Dihydrobenzofuran Analogues of Hallucinogens. 4. Mescaline Derivatives


Department of Medicinal Chemistry and Molecular Pharmacology, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, Indiana 47907, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46285, and Department of Pharmacology, School of Medicine, Vanderbilt University Medical Center, Nashville, Tennessee 37232

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Dihydrobenzofuran and tetrahydrobenzodifuran functionalities were employed as conformationally restricted bioisosteres of the aromatic methoxy groups in the prototypical hallucinogen, mescaline (1). Thus, 4-(2-aminoethyl)-6,7-dimethoxy-2,3-dihydrobenzofuran hydrochloride (8) and 1-(8-methoxy-2,3,5,6-tetrahydrobenzo[1,2-b:5,4-b']difuran-4-yl)-2-aminoethane hydrochloride (9) were prepared and evaluated along with 1 for activity in the two-lever drug discrimination (DD) paradigm in rats trained to discriminate saline from LSD tartrate (0.08 mg/kg). Also, 1, 8, and 9 were assayed for their ability to displace [3H]ketanserin from rat cortical homogenate 5-HT2A receptors and [3H]8-OH-DPAT from rat hippocampal homogenate 5-HT2A receptors. In addition, these compounds were evaluated for their ability to compete for antagonist and agonist binding to cells expressing cloned human 5-HT2A, 5-HT2B, and 5-HT2C receptors. Finally, agonist efficacy was assessed by measurement of phosphoinositide hydrolysis in NIH 3T3 cells expressing the rat 5-HT2A or 5-HT2C receptors. Although 1 fully substituted for LSD in the DD assays (ED50 = 33.5 µmol/kg), neither 8 nor 9 substituted for LSD, with just 50% of the rats administered 8 selecting the drug lever, and only 29% of the rats administered 9 selecting the drug lever. All of the test compounds had micromolar affinity for the 5-HT2A and 5-HT2A receptors in rat brain homogenate. Curiously, the rank order of affinities of the compounds at 5-HT2A sites was opposite their order of potency in the behavioral assay. An evaluation for ability to stimulate phosphoinositide turnover as a measure of functional efficacy revealed that all the compounds were of approximately equal efficacy to serotonin in 5-HT2C receptors. At 5-HT2A receptors, however, 8 and 9 were significantly less efficacious, eliciting only 61 and 45%, respectively, of the maximal response. These results are consistent with the proposed mechanism of action for phenethylamine hallucinogens, that such compounds must be full agonists at the 5-HT2A receptor subtype. In contrast to the 2,5-dimethoxy-substituted phenethylamines, where rigidification of the methoxy groups had no deleterious effect on activity, the loss of activity in the 3,4,5-trioxygenated mescaline analogues may suggest that the 3 and 5 methoxy groups must remain conformationally mobile to enable receptor activation.

Introduction

Peyote (Lophophora williamsii), a member of the Cactaceae family, represents one of the earliest known hallucinogenic plants, the use of which was exclusively limited to the New World. The main constituent of peyote, mescaline (3,4,5-trimethoxy-β-phenethylamine, 1), was first identified by Heffter in 1896 and was recognized as the active hallucinatory principle on the basis of rudimentary pharmacological testing and self experiments. Following the first chemical synthesis of mescaline in 1919, this alkaloid served as the prototypical structure in more than 75 years of structure–activity relationship (SAR) studies linking molecular structure to hallucinogen-like, or psychedelic, activity. In addition, the psychic–behavioral effects produced by mescaline were frequently used as a benchmark against which the effects of other hallucinogens were compared, and the potencies of novel compounds prepared in SAR investigations were for many years expressed in terms of “mescaline units.”

The successive SAR studies of 1-like hallucinogens have ultimately led to agents of general structure 2 (R = H, CH3; X = alkyl, halo, alkoxy, alkylthio, etc.) which currently are among some of the most potent hallucinogens known. As such, compounds 2 have
typically been shown to have low nanomolar affinities for serotonin 5-HT$_2A$ and 5-HT$_2C$ receptors,\textsuperscript{17-25} the primary sites believed to be involved in the mediation of the unique behavioral effects of hallucinogenic drugs.\textsuperscript{26-29} Given the high potency and site-selectivity of 2 derivatives, they have served as convenient prototypes for further SAR studies using rigid analogs to probe the topography of the serotonin 5-HT$_2$ agonist binding sites.\textsuperscript{30,31} Recently, compounds 3,\textsuperscript{32} 4,\textsuperscript{33,34} 5,\textsuperscript{35} and 6 and 7\textsuperscript{36} have all been synthesized and evaluated pharmacologically in our laboratory for hallucinogen-like behavioral activity and serotonin receptor affinities, to study the possible “active” conformations of the aromatic methoxy groups of 2 at the serotonin agonist binding site. We have reported that the dihydrobenzofuran structure serves as an effective, conformationally-restricted bioisostere of the aromatic methoxy group, and that the orientation of alkoxy groups exemplified by compound 7 most likely represents the active conformation of these groups during serotonin receptor activation.\textsuperscript{36}

As an extension of our previous efforts with dihydrobenzofuranyl analogs of hallucinogenic phenethylamines, we sought to apply a similar design rationale and our established synthetic methodology to the mescaline prototype 1, a structure containing three aromatic methoxy groups. Because 1 has been shown to have action at both serotonin and dopamine (DA) receptors,\textsuperscript{37,38} and because hallucinogenic activity has generally been correlated with the overall hydrophobicity of the drug molecule,\textsuperscript{39-41} it was hypothesized that 8 and 9 could ultimately have potential as probes of 5-HT, DA, or other central neurotransmitter receptors. As molecules with increased hydrophobicity relative to 1, they might also be useful in evaluating the role of this physicochemical property in the production of hallucinogenic effects. Thus, we now report on the synthesis and preliminary pharmacological evaluation of rigid analogs of mescaline in which one (8) or two (9) of the three aromatic methoxy groups of 1 are tethered into rotationally constrained dihydrofuran rings.

**Chemistry**

The dihydrobenzofuranyl mescaline analog 8 was synthesized successfully as shown in Scheme 1. Construction of the furan ring was accomplished using the method of Jung and Abrecht.\textsuperscript{42} Thus, 2,3,4-trimethoxybenzoic acid was converted to its corresponding diazomethylketone which was cyclized to the benzofuranone 10 upon treatment with acetic acid. Catalytic hydrogenation of the ketone then afforded the parent dihydrobenzofuran 11 in excellent yield. Initial unsuccessful attempts to elaborate the side chain of 11 involved the standard sequential procedure of electrophilic formylation, condensation of the aldehyde with nitromethane, and subsequent reduction of the nitrostyrene with

\begin{center}
\textbf{Scheme 1}\textsuperscript{a}
\end{center}

\begin{center}
\begin{align*}
\text{H}_2\text{CO} & \xrightarrow{\text{a}} \text{H}_2\text{CO} \xrightarrow{\text{b}} \text{H}_2\text{CO} \xrightarrow{\text{c}} \\
\text{H}_2\text{O} \xrightarrow{\text{d}} \text{H}_2\text{O} \xrightarrow{\text{e}} \text{H}_2\text{O} \xrightarrow{\text{f}} \\
\text{CN} \xrightarrow{\text{g}} \text{CN} \xrightarrow{\text{h}} \text{CN}
\end{align*}
\end{center}

\textsuperscript{a} Reagents: (a) (COCl)$_2$, PhH; CH$_2$N$_2$, Et$_2$O; (b) AcOH; (c) Pd/C, H$_2$, EtOH; (d) BCl$_3$, CH$_2$Cl$_2$; (e) H$_2$CO, (CH$_3$)$_2$NH (aqueous), heat; CH$_3$I, iPrOH; (f) KCN, H$_2$O, heat; (g) CH$_3$I, K$_2$CO$_3$, Me$_2$CO, reflux; (h) Pd/C, H$_2$, EtOH, HCl.
LiAlH₄ as described previously. Because the 5-position of 11 was more susceptible to electrophilic attack, this position was first blocked by aromatic bromination which occurred in a facile and near-quantitative manner. (The bromine atom would then be removed during the subsequent LAH reduction step). Attempts to formylate the 5-bromo compound at the 4-position, however, gave complex mixtures of products that were extremely difficult to purify, and this method was abandoned. A possible explanation for the difficulties with this formylation may lie in the fact that some demethylation of the 7-methoxy group may have occurred in the presence of the Lewis acid catalyst. The steric crowding of this group causes it to lie out of plane, thus decreasing the overlap between the 7-methoxylone pair electrons and the aromatic π system. Consequently, the 7-methoxy group is more basic than a planar aromatic ether and would be expected to be more labile to cleavage by Lewis acids such as the tin(IV) chloride used in this formylation step.

Fortunately, an alternative method for constructing phenethylamines existed in the literature that was well suited for mescaline-like compounds. This methodology effects direct aromatic substitution at the 4-position as shown in Scheme 1. Thus, the 7-methoxy group of 11 was regioselectively demethylated using boron trichloride in dichloromethane. The ring was functionalized at the 4-position of 12 under mild Mannich conditions, and the resulting crude Mannich base was quaternized to the methiodide salt 13 by addition of iodomethane to the reaction mixture. Displacement of trimethylamine from 13 with cyanide ion produced the phenylacetonitrile 14 in good yield. This phenol was then remethylated with iodomethane to give 15, and catalytic hydrogenation of the nitrile in the presence of HCl afforded the desired target compound 8 in good overall yield.

The tetrahydrobenzofuran mescaline analog 9 was synthesized successfully as shown in Scheme 2. Chloromethylation of 1,2,3-trimethoxybenzene according to the method of Dallacker et al. proceeded in fair yield to give the bis-chloromethyl compound 16. Both chlorine atoms of 16 were displaced with cyanide using KCN in DMSO to afford the bis-nitrile 17. After acidic hydrolysis to the corresponding bis-acetic acid 18, reduction using the selective borane-THF method of Yoon et al. gave diol 19 in reasonable yield considering the difficulty of workup for this water-soluble intermediate.

The two dihydrofuran rings were formed simultaneously employing a methodology developed recently in our laboratory. Thus, essentially conducting three steps in one pot, 19 was first tri-demethylated and dibrominated with excess BBr₃, quenching with methanol to avoid aqueous workup of the highly water soluble and unstable pyrogallol intermediate. Removal of methanol under vacuum and immediate reflux with potassium carbonate in acetone led to ring closure to give the cyclized, phenolic intermediate. Once the complete disappearance of the dibromo compound was noted by TLC, iodomethane was added to the reaction mixture to O-methylate the remaining phenolic oxygen, giving 20 in excellent overall yield from 19. Final elaboration of the sidechain then proceeded according to standard methods. Friedel–Crafts formylation of 20 using tin-(IV) chloride catalyst gave aldehyde 21 in modest yield. The low yield was likely due to the partial cleavage of the out-of-plane 8-methoxy group, as discussed above for the synthesis of the monofuran derivative 8. Nitrostyrene 22 was formed by condensation of 21 with nitromethane using ammonium acetate as the catalyst, and the final target compound 9 was obtained in good yield by LAH reduction of 22.
have somewhat increased the necessary pharmacophoric elements, they might substituting for LSD with an ED50 of 33.5 µM for radioligand binding to 5-HT1A and 5-HT2A receptor cAMPal homogenate and 0.75 nM [3H]ketanserin from 5-HT2A and [3H]8-OH-DPAT-labeled rat 5-HT1A receptor competition experiments at [3H]ketanserin-labeled rat in LSD-trained rats and the results of the radioligand competition studies at [3H]ketanserin-labeled rats.

**Results and Discussion**

Table 1. Results of the Drug Discrimination Studies in LSD-Trained Rats and Radioligand Competition Studies at [3H]Ketanserin-Labeled Rat 5-HT2A, and [3H]8-OH-DPAT-Labeled Rat 5-HT1A Receptors

<table>
<thead>
<tr>
<th>Drug</th>
<th>ED50 (µM/kg)</th>
<th>95% CI</th>
<th>n</th>
<th>5-HT2A sites</th>
<th>5-HT1A sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>33.5</td>
<td>(20.9–53.5)</td>
<td>8–12</td>
<td>5500 ± 600</td>
<td>6900 ± 800</td>
</tr>
<tr>
<td>8</td>
<td>50% @ 57.8 NSb</td>
<td>8–12</td>
<td>2500 ± 300</td>
<td>6200 ± 400</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>29% @ 55.2 NSb</td>
<td>8–12</td>
<td>NTc</td>
<td>NTc</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>50% @ 16.2 NSb</td>
<td>6–15</td>
<td>2100 ± 200</td>
<td>6900 ± 800</td>
<td></td>
</tr>
</tbody>
</table>

a Number of animals tested at each dose. b NS = no substitution. c Not tested in this preparation.

**Pharmacology**

Compounds 1, 8, and 9 were initially evaluated in the two-lever drug discrimination assay in a group of rats trained to discriminate the effects of ip injections of saline from those of LSD tartrate (0.08 mg/kg), according to methods described previously (Table 1). For the compounds not fully substituting for LSD in this assay, the percentage of rats selecting the drug lever at the given dosage levels is reported in the table. Additionally, 1, 8, and 9 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 displace 0.75 nM [3H]8-OH-DPAT from rat hippocampal homogenate and 0.75 nM [3H]ketanserin from rat frontal cortex homogenate was measured. In addition, the compounds were evaluated in cells expressing cloned human 5-HT2A, 5-HT2B, and 5-HT2C receptors that were labeled either with [3H]antagonists or radiolabeled agonists (Table 2). Finally, as a measure of efficacy, compounds were assessed for their ability to stimulate phosphoinositide hydrolysis in cell lines expressing the rat 5-HT2A and 5-HT2C receptors (Figures 1 and 2).

**Results and Discussion**

The results of the drug discrimination (DD) studies in LSD-trained rats and the results of the radioligand competition experiments at [3H]Ketanserin-labeled rat 5-HT2A and [3H]8-OH-DPAT-labeled rat 5-HT1A receptors are presented in Table 1. In the behavioral assays, mescaline 1 was the most potent of the test compounds, substituting for LSD with an ED50 of 33.5 µmol/kg. Neither of the new rigid analogs fully substituted for LSD. For the dihydrobenzofuran 8, only 50% of the rats selected the drug lever when administered 57.8 µmol/kg of test drug. When a similar dosage of the tetrahydrobenzodifuran 9 was administered, only 29% of the rats selected the drug lever.

It was initially envisioned that if 8 and 9 retained the necessary pharmacophoric elements, they might have somewhat increased in vivo hallucinogenic-like activity compared to 1 due to the slightly greater increase resulting from the added methylene groups is molecular hydrophobilities anticipated. Barfknecht et al. previously correlated hallucinogenic activity with octanol–water partition coefficients for a series of substituted phenylalkylamines and found that the optimum hydrophobicity was centered at log P = 3.15. Since mescaline has a log P of only 1.18, it was felt that increased hydrophobilities of 8 and 9 (however, the increase resulting from the added methylene groups is

<table>
<thead>
<tr>
<th>DOSE RESPONSE IN NIH 3T3 (5-HT2A) RECEPTORS</th>
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<tbody>
<tr>
<td><img src="image" alt="Graph" /></td>
</tr>
</tbody>
</table>

**Figure 1.** Effects of mescaline 1 (○), 8 (■), 9 (○), and 23 (□) on phosphoinositide hydrolysis in NIH 3T3 fibroblasts expressing the rat 5-HT2A receptor. The curves are representative of experiments carried out on the same day. The mean Emax values (3–5 determinations) for 1, 23, 8, and 9 were 92, 96, 61, and 45%, respectively, of the maximum 5-HT response. The mean E50 values ± SEM were 4 ± 1, 4 ± 2, 3 ± 1, and 2 ± 1 µM, respectively.

**Figure 2.** Effects of mescaline 1 (○), 8 (■), 9 (○), and 23 (□) on phosphoinositide hydrolysis in NIH 3T3 fibroblasts expressing the rat 5-HT2C receptor. The curves are representative of experiments carried out on the same day. The mean Emax values (three determinations) for 1, 23, 8, and 9 were 97, 95, 82, and 79% respectively, of the maximum 5-HT response. The mean E50 values ± SEM were 24 ± 1, 2 ± 1, 2 ± 1, and 1 ± 0.2 µM, respectively.

**Table 2.** Results of the Radioligand Competition Studies of 8 and 9 at Cloned Human 5-HT2A, 5-HT2B, and 5-HT2C Receptors (Ki values ± SEM in nanomolar)

<table>
<thead>
<tr>
<th>Agonist radioligands</th>
<th>3H]-DOE</th>
<th>[3H]-serotonin</th>
<th>3H]-Ketanserin</th>
<th>3H]-Rauwolscine</th>
<th>[3H]-Mesulergine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound</td>
<td>5-HT2A</td>
<td>5-HT2B</td>
<td>5-HT2A</td>
<td>5-HT2B</td>
<td>5-HT2A</td>
</tr>
<tr>
<td>1</td>
<td>551.0 ± 35.3</td>
<td>320.8 ± 2.9</td>
<td>795.1 ± 65.5</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>8</td>
<td>368.4 ± 17.9</td>
<td>118.3 ± 16.0</td>
<td>523.0 ± 53.0</td>
<td>6405 ± 834</td>
<td>311 ± 38</td>
</tr>
<tr>
<td>9</td>
<td>335.3 ± 35.7</td>
<td>61.5 ± 5.3</td>
<td>301.5 ± 70.0</td>
<td>4443 ± 319</td>
<td>205 ± 7.6</td>
</tr>
<tr>
<td>23</td>
<td>215.6 ± 25.0</td>
<td>177.2 ± 18.0</td>
<td>555.1 ± 41.0</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>
somewhat offset by the more effective hydrogen-bonding of the constrained heterocyclic oxygens) would enhance their in vivo activity. Nevertheless, in the behavioral assays, the rank order of potency for this series decreased with increasing molecular hydrophobicity. In view of the somewhat greater brain concentrations of \( \mathbf{8} \) and \( \mathbf{9} \) that should be achieved, relative to \( \mathbf{1} \), the ability to activate some essential biological target appeared to have been lost by compounds \( \mathbf{8} \) and \( \mathbf{9} \). These results are even more surprising when it is recalled that similar transformations leading to molecules such as \( \mathbf{7} \) afforded extremely potent compounds.\(^{36} \) Clearly, there seem to be some fundamental differences between the molecular mechanisms of action of the 3,4,5- and 2,4,5-substituted phenethylamine hallucinogens.

At this point, it was decided to assay a related compound, 3,5-dimethoxy-4-ethoxyphenethylamine (esclaline; \( \mathbf{23} \)), reported to be significantly more potent than mescaline in humans.\(^{10} \) To our surprise, \( \mathbf{23} \) also failed to produce generalization in LSD-trained rats, but did produce LSD-appropriate responding in 50% of the rats at a dose that was approximately one-half the ED\(_{50} \) of mescaline.

![Dihydrobenzofuran Analogues of Hallucinogens](image)

\[ \text{H}_2\text{C} \quad \text{O} \quad \text{NH}_2 \quad \text{CH}_3 \text{CH}_2 \text{O} \quad \text{OCH}_3 \]

\[ \text{E} \]

\[ \text{C} \]

\[ \text{O} \]

\[ \text{N} \]

\[ \text{C} \]

\[ \text{H}_2\text{C} \quad \text{O} \quad \text{NH}_2 \quad \text{CH}_3 \text{CH}_2 \text{O} \quad \text{OCH}_3 \]

\[ \text{23. Escaline} \]

In the in vitro assays, none of the test compounds had significant affinity for 5-HT\(_{2A} \) receptors, results that are consistent with what is known of the general SAR of hallucinogenic phenylalkylamines. Curiously, however, the rank order of affinities of the test compounds for 5-HT\(_{2A} \) receptors was opposite the potencies obtained in the behavioral assays. Thus, while mescaline \( \mathbf{1} \) had the lowest affinity for 5-HT\(_{2A} \) sites, the rigid analog \( \mathbf{8} \) had more than twice the potency at these sites. These results are unusual because 5-HT\(_{2A} \) receptor affinity of similar phenethylamines typically parallels the potency in behavioral assays for hallucinogenic activity. Indeed, the 5-HT\(_{2} \) receptor subtype is commonly accepted as the primary site of action through which the unique behavioral effects of this class of drugs are mediated.\(^{26 - 29} \)

Although mescaline represents one of the least potent hallucinogens, one would still expect its in vitro and in vivo pharmacological profiles to be in agreement with the known phenylalkylamine SAR. Furthermore, the related 3,5-dimethoxy-4-ethoxyphenethylamine (\( \mathbf{23} \)) had a \( K_i \) of 2.1 \( \mu \)M at these 5-HT\(_{2A} \) sites, virtually identical to \( \mathbf{8} \), yet is reported to be 6-8 times more potent than mescaline \( \mathbf{1} \) as an hallucinogen in humans.\(^{10} \)

The data from the binding studies in denon human receptors are no more revealing (Table 2). At \( [\text{H}] \)-agonist-labeled 5-HT\(_{2A} \), 5-HT\(_{2B} \), or 5-HT\(_{2C} \) receptors, increased rigification in going from \( \mathbf{8} \) to \( \mathbf{9} \) led to increased affinity. Hallucinogens are generally considered to act as agonists, so it was even more surprising when data from the competition studies employing the agonist ligands \([125] \text{I} \text{DOI}\) and \([3H] \text{serotonin}\) were obtained also showing increased affinity as the molecules become more rigid. When one considers that the 4-ethoxy homologue \( \mathbf{23} \) is considerably more potent than \( \mathbf{1} \) as an hallucinogen,\(^{10} \) a comparison of its affinities for the three 5-HT\(_{2} \) receptor subtypes with those of \( \mathbf{1}, \mathbf{8}, \) and \( \mathbf{9} \) revealed no obvious basis for the decreased in vivo potencies of \( \mathbf{8} \) and \( \mathbf{9} \).

This prompted us to examine the relative efficacies of the compounds in activating phosphoinositide turnover in cells expressing the 5-HT\(_{2A} \) receptor, anticipating that perhaps compounds \( \mathbf{8} \) and \( \mathbf{9} \) might be partial agonists or antagonists. These compounds were compared to mescaline \( \mathbf{1} \) and \( \mathbf{23} \) (Figure 1). All four phenethylamine derivatives stimulated \([\text{H}] \text{IP}_3 \) hydrolysis in a transfected cell line expressing the 5-HT\(_{2A} \) receptor. Consistent with the drug discrimination data, compounds \( \mathbf{8} \) and \( \mathbf{9} \) were partial agonists, eliciting a maximum response less than that obtained with serotonin. Also in agreement with the drug discrimination data, compound \( \mathbf{9} \) was a weaker agonist than was \( \mathbf{8} \). Curiously, however, in these studies, escaline was a full agonist, in contrast to its low efficacy in the drug discrimination paradigm, with an \( E_{\text{max}} \) virtually identical to that of mescaline. This latter result contrasts with the partial substitution for \( \mathbf{23} \) in the drug discrimination assay.

Similar phosphoinositide turnover experiments were then carried out in cells expressing the rat 5-HT\(_{2C} \) receptor (Figure 2) to determine whether agonist properties at this receptor might provide more insight into the behavioral effects of the four drugs. The profile was very similar to that found for the 5-HT\(_{2A} \) receptor. While mescaline was a full agonist at the 5-HT\(_{2C} \) receptor, compounds \( \mathbf{8} \) and \( \mathbf{9} \) had somewhat reduced efficacy. Escaline \( \mathbf{23} \), once again, appeared to be equally efficacious to mescaline \( \mathbf{1} \). At this receptor, however, \( \mathbf{1} \) had a much larger EC\(_{50} \) than did the other three compounds. We believe this latter result has bearing on the drug discrimination results, especially for \( \mathbf{23} \), as discussed later.

As one final assay to compare these compounds and attempt to identify additional features in their in vitro pharmacology that might serve to explain the behavioral results, we evaluated the ability of all four compounds to inhibit the accumulation of \([\text{H}] \text{DA} \) or \([\text{H}] \text{5-HT} \) in rat whole brain synaptosomes. The IC\(_{50} \) values of all compounds were > 10 \( \mu \)M, with no obvious trends to parallel the observed behavioral potencies (unpublished results).

We believe that the loss of LSD-like activity in the drug discrimination assay for compounds \( \mathbf{8} \) and \( \mathbf{9} \) may lie in their reduced 5-HT\(_{2A} \) agonist efficacies, compared to \( \mathbf{1} \). This point is particularly emphasized by the lowered degree of substitution of \( \mathbf{9} \) relative to \( \mathbf{8} \), and the parallel reduction of agonist efficacy. These results support the current hypothesis that hallucinogens are agonists at 5-HT\(_{2A} \) receptors.

Somewhat more problematic, however, is the interpretation of the behavioral data for compound \( \mathbf{23} \). To our knowledge, examples of false negatives among hallucinogen analogues in this assay have been unknown up to the present time. The affinity of the 4-ethoxy homologue \( \mathbf{23} \) for the 5-HT\(_{2A} \) receptor, as well as its ability to stimulate phosphoinositide turnover in the denon receptor, are consistent with its activity as a mescaline-like agent in man. Although the radioligand competition experiments indicate only about a two-fold increase of affinity over \( \mathbf{1} \), the enhanced hydrophobicity
of 23 could be part of the explanation for its 6–8 fold higher clinical potency.

Escaline 23 also has full efficacy at the 5-HT₂C receptor and furthermore has significantly greater potency than mescaline at that site, at least in the cloned rat receptor. That result suggests that 5-HT₂C agonism should be a relatively more prominent feature of the pharmacology of 23 compared with 1. No studies have been carried out to compare the relative strengths of the interoceptive cues produced in rats by 5-HT₂A and 5-HT₂C receptor agonists. It seems possible, therefore, that the interoceptive cue induced by the increased intensity of the 5-HT₂C agonist action of 23 may be masking its 5-HT₂A stimulus properties. It might be noted in this context that the EC₅₀ values for compounds 8 and 9 (Figure 2) are also significantly lower than for 1. Thus, while we believe that lowered 5-HT₂A efficacy of 8 and 9 is sufficient to explain their behavioral effects, stimulus masking by agonism at the 5-HT₂C receptor could also be invoked as a partial explanation. Of course, both mechanisms may be important.

It is possible that other pharmacological factors might be involved in the different behavioral effects of these compounds. For example, it has been reported previously that the behavioral effects of 1 are mediated through both 5-HT and dopamine (DA) receptors. Although we have not examined dopamine receptor affinities for any of these molecules, their ability to inhibit accumulation of dopamine into rat whole brain synaptosomes did not differ significantly (unpublished study). It is still possible that other unidentified variables that we did not consider may be important. The efficacy data at the 5-HT₂A receptor, however, seem adequate to explain the decreased LSD-like effects of compounds 8 and 9.

The decreased efficacy of compounds 8 and 9 at the 5-HT₂A receptor relative to 1 or 23 is most interesting. When one incorporates the 2- and 5-methoxy groups of 2,4,5-substituted hallucinogenic amphetamines into dihydrofurans rings, activity is completely retained. In the present instance, however, the same type of increased rigidification leads to decreased agonist efficacy, while at the same time increasing affinity. If the previously proposed receptor model is correct, involving hydrogen bonding sites for the methoxy oxygens, the present results suggest that the 3- and 5-methoxy groups in mescaline-related compounds may not lie in conformations that place them in the plane of the aromatic ring. Implicit in this observation is the possibility that these methoxy groups may reorient themselves upon receptor binding, to achieve a complementary fit and induce an activated conformational state of the receptor. This would necessarily involve out-of-plane conformations for the methoxy groups. Therefore, future molecular modeling studies involving comparisons of 3,4,5- and 2,4,5-trisubstituted phenethylamines should take this possibility into account.

Further pharmacological evaluation of 1, 8, 9, and 23 appears warranted to elucidate fully the mechanism of action of these mescaline analogues, and to understand how the mechanism(s) of action for mescaline might differ from the action of hallucinogenic 2,5-dimethoxyphenethylalkylamines. These results also suggest the possibility that 5-HT₂C agonists may produce a more robust interoceptive cue than do 5-HT₂A agonists, at least in rats.

In summary, we have extended our previously described rigid analog approach, using tetrahydrobenzodiazepine functionalities as rotationally restricted bioisosteres of the aromatic methoxy groups of mescaline. Although the rigid analogs synthesized here had increased overall hydrophobicity and enhanced affinity for serotonin 5-HT₂ receptors, they had decreased efficacy and were not as potent as 1 in a behavioral assay for hallucinogen-like activity. These results may point to different (i.e. out-of-plane) methoxy group conformations for 3,4,5-trimethoxyphenethylalkylamines related to mescaline as compared with the 2,5-dimethoxyphenethylalkylamines. The novel rigid analogs 8 and 9 may serve as useful ligands for probing possible alternate mechanisms of action for mescaline and similar structures, and will be valuable tools in future pharmacological investigations of the SAR of hallucinogenic agents.

**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 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% w/w). 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 Microanalytical 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 CH₂Cl₂–C₄H₈O₂, and visualizing with UV light at 254 nm and/or f₂ vapor unless otherwise noted. Plates used for radial centrifugal chromatography ("Chromatotron", 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.

**6,7-Dimethoxybenzofuran-3-one (10).** Oxalyl chloride (7.2 mL, 81.0 mmol) in 27 mL of dry benzene was added dropwise to a solution of 12.05 g (56.8 mmol) of 2,3,4-trimethoxybenzoic acid (Aldrich, Milwaukee, WI) in 150 mL of benzene, with two drops of DMF added as a catalyst. The mixture was stirred for 3.5 h, and the volatiles were removed under reduced pressure to give a quantitative yield of crude benzoyl chloride as an oil that spontaneously crystallized to a white solid.

Following literature methods, a etherial solution of diazomethane was cautiously prepared from Diazald (N-methyl-N-nitrosamidomethylamine) in a well-ventilated fume hood. A solution of 17 g of KOH in 23 mL of water, 66 mL of carbitol (2-ethoxyethoxyethanol), and 20 mL of ether was stirred in a Diazald apparatus (Aldrich, Milwaukee, WI) and warmed to 60 °C over an oil bath. Diazald (48.42 g, 226.1 mmol) in 150 mL of anhydrous ether was added slowly to the warm solution, and the clear, yellow distillate that formed was collected and kept cold in an ice–salt bath. The crude benzoyl chloride prepared above was dissolved in 50 mL of anhydrous ether and added dropwise to the cold diazomethane solution. After 10 min, the ice–salt bath was removed, and the mixture was stirred for an additional 2 h. The solvent and excess diazomethane were removed by stirring under reduced pressure (water aspirator), to afford the intermediate diazomethylketone as a viscous yellow-orange oil. To the oil was added...
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80 mL of glacial acetic acid, giving an immediate, strong evolution of gas and heat. The mixture was allowed to stir in a room temperature water bath for 1.5 h. Solvent removal under reduced pressure gave a transparent red-brown benzoic acid that crystallized spontaneously. Recrystallization from ethyl ketone gave 8.30 g (77% from 2,3,4-trimethoxybenzoic acid) of pure product 10 as fluffy, white needles: mp 122–124°C; CIMS m/z 206 (M+1), 195 (M+1). Anal. (C12H17NO3) C, H, N.

7-Hydroxy-6-methoxy-2,3-dihydrobenzofuran (12). The dimethoxy benzofuran 11 (5.72 g, 30.4 mmol) was dissolved in 84 mL of methylene chloride and stirred under a reduced pressure, leaving the crude benzylamine as a white solution, 0.8 mmol) were added to drive the reaction to completion. Water (40 mL of a 37% aqueous solution, 3.7 mmol). The mixture in 5 mL of methanol was added dimethylamine (0.47 mL of a 37% aqueous solution) and stirred under nitrogen for 2 h and was then acidified with 6 N HCl (warning! HCN produced), giving a colorless oil that spontaneously crystallized on standing. The crude methiodide salt was immediately taken up in 63 mL of water containing 7.41 g (113.8 mmol) of potassium cyanide, and the mixture was heated to reflux. All solids dissolved on heating, but as the reaction proceeded a small amount of precipitate became visible. After 5 h, the reaction was cooled and cautiously acidified with 6 N HCl (warning! HCN produced), giving a substantial quantity of solid. The acidified mixture was stirred for 2 h with the flame hood and the solid was filtered by suction. Aspirated air drying on the filter paper afforded 2.54 g (79%) of the title compound 13 as a yellow solid: mp 87–89°C; 1HNMR (CDCl3) δ 3.2 (t, 2, ArCH2CH3O), 3.6 (s, 3, CH3O), 3.9 (s, 3, CH3O), 4.6 (t, 2, ArCH2CH3O), 6.4 (d, 1, ArH), 6.8 (d, 1, ArH); CIMS m/z 206 (M+1), 195 (M+1). Anal. (C12H15NO3) C, H, N.

4-(Cyanomethyl)-6,7-dimethoxy-2,3-dihydrobenzofuran (15). A mixture of 4-(cyanomethyl)-7-hydroxy-2,3-dihydrobenzofuran (100 mg, 0.5 mmol), powdered anhydrous potassium carbonate (539 mg, 3.9 mmol), and methyl iodide (0.24 mL, 3.9 mmol) in 4.6 mL of acetone was heated at reflux and stirred under N2 for 20 h. The reaction mixture was cooled, filtered through Celite, and evaporated to give an oily yellow solid. This was taken up in ethyl acetate, washed with 10% sodium bicarbonate (2 × 25 mL) and brine (25 mL), and dried (MgSO4). Filtration, followed by solvent removal under reduced pressure afforded a dark yellow oil that crystallized spontaneously. The oil was purified on the Chromatotron (4 mm silica plate, CH2Cl2) to give 345 mg (81%) of 14 as a yellow solid: mp 87–89°C; 1HNMR (CDCl3) δ 3.2 (t, 2, ArCH2CH3O), 3.6 (s, 2, ArCH(CH3)O), 3.8 (s, 3, CH3O), 3.9 (s, 3, CH3O), 4.7 (t, 2, ArCH2CH3O), 6.4 (s, 1, ArH); CIMS m/z 220 (M+1), 193. Anal. (C12H14O5) C, H, N.

4-(Aminooxy)-6,7-dimethoxy-2,3-dihydrobenzofuran Hydrochloride (8). In a Parr hydrogenation flask, 1.85 g (6.44 mmol) of 4,6,7-dimethoxy-4-(cyanomethyl)-2,3-dihydrobenzofuran 15 (40% purity) was reduced to 150 mL of a 10% Pd/C and Filter paper afforded 2.54 g (79%) of the title compound 16 as a colorless oil that spontaneously crystallized on standing. The crude methiodide salt was immediately taken up in 63 mL of water containing 7.41 g (113.8 mmol) of potassium cyanide, and the mixture was heated to reflux. All solids dissolved on heating, but as the reaction proceeded a small amount of precipitate became visible. After 5 h, the reaction was cooled and cautiously acidified with 6 N HCl (warning! HCN produced), giving a substantial quantity of solid. The acidified mixture was stirred for 2 h with the flame hood and the solid was filtered by suction. Aspirated air drying on the filter paper afforded 2.54 g (79%) of the title compound 14 as an off-white solid: mp 100–104°C; 1HNMR (CDCl3) δ 3.2 (t, 2, ArCH2CH3O), 3.6 (s, 2, ArCH(CH3)O), 3.9 (s, 3, CH3O), 4.7 (t, 2, ArCH2CH3O), 5.3 (s, 1, ArOH), 6.4 (s, 1, ArH); CIMS m/z 206 (M+1), 195. Anal. (C12H15NO3) C, H, N.

4-(Cyanomethyl)-2,3,4-trimethoxybenzene (16). A mixture of 49.0 g (0.29 mol) of 1,2,3-trimethoxybenzene (4.44 mol) and 0.5% Pd/C was stirred under 70 psi of H2 for 80 h. The reaction mixture was cooled, filtered through Celite, and evaporated to yield a red-orange oil. Kugelrohr distillation gave a colorless oil that spontaneously condensed on a white solid upon cooling. Recrystallization of this solid from ethyl acetate and hexane afforded 2.92 g (59%) of 15 as colorless, needles: mp 71–72°C; 1HNMR (CDCl3) δ 3.2 (t, 2, ArCH2CH3O), 3.9 (s, 3, CH3O), 4.6 (t, 2, ArCH2CH3O), 5.3 (s, 1, ArOH), 6.4 (d, 1, ArH), 6.7 (d, 1, ArH); CIMS m/z 167 (M+1), 166 (M+1). Anal. (C12H16O3) C, H, N.

1,5-Bis(chloromethyl)-2,3,4-trimethoxybenzene (17). Following the method of Friedman and Schecter, 20 g (0.075 mol) of the bis-chloro compound 16 was added portionwise to a stirred mixture of 8.5 g (0.17 mol) of sodium cyanide in 65
The aqueous residue was saturated with K₂CO₃ and extracted with 5 × 100 mL of ethyl acetate. The extracts were combined and washed with 2 × 50 mL of 3 N HCl, 2 × 50 mL of 6 N HCl, and 50 mL of brine. After drying (MgSO₄), and filtering through Celite, washing the filter cake with ether, and the volatiles were removed on the rotary evaporator. The residue was taken up in 10% CH₂Cl₂ in ether, precipitated by the addition of 1 N NaOH, and the precipitate was washed with 10% CH₂Cl₂ in ether, dried (MgSO₄), and filtered through Celite and a thin pad of silica gel. Removal of solvent under reduced pressure gave a yellow oil that crystallized spontaneously. The solid was recrystallized from ethyl acetate–hexane to give 1.3 g (40%) of 21 as pale yellow needles: mp 152–156 °C; ¹H NMR (CDCl₃) δ 3.44 (t, 4, ArOCH₂CH₂J = 8.7 Hz), 4.10 (s, 3, ArOCH₃), 4.68 (t, 4, ArOCH₂CH₂J = 8.7 Hz), 10.08 (s, 1, ArCHO); CIMS m/z 221 (M + 1). Anal. (C₁₆H₁₂O₆) C, H, N.

1-Me-hex-2-yl-2,3,5,6-tetrahydrobenzofuran[1,2-b:5,4-b]difuuran (21). A solution of 2.8 g (0.015 mol) of 20 was dissolved in 50 mL of dry CH₂Cl₂ and cooled over an ice bath. Tin(IV) chloride (3.4 mL, 0.029 mol) was added to the mixture by syringe, and the reaction was allowed to stir at room temperature for 4 h. The reaction was quenched by the addition of 50 mL of ice cold 3 N HCl and diluted with 100 mL of ether, and the layers were separated. The organic phase was washed with 5 × 50 mL of 3 N HCl, 2 × 50 mL of 5% NaHCO₃, and brine, dried (MgSO₄), and filtered through Celite, washing the filter cake with ether, and the volatiles were removed on the rotary evaporator. The residue was taken up in 10% CH₂Cl₂ in ether, precipitated by the addition of 1 N NaOH, and the precipitate was washed with 10% CH₂Cl₂ in ether, dried (MgSO₄), and filtered through Celite and a thin pad of silica gel. Removal of solvent under reduced pressure gave a yellow oil that crystallized spontaneously. The solid was recrystallized from ethyl acetate–hexane to give 1.3 g (40%) of 21 as pale yellow needles: mp 152–156 °C; ¹H NMR (CDCl₃) δ 3.44 (t, 4, ArOCH₂CH₂J = 8.7 Hz), 4.10 (s, 3, ArOCH₃), 4.68 (t, 4, ArOCH₂CH₂J = 8.7 Hz), 10.08 (s, 1, ArCHO); CIMS m/z 221 (M + 1). Anal. (C₁₆H₁₂O₆) C, H, N.

Pharmacology Methods. Drug Discrimination Studies. The procedures for the drug discrimination assays were essentially as described in previous reports. Twenty-four 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 home cage.
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) consisted of modular test cages enclosed within sound-attenuated cubicles with fans for ventilation and background white noise. A white house light was centered near the top of the front panel of the cage, which was also equipped with two response levers, separated by a food hopper (combination dipper pellet trough, Model E14-06, module size 1/2), all positioned 2.5 cm above the floor. Solid state logic in an adjacent room, interfaced through a Med Associates interface to a 486-based microcomputer, controlled training parameters 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 (one week after isolation in the individual home-cages and at the beginning of the food deprivation), the rat’s initial shaping was started. During the first 2–3 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. Half of the rats were trained on drug-L (left), saline-R (right) and the other 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 presented during the first 10 sessions of initial learning (after beginning to administer saline or training drug, i.p. 30 min before sessions). Afterwards, both levers were present during all following phases of training, but reinforcements were delivered only after responses on the appropriate lever. Presses on the incorrect lever had no programmed consequences. As responding rates stabilized (during the next 15 sessions), the schedule of reinforcement was gradually increased to a FR50. Once at the FR50, trials continued until at least 95% (number of correct presses × 100/number of total presses) was attained for eight of ten consecutive sessions (approximately 40–60 sessions). Once criterion performance was attained, test sessions were interspersed between training sessions, either one or two times per week. At least one drug and one saline session separated each test session. Rats were required to maintain the 85% correct responding criterion on training days in order to be tested. In addition, test data were discarded when the accuracy criterion of 85% was not achieved on the two training sessions following a test session. Test drugs were administered ip 30 min prior to the sessions; test sessions were run under conditions of extinction, with rats removed from the operant chamber when fifty presses were emitted on one lever. If fifty presses on a 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. “No substitution” was determined by the maximum %SDL for all doses of the test drug. “No substitution” is defined as 59% SDL or less, and “partial” substitution is 60–79% SDL. If the drug was one which completely substituted for the training drug (at least one dose resulted in a %SDL = 80% or higher) the method of Litchfield and Wilcoxon was used to determine the ED50 (log–probit analysis as the dose producing 50% drug-lever responding) and 95% confidence interval (%5 CI). This method also allowed for tests of parallelism between dose–response curves of the drug and the training drug. If 50% or more of the animals tested were disrupted at a dose where the nondisrupted rats gave 80% SDL, no ED50 was calculated.

Pharmacological Methods. Radioligand Competition Assays in Rat Brain Homogenates. All key rats (Harlan Laboratories, Indianapolis, IN) weighing 175–199 g were used. The animals were kept in groups of five rats per cage, at the same conditions described above, but with free access to food and water.

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

The procedure of Johnson et al. 60 was 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 36000g for 10 min, and the resulting pellets were resuspended in the same volume of sucrose. Separate aliquots of tissue suspension were then frozen at −70 °C until assay.

For each separate experiment, a tissue aliquot was thawed slowly and diluted 1:25 with 50 mM Tris HCl (pH = 7.4). The homogenate was then incubated at 37 °C for 10 min and centrifuged twice at 36000g for 20 min with an intermittent wash. The resulting pellet was resuspended in 50 mM Tris HCl with 0.5 mM Na2EDTA, 0.1% Na ascorbate, and 10 mM pargyline HCl (pH 7.4). A second preincubation for 10 min at 37 °C was conducted, and the tissues were then cooled in an ice bath.

All experiments were performed with triplicate determinations using the appropriate buffer to which 200–400 µg of protein was added, giving a final volume of 1 ml. The tubes were allowed to equilibrate for 15 minutes at 37 °C before filtering through Whatman GF/C filters using a cell harvester (Bioserv, Frenchtown, NJ) at 22.8 ± 0.4 Ci/mmol and directly counted in a γ-counter. Active specific binding was defined as that displaced with 10 nM cinnarizine in the [3H]ketanserin binding study and with 10 nM 5-HT in the [3H]-8-OH-DPAT binding study. Filters were air-dried, placed into scintillation vials with 10 ml of Ecolite scintillation cocktail, allowed to sit overnight before counting at an efficiency of 37% for tritium, and directly counted in a γ counter for [3H]-ligand 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 Corporation (Arlington Heights, IL) at 22.8–26.7 or 81–91 Ci/mmol, respectively. [125]iodotryptamine (5200 Ci/mmol), [3H]frauwlscine (70–90 Ci/mmol), and [3H]-5-HT. Cell Membrane Preparations from Transfected Cell Lines. Membranes were prepared essentially as previously described using AV12 cell lines (Syrian hamster fibroblast, ATCC no. CRL 9595) stably transfected with the human 5-HT6A, 5-HT6B, or 5-HT6C receptors. 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 × 107 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 39000g for 10 min at 4 °C, and the resulting membrane pellet was resus-
pended and incubated at 37 °C for 10 minutes 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-HT2AR receptor, in Tris-HCl, pH 7.4, containing MgCl2 and EDTA for [3H]DOI binding to 5-HT2A or 5-HT2C receptors, or in Tris-HCl, pH 7.6, for [3H]ketanserin and [3H]mesulergine binding to 5-HT2AR receptors. Incubation was at 37 °C.

5-HT2A [3H]DOI Binding Studies. Human 5-HT2A receptor binding assays using [3H]5-HT were performed as previously described.64 The assay was automated using a Biomek 1000 (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 six log units, in water. Then 200 µL of membrane resuspension (approximately 100–150 µg protein) was added with mixing and incubated for 15 min at 37 °C. Nonspecific binding was defined as the [3H]-5-HT binding in the presence of 100 nM prazosin (to block potential binding of [3H] DOI). Incubations were essentially as described above. Conditions specific to this assay were as follows (all concentrations given as final concentrations): 2 nM [3H]rauwolscine, 500 nM etanercept (to mask [3H]rauwolscine binding to α1-adrenergic receptors), and 50 mM Tris-HCl, pH 7.4. Tubes were incubated at 37 °C for 30 min and then rapidly filtered and washed, and radioactivity determined as for the [3H]ketanserin binding assay described above.

Statistical Analysis for Radioligand Binding. Nonlinear regression analysis for the competition curves was performed as previously described67 to determine IC50 values. These were converted to Ki values by the method of Cheng and Prusoff.68

Measurement of Phosphoinositide Turnover in NIH 3T3 Cells. 5-HT2A and 5-HT2C receptor cDNAs were gift of David J. Julius (UCSF, San Francisco, CA). Clonal cell lines were generated in NIH 3T3 fibroblasts (American Type Culture Collection, Rockville, MD) by calcium phosphate precipitation and screening by radioligand binding. Cell lines expressing 5-HT2A receptors (~5000 fmoI/mg protein) and 5-HT2C receptors (~3000 fmoI/mg protein) are referred as 3T3-2A and 3T3-2C, respectively. [3H]Mymo-inositol (20–25 Ci/ mmol) was purchased from DuPont/MEN Corporation (Boston, MA), MDA-286, and Dulsobur (Dulbecco's modified medium, DMEM) were obtained from Gibco/BRL (Grand Island, NY). Bovine serum was purchased from Hyclone (Logan, UT) and tissue culture plates, from Falcon (Lincoln Park, NJ).

Fibroblasts were grown in DMEM supplemented with penicillin (5 U/ml), streptomycin (5 µg/ml), and calf serum (9%). For the PI hydrolysis assay, cells were plated into 24-well culture plates and allowed to adhere for 30 h in DMEM supplemented with 9% calf serum. Cells were subsequently washed once with 0.5 mL serum-free DMEM and then incubated for 18 h in 0.5 mL serum-free, inositol-free DMEM supplemented with 5 µCi/mL [3H]myo-inositol. Labeled cells were subsequently used for determination of [3H]inositol phosphate (IP) formation as described originally by Berridge et al.69 and modified by Grotowel and Sanders-Bush.60 Dose–response curves were fit using Prism, a commercially available computer graphics/analysis program (GraphPad, San Diego, CA).

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References
(1) For parts 1–3 in this series, see refs 32, 33, and 36, respectively.
(2) Abstracted in part from the Ph.D. thesis of A. Monte, Purdue University, 1995.
(8) Nichols, D. E.; Giannini, J. Myo-Inositol phosphate (IP) formation as described originally by Berridge et al.69 and modified by Grotowel and Sanders-Bush.60 Dose–response curves were fit using Prism, a commercially available computer graphics/analysis program (GraphPad, San Diego, CA).

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