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

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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-HT2A and 5-HT2C receptor subtypes.1–10 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.11–13 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-HT2A 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, 1a has been incorporated into amino-tetralin 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-HT2A 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...
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. In 4, these electron pairs are directed anti to the side chain, and this compound was equipotent to the untethered 5-HT₂ agonist (unpublished data). These results prompted us to synthesize the highly rigidified difuranyl derivatives 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-(o-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 nitropropane 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
as before afforded 8-methyl compound to methods described previously (Table 1). For those saline from those of LSD tartrate (0.08 mg/kg) according trained to discriminate the effects of ip injections of lever drug discrimination assay in a group of rats Pharmacology benzodifuran completely reduced to a methyl, and the resulting compound was found to be exceptionally potent in the initial behavioral and in vitro assays, was also evaluated in cells expressing cloned human 5-HT2A, 5-HT2B, and 5-HT2C 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. This assay system has been used extensively to model human hallucinogenic effects by studying the LSD-like discriminative stimulus properties produced in animals. Previous DD studies have established that the discriminative behavioral cue is mediated primarily by the stimulation of 5-HT2 receptors. Thus, agents exhibiting in vivo hallucinogenic activity typically can be shown to activate serotonin 5-HT2A and 5-HT2C receptors in vitro, and no agonists have yet been found that can significantly differentiate between these subtypes.

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 Rat Cortical 5-HT2A and [3H]8-OH-DPAT-labeled Rat Hippocampal 5-HT1A Receptors (Kd ± SEM)

<table>
<thead>
<tr>
<th>Drug</th>
<th>ED50 (mmol/kg)</th>
<th>95% CI</th>
<th>n</th>
<th>5-HT2A sites (nM)</th>
<th>5-HT1A sites (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1b</td>
<td>1.12</td>
<td>0.86–1.46</td>
<td>8–12</td>
<td>22 ± 3</td>
<td>6.1 ± 0.5</td>
</tr>
<tr>
<td>4</td>
<td>0.57b</td>
<td>0.29–1.09</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>7a</td>
<td>64% @4.0 pSd</td>
<td>10–13</td>
<td>2010 ± 83</td>
<td>29 ± 2</td>
<td></td>
</tr>
<tr>
<td>7b</td>
<td>0.061</td>
<td>0.031–0.12</td>
<td>7–15</td>
<td>18 ± 1</td>
<td>4.3 ± 0.4</td>
</tr>
<tr>
<td>7c</td>
<td>1.43</td>
<td>0.45–4.5</td>
<td>7–14</td>
<td>2300 ± 170</td>
<td>5.2 ± 0.4</td>
</tr>
<tr>
<td>7d</td>
<td>0.31</td>
<td>0.08–1.14</td>
<td>8–12</td>
<td>34 ± 2</td>
<td>0.80 ± 0.06</td>
</tr>
<tr>
<td>7e</td>
<td>0.78</td>
<td>0.48–1.22</td>
<td>8–9</td>
<td>NT*</td>
<td>NT</td>
</tr>
<tr>
<td>LSD</td>
<td>0.040</td>
<td>0.03–0.05</td>
<td>15</td>
<td>4.4 ± 0.2</td>
<td>5.1 ± 0.4 nM</td>
</tr>
</tbody>
</table>

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

Pharmacology

Compounds 7a–e were initially evaluated in a 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). For those compounds that completely substituted for LSD, potencies 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-HT1A, and 5-HT2A receptor sites (Table 1). 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 Kd.

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-HT2A and 5-HT1A 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-HT2A and 5-HT1A receptors. None of the compounds had significant affinity for 5-HT1A sites. In agreement with the established hallucinogenic phenylalkylamine 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-HT2A 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, 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...
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-HT2A receptors, indicating that the benzodifuran structure must be highly complementary to these antagonist-labeled binding sites.

Given the exceptional activity of the benzodifuran analogues in the rat assays, the most potent compound of the series (7b) was also evaluated in cells expressing cloned human 5-HT2A, 5-HT2B, and 5-HT2C 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-HT2A site, the Ki 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 (TM3), 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-HT2A 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 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 (0.00 ppm, δ). Abbreviations used in NMR analyses are as follows: s = singlet, d = doublet, t = triplet, dq = 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 ±0.4% of the calculated values unless otherwise noted. Thin-layer chromatography (TLC) was typically performed using Baker-flex silica gel (IB2, plastic-backed plates with fluorescent indicator (2.5 × 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 (Chromatron; Harrison Research, Palo Alto, CA) were prepared from silica gel 60 PF254 containing gypsum. Most reactions were carried out under an inert atmosphere of dry nitrogen.

**1,4-Bis(2-chloroethoxy)benzene (8).** 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 EtO and H2O. The EtO 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 MgSO₄ and filtered. The solvent was removed in vacuo, yielding 1.5 g (92%) of an off-white solid. An analytical sample was recrystallized from chloroform (m.p. 156 °C, lit. mp 153 °C). Removal of solvent under reduced pressure and the residue was taken up in 100 mL of EtOH. After 1 h, the mixture was filtered and the solid was washed with 3 × 50 mL of EtOH, and the organic fractions were combined. The organic extract was washed with 3 × 50 mL of H₂O, and 100 mL of brine. The organic phase was washed with 3 × 50 mL of CH₂Cl₂, and the organic fractions were combined, washed with brine, dried (MgSO₄), and filtered through a pad of Celite and silica gel. Removal of solvent under reduced pressure and under high vacuum gave 2.9 g (62%) 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 (CDCl₃) δ 3.20 (td, J = 8.9, 1.1 Hz), 3.45 (t, J = 8.9 Hz), 4.70 (t, J = 8.9 Hz), 6.80 (s, 1 H, ArCCH₂), 6.60 (s, 1 H, CH₃), 6.90 (s, 1 H, CH₃), 7.50 (s, 1 H, CH₃), 7.70 (s, 1 H, CH₃), 7.90 (s, 1 H, CH₃), 8.00 (s, 1 H, CH₃), 10.25 (s, 1 H, COOH); CIMS m/z 191 (M + 1). Anal. (C₁₃H₁₀O₅)C, H, N.

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 nitroethane 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 M H₂O, 3 × 50 mL of NaHCO₃, and 50 mL of brine. The organic phase was washed with 3 × 50 mL of CH₂Cl₂, and the organic fractions were combined, washed with brine, dried (MgSO₄), and filtered through a pad of Celite and silica gel. Removal of solvent under reduced pressure and 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 (CDCl₃) δ 3.20 (td, J = 8.9, 1.1 Hz), 3.45 (t, J = 8.9 Hz), 4.70 (t, J = 8.9 Hz), 6.80 (s, 1 H, ArCCH₂), 6.60 (s, 1 H, CH₃), 6.90 (s, 1 H, CH₃), 7.50 (s, 1 H, CH₃), 7.70 (s, 1 H, CH₃), 7.90 (s, 1 H, CH₃), 8.00 (s, 1 H, CH₃), 10.25 (s, 1 H, COOH); CIMS m/z 191 (M + 1). Anal. (C₁₃H₁₀O₅)C, H, N.

4-(2-Nitro-1-ethyl)-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 bath of 100 °C for 1 h. The nitromethane was then removed under reduced pressure, and the solid residue was partitioned between CH₂Cl₂ and water. The layers were separated, and the organic phase was washed with 3 × 50 mL of 3 N HCl and 2 × 50 mL of water, and 50 mL of brine. The ether solution was dried over MgSO₄ 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 (93%) of the nitrothene 13 as pumpkin-orange needles: mp 134 °C; ¹H NMR (CDCl₃) δ 3.25 (t, J = 8.7 Hz), 3.28 (t, 2 ArOCH₂CH₂J = 8.7 Hz), 4.73 (t, 2 ArOCH₂CH₂J = 8.7 Hz), 4.75 (t, 2 ArOCH₂CH₂J = 8.7 Hz), 6.70 (s, 1 H, Ar), 7.85 (d, 1 H, Ar), 13.4 °C, 8.05 (d, 1 H, Ar), 13.4 °C; HCl m/z 234 (M + 1), 216. Anal. (C₁₂H₁₁NO₄)C, H, N.

1-(2,3,6,7-Tetrahydrobenzoyl)[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 nitrothene 13 in 25 mL of dry THF was added dropwise to a stirred suspension of 0.31 g (7.58 mmol) of LiAlH₄ in 50 mL of dry THF under N₂. The reaction mixture was heated at reflux over an oil bath for 3 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 CH₂Cl₂. The volatiles were removed from the rotary evaporator, 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 CH₂Cl₂. The organic extracts were combined, washed with brine, dried (MgSO₄), 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 under reduced pressure gave 0.56 g (85%) of the free amine 8 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 give 7b–HCl as a yellow crystalline solid: mp 244–245 °C; ¹H NMR (free base in CDCl₃) δ 1.16 (d, J = 6.3 Hz, 3 ArCH₂CH₂), 1.55 (bs, 2 N₃H₃), 2.58 (m, 2 ArOCH₂CH₂), 3.13 (q, J = 4.0, ArOCH₂CH₂), 3.22 (m, 1 H, ArOCH₂CH₂), 4.50 (q, 4 ArOCH₂CH₂), 6.52 (s, 1, ArH); CIMS m/z 220 (M+1), 203, 176. Anal. (C₁₂H₁₁NO₄)HCl C, H, N.
acid was treated with 2.80 mL of a 0.885 N Br2 in HOAc under 60 psig of H2 for 24 h and then filtered through Celite to give (0.037 mol) of the benzaldehyde.

Absolute ethanol in a Parr hydrogenation flask was added 7.0 g (0.037 mol) of the benzaldehyde. The mixture was stirred under N2 for 5 min, and 1.33 mL of ice and then the reaction quenched with the addition of 50 mL of 3 N HCl. The hydrochloride salt was precipitated from the filter, and dissolved in 75 mL of 3 N HCl. The acidic solution was washed with 3 (× 30 mL of 3% sodium bicarbonate, and brine, dried over MgSO4, × 20 mL of CH2Cl2. The combined extracts were washed with 3 N HCl, 2 (× 20 mL of 3NHCl, 2 (× 30 mL of 3NHCl, 2 (× 20 mL of CH2Cl2. The combined extracts were washed with brine, dried (MgSO4), 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 hydrayous 1 M solution in ethanol. The salt was recrystallized from ethanol–ethyl acetate to give 7e-HCl as very flufly, white crystals: mp 275–276 °C; H NMR (free base in CDCl3) δ 1.52 (bs, 2, NH2), 2.65 (t, 2, ArCH2-CH2-N, J = 6.9 Hz), 2.90 (t, 2, ArCH2CH2-N, J = 6.9 Hz), 3.20 (m, 4, ArOCH2CH2), 4.60 (pair of superimposed triplets, 4, ArOCH2CH2); CIMS m/z 284, 286 (M + 1), 267, 269, 254, 256, 247. Anal. (C12H12NO2 BrCl) C, H, N.

**Pharmacology Methods: Drug Discrimination Studies.** The procedures for the drug discrimination assays were essentially as described in previous reports. 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 supplement of Purina Lab Blox was used 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) consisted of modular two-lever, two-food-reward enclosures. This equipment was used 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. 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 (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. A partial substitution was defined as 60% of the test drug. "No substitution" is defined as 59% saturation of the receptor of interest were grown in suspension and harvested by centrifugation. The homogenate was then incubated at 37 °C for 10 min and the resulting pellets were suspended using a Tissumizer, setting 150 µg of protein. The homogenate was then incubated at 37 °C. The combination of hypotonic buffer 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 suspension (approximately 100–150 µg of protein) was added with mixing and incubated for 15 min at 37 °C. The total incubation volume was 800 µL, and all incubations were performed in triplicate. The final concentration of CaCl2, 5-HT in the [3H]-8-OH-DPAT binding study. The assay was terminated by vacuum filtration through Whatman GF/F filters which had been presoaked with 0.5% poly(ethyleneimine) (w/v) and precooled to +4 °C. The filter paper was removed from the filter holder, dried, and placed in scintillation vials with 10 mL of EcoLite scintillation cocktail, allowed to sit overnight, and counted at an efficiency of 37% for tritium, and directly counted in a gamma counter for [125I]ligand and at an efficiency of 79.4%.

Radioligand Competition Experiments Using Cloned Human Receptors. All chemicals were obtained from the sources previously described. [3H]-5-HT was purchased from DuPont-NEN (Wilmington, DE) or Amersham Corp. (Arlington Heights, IL) at 22.8–26.7 Ci/mmol, respectively. [3H]-8-OH-DPAT (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 described using AV12 cell lines (Syrian hamster fibroblast, ATCC no. CRL 9595) stably transformed with the human 5-HT2A, 5-HT2B, or 5-HT2C 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 −78 °C. The tissue 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-HT2B receptor, in Tris HCl, pH 7.4, containing MgCl2 and EDTA for 5-HT2A or 5-HT2C receptors, or in Tris HCl, pH 7.6, for [3H]ketanserin and [3H]mesulergine binding to 5-HT2A and 5-HT2C receptors respectively.

5-HT2B [3H]-5-HT Binding Studies. Human 5-HT2B receptor binding assays were performed using [3H]-5-HT as previously described. 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 µL of membrane suspension (approximately 100–150 µg of protein) was added with mixing and incubated for 15 min at 37 °C. The total incubation volume was 800 µL, and all incubations were performed in triplicate. The final concentration of [3H]-5-HT was then filtered and analyzed using radioligand displacement spectrometry (Ready Protein, LS 6000C, 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 µM 5-HT or 10 µM 1-naphthalene acetic acid (1-NP). The amount of protein was determined by the method of Bradford, with bovine serum albumin as the standard.
5-HT<sub>2A</sub> and 5-HT<sub>2C</sub> [¹²⁵I]DOI Binding Studies. Human 5-HT<sub>2A</sub> or 5-HT<sub>2C</sub> binding studies were performed essentially as described for [³H]-5-HT binding to the 5-HT<sub>2A</sub> receptor with the following exceptions. The assay buffer contained, in final concentration, 10 μM pargylene, 9.75 mM MgCl<sub>2</sub>, 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 μg of protein for the 5-HT<sub>2A</sub> and 5-HT<sub>2C</sub> 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 μM mianserin for 5-HT<sub>2A</sub> and 1 μM ketanserin for 5-HT<sub>2A</sub> receptors. The final concentration of [¹²⁵I]DOI was approximately 0.07–0.15 nM.

[¹²⁵I]Ketanserin Binding to the Human 5-HT<sub>2A</sub> Receptor. Membranes were prepared as described above, and the assay conditions were essentially as previously described. Assays consisted of 0.8 mL total volume containing 50 mM Tris HCL, 100 mM prazosin (to block potential binding of [³H]-ketanserin to α<sub>1</sub>-adrenergic receptors), 0.4–0.5 nM [¹²⁵I]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 30 min and then rapidly filtered and washed as described above. The amount of [¹²⁵I]ketanserin trapped on the filters was determined by liquid scintillation spectrometry.

[¹²⁵I]Rauwolscine Binding to the Human 5-HT<sub>2B</sub> Receptor. This assay was based on a previously described procedure. Membrane preparation and the filtering binding assay were essentially as described above. Conditions specific to this assay were as follows (all concentrations given as final concentrations): 2 nM [¹²⁵I]rauwolscine, 500 nM efaroxan (to mask rauwolscine binding to α<sub>2</sub>-adrenergic receptors), and 50 mM Tris HCl, final pH 7.4. Tubes were incubated at 37 °C for 20 min and then rapidly filtered and washed as described above. Nonspecific binding was defined in the presence of 10 μM 1-naphthylepiperazone.

[¹²⁵I]Mesulergine Binding to the Human 5-HT<sub>2C</sub> Receptor. This assay was adapted from that described by Pazos et al. Membranes were prepared as described above. Final concentrations for the 0.8 mL assays were 0.74–0.82 nM [¹²⁵I]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 described above. Nonspecific binding was trapped for 30 min at 37 °C, after which the samples were filtered and washed, and radioactivity was determined as described above. Non-selective binding was defined in the presence of 10 μM 5-HT<sub>2C</sub> specific ligands.

Statistical Analysis. Nonlinear regression analysis for the competition curves was performed as previously described to determine IC<sub>50</sub> values. These were converted to K<sub>i</sub> values by the method of Cheng and Prusoff.

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References

(1) For parts 1 and 2 in this series, see refs 18 and 19, respectively.
(2) Abstracted in part from the Ph.D. Thesis submitted by A.P.M. to Purdue University, 1995.


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