Nile Red-Based GPCR Ligands as Ultrasensitive Probes of the Local Lipid Microenvironment of the Receptor

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Materials and Methods

Reagents were obtained from commercial sources and used without any further purification. Compounds **5**, **6a**, **6b**, **7a**, **7b**, **8a** and **8b** were synthesized following the previously published protocols.[1] Compound **6c** was synthesized according to Zhang, et al.[2] Thin-layer chromatography was performed on Merck silica gel 60F254 plates. VWR silica gel (40-63 µm) was used for chromatography columns. Semi-preparative reverse-phase HPLC purifications were performed on a Waters SunFire C18 OBD Prep column (5 µm, 19 × 150 mm) on a Gilson PLC2020 system. Reverse-phase flash purifications were performed on prepacked Puriflash C18 columns from Interchim on a Gilson PLC2020 system. Analytical reverse-phase HPLC were performed on an Ascentis Express C18 column (2.7 µm, 4.6 mm × 75 mm) on an Agilent Technologies 1200 series HPLC system using a linear gradient (5% to 100% v/v in 7.3 min, flow rate of 1.6 $mL.min^{-1}$) of solvent B (0.1% v/v TFA in ACN) in solvent A (0.1% v/v TFA in H_2O). ^1H NMR spectra were recorded at 400 MHz and 500 MHz and ^{13}C NMR spectra were recorded at 101 MHz and 126 MHz, on a Bruker Advance spectrometer. Chemical shifts are reported in parts per million (ppm), coupling constants (J) are reported in hertz (Hz). Signals are described as s (singlet), d (doublet), t (triplet), q (quadruplet), p (pentuplet) and m (multiplet). Low resolution mass spectra (LRMS) and high resolution mass spectra (HRMS) were obtained on an Agilent Technologie 6520 Accurare-Mass Q.Tof LC/MS apparatus equipped with a Zorbax SB C_{18} column (1.8 µm, 2.1 × 50 mm) using electrospray ionization (ESI) and a time-of-flight analyzer (TOF). Absorption and fluorescence measurement were performed in 114F-QS 10 mm quartz fluorescence cuvettes (Hellma Analytics). Absorption spectra were recorded on a Cary 4000-HP spectrophotometer (Varian) and fluorescence spectra on a FluoroMax 4 (Jobin Yvon, Horiba) spectrofluorometer equipped with a thermostated cuvette holder at 20 °C. The solvents used were either of spectroscopy or HPLC grade. Fluorescence confocal microscopy experiments were performed on a Leica TCS SPE-II microscope with an HXC PL APO 63x/1.40 OIL CS objective.

Chemical Synthesis

5-(diethylamino)-2-nitrosophenol (2). To a solution of **1** (1.00 g, 5.87 mmol, 1 eq.) in a mixture of water (6 mL) and concentrated HCl (6 mL, 70.5 mmol, 12 eq.) at iced water temperature was dropwise added a solution of $NaNO_2$ (418 mg, 5.87 mmol, 1 eq.) in water (4 mL) over a period of 30 min. The mixture was stirred at iced water temperature for 2 h and then concentrated under vacuum to afford a brown solid (1.14 g). This unstable product was directly used for the next step. HPLC t_R = 2.22 min (> 95% purity [220.8 nm]). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.31 (d, *J* = 9.9 Hz, 1H), 6.53 (dd, *J* = 9.9, 2.5 Hz, 1H), 5.66 (d, *J* = 2.6 Hz, 1H), 3.48 (q, *J* = 7.0 Hz, 4H), 1.26 (t, *J* = 7.2 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.36, 157.23, 149.68, 135.57, 113.69, 96.12, 46.07, 13.00.

9-(diethylamino)-2-hydroxy-5*H*-benzo[*a*]phenoxazin-5-one (3). A solution of naphthalene-1,6-diol (73.0 mg, 0.45 mmol, 1 eq.) in anhydrous DMF (4 mL) was added to a solution of **2** (175 mg, 0.90 mmol, 2 eq.) in anhydrous DMF (1 mL). The mixture was stirred at 110 °C for 45 min. DMF was removed under reduced pressure and the crude product was purified by reverse-phase flash chromatography using a linear gradient of 15-70% ACN (0.1%TFA) in H_2O (0.1% TFA) in 30 min to afford after lyophilization a dark purple solid (66.5 mg, 44%). HPLC t_R = 4.57 min (> 95% purity [220.8 nm]). ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.40 (s, 1H), 7.96 (d, *J* = 8.6 Hz, 1H), 7.87 (d, *J* = 2.5 Hz, 1H), 7.56 (d, *J* = 9.0 Hz, 1H), 7.08 (dd, *J* = 8.6, 2.5 Hz, 1H), 6.78 (dd, *J* = 9.1, 2.7 Hz, 1H), 6.62 (d, *J* = 2. 7 Hz, 1H), 6.14 (s, 1H), 3.48 (q, *J* = 7.1 Hz, 4H), 1.15 (t, *J* = 7.0 Hz, 6H). ^{13}C NMR (126 MHz, DMSO) δ 181.37, 159.78, 151.56, 150.71, 144.95, 138.59, 134.87, 132.01, 128.40, 125.16, 123.11, 118.34, 110.01, 107.47, 105.80, 95.33, 45.28, 12.98.

2-((9-(diethylamino)-5-oxo-5*H*-benzo[*a*]phenoxazin-2-yl)oxy)acetic acid (4). To a solution of **3** (537 mg, 1.60 mmol, 1 eq.) and K_2CO_3 (1.32 g, 9.58 mmol, 6 eq.) in acetone (16 mL) was added *tert*-butyl chloroacetate (0.7 mL, 4.79 mmol, 3 eq.). The mixture was stirred under reflux for 6.5 h. The solvent was evaporated under reduced pressure and the residue was dissolved in ethyl acetate (50 mL), washed with water and brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The obtained residue was dissolved in DCM (9 mL) and TFA (6 mL) was added. The mixture was stirred at 25 °C for 1.5 h. After evaporation of the volatiles under reduced pressure, ethyl acetate (50 mL) was added and the organic layer was washed with water and brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by reverse-phase flash chromatography using a linear gradient of 30-80% ACN (0.1% TFA) in H_2O (0.1% TFA) in 30 min to afford after lyophilization a dark purple solid (721 mg, 84%). HPLC t_R = 4.57 min (> 95% purity [220.8 nm]). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.01 (d, *J* = 8.7, 1H), 7.84 (d, *J* = 2.7 Hz, 1H), 7.53 (d, *J* = 9.1 Hz, 1H), 7.24 (dd, *J* = 8.7, 2.7 Hz, 1H), 6.79 - 6.72 (m, 1H), 6.57 (d, *J* = 2.6 Hz, 1H), 6.14 (s, 1H), 4.87 (s, 2H), 3.46 (m, 4H), 1.15 (t, *J* = 7.0 Hz, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 181.12, 169.74, 160.26, 151.68, 150.86, 146.42, 137.97, 133.40, 130.91, 127.19, 125.23, 124.00, 117.80, 110.14, 106.51, 103.98, 95.94, 64.74, 44.43, 12.43.

59-azido-3,6,9,12,15,18,21,24,27,30,33,36,39,42,45,48,51,54,57-nonadecaoxanonapentacontyl 4-methylbenzenesulfonate (6d). To a solution of N_3-$PEG_{(20)}$-OH (50.0 mg, 54.1 µmol, 1 eq.) in anhydrous DCM (1 mL) were added 4-methylbenzenesulfonyl chloride (21 mg, 108 µmol, 2 eq.) and KOH (18.2 mg, 325 µmol, 6 eq.). The mixture was stirred at iced water temperature for 1 h then at room temperature for 3 days. DCM (50 mL) was added and the organic layer was washed with water, brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by flash chromatography eluted with 5-10% v/v MeOH in DCM to afford a colorless oil (47.2 mg, 81%). HPLC t_R = 4.59 min (> 95% purity [220.8 nm]). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 (d, *J* =

7.0 Hz, 2H), 7.30 (d, *J* = 7.9 Hz, 2H), 4.11 (t, *J* = 4.4 Hz, 2H), 3.67 – 3.52 (m, 76H), 3.34 (t, *J* = 5.1 Hz, 2H), 2.41 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 144.79, 133.15, 129.85, 128.00, 70.77, 70.73, 70.70, 70.67, 70.60, 70.55, 70.05, 69.27, 68.71, 50.74, 21.65.

5-(3-(37-azido-2,5,8,11,14,17,20,23,26,29,32,35-dodecaoxahepta triacontyl)-5-(3-(2-chloro-4-fluorophenoxy)azetidin-1-yl)-4*H*-1,2,4-triazol-4-yl)-2-methoxypyridine (7c). To a solution of **5** (19.5 mg, 47.9 µmol, 1 eq.) in anhydrous DMF (3 mL) at iced water temperature was added KOH (19 mg, 302 µmol, 6.3 eq.). The solution was stirred for 5 min before a solution of **6c** (34.8 mg, 47.9 µmol, 1 eq.) in anhydrous DMF (3 mL) was added. The mixture was stirred at iced water temperature for 1.5 h and at 25 °C for 1 h. DMF was evaporated, to the residue DCM (70 mL) was added and the organic layer was washed with water, brine, dried over anhydrous Na_2SO_4, concentrated under vacuum and lyophilized to afford a yellow oil (42,5 mg, 92%). HPLC t_R = 4.56 min (> 95% purity [220.8 nm]). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.22 (d, *J* = 2.7 Hz, 1H), 7.67 (dd, *J* = 8.9, 2.7 Hz, 1H), 7.13 (dd, *J* = 8.0, 3.0 Hz, 1H), 6.86 (d, *J* = 8.7 Hz, 2H), 6.51 (dd, *J* = 9.0, 4.7 Hz, 1H), 4.90 (p, *J* = 5.7 Hz, 1H), 4.42 (s, 2H), 4.18 – 4.10 (m, 2H), 4.05 – 4.00 (m, 2H), 3.99 (s, 3H), 3.70 – 3.53 (m, 46H), 3.38 (t, *J* = 5.0 Hz, 2H).^{13}C NMR (101 MHz, $CDCl_3$) δ 164.42, 158.34, 149.74, 148.88, 145.56, 137.74, 123.58, 118.26, 118.00, 114.31, 114.09, 111.78, 70.72, 70.70, 70.66, 70.59, 70.52, 70.27, 70.05, 69.30, 68.86, 62,45, 59.46, 54.09, 50.74.

5-(3-(3-((λ^1-oxidaneyl)methyl)-59-azido-2,6,9,12,15,18,21,24,27, 30,33,36,39,42,45,48,51,54,57-nonadecaoxanonapentacontyl)-5-(3-(2-chloro-4-fluorophenoxy)azetidin-1-yl)-4*H*-1,2,4-triazol-4-yl)-2-methoxypyridine (7d). To a solution of **5** (10.0 mg, 24.6 µmol, 1 eq.) in anhydrous DMF (1.7 mL) at iced water temperature was added KOH (19 mg, 295 µmol, 14 eq.). The solution was stirred for 10 min before a solution of **6d** (26.6 mg, 24.6 µmol, 1 eq.) in anhydrous DMF (1.6 mL) was added. The mixture was stirred at iced water temperature for 30 min and at 25 °C for 2.5 h. DCM (70 mL) was added and the organic layer was washed with water, brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by semi-preparative HPLC using a linear gradient of 20-50% ACN (0.1% v/v TFA) in H_2O (0.1% v/v TFA) in 30 min to afford after lyophilization a colorless oil (23.2 mg, 72%). HPLC t_R = 4.67 min (> 95% purity [220.8 nm]). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.32 (s, 1H), 7.81 (d, *J* = 8.7 Hz, 1H), 7.12 (dd, *J* = 7.9, 2.7 Hz, 1H), 6.95 – 6.85 (m, 2H), 6.55 (dd, *J* = 9.2, 4.6 Hz, 1H), 4.99 (s, 1H), 4.49 (s, 2H), 4.40 (s, 2H), 4.13 (d, *J* = 7.4 Hz, 2H), 4.01 (s, 3H), 3.64 (s, 78H), 3.38 (t, *J* = 5.0 Hz, 2H). LRMS (ESI) calcd for $C_{58}H_{98}ClFN_8O_{22}$ $[M+2H]^{2+}/2$: 656.323, found: 656.283.

3-((λ^1-oxidaneyl)methyl)-1-(5-(3-(2-chloro-4-fluorophenoxy)azetidin-1-yl)-4-(6-methoxypyridin-3-yl)-4*H*-1,2,4-triazol-3-yl)-2,6,9,12,15,18,21, 24,27,30,33-undecaoxapentatriacontan-35-aminium 2,2,2-trifluoroacetate (8c). Polymer-bound triphenylphosphine (70.8 mg, 3 mmol/g, 8 eq.) was added to a solution of **7c** (25.5 mg, 26.6 µmol, 1 eq.) in anhydrous THF (1 mL). The mixture was stirred under reflux for 3.5 h, then water (400 µL) was added and the obtained mixture was stirred under reflux for another 1.5 h. The mixture was filtered and the filtrate was concentrated under reduced pressure and lyophilized. The crude product was purified by semi-preparative HPLC using a linear gradient of 20-45% ACN (0.1% v/v TFA) in H_2O (0.1% v/v TFA) in 30 min to afford after lyophilization a yellow oil (11.9 mg, 43%). HPLC t_R = 3.94 min (> 95% purity [280.8 nm]). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.27 (d, *J* = 2.7 Hz, 1H), 7.77 (dd, *J* = 8.9, 2.7 Hz, 1H), 7.68 (s, 2H), 7.12 (dd, *J* = 8.0, 3.1 Hz, 1H), 6.90 (d, *J* = 8.7 Hz, 2H), 6.53 (dd, *J* = 9.1, 4.7 Hz, 1H), 5.02 – 4.89 (m, 1H), 4.43 (s, 2H), 4.39 – 4.31 (m, 2H), 4.08 (dd, *J* = 8.7, 3.6 Hz, 2H), 4.00 (s, 3H), 3.80 (t, *J* = 4.9 Hz, 2H), 3.71 – 3.56 (m, 44H), 3.23 – 3.12 (m, 2H).

3-((λ^1-oxidaneyl)methyl)-1-(5-(3-(2-chloro-4-fluorophenoxy)azetidin-1-yl)-4-(6-methoxypyridin-3-yl)-4*H*-1,2,4-triazol-3-yl)-2,6,9,12,15,18,21, 24,27,30,33,36,39,42,45,48,51,54,57-nonadecaoxanonapentacontan-59-aminium 2,2,2-trifluoroacetate (8d). Polymer-bound triphenylphosphine (33.8 mg, 3 mmol/g, 10 eq.) was added to a solution of **7d** (13.3 mg, 10.1 µmol, 1 eq.) in anhydrous THF (500 µL). The mixture was refluxed for 5 h, then water (200 µL) was added and the obtained mixture was stirred under reflux for another 2 h. The mixture was filtered and the filtrate was concentrated under reduced pressure and lyophilized. The crude product was purified by semi-preparative HPLC using a linear gradient of 20-45% ACN (0.1% v/v TFA) in H_2O (0.1% v/v TFA) in 30 min to afford after lyophilization a colorless oil (5.8 mg, 44%). HPLC t_R = 4.32 min (> 95% purity [220.8 nm]). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.20 (d, *J* = 2.7 Hz, 1H), 7.65 (dd, *J* = 8.8, 2.7 Hz, 1H), 7.10 (dd, *J* = 8.0, 3.0 Hz, 1H), 6.89 – 6.77 (m, 2H), 6.49 (dd, *J* = 9.0, 4.7 Hz, 1H), 4.88 (p, *J* = 6.2, 5.6 Hz, 1H), 4.39 (s, 2H), 4.17 – 4.05 (m, 2H), 4.04 – 3.97 (m, 2H), 3.97 (s, 3H), 3.66 – 3.58 (m, 78H), 2.99 (t, *J* = 5.0 Hz, 2H). LRMS (ESI) calcd for $C_{58}H_{100}ClFN_6O_{22}$ $[M+2H]^{2+}/2$: 643.328, found: 643.764.

Pf-PEG$_4$-NR. To a solution of **4** (3.6 mg, 9.17 µmol, 1 eq.) in anhydrous DMF (300 µL) were added a solution of **8a** (7.0 mg, 10.1 µmol, 1.1 eq.) in anhydrous DMF (300 µL), a solution of PyBOP (13.6 mg, 26.3 µmol, 2.6 eq.) in anhydrous DMF (300 µL) and DIPEA (13.3 µL, 80.6 µmol, 8 eq.). The mixture was stirred at 25 °C for 1 h and the product was then isolated by semi-preparative HPLC using a linear gradient of 20-60% ACN (0.1% v/v TFA) in H_2O (0.1% v/v TFA) in 30 min to afford after lyophilization a dark purple solid (6.8 mg, 78 %). HPLC t_R = 5.37 min (> 95% purity [220.8 nm]). HRMS (ESI) calcd for $C_{48}H_{54}ClFN_8O_{10}$ $[M+2H]^{2+}/2$: 478.1818; found: 478.1824.

Pf-PEG$_8$-NR. To a solution of **4** (0.63 mg, 1.61 µmol, 1 eq.) in anhydrous DMF (65 µL) were added a solution of **8b** (1.40 mg, 1.61 µmol, 1 eq.) in anhydrous DMF (65 µL), a solution of PyBOP (2.09 mg, 4.02 µmol, 2.5 eq.) in anhydrous DMF (65 µL) and DIPEA (2.13 µL, 12.9 µmol, 8 eq.). The mixture was stirred at 25 °C for 30 min and then were added PyBOP (2.09 mg, 4.02 µmol, 2.5 eq.) and DIPEA (2.13 µL, 12.9 µmol, 8 eq.). The mixture was stirred at 25 °C for 2 h and the product was then isolated by semi-preparative HPLC using a linear gradient of 20-60% ACN (0.1% v/v TFA) in H_2O (0.1% v/v TFA) in 30 min to afford after lyophilization a black purple solid (1.3 mg, 71%). HPLC t_R = 5.39 min (> 95% purity [220.8 nm]). HRMS (ESI) calcd for $C_{56}H_{70}ClFN_8O_{14}$ $[M+2H]^{2+}/2$: 566.2342, found: 566.2341.

Pf-PEG$_{12}$-NR. To a solution of **8c** (3.40 mg, 3.25 µmol, 1.1 eq.) in anhydrous DMF (290 µL) were added **4** (1.16 mg, 2.96 µmol, 1 eq.), PyBOP (4.38 mg, 8.42 µmol, 2.6 eq.) and DIPEA (4.29 µL, 26.0 µmol, 8 eq.). The mixture was stirred at 25 °C for 30 min and the product was then isolated by semi-preparative HPLC using a linear gradient of 20-55% ACN (0.1% v/v TFA) in H_2O (0.1% v/v TFA) in 30 min to afford after lyophilization a dark purple solid (1.50 mg, 39%). HPLC t_R = 5.37 min (> 95% purity [220.8 nm]). HRMS (ESI) calcd for $C_{64}H_{86}ClFN_8O_{18}$ $[M+2H]^{2+}/2$: 654.2866, found: 654.2871.

Pf-PEG$_{20}$-NR. To a solution of **8d** (2.70 mg, 1.93 µmol, 1 eq.) in anhydrous DMF (172 µL) were added **4** (1.51 mg, 3.86 µmol, 2 eq.), PyBOP (7.53 mg, 14.5 µmol, 7 eq.) and DIPEA (10.2 µL, 61.7 µmol, 32 eq.). The mixture was stirred at 25 °C for 25 h and the product was then isolated by semi-preparative HPLC using a linear gradient of 20-60% ACN (0.1% v/v TFA) in H_2O (0.1% v/v TFA) to afford after lyophilization a dark purple solid (1.60 mg, 50%). HPLC t_R = 5.58 min (> 95% purity [220.8 nm]). HRMS (ESI) calcd for $C_{80}H_{119}ClFN_8O_{26}$ $[M+3H]^{3+}/3$: 554.2583, found: 554.2582.

Absorption and Steady-State Fluorescence Measurements

General information. The excitation wavelength was 520 nm and the recorded emission spectral range was 530 – 800 nm. Unless specified, all fluorescence spectra were corrected for instrumental factors. The absorbance of sample solutions at the excitation wavelength were kept below 0.05 to avoid inner filter effects. Data treatment was performed using OriginPro 8.6.

Determination of fluorescence quantum yields. Determination of relative fluorescence quantum yields (QY) was performed using Nile Red in 1,4-dioxane as a reference (QY = 70%).[3]

Preparation of 1 mM DOPC/cholesterol (2:1) liposomes. To a solution of DOPC (667 µL, 5 mM) in $CHCl_3$ was added a solution of cholesterol (333 µL, 5 mM) in $CHCl_3$ and the solvent was slowly evaporated under reduced pressure. The obtained film was rehydrated with PBS (5 mL, pH 7.1 - 7.5), vortexed for 5 min and kept at 25 °C for 30 min. The suspension of multilamellar vesicles was extruded using a Lipex Biomembranes extruder with a 0.2 µm filter for 7 passages and then with 0.1 µm filter for 10 passages. The generated monodisperse LUVs were characterized by a mean diameter of 0.15 µm as measured with a Malvern Zetamaster 300 particle size analyser.

Preparation of 1 mM DOPC liposomes. A solution of DOPC (1 mL, 5 mM) in $CHCl_3$ was slowly concentrated under reduced pressure. The obtained film was rehydrated with PBS (5 mL, pH 7.1 - 7.5), vortexed for 5 min and kept at 25 °C for 30 min. The suspension of multilamellar vesicles was extruded using a Lipex Biomembranes extruder with a 0.2 µm filter for 7 passages and then with 0.1 µm filter for 10 passages. The generated monodisperse LUVs were characterized by a mean diameter of 0.13 µm as measured with a Malvern Zetamaster 300 particle size analyser.

Evaluation of non-specific interactions with liposomes. The 1 µM solutions of the studied probes in PBS with increasing amounts of DOPC/cholesterol (2:1) liposomes were prepared as follows. 5 µL of 0.2 mM stock DMSO solution of a studied probe were added accordingly to 995 µL, 993 µL, 991 µL, 985 µL, 975 µL, 945 µL, 895 µL and 795 µL of PBS. Then 0 µL, 2 µL, 4 µL, 10 µL, 20 µL, 50 µL, 100 µL and 200 µL of 1 mM DOPC/cholesterol (2:1) liposomes were respectively added and the solutions were carefully mixed.

Evaluation of non-specific interactions with BSA. The titrations of the studied probes with BSA were performed as follows. 1 µM solution of the studied probes in PBS were prepared by diluting 5 µL of 0.2 mM DMSO stock solutions in 995 µL of PBS. Then successively 5 µL, 5 µL, 15 µL, 25 µL, 60 µL of 0.2 mM stock solution of BSA in PBS were added and the solutions were carefully mixed.

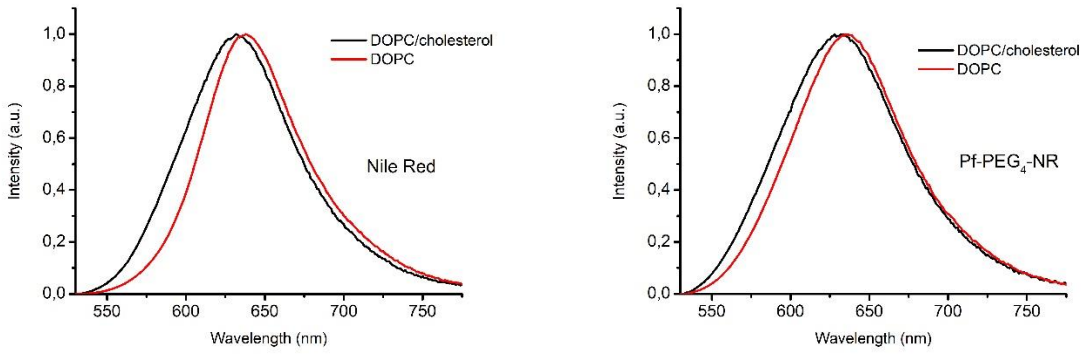

Figure S1. Fluorescence spectra of Nile Red and Pf-PEG_4-NR (1 µM) in the presence of DOPC and DOPC/cholesterol (2:1) liposomes at 200 µM lipid concentration.

Functional Characterization of the OTR ligands

Antagonist properties of the OTR ligands were determined by measuring their capacities to inhibit oxytocin-induced intracellular Ca^{2+} release (according to Weill et al[4] with modifications). HEK293 cells stably overexpressing the OTR were first incubated with 5 µM of Indo-1 AM in HEPES buffer (10 mM HEPES, 137.5 mM NaCl, 1.25 mM $MgCl_2$, 1.25 mM $CaCl_2$, 6 mM KCl, 0.4 mM NaH_2PO_4 and 5.6 mM glucose, pH 7.4) supplemented with 0.1% BSA for 45 minutes at 37 °C and 5% CO_2. The cells were then washed, resuspended in HEPES buffer supplemented with 0.1% BSA buffer, seeded into 384 well polystyrene plates (Greiner 781091) at 50000 cells/well and then centrifuged for 5 min at 800 rpm. Addition of compounds was performed using the FlexStation® 3 automated pipettor. Compounds were tested at concentrations from 0.3 pM to 3 µM in the presence of 10 nM of oxytocin. Intracellular Ca^{2+} release measurements were performed by monitoring the fluorescence of Indo-1 using a FlexStation® 3 microplate reader (Molecular Devices Corp.). Excitation was set at 338 nm, emissions were recorded at 401 nm and 475 nm at 25 °C. Fluorescence emission ratio 401 nm/ 475 nm was calculated in order to follow the calcium release. In order to assess the maximum calcium level, addition of digitonin (Sigma Aldrich) at 100 µM was performed 110 s after the addition of the compounds. Dose-response curves were obtained by plotting the normalized signal of calcium release against the logarithmic values of compound concentration. The half maximal inhibitory concentration (IC_{50}) were calculated using GraphPad Prism 6. Data resulted from two independent experiments performed in duplicate. Values are means ± SEM.

Table S1. Functional activities of the NR conjugates

Compound	IC_{50}, nM
Pf-PEG_4-NR	132 ± 48
Pf-PEG_8-NR	92 ± 20
Pf-PEG_{12}-NR	83 ± 9
Pf-PEG_{20}-NR	30 ± 3

[a] Results are expressed as mean ± SD of two independent separate experiments performed in duplicate.

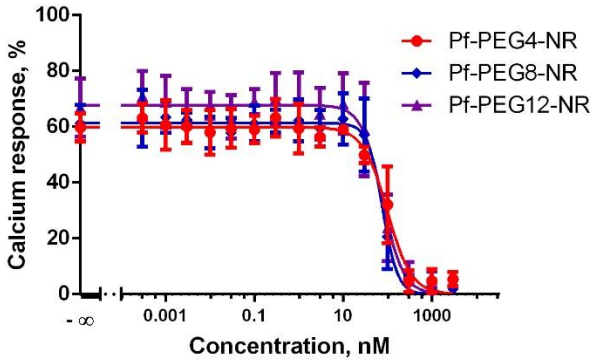

Figure S2. Representative dose-response curves for OTR antagonists Pf-PEG_4-NR, Pf-PEG_8-NR and Pf-PEG_{12}-NR. Values are means ± SEM.

Cytotoxicity Assay (WST-1)

Cell cytotoxicity was performed according to Houel et al[5] with modifications. HEK293 cells were cultured in Eagle's minimal essential medium (MEM, Invitrogen 21090) with 10% FBS, 100 U mL^{-1} of penicillin, 100 µg mL^{-1} of streptomycin, 2 mM of glutamine at 37 °C in a humidified 5% CO_2 atmosphere. Cells were seeded in a 96-well plate at 2.5×10^4 cells per well and incubated for 20 hours at 37 °C in a humidified 5% CO_2 atmosphere. Pf-PEG_{20}-NR was then added to the wells at a final concentration of 10 nM, 100 nM or 1 µM (0.1% DMSO) and a final volume of 200 µL/well. Chlorpromazine at 50 µM (0.1% DMSO) was used as a positive control. Cells were then incubated at 37 °C in a humidified 5% CO_2 atmosphere for 24 hours. The WST-1 viability assay was performed after 1 hour and 24 hours of incubation as follows. 100 µL of cell medium was replaced by 100 µL of WST-1 2x reagent, the cells were incubated for 30 min at 37 °C and 5% CO_2 and then the absorbance at 450 nm was measured. Viability of the treated cells was expressed as a percentage of the untreated control (0.1% DMSO).

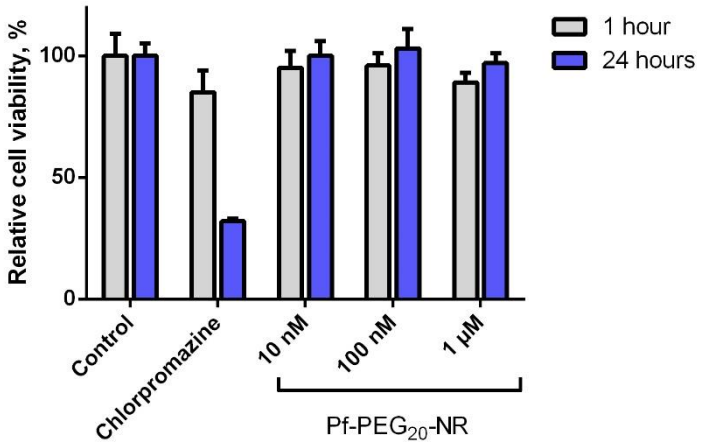

Figure S3. Cytotoxicity of Pf-PEG_{20}-NR measured using the WST-1 cell viability assay. Viability of HEK293 cells (%) relative to the control (0.1% of DMSO) is shown; values are means ± SD.

LC-HRMS spectra

Qualitative Analysis Report

Data Filename	QT13618.d	**Sample Name**	FH42
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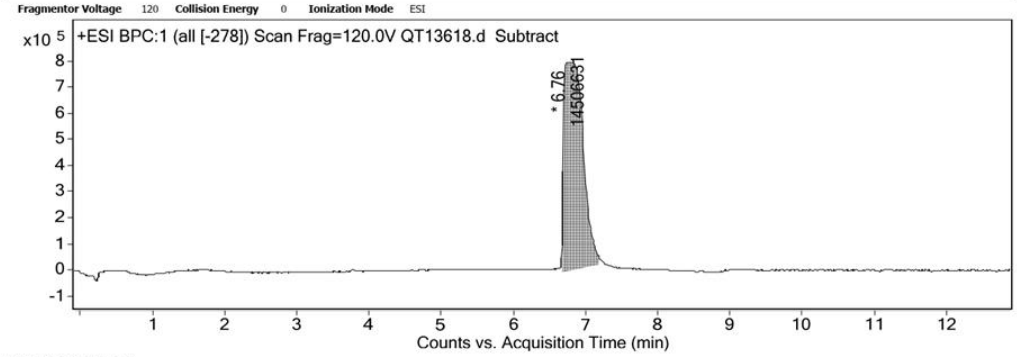
Instrument Name	SCA Illkirch QToF	**User Name**	
Acq Method	C18-2,1x5x1,8-126+.m	**Acquired Time**	3/29/2017 4:18:18 PM
IRM Calibration Status	Success	**DA Method**	C18-2,1x5x1,8.m
Comment			

Pf-PEG4-NR

Sample Group **Info.**

User Chromatograms

Fragmentor Voltage 120 **Collision Energy** 0 **Ionization Mode** ESI

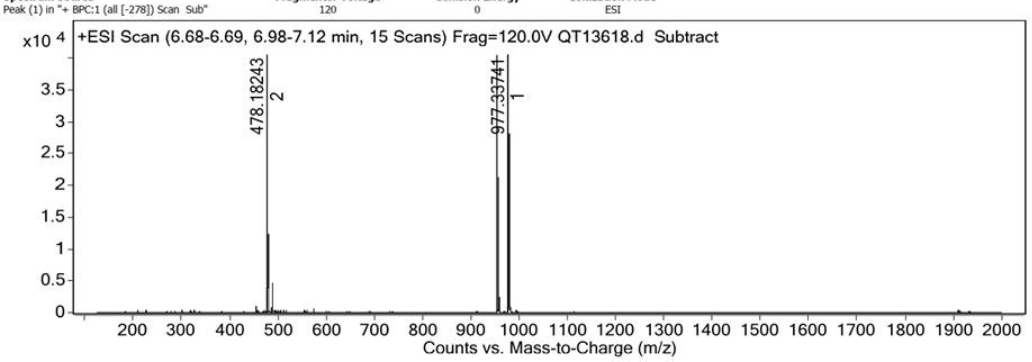


Integration Peak List

Start	RT	End	Height	Area	Area %	AreaSum%
6.68	6.76	7.19	799458	14506631	100	100

Qualitative Analysis Report

Spectrum Source Peak (1) in "+ BPC:1 (all [-278]) Scan Sub" **Fragmentor Voltage** 120 **Collision Energy** 0 **Ionization Mode** ESI



Qualitative Analysis Report

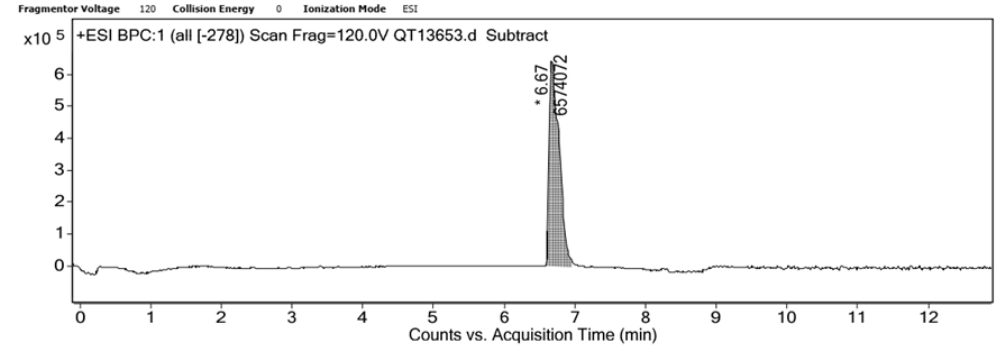
Data Filename	QT13653.d	**Sample Name**	FH48-6
Inj. Vol.	0.3	**Position**	P1-E5
Instrument Name	SCA Illkirch QToF	**User Name**	
Acq Method	C18-2,1x5x1,8-126+.m	**Acquired Time**	4/6/2017 10:59:00 AM
IRM Calibration Status	Success	**DA Method**	C18-2,1x5x1,8.m
Comment			

Pf-PEG8-NR

Sample Group **Info.**

User Chromatograms

Fragmentor Voltage 120 **Collision Energy** 0 **Ionization Mode** ESI

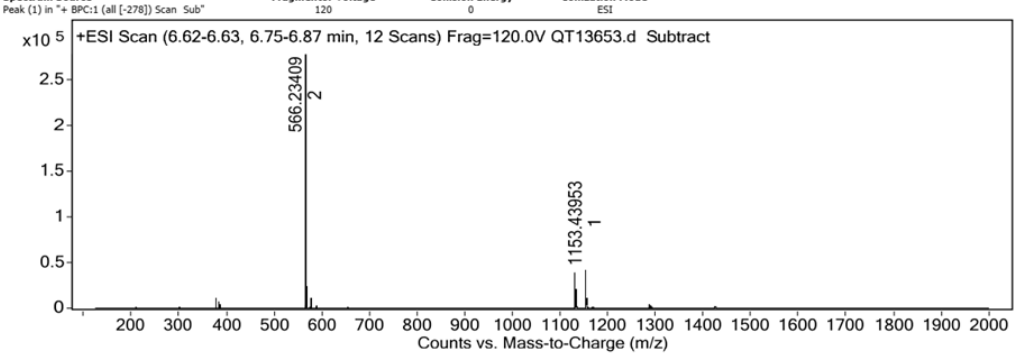


Integration Peak List

Start	RT	End	Height	Area	Area %	AreaSum%
6.57	6.67	6.96	643001	6574072	100	100

Qualitative Analysis Report

Spectrum Source	**Fragmentor Voltage**	**Collision Energy**	**Ionization Mode**
Peak (1) in "+ BPC:1 (all [-278]) Scan Sub"	120	0	ESI



Qualitative Analysis Report

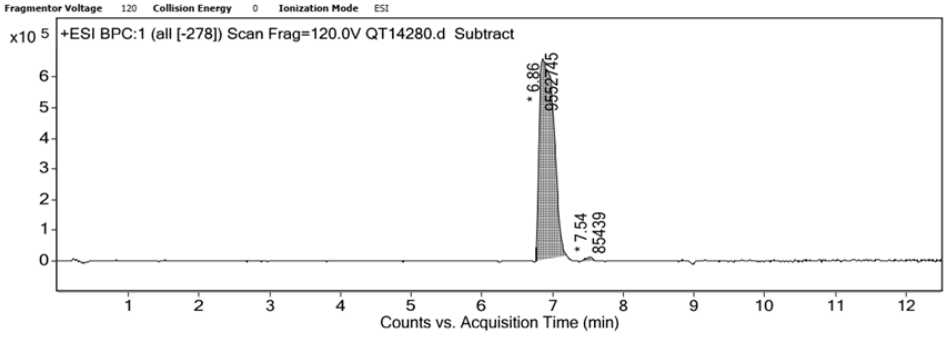
Pf-PEG12-NR

Data Filename	QT14280.d	**Sample Name**	FH74-6
Inj. Vol.	0.8	**Position**	P1-F9
Instrument Name	SCA Illkirch QToF	**User Name**	
Acq Method	C18-2,1x5x1,8-126+.m	**Acquired Time**	9/22/2017 2:07:46 PM
IRM Calibration Status	Success	**DA Method**	C18-2,1x5x1,8.m
Comment			

Sample Group **Info.**

User Chromatograms

Fragmentor Voltage 120 **Collision Energy** 0 **Ionization Mode** ESI



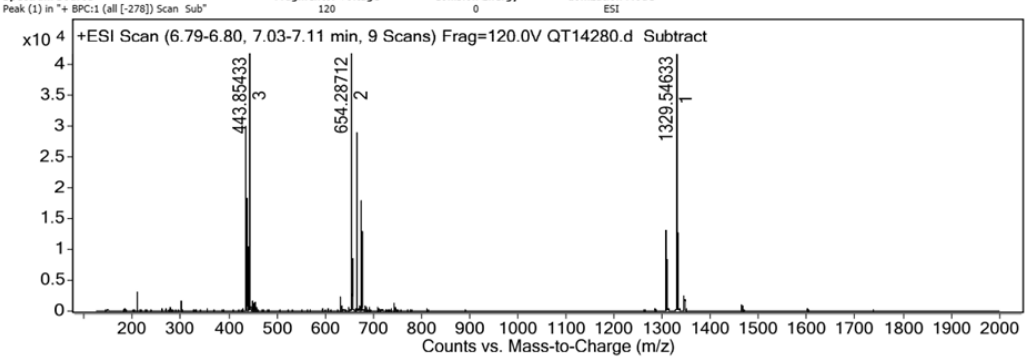
Integration Peak List

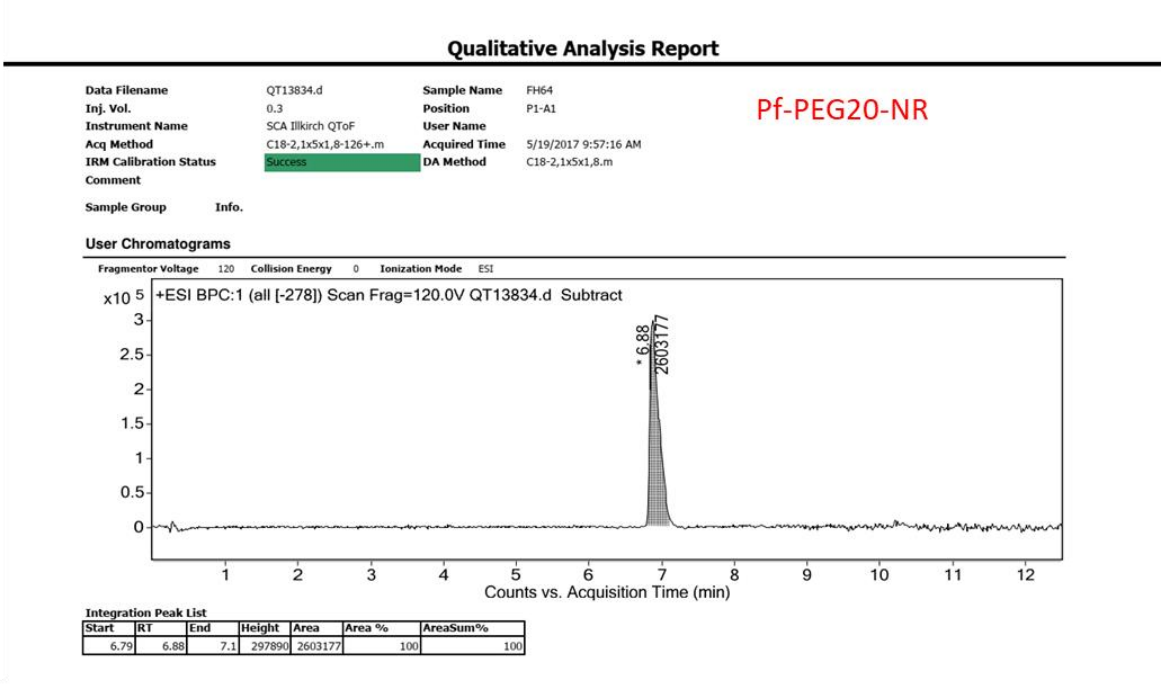
Start	RT	End	Height	Area	Area %	AreaSum%
6.72	6.86	7.2	656167	9552745	100	99.11
7.37	7.54	7.59	12796	85439	0.89	0.89

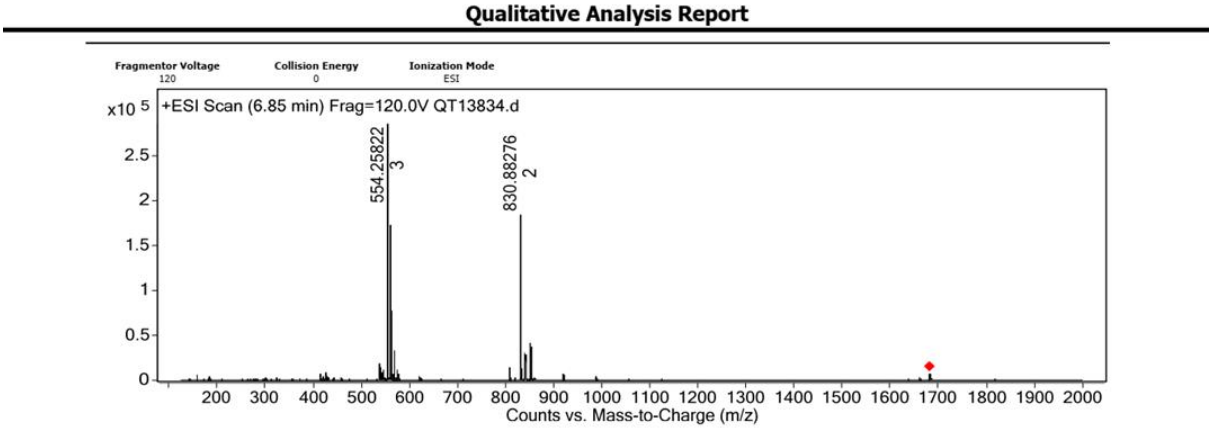
Qualitative Analysis Report

Spectrum Source Peak (1) in "+ BPC:1 (all [-278]) Scan Sub"

Fragmentor Voltage 120 **Collision Energy** 0 **Ionization Mode** ESI







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