Isothiocyanate Derivatives of 9-[3-(*cis*-3,5-Dimethyl-1-piperazinyl)propyl]-carbazole (Rimcazole): Irreversible Ligands for the Dopamine Transporter

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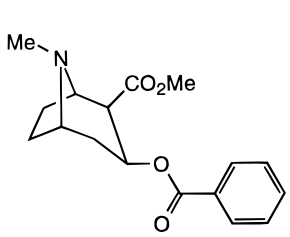
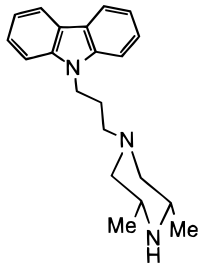
Cocaine has been reported to bind to the dopamine transporter in a biphasic fashion, and it has been hypothesized that the low-affinity component may play a modulatory role in cocaine's psychomotor stimulant effects. In an effort to gain further insight into the roles of the two sites, we have prepared a series of irreversible ligands based on rimcazole (9-[3-(*cis*-3,5-dimethyl-1-piperazinyl)propyl]carbazole, **2**), a compound that has been postulated to bind only to the low-affinity site. The alkylating moiety (isothiocyanate) is attached to the distal nitrogen of the piperazine ring via alkyl chains of varying lengths or directly attached to one of the aromatic groups. It was found that substitution on the piperazine nitrogen caused an initial decrease in affinity that was recovered as the alkyl chain length increased. Importantly, the analogue **16**, with the highest affinity for the dopamine transporter (DAT), binds in a monophasic and irreversible manner, as evidenced by the greatly diminished binding of [^{3}H]WIN 35,428 in tissue that had been preincubated with the ligand and then thoroughly washed using centrifugation. The dose-dependent reduction in B_{max} was accompanied by a concentration-related decrease in K_D values. This shift in K_D to a lower value suggests that the preincubation with **16** produced a preferential irreversible binding to the low-affinity [^{3}H]WIN 35,428 site on the dopamine transporter. These ligands may prove to be important tools with which to study the significance of the low-affinity site on the DAT. Since rimcazole does not share the behavioral profile of cocaine, and in fact appears to play a modulatory role, these compounds may provide leads for a novel cocaine-abuse treatment.

The social and economic consequences of increasing illicit drug use, and in particular cocaine (**1**) abuse, emphasize the need for pharmacological intervention. A wide range of potential medications have been studied in clinical settings as possible pharmacotherapeutics, from a number of diverse therapeutic classes, including dopamine agonists and antagonists, dopamine reuptake inhibitors, anticonvulsants, antidepressants, and opioid agonists, antagonists, and partial agonists.[1] The results of trials with these compounds have often proved inconclusive, with no single class appearing to be fully effective across the range of cocaine addict profiles. Consequently, there has been significant interest in the design of novel compounds and, in particular, compounds that could act as substitution therapies or cocaine antagonists.[2]

Although there are as yet no compounds that have been proven to act as cocaine antagonists, there are several compounds known that appear to attenuate, rather than potentiate, cocaine's effects in animal models of psychomotor stimulant abuse. These include compounds from the GBR[3] series and various sigma (σ) ligands.[4] Interestingly, certain σ-ligands such as rimcazole (9-[3-(*cis*-3,5-dimethyl-1-piperazinyl)propyl]carbazole, **2**) and BMY 14802 (4-(fluorophenyl)-4-(5-fluoro-2-pyrimidinyl)-1-piperazinebutanol) have been shown to attenuate the locomotor stimulant effects of cocaine at doses that do not have marked behavioral effects of their own.[4] Results from research in a number of laboratories



1: Cocaine



2: Rimcazole

have suggested a relationship between σ-receptors and the dopamine transporter (DAT). For example, the low-affinity binding of cocaine to σ-receptors labeled with [^{3}H]haloperidol in rats has been demonstrated,[5] and the photoaffinity label [^{125}I]iodoazidococaine, that recognizes both the high- and low-affinity cocaine binding sites on the DAT, appears to label a σ-receptor when photoactivated.[6] From these studies, a relationship between σ-sites and cocaine binding sites is apparent. In addition, many of the GBR series of DAT ligands bind with high affinity to both the DAT and also to σ-receptors, suggesting an overlap of structural requirements for binding to the DAT and σ-sites.[7,8] Recently, GBR-like ligands have been prepared with greater selectivity for the DAT; however, it is clear that there is still significant overlap in the binding requirements.[8]

A number of σ-ligands were previously tested for their ability to inhibit dopamine uptake and [^{3}H]WIN 35,428 binding to the DAT.[9] Many of these ligands inhibited dopamine uptake and displaced [^{3}H]WIN 35,428 binding

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in a dose-dependent manner. They also displayed monophasic binding, in contrast to cocaine and many cocaine analogues, which recognize both high- and low-affinity binding sites. Further the σ-ligands also inhibited dopamine uptake in a monophasic manner, again in contrast to cocaine. These differences in actions at the DAT, in comparison to cocaine, may suggest alternative interactions at the molecular level. As these compounds do not produce cocaine-like psychomotor stimulant effects and some attenuate locomotor stimulant effects of cocaine, it is possible that a novel action at the DAT may be responsible for the lack of cocaine-like pharmacology.[10]

One approach to further characterize these actions is to prepare an irreversible ligand that might bind covalently to the low-affinity component of the transporter and therefore eliminate those sites. Affinity labels have proved to be immensely valuable pharmacological tools to study receptor and transporter pharmacology.[11,12] An irreversible ligand first binds in a reversible manner to the protein; irreversibility is the result of subsequent covalent bond formation between the ligand and protein. To be of use as a pharmacological tool, the proposed irreversible ligand must bind with high affinity and selectivity to the target protein. On binding, the reactive group (typically an electrophile) present on the ligand must be located close to a suitable nucleophile on the protein. In this particular case, little is known about the relative positions of suitable nucleophiles on the DAT. It is therefore necessary to synthesize a number of potential irreversible ligands in the hope that one will meet the above criteria.

The isothiocyanate is the most widely employed moiety in the design of irreversible ligands for receptors or transporters. This group is fairly small and so has minimum impact on the binding affinity and selectivity of the parent ligand, though examples are known where a change in selectivity has occurred.[13] The isothiocyanate is also readily introduced into a molecule, being synthesized from the corresponding primary amine. This group is highly reactive toward amino and sulfhydryl groups, while displaying low reactivity toward water and other hydroxyl functions. Thus there is less opportunity for nonspecific binding with the isothiocyanate group compared to other, more reactive electrophiles.

Of the σ-ligands studied, rimcazole had the highest affinity for the DAT (K_i = 103 nM)[9] and displayed significant activity in behavioral tests, antagonizing the locomotor stimulant effects of cocaine at a dose that displayed no significant activity of its own.[4] Furthermore, rimcazole demonstrated a significantly lower potency for inhibiting dopamine uptake (IC_{50} = 4.22 μM)[9] as compared to its binding affinity than any of the other ligands evaluated, or any other dopamine transporter ligands reported to date. This novel neurochemical and behavioral profile provided interest in choosing rimcazole as a suitable candidate for further chemical modification, particularly with the aim of developing potential irreversible ligands that might allow the functional significance of the high- and low-affinity sites to be more clearly defined.

Examining the rimcazole structure, it is clear that there is the possibility of placing the isothiocyanate group on the carbazole ring system or attaching, via an alkyl chain, to the secondary nitrogen of the piperazine ring.

Chemistry

Initially our work centered on the synthesis of the carbazole-substituted isothiocyanate. By the selective mononitration of rimcazole, using conditions developed for the nitration of imipramine,[14] the nitro derivative **4a** was formed. Reduction using H_2 over a Pd/C catalyst resulted in the *p*-amino analogue **5a** in good yield. Due to the presence of the secondary amine, and its interference in the reaction with thiophosgene, a mixture of products was formed, from which the isothiocyanate derivative could not be isolated. By reductive methylation, using the standard conditions of paraformaldehyde and $NaCNBH_3$[15] to give **3**, this secondary amine was converted to a tertiary amine and the possibility of interference in the reaction was removed. Nitration and reduction were carried out as before, but now the reaction of the amine with thiophosgene[16] proceeded smoothly to yield the isothiocyanate **6** (Scheme 1).

For the alkyl-substituted isothiocyanates, two procedures were employed depending on the length of the alkyl chain. For chain lengths of two or three carbons, simple alkylation of rimcazole with the carbobenzoxy-protected aminobromoalkane gave the best results. Yields were optimal when potassium carbonate was used as the base, with dimethylformamide as the solvent. Deprotection was then carried out with TMSI[16] to give the primary amines **9** and **10**, and the isothiocyanates **11** and **12** were then formed by reaction with thiophosgene (Scheme 2). For the six-carbon-chain analogue, simple alkylation, as just described in the synthesis of **11** and **12**, resulted in little or no reaction. Better yields were obtained by first acylating to give the amide **13**. This intermediate was deprotected with TMSI and then reduced using $LiAlH_4$ to **15**. Treatment with thiophosgene again gave the desired isothiocyanate **16 (**Scheme 3).

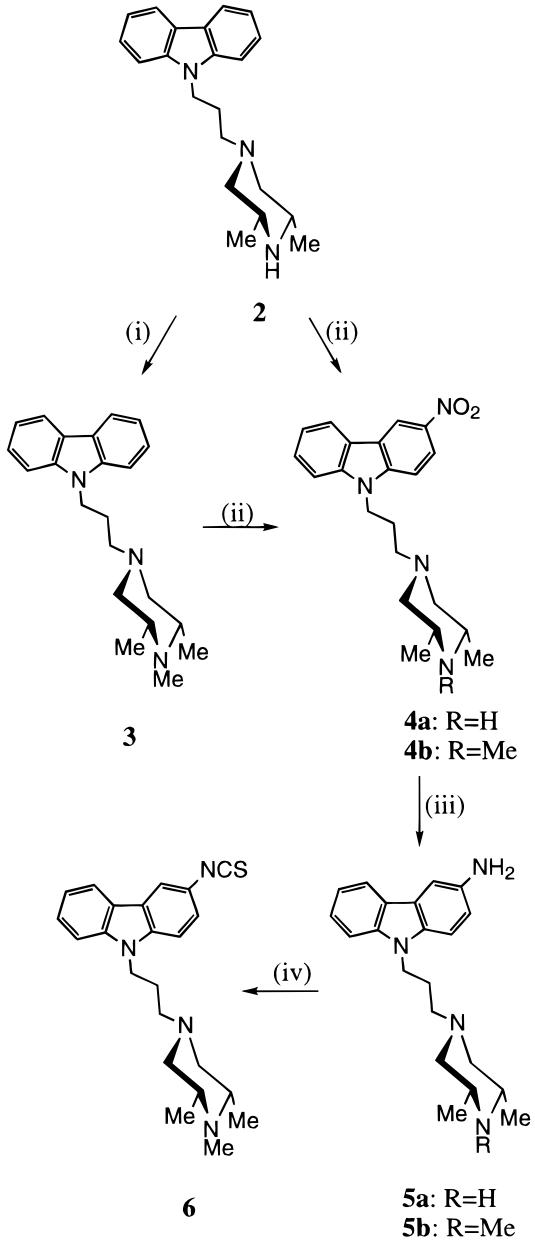
Results and Discussion

Binding affinities of the synthesized compounds for the DAT in rat caudate-putamen and the σ_1- and σ_2-receptors in guinea pig brain and rat liver, respectively, were determined and are shown in Table 1. [^{3}H]WIN 35,425 was used to label the DAT, while [^{3}H]pentazocine and [^{3}H]DTG in the presence of dextrallorphan were used for σ_1- and σ_2-sites, respectively. Additionally, one compound (**16**) was tested for irreversible binding to the DAT.

Methylation of rimcazole to **3** caused a decrease in affinity for the DAT of approximately 4-fold. This could be attributed either to the loss of hydrogen bond donor capabilities or as a direct result of the increase in steric bulk at this position. The carbazole-substituted isothiocyanate **6** was a further 5-fold lower in affinity at the DAT, resulting in a compound with too low affinity (K_i = 2374 nM) to be of further interest as a pharmacological probe.

With the three alkylisothiocyanates prepared (**11**, **12**, **16**), affinity for the transporter proved to be directly proportional to the length of the alkyl chain used. There is a >2-fold increase in affinity on going from the two-

Scheme 1[a]

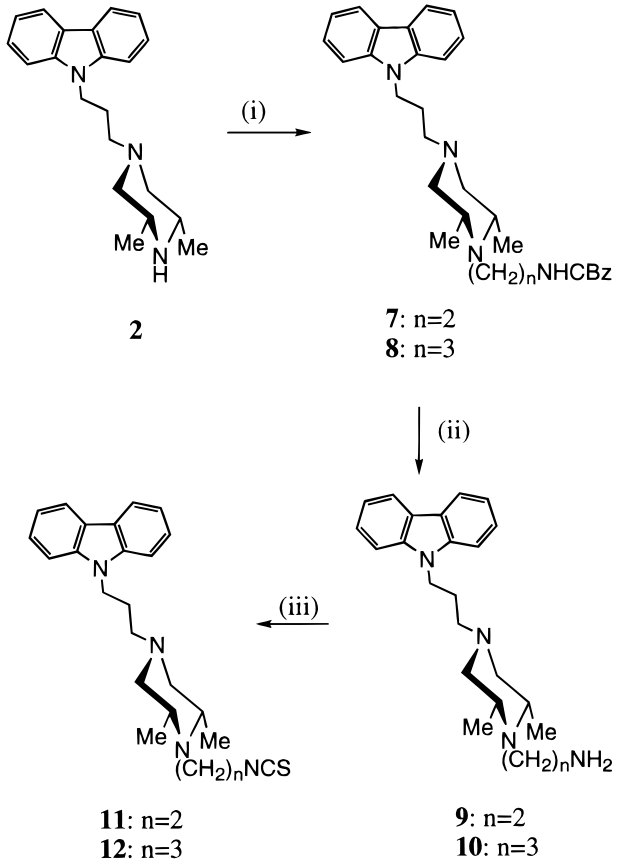


[a] (i) $(CH_2O)_n$, $NaBH_3CN$; (ii) AcOH, HNO_3; (iii) H_2, Pd/C; (iv) Cl_2CS.

carbon chain (**11**: K_i = 2241 nM) to the three-carbon chain (**12**: K_i = 935 nM) and a further 6-fold improvement on going from three to six carbons (**16**: K_i = 155 nM) in length (Table 1).

It thus appears that additional bulk (or loss of H-bond-donating capability) at the secondary nitrogen causes an initial drop in affinity for the DAT. It seems most likely that this negative interaction has at least a large steric component, as there is a further significant drop (5-fold) in affinity on going from NMe (**3**) to $N(CH_2)_2NCS$ (**11**). This could not be explained by exclusively H-bonding interactions. As the chain length increases further (to three and then six carbons) the affinity begins to increase, presumably due to interactions at another binding domain. The initial decrease, followed by an increase in affinity on lengthening of the tether between the functional group and the pharmacophore, has previously been observed in other series of receptor ligands.[17]

Scheme 2[a]

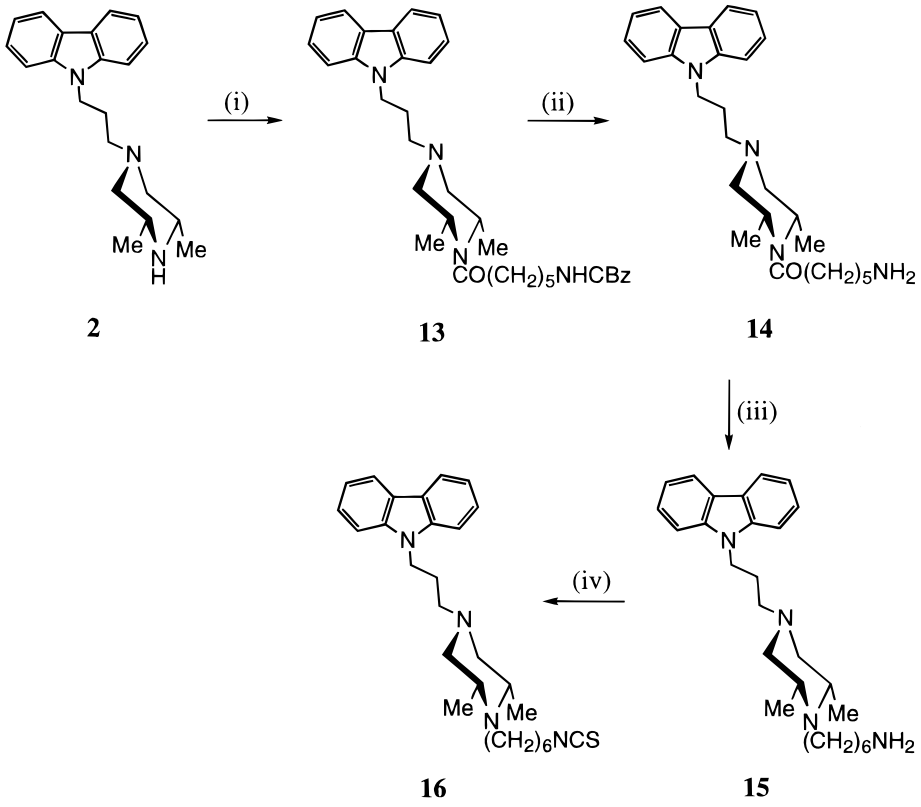


[a] (i) $Br(CH_2)_nNHCBz$; (ii) TMSI; (iii) Cl_2CS.

As previously discussed, rimcazole binds with moderate affinity to σ-receptors. The new analogues, with the exception of **6**, were evaluated for their binding affinity to σ-receptors (Table 1). There appeared to be a good correlation between σ_2-affinity and affinity at the DAT. Thus, increasing chain length caused an initial drop in affinity followed by an increase in affinity as the chain length increased further. The significant exception to this appears to be rimcazole itself, it having relatively low affinity for σ_2-sites and about 14-fold selectivity for the DAT. With σ_1-sites there was less of a variation in affinity, with only **12** (three-carbon chain) showing a drop in affinity. The approximately 3-fold selectivity of **16** for the DAT over σ_1-sites is similar to the selectivity displayed by various GBR analogues, including GBR 12935 (~4-fold).[8]

Because **16** displayed the highest affinity for the DAT, it was chosen for further evaluation to determine its potential for irreversible binding. As shown in Table 2, there was a concentration-dependent decrease in B_{max} values for [^{3}H]WIN 35,428 binding following incubation with 100 nM or 10 μM **16**, as compared to buffer, despite two washes with centrifugation. This result is indicative of an irreversible interaction of **16** with the dopamine transporter. Figure 1 shows representative Scatchard analyses of the binding of [^{3}H]WIN 35,428 under the different preincubation conditions. In contrast to what typically has been reported, the binding of [^{3}H]-WIN 35,428 in tissue preincubated in buffer did not model better for two sites than for one binding site. It has previously been shown that the conditions used in this assay can critically affect the ability to delineate two binding sites.[18] It is likely that the added prein-

Scheme 3[a]



[a] (i) $ClCO(CH_2)_5NHCBz$; (ii) TMSI; (iii) $LaAlH_4$; (iv) Cl_2CS.

Table 1. Binding Affinities at the DAT and σ_1- and σ_2-Receptors

		IC_{50} (nM ± SEM)[a]		
compound	substitution	[^{3}H]WIN 35,428 binding (DAT)	[^{3}H]pentazocine binding (σ_1)	[^{3}H]DTG binding (σ_2)[b]
2, rimcazole	H	57.6 ± 14	1480 ± 160	386 ± 48
3	NMe	244 ± 24	897 ± 180	294 ± 26
6	NMe, 4-NCS	1330 ± 40	nd[c]	nd[c]
11	$N(CH_2)_2NCS$	1250 ± 100	949 ± 220	1440 ± 44
12	$N(CH_2)_3NCS$	523 ± 52	1830 ± 320	877 ± 120
16	$N(CH_2)_6NCS$	86.7 ± 14	707 ± 29	211 ± 15

[a] IC_{50}'s were determined as described in the Experimental Section. [b] [^{3}H]DTG was used in the presence of dextrallorphan. [c] nd = not determined.

Table 2. Binding Constants (B_{max} and K_D) for Studies of [^{3}H]WIN 35,428 Saturation in Rat Caudate-Putamen Tissue Preincubated in Either Buffer or Buffer with Different Concentrations of **16**

	B_{max}		K_D	
condition	fmol/mg of prot	% control	nM	% control
buffer	537 ± 19.6	100	16.6 ± 1.09	100
100 nM	167 ± 69.3	31	7.61 ± 4.60	45
10 μM	6.65 ± 1.66	1.2	5.60 ± 2.52	34

cubation period of the present study altered the conditions sufficiently to affect this outcome. Despite not fitting a two-site model significantly better than a one-site model, the Scatchard plot showed a significant deviation from linearity as assessed by a Runs test (p = 0.0367). This result was evidence of heterogeneity of binding which was not true for the tissues that had been pretreated with **16**.

In addition to changes in B_{max} values, preincubation with **16** also produced a concentration-related decrease in K_D values (Table 2). These decreases in K_D values can be seen in the altered slopes of the Scatchard plots in Figure 1. This shift in K_D to a lower value is evidence of a loss of low-affinity binding. The decrease in K_D values indicates that the preincubation with **16** produced a preferential irreversible binding to the low-affinity [^{3}H]WIN 35,428-labeled site on the dopamine transporter.

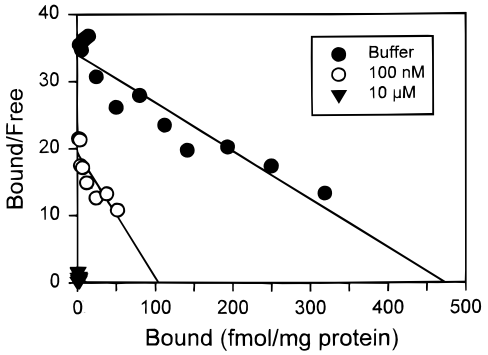


Figure 1. Representative Scatchard analyses of the binding of [^{3}H]WIN 35,428 after preincubation with buffer or buffer with **16**.

Conclusions

A series of potential irreversible ligands were synthesized and evaluated for their DAT and σ-site affini-

ties. From the structural modifications made, it appears that rimcazole is very sensitive to alterations in its structure, with fairly large changes in affinity occurring with each of the substitutions made. Although σ_1-affinity did not vary significantly within this series of compounds, there seemed to be a good correlation between σ_2-affinity and affinity at the DAT for the N-substituted analogues. In each case there was an initial lowering of affinity on going from NMe to $N(CH_2)_2NCS$; then affinity rose as the length of the side chain was increased. This is further evidence of the very similar structural requirements for binding to the DAT and σ-sites.

One of the potential irreversible ligands, **16**, was found to have similar affinity to rimcazole at the DAT and was thus deemed a suitable candidate for further characterization aimed at determining its ability to bind in an irreversible manner. Binding studies have shown that **16** is binding irreversibly, and additionally, it appears that **16** may be labeling only one binding site on the DAT. This result is important, in that it may allow the functional significance of the low- and high-affinity binding sites on the DAT to be separated. Experiments are being designed for further characterization in both in vitro and behavioral assays.

Experimental Section

Chemistry. Melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded using a Bruker (Billerica, MA) AC-300 spectrometer. Samples were dissolved in $CDCl_3$, and the chemical shifts are reported as parts per million (δ) relative to tetramethylsilane (Me_4Si, 0.00 ppm) as internal standard. Chemical shifts for ^{13}C NMR spectra are reported as δ relative to deuterated chloroform ($CDCl_3$, 77.0 ppm). Infrared spectra were recorded with a Perkin-Elmer 1600 series FTIR spectrophotometer with KBr disks. Thin-layer chromatography was performed on silica gel plates (Uniplates, Analtech, Inc.) with 10% CMA (89% $CHCl_3$, 10% MeOH, 1% NH_4OH) as solvent unless noted otherwise. Compounds for in vitro testing were converted to their HCl salts by dissolving in a minimum volume of MeOH and slowly adding a saturated solution of HCl in MeOH until acidic. Elemental analysis performed by Atlantic Microlab, Inc. (Norcross, GA) agreed to within 0.4% of the calculated values. All chemicals and reagents were purchased from Lancaster Synthesis, Inc. or Aldrich Chemical Co.

General Procedures. Method A: Nitrogen Alkylation. Rimcazole, the alkyl bromide (1.1 equiv), and K_2CO_3 (2 equiv) were heated to 110 °C in DMF (3 mL/mmol of rimcazole) for 6 days. On days 2 and 4 an extra 0.5 equiv of the alkyl bromide was added. The mixture was cooled to room temperature and H_2O added. The organics were extracted with EtOAc:Et_2O (1:1), dried (Na_2SO_4), and evaporated to yield the crude product.

Method B: CBz Deprotection.[17] The CBz-protected amine was dissolved in CH_3CN (5 mL/mmol) and the iodotrimethylsilane then added (3.6 equiv). The solution was stirred at room temperature for 1 h before quenching with MeOH (0.5× CH_3CN vol). The solution was reduced to dryness in vacuo and the solid then taken up in ether and 1 N HCl. After separation, the aqueous layer was washed with further Et_2O (6 × 10 mL/mmol), before then basifying the aqueous layer with NH_4OH and extracting the product into $CHCl_3$:2-PrOH (7:3). This solution was dried (Na_2SO_4) and evaporated to yield crude product.

Method C: Isothiocyanate (NCS) Formation. The amine was dissolved in a mixture of $CHCl_3$ (60 mL/mmol) and water (25 mL/mmol) with $NaHCO_3$ (4.5 equiv). This mixture was cooled in an ice bath before adding freshly distilled thiophosgene (1.3 equiv). Stirring was continued for 2 h before pouring the mixture into a separatory funnel. The organic layer was collected and the aqueous layer extracted with $CHCl_3$ (2 × 20 mL/mmol). The organic layers were combined, dried (Na_2SO_4), and evaporated to yield crude isothiocyanate product. The product was then filtered through silica gel using EtOAc:$CHCl_3$ (4:1) as the eluent. This gave products that were pure by TLC.

9-[3-(*cis*-3,4,5-Trimethyl-1-piperazinyl)propyl]carbazole (3). Rimcazole (1.41 g, 4.4 mmol) was dissolved in a mixture of CH_3CN (9 mL) and EtOH (9 mL) before adding formaldehyde (0.72 mL of 37%). $NaBH_3CN$ (0.28 g, 4.4 mmol) was then added slowly and stirring continued for 2 h. Water was added to quench the reaction and the mixture evaporated to dryness. The product was extracted into $CHCl_3$ and washed with water (2 × 20 mL). Drying (Na_2SO_4) and evaporation yielded an oil. Column chromatography (7% CMA) gave a white foam, 1.25 g (85%). The HCl salt was formed and recrystallized from EtOH:2-PrOH: mp 214−215 °C; R_f 0.5 (10% CMA); IR ($CHCl_3$) 742, 719 (Ph) cm^{-1}; ^{1}H NMR δ 1.03 (6H, d, J = 6.1 Hz, 2 × Me), 2.19 (2H, t, J = 6.8 Hz, 2H of piperazine), 2.26 (5H, t, s, NMe, 2H of piperazine), 2.63 (2H, d, J = 9.6 Hz, 2H of piperazine), 4.32 (2H, t, J = 6.6 Hz, CH_2N); ^{13}C NMR δ 18.21, 25.78, 37.92, 40.35, 54.55, 57.96, 60.93, 108.81, 118.69, 120.19, 122.76, 125.43, 140.43. Anal. ($C_{22}H_{31}N_3$·2HCl·2-PrOH) C, H, N.

4-Nitro-9-[3-(*cis*-3,4,5-trimethyl-1-piperazinyl)propyl]carbazole (4b). Compound **3** (2.30 g, 6.9 mmol) was taken up in glacial acetic acid (52 mL) and then nitric acid (1.04 mL of 70%) slowly added at room temperature. Stirring was continued for 2.5 h before diluting the reaction with water (100 mL) and HCl (4 mL of 37%). The solution was washed with ether (2 × 40 mL) and basified with NH_4OH, and the organics were extracted into $CHCl_3$. The organic layer was dried (Na_2SO_4) and evaporated to give a red oil: 2.3 g (88%); IR ($CHCl_3$) 1515 (NO_2, asymm), 1330 (NO_2, symm) cm^{-1}; ^{1}H NMR δ 1.03 (6H, d, 2 × Me), 2.25 (3H, s, NMe), 2.64 (2H, d, 2H of piperazine), 4.45 (2H, t, CH_2N), 7.29 (1H, m, Ph), 7.48 (3H, m, Ph), 8.12 (1H, d, Ph *m*- to NO_2), 8.36 (1H, d, Ph *o*- to NO_2), 8.99 (1H, s, Ph *o*- to NO_2).

4-Amino-9-[3-(*cis*-3,4,5-trimethyl-1-piperazinyl)propyl]carbazole (5b). Compound **4b** (380 mg, 1.0 mmol) was dissolved in MeOH (25 mL) and hydrogenated under an atmosphere of H_2 at 30 psi, for 3 h, over a 10% Pd/C catalyst (60 mg). Filtration through Celite followed by column chromatography (5−10% CMA) yielded a red oil: 190 mg (54%); R_f 0.38 (10% CMA); IR ($CHCl_3$) 3413, 3346 (NH_2) cm^{-1}; ^{1}H NMR δ 1.07 (6H, d, 2 × CH_3), 2.30 (3H, s, NMe), 2.64 (2H, d, 2H of piperazine), 4.32 (2H, t, CH_2N), 6.89 (1H, m, Ph), 7.17 (1H, m, Ph), 7.28 (1H, s, Ph), 7.38 (3H, m, Ph), 7.94 (1H, d, Ph).

4-Isothiocyanato-9-[3-(*cis*-3,4,5-trimethyl-1-piperazinyl)propyl]carbazole (6). Using general procedure C with **5b** (180 mg, 0.51 mmol), but with EtOH as the eluent for purification, the isothiocyanate **6** was obtained as an oily red solid,, 160 mg (79%). The HCl salt was formed and recrystallized in 2-PrOH:THF: mp 188−190 °C dec; R_f 0.05 (10% EtOH in EtOAc); IR ($CHCl_3$) 2108 (NCS) cm^{-1}; ^{1}H NMR δ 1.03 (6H, d, J = 6.4 Hz, 2 × Me), 2.22 (3H, s, NMe), 2.67 (2H, d, J = 10.5 Hz, 2H of piperazine), 4.37 (2H, t, J = 6.5 Hz, CH_2N), 7.28 (2H, m, aryl), 7.48 (3H, m, aryl), 7.92 (1H, d, J = 2.0 Hz, aryl-H *o*- to NCS), 8.04 (1H, d, J = 7.5 Hz, aryl-H *o*- to NCS); ^{13}C NMR δ 16.26, 25.78, 37.98, 41.12, 54.44, 58.04, 60.95, 109.36, 109.69, 117.73, 119.59, 120.60, 121.95, 123.31, 126.58, 139.50, 141.82. Anal. ($C_{23}H_{28}N_4S$·2HCl·0.5H_2O) C, H, N.

9-[3-[*cis*-3,5-Dimethyl-4-[2-(carbobenzoxyamino)ethyl]-1-piperazinyl]propyl]carbazole (7). Using general procedure A with benzyl 2-bromoethylcarbamate.[18] Column chromatography (7% CMA) of the crude product yielded an off-white solid: 83%; R_f 0.55 (10% CMA); IR ($CHCl_3$) 1715 (CO of CBz) cm^{-1}; ^{1}H NMR δ 1.06 (6H, d, 2 × Me), 2.6−2.8 (6H, m), 3.21 (2H, t, CH_2NHCBz), 4.38 (2H, t, CH_2N), 5.09 (2H, s, $PhCH_2$).

9-[3-[*cis*-3,5-Dimethyl-4-[3-(carbobenzoxyamino)propyl]-1-piperazinyl]propyl]carbazole (8). Using general procedure A with benzyl 3-bromopropylcarbamate.[18] Column chromatography (7% CMA) yielded an oil: 35%; R_f 0.5 (10% CMA); IR ($CHCl_3$) 1713 (CO of CBz) cm^{-1}; ^{1}H NMR δ 1.06 (6H, d, 2

× Me), 2.59 (4H, m), 2.78 (2H, m), 4.37 (2H, t, CH_2N), 5.13 (2H, s, CH_2Ph).

9-[3-[*cis*-3,5-Dimethyl-4-[6-(carbobenzoxyamino)-1-oxohexyl]-1-piperazinyl]propyl]carbazole (13). Rimcazole (3.53 g, 11 mmol) was dissolved in toluene (90 mL) before adding *N*-CBz-6-aminohexanoyl chloride (5.10 g, 18 mmol) and triethylamine (2.6 mL, 18.7 mmol) and warming to 60 °C overnight. The solution was cooled, and the organics were extracted in EtOAc (3 × 50 mL) from a Na_2CO_3 solution. Drying (Na_2SO_4) and evaporation gave a brown foam. Column chromatography (2% CMA) yielded a cream-colored solid: 2.8 g (45%); R_f 0.83 (10% CMA); IR ($CHCl_3$) 3317 (NH), 1713 (CO of carbamate), 1625 (CO of amide) cm^{-1}; ^1H NMR δ 1.43 (6H, m, 2 × Me), 2.76 (2H, d, 2H of piperazine), 3.20 (2H, t), 4.43 (2H, t, CH_2N), 5.11 (2H, s, CH_2Ph), 7.2−7.5 (11H, m, CBz, carbazole H's), 8.09 (2H, m, 2H of carbazole).

9-[3-[*cis*-3,5-Dimethyl-4-(6-amino-1-oxohexyl)-1-piperazinyl]propyl]carbazole (14). Using general procedure B with the CBz-protected amine **13**. Column chromatography (5−10% CMA) of the crude product gave an oil: 75%; R_f 0.15 (10% CMA); IR ($CHCl_3$) 3373 (NH), 3297 (NH), 1632 (CO of amide) cm^{-1}; ^1H NMR δ 1.42 (6H, m, 2 6 × Me), 2.78 (4H, m, 2H of piperazine plus CH_2N of side chain), 4.47 (2H, t, CH_2N).

9-[3-[*cis*-3,5-Dimethyl-4-(2-aminoethyl)-1-piperazinyl]-propyl]carbazole (9). Using general procedure B with the CBz-protected amine **7**. The gummy white foam product was purified by column chromatography (5−10% CMA) to give a white solid: 75%; R_f 0.1 (10% CMA); IR ($CHCl_3$) 3404, 3179 (NH) cm^{-1}; ^1H NMR δ 1.07 (6H, d, 2 × Me), 1.75 (4H, m), 2.08 (2H, t), 2.24 (2H, t), 2.6−2.8 (6H, m), 4.39 (2H, t, CH_2N).

9-[3-[*cis*-3,5-Dimethyl-4-(3-aminopropyl)-1-piperazinyl]-propyl]carbazole (10). Using general procedure B with the CBz-protected amine **8**. The off-white, foam product was purified by column chromatography (10% CMA) to yield a white foam: 62%; R_f 0.1 (10% CMA); IR ($CHCl_3$) 3400, 3200 (NH) cm^{-1}; ^1H NMR δ 1.01 (6H, d, 2 × Me), 2.69 (4H, m), 2.85 (2H, m), 4.39 (2H, t, CH_2N).

9-[3-[*cis*-3,5-Dimethyl-4-(6-aminohexyl)-1-piperazinyl]-propyl]carbazole (15). The amide **14** was dissolved in dry THF (1 mL/mmol) and added to a cooled mixture of $LiAlH_4$ (1.3 equiv) in dry THF (1 mL/mmol). The mixture was allowed to warm to room temperature and stirring continued overnight. The reaction was quenched with solid Rochelle's salt before adding water (1 mL/2 mmol). The mixture was evaporated to dryness, the product was taken up in 7:3 $CHCl_3$:2-PrOH, and the inorganic salts were filtered off. Drying (Na_2SO_4) and evaporation gave the crude product as a thick oil; crude yield, 95%. Column chromatography gave the pure amine as a sticky solid: 70%; R_f 0.3 (10% CMA); IR ($CHCl_3$) 3356, 3282 (NH) cm^{-1}; ^1H NMR δ 1.07 (6H, d, 2 × Me), 1.3−1.5 (6H, m), 2.70 (6H, m), 4.38 (2H, t, CH_2N).

9-[3-[*cis*-3,5-Dimethyl-4-(6-isothiocyanatohexyl)-1-piperazinyl]propyl]carbazole (16). Using general procedure C with amine **15** yielded the product as an oily solid, 50%. The HCl salt was formed and recrystallized from 2-PrOH: Et_2O: mp 210−212 °C dec; IR ($CHCl_3$) 2102 (NCS) cm^{-1}; ^1H NMR δ 1.05 (6H, d, J = 6 Hz, 2 × Me), 2.68 (4H, d, J = 9.8 Hz, 2H of piperazine; t, J = 7.8 Hz, CH_2N of side chain), 3.52 (2H, t, J = 6.5 Hz, CH_2NCS), 4.38 (2H, t, J = 6.6 Hz, CH_2N at carbazole); ^{13}C NMR δ 17.98, 23.21, 25.76, 26.48, 26.78, 29.88, 40.45, 44.85, 47.88, 53.62, 54.60, 61.13, 108.72, 118.62, 120.14, 122.90, 125.37, 140.37. Anal. ($C_{28}H_{38}N_4S$·2HCl·0.25H_2O) C, H, N.

9-[3-[*cis*-3,5-Dimethyl-4-(2-isothiocyanatoethyl)-1-piperazinyl]propyl]carbazole (11). Using general procedure C with amine **9** yielded the product as an off-white solid, 55%. The HCl salt was formed and recrystallized in EtOH:2-PrOH: mp 203−204 °C dec; IR ($CHCl_3$) 2108 (NCS) cm^{-1}; ^1H NMR δ 1.07 (6H, d, J = 6.2 Hz, 2 × Me), 2.66 (4H, d, J = 9 Hz, 2H of piperazine, CH_2N of side chain), 2.95 (2H, dd, J = 7.3 Hz, 2H of piperazine), 3.52 (2H, t, J = 7.7 Hz, CH_2NCS), 4.39 (2H, t, J = 6.5 Hz, NCH_2 at carbazole); ^{13}C NMR δ 18.37, 25.64, 40.37, 43.10, 48.78, 54.55, 55.24, 60.69, 108.72, 118.68, 120.18, 122.71, 125.41, 140.36. Anal. ($C_{24}H_{30}N_4S$·2HCl·0.5H_2O) C, H, N.

9-[3-[*cis*-3,5-Dimethyl-4-(3-isothiocyanatopropyl)-1-piperazinyl]propyl]carbazole (12). Using general procedure C with the amine **10** yielded the product as a white solid, 81%. The HCl salt was formed and recrystallized in 2-PrOH:THF: mp 230−232 °C; IR ($CHCl_3$) 2110 (NCS) cm^{-1}; ^1H NMR δ 1.05 (6H, d, J = 6.2 Hz, 2 × Me), 2.63 (4H, d, J = 8.7 Hz, 2H of piperazine, CH_2N of side chain), 2.83 (2H, dd, J = 7.9 Hz, 2H of piperazine), 3.49 (2H, t, J = 6.4 Hz, CH_2NCS), 4.41 (2H, t, J = 6.5 Hz, CH_2N at carbazole); ^{13}C NMR δ 18.29, 25.69, 25.84, 40.53, 43.49, 45.77, 54.51, 54.74, 61.09, 108.82, 118.75, 120.26, 122.81, 125.49, 140.48. Anal. ($C_{25}H_{32}N_4S$·2HCl) C, H, N.

Binding Methods. 1. Chemicals. Chemicals and reagents were obtained from the following sources: [^{3}H]WIN 35,-428 (2β-carbomethoxy-3β-(4-fluorophenyl)tropane 1,5-naphthalene disulfonate; specific activity 83.5 Ci/mmol) from New England Nuclear (Boston, MA); cocaine hydrochloride from Sigma Chemical Co. (St. Louis, MO). [^{3}H]-(+)-Pentazocine was synthesized as described previously.[19,20] [^{3}H]DTG (39.1 Ci/mmol) was purchased from DuPont/New England Nuclear (Boston, MA). Dextrallorphan was provided by Dr. F. I. Carroll (Research Triangle Institute, Research Triangle Park, NC). Haloperidol and poly(ethylenimine) were purchased from Sigma Chemicals (St. Louis, MO).

2. Dopamine Transporter Binding Assay. Male Sprague−Dawley rats (200−250 g; Taconic, Germantown, NY) were decapitated and their brains removed to an ice-cooled dish for dissection of the caudate-putamen. The tissue was homogenized in 30 vol of ice-cold modified Krebs-HEPES buffer (15 mM HEPES, 127 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.3 mM NaH_2PO_4, 10 mM D-glucose, pH adjusted to 7.4) using a Brinkman polytron and centrifuged at 20000*g* for 10 min at 4 °C. The resulting pellet was then washed two more times by resuspension in ice-cold buffer and centrifugation at 20000*g* for 10 min at 4 °C. Fresh homogenates were used in all experiments.

Binding assays were conducted in modified Krebs-HEPES buffer on ice. The total volume in each tube was 0.5 mL, and the final concentration of membrane after all additions was 0.5% (w/v) corresponding to 200−300 mg of protein/sample. [^{3}H]WIN 35,428 (specific activity 82.4 Ci/mmol) was added, and the incubation was continued for 1 h on ice. The incubation was terminated by the addition of 3 mL of ice-cold buffer and rapid filtration through Whatman GF/B glass fiber filter paper (presoaked in 0.1% BSA in water to reduce nonspecific binding) using a Brandel cell harvester (Gaithersburg, MD). The filters were washed with three additional 3-mL washes and transferred to scintillation vials. Absolute ethanol (0.5 mL) and Beckman Ready Value scintillation cocktail (2.75 mL) were added to the vials which were counted the next day at an efficiency of about 36%. For determination of IC_{50} values, triplicate samples of membrane suspension were preincubated for 5 min in the presence or absence of the compound being tested. Each compound was tested with concentrations ranging from 0.01 nM to 100 μM for competition against binding of [^{3}H]WIN 35,428 (final concentration 1.5 nM), in three independent experiments. Under these assay conditions, an average experiment yielded approximately 6000 dpm total binding per sample and approximately 250 dpm nonspecific binding, defined as binding in the presence of 100 μM cocaine.

3. Irreversible Binding Assay. Caudate-putamen tissue from rat brain was homogenized in 30 vol of ice-cold modified Krebs-HEPES buffer (see above) and centrifuged at 20000*g* for 10 min at 4 °C. The resulting pellet was then washed one additional time by resuspension in ice-cold buffer and centrifugation. The tissue was then incubated on ice for 1 h in the presence of drug-free buffer, buffer with 100 nM **16**, or buffer containing 10 μM **16**. After this, the tissue was resuspended in cold buffer and subjected to two more centrifugations, each in 30 vol of buffer, as above. Twelve-point [^{3}H]WIN 35,428 saturation curves were performed over a concentration range of 0.1−25 nM [^{3}H]WIN 35,428, which was added at the beginning of a 1-h incubation on ice, as described above.

4. Data Analysis. Saturation data were analyzed by the use of the nonlinear least-squares curve-fitting computer

program LIGAND.[21] Saturation data from replicate experiments were modeled together to produce a set of parameter estimates (K_D and B_{max} values) and the associated standard errors of these estimates. Protein values were determined using a modification of the Lowry procedure.[22]

5. σ-Binding Assays. σ_1-Receptors were labeled as described previously, using the σ_1-selective probe [^{3}H]-(+)-pentazocine and guinea pig brain membranes.[20] Guinea pig membranes (350−500 μg of membrane protein) were incubated with 3 nM [^{3}H]-(+)-pentazocine in a total volume of 0.5 mL of 50 mM Tris-HCl, pH 8.0. Incubations were carried out for 120 min at 25 °C. Nonspecific binding was determined in the presence of 10 μM unlabeled haloperidol. Assays were terminated by dilution with 5 mL of ice-cold Tris-HCl, pH 8.0, and vacuum filtration through glass fiber filters using a Brandel cell harvester. Filters were then washed twice with 5 mL of ice-cold 10 mM Tris-HCl, pH 8.0. Filters were soaked in 0.5% poly(ethylenimine) for at least 30 min at 25 °C prior to use. Filters were counted in CytoScint cocktail (ICN, Costa Mesa, CA) after an overnight extraction of counts. Membranes were prepared from frozen guinea pig brains (minus cerebella) as previously described.[23]

σ_2-Receptors were labeled as previously described using rat liver membranes, a rich source of σ_2-sites, and [^{3}H]-1,3-di-*o*-tolylguanidine ([^{3}H]DTG) in the presence of 1 μM dextrallorphan to mask σ_1-receptors.[23] Assays were performed in 50 mM Tris-HCl, pH 8.0, for 120 min at 25 °C in a volume of 0.5 mL with 160 μg of membrane protein and 5 nM radioligand. Nonspecific binding was determined in the presence of 10 μM haloperidol. All other manipulations were as described for the σ_1-receptor assay. Rat liver membranes were prepared from the livers of male Sprague−Dawley rats as previously described.[23]

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