Journal of Medicinal Chemistry



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*J. Med. Chem.*, **Just Accepted Manuscript** • DOI: 10.1021/acs.jmedchem.8b00642 • Publication Date (Web): 20 Jul 2018

# Discovery of β-arrestin biased ligands of 5-HT$_7$R

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**Abstract.** Though many studies have been published about therapeutic potentials of selective 5-$HT_7$R ligands, there have been few biased ligands of 5-$HT_7$R. The development of potent and selective biased ligands of 5-$HT_7$R would be of great help in understanding the relationship between pharmacological effects and G protein/β-arrestin signaling pathways of 5-$HT_7$R. In order to identify 5-$HT_7$R ligands with biased agonism, we designed and synthesized a series of tetrahydroazepine derivatives **1** and **2** with arylpyrazolo moiety or arylisoxazolo moiety. Through several biological evaluations such as binding affinity, selectivity profile, and functions in G protein and β-arrestin signaling pathways, (3-(4-chlorophenyl)-1,4,5,6,7,8-hexahydropyrazolo[3,4-d]azepine) **1g** was discovered as the β-arrestin biased ligand of 5-$HT_7$R. In electroencephalogram (EEG) test, **1g** increased total non-rapid eye movement (NREM) sleep time and decreased total rapid eye movement (REM) sleep time.

## ■ INTRODUCTION

G protein-coupled receptors (GPCRs) are seven transmembrane helical proteins and there are over 800 members of GPCRs in the human genome with diverse structures and functions.[1-4] These receptors are generally involved in regulation of various physiological signal transductions through G proteins stimulated by diverse ligands including biogenic amines, amino acids, and peptides.[5-7] Since GPCRs mediate many cellular signaling pathways, they have become targets of numerous therapeutic drugs and still considered as one of the most important classes of pharmacological targets.[8] However, there have been difficulties in elucidating *in vivo* pharmacological effects of GPCR ligands through an *in vitro* assay for G protein activation, which resulted in limitations in discovering GPCR drugs.[9-11] As GPCR biology advances, these problems have been shown to be associated with the complexity of the GPCR-mediated signaling. Particularly, not only G proteins but also non-G protein effectors such as β-arrestin are involved in GPCR signaling. Thus, to understand the mechanism of GPCR drugs and to develop more efficacious drugs with fewer side effects, ligands with functional selectivity or signaling bias has been actively pursued. The so-called "functionally selective" or "biased" ligands are believed to stimulate either G protein signaling pathway or β-arrestin signaling pathway independently by binding and stabilizing different conformations of GPCR resulting in ligand-specific cellular responses.

5-$HT_7$ receptor (5-$HT_7$R) is the most recently identified serotonin receptor among serotonin receptor subtypes, which is mainly detected in central nervous system (CNS) including prefrontal cortex, spinal cord, thalamus, hippocampus, hypothalamus, and suprachiasmatic nucleus.[12] There are four isoforms of 5-$HT_7$R such as 5-$HT_{7a}$R, 5-$HT_{7b}$R, 5-$HT_{7c}$R, and 5-$HT_{7d}$R. All of 5-$HT_7$R isoforms belong to GPCRs and are coupled to Gs protein, which activate adenylyl cyclase (AC) with a resultant increase of the intracellular level of a

secondary messenger cyclic AMP (cAMP).[13-18] The pathophysiological function of 5-$HT_7$R is known to be associated with circadian rhythm, learning and memory as well as several diseases such as migraine, mental fatigue, sleep disturbance, anxiety, depression, and schizophrenia.[19-21]

Until now, many potent 5-$HT_7$R ligands have been reported and classified according to activities on G protein signaling pathway, the two main classes being agonists (AS-19,[22,23] E55888,[24] 8OH-DPAT,[25] and LP-211[26]) and antagonists (SB269970,[27] SB258719,[28,29] DR4485,[30] and JNJ18038683[31]) (Figure 1). It was reported that 5-$HT_7$R antagonists, SB269970 and JNJ18038683, showed antidepressant-like activities in forced swimming test (FST).[31] In addition, administration of the 5-$HT_7$R antagonists caused modulation of sleep patterns, especially decrease of rapid eye movement (REM) duration and increase of REM latency.[32] However, there have been controversial results about *in vivo* effects of 5-$HT_7$R agonists and antagonists. LP-211, a 5-$HT_7$R agonist, induced REM sleep suppression like 5-$HT_7$R antagonists,[33,34] while both LP-211 and SB269970 (an antagonist) exhibited anxiolytic-like effects.[35,36] Although the role of 5-$HT_7$R may depend on brain regions and neurochemical environment, the controversial pharmacological effects of 5-$HT_7$R ligands might be explained by concurrent elucidation of their activities on β-arrestin signaling pathway: 5-$HT_7$R ligands could be evaluated on signaling pathways of G protein and β-arrestin, and categorized as G protein biased ligands, β-arrestin biased ligands, and balanced agonists/antagonists on both pathways. It was reported that 5-$HT_7$R indeed activates not only G protein signaling pathway but also β-arrestin signaling pathway.[37]

Recently, ergotamine and a compound with indole-aripiprazole moiety were reported as β-arrestin biased ligand of 5-$HT_7$R,[38,39] which, however, were not selective 5-$HT_7$R ligands. Biased ligands which selectively bind to 5-$HT_7$R have not been developed yet. Thus,

developing potent and selective biased ligands against 5-HT$_7$R would be of great help in understanding the relationship between physiological effects and G protein/β-arrestin signaling pathways of 5-HT$_7$R. In this study, we designed and synthesized tetrahydroazepine derivatives **1** and **2** to discover novel G protein or β-arrestin biased ligands, resulting in discovery of the β-arrestin biased ligands of 5-HT$_7$R which modulated sleep patterns like SB-269970.

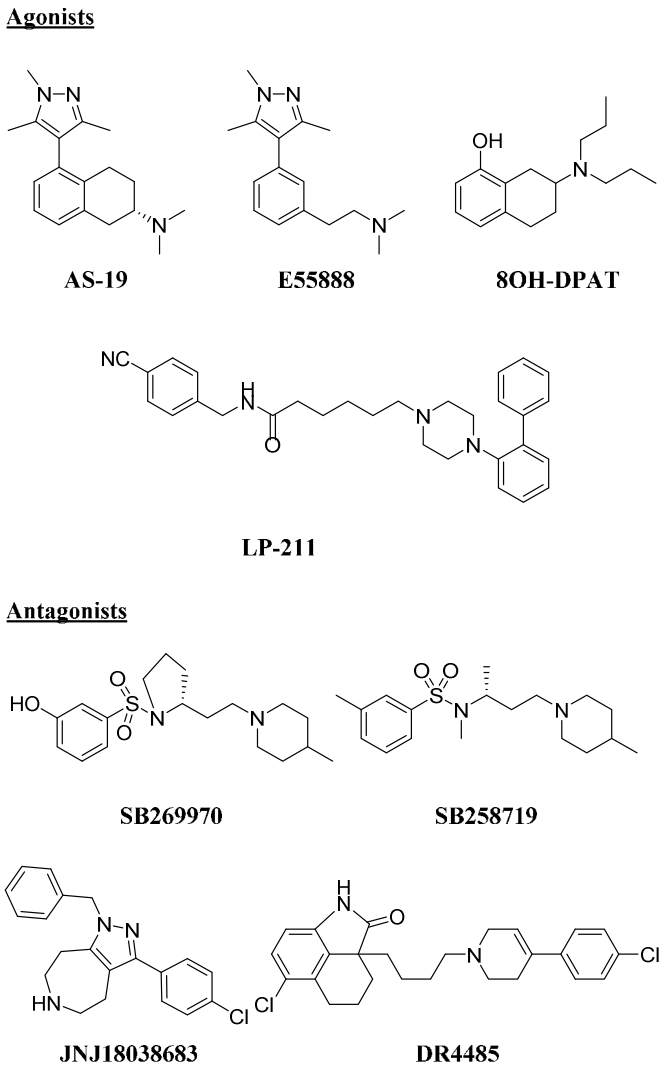



**Figure 1.** Structures of representative 5-HT$_7$R agonists and antagonists

## ■ RESULTS AND DISCUSSION

Due to the lack of 5-$HT_7$R biased ligands, the approach to develop a novel biased ligand entailed of identifying the common structural features of the known 5-$HT_7$R ligands. Two agonists (AS-19 and E55888) and one antagonist (JNJ18038683) (Figure 2) were chosen as the representatives for 5-$HT_7$R ligands, which showed two common features such as a biaryl group (red color in Figure 2) and ethyleneamine moiety (blue in Figure 2). Based on these common features, we designed tetrahydropyrazoloazepines **1** and tetrahydroisoxazoloazepines **2** which have various aryl substituent (R) including hydrogen, halogen and methyl group.

**Synthesis.** The tetrahydropyrazoloazepine derivatives **1** with R substituents were synthesized in total 6 steps (R = H, 2-F, 3-F, 4-F, 2-Cl, 3-Cl, 4-Cl, 2-Me, 3-Me, and 4-Me) (Scheme 1).[40] 1-*Boc*-4-piperidone **3** underwent ring expansion by reaction with ethyldiazoacetate and $BF_3 \cdot OEt_2$ at -20°C to give oxoazepane **4**, which was reacted with hydrazine to afford dihydropyrazolone **5** (97% yield in two steps). The dihydropyrazolone **5** underwent *Boc* protection of the secondary amino group in dihydropyrazolone moiety in 63% yield, and the resulting compound **6** was treated with *N*-phenyl-bis(trifluoromethanesulfonimide) ($Tf_2NPh$) under basic conditions to give a triflate **7** in 93% yield. We confirmed the structure of the compound **7** through X-ray crystallographic analysis (Supplementary Figure S1). Suzuki coupling reactions between **7** and variously substituted phenylboronic acids were carried out by using palladium catalyst, $Pd(dppf)Cl_2$, to afford *Boc*-protected tetrahydropyrazoloazepines **8a**~**8j** in 11-47% yields. Finally, **8a**~**8j** were treated with 1N HCl to obtain the desired aryl tetrahydropyrazoloazepines **1a**~**1j** in 16-81% yields.

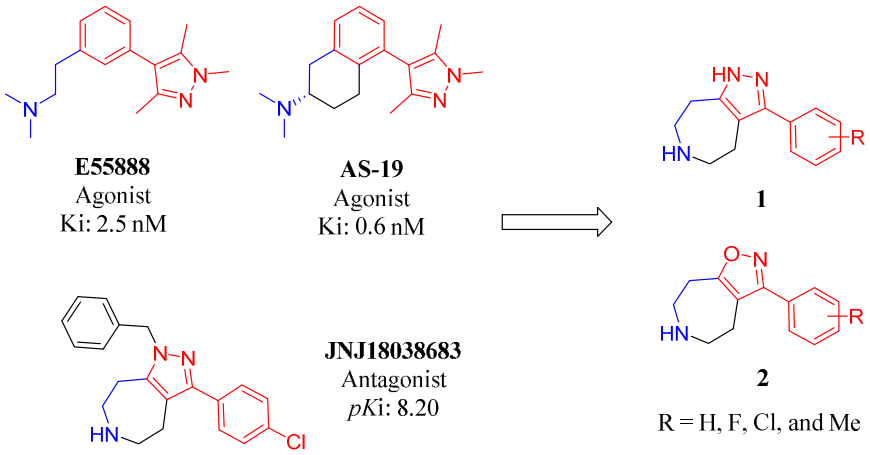
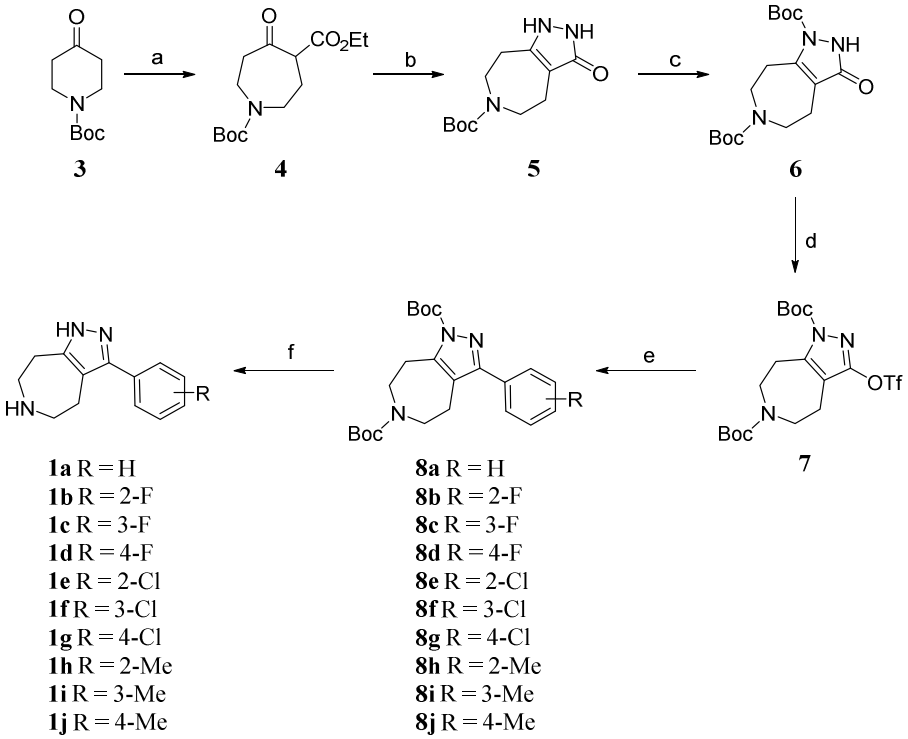



**Figure 2.** Designed tetrahydropyrazoloazepines **1** and tetrahydroisoxazoloazepines **2**

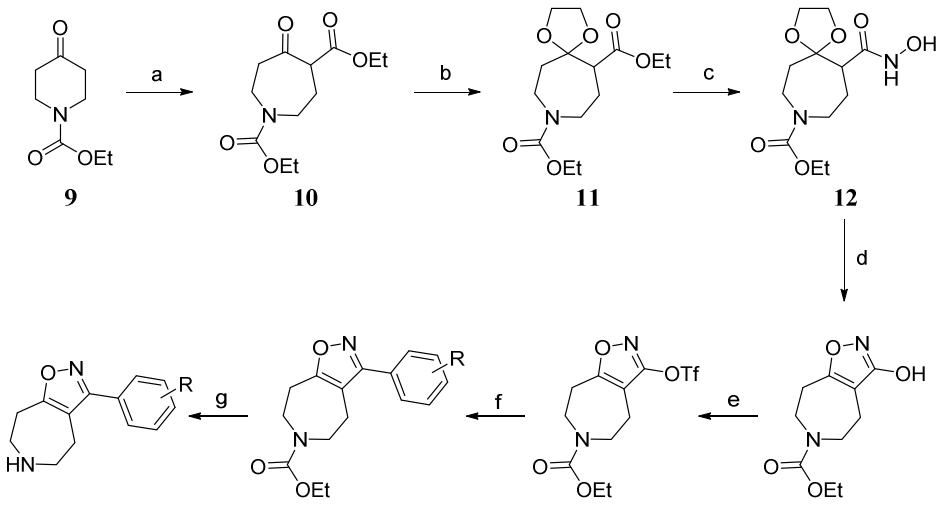
We also synthesized the aryl tetrahydroisoxazoloazepine derivatives **2** with various substituents (R) in 7 steps starting from ethyl 4-oxopiperidine-1-carboxylate **9** (Scheme 2). The 4-oxopiperidine **9** underwent ring expansion followed by acetal protection to give **11** in 46% yield in two steps. The compound **11** was treated with hydroxylamine to afford **12** in 31% yield, which underwent deprotection of acetal group followed by subsequent cyclization under acidic conditions to give 3-hydroxyisoxazole **13** in 94% yield.[40] The 3-hydroxyisoxazole **13** was treated with $TF_2NPh$ to give the corresponding triflate **14** in 86% yield. Suzuki coupling of **14** with various phenylboronic acids afforded aryl tetrahydroisoxazoloazepines **15a**~**15j** in 19-33% yields. After deprotection of ethyl carbamate group by treatment with HBr in acetic acid, the final compounds **2a**~**2j** were obtained in 15-70% yields.

**Scheme 1.** Synthesis of tetrahydropyrazoloazepine **1a-1j**[a]




[a]*Reagents and conditions*: a) ethyldiazoacetate, $BF_3OEt_2$, $Et_2O$, -20 °C to rt; b) hydrazine, EtOH, reflux, 97% in 2 steps; c) $Boc_2O$, TEA, DCM, DMF, rt, 63%; d) $Tf_2NPh$, DIPEA, DCM, rt, 93%; e) R-$PhB(OH)_2$, $Pd(dppf)Cl_2$, $K_2PO_4$, THF, reflux, 11-47%; f) i) 1 N HCl in $Et_2O$, DCM, MeOH, ii) 10 N NaOH, rt, 16-81%.

**Scheme 2.** Synthesis of tetrahydroisoxazoloazepines **2a-2j**[a]




| | |
|---|---|
| **2a** R = H | **15a** R = H |
| **2b** R = 2-F | **15b** R = 2-F |
| **2c** R = 3-F | **15c** R = 3-F |
| **2d** R = 4-F | **15d** R = 4-F |
| **2e** R = 2-Cl | **15e** R = 2-Cl |
| **2f** R = 3-Cl | **15f** R = 3-Cl |
| **2g** R = 4-Cl | **15g** R = 4-Cl |
| **2h** R = 2-Me | **15h** R = 2-Me |
| **2i** R = 3-Me | **15i** R = 3-Me |
| **2j** R = 4-Me | **15j** R = 4-Me |

[a]*Reagents and conditions*: a) ethyldiazoacetate, $BF_3OEt_2$, $Et_2O$, -20 °C to rt; b) ethylene glycol, p-TsOH, benzene, reflux, 46% in 2 steps; c) $NH_2OH$/HCl, Na, MeOH, 40 °C to rt, 31%; d) HCl, MeOH, reflux, 94%; e) $Tf_2NPh$, DIPEA, DCM, rt, 86%; f) R-$PhB(OH)_2$, $Pd(dppf)Cl_2$, $K_2PO_4$, KBr, THF, reflux, 19-33%; g) i) HBr in AcOH, ii) 1 N NaOH, rt, 15-70%.

**Binding affinities.** Binding affinities ($K_i$'s) of the synthesized tetrahydropyrazoloazepines **1** and tetrahydroisoxazoloazepines **2** against 5-$HT_7$R were determined by [$^3$H]LSD radioligand binding assay in transfected HEK293 cells (Table 1). SB269970 was used as a reference compound. Compound **1a** without any substituent in phenyl moiety showed moderate binding affinity with a $K_i$ value of 322 nM. Among **1b-1j** (R = F, Cl, Me), the *ortho*-substituted compounds **1b**, **1e,** and **1h** showed moderate binding affinities with $K_i$ values of 714 nM, 386 nM, and 881 nM, respectively. Compounds **1c**, **1f**, and **1i** with R's at *meta*-position were less

active than the corresponding *ortho*-substituted congeners, and they showed $K_i$ values of 1,243 nM, 883 nM, and 1,436 nM, respectively. On the other hands, the *para*-substituted compounds **1d**, **1g,** and **1j** showed significantly improved binding affinities with $K_i$ values of 104 nM, 30 nM, and 47 nM, respectively. These results clearly show that the position of a substituent in phenyl ring is very important in the binding affinity to 5-$HT_7$R. Specifically, introduction of a substituent at the *para*-position of the aromatic ring potentiated binding of the resulting compounds (**1d**, **1g**, and **1j**) to 5-$HT_7$R. Among the *para*-substituted compounds, **1g** (R = *p*-Cl) and **1j** (R = *p*-Me) showed better binding affinities than the *para*-fluorinated compound **1d**. Considering the similar size of chlorine and methyl group, it could be suggested that size of the *para*-substituent matters for binding to 5-$HT_7$R.

Next, we introduced an isoxazole group instead of the pyrazole group to investigate changes in binding affinity to 5-$HT_7$R. Most of the compounds **2** showed moderate to potent binding affinities against 5-$HT_7$R with exception of **2h** ($K_i$ > 10,000 nM) and **2c** ($K_i$ = 1,407 nM). As in the case of **1**, *para*-substituted compounds **2d**, **2g**, and **2j** showed much better binding affinities than *ortho*-substituted (**2b**, **2e**, and **2h**) and *meta*-substituted (**2c**, **2g**, and **2j**) counterparts. The binding affinities ($K_i$'s) of *para*-substituted compounds **2d**, **2g**, and **2j** were 53 nM, 70 nM, and 16 nM, respectively. Like **1a**, the compound **2a** with no substitution at the phenyl group showed only moderate binding affinity against 5-$HT_7$R with a $K_i$ value of 319 nM.

**Table 1.** Binding affinities of tetrahydropyrazoloazepines **1** and tetrahydroisoxazoloazepines **2** to 5-$HT_7$R

| Compound | X | R | 5-$HT_7$R | |
|---|---|---|---|---|
| | | | p$K_i$[a] | $K_i$[b] in nM |
| **1a** | NH | H | 6.49 ± 0.07 | 322 |
| **1b** | | 2F | 6.15 ± 0.05 | 714 |
| **1c** | | 3F | 5.91 ± 0.05 | 1,243 |
| **1d** | | 4F | 6.98 ± 0.06 | 104 |
| **1e** | | 2Cl | 6.41 ± 0.05 | 386 |
| **1f** | | 3Cl | 6.05 ± 0.05 | 883 |
| **1g** | | 4Cl | 7.50 ± 0.10 | 30 |
| **1h** | | 2Me | 6.05 ± 0.05 | 881 |
| **1i** | | 3Me | 5.84 ± 0.06 | 1,436 |
| **1j** | | 4Me | 7.33 ± 0.06 | 47 |
| **2a** | O | H | 6.50 ± 0.06 | 319 |
| **2b** | | 2F | 6.44 ± 0.08 | 366 |
| **2c** | | 3F | 5.85 ± 0.06 | 1,407 |
| **2d** | | 4F | 7.28 ± 0.06 | 53 |
| **2e** | | 2Cl | 6.37 ± 0.05 | 425 |
| **2f** | | 3Cl | 6.43 ± 0.05 | 369 |
| **2g** | | 4Cl | 7.15 ± 0.05 | 70 |
| **2h** | | 2Me | --[c] | --[c] |
| **2i** | | 3Me | 6.30 ± 0.05 | 506 |
| **2j** | | 4Me | 7.79 ± 0.05 | 16 |
| SB269970 | | | 9.47 ± 0.05 | 0.34 |

[a]Values are the mean ± SEM of at least three independent experiments performed in triplicate.
[b]Values calculated from p$K_i$.
[c]Not determined due to <50% inhibition at 10 μM of the compound against 5-$HT_7$R.

**Selectivity Profiles.** The best 5-$HT_7$R binders (**1d**, **1g**, **1j**, **2d**, **2g**, and **2j**) were then evaluated for their selectivity over other serotonin receptor subtypes 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-$HT_4$R, 5-$HT_{5A}$R, and 5-$HT_{6R}$. The selectivity for 5-$HT_7$R over other serotonin receptor subtypes was expressed as selectivity index (SI) which can be calculated by dividing $K_i$ for other serotonin receptor subtypes by $K_i$ for 5-$HT_7$R (Table 2). Compound **1d** showed good selectivity (SI > 5) over 5-$HT_{1A}$R, 5-$HT_{1B}$R, 5-$HT_{1D}$R, 5-$HT_{1E}$R, 5-$HT_3$R, 5-$HT_4$R, and 5-$HT_{5A}$R. However, **1d** exhibited no selectivity (SI < 2) or only moderate (2 < SI < 5) 5-$HT_7$R selectivity over 5-

$HT_{2A}R$, 5-$HT_{2B}R$, 5-$HT_{2C}R$, and 5-$HT_6R$. Compound **1g** was very selective to almost 5-HTR subtypes except 5-$HT_{2B}R$, over which it showed only moderate selectivity for 5-$HT_7R$. Compound **1j** also showed good selectivity over 5-HTR subtypes, except 5-$HT_{2A}R$, 5-$HT_{2B}R$, and 5-$HT_6R$. In the case of the isoxazoles, compound **2d** showed the best selectivity profiles with all SI values over 5, while compounds **2g** and **2j** showed good selectivity to almost 5-HTR subtypes, except 5-$HT_{2B}R$, 5-$HT_{2C}R$, and/or 5-$HT_3R$. Compound **2g** showed moderate selectivity over 5-$HT_{2B}R$ and 5-$HT_3R$, and compound **2j** showed very low selectivity over 5-$HT_{2C}R$ with an SI value of 1.3. Based on selectivity profiles, the compounds **1g** and **2d** were considered as the most potent and selective ligands for 5-$HT_7R$.

**Table 2.** Selectivities of compounds **1d**, **1g**, **1j**, **2d**, **2g**, and **2j** over other serotonin receptor subtypes

| 5-HTR subtypes | **1d** | | **1g** | | **1j** | | **2d** | | **2g** | | **2j** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $K_i$[a] in nM | SI[b] | $K_i$[a] in nM | SI[b] | $K_i$[a] in nM | SI[b] | $K_i$[a] in nM | SI[b] | $K_i$[a] in nM | SI[b] | $K_i$[a] in nM | SI[b] |
| 5-$HT_7$ | 104 | 1.0 | 30 | 1.0 | 47 | 1.0 | 53 | 1.0 | 70 | 1.0 | 16 | 1.0 |
| 5-$HT_{1A}$ | 1,866 | 18.0 | > 10,000 | --[c] | > 10,000 | --[c] | > 10,000 | --[c] | > 10,000 | --[c] | > 10,000 | --[c] |
| 5-$HT_{1B}$ | > 10,000 | --[c] | > 10,000 | --[c] | > 10,000 | --[c] | > 10,000 | --[c] | > 10,000 | --[c] | > 10,000 | --[c] |
| 5-$HT_{1D}$ | 636 | 6.1 | 2,112 | 70.5 | 165 | 3.5 | > 10,000 | --[c] | > 10,000 | --[c] | 147 | 9.2 |
| 5-$HT_{1E}$ | 793 | 7.6 | 1,366 | 45.5 | 890 | 18.9 | > 10,000 | --[c] | > 10,000 | --[c] | 1,787 | 111.7 |
| 5-$HT_{2A}$ | 175 | 1.7 | 187 | 6.2 | 159 | 3.4 | 471 | 8.9 | 399 | 5.7 | 138 | 8.6 |
| 5-$HT_{2B}$ | 97 | 0.9 | 100 | 3.3 | 106 | 2.3 | 505 | 9.5 | 247 | 3.5 | 150 | 9.4 |
| 5-$HT_{2C}$ | 200 | 1.9 | 799 | 26.6 | 296 | 6.3 | 785 | 14.8 | 1,099 | 15.7 | 20 | 1.3 |
| 5-$HT_3$ | > 10,000 | --[c] | > 10,000 | --[c] | > 10,000 | --[c] | 703 | 13.3 | 303 | 4.3 | 420 | 26.3 |
| 5-$HT_4$ | > 10,000 | --[c] | > 10,000 | --[c] | > 10,000 | --[c] | > 10,000 | --[c] | > 10,000 | --[c] | > 10,000 | --[c] |
| 5-$HT_{5A}$ | > 10,000 | --[c] | > 10,000 | --[c] | > 10,000 | --[c] | 1,231 | 23.2 | > 10,000 | --[c] | 1,135 | 70.9 |
| 5-$HT_6$ | 416 | 4.0 | 248 | 8.3 | 109 | 2.3 | > 10,000 | --[c] | 360 | 5.1 | 492 | 30.8 |

[a]Values are the mean of at least three independent experiments performed in triplicate.
[b]Selectivity indexes = $K_i$ for other serotonin receptor subtype/Ki for 5-$HT_7$R.
[c]Not determined due to > 10 μM $K_i$ value of the compound against each 5-HTR.

**Activities on G Protein/β-Arrestin Signaling Pathway.** We tested functional activities of G protein pathway and β-arrestin pathway, respectively, of the selected compounds **1d**, **1g**, **1j**, **2d**, **2g**, and **2j**. Bioluminescence-based assays of all selected ligands have been carried out by measuring Gs-mediated cAMP production (cAMP assay)[41,42] and β-arrestin recruitment (Tango assay). An endogenous agonist 5-HT (serotonin) and a selective 5-$HT_7$R antagonist SB269970 were used as controls.[37,43] In cAMP assay for measuring G protein activation, all compounds tested (**1d**, **1g**, **1j**, **2d**, **2g**, and **2j**) acted as inverse agonists with the same result of SB269970, while the endogenous agonist 5-HT showed agonistic effect in G protein pathway (Figure 3a). In 5-HT (100 nM)-stimulated antagonist assay, the test compounds showed moderate antagonistic potencies with $IC_{50}$ values of from 844 nM to 7,788 nM (Figure 3b, Table 3). The $IC_{50}$ value means the concentration of a compound as antagonist required to produce 50% reduction of maximal response of a control agonist (in this receptor, 5-HT). Maximum %-inhibition ($I_{max}$) was calculated based on maximum inhibition by SB269970 as 100%. Because all the $I_{max}$ values were over 94.76%, the compounds **1d**, **1g**, **1j**, **2d**, **2g**, and **2j** could be defined as full antagonists in G protein signaling pathway.

Tango assay is an experimental tool to monitor protein interactions between GPCR and β-arrestin in a living cell,[44] and this assay requires interaction of the two proteins to stimulate a cellular response. For Tango assay, HEK293-derived cell line containing stable interactions of a tTA-dependent luciferase reporter and a β-arrestin2-TEV fusion gene (HTLA cells) was used. SB269980 showed a slight increase in the cellular response in Tango assay with negligible (less than 20%) activation, which cannot be considered as an agonist in β-arrestin signaling pathway (Figure 3c). However, all the test compounds (**1d**, **1g**, **1j**, **2d**, **2g**, and **2j**) showed partial agonistic effect in Tango assays with $EC_{50}$ values of 188 nM, 162 nM, 165 nM, 135 nM, 113 nM, 40.8 nM, and 48 nM, respectively, and with $E_{max}$ values of between 38.23%

and 62.75% (Figure 3c, Table 3). They showed no antagonistic activities in 5-HT (1 μM)-stimulated Tango assays, but SB269970 acted as an antagonist in Tango assay.

Taken together, Compounds **1d**, **1g**, **1j**, **2d**, **2g**, and **2j** with tetrahydropyrazoloazepine or tetrahydroisoxazoloazepine scaffold act as potent partial agonists in β-arrestin signaling pathway of 5-$HT_7$R, and as full antagonists in G protein signaling pathway. Among them, compound **1g**, 3-(4-chlorophenyl)-1,4,5,6,7,8-hexahydropyrazolo[3,4-d]azepine, is the most effective β-arrestin partial agonist ($E_{max}$ 62.75%, $EC_{50}$ 162 nM), which is the β-arrestin biased ligand of 5-$HT_7$R.

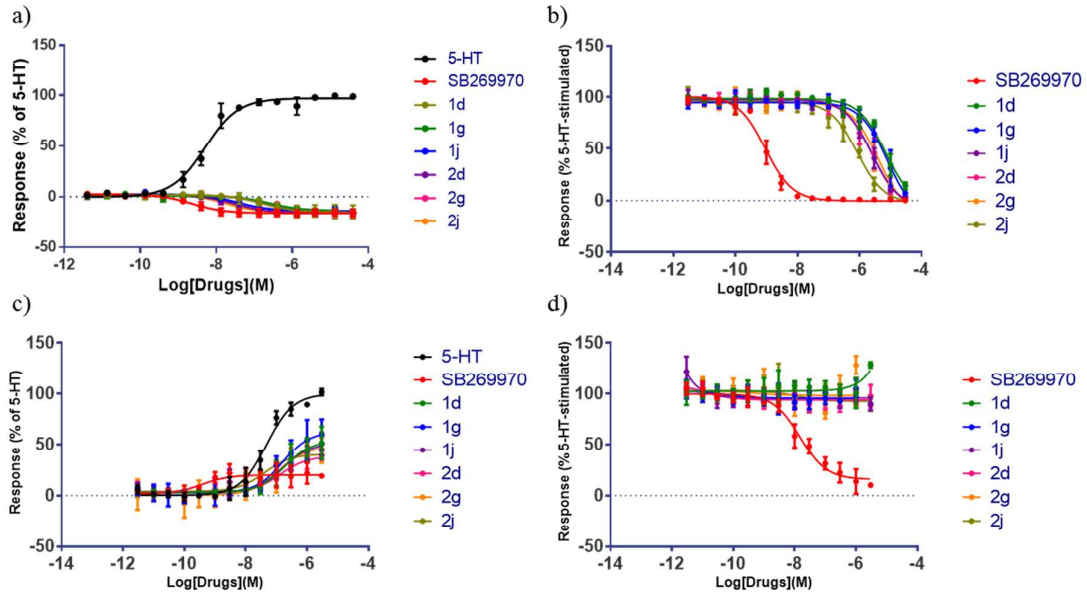



**Figure 3.** Dose-response curves of a) cAMP agonist assay, b) cAMP antagonist assay, c) Tango agonist assay, and d) Tango antagonist assay of compounds **1d**, **1g**, **1j**, **2d**, **2g**, and **2j**. Activation of Gs protein was determined by bioluminescence-based cAMP assay using D-luciferin. Tango assay was selected to determine potency and efficacy for β-arrestin recruitment. Data were normalized relative to the response of 5-HT (100%) or SB269970 (100% at antagonist assays) and vehicle (0%) and represent mean ± SEM from three independent experiments, each performed at least in triplicate.

**Table 3**. $IC_{50}$ and $I_{max}$ values of compounds **1d**, **1g**, **1j**, **2d**, **2g**, and **2j** in cAMP assay and their $EC_{50}$ and $E_{max}$ values in Tango assay

| Compound | cAMP | | | β-arrestin | | |
|---|---|---|---|---|---|---|
| | $pIC_{50}$ [a] | $IC_{50}$ (nM) [b] | $I_{max}$ (%) | $pEC_{50}$ [a] | $EC_{50}$ (nM) [b] | $E_{max}$ (%) |
| **1d** | 5.16 ± 0.10 | 6,978 | 98.41 | 6.73 ± 0.15 | 188 | 53.86 |
| **1g** | 5.11 ± 0.08 | 7,788 | 94.76 | 6.79 ± 0.16 | 162 | 62.75 |
| **1j** | 5.59 ± 0.05 | 2,556 | 97.41 | 6.78 ± 0.11 | 165 | 50.94 |
| **2d** | 5.61 ± 0.06 | 2,463 | 97.96 | 6.87 ± 0.13 | 135 | 38.23 |
| **2g** | 5.40 ± 0.07 | 3,973 | 94.92 | 6.95 ± 0.21 | 113 | 47.11 |
| **2j** | 6.07 ± 0.07 | 844 | 95.79 | 7.39 ± 0.12 | 40.8 | 40.33 |
| 5-HT | --[c] | --[c] | --[c] | 7.32 ± 0.06 | 48 | 100 |
| SB269970 | 9.03 ± 0.04 | 0.9 | 100.5 | --[c] | --[c] | < 20% |

[a]Values are the mean ± SEM of three independent experiments performed at least in triplicate.
[b]Values calculated from $pIC_{50}$ or $pEC_{50}$.
[c]Not determined.

**Molecular Docking Study.** We analyzed docking poses of compound **1g** into the binding site of 5-$HT_7$R to identify residues implicated in β-arrestin signaling pathway in relation to G protein signaling pathway. We constructed a comparative model structure of 5-$HT_7$R by the SwissModel server using the 5-$HT_{1B}$ receptor structure (PDB: 4IAR) as a template.[45-47] Recently, it has been uncovered that signal biases of several kinds of aminergic GPCRs are induced by competitive interactions between a ligand and Ile residue in extracellular loop 2 (EL2) or Ser residue in transmembrane helix 5 (TM5).[38,48] That is, the closer the distance between a ligand and EL2 is, the more β-arrestin biased activity is enhanced. Also, a hydrogen bond of a ligand with Ser residue in TM5 stabilizes the conformation, resulting in moving intracellular loop 2 (IL2) and TM6 regions that are involved in G protein activation. Until now, there has been no biased ligand of 5-$HT_7$R and thus, no explanation of interactions between a biased ligand and the binding site of 5-$HT_7$R. In this molecular docking study, we tried to explain the β-arrestin bias of **1g** by specifying residues in the binding site of 5-$HT_7$R. According to the literature,[49-53] the binding site of 5-$HT_7$R consisted of two hydrophobic binding pockets, HPP1 (Val163/Thr214/Phe344) and HPP2 (Leu232/Ile233/Arg367), and an

ionic interaction residue (Asp162) (Figure 4). An agonist generally docked into only one hydrophobic pocket (HPP1), while an antagonist occupied both the hydrophobic pockets HPP1 and HPP2. In docking study of compound **1g**, it occupied only one hydrophobic pocket (HPP1) of the two hydrophobic pockets by forming ionic interaction with Asp162 and π-alkyl interaction with Ile233 in EL2 (Figure 4, 5a). Interestingly, it did not occupy the other hydrophobic pocket (HPP2) even though the compound **1g** was identified as a full antagonist in cAMP assays. This might be explained by the lack of interaction of compound **1g** with Ser243 in TM5 and other residues in TM6, which are known to be critical in activation of G protein signaling pathway. Collectively, these results show that, if a ligand is not close enough to TM5 or TM6, it can block G protein signaling of 5-$HT_7$R even in the absence of occupation of the HPP2 region.

To verify an effect of the key residue Ile233 in EL2 on β-arrestin signaling pathway, we also studied docking pose of AS-19 to compare with that of **1g** (Figure 5b). AS-19 contains pyrazole group and tetralin moiety, which acts as a partial agonist of 5-$HT_7$R to enhance G protein as well as β-arrestin signaling (Supplementary Figure S2, Table S1). In the docking mode of AS-19, there is no interaction with Ser243 in TM5, but T-shape π-π interaction with Arg350 direct affects TM6 which is involved in activation of G protein signaling pathway. The hydrophobic interaction with Ile233 in EL2 is also found in the docking mode, which makes AS-19 a partial β-arrestin agonist. Overall, compound **1g** might be able to stimulate only β-arrestin signaling pathway because it makes the hydrophobic interaction with Ile233 in EL2 and no interaction with Ser243 in TM5 and other residues in TM6. Taken together, it could be proposed that engagement of **1g** with Ile233 in 5-$HT_7$R lead to activation of β-arrestin signaling pathway.

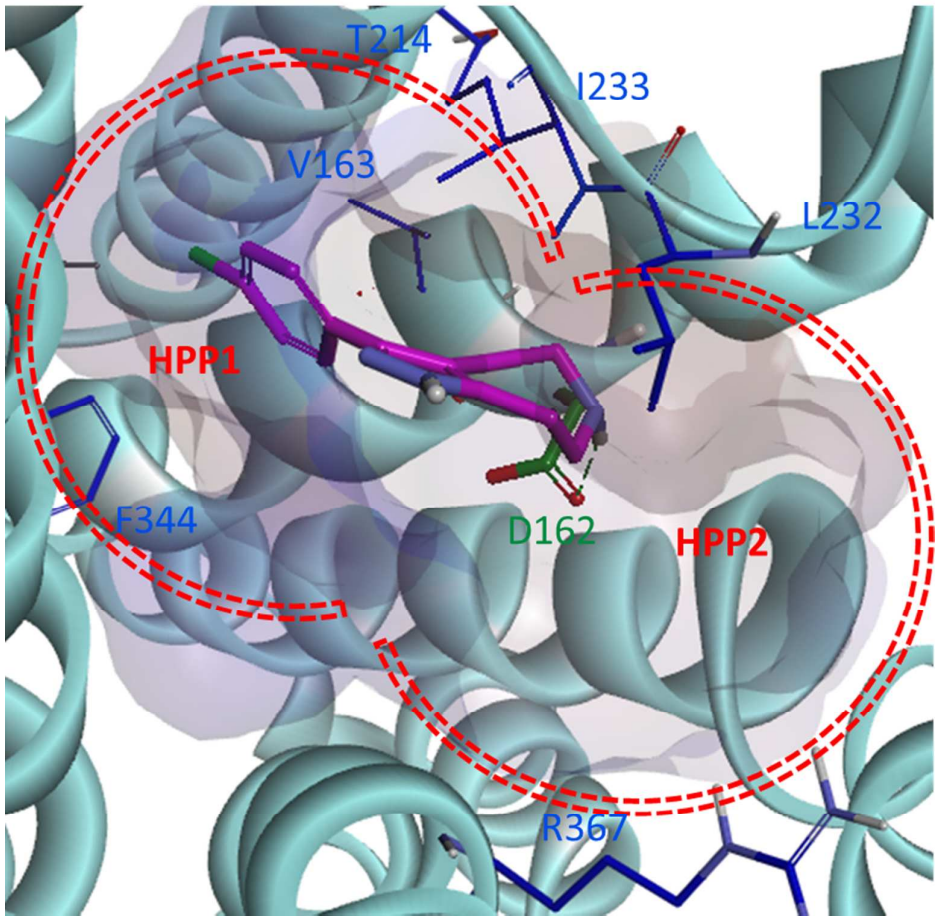



**Figure 4.** Docking pose of **1g** (pink) and hydrophobic pockets (HPP1 and HPP2) at ligand binding site of 5-$HT_7$R constructed by homology modeling using the 5-$HT_{1B}$R structure (PDB: 4IAR). The two hydrophobic pockets HPP1 and HPP2 are shown in molecular surface representation, respectively highlighted with red dotted half-circles. HPP1 consists of Val163/Thr214/Phe344 and HPP2 includes Leu232/Ile233/Arg367 residues, which are expressed by blue color sticks. Compound **1g** formed ionic interaction with Asp162 that is shown by the green color stick.

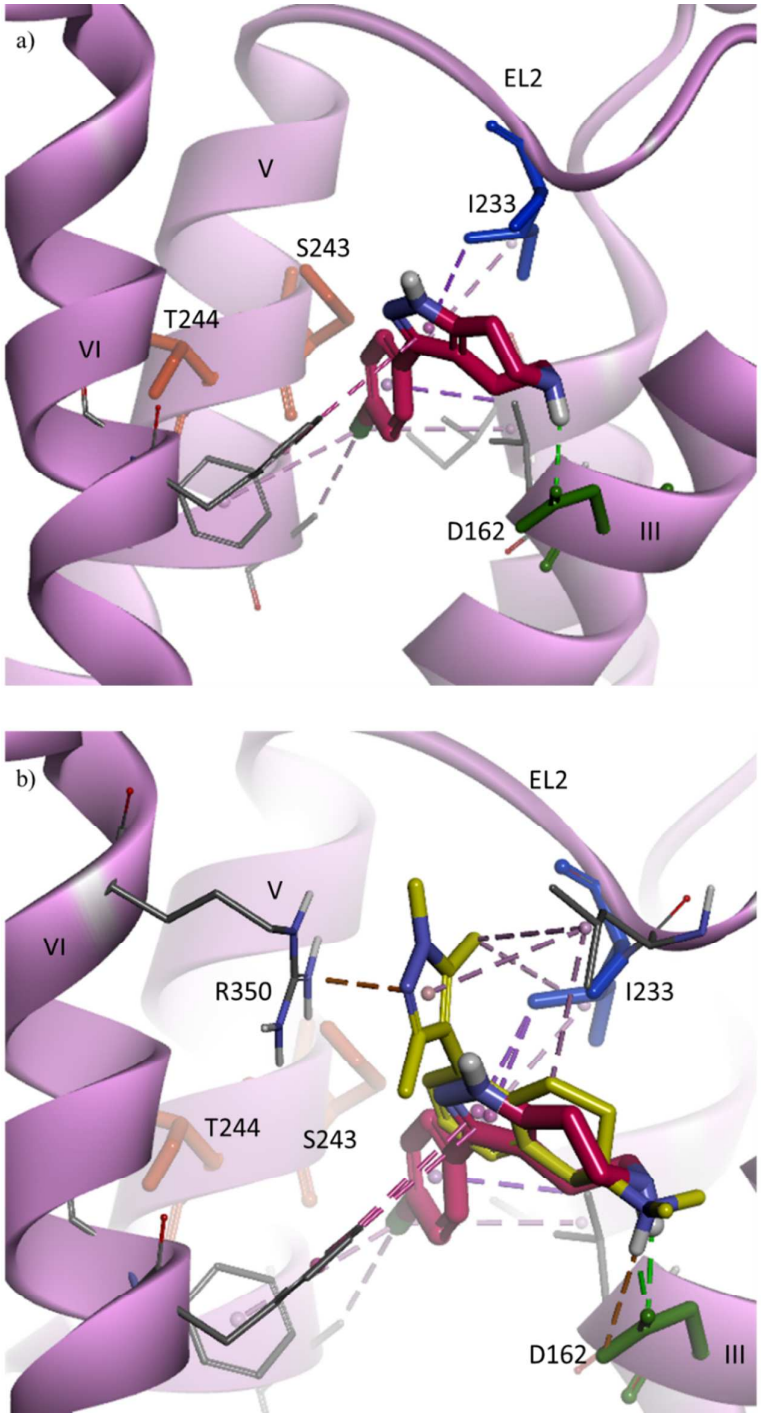



**Figure 5.** a) Binding interaction between **1g** and several amino acids in binding site of 5-$HT_7R$ and b) superimposed structures of **1g** and AS-19 shown in red and yellow, respectively. The important amino acid residues such as Ser243 and Thr244 in TM5, Ile233 in EL2 and Asp162 in TM3 were expressed by orange, blue, and green color, respectively.

**Cytochrome P450 (CYP) Isozyme Activity and Microsomal Stability.** To avoid drug-drug interactions, inhibitory activities of **1g** against cytochrome P450 (CYP) isozymes such as CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 were examined (Table 4). Compound **1g** has little effects on five CYP isozymes (%-remaining activities > 75.7%) implying that **1g** has no high risk of drug-drug interaction. To test metabolic stability of **1g**, human liver microsomal stability assay was carried out. The microsomal stability was shown in %-remaining concentration after incubation with human liver microsomes for 30 min. The stability is a factor that affects pharmacokinetics (PK) parameters such as clearance, a half-life and oral bioavailability of the drug, which can be easily tested in *in vitro* assays before *in vivo* animal study. Compound **1g** showed 82.4% recovery after incubation with human liver microsomes. Overall, **1g** displayed excellent *in vitro* pharmacological properties regarding selectivity over other serotonin receptor subtypes, CYP isozyme activity, and microsomal stability.

PK parameters for **1g** were then evaluated after intravenous and oral administration in male ICR mice (Table 5). No mortality occurred when **1g** was administered orally and intravenously. After intravenous injection, **1g** exhibited drug-like PK parameters. It was present in the blood at the highest concentration at 0.11 h and the maximum plasma concentration ($C_{max}$) was 2198.00 ng/ml. In case of clearance, the mean clearance rate (CL) was 0.96 l/hr/kg and the terminal half-life was 1.78 h. In comparison, after oral administration, the oral bioavailability of **1g** was excellent (F = 90.79%). Thus, the PK properties of **1g** strongly support its favorable druggable properties.

**Table 4.** Results of CYP450 and microsomal stability tests

| Compound | CYP450 screening[a] | | | | | Human Microsomal stability (%)[b] |
|---|---|---|---|---|---|---|
| | CYP1A2 | CYP2C9 | CYP2C19 | CYP2D6 | CYP3A4 | |
| **1g** | 86.0 | > 100 | > 100 | 75.7 | 95.4 | 82.4 |

[a]%-remaining activity.
[b]Human liver microsomal stability (% remaining duing 30 min).

**Table 5.** Mean (± SD[a]) pharmacokinetic parameters after intravenous (IV, n = 5) and oral (PO, n = 4) administration (5 mg/kg for IV, 50 mg/kg for PO) of **1g** to ICR mice

| Plasma | Intravenous | | | Oral | | |
|---|---|---|---|---|---|---|
| $AUC_{0-\infty}$ (hr ng/ml) | 5210.48 | ± | 297.09 | 64319.56 | ± | 3601.43 |
| $AUC_{last}$ (hr ng/ml) | 4897.50 | ± | 391.73 | 53688.13 | ± | 4433.79 |
| Terminal half-life (hr) | 1.78 | ± | 0.35 | 2.77 | ± | 0.55 |
| $C_{max}$ (ng/ml) | 2198.00 | ± | 211.12 | 11782.50 | ± | 1773.48 |
| $T_{max}$ (h) | 0.11 | ± | 0.08 | 1.37 | ± | 0.75 |
| CL (l/hr/kg) | 0.96 | ± | 0.06 | | -- | |
| MRT (hr) | 2.50 | ± | 0.19 | | -- | |
| $V_{dss}$ (l/kg) | 2.47 | ± | 0.52 | | -- | |
| F (%) | | -- | | 90.79 | ± | 12.69 |

$AUC_{0-\infty}$, total area under the plasma concentrationetime curve from time zero to time infinity; $AUC_{last}$, total area under the plasma concentrationetime curve from time zero to last measured time; $C_{max}$, peak plasma concentration; $T_{max}$, time to reach Cmax; CL, time-averaged total body clearance; MRT, mean residence time; $V_{dss}$, apparent volume of distribution at steady state; F, oral bioavailability
[a]SD: Standard deviations.

**Effect on NREM/REM Sleep.** REM sleep pattern could be changed in several psychiatric disorders such as depression and autism.[54,55] Recently, preclinical data indicated that the total amount of REM sleep was reduced in the 5-$HT_7$R knock-out models.[56,57] In addition, selective 5-$HT_7$R antagonists and selective serotonin reuptake inhibitors (SSRIs) could increase the latency of REM sleep and decrease the total time of REM sleep with little change in non-rapid eye movement (NREM) sleep pattern.[31,56,58] Thus, we evaluated the effect of **1g** on electroencephalogram (EEG) sleep architecture to investigate a correlation between β-arrestin biased activity of **1g** and NREM/REM sleep pattern in male mice with SB269970 as a control (Figure 6, Table 6). All of the tested drugs were intraperitoneally (ip) administered at the beginning of the light phase. After administration of SB269970 at a dose of 30 mg/kg to EEG mouse models, the duration of NREM sleep was not altered compared with NREM sleep in the absence of a drug. Compound **1g**, however, elevated NREM sleep duration during all of the time intervals and total NREM time about 21.4%.

Next, the total REM sleep after injection of SB269970 was decreased to 14.4 min, which was consistent with previous pharmacological data (Figure 6b, Table 6). In total, about 37.1% of total REM sleep was reduced for tested 8 h compared to 22.9 min of total REM sleep in the absence of a drug. In sleep EEG experiments of **1g**, a more notable decrease in REM sleep duration was measured during the first 4 h after administration of **1g** (30 mg/kg). Only 3.3 min were spent for REM sleep in these time intervals, which means that about 69.4 % of REM sleep time was decreased compared to the vehicle-injected mice in the same time. This suppression of REM sleep was prolonged to third time interval (5~6 h) and not observed at fourth time interval (7~8 h). Total REM sleep time (14.5 min) was also decreased by about 36.7%. Taken together, the β-arrestin partial agonist **1g** increased NREM sleep duration and decreased REM sleep duration, while the antagonist SB269970 in both pathways decreased

REM sleep duration without change of NREM sleep. Though there are more experimental data needed on sleep patterns of 5-$HT_7$R biased ligands, it could be suggested that β-arrestin activity of 5-$HT_7$R might modulate NREM sleep patterns.

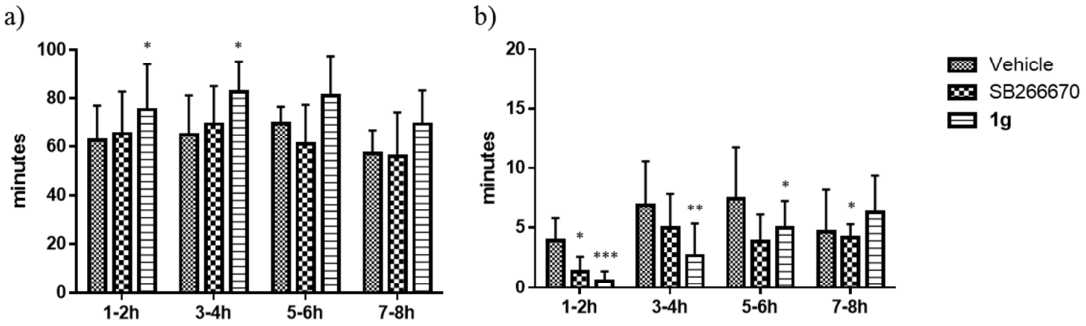



**Figure 6.** Effect of **1g** on a) NREM sleep and b) REM sleep duration. Data bars represent means ± SEM. REM sleep duration was compiled for the 8 h following the intraperitoneal administration of vehicle, SB269970 and **1g**: *P < 0.05, **P < 0.01, and ***P < 0.001 versus vehicle based on two-way ANOVA followed by Bonferroni *post hoc* test.

**Table 6.** Duration of REM sleep and total time spent in REM sleep after treatment with vehicle, SB269970 and **1g** (30 mg/kg) for 8 hours measured in healthy subjects (n = 8, 6, and 9, respectively). All data are expressed as mean ± SEM (minutes).

| | NREM duration | | | | Total NREM |
|---|---|---|---|---|---|
| | 1-2 h | 3-4 h | 5-6 h | 7-8 h | |
| Vehicle | 62.7 ± 5.1 | 65.0 ± 5.7 | 69.5 ± 2.5 | 57.0 ± 3.5 | 254.2 ± 12.7 |
| SB269970 | 65.5 ± 7.0 | 69.4 ± 6.4 | 61.1 ± 6.6 | 55.9 ± 7.4 | 252.0 ± 22.7 |
| **1g** | 75.3 ± 6.2* | 82.7 ± 4.1* | 80.9 ± 5.5 | 69.5 ± 4.6 | 308.5 ± 14.5* |
| | REM duration | | | | Total REM |
| | 1-2 h | 3-4 h | 5-6 h | 7-8 h | |
| Vehicle | 3.9 ± 0.7 | 6.9 ± 1.3 | 7.5 ± 1.5 | 4.6 ± 1.3 | 22.9 ± 3.9 |
| SB269970 | 1.4 ± 0.5* | 5.0 ± 1.2 | 3.8 ± 0.9 | 4.2 ± 0.5* | 14.4 ± 2.3* |
| **1g** | 0.6 ± 0.3*** | 2.7 ± 0.9** | 4.9 ± 0.8* | 6.3 ± 1.0 | 14.5 ± 2.7** |

*P < 0.05, **P < 0.01, and ***P < 0.001 versus vehicle based on two-way ANOVA followed by Bonferroni *post hoc* test.

## ■ CONCLUSIONS

We designed and synthesized a series of tetrahydroazepines **1** and **2** with arylpyrazolo moiety or arylisoxazolo moiety. With total 20 synthesized compounds (**1** and **2**), biological evaluations such as binding affinity, selectivity profile, and functions in G protein/β-arrestin signaling pathway were carried out. SAR studies of **1** and **2** revealed that compounds **1d**, **1g**, **1j**, **2d**, **2g**, and **2j** with *para*-substitutent in phenyl group more strongly bind to 5-$HT_7$R than the corresponding *ortho* or *meta*-substituted congeners. In cAMP and Tango assays, we have discovered that compounds **1d**, **1g**, **1j**, **2d**, **2g**, and **2j** acted as β-arrestin biased ligands of 5-$HT_7$R ($EC_{50}$ < 188 nM, $E_{max}$ > 38.23%). Among those 6 compounds, the compound **1g** was the most effective β-arrestin partial agonist with 5-$HT_7$R selectivity over other serotonin receptor subtypes. Molecular docking studies suggested that **1g** could activate only β-arrestin recruitment, where **1g** has π-alkyl interaction with Ile233, a key residue for β-arrestin activation, but no interaction with residues for G protein activation such as Ser243 in TM5 and other residues in TM6. According to assessment on EEG sleep architecture, the β-arrestin biased ligand **1g** increased NREM sleep duration during all of the time intervals after intraperitoneal administration unlike a balanced antagonist SB269970, while both **1g** and SB269970 decreased REM sleep time. The novel β-arrestin biased ligand **1g** with good druggable properties would be a lead compound to develop drugs targeting 5-$HT_7$R for treatment of sleep disorders.

## ■ 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. $^1H$ and $^{13}C$ NMR spectra were recorded on Bruker 300, or 400 NMR spectrometers. $^1H$ NMR spectra are represented as follows: chemical shift, multiplicity (s = singlet, brs = broad singlet, d = doublet, t = triplet, q = quartet, m = multiplet), integration, and coupling constant (*J*) in Hertz (Hz). LC/MS and HRMS analyses were performed on Agilent 6410 Triple Quad system and Bruker Compact $ESI^+$ positive mode, respectively. The purities of all final compounds were ≥95% and 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 x 75 mm) column. Standard conditions were as follows: solvent A ($CH_3CN$), solvent B (0.1% AcOH in $H_2O$), a flow rate of 1 mL/min, and a gradient of solvent A (10–100%) in solvent B over 20 min. The detector wavelength was set at 254 nm or 280 nm.

**3-Phenyl-1,4,5,6,7,8-hexahydropyrazolo[3,4-d]azepine** (**1a**). To a solution of compound **8a** (0.27 mmol) in DCM/MeOH (9:1, 5 ml), the excess amount of 1 N HCl in $Et_2O$ (3 ml) was added. The reaction mixture was stirred at room temperature for 12 h. After reaction termination, the reaction solution extracted with a minimum volume of water, and basified by

10 N NaOH. The aqueous layer was extracted with DCM again, and dried over $MgSO_4$, filtered, and concentrated to obtain desired final compounds **1a** (0.042 mmol, 16% yield). HPLC: purity 99 %, $t_R$=6.1 min; $^1$HNMR (300 MHz, $CDCl_3$) δ 7.47-7.35 (m, 5H), 3.00-2.93 (m, 4H), 2.86-2.82 (m, 2H), 2.78-2.75 (m, 2H); $^{13}$CNMR (75 MHz, $CDCl_3$) δ 149.75, 144.84, 131.85, 128.64, 128.12, 127.90, 116.79, 50.51, 48.69, 32.13, 28.12; HRMS ($ESI^+$) calcd for $C_{13}H_{16}N_3^+$ $[M+H]^+$ 214.1344, found 214.1349.

**3-(2-Fluorophenyl)-1,4,5,6,7,8-hexahydropyrazolo[3,4-d]azepine** (**1b**). Compound **1b** was synthesized according to the procedure of **1a** using a solution of **8b** (0.093 mmol) and 1N HCl in $Et_2O$ (2 ml) in DCM/MeOH (9:1, 2 ml). Yield: 28% (0.026 mmol); HPLC: purity 98 %, $t_R$=6.1 min; $^1$HNMR (300 MHz, $CDCl_3$) δ 7.43-7.31 (m, 2H), 7.21-7.11 (m, 2H), 3.02-2.93 (m, 4H), 2.87-2.84 (m, 2H), 2.67-2.63 (m, 2H); $^{13}$CNMR (75 MHz, $CDCl_3$) δ 159.77 (d, $J$ = 246 Hz), 149.09, 139.44, 131.02 (d, $J$ = 3.7 Hz), 129.92 (d, $J$ = 8.2 Hz), 124.29 (d, $J$ = 3.0 Hz), 119.65 (d, $J$ = 15 Hz), 118.50, 116.05 (d, $J$ = 22.5 Hz), 50.32, 48.67, 32.12, 28.37; HRMS ($ESI^+$) calcd for $C_{13}H_{15}FN_3^+$ $[M+H]^+$ 232.1250, found 232.1252.

**3-(3-Fluorophenyl)-1,4,5,6,7,8-hexahydropyrazolo[3,4-d]azepine** (**1c**). Compound **1c** was synthesized according to the procedure of **1a** using a solution of **8c** (0.43 mmol) and 1N HCl in $Et_2O$ (10 ml) in DCM/MeOH (9:1, 10 ml). Yield: 72% (0.31 mmol); HPLC: purity 99 %, $t_R$=6.3 min; $^1$HNMR (300 MHz, $CDCl_3$) δ 7.42-7.15 (m, 3H), 7.05-6.99 (m, 1H), 2.98-2.92 (m, 4H), 2.79-2.70 (m, 4H); $^{13}$CNMR (75 MHz, $CDCl_3$) δ 162.75 (d, $J$ = 224.5 Hz), 147.36, 145.70, 134.83 (d, $J$ = 7.5 Hz), 130.02 (d, $J$ = 8.25 Hz), 124.00 (d, $J$ = 2.25 Hz), 116.77, 115.14 (d, $J$ = 22.5 Hz), 114.49 (d, $J$ = 21 Hz), 50.33, 48.41, 31.19, 28.02; HRMS ($ESI^+$) calcd for $C_{13}H_{15}FN_3^+$ $[M+H]^+$ 232.1250, found 232.1247.

**3-(4-Fluorophenyl)-1,4,5,6,7,8-hexahydropyrazolo[3,4-d]azepine** (**1d**). Compound **1d** was synthesized according to the procedure of **1a** using a solution of **8d** (0.23 mmol) and 1N HCl

in $Et_2O$ (10 ml) in DCM/MeOH (9:1, 5 ml). Yield: 75% (0.17 mmol); HPLC: purity 97 %, $t_R$=6.3 min; $^1$HNMR (300 MHz, $CDCl_3$) δ 7.41-7.36 (m, 2H), 7.07-7.01 (m, 2H), 2.95-2.90 (m, 4H), 2.72-2.69 (m, 4H); $^{13}$CNMR (75 MHz, $CDCl_3$) δ 162.45 (d, $J$ = 245.25 Hz), 147.91, 145.50, 129.99 (d, $J$ = 8.25 Hz), 128.55, 116.60, 115.43 (d, $J$ = 21 Hz), 50.46, 48.58, 31.54, 28.14; HRMS ($ESI^+$) calcd for $C_{13}H_{15}FN_3^+$ $[M+H]^+$ 232.1250, found 232.1247.

**3-(2-Chlorophenyl)-1,4,5,6,7,8-hexahydropyrazolo[3,4-d]azepine** (**1e**). Compound **1e** was synthesized according to the procedure of **1a** using a solution of **8e** (0.23 mmol) and 1N HCl in $Et_2O$ (10 ml) in DCM/MeOH (9:1, 5 ml). Yield: 81% (0.19 mmol); HPLC: purity 97 %, $t_R$=6.4 min; $^1$HNMR (300 MHz, $CDCl_3$) δ 7.45-7.42 (m, 1H), 7.36-7.24 (m, 3H), 2.94-2.90 (m, 4H), 2.69 (t, $J$ = 4.9 Hz, 2H), 2.51 (t, $J$ = 4.9 Hz, 2H); $^{13}$CNMR (75 MHz, $CDCl_3$) δ 147.62, 143.42, 133.92, 132.12, 131.45, 129.76, 129.57, 126.61, 118.38, 50.13, 48.64, 31.57, 28.42; HRMS ($ESI^+$) calcd for $C_{13}H_{15}ClN_3^+$ $[M+H]^+$ 248.0955, found 248.0958.

**3-(3-Chlorophenyl)-1,4,5,6,7,8-hexahydropyrazolo[3,4-d]azepine** (**1f**). Compound **1f** was synthesized according to the procedure of **1a** using a solution of **8f** (0.56 mmol) and 1N HCl in $Et_2O$ (15 ml) in DCM/MeOH (9:1, 5 ml). Yield: 49% (0.27 mmol); HPLC: purity 99 %, $t_R$=6.8 min; $^1$HNMR (300 MHz, $CDCl_3$) δ 7.43 (s, 1H), 7.32-7.27 (m, 3H), 2.95-2.90 (m, 4H), 2.74-2.68 (m, 4H); $^{13}$CNMR (75 MHz, $CDCl_3$) δ 147.35, 145.62, 134.53, 134.37, 129.72, 128.26, 127.68, 126.45, 116.87, 50.39, 48.50, 31.32, 28.14; HRMS ($ESI^+$) calcd for $C_{13}H_{15}ClN_3^+$ $[M+H]^+$ 248.0955, found 248.0959.

**3-(4-Chlorophenyl)-1,4,5,6,7,8-hexahydropyrazolo[3,4-d]azepine** (**1g**). Compound **1g** was synthesized according to the procedure of **1a** using a solution of **8g** (0.78 mmol) and 1N HCl in $Et_2O$ (15 ml) in DCM/MeOH (9:1, 5 ml). Yield: 73% (0.57 mmol); HPLC: purity 99 %, $t_R$= 6.5 min; $^1$HNMR (300 MHz, $CDCl_3$) δ 7.41-7.30 (m, 4H), 2.99-2.94 (m, 4H), 2.80-2.71 (m, 4H); $^{13}$CNMR (75 MHz, $CDCl_3$) δ 147.88, 145.42, 133.77, 130.88, 129.47, 128.72,

116.81, 50.45, 48.56, 31.55, 28.14; HRMS ($ESI^+$) calcd for $C_{13}H_{15}ClN_3^+$ $[M+H]^+$ 248.0955, found 248.0962.

**3-(o-Tolyl)-1,4,5,6,7,8-hexahydropyrazolo[3,4-d]azepine** (**1h**). Compound **1h** was synthesized according to the procedure of **1a** using a solution of **8h** (0.50 mmol) and 1N HCl in $Et_2O$ (15 ml) in DCM/MeOH (9:1, 5 ml). Yield: 55% (0.27 mmol); HPLC: purity 96 %, $t_R$= 6.4 min; $^1$HNMR (300 MHz, $CDCl_3$) δ 7.26-7.17 (m, 4H), 2.83 (q, *J* = 4.9 Hz, 4H), 2.53 (t, *J* = 4.7 Hz, 2H), 2.41 (t, *J* = 4.6 Hz, 2H), 2.17 (s, 3H); $^{13}$CNMR (75 MHz, $CDCl_3$) δ 149.32, 143.75, 137.51, 131.35, 130.58, 130.12, 128.41, 125.50, 117.53, 50.40, 48.86, 31.83, 28.10, 19.87; HRMS ($ESI^+$) calcd for $C_{14}H_{18}N_3^+$ $[M+H]^+$ 228.1501, found 228.1504.

**3-(m-Tolyl)-1,4,5,6,7,8-hexahydropyrazolo[3,4-d]azepine** (**1i**). Compound **1i** was synthesized according to the procedure of **1a** using a solution of **8i** (0.28 mmol) and 1N HCl in $Et_2O$ (10 ml) in DCM/MeOH (9:1, 5 ml). Yield: 33% (0.092 mmol); HPLC: purity 98 %, $t_R$= 6.7 min; $^1$HNMR (300 MHz, $CDCl_3$) δ 7.35-7.30 (m, 1H), 7.26-7.17 (m, 3H), 3.05-3.02 (m, 2H), 2.98-2.90 (m, 4H), 2.79-2.76 (m, 2H), 2.40 (s, 3H); $^{13}$CNMR (75 MHz, $CDCl_3$) δ 149.30, 144.43, 138.46, 131.29, 128.87, 128.69, 128.63, 125.18, 116.09, 49.81, 48.00, 30.61, 26.60, 21.45; HRMS ($ESI^+$) calcd for $C_{14}H_{18}N_3^+$ $[M+H]^+$ 228.1501, found 228.1501.

**3-(p-Tolyl)-1,4,5,6,7,8-hexahydropyrazolo[3,4-d]azepine** (**1j**). Compound **1j** was synthesized according to the procedure of **1a** using a solution of **8j** (0.95 mmol) and 1N HCl in $Et_2O$ (15 ml) in DCM/MeOH (9:1, 5 ml). Yield: 58% (0.55 mmol); HPLC: purity 100 %, $t_R$= 6.6 min; $^1$HNMR (300 MHz, $CDCl_3$) δ 7.51 (brs, 1H), 7.29 (d, *J* = 7.8 Hz, 2H), 7.12 (d, *J* = 7.5 Hz, 2H), 2.87 (brs, 4H), 2.71-2.69 (m, 4H), 2.32 (s, 3H); $^{13}$CNMR (75 MHz, $CDCl_3$) δ 148.81, 145.19, 137.36, 129.37, 129.16, 128.22, 116.30, 50.44, 48.58, 31.77, 28.16, 21.26; HRMS ($ESI^+$) calcd for $C_{14}H_{18}N_3^+$ $[M+H]^+$ 228.1501, found 228.1505.

**3-Phenyl-5,6,7,8-tetrahydro-4H-isoxazolo[4,5-d]azepine** (**2a**). To a solution of HBr in

AcOH (33%, 0.15 ml), compound **15a** (0.15 mmol) was added. The reaction mixture was stirred at room temperature for 2 h. During the reaction time, HBr in AcOH (33%, 0.15 x 2 ml) was additionally added twice. After reaction termination, the reaction solution extracted with a minimum volume of water, and basified by 10 N NaOH. The aqueous layer was extracted with DCM again, and dried over $MgSO_4$, filtered, and concentrated to obtain desired final compounds **2a** (0.042 mmol, 28%). HPLC: purity 96 %, $t_R$= 6.5 min; $^1$HNMR (300 MHz, $CDCl_3$) δ 7.62-7.35 (m, 5H), 3.20-2.95 (m, 4H), 2.94-2.80 (m, 2H), 2.79-2.71 (m, 2H); $^{13}$CNMR (75 MHz, $CDCl_3$) δ 161.89, 141.20, 128.95, 128.55, 127.12, 120.46, 112.11, 47.05, 46.58, 40.50, 36.0; HRMS ($ESI^+$) calcd for $C_{13}H_{15}N_2O^+$ $[M+H]^+$ 215.1184, found 215.1181.

**3-(2-Fluorophenyl)-5,6,7,8-tetrahydro-4H-isoxazolo[4,5-d]azepine** (**2b**). Compound **2b** was synthesized according to the procedure of **2a** using a solution of **15b** (0.31 mmol) and HBr in AcOH (33%, 0.15 x 3 ml). Yield: 79% (0.10 mmol); HPLC: purity 98 %, $t_R$= 6.4 min; $^1$HNMR (300 MHz, $CDCl_3$) δ 7.40-7.26 (m, 2H), 7.25-7.07 (m. 2H), 3.15-2.92 (m, 4H), 2.90-2.62 (m 4H); ; $^{13}$CNMR (75 MHz, $CDCl_3$) δ 159.00 (d, $J$ = 236.25 Hz), 130.72 (d, $J$ = 8.25 Hz), 129.57, 129.53, 129.26 (d, $J$ = 14.25 Hz), 124.29 (d, $J$ = 3 Hz), 119.61, 116.05 (d, $J$ = 21 Hz), 115.25, 47.04, 46.38, 40.40, 36.18; HRMS ($ESI^+$) calcd for $C_{13}H_{14}FN_2O^+$ $[M+H]^+$ 233.1090, found 233.1084.

**3-(3-Fluorophenyl)-5,6,7,8-tetrahydro-4H-isoxazolo[4,5-d]azepine** (**2c**). Compound **2c** was synthesized according to the procedure of **2a** using a solution of **15c** (0.20 mmol) and HBr in AcOH (33%, 0.15 x 3 ml). Yield: 95% (0.19 mmol); HPLC: purity 98 %, $t_R$= 6.6 min; $^1$HNMR (400 MHz, $CDCl_3$) δ 7.38-7.06 (m, 4H), 2.99 (brs, 4H), 2.83 (brs, 2H), 2.72 (brs, 2H); $^{13}$CNMR (100 MHz, $CDCl_3$) δ 162.51 (d, $J$ = 245 Hz), 160.48, 143.30 (d, $J$ = 7 Hz), 130.22 (d, $J$ = 8 Hz), 122.97 (d, $J$ = 3 Hz), 120.01, 115.78 (d, $J$ = 21 Hz), 114.19 (d, J = 22

Hz), 113.15, 47.02, 46.59, 40.54, 36.19; HRMS ($ESI^+$) calcd for $C_{13}H_{14}FN_2O^+$ $[M+H]^+$ 233.1090, found 233.1082.

**3-(4-Fluorophenyl)-5,6,7,8-tetrahydro-4H-isoxazolo[4,5-d]azepine** (**2d**). Compound **2d** was synthesized according to the procedure of **2a** using a solution of **15d** (0.36 mmol) and HBr in AcOH (33%, 0.25 x 3 ml). Yield: 50% (0.18 mmol); HPLC: purity 99 %, $t_R$= 6.6 min; $^1$HNMR (300 MHz, $CDCl_3$) δ 7.41-7.32 (m, 2H), 7.15-7.03 (m, 2H), 3.09-2.92 (m, 4H), 2.90-2.80 (m, 2H), 2.79-2.69 (m, 2H); $^{13}$CNMR (75 MHz, $CDCl_3$) δ 162.85 (d, $J$ = 247.5 Hz), 160.86, 137.26 (d, $J$ = 3.75 Hz), 129.15 (d, $J$ = 8.25 Hz), 120.35, 115.58 (d, $J$ = 11.25 Hz), 112.40, 47.06, 46.58, 40.40, 36.11; HRMS ($ESI^+$) calcd for $C_{13}H_{14}FN_2O^+$ $[M+H]^+$ 233.1090, found 233.1086.

**3-(2-Chlorophenyl)-5,6,7,8-tetrahydro-4H-isoxazolo[4,5-d]azepine** (**2e**). Compound **2e** was synthesized according to the procedure of **2a** using a solution of **15e** (0.14 mmol) and HBr in AcOH (33%, 0.15 x 3 ml). Yield: 17% (0.024 mmol); HPLC: purity 96 %, $t_R$= 5.2 min; $^1$HNMR (300 MHz, $CDCl_3$) δ 7.49-7.40 (m, 1H), 7.33-7.23 (m, 3H), 3.10-2.90 (m, 4H), 2.80-2.65 (m, 4H); $^{13}$CNMR (75 MHz, $CDCl_3$) δ 160.38, 140.77, 131.41, 130.00, 129.84, 129.32, 127.14, 119.30, 115.34, 47.27, 46.77, 40.27, 36.03; HRMS ($ESI^+$) calcd for $C_{13}H_{14}ClN_2O^+$ $[M+H]^+$ 249.0795, found 249.0792.

**3-(3-Chlorophenyl)-5,6,7,8-tetrahydro-4H-isoxazolo[4,5-d]azepine** (**2f**). Compound **2f** was synthesized according to the procedure of **2a** using a solution of **15f** (0.075 mmol) and HBr in AcOH (33%, 0.10 x 3 ml). Yield: 86% (0.064 mmol); HPLC: purity 97 %, $t_R$= 5.4 min; $^1$HNMR (300 MHz, $CDCl_3$) δ 7.45-7.25 (m, 4H), 3.12-2.95 (m, 4H), 2.87-2.80 (m, 2H), 2.79-2.71 (m, 2H); $^{13}$CNMR (75 MHz, $CDCl_3$) δ 160.27, 143.02, 134.49, 129.89, 128.94, 127.15, 125.42, 119.90, 113.39, 47.08, 46.64, 40.53, 36.21; HRMS ($ESI^+$) calcd for $C_{13}H_{14}ClN_2O^+$ $[M+H]^+$ 249.0795, found 249.0757.

**3-(4-Chlorophenyl)-5,6,7,8-tetrahydro-4H-isoxazolo[4,5-d]azepine** (**2g**). Compound **2g** was synthesized according to the procedure of **2a** using a solution of **15g** (0.12 mmol) and HBr in AcOH (33%, 0.15 x 3 ml). Yield: 70% (0.084 mmol); HPLC: purity 96 %, $t_R$= 5.7 min; $^1$HNMR (400 MHz, $CDCl_3$) δ 7.37 (d, $J$ = 8.4 Hz, 2H), 7.31 (d, $J$ = 8.4 Hz, 2H), 3.10-2.94 (m, 4H), 2.88-2.79 (m, 2H), 2.78-2.65 (m, 2H); $^{13}$CNMR (100 MHz, $CDCl_3$) δ 160.64, 139.59, 134.87, 128.78, 128.56, 120.19, 112.77, 47.02, 46.57, 40.51, 36.13; HRMS ($ESI^+$) calcd for $C_{13}H_{14}ClN_2O^+$ $[M+H]^+$ 249.0795, found 249.0793.

**3-(o-Tolyl)-5,6,7,8-tetrahydro-4H-isoxazolo[4,5-d]azepine** (**2h**). Compound **2h** was synthesized according to the procedure of **2a** using a solution of **15h** (0.30 mmol) and HBr in AcOH (33%, 0.30 x 3 ml). Yield: 61% (0.18 mmol); HPLC: purity 97 %, $t_R$= 6.8 min; $^1$HNMR (400 MHz, $CDCl_3$) δ 7.29-7.21 (m, 3H), 7.13-7.02 (m, 1H), 3.04-2.95 (m, 4H), 2.80-2.59 (m, 4H), 2.28 (s, 3H); $^{13}$CNMR (100 MHz, $CDCl_3$) δ 162.98, 141.96, 133.76, 130.58, 128.42, 127.15, 126.15, 119.72, 114.07, 47.61, 46.77, 40.75, 35.80, 19.78; HRMS ($ESI^+$) calcd for $C_{14}H_{17}N_2O^+$ $[M+H]^+$ 229.1341, found 229.1335.

**3-(m-Tolyl)-5,6,7,8-tetrahydro-4H-isoxazolo[4,5-d]azepine** (**2i**). Compound **2i** was synthesized according to the procedure of **2a** using a solution of **15i** (0.20 mmol) and HBr in AcOH (33%, 0.20 x 3 ml). Yield: 44% (0.088 mmol); HPLC: purity 96 %, $t_R$= 5.0 min; $^1$HNMR (400 MHz, $CDCl_3$) δ 7.33-7.29 (m, 1H), 7.23-7.17 (m, 3H), 3.04-3.00 (m, 4H), 2.91-2.83 (m, 2H), 2.76-2.71 (m, 2H), 2.40 (s, 3H); $^{13}$CNMR (100 MHz, $CDCl_3$) δ 162.00, 141.14, 138.21, 129.69, 128.43, 127.66, 124.20, 120.43, 111.79, 46.91, 46.42, 40.21, 35.77, 21.40; HRMS ($ESI^+$) calcd for $C_{14}H_{17}N_2O^+$ $[M+H]^+$ 229.1341, found 229.1338.

**3-(p-Tolyl)-5,6,7,8-tetrahydro-4H-isoxazolo[4,5-d]azepine** (**2j**). Compound **2j** was synthesized according to the procedure of **2a** using a solution of **15j** (0.20 mmol) and HBr in AcOH (33%, 0.20 x 3 ml). Yield: 44% (0.088 mmol); HPLC: purity 98 %, $t_R$= 7.0 min;

$^{1}$HNMR (300 MHz, $CDCl_3$) δ 7.28 (d, *J* = 8.2 Hz, 2H), 7.19 (d, *J* = 8.0 Hz, 2H), 3.03-2.94 (m, 4H), 2.88-2.78 (m, 2H), 2.75-2.64 (m, 2H), 2.13 (s, 3H); $^{13}$CNMR (75 MHz, $CDCl_3$) δ 161.96, 139.02, 138.33, 129.18, 127.10, 120.76, 111.47, 47.13, 46.64, 40.67, 36.21, 21.30; HRMS ($ESI^+$) calcd for $C_{14}H_{17}N_2O^+$ $[M+H]^+$ 229.1341, found 229.1338.

**1-(Tert-butyl) 4-ethyl 5-oxoazepane-1,4-dicarboxylate** (**4**). Solution of ethyldiazoacetate in $Et_2O$ (1.3 mmol) and $BF_3OEt_2$ in $Et_2O$ (1.1 mmol) were independently and slowly added to the mixture of compound **3** (1.0 mmol) in $Et_2O$ (3 ml) at -20 ˚C. The reaction mixture was stirred at room temperature for 1 h. The reaction mixture was quenched with 30% $K_2CO_3$ and then extracted with $Et_2O$. The combined organic layers were dried over $MgSO_4$, filtered, and evaporated to obtain desired product **4**. The residue went to the next step without further purification.

**Tert-butyl 3-oxo-2,3,4,5,7,8-hexahydropyrazolo[3,4-d]azepine-6(1H)-carboxylate** (**5**). Hydrazine in 35% water (85.2 mmol) were added to a solution of compound **4** (50.1 mmol) in EtOH (100 ml). The mixture was stirred and refluxed for 2 days. The reaction mixture was cooled to room temperature and stored at -15 ˚C for 16 h. The white solid was then filtered to obtain desired product **5** (16.98 mmol, 97% in 2 steps) as two rotamers. $^{1}$H NMR (400 MHz, MeOD) δ 3.63-3.52 (m, 4H), 2.78-2.76 (m, 2H), 2.58-2.55 (m, 2H), 1.52 (s, 9H); $^{13}$CNMR (100 MHz, DMSO) δ 159.56, 154.52 and 154.38, 141.35, 100.94, 79.17, 49.44 and 48.85, 47.47 and 46.97, 28.56, 28.04 and 27.33, 23.59 and 22.85; LC/MS ($ESI^+$) calcd for $C_{12}H_{20}N_3O_3^+$ $[M+H]^+$ 254.15, found 254.10.

**Di-tert-butyl 3-oxo-2,3,4,5,7,8-hexahydropyrazolo[3,4-d]azepine-1,6-dicarboxylate** (**6**). $Boc_2O$ (39.5 mmol) and TEA (39.5 mmol) were added to a solution of compound **5** (39.5 mmol) in DCM/DMF (100/10 ml). The mixture was stirred at room temperature for 18 h. The reaction mixture was cooled to room temperature and stored at -15 ˚C for 16 h. After reaction

termination, the reaction mixture was evaporated and the white solid was then filtered and washed with $Et_2O$ to obtain desired product **6** (24.9 mmol, 63%) as two rotamers. $^1$HNMR (400 MHz, $CDCl_3$) δ 3.67-3.55 (m, 4H), 3.31 (brs, 2H), 2.67-2.65 (m, 2H), 1.61 (s, 9H), 1.47 (s, 9H); $^{13}$CNMR (100 MHz, $CDCl_3$) δ 162.36 and 162.22, 154.81, 148.75 and 148.44, 145.25 and 145.05, 110.75 and 110.39, 85.01, 79.76, 47.81 and 47.26, 45.24 and 44.37, 29.26 and 28.82, 28.50, 28.06, 22.17 and 21.84; LC/MS ($ESI^+$) calcd for $C_{17}H_{28}N_3O_5^+$ $[M+H]^+$ 354.20, found 254.10.

**Di-tert-butyl 3-(((trifluoromethyl)sulfonyl)oxy)-4,5,7,8-tetrahydropyrazolo[3,4-d]azepine-1,6-dicarboxylate** (**7**). $Tf_2NPh$ (37.35 mmol) and DIPEA (29.88 mmol) were added to the solution of compound **6** (24.90 mmol) in DCM (100 ml). The resulting mixture was refluxed and stirred for 12 h. After reaction termination, the reaction solution was evaporated. The residue was purified by column chromatography on silica gel (Hex:EA = 10:1) to obtain desired product **7** (23.15 mmol, 93%) as two rotamers. $^1$HNMR (300 MHz, $CDCl_3$) δ 3.72-3.55 (m, 4H), 3.38 (brs, 2H), 2.71 (t, $J$ = 4.8 Hz, 2H), 1.62 (s, 9H), 1.48 (s, 9H); $^{13}$CNMR (100 MHz, $CDCl_3$) δ 154.71 and 154.56, 152.73, 147.99 and 147.64, 146.64 and 146.55, 118.55 (q, $J$ = 319 Hz), 112.94 and 112.67, 86.39 and 86.23, 80.10, 47.32 and 46.76, 45.03 and 44.21, 29.51 and 29.08, 28.41, 27.78, 22.75 and 22.33; LC/MS ($ESI^+$) calcd for $C_{18}H_{26}F_3N_3NaO_7S^+$ $[M+Na]^+$ 508.13 found 508.20.

**Di-tert-butyl 3-phenyl-4,5,7,8-tetrahydropyrazolo[3,4-d]azepine-1,6-dicarboxylate** (**8a**). Phenylboronic acid (0.93 mmol), $Pd(dppf)Cl_2$ (0.062 mmol), and $K_2PO_4$ (1.24 mmol) were added to a solution of compound **7** (0.62 mmol) in THF (10 ml). The mixture was refluxed and stirred for overnight. After cooling down to room temperature, the reaction mixture was quenched with saturated $NaHCO_3$ and then extracted with EA. The combined organic layers were dried over $MgSO_4$, filtered, and evaporated. The residue was purified by column

chromatography on silica gel (Hex:EA = 15:1) to obtain desired products **8a** (0.29 mmol, 47%). $^1$HNMR (300 MHz, $CDCl_3$) δ 7.54 (d, *J* = 6.3 Hz, 2H), 7.44-7.38 (m, 3H), 3.74-3.68 (m, 2H), 3.55 (brs, 2H), 3.39 (t, *J* = 5.4 Hz, 2H), 2.85 (brs, 2H), 1.65 (s, 9H), 1.48 (s, 9H)

**Di-tert-butyl 3-(2-fluorophenyl)-4,5,7,8-tetrahydropyrazolo[3,4-d]azepine-1,6-dicarboxylate** (**8b**). Compound **8b** was synthesized according to the procedure of **8a** using a solution of **7** (0.62 mmol), 2-fluorophenylboronic acid (0.93 mmol), $Pd(dppf)Cl_2$ (0.062 mmol), and $K_2PO_4$ (1.24 mmol) in THF (10 mL). Yield: 15% (0.093 mmol); $^1$HNMR (400 MHz, $CDCl_3$) δ 7.57-7.55 (m, 1H), 7.45-7.40 (m, 1H), 7.25 (t, *J* = 7.4 Hz, 1H), 7.15 (t, *J* = 9.2, 1H), 3.81-3.70 (m, 2H), 3.59-3.54 (m, 2H), 3.41 (t, *J* = 5.2 Hz, 2H), 2.71-2.68 (m, 2H), 1.68 (s, 9H), 1.45 (d, *J* = 5.7 Hz, 9H).

**Di-tert-butyl 3-(3-fluorophenyl)-4,5,7,8-tetrahydropyrazolo[3,4-d]azepine-1,6-dicarboxylate** (**8c**). Compound **8c** was synthesized according to the procedure of **8a** using a solution of **7** (2.06 mmol), 3-fluorophenylboronic acid (3.09 mmol), $Pd(dppf)Cl_2$ (0.21 mmol), and $K_2PO_4$ (4.12 mmol) in THF (20 mL). Yield: 39% (0.81 mmol); $^1$HNMR (300 MHz, $CDCl_3$) δ 7.46-7.29 (m, 3H), 7.14-7.09 (m, 1H), 3.77-3.72 (m, 2H), 3.60 (brs, 2H), 3.43 (t, *J* = 5.3 Hz, 2H), 2.88 (brs, 2H), 1.68 (s, 9H), 1.52 (s, 9H).

**Di-tert-butyl 3-(4-fluorophenyl)-4,5,7,8-tetrahydropyrazolo[3,4-d]azepine-1,6-dicarboxylate** (**8d**). Compound **8d** was synthesized according to the procedure of **8a** using a solution of **7** (2.06 mmol), 4-fluorophenylboronic acid (3.09 mmol), $Pd(dppf)Cl_2$ (0.21 mmol), and $K_2PO_4$ (4.12 mmol) in THF (20 mL). Yield: 24% (0.49 mmol); $^1$HNMR (300 MHz, $CDCl_3$) δ 7.53-7.49 (m, 2H), 7.11 (t, *J* = 8.7 Hz, 2H), 3.73-3.68 (m, 2H), 3.55 (brs, 2H), 3.38 (t, *J* = 5.3 Hz, 2H), 2.82 (brs, 2H), 1.64 (s, 9H), 1.48 (s, 9H).

**Di-tert-butyl 3-(2-chlorophenyl)-4,5,7,8-tetrahydropyrazolo[3,4-d]azepine-1,6-dicarboxylate** (**8e**). Compound **8e** was synthesized according to the procedure of **8a** using a

solution of **7** (2.06 mmol), 2-chlorophenylboronic acid (3.09 mmol), Pd(dppf)$Cl_2$ (0.21 mmol), and $K_2PO_4$ (4.12 mmol) in THF (20 mL). Yield: 11% (0.23 mmol); $^1$HNMR (300 MHz, $CDCl_3$) δ 7.45-7.28 (m, 4H), 3.72 (brs, 2H), 3.54-3.52 (m, 2H), 3.38 (t, *J* = 4.9 Hz, 2H), 2.59 (brs, 2H), 1.64 (s, 9H), 1.47 (d, *J* = 7.5 Hz, 9H).

**Di-tert-butyl 3-(3-chlorophenyl)-4,5,7,8-tetrahydropyrazolo[3,4-d]azepine-1,6-dicarboxylate** (**8f**). Compound **8f** was synthesized according to the procedure of **8a** using a solution of **7** (2.06 mmol), 3-chlorophenylboronic acid (3.09 mmol), Pd(dppf)$Cl_2$ (0.21 mmol), and $K_2PO_4$ (4.12 mmol) in THF (20 mL). Yield: 33% (0.68 mmol); $^1$HNMR (300 MHz, $CDCl_3$) δ 7.56 (s, 1H), 7.39-7.34 (m, 3H), 3.72-3.68 (m, 2H), 3.56 (brs, 2H), 3.39 (t, *J* = 5.4 Hz, 2H), 2.83 (brs, 2H), 1.65 (s, 9H), 1.48 (s, 9H).

**Di-tert-butyl 3-(4-chlorophenyl)-4,5,7,8-tetrahydropyrazolo[3,4-d]azepine-1,6-dicarboxylate** (**8g**). Compound **8g** was synthesized according to the procedure of **8a** using a solution of **7** (2.06 mmol), 4-chlorophenylboronic acid (3.09 mmol), Pd(dppf)$Cl_2$ (0.21 mmol), and $K_2PO_4$ (4.12 mmol) in THF (20 mL). Yield: 38% (0.78 mmol); $^1$HNMR (400 MHz, $CDCl_3$) δ 7.48 (d, *J* = 8.0 Hz, 2H), 7.40 (d, *J* = 8.4 Hz, 2H), 3.79-3.64 (m, 2H), 3.52-3.49 (m, 2H), 3.39 (t, *J* = 2.0 Hz, 2H), 2.90-2.75 (m, 2H), 1.65 (s, 9H), 1.48 (s, 9H).

**Di-tert-butyl 3-(o-tolyl)-4,5,7,8-tetrahydropyrazolo[3,4-d]azepine-1,6-dicarboxylate** (**8h**). Compound **8h** was synthesized according to the procedure of **8a** using a solution of **7** (2.06 mmol), 2-methylphenylboronic acid (3.09 mmol), Pd(dppf)$Cl_2$ (0.21 mmol), and $K_2PO_4$ (4.12 mmol) in THF (20 mL). Yield: 24% (0.50 mmol); $^1$HNMR (300 MHz, $CDCl_3$) δ 7.30-7.17 (m, 4H), 3.73-3.69 (m, 2H), 3.50 (brs, 2H), 3.39 (t, *J* = 5.1 Hz, 2H), 2.54 (brs, 2H), 2.23 (s, 3H), 1.64 (s, 9H), 1.47 (d, *J* = 8.7 Hz, 9H).

**Di-tert-butyl 3-(m-tolyl)-4,5,7,8-tetrahydropyrazolo[3,4-d]azepine-1,6-dicarboxylate** (**8i**). Compound **8i** was synthesized according to the procedure of **8a** using a solution of **7** (2.06

mmol), 3-methylphenylboronic acid (3.09 mmol), Pd(dppf)$Cl_2$ (0.21 mmol), and $K_2PO_4$ (4.12 mmol) in THF (20 mL). Yield: 14% (0.28 mmol); $^1$HNMR (400 MHz, $CDCl_3$) δ 7.41 (d, *J* = 10.6 Hz, 1H), 7.35-7.32 (m, 2H), 7.23-7.22 (m, 1H), 3.79-3.70 (m, 2H), 3.59-3.57 (m, 2H), 3.41 (t, *J* = 5.1 Hz, 2H), 2.90-2.86 (m, 2H), 2.42 (s, 3H), 1.68 (s, 9H), 1.51 (d, *J* = 6.1 Hz, 9H).

**Di-tert-butyl 3-(p-tolyl)-4,5,7,8-tetrahydropyrazolo[3,4-d]azepine-1,6-dicarboxylate** (**8j**). Compound **8j** was synthesized according to the procedure of **8a** using a solution of **7** (2.06 mmol), 4-methylphenylboronic acid (3.09 mmol), Pd(dppf)$Cl_2$ (0.21 mmol), and $K_2PO_4$ (4.12 mmol) in THF (20 mL). Yield: 46% (0.95 mmol); $^1$HNMR (300 MHz, $CDCl_3$) δ 7.42 (d, *J* = 7.8 Hz, 2H), 7.22 (d, *J* = 7.8 Hz, 2H), 3.73-3.68 (m, 2H), 3.54 (brs, 2H), 3.38 (t, *J* = 5.4 Hz, 2H), 2.84 (brs, 2H), 2.38 (s, 3H), 1.64 (s, 9H), 1.48 (s, 9H).

**Diethyl 5-oxoazepane-1,4-dicarboxylate** (**10**). Compound **10** was synthesized according to the procedure of **4** using a solution of **9** (87.6 mmol), ethyldiazoacetate (113.9 mmol), and $BF_3OEt_2$ (92.0 mmol) in $Et_2O$ (120 ml). The residue went to the next step without further purification.

**Diethyl 1,4-dioxa-8-azaspiro[4.6]undecane-8,11-dicarboxylate** (**11**). Ethylene glycol (437 mmol) and p-TsOH (6.12 mmol) were added to a solution of compound **10** (87.40 mmol) in benzene (200 ml). The mixture was stirred and refluxed for 78 h by using Dean-Stark water separator. After cooling down to room temperature, the reaction mixture was washed with brine and extracted with EA. The combined organic layers were dried over $MgSO_4$, filtered, and evaporated. The residue was purified by column chromatography on silica gel (DCM:EA = 3:1) to obtain desired product **11** (39.8 mmol, 46% in 2 steps) as two rotamers. $^1$HNMR (400 MHz, $CDCl_3$) δ 4.16-4.09 (m, 4H), 4.03-3.88 (m, 4H), 3.56-3.31 (m, 4H), 2.83 (td, *J* = 9.2, 3.6 Hz, 1H), 2.24-1.75 (m, 4H), 1.28-1.22 (m, 6H); $^{13}$CNMR (100 MHz, $CDCl_3$) δ

171.96 and 171.81, 155.99, 109.94, 65.11 and 64.68, 61.18, 60.47, 51.50 and 50.99, 44.22 and 44.08, 41.02 and 40.77, 37.62 and 37.54, 25.11 and 25.00, 14.75, 14.16; LC/MS ($ESI^+$) calcd for $C_{14}H_{24}NO_6^+$ $[M+H]^+$ 302.16, found 302.10.

**Ethyl 11-(hydroxycarbamoyl)-1,4-dioxa-8-azaspiro[4.6]undecane-8-carboxylate** (**12**). At 50 ˚C, sodium (19.92 mmol) was added to MeOH (10 ml) to make NaOMe solution *in situ*. This solution was added to the mixture of $NH_2OH$/HCl (10.22 mmol) in MeOH at 50 ˚C. In this reaction mixture, compound **11** was then added at room temperature. The reaction mixture was stirred at room temperature for 24 h. After reaction termination, 2.5 M HCl was added to the reaction mixture until pH = 3 and extracted with EA. The combined organic layers were dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (EA:MeOH:formic acid = 150:10:1) to obtain desired product **12** (2.06 mmol, 31%) as two rotamers. $^1$HNMR (400 MHz, $CDCl_3$) δ 9.23 (brs, 1H), 4.18-4.07 (m, 2H), 4.06-4.00 (m, 1H), 3.99-3.86 (m, 3H), 3.67-3.45 (m, 3H), 3.30-3.15 (m, 1H), 2.66 (t, *J* = 8.4 Hz, 1H), 2.20-2.09 (m, 1H), 2.08-1.89 (m, 2H), 1.88-1.71 (m, 1H), 1.24 (t, *J* = 6.8 Hz, 3H); $^{13}$CNMR (100 MHz, $CDCl_3$) δ 170.13, 156.30, 109.86, 65.02 and 64.85, 61.37, 49.75 and 49.42, 44.46, 40.72 and 40.35, 37.75, 37.28 and 37.12, 25.21 and 25.00, 14.68; LC/MS ($ESI^+$) calcd for $C_{12}H_{21}N_2O_6^+$ $[M+H]^+$ 289.14, found 289.10.

**Ethyl 3-hydroxy-4,5,7,8-tetrahydro-6H-isoxazolo-[4,5-d]azepine-6-carboxylate** (**13**). Concentrated HCl (33-36%, 0.25 ml) was added to the solution of compound **12** (0.52 mmol) in MeOH (3 ml). The resulting mixture was stirred at 70 ˚C for 10 min. After reaction termination, the reaction mixture was evaporated. The residue was purified by column chromatography on silica gel (EA:MeOH = 20:1) to obtain desired product **13** (0.49 mmol, 94%) as two rotamers. $^1$HNMR (400 MHz, $CDCl_3$) δ 11.10 (s, 1H), 4.12 (q, *J* = 7.2 Hz, 2H), 3.67-3.52 (m, 4H), 2.92-2.80 (m, 2H), 2.55-2.44 (m, 2H), 1.21 (t, *J* = 7.2 Hz, 3H); $^{13}$CNMR

(100 MHz, $CDCl_3$) δ 169.81 and 169.76, 168.97 and 168.56, 155.52 and 155.31, 105.83 and 105.51, 61.55, 48.65 and 48.45, 45.85 and 45.63, 28.98 and 28.33, 21.37 and 20.69, 14.60; LC/MS ($ESI^-$) calcd for $C_{10}H_{13}N_2O_4^-$ $[M-H]^-$ 225.09, found: 225.15.

**Ethyl 3-(((trifluoromethyl)sulfonyl)oxy)-4,5,7,8-tetrahydro-6H-isoxazolo[4,5-d]azepine-6-carboxylate** (**14**). Compound **14** was synthesized according to the procedure of **7** using a solution of **13** (3.89 mmol), $Tf_2NPh$ (5.83 mmol). and DIPEA (4.67 mmol) in DCM (20 ml) as two rotamers. Yield: 86% (3.35 mmol); $^1$HNMR (400 MHz, $CDCl_3$) δ 4.20 (q, *J* = 6.9 Hz, 2H), 3.75-3.55 (m, 4H), 3.15-3.05 (m, 2H), 2.69-2.59 (m, 2H), 1.29 (t, *J* = 6.9 Hz, 3H); $^{13}$CNMR (100 MHz, $CDCl_3$) δ 173.21 and 172.72, 163.06, 155.28 and 155.12, 118.50 (q, J = 319 Hz), 108.56 and 108.20, 61.81, 48.29 and 48.06, 45.66 and 45.47, 29.77 and 29.14, 21.82 and 21.08, 14.67; LC/MS ($ESI^+$): m/z: calcd for $C_{11}H_{14}F_3N_2O_6S^+$ $[M+H]^+$ 359.05, found 359.25.

**Ethyl 3-phenyl-4,5,7,8-tetrahydro-6H-isoxazolo[4,5-d]azepine-6-carboxylate** (**15a**). Phenylboronic acid (0.83 mmol), $Pd(dppf)Cl_2$ (0.056 mmol), KBr (0.62 mmol), and $K_2PO_4$ (1.13 mmol) were added to a solution of compound **14** (0.56 mmol) in THF (10 ml). The mixture was refluxed and stirred for overnight. After cooling down to room temperature, the reaction mixture was quenched with saturated $NaHCO_3$ and then extracted with EA. The combined organic layers were dried over $MgSO_4$, filtered, and evaporated. The residue was purified by column chromatography on silica gel (Hex:EA = 10:1) to obtain desired products **15a** (0.16 mmol, 28%). $^1$HNMR (400 MHz, $CDCl_3$) δ 7.43-7.30 (m, 5H), 4.21-4.12 (m, 2H), 3.68 (brs, 4H), 2.86 (brs, 2H), 2.74 (brs, 2H), 1.31-1.19 (m, 3H).

**Ethyl 3-(2-fluorophenyl)-4,5,7,8-tetrahydro-6H-isoxazolo[4,5-d]azepine-6-carboxylate** (**15b**). Compound **15b** was synthesized according to the procedure of **15a** using a solution of **14** (1.12 mmol), 2-fluorophenylboronic acid (1.34 mmol), $Pd(dppf)Cl_2$ (0.11 mmol), KBr

(1.23 mmol), and $K_2PO_4$ (3.36 mmol) in THF (10 ml). Yield: 23% (0.26 mmol); $^1$HNMR (400 MHz, $CDCl_3$) δ 7.21-7.06 (m, 4H), 4.25-4.15 (m, 2H), 3.72 (brs, 4H), 2.79 (brs, 4H), 1.38-1.25 (m, 3H).

**Ethyl 3-(3-fluorophenyl)-4,5,7,8-tetrahydro-6H-isoxazolo[4,5-d]azepine-6-carboxylate** (**15c**). Compound **15c** was synthesized according to the procedure of **15a** using a solution of **14** (1.12 mmol), 3-fluorophenylboronic acid (1.34 mmol), Pd(dppf)$Cl_2$ (0.11 mmol), KBr (1.23 mmol), and $K_2PO_4$ (3.36 mmol) in THF (10 ml). Yield: 29% (0.32 mmol); $^1$HNMR (400 MHz, $CDCl_3$) δ 7.40 (q, $J$ = 6.1 Hz, 1H), 7.20-7.04 (m, 3H), 4.22 (q, $J$ = 7.0 Hz, 2H), 3.71 (brs, 4H), 2.86 (brs, 2H), 2.77 (brs, 2H), 1.31 (t, $J$ = 6.0 Hz, 3H).

**Ethyl 3-(4-fluorophenyl)-4,5,7,8-tetrahydro-6H-isoxazolo[4,5-d]azepine-6-carboxylate** (**15d**). Compound **15d** was synthesized according to the procedure of **15a** using a solution of **14** (1.12 mmol), 4-fluorophenylboronic acid (1.34 mmol), Pd(dppf)$Cl_2$ (0.11 mmol), KBr (1.23 mmol), and $K_2PO_4$ (3.36 mmol) in THF (10 ml). Yield: 33% (0.37 mmol); $^1$HNMR (400 MHz, $CDCl_3$) δ 7.39-7.25 (m, 2H), 7.14-7.03 (m, 2H), 4.24-4.11 (m, 2H), 3.68 (brs, 4H), 2.83 (brs, 2H), 2.73 (brs, 2H), 1.32-1.20 (m, 3H).

**Ethyl 3-(2-chlorophenyl)-4,5,7,8-tetrahydro-6H-isoxazolo[4,5-d]azepine-6-carboxylate** (**15e**). Compound **15e** was synthesized according to the procedure of **15a** using a solution of **14** (0.28 mmol), 2-chlorophenylboronic acid (0.33 mmol), Pd(dppf)$Cl_2$ (0.022 mmol), KBr (0.31 mmol), and $K_2PO_4$ (0.84 mmol) in THF (3 ml). Yield: 12% (0.032 mmol); $^1$HNMR (400 MHz, $CDCl_3$) δ 7.46-7.41 (m, 1H), 7.35-7.28 (m, 2H), 7.25-7.18 (m, 1H), 4.20 (q, $J$ = 3.4 Hz, 2H), 3.83 (brs, 2H), 3.69-3.52 (m, 2H), 2.75 (brs, 4H), 1.35-1.27 (m, 3H).

**Ethyl 3-(3-chlorophenyl)-4,5,7,8-tetrahydro-6H-isoxazolo[4,5-d]azepine-6-carboxylate** (**15f**). Compound **15f** was synthesized according to the procedure of **15a** using a solution of **14** (0.28 mmol), 3-chlorophenylboronic acid (0.33 mmol), Pd(dppf)$Cl_2$ (0.028 mmol), KBr

(0.31 mmol), and $K_2PO_4$ (0.84 mmol) in THF (3 ml). Yield: 28% (0.078 mmol); $^1$HNMR (400 MHz, $CDCl_3$) δ 7.38-7.31 (m, 3H), 7.27-7.23 (m, 1H), 4.22 (q, *J* = 7.1 Hz, 2H), 3.71 (brs, 4H), 2.85 (brs, 2H), 2.77 (brs, 2H), 1.31 (t, *J* = 7.0 Hz, 3H).

**Ethyl 3-(4-chlorophenyl)-4,5,7,8-tetrahydro-6H-isoxazolo[4,5-d]azepine-6-carboxylate** (**15g**). Compound **15g** was synthesized according to the procedure of **15a** using a solution of **14** (0.32 mmol), 4-chlorophenylboronic acid (0.48 mmol), Pd(dppf)$Cl_2$ (0.032 mmol), and $K_2PO_4$ (0.64 mmol) in THF (3 ml). Yield: 37% (0.12 mmol); $^1$HNMR (300 MHz, $CDCl_3$) δ 7.42 (d, *J* = 8.4 Hz, 2H), 7.32 (d, *J* = 9.3 Hz, 2H), 4.23 (q, *J* = 7.2 Hz, 2H), 3.72 (brs, 4H), 2.86 (brs, 2H), 2.79 (brs, 2H), 1.32 (t, *J* = 6.9 Hz, 4H).

**Ethyl 3-(o-tolyl)-4,5,7,8-tetrahydro-6H-isoxazolo[4,5-d]azepine-6-carboxylate** (**15h**). Compound **15h** was synthesized according to the procedure of **15a** using a solution of **14** (1.12 mmol), 2-methylphenylboronic acid (1.34 mmol), Pd(dppf)$Cl_2$ (0.11 mmol), KBr (1.23 mmol), and $K_2PO_4$ (3.36 mmol) in THF (10 ml). Yield: 19% (0.22 mmol); $^1$HNMR (400 MHz, $CDCl_3$) δ 7.39-7.20 (m, 3H), 7.08 (d, *J* = 7.1 Hz, 1H), 4.23 (q, *J* = 7.1 Hz, 2H), 3.73 (brs, 4H), 2.56 (brs, 4H), 2.29 (s, 3H), 1.38-1.26 (m, 3H).

**Ethyl 3-(m-tolyl)-4,5,7,8-tetrahydro-6H-isoxazolo[4,5-d]azepine-6-carboxylate** (**15i**). Compound **15i** was synthesized according to the procedure of **15a** using a solution of **14** (1.12 mmol), 3-methylphenylboronic acid (1.34 mmol), Pd(dppf)$Cl_2$ (0.11 mmol), KBr (1.23 mmol), and $K_2PO_4$ (3.36 mmol) in THF (10 ml). Yield: 19% (0.21 mmol); $^1$HNMR (300 MHz, $CDCl_3$) δ 7.36-7.30 (m, 1H), 7.24-7.15 (m, 3H), 4.23 (q, *J* = 7.2 Hz, 2H), 3.72 (brs, 4H), 2.88 (brs, 2H), 2.77 (brs, 2H), 2.41 (s, 3H), 1.33 (t, *J* = 6.9 Hz, 3H).

**Ethyl 3-(p-tolyl)-4,5,7,8-tetrahydro-6H-isoxazolo[4,5-d]azepine-6-carboxylate** (**15j**). Compound **15j** was synthesized according to the procedure of **15a** using a solution of **14** (1.12 mmol), 4-methylphenylboronic acid (1.34 mmol), Pd(dppf)$Cl_2$ (0.11 mmol), KBr (1.23

mmol), and $K_2PO_4$ (3.36 mmol) in THF (10 ml). Yield: 25% (0.28 mmol); $^1$HNMR (300 MHz, $CDCl_3$) δ 7.32-7.20 (m, 4H), 4.22 (q, *J* = 7.2 Hz, 2H), 3.71 (brs, 4H), 2.88 (brs, 2H), 2.77 (brs, 2H), 2.40 (s, 3H), 2.31 (t, *J* = 7.2 Hz, 3H).

**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 (5x 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 (EDTA), 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] D-lysergic acid diethylamide ([$^3$H]LSD) radioligand was diluted to five 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 recombinant target were dispensed into each well. Total 250ml of the reaction mixtures were incubated at room temperature and shielded from light for 1.5 h, 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, 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 150 mm dishes and changed medium from DMEM with 10% 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 recovery, the medium was removed from the wells and cells were treated with 3% Glosensor cAMP reagent, luciferin (Promega) 20 μL in filter-sterilized assay buffer including 1X HBSS, 20 mM HEPES, and 3$^{rd}$ distilled water, pH 7.4. Then, reference agonist (5-HT, 400 nM) or the compounds to be tested 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, 120 μM, and 400 μM) in above assay buffer with 0.1% bovine serum albumin (BSA). After 30 min, cells were treated with 10 μL of drugs prepared above (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, 300 nM, 100 nM, 300 nM, 1 μM, 3 μM, 10 μM, 30 μM, and 100 μM of test compounds). The luminescence intensity of the accumulated cAMP level was measured by using a microplate reader (Flexstation3 or SpectraMax® i3, Molecular Devices). The sigmoidal dose-response graph of 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-arrestin2-TEV fusion gene) were plated in 150 mm

dishes and changed medium from DMEM with 10% FBS, 100 U/ml penicillin and 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 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, 180 μM, and 600 μM) in HBSS, 20 mM HEPES, pH 7.4 (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, 300 nM, 100 nM, 300 nM, 1 μM, 3 μM, 10 μM, 30 μM, and 100 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 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), 0.1M 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,

NADPH generation system solution was also added and incubated at 37 ˚C for 15 min again. To terminate the reaction, acetonitrile including 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), 0.1M phosphate buffer (pH 7.4) and tested compounds (1 μM) were added. After incubation at 37 ˚C for 5 min, NADPH generation system solution was also added and incubated at 37 ˚C for 30 min again. To terminate 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.

**Pharmacokinetic 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 pharmacokinetic and tissue distribution studies, and were purchased from Orient Co. (South Korea). The mice were kept at a room temperature controlled at 23 ± 3° with relative humidity controlled at about 55% ± 10%, fed with standard solid composite feedstuff, and received tap water. Compound **1g** at a dose of 5 mg/kg or 50 mg/kg was administered intravenously or orally, 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 12000 rpm for 3 min, plasma samples were stored at -70 ˚C until analysis. Pharmacokinetic 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 last measured time ($AUC_{last}$t) was calculated by the trapezoidal rule-extrapolation method.

Standard methods were used to calculate the following pharmacokinetic parameters[59,60]; the time-averaged total body clearance (CL), total area under the first moment of plasma concentration e time curve from time zero to time infinity ($AUC_{0-\infty}$), terminal half-life, mean residence time (MRT), apparent volume of distribution at steady state ($V_{dss}$). 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 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 x 2.1 mm, 1.7um, 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.

**Sleep EEG Test.** All mice experiments conducted for this study were approved by the Korea Advanced Institute of Science and Technology (KAIST, Daejeon, Korea) and were performed in accordance with the ethical standards stated in the Animal Care and Use Guidelines of KAIST. Adult male wild-type mice (C57B1/6J; 10 weeks; n = 23) were used, and animals were provided with unrestricted access to food and water throughout the experiments. For EEG recording, two stainless steel screw (diameter 1 mm) electrodes were implanted at the lateral parietal association cortex (Anterior-Posterior, AP: 2 mm, Medial-Lateral, ML: 1.5 mm) and the cerebellum for animal ground, respectively, under ketamine anesthesia. For the

electromyogram (EMG) recording, a stainless steel fine wire was inserted into the trapezius muscle. The electrodes and wires were fixed to the skull with dental cement. During the recovery period of 7 days, animals were housed in the sound chamber individually with 12:12 light/dark schedule (lights on at 6 AM) to allow for adaptation. One day prior to the vehicle/compound administration, EEG and EMG signals were recorded for an 8-h time interval using SmarBox$^{TM}$ Kit of 32 channels (NeuroNexus, USA) from 9 AM. On the designated day of vehicle/compound administration, vehicle/compound was administrated through intraperitoneal injection at 9 AM, and EEG and EMG signals were recorded for an 8-h time interval. Signals were acquired at a sampling rate of 1000 Hz, and no filter was applied to both the EEG and EMG signals during the recording.

We developed a custom MATLAB program that divides EEG and EMG signals into 10-s epochs, categorizes the states between active wake, quiet wake, NREM sleep, and REM sleep, and extracts important parameters such as latency to REM/NREM sleep and duration of each state.[61] Fast Fourier transform (FFT) analyses were applied on the acquired EEG signals to extract delta (0.5 - 4 Hz) and theta frequency bands (6 - 10 Hz) and on the EMG signals to extract 20 - 100 Hz band. Active wake was characterized by significant EMG activity. When the spectral power of the EMG signals was low, the EEG signals in both delta and theta bands were examined. When the EEG delta to theta ratio was larger than a threshold, the state as categorized as NREM sleep (dominant of EEG delta band). On the other hand, REM was specified by a higher theta to delta ratio and a lower EMG integral power than threshold values. The extra undefined epochs were marked as the quiet wake state.[62] After scoring, parameters such as latency to REM/NREM sleep and time spent in the wake, NREM, and REM states were calculated at 2-h time intervals. Results were expressed as the mean ± standard error of the mean (S.E.M.) for each set of experiments. For comparison between

vehicle and compound administrations, an unpaired *t*-test was executed.

**Molecular docking study.** For molecular docking study, the homology model structure of the 5-$HT_7$R was constructed through the SwissModel server using the 5-$HT_{1B}$R structure (PDB: 4IAR).[45-47] The protein structures were prepared under the standard procedure of the Prepare Protein module (CHARMm force field). The binding site was defined and edited by the centroid of co-crystal ligand, ergotamine. Ligands were prepared with energy minimization at pH 7.4. The molecular docking poses of ligands were obtained using CDOCKER in Discovery Studio 2017 (Accelrys, Inc.) based on the CHARMm docking algorithm. The docking poses of ligands were saved for analysis up to 10 poses.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

$^{1}$H and $^{13}$C NMR spectra and Supplementary figures (PDF)

Molecular formula strings (CSV)

Homology model of 5-$HT_7$R (PDB)

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### Author Contributions

The first two authors Y. Kim and H. Kim contributed equally to this work. The manuscript

was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

**Notes**

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

We are grateful to the US National Institute of Mental Health (NIMH) Psychoactive Drug Screening Program (contract: HHSN-271-2008-00025-C) for providing binding affinity data. This research is supported by the Original Technology Research Program (NRF-2016M3C7A1904344 and 2016M3C7A1904343) funded by the National Research Foundation of Korea (NRF). And this work is additionally funded by the Korea Institute of Science and Technology (KIST) Institutional Program (2E27870 and 2E28412).

## ■ ABBREVIATIONS USED

AC, adenylyl cyclase; $BF_3OEt_2$, boron trifluoride diethyl etherate; $Boc_2O$, di-tert-butyl dicarbonate; cAMP, cyclic adenosine monophosphate; CNS, central nervous system; CYP450, cytochrome P450; DIPEA, diisopropylethylamine; $EC_{50}$, half maximal effective concentration; EEG, electroencephalogram (EEG); GPCRs, G protein coupled receptors; HEK293, human embryonic kidney cells 293; [$^3$H]LSD, [$^3$H]lysergic acid diethylamide; 5-HT, 5-hydroxytryptamine; 5-$HT_7$R, 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; $Pd(dppf)Cl_2$, [1,1′-bis(diphenylphosphino)ferrocene]dichloropalladium(II); NREM, non-rapid eye movement; PKs, pharmacokinetic parameters; *p*-TsOH, *para*-toluenesulfonic acid; REM,

rapid eye movement; SAR, structure-activity relationship; SI, selectivity index; SSRIs, selective serotonin reuptake inhibitors; $Tf_2NPh$, N-phenyl-bis(trifluoromethanesulfonimide); THF, tetrahydrofuran.

## ■ REFERENCES

1. Fredriksson, R.; Lagerström, M. C.; Lundin, L. G.; Schiöth, H. B. The G-protein-coupled Receptors in the Human Genome form Five Main Families. Phylogenetic analysis, paralogon groups, and fingerprints. *Mol. Pharmacol.* **2003,** *63,* 1256-1272

2. Sakmar, T. P. Introduction: G-protein Coupled Receptors. *Chem. Rev*. **2017**, *117*, 1–3

3. Rasmussen, S. G.; DeVree, B. T.; Zou, Y.; Kruse, A. C.; Chung, K. Y.; Kobilka, T. S.; Thian, F. S.; Chae, P. S.; Pardon, E.; Calinski, D.; Mathiesen, J. M.; Shah, S. T.; Lyons, J. A.; Caffrey, M.; Gellman, S. H.; Steyaert, J.; Skiniotis, G.; Weis, W. I.; Sunahara, R. K.; Kobilka, B. K. Crystal Structure of the β2 Adrenergic Receptor-Gs Protein Complex. *Nature* **2011**, *477*, 549−555

4. Shukla, A. K.; Manglik, A.; Kruse, A. C.; Xiao, K.; Reis, R. I.; Tseng, W. C.; Staus, D. P.; Hilger, D.; Uysal, S.; Huang, L. Y.; Paduch, M.; Tripathi-Shukla, O.; Koide, A.; Koide, S.; Weis, W. I.; Kossiasoff, A. A.; Kobilka, B. K.; Lefkowitz, R. J. Structure of Active β-arrestin-1 Bound to a G-protein-coupled Receptor Phosphopeptide. *Nature* **2013**, *497*, 137−141

5. Kristiansen, K. Molecular Mechanisms of Ligand Binding, Signaling, and Regulation within the Superfamily of G-protein-coupled Receptors: Molecular Modeling and Mutagenesis Approaches to Receptor Structure and Function. *Pharmacol. Ther.* **2004,** *103,* 21-80

6. Klabunde, T.; Hessler, G. Drug Design Strategies for Targeting G-protein-coupled Receptors. *Chembiochem.* **2002**, *3,* 928-924

7. Lappano, R.; Maggiolini, M. G Protein-coupled Receptors: Novel Targets for Drug Discovery in Cancer. *Nat. Rev. Drug Discov.* **2011**, *10*, 47-60

8. Wacker D.; Stevens R. C.; Roth B. L. How Ligands Illuminate GPCR Molecular Pharmacology. *Cell* **2017**, *170*, 414-427.

9. Jacobson, K. A. New Paradigms in GPCR Drug Discovery. *Biochem. Pharmacol.* **2015,** *98,* 541-555

10. Langenhan, T.; Barr, M. M.; Bruchas, M. R.; Ewer, J.; Griffith, L. C.; Maiellaro, I.; Taghert, P. H.; White, B. H.; Monk, K. R. Model Organisms in G Protein-coupled Receptor Research. *Mol. Pharmacol.* **2015**, *88*, 596-603

11. Jacobson, K. A.; von Lubitz, D. K. J. E.; Daly, J. W.; Fredholm, B. B. Adenosine Receptor Ligands: Differences with Acute Versus Chronic Treatment. *Trends Pharmacol. Sci.* **1996**, *17*, 108–113

12. Hoyer, D.; Clarke, D. E.; Fozard, J. R.; Hartig, P. R.; Martin, G. R.; Mylecharane, E. J.; Saxena, F. R.; Humphrey, P. P. A. International Union of Pharmacology Classification of Receptors for 5-Hydroxytryptamine (Serotonin). *Pharmacol. Rev.* **1994**, *46*, 157-203

13. Lovenberg, T. W.; Baron, B. M.; de Lecea, L.; Miller, J. O.; Prosser, R. A.; Rea, M. A.; Foye, P. E.; Rucke, M.; Slone, A. L.; Siegel, B. W.; Danielson, P. E.; Sutcliffe, J. G.; Erlander, M. G. A Novel Adenylyl Cyclase-activating Serotonin Receptor (5-HT7) Implicated in the Regulation of Mammalian Circadian Rhythms. *Neuron.* **1993**, *11*, 449-458

14. Meyerhof, W.; Obermuller, F.; Fehr, S.; Richter, D. A Novel Rat Serotonin Receptor: Primary Structure, Pharmacology, and Expression Pattern in Distinct Brain Regions. *DNA Cell Biol.* **1993**, *12*, 401-409

15. Ruat, M.; Traiffort, E.; Leurs, R.; Tardivel-Lacombe, J.; Diaz, J.; Arrang, J.; Schwartz, J.

C. Molecular Cloning, Characterization, and Localization of a High-affinity Serotonin Receptor (5-HT7) Activating cAMP Formation. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 8547-8551

16. Shen Y.; Monsma F. J.; Metcalf M. A.; Jose P. A.; Hamblin M. W.; Sibley D. R. Molecular Cloning and Expression of a 5-Hydroxytryptamine7 Serotonin Receptor Subtype. *J. Biol. Chem.* **1993**, *268*, 18200-18204

17. Plassat, J. L.; Amlaiky, N.; Hen, R. Molecular Cloning of a Mammalian Serotonin Receptor That Activates Adenylate Cyclase. *Mol. Pharmacol.* **1993**, *44*, 229-236

18. Gustafson, E. L.; Durkin, M. M.; Bard, J. A.; Zgombick, J.; Branchek, T. A. A Receptor Autoradiographic and In Situ Hybridization Analysis of the Distribution of the 5-HT7 Receptor in Rat Brain. *Br. J. Pharmacol.* **1996**, *117*, 657-666

19. Vanhoenacker, P.; Haegeman, G.; Leysen, J. E. 5-HT7 Receptors: Current Knowledge and Future Prospects. *Trends Pharmacol. Sci.* **2000**, *21*, 70-77

20. Sleight, A. J.; Carolo, C.; Petit, N.; Zwingelstein, C.; Bourson, A. Identification of 5-Hydroxytryptamine7 Receptor Binding Sites in Rat Hypothalamus: Sensitivity to Chronic Antidepressant Treatment. *Mol. Pharmacol.* **1995**, *47,* 99-103

21. Brenchat, A.; Nadal, X.; Romero, L.; Ovalle, S.; Muro, A.; Sanchez-Arroyos, R.; Portillo-Salido, E.; Pujol, M.; Montero, A.; Codony, X.; Burgueno, J.; Zamanillo, D.; Hamon, M.; Maldonado, R.; Vela, J. M. Pharmacological Activation of 5-HT7 Receptors Reduces Nerve Injury-induced Mechanical and Thermal Hypersensitivity. *Pain* **2010**, *149*, 483-494

22. Meneses, A.; Perez-Garcia, G.; Liy-Salmeron, G.; Flores-Galvez, D.; Castillo, C.; Castillo, E. The Effects of The 5-HT(6) Receptor Agonist EMD and the 5-HT(7) Receptor Agonist AS19 on Memory Formation. *Behav. Brain Res.* **2008**, *195*, 112-119

23. Brenchat, A.; Romero, L.; Garcia, M.; Pujol, M.; Burgueno, J.; Torrens, A.; Hamon, M.; Baeyens, J. M.; Buschmann, H.; Zamaillo, D.; Vela, J. M. 5-HT7 Receptor Activation Inhibits Mechanical Hypersensitivity Secondary to Capsaicin Sensitization in Mice. *Pain* **2009**, *141*, 239-247

24. Brenchat, A.; Ejarque, M.; Zamanillo, D.; Vela, J. M.; Romero, L. Potentiation of Morphine Analgesia by Adjuvant Activation of 5-HT7 Receptors. *J. Pharmacol. Sci.* **2011**, *116*, 388-391

25. Sprouse, J.; Reynolds, L.; Li, X.; Braselton, J.; Schmidt, A. 8-OH-DPAT as a 5-HT7 Agonist: Phase Shifts of the Circadian Biological Clock through Increases in cAMP Production. *Neuropharmacology* **2004**, *46*, 52–62

26. Nikiforuk, A. Targeting the Serotonin 5-HT7 Receptor in the Search for Treatments for CNS Disorders: Rationale and Progress to Date. *CNS drugs.* **2015**, *29*, 265-275

27. Hagan, J. J.; Price, G. W.; Jeffrey, P.; Deeks, N. J.; Stean, T.; Piper, D.; Smith, M. I.; Upton, N.; Medhurst, A. D.; Middlemiss, D. N.; Riley, G. J.; Lovell, P. J.; Bromidge, S. M.; Thomas, D. R. Characterization of SB-269970-A, a Selective 5-HT(7) Receptor Antagonist. *Br. J. Pharmacol.* **2000**, *130*, 539-548

28. Mahé, C.; Loetscher, C.; Feuerbach, D.; Müller, W.; Seiler, M. P.; Schoeffter, P. Differential Inverse Agonist Efficacies of SB-258719, SB-258741 and SB-269970 at Human Recombinant Serotonin 5-HT7 Receptors. *Eur. J. Phramcol.* **2004**, *495*, 97-102

29. Linnanen, T.; Brisander, M.; Unelius, L.; Rosqvist, S.; Nordvall, G.; Hacksell, U.; Johansson, A. M. Atropisomeric Derivatives of 2',6'-Disubstituted (R)-11-phenylaporphine: Selective Serotonin 5-HT(7) Receptor Antagonists. *J. Med. Chem.* **2001**, *44*, 1337-1340

30. Nicholas, I. C.; Wenying, C.; Xiaohu, D.; Curt, A. D.; Annette, K. K.; Jimmy, T. L.;

Neelakandha, M.; Dale, A. R. Fused Heterocyclic Compounds as Serotonin Receptor Modulators. *WO2005/040169A2*, **2005**

31. Wesolowska, A.; Nikiforuk, A.; Stachowicz, K.; Tatarczynska, E. Effect of the Selective 5-HT7 Receptor Antagonist SB 269970 in Animal Models of Anxiety and Depression. *Neuropharmacology* **2006**, *51*, 578-586

32. Bonaventure, P.; Dugovic, C.; Kramer, M.; De Boer, P.; Singh, J.; Wilson, S.; Bertelsen, K.; Di, J.; Shelton, J.; Aluisio, L.; Dvorak, L.; Fraser, I.; Lord, B.; Nepomuceno, D.; Ahnaou, A.; Drinkenburg, W.; Chai, W.; Dvorak, C.; Sands, S.; Carruthers, N.; Lovenberg, T. W. Translational Evaluation of JNJ-18038683, a 5-Hydroxytryptamine Type 7 Receptor Antagonist, on Rapid Eye Movement Sleep and in Major Depressive Disorder. *J. Pharmacol. Exp. Ther.* **2012**, *342,* 429-440

33. Monti, J. M.; Jantos, H. The Role of Serotonin 5-HT7 Receptor in Regulating Sleep and Wakefulness. *Rev. Neurosci*. **2014**, *25*, 429–437

34. Monti, J. M.; Leopoldo, M.; Jantos, H. Systemic Administration and Local Microinjection into the Central Nervous System of the 5-HT(7) Receptor Agonist LP-211 Modify the Sleep-wake Cycle in the Rat. *Behav. Brain Res.* **2014**, *259*, 321-329

35. Wesolowska, A.; Nikiforuk, A.; Stachowicz, K.; Tatarczynska, E. Effect of the Selective 5-HT7 Receptor Antagonist SB 269970 in Animal Models of Anxiety and Depression. *Neuropharmacology*. **2006**, *51*, 578–586

36. Adriani, W.; Travaglini, D.; Lacivita, E.; Saso, L.; Leopoldo, M.; Laviola, G. Modulatory Effects of Two Novel Agonists for Serotonin Receptor 7 on Emotion, Motivation and Circadian Rhythm Profiles in Mice. *Neuropharmacology.* **2012**, *62*, 833–842

37. Kroeze, W. K.; Sassano, M. F.; Huang, X. P.; Lansu, K.; McCorvy, J. D.; Giguere, P. M.;

Sciaky, N.; Roth, B. L. PRESTO-Tango as an Open-source Resource for Interrogation of the Druggable Human GPCRome. *Nat. Struct. Mol. Biol*. **2015**, *22*, 362-369

38. McCorvy J. D.; Butler K. V.; Kelly B.; Rechsteiner K.; Karpiak J.; Betz R. M.; Kormos B. L.; Shoichet B. K.; Dror R. O.; Jin J.; Roth B. L. Structure-inspired Design of β-Arrestin-biased Ligands for Aminergic GPCRs. *Nat. Chem. Biol.* **2018**, *14*, 126-134

39. Peng Y.; McCorvy J. D.; Harpsøe K.; Lansu K.; Yuan S.; Popov P.; Qu L.; Pu M.; Che T.; Nikolajsen L. F.; Huang X. P.; Wu Y.; Shen L.; Bjørn-Yoshimoto W. E.; Ding K.; Wacker D.; Han G. W.; Cheng J.; Katritch V.; Jensen A. A.; Hanson M. A.; Zhao S.; Gloriam D. E.; Roth B. L.; Stevens R. C.; Liu Z. J. 5-HT2C Receptor Structures Reveal the Structural Basis of GPCR Polypharmacology. *Cell* **2018**, *172*, 719-730

40. Krogsgaard-Larsen, P.; Hjeds, H. Organic Hydroxylamine Derivatives. X. Structural Analogues of Aamma-aminobutyric Acid (GABA) of the Isoxazole Enol-betaine Type. Synthesis of 5,6,7,8-Tetrahydro-4H-isoxazolo (4,5-D) Azepin-3-ol Zwitterion and 4,5,6,7-Tetrahydroisoxazole (4,5-c) Pyridin-3-ol Zwitterion. *Acta. Chem. Scand. B.* **1974**, *28*, 533-538

41. DiRaddo, J. O.; Miller, E. J.; Hathaway, H. A.; Grajkowska, E.; Wroblewska, B.; Wolfe, B. B.; Liotta, D. C.; Wroblewski, J. T. A Real-time Method for Measuring cAMP Production Modulated by Gαi/o-coupled Metabotropic Glutamate Receptors. *J. Pharmacol. Exp. Ther.* **2014**, *349,* 373-382

42. Binkowski, B. F.; Fan, F.; Wood, K. V. Luminescent Biosensors for Real-time Monitoring of Intracellular cAMP. *Methods Mol. Biol.* **2011**, *756*, 263-271

43. Barnea, G.; Strapps, W.; Herrada, G.; Berman, Y.; Ong, J.; Kloss, B.; Axel, R.; Lee, K. J. The Genetic Design of Signaling Cascades to Record Receptor Activation. *Proc. Natl. Acad. Sci.* **2008**, *105*, 64–69

44. Barnea, G.; Strapps, W.; Herrada, G.; Berman, Y.; Ong, J.; Kloss, B.; Axel, R.; Lee, K. J. The Genetic Design of Signaling Cascades to Record Receptor Activation. *Proc. Natl. Acad. Sci.* **2008**, *105*, 64–69

45. Wang, C.; Jiang, Y.; Ma, J.; Wu, H.; Wacker, D.; Katritch, V.; Han, G. W.; Liu, W.; Huang, X. P.; Vardy, E.; McCorvy, J. D.; Gao, X.; Zhou, X. E.; Melcher, K.; Zhang, C.; Bai, F.; Yang, H.; Yang, L.; Jiang, H.; Roth, B. L.; Cherezov, V.; Stevens, R. C.; Xu, H. E. Structural Basis for Molecular Recognition at Serotonin Receptors. *Science* **2013**, *340*, 610−614

46. Wacker, D.; Wang, C.; Katritch, V.; Han, G. W.; Huang, X. P.; Vardy, E.; McCorvy, J. D.; Jiang, Y.; Chu, M.; Siu, F. Y.; Liu, W.; Xu, H. E.; Cherezov, V.; Roth, B. L.; Stevens, R. C. Structural Features for Functional Selectivity at Serotonin Receptors. *Science* **2013**, *340*, 615−619

47. Manfra, O.; Van Craenenbroeck, K.; Skieterska, K.; Frimurer, T.; Schwartz, T. W.; Levy, F. O.; Andressen, K. W. Downregulation of 5‑HT7 Serotonin Receptors by the Atypical Antipsychotics Clozapine and Olanzapine. Role of Motifs in the C •terminal Domain and Interaction with GASP‑1. *ACS. Chem. Neurosci.* **2015**, *6*, 1206−1218

48. Wacker, D.; Wang, S.; McCorvy, J. D.; Betz, R. M.; Venkatakrishnan, A. J.; Levit, A.; Lansu, K.; Schools, Z. L.; Che, T.; Nichols, D. E.; Shoichet, B. K.; Dror, R. O.; Roth, B. L. Crystal Structure of an LSD-bound Human Serotonin Receptor. *Cell* **2017**, *168*, 377–389

49. Bielenica, A,; Kozioł, A. E.; Struga, M. Binding Modes of Chain Arylpiperazines to 5-HT1a, 5-HT2a and 5-HT7 Receptors. *Mini Rev. Med. Chem.* **2013**, *13*, 1516-1539

50. Kołaczkowski, M.; Nowak, M.; Pawłowski, M.; Bojarski, A. J. Receptor-based Pharmacophores for Serotonin 5-HT7R Antagonists-implications to Selectivity. *J. Med. Chem.* **2006**, *49*, 6732-6741

51. Vermeulen, E. S.; Schmidt, A. W.; Sprouse, J. S.; Wikstrom, H. V.; Grol, C. J.

Characterization of the 5-HT(7) Receptor. Determination of the Pharmacophore for 5-HT(7) Receptor Agonism and CoMFA-based Modeling of the Agonist Binding Site. *J. Med. Chem.* **2003**, *46*, 5365-5374

52. Lopez-Rodríguez, M. L.; Porras, E.; Morcillo, M. J.; Benhamú, B.; Soto, L. J.; Lavandera, J. L.; Ramos, J. A.; Olivella, M.; Campillo, M.; Pardo, L. Optimization of the Pharmacophore Model for 5-HT7R Antagonism. Design and Synthesis of New Naphtholactam and Naphthosultam Derivatives. *J. Med. Chem.* **2003**, *46*, 5638-5650

53. Kim, J.; Kim, Y.; Tae, T.; Yeom, M.; Moon, B.; Huang, X. P.; Roth, B. L,; Lee, K.; Rhim, H.; Choo, I. H.; Chong, Y.; Keum, G.; Nam, G.; Choo, H. Aryl Biphenyl-3-ylmethylpiperazines as 5-HT7 Receptor Antagonists. *ChemMedChem.* **2013**, *8*, 1855-1864

54. Mayers, A. G.; Baldwin, D. S. Antidepressants and Their Effect on Sleep. *Hum. Psychopharmacol.* **2005**, *20*, 533–559

55. Wilson, S.; Argyropoulos, S. Antidepressants and Sleep: a Qualitative Review of the Literature. *Drugs* **2005**, *65*, 927–947.

56. Hedlund, P. B.; Huitron-Resendiz, S.; Henriksen, S. J.; Sutcliffe, J. G. 5-HT7 Receptor Inhibition and Inactivation Induce Antidepressantlike Behavior and Sleep Pattern. *Biol. Psychiatry* **2005**, *58*, 831–837

57. Shelton, J.; Bonaventure, P.; Li, X.; Yun, S.; Lovenberg, T.; Dugovic, C. 5-HT7 Receptor Deletion Enhances REM Sleep Suppression Induced by Selective Serotonin Reuptake Inhibitors, But Not by Direct Stimulation of 5-HT1A Receptor. *Neuropharmacology* **2009**, *56*, 448–454

58. Hagan, J. J.; Price, G. W.; Jeffrey, P.; Deeks, N. J.; Stean, T.; Piper, D.; Smith, M. I.; Upton, N.; Medhurst, A. D.; Middlemiss, D. N.; Riley, G. J.; Lovell, P. J.; Bromidge, S. M.; Thomas, D. R. Characterization of SB-269970-A, A Selective 5-HT(7) Receptor Antagonist.

*Br. J. Pharmacol.* **2000**, *130*, 539–548

59. Gibaldi, M.; Perrier, D.; Pharmacokinetics, second ed., *Marcele-Dekker*, New York, **1982**

60. Kerns, E.H.; Di, L. Drug-like Properties: Concepts, Structure Design and Methods: From ADME to Toxicity Optimization, First Ed. *Academic Press*, UK, **2008**

61. Oishi, Y.; Takata, Y.; Taguchi, Y.; Kohtoh, S.; Urade, Y.; Lazarus, M. Polygraphic Recording Procedure for Measuring Sleep in Mice. *J. Vis. Exp*. **2016**, *107*, e53678

62. Kohtoh, S.; Taguchi, Y.; Matsumoto, N.; Wada, M.; Huang, Z.; Urade, Y. Algorithm for Sleep Scoring in Experimental Animals Based on Fast Fourier Transform Power Spectrum Analysis of the Electroencephalogram. *Sleep. Biol. Rhythms*, **2008**, *6*, 163-171.

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