Supporting information

Squaraine as bright, stable and environment-sensitive far-red label for receptor-specific cellular imaging

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1. Chemical Synthesis

1.1. General Information

Reagents were obtained from commercial sources and used without any further purification. Thin-layer chromatography was performed on silica gel 60F_{254} plates. Merck silica gel (Kieselgel 60; 230–400 mesh) was used for chromatography columns. Flash chromatography was performed on RP18 (25–40 μm, Merck) prepacked columns on a SPOT II ultima from Armen. Semi-preparative HPLC chromatography was performed on SunFire C18 column (5 μm, 19 × 150 mm) on Gilson PLC2020. Analytical RP-HPLC separations were performed on Ascentis Express C18 column (2.7 μm, 4.6 mm × 75 mm) using a linear gradient (5% to 100% in 7.4 min, flow rate of 1.6 mL/min) of solvent B (0.1% TFA in CH₃CN, v/v) in solvent A (0.1% TFA in H₂O, v/v). Detection was set at 220 and 254 nm. NMR spectra were recorded at 400 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), m (multiplet), and br (broad singlet). LC-MS spectra were obtained on a ZQ (Z quadripole) Waters/Micromass spectrometer equipped with an X-Terra C18 column (3.5 μm, 4.6 × 50 mm) using electrospray ionization mode (ESI). High resolution mass spectra (HRMS) were acquired on a Bruker MicroTof mass spectrometer, using electrospray ionization (ESI) and a time-of-flight analyzer (TOF).

1.2. Synthetic Procedures

Scheme S1. Synthesis of SQ-COOH.
**4-((1-(4-Ethoxy-4-oxobutyl)-3,3-dimethyl-3H-indol-1-ium-2-yl)methylene)-2-((1-ethyl-3,3-dimethylindolin-2-ylidene)methyl)-3-oxocyclobut-1-en-1-olate (5).**

To a refluxed solution of 3\(^1\) (1 equiv., 2.83 mmol, 881 mg) in ethanol (10 mL) a water solution of NaOH (1 mL of 40%) was added and the reaction mixture was heated for 5 min. The resulted mixture was cooled down, concentrated under vacuum and 5 mL of cold ethanol was added. The yellow precipitated was washed three times with cold ethanol and dissolved in a mixture of n-butanol (25 mL) and toluene (25 mL). To the solution 4\(^2\) (1 equiv., 2.83 mmol, 1004 mg) was added and the reaction mixture was refluxed for 18 hours in a flask equipped with a Dean-Stark apparatus.

The solvents were removed under vacuum. The residue was purified by column chromatography on silica gel (eluted first with CH\(_2\)Cl\(_2\)/EtOAc 25/75, then with CH\(_2\)Cl\(_2\)/MeOH 98/2) to obtain 5 as blue fluffy solid (1.30 g). Mass spectra analysis revealed that a mixture of ethyl and butyl esters was obtained respectively in a ~4:1 ratio. Ethyl ester: MS (ESI), calcd for C\(_{35}\)H\(_{38}\)N\(_2\)O\(_8\)\(^+\) [M+H]\(^+\) 539.29, found 539.29. Butyl ester: MS (ESI), calcd for C\(_{36}\)H\(_{43}\)N\(_2\)O\(_4\)\(^+\) [M+H]\(^+\) 567.32, found 567.32.

**4-((1-(3-Carboxypropyl)-3,3-dimethyl-3H-indol-1-ium-2-yl)methylene)-2-((1-ethyl-3,3-dimethylindolin-2-ylidene)methyl)-3-oxocyclobut-1-en-1-olate (SQ-COOH).**

5 (1 equiv., 0.706 mmol, 384 mg) was dissolved in an aqueous solution of HBr (48%, 5 mL) and the reaction mixture was allowed to stir at 60°C overnight. The solution was cooled down to room temperature and water was added. The product was extracted three times with EtOAc. The combined organic fractions were washed with brine, dried over MgSO\(_4\) and concentrated under vacuum. The residue was purified by semi-preparative HPLC eluted with solvent B in solvent A (from 30% to 100% in 30 min) to afford SQ-COOH as a blue foam (219 mg, 61%). \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 7.38 – 7.28 (m, 4H), 7.19 – 7.11 (m, 3H), 7.03 – 6.97 (m, 1H), 6.04 (s, 1H), 5.94 (s, 1H), 4.20 – 4.01 (m, 4H), 2.64 (t, \(J = 6.9\) Hz, 2H), 2.22 – 2.09 (m, 2H), 1.77 (br, 12H), 1.43 – 1.36 (m, 4H). \(^13\)C NMR (101 MHz, CDCl\(_3\)): \(\delta\) 177.01, 174.81, 170.43, 142.32, 142.02, 128.14, 128.00, 124.12, 122.50, 122.36, 109.99, 109.46, 90.91, 86.33, 70.24, 67.23, 66.29, 62.43, 49.56, 42.94, 38.68, 31.44, 27.15, 27.04, 22.48, 12.19. HRMS (ESI), calcd for C\(_{32}\)H\(_{35}\)N\(_2\)O\(_4\)\(^+\) [M+H]\(^+\) 511.2597, found 511.2593.
**Scheme S2.** Synthesis of 1.

*N,N-bis(2-ethylhexyl)aniline 7.*

A mixture of aniline (1 equiv., 21.9 mmol, 2 mL), 2-ethylhexyl iodide (2.5 equiv., 54.9 mmol, 9.85 mL) and potassium carbonate (3 equiv., 65.8 mmol, 9.1 g) in dry DMF (50 mL) was stirred upon heating at 90°C for 4 hours. After cooling to room temperature the excess of potassium carbonate was filtered off and the volatiles were removed under vacuum. To the residue water (100 mL) was added and the mixture was extracted three times with CH$_2$Cl$_2$. The organic layer was dried over sodium sulfate and concentrated under vacuum. The residue was purified by column chromatography on silica gel (eluted with heptane) to give 7 as a colorless liquid (4.68 g, 67%). $^1$H NMR (400 MHz, CDCl$_3$): δ 7.24 – 7.13 (m, 2H), 6.66 (d, $J$ = 8.0 Hz, 2H), 6.61 (m, 1H), 3.29 – 3.09 (m, 4H), 1.85 – 1.71 (m, 2H), 1.45 – 1.15 (m, 16H), 0.96 – 0.82 (m, 12H). $^{13}$C NMR (101 MHz, CDCl$_3$): δ 148.58, 129.04, 115.31, 113.02, 56.58, 36.87, 30.88, 28.88, 24.12, 23.38, 14.22, 10.82. HRMS (ESI), calcd for C$_{22}$H$_{40}$N$^+$ [M+H]$^+$ 318.3161, found 318.3159.

2-(4-(bis(2-ethylhexyl)amino)phenyl)-4-(4-(bis(2-ethylhexyl)iminio)cyclohexa-2,5-dien-1-ylidene)-3-oxocyclobut-1-en-1-olate 1.

A solution of 7 (2 equiv., 3.16 mmol, 1002 mg) and squaric acid (1 equiv., 1.58 mmol, 180 mg) in a mixture of 1-butanol (20 mL) and toluene (20 mL) was refluxed overnight. After cooling to room temperature the solvents were removed under vacuum. The residue was purified by column chromatography on silica gel (eluted with EtOAc/heptane 2/8) to give 1 as a dark solid (957 mg, 85%). $^1$H NMR (400 MHz, CDCl$_3$): δ 8.35 (d, $J$ = 9.3 Hz, 4H), 6.75 (d, $J$ = 9.3 Hz, 4H), 3.53 – 3.11 (m, 8H), 1.92 – 1.77 (m, 4H), 1.43 – 1.11 (m, 32H), 1.08 – 0.62 (m, 24H). $^{13}$C NMR (101 MHz, CDCl$_3$): δ 187.49, 183.63, 153.74, 133.02, 119.61, 113.23, 56.66, 37.72, 30.69, 28.72, 24.04, 23.16, 14.12, 10.77. HRMS (ESI), calcd for C$_{48}$H$_{77}$N$_2$O$_2$$^+$ [M+H]$^+$ 713.5985, found 713.5957.
Scheme S3. Synthesis of SQ-PEG-CBT.


To a stirred solution of SQ-COOH (1 equiv., 0.0333 mmol, 17 mg,) and 8 (1.5 equiv., 0.0499 mmol, 23.4 mg) in dry DMF (1 mL) PyBOP (1.5 equiv., 0.0499 mmol, 26 mg) and DIEA (8 equiv., 0.266 mmol, 34.4 mg, 44 μL) were added. The reaction mixture was stirred at room temperature overnight. The resulting mixture was purified by reverse phase flash chromatography eluted with solvent B in solvent A (from 30% to 100% in 30 min). The volatiles were removed under vacuum and the residue was dissolved in CH₂Cl₂ (1 mL) and treated with TFA (1 mL) for 30 min. The volatiles were removed under vacuum and the residue was lyophilized from ACN/H₂O mixture to give 9 as a dark taffy (15 mg, 46%). ¹H-NMR (500 MHz, DMSO-d₆) δ 7.97 (t, J = 5.4 Hz, 1H), 7.82 (br, 3H), 7.56 – 7.45 (m, 2H), 7.39 – 7.27 (m, 4H), 7.20 – 7.11 (m, 2H), 5.84 – 5.77 (m, 2H), 4.20 – 3.99 (m, 4H), 3.61 – 3.57 (m, 2H), 3.57 – 3.52 (m, 4H), 3.51 – 3.46 (m, 20H), 3.43 – 3.36 (m, 2H), 3.26 – 3.15 (m, 2H), 3.00 – 2.93 (m, 2H), 2.25 (t, J = 7.1 Hz, 2H), 1.99 – 1.87 (m, 2H), 1.73 – 1.62 (m, 12H), 1.32 – 1.19 (m, 3H). ¹³C-NMR (126 MHz, DMSO-d₆)
δ 180.7, 178.73, 178.45, 171.3, 169.01, 168.78, 142.23, 141.69, 141.58, 141.43, 128.01, 127.95, 123.74, 123.64, 122.27, 122.22, 110.24, 110.12, 86.04, 85.75, 69.75, 69.66, 69.62, 69.55, 69.07, 66.7, 54.9, 48.7, 42.5, 38.6, 38.5, 38.0, 31.8, 26.53, 26.41, 22.5, 11.7. BRMS (ESI): (M+H)+ calcd: 861.5, found: 861.5.


To a solution of 9 (1 equiv., 0.0154 mmol, 15 mg) in dry MeOH (1 mL) succinic anhydride (5 equiv., 0.0769 mmol, 7.7 mg) was added followed by DIEA (10 equiv., 0.154 mmol, 19.9 mg, 25.4 µL). The reaction mixture was stirred at room temperature for 1 hour. MeOH was evaporated under vacuum and the residue was purified by semi-preparative HPLC eluted with solvent B in solvent A (from 30% to 100% in 30 min) to give 2 as a dark taffy (10.4 mg, 70%).

1H NMR (500 MHz, DMSO-d₆) δ 8.01–7.93 (m, 1H), 7.92–7.84 (m, 1H), 7.57–7.47 (m, 2H), 7.40–7.28 (m, 4H), 7.22–7.12 (m, 2H), 5.87–5.74 (m, 2H), 4.23–3.99 (m, 4H), 3.63–3.27 (m, 28H), 3.25–3.12 (m, 4H), 2.40 (t, J = 6.8 Hz, 2H), 2.30 (t, J = 6.8 Hz, 2H), 2.28–2.20 (m, 2H), 1.98–1.86 (m, 2H), 1.68 (s, 12H), 1.28 (t, J = 7.1 Hz, 3H). 13C NMR (126 MHz, DMSO-d₆) δ 180.6, 178.84, 178.59, 173.8, 171.21, 171.00, 169.01, 168.72, 142.27, 141.68, 141.59, 141.42, 127.99, 127.95, 123.71, 123.62, 122.28, 122.22, 110.23, 110.10, 86.02, 85.73, 69.76, 69.58, 69.54, 69.10, 69.08, 48.7, 42.5, 38.57, 38.54, 37.97, 31.8, 29.91, 29.12, 26.53, 26.41, 22.5, 11.7. HRMS (ESI): calcd for C₅₂H₇₄N₄O₁₃ (M+2H)⁺/2 481.2626, found: 481.2623.

SQ-PEG-CBT

To a solution of 2 (1.1 equiv., 0.01 mmol, 10 mg) and Lys⁸-CBT³ (1 equiv., 0.0948 mmol, 10.5 mg) in dry DMF (1 mL) a solution of PyBOP (2.7 equiv., 0.026 mmol, 13.5 mg) in dry DMF (1 mL) was added followed by DIEA (9 equiv., 0.083 mmol, 13.7 µL). The reaction mixture was stirred at room temperature for 40 min. The resulting mixture was purified by semi-preparative HPLC eluted with solvent B in solvent A (5 min at 20% then 5-35 min from 20 to 50%) to give SQ-PEG-CBT as a dark taffy (5.3 mg, 30%). HRMS (ESI): calcd for C₉₇H₁₄₂N₁₆O₂₄S (M+2H)⁺/2 973.50518, found: 973.50242.
2. **Cell Lines, Culture Conditions, and Treatment**

HEK293 cells expressing the wild-type (wtOTR) or the GFP-coupled oxytocin receptor (GFP-OTR) were cultured in Eagle’s minimal essential medium (MEM, Invitrogen 21090) with 10% heat-inactivated fetal bovine serum, 100 U/mL of penicillin, 100 µg/mL of streptomycin, 2 mM of glutamine and 500 µg/mL of G418 for wtOTR cells or 50 µg/mL of hygromycin B for GFP-OTR cells at 37 °C in a humidified 5% CO₂ atmosphere. 70-80% cell confluence was maintained by removal of a portion of the culture and replacement with fresh medium twice a week.

For fluorescence spectroscopy experiments (Table S2), wtOTR cells were detached by treatment with a trypsin solution (Aldrich). The solution of detached cells was diluted with MEM, transferred to Falcon tubes and centrifuged at 900 rpm for 5 min. Cells were then washed twice with PBS. Finally, cells were suspended in Leibovitz’s L-15 medium (no phenol red) at 10⁶ cells/mL.

For microscopy studies, wtOTR or GFP-OTR cells were seeded onto a chambered cover glass (IBiDi) at a density of 5×10⁴ cells/IBiDi. Cells were washed two times by gentle rinsing with PBS, then solutions of fluorescent ligands in 1 mL of Leibovitz’s L-15 medium (no phenol red) were added.

3. **Pharmacological characterizations**

These assays were performed as previously described.² Briefly, 10 µL of a 1×10⁶ cells/mL suspension of defreezed Lumi4-Tb-SNAP-OTR cells in Tag-lite buffer were distributed in each well of a 384-well plate (CORNING 3824). Increasing concentrations of SQ-PEG-CBT were diluted in 5 µL of Tag-lite buffer and added to the cells together with 5 µL of Tag-lite buffer (total binding) or 5 µL of a carbetocin solution at final concentration of 10 µM (non-specific binding).

The plate was incubated for 3 hours at room temperature before reading on the Envision plate reader (Perkin Elmer) with a classical HTRF protocol (excitation at 337 nm, emission at 665 and 615 nm). The dissociation constant (Kₐ) was calculated using GraphPad Prism Software (GraphPad, San Diego, CA, USA).
4. Steady-State Fluorescence Spectroscopy

Absorption spectra were recorded on a Cary 4000 spectrophotometer (Varian) and fluorescence spectra on a FluoroMax 3 (Jobin Yvon, Horiba) spectrofluorometer. Fluorescence spectra in solvents were recorded at 600 nm excitation wavelength, fluorescence spectra in wtOTR cells were recorded at 620 nm excitation wavelength at 20°C. All spectra were corrected for instrumental effects. Fluorescence quantum yields (QY) were measured using DID in MeOH as a reference (QY = 33%).

Figure S1. Saturation experiments with SQ-PEG-CBT on the cells expressing Lumi4-Tb-SNAP-OTR.
### Table S1. Spectroscopic properties of SQ-COOH, 2 and SQ-PEG-CBT.\[^a\]

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<th>Compound</th>
<th>Solvent</th>
<th>$\varepsilon$, M$^{-1}$cm$^{-1}$</th>
<th>$\lambda_{\text{max}}$ abs, nm</th>
<th>$\lambda_{\text{max}}$ fluor, nm</th>
<th>QY, %</th>
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<td>SQ-COOH</td>
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<td>51</td>
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\[^a\] $\varepsilon$ is molar absorption coefficient; $\lambda_{\text{max}}$ abs and $\lambda_{\text{max}}$ fluor are positions of the absorption and the emission maximum, respectively; QY is fluorescence quantum yield.

**Figure S2.** Absorption spectra of SQ-COOH (A) and 1 (B) in EtOH before and after the addition of 10 mM of dimercaprol (DMP).
5. Fluorescence Confocal Microscopy and Spectroscopic Data

Fluorescence confocal microscopy experiments were performed on a Leica TCS SPE-II microscope with a HXC PL APO 63x/1.40 OIL CS objective. GFP excitation ("GFP" channel) was performed with a 488 nm 10 mW laser (30% intensity), the excitation of SQ-PEG-CBT ("squaraine" channel) was performed with a 635 nm 18 mW laser (30% intensity).

Figure S3. Confocal studies of SQ-PEG-CBT. Images of adherent GFP-OTR cells in the presence of 20 nM of SQ-PEG-CBT without (A) and with (B) the unlabeled competitor CBT.
Figure S4. Spectroscopic response of 5 nM SQ-PEG-CBT in Leibovitz’s L-15 medium to the presence of 1 mln/ mL suspension of wtOTR cells (without and with 2 µM CBT competitor). Inset table shows the spectroscopic parameters of the probe with and without cells.
6. Photobleaching Experiments

Photobleaching experiments (Figure 4 and Figure S3) were conducted by exciting NR-PEG-CBT with a 488 nm laser at 100% intensity and SQ-PEG-CBT with a 635 nm laser at 25% intensity. Timelaps experiments of 50 frames (2 scans per frame, minimal time between frames) were performed. The image treatment as well as the quantification of the average membrane intensity was performed using ImageJ software (Wayne Rasband, National Institute of Mental Health, Bethesda).

Figure S5. Photostability of NR-PEG-CBT and SQ-PEG-CBT under the laser irradiation.
7. NMR Spectra
8. LC-HRMS Spectra


