Possible Contribution of a Glutathione Conjugate to the Long-Duration Action of β-Funaltrexamine

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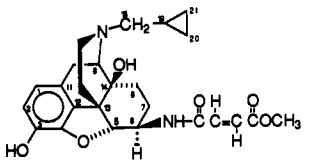
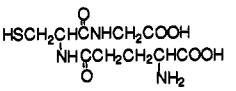
Received June 21, 1993•

The fumaramate derivative of naltrexone, β-funaltrexamine (β-FNA), is a highly selective long-lasting μ opioid receptor antagonist that is active both *in vitro* and *in vivo*, presumably as a result of covalent binding to a μ receptor-based sulfhydryl group. Glutathione, which occurs in significant levels in brain and liver, was found to undergo a Michael-type reaction with β-FNA in the test tube to give a stable conjugate **3** which occurred as an isomeric mixture. When tested in the GPI and MVD smooth muscle preparations, **3** was found to possess one-tenth the agonist activity of β-FNA is both tissues, but showed no irreversible antagonist activity. The same result was found for the cysteine conjugate **4**, except for some irreversible antagonism in the MVD. Both conjugates antagonize the antinociceptive effect of morphine in the mouse radiant heat tail-flick assay on icv administration. This antagonism persisted and actually increased over 24 h and generally paralleled the duration profile of β-FNA. On sc administration, β-FNA and **3** showed similar duration of antagonistic effect, while **4** exhibited only marginal activity at the early time interval. When the compounds are compared by the dose to produce equivalent antagonism, β-FNA and **3** appeared equally effective and accessible by either route, whereas **4** showed a large difference between the two routes. It is possible that the ultra-long antagonism of the conjugates may result from their enzymatic conversion to β-FNA in the central nervous system in view of the fact that conjugate **5**, which cannot be converted to β-FNA, did not produce antagonism of long duration *in vivo*. Alternatively, the protracted antagonism could arise from sequestration of **3** and **4** in tissue compartments that interface with μ opioid receptors.

The nonequilibrium opioid receptor antagonist, β-funaltrexamine[1] (**1**, β-FNA) is an important pharmacologic tool in opioid research.[2,3] In addition to being highly selective for the μ opioid receptor in smooth muscle preparations, it possesses an ultralong antagonism of μ agonist activity *in vivo*, presumably by covalent binding to a receptor-based sulfhydryl group. This may occur through Michael addition to the fumaramate moiety of **1**.



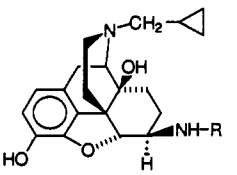
1, β-FNA



2, Glutathione

In view of the high levels of glutathione (**2**, GSH) in the central nervous system (CNS)[4] and the fact that β-FNA has been reported[5] to react with Cys at physiological pH *in vitro*, it is likely that GSH conjugates of β-FNA may be formed in the brain. The pharmacologic effect of such a β-FNA conjugate **3** in the brain was not known, and one can envisage several possible consequences of conjugation in the CNS. First, the conjugate **3** may not behave as an opioid antagonist at opioid receptors. Secondly, **3** may act as an antagonist, but due to its zwitterionic character it may not exit the CNS freely. Under such circumstances, **3** could possess a long duration of action if the intact molecule is an active opioid antagonist. Thirdly, **3** might undergo a retro-Michael addition reaction to regenerate β-FNA **1** which would afford long-lasting antagonism due to alkylation of μ opioid receptors.

Here we report on the pharmacologic evaluation of a β-FNA–GSH conjugate **3** and related conjugates (**4**, **5**) in order to obtain insight into the possible role of GSH on the action of β-FNA.



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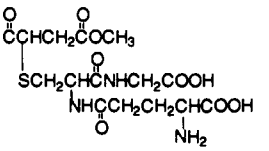
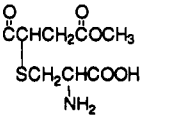
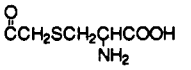
3	
4	
5	
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• Abstract published in *Advance ACS Abstracts*, October 15, 1993.

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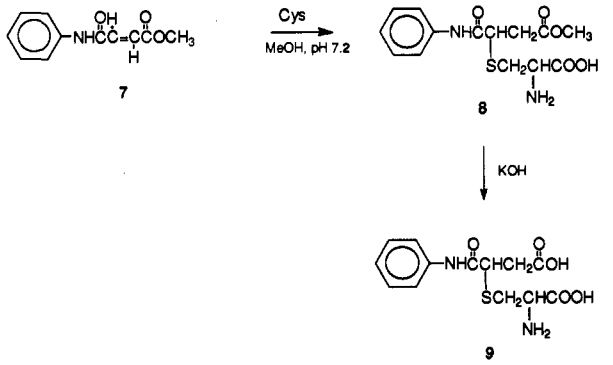
Scheme I



7 8 9

Chemistry

The conjugates 3 and 4 were synthesized from β-FNA (1) and GSH (2) or Cys in aqueous methanol at 25 °C and physiologic pH. The condensation involved Michael addition of the sulfhydryl of GSH or Cys to the fumaramate moiety. Conjugate 5 was synthesized by the reaction of the iodoacetamide derivative 6 with Cys.

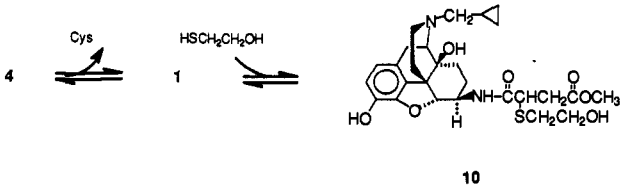
All three products (3–5) were purified by preparative reverse-phase HPLC. Products 3 and 4 each were bicomponent isomeric mixtures. The close retention times of each of the isomeric components, their identical UV spectra using a photodiode detector, and the NMR and MS data suggested that they are epimers rather than regioisomers.

The stereochemistry and regiochemistry of the newly created chiral center in 3 and 4 has not yet been rigorously established. Although the cross-conjugation in the fumaramate moiety can potentially lead to Michael addition at either of the ethylene carbons to afford a mixture of epimeric regioisomers, it seems reasonable that the major mode of addition is β to the ester function, as acyclic α,β-unsaturated amides are usually less reactive. The suggested regioselectivity may be due to the greater resonance interaction between the double bond and the ester group.

Model studies employing the methyl fumaramate anilide 7 have provided some support for this suggestion. Reaction of 7 with Cys gave a single addition product 8 (Scheme I). Attachment of the Cys sulfur atom to the carbon β to the ester moiety, as opposed to β to the amide group, was suggested by the paramagnetic effect of $Gd(NO_3)_3$ on the relaxation times in the ^{13}C NMR spectrum of 9 derived from base hydrolysis of 8. The addition of increasing concentrations of $Gd(NO_3)_3$ caused first the disappearance of the methylene carbon absorption and then that of the methine carbon. This suggested that the Cys moiety is attached to the carbon β to the fumaramate carboxyl group of 9 and that Michael addition of Cys occurred at the same carbon of the parent ester 8. It is reasonable that an identical outcome occurred with the addition of GSH and Cys to β-FNA.

As it was of interest to assess the likelihood of a retro-Michael reaction of the conjugates (3, 4) occurring at physiologic pH, the effect of 24-fold excess of mercaptoethanol in phosphate buffer on the stability of the Cys conjugate 4 was investigated. With this large excess of mercaptan, it was conceivable that a small amount of β-FNA generated from 4 in a retro-Michael reaction could lead to the accumulation of adduct 10 (Scheme II). The fact that 10 was not detected over a 72-h period, with periodic monitoring by HPLC, suggests that at physiologic pH a nonenzymatic retro-Michael reaction of 3 or 4 is not likely.

Scheme II



4 1 10

Pharmacology

The Michael addition products 3 and 4 were tested for opioid agonist and antagonist activities in the electrically stimulated guinea pig longitudinal muscle[6] (GPI) and mouse vas deferens[7] (MVD) as described previously (Table I).[8] The irreversible antagonism was evaluated after incubating the conjugate (1 μM) with the smooth muscle preparation for 3.0 min followed by ≥40 washes with buffer. The IC_{50} of the agonist after this treatment was expressed as an IC_{50} ratio by dividing by the control IC_{50} value in the same preparation. Both conjugates were found to possess lower agonist potency than that of β-FNA. However, in contrast to the nonequilibrium opioid antagonism of morphine produced by β-FNA in the GPI, 3 and 4 showed no irreversible antagonist activity. In the MVD, neither of the β-FNA conjugates exhibited irreversible antagonism of [D-Ala^2,D-Leu^5]enkephalin[9] (DADLE), but 4 shifted the IC_{50} of morphine by 5-fold. This amounted to approximately half of the IC_{50} ratio observed with β-FNA. The GSH conjugate 3 exhibited no significant antagonism of morphine in the MVD.

Interestingly, all three conjugates (3–5) antagonized the antinociceptive effect of morphine in the mouse radiant heat tail-flick assay[10,11] on icv administration (Table II). Two hours after administration, they produced a 3–5-fold increase in the ED_{50} ratio of morphine as compared to the 10-fold shift produced by β-FNA. The conjugates differed in their duration of action in that the morphine antagonism produced by 3 and 4 persisted, while that of 5 rapidly disappeared. Thus, 24 h after administration of 3, the ED_{50} ratio of morphine increased to 6 and that of 4 showed a 46-fold increase. Although the peak antagonist effect of 4 was somewhat later than that of β-FNA, the duration of action of the conjugates (3, 4) appears to parallel that of β-FNA.

On sc administration, β-FNA 1, 3, and 5 showed similar antagonism of morphine antinociception at the 30-min time interval (Table III). However, 4 exhibited marginal antagonism at that time, and only the GSH adduct 3 significantly antagonized the agonist effect of morphine at 24 h.

When 1, 3, and 4 are compared by the icv or sc dose to produce equivalent antagonism of morphine at the 24-h interval, β-FNA (1) and the GSH conjugate 3 appear to be equally effective by either route of administration (Table IV). Thus, the sc dose of β-FNA and 3 to produce a 2-fold shift of the morphine ED_{50} was 74 and 83 times greater, respectively, than by the icv route. By comparison, the Cys conjugate 4 exhibited a very large difference (1250-fold) between the two routes.

Discussion

Binding and biochemical studies suggest that β-FNA (1) affords its nonequilibrium μ antagonist effect *in vitro*

Table I. Activities of β-FNA Conjugates in Smooth Muscle Assays

	GPI			MVD		
		irreversible antagonism IC_{50} ratio[a] ± se			irreversible antagonism IC_{50} ratio[a] ± se	
compd	agonism IC_{50} (nM) ± se	M	EK	agonism IC_{50} (nM) ± se	DADLE	M
1	4.8 ± 1.2[b]	19.5 ± 4.2	1.1 ± 0.1	82 ± 35[c]	1.1 ± 0.2	10.6 ± 0.4
3	41 ± 9	1.2 ± 0.9	0.3 ± 0.2	280 ± 80	0.8 ± 0.1	1.9 ± 0.6
4	22 ± 7	1.7 ± 1.0	1.0 ± 0.5	140 ± 60	1.4 ± 0.5	5.2 ± 1.2

[a] The IC_{50} of morphine (M), ethylketazocine (EK), or DADLE after treatment with the conjugate (1×10^{-6} M) for 60 min (GPI) or 30 min (MVD) and washing, divided by the control IC_{50} in the same preparation. All se values based on triplicate assays. [b] Value from ref 21. [c] Value from ref 18.

Table II. Time Course of Antagonism of Morphine Antinociception[a] by icv Administered β-FNA Conjugates

	morphine ED_{50} ratio[b]			
compd	2 h	24 h	48 h	72 h
1 (β-FNA)[c]	9.7 (7.3–13.2)	6.6 (5.3–8.2)		2.4 (1.5–3.1)
3	3.1 (3.0–5.3)	6.3 (3.5–14.3)		
4	4.8 (1.5–24.5)[d]	42.2 (33.8–54.0)	2.8 (1.4–5.5)	1.3 (0.6–4.0)
5	3.0 (1.0–8.8)	0.57 (0.5–0.7)		

[a] Measured 30 min after sc administration of morphine in mice. [b] Ratio of morphine ED_{50} at indicated time after antagonist treatment (4.8 nmol/mouse, icv) divided by its control ED_{50}. Values in parentheses are 95% confidence limits from three or four animals. [c] Values for β-FNA are calculated from ref 22. [d] At 3 h.

Table III. Antagonism[a] of Morphine Antinociception by sc Administration of β-FNA Conjugates

	morphine ED_{50} ratio[b]	
compd	30 min	24 h
1 (β-FNA)	3.9[c]	3.9[c]
3	3.8 (3.8–4.5)	2.6 (0.5–3.7)
4	1.4 (1.2–1.7)	1.3 (0.9–2.1)
5	3.0 (2.6–3.5)	1.3 (0.9–1.9)

[a] Evaluated by the radiant heat tail-flick method in mice using sc morphine administered 30 min prior to testing. Values in parentheses are 95% confidence limits from three or four animals. [b] The ED_{50} of sc morphine after treatment with the antagonist (5 mg/kg) divided by the control ED_{50}. [c] Values calculated from ref 22.

Table IV. Comparison of Equivalent Antagonist Potencies 24 h after Administration

	equivalent antagonist dose (μmol/kg)[a]		
antagonist[b]	icv	sc	sc/icv ratio
1	0.034	2.5	74
3	0.036	3.0	83
4	0.004	5.0	1250

[a] Calculated dose to produce a 2-fold shift in the morphine ED_{50} in an average-weight mouse of 25 g. [b] Compound 5 exhibited no significant potency by either route at 24 h.

and *in vivo* by covalent bonding to μ opioid receptors. In view of the facility with which β-FNA forms Michael addition products with Cys and other sulfhydryl-containing compounds in model studies, it has been suggested that a Cys residue of the μ opioid receptor is alkylated by the fumaramate moiety of β-FNA.[5]

However, as there are relatively high concentrations of GSH in the brain and liver, it is possible that the GSH conjugate 3 might play a role in the pharmacologic profile or duration of action of β-FNA. The fact that no irreversible antagonism of morphine was observed with either the conjugates 3 and 4 in the GPI (Table I) is in marked contrast to that of β-FNA. This has suggested that, under the conditions of this *in vitro* assay, neither 3 nor 4 bind irreversibly to the μ opioid receptor. In this regard, the significant morphine IC_{50} ratio (5.2) produced by 4 in the MVD is perplexing. Is it possible that 4 is transformed to β-FNA in this tissue? As we have demonstrated in the present study that 4 does not dissociate significantly to β-FNA and Cys under physiologic conditions, such a transformation would have to be enzyme-catalyzed, if a retro-Michael reaction occurs at all. Examples of biological retro-Michael reactions have been reported for the GSH conjugate of a reactive acrylonitrile metabolite of furazolidone by a swine liver microsomal preparation,[12] and for the glutathione S-conjugate of acrolein.[13] The pertinent aspect of the latter case is that the mechanism for the retro-reaction involved bioactivation of the conjugate to an S-oxide derivative by kidney cells, followed by a general-base-catalyzed elimination to acrolein. It seems reasonable that 3 and 4 could also undergo a retro-Michael reaction by the same bioactivation mechanism. An alternative possibility is that 4 is sequestered in an MVD tissue compartment that is not accessible to washing, but which interfaces with μ receptors. If either of these possibilities was found to be the case, the MVD would be a better *in vitro* model than the GPI to study the μ opioid receptor.

The duration of the antagonism of morphine antinociception after icv administration of the conjugates was noteworthy in that both 3 and 4 exhibited ultralong antagonist action (Table II). Remarkably, the Cys conjugate 4 was substantially more potent than β-FNA 24 h after administration, while 3 was equipotent. The fact that conjugate 5 was inactive as an antagonist at this time period may be significant because, unlike 3 and 4, its structure is incapable of generating β-FNA. Thus, as was discussed earlier in connection with the irreversible antagonist activity of 4 in the MVD, one possibility for the ultralong antagonism may be an enzyme-catalyzed reversed Micheal addition which generates β-FNA. If 4 were a better substrate than 3, this could explain its lower potency. An alternative explanation could be entrapment of 3 and 4 in a tissue compartment which interfaces with the μ receptor. However, this does not explain why 5 is not similarly trapped.

The fact that 3 and 5 were at least as potent as β-FNA by the sc route was surprising in view of their zwitterionic nature. Based on the sc/icv ratio it appears that the GSH conjugate 3 and β-FNA are nearly equally accessible by the sc route (Table IV). The Cys conjugate 4 is considerably less accessible, but is considerably more potent than β-FNA by the icv route. These data suggest that the GSH conjugate 3 may enter the brain by a transport system after sc administration. Glutathione itself is normally synthesized intracellularly and exported from cells,[14] but only poorly transported into cells.[15] However, glutathione S-conjugates have been reported to be taken up into kidney cells[16] and human neutrophil leukocytes[17] by membrane GSH transport systems. It is conceivable that the GSH

moiety of 3 is the recognition element for similar uptake into brain endothelial cells, if such transport occurs.

In summary, the results of the present study run counter to the dogma concerning the inability of highly polar exogenous molecules to penetrate the blood–brain barrier. Moreover, the finding that in contrast to 5, the conjugates 3 and 4 derived from β-FNA (1) possess ultralong-acting antagonist activity *in vivo*, may mean that 3 and 4 may be enzymatically converted to β-FNA in the CNS. Alternatively, this may represent a sequestering of these ligands in tissue compartments that interface with μ opioid receptors. If this is the case, the ultralong duration of antagonist action observed with β-FNA *in vivo* may in part be mediated by the GSH conjugate 3 or its metabolites. Further studies with labeled compounds may provide a definitive answer regarding the mechanism of the prolonged activity.

Experimental Section

Melting points were determined in open capillary tubes with a Thomas-Hoover melting point apparatus and are uncorrected. Analysis were performed by M-H-W Laboratories, Phoenix, AZ, and were within ±0.4% of the theoretical values. Mass spectra were obtained on a AEIMS-30, Finnigan 4000, or VG 7070E-HF spectrometer. IR spectra were obtained from KBr pellets with a Nicolet 5DXB FTIR spectrometer. NMR spectra were recorded on an IBM Bruker AC-200, IBM Bruker AC-300, or Nicolet 300-MHz spectrometer using $CDCl_3$, DMSO-d_6, or D_2O as solvent and were referenced to the deuterium lock frequency of the spectrometer for each solvent (values found: $CHCl_3$, 7.27 ppm; DMSO-d_6, 2.51 ppm; D_2O, 4.79 ppm). TLC data were obtained on glass-backed silica gel plates (Analtech, 0.25 m, F254). Column chromatography was performed on silica gel (Woelm DCC, 120–200 mesh). HPLC separations were performed on a semipreparative C_{16} column (Alltech, 10 μm, 10-mm i.d. × 25 cm; mobile phases at 2 mL/min) with detection at 254 nm. UV spectra were recorded of HPLC peaks using a photodiode-array detector (LKB 2140) and 200–370-nm scan range. All reagents and solvents employed were reagent or HPLC grade and were used without purification.

β-Funaltrexamine–Glutathione Conjugate (3). Compound 1·HCl (100 mg, 0.020 mmol) was added to a solution (4 mL) of glutathione (56 mg, 0.18 mmol) in 50% aqueous MeOH and adjusted to pH 7.3 with 1 N KOH. The mixture was stirred for 4 h at room temperature, at which time TLC (CH_3CN/MeOH/H_2O/HOAc, 50:30:15:5) showed only a single new component (R_f 0.42) and β-FNA (R_f 0.82). Methanol was removed on a rotary evaporator, and the aqueous residual was extracted with 2 volumes of $CHCl_3$. The aqueous layer was processed by HPLC (0.1% TFA/MeOH, 70:30) to separate a minor impurity (t_R = 6.8 min, 25%) from the product which appeared as two peaks (t_R = 11.0 and 11.6 min, 75%). MeOH was removed from the eluate, and the aqueous residual was shell-frozen and lyophilized to give the trifluoroacetate salt: yield 74 mg (49%); mp 185–205 °C; TLC R_f 0.42 (CH_3CN/MeOH/H_2O/HOAc, 50:30:15:5); HPLC (as above) mixture of two isomers (ratio 1:1.8); UV λ_{max} 221 (abs2.05), 279 (abs 0.86), both isomeric materials exhibiting completely coincidental spectra; IR (KBr) ν_{max} 3360–2910 (s), 1730–1610 (s), 1590–1500 (m), 1380 (m), 1205–1180 (s), 1125 (s), 1035 (m), 800 (m), 720 (m) cm^{-1}; FAB-MS (thioglycerol) + Ve *m/e* 762 $(M + H)^+$, −Ve 760 $(M - H)^-$; ^1H NMR (DMSO-d_6), δ 6.75 (d, 1H, *J* = 8.1 Hz, H-2), 6.67 (d, 1H, *J* = 8.1 Hz, H-1), 4.62 (m, 2H, H-5 and H-9), 2.07 (m, 2H, Glu ββ), 1.08 (m, 1H, H-19), 0.67 (m, 2H, H-20 and H-21), 0.46 (m, 2H, H-20 and H-21); ^{13}C (D_2O) δ 178.5 (ester C=O), 177.6, 176.6, 174.4, 174.2 (C=O), 144.6, 144.2 (split C4), 143.3, 142.9 (split C3), 132.2 (C12), 124.7 (C11), 123.5, 123.2 (split C1), 121.3 (br C2), 90.1 (br C5), 73.5, 73.4 (split C14), 65.1 (Cys α), 61.2 (C9), 60.5 (C18), 56.9 (Glu α), 47.6 (OCH_3), 46.0 (Gly α), 37.4 (succ CH), 34.3 (Glu γ), 29.1 (Glu β), 25.8 (Cys β), 8.3 (C19), 7.8, 5.3 (C20 or C21). Anal. ($C_{35}H_{47}N_5O_{12}S·2CF_3COOH$) C, H, N.

β-Funaltrexamine–Cysteine Conjugate (4). Compound 1·HCl (183 mg, 0.37 mmol) was dissolved in MeOH (4 mL) and mixed with cysteine (43 mg, 0.36 mmol) dissolved in H_2O (4 mL). The mixture was adjusted to pH 7.4 with aqueous trimethylamine, and the solution was stirred at room temperature overnight. Evaporation of the reaction mixture via CH_3CN–H_2O azeotrope gave 200 mg of a solid, which was dissolved in 0.1% aqueous TFA and processed by HPLC (0.1% aqueous TFA/MeOH, 70:30) to separate the first pair of overlapping peaks from residual β-FNA (1). The product eluate was concentrated on a rotary evaporator at ambient temperature to remove MeOH and then shell-frozen and lyophilized to give 116 mg (55%) of 4 trifluoroacetate salt: mp >260 °C (darkens at 209 °C); TLC R_f 0.30 (CH_3CN/H_2O/HOAc, 75:25:5); HPLC (above mobile phase) doublet peak, t_R = 8.9 and 10.1 (ratio = 1:2); IR (KBr) ν_{max} 3400–2900 (m), 1674 (s), 1507 (w), 1201 (s), 1128 (s) cm^{-1}; UV λ_{max} 204 (abs 1.09), 230 (sh, abs 0.282), 279 (abs 0.134); FAB-MS (glycerol) + Ve *m/e* 576 $(M + H)^+$, 457 $(M + H - Cys)^+$, −Ve *m/z* 574 $(M - H)^-$; MS–ion spray (1% HOAc in CH_3CN) 575.6 $(M + H)^+$; ^1H NMR (D_2O), δ 6.80 (d, 2H, *J* = 8.5 Hz, H-2), 6.63 (d, 2H, *J* = 7.9 Hz, H-1), 4.69–4.62 (m, 1H, H-5), 4.03 (m, 2H, H-9 and Cys α), 3.78 (m, 1H, H-6), 3.67 (br s, <3H, OCH_3), 1.08 (m, 1H, H-19), 0.69, 0.40 (2 m, 2H, H-20), 0.73, 0.41 (2 m, 2H, H-21); ^{13}C NMR (D_2O) δ 175.7, 175.5, 175.2 (C=O), 142.9 (br C3), 123.2 (br C1), 121.1 (br C2), 94.1 (br C5), 73.6 (split C14), 64.9 (Cys α), 60.4 (C9), 47.4 (OCH_3), 38.4 (succ CH), 33.8 (succ CH_2), 25.7 (Cys β), 8.2 (C19), 7.7 (C20), 5.1 (C21). Anal. ($C_{28}H_{37}N_3O_8S·2CF_3COOH$) C, H, N.

6β-*N*-(Cystein-*S*-ylacetyl)naltrexamine (5). 6β-*N*-(Iodoacetyl)naltrexamine (6)[18] (112 mg, 0.20 mmol) was mixed with cysteine (29 mg, 0.23 mmol) in 2.5 mL of H_2O–MeOH (20%), and to the stirred suspension was added aqueous trimethylamine dropwise to pH 7.2. The resulting mixture was stirred at room temperature for 2 h, and a TLC (CH_3CN/H_2O/HOAc, 75:20:5) showed one major and two minor products. The reaction mixture was passed through a C_{18} Sep Pak to remove nonmobile material, and the eluate was adjusted to pH 6 in an unsuccessful attempt to precipitate the amino acid product. The clear aqueous solution was then subjected to reverse-phase HPLC (0.1% aqueous TFA/MeOH, 80:20) which gave the desired product 5 (50%) at t_R = 10.7 min. This fraction was collected from seven runs, concentrated at ambient temperature to remove MeOH, and lyophilized to give 71 mg (75%) of trifluoroacetate salt: mp darkens 180 °C (foams 207 °C); TLC R_f 0.30 (CH_3CN/H_2O/HOAc, 75:25:5), HPLC (0.1% aqueous TFA/MeOH, 80:20) t_R = 10.5 min; IR (KBr) ν_{max} 3425 (m), 2924 (m), 1675–1646 (s), 1201 (s), 1129 (s), 1033 (w), 800 (w), 723 (w) cm^{-1}; FAB-MS (thioglycerol) + Ve *m/z* 504 $(M + H)^+$, 385 $(M + H - Cys)^+$, −Ve *m/z* 502 $(M - H)^-$; ^1H NMR (DMSO-d_6, 300 MHz) δ 8.83 (m, 1H, ArOH), 8.57 (d, 1H, *J* = 8.0 Hz, amide NH), 6.72, 6.68 (2 d, 2H, *J* = 8.2 Hz, ArH), 6.19 (br s, 1H, 14-OH), 4.58 (d, 1H, *J* = 7.9 Hz, H-5), 4.18 (m, 1H, Cys α), 3.84 (d, 1H, *J* = 5.4 Hz, H-9), 3.78 (m, 1H, H-6), 3.45 (s, 2H, acetyl CH_2), 3.23, 3.18 (2 d, 2H, *J* = 4.4, 5.9 Hz, Cys ββ), 1.05 (m, 1H, H-19), 0.67, 0.59 (2 m, 2H, H-20 or H-21), 0.49, 0.41 (2 m, 2H, H-20, or H-21); ^{13}C NMR (D_2O, 300 MHz) δ 174.4 (Cys C=O), 173.2 (acetyl C=O), 144.6, 143.0 (C3 or C4), 123.2, 121.3 (C1 or C2), 94.2 (C5), 73.6 (C14), 65.5 (C9), 60.6 (C18), 54.0, 55.3 (C6 or Cys α), 38.6 (acetyl CH_2), 35.1 (Cys β), 25.9 (C10), 8.2 (C19), 7.6, 5.2 (C20 or C21). Anal. ($C_{25}H_{33}N_3O_8S·2CF_3COOH$) C, H, N.

Stability Study of 4. The stability of 4 under aqueous conditions at near neutral conditions was examined with regard to a reverse Michael reaction. Twenty milliliters of aqueous β-FNA·HCl (0.02 M) was mixed with a 5-fold molar excess cysteine (10 mL, 0.2 M) in 100 mL of NaH_2PO_4–Na_2HPO_4 buffer, pH 8.6. The mixture was rapidly stirred for 5 min at room temperature, and then a 7-mL aliquot was injected onto an analytical HPLC column (Ultrasphere C_{18}, 5 μm, 4.6-mm i.d. × 25 cm; 0.05 M KH_2PO_4–K_2HPO_4 buffer, pH 5.5/MeOH (65:35), isocratic at 1 mL/min; detection at 254 nm) to monitor the disappearance of β-FNA and the formation of 4. β-FNA (t_R = 9.6 min) disappeared completely in 4 min with concurrent formation of 4 (t_R = 5.1 min). Adduct formation could be completely blocked by preacidification with 20 mL of 1 N HCl. After an additional 30 min, a 24-fold excess of aqueous mercaptoethanol (0.48 M, 100 mL) was added to the stirred mixture maintained at room temperature. At intervals of 1, 2, 3, 21, and 72 h, a 5–7-mL aliquot was removed from the reaction mixture and examined by HPLC for formation of β-FNA–mercaptoethanol adduct 10 (Scheme II) (t_R = 7.8 min). No appearance of 10 was detected at any of the intervals; its

expected retention time in the mixture chromatogram was determined from the chromatographic profile of 10 prepared *in situ* from β-FNA and mercaptoethanol under the same experimental conditions.

Methyl 3-(Cystein-*S*-yl)succinanilate (8). Fumaranilic acid methyl ester (7)[19] (410 mg, 2.0 mmol) in MeOH (10 mL) was mixed with cysteine (242 mg, 2.0 mmol) in H_2O, and the solution was adjusted to pH 7 with aqueous trimethylamine. The mixture was stirred overnight, the solvent was evaporated, and the residue (0.62 g) was washed once with Et_2O. Since the dried solid was impure on TLC analysis (1-butanol/HOAc/H_2O, 3:1:1), a portion (400 mg) was chromatographed on a column of silica gel (100 g, Woelm for DCC) using CH_3CN/H_2O (90:10) to give 0.28 g (54%) of 8: mp 147–148 °C; TLC R_f 0.51 (CH_3CN/H_2O, 90:10): FAB-MS (*m*-nitrobenzoic acid) + Ve *m/z* 327 $(M + H)^+$, -Ve *m/z* 325 $(M - H)^-$; ^1H NMR (D_2O) δ 7.44 (m, 4H, ArH), 7.28 (m, 1H, Ar-*p*-H), 4.02 (m, 2H, succ CH and Cys α), 3.69 (s, 3H, OCH_3), 3.47–2.87 (m, 4H, succ CH_2 and Cys β); ^{13}C NMR (D_2O) δ 175.9, 175.0, 174.4 (C=O), 139.2 (ArCN), 132.1, 128.8, 124.8 (ArCH), 56.6 (Cys α), 55.4 (OCH_3), 46.7, 46.4 (succ CH), 39.0 (succ CH_2), 34.3 (Cys β). Anal. ($C_{14}H_{18}N_2O_5S$) C, H, N.

3-(Cystein-*S*-yl)succinanilic Acid (9). The ester 8 (100 mg, 0.31 mmol) was dissolved in 6 mL of 0.5 N KOH, and the solution was stirred at room temperature overnight. The reaction mixture was neutralized to slight acidity with 1 N HCl in MeOH, and then the solvent was exchanged for EtOH. The EtOH solution was concentrated, the KCl precipitate was filtered off, and the filtrate was diluted with EtOAc and again concentrated. The product 9 was isolated on a filter and dried under vacuum overnight: yield 90 mg (94%); TLC R_f 0.42 (CH_3CN/H_2O/HOAc, 90:5:5); mp >210 °C; FAB-MS (glycerol) + Ve *m/z* 295 $(M - H_2O + H)^+$, -Ve *m/z* 293 $(M - H_2O - 1)^-$; IR (KBr) ν_{max} 3310 (s), 1734–1717 (m), 1662–1636 (m), 1445 (m), 1310 (w), 1256 (w); ^1H NMR (D_2O) δ 7.42 (m, 4H, Ar-*o*,*m*-H), 7.26 (m, 1H, Ar-*p*-H), 4.32–3.86 (m, 2H, succ CH and Cys α), 3.00–2.70 (m, 4H, succ CH_2 and Cys ββ); ^1H NMR (DMSO-d_6) δ 10.03 (s, 1H, amide NH) exchangeable with D_2O, 7.58 (d, 2H, J = 7.9 Hz, Ar-*o*-H), 7.30 (t, 2H, J = 8.0), 7.04 (t, 1H, J = 7.3 Hz, Ar-*p*-H), ^{13}C NMR (D_2O) δ 174.2, 174.1, 173.8 (C=O), 139.3 (ArCN), 132.0, 128.4, 124.7 (ArCH), 60.5 (Cys α), 40.9 (succ CH_2), 40.3 (split, succ CH), 30.6 (Cys β). Sequential addition of 10-, 30-, and 80-μL volumes of 0.025 M $Gd(NO_3)_3$[20] in D_2O to 0.3 M 9 in D_2O caused disappearance of succinyl methylene (40.9 ppm) and then methine (40.3 ppm) absorbances in the DEPT[23] spectrum.

Acknowledgment. We thank Mary Lunzer for *in vitro* and *in vivo* testing. This work was supported by the National Institute on Drug Abuse.

References

(1) Portoghese, P. S.; Larson, D. L.; Sayre, L. M.; Fries, D. S.; Takemori, A. E. A Novel Opioid Receptor Site-Directed Alkylating Agent with Irreversible Narcotic Antagonistic and Reversible Agonistic Activities. *J. Med. Chem.* **1980**, *23*, 233–234.

(2) Takemori, A. E.; Portoghese, P. S. Affinity Labels for Opioid Receptors. *Annu. Rev. Pharmacol. Toxicol.* **1985**, *25*, 193–223.

(3) Zimmerman, D. M.; Leander, J. D. Selective Opioid Receptor Agonists and Antagonists: Research Tools and Potential Therapeutic Agents. *J. Med. Chem.* **1990**, *33*, 895–902.

(4) *Geigy Scientific Tables*; Lentner, C., Ed.; CIBA-GEIGY, Ltd.: Basle, 1981; Vol. 1, Units of Measurement, Body Fluids, Composition of the Body, Nutrition; p 222.

(5) Sayre, L. M.; Larson, D. L.; Takemori, A. E.; Portoghese, P. S. Design and Synthesis of Naltrexone-Derived Affinity Labels with Nonequilibrium Opioid Agonist and Antagonist Activities. Evidence for the Existence of Different μ Receptor Subtypes in Different Tissues. *J. Med. Chem.* **1984**, *27*, 1325–1335.

(6) Rang, H. B. Stimulant Actions of Volatile Anaesthetics. *Br. J. Pharmacol.* **1964**, *22*, 356–365.

(7) Henderson, G.; Hughes, J.; Kosterlitz, H. N. A New Example of a Morphine-Sensitive Neuro-Effector Junction: Adrenergic Transmission in the Mouse vas Deferens. *Br. J. Pharmacol.* **1972**, *46*, 764–766.

(8) Sayre, L. M.; Takemori, A. E.; Portoghese, P. S. Alkylation of Opioid Receptor Subtypes by α-Chlornaltrexamine Produces Concurrent Irreversible Agonistic and Irreversible Antagonistic Activities. *J. Med. Chem.* **1983**, *26*, 503–506.

(9) Fournie-Zaluskie, M.-C.; Gacel, G.; Maigret, B.; Premilat, S.; Roques, B. P. Structural Requirements for Specific Recognition of Mu or Delta Opiate Receptors. *Mol. Pharmacol.* **1981**, *20*, 484–491.

(10) D'Amour, F. E.; Smith, D. L. A Method of Determining Loss of Pain Sensation. *J. Pharmacol. Exp. Ther.* **1941**, *72*, 74–79.

(11) Hayashi, G.; Takemori, A. E. The Type of Analgesic-Receptor Interaction Involved in Certain Analgesic Assays. *Eur. J. Pharmacol.* **1971**, *16*, 63–66.

(12) Vroomen, L. H. M.; Berghmans, M. C. J.; Groten, J. P.; Koeman, J. H.; van Bladeren, P. J. Reversible Interaction of a Reactive Intermediate Derived from Furazolidone with Glutathione and Protein. *Toxicol. Appl. Pharmacol.* **1988**, *95*, 53–60.

(13) Hashmi, M.; Vamvakas, S.; Anders, M. W. Bioactivation Mechanism of S-(3-Oxopropyl)-N-acetyl-L-cysteine, the Mercapturic Acid of Acrolein. *Chem. Res. Toxicol.* **1992**, *5*, 360–365.

(14) Meister, A. Metabolism and Function of Glutathione. In *Glutathione: Chemical, Biochemical, and Medical Aspects. Part A*; Dolphin, D., Poulson, R., Auramovic, O., Eds.; Wiley & Sons: New York, 1989; pp 368–457.

(15) Anderson, M. E.; Underwood, M.; Bridges, R. J.; Meister, A. Glutathione Metabolism at the Blood Cerebrospinal Fluid Barrier. *FASEB J.* **1989**, *3*, 2527–2531.

(16) Lash, L. H.; Jones, D. P. Uptake of the Glutathione Conjugate S-(1,2-Dichlorovinyl)glutathione by Renal Basal-Lateral Membrane Vesicles and Isolated Kidney Cells. *Mol. Pharmacol.* **1985**, *28*, 278–282.

(17) Scott, R. B.; Matin, S.; Hamilton, S. C. Glutathione, Glutathione S-Transferase, and Transmembrane Transport of Glutathione Conjugate in Human Neutrophil Leukocytes. *J. Lab. Clin. Med.* **1990**, *116*, 674–680.

(18) Sayre, L. M.; Larson, D. L.; Fries, D. S.; Takemori, A. E.; Portoghese, P. S. Importance of C-6 Chirality in Conferring Irreversible Opioid Antagonism to Naltrexone-Derived Affinity Labels. *J. Med. Chem.* **1983**, *26*, 1229–1235.

(19) Patel, M. V.; Balasubramaniyan, V. Reactions of Cyclic Anhydrides: Part V. Acid Catalyzed Esterification of Maleanilic and Fumaranilic Acids and Evidence of Activation by Carboxamido Function. *Indian J. Chem.* **1981**, *20B*, 804–805.

(20) Bratt, G. T.; Hogenkamp, H. P. C. The Interaction of Cyanocobalamin and Some of Its Analogs with Manganese(II) and Gadolinium(III). *Arch. Biochem. Biophys.* **1982**, *218*, 225–232.

(21) Takemori, A. E.; Larson, D. L.; Portoghese, P. S. The Irreversible Narcotic Antagonistic and Reversible Agonistic Properties of the Fumaramide Methyl Ester Derivative of Naltrexone. *Eur. J. Pharmacol.* **1981**, *70*, 445–451.

(22) Ward, S. J.; Portoghese, P. S.; Takemori, A. E. Pharmacological Characterization *In Vivo* of the Novel Opiate, β-Funaltrexamine. *J. Pharmacol. Exp. Ther.* **1982**, *220*, 494–498.

(23) (a) Sanders, J. K. M.; Hunter, B. K. *Modern NMR Spectroscopy*; Oxford University Press: New York, 1987; pp 253–256. (b) Derome, A. E. *Modern NMR Techniques for Chemistry Research*; Pergamon Press: New York, 1987; pp 143–151.