Activity of *N*-Methyl-α- and -β-funaltrexamine at Opioid Receptors

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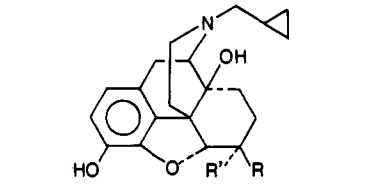
The *N*-methyl analogues (**2a, 2b**) of the nonequilibrium μ opioid receptor antagonist β-funaltrexamine (**1b**) were synthesized and evaluated in the guinea pig ileum preparation (GPI). These analogues are highly potent, reversible opioid agonists and possess no nonequilibrium antagonist activity. The ineffectiveness of **2b** in protecting against irreversible blockage of μ opioid receptors by **1b** and the fivefold lower reactivity of **2b** with cysteine suggest that *N*-methyl substitution adversely affects both the first and second recognition steps that are essential for effective covalent blockage of opioid receptors.

The nonequilibrium receptor antagonist β-funaltrexamine[1] **1b** (β-FNA) is employed widely as a pharmacologic tool in opioid research.[2,3] Its high selectivity for μ opioid receptors has been attributed to two sequential recognition steps.[4] The first is manifested by the affinity of the ligand for the recognition site; the second involves proper juxtaposition of a proximal nucleophile (most likely an SH group) and the Michael acceptor moiety of β-FNA in its reversibly bound state.

It is thought that the orientation of the Michael acceptor group at the binding site is important for the second recognition step. In this regard, it has been reported that the configuration of the C-6 chiral center of the opiate and the constitution of the Michael acceptor both affect the facility with which covalent bonding occurs.[4] Moreover, the results from X-ray studies[5] of the C-6 epimers suggest that the inability of α-FNA (**1a**) to irreversibly block the effects of morphine is related to different preferred conformations of the electrophilic fumaramate methyl ester moiety.

In the present study, we have prepared the *N*-Me derivatives of funaltrexamine **2a** and **2b** in an effort to alter the conformational preference of the electrophilic moiety, as this should be mirrored by a change in the efficiency to irreversibly block μ receptor activity.



1a: R = H; R' = NHCOC(H)=C(H)CO$_2$Me

1b: R = NHCOC(H)=C(H)CO$_2$Me; R' = H

2a: R = H; R' = N(CH$_3$)COC(H)=C(H)CO$_2$Me

2b: R = N(CH$_3$)COC(H)=C(H)CO$_2$Me; R' = H

3a: R = H; R' = NHCH$_3$

3b: R = NHCH$_3$; R' = H

Chemistry

The target compounds, **2a** and **2b**, were synthesized from naltrexone via the epimeric methylamino intermediates **3a** and **3b**. These intermediates were obtained by reductive amination of naltrexone hydrochloride with use of methylamine hydrochloride and $NaCNBH_3$. The 6α and 6β epimers (**3a** and **3b**, respectively) were formed in a ratio of approximately 4:1 and separated by chromatography. The stereochemical assignments of **3a** and **3b** were based on the relative magnitude of the NMR vicinal coupling constants, as it is known[6] that $J_{5,6}$ of the 6α isomers of closely related compounds is of smaller magnitude (ca. 4 Hz) than that of the 6β isomers (ca. 7 Hz). The methylamino intermediates (**3a, 3b**) were converted to the corresponding amides (**2a, 2b**) by reaction with *trans*-3-(methoxycarbonyl)acroyl chloride.

In order to determine the reactivity of **2b** relative to that of β-FNA (**1b**), we measured their pseudo-first-order rates of reaction with a 30-fold excess of cysteine in aqueous medium. The rate of **1b** was 5.5-fold greater than that of **2b**.

Pharmacology

The *N*-methyl-β-FNA analogues (**2a, 2b**) were tested on the electrically stimulated longitudinal muscle of the guinea pig ileum[7] (GPI) (Table I). Both epimers behaved as agonists with potencies 600 (**2a**) and 20 times greater than that of morphine. The agonist effect of **2a** and **2b** could be reversed with 500 nM naloxone, but the recovery of the GPI treated with **2a** was slow.

Compounds **2a** and **2b** were evaluated in the GPI for irreversible blockage of agonism produced by morphine or ethylketazocine (Table I). Because of the extreme agonist potency of **2a**, its incubation concentration was limited to 20 nM, as compared to 200 nM for **2b**. Naloxone (500 nM) was employed to displace reversibly bound **2a** or **2b** during the washing (30 times) process in order to ensure at least 80% recovery of the GPI. Neither **2a** nor **2b** appeared to irreversibly block the agonist effect of morphine or ethylketazocine. In the mouse vas deferens preparation[8] (MVD) no significant irreversible blockage of the agonist effect of morphine or [D-Ala^2,D-Leu^5]enkephalin by **2a** or **2b** (200 nM) was observed.

In order to determine whether or not the *N*-methyl-β-FNA analogues (**2a, 2b**) effectively interact with the same

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Table I. Activities of *N*-Methyl Derivatives of α- and β-Funaltrexamine in the GPI

compd	agonist potency[a]	concn, nM	irreversible antagonist potency: morphine: IC_{50} ratio[b] ± SE (*n*)	irreversible antagonist potency: ethylketazocine: IC_{50} ratio[b] ± SE (*n*)
2a	597	1	1.7 ± 0.5 (3)	1.8 ± 0.3 (4)
		20	1.3 ± 0.4 (4)	1.0 ± 0.3 (5)
2b	21	20	1.3 ± 0.4 (4)	0.8 ± 0.1 (3)
		200	0.9 ± 0.2 (4)	0.5 ± 0.2 (3)

[a] Relative to morphine (1.53×10^{-7}; $n = 12$) on the same ileum preparation. The values are averages of three determinations. [b] Agonist IC_{50} (after 30-min incubation with **2a** or **2b** following by 30 washes) divided by the control IC_{50} value in the same preparation.

Table II. β-Funaltrexamine (β-FNA) Antagonism of Morphine Agonism in the Presence of **2a** or **2b** in the GPI[a]

protector	concn, nM	morphine: IC_{50} ratio ± SE (*n*)[b]
2a	20	5.4 ± 0.8 (4)
	200	3.3 ± 0.7 (4)
2b	20	4.0 ± 0.6 (6)
	200	6.5 ± 1.5 (6)
none		6.5 ± 0.6 (4)
naltrexone	20	2.1 ± 0.7 (3)

[a] Protectors **2a** or **2b** were added to the tissue 10 min prior to incubation with 20 nM β-FNA for 30 min, and the preparation was then washed 30 times with intermittent "chasing" with 500 nM naloxone. [b] Morphine IC_{50} (treated) divided by morphine IC_{50} (control) in the same preparation.

recognition site as β-FNA (**1b**), protection studies were carried out in the GPI (Table II). In the absence of a protector against irreversible blockage of μ opioid receptors by β-FNA, the morphine IC_{50} ratio was 6.5; this was reduced to 2.1 when protected with 20 nM naltrexone. By comparison, **2b** appeared to afford marginal protection at 20 nM but not at 200 nM. Its epimer, **2a**, offered some protection at 200 nM.

Discussion

The fact that the *N*-methyl analogue **2b** is unable to irreversibly block μ opioid receptors (Table I), even at high concentration, may be a consequence of low affinity and/or a deficient second recognition step. Since the protection studies (Table II) demonstrate that **2b** is ineffective at protecting μ receptors against irreversible blockage by 200 nM β-FNA (**1b**), it is apparent that the *N*-methyl group in **2b** has greatly reduced the affinity of this ligand for the antagonist recognition site. The reason for this reduced affinity may be related to steric hindrance between the *N*-methyl group and the receptor, to possible alteration of the preferred conformation of the fumaramate group, or to loss of a hydrogen-bonding proton donor group (replacement of NH with NMe). Any one or a combination of these factors might contribute to loss of affinity at the μ antagonist recognition site.

The extent to which the second recognition step is affected by this modification is difficult to evaluate because of the apparent low affinity of **2b** for the μ antagonist recognition site. However, in view of the the 5.5-fold lower electrophilicity of the fumaramate moiety in **2b** relative to that of **1b**, it is likely that covalent bond formation at the recognition site would be slower for **2b**, even if the fumaramate group in both ligands were in the same conformation at the recognition site. Thus it appears that the methyl group on the 6β-nitrogen adversely affects both the first and second recognition steps.

Experimental Section

General Procedures. Melting points were determined in open capillary tubes with a Thomas-Hoover melting point apparatus and are uncorrected. Elemental analyses were performed by M-H-W Laboratories, Phoenix, AZ, and were within ±0.4% of the theoretical values. IR spectra were obtained from KBr pellets with a Perkin-Elmer 281 instrument. NMR spectra were recorded at ambient temperature on JEOL instruments with Me_4Si as internal standard in chloroform. Optical rotations were determined with a Perkin-Elmer 141 polarimeter. Mass spectra were obtained on an AEI MS-30 instrument. All TLC data were obtained on Analtech silica gel chromatography plates. The eluent EMA refers to EtOAc–MeOH–NH_4OH. All reagents were used without subsequent purification and were reagent grade.

17-(Cyclopropylmethyl)-4,5α-epoxy-3,14-dihydroxy-6-(methylamino)morphinan (3a and 3b). To a mixture of naltrexone hydrochloride (3.74 g, 10 mmol) and methylamine hydrochloride (6.7 g, 100 mmol) in methanol (25 mL) was added a methanolic solution (20 mL) of $NaCNBH_3$ (0.385 g, 6.3 mmol). The resulting solution was stirred for 72 h, followed by acidification with concentrated hydrochloric acid to pH 1. The solvent was removed and the residue was dissolved in water and extracted with chloroform to remove the water-insoluble material. The solution was then adjusted to pH 9 with sodium carbonate. This mixture was extracted with chloroform and dried (Na_2SO_4), and the chloroform was removed in vacuo. Medium performance liquid chromatography was used to separate the isomers (**3a** and **3b**), (silica gel 32–63 μm). Epimer **3b** was obtained in 27% yield (990 mg): mp 214–215 °C; $[\alpha]^{25}_D$ −161.9° (*c* 1, MeOH); R_f 0.37 (EMA 10:8 0.3); EIMS (70 eV), *m/e* 356 [M^+]; NMR (deuterium-exchanged free base in $CDCl_3$) δ 6.52 and 6.67 (1 H each, 2 d, J = 8.1 Hz, aromatic H), 4.5 (1 H, d, $J_{5,6}$ = 7.5 Hz, C_5-H). Anal. ($C_{21}H_{28}N_2O_3$).

Epimer **3a** was obtained in 6.3% yield (225 mg); 235–236 °C; $[\alpha]^{25}_D$ −125.4° (*c* 2, methanol); R_f 0.24 (EMA 10:8:0.3); EIMS (20 eV), *m/e* 356 [M^+]; NMR (deuterium-exchanged free base in $CDCl_3$) δ 6.54 and 6.75 (1 H each, 2 d, J = 8.14 Hz, aromatic H), 4.72 (1 H, $J_{5,6}$ = 3.76 Hz, C_5-H). Anal. ($C_{21}H_{28}N_2O_3$).

17-(Cyclopropylmethyl)-4,5α-epoxy-3,14-dihydroxy-6-[*trans*-3-(methoxycarbonyl)-*N*-methylacrylamido]morphinan (2a and 2b). To a solution containing either **3a** or **3b** (118 mg, 0.3 mmol) in THF (5 mL) and K_2CO_3 (150 mg) in water (1 mL) was added *trans*-3-(methoxycarbonyl)acroyl chloride[4] (49 mg, 0.3 mmol) with stirring. The acid chloride was added over a 15-min period and stirring was continued for an additional hour. The solvent was removed and the residue was dissolved in water (10 mL) and extracted with chloroform (4 × 25 mL). The combined extract was dried (Na_2SO_4) and chloroform was removed under reduced pressure. The residue was crystallized from methanol–ether to afford the desired products in a yield of about 77%. **2b**: mp 136–137 °C; $[\alpha]^{25}_D$ −108° (*c* 1, MeOH); R_f 0.9 (EMA 10:8:0.3); EIMS (30 eV), *m/e* 468 [M^+]; NMR (deuterium-exchanged free base in $CDCl_3$) δ 6.76 and 7.38 (2 d, J = 15.29 Hz, 2 H, vinyl), 6.56 and 6.6 (1 H each, 2 d, J = 8.13 Hz, aromatic H), 4.56 (1 H, d, J = 7.75 Hz, C_5-H). Anal. ($C_{26}H_{32}N_2O_6 \cdot {}^1/_4H_2O$).

2a: mp 225 °C; $[\alpha]^{25}_D$ −214.8° (*c* 0.5, methanol); R_f 0.68 (EMA 10:8:0.3); EIMS (20 eV), *m/e* 468 [M^+]; NMR (deuterium-exchanged free base in $CDCl_3$) 6.77 and 7.43 (2 d, J = 15.3 Hz, 2 H, vinyl), 6.52 and 6.7 (1 H each, 2 d, J = 8 Hz, aromatic H), 4.88 (1 H, d, $J_{5,6}$ = 3.4 Hz, C_5-H). Anal. ($C_{26}H_{32}N_2O_6 \cdot {}^2/_3H_2O$).

Kinetic Studies. A rapidly stirred 0.006 M solution (100 μL) of **2b** in 0.025 M phosphate buffer (pH 6.0, 6.6, or 7.1) was mixed with 10 μL of 0.76 M aqueous cysteine. After various time intervals (1, 2, 5, 10, 20 min), the reaction mixture was quenched with 1 N HCl, and 100 μL of aqueous naltrexamine hydrochloride[6] (6 mM) was added to serve as an internal standard. An aliquot (5–10 μL) of the quenched reaction mixture was assayed in triplicate by HPLC [R-P C8; MeOH–aqueous 0.1% TFA (45:55) at 1.0 mL/min, 254 nm; **2b** t_R = 8.6 min, β-naltrexamine t_R = 5.7 min] to quantitate the amount of **2b** remaining. For each time interval the mean peak height ratio (**2b**/β-naltrexamine) was calculated, and a linear regression of the log peak height ratio

vs. time was determined. A half-life (minutes) was calculated from the slope of the regression line. The half-life values ($t_{1/2}$) for **2b** are 9.9 min (pH 6.0), 3.9 min (pH 6.6), 1.8 min (pH 7.1); for **1b** the values are 5.8 min (pH 5.1) and 1.8 min (pH 6.0). A statistical comparison between the two compounds shows a significant difference ($p < 0.05$) in the $t_{1/2}$ values at pH 6.0 but no significant difference in the dependence of half-life on pH. This indicates that although the two compounds exhibit a difference in their rate of reaction with cysteine, the mode of the reaction is the same.

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Plant Antitumor Agents. 22.[1] Isolation of 11-Hydroxycamptothecin from *Camptotheca acuminata* Decne: Total Synthesis and Biological Activity

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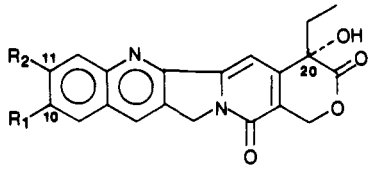
The antitumor alkaloid 11-hydroxy-(20*S*)-camptothecin (**4**) was isolated from the woody tissue of *Camptotheca acuminata*. Structural proof derived from comparison with racemic **4** prepared by total synthesis. Antitumor activity of racemic **4** in L1210 leukemia in mice was considerably greater than that of natural (20*S*)-camptothecin (**1**) and its sodium salt. There was also no toxic effect observed even at relatively high doses.

Since the initial isolation from *Camptotheca acuminata* of the novel alkaloid camptothecin (**1**) in 1966,[2] there has been a continuing search for related compounds. Moreover, because of the potent activity of **1** in the resistant L1210 mouse leukemia assay, as well as in numerous other rodent leukemia and solid tumor systems,[3] there has also been interest in the total synthesis of **1** and analogues.[4] In addition to **1**, we have previously isolated as minor components 10-hydroxycamptothecin (**2**) and its 10-methoxy analogue **3** from *C. acuminata* bark and wood.[5] Other minor camptothecin analogues with structural differences at C-20 have been isolated by Hutchinson's group.[6] 9-Methoxycamptothecin has been isolated as a minor fraction from *Nothapodytes foetida* (Wight) Sleumer (Icacinaceae) (formerly *Mappia foetida* Miers).[7]

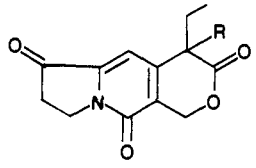


1, $R_1 = R_2 = H$
2, $R_1 = OH$; $R_2 = H$
3, $R_1 = OCH_3$; $R_2 = H$
4, $R_1 = H$; $R_2 = OH$
5, $R_1 = H$; $R_2 = OCH_3$

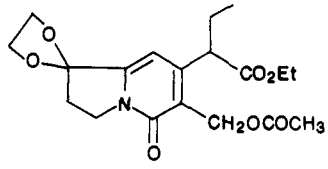
We now report the isolation of a new alkaloid 11-hydroxy-(20*S*)-camptothecin (**4**) in trace quantities from residual fractions of a large-scale extraction of *C. acuminata* bark and wood and the synthesis of the 20*R*,*S*-analogue.

Residual fractions remaining after a large-scale extraction and isolation of **1** from *C. acuminata* were analyzed by HPLC, primarily in search of the 10-hydroxy analogue **2**. A minor impurity was present with R_T values close to those of **2**, which could be concentrated to some extent in mother liquors after recrystallization. After repetitive HPLC, approximately 2 mg of pure compound was obtained. The exact mass determination of 364.1054 ($C_{20}H_{16}N_2O_5$) suggested a positional isomer of hydroxycamptothecin. Direct comparisons of the unknown with authenic 9-hydroxycamptothecin prepared by demethylation of 9-methoxycamptothecin eliminated the 9-position from consideration. The limited ^1H NMR analysis that could be conducted with the small available sample suggested that the compound might have structure **4**.

To further confirm structure **4**, racemic 11-methoxycamptothecin (**5**) was prepared by the acid-catalyzed Friedlander condensation of 4-methoxy-2-aminobenzaldehyde[5] with the key tricyclic ketone **7** in a manner previously described for the deoxy tricyclic ketone **6**.[8] The intermediate **7** was prepared from the bicyclic pyridone **8**[8] in two steps. Thus, simultaneous ring closure and oxygenation of **8** gave the tricyclic ketal **9**. Hydrolysis of the ketal moiety in **9** gave compound **7**. The methoxy functionality of **5** was cleaved in refluxing 48% aqueous HBr to afford racemic **4** after chromatographic purification. The 250-MHz ^1H NMR spectra (Me_2SO-d_6) of synthetic and natural **4** were superimposable. The melting points and TLC and HPLC properties were identical, as well as the UV and IR spectra.



6, R = H
7, R = OH



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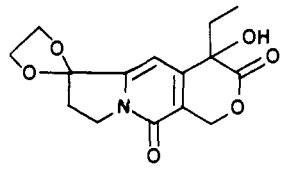

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After our work was completed, a review by Cai and Hutchinson[4] noted that **4** was isolated by Chinese workers

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