Aspartic Acid Conjugates of 2-(3,4-Dichlorophenyl)-*N*-methyl-*N*-[(1*S*)-1-(3-aminophenyl)-2-(1-pyrrolidinyl)ethyl]acetamide: κ Opioid Receptor Agonists with Limited Access to the Central Nervous System

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Received June 25, 1996[⊗]

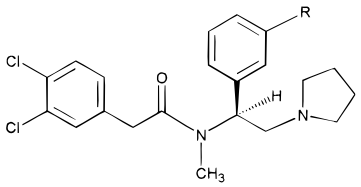
Aspartic acid conjugates of 2-(3,4-dichlorophenyl)-*N*-methyl-*N*-[(1S)-1-(3-aminophenyl)-2-(1-pyrrolidinyl)ethyl]acetamide (**5**) were synthesized and evaluated in mice for antinociceptive activity by intravenous and intracerebroventricular routes of administration. The intravenously-administered α-conjugate of L-Asp (**2**), its D-Asp diastereomer (**3**), and the *β*-conjugate of L-Asp (**4**) were found to be 11-, 31-, and 40-fold, respectively, less effective than the parent ligand **1** (ICI 199,441) in producing central nervous system mediated antinociception in the mouse abdominal stretch assay. In addition, iv-administered **2** and **3** were found to also produce potent antinociception in the tonic phase of the mouse formalin assay, which is a model of tonic rather than acute pain. This study suggests that the attachment of a zwitterionic moiety to a position in the molecule that exhibits bulk tolerance is a viable strategy for the design of peripherally-selective and peripherally-active opioids.

Introduction

In view of the pain and suffering inflicted by chronic inflammatory diseases such as rheumatoid arthritis, there is an urgent need for new and improved methods of treatment. Now there is considerable evidence indicating that opioid ligands can produce opioid receptor-mediated antinociception outside the central nervous system (CNS).[1−3] Furthermore, the peripheral analgesic effects of opioids are enhanced under inflammatory conditions. However, the use of opioids for alleviating inflammatory pain has been limited because of the variety of CNS side effects produced by opioids. There has been an interest in the preparation of peripherally-acting opioid agonists that have limited or no access to the CNS in an effort to reduce or eliminate these side effects. Another potential application of peripherally-acting opioids is the treatment of gastrointestinal motility disorders.[4−6]

Previous work in our laboratory has shown that aspartic acid conjugates of opioid ligands naltrexamine and oxymorphamine crossed the blood−brain barrier (BBB) poorly, as indicated by very large iv/icv dose ratios in mice.[7] The poor CNS penetration of these conjugates was attributed to the highly charged nature of the zwitterionic group, which decreases the lipophilicity of the conjugates.

There has been a limited number of reports of *κ*-selective agonists with restricted access to the CNS.[8−11] Because the coupling of a zwitterionic group to naltrexamine has been shown to greatly reduce its ability to produce CNS-mediated effects,[7] we sought to produce peripherally-selective *κ* opioid receptor agonists by the introduction of zwitterionic groups on the central phenyl group of the potent and selective *κ* agonist 2-(3,4-dichlorophenyl)-*N*-methyl-*N*-[(1S)-1-phenyl-2-(1-pyrrolidinyl)ethyl]acetamide (**1**, ICI 199,441).[12,13]



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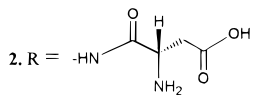

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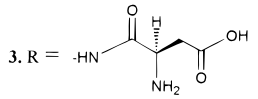

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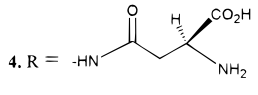

4. R =

Chemistry

Aspartic acid conjugates **2**−**4** were prepared via DCC/HOBT coupling of 2-(3,4-dichlorophenyl)-*N*-methyl-*N*-[(1S)-1-(3-aminophenyl)-2-(1-pyrrolidinyl)ethyl]acetamide[14,15] (**5**) and the suitably protected aspartic acid (Scheme 1). The protected conjugates **6**−**8** (not characterized) were deprotected by treatment with a 1:1 mixture of 2 N HCl and glacial acetic acid to remove the Boc group, followed by Pd/C-catalyzed hydrogenolysis of the benzyl ester.

Results

Smooth Muscle Preparations. Compounds **2**−**4** were tested on the electrically stimulated guinea pig ileal longitudinal muscle[16] (GPI) and mouse vas deferens[17] (MVD) preparations as described previously.[18] All three conjugates were found to be potent agonists in

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[⊗] Abstract published in *Advance ACS Abstracts,* September 15, 1996.

S0022-2623(96)00459-1 CCC: $12.00

Scheme 1

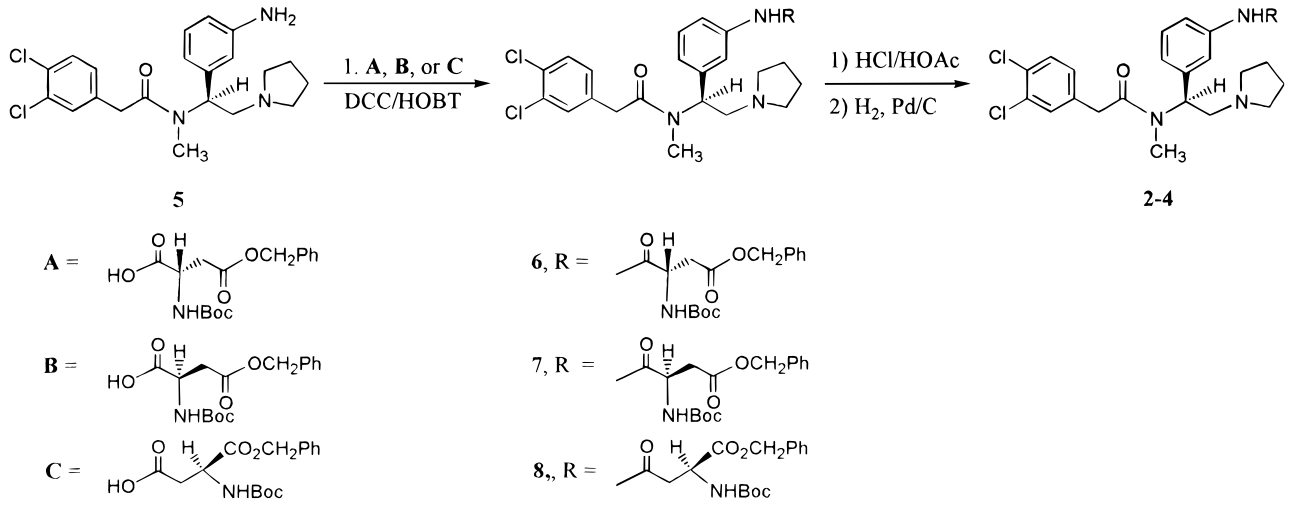

Table 1. Agonist Potencies in Smooth Muscle Preparations

	IC_{50} (nM) ± SEM[a]	
compd	GPI	MVD
1	0.27 ± 0.09	2.50 ± 1.65
2	0.84 ± 0.30	2.49 ± 0.64
3	4.59 ± 1.11	4.06 ± 1.46
4	0.55 ± 0.16	6.36 ± 1.44

[a] Values are arithmetic means of three experiments.

Table 2. Opioid Receptor Binding Affinities

	K_i (nM)[a]		
compd	κ	μ	δ
2	0.34 ± 0.23	462 ± 80	1072 ± 157
3	1.20 ± 0.43	280 ± 84	573 ± 33

[a] Values are arithmetic means of three experiments.

smooth muscle preparations (Table 1). While they have similar potencies in MVD, the D-aspartic acid conjugate **3** is significantly less potent than **2** and **4** in GPI.

Binding. The opioid receptor binding affinity and selectivity of diastereomers **2** and **3** were determined by competition with radioligands in guinea pig brain membranes employing a modification of the method of Werling et al.[19] Binding to κ receptors was evaluated with 1 nM [^{3}H]-(5α,7α,8β)-(−)-*N*-methyl-*N*-1-pyrrolidinyl-1-oxaspiro[4.5]dec-8-ylbenzeneacetamide[20] ([^{3}H]-U69,593), to μ receptors with 2 nM [^{3}H][D-Ala^2,MePhe^4,-Gly-ol^6]enkephalin[21] ([^{3}H]DAMGO), and to δ receptors with 5 nM [^{3}H][D-Pen^2,D-Pen^5]enkephalin[22] ([^{3}H]DP-DPE). Both **2** and **3** were found to bind κ opioid receptors with high affinity and selectivity (Table 2).

In Vivo Studies. In order to determine their peripheral selectivity, compounds **1**−**4** were evaluated for in vivo activity using either the mouse tail-flick[23] or the mouse abdominal stretch[24] assay via both iv and icv routes. The analgesic effects of opioid agonists in these models of acute pain are believed to be mediated through opioid receptors in the CNS. The fact that the compounds are more potent in the mouse abdominal stretch assay also suggests the involvement of opioid receptors, inasmuch as this assay is known to be more sensitive to κ agonists.[25] In evaluating agonist selectivity, nor-BNI,[26] naltrindole[27] (NTI), and β-funaltrexamine[28] (β-FNA) were employed as κ-, δ-, and μ-selective antagonists, respectively. Intravenously administered **2** and **3** were also evaluated for analgesic activity in the second (tonic) phase of the mouse formalin assay, which is a model for tonic inflammatory pain.[29] Attempts to determine the icv potencies of these compounds in the mouse formalin assay were unsuccessful because of interference from either ether or halothane anesthesia.

Table 3. Antinociceptive Potencies of **1**−**4** in Mice

		ED_{50} (nmol/kg)		dose ratio
compd	assay	icv[a]	iv	iv/icv
1	writhing	11.8 (11.7−11.9)	16.5 (10.8−23.8)	1.4
2	writhing	6.8 (4.8−9.2)	105 (90−120)	15.7
	tail-flick	335 (265−412)	850 (340−2070)	2.5
	formalin	ND	10 (4−16)	
3	writhing	46 (31−72)	2015 (1850−2180)	43.6
	tail-flick	no effect found[b]		
	formalin	ND	1050 (510−1590)	
4	writhing	11 (8−15)	628 (360−960)	56.5
	tail-flick	502 (460−542)	5120 (3940−6660)	10.2

[a] Dose is converted from units of nmol/mouse to nmol/kg by employing the average weight of 25 g/mouse. The icv potencies in nmol/mouse were the following: **1** (0.3), **2** (0.17 in writhing, 8.37 in tail-flick), **3** (1.15), **4** (0.28 in writhing, 12.54 in tail-flick). [b] No effect was found at icv doses of up to 160 nmol/mouse or 6.4 μmol/kg.

When tested in the abdominal stretch assay, the derivatives **2**−**4** had iv/icv dose ratios of 11, 31, and 40-fold, respectively, greater than the iv/icv dose ratio of **1** (Table 3). In addition, the ability of the iv-administered L-aspartic acid conjugate **2** to produce CNS effects was enhanced 3-fold relative to that of the corresponding D-aspartic acid conjugate **3**. A similar, but less pronounced, reduction in the abilities of intravenously administered **2** and **4** to produce CNS-mediated effects was also observed in the mouse tail-flick assay (Table 3). Interestingly, the D-aspartyl conjugate **3** was inactive at icv doses of up to 160 nmol/mouse (6.4 μmol/kg) in the tail-flick assay. In the mouse formalin assay, iv-administered **2** and **3** were found to have ED_{50} doses of 10 and 1050 nmol/kg, respectively.

The antinociceptive effects of compounds **2** and **4** in the mouse abdominal stretch assay were found to be κ-selective, as indicated by the fact that the κ-selective antagonist nor-BNI[26] significantly increased the ED_{50} values of **2** and **4**, while the μ and δ antagonists, β-FNA[28] and NTI,[27] were less effective or ineffective in this regard (Table 4). Both **2** and **4** also exhibited weak agonist activity at the μ receptors. Surprisingly, the ED_{50} of **3** was not affected significantly by any of the three opioid receptor selective antagonists.

Table 4. Receptor Selectivity of Antinociception in the Mouse Abdominal Stretch Assay

		ED_{50} ratio[a]			
antagonist	selectivity	**1**	**2**	**3**	**4**
nor-BNI[b]	κ	2.31 (1.6−3.3)	5.00 (3.13−8.33)	1.69 (0.83−2.94)	7.14 (5.00−11.11)
β-FNA[c]	μ	1.72 (1.00−2.86)	2.86 (1.69−4.76)	1.75 (0.88−3.23)	2.56 (1.67−4.00)
NTI[d]	δ	1.19 (0.55−2.77)	0.92 (0.56−1.49)	0.55 (0.28−0.96)	1.01 (0.66−1.56)

[a] The ED_{50} of the agonist (**2**−**4** were given icv; **1** was given sc) in the antagonist-treated mice divided by the control ED_{50} (determined in the absence of antagonist); numbers in parentheses are 95% confidence levels. [b] 12.25 μmol/kg, sc, 2 h after administration. [c] 20.37 μmol/kg, sc, 24 h after administration. [d] 44.44 μmol/kg, sc, 30 min after administration.

Table 5. Peak Times for Antinociception of **2**−**4**

	writhing (min)		tail-flick (min)	
compd	icv	iv	icv	iv
2	10	10	30	20
3	10	10	no effect was found[a]	
4	30[b]	10[c]	30	30

[a] No effect was found for icv doses of up to 160 nmol/mouse or 6.4 μmol/kg. [b] Antinociceptive activity evaluated at 10, 20, 30, and 60 min after administration. [c] Antinociceptive activity evaluated at 10, 20, and 40 min after administration.

While **2** and **3** have identical peak times (10 min) in the abdominal stretch assay via iv and icv routes, the antinociceptive effect of **4** administered iv peaked significantly earlier relative to icv administration (Table 5).

Discussion

Previous work in our laboratory has shown that aspartate conjugates of opioid ligands naltrexamine and oxymorphamine crossed the BBB poorly, as indicated by very large iv/icv ED_{50} dose ratios in mice.[7] Peripherally administered aspartic acid conjugates **2**−**4** were also found to have reduced antinociceptive potencies, but the dose ratios were smaller than those of the opiates. This was indicated by much smaller iv/icv ED_{50} dose ratios in the mouse abdominal stretch and tail-flick assays. In this regard, the iv-administered aspartate conjugates **2**−**4** were 11-, 31-, and 40-fold, respectively, less effective than the parent compound **1** in producing CNS-mediated antinociception in the mouse abdominal stretch assay.

There is a possibility that amino acid conjugates may be transported across the BBB. As a matter of fact, γ-glutamyl transpeptidase (γ-GT), which is present in the BBB, facilitates CNS uptake of polar substances by conjugation with L-Glu through the γ-carboxyl group.[30,31] Furthermore, transporters specific for one of three classes of α-amino acids (acidic,[32] basic,[33,34] and neutral[35]) are also present in the BBB, and competition for transport occurs among both natural and unnatural amino acids of the same class.[36,37] In addition to broad substrate specificity,[38] the neutral amino acid (NAA) transporter has varying degrees of stereoselectivity in its transport of the L and D isomers of different amino acids.[39] There are examples of small unnatural amino acids or amino acid conjugates being transported by the NAA transporter.[40−43]

The antinociceptive action of iv administered aspartate conjugates **2**−**4** may in part be facilitated by transport across the BBB. Alternately, the relative ease with which iv-administered **2**−**4** produce CNS-mediated effects may be a result of hydrolysis of the conjugates to yield the parent amine **5**, which would easily penetrate the BBB. However, this latter possibility appears less likely in view of the short time intervals involved in the in vivo testing.

A comparison of their iv potencies shows that compounds **2** and **3** are 10- and 2-fold, respectively, more potent in the mouse formalin assay than in the writhing assay. This may indicate that both **2** and **3**, especially **2**, are more effective analgesics against tonic pain than acute pain. In view of the well-documented evidence indicating that peripheral opioid receptor mediated antinociception is manifested during inflammation,[1−3] however, the greater potencies of **2** and **3** in the mouse formalin assay may indicate that a greater portion of the antinociception produced by **2** and **3** in the formalin assay is mediated by localized peripheral opioid receptors at the site of inflammation. Assuming the iv administration of **2** results in the same CNS penetration in both formalin and writhing assays, the amount of CNS-mediated analgesia by **2** should be the same in both assays. The greater potency of iv-administered **2** in the formalin assay may therefore reflect increased contribution to the overall antinociception by peripheral opioid receptors.

While both **2** and **3** exhibited high affinity and selectivity binding to κ opioid receptors in competitive binding assays against [^{3}H]U69,593, their in vivo activities are significantly different. This may be due to different pharmacokinetic properties that may result from their diastereomeric relationship. Alternatively, the low potency and low ED_{50} ratios of **3** suggest that **3** may be producing its antinociceptive effect via a non-opioid mechanism of action or by interacting with a κ subtype that is not effectively antagonized by nor-BNI.

The unexpected finding that the peak time of antinociception of **4** by the icv route in the mouse abdominal stretch assay was later than that when administered iv is significant. The faster onset of action suggests the possibility of a peripheral site of action for **4** at the high doses required for iv administration.

In conclusion, the present study has been successful in the development of κ agonists that have reduced access into the CNS. In addition to retaining κ receptor affinity and selectivity, the aspartate conjugates **2**−**4** were found to be significantly less effective than the parent compound **1** in producing CNS-mediated opioid antinociception after peripheral administration, and they may be useful as potential therapeutic agents and as pharmacologic tools in studying the peripheral opioid receptors. Furthermore, the actions of peripherally-administered **2**−**4**, though limited, hint of the possible involvement of an amino acid transporter system in their CNS penetration.

Experimental Section

All NMR spectra were recorded on a GE 300 MHz spectrometer at room temperature. Optical rotations were measured using a Rudolph Research Autopol III automatic pola-

rimeter. Melting points were determined using a Thomas Hoover capillary melting point apparatus and are uncorrected. Elemental analyses were conducted by M-H-W Laboratories in Phoenix, AZ. Mass spectra were recorded by the Chemistry Mass Spec Labs at the University of Minnesota's Chemistry Department. The diprotected aspartic acid derivatives were purchased from Sigma Chemical Co., St Louis, MO 63178.

2-(3,4-Dichlorophenyl)-*N*-methyl-*N*-{(1*S*)-1-[3-*N*-(*S*)-aspartylamino]phenyl]-2-(1-pyrrolidinyl)ethyl}acetamide (2). To an ice-cold mixture of *N*-*t*-Boc-L-aspartic acid-β benzyl ester (0.3541 g, 1.095 mmol) and 1-hydroxybenzotriazole (HOBT) (0.1495 g, 1.106 mmol) in dry CH_2Cl_2 (20 mL) was added with stirring under N_2 a solution of *N*,*N*′-dicyclohexylcarbodiimide (DCC) (0.2331 g, 1.130 mmol) in dry CH_2Cl_2 (10 mL). After 1 h of stirring at 0 °C, a solution of **5**[14,15] (0.2228 g, 0.5483 mmol) in dry CH_2Cl_2 (6 mL) was added, and the reaction mixture was stirred at 25 °C under N_2 for 23 h before it was filtered. The filtrate was then washed with saturated $NaHCO_3$ before it was dried (Na_2SO_4), filtered, and evaporated under reduced pressure. Flash column chromatography with $CHCl_3$:2% NH_3:2% MeOH yielded 0.3315 g (85%) of the protected intermediate, which was further purified on HPLC using $CHCl_3$:2% NH_3:1% MeOH. After the protected intermediate (0.0905 g, 0.1272 mmol) was stirred in 1 mL of 2 N HCl, 1 mL of AcOH, and 1 drop of anisole at 25 °C for 20 min, 2 mL of MeOH and 10% Pd/C (about 15 mg) were added, and the mixture was hydrogenated at 25 °C using a hydrogen balloon. After 1 h, more Pd/C (about 10 mg) was added, and 20 min later the mixture was filtered through Celite and evaporated in vacuo. The clear film which remained was converted to a white solid by addition of iPrOH, and evaporation to dryness in vacuo yielded **2**·2HCl (50.6 mg, 66.9%): mp 170 °C dec; $[\alpha]^{25}_D$ +113° (c = 0.2, MeOH); ^{1}H NMR (DMSO-d_6) δ 1.97 (br s, 4H, CH_2CH_2), δ 2.82 (s, 3H, NCH_3), 2.85−4.23 (complex, 11H, 5 CH_2 and 1 CH), 6.10 (m, 1H, CH), 7.04−7.67 (complex, 7H, aromatic), 8.3 (s, exchangeable proton), 8.2−8.8 (br s, exchangeable proton), 10.6−10.8 (br s, exchangeable proton); MS (FAB) m/z 521.3. Anal. ($C_{25}H_{30}N_4O_4Cl_2$·2HCl) C, H, N, Cl.

2-(3,4-Dichlorophenyl)-*N*-methyl-*N*-{(1*S*)-1-[3-[*N*-(*R*)-aspartylamino]phenyl]-2-(1-pyrrolidinyl)ethyl}acetamide (3). Compound **3** was prepared from **5**[14,15] (0.2259 g, 0.5559 mmol), *N*-*t*-Boc-D-aspartic acid β-benzyl ester (0.3598 g, 1.113 mmol), HOBT (0.1520 g, 1.125 mmol), and DCC (0.2363 g, 1.145 mmol) in dry CH_2Cl_2 (23 mL). The procedure and reaction conditions are similar to those employed for the preparation of **2**. After 23 h, the reaction was worked up in a manner similar to that of **2**, and purification by flash column chromatography with CH_2Cl_2:2% NH_3:3% MeOH yielded 0.3845 g (97%) of the protected intermediate, which eluted off the HPLC as a single sharp peak with $CHCl_3$:2% NH_3:1% MeOH. After the protected intermediate in 2 N HCl (3 mL), AcOH (3 mL), and 2 drops of anisole was stirred at 25 °C for 1 h, the mixture was evaporated in vacuo, and the residue was partitioned between CH_2Cl_2 and saturated $NaHCO_3$. The organic fraction was dried (Na_2SO_4), filtered through Celite, and evaporated to yield the benzyl ester intermediate which was purified by gravity column chromatography with CH_2Cl_2: 2% NH_3:3% MeOH. The benzyl ester intermediate was then converted to the 2HCl salt with Et_2O·HCl and hydrogenated at 40 psi with 35 mg of 10% Pd/C in MeOH (8 mL). After 45 min, the mixture was filtered through Celite, and the Pd/C was washed thoroughly with hot MeOH. The solvent from the combined filtrates was removed, the residue was then taken up in iPrOH, and the solvent was evaporated to yield **3**·2HCl (0.1012 g, 30.6%): mp 200 °C dec; $[\alpha]^{25}_D$ +103.9° (c = 0.23, MeOH); ^{1}H NMR (DMSO-d_6) δ 1.94 (br s, 4H, CH_2CH_2), 2.808 (s, 3H, NCH_3), 2.77−4.24 (complex, 11H, 5 CH_2 and 1 CH), 6.076 (m, 1H, CH), 7.014−7.658 (complex, 7H, aromatic), 8.6 (br s, exchangeable proton), δ10.9 (br s, exchangeable proton); MS (FAB) m/z 521.2. Anal. ($C_{25}H_{30}N_4O_4Cl_2$·2HCl) C, H, N, Cl.

2-(3,4-Dichlorophenyl)-*N*-methyl-*N*-{(1*S*)-1-[*N*-(*S*)-aspartylamino]phenyl]-2-(1-pyrrolidinyl)ethyl}acetamide (4). Compound **4** was prepared from **5**[14,15] (0.2379 g, 0.5855 mmol), *N*-*t*-Boc-L-aspartic acid α-benzyl ester (1.8933 g, 5.855 mmol), HOBT (0.8070 g, 5.972 mmol), and DCC (1.2441 g, 6.030 mmol) in dry CH_2Cl_2 (31 mL). The procedure and reaction conditions are similar to those employed for the preparation of **2**, but more than 2 equiv of the aspartic acid derivative, DCC, and HOBT were needed to drive the reaction to completion. After 17 h, the reaction was worked up in a manner similar to that of **2**, and purification by flash column chromatography with CH_2Cl_2:2% NH_3:3% MeOH yielded 0.3 g (72%) of the protected intermediate. After removal of the Boc group by treatment with 2 N HCl (3 mL), AcOH (3 mL), and 3 drops of anisole at 25 °C for 105 min, the benzyl ester protected intermediate was worked up as the Boc deprotection of **3** and purified by flash column chromatography with CH_2Cl_2:2% NH_3:5% MeOH. The benzyl ester was converted to the 2HCl salt with Et_2O·HCl and cleaved by hydrogenation at 39 psi with 25 mg of 10% Pd/C in MeOH (8 mL) for 1 h, followed by the workup analogous to the benzyl ester deprotection of **3** to yield **4**·2HCl (0.0714 g, 20.5%): mp 185 °C dec; $[\alpha]^{25}_D$ +147° (c = 0.10, MeOH); ^{1}H NMR (DMSO-d_6) δ 1.937 (br s, 4H, CH_2CH_2), 2.795 (s, 3H, NCH_3), 2.967−4.170 (complex, 11H, 5 CH_2 and 1 CH), 6.06 (m, 1H, CH), 6.965−7.611 (complex, 7H, aromatic), 8.4 (br s, exchangeable proton), 10.5 (s, exchangeable proton), 10.9 (br s, exchangeable proton); MS (FAB) m/z 521.2. Anal. ($C_{25}H_{30}N_4O_4Cl_2$·2HCl) C, H, N, Cl.

Acknowledgment. We thank Veronika Doty, Michael Powers, Joan Naeseth, and Idalia Sanchez for in vitro and in vivo testing of the compounds. We also thank Dr. Diane DeHaven-Hudkins for pleasant and insightful discussions of the results.

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JM960459X