Molecular interaction of serotonin 5-HT$_{2A}$ receptor residues Phe339$^{(6.51)}$ and Phe340$^{(6.52)}$ with super-potent *N*-benzyl phenethylamine agonists

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Abbreviations: 5-HT, 5-hydroxytryptamine, serotonin; **dLSD**, d-lysergic acid diethylamide; **psilocin**, 4-hydroxy-*N,N*-dimethyltryptamine; **5-MeO-DMT,** 5-methoxy-*N,N*-dimethyltryptamine; **mescaline**, 3,4,5-trimethoxyphenethylamine; **DOI,** 4-iodo-2,5-dimethoxyphenylisopropylamine; **DOI-NBOMe**, *N*-(2-methoxybenzyl)-4-iodo-2,5-dimethoxyphenylisopropylamine; **25H,** 2,5-dimethoxyphenethylamine; **25H-NMe,** *N*-methyl-2,5-dimethoxyphenethylamine; **25H-NPr,** *N*-propyl-2,5-dimethoxyphenethylamine; **25H-NB,** *N*-benzyl-2,5-dimethoxyphenethylamine; **25H-NBOMe,** *N*-(2-methoxybenzyl)-2,5-dimethoxyphenethylamine; **25H-NBOH,** N-(2-hydroxybenzyl)-2,5-dimethoxyphenethylamine; **24,** 2,4-dimethoxyphenethylamine; **24-NB,** *N*-benzyl-2,4-dimethoxyphenethylamine; **24-NBOMe,** *N*-(2-methoxybenzyl)-2,4-dimethoxyphenethylamine; **24-NBOH,** *N*-(2-hydroxybenzyl)-2,4-dimethoxyphenethylamine; **25I, 2CI,** 4-iodo-2,5-dimethoxyphenethylamine; **25I-NB,** *N*-benzyl-4-iodo-2,5-dimethoxyphenethylamine; **25I-NNap,** *N*-methylnapthyl-4-iodo-2,5-dimethoxyphenethylamine; **25I-NBOMe,** *N*-(2-methoxybenzyl)-4-iodo-2,5-dimethoxyphenethylamine; **25I-NBOH,** *N*-(2-hydroxybenzyl)-4-iodo-2,5-dimethoxyphenethylamine; **25I-NBF,** *N*-(2-fluorobenzyl)-4-iodo-2,5-dimethoxyphenethylamine; **25I-NBMD,** *N*-(2,3-methylenedioxybenzyl)-4-iodo-2,5-dimethoxyphenethylamine; **8-OH-DPAT,** 8-hydroxy-2-(dipropylamino)tetralin; **PI,** phosphatidylinositide(s); **TM,** transmembrane.

ABSTRACT

Experiments were conducted to examine the molecular basis for the high affinity and potency of a new class of 5-HT$_{2A}$ receptor agonists, *N*-benzyl phenethylamines. Competition binding assays at several serotonin receptors confirmed that an *N*-arylmethyl substitution was necessary for affinity increases up to 300-fold over simple *N*-alkyl homologues, as well as enhanced selectivity for 5-HT$_{2A}$ vs. 5-HT$_{2C}$ and 5-HT$_{1A}$ receptors. PI hydrolysis functional assays confirmed that these *N*-benzyl phenethylamines are potent and highly efficacious agonists at the rat 5-HT$_{2A}$ receptor. Virtual docking of these compounds into a human 5-HT$_{2A}$ receptor homology model indicated that the *N*-benzyl moiety might be interacting with F339$^{(6.51)}$, whereas the phenethylamine portion was likely interacting with F340$^{(6.52)}$. Experiments in h5-HT$_{2A}$ receptors with F339$^{(6.51)}$L and F340$^{(6.52)}$L mutations appear to support this hypothesis. Dramatic detrimental effects on affinity, potency, and intrinsic activity were observed with the F339$^{(6.51)}$L mutation for all *N*-benzyl analogues, whereas most *N*-unsubstituted phenethylamines and traditional agonists were only weakly affected, if at all. Consistent with other published studies, the F340$^{(6.52)}$L mutation detrimentally affected affinity, potency, and intrinsic activity of nearly all compounds tested, although a strong change in intrinsic activity was not seen with most *N*-aryl analogues. These data further validate the topology of our h5-HT$_{2A}$ receptor homology model. Importantly, this study is the first to identify a hitherto unrecognized role for residue 6.51 in agonist activation of a serotonin GPCR, whereas most previous reports have suggested a varied and sometimes contradictory role in homologous GPCRs.

Agonist activity at the serotonin 2A (5-HT$_{2A}$) receptor is essential for the psychopharmacology of serotonergic psychedelics such as LSD, DOI, psilocin, and 5-MeO-DMT, compounds with unique and dramatic effects on certain aspects of consciousness (Nichols 2004). Moreover, we have recently identified a functionally-selective 5-HT$_{2A}$ receptor agonist that selectively activates phosphoinositide turnover over production of eicosanoids (McLean *et al.*, 2006a). A key aspect to understanding the effects on consciousness of psychedelics is the study of the receptor-ligand interaction at the molecular level, and how it modulates second messenger generation subsequent to receptor activation.

Recently, we have been particularly interested in experimental validation of our h5-HT$_{2A}$ receptor homology model (Chambers and Nichols, 2002). Although the model has proven to have predictive power in designing simple conformationally-constrained phenethylamine agonists for this receptor (McLean et al., 2006a, 2006b), we were interested in extending it to provide a more comprehensive description of key residues involved in binding and activation. As a member of the type A GPCR family, we reasoned that understanding this receptor would provide general concepts that might extend across the GPCR field.

Thus, we were intrigued when we were alerted to a new class of extremely potent 5-HT$_{2A}$ receptor agonists that are derivatives of the phenethylamines mescaline and 2C-B (Pertz *et al.*, 1999; Elz *et al.*, 2002; K. Ratzeburg, personal communication). Although these novel agonists were characterized in a rat-tail artery model, we decided to examine whether these *N*-benzyl and related analogues had high affinity and potency in our heterologous cell-based assay systems. Furthermore, based on the fact that *N*-alkyl substitution was known to abolish hallucinogenic activity and binding affinity of phenethylamines (Shulgin and Shulgin, 1991; Glennon *et al.*, 1994), we were particularly curious about the contribution of the *N*-benzyl group, which seemed

most likely to be a π-π interaction of some type with an aromatic residue within the receptor.

Based on virtual docking into our homology model of the h5-HT$_{2A}$ receptor, we identified two highly conserved aromatic amino acids, F339$^{(6.51)}$ and F340$^{(6.52)}$, as likely interacting with these novel ligands. Previous site-directed mutagenesis studies had identified these residues to be within the 5-HT$_{2A}$ agonist binding pocket (Choudhary *et al.*, 1993; Choudhary *et al.*, 1995; Roth *et al.*, 1997). F339$^{(6.51)}$, however, had been indicated to be more important for antagonist than for agonist binding. SCAM studies in the human dopamine D$_2$ (Javitch *et al.*, 1998) and hamster adrenergic β_2 (Chen et. *al.*, 1999) receptors provided evidence supporting the idea that the F339$^{(6.51)}$ cognate residue is solvent accessible and likely part of the ligand binding pocket. These two studies disagree, however, as to whether the F340$^{(6.52)}$ cognate residue is solvent accessible and thus within the binding pocket or is involved in helical packing. Mutations of the cognate residues in other amine-binding type A GPCRs have provided varied and disparate support for the involvement of either residue 6.51 or 6.52 in the binding and activity of agonists and antagonists at muscarinic acetylcholine (Ward *et al.*, 1999), adrenergic (Chen et. *al.*, 1999), bradykinin (Nardone *et al.*, 1994), histamine (Wieland *et al.*, 1999), neurokinin (Huang *et al.*, 1995), and dopamine receptors (Cho *et al.*, 1995; Brown *et al.*, 2004).

The present paper describes our examination of a series of 5-HT$_{2A}$ receptor agonists, including a new series of *N*-benzyl analogues, and the effects of the mutations F339L and F340L on binding affinity to and functional activity at 5-HT$_{2A}$ receptors. We provide additional new evidence for the role of these two residues in GPCR receptor function.

MATERIALS AND METHODS

<u>Materials</u>

[^{3}H]Ketanserin, [^{3}H]8-hydroxy-2-(dipropylamino)tetralin (8-OH-DPAT), [^{125}I]4-iodo-2,5-dimethoxyphenylisopropylamine (DOI), and [^{3}H]*myo*-inositol were obtained from Perkin Elmer (Wellesley, MA). Serotonin (5-HT) was obtained from Sigma-Aldrich (St. Louis, MO). All other test ligands used in this study were synthesized in our laboratory using standard methods. The purity and identity of synthesized compounds were verified with TLC, melting point, NMR, mass spectrometry, and elemental analysis. Full details of chemical syntheses of all novel compounds and characterization of additional analogues will be described elsewhere. Structures of novel compounds used in this study and their abbreviations are shown in Figure 1. Stock solutions of 5-HT were prepared as the creatine sulfate salt, 5-MeO-DMT and psilocin as the maleate salts, and all other compounds as their HCl salts.

<u>Cell culture methods</u>

NIH-3T3 cells stably expressing either the rat 5-HT$_{2A}$ receptor (GF-6; 5500 fmol/mg) or the r5-HT$_{2C}$ receptor (PØ; 7500 fmol/mg) were the kind gift of Dr. David Julius and within this article will hereafter be referred to as Nr2A and Nr2C respectively. CHO cells stably expressing the human 5-HT$_{1A}$ receptor (CHO-1A; 500 fmol/mg) were the kind gift of Upjohn and will be referred to as Ch1A. The construction of HEK-293 human embryonic kidney cells with high stable expression of wild type h5-HT$_{2A}$ receptors (Hh2A; 8000 fmol/mg) has been described previously (Parrish *et al.*, 2005) and will be referred to as Hh2A$_{hi}$. Human 5-HT$_{2C}$ receptors were transiently expressed in HEK-293 cells by transfection with a 3:1 (v:w) ratio of Fugene 6 (Roche Biomolecules, Indianapolis, IN) to pcDNA3.1-h5HT$_{2C}$R (Guthrie cDNA resource center, Sayre, PA; http://www.cdna.org) mixture in Optimem (Invitrogen, Carlsbad, CA). These membrane

preparations will be referred to as Hh2C. All cell types were maintained as previously described (Parrish *et al.*, 2005), with the exception that media for regular growth of cells contained 5% (v/v) bovine calf serum and 5% (v/v) fetal clone serum (VWR, West Chester, PA), whereas media for propagating cells for membrane preparations and PI hydrolysis and eicosanoid release assays contained 10% (v/v) dialyzed fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA). All DMEM media for stably expressing cell lines contained 300 µg/mL G-418 (Sigma-Aldrich) for Nr2A and Nr2C cells, 22.5 units/mL Hygromycin B (Invitrogen) for CHO-1A cells, and 30 µg/mL Zeocin (Invitrogen) for Hh2A, Hh2A/F339L, and Hh2A/F340L (described below) cells.

Establishing stable wild type and mutant human 5-HT$_{2A}$R cell lines

A heterologous cell population with lower expression of the wild type h5-HT$_{2A}$ receptor (Hh2Ahet3; 1600 fmol/mg) was obtained in a manner similar to the Hh2A cell line described previously (Parrish *et al.*, 2005) without clonal selection and within this article will hereafter be referred to as Hh2A$_{lo}$. The h5HT$_{2A}$R insert was excised from pcDNA3.1-h5HT$_{2A}$R (Guthrie cDNA resource center, Sayre, PA, USA; http://www.cdna.org) and subcloned into the pLNCX2 vector (Clontech, Mountain View, CA). Site-directed mutagenesis of pLNCX2-h5HT$_{2A}$R was performed using the QuikChange Kit (Stratagene, La Jolla, CA) method of dual complementary mutant primer PCR followed by DpnI digestion of parental vector DNA. The following sense primers were used, along with their corresponding anti-sense primers (Integrated DNA Technologies, Coralville, IA):

GGTGATGTGGTGCCCTTTGTTCATCACAAACATCATGGCCG for F339L and

GGTGATGTGGTGCCCTTTCTTGATCACAAACATCATGGCCG for F340L. Mutant inserts verified by primer directed sequencing (Retrogen, San Diego, CA) were then subcloned into the pBudCE4 vector (Invitrogen, Carlsbad, CA). HEK-293 cells were transfected, colonies selected,

and receiver expression verified as previously described (Parrish *et al.,* 2005). The Hh2A/F339L

and Hh2A/F340L cell lines were chosen for moderate expression (2200 and 2500 fmol/mg,

respectively).

Radioligand binding assays

Membrane preparations, saturation isotherm, and competition binding assays were

performed as described in detail previously (Marona-Lewicka *et al.*, 2002; Chambers *et al.,*

2002). Saturation isotherm binding assays utilized 0.25-10 nM [^{3}H]ketanserin or 0.125-5 nM

($\pm$)-[^{125}I]DOI. Ligands were tested in competition binding assays for their ability to displace 0.25

nM [^{125}I]DOI at Nr2A, Nr2C, Hh2A$_{hi}$, and Hh2A/F339L membranes, 0.5 nM [^{3}H]ketanserin at

Hh2A$_{hi}$ and Hh2A/F340L membranes, and 0.5 nM [^{3}H]8-OH-DPAT at Ch1A membranes.

Inositol phosphate accumulation assays

Compounds were tested for their ability to stimulate hydrolysis of radiolabeled

phosphatidylinositides (PI) by measurement of radiolabeled inositol phosphates accumulation in

Nr2A, Hh2Ahet3, Hh2A/F339L, and Hh2A/F340L cells, as described previously in detail

(Marona-Lewicka *et al.*, 2002; Kurrasch-Orbaugh *et al.*, 2003). Primary comparison of EC50 and

intrinsic activity was performed between cell lines with approximately equivalent expression of

h5-HT$_{2A}$ wild type (1600 fmol/mg), F339L mutant (2000 fmol/mg), and F340L mutant (2500

fmol/mg) receptors. Each assay plate was normalized to wells stimulated with water (0%) and a

concentration of serotonin chosen to be maximally stimulating (100%).

Computational modeling/virtual docking

Ligand structures were prepared, virtually docked into an h5-HT$_{2A}$R homology model

(Chambers and Nichols, 2002), and local energy minimized-ensemble structures obtained as

previously described (Parrish *et al.*, 2005). Briefly, energy minimized structures were virtually

docked using the GOLD software package (Cambridge Crystallographic Data Center, Cambridge,

UK). Docking algorithms were performed without any constraints. Ligand-receptor ensemble

structures were each obtained by merging the highest ranked output ligand orientation structures

with the input h5-HT$_{2A}$ homology model structure using the SYBYL software package (Tripos,

St. Louis, MO), followed by energy minimization, molecular dynamics, and a final energy

minimization simulation. The molecular dynamics and minimization simulations were performed

with constraints only between D155$^{(3.32)}$ and the amine nitrogen of the ligand, and S239$^{(5.43)}$ and

the nearest polar group of the ligand.

Data Analysis

GraphPad Prism Software (GraphPad Software Inc. San Diego, CA) was used to calculate

non-linear regression curves for a one-site model in order to obtain K$_i$ (affinity) values for

radioligand displacement and variable slope sigmoidal dose-response curves for EC50 (potency)

and intrinsic activity from PI hydrolysis assays. This software also was utilized to perform two-

way ANOVAs on pEC50 and intrinsic activity values of human wild type and mutant receptors,

with a Bonferroni post-test to compare replicate mean values of mutant receptors to the wild type.

Unpaired two-tailed Student's T-tests were used to compare the pK$_i$ at the mutant receptors to the

corresponding wild type for the same competing radioligand. Values obtained from mutant

receptors were considered statistically distinguishable from wild type if the models generated

p<0.01. Changes in the standard Gibbs free energy ($\Delta\Delta G°$) of binding due to the mutations were

calculated from the K$_i$ values at 25 °C with equation 1, as follows:

$$\Delta(\Delta G°) = \Delta G°_{mutant} - \Delta G°_{wild\ type} = RT \ln(K_{mutant}/K_{wild\ type}) \qquad (1)$$

Changes in binding affinity and EC50 values were transformed to normalize the scale by taking

the difference of the $\log_{10}$ value (ΔpK_i and $\Delta EC50$ respectively) with equations 2 and 3 as

follows:

$$\Delta pK_i = pK_{i\text{-mutant}} - pK_{i\text{-WT}} = -logK_{i\text{-mutant}} - (-logK_{i\text{-WT}}) \tag{2}$$

$$\Delta pEC50 = pEC50_{mutant} - pEC50_{WT} = -logEC50_{mutant} - (-logEC50_{WT})$$

(3)

Changes in intrinsic activity (ΔInt.Act.) were calculated with equation 4 as follows:

$$\Delta Int.Act. = Int.Act._{mutant} - Int.Act._{WT} \tag{4}$$

An arbitrary threshold of "weak effect" was defined as a change in pK_i or pEC50 of *ca.* 1 log order (10-fold) or less, and a change in intrinsic activity of $\leq$25%. Virtual docking figures were generated using PyMol (DeLano Scientific, San Carlos, CA; http://www.pymol.org). Amino acid residues are numbered with their position in the h5-HT$_{2A}$ receptor and their position relative to the most highly conserved residue of that transmembrane region, as per Ballesteros and Weinstein (1995).

RESULTS

The effect of the *N*-benzyl moiety does not simply result from a hydrophobic interaction. As shown in Table 1, increasing hydrophobic bulk of the *N*-substituent on 25H (2,5-dimethoxyphenethylamine) by adding a methyl or *n*-propyl group to yield 25H-NMe and 25H-NPr was detrimental to the binding affinity at all wild type receptors tested. By contrast, the *N*-benzyl group produced a profound increase in affinity (*ca.* 7- to 300-fold) and potency (*ca.* 100- to 200-fold) at the r5-HT$_{2A}$ receptor. Addition of a polar methoxy or hydroxy group at the 2-position of the benzyl group further increased affinities.

Increases in affinity for *N*-benzyl analogs of 25I (4-iodo-2,5-dimethoxyphenethylamine; 2CI) were not as dramatic (*ca.* 2- to 7-fold) as the other phenethylamines tested, yet were still significant. Addition of an alpha-methyl to the side chain (DOI-NBOMe) or the large *N*-naphthyl analogue (25I-NNap) provided the only *N*-benzyl compounds tested that had decreased activity. Most of the test compounds also were assayed at the r5-HT$_{2C}$, h5-HT$_{2C}$, and 5-HT$_{1A}$ receptors (see supplemental material). *N*-substitution with an arylmethyl group appears to maintain if not slightly increase the modest 2A vs. 2C selectivity of these compounds, but the *N*-benzyl had no effect on 5-HT$_{1A}$ receptor affinity.

N-Benzyl analogues of phenethylamines are potent agonists at the r5-HT$_{2A}$ receptor. Several of the test ligands were examined at cloned r5-HT$_{2A}$ receptors for their ability to stimulate the hydrolysis of radiolabeled phosphatidylinositides (PI), as also shown in Table 1. All compounds were relatively potent with high intrinsic activity in the PI hydrolysis assay, with the exception of DOI-NBOMe, 25I-NNap, 25I-NB, and 25I-NBF, which were partial agonists. Again, the *N*-benzyl analogs of 25H and 24 displayed dramatic increases in PI hydrolysis potency (*ca.* 100- to 200-fold), relative to their simple phenethylamine counterparts. Only modest

improvement of potency and efficacy were generally observed in the series of *N*-arylmethyl

substituted of 25I (*ca.* 2- to 8-fold), with the exception of 25I-NNap and 25I-NBF which both lost

potency and efficacy. The *N*-(2-methoxy)benzyl analog of DOI, DOI-NBOMe, did not have

improved potency or efficacy.

Virtual docking of *N*-benzyl analogues to an *in silico*-activated h5-HT$_{2A}$ receptor

homology model reveals potential aromatic residues interacting with these ligands. As the

example in Figure 2 illustrates, unconstrained virtual docking and subsequent energy

minimization simulations of several *N*-benzyl analogues of phenethylamines produced

orientations that placed the aromatic ring of the classical phenethylamine pharmacophore in a

position to interact with F340$^{(6.52)}$, similar to previously observed orientations (Parrish *et al.*,

2005). A further potential π-π interaction was identified between the novel aromatic *N*-benzyl

moiety and F339$^{(6.51)}$, a residue previously identified only to affect antagonist binding in the 5-

HT$_{2A}$ receptor (Roth *et al*., 1997). It was predicted that this residue might play a key role in

interacting with this new class of 5-HT$_{2A}$ agonists.

The F339$^{(6.51)}$L and F340$^{(6.52)}$L mutations within the h5-HT$_{2A}$ receptor both strongly

affect the binding affinities of the *N*-benzyl analogues, whereas F340$^{(6.52)}$L strongly affects

only the smaller classic ligands. K$_D$ values for [^{125}I]DOI at Hh2A and Hh2A/F339L membrane

preparations were 0.78 ± 0.01 nM and 0.86 ± 0.14 nM, respectively. K$_D$ values for [^{3}H]ketanserin

at Hh2A, Hh2A/F339L, and Hh2A/F340L membrane preparations were 1.10 ± 0.12 nM, $11.1 \pm$

3.6 nM, and 0.40 ± 0.02 nM, respectively. As anticipated from the work of Roth and colleagues

(1997), it was not possible to determine a K$_D$ value for [^{125}I]DOI at h5-HT$_{2A}$/F340L receptors.

Furthermore, due to the high (11 nM) K$_D$ for [^{3}H]ketanserin at h5-HT$_{2A}$/F339L, it was not

practical to use this radioligand for competition binding at this receptor. Therefore, competition

binding assays were performed with $[^{125}I]$DOI at h5-HT$_{2A}$/F339L receptors, $[^3H]$ketanserin at

h5-HT$_{2A}$/F340L receptors, and both radioligands at wild type receptors. In order to control for

differences in wild type K$_i$ values from different radioligand displacement, K$_i$ values were

compared only to wild type receptors utilizing the same radioligand.

As Table 2 and Figure 3 show, the F339$^{(6.51)}$L mutation had relatively weak (<10-fold)

detrimental effects on the affinities of all the classical and smaller ligands except for the

phenethylamine 25H and the 5-substituted tryptamines 5-HT and 5-MeO-DMT. With the

exception of LSD, all compounds tested had binding affinities that were statistically

distinguishable from wild type. The mutation had weak detrimental effects on binding when a

hydrophobic *N*-alkyl substitution on the amine of 25H was present (*ca.* 5- to 6-fold), but when an

N-arylmethyl group was attached, as seen with 25H-NB, 25H-NBOMe, and 25H-NBOH, there

was a dramatic *ca.* 40- to 700-fold decrease in affinity compared to the wild type. A similar

marked detrimental effect was observed between 24 and 24-NB, 24-NBOMe, and 24-NBOH,

with a *ca.* 100-fold decrease in affinity for the latter three *N*-benzyl analogues. The *N*-benzyl

analogues of 25I were not as strongly affected, with 25I-NNap, 25I-NBOMe, 25I-NBOH, and

25I-NBF showing 30- to 50-fold decreases in binding affinities, and 25I-NB and 25I-NMD

showing only *ca.* 10-fold decreases in affinity.

The F340$^{(6.52)}$L mutation had comparatively weak (<10-fold) detrimental effects only on

the phenethylamines mescaline, 25H, 24, and 25I, in addition to the aliphatic analogues 25H-NMe

and 25H-NPro, and the aromatic analogue 25I-NBMD. Most of the other *N*-arylmethyl

derivatives had *ca.* 100-fold decreases in binding affinities. The strongest detrimental effects

were seen with 5-HT (>1000-fold), psilocin (160-fold), and 5-MeO-DMT (500-fold). The

aromatic analogues followed a trend similar to that seen with the phenethylamines in the

F339$^{(6.51)}$L mutation. All compounds tested had binding affinities that were statistically

distinguishable from the wild type.

Both the F339$^{(6.51)}$L and F340$^{(6.52)}$L mutations within the h5-HT$_{2A}$ receptor adversely

affect to different degrees the ability of compounds tested to stimulate PI hydrolysis. It has

been shown that large differences in expression level can have marked effects on potency and

intrinsic activity (Esbenshade *et al.*, 1995; Zong *et al.*, 1996; Roth *et al.*, 1997; Kenakin 1997), so

a new HEK-293 cell line was developed with wild type expression comparable to the mutant

h5-HT$_{2A}$ receptor expressing cell lines. The Hh2A$_{hi}$ cell line established previously (Parrish *et*

al., 2005) has a [^{3}H]ketanserin B$_{max}$ of *ca.* 9000 fmol/mg. The newly constructed Hh2A$_{lo}$ cell

population had a B$_{max}$ of *ca.* 1600 fmol/mg, compared to expression levels of *ca.* 2200 and 2500

fmol/mg, respectively, for the mutant Hh2A/F339L and Hh2A/F340L cell lines.

As Table 3 and Figure 4 indicate, the F339$^{(6.51)}$L mutation had relatively weak detrimental

effects on the potency of LSD and the unsubstituted phenethylamine agonists mescaline, 25H, 24,

and 25I. The tryptamines 5-HT, psilocin, and 5-MeO-DMT were moderately affected. This

mutation produced strong detrimental effects (*ca.* 100-fold or greater), however, with all of the *N*-

benzyl phenethylamine analogues. Some detrimental effects on intrinsic activity of the smaller

agonists were observed with this mutation, but generally were weak (<10-fold). As evident in the

EC50 values, stronger detrimental effects on intrinsic activity were observed with all of the *N*-

benzyl analogues of phenethylamines. For some of the *N*-benzyl analogues, this change is so

dramatic that they are transformed from nearly full agonists at the wild type receptor into very

weak partial agonists by the mutation.

The F340$^{(6.52)}$L mutation had marked detrimental effects on the potency of all compounds

tested, particularly the tryptamines 5-HT, psilocin, and 5-MeO-DMT. Due to the extreme loss of

potency for 5-HT at this mutant receptor and solubility limitations of 5-HT, the normalization

concentration of serotonin may not be maximally stimulating and thus all intrinsic activity values

indicated at this mutant receptor may be slightly elevated and all negative ΔInt.Act. values may

therefore be underestimated as well. With this caveat, the F340$^{(6.52)}$L mutation had strong

detrimental effects on intrinsic activity (efficacy) of all the smaller classic agonists. Interestingly,

the *N*-benzyl analogues of 25H and one analogue of 24 also showed relatively strong (*ca.* 100-

fold or greater) decreases in intrinsic activity, whereas none of the 25I analogues showed any

strong decreases. As with the F339$^{(6.51)}$L mutation, the compounds most dramatically affected by

the F340$^{(6.52)}$L mutation were transformed from nearly full agonists at the wild type receptor to

very weak partial agonists in the mutant receptor.

DISCUSSION

Our primary goal with this study was to explore further the topology of the human 5-HT$_{2A}$ receptor by characterizing the pharmacology of a new class of 5-HT$_2$ receptor agonists. Initially, we wished to determine whether the beneficial effects of *N*-(2-methoxy)-benzyl substitution on phenethylamines reported by Pertz *et al.* (1999) and Elz *et al.* (2002) would be observed in our heterologous expression systems. We also compared two *N*-alkyl substituted phenethylamines to ascertain that the effect of the *N*-benzyl was not simply due to a hydrophobic interaction.

As Table 1 indicates, these analogues are high affinity, selective, potent, and highly efficacious agonists that depend on an *N*-arylmethyl substitution for their exceptionally high activity. The *N*-methyl and *n*-propyl analogues of 25H had decreased affinity at all receptors tested. Yet, *N*-benzyl compound 25H-NBOMe had more than 100-fold increased affinity compared to the unsubstituted 25H. *N*-(2-methoxy)-benzyl compound 24-NBOMe, displayed an increase in affinity of up to 300-fold compared to the unsubstituted 24. We recognized, therefore, that the aromatic ring of the *N*-benzyl moiety was enhancing activity through some specific biophysical property, which we believe to be a π-π interaction with an aromatic residue in the receptor. These data are consistent with a previous study evaluating amine substitution on phenylalkylamines and indolealkylamines (Glennon *et al.*, 1994). In that study, however, *N*-arylmethyl analogues of the phenethylamine 2C-B showed only *ca.* 2- to 3-fold increased affinity. The underlying basis for this effect was not evaluated, and none of their ligands were tested for functional activity.

Dramatic increases also were observed in the potency of these compounds to stimulate PI hydrolysis. Although *N*-arylmethyl analogues of 25I did not show as marked an increase in affinity or potency, they still possessed affinities at the h5-HT$_{2A}$ receptor as high as 40 pM. We

speculate that the decreased sensitivity of the 4-iodo series to *N*-benzyl substitution may be due to

a reorientation of the ligand within the receptor binding domain in order to accommodate the

large iodine atom. This change may force the ligand to adopt a binding pose that displaces the *N*-

benzyl from an optimal position for interaction with F339$^{(6.51)}$.

The *in silico*-activated homology model of the h5-HT$_{2A}$ receptor developed in our

laboratory (Chambers and Nichols 2002) was utilized in an attempt to provide some qualitative

explanation of the high affinity, potency, and intrinsic activity of this novel class of compounds.

Previous virtual dockings of phenethylamines and related classic agonists have produced low-

energy ensemble structures with orientations placing F340$^{(6.52)}$ in a position to interact with the

aromatic ring of the ligand through a π-π interaction (Parrish *et al.*, 2005). Those findings were

consistent with previous mutagenesis results indicating the cognate residue in other receptors is

solvent-accessible (Javitch *et al.*, 1998) and is involved in an essential π-π interaction with

agonists (Choudhary *et al.*, 1993; Cho *et al.*, 1995; Choudhary *et al.*, 1995; Huang *et al.*, 1995;

Roth *et al.*, 1997). Other mutagenesis studies, however, indicate that this residue is not solvent

accessible (Chen *et al.*, 1999), does not interact with agonists, and/or may be interacting with

some antagonists (Choudhary *et al.*, 1993; Cho *et al.*, 1995; Choudhary *et al.*, 1995; Wieland *et

al.*, 1999), or is not involved in receptor activation (Ward *et al.*, 1999). Docking orientations

produced in this study again positioned the aromatic ring of the phenethylamine pharmacophore

in a position to interact with F340$^{(6.52)}$, as well as positioning the polar methoxy groups in ways

that indicated interactions with other residues identified by site-directed mutagenesis to be

involved in 5-HT$_{2A}$ agonist binding (Wang *et al.*, 1993; Kristiansen *et al.*, 2000; Shapiro *et al.*,

2000) and observed previously in our work (Parrish *et al.*, 2005).

In the present work a potential π-π interaction was identified between F339$^{(6.51)}$ and the

novel *N*-benzyl moiety of the ligand. Mutation of the cognate residue in a variety of other GPCRs

has indicated that residue 6.51 is (1) located near retinal in bovine rhodopsin (Nakayama and

Khorana, 1991), (2) solvent accessible (Javitch *et al.*, 1999; Chen *et al.*, 1999), and (3) may be

essential for a π-π interaction with both agonists and antagonists (Nardone *et al.*, 1994; Chen *et

al.*, 1999; Cho *et al.*, 1995; Huang *et al.*, 1995; Ward *et al.*, 1999; Brown *et al.*, 2004). With

respect specifically to the 5-HT$_{2A}$ receptor, previous data seemed to indicate that this residue was

not within the agonist-binding pocket and was not involved in the binding of agonists or partial

agonists (Choudhary *et al.*, 1993), but rather stabilized binding of the antagonist ketanserin

(Choudhary *et al.*, 1995; Roth *et al,*. 1997).

In order to test the hypothesis that both F339$^{(6.51)}$ and F340$^{(6.52)}$ might be contributing to a

π-π interaction with the *N*-benzyl analogues of phenethylamines, mutations of these two residues

were performed in the h5-HT$_{2A}$ receptor. A semiconservative phenylalanine to leucine mutation

was chosen to eliminate aromaticity, while conserving steric bulk and hydrophobicity of the

residues to reduce possible global effects of the mutations (Fersht *et al.*, 1987). Concerns about

potential global structural change were addressed by including several different chemical classes

of traditional 5-HT$_2$ agonists. Differential shifts in binding affinities between ligand classes

should indicate more regional changes in the receptor structure rather than a global one affecting

all ligand classes.

Differences in binding affinities between mutant and wild type receptors were assessed, as

well as the functional repercussions of the F339$^{(6.51)}$L and F340$^{(6.52)}$L mutations in the PI

hydrolysis second messenger system. The results of these experiments are illustrated in Figures 3

and 4, based on the data from Tables 2 and 3. The trends observed in these figures seem to

indicate that both F339$^{(6.51)}$ and F340$^{(6.52)}$ are interacting with the *N*-benzyl phenethylamines,

whereas F340$^{(6.52)}$ appears to be interacting mainly with *N*-unsubstituted phenethylamines and the other classical agonists. The $\Delta\Delta G°$ values of *ca.* 2-4 kcal/mol are in agreement with the energy of a "stacked" or "T-shape" π-π interaction (Jorgensen and Severance, 1990). This trend is particularly evident in the weak effects of the F339$^{(6.51)}$L mutation on EC50 and near absence of effects on intrinsic activity of the *N*-unsubstituted phenethylamines 25H, 24, and 25I. In stark contrast, the F339$^{(6.51)}$L mutation had profound effects on both measures of function for the *N*-benzyl analogues of these phenethylamines, which were nearly converted into antagonists in this mutant receptor.

The F340$^{(6.52)}$L mutation appears to affect the affinity and potency of nearly all the ligands tested, although to a different degree across different ligand classes. It appears still able to couple to activation of PI hydrolysis, thus reducing concerns of massive global change of the receptor structure. The simple classic agonists also are turned nearly into antagonists by this mutation. Surprisingly, the F340$^{(6.52)}$L mutation does not appear to have an effect on intrinsic activity of the *N*-benzyl analogues of 25I, yet the binding and potency are shifted to a degree similar to the other *N*-benzyl analogues. Thus, an interaction with F339$^{(6.51)}$ in this series may be sufficient to produce a fully "active" receptor state.

F339$^{(6.51)}$ and F340$^{(6.52)}$ are highly conserved and make up an evolutionarily constrained cascade of aromatic residues that mediate allosteric communication and receptor activation in GPCRs (Süel *et al.*, 2003). These residues reside within transmembrane domain 6 (TM6), which is directly coupled to the third intracellular loop (IL3), between TM5 and TM6. This loop is implicated as being important for the ability of the 5-HT$_{2A}$ receptor (and all related GPCRs) to interact with and activate the appropriate G-proteins, and thus affect second messenger production, particularly Gα_q activation of PI hydrolysis (Kubo *et al.*, 1988; Wess *et al.*, 1989,

1990; Oksenberg *et al*., 1995; Hill-Eubanks *et al.,* 1996). It would appear that agonist or

antagonist character, affinity, and potency of a particular ligand is dependent on the ability of the

ligand to interact with specific TM6 residues and induce or suppress movement of this helix.

Although there is disagreement about the individual contributions of $F^{(6.51)}$ and $F^{(6.52)}$ toward the

binding and activity of agonists and antagonists across several amine-binding type A GPCRs, it

appears from our data that within the h5-HT$_{2A}$ receptor, involvement of either residue is sufficient

for agonist binding and receptor activation.

Moreover, although we have provided further evidence that F340$^{(6.52)}$ is likely not

involved in the binding of the antagonist ketanserin (Roth *et al.*, 1997), our results do not preclude

the possibility that this residue interacts with other classes of structurally diverse antagonists or

partial agonists. We also have expanded and extended the findings of Pertz *et al.* (1999), and Elz

et al. (2002), that *N*-benzyl phenethylamines, particularly those with a 2-methoxy or 2-hydroxy

function on the benzyl moiety, represent a novel class of high affinity, potent, and modestly

selective 5-HT$_{2A}$ receptor agonists. We believe our data are consistent with the general topology

for the h5-HT$_{2A}$ receptor reflected in our *in silico*-activated homology model of this receptor

(Chambers and Nichols, 2002). Finally, and perhaps most important, we believe it may be

possible to exploit cognate residue in 6.51 in other GPCRs to design agonists with increased

potency and intrinsic activity.

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Footnotes

LEGENDS FOR FIGURES

Figure 1: Structures of molecules used in this study.

Figure 2: Illustrative cross-eyed stereopair representation of ligand pose from virtual docking experiments with *(A)*, 25H-NBOMe, and *(B)*, 25I-NBOH in the h5-HT$_{2A}$ receptor, showing proposed π-π interactions between the *N*-benzyl moiety and F339, and the aryl portion of the phenethylamine and F340. Ligands are shown as space-filling spheres, and receptor residues believed to be interacting with the ligand are displayed as sticks. The view is within the membrane, with TM6 in the left foreground, TM5 in the left background, TM3 on the right, and the extracellular face of the receptor toward the top of the figure. TMs 1, 2, 4, and 7 are not displayed. Energy minimized ensemble structures indicate that F340$^{(6.52)}$ may interact with the aryl ring of the phenethylamine pharmacophore and F339$^{(6.51)}$ may interact with the *N*-benzyl moiety.

Figure 3: Effects on binding affinities of the (A) F339$^{(6.51)}$L, and (B) F340$^{(6.52)}$L mutations in the h5-HT$_{2A}$ receptor. These bar graphs display the ΔpK$_i$ values derived from the data of Table 3 (see Methods). Larger negative (upward) values in these graphs indicate a greater negative effect of the mutation on binding affinity. The dashed line at -1.0 indicates an arbitrary threshold for "weak" effects. ** indicates $p < 0.01$ for values of ΔpK$_i$ from unpaired two-tailed Student T-tests.

Figure 4: Effects on EC50 and intrinsic activity of PI hydrolysis functional activity by F339$^{(6.51)}$L (open bars) and F340$^{(6.52)}$L (solid bars) mutations in the h5-HT$_{2A}$ receptor. These bar

graphs display the ΔpEC50 and ΔInt. Act. values derived from the data of Table 4 (see Methods).

All intrinsic activity values are normalized to serotonin stimulation and thus it is not possible to

obtain ΔInt.Act. values for 5-HT. Positive ΔInt.Act. values for 25I-NB and 25I-NBOMe at

h5-HT$_{2A}$/F340L receptors were not significantly different from WT and are not shown for

formatting reasons. Dashed lines at -1.0 and -25% indicate an arbitrary threshold for "weak"

effects. ** indicates p<0.01 for values of ΔpEC50 and ΔInt.Act. from two-way ANOVA tests

with Bonferroni post-tests.

Table 1. Abilities of test compounds to displace (±)-[^{125}I]DOI and activate PI hydrolysis at rat 5-HT$_{2A}$ receptors.

Data are represented as the mean and (SEM) from non-linear regression fits of a single binding site model for K_i values and normalize variable slope sigmoidal dosage-response curves for estimates of EC50 and intrinsic activity. All data are from at least three independent experiments. A typical experiment would show 10-20 fold stimulation by 5-HT over basal for PI hydrolysis assays.

Drug	K_i	r5-HT$_{2A}$R PI Hydrolysis	
	r5-HT$_{2A}$ (nM) (±)-[^{125}I]DOI	EC50 (nM)	Intrinsic Activity (% 5-HT)
25H	227 (39)	12877 (1930)	82(8)
25H-NMe	1286 (64)		
25H-NPr	734 (30)		
25H-NB	17.5 (1.9)		
25H-NBOMe	1.19 (0.17)	81.2 (3.8)	81 (0.4)
25H-NBOH	2.76 (0.40)	141 (21)	66 (2)
24	202 (19)	4034 (260)	67 (8)
24-NB	28.5 (2.9)		
24-NBOMe	0.68 (0.12)	51.0 (6.7)	72(1)
24-NBOH	0.67 (0.01)	74.0 (6.7)	82(4)
DOI	0.58 (0.06)	19.2 (2.6)	77 (3)
DOI-NBOMe	1.08 (0.21)	36.1 (2.7)	43 (3)
25I	0.62 (0.08)	19.0 (2.6)	59 (4)
25I-NB	0.31 (0.03)	12.0 (0.7)	37 (2)
25I-NNap	3.74 (0.52)	> 1 µM	25 @ 10 µM
25I-NBOMe	0.087 (0.010)	2.50 (0.55)	78 (6)
25I-NBOH	0.12 (0.02)	6.34 (0.18)	71 (2)
25I-NBF	0.28 (0.04)	23.2 (1.2)	32 (3)
25I-NBMD	0.19 (0.02)	8.2 (1.6)	68 (7)

Table 2. Abilities of test compounds to displace (±)-[^{125}I]DOI or [^{3}H]ketanserin at wild type and mutant h5-HT$_{2A}$ receptors.
Data are represented as the mean and (SEM) in nM of K$_i$ values from non-linear regression fits of a single binding site model from at least three independent experiments. ΔΔG° values are calculated from K$_i$ values at 25°C. ** indicates p<0.01 for values of ΔpK$_i$ from unpaired two-tailed Student T-tests between mutant and wild type receptors tested with the same radioligand.

| Drug	(±)-[^{125}I]DOI			[^{3}H]Ketanserin		
	h5-HT$_{2A}$ K$_i$ (nM)	h5-HT$_{2A}$/F339L K$_i$ (nM)	ΔΔG° (kcal/mol)	h5-HT$_{2A}$ K$_i$ (nM)	h5-HT$_{2A}$/F340L K$_i$ (nM)	ΔΔG° (kcal/mol)
5-HT	4.84 (0.2)	59.6 (10.0)**	1.5	77.6 (13.8)	192725 (36305)**	4.6
d-LSD	0.40 (0.02)	0.60 (0.12)	0.2	0.81 (0.16)	13.01 (1.09)**	1.6
psilocin	11.8 (1.2)	28.6 (4.3)**	0.5	22.8 (4.0)	3659 (243)**	3.0
5-MeO-DMT	7.54 (1.06)	129 (15)**	1.7	49.2 (3.2)	23726 (4726)**	3.7
mescaline	1499 (245)	4488 (608)	0.6	14640 (2447)	62425 (10485)**	0.9
25H	377 (67)	5786 (734)**	1.6	1999 (311)	16001 (3163)**	1.2
25H-NMe	1907 (254)	8719 (671)**	0.9	5934 (92)	43918 (2271)**	1.2
25H-NPr	1295 (151)	7863 (769)**	1.1	3597 (642)	9815 (943)**	0.6
25H-NB	68.1 (10.6)	2722 (470)**	2.2	184 (33)	6698 (1031)**	2.1
25H-NBOMe	2.83 (0.31)	1435 (192)**	3.5	11.0 (0.5)	689 (107)**	2.5
25H-NBOH	3.73 (0.45)	2642 (455)**	3.9	11.6 (1.7)	277 (40)**	1.9
24	298 (29)	1013 (190)**	0.7	999 (182)	8391 (1200)**	1.3
24-NB	26.6 (2.7)	1768 (339)**	2.5	71.9 (3.0)	3316 (356)**	2.3
24-NBOMe	1.71 (0.34)	252 (49)**	3.0	5.24 (1.01)	703 (14)**	2.9
24-NBOH	1.51 (0.20)	306 (57)**	3.1	2.83 (0.36)	292 (14)**	2.7
25I	0.73 (0.06)	2.63 (0.32)**	0.8	4.52 (0.30)	28.9 (4.8)**	1.1
25I-NB	0.25 (0.05)	3.1 (0.1)**	1.3	0.28 (0.02)	27.0 (1.8)**	2.7
25I-NNap	4.83 (0.55)	157 (31)**	2.1	6.68 (1.02)	268 (268)**	2.1
25I-NBOMe	0.044 (0.006)	2.08 (0.35)**	2.3	0.15 (0.03)	4.3 (0.76)**	2.1
25I-NBOH	0.061 (0.012)	1.84 (0.16)**	2.0	0.068 (0.012)	1.58 (0.17)**	1.9
25I-NBF	0.26 (0.05)	15.2 (1.7)**	2.4	0.19 (0.03)	37.9 (1.3)**	3.1
25I-NBMD	0.049 (0.008)	0.29 (0.03)**	1.1	0.21 (0.03)	0.94 (0.17)**	0.9

Table 3. Ability of compounds to activate PI hydrolysis at wild type and mutant h5-HT$_{2A}$ receptors.

Data are represented as the mean and (SEM) of computer-derived estimates of EC50 and Intrinsic Activity values from at least three independent experiments. A typical experiment would show 4-10 fold stimulation by 5-HT over basal. ** indicates p<0.01 values for ΔpEC50 and ΔInt.Act. from two-way ANOVA tests with Bonferroni post-tests.

| Drug	h5-HT$_{2A}$		h5-HT$_{2A}$/F339L		h5-HT$_{2A}$/F340L	
	EC50 PI Hydrolysis (nM)	Intrinsic Activity (% 5-HT)	EC50 PI Hydrolysis (nM)	Intrinsic Activity (% 5-HT)	EC50 PI Hydrolysis (nM)	Intrinsic Activity (% 5-HT)
5-HT	5.17 (0.97)	100	92.4 (10.5)**	100	9840 (458)**	100
d-LSD	0.22 (0.04)	84 (3)	1.36 (0.23)**	55 (5)**	15.7 (2.9)**	20 (5)**
psilocin	7.29 (0.72)	105 (9)	129 (18)**	44 (8)**	4529 (813)**	9 (1)**
5-MeO-DMT	4.33 (0.78)	98 (4)	416 (71)**	74 (5)**	5255 (969)**	15 (4)**
mescaline	1117 (223)	83 (5)	11333 (991)**	82 (7)	78795 (3869)**	30 (1)**
25H	1021 (14)	96 (10)	10353 (1652)**	78 (1)	141033 (39537)**	12 (4)**
25H-NBOMe	15.3 (3.7)	88 (6)	3407 (390)**	27 (4)**	1341 (53)**	43 (5)**
25H-NBOH	23.5 (1.8)	100 (6)	11267 (758)**	32 (6)**	2156 (503)**	28 (3)**
24	832 (200)	83 (5)	4077 (579)**	66 (4)	109311 (37671)**	17 (1)**
24-NBOMe	4.00 (0.80)	89 (6)	1436 (281)**	55 (5)**	2029 (199)**	66 (8)
24-NBOH	5.42 (0.66)	84 (4)	5623 (29)**	49 (8)**	696 (139)**	31 (3)**
25I	2.54 (0.18)	82 (3)	22.8 (2.7)**	72 (5)	99.5 (5.3)**	38 (2)**
25I-NB	1.96 (0.12)	66 (2)	1093 (353)**	14 (2)**	263 (40)**	82 (1)
25I-NBOMe	0.44 (0.07)	81 (4)	28.0 (5.2)**	51 (4)**	26.8 (4.2)**	84 (7)
25I-NBOH	0.19 (0.03)	86 (5)	42.3 (6.5)**	45 (6)**	14.6 (2.9)**	82 (7)
25I-NBF	1.55 (0.21)	87 (11)	150 (25)**	8 (1)**	410 (33)**	81 (6)
25I-NBMD	1.07 (0.20)	72 (3)	91.0 (30.9)**	11 (1)**	145 (25)**	70 (5)
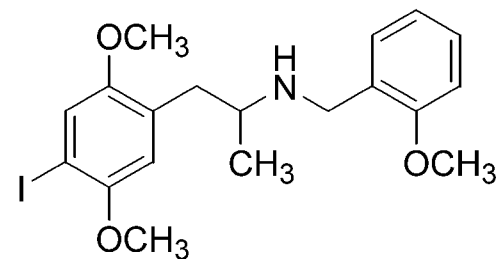
DOI-NBOMe
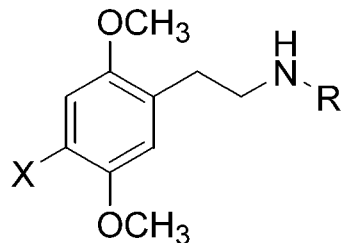
25I (2C-I); X = I, R = H
25H;   X = H, R = H
25H-NMe; X = H, R = CH₃
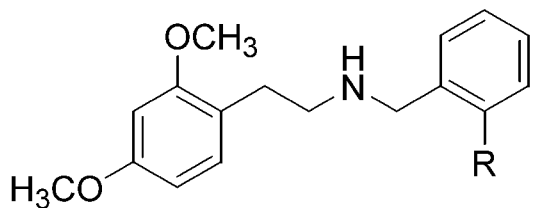25H-NPro; X = H, R = nPr
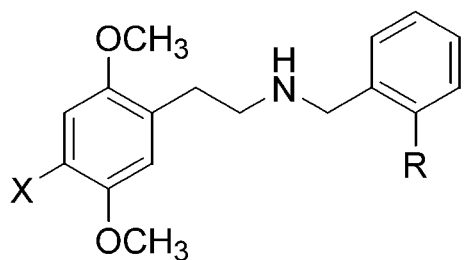
25H-NB;   X = H, R = H
25H-NBMeO; X = H, R = OCH₃
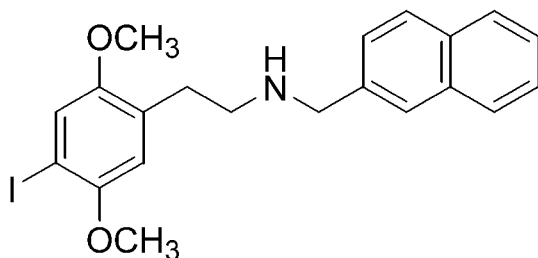25H-NBOH;   X = H, R = OH

25I-NB;   X = I, R = H
25I-NBMeO; X = I, R = OCH₃
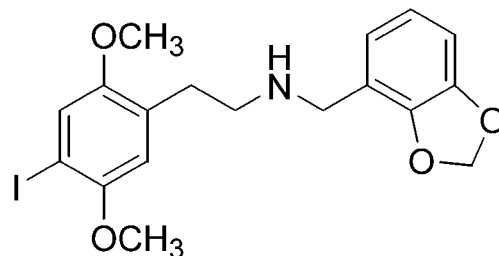25I-NBOH;   X = I, R = OH
25I-NBF;   X = I, R = F
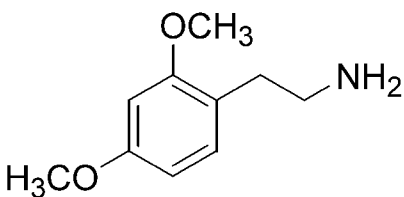
24-NB;   R = H
24-NBMeO; R = OCH₃
24-NBOH;   R = OH

24

25I-NNap

25I-NBMD