ACS Chemical Neuroscience

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5-HT$_{2A}$/5-HT$_{2C}$ Receptor Pharmacology and Intrinsic Clearance of *N*-Benzylphenethylamines Modified at the Primary Site of Metabolism

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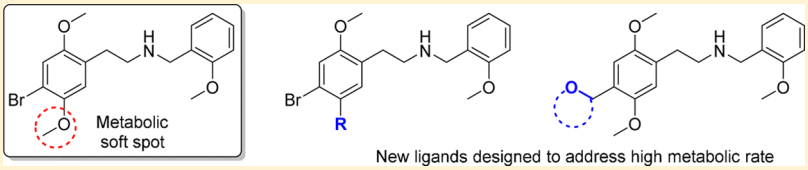
Supporting Information



ABSTRACT: The toxic hallucinogen 25B-NBOMe is very rapidly degraded by human liver microsomes and has low oral bioavailability. Herein we report on the synthesis, microsomal stability, and 5-HT$_{2A}$/5-HT$_{2C}$ receptor profile of novel analogues of 25B-NBOMe modified at the primary site of metabolism. Although microsomal stability could be increased while maintaining potent 5-HT$_2$ receptor agonist properties, all analogues had an intrinsic clearance above 1.3 L/kg/h predictive of high first-pass metabolism.

KEYWORDS: *Phenethylamines, hallucinogens, microsomal stability, 5-HT$_{2A}$ receptor agonists*

The "NBOMes" constitute a novel class of phenethylamine hallucinogens that are increasingly being used as pharmacological and neuroimaging tools in the serotonin field.[1] In recent years, the NBOMes have also gained notoriety within forensic science, as the recreational use of the drugs have led to several hospitalizations and deaths in the United States and in Western Europe, albeit the exact mechanism underlying the toxicity of the drugs remains unresolved.[2]

In a previous study, the metabolism of one of these ligands 25B-NBOMe was investigated as this compound is currently being used in its [^{11}C]-labeled form as a PET-ligand to visualize serotonin 2A receptors (5-HT$_{2A}$R) in the brain as "CIMBI-36" (**1**).[3] The primary route of degradation of **1** in the body is via demethylation of the 5′-methoxy of the phenethylamine core (Scheme 1).[4]

We have previously investigated the human liver microsomal stability of a series of NBOMes and found them to have very high intrinsic clearance compared to the classic phenethylamine hallucinogens (2C-B, 2C-I, etc.).[5] Whereas the 2-methoxybenzyl moiety confers high binding affinity toward 5-HT$_2$Rs and increases the agonist potencies of the compounds at these receptors, it also increases intrinsic clearance to beyond the human liver blood flow (1.3 L/kg/h), which is a predictor for high first-pass metabolism and low oral bioavailability.[6] With the knowledge that **1** has a single primary metabolic soft spot in pigs and humans, we hypothesized that structural modifications in this part of the molecule would decrease intrinsic clearance. To test this hypothesis we synthesized a small library of 8 25B-NBOMe analogues altered at the position of the 5′-methoxy (compounds **2**–**9** in Chart 1). These structural modifications were made knowing that previous structure–activity relationship (SAR) studies of this compound class indicate that such modifications could be detrimental to the binding affinities of the ligands to the 5HT$_{2A}$R.[7−9] In addition to assessing stability of the compounds toward human liver microsomes (intrinsic clearance), the binding affinities and functional properties of

Scheme 1. Metabolic "Soft Spot" of 25B-NBOMe (1)

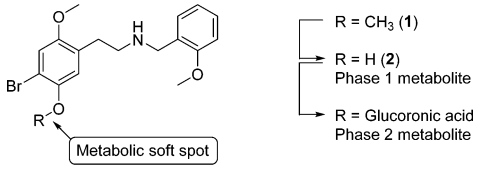
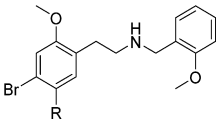


Chart 1. 25B-NBOMe Analogues (1−9)



1: R = OMe
2: R = OH
3: R = H
4: R = F
5: R = Cl
6: R = CF_3
7: R = I
8: R = OiPr
9: R = OCH_2CH_2OMe

Received: August 26, 2016
Accepted: August 26, 2016

DOI: 10.1021/acschemneuro.6b00265
ACS Chem. Neurosci. XXXX, XXX, XXX−XXX

Table 1. Intrinsic Clearance (HCl_{Int}), Binding Affinities, and Agonist Properties of Analogues 1−9 at 5-HT_{2A} and 5-HT_{2C} Receptors[a]

compd	affinity (pK_i)		pEC_{50}		R_{max}		HCl_{Int} (L/kg/h)	binding selectivity (2A/2C)	functional selectivity (2A/2C)
	5-HT_{2A}	5-HT_{2C}	5-HT_{2A}	5-HT_{2C}	5-HT_{2A}R	5-HT_{2C}R			
1 OMe	9.68 (±0.09)	8.60 (±0.07)	9.02 (±0.09)	9.04 (±0.06)	83 (±3)	99 (±7)	3.3	12	0.9
2 OH	8.65 (±0.08)	7.98 (±0.11)	8.92 (±0.09)	9.21 (±0.09)	61 (±3)	73 (±6)	1.9	4.3	0.5
3 H	8.68 (±0.10)	7.82 (±0.02)	8.87 (±0.14)	8.09 (±0.09)	70 (±4)	84 (±4)	2.0	7.1	6.2
4 F	9.01 (±0.11)	8.00 (±0.04)	8.55 (±0.13)	7.87 (±0.06)	63 (±6)	85 (±2)	2.5	10	4.3
5 Cl	9.19 (±0.12)	7.36 (±0.05)	8.35 (±0.10)	8.03 (±0.13)	62 (±6)	88 (±5)	2.5	66	2.1
6 CF_3	9.34 (±0.06)	8.24 (±0.11)	7.65 (±0.10)	7.65 (±0.11)	75 (±6)	88 (±2)	2.3	13	1.0
7 I	8.15 (±0.12)	8.14 (±0.06)	7.91 (±0.13)	7.75 (±0.09)	69 (±5)	85 (±2)	4.6	1.0	1.5
8 OiPr	8.37 (±0.13)	7.38 (±0.12)	8.17 (±0.12)	7.69 (±0.10)	71 (±4)	71 (±3)	3.0	25	3.1
9 OCH_2CH_2OMe	8.18 (±0.07)	6.58 (±0.09)	8.66 (±0.06)	8.30 (±0.10)	69 (±3)	78 (±4)	4.4	39	2.3

[a]Binding affinities (pK_i ± SEM) of the analogues were determined in a [^{3}H]CIMBI-36 binding assay using membranes from tsA201 cells transiently expressing human 5-HT_{2A}R and 5-HT_{2C}R, and their agonist potencies (pEC_{50} ± SEM) and efficacies (R_{max} ± SEM values, given in % of R_{max} of 5-HT) were determined in a Ca^{2+}/Fluo-4 assay using stable 5-HT_{2A}R- and 5-HT_{2C}R-HEK293 cell lines. All pK_i ± SEM, pEC_{50} ± SEM, and R_{max} ± SEM values in the table are based on at least three independent experiments.

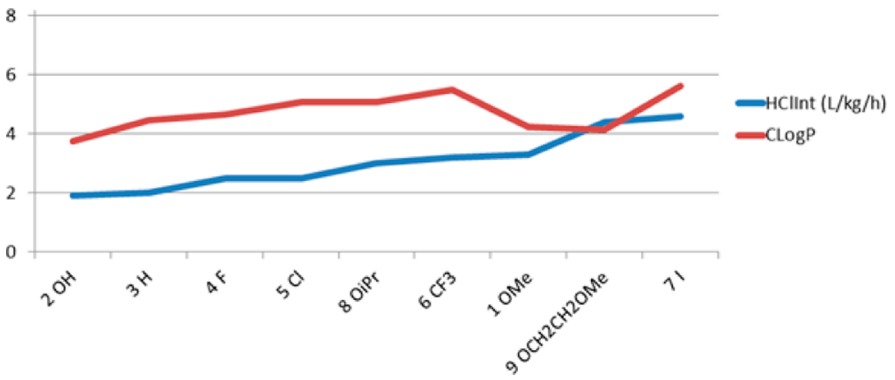


Figure 1. cLogP and Intrinsic clearence of analogues 1−9.

the compounds at the human 5-HT_{2A} and 5-HT_{2C} receptors were also determined (Table 1; see the Supporting Information for K_i and EC_{50} values reported in nM).

RESULTS AND DISCUSSION

Substituting a hydroxyl for the methoxy of **1** (in effect a demethylation, arriving at **2**) decreased intrinsic clearance (1.9 L/kg/h) by approximately 40% - insufficient to significantly remedy high first pass metabolism. Somewhat surprisingly **2** retained high binding affinity to 5-HT_{2A}R (2.3 nM) and 5-HT_{2C}R (10 nM) and remained a potent partial agonist at the receptors. The 2,5-dimethoxy motif is imperative to the 5-HT_2R affinity and potency displayed by the *N*-unsubstituted "classic" phenethylamine hallucinogens,[7] but in the NBOMe class this does not appear to be the case, as omission of the methoxy of **1** completely (arriving at **3**) only reduced the 5-HT_{2A}R binding affinity approximately 10-fold, whereas both agonist potencies and efficacies of the two analogues were comparable. The intrinsic clearance of **3** (2.0 L/kg/h) was not substantially different from that of **2**. Substituting a fluorine for the methoxy of **1** (arriving at **4**) did not remedy the high intrinsic clearance (2.5 L/kg/h), but 5-HT_{2A}R affinity and potency was retained whereas 5-HT_{2C}R affinity and potency was slightly decreased in this analogue.

Increasing the size of the halogen of **4** by substituting chlorine for fluorine (arriving at **5**) had negligible effect on intrinsic clearance (2.5 L/kg/h) and only modest effects on the binding affinities and functional potencies displayed by the analogue at 5-HT_{2A}R and 5-HT_{2C}R. Similar modest changes in intrinsic clearance and pharmacological properties were observed when substituting CF_3 for the fluorine of **4** (arriving at **6**). Increasing the size of the halogen of **4** even more by substituting iodine for fluorine resulted in an analogue (**7**) exhibiting higher clearance and relatively unaltered pharmacological properties compared to **4**.

Keeping the 5′-alkoxy motif of **1** while increasing steric bulk by substituting methoxy for isopropoxy (arriving at **8**) had negligible effect on intrinsic clearance, but **8** exhibited substantially lower binding affinities and agonist potencies at 5-HT_{2A}R and 5-HT_{2C}R. Substituting methoxy for methoxyethoxy (**9**) increased intrinsic clearance somewhat and also decreased the binding affinities and agonist potencies at 5-HT_{2A}R and 5-HT_{2C}R. The fact that in particular the binding affinity to 5-HT_{2C}R is decreased, results in an increased 2*A*/2C binding-selectivity of **9** compared to **1** (Table 1).

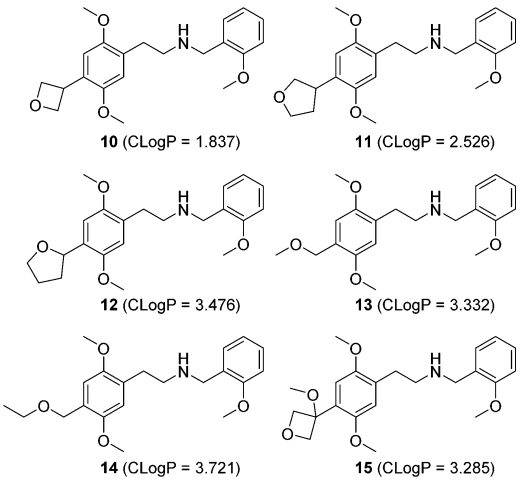
Whereas the 5′-methoxy at the metabolic soft spot of 25B-NBOMe thus apparently is not required for high agonist potency at 5-HT_{2A}R and 5-HT_{2C}R, our attempts to significantly decrease intrinsic clearance by modications of this part of the scaffold were unsuccessful. Even though 5′-demethylation is the preferred degradation pathway of **1**, the site of oxidation presumably changes in **2**-**9** retaining high intrinsic clearance (above 1.3 L/kg/h).

DOI: 10.1021/acschemneuro.6b00265
ACS Chem. Neurosci. XXXX, XXX, XXX−XXX

The fact that analogues **1**−**9** are characterized by cLogP values of 4.94 ± 0.21 on average (ChemBioDraw Ultra 14.0, PerkinElmer) could explain their high clearance (Figure 1), as very lipophilic compounds are known to be prime targets for cytochrome P450 (CYP450).[10]

We speculated that the lipophilic nature of the compounds possibly could target them to degradation by CYP450 regardless of blocking any specific C−H bond from oxidation/hydroxylation. Thus, decreasing cLogP of the compounds should, theoretically, decrease their intrinsic clearance. The most lipophilic substituent in **1** is the bromide in the 4′-position of the phenethylamine core, and this position has in a previous study been found to be amenable to substitution without concomitant loss of 5-HT$_2$R activity.[11] In an effort to reduce cLogP of the 25B-NBOMe scaffold while retaining 5-HT$_2$R activity, we synthesized a small set of analogues (Chart 2) with Br substituted for polar aliphatic

Chart 2. 25B-NBOMe Analogues (10−15)



open-chained or cyclic ethers. The resulting compounds (**10**−**15**) have cLogP values of 3.03 ± 0.29 on average (Chart 2), and we measured the stability of analogues **10**−**15** toward human liver microsomes and their pharmacological properties at 5-HT$_{2A}$R and 5-HT$_{2C}$R (Table 2).

The oxetane structural motif has gained popularity in medicinal chemistry research as its addition to a molecule has been shown to increase aqueous solubility and stability toward liver enzymes.[12] Unfortunately, the marked drop in cLogP of **10** was not accompanied by a reduction in intrinsic clearance (2.8 L/kg/h) and **10** displayed substantially reduced binding affinities to 5-HT$_{2A}$R (18 nM) and 5-HT$_{2C}$R (120 nM) compared to **1**. Increasing the ring-size of **10** by one methylene unit (arriving at **11**) increased 5-HT$_{2A}$R binding affinity substantially (1.2 nM) whereas the 5-HT$_{2C}$R affinity (100 nM) was comparable to that exhibited by **10**. Thus, compound **11** exhibited the desired high 5-HT$_{2A}$R binding affinity and 2*A*/2C-binding selectivity compared to **10**, but the effect on intrinsic clearance (13 L/kg/h) was detrimental.

Moving the oxygen in the THF-ring of **11** one atom closer to the phenyl core (**12**) had little effect on 5-HT$_{2A}$R and 5-HT$_{2C}$R affinity or potency, but the intrinsic clearance (17 L/kg/h) was increased compared to **11**.

The simplest open-chain aliphatic ether analogue in this series was the "methoxy-methyl" **13** which displayed ∼10-fold decreased binding affinities at 5-HT$_{2A}$R and 5-HT$_{2C}$R compared to **1**, whereas the agonist properties of the two analogues were comparable (Table 2). Interestingly, the intrinsic clearance of **13** (13 L/kg/h) was considerably increased compared to **1**. Extending the methoxy-methyl group of **13** to ethoxy-methyl yielded analogue **14** characterized by similar pharmacological properties and intrinsic clearance. Adding "methoxymethyl" to the oxetane of **10** (arriving at **15**) did not substantially change the intrinsic clearance, but the binding affinity of **15** at 5-HT$_{2A}$R was ∼10-fold higher than that of **10** resulting in an increased 2*A*/2C-binding selectivity ratio. The lowered cLogP values of **10**−**15** compared to **1**-**9** had no discernible effect on intrinsic clearance (Figure 2), and cLogP thus seems to be a poor predictor of microsomal stability in the case of the NBOMe hallucinogens.

While our attempts to increase the microsomal stability of **1** by blocking the primary metabolic "soft spot" also were unsuccessful, this SAR study did lead to some surprising discoveries. Most notably, in contrast to the observed SAR for the "classic" *N*-unsubstituted phenethylamine hallucinogens, a 2,5-dimethoxy pattern on the phenyl core of the NBOMes is not required for high binding affinity and agonist potency at 5-HT$_2$R. Interestingly, the 5′-position tolerates a comparatively large variety of substituents ranging from small (e.g., H) to large (e.g., CH_2CH_2OMe) and from polar (e.g., OH) to apolar (e.g., Oi-Pr).

Table 2. Intrinsic Clearance, Binding Affinities, and Functional Properties of Analogues 10−15 at 5-HT$_{2A}$ and 5-HT$_{2C}$ Receptors[a]

	affinity (pK_i)		pEC$_{50}$		R_{max}				
compd	5-HT$_{2A}$R	5-HT$_{2C}$R	5-HT$_{2A}$R	5-HT$_{2C}$R	5-HT$_{2A}$R	5-HT$_{2C}$R	HCl$_{Int}$ (L/kg/h)	binding selectivity (2A/2C)	functional selectivity (2A/2C)
1 OMe	9.68 (±0.09)	8.60 (±0.07)	9.02 (±0.09)	9.04 (±0.06)	83 (±3)	99 (±7)	3.3	12	0.9
10 oxetan-3-yl	7.74 (±0.01)	6.91 (±0.05)	8.93 (±0.04)	8.21 (±0.04)	85 (±4)	86 (±3)	2.8	6.7	5.2
11 tetrahydrofuran-3-yl	8.64 (±0.11)	7.00 (±0.09)	8.94 (±0.03)	7.80 (±0.02)	79 (±4)	85 (±1)	13	43	13
12 tetrahydrofuran-2-yl	8.59 (±0.08)	6.71 (±0.11)	8.81 (±0.13)	8.48 (±0.08)	85 (±5)	99 (±6)	17	73	2.2
13 CH_2OMe	8.59 (±0.07)	7.34 (±0.10)	9.12 (±0.07)	8.81 (±0.09)	67 (±4)	88 (±4)	13	18	2.0
14 CH_2OEt	8.62 (±0.06)	7.54 (±0.08)	9.16 (±0.13)	8.72 (±0.08)	72 (±4)	85 (±2)	16	12	2.8
15 (3-OMe)oxetan-3-yl	8.58 (±0.08)	6.92 (±0.08)	9.04 (±0.06)	8.20 (±0.06)	68 (±3)	83 (±3)	3.7	46	7.0

[a]Binding affinities (pK_i ± SEM) of the analogues were determined in a [^{3}H]Cimbi-36 binding assay using membranes from tsA201 cells transiently expressing human 5-HT$_{2A}$R and 5-HT$_{2C}$R and their agonist potencies (pEC$_{50}$ ± SEM) and efficacies (R_{max} ± SEM values, given in % of R_{max} of 5-HT) were determined in a Ca^{2+}/Fluo-4 assay using stable 5-HT$_{2A}$R- and 5-HT$_{2C}$R-HEK293 cell lines. All pK_i ± SEM, pEC$_{50}$ ± SEM, and R_{max} ± SEM values in the table are based on at least three independent experiments.

DOI: 10.1021/acschemneuro.6b00265
ACS Chem. Neurosci. XXXX, XXX, XXX−XXX

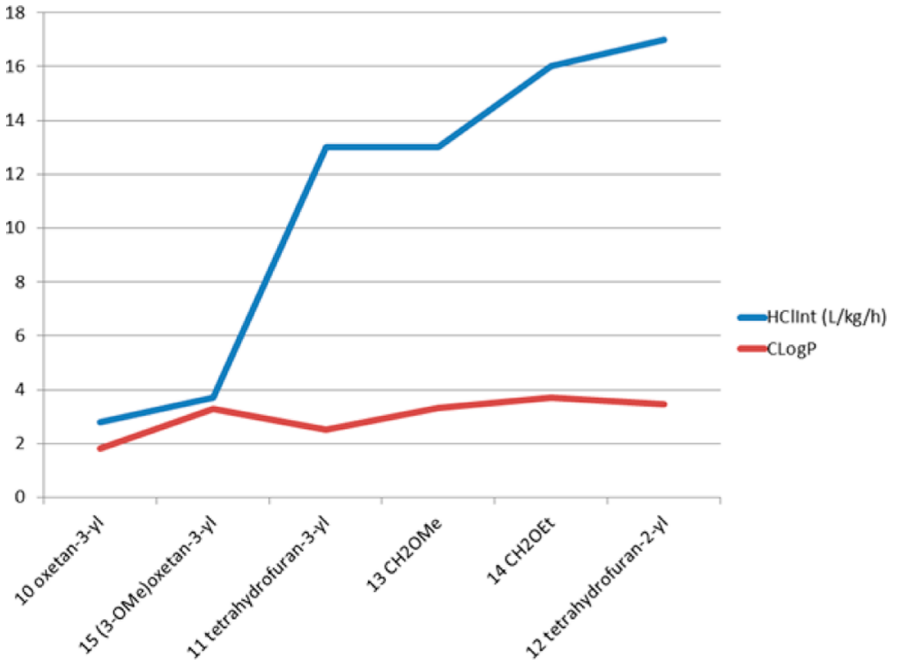


Figure 2. cLogP and Intrinsic clearance of **10**–**15**.

The case of the phenol analogue **2** is particularly interesting as it is the primary phase-I metabolite of **1** in vivo. Since **2** is a potent 5-HT_{2A}R/5-HT_{2C}R agonist in its own right, it could very feasibly play a role in the in vivo effects of **1**.

The analogues of **1** modified at the position of the 5′-methoxy of the phenethylamine core (**2**–**9**) were designed with optimization of intrinsic clearance as the primary goal, but at the same time we anticipated a marked drop in agonist potency at 5-HT_{2A}R. Glennon et al. reported that the deletion of the 5′-Methoxy-group in the parent phenethylamines and the corresponding amphetamines decreased the binding affinity for 5-HT_2R 15–30-fold.[9] Furthermore, Braden and Nichols have investigated the interaction of the receptor with a variety of agonists of the ergoline, tryptamine and phenethylamine classes in a mutagenesis study.[13] Here the 5′-methoxy group of the phenethylamines was found to be important for 5-HT_{2A}R binding and activation due to its hydrogen bonding with the Ser5.43(239) residue in the receptor, and in support of this 2-methoxy-4-methylamphetamine and 5-ethyl-2-methoxy-4-methylamphetamine were found to be 190-fold and 26-fold less potent agonists at the receptor than 2,5-dimethoxy-4-methylamphetamine, respectively.[13] In another study, the Nichols group examined the pharmacology of a variety of primary and *N*-benzylated phenethylamines at 5-HT_{2A}R by use of mutagenesis and virtual docking.[14] This study revealed that while Ser5.43(239) is predicted to interact with the 5′-methoxy of the *N*-benzylphenethylamines, the Phe6.51(339) residue is more important for the binding affinities and agonist potencies of this compound class, while not being essential for the binding affinity and potency of the *N*-unsubstituted "classic" phenethylamines. Moreover, a substituent (other than −H) at the 4′-position of the phenethylamine core of the *N*-benzylphenethylamines was found to be more important for 5-HT_{2A}R binding and potency than the 5′-methoxy.[14] This observation might explain the observed tolerance to substituent size and topology at the 5′-position of the phenethylamines in the present study, as compared to what has previously been reported for the "classic" phenethylamine 5-HT_{2A}R agonists.

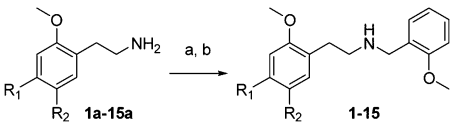
CONCLUSION

In the present study, the SAR and metabolic properties of two series of 5HT_{2A}R agonists from the *N*-benzylphenethylamine structural class have been delineated. In the first series of analogues, modifications to the metabolic softspot of **1** yielded analogues which had preserved their pharmacological profile at 5-HT_2Rs, but the high intrinsic clearance characterizing the lead compound was retained in the analogues. Thus, a different approach was taken and less lipophilic analogueues where pursued by replacing the bromide at the 4′-position of the phenethylamine core of the scaffold. This series also comprised several potent 5-HT_2R agonists, but a correlation between lipophilicity and metabolic liability was not observed. Thus, it appears to be challenging to develop metabolically stable 5-HT_2R agonists with an *N*-benzylphenethylamine scaffold. Nonetheless, important information about the SAR of this compound class was obtained in the study, especially the fact that the 5′-methoxy-substituent is not crucial for binding to and activation of the 5-HT_{2A}R as previous investigations have suggested.

METHODS

Chemistry. Compounds (**1**–**15**) were synthesized by condensation of a suitable phenethylamine (compounds **1a**–**15a**) with 2-methoxybenzaldehyde, followed by sodium borohydride reduction of the resulting imine, in one-pot fashion (Scheme 2).

Scheme 2. Synthesis of Compounds 1–15[a]

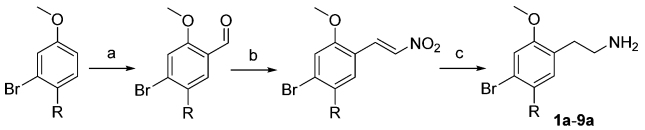


[a]Reaction conditions (see the Supporting Information for full experimental details): (a) 2-methoxybenzaldehyde, $N(Et)_3$, 3 Å molecular sieves, EtOH, reflux; (b) $NaBH_4$, EtOH, rt, 30–80% over two steps.

DOI: 10.1021/acschemneuro.6b00265
ACS Chem. Neurosci. XXXX, XXX, XXX−XXX

The phenethylamine precursors substituted at the 5′-position of the phenyl core (**1a**−**9a**) were generally synthesized via Rieche formylation of a suitable precursor, Henry condensation of the resulting benzaldehyde, and DIBAL-H reduction of the resulting nitrostyrene (Scheme 3).

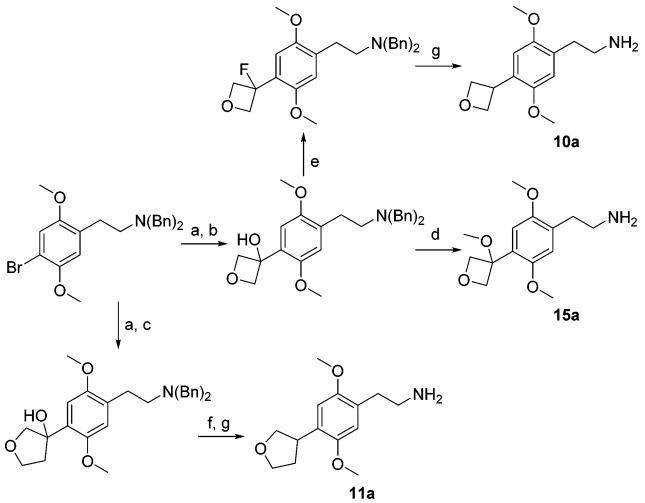
Scheme 3. Synthesis of Compounds 1−9[a]



[a]Reaction conditions (see the Supporting Information for full experimental details): (a) $TiCl_4$, dichloromethyl methyl ether, CH_2Cl_2, −10 to 0 °C, 30−70%; (b) propylene diammonium diacetate, $MeNO_2$, iPrOH, rt to reflux, 30−71%; (c) neat DIBAL-H, THF, rt to reflux 32−80%.

10a, **11a**, and **15a** were synthesized (Scheme 4) by lithium-halogen exchange of *N*,*N*-dibenzyl-"2C−B" followed by quench with a suitable

Scheme 4. Synthesis of Compounds 10a, 11a, and 15a[a]



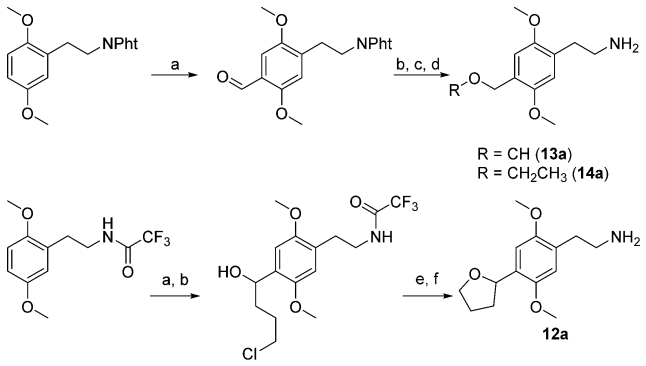
[a]Reaction conditions: (a) *n*-BuLi, THF, −78 °C; (b) 3-oxetanone, −78 °C to rt, 57% over two steps; (c) dihydrofuran-3(2H)-one, −78 °C to rt, 46% over two steps; (d) NaH, MeI, DMF, 0 °C to rt, 68%; (e) DAST, CH_2Cl_2, −78 °C, 54%; (f) $SiH(Et)_3$, TFA, DCM, 100%; (g) $Pd(OH)_2$, H_2 (10 bar), MeOH, 50 °C, 64−78%.

ketone. The resulting benzylic alcohol was either methylated using strong base and MeI, removed directly in a reductive dehydroxylation reaction or removed indirectly by conversion to the fluoride, using DAST, followed by reductive dehalogenation using $H/Pd(OH)_2$. Debenzylation using $H/Pd(OH)_2$ furnished the primary phenethylamines.

12a, **13a**, and **14a** were synthesized (Scheme 5) by a Friedel−Crafts type acylation of "2C−H" protected on the nitrogen as either the phthalimide or trifluoroacetamide. The resulting carbonyl was reduced using $NaBH_4$, and the resulting benzylic alcohol was alkylated using NaH as base and a suitable alkyl halide. Deprotection by either hydrazine or KOH furnished the primary phenethylamines.

In Vitro Pharmacology. *[^{3}H]Cimbi-36 Binding Assay.* The binding affinities of the analogues were determined at membranes from tsA201 cells transiently expressing the human 5-HT_{2A} or 5-HT_{2C} receptors using [^{3}H]Cimbi-36 as radioligand. The tsA201 cells (2×10^6) were split into a 10 cm tissue culture plate a day before transfection and transfected with 8 μg 5-HT_{2A}R-pCDNA3.1 or 5-HT_{2C}R-pCDNA3.1 using Polyfect (Qiagen) as a DNA carrier. The day after the transfection the culture medium was changed. 36−48 h after

Scheme 5. Synthesis of Compounds 12a−14a[a]



[a]Reaction conditions: (a) $TiCl_4$, dichloromethyl methyl ether/4-chlorobutanoyl chloride, CH_2Cl_2, −10 to 0 °C, 43−61%; (b) $NaBH_4$, EtOH, rt, 33−79%; (c) NaH, EtI/MeI, DMF, 0 °C to rt; (d) hydrazine (aq.), THF, rt, 33−42% over two steps; (e) NaH, THF, −10 °C, 84%; (f) KOH, MeOH, H_2O, rt, 62%.

the transfection, the cells were harvested and scraped into ice-cold assay buffer (50 mM Tris-HCl, 4 mM $CaCl_2$, pH 7.4), homogenized with a Polytron homogenizer for 10 s, and centrifuged for 20 min at 50 000*g* at 4 °C. Cell pellets were resuspended in fresh assay buffer, homogenized, and centrifuged at 50 000*g* for another 20 min, after which the membranes were stored at −80 °C until use.

At the day of the assay, cell membranes were resuspended in assay buffer and incubated with [^{3}H]Cimbi-36 (using assay concentrations of 0.1 nM and 0.2 nM for 5-HT_{2A}R and 5-HT_{2C}R membranes, respectively) and various concentrations of the test compounds. Nonspecific binding was determined using 20 μM mianserin. The total assay volume was 1 mL, and the reactions were incubated for 2 h at room temperature while shaking. Whatman GF/C filters were presoaked for 1 h in a 0.2% polyethylenimine solution, and binding was terminated by filtration through the filters using a 48-well Brandel cell harvester and four washes with 4 mL of ice-cold isotonic NaCl solution. After this, the filters were dried, 3 mL of Opti-Fluor (PerkinElmer) was added, and the amount of bound radioactivity was determined in a scintillation counter.

K_i values for the analogues were calculated from the IC_{50} values determined in the assay using the Cheng−Prusoff equation: $K_i = [IC_{50}/(1 + [RL]/K_D)]$, where K_D and [RL] are equilibrium dissociation constant for [^{3}H]Cimbi-36 at the specific receptor and the radioligand concentration used in the assay, respectively.

Ca^{2+}/Fluo-4 Assay. The functional properties of the analogues were characterized at stable HEK293 cell lines stably expressing the human 5-HT_{2A} and 5-HT_{2C} receptors in a fluorescence-based Ca^{2+} imaging assay using the fluorophore Fluo-4 essentially as previously described.[15] The cells were split into poly-D-lysine-coated black 96-well plates with clear bottom. The following day, the culture medium was aspirated and the cells were incubated in 50 μL of assay buffer [Hank's Buffered Saline Solution containing 20 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 2.5 mM probenecid, pH 7.4] supplemented with 6 μM Fluo−4/AM at 37 °C for 1 h. Then the buffer was aspirated, the cells were washed once with 100 μL assay buffer, after which 100 μL assay buffer was added to the cells. The 96-well plate was assayed in a FLEXStation (Molecular Devices, Crawley, UK) measuring emission [in fluorescence units (FU)] at 525 nm caused by excitation at 485 nm a total of 90 s before and after addition of 33.3 μL of agonist solution in assay buffer. Experiments were performed in duplicate at least three times for each compound at each receptor. Concentration−response curves were constructed based on the differences in the fluorescence units (ΔFU) between the maximal fluorescence levels recorded before and after addition of the ligands, and EC_{50} and R_{max} values for the agonists were extracted from the curve fits.

Metabolic Stability. Intrinsic clearance in human liver microsmes was determined by assessing the depletion of test compound over time

DOI: 10.1021/acschemneuro.6b00265
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as described previously.[5] Test compounds were incubated at 1 μM. Intrinsic clearance were calculated from the slope of the linear regression of percentage of compound remaining in the incubation against time.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschemneuro.6b00265.

Full experimetal details (PDF)

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Author Contributions

S.L.P., I.N.P., and M.B. synthesized the compounds, A.J.J. performed the in vitro pharmacology and C.B. determined the intrinsic clearance. The manuscript was written through contributions of all the authors who have given approval to the final version of the manuscript.

Author Contributions

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Funding

The Novo Nordisk, Carlsberg and Lundbeck Foundation.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

5-HT_{2A}R, serotonin 2A receptor; 5-HT_{2C}R, serotonin 2C receptor; HCl_{INT}, human intrinsic clearence; DIBAL-H, diisobutylaluminum hydride; DAST, diethylaminosulfur trifluoride

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DOI: 10.1021/acschemneuro.6b00265
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