Supporting Information

Cell-Penetrating Streptavidin: A General Tool for Bifunctional Delivery with Spatiotemporal Control, Mediated by Transport Systems such as Adaptive Benzopolysulfane Networks

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1. Materials and Methods

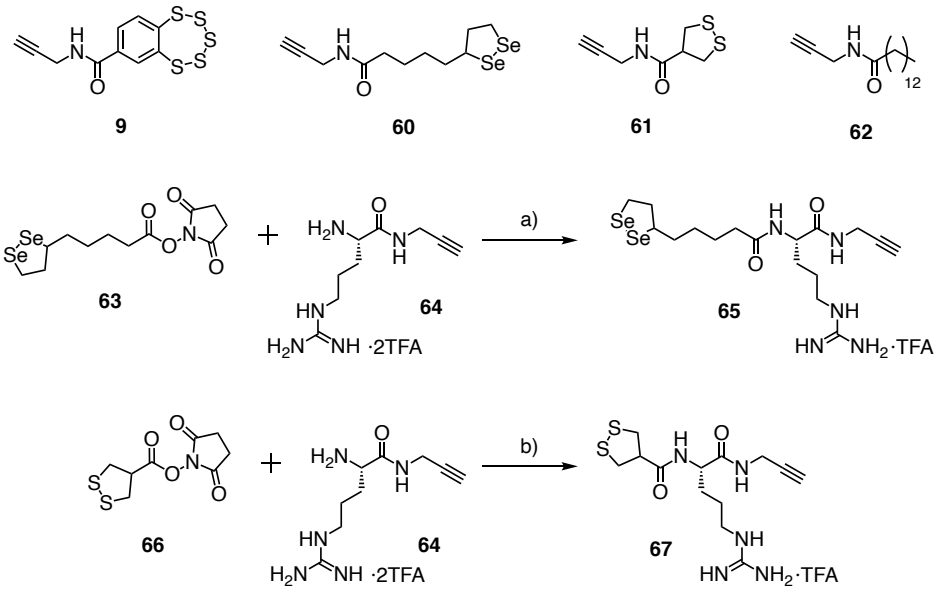
As in ref. S1. Briefly, reagents for synthesis and commercially available final compounds were purchased from Sigma-Aldrich, Brunschwig, Alfa Aesar, Merck, TCI, Acros, Iris Biotech and Click Chemistry Tools. Wild-type streptavidin was a generous gift from Prof. Thomas R. Ward (University of Basel). Column chromatography was carried out on silica gel (SiliaFlash® P60, SILICYCLE, 230–400 mesh). Analytical thin layer chromatography (TLC) was performed on silica gel 60 F254 (Merck). Activated Thiol Sepharose™ 4B was acquired from GE Healthcare. Phosphate buffered saline (PBS, pH = 7.4), DMEM (GlutaMAX, 4.5 g/L D-glucose, with phenol red) medium, FluoroBrite DMEM (high D-Glucose) medium, Leibovitz's L-15 medium, Penicillin-Streptomycin, Fetal Bovine Serum and TrypLE Express Enzyme were obtained from Thermo Fisher Scientific. Xfect™ transfection reagent was obtained from Takara Bio. μ-Slide 8-Well Glass Bottom and μ-Plate 96-Well were obtained from Ibidi.

Reverse phase flash chromatography was performed on Biotage Isolera™ Four. PNAs were synthesized in 500 μL fritted tubes using an Intavis MultiPep instrument in a fully automated fashion. IR spectra were recorded on a Perkin Elmer Spectrum One FT-IR spectrometer (ATR, Golden Gate) and are reported as wavenumbers ν in cm^{-1} with band intensities indicated as s (strong), m (medium), w (weak). ^{1}H and ^{13}C spectra were recorded (as indicated) either on a Bruker 400 MHz or 500 MHz spectrometer and are reported as chemical shifts (δ) in ppm relative to TMS ($\delta = 0$). Spin multiplicities are reported as a singlet (s), doublet (d), triplet (t) and quartet (q) with coupling constants (J) given in Hz, or multiplet (m). Broad peaks are marked as br. ^{1}H and ^{13}C resonances were assigned with the aid of additional information from 1D and 2D NMR spectra (H,H-COSY, DEPT 135, HSQC and HMBC). HPLC-MS were recorded using a Thermo Scientific Accela HPLC equipped with a Thermo C18 (5 cm x 2.1 mm, 1.9 μm particles) Hypersil gold column coupled with an LCQ Fleet three-dimensional ion trap mass spectrometer (ESI, Thermo Scientific) with a linear elution gradient from 95% H_2O / 5% CH_3CN + 0.1% TFA to 10% H_2O / 90% CH_3CN + 0.1% TFA in 4.0 min at a flow rate of 0.75 mL/min. Retention times R_t are reported in minutes. HR ESI-MS for the characterization of new compounds were performed on a Xevo G2-S Tof (Waters) and are reported as mass-per-charge ratio m/z calculated and observed. Fluorescence cellular imaging was performed using Leica SP5 confocal equipped with 63x oil immersion objective lens, or an IXM-C Automated microscope from ImageXpress equipped with a Lumencor Aura III with 5 independently selectable solid-state light sources, bandpass filters and 5 objectives (4x to 60x).

Sample preparation and washing on 96-well plates was performed using a Plate washer Biotek EL406®.

Abbreviations. BTTAA: 2-(4-((Bis((1-(tert-butyl)-1H-1,2,3-triazol-4-yl)methyl)amino)methyl)-1H-1,2,3-triazol-1-yl)acetic acid; CAPA: Chloroalkane penetration assay; CLSM: Confocal laser scanning microscopy; CPS: Cell penetrating streptavidin; CP_{50}: half maximal cell penetration concentration; DCE: 1,2-Dichloroethane; DIC: *N*,*N*′-Diisopropylcarbodiimide; DIPEA: *N*,*N*-Diisopropylethylamine; DMEM: Dulbecco's modified eagle medium; DMF: Dimethylformamide; DMSO: Dimethyl sulfoxide; EDC: 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide; GFP: Green fluorescent protein; HATU: 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate; HBTU: *N*,*N*,*N*′,*N*′-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate; HC: High content; HFIP: Hexafluoro-2-propanol; HOBt: Hydroxybenzotriazole; LP: Laser power; NHS: *N*-Hydroxysuccinimide; NMP: *N*-Methyl-2-pyrrolidone; on: overnight; PBS: Phosphate-buffered saline; PNA: Peptide nucleic acid; RP: Reverse phase; rt: Room temperature; TAMRA: 5-Carboxytetramethylrhodamine; TEA: Triethylamine; TFA: Trifluoroacetic acid; TNBSA: 2,4,6-Trinitrobenzene sulfonic acid.

2. Small-Molecule Synthesis



Scheme S1. (a) DIPEA, DMF, rt, on, 76%. (b) DIPEA, DMF, rt, 2 h, 68%.

Compound 9 was prepared following the reported procedure.[S1]

Compound 60 was prepared following the reported procedure.[S2]

Compound 61 was prepared following the reported procedure.[S3]

Compound 62 was prepared following the reported procedure.[S4]

Compound 63 was prepared following the reported procedure.[S5]

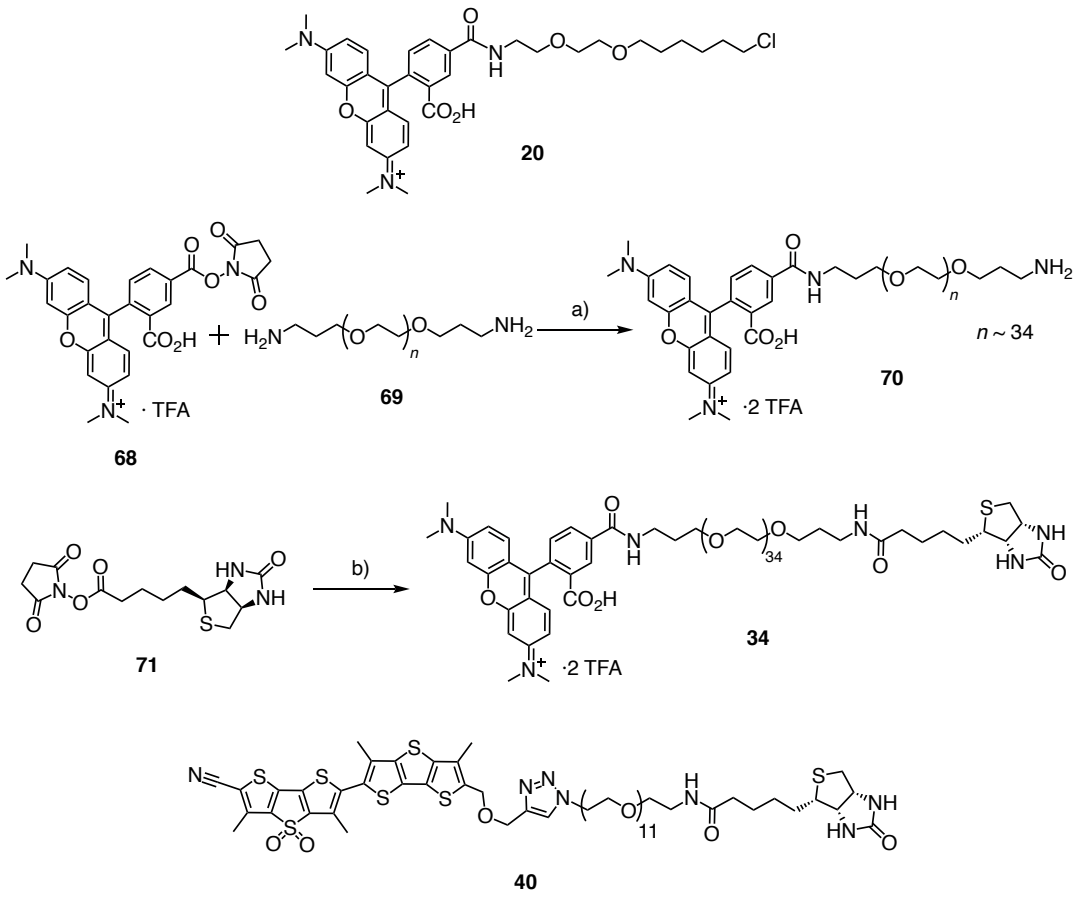
Compound 64 was prepared following the reported procedure.[S3]

Compound 66 was prepared following the reported procedure.[S3]

Compound 65. To a solution of **63** (40 mg, 0.091 mmol) in dry DMF (1 mL), DIPEA (16 μL, 0.091 mmol) was added, followed by **64** (36 mg, 0.091 mmol) and the mixture was allowed to react at rt under N_2 atmosphere. After one hour, more DIPEA (3.0 μL, 0.017 mmol) was added. After overnight reaction, the mixture was concentrated *in vacuo* and the crude product

was purified by RP flash chromatography (Claricep C18, linear gradient 0 – 100% CH_3CN in H_2O with 0.1% TFA) to give **65** (45 mg, 76%) as a brown oil. IR (neat): 3269 (br), 2932 (w), 1651 (s), 1533 (m), 1423 (w), 1200 (s), 1175 (s), 1129 (s), 1024 (s), 1004 (s), 824 (m); ^{1}H NMR (400 MHz, DMSO-d_6): 8.40 (t, $^3J_{H\text{-}H}$ = 5.5 Hz, 1H), 8.01 (d, $^3J_{H\text{-}H}$ = 8.0 Hz, 1H), 7.64 (t, $^3J_{H\text{-}H}$ = 5.7 Hz, 1H), 7.33 (s, 2H), 6.99 (s, 2H), 4.24 (dt, $^3J_{H\text{-}H}$ = 8.0, 5.2 Hz, 1H), 3.98 – 3.88 (m, 1H), 3.88 – 3.80 (m, 2H), 3.30 (d, $^3J_{H\text{-}H}$ = 6.4 Hz, 2H), 3.13 – 3.04 (m, 3H), 2.92 – 2.80 (m, 1H), 2.48 – 2.35 (m, 1H), 2.13 (t, ^3J (H,H) = 7.4 Hz, 2H), 1.83 – 1.27 (m, 10H); ^{13}C NMR (100 MHz, DMSO-d_6): 172.3 (C), 171.4 (C), 156.8 (C), 81.1 (C), 73.1 (CH), 52.9 (CH), 51.8 (CH), 45.2 (CH_2), 40.4 (CH_2), 35.0 (CH_2), 34.9 (CH_2), 34.9 (CH_2), 29.8 (CH_2), 29.4 (CH_2), 29.3 (CH_2), 29.3 (CH_2), 28.0 (CH_2), 25.2 (CH_2), 25.0 (CH_2), 25.0 (CH_2); HRMS (ESI, +ve): calcd for $C_{17}H_{29}N_5O_2Se_2$ ($[M - TFA]^+$): 496.0728, found: 496.0721.

Compound 67. To a solution of **64** (50 mg, 0.11 mmol) in dry DMF (1 mL), DIPEA (20 μL, 0.11 mmol) was added, followed by **66** (28 mg, 0.11 mmol) and the mixture was allowed to react at rt under N_2 atmosphere for two hours. Then, the mixture was concentrated *in vacuo* and the crude product was purified by RP flash chromatography (Claricep C18, linear gradient 0 – 40% CH_3CN in H_2O with 0.1% TFA) to give **67** (34 mg, 68%) as a colorless solid. IR (neat): 3279 (br), 2947 (w), 1631 (s), 1535 (m), 1432 (w), 1181 (s), 1128 (s), 838 (m); ^{1}H NMR (400 MHz, CD_3OD): 4.42 – 4.32 (m, 1H), 4.05 – 3.91 (m, 2H), 3.45 – 3.16 (m, 7H), 2.61 (t, $^4J_{H\text{-}H}$ = 2.6 Hz, 1H), 1.90 – 1.81 (m, 1H), 1.75 – 1.56 (m, 3H); ^{13}C NMR (100 MHz, CD_3OD): 174.4 (C), 173.3 (C), 158.6 (C), 80.4 (C), 72.4 (CH), 54.1 (CH), 52.8 (CH), 43.8 (CH_2), 43.3 (CH_2), 41.9 (CH_2), 30.3 (CH_2), 29.5 (CH_2), 26.3 (CH_2); HRMS (ESI, +ve): calcd for $C_{13}H_{21}N_5O_2S_2$ ($[M - TFA]^+$): 344.1210, found: 344.1199.



Scheme S2. (a) DMF, rt, 1.5 h, 42%; (b) TEA, DMF, rt, 2 h, 82%; (c) TEA, DMF, rt, on, 76%.

Compound 20 was prepared following the reported procedure.[S6]

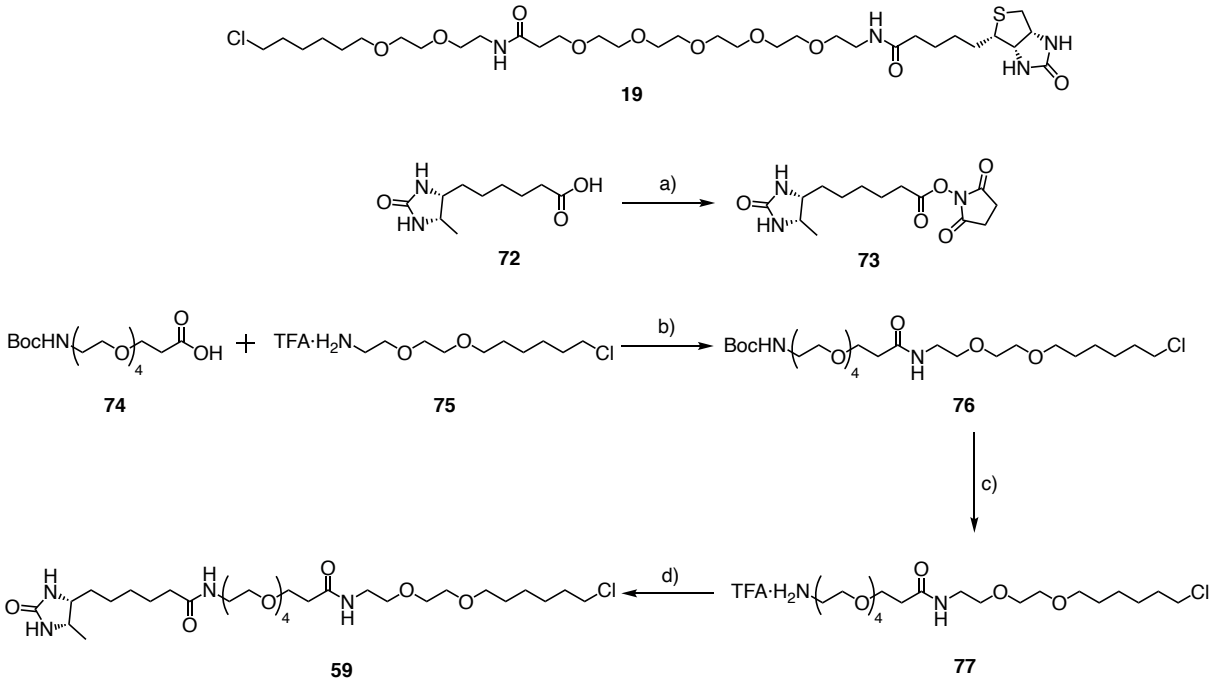
Compound 71 was prepared following the reported procedure.[S7]

Compound 40 was prepared following the reported procedure.[S8]

Compound 70. To a solution of **69** (74 mg, 0.050 mmol) in dry DMF (0.5 mL), a solution of **68** (13 mg, 0.024 mmol) in dry DMF (4 mL) was added dropwise over a period of 5 min. The mixture was stirred for one hour and a half at rt and then concentrated *in vacuo*. The crude product was purified by RP flash chromatography (Claricep C18, linear gradient 0 – 60% CH_3CN in H_2O with 0.1% TFA) to give **70** (22 mg, 42%) as a deep red solid. The product contained around 10% of hydrolyzed **68**, that was removed in the next step. ^{1}H NMR (400 MHz, CD_3OD): 8.78 (d, $^4J_{H\text{-}H}$ = 1.8 Hz, 1H), 8.28 (dd, $^3J_{H\text{-}H}$ = 7.9, $^4J_{H\text{-}H}$ = 1.8 Hz, 1H), 7.55 (d, $^3J_{H\text{-}H}$ = 7.9 Hz, 1H), 7.15 (d, $^3J_{H\text{-}H}$ = 9.5 Hz, 2H), 7.08 (dd, $^3J_{H\text{-}H}$ = 9.5, $^4J_{H\text{-}H}$ = 2.5 Hz, 2H), 7.00 (d,

$^4J_{H\text{-}H}$ = 2.5 Hz, 2H), 3.84 – 3.48 (m, 183H), 3.32 (s, 12H), 3.17 (t, $^3J_{H\text{-}H}$ = 6.0 Hz, 2H), 2.04 – 1.86 (m, 4H); HPLC-MS (ESI, +ve): R_t = 2.06 min, 544 (100, $[M + 4H]^{4+}$).

Compound 34. To a solution of **70** (9.8 mg, 5.2 μmol) and **40** (2.6 mg, 7.7 μmol) in dry DMF (1 mL), TEA (1.1 μL, 7.7 μmol) was added and the mixture was stirred for two hours at rt. Then, the crude product was concentrated *in vacuo* and purified by RP flash chromatography (Claricep C18, linear gradient 25 – 50% CH_3CN in H_2O with 0.1% TFA) to give **34** (10.0 mg, 82%) as a deep red solid. IR (neat): 2869 (m), 1693 (w), 1649 (w), 1594 (m), 1347 (m), 1187 (m), 1094 (s), 928 (m), 729 (s), 699 (m); ^{1}H NMR (400 MHz, CD_3OD): 8.78 (d, $^4J_{H\text{-}H}$ = 1.8 Hz, 1H), 8.28 (dd, $^3J_{H\text{-}H}$ = 7.9, $^4J_{H\text{-}H}$ = 1.8 Hz, 1H), 7.55 (d, $^3J_{H\text{-}H}$ = 7.9 Hz, 1H), 7.15 (d, $^3J_{H\text{-}H}$ = 9.5 Hz, 2H), 7.08 (dd, $^3J_{H\text{-}H}$ = 9.5, $^4J_{H\text{-}H}$ = 2.4 Hz, 2H), 7.01 (d, $^4J_{H\text{-}H}$ = 2.4 Hz, 2H), 4.53 – 4.46 (m, 1H), 4.36 – 4.27 (m, 1H), 3.72 – 3.56 (m, 186H), 3.52 (t, $^3J_{H\text{-}H}$ = 6.1 Hz, 2H), 3.33 (s, 12H), 3.26 (t, $^3J_{H\text{-}H}$ = 6.7 Hz, 2H), 3.24 – 3.17 (m, 1H), 2.93 (dd, $^2J_{H\text{-}H}$ = 12.7, $^3J_{H\text{-}H}$ = 5.0 Hz, 1H), 2.71 (d, $^2J_{H\text{-}H}$ = 12.7 Hz, 1H), 2.20 (t, $^3J_{H\text{-}H}$ = 7.3 Hz, 2H), 1.99 – 1.92 (m, 2H), 1.91 – 1.52 (m, 6H), 1.53 – 1.37 (m, 2H); HRMS (ESI, +ve): calcd for $C_{109}H_{186}N_6O_{41}S$ ($[M + 2H]^{2+}$): 1134.6261, found: 1134.6284.



Scheme S3. (a) NHS, EDC, DMF, rt, on, 89%; (b) HBTU, DIPEA, CH_2Cl_2, rt, 1 h, 56%; (c) TFA, CH_2Cl_2, rt, 2 h, *quant.*; (d) **73**, TEA, CH_2Cl_2, rt, 1 h, 77%.

Compound 19 was prepared following the reported procedure.[S6]

Compound 75 was prepared following the reported procedure.[S9]

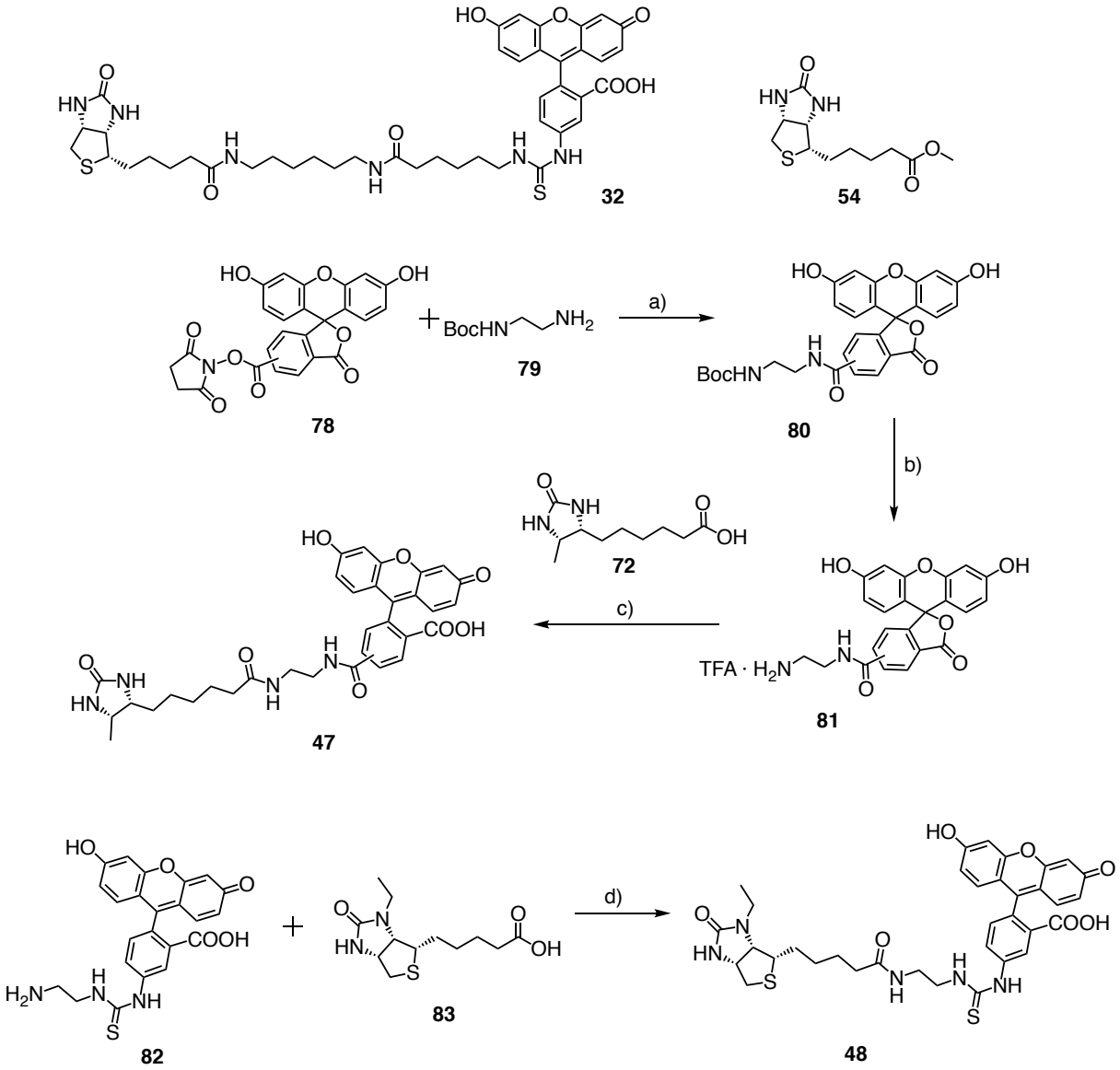
Compound 73. To a solution of **72** (100 mg, 0.470 mmol) in dry DMF (1 mL), NHS (65 mg, 0.56 mmol) and EDC (110 mg, 0.560 mmol) were added and the mixture was allowed to react overnight at rt under Argon atmosphere. Then, the crude product was concentrated *in vacuo* and purified by flash chromatography (silica gel, linear gradient 2 – 100% acetone in CH_2Cl_2) to give **73** (129 mg, 89%) as a colorless solid. IR (neat): 3194 (w), 2923 (w), 1790 (m), 1745 (s), 1694 (s), 1461 (m), 1371 (m), 1207 (s), 1062 (s), 861 (m); ^{1}H NMR (400 MHz, $CDCl_3$): 5.18 (s, 1H), 4.79 (s, 1H), 3.91 – 3.77 (m, 1H), 3.77 – 3.64 (m, 1H), 2.84 (s, 4H), 2.62 (t, $^3J_{H\text{-}H}$ = 7.2 Hz, 2H), 1.77 (p, $^3J_{H\text{-}H}$ = 7.3 Hz, 2H), 1.57 – 1.36 (m, 5H), 1.35 – 1.26 (m, 1H), 1.12 (d, $^3J_{H\text{-}H}$ = 6.5 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$): 169.4 (C), 168.7 (C), 163.6 (C), 56.0 (CH), 51.5 (CH), 30.9 (CH_2), 29.6 (CH_2), 28.7 (CH_2), 26.0 (CH_2), 25.7 (CH_2), 24.4 (CH_2), 15.9 (CH_3); HPLC-MS (ESI, +ve): R_t = 1.70 min, 312 (100, $[M + H]^+$).

Compound 76. To a solution of **74** (100 mg, 0.270 mmol) and **75** (91 mg, 0.27 mmol) in dry CH_2Cl_2 (5 mL), DIPEA (145 μL, 0.820 mmol) and HBTU (128 mg, 0.330 mmol) were added and the mixture was allowed to react one hour at rt. The reaction mixture was diluted with CH_2Cl_2 (50 mL), washed with aq. K_2CO_3 (sat. 3 x 20 mL), aq. NH_4Cl (sat. 2 x 20 mL) and aq. NaCl (sat. 1 x 20 mL), dried over Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by flash chromatography (silica gel, linear gradient 2 – 15% MeOH in CH_2Cl_2) to yield compound **76** (86 mg, 56%) as a colorless liquid. IR (neat): 3347 (w), 2931 (m), 2865 (m), 1711 (m), 1663 (m), 1531 (m), 1249 (m), 1170 (m), 1099 (s), 865 (w); ^{1}H NMR (400 MHz, $CDCl_3$): 6.61 (br s, 1H), 5.10 (br s, 1H), 3.71 (t, ^3J (H,H) = 6.0 Hz, 2H), 3.63 – 3.57 (m, 14H), 3.55 – 3.48 (m, 8H), 3.45 – 3.39 (m, 4H), 3.28 (q, $^3J_{H\text{-}H}$ = 5.4 Hz, 2H), 2.44 (t, $^3J_{H\text{-}H}$ = 6.0 Hz, 2H), 1.75 (p, $^3J_{H\text{-}H}$ = 6.8 Hz, 2H), 1.57 (p, $^3J_{H\text{-}H}$ = 6.8 Hz, 2H), 1.44 – 1.39 (m, 11H), 1.38 – 1.30 (m, 2H); ^{13}C NMR (101 MHz, $CDCl_3$): 171.4 (C), 156.1 (C), 79.2 (C), 71.3 (CH_2), 70.7 (CH_2), 70.6 (CH_2), 70.5 (CH_2), 70.4 (CH_2), 70.3 (CH_2), 70.1 (CH_2), 69.9 (CH_2), 67.4 (CH_2), 45.1 (CH_2), 40.4 (CH_2), 39.2 (CH_2), 37.1 (CH_2), 32.6 (CH_2), 29.5 (CH_2), 28.5 (CH_2), 26.8 (CH_2), 25.5 (CH_2); HPLC-MS (ESI, +ve): R_t = 2.54 min, 571 (100, $[M + H]^+$).

Compound 77. To a solution of compound **76** (80 mg, 0.14 mmol) in CH_2Cl_2 (5 mL), TFA (1 mL) is added and the mixture is stirred for two hours. Then, solvent is removed, yielding compound **77** as a colorless oil (82 mg, *quant.*) without any further purification. IR (neat): 2918 (m), 2869 (m), 1650 (m), 1352 (w), 1199 (m), 1176 (m), 1099 (s), 832 (w), 798 (m); ^{1}H NMR

(400 MHz, $CDCl_3$): 7.83 (br s, 3H), 7.11 (br s, 1H), 3.79 (d, $^3J_{H\text{-}H}$ = 5.6 Hz, 2H), 3.75 – 3.66 (m, 5H), 3.65 – 3.57 (m, 12H), 3.56 – 3.49 (m, 6H), 3.47 – 3.36 (m, 4H), 3.24 – 3.10 (m, 2H), 2.49 (t, $^3J_{H\text{-}H}$ = 5.6 Hz, 2H), 1.76 (d, $^3J_{H\text{-}H}$ = 6.9 Hz, 2H), 1.57 (p, $^3J_{H\text{-}H}$ = 6.9 Hz, 2H), 1.48 – 1.30 (m, 4H); ^{13}C NMR (101 MHz, $CDCl_3$): 172.0 (C), 71.3 (CH_2), 70.4 (CH_2), 70.1 (CH_2), 70.1 (CH_2), 70.0 (CH_2), 69.9 (CH_2), 69.9 (CH_2), 69.7 (CH_2), 69.5 (CH_2), 67.4 (CH_2), 67.0 (CH_2), 45.2 (CH_2), 39.7 (CH_2), 39.3 (CH_2), 36.1 (CH_2), 32.6 (CH_2), 29.5 (CH_2), 26.7 (CH_2), 25.4 (CH_2); HPLC-MS (ESI, +ve): R_t = 1.76 min, 471 (100, $[M + H]^+$).

Compound 59. To a solution of **77** (60 mg, 0.10 mmol) and **73** (32 mg, 0.10 mmol) in dry CH_2Cl_2 (2 mL), TEA (22 μL, 0.15 mmol) was added and the mixture was allowed to react for one hour at rt. Then, the mixture was concentrated *in vacuo* and the crude product was purified by RP flash chromatography (Claricep C18, linear gradient 30 – 60% CH_3CN in H_2O with 0.1% TFA) to give **59** (53 mg, 77%) as a colorless oil. IR (neat): 3301 (br), 2932 (m), 2864 (m), 1695 (m), 1648 (s), 1550 (m), 1447 (w), 1351 (w), 1094 (s); ^{1}H NMR (400 MHz, $CDCl_3$): 6.77 (t, $^3J_{H\text{-}H}$ = 6.3 Hz, 1H), 6.70 (t, $^3J_{H\text{-}H}$ = 6.3 Hz, 1H), 6.07 (br s, 1H), 5.55 (br s, 1H), 3.94 – 3.83 (m, 1H), 3.78 – 3.69 (m, 3H), 3.65 – 3.58 (m, 14H), 3.57 – 3.50 (m, 8H), 3.47 – 3.40 (m, 6H), 2.47 (t, $^3J_{H\text{-}H}$ = 6.0 Hz, 2H), 2.20 (t, $^3J_{H\text{-}H}$ = 7.4 Hz, 2H), 1.76 (q, $^3J_{H\text{-}H}$ = 6.7 Hz, 2H), 1.69 – 1.54 (m, 4H), 1.53 – 1.23 (m, 10H), 1.13 (d, $^3J_{H\text{-}H}$ = 6.4 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$): 173.6 (C), 171.7 (C), 164.4 (C), 71.4 (CH_2), 70.5 (CH_2), 70.4 (CH_2), 70.3 (CH_2), 70.3 (CH_2), 70.2 (CH_2), 70.1 (CH_2), 70.1 (CH_2), 69.8 (CH_2), 67.3 (CH_2), 56.4 (CH), 51.9 (CH), 45.2 (CH_2), 39.3 (CH_2), 36.9 (CH_2), 36.0 (CH_2), 32.6 (CH_2), 29.5 (CH_2), 29.4 (CH_2), 28.8 (CH_2), 26.0 (CH_2), 25.5 (CH_2), 25.4 (CH_2), 15.7 (CH_3); HRMS (ESI, +ve): calcd for $C_{31}H_{59}ClN_4O_9$ ($[M + H]^+$): 667.4049, found: 667.4044.



Scheme S4. (a) TEA, DMF, rt, 0.5 h, 75%; (b) TFA, CH_2Cl_2, rt, 20 min; (c) 1. HBTU, TEA, DMF, rt, 0.5 h; 2. NH_3, MeOH, rt, 3 h, 3 steps, 47%; (d) HBTU, HOBt, DIPEA, DMF, rt, 2 h, 29%.

Compound 32 was prepared following the reported procedures.[S10]

Compound 54 was prepared following the reported procedures.[S11]

Compound 78 was prepared following the reported procedures.[S12] 5- and 6-isomers (≈ 2:1) were not separated.

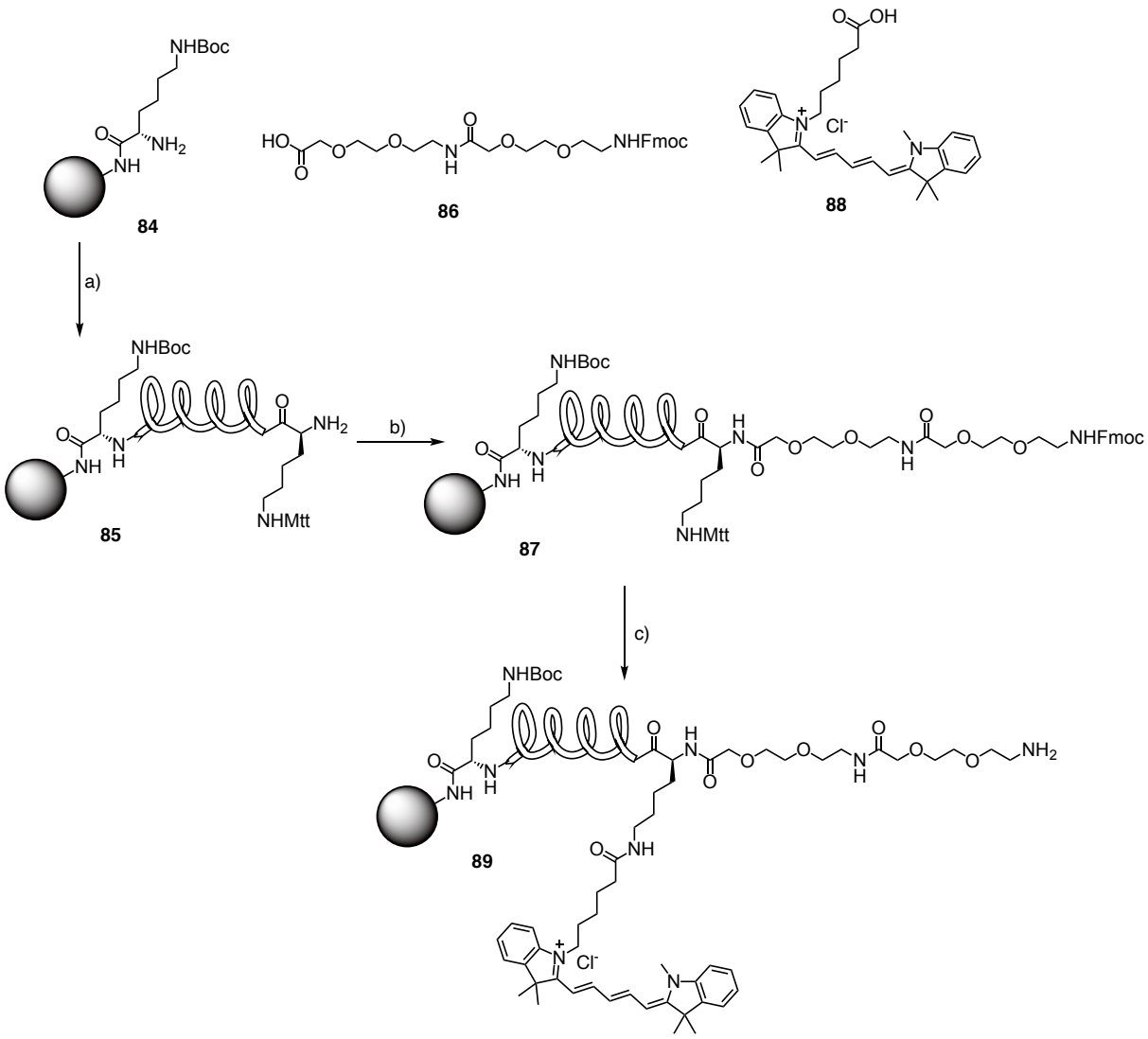
Compound 83 was prepared following the reported procedures.[S13]

Compound 80. To a solution of **78** (11 mg, 23 µmol) in DMF (0.25 mL) at rt were added **79** (6.0 µL, 40 µmol) followed by TEA (10 µL, 72 µmol). The mixture was stirred for 30 min, then diluted with EtOAc, washed with aq. $KHSO_4$ (1 M) and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was triturated with pentane, and the remaining solid was purified by column chromatography (silica gel, pentane / acetone 1:1 to 0:1; R_f = 0.37 with pentane / acetone 1:1) to give pure **80** (9 mg, 75%) as a yellow solid. Spectroscopic data are consistent with those in the literature.[S14]

Compound 47. A solution of **80** (9.0 mg, 17 µmol) in TFA (0.5 mL) and CH_2Cl_2 (0.5 mL) was stirred for 20 min at rt, and then concentrated to give **81**. To a solution of the obtained **81** and TEA (8 µL, 60 µmol) in DMF (0.1 mL) at rt was added a solution of **72** (12 mg, 56 µmol), HBTU (20 mg, 53 µmol) and TEA (8 µL, 60 µmol) in DMF (0.3 mL). The mixture was stirred for 15 min at rt and concentrated *in vacuo*. To a solution of the residue in MeOH (1 mL) was added a solution of NH_3 in MeOH (40 µL of 7 M, 0.14 mmol). The resulting mixture was stirred for 3 h at rt and concentrated *in vacuo*. The residue was triturated with Et_2O and 0.1% TFA in H_2O, and purified by RP flash chromatography (Claricep C18, linear gradient 10 – 60% CH_3CN in H_2O with 0.1% TFA) to give **47** (5 mg, 47%, a mixture of ≈ 3:2 5- and 6- isomers) as a yellow oil. IR (neat): 3294 (br), 2940 (w), 1675 (s), 1543 (m), 1454 (m), 1385 (w), 1277 (m), 1179 (s), 1135 (m), 852 (w), 721 (w); ^{1}H NMR (400 MHz, CD_3OD, *n*/*n*: 5-/6-regio isomeric peaks): 8.83/8.68 (br t, $^3J_{H\text{-}H}$ = 6.0 Hz, NH), 8.44/7.61 (br s, 1H), 8.20/8.13 (dd, $^3J_{H\text{-}H}$ = 8.1 Hz, $^4J_{H\text{-}H}$ = 1.6 Hz, 1H), 7.32/8.09 (d, $^3J_{H\text{-}H}$ = 8.1 Hz, 1H), 6.72 (d, $^4J_{H\text{-}H}$ = 2.3 Hz, 2H), 6.68 – 6.54 (m, 4H), 3.84 – 3.75 (m, 1H), 3.70 – 3.61 (m, 1H), 3.58 – 3.52 (m, 1H), 3.48 – 3.39 (m, 3H), 2.22/2.10 (t, $^3J_{H\text{-}H}$ = 7.5 Hz, 2H), 1.68 – 1.16 (m, 8H), 1.07 (d, $^3J_{H\text{-}H}$ = 6.4 Hz, 3H); ^{13}C NMR (101 MHz, CD_3OD): 176.9 (C), 169.6 (C), 168.5 (C), 166.2 (C), 164.7 (2×C), 156.1 (2×C), 138.0 (C), 134.6 (C), 131.3 (3×CH), 130.5 (C), 127.5 (CH), 126.9 (CH), 116.0 (2×CH), 113.0 (2×C), 103.6 (2×CH), 57.4 (CH), 52.7 (CH), 41.2 (CH_2), 39.9 (CH_2), 37.1 (CH_2), 30.7 (CH_2), 30.3 (CH_2), 27.2 (CH_2), 26.8 (CH_2), 15.6 (CH_3); HRMS (ESI, +ve): calcd for $C_{33}H_{35}N_4O_8$ ([M + H]$^+$) 615.2450, found: 615.2430.

Compound 48. To a solution of compound **83** (7.0 mg, 25 µmol), HBTU (19 mg, 50 µmol) and HOBt (7.0 mg, 50 µmol) in DMF (200 µL), a solution of **82** (17 mg, 38 µmol) and DIPEA (9.0 µL, 50 µmol) in DMF (200 µL) was added. The reaction mixture was stirred at rt for 2 h. The crude reaction mixture was concentrated under reduced pressure and purified by RP flash chromatography (Claricep C18, linear gradient 5 – 60% CH_3CN in H_2O with 0.1% TFA) to

yield **48** as a yellow solid (5.1 mg, 29%). IR (neat): 3382 (br), 2927 (m), 2312 (w), 2247 (w), 2208 (w), 1659 (s), 1446 (m), 1203 (m), 759 (s), 699 (s), 638 (s); ^{1}H NMR (300 MHz, CD_3OD): 8.23 (s, 1H), 7.86 (d, $^{3}J_{H\text{-}H}$ = 7.6 Hz, 1H), 7.27 (d, $^{3}J_{H\text{-}H}$ = 8.3 Hz, 1H), 7.07 (d, $^{3}J_{H\text{-}H}$ = 8.8 Hz, 2H), 6.94 (s, 2H), 6.80 (d, $^{3}J_{H\text{-}H}$ = 9.0 Hz, 2H), 4.38 – 4.32 (m, 1H), 4.25 (dd, $^{3}J_{H\text{-}H}$ = 8.9, 4J = 5.0 Hz, 1H), 3.80 – 3.71 (m, 2H), 3.60 – 3.48 (m, 1H), 3.45 (t, $^{3}J_{H\text{-}H}$ = 6.0 Hz, 2H), 3.30 – 3.20 (m, 1H), 2.96 – 2.85 (m, 2H), 2.69 (dd, $^{3}J_{H\text{-}H}$ = 12.5, $^{4}J_{H\text{-}H}$ = 2.7 Hz, 1H), 2.22 (t, $^{3}J_{H\text{-}H}$ = 7.2 Hz, 2H), 1.76 – 1.30 (m, 8H), 1.07 (t, $^{3}J_{H\text{-}H}$ = 7.1 Hz, 3H); ^{13}C NMR (126 MHz, CD_3OD): 183.2 (C), 176.8 (C), 169.8 (C), 164.8 (C), 160.5 (C), 156.6 (C), 142.4 (C), 131.9 (CH), 130.9 (CH), 130.5 (C), 128.0 (C), 122.9 (CH), 116.3 (CH), 113.9 (C), 103.5 (CH), 66.2 (CH), 59.4 (CH), 56.7 (CH), 56.6 (CH), 45.9 (CH_2), 39.7 (CH_2), 39.5 (CH_2), 39.1 (CH_2), 36.8 (CH_2), 30.0 (CH_2), 29.7 (CH_2), 26.6 (CH_2), 12.5 (CH_3); HRMS (ESI, +ve) calc. for $C_{35}H_{38}N_5O_7S_2$: 704.2208, found: 704.2201.



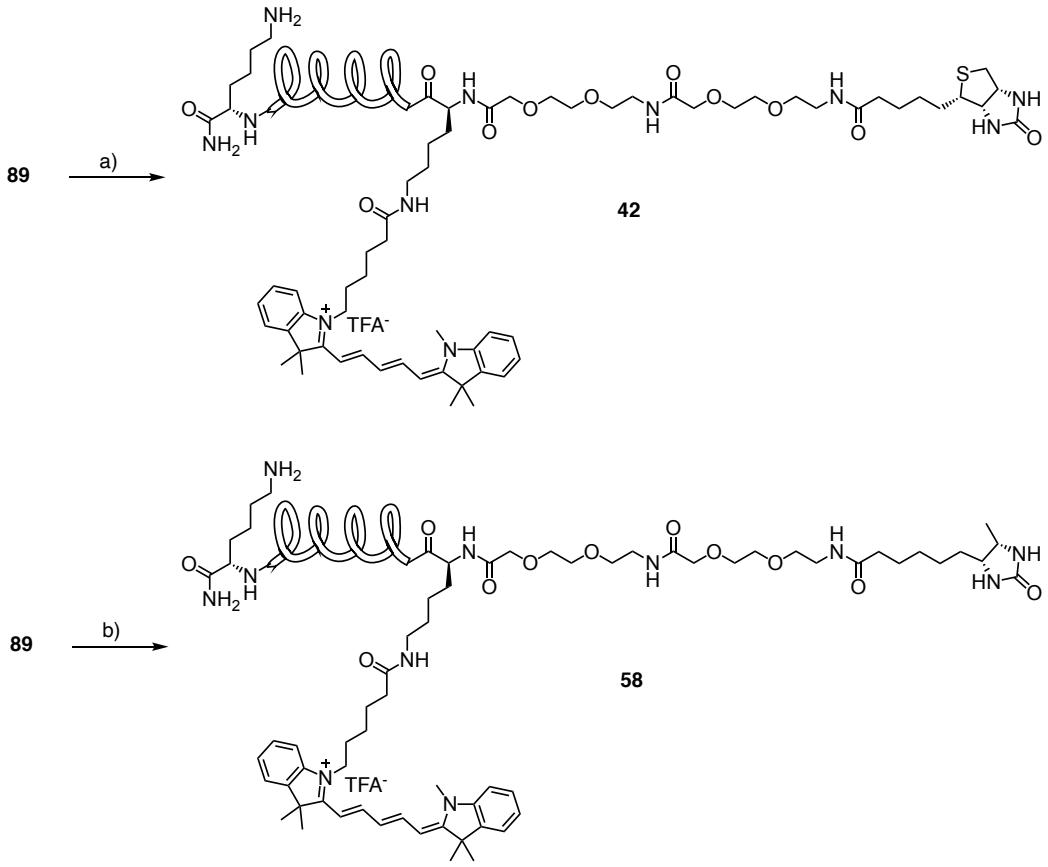
Scheme S5. (a) 1. Mtt-PNA(Boc)-OH monomers, HATU, DIPEA, 2,6-lutidine, NMP, rt, 20 min; 2. Ac_2O, 2,6-lutidine, DMF, 5 min; 3. HOBt, HFIP/DCE, rt, 3 min; 4. Fmoc-L-Lys(Mtt)-OH, HATU, DIPEA, 2,6-lutidine, NMP, rt, 1 h; 5. Ac_2O, 2,6-lutidine, DMF, 5 min; 6. piperidine, DMF, rt, 10 min; b) **86**, HATU, DIPEA, 2,6-lutidine, NMP, rt, 1 h; (c) 1. HOBt, HFIP/DCE, rt; 2. **88**, HOBt, DIC, NMP, rt, 12 h; 3. piperidine, DMF, rt, 10 min (complete structure of PNAs in Figure S34).

Compound 85. As in ref. S15, the Boc-Lys-preloaded resin **84** (5 mg, 1 μmol per column) was washed with DMF and CH_2Cl_2 and treated with a pre-activated (5 min) solution of the corresponding Mtt-PNA(Boc)-OH monomer (5.0 equiv, 5.0 μmol), HATU (1.5 mg, 4.0 μmol), DIPEA (0.9 μL, 5.0 μmol), and 2,6-lutidine (0.9 μL, 7.5 μmol) in NMP (100 μL). This process was repeated twice. Each coupling sequence was followed by a capping step with Ac_2O (0.5 μL, 5.3 μmol) and 2,6-lutidine (0.8 μL, 6.7 μmol) in DMF (150 μL per column) for 5 min. The

resin was subsequently washed with DMF (3 x 1.8 mL) and CH_2Cl_2 (3 x 1.8 mL). Then, the corresponding monomer was deprotected using a solution of HOBt (2 x 1.8 mg, 2 x 13.6 μmol) in HFIP/DCE (2 x 150 μL, 1/1 v:v) for 3 min followed by washing with CH_2Cl_2 (1.8 mL). This process was repeated a second time (HFIP deprotection 2 x 3 min and washing). All the coupling process was repeated for each Mtt-PNA(Boc)-OH monomer. Finally, the resin was treated with a solution of Fmoc-L-Lys(Mtt)-OH (3.1 mg, 5.0 μmol) activated with HATU (1.5 mg, 4.0 μmol), DIPEA (0.9 μL, 5.0 μmol), and 2,6-lutidine (0.9 μL, 7.5 μmol) in NMP (100 μL). The reaction was shaken for 1 h at 25 °C followed by washing with DMF (3 x 1.8 mL) and CH_2Cl_2 (3 x 1.8 mL). The coupling was followed by an acetylation step with Ac_2O (0.5 μL, 5.3 μmol) and 2,6-lutidine (0.8 μL, 6.7 μmol) in DMF (150 μL per column) for 5 min. The resin was washed with DMF (3 x 1.8 mL) and CH_2Cl_2 (3 x 1.8 mL) and the Fmoc protecting group was removed using a solution of piperidine in DMF (20%, 200 μL) for 10 min. This last step was repeated a second time and washed again.

Compound 87. To the rink amide resin **85** loaded with 18-mer PNA (5.0 mg, 0.2 mmol/g, 1.0 μmol) a solution of **86** (1.9 mg, 5 μmol), HATU (9.1 mg, 4.8 μmol) and DIPEA (1.0 μL, 5 μmol) and 2,6-lutidine (1.0 μL, 8.0 μmol) in NMP (100 μL) was added. The reaction was shaken for 1 h at room temperature. The resin was subsequently washed with DMF (3 x 1.8 mL) and CH_2Cl_2 (3 x 1.8 mL). All the process was repeated a second time.

Compound 89. To the rink amide resin **87** (5.0 mg, 0.2 mmol/g, 1.0 μmol) a solution of HOBt (2 x 13.6 equiv) in HFIP/DCE (2 x 150 μL, 1/1 v:v) was added and the reaction was shaken for 3 min, followed by washing with CH_2Cl_2 (1.8 mL). This process was repeated a second time (HFIP deprotection 2 x 3 min and washing). Then, a solution of **88** (5.0 equiv, 1.92 mg, 5 μmol), HOBt (10.0 equiv, 1.3 mg,10.0 μmol) and DIC (15.0 equiv, 2.4 μL, 15.0 μmol) in NMP (100 μL) was added. The reaction was shaken for 12 h at room temperature. The resin was subsequently washed with DMF (3 x 1.8 mL) and CH_2Cl_2 (3 x 1.8 mL). The coupling step was repeated a second time. Finally, the Fmoc protecting group was removed using a solution of piperidine in DMF (20%, 200 μL) for 10 min (the process was repeated twice) and washed again.



Scheme S6. (a) 1. **53**, HOBt, DIC, NMP, rt, 12 h; 2. TFA, rt, 45 min; (b) 1. **41**, HOBt, DIC, NMP, rt, 12 h; 2. TFA, rt, 45 min (complete structure of PNAs in Figure S34).

Compound 42. To the rink amide resin **89** (5.0 mg, 0.2 mmol/g, 1.0 µmol) a solution of biotin **53** (1.2 mg, 5.0 µmol), HOBt (1.4 mg, 10 µmol) and DIC (2.4 µL, 15 µmol) in NMP (100 µL) was added. The reaction was shaken for 12 h at room temperature. The resin was subsequently washed with DMF (3 x 1.8 mL) and CH_2Cl_2 (3 x 1.8 mL). This coupling step was repeated a second time. Finally, the resin was treated with TFA (300 µL), collecting the solution after 45 min of reaction. This process was repeated a second time. Then, ice cold diethyl ether (12 mL) was added to the combined TFA solution in order to precipitate the final product **42**. The dispersion was centrifuged at 4 °C (10000 rpm, 5 min) and the precipitate was washed and centrifuged a second time with ice cold diethyl ether (12 mL). The pellet was dried under a nitrogen flux, redissolved in the minimum amount of DMSO/water 1:1 and purified using RP HPLC (linear gradient 5 – 90% CH_3CN in H_2O with 0.1% TFA). HPLC-MS (ESI, +ve): R_t = 1.94 min, 1370 (35, $[M + 4H]^{4+}$), 1142 (95, $[M + 5H]^{5+}$), 979 (100, $[M + 6H]^{6+}$), 857 (65, $[M + 7H]^{7+}$), 762 (20, $[M + 8H]^{8+}$); MS (MALDI, +ve) calc. for $C_{294}H_{410}N_{110}O_{83}S$: 6845.26, found: 6845.03.

Compound 58. Same protocol as compound **42**, exchanging biotin for desthiobiotin **72** (1.1 mg, 5.0 μmol). HPLC-MS (ESI, +ve): R_t = 1.95 min, 1364 (55, $[M + 4H]^{4+}$), 1137 (90, $[M + 5H]^{5+}$), 975 (100, $[M + 6H]^{6+}$), 853 (65, $[M + 7H]^{7+}$), 758 (15, $[M + 8H]^{8+}$); MS (MALDI, +ve) calc. for $C_{294}H_{412}N_{110}O_{83}$: 6815.22, found: 6816.65.

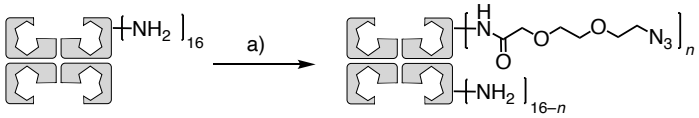
3. Cell Lines and Plasmids

HeLa Kyoto, C2C12, MCF7 and MDCK cells: The cells were cultured in 25 cm^2 cell culture flasks with a vent cap and grew in DMEM + 10% FBS + 1% Pen/Strep.

HeLa cells stably expressing the HaloTag-GFP-Mito construct (*HGM*): The cells were originally designed by the Chenoweth lab.[S16] They were cultured using the described procedure.[S17]

Halo-GFP-Mito mammalian expression vector (*pERB254*): The transfection vector was a gift from Prof. Michael Lampson (Addgene plasmid # 67762).[S18] For transfection, HeLa cells were seeded at 5 $\times 10^5$ cells/mL in DMEM + 10% FBS + 1% Pen/Strep on 6-well plates (2 mL/well) and kept at 37 °C with 5% CO_2 for 8 h. Then, transient transfection was performed by diluting Halo-GFP-Mito mammalian expression vector (5.0 μg) and Xfect™ transfection reagent (1.5 μL) in Xfect™ reaction buffer (100 μL) and directly applying to the cells in one well (1 mL of growing media), incubating overnight.

4. CPS Synthesis



2 (WTS) **8**

Scheme S7. (a) **7**, PBS, rt, 2 h.

As in S1, a stock solution of **2** (WTS, 3 mL, 10 μM in PBS buffer) was distributed in 20 Eppendorf tubes (150 μL/tube) containing different amounts of compound **7**,[S19] reaching a final concentration of **7** as described in Table S1. The resulting mixtures were shaken (1000 rpm) for 2 h at rt, filtered through Amicon® Ultra 0.5 mL centrifugal filters (cut off: 10 kDa, 5 min, 14.0 krpm) and washed twice with a bicarbonate buffer solution (0.1 M, pH 8.5, 0.4 mL each) and filtered again (cut off: 10 kDa, 10 min, 14.0 krpm). The material was recovered from the centrifugal filter and diluted again in the bicarbonate buffer solution (1.5 mL). Final

concentration of pre-CPS **8** was calculated by UV-vis absorption spectroscopy at λ = 280 nm using the calibration curve in Figure S1A. Number of functionalized lysines was calculated using TNBSA colorimetric assay, by UV-vis absorption spectroscopy at λ = 335 nm, using the calibration curve in Figure S1B and the following procedure: To the protein solution (0.5 mL), TNBSA solution (0.25 mL, 0.01% in bicarbonate buffer) was added and the mixture was stirred for 2 h at 37 °C. Then, a solution of SDS (0.25 mL, 10% in bidistilled water) and aq. HCl (0.125 mL, 1 M) were added. Afterward, the absorption at λ = 335 nm was directly recorded. The dependence on the loading of **7** in **8** (n) with the concentration of **7** in the reaction mixture is depicted in Table S1 and Figure S1C.

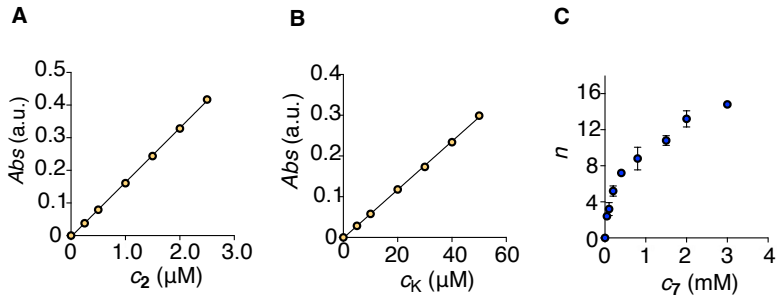


Figure S1. (A) Absorbance of **2** at λ = 280 nm depending on the concentration of **2**. (B) Absorbance of **2** at λ = 335 nm depending on the concentration of lysine residues (K, c_K = 16 × c_2), after treatment with TNBSA. (C) Number of azide linkers **7** (n) introduced in the structure of **8**, depending on the concentration of **7** in the reaction mixture (Scheme S7), calculated using Equation S1.

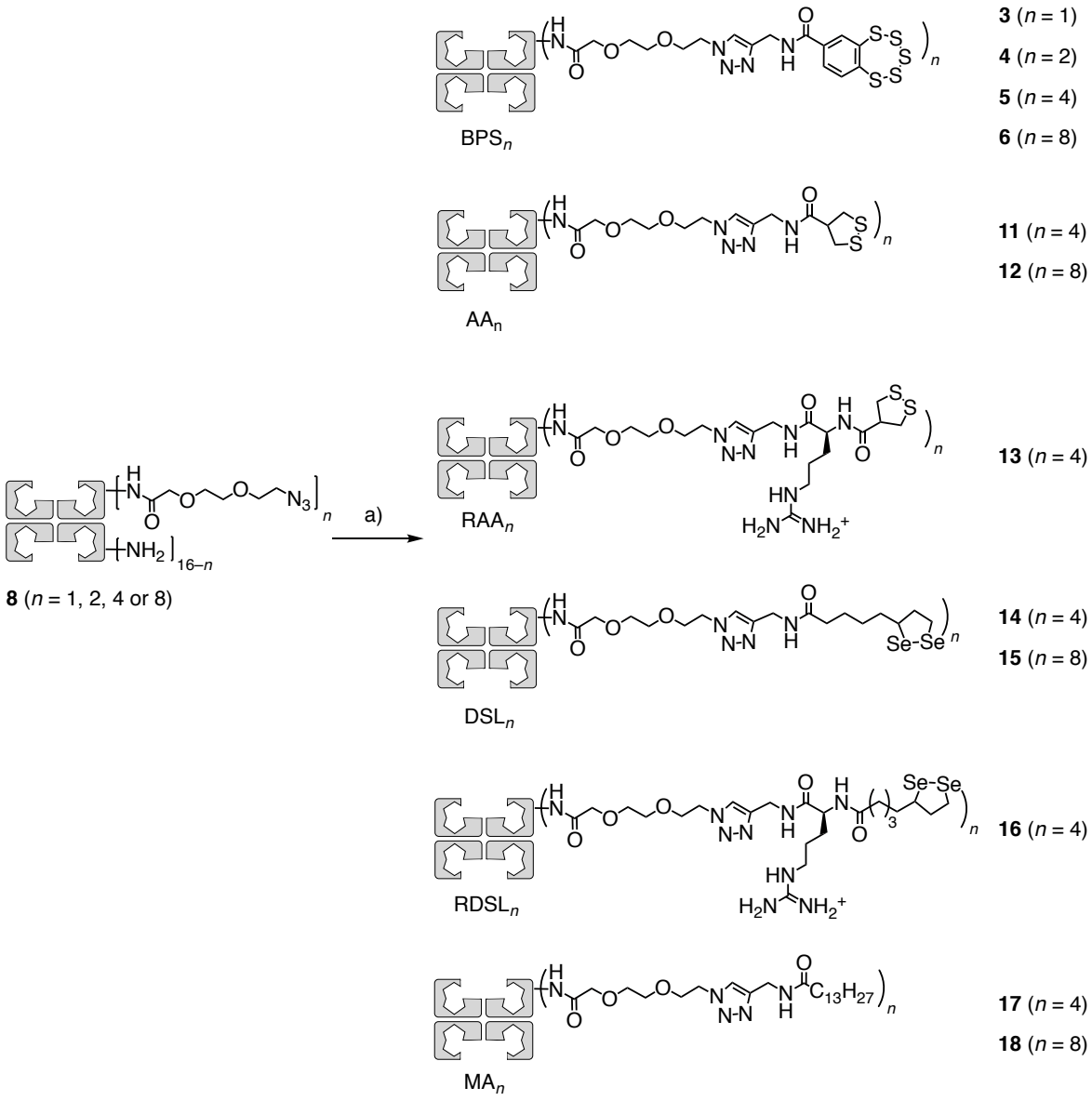
Loading of **8** with azide linker **7** (n) can be calculated from Equation S1.

$$n = 16 - (c_K / c_2) \qquad \text{S1}$$

Table S1. Summary of conditions used for Figure S1C.

	c_7 (μM)	n[a]
1	0	0
2	48	2.4 ± 0.3
3	100	3.2 ± 0.7
4	200	5.2 ± 0.6
5	400	7.2 ± 0.2
6	800	8.8 ± 1.3
7	1500	10.8 ± 0.5
8	2000	13.2 ± 0.9
9	3000	14.8 ± 0.3

[a]Loading of azide **7** per streptavidin tetramer, in **8**. Values calculated based on two independent experiments using Equation S1.



Scheme S8. (a) **9**, **60**, **61**, **62**, **65** or **67**, $CuSO_4 \cdot 5H_2O$, BTTAA, sodium ascorbate, aminoguanidine hydrochloride, PBS, rt, 1 h.

Pre-CPS **8** were prepared with different loading of azide linker **7** (n = 1, 2, 4 and 8), according to Table S2, using the calibration curve in Figure S1C. After this, the loading of the protein with the different transporters was performed under previously optimized Cu-catalyzed click reaction conditions:[S1] To an Eppendorf tube containing a solution of **8** (12.5 μM, 800 μL) and the corresponding alkyne-transporter (**9**, **60**, **61**, **62**, **65** or **67**, 20 mM, 3.75 μL – 15 μL) in PBS buffer, the premixed click reagents ($CuSO_4 \cdot 5H_2O$ 1.0 mM; BTTAA 7.5 mM; sodium ascorbate 15.0 mM; aminoguanidine hydrochloride 15.0 mM, in 200 μL in bidistilled water) were added, reaching a total volume of approximately 1 mL and the following final concentrations:

Pre-CPS **8**: 10 μM;

Alkyne transporter: see Table S2;

$CuSO_4 \cdot 5H_2O$: 0.2 mM;

Ligand BTTAA: 1.5 mM;

Sodium ascorbate: 3.0 mM;

Aminoguanidine hydrochloride: 3.0 mM

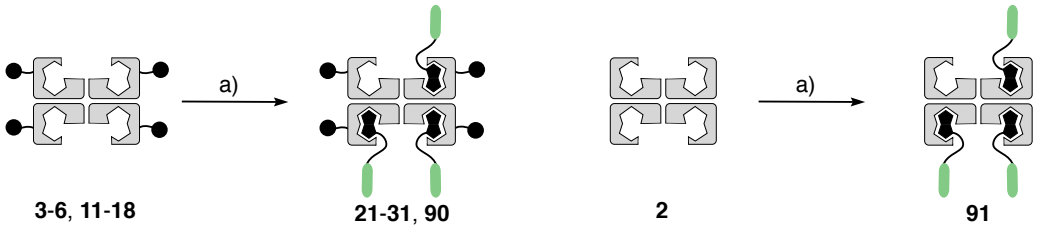
The resulting mixture was shaken (1000 rpm) for 1 h at 25 °C. During this time, the premixed click reagents were added twice again (200 μL each, t = 20 min, t = 40 min). After shaking, the mixture was filtered through an Amicon® Ultra 0.5 mL centrifugal filter (cut off: 30 kDa, 5 min, 14.0 krpm) and washed 5 times with PBS buffer (0.4 mL each). If necessary, volumes of the reaction were scaled-up to 9 mL maximum, depending on the amount of material needed for the corresponding experiment, keeping the concentration of the substrates and reagents constant. Depending the scale, reactions were carried out in Falcon™ 15 mL conical centrifuge tubes and the washings in Amicon® Ultra 2 mL or 15 mL centrifugal filters. Based on several experiments, the loss of material during the washing cycles was estimated to be 20% average, which was considered in the final concentration of the corresponding CPS.

Table S2. Concentrations of **7** and alkyne transporters (**9**, **60**, **61**, **62**, **65** or **67**) for the synthesis of **8** and the different CPS candidates, respectively.

	n	c_7 (μM)	c_{alkyne} (μM)
1	1	20	75
2	2	45	75
3	4	180	150
4	8	700	300

5. Combining CAPA with Automated HC Microscopy (HC-CAPA)

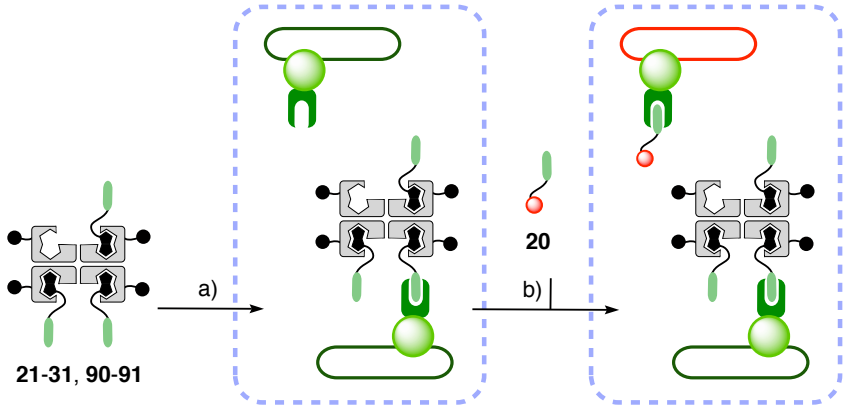
5.1. Monofunctional Non-Fluorescent CPS Complexes



Scheme S9. (a) **19**, PBS, rt, 10 min.

Complex 23. To a freshly prepared solution of **5** (4 mL in PBS, 8 µM), **19** (48 µL, 2 mM in DMSO, 3 equivalents) was added and the mixture was shaken for 10 min at rt. Then, the CPS complex was concentrated and washed with PBS (2 x 0.4 mL) using Amicon® Ultra 0.5 mL centrifugal filters (cut off: 30 kDa, 10 min, 14.0 krpm). The material was recovered from the centrifugal filter and diluted again with PBS to the required concentration (i.e. 200 µM, 160 µL). The same synthetic protocol was applied for the other complexes **21-31**, **90** and **91**, as in Scheme S9.

5.2. Assay Protocol for HC-CAPA



Scheme S10. (a) Leibovitz's, 37 °C, 5% CO_2, 4 h. (b) Leibovitz's, 37 °C, 5% CO_2, 15 min.

For the assay, HGM cells were seeded at 8 $\times 10^4$ cells/mL in FluoroBrite DMEM + 10% FBS on µ-Plate 96-well ibiTreat sterile and kept at 37 °C with 5% CO_2 overnight. Next day, cells were washed with PBS (3 x 3 mL/well) and the media was exchanged to Leibovitz's (4 x 150 µL/well) using a plate washer, keeping a final volume of 135 µL/well. Then, serial dilutions of the corresponding CPS **21-31**, **90-91** in PBS were prepared in a 96-well V-bottom plate and added to the µ-Plate containing the cells (15 µL/well, 10x final concentration in PBS) to reach

a final volume of 150 µL/well (0 to 20 µM). Cells were incubated for 4 h at 37 °C with 5% CO_2. After this, cells were washed again and **20** was added (15 µL/well, 50 µM in PBS) to reach a final volume of 150 µL/well (5 µM), except for the control wells, where only PBS was added (15 µL/well). After 15 min of incubation at 37 °C with 5% CO_2, the plate was washed again. Then, Hoechst 33342 was added (15 µL/well, 170 µM in PBS) to reach a final volume of 150 µL/well (17 µM). After 15 min of incubation at 37 °C with 5% CO_2, the plate was washed one last time and the cells were kept in clean Leibovitz's media. During imaging, samples were kept at 37 °C with 5% CO_2.

A total of 25 images/well at 63x were recorded, using three channels: blue (excitation filter: 377/50 nm, emission filter: 477/60 nm, exposure time: 10 ms), green (excitation filter: 475/34 nm, emission filter: 536/40 nm, exposure time: 20 ms) and red (excitation filter: 531/40 nm, emission filter: 593/40 nm, exposure time: 15 ms), as shown in Figure S2. Duplicates were performed for each condition.

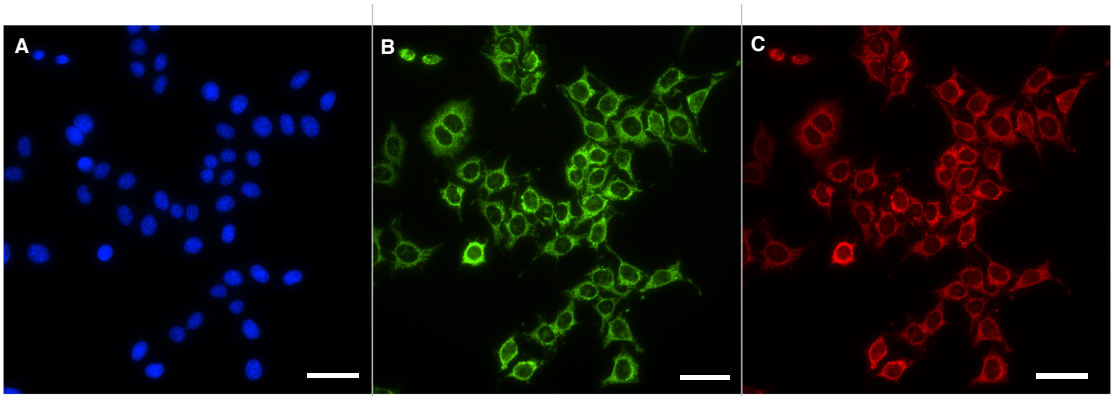


Figure S2. (A) Blue channel recording the Hoechst 33342 localization in the nuclei. (B) Green channel recording the GFP signal in the mitochondria. (C) Red channel recording the signal from **20** covalently attached to the free HaloTag protein in the mitochondria. Scale bar: 50 µm.

5.3. Data Analysis for HC-CAPA

For each cell, the blue channel image is used for the segmentation of nuclei while the green channel image is used for the segmentation of the whole cell body.

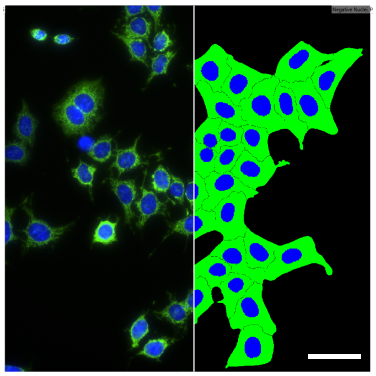


Figure S3. Segmentation of nuclei and cell body (right) using the blue and green channels (left), respectively. Scale bar: 50 μm.

Top-hat transform of the green channel image is used to lower the background and to facilitate the segmentation of the mitochondria.

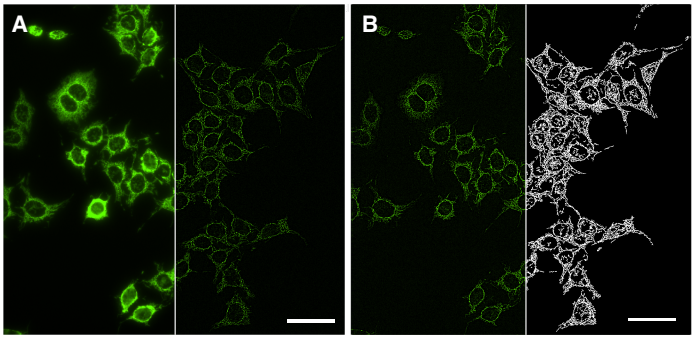


Figure S4. (A) *Top-hat* transform (right) of the green channel image (left). (B) Segmentation of the mitochondria (right) using the *top-hat* transformed image (left). Scale bar: 50 μm.

Cell body and mitochondria masks are applied to extract the integrated intensity values (sum of the intensities of the pixels included in the mask) in the red channel image, from the labeling with compound **20**.

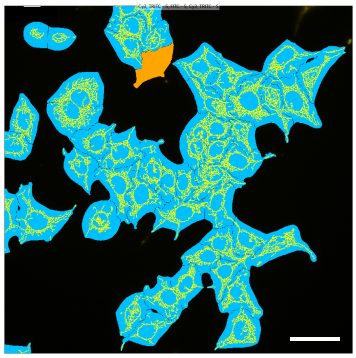


Figure S5. Masks applied for the quantification of the fluorescence intensity of **20**, in the red channel image. Cell body (light blue) and mitochondria (yellow). Orange area represents one cell. Scale bar: 50 μm.

5.4. HC-CAPA Results

Integrated intensity values for each condition were normalized using the value of integrated intensity with addition of **20** (0 μM of CPS) as maximum signal (I_{rel} = 1) and the value of integrated intensity without addition of **20** (0 μM of CPS) as minimum (I_{rel} = 0), for each set of experiments. Duplicates were performed for each condition.

The resulting dependence of the relative intensity values (I_{rel}) to the concentration of CPS (c_{CPS}) was plotted and fitted with Equation S2 to retrieve the half maximal cell penetration (CP_{50}) value and the Hill coefficient (n).

$$I_{rel} = 1 / (1 + (c_{CPS} / CP_{50})^{n}) \qquad \text{S2}$$

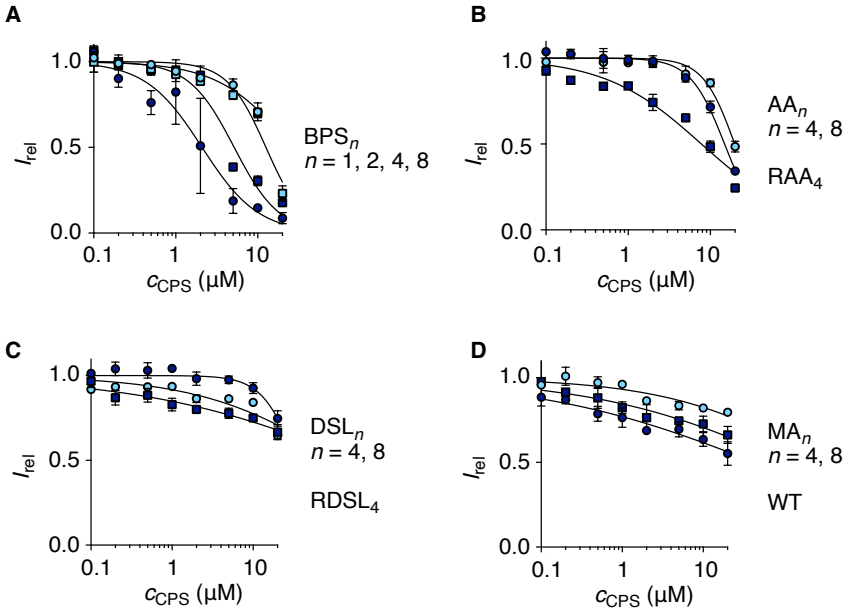


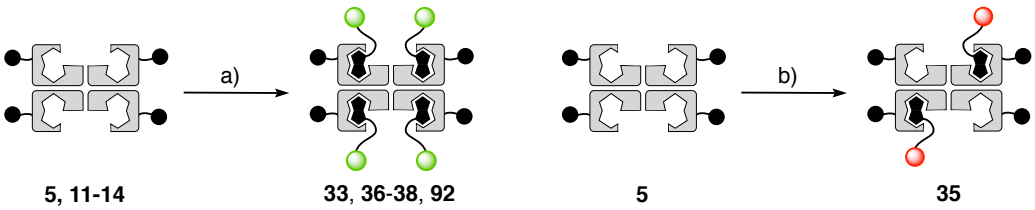
Figure S6. Dose response curves obtained from HC-CAPA assay: (A) **21** (light blue circle), **22** (light blue square), **23** (dark blue circle) and **24** (dark blue square); (B) **25** (dark blue circle), **26** (dark blue square), **27** (light blue circle); (C) **28** (dark blue circle), **29** (dark blue square), **30** (light blue circle); (D) **31**(dark blue circle), **90** (dark blue square), **91** (light blue circle).

Table S3. CP_{50} values of each complex.

	Complex	CP_{50} (μM)	*n*
1	**21** (BPS_1)	26 ± 5	1.0 ± 0.1
2	**22** (BPS_2)	13 ± 1	1.9 ± 0.4
3	**23** (BPS_4)	2.1 ± 0.3	1.3 ± 0.2
4	**24** (BPS_8)	5.2 ± 0.7	1.6 ± 0.3
5	**25** (AA_4)	14.9 ± 0.5	2.1 ± 0.1
6	**26** (AA_8)	8 ± 1	0.7 ± 0.1
7	**27** (RAA_4)	20 ± 1	2.2 ± 0.3
8	**28** (DSL_4)	34 ± 6	2.0 ± 0.5
9	**29** (DSL_8)	>> 35	-
10	**30** ($RDSL_4$)	>> 35	-
11	**31** (MA_4)	>> 35	-
12	**90** (MA_8)	>> 35	-
13	**91** (WT)	>> 35	-

6. Cellular Uptake of CPS

6.1. Monofunctional Fluorescent CPS Complexes



Scheme S11. (a) **32**, PBS, rt, 10 min. (b) **34**, PBS, rt, 10 min.

Complex 33 (BPS_4-32_4). ***General procedure of complex formation.*** To a freshly prepared solution of **5** (BPS_4, 1 mL in PBS, 8 μM), **32** (16 μL, 2 mM in DMSO, 4 equivalents) was added and the mixture was shaken for 10 min at rt. Then, the CPS complex was concentrated and washed with PBS (2 x 0.4 mL) using Amicon® Ultra 0.5 mL centrifugal filters (cut off: 30 kDa, 10 min, 14.0 krpm). The material was recovered from the centrifugal filter (130 μM, 60 μL) and used directly for the cell experiments. The same synthetic protocol is applied for the other CPS complexes to generate complexes **36** (AA_4-$\mathbf{32}_4$), **37** (RAA_4-$\mathbf{32}_4$), **92** (DSL_4-$\mathbf{32}_4$) and **38** ($RDSL_4$-$\mathbf{32}_4$) respectively.

Complex 35 (BPS_4-34_2) was prepared following the *general procedure of complex formation* using **34** (8 µL, 2 mM in H_2O, 2 equivalents), instead of **32**.

6.2. Thiol Exchange Column Chromatography

The medium was prepared following the reported procedures with modifications.[S1] Namely, activated thiol Sepharose™ 4B (3 g) was suspended in bidistilled water and filtered. The swollen medium was then suspended in an aqueous solution of DTT (1% w/v), EDTA (1 mM) and $NaHCO_3$ (0.3 M) at pH 8.4. The mixture was shaken for 1 h at rt, filtered and rinsed with an aqueous solution of AcOH (0.1 M), NaCl (0.5 M) and EDTA (1 mM). Resulting medium was suspended in a buffer (10 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.5), and packed in a column (Omnifit EZ, 10 x 110 mm). Analyses were performed using JASCO LC-2000 systems at rt, under the following conditions: 0.4 mL/min, a buffer (10 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.5) with DTT (gradient: 0 – 60 min: 0 mM, 60 – 70 min: 0 – 50 mM, 70 – 210 min: 50 mM; or isocratic: 50 mM) as an eluent, λ_{abs} at 560 nm for detection. Sample solutions of **35** (20 µM) were prepared in PBS without DTT, and injected (50 µL) for analyses.

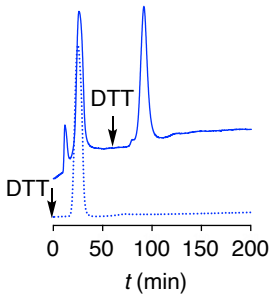


Figure S7. Thiol-exchange affinity column chromatograms of **35** with a 0 – 50 mM DTT gradient at t = 60 – 70 min (solid) and constant 50 mM DTT from t = 0 (dashed).

6.3. Protocol for Incubation and Microscopy

General procedure. For the delivery of complexes **33**, **36**, **37**, **92** and **38**, HeLa cells were seeded at 8 $\times 10^4$ cells/mL in DMEM + 10% FBS + 1% Pen/Strep on µ-Slide 8-Well Glass Bottom (ibidi) and kept at 37 °C with 5% CO_2 overnight (or at least for a period of 15 h). The day of the experiment, the media was removed and the cells were washed with PBS (2 x 200 µL) and Leibovitz's medium (1 x 200 µL) and treated with a solution of the corresponding complex (150 µL, 5 or 10 µM in Leibovitz's medium). The cells were incubated for 8 h at 37 °C under 5% CO_2, then the medium was removed. The cells were washed with Leibovitz's medium (3 x 200 µL) and finally kept in clean Leibovitz's medium. Distribution of fluorescent

compounds was analyzed without fixing using confocal laser scanning microscope. Argon laser was used as light source with excitation wavelength 488 nm and emission 499 – 565 nm, LP: 30% (Leica HyDTM detector). During the imaging, the samples were kept at 37 °C. Brightness and contrast were adjusted equally in all images using ImageJ software.

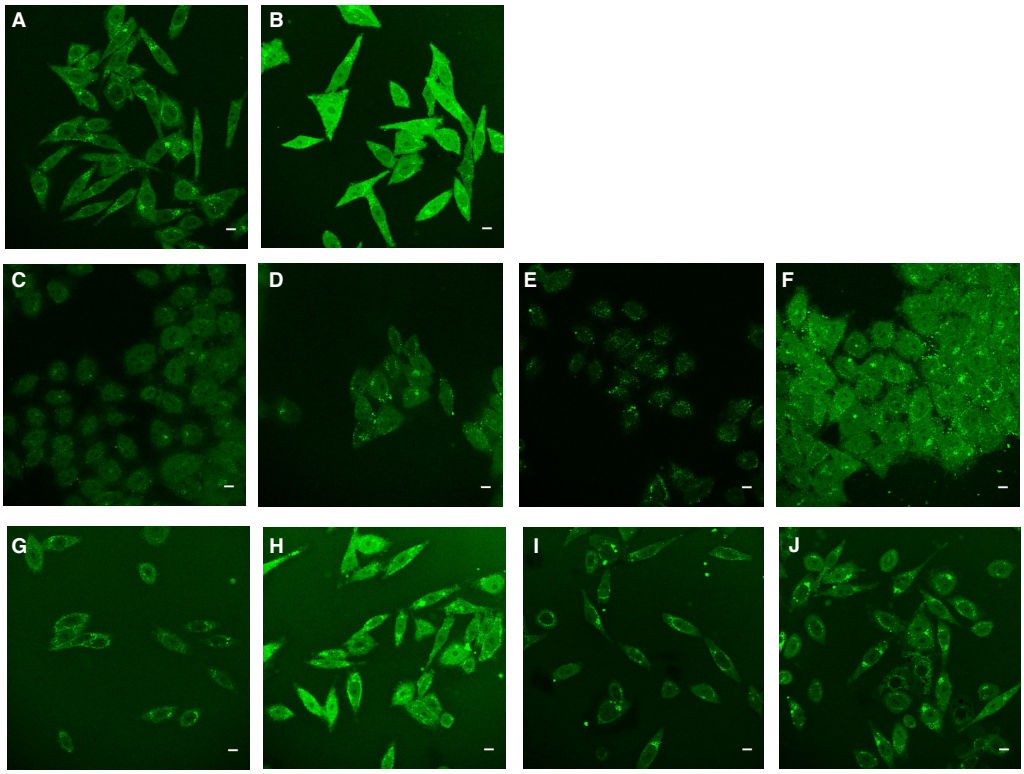


Figure S8. CLSM images of Hela Kyoto cells after incubation with (A) 5 μM and (B) 10 μM of **33**,[S1] (C) 5 μM and (D) 10 μM of **36**, (E) 5 μM and (F) 10 μM of **37**, (G) 5 μM and (H) 10 μM of **92**, (I) 5 μM and (J) 10 μM of **38** for 8 h. Scale bar: 10 μm.

Complex **35** was delivered following the *general procedure* using HeLa, C2C12, MCF7 and MDCK cells. DPSS laser was used as light source with excitation wavelength 561 nm and emission 571 – 620 nm, LP: 30% (Leica HyDTM detector).

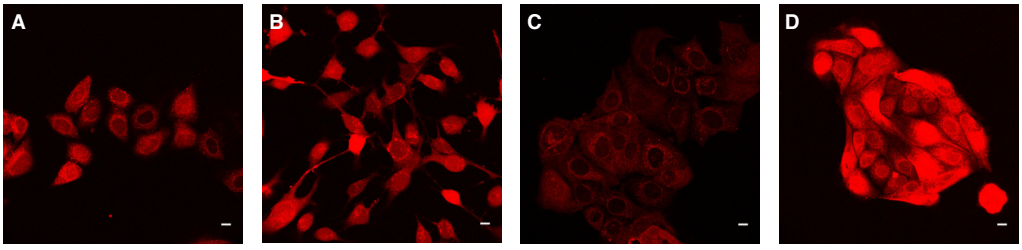
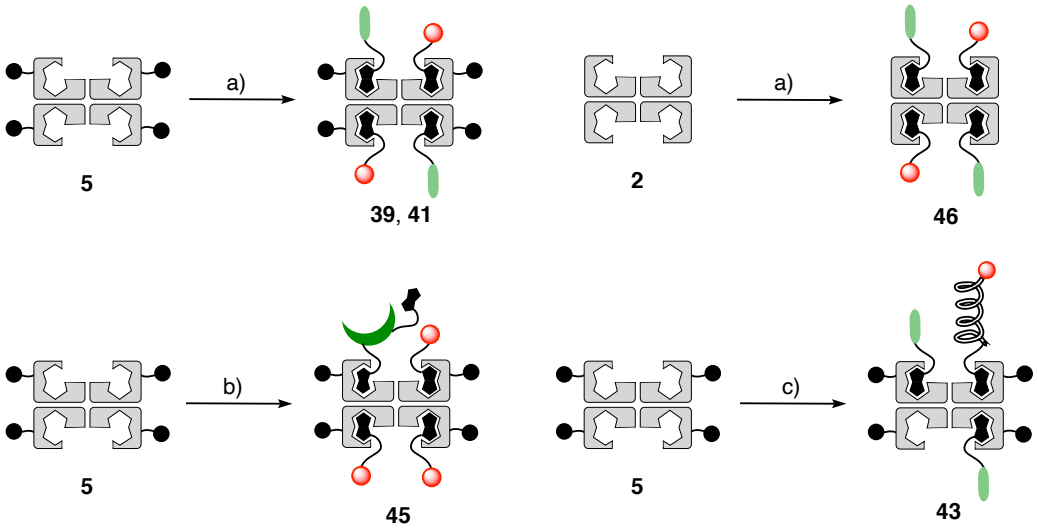


Figure S9. CLSM images of (A) Hela Kyoto (B) C2C12, (C) MCF7 and (D) MDCK cells after incubation of **35** at 10 μM for 2 h. Scale bar: 10 μm.

7. Targeted Delivery

7.1. Bifunctional CPS Complexes



Scheme S12. (a) **34** or **40**, and **19**, PBS, rt, 10 min. (b) 1. **34**, PBS, 20 °C, 10 min; 2. **44**, Leibovitz's, 37 °C, *in situ* with cells. (c) **42**, **19**, PBS, 20 °C, 10 min.

Complex 39 (BPS_4-34_2-19_2) was prepared following the *general procedure of complex formation* (6.1), exchanging compound **32** for **34** (8 μL, 2 mM in H_2O, 2 equivalents) and **19** (8 μL, 2 mM in DMSO, 2 equivalents).

Complex 41 (BPS_4-40_2-19_2). Same procedure as that for **39**, exchanging compound **34** for **40** (8 μL, 2 mM in DMSO, 2 equivalents).

Complex 46 (WT-34_2-19_2). Same procedure as that for **39**, using **2** (1 mL in PBS, 8 μM) instead of **5**.

Complex 45 (BPS_4-34_3-44_1) was prepared following the *general procedure of complex formation* (6.1), using 3 equivalents of **34** instead of **32**. Addition of **44** was done in the next step, directly in the plate containing the cells.

Complex 43 (BPS_4-42_1-19_2). Same procedure as that for **39**, exchanging compound **34** for **42** (2.6 μL, 3 mM in H_2O, 1 equivalent).

7.2. Protocol for Incubation and Microscopy

Targeted delivery of complex 39. The *general procedure* (6.3) was followed, using transiently transfected Hela cells (pERB254 expression vector). For the acquisition of fluorescence coming from GFP, Argon laser was used as light source with excitation

wavelength 488 nm and emission 492 – 534 nm, LP: 20%. For the acquisition of the fluorescence coming from rhodamine, DPSS laser was used as light source with excitation wavelength 561 nm and emission 571 – 620 nm, LP: 30% (Leica HyDTM detector). Brightness and contrast were adjusted equally in all images using ImageJ software.

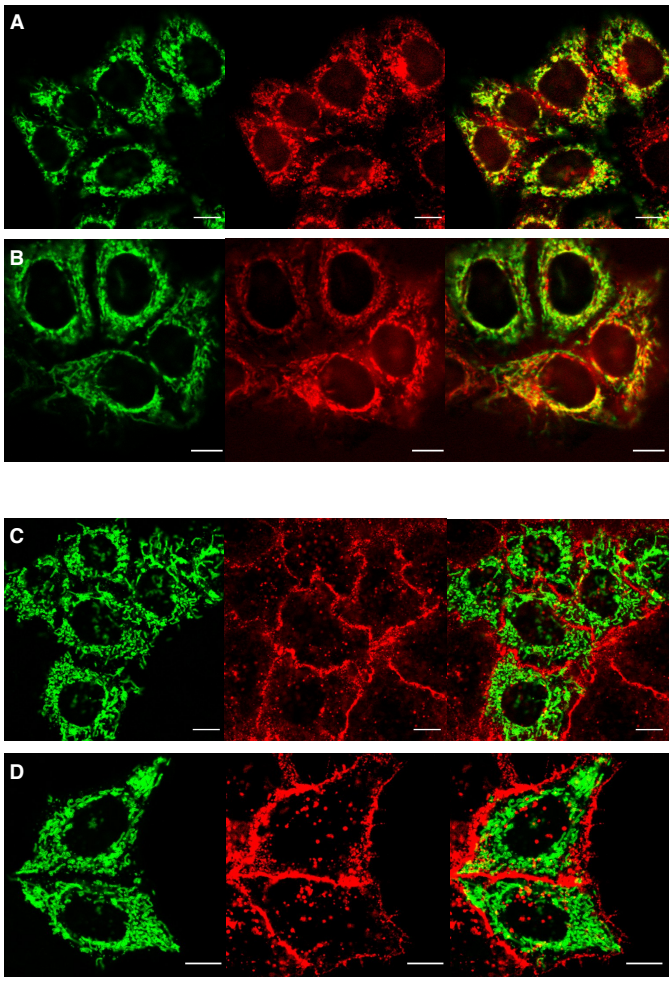


Figure S10. CLSM images of transfected cells expressing GFP and HaloTags on the mitochondria, after incubation of (A,B) **39** and (C,D) control **46** at 5 μM for 2 h. Left to right: GFP channel, rhodamine channel and merge. Scale bar: 10 μm.

Targeted delivery of complex 41. The *general procedure* (6.3) was followed, using HGM cells. For the acquisition of fluorescence coming from GFP, Argon laser was used as light source with excitation wavelength 488 nm for both channels, LP: 15%, recording the emission from GFP between 492 – 534 nm and the emission from flipper probe between 618 – 737 nm.

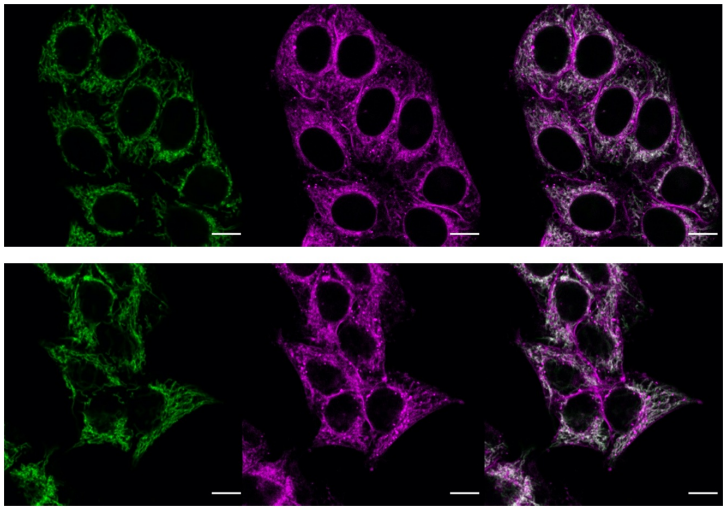


Figure S11. CLSM images of HGM cells expressing GFP and HaloTags on the mitochondria, after incubation of **41** at 5 μM for 2 h. Left to right: GFP channel, flipper probe channel and merge. Scale bar: 10 μm.

Targeted delivery of complex 45. Transiently transfected Hela cells (pERB254 expression vector) were seeded at 8 $\times 10^4$ cells/mL in DMEM + 10% FBS + 1% Pen/Strep on μ-Slide 8-Well Glass Bottom (ibidi) and kept at 37 °C with 5% CO_2 overnight. Next day, the media was removed and the cells were washed with PBS (2 x 200 μL) and Leibovitz's medium (1 x 200 μL) and treated with a solution of **45** (1.0 or 2.3 μM), which was formed *in situ* after addition of **44** (7.5 or 15.0 μL, 20 μM in HEPES, 1 equivalent) to the complex. The cells were incubated for 2 h or 4 h at 37 °C under 5% CO_2. Then, the medium was removed and the cells were washed with Leibovitz's medium (3 x 200 μL) and incubate in clean medium (200 μL) for extra 15 min at 37 °C under 5% CO_2. The medium was removed and the cells were finally kept in clean Leibovitz's medium (200 μL) for imaging. Distribution of fluorescent compounds was analyzed without fixing using confocal laser scanning microscope. For the acquisition of fluorescence coming from GFP, Argon laser was used as light source with excitation wavelength 488 nm and emission 492 – 534 nm, LP: 6%. For the acquisition of the fluorescence coming from rhodamine, DPSS laser was used as light source with excitation wavelength 561 nm and emission 571 – 620 nm, LP: 30% (Leica HyDTM detector). During the imaging, the samples were kept at 37 °C.

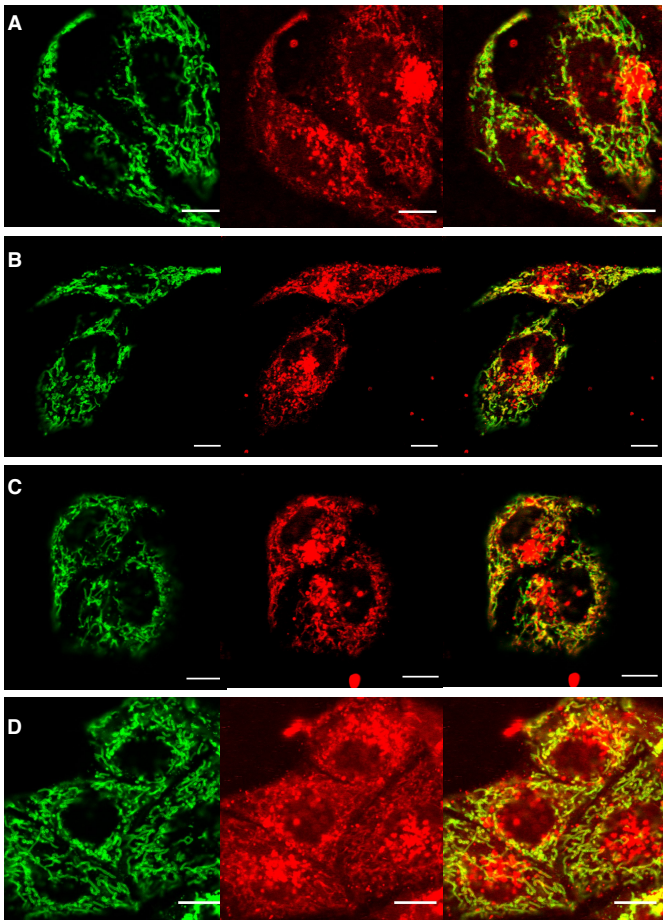


Figure S12. CLSM images of transfected cells expressing GFP and HaloTags on the mitochondria, after incubation of **45** at (A) 1 μM for 2 h, (B) 1 μM for 4 h, (C) 2.5 μM for 2 h (D) 5.0 μM for 4 h. Left to right: GFP channel, rhodamine channel and merge. Scale bar: 10 μm.

Targeted delivery of complex 43. HGM cells were seeded in FluoroBrite DMEM + 10% FBS + 1% Pen/Strep on μ-Plate 96-Well Plastic Bottom (ibidi) and kept at 37 °C with 5% CO_2 overnight. The day of the experiment, the media was removed and the cells were washed with PBS (3 x 3 mL/well) and the media was exchanged to Leibovitz's (4 x 150 mL/well) using a plate washer, keeping a final volume of 135 μL/well. Then, **43** was added (15 μL/well, 50 μM in PBS) to reach a final volume of 150 μL/well (5 μM). The cells were incubated for 2 h at 37 °C under 5% CO_2, then the cells were washed again and finally kept in clean Leibovitz's medium (135 μL) for imaging. Distribution of fluorescent compounds was analyzed without fixing using automated fluorescence microscope. A total of 16 images/well at 40x were recorded, using two channels: green (excitation filter: 475/34 nm, emission filter: 536/40 nm,

exposure time: 30 ms) and red (excitation filter: 631/28 nm, emission filter: 692/40 nm, exposure time: 150 ms).

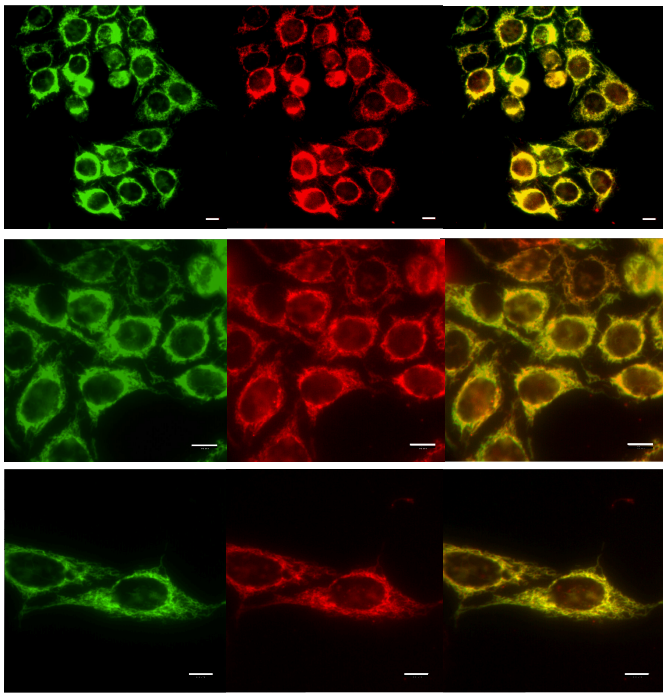
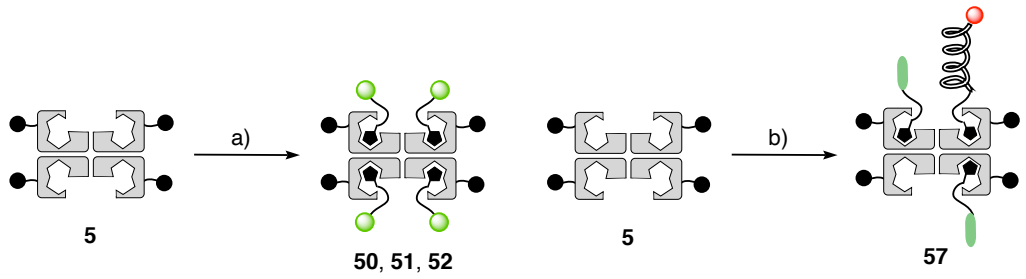


Figure S13. Micrographs of HGM cells expressing GFP and HaloTags on the mitochondria, after incubation of **43** at 5 μM for 2 h. Left to right: green channel, red channel and merge. Scale bar: 10 μm.

8. Controlled Release

8.1. Exchangeable CPS Complexes

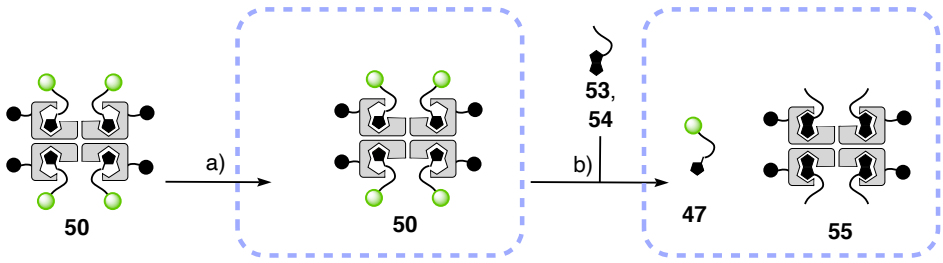


Scheme S13. (a) **47** or **48** or **49**, PBS, rt, 10 min. (b) **58**, **59**, PBS, 20 °C, 10 min.

Complexes 50 (BPS_4-47_4), 51 (BPS_4-48_4) and 52 (BPS_4-49_4) were prepared following the *general procedure of complex formation* (6.1) using **47** (32 µL, 1 mM in DMSO, 4 equivalents), **48** (32 µL, 1 mM in DMSO, 4 equivalents) and **49** (32 µL, 1 mM in DMSO, 4 equivalents), respectively, instead of **32**.

Complex 57 (BPS_4-58_1-59_2) was prepared following the *general procedure of complex formation* (6.1) using **58** (2.6 µL, 3.4 mM in H_2O, 1 equivalent) and **59** (8 µL, 2 mM in DMSO, 2 equivalents) instead of **32**.

8.2. Assay Protocol for Controlled Release



Scheme S14. (a) Leibovitz's medium, 37 °C, 6 h. (b) Leibovitz's medium (or FluoroBrite DMEM), 37 °C, 6 h (example for **50**, same protocol for **51** and **52**).

In the morning, HeLa Kyoto cells were seeded at 8 ×10^4 cells/mL in FluoroBrite DMEM + 10% FBS on µ-Plate 96-well ibiTreat sterile and kept at 37 °C with 5% CO_2. Late in the afternoon, media was exchanged to FluoroBrite DMEM (no FBS) and the cells were kept at 37 °C with 5% CO_2 overnight. Next day, cells were washed and the media was exchanged to Leibovitz's using a plate washer keeping a final volume of 135 µL/well. Then, CPS complex **50**, **51** or **52** was added to 6 different wells (15 µL/well, 100 µM in PBS) to reach a final volume of 150 µL/well (10 µM) and the cells were incubated for 6 h at 37 °C with 5% CO_2. After this, cells were washed and Hoechst 33342 was added (15 µL/well, 170 µM in PBS) to reach a final volume of 150 µL/well (17 µM). After 15 min of incubation at 37 °C with 5% CO_2, the plate was washed again and kept in FluoroBrite DMEM and it was directly imaged (t = 0 min). Finally, biotin **53** or **54** was added to 2 wells/complex (15 µL/well, 400 µM in PBS) to reach a final volume of 150 µL/well (40 µM). The plate was recorded for 360 min, in intervals of 12 min. During imaging, samples were kept at 37 °C with 5% CO_2. A total of 16 images/well and timepoint at 60x were recorded (excitation filter: 475/34 nm, emission filter: 536/40 nm, exposure time: 30 ms), for the quantification of cargo release from the fluorescence intensity of **47**, **48** or **49**. Duplicates were performed for each condition.

The resulting time dependence of the average intensity in each well (I) after addition of biotin **53** or **54**, i.e. the kinetics of cargo release, was plotted and the results were compared with the control experiments without addition of **53** or **54**.

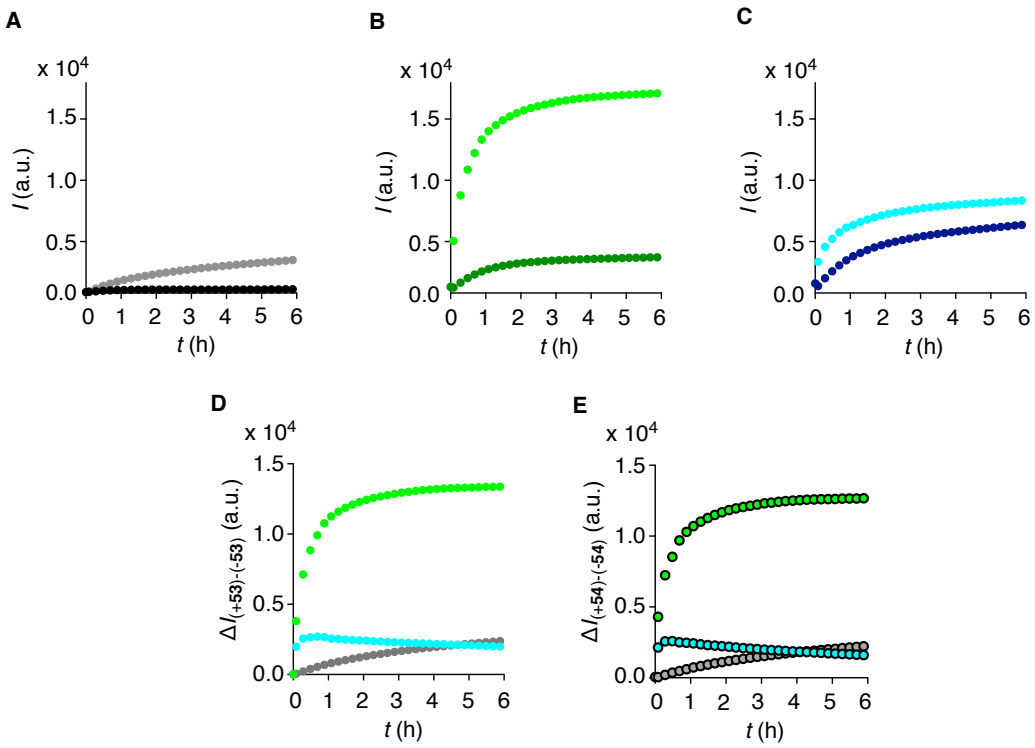


Figure S14. (A) Kinetics of release of **49** inside the cells with (grey) and without (black) addition of **53**. (B) Kinetics of release of **47** inside the cells with (light green) and without (dark green) addition of **53**. (C) Kinetics of release of **48** inside the cells with (light blue) and without (dark blue) addition of **53**. (D) Difference between kinetics of release of **49** (grey), **47** (light green) and **48** (light blue) with and without addition of **53**. (E) Difference between kinetics of release of **49** (grey), **47** (light green) and **48** (light blue) with and without addition of **54**.

8.3. Targeted Delivery and Controlled Release of PNA

The same procedure as that for **43** was followed (*protocol* 7.2.) using **57**. After incubation for 2 h at 37 °C under 5% CO_2, the cells were kept in clean Leibovitz's medium and the plate was directly imaged (t = 0 h). Then, biotin was added (15 μL/well, 400 μM in PBS) to reach a final volume of 150 μL/well (40 μM). The plate was incubated for 2 h at 37 °C under 5% CO_2 and then directly imaged (t = 2 h). During imaging, samples were kept at 37 °C with 5% CO_2. A total of 16 images/well at 40x were recorded, using two channels: green (excitation filter: 475/34 nm, emission filter: 536/40 nm, exposure time: 30 ms) and red (excitation filter: 631/28 nm, emission filter: 692/40 nm, exposure time: 150 ms).

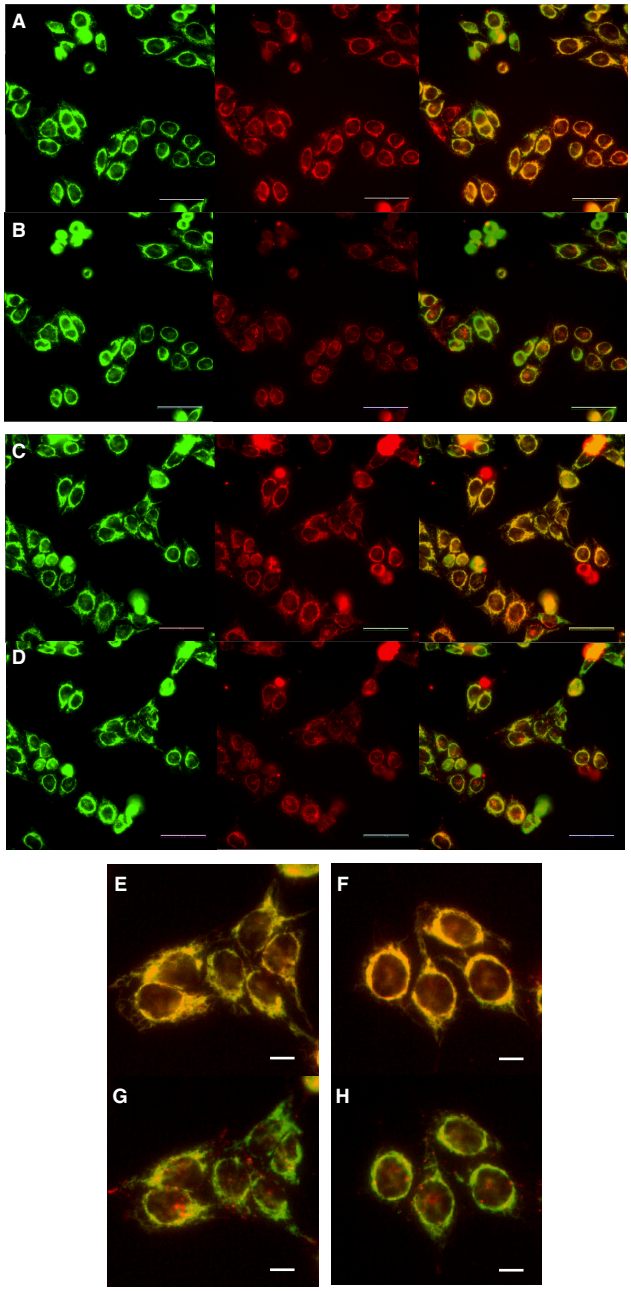


Figure S15. (A,C) Micrographs of HGM cells expressing GFP and HaloTags on the mitochondria, after incubation with **57** at 5 μM for 2 h and (B,D) after additional 2 h in the presence of biotin **53**. Left to right: GFP channel, Cy5 channel and merge. Scale bar: 50 μm. (E,F) Zoom micrographs of the merge channel before the addition and (G,H) after the addition of biotin. Scale bar: 10 μm.

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The original data can be found at:

https://dx.doi.org/10.26037/yareta:v7airh7g7rfrlpa3s7m64unefy

10. NMR Spectra

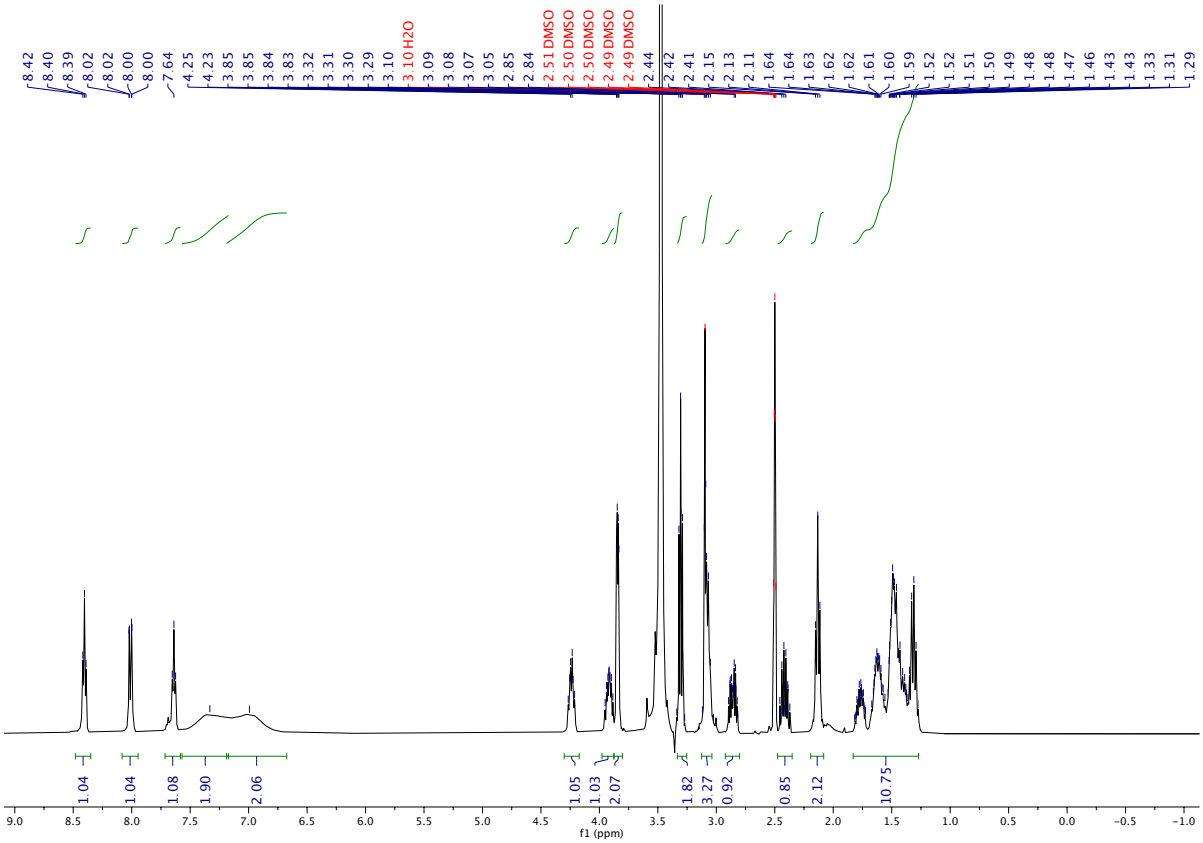

Figure S16. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of **65**.

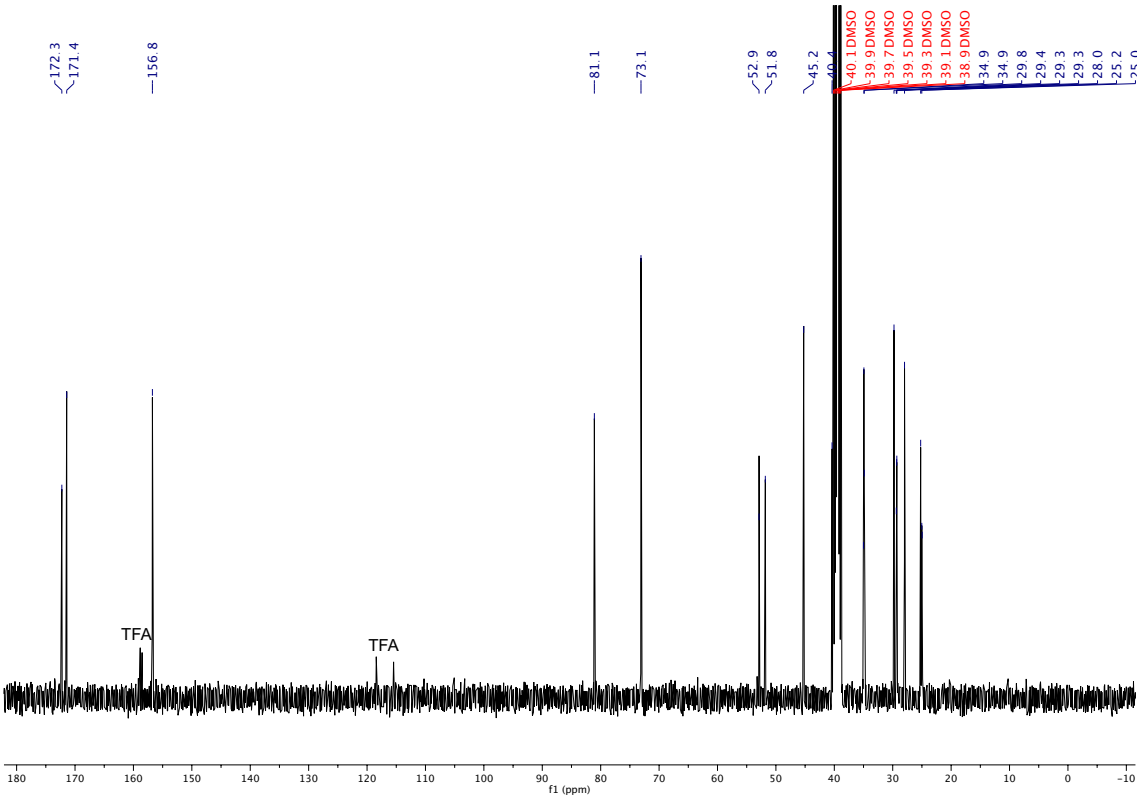

Figure S17. ^{13}C NMR (101 MHz, DMSO-d_6) spectrum of **65**.

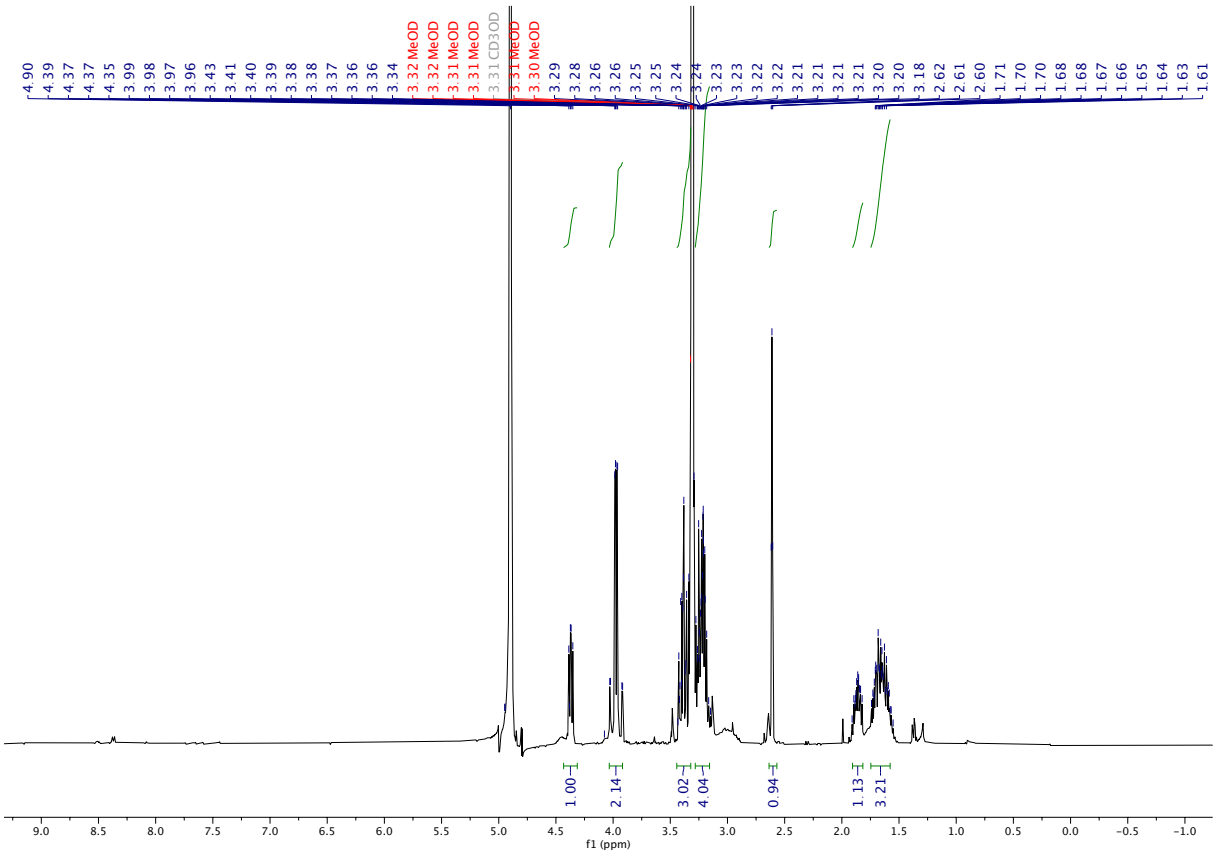

Figure S18. ^{1}H NMR (400 MHz, CD_3OD) spectrum of **67**.

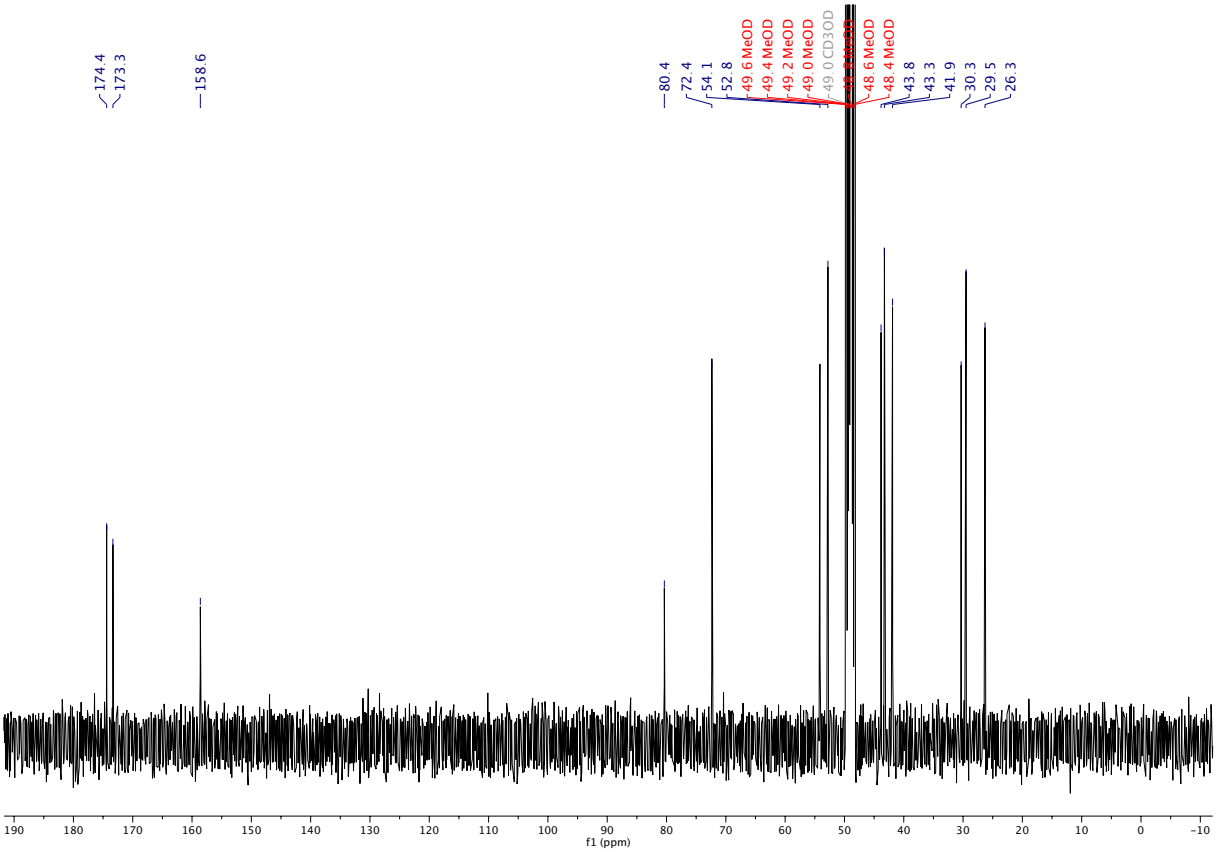

Figure S19. ^{13}C NMR (101 MHz, CD_3OD) spectrum of **67**.

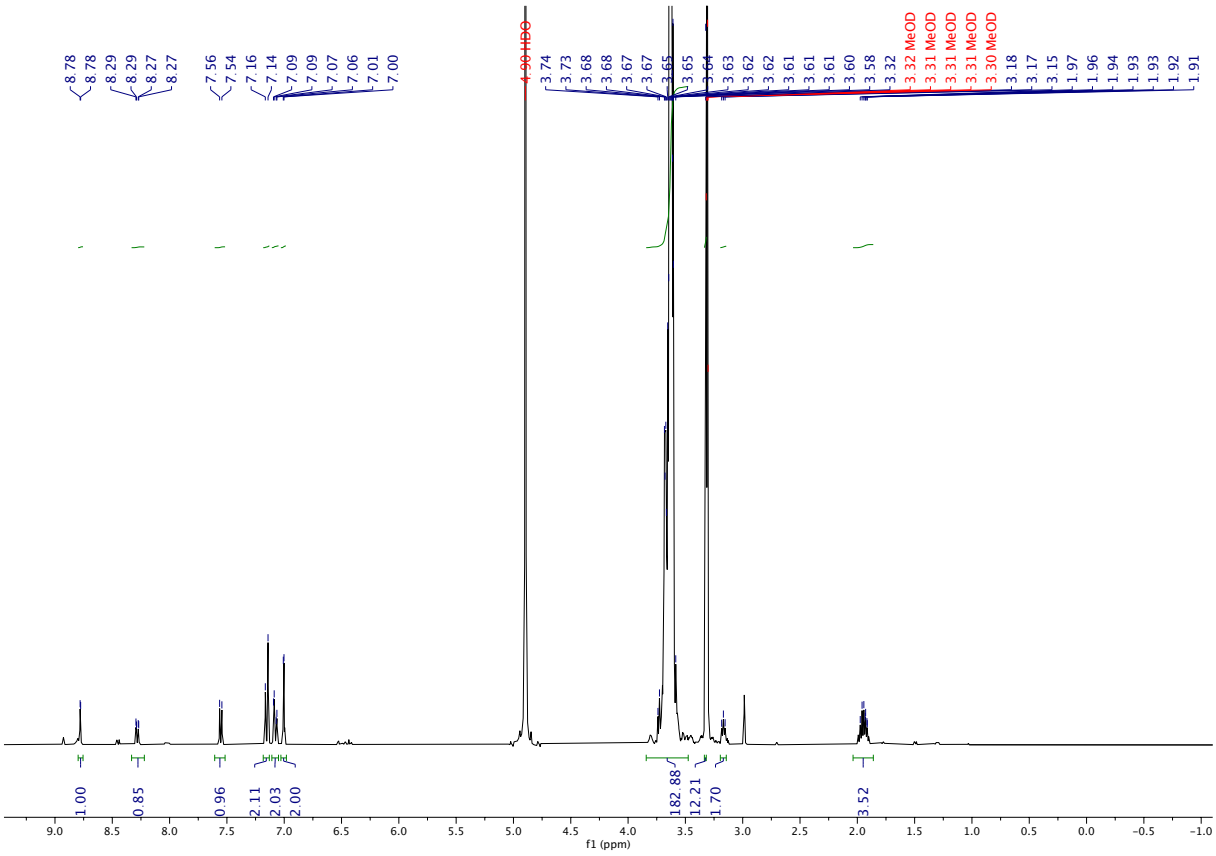

Figure S20. ^{1}H NMR (400 MHz, CD_3OD) spectrum of **70** with ≈10% TAMRA.

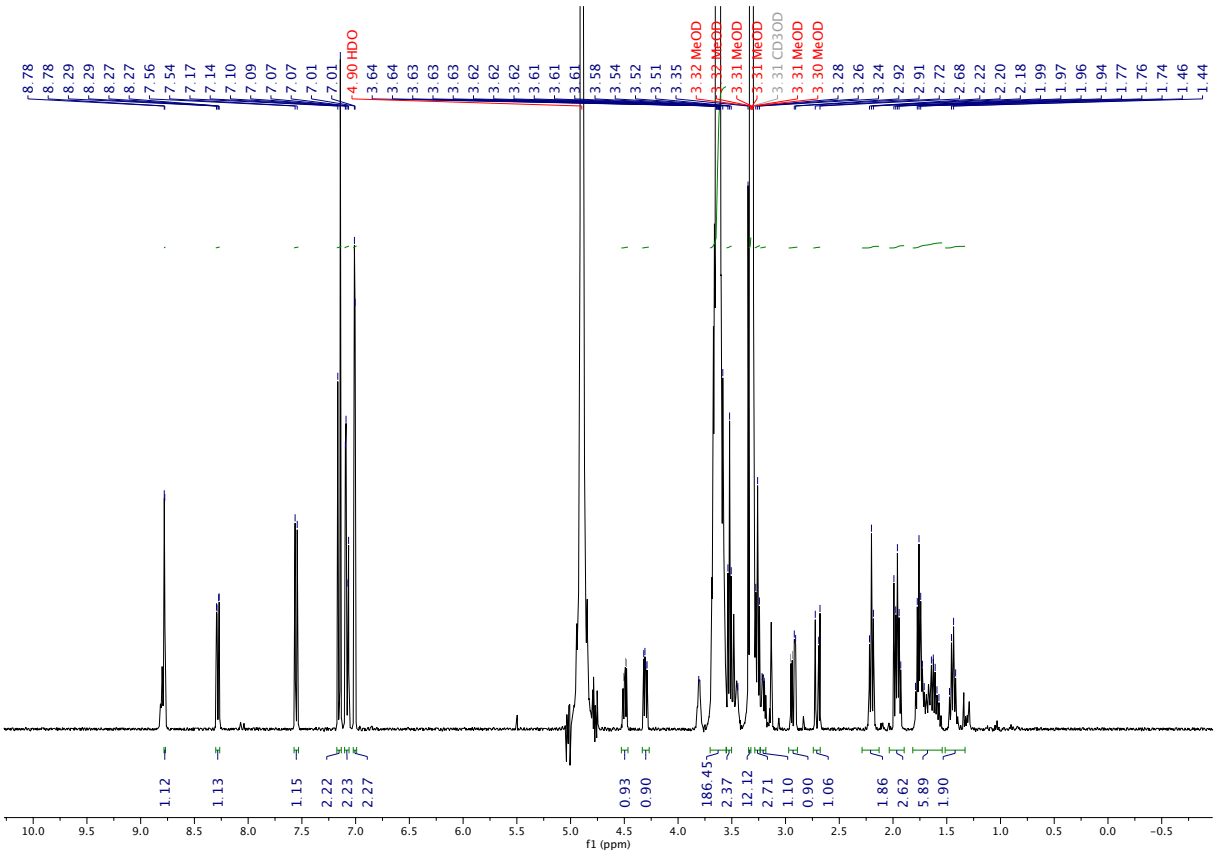

Figure S21. ^{1}H NMR (400 MHz, CD_3OD) spectrum of **34**.

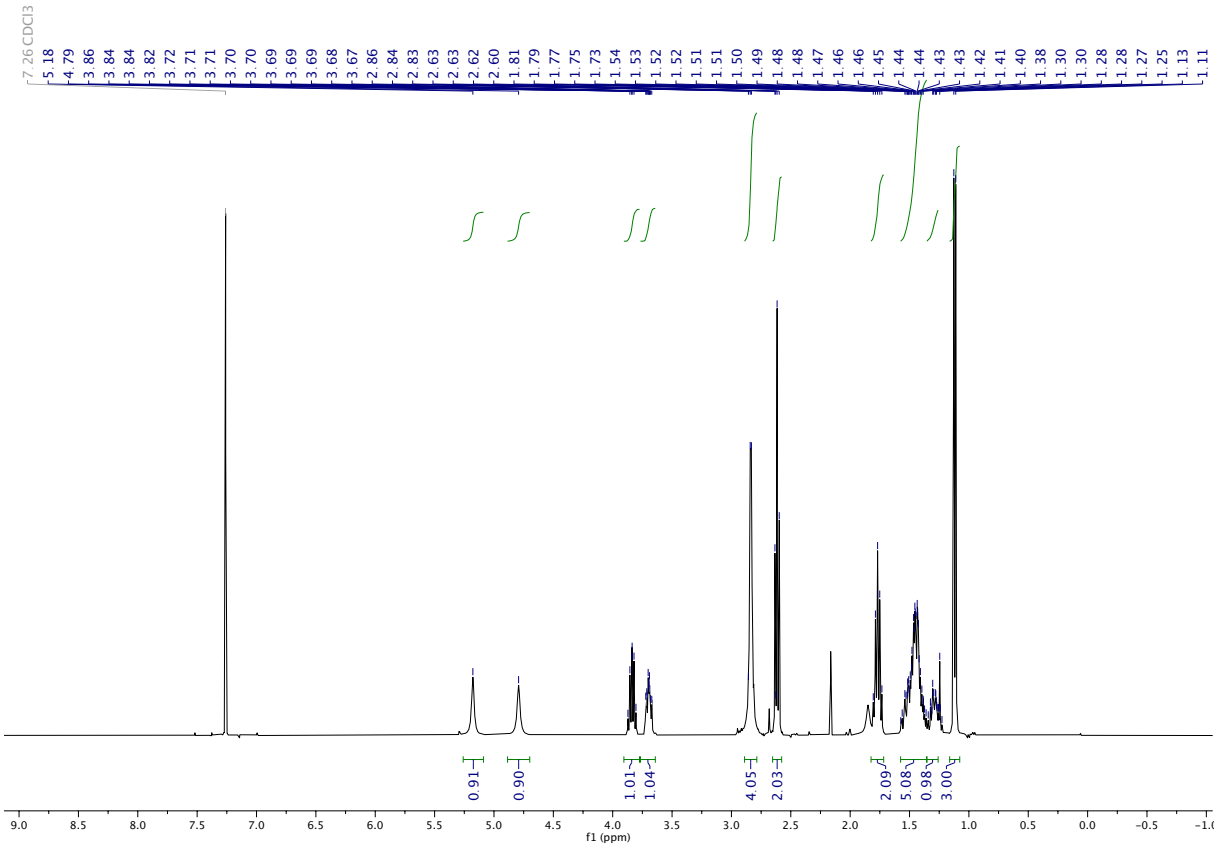

Figure S22. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of **73**.

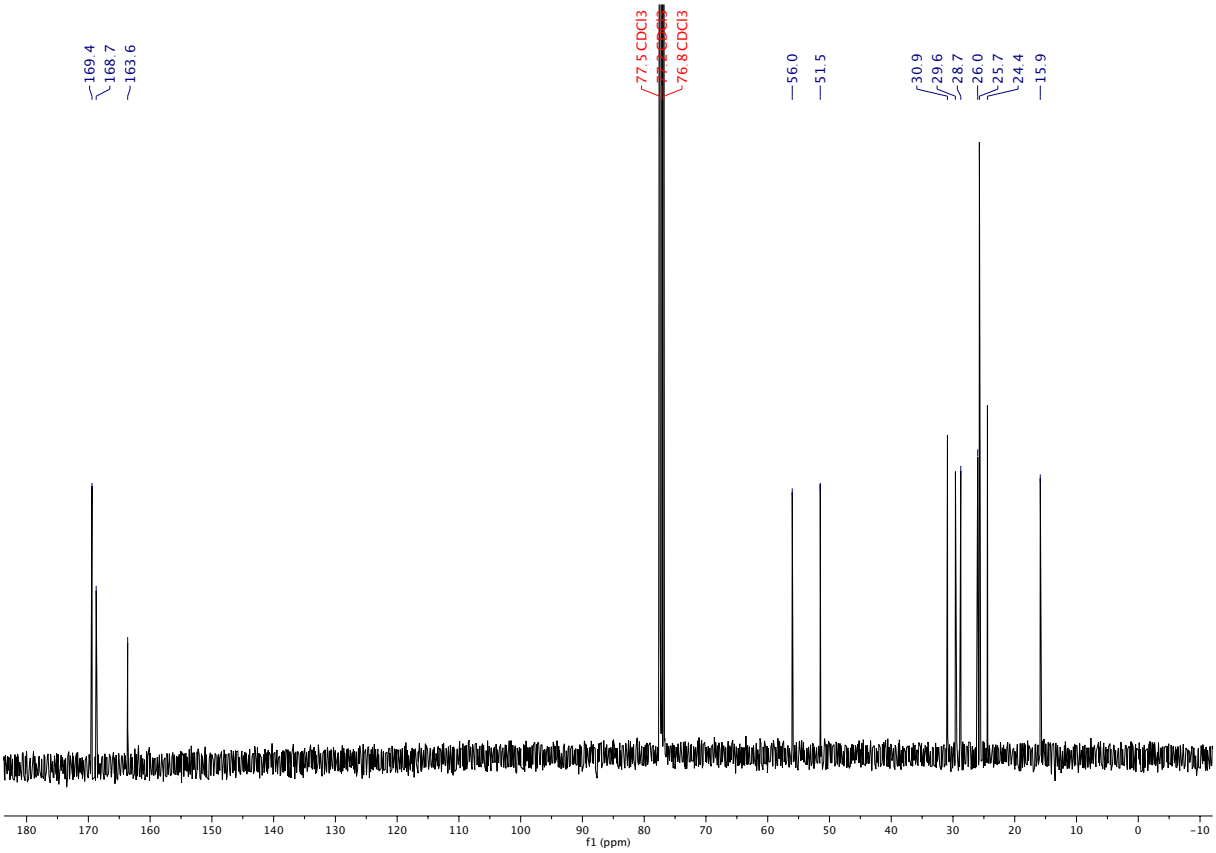

Figure S23. ^{13}C NMR (101 MHz, $CDCl_3$) spectrum of **73**.

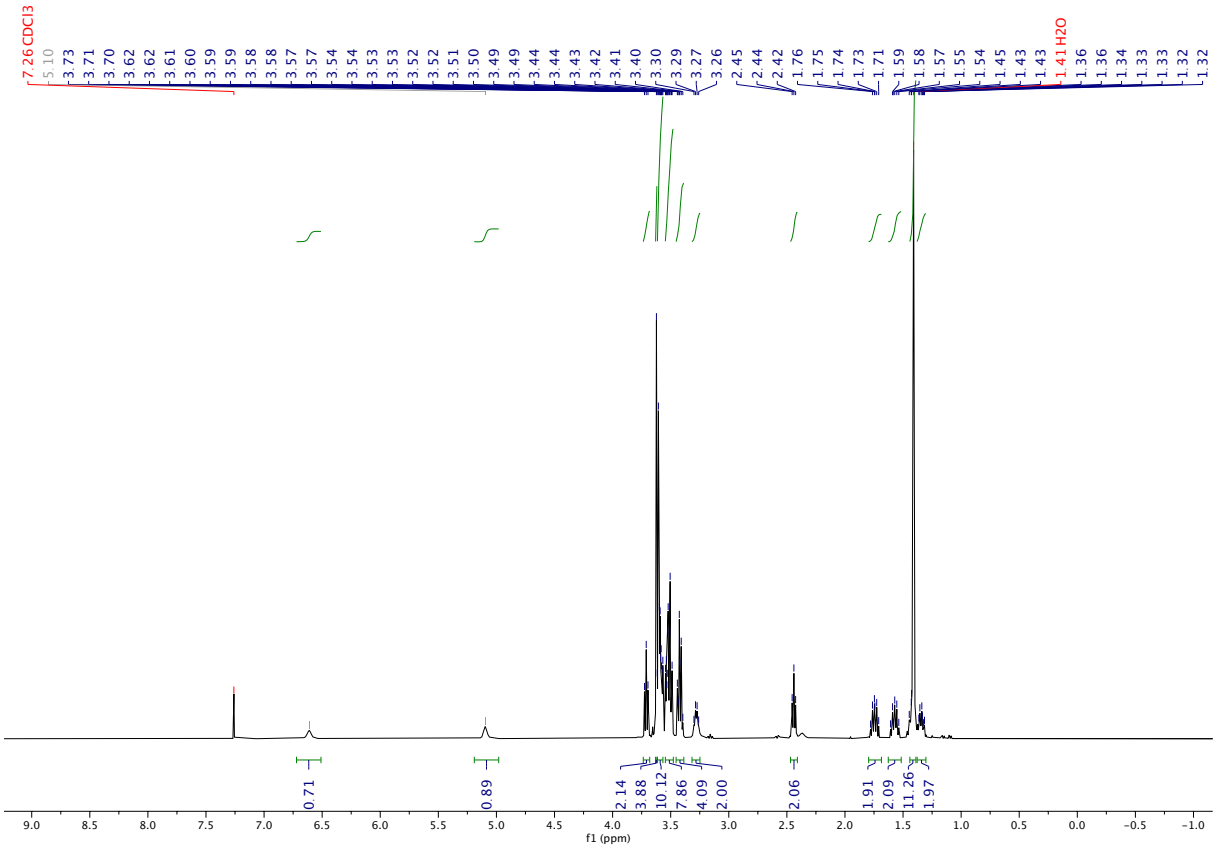

Figure S24. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of **76**.

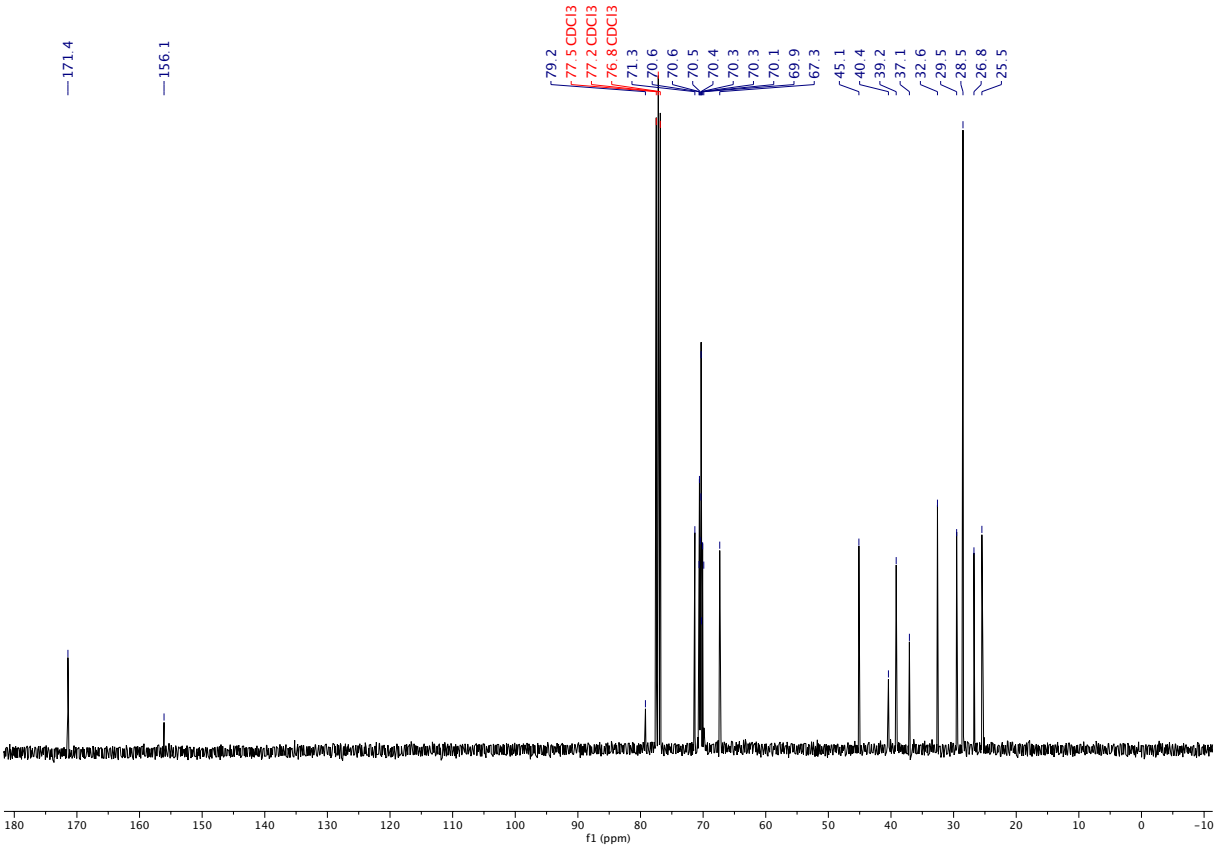

Figure S25. ^{13}C NMR (101 MHz, $CDCl_3$) spectrum of **76**.

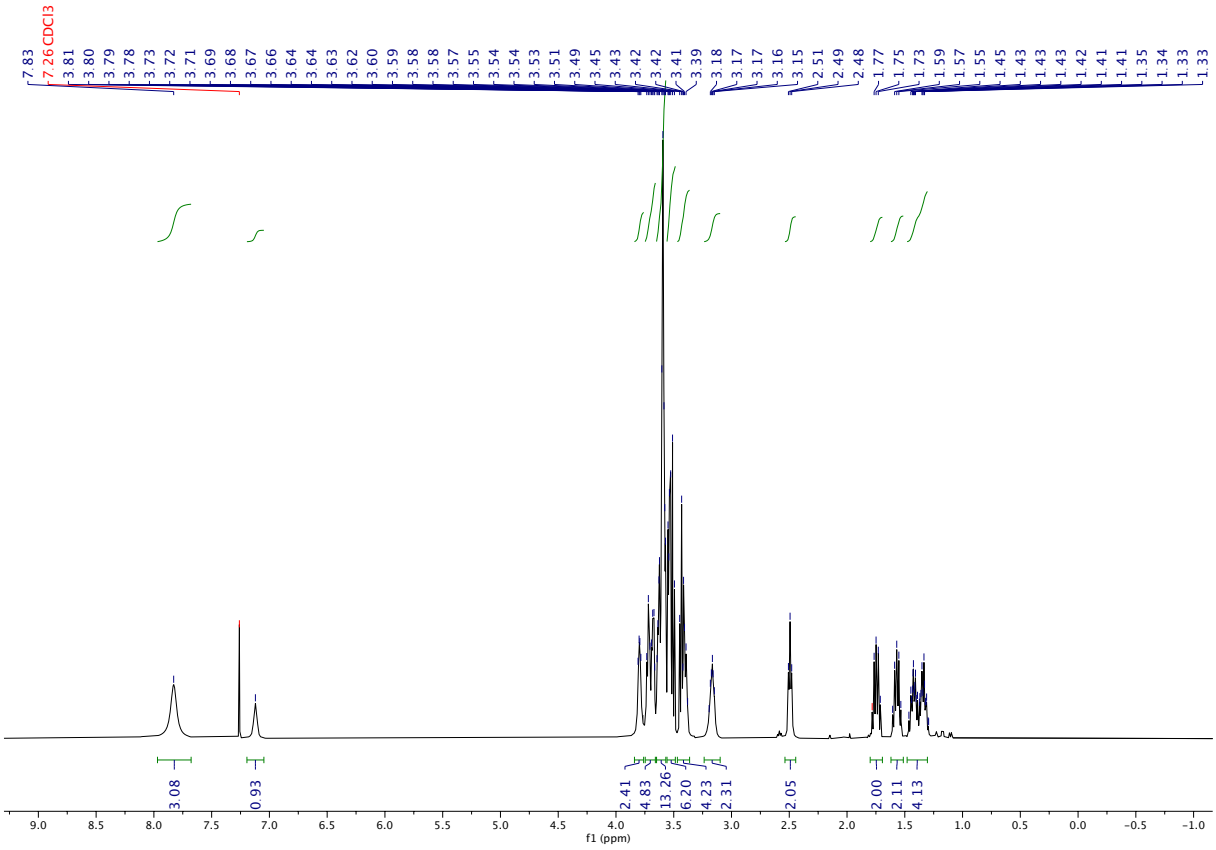

Figure S26. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of **77**.

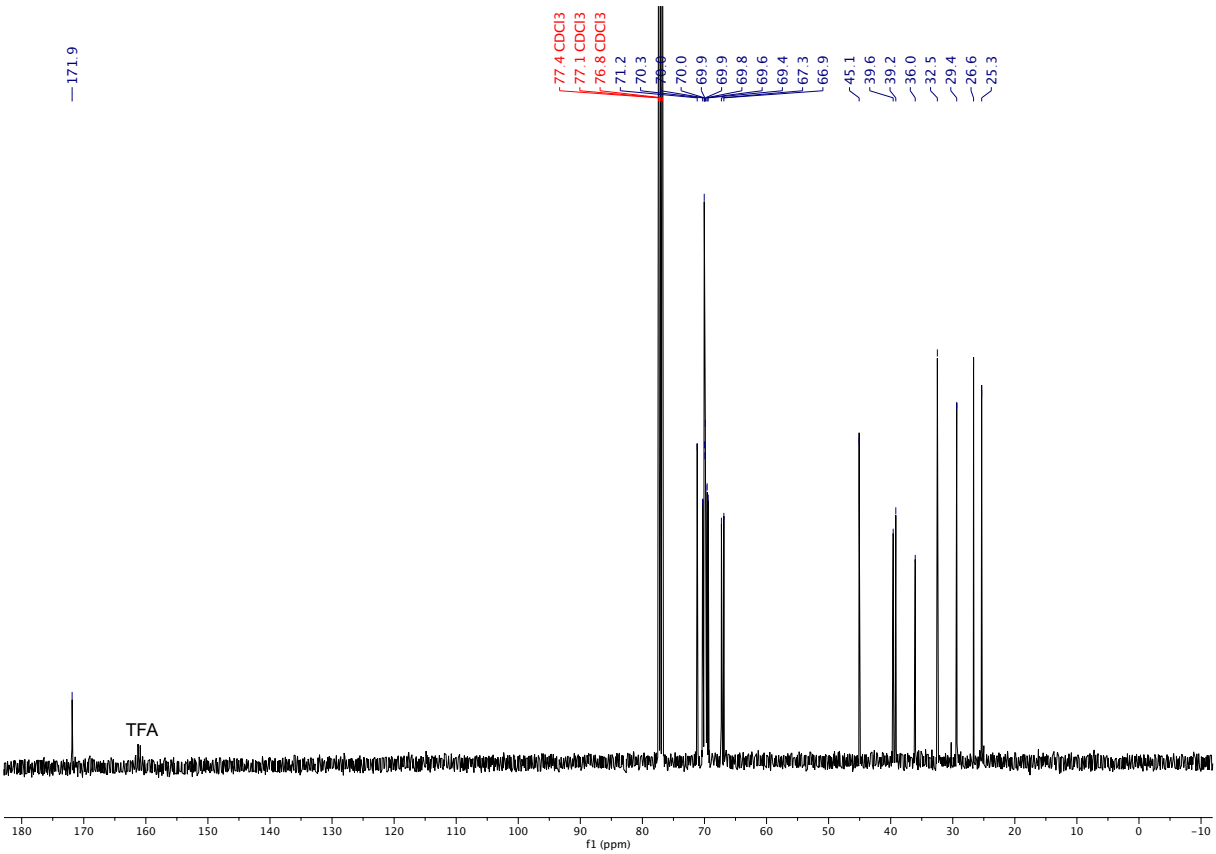

Figure S27. ^{13}C NMR (101 MHz, $CDCl_3$) spectrum of **77**.

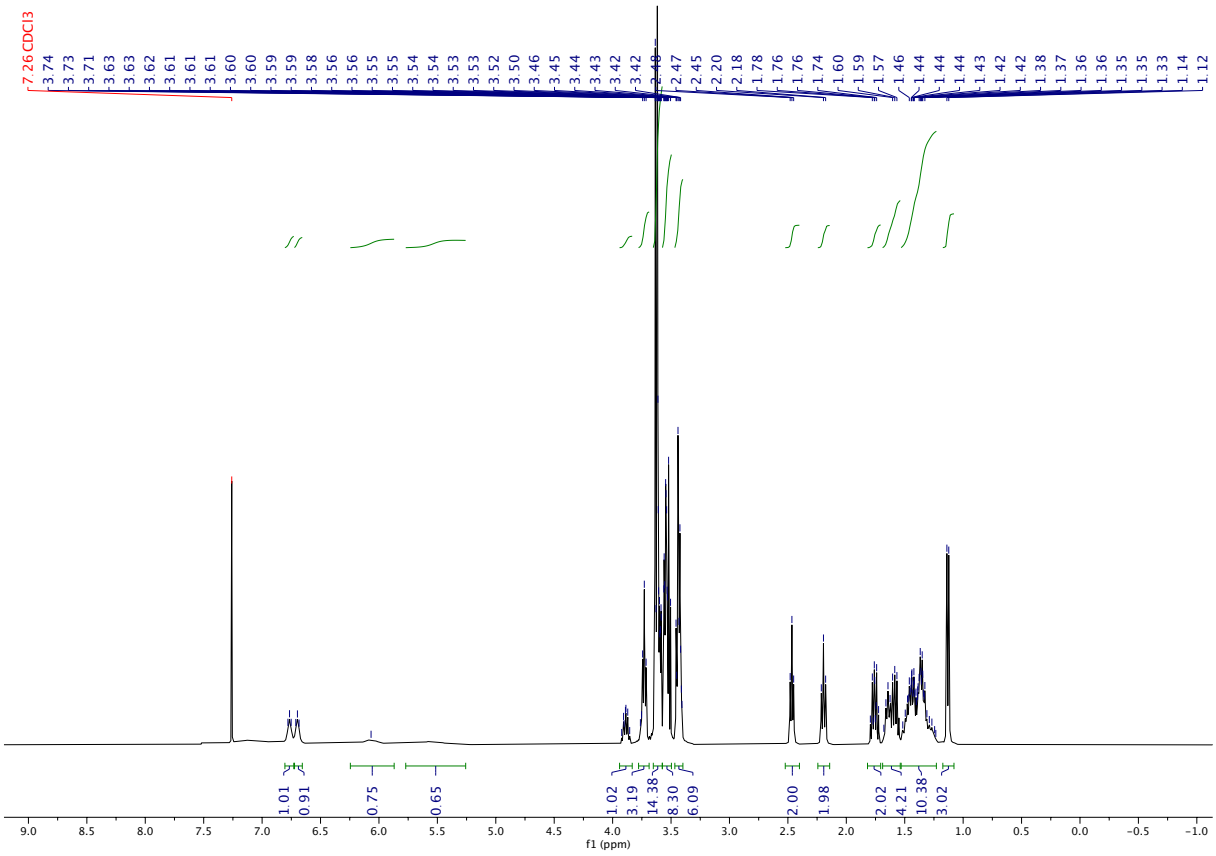

Figure S28. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of **59**.

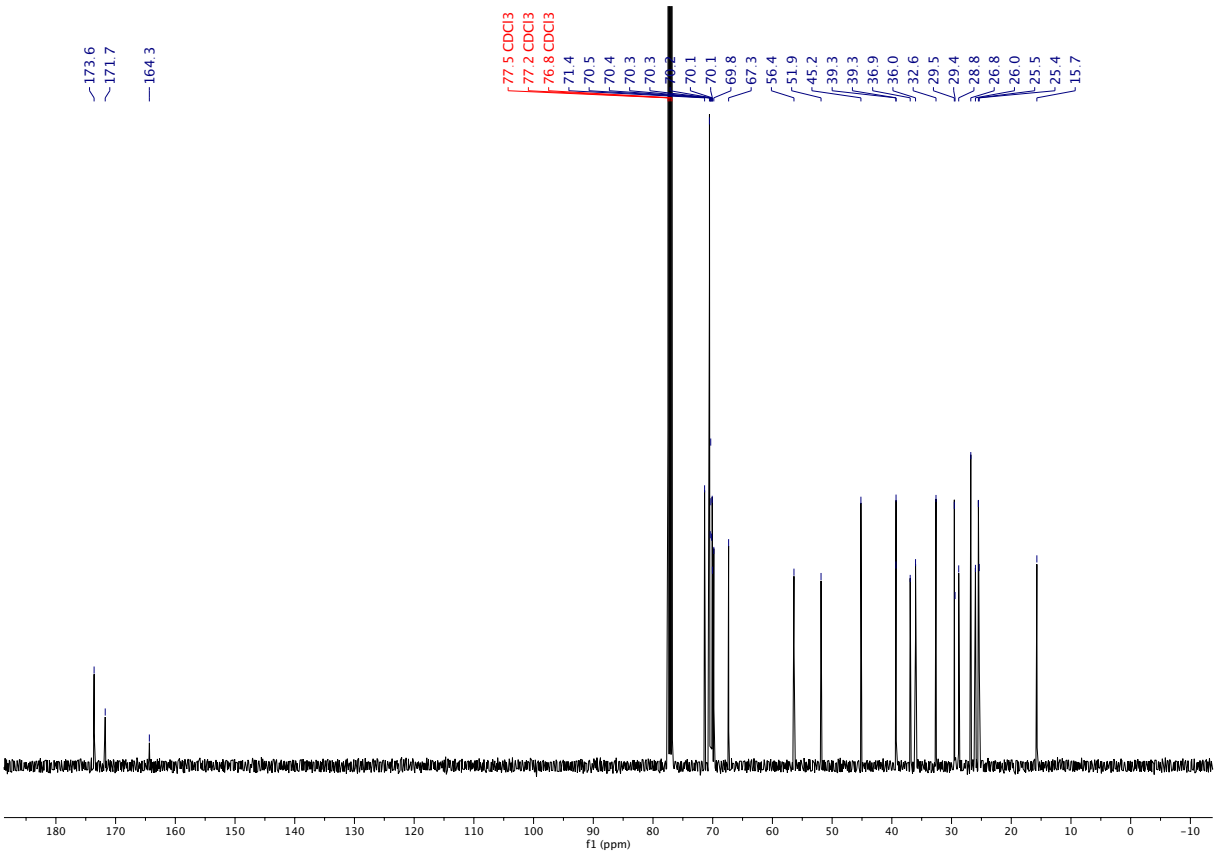

Figure S29. ^{13}C NMR (101 MHz, $CDCl_3$) spectrum of **59**.

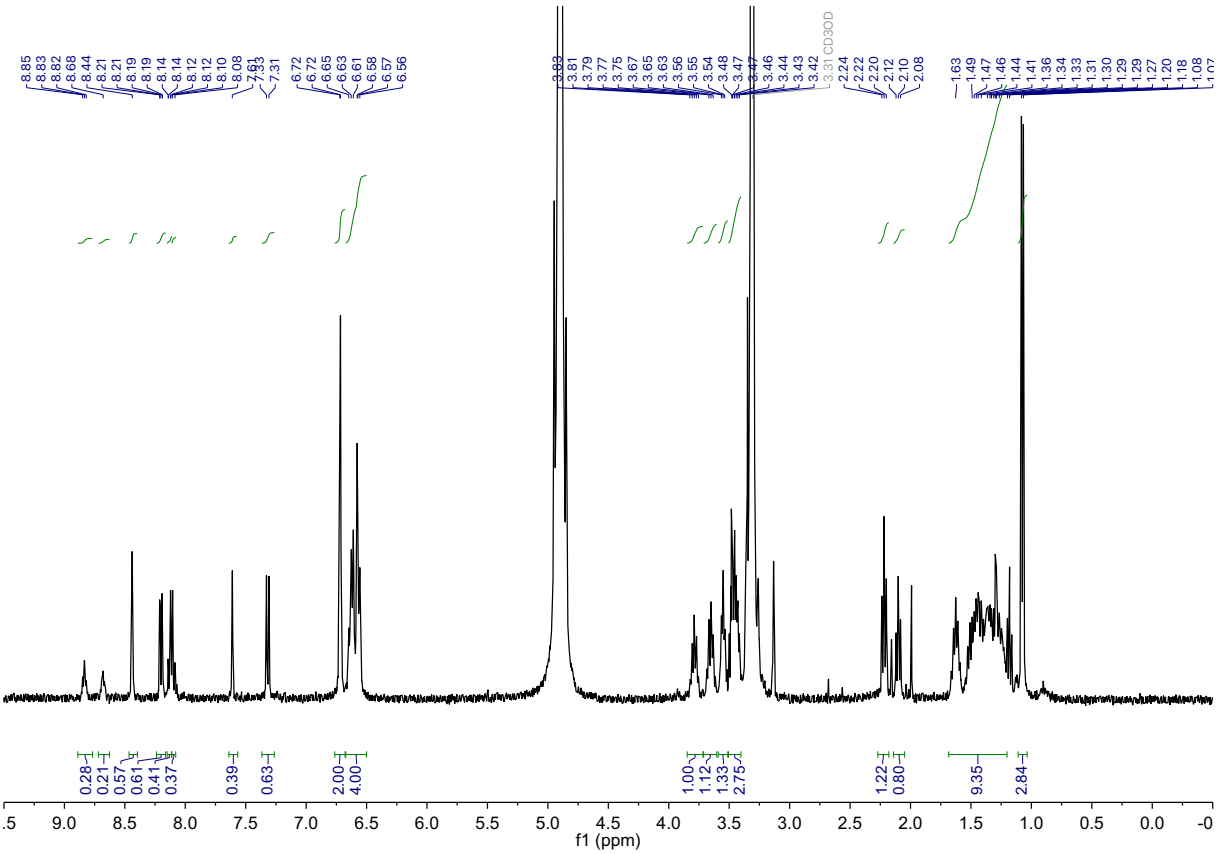

Figure S30. ^{1}H NMR (400 MHz, CD_3OD) spectrum of **47**.

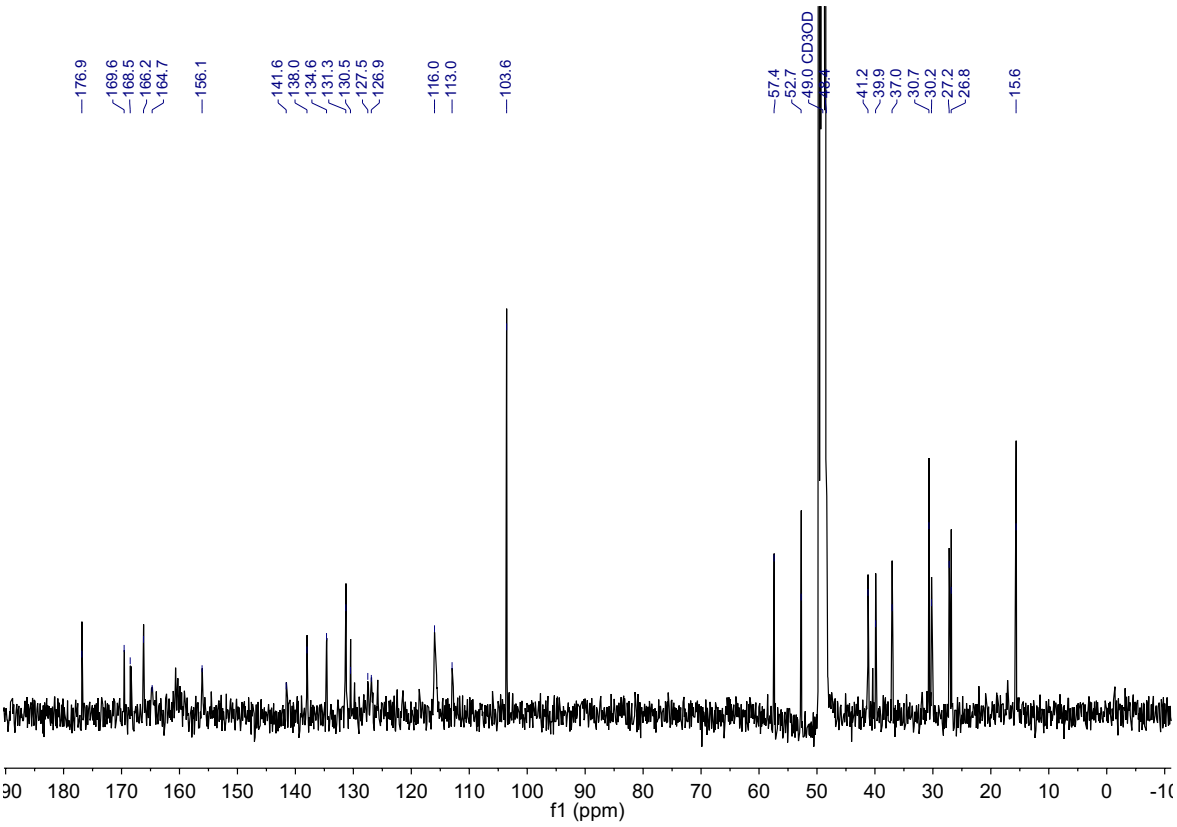

Figure S31. ^{13}C NMR (101 MHz, CD_3OD) spectrum of **47.**

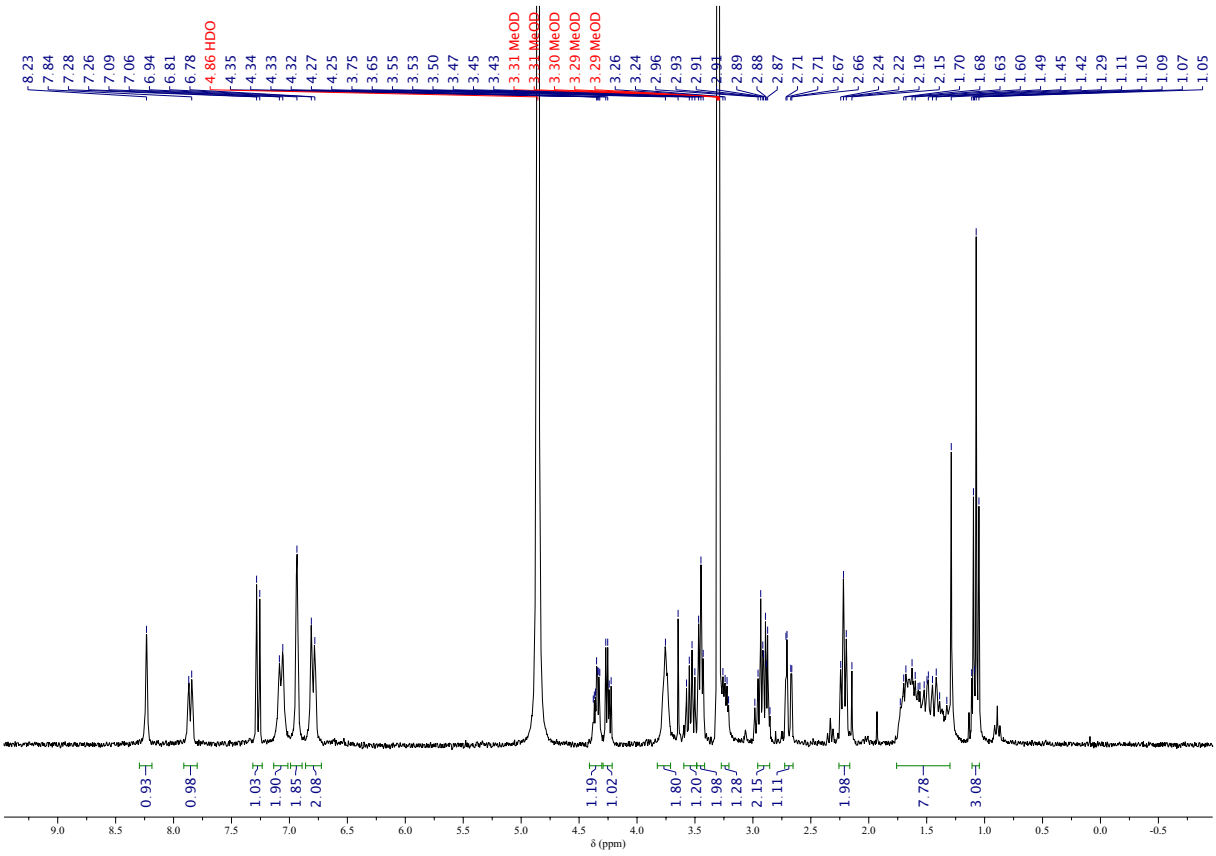

Figure S32. ^{1}H NMR (400 MHz, CD_3OD) spectrum of **48**.

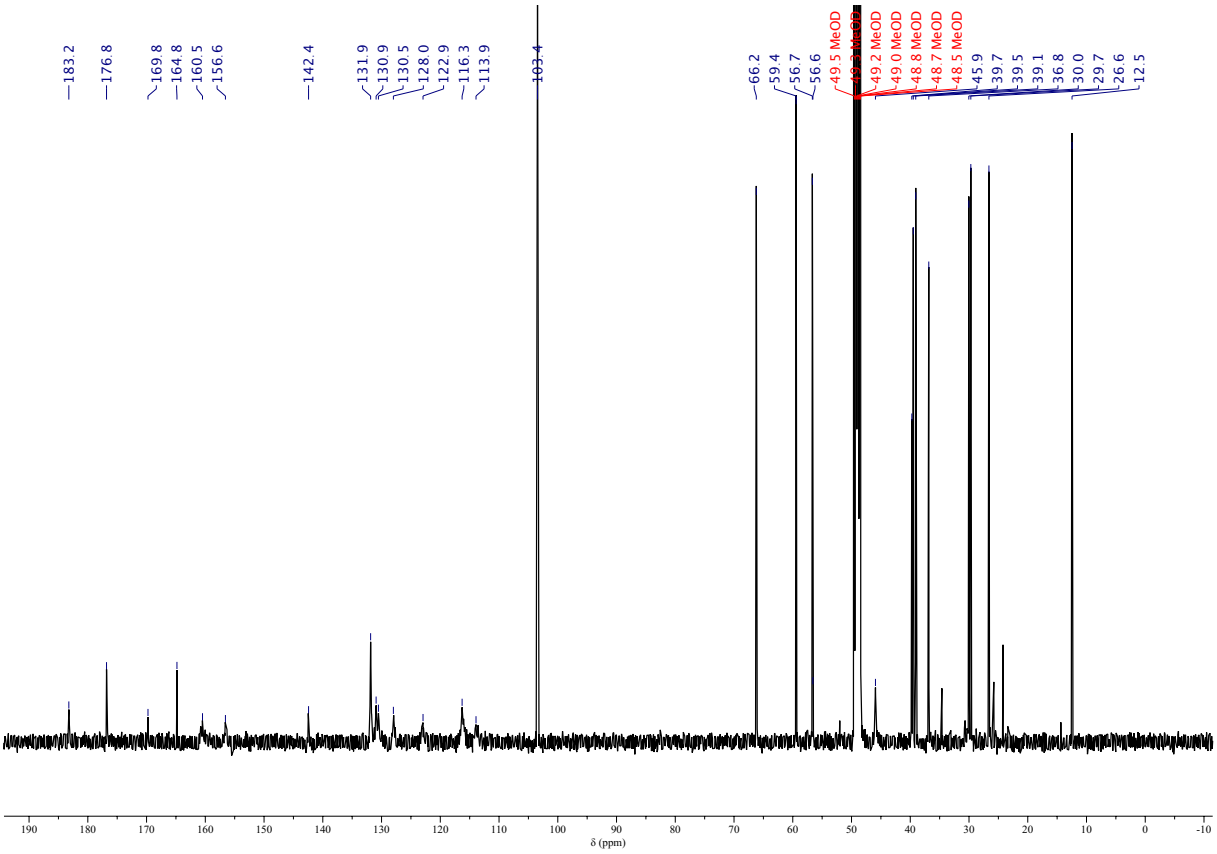

Figure S33. ^{13}C NMR (101 MHz, CD_3OD) spectrum of **48**.

11. Characterization of PNA Derivatives

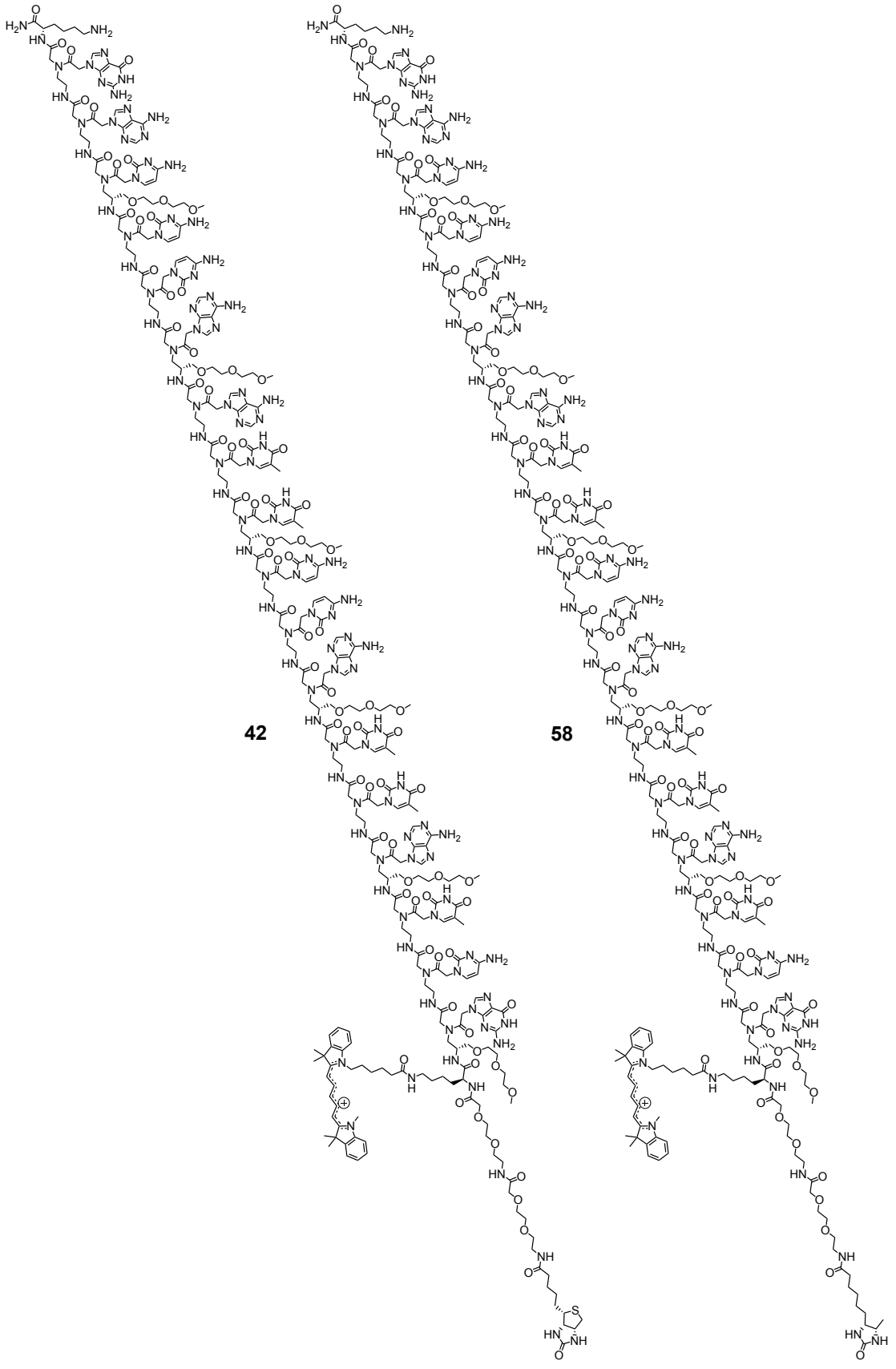

Figure S34. Chemical structures of PNA derivatives **42** and **58**.

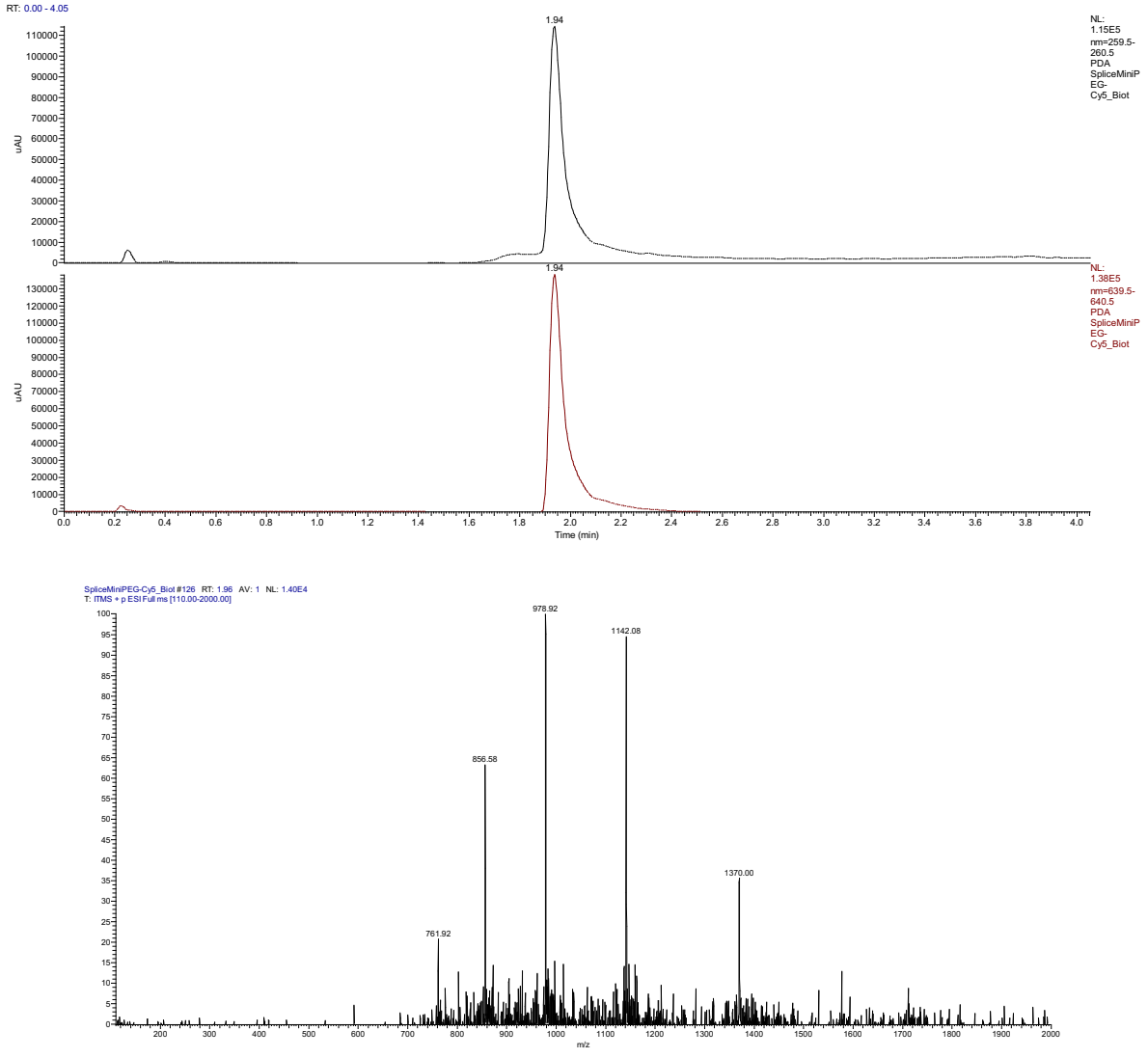

Figure S35. HPLC-MS trace of **42**. Chromatograms at λ = 260 nm (top) and λ = 640 nm (middle) and mass distribution at R_t = 1.96 min (bottom).

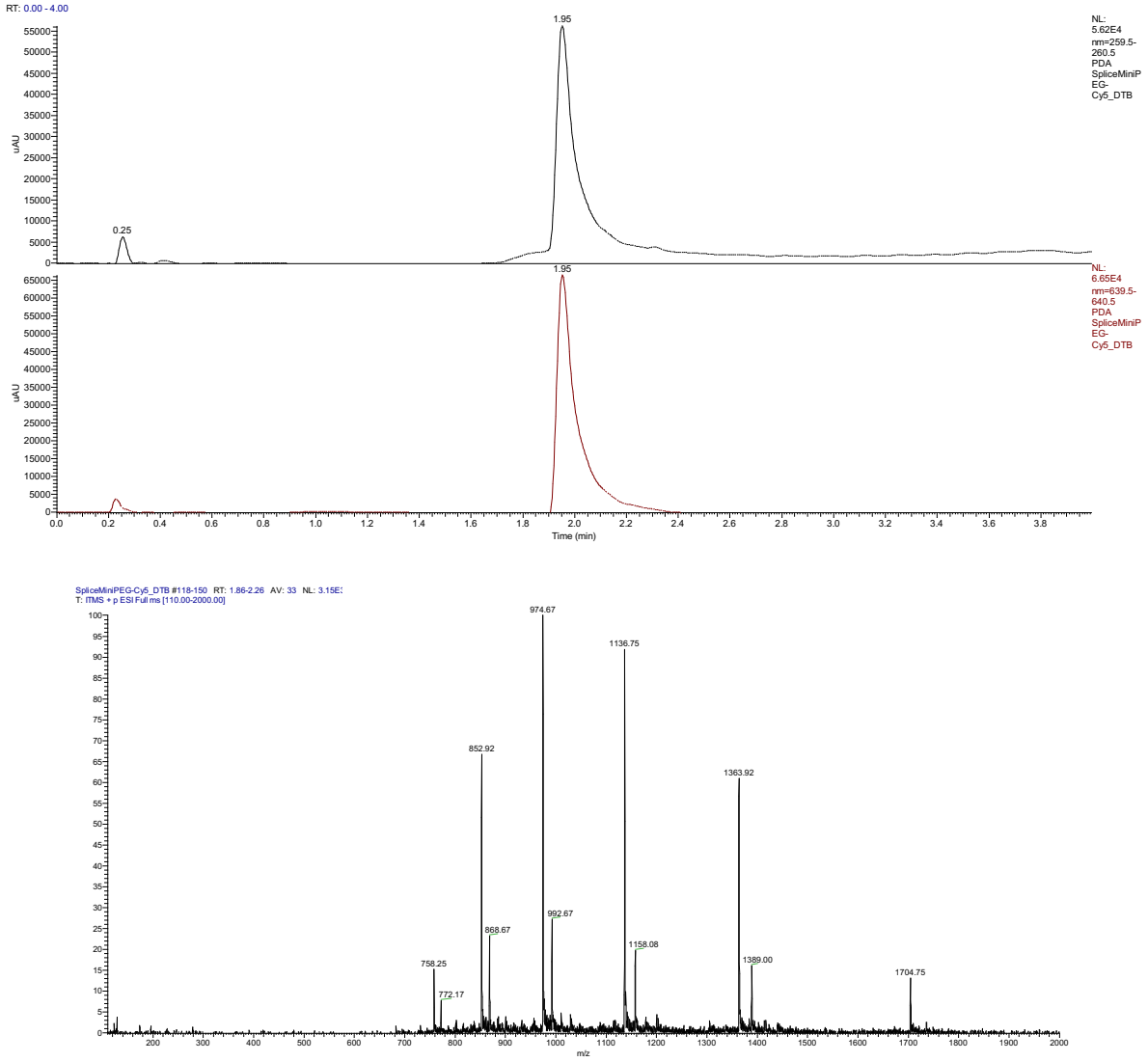

Figure S36. HPLC-MS trace of **58**. Chromatograms at λ = 260 nm (top) and λ = 640 nm (middle) and mass distribution at R_t = 1.86 ~ 2.26 min (bottom).

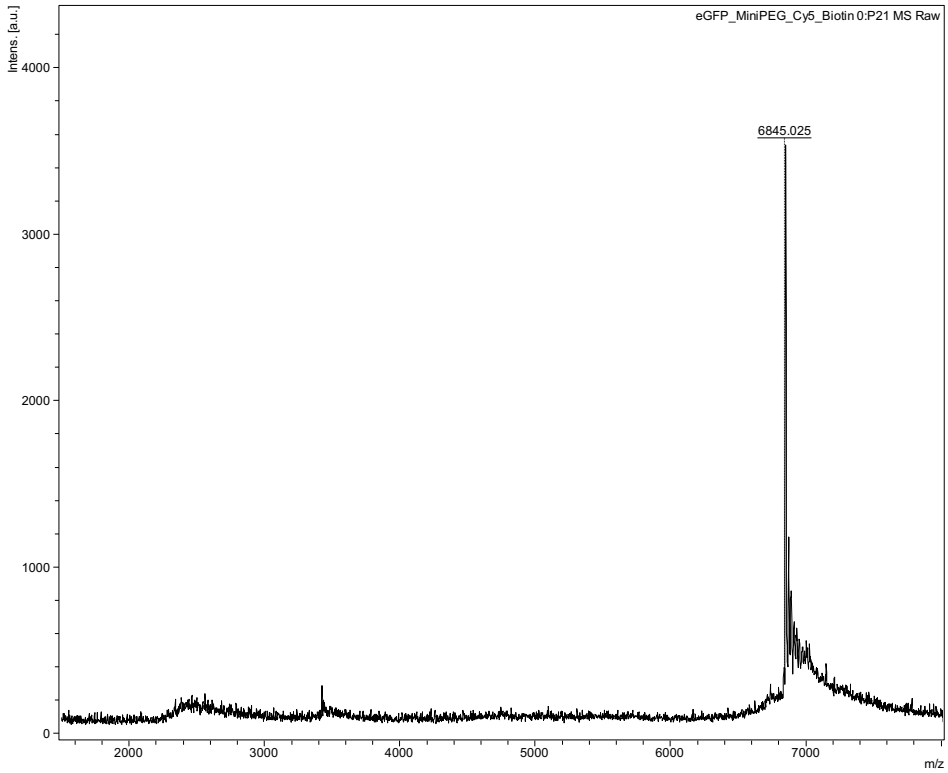

Figure S37. MALDI spectrum of **42**.

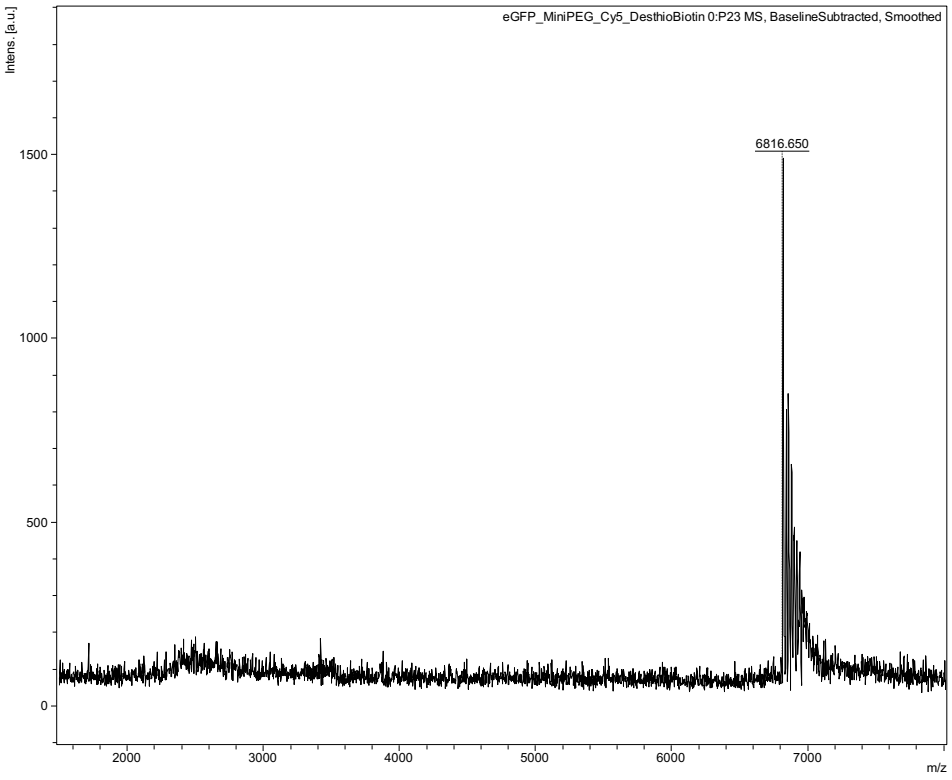

Figure S38. MALDI spectrum of **58**.

12. Summary of Structures

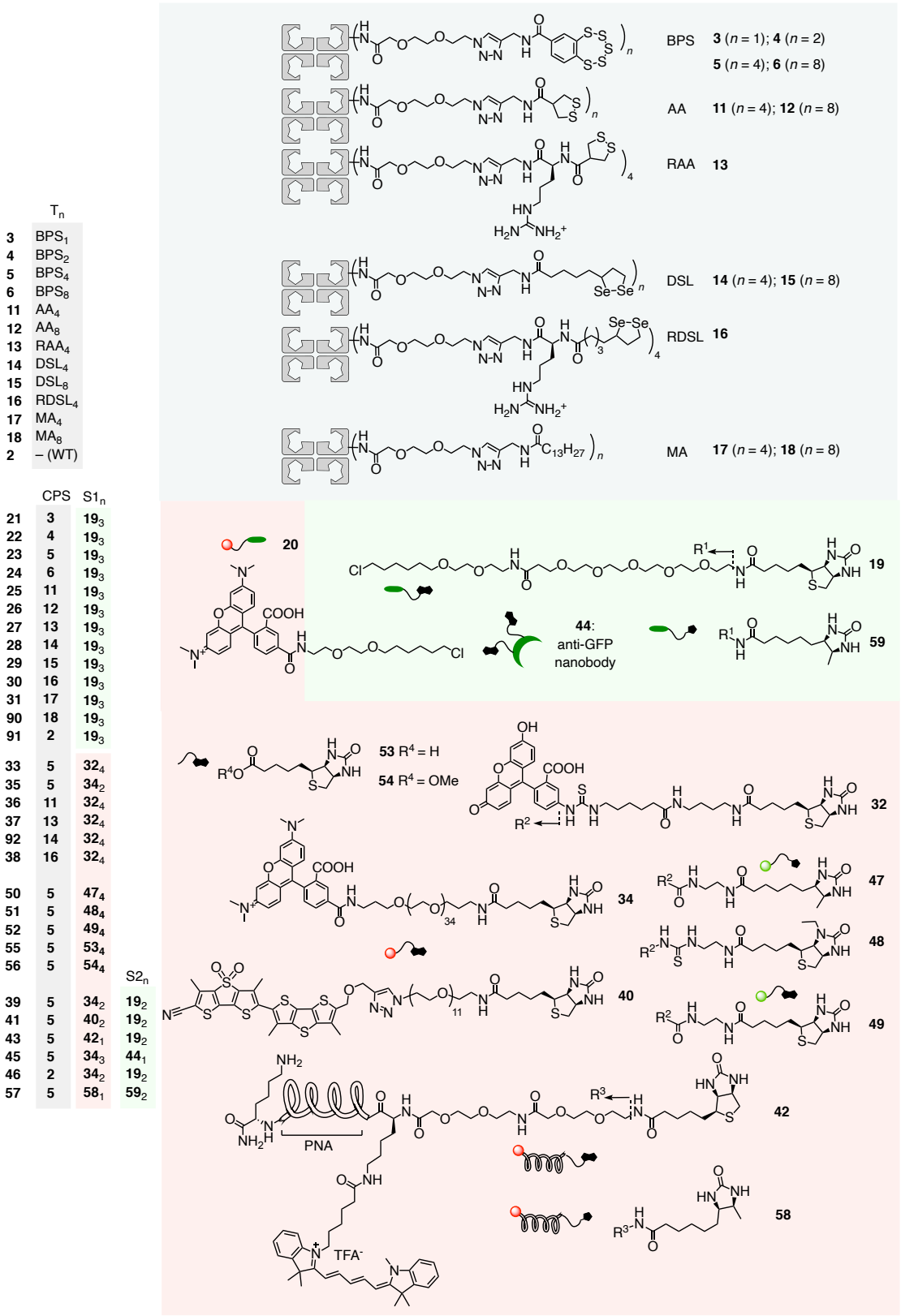

Figure S39. Summary of all substrates and CPS complexes used in this study. See Figure S34 for the complete structure of PNA.