Supporting Information

BTDAzo: A Photoswitchable TRPC5 Channel Activator

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Supporting Information

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Figure S1. Structures of btda1-7.
Materials and Methods Chemistry

**Reagents and Conditions.** Unless stated otherwise, (a) all reactions and characterisations were performed with unpurified, undried, non-degassed solvents and reagents, used as obtained, under closed air atmosphere without special precautions; (b) “hexane” used for chromatography was distilled from commercial crude isohexane fraction by rotary evaporation; (c) “column” and “chromatography” refer to manual flash column chromatography on Merck silica gel Si-60 (40–63 μm); (d) “MPLC” refers to flash column chromatography purification on a Biotage Selekt system, using prepacked silica cartridges purchased from Biotage; (e) procedures and yields are unoptimized; (f) yields refer to isolated chromatographically and spectroscopically pure materials; (g) all eluent and solvent mixtures are given as volume ratios unless otherwise specified. (h) Thin-layer chromatography (TLC) was run on 0.25 mm Merck silica gel plates (60, F-254). UV light (254 nm) was used as a visualising agent. (i) "st" or "sat. aq." refer to saturated aqueous solutions of the given salt.

**Nuclear magnetic resonance (NMR) spectroscopy.** Standard NMR characterisation was by ¹H- and ¹³C-NMR spectra on a Bruker Ascend 400 (400 MHz & 101 MHz for ¹H and ¹³C respectively) and on a Bruker Ascend 500 (500 MHz & 126 MHz for ¹H and ¹³C respectively). Chemical shifts (δ) are reported in ppm calibrated to residual non-perdeuterated solvent as an internal reference. Peak descriptions singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m) and broad (br) are used. NMR spectra are given at the end of this document.

**High resolution mass spectrometry (HRMS).** HRMS was carried out by the Zentrale Analytik of the LMU Munich using ESI ionisation on a Thermo Finnigan LTQ FT Ultra Fourier Transform Ion Cyclotron Resonance Spectrometer.

**High Performance liquid chromatography (HPLC) coupled with mass spectrometry (MS).** Analytical HPLC-MS was performed on an Agilent 1100 SL with (a) a binary pump to deliver H₂O:MeCN eluent mixtures containing 0.1% formic acid at a 0.4 mL/min flow rate, (b) YMC-Triart C18 column (3.0 μm; 50 mm × 3 mm) maintained at 40 °C (c) an Agilent 1100 series diode array detector, (d) an Agilent LC/MSD iQ mass spectrometer. Typical run conditions were a linear gradient of H₂O:MeCN from 90:10 to 0:100 (first 5 min), then 0:100 for 2 min for flushing; then the column was (re)equilibrated with 90:10 eluent mixture for 2 min.

**HPLC.** Analytical HPLC was performed on an Agilent 1100 SL with (a) a binary pump to deliver H₂O:MeCN eluent mixtures containing 0.1% formic acid at a 0.4 mL/min flow rate, (b) Agilent-Zorbax C18 column (10 μm; 250 mm × 4.6 mm) maintained at 40 °C (c) an Agilent 1100 series diode array detector. Typical run conditions were a linear gradient of H₂O:MeCN from 75:25 to 2:98 (20 min), then 2:98 for 5 min for flushing; then the column was (re)equilibrated with 75:25 eluent mixture for 3 min.
**Preparative HPLC.** Preparative HPLC was performed on an Agilent 1200 SL with (a) a binary pump to deliver H2O:MeCN eluent mixtures containing 0.1% formic acid at 20 mL/min flow rate, (b) Agilent-Zorbax C18 column (10.0 μm; 250 mm × 30 mm) maintained at room temperature, (c) an Agilent 1200 series diode array detector, (d) and Agilent 1200 series fraction collector.
Synthetic procedures

Scheme S1. Synthetic overview on btada1-7. Conditions: a) succinic anhydride, AcOH; b) NaOH, reflux; c) HNO3; d) S4, HBTU, DIPEA, DMF; e) SN2, EtOH, 60°C. f) i) Azo coupling/Mills reaction, ii) MeI, K2CO3, DMF, 40 °C; g) i) K2CO3, acetone, 60 °C; ii) HCl in Dioxane, CH2Cl2; h) HBTU, DIPEA, S1, DMF.

Synthesis of Precursors

3-(1,1-dioxido-4H-benzo[e][1,2,4]thiadiazin-3-yl)propanoic acid (S1)

The synthesis was conducted according to a modified literature procedure.\textsuperscript{[1]}

2-Aminobenzenesulfonamide (1.0 g, 5.81 mmol, 1.0 eq) and succinic acid (872 mg, 8.71 mmol, 1.5 eq) were dissolved in AcOH (60 mL) and stirred at room temperature for 16 h. After removal of solvent under vacuum, the colourless residue was dissolved in 2 M NaOH (30 mL) and heated to 100°C for 2 h. The reaction mixture was cooled down, neutralized with 2 M HCl and the precipitate was filtered and washed with water to afford S1 as a colourless solid (1.31 g, 5.16 mmol, 89%). The compound is literature known but NMR spectra were not published.

\textsuperscript{1}H NMR (400 MHz, DMSO-d6) δ: 12.09 (bs, 1H), 7.77 (dd, J = 8.0, 1.4 Hz, 1H), 7.71 – 7.62 (m, 1H), 7.47 – 7.39 (m, 1H), 7.32 (d, J = 8.3 Hz, 1H), 2.81 (t, J = 6.7 Hz, 2H), 2.66 (t, J = 6.7 Hz, 2H). \textsuperscript{13}C NMR (126 MHz, DMSO-d6) δ: 173.15, 159.34, 135.15, 133.12, 126.22, 123.53, 121.28, 117.28, 30.03, 29.28. HRMS (ESI, m/z): [M-H] calcd for C_{10}H_{9}N_{2}O_{4}S: 253.0289; found: 253.0289.
3-(7-nitro-1,1-dioxido-4H-benzo[e][1,2,4]thiadiazin-3-yl)propanoic acid (S2)

S1 (2.03 g, 8.00 mmol, 1 eq) was added in small portions to nitric acid (86%, 24 mL) at 0 °C and further stirred for 3 h at 0 °C. The reaction mixture was poured carefully on ice (60 g) and the resulting precipitate was collected by filtration and washed with water to afford S2 as a colourless solid (1.55 g, 5.18 mmol, 65%).

$^1$H NMR (400 MHz, DMSO-$d_6$) δ: 12.64 (s, 1H), 8.70 – 8.30 (m, 2H), 7.54 (d, $J = 9.0$ Hz, 1H), 2.88 (t, $J = 6.7$ Hz, 2H), 2.68 (t, $J = 6.7$ Hz, 2H).

$^{13}$C NMR (101 MHz, DMSO-$d_6$) δ: 173.06, 160.58, 144.25, 139.89, 128.02, 120.84, 120.16, 119.20, 30.20, 29.16.

HRMS (ESI, m/z): [M-H] calcd for C$_{10}$H$_8$N$_3$O$_6$S: 298.0139; found: 298.0139.

3-((adamantan-1-yl)oxy)propan-1-amine (major product, S3)

The synthesis was conducted according to a literature procedure.$^{[2]}$

A mixture of 1-bromoadamantane (4.35 g, 20 mmol, 1.0 eq), 3-amino-1-propanol (30.6 mL, 400 mmol, 20 eq), and triethylamine (5.62 mL, 40 mmol, 2 eq) was heated to 180 °C for 16 h. After cooling to room temperature, the mixture was diluted with CH$_2$Cl$_2$ (150 mL), washed with 0.1 M NaOH (5 x 100 mL) and with a mixture of brine/ 1 M NaOH (3:1, 100 mL). The organic layer was dried over anhydrous Na$_2$SO$_4$, filtered and concentrated to afford a crude reaction mixture containing S3 and its minor regioisomer (overall 3.75 g, 17.9 mmol, 90 %; $^1$H NMR analysis shows major desired:minor undesired regioisomers in ratio 74:26 which is in accordance with the literature (~ 70:30)$^{[2]}$.) The mixture was used without further purification as the minor isomer does not couple in the next step and so is removed in purification of S4.

$^1$H NMR of crude S3 mixture: (400 MHz, CDCl$_3$) δ: 3.80 (t, $J = 5.1$ Hz, 2H, minor), 3.48 (t, $J = 6.2$ Hz, 2H, major), 2.87 (t, $J = 5.6$ Hz, 2H, minor), 2.78 (t, $J = 6.7$ Hz, 2H, major), 2.13 (br, 3H, major), 2.06 (br, 3H, minor), 1.76 – 1.54 (m, 14H major + 14H minor).

N-(3-((adamantan-1-yl)oxy)propyl)-3-(7-nitro-1,1-dioxido-4H-benzo[e][1,2,4]thiadiazin-3-yl)propanamide (S4)
HBTU (2.13 g, 5.62 mmol, 1.1 eq) was added to a solution of S2 (1.53 g, 5.11 mmol, 1.0 eq) and DIPEA (2.70 ml, 15.3 mmol, 3.0 eq) in DMF (50 mL) and stirred for 10 min followed by addition of the crude mixture of S3 (2.14 g, 10.2 mmol, 2.0 eq). After 2 h the reaction was quenched with st. NH4Cl (100 mL) and st. NaHCO3 (100 mL), the pH was set to 7 and the mixture was extracted with EtOAc (2 x 200 mL). The combined organic layers were washed with brine (5 x 100 mL), dried over anhydrous Na2SO4, filtered, and concentrated. The crude product was purified by flash chromatography (DCM/MeOH 100:0 to 95:5) to afford S4 (2.30 g, 4.69 mmol, 93 %) as a slightly brown solid.

TLC (DCM/MeOH 95:5): Rf = 0.40. 1H NMR (400 MHz, DMSO-d6) δ: 12.63 (br, 1H), 8.52 – 8.35 (m, 2H), 7.91 (t, J = 5.5 Hz, 1H), 7.54 (d, J = 8.9 Hz, 1H), 3.36 – 3.26 (m, 2H), 3.11 – 3.03 (m, 2H), 2.86 (t, J = 7.0 Hz, 2H), 2.55 (t, J = 7.0 Hz, 2H), 2.05 (br, 3H), 1.67 – 1.60 (m, 6H), 1.60 – 1.45 (m, 8H). 13C NMR (101 MHz, DMSO-d6) δ: 170.06, 160.97, 144.16, 139.94, 127.93, 120.82, 120.11, 119.18, 71.05, 56.73, 41.10, 36.13, 35.94, 30.78, 30.54, 30.15, 29.83. HRMS (ESI, m/z): [M-H] calcd for C23H29N4O6S: 489.1813; found: 489.1814.

N-(3-((adamantan-1-yl)oxy)propyl)-3-(7-amino-1,1-dioxido-4H-benzo[e][1,2,4]thiadiazin-3-yl)propenamide (S5)

A flask was charged with S4 (1.36 g, 2.77 mmol, 1.0 eq), tin(II)chloride (2.10 g, 11.1 mmol, 4 eq) and EtOH (30 mL). The reaction mixture was heated to 60 °C for 2 h and quenched by addition of water, st. aq. NaHCO3 and st. aq. NH4Cl. PH was set to 7 and the suspension was extracted with EA (6 x 100 mL). The combined organic layers were dried over anhydrous Na2SO4, filtered, and concentrated. The crude product was purified by flash chromatography (DCM/MeOH 100:0 to 90:10) to afford S5 (670 mg, 1.45 mmol, 53 %) as a slightly brown solid.

TLC (DCM/MeOH 95:5): Rf = 0.29. 1H NMR (400 MHz, DMSO- d6) δ: 11.67 (s, 1H), 7.86 (t, J = 5.5 Hz, 1H), 7.01 (dd, J = 8.8, 1.1 Hz, 1H), 6.89 – 6.81 (m, 2H), 5.54 (s, 2H), 3.33 – 3.30 (m, 2H), 3.07 (dd, J = 6.5 Hz, 2H), 2.69 (t, J = 7.3 Hz, 2H), 2.51 – 2.44 (m, 2H), 2.06 (br, 3H), 1.69 – 1.62 (m, 6H), 1.60 – 1.47 (m, 8H). 13C NMR (101 MHz, DMSO-d6) δ: 170.27, 160.97, 144.16, 139.94, 124.64, 122.50, 119.46, 118.26, 104.51, 71.07, 56.75, 41.12, 36.12, 35.97, 30.78, 30.54, 30.15, 29.84. HRMS (ESI, m/z): [M+H]+ calcd for C23H33N4O6S: 461.2217; found: 461.2218.
N-(3-((adamantan-1-yl)oxy)propyl)-3-(7-((E)-(4-hydroxy-3,5-dimethoxyphenyl)diazenyl)-1,1-dioxido-4H-benzo[e][1,2,4]thiadiazin-3-yl)propenamide (S6)

NaNO₂ (550 µL, 2 M, 1.10 mmol, 1.1 eq) was added to a solution of S5 (461 mg, 1.00 mmol, 1.0 eq) in Hydrochloric acid (4 mL, 2M) and MeOH (4 mL) at 0 °C. The reaction mixture was allowed to stir for 15 min at 0 °C and was subsequently added to a solution of 2,6-Dimethoxyphenol (185 mg, 1.2 mmol, 1.2 eq) in MeOH (3 mL) and Buffer (3 mL of 0.5 M K₂HPO₄ and 1 mL of 1 M KOH). The pH was adjusted to 9 - 11 and the reaction was stirred for 45 min at 0°C. The reaction was quenched by addition of st. NH₄Cl, pH was adjusted to 7 with st. NaHCO₃ and the mixture was extracted with EA (3 x 60 mL). The combined organic layers were dried over anhydrous Na₂SO₄, filtered, and concentrated. The crude product was purified by flash chromatography (DCM/MeOH 100:0 to 95:5) to afford S6 (330 mg, 527 µmol, 53 %) as a deep red oil which was used directly without further purification.

A representative sample was further purified by preparative HPLC (gradient: H₂O/ACN 75:25 → 10:90 in 25 minutes) to obtain an analytical sample.

**TLC** (DCM/MeOH 95:5): Rᵥ = 0.36. **¹H NMR** (500 MHz, DMSO-d₆) δ: 8.17 – 8.12 (m, 2H), 7.90 (t, J = 5.5 Hz, 1H), 7.49 (d, J = 8.5 Hz, 1H), 7.32 (s, 2H), 3.88 (s, 6H), 3.37 – 3.28 (m, 2H), 3.11 – 3.03 (m, 2H), 2.82 (t, J = 7.2 Hz, 2H), 2.55 (t, J = 7.1 Hz, 2H), 2.05 (br, 3H), 1.65 – 1.59 (m, 6H), 1.60 – 1.48 (m, 8H). **¹³C NMR** (126 MHz, DMSO-d₆) δ: 170.22, 160.15, 149.12, 148.25, 143.87, 140.14, 136.78, 127.56, 121.93, 119.01, 116.05, 101.11, 71.06, 56.73, 56.06, 41.11, 36.12, 35.95, 30.95, 30.83, 30.16, 29.82. **HRMS** (ESI, m/z): [M+H]⁺ calcd for C₃₁H₄₀N₅O₄S: 626.2643; found: 626.2650.

**3-(4-(phenyldiazenyl)phenoxy)propan-1-amine (S7)**

The synthesis was conducted according to a modified literature procedure.[³]

A screw cap vial was charged with 4-Phenylazophenol (49 mg, 0.25 mmol, 1.0 eq), 3-(Boc-amino)propyl bromide (89 mg, 0.38 mmol, 1.5 eq), K₂CO₃ (69 mg, 0.50 mmol, 2 eq) and acetone (2.5 mL), locked and stirred at 50°C for 16 h. The mixture was diluted with EA (20 mL),
quenched with st. NH₄Cl (10 mL), the layers separated, and the organic layer washed with 
brine (3 x 20 mL). The organic layers were dried over anhydrous Na₂SO₄, filtered, and 
concentrated. The residue was filtered through a short silica column (isoheaxane/EA 90:10), 
concentrated, dissolved in abs DCM (5 mL) and HCl (4 M in dioxane, 625 µL, 2.5 mmol, 10 
eq) was added dropwise to the solution. After 1 h stirring Et₂O was added, the product 
precipitated as hydrochloride, filtered off, washed with Et₂O and air-dried to afford S7 (58 mg, 
0.20 mmol, 80 %) as a yellow solid.

¹H NMR (400 MHz, DMSO-d6) δ 8.15 (br, 3H), 7.90 (d, J = 9.0 Hz, 2H), 7.87 – 7.81 (m, 2H), 
7.62 – 7.47 (m, 3H), 7.15 (d, J = 9.0 Hz, 2H), 4.20 (t, J = 6.1 Hz, 2H), 3.04 – 2.92 (m, 2H), 2.15 
– 2.03 (m, 2H). ¹³C NMR (101 MHz, DMSO) δ 161.11, 151.98, 146.22, 130.87, 129.40, 124.56, 
122.25, 115.12, 65.17, 36.10, 26.72.

4-((4-methoxyphenyl)diazenyl)phenol (S8)

The synthesis was conducted according to a literature procedure.[⁴]

NaNO₂ (1.10 mL, 2 M, 2.20 mmol, 1.1 eq) was added to a solution of p-anisidine (246 mg, 2.00 
mmol, 1.0 eq) in hydrochloric acid (4 mL, 2M) and MeOH (6 mL) at 0 °C. The reaction mixture 
was allowed to stir for 15 min at 0 °C and was subsequently added to a solution of phenol (226 
mg, 2.40 mmol, 1.2 eq) in MeOH (6 mL) and buffer (6 mL of 0.5 M K₂HPO₄ and 1 mL of 1 M 
KOH). The pH was adjusted to 9 - 11 and the reaction was stirred for 45 min at 0°C. The 
reaction was quenched by addition of st. NH₄Cl, pH was adjusted to 7 with st. NaHCO₃ 
and the mixture was extracted with EA (3 x 60 mL). The combined organic layers were dried over 
anhydrous Na₂SO₄, filtered, and concentrated. The crude product was purified by flash 
chromatography (Isohexane/EA 80:20) to afford S8 (248 mg, 1.09 mmol, 54 %) as a brown oil.

Spectral data are in accordance with the literature.[⁴]

TLC (Hex/EtOAc 8:2): Rₜ = 0.35. ¹H NMR (400 MHz, CDCl₃) δ: 7.88 (d, J = 9.0 Hz, 2H), 7.83 
(d, J = 8.8 Hz, 2H), 7.00 (d, J = 9.0 Hz, 2H), 6.93 (d, J = 8.8 Hz, 2H), 3.89 (s, 3H). ¹³C NMR 
(101 MHz, CDCl₃) δ: 161.76, 157.87, 147.38, 147.15, 124.72, 124.53, 115.90, 114.34, 55.72.
3-(4-((4-methoxyphenyl)diazenyl)phenoxy)propan-1-amine (S9)

The synthesis was conducted according to a modified literature procedure.\[4\]

A screw cap vial was charged with S8 (57 mg, 0.25 mmol, 1.0 eq), 3-(Boc-amino)propyl bromide (89 mg, 0.38 mmol, 1.5 eq), K$_2$CO$_3$ (69 mg, 0.50 mmol, 2 eq) and acetone (2.5 mL), locked and stirred at 50°C for 16 h. The mixture was diluted with EA (20 mL), quenched with st. NH$_4$Cl (10 mL), the layers separated, and the organic layer washed with brine (3 x 20 mL). The organic layers were dried over anhydrous Na$_2$SO$_4$, filtered, and concentrated. The residue was filtered through a short silica column (Isohexane/EA 90:10), concentrated, dissolved in abs DCM (5 mL) and HCl (4 M in Dioxane, 625 µL, 2.5 mmol, 10 eq) was added dropwise to the solution. After 1 h stirring Et$_2$O was added, the product precipitated as hydrochloride, filtered off, washed with Et$_2$O and dried to afford S9 (25 mg, 78 µmol, 31 %) as a yellow solid.

Spectral data are in accordance with the literature.\[4\]

$^1$H NMR (400 MHz, DMSO-d$_6$) δ: 8.12 (br, 3H), 7.89 – 7.81 (m, 4H), 7.19 – 7.05 (m, 4H), 4.18 (t, J = 6.1 Hz, 2H), 3.85 (s, 3H), 3.05 – 2.90 (m, 2H), 2.17 – 2.01 (m, 2H). $^{13}$C NMR (101 MHz, DMSO) δ 161.49, 160.52, 146.27, 146.17, 124.14, 124.10, 115.03, 114.54, 65.07, 55.60, 36.13, 26.75. HRMS (ESI, m/z): [M+H]$^+$ calcd for C$_{16}$H$_{20}$O$_2$N$_3$: 286.1550; found: 286.1552.

2,6-diisopropyl-4-((4-methoxyphenyl)diazenyl)phenol (S10)

The synthesis was conducted according to a literature procedure.\[5\]

NaNO$_2$ solution (550 µL, 2 M, 1.10 mmol, 1.1 eq) was added to a solution of p-anisidine (123 mg, 1.00 mmol, 1.0 eq) in hydrochloric acid (2 mL, 2M) and MeOH (3 mL) at 0 °C. The reaction mixture was allowed to stir for 15 min at 0 °C and was subsequently added to a solution of 2,6-diisopropylphenol (123 mg, 1.2 mmol, 1.2 eq) in MeOH (3 mL) and buffer (3 mL of 0.5 M K$_2$HPO$_4$ and 1 mL of 1 M KOH). The pH was adjusted to 9 - 11 and the reaction was stirred for 45 min at 0°C. The reaction was quenched by addition of st. NH$_4$Cl, pH was adjusted to 7 with st. NaHCO$_3$ and the mixture was extracted with EA (3 x 60 mL). The combined organic layers
were dried over anhydrous Na$_2$SO$_4$, filtered, and concentrated. The crude product was purified by flash chromatography (Isohexane/EA 90:10 to 65:35) to afford S10 (148 mg, 474 µmol, 47%) as a deep red oil.

Spectral data are in accordance with the literature.$^5$

**TLC** (Hex/EtOAc 8:2): R$_f$ = 0.45. **$^1$H NMR** (400 MHz, CDCl$_3$) δ: 7.94 – 7.83 (m, 2H), 7.69 (s, 2H), 7.04 – 6.96 (m, 2H), 5.13 (s, 1H), 3.89 (s, 3H), 3.20 (hept, $J$ = 6.8 Hz, 2H), 1.34 (d, $J$ = 6.8 Hz, 12H). **$^{13}$C NMR** (101 MHz, CDCl$_3$) δ (ppm): 161.51, 152.61, 147.37, 147.14, 134.37, 124.35, 118.81, 114.27, 55.68, 27.54, 22.80.

3-(2,6-diisopropyl-4-((4-methoxyphenyl)diazenyl)phenoxy)propan-1-amine (S11)

![Chemical structure of S11](image)

A screw cap vial was charged with S10 (78 mg, 0.25 mmol, 1.0 eq), 3-(Boc-amino)propyl bromide (89 mg, 0.38 mmol, 1.5 eq), K$_2$CO$_3$ (69 mg, 0.50 mmol, 2 eq) and acetone (2.5 mL), locked and stirred at 50°C for 16 h. The mixture was diluted with EA (20 mL), quenched with st. NH$_4$Cl (10 mL), the layers separated, and the organic layer washed with brine (3 x 20 mL). The organic layers were dried over anhydrous Na$_2$SO$_4$, filtered, and concentrated. The residue was filtered through a short silica column (hexane/EtOAc 90:10), concentrated, dissolved in abs DCM (5 mL) and HCl (4 M in dioxane, 625 µL, 2.5 mmol, 10 eq) was added dropwise to the solution. After 1 h stirring Et$_2$O was added, the product precipitated as hydrochloride, filtered off, washed with Et$_2$O and air-dried to afford S11 (36 mg, 0.089 mmol, 36%) as a yellow solid which was used without further purification.

A representative sample was further purified by reverse phase flash chromatography (Biotage Sfär C18, H$_2$O/MeCN +0.1% formic acid 90:10 to 5:95) to obtain spectroscopically pure material.

**$^1$H NMR** (500 MHz, DMSO-$d_6$) δ: 7.88 (d, $J$ = 9.0 Hz, 2H), 7.63 (s, 2H), 7.12 (d, $J$ = 9.0 Hz, 2H), 3.93 – 3.74 (m, 5H), 3.29 (p, $J$ = 6.8 Hz, 2H), 2.97 (br, 2H), 2.05 (t, $J$ = 7.2 Hz, 2H), 1.25 (d, $J$ = 6.9 Hz, 12H). **$^{13}$C NMR** (126 MHz, DMSO-$d_6$) δ 161.71, 155.24, 148.86, 146.21, 142.57, 124.38, 118.22, 114.54, 72.13, 55.63, 36.63, 29.61, 26.29, 23.77. **HRMS** (ESI, m/z): [M+H]$^+$ calcd for C$_{22}$H$_{32}$O$_2$N$_3$: 370.2489; found: 370.2493.
**Synthesis of btda1-7**

**BTDAzo (btda3)**

A screw cap vial was charged with S6 (95 mg, 152 µmol, 1.0 eq), K$_2$CO$_3$ (42 mg, 304 µmol, 2.0 eq), DMF (1.5 mL) and iodomethane (10.4 µL, 167 µmol, 1.1 eq), locked and stirred 1 h at 40 °C. The mixture was diluted with EA (30 mL), quenched with st. NH$_4$Cl (20 mL), the layers separated, the organic layer washed with brine (3 x 20 mL). The organic layers were dried over anhydrous Na$_2$SO$_4$, filtered, and concentrated. The crude product was pre-purified by flash chromatography (DCM/MeOH 100:0 to 95:5) and further purified by recrystallization (EtOH/H$_2$O 2:1) to afford **BTDAzo** (25 mg, 39 µmol, 26 %) as an orange solid. Recrystallization was necessary to separate the double methylated side product.

**TLC** (DCM/MeOH 95:5): R$_f$ = 0.40. **$^1$H NMR** (400 MHz, DMSO-$d_6$) δ: 12.38 (s, 1H), 8.22 – 8.18 (m, 2H), 7.98 – 7.87 (m, 3H), 7.66 – 7.58 (m, 3H), 7.56 – 7.51 (m, 1H), 3.38 – 3.28 (m, 2H), 3.08 (q, J = 6.5 Hz, 2H), 2.84 (t, J = 7.1 Hz, 2H), 2.56 (t, J = 7.1 Hz, 2H), 2.04 (br, 3H), 1.65 – 1.46 (m, 8H). **$^{13}$C NMR** (101 MHz, DMSO-$d_6$) δ: 170.17, 160.18, 153.38, 148.92, 147.55, 140.81, 137.09, 127.77, 121.86, 118.93, 116.81, 100.66, 71.05, 60.29, 56.72, 56.07, 41.10, 36.13, 35.94, 30.83, 30.73, 30.16, 29.82. **HRMS** (ESI, m/z): [M+H]$^+$ calcd for C$_{32}$H$_{42}$O$_7$N$_5$S: 640.2800; found: 640.2804.

**btda1**

A screw cap vial was charged with S4 (98 mg, 0.21 mmol, 1.0 eq), nitrosobenzene (25 mg, 0.23 mmol, 1.1 eq) and acetic acid (2 mL), locked and stirred for 16 h at room temperature. The resulting yellow suspension was diluted with EA, neutralized with st NaHCO$_3$, the layers separated and the organic layer was washed with st NaHCO$_3$ (2 x 20 mL) and brine (20 mL). The organic layer was dried over anhydrous Na$_2$SO$_4$, filtered, and concentrated. The crude product was purified by flash chromatography (DCM/MeOH 100:0 to 95:5) to afford **btda1** (32 mg, 67 µmol, 37%) as a yellow solid.

**TLC** (DCM/MeOH 95:5): R$_f$ = 0.47. **$^1$H NMR** (400 MHz, DMSO-$d_6$) δ: 12.39 (br s, 1H), 8.25 – 8.19 (m, 2H), 7.98 – 7.87 (m, 3H), 7.66 – 7.58 (m, 3H), 7.56 – 7.51 (m, 1H), 3.38 – 3.28 (m, 2H), 3.08 (q, J = 6.5 Hz, 2H), 2.84 (t, J = 7.1 Hz, 2H), 2.56 (t, J = 7.1 Hz, 2H), 2.04 (br, 3H).
1.68 – 1.60 (m, 6H), 1.59 – 1.45 (m, 9H). $^{13}$C NMR (101 MHz, DMSO d6) δ 170.14, 160.17, 151.69, 149.00, 137.26, 132.04, 129.57, 127.39, 122.80, 121.78, 118.96, 117.52, 71.04, 56.71, 41.09, 36.11, 35.93, 30.78, 30.71, 30.16, 29.81. HRMS (ESI, m/z): [M+H]$^+$ calcd for C$_{29}$H$_{36}$N$_5$O$_4$S: 550.2483; found: 550.2483.

**btda4**

NaNO$_2$ (113 µL, 2 M, 0.227 mmol, 1.1 eq) was added to a solution of S4 (95 mg, 0.206 mmol, 1.0 eq) in hydrochloric acid (413 µL, 2M) and MeOH (0.4 mL) at 0 °C. The reaction mixture was allowed to stir for 15 min at 0 °C and was subsequently added to a solution of phenol (23.3 mg, 0.248 mmol, 1.2 eq) in MeOH (0.4 mL) and buffer (0.4 mL of 0.5 M K$_2$HPO$_4$ and 0.1 mL of 1 M KOH). The pH was adjusted to 9 - 11 and the reaction was stirred for 45 min at 0°C. The reaction was quenched by addition of st. NH$_4$Cl, pH was adjusted to 7 with st. NaHCO$_3$ and the mixture was extracted with EA (3 x 20 mL). The combined organic layers were dried over anhydrous Na$_2$SO$_4$, filtered, and concentrated. The crude product was purified by flash chromatography (DCM/MeOH 100:0 to 90:10) to afford **btda4** (34 mg, 60.1 µmol, 34 %) as a yellow solid.

**TLC** (DCM/MeOH 95:5): $R_f = 0.34$. $^1$H NMR (400 MHz, DMSO d6) δ: 12.33 (br s, 1H), 10.42 (br s, 1H), 8.13 (dd, $J = 8.7$, 2.2 Hz, 1H), 8.10 (d, $J = 2.2$ Hz, 1H), 7.90 (t, $J = 5.6$ Hz, 1H), 7.85 (d, $J = 8.9$ Hz, 2H), 7.49 (d, $J = 8.7$ Hz, 1H), 6.96 (d, $J = 8.9$ Hz, 2H), 3.34 – 3.28 (m, 2H), 3.08 (q, $J = 6.5$ Hz, 2H), 2.83 (t, $J = 7.1$ Hz, 2H), 2.55 (t, $J = 7.1$ Hz, 2H), 2.04 (br, 3H), 1.67 – 1.59 (m, 6H), 1.59 – 1.47 (m, 9H). $^{13}$C NMR (101 MHz, DMSO d6) δ: 170.17, 161.51, 160.01, 149.33, 145.04, 136.43, 127.23, 125.25, 121.81, 118.84, 116.43, 116.09, 71.06, 56.72, 41.10, 36.12, 35.95, 30.78, 30.75, 30.16, 29.82. HRMS (ESI, m/z): [M+H]$^+$ calcd for C$_{29}$H$_{36}$N$_5$O$_4$S: 566.2432; found: 566.2432.

**btda2**

A screw cap vial was charged with **btda4** (27 mg, 48 µmol, 1.0 eq), K$_2$CO$_3$ (13 mg, 96 µmol, 2.0 eq), DMF (0.5 mL) and iodomethane (3.3 µL, 53 µmol, 1.1 eq), locked and stirred 1 h at 40 °C. The mixture was diluted with EA (20 mL), quenched with st. NH$_4$Cl (10 mL), the layers separated, the organic layer washed with brine (3 x 20 mL). The organic layers were dried over
anhydrous Na$_2$SO$_4$, filtered, and concentrated. The crude product was pre-purified by flash chromatography (DCM/MeOH 100:0 to 95:5) and further purified by preparative HPLC (gradient: H$_2$O/ACN 75:25 → 10:90 in 25 minutes) to afford btda2 (7 mg, 12 µmol, 25 %) as a yellow solid.

$^1$H NMR (400 MHz, DMSO-d$_6$) δ: 12.29 (br, 1H), 8.20 – 8.10 (m, 2H), 7.98 – 7.85 (m, 3H), 7.50 (d, $J = 8.6$ Hz, 1H), 7.20 – 7.09 (m, 2H), 3.88 (s, 3H), 3.33 – 3.27 (m, 2H), 3.11 – 3.05 (m, 2H), 2.83 (t, $J = 7.1$ Hz, 2H), 2.55 (t, $J = 7.3$ Hz, 2H), 2.04 (br, 3H), 1.68 – 1.59 (m, 6H), 1.60 – 1.46 (m, 9H). $^{13}$C NMR (101 MHz, DMSO-d$_6$) δ: 170.23, 162.46, 160.20, 149.14, 145.99, 137.03, 127.22, 124.93, 119.08, 116.77, 114.77, 71.06, 56.73, 55.74, 41.11, 36.13, 35.95, 30.93, 30.82, 30.17, 29.83. $^{1}$D NOE (400 MHz, DMSO-d$_6$, pulse at 3.89 ppm) δ: 7.17, 7.14. HRMS (ESI, m/z): [M+H]$^+$ calcd for C$_{30}$H$_{38}$O$_5$N$_5$S: 580.2588; found: 580.2588.

Note: The exchangeable NH-proton on the benzothiadiazine core at 12.29 ppm is only a very faint signal in the NMR.

btda5

HBTU (42. mg, 0.11 mmol, 1.1 eq) was added to a solution of S1 (28 mg, 0.11 mmol, 1.1 eq) and DIPEA (73 µL, 0.40 mmol, 4.0 eq) in DMF (1 mL) and stirred for 10 min followed by addition of S7 (29 mg, 0.10 mmol, 1.0 eq). After 1 h the reaction was quenched with st. NH$_4$Cl (10 mL) and the mixture was extracted with EtOAc (2 x 20 mL). The combined organic layers were washed with brine (5 x 10 mL), dried over anhydrous Na$_2$SO$_4$, filtered, and concentrated. The crude product was purified by flash chromatography (DCM/MeOH 100:0 to 95:5) to afford btda5 (32 mg, 65 µmol, 65 %) as a yellow solid.

$^1$H NMR (400 MHz, DMSO-d$_6$) δ: 12.05 (br, 1H), 8.10 (t, $J = 5.7$ Hz, 1H), 7.88 – 7.82 (m, 4H), 7.78 (dd, $J = 7.9$, 1.4 Hz, 1H), 7.66 (ddd, $J = 8.5$, 7.3, 1.4 Hz, 1H), 7.61 – 7.55 (m, 2H), 7.54 – 7.50 (m, 1H), 7.43 (ddd, $J = 8.2$, 7.3, 1.1 Hz, 1H), 7.31 (ddd, $J = 8.3$, 1.1 Hz, 1H), 7.10 (d, $J = 9.0$ Hz, 2H), 4.08 (t, $J = 6.2$ Hz, 2H), 3.28 – 3.19 (m, 2H), 2.81 (t, $J = 7.1$ Hz, 2H), 2.55 (t, $J = 7.1$ Hz, 2H), 1.88 (t, $J = 6.4$ Hz, 2H). $^{13}$C NMR (101 MHz, DMSO-d$_6$) δ: 170.54, 161.48, 159.72, 152.04, 146.09, 135.18, 133.08, 130.85, 129.43, 126.19, 124.58, 123.52, 122.27, 121.27, 117.30, 115.08, 65.73, 35.46, 30.71, 30.60, 28.82.

HRMS (ESI, m/z): [M+H]$^+$ calcd for C$_{25}$H$_{36}$O$_4$N$_5$S: 492.1700; found: 492.1700.
**btda6**

HBTU (26 mg, 69 µmol, 1.1 eq) was added to a solution of S1 (18 mg, 69 µmol, 1.1 eq) and DIPEA (44 µL, 0.25 mmol, 4.0 eq) in DMF (0.6 mL) and stirred for 10 min followed by addition of S9 (29 mg, 0.10 mmol, 1.0 eq). After 1 h the reaction was quenched with st. NH₄Cl (10 mL) and the mixture was extracted with EtOAc (2 x 20 mL). The combined organic layers were washed with brine (5 x 10 mL), dried over anhydrous Na₂SO₄, filtered, and concentrated. The crude product was purified by flash chromatography (DCM/MeOH 100:0 to 95:5) to afford **btda6** (30 mg, 58 µmol, 55%) as a yellow solid.

**¹H NMR** (400 MHz, DMSO-d₆) δ: 12.04 (br, 1H), 8.09 (t, J = 5.7 Hz, 1H), 7.84 (d, J = 8.9 Hz, 2H), 7.81 – 7.76 (m, 3H), 7.66 (dd, J = 8.7, 7.3, 1.5 Hz, 1H), 7.43 (t, J = 7.7 Hz, 1H), 7.31 (d, J = 8.2 Hz, 1H), 7.15 – 7.00 (m, 4H), 4.06 (t, J = 6.2 Hz, 2H), 3.85 (s, 3H), 3.27 – 3.19 (m, 2H), 2.81 (t, J = 7.1 Hz, 2H), 2.55 (t, J = 7.1 Hz, 2H), 1.93 – 1.82 (m, 2H). ¹³C NMR (101 MHz, DMSO-d₆) δ: 170.50, 161.46, 160.87, 159.69, 146.22, 146.11, 135.16, 133.05, 126.16, 124.10, 123.50, 121.25, 117.28, 114.97, 114.55, 65.63, 55.61, 35.45, 30.70, 30.59, 28.82.

**HRMS (ESI, m/z):** [M+H]⁺ calcd for C₂₆H₂₈O₅N₅S: 522.1806; found: 522.1807.

**btda7**

HBTU (37 mg, 98 µmol, 1.1 eq) was added to a solution of S1 (25 mg, 98 µmol, 1.1 eq) and DIPEA (64 µL, 0.36 mmol, 4.0 eq) in DMF (1 mL) and stirred for 10 min followed by addition of S9 (36 mg, 89 µmol, 1.0 eq). After 1 h the reaction was quenched with st. NH₄Cl (10 mL) and the mixture was extracted with EtOAc (2 x 20 mL). The combined organic layers were washed with brine (5 x 10 mL), dried over anhydrous Na₂SO₄, filtered, and concentrated. The crude product was purified by flash chromatography (DCM/MeOH 100:0 to 95:5) to afford **btda7** (42 mg, 69 µmol, 78%) as a yellow solid.

**¹H NMR** (400 MHz, DMSO-d₆) δ: 12.05 (br, 1H), 8.06 (t, J = 5.5 Hz, 1H), 7.92 – 7.86 (m, 2H), 7.77 (dd, J = 8.0, 1.4 Hz, 1H), 7.71 – 7.62 (m, 1H), 7.62 (s, 2H), 7.47 – 7.38 (m, 1H), 7.32 (d, J = 8.2 Hz, 1H), 7.16 – 7.09 (m, 2H), 3.86 (s, 3H), 3.78 (t, J = 6.3 Hz, 2H), 3.32 – 3.23 (m, 4H), 2.82 (t, J = 7.2 Hz, 2H), 2.57 (t, J = 7.2 Hz, 2H), 2.01 – 1.85 (m, 2H), 1.23 (d, J = 6.8 Hz, 12H).
\textbf{13C NMR} (101 MHz, DMSO) δ: 170.42, 161.68, 159.66, 155.36, 148.81, 146.22, 142.61, 135.18, 133.01, 126.12, 124.36, 123.47, 121.26, 118.21, 117.28, 114.53, 72.46, 55.62, 35.74, 30.72, 30.63, 29.98, 26.23, 23.79. \textbf{HRMS} (ESI, m/z): [M+H]^+ calcd for C_{32}H_{46}O_8N_5S: 606.2745; found: 606.2747.

\textbf{Photocharacterization}

\textbf{UV-VIS.} UV-Vis spectra were recorded on an Agilent Cary 60 UV-Vis spectrophotometer using 1 cm quartz or PMMA cuvettes. All photoisomerisations and relaxation rate measurements were performed at room temperature in non-degassed solvents unless stated differently. Samples were irradiated with a Polycon V monochromator (TILL Photonics, Gräfelfing, Germany) by shining from the top of the cuvette, until the spectra did not change further (photoequilbrium). Unless stated differently, all measurements were performed at a default concentration of 25 μM. Dark state refers to stocks in DMSO kept at 60 °C for >14 h prior to measurements (all-trans).

\textbf{PSS as Ratio of Z/E.} Samples were irradiated until PSS was reached; (a) for BTDAzo in different solvents PSS was calculated from the UV-Vis spectrum under the assumption that the absorbance of the Z isomer at 378-384 nm is effectively zero compared to that of the E isomer, so that the absorbance at this wavelength gives the fractional content of E isomer, which is then used to back-calculate the fraction of Z as (1 - E). In the important solvents for this paper, which are aprotic apolar (e.g. EtOAc/hexanes), the Z-absorbance is ca. 3% of the E-isomer’s at this wavelength: so results at 100% E isomer are ~100% accurate, while results at 100% Z isomer would be calculated as 3% E therefore 97% Z, ie. a maximal error of 3%, which we considered entirely acceptable. (b) PSS determined from HPLC: analysis by integrating the signal at the respective isosbestic point derived from UV/VIS and calculating the ratio from the integrals.
Solvent dependent photoisomerization of BTDAzo

Figure S2. PSS spectra of BTDAzo in different solvents. ACN+FA is acetonitrile with 0.1% formic acid, useful in calibrating the spectra obtained from the HPLC DAD. H2O/DMSO is a 90/10 mixture with a reduced concentration of 10 µM.

Table S1. Ratios of Z/E at PSS in different solvents calculated from UV/VIS spectra.

| λ [nm] | 90% H2O 10%DMSO | 50% EtOAc 50% Hexane | MeCN | EtOH | EtOAc |
|--------|------------------|----------------------|------|------|-------|
| 360    | 77:23            | 86:14                | 87:13| 72:28| 78:22 |
| 380    | 85:15            | 94:6                 | 93:7 | 88:12| 92:8  |
| 400    | 77:23            | 90:10                | 84:16| 86:14| 89:11 |
| 440    | 42:58            | 43:57                | 46:54| 38:62| 43:57 |
| 480    | 23:77            | 20:80                | 29:71| 19:81| 20:80 |
| 520    | 18:82            | 13:87                | 20:80| 16:84| 11:89 |
Relaxation of BTDAzo

![Graphs](image)

Fig S3. Spontaneous thermal relaxation a) of BTDAzo in polar protic solvent, b) of BTDAzo in apolar aprotic solvent, c) and of btda4 in polar protic/apolar aprotic solvent, at 25°C.

Relaxation of Z-BTDAzo

The thermal relaxation of Z-BTDAzo to E-BTDAzo was determined in a) polar protic environment and b) apolar solvent mixture to mimic cellular lipid environment. In both cases spontaneous relaxation ($t_{1/2} >> 4$ h; Fig S3a-b) is negligible on the time scale of biological experiments (seconds-scale switching, minutes-scale experiments).

Relaxation of Z-btda4

In contrast to BTDAzo, para-hydroxy btda4 can relax "quickly" on the biological timescale depending on the solvent environment. In polar protic media the thermal relaxation from Z-btda4 to E-btda4 takes place in the ms time scale (Fig. S3c). Therefore, a “real” photostationary state is not obtained in our cuvette switching experiments because the rate of thermal back-reaction to E-btda4 ($\tau=44$ ms) is much faster than the photon encounter rate. However, in aprotic apolar, membrane-mimicking environment (hexane/ethyl acetate 1:1) the thermal relaxation from Z-btda4 to E-btda4 at room temperature is slowed to $\tau=134$ s; therefore, a photostationary state is obtained. We believed that this would allow us to estimate the effective cellular environment that btda4 (and hence also BTDAzo) is located in:

In cellular FLIPR studies btda4 was found to reversibly activate TRPC5 under the same illumination conditions as BTDAzo (Fig. S5). With an EC$_{50}$ of 45 µM under 365 nm illumination btda4 is only a weak photoswitchable agonist, but we suggest that the photoswitch behaviour enables useful conclusions on the environment. In a polar protic environment, >90% relaxation should occur within 150 ms: but instead we observed similar on-and-off kinetics of cellular current responses as with slow-relaxing BTDAzo. We believe this indicates that the Z-to-E "off-switching" of btda4 is likewise majorly under photocontrol, not thermal relaxation: therefore, the environment relevant for action of btda4 is lipophilic. A similar solvent dependent behaviour for azobenzene-based photoswitches in membranes has been observed elsewhere,
e.g. in Lanzani's studies,[6] further experimental discussions of microscopic localisation and inhomogenous environment effects[7,8] are given in the section **Comment on Action Spectra** below; and although an alternative explanation could be that (very) slow off-rates could combine with suppression of thermal relaxation in the bound state to reproduce similar effects, and although conclusive investigations would require much further work beyond the scope of this paper, we note recent work showing that simply binding to a protein does not impede (photo)isomerisation of photoswitches[9] and we believe that for compounds where both $E$ and $Z$ isomers are capable of binding (certainly this is the case with the sterically larger BTDAzo, revealed through the competition experiment use of $E$-BTDAzo as well as the channel activation use of $Z$-BTDAzo), it is likely that the isomerisation process is at least not sterically forbidden in the protein.

**PSS spectra of btda1-7**

![PSS spectra of btda1-7](image)

*Figure S4* PSS spectra of **btda1-7** (in MeCN).
**Table S2.** Ratios of Z/E at relevant PSSs in ACN determined by HPLC at the isosbestic point.

| Isosbestic point [nm] | PSS at 365 nm | PSS at 440 nm |
|-----------------------|---------------|---------------|
| btda1 403             | 92:8          | 28:72         |
| btda2 434             | 86:14         | 6:94          |
| btda3 450             | 89:11         | 44:56         |
| btda5 405             | 96:4          | 26:74         |
| btda6 424             | 87:13         | 28:72         |
| btda7 417             | 96:4          | 30:70         |

*btda4* was not determined because its fast relaxation.
Cell biology

**Ca\(^{2+}\) influx assays in cell suspensions.** The initial characterisation of biological activity of bt\(\text{da}_{1-7}\) on TRPC5 was performed in a stably transfected HEK293 cell line that expresses TRPC5 after induction with 1 \(\mu\)M tetracycline (HEK\(\text{TRPC5-CFP}\)). Cells were induced to express TRPC5, harvested and loaded with the Ca\(^{2+}\) indicator dye as described earlier.\(^{[10]}\) The fluorescence imaging plate reader was modified with an additional high-power light-emitting diode (LED) to allow for an even illumination of the microplate at 365 nm. The original BTD compound and newly synthesised bt\(\text{da}_{1-7}\) compounds were serially diluted and applied to the microplate during fluorescence imaging by a liquid handling device equipped with a 384-well multichannel head (Fluent 480 with MCA384, Tecan, Männedorf, Switzerland). Fluorescence emission was continuously recorded with an Andor Zyla 5.5 camera (Andor, Belfast, UK) under the control of a \(\mu\)Manager 2.0 gamma imaging software.\(^{[11]}\) Data were averaged over single wells, and background signals were subtracted prior to export to MS Excel (Microsoft, Redmond, WA, U.S.A.). Graphs and concentration response curves were generated by fitting a four-parameter Hill equation to the experimental data (MS Excel Solver function).

**Microfluorometric Ca\(^{2+}\) influx assays in single cells.** To obtain action spectra and illumination-dependencies of on- and off-switching of functional responses in HEK\(\text{TRPC5-CFP}\) cells, we applied single-cell imaging of fluo-4-loaded adherent cells in an inverted microscope (Zeiss Axio, Zeiss AG, Jena, Germany) equipped with a 10x/0.5 Fluar objective (Zeiss AG) monochromator (Polychrome V)-assisted imaging system (TILL Photonics, Gräfelfing, Germany). The monochromator switching protocols were programmed as illustrated in Figure 3, and data were collected and processed in TILLVision 4.2 and MS Excel. After background subtraction, fluorescence intensity data were corrected for partial photobleaching of the single-wavelength Ca\(^{2+}\) indicator dye. Correction factors were derived from imaging untreated, fluo-4-loaded cells with the identical illumination protocols. The wavelength dependence of on- and off-switching was sampled in 10-nm-steps, and data from single cells as well as averaged data of all cells within a single experiment were obtained and displayed. To obtain the off-switching action spectrum, cells were exposed to a defined on-switching step (500 ms exposure to 360 nm light) followed by 200 ms of either no illumination, or illumination at the indicated wavelengths. The following rise in fluo-4-fluorescence imaged at short pulses of 480-nm light showed the functional response to the remaining fraction of active BTDAzo compound, and subsequent longer exposure (1.5 s) to 440 nm light served to switch the compound off again before repeating the cycle with another off-switching wavelength. The Xe lamp in the Polychrome V monochromator has no tunable intensity, therefore, the light power was not adjusted between the different wavelengths. Further discussion in section: Photoswitch-on and Photoswitch-off: extended characterisations.
Characterisation of BTDAzo-induced ionic currents. Electrophysiological recordings of TRPC5 currents were performed on adherent HEK<sub>TRPC5-CFP</sub> cells with an Axopatch 200B amplifier connected to a Digidata 1440A digitizer (Axon Instruments, Molecular Devices, San Jose, CA, U.S.A.). Borosilicate filaments, pipette and bath solutions were prepared as described<sup>10</sup> and supplemented with 10 µM BTDAzo for subsequent photoswitching during the recording of whole cell currents at -80 mV. A Polychrome II monochromator (TILL Photonics) was coupled to the inverted microscope (Zeiss Axiovert 100 with α Plan-Fluar 100x/1.45 objective) and controlled by the analog output of the Digidata 1440A device to apply 360 nm and 440 nm light in a timely fashion during the recording sweeps. Current voltage relationships were measured by applying slow (200 ms/100 mV) voltage ramps to the cells after illumination with 360 nm or 440 nm as illustrated. Holding currents were filtered at 1 kHz (four-pole Bessel filter) and sampled at 5 kHz. To characterize currents through heteromeric TRPC1:TRPC5 channel complexes, a stably transfected HEK<sub>TRPC5-CFP,TRPC1-YFP</sub> cell line was generated by transfecting the HEK<sub>TRPC5-CFP</sub> cell line with an expression plasmid encoding TRPC1-YFP, and isolating transfected colonies after selection with G418 (1 µg/ml). All results are expressed as current densities (pA/pF), the cell capacitance in the experiments shown was 10-12 pF.
FLIPR assays of btdas reveal good photoswitchability of Ca2+ currents with btda3

Figure S5 Comparative analysis of biological activity of btd1-btd7 (FLIPR). The btdas were serially diluted and applied to flou-4-loaded HEKmTRPC5-CFP cell suspensions in a fluorescence imaging plate reader device built into a robotic liquid handling system (Tecan Fluent 480). (a) Fluorescence intensities (readout at 480 nm excitation) were continuously recorded during application of the indicated concentrations of the compounds and subsequent alternating illumination with 365 nm and 447 nm light. Results for btda5-btda7 are not shown since they had no activity in TRPC5-expressing cells. (b) Concentration response curves were constructed from peak responses during the 5th illumination cycle (indicated by the dotted bracketing lines in panels (a)). All experiments conducted as minimum of 3 independent experiments with each experiment averaging data in technical duplicates.
Photoswitch-on and Photoswitch-off: extended characterisations

Setup: with the Polychrome V monochromator, the maximum intensity (Xenon light source) is at 470 nm with a maximum intensity drop at 400 nm of only 25% and power (as measured by output from a coupled fibre) is constant to within a factor of 2 over the full range of interest (370-480 nm), which should translate to fairly similar intensities also on the stage. Thus, the action spectra are so slightly biased towards longer wavelengths, that to best approximation the optimal wavelengths at identical intensities are indeed returned by our data.

Off-switching assay (Fig 3e-f): The off-switching response assay measures how completely and how photon-efficiently a given switchoff wavelength (200 ms) counteracts the $E\rightarrow Z$ isomerisation in an immediately preceding 360 nm phase (500 ms). The 360 nm part establishes a fixed Z/E ratio, which by itself would cause strong channel activation (see first cycle in Fig 3e, "none"). But if the switchoff pulse efficiently performs $Z\rightarrow E$ isomerisation, this Z/E ratio is reduced and so is channel activation as revealed during the next 20 seconds of imaging, seen as the peak height reached in each cycle: more peak suppression = better switchoff. After this 20 s imaging that reaches the peak plateau, the cells are reset towards a non-activated basal level to enable the next test round by the 1.5 seconds of 440 nm in the middle of the cycle does (validated in Fig S6); the 60 s imaging after that confirms that we are nearing or have reached a plateau. Thus each cycle tells us about the efficiency of the tested switchoff wavelength.

On-switching assay (Fig 3c-d): Conceptually similar to the off-switching assay, but somewhat simpler, here an activation wavelength is tested against a bracketing background of 440 nm switchoff phases, to see which activation wavelength most efficiently causes $E\rightarrow Z$ isomerisation and channel activation in these flux settings.

Practical Photoswitching: these tests are designed to operate with minimal flux, identifying effective wavelengths that will later allow a maximum of photoswitching cycles before bleaching and photodamage. Effective wavelengths (360/440 nm) are therefore identified, rather than theoretically more complete wavelengths that would however require prohibitively high light fluxes to be used, rendering them suboptimal in practice (370/480-520 nm).

Dark Delay in Imaging Experiments: Any excitation wavelength for imaging Fluo-4 intensities will cause at least a partial change of E/Z ratios. Dark periods were therefore included to allow $Ca^{2+}$ signals in TRPC5-expressing cells to develop for some time towards their plateau without such interference that would depress peak signals (there is a much slower response of the $Ca^{2+}$-imaging agent, on a seconds scale, than electrophysiology readouts that are on the millisecond scale). The data points used to construct the action spectrum were collected from
the very first images after the dark phase, reflecting the Ca\textsuperscript{2+} influx induced by the applied on-switching wavelength during a defined dark time interval.

Figure S6 Light energy dependence (power spectra) of BTDAzo-induced Ca\textsuperscript{2+} influx responses in single HEK\textsubscript{TRPC5-CFP} cells, reflecting E→Z and Z→E photoisomerisation in cells. Fluo-4-loaded adherent HEK\textsubscript{TRPC5-CFP} cells were imaged through a 10x/0.5 Fluar objective (Zeiss) in a monochromator (Polychrome V, TILL Photonics)-equipped imaging system built around an inverted microscope. Based on the action spectra shown in Fig. 3 of the main manuscript, the on- and off-photoswitching efficiency was assayed at optimal illumination wavelengths of 360 nm and 440 nm, respectively. (a) On-photoswitching responses in TRPC5-expressing cells in the presence of 1 µM BTDAzo. The on-photoswitching was induced by illuminating the visual field with 360 nm light for the indicated times. Imaging of fluo-4 fluorescence intensities was initiated 20 s after the onset of the on-switching illumination pulse. Shown are background- and photobleaching-corrected fluorescence intensities F after normalisation to the respective initial intensities F\textsubscript{0} determined in regions of interest that covered single cells, each (grey lines). Black line: averaged response of all cells measured in the experiment. (b) Semilogarithmic plot of on-switching illumination times and resulting peak fluorescence intensities in first images following the on-photoswitching illumination derived from data shown in (a). The second x axis depicts the energy of applied 360 nm light as calculated from the excitation power density of the imaging system and the respective illumination times. (c) Similar experiment as in (a), but with 10 µM BTDAzo in the bath solution and with a constant on-switching illumination time of 100 ms, immediately followed by illumination at 440 nm for the indicated times. Imaging of fluo-4 fluorescence intensities was done by applying short (40 ms) pulses of 480 nm excitation to minimize off-switching by the imaging procedure. After 30 s of imaging, 440 nm light was applied for 2 s to intentionally switch the compound back to its off-state. (d) Semilogarithmic plot of off-switching illumination times and resulting attenuation of Ca\textsuperscript{2+} influx signals in TRPC5-expressing HEK cells.

Comment on action spectra: The E→Z action spectrum scan (Fig 3d) used 100 ms pulses, which we know from the 360 nm E→Z power spectrum does not deliver PSS at this wavelength, or any other (as they do not have significantly greater bulk photoconversion rates). Therefore Fig 3d presents photon-flux-limited results that depend not only on the final PSS values that would eventually be reached after saturating illumination, but also on the rapidity
of bulk $E \rightarrow Z$ isomerisation, which is a practical parameter of great interest for biological use (to minimise sample bleaching etc). We believe that the PSS spectra and values that were measured in homogenous media (e.g. Fig 2d) do not sufficiently predict the narrowness of this action spectrum peak. Noting that bulk $E \rightarrow Z$ isomerisation rates depend on the relative magnitudes of the $E$ and $Z$ extinction coefficients, so that this ratio can be strongly affected by environment-dependency of absorption band breadth particularly in the $Z$ isomer, we believe the narrowed action spectrum reflects narrowed bands in the cellular setting, due to concentration of this lipophilic photoswitch into water-excluded cellular environments. Though measurements to study this appropriately would be outside the scope of this paper, we refer the interested reader to other examples of photoswitch performance being dependent on microscopic localisation and inhomogenous environment effects[7,8].

**E-BTDAzo antagonises channel activation by BTD**

![Graph showing dose-response of effect/baseline](image)

**Figure S7** E-BTDAzo dose-dependently inhibits BTD-induced Ca^{2+} currents. (Zero-concentration curve in panel b measured at edge of plate, magnitude of effect ca. 20% lower than other curves which are from interior wells)
btda compounds do not activate TRPC4

Figure S8 Lack of biological activity of btda1-btda7 compounds on TRPC4. The selectivity of btda1-btda7 was assessed by FLIPR in fluo-4-loaded HEK cell suspensions stably overexpressing mouse TRPC4β C-terminally fused to cyan fluorescent protein (HEKTRPC4-CFP). TRPC4 is the closest relative of TRPC5 and capable of forming functionally active homotetrameric channel complexes. The experimental procedure followed that described in Fig. 3a,b of the main manuscript.

Mouse experiments

Mice. Adult female mice (7 - 20 weeks old) were kept under standard light/dark cycle (12:12; lights on 0600; lights off 1800) with food (Ssniff feed containing 9% fat, 24% protein, and 67% carbohydrate) and water ad libitum. Mice were maintained in IVC housing containing enrichment (nesting, bedding and other material). We used the following mouse strains: B6.Cg-7630403G23RikTg(Th-cre)1Tmd/J (RRID:IMSR_JAX:008601, referred to as Th-Cre mice) B6;129S-Gt(Rosa)26Sortm95.1(CAG-GCaMP6f)Hze/J (RRID:IMSR_JAX:024105, referred to as R26-GCaMP6f or Ai95D mice) and Trpc5tm1.1Lbi (RRID:IMSR_JAX:024535; MMRRC Stock No: 37349-JAX, referred to as Trpc5-E5−/− mice. We crossed Th-Cre mice with R26-GCaMP6f reporter mice resulting in a strain in which all Th+ cells are identifiable through their green fluorescence (referred to as Th-GCaMP6f mice). These mice were also crossed with Trpc5-E5−/− mice, resulting in a strain in which all Th+ cells, identifiable through their green fluorescence, are deficient for Trpc5-E5 (referred to as Th-GCaMP6f-Trpc5). Th-GCaMP6f and Th-GCaMP6f-Trpc5 mice were heterozygous for Cre and GCaMP6f.

Animal care and experimental procedures were performed in accordance with the guidelines established by the German Animal Welfare Act, European Communities Council Directive...
2010/63/EU, the institutional ethical and animal welfare guidelines of the Saarland University (approval number of the Institutional Animal Care and Use Committee: H-2.2.4.1.1). The number of animals used is a minimum necessary to provide adequate data to test the hypotheses of this project. We minimized the number of animals required by the animal welfare committees wherever possible.

**Solutions and Chemicals.** Oxygenated extracellular solution aCSF (95% O₂/5% CO₂) contained (in mM): 120 NaCl (Gruessing, Germany), 25 NaHCO₃ (Merck, Darmstadt, Germany), 5 KCl (Gruessing, Germany), 5 N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), 1 MgSO₄, 1 CaCl₂ (Gruessing), 10 glucose (Merck). Prolactin stock solutions were prepared in distilled water (1 mg/ml), diluted in aCSF and stored at -20 °C. BTD (Bio-Technne GmbH, Germany) stock solution was prepared in dimethyl sulfoxide (DMSO; 5 mM) and stored at -20 °C. The BTD stock solution was then diluted in aCSF to make final working concentrations of 1, 5, and 10 µM on the day of the experiment. BTDAzo (btda3) was prepared in DMSO (20 mM) and diluted in aCSF to 100 µM. Warming the solution to 40 °C combined with a short sonication yielded a clear yellowish solution of the BTDAzo. The BTDAzo stock was further diluted in aCSF to yield 10 µM BTDAzo at the day of the experiment. DMSO concentrations were ≤ 0.05 % (vol/vol) in the final working solution. Unless stated otherwise, all chemicals were purchased from Sigma (Munich, Germany). Chemicals were of analytical or higher grade.

**Preparation of Hypothalamic Brain Tissue Slices.** All experiments were performed on coronal brain slices (Bregma -1.6 and -2.2 mm) freshly prepared from female mice adapting previously described methods. Mice were anesthetized with isoflurane, followed by decapitation. Brains were removed quickly, submerged in ice-cold extracellular aCSF solution, and sliced (275 µm thick) using a vibrating-blade microtome (Leica, Germany). Slices were
kept in oxygenated aCSF (95% O\textsubscript{2}/5% CO\textsubscript{2}) at 31.5 °C for 15 min and then at RT for 30 min before starting an experiment.

**Ca\textsuperscript{2+} Imaging and Combined Laser Scanning-controlled Photoswitching.** We used an upright scanning confocal microscope (Zeiss LSM 880 Indimo) equipped with a standard Argon laser for excitation at a wavelength of 488 nm and a UV laser (Coherent) emitting 355 nm.\textsuperscript{18–20} Emitted fluorescence was collected between 500 and 560 nm. All scanning head settings were kept constant during each experiment. Images were acquired at 1.7 Hz and analysed using a combination of Zen (Zeiss), ImageJ (NIH), Igor (Wavemetrics) and OriginLab (OriginLab Corporation) software.

For laser scanning-controlled photoswitching of BTDAzo, the UV laser light coupled to the confocal microscope was focused onto the image plane through a 20 x 1.0 NA Plan-Apochromat water immersion objective (Zeiss). The depth of focus was 16 µm which ensured, together with the region of interest (ROI) diameter, illumination of individual cells. Before photoswitching, UV laser light was optimally focused using 18 µm thick brain tissue sections loaded with Hoechst 33342 (1:10000; ThermoFisher) and the semi-automated correction tool of the Zen software (Zeiss). BTDAzo (10 µM) was added to the bath chamber containing the brain slice and subsequently incubated for about 15 min in the dark. GCaMP6f fluorescence was then measured in the presence of BTDAzo using the 488 nm Argon laser (2% 25 mW). Photoswitching was achieved by directing UV laser light (6, 10 or 15 mW) on preselected ROIs using the Zen software (Zeiss), followed by exposure to the Argon laser light to resume monitoring of GCaMP6f fluorescence and switching BTDAzo back into the inactive state. For a typical single Th+ neuron with a size of approximately 154 µm\textsuperscript{2}, a UV stimulation consisted of 10 individual scans of this ROI (647 pixels with a pixel size of 0.238 µm\textsuperscript{2}). Given a laser dwell time of 2.05 µs/pixel, the total UV exposure (= number of scans x area x dwell time/pixel) was thus 13.3 ms in this example. Due to slight changes in individual neuron size, UV exposure
ranged from 10 to 20 ms in the experiments shown in Fig 4. Depending on the depth of a given cell within the tissue slice, UV laser power was set to either 6, 10 or 15 mW.

Additional experiments analyzed the response delay as a function of total UV exposure time (Fig S9d-e). Here, the UV stimulation (6 mW or 10 mW 355 nm) consisted of either (i) 300 individual scans across the cell soma with a pixel dwell time of 0.82 μs or 1.02 μs, or (ii) 100 individual scans with a pixel dwell time of 2.05 μs.

To protect the ultrasensitive GaAsP photomultiplier tubes from the 355 nm light, high-speed shutters closed and opened at the beginning and end of the UV exposure. Hence, GCaMP6f fluorescence could not be collected during this time (see example Fig S9d). The response delay was calculated from the end of the UV stimulation protocol until the time when the GCaMP6f fluorescence baseline or peak values increased their previous values (i.e. before UV stimulation) by 2 x SD.

Changes in GCaMP6f fluorescence were expressed as relative fluorescence changes, i.e. \( \Delta F/F_0 \) (\( F_0 \) was the average of the fluorescence values of 30 frames before stimulation). Fluorescence (F) data were normalized to the Peak_{max} obtained during control measurements and plotted as a function of time (t). To quantify the changes in the dynamics of individual \( \mathrm{Ca}^{2+} \) responses, we calculated the area under the curve (AUC) as a measure for the increase in intracellular \( \mathrm{Ca}^{2+} \). A control AUC was taken at the beginning of each recording for 3 min. The AUCs of BTD and BTDAzo were calculated from the last 3 min of the recording. The duration of the recordings was 9 min. Duration of the \( \mathrm{Ca}^{2+} \) recordings after prolactin treatment lasted 15 min because more time is needed to induce an increase in calcium fluorescence. Therefore, control AUCs in these recordings were taken for 5 min at the beginning of the recording. Prolactin-induced AUCs were taken from the last 5 min of the recording.

**Quantification and Statistical Analysis.** Statistical analyses were performed using Origin Pro 2017G (OriginLab Corporation, Northampton, MA, USA). Assumptions of normality and
homogeneity of variance were tested before conducting the following statistical approaches. A paired two-tailed Student's t-test was used to measure the significance of the differences between two distributions (treatments) of the same Th+ neuron. Multiple groups were compared using a two-way analysis of variance (ANOVA) with Tukey's multiple comparison test as a posthoc comparison. In case the results failed the test of normality, the Kruskal-Wallis ANOVA in combination with the Dunn's test were performed. The probability of error level (alpha) was chosen to be 0.05. Unless otherwise stated, data are expressed as means ± SD. Legends of the figures indicate the number of independent measurements. We used at least 3 mice per genotype, as required by the animal welfare committee.

![Figure S9](image)

**Figure S9:** (a) AUC (area under the curve) plots from individual Th+ neurons shown in Fig. 4h. In the presence of 10 µM BTDAzo, AUC values increased after 355 nm UV light illumination in Th+ neurons of Th-GCaMP6f mice (n = 5). Paired t-test: \( t(34) = 8.183, p < 0.0001 \). (b) TRPC5 channel knockout prevented the UV light-induced increase in AUC in Th+ neurons of Th-GCaMP6f-∆Trpc5 mice (n = 3). Paired t-test: \( t(21) = 0.289, p = 0.78 \). (c) Application of 10 µM BTD produced very similar results as UV stimulation of BTDAzo in Th+ neurons of Th-GCaMP6f mice (n = 4). Paired t-test: \( t(25) = 5.530, p < 0.0001 \). Number of individual cells is indicated in parentheses above each bar. (d) Example showing BTDAzo stimulation with a longer total UV exposure time resulting in a much shorter delay (6.2 s) of the TRPC5-dependent Ca\(^{2+}\) response. The UV stimulation (6 mW 355 nm) consisted of 300 individual scans of the cell soma with a pixel dwell time of 0.82 µs which resulted in a total UV exposure time of 159 ms.
The time required for the repeated UV laser stimulation protocol and the opening/closing of the laser shutter was 11.5 s. (e) Group data showing a plot of response delay vs. total UV exposure time. With exposure times > 120 ms, the delays ranged between 0.7 - 27.5 s (mean ± SD: 10.6 ± 7.5 s, n = 14). Dashed line is an exponential fit of the datapoints to determine the horizontal asymptote reflecting the mean response delay. The pink data point labelled (d) represents the example shown in (d).
HPLC & NMR Spectra

HPLC traces of E-btda1-7 with absorption at 254 nm.
$^1$H NMR (400 MHz, DMSO $d_6$)

$^{13}$C NMR (101 MHz, DMSO $d_6$)
$^1$H NMR (500 MHz, DMSO d6)

$^{13}$C NMR (126 MHz, DMSO d6)
NMMCO

$^1$H NMR (400 MHz, DMSO $d_6$)

$^{13}$C NMR (101 MHz, DMSO $d_6$)
$^1$H NMR (400 MHz, DMSO d6)

$^{13}$C NMR (101 MHz, DMSO d6)
$^1$H NMR (400 MHz, DMSO d6)

$^{13}$C NMR (101 MHz, DMSO d6)
\[ ^1H \text{NMR} \ (400 \text{ MHz, DMSO } d6) \]

\[ ^{13}C \text{NMR} \ (101 \text{ MHz, DMSO } d6) \]
2D NOE spectrum of BTDAzo (btda3): NOE cross peak between methoxy groups at 3.90 ppm and 3.78 ppm indicating the desired O-alkylated product.
1D NOE spectrum of btda2: pulse at 3.89 ppm on the methoxy group and NOE cross peak at 7.17 and 7.14 ppm with ortho protons indicating the desired O-alkylated product.
$^1$H NMR (400 MHz, DMSO $d_6$)

$^{13}$C NMR (101 MHz, DMSO $d_6$)
$^1$H NMR (400 MHz, DMSO d6)

$^13$C NMR (101 MHz, DMSO d6)
1-H,1H COSY of btda7 to resolve the CH group of the isopropyl and the CH₂ group connected to the amide from the overlaying water solvent peak.
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Figure 1

Scheme 1
Figure 2

a) E-BTDAzo

440 - 520 nm: up to 87% and thermal

Z-BTDAzo

b) PSS Spectra (EtOAc/Hex 1:1)

c) Z:E ratio at PSS in λ [nm] 90/10 H2O/DMSO 50/50 DMSO EtOAc/Hex

| PSS | 360  | 380  | 400  | 440  | 480  | 520  |
|-----|------|------|------|------|------|------|
| 360 | 77:23| 85:15| 77:23| 42:58| 23:77| 18:82|
| 380 | 86:14| 94:6 | 90:10| 43:57| 20:80| 13:87|
| 400 |      |      |      |      |      |      |
| 440 |      |      |      |      |      |      |
| 480 |      |      |      |      |      |      |
| 520 |      |      |      |      |      |      |

d) Isolated Spectra (MeCN)

e) Photoswitching (H2O/DMSO)
Figure 3

**HEK cell Ca\(^{2+}\) influx photomodulation with BTDazo (365/447 nm illumination cycles) - plate imager**

**HEK cell Ca\(^{2+}\) influx photomodulation with BTDazo - single cell fluorescence microscopy analysis**

**Whole-cell recordings - BTDazo-photomodulated electrophysiology**

*Figure 3 diagrams and data*
