H, s, H_5), 6.2–6.3 (2H, ABq, H_7 and H_8), 6.58–6.80 (3H, m, H_1, H_2 and NHCOCH=), 7.60–7.75 (3H, m, =*CH*Ar and ArH), 8.20–8.25 (2H, d, ArH). Anal. ($C_{28}H_{23}N_3O_8$·0.5H_2O) C, H, N.

14β-[(*p*-Nitrocinnamoyl)amino]-*N*-(cyclopropylmethyl)-normorphinone (6). To a cooled solution (−20 °C) of **4** (90 mg, 0.17 mmol) in CH_2Cl_2 was added BBr_3 (63.6 μL) in CH_2Cl_2 dropwise with stirring. The reaction mixture was stirred at this temperature for 4 h and at room temperature for 30 min. After this period 2 mL of MeOH and saturated $NaHCO_3$ solution were added, and the organic layer was separated. The aqueous layer was extracted two times with a $CHCl_3$/MeOH (4:1) mixture. The combined extracts were then washed with brine (20 mL) and dried over anhydrous Na_2SO_4, and the solvent was removed under reduced pressure. The residue was then chromatographed on silica gel with EtOAc/MeOH (4:1) as the eluant to furnish pure **6** (65 mg) as a pale yellow solid, mp 305–310 °C dec. ^{1}H NMR (DMSO-d_6) δ 4.81 (1H, s, H_5), 6.01–6.07 (1H, d, H_8), 6.42–6.57 (1H, m, H_1, H_2 and H_7), 7.14–7.21 (1H, d, NHCOC*H*=), 7.56–7.58 (1H, d, =*CH*Ar), 7.85–7.89 (2H, d, ArH), 8.25–8.29 (2H, d, ArH), 9.12 (1H, s, HOAr). Anal. ($C_{26}H_{27}N_3O_8$) C, H, N.

14β-[(*p*-Nitrocinnamoyl)amino]-7-8-dihydrocodeinone (9). A solution of 200 mg of dihydrocodeinone (**7**) and triethylamine (95 μL) in 40 mL of dry CH_2Cl_2 was cooled to −20 °C. To this solution was added *p*-nitrocinnamoyl chloride, freshly prepared from 193 mg of *p*-nitrocinnamic acid and $SOCl_2$, in 15 mL of CH_2Cl_2 dropwise with stirring. The reaction mixture was slowly brought to room temperature and stirred for 2.5 h. The a solution of saturated $NaHCO_3$ was added, and the organic layer was separated. It was washed with saturated $NaHCO_3$ solution (15 mL) and water (2 × 15 mL) and dried (Na_2SO_4), and the solvent was evaporated under reduced pressure. The solid residue was chromatographed on silica gel using EtOAc as the eluant to furnish 150 mg of the desired product **9**, mp 278–280 °C. IR (KBr): 3350, 2920, 1710, 1670, 1620, 1500 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 2.44 (3H, s, NCH_3), 3.06–3.07 (1H, d, H_9), 3.23–3.27 (1H, d, H_{10}), 3.89 (3H, s, $ArOCH_3$), 4.95 (1H, s, H_5), 6.68–6.77 (3H, m, H_1, H_2, and NHCOC*H*=), 7.70–7.73 (3H, m, =*CH*Ar and ArH), 8.25–8.27 (2H, d, ArH). Anal. ($C_{27}H_{27}N_3O_8$·0.25H_2O) C, H, N.

14β-[(*p*-Nitrocinnamoyl)amino]-7-8-dihydro-*N*-(cyclopropylmethyl)norcodeinone (10). A solution of 250 mg of **8**[14] and triethylamine (100 μL) in dry CH_2Cl_2 was cooled to −20 °C. A solution of *p*-nitrocinnamoyl chloride, freshly prepared from *p*-nitrocinnamic acid (200 mg) and $SOCl_2$, in CH_2Cl_2 was added dropwise with stirring. The reaction mixture was slowly brought to room temperature and stirred for 1 h. After this period the reaction mixture was washed with saturated $NaHCO_3$ solution and water and dried (Na_2SO_4). The solvent was evaporated to leave a solid residue, which was chromatographed on silica gel using EtOAc/hexane (3:2) as the eluant. There was obtained 230 mg of **10**, mp 130–133 °C. IR (KBr): 3400, 2910, 2810, 1710, 1660, 1620, 1600, 1500 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 3.10–3.14 (1H, d, H_{10}), 3.35–3.36 (1H, d, H_9), 3.88 (3H, s, OCH_3), 4.93 (1H, s, H_5), 6.63–6.76 (3H, m, H_1, H_2 and NHCOCH=), 7.67–7.72 (3H, m, =*CH*Ar and ArH), 8.25–8.26 (2H, d, ArH). Anal. ($C_{30}H_{31}N_3O_6$·H_2O) C, H, N.

14β-[(*p*-Nitrocinnamoyl)amino]-7-8-dihydromorphinone (11). To a cooled (−20 °C) solution of **9** (120 mg) in dry CH_2Cl_2 (40 mL) was added BBr_3 (93 μL) dissolved in CH_2Cl_2 dropwise with stirring. The mixture was slowly brought to room temperature and stirred for 1.5 h. After this period MeOH (5 mL) and saturated $NaHCO_3$ solution were added, and the organic layer was separated. The aqueous layer was further extracted twice with $CHCl_3$/MeOH (3:1) (2 × 40 mL). The combined extracts were washed with saturated $NaHCO_3$ solution (15 mL) and water (15 mL) and dried, and the solvent was removed under reduced pressure. The solid residue was chromatographed on silica gel using EtOAc/MeOH (19:1) as the eluant to yield 50 mg of **11** as a light yellow solid, mp 245–247 °C. IR (KBr): 3350, 2910, 1705, 1660, 1620, 1500 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 2.42 (3H, s, NCH_3), 5.07 (1H, s, H_5), 6.59–6.75 (3H, m, H_1, H_2 and NHCOCH=), 7.49–7.51 (2H, d, ArH), 7.62–7.65 (1H, d, =*CH*Ar), 8.16–8.18 (2H, d, ArH). Anal. ($C_{28}H_{25}N_3O_8$·0.5H_2O) C, H, N.

5β-Methylthebaine (13). A solution of 10 g of thebaine in 500 mL of dry THF was stirred and cooled to −78 °C in a nitrogen atmosphere, and to it was added a hexane solution of 3.08 g of n-BuLi. After the solution was stirred for 20 min, 6.1 g of $(CH_3)_2SO_4$ was added in one portion, and the reaction mixture was allowed to warm to room temperature over a 3-h period. Water was added, and the THF was removed under reduced pressure to leave a suspension which was extracted with $CHCl_3$. The extract was washed with H_2O and dried. The residue left after evaporation of the $CHCl_3$ was crystallized from EtOH to give 7.5 g (72%) of the desired product, mp 159–160 °C (lit.[9] mp 154–157 °C).

***N*-(Cyclopropylmethyl)-5β-methylnorthebaine (14).** This compound was prepared from 9.1 g of *N*-cyclopropylnorthebaine, 2.5 g of BuLi, and 4.9 g of dimethyl sulfate by the same procedure described above in 95% yield, mp 116 °C, after crystallization from EtOH. IR (KBr): 3000, 2900, 2800, 1600, 1450, 1250 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 1.72 (3H, s, 5β-CH_3), 2.43–2.46 (2H, d, NCH_2), 3.53 (3H, s, C_6-OCH_3), 3.80 (3H, s, C_3-OCH_3), 4.88–4.92 (1H, d, H_8), 5.51–5.59 (1H, d, H_7), 6.55–6.63 (2H, m, H_1 and H_2). Anal. Calcd for ($C_{23}H_{27}NO_3$) C, H, N.

14β-[(*p*-Nitrocinnamoyl)amino]-7,8-dihydro-*N*-(cyclopropylmethyl)normorphinone (12). To a cooled (−20 °C) solution of **10** (100 mg) in CH_2Cl_2 was added BBr_3 (72 μL) dissolved in CH_2Cl_2 dropwise with stirring. The reaction mixture was slowly brought to room temperature and stirred for 2 h. After this period MeOH (3 mL) and a saturated solution of $NaHCO_3$ were added, and the organic layer was separated. The aqueous layer was extracted again with $CHCl_3$/MeOH (3:1) (2 × 25 mL), the combined extracts were washed with saturated $NaHCO_3$ solution (15 mL) and water (15 mL) and dried (Na_2SO_4), and the solvent was evaporated. The residue was chromatographed on silica gel using EtOAc/hexane (3:2) as the eluant to give the desired product **12** (45 mg), mp 260–263 °C. IR (KBr): 3250, 2910, 1710, 1660, 1620, 1500 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 4.9 (1H, s, H_5), 6.58–6.73 (3H, m, H_1, H_2 and NHCOC*H*=), 7.64–7.67 (3H, m, =*CH*Ar, ArH), 8.22–8.23 (2H, d, ArH). Anal. ($C_{29}H_{29}N_3O_8$·0.75H_2O) C, H, N.

5β-Methyl-19-[(2,2,2-trichloroethoxy)carbonyl]-6,14-dihydro-6,14-(epoxyimino)thebaine (15). A solution of 6.0 g of 5β-methylthebaine (**13**) in 200 mL of EtOAc was added to a cooled (0 °C) solution of 5.89 g of $NaIO_4$ in 150 mL of 0.5 M NaOAc solution at pH 6.0. To the vigorously stirred solution there was added 3.8 g of 2,2,2-trichloroethyl *N*-hydroxycarbamate in small portions over a period of 30 min. The mixture was stirred at 0 °C for 3 h and then treated with excess aqueous $NaHCO_3$. The organic layer was separated, washed with a solution of $Na_2S_2O_3$ and brine, and dried (Na_2SO_4). Evaporation of the EtOAc solution left an oily residue which was covered with a small amount of MeOH and stored at 0 °C. The adduct, which crystallized, was filtered and dried, 7.2 g (mp 157–158 °C). IR (KBr): 2910, 1710, 1610 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 1.69 (3H, s, 5β-CH_3), 3.48 (3H, s, OCH_3), 3.79 (3H, s, $ArOCH_3$), 4.59–4.62 (2H, q, OCH_2CCl_3), 5.97–6.17 (2H, ABq, H_8 and H_7), 6.52–6.56 (2H, H_1 and H_2). Anal. ($C_{23}H_{25}Cl_3N_2O_6$) C, H, N.

***N*-(Cyclopropylmethyl)-5β-methyl-19-[(trichloroethoxy)carbonyl]-6,14-dihydro-6,14-(epoxyimino)northebaine (16).** Using the procedure described above, 6 g of 5β-methyl-*N*-(cyclopropylmethyl)northebaine (**14**) afforded 5.7 g of the adduct **16**, mp 132–134 °C. IR (KBr): 2990, 2915, 1720 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 1.67 (3H, s, 5β-CH_3), 3.57 (3H, s, OCH_3), 3.77 (3H, s, $ArOCH_3$), 4.95–5.12 (2H, ABq, OCH_2CCl_3), 6.01–6.17 (2H, ABq, H_8 and H_7), 6.51–6.95 (2H, ABq, H_1 and H_2). Anal. ($C_{26}H_{29}Cl_3N_2O_6$·0.5H_2O) C, H, N.

14β-Amino-5β-methylcodeinone (23, and 29a, and 30a). To a solution of 0.26 M HCl in 40 mL of ethylene glycol was added 2.0 g of the adduct **15**. After the solution was stirred for 3 h at room temperature there was added slowly 900 mg of $(NH_4)_2CO_3$ and 1.7 g of zinc powder, and the suspension was stirred at 70 °C for 1 h. The excess zinc was filtered off, and the filtrate was made alkaline with $NaHCO_3$ solution. After dilution with H_2O, the reaction mixture was extracted with 3 × 50 mL of $CHCl_3$.

The combined extracts were washed with brine, dried, and evaporated. The residue was dissolved in 20 mL of MeOH, 10 mL of H_2O, and 1 mL of 6 N HCl and heated under reflux for 30 min. After cooling, the reaction mixture was made alkaline with $NaHCO_3$ solution, washed with H_2O, and dried. Evaporation of the $CHCl_3$ left an oil which was chromatographed on a silica gel column using EtOAc/MeOH (9:1) as the eluant. There was obtained 327 mg (25%) of **23**, mp 149–150 °C, 528 mg (40%) of **29a**, mp 170–172 °C, and 62 mg (5%) of **30a**, mp 175–177 °C. Spectral data for **27**. IR (KBr): 3350, 2950, 1675, 1600 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.72 (3H, s, 5β-CH_3), 2.40 (3H, s, NCH_3), 2.83–2.86 (1H, d, H_9), 3.16–3.25 (1H, d, H_{10}), 3.80 (3H, s, $ArOCH_3$), 5.99–6.04 (1H, d, H_8), 6.58–6.66 (3H, m, H_7, H_1 and H_2). Anal. ($C_{19}H_{22}N_2O_3 \cdot 0.25H_2O$) C, H, N.

For **29a**. IR (KBr): 3480, 2910, 1655 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.51–1.55 (3H, d, 5β-CH_3), 2.32 (3H, s, NCH_3), 3.81 (3H, s, OCH_3), 4.52–4.55 (1H, q, H_5), 5.75–5.8 (1H, d, H_8), 6.63–6.64 (1H, d, H_7), 6.67–6.69 (2H, m, H_1, H_2). Anal. ($C_{19}H_{20}N_2O_3 \cdot 0.25H_2O$) C, H, N.

For **30a**. IR (KBr) 2900, 1665, 1600 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.61 (3H, s, 5β-CH_3), 2.45 (3H, s, NCH_3), 3.82 (3H, s, OCH_3), 5.98–6.04 (1H, dd, H_8), 6.56–6.67 (3H, m, H_7, H_1 and H_2).

14β-Amino-5β-methyl-7,8-dihydrocodeinone (17). A solution of 500 mg of the adduct **15** in 15 mL of MeOH and 25 mL of a 2 N HOAc/1.5 N NaOAc solution was hydrogenated in the presence of 150 mg of 5% Pd/C at 50 psi. After 10 h, the catalyst was filtered off and washed with a small amount of 2 N HOAc. The combined filtrates were made alkaline with NH_4OH and extracted with 3 × 25 mL of $CHCl_3$. The extracts were combined, washed with H_2O, dried, and evaporated. The residue was chromatographed on a silica gel column using EtOAc/MeOH (8:1) as the eluant. There was obtained 220 mg of pure **17**, mp 168–169 °C. IR (KBr): 3380, 3340, 1710 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.63 (3H, s, 5β-CH_3), 2.33 (3H, s, NCH_3), 3.03–3.12 (2H, m, H_9, H_{10}), 3.82 (3H, s, OCH_3), 6.51–6.64 (2H, m, H_1, H_2). Anal. ($C_{19}H_{22}N_2O_3$): C, H, N.

14β-Amino-5β-methyl-7,8-dihydro-*N*-(cyclopropylmethyl)norcodeinone (18). A solution of 2 g of the adduct **16** dissolved in 20 mL of MeOH and 25 mL of 2 N AcOH/1.3N NaOAc was hydrogenated at 50 psi in the presence of 5% Pd/C (500 mg) as the catalyst. After 8 h, the catalyst was filtered off, washed with 2 N AcOH, treated with concentrated NH_4OH solution, and extracted with $CHCl_3$ (3 × 30 mL), and the extract was washed with water (2 × 20 mL) and dried. The solvent was removed in vacuo. The residue was chromatographed on silica gel using EtOAc as the eluant. There was obtained 700 mg of **18**, mp 92–95 °C. IR (KBr): 3340, 3000, 2940, 1710, 1600 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.68 (1H, s, 5β-CH_3), 3.86 (3H, s, OCH_3), 6.56–6.58 (1H, d, H_2), 6.64–6.66 (1H, d, H_1). Anal. ($C_{22}H_{23}N_2O_3$) C, H, N.

5β-Methyl-14β-[(*p*-nitrocinnamoyl)amino]-7,8-dihydrocodeinone (19). A solution of 14β-amino-5β-methyl-7,8-dihydrocodeinone **17** (400 mg, 1.21 mmol) in dry CH_2Cl_2 (50 mL) and triethylamine (203 μL) was cooled to −60 °C with stirring. A solution of *p*-nitrocinnamoyl chloride, freshly prepared from the acid (353 mg) and thionyl chloride in dry CH_2Cl_2, was added dropwise to the stirred solution, and the mixture was slowly brought to room temperature and stirred for 3 h. Aqueous saturated $NaHCO_3$ was added, and the separated organic layer was washed with $NaHCO_3$ solution (2 × 15 mL) and water (2 × 15 mL) and dried (Na_2SO_4). The crude product obtained after the evaporation of the solvent was chromatographed on silica gel using EtOAc/hexane (9:1) as the eluant to furnish 350 mg (57%) of **19**, which was further crystallized from MeOH, mp 283–285 °C. IR (KBr): 3240, 2920, 1700, 1665, 1625, 1590 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.63 (3H, s, 5β-CH_3), 2.42 (3H, s, NCH_3), 3.86 (3H, s, OCH_3), 6.67–6.70 (3H, m, H_1, H_2 and NHCOC*H*=), 7.64–7.71 (m, 3H, COCH=C*H* and ArH), 8.21–8.26 (2H, d, ArH). Anal. ($C_{28}H_{29}N_3O_6$) C, H, N.

5β-Methyl-14β-[(*p*-nitrocinnamoyl)amino]-7,8-dihydromorphinone (20). The procedure described for **26** was employed for preparing **20**. A 130-mg portion of **19** gave 105 mg of **20** after chromatography over silica gel using EtOAc as the eluant: mp 296–300 °C dec. IR (KBr): 1700, 1660 cm^{-1}. ^1H NMR (DMSO-d_6): δ 1.56 (3H, s, 5β-CH_3), 2.25 (3H, s, NMe), 3.02–3.07 (1H, d, H_9), 6.51–6.57 (2H, ABq, H_1, H_2), 7.82–7.86 (2H, d, ArH), 8.23–8.28 (2H, d, ArH). Anal. ($C_{27}H_{27}N_3O_6 \cdot 0.25H_2O$) C, H, N.

5β-Methyl-14β-[(*p*-nitrocinnamoyl)amino]-7,8-dihydro-*N*-(cyclopropylmethyl)norcodeinone (21). This compound was prepared by the procedure described for **19**; 350 mg (0.953 mmol) of **18** was treated with the acid chloride obtained from 280 mg of *p*-nitrocinnamic acid to furnish 300 mg of **21** after chromatography over silica gel with EtOAc/hexane (7:3) as eluant, mp 265–268 °C. IR (KBr): 3250, 1700, 1660, 1620 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.63 (3H, s, 5β-CH_3), 2.36 (2H, d, NCH_2), 3.81 (3H, s, OCH_3), 6.64–6.77 (3H, m, H_1, H_2 and NHCOCH=), 7.60–7.64 (3H, m, =C*H*Ar and ArH), 8.17–8.21 (2H, d, ArH). Anal. ($C_{31}H_{33}N_3O_6$) C, H, N.

5β-Methyl-14β-[(*p*-nitrocinnamoyl)amino]-7,8-dihydro-*N*-(cyclopropylmethyl)normorphinone (22). The procedure described for **20** was used to prepare **22**. One hundred milligrams of **21** was treated with BBr_3 (52 μL). The yellow residue obtained after evaporation of the solvent was purified by chromatography on silica gel with EtOAc/hexane (7:3), which was crystallized from $CHCl_3$ to yield 57 mg of **22**, mp 210–215 °C. IR (KBr): 1700, 1665 cm^{-1}; ^1H NMR (DMSO-d_6): δ 1.4 (3H, s, 5β-CH_3), 2.77–2.88 (1H, d, H-9), 6.32–6.46 (2H, ABq, H_1, H_2), 7.07–7.14 (1H, d, NHCOC*H*=), 7.36–7.44 (1H, d, =C*H*Ar), 7.60–7.64 (2H, d, ArH), 8.05–8.09 (2H, d, ArH). Anal. ($C_{30}H_{31}N_3O_6 \cdot 0.25H_2O$) C, H, N.

5β-Methyl-14β-[(nitrodihydrocinnamoyl)amino]-7,8-dihydro-*N*-(cyclopropylmethyl)normorphinone (32). Using the procedure described for the preparation of **19**, 150 mg of the codeinone **18** and 130 mg of *p*-nitrodihydrocinnamoyl chloride furnished 60 mg of the *p*-nitrodihydrocinnamoyl derivative. Demethylation of the amide with 45 μL of BBr_3 in 30 mL of dry $CHCl_3$ at −20 °C gave a crude product which was purified on a preparative TLC plate using EtOH/hexane (3:2) as the developing solvent. There was obtained 15 mg of pure **32**, mp 264–266 °C dec. ^1H NMR ($CDCl_3$): δ 1.55 (3H, s, 5β-CH_3), 6.54–6.56 (1H, d, H_1), 6.71–6.73 (1H, d, H_2), 7.54–7.56 (1H, d, ArH), 8.00–8.10 (1H, d, ArH). Anal. ($C_{30}H_3N_3O_6 \cdot 0.5H_2O$) C, H, N.

14β-Amino-5β-methyl-*N*-(cyclopropylmethyl)norcodeinone (24), 29, and 30b). Using the same procedure employed for the preparation of **23**, there was obtained 170 mg (20%) of **24**, mp 150–153 °C, 300 mg (30%) of **29b**, mp 178–180 °C, and 85 mg (10%) of **30b**, mp 85–86 °C. Spectral data for **24**. IR (KBr): 3350, 2920, 1660, 1630 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.77 (3H, s, 5β-CH_3), 3.8 (3H, s, $ArOCH_3$), 6.04–6.06 (1H, d, H_8), 6.59 (1H, d, H_7), 6.63–6.66 (2H, m, H_1 and H_2). Compound **24** was used without further purification for the preparation of **27**.

For **29b**. IR (KBr): 3400, 2910, 1660 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.51–1.53 (3H, d, 5β-CH_3), 3.78 (3H, s, OCH_3), 4.50–4.52 (1H, q, 5-H), 5.75–5.77 (1H, d, H_8), 6.47–6.49 (1H, d, H_7), 6.60–6.66 (2H, m, H_1 and H_2). Anal. ($C_{22}H_{28}N_2O_3$): C, H, N.

For **30b**. IR (KBr): 2910, 1665, 1600 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.60 (3H, s, 5β-CH_3), 3.80 (3H, s, $ArOCH_3$), 5.98–6.01 (1H, dd, H_8), 6.53–6.62 (3H, m, H, H_1 and H_7). Anal. ($C_{22}H_{25}N_2O_3$) C, H, N.

5β-Methyl-14β-[(*p*-nitrocinnamoyl)amino]codeinone (25). A solution of 100 mg of **23** in 20 mL of dry CH_2Cl_2 and 55 μL of triethylamine was cooled to −60 °C. A solution of *p*-nitrocinnamoyl chloride prepared from 118.5 mg of *p*-nitrocinnamic acid and $SOCl_2$ in 10 mL of CH_2Cl_2 was added dropwise with stirring. The reaction mixture was allowed to warm to room temperature, and after 5 h a solution of saturated $NaHCO_3$ was added. The layers were separated, and the organic layer was washed with H_2O and dried (Na_2SO_4). The solvent was evaporated, and the residue was chromatographed on a column of silica gel using EtOAc/hexane (9:1) as the eluant. There was obtained 70 mg of **25**, mp 310 °C dec, after crystallization from MeOH. IR (KBr): 3280, 1670, 1660, 1590 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.73 (3H, s, 5β-CH_3), 2.51 (3H, s, NCH_3), 3.82 (3H, s, OCH_3), 6.34 (2H, d, H_8, NHCOC*H*=), 6.59–6.71 (3H, m, H_7, H_1, H_2), 7.58–7.69 (3H, m, ArH, =C*H*Ar), 8.21–8.25 (2H, d, ArH). Anal. ($C_{28}H_{27}N_3O_6$) C, H, N.

5β-Methyl-14β-[(*p*-nitrocinnamoyl)amino]normorphinone (26). To a cooled (−20 °C) solution of **25** (100 mg) in CH_2Cl_2 was added a solution of BBr_3 (75 μL) in CH_2Cl_2 dropwise with stirring. The mixture was slowly brought to room temperature and stirred for 1.5 h; after this period 2 mL of MeOH

and $NaHCO_3$ solutions were added and the organic layer separated. The aqueous layer was extracted further with $CHCl_3$/MeOH (3:1) (2 × 25 mL), the combined extracts were washed with water and dried, and the solvent was evaporated using a rotary evaporator. The residue obtained was chromatographed on silica gel using EtOAc/hexane (3:2) as the eluant to furnish pure **26** (75 mg), mp 315 °C dec. IR (KBr): 3350, 2910, 1660, 1630, 1590 cm^{-1}. ^1H NMR (DMSO-d_6): δ 1.59 (3H, s, 5β-CH_3), 6.04–6.06 (1H, d, H_8), 6.49–6.51 (2H, m, H_1 and H_2), 6.88–6.90 (1H, d, H_7), 7.26–7.29 (1H, d, =C*H*Ar), 7.46–7.49 (1H, d, NHCOC*H*=), 7.86–7.88 (1H, d, ArH), 8.26–8.28 (1H, d, ArH). Anal. ($C_{27}H_{25}N_3O_8$·0.75H_2O) C, H, N.

5β-Methyl-14β-[(*p*-nitrocinnamoyl)amino]-*N*-(cyclopropylmethyl)norcodeinone (27). The method described for the preparation of **25** was used. Two hundred milligrams of **24** furnished 120 mg of the desired amide **27**, mp 293–295 °C dec. IR (KBr): 3280, 1670, 1630 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.71 (3H, s, 5β-CH_3), 3.81 (3H, s, OCH_3), 6.30–6.71 (5H, m, H_1, H_2, H_7, H_8 and COC*H*=), 7.61–7.71 (3H, m, ArH, =C*H*Ar), 8.19–8.20 (2H, d, ArH). Anal. ($C_{31}H_{31}N_3O_8$·H_2O) C, H, N.

5β-Methyl-14β-[(*p*-nitrocinnamoyl)amino]-*N*-(cyclopropylmethyl)normorphinone (28). To a solution of 60 mg of the codeinone **27** in 40 mL of CH_2Cl_2 was added dropwise with stirring a solution of 83 mg of BBr_3 in 20 mL of CH_2Cl_2. The mixture was allowed to warm to room temperature and was stirred for 1 additional h. A solution of saturated $NaHCO_3$ was added cautiously, and the organic layer was separated. It was washed with $NaHCO_3$ solution and H_2O and dried. After chromatography on a silica gel column using EtOAc/hexane (3:2) as the eluant there was obtained 20 mg of pure **28**, mp 265–270 °C dec. IR (KBr): 3250, 1660 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.67 (3H, s, β-CH_3), 3.05–3.15 (1H, d, H_{10}), 6.26–6.31 (1H, d, H_8), 6.44–6.66 (4H, m, H_7, NHCOC*H*=, H_1 and H_2), 7.59–7.61 (3H, m, =C*H*Ar, ArH), 8.17–8.22 (2H, d, ArH). Anal. ($C_{30}H_{29}N_3O_8$·1.2H_2O) C, H, N.

Addition Reaction of *N*-Acetylcysteine to 22. Twenty-five milligrams of **22** was dissolved in 3 mL of THF and 3 mL of tissue buffer pH 8.0 and stirred at 37 °C. To this solution was added *N*-acetylcysteine (7.8 mg) dissolved in buffer and the resulting solution stirred at 37 °C for 24 h. The reaction mixture was then neutralized with pH 5.5 buffer and extracted with $CHCl_3$. The $CHCl_3$ extracts were washed with water, and the residue obtained after the evaporation of the solvent was found to be the starting material **22** (17 mg, 76% recovery) from TLC. The aqueous layer was evaporated, and the residue was dissolved in MeOH. Analysis (TLC, IR) of the residue after evaporation of the MeOH showed that no adduct between *N*-acetylcysteine and **22** was formed.

The addition of *N*-acetylcysteine to **22** was followed spectrophotometrically. A 10^{-3} M stock solution of *N*-acetylcysteine and **22** were made in 50% THF and buffer (pH 7.4). One mL of each were then mixed and diluted to give a 10^{-4} M solution. The λ_{max} of **22** was 314 nm. Ultraviolet spectra of this solution were taken periodically and compared to the spectra of starting materials. There was no shift in the absorption peaks due to (*p*-nitrocinnamoyl)amino group after 24 h indicating that no Michael addition had occurred.

Radioligand Binding Assays. Calf frontal cortex or calf caudate tissue was obtained by dissection of calf brain over ice and was immediately homogenized. Two grams of tissue was suspended in 30 mL of 50 mM Tris–HCl buffer, pH 7.7, at 25 °C containing 10 mM $MgSO_4$ and 0.5 mM EDTA. Following centrifugation for 15 min at 14000*g* the samples were resuspended in buffer and incubated at 37 °C for 15 min. The homogenates were centrifuged for 10 min at 14000*g*, resuspended in buffer, and recentrifuged and stored as a pellet at −30 °C until use.

Radioligand binding assays were performed in triplicate in a final volume of 2 mL of 50 mM Tris–HCl, pH 7.5, 10 mM $MgSO_4$, and 0.5 mM EDTA. The tissue concentrations used were 10 mg wet weight of calf frontal cortex for μ and κ binding sites and 12 mg wet weight of calf caudate for the δ binding site; tissue was added last. Nonspecific binding was determined using 1 μM naloxone for [^{3}H]DAMGO,[16] [^{3}H]DPDPE,[17] and [^{3}H]U-69,593.[18] Incubation times used were 30 min for the μ-selective peptide [^{3}H]DAMGO and the κ-selective ligand [^{3}H]U69,593 at 37 °C and 4 h for the δ-selective peptide [^{3}H]DPDPE at 25 °C. The IC_{50} values for all the compounds, except for **20** and **22** (Table I and Table II), were determined by incubating membranes with eight concentrations of the ligands in the presence of either 0.8 nM [^{3}H]DAMGO, 1 nM [^{3}H]DPDPE, or 1 nM [^{3}H]U69,593 to measure their affinity for μ, δ, and κ receptor sites, respectively. The binding was terminated by rapid filtration through Whatman GF/B glass fiber filters presoaked in 0.1% polyethyleneimine for 30 min for [^{3}H]DPDPE and [^{3}H]U69,593 binding and rinsed with two 2-mL aliquots of cold 50 mM Tris–HCl buffer, pH 7.5. Filters were counted in 5 mL of Ecoscint A scintillation fluid.

The IC_{50} values for **20** and **22** were determined as described previously.[10]

[^{3}H]DAMGO Saturation Binding to Bovine Striatal Membranes Treated in the Absence and Presence of 20 nM N-CPM-MET-CAMO. In a final volume of 2 mL of 50 mM Tris–HCl, pH 7.5, 10 mg of bovine striatal membrane protein was incubated with 20 nM **22** for 60 min at 25 °C. Control membranes were treated under identical conditions except for the omission of **22**. The reaction was terminated by diluting the contents of the tubes to 40 mL with cold 50 mM Tris–HCl, pH 7.5. The membranes were centrifuged at 39000*g* for 15 min at 4 °C, and the wash step was repeated four times. The membranes were resuspended in 2 mL of 50 mM Tris–HCl, pH 7.5, and were used in [^{3}H]DAMGO saturation binding assays.

[^{3}H]DAMGO saturation binding was measured by incubating 200 μL of resuspended membranes with [^{3}H]DAMGO at concentrations ranging from 0.05 to 6.4 nM in a final volume of 1 mL of 50 mM Tris–HCl, pH 7.5. After a 60-min incubation at 25 °C, the membranes were filtered through Schleicher & Schuell No. 32 glass fiber filters. The filters were washed three times with 4 mL of cold 50 mM Tris–HCl, pH 7.5. Bound radioactivity was determined by liquid scintillation spectrophotometry in 2 mL of Ecoscint A scintillation fluid (National Diagnostics). Nonspecific binding was measured by inclusion of 10 μM naloxone. Protein concentration was determined by the method of Bradford.[19] Saturation data were analyzed by nonlinear regression analysis using the LIGAND program.[20] The experiment was performed in triplicate and was replicated three times.

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