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Synthesis and Functional Characterization of 2-(2,5-Dimethoxyphenyl)-*N*-(2-fluorobenzyl)ethanamine (25H-NBF) Positional Isomers

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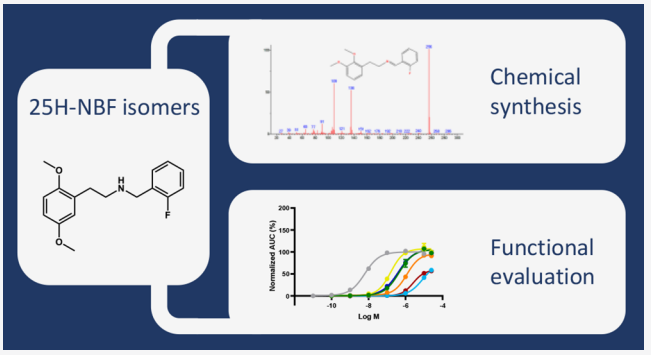
ABSTRACT: Serotonergic psychedelics, substances exerting their pharmacological action through activation of the serotonin 2A receptor (5-HT_{2A}R), have continuously comprised a substantial fraction of the over 1000 reported New Psychoactive Substances (NPS) so far. Within this category, *N*-benzyl derived phenethylamines, such as NBOMes and NBFs, have shown to be of particular relevance. As these substances remain incompletely characterized, this study aimed at synthesizing positional isomers of 25H-NBF, with two methoxy groups placed on different positions of the phenyl group of the phenethylamine moiety. These isomers were then functionally characterized in an *in vitro* bioassay monitoring the recruitment of *β*-arrestin 2 to the 5-HT_{2A}R through luminescent readout via the NanoBiT technology. The obtained results provide insight into the optimal substitution pattern of the phenyl group of the phenethylamine moiety of *N*-benzyl derived substances, a feature so far mostly explored in the phenethylamines underived at the *N*-position. In the employed bioassay, the most potent substances were 24H-NBF (EC_{50} value of 158 nM), 26H-NBF (397 nM), and 25H-NBF (448 nM), with 23H-NBF, 35H-NBF, and 34H-NBF yielding *μ*M EC_{50} values. A similar ranking was obtained for the compounds' efficacy: taking as a reference LSD (lysergic acid diethylamide), 24H-, 26H-, and 25H-NBF had an efficacy of 106−107%, followed by 23H-NBF (96.1%), 34H-NBF (75.2%), and 35H-NBF (58.9%). The stronger activity of 24H-, 25H-, and 26H-NBF emphasizes the important role of the methoxy group at position 2 of the phenethylamine moiety for the *in vitro* functionality of NBF substances.



KEYWORDS: *Psychedelic, new psychoactive substances, synthesis, bioassay, structure−activity relationship, serotonin receptor*

INTRODUCTION

At the beginning of 2021, a cumulative total of more than 1000 New Psychoactive Substances (NPS) had been reported to the United Nations Office on Drugs and Crime (UNODC).[1] When looking at the classification of NPS based on their pharmacological mechanism, one of the consistently larger groups is that of the classic hallucinogens.[1] Classic hallucinogens, or serotonergic psychedelics, are substances that exert their main pharmacological effects through activation of the serotonin 2A receptor (5-HT_{2A}R), a G protein-coupled receptor (GPCR).[2] Effects typically sought for by users of psychedelics include, *inter alia*, mystical experiences, empathic feelings, alterations in consciousness, and sensory and somatic effects. However, the use of these substances can result in severe side effects, such as tachycardia, hyperthermia, agitation, rhabdomyolysis, hypertension, and seizures, and with the more recent group of NBOMes even deaths have been reported.[3,4] On the other hand, interest in the potential therapeutic benefits of psychedelics is increasing. This translates into clinical trials in, *e.g.*, the context of treatment of addiction and in psychotherapy, mainly with LSD (lysergic acid diethylamide) and psilocybin.[5,6]

Serotonergic psychedelics (not limited to the NPS) can be categorized into three main structural classes, more specifically the ergolines (such as the prototypical psychedelic substance LSD), tryptamines (such as psilocybin), and phenylalkylamines (with mescaline as the prototypical substance). This latter group comprises subcategories such as the phenethylamines (among which 2C-X substances), the phenylisopropylamines (DOx substances) and, more recently, *N*-benzylderivatives of the former group (*e.g.*, *N*-methoxybenzylderivatives or NBOMes).[3,7] Certain representatives of the *N*-benzyl derived substances, such as 25B-NBOMe, have been explored as

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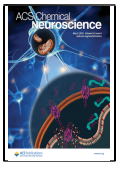

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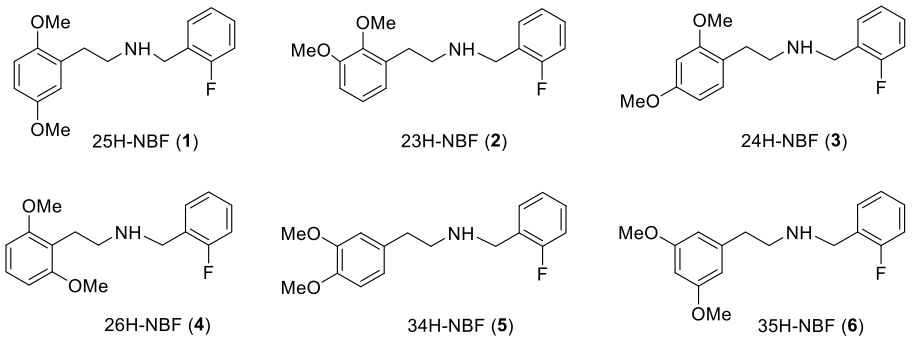


Figure 1. Structures of compounds **1**−**6** investigated in the present study.

neuroimaging tools.[8] For the group of phenethylamine psychedelics, several Risk Assessment Reports have been issued by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA). These reports specifically emphasize the relevance and risk of the specific substances 2C-I, 2C-T-2 and 2C-T-7, and 25I-NBOMe.[9,10]

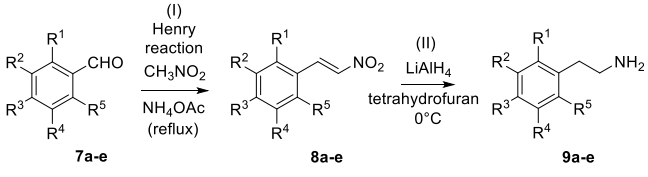
Despite their relevance, psychedelic NPS still remain incompletely characterized. One example of a scarcely described group is that of the 25X-NBF substances, structurally related to the NBOMes, only differing in a fluorine substituent instead of a methoxy group at position 2 of the *N*-benzyl group. The recreational use of these substances is suggested by the identification of 25B/C-NBF on the illegal drug market in Japan and by the mention of 25I-NBF on drug forums.[11,12] Additionally, several 25X-NBF substances are subject to legislative control, for example, in Sweden, Japan, and the United Kingdom.[11,13,14] However, little is known about the pharmacology and toxicology of NBF substances. In rodent *in vivo* studies, 25C-NBF was shown to initiate neurotoxicity and addiction potential. Therefore, Hur et al. emphasize the danger that lies with the use of 25C-NBF.[15]

As the NBF substances, either with 4-substituent (25X-NBF) or without 4-substituent (25H-NBF) on the phenyl ring of the phenethylamine moiety, are pharmacologically scarcely described, this study aimed to provide an estimation of what substitution patterns are favorable for their *in vitro* receptor activation. Therefore, we here describe the synthesis and functional characterization of five positional isomers of 2-(2,5-dimethoxyphenyl)-*N*-(2-fluorobenzyl)ethanamine (25H-NBF (**1**)), namely, 2-(2,3-dimethoxyphenyl)-*N*-(2-fluorobenzyl)-ethanamine (23H-NBF (**2**)), 2-(2,4-dimethoxyphenyl)-*N*-(2-fluorobenzyl)ethanamine (24H-NBF (**3**)), 2-(2,6-dimethoxyphenyl)-*N*-(2-fluorobenzyl)ethanamine (26H-NBF (**4**)), 2-(3,4-dimethoxyphenyl)-*N*-(2-fluorobenzyl)ethanamine (34H-NBF (**5**)), and 2-(3,5-dimethoxyphenyl)-*N*-(2-fluorobenzyl)-ethanamine (35H-NBF (**6**)). The structures of these compounds are shown in Figure 1. In these isomers, the two methoxy groups on the phenylethylamine moiety, which are situated at positions 2 and 5 in the original structure (hence 25H-NBF), are switched to different positions on the benzene ring. Typically, the X in the 25X-NBF conventional nomenclature (in this case, H for hydrogen) points at the substituent at position 4 of the phenethylamine moiety. As 24H- and 34H-NBF carry a methoxy group at position 4, these substances can theoretically also be denoted as 24-NBF and 34-NBF. In this study, we opted to refer to these compounds as 24H-NBF and 34H-NBF, to emphasize that no other substituents are introduced on the phenyl ring of the phenethylamine moiety, besides the two methoxy groups. As far as we know, synthesis, analytical data, and biological studies for compounds **2**, **3**, **4**, and **6** have not been previously described in the scientific literature. For compound **5**, data on testing its antibacterial activity were published.[16] Compounds **1** and **5** are commercially available. The activation potential of each of these isomers was determined in this study by means of their ability to induce recruitment of the cytosolic scaffolding protein β-arrestin 2 (βarr2) to the activated target receptor 5-HT_{2A}R.

RESULTS AND DISCUSSION

Chemistry. Synthesis of compounds **2**−**6** was achieved in three steps. The first two steps (synthesis of β-nitrostyrene derivatives **8a**−**e** by nitroaldol condensation reaction (Henry reaction[17]) with subsequent reduction to substituted phenethylamines **9a**−**e**) were carried out using previously reported procedures[18] (Scheme 1). Target compounds were obtained

Scheme 1. Two Step Synthesis of Phenethylamines 9a−e



7a, **8a**, **9a**: R^1=R^2=OMe; R^3=R^4=R^5=H
7b, **8b**, **9b**: R^1=R^3=OMe; R^2=R^4=R^5=H
7c, **8c**, **9c**: R^1=R^5=OMe; R^2=R^3=R^4=H
7d, **8d**, **9d**: R^2=R^3=OMe; R^1=R^4=R^5=H
7e, **8e**, **9e**: R^2=R^4=OMe; R^1=R^3=R^5=H

by a stepwise reductive *N*-amination from dimethoxyphenethylamines **9a**−**e** via the formation of the corresponding imines **10a**−**e** (Scheme 2), using the reported earlier method[18] which we adapted to the synthesized class of compounds. In the final version, the synthesis of compounds **2**−**6** was carried out according to Schemes 1 and 2.

Functional Evaluation. As this is the first report describing the synthesis of this full set of positional isomers of 25H-NBF, the pharmacological profile of these substances was also unknown. Therefore, they were functionally characterized by means of their potential to induce recruitment of the cytosolic protein β-arrestin 2 (βarr2) to the target receptor 5-HT_{2A}R. To this end, a NanoBiT functional complementation assay was employed, of which the assay components were stably expressed in a previously developed stable cell system.[19] In this assay, each of two (inactive) parts of a split-nanoluciferase (LgBiT and SmBiT) is fused to one of two potentially interacting proteins, in this case the receptor and βarr2. When the receptor (C-terminally fused to LgBit) is

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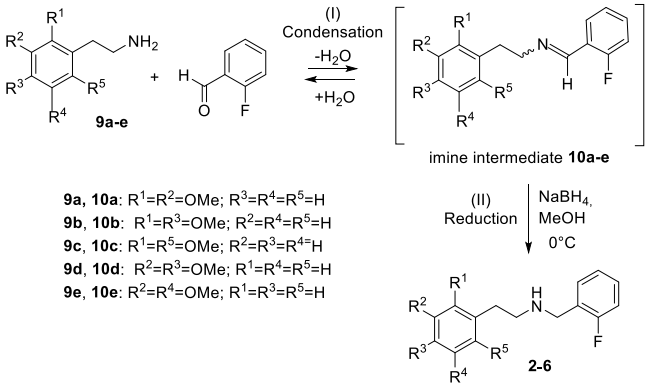
Scheme 2. Synthesis of Substituted *N*-(2-Fluorobenzyl)phenylethanamines (2−6) via Reductive Amination



activated, βarr2 (N-terminally fused to SmBiT) is recruited, leading to a reassociation of the split fragments. In the presence of the enzyme's substrate, this can then be monitored via a luminescent readout, in which the luminescence is related to the activity of the substance in a concentration-dependent manner.[20] This approach enabled the establishment of concentration−response curves, as depicted in Figure 2, and

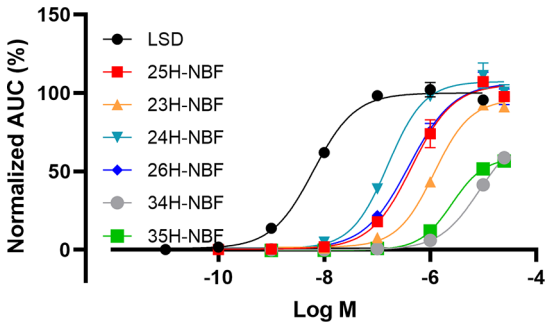


Figure 2. Concentration−response curves of each of the tested 25H-NBF isomers, together with that of the reference psychedelic substance LSD. Each point represents the mean of three independent experiments, each performed in duplicate ± SEM (standard error of the mean).

the calculation of EC_{50} (as a measure of potency) and E_{max} values (as a measure of efficacy, relative to the reference agonist LSD). These latter values can be found in Table 1.

Data obtained for LSD and 25H-NBF are in the same range as those previously reported employing our βarr2 recruitment assays (in both transiently transfected and stably expressing cells). For the reference agonist LSD, we have previously reported EC_{50} values between 5.95 and 7.43 nM for the recruitment of βarr2 to the 5-HT_{2A}R.[19,21−23] The value obtained in this study, 6.23 nM, perfectly aligns with those results. For 25H-NBF, we have previously reported values in the high nanomolar range, with an efficacy that was similar (104−107%) to that of the reference psychedelic substance LSD.[19,22] Also here, the EC_{50} value of the conventionally substituted 25H-NBF lies in the higher nanomolar range (448 nM), with an efficacy of 106%, where that of LSD is set at 100%.

When looking at the data in Table 1, several trends can be observed concerning the activity of the isomers, considering either the potency or the efficacy of the substances. When

Table 1. Quantitative Data Obtained for the Potency (EC_{50}) and Efficacy (E_{max}) for Each of the Tested Substances and for the Reference Agonist LSD[a]

compd	EC_{50} (nM, 95% CI)	E_{max} (%, 95% CI)
LSD	6.23 (4.78−7.94)	100 (96.5−104)
25H-NBF	448 (304−647)	106 (98.0−115)
23H-NBF	1175 (978−1533)	96.1 (90.1−105)
24H-NBF	158 (120−219)	107 (101−114)
26H-NBF	397 (261−596)	107 (98.7−116)
34H-NBF[b]	*8382 (6566−12 000)*	*75.2 (67.0−89.0)*
35H-NBF	2566 (1889−3477)	58.9 (54.6−64.9)

[a]Data are from three independent experiments, each performed in duplicate. The maximal response in each individual experiment is normalized to the maximal response of LSD (set at 100%). CI: confidence interval. [b]As the maximum of the sigmoidal curve was not reached in the tested concentration range, care must be taken when interpreting these values.

looking at the EC_{50} values, the most potent substance is 24H-NBF, with 25H-NBF and 26H-NBF having potencies that are 2−3 times lower. Substantially lower potencies are then found in 23H-NBF and 35H-NBF. 34H-NBF had the lowest observed potency in this set of substances, not reaching the maximal plateau of the concentration−response curve in the tested concentration range. The E_{max} values, as a measure of the efficacy of the substances, show values that are similar for 25H-, 24H-, and 26H-NBF, lying in the same range as that of the reference agonist LSD. 23H-NBF tended to have a slightly lower efficacy, while substantially lower values were obtained for 35H- and 34H-NBF. The data obtained for the latter substance have to be interpreted with caution, as no maximal response was reached in the tested concentration range. Overall, it can be concluded that 24H-NBF is the most active substance in the *in vitro* bioassay, followed by 25H-NBF and 26H-NBF, with similar potencies and efficacies. Of the substances carrying a methoxy group at position 2 of the phenethylamine moiety, 23H-NBF is the least active substance. The overall least active substances are 34H-NBF and 35H-NBF.

The positioning of the two methoxy groups on the phenethylamine moiety has been described mostly in substances unsubstituted at the *N*-position and recently also in their *N*-methoxybenzyl counterparts, the NBOMes.[23] For the former group, PiHKAL describes the 2,4,5-substitution of the phenyl ring to be more effective than the 3,4,5-substitution pattern, with the suggestion of a 2,4,6-substitution pattern to be effective as well. In this first substitution pattern, positions 2 and 5 ideally carry a methoxy group, with more flexibility possible at position 4.[24] When testing desmethoxy analogues of 2C-B and DOB, the omission of either group appeared to more dramatically impact the *in vivo* effects than the *in vitro* effects of the substances. In *in vitro* functional assays, the removal of the 2-methoxy group resulted in a stronger decrease in activity than that of the 5-methoxy group.[25]

In previous work, we have evaluated the potencies and efficacies of five positional isomers of 25H-NBOMe, where the positions of the methoxy groups on the phenyl group of the phenethylamine moiety were explored similarly as in this set of substances.[23] Overall, the observed trends of the -NBOMe isomers were largely the same, irrespective of the reference agonist (LSD versus serotonin), the calculation time point (2 h versus 30 min AUC), or the bioassay used (recruitment of

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βarr2 versus miniGα_q). The ranking based on potency was 24H > 25H − 26H > 23H > 35H − 34H-NBOMe, and 24H-, 25H-, 26H-, and 34H-NBOMe were categorized as more efficacious substances, with 35H- and 23H-NBOMe being less efficacious. Therefore, the data in the present study are in line with our earlier findings, hinting at the importance of the methoxy group at position 2 in both NBOMe and NBF substances. However, combining this methoxy group at position 2 with a 3-methoxy group negatively impacts activity at the 5-HT_{2A}R. Overall, our data also hint at the fact that introducing a methoxy group at position 3 strongly decreases the activity of a substance, likely owing to an altered interaction with specific 5-HT_{2A}R residues, as suggested by molecular docking of the corresponding panel of -NBOMes into a 5-HT_{2A}R model, based on the cryo-EM structure of the receptor with 25CN-NBOH.[23,26]

In terms of structure−activity relationship, introducing an *N*-benzyl substituent at the amine function of a phenethylamine has been reported to increase the activity of substances.[27,28] The best explored groups having this substitution are -NBOMe, -NBMD, -NBOH, and -NBF derivatives of 2C-X substances as *N*-unsubstituted counterparts. Consistent with our previously reported data, also here 25H-NBF is more potent and efficacious than 2C-H (comparison with our historical data from 2C-H),[19,22] the counterpart that does not have the *N*-fluorobenzyl group at its amine function. On the other hand, the -NBF isomers tested here were consistently less potent than the corresponding -NBOMe isomers. Jensen et al. ascribes this low potency and efficacy for the -NBF substances to the absence of an oxygen atom at position 2 on the *N*-benzyl group, that is present in the corresponding -NBOMe, -NBMD, and -NBOH derivatives.[29]

Analogous to the NBOMe substances, *O*-demethylation of methoxy groups has been reported to be an important metabolic pathway in the degradation of 25X-NBF substances. At this moment, it is unknown what the impact on metabolism is of the altered positions of the methoxy groups, compared to the 2,5-dimethoxy pattern.[30] This is one of the limitations that complicates the interpretation of data obtained in *in vitro* functional assays and their potential translation to *in vivo* outcomes. Additionally, the presented data do not allow for the assessment of the binding affinities of the synthesized isomers, which could provide more insight on how these ligands behave in the presence of other (endogenous) ligands. The use of other *in vitro* bioassays, monitoring different events along the signaling cascade (such as Ca^{2+}, miniGα_q, PLC or PLA assays), could potentially yield additional insights into the molecular pharmacology of these substances. As the precise mechanism of action of psychedelics at their (main) pharmacological target, 5-HT_{2A}R, has not been fully elucidated, it remains unclear how the obtained results correlate with the *in vivo* situation.[31]

In conclusion, this study is the first report describing the synthesis of all five 25H-NBF isomers with different positions of two methoxy groups in the phenethylamine moiety. Functional evaluation of these isomers in an *in vitro* βarr2 recruitment assay yielded marked differences, based on either the EC_{50} or E_{max} values. Focusing on the potencies of the substances, 24H-NBF was found to be the most potent, followed by 25H- and 26H-NBF, with 23H-NBF being the least potent substance with a 2-methoxy group. 34H- and 35H-NBF are the least potent substances in the tested panel. Looking at the efficacies, a highly similar ranking is obtained: 24H − 25H − 26H ≥ 23H > 34H and 35H-NBF. Together with data from previously published NBOMe positional isomers, these data emphasize the importance of the methoxy group at position 2 of the phenyl group of the phenethylamine moiety for the *in vitro* activity of *N*-benzyl derived substances.

EXPERIMENTAL PROCEDURES

Materials, Solvents, and Reagents. The following starting materials, reagents, and solvents were used for the synthesis of compounds **2**−**6**: 2-fluorobenzaldehyde (97%), 2,4-dimethoxybenzaldehyde (98%), 2,6-dimethoxybenzaldehyde (97%), 3,4-dimethoxybenzaldehyde (99%), and 3,5-dimethoxybenzaldehyde (98%), all from Alfa Aesar (Kandel, Germany); 2,3-dimethoxybenzaldehyde (97%), nitromethane (96%), lithium aluminum hydride (95%), and sodium borohydride (99%), all from Acros Organics (Fair Lawn, NJ); solvents: methylene chloride (≥99%, TU 2631-019-44493179-98), acetone (≥99%, TU 2633-018-44493179-98), propanol-2 (≥99%, TU 2632-181-44493179-14), all from EKOS-1 (Moscow, Russia); diethyl ether (≥99%, TU 2600-001-45286126-11) from Medhimprom (Moscow, Russia); and methanol (≥99%, Russian GOST 6995-77) from Vekton (St. Petersburg, Russia). Other reagents used were as follows: glacial acetic acid (≥99%, Russian GOST 61-75), hydrochloric acid (≥99%, Russian GOST 3118-77), sodium chloride (≥99%, Russian GOST 4233-77), ammonium acetate (≥98.5%, Russian GOST 3117-78), sodium hydroxide (≥99%, Russian GOST 4328-77), all from Reachem (Moscow, Russia); and anhydrous magnesium sulfate (≥99.0%, Bioshop, Canada), purchased commercially and used without further purification. Tetrahydrofuran (99.8%, Tathimprodukt, Kazan, Russia) was dried and purified using standard procedures.[32] Flash chromatography was carried out using high-purity grade Merck silica gel (9385, 60 Å, 230−400 mesh). The analytical standard of 25H-NBF hydrochloride was from Chiron AS (Trondheim, Norway). Poly-D-lysine hydrobromide and the analytical standard of LSD were from Sigma-Aldrich (Overijse, Belgium). Dulbecco's modified Eagle's medium (DMEM, GlutaMAX), fetal bovine serum (FBS), Hank's balanced salt solution (HBSS), penicillin/streptomycin, and amphotericin B were purchased from Thermo Fisher Scientific (Pittsburgh, PA). Nano-Glo Live Cell Reagent was from Promega (Madison, WI).

Instrumentation. *NMR Spectroscopy.* ^{1}H, ^{13}C, and ^{19}F NMR spectra were acquired in CD_3OD at 400, 101, and 376 MHz, respectively, using a Bruker Avance II spectrometer (Switzerland). For ^{1}H and ^{13}C spectra, signals of residual protons and carbons from the solvent were used as internal standards. To record the ^{19}F spectra, trichlorofluoromethane was used as an internal standard. For NMR spectroscopy, CD_3OD of isotopic purity ≥ 99.5% (Russian TU 95-669-79, FSUE RSC "Applied Chemistry," St. Petersburg, Russia) was used.

Melting Point. The melting points of compounds **2**−**6** were obtained using a Stuart SMP 30 (Bibby Scientific, Ltd., UK) digital melting temperature measuring instrument at a rate of 1 °C/min.

Gas Chromatography−Mass Spectrometry (GC−MS). GC−MS analysis was performed using an Agilent 7890B/5977A system (Agilent Technologies, USA) with a HP-5 ms (30.0 m × 0.25 mm × 0.25 μm; 19091S-433; Agilent) capillary column. The oven temperature was maintained at 70 °C for 1.0 min and then programmed at 15 °C/min to 295 °C, which was maintained for 15 min. The injector temperature and the interface temperature were 280 and 290 °C, respectively. Helium with flow rate of 1.0 mL/min was used as the carrier gas.

Ultrahigh-Performance Liquid Chromatography−High Resolution Mass Spectrometry (UHPLC−HRMS). High resolution mass spectra were recorded using an Agilent 1290 Infinity II UHPLC system with a tandem quadrupole time-of-flight (Q-TOF) accurate mass detector Agilent 6545 Q-TOF LC−MS (Agilent Technologies, USA). The Q-TOF instrument was operated with an electrospray ion source in positive ion mode. The mobile phase was prepared from 0.1% aqueous formic acid (solvent A) and 0.1% formic acid in acetonitrile (solvent B). A gradient of 5% solvent B to 100% solvent B

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at 5 min, with a flow rate of 0.4 mL/min, was employed. UHPLC analysis was performed on a Zorbax Eclipse Plus C18 RRHD (2.1 mm × 50 mm × 1.8 μm) column with additional 5 mm guard column. Acetonitrile (HPLC-gradient grade, Panreac, Barcelona, Spain), water (for GC, HPLC, and spectrophotometry grade, Honeywell, Burdick, and Jackson, Muskegon, MI) and formic acid (≥98.0%) from Sigma-Aldrich (Steinheim, Germany) were used for UHPLC-HRMS.

Synthetic Procedures. *General Procedure for the Synthesis of 2-Dimethoxyphenyl-N-(2-fluorobenzyl)ethanamines (**2**–**6**).* To a solution of substituted phenethylamine **9a**–**e** (1.0 g, 5.52 mmol) in CH_2Cl_2 (40 mL) with cooling to 0 °C was added a solution of 2-fluorobenzaldehyde (0.788 g, 6.35 mmol) in CH_2Cl_2 (40 mL) dropwise over 2 h. Stirring with cooling was continued for another 2 h. The completeness of the reaction and identification of the formed imines **10a**–**e** were monitored using GC–MS. Then the solvent was evaporated, the residue was dissolved in MeOH abs. (80 mL), followed by the addition of $NaBH_4$ (0.839 g, 22.08 mmol) over 1 h. The reaction was quenched with distilled H_2O (20 mL), the organic phase was evaporated to dryness, and the pH of the residual solution was adjusted to 11 with 3 M aqueous NaOH. Products-bases **2**–**6** were extracted with CH_2Cl_2 (3 × 40 mL), the combined extracts were washed with distilled H_2O (2 × 30 mL) and brine (40 mL), dried over anhydrous $MgSO_4$, and evaporated. Then, with stirring, 5 mL of propan-2-ol saturated with 5 M hydrochloric acid was added dropwise. After filtering off, washing with diethyl ether, and drying, hydrochlorides **2**–**6** were obtained as white powders. Compounds **2**–**6** were purified by flash chromatography and extensively analytically characterized, with no detectable impurities present.

Analytical data. *23H-NBF Imine (**10a**).* MS (EI, 70 eV), *m*/*z* (%): 287 ($M^{+\bullet}$), 256 (100), 109 (60), 136 (51), 257 (20), 91 (12), 107 (8), 135 (8), 65 (6), 108 (6), 77 (5).

*24H-NBF Imine (**10b**).* MS (EI, 70 eV), *m*/*z* (%): 287 ($M^{+\bullet}$), 151 (100), 109 (28), 121 (28), 91 (12), 152 (10), 136 (9), 77 (8), 108 (7), 78 (7), 107 (7).

*26H-NBF Imine (**10c**).* MS (EI, 70 eV), *m*/*z* (%): 287 ($M^{+\bullet}$), 151 (100), 256 (78), 109 (62), 136 (43), 91 (39), 257 (15), 77 (11), 107 (11), 108 (11), 152 (11).

*34H-NBF Imine (**10d**).* MS (EI, 70 eV), *m*/*z* (%): 287 ($M^{+\bullet}$), 151 (100), 109 (51), 136 (35), 287 (18), 107 (15), 152 (10), 108 (8), 135 (7), 106 (6), 78 (6).

*35H-NBF Imine (**10e**).* MS (EI, 70 eV), *m*/*z* (%): 287 ($M^{+\bullet}$), 109 (100), 136 (94), 192 (30), 287 (29), 166 (17), 137 (11), 77 (10), 108 (10), 151 (9), 91 (9).

*2-(2,3-Dimethoxyphenyl)-N-(2-fluorobenzyl)ethanamine Hydrochloride (23H-NBF, (**2**)).* White powder, yield 87%; ^{1}H NMR (CD_3OD) *δ*, ppm: 3.05–3.14 (m, 2H), 3.23–3.31 (m, 2H), 3.85 (s, 3H), 3.86 (s, 3H), 4.36 (s, 2H), 6.87 (d, ^3J = 7.6 Hz, 1H), 6.97 (d, ^3J = 7.2 Hz), 7.05 (t, ^3J = 8.0 Hz, 1H), 7.21–7.34 (m, 2H), 7.48–7.56 (m, 1H); 7.61–7.68 (m, 1H); ^{13}C NMR (CD_3OD) *δ*, ppm: 28.19, 45.48 (d, $^3J_{C-F}$ = 4.0 Hz), 49.05, 56.35, 61.25, 113.38, 116.97 (d, $^2J_{C-F}$ = 21.5 Hz), 119.79 (d, $^2J_{C-F}$ = 14.9 Hz), 123.23, 125.64, 126.24 (d, $^3J_{C-F}$ = 3.7 Hz), 131.05, 133.30 (d, $^3J_{C-F}$ = 8.3 Hz), 133.48 (d, $^4J_{C-F}$ = 2.8 Hz), 148.66, 154.28, 162.71 (d, $^1J_{C-F}$ = 247.8 Hz); ^{19}F NMR (CD_3OD) *δ*, ppm: −117.96 (1F); HRMS (ESI, $[M + H]^+$), *m*/*z*: accurate mass 290.1552, exact mass 290.1551, Δ = −0.57 ppm, $C_{17}H_{20}FNO_2$; melting point 153–155 °C.

*2-(2,4-Dimethoxyphenyl)-N-(2-fluorobenzyl)ethanamine Hydrochloride (24H-NBF, (**3**)).* White powder, yield 72%; ^{1}H NMR (CD_3OD) *δ*, ppm: 2.96–3.04 (m, 2H), 3.19–3.28 (m, 2H), 3.80 (s, 3H), 3.83 (s, 3H), 4.33 (s, 2H), 6.50 (dd, ^3J = 8.2 Hz/^4J = 2.4 Hz, 1H), 6.56 (d, ^4J = 2.4 Hz, 1H), 7.13 (d, ^3J = 8.2 Hz, 1H), 7.22–7.35 (m, 2H), 7.48–7.56 (m, 1H), 7.62 (td, ^3J = 7.6 Hz/^4J = 1.6 Hz, 1H); ^{13}C NMR (CD_3OD) *δ*, ppm: 27.91, 45.45 (d, $^3J_{C-F}$ = 4.0 Hz), 48.67, 55.90, 55.93, 99.62, 105.96, 116.95 (d, $^2J_{C-F}$ = 21.6 Hz), 117.73, 119.85 (d, $^2J_{C-F}$ = 14.8 Hz), 126.21 (d, $^3J_{C-F}$ = 3.7 Hz), 132.04, 133.27 (d, $^3J_{C-F}$ = 8.4 Hz), 133.42 (d, $^4J_{C-F}$ = 2.8 Hz), 159.90, 162.13, 162.72 (d, $^1J_{C-F}$ = 247.8 Hz); ^{19}F NMR (CD_3OD) *δ*, ppm: −118.31 (1F); HRMS (ESI, $[M + H]^+$), *m*/*z*: accurate mass 290.1553, exact mass 290.1551, Δ = −0.79 ppm, $C_{17}H_{20}FNO_2$; melting point 123–124 °C.

*2-(2,6-Dimethoxyphenyl)-N-(2-fluorobenzyl)ethanamine Hydrochloride (26H-NBF, (**4**)).* White powder, yield 83%; ^{1}H NMR (CD_3OD) *δ*, ppm: 3.06–3.22 (m, 4H), 3.85 (s, 6H), 4.34 (s, 2H), 6.67 (d, ^3J = 8.4 Hz, 2H), 7.19–7.35 (m, 3H), 7.48–7.62 (m, 2H); ^{13}C NMR (CD_3OD) *δ*, ppm: 20.85, 45.38 (d, $^3J_{C-F}$ = 3.9 Hz), 47.76; 56.26 (2C), 104.95 (2C), 113.07, 116.96 (d, $^2J_{C-F}$ = 21.5 Hz), 119.85 (d, $^2J_{C-F}$ = 14.7 Hz), 126.20 (d, $^3J_{C-F}$ = 3.7 Hz), 130.01, 133.20 (d, $^3J_{C-F}$ = 8.4 Hz), 133.28 (d, $^4J_{C-F}$ = 2.8 Hz), 159.75 (2C), 162.72 (d, $^1J_{C-F}$ = 247.6 Hz); ^{19}F NMR (CD_3OD) *δ*, ppm: −118.16 (1F); HRMS (ESI, $[M + H]^+$), *m*/*z*: accurate mass 290.1555, exact mass 290.1551, Δ = −1.27 ppm, $C_{17}H_{20}FNO_2$; melting point 118–120 °C.

*2-(3,4-Dimethoxyphenyl)-N-(2-fluorobenzyl)ethanamine Hydrochloride (34H-NBF, (**5**)).* White powder, yield 82%; ^{1}H NMR (CD_3OD) *δ*, ppm: 2.99–3.09 (m, 2H), 3.28–3.38 (m, 2H), 3.82 (s, 3H), 3.85 (s, 3H), 4.35 (s, 2H), 6.86 (d, ^3J = 8.0 Hz, 1H), 6.89–6.97 (m, 2H), 7.22–7.34 (m, 2H), 7.48–7.57 (m, 1H), 7.65 (td, ^3J = 7.6 Hz/^4J = 1.6 Hz, 1H); ^{13}C NMR (CD_3OD) *δ*, ppm: 32.76, 45.59 (d, $^3J_{C-F}$ = 4.0 Hz), 50.13; 56.60 (2C), 113.50, 113.80, 116.96 (d, $^2J_{C-F}$ = 21.5 Hz), 119.84 (d, $^2J_{C-F}$ = 14.8 Hz), 122.25, 126.23 (d, $^3J_{C-F}$ = 3.7 Hz), 130.50, 133.32 (d, $^3J_{C-F}$ = 8.4 Hz), 133.50 (d, $^4J_{C-F}$ = 2.7 Hz), 149.79, 150.84, 162.71 (d, $^1J_{C-F}$ = 247.7 Hz); ^{19}F NMR (CD_3OD) *δ*, ppm: −118.04 (1F); HRMS (ESI, $[M + H]^+$), *m*/*z*: accurate mass 290.1553, exact mass 290.1551, Δ = −0.87 ppm, $C_{17}H_{20}FNO_2$; melting point 163–165 °C.

*2-(3,5-Dimethoxyphenyl)-N-(2-fluorobenzyl)ethanamine Hydrochloride (35H-NBF, (**6**)).* White powder, yield 77%; ^{1}H NMR (CD_3OD) *δ*, ppm: 2.98–3.06 (m, 2H), 3.29–3.36 (m, 2H), 3.78 (s, 6H), 4.34 (s, 2H), 6.40 (t, ^3J = 2.2 Hz, 1H), 6.48 (d, ^3J = 2.2 Hz, 2H), 7.22–7.34 (m, 2H), 7.49–7.56 (m, 1H), 7.64 (td, ^3J = 7.6 Hz/^4J = 1.6 Hz, 1H); ^{13}C NMR (CD_3OD) *δ*, ppm: 33.40, 45.60 (d, $^3J_{C-F}$ = 4.0 Hz), 49.82; 56.87 (2C), 100.11, 107.85 (2C), 116.97 (d, $^2J_{C-F}$ = 21.4 Hz), 119.79 (d, $^2J_{C-F}$ = 14.8 Hz), 126.23 (d, $^3J_{C-F}$ = 3.7 Hz), 133.33 (d, $^3J_{C-F}$ = 8.4 Hz), 133.49 (d, $^4J_{C-F}$ = 2.8 Hz), 139.96, 162.71 (d, $^1J_{C-F}$ = 247.8 Hz), 162.80 (2C); ^{19}F NMR (CD_3OD) *δ*, ppm: −118.14 (1F); HRMS (ESI, $[M + H]^+$), *m*/*z*: accurate mass 290.1553, exact mass 290.1551, Δ = −0.88 ppm, $C_{17}H_{20}FNO_2$; melting point 126–128 °C.

Routine Cell Culture and NanoBiT βarr2 Recruitment Assay. The generation of human embryonic kidney (HEK) 293T cells that stably express the components of the NanoBiT functional complementation assay has been previously described.[19] The cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM, GlutaMAX) supplemented with 10% heat-inactivated FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B and maintained in a humidified atmosphere at 37 °C and 5% CO_2. For the implementation of the *in vitro* functional assay, the cells were seeded in poly-D-lysine coated 96-well plates and incubated overnight. The cells were then rinsed twice with HBSS, and 100 μL of HBSS was added to each of the wells, followed by the addition of 25 μL of the Nano-Glo Live Cell Reagent (diluted 1/20 in the provided LCS buffer, according to the manufacturer's protocol) and equilibration in the Tristar^2 LB 942 multimode microplate reader (Berthold Technologies GmbH & Co, Germany). After stabilization of the luminescent signal, a series of 13.5× concentrated agonist dilutions was added to the wells, followed by a continuous readout for 2 h. The measured concentrations were (100 μM) − 25 μM − 10 μM − 1 μM − 10^{-7} M − 10^{-8} M − 10^{-9} M − (10^{-10} M). In total, three independent experiments were carried out, each performed in duplicate, including LSD as a reference agonist for the purpose of normalization and the appropriate solvent controls for blank correction.

Data Analysis. The data were analyzed as previously described in more detail.[33] In brief, the obtained activation profiles are plotted, data are corrected for interwell variability, and the corresponding AUC (area under the curve) values are calculated. After subtraction of the AUC value of the corresponding solvent control ("blank"), the data are normalized in GraphPad (San Diego, CA) and sigmoidal curves are fit through the four-parameter nonlinear regression model. The data of the three independent experiments are then used to calculate potency (EC_{50}) and efficacy (E_{max}) parameters.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschemneuro.1c00124.

EI spectra of imine intermediates, the ^{1}H, ^{13}C, ^{19}F NMR spectra, data of elemental analysis and LC-MS chromatograms for the final synthesized compounds (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) UNODC (2020) *Current NPS Threats.*

(2) Nichols, D. E. (2004) Hallucinogens. *Pharmacol. Ther. 101*, 131−181.

(3) Poulie, C. B. M., Jensen, A. A., Halberstadt, A. L., and Kristensen, J. L. (2020) DARK Classics in Chemical Neuroscience: NBOMes. *ACS Chem. Neurosci. 11*, 3860.

(4) Hondebrink, L., Zwartsen, A., and Westerink, R. H. S. (2018) Effect Fingerprinting of New Psychoactive Substances (NPS): What can we Learn from In Vitro Data? *Pharmacol. Ther. 182*, 193−224.

(5) Bogenschutz, M. P., and Ross, S. (2016) Therapeutic Applications of Classic Hallucinogens. *Curr. Top. Behav. Neurosci. 36*, 361−391.

(6) Davis, A. K., Barrett, F. S., May, D. G., Cosimano, M. P., Sepeda, N. D., Johnson, M. W., Finan, P. H., and Griffiths, R. R. (2020) Effects of Psilocybin-Assisted Therapy on Major Depressive Disorder: A Randomized Clinical Trial. *JAMA psychiatry*, DOI: 10.1001/jamapsychiatry.2020.3285.

(7) Nichols, D. E. (2017) Chemistry and Structure-Activity Relationships of Psychedelics. *Curr. Top. Behav. Neurosci. 36*, 1−43.

(8) Jacobsen, S. C., Speth, N. R., Xiong, M., Herth, M. M., Kristensen, J. L., Palner, M., and Janfelt, C. (2021) Desorption Electrospray Ionization Mass Spectrometry Imaging of Cimbi-36, a 5-HT2A Receptor Agonist, with Direct Comparison to Autoradiography and Positron Emission Tomography. *Mol. Imaging Biol.*, DOI: 10.1007/s11307-021-01592-2.

(9) EMCDDA (2004) *Report on the risk assessment of 2C-I, 2C-T-2 and 2C-T-7 in the framework of the joint action on new synthetic drugs.*

(10) EMCDDA-Europol (2014) *EMCDDA−Europol Joint Report on a New Psychoactive Substance 4-Iodo-2,5-Dimethoxy-N-(2-Methoxybenzyl)phenethylamine (25I-NBOMe).*

(11) Kikura-Hanajiri, R. In *Neuropathology of Drug Addictions and Substance Misuse*, Preedy, V. R., Ed.; Academic Press, 2016; pp 1055−1065.

(12) *Tripsit.* www.tripsit.me (accessed February 2021).

(13) Swedish Government (2015) Ordinance amending the Ordinance (1992:1554) on the control of narcotic drugs. https://ec.europa.eu/growth/tools-databases/tris/nl/index.cfm/search/?trisaction=search.detail&year=2015&num=686&mLang=EN (accessed February 2021).

(14) UK Statutory Instruments (2014) *The Misuse of Drugs Act 1971 (Ketamine etc.) (Amendment) Order 2014.* https://www.legislation.gov.uk/uksi/2014/1106/made (accessed February 2021).

(15) Hur, K. H., Kim, S. E., Lee, B. R., Ko, Y. H., Seo, J. Y., Kim, S. K., Ma, S. X., Kim, Y. J., Jeong, Y., Pham, D. T., Trinh, Q. D., Shin, E. J., Kim, H. C., Lee, Y. S., Lee, S. Y., and Jang, C. G. (2020) 25C-NBF, a New Psychoactive Substance, has Addictive and Neurotoxic Potential in Rodents. *Arch. Toxicol. 94*, 2505−2516.

(16) De La Fuente, R., Sonawane, N. D., Arumainayagam, D., and Verkman, A. S. (2006) Small Molecules with Antimicrobial Activity against E. coli and P. aeruginosa Identified by High-Throughput Screening. *Br. J. Pharmacol. 149*, 551−559.

(17) Guy, M., Freeman, S., Alder, J., and Brandt, S. (2008) The Henry Reaction: Spectroscopic Studies of Nitrile and Hydroxylamine By-Products Formed during Synthesis of Psychoactive Phenylalkylamines. *Cent. Eur. J. Chem. 6*, 526−534.

(18) Kupriyanova, O. V., Shevyrin, V. A., Shafran, Y. M., Lebedev, A. T., Milyukov, V. A., and Rusinov, V. L. (2020) Synthesis and Determination of Analytical Characteristics and Differentiation of Positional Isomers in the Series of N-(2-Methoxybenzyl)-2-(Dimethoxyphenyl)ethanamine using Chromatography-Mass Spectrometry. *Drug Test. Anal. 12*, 1154−1170.

(19) Pottie, E., Cannaert, A., and Stove, C. P. (2020) In Vitro Structure-activity Relationship Determination of 30 Psychedelic New Psychoactive Substances by means of Beta-arrestin 2 Recruitment to the Serotonin 2A Receptor. *Arch. Toxicol. 94*, 3449.

(20) Dixon, A. S., Schwinn, M. K., Hall, M. P., Zimmerman, K., Otto, P., Lubben, T. H., Butler, B. L., Binkowski, B. F., Machleidt, T., Kirkland, T. A., Wood, M. G., Eggers, C. T., Encell, L. P., and Wood, K. V. (2016) NanoLuc Complementation Reporter Optimized for Accurate Measurement of Protein Interactions in Cells. *ACS Chem. Biol. 11*, 400−408.

(21) Pottie, E., Cannaert, A., Van Uytfanghe, K., and Stove, C. P. (2019) Setup of a Serotonin 2A Receptor (5-HT2AR) Bioassay: Demonstration of Its Applicability To Functionally Characterize Hallucinogenic New Psychoactive Substances and an Explanation Why 5-HT2AR Bioassays Are Not Suited for Universal Activity-Based Screening of Biofluids for New Psychoactive Substances. *Anal. Chem. 91*, 15444−15452.

(22) Pottie, E., Dedecker, P., and Stove, C. P. (2020) Identification of Psychedelic New Psychoactive Substances (NPS) showing Biased Agonism at the 5-HT2AR through Simultaneous Use of Beta-arrestin 2 and MiniGalphaq Bioassays. *Biochem. Pharmacol. 182*, 114251.

(23) Pottie, E., Kupriyanova, O. V., Brandt, A. L., Laprairie, R. B., Shevyrin, V. A., and Stove, C. P. (2021) Serotonin 2A Receptor (5-

HT2AR) Activation by 25H-NBOMe Positional Isomers: In Vitro Functional Evaluation and Molecular Docking. *ACS Pharmacol. Transl. Sci. 4*, 479.
(24) Shulgin, A., and Shulgin, A. (1991) *PiHKAL: A Chemical Love Story*, Transform Press, Berkeley, CA.
(25) Marcher-Rorsted, E., Halberstadt, A. L., Klein, A. K., Chatha, M., Jademyr, S., Jensen, A. A., and Kristensen, J. L. (2020) Investigation of the 2,5-Dimethoxy Motif in Phenethylamine Serotonin 2A Receptor Agonists. *ACS Chem. Neurosci. 11*, 1238−1244.
(26) Kim, K., Che, T., Panova, O., DiBerto, J. F., Lyu, J., Krumm, B. E., Wacker, D., Robertson, M. J., Seven, A. B., Nichols, D. E., Shoichet, B. K., Skiniotis, G., and Roth, B. L. (2020) Structure of a Hallucinogen-Activated Gq-Coupled 5-HT2A Serotonin Receptor. *Cell 182*, 1574−1588.e19.
(27) Braden, M. R., Parrish, J. C., Naylor, J. C., and Nichols, D. E. (2006) Molecular Interaction of Serotonin 5-HT2A Receptor Residues Phe339(6.51) and Phe340(6.52) with Superpotent N-Benzyl Phenethylamine Agonists. *Mol. Pharmacol. 70*, 1956−1964.
(28) Hansen, M., Phonekeo, K., Paine, J. S., Leth-Petersen, S., Begtrup, M., Brauner-Osborne, H., and Kristensen, J. L. (2014) Synthesis and Structure-Activity Relationships of N-Benzyl Phenethylamines as 5-HT2A/2C Agonists. *ACS Chem. Neurosci. 5*, 243−249.
(29) Jensen, A. A., McCorvy, J. D., Leth-Petersen, S., Bundgaard, C., Liebscher, G., Kenakin, T. P., Brauner-Osborne, H., Kehler, J., and Kristensen, J. L. (2017) Detailed Characterization of the In Vitro Pharmacological and Pharmacokinetic Properties of N-(2-Hydroxybenzyl)-2,5-Dimethoxy-4-Cyanophenylethylamine (25CN-NBOH), a Highly Selective and Brain-Penetrant 5-HT2A Receptor Agonist. *J. Pharmacol. Exp. Ther. 361*, 441−453.
(30) Kim, J. H., Kim, S., Lee, J., In, S., Cho, Y. Y., Kang, H. C., Lee, J. Y., and Lee, H. S. (2019) In Vitro Metabolism of 25B-NBF, 2-(4-Bromo-2,5-Dimethoxyphenyl)-N-(2-Fluorobenzyl)ethanamine, in Human Hepatocytes Using Liquid Chromatography(−)Mass Spectrometry. *Molecules (Basel, Switzerland) 24*, 818.
(31) Canal, C. E. (2018) Serotonergic Psychedelics: Experimental Approaches for Assessing Mechanisms of Action. *Handb. Exp. Pharmacol. 252*, 227−260.
(32) Simas, A. B. C., Pereira, V. L. P., Barreto, C. B., Jr, Sales, D. L. d., and Carvalho, L. L. d. (2009) An Expeditious and Consistent Procedure for Tetrahydrofuran (THF) Drying and Deoxygenation by the Still Apparatus. *Quim. Nova 32*, 2473−2475.
(33) Pottie, E., Tosh, D. K., Gao, Z. G., Jacobson, K. A., and Stove, C. P. (2020) Assessment of Biased Agonism at the A3 Adenosine Receptor using Beta-arrestin and MiniGalphai Recruitment Assays. *Biochem. Pharmacol. 177*, 113934.

https://doi.org/10.1021/acschemneuro.1c00124
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