Novel Tropane-Based Irreversible Ligands for the Dopamine Transporter

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3α-(Diphenylmethoxy)tropane (benztropine) and its analogues are tropane ring-containing dopamine uptake inhibitors that display binding and behavioral profiles that are distinct from cocaine. We previously prepared a benztropine-based photoaffinity label [^{125}I]-(*N*-[4-(4′-azido-3′-iodophenyl)butyl]-3α-[bis(4′-fluorophenyl)methoxy]tropane, [^{125}I]**1**, that covalently attached to the 1−2 transmembrane spanning region of the dopamine transporter (DAT). This was in contrast to the 4−7 transmembrane spanning region labeled by a cocaine-based photoaffinity label, [^{125}I] **2** (RTI 82). To characterize further these different binding domains, photoaffinity ligands that had the 4′-azido-3′-iodophenyl substituent extended from the same position on the tropane ring were desirable. Thus, identification of the optimal alkyl linker between this substituent and the tropane nitrogen in the benztropine series was investigated to ultimately prepare the identical N-substituted analogue of **2**. In this pursuit, the *N*-[4-(4′-azido-3′-iodophenyl)propyl] analogue of 3α-[bis(4′-fluorophenyl)methoxy]tropane (**9a**) was synthesized as well as two isothiocyanate analogues that do not require photoactivation (**10a**,**b**) for irreversible binding. The synthesis of these target compounds was achieved using a modification of the strategy developed for **1**. Evaluation of these compounds for displacing [^{3}H]WIN 35 428 binding at DAT in rat caudate putamen revealed that the 4′-azido-3′-iodophenylbutyl substituent, found in **1**, provided optimal binding affinity and was chosen to replace the N−CH_3 group on **2**. Both the 4′-azido-3′-iodophenyl- and the 4′-isothiocyanatophenylbutyl analogues of **2** (**25** and **26**, respectively) were synthesized. Both products bound to DAT with comparable potency (IC_{50} = 30 nM) to RTI 82 (**2**). In addition, compound **26** demonstrated wash-resistant displacement of [^{3}H]WIN 35 428 in HEK 293 cells stably transfected with hDAT. These ligands will provide important tools for further characterizing the binding domains for tropane-based dopamine uptake inhibitors at the DAT.

Introduction

Reinforcing and stimulant effects of cocaine have been associated with its propensity to bind to the dopamine transporter (DAT) and inhibit the reuptake of dopamine into dopaminergic neurons.[1−3] Cocaine also binds to the other monoamine transporters; however, their roles in its pharmacological actions are less well defined than that of the DAT. The synthesis of novel ligands, based on cocaine, as well as other structurally diverse dopamine uptake inhibitors has been undertaken to further characterize the DAT and its role in the pharmacology of cocaine.[4−6] These studies have provided structure−activity relationships at the DAT as well as numerous highly potent and selective DAT ligands. Many of these compounds have been evaluated preclinically, and several are candidates for clinical trials in the treatment of cocaine abuse.[7,8] It has become clear, from these studies, that not only does the DAT play an important role in the psychostimulant actions of cocaine but a cocaine abuse treatment may be developed to target this system as an indirect agonist-approach medication.

The identification and characterization of tropane-based ligand binding sites on the DAT are being pursued in an attempt to characterize its structure and function and to further elucidate its role in the pharmacology of cocaine.[9−11] Site-directed mutagenesis and chimera studies suggest that inhibitors such as cocaine and substrates such as dopamine and amphetamine bind to different sites on the DAT.[12−16]

The development of molecular probes that include irreversible (N_3, NCS) and radiolabeled (^{3}H, ^{125}I) ligands provides important molecular tools with which to identify binding sites of both DAT substrates and inhibitors at a molecular level.[17,18] In combination with an arsenal of molecular biological and immunological techniques, these ligands can be used to characterize the molecular structure of the DAT protein and to delineate the differences between structurally diverse inhibitor binding sites. For example, the photoaffinity ligand [^{125}I]**1**, based on the benztropine series of compounds, photolabeled an 80 kDa protein in rat striatal membranes that was identified as the DAT by immunoprecipitation.[9] Proteolytic and immunological peptide mapping studies showed that [^{125}I]**1** was incorporated into a membrane-bound 14 kDa fragment predicted to contain transmembrane (TM) domains 1 and 2, whereas the cocaine analogue [^{125}I]**2** binds closer to the C-terminus in a domain containing TM helices 4−7.[10] On the basis of these studies, it was hypothesized that these tropane-based dopamine uptake inhibitors may be interacting

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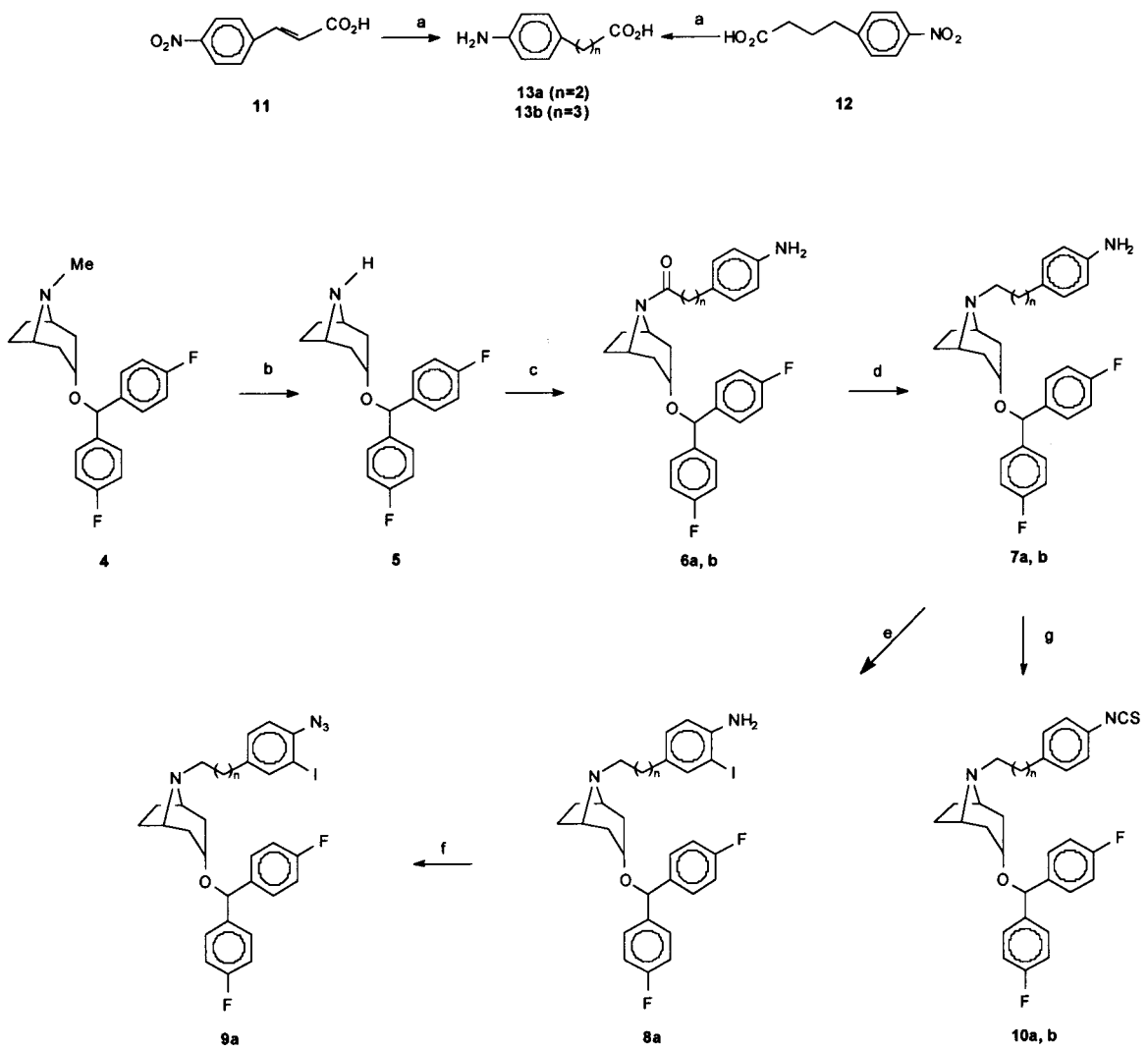
Scheme 1[a]

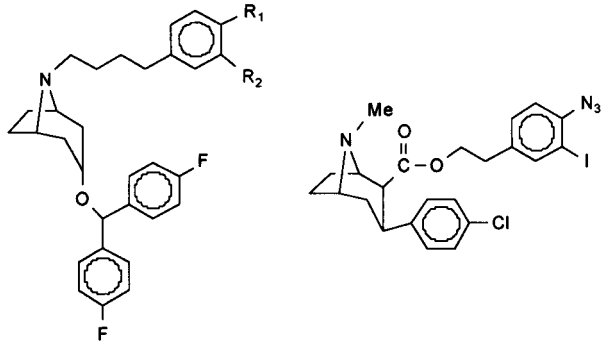


[a] Reagents and conditions: (a) H_2 (40 psi), Pd/C (10%), MeOH; (b) (1) ACE-Cl, Na_2CO_3, 1,2-dichloroethane, reflux, (2) MeOH, room temp, overnight; (c) **13**, DCC, HOBt, Et_3N, DMF, room temp, 48 h; (d) $LiAlH_4$, THF, reflux, 3 h; (e) ICl, glacial acetic acid, room temp, 3 h; (f) (1) $NaNO_2$, aqueous HOAc, 0 °C, 30 min, (2) NaN_3, 0 °C, 30 min; (g) $CSCl_2$, $NaHCO_3$, $CHCl_3/H_2O$, 0 °C, 3 h.

with distinctive binding domains on the DAT. These results supported structure−activity and CoMFA studies[19−24] that demonstrated that although benztropine and cocaine are both tropane-based dopamine uptake inhibitors, they have distinctive structural requirements for binding to DAT. However, in the previous studies comparing the photoaffinity ligands [^{125}I]**1** and [^{125}I]**2**, the azido (N_3) group, responsible for covalent bonding, was placed at a different position on the tropane ring of these two molecules.[9,10] As a result, it might be possible for the pharmacophore of these two molecules to be binding at the same site on the DAT but simply covalently attached to different sites because of the placement of the photoactivated azido group.

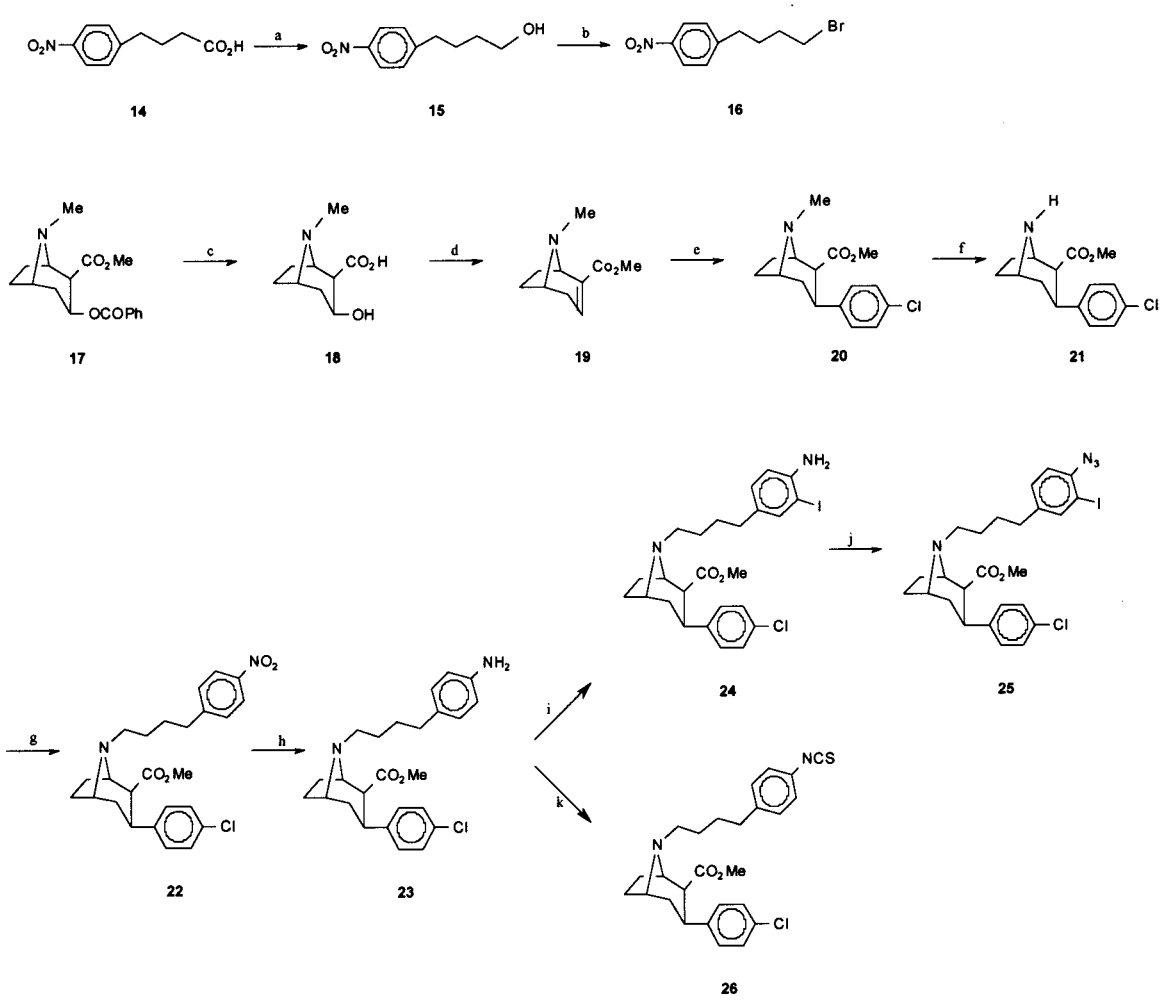
The binding affinity of **1** (K_i = 159 nM) was approximately 20-fold less than the unsubstituted *N*-butylphenyl analogue **3** (K_i = 8.5 nM)[25] (Chart 1), warranting an investigation into N-substitution modification toward optimization. The rationale for decreasing the alkyl chain linker between the tropane nitrogen and the pendant phenyl ring was based on the hypothesis that the steric bulk of the iodo and azido functional groups was not well tolerated at DAT when appended

Chart 1



to the butylphenyl substituent. In support of this, Meltzer and colleagues had previously shown, in a related series of compounds, that the *N*-pentylphenyl substituent significantly decreased binding affinity compared to the propylphenyl substituent.[26] Additional structure−activity relationship (SAR) studies have shown

Scheme 2[a]



[a] Reagents and conditions: (a) BH_3·SMe_2, THF, room temp, 3 h, 98%; (b) CBr_4, PPh_3, acetonitrile, room temp, 1 h, 94%; (c) aqueous HCl (6 N), reflux, 6 h; (d) (1) $POCl_3$, reflux, 4 h, (2) MeOH, −40 °C, 73.5% from **17** to **19**; (e) *p*-ClPhMgBr, ether, −40 °C, 2 h, 49%; (f) (1) ACE-Cl, Na_2CO_3, 1,2-dichloroethane, reflux, 3 h, (2) MeOH, room temp, overnight, 86%; (g) **21**, K_2CO_3, DMF, room temp, overnight, 87%; (h) H_2 (40 psi), Pd/C (10%), EtOAc/MeOH (1:1), room temp, 1.5 h, 70%; (i) ICl, HOAc (glacial), room temp, 3 h, 60%; (j) (1) $NaNO_2$, HOAc/H_2O (1:1), 0 °C, 30 min, (2) NaN_3, 0 °C, 30 min, 92%; (k) $CSCl_2$, $NaHCO_3$, $CHCl_3$/H_2O, 0 °C, 3 h, 87%.

that although the *N*-propylphenyl-substituted 3α-[bis-(4′-fluorophenyl)methoxy]tropane binds with lower affinity than the *N*-butylphenyl-substituted analogue (K_i = 41 vs 8.5 nM), the *N*-*p*-F-propylphenyl analogue did not show a significant further decrease in affinity (K_i = 60 nM).[23,24] This suggested that 4′-azido-3′-iodo substitution on the phenyl ring with the propyl linker may not further reduce DAT binding affinity, and the resulting compound might prove to be superior to **1**.

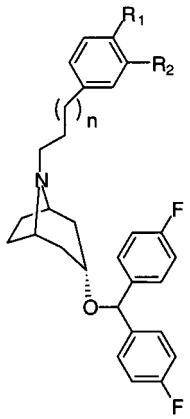
Thus, in this study, synthesis of novel irreversible ligands, based on both **1** and **2**, were pursued in an attempt (1) to identify the optimal chain length between the tropane nitrogen and the pendant phenyl ring of **1**, (2) to replace the N−CH_3 group on **2** with this N-substituent so that both photoaffinity ligands would have the azido function in the identical position on the tropane ring, and (3) to prepare the isothiocyanate analogues for future utilization as irreversible ligands under nonphotoactivation conditions.

Chemistry

The synthesis of the benztropine-based irreversible ligands is shown in Scheme 1. Our synthetic strategy was based on the coupling reaction of nor-3α-[bis(4′-fluorophenyl)methoxy]tropane (**5**)[25] with 3-(*p*-amino)-phenylpropionic acid (**13a**) or 4-(*p*-amino)phenylbutyric acid (**13b**). The side chains **13a** or **13b** were synthesized by catalytic hydrogenation (10% Pd/C) of 4-nitrocinnamic acid (**11**) or 4-(*p*-nitro)phenylbutyric acid (**12**), respectively, in nearly quantitative yield. 3α-[Bis-(4′-fluorophenyl)methoxy]tropane (**4**)[20] was treated with 1-chloroethylchloroformate (ACE-Cl) as previously described[25] to afford the N-demethylated product (**5**). Compound **5** was reacted with either **13a** or **13b** in the presence of 1, 3-dicyclohexylcarbodiimide (DCC), 1-hydroxybenzotriazole hydrate (HOBt), and triethylamine, in DMF, to give the amide **6a** or **6b**, which was then converted to amine **7a** or **7b** by reduction with $LiAlH_4$. The yields of **7a** and **7b** from **5** were 74% and 68%, respectively. Compound **7a** was reacted with ICl in glacial acetic acid to give iodo-substituted product **8a** in 75% yield, which was then treated with $NaNO_2$ followed by NaN_3 to give the azidoiodo product **9a** in 80% yield. Amines **7a** and **7b** were reacted with $CSCl_2$ to give **10a** and **10b** in 80% and 95% yield, respectively.

The synthesis of ligands based on **2** is shown in Scheme 2. The strategy for the synthesis of these compounds could not be directly adapted from that

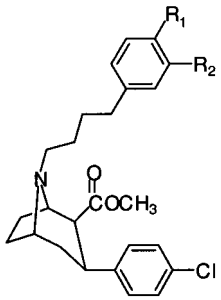
Table 1. Binding Data for N-Substituted 3α-[Bis(4′-fluorophenyl)methoxy]tropanes at DAT



compd	*n*	R_1, R_2	DAT, $K_i \pm$ SEM,[a] nM
1	2	N_3, I	159 ± 21[b]
3	2	H, H	8.51 ± 1.2[c]
27	2	NO_2, H	20.2 ± 2.2[b]
28	2	NH_2, H	29.7 ± 3.6[b]
9a	1	N_3, I	284 ± 34
10a	1	NCS, H	611 ± 81[d]
10b	2	NCS, H	420 ± 52[d]
WIN 35 428			43.7 ± 3.0[e]

[a] Each K_i value represents data from at least three independent experiments, each performed in triplicate. K_i is analyzed by LIGAND. [b] Reference 9. [c] Reference 25. [d] IC_{50} values. [e] Included for reference.

Table 2. Binding Data at DAT for Irreversible Ligands Based on RTI 82 (**2**)



compd	R_1, R_2	DAT, $IC_{50} \pm$ SEM,[a] nM
2		14.5 ± 0.94[b]
25	N_3, I	33.0 ± 4.7
26	NCS, H	31.5 ± 3.1

[a] Each IC_{50} value represents data from at least three independent experiments, each performed in triplicate. [b] Reference 17.

strategy depicted in Scheme 1. Since both amide and ester functional groups exist in the coupling product, it would be difficult to reduce the amide to the amine without also reducing the ester simultaneously. Therefore, the synthetic strategy was modified to incorporate the coupling of nor-2*β*-carbomethoxy-3*β*-(4-chlorophenyl)tropane (**21**) with 4-(*p*-nitrophenyl)butyl bromide (**16**). Compound **16** was prepared from 4-(*p*-nitrophenyl)butyric acid (**14**) by reduction with borane in THF to alcohol **15** in 98% yield. Compound **15** was then converted to **16** using the Corey−Fouchs reaction, in 94% yield. (−)-Cocaine (**17**) was converted to 2*β*-carbomethoxy-3*β*-(4-chlorophenyl)tropane (**20**), following a modification of the procedures described previously.[27,28] Compound **20** was then N-demethylated with ACE-Cl to give nor-2*β*-carbomethoxy-3*β*-(4-chlorophenyl)tropane (**21**). Compound **21** was reacted with bromide **16** to give the coupling product **22** in 87% yield. Compound **22** was then converted to **23** by catalytic hydrogenation (10% Pd/C). The synthetic strategy for converting compound **23** to the target products **25** and **26** was essentially identical to that described for **9a** and **10** from amine **7** (Scheme 1).

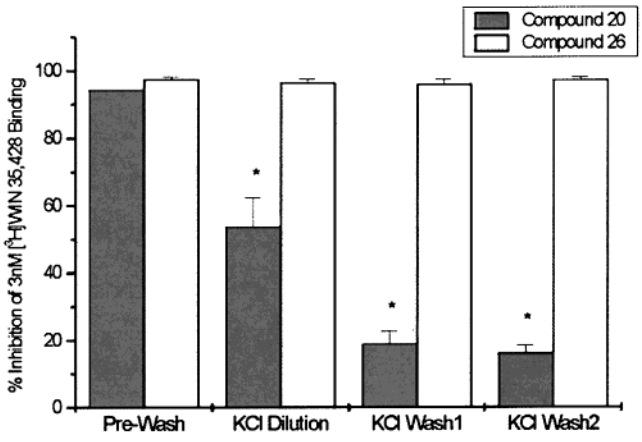


Figure 1. Differential effects of washing on the ability to inhibit radioligand binding by compounds **20** and **26**. Values shown are the means of at least four independent experiments. The ∗ indicates a significant difference (*t* test, $p < 0.01$) in [^{3}H]WIN 35 428 binding inhibition compared to the results of compound **20** sample that did not undergo washing. With each successive wash, compound **20** produced reduced radioligand binding inhibition suggestive of reversible labeling. In contrast, compound **26** maintained a level of 98% inhibition regardless of washing. This wash-resistant radioligand binding inhibition suggested that compound **26** is bound irreversibly.

Results and Discussion

Three potential irreversible ligands, based on benztropine, were prepared and evaluated for displacement of [^{3}H]WIN 35 428 binding at DAT, in rat caudate putamen. The K_i value for **9a** (nonphotoactivated) and the IC_{50} values for **10a** and **10b** can be seen in Table 1 and are compared to the previously published K_i values for **1**, **3**, **27**, and **28**. As discussed previously, addition of the 4′-azido-3′-iodo groups on the *N*-butylphenyl ring significantly decreased binding affinity of compound **1** compared to the unsubstituted **3**. This was somewhat surprising because the addition of either a 4′-NO_2 (**27**) or 4′-NH_2 (**28**) decreased binding affinity by only <3-fold.[9] When the alkyl linker between the tropane nitrogen and the pendant phenyl ring was decreased to propyl, the binding affinities of the resulting compounds were further reduced to $K_i = 284$ nM for **9a** compared to that for **1** ($K_i = 159$ nM). The 4′-NCS group also reduced binding potency at DAT in both series for **10a** ($IC_{50} = 611$ nM) and **10b** ($IC_{50} = 420$ nM). Although washout experiments were not conducted on these compounds, reversible binding could not be assumed, and thus, IC_{50} values are reported.

These results suggest that the linear steric bulk of both the N_3 and NCS groups have deleterious effects on binding affinity and that moving the terminal phenyl ring closer to the tropane nitrogen only compounds these negative effects. Therefore, the results of these and previous SAR studies[24] confirmed that the butyl

linker provided the optimal chain length, and this was then used for the irreversible ligands based on compound **2**.

Two novel irreversible ligands based on **2** were prepared and evaluated for displacement of [^{3}H]WIN 35 428 binding at DAT in rat caudate putamen. The IC_{50} values of compounds **25** and **26** can be seen in Table 2 and are compared to the parent ligand, **2**. Both of these compounds demonstrated DAT binding potency comparable to the potency of the parent compound.

Washout experiments were performed on the isothiocyanato derivative, compound **26**. In our experience, the isothiocyanates have a significantly higher incorporation into the cell membrane preparation, and thus, wash-resistant binding is more readily detected and quantitated. Further, when these two types of irreversible ligands are compared, the isothiocyanate, which depends on reaction with a nucleophile (SH− or NH_2−) for covalent attachment to the binding site, is less reactive than the photoactivated azido group. As a result, we make the assumption that if the isothiocyanato analogue binds in a wash-resistant manner, then the photoactivated azide will do so as well. The irreversible binding of compound **26** was determined according to the methods described in Experimental Methods. Figure 1 represents the percent inhibition of [^{3}H]WIN 35 428 binding in HEK 293 cells stably transfected with hDAT, at each step of the washout experiment. Samples were preincubated with saturating concentrations of compound **20**, which was used as a control for reversible binding at DAT (K_i = 1.2 nM[30]) and compound **26**. Values are expressed as percentages of inhibition produced by controls that were incubated with vehicle. With each successive washing step, **20** produced decreased radioligand binding inhibition. In contrast, **26** maintained a level of 98% inhibition despite repeated washes. These data are consistent with irreversible labeling hDAT by **26** because the compound is retained by methods that wash out **20**.

Summary

In summary, novel irreversible ligands based on 3α-(diphenylmethoxy)tropane (benztropine) and RTI 82 (**2**) have been prepared. The optimal N-substituent in the benztropine series was determined to be *N*-butylphenyl, and this was used in the two ligands in the series based on compound **2** (RTI 82). Washout experiments demonstrated that the isothiocyanato analogue, **26**, did indeed bind to DAT in a wash-resistant manner. This experiment suggests that the isothiocyanate functional group covalently attached to an amino acid residue in proximity to the binding site of the tropane-based pharmacophore. Radiodination of the photoaffinity ligand, **25**, is underway. Immunoprecipitation and proteolysis studies will be used with [^{125}I]**25** to determine the TM binding domain of this novel ligand and to compare it to those of **1** and **2**. These studies will provide further characterization of the binding domains of tropane-based dopamine uptake inhibitors on the DAT.

Experimental Methods

Chemistry. All melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker (Billerica, MA) AC-300 instrument. Samples were dissolved in an appropriate deuterated solvent ($CDCl_3$ or CD_3OD). Proton chemical shifts are reported as parts per million (δ) relative to tetramethylsilane (Me_4Si; 0.00 ppm), which was used as an internal standard. Carbon chemical shift values (δ) are recorded in parts per million (ppm) relative to deuterated chloroform ($CDCl_3$; 77.0 ppm). Mass spectra were recorded on a Hewlett-Packard (Palo Alto, CA) 5971A mass-selective ion detector in the electron-impact mode with sample introduction via an HP-1 (cross-linked methylsilicone gum) 25 m × 0.2 mm i.d., 50 μm film thickness. 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 oven temperature was 100 °C, held for 3.0 min, programmed to 295 °C at 15.0 °C/ min, and maintained at 295 °C for 10.0 min. Infrared spectra were recorded as a neat film on NaCl plates with a Perkin-Elmer 1600 series FTIR spectrometer. Microanalyses were performed by Atlantic Microlab, Inc. (Norcross, GA) and agree within ±0.4% of calculated values. All flash column chromatography experiments were performed using the flash-grade silica gel (Aldrich, 230−400 mesh, 60 Å). All chemical and reagents were purchased from Aldrich Chemical Co. and Lancaster Synthesis, Inc., unless otherwise indicated, and used without further purification.

3-(4-Aminophenyl)propionic Acid (13a). *p*-Nitrocinnamic acid (**11**, 1.93 g, 10 mmol) in MeOH (80 mL) was reduced on a Parr hydrogenator at 40 psi for 1 h over a catalytic amount (50 mg) of Pd/C (10%). The reaction mixture was filtered through Celite. The filtrate was concentrated to dryness to give 1.51 g (91%) of 3-(4′-amino)phenylpropionic acid (**13a**) as a solid; mp 124−126 °C. ^{1}H NMR (CD_3OD): δ 6.97 (d, J = 8.1 Hz, 2H), 6.65 (d, J = 8.1 Hz, 2H), 2.76 (t, J = 7.8 Hz, 2H), 2.50 (t, J = 7.8 Hz, 2H).

4-(4-Aminophenyl)butyric Acid (13b). 4-(4-Nitrophenyl)-butyric acid (**12**, 523 mg, 2.5 mmol) in MeOH (20 mL) was reduced on a Parr hydrogenator at 40 psi for 1 h over a catalytic amount (50 mg) of Pd/C (10%). The reaction mixture was filtered through Celite. The filtrate was concentrated to dryness to give 429 mg (96%) of 4-(4-aminophenyl)butyric acid (**3b**) as a solid; mp 120−123 °C. ^{1}H NMR (CD_3OD): δ 6.93 (d, J = 8.0 Hz, 2H), 6.68 (d, J = 8.0 Hz, 2H), 2.50 (t, J = 7.5 Hz, 2H), 2.24 (t, J = 7.5 Hz, 2H), 1.83(m, 2H).

***N*-[3-(4-Aminophenyl)propyl]-3α-[bis(4′-fluorophenyl)-methoxy]tropane (7a).** To a solution of **5**[25] (4.60 g, 12.58 mmol) in DMF (125 mL) were added acid **13a** (2.08 g, 13.84 mmol), 1,3-dicyclohexylcarbodiimide (DCC, 2.85 g, 13.84 mmol), 1-hydroxybenzotriazole (HOBt, 1.87 g, 13.84 mmol), and triethylamine (3.78 mL, 27 mmol) under argon. The reaction mixture was allowed to stir at room temperature for 48 h. DMF was removed in vacuo, and the residue was dissolved in 200 mL of water and extracted with chloroform (3 × 50 mL). The combined organic layer was dried over anhydrous $MgSO_4$ and concentrated. The crude product was purified by flash column chromatography [$CHCl_3$/MeOH/NH_4OH, 95:5:1 (5% CMA)] to give 4.75 g of product **6a**. IR: 3343(br), 1630(s) cm^{-1}. Amide **6a** (4.75 g) was dissolved in dry THF (20 mL) and added dropwise to $LiAlH_4$ (0.76 g, 20.0 mmol) in THF (10 mL) under argon. After the addition, the mixture was heated to gentle reflux for 3h. The reaction mixture was then cooled in an ice bath and then slowly quenched with H_2O (0.75 mL), aqueous NaOH (15%, 0.75 mL), and H_2O (2.2 mL) successively. The mixture was filtered. The filtrate was concentrated and purified by flash column chromatography (10% CMA) to give 4.38 g of amine **7a** (74% yield from **5**). ^{1}H NMR ($CDCl_3$): δ 7.24−7.30 (m, 4H), 6.93−7.02 (m, 6H), 6.10 (d, J = 8.1 Hz, 2H), 5.36 (s, 1H), 3.52 (br s, 2H), 3.15 (br s, 1H), 2.52 (t, J = 7.7 Hz, 2H), 2.34 (m, 2H), 2.09 (m, 2H), 1.70−1.98 (m, 8H). ^{13}C NMR ($CDCl_3$): δ 26.1, 30.6, 32.8, 35.6, 51.4, 57.9, 69.6, 79.2, 115.1, 128.2, 129.0, 132.2, 138.6, 144.0, 160.2, 163.5. IR: 3373 (br), 1603 (m) cm^{-1}. EIMS m/z: 462 (M^+).

***N*-[4-(4-Aminophenyl)butyl]-3α-[bis(4′-fluorophenyl)-methoxy]tropane (7b).** Compound **7b** was prepared as described above for **7a**, giving 68% yield. ^{1}H NMR ($CDCl_3$): δ

7.31−7.21(m, 4H), 7.06−6.90 (m, 6H), 6.62 (d, J = 8.1 Hz, 2H), 5.34 (s, 1H), 3.65−3.43 (m, 3H), 3.17 (m, 2H), 2.47 (t, J = 7.5 Hz, 2H), 2.35 (t, J = 7.5 Hz, 2H), 2.20−1.74 (m, 8H), 1.63−1.52 (m, 4H).

***N*-[3-(3-Iodo-4-aminophenyl)propyl]-3α-[bis(4′-fluorophenyl)methoxy]tropane (8a).** ICl (285 mg, 1.75 mmol) in 5 mL of glacial acetic acid was added very slowly to a solution of amine **7a** (737 mg, 1.59 mmol) in 35 mL of glacial acetic acid at room temperature over a period of 3 h. Acetic acid was then removed in vacuo. The residue was diluted with H_2O (50 mL), basified to pH 9 with NH_4OH, and extracted with $CHCl_3$ (3 × 25 mL). The combined organic layer was dried over anhydrous $MgSO_4$ and concentrated. The residue was purified by flash column chromatography (10% CMA) to produce 697 mg (74.5%) of **8a** as an oil. ^{1}H NMR ($CDCl_3$): δ 7.47 (s, 1H), 7.35−7.25 (m, 4H), 7.03−6.95 (m, 5H), 5.36 (s, 1H), 3.94 (br, s, 2H), 3.56 (t, J = 4.8 Hz, 1H), 3.18 (br s, 2H), 2.44 (t, J = 7.5 Hz, 2H), 2.36 (t, J = 7.5 Hz, 2H), 2.15−1.73 (m, 10H). IR: 3373 (br), 1605 (m) cm^{-1}.

***N*-[3-(3-Iodo-4-azidophenyl)propyl]-3α-[bis(4′-fluorophenyl)methoxy]tropane (9a).** To a solution of compound **8a** (325 mg, 0.55 mmol) in a mixture of acetic acid (2 mL) and H_2O (6 mL) was added $NaNO_2$ (53.4 mg, 0.77 mmol), and the mixture was stirred at 0 °C for 30 min. NaN_3 (54 mg, 0.83 mmol) was added, and the mixture was stirred for another 30 min at 0 °C. The mixture was diluted with water (30 mL), basified with saturated $NaHCO_3$ solution to pH 9, and extracted with $CHCl_3$. The combined organic layer was dried over $MgSO_4$ and concentrated. The residue was purified by column chromatography (3% CMA) to yield 271 mg (80%) of **9a** as an oil. The product was converted to its oxalate salt and recrystallized from acetone to give the oxalate salt of **9a** as a crystalline product, mp 105 °C (dec). ^{1}H NMR: δ 7.62 (s, 1H), 7.18−7.30 (m, 5H), 6.94−7.04 (m, 5H), 5.36 (s, 1H), 3.56 (t, J = 4.8 Hz, 1H), 3.20 (br s, 2H), 2.58 (t, J = 7.5 Hz, 2H), 2.36 (t, J = 7.5 Hz, 2H), 1.73−2.15 (m, 10H). ^{13}C NMR: δ 26.0, 29.7, 32.3, 35.6, 51.0, 58.2, 69.4, 79.3, 87.5, 115.0, 115.2, 118.1, 128.2, 129.5, 138.4, 139.1, 139.7, 140.6, 160.3, 163.5. IR: 2117(s), 1603(m), 1069 cm^{-1}. Anal. ($C_{29}H_{29}N_4OF_2I\cdot{}^1/_2C_2H_2O_4\cdot C_3H_6O\cdot H_2O$) C, H, N.

***N*-[3-(4-Isothiocyanatophenyl)propyl]-3α-[bis(4′-fluorophenyl)methoxy]tropane (10a).** Amine **8a** (585 mg, 1.27 mmol) was dissolved in a mixture of $CHCl_3$ (76 mL) and aqueous $NaHCO_3$ solution (479 mg in 32 mL of H_2O), and the mixture was vigorously stirred. Freshly distilled $CSCl_2$ (0.126 mL, 1.65 mmol) was added dropwise at 0 °C. After the addition, the reaction mixture was allowed to stir for 3 h. The two layers were separated, and the aqueous layer was extracted with $CHCl_3$ (3 × 25 mL). The combined organic layer was dried over $MgSO_4$ and concentrated. The residue was purified by flash column chromatography (2% CMA) to provide 515 mg (80%) of product **10a** as an oil, which was converted to its HCl salt in HCl-saturated 2-PrOH and recrystallized from acetone to give **10a**·HCl, mp 205.5−207 °C. ^{1}H NMR: δ 7.21−7.30 (m, 4H), 7.10−7.18 (m, 4H), 6.94−7.02 (m, 4H), 5.36 (s, 1H), 3.57 (t, J = 4.8 Hz, 1H), 3.24 (br s, 2H), 2.64 (t, J = 7.5 Hz, 2H), 2.40 (t, J = 7.5 Hz, 2H), 1.80−2.17 (m, 10H). ^{13}C NMR: δ 26.0, 29.6, 33.2, 35.6, 51.2, 58.5, 69.4, 79.6, 115.1, 115.4, 125.7, 128.4, 128.8, 129.5, 138.5, 141.6, 160.4, 163.6. IR: 2103.8 (s, br), 1603 (m), 1069 cm^{-1}. EIMS m/z: 504 (M^+). Anal. ($C_{30}H_{30}N_2OFS\cdot$HCl) C, H, N.

***N*-[4-(4-Isothiocyanatophenyl)butyl]-3α-[bis(4′fluorophenyl)methoxy]tropane (10b).** Compound **10b** was prepared as described above for **10a** from amine **8b** (541 mg, 1.14 mmol) to give 562 mg (95%) of product, which was converted the HCl salt and recrystallized from acetone, mp 166−168 °C. ^{1}H NMR: δ 7.22−7.30 (m, 4H), 7.10−7.15 (m, 4H), 6.93−7.01-(m, 4H), 5.35 (s, 1H), 3.52 (t, J = 4.6 Hz, 1H), 3.13 (br s, 2H), 2.60 (t, J = 7.5 Hz), 2.31 (t, J = 7.5 Hz, 2H), 2.03 (m, 2H), 1.78−1.91 (m, 6H), 1.43−1.63 (m, 4H). ^{13}C NMR: δ 26.2, 28.5, 29.2, 35.5, 35.9, 52.0, 58.1, 69.8, 79.3, 115.1, 115.4, 125.6, 128.3, 128.6, 129.5, 138.7, 142.2, 160.4, 163.6. IR: 2099.4 (s, br), 1602.6 (m), 1069 cm^{-1}. EIMS: m/z 518(M^+). Anal. ($C_{30}H_{32}N_2OF_2S\cdot HCl\cdot{}^1/_2C_3H_6O$) C, H, N.

4-(*p*-Nitrophenyl)butanol (15). To a solution of 4-(*p*-nitrophenyl)butyric acid **14** (3.14 g, 15 mmol) in dry THF (40 mL), 10 mL of $BH_3\cdot SMe_2$ (2.0 M in THF, 20 mmol) was added dropwise at room temperature under argon. The solution was stirred for 1 h after the addition was complete, and MeOH (20 mL) was then carefully added to destroy the excess of $BH_3\cdot SMe_2$. All solvents were removed in vacuo. The residue was diluted with H_2O (20 mL) and extracted with $CHCl_3$ (3 × 20 mL). The combined organic layer was dried ($MgSO_4$), After removal of the solvent, the crude product was purified by flash chromatography [hexanes/ethyl acetate (5:1)] to give 2.87 g (98%) of pure product **15** as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 8.15 (d, J = 8.7 Hz, 2H), 7.33 (d, J = 8.7 Hz, 2H), 3.68 (t, J = 6.3 Hz, 2H), 2.76 (t, J = 7.4 Hz, 2H), 1.82−1.20 (m, 5H). IR: 3500−3100 (br), 1599 cm^{-1}.

1-Bromo-4-(4-nitrophenyl)butane (16). 4-(4-Nitrophenyl)butanol (**15**) (2.87 g, 14.7 mmol) was dissolved in acetonitrile (50 mL). Triphenylphosphine (5.78 g, 22.1 mmol) was added, followed by addition of carbon tetrabromide (7.31 g, 22.1 mmol). The reaction mixture was stirred at room temperature for 1 h, then basified with 15% NaOH to pH 9. The mixture was then poured into a separatory funnel, and the aqueous layer was extracted with ether (3 × 50 mL). The combined organic layer was dried ($MgSO_4$) and concentrated. The residue was purified by flash column chromatography [hexanes/ethyl acetate (10/1)] to give 3.57 g (94%) of compound **16** as an oil. ^{1}H NMR ($CDCl_3$): δ 8.15 (d, J = 8.7 Hz, 2H), 7.33 (d, J = 8.7 Hz, 2H), 3.43 (t, J = 8.7 Hz, 2H), 3.68 (t, J = 6.3 Hz, 2H), 2.76 (t, J = 7.4 Hz, 2H), 2.00−1.76 (m, 4H). EIMS m/z: 257 (M^+), 259 (M^+).

(*R*)-(−)-Anhydroecgonine Methyl Ester (19). (−)-Cocaine HCl salt (20.0 g, 58.6 mmol) was dissolved in 6 N HCl (130 mL), and the mixture was heated to reflux for 6 h.[27] The solution was then cooled to room temperature and extracted with ether (2 × 100 mL). The aqueous phase was concentrated and then lyophilized to dryness. $POCl_3$ (80 mL) was added to the residue, and the mixture was stirred at reflux for 4 h. The excess $POCl_3$ was removed under reduced pressure, and the resulting dark oil was cooled to −40 °C and carefully treated with anhydrous MeOH (100 mL). The mixture was then warmed to room temperature and concentrated under reduced pressure. The residue was dissolved in water (150 mL), and the resulting solution was adjusted to pH 9 with NH_4OH, extracted with $CHCl_3$, and dried over K_2CO_3. After removal of the solvents, the residue was distilled under reduced pressure to afford 7.80 g (73.5%) of **19** as a clear oil. ^{1}H NMR ($CDCl_3$): δ 6.87 (m, 1H, H-3), 3.77 (m, 1H, H-1), 3.75 (s, 3H, CO_2CH_3), 3.23 (m, 1H, H-5), 2.62 (m, 1H, H-4ax), 2.34 (s, 3H, NCH_3), 2.13 (m, 1H, H-4eq), 1.86 (m, 4H, H-6 and H-7).

2β-Carbomethoxy-3β-(4-chlorophenyl)tropane (20). A solution of anhydroecgonine methyl ester (**19**, 2.81 g, 15.5 mmol) in anhydrous ether (90 mL) was added slowly to a solution of (4-chlorophenyl)magnesium bromide (1 M in ether, 31 mL, 31 mmol) in anhydrous ether (90 mL) at −40 °C under argon.[28] The reaction mixture was stirred for 2 h at −40 °C, then cooled to −78 °C, quenched with trifluoroacetic acid (10 mL), and allowed to warm to room temperature. The yellow mixture was then diluted with H_2O, basified with NH_4OH to pH 9, and extracted with $CHCl_3$. The combined organic layer was dried over K_2CO_3, concentrated, and purified by column chromatography (Et_2O/Et_3N, 95:5) to give 2.23 g (49%) of the β-isomer **20** as a white solid, mp 118.5−119.5 °C (lit.[28] 120−121 °C). $[\alpha]^{25}_D$ −42.0° (*c* 1, MeOH) (lit.[31] $[\alpha]^{21}_D$ −44.0°). ^{1}H NMR ($CDCl_3$): δ 7.30−7.15 (m, 4H), 3.54 (m, 1H), 3.47 (s, 3H), 3.38 (m, 1H), 3.00−2.87 (m, 2H), 2.55 (m, 1H), 2.18 (s, 3H), 2.18−2.05 (m, 1H), 1.74−1.58 (m, 4H). EIMS m/z: 293 (M^+).

Nor-2β-carbomethoxy-3β-(4-chlorophenyl)tropane (21). 2β-Carbomethoxy-3β-(4-chlorophenyl)tropane **20** (1.44 g, 4.91 mmol) was dissolved in 20 mL of 1,2-dichloroethane. To the solution was added Na_2CO_3 (2.09 g, 19.65 mmol) and 1-chloroethyl chloroformate (ACE-Cl, 2.11 mL, 19.7 mmol), and the mixture was stirred at reflux for 3 h. After cooling to room temperature, the reaction mixture was filtered, and the filtrate was concentrated under reduced pressure. To the residue was

added MeOH (15 mL), and the solution was stirred at room temperature overnight. The volatiles were removed under reduced pressure. The residue was dissolved in H_2O (50 mL), basified with NH_4OH, and extracted with $CHCl_3$. The organic phase was dried over K_2CO_3, concentrated, and purified by column chromatography (5% CMA) to afford 1.19 g (86%) of **21** as a white solid, mp 91−92 °C. $[\alpha]^{25}_D$ −116.7° (*c* 1, MeOH). ^{1}H NMR ($CDCl_3$): δ 7.21 (d, J = 8.5 Hz, 2H), 7.12 (d, J = 8.5 Hz, 2H), 3.74 (m, 2H), 3.40 (s, 3H), 3.21 (m, 1H), 2.72 (m, 1H), 2.39 (m, 1H), 2.20−1.92 (m, 4H), 1.30−1.08 (m, 3H).

***N*-[4-(4-Nitrophenyl)butyl]-2β-Carbomethoxy-3β-(4-chlorophenyl)tropane (22).** Nor-2β-carbomethoxy-3β-(4-chlorophenyl)tropane (**21**, 100 mg, 0.36 mmol) was dissolved in DMF (3 mL). To the solution was added 1-bromo-4-(*p*-nitrophenyl)butane (111 mg, 0.43 mmol) followed by the addition of K_2CO_3 (99 mg, 0.72 mmol). The mixture was stirred overnight and filtered. The volatiles were removed under reduced pressure. The residue was taken into H_2O (10 mL) and extracted with $CHCl_3$ (3 × 10 mL). The organic layer was dried over K_2CO_3 and concentrated. The crude product was purified by column chromatography (1% CMA) to give 153 mg (94%) of product **22**. $[\alpha]^{26}_D$ −22.1° (*c* 1, $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 8.15 (d, J = 8.7 Hz, 2H), 7.33 (d, J = 8.7 Hz, 2H), 7.25−7.15 (m, 4H), 3.68 (m, 1H), 3.43 (s, 3H), 3.38(m, 1H), 2.98−2.87 (m, 2H), 2.71 (t, J = 7.4 Hz, 2H), 2.55 (m, 1H), 2.33−1.98 (m, 4H), 1.74−1.58 (m, 5H), 1.46 (m, 2H). IR: 1745, 1598, 1516, 1344 cm^{-1}. EIMS *m*/*z*: 456 (M^+).

***N*-[4-(4-Aminophenyl)butyl]-2β-carbomethoxy-3β-(4-chlorophenyl)tropane (23).** Compound **22** (1.54 g, 3.38 mmol) was dissolved in a mixture of MeOH (30 mL) and ethyl acetate (30 mL) to which a catalytic amount of Pd/C (10%) was added. The mixture was reduced on a Parr hydrogenator at 40 psi for 3 h and then filtered over Celite. The filtrate was concentrated. The residue was purified by column chromatography (eluting with 3% CMA) to give 1.01 g (70%) of product **23**. $[\alpha]^{23}_D$ −18.1° (*c* 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 7.26−7.17 (m, 4H), 6.97 (d, J = 8.1 Hz, 2H), 6.61 (d, J = 8.1 Hz, 2H), 3.78 (m, 1H), 3.48 (s, 3H), 3.40 (m, 1H), 2.97−2.86 (m, 2H), 2.60−2.48 (m, 3H), 2.33−1.95 (m, 4H), 1.70−1.48 (m, 5H), 1.48−1.32 (m, 2H). EIMS *m*/*z*: 426 (M^+).

***N*-[4-(3-Iodo-4-aminophenyl)butyl]-2β-carbomethoxy-3β-(4-chlorophenyl)tropane (24).** Compound **23** (514 mg, 1.20 mmol) was dissolved in 26 mL of glacial acetic acid. To this reaction mixture, the solution of ICl (215 mg, 1.33 mmol) in glacial acetic acid (4 mL) was added with extreme caution at room temperature over a period of 3 h. Acetic acid was then removed under reduced pressure. The residue was then taken into H_2O (30 mL), basified with NH_4OH to pH 9, and extracted with $CHCl_3$ (3 × 10 mL). The organic layer was dried over K_2CO_3 and concentrated. The residue was purified by column chromatography (1% CMA) to give 399 mg (60%) of product **24** as an oil. $[\alpha]^{24}_D$ −14.8° (*c* 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 7.45 (s, 1H), 7.26−7.15 (m, 4H), 6.94 (d, J = 8.1 Hz, 1H), 6.78 (d, J = 8.1 Hz, 1H), 3.93 (s, 2H), 3.69 (m, 1H), 3.48 (s, 3H), 3.38 (m, 1H), 3.00−2.85 (m, 2H), 2.62−2.45 (m, 3H), 2.35−1.96 (m, 4H), 1.76−1.54 (m, 5H), 1.42 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 25.9, 26.1, 28.6, 29.0, 33.9, 34.1, 34.3, 51.0, 52.8, 53.4, 61.6, 62.8, 84.3, 114.7, 128.0, 128.7, 129.4, 131.4, 138.5, 141.9, 144.5, 171.9.

***N*-[4-(3-Iodo-4-azidophenyl)butyl]-2β-carbomethoxy-3β-(4-chlorophenyl)tropane (25).** Compound **24** (351 mg, 0.63 mmol) was dissolved in acetic acid (2 mL) and H_2O (2 mL). To the solution, $NaNO_2$ (61.3 mg, 0.89 mmol) was added at 0 °C, and the mixture was allowed to stir at 0 °C for 30 min. NaN_3 (60 mg, 0.92 mmol) was added, and the mixture was allowed to stir for another 30 min at 0 °C. The reaction mixture was diluted with water, basified with saturated $NaHCO_3$ solution to pH 9, and extracted with $CHCl_3$ (3 × 10 mL). The combined organic layer was dried over K_2CO_3 and concentrated. The residue was purified by column chromatography (1% CMA) to yield 335 mg (92%) of product **25**. Compound **25** was converted to the HBr salt in MeOH and dried in vacuo, mp 75 °C (dec). $[\alpha]^{25}_D$ −71° (*c* 1.0, MeOH). ^{1}H NMR ($CDCl_3$): δ 7.60 (s, 1H), 7.27−7.16 (m, 4H), 7.06 (d, J = 8.1 Hz, 2H), 3.68 (m, 1H), 3.46 (s, 3H), 3.38 (m, 1H), 3.05−2.88 (m, 2H), 2.63−2.50 (m, 3H), 2.33−2.16 (m, 2H), 2.16−1.90 (m, 2H), 1.78−1.55 (m, 5H), 1.40 (m, 2H). ^{13}C NMR: δ 25.8, 25.9, 28.3, 28.5, 33.7, 34.0, 34.5, 61.3, 72.8, 87.5, 118.0, 127.8, 128.6, 129.5, 131.3, 138.9, 139.6, 141.2, 141.7, 171.7. IR: 2116, 1745 cm^{-1}. Anal. ($C_{25}H_{28}N_4O_2BrClI$·HBr) for C, H, N.

***N*-[4-(4-Isothiocyanatophenyl)butyl]-2β-carbomethoxy-3β-(4-chlorophenyl)tropane (26).** Compound **23** (355 mg, 0.83 mmol) was dissolved in a $CHCl_3$ (50 mL) and aqueous $NaHCO_3$ (315 mg, 20 mL of H_2O) solution. To the vigorously stirring mixture was added 0.083 mL (1.08 mmol) of freshly distilled thiophosgene at 0 °C. The reaction mixture was allowed to stir for 3 h at 0 °C. The two layers were separated, and the aqueous phase was extracted with $CHCl_3$ (3 × 20 mL). The combined organic layer was dried over K_2CO_3 and concentrated. The residue was purified by column chromatography (1% CMA) to afford 340 mg (87%) of product **26**. Compound **26** was converted to the HBr salt in MeOH and dried in vacuo, mp 72 °C (dec). $[\alpha]^{24}_D$ −89.6° (*c* 1.0, MeOH). ^{1}H NMR ($CDCl_3$): δ 7.26−7.10 (m, 8H), 3.68 (m, 1H), 3.46 (s, 3H), 3.38 (m, 1H), 3.05−2.88 (m, 2H), 2.63−2.50 (m, 3H), 2.33−2.16 (m, 2H), 2.16−1.90 (m, 2H), 1.78−1.55 (m, 5H), 1.40 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 26.0, 28.6, 33.8, 34.1, 35.4, 50.9, 52.8, 53.2, 61.4, 62.9, 87.5, 125.6, 128.0, 128.6, 128.7, 129.5, 131.4, 134.5, 141.8, 142.3, 171.8. IR: 2108 (br, s), 1746 (s) cm^{-1}. EIMS *m*/*z*: 478 (M^+). Anal.($C_{26}H_{29}ClN_2O_2S$·HBr) for C, H, N.

Pharmacology. 1. Dopamine Transporter Binding Assay in Rat Caudate Putamen. Male Sprague−Dawley rats (200−250 g, Taconic, Germantown, NY) were decapitated, and their brains were removed to an ice-cooled dish for dissection of the caudate putamen. The tissue was homogenized in 30 volumes 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. 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 (2-β-carbomethoxy-3-β-(4-fluorophenyl)tropane 1,5-naphthalene disulfonate; specific activity 82.4 Ci/mmol, from New England Nuclear, Boston, MA, final concentration 1.5 nM) 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 per 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.

In both saturation and competition experiments, two components of [^{3}H]WIN 35 428 binding were apparent. Analysis of the data 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.

Saturation and displacement data were analyzed by the use of the nonlinear least-squares curve-fitting computer program

LIGAND.[32] 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 a 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.

2. Membrane Preparation. Cells from a mammalian line (HEK 293) expressing the human dopamine transporter (hDAT) were grown in 150 mm tissue culture plates in Dulbecco's modified eagle's medium (supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 90 μg/mL penicillin/streptomycin, and 0.002% G418). Cells were maintained at 37 °C in a 5% CO_2 water-jacketed incubator. To prepare the membranes, the medium was aspirated from plate and cells were washed with KRH buffer (120 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM HEPES, 10 mM D-glucose, pH 7.4). Lysis buffer (2 mM HEPES, 1 mM EDTA) was added, and cells were incubated for 10 min at 4 °C. Membranes were scraped, centrifuged at 31000*g* for 20 min at 4 °C, and the supernatant was discarded.

3. Radioligand Binding Assay in HEK 293 Cells. Radioligand binding was performed on membranes in incubation buffer (100 mM NaCl, 50 mM Tris, pH 8.0) for 1 h at 23 °C with shaking (100 rpm). Each sample was run in triplicate, and the total volume in each tube was 0.5 mL. Bound [^{3}H]-WIN 35 428 (DuPont NEN Research Products, Boston, MA, final concentration 3 nM) was harvested using a Brandel cell harvester through Whatman GF/B filter paper presoaked in 0.3% polyethylenimine, followed by two 5 mL washes with incubation buffer. The filter paper was then incubated with 5 mL of Ultima Gold MV liquid scintillation fluid (Packard) for 10 h, followed by counting for 5 min each with a Beckman LS6500 scintillation counter, at 63% efficiency. Triplicate tubes containing 0.1 mM unlabeled WIN 35 428 were used to determine nonspecific binding of [^{3}H]WIN 35 428.

4. Washout Studies. Washout studies to evaluate the irreversible inhibition of 3 nM [^{3}H]WIN 35 428 binding at hDAT were conducted. Pellets resulting from membrane preparation were resuspended in cold incubation buffer and sonicated for 5 s, and appropriate volumes of compounds **26** and **20** were added to reach a saturating final concentration of 1 μM. Control samples, run in parallel, were combined with an equivalent volume of dH_2O. Binding was carried out for 1 h on ice with shaking (200 rpm). Immediately following the incubation, aliquots were removed from each sample ("pre-wash") and stored on ice. Each was diluted 1:1 with 100 mM KCl and centrifuged at 31000*g* for 20 min at 0 °C. The supernatant was discarded to remove any unreacted compound, and the pellet was resuspended in 100 mM KCl and sonicated for 5 s ("KCl dilution"). An aliquot was removed from each sample and stored on ice. The samples were incubated for 10 min at room temperature with shaking to promote dissociation of reversibly bound material, and centrifuged again. This washing procedure was repeated for a total of two KCl washes. The membrane pellets resulting from washout experiments were resuspended in incubation buffer and sonicated prior to adding them to the test tubes containing 3 nM [^{3}H]WIN 35 428 and incubation buffer.

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