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-HT2A/C 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-2H-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 denoned human 5-HT2A, 5-HT2B, and 5-HT2C 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-2H-naphtho[1,8-bc]furan (4e) and syn-N,N-dipropyl-4-amino-6-methoxy-2a,3,4,5-tetrahydro-2H-naphtho[1,8-bc]furan (4f) were prepared and assayed for their affinities at [3H]ketanserin-labeled 5-HT2A and [3H]-8-OH-DPAT-labeled 5-HT1A 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 M1–M5 receptors and also assessed for their functional characteristics at the M1 and M2 isomers. 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 M1 receptor, while only 4d completely antagonized carbachol at the M2 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-HT2 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 oxtocic 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 Asp152 to an Asn in the 5-HT2A Receptor gave a mutant with greatly decreased affinity for 5-HT2 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-HT2A receptor, possibly Ser242 in TM III,6 while the 2-methoxy oxygen atom of 1 and the carbonyl oxygen.
of 2 could interact with a common H-bond donor within the 5-HT2 binding site. This hypothesis is supported by the finding that it is the R enantiomer of the phenylisopropylamines 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 dihydronaphthofuran moieties (as in 3) provided compounds of high potency and selectivity for 5-HT2 receptors that exhibited behavioral effects similar to 2 in the rat drug discrimination assay.10 Thus, 3 clearly established the binding conformation of the alkox substituents at 5-HT2 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 dihydronafuran ring. This strategy would produce a molecule that is, in essence, a “hybrid” of molecules 1 and 2, in which the indolic and B rings of 2 are mimicked by the benzodihydronfuran 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., 4a, 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-HT2 receptors. After a receptor screen demonstrated that 4d had affinity for muscarinic receptors, the syn diastereomers were evaluated at cloned human M1–M5 muscarinic receptor subtypes.

Chemistry

The series of target racemic tetrahydronapthofurans 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 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(I) 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 N2.15 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
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.

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. 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-HT2A receptor affinity or lead to ligands with higher affinity for the 5-HT1A receptor.
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-HT2 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 M1—M5 muscarinic receptors. These agents also were evaluated functionally by assessing the ability of the compounds to stimulate phosphoinositide hydrolysis in muscarinic M1 and M2 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-HT2A 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 [3H]ketanserin-labeled 5-HT2A 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-HT2A receptor than the anti diastereomer.

Table 2 shows radioligand competition data for compounds 4c, d, and comparison data for 3, in the cloned human 5-HT2 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-HT2A and 5-HT2C receptors and a 164-fold lower affinity for the 5-HT2B 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-HT2A receptor, it does have significant affinity for the agonist-labeled high-affinity states of both the 5-HT2A and 5-HT2C human receptors. 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]IDA1-labeled 5-HT2A 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, these results lead us to conclude that the active conformation of the hallucino-
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-HT1A and 5-HT2A receptors in rat brain homogenate (Table 2). The results show that neither of these target naphthofurans have significant affinity for [3H]ketanserin-labeled 5-HT2A sites or [3H]-8-OH-DPAT-labeled 5-HT1A sites. It is curious to note, however, that the affinity of the syn diastereomers for 5-HT2A 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-HT2A 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 M2 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, M1–M5.

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 recep-
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 (25 g/lit), plastic-backed plates with fluorescent indicator (L. Baker), eluting with CH₂Cl₂, and visualizing with UV light at 254 nm and/or I₂ vapor unless otherwise noted. Plates used for radial centrifugal chromatography (Chromatotron; Harrison Research, Palo Alto, CA) were prepared from silica gel 60 PF2-54 containing gypsum. Reactions were carried out under an atmosphere of dry nitrogen.

5-Methoxybenzofuran-3(2H)-one (6). Oxyal 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-toluensulfonamide (Di-azaid) in 250 mL of anhydrous ethanol to a stirred solution of 10 g of KOH, 17 mL of H₂O, 50 mL of 2-(2-ethoxyethoxy)-ethanol (carbitol), and 15 mL of ether held over a 40 °C oil bath. 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 (1:5) to give 7.5 g (82%) of 6 as a fluffy, pale-yellow flakes: mp 92 °C (lit. mp 88 °C); 1H NMR (CDCl₃) δ 3.86 (s, 3, ArOC₂), 7.07 (d, 2, ArH), 9.0 Hz, 7.07 (d, 2, ArH), J = 9.0 Hz, 7.25 (dd, 1, ArH, J = 9.0, 2.8 Hz).

Methyl 2-(5-Methoxybenzofuran-3-yl)acetate (7a). Following the procedure of Chan et al., a mixture of 20.0 g (0.122 mol) of ketone 6 and 41.54 g (0.124 mol) of methyl (triphenylphosphanylidene)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 remaining 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 1H NMR spectra) as a yellow solid that weighed 15.1 g (68%). The mixture was stirred in a solution of CHCl₃ 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; 1H NMR (CDCl₃) δ 3.70 (s, 2, CH₃COOC₂H), 3.75 (s, 3, CH₂COOCH₃), 3.83 (s, 3, ArOCH₃), 6.90 (dd, 1, ArH, 

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 (0.00 ppm). 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
Methyl 2-(2,3-Dihydro-5-methoxy-3-benzofuranyl)acetate (8). 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 H2. After 34 h, the theoretical amount of H2 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 sufficiently pure by TLC and 'H NMR analyses: 'H NMR (CDCl3) δ 2.60 (dd, 1, CH2COOCH3) J = 16.6, 9.4 Hz), 2.80 (dd, 1, CH2COOCH3) J = 16.5, 5.3 Hz), 3.70 (s, 3, COOCH3), 3.75 (s, 3, ArOCH3), 3.82 (m, 1, ArCH), 4.22 (dd, 1, ArOCH3) J = 9.2, 6.4 Hz), 4.73 (t, 1, ArOCH3) J = 8.9 Hz), 6.68 (dd, 1, ArH) J = 8.6, 2.4 Hz), 6.71 (d, 1, Ar) J = 8.6 Hz), 6.74 (d, 1, ArH) 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 H2O 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 mixture was extracted with 30 mL of CH2Cl2. The organic extracts were combined, washed with water, and the hydrochloride salt was crystallized as a yellow oil. An analytical sample was prepared by recrystallization from ethyl ether as needed to maintain about pH 5. In a separate preparation, the hydrochloride salt was treated with excess 5 N HCl in ether to afford 3.38 g of the acid as a yellow oil weighing 0.35 g (26%). It was taken up into dry HCl ether as needed to maintain about pH 5, and the hydrochloride salt was crystallized as a yellow oil. An analytical sample was prepared by recrystallization from ethyl ether. The first band to elute from the Chromatotron contained 3.5 g of unreacted 6. The second band to elute from the column contained 3.5 g of reacted 6 that could be recycled for subsequent runs.

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Anti-N-Benzyl-4-amino-6-methoxy-2,3,4,5-tetrahydro-2H-naphtho[1,8-b]furan (12a) and syn-N-Benzyl-4-amino-6-methoxy-2,3,4,5-tetrahydro-2H-naphtho[1,8-b,c]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 of 1 N HCl in ether. The solution was then taken up into dry ether and the hydrochloride salt was removed under reduced pressure and the dark red residue was taken up and shaken gently between 25% N K2HPO4 and ether. The layers were separated, and the aqueous phase was then extracted with 3 x 20 mL of ether. The organic fractions were combined, washed with 3 x 20 mL of H2O and 50 mL of Brine, dried (MgSO4), 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 removed under reduced pressure. The first band to elute from the Chromatotron 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 ether. After solvent removal in vacuo, the solid was crystallized from ethanol–ether to give light yellow crystals: mp 114°C (lit. mp 111°C), [α]D +2.5° (c 1, CHCl3) H Rf = 0.95, 0.8, 0.5, 0.4, 0.3, 0.2, 0.1 (CH2Cl2). The organic extracts were combined, washed with 3 x 20 mL of H2O and 50 mL of Brine, dried (MgSO4), 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 removed under reduced pressure. The first band to elute from the Chromatotron 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 ether. After solvent removal in vacuo, the solid was crystallized from ethanol–ether to give light yellow crystals: mp 114°C (lit. mp 111°C), [α]D +2.5° (c 1, CHCl3) H Rf = 0.95, 0.8, 0.5, 0.4, 0.3, 0.2, 0.1 (CH2Cl2). The organic extracts were combined, washed with 3 x 20 mL of H2O and 50 mL of Brine, dried (MgSO4), 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 removed under reduced pressure. The first band to elute from the Chromatotron 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 ether. After solvent removal in vacuo, the solid was crystallized from ethanol–ether to give light yellow crystals: mp 114°C (lit. mp 111°C), [α]D +2.5° (c 1, CHCl3) H Rf = 0.95, 0.8, 0.5, 0.4, 0.3, 0.2, 0.1 (CH2Cl2). The organic extracts were combined, washed with 3 x 20 mL of H2O and 50 mL of Brine, dried (MgSO4), 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 removed under reduced pressure. The first band to elute from the Chromatotron 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 ether. After solvent removal in vacuo, the solid was crystallized from ethanol–ether to give light yellow crystals: mp 114°C (lit. mp 111°C), [α]D +2.5° (c 1, CHCl3) H Rf = 0.95, 0.8, 0.5, 0.4, 0.3, 0.2, 0.1 (CH2Cl2). The organic extracts were combined, washed with 3 x 20 mL of H2O and 50 mL of Brine, dried (MgSO4), 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 removed under reduced pressure. The first band to elute from the Chromatotron 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 ether. After solvent removal in vacuo, the solid was crystallized from ethanol–ether to give light yellow crystals: mp 114°C (lit. mp 111°C), [α]D +2.5° (c 1, CHCl3) H Rf = 0.95, 0.8, 0.5, 0.4, 0.3, 0.2, 0.1 (CH2Cl2).


J = 8.5 Hz, 6.58 (d, 1, ArH, J = 8.5 Hz), 7.30 (m, 5, ArH, benzylidene); CIMS m/z 296 (M + 1). Anal. (C12H10NO2·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 redissolved in ether–ether to give a fine white crystals of 12b·HCl: mp 269–270 °C; H NMR (free base in CDCl3) δ 1.33 (q, 1, ArCH2CH2CH3, J = 11.1 Hz), 1.80 (bs, 1, NH), 2.29–2.39 (m, 2, a mixture of ArCH2CH2CH3 and ArCH2CHN), 3.06–3.26 (m, a mixture of ArCH2CHN and ArCH2CHN), 3.38 (m, 1, ArCH3), 3.78 (s, 3, ArOCH3), 3.9–4.1 (a mixture of s, 2, a mixture of OCH2 and the benzylidene methylene), 4.73 (t, 1, OCH2, J = 8.2 Hz), 6.53 (d, 1, ArH, J = 8.4 Hz), 7.32 (d, 5, ArH, benzylidene); CIMS m/z 296 (M + 1). Anal. (C12H10NO2·HCl) C, H, N.

12b·HCl to that for the preparation of the anti isomer 10,11

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 H2 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; H NMR (free base in CDCl3) δ 1.35 (bs, 2, NH2), 1.45 (q, 1, ArCH2CH2CH3, J = 12.1, 2.9 Hz), 1.98 (dt, 1, ArCH2CH2CHN, J = 12.5, 4.5 Hz), 2.53 (d, 1, ArCH2CHN, J = 18.2 Hz), 2.71 (dd, 1, ArCH2CHN, J = 18.2, 4.5 Hz), 3.59 (m, 1, ArCH3), 3.62 (m, 1, ArCH2CHN), 3.69 (s, 3, ArOCH3), 3.9 (dd, 1, OCH2, J = 12.3, 8.0 Hz), 4.70 (t, 1, OCH2, J = 8.0 Hz), 6.65 (d, 1, ArH, J = 8.5 Hz), 6.50 (d, 1, ArH, J = 8.5 Hz); CIMS m/z 206 (M + 1), 189. Anal. (C6H12NO·HCl) C, H, N.

syn-N,N-Dimethyl-4-amino-6-methoxy-2a,3,4,5-tetrahydro-2H-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 H2 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; H NMR (free base in CDCl3) δ 1.19 (q, 1, ArCH2CH2CH3, J = 11.6 Hz), 1.42 (bs, 2, NH2), 2.09 (dt, 1, ArCH2CH2CHN, J = 11.6, 3.9 Hz), 2.38 (dd, 1, ArCH2CH2CH3, J = 17.3, 11.9 Hz), 3.01 (dd, 1, ArCH2CHN, J = 17.4, 6.3 Hz), 3.21 (m, 1, ArCH2CHN), 3.38 (m, 1, ArCH3), 3.69 (s, 3, ArOCH3), 3.9 (dd, 1, OCH2, J = 12.3, 8.3 Hz), 4.70 (t, 1, OCH2, J = 8.2 Hz), 6.45 (d, 1, ArH, J = 8.5 Hz), 6.49 (d, 1, ArH, J = 8.5 Hz); CIMS m/z 206 (M + 1), 189. Anal. (C6H12NO·HCl) C, H, N.

syn-N,N-Dipropyl-4-amino-6-methoxy-2a,3,4,5-tetrahydro-2H-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 of 25% NaOH solution of 25 mL of brine, dried over MgSO4, and filtered through Celite. Removal of solvent on the rotary evaporator gave a yellow oil that was purified by applying it to a 1-mm Chromatotron plate and eluting with CHCl3 under a N2–NH3 atmosphere. This gave 36 mg (50%) of the free base 4f 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 filtered off, washed with ether and ether with yield 49% of 4f·H2O·HCl as a white solid: mp 186–187 °C; H NMR (free base in CDCl3) δ 1.35 (q, 1, ArCH2CH2CH3, J = 11.4 Hz), 2.24 (ddd, 1, ArCH2CH2CHN, J = 11.3, 4.2, 3.2 Hz), 2.40 (s, 6, N(CH3)2), 2.52 (dd, 1, ArCH2CHN, J = 15.8, 10.6 Hz), 2.9–3.1 (m, 2, a mixture of ArCH2CH2CHN and ArCH2CHN), 3.40 (m, 1, ArCH3), 3.77 (s, 3, ArOCH3), 4.0 (dd, 1, OCH2, J = 12.1, 8.2 Hz), 4.82 (t, 1, OCH2, J = 8.2 Hz), 6.52 (d, 1, ArH, J = 8.6 Hz), 6.56 (d, 1, ArH, J = 8.6 Hz); CIMS m/z 234 (M + 1), 205, 162. Anal. (C12H14N2O2·HCl·H2O) 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


2141
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, Coul- bourn 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 trained to lever press on an FR 1 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 day. 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. However, 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 FFR50, 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 for 10 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/g; NDMA). 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. 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 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 Radioimager 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.

{[H]}Ketanserin and {[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. were employed. Briefly, the frontal cortex or hippocampal brain regions from 20–40 rats were pooled and homogenized (Brinkman Polytron, setting 6 for 2 x 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 36500g for 20 min with an interminimal wash. The resulting pellet was resuspended in 50 mM Tris-HCl with 0.5 mM Na_{2}EDTA, 0.1% Na ascorbate, and 10 mM p-aminohippuric acid (pH 7.4). In experiments with {[H]}ketanserin or {[H]}-8-OH-DPAT, either 5.7 μM CG or 10 μM MGC 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 {[H]}ketanserin binding study and 10 μM 5-HT in the {[H]}-8-OH-DPAT binding study. Filters were air-dried, placed into scintillation vials with 10 mL of Eclite scintillation cocktail, and allowed to sit overnight before counting for tritium.
in Tris-HCl, pH 7.4, containing MgCl₂ and EDTA for [125I]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-HT₂A and 5-HT₂C receptors, respectively.

5-HT₂B [(H)]-5-HT Binding Studies. Human 5-HT₂B receptor binding assays using [(H)]-5-HT were performed as previously described.24 The assay was automated using a Biomek 1000 (Beckman Instruments, Fullerton, CA). [(H)]-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 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 CaCl₂, 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(ethyleneimine) (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 [(H)]-5-HT trapped on the filters was determined by liquid scintillation spectrometry (Ready Protein, LS 6000 CI, Beckman Instruments, Fullerton, CA). The final [(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-naphthyliperazine (1-NP). The amount of protein was determined by the method of Bradford, with bovine serum albumin as the standard.30

5-HT₂A/₂C [(125)I]DOI Binding Studies. Human 5-HT₂A or 5-HT₂C binding studies were performed essentially as described for [(H)]-5-HT binding to the 5-HT₂A receptor with the following exceptions. The assay buffer contained, in final concentration, 10 μM pargyline, 9.75 mM MgCl₂, 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-HT₂A and 5-HT₂C 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-HT₂A and 1 μM ketanserin for 5-HT₂C receptors. The final concentration of [(125)I]DOI was approximately 0.07–0.15 nM.

[(H)]Ketanserin Binding to the Human 5-HT₂A Receptor. The assay conditions were essentially as previously described.36 Assays consisted of 0.8–0.8 mL total volume containing 50 mM Tris-HCl, 100 nM prazosin (to block potential binding of [(H)]ketanserin to α₁-adrenergic receptors), 0.4–0.5 nM [(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 [(H)]ketanserin trapped on the filters was determined by liquid scintillation spectrometry.

[(H)]Rauwolscine Binding to the Human 5-HT₂A 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 [(H)]rauwolscine, 500 nM efaroxan (to mask rauwolscine binding to α₂-adrenergic receptors), and 50 mM Tris-HCl, pH 7.4. Tubes were incubated at 37 °C for 10 min. Incubations were performed in triplicate. Nonspecific binding was defined in the presence of 0.5 μM 1-naphthyliperazine.

[(H)]Mesulergine Binding to the Human 5-HT₂C Receptor. This assay was adapted from that described by Pazos et al.38 Membranes were prepared as described above. Final concentrations for the 0.8–0.8 mL assays were 0.74–0.82 nM [(H)]mesulergine, varying concentrations of competing compound, and 50 mM 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 [(H)]ketanserin binding assay described above.

Muscarinic Receptor Studies: 1. Cell Culture. B2B (murine fibroblasts) transfected with the muscarinic M₁–M₅ receptors were prepared as previously described by Lai et al.29 (for the M₁ receptor), Lai et al.28 (for the M₂ receptor), and Kashani et al.41 (for the M₂–M₅ 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-cm² flasks (binding studies) or 24-well plates (functional studies) and kept in a humidified incubator maintained at 37 °C with 95% O₂ and 5% CO₂ levels.

2. Membrane Preparation. Confluent flasks of cells were incubated with 7 mL of 5 mM Hapes, 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 Hapes, 1 mM MgCl₂, 10 mM Na₅PO₄ 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. [(H)]QNB binding was performed essentially as described in Lai et al.36 with slight modifications. Membranes were diluted with 50 mM Hapes, 4 mM MgCl₂ (pH 7.4), and 100 μL (approximately 25 μg of protein) was incubated with 0.25 nM [(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, M₁B-B2B cells were plated onto 24-well plates and grown to ~50% confluency. Cells were then incubated overnight with 0.2 μM myo-2-[3H]inositol (15 Ci/mmol; New England Nuclear) in 0.5 mL of media. Excess myo-2-[3H]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 carbacbol 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 [(H)]IP₃ 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 spectrometry.
cAMP Assay. B82 cells transfected with the M2 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 14000g 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 assess 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), 100 μL of [3H]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 1700g 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 IC50 values and a Hill coefficient for each curve. The apparent Hill coefficient was determined using an LKB Wallac γ-counter (Wallac Inc., Gaithersburg, MD).

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Substituted Naphthofurans as Hybrid Molecules


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