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Structure−Activity Relationships for Psilocybin, Baeocystin, Aeruginascin, and Related Analogues to Produce Pharmacological Effects in Mice

Grant C. Glatfelter,* Eline Pottie, John S. Partilla, Alexander M. Sherwood, Kristi Kaylo, Duyen N. K. Pham, Marilyn Naeem, Vamshikrishna Reddy Sammeta, Stacie DeBoer, James A. Golen, Elliott B. Hulley, Christophe P. Stove, Andrew R. Chadeayne, David R. Manke, and Michael H. Baumann

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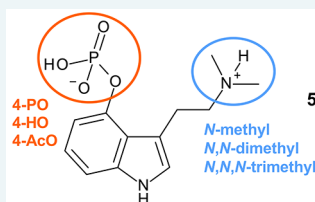ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: 4-Phosphoryloxy-N,N-dimethyltryptamine (psilocybin) is a naturally occurring tertiary amine found in many mushroom species. Psilocybin is a prodrug for 4-hydroxy-N,N-dimethyltryptamine (psilocin), which induces psychedelic effects via agonist activity at the serotonin (5-HT) 2A receptor (5-HT$_{2A}$). Several other 4-position ring-substituted tryptamines are present in psilocybin-containing mushrooms, including the secondary amine 4-phosphoryloxy-N-methyltryptamine (baeocystin) and the quaternary ammonium 4-phosphoryloxy-N,N,N-trimethyltryptamine (aeruginascin), but these compounds are not well studied. Here, we investigated the structure− activity relationships for psilocybin, baeocystin, and aeruginascin, as compared to their 4-acetoxy and 4-hydroxy analogues, using *in vitro* and *in vivo* methods. Broad receptor screening using radioligand binding assays in transfected cells revealed that secondary and tertiary tryptamines with either 4-acetoxy or 4-hydroxy substitutions display nanomolar affinity for most human 5-HT receptor subtypes tested, including the 5-HT$_{2A}$ and the serotonin 1A receptor (5-HT$_{1A}$). The same compounds displayed affinity for 5-HT$_{2A}$ and 5-HT$_{1A}$ in mouse brain tissue *in vitro* and exhibited agonist efficacy in assays examining 5-HT$_{2A}$-mediated calcium mobilization and β-arrestin 2 recruitment. In mouse experiments, only the tertiary amines psilocin, psilocybin, and 4-acetoxy-N,N-dimethyltryptamine (psilacetin) induced head twitch responses (ED$_{50}$ 0.11−0.29 mg/kg) indicative of psychedelic-like activity. Head twitches were blocked by 5-HT$_{2A}$ antagonist pretreatment, supporting 5-HT$_{2A}$ involvement. Both secondary and tertiary amines decreased body temperature and locomotor activity at higher doses, the effects of which were blocked by 5-HT$_{1A}$ antagonist pretreatment. Across all assays, the pharmacological effects of 4-acetoxy and 4-hydroxy compounds were similar, and these compounds were more potent than their 4-phosphoryloxy counterparts. Importantly, psilacetin appears to be a prodrug for psilocin that displays substantial serotonin receptor activities of its own.

Target Validation
Binding profiles
Binding affinities
5-HT$_{2A}$ Functional Activities
β-arrestin 2 recruitment
G$_q$-calcium signaling
Mouse Studies
Head twitch
Thermoregulation
Locomotor activity

SARs for Psilocybin Analogues

KEYWORDS: *psilocybin, baeocystin, aeruginascin, head twitch response, hypothermia, mice*

4-Phosphoryloxy-N,N-dimethyltryptamine (psilocybin) is a naturally occurring tertiary amine found in diverse mushroom species throughout the world.[1−4] Psilocybin-containing mushrooms, sometimes colloquially called "magic mushrooms", have been used for centuries as religious sacraments to facilitate spiritual or mystical-type subjective experiences.[2−5] Additionally, psilocybin-containing mushrooms are used in various recreational contexts, which can pose risks to public health.[1,6] Psychedelics like psilocybin,[7−10] its active metabolite 4-hydroxy-N,N-dimethyltryptamine (psilocin),[11−14] and some other related compounds are designated as controlled substances internationally. Legal restrictions on psychedelic compounds have significantly hindered research efforts aimed at understanding their complex pharmacology.[3,4,15] However, in recent times, there is renewed interest in the therapeutic potential of psychedelics, and psilocybin has been investigated as an adjunct to psychotherapy in the treatment of psychological distress in end stage cancer, depression, substance use disorders, chronic pain, and obsessive-compulsive disorders.[3,16−20]

The subjective effects of psilocybin administration in humans are blocked by pretreatment with antagonists of the serotonin (5-HT) 2A receptor (5-HT$_{2A}$) and are positively correlated with occupancy of this receptor in the brain,[11,21,22] supporting a primary role in mediating psychedelic subjective effects. Likewise, in rodent behavioral models, many effects of psilocybin and psilocin are reversed by 5-HT$_{2A}$ antagonists or
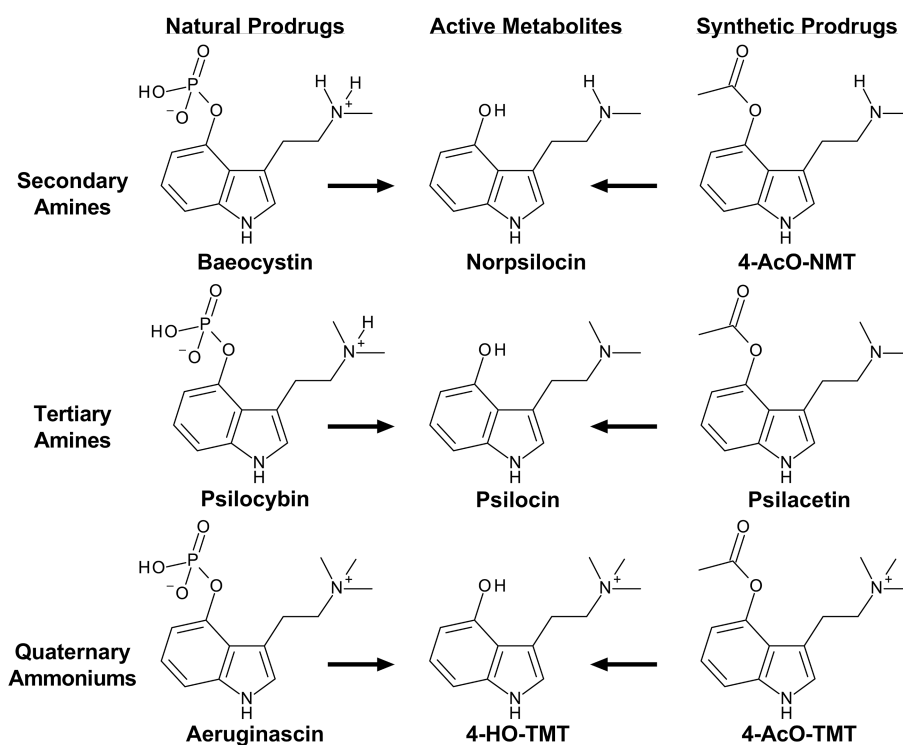
Figure 1. Chemical structures of the natural prodrugs baeocystin, psilocybin, and aeruginascin as well as their 4-hydroxy active metabolites and synthetic 4-acetoxy analogues.

genetic deletion of the receptor.[23−25] On the other hand, it is well known that psilocin acts as a nonselective serotonin receptor agonist.[26] Indeed, some effects of psilocybin in humans and rodents are modulated by actions at non-5HT$_{2A}$ sites, like the serotonin 1A receptor (5-HT$_{1A}$) and the serotonin 2C receptor (5-HT$_{2C}$).[23,26−30] Thus, some effects of psilocybin and related tryptamine compounds likely involve multiple serotonergic receptor sites of action.

Some psychoactive mushroom species contain other potentially bioactive compounds in addition to psilocybin, including various tryptamines, β-carbolines, and terpenes.[5,31,32] Underexplored tryptamines present in psilocybin-containing mushrooms include 4-phosphoryloxy-N-methyltryptamine (baeocystin), 4-phosphoryloxy-N,N,N-trimethyltryptamine (aeruginascin), 4-hydroxy-N-methyltryptamine (norpsilocin), and 4-phosphoryloxytryptamine (norbaeocystin)[33−39] (see Figure 1). The precise amounts of these tryptamines relative to that of psilocybin vary across mushroom species, but the compounds are found in mushroom species commonly ingested by humans, such as *Psilocybe cubensis*.[33] There is scant information about the pharmacological effects of most tryptamines found in psilocybin-containing mushrooms, with the exception of psilocybin. Preclinical evaluation of baeocystin in the mouse head twitch response (HTR) assay, which predicts psychedelic subjective effects in humans,[40] shows that the substance does not produce effects *in vivo*.[41] Pharmacological evaluations of tryptamines other than psilocybin and psilocin are lacking, despite initiatives in the USA to legalize or decriminalize psilocybin-containing mushrooms,[42] many of which contain the aforementioned psilocybin analogues.[5,33,35−38]

Beyond naturally occurring psilocybin analogues, synthetic 4-acetoxy derivatives of psilocybin and aeruginascin have been previously synthesized by members of our team and are thought to be prodrugs for corresponding 4-hydroxy active metabolites.[43,44] The first synthetic prodrug identified was 4-acetoxy-N,N-dimethyltryptamine (psilacetin), which was originally reported by Hofmann and Troxler,[45] discussed by the Shulgins in TiHKAL as a likely psilocin prodrug ester,[46] and suggested as an alternative to psilocybin based on a more efficient synthesis route by Nichols and Frescas.[47] More recently, psilacetin has emerged as a popular new psychoactive substance (NPS) on recreational drug markets, where it is sold as a psilocybin alternative.[48−52] Analogous to the metabolism of psilocybin to psilocin, 4-acetoxy compounds are thought to hydrolyze to their corresponding 4-hydroxy compounds.[43,44,51] While definitive biotransformation studies in animals are lacking, *in vitro* evidence from liver microsomes supports the metabolic conversion of 4-acetoxy compounds to their 4-hydroxy analogues.[53] This biotransformation reaction probably also occurs with secondary amines like 4-acetoxy-N-methyltryptamine (4-AcO-NMT) and quaternary ammoniums like 4-acetoxy-N,N,N-trimethyltryptamine (4-AcO-TMT), the synthetic analogues of baeocystin and aeruginascin.[41,43,54] Importantly, psilacetin is less potent than psilocin in producing psychedelic-like effects in rodents,[52,55] which is consistent with the expected profile of a prodrug. As with the other constituents of psilocybin-containing mushrooms, the pharmacology of 4-acetoxy analogues of psilocybin, baeocystin, and aeruginascin is largely uncharacterized, and a side-by-side comparison of the natural products and their corresponding active metabolites has not been performed.

Herein, we investigated the receptor binding profiles and 5-HT$_2$ receptor functional activities for a series of psilocybin analogues which vary in their degree of N-methylation and 4-position indole ring substitution. Specifically, we examined the

Table 1. Affinities of Psilocybin Analogues in Radioligand Binding Assays for Human 5-HT Receptors[a]

Drug	5-HT$_{1A}$ K_i (nM)	5-HT$_{1B}$ K_i (nM)	5-HT$_{1D}$ K_i (nM)	5-ht$_{1e}$ K_i (nM)	5-HT$_{2A}$ K_i (nM)	5-HT$_{2B}$ K_i (nM)	5-HT$_{2C}$ K_i (nM)	5-HT$_{5A}$ K_i (nM)	5-HT$_6$ K_i (nM)	5-HT$_{7a}$ K_i (nM)
norpsilocin	86	99	194	161	391	57	243	365	54	68
baeocystin		370	394	1,352		134				117
4-AcO-NMT	141	994	150	522	1,512	76	729	389	82	1,766
psilocin	164	580	130	155	180	8	175	116	38	75
psilocybin	5,284	4,983	186	601		259	808		89	138
psilacetin	337	1,178	108	335	395	9	210	350	112	157
4-HO-TMT						720			2,267	
aeruginascin										
4-AcO-TMT						891				
DOI			607	713	11	5	41		2,254	1,857

[a]Radioligands used, reference control compound used, and control K_i values for each 5-HT receptor were 5-HT$_{1A}$ = [^{3}H]WAY100635 vs. 8-HO-DPAT (K_i = 0.6 nM), 5-HT$_{1B}$ = [^{3}H]GR125743 vs. ergotamine tartrate (K_i = 4 nM), 5-HT$_{1D}$ = [^{3}H]GR125743 vs. ergotamine tartrate (K_i = 4 nM), 5-ht$_{1e}$ = [^{3}H]5-HT vs. 5-HT (K_i = 2 nM), 5-HT$_{2A}$ = [^{3}H]ketanserin vs. clozapine (K_i = 4 nM), 5-HT$_{2B}$ = [^{3}H]LSD vs. SB206553 (K_i = 18 nM), 5-HT$_{2C}$ = [^{3}H]mesulergine vs. ritanserin (K_i = 1 nM), 5-HT$_{5A}$ = [^{3}H]LSD vs. ergotamine tartrate (K_i = 12 nM), 5-HT$_6$ = [^{3}H]LSD vs. clozapine (K_i = 7 nM), 5-HT$_{7a}$ = [^{3}H]LSD vs. clozapine (K_i = 20 nM). No value = <50% inhibition of radioligand binding in the 10 μM primary screening. DOI = (±)-2,5-dimethoxy-4-iodoamphetamine.

in vitro effects of naturally occurring 4-phosphoryloxy prodrugs, synthetic 4-acetoxy prodrugs, and their shared 4-hydroxy metabolites (see Figure 1 for chemical structures). We further tested the ability of these compounds to compete for 5-HT$_{2A}$ and 5-HT$_{1A}$ binding in mouse brain, as well as to produce behavioral and physiological effects in mice. Lastly, we determined the role of 5-HT$_{2A}$ and 5-HT$_{1A}$ in mediating behavioral and physiological effects using antagonist reversal studies with M100907 and WAY100635, respectively. Overall, the data reveal that the degree of *N*-methylation and 4-position ring substitution powerfully influence receptor binding profiles and the potential for inducing psychedelic-like effects in mice.

RESULTS AND DISCUSSION

Radioligand Binding Assays in Cells Transfected with Human Receptors. At a 10 μM screening concentration, two of three secondary (norpsilocin, 4-AcO-NMT) and tertiary amines (psilocin, psilacetin) displayed >50% inhibition of radioligand binding at all human 5-HT receptor subtypes tested, except serotonin 3 receptors (5-HT$_3$). The 4-position constituent impacted receptor affinities, with the 4-phosphoryloxy compounds binding to fewer targets than 4-hydroxy and 4-acetoxy analogues. Notably, psilocybin did not exhibit >50% inhibition of radioligand binding at 5-HT$_{2A}$, while psilocin and psilacetin did. For secondary and tertiary amine compounds, non-5-HT receptor targets included alpha adrenergic receptors (alpha$_{2A}$ and alpha$_{2C}$), the dopamine transporter (DAT), the histamine receptor 1 (H$_1$), and the serotonin transporter (SERT). Quaternary ammonium compounds (4-HO-TMT, aeruginascin, 4-AcO-TMT) displayed little activity at 10 μM in receptor assays and only displayed >50% inhibition of radioligand binding at serotonin 2B (5-HT$_{2B}$), the serotonin 6 receptor (5-HT$_6$), the sigma 1 receptor, and SERT.

Compounds that displayed >50% inhibition for any potential target at 10 μM were further assessed to determine binding affinities at these sites. Affinities of all 9 compounds for human 5-HT receptor subtypes are listed in Table 1, while affinities for non-5-HT human targets are listed in Table S1. For secondary amines, the screening data revealed that norpsilocin and 4-AcO-NMT share similar 5-HT receptor targets. However, when compared to that of norpsilocin, the affinity of 4-AcO-NMT was weaker for the serotonin 1B

receptor (5-HT$_{1B}$), the serotonin 1e receptor (5ht$_{1e}$), 5-HT$_{2A}$, 5-HT$_{2C}$, and the serotonin 7A receptor (5-HT$_{7a}$), with high nanomolar to low micromolar affinities. Baeocystin inhibited binding to fewer 5-HT receptors than either norpsilocin or 4-AcO-NMT, displaying variable affinities for 5-HT$_{1B}$, the serotonin 1D receptor (5-HT$_{1D}$), 5-ht$_{1e}$, 5-HT$_{2B}$, and 5-HT$_{7a}$. Regarding non-5-HT receptors, norpsilocin displayed low micromolar affinity for alpha$_{2A}$ and SERT. Overall, the 4-acetoxy prodrug had a receptor binding profile more similar to norpsilocin than the 4-phosphoryloxy prodrug, and all three compounds were nonselective 5-HT receptor ligands.

Tertiary amine compounds displayed near identical target profiles to secondary amines across human 5-HT receptor subtypes. For tertiary amine compounds, psilocybin did not compete for binding to 5-HT$_{2A}$ or serotonin 5A (5-HT$_{5A}$) receptors in primary screens, in contrast to psilocin and psilacetin. In addition, psilocybin generally had weaker affinity across human 5-HT receptor subtypes compared to both psilocin and psilacetin. Beyond these differences with psilocin and psilacetin, psilocybin displayed nearly equivalent binding affinities for 5-HT$_6$ and 5-HT$_{7a}$ receptors. Psilocin displayed binding affinities of <200 nM across nearly all 5-HT receptor subtypes examined. Affinities for psilacetin at 5-HT receptor subtypes were nearly all <400 nM, and psilacetin had no additional receptor binding activity in the target validation screen. Outside of 5-HT receptors, psilocin displayed high nanomolar affinity at H$_1$ and low micromolar affinity at SERT, but affinity at SERT was >100 times weaker than the highest affinity displayed by psilocin at 5-HT$_{2B}$. Similar to secondary amines, tertiary amines were overall nonselective 5-HT receptor ligands with nanomolar affinities and limited non-5-HT receptor binding activities. 4-Phosphoryloxy substitution was associated with fewer 5-HT receptor targets and decreased affinity at those receptor subtypes.

Quaternary ammonium compounds interacted with few receptor targets in primary screens, and secondary binding analysis revealed that only 4-HO-TMT and 4-AcO-TMT displayed 5-HT receptor activity, with high nanomolar affinities for both compounds at 5-HT$_{2B}$ and low micromolar affinity for 4-HO-TMT at 5-HT$_6$. Beyond the 5-HT receptors, quaternary ammonium compounds 4-HO-TMT and aeruginascin had micromolar affinities at SERT and sigma 1

Table 2. Affinity (K_i), Potency (EC_{50} or ED_{50}), and Maximum Efficacy (E_{max}) of Psilocybin Analogues in 5-HT$_{2A}$ Binding Assays, 5-HT$_{2A}$ Functional Assays, and *In Vivo* Tests in Mice[a]

| | affinity—mouse brain | potency and efficacy—cells | | potency and efficacy—mice | | |
| | [³H]M100907 binding m5-HT$_{2A}$ | calcium mobilization h5-HT$_{2A}$ | β-arrestin 2 recruitment h5-HT$_{2A}$ | HTR | hypolocomotion | temperature Δ |
ligand	K_i (nM)	EC_{50} (nM) E_{max} (% 5-HT)	EC_{50} (nM) E_{max} (% LSD)	ED_{50} (mg/kg s.c.)	ED_{50} (mg/kg s.c.)	ED_{50} (mg/kg s.c.)
norpsilocin	706 (525–948)	22 (17–28) $E_{max} = 89$	140 (92–228) $E_{max} = 85$	>30	7.6 (7.3–14.1) $E_{max} = 585$ cm	13.7 (10.9–16.5) $E_{max} = -2.8$ °C
baeocystin	>5,000	n.d.	1,202 (575–8,276) $E_{max} = 82$	>30	18.5 (14.9–20.9) $E_{max} = 506$ cm	20.9 (10.4–31.5) $E_{max} = -2.5$ °C
4-AcO-NMT	1,334 (1,019–1,773)	312 (214–456) $E_{max} = 96$	408 (297–562) $E_{max} = 74$	>30	11.7 (10.7–21.5) $E_{max} = 843$ cm	17.7 (13.5–23.7) $E_{max} = -3.9$ °C
psilocin	235 (187–296)	13 (10–17) $E_{max} = 67$	81 (54–123) $E_{max} = 76$	0.11 (0.09–0.19) $E_{max} = 23$ HTR events	1.8 (0.9–3.5) $E_{max} = 303$ cm	2.5 (1.9–3.5) $E_{max} = -5.6$ °C
psilocybin	2,096 (1,209–3,635)	2,132 (1,786–2545) $E_{max} = 88$	1,242 (771–2,538) $E_{max} = 74$	0.29 (0.17–0.53) $E_{max} = 30$ HTR events	2.8 (1.5–5.6) $E_{max} = 605$ cm	5.4 (2.8–10.9) $E_{max} = -6.3$ °C
psilacetin	649 (430–980)	328 (227–474) $E_{max} = 54$	95 (66–138) $E_{max} = 74$	0.21 (0.08–0.34) $E_{max} = 27$ HTR events	1.4 (0.7–2.9) $E_{max} = 670$ cm	5.0 (2.9–9.2) $E_{max} = -6.1$ °C
4-HO-TMT	>5,000	2,312 (1,754–3,048) $E_{max} = 86$	2,506 (1,010–6,216) $E_{max} = 46$	>30	>30	>30
aeruginascin	>5,000	n.d.	>10,000	>30	>30	>30
4-AcO-TMT	>5,000	n.d.	6,564 (3,687–18,350) $E_{max} = 65$	>30	>30	>30

[a]m5-HT$_{2A}$ and h5-HT$_{2A}$ = mouse and human 5-HT$_{2A}$. N.d. = not determined. ED_{50} values for hypolocomotion and temperature change for secondary amines are approximate as full sigmoidal curves were not exhibited up to 30 mg/kg for these compounds. Potency and affinity values are shown with 95% confidence intervals noted below in parentheses. Affinities and potencies of reference compounds in binding and functional assays are listed in the text of the Results and Discussion section.

receptors, respectively. The standard 5-HT$_2$ ligand, DOI, displayed expected high affinity and selectivity at 5-HT$_2$ receptors relative to most other targets tested. Despite some other listed affinities of DOI across 5-HT receptor subtypes and other targets, only alpha$_{2A}$ binding was <100 times lower than the affinity of DOI at 5-HT$_2$ receptors.

A separate radioligand binding screen was utilized to assess competition binding of all the compounds using agonist radioligands (data shown in Table S2). Compounds were screened using 100 nM and 10 μM concentrations to assess percent reduction of agonist radioligand binding at several 5-HT receptors, monoamine transporters, and opioid receptors. Norpsilocin, psilocin, and psilacetin were the only compounds assessed at 100 nM to display >50% reduction in radioligand binding at 5-HT$_{1A}$, 5-HT$_{2B}$, and/or 5-HT$_{2C}$ receptors. At 10 μM, secondary amines displayed nonselective 5-HT receptor activity, with norpsilocin showing additional activity at SERT at this concentration. Psilacetin and psilocybin also displayed nonselective 5-HT receptor activity at this concentration, while quaternary ammonium 4-HO-TMT showed 5-HT$_{2A}$, 5-HT$_{2B}$, and SERT activity. The quaternary ammonium prodrugs showed reduced receptor binding targets, with only 4-AcO-TMT showing weak 5-HT$_{2B}$ activity at the high 10 μM screening concentration. In general, the findings from the

additional agonist radioligand binding screen agreed with the findings from the more comprehensive target screening.

Radioligand Binding at 5-HT$_{1A}$ and 5-HT$_{2A}$ Receptors in Mouse Brain. As a means to characterize the receptor binding of psilocybin analogues in native tissue preparations, we examined binding affinities of test compounds at 5-HT$_{1A}$ ([³H]8-OH-DPAT binding) and 5-HT$_{2A}$ ([³H]M100907 binding) in mouse brain membranes. Affinity values at mouse 5-HT$_{2A}$ are shown in Table 2, whereas affinity values at mouse 5-HT$_{1A}$ can be found in Table 3. Binding curves for 5-HT$_{2A}$ and 5-HT$_{1A}$ assays are depicted in Figure S1. At 5-HT$_{1A}$ receptors, secondary amine compounds displayed high affinities for [³H]8-OH-DPAT-labeled binding sites (25–50 nM). Tertiary amine compounds also competed for [³H]8-OH-DPAT binding in mouse brain, but affinities were right-shifted when compared to the secondary amines (118–299 nM). Quaternary ammonium compounds displayed little competition for [³H]8-OH-DPAT binding in mouse brain up to 10 μM. As expected, standard reference compounds 5-HT and WAY-100635 displayed high affinity for 5-HT$_{1A}$ binding in mouse brain (i.e., 3.2–4.4 nM).

At 5-HT$_{2A}$ receptors in mouse brain, psilocin displayed the highest affinity for [³H]M100907-labeled sites, with a K_i in the low nanomolar range. Psilocybin, psilacetin, norpsilocin, and 4-AcO-NMT all displayed high nanomolar to low micromolar

Table 3. Affinity of Psilocybin Analogues at 5-HT$_{1A}$ Receptors in Mouse Brain[a]

ligand	affinity—mouse brain
	[^{3}H]8-OH-DPAT binding m5-HT$_{1A}$
	K_i (nM)
norpsilocin	29
	(22−39)
baeocystin	25
	(21−29)
4-AcO-NMT	50
	(39−65)
psilocin	118
	(84−165)
psilocybin	197
	(118−328)
psilacetin	299
	(205−438)
4-HO-TMT	>10,000
aeruginascin	>10,000
4-AcO-TMT	>10,000

[a]Reference compounds WAY100635 and 5-HT displayed affinities of 4.4 (3.3−5.8) nM and 3.2 (2.4−4.1) nM, respectively. Affinity values are accompanied by 95% CI in parentheses below.

affinities at 5-HT$_{2A}$. The affinity of psilocybin for 5-HT$_{2A}$ binding was 3−4-fold weaker than that of psilacetin. Baeocystin and the quaternary ammonium analogues showed little competition for [^{3}H]M100907 binding up to 5 μM. Standard reference compounds M100907 and DOI displayed the expected high affinities (0.7 and 12.5 nM, respectively). Affinities for all compounds at 5-HT$_{2A}$ in mouse brain were relatively similar to results from transfected cells reported in Table 1. For example, the reference compounds DOI displayed an affinity of 12 nM at mouse 5-HT$_{2A}$ labeled with [^{3}H]M100907, whereas this same compound displayed an affinity of 11 nM at human 5-HT$_{2A}$ labeled with [^{3}H]-ketanserin. Regardless of any differences in absolute K_i values between radioligands used in tissue-based and cell-based assays, the rank order of drug potencies at 5-HT$_{2A}$ was similar for both methods.

Functional Activities at Identified 5-HT Receptor Targets. Calcium mobilization assays were also carried out to assess functional potency (EC$_{50}$) and efficacy (E$_{max}$) for six of the nine compounds at 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$. Potency values for 5-HT$_{2A}$ receptor assays are listed in Table 2, while potency values for 5-HT$_{2B}$ and 5-HT$_{2C}$ assays are listed in Table 4. Concentration−response curves for 5-HT$_2$ subtypes are shown in Figure S2. The reference agonist used for each assay was 5-HT (E$_{max}$ = 100%), which had EC$_{50}$ values of 2.4, 0.9, and 0.4 nM at 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$, respectively. Psilocin and norpsilocin were the most potent ligands for calcium mobilization at 5-HT$_{2A}$ (EC$_{50}$ = 13 and 22 nM). Secondary amines displayed E$_{max}$ values more akin to full agonists (89−96%), while tertiary amines exhibited partial agonist E$_{max}$ values (54−88%). 4-HO-TMT displayed weak agonist potency for calcium mobilization at 5-HT$_{2A}$ (EC$_{50}$ = 2,300 nM, E$_{max}$ = 86%).

Norpsilocin and psilocin exhibited low nanomolar potencies for calcium mobilization at 5-HT$_{2B}$ (EC$_{50}$ = 8−13 nM). Psilocin was a weak partial agonist (E$_{max}$ = 38%), while norpsilocin displayed the highest partial agonist activity (E$_{max}$ = 82%). Similar to psilocin, psilacetin and psilocybin displayed

Table 4. Functional Activity at Human 5-HT$_{2B}$ and 5-HT$_{2C}$ Receptors in Cellular Assays[a]

	potency and efficacy—cells	
	calcium mobilization h5-HT$_{2B}$	calcium mobilization h5-HT$_{2C}$
ligand	EC$_{50}$ (nM) E$_{max}$(% 5-HT)	EC$_{50}$ (nM) E$_{max}$(% 5-HT)
norpsilocin	13	32
	(12−15)	(28−37)
	E$_{max}$ = 82	E$_{max}$ = 96
4-AcO-NMT	153	296
	(135−173)	(254−345)
	E$_{max}$ = 74	E$_{max}$ = 84
psilocin	8	34
	(7−10)	(28−40)
	E$_{max}$ = 38	E$_{max}$ = 84
psilocybin	612	3,741
	(504−743)	(3,120−4,486)
	E$_{max}$ = 38	E$_{max}$ = 88
psilacetin	88	408
	(69−114)	(318−525)
	E$_{max}$ = 29	E$_{max}$ = 32
4-HO-TMT	845	8,940
	(675−1,059)	(7,592−10,520)
	E$_{max}$ = 32	E$_{max}$ = 64

[a]Potency (EC$_{50}$) with 95% CI values (parentheses) and maximum efficacy (E$_{max}$) for each are listed. Reference compound 5-HT displayed potency values of 0.9 nM (0.7−1.1) and 0.4 nM (0.3−0.5) at 5-HT$_{2B}$ and 5-HT$_{2C}$, respectively.

weak partial agonist efficacy at 5-HT$_{2B}$ (E$_{max}$ = 29 and 38%) for calcium mobilization with potencies in the low or mid nanomolar range (EC$_{50}$ = 88 and 612 nM, respectively). 4-AcO-NMT had similar efficacy and reduced potency compared to norpsilocin (EC$_{50 =}$ 153 nM, E$_{max}$ = 74%). 4-HO-TMT displayed high nanomolar potency (EC$_{50}$ = 845 nM) and partial agonist efficacy (E$_{max}$ = 32%) for calcium mobilization at 5-HT$_{2B}$.

In calcium mobilization assays at 5-HT$_{2C}$, norpsilocin and psilocin again had similar potencies (EC$_{50}$ = 32 and 34 nM), but norpsilocin produced full agonist efficacy (E$_{max}$ = 96%), whereas psilocin had slightly lower efficacy (E$_{max}$ = 84%). Both 4-AcO-NMT and psilacetin had similar potencies for calcium mobilization at 5-HT$_{2C}$ (EC$_{50}$ = 296 and 408 nM), but psilacetin was a weak partial agonist (E$_{max}$ = 32%) in contrast to 4-AcO-NMT (E$_{max}$ = 84%) and psilocybin (EC$_{50}$ = 3,741 nM, E$_{max}$ = 88%). 4-HO-TMT also displayed weak partial agonist potency and efficacy for calcium mobilization at 5-HT$_{2C}$ (EC$_{50}$ = 8,940 nM, E$_{max}$ = 64%).

Next, six of the nine compounds were also screened for potential functional activities for β-arrestin translocation across various 5-HT receptors (Table S3). In the Tango G protein-independent β-arrestin recruitment assay, all compounds except psilocybin and the quaternary ammoniums displayed agonist-like activity at most 5-HT receptors tested. None of the compounds displayed agonist-like activity at 5-hydroxytrypt-amine 4 receptors (5-HT$_4$), and as expected, DOI exhibited little to no agonist-like activity at these receptors, consistent with its known selectivity at 5-HT$_2$ receptor subtypes. Interestingly, only norpsilocin and 4-AcO-NMT displayed weak 5-HT$_{1A}$ agonist-like activity (34−39% of 5-HT response) at the highest concentration tested.
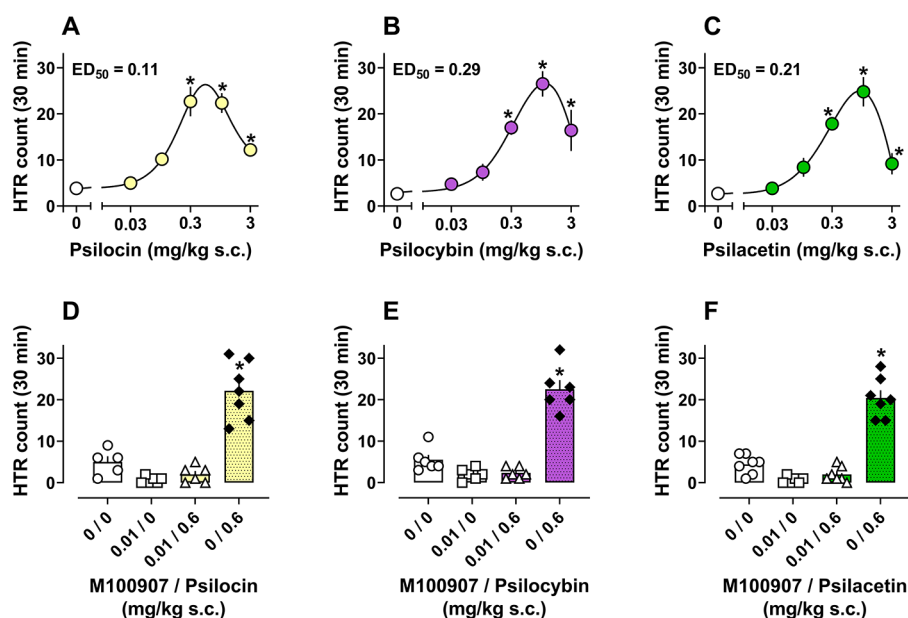
Figure 2. Dose−response curves (A−C) and antagonist reversal plots (D−F) for a total number of HTR produced by psilocin, psilocybin, and psilacetin in mice. *$p < 0.05$ vs vehicle control group (0 or 0, 0). All values are mean ± SEM of $n = 4$−6 (A−C) and $n = 5$−7 (D−F) per condition.
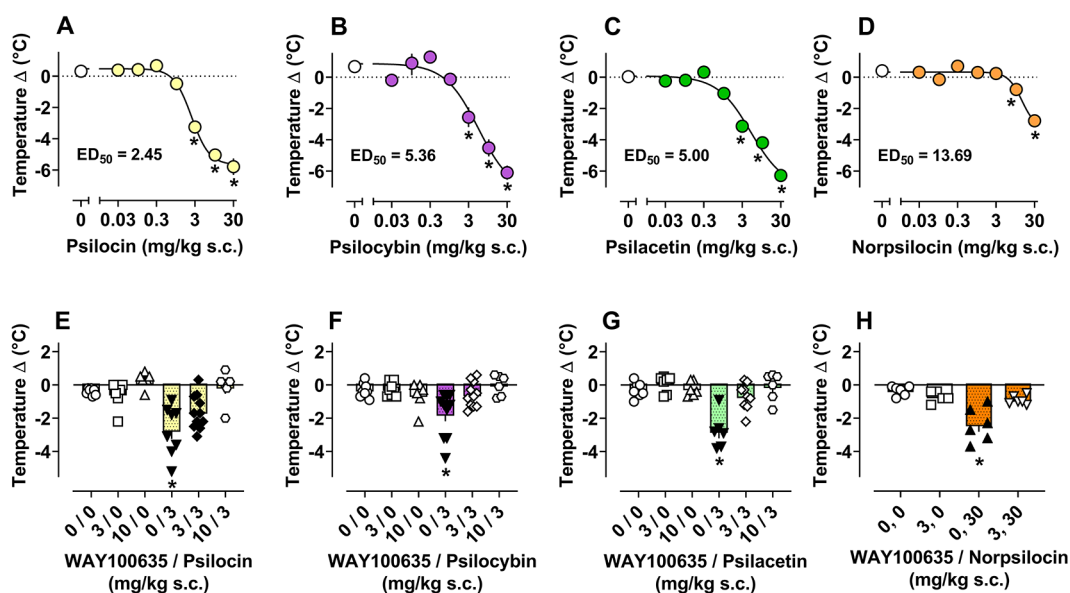

Figure 3. Dose−response curves (A−D) and antagonist reversal plots (E−H) of pre- vs. post-session temperature change produced by psilocin, psilocybin, psilacetin, and norpsilocin in mice. *$p < 0.05$ vs. vehicle control group (0 or 0, 0). All values are mean ± SEM of $n = 4$−6 (A−D), $n = 6$−11 (E−G), and $n = 5$−6 (H) per condition.

Functional activity of psilocybin analogues was further examined in a NanoBiT system-based assay measuring 5-HT$_{2A}$-mediated β-arrestin 2 recruitment *in vitro*. Potency (EC$_{50}$) and efficacy (E_{max}) values for all test compounds and controls are listed in Table 2, while concentration−response curves can be found in Figure S3. Lysergic acid diethylamide (LSD) was used as the reference ligand ($E_{max} = 100\%$) for the assays to determine the relative efficacy of the test compounds and displayed a potency of 7.7 nM. The positive control compound DOI also displayed potent (EC$_{50}$ = 5.5 nM) stimulation of β-arrestin 2 recruitment *in vitro*, with maximal efficacy similar to LSD (106%). Secondary amines all induced β-arrestin 2 recruitment, with norpsilocin and 4-AcO-NMT

exhibiting higher potencies (EC$_{50}$ = 140 and 408 nM) than baeocystin (EC$_{50}$ = 1.20 μM). Tertiary amines similarly all induced β-arrestin 2 recruitment, with psilocybin producing the weakest potency (EC$_{50}$ = 1.24 μM) versus psilocin and psilacetin, which displayed similar potencies (EC$_{50}$ = 81 and 95 nM). Quaternary ammoniums displayed weak micromolar potencies for β-arrestin 2 recruitment. 4-HO-TMT and 4-AcO-TMT exhibited EC$_{50}$ values of 2.5 and 6.6 μM, while aeruginascin did not produce a sigmoidal concentration effect curve up to 10 μM. Lastly, secondary and tertiary amines tested were all partial agonists, with E_{max} values of 73.5−85.3% relative to LSD, whereas quaternary compounds were partial agonists with low potencies.

Dose−Response Effects of Psilocybin Analogues on HTR, Locomotor Activity, and Body Temperature in Mice. Based on our *in vitro* pharmacological data and the known behavioral effects of psilocybin, we sought to examine SAR for psilocybin analogues to produce psychedelic-like, as well as other effects, in mice. To test for pharmacological effects *in vivo*, each compound was administered subcutaneously (s.c.) to mice at doses ranging from 0.03 to 30 mg/kg to measure HTR, temperature change, and locomotor activity over a 30 min testing session. Dose−response data for HTR induced by psilocin, psilocybin, and psilacetin over the testing session are shown in Figure 2A−C while time-course data are shown in Figure S4. Potency (ED_{50}) values are shown in Table 2, while descriptive statistics and additional details of statistical comparisons can be found in Table S4. Tertiary amines displayed roughly similar potencies and efficacies for producing HTR in mice, with the rank order of potencies being psilocin > psilacetin > psilocybin (Figure 2A−C). This is further demonstrated when considering the salt used: psilocin (0.54 μmol/kg) > psilacetin (0.58 μmol/kg) > psilocybin (1.0 μmol/kg). In contrast to tertiary amines, secondary amines and quaternary ammoniums produced little to no HTR in mice (Figure S5). Total HTR counts over the session for psilocin, psilocybin, and psilacetin were significantly increased versus vehicle controls at 0.3−3 mg/kg ($F_{5,30}$ = 19.09, p < 0.0001; $F_{5,27}$ = 15.16, p < 0.0001; $F_{5,29}$ = 22.30, p < 0.0001). All three tertiary amine compounds produced peak effects on HTR within the first 5−10 min after administration that waned to vehicle control levels by the end of the session. Inverted U dose-related effects were evident for psilocin, psilocybin, and psilacetin, with a downward slope in activity at doses above 1 mg/kg. At the highest doses tested (3−30 mg/kg), HTR was rapidly reduced to vehicle control levels within the first 10−15 min for all three tertiary amine compounds.

In conjunction with measuring HTR, we monitored body temperature change and locomotor activity during all experimental drug administration sessions. Dose−response effects for tertiary amines and norpsilocin to affect body temperature are depicted in Figure 3A−D, whereas temperature data for other compounds can be found in Figure S6 and Table 2. Dose−response and time-course of effects for all compounds tested to affect locomotor activity are shown in Figures S7−S9 and Table 2. Psilocin, psilocybin, and psilacetin displayed the most potent effects on body temperature ($F_{7,40}$ = 58.19, p < 0.0001; $F_{7,37}$ = 33.63, p < 0.0001; $F_{7,36}$ = 53.73, p < 0.0001) and locomotor activity ($F_{7,40}$ = 14.90, p < 0.0001; $F_{7,36}$ = 11.20, p < 0.0001; $F_{7,38}$ = 15.93, p < 0.0001), decreasing both measures significantly versus vehicle controls from 3 to 30 mg/kg. It is noteworthy that the potencies of psilocin, psilocybin, and psilacetin to reduce body temperature and locomotor activity were ~20-fold weaker than their potencies to elicit HTR. Time-course data for locomotor effects of tertiary amines showed that at the same doses that reduced HTR, mice displayed reduced forward locomotion relative to vehicle controls over the last 15−20 min of the session. Secondary amines displayed weak potencies for reducing body temperature (14−21 mg/kg) and locomotor activity (8−19 mg/kg), while quaternary ammonium compounds had little to no effects on these measures. Overall, dose−response mouse studies revealed that only tertiary amines produced measurable effects on HTR in mice, whereas both secondary and tertiary amines reduced body temperature and locomotor activity at

sufficient doses. By contrast, quaternary ammonium psilocybin analogues were essentially inactive *in vivo*.

5-HT$_{2A}$ Antagonist Pretreatment Effects in Mice. Since the effects of psilocybin and related psychedelics to produce HTR in rodents depend on 5-HT$_{2A}$ activation, we hypothesized that HTR produced by psilocybin analogues would be attenuated by the blockade of this receptor. To test whether the HTR produced by psilocin, psilocybin, and psilacetin was mediated by actions at 5-HT$_{2A}$ receptors, we pretreated mice with 5-HT$_{2A}$ antagonist M100907 prior to administration of each compound. The dose of M100907 was chosen based on a small pilot experiment showing no reduction of locomotor activity by 0.01 or 0.1 mg/kg doses relative to 1 mg/kg ($F_{3,16}$ = 4.69, p = 0.015, Figure S10). The 0.01 mg/kg dose of M100907 did not significantly change body temperature or locomotor activity relative to vehicle controls (Figure S11).

Overall, treatment differences were observed in M100907 pretreatment experiments with psilocin, psilocybin, and psilacetin ($F_{3,20}$ = 37.33, p < 0.0001; $F_{3,20}$ = 54.64, p < 0.0001; $F_{3,23}$ = 67.86, p < 0.0001). Specifically, the HTR produced by psilocin, psilocybin, and psilacetin at a near maximal dose of 0.6 mg/kg was blocked by M100907 pretreatment as this condition was not significantly different from vehicle controls in contrast to the vehicle + drug conditions (Figure 2D,E, Table S5). These results demonstrate that drug-induced HTR is dependent upon 5-HT$_{2A}$ activation.

5-HT$_{1A}$ Antagonist Pretreatment Effects in Mice. Given that the highest doses of psilocybin analogues tested (3−30 mg/kg) produced reductions in HTR and induced physiological (body temperature decrease) and behavioral (locomotor activity decrease) responses indicative of the 5-HT$_{1A}$ receptor agonist-induced syndrome, we surmised that these effects might be reversible by pretreatment with a 5-HT$_{1A}$ receptor antagonist. To evaluate this hypothesis, we pretreated mice with the 5-HT$_{1A}$ antagonist WAY100635 prior to administration of psilocin, psilocybin, and psilacetin. Mean effects of WAY100635 pretreatment on body temperature changes induced by psilocybin analogues are shown in Figure 3E−H and Table S6, while effects on HTR and locomotor activity are shown in Figure S12 and Table S6. Overall, for psilocin, psilocybin, and psilacetin, significant main effects were observed for HTR ($F_{5,39}$ = 13.99, p < 0.0001; $F_{5,43}$ = 32.95, p < 0.0001; $F_{5,35}$ = 15.43, p < 0.0001; $F_{3,19}$ = 5.12, p = 0.0092), body temperature ($F_{5,39}$ = 10.24, p < 0.0001; $F_{5,43}$ = 4.88, p = 0.0013; $F_{5,36}$ = 14.37, p < 0.0001), and locomotor activity ($F_{5,43}$ = 5.03, p = 0.001; $F_{5,42}$ = 4.15, p = 0.0037; $F_{5,37}$ = 3.72, p = 0.0080) measures in WAY100635 experiments. Main effects were also observed for norpsilocin in locomotor activity ($F_{3,19}$ = 4.59, p = 0.0139) and body temperature change ($F_{3,19}$ = 14.26, p < 0.0001) data sets from WAY100635 experiments. Specifically, the effects of psilocin, psilocybin, psilacetin, and norpsilocin on body temperature and locomotor activity were blocked by WAY100635 treatment, suggesting a 5-HT$_{1A}$-mediated mechanism of action. Psilocin required the highest dose of WAY100635 (10 mg/kg) to block reductions of body temperature and locomotor activity, while the effects of the other compounds were blocked by 3 and 10 mg/kg WAY1003635. WAY100635 pretreatment seemed to slightly increase the HTR produced by psilocin, psilocybin, and psilacetin at 3 mg/kg; however, the currently employed design did not assess these potential treatment effects. WAY100635 pretreatment prior to norpsilocin and norpsilocin alone

significantly reduced basal vehicle-induced HTR (Figure S12D).

DISCUSSION

In the present study, we examined the preclinical pharmacology of naturally occurring and synthetic psilocybin analogues with varying degrees of *N*-methylation and differing 4-position ring substitutions. Determining the biological effects of psilocybin and its analogues is critical for understanding their potential therapeutic utility and possible health risks. Our *in vitro* data show that secondary (i.e., norpsilocin, baeocystin, 4-AcO-NMT) and tertiary amines (i.e., psilocin, psilocybin, psilacetin) display nanomolar affinity for most 5-HT receptor subtypes, while quaternary ammoniums (i.e., 4-HO-TMT, aeruginascin, 4-AcO-TMT) do not. In mouse brain tissue, secondary and tertiary amines inhibited radioligand binding at 5-HT$_{2A}$ and 5-HT$_{1A}$, analogous to their effects in transfected cells. Secondary and tertiary amine compounds also displayed agonist activity at 5-HT$_{2A}$ in functional assays assessing G$_{\alpha q}$-dependent calcium mobilization and β-arrestin 2 recruitment *in vitro*. Importantly, only the tertiary amine compounds produced 5-HT$_{2A}$-mediated HTR, illustrating the importance of an *N,N*-dimethyl moiety for enabling psychedelic-like effects of these compounds *in vivo*.[23,40,41,55] Both secondary and tertiary amines caused 5-HT$_{1A}$-mediated suppression of locomotion and body temperature *in vivo*, but these effects required high drug doses. 4-Acetoxy compounds, regardless of the degree of *N*-methylation, had effects that were more similar to 4-hydroxy compounds when compared to 4-phosphoryloxy compounds. In this regard, our findings support the notion that psilacetin is a prodrug which displays its own agonist activity at 5-HT receptors.[46,47] Overall, the results suggest that secondary amines and quaternary ammoniums tested in the present study may lack psychoactive effects in humans, in contrast to the established psychedelic effects of tertiary amines like psilocin and its prodrugs.

We provide the first comprehensive receptor binding screen comparing the effects of psilocybin, baeocystin, aeruginascin, and their analogues. Secondary and tertiary amine compounds displayed nanomolar affinities for most 5-HT receptor subtypes, while quaternary ammonium compounds had weak affinities for only 5-HT$_{2B}$ and SERT, as previously reported.[56] Our findings with 4-HO-TMT might seem inconsistent with a previous study which showed that this compound has affinity (i.e., 670 nM) for 5-HT$_{2A}$ in transfected cells.[43] However, the present study used the antagonist [³H]ketanserin to label 5-HT$_{2A}$, while the prior work used the agonist [¹²⁵I]DOI. It is well known that agonists preferentially bind to the high affinity state of the receptor compared to antagonists, and radiolabeled agonists will yield K_i values for the high affinity state.[57] 4-Hydroxy and 4-acetoxy compounds displayed higher affinities across all 5-HT receptors when compared to their 4-phosphoryloxy counterparts. Notably, our binding data agree with previous studies showing that psilocin is a nonselective 5-HT receptor ligand.[26,58] Drug binding affinities at human 5-HT$_{2A}$ and 5-HT$_{1A}$ in transfected cells were subsequently verified in native tissue preparations using radioligand binding assays in mouse brain membranes consistent with another recent study.[59] The present studies also determined the potency and efficacy of psilocybin analogues for 5-HT$_{2A}$-mediated calcium mobilization and β-arrestin 2 recruitment *in vitro*. Importantly, the rank order of drug potencies in the 5-HT$_{2A}$ functional assays was similar to the rank order of

affinities in 5-HT$_{2A}$ binding at human and mouse receptors. In both functional assays, secondary and tertiary amine compounds displayed agonist efficacies relative to those of 5-HT and LSD.

5-HT$_{2A}$ and other 5-HT receptors couple to many canonical and noncanonical intracellular signaling pathways, depending on biological context, which could mediate distinct pharmacological effects of psychedelics.[60,61] In cells transfected with rat 5-HT$_{2A}$, drug potencies in the 5-HT$_{2A}$ calcium mobilization assay associated with G$_{\alpha q}$ signaling have been shown to predict potency for phenethylamine psychedelic-induced HTR in mice.[57] However, a previous mouse study showed that genetic deletion of G$_{\alpha q}$ only partially reduces drug-induced HTR,[62] while another mouse study pointed to a possible role for G$_{\alpha s}$ in producing psychedelic-like drug effects.[63] We found that psilocin and norpsilocin were both potent agonists in the calcium mobilization assay, and our results generally agree with those of Sherwood and colleagues.[41] Although many assays can be used to study the functional potency and efficacy of psychedelics at 5-HT$_{2A}$,[60] it is unclear which signaling pathway(s) are predictive for therapeutic or other effects. The usefulness of *in vitro* functional potencies may be limited as these values do not always relate to binding affinities at 5-HT$_{2A}$[3,64−66] or *in vivo* potencies in rodent behavioral models.[67] On the other hand, the binding affinities of various 5-HT$_{2A}$ agonists at [³H]ketanserin-labeled 5-HT$_{2A}$ are positively correlated with bioactive doses of psychedelics in humans[68−70] and the potencies for inducing effects in rodents.[66,71,72] Such observations support the idea that 5-HT$_{2A}$ binding affinity is at least one useful metric for predicting possible psychedelic activity of novel compounds.

5-HT$_{2A}$ is known to form complexes with β-arrestin 2 in cortical neurons *in vivo*.[73] Here, we demonstrate that both secondary and tertiary amines act as potent partial agonists in an assay measuring 5-HT$_{2A}$-mediated β-arrestin 2 recruitment. Prior studies show that genetic deletion of β-arrestin 2 in mice reduces HTR induced by 5-hydroxytryptophan (5-HTP) and LSD, but not DOI.[74,75] Paradoxically, the HTR activities of 5-methoxy-*N,N*-dimethyltryptamine (5-MeO-DMT) and 5-HT are reportedly enhanced in β-arrestin 2 knockout mice.[76] Despite the uncertain role of β-arrestin 2 signaling for psychedelic drug action in mice, potencies for 30 phenethylamine psychedelics in the β-arrestin 2 assay used here were shown to positively correlate with doses that produce psychedelic subjective effects in humans.[77] Whether 5-HT$_{2A}$-mediated β-arrestin recruitment underlies the psychedelic effects of tryptamine or LSD-like compounds warrants further investigation. Some data indicate that LSD and related psychedelics exhibit preferential β-arrestin 2 bias at 5-HT$_{2A}$ receptors,[78,79] but other investigators found no evidence for such bias when directly comparing the effects of compounds in assays measuring 5-HT$_{2A}$-mediated β-arrestin 2 versus mini-G$_{\alpha q}$ recruitment.[60,80] Unraveling which *in vitro* assay(s) and receptor signaling pathway(s) are predictive for pharmacological and potential clinical effects of psychedelics will be crucial for future drug development efforts.

The HTR is a widely used behavioral proxy for 5-HT$_{2A}$-mediated psychedelic-like drug activity in rodent models.[40,61,81] Here, we quantified HTR in mice using a novel automated computer software-based scoring method recently validated in our laboratory.[82] We found that tertiary amines—psilocin, psilocybin, and psilacetin—induce dose-related increases in HTR, whereas secondary amines and quaternary

ammoniums do not. The HTR produced by tertiary amines was completely blocked by pretreatment with the 5-HT$_{2A}$ antagonist M100907. Our *in vivo* findings with psilocybin and psilocin agree with previous results showing that these compounds, and related *N,N*-disubstituted tryptamines, induce HTR in mice.[23,40,41,55,59] It is noteworthy that ED$_{50}$ potency values reported here for psilocybin (i.e., 0.29 mg/kg, s.c.) and psilocin (i.e., 0.11 mg/kg, s.c.) to induce HTR are nearly identical to those reported by others who used magnetometer-based scoring methods to quantify HTR.[40,55,83] It is not surprising that quaternary ammoniums failed to induce HTR because these compounds display weak or no affinity for 5-HT$_{2A}$. By contrast, the secondary amines norpsilocin and 4-AcO-NMT are potent agonists at 5-HT$_{2A}$ *in vitro*, yet they did not induce HTR at any dose tested. Possible reasons for the lack of *in vivo* activity for secondary amines could be poor blood−brain barrier penetration or rapid metabolism by monoamine oxidase.[84,85] Another feasible explanation is that norpsilocin and 4-AcO-NMT exhibit agonist effects at non-5-HT$_{2A}$ sites which serve to dampen the expression of HTR. Indeed, there are many examples of 5-HT$_{2A}$ agonists that do not produce psychedelic-like effects in rodents,[24,86−88] suggesting the possibility that competing receptor actions, or perhaps biased agonism at 5-HT$_{2A}$,[89] could underlie the lack of HTR associated with these compounds.[3,26,81,90]

An important non-5HT$_{2A}$ site of action for psilocybin analogues and other tryptamine psychedelics is the 5-HT$_{1A}$ receptor.[3,70,91] It is well established that 5-HT$_{1A}$ ligands can modulate 5-HT$_{2A}$-mediated pharmacological effects. For example, 5-HT$_{1A}$ agonist pretreatment reduces 5-HT$_{2A}$-mediated effects of psychedelics in rodents and humans, while 5-HT$_{1A}$ antagonists have the opposite effect.[27,92−96] Here, we show that secondary and tertiary amine psilocybin analogues produce 5-HT$_{1A}$-mediated suppression of locomotor activity and body temperature. These effects are consistent with other studies showing similar effects of psilocybin and psilocin at high doses in rodents that were reversed by 5-HT$_{1A}$ antagonist pretreatment.[59,97] In the case of tertiary amines, the induction of 5-HT$_{1A}$ effects *in vivo* required ∼10-fold higher doses than those eliciting 5-HT$_{2A}$-mediated HTR. In fact, the descending limb of the HTR dose−response curve for all drugs coincided with the expression of 5-HT$_{1A}$-mediated hypoloco-motion and hypothermia, suggesting that 5-HT$_{1A}$ activation counteracted HTR. In the case of secondary amines, the induction of 5-HT$_{1A}$ effects *in vivo* required even higher doses than those observed with the tertiary amines. It is tempting to speculate that the low *in vivo* potency of secondary amines could result from poor brain penetrance or rapid metabolism, as noted above. Given that 5-HT$_{1A}$-mediated effects were only observed at high drug doses in mice, these effects might only be relevant to overdose situations in humans. However, the ability of psychedelic compounds to cause long-term changes in brain neuronal structure (i.e., neuroplasticity) is thought to be mediated in part by 5-HT$_{1A}$ receptors.[29,98] Future preclinical and clinical investigations should examine the role of 5-HT$_{1A}$ and other 5-HT receptor sites in modulating the pharmacological effects of psychedelic compounds at clinically relevant doses.

CONCLUSIONS

In summary, the SAR findings reported here show that the degree of *N*-methylation and 4-position ring substitution can powerfully influence pharmacological effects of psilocybin analogues. Secondary and tertiary amine compounds are nonselective 5-HT receptor ligands *in vitro*, whereas quaternary ammonium compounds bind to few receptor targets. Secondary and tertiary amines act as potent partial agonists at 5-HT$_{2A}$ *in vitro*, but only tertiary amine compounds produce 5-HT$_{2A}$-mediated HTR *in vivo*. Secondary and tertiary amine compounds also induce 5-HT$_{1A}$-mediated suppression of locomotion and body temperature, but these effects require high drug doses. While our studies focused on the role of 5-HT$_{2A}$ and 5-HT$_{1A}$, further research is warranted to examine the role of other 5-HT receptors in modulating effect of psilocybin analogues. Across all assays, the effects of 4-acetoxy and 4-hydroxy compounds were more potent than those of 4-phosphoryloxy compounds. In this regard, psilacetin appears to be a potential prodrug which displays its own serotonergic receptor activities. Future studies should directly test the hypothesis that psilacetin is hydrolyzed to psilocin *in vivo*. Importantly, our data indicate that the naturally occurring mushroom constituents, baeocystin and aeruginascin, are devoid of *in vivo* psychedelic-like effects in mice, but the pharmacology of other compounds from psilocybin-containing mushrooms or combinations thereof remain unexplored. As decriminalization or legalization efforts continue to allow personal use of psilocybin-containing mushrooms, examining the pharmacology of different constituents will be crucial for understanding their potential therapeutic and adverse effects.

MATERIALS AND METHODS

Drugs. 4-AcO-NMT hydrochloride was synthesized and structurally characterized using the scheme described in Supporting Information (Figure S13, Tables S7−S11). Baeocystin (zwitterion) and aeruginascin were synthesized as previously described.[41] The solid-state structure of synthesized aeruginascin was determined by single-crystal X-ray diffraction, revealing stoichiometric hydration and ammonium iodide co-crystallization for a bis(aeruginascin) bis(ammonium) iodide salt; thus, the corrected aeruginascin molecular weight (379.26 g/mol) was calculated for the lot used in these experiments. Structural confirmation of baeocystin has been reported by members of our team previously,[99] and structural data for aeruginascin are described in Supporting Information (Figure S14, Tables 12−16). Psilacetin hydrofumarate, norpsilocin hydrofumarate, 4-HO-TMT iodide, and 4-AcO-TMT iodide were synthesized previously.[43,44,54] Psilocin freebase and psilocybin (zwitterion) were generously provided by the National Institute on Drug Abuse (NIDA), Drug Supply Program (Rockville, MD, USA). For *β*-arrestin functional assays, psilocybin was obtained from Cayman Chemical (Ann Arbor, MI, USA), whereas psilocin freebase was obtained from Chiron (Trondheim, Norway). DOI hydrochloride and WAY100635 maleate (WAY100635) were purchased from Cayman Chemical. DOI and LSD for functional studies were purchased from Chiron. 5-HT hydrochloride was purchased from MilliporeSigma (St. Louis, MO, USA). (+)-M100907 freebase (M100907) was generously provided by Kenner Rice, Ph.D., and Agnieszka Sulima, Ph.D. (NIDA). For all *in vitro* and mouse brain tissue studies, compounds were initially dissolved in 100% DMSO and were subsequently diluted in respective assay buffers. The nonenzymatic hydrolysis rates for the stability of psilocybin and psilacetin in aqueous solution were determined to address concerns of background hydrolysis rates, and results are shown in Figure S15. For mouse behavior

studies, all drug doses represent the weight of the salt dissolved in 0.9% saline vehicle.

Animals. Mice (C57BL/6J males) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) at 5−6 weeks of age and allowed at least 1−2 weeks to acclimate to the NIDA, Intramural Research Program (IRP), animal research facility in Baltimore, MD, USA. The animal facility is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, and all procedures were approved by the NIDA IRP Animal Care and Use Committee. Mice were initially group housed 3−5 per cage during acclimation and housed in a 12 h light−dark cycle throughout the study, with lights on at 0700 h. Food and water were available *ad libitum* except during testing.

Receptor Binding and Functional Screening. Receptor binding and functional screening assays were generously conducted by the National Institute of Mental Health (NIMH) Psychoactive Drug Screening Program (PDSP) in the laboratory of Dr. Bryan Roth (University of North Carolina, Chapel Hill, NC, USA). All 9 test compounds (depicted in Figure 1) were screened at 46 potential binding sites using radioligand binding assays in cells transfected with human G protein-coupled receptors (GPCRs). Initial screening was carried out at a fixed 10 μM concentration using quadruplicate determinations from a single experiment for each compound. For any receptor site where binding was inhibited by 50% or greater in the 10 μM screen, full concentration−effect curves were constructed using triplicate determinations from 1 to 2 independent experiments, and binding affinities (i.e., K_i) were calculated. Primary functional studies were conducted for six of the nine compounds at 3 and 10 μM concentrations, based on the receptors identified from the binding screens, using the GPCR Tango assay for G-protein-independent β-arrestin translocation.[78,100] Lastly, secondary full concentration effect curves were also generated for the same compounds at selected identified targets in primary functional screens using a calcium mobilization assay. Relevant details about the assay procedures, including radioligands used, are provided in the text or table legends. Further details (cell types used, etc.) about the binding and functional assays carried out by PDSP can be found online.[101]

Additional receptor screening was conducted by the Addiction Treatment Discovery Program of NIDA (Rockville, MD, USA). Briefly, all nine compounds were screened at two concentrations (100 nM and 10 μM) to determine % inhibition of agonist radioligand binding at the following sites: 5-HT$_{1A}$, 5-HT$_{2A}$, serotonin 2B receptor (5-HT$_{2B}$), 5-HT$_{2C}$, DAT, norepinephrine transporter (NET), SERT, mu-opioid receptor (MOR), delta opioid receptor (DOR), and kappa opioid receptor (KOR). Radioligand binding methods are similar to those previously described for [³H]8-OH-DPAT,[102] [³H]5-HT,[103] [³H]RTI-55,[104] [³H]DAMGO, [³H]-DPDPE, and [³H]U69,593.[105] Data represent mean inhibition of binding, as assessed by triplicate determinations from two independent experiments.

Radioligand Binding Assays in Mouse Brain. Dose−response curves for inhibition of radioligand binding in mouse brain tissue were constructed using triplicate determinations from three independent experiments. Effects of test compounds on [³H]8-OH-DPAT or [³H]M100907 binding are expressed as percent inhibition of specific binding versus log of compound concentration in graphical plots and as affinity values in relevant tables.

Inhibition of [³H]8-OH-DPAT Binding to 5-HT$_{1A}$. Radioligand binding to mouse brain 5-HT$_{1A}$ was performed as previously described with minor modifications.[106] Briefly, for each experiment, two frozen mouse brains (C57BL/6 strain, BioIVT, Westbury, NY, USA) were each homogenized in 10 mL of ice cold 50 mM Tris−HCl pH 7.7 using a polytron at 15,000 rpm for 7 s. Homogenates were centrifuged at 40,000g for 10 min at 4 °C, the supernatants were discarded, and each pellet was resuspended in 10 mL of 50 mM Tris−HCl pH 7.7 by vigorous vortexing. The tissue suspensions were incubated at 37 °C for 10 min and were then centrifuged at 40,000g for 10 min at 4 °C. Each pellet was resuspended in 10 mL assay buffer (50 mM Tris−HCl, 4 mM CaCl$_2$, 10 μM pargyline, 0.1% ascorbic acid, pH 7.7), and the tissue suspensions were combined, diluted to 150 mL in assay buffer (22 °C), and used immediately.

Binding assays were performed in 12 × 75 mm polystyrene test tubes that contained 800 μL of tissue prep, 100 μL of 5 nM [³H]8-OH-DPAT (129 Ci/mmol, PerkinElmer, Boston, MA, USA) diluted in assay buffer, and 100 μL of the test compound diluted in assay buffer. Assays were initiated by the addition of tissue and were terminated after 30 min at 22 °C by rapid vacuum filtration through GF/B filter paper (Brandel, Gaithersburg, MD, USA) mounted on a cell harvester (Brandel). Filters were rinsed with 5 mL of ice cold wash buffer (50 mM Tris−HCl, pH 7.7) and placed into 24-well flex plates (PerkinElmer) with 500 μL/well CytoScint (MP Biomedical, Irvine, California, USA). After an overnight incubation, radioactivity was quantitated using a MicroBeta2 liquid scintillation counter (PerkinElmer) at 40% efficiency. Nonspecific binding was determined in the presence of 10 μM serotonin, and maximal binding was determined in the absence of any competing drug.

Inhibition of [³H]M100907 Binding to 5-HT$_{2A}$. Radioligand binding to mouse brain 5-HT$_{2A}$ was performed as previously described with minor modifications.[66] For each experiment, two frozen mouse brains (C57BL/6 strain, BioIVT) were each homogenized in 10 mL of ice cold 0.25 M sucrose using a polytron at 15,000 rpm for 7 s. Homogenates were centrifuged at 40,000g for 10 min at 4 °C, the supernatants were discarded, and each pellet was resuspended in 10 mL of ice cold 50 mM Tris−HCl pH 7.0 by vigorous vortexing. The tissue suspensions were incubated at 37 °C for 10 min and were then centrifuged at 40,000g for 10 min at 4 °C. Pellets were resuspended to a final volume of 16 mL in assay buffer (50 mM Tris−HCl, 0.5 mM EDTA, 10 mM MgSO$_4$, 0.1% ascorbic acid, pH 7.4) and were used immediately.

Binding assays were performed in 12 × 75 mm polystyrene test tubes that contained 700 μL of assay buffer, 100 μL of 10 nM [³H]M100,907 (87 Ci/mmol, Novandi, Sodertalje, Sweden) diluted in assay buffer, 100 μL of the test drug diluted in assay buffer, and 100 μL of tissue preparation for a total volume of 1 mL. Assays were initiated by the addition of tissue and were terminated after 30 min at 37 °C by rapid vacuum filtration as described above. Filters were rinsed with 5 mL of ice cold wash buffer (10 mM Tris−HCl, pH 7.0) and processed as described above. Nonspecific binding was determined in the presence of 10 μM ketanserin, and maximal binding was determined in the absence of any competing drug.

5-HT$_{2A}$-Mediated β-Arrestin 2 Recruitment. β-Arrestin 2 recruitment at the human 5-HT$_{2A}$ was assessed as previously described using Human Embryonic Kidney (HEK) 293T cells

stably expressing the SmBiT-βarr2 and 5-HT$_{2A}$-LgBiT constructs.[60,77] Briefly, the dilutions for functional activity assessments were made in Hank's buffered saline solution (HBSS) by means of serial dilution, while keeping the solvent concentration constant, yielding in-well concentration ranges from 10 μM to 10^{-11} M. One day prior to the experiment, cultured cells stably expressing 5-HT$_{2A}$-LgBiT and SmBiT-β-arrestin 2 (LgBiT and SmBiT being the enzyme fragments of the NanoBiT system, initially developed by Promega)[107] were seeded at a density of 50,000 cells per well in 96-well plates coated with poly-D-lysine for optimal cell adhesion. Plates were incubated at 37 °C and 5% CO$_2$ until the readout on day 2. On the second day, the cells were washed twice with 150 μL of HBSS to remove all remaining medium/serum, and 100 μL of HBSS was pipetted into each well. Next, 25 μL of the NanoGlo Live Cell substrate was added (1/20 diluted in the LCS dilution buffer provided by the manufacturer), and plates were transferred to a plate reader (Tristar^2—Berthold Technologies GmbH and Co, Germany). After equilibration of the luminescent signal, 10 μL of each agonist solution was added, and the signal was monitored for 2 h. All concentrations were tested in duplicate in three independent experiments, and solvent controls were run for all conditions. LSD was used as the reference compound, and DOI was also used as a standard comparator positive control.

Mouse Studies. *Details and Design.* For the mouse studies, cohorts of 9−12 mice were used for each test drug. The mice were subjected to experimental testing once every 1−2 weeks for 2−3 months to complete dose−effect curves and antagonist experiments. A minimum of 7 days between treatments was utilized to avoid any tolerance to effects of repeated drug administration.[81,108] Mice were tested first in dose−response studies to assess the effects of each compound at doses from 0.03 to 30 mg/kg s.c. and were subsequently tested in antagonist reversal studies utilizing pretreatment with M100907 and WAY100635. All experiments were conducted from 0900 to 1700 local time during the light phase, as sensitivity of rodents to other tryptamine psychedelics is diurnal, with maximal HTR observed in the middle of the light phase.[109,110] Experiments were run during the light phase also to avoid any potential influence of melatonin receptor activity on HTR as melatonin and related agonists are known to reduce HTR induced by DOI in rats.[111,112]

For each experiment, mice were acclimated to the testing room in their home cage for at least 1 h prior to experimental sessions. Behavioral test sessions were carried out in Tru Scan mouse locomotor arenas equipped with photobeam arrays (Coulbourn Instruments, Holliston, MA, USA), which were modified with cylindrical inserts and transparent floors useful in detecting mouse HTR as described previously.[82]

Subcutaneous Temperature Transponder Implants. At least 1 week prior to the start of the experiments, mice received s.c. implanted temperature transponders (14 × 2 mm, model IPTT-300, Bio Medic Data Systems, Inc., Seaford, DE, USA) under brief isoflurane anesthesia as previously described.[113] Mice were single housed post implant for the remainder of the study to protect the transponder from removal by cage mates. Temperature was determined noninvasively using a handheld receiver that is sensitive to signals emitted from the implanted transponders.

Behavioral Testing. Prior to each experiment, mouse body weight and temperature were recorded. Mice were then placed into testing chambers for acclimation. In dose−response studies, after a brief 5 min acclimation, mouse body temperature was recorded for baseline measurement, mice received s.c. injection of test substance or vehicle, and animals were returned to the testing arena for 30 min. During the session, locomotor activity was monitored via photobeam tracking of movements in the horizontal plane to yield distance traveled in centimeter. HTR was monitored by the analysis of GoPro Hero Black 7 video recordings (120 frames per sec and 960p resolution) using a commercially available software package from Clever Sys Inc. (Reston, VA, USA).[82] Post-treatment body temperature values were also recorded, and temperature data are represented as change from pretreatment baseline.

Antagonist Studies. In antagonist reversal experiments, mice received a s.c. injection of either receptor antagonists or vehicle and were returned to the testing chamber for 30 min. During this period, locomotor activity was monitored to examine the potential effects of antagonist treatment on general behavior or movement. At 30 min after antagonist administration, mice were given test drug or vehicle and returned to the chambers for an additional 30 min of video recording used for analyses.

Data Analyses. All statistical analyses were conducted using GraphPad Prism 9 (La Jolla, CA, USA). Nonlinear regression was utilized to determine receptor affinity (K_i), potency (EC$_{50}$ or ED$_{50}$), and efficacy (E_{max}) for in vitro and *in vivo* experiments. K_i values were determined in mouse brain experiments using previously reported K_d values for these radioligands in mouse brain.[106,114] For the β-arrestin recruitment assays, the full 2 h time−luminescence profile was corrected for inter-well variability and used to calculate the area under the curve (AUC). The AUC of the corresponding solvent control was subtracted, and the obtained values were used to fit sigmoidal concentration−response curves as well as to determine EC$_{50}$ and E_{max} values relative to the E_{max} of LSD (set at 100%). Dose−response data from mouse experiments were analyzed using nonlinear regression, and potency values were determined from the rising phase of the curves for HTR measures. For mouse studies, one-way ANOVA with Dunnett's post hoc test was used to compare all conditions to vehicle controls (0 or 0,0) in dose−response and antagonist experiments. Time-course drug effects for all parameters in mouse studies are shown for reference. Mean HTR count, distance traveled, and temperature change for each condition were used for statistical comparisons. Alpha was set at 0.05 for all analyses.

■ ASSOCIATED CONTENT

ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsptsci.2c00177.

> Affinity values of various psilocybin analogues for sites other than 5-HT receptors; competition of psilocybin analogues for agonist radioligand binding *in vitro*; concentration effect curves of psilocybin analogues in mouse brain; concentration−response curves for psilocybin analogues in 5-HT$_2$ receptor calcium mobilization assays; functional agonist activity screening for psilocybin analogues across 5-HT receptors; concentration effect curves of psilocybin analogues at 5-HT$_{2A}$ receptors in β arrestin 2 functional assays; time-course for HTR by psilocin, psilocybin, psilacetin, and norpsilocin in mice;

statistical information and descriptive statistics for dose−response mouse studies; dose−response curves of secondary amine and quaternary ammonium psilocybin analogues for HTR count in mice; dose−response curves of psilocybin analogues for temperature change in mice; psilocin, psilocybin, and psilacetin locomotor plots; secondary amine locomotor plots; locomotor plots for quaternary ammonium psilocybin analogues; dose-related effects of M100907 on locomotor activity in mice; M100907 experiment control plots; descriptive statistics for HTR data from M100907 experiments; descriptive statistics for all data from WAY100635 experiments; WAY100635 effects on HTR and locomotor activity in mice; X-ray crystal structures of salts used of 4-AcO-NMT and aeruginascin; extended information and data related to crystallographic analyses of 4-AcO-NMT and aeruginascin; and nonenzymatic hydrolysis rates of psilocybin and psilacetin in aqueous buffer (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Grant C. Glatfelter − *Designer Drug Research Unit, National Institute on Drug Abuse Intramural Research Program, Baltimore, Maryland 21224, United States;* ● orcid.org/0000-0003-1011-9083; Email: grant.glatfelter@nih.gov

Authors

Eline Pottie − *Laboratory of Toxicology, Department of Bioanalysis, Faculty of Pharmaceutical Sciences, Ghent University, 9000 Ghent, Belgium*

John S. Partilla − *Designer Drug Research Unit, National Institute on Drug Abuse Intramural Research Program, Baltimore, Maryland 21224, United States*

Alexander M. Sherwood − *Usona Institute, Madison, Wisconsin 53711, United States;* ● orcid.org/0000-0003-0895-0791

Kristi Kaylo − *Usona Institute, Madison, Wisconsin 53711, United States*

Duyen N. K. Pham − *Department of Chemistry & Biochemistry, University of Massachusetts Dartmouth, North Dartmouth, Massachusetts 02747, United States*

Marilyn Naeem − *Department of Chemistry & Biochemistry, University of Massachusetts Dartmouth, North Dartmouth, Massachusetts 02747, United States*

Vamshikrishna Reddy Sammeta − *Department of Chemistry & Biochemistry, University of Massachusetts Dartmouth, North Dartmouth, Massachusetts 02747, United States*

Stacie DeBoer − *Department of Chemistry, University of Wyoming, Laramie, Wyoming 82071, United States*

James A. Golen − *Department of Chemistry & Biochemistry, University of Massachusetts Dartmouth, North Dartmouth, Massachusetts 02747, United States*

Elliott B. Hulley − *Department of Chemistry, University of Wyoming, Laramie, Wyoming 82071, United States;* ● orcid.org/0000-0002-2630-3689

Christophe P. Stove − *Laboratory of Toxicology, Department of Bioanalysis, Faculty of Pharmaceutical Sciences, Ghent University, 9000 Ghent, Belgium;* ● orcid.org/0000-0001-7126-348X

Andrew R. Chadeayne − *CaaMTech, Inc., Issaquah, Washington 98027, United States*

David R. Manke − *Department of Chemistry & Biochemistry, University of Massachusetts Dartmouth, North Dartmouth, Massachusetts 02747, United States;* ● orcid.org/0000-0002-2513-4801

Michael H. Baumann − *Designer Drug Research Unit, National Institute on Drug Abuse Intramural Research Program, Baltimore, Maryland 21224, United States;* ● orcid.org/0000-0001-7758-1470

Complete contact information is available at:
https://pubs.acs.org/10.1021/acsptsci.2c00177

Author Contributions

Study design: G.C.G. and M.H.B. Chemical synthesis and analysis: D.R.M., J.A.G., A.M.S., D.N.K.P., V.R.S., and M.N. Hydrolysis experiments: S.D. and E.B.H. Mouse brain binding assays: J.P. 5-HT$_{2A}$ β-arrestin 2 assays: E.P. Mouse experiments: G.C.G. Manuscript was drafted by G.C.G. and critically reviewed by M.H.B., A.R.C., D.R.M., A.M.S., K.K., E.B.H., E.P., and C.P.S., and final version approved by all authors.

Notes

The authors declare the following competing financial interest(s): A.R.C. has an ownership stake in CaaMTech, Inc., which owns patent applications covering new tryptamine compounds, their compositions, formulations, novel crystalline forms, methods of treatment, and methods for synthesis. No other authors report any competing financial interests related to the present work.

■ ABBREVIATIONS

5-HT, serotonin; 5-HT$_{1A}$, serotonin 1A receptor; 5-HT$_{1B}$, serotonin 1B receptor; 5-HT$_{1D}$, serotonin 1D receptor; 5-ht$_{1e}$, serotonin 1e receptor; 5-HT$_{2A}$, serotonin 2A receptor; 5-HT$_{2B}$, serotonin 2B receptor; 5-HT$_{2C}$, serotonin 2C receptor; 5-HT$_3$, serotonin 3 receptor; 5-HT$_4$, serotonin 4 receptor; 5-HT$_{5A}$, serotonin 5A receptor; 5-HT$_6$, serotonin 6 receptor; 5-HT$_{7a}$, serotonin 7a receptor; Alpha$_{2A}$, alpha 2A receptor; Alpha$_{2C}$, alpha 2C receptor; DAT, dopamine transporter; SERT, serotonin transporter; H$_1$, histamine H1 receptor; norbaeocystin, 4-phosphoryloxytryptamine; baeocystin, 4-phosphoryloxy-*N*-methyltryptamine; psilocybin, 4-phosphoryloxy-*N,N*-

dimethyltryptamine; aeruginascin, 4-phosphoryloxy-N,N,N-trimethyltryptamine; norpsilocin, 4-hydroxy-N-methyltryptamine; psilocin, 4-hydroxy-N,N-dimethyltryptamine; 4-HO-TMT, 4-hydroxy-N,N,N-trimethyltryptamine; 4-AcO-NMT, 4-acetoxy-N-methyltryptamine; psilacetin or 4-AcO-DMT, 4-acetoxy-N,N-dimethyltryptamine; 4-AcO-TMT, 4-acetoxy-N,N,N-trimethyltryptamine; M100907, (+)MDL 100907; WAY100635, WAY 100653; DOI, (±)-2,5-dimethoxy-4-iodoamphetamine; LSD, lysergic acid diethylamide; 5-MeO-DMT, 5-methoxy-N,N-dimethyltryptamine; 5-HTP, 5-hydroxytryptophan; NIMH PDSP, National Institute of Mental Health Psychoactive Drug Screening Program

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