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Article

Discovery of G Protein-Biased Ligands against 5-HT$_7$R

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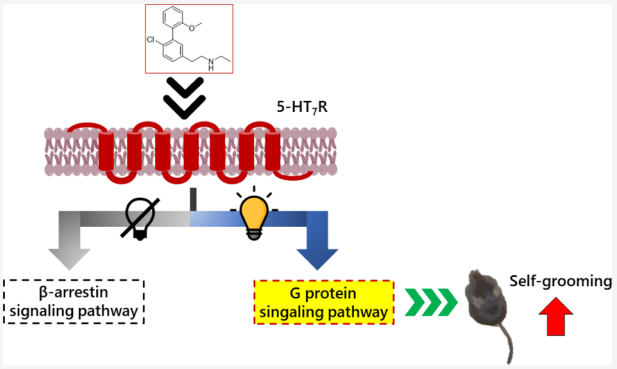
ABSTRACT: There has been significant attention concerning the biased agonism of G protein-coupled receptors (GPCRs), and it has resulted in various pharmacological benefits. 5-HT$_7$R belongs to a GPCR, and it is a promising pharmaceutical target for the treatment of neurodevelopmental and neuropsychiatric disorders. Based on our previous research, we synthesized a series of 6-chloro-2′-methoxy biphenyl derivatives **1**, **2**, and **3** with a variety of amine scaffolds. These compounds were evaluated for their binding affinities to 5-HTR subtypes and their functional selectivity toward the Gs protein and the β-arrestin signaling pathways of 5-HT$_7$R. Among them, 2-(6-chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)-*N*-ethylethan-1-amine, **2b**, was found to be a G-protein-biased ligand of 5-HT$_7$R. In an *in vivo* study with *Shank3* transgenic mice, the self-grooming behavior test was performed with **2b**, which increased the duration of self-grooming. The experiments further suggested that 5-HT$_7$R is associated with autism spectrum disorders (ASDs) and could be a therapeutic target for the treatment of stereotypy in ASDs.



INTRODUCTION

G protein-coupled receptors (GPCRs) are a family of cell surface receptors with seven highly conserved, transmembrane helical proteins, and there are over 800 individual genes encoded in the human genome.[1−3] These receptors are expressed extensively in various tissues, and they participate in multiple physiological signal transductions through interactions with the heterotrimeric G proteins that transfer the signals to canonical transducer proteins, arrestins, kinases, ion channels, and scaffolding proteins. Thus, they have been the pharmaceutical targets of various drugs.[4−7] Canonical GPCR signaling is mediated via coupling to intracellular transducers, that is, G proteins, which are formed by G_α, G_β, and G_γ subunits.[8] A ligand binds to a GPCR and activates the G protein, which is followed by the activation of the downstream signaling known as the G protein signaling pathway. Then, GPCR recruits β-arrestin to block or desensitize the activated signal. In addition to the classical G protein signaling pathway mediated by G proteins, it has been suggested recently that β-arrestin is able to initiate G protein-independent cellular signals, including the activation of various MAPKs, such as ERK,[5,9] which is referred to as the β-arrestin signaling pathway. Thus, there have been active efforts to produce biased ligands in order to elucidate the complicated mechanism of GPCRs and to develop novel GPCR drugs with fewer side effects. These biased ligands can activate either the G protein or the β-arrestin signaling pathway selectively, thereby yielding the desired effects of drugs and blocking unwanted side effects due to the stimulation of other signaling pathways.[5,10]

There are 14 distinct serotonin receptors (5-HTRs) encoded in the human genome. Among the 5-HTR subtypes, the 5-HT$_7$ receptor (5-HT$_7$R) was the last to be identified, and it has been shown to be highly expressed in the central nervous system (CNS), for example, in the hypothalamus, hippocampus, and cortex. 5-HT$_7$R belongs to a family of GPCRs and binds positively to adenylate cyclase (AC) through the activation of Gs protein, resulting in an extracellular increase of cyclic adenosine monophosphate (cAMP), and it also displays a high constitutive AC activity.[11−17] On the basis of the distribution of 5-HT$_7$R in the CNS, it has been proposed that it is involved in various important functional roles, such as thermoregulation, circadian rhythm, sleep, learning and memory, autism, cognition, and schizophrenia.[18] In addition, numerous studies have suggested that the altered 5-HT system that includes abnormal levels of 5-HT, morphological changes in the serotonergic fibers, and decreased expression of 5-HTR might be a major marker of abnormalities in autism spectrum disorders (ASDs).[19,20] Recent reports have provided evidence that 5-HT$_7$R has an essential role in regulating severe behavioral symptoms, which are represented as autistic-like behaviors in animal models, such as

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the *Fmr1* KO mouse model of the fragile X syndrome and the *Mecp2* KO mouse model of the Rett syndrome. The 5-HT$_7$R agonist restored the long-term depression level in an *Fmr1* KO mouse to the level of wild type (WT), which is applicable to ASDs; also, the treatment of 5-HT$_7$R agonist improved the behavioral impairments and cognition in *Mecp2* KO mice.[21−24] However, no studies have been conducted in which the 5-HT$_7$R-biased ligand was used in testing autism behavior.

Although many potent agonists (E-55888,[25] AS-19,[26,27] and **1a**[28]) and antagonists (SB-269970[29]) against 5-HT$_7$R have been reported, they are considered to be agonists/antagonists that activate/inhibit the G protein signaling pathway, while no activation/inhibition of the β-arrestin signaling pathway other than SB-269970 has been reported[30] (Figure 1). Recently, Kim

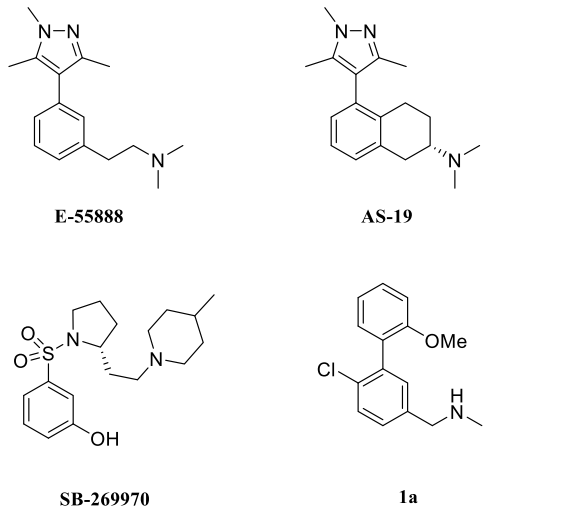

Figure 1. Structures of representative 5-HT$_7$R agonists (E-55888, AS-19, and **1a**) and antagonist (SB-269970).

et al.[30] have discovered an azepine derivative, that is, 3-(4-chlorophenyl)-1,4,5,6,7,8-hexahydropyrazolo[3,4-*d*]azepine, as a β-arrestin-biased ligand against 5-HT$_7$R (K_i 30 nM), IC$_{50}$ (G protein) 7800 nM, and EC$_{50}$ (β-arrestin) 162 nM, whereas, to date, no Gs protein-biased ligand of 5-HT$_7$R has been discovered. In this study, we designed and synthesized biphenyl derivatives **1**, **2**, and **3** (Figure 2), and **2b** was found to be a potent Gs protein-biased agonist of 5-HT$_7$R, and it was used in

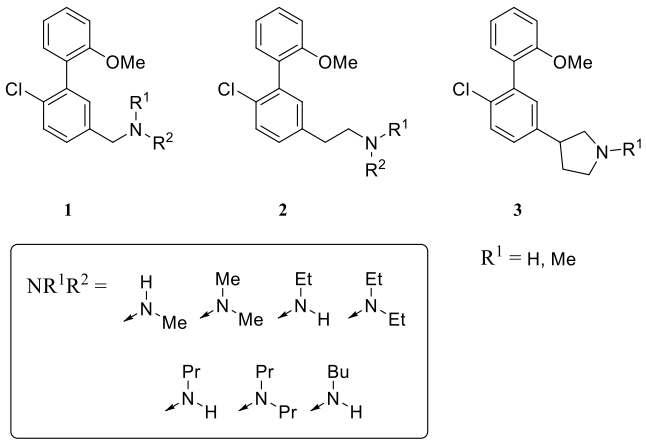

Figure 2. Structures of 6-chloro-2′-methoxy biphenyl derivatives **1**, **2**, and **3** under this study.

the self-grooming behavior test that was conducted with *Shank3* transgenic (TG) mice to investigate the association between 5-HT$_7$R and ASD.

RESULTS AND DISCUSSION

To discover Gs protein-biased ligands of 5-HT$_7$R, we referred three structurally similar agonists: well-known agonists AS-19 and E-55888, with a biaryl core and an ethyleneamine moiety, and **1a** with a biphenyl core and a methyleneamine moiety, which was identified in our previous work as a potent and selective agonist against 5-HT$_7$R[28] (Figure 1). According to our study of the structure−activity relationship (SAR), compounds with a 6-chloro-2′-methoxy biphenyl core showed good binding affinities, so this core structure was retained in this study. We planned to synthesize and biologically evaluate 6-chloro-2′-methoxy-[1,1′-biphenyl]-3-yl-*N*-alkylmethanamine derivatives **1**, 2-(6-chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)-*N*-alkylethan-1-amine derivatives **2** and 3-(6-chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)pyrrolidine derivatives **3** as shown in Figure 2, and the introduction of secondary/tertiary amines with alkyl chains and cyclic amines to the amine moiety followed by SAR study was our main concern.

Synthesis. 6-Chloro-2′-methoxy-[1,1′-biphenyl]-3-yl-*N*-alkylmethanamine derivatives **1** were synthesized in three steps starting from 3-chloro-4-iodobenzaldehyde **4** (Scheme 1), which is iodinated from 4-chlorobenzaldehyde **3** through electrophilic aromatic substitution of aldehyde by using I_2, $NaIO_3$, and sulfuric acid.[31,32] Suzuki cross-coupling reaction between **4** and 2-methoxyphenylboronic acid **5** provided biphenyl core **6** in 64−98% yields. This biphenyl benzaldehyde **6** was then converted to *N*-alkylamines **1a−1g** via reductive amination by the treatment of NaBH(OAc)$_3$ and various alkylamines in 16−93% yields.

With iodinated starting material **4**, the syntheses of 2-(6-chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)-*N*-alkylethan-1-amine derivatives **2** were accomplished efficiently, as shown in Scheme 2. One carbon homologation of the iodoaldehyde **4** was obtained by the introduction of cyanide and reduction. The iodobenzaldehyde **4** was reduced by $NaBH_4$, which is brominated, and this was followed by the S_N2 reaction with NaCN to provide an intermediate **9** with the cyanide group. Next, the Suzuki cross-coupling of **9** produced biphenyl cyanide **10**, which subsequently was reduced by $LiAlH_4$ and $AlCl_3$ to produce one-carbon-homologated ethyleneamine **11**. Preparation of dialkyl compounds was established by reductive amination using formaldehyde/formic acid or the S_N2 reaction using alkyliodide/K_2CO_3. Reductive amination was conducted to synthesize the dimethyl derivative **2e**, and the S_N2 reaction was performed to obtain diethyl compound **2f** and dipropyl compound **2g**. In order to obtain monoalkylated compounds, ethyleneamine **11** was protected by the Boc group to afford an intermediate **12**, which was treated with NaH and alkyliodide to provide Boc-protected monoalkyl compounds **13**. The use of 1 N HCl in diethyl ether to eliminate the protection of the Boc group allowed us to obtain the final monoalkylated compounds **2a−2d**.

We also synthesized 3-(6-chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)pyrrolidine derivatives **3a** and **3b**, as shown in Scheme 3. Biphenylaldehyde **6** underwent the nitroaldol reaction by treatment with nitromethane and ammonium acetate at 100 °C to afford a nitrovinyl derivative **14**. To incorporate the malonate ester into the nitrovinyl position by Michael addition, NaH and diethyl malonate were used to provide a malonated

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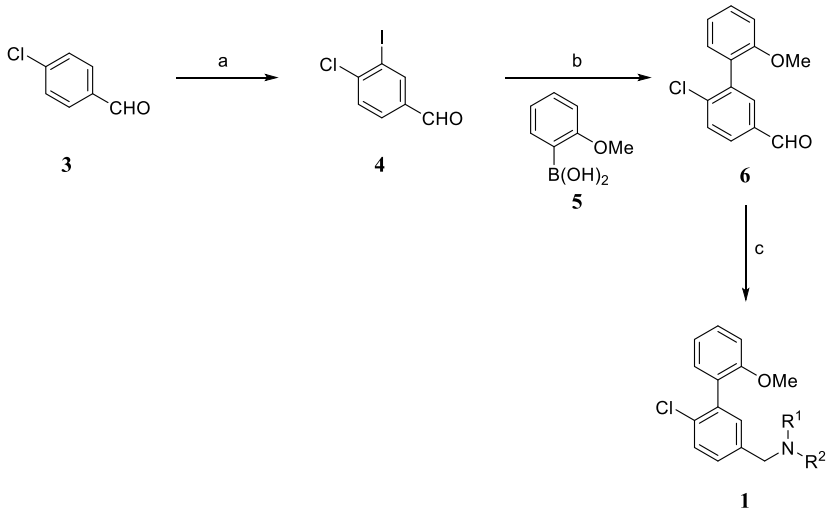
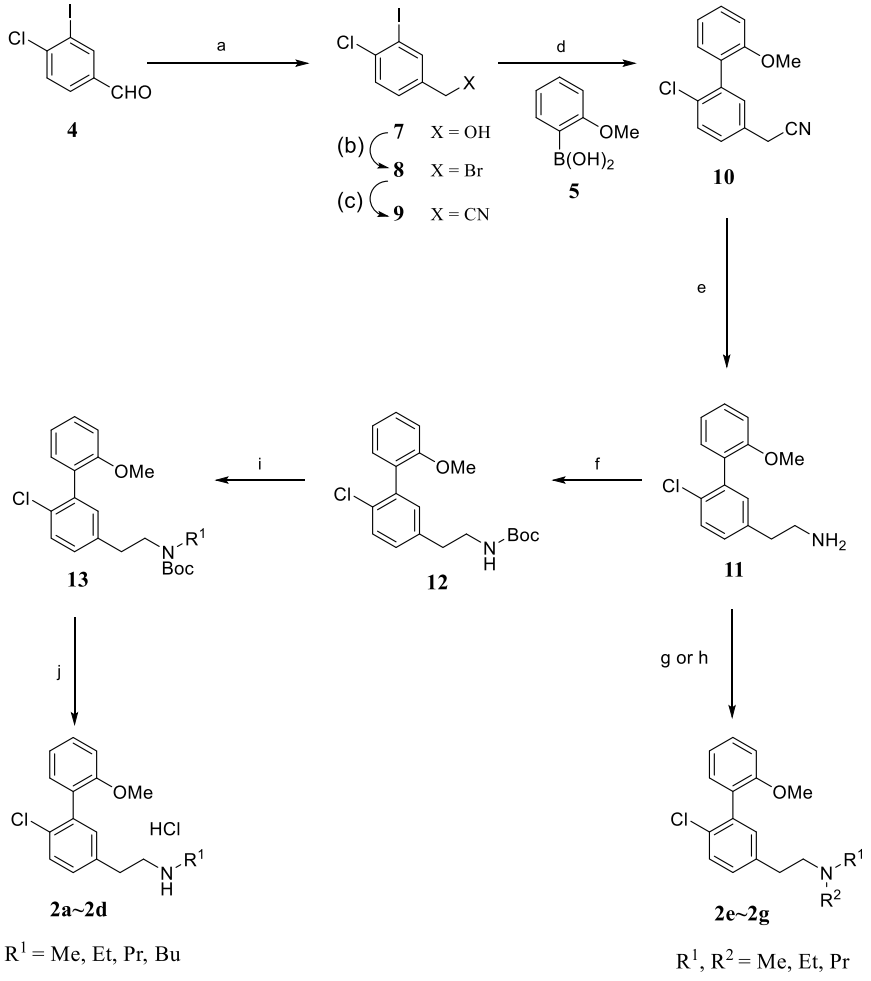
Scheme 1. Synthesis of Biphenyl-3-yl-methanamines 1[a]



[a]Reagents and conditions: (a) I_2, $NaIO_3$, H_2SO_4, rt, and 33−64%; (b) 2-methoxyphenylbononic acid **5**, $Pd(PPh_3)_4$, Na_2CO_3, *N*,*N*-dimethylformamide (DMF), reflux, and 64−98%; (c) HNR^1R^2, $NaBH(OAc)_3$, MeOH, rt, and 16−93%.

Scheme 2. Synthesis of Biphenyl-3-yl-methanamines 2[a]



[a]Reagents and conditions: (a) $NaBH_4$, MeOH, rt, and 88%; (b) Ph_3P, CBr_4, CH_2Cl_2, rt, and 93%; (c) NaCN, TBAB, toluene, 50 °C, and 77%; (d) 2-methoxyphenylboronic acid **5**, $Pd(PPh_3)_4$, Na_2CO_3, DMF, reflux, and 60−95%; (e) 1 M $LiAlH_4$ in THF, $AlCl_3$, THF, 0 °C to rt, and 43−69%; (f) Boc_2O, $NaHCO_3$, H_2O, THF, rt, and 77%; (g) HCOOH, HCOH, reflux, and 70%; (h) K_2CO_3, alkyliodide, MeCN, 65 °C, and 40−63%; (i) NaH, alkyliodide, DMF, 0 °C to rt, and 41−57%; and (j) 1 N HCl in diethyl ether, rt, and 80−95%.

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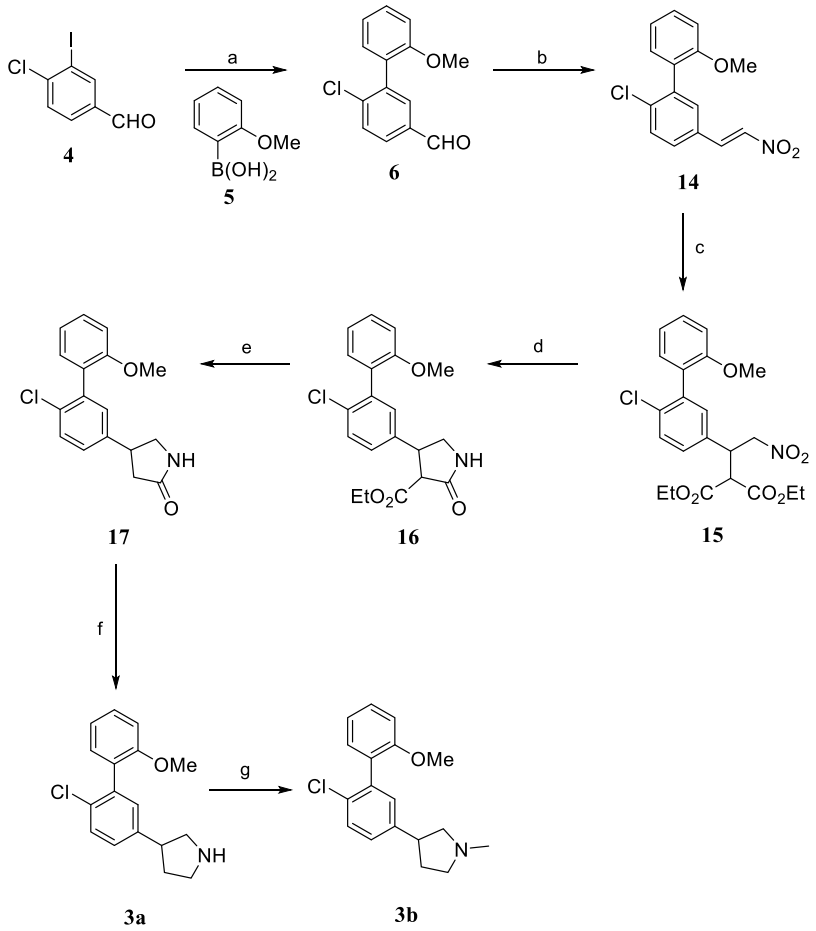
Scheme 3. Synthesis of Biphenyl-3-ylpyrrolidine Compounds 3[a]



[a]Reagents and conditions: (a) 2-methoxyphenylboronic acid **5**, $Pd(PPh_3)_4$, Na_2CO_3, DMF, reflux, and 60%−95%; (b) CH_3NO_2, NH_4OAc, 100 °C, and 66%; (c) diethylmalonate, NaH, THF, rt, and 48%; (d) $NiCl_2{\cdot}6H_2O$, $NaBH_4$, MeOH, 0 °C to rt, and 55%; (e) (i) NaOH and EtOH; (ii) toluene, reflux, and 67%; (f) 1 M $LiAlH_4$ in THF, THF, 0 °C to rt, and 73%; (g) HCOOH, HCOH, reflux, and 37%.

compound **15**, which was subjected to ring formation by using $NiCl_2{\cdot}6H_2O/NaBH_4$ to provide a carboxylated oxopyrrolidine **16**. The decarboxylation of **16** produced oxopyrrolidine **17**, which was subsequently reduced by $LiAlH_4$ to obtain a pyrrolidine compound **3a**. Synthesis of methylpyrrolidine **3b** was accomplished by reductive amination with formaldehyde/formic acid.

Binding Affinity and Functional Activity against 5-HT$_7$R. Binding affinities of **1**, **2**, and **3** for 5-HT$_7$R were determined by the [^{3}H] D-lysergic acid diethylamide ([^{3}H]LSD) radioligand binding assay in transfected HEK293 cells.[33] Table 1 shows the binding affinities (K_i) and functional activities in the Gs protein/β-arrestin signaling pathways against 5-HT$_7$R. Among the biphenyl derivatives **1** with the methyleneamine moiety, compounds **1a−c** (R^1 = Me, Et, and Pr, R^2 = H) showed binding affinities against 5-HT$_7$R with K_i values of 5.2, 9.3, and 6.8 nM, respectively. However, the longer carbon chain of the *N*-alkyl moiety, like a butyl chain, reduces the affinity for 5-HT$_7$R. Compound **1d** with the monobutyl amine moiety (R^1 = Bu, R^2 = H) had a K_i value of 52 nM. Dimethyl derivative **1e** (R^1 = Me, R^2 = Me) showed binding affinity with a K_i value of 18 nM, while the diethyl and dipropyl compounds **1f** and **1g** had decreased affinity (K_i = 110 nM) or no activity up to 10 μM, respectively. Interestingly, compared with the binding affinities of compounds **1**, one-carbon-elongated compounds **2** mostly maintained potency of binding affinities to 5-HT$_7$R. Compounds **2a** and **2b** (R^1 = Me and Et) showed binding affinity with K_i values of 1.6 and 2.8 nM for 5-HT$_7$R, respectively, comparable to those of **1a** and **1b**. Compounds **2c** and **2d** (R^1 = Pr and Bu) had almost the same binding affinities with K_i values of 8.7 and 64 nM in comparison to the corresponding compounds **1c** and **1d**, while dialkyl compounds **2e−g** (R^1, R^2 = Me, Et, and Pr) showed at least 4 times better binding affinities with K_i values of 2.5, 25, and 20 nM, respectively, than the corresponding compounds **1e−g**. Compounds **3a** and **3b** (R^1 = H and Me) with a pyrrolidine moiety showed binding affinities with K_i values of 2.8 and 30 nM, respectively. Among the synthesized compounds, compound **2a** had the best binding affinity value (K_i = 1.6 nM), which was comparable to that of E-55888 (K_i = 1.3 nM).

To investigate the functional activities of compounds on the Gs protein and β-arrestin signaling pathways, we conducted two types of cell-based functional assays, that is, bioluminescence-based assays to measure Gs-mediated cAMP production (cAMP assay[34,35]) and β-arrestin recruitment (Tango assay[36,37]) with synthesized compounds **1**, **2**, and **3**, and an endogenous agonist 5-HT and a 5-HT$_7$R agonist E-55888 were used as reference compounds. All compounds were evaluated by both cAMP assay and Tango assay. The cAMP assay was done in transiently transfected HEK293 cells with 5-HT$_7$R plasmid/GloSensor plasmid, while, in the Tango assay, the HEK293-derived cell line

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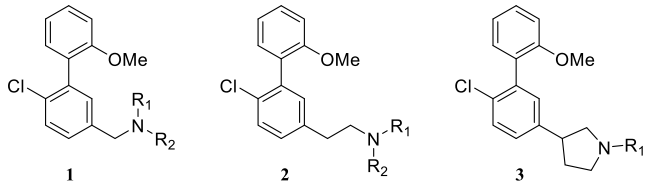
Table 1. Results of Biological Activity Assays of Compounds 1, 2, and 3 against 5-HT$_7$R



			binding affinity[a]		cAMP production			β-arrestin recruitment		
compound	R^1	R^2	pK_i[b]	K_i (nM)	pEC$_{50}$[b]	EC$_{50}$ (nM)	E_{max} (%)	pEC$_{50}$[b]	EC$_{50}$ (nM)	E_{max} (%)
1a[28]	Me	H	8.28 ± 0.07	5.2	6.34 ± 0.04	460	72	6.83 ± 0.08	150	61
1b	Et	H	8.00 ± 0.20	9.3	6.12 ± 0.11	770	71	[e]	[e]	[e]
1c	Pr	H	8.20 ± 0.10	6.8	5.93 ± 0.14	1200	68	[e]	[e]	[e]
1d	Bu	H	7.28 ± 0.06	52	[e]	[e]	[e]	[e]	[e]	[e]
1e	Me	Me	7.70 ± 0.10	18	5.94 ± 0.09	1100	11	6.32 ± 0.13	480	43
1f	Et	Et	7.00 ± 0.10	110	[e]	[e]	[e]	[e]	[e]	[e]
1g	Pr	Pr	[c]	[c]	[e]	[e]	[e]	[e]	[e]	[e]
2a	Me	H	8.55 ± 0.10	1.6	7.14 ± 0.12	73	77	7.15 ± 0.12	71	67
2b	Et	H	8.80 ± 0.10	2.8	6.74 ± 0.04	180	91	[e]	[e]	[e]
2c	Pr	H	8.07 ± 0.12	8.7	5.98 ± 0.06	1000	53	[e]	[e]	[e]
2d	Bu	H	7.19 ± 0.11	64	[e]	[e]	[e]	[e]	[e]	[e]
2e	Me	Me	8.60 ± 0.09	2.5	7.08 ± 0.10	84	67	7.25 ± 0.18	56	53
2f	Et	Et	7.60 ± 0.12	25	5.73 ± 0.04	1900	76	[e]	[e]	[e]
2g	Pr	Pr	7.70 ± 0.12	20	5.86 ± 0.06	1400	72	[e]	[e]	[e]
3a	H		8.56 ± 0.13	2.8	7.04 ± 0.14	92	29	7.12 ± 0.09	75	54
3b	Me		7.52 ± 0.10	30	6.25 ± 0.24	560	9.8	6.19 ± 0.13	650	51
5-HT			[d]	[d]	8.03 ± 0.06	4.5	100	6.95 ± 0.06	110	100
E-55888			8.89 ± 0.05	1.3	7.08 ± 0.04	83	99	7.68 ± 0.08	21	54

[a]Binding affinity data were generously provided by the US National Institute of Mental Health (NIMH) Psychoactive Drug Screening Program (University of North Carolina). [b]Values are the mean ± SEM of at least three independent experiments performed in triplicate. [c]K_i > 10,000 nM. [d]Not determined. [e]No activity.

was used, and it contained stable interactions of a tTA-dependent luciferase reporter and a β-arrestin 2−TEV fusion gene (HTLA cells). Compounds **1a−c** showed potency and efficacy in the cAMP assay (EC$_{50}$ = 460, 770, and 1200 nM, respectively, and E_{max} = 72, 71, and 68%, respectively), while the E_{max} value of compound **1e** is relatively low with 11%, even though the EC$_{50}$ value of **1e** was 1100 nM. Compounds **1d**, **1f**, and **1g** show no functional activities in the cAMP assay. In β-arrestin recruitment Tango assay, only compounds **1a** and **1e** showed functional activities with EC$_{50}$ values of 150 and 480 nM, respectively. Compounds **1b** and **1c** had activities in cAMP production but no activities in β-arrestin recruitment, which can be called Gs protein-biased ligands. In the series of compounds **2**, compounds **2a** and **2b** had EC$_{50}$ values of 73 and 180 nM, which showed better activities than compounds **1a** and **1b** in the cAMP assay. Efficacy of **2b** in cAMP production is the best with an E_{max} value of 91% among the synthesized compounds. Compound **2c** showed marginal activity in cAMP production with an EC$_{50}$ value of 1000 nM and an E_{max} value of 53% and compound **2d** showed no activity. Compared with compounds **1e−g**, compounds **2e−g** showed improved activities in the cAMP assay (EC$_{50}$ = 84, 1900, and 1400 nM, E_{max} = 67, 76, and 72%). In β-arrestin recruitment Tango assay, only compounds **2a** and **2e** showed functional activities with EC$_{50}$ values of 71 and 56 nM. Therefore, compounds **2b**, **2c**, **2f**, and **2g** had activities in cAMP production but no activities in β-arrestin recruitment, which can also be called Gs protein-biased ligands. Compounds **3a** and **3b** displayed better activities in the Tango assay (EC$_{50}$ = 75 and 650 nM, E_{max} = 54 and 51%) than they did in the cAMP assay (EC$_{50}$ = 92 and 560 nM, E_{max} = 29 and 9.8%). There are three balanced agonists **1a**, **2a**, and **2e** like E-55888, among which **2a** is the best in the view of potency and efficacy in both assays, while there are six G protein-biased ligands **1b**, **1c**, **2b**, **2c**, **2f**, and **2g**, among which **2b** is the best in the cAMP assay (EC$_{50}$ = 180 nM, E_{max} = 91%) and **1b** is the next (EC$_{50}$ = 770 nM, E_{max} = 71%) (Figure 3).

Among 16 synthesized compounds, 15 compounds bound to 5-HT$_7$R with K_i values between 1.6 and 110 nM, and 12 compounds showed potency and efficacy in the Gs protein signaling pathway, while only six compounds were active in β-arrestin signaling pathway. Among those, **2a** is the best balanced agonist and **2b** is the best G protein-biased ligand.

Selectivity over Other 5-HTR Subtypes. We investigated the binding affinities of compounds **1**, **2**, and **3** for other serotonin subtype receptors, such as 5-HT$_{1A}$R, 5-HT$_{1B}$R, 5-HT$_{1D}$R, 5-HT$_{1E}$R, 5-HT$_{2A}$R, 5-HT$_{2B}$R, 5-HT$_{2C}$R, 5-HT$_3$R, 5-HT$_{5A}$R, and 5-HT$_6$R to determine their selectivity for 5-HT$_7$R. Compounds **1a−e** showed selectivity over 5-HT$_{1A}$R, 5-HT$_{1B}$R, 5-HT$_{1D}$R, 5-HT$_{1E}$R, 5-HT$_{2A}$R, 5-HT$_3$R, 5-HT$_{5A}$R, and 5-HT$_6$R, except 5-HT$_{2B}$R and some cases of 5-HT$_{2C}$R. Compounds **1a−e** had binding affinities for 5-HT$_{2B}$R with K_i values of 13, 19, 12, 15, and 11 nM, respectively, resulting in little selectivity of compounds **1a−e** over 5-HT$_{2B}$R. Compounds **2a** and **2b** had at least 19-fold selectivity over other 5-HTR subtypes except 5-HT$_{1D}$R. Compounds **2d**, **2f**, and **2g** had almost no selectivity over 5-HT$_{1A}$R and 5-HT$_{1D}$R. It is very interesting that compounds **1** had little selectivity over 5-HT$_{2B}$R, while compounds **2** except **2d** had at least 12-fold selectivity over 5-HT$_{2B}$R, but little selectivity over 5-HT$_{1D}$R. Specially, compounds **2a** and **2b** showed more than 40-fold selectivity over 5-

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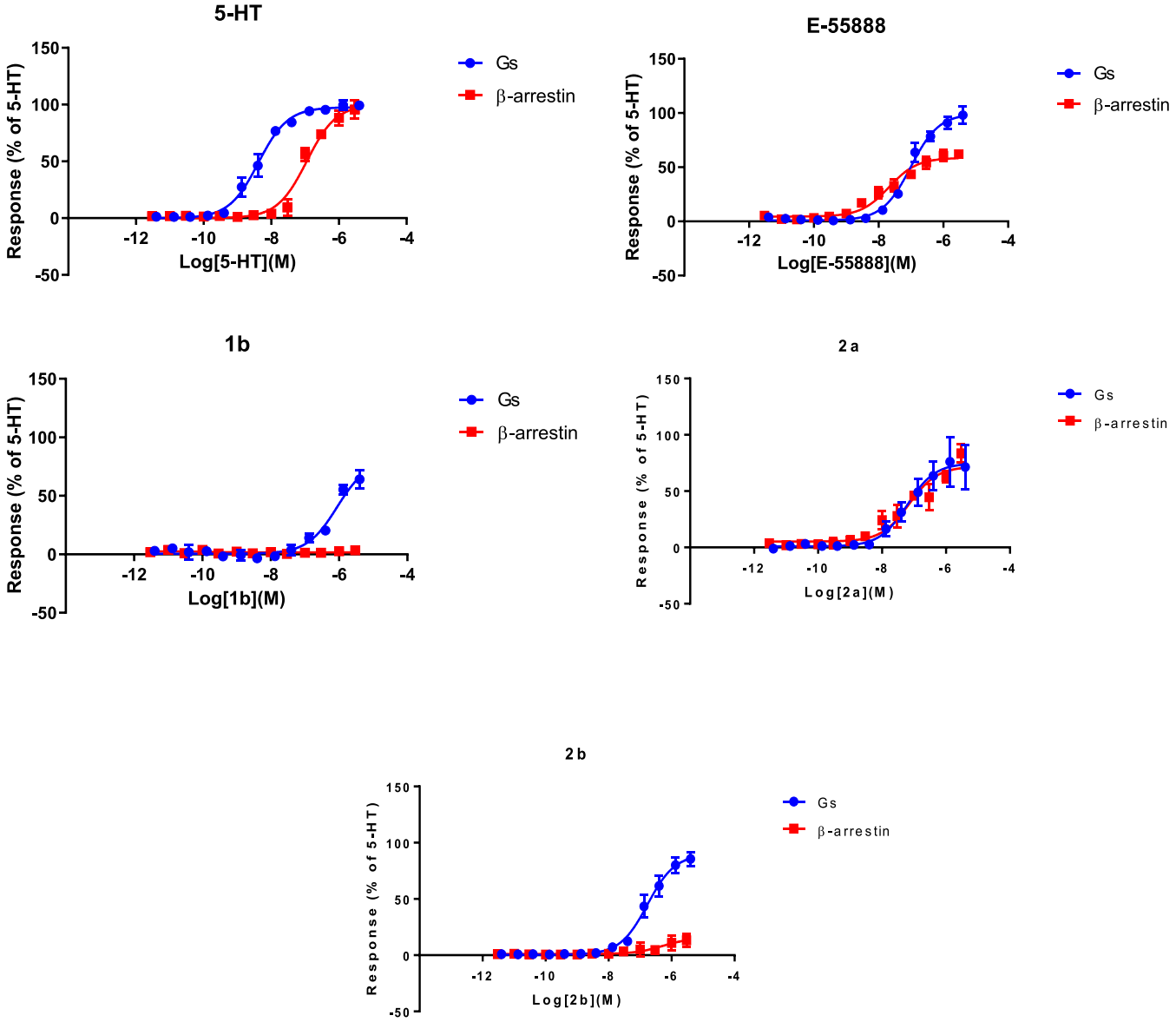

Figure 3. Dose−response curves of Gs protein activation assay (cAMP agonist assay) and *β*-arrestin recruitment assay (Tango agonist assay) of compounds 5-HT, E-55888, **1b**, **2a**, and **2b**.

$HT_{2B}R$. Compound **3a** indicated at least 9-fold selectivity over other 5-HTR subtypes, except for $5\text{-}HT_{1D}R$, while compound **3b** showed more than 30-fold selectivity over $5\text{-}HT_{1E}R$, $5\text{-}HT_{2A}R$, $5\text{-}HT_{2C}R$, $5\text{-}HT_{3}R$, and $5\text{-}HT_{5A}R$. It was reported that $5\text{-}HT_{2B}R$ was associated with cardiac valvulopathy, resulting in potential cardotoxicity,[38] while the $5\text{-}HT_{1D}R$ agonist, triptan, shows a pharmacological benefit in migraine pain.[39]

Functional Selectivity. Based on their pharmacological profiles (Table 1) and selectivity profiles (Table 2), we selected potent and functionally distinct ligands **1b**, **2a**, and **2b** to determine ligand bias toward the Gs protein signaling pathway or the *β*-arrestin signaling pathway compared with their functional selectivity profile of 5-HT and E-55888. The results indicated that E-55888 had almost full efficacy in the Gs protein pathway (EC_{50} = 83 nM, E_{max} = 99%) compared to 5-HT, while showed a partial agonistic effect in the *β*-arrestin pathway (EC_{50} = 21 nM, E_{max} = 54%). Since compound **2a** activated both the Gs protein pathway and the *β*-arrestin pathway, **2a** was shown to be a balanced agonist with good potency and efficacy (cAMP assay: EC_{50} = 73 nM, E_{max} = 77%, and Tango assay: EC_{50} = 71 nM, E_{max} = 67%). By contrast, compounds **1b** and **2b**, both of which have a mono-ethyl group as the alkyl substitution at the methylene-amine and ethyleneamine moieties, exhibited no activity in the *β*-arrestin pathway compared to 5-HT (100%) and E-55888(54%). Compound **2b** acted as almost full agonist in the Gs protein pathway with an E_{max} of 91%, while compound **1b** had a lower E_{max} value with 71% than compound **2b**.

Using 5-HT as a positive control and E-55888 as a reference ligand to evaluate our compounds, we found that compound **2a** with mono-methyl moiety acted as a balanced agonist, whereas compounds **1b** and **2b**, with mono-ethyl moiety, showed functional selectivity with preference to the production of Gs protein-mediated cAMP, which means the two compounds, that is, **1b** and **2b**, are Gs protein-biased ligands. Based on the potency and efficacy, **2b** exhibited a better Gs protein-biased ligand, that is, $5\text{-}HT_{7}R$, than compound **1b**. It could be suggested that alkyl substitution is important to selectively activate the Gs protein signaling pathway, and in particular, ethyl

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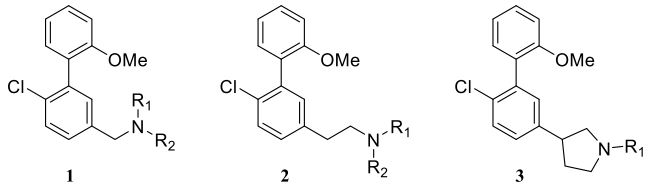
Table 2. Binding Affinities (K_i) of Compounds 1, 2, and 3 against 5-HTRs



			K_i in nM[a,b]										
compounds	R^1	R^2	5-HT_7	5-HT_{1A}	5-HT_{1B}	5-HT_{1D}	5-HT_{1E}	5-HT_{2A}	5-HT_{2B}	5-HT_{2C}	5-HT_3	5-HT_{5A}	5-HT_6
1a[28]	Me	H	5.2	1700	1200	1200	2400	380	13	200	[c]	420	1300
1b	Et	H	9.3	840	210	220	[c]	910	19	45	[c]	1800	1400
1c	Pr	H	6.8	420	640	220	[c]	670	12	200	[c]	120	350
1d	Bu	H	52	320	[c]	2000	[c]	280	15	310	[c]	360	240
1e	Me	Me	18	2100	830	1300	4100	360	11	270	1000	220	440
1f	Et	Et	110	1700	[c]	[c]	[c]	[c]	67	820	[c]	230	2000
1g	Pr	Pr	[c]	400	[c]	[c]	[c]	[c]	80	2600	[c]	[c]	[c]
2a	Me	H	1.6	280	84	3.3	180	170	69	[c]	[c]	910	740
2b	Et	H	2.8	52	580	5.3	2600	520	130	[c]	[c]	700	[c]
2c	Pr	H	8.7	140	250	41	[c]	600	170	[c]	[c]	240	[c]
2d	Bu	H	64	220	280	110	[c]	570	130	[c]	[c]	2400	[c]
2e	Me	Me	2.5	280	74	86	2200	250	280	[c]	[c]	380	1000
2f	Et	Et	25	84	160	8.4	6700	680	320	[c]	[c]	2100	[c]
2g	Pr	Pr	20	65	160	38	[c]	1200	230	[c]	[c]	2900	[c]
3a	H		2.8	96	410	3.4	770	340	50	[c]	590	970	400
3b	Me		30	200	12	19	4100	1200	170	920	420	1000	[c]

[a]Binding affinity data were generously provided by the US National Institute of Mental Health (NIMH) Psychoactive Drug Screening Program (University of North Carolina). [b]Values are the mean of at least three independent experiments performed in triplicate. [c]K_i > 10,000 nM.

group at the amine in this series of compounds leads to the Gs protein bias.

To determine ligand biasness of a better G protein-biased ligand **2b**, we evaluated the differences $\Delta\Delta\log(\tau/K_A)$ between the $\Delta\log(\tau/K_A)$ values of **2b** and E-55888 in both G protein signaling pathway and β-arrestin signaling pathway.[40] As shown in Table 3, E-55888 had $\Delta\Delta\log(\tau/K_A) = -0.47$, which was converted to a bias factor. The bias factor of E-55888 is less than 1, which means that E-55888 is more biased to the β-arrestin signaling pathway, not the Gs protein signaling pathway. **2b** had $\Delta\Delta\log(\tau/K_A) = 2.66$, of which the bias factor is 461. By the calculation of the bias factors of those two compounds E-55888 and **2b**, it became more obvious that **2b** is a Gs protein-biased ligand of 5-HT_7R.

Table 3. Bias Factor[a] of E-55888 and Compound 2b toward Gs Protein

signaling pathway	parameters	5-HT[b]	E-55888	**2b**
Gs protein	$\log\tau$	0.71	1.22	0.68
	$\log K_A$	−7.46	−5.84	−6.11
	$\Delta\log(\tau/K_A)$	0	−1.12	−1.39
β-arrestin	$\log\tau$	0.22	0.33	−1.61
	$\log K_A$	−6.14	−5.39	−3.92
	$\Delta\log(\tau/K_A)$	0	−0.65	−4.05
	$\Delta\Delta\log(\tau/K_A)$[c]	0	−0.47	2.66
	bias factor (toward Gs protein)	1	0.34	461

[a]Bias factor was calculated with Prism 4.0 program (GraphPad software, San Diego). [b]5-HT was used as the reference agonist. [c]$\Delta\Delta\log(\tau/K_A) = \Delta\log(\tau/K_A)$(Gs protein) − $\Delta\log(\tau/K_A)$(β-arrestin).

CYP Activity and Microsomal Stability. The *in vitro* cytochrome P450 (CYP450) inhibition assay, including five major isoforms, that is, CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4, was performed to assess the potential drug−drug interaction of **2b** (Table 4). As shown in Table 4, the percentage of remaining activities of **2b** in the five CYP isozymes is greater than 88.7%, and compound **2b** has little effect on the CYP isozymes, indicating that it might not have a drug−drug interaction. In addition, the metabolic stability of compound **2b** was determined through the human liver microsomal stability test. Since the metabolic stability of drug is a key factor that affects both the efficacy and toxicity of the drug, as well as its pharmacokinetic (PK) parameters, it can be tested in an *in vitro* assay prior to conducting an *in vivo* study. Compound **2b** showed 71.5% as the percent-remaining concentration, which means that **2b** is barely decomposed and is stable after incubation with human liver microsomes. Thus, the PK

Table 4. Results of CYP450 Remaining Activity and Microsomal Stability Test

	CYP450 activity[a]					
compd	CYP1A2	CYP2C9	CYP2C19	CYP2D6	CYP3A4	human liver microsomal stability (%)[b,c]
2b	94.3	>100	90.3	88.7	98.5	71.5

[a]%-Remaining cytochrome activity. [b]%-Remaining amount of **2b** after 30 min of treatment with human liver microsomes. [c]Verapamil was used as a reference compound and the microsomal stability was 16.8%.

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parameters of **2b** were evaluated after intravenous injection (dose 1 mg/kg) and intraperitoneal (i.p.) administration (dose 10 mg/kg) in male ICR mice (Table 5). After the intravenous injection, the mean clearance rate (CL) was measured as 477.7 mL/min/kg, the half-life ($T_{1/2}$) of **2b** was 0.5 h, and the AUC_{last} was 32.9 h ng/mL. According to the half-life and CL, **2b** seems to be rapidly eliminated from the body. In i.p. administration, the half-life ($T_{1/2}$) was 1.0 h, and AUC_{last} was 442.3 h ng/mL. The bioavailability of **2b** was about 134%.

Table 5. Mean (±SD[b]) PK Parameters after Intravenous Injection at a Dose of 1 mg/kg (n = 4) and Intraperitoneal Administration at a Dose of 10 mg/kg (n = 4) of 2b to ICR Mice[a]

plasma	intravenous injection	intraperitoneal administration
T_{max} (h)		0.4 ± 0.1
C_{max} (ng/mL)		347.7 ± 69.9
$T_{1/2}$ (h)	0.5 ± 0.2	1.0 ± 0.3
AUC_{last} (h ng/mL)	32.9 ± 2.5	442.3 ± 71.7
Cl_obs (mL/min/kg)	477.7 ± 60.3	
MRT_{inf}_obs (h)	0.7 ± 0.3	1.3 ± 0.4
Vss_obs (L/kg)	19.5 ± 4.7	
BA (%)		134.4

[a]T_{max}, time to reach C_{max}; C_{max}, peak plasma concentration; $T_{1/2}$, terminal half-life; AUC_{last}, total area under the plasma concentration time curve from time zero to the last measured time; Cl, time-averaged total body clearance; MRT, mean residence time; V_{dss}, apparent volume of distribution at steady state; BA, bioavailability. [b]SD: standard deviations.

Self-Grooming Behavior. One of the symptoms in patients with ASD is stereotypy, which is observed as restricted and repetitive patterns of behavior.[41] Currently, studies of the correlation between ASD and 5-HT_7R are emerging, and there are several reported results indicating that 5-HT_7R ligands correct phenotypic deficits in various heterogeneous mouse models, such as *Fmr1* KO mice,[22] *Mecp2* TG mice,[23] and C58/J mice,[42] which exhibit spontaneous self-grooming. Thus, we conducted a self-grooming behavior test using *Shank3*$^{-/-}$ (*Shank3* TG) male mice[43−45] that are a well-known ASD animal model. We tested SB269970, also known as 5-HT_7R selective antagonist, at a dose of 30 mg/kg in i.p. administration to WT and *Shank3* TG mice. The administration of SB269970 showed duration times in both WT (t = 29.3 s) and TG mice (t = 181 s) without any significant changes compared with those of saline (Figure 4a). After i.p. administration, the Gs protein-biased agonist **2b** with a dose of 5 mg/kg showed a highly increased duration of the self-grooming time in *Shank3* TG mice (t = 553 s) compared to that of saline (Figure 4b). To determine the direct effect of 5-HT_7R on self-grooming behavior, SB269970 was administered 10 min prior to the injection of **2b**. Figure 4b shows that the co-treatment of SB269970 (30 mg/kg) and **2b** (5 mg/kg) reduced the duration of self-grooming (t = 70.8 s in WT mice and 166 s in TG mice) to the normal duration with saline administration, which demonstrates that SB269970 inhibits the effect of **2b**. Overall, the Gs protein-biased agonist **2b** prominently increased the duration time of self-grooming, which was suppressed by the effect of the 5-HT_7R selective antagonist, SB269970. Although more experiments are needed regarding the association of 5-HT_7R and autism, these results support the conclusion that 5-HT_7R is involved in a modulatory role to stereotypy in ASD (Table 6).

Table 6. Duration Time of Self-Grooming Behavior in WT and *Shank3* TG Mice after Treatment with Saline, SB269970 (30 mg/kg), 2b (5 mg/kg), and SB269970 + 2b for 30 min Measured in Healthy Subjects (n = 6)[a]

	duration time (t) of grooming (in seconds)	
compounds	WT	*Shank3* TG
SB269970	29.3 ± 6.90	181 ± 37.8
2b	107 ± 41.6	553 ± 58.0
SB269970 + **2b**	70.8 ± 9.20	166 ± 35.0
saline	43.7 ± 10.8	224 ± 47.4

[a]All data are expressed as mean ± SEM (seconds).

CONCLUSIONS

We synthesized a series of 6-chloro-2'-methoxy biphenyl derivatives **1**, **2**, and **3** bearing various amine moieties and evaluated binding affinities of these synthesized compounds for 5-HT_7R along with other serotonin receptor subtypes. Most of the compounds had binding affinities to 5-HT_7R. By conducting cAMP production assay and β-arrestin recruitment Tango assay, three balanced agonists **1a**, **2a**, and **2e** like E-55888 were found, among which **2a** is the best in the view of potency and efficacy in both assays, while there are six G protein-biased ligands **1b**, **1c**, **2b**, **2c**, **2f**, and **2g**, among which **2b** is the best in the cAMP assay

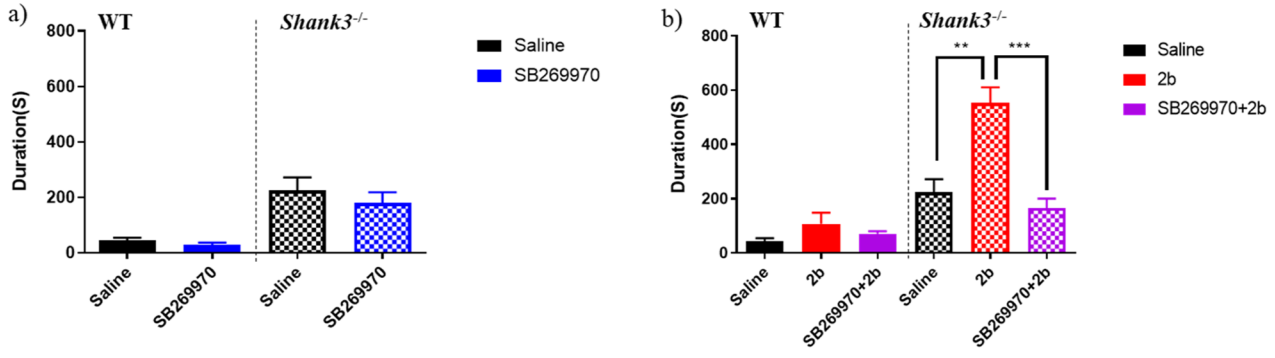

Figure 4. (a) Duration time of self-grooming behavior in WT and *Shank3* TG mice treated with saline and SB269970 (30 mg/kg) and (b) duration of self-grooming behavior in WT and *Shank3* TG mice co-treated with SB269970 (30 mg/kg) and **2b** (5 mg/kg). Data bars represent means ± SEM. Duration compiled for the 30 min following the intraperitoneal administration of drugs: *P < 0.05, **P < 0.01, and ***P < 0.001 *vs* vehicle based on Student's t-test.

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(EC_{50} = 180 nM, E_{max} = 91%). In the animal study with *Shank3* TG mice, the self-grooming behavior test of **2b** was performed, and **2b** showed an increased duration of self-grooming, which was reversed by the selective 5-HT_7R antagonist, SB269970. Based on the results of the self-grooming behavior, we further suggested that 5-HT_7R is associated with ASD and could be a therapeutic target for the treatment of stereotypy in ASD.

EXPERIMENTAL SECTION

Chemistry. All reactions were carried out under dry nitrogen unless otherwise indicated. Commercially available reagents were used without further purification. Solvents and gases were dried according to standard procedures. Organic solvents were evaporated with reduced pressure using a rotary evaporator. Analytical thin layer chromatography (TLC) was performed using glass plates pre-coated with silica gel (0.25 mm). TLC plates were visualized by exposure to UV light and then were visualized with a $KMnO_4$, ninhydrin, and *p*-anisaldehyde staining solution followed by brief heating on a hot plate. Flash column chromatography was performed using silica gel 60 (230−400 mesh, Merck) with the indicated solvents. ^{1}H and ^{13}C NMR spectra were recorded on Bruker 300 or 400 NMR spectrometers. ^{1}H NMR spectra are represented as follows: chemical shift, multiplicity (s = singlet, br s = broad singlet, d = doublet, t = triplet, q = quartet, m = multiplet), integration, and coupling constant (*J*) in hertz. Liquid chromatography−mass spectrometry (LC/MS) and high-resolution mass spectrometry (HRMS) analyses were performed on an Agilent 6410 Triple Quad system and the Bruker Compact ESI+ positive mode, respectively. The purity of all the tested compounds was checked on a Waters HPLC e2695 instrument equipped with a UV/vis 2489 detector and a Capcell Pak 3 μm C18 MG-II (4.6 × 75 mm) column and was at least 95% for all tested compounds. Standard conditions were as follows: eluents system A (CH_3CN), system B (H_2O/0.1 M AcOH); a flow rate of 1 mL/min; a gradient of (10−100%) A over 20 min; and detection at 254 and 280 nm.

*4-Chloro-3-iodobenzaldehyde (**4**).* Iodine (1.59 g, 6.26 mmol) and $NaIO_3$ (620 mg, 3.13 mmol) were suspended in 95% concentrated H_2SO_4. The solution was stirred for 30 min at room temperature (rt). 4-Chlorobenzaldehyde **3** (2 g, 14.23 mmol) was added to the dark brown iodinating solution and the mixture was stirred for an additional 1 h at rt. The reaction mixture was poured slowly into ice−water. The crude solid product was collected by filtration with EtOH to afford the desired product **4** (2.43 g, 9.12 mmol) in 64% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.92 (s, 1H), 8.34 (d, *J* = 1.9 Hz, 1H), 7.80 (dd, *J* = 8.2, 1.9 Hz, 1H), 7.61 (d, *J* = 8.2 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 189.41, 145.07, 141.37, 135.68, 130.00, 129.95, 98.83.

*6-Chloro-2′-methoxy-[1,1′-biphenyl]-3-carbaldehyde (**6**).* 2-Methoxyphenylboronic acid **5** (2.25 mmol), $Pd(PPh_3)_4$ (0.02 mmol), and Na_2CO_3 (2.82 mmol) were added to a solution of benzaldehyde **4** (1.88 mmol) in DMF (15 mL). The mixture was stirred overnight at 80 °C. After cooling down to rt, the reaction mixture was quenched by the addition of the saturated solution of $NaHCO_3$ and then partitioned with EtOAc. The combined organic layers were dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel (hexanes/EtOAc = 10:1) to obtain desired product **6** in 82% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 10.00 (s, 1H), 7.82−7.79 (m, 2H), 7.62 (dd, *J* = 6.2, 2.6 Hz, 1H), 7.42 (m, 1H), 7.20 (dd, *J* = 7.4, 1.8 Hz, 1H), 7.05 (td, *J* = 7.5, 1.2 Hz, 1H), 7.01 (dd, *J* = 8.4, 0.8 Hz, 1H), 3.79 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 191.1, 156.6, 140.8, 138.9, 134.7, 133.3, 130.1, 130.2, 130.0, 129.0, 127.2, 120.5, 111.0, 55.6.

*6-Chloro-2′-methoxy-[1,1′-biphenyl]-3-yl-methanamine Derivatives (**1**).* The mixture of 6-chloro-2′-methoxy-[1,1′-biphenyl]-3-carbaldehyde **6** (1 equiv) and appropriate amine (2 equiv) in MeOH (20 mL) was stirred at rt for 2 h. $NaBH(OAc)_3$ (3 equiv) was added and the solution was stirred overnight at rt. The reaction mixture was quenched by the addition of a saturated solution of $NaHCO_3$ and extracted with dichloromethane (DCM). The combined organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (DCM/MeOH = 10:1) to afford the desired final product **1** in 16−93% yields.

*1-(2′-Methoxy-[1,1′-biphenyl]-3-yl)-N-methylmethanamine (**1a**).* Compound **1a** was synthesized according to the general procedure of **1**. Yield: 37%; HPLC: purity 100%, t_R = 5.5 min; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.51−7.30 (m, 6H), 7.09−7.01 (m, 2H), 3.86 (s, 2H), 3.85 (s, 3H), 2.53 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 156.49, 139.19, 138.72, 130.93, 130.64, 127.48, 128.65, 128.41, 128.10, 126.88, 120.85, 111.29, 55.88, 55.60, 35.71; HRMS (ESI+): calcd for $C_{15}H_{17}ClNO^+$ $[M + H]^+$, 262.0999; found, 262.0996.

*N-((6-Chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)methyl)-ethanamine (**1b**).* Compound **1b** was synthesized according to the general procedure of **1**. Yield: 57%; HPLC: purity 100%, t_R = 5.8 min; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.41−7.35 (m, 2H), 7.26 (dd, *J* = 5.8, 1.6 Hz, 2H), 7.12 (dd, *J* = 7.6, 1.8 Hz, 1H), 7.01 (td, *J* = 7.6, 1.1, 1H), 6.98 (dd, *J* = 8.4, 0.8 Hz, 1H), 3.80 (s, 2H), 3.78 (s, 3H), 2.70 (q, *J* = 7.2 Hz, 2H), 1.14 (t, *J* = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 156.7, 138.5, 137.6, 132.4, 131.4, 131.0, 129.4, 129.2, 128.6, 128.3, 120.4, 111.0, 55.6, 53.1, 43.6, 15.1; HRMS (ESI+): calcd for $C_{16}H_{19}ClNO^+$ $[M + H]^+$, 276.1155; found, 276.1150.

*N-((6-Chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)methyl)propan-1-amine (**1c**).* Compound **1c** was synthesized according to the general procedure of **1**. Yield: 80%; HPLC: purity 100%, t_R = 5.7 min; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.41−7.35 (m, 2H), 7.27−7.25 (m, 2H), 7.02 (td, *J* = 7.4, 1.2 Hz, 1H), 6.98 (d, *J* = 0.8 Hz, 1H), 3.79 (s, 1H), 3.78 (s, 3H), 1.54 (m, *J* = 3.4 Hz, 2H), 0.92 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 156.8, 138.7, 137.6, 132.4, 131.4, 131.0, 129.4, 129.2, 128.6, 128.3, 120.4, 111.0, 55.6, 53.2, 51.3, 23.1, 11.8; HRMS (ESI+): calcd for $C_{17}H_{21}ClNO^+$ $[M + H]^+$, 190.1312; found, 190.1306.

*N-((6-Chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)methyl)butan-1-amine (**1d**).* Compound **1d** was synthesized according to the general procedure of **1**. Yield: 93%; HPLC: purity 100%, t_R = 6.1 min; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.41−7.35 (m, 2H), 7.25−7.23 (m, 2H), 7.19 (dd, *J* = 7.2, 1.6 Hz, 1H), 7.04−6.97 (m, 2H), 3.78 (s, 5H), 2.64 (t, *J* = 7.2 Hz, 2H), 1.48 (m, *J* = 4.3 Hz, 2H), 1.35 (m, *J* = 7.5 Hz, 2H), 0.91 (t, *J* = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 156.8, 139.0, 137.6, 132.2, 131.3, 131.0, 129.3, 129.2, 128.6, 128.2, 120.3, 111.0, 55.6, 53.4, 49.2, 32.3, 20.5, 14.0; HRMS (ESI+): calcd for $C_{18}H_{23}ClNO^+$ $[M + H]^+$, 304.1468; found, 304.1466.

*1-(2′-Methoxy-[1,1′-biphenyl]-3-yl)-N,N-dimethylmethanamine (**1e**).* Compound **1e** was synthesized according to the general procedure of **1**. Yield: 74%; HPLC: purity 100%, t_R = 5.6 min; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.52−7.30 (m, 6H), 7.10−7.01 (m, 2H), 3.85 (s, 3H), 3.61 (s, 2H), 2.37 (s, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 156.50, 138.60, 137.03, 130.94, 130.66, 130.56, 128.68, 128.04, 128.01, 120.88, 111.33, 63.96, 55.60, 44.85; HRMS (ESI+): calcd for $C_{16}H_{19}ClNO^+$ $[M + H]^+$, 276.1155; found, 276.1152.

*N-((6-Chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)methyl)-N-ethylethanamine (**1f**).* Compound **1f** was synthesized according to the general procedure of **1**. Yield: 20%; HPLC: purity 100%, t_R = 6.1 min; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.40−7.35 (m, 2H), 7.28−7.26 (m, 2H), 7.20 (dd, *J* = 7.4, 1.8 Hz, 1H), 7.04−6.97 (m, 2H), 3.78 (d, 3H), 7.58 (s, 2H), 2.55 (q, *J* = 7.4 Hz, 4H), 1.04 (t, *J* = 7.0 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 156.8, 138.2, 137.3, 132.1, 132.0, 131.1, 129.3, 129.0, 128.7, 120.3, 111.0, 56.8, 55.6, 46.8, 11.8; HRMS (ESI+): calcd for $C_{18}H_{23}ClNO^+$ $[M + H]^+$, 304.1468; found, 304.1467.

*N-((6-Chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)methyl)-N-propylpropan-1-amine (**1g**).* Compound **1g** was synthesized according to the general procedure of **1**. Yield: 16%; HPLC: purity 100%, t_R = 6.8 min; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.38−7.34 (m, 2H), 7.26−7.23 (m, 2H), 7.19 (dd, *J* = 7.4, 1.8 Hz, 1H), 7.01 (td, *J* = 7.4, 1.2 Hz, 1H), 6.97 (d, *J* = 8.0 Hz, 1H), 3.77 (s, 3H), 3.54 (s, 2H), 2.39−2.34 (m, 4H), 1.47 (m, *J* = 3.2 Hz, 4H), 0.86 (t, *J* = 7.2 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 156.8, 138.8, 137.2, 132.0, 131.9, 131.1, 129.3, 128.9, 128.9, 120.3, 111.0, 58.0, 55.9, 55.6, 20.3, 11.9; HRMS (ESI+): calcd for $C_{20}H_{27}ClNO^+$ $[M + H]^+$, 332.1781; found, 332.1779.

*(4-Chloro-3-iodophenyl)methanol (**7**).* 4-Chloro-3-iodophenylbenzaldehyde **6** (7.62 mmol) was added in portions to a suspension of sodium borohydride (7.62 mmol) in methanol (50 mL). The mixture was stirred for 2 h at rt. The solution was quenched by the addition of

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water and then extracted with DCM. The combined organic layers were dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography (hexanes/EtOAc = 5:1) to obtain the desired product **7** (6.70 mmol, 88%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.85 (d, J = 1.9 Hz, 1H), 7.41 (d, J = 8.0 Hz, 1H), 7.26 (dd, J = 8.2, 2.0 Hz, 1H), 4.62 (d, J = 4.0 Hz, 2H), 1.98 (t, J = 6.0 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 140.98, 138.45, 137.51, 129.26, 127.86, 98.12, 63.59.

*4-(Bromomethyl)-1-chloro-2-iodobenzene (**8**).* (4-Chloro-3-iodophenyl)methanol **7** (7.45 mmol) was dissolved in DCM (50 mL) and triphenylphosphine (8.94 mmol) was added to the solution at rt and then carbon tetrabromide (8.94 mmol) was slowly added to the mixture. The reaction mixture was stirred for 2 h at rt, at which time, the reaction mixture was concentrated under reduced pressure. The residue was purified by column chromatography (hexanes/EtOAc = 50:1) to obtain the desired product **8** (6.94 mmol, 93%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.87 (d, J = 2.1 Hz, 1H), 7.40 (d, J = 8.2 Hz, 1H), 7.30 (dd, J = 7.9, 2.1 Hz, 1H), 4.37 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 140.50, 138.60, 137.84, 130.10, 129.50, 98.21, 30.94.

*2-(4-Chloro-3-iodophenyl)acetonitrile (**9**).* 4-(Bromomethyl)-1-chloro-2-iodobenzene **8** (4.71 mmol) was dissolved in toluene (30 mL) and sodium cyanide (7.06 mmol) was added to the solution. Tetrabutylammonium bromide (0.047 mmol) dissolved in water (1 mL) was added to the mixture and then the reaction mixture was stirred overnight at 50 °C. Based on TLC analysis, sodium cyanide (2.36 mmol) and water were additionally added to the mixture. After stirring for 5 h, the solution was quenched by the addition of water and extracted with EtOAc. The combined organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography (hexanes/EtOAc = 7:1) to afford the desired final product **9** (3.60 mmol, 77%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.87 (d, J = 2.1 Hz, 1H), 7.40 (d, J = 8.2 Hz, 1H), 7.25 (dd, J = 8.2, 2.2 Hz, 1H), 3.70 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 139.50, 138.55, 130.01, 129.73, 129.08, 117.02, 98.75, 22.49.

*2-(6-Chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)acetonitrile (**10**).* 2-Methoxyphenylboronic acid **5** (7.57 mmol), $Pd(PPh_3)_4$ (0.06 mmol), and Na_2CO_3 (9.46 mmol) were added to a solution of 2-(4-chloro-3-iodophenyl)acetonitrile **9** (6.31 mmol) in DMF (30 mL). The mixture was stirred overnight at 110 °C. After cooling down to rt, the reaction mixture was quenched with a saturated solution of $NaHCO_3$ and then extracted with EtOAc. The combined organic layers were dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (hexanes/EtOAc = 10:1) to afford the desired product **10** (5.04 mmol, 80%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.47−7.44 (m, 1H), 7.39 (ddd, J = 8.1, 7.8, 1.8 Hz, 1H), 7.25−7.22 (m, 2H), 7.16 (dd, J = 7.4, 1.8 Hz, 1H), 7.02 (td, J = 7.4, 1 Hz, 1H), 6.98 (d, J = 8.3 Hz, 1H), 3.77 (s, 3H), 3.73 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 156.65, 138.70, 133.95, 131.25, 130.83, 130.05, 129.81, 128.27, 127.97, 127.68, 120.49, 117.54, 111.06, 55.64, 23.11.

*2-(6-Chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)ethan-1-amine (**11**).* 1 M $LiAlH_4$ in anhydrous THF (4.3 mL) was added dropwise under N_2 to the solution of $AlCl_3$ (3.49 mmol) in THF while stirring. The mixture was cooled down to 0 °C before dropwise addition of the solution of 2-(6-chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)acetonitrile **10** (3.88 mmol) in THF (10 mL). The solution was kept stirring for 2 h at 0 °C, at which time, MeOH was slowly added to quench the reaction. The mixture was concentrated under reduced pressure and the residue was dissolved in DCM and washed with water. The organic phase was partitioned with 1 N solution of HCl. The combined aqueous layer was washed with DCM and neutralized by the addition of 10 N solution of NaOH. The mixture was partitioned with DCM and the pooled organic layer was dried over Na_2SO_4, filtered, and concentrated under reduced pressure to obtain the desired product **11** (3.88 mmol, 68.9%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.35−7.30 (m, 2H), 7.15 (dd, J = 7.5, 1.7 Hz, 2H), 7.12 (br s, 2H), 7.10 (d, J = 2.2 Hz, 1H), 6.97 (td, J = 7.4, 0.9 Hz, 1H), 6.93 (d, J = 8.3 Hz, 1H), 3.73 (s, 3H), 3.16 (t, J = 7.8 Hz, 2H), 3.01 (t, J = 7.8 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 156.63, 138.16, 135.12, 132.70, 131.99, 130.95, 129.64, 129.50, 128.91, 128.19, 120.41, 111.00, 55.60, 41.32, 34.10.

*tert-Butyl (2-(6-Chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)ethyl)-carbamate (**12**).* 2-(6-Chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)ethan-1-amine **11** (2.67 mmol) and di-*tert*-butyl dicarbonate (2.67 mmol) were dissolved in anhydrous THF (10 mL). An aqueous solution of $NaHCO_3$ (2 mL) was added to a mixture and stirred overnight at rt. The mixture was partitioned with water and DCM and the combined organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography (hexanes/EtOAc = 5:1) to afford the desired product **12** (2.67 mmol, 77%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.39−7.35 (m, 2H), 7.18 (dd, J = 7.5, 1.7 Hz, 1H), 7.12 (d, J = 2.0 Hz, 1H), 7.10 (dd, J = 8.1, 2.2 Hz, 1H), 7.01 (td, J = 7.4, 1.0 Hz, 1H), 6.98 (d, J = 8.3 Hz, 1H), 4.58 (br s, 1H), 3.78 (s, 3H), 3.38 (q, J = 6.5 Hz, 2H), 2.79 (t, J = 7.0 Hz, 2H), 1.43 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 156.74, 155.83, 137.78, 137.32, 132.15, 131.95, 131.00, 129.39, 128.89, 128.47, 120.36, 111.05, 55.63, 41.62, 35.56, 35.53, 35.52, 28.41.

*tert-Butyl (2-(6-Chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)ethyl)-(alkyl)carbamate Derivatives (**13**).* A solution of *tert*-butyl (2-(6-chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)ethyl)carbamate **12** (1 equiv) in DMF (15 mL) was added dropwise to a sodium hydride 60% in oil (1.25 equiv) in DMF at 0 °C and stirred for an hour. Alkyliodide (2.4 equiv) was then slowly added to a mixture at 0 °C and the stirring was continued for an additional 2 h. The reaction mixture was quenched by the addition of methanol and partitioned with water and EtOAc. The combined organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography (hexanes/EtOAc = 5:1) to obtain the desired product **13** in 41−57% yields.

*tert-Butyl (2-(6-Chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)ethyl)-(methyl)carbamate (**13a**).* Compound **13a** was synthesized according to the general procedure of **13**. Yield: 50%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.39−7.35 (m, 2H), 7.17 (dd, J = 7.4, 1.6 Hz, 1H), 7.11 (br s, 2H), 7.01 (t, J = 7.4 Hz, 1H), 6.97 (d, J = 8.3 Hz, 1H), 3.78 (s, 3H), 3.44 (t, J = 8.0 Hz, 2H), 2.84−2.79 (m, 5H), 1.40 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 156.74, 155.56, 137.70, 137.61, 132.15, 131.81, 130.97, 130.22, 129.35, 129.24, 128.98, 128.54, 120.35, 110.97, 79.35, 55.58, 50.62, 33.95, 28.39.

*tert-Butyl (2-(6-Chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)ethyl)-(ethyl)carbamate (**13b**).* Compound **13b** was synthesized according to the general procedure of **13**. Yield: 57.1%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.43−7.39 (m, 2H), 7.21 (dd, J = 7.4, 1.5 Hz, 1H), 7.16 (br s, 2H), 7.06 (t, J = 7.5 Hz, 1H), 7.02 (d, J = 8.3 Hz, 1H), 3.82 (s, 3H), 3.43 (t, J = 7.4 Hz, 2H), 3.24 (br s, 2H), 2.86 (br s, 2H), 1.48 (s, 9H), 1.11 (t, J = 6.3 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 156.75, 155.25, 137.78, 137.70, 132.16, 131.76, 130.97, 129.35, 129.23, 129.00, 128.57, 120.35, 110.98, 55.60, 48.56, 42.74, 41.92, 34.74, 34.17, 28.48, 14.23, 13.54.

*tert-Butyl (2-(6-Chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)ethyl)-(propyl)carbamate (**13c**).* Compound **13c** was synthesized according to the general procedure of **13**. Yield: 42.4%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.42−7.38 (m, 2H), 7.21 (dd, J = 7.4, 1.3 Hz, 1H), 7.15 (br s, 2H), 7.05 (t, J = 7.4 Hz, 1H), 7.01 (d, J = 8.3 Hz, 1H), 3.81 (s, 3H), 3.42 (t, J = 6.5 Hz, 2H), 3.13 (d, J = 19.4 Hz, 2H), 2.85 (br s, 2H), 1.54 (br s, 2H), 1.47 (s, 9H), 0.89 (t, J = 7.4 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 156.76, 155.50, 137.78, 137.71, 132.15, 131.77, 130.97, 129.36, 129.23, 129.01, 128.58, 120.36, 110.98, 79.22, 55.60, 49.01, 34.70, 34.04, 28.47, 21.97, 21.55, 11.30.

*tert-Butyl (2-(6-Chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)ethyl)-(butyl)carbamate (**13d**).* Compound **13d** was synthesized according to the general procedure of **13**. Yield: 41%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.41 (t, J = 7.6 Hz, 2H), 7.21 (d, J = 4.9 Hz, 1H), 7.15 (br s, 2H), 7.05 (t, J = 7.4 Hz, 1H), 7.01 (d, J = 8.3 Hz, 1H), 3.81 (s, 3H), 3.42 (br s, 2H), 3.16 (d, J = 21.4 Hz, 2H), 2.85 (s, 2H), 1.47 (s, 9H), 1.31 (q, J = 7.4 Hz, 2H), 0.94 (t, J = 7.4 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 156.77, 155.46, 137.79, 137.72, 132.15, 131.78, 130.97, 129.36, 129.23, 129.02, 128.59, 120.36, 110.99, 79.23, 55.36, 48.96, 47.11, 34.71, 30.88, 30.52, 28.48, 20.06, 13.91.

*2-(6-Chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)-N-alkylethan-1-amine (**2**).* The protected amine was dissolved in 1 N HCl solution (1.5 equiv) in diethyl ether and the reaction mixture was stirred at rt

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overnight. A white precipitate formed after several minutes and additional HCl was added as needed. After completing the reaction, the precipitate was filtered, washed with diethyl ether, and concentrated under reduced pressure. The desired products were obtained in 80−95% yields.

*2-(6-Chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)-N-methylethan-1-amine (**2a**).* Compound **2a** was synthesized according to the general procedure of **2**. Yield: 93%; HPLC: purity 100%, t_R = 6.0 min; ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.69 (br s, 2H), 7.54 (d, J = 8.1 Hz, 1H), 7.41 (t, J = 7.8 Hz, 1H), 7.36 (d, J = 2.1 Hz, 1H), 7.32 (dd, J = 8.2, 2.2 Hz, 1H), 7.02 (d, J = 2.5 Hz, 1H), 7.01−6.99 (m, 2H), 3.81 (s, 3H), 3.20 (t, J = 7.8 Hz, 2H), 2.98 (t, J = 7.8 Hz, 2H), 2.58 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 156.77, 138.05, 136.53, 132.46, 131.77, 131.00, 130.10, 129.68, 129.63, 128.12, 120.71, 111.78, 55.84, 49.20, 32.74, 31.06; HRMS (ESI+): calcd for $C_{16}H_{19}ClNO^+$ [M + H]$^+$, 276.1155; found, 276.1152.

*2-(6-Chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)-N-ethylethan-1-amine (**2b**).* Compound **2b** was synthesized according to the general procedure of **2**. Yield: 91%; HPLC: purity 100%, t_R = 6.1 min; ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.83 (br s, 2H), 7.47 (d, J = 8.2 Hz, 1H), 7.41 (ddd, J = 8.2, 7.4, 1.7 Hz, 1H), 7.28 (dd, J = 8.2, 2.2 Hz, 1H), 7.23 (d, J = 2.2 Hz, 1H), 7.15 (dd, J = 7.4, 1.7 Hz, 1H), 7.12 (d, J = 8.1 Hz, 1H), 7.03 (td, J = 7.3, 0.7 Hz, 1H), 3.73 (s, 3H), 3.16 (t, J = 7.9 Hz, 2H), 2.98−2.94 (m, 4H), 1.20 (t, J = 7.3 Hz, 3H); ^{13}C NMR (100 MHz, MeOD): δ 156.75, 138.51, 134.95, 132.64, 131.85, 130.31, 129.30, 128.62, 128.21, 120.03, 110.74, 54.56, 47.93, 42.81, 31.32, 10.13; HRMS (ESI+): calcd for $C_{17}H_{21}ClNO^+$ [M + H]$^+$, 290.1312; found, 290.1307.

*2-(6-Chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)-N-propylethan-1-amine (**2c**).* Compound **2c** was synthesized according to the general procedure of **2**. Yield: 92.2%; HPLC: purity 100%, t_R = 6.5 min; ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.70 (br s, 2H), 7.47 (d, J = 8.2 Hz, 1H), 7.41 (ddd, J = 8.0, 7.3, 1.8 Hz, 2H), 7.28 (dd, J = 8.2, 2.2 Hz, 1H), 7.23 (d, J = 2.1 Hz, 1H), 7.15 (dd, J = 7.5, 1.8 Hz, 1H), 7.12 (d, J = 8.2 Hz, 1H), 7.03 (t, J = 7.4 Hz, 1H), 3.73 (s, 3H), 3.17 (t, J = 8.1 Hz, 2H), 2.96 (t, J = 8.1 Hz, 2H), 2.88 (t, J = 7.7 Hz, 2H), 1.62 (sext, J = 7.6 Hz, 2H), 0.92 (t, J = 7.4 Hz, 3H); ^{13}C NMR (100 MHz, MeOD): δ 156.75, 138.51, 137.89, 134.97, 132.63, 131.83, 130.30, 129.29, 128.60, 128.21, 120.02, 110.74, 54.56, 48.38, 47.49, 31.28, 27.87, 19.42; HRMS (ESI+): calcd for $C_{18}H_{23}ClNO^+$ [M + H]$^+$, 304.1468; found, 304.1464.

*2-(6-Chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)-N-butyllethan-1-amine (**2d**).* Compound **2d** was synthesized according to the general procedure of **2**. Yield: 95%; HPLC: purity 100%, t_R = 6.3 min; ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.66 (br s, 2H), 7.49 (d, J = 8.1 Hz, 1H), 7.44−7.40 (m, 1H), 7.29 (dd, J = 8.3, 1.7 Hz, 1H), 7.24 (d, J = 1.6 Hz, 1H), 7.17−7.12 (m, 2H), 7.04 (t, J = 7.4 Hz, 1H), 3.74 (s, 3H), 3.19 (t, J = 8.1 Hz, 2H), 2.98−2.90 (m, 4H), 1.63−1.55 (m, 2H), 1.34 (t, J = 7.3 Hz, 2H), 0.91 (t, J = 7.3 Hz, 3H); ^{13}C NMR (100 MHz, MeOD): δ 156.75, 138.51, 137.89, 134.97, 132.63, 131.83, 130.30, 129.29, 128.60, 128.21, 120.02, 110.74, 54.56, 48.38, 47.49, 31.28, 27.87, 19.42, 12.47; HRMS (ESI+): calcd for $C_{19}H_{25}ClNO^+$ [M + H]$^+$, 318.1625; found, 318.1621.

*2-(6-Chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)-N,N-dimethylethan-1-amine (**2e**).* A solution of 2-(6-chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)ethan-1-amine **11** (0.44 mmol) in formic acid (2.22 mmol, 88% in water solution) and formaldehyde (2.22 mmol, 37% in water solution) was stirred at 80 °C for 20 h. The reaction mixture was cooled down to rt, and the mixture was diluted with water, adjusted to pH 10 with K_2CO_3, and extracted with DCM. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by column chromatography (DCM/MeOH = 10:1) to obtain the desired product **2e** (0.19 mmol, 43%); HPLC: purity 100%, t_R = 5.4 min; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.37 (t, 2H), 7.19−7.17 (m, 3H), 7.03−6.97 (m, 2H), 3.78 (s, 3H), 2.81 (t, J = 8.0 Hz, 2H), 2.61 (t, J = 8.0 Hz, 2H), 2.33 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 156.76, 138.28, 137.66, 131.91, 131.65, 130.98, 129.36, 129.24, 128.82, 128.60, 120.36, 111.03, 60.94, 55.65, 45.13, 33.27; HRMS (ESI+): calcd for $C_{16}H_{19}ClNO^+$ [M + H]$^+$, 276.1155; found, 276.1156.

*2-(6-Chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)-N,N-diethylethan-1-amine (**2f**).* 2-(6-Chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)ethan-1-amine **11** (0.50 mmol) was dissolved in acetonitrile (3 mL). Iodoethane (0.6 mmol) and K_2CO_3 (1.99 mmol) were added to the solution and the reaction mixture was stirred for 15 h at 65 °C. The mixture was cooled down to rt and quenched with saturated solution of $NaHCO_3$ and then extracted with EtOAc. The combined organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography (DCM/MeOH = 20:1) to obtain the desired product **2f** (0.32 mmol, 63%); HPLC: purity 100%, t_R = 6.5 min; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.40−7.36 (m, 2H), 7.19−7.15 (m, 3H), 7.02 (td, J = 7.5, 1.7 Hz, 1H), 6.98 (d, J = 8.3 Hz, 1H), 3.78 (s, 3H), 3.10−3.05 (m, 4H), 3.02 (q, J = 7.3 Hz, 4H), 1.32 (t, J = 7.3 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 156.6, 138.3, 135.1, 132.8, 130.9, 129.8, 129.6, 128.9, 128.0, 120.4, 111.0, 55.7, 55.6, 46.9, 40.2, 30.1, 9.2; HRMS (ESI+): calcd for $C_{19}H_{25}ClNO^+$ [M + H]$^+$, 318.1625; found, 318.1621.

*2-(6-Chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)-N,N-dipropylethan-1-amine (**2g**).* Compound **2g** was synthesized according to the general procedure of **2f**. Yield: 30%; HPLC: purity 100%, t_R = 7.9 min; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.43−7.38 (m, 2H), 7.22 (dd, J = 7.4, 1.2 Hz, 1H), 7.16−7.14 (m, 2H), 7.06 (t, J = 7.4 Hz, 1H), 7.02 (d, J = 8.4 Hz, 1H), 3.82 (s, 3H), 2.82−2.73 (m, 4H), 2.49 (t, J = 7.6 Hz, 4H), 1.52 (sext, J = 7.5 Hz, 4H), 0.91 (t, J = 7.4 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 156.80, 139.15, 137.52, 131.97, 131.36, 130.99, 129.31, 129.07, 128.95, 128.75, 120.36, 111.02, 56.14, 55.87, 55.65, 32.84, 20.29, 11.97; HRMS (ESI+): calcd for $C_{21}H_{29}ClNO^+$ [M + H]$^+$, 346.1938; found, 346.1934.

*2-Chloro-2′-methoxy-5-(2-nitrovinyl)-1,1′-biphenyl (**14**).* Ammonium acetate (3.91 mmol) was added to a solution of 6-chloro-2′-methoxy-[1,1′-biphenyl]-3-carbaldehyde **6** (3.91 mmol) in nitromethane (31.3 mL). The mixture was stirred for 6 h at 100 °C. After cooling down to rt, the reaction mixture was concentrated under reduced pressure and then the residue was purified by flash column chromatography (hexanes/EtOAc = 20:1) to afford the desired product **14** (2.59 mmol, 66.2%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.01 (d, J = 13.7 Hz, 1H), 7.61−7.56 (m, 2H), 7.50 (td, J = 8.2, 2.0 Hz, 2H), 7.45−7.43 (m, 1H), 7.21 (dd, J = 7.4, 1.6 Hz, 1H), 7.08 (t, J = 7.5 Hz, 1H), 7.04 (d, J = 8.3 Hz, 1H), 3.82 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 156.59, 139.20, 138.27, 137.96, 137.40, 132.28, 130.72, 130.50, 130.08, 128.84, 128.50, 127.08, 120.55, 111.08, 55.62.

*Diethyl 2-(1-(6-Chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)-2-nitroethyl)malonate (**15**).* Sodium hydride (1.38 mmol) was added to a solution of diethyl malonate (2.76 mmol) in THF (3 mL) and then the mixture was stirred for 15 min at rt. A solution of 2-chloro-2′-methoxy-5-(2-nitrovinyl)-1,1′-biphenyl **14** (0.69 mmol) in THF (2 mL) was then added to the mixture and the mixture was stirred for 3 h at rt. The reaction mixture was quenched by the addition of saturated solution of NH_4Cl and then extracted with EtOAc. The combined organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (hexanes/EtOAc = 10:1) to obtain the desired product **15** (0.33 mmol, 48.3%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.43−7.41 (m, 1H), 7.40−7.38 (m, 1H), 7.20 (d, J = 7.4 Hz, 2H), 7.17 (dd, J = 7.5, 1.8 Hz, 1H), 7.04 (t, J = 7.4 Hz, 1H), 7.00 (d, J = 8.4 Hz, 1H), 4.95 (dd, J = 13.2, 4.9 Hz, 1H), 4.88 (dd, J = 9.1, 13.3 Hz), 4.30−4.20 (m, 3H), 4.08 (q, J = 7.1 Hz, 2H), 3.83 (d, J = 9.2 Hz, 1H), 3.78 (s, 3H), 1.28 (t, J = 7.1 Hz, 3H), 1.11 (t, J = 7.1 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 167.33, 166.76, 156.67, 138.31, 134.56, 133.97, 130.92, 129.82, 129.64, 128.18, 127.84, 120.34, 111.08, 62.24, 62.04, 55.48, 54.89, 42.33, 13.96, 13.74.

*Ethyl 4-(6-Chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)-2-oxopyrrolidine-3-carboxylate (**16**).* To a solution of diethyl 2-(1-(6-chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)-2-nitroethyl)malonate **15** (3.8 mmol) and $NiCl_2{\cdot}6H_2O$ (3.8 mmol) in MeOH (30 mL) was added sodium borohydride (45.5 mmol) slowly at 0 °C. After the reaction mixture was stirred overnight at rt, the mixture was quenched with a saturated solution of NH_4Cl and extracted with DCM. The combined organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (DCM/MeOH = 20:1) to obtain the desired product **16** (1.78 mmol, 55%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.47 (d, J = 8.1 Hz, 1H), 7.45−

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7.40 (m, 1H), 7.23−7.19 (m, 3H), 7.06 (td, J = 7.4, 1.0 Hz, 1H), 7.02 (d, J = 8.3 Hz, 1H), 4.31−4.25 (m, 2H), 4.16 (q, J = 8.0 Hz, 1H), 3.89−3.86 (m, 1H), 3.82 (s, 3H), 3.57 (d, J = 9.3 Hz, 1H), 3.50−3.46 (m, 1H), 1.32 (t, J = 7.1 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 175.7, 169.9, 157.7, 147.0, 137.1, 130.8, 129.8, 129.3, 129.0, 128.6, 125.6, 121.5, 116.6, 60.9, 60.6, 56.1, 39.8, 29.4, 14.1.

*4-(6-Chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)pyrrolidin-2-one (**17**).* To the solution of ethyl 4-(6-chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)-2-oxopyrrolidine-3-carboxylate **16** (0.90 mmol) in EtOH (3 mL) was added 1 N NaOH (1 mL) at rt. After the mixture was stirred for an hour, the reaction mixture was concentrated under reduced pressure and the residue was added to 5 N HCl and the aqueous phase was partitioned with DCM. The combined organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure to obtain corresponding carboxylic acid (0.81 mmol, 90%). The solution of carboxylic acid (0.81 mmol) in toluene (10 mL) was refluxed at 140 °C for 6 h. After cooling down to rt, the mixture was concentrated under reduced pressure and the residue was purified by flash column chromatography (DCM/MeOH = 7:1) to obtain the desired product **17** (0.43 mmol, 67%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.42−7.35 (m, 2H), 7.17−7.14 (m, 3H), 7.01 (td, J = 7.5, 0.8 Hz, 1H), 6.98 (d, J = 8.3 Hz, 1H), 3.77 (s, 3H), 3.67 (t, J = 7.9 Hz, 1H), 3.44−3.40 (m, 1H), 2.73 (q, J = 8.6 Hz, 1H), 2.49 (q, J = 8.6 Hz, 1H), 2.28 (br s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.8, 157.7, 147.0, 137.1, 130.8, 129.8, 129.3, 129.0, 128.6, 126.4, 121.5, 116.6, 56.1, 43.3, 42.4, 32.9.

*3-(6-Chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)pyrrolidine (**3a**).* Aluminum chloride (3.38 mmol) in THF (2 mL) was mixed with lithium aluminum hydride 1.0 M in THF (3.38 mmol) at 0 °C. 4-(6-Chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)pyrrolidin-2-one **17** (0.85 mmol) was added dropwise to the mixture at 0 °C and then the reaction mixture was stirred at rt for an hour. After an hour, the reaction mixture refluxed at 80 °C overnight. The mixture was carefully quenched by the addition of MeOH at 0 °C and concentrated under reduced pressure. The residue was diluted by DCM and washed with water. The organic phase was partitioned with 1 N HCl aqueous solution. The combined aqueous layer was washed with DCM and neutralized by the addition of 10 N solution of NaOH. The mixture was extracted with DCM and the combined organic layer was dried over Na_2SO_4, filtered, and concentrated under reduced pressure to afford the desired product **3a** (0.62 mmol, 73%); HPLC: purity 100%, t_R = 6.4 min; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.34 (br s, 1H), 7.44−7.37 (m, 2H), 7.29 (t, J = 9.0 Hz, 1H), 7.24−7.19 (m, 1H), 7.17−7.14 (m, 1H), 7.02 (dd, J = 7.4, 3.1 Hz, 1H), 6.97 (d, J = 8.3 Hz, 1H), 3.78 (d, J = 11.5 Hz, 3H), 3.64−3.50 (m, 2H), 3.45−3.37 (m, 1H), 3.31−3.20 (m, 1H), 2.48−2.39 (m, 1H), 2.13 (sep, J = 10.5 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 156.63, 139.27, 138.32, 136.69, 133.16, 130.89, 129.86, 128.53, 127.18, 125.58, 120.43, 111.03, 55.61, 53.48, 50.55, 45.19, 43.75, 43.08, 32.43; HRMS (ESI+): calcd for $C_{17}H_{19}ClNO^+$ $[M + H]^+$, 288.1155; found, 288.1153.

*3-(6-Chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)-1-methylpyrrolidine (**3b**).* A solution of 3-(6-chloro-2′-methoxy-[1,1′-biphenyl]-3-yl)pyrrolidine **3a** (0.45 mmol) in formic acid (3.16 mmol, 88% in water solution) and formaldehyde (3.16 mmol, 37% in water solution) was stirred at 80 °C for 20 h. After cooling down to rt, the reaction mixture was diluted with water, adjusted to pH 10 with K_2CO_3, and extracted with DCM. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by flash column chromatography (DCM/MeOH = 10:1) to obtain the desired product **3b** (0.17 mmol, 37%); HPLC: purity 100%, t_R = 6.2 min; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.13 (s, 1H), 7.41−7.36 (m, 2H), 7.23−7.16 (m, 3H), 6.97 (dd, J = 8.4, 3.3 Hz, 1H), 3.79 (d, J = 4.3 Hz, 3H), 3.48−3.40 (m, 1H), 3.11 (t, J = 8.7 Hz, 1H), 2.89 (t, J = 7.2 Hz, 1H), 2.73 (q, J = 7.7 Hz, 1H), 2.59 (t, J = 8.8 Hz, 1H), 2.47 (s, 3H), 2.43−2.34 (m, 1H), 1.98−1.90 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 156.63, 139.27, 138.32, 136.69, 133.16, 130.89, 129.86, 128.53, 127.18, 125.58, 120.43, 111.03, 55.61, 53.48, 50.55, 45.19, 43.75, 43.08, 32.43; HRMS (ESI+): calcd for $C_{19}H_{23}ClNO_2^+$ $[M + H]^+$, 332.1417; found, 332.1411.

Experimental Procedure for Binding Affinity Assay. All of the binding affinity data were generously provided by the US National Institute of Mental Health (NIMH) Psychoactive Drug Screening Program (University of North Carolina, US). Eleven dilutions (5× assay concentration) of the test and reference compounds were prepared in standard binding buffer [50 mM tris(hydroxymethyl)-aminomethane-HCl (Tris-HCl), 10 mM $MgCl_2$, 1 mM ethylenediaminetetraacetate, and pH 7.4] by serial dilution: 0.05 nM, 0.5 nM, 1.5 nM, 5 nM, 15 nM, 50 nM, 150 nM, 500 nM, 1.5 μM, 5 μM, and 50 μM. The [^{3}H]LSD radioligand was diluted to 5 times the assay concentration in standard binding buffer. Aliquots (50 mL) of the radioligand were dispensed into the wells of a 96-well plate containing 100 mL of standard binding buffer. Triplicate aliquots (50 mL) of the test and reference compound dilutions were then added. Finally, crude membrane fractions (50 mL) of cells expressing a recombinant target were dispensed into each well. Totally, 250 mL of the reaction mixtures was incubated at rt and shielded from light for 1.5 h and then harvested by rapid filtration onto Whatman GF/B glass fiber filters presoaked with 0.3% polyethyleneimine, by using a 96-well Brandel harvester.

Four rapid washes were performed with chilled standard binding buffer (500 mL) to decrease nonspecific binding. Filters were placed in 6 mL scintillation tubes and allowed to dry overnight. The next day, 4 mL of EcoScint scintillation cocktail (National Diagnostics) was added to each tube. The tubes were capped, labeled, and counted by liquid scintillation counting. The filter mats were dried, and the scintillant was melted onto the filters, and then the radioactivity retained on the filters was counted in a MicroBeta scintillation counter. The IC_{50} values were obtained by using the Prism 4.0 program (GraphPad software) and converted into K_i values. Each compound was tested in triplicate at least.

Gs Protein-Mediated cAMP Assay. HEK293 cells were harvested in 150 mm dishes and the medium was changed from Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin to DMEM with 10% dialyzed FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. After 5−6 h, those cells were transfected (via calcium phosphate) with 10 μg of human 5-HT$_7$R plasmid and 10 μg of GloSensor-22F plasmid (Promega). Transiently transfected HEK293 cells were seeded (20,000−30,000 cells/20 μL/well) into white, clear-bottom 384-well plates (Greiner) in the same medium. After 5−6 h of recovery, the medium was removed from the wells and the cells were treated with 3% GloSensor cAMP reagent, luciferin (Promega) 20 μL in filter-sterilized assay buffer including 1× Hanks' balanced salt solution (HBSS), 20 mM *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid (HEPES), and 3rd distilled water, pH 7.4. Then, the reference agonist (5-HT, 400 nM) or the compounds to be tested were prepared by serial dilution (0.04 nM, 0.12 nM, 0.4 nM, 1.2 nM, 4 nM, 12 nM, 40 nM, 120 nM, 400 nM, 1.2 μM, 4 μM, 12 μM, 40 μM, and 120 μM) in the above assay buffer with 0.1% bovine serum albumin. After 30 min, the cells were treated with 10 μL of drugs prepared above (the final ligand concentrations are 100 nM of serotonin and 0.01 nM, 0.03 nM, 0.1 nM, 0.3 nM, 1 nM, 3 nM, 10 nM, 100 nM, 300 nM, 1 μM, 3 μM, 10 μM, and 30 μM of test compounds). The luminescence intensity of the accumulated cAMP level was measured by using a microplate reader (FlexStation 3 or SpectraMax i3, Molecular Devices). The sigmoidal dose−response graph of the obtained data was obtained by using the Prism 6.0 program (GraphPad software) to calculate the EC_{50} and IC_{50} values. Each compound was tested in triplicate at least.

β-Arrestin Recruitment Tango Assay. HTLA cells (a HEK293 cell line stably expressing a tTA-dependent luciferase reporter and a β-arrestin 2−TEV fusion gene) were plated in 150 mm dishes and the medium was changed from DMEM with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 μg/mL puromycin, and 100 μg/mL hygromycin B to DMEM with 10% dialyzed FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin to transfect the plasmid. After 5−6 h, those HTLA cells were transfected (via calcium phosphate) with 20 μg of a 5-HT$_7$R-TCS-tTA construct. The next day, transiently transfected HTLA cells were plated in white, clear-bottom, 384-well plates (Greiner; 30,000−40,000 cells/well, 50 μL/well) in DMEM containing 1% dialyzed FBS, 100 U/mL penicillin, and 100 μg/mL. After 6 h, the cells were challenged with 10 μL/well of reference agonist (serotonin, 6 μM) or the compounds to be tested prepared by serial

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dilution (0.06 nM, 0.18 nM, 0.6 nM, 1.8 nM, 6 nM, 18 nM, 60 nM, 180 nM, 600 nM, 1.8 μM, 6 μM, 18 μM, 60 μM, and 180 μM) in HBSS, 20 mM HEPES, and pH 7.4 (the final ligand concentrations are 1 μM of serotonin and 0.01 nM, 0.03 nM, 0.1 nM, 0.3 nM, 1 nM, 3 nM, 10 nM, 30 nM, 100 nM, 300 nM, 1 μM, 3 μM, 10 μM, and 30 μM of test compounds) and incubated for about 22 h. The medium was removed and replaced with 1× BrightGlo reagent (Promega), and luminescence was read using a SpectraMax i3 (Molecular Devices). The sigmoidal dose−response graph of the obtained data was obtained by using the Prism 6.0 program (GraphPad software) to calculate the EC_{50} and IC_{50} values. Each compound was tested in triplicate at least.

CYP450 Assay. All of CYP450, microsomal stability, and PK data were provided by the New Drug Development Center or Institutional Animal Care and Use Committees (IACUC) in Daegu-Gyeongbuk Medical Innovation Foundation (DGMIF) (Daegu, Republic of Korea). To human liver microsomes (0.25 mg/mL), 0.1 M phosphate buffer (pH 7.4), a cocktail of five probe substrates (phenacetin 50 μM, diclofenac 10 μM, S-mephenytoin 100 μM, dextromethorphan 5 μM, and midazolam 2.5 μM), and tested compounds were added at concentrations of 0 μM (as a control) and 10 μM. After incubation at 37 °C for 5 min, reduced nicotinamide adenine dinucleotide phosphate (NADPH) generation system solution was also added and incubated at 37 °C for 15 min again. To terminate the reaction, acetonitrile including the internal standard (terfenadine) was added and the solution was centrifuged for 5 min (14,000 rpm, 4 °C). The supernatant was then injected into the LC/MS system to simultaneously analyze the metabolites of the probe substrates and evaluate the % CYP inhibition of the tested compounds.

Microsomal Stability Assay. To human liver microsomes (0.5 mg/mL), 0.1 M phosphate buffer (pH 7.4) and tested compounds (1 μM) were added. After incubation at 37 °C for 5 min, the NADPH generation system solution was also added and incubated at 37 °C for 30 min again. To terminate the reaction, acetonitrile including internal standard (chloprapamide) was added and the solution was centrifuged for 5 min (14,000 rpm, 4 °C). The supernatant was then injected into the LC/MS system to analyze the microsomal stability of the tested compounds.

PK Studies. All animal experiments were evaluated and approved by the DGMIF IACUC. ICR mice (7−8 weeks of age) weighing 30 ± 5 g were used for the PK and tissue distribution studies and were purchased from Orient Co. (South Korea). The mice were kept at rt controlled at 23 ± 3 °C with relative humidity controlled at about 55 ± 10%, fed with standard solid composite feedstuff, and received tap water. Compound **2b** at a dose of 1 or 10 mg/kg was administered intravenously or intraperitoneally, respectively, to male ICR mice. Blood samples were collected via carotid artery at 0 (to serve as a control), 0.08 (IV only), 0.25, 0.5, 1, 2, 4, 6, and 8 h after administration of each compound. After centrifugation at 12,000 rpm for 3 min, plasma samples were stored at −70 °C until analysis. PK parameters were determined by a non-compartmental analysis using WinNonlin v6.4 (Pharsight Corporation, Mountain View, CA) program. The total area under the plasma concentration−time curve from time zero to the last measured time (AUC_{last}) was calculated by the trapezoidal rule-extrapolation method. Standard methods were used to calculate the following PK parameters:[46,47] the time-averaged total body clearance (CL), total area under the first moment of plasma concentration and time curve from time zero to time infinity ($AUC_{0-\infty}$), terminal half-life, mean residence time (MRT), and apparent volume of distribution at steady state (V_{dss}). The concentrations of each compound in the above samples were analyzed using LC−MS/MS. To a 20 μL aliquot of plasma sample, an 80 μL aliquot of acetonitrile containing 2 μM of internal standard (chloropropramide) was added. After vortex mixing and centrifugation at 15,000 rpm for 5 min, a 2 μL of supernatant was injected into the LC−MS/MS system. The LC−MS/MS system consisted of an Agilent 1290 infinity series HPLC system (Agilent, Santa Clara, CA) and API5500 triple-quadrupole mass spectrometer (Applied Biosystems-SCIEX, Concord, Canada). The HPLC mobile phases consisted of 0.1% formic acid in 100% deionized water (A) and 0.1% formic acid in 100% acetonitrile (B). Chromatographic separation was achieved on a reversed-phase Kinetex C18 column (100 × 2.1 mm, 1.7 μm, Phenomenex) using gradient elution at a flow rate of 0.3 mL/min. The lower limit of quantitation of each compound in rat plasma was 100 ng/mL. The values of coefficients of correlation (R) were more than 0.9971.

Self-Grooming Behavior Test. All animal experiments were conducted according to the guidelines of the KIST IACUC. WT and *Shank3* TG male mice, 7 to 9 week old, were obtained from KIST (Korea Institute of Science and Technology, Seoul, Korea). They were housed at a temperature of 22 ± 2 °C with a humidity of 55 ± 5% and maintained in a 12/12 h light/dark cycle under 5 dB. All mice had ad libitum access to food and water. Drugs were dissolved in 5% dimethyl sulfoxide in saline solution and injected into the peritoneal cavity before 30 min starting the experiment. Each mouse was placed in a 30 × 20 cm opaque acrylic container with 1−2 cm bedding on the floor under 40 lux lighting. The total duration and number of self-grooming behavior were recorded for 30 min. After the test, the container was cleaned with 70% ethanol, and the floor was covered with new bedding for the next mouse recording. Analyze the video by stopwatch program with the function of snap, being careful not to count sniffing behavior. In case of co-treatment, after 10 min of SB269970 injection, **2b** was administrated. The behavior started to be recorded after 30 min of **2b** injection.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.1c00110.

^{1}H and ^{13}C NMR spectra, HPLC spectra, dose−response curves, pIC_{50} and IC_{50} values, and pK_i ± SEM values (PDF)

Molecular formula strings (CSV)

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Author Contributions

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AC, adenylyl cyclase; $AlCl_3$, aluminum chloride; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HBSS, Hanks' balanced salt solution; $LiAlH_4$, lithium aluminum hydride; Boc_2O, di-*tert*-butyl dicarbonate; cAMP, cyclic adenosine monophosphate; CNS, central nervous system; CYP450, cytochrome P450; I_2, iodine; EC_{50}, half maximal effective concentration; H_2SO_4, sulfuric acid; GPCRs, G protein-coupled receptors; HEK293, human embryonic kidney cells 293; [^{3}H]LSD, [^{3}H]lysergic acid diethylamide; 5-HT, 5-hydroxytryptamine; 5-HT_7R, 5-HT_7 receptor; HPP, hydrophobic binding pocket; HTLA, HEK293-derived cell line containing stable integrations of a tTA-dependent; IC_{50}, half maximal inhibitory concentration; K_i, binding affinity value; $NaIO_3$, sodium iodate; $Pd(PPh_3)_4$, tetrakis-(triphenylphosphine)palladium(0); PKs, pharmacokinetic parameters; $NaBH(OAc)_3$, sodium triacetoxyborohydride; NaH, sodium hydride; SAR, structure−activity relationship; $NaHCO_3$, sodium bicarbonate; K_2CO_3, potassium carbonate; THF, tetrahydrofuran; DMF, *N*,*N*-dimethylformamide; NaCN, sodium cyanide; $NaBH_4$, sodium borohydride; $NiCl_2{\cdot}6H_2O$, nickel(II) chloride hexahydrate

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