Substituted Naphthofurans as Hallucinogenic Phenethylamine−Ergoline Hybrid Molecules with Unexpected Muscarinic Antagonist Activity

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A series of substituted racemic naphthofurans were synthesized as "hybrid" molecules of the two major prototypical hallucinogenic drug classes, the phenethylamines and the tryptamines/ergolines. Although it was hypothesized that these new agents might possess high affinity for the serotonin 5-$HT_{2A/2C}$ receptor subtypes, unexpected affinity for muscarinic receptors was observed. The compounds initially synthesized for this study were (±)-*anti*- and *syn*-4-amino-6-methoxy-2a,3,4,5-tetrahydro-2*H*-naphtho[1,8-*bc*]furan (**4a**,**b**), respectively, and their 8-bromo derivatives **4c**,**d**, respectively. The brominated primary amines **4c**,**d** were assayed initially for activity in the two-lever drug discrimination (DD) paradigm in rats trained to discriminate saline from LSD tartrate (0.08 mg/kg). Also, **4c**,**d** were evaluated for their ability to compete against agonist and antagonist radioligands at cloned human 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors. After the syn diastereomers were found to have the highest activity in these preliminary assays, the N-alkylated analogues *syn-N,N*-dimethyl-4-amino-6-methoxy-2a,3,4,5-tetrahydro-2*H*-naphtho[1,8-*bc*]furan (**4e**) and *syn-N,N*-dipropyl-4-amino-6-methoxy-2a,3,4,5-tetrahydro-2*H*-naphtho[1,8-*bc*]furan (**4f**) were prepared and assayed for their affinities at [^{3}H]ketanserin-labeled 5-HT_{2A} and [^{3}H]-8-OH-DPAT-labeled 5-HT_{1A} sites. All of the molecules tested had relatively low affinity for serotonin receptors, yet a preliminary screen indicated that compound **4d** had affinity for muscarinic receptors. Thus, **4b**,**d**,**e** were evaluated for their affinity at muscarinic M_1−M_5 receptors and also assessed for their functional characteristics at the M_1 and M_2 isoforms. Compound **4d** had affinities of 12−33 nM at all of the muscarinic sites, with **4b**,**e** having much lower affinity. All three compounds fully antagonized the effects of carbachol at the M_1 receptor, while only **4d** completely antagonized carbachol at the M_2 receptor. The fact that the naphthofurans lack LSD-like activity suggests that they do not bind to the serotonin receptor in a way such that the tricyclic naphthofuran nucleus is bioisosteric with, and directly superimposable upon, the A, B, and C rings of LSD. This also implies, therefore, that the hallucinogenic phenethylamines *cannot* be directly superimposed on LSD in a common binding orientation for these two chemical classes, contrary to previous hypotheses.

Introduction

One of the most intriguing issues in exploring the structure−activity relationships (SAR) of psychedelic (hallucinogenic) agents has been to determine how different chemical classes of hallucinogens with similar pharmacologic and behavioral effects may act through a common receptor. Much of our previous work on the SAR of hallucinogens has been driven by our efforts to discern the common pharmacophoric elements within the phenethylamine (e.g., **1**) and tryptamine/ergoline (e.g., **2**) classes as they relate to binding interactions within the serotonin 5-HT_2 family of receptors, the putative primary site of action of these drugs.[1−3] To date, however, no entirely satisfactory model correlates the activity of these hallucinogens with their unique structural features.

The most widely accepted pharmacophoric hypothesis is one in which the aryl ring of a phenethylamine, such as **1**, is superimposed over the A ring of LSD **2**. This idea was first developed in the 1950s by Marini-Bettolo, Bovet, and co-workers as it related to the oxytocic properties of the ergolines.[4] In this model, the protonated primary amine of **1** and N(6) of **2** would bind to a common anionic residue within the receptor binding site, now thought to be a conserved aspartate present in transmembrane helix 3 of all characterized monoamine receptors. Indeed, Wang et al.[5] have shown that mutation of Asp^{155} to an Asn in the 5-HT_{2A} receptor gave a mutant with greatly decreased affinity for 5-HT_2 agonists and antagonists. This model also suggests that the 5-methoxy oxygen of **1** and the indolic NH of **2** might hydrogen-bond to a common serine residue in the human 5-HT_{2A} receptor, possibly Ser^{242} in TM III,[6] while the 2-methoxy oxygen atom of **1** and the carbonyl oxygen

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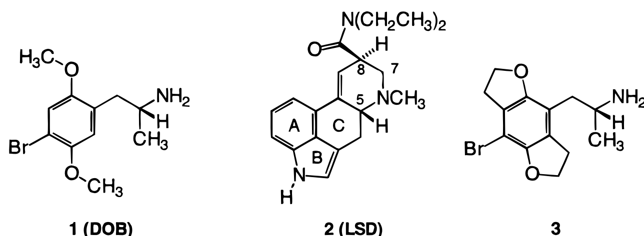
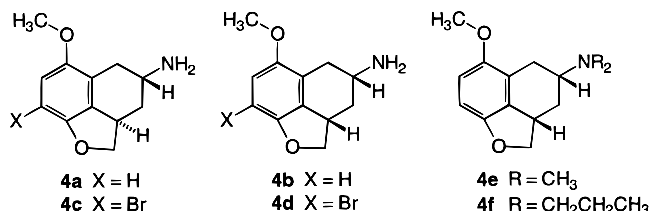
of **2** could interact with a common H-bond donor within the 5-HT_2 binding site. This hypothesis is supported by the finding that it is the *R* enantiomer of the phenylispropylamines that is the more potent hallucinogen, and the asymmetric center in these agents is homochiral with C(5) of the ergolines in this superimposition.[7,8] Later studies that compared chiral tryptamines with phenethylamines gave data consistent with this notion.[9]



Recently, we have shown that constraining the methoxy groups of the 2,5-dimethoxy-substituted hallucinogenic phenethylamines into dihydrofuran moieties (as in **3**) provided compounds of high potency and selectivity for 5-$HT_{2A/2C}$ receptors that exhibited behavioral effects similar to **2** in the rat drug discrimination assay.[10] Thus, **3** clearly established the binding conformation of the alkoxy substituents at 5-HT_2 sites in this class of compounds. As an extension of that work, and as a means to test the pharmacophoric hypotheses discussed above, it was reasoned that an extremely rigidified phenethylamine-like species could be constructed by tethering the side chain of **3** to the adjacent dihydrofuran ring. This strategy would produce a molecule that is, in essence, a "hybrid" of molecules **1** and **2**, in which the indolic A and B rings of **2** are mimicked by the benzodihydrofuran portion of **3** and the C ring of **2** is equivalent to the tethered side chain of the new hybrid. Thus, a series of compounds (**4**) was proposed as methoxylated phenethylamine analogues that potentially would be bioisosteric with rings A, B, and C of the ergolines. Following an evaluation of physical models, molecular modeling studies were initially carried out to examine conformations of the syn isomers (e.g., **4b**,**d**) and to determine whether they were superimposable on the A, B, and C rings of **2**. Energy minimization using semiempirical methods (AM1 Hamiltonian) gave low-energy conformational minima for **2** and **4d**. A least-squares fit of the framework atoms of **4d** to the A/B/C rings and N(6) of **2** gave a superposition where the distance between any two paired atoms was generally less than 0.1 Å, with the exceptions of the basic nitrogens and atoms C(2a) in both molecules, where the corresponding distances between them were 0.44 and 0.48 Å, respectively. Given that a small degree of conformational flexibility exists in both molecules, we believe that the fit between molecules **4** and the A, B, and C rings of **2** can be considered to be good. Here we report on the synthesis and pharmacological evaluation of both the anti (**4a**,**c**) and syn (**4b**,**d**–**f**) racemic diastereomeric naphthofurans as phenethylamine–ergoline hybrid molecules.

Each of the compounds **4a**–**f** synthesized in this study was assayed for LSD-like behavioral activity in the drug discrimination model, a paradigm that has been correlated with hallucinogenic activity in man.[11] Radioligand binding studies initially were carried out using both rat brain and cloned human 5-HT_2 receptors. After a receptor screen demonstrated that **4d** had affinity for muscarinic receptors, the syn diastereomers were evaluated at cloned human M_1–M_5 muscarinic receptor subtypes.

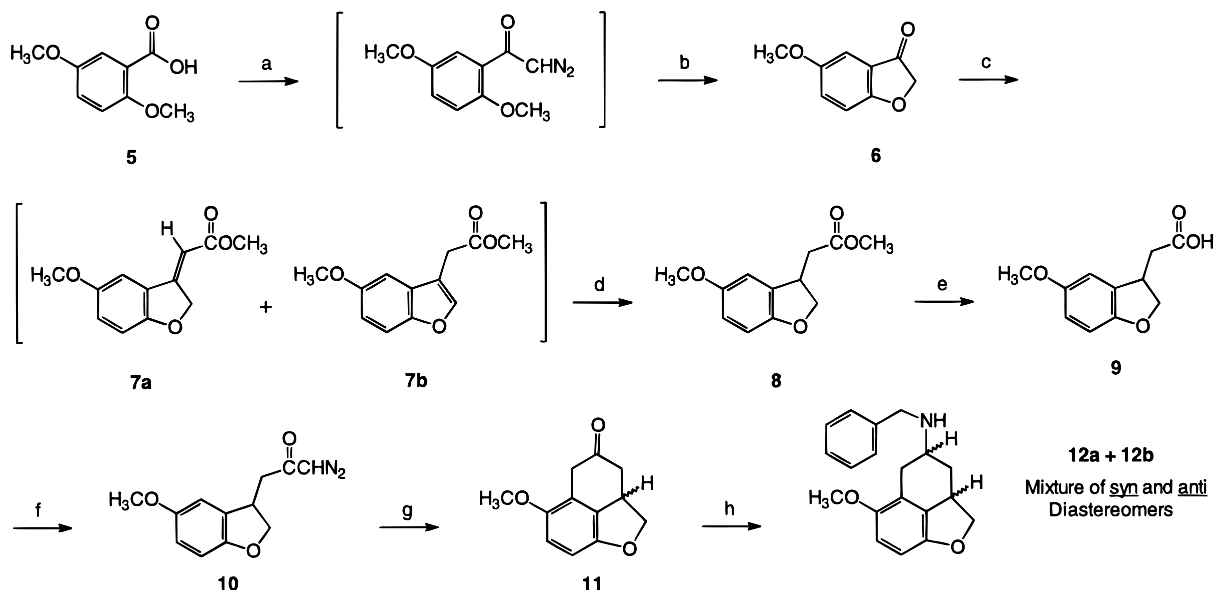


Chemistry

The series of target racemic tetrahydronaphthofurans **4a**–**f** was synthesized successfully as illustrated in Schemes 1 and 2. The early stages of the synthesis are presented in Scheme 1. The acyl chloride of 2,5-dimethoxybenzoic acid (**5**) was converted to the diazomethyl ketone and efficiently cyclized to the benzofuranone **6** using the method of Jung and Abrecht.[12] This method provided an excellent means of producing large quantities of **6**. Following the procedure of Chan et al.,[13] **6** underwent Wittig reaction with the ylide, methyl (triphenylphosphorylidene)acetate. These authors claimed that for the analogous Wittig adduct using the ethyl ester ylide, the mixture of dihydrobenzofuran (exo double bond) and benzofuran products could be isomerized to the more stable benzofuran isomer by storing the mixture in chloroform overnight. While a similar mixture of products (**7a**,**b**) was obtained using the methyl ester ylide, isomerization to the reported more stable benzofuran **7b** did not always proceed to completion. Nonetheless, the benzofuran alone, or the mixture of isomers, could be reduced catalytically in the next step to afford the 2,3-dihydrobenzofuran ester **8**. Basic hydrolysis then gave the benzofuranylacetic acid **9** in excellent yield.

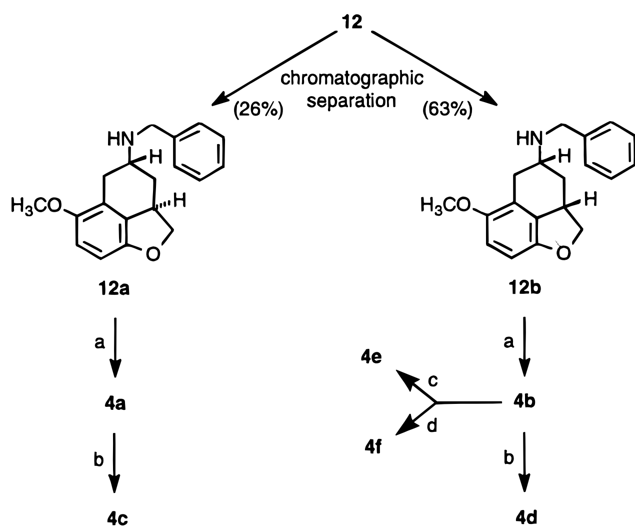
Homologation of the side chain of **9** by one carbon, and formation of the third ring, was accomplished by initially forming diazomethyl ketone **10**. Cyclization to **11** was accomplished by reflux of **10** in dichloromethane with a catalytic amount of rhodium(II) acetate dimer, followed by treatment with trifluoroacetic acid, according to the method of McKervey et al.[14] Because there was only one possible aromatic insertion point in **10**, it was anticipated that this cyclization would proceed in good yield. Nevertheless, pure naphthofuranone **11** was obtained only in modest amounts (30–35% yields). Therefore, an alternate cyclization was attempted in which trifluoroacetic acid (TFA) was used to protonate the diazomethyl ketone and to catalyze intramolecular attack of the arene on the diazonium moiety with concomitant displacement of N_2.[15] Cushman et al.[16,17] had previously used this cyclization method in a more complex system with some success, attaining yields of up to 37%. When **10** was treated with TFA, however, the only product isolated (55%) arose from direct nucleophilic displacement of the diazo group by trifluoroacetate anion. Similar discouraging results were obtained when methanesulfonic acid was used in place of

Scheme 1[a]



[a] Reagents: (a) 1. ClCOCOCl, benzene, DMF, 2. CH_2N_2, $(CH_3CH_2)_2O$; (b) CH_3COOH; (c) $Ph_3PCHCOOCH_3$, xylene, Δ; (d) 10% Pd−C/H_2, CH_3CH_2OH; (e) KOH, CH_3CH_2OH, Δ; (f) 1. ClCOCOCl, benzene, DMF, 2. CH_2N_2, $(CH_3CH_2)_2O$; (g) 1. $Rh_2(OAc)_4$, CH_2Cl_2, Δ, 2. CF_3COOH; (h) benzylamine, benzene, 2. $NaCNBH_3$, CH_3CH_2OH.

Scheme 2[a]



[a] Reagents: (a) 10% Pd−C/H_2, CH_3OH; (b) Br_2, $CHCl_3$; (c) HCHO, $NaCNBH_3$, CH_3OH; (d) CH_3CH_2CHO, $NaCNBH_3$, CH_3OH.

TFA. It was concluded that the rhodium-catalyzed cyclization approach gave the best results for this system, and this method was used to produce ketone **11**. Reductive amination of **11** with benzylamine in the presence of sodium cyanoborohydride readily gave the diastereomeric mixture of *anti-* and *syn-N*-benzyltetrahydronaphthofurans **12a**,**b**, respectively.

Final elaboration of the 4-aminonaphthofuran nucleus was carried out as illustrated in Scheme 2. The diastereomeric 1:2.4 mixture of *anti*-**12a**:*syn*-**12b** *N*-benzyl products was separated by column chromatography. The absorptions and coupling constants of all hydrogens were assigned following 2D-NOESY NMR experiments. The racemic diastereomers were differentiated by observing the presence or absence of a nuclear Overhauser effect (NOE) between hydrogens H(2a) and H(4). The 2D-NOESY NMR spectrum of the syn compound **12b** clearly showed a small cross-peak between these protons, but this coupling was completely absent in the 2D-NOESY spectrum of **12a**, indicating that the protons were on opposite faces of the planar ring system in this diastereomer. These initial NMR experiments were confirmed later by comparing the 2D-NMR spectra of the subsequent diastereomeric pairs (**4a** with **4b**; **4c** with **4d**). The coupling constants for the methylene hydrogens at C(3) in **4a**,**b** were also diagnostic. In **4b** $H(3)_a$ is pseudoaxial, observed as a quartet at δ 1.19, with dihedral angles to both H(2a) and H(4) of approximately 175°, giving large and essentially identical vicinal coupling constants. The geminal coupling constant to $H(3)_{eq}$ is large and also virtually identical to the two vicinal coupling constants. Thus, $H(3)_a$ is coupled to three protons with nearly identical coupling constants. In **4a** no clear anti relationships exist between vicinal hydrogens, with the dihedral angles between $H(3)_a$−H(2a) and $H(3)_a$−H(4) of 139° and 29°, respectively, and between $H(3)_{eq}$−H(2a) and $H(3)_{eq}$−H(4) of 22° and 145°, respectively, with the resonance at δ 1.45 observed as a doublet of quartets.

Once the stereochemistry of the pure diastereomers **12a**,**b** was established, each was carried through a series of analogous reactions as shown in Scheme 2 to afford the desired target compounds **4a**−**d**. Both the catalytic hydrogenolysis to give primary amines **4a**,**b** and the subsequent aromatic brominations affording **4c**,**d**, proceeded smoothly for each diastereomer, giving the desired target molecules in good yield. Finally, the *syn*-tetrahydronaphthofuran **4b** was converted to its *N*,*N*-dimethyl (**4e**) and *N*,*N*-dipropyl (**4f**) congeners using previously described methods with the appropriate aldehydes and sodium cyanoborohydride.[18] After chromatographic purification, oxalate salts of both **4e**,**f** were prepared, and these were crystallized from ethanol. The intent of preparing these compounds was to ascertain whether N-alkylation might either abolish 5-HT_{2A} receptor affinity or lead to ligands with higher affinity for the 5-HT_{1A} receptor.

Table 1. Data from Drug Discrimination Tests in Rats Trained To Discriminate Saline from LSD Tartrate (0.08 mg/kg ip)

compd	mg/kg	μmol/kg	*N*	%D[a]	%SDL[b]	ED50 μmol/kg	ED50 mg/kg
LSD (**2**)	0.01	0.023	13	0	31	0.037	0.016
	0.02	0.046	14	7	62	(0.023−0.057)	(0.01−0.025)
	0.04	0.093	15	0	81		
	0.08	0.186	16	0	100		
1[c]			8−12			1.12	
						(0.086−1.46)	
3[c]			7−15			0.061	
						(0.031−0.12)	
4c	0.365	1	7	0	0		
	0.78	2	9	0	11		
	1.46	4	10	0	20	NS	NS
	2.92	8	11	0	27		
	5.84	16	9	11	37.5		
4d	0.365	1	10	0	0		
	0.78	2	11	0	0		
	1.46	4	10	0	10	NS	NS
	2.92	8	12	0	33		
	5.84	16	13	15	44		

[a] Percentage of animals failing to emit 50 presses on either lever within the 15-min test period. [b] Percentage of animals emitting 50 presses on the LSD appropriate lever during the test period. [c] Data from Monte et al.[10]

Pharmacology

Compounds **4c**,**d** initially were screened for in vivo activity using the two-lever drug discrimination assay in rats trained to discriminate saline from LSD tartrate (0.08 mg/kg). Subsequently, the new compounds were assessed for affinity at the serotonin 5-HT_2 family of receptors. The *syn*-naphthofuran **4d**, shown to have highest affinity in the serotonin receptor assays, then was submitted to NovaScreen to assess its affinity for other monoamine receptor subtypes. Following identification of possible high affinity for muscarinic receptors through this screen, compounds **4c**,**d** were tested for their affinity at the cloned M_1−M_5 muscarinic receptors. These agents also were evaluated functionally by assessing the ability of the compounds to stimulate phosphoinositide hydrolysis in muscarinic M_1 and M_2 receptor systems.

Results and Discussion

Drug Discrimination and Serotonin Receptor Affinity. Table 1 shows the drug discrimination data for compounds **4c**,**d**, and includes comparison data that we had obtained previously for compounds **1** and **3**. Clearly, the rigid naphthofurans have lost the LSD-like behavioral effects that are characteristic of compounds **1** and **3**. This result was surprising because we had previously shown that LSD-like behavioral potency was increased in the rigid analogue **3** compared with **1**. Based on the hypothesis that the hallucinogenic amphetamines superimpose over the ergoline structure, with correspondence between the phenyl ring of the amphetamines and ring A of the ergolines, it had been anticipated that **4d** might have enhanced LSD-like effects, relative to **3**. Although design of rigid analogues can be problematic, and data for inactive structures can be difficult to interpret, based on our experience to date, it seemed the most likely explanation for the lack of activity was simply that the hallucinogenic amphetamines do not bind to the 5-HT_{2A} receptor in a conformation that resembles the ergolines, as described above, and as embodied within the structure of **4d**.

The two diastereomers **4c**,**d** then were assessed for their affinity at [^{3}H]ketanserin-labeled 5-HT_{2A} receptors in rat brain homogenate (Table 2). Their affinities were very low, especially when compared with compounds such as **1** and **3**. Nonetheless, these data did establish that the syn diastereomer **4d** had greater complementarity to the 5-HT_{2A} receptor than the anti diastereomer.

Table 2. Results of the Radioligand Competition Studies of **4c**−**f** at 5-HT_{2A} and 5-HT_{1A} Receptors in Rat Brain Homogenate (K_i values ± SEM)

drug	[^{3}H]ketanserin-labeled 5-HT_{2A} sites (nM)	[^{3}H]-8-OH-DPAT-labeled 5-HT_{1A} sites (μM)
1[a]	22 ± 3	6.1 ± 0.5
3[a]	18 ± 1	4.3 ± 0.4
4c	3880 ± 170	NT
4d	1050 ± 170	NT
4e	967 ± 124	38 ± 6
4f	644 ± 80	144 ± 10

[a] Data from Monte et al.[10]

Table 3 shows radioligand competition data for compounds **4c**,**d**, and comparison data for **3**, in the cloned human 5-HT_2 receptor family. From the affinity data, it can be clearly seen that the syn compound **4d** again gives a markedly better fit to the receptor. When compared with **3**, however, **4d** has approximately 20−30-fold less affinity at the antagonist-labeled 5-HT_{2A} and 5-HT_{2C} receptors and a 164-fold lower affinity for the 5-HT_{2B} receptor. These data are consistent with the observed loss of behavioral activity in the drug discrimination assay and reinforce the conclusion that **4d** does not represent an active conformation for hallucinogenic amphetamines such as **1**.

Although naphthofuran **4d** has low affinity for the antagonist-labeled 5-HT_{2A} receptor, it does have significant affinity for the agonist-labeled high-affinity states of both the 5-HT_{2A} and 5-HT_{2C} human receptors.[1,19] The absence of an LSD-like interoceptive cue leads us to speculate that **4d** may be a partial agonist or antagonist, since all full agonists with high affinity for [^{125}I]DOI-labeled 5-HT_{2A} receptors that we have examined so far have possessed LSD-like behavioral activity in the drug discrimination paradigm. Since we have previously elucidated the active conformations of the methoxy groups for **1**,[10] these results lead us to conclude that the active conformation of the hallucino-

Table 3. Results of the Radioligand Competition Studies of **4c**,**d** at Cloned Human 5-HT_{2A}, 5-Ht_{2B}, and 5-HT_{2C} Receptors (K_i values in nM ± SEM)

	[³H]antagonist radioligands			agonist radioligands		
compd	ketanserin 5-HT_{2A}	rauwolscine 5-HT_{2B}	mesulergine 5-HT_{2C}	[¹²⁵I]DOI 5-HT_{2A}	[³H]-5-HT 5-HT_{2B}	[¹²⁵I]DOI 5-HT_{2C}
3[a]	10.7 ± 1.0	1.15 ± 0.04	2.28 ± 0.38	0.48 ± 0.03	1.60 ± 0.25	0.30 ± 0.02
4c	NT	NT	NT	8500 ± 150	11200 ± 1800	4190 ± 120
4d	240 ± 16	255 ± 6	89.6 ± 21.1	13.0 ± 1.6	263 ± 32	5.96 ± 0.62

[a] Data from Monte et al.[10]

genic amphetamines may not be one where the ethylamine side chain lies in an approximately coplanar arrangement with the aromatic system. It also seems possible that side-chain flexibility may be required to accommodate the dynamics of receptor activation for phenethylamine type agonists, but not ergolines. This conclusion would also imply, however, that the classical view of the relationship between the ergolines and the binding conformation of the phenethylamine hallucinogens is incorrect.

Following these investigations, the nonbrominated *syn-N,N*-dimethyl (**4e**) and *syn-N,N*-dipropyl (**4f**) naphthofurans were prepared and assayed for affinity at the 5-HT_{1A} and 5-HT_{2A} receptors in rat brain homogenate (Table 2). The results show that neither of these target naphthofurans have significant affinity for [³H]ketanserin-labeled 5-HT_{2A} sites or [³H]-8-OH-DPAT-labeled 5-HT_{1A} sites. It is curious to note, however, that the affinity of the syn diastereomers for 5-HT_{2A} sites increases slightly with N-alkylation and with extension of the *N*-alkyl groups from methyl to propyl groups, an observation that is contrary to the known SAR of phenethylamine type 5-HT_{2A} agonists.

Effects at Muscarinic Receptors. One unexpected finding resulted from a radioreceptor screen of the most active *syn*-bromonaphthofuran **4d**. This compound had significant affinity for muscarinic receptors, a surprising finding in light of the fact that **4d** did not resemble any known muscarinic ligands.[20−23] Although new muscarinic ligands have been developed that can cross the blood−brain barrier, many of the prototypical muscarinics possess a quaternary ammonium functionality that prevents their penetration into the central nervous system (CNS).[24] Muscarinic agonists with CNS activity are currently highly sought, since it is now known that patients suffering from Alzheimer's disease exhibit selective degeneration of the muscarinic M_2 subtype receptors in the posterior parietal cortex.[24] Because the molecular and three-dimensional structures of muscarinic receptors are believed to resemble closely those of the other GPCRs,[23] it is possible that compounds designed as ligands for a different receptor type may actually have affinity for any one of the muscarinic receptor subtypes. With this in mind, the *syn*-naphthofurans **4b**,**d**,**e** were subjected to comprehensive radioligand competition and functional studies at all five of the muscarinic receptor subtypes, M_1−M_5.

The receptor binding results obtained for **4b**,**d**,**e** at the five cloned human muscarinic subtypes are shown in Table 4. In agreement with the preliminary NovaScreen data, compound **4d** has significant affinity for several of the muscarinic receptor subtypes. In addition, an important and striking structural feature is the hydrophobic aromatic substituent para to the alkylamine side chain (compare **4b**,**d**) that increases receptor affinity. This substituent is also an important recognition element at the 5-HT_{2A} receptor. Thus, there may be a region of hydrophobic interaction within the muscarinic binding site analogous to the one that is proposed to exist in the serotonin 5-HT_{2A} receptor. The results also show that N-substitution greatly attenuates ligand affinity for all subtypes, a structural feature that is divergent from the SAR of most other muscarinic receptor ligands.

Table 4. Results of Radioligand Competition Studies at Cloned Muscarinic Receptors ($K_{0.5}$ values in nM ± SEM)

drug	M_1	M_2	M_3	M_4	M_5
4b	149 ± 9	33400 ± 5	129 ± 6	240 ± 25	236 ± 22
4d	20 ± 8	41 ± 18	12 ± 3	30 ± 8	33 ± 5
4e	846 ± 26	953 ± 74	654 ± 56	913 ± 42	1100 ± 50
atropine	0.6 ± 0.1	1.8 ± 0.2	0.6 ± 0.1	0.5 ± 0.1	0.7 ± 0.1

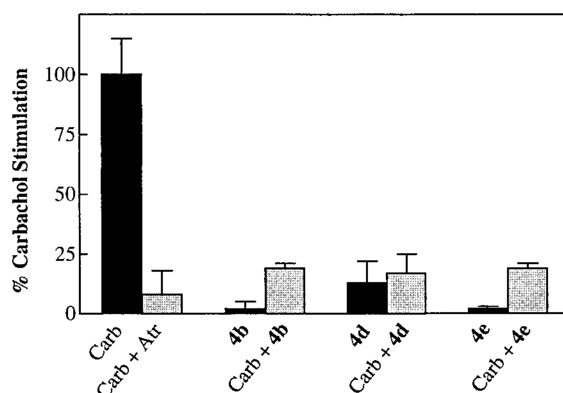


Figure 1. Functional activity of **4** derivatives at the cloned M_1 muscarinic receptor. B82 cells tranfected with the M_1 receptor were incubated with carbachol (Carb; 1 mM) or carbachol plus drug. Atropine (1 μM) was able to inhibit the carbachol-stimulated response. Compounds **4b**,**d**,**e** tested at 10 μM had little activity on their own but were able to attenuate the carbachol response to a similar degree as atropine. Values represent the mean ± SEM for duplicates run at three different times.

Figures 1 and 2 illustrate that **4b**,**d** are antagonists at the M_1 and M_2 receptors, respectively. While the affinity of **4d** for the various muscarinic receptor subtypes is lower than that of atropine, it is similar to several currently available, hydrophobic M_2 agonists and antagonists.[20,21] Furthermore, while it has approximately one-tenth the affinity of atropine, it is an effective muscarinic antagonist, at least at the M_1 and M_2 subtypes. As a potential muscarinic ligand, **4d** represents a compound of novel molecular structure that is of sufficient hydrophobicity to cross the blood−brain barrier and enter the CNS. Specifically, **4d** may serve as a valuable lead compound in the future development of muscarinic ligands and generally may lead to a better understanding of the SAR of muscarinic receptors.

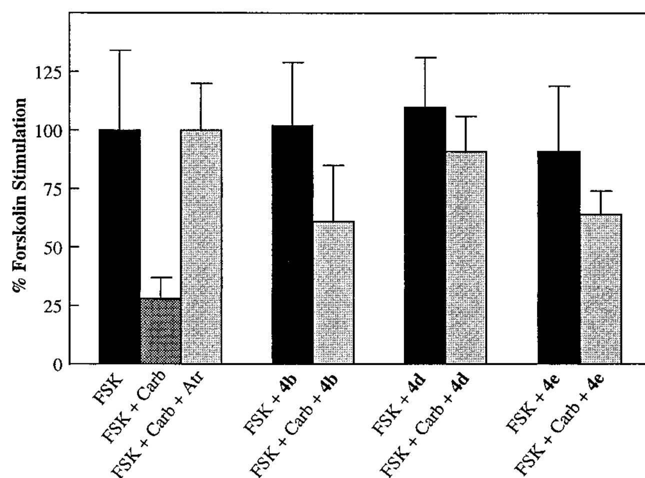


Figure 2. Functional assessment of **4** derivatives at the cloned M_2 muscarinic receptor. B82 cells transfected with the M_2 receptor were stimulated with forskolin (FSK; 10 μM), and then cyclase activity was inhibited by coincubation with carbachol (FSK + Carb (1 mM)). Addition of atropine (1 μM) was able to reverse the carbachol-induced inhibition of FSK stimulation. The new compounds had no effect by themselves, but **4d** is able to reverse totally the carbachol-induced inhibition, while **4b**,**e** are able to reverse this effect only partially. Values represent the mean ± SEM for duplicates run at two different times. All **4** analogues were tested at 10 μM.

From a molecular standpoint, **4d** was designed to be a hybrid of the hallucinogenic phenethylamines, exemplified by DOB (**1**) or the rigid congener **3**, and the ergolines, exemplified by LSD (**2**). Because **4d** is less potent than **1** or **3** and appears to lack the LSD-like activity that is characteristic of the latter two molecules, one hypothesis to be drawn from these data is that **4d** does not bind to the serotonin receptor in a way that the tricyclic naphthofuran nucleus is bioisosteric with, and directly superimposable upon, the A, B, and C rings of LSD. This also implies, therefore, that the hallucinogenic phenethylamines *cannot* be directly superimposed on LSD in a common binding orientation for these two chemical classes, contrary to previous hypotheses. The loss of activity for **4d** relative to **1** and **3** also suggests that the flexible aminoalkyl side chain in the phenethylamines does not adopt a conformation that is relatively coplanar with the aromatic ring system, as is the case with the ergolines. While the present data do not absolutely prove such an assertion, it is the conclusion most parsimonious with our results. If true, one could infer that the agonist site of the 5-HT_{2A} receptor accommodates either the tryptamine/ergoline or the oxygenated phenethylamine templates in different binding orientations, in which at least some distinct amino acid residues are involved in the recognition of each molecular species.

Experimental Section

Chemistry. Melting points were determined using a Thomas-Hoover apparatus and are uncorrected. ^{1}H NMR spectra were recorded using either a 500-MHz Varian VXR-500S or 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. 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 were within ±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 fluorescent indicator (2.5 × 7.5 cm; J. T. Baker), eluting with CH_2Cl_2, and visualizing with UV light at 254 nm and/or I_2 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. Reactions were carried out under an atmosphere of dry nitrogen.

5-Methoxybenzofuran-3(2*H*)-one (6).[25] Oxalyl chloride (36 mL, 0.41 mol) was added dropwise over 1 h to a stirred suspension of 57.5 g (0.32 mol) of 2,5-dimethoxybenzoic acid (**5**) in 250 mL of benzene and drops of DMF. As the reaction progressed, the acid gradually dissolved to give a clear-yellow solution. After 2.5 h, volatiles were removed on the rotary evaporator, and the acyl chloride was distilled in a Kugelrohr apparatus (bp 105−110 °C at 0.05 mmHg) to give 61.3 g (97%) of a pale-yellow oil. The pure acyl chloride was used portionwise in the following diazomethane reaction.

Using a large diazomethane apparatus (Aldrich, Milwaukee, WI), diazomethane was generated by adding a solution of 38 g (0.18 mol) of *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (Diazald) in 250 mL of anhydrous ether to a stirred solution of 10 g of KOH, 17 mL of H_2O, 50 mL of 2-(2-ethoxyethoxy)-ethanol (carbitol), and 15 mL of ether held over a 40 °C oil bath.[26,27] The distilled ether−diazomethane was cooled in an ice−salt bath and stirred while 11 g (0.055 mol) of the 2,5-dimethoxybenzoyl chloride (see above) in 30 mL of ether was cautiously added over a 10-min period. After the vigorous evolution of HCl gas had subsided, the ice−salt bath was removed, and the mixture was allowed to warm to room temperature while stirring in the hood. Remaining volatiles were removed by attaching the reaction flask directly to a water aspirator in the hood and immersing the flask in a warm water bath. The intermediate diazoketone was obtained as a bright-yellow solid caked to the walls of the flask.

Glacial acetic acid (70 mL) then was added cautiously to the intermediate diazoketone, causing the solid to dissolve with the vigorous evolution of nitrogen. The mixture was stirred for 1.5 h, and the solvent was removed on the rotary evaporator. Further drying under high vacuum produced a yellow solid that was recrystallized from ethyl acetate−petroleum ether to give 7.3 g (82%) of **6** as fluffy, pale-yellow flakes: mp 92 °C (lit.[25] mp 88 °C); ^{1}H NMR ($CDCl_3$) δ 3.80 (s, 3, ArOCH_3), 4.64 (s, 2, ArOCH_2CO), 7.06 (d, 1, Ar*H*, J = 2.5 Hz), 7.07 (d, 2, Ar*H*, J = 9.0 Hz), 7.25 (dd, 1, Ar*H*, J = 9.0, 2.8 Hz).

Methyl 2-(5-Methoxybenzofuran-3-yl)acetate (7a).[25] Following the procedure of Chan et al.,[13] a mixture of 20.0 g (0.122 mol) of ketone **6** and 41.54 g (0.124 mol) of methyl (triphenylphosphoranylidene)acetate (Aldrich, Milwaukee, WI) was stirred vigorously in 400 mL of toluene at reflux for 50 h. The solvent was then removed on the rotary evaporator, and the residue was dissolved in a large quantity of ether. The triphenylphosphine oxide that did not dissolve was filtered and discarded, and additional oxide was removed by allowing it to crystallize out of the ether solution in the freezer with collection by vacuum filtration. Final purification was accomplished by repeated flash column chromatography, eluting with 10% ethyl acetate−petroleum ether until the product was free of triphenylphosphine oxide and appeared as a single spot on TLC. The first band to elute from the columns was a mixture of predominantly **7a** and its isomer **7b** (assignments based on ^{1}H NMR spectra) as a yellow solid that weighed 15.1 g (68%). The mixture was stirred in a solution of $CHCl_3$ at room temperature for 12 h to isomerize the product mixture to the more stable isomer **7b** that was a yellow oil: bp 103 °C at 0.01 mmHg; ^{1}H NMR ($CDCl_3$) δ 3.70 (s, 2, CH_2COOCH$_3$), 3.75 (s, 3, CH$_2$COOCH_3), 3.83 (s, 3, ArOCH_3), 6.90 (dd, 1, Ar*H*,

J = 8.9, 2.5 Hz), 7.00 (d, 1, Ar*H*, J = 2.1 Hz), 7.36 (d, 1H, Ar*H*, J = 8.9 Hz), 7.60 (s, 1H, ArCC*H*); CIMS *m/z* 221 (M + 1). The second band to elute from the column contained 3.5 g of unreacted **6** that could be recycled for subsequent runs.

Methyl 2-(2,3-Dihydro-5-methoxy-3-benzofuranyl)acetate (8).[28] A solution of 4.7 g (0.021 mol) of the Wittig adduct **7b** was shaken in 55 mL of absolute ethanol containing 750 mg of 10% Pd−C in a Parr hydrogenation apparatus at 50 psi of H_2. After 34 h, the theoretical amount of H_2 had been taken up, and the reaction mixture was filtered through Celite. Solvent removal under reduced pressure gave 4.4 g (92%) of **8** as a clear oil that was satisfactorily pure by TLC and ^{1}H NMR analyses: ^{1}H NMR ($CDCl_3$) δ 2.60 (dd, 1, C*H*$_2$COOCH$_3$, J = 16.6, 9.4 Hz), 2.80 (dd, 1, C*H*$_2$COOCH$_3$, J = 16.5, 5.3 Hz), 3.70 (s, 3, COOC*H*$_3$), 3.75 (s, 3, ArOC*H*$_3$), 3.82 (m, 1, ArC*H*), 4.22 (dd, 1, ArOC*H*$_2$, J = 9.2, 6.4 Hz), 4.73 (t, 1, ArOC*H*$_2$, J = 8.9 Hz), 6.68 (dd, 1, Ar*H*, J = 8.6, 2.4 Hz), 6.71 (d, 1, Ar*H*, J = 8.6 Hz), 6.74 (d, 1, Ar*H*, J = 2.4 Hz).

(2,3-Dihydro-5-methoxy-3-benzofuranyl)acetic Acid (9). A solution of 4.2 g (0.019 mol) of the reduced methyl ester **8** in 20 mL of ethanol was added to a solution of 2.5 g of KOH in 5 mL of H_2O and 20 mL of ethanol. The mixture was stirred over a steam bath for 30 min, and the alcohol then was removed under reduced pressure. The residue was diluted with 30 mL of water and cooled on an ice bath. Cold, concentrated HCl was added, and the acidic mixture was extracted with 3 × 20 mL of CH_2Cl_2. The organic extracts were combined, washed once with brine, dried over $MgSO_4$, and filtered through Celite. Solvent removal on the rotary evaporator gave 3.82 g (97%) of a white solid that was purified by recrystallization from ethyl acetate−ether to afford 3.38 g (86%) of **9** as heavy white crystals: mp 114 °C (lit.[28] mp 111−112 °C); ^{1}H NMR (DMSO-d_6) δ 2.50 (dd, 1, C*H*$_2$COOCH$_3$, J = 16.7, 9.4 Hz), 2.78 (dd, 1, C*H*$_2$COOCH$_3$, J = 16.7, 5.3 Hz), 3.66 (s, 3, ArOC*H*$_3$), 3.70 (m, 1, ArC*H*), 4.12 (dd, 1, ArOC*H*$_2$, J = 8.8, 7.2 Hz), 4.64 (t, 1, ArOC*H*$_2$, J = 8.9 Hz), 6.65 (s, 2, Ar*H*), 6.85 (s, 1, Ar*H*); CIMS *m/z* 209 (M + 1), 191, 149.

1-Diazo-3-(2,3-dihydro-5-methoxy-3-benzofuranyl)propan-2-one (10). The acyl chloride was prepared by slowly adding 1.37 mL (0.016 mol) of oxalyl chloride to a stirred mixture of 2.5 g (0.012 mol) of the acid **9** in 20 mL of benzene and 1 drop of DMF. After stirring at room temperature for 1 h, the acid had completely dissolved and the solution was orange in color. The volatiles were removed under reduced pressure to give, quantitatively, 2.7 g of a clear-yellow oil that crystallized to a yellow solid on further drying under high vacuum.

The acyl chloride was dissolved in 30 mL of anhydrous ether and was cautiously added to a stirred, ice−salt bath-cooled solution of ethereal diazomethane that had been prepared and distilled from 8.3 g (0.038 mol) of Diazald (Aldrich, Milwaukee, WI), 15 mL of carbitol, 2.5 g of solid KOH, 4 mL of H_2O, and 100 mL of anhydrous ether. Once addition was complete, the ice−salt bath was removed, and the mixture was allowed to warm to room temperature over the next hour. The volatiles were then removed under reduced pressure to give the diazoketone **10** as a pale-yellow solid that weighed 2.71 g (98%) and could be used without further purification. An analytical sample was prepared by recrystallization from ether−petroleum ether to give fine yellow needles: mp 73−74 °C; ^{1}H NMR ($CDCl_3$) δ 2.74 (m, 2, C*H*$_2$COCHN$_2$), 3.75 (s, 3, ArOC*H*$_3$), 3.90 (m, 1, ArC*H*), 4.20 (dd, 1, ArOC*H*$_2$, J = 9.1, 6.0 Hz), 4.70 (t, 1, ArOC*H*$_2$, J = 9.0 Hz), 5.25 (s, 1, CH$_2$COC*H*N$_2$), 6.73 (m, 3, Ar*H*); CIMS *m/z* 233 (M + 1), 205, 161, 149. Anal. ($C_{12}H_{12}O_3N_2$) C, H, N.

6-Methoxy-2a,3,4,5-tetrahydro-2*H*-naphtho[1,8-*bc*]furan-4-one (11). Two grams (8.61 mmol) of the diazoketone **10** was dissolved in 150 mL of CH_2Cl_2 and added dropwise over 2 h to a suspension of 5 mg (0.132 mol %) of $Rh_2(OAc)_4$ catalyst in 150 mL of CH_2Cl_2 at reflux. After addition was complete, the mixture was held at reflux for an additional 1.5 h, and then 1 drop of trifluoroacetic acid was added. The mixture was stirred and allowed to cool to room temperature over 1 h. The cooled organic solution was washed with 150 mL of H_2O, 2 × 150 mL of 5% $NaHCO_3$, and 150 mL of brine, dried over $MgSO_4$, and filtered through a pad of Celite and silica gel. Removal of solvent under reduced pressure yielded 1.25 g (72%) of a yellow oil that appeared to be nearly homogeneous on TLC. The crude product was purified by centrifugal radial chromatography (Chromatotron; Harrison Research, Palo Alto, CA) on a 4-mm silica gel plate, eluting with 15% ethyl acetate−hexane, to afford 600 mg (34%) of **11** as a yellow oil. An analytical sample was prepared by crystallizing a small portion from ether−hexane to give a waxy, off-white solid: mp 83 °C; ^{1}H NMR ($CDCl_3$) δ 2.36 (t, 1, ArCHC*H*$_2$CO), 2.90 (dd, 1, ArCHC*H*$_2$CO), 3.30 (d, 1, ArC*H*$_2$CO), 3.62 (d, 1, ArC*H*$_2$CO), 3.78 (s, 3, ArOC*H*$_3$), 3.82 (m, 1, ArC*H*, methine), 4.15 (t, 1, ArOC*H*$_2$), 4.88 (t, 1, ArOC*H*$_2$), 6.65 (dd, 2, Ar*H*); IR (neat) (C=O) 1711 cm^{-1}; CIMS *m/z* 205 (M + 1). Anal. ($C_{12}H_{12}O_3$) C, H.

***anti*-*N*-Benzyl-4-amino-6-methoxy-2a,3,4,5-tetrahydro-2*H*-naphtho[1,8-*bc*]furan (12a) and *syn*-*N*-Benzyl-4-amino-6-methoxy-2a,3,4,5-tetrahydro-2*H*-naphtho[1,8-*bc*]furan (12b).** In a typical procedure for the preparation of a diastereomeric mixture of the *N*-benzylamines, the naphthofuranone **11** (1.1 g, 5.40 mmol) was stirred for 7 h under a nitrogen atmosphere in 20 mL of benzene containing 0.58 mL (5.40 mmol) of benzylamine and 0.5 g of 4-Å molecular sieves. Reaction completion was indicated by the disappearance of the carbonyl IR band at 1711 cm^{-1}. The dark mixture was filtered through Celite, and the solvent was removed on the rotary evaporator to afford a red oil. Drying under high vacuum overnight gave the enamine as a red solid. The enamine was then taken up into 15 mL of absolute ethanol and stirred under N_2, while anhydrous 1 N HCl in ether was added via syringe to bring the reaction mixture to about pH 4 on moist pH paper. Sodium cyanoborohydride, 0.28 g (4.04 mmol), was added, and the mixture was stirred at room temperature for 5 h, adding dry HCl−ether as needed to maintain about pH 5 (approximately 6 mL of 1 N HCl−ether was used.) The volatiles were removed under reduced pressure and the dark red residue was taken up and shaken gently between 2.5 N KOH and ether. The layers were separated, and the aqueous phase was extracted with 3 × 20 mL of ether. The organic fractions were combined, washed with 3 × 20 mL of H_2O and 50 mL of brine, dried ($MgSO_4$), and filtered through Celite. Solvent removal under reduced pressure gave a dark-brown oil. The oil was taken up into dry ether, and the hydrochloride salt was formed by the addition of 1 N HCl in anhydrous ethanol. Removal of the solvent on the rotary evaporator gave a dark solid that was triturated with acetone−ether and collected on a Büchner funnel, a procedure that removed much of the dark color. The tan-gray solid collected was a mixture of diastereomeric *N*-benzylamine hydrochlorides **12a**,**b** that weighed 0.90 g (50%).

The crude products from this and other runs were purified on the Chromatotron. In a typical separation, 1.5 g of a crude mixture of **12a**,**b** hydrochlorides was treated with excess 5 N KOH, and the free bases were extracted into 4 × 25 mL of CH_2Cl_2. The organic extracts were combined, washed with brine, dried over $MgSO_4$, and filtered through Celite. After concentration on the rotary evaporator, the residue was dissolved in ethyl acetate, applied to a 4-mm silica plate, and eluted with 15% ethyl acetate−hexane under an ammonia atmosphere. The first band to elute from the Chromatotron plate was anti isomer **12a**, the minor product whose identity was confirmed subsequently by NOESY NMR. The free base was a yellow oil weighing 0.35 g (26%). It was taken up into dry ether and converted to its hydrochloride salt by the addition of 1 N HCl in anhydrous ethanol. After solvent removal in vacuo, the solid was crystallized from ethanol−ether to give fine white crystals of **12a**·HCl: mp 235−236 °C; ^{1}H NMR (free base in $CDCl_3$) δ 1.40 (td, 1, ArCHC*H*$_2$CHN, J = 12.5, 2.7 Hz), 1.50 (bs, 1, N*H*), 2.25 (dt, 1, ArCHC*H*$_2$CHN, J = 12.8, 4.2 Hz), 2.75 (bs, 2, ArC*H*$_2$CHN), 3.40 (bt, 1, ArCH$_2$C*H*N, J = unidentifiable), 3.65 (m, 1, ArC*H*), 3.78 (s, 3, ArOC*H*$_3$), 3.9 (s, 2, benzylic methylene), 4.0 (dd, 1, OC*H*$_2$, J = 12.2, 8.0 Hz), 4.78 (t, 1, OC*H*$_2$, J = 8.1 Hz), 6.52 (d, 1, Ar*H*,

J = 8.5 Hz), 6.58 (d, 1, Ar*H*, *J* = 8.5 Hz), 7.30 (m, 5, Ar*H*, benzylic); CIMS *m*/*z* 296 (M + 1). Anal. ($C_{19}H_{21}NO_2$·HCl) C, H, N.

The second band to elute from the Chromatotron was the major product and was the syn isomer **12b**, as confirmed by subsequent NOESY NMR experiments. The free base was a yellow oil weighing 0.84 g (63%). It was taken up into dry ether and converted to its hydrochloride salt by the addition of 1 N HCl in anhydrous ethanol. After solvent removal in vacuo, the solid was crystallized from ethanol−ether to give fine white crystals of **12b**·HCl: mp 269−270 °C; ^{1}H NMR (free base in $CDCl_3$) δ 1.33 (q, 1, ArCHC*H*$_2$CHN, *J* = 11.1 Hz), 1.80 (bs, 1, N*H*), 2.29−2.39 (m, 2, a mixture of ArCHC*H*$_2$CHN and ArC*H*$_2$CHN), 3.06−3.26 (m, a mixture of ArC*H*$_2$CHN and ArCH$_2$C*H*N), 3.38 (m, 1, ArC*H*), 3.78 (s, 3, ArOC*H*$_3$), 3.9−4.1 (a mixture of s, 2, a mixture of OC*H*$_2$ and the benzylic methylene), 4.73 (t, 1, OC*H*$_2$, *J* = 8.2 Hz), 6.53 (d, 1, Ar*H*, *J* = 8.4 Hz), 6.57 (d, 1, Ar*H*, *J* = 8.4 Hz), 7.32 (m, 5, Ar*H*, benzylic); CIMS *m*/*z* 296 (M + 1). Anal. ($C_{19}H_{21}NO_2$·HCl) C, H, N.

***anti*-4-Amino-6-methoxy-2a,3,4,5-tetrahydro-2*H*-naphtho[1,8-*bc*]furan Hydrochloride (4a).** To a suspension of 100 mg of 10% Pd−C in 100 mL of absolute methanol was added 160 mg (0.48 mmol) of **12a**·HCl in a Parr flask. The mixture was shaken under 60 psi of H_2 for 12 h and then filtered through Celite. Removal of solvent under reduced pressure gave a clean-white solid that was recrystallized from methanol−ethyl acetate−ether to yield 97 mg (84%) of **4a**·HCl as fine white crystals: mp 229 °C; ^{1}H NMR (free base in $CDCl_3$) δ 1.35 (bs, 2, N*H*$_2$), 1.45 (qd, 1, ArCHC*H*$_2$CHN, *J* = 12.1, 2.9 Hz), 1.98 (dt, 1, ArCHC*H*$_2$CHN, *J* = 12.5, 4.5 Hz), 2.53 (d, 1, ArC*H*$_2$CHN, *J* = 18.2 Hz), 2.71 (dd, 1, ArC*H*$_2$CHN, *J* = 18.2, 4.5 Hz), 3.59 (m, 1, ArC*H*), 3.62 (m, 1, ArCH$_2$C*H*N), 3.69 (s, 3, ArOC*H*$_3$), 3.9 (dd, 1, OC*H*$_2$, *J* = 12.3, 8.0 Hz), 4.70 (t, 1, OC*H*$_2$, *J* = 8.0 Hz), 6.45 (d, 1, Ar*H*, *J* = 8.5 Hz), 6.50 (d, 1, Ar*H*, *J* = 8.5 Hz); CIMS *m*/*z* 206 (M + 1), 189. Anal. ($C_{12}H_{15}NO_2$·HCl) C, H, N.

***syn*-4-Amino-6-methoxy-2a,3,4,5-tetrahydro-2*H*-naphtho[1,8-*bc*]furan Hydrochloride (4b).** In a method identical to that for the preparation of the anti isomer **4a**·HCl above, 550 mg (1.66 mmol) of **12b**·HCl was added to a suspension of 150 mg of 10% Pd−C in 100 mL of absolute methanol and shaken under 60 psi of H_2 for 12 h. Filtration through Celite and removal of solvent under reduced pressure gave a clean-white solid that was recrystallized from methanol−ethyl acetate−ether to yield 381 mg (95%) of **4b**·HCl as fine white crystals: mp >310 °C; ^{1}H NMR (free base in $CDCl_3$) δ 1.19 (q, 1, ArCHC*H*$_2$CHN, *J* = 11.6 Hz), 1.42 (bs, 2, N*H*$_2$), 2.09 (dt, 1, ArCHC*H*$_2$CHN, *J* = 11.6, 3.9 Hz), 2.18 (dd, 1, ArC*H*$_2$CHN, *J* = 17.3, 11.9 Hz), 3.01 (dd, 1, ArC*H*$_2$CHN, *J* = 17.4, 6.3 Hz), 3.21 (m, 1, ArCH$_2$C*H*N), 3.38 (m, 1, ArC*H*), 3.69 (s, 3, ArOC*H*$_3$), 3.9 (dd, 1, OC*H*$_2$, *J* = 12.3, 8.3 Hz), 4.70 (t, 1, OC*H*$_2$, *J* = 8.2 Hz), 6.45 (d, 1, Ar*H*, *J* = 8.5 Hz), 6.49 (d, 1, Ar*H*, *J* = 8.5 Hz); CIMS *m*/*z* 206 (M + 1), 189. Anal. ($C_{12}H_{15}NO_2$·HCl) C, H, N.

***syn*-4-Amino-8-bromo-6-methoxy-2a,3,4,5-tetrahydro-2*H*-naphtho[1,8-*bc*]furan (4d).** To an ice-bath-cooled solution of 85 mg (0.41 mmol) of the naphthofuran **4b** in 5 mL of $CHCl_3$ was slowly added via syringe through a septum 2.1 mL of a 0.20 N solution of Br_2 in $CHCl_3$. The mixture was stirred for 30 min, during which time the solution turned orange and a white precipitate formed. Ether was added, and the solid was collected by suction filtration, rinsing well with ether on the filter to afford 119 mg (79%) of **4d**·HBr as a pristine-white solid. The solid was recrystallized from methanol to give fine white crystals: mp 277−278 °C; ^{1}H NMR (HBr salt in CD_3OD) δ 1.45 (q, 1, ArCHC*H*$_2$CHN, *J* = 11.7 Hz), 2.35 (m, 1, ArCHC*H*$_2$CHN), 2.40 (ddd, 1, ArC*H*$_2$CHN, *J* = 17.4, 10.3, 1.9 Hz), 3.1 (dd, 1, ArC*H*$_2$CHN, *J* = 17.4, 6.6 Hz), 3.55 (m, 2, a mixture of ArC*H* and ArCH$_2$C*H*N), 3.70 (s, 3, ArOC*H*$_3$), 3.95 (dd, 1, OC*H*$_2$, *J* = 12.2, 8.4 Hz), 4.82 (t, 1, OC*H*$_2$, *J* = 8.4 Hz), 6.68 (s, 1, Ar*H*); CIMS *m*/*z* 284, 286 (M + 1), 206. Anal. ($C_{12}H_{14}BrNO_2$·HBr) C, H, N.

***anti*-4-Amino-8-bromo-6-methoxy-2a,3,4,5-tetrahydro-2*H*-naphtho[1,8-*bc*]furan (4c).** In a manner identical to that for the preparation of the syn isomer **4d** above, 30 mg (0.16 mmol) of *anti*-naphthofuran **4a** and 1.5 mL of 0.11 N Br_2−$CHCl_3$ solution in 5 mL of $CHCl_3$ gave 40 mg (69%) of **4c**·HBr as a white solid that was purified by recrystallization from methanol: mp 269−270 °C; ^{1}H NMR (HBr salt in CD_3OD) δ 1.65 (ddd, 1, ArCHC*H*$_2$CHN, *J* = 15.9, 12.4, 3.6 Hz), 2.30 (dt, 1, ArCHC*H*$_2$CHN, *J* = 14, 4.4 Hz), 2.65 (d, 1, ArC*H*$_2$CHN, *J* = 19.0 Hz), 2.92 (ddd, 1, ArC*H*$_2$CHN, *J* = 19, 6.5, 1.8 Hz), 3.45−3.65 (m, 1, ArC*H*), 3.68 (s, 3, ArOC*H*$_3$), 3.92 (m, 1, ArCH$_2$C*H*N), 4.0 (dd, 1, OC*H*$_2$, *J* = 11.8, 8.4 Hz), 4.82 (t, 1, OC*H*$_2$, *J* = 8.4 Hz), 6.70 (s, 1, Ar*H*); CIMS *m*/*z* 284, 286 (M + 1), 206. Anal. ($C_{12}H_{14}BrNO_2$·HBr) C, H, N.

***syn*-*N*,*N*-Dimethyl-4-amino-6-methoxy-2a,3,4,5-tetrahydro-2*H*-naphtho[1,8-*bc*]furan (4e).** Sodium cyanoborohydride, 78 mg (1.24 mmol), was added to a stirred mixture of 70 mg (0.31 mmol) of **4b**·HCl in 5 mL of methanol and 0.5 mL of 37% aqueous formaldehyde under N_2. The clear mixture was stirred at room temperature for 26 h, and the methanol was removed under reduced pressure. The aqueous residue was diluted with 2.5 N NaOH and extracted with 4 × 15 mL of CH_2Cl_2. The extracts were combined, washed with 2 × 15 mL of 5% $NaHCO_3$ solution and 25 mL of brine, dried over $MgSO_4$, and filtered through Celite. Removal of the solvent on the rotary evaporator gave a yellow oil that was purified by applying it to a 1-mm Chromatotron plate and eluting with CH_2Cl_2 under a N_2−NH_3 atmosphere. This gave 36 mg (50%) of the free base **4e** as a pale-yellow oil. The oxalate salt was formed by dissolving the base in ether and adding 1 equiv of oxalic acid that had been dissolved in a small amount of ether. The precipitated salt was collected on a Büchner funnel and washed well with ether to yield 48 mg (49%) of **4e**·$C_2H_2O_4$ as a white solid: mp 186−187 °C; ^{1}H NMR (free base in $CDCl_3$) δ 1.35 (q, 1, ArCHC*H*$_2$CHN, *J* = 11.4 Hz), 2.24 (ddd, 1, ArCHC*H*$_2$CHN, *J* = 11.3, 4.2, 3 Hz), 2.40 (s, 6, N(C*H*$_3$)$_2$), 2.52 (dd, 1, ArC*H*$_2$CHN, *J* = 15.8, 10.6 Hz), 2.9−3.1 (m, 2, a mixture of ArC*H*$_2$CHN and ArCH$_2$C*H*N), 3.40 (m, 1, ArC*H*), 3.77 (s, 3, ArOC*H*$_3$), 4.0 (dd, 1, OC*H*$_2$, *J* = 12.1, 8.2 Hz), 4.82 (t, 1, OC*H*$_2$, *J* = 8.2 Hz), 6.52 (d, 1, Ar*H*, *J* = 8.6 Hz), 6.56 (d, 1, Ar*H*, *J* = 8.6 Hz); CIMS *m*/*z* 234 (M + 1), 205, 162. Anal. ($C_{14}H_{19}NO_2$·$C_2H_2O_4$) C, H, N.

***syn*-*N*,*N*-Dipropyl-4-amino-6-methoxy-2a,3,4,5-tetrahydro-2*H*-naphtho[1,8-*bc*]furan (4f).** Sodium cyanoborohydride, 130 mg (2.07 mmol), was added to a stirred mixture of 100 mg of **4b**·HCl in 3 mL of methanol and 0.3 mL (4.14 mmol) of propionaldehyde under N_2. The white slurry was stirred at room temperature for 72 h, and the methanol was removed under reduced pressure. The residue was diluted with 2.5 N NaOH and extracted with 4 × 15 mL of CH_2Cl_2. The extracts were combined, washed with 2 × 15 mL of 5% $NaHCO_3$ solution and 25 mL of brine, dried over $MgSO_4$, and filtered through Celite. Removal of solvent on the rotary evaporator gave an oil that was purified by applying it to a 1-mm Chromatotron plate and eluting with CH_2Cl_2 under a N_2−NH_3 atmosphere. Concentration of the eluted fractions gave a quantitative yield of the free base as a yellow oil. Several attempts were made to crystallize the product as its hydrochloride or methanesulfonate salt, but these formed gumlike solids that could not be induced to crystallize. The product finally was precipitated as its oxalate salt and recrystallized from ethyl acetate−ether−hexane to give **4f**·$C_2H_2O_4$ as a white granular solid: mp 132−133 °C; ^{1}H NMR (oxalate in CD_3OD) δ 1.01 (t, 6, N(CH$_2$CH$_2$C*H*$_3$)$_2$, *J* = 7.4 Hz), 1.7 (q, 1, ArCHC*H*$_2$CHN, *J* = 11.4 Hz), 1.73 (m, 4, N(CH$_2$C*H*$_2$CH$_3$)$_2$) 2.45 (dt, 1, ArCHC*H*$_2$CHN, *J* = 10.9, 3.8 Hz), 2.78 (dd, 1, ArC*H*$_2$CHN, *J* = 17.3, 11.8 Hz), 3.1−3.26 (m, 5, a mixture of ArC*H*$_2$CHN and N(C*H*$_2$CH$_2$CH$_3$)$_2$), 3.5 (m, 1, ArCH$_2$C*H*N), 3.77 (s, 3, ArOC*H*$_3$), 4.01 (m, 1, ArC*H* and OC*H*$_2$), 4.78 (t, 1, OC*H*$_2$, *J* = 8.2 Hz), 6.54 (d, 1, Ar*H*, *J* = 8.5 Hz), 6.63 (d, 1, Ar*H*, *J* = 8.5 Hz); CIMS *m*/*z* 290 (M + 1). Anal. ($C_{18}H_{27}NO_2$·$C_2H_2O_4$) C, H, N.

Pharmacological Methods. Drug Discrimination Studies. The procedures for the drug discrimination assays were essentially as described in previous reports.[10,29−31] Twenty male Sprague−Dawley rats (Harlan Laboratories, Indianapolis, IN), weighing 200−220 g at the beginning of the study, were 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 °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) were enclosed within sound-attenuated cubicles with fans both for ventilation and as a source of background white noise. A white 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. Control of reinforcement and acquisition of data was accomplished through a Med Associates interface to an ISA microcomputer, using custom software written in this laboratory.

A fixed ratio (FR) 50 schedule of food reinforcement (Bioserv 45 mg of dustless pellets) in a two-lever paradigm was used. Initially, rats were shaped to lever-press on an FR1 schedule so that one food pellet was dispensed for each press. To avoid positional preference, half of the rats were trained on drug-L (left), saline-R (right) and the other half on drug-R, saline-L. Training sessions lasted 15 min and were conducted at the same time each 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). Afterward, 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 a FR50. Once at the FR50, training continued until an accuracy of at least 85% (number of correct presses × 100/number 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 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 that completely substituted for the training drug (at least one dose resulted in a %SDL = 80% or higher), the method of Litchfield and Wilcoxon[32] was used to determine the ED_{50} (log−probit analysis as the dose producing 50% 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 ED_{50} was calculated.

Serotonin Radioreceptor Assays Using Rat Brain Homogenate. Male Sprague−Dawley rats (Harlan Laboratories, Indianapolis, IN) weighing 175−199 g were used. The animals were kept in groups of five rats per cage, using the conditions described above, except for free access to food and water.

[^{3}H]Ketanserin and [^{3}H]-8-OH-DPAT were purchased from New England Nuclear (Boston, MA) at specific activities of 61 and 135.5−216 Ci/mmol, respectively. Cinanserin was a gift from the E. R. Squibb & Sons, Inc. (New Brunswick, NJ), and 5-HT was purchased from Sigma Chemical Co. (St. Louis, MO).

The procedures of Johnson et al.[33] were employed. Briefly, the frontal cortex or hippocampal brain regions from 20−40 rats were pooled and homogenized (Brinkman Polytron, setting 6 for 2 × 20 s) in 4 or 8 volumes of 0.32 M sucrose for frontal cortex or hippocampus, respectively. The homogenates were centrifuged at 36000*g* 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 36500*g* for 20 min with an intermittent wash. The resulting pellet was resuspended in 50 mM Tris-HCl with 0.5 mM Na_2EDTA, 0.1% Na ascorbate, and 10 mM pargyline HCl (pH 7.4). In experiments with [^{3}H]ketanserin or [^{3}H]-8-OH-DPAT, either 5.7 mM $CaCl_2$ or 10 mM $MgCl_2$ 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 in triplicate using the appropriate buffer to which 200−400 μg 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 using 10 μM cinanserin in the [^{3}H]ketanserin binding study and 10 μM 5-HT in the [^{3}H]-8-OH-DPAT binding study. Filters were air-dried, placed into scintillation vials with 10 mL of Ecolite scintillation cocktail, and allowed to sit overnight before counting for tritium.

Radioligand Competition Experiments Using Cloned Human 5-HT$_2$ Receptors. All chemicals were obtained from the sources previously described.[34] [^{3}H]-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. [^{3}H]Mesulergine (76−83 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, IL). [^{125}I]-DOI (2200 Ci/mmol), [^{3}H]rauwolscine (70−90 Ci/mmol), and [^{3}H]ketanserin (60−78.7 Ci/mmol) were purchased from DuPont-NEN (Wilmington, DE).

Membranes were prepared essentially as previously described 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$_{2C}$ receptors.[34] 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 per 0.5 × 10^9 cells 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 39000*g* 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 39000*g* for 10 min at 4 °C. This pellet was resuspended and centrifuged one more time, and the final membrane pellet was resupended (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 $MgCl_2$ and EDTA for [^{125}I]DOI binding to 5-HT$_{2A}$ or 5-HT$_{2C}$ receptors, or in Tris-HCl, pH 7.6, for [^{3}H]ketanserin and [^{3}H]mesulergine binding to 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors, respectively.

5-HT$_{2B}$ [^{3}H]-5-HT Binding Studies. Human 5-HT$_{2B}$ receptor binding assays using [^{3}H]-5-HT were performed as previously described.[34] The assay was automated using a Biomek 1000 (Beckman Instruments, Fullerton, CA). [^{3}H]-5-HT in Tris-HCl containing $CaCl_2$, pargyline, and L-ascorbic acid, adjusted to pH 7.4, was added to drug dilutions, spanning 6 log units, in water. Then 200 μL of membrane resuspension (approximately 100−150 μg of protein) was added with mixing followed by incubation for 15 min at 37 °C. The total incubation volume was 800 μL, and all incubations were performed in triplicate. The final concentration of $CaCl_2$, pargyline, Tris, and L-ascorbic acid was 3 mM, 10 μM, 50 mM, and 0.1%, respectively. The assay was terminated by vacuum filtration through Whatman GF/B filters that 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 then were washed rapidly four times with 1 mL of ice-cold wash buffer. The amount of [^{3}H]-5-HT trapped on the filters was determined by liquid scintillation spectrometry (Ready Protein, LS 6000IC, Beckman Instruments, Fullerton, CA). The final [^{3}H]-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 μM 5-HT or 10 μM 1-naphthylpiperazine (1-NP). The amount of protein was determined by the method of Bradford, with bovine serum albumin as the standard.[35]

5-HT$_{2A/2C}$ [^{125}I]DOI Binding Studies. Human 5-HT$_{2A}$ or 5-HT$_{2C}$ binding studies were performed essentially as described for [^{3}H]-5-HT binding to the 5-HT$_{2B}$ receptor with the following exceptions. The assay buffer contained, in final concentration, 10 μM pargyline, 9.75 mM $MgCl_2$, 0.5 mM disodium 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 μg of protein for the 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors, respectively, followed by filtration and washing as described above. The amount of [^{125}I]DOI trapped on the filters was determined using a γ-counter. Nonspecific binding was determined with 10 μM mianserin for 5-HT$_{2C}$ and 1 μM ketanserin for 5-HT$_{2A}$ receptors. The final concentration of [^{125}I]DOI was approximately 0.07−0.15 nM.

[^{3}H]Ketanserin Binding to the Human 5-HT$_{2A}$ Receptor. The assay conditions were essentially as previously described.[36] Assays consisted of 0.8-mL total volume containing 50 mM Tris-HCl, 100 nM prazosin (to block potential binding of [^{3}H]ketanserin to α_1-adrenergic receptors), 0.4−0.5 nM [^{3}H]ketanserin, and varying concentrations of the competing compound of interest (final pH 7.6). Mianserin, 3 μM, 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 [^{3}H]ketanserin trapped on the filters was determined by liquid scintillation spectrometry.

[^{3}H]Rauwolscine Binding to the Human 5-HT$_{2B}$ Receptor. This assay is based on a previously described procedure.[37] Conditions specific to this assay were as follows (all concentrations given as final concentrations): 2 nM [^{3}H]-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 μM 1-naphthylpiperazine.

[^{3}H]Mesulergine Binding to the Human 5-HT$_{2C}$ Receptor. This assay was adapted from that described by Pazos et al.[38] Membranes were prepared as described above. Final concentrations for the 0.8-mL assays were 0.74−0.82 nM [^{3}H]-mesulergine, varying concentrations of competing compound, and 50 nM Tris-HCl, final pH 7.6. Nonspecific binding was determined using 3 μM 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 [^{3}H]ketanserin binding assay described above.

Muscarinic Receptor Studies: 1. Cell Culture. B82 (murine fibroblasts) transfected with the muscarinic M_1−M_5 receptors were prepared as previously described by Lai et al.[39] (for the M_1 receptor), Lai et al.[40] (for the M_2 receptor), and Kashihara et al.[41] (for the M_3−M_5 receptors) and were a generous gift from Dr. Henry Yamamura (University of Arizona). Cells were maintained in DMEM/F12 media supplemented with 5% fetal calf serum, 5% newborn calf serum, and pen/strep. Cells were grown in media supplemented with 500 ng/mL G418 every fourth passage to maintain receptor expression. Cells were grown in either Costar 75-cm^2 flasks (binding studies) or 24-well plates (functional studies) and kept in a humidified incubator maintained at 37 °C with 95% O_2 and 5% CO_2 levels.

2. Membrane Preparation. Confluent flasks of cells were incubated with 7 mL of 5 mM Hepes, 5 mM EDTA (pH 7.4) buffer, homogenized with a Wheaton Teflon-glass homogenizer (7 strokes), and centrifuged at 1000g for 10 min at 4 °C. The resulting supernatant was centrifuged at 27000g for 30 min at 4 °C, and the pellet was resuspended in 10 mM Hepes, 1 mM $MgCl_2$, 10 mM Na/K-PO_4 buffer, pH 7.4, and stored in 1-mL aliquots at −80 °C until use in the binding assays. Aliquots contained approximately 1 mg/mL protein, as measured using the BCA protein assay reagent (Pierce, Rockford, IL).

3. Muscarinic Radioligand Binding Studies. [^{3}H]QNB binding was performed essentially as described in Lai et al.[39] with slight modifications. Membranes were diluted with 50 mM Hepes, 4 mM $MgCl_2$ (pH 7.4), and 100 μL (approximately 25 μg of protein) was incubated with 0.25 nM [^{3}H]QNB and increasing concentrations of competing drugs. Nonspecific binding was determined using 1 μM atropine. Incubations were run in duplicate at 37 °C for 60 min in a final volume of 1 mL. Tubes then were filtered rapidly through Skatron glass fiber filter mats (cat. 11734), and rinsed with 5 mL of ice-cold buffer using a Skatron micro cell harvester (Skatron Instruments Inc., Sterling, VA). Filters were allowed to dry and then punched into scintillation vials (Skatron Instruments Inc., Sterling, VA). OptiPhase HiSafe II scintillation cocktail (1 mL) was added to each vial. After the vials shook for 30 min, radioactivity in each sample was determined on an LKB Wallac 1219 Rackbeta liquid scintillation counter (Wallac Inc., Gaithersburg, MD).

Inositol Lipid Hydrolysis Studies. These experiments were performed as described by Mei et al.[42] Briefly, M_1-B82 cells were plated onto 24-well plates and grown to ~50% confluency. Cells then were incubated overnight with 0.2 μM myo-2-[^{3}H]inositol (15 Ci/mmol; New England Nuclear) in 0.5 mL of media. Excess myo-2-[^{3}H]inositol was removed, and cells were rinsed with 1 mL of plain media. Following this rinse, cells were incubated with 10 mM LiCl for 10 min, and the reaction was initiated by the addition of carbachol or other test drugs. The reaction was terminated 60 min later by aspirating the media and adding 0.31 mL of methanol. The cells were scraped and placed in 1.7-mL centrifuge tubes. The wells were rinsed with an additional 0.31 mL of methanol, which was then added to the centrifuge tubes. Chloroform (0.62 mL) and distilled water (0.31 mL) were added to each tube; the tubes were shaken for 15 s and then centrifuged for 5 min at 1000g. The top phase (0.7 mL) was then added to 2 mL of distilled water, and this mixture was passed over minicolumns consisting of 2 mL of a 10% slurry of anion-exchange resin in formate form (AG 1-X8, 100−200 mesh; Bio-Rad Labs). The columns were washed five times with 5 mL of distilled water. The [^{3}H]IP$_1$ was then eluted using 2 mL of 0.2 M ammonium formate/0.1 M formic acid. Nine milliliters of AquaMix (ICN Radiochemicals, San Diego, CA) then was added to the eluates that were then quantified by liquid scintillation spectroscopy.

cAMP Assay. B82 cells transfected with the M_2 receptor were plated onto 24-well plates and grown to ca. 90% confluency. Duplicate wells were run for each drug concentration. cAMP levels were increased by the addition of 100 μM forskolin in media supplemented by 500 μM isobutyl methylxanthine (IBMX), after which the ability of drugs to inhibit cAMP synthesis elevation was assessed. Carbachol was run as a control in each assay. Cells were incubated with drugs for 10 min at 37 °C, after which the reaction was terminated by the addition of 0.5 mL of ice-cold 10 mM Tris/2 mM EDTA. Cells were then scraped and placed into 1.7-mL centrifuge tubes. The wells were rinsed with an additional 0.5 mL of Tris/EDTA buffer that was added to the centrifuge tubes before they were spun at 14000*g* for 5 min to pellet large debris. The level of cAMP was determined as described below.

Radioimmunoassay of cAMP. The concentration of cAMP in each sample was determined with an RIA of acetylated cAMP, modified from that previously described.[43] Iodination of cAMP was performed using a method described previously.[44] Assay buffer was 50 mM sodium acetate buffer with 0.1% sodium azide (pH 4.75). Standard curves of cAMP were prepared in buffer at concentrations of 2−500 fmol/assay tube. To improve assay sensitivity, all samples and standards were acetylated with 10 μL of a 2:1 solution of triethylamine−acetic anhydride. Samples were assayed in duplicate. Each assay tube contained 10 μL of sample, 100 μL of buffer, 100 μL of primary antibody (sheep anti-cAMP, 1:100000 dilution with 1% BSA in buffer), and 100 μL of [^{125}I]cAMP (50 000 dpm/100 μL of buffer) giving a total assay volume of ca. 300 μL. Tubes were vortexed and stored at 4 °C overnight (approximately 18 h). Antibody-bound radioactivity was then separated by the addition of 10 μL of BioMag rabbit anti-goat IgG (Advanced Magnetics, Cambridge, MA), followed by vortexing and further incubation at 4 °C for 1 h. To these samples was added 1 mL of 12% poly(ethylene glycol)/50 mM sodium acetate buffer (pH 6.75), and all tubes were centrifuged at 1700*g* for 10 min. Supernatants were aspirated, and radioactivity in the resulting pellet was determined using an LKB Wallac γ-counter (Wallac Inc., Gaithersburg, MD).

Data Analysis. The resulting data from each competition assay were analyzed by nonlinear regression using the model for sigmoid curves in the curve-fitting program Prism (Graphpad Inc., San Francisco, CA). This program generated IC_{50} values and a Hill coefficient for each curve. The apparent affinities ($K_{0.5}$) were calculated by the Cheng−Prusoff equation.[45]

For M_1 functional data, basal levels of IP_1 were subtracted and the data were then expressed as percent carbachol stimulation at 100 μM, to minimize interassay variability. Values were then averaged across assays. For M_2 functional data, baseline values of cAMP were subtracted from the total amount of cAMP produced for each drug condition. To minimize interassay variation, data for each drug were expressed relative to the percentage of the stimulation produced by 100 μM forskolin in each assay. These values then were averaged to obtain the cAMP stimulation for each drug.

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