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3′-Chloro-3α-(diphenylmethoxy)tropane But Not 4′-Chloro-3α-(diphenylmethoxy)tropane Produces a Cocaine-like Behavioral Profile†

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A series of 2′- and 3′-substituted and 3′,3″-disubstituted 3α-(diphenylmethoxy)tropane analogs were designed and synthesized as novel probes for the dopamine transporter. All the analogs were evaluated for displacement of [^{3}H]WIN 35,428 binding at the dopamine transporter and for inhibition of [^{3}H]dopamine uptake in rat caudate putamen. Compounds were observed to monophasically displace [^{3}H]WIN 35,428 binding to the dopamine transporter with affinities of 21.6−1836 nM (K_i). Generally, *meta*-substituted compounds were more potent than benztropine and equipotent to or slightly less potent than their previously reported *para*-substituted homologs in inhibiting [^{3}H]WIN 35,428 binding. However, these same *meta*-substituted analogs were typically less potent than the 4′-substituted analogs in inhibiting [^{3}H]dopamine uptake. *Ortho*-substituted analogs were generally less potent in both binding and inhibition of uptake at the dopamine transporter than either benztropine or other aryl-substituted homologs. The analogs were also tested for binding at norepinephrine and serotonin transporters as well as muscarinic m_1 receptors. None of the compounds in the present study bound with high affinity to either the norepinephrine or serotonin transporters, but all bound to muscarinic m_1 receptors with high affinity (K_i = 0.41−2.52 nM). Interestingly, 3′-chloro-3α-(diphenylmethoxy)tropane (**5c**) produced effects like cocaine in animals trained to discriminate 10 mg/kg cocaine from saline, unlike its 4′-Cl homolog and all of the previously evaluated benztropine analogs. Further evaluation of compound **5c** and the other benztropine analogs will undoubtedly prove useful in the elucidation of the role of the dopamine transporter in the reinforcing effects of cocaine and the ultimate identification of a cocaine-abuse treatment.

Introduction

Cocaine binds to discrete recognition sites on the dopamine transporter, blocking the uptake of dopamine and resulting in the accumulation of dopamine in the synapse. This mechanism of action has been determined to be of primary importance in the stimulant and reinforcing effects of this widely abused drug.[1,2] Elucidation of the binding domains of this recognition site through the use of novel, high-affinity ligands has provided further insight into the role of the dopamine transporter in the pharmacology of cocaine[3−13] and will ultimately guide the design of future drugs that may serve as potential medical treatments for cocaine abuse.

Benztropine (3α-(diphenylmethoxy)-1α*H*,5α*H*-tropane) is the parent compound in a class of dopamine uptake inhibitors.[14−16] This compound is equipotent to cocaine at the dopamine transporter and has been shown to be a central nervous system (CNS) stimulant in animal models.[17−20] Benztropine is interesting in that it shares some structural homology with cocaine (and numerous analogs of cocaine, e.g., WIN 35,428) as well as the 1,4-dialky(en)ylpiperazine compounds (e.g., GBR 12909). Previous reports from this laboratory[12,13] demonstrated the effects of various *para* aromatic substitutions on the structure−activity relationships of benztropine at the dopamine transporter. It was discovered that these 4′-substituted and 4′,4″-disubstituted 3α-(diphenylmethoxy)-tropane analogs bound with high affinity at the dopamine transporter and were selective at this transporter over other monoamine transporters.[13] More specifically, 4′-halogen- and 4′,4″-dihalogen-substituted compounds were found to be more potent than the parent compound, benztropine, with F > Cl > Br. Conversely, additional electron-withdrawing substituents resulted in compounds with greatly decreased potencies at the dopamine transporter. The structure−activity relationships deduced from these compounds were very different from those found with previously reported cocaine analogs.[3] These data coupled with the fact that a representative of this group of compounds, 4′-chloro-3α-(diphenylmethoxy)tropine (4′-Cl benztropine), did not demonstrate a cocaine-like behavioral profile suggested that a new binding domain with unique structure−activity relationships may exist on the dopamine transporter.[12] The present study was undertaken to further explore the structure−activity relationships of these 3α-(diphenylmethoxy)tropane analogs by investigating the effects of *meta* and *ortho* aromatic substitution. Due to steric and electronic differences between the proposed analogs and those previously reported,[12,13] it was envisioned that these compounds would provide further information about the relationship between binding to the dopamine transporter, inhibition of dopamine uptake, and cocaine-like behavior.

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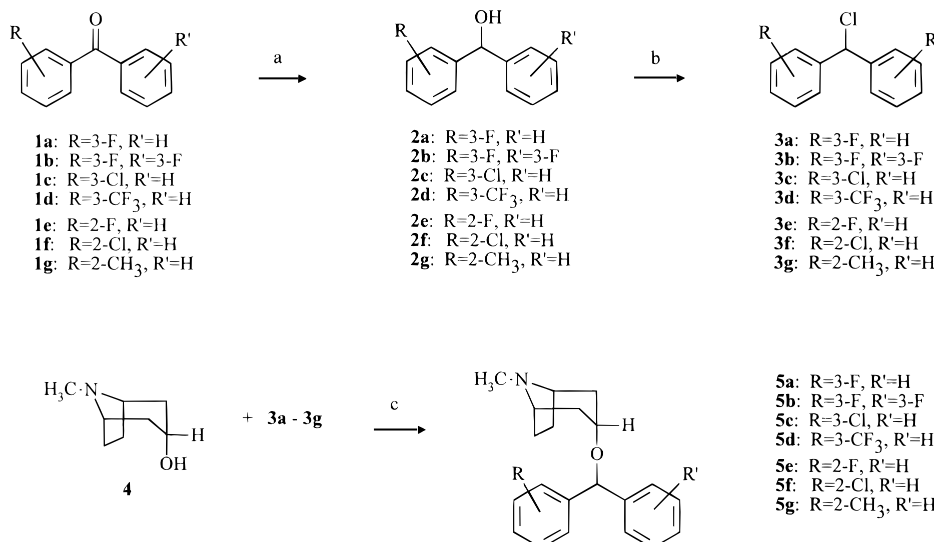
Scheme 1[a]



[a] (a) $NaBH_4$; (b) $SOCl_2$; (c) 160 °C.

Table 1. Physical Properties of Compounds **5a–g**

compd no.	recryst solvent	mp, °C	MS (M^+)	formula	% yield
5a	acetone	177–179	325	$C_{21}H_{25}NOFCl$	38
5b	acetone/EtOAc	197–199	343	$C_{21}H_{24}NOF_2Cl$	32
5c	EtOAc	172–174	341	$C_{21}H_{25}NOCl_2$	31
5d	EtOAc	87–89	375	$C_{22}H_{25}NOF_3Cl{\cdot}H_2O$	38
5e	acetone	204–206	325	$C_{21}H_{25}NOFCl{\cdot}H_2O$	31
5f	EtOAc	84–86.5	341	$C_{21}H_{25}NOCl_2{\cdot}H_2O$	18
5g	EtOAc	182–183	321	$C_{22}H_{28}NOCl$	29

Chemistry

The synthesis of compounds **5a–g** is presented in Scheme 1. Commercially available 3- or 2-substituted benzophenones **1a–g** were first reduced to the corresponding benzhydrols **2a–g** using $NaBH_4$ which were then converted to the benzhydryl chlorides **3a–g** in either refluxing or ambient thionyl chloride.[13] The resulting benzhydryl chlorides were added, either neat or in a minimal amount of ether, to tropane (**4**) at 160 °C.[13] The overall yields of this melt reaction ranged from 18% to 38% and were lower than those reported for the 4′-substituted and 4′,4″-disubstituted 3α-(diphenylmethoxy)tropane analogs.[13] While the reasons for this are unclear, it is postulated that the formation of the carbocation intermediate may be hindered by electron-withdrawing groups and that nucleophilic attack of the cation may be sterically hindered by *ortho*-, and to a lesser extent *meta*-, substituted aromatic rings. Physical properties of the compounds, isolated and purified as the HCl salts, are listed in Table 1.

Pharmacology

All the 2′- and 3′-substituted and 3′,3″-disubstituted 3α-(diphenylmethoxy)tropane analogs were evaluated for displacement of [^{3}H]WIN 35,428 binding to the dopamine transporter and for inhibition of [^{3}H]dopamine uptake in rat caudate putamen (Table 2). All of the compounds monophasically displaced [^{3}H]WIN 35,428 binding with affinities of 21.6–1840 nM (K_i). Generally, compounds containing *meta*-substituted halogens (**5a–c**) were more potent than benztropine and equipotent to or slightly less potent than their *para*-substituted homologs (4′-F, 4′,4″-diF, 4′-Cl)[12,13] in displacing [^{3}H]WIN 35,428 binding to the dopamine transporter. The most potent compound in this series was the 3′-chloro-3α-(diphenylmethoxy)tropane analog (**5c**, 3′-Cl benztropine) (K_i = 21.6 nM).

Table 2. Affinities of Analogs for Binding to the DAT and IC_{50} Values for Blockade of [^{3}H]Dopamine Uptake in Rat Caudate Putamen[a]

compd	substitution	DAT K_i, nM (% error)	[^{3}H]DA uptake inhibition IC_{50}, nM (±SEM)	[^{3}H]DA uptake inhibition IC_{50}/DAT binding
5c	3′-Cl	21.6 (7)	228 (77.1)	10.6
6	4′-Cl	30.0 (12)[b]	115[b] (27.6)	3.8
5b	3′,3″-diF	47.4 (11)	407 (63.9)	8.6
5e	2′-F	50.0 (12)	140 (17.2)	2.8
5a	3′-F	68.5 (12)	250 (64.7)	3.7
7		118 (9)[b]	403[b] (115)	3.4
5d	3′-CF_3	187 (5)	457 (72.0)	2.4
5f	2′-Cl	228 (9)	997 (109)	4.4
5g	2′-CH_3	309 (6)	1200 (164)	3.9
8	2′-NH_2	1840 (8)	373 (117)	0.20
1,[c,d] cocaine		32 (16), 388 (47)	12, 3250	

[a] Each experimental value represents data from at least three independent experiments, each performed in triplicate. [b] Data from ref 13. [c] The binding and uptake inhibition curves for cocaine were best fit by two-site models (p < 0.01), so both high- and low-affinity values are reported. Single-site models provided the best fit for all other compounds, and therefore a single value is reported. [d] Data from ref 24. **6** = 4′-chloro-3α-(diphenylmethoxy)tropane, **7** = benzotropine, **8** = 2′-amino-3α-(diphenylmethoxy)tropane (Molecular Probes, Inc.).

The 3′-CF_3 substitution resulted in a compound (**5d**) with a binding affinity at the dopamine transporter that was lower than that of benztropine but significantly higher than that of its *para*-substituted homolog 4′-CF_3 (K_i = 635 nM).[13] This may indicate that electron-withdrawing groups are not favored and that steric bulk is better tolerated in the *meta* than in the *para* position. Analogs containing *ortho* substituents with larger steric bulk than F (**5g**,**f**, **8**) were generally less potent than benztropine. This lower potency might be due to a restriction of the phenyl ring rotation by the 2′-substituent that does not allow optimum binding.

The analogs were also tested for (1) displacement of [^{3}H]citalopram binding at the serotonin transporter, (2) displacement of [^{3}H]desmethylimipramine binding at

Table 3. Results of Radiolabeled Binding Experiments on 2′- and 3′-Substituted and 3′,3″-Disubstituted 3α-(Diphenylmethoxy)tropane Analogs

compd	substitution	% *I* at 10 μM[a,b] NET	5HTT	m_1 K_i, nM (±SEM)
5c	3′-Cl	74.7	24.5	0.98 (0.01)
6[c]	4′-Cl	32.0	34.0	1.48 (0.02)
5b	3′,3″-diF	75.6	72.6	0.85 (0.01)
5e	2′-F	17.8	22.3	0.43 (0.02)
5a	3′-F	82.4	42.2	0.60 (0.05)
7[c]		18.3	17.2	0.59 (0.01)
5d	3′-CF_3	78.5	59.8	2.52 (0.28)
5f	2′-Cl	76.1	55.4	0.41 (0.01)
5g	2′-CH_3	66.5	35.7	0.50 (0.01)
8[c]	2′-NH_2	14.0	16.0	1.12 (0.04)

[a] Percent inhibition at 10 μM. [b] Data provided by NOVA-SCREEN. [c] Data from ref 13. **6** = 4′-chloro-3α-(diphenylmethoxy)tropane, **7** = benztropine, **8** = 2′-amino-3α-(diphenylmethoxy)tropane (Molecular Probes, Inc.).

the norepinephrine transporter in rat cortex, and (3) displacement of [^{3}H]pirenzepine binding at muscarinic m_1 receptors in rat brain P_2 membranes (Table 3). As with the previously reported 4′-substituted and 4′,4″-disubstituted 3α-(diphenylmethoxy)tropane analogs,[13] none of the compounds in the present study bound with high affinity to either the norepinephrine or serotonin transporters, but all were potent inhibitors of binding at muscarinic m_1 receptors (K_i = 0.41−2.52 nM). Of particular notice were the 2′-substituted 3α-(diphenylmethoxy)tropane analogs **5e**−**g** and **8** which all showed extremely high affinity for muscarinic m_1 receptors. Thus, these 2′- and 3′-substituted and 3′,3″-disubstituted 3α-(diphenylmethoxy)tropane analogs were selective for the dopamine transporter over the other monoamine transporters. However, they gave no better selectivity for the dopamine transporter over muscarinic m_1 receptors than the 4′-substituted and 4′,4″-disubstituted analogs. It is possible the restriction of phenyl ring rotation by the 2′-substituent, which is postulated to reduce the affinity of these compounds at the dopamine transporter, leads to compounds sterically favored at the muscarinic m_1 receptors. Molecular modeling studies investigating this hypothesis are currently underway.

Despite the fact that some of the 3′-substituted 3α-(diphenylmethoxy)tropane analogs demonstrated high-affinity binding to the dopamine transporter, they were generally less potent in inhibiting [^{3}H]dopamine uptake than their 4′-substituted homologs.[12,13] Notably, 3′-chloro-3α-(diphenylmethoxy)tropane (**5c**) which potently displaced [^{3}H]WIN 35,428 binding (K_i = 21.6 nM) was approximately equipotent to the 4′-Cl benztropine analog (K_i = 30.0 nM). Interestingly, compound **5c** was less potent than 4′-Cl benztropine in inhibiting [^{3}H]dopamine uptake (IC_{50} = 228 and 115 nM for 3′-Cl and 4′-Cl analogs, respectively), suggesting that these compounds may give some clues to separability of binding and inhibition of uptake at the dopamine transporter.

Compound **5c** was selected for behavioral evaluation and compared to cocaine and 4′-Cl benztropine in two models of psychostimulant action. Cocaine produced a dose-related increase in locomotor activity in mice (Figure 1), as has been demonstrated previously with it and other classes of dopamine uptake inhibitors.[24] Significant effects were obtained at doses of 5−40 mg/kg, and the maximal increase was approximately 267% of control values. In contrast, both compound **5c** (Figure 1) and its 4′-Cl analog, **6** (Figure 1), had much lower

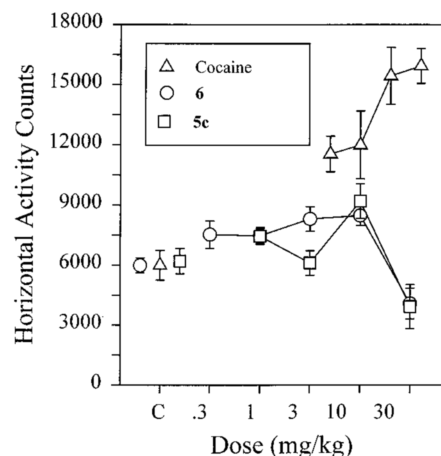


Figure 1. Dose-dependent effects of cocaine (△), 4′-Cl benzotropine (**6**) (○), and **5c** (□) on locomotor activity in mice. Ordinates: horizontal activity counts after drug administration. Abscissae: dose of drug in mg/kg, log scale. Unfilled points above C represent the effects of saline vehicle controls. Each point represents the average effect determined in 6−8 mice. The data are from the first 30 min period after drug administration, in which the greatest stimulant effects were obtained.

efficacies in stimulating locomotor activity. For compound **5c**, significant effects were only obtained at a dose of 10 mg/kg (Dunnetts t = 2.8094, critical value 2.60), and the increase was 148% of control values. For 4′-Cl benztropine, significant effects were obtained at doses of 3 and 10 mg/kg (Dunnetts t = 2.8447 and 3.0532, respectively, critical value 2.67), and the increases were 139% and 142% of control values, respectively.[12] These limited increases were of a similar magnitude to those obtained previously with other benztropine analogs.[21]

Each of the compounds was also evaluated in rats trained to discriminate ip injections of 10 mg/kg cocaine from saline. As has been reported previously, cocaine produced a dose-related substitution, whereas 4′-Cl benztropine did not produce an effect over the range of behaviorally active doses (Figure 2). In contrast to the effects of its 4′-Cl analog, compound **5c** produced discriminative stimulus effects similar to those of cocaine (Figure 2). This finding is different from all previously evaluated benztropine analogs that were like the 4′-Cl analog in that these compounds did not produce cocaine-like discriminative effects over the range of behaviorally active doses.[21] These differences between **5c** and its *para*-substituted analog occurred despite similar biochemical effects (Tables 2 and 3). The difference between the *meta*- and *para*-substituted compounds was particularly surprising considering the fact that the only structural difference between the compounds is the placement of the chloro substituent.

The substitution of **5c** for the discriminative stimulus effects of cocaine has implications for the interpretation of the lack of cocaine-like behavioral effects of 4′-Cl benztropine and other analogs. Because the previously reported benztropine analogs[12,13] generally have high affinity for muscarcinic m_1 receptors, the prepotent antimuscarinic actions might be implicated in disrupting behavior at doses lower than those necessary for the expression of the cocaine-like activity. For example, 4′-Cl benztropine has approximately an 8-fold higher affinity for muscarinic m_1 receptors than it does for the

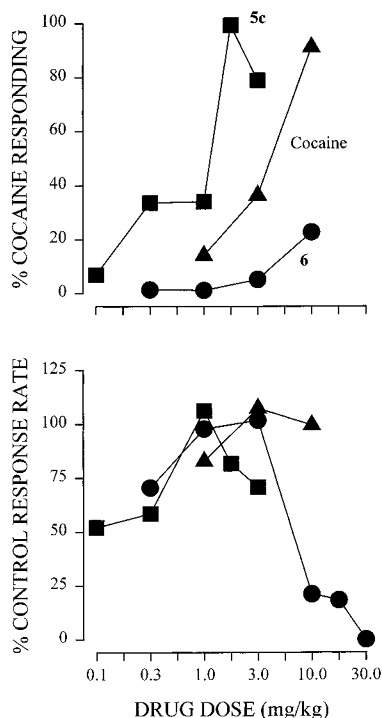


Figure 2. Effects of cocaine (▲), **6** (●), and **5c** (■) in rats trained to discriminate injections of cocaine from saline. Top panel ordinates: percentage of responses emitted on the cocaine-appropriate lever. Bottom panel ordinates: rates at which responses were emitted as a percentage of response rate after saline administration. Abscissae: drug dose in mg/kg, log scale. Each point represents the effect in 6 rats.

dopamine transporter. However, compound **5c** has about a 22-fold higher affinity for muscarinic m_1 receptors than it does for the dopamine transporter, yet it generalizes to cocaine. These findings indicate that alone, a high affinity for muscarinic m_1 receptors does not preclude the expression of cocaine-like behavioral activity.

Several investigators have suggested that a high ratio of potency for inhibiting dopamine uptake to that for binding to the dopamine transporter is indicative of a functional cocaine antagonist.[9,22] However, cocaine antagonist activity has not been demonstrated for many compounds with ratio values greater than 1.[10] In examining these ratios for the present set of compounds (Table 2), we found that the ratio for compound **5c** (10.6) was larger than that for its 4′-Cl analog (3.83) and, to our knowledge, was one of the highest ratios of this type reported to date. Seemingly in contradistinction to this relatively high ratio value, compound **5c** produced a cocaine-like profile in the drug discrimination paradigm. This finding further supports a suggestion that the significance of the differences between dopamine transporter binding affinities and inhibition of dopamine uptake potencies needs to be interpreted with caution because of their potential influence by methodological factors,[10,12,23−25] as well as the absence of demonstrated antagonist activity or limited intrinsic efficacy for these compounds.

One approach to a potential treatment modality for cocaine abuse would be a drug that has some of the behavioral effects of cocaine but is not self-administered. While compound **5c** was not an efficacious locomotor stimulant, it did produce generalization in rats trained to discriminate cocaine from saline. Studies of the self-administration of this compound are underway to further characterize its behavioral profile, in order to better delineate spectrums of behavioral activity that may provide leads for the development of therapeutics directed at cocaine abuse.

In summary, a series of 2′- and 3′-substituted and 3′,3″-disubstituted 3α-(diphenylmethoxy)tropane analogs were synthesized and found to displace [^{3}H]WIN 35,428 binding at the dopamine transporter and to inhibit [^{3}H]dopamine uptake in rat caudate putamen. Additionally, these compounds were selective for the dopamine transporter over other monoamine transporters but retained potency at muscarinic m_1 receptors. However, unlike its 4′-Cl benztropine homolog and every other analog in this series of compounds tested, this compound did generalize in animals trained to discriminate cocaine from saline. The continued investigation of compound **5c** and its congeners will undoubtedly prove useful in the further characterization of functional differences between cocaine-like dopamine uptake inhibitors and the benztropine analogs that do not share subjective effects with cocaine and may provide leads for the identification of medical treatments for cocaine abusers.

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 the indicated deuterated solvents, and 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). Mass spectra were obtained using a Hewlett Packard (Palo Alto, CA) 5971A mass selective ion detector (MSD) in the electron-impact mode. Samples were introduced into the MSD via an HP-5890 series II gas chromatograph (GC) fitted with an HP-1 (cross-linked methyl silicone gum, 25 m × 0.2 mm i.d., 50 μm film thickness) column. Ultrapure grade helium was used as the carrier gas at a flow rate of 1.2 mL/min. The injection port and transfer line temperatures were 250 and 280 °C, respectively. The initial GC oven temperature was 100 °C, held for 3.0 min, increased to 295 °C at a rate of 15 °C/min, and held at this final temperature for 10 min. Infrared spectra were recorded with a Perkin-Elmer 1600 series FTIR spectrophotometer with KBr disks. Elemental analysis performed by Atlantic Microlab, Inc. (Norcross, GA) agreed to within 0.4% of the calculated values. TLC solvent used was $CHCl_3/CH_3OH/NH_4OH$ (89:10:1), unless otherwise indicated. All chemicals and reagents were purchased from Lancaster Synthesis, Inc. or Aldrich Chemical Co.

General Synthetic Method for Substituted 3α-(Diphenylmethoxy)tropanes 5a−g. Commercially available benzophenones **1a−g** (10 mmol) were dissolved in absolute ethanol or methanol (75−100 mL), and $NaBH_4$ (10 mmol) was added. Reactions were typically allowed to proceed for 1−2 h, after which time the excess alcohol was removed *in vacuo*. The dry white residue was taken up in H_2O (50 mL) and ether (50 mL), and after the two phases were mixed and separated, the aqueous phase was further extracted with ether (2 × 25 mL). The ether fractions were combined, washed with H_2O (2 × 20 mL), dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. Reaction products were obtained in yields of 93−98%.

Resultant benzhydrols **2a−g** (5−10 mmol) were dissolved in $SOCl_2$ (10 mL) in an atmosphere of Ar. The reaction was allowed to proceed overnight at ambient temperature. Excess $SOCl_2$ was removed *in vacuo* (addition of two small portions of dry toluene and removal *in vacuo* ensured removal of all $SOCl_2$). Resultant oils were determined spectroscopically to be the desired benzhydryl chlorides **3a−g**.

These benzhydryl chlorides were individually added to a tropine (5−10 mmol) melt (160 °C) over 5 min. The reactions were allowed to proceed for 10−45 min resulting in brown oils which solidified upon cooling. The crude products were dissolved in $CHCl_3$ (30 mL) and transferred to separatory funnels. Ion pairing of the desired products was carried out via extraction with 2.8 N HCl (30 mL) as previously described.[13] The phases were mixed and separated, and the aqueous phase was further extracted with $CHCl_3$ (2 × 25 mL). The organic fractions were combined, washed with 2.8 N HCl (2 × 25 mL), dried over anhydrous Na_2SO_4, filtered, and concentrated *in vacuo* resulting in light brown foams which were recrystallized to give the pure products as HCl salts (18−38% yield).

Representative Spectral Data: Compound 5a. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.81−1.91 (m, 4H, H-2, 4_{ax}, 4_{eq}), 1.93−1.98 (m, 2H, H-6,7_{exo}), 2.08−2.17 (m, 2H, H-6,7_{endo}), 2.25 (s, 3H, N-CH_3), 3.06 (br t, 2H, H-1,5), 3.55 (t, J = 4.6 Hz, 1H, H-3), 5.39 (s, 1H, Ph-CH-Ph), 6.87−7.34 (m, 9H, aromatics). ^{13}C NMR (75 MHz, $CDCl_3$): δ 25.65 (t, C-6,7), 36.19 (t, C-1,5), 40.40 (q, N-CH_3), 59.99 (d, C-1,5), 69.16 (d, C-3), 79.92 (d, C-9), 113.25, 113.54, 113.74, 114.02, 122.27, 126.71, 127.33, 128.29, 129.54 (d, C-protonated aromatic), 129.65, 141.55, 146.25 (s, C-nonprotonated aromatic). IR (KBr): 735 (aro-H out of plane), 945, 1035, 1056, 1084, 1238, 1447, 1484, 1590, 1612, 2401, 2957, 3017, 3430 cm^{-1}. EIMS m/z (rel intensity): 325 (M^+, 3.5), 219 (9.8), 201 (13), 183 (18), 165 (16), 140 (100), 124 (52), 96 (29), 83 (93).

Pharmacology. 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 homogenizer and centrifuged at 20000g for 10 min at 4 °C. The resulting pellet was then washed two more times by resuspension in ice-cold buffer and centrifugation at 20000g 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. Triplicate samples of membrane suspension were preincubated for 5 min in the presence or absence of the compound being tested. [^{3}H]WIN 35,428 (2β-carbomethoxy-3β-(4-fluorophenyl)-tropane 1,5-naphthalenedisulfonate, specific activity 82.4 Ci/mmol, final concentration 1.5 nM; New England Nuclear, Boston, MA) 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%. Under these assay conditions, an average experiment yielded approximately 6000 dpm total binding/sample and approximately 250 dpm nonspecific binding, defined as binding in the presence of 100 μM cocaine. Each compound was tested with concentrations ranging from 0.01 nM to 100 μM for competition against binding of [^{3}H]WIN 35,428, in three independent experiments, each performed in triplicate.

[^{3}H]Dopamine Uptake Assay. Rats were sacrificed by decapitation and their brains removed to an ice-cooled dish for dissection of the caudate putamen. [^{3}H]Dopamine uptake was measured in a chopped tissue preparation as described previously.[26] Briefly, the tissue was chopped into 225 μm slices on a McIllwain tissue slicer with two successive cuts at an angle of 90°. The strips of tissue were suspended in oxygenated modified Krebs-HEPES buffer (see above), which was pregassed with 95% O_2/5% CO_2 and warmed to 37 °C. After rinsing, aliquots of tissue slice suspensions were incubated in buffer in glass test tubes at 37 °C to which either the drug being tested or no drug was added, as appropriate. After a 5 min incubation period in the presence of drug, [^{3}H]dopamine (final concentration 15 nM, specific activity 50 Ci/mmol; Amersham Corp., Arlington Heights, IL) was added to each tube. After 5 min the incubation was terminated by the addition of 2 mL of ice-cold buffer to each tube and filtration under reduced pressure over glass fiber filters (presoaked in 0.1% poly(ethylenimine) in water). The filters were rinsed and placed in scintillation vials to which 1 mL of methanol and 2 mL of 0.2 M HCl were added to extract the accumulated [^{3}H]-dopamine. Radioactivity was determined by liquid scintillation spectrometry at an efficiency of approximately 30%. The reported values represent specific uptake from which nonspecific binding to filters was subtracted.

Analysis of Data. Saturation and displacement data were analyzed by the use of the nonlinear least-squares curve-fitting computer program LIGAND.[27] Data from replicate experiments were modeled together to produce a set of parameter estimates and the associated standard errors of these estimates. In each case, the model reported fit significantly better than all others according to the F test at $p < 0.05$. The K_i values reported are the dissociation constants derived for the unlabeled ligands. Uptake data were analyzed using standard analysis of variance and linear regression techniques.[28] IC_{50} values were calculated using the linear portion of the concentration−response curve (linear regression $p < 0.05$).

In both saturation and competition experiments, two components of [^{3}H]WIN 35,428 binding were apparent. Analysis of the data utilizing the LIGAND program revealed a high-affinity component with a K_D of 7 ± 5 nM and a B_{max} of 445 ± 338 fmol/mg of protein and a low-affinity component with a K_D of 126 ± 115 nM and a B_{max} of 1995 ± 559 fmol/mg of protein.[24] Competition of [^{3}H]WIN 35,428 binding by cocaine also revealed two binding sites (Table 2).

Serotonin (5-HTT) and norepinephrine transporter (NET) receptor binding data were provided by NOVASCREEN. The radiolabeled ligands used and the methods were from the following published procedures: 5-HTT, [^{3}H]citalopram (specific activity 70−87 Ci/mmol, final ligand concentration 0.7 nM);[29] NET, [^{3}H]desmethylimipramine (specific activity 40−70 Ci/mmol, final ligand concentration 3.0 nM).[30]

Muscarinic m_1 Receptor Binding. Rat brain P_2 membranes were prepared as previously described.[31] Competition binding assays were performed in a 0.5 mL vol of 50 mM Tris-HCl, pH 7.4, containing 5 mM $MgCl_2$, 5 nM [^{3}H]pirenzepine, nine concentrations of competing compound (0.03−300 nM), and 0.3 mg of membrane protein. Nonspecific binding was measured in the presence of 10 μM QNB. Samples were incubated for 60 min at 37 °C followed by filtration over glass fiber filters. Filters were washed three times with 5 mL of 10 mM Tris-HCl, pH 7.4, and counted in 4 mL of Cytoscint. Prior to use, filters were soaked in 0.5% poly(ethylenimine) for at least 30 min at 25 °C. Competition binding data were analyzed using the nonlinear curve-fitting program GraphPAD InPlot,[32] and K_i values were calculated using the Cheng−Prusoff equation.[33] The K_D of [^{3}H]pirenzepine, 13.6 nM, was estimated from saturation binding analysis using the nonlinear curve-fitting program LIGAND.[27]

Locomotor Activity. Ambulatory activity of male Swiss Webster mice (Taconic Farms) were studied in 40 cm^3 clear acrylic chambers. The acrylic chambers were placed inside monitors (Omnitech Electronics, Columbus, OH) that were equipped with light sensitive detectors, spaced 2.5 cm apart along two perpendicular walls. Mounted on the opposing walls were infrared light sources that were directed at the detectors. One count of horizontal activity was registered each time the subject interrupted two successive beams. Repetitive interruptions of the same beam due to behaviors, such as head bobbing or grooming, were not counted. Mice were injected and immediately placed in the apparatus for 60 min. Injections were administered ip in volumes of 1 mL/100 g. Each dose was studied in eight mice, and mice were used only once.

Horizontal activity counts are shown for the first 30 min of the session in which the maximal stimulation of activity was

obtained. Each dose–effect curve was analyzed using standard analysis of variance (ANOVA) and post-hoc testing to determine significance of effects at individual doses.

Drug Discrimination. Six adult male Sprague–Dawley rats (Charles River, Wilmington, MA) were maintained at 350 g by postsession feeding. All subjects had unrestricted access to water within a temperature-controlled animal-housing room under a 12 h light/dark cycle. All testing was conducted during the light phase.

Rats were studied in operant conditioning test chambers (BRS/LVE, model RTC-022, Laurel, MD) which contained two response keys. Responses were recorded as depressions of the keys with a downward force of 0.2 N. Each response produced an audible click when the house light and the stimulus lamps over either lever were illuminated.

Experimental sessions were conducted at approximately the same time daily, 5 days/week. Before each training session, subjects received either saline or cocaine (10 mg/kg, ip) which was given in a mixed sequence. When subjects received cocaine, they were trained with food reinforcement to emit 20 consecutive responses on only one of the two response keys. Food presentation was followed by a 20 s time out period during which all stimulus lights were out and responses had no scheduled consequences. When subjects received saline, responses on the alternate key produced a food pellet according to the same schedule. Injections were given 5 min before sessions started. Sessions ended after 20 food presentations or 15 min, whichever occurred first. Since subjects had been trained previously, no further training was necessary prior to conducting test sessions, in which the effects of various doses of cocaine or test compounds were examined. Test sessions were conducted after each two training sessions and were identical with those training sessions with the exception that 20 consecutive responses on either of the response keys produced a food pellet. Subjects were injected ip with one of several doses of cocaine or test compound. Each dose was typically examined once in each subject. Test sessions were conducted if subjects met criteria of emitting greater than 85% correct responses in the entire session and before the first food pellet in each of the two training sessions preceding the test session. When subjects failed to meet the criteria, training continued until they did so for two consecutive sessions before testing resumed.

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