Synthesis of Substituted 3-Carbamoylecgonine Methyl Ester Analogues: Irreversible and Photoaffinity Ligands for the Cocaine Receptor/Dopamine Transporter

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Received April 26, 1993[●]

Photoaffinity ligands are useful tools for the isolation, purification, and characterization of proteins. As a step toward the goal of producing a photoaffinity probe for the dopamine transporter, isocyanato and azido derivatives of 3-[(phenylcarbamoyl)oxy]ecgonine methyl ester were synthesized and tested for their ability to interact with the cocaine receptor of mammalian brain *via* two different assays. The ability of two isothiocyanato (N=C=S) (para and meta) and two azido (N_3) (para and meta) derivatives, as well as (−)-cocaine, to inhibit [^{3}H]cocaine binding and [^{3}H]dopamine uptake and to covalently interact with the cocaine-binding site was tested. The *p*-N=C=S was the most potent, with IC_{50} values of 0.23 and 0.49 μM for [^{3}H]cocaine binding and [^{3}H]dopamine uptake. The *m*-N_3 and *p*-N_3 inhibited [^{3}H]cocaine binding with IC_{50} values of 0.63 and 1.00 μM and inhibited [^{3}H]dopamine uptake with IC_{50} values of 5.08 and 1.32 μM, respectively. Preincubation of synaptosomal membranes with the *m*- or *p*-N=C=S isomer either in reduced lighting or under ultraviolet light followed by two washes resulted in inhibition of 70% and 85% of [^{3}H]cocaine binding, respectively, indicating the highly reactive properties of these compounds. After preincubation in reduced lighting, *m*-N_3 and *p*-N_3 inhibited 0% and 13% of [^{3}H]cocaine binding, while following preincubation under ultraviolet light, the inhibition increased to 61% and 68%, respectively. Thus, the isothiocyanato derivatives appear to bind irreversibly to the cocaine receptor in the presence or absence of ultraviolet light, whereas the azido derivatives are photoreactive compounds which may prove useful in the purification of the receptor.

Introduction

Cocaine is a widely used recreational drug which is of concern as a drug of abuse due to its powerful reinforcing properties[1] and toxic side effects.[2] The central nervous system effects which lead to abuse include feelings of well-being and euphoria which occur without hampering motor activity.[3] A primary target for cocaine in the central nervous system is the dopamine transporter of the caudate nucleus and the mesocorticolimbic system.[4–6] This transmembrane protein transports dopamine into the presynaptic nerve terminal and thus is responsible for the primary mechanism for termination of neurotransmission at dopaminergic synapses. Cocaine inhibits dopamine uptake *in vitro*,[7] and dopamine transport inhibition has been shown to be associated with the reinforcing effects of cocaine.[8] Further evidence that the central stimulant and reinforcing properties of cocaine are linked to its ability to inhibit dopamine uptake includes (1) the administration of dopamine receptor antagonists inhibits the reinforcing efficacy of cocaine,[9] (2) a chemically induced lesion of dopamine nerve cells blocks self-administration of cocaine,[10–12] and (3) depletion of dopamine reserves in the brain by treatment with reserpine and tyrosine hydroxylase inhibitors prevents cocaine-induced stereotypy and hyperkinesis.[13]

Due to this strong link between cocaine-induced behaviors and the dopamine transporter, the isolation, purification, and characterization of the protein may lead to better understanding of the molecular properties of the transporter and how it interacts with substrate and inhibitors. Significant progress toward this goal has been facilitated by the development of several series of cocaine analogues.[14–19] Recent work has focused on the 3-arylecgonine methyl ester analogues first reported by Clarke,[15] and later by Kline *et al.*[18] and Carroll and co-workers.[17] Compounds of this group proved to be potent inhibitors of [^{3}H]cocaine binding and [^{3}H]dopamine uptake.

Several potentially irreversible ligands for the cocaine receptor site on the dopamine transporter, derived from cocaine or 3β-phenyltropane-2β-carboxylic acid methyl ester (WIN 35.065-2), were described recently by Carroll *et al.*[19] These included isothiocyanate and azide derivatives. The irreversible ligands were assayed as inhibitors of the reversible binding of [^{3}H]WIN 35,428 only. The isothiocyanate and azide derivatives of WIN 35,065-2 were 2 orders of magnitude more potent than the derivatives of cocaine.[20]

In studies intended to further delineate the substituent requirements of the 3-position, we synthesized a series of 3-[(phenylcarbamoyl)oxy]ecgonine methyl ester analogues which potently interacted with the cocaine-binding site and were readily obtained in good yield from ecgonine methyl ester.[21] The phenylisothiocyanato and phenylazido derivatives in this series were tested for their ability to interact with the dopamine transporter by the assays which test location (inhibition of [^{3}H]cocaine binding) and function (inhibition of [^{3}H]dopamine uptake). These compounds were then tested for their ability to irreversibly bind to the cocaine receptor, either spontaneously or following activation by ultraviolet light.

Results and Discussion

Chemistry. (1*R*)-Ecgonine methyl ester (1) was obtained from (1*R*)-cocaine hydrochloride by known pro-

● Abstract published in *Advance ACS Abstracts*, May 15, 1994.

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Scheme 1

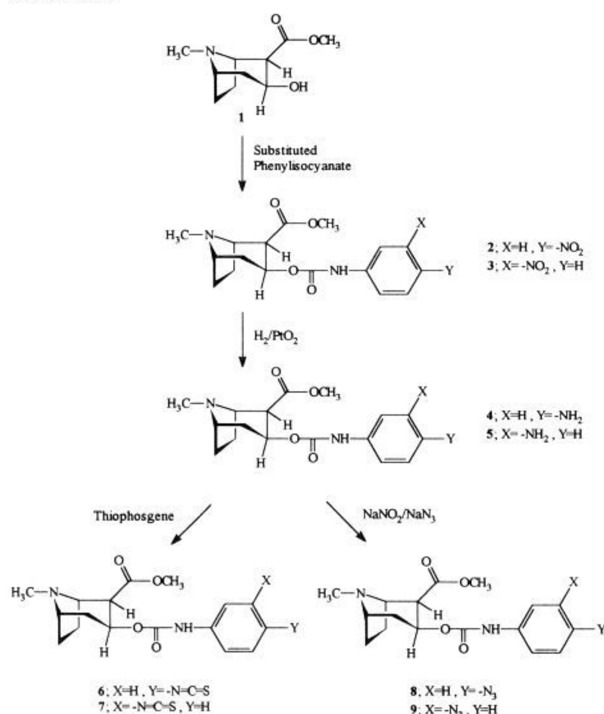

Table 1. Inhibition of [^{3}H]Cocaine Binding and [^{3}H]Dopamine Uptake in Rat Striatal Tissue

corresponding analogue	IC_{50}[a] (μM) cocaine binding	dopamine uptake
m-N=C=S	0.96 ± 0.21	4.90 ± 0.42
p-N=C=S	0.26 ± 0.06	0.49 ± 0.08
m-N_3	0.63 ± 0.15	3.90 ± 1.59
p-N_3	1.00 ± 0.24	1.18 ± 0.36
cocaine	0.07 ± 0.01	0.21 ± 0.07

[a] Values are the mean ±SEM for three to five experiments, each performed in triplicate.

cedures.[22,23] Thus, (1*R*)-cocaine hydrochloride was hydrolyzed in dilute HCl and then reesterified at the C_2 position in CH_3OH/H_2SO_4. Overall yield from (1*R*)-cocaine hydrochloride to (1*R*)-ecgonine methyl ester was 85%.

The preparation of compounds **2**, **3**, **4**, and **5** (Scheme 1) was reported in a previous volume of this journal.[21] Isothiocyanato derivatives **6** and **7** (Scheme 1) were obtained by reacting compounds **4** and **5** with thiophosgene, while the azido analogues **8** and **9** were prepared from the same compounds *via* a diazotization reaction ($NaNO_2/NaN_3$).[24]

Pharmacology. The ability of these derivatives to inhibit [^{3}H]cocaine binding to rat striatal P_2 membranes and [^{3}H]dopamine uptake into rat striatal synaptosomes was tested. All compounds tested were found to be inhibitory in both assays at high nanomolar or low micromolar concentrations, as shown in Table 1. These results indicate that the compounds have high affinity for the dopamine transporter.

Previously reported data[21] indicated a possible connection between the electron density of the aromatic ring in the carbamate side chain and the potency of the compound. It was observed that the more potent derivatives of this series were those which had electron-withdrawing substituents on the phenyl ring. Introduction

Table 2. Irreversible Binding to the Cocaine Receptor: Inhibition of [^{3}H]Cocaine Binding

compound	percent of dark control[a] reduced lighting	ultraviolet light
control	100	89.9 ± 9.2
cocaine	137.9 ± 18.9	74.1 ± 17.5
m-N=C=S	33.4 ± 9.1[b]	29.1 ± 4.7[b]
p-N=C=S	12.3 ± 1.3[b]	18.5 ± 5.6[b]
m-N_3	101.9 ± 22.4	39.1 ± 3.2[b]
p-N_3	86.8 ± 20.2	32.5 ± 6.8[b]

[a] All binding data are expressed as percent of the dark control for each experiment. Each number represents the average of three experiments, each performed in triplicate. [b] Binding was significantly less than control binding in every experiment.

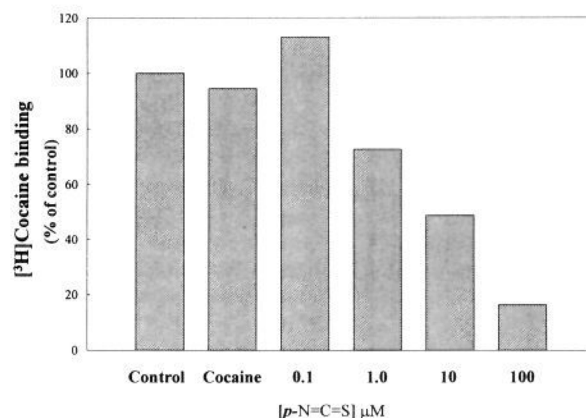

Figure 1. Dose dependency of irreversible inhibition of [^{3}H]-cocaine binding by *p*-isothiocyanate.

of an electron-donating group such as an amino moiety resulted in a much less potent compound. It is interesting to note that by reducing the electron-donating effect of the amino substituent *via* conversion to either isothiocyanato or azido groups, the affinity for the cocaine-binding site is greatly increased.

The ability of these compounds to bind irreversibly to the cocaine receptor was assayed as detailed in the methods. As shown in Table 2, the *p*- and *m*-isothiocyanato derivatives inhibited cocaine binding following both preincubation conditions and the washes, indicating the spontaneous irreversible binding of these two compounds. Inhibition was almost complete with *p*-N=C=S, with only 12% of [^{3}H]cocaine binding remaining. The higher potency of *p*-N=C=S in this assay was consistent with its lower IC_{50} values in the [^{3}H]cocaine and [^{3}H]dopamine assays. This irreversible binding was dose-dependent for *p*-N=C=S, as shown in Figure 1, with 50% inhibition of [^{3}H]cocaine binding at a concentration of 10 μM.

The two azido derivatives displayed little or no inhibitory activity of [^{3}H]cocaine binding when preincubation was conducted under reduced lighting. However, following exposure to ultraviolet light, these compounds inhibited 61% (meta) and 68% (para) of [^{3}H]cocaine binding in contrast to cocaine which showed no significant irreversible binding. The observation that there was a reduction in percent control of [^{3}H]cocaine binding with cocaine preincubation was attributed to the possible sensitivity of the receptor to ultraviolet light. These results demonstrated that binding of the azido compounds to the cocaine receptor became irreversible following exposure to UV light, indicating that these compounds may prove to be useful photoaffinity ligands for the cocaine receptor of the dopamine transporter.

Experimental Section

Chemistry. Melting points were determined with a Thomas-Hoover capillary melting point apparatus and are uncorrected. NMR spectra were obtained with a General Electric QE-300 spectrometer. Spectra were obtained using $CDCl_3$ with TMS as an internal standard. Infrared spectra were recorded on an Analect FX-6160 FT-IR spectrophotometer with KBr disks. All optical rotations were determined at the sodium D line using a Perkin-Elmer (Model 141) polarimeter (1-dm cell). Elemental analyses performed (on samples dried to constant weight) by Micro Analysis, Inc., agreed to within 0.4% of the calculated values.

(1*R*,2-*exo*,3-*exo*)-3-[[*N*′-(4′-Isothiocyanatophenyl)carbamoyl]oxy]-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylic Acid Methyl Ester (6). Compound 4 (0.10 g, 0.30 mmol) and $NaHCO_3$ (0.50 g) were suspended in dry toluene (50 mL). To this mixture was added thiophosgene (65.7 μL, 0.90 mmol), and the reaction was stirred at room temperature for 45 min. Ice-cold H_2O (50 mL) was then added, and the reaction mixture was stirred vigorously for an additional 10 min. The two phases were separated, and the aqueous phase was extracted with additional toluene (2 × 30 mL). The organic fractions were combined and concentrated *in vacuo*. Crystallization of the residue in EtAc/pentane resulted in a nearly quantitative yield of the desired product: small white crystals; mp = 118–121 °C; $[\alpha]^{23}_D = -15.67°$ (c = 0.30, $CHCl_3$); IR (KBr pellet) 3318 (ν_{N-H}), 2953 (ν_{C-H}), 2130 ($\nu_{N=C=S}$), 2047 ($\nu_{N=C=S}$), 1730 ($\nu_{C=O}$, ester), 1698 ($\nu_{C=O}$, carbamate), 1600 ($\nu_{C=C}$), 1536 (amide II), 1231 (amide III) cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1..62–1.74 (m, 2H, H_{6ax}, H_{7ax}), 1.78–1.88 (m, 1H, H_{4eq}), 2.04–2.20 (m, 2H, H_{6eq}, H_{7eq}), 2.22 (s, 3H, NCH_3), 2.32–2.41 (dt, J = 3 Hz (vic), 9 Hz (gem), 1H, H_{4ax}), 2.97–3.00 (m, 1H, H_2), 3.25–3.32 (m, 1H, H_5) 3.51–3.57 (m, 1H, H_1), 3.72 (s, 3H, OCH_3), 4.97–5.05 (m, 1H, H_3), 6.98 (br s, 1H, NH), 7.13–7.18 (dt, J = 2.7 Hz (meta), 9 Hz (ortho), 2H, $H_{2'}$, $H_{6'}$), 7.34–7.38 (dt, J = 2.7 Hz (meta), 9 Hz (ortho), 2H, $H_{3'}$, $H_{5'}$); ^{13}C NMR ($CDCl_3$) δ 24.71, 24.82 (C_6, C_7), 35.16 (C_4), 40.63 (NCH_3), 49.67 (C_2), 51.04 (OCH_3), 61.06 (C_5), 64.30 (C_1), 67.16 (C_3), 118.53 ($C_{2'}$, $C_{6'}$), 125.96 ($C_{3'}$, $C_{5'}$), 134.11 ($C_{1'}$), 136.56 ($C_{4'}$), 152.23 (C=O, carbamate), 170.47 (C=O, ester). Anal. ($C_{18}H_{21}N_3O_4S{\cdot}0.25H_2O$) C, H, N.

(1*R*,2-*exo*,3-*exo*)-3-[[*N*′-(3′-Isothiocyanatophenyl)carbamoyl]oxy]-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylic Acid Methyl Ester (7). Compound 5 (0.10 g, 0.30 mmol) and $NaHCO_3$ (0.50 g) were suspended in dry toluene (50 mL). To this stirring mixture was added thiophosgene (65.7 μL, 0.90 mmol), and the reaction was allowed to proceed at room temperature for 45 min. Ice-cold H_2O (50 mL) was then added, and the reaction vessel was shaken vigorously. The two phases were then separated, and the aqueous phase was extracted with additional toluene (2 × 30 mL). The organic fractions were combined, dried over anhydrous Na_2SO_4, filtered, and concentrated *in vacuo*. Crystallization of the residue from EtAc/pentane resulted in small cream-colored crystals: mp = 125–127 °C; $[\alpha]^{23}_D = -21.55°$ (c = 0.30, $CHCl_3$); IR (KBr pellet) 3333 (ν_{N-H}), 3050 (ν_{C-H}, aromatic), 2942 (ν_{C-H}, aliphatic), 1737 ($\nu_{C=O}$, ester), 1722 ($\nu_{C=O}$, carbamate), 1650 ($\nu_{C=C}$), 1539 (amide II), 1231 (amide III) cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.62–1.74 (m, 2H, H_{6ax}, H_{7ax}), 1.78–1.84 (m, 1H, H_{4eq}), 2.05–2.17 (m, 2H, H_{6eq}, H_{7eq}), 2.21 (s, 3H, NCH_3), 2.32–2.41 (dt, J = 3 Hz (vic), 11.7 Hz (gem), 1H, H_{4ax}), 2.98–3.01 (m, 1H, H_2), 3.26–3.30 (m, 1H, H_5), 3.51–3.55 (m, 1H, H_1), 3.72 (s, 3H, OCH_3), 4.97–5.05 (m, 1H, H_3), 6.89–6.91 (dt, J = 1.8 Hz (meta), 9.3 Hz (ortho), 1H, $H_{6'}$), 6.98 (br s, 1H, NH), 7.11–7.14 (dt, J = 1.8 Hz (meta), 9.3 Hz (ortho), 1H, $H_{4'}$), 7.21–7.27 (dd, J = 9.3 Hz (ortho), 1H, 1$H_{5'}$), 7.46–7.47 (t, J = 1.8 Hz (meta), 1H, $H_{2'}$); ^{13}C NMR ($CDCl_3$) δ 24.71, 24.83 (C_6, C_7), 35.15 (C_4), 40.63 (NCH_3), 49.67 (C_2), 51.04 (OCH_3), 61.05 (C_5), 64.30 (C_1), 67.26 (C_3), 114.99 ($C_{5'}$), 116.55 ($C_{6'}$), 119.96 ($C_{4'}$), 129.39 ($C_{2'}$), 131.38 ($C_{1'}$), 138.62 ($C_{3'}$), 152.27 (C=O, carbamate), 170.46 (C=O, ester). Anal. ($C_{18}H_{21}N_3O_4S$) C, H, N.

(1*R*,2-*exo*,3-*exo*)-3-[[*N*′-(4′-Azidophenyl)carbamoyl]oxy]-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylic Acid Methyl Ester (8). Compound 8 was prepared following the general procedure of Smith and Boyer.[24] The entire reaction and workup was carried out in subdued light. Compound 4 (0.10 g, 0.30 mmol) was suspended in H_2O (10 mL), and concentrated HCl (1 mL) was added. The light brown solution was then cooled to 0–5 °C in an ice–salt bath. $NaNO_2$ (25 mg, 0.36 mmol) was dissolved in H_2O (1 mL) and added to the reaction mixture dropwise, maintaining the temperature between 0–5 °C. The now yellow-green solution was allowed to stir at the indicated temperature for 1 h. A solution of NaN_3 (24 mg, 0.36 mmol) in H_2O (1 mL) was added dropwise in a similar fashion to the described above. The solution was stirred for an additional 30 min. The reaction mixture was then neutralized with Na_2CO_3 and immediately extracted with $CHCl_3$ (4 × 20 mL). The organic fractions were combined, dried over anhydrous Na_2SO_4, filtered, and concentrated *in vacuo*. Crystallization of the residue from pentane resulted in light brown crystals: mp = 110–112.5 °C; IR (KBr pellet) 3350 (ν_{N-H}), 3028 (ν_{C-H}, aromatic), 2945 (ν_{C-H}, aliphatic), 2116 ($\nu_{N=N}$), 1739 ($\nu_{C=O}$, ester), 1726 ($\nu_{C=O}$, carbamate), 1598 ($\nu_{C=C}$), 1544 (amide II), 1225 (amide III) cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.62–1.74 (m, 2H, H_{6ax}, H_{7ax}), 1.78–1.85 (m, 1H, H_{4eq}), 2.03–2.25 (m, 2H, H_{6eq}, H_{7eq}), 2.21 (s, 3H, NCH_3), 2.32–2.41 (dt, J = 3 Hz (vic), 12 Hz (gem), 1H, H_{4ax}), 2.98–3.01 (m, 1H, H_2), 3.25–3.31 (m, 1H, H_5), 3.50–3.56 (m, 1H, H_1), 3.72 (s, 3H, OCH_3), 4.96–5.04 (m, 1H, H_3), 6.89 (br s, 1H, NH), 6.91–6.98 (dt, J = 2.1 Hz (meta), 8.7 Hz (ortho), 2H, $H_{2'}$, $H_{6'}$), 7.34–7.38 (d, J = 8.7 Hz (ortho), 2H, $H_{3'}$, $H_{5'}$); ^{13}C NMR ($CDCl_3$) δ 24.70, 24.82 (C_6, C_7), 35.21 (C_4), 40.65 (NCH_3), 49.74 (C_2), 50.99 (OCH_3), 61.06 (C_5), 64.32 (C_1), 66.92 (C_3), 119.03 ($C_{2'}$, $C_{6'}$), 119.27 ($C_{3'}$, $C_{5'}$), 134.34 ($C_{1'}$), 134.44 ($C_{4'}$), 152.52 (C=O, carbamate), 170.54 (C=O, ester). Anal. ($C_{17}H_{21}H_5O_4$) C, H, N.

(1*R*,2-*exo*,3-*exo*)-3-[[*N*′-(3′-Azidophenyl)carbamoyl]oxy]-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylic Acid Methyl Ester (9). This compound was synthesized following the general methods of Smith and Boyer.[24] Compound 5 (0.10 g, 0.30 mmol) was the starting material. Experimental procedures are identical to those reported for compound 8. Recrystallization from pentane resulted in fluffy yellow crystals: yield = 79.8%; mp = 116.5–118 °C; IR (KBr pellet) 3328 (ν_{N-H}), 3076 (ν_{C-H}, aromatic), 2947 (ν_{C-H}, aliphatic), 2112 ($\nu_{N=N}$), 1733 ($\nu_{C=O}$, ester), 1721 ($\nu_{C=O}$, carbamate), 1604 ($\nu_{C=C}$), 1540 (amide II), 1231 (amide III) cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.60–1.75 (m, 2H, H_{6ax}, H_{7ax}), 1.78–1.85 (m, 1H, H_{4eq}), 2.00–2.25 (m, 2H, H_{6eq}, H_{7eq}), 2.21 (s 3H, NCH_3), 2.32–2.41 (dt, J = 2.7 Hz (vic), 11.7 Hz (gem), 1H, H_{4ax}), 2.98–3.01 (m, 1H, H_2), 3.25–3.31 (m, 1H, H_5), 3.50–3.56 (m, 1H, H_1), 3.72 (s, 3H, OCH_3), 4.97–5.05 (m, 1H, H_3), 6.70–6.73 (dd, J = 2 Hz (meta), 7.8 Hz (ortho), 1H, $H_{6'}$), 6.97 (br s, 1H, NH), 7.01–7.05 (dd, J = 2 Hz (meta), 7.8 Hz (ortho), 1H, $H_{4'}$), 7.21–7.27 (dd, J = 7.8 Hz (ortho), 2H, $H_{5'}$, $H_{2'}$); ^{13}C NMR ($CDCl_3$) δ 24.70, 24.82 (C_6, C_7), 35.18 (C_4), 40.63 (NCH_3), 49.70 (C_2), 50.99 (OCH_3), 61.04 (C_5), 64.31 (C_1), 67.05 (C_3), 108.36 ($C_{5'}$), 113.22 ($C_{6'}$), 114.18 ($C_{4'}$), 129.61 ($C_{2'}$), 138.91 ($C_{1'}$), 140.43 ($C_{3'}$), 152.33 (C=O, carbamate), 170.50 (C=O, ester). Anal. ($C_{17}H_{21}H_5O_4{\cdot}H_2O$) C, H, N.

Biology. Membrane Preparation. Both binding and uptake assays were done on synaptosomes prepared from striata dissected from rat brains. The dissected tissue included caudate, globus pallidus, and putamen as well as nucleus accumbens. All experiments were conducted with fresh tissue, and all subsequent steps for membrane preparation were performed at 0–4 °C.

For [^{3}H]cocaine-binding experiments, the striatal tissue was homogenized in ice-cold 10 mM Na_2HPO_4 buffer containing 0.25 M sucrose, pH = 7.4, using a glass Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was centrifuged at 1000*g* for 10 min, and the supernatant was centrifuged at 48000*g* for 20 min. The pellet was resuspended in the same buffer with a P-10 Kinematica Polytron homogenizer (setting 7) for 20 s. The homogenate was centrifuged at 48000*g* for 20 min. The resulting pellet was resuspended in 40 volumes (vol/wt) of the same phosphate–sucrose buffer to yield approximate protein concentrations of 1 mg/mL. The method of Lowry *et al.*[25] was utilized to determine all final protein concentrations.

For covalent and photoaffinity assays, aliquots of ligand (final concentration 100 μM or as indicated) in DMSO or H_2O (20 μL) or vehicle alone were added to aliquots of membrane suspension to reach a final volume of 2 mL. Mixtures were incubated for 5 min either in reduced lighting or 4 in. from an ultraviolet light source. Incubation was terminated by the addition of ice-cold buffer (10 mL), and the membrane suspensions were centrifuged at 48000*g* for 20 min. The pellet was washed by centrifugation and resuspended in 30 volumes of buffer and used for [^{3}H]cocaine binding.

Tissue used in [^{3}H]dopamine-uptake experiments was pre-

pared according to the procedure of Richelson and Pfenning[7] with slight modifications. Homogenization of the striatal tissue was performed in ice-cold 0.25 M sucrose, 11 mM glucose (pH = 7.4) buffer using a glass Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was centrifuged for 10 min at 1000*g* in a Beckman centrifuge, and then, the supernatant was decanted and centrifuged for 20 min at 48000*g*. The resultant P_2 pellet was washed by centrifugation in the same sucrose–glucose solution, and the final pellet was resuspended in 20 volumes (vol/wt) of the same solution.

[^{3}H]Cocaine Binding. Binding of [^{3}H]cocaine (1-[*benzoyl*-3,4-^{3}H(N)], 29.7 Ci/mmol; New England Nuclear, Boston, MA) to rat striatal membranes was measured by filtration assay using a previously reported method.[21] At least eight concentrations of each ligand were tested, ranging from 10 nM to 100 μM. In most cases, these concentrations displaced specific [^{3}H]cocaine binding from 2% to 98%. The assay included 200 μL of membrane preparation (200 μg of protein, 25 μL of [^{3}H]cocaine (6 nM), 2.5 μL of cocaine analogue in dimethyl sulfoxide (DMSO) or solvent alone, and buffer to reach a final volume of 250 μL. Nonspecific binding was defined as that measured in the presence of 100 μM unlabeled cocaine and was typically less than 15% of total binding (total dpm bound was 3900; dpm bound in the presence of 100 μM cocaine was 430). After 20 min of incubation, the reaction was terminated by the addition of 4 mL of ice-cold saline and filtration through Whatman GF/B filters which were presoaked in 0.05% poly(ethyleneimine). Filters were washed once with 4 mL of ice-cold saline, and the radioactivity remaining on the filters was counted.

[^{3}H]Dopamine Uptake. Uptake of [^{3}H]dopamine ((dihydroxyphenyl)ethylamine-*3,4-t_2*, 36.9 Ci/mmol; New England Nuclear, Boston, MA) into rat striatal synaptosomes was measured at 37 °C using a previously described method.[21] In brief, 50 μL of striatal synaptosomes (100 μg of protein) was preincubated with 5 μL of cocaine analogue in DMSO or solvent alone for 10 min in a final volume of 500 μL; uptake was initiated by the addition of 4 nM [^{3}H]dopamine and terminated after 5 min by the addition of 4 mL of ice-cold saline and filtration through Whatman GF/B filters. Nonspecific binding was measured at 37 °C using 100 μM cocaine in buffer in which choline was substituted equimolar for sodium, which defined sodium-dependent, cocaine-sensitive [^{3}H]dopamine uptake. Nonspecific uptake was typically 3–5% of total (total and nonspecific dpm were 38 600 and 1360, respectively).

Data Analysis. All assays were performed in triplicate, and the mean values of at least three separate experiments were used. The IC_{50} values for inhibition of [^{3}H]cocaine binding were determined using Ligand EBDA, an iterative nonlinear curve-fitting routine for an IBM-PC.[26] The IC_{50} values for inhibition of [^{3}H]dopamine uptake were determined by transformation of data to log–logit coordinates.

Acknowledgment. Supported in part by NIH Grant No. 03680.

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