Tetrazine-Triggered Bioorthogonal Cleavage of trans-Cyclooctene-Caged Phenols Using a Minimal Self-Immolative Linker Strategy**

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1 General Methods

Unless otherwise noted, reactions were carried out under an atmosphere of argon in air-dried glassware with magnetic stirring. Air- and/or moisture-sensitive liquids were transferred via syringe. All reagents were purchased from commercial suppliers and used without further purification. Combretastatin A-4 (CA4) was obtained from BLD Pharmatech. Amberlite IR120 hydrogen form ion-exchange resin was purchased from Sigma Aldrich and washed prior use. Dichloromethane, ethyl acetate and methanol were obtained from Donau Chemie AG. Dichloromethane was dried using PURESOLV-columns (Innovative Technology). Dry DMSO and DMF were obtained from ACROS Organics and stored under argon. Organic solvents used for preparative HPLC were purchased from VWR (acetonitrile) and Sigma Aldrich (hexane). HPLC-grade water was obtained by using a Purelab Chorus 1 water purification system (ELGA).

Thin layer chromatography was performed using TLC alumina plates (Merck, silica gel 60, fluorescence indicator F254). Visualization of the spots was achieved either by UV irradiation (254 or 365 nm) or by heat staining with ceric ammonium molybdate in ethanol/sulfuric acid.

Preparative HPLC and flash chromatography were carried out on a Grace REVELERIS Prep purification system using a Kinex 5µm C18 100Å, AXIA Packed LC Column 100 x 30.0mm (Phenomenex) for preparative RP-HPLC or a Luna 10µm Silica (2) 100Å, LC Column 250 x 21.2 mm (Phenomenex) for preparative NP-HPLC purifications. Silica gel 60 (40-63µm) was purchased from Merck.

HPLC-MS (LCMS) analysis was performed on a Nexera X2 system (Shimadzu) comprised of LC-30AD pumps, a SIL-30AC autosampler, a CTO-20AC column oven, and a DGU-20A5/3 degasser module. Detection was done using an SPD-M20A photo diode array, an RF-20Ax fluorescence detector, an ELS-2041 evaporative light scattering detector (JASCO) and an LCMS-2020 mass spectrometer (ESI/APCI). If not stated otherwise, all separations were performed using a Waters XSelect CSH™ C18 2.5 µm (3.0 x 50 mm) column XP at 40 °C and a flowrate of 1.7 mL/min with 0.1% aqueous formic acid or ammonium formate buffer (2.5 mM, pH8.4) and acetonitrile (gradient elution). Acidic HPLC conditions (acetonitrile/0.1% formic acid) 0 min: 5%, 0.15 min: 5%, 2.20 min: 98%, 2.50 min: 98%; Buffered HPLC conditions (acetonitrile/2.5 mM ammonium formate buffer, pH8.4) 0 min: 5%, 0.15 min: 5%, 2.20 min: 98%, 2.50 min: 98%. See section 3 for further details on buffer preparation.

$^1$H and $^{13}$C NMR spectra were recorded on a Bruker Ascend 600 MHz spectrometer at 20 °C. Chemical shifts (δ) are reported in ppm relative to tetramethylsilane and calibrated using solvent residual peaks. Data is shown as follows: Chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, m = multiplet, br = broad signal), coupling constants (J, Hz) and integration.

HRMS analysis of aqueous or acetonitrile solutions of the compounds (sample concentration: 10 ppm) was carried out on an Agilent 6545 Q-TOF mass spectrometer equipped with an Agilent Dual AJS ESI source. The mass spectrometer was connected to a liquid chromatography system comprised of an Agilent G7167B multi sampler, an Agilent G7120A binary pump with degasser and an Agilent G7116B oven (Agilent Technologies, Palo Alto, CA, USA). A SecurityGuard Cartridge (Phenomenex) was used as a stationary phase. Data evaluation was performed using Agilent MassHunter Workstation Qualitative Analysis 10.0. Identification was based on peaks obtained from extracted ion chromatograms (extraction width ± 20 ppm).
2 Synthesis

rTCO-caged BODIPY-labeled released probe

Tyr-BODIPY (S1)

To a solution of BODIPY-acid\[^{[1]}\] (20 mg, 62.5 µmol) in DMSO (4 mL) were added L-tyrosine methyl ester hydrochloride (40.5 mg, 174.9 µmol), HBTU (35.5 mg, 93.7 µmol) and DIPEA (53.1 µL, 40.3 mg, 312 µmol). LCMS analysis indicated complete conversion to the desired product after stirring for 1 h at room temperature (rt). Purification by preparative RP-HPLC (H₂O/MeCN gradient elution, 0.1% formic acid) afforded S1 as an orange solid (28.0 mg, 90%). \[^{[1]}\]H NMR (600 MHz, CD₂Cl₂) δ 6.91 (d, J = 8.4 Hz, 2H), 6.69 (d, J = 8.5 Hz, 2H), 6.13 – 6.01 (m, 2H), 4.85 – 4.79 (m, 1H), 3.72 (s, 3H), 3.30 – 3.17 (m, 2H), 3.08 (dd, J = 14.1, 5.6 Hz, 1H), 2.93 (dd, J = 14.1, 6.5 Hz, 1H), 2.48 – 2.33 (m, 14H); \[^{13}\]C NMR (151 MHz, CD₂Cl₂) δ 172.36, 170.63, 155.70, 154.82, 144.69, 141.22, 131.58, 130.69, 127.86, 122.21, 115.80, 53.78, 52.76, 37.36, 37.25, 24.06, 16.61, 14.59; HRMS [M+Na]^+ calcd. 520.2189 for C₂₆H₂₆BF₄N₂O₃Na⁺, found 520.2210.

PNP-Tyr-BODIPY (3)

To a solution of S1 (28.0 mg, 56.3 µmol) in CH₂Cl₂ (4 mL) was added a solution of 4-nitrophenyl chloroformate (PNP chloroformate) (12.5 mg, 61.9 µmol) in CH₂Cl₂ (1 mL) and Et₃N (18.8 µL, 13.7 mg, 135.1 µmol). The reaction mixture was heated to reflux for 1 h after which TLC indicated full consumption of the starting material. Purification by preparative HPLC (hexane/EtOAc gradient elution) afforded 3 as an orange solid (34.5 mg, 93%). \[^{1}\]H NMR (600 MHz, CD₂Cl₂) δ 8.33 – 8.27 (m, 2H), 7.51 – 7.46 (m, 2H), 7.23 – 7.19 (m, 2H), 7.19 – 7.15 (m, 2H), 6.08 (s, 2H), 6.04 (d, J = 7.8 Hz, 1H), 4.88 (dt, J = 7.8, 6.1 Hz, 1H), 3.73 (s, 3H), 3.31 – 3.18 (m, 3H), 3.07 (dd, J = 14.0, 6.4 Hz, 1H), 2.51 – 2.39 (m, 14H); \[^{13}\]C NMR (151 MHz, CD₂Cl₂) δ 172.00, 170.27, 155.67, 154.80, 151.50, 150.22, 146.09, 144.82, 141.19, 135.25, 131.60, 130.92, 125.75, 122.25, 122.18, 121.30, 53.51, 52.81, 37.57, 37.35, 24.11, 16.63, 14.59; HRMS [M+Na]^+ calcd. 685.2252 for C₃₃H₂₂BF₄N₂O₆Na⁺, found 685.2280.
A solution of rTCO-PNP (1)\(^{[2]}\) (axial isomer, 20.2 mg, 69.5 µmol) in CH\(_2\)Cl\(_2\) (4 mL) was added via syringe pump (30 µL/min) to a solution of N,N'-dimethylethlenediamine (DMEDA) (74.9 µL, 61.3 mg, 695 µmol) in CH\(_2\)Cl\(_2\) (1.2 mL) and DMF (0.4 mL) at 0 °C. Upon complete addition after 2h, stirring at 0 °C was continued for 1 h. Excess of DMEDA was then removed under high vacuum (3 h) to obtain crude rTCO-DMEDA (2), which was then redissolved in CH\(_2\)Cl\(_2\) (860 µL) and added to a solution of 3 (30.7 mg, 46.3 µmol) in DMF (430 µL) at rt. Excess of DMEDA was then removed under high vacuum (3 h) to obtain crude rTCO-DMEDA (2), which was then redissolved in CH\(_2\)Cl\(_2\) (860 µL) and added to a solution of 3 (30.7 mg, 46.3 µmol) in DMF (430 µL) at rt. LCMS indicated complete conversion after a reaction time of 1 h. Purification by preparative HPLC (hexane/EtOAc gradient elution) afforded 4 as an orange solid (24.0 mg, 68%). \(^1\)H NMR (600 MHz, CD\(_2\)Cl\(_2\), mixture of rotamers) δ 7.10 – 7.04 (m, 2H), 7.03 – 6.96 (m, 2H), 6.09 (s, 2H), 6.06 (s, 1H), 5.85 – 5.71 (m, 1H), 5.58 – 5.46 (m, 1H), 4.87 – 4.80 (m, 1H), 3.72 (s, 3H), 3.68 – 3.38 (m, 4H), 3.33 – 3.21 (m, 2H), 3.18 – 3.11 (m, 1H), 3.11 – 2.89 (m, 7H), 2.53 – 2.34 (m, 15H), 2.19 – 1.62 (m, 6H), 1.56 – 1.41 (m, 1H), 1.15 – 0.98 (m, 1H), 0.86 – 0.72 (m, 1H); \(^1\)C NMR (151 MHz, CD\(_2\)Cl\(_2\), mixture of rotamers) δ 172.11, 170.28, 170.26, 156.00, 155.60, 155.08, 154.92, 154.79, 154.73, 151.16, 151.12, 151.06, 151.04, 144.95, 141.26, 133.47, 133.35, 133.21, 132.10, 131.98, 131.62, 130.40, 130.33, 122.41, 122.36, 122.31, 122.23, 122.15, 74.98, 74.91, 74.76, 74.66, 53.63, 52.72, 47.85, 47.70, 47.45, 47.35, 47.20, 47.15, 46.71, 46.58, 41.17, 41.05, 37.47, 37.44, 37.30, 36.41, 36.36, 36.23, 36.15, 36.07, 35.64, 35.56, 35.52, 35.46, 35.30, 35.24, 34.98, 34.57, 32.31, 30.18, 30.13, 30.09, 29.91, 29.76, 29.72, 29.65, 29.61, 29.56, 29.45, 29.38, 27.57, 27.55, 25.89, 24.74, 24.55, 24.16, 23.09, 16.66, 14.59; HRMS [M+Na]\(^+\) calcd. 786.3820 for C\(_{40}\)H\(_{52}\)BF\(_2\)N\(_{6}\)O\(_{10}\)Na\(^+\), found 786.3849.

To a solution of 4 (21.7 mg, 28.4 µmol) in isopropanol (IPA) (2.84 mL) was added 1M aqueous LiOH solution (2.84 mL) and the mixture was stirred for 20 min at rt, after which LCMS indicated complete saponification. A mixture of IPA and H\(_2\)O (4 mL, IPA/H\(_2\)O = 1:1) was added followed by addition of Amberlite IR120 (1.70 mL) and the suspension was rigorously stirred for 2 min. The solvent was removed upon filtration to obtain the free acid 5, which was used without further purification. To a solution of crude 5 in DMF (1.6 mL) was added HBTU (21.5 mg, 56.8 µmol), DIPEA (24.6 µL, 18.4 µmol, 142.0 µmol) and mPEG\(_7\)-NH\(_2\) (18.4 µL, 19.3 mg, 56.8 µmol) and the mixture was stirred for 1 h at rt. LCMS indicated complete conversion to the desired product. Purification by preparative RP-HPLC (H\(_2\)O/McCN gradient elution, 0.1% formic acid) afforded 6 as a red-brown solid (23.1 mg, 76%). \(^1\)H NMR (600 MHz, CD\(_2\)Cl\(_2\), mixture of rotamers) δ 7.22 – 7.10 (m, 2H), 7.06 – 6.93 (m, 2H), 6.57 (s, 2H), 6.08 (s, 2H), 5.87 – 5.71 (m, 1H), 5.59 – 5.46 (m, 1H), 4.64 (q, J = 7.1 Hz, 1H), 3.72 – 2.89 (m, 45H), 2.58 – 2.28 (m, 15H), 2.05 – 1.60 (m, 6H), 1.54 – 1.35 (m, 1H), 1.16 – 0.99 (m, 1H), 0.87 – 0.74 (m, 1H); \(^1\)C NMR (151 MHz, CD\(_2\)Cl\(_2\), mixture of rotamers) δ 170.80, 170.32, 170.30, 155.98, 155.86, 155.58, 155.12, 154.95, 154.82, 154.65, 150.96, 150.92, 150.87, 150.83, 145.17, 141.29, 134.24, 134.14, 134.03, 132.08, 132.00, 131.96, 131.62, 130.53, 130.46, 122.26, 122.20, 122.10, 74.97, 74.88, 74.75, 74.65, 72.28, 70.89, 70.87, 70.86, 70.84, 70.76, 70.62, 69.78, 59.00, 54.65, 54.57, 47.86, 47.70, 47.48, 47.34, 47.23, 47.16, 46.72, 46.61, 41.17, 41.06, 39.73, 38.40, 38.34, 38.29, 37.32, 36.41, 36.38, 36.23, 36.16, 35.65, 35.58, 35.55, 35.45, 35.32, 35.26, 34.98, 34.59, 29.45, 29.39, 24.75, 24.55, 24.25, 16.69, 14.58; HRMS [M+H]\(^+\) calcd. 1071.5996 for C\(_{46}\)H\(_{62}\)BF\(_2\)N\(_{6}\)O\(_{15}\)\(^+\), found 1071.6026.

**Synthesis of tetrazines**

DMT (7)\(^{[2]}\), PA\(_2\) (8)\(^{[3]}\) and PymK (9)\(^{[4]}\) were prepared according to known procedures.
Synthesis of sulfo-cTCO-DMEDA-caged CA4-prodrug 12

To a solution of CA4 (27.0 mg, 85.4 μmol) in CH₂Cl₂ (1 mL) was added a solution of PNP chloroformate (20.7 mg, 102.5 μmol) in CH₂Cl₂ (1 mL) and Et₃N (28.6 μL, 207 μmol). The mixture was heated to reflux for 1 h, after which TLC indicated full consumption of the starting material. Purification by preparative HPLC (hexane/EtOAc gradient elution) afforded 10 as an off-white solid (38.0 mg, 92%).¹H NMR (600 MHz, CDCl₃) δ 8.32 – 8.27 (m, 2H), 7.48 – 7.42 (m, 2H), 7.21 (dd, J = 8.5, 2.1 Hz, 1H), 7.17 (d, J = 2.1 Hz, 1H), 6.95 (d, J = 8.5 Hz, 1H), 6.54 – 6.48 (m, 4H), 3.88 (s, 3H), 3.75 (s, 3H), 3.67 (s, 6H); ¹³C NMR (151 MHz, CDCl₃) δ 155.89, 153.57, 150.97, 150.37, 146.02, 139.75, 137.81, 132.64, 130.71, 130.47, 128.95, 128.42, 125.72, 122.76, 122.19, 112.77, 106.31, 60.84, 56.49, 56.19; HRMS [M+H]⁺ calcd. 482.1446 for C₂₃H₂₈NO₅⁺, found 482.1447.

cTCO-PNP (11)

To an ice-cooled solution of cTCO-OH[5] (axial isomer, 80.0 mg, 404 μmol) in CH₂Cl₂ (2 mL), 4-((N,N-Dimethylaminopyridino)pyridine (DMAP) (197.3 mg, 1615 μmol) in CH₂Cl₂ (1 mL) and PNP chloroformate (162.8 mg, 808 μmol) in CH₂Cl₂ (1 mL) were added and the mixture was stirred at rt for 4 h. Purification by column chromatography (CH₂Cl₂, isocratic elution) afforded 11 as an off-white solid (73.2 mg, 50%).¹H NMR (600 MHz, CDCl₃) δ 8.32 – 8.23 (m, 2H), 7.46 – 7.36 (m, 2H), 6.09 – 5.98 (m, 1H), 5.65 (dd, J = 16.8, 2.6 Hz, 1H), 5.26 (s, 1H), 3.62 (s, 3H), 2.37 – 2.25 (m, 2H), 2.17 (ddd, J = 14.0, 12.1, 5.0 Hz, 2H), 1.98 – 1.85 (m, 3H), 1.70 (ddd, J = 12.5, 9.0, 5.4 Hz, 1H), 1.13 (s, 3H); ¹³C NMR (151 MHz, CDCl₃) δ 180.00, 156.05, 151.98, 145.79, 133.33, 129.81, 125.62, 122.29, 77.48, 52.24, 45.15, 44.92, 35.95, 31.24, 30.90, 18.47; HRMS [M+H]⁺ calcd. 364.1319 for C₁₈H₂₆NO₅⁺, found 364.1319.

cTCO-DMEDA-CA4 (S3)

A solution of cTCO-PNP (11) (axial isomer, 24.2 mg, 66.6 μmol) in CH₂Cl₂ (3.8 mL) was added via syringe pump (30 μL/min) to a solution of DMEDA (71.7 μL, 58.7 mg, 666 μmol) in CH₂Cl₂ (1.15 mL) and DMF (0.37 mL) at 0 °C. Upon complete addition after 2 hours, stirring at 0 °C was continued for 1 h. Excess of DMEDA was removed under high vacuum (3 h) to obtain crude cTCO-DMEDA (S2), which was redissolved in CH₂Cl₂ (840 µL) and added to a solution of 10 (22.7 mg, 47.2 μmol) in DMF (420 µL) at rt. LCMS indicated complete conversion after a reaction time of 1 h. Purification by preparative HPLC (CH₂Cl₂/MeOH
gradient elution) afforded S3 as a colorless oil (19.0 mg, 61%). 1H NMR (600 MHz, CD3Cl, mixture of rotamers) δ 7.12 (d, J = 8.5 Hz, 1H), 7.07 – 7.01 (m, 1H), 6.85 (d, J = 8.5 Hz, 1H), 6.52 (s, 2H), 6.50 – 6.42 (m, 2H), 5.94 – 5.76 (m, 1H), 5.67 – 5.54 (m, 1H), 5.15 (d, J = 17.7 Hz, 1H), 3.79 (d, J = 5.5 Hz, 3H), 3.75 (s, 3H), 3.68 (s, 6H), 3.64 – 3.42 (m, 7H), 3.12 – 2.89 (m, 6H), 2.30 – 1.58 (m, 8H), 1.14 – 1.05 (m, 3H); 13C NMR (151 MHz, CD3Cl, mixture of rotamers) δ 180.32, 180.24, 180.21, 155.75, 155.68, 155.40, 154.65, 154.60, 154.42, 153.47, 151.38, 151.33, 140.72, 140.65, 140.59, 137.68, 132.90, 132.87, 132.86, 131.91, 131.86, 131.81, 131.77, 130.32, 130.28, 129.72, 129.67, 128.96, 127.48, 127.43, 127.39, 124.26, 124.17, 124.10, 124.00, 112.41, 112.30, 112.20, 106.37, 73.22, 73.07, 73.01, 60.82, 56.33, 56.29, 56.24, 56.19, 52.15, 48.18, 47.96, 47.69, 47.60, 47.53, 47.15, 46.68, 45.27, 45.22, 44.98, 36.21, 36.17, 35.79, 35.57, 35.45, 35.21, 34.95, 31.31, 31.22, 31.17, 18.37; HRMS [M+H]+ calcd. 655.3225 for C35H37N2O10+, found 655.3216.

sulfo-CTCO-DMEDA-CA4 (12)

To a solution of S3 (11.7 mg, 17.8 µmol) in IPA (0.5 mL) was added 5 M aqueous KOH solution (0.5 mL) and the mixture was stirred for 43 h at rt, after which LCMS indicated complete saponification. IPA/H2O (2 mL, 1:1) and Amberlite IR120 (1.50 mL) were added, and the solution was rigorously stirred for 1 min. The mixture was filtered and concentrated to obtain free acid S4, which was used without further purification. To a solution of S4 in DMSO (1.0 mL), HBTU (10.1 mg, 26.7 µmol), DIPEA (15.3 µL, 11.5 mg, 89.6 µmol) and taurine (6.2 mg, 49.8 µmol) were added, and the mixture was stirred at rt for 1 h. Purification by preparative RP-HPLC (phosphate buffer/MeCN gradient elution) and subsequent solid phase extraction afforded the sodium salt of 12 as a colorless oil (7.6 mg, 56%). Solid phase extraction was performed using a Strata® C18-E (55 µm, 70 Å), 50 mg / 1 mL tube (Phenomenex). The tube was conditioned with MeCN (600 µL) and equilibrated with H2O (1800 µL). After preparative HPLC and evaporation of the solvent, the residue (containing the product and buffer salts) was dissolved in H2O (300 µL) and loaded onto the tube. After washing with H2O (600 µL), 12 was eluted with MeCN (600 µL) and the solvent was removed. 1H NMR (600 MHz, DMSO-d6, mixture of rotamers) δ 7.54 – 7.40 (m, 1H), 7.11 (dd, J = 8.5, 2.2 Hz, 1H), 7.07 – 6.92 (m, 2H), 6.62 – 6.53 (m, 2H), 6.53 – 6.42 (m, 2H), 5.80 – 5.65 (m, 1H), 5.65 – 5.52 (m, 1H), 5.00 (dd, J = 27.4, 15.3 Hz, 1H), 3.79 – 3.72 (m, 3H), 3.69 – 3.60 (m, 9H), 3.58 – 3.35 (m, 4H), 3.27 (s, 2H), 3.12 – 2.78 (m, 6H), 2.55 – 2.51 (m, 2H), 2.18 – 1.32 (m, 8H), 0.95 (d, J = 23.9 Hz, 3H); 13C NMR (151 MHz, DMSO-d6, mixture of rotamers) δ 178.99, 178.90, 154.69, 154.64, 154.46, 154.40, 153.57, 153.43, 153.32, 153.03, 152.59, 150.71, 150.63, 139.69, 139.65, 136.77, 136.71, 132.05, 132.01, 131.65, 131.33, 130.97, 130.90, 130.83, 130.79, 129.20, 129.14, 129.07, 128.35, 128.28, 128.61, 126.68, 123.47, 123.21, 112.48, 112.32, 105.93, 103.66, 72.38, 72.20, 72.15, 60.05, 60.01, 55.85, 55.80, 55.62, 55.59, 50.08, 46.79, 46.69, 46.45, 46.34, 46.27, 45.99, 45.65, 45.58, 45.01, 43.48, 43.44, 35.68, 35.35, 35.30, 35.25, 35.20, 34.82, 34.70, 34.65, 34.60, 34.40, 34.20, 33.97, 30.85, 30.75, 30.63, 30.58, 17.82; HRMS [M+H]+ calcd. 748.3110 for C36H36N2O15S4, found 748.3095.
3 Release Experiments

Instrument and solvents
Reaction monitoring of release experiments was performed on a Nexera X2® UHPLC system (Shimadzu®) with a temperature-controlled autosampler at 37°C. For acidic HPLC conditions, the aqueous solvent was prepared by addition of 2.5 mL of neat formic acid to 2.5 L of HPLC-water to yield a final concentration of 0.1% formic acid. For buffered HPLC conditions, the aqueous solvent was prepared by addition of 625 µL of 10 M ammonium formate (BioUltra, Sigma-Aldrich) to 2.5 L of HPLC-grade water followed by adjusting the pH to 8.4 by addition of 25% aqueous ammonia (for HPLC, LiChropur, Merck). Since its pH declines over time, this volatile buffer was freshly prepared each day. HPLC-grade acetonitrile was used without any additives.

Stock solutions
Stock solutions of rTCO-DMEDA-Tyr-BODIPY (6) and sulfo-cTCO-DMEDA-CA4 (12) were prepared at a concentration of 20 mM in DMSO. Tetrazine stock solutions of DMT (7), PA2 (8) and PymK (9) were prepared at a concentration of 10 mM in DMSO.

Release kinetics measurements
rTCO-DMEDA-Tyr-BODIPY (6): The stock solution of 6 was diluted with PBS to a concentration of 100 µM in an HPLC vial (2.5 µL 20 mM TCO stock, 497.5 µL PBS). Tetrazine stock solutions were diluted with PBS to a concentration of 200 µM in an HPLC vial (2.5 µL Tz stock, 122.5 µL PBS), and the click-to-release reaction was initiated by addition of the diluted TCO solution (125 µL) to obtain starting concentrations of 50 µM TCO and 100 µM Tz. The samples were immediately incubated at 37°C in the autosampler and subjected to serial HPLC analysis. All measurements were conducted in triplicates.

sulfo-cTCO-DMEDA-CA4 (12): The stock solution of 12 was diluted with DMSO (30 µL TCO stock, 30 µL DMSO) to give a 10 mM stock solution. Tz stock solution (10 µL) was added to PBS (985 µL, containing 8.6% DMSO), and the click-to-release reaction was initiated by addition of the stock solution of 12 (5.24 µL) to obtain starting concentrations of 50 µM TCO and 100 µM Tz (in 10% DMSO/PBS). The samples were immediately incubated at 37°C in the autosampler and subjected to serial HPLC analysis in intervals of 30 min. All measurements were conducted in triplicates.

Determination of exact TCO stock concentrations
The exact TCO stock concentrations were determined by absorbance titration (535 nm) with a freshly prepared stock solution of 3,6-bis(2-pyridyl)tetrazine 2Pyr2 (Sigma Aldrich) in DMSO using a Thermo Fisher Scientific NanoDrop One® Microvolume UV-Vis Spectrophotometer in cuvette mode at 25°C. The TCO stock solution (20 mM) was diluted with DMSO to reach a concentration of 1 mM, and then spiked with an excess of 2Pyr2 stock solution (20.4 mM). Upon IEDDA reaction, the remaining tetrazine absorbance at 535 nm was measured. This procedure was repeated twice (standard addition) to determine the exact TCO stock concentration.

Analytical HPLC analysis
rTCO-DMEDA-Tyr-BODIPY (6): PDA data was collected for all samples and used to identify all signals showing a characteristic BODIPY absorption. Relative quantification of reactants, intermediates and products was done using extracted chromatograms (wavelength: 500 nm).

sulfo-cTCO-DMEDA-CA4 (12): PDA data was collected for all samples. Relative quantification of intermediates and products was done using extracted chromatograms (wavelength: 254 nm). In addition, released CA4 was quantified via external calibration.
External CA4 calibration
A CA4 stock solution (20 mM) was prepared in DMSO and diluted with PBS (containing 10% DMSO) to reach a concentration of 100 µM. CA4 standard solutions (1 µM - 75 µM) were prepared by serial dilution into PBS (containing 10% DMSO). All measurements were conducted in triplicates.

Selected chromatograms and MS data

Tz-triggered cleavage of rTCO-DMEDA-Tyr-BODIPY (6)

rTCO-DMEDA-Tyr-BODIPY (6) + DMT (7), 65 min reaction time, 15% phenol release

HPLC gradient (% acetonitrile in 2.5 mM ammonium formate buffer, pH 8.4) 0 min: 2%, 0.25 min: 2%, 0.27 min: 35%, 2.50 min: 50%, 3.56 min: 85%.

Datafile Name: DMT_1_DMT_65min_1,08h_method45_37C_basic_C18_5min_493_503BODIPY_port6_GAINlowx1_23.08.2020_019.lcd
Sample Name: 1_DMT
Sample ID: 65min_1,08h
rTCO-DMEDA-Tyr-BODIPY (6) + PA₂ (8), 4h 5 min reaction time, 52% phenol release

HPLC gradient (% acetonitrile in 2.5 mM ammonium formate buffer, pH8.4) 0 min: 5%, 0.15 min: 5%, 0.17 min: 20%, 2.30 min: 25%, 3.51 min: 98%, 3.76 min: 98%.
rTCO-DMEDA-Tyr-BODIPY (6) + PymK (9), 65 min reaction time, 44% phenol release

HPLC gradient (% acetonitrile in 0.1% formic acid) 0 min: 5%, 0.15 min: 5%, 3.20 min: 98%, 3.50 min: 98%.
Tz-triggered cleavage of sulfo-cTCO-DMEDA-CA4 (12)

sulfo-cTCO-DMEDA-CA4 (12) + DMT (7), 35 min reaction time, 47% phenol release

HPLC gradient (% acetonitrile in 2.5 mM ammonium formate buffer, pH 8.4) 0 min: 5%, 0.15 min: 5%, 1.20 min: 30%, 3.45 min: 50%, 3.56 min: 50%.
sulfo-cTCO-DMEDA-CA4 (12) + PymK (9), 35 min reaction time, 31% phenol release

HPLC gradient (% acetonitrile in 0.1% formic acid) 0min: 5%, 0.15min: 5%, 3.20min: 98%, 3.50min: 98%.
Release performance of sulfo-cTCO-DMEDA-CA4 (12) upon reaction with DMT (7) or PymK (9) in PBS at 37°C. Relative quantification was done using extracted chromatograms (254 nm, left). In addition, released CA4 was quantified via external calibration (right) to correct for the different absorption of intermediates/products at 254 nm.
4 Click Kinetics

A stock solution of rTCO-PEG4 in DMSO was prepared at an approximate concentration of 20 mM. The exact concentration was determined by absorbance titration with DMT (7) (extinction coefficient 510 M⁻¹cm⁻¹ at 520 nm), quantifying the decrease in tetrazine absorbance upon reaction with TCO. The initial DMSO stock was diluted with PBS to prepare solutions for stopped-flow analysis at a final TCO concentration of 1 mM.

20 mM stock solutions of DMT (7), PA2 (8), and PymK (9) in DMSO were prepared. Serial dilution into PBS gave solutions for stopped-flow analysis at a final TCO concentration of 1 mM.

Stopped-flow measurements were performed using an SX20-LED stopped-flow spectrophotometer (Applied Photophysics) equipped with a 535nm LED (optical pathlength 10 mm, full width half-maximum 34 nm) to monitor the characteristic tetrazine visible light absorbance (520-540 nm). The reagent syringes were loaded with tetrazine and TCO solutions and the instrument was primed. Measurements were done in sextuplicate for each Tz. Reactions were conducted at 37 °C and recorded automatically at the time of acquisition.

Data sets were analyzed by fitting an exponential decay using Prism 6 (GraphPad) to calculate the observed pseudo-first-order rate constants that were converted into second-order rate constants (Table S1) by dividing through the TCO concentration.

Table S1. Second-order rate constants (k₂) of Tz with rTCO-PEG₄ (PBS, 37 °C), in comparison to the observed release yields.

| Tz        | k₂ (M⁻¹s⁻¹) with rTCO-PEG₄ | Release yield with 6 | Release yield with 12 |
|-----------|-----------------------------|----------------------|-----------------------|
| DMT (7)   | 82 ± 9                      | 89%                  | 98%                   |
| PA2 (8)   | 12 ± 1                      | 92%                  | n.d.                  |
| PymK (9)  | 1420 ± 160                  | 70%                  | 50%                   |

5 Cell Viability Assays

HT1080 human fibrosarcoma cells (ATCC) were cultivated in EMEM (Minimum Essential Medium Eagle, with Earle’s salts, L-glutamine and sodium bicarbonate; Sigma Aldrich) supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic solution (100X, Sigma-Aldrich) at 37 °C and 5% CO₂. HT1080 cells were seeded into 96-well plates (triplicates for each group) at 10,000 cells per well and allowed to grow overnight.

The medium was removed and a dilution series of sulfo-cTCO-DMEDA-CA4 (12) or the parent drug CA4 in growth medium (10µM, 2µM, 0.4µM, 0.08µM, 0.016µM, 0.0032µM, 0.00064µM, 0.000128µM, 0.0000256µM) was added to the cells (0.1% final DMSO concentration). For release experiments the same concentrations of 12 were used, while a stock solution of DMT (7) or PymK (9) was added to obtain final concentration of 5 µM of the respective tetrazine. Incubation was carried out for 72h.

Cell viability was assessed by replacing the medium with 100µL of PrestoBlue solution (Invitrogen, 1:9 in growth medium) followed by incubation for 30 minutes at 37 °C. Read-out of the fluorescence signal was carried out using a PerkinElmer EnSpire Multimode Plate Reader and data processing was done in GraphPad Prism.

Following the same procedure, cells were treated with DMT (7), PymK (9), or 1,3-dimethylimidazolidin-2-one (= byproduct of the self-immolation process) with concentrations of up to 10 µM, revealing no significant effect on cell viability.
6 Cell Imaging

HT1080 cells were seeded into a 96-well plate at 3,000 cells per well and allowed to grow overnight. The medium was removed, and the cells were treated with a 200 nM solution of sulfo-ctCO-DMEDA-CA4 (12) in media. In situ click-to-release was initiated by addition of DMT (7) at a final concentration of 10 μM. As controls, cells were left untreated or incubated with either the parent drug CA4 (200 nM) or 10 μM DMT (7). After an incubation time of 6 h cells were stained with SiR-tubulin (a fluorogenic, cell permeable and highly specific probe for microtubules).[6] An 11X stock solution of the probe was directly added to the growth medium to obtain a final concentration of 1 μM and incubation was carried out for 1 h. Subsequently, the medium was removed and cells were stained with Hoechst 33342 nuclear dye (Invitrogen, 5 μM in growth medium) for 10 minutes and washed once with PBS. Multichannel imaging of the cells was carried out in FluoroBrite DMEM medium (Gibco) on an Olympus IX82 microscope (Fig. S1).

Figure S1. Cell imaging via fluorescence microscopy (scale bars: 50 μm) upon staining with Hoechst 33342 (blue, nuclei) and SiR-tubulin[6] (red, microtubules) shows comparable depletion of tubulin signals after 6 h treatment with CA4 (200 nM) or bioorthogonal activation of prodrug 12 (200 nM) by in situ reaction with DMT (7). No significant change (compared to untreated cells) was observed after treatment with DMT (7) or prodrug 12.
7 Prodrug Stability

Prodrug 12 was incubated in (i) PBS and (ii) cell growth medium (DMEM + 10% fetal bovine serum) at 37 °C at a concentration of 100 µM (1 µL 10 mM stock in DMSO + 99 µL PBS/medium). Samples in PBS were analyzed by serial HPLC measurements (n=3) for 120 h. No degradation of the DMEDA bis(carbamate)-linkage and <5% isomerization of the cTCO linker was observed.

Aliquots (50 µL) of samples in cell growth medium were diluted with ice cold MeCN (200 µL) followed by centrifugation at 14,000 rpm for 8 min at 4 °C. A sample of CA4 (100 µM in cell growth medium) was prepared following the same procedure as a control. HPLC analysis of the supernatants (n=3) revealed 33.0 ± 0.2% trans-to-cis isomerization of the cTCO linker after 72 h and 43.6 ± 1.0% after 120 h (as verified by addition of 2Pyr2), but no release of CA4, confirming integrity of the DMEDA bis(carbamate)-linkage (Fig. S2).

Figure S2. HPLC chromatograms (254 nm) of (a) CA4 (control), and (b) prodrug 12 incubated in cell growth medium (DMEM + 10% fetal bovine serum) at 37 °C for 72 h and 120 h. HPLC gradient (%MeCN in 2.5 mM ammonium formate buffer, pH 8.4): 0 min 5% - 0.15 min, 5% - 1.20 min, 30% - 3.45 min, 50% - 3.56 min, 50%.
8 NMR Spectra, Chromatograms and MS Data

S1, $^1$H NMR

S1, $^{13}$C NMR
S1, HPLC (acidic conditions)

3, $^1$H NMR
3, $^{13}$C NMR

![Carbon-13 NMR spectrum](image)

3, HPLC (acidic conditions)

![HPLC spectrum](image)
4, $^1$H NMR

4, $^{13}$C NMR
4, HPLC (acidic conditions)

PDA

mAU

Exact Mass. 763.39

ESI (+)

m/z

Inten. (x100,000)

8.8

0.0

122.2 224.3 326.3 428.0 531.4 634.7 738.3 841.0 943.2

ESI (-)

m/z

Inten. (x100,000)

5.0

0.0

700.3 701.4 702.5 703.6 704.7 705.8 706.9 707.0 708.1

ESI (+)

m/z

Inten. (x100,000)

8.8

0.0

700.3 701.4 702.5 703.6 704.7 705.8 706.9 707.0 708.1

ESI (-)

m/z

Inten. (x100,000)

5.0

0.0

700.5 701.6 702.7 703.8 704.9 705.0 706.1 707.2 708.3

6, $^1$H NMR

m/z

Inten. (x100,000)

8.8

0.0

700.5 701.6 702.7 703.8 704.9 705.0 706.1 707.2 708.3

m/z

Inten. (x100,000)

5.0

0.0

700.5 701.6 702.7 703.8 704.9 705.0 706.1 707.2 708.3

m/z

Inten. (x100,000)

8.8

0.0

700.5 701.6 702.7 703.8 704.9 705.0 706.1 707.2 708.3

m/z

Inten. (x100,000)

5.0

0.0

700.5 701.6 702.7 703.8 704.9 705.0 706.1 707.2 708.3
6, $^{13}$C NMR

![$^{13}$C NMR spectrum](image)

6, HPLC (acidic conditions)

![HPLC chromatogram](image)
10, HPLC (acidic conditions)

11, $^1$H NMR
11, $^{13}$C NMR

![Carbon NMR spectrum](image)

11, HPLC (acidic conditions)

![HPLC chromatogram](image)

**ESI (+)**

Inten. (x10,000)

| m/z | Inten. |
|-----|--------|
| 269.3 | 329.1 |
| 361.2 | 439.2 |
| 484.4 | 544.2 |
| 646.1 | 717.2 |
| 833.5 | 906.8 |
| 920.0 | 1000.0 |

**ESI (-)**

Inten. (x10,000)

| m/z | Inten. |
|-----|--------|
| 301.1 | 311.1 |
| 317.0 | 329.1 |
| 339.2 | 343.5 |
| 353.0 | 365.3 |
| 375.8 | 380.2 |
| 392.1 | 396.8 |

**ESI (+)**

Inten. (x10,000)

| m/z | Inten. |
|-----|--------|
| 201.5 | 221.0 |
| 309.9 | 411.6 |
| 533.0 | 587.9 |
| 643.9 | 696.7 |
| 768.4 | 897.1 |
| 950.5 | 1000.0 |

**ESI (-)**

Inten. (x10,000)

| m/z | Inten. |
|-----|--------|
| 301.1 | 311.1 |
| 317.1 | 326.8 |
| 339.2 | 344.3 |
| 354.0 | 361.9 |
| 396.2 | 379.0 |
| 386.7 | 391.3 |

Datafile Name: DMEDA-SIL_ACE014_cTCO-pNP_6_C18_acidic_ESI_14.04.2022_003.lcd

Sample Name: ACE014

Sample ID: cTCO-pNP

0,00 0,25 0,50 0,75 1,00 1,25 1,50 1,75 2,00 2,25 min

0 100 200 300 mAU

254nm, 4nm

0,00 0,25 0,50 0,75 1,00 1,25 1,50 1,75 2,00 2,25 min

0 100 200 300 mAU

PDA

ESI (+)

ESI (-)
S3, $^1$H NMR

S3, $^{13}$C NMR
S3, HPLC (acidic conditions)

ESI (+)

ESI (-)

12, $^1$H NMR
12, $^{13}$C NMR

12, HPLC (basic conditions)
9 References

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