

Dihydrobenzofuran Analogues of Hallucinogens. 3.¹ Models of 4-Substituted (2,5-Dimethoxyphenyl)alkylamine Derivatives with Rigidified Methoxy Groups²

Aaron P. Monte,[‡] Danuta Marona-Lewicka, Matthew A. Parker, David B. Wainscott,[†] David L. Nelson,[†] and David E. Nichols*

Department of Medicinal Chemistry and Molecular Pharmacology, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, Indiana 47907, and Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46285

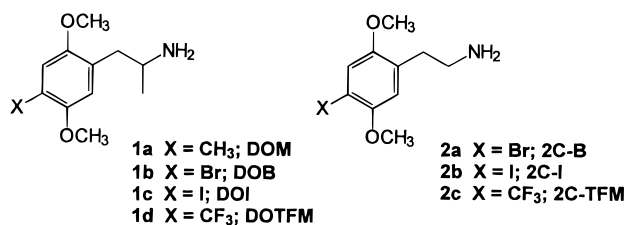
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Tetrahydrobenzodifuran functionalities were employed as conformationally restricted bioisosteres of the aromatic methoxy groups in prototypical hallucinogenic phenylalkylamines **1** and **2**. Thus, a series of 8-substituted 1-(2,3,6,7-tetrahydrobenzo[1,2-*b*:4,5-*b'*]difuran-4-yl)-2-aminoalkanes (**7a–e**) were prepared and evaluated for activity in the two-lever drug discrimination paradigm in rats trained to discriminate saline from LSD tartrate (0.08 mg/kg) and for the ability to displace [³H]ketanserin from rat cortical homogenate 5-HT_{2A} receptors and [³H]-8-OH-DPAT from rat hippocampal homogenate 5-HT_{1A} receptors. In addition, 1-(8-bromo-2,3,6,7-tetrahydrobenzo[1,2-*b*:4,5-*b'*]difuran-4-yl)-2-aminopropane (**7b**), which was found to be extremely potent in the rat *in vivo* assays, was evaluated for its ability to compete with [¹²⁵I]DOI and [³H]ketanserin binding to cells expressing cloned human 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors. All of the dihydrofuranyl compounds having a hydrophobic substituent para to the alkylamine side chain had activities in both the *in vitro* and *in vivo* assays that equaled or surpassed the activity of the analogous conformationally flexible parent compounds. For example, **7b** substituted for LSD in the drug discrimination assay with an ED₅₀ of 61 nmol/kg and had K_i values in the nanomolar to subnanomolar range for the displacement of radioligand from rat and human 5-HT₂ receptors, making it one of the most potent hallucinogen-like phenylalkylamine derivatives reported to date. The results suggest that the dihydrofuran rings in these new analogues effectively model the active binding conformations of the methoxy groups of the parent compounds **1** and **2**. In addition, the results provide information about the topography and relative orientation of residues involved in agonist binding in the serotonin 5-HT₂ receptors.

Introduction

Representative phenylisopropylamines **1a–d** and phenethylamines **2a–c** remain some of the most potent and selective serotonin 5-HT₂ agonists available, having nanomolar affinity for both 5-HT_{2A} and 5-HT_{2C} receptor subtypes.^{3–10} With the exception of the hallucinogenic ergolines such as *d*-lysergic acid *N,N*-diethylamide (LSD), these agents also represent some of the most potent compounds in behavioral assays for hallucinogen-like activity.^{3,10–12} Because we have been interested in exploring the molecular mechanisms of action of hallucinogens for many years,^{13,14} compounds of general structure **1** and **2** seemed logical starting points for further structure–activity relationship (SAR) studies involving a rigid analogue approach to probe the topography of the serotonin receptor agonist binding site(s).

Previous SAR investigations have established that three main structural features of **1** and **2** are required for optimal *in vivo* and *in vitro* hallucinogen-like activity.^{13,14} These are (1) the primary amine functionality separated from the phenyl ring by two carbon atoms, (2) the presence of the 2- and 5-aromatic methoxy groups, and (3) a hydrophobic 4-substituent (alkyl, halo, alkylthio, trifluoromethyl, etc.). The presence of the



methyl group α to the amine in compounds such as **1** does not enhance *in vitro* receptor affinity but does lend increased *in vivo* potency and duration of action to these molecules, possibly due to the inhibition of metabolism by deamination. We have recently suggested that the α -methyl may also increase intrinsic efficacy at the 5-HT_{2A} receptor.¹⁰ Given these basic structural requirements, rigid analogues of **1** and **2** have previously been designed to examine the pharmacological consequences of locking the various functional groups of these molecules into restricted conformations. For example, the aminopropyl side chain of **1a** has been incorporated into aminotetralin and aminoindan rings to restrict mobility of the alkylamine side chain, but these modifications generally lead to inactive compounds.¹⁵

It has been proposed that serine residues are key recognition elements within the ligand binding domain of the serotonin 5-HT_{2A} receptor.¹⁶ Indeed, Westkaemper and Glennon¹⁷ have identified specific serine residues in transmembrane helices 4 and 5 of this receptor that are hypothesized to interact with the 2- and 5-methoxy groups of **1** and **2**. If the heterocyclic oxygen

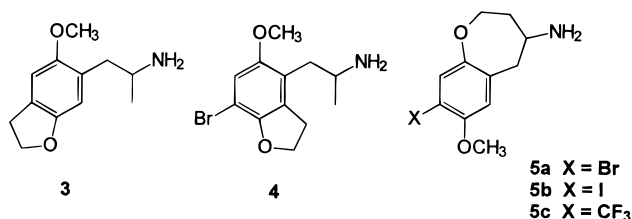
* Author for correspondence. Phone: (317) 494-1461. Fax: (317) 494-6790. E-mail: drdave@pharmacy.purdue.edu.

[†] Eli Lilly and Co.

[‡] Current address: Department of Chemistry, Cowley Hall, University of Wisconsin-La Crosse, La Crosse, WI 54601.

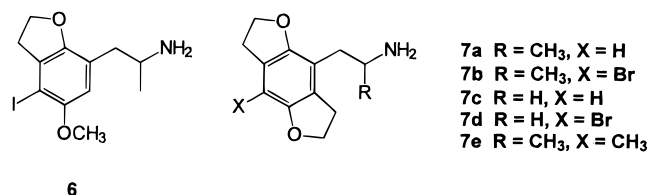
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atoms of compounds such as **3–5** undergo similar H-bonding interactions, these rigid analogues of **1** should offer valuable insight into the location of the putative H-bond donors and into the overall topography of the 5-HT₂ agonist binding site.



We recently began a series of investigations involving **3–5** that would address the binding conformations of the aromatic methoxy groups in this class of compounds. Thus, dihydrobenzofurans **3** and **4** were synthesized and evaluated in assays for hallucinogen-like activity.^{18–20} In these studies, compound **3**, which has the lone pair electrons on the oxygen atom meta to the side chain directed *syn* to the alkylamine chain, had dramatically attenuated LSD-like behavioral effects in rats.¹⁸ In **4**, these electron pairs are directed *anti* to the side chain, and this compound was equipotent to the untethered parent compound **1b**, in both *in vivo* and *in vitro* pharmacological assays.¹⁹ Additionally, the benzoxepins **5a–c** were prepared as rigid analogues of **1b–d** to evaluate the possible active binding conformations of the 2-methoxy group at serotonin receptors.²¹ In that study, **5a–c** all had low affinities for 5-HT_{2A} sites, possibly indicating that the *anti* orientation of the lone pair electrons on the oxygen adjacent to the side chain in these compounds may not be complementary to the agonist binding site and that compounds having the opposite (*syn*) lone pair orientation might provide a better fit to the receptor.

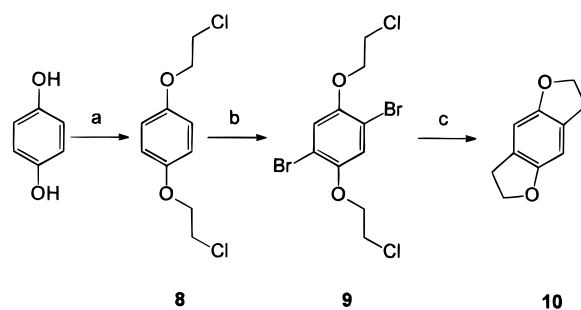
As an extension of those earlier efforts, we present here a series of rigid analogues of **1** and **2** in which the remaining possible 2-methoxy group conformation is explored. Thus **6**, which possesses the *syn* orientation of lone pair electrons on the O adjacent to the side chain ("opposite" to the analogous O2 conformation of compounds **5a–c**), was initially synthesized and shown in preliminary pharmacological screens to be a potent serotonin agonist (unpublished data). These results prompted us to synthesize the highly rigidified difuranyl compounds **7a–e**, which have both aromatic methoxy groups tethered into rotationally restricted dihydrofuran rings. We describe here the synthesis, hallucinogen-like behavioral effects, and rat and human serotonin 5-HT_{2A}, 5-HT_{2C}, and 5-HT_{1A} receptor affinities for these compounds.



Chemistry

In considering the development of the benzodifuran derivatives **7a–e**, it was apparent that each of these target compounds could be prepared from the common

Scheme 1^a



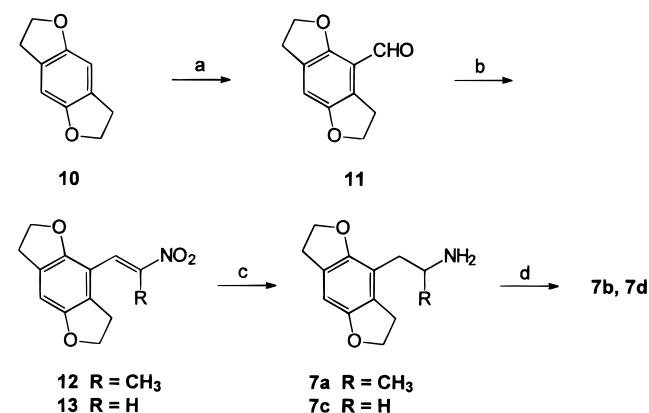
^a (a) BrCH₂CH₂Cl; (b) Br₂, CCl₄; (c) *n*-BuLi, THF, 0 °C.

intermediate 2,3,6,7-tetrahydrobenzo[1,2-*b*:4,5-*b'*]difuran (**10**) (Scheme 2). The synthesis of this simple coumaran was reported to be difficult, and the existing literature method, although it involved only three steps, remained undesirable because it afforded very poor overall yields of **10**.²¹ Thus, our initial goal was to develop an improved synthesis of **10** as a starting point for the construction of the series of (tetrahydrobenzodifuranyl)phenethylamine derivatives.

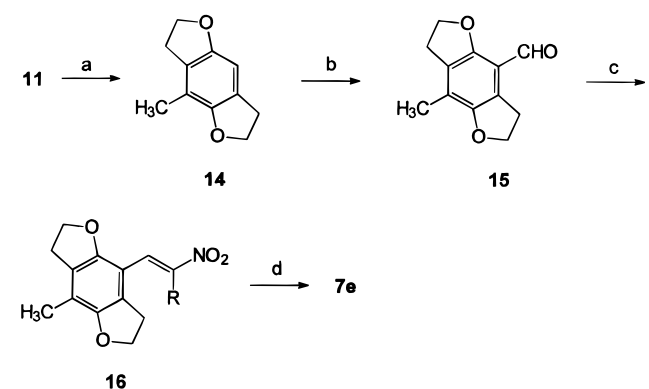
Our synthesis of **10**, shown in Scheme 1, is based on the work of Parham *et al.*,²² who achieved efficient syntheses of benzocyclobutene and indan by lithiation of the appropriate 1-bromo-2-(ω -chloroalkyl)benzenes at –100 °C followed by intramolecular cyclization upon warming to room temperature. Thus, hydroquinone was alkylated with 1-bromo-2-chloroethane and potassium carbonate in acetone to give the bis(2-chloroethyl) ether **8**. This procedure was preferred to the most recent literature preparation of this compound²³ in which isolation of product was hampered by the presence of large amounts of phase-transfer catalyst. Bromination in carbon tetrachloride, a reaction that was readily carried out on a preparative scale, gave the dibromo compound **9**. Cyclization of **9** with 2 equiv of *n*-butyllithium in THF then directly gave the benzodifuran **10** in excellent yield. We also discovered that the lithiation and cyclization could be performed at 0 °C with good results if the *n*-butyllithium was added quickly to a rapidly stirred solution of **9** in THF.

With an efficient synthesis of **10** established, this tricyclic nucleus was readily elaborated using conventional procedures described previously²⁴ to give the desired series of 1-(2,5-dimethoxyphenyl)-2-aminopropane analogues **7a–e**. As shown in Scheme 2, **10** was formylated according to our previously described methods²⁵ to give the 4-formyl compound **11** in good yield. This aldehyde was then condensed with nitroethane, and the intermediate nitropropene **12** was reduced with lithium aluminum hydride to afford **7a**. As there was only one remaining aromatic position, **7a** was easily brominated by treatment with elemental bromine in acetic acid to afford the target compound **7b**.²⁶ Similarly, the aminoethane analogues **7c,d** were also synthesized as shown in Scheme 2. Thus, **11** was condensed with nitromethane to give the nitrostyrene derivative **13** in excellent yield, and this was reduced with lithium aluminum hydride to afford the aminoethane **7c**. Bromination of **7c** in acetic acid²⁶ then gave the desired target compound **7d** in good yield.

The 8-methyl analogue **7e** was constructed as shown in Scheme 3. In this case, the formyl group of **11** was

Scheme 2^a

^a (a) Cl₂CHOCH₃, SnCl₄, CH₂Cl₂; (b) RCH₂NO₂, NH₄OAc, Δ; (c) LiAlH₄, THF, reflux; (d) Br₂, AcOH.

Scheme 3^a

^a (a) H₂ (60 psig), Pd-C, EtOH; (b) Cl₂CHOCH₃, SnCl₄, CH₂Cl₂; (c) RCH₂NO₂, NH₄OAc, Δ; (d) LiAlH₄, THF, HCl.

Table 1. Results of the Drug Discrimination Studies in LSD-Trained Rats and of the Radioligand Competition Experiments at [³H]Ketanserin-Labeled Rat Cortical 5-HT_{2A} and [³H]-8-OH-DPAT-Labeled Rat Hippocampal 5-HT_{1A} Receptors ($K_i \pm$ SEM)

drug	DD Studies			5-HT _{2A} sites (nM)	5-HT _{1A} sites (μM)
	ED ₅₀ (mmol/kg)	95% CI	n ^a		
1b	1.12	0.86–1.46	8–12	22 ± 3	6.1 ± 0.5
4	0.57 ^b	0.29–1.09	—	— ^c	—
7a	64% @ 4.0	PS ^d	10–13	2010 ± 83	29 ± 2
7b	0.061	0.031–0.12	7–15	18 ± 1	4.3 ± 0.4
7c	1.43	0.45–4.5	7–14	2300 ± 170	5.2 ± 0.4
7d	0.31	0.08–1.14	8–12	34 ± 2	0.80 ± 0.06
7e	0.78	0.48–1.22	8–9	NT ^e	NT
LSD	0.040	0.03–0.05	15	4.4 ± 0.2	5.1 ± 0.4 nM

^a Number of animals tested at each dose. ^b Data taken from ref 19. ^c Affinity at [³H]ketanserin sites has not been measured. Affinity at [¹²⁵I]DOI-labeled sites was virtually identical with that of compound 1b.¹⁹ ^d PS = partial substitution. ^e Not tested.

completely reduced to a methyl, and the resulting benzodifuran **14** was reformylated to give the 4-formyl-8-methyl compound **15**. Elaboration of the side chain as before afforded **7e** via its nitropropene **16**.

Pharmacology

Compounds **7a–e** were initially evaluated in the two-lever drug discrimination assay in a group of rats trained to discriminate the effects of ip injections of saline from those of LSD tartrate (0.08 mg/kg) according to methods described previously (Table 1).²⁷ For those compounds that completely substituted for LSD, poten-

cies were measured using ED₅₀ values with 95% confidence intervals (CI). Additionally, **7a–d** were tested for their ability to compete for radioligand binding to 5-HT_{1A} and 5-HT_{2A} receptor sites (Table 1).²⁷ Briefly, the ability of test compounds to compete for 0.75 nM [³H]-8-OH-DPAT at rat hippocampal homogenate binding sites and 0.75 nM [³H]ketanserin in rat frontal cortex homogenate was measured, with the affinities of the compounds for the receptor sites expressed as K_i . **7b**, which was found to be exceptionally potent in the initial behavioral and *in vitro* assays, was also evaluated in cells expressing cloned human 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors that were labeled with agonist or antagonist radioligands (Table 2).

Results and Discussion

The drug discrimination (DD) paradigm is routinely used in our laboratory as an initial screen for evaluating the behavioral activity or hallucinogenic potential of newly synthesized molecules.^{12,28–30} This assay system has been used extensively to model human hallucinogenic effects by studying the LSD-like discriminative stimulus properties produced in animals.^{31,32} Previous DD studies have established that the discriminative behavioral cue is mediated primarily by the stimulation of 5-HT₂ receptors.^{9,29} Thus, agents exhibiting *in vivo* hallucinogenic activity typically can be shown to activate serotonin 5-HT_{2A} and 5-HT_{2C} receptors *in vitro*, and no agonists have yet been found that can significantly differentiate between these subtypes.^{4,7,33}

Table 1 shows the results of the DD studies in LSD-trained rats and the results of the radioligand competition experiments at [³H]ketanserin-labeled and [³H]-8-OH-DPAT-labeled rat 5-HT_{2A} and 5-HT_{1A} receptors, respectively. As expected, the *in vivo* ED₅₀ values of **7a–d** in the DD assay closely parallel the affinities of these compounds for 5-HT_{2A} receptors. None of the compounds had significant affinity for 5-HT_{1A} sites. In agreement with the established hallucinogenic phenyl-alkylamine SAR, the agents not having a hydrophobic substituent para to the alkylamine side chain (**7a,c**) were much less potent than those that did, providing evidence that the benzofuranyl compounds bind to the same agonist site as the untethered parent compounds. Also in line with established SAR requirements, the isopropylamine **7b** was considerably more potent *in vivo* than the phenethylamine **7d**, but these compounds were of similar potencies in the *in vitro* binding assay at 5-HT_{2A} sites.

The most significant findings of the present report are related to the extremely high potencies of benzodifurans **7b,d**, in both the *in vivo* and *in vitro* assays. These results support the idea that the O2 lone pair electron *syn* orientation with respect to the alkylamine side chain is optimal in this class of compounds. Thus, while the benzoxepins **5**, having the *anti* orientation of O2 lone pair electrons, were essentially inactive, analogous compounds having the *syn* orientation of these electrons were quite potent. Since the optimal orientation of the 5-alkoxy group had already been established in our previous study,¹⁹ it was predicted that molecules in which both the 2- and 5-methoxy groups were tethered into “active” conformations using dihydrofuran rings would also be potent serotonin agonists. Thus, **7a–e** were synthesized as novel, highly rigidified analogues

Table 2. Results of the Radioligand Competition Studies of **7b** at Cloned Human 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} Receptors (K_i values in nM $\pm$ SEM)

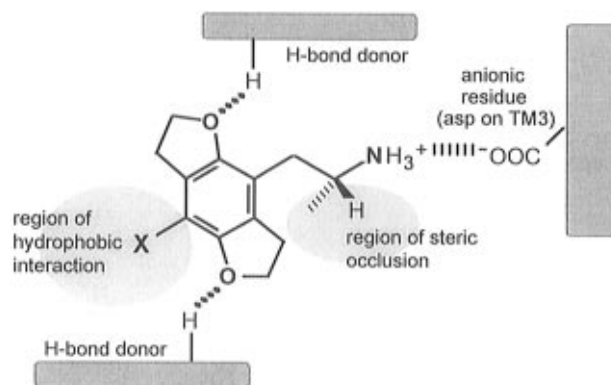
[³ H]antagonist radioligands			agonist radioligands		
ketanserin (5-HT _{2A})	rauwolscine (5-HT _{2B})	mesulergine (5-HT _{2C})	[¹²⁵ I]DOI (5-HT _{2A})	[³ H]-5-HT (5-HT _{2B})	[¹²⁵ I]DOI (5-HT _{2C})
10.73 $\pm$ 0.95	1.15 $\pm$ 0.04	2.28 $\pm$ 0.38	0.48 $\pm$ 0.03	1.60 $\pm$ 0.25	0.30 $\pm$ 0.02

of general structure **1**. Confirming our hypothesis, **7b** was found to be nearly equipotent to LSD in the rat behavioral assay, a result unparalleled by existing substituted phenylalkylamines. Additionally, **7b,d** had nanomolar affinity for rat cortical 5-HT_{2A} receptors, indicating that the benzodifuran structure must be highly complementary to these antagonist-labeled binding sites.

Given the exceptional activity of the benzodifuranyl analogues in the rat assays, the most potent compound of the series (**7b**) was also evaluated in cells expressing cloned human 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptor subtypes. The results of these studies are shown in Table 2. At the agonist-labeled human receptors, **7b** had very high, subnanomolar affinities, while at the antagonist-labeled 5-HT_{2A} site, the K_i value was comparable to the value obtained at the rat receptor.

Consideration of all the pharmacological results provides some definite clues about the topography of the serotonin 5-HT₂ agonist binding site for this series of compounds. If the protonated amine undergoes an electrostatic binding interaction with the conserved aspartate residue in transmembrane helix 3 (TM 3), as suggested in the molecular modeling studies of several G-protein-coupled receptors,^{16,34} the direction of approach of the putative H-bond donors that might interact with O2 and O5 of the prototypical (dimethoxyphenyl)alkylamines can now be established with respect to this aspartate-amine interaction site. At O2 the H-bond donor likely approaches from a direction *syn* to the side chain, and at O5 the donor approaches from a direction *anti* to the side chain, near the para substituent. Also, the importance of the hydrophobic para substituent in activating the 5-HT₂ receptor is further emphasized by the present study. A comparison of the 5-HT_{2A} receptor affinities of **7a** with **7b** and **7c** with **7d** indicates that the brominated analogues are approximately 100 times more potent in this assay than the compounds lacking the hydrophobic substituent. These new SAR data are summarized in the schematic in Figure 1, adapted from the model proposed by Westkaemper and Glennon¹⁷ which illustrates the relative location of proposed binding interactions within the 5-HT₂ agonist site based on the generalized (benzodifuranyl)isopropylamine structure.

In conclusion, we have effectively utilized the dihydrobenzofuran functionality as a rigid conformer of the aromatic methoxy group in hallucinogenic phenylalkylamines. By locking both methoxy groups of this chemical class into "active" conformations using the tetrahydrobenzodifuran nucleus, we have constructed the most potent and selective phenylalkylamine-type serotonin 5-HT₂ agonists yet reported. In so doing, a clearer picture of the topography of the serotonin agonist binding site is emerging. The use of the dihydrobenzofuran functionality may have general applicability in SAR investigations of other drug classes and should serve as a powerful tool in exploring the relative

**Figure 1.** Schematic representation of the 5-HT₂ agonist binding site, modified from the proposal of Westkaemper and Glennon.¹⁷

orientation of the residues within various receptors or enzymes that bind molecules containing aromatic methoxy groups.

Experimental Section

Chemistry. Melting points were determined using a Thomas-Hoover apparatus and are uncorrected. ¹H-NMR spectra were recorded using either a 500 MHz Varian VXR-500S or a 300 MHz Bruker ARX-300 NMR spectrometer. Chemical shifts are reported in δ values ppm relative to tetramethylsilane (TMS) as an internal reference (0.03%, v/v). Abbreviations used in NMR analyses are as follows: s = singlet, d = doublet, t = triplet, dd = doublet of doublets, dt = doublet of triplets, td = triplet of doublets, q = quartet, p = pentet, m = multiplet, b = broad, Ar = aromatic, cp = cyclopropyl. Chemical ionization mass spectra (CIMS) using methane as the carrier gas were obtained with a Finnigan 4000 spectrometer. IR measurements were taken with a Perkin-Elmer 1600 Series FTIR spectrophotometer. Elemental analyses were performed by the Purdue University Microanalysis Laboratory and are within $\pm 0.4\%$ of the calculated values unless otherwise noted. Thin-layer chromatography (TLC) was typically performed using Baker-flex silica gel IB2-F, plastic-backed plates with fluoresecent indicator (2.5 $\times$ 7.5 cm; J. T. Baker), eluting with CH₂Cl₂, and visualizing with UV light at 254 nm and/or I₂ vapor unless otherwise noted. Plates used for radial centrifugal chromatography (Chromatotron; Harrison Research, Palo Alto, CA) were prepared from silica gel 60 PF2-54 containing gypsum. Most reactions were carried out under an inert atmosphere of dry nitrogen.

1,4-Bis(2-chloroethoxy)benzene (8).²³ A mixture of 50 g (0.455 mol) of hydroquinone, 196 g (1.37 mol) of 1-bromo-2-chloroethane, 190 g (1.37 mol) of finely powdered anhydrous potassium carbonate, and 300 mL of acetone was stirred and heated at reflux under N₂ for 24 h. Acetone and excess dihaloethane were removed *in vacuo*, and the residue was partitioned between Et₂O and H₂O. The Et₂O phase was extracted three times with 4 M NaOH, dried with anhydrous MgSO₄, and filtered. Solvent was removed *in vacuo*, yielding 33.7 g (32%) of a white solid with a ¹H-NMR spectrum identical with that reported in ref 23.

1,4-Bis(2-chloroethoxy)-2,5-dibromobenzene (9). To a stirred suspension of 1.0 g (4.26 mmol) of the bis-ether **8** in 20 mL of CCl₄ was added 12 mg (0.21 mmol) of Fe granules. Bromine (1.50 g, 9.37 mmol) in 5 mL of CCl₄ was added dropwise, and the reaction mixture was stirred for 4.5 h at room temperature. The mixture was washed twice with H₂O,

dried with MgSO_4 , and filtered. The solvent was removed *in vacuo*, yielding 1.54 g (92%) of an off-white solid. An analytical sample was recrystallized from EtOH: mp 119 °C; ^1H NMR (CDCl_3) δ 3.83 (t, 4, $\text{ArOCH}_2\text{CH}_2\text{Cl}$, $J = 6$ Hz), 4.23 (t, 4, $\text{ArOCH}_2\text{CH}_2\text{Cl}$, $J = 6$ Hz), 7.15 (s, 2, ArH).

2,3,6,7-Tetrahydrobenzo[1,2-*b*:4,5-*b'*]difuran (10).²¹ A solution of 10 g (25.4 mmol) of the dibromo bis-ether **9** in 250 mL of dry THF was placed in a N_2 atmosphere and cooled to 0 °C. A solution of 2.5 M *n*-butyllithium in hexanes (21.4 mL, 2.1 equiv) was added very quickly (addition time: 7 s) to the rapidly stirred solution using a syringe with a large gauge needle. (Fast addition of *n*-BuLi is critical. An otherwise identical run in which this reagent was added over 40 s resulted in a 25% reduction in yield.) The reaction mixture was stirred for 10 min, and solvent was removed *in vacuo*. The residue was partitioned between Et_2O and H_2O , and the organic phase was dried with MgSO_4 and filtered. The resulting solution was evaporated *in vacuo* until crystallization occurred. The crystals were filtered off, the filtrate was again evaporated, and a second crop of crystals was collected. After executing this procedure a third time, a total of 3.30 g (80%) of off-white crystals was obtained. Recrystallization from ether gave fluffy white crystals: mp 155–156 °C (lit.²¹ mp 153 °C); ^1H NMR (CDCl_3) δ 3.13 (t, 4, $\text{ArOCH}_2\text{CH}_2$, $J = 8.5$ Hz), 4.52 (t, 4, $\text{ArOCH}_2\text{CH}_2$, $J = 8.5$ Hz), 6.63 (s, 2, ArH); CIMS m/z 163 ($M + 1$).

4-Formyl-2,3,6,7-tetrahydrobenzo[1,2-*b*:4,5-*b'*]difuran (11). A solution of 3.0 g (0.019 mol) of the tetrahydrobenzodifuran **10** in 100 mL of dry CH_2Cl_2 was stirred under N_2 and cooled over an ice bath. Tin(IV) chloride (2.81 mL, 0.024 mol) was added to the solution, and the mixture was stirred for 5 min. Dichloromethyl methyl ether (1.67 mL, 0.019 mol) in 5 mL of CH_2Cl_2 was then introduced into the mixture dropwise over a 5 min period. After the mixture had stirred for 30 min, the reaction was quenched by the addition of 50 mL of ice- H_2O , and the layers were separated. The aqueous phase was extracted with 2×30 mL of CH_2Cl_2 , and the organic fractions were combined. The organic extract was washed with 3 N HCl (3×100 mL), 100 mL of H_2O , and 100 mL of brine, dried over MgSO_4 , and filtered through a pad of Celite and silica gel. Removal of solvent under reduced pressure and drying under high vacuum gave 2.9 g (82%) of a yellow oil that spontaneously crystallized upon standing. The solid was recrystallized from chloroform-hexane to yield 2.51 g (71%) of pure product **11** as light yellow crystals: mp 86–87 °C; ^1H NMR (CDCl_3) δ 3.20 (td, 2, $\text{ArOCH}_2\text{CH}_2$, $J = 8.6$ Hz, 1.1 Hz), 3.45 (t, 2, $\text{ArOCH}_2\text{CH}_2$, $J = 8.8$ Hz), 4.60 (t, 2, $\text{ArOCH}_2\text{CH}_2$, $J = 8.8$ Hz), 4.70 (t, 2, $\text{ArOCH}_2\text{CH}_2$, $J = 8.8$ Hz), 6.80 (s, 1, ArH), 10.25 (s, 1, ArCHO); CIMS m/z 191 ($M + 1$), Anal. ($\text{C}_{11}\text{H}_{10}\text{O}_3$) C, H.

4-(2-Nitro-1-propenyl)-2,3,6,7-tetrahydrobenzo[1,2-*b*:4,5-*b'*]difuran (12). The aldehyde **11** (2.3 g, 0.012 mol) and ammonium acetate (0.93 g, 0.012 mol) were dissolved in 10 mL of nitromethane and stirred under a nitrogen atmosphere at 90 °C for 15 h. The volatiles were removed *in vacuo*, and the residue was taken up in ether-ethyl acetate and washed with 3×50 mL of 3 M HCl, 3×50 mL of 5% NaHCO_3 , and 50 mL of brine. The organic phase was dried over MgSO_4 and filtered through Celite-silica gel, and the solvent was removed under reduced pressure to yield a thick red oil. The oil was purified on the Chromatotron (4 mm silica plate, CH_2Cl_2) to give 1.74 g (60%) of the desired product **12** as an orange oil. The oil was crystallized from methanol to give yellow-orange crystals: mp 94 °C; ^1H NMR (CDCl_3) δ 2.24 (s, 3, CH_3), 3.10 (t, 2, $\text{ArOCH}_2\text{CH}_2$, $J = 8.6$ Hz), 3.20 (t, 2, $\text{ArOCH}_2\text{CH}_2$, $J = 8.7$ Hz), 4.60 (t, 4, $\text{ArOCH}_2\text{CH}_2$, $J = 8.6$ Hz), 6.73 (s, 1, ArH), 7.80 (s, 1, ArCH=C); CIMS m/z 248 ($M + 1$), 230, 191. Anal. ($\text{C}_{13}\text{H}_{11}\text{NO}_4$) C, H, N.

1-(2,3,6,7-Tetrahydrobenzo[1,2-*b*:4,5-*b'*]difuran-4-yl)-2-aminopropane Hydrochloride (7a). A solution of 0.75 g (3.03 mmol) of the nitropropene **12** in 25 mL of dry THF was added dropwise to a stirred suspension of 0.31 g (7.58 mmol) of LiAlH_4 in 50 mL of dry THF under N_2 . The reaction mixture was heated at reflux over an oil bath for 3.5 h and then cooled on an ice bath and the reaction quenched by the cautious addition of 2 mL of water in 10 mL of THF. Celite and 5 mL

of 5 N KOH were added, and the mixture was filtered through Celite, rinsing the filter cake well with ether and CH_2Cl_2 . The volatiles were removed on the rotary evaporator, and the residue was taken up in 100 mL of ether and extracted with 4×20 mL of 3 N HCl. The aqueous extracts were combined, made strongly basic with 5 N KOH, and extracted with 5×20 mL of CH_2Cl_2 . The organic extracts were combined, washed with brine, dried (MgSO_4), and filtered through Celite. Removal of solvent under reduced pressure gave 0.56 g (85%) of the free amine **8** as a pale yellow oil. The hydrochloride salt was formed by taking up the oil in ether and adding 1 N HCl in anhydrous ethanol. Removal of solvent and crystallization from ethanol-ethyl acetate afforded 0.49 g (64%) of **7a**·HCl as a white, crystalline solid: mp 267–269 °C; ^1H NMR (free base in CDCl_3) δ 1.16 (d, 3, $\text{ArCH}_2\text{CHCH}_3$, $J = 6.3$ Hz), 1.55 (bs, 2, NH_2), 2.58 (m, 2, $\text{ArCH}_2\text{CHCH}_3$), 3.13 (q, 4, $\text{ArOCH}_2\text{CH}_2$), 3.22 (m, 1, $\text{ArCH}_2\text{CHCH}_3$), 4.50 (q, 4, $\text{ArOCH}_2\text{CH}_2$), 6.52 (s, 1, ArH); CIMS m/z 220 ($M + 1$), 203, 176. Anal. ($\text{C}_{13}\text{H}_{17}\text{NO}_2\text{HCl}$) C, H, N.

1-(8-Bromo-2,3,6,7-tetrahydrobenzo[1,2-*b*:4,5-*b'*]difuran-4-yl)-2-aminopropane Hydrochloride (7b). A mixture of 200 mg (0.91 mmol) of **7a**·HCl in 4 mL of glacial acetic acid was stirred under N_2 , and 0.51 mL of 1.79 N Br_2 in HOAc solution was added dropwise. After 5 min the salt had completely dissolved to give a clear orange solution. Subsequently, a precipitate began to form, and after 1.5 h the volatiles were removed on the rotary evaporator. The solid residue was taken up in 3 N HCl and washed with 2×20 mL of ether. The aqueous phase was made strongly basic by the addition of 5 N KOH and extracted with 5×15 mL of CH_2Cl_2 . The organic fractions were combined, washed with brine, dried (MgSO_4), and filtered through Celite. Removal of solvent under reduced pressure gave 199 mg (67%) of the free amine as a pale yellow oil. The hydrochloride salt was formed by the addition of 1 N HCl in anhydrous ethanol, and, after solvent removal, this was crystallized from ethanol-ethyl acetate to yield **7b**·HCl as a white crystalline solid: mp 244–245 °C; ^1H NMR (free base in CDCl_3) δ 1.16 (d, 3, $\text{ArCH}_2\text{CHCH}_3$, $J = 6.3$ Hz), 1.78 (bs, 2, NH_2), 2.51 (t, 2, $\text{ArCH}_2\text{CHCH}_3$), 3.23 (m, 4, $\text{ArOCH}_2\text{CH}_2$), superimposed upon 3.25 (m, 1, $\text{ArCH}_2\text{CHCH}_3$), 4.60 (t, 2, $\text{ArOCH}_2\text{CH}_2$, $J = 8.8$ Hz), 4.65 (t, 2, $\text{ArOCH}_2\text{CH}_2$, $J = 8.8$ Hz); CIMS m/z 298 ($M + 1$), 281, 254, 218. Anal. ($\text{C}_{13}\text{H}_{16}\text{NO}_2\text{BrHCl}$) C, H, N.

4-(2-Nitro-1-ethenyl)-2,3,6,7-tetrahydrobenzo[1,2-*b*:4,5-*b'*]difuran (13). A mixture of 3.2 g (0.017 mol) of the aldehyde **11**, 1.3 g (0.017 mol) of ammonium acetate, and 8 mL of nitromethane was stirred under nitrogen while heating over a 96 °C oil bath for 1 h. The nitromethane was then removed under reduced pressure, and the solid residue was partitioned between CH_2Cl_2 and water. The layers were separated, and the organic phase was washed with 3×50 mL of 3 N HCl, 2×50 mL of water, and 50 mL of brine. The ether solution was dried over MgSO_4 and filtered through Celite, and the solvent was removed on the rotary evaporator to give 3.7 g (94%) of a bright red-orange solid. The solid was recrystallized from methanol to give 3.5 g (91%) of the nitroethene **13** as pumpkin-orange needles: mp 134 °C; ^1H NMR (CDCl_3) δ 3.25 (t, 2, $\text{ArOCH}_2\text{CH}_2$, $J = 8.7$ Hz), 3.28 (t, 2, $\text{ArOCH}_2\text{CH}_2$, $J = 8.7$ Hz), 4.73 (t, 2, $\text{ArOCH}_2\text{CH}_2$, $J = 8.7$ Hz), 4.75 (t, 2, $\text{ArOCH}_2\text{CH}_2$, $J = 8.7$ Hz), 6.76 (s, 1, ArH), 7.85 (d, 1, $\text{ArCH}=\text{CH}$, $J = 13.4$ Hz), 8.05 (d, 1, $\text{ArCH}=\text{CHNO}_2$, $J = 13.5$ Hz); CIMS m/z 234 ($M + 1$), 216. Anal. ($\text{C}_{12}\text{H}_{11}\text{NO}_4$) C, H, N.

1-(2,3,6,7-Tetrahydrobenzo[1,2-*b*:4,5-*b'*]difuran-4-yl)-2-aminoethane hydrochloride (7c). In a manner identical with that for the reduction of compound **12** above, 3.0 g (0.013 mol) of nitroethene **13** in 100 mL of dry THF was added to a stirred suspension of 1.31 g (0.032 mol) of LiAlH_4 in 200 mL of THF and heated at reflux for 2 h. After standard workup, 1.7 g (69%) of the free amine **7c** was obtained as a yellow oil. This was precipitated as its hydrochloride salt with the addition of 1 N HCl in anhydrous ethanol and recrystallized from ethanol to yield 1.68 g (54%) of **7c**·HCl as a white crystalline solid: mp 295–296 °C dec; ^1H NMR (free base in CDCl_3) δ 1.48 (bs, 2, NH_2), 2.75 (t, 2, $\text{ArCH}_2\text{CH}_2\text{N}$, $J = 6.95$ Hz), 2.95 (t, 2, $\text{ArCH}_2\text{CH}_2\text{N}$, $J = 6.95$ Hz), 3.15 (q, 4, $\text{ArOCH}_2\text{CH}_2$, $J = 7.4$ Hz), 4.58 (q, 4, $\text{ArOCH}_2\text{CH}_2$, $J = 8.5$ Hz),

6.58 (s, 1, ArH); CIMS m/z 206 ($M + 1$), 189, 176. Anal. ($C_{12}H_{15}NO_2 \cdot HCl$) C, H, N.

1-(8-Bromo-2,3,6,7-tetrahydrobenzo[1,2-*b*:4,5-*b'*]difuran-4-yl)-2-aminoethane Hydrochloride (7d). A solution of 0.51 g (2.48 mmol) of the free amine **7c** in 6 mL of glacial acetic acid was treated with 2.80 mL of a 0.885 N Br_2 in HOAc solution. The bromine color rapidly dissipated, and the hydrobromide salt precipitated after 15 min. The mixture was stirred for 2 h and then diluted with dry ether. The precipitate was collected by suction filtration, rinsed well with ether on the filter, and dissolved in 75 mL of 3 N HCl. The acidic solution was washed with 3×50 mL of CH_2Cl_2 and then made strongly basic with the addition of 5 N KOH. The basic phase was extracted with 4×30 mL of CH_2Cl_2 , and the extracts were combined and washed with brine. After drying ($MgSO_4$) and filtration through Celite, the solvent was removed under reduced pressure to afford 0.52 g (73%) of the free amine **7d** as a yellow oil. The hydrochloride salt was precipitated from an ether solution by adding 1 N HCl in anhydrous ethanol. The salt was freed of solvent under reduced pressure and recrystallized from ethanol–ethyl acetate to give the desired product **7d**·HCl as a white crystalline solid: mp $>310^\circ C$; 1H NMR (free base in $CDCl_3$) δ 1.52 (bs, 2, NH_2), 2.65 (t, 2, $ArCH_2CH_2N$, $J = 6.9$ Hz), 2.90 (t, 2, $ArCH_2CH_2N$, $J = 6.9$ Hz), 3.20 (m, 4, $ArOCH_2CH_2$), 4.60 (pair of superimposed triplets, 4, $ArOCH_2CH_2$); CIMS m/z 284, 286 ($M + 1$), 267, 269, 254, 256, 247. Anal. ($C_{12}H_{14}NO_2 \cdot Br \cdot HCl$) C, H, N.

4-Methyl-2,3,6,7-tetrahydrobenzo[1,2-*b*:4,5-*b'*]difuran (14). To a suspension of 1.0 g of 10% Pd–C in 250 mL of absolute ethanol in a Parr hydrogenation flask was added 7.0 g (0.037 mol) of the benzaldehyde **11**. The mixture was shaken under 60 psi of H_2 for 24 h and then filtered through Celite to remove the catalyst. Evaporation of the solvent under reduced pressure and recrystallization of the white, solid residue from ethyl acetate–hexane gave 6.18 g (95%) of pure product **14** as fluffy, white crystals: mp $78^\circ C$; 1H NMR ($CDCl_3$) δ 2.12 (s, 3, $ArCH_3$), 3.07 (t, 2, $ArOCH_2CH_2$, $J = 8.7$ Hz), 3.13 (t, 2, $ArOCH_2CH_2$, $J = 8.7$ Hz), 4.53 (m, 4, $ArOCH_2CH_2$), 6.62 (s, 1, ArH); CIMS m/z 177 ($M + 1$). Anal. ($C_{11}H_{12}O_2$) C, H.

4-Formyl-8-methyl-2,3,6,7-tetrahydrobenzo[1,2-*b*:4,5-*b'*]difuran (15). To an ice–salt bath-cooled solution of 2.60 g (0.015 mol) of **14** in 30 mL of dry CH_2Cl_2 was added 2.24 mL (0.019 mol) of tin(IV) chloride via syringe through septum. The mixture was stirred under N_2 for 5 min, and 1.33 mL (0.015 mol) of α,α -dichloromethyl methyl ether was added dropwise over 5 min. The mixture was stirred for 5 min more and then the reaction quenched with the addition of 50 mL of ice–water. The layers were separated, and the aqueous phase was extracted with 3×20 mL of CH_2Cl_2 . The combined organic extracts were washed with 3×50 mL of 6 N HCl, 2×50 mL of 5% sodium bicarbonate, and brine, dried over $MgSO_4$, and filtered through a pad of Celite and silica gel. Removal of solvent under reduced pressure gave a bright yellow solid that weighed 2.99 g (99%). The solid was recrystallized from ethyl acetate–hexane to give 2.82 g (94%) of the product **15** as yellow needles: mp $152^\circ C$; 1H NMR ($CDCl_3$) δ 2.18 (s, 3, $ArCH_3$), 3.09 (t, 2, $ArOCH_2CH_2$, $J = 8.6$ Hz), 3.45 (t, 2, $ArOCH_2CH_2$, $J = 8.7$ Hz), 4.57 (t, 2, $ArOCH_2CH_2$, $J = 8.7$ Hz), 4.70 (t, 2, $ArOCH_2CH_2$, $J = 8.7$ Hz), 10.21 (s, 1, $ArCHO$); CIMS m/z 205 ($M + 1$). Anal. ($C_{12}H_{12}O_3$) C, H.

8-Methyl-4-(2-nitro-1-propenyl)-2,3,6,7-tetrahydrobenzo[1,2-*b*:4,5-*b'*]difuran (16). The aldehyde **15** (1.0 g, 4.90 mmol) was stirred with 0.40 g (4.90 mmol) of ammonium acetate in 5 mL of nitroethane over an $80^\circ C$ oil bath for 19 h. The volatiles were removed on the rotary evaporator, and the orange residue was taken up in ether and washed with 4×30 mL of 3 N HCl, 2×30 mL of 5% $NaHCO_3$, and brine. The organic phase was dried over $MgSO_4$, filtered through a pad of Celite–silica gel, and concentrated on the rotary evaporator to give 1.2 g of an impure (by TLC) orange gum. The crude material was purified on the Chromatotron (4 mm silica plate, CH_2Cl_2) to afford 0.60 g (46%) of pure nitropropene **16** as a bright, pumpkin-orange solid that was crystallized from methanol: mp $105^\circ C$; 1H NMR ($CDCl_3$) δ 2.12 (s, 3, $ArCH=CCH_3$), 2.22 (s, 3, $ArCH_3$), 3.11 (m, 4, $ArOCH_2CH_2$), 4.59 (t, 2, $ArOCH_2CH_2$, $J = 8.6$ Hz) superimposed upon 4.60

(t, 2, $ArOCH_2CH_2$, $J = 8.6$ Hz), 7.83 (s, 1, $ArCH=C$); CIMS m/z 262 ($M + 1$), 244, 220, 205. Anal. ($C_{14}H_{15}NO_4$) C, H, N.

1-(8-Methyl-2,3,6,7-tetrahydrobenzo[1,2-*b*:4,5-*b'*]difuran-4-yl)-2-aminopropane Hydrochloride (7e). The nitropropene **16** (0.50 g, 1.90 mmol) was dissolved in 40 mL of dry THF and added dropwise over 40 min to a stirred slurry of 0.20 g (4.80 mmol) of $LiAlH_4$ in 75 mL of dry THF. The mixture was heated at reflux over an oil bath for 3 h, and the reaction was quenched by the cautious addition of 10 mL of 5 N KOH while cooling over an ice bath. The reaction mixture was filtered through a Celite pad, rinsing the filter cake well with dichloromethane. The volatiles were removed on the rotary evaporator, and the aqueous residue was taken up in ether and extracted with 4×20 mL of 3 N HCl. The extracts were combined and made strongly basic by the addition of 5 N KOH while cooling the mixture on an ice bath. The basic phase was extracted with 4×20 mL of CH_2Cl_2 , and the combined extracts were washed with brine, dried ($MgSO_4$), and filtered through Celite. Removal of the solvent *in vacuo* afforded 0.39 g (88%) of the free base **7e** as a yellow oil. The base was converted to its hydrochloride salt by adding exactly 1 equiv of HCl as an anhydrous 1 M solution in ethanol. The salt was recrystallized from ethanol–ethyl acetate to give **7e**·HCl as very fluffy, white crystals: mp 275 – $276^\circ C$; 1H NMR (free base in $CDCl_3$) δ 1.14 (d, 3, $ArCH_2CHCH_3$, $J = 6.3$ Hz), 1.48 (bs, 2, NH_2), 2.10 (s, 3, $ArCH_3$), 2.52 (m, 2, $ArCH_2CHCH_3$), 3.19 (m, 4, $ArOCH_2CH_2$), 3.23 (m, 1, $ArCH_2CHCH_3$), 4.55 (pair of nearly superimposed triplets, 4, $ArOCH_2CH_2$, $J = 8.7$ Hz); CIMS m/z 234 ($M + 1$), 217, 190. Anal. ($C_{14}H_{19}NO_2 \cdot HCl$) C, H, N.

Pharmacology Methods: Drug Discrimination Studies. The procedures for the drug discrimination assays were essentially as described in previous reports.^{10,27} Twenty male Sprague–Dawley rats (Harlan Laboratories, Indianapolis, IN) weighing 200–220 g at the beginning of the drug discrimination study were used as subjects trained to discriminate LSD tartrate from saline. None of the rats had previously received drugs or behavioral training. Water was freely available in the individual home cages, and a rationed amount of supplemental feed (Purina Lab Blox) was made available after experimental sessions to maintain approximately 80% of free-feeding weight. Lights were on from 0700 to 1900. The laboratory and animal facility temperature was 22 – $24^\circ C$, and the relative humidity was 40–50%. Experiments were performed between 0830 and 1700 each day, Monday–Friday.

Six standard operant chambers (Model E10–10RF; Coulbourn Instruments, Lehigh Valley, PA) consisted of modular test cages enclosed within sound-attenuated cubicles with fans for ventilation and background white noise. A white house light was centered near the top of the front panel of the cage, which was also equipped with two response levers, separated by a food hopper (combination dipper–pellet trough, Model E14-06, module size 1/2), all positioned 2.5 cm above the floor. Solid state logic in an adjacent room, interfaced through a Med Associates interface to a 486-based microcomputer, controlled reinforcement and data acquisition with a locally written program.

A fixed ratio (FR) 50 schedule of food reinforcement (Bioserv 45 mg dustless pellets) in a two-lever paradigm was used. The drug discrimination procedure details have been described elsewhere.^{35,36} After habituation to the experimental conditions (1 week after isolation in the individual home cages and at the beginning of the food deprivation), the rats' initial shaping was started. During the first two to three sessions, rats were trained only to associate a characteristic noise (click) after lever pressing with a delivered food pellet (without drug injections). Initially, rats were shaped to lever press on an FR1 schedule so that one food pellet was dispensed for each press. One-half of the rats were trained on drug-L (left), saline-R (right) and the other one-half on drug-R, saline-L to avoid positional preference. Training sessions lasted 15 min and were conducted at the same time each day. Levers were cleaned between animals with 10% ethanol solution to avoid olfactory cues. Only one appropriate lever was present during the first 10 sessions of initial learning (after beginning to administer saline or training drug ip 30 min before sessions).

Afterwards both levers were present during all following phases of training, but reinforcements were delivered only after responses on the appropriate lever. Presses on the incorrect lever had no programmed consequences. As responding rates stabilized (during the next 15 sessions), the schedule of reinforcement was gradually increased to an FR50. Once at the FR50, training continued until an accuracy of at least 85% (no. of correct presses $\times$ 100/no. of total presses) was attained for 8 of 10 consecutive sessions (approximately 40–60 sessions). Once criterion performance was attained, test sessions were interspersed between training sessions, either one or two times per week. At least one drug and one saline session separated each test session. Rats were required to maintain the 85% correct responding criterion on training days in order to be tested. In addition, test data were discarded when the accuracy criterion of 85% was not achieved on the two training sessions following a test session. Test drugs were administered ip 30 min prior to the sessions; test sessions were run under conditions of extinction, with rats removed from the operant chamber when 50 presses were emitted on one lever. If 50 presses on one lever were not completed within 5 min, the session was ended and scored as a disruption. Treatments were randomized at the beginning of the study.

The training drug was (+)-lysergic acid diethylamide tartrate (LSD; 0.08 mg/kg, 186 nmol/kg; NIDA). All drugs were dissolved in 0.9% saline and were injected intraperitoneally in a volume of 1 mL/kg 30 min before the sessions.

Data from the drug discrimination study were scored in a quantal fashion, with the lever on which the rat first emitted 50 presses in a test session scored as the "selected" lever. The percentage of rats selecting the drug lever (% SDL) for each dose of test compound was determined. The degree of substitution was determined by the maximum % SDL for all doses of the test drug. "No substitution" is defined as 59% SDL or less, and "partial" substitution is 60–79% SDL. If the drug was one which completely substituted for the training drug (at least one dose resulted in a % SDL = 80% or higher), the method of Litchfield and Wilcoxon³⁷ was used to determine the ED₅₀ (log–probit analysis as the dose producing 50 percent drug–lever responding) and 95% confidence interval (95% CI). This method also allowed for tests of parallelism between dose–response curves of the drug and the training drug. If 50% or more of the animals tested were disrupted at a dose where the nondisrupted rats gave 80% SDL, no ED₅₀ was calculated.

Radioligand Competition Assays in Rat Brain Homogenate. Male Sprague–Dawley rats (Harlan Laboratories, Indianapolis, IN) weighing 175–199 g were used. The animals were kept in groups of 5 rats/cage, at the same conditions described above, but with free access to food and water.

[³H]Ketanserin and [³H]-8-OH-DPAT were purchased from New England Nuclear (Boston, MA) at specific activities of 61 and 135.5–216 Ci/mmol, respectively. (+)-LSD tartrate was obtained from the National Institute on Drug Abuse. Cinanserin was a gift from the SQUIBB Institute for Medical Research, and 5-HT was purchased from Sigma (St. Louis, MO).

The procedures of Johnson *et al.*³⁸ were employed. Briefly, the frontal cortex or hippocampal brain regions from 20 to 40 rats were pooled and homogenized (Brinkman Polytron, setting 6 for 2 $\times$ 20 s) in 4 or 8 vol of 0.32 M sucrose for frontal cortex or hippocampus, respectively. The homogenates were centrifuged at 36000g for 10 min, and the resulting pellets were resuspended in the same volume of sucrose. Separate aliquots of tissue suspension were then frozen at –70 °C until assay.

For each separate experiment, a tissue aliquot was thawed slowly and diluted 1:25 with 50 mM Tris HCl (pH = 7.4). The homogenate was then incubated at 37 °C for 10 min and centrifuged twice at 36500g for 20 min with an intermittent wash. The resulting pellet was resuspended in 50 mM Tris HCl with 0.5 mM Na₂EDTA, 0.1% Na ascorbate, and 10 mM pargyline HCl (pH = 7.4). In experiments with [³H]ketanserin, either 10 mM MgCl₂ or 5.7 mM CaCl₂ was included, respectively. A second preincubation for 10 min at 37 °C was conducted, and the tissues were then cooled in an ice bath.

All experiments were performed with triplicate determinations using the appropriate buffer to which 200–400 μ g of protein was added, giving a final volume of 1 mL. The tubes were allowed to equilibrate for 15 min at 37 °C before filtering through Whatman GF/C filters using a cell harvester (Brandel, Gaithersburg, MD) followed by two 5 mL washes using ice-cold Tris buffer. Specific binding was defined as that displaceable with 10 μ M cinanserin in the [³H]ketanserin binding study and with 10 μ M 5-HT in the [³H]-8-OH-DPAT binding study. Filters were air-dried, placed into scintillation vials with 10 mL of Ecolite scintillation cocktail, allowed to sit overnight before counting at an efficiency of 37% for tritium, and directly counted in a gamma counter for [¹²⁵I]ligand at an efficiency of 79.4%.

Radioligand Competition Experiments Using Cloned Human Receptors. All chemicals were obtained from the sources previously described.³⁹ [³H]-5-HT was purchased from DuPont-NEN (Wilmington, DE) or Amersham Corp. (Arlington Heights, IL) at 22.8–26.7 or 81–91 Ci/mmol, respectively. [¹²⁵I]-DOI (2200 Ci/mmol), [³H]rauwolscine (70–90 Ci/mmol), and [³H]ketanserin (60–78.7 Ci/mmol) were purchased from DuPont-NEN (Wilmington, DE).

Membrane Preparation from Transformed Cell Lines. Membranes were prepared essentially as previously described³⁹ using AV12 cell lines (Syrian hamster fibroblast, ATCC no. CRL 9595) stably transformed with the human 5-HT_{2A}, 5-HT_{2B}, or 5-HT_{2C} receptor. In brief, cells expressing the receptor of interest were grown in suspension and harvested by centrifugation. The cell pellets were then resuspended in a minimal volume of a hypotonic buffer, 50 mM Tris HCl, pH 7.4, and frozen at –70 °C until needed. On the day of the assay, the membrane suspension was thawed and diluted to 35 mL/0.5 $\times$ 10⁹ cells, original cell number, with 50 mM Tris HCl, pH 7.4. The combination of hypotonic buffer and vortexing was sufficient to lyse the cells for the membrane preparation. After vortexing, the preparation was centrifuged at 39000g for 10 min at 4 °C, and the resulting membrane pellet was resuspended and incubated at 37 °C for 10 min and then centrifuged at 39000g for 10 min at 4 °C. This pellet was resuspended and centrifuged one more time, and the final membrane pellet was resuspended (using a Tissumizer, setting 65 for 15 s) in Tris HCl, pH 7.4, for cells expressing the human 5-HT_{2B} receptor, in Tris HCl, pH 7.4, containing MgCl₂ and EDTA for [¹²⁵I]DOI binding to 5-HT_{2A} or 5-HT_{2C} receptors, or in Tris HCl, pH 7.6, for [³H]ketanserin and [³H]mesulergine binding to 5-HT_{2A} and 5-HT_{2C} receptors respectively.

5-HT_{2B} [³H]-5-HT Binding Studies. Human 5-HT_{2B} receptor binding assays using [³H]-5-HT were performed as previously described.³⁹ The assay was automated using a Biomek 1000 instrument (Beckman Instruments, Fullerton, CA). [³H]-5-HT in Tris HCl containing CaCl₂, pargyline, and L-ascorbic acid, adjusted to pH 7.4, was added to drug dilutions, spanning 6 log units, in water. Then 200 μ L of membrane suspension (approximately 100–150 μ g of protein) was added with mixing and incubated for 15 min at 37 °C. The total incubation volume was 800 μ L, and all incubations were performed in triplicate. The final concentration of CaCl₂, pargyline, Tris, and L-ascorbic acid was 3 mM, 10 μ M, 50 mM, and 0.1% respectively. The assay was terminated by vacuum filtration through Whatman GF/B filters which had been presoaked with 0.5% poly(ethylenimine) (w/v) and precooled with 4 mL of ice-cold wash buffer (50 mM Tris HCl, pH 7.4), using a Brandel cell harvester (Model MB-48R, Brandel, Gaithersburg, MD). The filters were then washed rapidly four times with 1 mL of ice-cold wash buffer. The amount of [³H]-5-HT trapped on the filters was determined by liquid scintillation spectrometry (Ready Protein, LS 6000IC, Beckman Instruments, Fullerton, CA). The final [³H]-5-HT concentration for competition studies was approximately 2 nM (range = 1.7–2.5 nM). The actual free radioligand concentration was determined by sampling the supernatant of identical tubes where bound ligand was removed by centrifugation. Nonspecific binding was defined with 10 μ M 5-HT or 10 μ M 1-naphthylpiperazine (1-NP). The amount of protein was determined by the method of Bradford,⁴⁰ with bovine serum albumin as the standard.

5-HT_{2A} and 5-HT_{2C} [¹²⁵I]DOI Binding Studies. Human 5-HT_{2A} or 5-HT_{2C} binding studies were performed essentially as described for [³H]-5-HT binding to the 5-HT_{2B} receptor with the following exceptions. The assay buffer contained, in final concentration, 10 μ M pargyline, 9.75 mM MgCl₂, 0.5 mM (ethylenedinitrilo)tetraacetic acid, disodium salt (EDTA), 0.1% sodium ascorbate and 50 mM Tris HCl, pH 7.4. Incubations were performed at 37 °C for 30 min with approximately 40 and 30 μ g of protein for the 5-HT_{2A} and 5-HT_{2C} receptors, respectively, and then filtered and washed as described above. The amount of [¹²⁵I]DOI trapped on the filters was determined using a gamma counter. Nonspecific binding was determined with 10 μ M mianserin for 5-HT_{2C} and 1 μ M ketanserin for 5-HT_{2A} receptors. The final concentration of [¹²⁵I]DOI was approximately 0.07–0.15 nM.

[³H]Ketanserin Binding to the Human 5-HT_{2A} Receptor. Membranes were prepared as described above, and the assay conditions were essentially as previously described.⁴¹ Assays consisted of 0.8 mL total volume containing 50 mM Tris HCl, 100 nM prazosin (to block potential binding of [³H]-ketanserin to α_1 -adrenergic receptors), 0.4–0.5 nM [³H]ketanserin, and varying concentrations of the competing compound of interest (final pH 7.6). Mianserin, 3 μ M, was used to define the level of nonspecific binding. Tubes were incubated at 37 °C for 15 min and then rapidly filtered and washed as described above. The amount of [³H]ketanserin trapped on the filters was determined by liquid scintillation spectrometry.

[³H]Rauwolscine Binding to the Human 5-HT_{2B} Receptor. This assay is based on a previously described procedure.⁴² Membrane preparation and the filtration binding assay were essentially as described above. Conditions specific to this assay were as follows (all concentrations given as final concentrations): 2 nM [³H]rauwolscine, 500 nM efaroxan (to mask rauwolscine binding to α_2 -adrenergic receptors), and 50 mM Tris HCl, pH 7.4. Tubes were incubated at 37 °C for 20 min and then rapidly filtered as described above. Nonspecific binding was defined in the presence of 10 μ M 1-naphthylpiperazine.

[³H]Mesulergine Binding to the Human 5-HT_{2C} Receptor. This assay was adapted from that described by Pazos et al.⁴³ Membranes were prepared as described above. Final concentrations for the 0.8 mL assays were 0.74–0.82 nM [³H]-mesulergine, varying concentrations of competing compound, and 50 mM Tris HCl, final pH 7.6. Nonspecific binding was determined using 3 mM mianserin. Assay tubes were incubated for 30 min at 37 °C, after which the samples were filtered and washed, and radioactivity was determined as for the [³H]-ketanserin binding assay described above.

Statistical Analysis. Nonlinear regression analysis for the competition curves was performed as previously described³⁹ to determine IC₅₀ values. These were converted to K_i values by the method of Cheng and Prusoff.⁴⁴

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