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Bivalent ligand approach on 4-[2-(3-methoxyphenyl)ethyl]-1-(2-methoxyphenyl)piperazine: Synthesis and binding affinities for 5-HT$_7$ and 5-HT$_{1A}$ receptors

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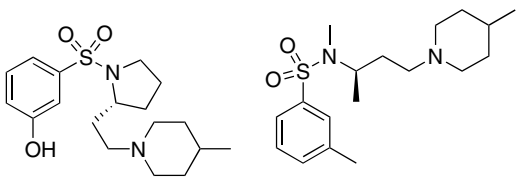
Abstract—We here report on the synthesis and binding properties at 5-HT$_7$ and 5-HT$_{1A}$ receptors of ligands **3**–**12**, that were designed according to the 'bivalent ligand' approach. Two moieties of the 5-HT$_7$/5-HT$_{1A}$ ligand 4-[2-(3-methoxyphenyl)ethyl]-1-(2-methoxyphenyl)piperazine (**1**) were linked through their 3-methoxy substituent by polymethylene chains of variable length, with the aim to increase the affinity for 5-HT$_7$ receptor and the selectivity over 5-HT$_{1A}$ receptors. In the best cases, the dimers showed affinities for 5-HT$_7$ receptors as high as the monomer with no improvement in selectivity. Some dimers displayed 5-HT$_{1A}$ receptor affinities slightly higher than monomer **1**.

1. Introduction

Structural, functional, and pharmacological characteristics identify seven major classes of 5-HT receptors. The 5-HT$_7$ receptor has been cloned from mouse, rat, guinea-pig, human, and pig. The receptor binding profile is consistent across species and between cloned and native 5-HT$_7$ receptors. 5-HT$_7$ receptors are defined pharmacologically by their high affinity for 5-CT, 5-HT, 5-MeOT, and methiothepin, moderate affinity for 8-OH-DPAT and ritanserin, and low affinity for pindolol, sumatriptan, and buspirone.[1] 5-HT$_7$ receptors couple positively to adenylyl cyclase when expressed in cell lines.[2,3] The distribution of 5-HT$_7$ receptor in the central nervous system and initial pharmacological studies using nonselective ligands have suggested that 5-HT$_7$ receptors may modulate 5-HT-induced effects on suprachiasmatic nucleus neuronal activity.[4] Thus, 5-HT$_7$ receptors may be implicated in the regulation of mammalian circadian rhythms[5] and, as such, be associated with sleep disorders and depression. In support of a role for 5-HT$_7$ receptors in depression, in vitro radioligand binding studies in rat suggested that chronic antidepressant treatment results in a functional downregulation of 5-HT$_7$-like binding sites in the hypothalamus.[6] New insights concerning the role of 5-HT$_7$ receptors come from studies performed with selective 5-HT$_7$ receptor antagonists or 5-HT$_7$ receptor knockout (KO) mice. 5-HT$_7$ receptor KO mice displayed behavioral and sleep patterns consistent with an antidepressant-like profile.[7]



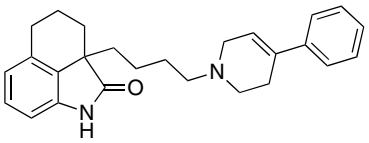


Figure 1. Structures of 5-HT$_7$ receptor antagonists.

Keywords: 5-HT$_7$ receptors; 5-HT$_{1A}$ receptors; Arylpiperazine; Structure–affinity relationships; Bivalent ligands.
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The same robust antidepressant-like effects can be generated in wild type mice by the 5-HT_7 receptor antagonists SB-258719 and SB-269970 (Fig. 1).[8–10] The involvement of 5-HT_7 receptors in other pathophysiological mechanisms has been highlighted by various studies. Administration of DR4004, a selective 5-HT_7 receptor antagonist (Fig. 1), significantly inhibited the exploratory behavior of mice, thus suggesting that 5-HT_7 receptor blockade might produce changes in some component of emotionality in a novel environment.[11] 5-HT_7 receptors are present in microglial cells that represent the resident immune cells of the brain. As such, they are involved in neuroinflammatory processes and can release inflammatory products like interleukin-6 (IL-6). It was found that activation of 5-HT_7 receptor stimulated the expression of IL-6 mRNA in MC-3 cells. This effect was abolished by the selective 5-HT_7 receptor antagonist SB-269970.[12] An immunocytochemical study of 5-HT_7 receptor distribution at the lumbar level of the spinal cord evidenced immunolabeling mainly localized in the two superficial laminae of the dorsal horn and in small and medium-sized dorsal root ganglion cells. Such localization is consistent with a predominant role in nociception.[13] Finally, analysis of 5-HT_7, receptors mRNA expression combined with selective stimulation or blockage of 5-HT_7 receptors demonstrated an association between 5-HT_7 receptors, mRNA expression, memory consolidation, amnesia, or recovery from amnesia, supporting the notion that 5-HT_7 receptors play a role in normal and impaired memory.[14,15] Therefore, it results clear that the 5-HT_7 receptor could serve as a putative target for novel drug development.

During the last decade a number of structurally diverse 5-HT_7 receptor ligands have been disclosed.[16] We have pointed our attention to arylpiperazine derivatives.[17–19] In particular, we have reported on the synthesis and initial pharmacological characterization of three distinct classes of 5-HT_7 receptor ligands with arylpiperazine structure (Fig. 2, general structures I, II, and III). A major issue of the arylpiperazine scaffold was the affinity also for other monoaminergic receptors. In particular, the 5-HT_{1A} receptor affinity is here relevant because it may interfere with the evaluation of pharmacological actions mediated by 5-HT_7 receptors.[20] Suitable structural modifications on structures I and II have led us to identify potent 5-HT_7 receptor ligands endowed with about 200-fold selectivity over 5-HT_{1A} receptor. By contrast, we failed to increase the selectivity over 5-HT_{1A} receptor of the compounds with structure III. Among the different methods currently available for medicinal chemists to design potent and selective receptor subtype ligands, the 'bivalent ligand' approach appears very promising. A bivalent ligand is defined as a molecule that contains two pharmacophores linked through a spacer. The rationale for employing the bivalent ligand approach stems from the possibility that dimeric structures may be capable of bridging independent recognition sites (i.e., two recognition sites on a receptor dimer or one receptor and an accessory site) resulting in a thermodinamically more favorable binding interaction than a monovalent binding of two molecules,[21–23] thus giving enhanced activity. Portoghese and coworkers applied first this approach in the field of opioid research, obtaining excellent results in terms of affinity and selectivity among opioid receptor subtypes.[24–27] This concept has been applied also to 5-$HT_{1B/1D}$ agonists,[28] 5-HT_4 ligands,[29] serotonin reuptake inhibitors,[30] muscarinic agonists,[31] and melatonergic ligands.[32] Therefore, with the aim to enhance the specificity for the 5-HT_7 receptor of compounds with general structure III, we decided to apply the bivalent ligand approach. To the best of our knowledge, this is the first time that this approach has been applied to 5-HT_7 receptor ligands. For our purpose, we have selected as pharmacophore the 5-HT_7/5-HT_{1A} ligand 4-[2-(3-methoxyphenyl)ethyl]-1-(2-methoxyphenyl)piperazine (**1**) (Fig. 2) in order to design selective 5-HT_7 receptor ligands.

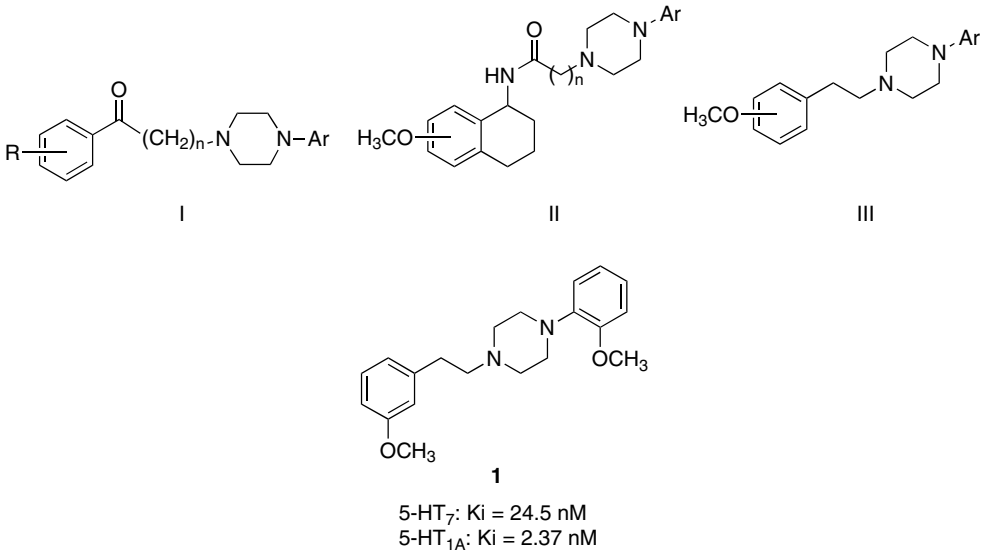
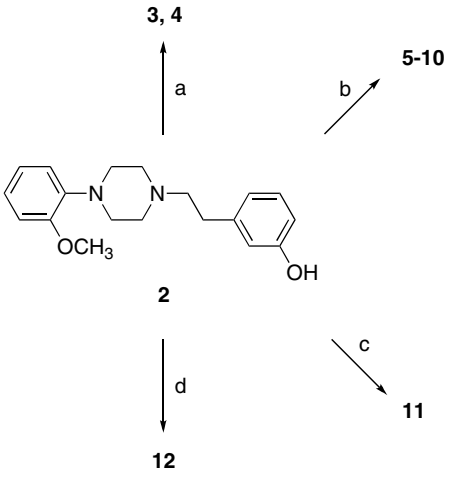


Figure 2. Structures of arylpiperazine-based 5-HT_7 receptor ligands.



Scheme 1. Synthesis of compounds **3**–**12**. Reagents and conditions: (a) 1,2-dibromoethane, or 1,3-dibromopropane KOH, 18-crown-6, toluene, reflux, overnight; (b) $Br(CH_2)_nBr$, NaH, anhydrous toluene, room temperature, 48 h; (c) diethylene glycol, triphenylphosphine, diethyl azodicarboxylate, anhydrous THF, room temperature 48 h; (d) diglycolyl chloride, Et_3N, CH_2Cl_2, room temperature, 48 h.

2. Chemistry

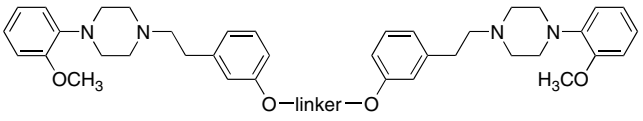
All the final compounds were prepared from the key derivative 4-[2-(3-hydroxyphenyl)ethyl]-1-(2-methoxyphenyl)piperazine (**2**), according to Scheme 1. The ligands **5**–**10**, containing from 4 to 12 methylene units, were prepared by coupling 2 equiv of **2** with the appropriate α,ω-dibromoalkane in the presence of sodium hydride in DMF, according to Neumayer et al.[33] This synthetic route was not useful for the preparation of the compounds with 2 or 3 methylene units (derivatives **3** and **4**, respectively). These compounds were obtained by the nucleophilic substitution reaction of 2 equiv of **2** with the appropriate α,ω-dibromoalkane under phase-transfer catalysis. The dimer **11**, bearing the alkyloxy linker, was synthesized from phenol **2** and diethylene glycol in the presence of triphenylphosphine and diethyl diazodicarboxylate, under Mitsunobu conditions. Finally, compound **13** was obtained from the condensation of 2 equiv of **2** with diglycolyl chloride, previously prepared from commercially available diglycolic acid by means of thionyl chloride.

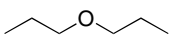
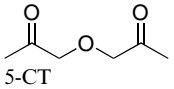
3. Results and discussion

Two potential bridging mechanisms have been proposed to rationalize bivalent ligand activity and selectivity.[26] First, if the spacer is of sufficient length, it may be possible for both pharmacophores in a bivalent ligand to occupy two recognition sites on a receptor dimer; a second possible mechanism involves the bridging of the second pharmacophore of a bivalent ligand to an accessory site adjacent to the receptor site. Under such circumstances, it is reasonable that the bridging of neighboring sites by a bivalent ligand would be dependent on the length and/or flexibility of the spacer. Several spacers have been used for dimer formation, including polymethylene, polyalkyloxy ether, polyglycine. We have selected a polymethylene spacer because of the ease of coupling of the pharmacophores to the spacer and the possibility to find the appropriate linker length by small increase. The final compounds originated by linking two moieties of pharmacophore **1** through their 3-methoxy rather than their 2-methoxy substituent, because structural modifications on the aryl linked to the piperazine nitrogen can result in a dramatic loss in 5-HT_7 receptor affinity.[19]

The chemical structures and binding affinities at 5-HT_7 and 5-HT_{1A} receptors of the dimers **3**–**12** are reported in Table 1. First, with the aim to find the appropriate linker length we evaluated the compounds **3**, **5**, **7**–**10** bearing a spacer of 2, 4, 6, 8, 10, and 12 methylene units, respectively. The 5-HT_7 affinity values revealed that dimerization of compound **1** was tolerated. In fact, dimers **3**, **5**, and **7** (n = 2, 4, and 6, respectively) displayed K_i values at 5-HT_7 receptor in the same range as **1**. By contrast, the higher homologues **8**–**10** proved to be considerably less potent than **1**. Because the best affinity values were displayed by the dimers with a shorter linker, we wanted to complete this series by preparing also the dimers with 3 and 5 methylene units (compounds **4** and **6**, respectively). Elongation of the spacer from 2 to 3 methylene units afforded compound **4** which was the most potent 5-HT_7 ligand within this series. Compound **6** (n = 5, K_i = 120 nM) was less potent than either the lower analogue **5** (n = 4, K_i = 28.5 nM) and the higher analogue **7** (K_i = 42.6 nM). Clearly, the 5-HT_7 affinity of dimers **3**–**10** decreased by increasing the spacer

Table 1. Binding affinities of the target compounds **3**–**12**



Compound	Linker	K_i (nM) ± SEM[a]	
		5-HT_7	5-HT_{1A}
3	$(CH_2)_2$	41.1 ± 2.6	2.2 ± 0.35
4	$(CH_2)_3$	25.0 ± 1.3	5.6 ± 0.7
5	$(CH_2)_4$	28.5 ± 6.4	0.90 ± 0.02
6	$(CH_2)_5$	120 ± 36	1.74 ± 0.80
7	$(CH_2)_6$	42.6 ± 2.4	1.6 ± 0.34
8	$(CH_2)_8$	573 ± 17	4.8 ± 0.50
9	$(CH_2)_{10}$	258 ± 34	28.3 ± 0.24
10	$(CH_2)_{12}$	(11%)[b]	253 ± 27
11		37.0 ± 1.4	0.62 ± 0.15
12		43.0 ± 2.3	2.74 ± 0.12
	5-CT	0.51 ± 0.01	
	5-HT		7.33 ± 0.25

[a] The values are means ± SEM from three independent experiments in triplicate ($P < 0.001$). Individual difference between the various compounds has been examined using Tukey's post hoc test ($P < 0.001$). Difference in the K_i values between the receptors for each compound has been analyzed using the Mann–Whitney U test ($P = 0.007$, $U = 8.000$).

[b] Full K_i not obtained. Percentage of inhibition measured at 10 μM.

length, but not linearly. At this point, we wondered if the hydrophobic nature of the spacer negatively affected the affinity for 5-HT_7 receptor, therefore we prepared compounds **11** and **12** which showed a hydrophilic linker. The impact on 5-HT_7 receptor affinity of a hydrophilic spacer was modest. In fact, K_i values of **11** and **12** (K_i = 37.0 and 43.0 nM, respectively) were comparable to those of **5** and **6**, which possessed a hydrophobic linker with comparable length as **11** and **12**. Taken together, the 5-HT_7 receptor affinity values of dimers **3**–**12** indicated that the two pharmacophores did not bind at two neighboring binding sites because none of the dimers showed higher HT_7 receptor affinity than that of the pharmacophore **1**. Therefore, on the basis of the above data, it can be supposed that both the linker and one pharmacophore are bound to the receptor in a region of steric tolerance.

Considering the affinities for 5-HT_{1A} receptor, dimers **3**–**8**, bearing a linker with 2–8 methylene units, displayed affinity comparable to that of the monomer **1** (K_i values ranging between 0.9 and 4.8 nM). Compound **9** displayed nearly the same 5-HT_{1A} affinity as **1**, whereas further linker elongation resulted in compound **10** which was 10-fold less potent than **1**. Finally, the 5-HT_{1A} receptor affinities of the hydrophilic linker bearing compounds **11** and **12** (K_i = 0.62 and 2.74 nM, respectively) were in the same range as **5** and **6** which were characterized by a polymethylene linker. Although the affinities of compounds **5** and **11** were slightly higher than that of the monomer **1** (2.6- and 3.8-fold, respectively), the observed increase cannot be accounted for by the interaction of **5** or **11** with two independent recognition sites. Similar results were obtained by Halazy et al. when studying dimers of 5-HT.[28] On the basis of affinity data for 5-HT_7 and 5-HT_{1A} receptors of dimers **3**–**12**, it is clear that dimerization of compound **1** did not lead to any improvement in specificity for 5-HT_7 receptor.

4. Conclusion

We have synthesized the ligands **3**–**12** that were designed according to the 'bivalent ligand' approach by linking two moieties of the 5-HT_7/5-HT_{1A} ligand 4-[2-(3-methoxyphenyl)ethyl]-1-(2-methoxyphenyl)piperazine (**1**) through their 3-methoxy substituent by polymethylene chains of variable length, with the aim to increase affinity and specificity for 5-HT_7 receptor. The dimers did not show 5-HT_7 receptor affinities higher than that of the pharmacophore **1** and did retain high 5-HT_{1A} receptor affinity. Therefore, the bivalent ligand approach failed, in this case, to achieve more selective compounds.

5. Experimental

Column chromatography was performed with 1:30 Merck silica gel 60A (63–200 μm) as the stationary phase. Melting points were determined in open capillaries on a Gallenkamp electrothermal apparatus. Elemental analyses (C, H, N) were performed on Eurovector Euro EA 3000 analyzer; the analytical results were within ±0.4% of the theoretical values for the formula given. ^{1}H NMR spectra were recorded at 300 MHz on a Varian Mercury-VX spectrometer. All spectra were recorded on free bases. All chemical shift values are reported in ppm (δ). ESI$^+$/MS/MS analyses were performed with an Agilent 1100 Series LC-MSD trap System VL workstation. All spectra were in accordance with the assigned structures. The purity of new compounds that was essential to the conclusions drawn in the text was determined by HPLC on a Perkin-Elmer series 200 LC instrument using a Phenomenex Prodigy ODS-3 RP-18 column, (250 × 4.6 mm, 5 μm particle size) and equipped with a Perkin-Elmer 785A UV/vis detector setting λ = 254 nm. All compounds were eluted with $CH_3CN/H_2O/Et_3N$, 9:1:0.01, v/v at a flow rate of 1 mL/min. A standard procedure was used to transform final compounds into their hydrochloride salts. 4-[2-(3-Hydroxyphenyl)ethyl]-1-(2-methoxyphenyl)piperazine (**2**) was prepared as previously reported.[19]

5.1. 1,2-Bis-[3-[2-[4-(2-methoxyphenyl)piperazin-1-yl]-ethyl]phenoxy]ethane (3)

A mixture of phenol **2** (0.25 g, 0.8 mmol), 1,2-dibromoethane (0.15 g, 0.8 mmol), powdered KOH (0.45 g, 8.0 mmol), and 18-crown-6 (0.085 g, 0.3 mmol) in toluene (15 mL) was vigorously stirred under reflux overnight. After cooling, the reaction mixture was concentrated and the residue was partitioned between H_2O (30 mL) and $CHCl_3$ (30 mL). The organic layer was separated, dried over Na_2SO_4, and then concentrated in vacuo. The crude residue was chromatographed ($CHCl_3$/AcOEt, 1:1 as eluent) to give the target compound (0.16 g, 31% yield). ^{1}H NMR: δ 2.66–2.71 (m, 4H), 2.76 (br s, 8H), 2.82–2.87 (m, 4H), 3.14 (br s, 8H), 3.87 (s, 6H), 4.32 (s, 4H), 6.79–6.90 (m, 8H), 6.92–7.04 (m. 6H), 7.22 (t, 2H, J = 8.0 Hz). ESI$^+$/MS m/z 651.4 (MH^+). ESI$^+$/MS/MS m/z 459 (100), 205 (59). The hydrochloride salt melted at 226 °C (from CH_3OH/diethyl ether). Anal ($C_{40}H_{50}N_4O_4 \cdot 4HCl \cdot H_2O$) C, H, N.

5.2. 1,3-Bis-[3-[2-[4-(2-methoxyphenyl)piperazin-1-yl]-ethyl]phenoxy]propane (4)

As described above, the final compound was prepared from **2** and 1,3-dibromopropane in 6% yield. ^{1}H NMR: δ 2.25 (quintet, 2H, J = 6.0 Hz), 2.98–3.28 (m, 16H), 3.46 (br s, 8H), 3.86 (s, 6H), 4.16 (t, 4H, J = 6.0 Hz), 6.79–7.09 (m, 14H), 7.23 (t, 2H, J = 8.2 Hz). ESI$^+$/MS m/z 665.4 (MH^+). ESI$^+$/MS/MS m/z 473 (100), 205 (54). The hydrochloride salt melted at 174–177 °C (from CH_3OH/diethyl ether). Anal ($C_{41}H_{52}N_4O_4 \cdot 4HCl$) C, H, N.

5.3. General procedure for the preparation of dimers 5–10

To a solution of **2** (0.80 mmol) in anhydrous DMF (3 mL), NaH powder (0.96 mmol) was carefully added. The mixture was stirred at room temperature for 1 h. Then, a solution of the appropriate dibromoalkane (0.53 mmol) in anhydrous DMF (1 mL) was added dropwise. The resulting mixture was stirred for 2 days at room temperature. Then, the mixture was evaporated to dryness and the residue was partitioned between H_2O

(30 mL) and $CHCl_3$ (30 mL). The organic phase was separated, dried over Na_2SO_4, and evaporated in vacuo. The crude residue was chromatographed ($CHCl_3$/AcOEt, 1:1 as eluent) to give the final compounds as pale yellow oils.

5.3.1. 1,4-Bis-[3-[2-[4-(2-methoxyphenyl)piperazin-1-yl]-ethyl]phenoxy]butane (5). Yield 14%. ^{1}H NMR: δ 1.98–2.02 (m, 4H), 2.80–2.86 (m, 4H), 2.88–2.99 (m, 12H), 3.21 (br s, 8H), 3.87 (s, 6H), 4.03 (br t, 4H), 6.74–7.05 (m, 14H), 7.21 (t, 2H, J = 8.0 Hz). ESI$^+$/MS m/z 679.5 (MH^+). ESI$^+$/MS/MS m/z 487 (100), 205 (38). The hydrochloride salt melted at 250 °C dec (from CH_3OH/diethyl ether). Anal ($C_{42}H_{54}N_4O_4\cdot4HCl\cdot H_2O$) C, H, N.

5.3.2. 1,5-Bis-[3-[2-[4-(2-methoxyphenyl)piperazin-1-yl]-ethyl]phenoxy]pentane (6). Yield 12%. ^{1}H NMR: δ 1.62–1.70 (m, 2H), 1.82–1.91 (m, 4H), 2.66–2.72 (m, 4H), 2.76 (br s, 8H), 2.80–2.86 (m, 4H), 3.14 (br s, 8H), 3.86 (s, 6H), 3.98 (t, 4H, J = 6.3 Hz), 6.65–7.04 (m, 14H), 7.13–7.22 (m, 2H). ESI$^+$/MS m/z 693.6 (MH^+). ESI$^+$/MS/MS m/z 501 (100), 205 (38). The hydrochloride salt melted at 170 °C dec (from CH_3OH/diethyl ether). Anal ($C_{43}H_{56}N_4O_4\cdot4HCl$) C, H, N.

5.3.3. 1,6-Bis-[3-[2-[4-(2-methoxyphenyl)piperazin-1-yl]-ethyl]phenoxy]hexane (7). Yield 38%. ^{1}H NMR: δ 1.52–1.58 (m, 4H), 1.78–1.82 (m, 4H), 2.66–2.71 (m, 4H), 2.76 (br s, 8H), 2.81–2.86 (m, 4H), 3.14 (br s, 8H), 3.86 (s, 6H), 3.96 (t, 4H, J = 6.3 Hz), 6.73–7.03 (m, 14H), 7.20 (t, 2H, J = 7.7 Hz). ESI$^+$/MS m/z 707.5 (MH^+). ESI$^+$/MS/MS m/z 515 (100), 205 (31). The hydrochloride salt melted at 238 °C dec (from CH_3OH/diethyl ether). Anal ($C_{44}H_{58}N_4O_4\cdot4HCl$) C, H, N.

5.3.4. 1,8-Bis-[3-[2-[4-(2-methoxyphenyl)piperazin-1-yl]-ethyl]phenoxy]octane (8). Yield 27%. ^{1}H NMR: δ 1.41–1.51 (m, 8H), 1.74–1.83 (m, 4H), 2.66–2.71 (m, 4H), 2.75 (br s, 8H), 2.80–2.86 (m, 4H), 3.14 (br s, 8H), 3.86 (s, 6H), 3.94 (t, 4H, J = 6.6 Hz), 6.72–7.03 (m, 14H), 7.21 (t, 2H, J = 7.7 Hz). ESI$^+$/MS m/z 735.5 (MH^+). ESI$^+$/MS/MS m/z 543 (100), 205 (21). The hydrochloride salt melted at 198–204 °C (from CH_3OH/diethyl ether). Anal ($C_{46}H_{62}N_4O_4\cdot4HCl\cdot H_2O$) C, H, N.

5.3.5. 1,10-Bis-[3-[2-[4-(2-methoxyphenyl)piperazin-1-yl]-ethyl]phenoxy]decane (9). Yield 29%. ^{1}H NMR: δ 1.32 (br s, 8H), 1.40–1.45 (m, 4H), 1.72–1.82 (m, 4H), 2.65–2.70 (m, 4H), 2.75 (br s, 8H), 2.80–2.85 (m, 4H), 3.13 (br s, 8H), 3.86 (s, 6H), 3.93 (t, 4H, J = 6.6 Hz), 6.72–7.03 (m, 14H), 7.19 (t, 2H, J = 7.7 Hz). ESI$^+$/MS m/z 763.6 (MH^+). ESI$^+$/MS/MS m/z 571 (100). The hydrochloride salt melted at 196–212 °C (from CH_3OH/diethyl ether). Anal ($C_{48}H_{66}N_4O_4\cdot4HCl$) C, H, N.

5.3.6. 1,12-Bis-[3-[2-[4-(2-methoxyphenyl)piperazin-1-yl]-ethyl]phenoxy]dodecane (10). Yield 33%. ^{1}H NMR: δ 1.29 (br s, 12H), 1.40–1.45 (m, 4H), 1.73–1.82 (m, 4H), 2.67–2.72 (m, 4H), 2.77 (br s, 8H), 2.81–2.88 (m, 4H), 3.15 (br s, 8H), 3.86 (s, 6H), 3.94 (t, 4H, J = 6.6 Hz), 6.73–7.04 (m, 14H), 7.19 (t, 2H, J = 7.7 Hz). ESI$^+$/MS m/z 791.6 (MH^+). ESI$^+$/MS/MS m/z 599 (100). The hydrochloride salt melted at 186–193 °C (from CH_3OH/diethyl ether). Anal ($C_{50}H_{70}N_4O_4\cdot4HCl\cdot H_2O$) C, H, N.

5.4. Di-2-[[3-[2-[4-(2-methoxyphenyl)piperazin-1-yl]-ethyl]phenoxy]ethyl] ether (11)

To a solution of **2** (0.50 g, 1.6 mmol), triphenylphosphine (0.42 g, 1.6 mmol), and diethylen glycol (0.10 g, 0.94 mmol) in anhydrous THF (20 mL) under stirring was added diethyl azodicarboxylate (0.28 g, 1.6 mmol). The resulting mixture was stirred at room temperature under anhydrous condition for 2 days. Then, the solvent was evaporated under reduced pressure and the residue was chromatographed ($CHCl_3$/AcOEt, 1:1 as eluent) to give a mixture of target compound and monosubstituted **2**. This mixture was then chromatographed with $CHCl_3$/MeOH, 49:1 as eluent, to afford pure **11** as a pale yellow oil (0.15 g, 14% yield). ^{1}H NMR: δ 2.66–2.72 (m, 4H), 2.77 (br s, 8H), 2.82–2.87 (m, 4H), 3.15 (br s, 8H), 3.86 (s, 6H), 3.94 (t, 4H, J = 5.2 Hz), 4.16 (t, 4H, J = 5.0 Hz), 6.75–7.04 (m, 14H), 7.20 (t, 2H, J = 8.0 Hz). ESI$^+$/MS m/z 695.1 (MH^+). ESI$^+$/MS/MS m/z 503 (100), 205 (31). The hydrochloride salt melted at 168–169 °C (from CH_3OH/diethyl ether). Anal ($C_{42}H_{54}N_4O_5\cdot4HCl$) C, H, N.

5.5. Bis[3-[2-[4-(2-methoxyphenyl)piperazin-1-yl]ethyl]-phenyl] diglycolate (12)

To an ice-cooled mixture containing **2** (0.30 g, 0.96 mmol), Et_3N (0.5 mL) in CH_2Cl_2 (30 mL) was added dropwise under vigorous stirring a solution of diglycolyl chloride, prepared by refluxing the corresponding acid (0.075 g, 0.56 mmol) and $SOCl_2$ (3 mL). Then, the cooling bath was removed and the mixture was stirred for 2 days at room temperature. Then, the reaction mixture was washed with H_2O. The separated organic was dried over Na_2SO_4 and evaporated to dryness under reduced pressure. The crude residue was chromatographed ($CHCl_3$/MeOH, 19:1 as eluent) to give target ester as a pale yellow liquid (0.15 g, 37% yield). ^{1}H NMR: δ 2.68–2.73 (m, 4H), 2.77 (br s, 8H), 2.86–2.91 (m, 4H), 3.15 (br s, 8H), 3.86 (s, 6H), 4.58 (s, 4H), 6.85–7.03 (m, 12H), 7.13 (d, 2H, J = 7.7 Hz), 7.32 (t, 2H, J = 7.7 Hz). ESI$^+$/MS m/z 723.0 (MH^+). ESI$^+$/MS/MS m/z 531 (100). The hydrochloride salt melted at 191 °C (from CH_3OH/diethyl ether). Anal ($C_{42}H_{50}N_4O_7\cdot4HCl\cdot H_2O$) C, H, N.

5.6. Pharmacology

Rat recombinant serotonin 5-HT$_7$ receptor expressed in HEK-293 cells, [^{3}H]LSD, and [^{3}H]-8-OH-DPAT were obtained from Perkin-Elmer (Zaventem, Belgium). 5-CT was purchased from Tocris Cookson Ltd (Bristol, UK). 5-HT was from Sigma–Aldrich RBI (Milan, Italy). For receptor binding studies, compounds **3**–**12** were dissolved in absolute ethanol.

5.6.1. Radioligand binding assay at rat cloned 5-HT$_7$ receptor. Binding of [^{3}H]LSD at rat cloned 5-HT$_7$ receptor was performed according to Jasper et al.[34] with minor modifications. In 1 mL of incubation buffer (50 mM Tris, 10 mM $MgCl_2$, and 0.5 mM EDTA, pH 7.4) were suspended 30 μg of membranes, 2.5 nM [^{3}H]LSD, and the drugs or reference compound (6–9 concentrations). The samples were incubated for 60 min at 37 °C.

The incubation was stopped by rapid filtration on GF/A glass fiber filters (pre-soaked in 0.5% polyethylenimine for 30 min). The filters were washed with 3×3 mL of ice-cold buffer (50 mM Tris, pH 7.4). Nonspecific binding was determined in the presence of 10 μM 5-CT. Approximately 90% of specific binding was determined under these conditions.

5.6.2. Radioligand binding assay at rat human cloned 5-HT_{1A} receptor. Human 5-HT_{1A} serotonin receptors stably expressed in HEK-293 cells were radiolabeled with 1.0 nM [^{3}H]-8-OH-DPAT.[35] Samples containing 40 μg of membrane protein, different concentrations of each compound ranging from 0.1 nM to 10 μM were incubated in a final volume of 500 μL of 50 mM Tris–HCl, pH 7.4, 5 mM $MgSO_4$ for 120 min at 37 °C. After this incubation time, samples were filtered through GF/C pre-soaked in polyethylenimine 0.5% for at least 30 min prior to use. The filters were washed twice with 1 mL of ice-cold buffer (50 mM Tris–HCl, pH 7.4). Nonspecific binding was determined in the presence of 10 μM 5-HT.

5.7. Statistical methods

The inhibition curves on the different binding sites of the compounds reported in Table 1 were analyzed by nonlinear curve fitting utilizing the GraphPad Prism® program. The value for the inhibition constant, K_i, was calculated by using the Cheng–Prusoff equation.[36] The values are means ± SEM from three experiments in triplicate. Individual differences between the various compounds have been examined using Tukey's post hoc test. Differences in K_i values between the receptors for each compound have been analyzed using the Mann–Whitney U test. A difference with $P < 0.05$ was considered statistically significant.

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