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Synthesis, binding, and functional properties of tetrahydroisoquinolino-2-alkyl phenones as selective σ_2R/TMEM97 ligands

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ABSTRACT

Sigma-2 receptor (σ_2R/TMEM97) has been implicated to play important roles in multiple cellular dysfunctions, such as cell neoplastic proliferation, neuro-inflammation, neurodegeneration, etc. Selective σ_2 ligands are believed to be promising pharmacological tools to regulate or diagnose various disorders. As an ongoing effort of discovery of new and selective σ_2 ligands, we have synthesized a series of tetrahydroisoquinolino-2-alkyl phenone analogs and identified that 10 of them have moderate to potent affinity and selectivity for σ_2R/TMEM97. Especially, 4 analogs showed K_i values ranging from 0.38 to 5.1 nM for σ_2R/TMEM97 with no or low affinity for sigma-1 receptor (σ_1R). Functional assays indicated that these 4 most potent analogs had no effects on intracellular calcium concentration and were classified as putative σ_2R/TMEM97 antagonists according to current understanding. The σ_2R/TMEM97 has been suggested to play important roles in the central nervous system. Based on published pharmacological and clinical results from several regarded σ_2R/TMEM97 antagonists, these analogs may potentially be useful for the treatment of various neurodegenerative diseases.

1. Introduction

Sigma receptors were historically misclassified as subtypes of opiate receptors but later distinguished as a new class of proteins [1]. Two subtypes of sigma receptors, termed σ_1R and σ_2R, were identified and implicated to be involved in various abnormal physiological conditions. σ_1R has been cloned and structurally characterized to be a ligand-regulated chaperone protein and mainly resides in the endoplasmic reticulum (ER) membrane to regulate calcium (Ca^{2+}) signaling, ion channels (Ca^{2+}, K^+) and G-protein-coupled receptors [2,3]. Due to its involvement in a variety of neurological disorders, σ_1R is viewed as a potentially useful therapeutic target for depression, amnesia, dementia, schizophrenia, addiction, rheumatoid arthritis, and neuropathic pain [4–7]. Studies indicated that the activation of σ_1R ameliorated neuronal survival and function in neurodegenerative diseases by modulation of calcium homeostasis, glutamate activities, ER and mitochondrial functions [8,9]; the inhibition or antagonism of σ_1R resulted in anti-proliferative and cytotoxic effects of neoplastic cells and the improvement of neuropathic pain induced by anticancer agents. Thus σ_1R agonists or ligands that activate the function of this receptor may potentially be useful as therapeutic agents for Alzheimer's disease, while σ_1R antagonists or ligands that inhibit the function of this receptor may be applied as anticancer agents [10,11].

The σ_2R is much less understood despite of being a more promising drug target due to its high expression in cells of abnormality and the potentials in such therapeutic fields as cancer, dementia, neuroprotection, anti-inflammatory and analgesic [12–16]. σ_2R was once believed to be a part or complex of the progesterone receptor membrane component 1 (PGRMC-1) protein [17–19], but subsequently confirmed to be a distinguished biomolecule [20,21].

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Recently, this receptor was purified and concluded to be identical to the endoplasmic reticulum-resident transmembrane protein 97 (TMEM97) that had been known to regulate the sterol transporter protein Niemann-Pick type C1 (NPC1) and consist of 176 amino acids with 4 transmembrane segments [22]. Thus, σ_2R has since been represented as σ_2R/TMEM97 (or TMEM97/σ_2R). More recently, σ_2R/TMEM97 and PGRMC-1 were demonstrated to regulate the LDL internalization by LDL receptor and this LDL internalization was inhibited by deletion of either σ_2R/TMEM97 gene or PGRMC-1 gene as well as by stipulated σ_2R/TMEM97 antagonists, suggesting that σ_2R/TMEM97, PGRMC-1 and LDL receptor form a ternary protein complex [23].

Extensive studies have shown that σ_2R/TMEM97 is highly expressed in a variety of rapidly proliferating cancer cells and thus has been recognized as a cell proliferative biomarker [24,25]. Small molecule σ_2 ligands have been evaluated as potentially useful imaging agents for solid tumors as well as anticancer drug delivery agents and anticancer agents [12,13]. It is now well established that compounds of stimulating σ_2R/TMEM97 activity inhibit cancer cell proliferation and promote cancer cell apoptosis via one or more of the pathways such as caspases, reactive oxygen species (ROS), or Ca^{2+} signaling [12], but their anticancer activities may not be directly correlated to either the σ_2R/TMEM97 or the PGRMC-1 protein as reported by Zeng and his coworkers [26]. More recently, σ_2R/TMEM97 was identified to regulate the metabolism of amyloid beta oligomers (AβO) in neurons and its antagonists/negative modulators were shown to inhibit, displace and reverse the binding of AβO to neurons and improve neurological functions [27,28]. Therefore, σ_2R/TMEM97 ligands are believed to be promising therapeutic agents for neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, depression, neuropathic pain, addiction, etc. [16,27–34]. At the present, it is generally agreed that the activation of σ_2R/TMEM97 promotes cancer cell apoptosis and thus has anticancer activity; while the inhibition or antagonism of the σ_2R/TMEM97 results in neuroprotection, neuroregeneration, improved cognition and anti-dementia [12,35–38]. Thus, the σ_1R and σ_2R/TMEM97 seem to have opposite regulatory effects at least in the fields of cancer cell proliferation and neurological disorders [8,14,39].

σ_2 selective ligands are promising therapeutic agents and useful pharmacological tools for the clarification and functional characterization of this receptor. Despite the tremendous efforts made in the last decade, the currently known selective σ_2 ligands are still very limited. Among the published σ_2 selective ligands, substituted indoles (such as siramesine), granatane derivatives (such as SW 43), 6,7-dimethoxytetrahydroisoquinoline analogs (such as RHM-4) and various substituted piperidines/piperazines (such as PB28) represent the most common structural elements [12,35]. More recently, several new classes of σ_2 selective ligands were just reported including norbenzomorphan derivatives (such as DKR-1677) [31,33,40–42], haloperidol analogs (such as SYA013) [43,44], benzoxazolones (such as CM398) [16,45], benzimidazolone and benzoxalole/benzothiazole derivatives (such as SN79) [46,47].

In our previous work, we modified the benzamide moiety of the N-(6,7-dimethoxy-1,2,3,4-tetrahydroisoquinolin-2-yl)alkyl benzamides to the corresponding phenones or their reduced hydroxyl derivatives and found several of the new structures showed high affinity and selectivity for σ_2R/TMEM97 [48,49]. Among them, analogs **1** and **2** had high affinities with K_i values around 5–6 nM for σ_2R/TMEM97 (Fig. 1), but analog **3** did not show measurable affinity for either σ receptor. It should be noted that the rationale of designing analog **3** was to have 4 methoxy substitutions on the aromatic moiety and later found to contain only 3 methoxy groups as determined by NMRs, LC/MS and elemental analysis. An attempt to methylate the phenol hydroxyl group did not work, indicating

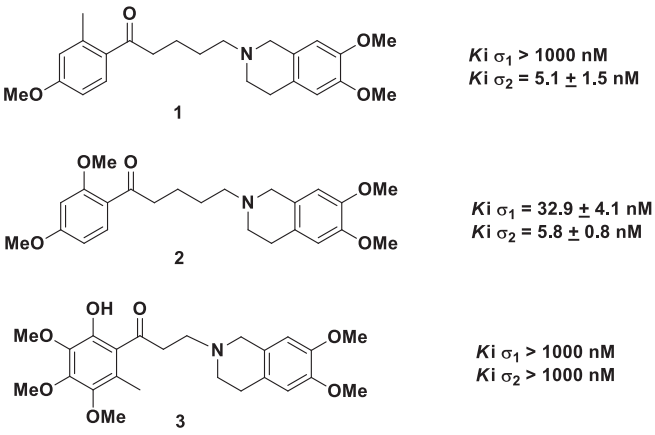


Fig. 1. Analogs 1, 2 and 3 and their affinities for sigma receptors.

that the ortho-methoxy had been demethylated as it intended to form intramolecular hydrogen bond with the adjacent ketone carbonyl group. It is well documented that σ_2R/TMEM97 is more tolerable to electron rich and bulky moieties than does σ_1R, thus analogs containing 2,3,4,5-tetramethoxytoluene may not bind to σ_1R and offer selective σ_2R/TMEM97 ligands. In this research, we set to exam the impact of the 2,3,4,5-tetramethoxytoluene moiety and increased methoxy substitution of tetrahydroisoquinoline moiety on the affinity for both σ receptors and to obtain potent and selective ligands for σ_2R/TMEM97. Thus, a series of di- and trimethoxy substituted tetrahydroisoquinolin-2-alkyl phenone analogs were prepared and evaluated for their affinity to both σ_1R and σ_2R/TMEM97, among which 10 analogs demonstrated moderate to excellent affinity and selectivity for σ_2R/TMEM97, with 4 of them displaying K_i values of 0.38–5.11 nM for σ_2R/TMEM97. Functional assays via measuring their effects on calcium mobilization indicated that these 4 most potent analogs had no effects on intracellular [Ca^{2+}] and thus were classified as putative σ_2R/TMEM97 antagonists, which, according to published pharmacological and clinical studies for similar σ_2 antagonists, are believed to be potentially useful as therapeutics for the treatment of neurological disorders.

2. Results and discussion

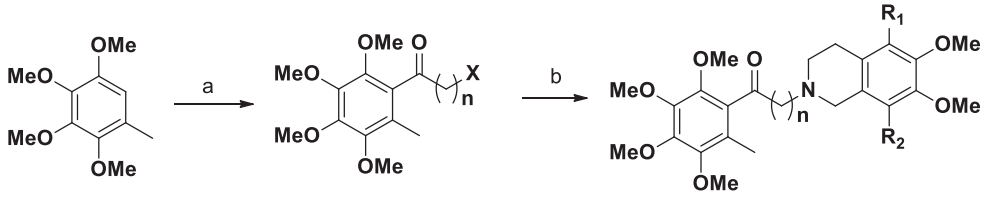
2.1. Chemistry

Though σ_1R and σ_2R/TMEM97 are coded by completely different genes, the binding requirements of them for small molecules are very similar. Both receptors require binding ligands to possess at least one basic nitrogen that is linked to one primary hydrophobic aromatic moiety with a proper length of alkyl chain and one or more additional hydrophobic elements [12,50]. Thus, many small molecules containing piperidine or piperazine scaffold showed very potent affinities for both receptors. As the molecular bulkiness increases, such as from monocyclo piperidine to bicyclononane or granatane, the affinity for σ_1R rapidly decreases with no significant change for σ_2R/TMEM97, resulting in σ_2R/TMEM97 selective ligands. In addition, the σ_2R/TMEM97 is more favorable for electron rich scaffolds than the σ_1R [12,49,51–54]. The 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline is one of the best known σ_2R/TMEM97 pharmacophores [12,48]. Except for the contraction of the piperidine ring or the cyclization of the 6,7-dihydroxy groups with a methylene, ethylene or other alkyl group within the tetrahydroisoquinoline structure, which both resulted in reduced or loss of

activity for σ_2R/TMEM97, no other modifications of the 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline moiety have been reported in the literature [12,49].

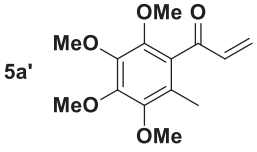
The purpose of this research was to further explore the electron rich aromatic moiety, the extramethoxy substitution on the tetrahydroisoquinoline and the length of the alkyl chain between them to find out the impact on affinity for σ_1R and σ_2R/TMEM97. Previously, we prepared an analog containing the 2,3,4,5-tetramethoxytoluene moiety that was electron rich but did not have affinity for either σ receptor most likely due to its demethylation of the ortho-methoxy group and too short alkyl chain length between the hydrophobic aromatic moiety and the basic nitrogen (Fig. 1, analog **3**). Many reported σ_2 selective ligands contained one or more methoxy substitution on the hydrophobic aromatic moiety; additional methoxy substitution usually results in an increase in the affinity and selectivity for σ_2R/TMEM97 [12,53]. On the other hand, one or more hydroxyl substitution on the hydrophobic aromatic moiety seems to be detrimental to the σ_2R/TMEM97 binding [1,53]. Methoxy substitution on the tetrahydroisoquinoline aromatic moiety is detrimental to σ_1R binding without influence on σ_2R/TMEM97 affinity, whereas electron withdrawing group substitution on the tetrahydroisoquinoline aromatic moiety is detrimental to σ_2R/TMEM97 binding [12,53]. Thus, this research intended to design and evaluate a series of compounds with more electron rich on the hydrophobic aromatic moiety, more methoxy substitution on the tetrahydroisoquinoline moiety and a variety of alkyl chain length between them to conduct a comprehensive structure activity relationship (SAR) to determine the optimum structure in this series as potent and selective σ_2 ligands.

Based on the above rationale, the electron rich 2,3,4,5-tetramethoxytoluene was selected as the hydrophobic aromatic moiety and derived with properly substituted tetrahydroisoquinoline intermediates into various analogs that were to be evaluated for their affinities for both σ receptors. Thus, the preparation of these analogs is highlighted in Scheme 1, which began with a Friedel-Crafts reaction between 2,3,4,5-tetramethoxytoluene (**4**) and properly halogen-substituted acyl chloride to form the corresponding haloalkyl phenone (Scheme 1, **5b-e**) and followed by substitution reaction between the substituted tetrahydroisoquinolines and the haloalkyl phenone intermediates to afford the tetrahydroisoquinolin-2-alkyl phenones (Scheme 1, **7** to **20**). It should be noted that the expected product of 3-chloro-1-(2,3,4,5-tetramethoxy-6-methylphenyl) propan-1-one (**5a**) was not obtained in the Friedel-Crafts acylation of 3-chloropropanoyl chloride and 2,3,4,5-tetramethoxytoluene. Instead, 1-(2,3,4,5-tetramethoxy-6-methylphenyl)propen-1-one (**5a**′) was obtained due to the elimination of HCl (most likely after the formation of **5a**). In this case, the next step was a Michael addition of the tetrahydroisoquinoline to the α,β-unsaturated phenylpropen-1-one to afford **6**. In addition to the commercially available 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline, two tetrahydroisoquinoline analogs, 5,6,7-trimethoxy-1,2,3,4-tetrahydroisoquinoline and 6,7,8-trimethoxy-1,2,3,4-tetrahydroisoquinoline (Scheme 2), were prepared and applied for the above substitution reaction in order to explore the impact of the trimethoxy substitution on the affinity for both σ receptors. The synthesis of **25a** and **25b** started with condensation between the properly substituted benzaldehyde (**21a** or **21b**) and nitromethane to afford the α,β-unsaturated (2-nitrovinyl)benzene (**22a** or **22b**) that was sequentially reduced by sodium borohydride to yield the corresponding (2-nitroethyl)benzene analog (**23a** or **23b**), then reduced by catalytic hydrogenation to give the 2-phenylethanamine (**24a** or **24b**), and finally treated with formaldehyde to form the trimethoxy substituted tetrahydroisoquinoline (**25a** or **25b**) in overall good yields (Scheme 2). The direct reduction of **22a** or **22b** to **24a** or **24b** with lithium aluminum hydride, lithium borohydride, Pd—C/H_2 and Raney Ni/H_2 catalytic hydrogenation all failed to give good results. It should be noted that the Friedel-Crafts acylation step may cause demethylation of the methoxy group especially if the reaction is carried out at above room temperature or with extended reaction time. Previously, the unexpected ortho-methoxy demethylation occurred at the ortho-methoxy group as demonstrated by not being able to be re-methylated with MeI or Me_2SO_4 [49]. However, demethylation and subsequent phenol esterification products were isolated and confirmed in the Friedel-Crafts acylation reaction (not shown). The 2,3,4,5-tetramethoxytoluene moiety was very sensitive to acid, especially at elevated temperature, thus the reaction should be under anhydrous condition and temperature below 0 °C. All the



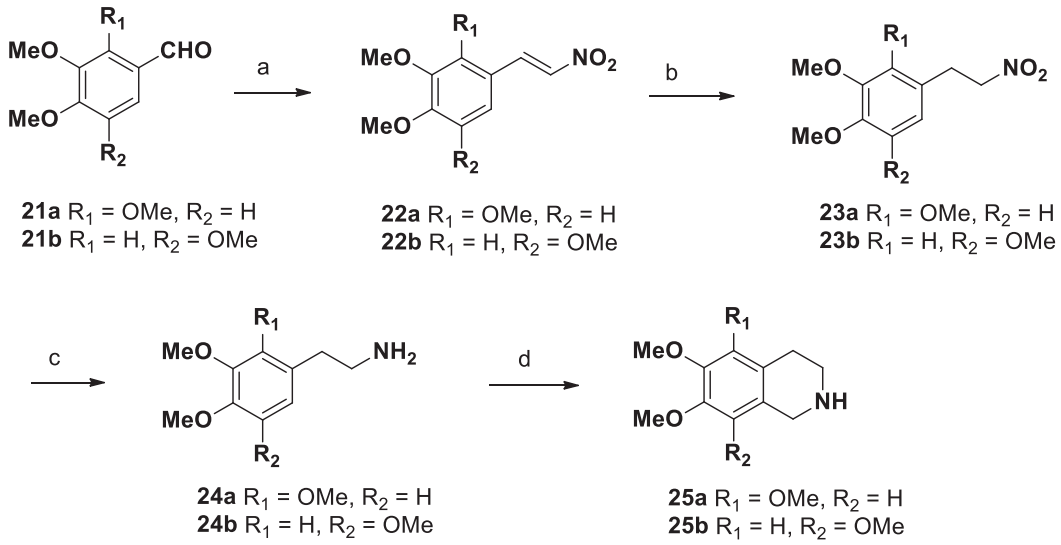
4

5a n = 2, X = Cl
5b n = 3, X = Cl
5c n = 4, X = Cl
5d n = 5, X = Br
5e n = 6, X = Br



6 n = 2, R_1,R_2 = H
7 n = 2, R_1 = OMe; R_2 = H
8 n = 2, R_1 = H; R_2 = OMe
9 n = 3, R_1,R_2 = H
10 n = 3, R_1 = OMe; R_2 = H
11 n = 3, R_1 = H; R_2 = OMe
12 n = 4, R_1,R_2 = H
13 n = 4, R_1 = OMe; R_2 = H
14 n = 4, R_1 = H; R_2 = OMe
15 n = 5, R_1,R_2 = H
16 n = 5, R_1 = OMe; R_2 = H
17 n = 5, R_1 = H; R_2 = OMe
18 n = 6, R_1,R_2 = H
19 n = 6, R_1 = OMe; R_2 = H
20 n = 6, R_1 = H; R_2 = OMe

Scheme 1. Synthesis of analogs **6**—**20**.
Reagents and conditions: a) haloalkyl chloride/$AlCl_3$, 0 °C; b) tetrahydroisoquinoline HCl/Et_3N.



Scheme 2. Synthesis of intermediates **25a** and **25b**.
Reagents and conditions: a) $MeNO_2/NH_4OAc/HOAc$; b) $NaBH_4/EtOH$; c) $NH_4OCHO/Pd-C$; d) HCHO/HCl/MeOH.

synthesized intermediates were characterized by ^1H NMR, ^{13}C NMR, LC-MS, and HPLC unless otherwise notified and all the test analogs were confirmed by ^1H NMR, ^{13}C NMR, LC-MS, HPLC, and elemental analysis.

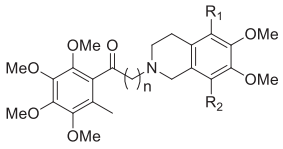
2.2. Pharmacology

Compounds **6**–**20** were measured their affinities for σ_1R and σ_2R/TMEM97 according to procedures described previously [49]. Guinea pig brain membrane homogenates were used for σ_1R binding assays and rat liver membrane homogenates were applied for the σ_2R/TMEM97 binding assays with haloperidol as the reference ligand for both receptors (Table 1). Binding curves for reference compound and active analogs are available in the supplemental file (Fig. S1).

The binding results indicated that most of these analogs had no or low affinity for σ_1R (Table 1, column 5) and moderate to high affinity for σ_2R/TMEM97 (Table 1, column 6). Ten of the analogs, **9**, **10**, **12**–**19,** showed K_i values ranging from 0.38 to 41.76 nM for σ_2R/TMEM97. In particular, analogs **9**, **12**, **15** and **18** had nanomolar or even sub-nanomolar affinity for σ_2R/TMEM97 with K_i values of 5.1, 3.0, 0.38 and 1.33 nM, respectively, with no affinity (**9**, **15** and **18**) or low affinity (**12**, $K_i = 160$ nM) for σ_1R. Analogs **10**, **13**, **14**, **16**, **17** and **19** showed moderate affinity for σ_2R/TMEM97 with K_i values of 41.76, 34.22, 22.37, 19.67, 12.58 and 40.04 nM, respectively, and no measurable affinity for σ_1R. Analog **6** showed low affinity for both σ_1R ($K_i = 3192$ nM) and σ_2R/TMEM97 ($K_i = 956$ nM). Analogs **7** and **8** had no affinity for σ_1R and very low affinity with K_i values of 11,500 and 3184 nM, respectively, for σ_2R/TMEM97. Analogs **11** and **20** did not have significant affinity for either σ receptor.

From structural point of view, these analogs can be classified into 3 categories: 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinolin-2-alkyl (2,3,4,5-tetramethoxy-6-methyl) phenones or 6,7-diMeO in brief (**6**, **9**, **12**, **15** and **18**), 5,6,7-trimethoxy-1,2,3,4- tetrahydroisoquinolin-2-alkyl (2,3,4,5-tetramethoxy-6-methyl) phenones or 5,6,7-triMeO in brief (**7**, **10**, **13**, **16** and **19**), and 6,7,8-trimethoxy-1,2,3,4-tetrahydroisoquinolin-2-alkyl (2,3,4,5-tetramethoxy-6-methyl) phenones or 6,7,8-triMeO in brief (**8**, **11**, **14**, **17** and **20**). For the 6,7-diMeO series, analog **6**, with 2 methylene units between the tetrahydroisoquinoline and the phenone carbonyl moiety, only had moderate or very weak affinity ($K_i = 956$ nM) for σ_2R/TMEM97. As the number of methylene units between the tetrahydroisoquinoline and the phenone carbonyl moiety increased to 3, 4 and 5, the affinity for σ_2R/TMEM97 significantly increased to 5.1, 3.0, 0.38 nM, respectively, for analogs **9**, **12**, and **15**. However, when the number of methylene units further increased to 6, the resultant analog **18** had a 3.5-fold decrease in affinity for σ_2R/TMEM97 (compared to that of the 5 methylene unit analog **15**) with a K_i value of 1.33 nM (Fig. 2). Analogs **9**, **12**, **15** and **18** showed no or low affinity for σ_1R and had selectivity ratios of >196, 53, >2631 and > 751 (Table 1, column 7), respectively, for σ_2R/TMEM97. For the 5,6,7-triMeO series, a very similar, but with less potency, pattern was observed. The 2 methylene unit analog **7** did not show significant affinity for σ_2R/TMEM97 ($Ki = 11{,}500$ nM), as the number of methylene units increased to 3, 4 and 5, the corresponding σ_2R/TMEM97 affinity increased to 41.76, 34.22, 19.67 nM, respectively, for analogs **10**, **13** and **16**. Again, when the number of methylene units further increased to 6, the resultant analog **19** had a K_i value of 40.04 nM, resulting in a 2-fold decrease in σ_2R/TMEM97 affinity (compared to that of the 5 methylene unit analog **16**). Analogs **10**, **13**, **16** and **19** showed no affinity for σ_1R and had selectivity ratios of >24, >29, >50 and > 24, respectively, for σ_2R/TMEM97. For the 6,7,8-triMeO series, a rather similar pattern of σ_2R/TMEM97 affinity can be seen except for analogs **11** and **20**, which, for some unknown reason, did not demonstrate any significant affinity for this receptor. Again, the 2 methylene unit analog **8** had weak or moderate affinity for σ_2R/TMEM97 with a Ki value of 3184 nM. The 4 and 5 methylene unit analogs, **14** and **17,** had excellent σ_2R affinity with K_i values of 22.3 and 12.58 nM, respectively, which were less potent than their corresponding 6,7-diMeO series (analogs **12** and **15**, respectively), but similar to or slightly more potent than their 5,6,7-triMeO series (analogs **13** and **16**, respectively) for σ_2R/TMEM97 (Fig. 2). Analogs **14** and **17** showed no affinity for σ_1R and had selectivity ratios of >44 and > 79, respectively, for σ_2R/TMEM97. Thus, based on these three multimethoxy substituted tetrahydroisoquinoline series, a structure activity relationship (SAR) can be concluded: the proper number of methylene units between the tetrahydroisoquinoline and the phenone carbonyl moiety is from 3 to 6 with 5 being the optimum number to offer the best affinity and selectivity for the σ_2R/TMEM97; the 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline moiety offers the best results (6,7-diMeO series) and additional

Table 1
Sigma receptor affinities and biological activities for analogs **6**–**20**



Analog#	n	R_1	R_2	*Ki* (nM ± SEM)[a]		*Ki* (σ_1)/*Ki*(σ_2)	Receptor
				σ_1R[b]	σ_2R/TMEM97[c]		function[d]
6	2	H	H	3192	956.0 ± 14.9	3	ND [e]
7	2	OMe	H	>1000 [f]	11,500		ND
8	2	H	OMe	>1000	3184		ND
9	3	H	H	>1000	5.1 ± 0.5	>196	antagonist
10	3	OMe	H	>1000	41.76 ± 11.28	>24	ND
11	3	H	OMe	>1000	>1000		ND
12	4	H	H	160.0 ± 35.4	3.00 ± 0.05	53	antagonist
13	4	OMe	H	>1000	34.22 ± 8.95	>29	ND
14	4	H	OMe	>1000	22.37 ± 9.22	>44	ND
15	5	H	H	>1000	0.38 ± 0.05	>2631	antagonist
16	5	OMe	H	>1000	19.67 ± 4.72	>50	ND
17	5	H	OMe	>1000	12.58 ± 3.80	>79	ND
18	6	H	H	>1000	1.33 ± 0.12	>751	antagonist
19	6	OMe	H	>1000	40.04 ± 15.61	>24	ND
20	6	H	OMe	>1000	>1000		ND
haloperidol				3.27 ± 1.02	35.22 ± 3.80		
siramesine							agonist
RHM-1							antagonist

[a] Mean (SEM), *Ki* values were determined by at least three experiments. Each inhibition curve consisted of 10 points from each binding assay.
[b] *Ki* values for σ_1R were measured on guinea pig brain membranes using [^{3}H](+)-pentazocine as the radioligand.
[c] *Ki* values for σ_2R/TMEM97 were measured on rat liver membranes using [^{3}H]-RHM-4 as the radioligand.
[d] Receptor function was solely based on Ca^{2+} assay: analogs that do not stimulate Ca^{2+} release from its storage are classified as σ_2R/TMEM97 antagonists in this study.
[e] ND indicates bioactivity not determined.
[f] Indicates no significant binding (up to 1000 nM of test compound) was noticed.

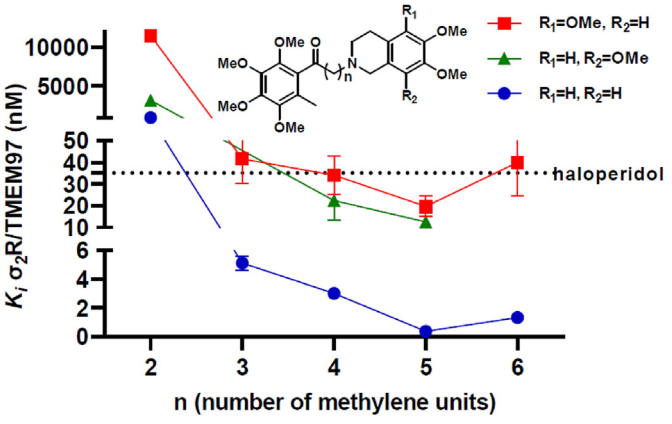


Fig. 2. The relationship between the number of methylene units (n) and the corresponding analog's affinity for σ_2R/TMEM97.

methoxy substitution on the tetrahydroisoquinoline aromatic moiety results in a decrease in affinity for σ_2R/TMEM97 (5,6,7-triMeO and 6,7,8-triMeO series).

It should be noted that the definition of an agonist or an antagonist for σ_2R is not entirely clear due to both an unknown endogenous ligand and the lack of a universally established functional assay. Thus, the current understanding is that σ_2R/TMEM97 agonists stimulate the release of calcium ion from its storage and increase the intracellular [Ca^{2+}] concentration [55]. Several methods have been published by various research groups in determining a σ_2R/TMEM97 ligand to be an agonist or an antagonist, including intracellular Ca^{2+} change, caspase-3 activity and cancer cell viability assays [56]. In this study, functional assays were conducted by measuring intracellular [Ca^{2+}] using the dual-wavelength fluorescence probe Fluo-3 AM by confocal laser scanning microscope. Images were acquired at 40X magnification. Siramesine and RHM-1 were used as the reference ligands for σ_2 agonist and antagonist, respectively (Table 1, column 8 and Fig. S2). Four of the most potent analogs in this series, **9**, **12**, **15** and **18**, were selected for the calcium assay at a concentration of 30 μM. The results indicated that siramesine (10 μM), a σ_2R/TMEM97 agonist [55], significantly stimulated the intracellular Ca^{2+} concentration as tracked by fluorescence probe Fluo-3 AM (Fig. 2S, bottom panel); RHM-1, a σ_2R/TMEM97 antagonist [56], did not affect the intracellular Ca^{2+} up to 30 μM (Fig. 2S, panel 4). Analogs **9**, **12**, **15** and **18** had no effect on the intracellular Ca^{2+} (Fig. 2S, panels 2–5) and thus were regarded by current understanding as putative σ_2R/TMEM97 antagonists (Table 1, column 8).

Cytotoxicity assays were conducted using human breast MCF-7 cancer cells and human breast MCF-10A normal cells (Table S1). Cisplatin was used as a reference cytotoxic anticancer agent and showed potent inhibitory effect against the growth of MCF-7 cancer cells and less effect against MCF-10A normal cells (Table S1, bottom row cisplatin). The σ_2R/TMEM97 potent antagonists **9**, **12** and **15** did not show significant inhibitory effect on both the MCF-7 cancer cells and the MCF-10A normal cells at concentrations ranging from 1 to 100 μM after 24 h and 48 h incubations (Table S1, rows under analogs **9**, **12** and **15**) with IC_{50} values greater than 100 μM. Another potent putative σ_2R/TMEM97 antagonist **18** showed minor cytotoxicity toward both MCF-7 and MCF-10A cells with IC_{50} values of 70.66 and 54.19 μM, respectively, after 24 h incubation and moderate cytotoxicity with IC_{50} values of 51.09 and 32.29 μM, respectively, after 48 h incubation (Table S1, row analog **18**). The

cytotoxicity results further confirmed that analogs **9**, **12**, **15** and **18** were classified as σ_2R/TMEM97 antagonists [56]. Analog **6** had very low affinity for either σ receptor but showed significant cytotoxicity against both MCF-7 and MCF-10A cells with IC_{50} values of 11.45 and 11.91 μM, respectively, after 24 h incubation, and 8.09 and 9.77 μM, respectively, after 48 h incubation (Table S1, row analog **6**). Analog **8** showed moderate cytotoxicity against both MCF-7 and MCF-10A cells with IC_{50} values of 25.52 and 45.85 μM, respectively, after 24 h incubation, and 19.71 and 27.44 μM, respectively, after 48 h incubation (Table S1, row analog **8**). Analog **20** that surprisingly did not have binding affinity for either σ receptor showed moderate cytotoxicity toward both MCF-7 and MCF-10A cells with IC_{50} values of 39.71 and 23.49 μM, respectively, after 48 h incubation, but no significant cytotoxicity after 24 h incubation (Table S1, row analog **20**). Overall, analog **6** was the most cytotoxic compound against both MCF-7 and MCF-10A cells among this series of analogs. An expanded cytotoxicity study was conducted for analog **6** along with cisplatin and showed that **6** had broad inhibitory effects against HEPG-2 (human liver cancer), KYSE-140 (human esophageal cancer) and MDA-MB-231 (human breast cancer) cells, in addition to human MCF-7 cancer cells, with IC_{50} values comparable to those of cisplatin (Table S2). Since analog **6** has no or very low affinity for either σ receptor, its cytotoxicity against the above cancer cell lines is not σ receptor based.

3. Conclusions

A series of tetrahydroisoquinolinoalkyl phenones were synthesized and evaluated for their affinities for σ_1 and σ_2 receptors. According to the substitution on the tetrahydroisoquinoline moiety, they were divided into 6,7-diMeO (**6**, **9**, **12**, **15** and **18**), 5,6,7-triMeO (**7**, **10**, **13**, **16** and **19**) and 6,7,8-triMeO (**8**, **11**, **14**, **17** and **20**) three categories. Among them, four 6,7-diMeO analogs, **9, 12**, **15** and **18**, demonstrated the best affinity with *K*i values of 5.1, 3.0, 0.38 and 1.33 nM, respectively, for σ_2R/TMEM97 and selectivity ratios of >196, 53, >2631 and > 751, respectively, for σ_2R/TMEM97 over σ_1R. Analog **15**, which showed the highest affinity for σ_2R/TMEM97 and no measureable affinity for σ_1R, is the most potent and selective σ_2 ligand identified in this study. Functional studies indicated that analogs **9, 12**, **15** and **18** did not affect the intracellular Ca^{2+} and thus were regarded as σ_2R/TMEM97 antagonists, which were further corroborated by their no significant cytotoxicity against MCF-7 cancer cells [56]. Thus, analogs **9, 12**, **15** and **18** are believed to be potentially useful therapeutics for neurodegenerative diseases such as Alzheimer's disease and are worth further pharmacological evaluation.

4. Experimental section

4.1. Chemistry

All reagents or solvents purchased commercially were used directly without further purification. Reactions were monitored by analytical thin-layer chromatography (TLC) on silica gel F254 glass plates and visualized under UV light (254 nm) and temperatures were recorded using regular thermometers without correction. Flash column chromatographies were performed on silica gel (200–300 mesh). Melting points were measured on a SGW X-4A melting point measurement apparatus (Inesa Instrument Inc, Shanghai, China) without correction. ^{1}H NMR spectra were recorded with a Bruker Avance III 400 MHz NMR spectrometer at room temperature. Chemical shifts were recorded as parts per million (ppm) downfield to tetramethylsilane (TMS). The following abbreviations are used for multiplicity of NMR signals: s) singlet; d) doublet; t) triplet; q) quartet; p) pentalet; m) multiplet; dd) doublet of doublets; dt) doublet of triplets; b) broad. ^{13}C NMR spectra were recorded with Bruker Avance III 400 MHz NMR spectrometer (100 MHz). LC-MS were analyzed on a SCIEX-Triple Quad 4500 system.

4.1.1. 1-(2,3,4,5-Tetramethoxy-6-methylphenyl)prop-2-en-1-one (5a′)

A solution of 2,3,4,5-tetramethoxytoluene (20.0 g, 94.2 mmol), 3-chloropropanoyl chloride (13.2 g, 103.7 mmol) in DCM (100 mL) was stirred in an ice bath and added anhydrous $AlCl_3$ (18.8 g, 141.3 mmol) portionwise. The mixture was stirred for additional 10 min in an ice bath and poured into ice-water (200 mL). The organic phase was separated, washed with saturated $NaHCO_3$ aqueous solution, brine and dried over sodium sulfate. After workup, the crude was purified by a silica gel column to give a product that was confirmed by ^{1}HNMR and ^{13}CNMR as well as LC/MS to be **5a′** due to elimination of HCl as a light yellow oil (12.3 g, yield 49%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.56–6.61 (m, *J* = 17.5, 10.4 Hz, 1H), 5.97–6.07 (m, *J* = 18.4, 14.0, 0.8 Hz, 2H), 3.95 (s, 3H), 3.90 (s, 3H), 3.80 (s, 3H), 3.77 (s, 3H), 2.04 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 197.5, 148.1, 147.9, 146.5, 144.6, 138.3, 131.6, 128.9, 123.8, 61.7, 61.1, 60.7, 12.1; LC/MS-m/z: 266.9 $[M+1]^+$ ($C_{14}H_{18}O_5$: calcd mass 266.1).

4.1.2. 4-Chloro-1-(2,3,4,5-tetramethoxy-6-methylphenyl)butan-1-one (5b)

4-Chloro-1-(2,3,4,5-tetramethoxy-6-methylphenyl)butan-1-one was prepared according to the procedure for the preparation of **5a′** and obtained as an oil in 73.7% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.93 (s, 3H), 3.91 (s, 3H), 3.83 (s, 3H), 3.80 (s, 3H), 3.66 (t, *J* = 6.4 Hz, 2H), 2.94 (t, *J* = 6.9 Hz, 2H), 2.16–2.22 (p, *J* = 6.7 Hz, 2H), 2.07 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 205.6, 148.2, 147.9, 145.8, 144.5, 131.3, 122.7, 61.8, 61.1, 60.7, 44.3, 41.7, 26.5, 11.9; LC/MS-m/z: 316.9 $[M+1]^+$ ($C_{15}H_{21}ClO_5$: calcd mass 316.1).

4.1.3. 5-Chloro-1-(2,3,4,5-tetramethoxy-6-methylphenyl)pentan-1-one (5c)

5-Chloro-1-(2,3,4,5-tetramethoxy-6-methylphenyl)pentan-1-one was prepared according to the procedure for the preparation of **5a′** and obtained as an oil in 62.9% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.92 (s, 3H), 3.90 (s, 3H), 3.81 (s, 3H), 3.79 (s, 3H), 3.57 (t, *J* = 6.3 Hz, 2H), 2.78 (t, *J* = 6.8 Hz, 2H), 2.05 (s, 3H), 1.83–1.88 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 206.2, 148.2, 147.8, 145.8, 144.6, 131.6, 122.6, 61.8, 61.1, 61.0, 60.6, 44.7, 43.9, 31.9, 20.9, 12.0; LC/MS-m/z: 330.9 $[M+1]^+$ ($C_{16}H_{23}ClO_5$: calcd mass 330.1).

4.1.4. 6-Bromo-1-(2,3,4,5-tetramethoxy-6-methylphenyl)hexan-1-one (5d)

A solution of 2,3,4,5-tetramethoxytoluene (5.0 g, 23.5 mmol), 6-bromohexanoyl chloride (4.8 g, 25.9 mmol) in DCM (25 mL) was stirred in an ice bath and added anhydrous $AlCl_3$ (3.5 g, 25.9 mmol). The mixture was stirred for 10 min in an ice bath and additional 30 min at room temperature, poured into ice-water (50 mL). The organic phase was separated, extracted with DCM, washed with saturated $NaHCO_3$ aqueous solution, brine and dried over sodium sulfate. After workup, the crude was purified by a silica gel column to give **5d** as a yellow oil (6.0 g, yield 65.5%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.93 (s, 3H), 3.90 (s, 3H), 3.81 (s, 3H), 3.79 (s, 3H), 3.43 (t, *J* = 6.8 Hz, 2H), 2.76 (t, *J* = 7.3 Hz, 2H), 2.06 (s, 3H), 1.88–1.94 (m, 2H), 1.69–1.75 (m, *J* = 15.2, 7.4 Hz, 2H), 1.49–1.55 (m, *J* = 10.0, 6.5 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 206.6, 148.2, 147.7, 145.7, 144.5, 131.7, 122.6, 61.8, 61.2, 61.1, 60.6, 44.7, 33.6, 32.6, 27.7, 22.6, 12.0.

4.1.5. 7-Bromo-1-(2,3,4,5-tetramethoxy-6-methylphenyl)heptan-1-one (5e)

To a flask was added 7-bromoheptanoic acid (5.0 g, 23.9 mmol) in DCM (25 mL) and oxalyl chloride (4.6 g, 35.8 mmol) and DMF (1 drop). The solution was stirred at room temperature overnight, concentrated under reduced pressure to give a yellow oil that was then dissolved in DCM (25 mL) and added with 2,3,4,5-tetramethoxytoluene (5.0 g, 23.5 mmol). The solution was then added with anhydrous $AlCl_3$ (3.5 g, 25.9 mmol) and stirred in ice-bath for 10 min and then at room temperature for 30 min and poured into ice water (50 mL). The organic phase was separated, extracted with DCM, washed with saturated $NaHCO_3$ aqueous solution, brine and dried over sodium sulfate. After workup, the crude was purified by a silica gel column to give **5e** as yellow oil (6.3 g, yield 66.5%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.92 (s, 3H), 3.90 (s, 3H), 3.80 (s, 3H), 3.79 (s, 3H), 3.41 (t, J = 6.8 Hz, 2H), 2.74 (t, J = 7.3 Hz, 2H), 2.05 (s, 3H), 1.85–1.91 (m, 2H), 1.70 (dt, J = 14.8, 7.3 Hz, 2H), 1.45–1.51 (m, 2H), 1.37–1.42 (m, J = 15.8, 7.3 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 206.8, 148.2, 147.7, 145.7, 144.6, 131.9, 122.6, 61.9, 61.2, 61.1, 60.7, 44.8, 33.8, 32.6, 28.3, 28.0, 23.3, 12.0.

4.1.6. 1,2,3-Trimethoxy-4-(2-nitrovinyl)benzene (22a)

A mixture of 2,3,4-trimethoxybenzaldehyde (10.0 g, 51.0 mmol), nitromethane (9.3 g, 152.9 mmol), ammonium acetate (3.9 g, 51.0 mmol), glacial acetic acid (10.0 mL) was stirred and heated at 90 °C for 10 h. The mixture was cooled to room temperature and poured into ice-water (100 mL) under vigorous stirring. The solid was filtered, washed with water and dried, recrystallized from petroleum ether/ethyl acetate to give a yellow solid (11.1 g, 91.1% yield). mp 77.8–79.1 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.10 (d, J = 13.6 Hz, 1H), 7.78 (d, J = 13.6 Hz, 1H), 7.22 (d, J = 8.8 Hz, 1H), 6.74 (d, J = 8.8 Hz, 1H), 4.01 (s, 3H), 3.94 (s, 3H), 3.89 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 157.3, 154.3, 142.4, 136.6, 135.3, 126.6, 117.1, 107.7, 61.2, 60.9, 56.2; LC/MS-m/z: 239.9 $[M+1]^+$ ($C_{11}H_{13}NO_5$: calcd mass 239.1).

4.1.7. 1,2,3-Trimethoxy-5-(2-nitrovinyl)benzene (22b)

1,2,3-Trimethoxy-5-(2-nitrovinyl)benzene was prepared according to the procedure for the preparation of **22a** and obtained in 98.4% yield. mp 121.0–122.1 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.95 (d, J = 13.6 Hz, 1H), 7.56 (d, J = 13.6 Hz, 1H), 6.78 (s, 2H), 3.91 (s, 3H), 3.91 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 153.7, 141.8, 139.3, 136.4, 125.3, 106.5, 61.0, 56.3; LC/MS-m/z: 240.2 $[M+1]^+$ ($C_{11}H_{13}NO_5$: calcd mass 239.1).

4.1.8. 1,2,3-Trimethoxy-4-(2-nitroethyl)benzene (23a)

To a stirred solution of **22a** (10.0 g, 41.8 mmol) in ethanol (100 mL) was added sodium borohydride (2.7 g, 62.7 mmol) portionwise under ice-bath cooling. The reaction mixture was then stirred at room temperature for 1 h, filtered off the solid, concentrated on a rotavap. The crude product was purified by a silica gel column to afford a colorless oil product (7.0 g, 69.5% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.83 (d, J = 8.5 Hz, 1H), 6.60 (d, J = 8.5 Hz, 1H), 4.58 (t, J = 7.3 Hz, 2H), 3.94 (s, 3H), 3.86 (s, 3H), 3.84 (s, 3H), 3.24 (t, J = 7.3 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 153.5, 151.9, 142.1, 124.5, 121.2, 107.1, 75.5, 60.9, 60.7, 56.0, 28.7; LC/MS-m/z: 242.2 $[M+1]^+$, 263.9 $[M+23]^+$ ($C_{11}H_{15}NO_5$: calcd mass 241.1).

4.1.9. 1,2,3-Trimethoxy-5-(2-nitroethyl)benzene (23b)

1,2,3-Trimethoxy-5-(2-nitroethyl)benzene was prepared according to the procedure for the preparation of **23a** and obtained as a white solid product in 54.8% yield. mp 82.6–83.0 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.43 (s, 2H), 4.63 (t, J = 7.4 Hz, 2H), 3.87 (s, 6H), 3.84 (s, 3H), 3.28 (t, J = 7.4 Hz, 2H); ^{13}C NMR (400 MHz, $CDCl_3$): δ 153.6, 137.4, 131.3, 105.6, 76.3, 60.8, 56.2, 33.8; LC/MS-m/z: 242.0 $[M+1]^+$, 263.9 $[M+23]^+$ ($C_{11}H_{15}NO_5$: calcd mass 241.1).

4.1.10. 5,6,7-Trimethoxy-1,2,3,4-tetrahydroisoquinoline (25a)

To a solution of **23a** (4.0 g, 16.6 mmol) in ethanol (30 mL) was added 5% Pd/C (0.5 g), ammonium formate (2.1 g, 33.2 mmol). The mixture was heated to reflux under stirring for 2 h. After cooled to room temperature, the solid was filtered off and the solution was concentrated to dryness to afford 2-(2,3,4-trimethoxyphenyl) ethanamine (**24a**) as an oil crude product (3.1g, ~yield 88%). LC/MS-m/z: 212.5 $[M+1]^+$ ($C_{11}H_{17}NO_3$: calcd mass 211.1), which was used directly for the next step without further purification.

To a flask was added **24a** (1.0 g, 4.7 mmol), methanol (10 mL), 37% formaldehyde (0.4 g, 5.2 mmol), and concentrated HCl (1.2 mL, 14.2 mmol). The solution was heated to reflux under stirring for 4 h and then stood at room temperature overnight. The product was filtered and dried as a white solid hydrochloride salt (0.9 g, 73.3% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.34 (s, 1H), 3.92 (s, 2H), 3.85 (6H), 3.82 (s, 3H), 3.10 (t, J = 6.0Hz, 2H), 2.66 (t, J = 6.0Hz, 2H), 1.92 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 151.6, 151.5, 140.3, 131.4, 120.9, 105.0, 60.9, 60.4, 56.0, 48.3, 43.6, 23.2; LC/MS-m/z: 224.1 $[M+1]^+$ ($C_{12}H_{17}NO_3$: calcd mass 223.1).

4.1.11. 6,7,8-Trimethoxy-1,2,3,4-tetrahydroisoquinoline (25b)

6,7,8-Trimethoxy-1,2,3,4-tetrahydroisoquinoline was prepared according to the procedure described for the preparation of **24a** to give **24b** as an oil (LC/MS-m/z: 212.2 $[M+1]^+$, $C_{11}H_{17}NO_3$: exact mass 211.1), which was then converted to **25b** according to the procedure of **25a** as HCl salt in 81.4% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.39 (s, 1H), 3.92 (s, 2H), 3.85 (s, 3H), 3.83 (s, 3H), 3.81 (s, 3H), 3.06 (t, J = 5.9 Hz, 2H), 2.70 (t, J = 5.7 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 151.8, 150.0, 139.9, 130.3, 121.8, 107.8, 60.8, 60.4, 56.0, 43.5, 43.2, 29.0; LC/MS-m/z: 224.2 $[M+1]^+$ ($C_{12}H_{17}NO_3$: calcd mass 223.1).

4.1.12. 3-(6,7-Dimethoxy-1,2,3,4-tetrahydroisoquinolin-2-yl)-1-(2,3,4,5-tetra- methoxy-6-methylphenyl)propan-1-one (6)

A solution of **5a'** (0.7 g, 2.2 mmol), 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline hydrochloride (0.5 g, 2.2 mmol) and triethylamine (1.1 g, 10.9 mmol) in DCM (10 mL) was stirred at room temperature overnight. The mixture was extracted with DCM, washed with brine and dried over anhydrous sodium sulfate. After workup, the crude was purified by a silica gel column to afford an oil product (0.7 g, 70% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.58 (s, 1H), 6.50 (s, 1H), 3.93 (s, 3H), 3.91 (s, 3H), 3.83 (s, 3H), 3.82 (6H, 2MeO), 3.76 (s, 3H), 3.57 (s, 2H), 3.06 (t, J = 7.1 Hz, 2H), 2.93 (t, J = 7.0 Hz, 2H), 2.79 (t, J = 5.6 Hz, 2H), 2.73 (t, J = 5.5 Hz, 2H), 2.07 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 205.8, 148.2, 147.9, 147.5, 147.2, 146.0, 144.4, 131.4, 126.4, 126.0, 123.3, 111.3, 109.4, 61.9, 61.1, 60.6, 55.9, 55.6, 52.5, 50.9, 43.0, 28.7, 12.0; LC/MS-m/z: 460.3 $[M+1]^+$ ($C_{25}H_{33}NO_7$: calcd mass 459.2); Anal: C 65.18, H 7.11, N 2.99 (Calcd: C 65.34, H 7.24, N 3.05).

4.1.13. 1-(2,3,4,5-Tetramethoxy-6-methylphenyl)-3-(5,6,7-trimethoxy-1,2,3,4-dihydroisoquinolin-2-yl)propan-1-one (7)

1-(2,3,4,5-Tetramethoxy-6-methylphenyl)-3-(5,6,7-trimethoxy-1,2,3,4-dihydroisoquinolin-2-yl)propan-1-one was prepared according to the procedure for the preparation of **6** by reacting **5a'** with 5,6,7-trimethoxy-1,2,3,4-tetrahydroisoquinoline and obtained as a light yellow oil in 54.2% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.35 (s, 1H), 3.95 (s, 3H), 3.93 (s, 3H), 3.87 (s, 3H), 3.86 (s, 3H), 3.85 (s, 3H), 3.83 (s, 3H), 3.79 (s, 3H), 3.59 (s, 2H), 3.07 (t, J = 7.1 Hz, 2H), 2.93 (t, J = 7.0 Hz, 2H), 2.70–2.80 (m, 4H), 2.09 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 205.7, 151.7, 151.2, 148.3, 147.9, 146.0, 144.5, 140.5, 131.4, 130.1, 123.2, 120.4, 105.3, 61.9, 61.2, 61.1, 60.9, 60.6, 60.4, 56.0, 55.9, 52.4, 50.6, 43.0, 23.5, 12.0; LC/MS-

m/z: 490.8 $[M+1]^+$ ($C_{26}H_{35}NO_8$: calcd mass 489.2); Anal: C 63.81, H 7.12, N 2.81 (Calcd: C 63.79, H 7.21, N 2.86).

4.1.14. 1-(2,3,4,5-Tetramethoxy-6-methylphenyl)-3-(6,7,8-trimethoxy-1,2,3,4-tetrahydroisoquinolin-2-yl)propan-1-one (8)

1-(2,3,4,5-Tetramethoxy-6-methylphenyl)-3-(6,7,8-trimethoxy-1,2,3,4-tetrahydroisoquinolin-2(1H)-yl)propan-1-one was prepared according to the procedure for the preparation of **6** by reacting **5a′** with 6,7,8-trimethoxy-1,2,3,4-tetrahydroisoquinoline and obtained as an oil in 70.4% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.42 (s, 1H), 3.94 (s, 3H), 3.92 (s, 3H), 3.87 (s, 3H), 3.85−3.82 (m, 9H), 3.78 (s, 3H), 3.57 (s, 2H), 3.09 (t, J = 7.0 Hz, 2H), 2.98 (t, J = 7.0 Hz, 2H), 2.82 (t, J = 5.6 Hz, 2H), 2.72 (t, J = 5.7 Hz, 2H), 2.09 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 205.8, 151.9, 150.1, 148.2, 147.9, 146.0, 144.4, 139.9, 131.4, 129.8, 123.3, 120.7, 107.2, 61.9, 61.2, 61.1, 60.8, 60.6, 60.5, 56.0, 52.67, 50.8, 50.6, 43.0, 29.2, 12.0; LC/MS-m/z: 490.1 $[M+1]^+$ ($C_{26}H_{35}NO_8$: calcd mass 489.2); Anal: C 63.65, H 7.17, N 2.69 (Calcd: C 63.79, H 7.21, N 2.86).

4.1.15. 4-(6,7-Dimethoxy-1,2,3,4-tetrahydroisoquinolin-2-yl)-1-(2,3,4,5-tetra-methoxy-6-methylphenyl)butan-1-one (9)

A solution of **5b** (0.7 g, 2.2 mmol), 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline hydrochloride (0.5 g, 2.2 mmol) and triethylamine (1.1 g, 10.9 mmol) in ethanol (10 mL) was heated to reflux under stirring overnight. The solvent was removed in vacuum and the crude produce was extracted with DCM, washed with brine and dried over anhydrous sodium sulfate. After workup, the product was purified by silica gel column to give an oil product (0.4 g, 40.7% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.60 (s, 1H), 6.53 (s, 1H), 3.92 (s, 3H), 3.90 (s, 3H), 3.84 (6H, 2MeO), 3.81 (s, 3H), 3.79 (s, 3H), 3.58 (s, 2H), 2.81−2.85 (m, 4H), 2.72−2.74 (m, 2H), 2.56−2.58 (m, 2H), 2.06 (s, 3H), 1.98−2.02 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 206.7, 148.2, 147.7, 147.5, 147.2, 145.7, 144.5, 131.8, 126.6, 126.2, 122.6, 111.3, 109.5, 61.8, 61.2, 61.1, 60.7, 57.2, 55.9, 55.6, 50.9, 42.7, 28.7, 21.0, 12.0; LC/MS-m/z: 474.0 $[M+1]^+$ ($C_{26}H_{35}NO_7$: calcd mass 473.2); Anal: C 65.83, H 7.44, N 3.03 (Calcd: C 65.94, H 7.45, N 2.96).

4.1.16. 1-(2,3,4,5-Tetramethoxy-6-methylphenyl)-4-(5,6,7-trimethoxy-3,4- dihydroisoquinolin-2-yl)butan-1-one (10)

1-(2,3,4,5-Tetramethoxy-6-methylphenyl)-4-(5,6,7-trimethoxy-3,4-dihydroisoquinolin-2-yl)butan-1-one was prepared according to the procedure for the preparation of **9** by reacting **5b** with **25a** and obtained as a yellowish oil in 60.5% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.36 (s, 1H), 3.92 (s, 3H), 3.89 (s, 3H), 3.85 (s, 3H), 3.84 (s, 3H), 3.82 (s, 3H), 3.80 (s, 3H), 3.78 (s, 3H), 3.56 (s, 2H), 2.83 (t, J = 7.2 Hz, 2H), 2.77 (t, J = 5.6 Hz, 2H), 2.71 (t, J = 5.6 Hz, 2H), 2.57 (t, J = 7.3 Hz, 2H), 2.05 (s, 3H), 1.97−2.02 (m, J = 14.5, 7.2 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 206.7, 151.7, 151.2, 148.2, 147.7, 145.7, 144.5, 140.4, 131.8, 122.6, 120.6, 105.3, 61.9, 61.2, 61.1, 60.1, 60.7, 60.4, 57.3, 56.0, 55.9, 50.7, 42.7, 29.7, 23.5, 21.0, 12.0; LC/MS-m/z: 504.2 $[M+1]^+$ ($C_{27}H_{37}NO_8$: calcd mass 503.3); Anal: C 64.29, H 7.30, N 2.63 (Calcd: C 64.40, H 7.41, N 2.78).

4.1.17. 1-(2,3,4,5-Tetramethoxy-6-methylphenyl)-4-(6,7,8-trimethoxy-3,4- dihydroisoquinolin-2(1H)-yl)butan-1-one (11)

1-(2,3,4,5-Tetramethoxy-6-methylphenyl)-4-(6,7,8-trimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)butan-1-one was prepared according to the procedure for the preparation of **9** by reacting **5b** with **25b** and obtained as a yellowish oil in 71.8% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.41 (s, 1H), 3.92 (s, 3H), 3.89 (s, 3H), 3.87 (s, 3H), 3.83 (s, 3H), 3.82 (s, 3H), 3.80 (s, 3H), 3.78 (s, 3H), 3.55 (s, 2H), 2.81−2.85 (m, 4H), 2.69 (t, J = 5.8 Hz, 2H), 2.60 (t, J = 7.3 Hz, 2H), 2.06 (s, 3H), 1.97−2.02 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 206.7, 151.9, 150.0, 148.2, 147.7, 145.7, 144.5, 139.9, 131.9, 130.0, 122.6, 121.0, 107.2, 61.9, 61.2, 61.1, 60.9, 60.7, 60.5, 57.5, 56.0, 50.9, 50.4, 42.8, 29.3, 21.0, 12.0; LC/MS-m/z: 504.9 $[M+1]^+$ ($C_{27}H_{37}NO_8$: calcd mass 503.3); Anal: C 64.33, H 7.27, N 2.72 (Calcd: C 64.40, H 7.41, N 2.78).

4.1.18. 5-(6,7-Dimethoxy-1,2,3,4-tetrahydroisoquinolin-2-yl)-1-(2,3,4,5-tetra-methoxy-6-methylphenyl)pentan-1-one (12)

A solution of **5c** (0.7 g, 2.2 mmol), 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline hydrochloride (0.5 g, 2.2 mmol), K_2CO_3 (0.3 g, 2.2 mmol) and triethylamine (1.1 g, 10.9 mmol) in n-butanol (10 mL) was stirred at 100 °C overnight. The mixture was cooled to room temperature, filtered off the solid and concentrated. The crude was purified by silica gel column to afford the product as a light yellow oil (0.65 g, 61.6% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.60 (s, 1H), 6.53 (s, 1H), 3.93 (s, 3H), 3.91 (s, 3H), 3.85 (s, 3H), 3.85 (s, 3H), 3.81 (s, 3H), 3.80 (s, 3H), 3.57 (s, 2H), 2.80−2.83 (m, 4H), 2.72 (t, 2H), 2.53−2.57 (m, 2H), 2.07 (s, 3H), 1.68−1.77 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 206.8, 148.2, 147.7, 147.5, 147.2, 145.8, 144.6, 131.8, 126.6, 126.2, 122.7, 111.3, 109.5, 61.9, 61.2, 61.1, 60.7, 58.2, 55.9, 55.8, 51.1, 44.9, 28.7, 26.8, 21.6, 12.1; LC/MS-m/z: 488.4 $[M+1]^+$ ($C_{27}H_{37}NO_7$: calcd mass 487.3); Anal: C 66.38, H 7.59, N 2.71 (Calcd: C 66.51, H 7.65, N 2.87).

4.1.19. 1-(2,3,4,5-Tetramethoxy-6-methylphenyl)-5-(5,6,7-trimethoxy-3,4- dihydroisoquinolin-2(1H)-yl)pentan-1-one (13)

1-(2,3,4,5-Tetramethoxy-6-methylphenyl)-5-(5,6,7-trimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)pentan-1-one was prepared according to the procedure for the preparation of **12** by reacting **5c** with **25a** and obtained as a yellowish oil in 57.8% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.36 (s, 1H), 3.92 (s, 3H), 3.90 (s, 3H), 3.85 (s, 3H), 3.84 (s, 3H), 3.82 (s, 3H), 3.80 (s, 3H), 3.79 (s, 3H), 3.54 (s, 2H), 2.76−2.81 (m, J = 14.5, 7.1 Hz, 4H), 2.69 (t, J = 5.9 Hz, 2H), 2.48−2.57 (m, 2H), 2.06 (s, 3H), 1.73−1.79 (m, J = 14.1, 6.9 Hz, 2H), 1.64−1.70 (m, J = 14.8, 8.6, 5.6 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 206.8, 151.7, 151.2, 148.2, 147.7, 145.8, 144.7, 140.4, 131.8, 122.7, 120.6, 105.4, 61.9, 61.2, 61.1, 60.9, 60.7, 60.4, 58.1, 56.1, 56.0, 50.9, 44.9, 26.8, 23.5, 21.6, 12.1; LC/MS-m/z: 518.2 $[M+1]^+$ ($C_{28}H_{39}NO_8$: calcd mass 517.3); Anal: C 64.84, H 7.51, N 2.66 (Calcd: C 64.97, H 7.59, N 2.71).

4.1.20. 1-(2,3,4,5-Tetramethoxy-6-methylphenyl)-5-(6,7,8-trimethoxy-3,4- dihydroisoquinolin-2(1H)-yl)pentan-1-one (14)

1-(2,3,4,5-Tetramethoxy-6-methylphenyl)-5-(6,7,8-trimethoxy-3,4- dihydroisoquinolin-2(1H)-yl)pentan-1-one was prepared according to the procedure for the preparation of **12** by reacting **5c** with **25b** and obtained as a yellowish oil in 59.5% yield. ^{1}H NMR (100 MHz, $CDCl_3$): δ 6.41 (s, 1H), 3.92 (s, 3H), 3.90 (s, 3H), 3.87 (s, 3H), 3.83 (s, 3H), 3.82 (s, 3H), 3.81 (s, 3H), 3.79 (s, 3H), 3.54 (s, 2H), 2.78−2.82 (m, 4H), 2.68 (t, J = 5.8 Hz, 2H), 2.51−2.61 (m, 2H), 2.06 (s, 3H), 1.73−1.79 (m, 2H), 1.66−1.71 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 206.8, 151.9, 150.0, 148.2, 147.7, 145.8, 144.6, 139.9, 131.9, 130.0, 122.7, 121.0, 107.2, 61.9, 61.2, 61.1, 60.8, 60.7, 60.5, 58.4, 56.0, 51.1, 50.7, 44.9, 29.3, 26.8, 21.6, 12.0; LC/MS-m/z: 518.0 $[M+1]^+$ ($C_{28}H_{39}NO_8$: calcd mass 517.3); Anal: C 64.81, H 7.57, N 2.48 (Calcd: C 64.97, H 7.59, N 2.71).

4.1.21. 6-(6,7-Dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)-1-(2,3,4,5- tetramethoxy-6-methylphenyl)hexan-1-one (15)

To a flask was added with 6-bromo-1-(2,3,4,5-tetramethoxy-6-methylphenyl) hexan-1-one **5d** (0.5 g, 1.28 mmol), 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline hydrochloride (0.29 g, 1.26 mmol), K_2CO_3 (0.35 g, 2.52 mmol) and triethylamine (0.26 g, 2.52 mmol) in isopropanol (15 mL) was stirred at refluxing overnight. The mixture was cooled to room temperature, filtered off the solid and concentrated. The crude was purified by silica gel column to afford the product as a light yellow oil (0.30 g, yield 47.9%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.59 (s, 1H), 6.52 (s, 1H), 3.92 (s, 3H), 3.90

(s, 3H), 3.84 (s, 3H), 3.83 (s, 3H), 3.80 (s, 3H), 3.79 (s, 3H), 3.55 (s, 2H), 2.82 (t, J = 5.8 Hz, 2H), 2.76 (t, J = 7.4 Hz, 2H), 2.70 (t, J = 5.9 Hz, 2H), 2.49–2.54 (m, 2H), 2.06 (s, 3H), 1.69–1.78 (m, 2H), 1.61–1.67 (m, J = 15.2, 7.6 Hz, 2H), 1.40–1.46 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 206.9, 148.2, 147.7, 147.5, 147.2, 145.8, 144.6, 131.9, 126.7, 126.2, 122.6, 111.4, 109.5, 61.8, 61.2, 61.1, 60.6, 58.2, 56.0, 55.9, 55.8, 51.1, 45.0, 28.7, 27.2, 27.1, 23.5, 12.0; LC/MS-m/z: 502.2 $[M+1]^+$ ($C_{28}H_{39}NO_7$: calcd mass 501.3); Anal: C 66.94, H 7.79, N 2.66 (Calcd: C 67.04, H 7.84, N 2.79).

4.1.22. 1-(2,3,4,5-Tetramethoxy-6-methylphenyl)-6-(5,6,7-trimethoxy-3,4- dihydroisoquinolin-2(1H)-yl)hexan-1-one (16)

1-(2,3,4,5-Tetramethoxy-6-methylphenyl)-6-(5,6,7-trimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)hexan-1-one was prepared according to the procedure for the preparation of **15** by reacting **5d** with **25a** and obtained as a yellowish oil in 64.5% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.41 (s, 1H), 3.92 (s, 3H), 3.90 (s, 3H), 3.87 (s, 3H), 3.83 (s, 3H), 3.82 (s, 3H), 3.80 (s, 3H), 3.79 (s, 3H), 3.54 (s, 2H), 2.82 (t, J = 5.7 Hz, 2H), 2.76 (t, J = 7.4 Hz, 2H), 2.67 (t, J = 5.9 Hz, 2H), 2.52–2.55 (m, 2H), 2.06 (s, 3H), 1.71–1.77 (m, J = 15.2, 7.4 Hz, 2H), 1.62–1.69 (m, J = 15.2, 7.6 Hz, 2H), 1.40–1.47 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 207.0, 151.8, 150.0, 148.2, 147.7, 145.8, 144.6, 139.9, 131.9, 129.9, 122.6, 121.0, 107.2, 61.9, 61.2, 61.1, 60.8, 60.7, 60.5, 58.4, 56.0, 51.1, 50.6, 45.0, 29.3, 27.2, 27.1, 23.5, 12.0; LC/MS-m/z: 532.3 $[M+1]^+$ ($C_{29}H_{41}NO_8$: calcd mass 531.3); Anal: C 65.50, H 7.69, N 2.57 (Calcd: C 65.52, H 7.77, N 2.63).

4.1.23. 1-(2,3,4,5-Tetramethoxy-6-methylphenyl)-6-(6,7,8-trimethoxy-3,4- dihydroisoquinolin-2(1H)-yl)hexan-1-one (17)

1-(2,3,4,5-Tetramethoxy-6-methylphenyl)-6-(6,7,8-trimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)hexan-1-one was prepared according to the procedure for the preparation of **15** by reacting **5d** with **25b** and obtained as a light yellow oil in 71.4% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.36 (s, 1H), 3.92 (s, 3H), 3.90 (s, 3H), 3.85 (s, 3H), 3.84 (s, 3H), 3.82 (s, 3H), 3.80 (s, 3H), 3.79 (s, 3H), 3.53 (s, 2H), 2.74–2.77 (m, 4H), 2.69 (t, J = 5.9 Hz, 2H), 2.47–2.53 (m, 2H), 2.06 (s, 3H), 1.69–1.77 (m, J = 15.2, 7.5 Hz, 2H), 1.61–1.67 (m, J = 15.2, 7.6 Hz, 2H), 1.40–1.46 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 206.9, 151.6, 151.2, 148.2, 147.7, 145.8, 144.6, 140.4, 131.9, 130.4, 122.6, 120.6, 105.4, 61.8, 61.2, 61.1, 60.9, 60.6, 60.4, 58.2, 56.1, 56.0, 50.8, 45.0, 27.2, 27.1, 23.5, 23.4, 12.0; LC/MS-m/z: 532.3 $[M+1]^+$ ($C_{29}H_{41}NO_8$: calcd mass 531.3); Anal: C 65.43, H 7.71, N 2.50 (Calcd: C 65.52, H 7.77, N 2.63).

4.1.24. 7-(6,7-Dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)-1-(2,3,4,5- tetramethoxy-6-methylphenyl)heptan-1-one (18)

7-(6,7-Dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)-1-(2,3,4,5-tetramethoxy-6-methylphenyl)heptan-1-one was prepared according to the procedure for the preparation of **15** by reacting 7-bromo-1-(2,3,4,5-tetramethoxy-6-methylphenyl)heptan-1-one (**5e**) with 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline hydrochloride and obtained as a light yellow oil in 63.8% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.59 (s, 1H), 6.52 (s, 1H), 3.92 (s, 3H), 3.90 (s, 3H), 3.84 (s, 3H), 3.83 (s, 3H), 3.80 (s, 3H), 3.79 (s, 3H), 3.53 (s, 2H), 2.82 (t, J = 5.8 Hz, 2H), 2.71–2.76 (m, J = 11.8, 6.7 Hz, 4H), 2.46–2.52 (m, 2H), 2.06 (s, 3H), 1.68–1.73 (m, 2H), 1.59–1.64 (m, 2H), 1.40–1.41 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 207.0, 148.2, 147.6, 147.5, 147.2, 145.7, 144.6, 132.0, 126.7, 126.2, 122.6, 111.4, 109.5, 61.9, 61.2, 61.1, 60.7, 58.4, 56.0, 55.9, 55.8, 51.1, 45.0, 29.2, 28.6, 27.5, 27.1, 23.5, 12.0; LC/MS-m/z: 516.2 $[M+1]^+$ ($C_{29}H_{41}NO_7$: calcd mass 515.3); Anal: C 67.39, H 7.86, N 2.64 (Calcd: C 67.55, H 8.01, N 2.72).

4.1.25. 1-(2,3,4,5-Tetramethoxy-6-methylphenyl)-7-(5,6,7-trimethoxy-3,4- dihydroisoquinolin-2(1H)-yl)heptan-1-one (19)

1-(2,3,4,5-Tetramethoxy-6-methylphenyl)-7-(5,6,7-trimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)heptan-1-one was prepared according to the procedure for the preparation of **15** by reacting **5e** with **25a** and obtained as a light yellow oil in 51.5% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.36 (s, 1H), 3.92 (s, 3H), 3.90 (s, 3H), 3.85 (s, 3H), 3.84 (s, 3H), 3.81 (s, 3H), 3.80 (s, 3H), 3.79 (s, 3H), 3.53 (s, 2H), 2.68 (t, J = 5.9 Hz, 4H), 2.45–2.52 (m, 2H), 2.06 (s, 3H), 1.68–1.73 (m, 2H), 1.58–1.64 (m, 2H), 1.36–1.44 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 207.0, 151.6, 151.2, 148.2, 147.6, 145.7, 144.5, 140.4, 131.9, 130.4, 122.6, 120.6, 105.4, 61.8, 61.2, 61.1 60.9, 60.6, 60.3, 58.4, 56.1, 56.0, 50.8, 45.0, 29.2, 27.4, 27.1, 23.6, 23.5, 12.0; LC/MS-m/z: 546.3 $[M+1]^+$ ($C_{30}H_{43}NO_8$: calcd mass 545.3); Anal: C 65.88, H 7.82, N 2.43 (Calcd: C 66.03, H 7.94, N 2.57).

4.1.26. 1-(2,3,4,5-Tetramethoxy-6-methylphenyl)-7-(6,7,8-trimethoxy-3,4- dihydroisoquinolin-2(1H)-yl)heptan-1-one (20)

1-(2,3,4,5-Tetramethoxy-6-methylphenyl)-7-(6,7,8-trimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)heptan-1-one was prepared according to the procedure for the preparation of **15** by reacting **5e** with **25b** and obtained as a light yellow oil in 51.4% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.41 (s, 1H), 3.92 (s, 3H), 3.90 (s, 3H), 3.87 (s, 3H), 3.83 (s, 3H), 3.82 (s, 3H), 3.80 (s, 3H), 3.79 (s, 3H), 3.53 (s, 2H), 2.82 (t, J = 5.7 Hz, 2H), 2.75 (t, J = 7.4 Hz, 2H), 2.67 (t, J = 5.9 Hz, 2H), 2.48–2.57 (m, 2H), 2.06 (s, 3H), 1.69–1.74 (m, 2H), 1.60–1.65 (m, J = 14.3, 7.3 Hz, 2H), 1.39–1.42 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 207.0, 151.8, 150.0, 148.2, 147.6, 145.7, 144.5, 139.9, 132.0, 130.0, 122.6, 121.0, 107.2, 61.8, 61.2, 61.1, 60.8, 60.6, 60.5, 58.6, 55.9, 51.1, 50.5, 45.0, 29.3, 29.2, 27.5, 27.1, 23.5, 12.0; LC/MS-m/z: 546.7 $[M+1]^+$ ($C_{30}H_{43}NO_8$: calcd mass 545.3); Anal: C 65.91, H 7.79, N 2.53 (Calcd: C 66.03, H 7.94, N 2.57).

4.2. Biological assays

4.2.1. σ receptor affinity assays

4.2.1.1. Tissue source and radioligands. σ_1R binding assays were conducted by using [^{3}H]-(+)-pentazocine (DuPont-NEN, Billerica, MA) in guinea pig brain membranes (Rockland Biological, Gilbertsville, PA). σ_2R/TMEM97 binding assays were conducted by using [^{125}I]-RHM-4 (America Radiolabeled Chemicals Inc., St. Louis, MO) in rat liver membranes.

4.2.1.2. σ_1R binding assay. Quinea pig brain membrane homogenates were prepared from frozen guinea pig brains without cerebellum. Guinea pig brain membrane homogenates (100 μL, 300 μg) were incubated with 50 μL of [^{3}H]-(+)-pentazocine (0.5–10 nM in 50 mM Tris, pH 8.0) and 50 μL of each test compound solution in 50 mM Tris-HCl (pH 8.0) for a total volume of 200 μL at 25 °C for 120 min. Test compounds were dissolved in DMSO and then diluted in buffer and added in concentrations ranging from 0.1 to 1000 nM. Incubations were terminated by the addition of ice-cold 10 mM Tris-HCl (pH 8.0) and followed by rapid filtration through Whatman GF/B glass fiber filters (presoaked in 0.5% polyethylenimine) using a Brandel cell harvester (Gaithersburg, MD). Filters were washed 3 times with 5 mL of ice cold buffer. Each filter was removed with a forcep and placed into 4 mL vials, added 3 mL microscintillation 20 and counted in microbeta counter with 45% efficiency. Nonspecific binding was determined in the presence of 10 μM haloperidol.

4.2.1.3. σ_2R/TMEM97 binding assay. Rat liver membrane homogenates were prepared from the livers of male Sprague-Dawley rats (300–350 g). Rat liver membrane homogenates (100 μL, 300 μg) were incubated with 50 μL of [^{125}I]RHM-4 (0.02–9 nM in 50 mM Tris, pH 7.4), 50 μL of each test compound solution and 50 μL of 100 nM (+)-pentazocine in 50 mM Tris-HCl (pH 8.0) for a total volume of 250 μL at 25 °C for 120 min. Each test compound was

dissolved in DMSO and then diluted in buffer and added in concentrations ranging from 0.1 to 1000 nM. Incubations were terminated by the addition of ice-cold 10 mM Tris-HCl (pH 8.0) and followed by rapid filtration through Whatman GF/B glass fiber filters (presoaked in 0.5% polyethylenimine) using a Brandel cell harvester (Gaithersburg, MD). Filters were washed 3 times with 5 mL of ice-cold buffer. Each filter was removed with a forcep placed into 4 mL vials, added 3 mL microscintillation 20 and counted in Wizard2 Automatic Gamma Counter 2470 with 45% efficiency. Nonspecific binding was determined in the presence of 10 μM RHM-4.

4.2.1.4. Data analysis. The IC_{50} values for σ receptors were determined in triplicate from nonlinear regression of binding data as analyzed by Graphpad Prism 7 (GraphPad Software, Inc., La Jolla, CA). *Ki* values were calculated using the method of Cheng-Prusoff and represent mean values.

4.2.2. Functional assay

4.2.2.1. Measurement of intracellular calcium. Intracellular Ca^{2+} concentration was measured according to the procedure as described in the user's manual of commercially available Fluo-3 AM assay kit (Beyotime Biotechnology, Beijing, China). Briefly, MCF-7 cells were seeded in 96-well plates (10^4 cells/mL) and incubated for 24 h, added with test or reference compounds in DMEM for 3 h incubation. The medium was removed, added with Fluo-3 AM (2.5 μM) and incubated at 37 °C for 30 min. The medium was again removed, added with new culture media and incubated for additional 20 min. The final medium was removed and washed with PBS for 3 times. The plates were read on a confocal laser scanning microscope (Weztlar, Leica, Germany) and images were acquired at 40X magnification.

4.2.2.2. Cytotoxicity assays. CCK-8 assays were carried out on human breast MCF-7 cancer cells and human breast MCF-10A normal cells (Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China) to primarily determine the cytotoxicity of test compounds. Briefly, MCF-7 and MCF-10A cells with exponential growth were seeded into 96-well culture plates at a density of 4000 cells/well and allowed to adhere overnight. The cells were then treated with a series of 5 concentrations of the test compounds or cisplatin (Sigma, USA) for 24 h and 48 h. Cells were treated with an equal volume of DMSO as the control. Thereafter, 10 μL CCK-8 solutions (Dojindo Laboratories, Kumamoto, Japan) were added to each well and the plates were incubated at 37 °C for 2 h in the dark. The UV absorbance was measured at 450 nm with an enzyme immunoassay reader (Bio-Rad, CA, USA). Cell viability was calculated as (optical density of experimental sample/optical density of control) × 100%. The IC50 values and standard deviation values were calculated using the Prism 7.0 program (GraphPad).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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