Electronic Supplementary Information for

Multivalent Ligands for the Serotonin 5-HT_4 Receptor

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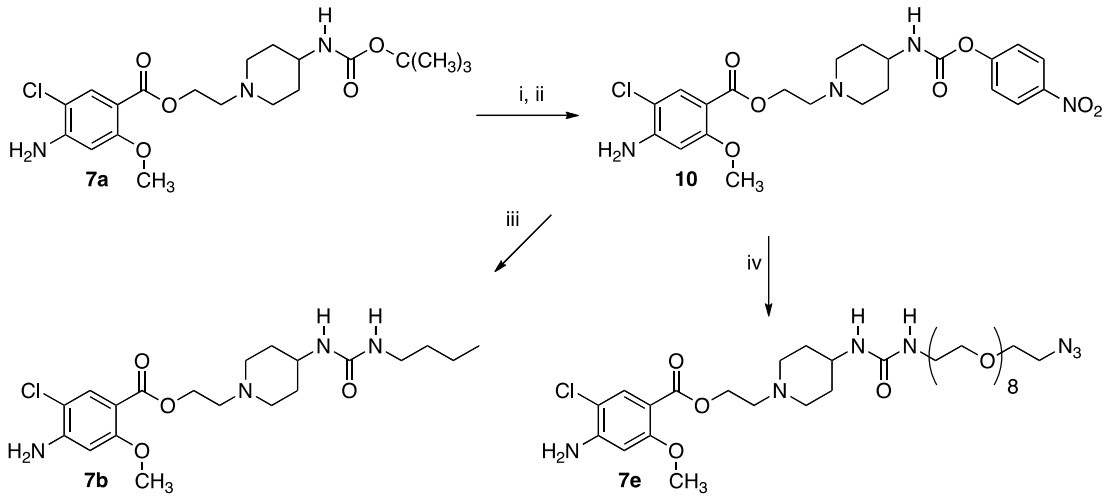
Synthesis

Target monovalent ligands **7b,e** were prepared according to the reaction sequence shown in Scheme 1. *N*-Boc amine **7a**[1] was deprotected in TFA and then reacted with bis (4-nitrophenyl) carbonate in $CHCl_3$ to obtain key activated carbamate **10**, which was reacted with the suitable amines to afford monovalent ligands **7b** and **7e.**

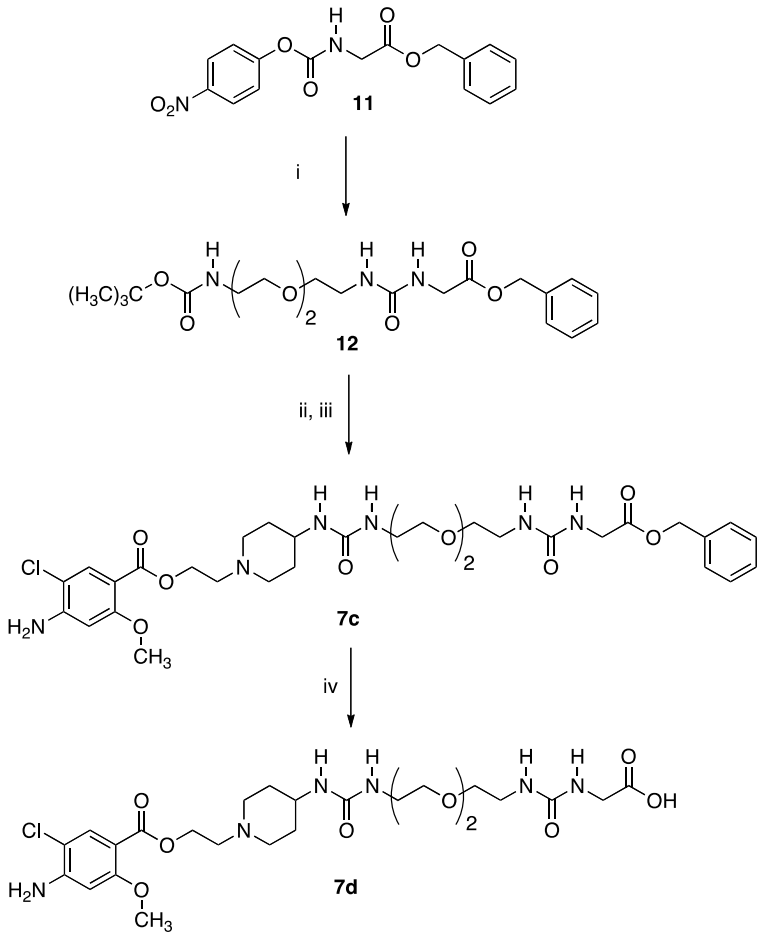
Scheme 1. Synthesis of monovalent ligands **7b,e**.



Reagents: (i) TFA; (ii) $CO(OC_6H_4NO_2)_2$, $CHCl_3$; (iii) $CH_3CH_2CH_2CH_2NH_2$, $CHCl_3$; (iv) $N_3(CH_2CH_2O)_8CH_2CH_2NH_2$, TEA, $CHCl_3$.

Activated carbamate **10** was also used in the synthesis of benzyl ester **7c** (Scheme 2). The reaction of **11**[2] with *tert*-butyl 2-[2-(2-aminoethoxy)ethoxy]ethylcarbamate gave intermediate **12**, which was deprotected with TFA and then reacted with the activated carbamate **10** to afford **7c**. Benzyl ester **7c** was finally submitted to catalytic hydrogenation to give acid **7d**.

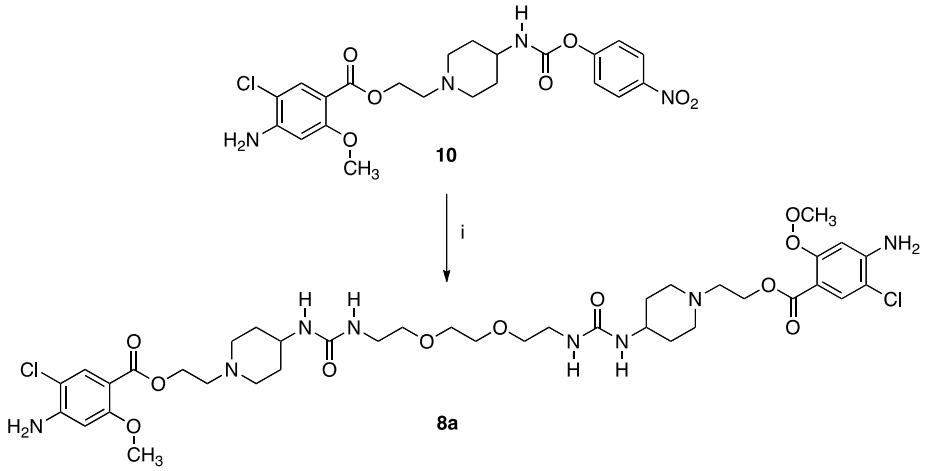
Scheme 2. Synthesis of monovalent ligands **7c**,**d**.



Reagents: (i) $(CH_3)_3OCONH(CH_2CH_2O)_2CH_2CH_2NH_2$, $CHCl_3$; (ii) TFA; (iii) **10**, $CHCl_3$; (iv) H_2, Pd/C, C_2H_5OH.

Shortly tethered homobivalent ligand **8a** was prepared starting from key intermediate **10** by reaction with tri(ethylene glycol) diamine in chloroform (Scheme 3).

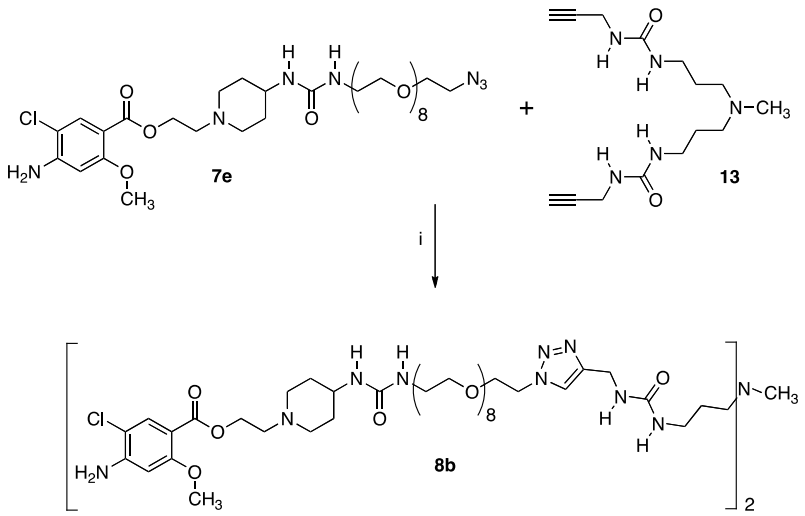
Scheme 3. Synthesis of homobivalent ligand **8a**.



Reagents: (i) $H_2N(CH_2CH_2O)_2CH_2CH_2NH_2$, TEA, $CHCl_3$.

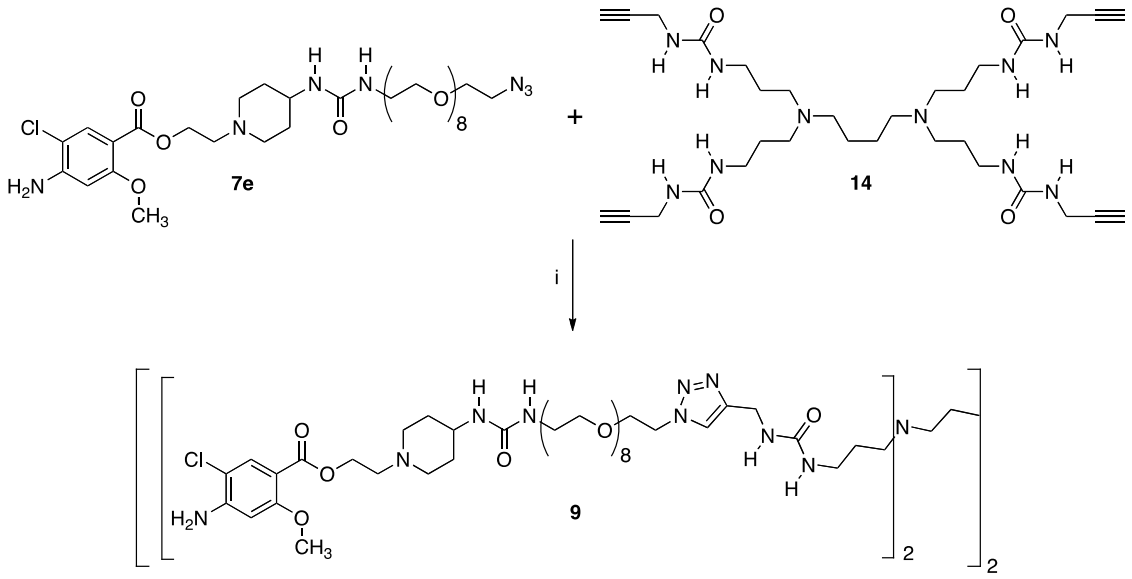
Bivalent ligand **8b** and the tetravalent ligand **9** were synthesized by microwave-assisted Cu(I)-catalyzed azide-alkyne 1,4-dipolar cycloaddition (CuAAC) reaction of ligand **7e** with the suitable multivalent alkynes (**13** or **14**) in DMF in the presence of CuBr and DIPEA (Schemes 4 and 5).

Scheme 4. Synthesis of bivalent ligand **8b**.



Reagents: (i) CuBr, DIPEA, DMF (MW).

Scheme 5. Synthesis of tetravalent ligand **9**.



Reagents: (i) CuBr, DIPEA, DMF (MW).

Experimental details

Chemistry

All chemicals used were of reagent grade. Yields refer to purified products and are not optimized. Melting points were determined in open capillaries on a Gallenkamp apparatus and are uncorrected. Merck silica gel 60 (230-400 mesh) was used for column chromatography. Merck TLC plates, silica gel 60 F_{254} were used for TLC. ^{1}H NMR spectra were recorded by means of a Bruker AC 200, a Bruker DRX 400 AVANCE, or a Bruker DRX 500 AVANCE spectrometers in the indicated solvents (TMS as internal standard); the values of the chemical shifts are expressed in ppm and the coupling constants (*J*) in Hz. Mass spectra were recorded on either a ThermoFinnigan LCQ-Deca or an Agilent 1100 LC/MSD. High-resolution mass spectrometry (HRMS) measurements were carried out on a Thermo LTQ Orbitrap instrument. Operating conditions for the ESI source were as follows: spray voltage + 4.2 kV; capillary temperature 275 °C; sheath gas (nitrogen) flow rate, ca. 0.75 L/min. Methanolic solutions of the different compounds (ca. 1 x 10^{-4} M) have been introduced via direct infusion at a flow rate of 5 μL/min. The purity of monovalent (**7a-e**) and bivalent (**8a,b**) ligands was assessed by RP-HPLC and was found to be higher than 95%. An Agilent 1100 Series system equipped with a Zorbax Eclipse XDB-C8 (4.6 x 150 mm, 5 μm) column was used in the HPLC analysis with methanol-(1% acetic acid-water) (50:50) as the mobile phase at a flow rate of 0.5 mL/min. UV detection was achieved at 254 nm.

2-[4-[(4-Nitrophenoxy)carbonylamino]piperidin-1-yl]ethyl 4-Amino-5-chloro-2-methoxybenzoate (10).

A solution of compound **7a**[1] (0.93 g, 2.17 mmol) in TFA (10 mL) was stirred at room temperature for 10 min. The excess of TFA was removed under reduced pressure by azeotropic distillation with toluene and the residue obtained was partitioned between ethyl acetate and a saturated solution of sodium carbonate. The organic layer was washed with water, dried over sodium sulfate, and evaporated under reduced pressure. The resulting residue (0.63 g, 1.92 mmol) was dissolved in chloroform (20 mL) and bis (4-nitrophenyl) carbonate (0.59 g, 1.94 mmol) was added. The reaction mixture was stirred at room

temperature for 4 days and then concentrated under reduced pressure. The residue was diluted with dichloromethane and washed with a saturated solution of sodium carbonate. The organic layer was dried over sodium sulfate and evaporated under reduced pressure. The residue was purified by flash chromatography with dichloromethane-methanol (9:1) as the eluent to give compound **10** as a yellow solid (0.59 g, yield 55%, mp 145-147° C). ^{1}H NMR (400 MHz, $CDCl_3$): 1.61 (m, 2H), 2.03 (d, *J* = 10.8, 2H), 2.31 (t, *J* = 10.7, 2H), 2.78 (t, *J* = 5.8, 2H), 2.99 (d, *J* = 11.7, 2H), 3.59 (m, 1H), 3.83 (s, 3H), 4.37 (t, *J* = 5.8, 2H), 4.45 (br s, 2H), 5.05 (d, *J* = 7.8, 1H), 6.27 (s, 1H), 7.29 (d, *J* = 9.1, 2H), 7.80 (s, 1H), 8.22 (d, *J* = 9.1, 2H). MS (ESI): *m/z* 493 ($M + H^+$).

2-[4-(3-Butylureido)piperidin-1-yl]ethyl 4-Amino-5-chloro-2-methoxybenzoate (7b).

A mixture of compound **10** (70 mg, 0.14 mmol) in chloroform (5.0 mL) with butylamine (0.027 mL, 0.27 mmol) was stirred at room temperature for 18 h. The reaction mixture was then concentrated under reduced pressure and the residue was partitioned between chloroform and a saturated ammonium chloride solution. The organic layer was dried over sodium sulfate and evaporated under reduced pressure. Purification of the residue obtained by washing with diethyl ether gave compound **7b** as an off-white solid (40 mg, yield 67%). An analytical sample was obtained by recrystallization from chloroform by slow evaporation (mp 177-178 °C). ^{1}H NMR (400 MHz, CD_3OD): 0.89 (t, *J* = 7.2, 3H), 1.30 (m, 2H), 1.39 (m, 2H), 1.52 (m, 2H), 1.92 (d, *J* = 12.4, 2H), 2.51 (m, 2H), 2.93 (t, *J* = 5.4, 2H), 3.07 (m, 4H), 3.58 (m, 1H), 3.78 (s, 3H), 4.37 (t, *J* = 5.4, 2H), 6.44 (s, 1H), 7.75 (s, 1H). MS (ESI): *m/z* 427 ($M + H^+$).

Benzyl 15-^{13}C-17,17-Dimethyl-4,15-dioxo-8,11,16-trioxa-3,5,14-triazaoctadecan-1-oate (12).

A mixture of compound **11** (0.20 g, 0.60 mmol) and *tert*-butyl 2-[2-(2-aminoethoxy)ethoxy]ethylcarbamate (0.14 mL, 0.60 mmol) in chloroform (8.0 mL) was stirred at room temperature for 18 h. The reaction mixture was concentrated under reduced pressure and the residue was purified by flash chromatography with ethyl acetate-methanol (9:1) as the eluent to give compound **12**

as a colorless oil (0.24 g, yield 91%). ^{1}H NMR (200 MHz, $CDCl_3$): 1.37 (s, 9H), 3.22 (m, 2H), 3.32 (t, *J* = 4.5, 2H), 3.44-3.58 (m, 8H), 3.95 (d, *J* = 5.8, 2H), 5.08 (d, *J* = 3.0, 2H), 5.45 (br s, 1H), 5.66 (br s, 2H), 7.27 (s, 5H). MS (ESI): m/z 463 ($M + Na^+$).

Benzyl 15-^{13}C-1-[1-[2-(4-Amino-5-chloro-2-methoxybenzoyloxy)ethyl]piperidin-4-ylamino]-1,12-dioxo-5,8-dioxa-2,11,13-triazapentadecan-15-oate (7c).

A mixture of compound **12** (60 mg, 0.136 mmol) in TFA was stirred at room temperature for 10 min. The excess of TFA was removed under reduced pressure by azeotropic distillation with toluene and the residue was dissolved into chloroform (15 mL). Compound **10** (65 mg, 0.13 mmol) and TEA (0.5 mL) were added to the resulting solution and the reaction mixture was stirred at room temperature for 18 h. The reaction mixture was then washed with water. The organic layer was dried over sodium sulfate and concentrated under reduced pressure. Purification of the residue by flash chromatography with ethyl acetate-triethylamine-methanol (7:2:1) as the eluent gave compound **7c** as a pale yellow oil (75 mg, yield 83%). ^{1}H NMR (200 MHz, $CDCl_3$): 1.51 (m, 2H), 1.90 (d, *J* = 12.9, 2H), 2.25 (t, *J* = 11.1, 2H), 2.73 (t, *J* = 5.8, 2H), 2.92 (d, *J* = 11.4, 2H), 3.30 (m, 4H), 3.45-3.62 (m, 9H), 3.78 (s, 3H), 3.99 (t, *J* = 5.7, 2H), 4.33 (t, *J* = 5.8, 2H), 4.51 (s, 2H), 5.11 (d, *J* = 2.9, 2H), 5.37 (br m, 2H), 5.73 (t, *J* = 5.0, 1H), 6.16 (br t, 1H), 6.25 (s, 1H), 7.30 (s, 5H), 7.77 (s, 1H). MS (ESI): m/z 694 ($M + H^+$).

15-^{13}C-1-[1-[2-(4-Amino-5-chloro-2-methoxybenzoyloxy)ethyl]piperidin-4-ylamino]-1,12-dioxo-5,8-dioxa-2,11,13-triazapentadecan-15-oic acid (7d).

A mixture of compound **7c** (55 mg, 0.079 mmol) in ethanol (20 mL) containing 10% Pd/C (10 mg) was hydrogenated at atmospheric pressure and room temperature for 3 h. The catalyst was then filtered off and the filtrate was concentrated under reduced pressure. The residue was purified by washing with chloroform to give acid **7d** as a yellow oil (40 mg, yield 84%). ^{1}H NMR (400 MHz, CD_3OD): 1.80 (m, 2H), 2.10 (m, 2H), 3.13 (m, 2H), 3.30 (m, 4H), 3.48-3.61 (m, 12H), 3.68 (m, 3H), 3.79 (s, 3H), 4.56 (m, 2H), 6.46 (s, 1H), 7.78 (s, 1H). MS (ESI, negative ions): m/z 602 ($M - H^+$).

Bivalent ligand 8a.

A mixture of compound **10** (0.10 g, 0.20 mmol) in chloroform (10 mL) containing 2,2'-(ethylenedioxy)bis(ethylamine) (0.015 mL, 0.10 mmol) and TEA (0.042 mL, 0.30 mmol) was stirred at room temperature for 18 h. The reaction mixture was then concentrated under reduced pressure and the resulting residue was partitioned between chloroform and water. The organic layer was washed with water, dried over sodium sulfate, and concentrated under reduced pressure. Purification of the residue by flash chromatography with dichloromethane-methanol (8:2) as the eluent gave pure compound **8a** as a pale yellow oil (0.050 g, yield 58%), which crystallized by treatment with diethyl ether to give a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): 1.46 (m, 4H), 1.92 (d, *J* = 10.6, 4H), 2.26 (t, *J* = 10.6, 4H), 2.73 (t, *J* = 5.7, 4H), 2.91 (d, *J* = 11.6, 4H), 3.31 (q, *J* = 5.1, 4H), 3.55 (t, *J* = 4.9, 4H), 3.59 (m, 6H), 3.81 (s, 6H), 4.35 (t, *J* = 5.8, 4H), 4.48 (br s, 4H), 5.14 (br d, 2H), 5.29 (br t, 2H), 6.27 (s, 2H), 7.79 (s, 2H). MS (ESI): *m/z* 877 ($M + Na^+$).

2-[4-[3-(26-Azido-3,6,9,12,15,18,21,24-octaoxahexacosyl)ureido]piperidin-1-yl]ethyl 4-Amino-5-chloro-2-methoxybenzoate (7e).

A mixture of compound **10** (0.11 g, 0.22 mmol) in chloroform (10 mL) containing TEA (0.064 mL, 0.46 mmol) and 26-azido-3,6,9,12,15,18,21,24-octaoxahexacosan-1-amine (0.19 g, 0.43 mmol) was stirred at room temperature for 4 h. The reaction mixture was concentrated under reduced pressure and the residue was diluted with dichloromethane and washed with water. The organic layer was dried over sodium sulfate and concentrated under reduced pressure. Purification of the residue by flash chromatography with dichloromethane-methanol (9:1) as the eluent gave pure compound **7e** as a pale yellow oil (0.14 g, yield 80%). ^{1}H NMR (500 MHz, DMSO-d_6): 1.21-1.33 (m, 2H), 1.67-1.75 (m, 2H), 2.07-2.15 (m, 2H), 2.59 (t, *J* = 5.9, 2H), 2.74-2.82 (m, 2H), 3.12 (q, *J* = 5.7, 2H), 3.30-3.42 (m, 5H), 3.47-3.56 (m, 28H), 3.59 (t, *J* = 5.2, 2H), 3.71 (s, 3H), 4.18 (t, *J* = 5.9, 2H), 5.75 (t, *J* = 5.7, 1H), 5.90 (d, *J* = 7.7, 1H), 6.15 (s, 2H), 6.44 (s, 1H), 7.57 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): 32.7, 39.3,

46.2, 50.1, 52.4, 55.7, 56.4, 61.8, 69.4, 69.7, 69.8, 69.9, 70.0, 70.3, 97.9, 107.0, 108.0, 132.5, 150.1, 157.4, 159.8, 163.9. HRMS (ESI): *m/z* calculated for [$C_{34}H_{58}ClN_7O_{12}$ + H^+] requires 792.3905, found 792.3894.

Bivalent ligand 8b.

In a microwave tube, a solution of **13** (25 mg, 0.081 mmol) in DMF (5.0 mL) containing **7e** (190 mg, 0.24 mmol), CuBr (5.7 mg, 0.040 mmol), and DIPEA (7.0 µL, 0.040 mmol) was exposed to microwave irradiation in a CEM Discover instrument for 50 min (5 x 10 min, T = 60 °C, W = 150) and finally evaporated under reduced pressure. The residue was dissolved with water (15 mL) and 33% NH_4OH (4.0 mL). The aqueous solution was extracted with dichloromethane and the organic layer was dried over sodium sulfate and concentrated under reduced pressure. The resulting residue was purified by washing with ethyl acetate to give dimer **8b** as a yellow oil (51 mg, yield 33%). ^{1}H NMR (500 MHz, DMSO-d_6): 1.19-1.32 (m, 4H), 1.43-1.52 (m, 4H), 1.64-1.74 (m, 4H), 2.04-2.16 (m, 7H), 2.28 (br s, 4H), 2.58 (t, *J* = 5.9, 4H), 2.73-2.81 (m, 4H), 3.00 (q, *J* = 6.5, 4H), 3.11 (q, *J* = 5.7, 4H), 3.30-3.36 (m, 6H), 3.45-3.51 (m, 56H), 3.70 (s, 6H), 3.77 (t, *J* = 5.2, 4H), 4.12-4.22 (m, 8H), 4.46 (t, *J* = 5.2, 4H), 5.75 (t, *J* = 5.7, 2H), 5.89 (d, *J* = 7.7, 2H), 6.01 (t, *J* = 5.8, 2H), 6.15 (s, 4H), 6.34 (t, *J* = 5.7, 2H), 6.43 (s, 2H), 7.56 (s, 2H), 7.83 (s, 2H). ^{13}C NMR (125 MHz, DMSO-d_6): 27.5, 32.5, 35.0, 37.4, 39.2, 41.6, 46.0, 49.2, 52.2, 54.5, 55.5, 56.2, 61.7, 68.8, 69.5, 69.6, 69.8, 70.1, 97.7, 106.9, 107.8, 122.8, 132.4, 145.8, 149.9, 157.3, 157.9, 159.7, 163.7. HRMS (ESI): *m/z* calculated for [$C_{83}H_{141}Cl_2N_{19}O_{26}$ + H^+] requires 1890.9745, found 1890.9718.

Tetravalent ligand 9.

In a microwave tube, a solution of **14** (27 mg, 0.042 mmol) in DMF (5.0 mL) containing **7e** (190 mg, 0.24 mmol), CuBr (11 mg, 0.077 mmol), and DIPEA (14 µL, 0.084 mmol) was exposed to microwave irradiation in a CEM Discover instrument for 50 min (5 x 10 min, T = 60 °C, W = 150) and finally evaporated under reduced pressure. The residue was dissolved with water (15 mL) and 33% NH_4OH

(4.0 mL). The aqueous solution was extracted with dichloromethane and the organic layer was dried over sodium sulfate and concentrated under reduced pressure. The resulting residue was purified by washing with ethyl acetate to give tetravalent ligand **9** as a yellow oil (55 mg, yield 34%). ^{1}H NMR (500 MHz, DMSO-d_6): 1.19-1.34 (m, 12H), 1.41-1.48 (m, 8H), 1.65-1.73 (m, 8H), 2.09 (t, *J* = 11.1, 8H), 2.29 (br s, 12H), 2.57 (t, *J* = 5.9, 8H), 2.74-2.80 (m, 8H), 2.98 (q, *J* = 6.6, 8H), 3.11 (q, *J* = 5.7, 8H), 3.34-3.37 (m, 12H), 3.44-3.51 (m, 112H), 3.70 (s, 12H), 3.77 (t, *J* = 5.1, 8H), 4.14-4.21 (m, 16H), 4.46 (t, *J* = 5.1, 8H), 5.75 (t, *J* = 5.6, 4H), 5.89 (d, *J* = 7.7, 4H), 5.99 (t, *J* = 5.7, 4H), 6.14 (s, 8H), 6.29 (t, *J* = 5.9, 4H), 6.43 (s, 4H), 7.56 (s, 4H), 7.83 (s, 4H). ^{13}C NMR (125 MHz, DMSO-d_6): 24.2, 27.6, 32.6, 35.0, 37.7, 39.6, 46.1, 49.2, 50.9, 52.2, 53.2, 55.5, 56.2, 61.7, 68.8, 69.5, 69.6, 69.8, 70.1, 97.7, 106.9, 107.8, 122.8, 132.4, 145.8, 149.9, 157.3, 157.9, 159.7, 163.8. HRMS (ESI): *m/z* calculated for [$C_{168}H_{284}Cl_4N_{38}O_{52}$ + $5H^+$] requires 762.1973, found 762.1950.

Binding assays

Male Dunkin-Hartley guinea pigs (Charles River Italia, Calco, CO, Italy) weighing 300-400 g were used. Animal care and handling throughout the experimental procedures were in accordance with both the Italian legislative decree DL 26/2014 and the European Communities Council Directive of 22 September 2010 (2010/63 UE).

Binding assays on striatal nuclei membrane preparations were performed according to Grossman et al. with slight modification.[3] Crude membranes were diluted in the binding buffer (Hepes 50 mM, pH 7.4) in order to obtain the final protein concentration. The incubation was performed in polystyrene 24-well multiwell plate at 37 °C for 30 min. The bound radioligand was separated by rapid filtration on glass fiber Unifilter GF/B 24w plate pre-treated with polyethyleneimine 0.1% in buffer. Filtrates were washed two times with 2 mL cold binding buffer, plates were dried for 30 min at room temperature, then 0.2 mL/well MICROSCINT-20 (Perkin Elmer Life and Analytical Sciences) and radioactivity measured after at least 2 h of stabilization. The specific binding of [^{3}H]GR113808 (final concentration

0.2 nM), defined as the difference between the total binding and the nonspecific binding determined in the presence of 30 μM 5-HT, represented about 70-80% of the total binding.

Competition experiments were analyzed by the GraphPad Prism software (version 6 for Windows) to obtain the concentration of unlabelled drug that caused 50% inhibition of [^{3}H]**17** specific binding (IC_{50}). Apparent affinity constants (K_i) were derived from the IC_{50} values according to the Cheng and Prusoff equation [$K_i = IC_{50}/(1+L/K_d)$].[4]

BRET recording of 5-HT$_4$R-G protein coupling

The preparation of retroviral vectors coding for Rluc-tagged human 5-HT$_4$ receptor and RGFP-fused to Gβ_1, and the transduction of SH-5YSY human neuroblastoma cells were done using procedures described previously.[5] The 5-HT$_4$R-Rluc fusion protein cDNA was engineered by replacing the stop codon of the receptor with a sequence encoding a 13-mer linker peptide (RTEEQKLISEEDL) and cloning it in frame with the Rluc sequence into the retroviral expression vector pQIXN (Clontech). Cells were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and F-12, with 10% (v/v) fetal calf serum, 100 μg/mL hygromycin B and 400 μg/mL G418 in a humidified atmosphere of 5% CO_2 at 37 °C. Enriched membranes from transfected cells were obtained by differential centrifugation[5] and stored in aliquots at -80 °C before use. Receptor/Gβ_1 interactions were measured in membrane preparations (5 μg of proteins in a total volume of 100 μL phosphate-buffered saline) placed into white plastic 96-well plates (Packard Opti-plate); BRET recording and computations methods were described previously.[5] The ligand concentration yielding 50% inhibition of coupling (IC_{50}) were computed from concentration-inhibition curves obtained using 8 log-spaced concentrations of the ligands in the presence of 100 nM serotonin. The data were analyzed by nonlinear curve fitting with a general 4-parametrs logistic function.[5]

cAMP measurements

GloSensor-22F[6] expressing 2B2 cell lines were generated by infecting the cells with a self-inactivating viral construct prepared after subcloning into the retroviral expression vector pQCIXN (Clontech Laboratories, Inc) the biosensor sequence excised from the GloSensor 22F plasmid (Promega, USA). Virally transduced cells were cloned and selected for neomycin resistance; the best biosensor expressing clone was then chosen after screening for forskolin responsiveness of luminescence signals. The selected cell line was further transduced with viral constructs encoding Gαs and human 5-HT_4R as described above for the BRET assay. To measure cAMP responsiveness of the compounds, cells were seeded in 96-well white-opaque plates at a density of 25 x 10^3 cells/well 24 h before the experiment. Two hours before the assay, cells were washed once with PBS, and further incubated 105 minutes in PBS containing 25 mM glucose and 2 mM luciferin in a total volume of 60 µL. Assay was started by addition of varying concentrations (up to 10 µM) of ligands and 1 mM (final) IBMX in a total volume of 100 µL/well. Luminescence form each well was then counted every 30s with an integration time of 0.5s for up to 60 minutes using a plate luminometr (Victoe EnLight, Perkin Elmer). cAMP response was determined as peak luminescence, area under the 60 min luminescence-time curves, or the initial rate of luminescence increase. All three methods gave similar results.

References

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