

Supporting Information

Spiro Di-orthoester (SpiDo), a Human Plasma Stable Acid-Sensitive Cleavable Linker for Lysosomal Release

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General experimental procedures: Unless otherwise indicated, reactions were carried out under argon atmosphere in flame-dried glassware equipped with magnetic stirring. Air or moisture-sensitive liquids were transferred *via* syringe. If required, solutions were degassed by passing a stream of argon through the solutions. Organic solutions were concentrated by rotary evaporation at 25-60 °C at 15-30 torr. Analytical thin layer chromatography (TLC) was performed using plates cut from glass sheets (silica gel 60F-254 from Merck). Visualization was achieved by 254 or 365 nm UV light and by immersion in an ethanolic solution of cerium sulfate, and subsequent treatment with a heat gun. Column chromatography was carried out as “Flash Chromatography” using silica gel G-25 (40-63 μM) from Macherey-Nagel.

Materials: All reagents were obtained from commercial sources and used without further purifications. All dry solvents were obtained from Aldrich or Alfa Aesar.

Instrumentation: ¹H and ¹³C NMR spectra were recorded at 23 °C on a Bruker 400 or 500 spectrometers. Recorded shifts are reported in parts per million (δ) and calibrated using residual undeuterated solvent signals. Data are represented as follows: Chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, m = multiplet, br = broad), coupling constant (*J*, Hz) and integration. High resolution mass spectra (HRMS) were obtained using a Agilent Q-TOF (time of flight) 6520 and low resolution mass spectra using a Agilent MSD 1200 SL (ESI/APCI) with a Agilent HPLC1200 SL. HPLC experiments were performed on a Shimadzu system (Pump: model LC 20-AD, UV-detector: SPD 20-A, Autosampler: SIL 20-A). Method: Column: XBridge C₁₈ (150 mm × 4.5 mm i.d., 5 μm, Waters). Flow: 1 mL/min. Injection volume = 10 μL. Eluent A/B NH₃/HCOOH solution (10 mM, pH 8.5)/ACN. Gradient: 5% B to 95% B in 20 minutes and 10 minutes of re-equilibration. Detection: 254 nm. Fluorescence kinetics were recorded using a multilabel plate reader (Victor X2, PerkinElmer - Excitation and emission used filters were 550/8 nm and 580/10 nm).

Semi-Preparative HPLC: The semi-preparative HPLC system consisted of a Waters 600 pump, a 2487 detector (Waters), a 5 mL sample loop.

Method 1 in acidic conditions: Column: Sunfire C₁₈ (150 mm × 19 mm i.d., 5 μm, Waters). Flow: 17 mL/min. Injection volume = 1 mL. Eluent A/B water/ACN, with 0.05% TFA.

Gradient: 5% B to 95% B in 40 minutes and 10 minutes of re-equilibration. Detection: 254 nm.

Method 2 in basic conditions: Column: XBridge C₁₈ (150 mm × 19 mm i.d., 5 μm, Waters). Flow: 17 mL/min. Injection volume = 1 mL. Eluent A/B NH₃/HCOOH solution (10 mM, pH 8.5)/ACN. Gradient: 5% B to 95% B in 40 minutes and 10 minutes of re-equilibration. Detection: 254 nm.

Method for hydrolysis of S3 at pH 5.5 and 7.4: A 10 mM solution of the S3 was prepared in DMSO and diluted in corresponding buffer to obtain a 1 mM solution (1 mL). For pH 7.4 and 5.5, phosphate buffers were used (NaH₂PO₄/Na₂HPO₄ pH 7.4 or KH₂PO₄/Na₂HPO₄ pH 5.5, 100 mM). Each solution was immediately analyzed by analytical HPLC (basic conditions, as mention above). Reaction was performed at 25 °C and crude was injected every 30 minutes for up to 15 hours.

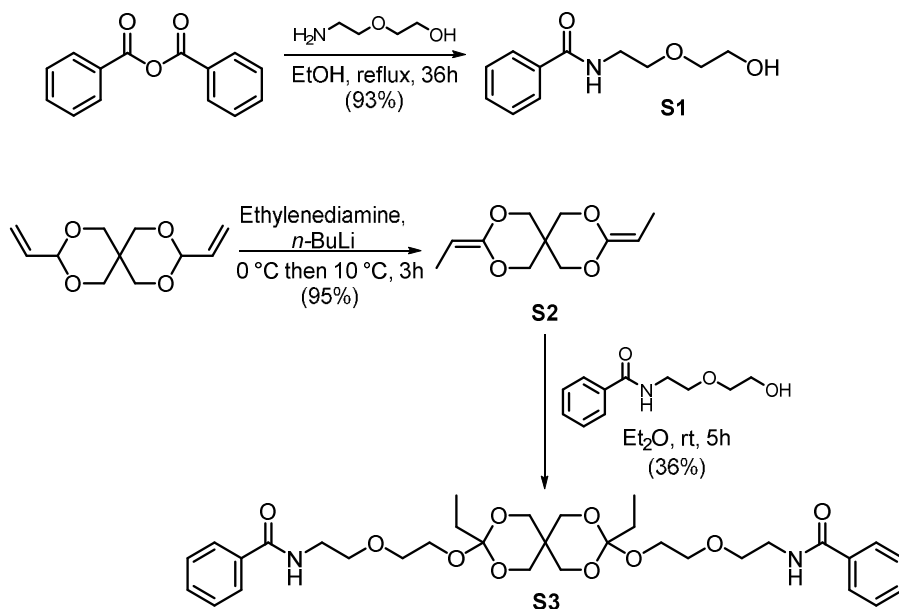


Figure S1: Synthesis of S3

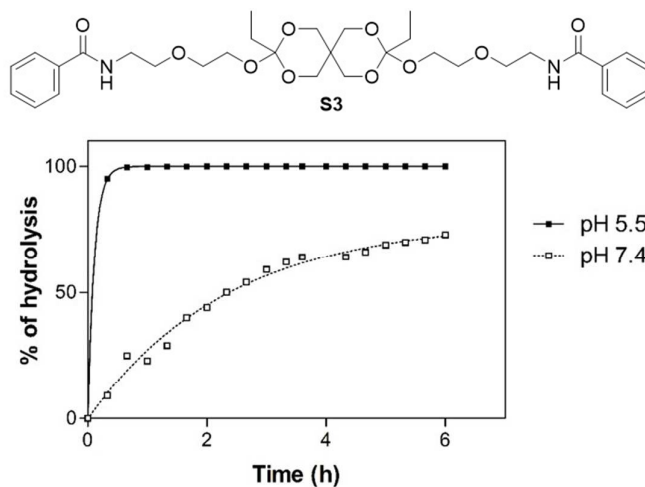


Figure S2: Hydrolysis kinetics of **S3** at pH 5.5 and 7.4

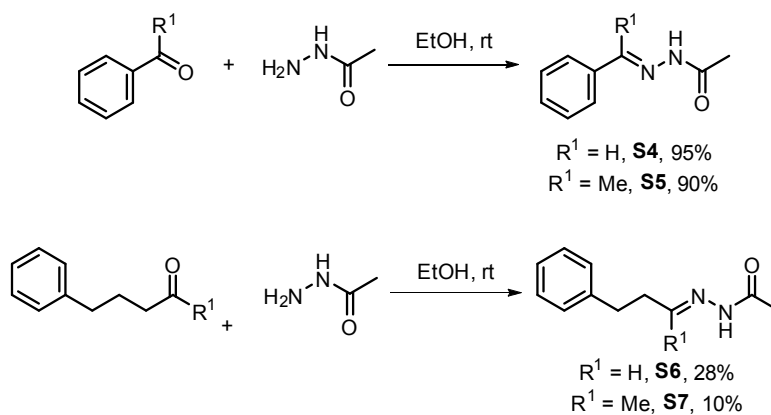


Figure S3: Synthesis of **S4-S7**

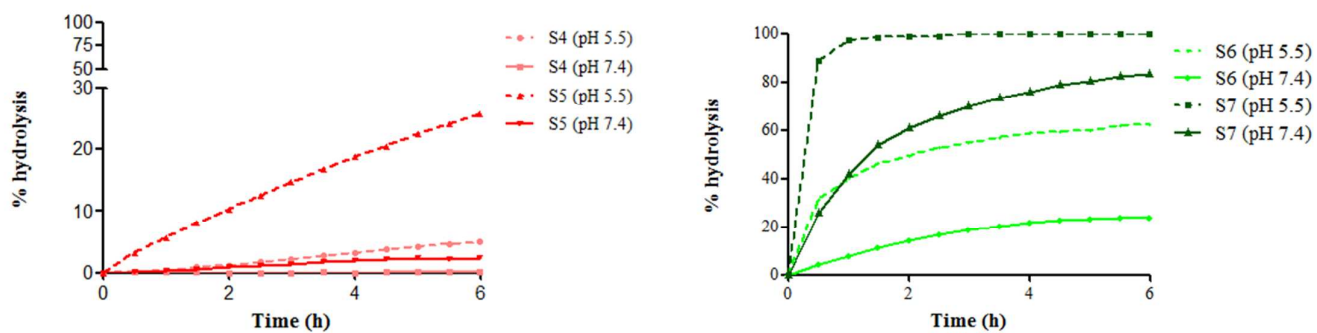


Figure S4: Hydrolysis kinetics of **S4-S7** at pH 5.5 and 7.4

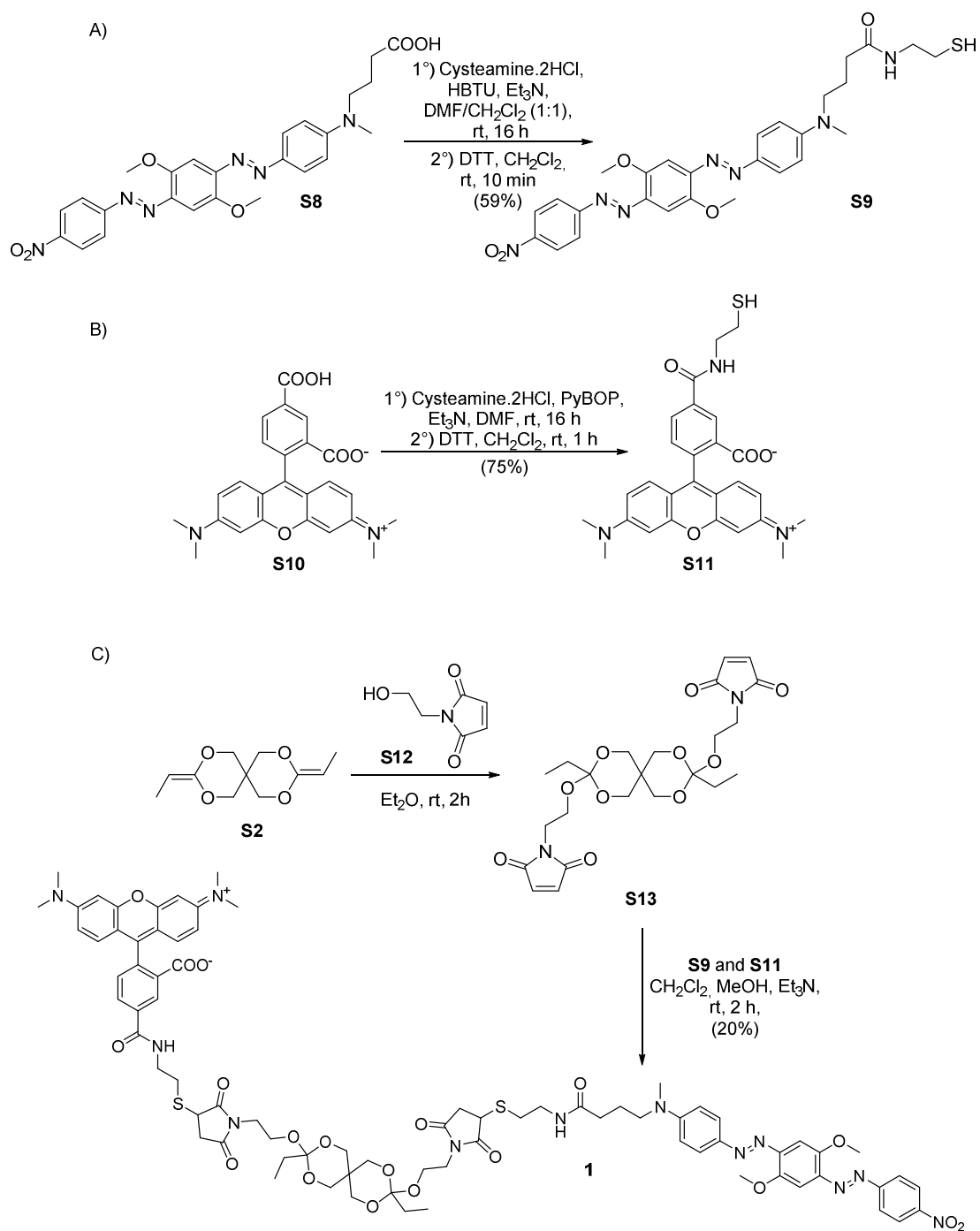


Figure S5: Synthesis of 1

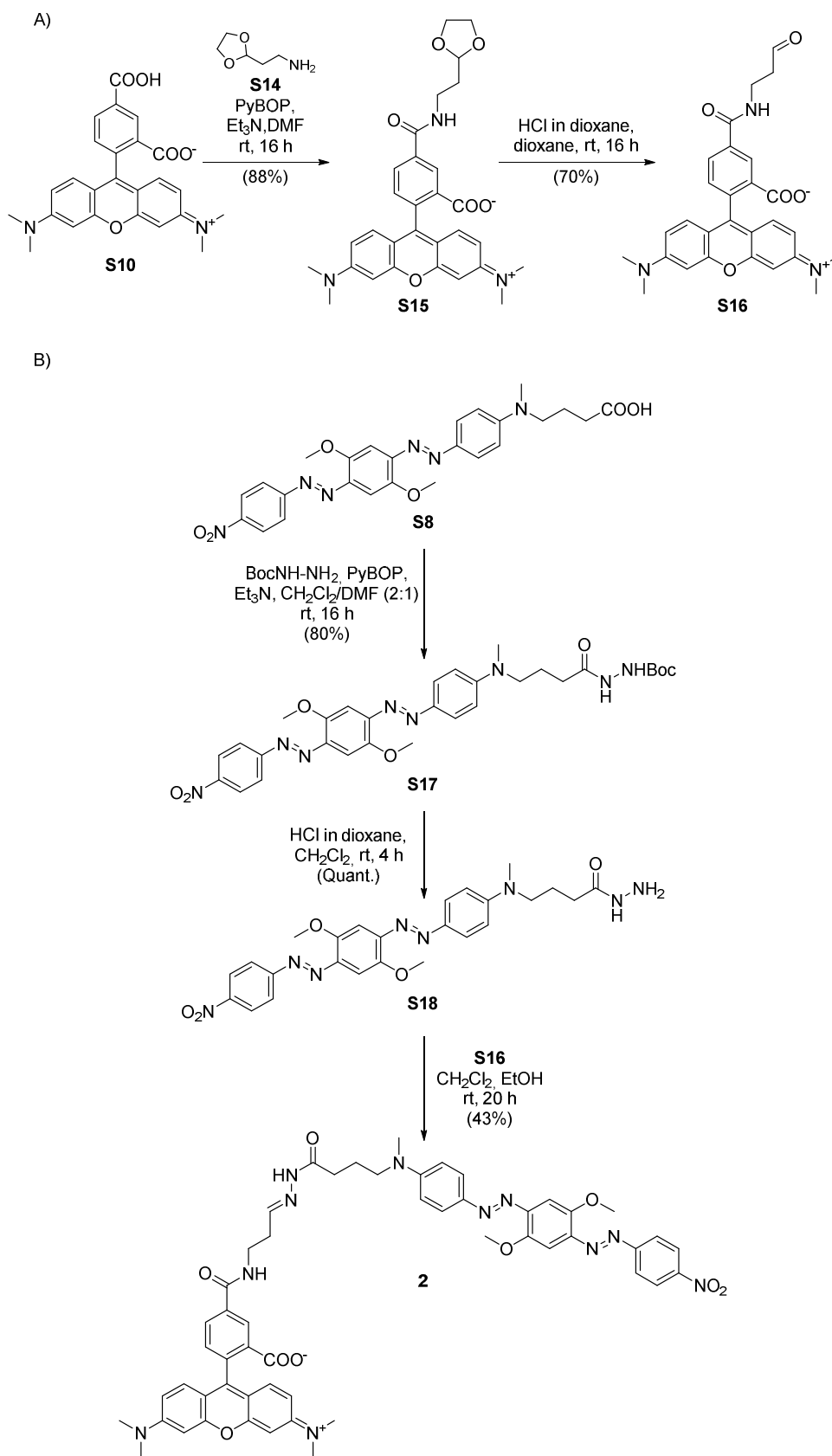


Figure S6: Synthesis of 2

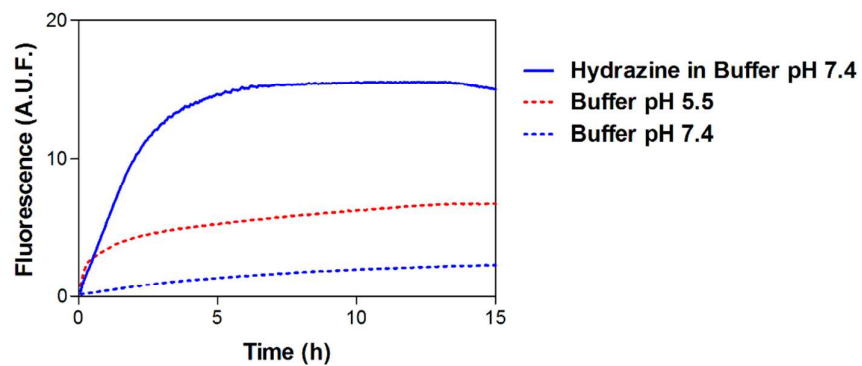


Figure S8: Effect of hydrazine on probe 2

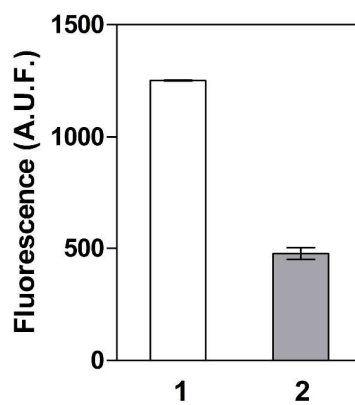


Figure S9: Flow cytometry analysis of 2000 BNL CL.2 cells loaded with probes 1 and 2 (1 μ M, 4 h). This experiment was performed on a PCA-96 Guava cytometer for which 2000 cells is sufficient to give accurate and reproducible results.

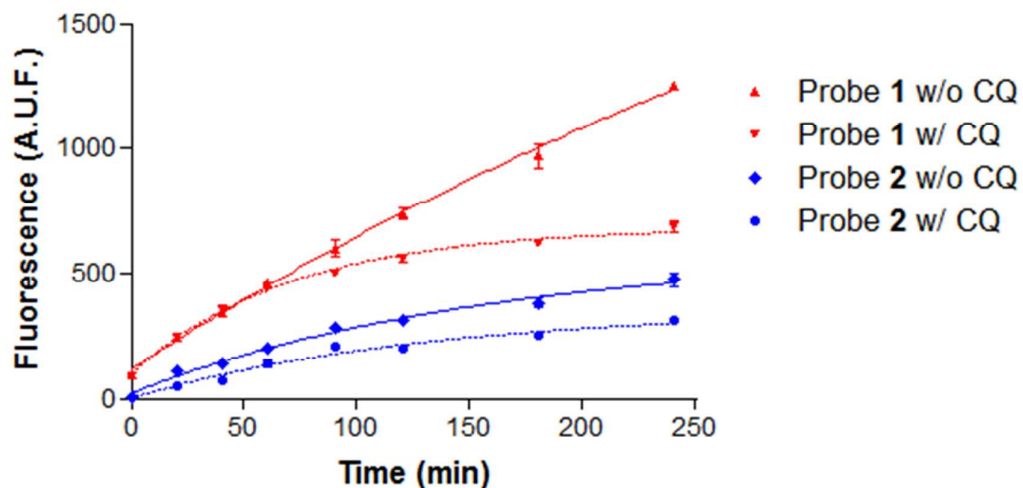


Figure S10: Chloroquine effect on FRET-based probes **1-2** activation using 2000 BNL CL.2 using flow cytometry analysis. Probe **1**: TAMRA-Orthoester-(BHQ-2) (1 μ M) (w/ QC) = cells pretreated with chloroquine (200 μ M, 2 h) and addition of chloroquine (200 μ M) during probe incubation. (w/o QC) = non-treated cells. Probe **2**: TAMRA-Acylhydrazon-(BHQ-2) (1 μ M) (w/ QC) = cells pretreated with chloroquine (200 μ M, 2 h) and addition of chloroquine (200 μ M) during probe incubation. (w/o QC) = non-treated cells.

Synthesis

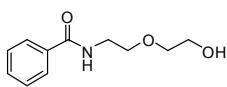
General procedure A: modification of S8

S8 (0.272 mmol) was dissolved in a mixture of DMF/CH₂Cl₂ (1:2) (9.0 mL) and Et₃N (0.816 mmol) and the amine (0.408 mmol) were added. The mixture was cooled to 0 °C and PyBOP (0.408 mmol) was added. The solution was allowed to warm up to room temperature and stirred for 16 hours. The reaction mixture was diluted with saturated NaHCO₃ solution (50 mL) and extracted with EtOAc (3 × 50 mL). The organic layers were combined, washed successively with saturated NaHCO₃ solution (50 mL), water (50 mL), brine (50 mL) and dried over Na₂SO₄. The crude product was purified by silica gel column chromatography (CH₂Cl₂/MeOH 100:0 to 95:5) to yield the desired product.

General procedure B: modification of S10

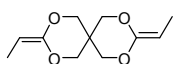
S10 (0.167 mmol) was dissolved in DMF (1.20 mL) and Et₃N (0.334 mmol) was added. The mixture was cooled to 0 °C and PyBOP (0.167 mmol) was added. After 10 minutes, the amine (0.200 mmol) was added to the mixture and stirred for additional 16 hours at room temperature. The crude mixture was purified by preparative HPLC (Method 1) and fractions were lyophilized to afford the desired product.

N-(2-(2-Hydroxyethoxy)ethyl)benzamide **S1**



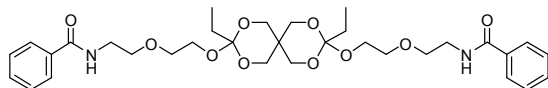
This compound was synthesized following a reported protocol.¹

3,9-Diethylidene-2,4,8,10-tetraoxaspiro[5.5]undecane **S2**



This compound was synthesized following a reported protocol.²

N,N'-(2,2'-(2,2'-(3,9-Diethyl-2,4,8,10-tetraoxaspiro[5.5]undecane-3,9-diyl)bis(oxy)bis(ethane-2,1-diyl))bis(oxy)bis(ethane-2,1-diyl))dibenzamide **S3**



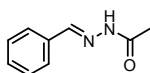
This compound was synthesized following a reported protocol for orthoester synthesis.³ **S1**

(425 mg, 2.03 mmol) was dissolved in anhydrous Et₂O (36 mL) and the spiro diketene acetal **S2** (196 mg, 0.92 mmol) was added. The reaction mixture was stirred for 5 hours at room

temperature and evaporated *in vacuo*. The obtained residue was then purified by preparative HPLC (method 2) to yield **S3** (210 mg, 36%) as a colorless oil.

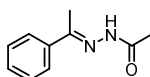
^1H NMR (400 MHz, $\text{CD}_3\text{CN}-d_3$) δ 7.78 (d, $J = 7.4$ Hz, 4H), 7.53-7.42 (m, 6H), 7.08 (brs, 2H), 3.96 (dd, $J = 2.4, 10.8$ Hz, 2H), 3.89 (d, $J = 11.3$ Hz, 2H), 3.66-3.62 (m, 10H), 3.55-3.49 (m, 8H), 3.17 (dd, $J = 2.4, 11.3$ Hz, 2H), 1.66 (q, $J = 7.5$ Hz, 2H), 0.85 (t, $J = 7.5$ Hz, 2H); ^{13}C NMR (100 MHz, $\text{CD}_3\text{CN}-d_3$) δ 132.3, 129.5, 128.0, 118.4, 113.5, 70.7, 70.3, 63.6, 63.5, 62.9, 40.5, 31.4, 29.2, 7.9.

(E)-*N'*-benzylideneacetohydrazide **S4**



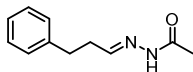
This compound was synthesized following a reported protocol.⁴

(E)-*N'*-(1-phenylethylidene)acetohydrazide **S5**



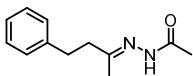
This compound was synthesized following a reported protocol.⁵

(E)-*N'*-(3-phenylpropylidene)acetohydrazide **S6**



This compound was synthesized following a reported protocol.⁶

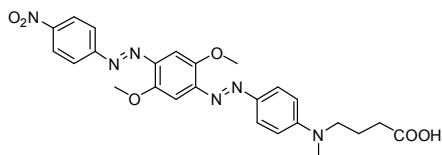
(E)-*N'*-(4-phenylbutan-2-ylidene)acetohydrazide **S7**



A solution of benzylacetone (742 μL , 4.95 mmol, 1.0 eq) and acetylhydrazine (366 mg, 4.95 mmol, 1.0 eq) in EtOH (10 mL) was stirred at rt for 12 hr. The reaction mixture was concentrated under reduced pressure and the crude material was purified by recrystallization in EtOH to give **S7** as a white solid (105 mg, 10%).

^1H NMR (400 MHz, CDCl_3) δ 8.50 (s, 1H), 7.31 – 7.26 (m, 2H), 7.21 – 7.19 (m, 3H), 2.89 (t, $J = 7.8$ Hz, 2H), 2.58 (t, $J = 7.8$ Hz, 2H), 2.24 (s, 3H), 1.82 (s, 3H); ^{13}C NMR (101 MHz, CDCl_3) δ 173.5, 150.8, 141.3, 128.5 (2C), 128.4 (2C), 126.2, 40.5, 32.3, 20.6, 15.5.

4-((4-((2,5-Dimethoxy-4-((4-nitrophenyl)diazenyl)phenyl)diazenyl)phenyl)(methyl)amino)butanoic acid **S8**

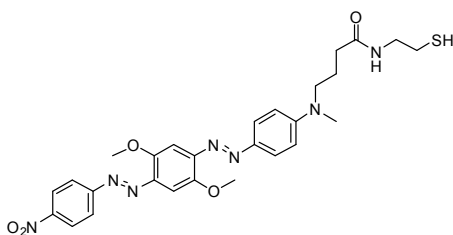


Fast Black K hemi(zinc chloride) salt (practical grade, \approx 30% dye content) (7.76 g) was suspended in cold water (150.0 mL, 0 °C) and stirred for 20 minutes. The suspension was filtered, and the red solution was

added dropwise to a cold (0 °C) mixture of 4-(methyl(phenyl)amino)butanoic acid (1.33 g, 6.88 mmol), concentrated hydrochloric acid (3.1 mL) and sodium acetate (3.6 g) in water and acetone (1:1) (150 mL). The reaction mixture was stirred at 10 °C for 15 minutes, then at room temperature for 2 hours. The reaction crude was extracted with EtOAc (3 \times 150 mL) and the combined organic layers were dried over Na₂SO₄. The crude product was purified by silica gel column chromatography (100% EtOAc, then 100% CH₂Cl₂ to CH₂Cl₂/MeOH (95:5)). **S8** (1.36 g, 39%) was obtained as a dark violet solid.

¹H NMR (400 MHz, MeOD-*d*₄) δ 8.31 (d, J = 9.0 Hz, 2H), 8.00 (d, J = 9.0 Hz, 2H), 7.86 (d, J = 9.0 Hz, 2H), 7.45 (s, 1H), 7.40 (s, 1H), 6.77 (d, J = 9.0 Hz, 2H), 4.05 (s, 3H), 4.00 (s, 3H), 3.5 (t, J = 7.1 Hz, 2H), 2.36 (t, J = 7.1 Hz, 2H), 1.98-1.90 (m, 2H); ¹³C NMR (101 MHz, MeOD-*d*₄) δ 176.2, 157.1, 154.3, 153.0, 151.4, 149.0, 147.4, 145.0, 142.6, 126.9, 125.3, 124.2, 112.1, 101.7, 100.7, 57.2, 52.3, 39.0, 31.6, 22.9.

4-((4-((2,5-Dimethoxy-4-((4-nitrophenyl)diazenyl)phenyl)diazenyl)phenyl)(methyl)amino)-N-(2-mercaptoethyl)butanamide **S9**



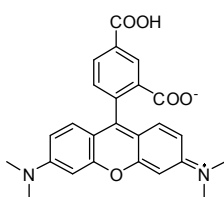
S8 (92 mg, 0.182 mmol) was dissolved in a mixture of DMF/ CH₂Cl₂ (1:2) (15 mL) and Et₃N (150 μ L, 1.09 mmol) and cystamine dichloride (138 mg, 0.95 mmol) were added. The mixture was cooled to 0 °C and HBTU (69 mg, 0.182 mmol) was added. The

solution was allowed to reach room temperature and stirred for 16 hours. When total conversion was reached, DTT (168 mg, 1.09 mmol) was added. After the resulting mixture has been stirred for 10 minutes at room temperature, the crude was diluted with saturated NaHCO₃ solution (75 mL) and extracted with EtOAc (2 \times 50 mL). The organic layers were combined, washed with water (50 mL), brine (50 mL) and dried over Na₂SO₄. The crude product was purified by column chromatography on silica gel (CH₂Cl₂/MeOH 100:0 to 95:5) to yield **S9** as a dark violet solid (61 mg, 59%).

R_f 0.33 (CH₂Cl₂/MeOH 95:5); ¹H NMR (400 MHz, CDCl₃-d₁) δ 8.33 (d, *J* = 9.0 Hz, 2H), 8.0 (d, *J* = 9.1 Hz, 2H), 7.9 (d, *J* = 9.1 Hz, 2H), 7.42 (s, 1H), 7.42 (s, 1H), 6.75 (d, *J* = 9.00 Hz, 2H), 5.90 (t, *J* = 5.6 Hz, 1H), 4.06 (s, 3H), 4.01 (s, 3H), 3.49 (t, *J* = 7.4 Hz, 2H), 3.41 (dt, *J* = 6.2, 6.4 Hz, 2H), 2.64 (td, *J* = 6.4, 8.47 Hz, 2H), 2.24 (t, *J* = 7.4 Hz, 2H), 2.01-1.94 (m, 2H); ¹³C NMR (101 MHz, CDCl₃-d₁) δ 172.2, 156.6, 153.8, 152.4, 151.1, 148.5, 147.0, 144.7, 142.3, 126.4, 124.9, 123.7, 111.6, 101.2, 100.3, 57.0, 56.9, 51.8, 42.5, 38.7, 33.4, 24.9, 23.0; IR (neat): 2939, 1594, 1518, 1339, 1155 cm⁻¹; ESI-MS: 566.2 [M+H]⁺. HRMS calcd 565.2107 for C₂₇H₃₁N₇O₅S, found 565.2105.

2-(6-(Dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl)-5-(prop-2-ynylcarbamoyl)

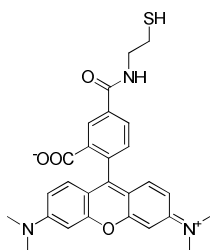
Benzoate **S10**



This compound was synthesized following a reported protocol.⁷

2-(6-(Dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl)-4-(2-mercaptoethylcarbamoyl)

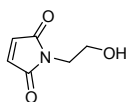
benzoate **S11**



S10 (92 mg, 0.214 mmol) was dissolved in DMF (1.60 mL) and Et₃N (208 μL, 1.50 mmol) was added. The mixture was cooled to 0 °C and PyBOP (111 mg, 0.214 mmol) was added. After 10 minutes, cystamine dihydrochloride (162 mg, 1.07 mmol) was added to the solution and the reaction mixture was stirred for 16 hours at room temperature. Then, the reaction mixture was diluted with CH₂Cl₂ (3.0 mL) and stirred for an additional 1 hour at room temperature after DTT (198 mg, 1.28 mmol) addition. CH₂Cl₂ was removed under reduce pressure, the crude purified by preparative HPLC (Method 1) and fractions were lyophilized to afford the desired **S11** as a fluffy pink solid (78 mg, 75%).

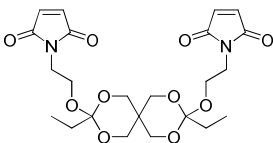
¹H NMR (400 MHz, DMSO-*d*₆) δ 9.06 (t, *J* = 5.4 Hz, 1H), 8.70 (d, *J* = 1.8 Hz, 1H), 8.30 (dd, *J* = 1.8, 8.0 Hz, 1H), 7.59 (d, *J* = 8.0 Hz, 1H), 7.08-7.02 (m, 4H), 6.95 (s, 2H), 3.52-3.42 (m, 2H), 3.26 (s, 12H), 2.72 (dt, *J* = 6.8, 8.0 Hz, 2H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 166.0, 164.7, 156.8, 156.6, 135.9, 131.2, 130.6, 114.6, 96.3, 42.9, 40.5, 23.3; ESI-MS: 490.2 [M+H]⁺. HRMS calcd 489.1722 for C₂₇H₂₇N₃O₄S, found 489.1723.

N-(2-Hydroxyethyl)maleimide **S12**



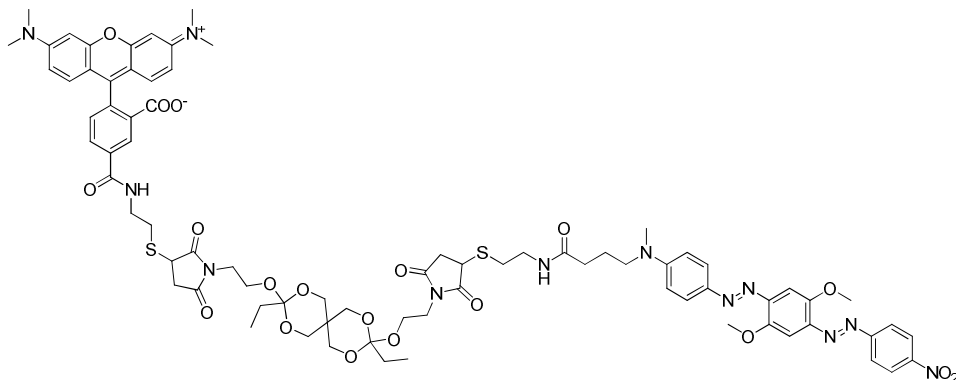
Starting from maleic anhydride, **S12** was synthesized in three steps according to a described procedure.⁸

3,9-Bis(maleimdioethoxy)-3,9-ethane-2,4,8,10-tetraoxaspiro[5.5]undecane **S13**



S13 was synthesized in one step according to a described procedure.³

4-(2-(1-(2-(9-(2-(3-(2-(4-((4-(2,5-Dimethoxy-4-((4-nitrophenyl)diazenyl)phenyl)diazenyl)phenyl)(methylamino)butanamido)ethylthio)-2,5-dioxopyrrolidin-1-yl)ethoxy)-3,9-diethyl-2,4,8,10-tetraoxaspiro[5.5]undecan-3-yloxy)ethyl)-2,5-dioxopyrrolidin-3-ylthio)ethylcarbamoyl)-2-(6-(dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl)benzoate **1**

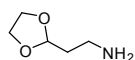


S11 (11.5 mg, 0.023 mmol) was dissolved in degassed anhydrous MeOH (1.7 mL) and Et₃N (20 μ L) was added to this solution. Then, a solution of **S9** (13.3 mg, 0.023 mmol) in degassed anhydrous CH₂Cl₂ (1.7 mL) was added to the previous solution. A solution of **S13** (11 mg, 0.023 mmol) in CH₂Cl₂ (0.2 mL) was added to the previous solution and the reaction mixture was stirred for 3 hours at room temperature. The solvents were removed under reduced pressure and the resulting residue was dissolved in DMSO (1.0 mL), purified by preparative HPLC (Method 2) and fractions were lyophilized to afford **1** (7 mg, 20%) as a fluffy violet-pink solid.

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.99 (t, *J* = 5.2 Hz, 1H), 8.44-8.42 (m, 3H), 8.22 (d, *J* = 8.3 Hz, 1H), 8.06-8.02 (m, 3H), 7.81 (d, *J* = 9.1 Hz, 2H), 7.44 (s, 1H), 7.37 (s, 1H), 7.32 (d, *J* = 8.4 Hz, 2H), 6.87 (d, *J* = 9.1 Hz, 2H), 6.52-6.45 (m, 6H), 4.11-4.03 (m, 2H), 3.99 (s, 3H), 3.94 (s, 3H), 3.87-3.85 (m, 4H), 3.77-3.75 (m, 4H), 3.63-3.55 (m, 6H), 3.48-3.47 (m, 6H), 3.29-3.17 (m, 6H), 3.06 (s, 3H), 2.93-2.87 (m, 14H), 2.58-2.54 (m, 2H), 2.16 (t, *J* = 6.8 Hz,

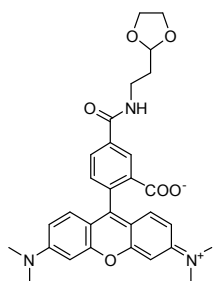
2H), 1.84-1.77 (m, 2H), 1.59 (q, $J = 7.4$ Hz, 4H), 0.78 (t, $J = 7.4$ Hz, 6H); ^{13}C NMR (400 MHz, DMSO- d_6) δ 176.7, 176.6, 171.7, 168.3, 164.8, 155.7, 154.9, 153.1, 152.2, 152.1, 152.0, 150.3, 148.1, 146.2, 143.5, 141.4, 135.9, 134.4, 128.3, 126.9, 125.7, 125.1, 124.2, 123.4, 123.1, 112.0, 111.5, 108.9, 105.5, 100.8, 100.1, 98.0, 62.1, 62.0, 58.2, 51.2, 38.3, 38.2, 38.0, 35.9, 32.2, 30.6, 30.3, 29.9, 27.7, 22.4, 7.3; MS analysis yield two products of orthoester hydrolysis : HRMS calcd 706.2533 for $\text{C}_{33}\text{H}_{38}\text{N}_8\text{O}_8\text{S}$, found 706.2523. HRMS calcd 630.2148 for $\text{C}_{33}\text{H}_{34}\text{N}_4\text{O}_7\text{S}$, found 630.2142.

2-(1,3-Dioxolan-2-yl)ethanamine S14



This compound was synthesized according to a described procedure.⁹

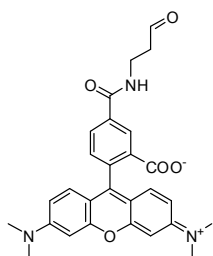
5-(2-(1,3-Dioxolan-2-yl)ethylcarbamoyl)-2-(6-(dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl)benzoate S15



S15 was synthesized according to the general procedure B. **S10** (81 mg, 0.189 mmol) reacted with **S14** (35 mg, 0.264 mmol) to give **S15** as a fluffy pink solid (88 mg, 88%).

^1H NMR (400 MHz, MeOD- d_4) δ 8.68 (d, $J = 1.8$ Hz, 1H), 8.15 (dd, $J = 1.8, 8.0$ Hz, 1H), 7.43 (d, $J = 8.0$ Hz, 1H), 7.06 (s, 1H), 7.04 (s, 1H), 6.96 (dd, $J = 2.4, 9.4$ Hz, 2H), 6.87 (d, $J = 2.4$ Hz, 2H), 4.91 (t, $J = 4.5$ Hz, 1H), 3.94-3.90 (m, 2H), 3.81-3.77 (m, 2H), 3.52 (t, $J = 7.0$ Hz, 2H), 3.21 (s, 12H), 1.95 (dt, $J = 4.5, 7.0$ Hz, 2H); ^{13}C NMR (101 MHz, MeOD- d_4) δ 168.1, 167.5, 160.2, 159.2, 159.1, 138.2, 138.0, 133.0, 132.4, 132.1, 131.4, 115.7, 114.9, 104.3, 97.6, 66.2, 41.1, 36.8, 34.4; IR (neat): 2930, 1646, 1594, 1408, 1346, 1186 cm^{-1} ; HRMS calcd 529.2213 for $\text{C}_{30}\text{H}_{31}\text{N}_3\text{O}_6$, found 529.2207.

2-(6-(Dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl)-5-(3-oxopropylcarbamoyl)benzoate S16

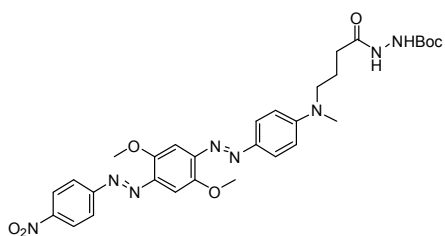


S15 (19 mg, 0.035 mmol) was dissolved in dioxane (0.7 mL) and concentrated HCl solution (0.3 mL) was added. The reaction was stirred at room temperature for 16 hours and the solvents were removed under reduced pressure. The residue was dissolved in DMSO (1.0 mL), purified by preparative HPLC (Method 1) and lyophilized to afford the desired

S16 as a fluffy pink solid (12 mg, 70%).

^1H NMR (400 MHz, DMSO- d_6) δ 9.74 (t, J = 1.7 Hz, 1H), 8.99 (t, J = 5.1 Hz, 1H), 6.45 (s, 1H), 8.27 (dd, J = 1.8, 8.0 Hz, 1H), 7.56 (d, J = 8.0 Hz, 1H), 7.00-6.92 (m, 6H), 3.63 (q, J = 6.3 Hz, 2H), 3.24 (s, 12H), 2.75 (dt, J = 1.8, 6.3 Hz, 2H); ^{13}C NMR (101 MHz, DMSO- d_6) δ 202.2, 164.7, 144.4, 135.7, 130.5, 130.4, 130.3, 114.7, 111.4, 101.6, 96.4, 43.0, 40.3, 33.6, HRMS calcd 485.1951 for $\text{C}_{28}\text{H}_{27}\text{N}_3\text{O}_5$, found 485.1951.

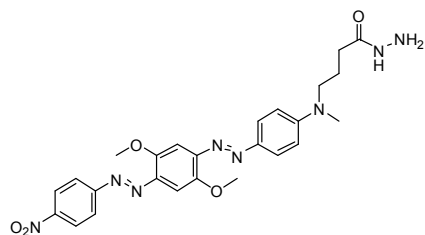
tert-Butyl 2-(4-((4-((2,5-dimethoxy-4-((4-nitrophenyl)diazenyl)phenyl)diazenyl)phenyl)
(methyl)amino)butanoyl)hydrazinecarboxylate **S17**



S17 was synthesized according to the general procedure A. **BHQ-2** (115 mg, 0.227 mmol) reacted with *t*-butyl carbazate (45 mg, 0.34 mmol) to give **S17** as a dark violet solid (112 mg, 80%).

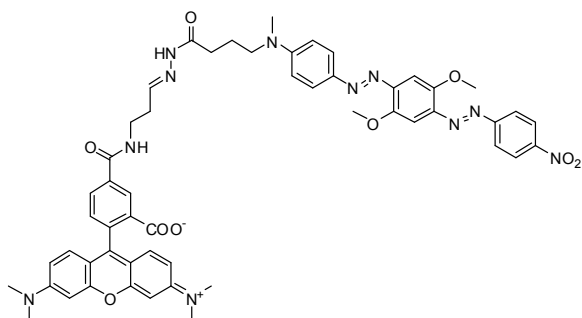
R_f 0.41 ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 95:5); ^1H NMR (400 MHz, CDCl_3 - d_1) δ 8.34 (d, J = 9.0 Hz, 2H), 8.01 (d, J = 9.0 Hz, 2H), 7.9 (d, J = 9.0 Hz, 2H), 7.47 (s, 1H), 7.43 (s, 1H), 6.75 (d, J = 9.0 Hz, 2H), 4.07 (s, 3H), 4.02 (s, 3H), 3.50 (t, J = 7.0 Hz, 2H), 3.06 (s, 3H), 2.26 (t, J = 7.0 Hz, 2H), 2.04-1.97 (m, 2H), 1.64 (brs, 2H), 1.46 (s, 9H); ^{13}C NMR (101 MHz, CDCl_3 - d_1) δ 172.0, 156.7, 153.8, 152.3, 151.2, 148.6, 147.0, 144.8, 142.3, 126.4, 124.9, 123.7, 111.7, 101.2, 100.3, 82.2, 57.0, 57.1, 51.7, 38.8, 30.9, 28.4, 22.7; IR (neat) : 3254, 1717, 1595, 1519, 1489, 1366, 1244, 1156 cm^{-1} ; ESI-MS: 620 $[\text{M}+\text{H}]^+$. HRMS calcd 620.2707 for $\text{C}_{30}\text{H}_{36}\text{N}_8\text{O}_7$, found 620.2709.

4-((4-((2,5-Dimethoxy-4-((*E*)-(4-nitrophenyl)diazenyl)phenyl)diazenyl)phenyl)
(methyl)amino)butanehydrazide **S18**



S73 (102 mg, 0.164 mmol) was dissolved in CH_2Cl_2 (4.0 mL) and 4N HCl in dioxane (2.0 mL) was added. The reaction was stirred at room temperature for 4 hours and the solvents were removed under reduced pressure. The resulting residue was extracted with CH_2Cl_2 (20 mL), the organic layer was washed with saturated NaHCO_3 solution (50 mL), water (50 mL) and dried over Na_2SO_4 . **S18** was obtained as a dark violet solid and directly engaged in the next step without further purification.

5-(3-(2-(4-((4-((2,5-Dimethoxy-4-((4-nitrophenyl)diazenyl)phenyl)diazenyl)phenyl)(methylamino)butanoyl)hydrazono)propylcarbamoyl)-2-(6-(dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl)benzoate **2**



A solution of **S16** (12 mg, 0.023 mmol) in anhydrous EtOH (0.7 mL) was added to a solution of **S18** (9.3 mg, 0.018 mmol) in anhydrous CH₂Cl₂ (0.7 mL). The reaction was stirred for 20 hours at room temperature. Then the solvents were removed under reduced pressure and the

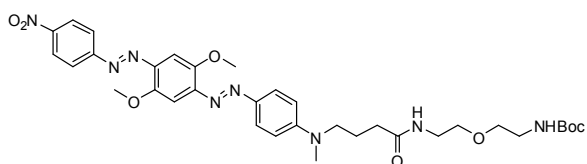
residue was dissolved in DMSO (1.0 mL), purified by preparative HPLC (Method 2) and fractions were lyophilized to afford **2** (7.6 mg, 43%) as a fluffy violet-pink solid.

¹H NMR (400 MHz, DMSO-*d*₆) δ 11.07 (s, 1H, minor isomer 43%), 10.90 (s, 1H, major isomer, 57%), 8.95-8.89 (m, 1H), 8.44-8.42 (m, 3H), 8.23-8.20 (m, 1H), 8.05 (d, *J* = 9.0 Hz, 2H), 7.79 (dd, *J* = 4.3, 9.00 Hz, 2H), 7.49 (t, *J* = 5.5 Hz, 1H minor isomer 43%), 7.43 (s, 1H), 7.38-7.32 (m, 3H include major isomer of one proton), 6.87 (d, *J* = 9.0 Hz, 2H), 6.55-6.45 (m, 6H), 3.99 (s, 3H), 3.93 (s, 3H), 3.55-3.43 (m, 4H), 3.05 (s, 3H minor isomer 43%), 3.03 (s, 3H major isomer 57%), 2.93 (s, 12H), 2.20 (t, *J* = 7.4 Hz, 2H), 1.87-1.77 (m, 2H); HRMS calcd 987.4028 for C₅₃H₅₃N₁₁O₉, found 987.4042.

tert-Butyl 2-(2-aminoethoxy)ethylcarbamate **S19**

This compound was synthesized according to a described procedure.¹⁰

tert-Butyl 2-(2-(4-((4-((2,5-dimethoxy-4-((4-nitrophenyl)diazenyl)phenyl)diazenyl)phenyl)(methylamino)butanamido)ethoxy)ethylcarbamate **S20**



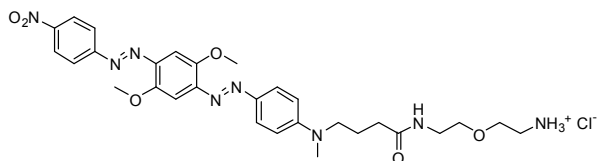
Compound **S20** was synthesized according to the general procedure A. **S8** (306 mg, 0.60 mmol) was reacted with **S19** (102 mg, 0.78 mmol) to give **S20** as a dark violet

solid (200 mg, 48%).

R_f 0.58 (CH₂Cl₂/MeOH 95:5); ¹H NMR (400 MHz, CDCl₃-*d*₁) δ 8.32 (d, *J* = 9.00 Hz, 2H), 8.00 (d, *J* = 9.00 Hz, 2H), 7.89 (d, *J* = 9.00 Hz, 2H), 7.45 (s, 1H), 7.42 (s, 1H), 6.74 (d, *J* = 9.0 Hz, 2H), 6.10 (brs, 1H), 4.87 (brs, 1H), 4.05 (s, 1H), 4.00 (s, 1H), 3.50-3.41 (m, 8H), 3.26 (dt, *J* = 4.6, 5.9 Hz, 2H), 3.05 (s, 3H), 2.24 (t, *J* = 7.0 Hz, 2H), 2.00-1.94 (m, 2H), 1.41 (s,

9H); ^{13}C NMR (101 MHz, CDCl_3 - d_1) δ 172.2, 156.6, 156.3, 153.8, 152.4, 151.1, 148.5, 147.0, 144.6, 142.2, 126.4, 124.9, 123.7, 111.6, 101.1, 100.2, 79.6, 70.4, 69.8, 56.9, 51.9, 40.5, 38.6, 33.3, 28.6, 23.0; ESI-MS: 693 $[\text{M}+\text{H}]^+$. HRMS calcd 692.3282 for $\text{C}_{34}\text{H}_{44}\text{N}_8\text{O}_8$, found 692.3290.

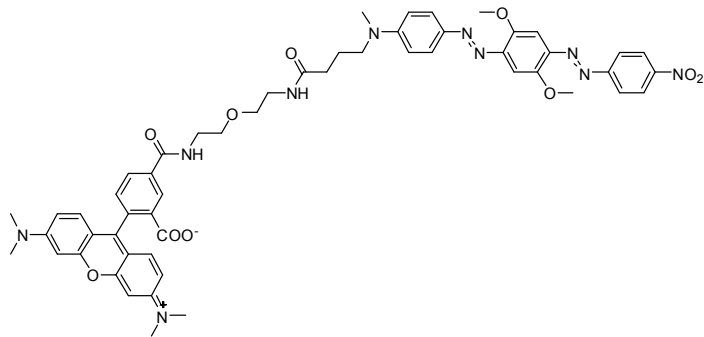
2-(2-(4-((4-((E)-(2,5-Dimethoxy-4-((E)-(4-nitrophenyl)diazenyl)phenyl)diazenyl)phenyl)(methyl)amino)butanamido)ethoxy)ethanaminium chloride **S21**



S20 (156 mg, 0.225 mmol) was dissolved in a mixture of $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (2:1) (2.7 mL) at 0 °C and a 4 M HCl solution in dioxane (1.8 mL) was added dropwise to

the solution. The reaction mixture was stirred at room temperature for 2 hours. When there was no more starting material left in the reaction mixture, the solvents were evaporated and gave **S21** as a dark violet solid. The crude was directly engaged in the next step without further purification.

4-(2-(2-(4-((4-((E)-(2,5-Dimethoxy-4-((E)-(4-nitrophenyl)diazenyl)phenyl)diazenyl)phenyl)(methyl)amino)butanamido)ethoxy)ethylcarbamoyl)-2-(6-(dimethylamino)-3(dimethyliminio)-3H-xanthen-9-yl)benzoate **3**



S21 (0.225 mmol) was dissolved in a mixture of DMF/ CH_2Cl_2 (1:1) (5.0 mL) and DIEA (117 μL , 0.675 mmol) and **S6** (97 mg, 0.225 mmol) were added. The mixture was cooled to 0 °C and PyBOP (117 mg, 0.225 mmol)

was added. The solution was allowed to reach room temperature and stirred for 16 hours. CH_2Cl_2 was evaporated under reduced pressure and reaction crude was purified by preparative HPLC (Method 1) without work-up. Probe **3** (79 mg, 35%) was obtained as a dark violet-pink solid.

^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 8.95 (t, J = 5.0 Hz, 1H), 8.70 (s, 1H), 8.41 (d, J = 9.0 Hz, 2H), 8.32 (dd, J = 2.1, 8.0 Hz, 1H), 8.03 (d, J = 9.0 Hz, 2H), 7.95 (t, J = 5.4 Hz, 1H), 7.75 (d, J = 9.0 Hz, 2H), 7.57 (d, J = 8.0 Hz, 1H), 7.38 (s, 1H), 7.31 (s, 1H), 6.98 (s, 4H), 6.87-6.82

(m, 4H), 3.98 (s, 3H), 3.91 (s, 3H), 3.61 (t, $J = 5.4$ Hz, 2H), 3.55-3.42 (m, 6H), 3.27 (dt, $J = 5.4$ Hz, 2H), 3.22 (s, 12H), 3.03 (s, 3H), 2.18 (t, $J = 7.5$ Hz, 2H), 1.82-1.76 (m, 2H); HRMS calcd 1004.4181 for $C_{54}H_{56}N_{10}O_{10}$, found 1004.4144.

Cell culture: Mice liver BNL CL.2 cells were grown in Dulbecco's MEM medium with 1 g/L glucose (Eurobio, Les Ulis, France) supplemented with 10% fetal bovine serum (Perbio, Brebieres, France), 2 mM L-Glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin (Eurobio). Cells were maintained in a 5% CO₂ humidified atmosphere at 37 °C.

Fluorescence imaging: 24 hours prior to experiment, 2.5×10^4 BNL CL.2 cells were seeded per well in 8-well Lab-Tek II Chambered coverglass plates (ref 155409, Nunc, Naperville, IL, USA). The fluorescent probes were diluted up to 300 μ L in MEM complete medium and then added onto cells, for a defined amount of time. After experiment completion the cells were washed twice with MEM and were incubated with a 5 μ g/mL of Hoechst 33258 (ref. H1399, Invitrogen) solution and a 100 nM LysoTracker green DND-26 solution (ref. L7526, Invitrogen). After washing with 10% FBS red phenol free Eagle's MEM medium, cell images were acquired on a confocal Leica TSC SPE II microscope (405, 488, or 561 nm) and controlled by image acquisition software (Leica confocal LAS AF, Leica). Two different objectives were used: HXC PL APO 20x/0.7 CS and HXC PL APO 63x/1.40 OIL CS.

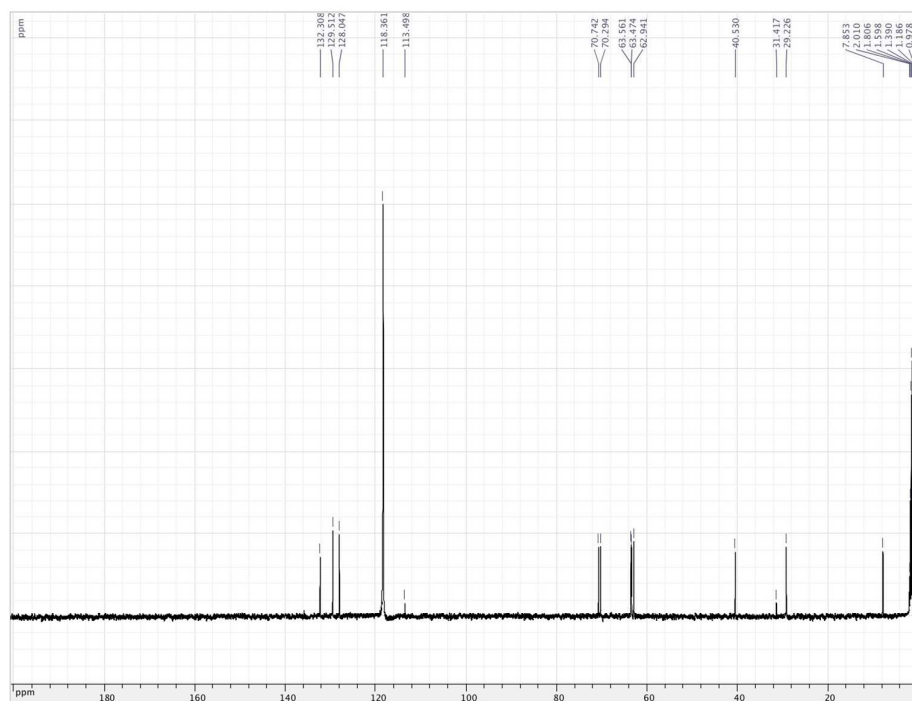
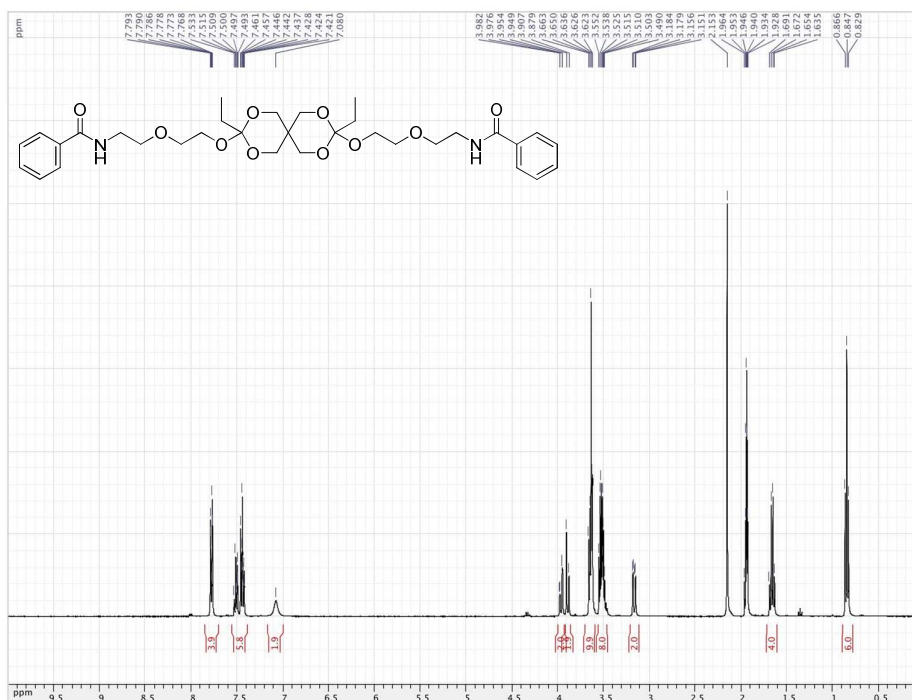
Cytometry: Twenty four hours before the experiment, BNL CL.2 cells were seeded in 96-well plates (Greiner Bio One, Frickenhausen, Germany) at 2.0×10^4 cells/well in Dulbecco's MEM complete medium. Cells were grown with or without 200 μ M chloroquine (SIGMA, St Louis, MO, USA) for 2 hours. The dyes were prepared at 1 μ M in Dulbecco's MEM and incubated with cells during 4 hours. After washing with PBS (Eurobio), 5 min incubation with 40 μ L trypsin, and addition of 160 μ L of PBS EDTA 5 mM, the suspended cells were analyzed by flow cytometry. A minimum of 2000 cells was acquired per sample and analyzed on the InCyte software (Guava/Millipore/Merck, CA, USA). Fluorescent cell rates were assessed on a capillary cytometry equipped with a blue (488 nm) and a red (630 nm) laser (Guava EasyCyte Plus 6C, Millipore Merck). Gates were drawn around the appropriate cell populations using a forward scatter (FSC) versus side scatter (SSC) acquisition dot plot to exclude debris. Cytometers performances are checked weekly using the Guava easyCheck Kit 4500-0025 (Merck/Millipore/Guava Hayward, CA, USA).

References

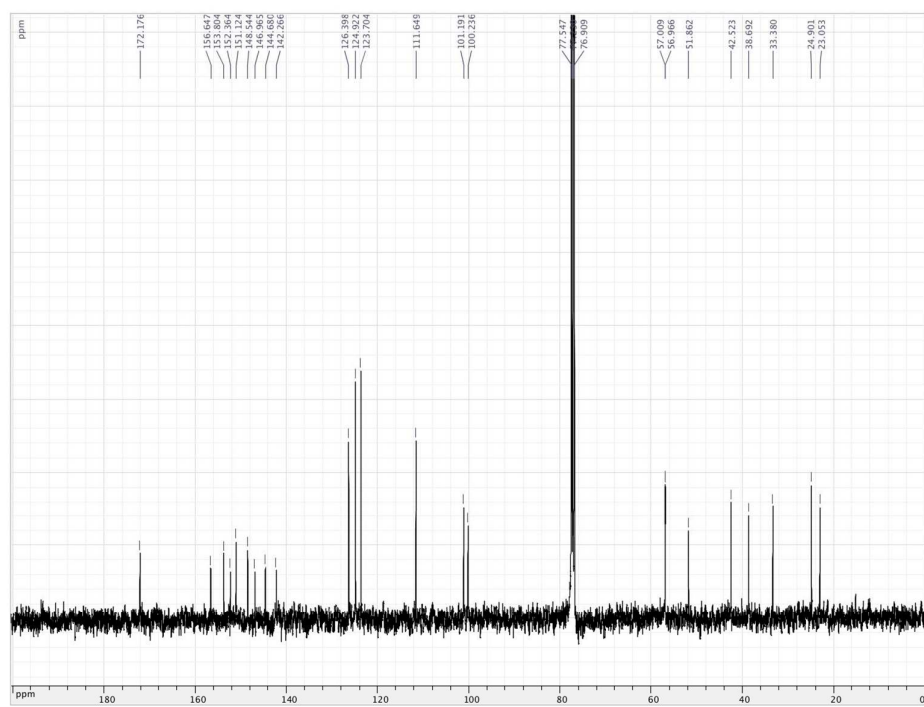
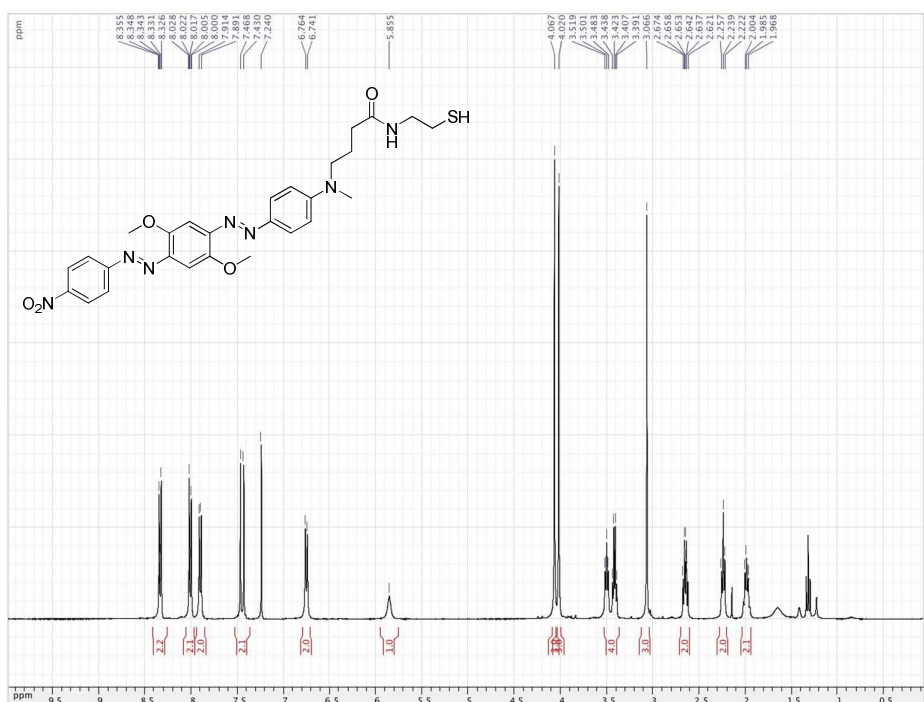
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NMR Spectra

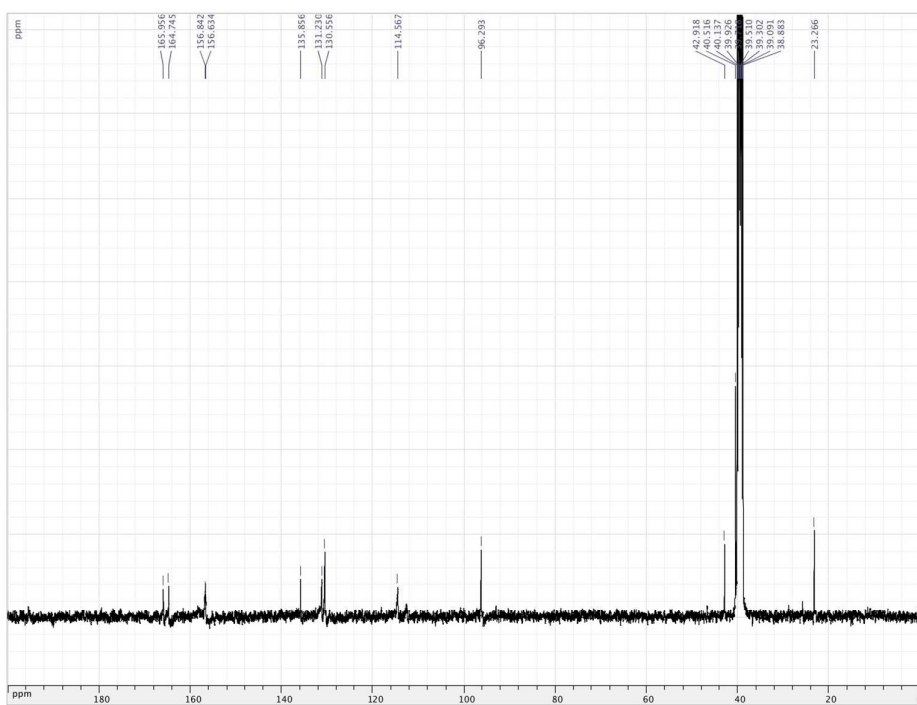
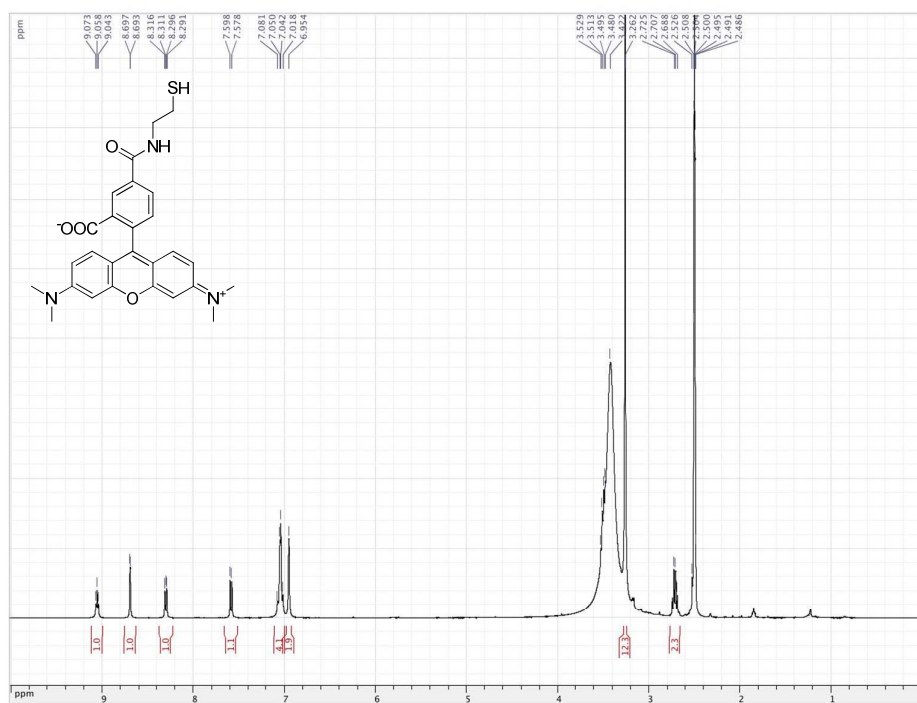
Compound S3



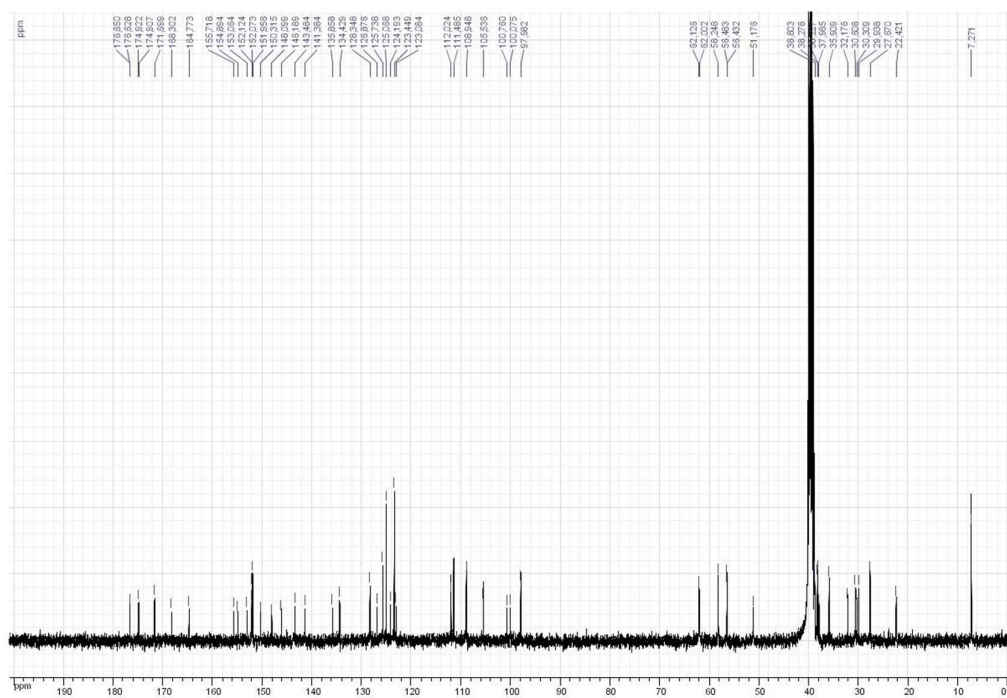
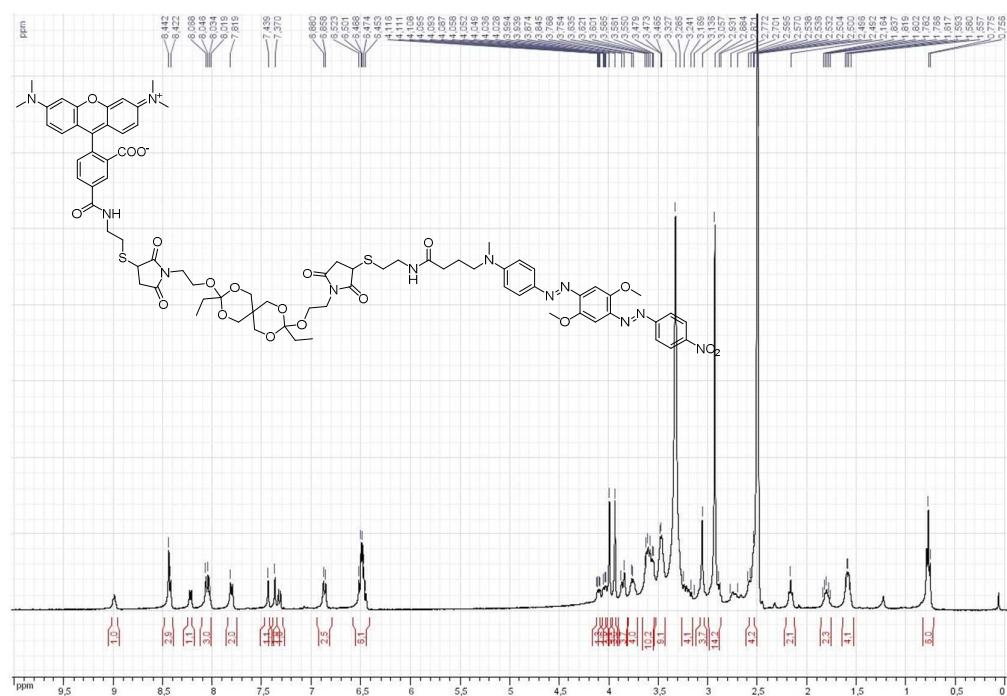
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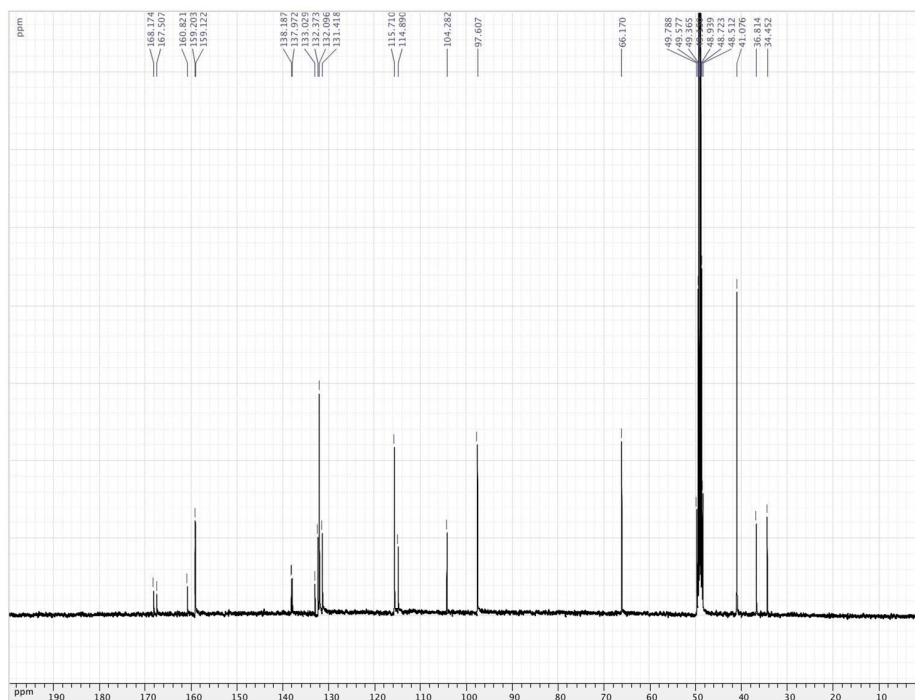
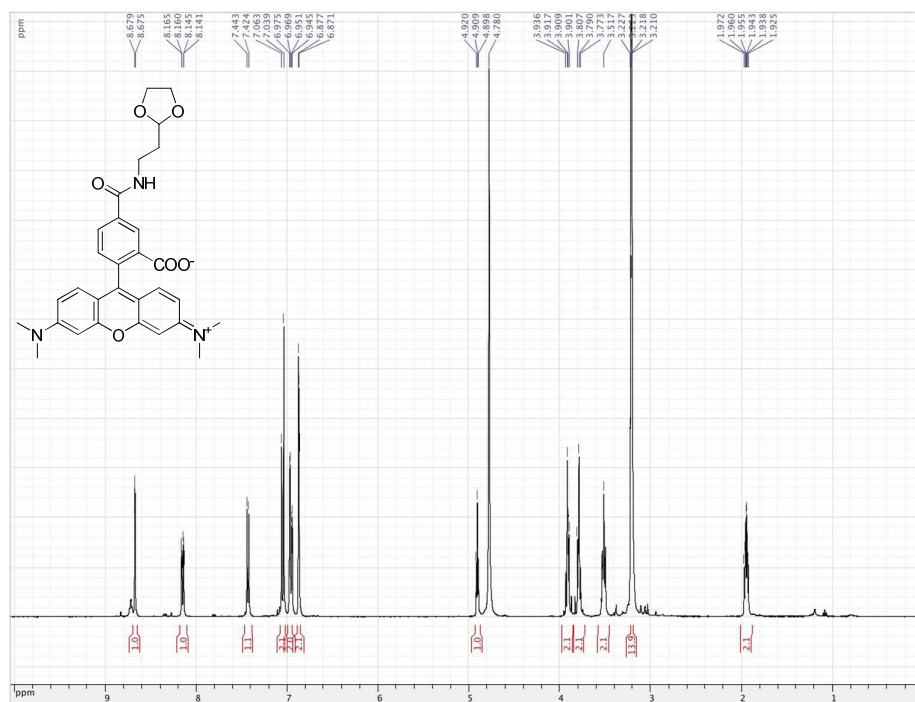
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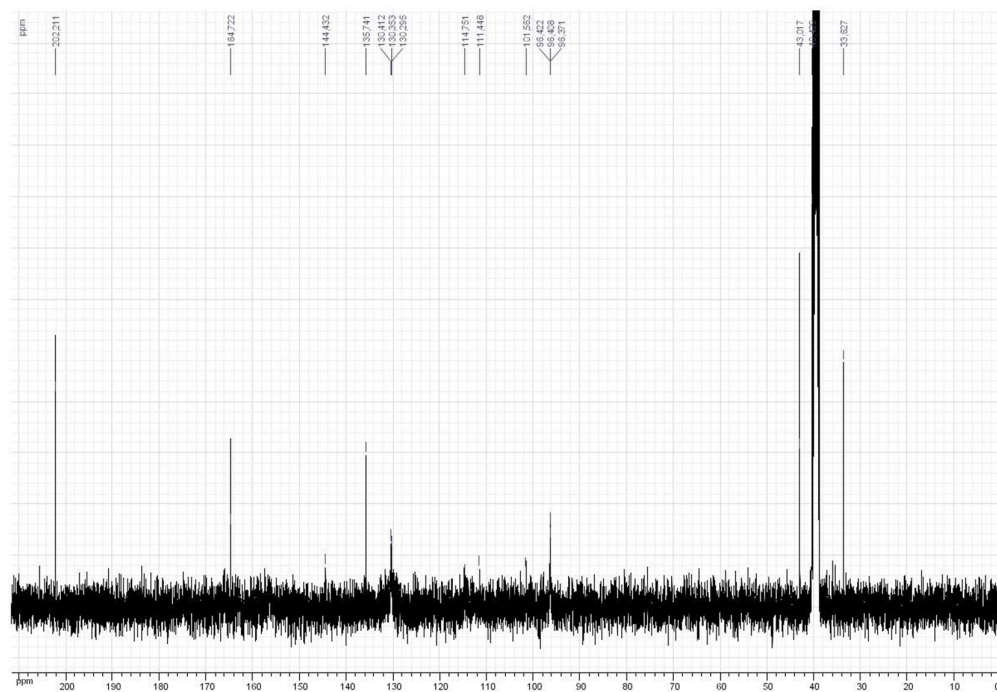
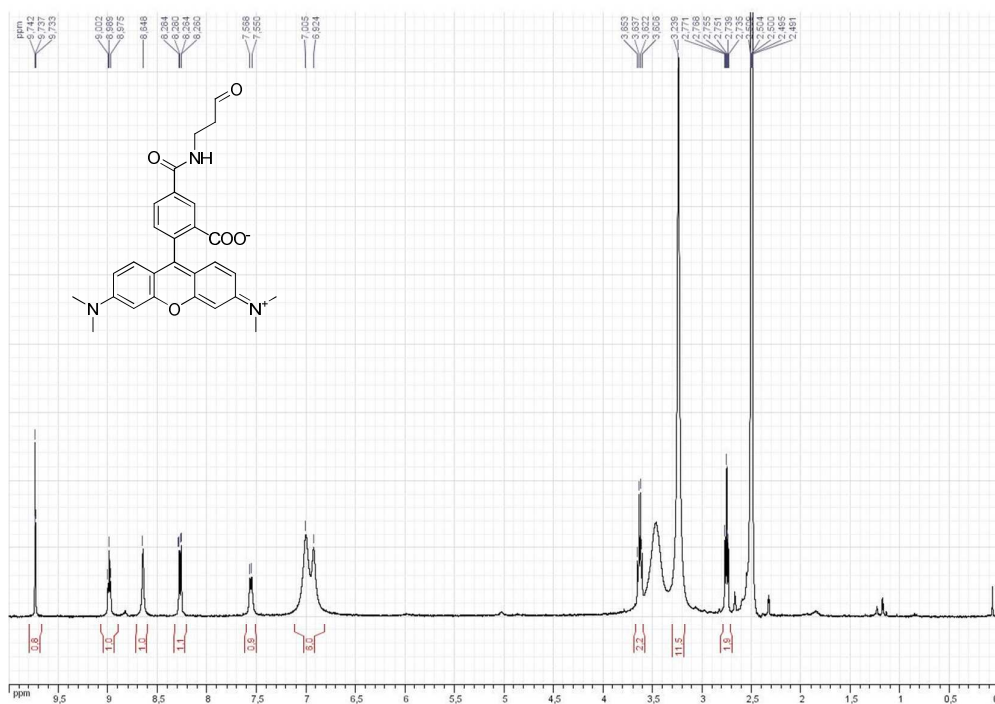
Compound 1



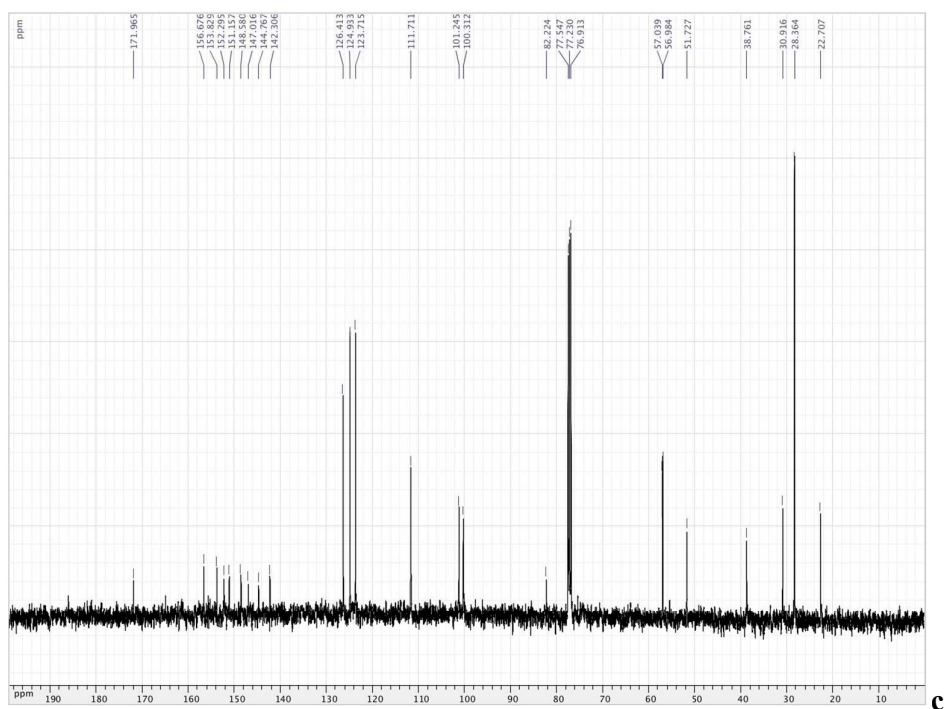
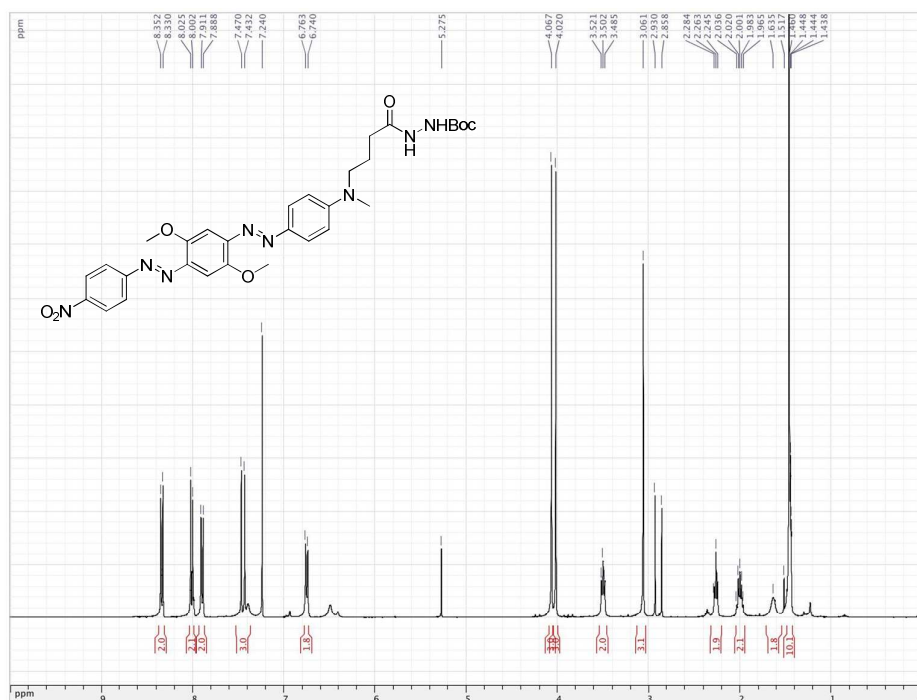
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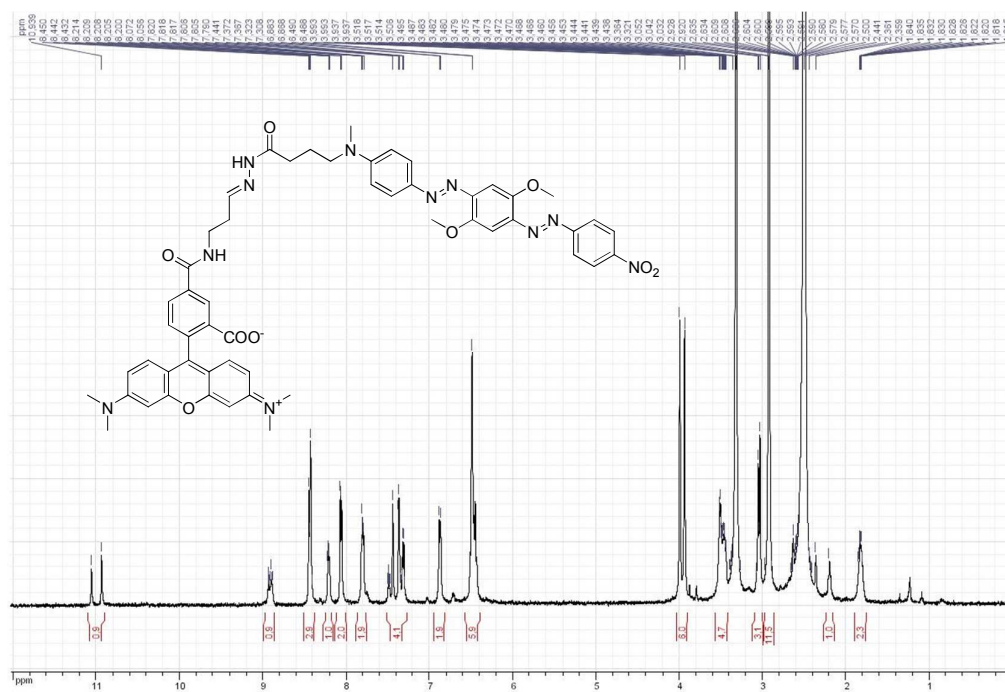
Compound S16



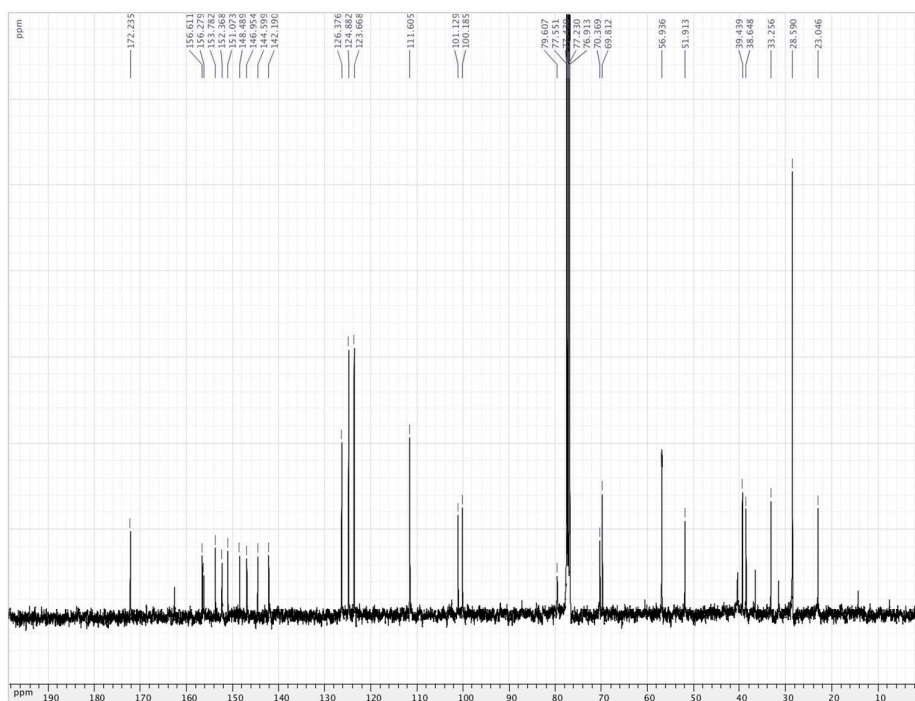
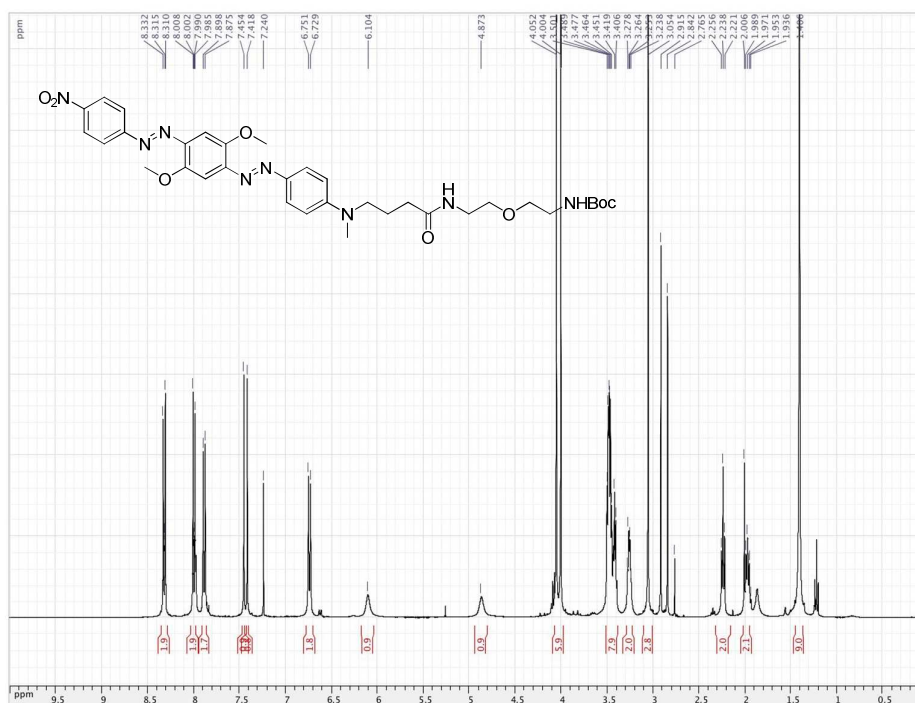
Compound S17



Compound 2



Compound S20



29

