Supporting Information

A Cleavable $C_2$-Symmetric trans-Cyclooctene Enables Fast and Complete Bioorthogonal Disassembly of Molecular Probes

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1) Synthesis

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 sources without further purification. Dichloromethane, methanol, THF and diethyl ether were dried using PURESOlv-columns (Inert Corporation, USA). Solvents used for flash column chromatography were purchased from Donau Chemie AG (Austria). Dry acetonitrile and dry DMF were obtained from Sigma-Aldrich (Germany) and ACROS Organics (Belgium), respectively, and stored under argon. Thin layer chromatography was performed using TLC plates on aluminum support (Merck, silica gel 60, fluorescent indicator 254). Column chromatography was performed using a BUCHI Sepacore Flash System (2 x BUCHI Pump Module C-605, BUCHI Pump Manager C-615, BUCHI UV Photometer C-635, and BUCHI Fraction Collector C-660) and a Reveleris® X2 Flash Chromatography/Prep Purification Systems (BUCHI). Silica gel 60 (40-63 µm) was obtained from Merck. A Kinetex® 5 µm C18 100 Å, AXIA LC column (100 x 30.0 mm, Phenomenex) was used for preparative HPLC. HPLC grade solvents were purchased from VWR (USA).

$^1$H and $^{13}$C NMR spectra were recorded on a Bruker AC 200 MHz, Bruker Avance UltraShield 400 MHz or Bruker Ascend 600 MHz spectrometer at 20 °C. Chemical shifts are reported in ppm (δ) 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, b = broad signal), coupling constants (J, Hz) and integration.

GC/MS experiments were done with a Thermo Finnigan GC 8000 Top gaschromatograph on a BGB5 column (l=30 m, di=0.32 mm, 1 µm coating thickness) coupled to a Voyager Quadrupol mass spectrometer (electron ionization, 70 eV).

HPLC analysis was performed on a Nexera X2® UHPLC system (Shimadzu®) comprised of LC-30AD pumps, SIL-30AC autosampler, CTO-20AC column oven and DGU-20A5/R degasser module. Detection was done using an SPD-M20A photo diode array, an RF-20Axs fluorescence detector, and 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 water/acetonitrile + 0.1% formic acid gradient elution.

HRMS analysis was carried out using methanol solutions (concentration: 10 ppm) on an Agilent 6230 LC TOFMS mass spectrometer equipped with an Agilent Dual AJS ESI-Source. The mass spectrometer was connected to a liquid chromatography system of the 1100/1200 series from Agilent Technologies (Palo Alto, CA, USA). The system consisted of a 1200SL binary gradient pump, a degasser, column thermostat, and an HTC PAL autosampler (CTC Analytics AG, Zwingen, Switzerland).
Synthesis of Sar-C₂TCO-Sar

4-Hydroxycyclooct-2-ene-1-one (9)

In a PESCHL UV-Photoreactor (Borosilicate glass; THQ 150 Z1 immersion lamp, 150 W) 1,3-cyclooctadiene B (4.3 g, 5 mL, 40.2 mmol) was dissolved in dry DCM (400 mL). Meso-tetraphenylporphyrin¹ (50 mg, 0.08 mmol) was added and the mixture was cooled to -5 °C. Molecular oxygen was bubbled through the solution and the UV-lamp was switched on. The solution was irradiated under constant oxygen flow, while the temperature was maintained between -10 °C and 0 °C. The reaction was monitored via ¹H NMR, drawing samples every hour. After 5 h further meso-tetraphenylporphyrin (50 mg, 0.08 mmol) was added to the brownish solution, as the photosensitizer had been degraded. After irradiating for additional 9 h the oxygen-bubbling was stopped and the solution was transferred to a 500 mL round bottom flask. DBU² (630 µL, 4.2 mmol) was added and the mixture was stirred at room temperature for 13 h. The solvent was evaporated and the residual solid was purified by column chromatography (33-50% EtOAc in hexanes, gradient elution) to give 9 (2.01 g, 63%) as a white solid. Analytical data matched that reported in the literature;²⁻³ ¹H NMR (200 MHz, CDCl₃) δ 6.0 (d, J = 5.3 Hz, 1H), 5.81 (d, J = 5.3 Hz, 1H), 4.98 (d, J = 6.1 Hz, 1H), 2.82 (bs, 1H), 2.02-1.42 (m, 8H).

anti-cyclooct-2-ene-1,4-diol (anti-10)

Under an atmosphere of argon, a solution of sodium triacetoxyborohydride (1.51 g, 7.13 mmol) in dry DCM (360 mL) was cooled to 0 °C. 4-Hydroxycyclooct-2-en-1-one 9 (500 mg, 5.13 mmol) dissolved in dry DCM (20 mL) was added followed by glacial acetic acid (500 µL). The slightly turbid solution was allowed to warm to room temperature and stirred for 8 days. Additional sodium triacetoxyborohydride (1.51 g, 7.13 mmol) and glacial acetic acid (500 µL) were added on days 3, 5, and 7. After complete consumption of the starting material, the reaction was neutralized by addition of Na₂CO₃, basified with 1N aqueous NaOH solution and extracted with DCM (3 x 500 mL). The combined organic layer was dried over Na₂SO₄ and concentrated. Column chromatography (50-100% EtOAc in hexanes, gradient elution) gave 10 (452.3 mg, 45%) as a white solid (mixture of diastereomers; anti-10/syn-10 = 10:1); syn-10: ¹H NMR (400 MHz, CD₃OD) δ 5.47 (dd, J = 4.4 Hz, J = 1.1 Hz, 0.2H), 4.47 (m, 0.2H), 1.82-1.75 (m, 0.2H), 1.72-1.61 (m, 0.4H), 1.59-1.51 (m, 0.2H); ¹³C NMR (150 MHz, CD₃OD) δ 133.94, 69.74, 39.6, 24.7; anti-10: ¹H NMR (400 MHz, CD₃OD) δ 5.55 (dd,
\[ J = 3.2 \text{ Hz}, J = 1.1 \text{ Hz}, 2\text{H}) , 4.72 \text{ (m, 2H)}, 1.82-1.75 \text{ (m, 2H)}, 1.72-1.61 \text{ (m, 4H)}, 1.59-1.51 \text{ (m, 2H)}; \]

\[^{13}\text{C NMR} \] (150 MHz, CD\text{OD}) \[ \delta \] 133.9, 69.7, 36.9, 24.1; GC-MS [M+H]^+ m/z calcd. 143.11 for C\text{8}H\text{13}O\text{2}^+. found 143.11.

**anti-cyclooct-2-ene-1,4-diol; C\text{2} TCO (bis-axial-11)**

A mixture of anti- and syn-10 (1.06 g, 7.45 mmol) and methyl benzoate (1.9 mL, 14.9 mmol) were dissolved in Et\text{2}O/i-PrOH = 10:1 (500 mL), and the mixture was purged with argon while sonicated for 30 min. The solution was irradiated in continuous flow in a UV-photoreactor\textsuperscript{4} equipped with a Biotaie SNAP cartridge containing 17.5 g of tosic silica\textsuperscript{5} for 21 h. After complete consumption of the starting material, the column was dried with air and then flushed with a mixture of Et\text{2}O/i-PrOH= 5:1 (500 mL). The crude product was eluted with 7 N methanolic ammonia solution (200 mL). After evaporation of the solvent, column chromatography (10-80% MTBE in n-hexane, gradient elution) yielded **bis-axial-11** (499 mg, 47%) and **bis-equatorial-11** (304 mg, 30%) as white solids; **bis-equatorial-11**: \[^{1}H\text{ NMR} \] (400 MHz, CD\text{OD}) \[ \delta \] 5.63 (dd, \( J = 5.8 \text{ Hz}, J = 2.8 \text{ Hz}, 2\text{H}) , 4.17 \text{ (m, 2H)}, 2.17-2.09 \text{ (m, 2H)}, 1.80-1.73 \text{ (m, 2H)}, 1.43 (dt, \( J = 12.2 \text{ Hz}, J = 10.3 \text{ Hz}, 2\text{H}) , 0.91-0.82 \text{ (m, 2H)}; \[^{13}\text{C NMR} \] (151 MHz, CD\text{OD}) \[ \delta \] 134.2, 77.2, 45.5, 28.1; **bis-axial-11**: \[^{1}H\text{ NMR} \] (400 MHz, CD\text{OD}) \[ \delta \] 6.00 (s, 2H), 4.61 (s, 2H), 2.00-1.92 (m, 2H), 1.76-1.67 (m, 2H), 1.60-1.53 (m, 2H), 1.22-1.12 (m, 2H); \[^{13}\text{C NMR} \] (151 MHz, CD\text{OD}) \[ \delta \] 133.1, 71.9, 44.5, 24.2; GC-MS [M+H]^+ m/z calcd. 143.11 for C\text{8}H\text{13}O\text{2}^+, found 143.08.

**Sar-C\text{2} TCO-Sar (13)**

**Bis-axial-11** (100 mg, 0.70 mmol) and CDI (342.1 mg, 2.10 mmol) were dissolved in dry THF (1.4 mL) and the solution was stirred for 3 h under an atmosphere of argon. The reaction mixture was diluted with Et\text{2}O (50 mL) and washed with H\text{2}O (3 x 50 mL) and brine (1 x 20 mL), dried over N\text{a}2SO\text{4} and concentrated. The residual white solid was dissolved in dry MeCN (2.8 mL), methyl iodide (435 \text{ mL}, 7.00 mmol) was added dropwise, and the mixture was stirred at room temperature for 19 h. The solvent and volatiles were evaporated to obtain the methylated imidazolium carbamate 12 as a yellow solid (343 mg). 12 was immediately dissolved in dry DMF (2.8 mL) and DIPEA (980 \text{ mL}, 5.6 mmol) followed by sarcosine methylester hydrochloride (390.8 mg, 2.8 mmol) were added. After stirring for 1 h, 4 M aqueous KOH solution (2.8 mL, 11.2 mmol) was added. Complete saponification was observed after 30 min (as confirmed by LC-MS). The mixture was cooled to 0 \textdegree C and acidified with formic acid (528 \text{ mL}, 14 mmol). The volatiles were evaporated and reversed phase chromatography (C18, H\text{2}O/MeCN gradient elution, 0.1% formic acid) gave 13 (152 mg, 58%) as a white solid after lyophilization; \[^{1}H\text{ NMR} \] (400 MHz, CD\text{OD}) \[ \delta \] 6.00-5.88 (m, 2H), 5.37-5.31 (m, 2H), 3.91-3.78 (m, 4H), 3.03-2.94 (m, 6H), 2.18-2.03 (m, 2H), 1.93-1.60 (m, 4H), 1.28-1.11 (m, 2H); \[^{13}\text{C NMR} \] (151 MHz, CD\text{OD}) \[ \delta \] 176.1 (2C), 157.8, 157.6, 131.2, 131.0, 76.1, 76.0, 53.4 (2C), 42.1, 42.0, 41.9, 41.9, 36.3, 35.6, 25.1, 25.0; ESI-MS [M+H]^+ m/z calcd. 373.16 for C\text{16}H\text{25}N\text{2}O\text{8}^+, found 373.15.
**Note:**
Initial attempts to activate both OH-functionalities as \( p \)-nitrophenyl (PNP) carbonates by reacting **bis-axial-11** (1 equ.), \( p \)-nitrophenyl chloroformate (4 equ.), and 4-(dimethylamino)-pyridine (DMAP, 8 equ.) in dry DMF afforded an inseparable mixture of the desired product and \( p \)-nitrophenol.

\[
\text{bis-axial-11} + p\text{-nitrophenyl chloroformate} + \text{DMAP} \rightarrow \text{inseparable mixture}
\]

\(^1\)H NMR of the obtained mixture (400 MHz, \( \text{CD}_2\text{Cl}_2 \)):

The obtained mixture could be used for the synthesis of Sar-C\( _2\)TCO-Sar (13) as described above by reaction with sarcosine methylester hydrochloride (4 equ. based on \( \text{C}_2\text{TCO-bis(PNP)} \)) and DIPEA (8 equ.) in dry DMF, followed by addition of 4 M aqueous KOH (16 equ.). Spectral data of the afforded product matched those of 13 as prepared via the bis-imidazolium-carbamate 12, but the overall yield starting from **bis-axial-11** was considerably lower (<20%).
**Synthesis of AF594-C₂TCO-AF594**

Sar-C₂TCO-Sar (13) (13.8 mg, 0.04 mmol) was dissolved in dry DMSO (375 µL) and TSTU (23.5 mg, 0.08 mmol) and DIPEA (27.2 µl, 0.16 mmol) were added. The mixture was stirred at room temperature for 10 min until LCMS showed full conversion (after quenching a sample by addition of an excess of 1,3-diaminopropane (DAP)). A solution of Fmoc-DAP hydrochloride (25.9 mg, 0.08 mmol) dissolved in dry DMSO (300 µL) was added and the mixture was stirred at room temperature for 15 min until LCMS showed full conversion (ESI-MS of the intermediate [Fmoc-DAP-C₂TCO-DAP-Fmoc+H]+ m/z calcd. 929.4 for C₅₂H₆₁N₆O₁₀+, found 929.5). Piperidine (220 µL) was added and the mixture was stirred for 20 min, quenched with formic acid (100 µL) and then centrifuged for 3 min at 6000 rpm. The supernatant was directly loaded onto a C18 column and reversed phase column chromatography (H₂O/MeCN gradient elution, 0.1% formic acid) gave the desired product as a pale-yellow oil (18 mg, 99%); ¹H NMR (600 MHz, D₂O, mixture of rotamers) δ 8.43 (bs, 2H), 5.96-5.75 (m, 2H), 5.27-5.26 (m, 2H), 4.07-3.90 (m, 4H), 3.45 (q, J = 5.8 Hz, 2H), 3.36-3.25 (m, 4H), 3.04-3.03 (m, 2H), 2.99-2.95 (m, 6H), 2.70-2.68 (m, 4H), 1.89-1.82 (m, 4H), 1.76-1.53 (m, 6H), 1.32-1.29 (m, 2H); ¹³C NMR (151 MHz, D₂O, mixture of rotamers) δ 172.24, 172.21, 171.8, 171.7, 170.6, 157.6, 157.6, 157.0, 156.9, 129.4, 129.3, 75.9, 75.8, 75.6, 54.3, 52.11, 52.1, 52.0, 47.0, 45.0, 44.5, 43.4, 42.5, 40.5, 40.4, 40.3, 40.2, 38.6, 37.9, 37.0, 36.9, 36.1, 35.9, 35.4, 27.7, 26.7, 26.6, 25.8, 25.2, 25.1, 23.7, 23.3, 23.2, 23.1, 22.2, 21.4, 17.6, 16.2, 12.1; HRMS [M+Na]+ m/z calcd. 507.2902 for C₂₂H₄₀N₆NaO₆+, found 507.2928.

**DAP-C₂TCO-DAP (14)**

To AF594-NHS (20.8 mg, 25.4 µmol) dissolved in dry DMSO (300 µL) were added DIPEA (14.1 µL, 80.5 µmol) and a solution of DAP-C₂TCO-DAP (14) (6 mg, 12.4 µmol) in dry DMSO (60 µL). The dark-blue mixture was stirred at room temperature for 80 min and then quenched by addition of formic acid (3.4 µL). Purification by reversed phase column chromatography (H₂O/MeCN gradient elution, 0.1% formic acid) afforded 15 as a dark-blue crystalline solid (5.3 mg, 23%);
$^1$H NMR (600 MHz, D$_2$O, mixture of rotamers) $\delta$ 8.57-8.46 (m, 2H), 8.15-8.02 (m, 2H), 7.31-7.15 (m, 2H), 6.98-6.90 (m, 4H), 6.48-6.38 (m, 4H), 5.83 (d, $J = 7.0$ Hz, 4H), 5.80 (s, 2H), 5.37-5.21 (m, 1H), 4.06-3.93 (m, 3H), 3.77-3.61 (m, 4H), 3.47-3.25 (m, 12H), 3.09-2.87 (m, 2H), 2.21-1.95 (m, 2H), 1.91-1.72 (m, 6H), 1.68-1.13 (m, 30 H), 1.10-0.86 (m, 2H); $^{13}$C NMR (151 MHz, D$_2$O, mixture of rotamers) $\delta$ 171.8, 168.2, 166.4, 157.3, 157.2, 157.0, 154.9, 153.2, 153.1, 138.3, 138.2, 136.1, 135.6, 131.8, 131.2, 129.4, 123.2, 122.9, 121.5, 121.4, 120.7, 120.6, 112.9, 95.1, 75.6, 60.0, 59.9, 59.9, 59.8, 52.6, 52.3, 40.5, 37.5, 37.0, 36.4, 32.7, 32.6, 32.5, 28.3, 27.9, 27.8, 27.8, 27.8, 23.7, 23.5; ESI-MS [(M+H)/2]$^+$ m/z calcd. 947.6 for [C$_{92}$H$_{105}$N$_{10}$O$_{26}$S$_4$]$^+$/2, found 947.8.

**Synthesis of DBCO-C$_2$TCO-AF594**

To a solution of Sar-C$_2$TCO-Sar (13) (20 mg, 0.05 mmol) in dry DMSO (560 µL) were added TSTU (34 mg, 0.11 mmol) and DIPEA (39.0 µL, 0.23 mmol). The mixture was stirred at room temperature for 40 min until LCMS analysis showed full conversion (after quenching a sample with an excess of 2-(2-aminoethoxy)ethanol). DBCO-PEG$_4$-amine (12.1 mg, 17.7 µmol) dissolved in dry DMSO (120 µL) was added and the mixture was stirred at room temperature for 2.5 h. A solution of NH$_2$-PEG$_7$-NH$_2$ (99 mg, 0.27 mmol) in dry DMSO (40 µL) was added and stirring was continued for 35 min. The solution was quenched with formic acid (9.1 µL) and directly loaded onto a C18 column. Purification by reversed phase column chromatography (H$_2$O/MeCN gradient elution, 0.1% formic acid) gave the desired product as an oil (4.8 mg, 20%; $^1$H NMR (600 MHz, D$_2$O) $\delta$ 7.65 (d, $J = 7.9$ Hz, 1H), 7.52-7.34 (m, 7H), 5.94-5.68 (m, 2H), 5.30-5.20 (m, 2H), 5.08 (d, $J = 14.1$ Hz, $J = 3.9$ Hz, 1H), 4.54-4.47 (m, 1H), 4.00-3.85 (m, 4H), 3.80-3.47 (m, 48H), 3.41-3.35 (m, 4H), 3.21-3.15 (m, 4H), 3.12-3.04 (m, 2H), 2.99-2.90 (m, 6H), 2.58-2.38 (m, 3H), 2.17-1.92 (m, 3H), 1.79-1.56 (m, 4H), 1.16-0.83 (m, 2H); ESI-MS [M]$^-$ m/z calcd. 1377.6 for C$_{66}$H$_{97}$N$_8$O$_{23}$S$^-$, found 1377.6.

**DBCO-C$_2$TCO-PEG$_7$ (S1)**

To a solution of Sar-C$_2$TCO-Sar (13) (20 mg, 0.05 mmol) in dry DMSO (560 µL) were added TSTU (34 mg, 0.11 mmol) and DIPEA (39.0 µL, 0.23 mmol). The mixture was stirred at room temperature for 40 min until LCMS analysis showed full conversion (after quenching a sample with an excess of 2-(2-aminoethoxy)ethanol). DBCO-PEG$_4$-amine (12.1 mg, 17.7 µmol) dissolved in dry DMSO (120 µL) was added and the mixture was stirred at room temperature for 2.5 h. A solution of NH$_2$-PEG$_7$-NH$_2$ (99 mg, 0.27 mmol) in dry DMSO (40 µL) was added and stirring was continued for 35 min. The solution was quenched with formic acid (9.1 µL) and directly loaded onto a C18 column. Purification by reversed phase column chromatography (H$_2$O/MeCN gradient elution, 0.1% formic acid) gave the desired product as an oil (4.8 mg, 20%; $^1$H NMR (600 MHz, D$_2$O) $\delta$ 7.65 (d, $J = 7.9$ Hz, 1H), 7.52-7.34 (m, 7H), 5.94-5.68 (m, 2H), 5.30-5.20 (m, 2H), 5.08 (d, $J = 14.1$ Hz, $J = 3.9$ Hz, 1H), 4.54-4.47 (m, 1H), 4.00-3.85 (m, 4H), 3.80-3.47 (m, 48H), 3.41-3.35 (m, 4H), 3.21-3.15 (m, 4H), 3.12-3.04 (m, 2H), 2.99-2.90 (m, 6H), 2.58-2.38 (m, 3H), 2.17-1.92 (m, 3H), 1.79-1.56 (m, 4H), 1.16-0.83 (m, 2H); ESI-MS [M]$^-$ m/z calcd. 1377.6 for C$_{66}$H$_{97}$N$_8$O$_{23}$S$^-$, found 1377.6.
To a solution of AF594-NHS (1.7 mg, 2.05 µmol) in dry DMSO (100 µL) were added DBCO-C₂TCO-PEG₇ (S1) (2.7 mg, 1.96 µmol) dissolved in dry DMSO (100 µL) and DIPEA (1.79 µL, 9.79 µmol). The mixture was stirred at room temperature and additional portions of AF594-NHS (0.2 mg each, 0.25 µmol) were added after 2, 5 and 22 h after which LCMS analysis showed full consumption of S1. The solution was quenched with formic acid (0.8 µL) and directly loaded onto a C18 column. Purification by reversed phase column chromatography (H₂O/MeCN gradient elution, 0.1% formic acid) afforded the desired product as a dark-blue solid (3.8 mg, 93%); ¹H NMR (600 MHz, DMSO-d₆) δ 8.99 (t, J = 5.1 Hz, 1H), 8.45 (bs, 1H), 8.19 (d, J = 7.6 Hz, 1H), 8.14 (s, 4H), 8.10-8.05 (m, 1H), 8.00-7.89 (m, 2H), 7.64-7.58 (m, 2H), 7.55-7.43 (m, 3H), 7.41-7.22 (m, 4H), 5.86-5.70 (m, 2H), 5.59 (bs, 1H), 5.37-5.17 (m, 2H), 5.08 (dd, J = 14.1, 3.9 Hz, 1H), 4.22-4.17 (m, 1H), 3.89-3.74 (m, 4H), 3.65-3.44 (m, 4H), 3.24-3.18 (m, 8H), 3.12-3.04 (m, 6H), 2.95-2.79 (m, 12H), 2.66-2.58 (m, 2H), 2.38 (s, 1H), 2.35-2.26 (m, 2H), 2.02-1.95 (m, 1H), 1.93-1.87 (m, 1H), 1.80-1.43 (m, 6H), 1.36-1.20 (m, 11H), 1.15-0.89 (m, 2H); ESI-MS [Fragment]⁺ m/z calcd. 1380.5 for C₆₆H₇₈N₆O₂₂S₂⁺, found 1380.6.

Synthesis of Ib-C₂TCO-SiR
**BocNMe-CH$_2$-Ib (S3)**

S$_2^6$ (44 mg, 64.1 µmol) was dissolved in dry DCM (800 µL) and TFA (200 µL) was added dropwise. The solution was stirred at room temperature for 90 min until LCMS analysis indicated complete deprotection. The reaction mixture was diluted with MeCN/DCM (1:1) and concentrated. (E)-4-(((tert-butoxycarbonyl)(methyl)amino)but-2-enoic acid (4-BocNMe-but-2-enoic acid)$^7$ (15.2 mg, 70.5 µmol) was dissolved in dry DMF (1 mL) and HBTU (26.8 mg, 70.5 µmol), HOBT (10 mg, 70.5 µmol) and NaHCO$_3$ (20 mg, 224.2 µmol) were added. The mixture was stirred at room temperature for 15 min. A solution of deprotected S$_2$ in dry DMF (500 µL) and NaHCO$_3$ (20 mg, 224.2 µmol) were added. The reaction mixture was stirred at room temperature for 43 h, acidified with formic acid (50 µL) and diluted with 0.1% formic acid in water (1 mL). Reversed phase column chromatography (H$_2$O/MeCN gradient elution, 0.1% formic acid) gave the product as a bright yellow solid (27 mg, 73%); $^1$H NMR (600 MHz, CDCl$_3$, mixture of rotamers) δ 8.34 (d, $J = 15.2$ Hz, 1H), 7.62 (d, $J = 8.3$ Hz, 2H), 7.39 (t, $J = 7.6$ Hz, 2H), 7.19-7.14 (m, 3H), 7.09 (d, $J = 7.8$ Hz, 2H), 6.81-6.69 (m, 1H), 6.37-6.23 (m, 1H), 6.20-5.73 (m, 1H), 4.91-4.81 (m, 1.5H), 4.66-4.53 (m, 0.5H), 4.15 (d, $J = 11.9$ Hz, 0.5H), 4.05-3.87 (m, 2.5H), 3.74 (t, $J = 11.1$ Hz, 0.5H), 3.36 (t, $J = 11.1$ Hz, 0.5H), 3.23-3.11 (m, 1H), 2.93-2.76 (m, 4H), 2.43-2.29 (m, 1H), 2.28-2.20 (m, 1H), 1.99 (d, $J = 13.7$ Hz, 1H), 1.78-1.67 (m, 1H), 1.50-1.42 (m, 5H), 1.39-1.30 (m, 4H); $^{13}$C NMR (151 MHz, CDCl$_3$, mixture of rotamers) δ 165.4, 159.0, 157.3, 156.8, 156.3, 155.7, 154.7, 154.1, 153.7, 153.5, 144.7, 144.5, 141.4, 140.8, 130.1, 130.0, 127.3, 124.3, 121.6, 120.3, 119.8, 119.3, 98.5, 98.3, 79.9, 53.7, 53.6, 52.9, 50.4, 50.2, 49.7, 46.2, 46.0, 42.3, 34.5, 34.4, 30.5, 30.2, 28.5, 28.4, 25.4, 24.0; ESI-MS [M+H]$^+$ m/z calcd. 584.3 for C$_{32}$H$_{38}$N$_7$O$_4^+$, found 584.0.

**Ib-C$_2$TCO-DAP (S4)**

Me-NBoc-CH$_2$-Ib (S3) (13 mg, 0.02 mmol) was dissolved in dry DCM (200 µL) and TFA (50 µL) was added dropwise. The solution was stirred at room temperature for 90 min until LCMS indicated complete deprotection. The reaction mixture was diluted with MeCN/DCM (1:1) and concentrated under reduced pressure three times. Sar-C$_2$TCO-Sar (13) (24.47 mg, 0.07 mmol) was dissolved in dry DMSO (490 µL) and TSTU (41.5 mg, 0.14 mmol) and DIPEA (71.0 µL, 0.41 mmol) were added. The mixture was stirred at room temperature for 80 min. Deprotected S$_3$ (10.6 mg, 0.02 mmol) dissolved in dry DMSO (100 µL) and DIPEA (11.5 µL, 0.07 mmol) were then added and stirring was continued for 1 h. DAP (123 mg, 1.65 mmol) was added and the mixture was stirred for 25 min. The solution was quenched with 10% formic acid in water (920 µL) while cooling in an ice bath. Purification by reversed phase column chromatography (H$_2$O/MeCN gradient elution, 0.1% formic acid) afforded the desired product as a pale-yellow oil (7.2 mg, 37%); $^1$H NMR (700 MHz, DMSO-d$_6$) δ 8.30-8.09 (m, 2H), 7.65 (d, $J = 8.1$ Hz, 2H), 7.44 (t, $J = 8.6$ Hz, 2H), 7.21-7.11 (m, 5H), 6.72-6.41 (m, 2H), 5.87-5.57 (m, 2H), 5.32-5.14 (m, 2H), 4.76-4.61 (m, 2H), 4.08-3.72 (m, 13H), 3.27-3.08 (m, 3H), 3.02-2.71 (m, 9H), 2.35-2.10 (m, 2H) 2.03-1.83 (m, 3H), 1.74-1.45 (m, 8H), 1.14-0.75 (m, 2H); ESI-MS [M+H]$^+$ m/z calcd. 894.5 for C$_{48}$H$_{60}$N$_{11}$O$_8^+$, found 894.5.
Ib-C\textsubscript{2}TCO-SiR (22) 

To a solution of SiR-COOH\textsuperscript{8} (4.95 mg, 10.5 µmol) in dry DMSO (100 µL) were added TSTU (3.4 mg, 11.3 µmol) and DIPEA (6.3 µL, 36.2 µmol) The mixture was stirred at room temperature for 3h.

Ib-C\textsubscript{2}-DAP (S4) (7.2 mg, 8.05 µmol) dissolved in dry DMSO (700 µL) was then added and the stirring was continued for 3.5 h. The solution was cooled to 0 °C and quenched by addition of 0.1% formic acid in water (1.2 mL). Purification by reversed phase column chromatography (H\textsubscript{2}O/MeCN gradient elution, 0.1% formic acid) gave the desired product as a cyan-blue solid (3.1 mg, 29%); \textsuperscript{1}H NMR (400 MHz, DMSO-d\textsubscript{6}) δ 8.72 (bs, 1H), 8.29-7.87 (m, 5H), 7.74-7.62 (m, 3H), 7.43 (t, J = 7.8 Hz, 2H), 7.22-7.10 (m, 5H), 7.00 (s, 2H), 6.72-6.39 (m, 6H), 5.87-5.56 (m, 2H), 5.42-5.04 (m, 2H), 4.67-4.47 (m, 1H), 4.28-3.55 (m, 9H), 3.12-3.05 (m, 3H), 2.40-2.05 (m, 2H), 2.00-1.74 (m, 3H), 1.67-1.36 (m, 6H), 1.29-0.73 (m, 4H), 0.63 (s, 3H), 0.50 (s, 3H); ESI-MS [M+H]\textsuperscript{+} m/z calcd. 1348.6 for C\textsubscript{73}H\textsubscript{86}N\textsubscript{13}O\textsubscript{11}Si\textsuperscript{+}, found 1348.7.

Synthesis of tetrazines

DMT (1),\textsuperscript{9} PymK (3),\textsuperscript{10} PyrMe (4a),\textsuperscript{11} PymMe (4b),\textsuperscript{11} MPA (17)\textsuperscript{12} and HK (20)\textsuperscript{10} were prepared according to known procedures.

General procedure for Tz-acids

A well-blended mixture of nitrile #1 (1 eq.) and nitrile #2 or formamidine acetate salt (5 eq.) and Zn(OTf)\textsubscript{2} (5 mol%) was treated dropwise with hydrazine monohydrate (25 eq.) while cooling in an ice bath. The mixture was allowed to warm up to room temperature and stirred for 10 min, after which it was stirred at 60 °C for the specified time. The crude reaction mixture was poured onto ice-water (50 mL). After addition of NaNO\textsubscript{2} (4 eq.) the solution was acidified with aqueous 2N HCl solution. The mixture was extracted with EtOAc, dried over Na\textsubscript{2}SO\textsubscript{4}, filtered and concentrated. The crude product was purified by column chromatography.

PyrPA (5)

Synthesis was performed according to the general procedure using 3-cyanopropanoic acid (200 mg, 1.65 mmol), 2-pyridinecarbonitrile (860 mg, 8.26 mmol), Zn(OTf)\textsubscript{2} (180 mg, 0.5 mmol) and hydrazine monohydrate (2 mL, 41.3 mmol). After stirring at 60 °C for 25 min the reaction mixture was oxidized using NaNO\textsubscript{2} (456 mg, 6.6 mmol) and 2N HCl, and extracted with EtOAc (4 x 120 mL). Purification was performed by reversed phase column chromatography (H\textsubscript{2}O/MeCN gradient elution, 0.1% formic
acid) to give the desired product as a dark-red solid (40 mg, 11%). $^1$H NMR (400 MHz, CD$_2$OD) $\delta$ 8.83 (d, $J = 4.3$ Hz, 1H), 8.67 (d, $J = 8.2$ Hz, 1H), 8.14 (td, $J = 7.8$, 1.6 Hz, 1H), 7.69 (dd, $J = 4.7$, 7.8 Hz, 1H), 3.69 (t, $J = 7.0$ Hz, 2H), 3.11 (t, $J = 7.0$ Hz, 2H); $^{13}$C NMR (100 MHz, CD$_2$OD) $\delta$ 176.0, 171.3, 164.7, 151.5, 128.0, 125.2, 31.5, 31.0; HRMS [M+H]$^+$ m/z calcd. 232.0829 for C$_{10}$H$_{10}$N$_5$O$_2^+$, found 232.0824.

**PymPA (18)**

Synthesis was performed according to the general procedure using 3-cyanopropanoic acid (472 mg, 4.76 mmol), 2-pyrimidinecarbonitrile (100 mg, 0.95 mmol), Zn(OTf)$_2$ (104 mg, 0.3 mmol) and hydrazine monohydrate (1.1 mL, 23.8 mmol). After stirring at 60 °C for 2.5 h the reaction mixture was oxidized using NaNO$_2$ (263 mg, 3.8 mmol) and 2N HCl, and extracted with EtOAc (7 x 80 mL). Purification was performed by reversed phase column chromatography (H$_2$O/MeCN gradient elution, 0.1% formic acid) to obtain the desired product as red solid (67 mg, 31%); $^1$H NMR (400 MHz, CD$_2$Cl$_2$) $\delta$ 9.08 (d, $J = 5.1$ Hz, 2H), 7.59 (t, $J = 4.9$ Hz, 1H), 3.78 (t, $J = 7.0$ Hz, 2H), 3.21 (t, $J = 7.0$ Hz, 2H); $^{13}$C NMR (100 MHz, CD$_2$Cl$_2$) $\delta$ 175.7, 171.8, 164.2, 159.7 (2C), 159.4, 124.4, 31.3, 31.0; HRMS [M+Na]$^+$ m/z calcd. 255.1922 for C$_9$H$_8$N$_6$NaO$_2^+$, found 255.1998.

**HPA (19)**

Synthesis was performed according to the general procedure using 3-cyanopropanoic acid (1 g, 8.26 mmol), formamidine acetate salt (4.3 g, 41.3 mmol), Zn(OTf)$_2$ (0.9 g, 2.5 mmol) and hydrazine monohydrate (10 mL, 206 mmol). After stirring at 60 °C for 60 min the reaction mixture was oxidized using NaNO$_2$ (2.3 g, 33 mmol) and 2N HCl, and extracted with EtOAc (6 x 150 mL). Purification was performed by reversed phase column chromatography (H$_2$O/MeCN gradient elution, 0.1% formic acid) to afford the desired product as red crystals (45 mg, 4%); $^1$H NMR (600 MHz, CD$_3$OD) $\delta$ 10.30 (s, 1H), 3.61 (t, $J = 6.9$ Hz, 2H), 3.06 (t, $J = 7.0$ Hz, 2H); $^{13}$C NMR (151 MHz, CD$_3$OD) $\delta$ 175.8, 173.1, 159.4, 31.3, 31.2; HRMS [M+Na]$^+$ m/z calcd. 177.1182 for C$_5$H$_8$N$_6$NaO$_2^+$, found 177.1136.
2) Click kinetics

Sample preparation
rTCO-dPEG₄-OH (6) and Sar-C₂TCO-Sar (13) were dissolved in PBS (pH 7.4, 10 mM) to reach an approximate concentration above 2 mM. The exact concentration was determined by absorbance titration with DMT (1) (extinction coefficient 510 M⁻¹ cm⁻¹ at 520 nm), quantifying the decrease in tetrazine absorbance upon reaction with TCO. The initial PBS stock was diluted to prepare solutions for stopped-flow analysis at a final TCO concentration of 2 mM. Stock solutions of tetrazines (1, 3, 4a, 4b, 5, 17, 18, 19, 20) were prepared in DMSO at a concentration of 10 mM. Serial dilution into PBS (pH 7.4, 10 mM) was used to prepare solutions for stopped-flow analysis at a tetrazine concentration of 100 µM.

Stopped-flow spectrophotometry
Stopped-flow measurements were performed using an SX20-LED stopped-flow spectrophotometer (Applied Photophysics) equipped with a 535nm LED (optical pathlength 10mm, full width half-maximum 34nm) to monitor the characteristic tetrazine visible light absorbance (520-540 nm). The reagent syringes were loaded with tetrazine and TCO compound (6 or 13) solutions and the instrument was primed. Subsequent data were collected in triplicate to sextuplicate for each tetrazine. Reactions were conducted at 37 °C and recorded automatically at the time of acquisition.

Data analysis
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 by dividing through the concentration of excess TCO compound (6, 13).
3) Release experiments

**Instrument & solvents**

All release experiments were performed on a Nexera X2® UHPLC system (Shimadzu®) with a temperature-controlled autosampler at 25 °C or 37 °C. For buffered LCMS 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 8.5 by addition of 100 µL of 25% aqueous ammonia (for HPLC, LiChropur, Merck). The pH of this volatile buffer declines over time and was thus freshly prepared each day. HPLC-grade acetonitrile was used without any additives.

**Release kinetics with rTCO-AF350 (7)**

A 20 mM stock solution of rTCO-Alexa Fluor 350 (rTCO-AF350, 7) in DMSO was diluted with citrate-phosphate buffer (10 mM, pH 7) to a final concentration of 100 µM directly in an LCMS sample vial. 20 mM tetrazine stock solutions were diluted with citrate-phosphate buffer (10 mM, pH 7) to give 200 µM concentrations. These solutions were added to the TCO sample and mixed (1:1) to initiate the cleavage/release reaction. Reactions were conducted at 25 °C and monitoring was performed by HPLC analysis (fluorescence and MS detection; ammonium formate 2.5 mM, pH 8.4 / acetonitrile gradient).

**Release measurements with AF594-C2TCO-AF594 (15) and AF350-cTCO-Sar-OEt**

**Analytical stock solutions and buffers.** 10 mM stock solutions of the release probes AF594-C2TCO-AF594 (15) and AF350-cTCO-Sar-OEt in DMSO were prepared and further diluted with citrate-phosphate buffer (as indicated below), so that the final concentration of DMSO in buffer was never higher than 2%. Tetrazine stock solutions were prepared in DMSO at concentrations ranging from 10-100 mM and diluted with citrate-phosphate buffer (as indicated below), so that the final concentration of DMSO in buffer was never higher than 5%. Citrate-phosphate buffer (10 mM) was prepared by dilution from standard stock solutions of 0.1 M citric acid and 0.2 M Na2HPO4 with water and the pH verified and adjusted as needed to within ±0.05 pH units by digital pH metering.

**Endpoint measurements with AF594-C2TCO-AF594 (15).** Analytical samples for endpoint-cleavage reactions were prepared as follows: AF594-C2TCO-AF594 (15) stock solution was diluted with citrate-phosphate buffer to a final concentration of 50 µM directly in an LCMS sample vial. Tetrazine stock solutions were diluted with aqueous buffer to give 100 or 200 µM concentrations, added to the TCO sample and mixed (1:1) to initiate the cleavage/release reaction. Samples were kept at 37 °C and HPLC analyses were done after 24 h. The released AF594-amine was detected using an RF-20Axs fluorescence detector and confirmed by a LCMS-2020 mass spectrometer (ESI) implemented in the Nexera X2® UHPLC system.

**Release kinetics measurements.** AF594-C2TCO-AF594 (15) and AF350-cTCO-Sar-OEt (12) stock solutions were diluted with citrate-phosphate buffer to a final concentration of 20 µM directly in an LCMS sample vial and mixed. Tetrazine stock solutions were diluted with aqueous buffer to give 40, 100, 200 and 1000 µM concentrations. These solutions were added to the TCO sample and mixed (1:1) to initiate the cleavage/release reaction. Monitoring of the reactions at 37 °C was performed by HPLC analysis (fluorescence and MS detection).
Selected chromatograms

All chromatograms were screened for m/z of starting materials, potential intermediates and products. No intermediates (= clicked but not released yet) could be detected, indicating instantaneous release (click = rate-determining step of overall cleavage).

- 10 µM AF594-C₂TCO-AF594 (15) + 20 µM HPA (19), 25 min reaction time, 67% cleavage

Fluorescence detection
Excitation: 594 nm
Emission: 619 nm

- 10 µM AF594-C₂TCO-AF594 (15) + 20 µM MPA (17), 50 min reaction time, 16% cleavage

Fluorescence detection
Excitation: 594 nm
Emission: 619 nm
Stability of C₂TCO and H-tetrazines

Stability of C₂TCO

Analytical stock solutions and solvents. The stability of C₂TCO was assessed in PBS buffer (10 mM), PBS buffer (10 mM) + 1 mM L-glutathione and full cell growth media (DMEM (fluorobrite) + 10% FBS). Therefore, a 200 mM stock solution of Sar-C₂TCO-Sar (13) in DMSO was prepared and further diluted to a concentration of 0.2 mM with the respective solvent (final concentration of DMSO = 0.1%).

Stability measurements. All solutions of Sar-C₂TCO-Sar (13) were incubated at 37 °C and aliquots were taken after 6, 12, 24, and 48 h. Triplicate aliquots of each solvent were spiked with excess 88.3 mM stock solution of DMT (1) in DMSO and mixed. Upon reaction with TCO, the resulting tetrazine absorbance was measured on a Thermo Fisher Scientific NanoDrop One C in cuvette mode at 25 °C at 520 nm. This procedure with adding excess tetrazine and measuring the absorbance of the solution upon reaction with TCO was repeated two more times (standard addition). The absorbance was further plotted against the concentration of tetrazine at each addition and the concentration of reacted tetrazine equated the corresponding concentration of TCO present in the solution at each time point. The stability was determined to be >97% for all analyzed samples, except for 48 h incubation in PBS + 1 mM L-glutathione (89±2%).

Stability of HPA (19) and HK (20)

We have not experienced any issues related to the stability of the H-tetrazines 19 and 20 when stored at -20 °C (either as solids or as DMSO stock solutions). When working with PBS solutions of both tetrazines at room temperature, we noticed that HPA (19) was significantly more stable than HK (20). To measure the stability of 19 and 20, both tetrazines were incubated in PBS (5% DMSO) at a concentration of 500 µM. The characteristic absorbance at 520 nm was then monitored by UV/Vis (Shimadzu UV1800 Spectrophotometer), showing that HPA (19) was stable in PBS (>97%) for at least 9 days. HK (20) was confirmed to be less stable, but still showed sufficient stability over several hours (e.g. 87% after 18 h in PBS).
PyrMe (4a) + rTCO-glycine

rTCO-glycine\textsuperscript{12} was dissolved in DMSO to prepare a 50 mM stock solution. 528 µL (26.4 µmol, 6 mg) of that solution were diluted into 50 mL of CitPhos buffer (10 mM, pH 7), for a final concentration of 0.528 mM rTCO-glycine. The solution was then sparged with argon for 15 minutes to reduce dissolved oxygen and minimize potential aromatization, whereupon 295 µL of a 98.3 mM stock solution of PyrMe tetrazine 4a (1.1 equiv., 5.03 mg) were then added. At these reagent concentrations the click reaction is expected to be done in <10 minutes. The release reaction was allowed to proceed under argon overnight, a timeline after which no further release was observed by serial HPLC analyses. The buffer solution was evaporated to near-dryness and then redissolved in 1 mL of DMSO. The DMSO solution was loaded directly onto a 30 g Biotage Snap C18 Ultra column and the non-releasing isomer isolated with an ammonium formate (2.5 mM, pH 8.4)/acetonitrile gradient. HPLC analysis indicated ≥ 99% purity (Figure S1). A single fraction containing the purified material was immediately concentrated, stored at -80 °C under argon, and 1 day later dissolved in CD\textsubscript{3}OD for NMR analysis; \textsuperscript{1}H NMR (600 MHz, CD\textsubscript{3}OD, mixture of rotamers) \(\delta\) 8.47 (d, \(J = 4.9\) Hz, 1H), 8.02 (dd, \(J = 7.4, 4.9, 1.2\) Hz, 1H), 4.93-4.90 (1H, under water signal), 4.43-4.40 (m, 1H), 3.90 (d, \(J = 17.6\) Hz, 0.5H), 3.68 (d, \(J = 17.4\) Hz, 0.5H), 3.66 (d, \(J = 17.6\) Hz, 0.5H), 3.54 (d, \(J = 17.4\) Hz, 0.5H), 2.62-2.55 (m, 1H), 2.23 (ddd, \(J = 16.5, 7.4\) Hz, 1H), 1.95 (s, 1H), 1.93 (s, 1H), 1.92-1.84 (m, 3H), 1.77-1.62 (m, 4H), 1.55-1.46 (m, 1H); \textsuperscript{13}C NMR (151 MHz, CD\textsubscript{3}OD, mixture of rotamers) \(\delta\) 174.2, 174.1, 156.8, 156.7, 155.9, 155.7, 147.7, 147.6, 136.2, 136.1, 135.8, 135.7, 131.8, 131.7, 120.8, 120.7, 99.4, 99.2, 76.5, 76.5, 44.0, 43.4, 36.9, 30.0, 29.9, 28.9, 28.7, 28.49, 24.0, 23.9, 20.3, 19.9, 12.9, 12.8; HRMS [M+H]\textsuperscript{+} m/z calcd. 373.1870 for C\textsubscript{19}H\textsubscript{25}N\textsubscript{4}O\textsubscript{4}, found 373.1879.

**Elucidated chemical structure** following 1D/2D NMR analysis (**\textsuperscript{1}H, \textsuperscript{13}C**, COSY, HSQC, HMBC):

**Blue:** \textsuperscript{1}H shifts (ppm)

**Red:** \textsuperscript{13}C shifts (ppm); double \textsuperscript{13}C signals were observed due to two rotamers. All differences were ≤ 0.2 ppm, except for the glycine-CH\textsubscript{2} (44.0 & 43.4 ppm).
$^1$H NMR

$^{13}$C NMR
PymMe (4b) + rTCO-glycine

rTCO-glycine\textsuperscript{12} was dissolved in DMSO to prepare a 62 mM stock solution. 1.29 mL (80.35 µmol, 18.26 mg) of that solution were diluted into 150.2 mL of CitPhos buffer (10 mM, pH 7), for a final concentration of 0.528 mM rTCO-glycine. The solution was then sparged with argon for 15 minutes to reduce dissolved oxygen and minimize potential aromatization, whereupon PymMe tetrazine 4b (1.1 equiv., 88.36 µmol, 15.39 mg) dissolved in 200 µL DMSO were added. At these reagent concentrations the click reaction is expected to be done in <10 minutes. The release reaction was allowed to proceed under argon for 48 h, a timeline after which no further release was observed by serial HPLC analyses. The buffer solution was evaporated to near-dryness and then redissolved in 1 mL of DMSO. The DMSO solution was loaded directly onto a 30 g Biotage Snap C18 SFÄR column and the non-releasing isomer isolated with an ammonium formate buffer (2.5 mM, pH 8.4) / acetonitrile gradient. A single fraction containing the purified material was immediately concentrated, stored at -20 °C under argon, and 1 day later dissolved in CD\textsubscript{3}OD for NMR analysis; \textsuperscript{1}H NMR (600 MHz, CD\textsubscript{3}OD, mixture of rotamers) \(\delta\) 8.74 (d, \(J = 4.9\) Hz, 2H), 7.29 (t, \(J = 4.8\) Hz, 1H), 5.02 – 4.94 (m, 1H), 4.44 (d, \(J = 2.9\) Hz, 1H), 3.79 (d, \(J = 17.5\) Hz, 0.3H), 3.68 – 3.59 (m, 1.1H), 3.52 (d, \(J = 17.5\) Hz, 0.6H), 2.63 – 2.54 (m, 1H), 2.30 – 2.23 (m, 1H), 1.97 (s, 1H), 1.95 (s, 2H), 1.92 – 1.82 (m, 3H), 1.79 – 1.73 (m, 1H), 1.73 – 1.62 (m, 3H), 1.55 – 1.47 (m, 1H); \textsuperscript{13}C NMR (151 MHz, CD\textsubscript{3}OD, mixture of rotamers) \(\delta\) 174.7, 174.6, 162.7, 162.6, 156.9, 156.7, 133.4, 131.5, 131.4, 118.9, 118.9, 100.6, 100.5, 76.3, 44.3, 43.7, 37.0, 36.9, 30.0, 30.0, 28.8, 28.4, 24.0, 20.5, 20.2, 12.8, 12.8; ESI-MS [M+H]\(^+\) m/z calcd. 374.18 for C\textsubscript{18}H\textsubscript{24}N\textsubscript{5}O\textsubscript{4}\(^+\), found 374.14.

Elucidated chemical structure following 1D/2D NMR analysis (\textsuperscript{1}H, \textsuperscript{13}C, COSY, HSQC, HMBC):

Blue: \textsuperscript{1}H shifts (ppm)
Red: \textsuperscript{13}C shifts (ppm); double \textsuperscript{13}C signals were observed due to two rotamers. All differences were \(\leq 0.3\) ppm, except for the glycine-CH\(_2\), (44.3 & 43.7 ppm).
Antibody Labeling

Cetuximab (2.0 mg/mL, as supplied by the manufacturer) was concentrated by centrifugal filtration (Amicon 100K filter) to a concentration of 4 mg/mL. The concentrated stock solution was then buffer-exchanged by 40K Zeba spin column into PBS-Bicarb buffer (phosphate buffered saline plus 100 mM NaHCO₃) that had been freshly adjusted to pH 8.4, as the pH of PBS-Bicarb stock solutions rises over time in storage. The concentration of the buffer-exchanged antibody was re-checked by Nanodrop (A280) and found to be 4.15 mg/mL (28.5 µM). In parallel, 6-azidohexanoic acid sulfoNHS ester (Click Chemistry Tools, 1251-5) was dissolved in MQ water to prepare a 500 µM stock solution.

Optimized Labeling Procedure

Labeling reactions were prepared by adding 1.2 – 1.6 – 2.0 equivalents of the sulfoNHS ester to 100 µL of the antibody solution in PBS-Bicarb. After 20 minutes, the reactions were purified by 40K Zeba spin column (pre-conditioned with PBS). The concentration of the antibody solutions was rechecked following the spin column to verify recovery and account for microscale dilution effects. Aliquots of each N₂-labeled antibody (12 µL) were then combined with 2 µL of a stock solution of DBCO-C₂TCO-AF594 (21) (1.7 mM in DMSO) for a final reaction concentration of 242 µM dye (~10X antibody concentration). The reactions were allowed to proceed at 4 °C overnight, then purified by two successive 40K Zeba spin columns (PBS). Nanodrop analysis of the fluorescently labeled antibodies was used to determine the final dye/label concentrations, per routine, with the following parameters for extinction coefficients and correction factors; cetuximab: \( E_{280} = 215,000 \text{ M}^{-1}\text{cm}^{-1} \), AF594: \( E_{594} = 93,000 \text{ M}^{-1}\text{cm}^{-1} \), correction factor: \( CF_{280} = 0.705 \). The experimentally determined DOL was consistent for the three labeling reactions, with ~0.7 dye molecules per equivalent of N₂-sulfoNHS, resulting in antibodies with a DOL of 0.87, 1.12, and 1.45 dyes/Ab respectively. [Note: the CF280 for DBCO-C₂TCO-AF594 in PBS was experimentally measured and gives consistent results for all AF594-labeled antibodies (irrespective of conjugation chemistry) in our hands; this differs from the CF280 value of 0.56 reported by ThermoFisher for AlexaFluor 594]. To verify intact click-reactivity of the C₂TCO probes and Tz/TCO cleavage in situ, these antibodies were combined on a microscale with HPA (19) and allowed to react for ten minutes, followed by a 40K Zeba column to remove free/cleaved dye. Nanodrop analysis revealed excellent cleavage efficiency for all three Ab-AF594 conjugates, with an inverse correlation between DOL and cleavage efficiency, ranging from ≥97% cleavage at a DOL of 0.87 to 92% cleavage at DOL 1.45. In earlier pilot experiments with a higher degree of N₂ labeling, we noted a consistent trend toward lower C₂TCO reactivity as the number of azides per antibody increased, suggestive of an intramolecular reaction of N₂ with C₂TCO.

Pilot Experiments: concern for C₂TCO – N₂ cross-reactivity

Initial Labeling reactions were prepared by adding 2 – 20 equiv. of the 6-azidohexanoic acid sulfoNHS ester to 100 µL of the antibody solution in PBS-Bicarb. After 20 minutes, the reactions were purified by 40K Zeba spin column (pre-conditioned with PBS), labeled with DBCO-C₂TCO-AF594 (21), 90 µM in the click reaction, 8-10 molar equiv. relative to the mAb) and then characterized as above. In these initial test experiments the efficiency of cleavage varied strikingly as a function of the number of equivalents of azide used in the initial labeling reaction. This was true irrespective of labeling time/temperature, suggesting that it was unlikely to be due to issues of general stability for C₂TCO (which is excellent in even more demanding biofluids/temperatures, see SI Section 4), and more likely to be related to an interaction between the azide tags and the C₂TCO. In order to test that hypothesis, we...
conducted additional follow-experiments systematically varying both the antibody DOL (by controlling the number of equivalents of 6-azidohexanoic acid sulfoNHS ester in the initial N₃-labeling reaction) and the concentration of DBCO-C₂TCO-AF594 (21) in the subsequent click reaction. Plotting the data by observed AF594 DOL (rather than equivalents azide) illustrates the trend:

Higher cleavage efficiency was observed: i) at lower DOL; ii) at higher concentrations of 21, consistent with more rapid reaction of the Ab-N₃ moieties with the DBCO in solution. At a concentration of 240 µM DBCO in excess and a rate constant of 0.3 M⁻¹s⁻¹, complete reaction (>99%) is expected to require ~18 hours. In both cases, cleavage efficiency is increased by conditions that reduce the likelihood (time/stoichiometry) of an intramolecular reaction between an antibody-linked C₂TCO and a neighboring azide, i.e. on a nearby lysine, within the ~3 nm molecular reach of the DBCO-C₂TCO linker.

C₂TCO – Azide reactivity and kinetics

To confirm cycloaddition of C₂TCO with azide-functionalities we reacted DBCO-C₂TCO-AF594 (21, 50 µM in PBS) with 50 mM 2-azidoethanol. While we observed an immediate reaction of DBCO and N₃ (in agreement with the reported second order rate constant of 0.3 M⁻¹s⁻¹, which predicts 99% conversion in ~5 min at these concentrations), extended LCMS monitoring demonstrated cycloaddition of N₃ to the C₂TCO moiety, with a second order rate constant of 0.0011 ± 0.0002 M⁻¹s⁻¹:

Taken together the results support slow reaction between Ab-linked C₂TCO and neighboring N₃-lysine residues due to increased local concentrations. We therefore recommend a switch to other bioconjugation methods when designing probes with a higher DOL.
Cell Culture

A-431 cells were purchased from the American Tissue Culture Collection (ATCC) and passaged in DMEM (10% FBS, 1% penicillin/streptomycin) according to the specifications from ATCC. Cells were first grown in a 150 mm cell culture dish and then seeded on Millicell 8-well EZ slides (Millipore) for imaging. After 24-48 hours confluency was assessed and cells were fixed with 4% paraformaldehyde in PBS (10 min) prior to EGFR imaging.

Cellular Imaging and in-situ cleavage

A-431 cells were stained with 70 nM cetuximab-C_2TCO-AFS94 at 4 °C for three hours, then rinsed with PBS three times. An Olympus BX-63 upright automated epifluorescence microscope was used to acquire fluorescent images. The stained cells were imaged to establish baseline brightness and register coordinates for serial imaging after Tz addition. After collecting the initial image, the slide was removed from the microscope and a solution of 500 µM HPA (19) in PBS was added to the well. The automatic stage was returned to the coordinates of the initial image collection and serial images collected (400 msec exposure) at two-minute intervals. FIJI was used to extract line-intensity profiles for each image for quantitative comparison of signal intensity vs. time (Figure S1).

Figure S1. Line-intensity profiles for HPA-triggered cleavage of Cetux-C_2TCO-AFS94 on A-431 cells.
Intracellular cleavage of Ib-C\textsubscript{2}TCO-SiR

**Cell culture**

HT1080 human fibrosarcoma cells (ATCC) and HT1080-BTK-mCherry cells\textsuperscript{6,14} 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% \text{CO}_2.

**Cellular imaging and in-situ cleavage**

HT1080-BTK-mCherry cells were seeded into a 96-well plate at 10,000 cells per well and allowed to grow overnight. Cells were incubated with Ib-C\textsubscript{2}TCO-SiR (22) at 2 \text{μM} final concentration in growth medium for five hours. They were washed three times with fresh medium for 30 minutes each, stained with Hoechst 33342 nuclear dye (Invitrogen) and washed twice with PBS. The cells were fixed and permeabilized by treatment with BD Cytofix\textsuperscript{TM} (contains 4.2 % paraformaldehyde) for 15 minutes at room temperature followed by two washes with BD Cytoperm\textsuperscript{TM} buffer. A solution of HK (20) (20 \text{μM} in PBS) or PBS only (control) was added to the cells and left for 30 minutes at room temperature. Cells were rinsed once with PBS and multichannel images were collected on an Olympus IX83 microscope.

In a control experiment, HT1080-BTK-mCherry and HT1080 cells were seeded into a 96-well plate at 10,000 cells per well, grown overnight and incubated with Ib-C\textsubscript{2}TCO-SiR (22) at 2 \text{μM} final concentration in growth medium for five hours. They were washed three times with fresh medium for 30 minutes each, stained with Hoechst 33342 nuclear dye (Invitrogen) and washed twice with PBS. Imaging of live cells was carried out in FluoroBrite\textsuperscript{TM} DMEM medium (Gibco) on an Olympus IX83 microscope equipped with a cellVivo incubation system.
8) NMR spectra and chromatograms

C$_2$TCO synthesis and modification

Compound 9, $^1$H NMR

Compound anti-10/syn-10 (10/1), $^1$H NMR
Compound **anti-10/syn-10 (10/1)**, $^1$H NMR, $^{13}$C NMR

Compound **anti-10/syn-10 (10/1)**, $^1$H NMR, GCMS
Compound bis-axial-11, $^1$H NMR

Compound bis-axial-11, $^{13}$C NMR
Compound **bis-axial-11**, GCMS

![GCMS spectrum of compound bis-axial-11](image)

Compound **bis-equatorial-11**, $^1$H NMR

![$^1$H NMR spectrum of compound bis-equatorial-11](image)
Compound **bis-equatorial-11**, $^{13}$C NMR

Compound **13**, $^1$H NMR
Compound 13, $^{13}$C NMR (APT)

(Sar-C$_2$TFO-Sar)

Compound 13, LCMS

extracted mass (m/z 373)
Compound 14, $^1$H NMR

![NMR spectrum of Compound 14, $^1$H NMR]}

Compound 14, $^{13}$C NMR

![NMR spectrum of Compound 14, $^{13}$C NMR]
Compound 15, $^1$H NMR

Compound 15, $^{13}$C NMR
Compound **15**, LCMS

![LCMS Graph]

**TIC(+)**

*extracted mass (m/z 948)*

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**Compound S1, $^1$H NMR**

![NMR Spectrogram]

**UV (590 nm)**

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Compound S1, LCMS


dendritic

UV (254 nm)

Compound 21, $^1$H NMR
Compound 21, LCMS

![LCMS graphs](image)

UV (594 nm)

Compound S3, $^1$H NMR

![NMR spectrum](image)

S38
Compound S3, $^{13}$C NMR

Compound S3, LCMS

UV (254 nm)
Compound S4, $^1$H NMR

![NMR Spectrum](image)

Compound S4, LCMS

![LCMS Spectrum](image)

UV (282 nm)
Compound 22, $^1$H NMR

UV (254 nm)

Compound 22, LCMS
Tetrazines

Compound 5, $^1$H NMR

Compound 5, $^{13}$C NMR
Compound 18, $^1$H NMR

Compound 18, $^{13}$C NMR
Compound 19, $^1H$ NMR

Compound 19, $^{13}C$ NMR
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