Supporting Information

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1. Supplementary Figures

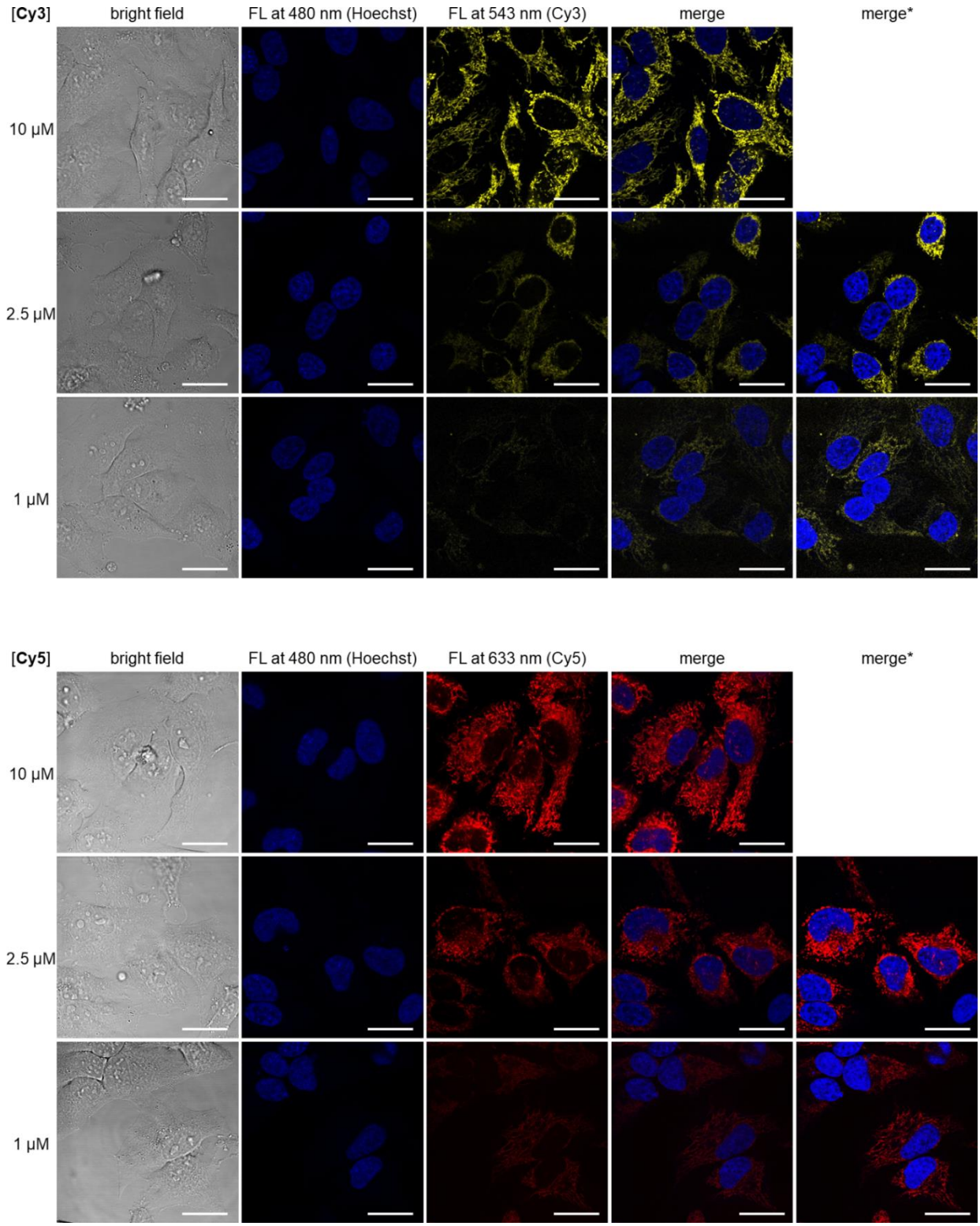


Figure S1. Concentration-dependent cellular uptake of **Cy3** and **Cy5**. HeLa cells were treated with **Cy3** or **Cy5** at the indicated concentration in serum free media at 37 °C for 1 h before staining the nucleus with Hoechst 33258 and imaging. Excitation wavelength for Hoechst 33258 (nucleus stain), **Cy3**, and **Cy5** is 405, 543, and 633 nm, respectively. Scale bars denote 25 µm. Images in merge* are adjusted with +40% brightness and +40% contrast to better visualize the fluorescence pattern. Representative (N = 10) confocal microscopy images are shown here.

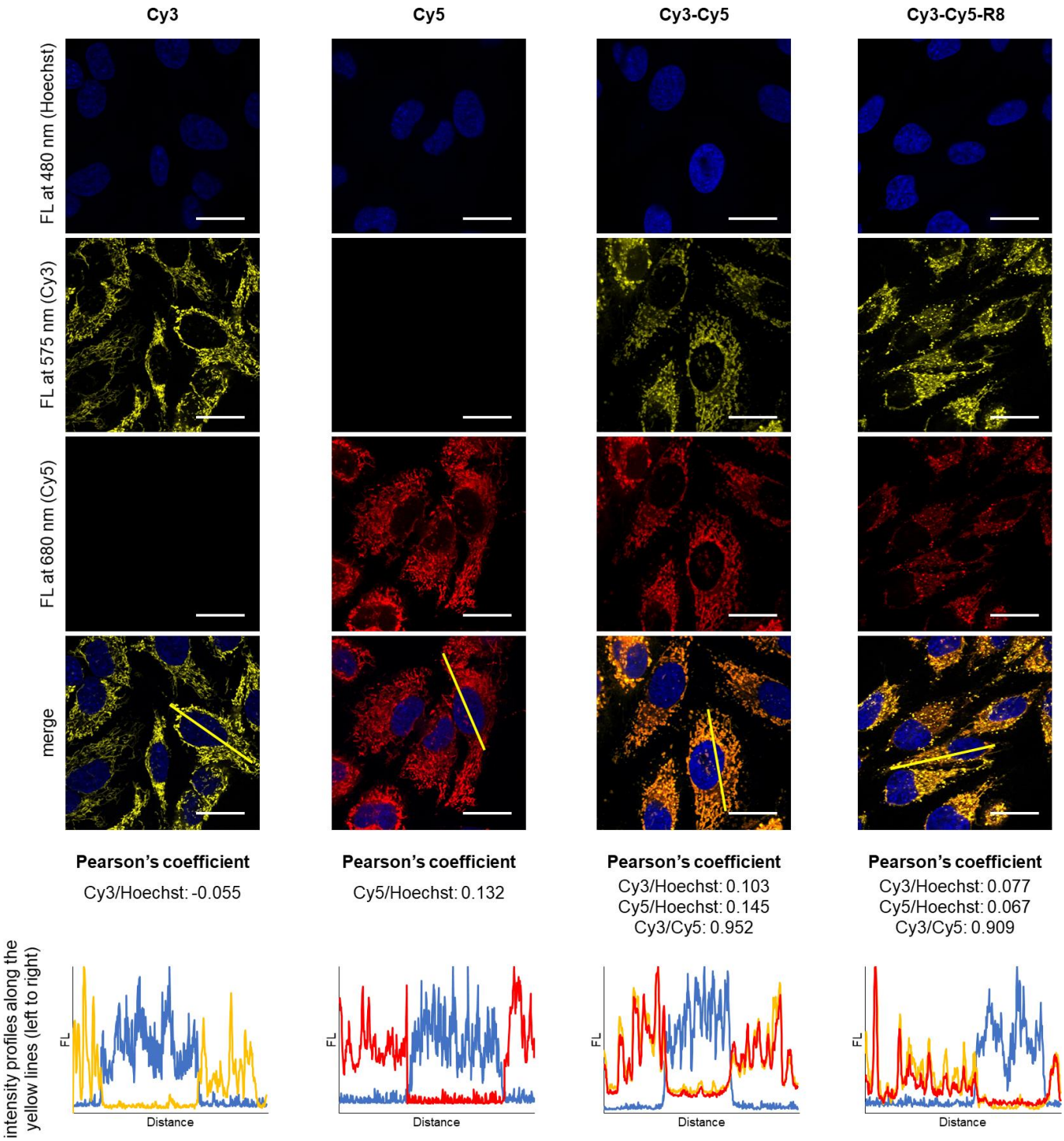


Figure S2. Cyanine dyes do not localize to the nucleus. HeLa cells were treated with 10 µM **Cy3**, **Cy5**, **Cy3-Cy5** or **Cy3-Cy5-R8** in serum free media at 37 °C for 1 h before staining the nucleus with Hoechst 33258 and imaging. Excitation wavelength for Hoechst 33258 (nucleus stain), **Cy3**, and **Cy5** is 405, 543, and 633 nm, respectively. Scale bars denote 25 µm. Representative (N = 10) confocal microscopy images are shown here.

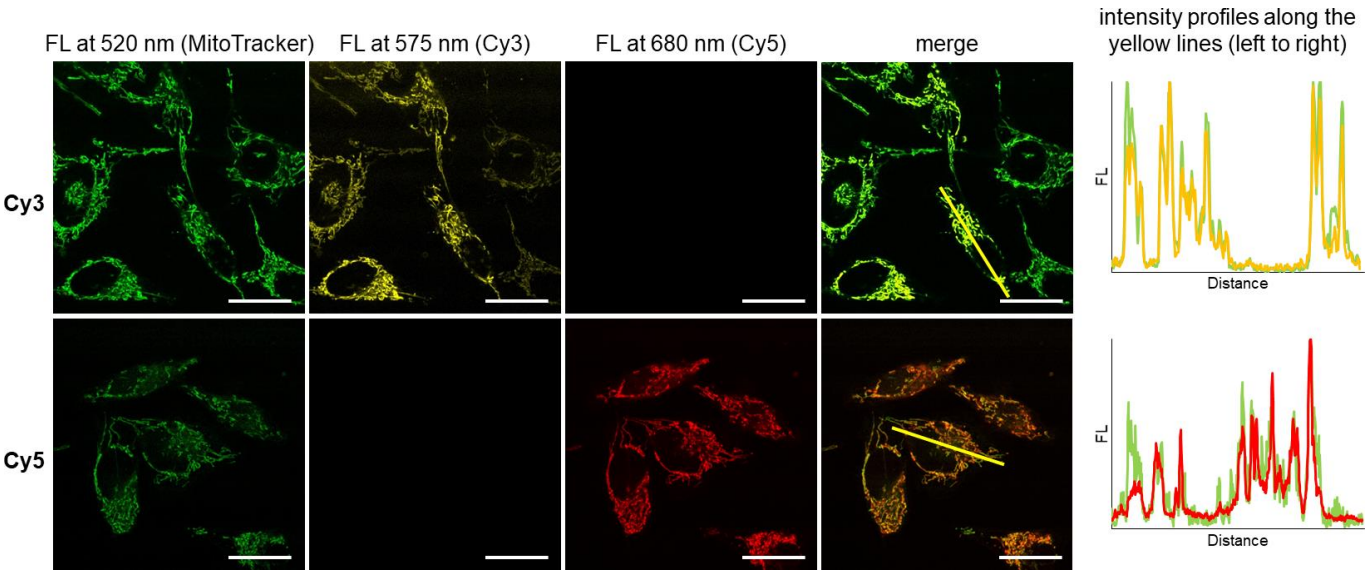
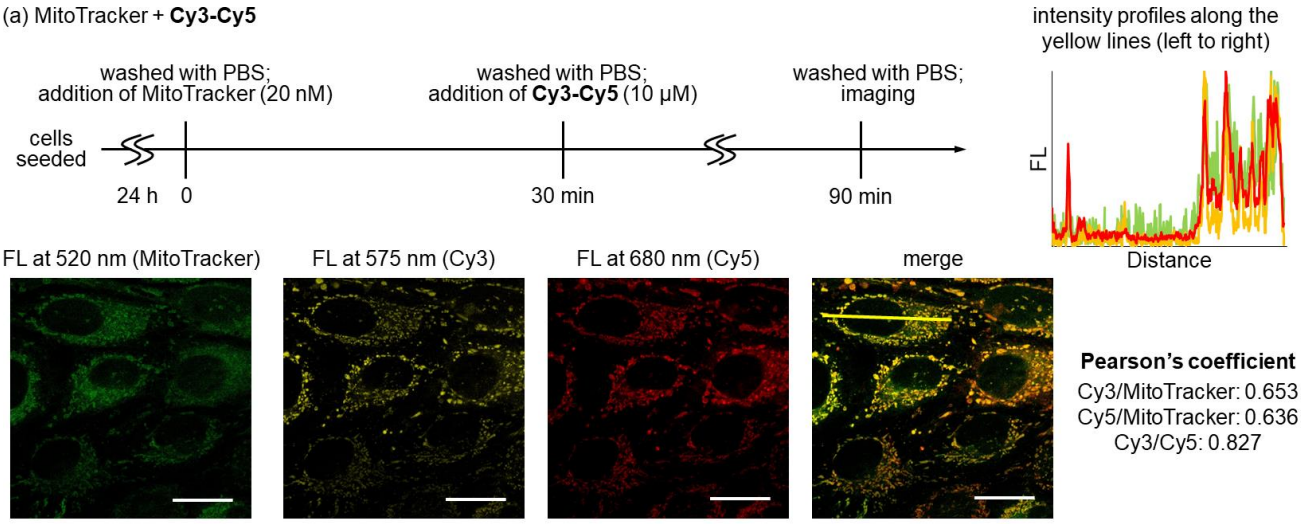
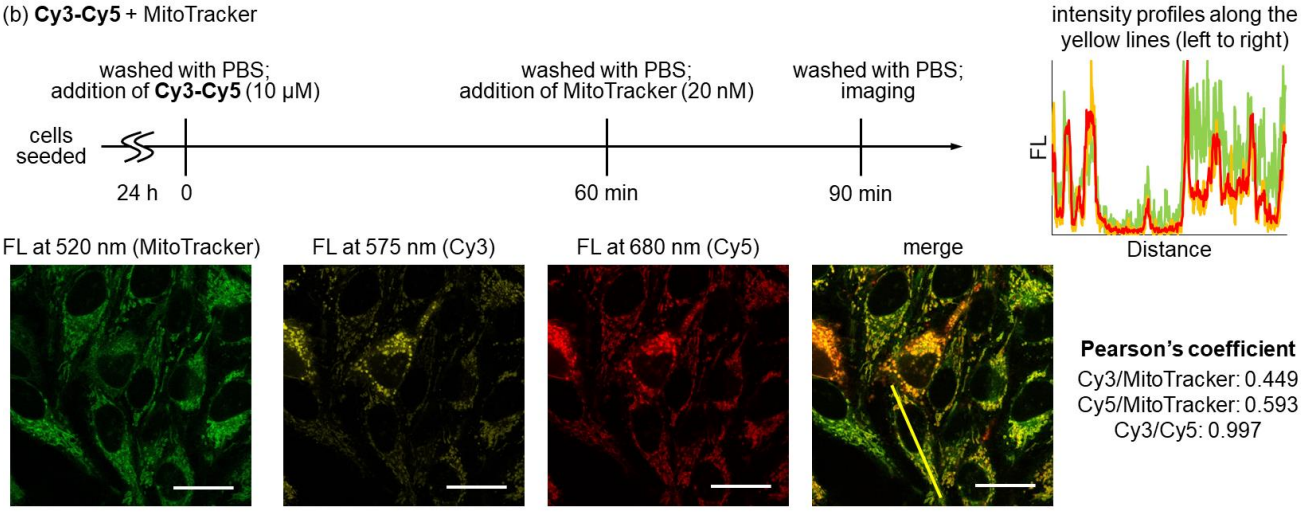
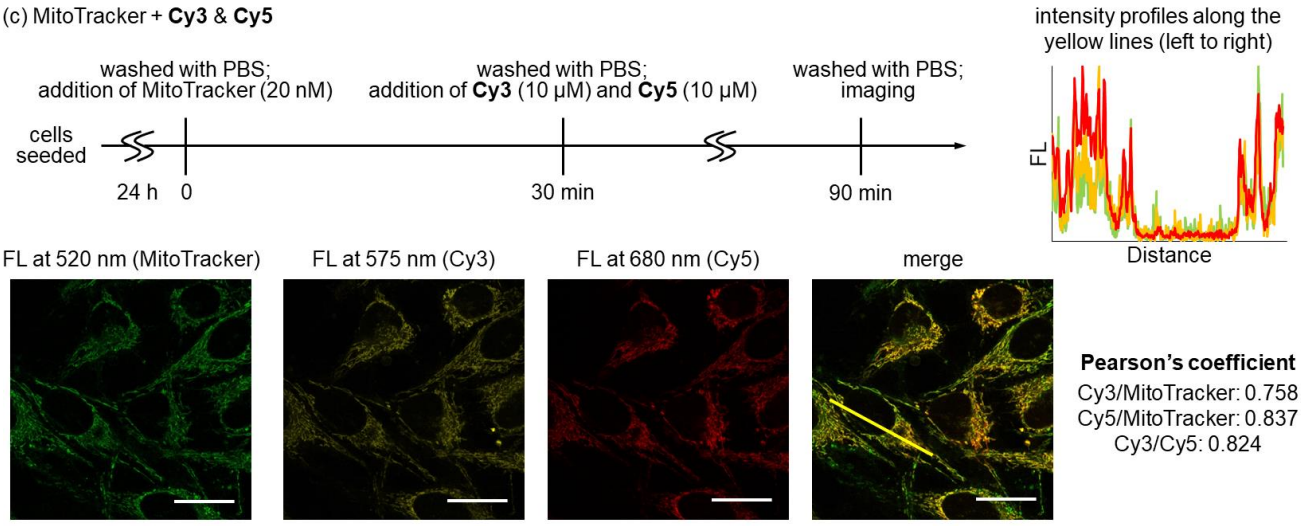


Figure S3. Mitochondrial localization of **Cy3** and **Cy5**. HeLa cells were treated with 10 µM **Cy3** or **Cy5** at 37 °C for 1 h before staining the mitochondria with 20 nM MitoTracker® Green and imaging. Pearson's correlation coefficients for **Cy3** or **Cy5** with MitoTracker® are 0.863 and 0.877, respectively. Excitation wavelength for MitoTracker® Green, Cy3, and Cy5 is 488, 543, and 633 nm, respectively. Scale bars denote 25 µm. Representative (N = 10) confocal microscopy images are shown here.


(a) MitoTracker + Cy3-Cy5
washed with PBS; addition of MitoTracker (20 nM)
washed with PBS; addition of Cy3-Cy5 (10 μM)
washed with PBS; imaging
cells seeded
24 h
0
30 min
90 min
intensity profiles along the yellow lines (left to right)
FL
Distance
FL at 520 nm (MitoTracker)
FL at 575 nm (Cy3)
FL at 680 nm (Cy5)
merge
Pearson's coefficient
Cy3/MitoTracker: 0.653
Cy5/MitoTracker: 0.636
Cy3/Cy5: 0.827


(b) Cy3-Cy5 + MitoTracker
washed with PBS; addition of Cy3-Cy5 (10 μM)
washed with PBS; addition of MitoTracker (20 nM)
washed with PBS; imaging
cells seeded
24 h
0
60 min
90 min
intensity profiles along the yellow lines (left to right)
FL
Distance
FL at 520 nm (MitoTracker)
FL at 575 nm (Cy3)
FL at 680 nm (Cy5)
merge
Pearson's coefficient
Cy3/MitoTracker: 0.449
Cy5/MitoTracker: 0.593
Cy3/Cy5: 0.997


(c) MitoTracker + Cy3 & Cy5
washed with PBS; addition of MitoTracker (20 nM)
washed with PBS; addition of Cy3 (10 μM) and Cy5 (10 μM)
washed with PBS; imaging
cells seeded
24 h
0
30 min
90 min
intensity profiles along the yellow lines (left to right)
FL
Distance
FL at 520 nm (MitoTracker)
FL at 575 nm (Cy3)
FL at 680 nm (Cy5)
merge
Pearson's coefficient
Cy3/MitoTracker: 0.758
Cy5/MitoTracker: 0.837
Cy3/Cy5: 0.824

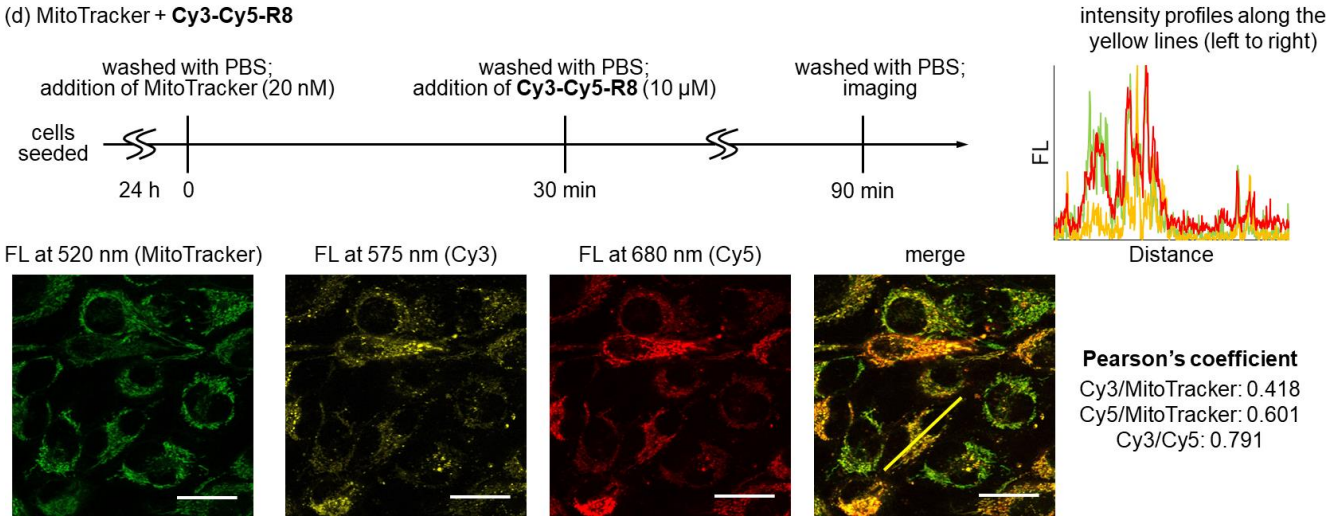


Figure S4. Colocalization of **Cy3-Cy5** or **Cy3-Cy5-R8** with MitoTracker® Green in HeLa cells. (a) Cells were treated with 20 nM MitoTracker® for 30 min in serum free DMEM, followed by incubation with 10 μM **Cy3-Cy5** in serum free DMEM for 1 h before imaging. (b) Same as (a) except cells were incubated with **Cy3-Cy5** first, followed by treatment with MitoTracker®. (c) Same as (b) except a mixture of **Cy3** and **Cy5** was used instead of **Cy3-Cy5**. (d) Same as (b) except **Cy3-Cy5-R8** was used instead of **Cy3-Cy5**. Excitation wavelength for MitoTracker® Green, Cy3, and Cy5 is 488, 543, and 633 nm, respectively. Scale bars denote 25 μm. Representative (N = 10) confocal microscopy images are shown here.

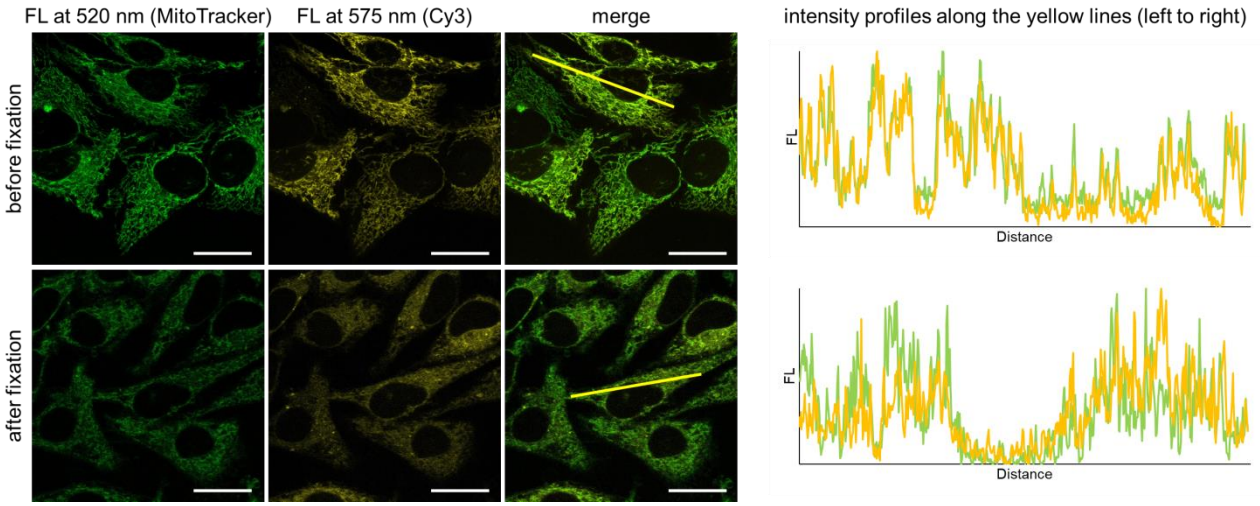


Figure S5. Mitochondrial localization of **Cy3** in fixed cells. HeLa cells were treated with 10 uM **Cy3** and 20 nM MitoTracker® and imaged before or after fixation with paraformaldehyde. Pearson's correlation coefficients for **Cy3** with MitoTracker® before and after fixation are 0.863 and 0.354, respectively. Excitation wavelength for MitoTracker® Green and Cy3 is 488 and 543 nm, respectively. Scale bars denote 25 µm. Representative (N = 10) confocal microscopy images are shown here.

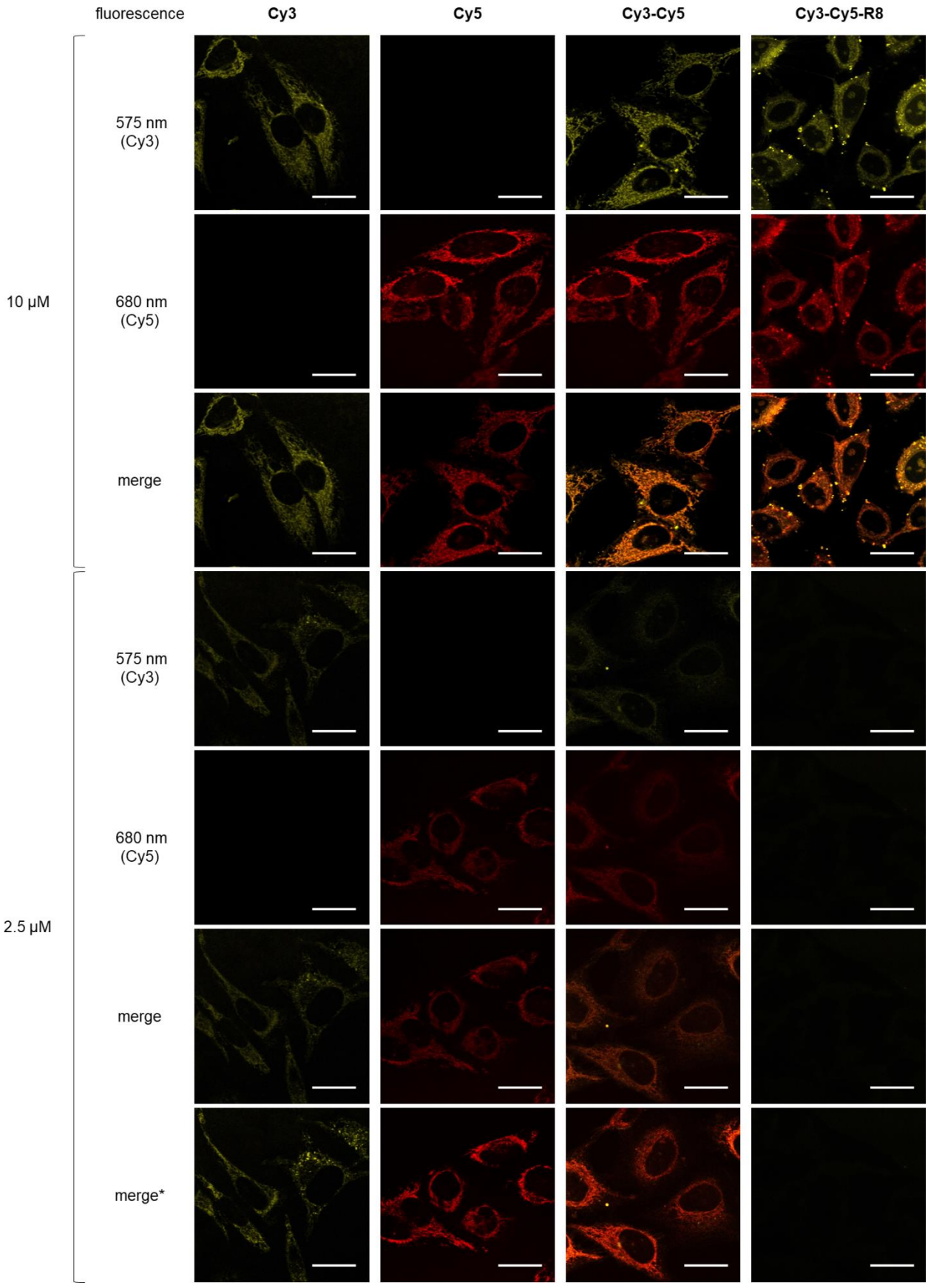


Figure S6. Concentration-dependent cellular uptake at 4 °C. HeLa cells were incubated in pre-chilled media at 4 °C for 10 min before addition of the indicated compound at either 10 µM or 2.5 µM. Cells were incubated at 4 °C for another 10 min before imaging. Excitation wavelength for Cy3, and Cy5 is 543, and 633 nm, respectively. Images in merge* are adjusted with +40% brightness and +40% contrast to better visualize the fluorescence pattern. Scale bars denote 25 µm. Representative (N = 10) confocal microscopy images are shown here.

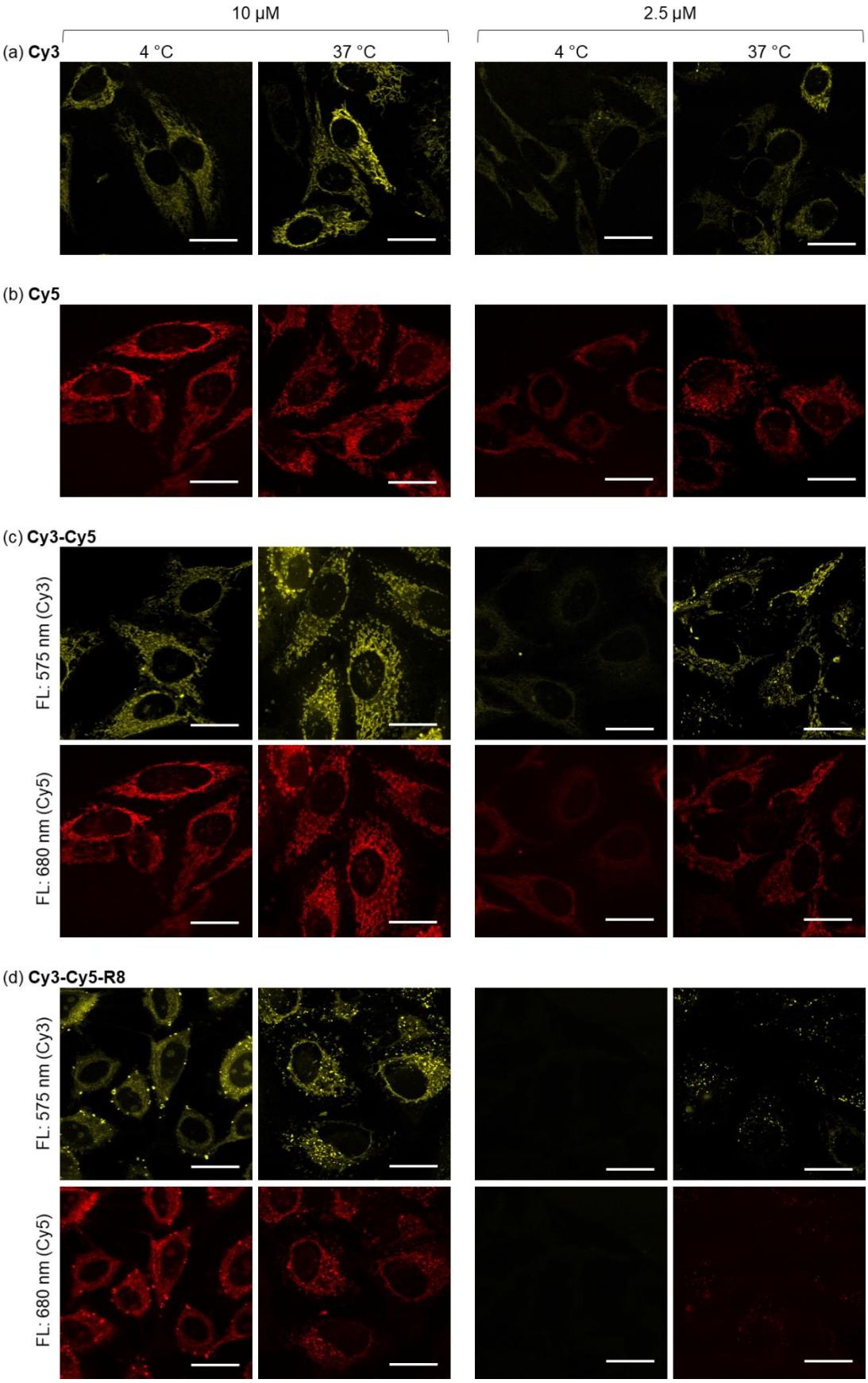


Figure S7. Concentration-dependent cellular uptake at 4 °C and 37 °C. HeLa cells were incubated in pre-chilled media at 4 °C for 10 min before addition of the indicated compound at either 10 µM or 2.5 µM for 10 min incubation before imaging. For 37 °C, HeLa cells were incubated with the indicated compound for 1 h before imaging. Excitation wavelength for Cy3, and Cy5 is 543, and 633 nm, respectively. Scale bars denote 25 µm.

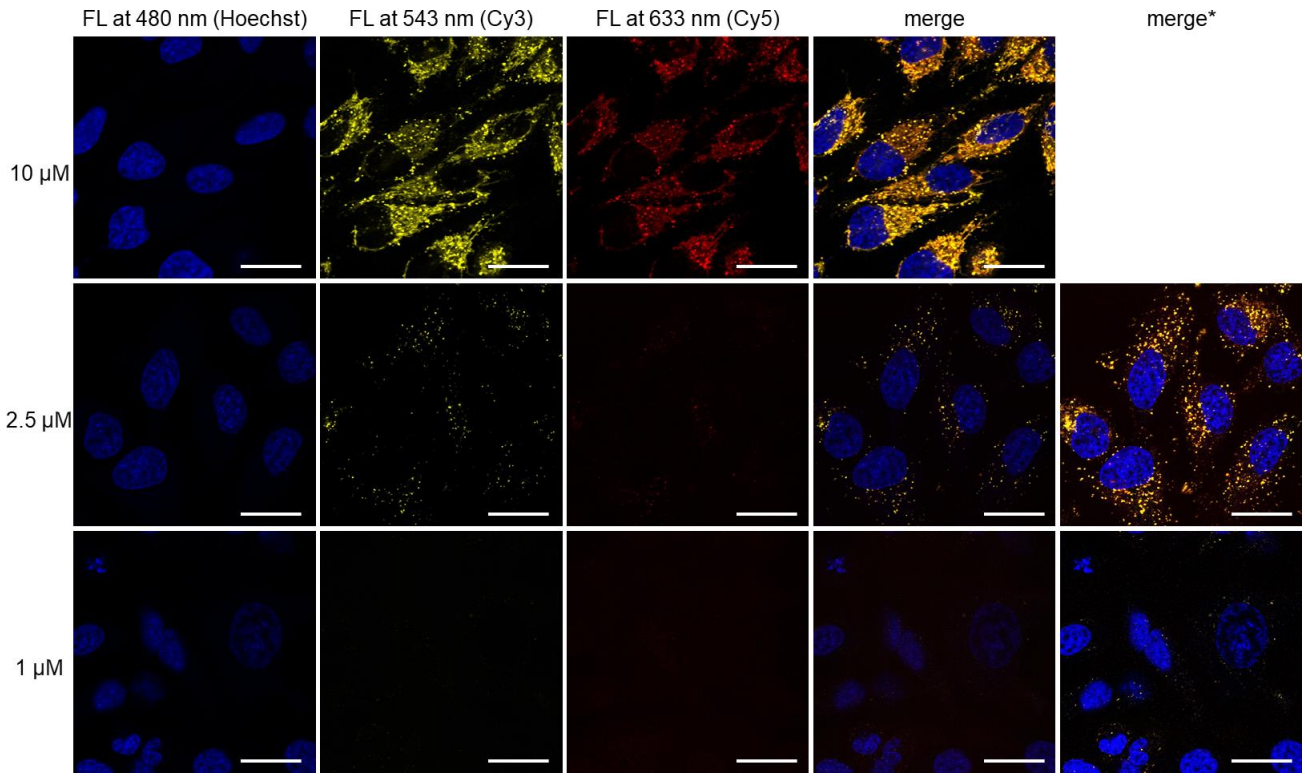
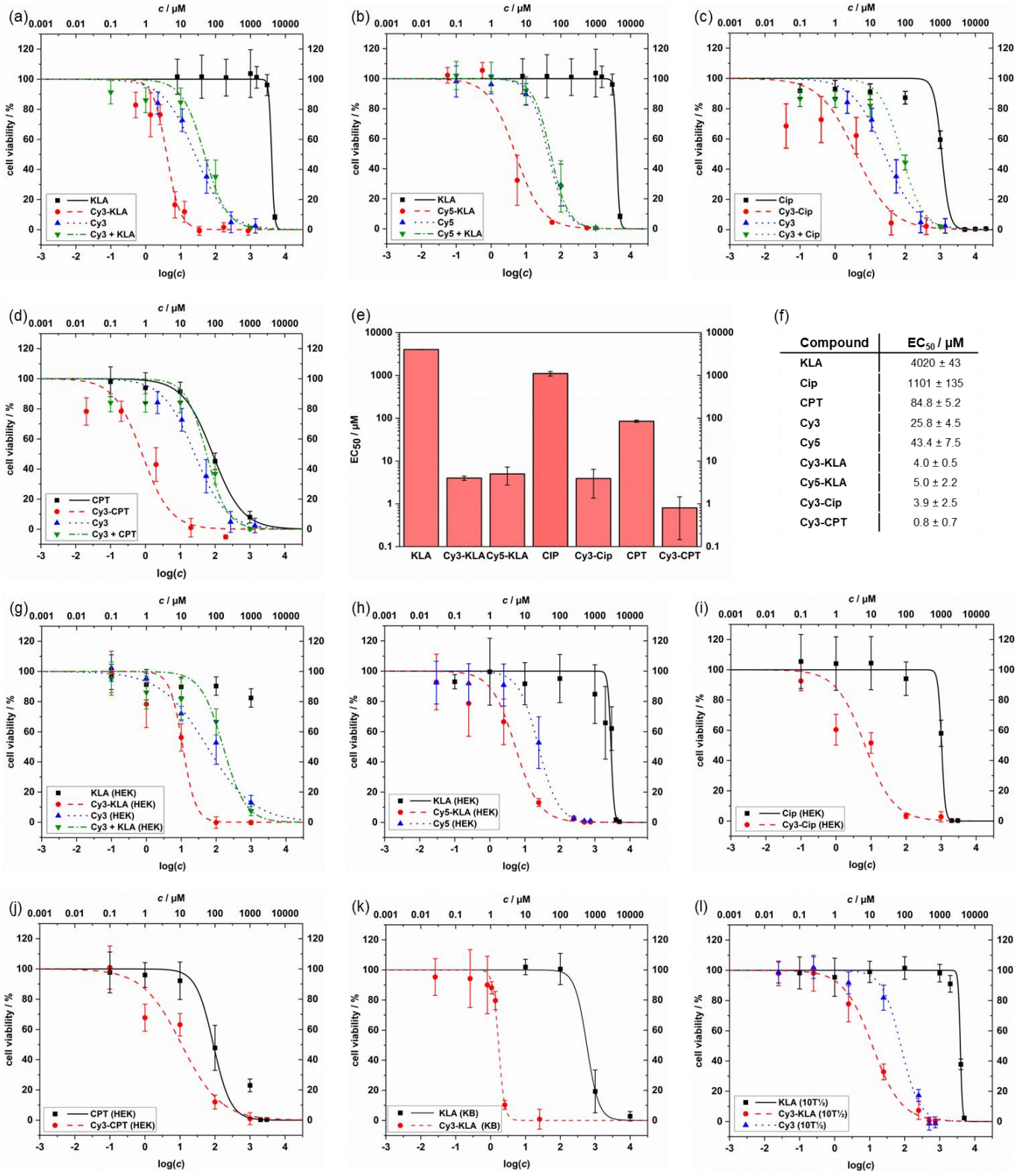
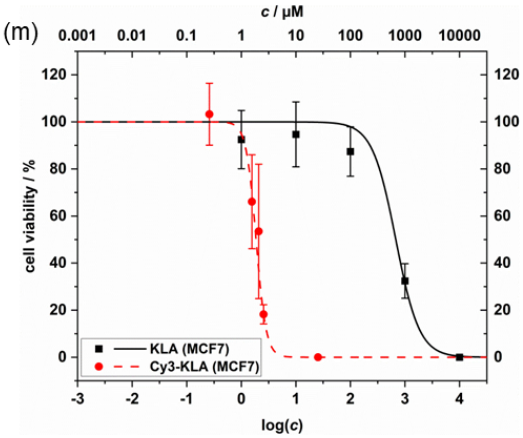


Figure S8. Concentration-dependent cellular uptake of **Cy3-Cy5-R8** at 37 °C. HeLa cells were treated with the compound at the indicated concentration in serum free media for 1 h before staining the nucleus with Hoechst 33258 and imaging. Excitation wavelength for Hoechst 33258 (nucleus stain), **Cy3**, and **Cy5** is 405, 543, and 633 nm, respectively. Images in merge* are adjusted with +40% brightness and +40% contrast to better visualize the fluorescence pattern. Scale bars denote 25 µm. Representative (N = 10) confocal microscopy images are shown here.



Compound	EC_{50} / µM
KLA	4020 ± 43
Cip	1101 ± 135
CPT	84.8 ± 5.2
Cy3	25.8 ± 4.5
Cy5	43.4 ± 7.5
Cy3-KLA	4.0 ± 0.5
Cy5-KLA	5.0 ± 2.2
Cy3-Cip	3.9 ± 2.5
Cy3-CPT	0.8 ± 0.7

(m)



(n)

Compound	EC_{50} / µM			
	HEK	KB	10T½	MCF7
KLA	2888 ± 169	575 ± 340	3865 ± 32	679 ± 132
Cip	1036 ± 51			
CPT	89.9 ± 71.9			
Cy3	55.3 ± 11.7		78.0 ± 19.7	
Cy5	24.7 ± 5			
Cy3-KLA	11.1 ± 1.5	1.7 ± 0.1	12.0 ± 1.5	1.87 ± 0.09
Cy5-KLA	5.3 ± 1.5			
Cy3-Cip	6.7 ± 3.3			
Cy3-CPT	11.1 ± 4.6			

Figure S9. (a–d) Effects of covalent conjugation of small molecules to cyanine dyes on HeLa cell viability: (a) **Cy3-KLA**, (b) **Cy5-KLA**, (c) **Cy3-Cip**, (d) **Cy3-CPT**. (e, f) Comparison of EC_{50} values of small molecules and their cyanine dye conjugates. (g–j) Effects of covalent conjugation of small molecules to cyanine dyes on HEK cell viability: (g) **Cy3-KLA**, (h) **Cy5-KLA**, (i) **Cy3-Cip**, (j) **Cy3-CPT**. (k) Effects of covalent conjugation of small molecules to cyanine dyes on KB cell viability: **Cy3-KLA**. (l) Effects of covalent conjugation of small molecules to cyanine dyes on 10T½ cell viability: **Cy3-KLA**. (m) Effects of covalent conjugation of small molecules to cyanine dyes on MCF7 cell viability: **Cy3-KLA**. (n) Comparison of EC_{50} values of small molecules and their cyanine dye conjugates in different cell lines. All cells were treated with the indicated compounds at the indicated concentration for 48 h before the quantification of cell viability using CellTiter-Blue®.

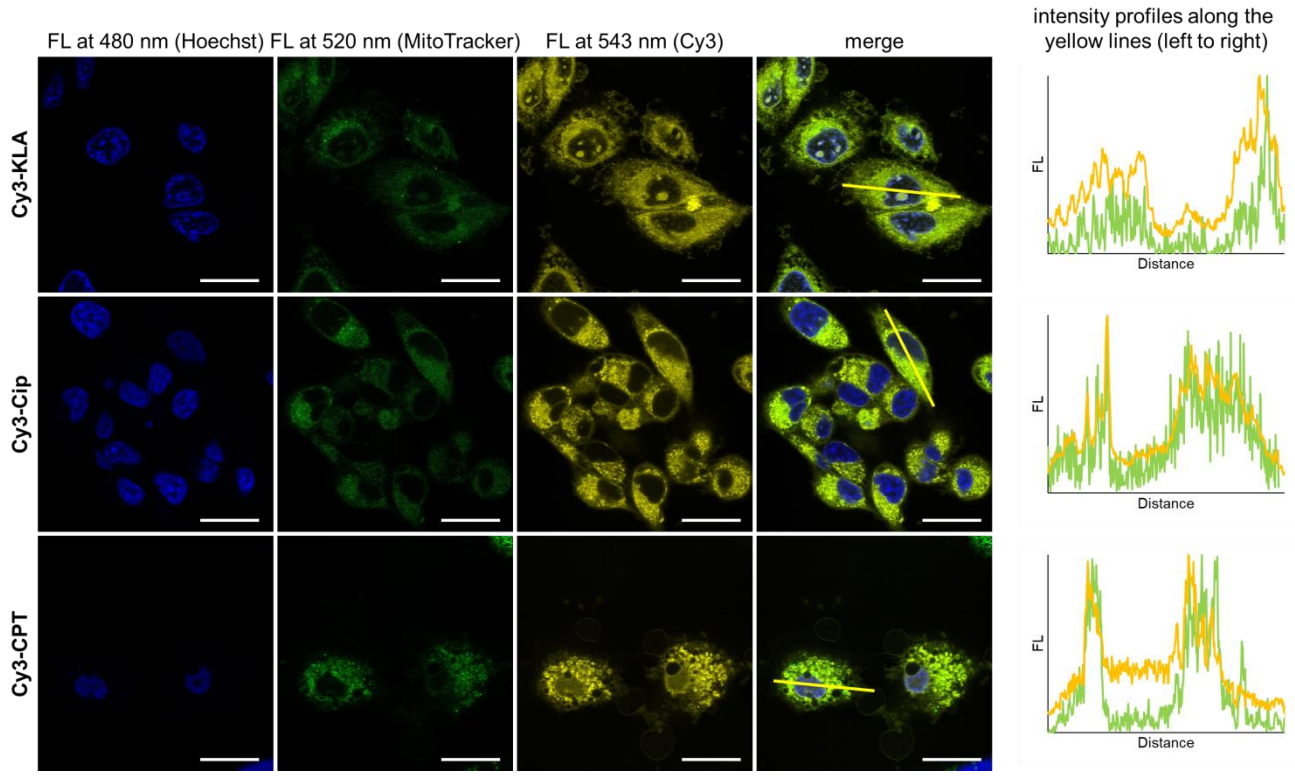


Figure S10. Colocalization of Cy3 conjugates with MitoTracker® Green in HeLa cells at 37 °C. Cells were treated with 20 nM MitoTracker® for 20 min, washed, treated with Hoechst 33258 for 10 min, washed, treated with 10 μM of the indicative Cy3 conjugate for 10 min, washed, and imaged. Pearson's correlation coefficients for **Cy3-KLA**, **Cy3-Cip** or **Cy3-CPT** with MitoTracker® are 0.830, 0.927 and 0.873, respectively. Excitation wavelength for Hoechst 33258, MitoTracker® Green and Cy3 is 405, 488, and 543 nm, respectively. Scale bars denote 25 μm. Representative (N = 10) confocal microscopy images are shown here.

2. Chemical Synthesis

Solvents and reagents were obtained from commercial sources (Sigma-Aldrich, Fisher Scientific, Fluorochem, Acros, Alfa Aesar) and used without further purification in reagent grade quality (> 98%). *N*,*N*′-diphenylformamide was used in HPLC grade quality. Reactions were performed in oven dried glassware without precautions to exclude air if nor otherwise stated. Reactions, work-ups, solvent removal and storage involving **Cy3-Cy5** conjugates were performed under exclusion of light. Syringe filters were obtained from Merck Millipore (#SLGP033RS, 0.22 µm, PES). Solid phase peptide synthesis (SPPS) was performed in TELOS® Filtration Columns (2 x 20 µm PE Frits, 15 mL, Kinesis, #900-0000-006T). Rink Amide MBHA resin (100-200 mesh) 0.3-0.8 mmol/g 1% DVB was obtained from Fluorochem, # M03536. Reaction temperatures are stated as heating device temperature (*e.g.* oil bath, shaker, *etc.*) or room temperature if not stated otherwise. Concentrations under reduced pressure were performed by rotary evaporation at 40 °C and appropriated pressure, unless otherwise noted. Deionized water was obtained by an Elga PURELAB 8 Option system (15 MΩ·cm). Compounds **Cy3**,[1] **Cy5**,[2] **mono-*N*-Boc-ethylenediamine**,[3] **Cip-OMe**,[4] and ***cis*-CBDCA-(HO-pda)-Pt**[5-7] were prepared according to literature procedures.

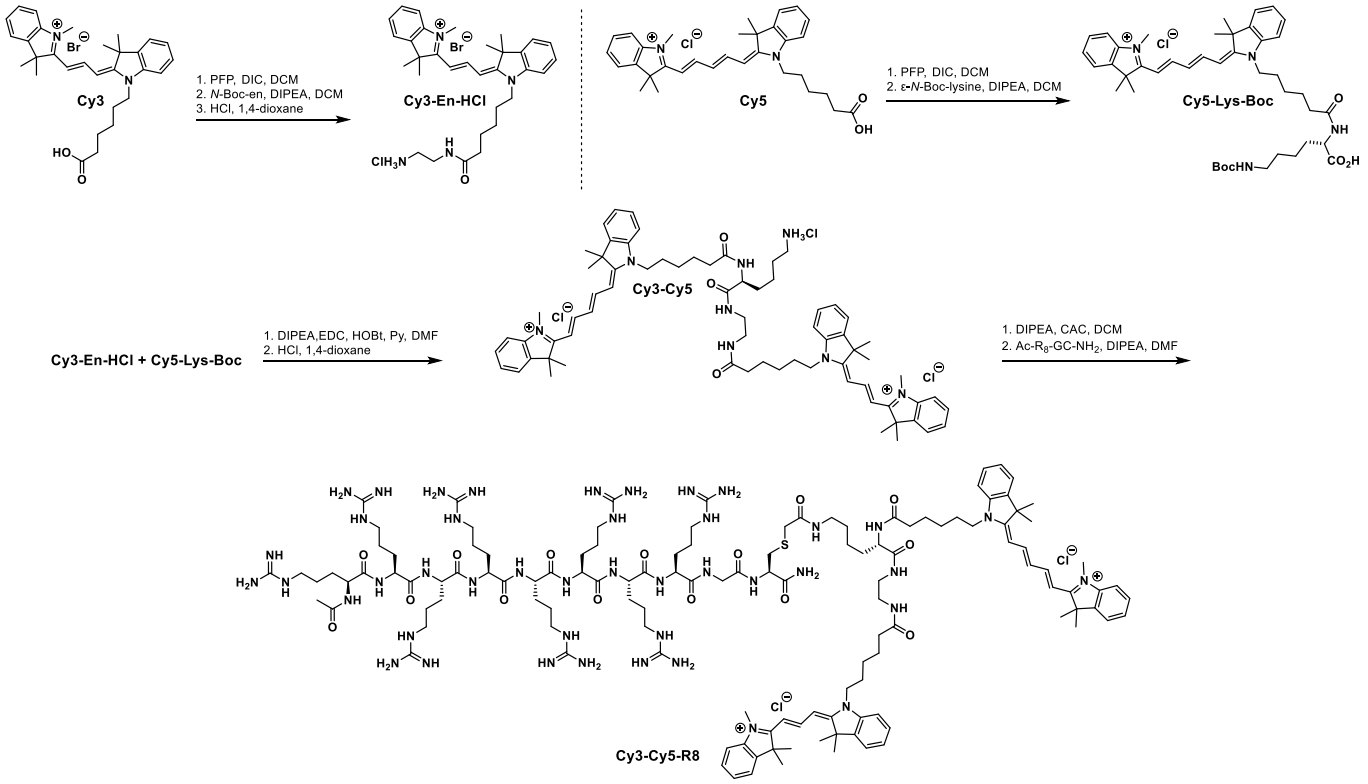
Analytical and preparative Thin Layer Chromatography (TLC) was carried out with silica gel 60 F254 aluminum sheets (Merck, #105554). Detection was carried out using UV light (λ = 254 nm and 366 nm), followed by immersion in permanganate (9 g $KMnO_4$, 60 g K_2CO_3, 1 mL AcOH, 900 mL H_2O) or cerium ammonium molybdate (5.0 g $Ce(SO_4)_2$, 25.0 g $(NH_4)_6Mo_7O_{24} \times 4{\cdot}H_2O$, 50 mL conc. H_2SO_4, 450 mL H_2O) staining solution with subsequent development via careful heating with a heat gun. Flash column chromatography was performed using silica gel (pore size 60 Å, 0.040-0.063 mm; Fisher Scientific, #84894290).

^1H and ^{13}C NMR spectra were recorded in $CDCl_3$, methanol-d_4, DMSO-d_6, or D_2O on Bruker Fourier 300, Ultrashield 400, or Ascend 500 instruments. Chemical shifts are reported in parts per million (ppm) and are referenced to the residual solvent resonance as the internal standard ($CHCl_3$: δ = 7.26 ppm for ^1H NMR and 77.2 ppm for ^{13}C NMR; methanol: δ = 3.31 ppm for ^1H NMR and 49.0 ppm for ^{13}C NMR; DMSO: δ = 2.50 ppm for ^1H NMR and 39.5 ppm for ^{13}C NMR; D_2O: δ = 4.79 ppm for ^1H NMR). Data are reported as follows: chemical shift, multiplicity (br s = broad singlet, s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, sept = septet, br m = broad multiplet, m = multiplet, mc = centrosymmetric multiplet), coupling constants (Hz) and integration.[8]

High Resolution Mass Spectrometry (HRMS) was performed on a Waters LCT Premier (ESI-(+) and APCI-(+)) or a Waters GCT Premier (EI) system.

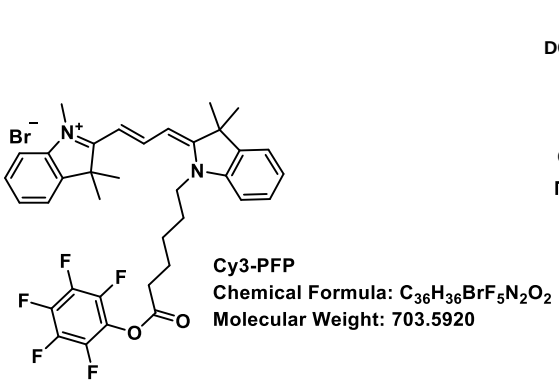
Liquid Chromatography–Mass Spectrometry (LCMS) was performed on a Dionex Ultimate 3000 LC system with a Bruker Amazon LS MS instrument employing a Waters Acquity BEH C18 column, 130 Å, 1.7 µm particle size, 2.1 mm × 100 mm. If not stated otherwise the **general method** was employed: 40 °C, flow rate = 0.3 mL · min^{-1}, H_2O:MeCN (0.1% HCO_2H) 97:3, 1 min, gradient to 3:97 over 18 min, 1 min hold, gradient to 97:3 over 1 min. Alternatively, an Agilent Infinity 1260 LC system with an Agilent B6120 quadrupole MS was employed, using the stated column and methods. Alternatively, an Agilent Infinity 1260 LC system with an Agilent B6120 quadrupole MS was employed, using the stated column and methods.

2.1 Synthesis of Cy3-Cy5 and Cy3-Cy5-R8



Scheme S1: Synthetic scheme of the four cyanine dye derivatives used in this study: **Cy3**; **Cy5**; **Cy3-Cy5**; **Cy3-C5-R8**. PFP: pentafluorophenol; DIC: di-*iso*-propylcarbodiimide; DCM: dichloromethane; DIPEA: di-*iso*-propylethylamine; EDC: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HOBt: 1-hydroxybenzotriazole; Py: pyridine; DMF: dimethylformamide; CAC: chloroacetyl chloride.

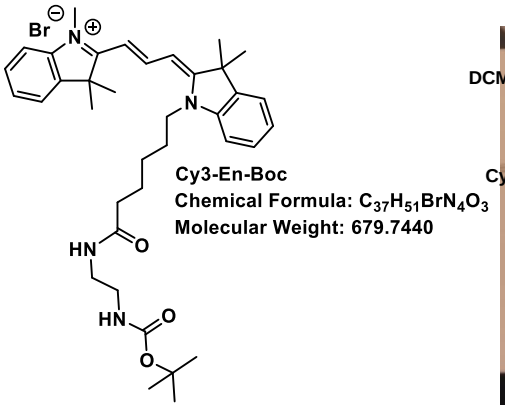
Cy3-PFP

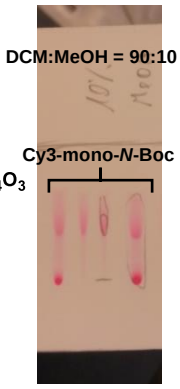


According to a procedure from *Silnikov*,[9] di-*iso*-propylcarbodiimide (DIC, 15 µL, 12 mg, 97 µmol, 3.2 eq) and pentafluorophenol (20 mg, 110 µmol, 3.7 eq) were added to a solution of **Cy3** (16 mg, 30 µmol, 1.0 eq) in CH_2Cl_2 (3.0 mL) at room temperature. After 1–2 h, TLC indicated full conversion (CH_2Cl_2:MeOH = 90:10, disappearance of pink starting material spot, and appearance of pink product spot). The reaction mixture was directly applied to a silica gel column for purification by flash column chromatography (SiO_2, CH_2Cl_2 → CH_2Cl_2:*i*-PrOH = 98:2 → CH_2Cl_2:*i*-PrOH = 95:5 → CH_2Cl_2:*i*-PrOH = 90:10). After removal of the solvent under reduced pressure the title compound **Cy3-PFP** (18 mg, 26 µmol, 87% yield) was obtained as red film. **^{1}H NMR** (500 MHz, $CDCl_3$): δ = 8.44 (t, *J* = 13.4 Hz, 1H),

7.44–7.31 (m, 6H), 7.24 (t, *J* = 7.9 Hz, 2H), 7.11 (t, *J* = 8.7 Hz, 2H), 4.26 (t, *J* = 7.5 Hz, 2H), 3.80 (s, 3H), 2.76 (t, *J* = 7.2 Hz, 2H), 1.95–1.83 (m, 4H), 1.81–1.74 (m, 2H), 1.70 (s, 12H) ppm; **^{13}C NMR** (125 MHz, $CDCl_3$): δ = 174.4, 173.7, 169.7, 151.0, 142.8, 142.1, 140.7, 140.6, 129.0, 125.5, 125.4, 122.3, 122.1, 110.9, 105.3, 104.8, 49.0, 48.9, 44.8, 33.1, 32.9, 28.3, 28.2, 27.4, 26.2, 24.6 ppm; **^{19}F NMR** (471 MHz, $CDCl_3$): δ = −152.6 (d, J = 18 Hz), −158.4 (t, J = 22 Hz), −162.6 (t, J = 23 Hz) ppm; **ESI-HRMS** (m/z): $[M]^+$ calcd. for $C_{36}H_{36}F_5N_2O_2^+$, 623.2697; found, 623.2702.

Cy3-mono-*N*-Boc-ethylenediamine

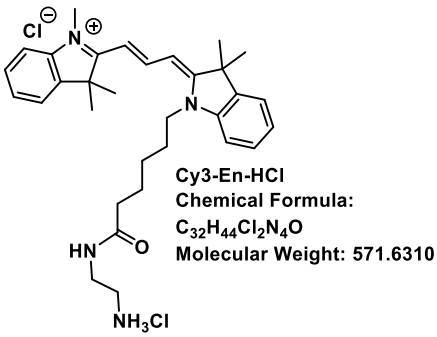




TLC of purified **Cy3-mono-*N*-Boc-ethylenediamine** at different concentrations. More concentrated samples partially stuck to the baseline. This was observed in flash column chromatography. Part of the compound stuck to the top and could only be eluted with MeOH:NH_3(35%, aq.) = 50:50. Pure fractions eluted with CH_2Cl_2:MeOH = 90:10 and MeOH:NH_3(35%, aq.) = 50:50 were both **Cy3-mono-*N*-Boc-ethylenediamine** according to LCMS and NMR analysis.

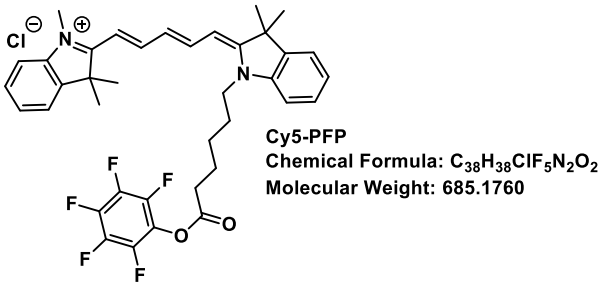
Mono-*N*-Boc-ethylenediamine (13 mg, 81 µmol, 4.8 eq) and di-*iso*-propylethylamine (DIPEA, 20 µL, 15 mg, 114 µmol, 6.7 eq) were added to a solution of **Cy3-PFP** (12 mg, 17 µmol, 1.0 eq) in CH_2Cl_2 (5.0 mL) at room temperature. After 16 h, TLC indicated full conversion (CH_2Cl_2:MeOH = 90:10, disappearance of pink starting material spot, and appearance of pink product spot). The reaction mixture was directly applied to a silica gel column for purification by flash column chromatography (SiO_2, CH_2Cl_2:MeOH = 80:20 → CH_2Cl_2:MeOH = 50:50 → MeOH → MeOH:NH_3(35%, aq.) = 50:50). The title compound **Cy3-En-Boc** (12 mg, 17 mmol, quantitative yield) was obtained as red film after removal of the solvent mixture under reduced pressure. **^{1}H NMR** (500 MHz, DMSO-d_6): δ = 8.34 (t, *J* = 13.5 Hz, 1H), 7.89 (t, *J* = 5.4 Hz, 1H), 7.63 (d, *J* = 7.5 Hz, 2H), 7.48 – 7.41 (m, 4H), 7.29 (ddd, *J* = 14.1, 7.3, 1.7 Hz, 3H), 6.79 (t, *J* = 5.6 Hz, 1H), 6.52 (d, *J* = 3.7 Hz, 1H), 6.50 (d, *J* = 3.6 Hz, 1H), 4.10 (t, *J* = 7.6 Hz, 2H), 3.66 (s, 3H), 3.02 (dd, *J* = 12.0, 5.8 Hz, 2H), 2.93 (dd, *J* = 12.4, 6.1 Hz, 2H), 2.06 (dd, J = 12.5, 5.2 Hz, 2H), 1.77–1.70 (m, J = 7.6 Hz, 2H), 1.69 (s, 10H), 1.56 (dt, *J* = 14.6, 7.3 Hz, 2H), 1.41–1.36 (m, 2H), 1.34 (s, 9H) ppm; **^{13}C NMR** (125 MHz, DMSO-d_6): δ = 174.5, 173.6, 172.0, 155.6, 149.7, 142.7, 141.9, 140.6, 131.6, 128.7, 128.6, 125.2, 125.1, 122.5, 122.4, 111.53, 111.48, 103.0, 102.4, 77.6, 48.9, 48.8, 43.7, 39.5, 38.6, 35.1, 31.4, 28.2, 27.5, 27.3, 26.7, 25.7, 24.9 ppm; **ESI-HRMS** (m/z): $[M]^+$ calcd. for $C_{37}H_{51}N_4O_3^+$, 599.3961; found, 599.3971.

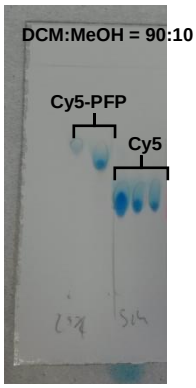
Cy3-ethylenediammonium chloride



Cy3-En-Boc[3] (12 mg, 17 μmol, 1.0 eq) was dissolved in CH_2Cl_2 (3.0 mL) and cooled with an ice bath. A solution of hydrogen chloride in 1,4-dioxane (4 M, 1 mL, 4 mmol, 235 eq) was added dropwise under fast stirring. The ice bath was removed after completed addition. After 1 h, TLC indicated full conversion (CH_2Cl_2:MeOH = 80:20, disappearance of pink starting material spot, and appearance of pink product spot). Solvents were removed under reduced pressure to yield crude **Cy3-En-HCl** as dark red solid which was used without further purification. **ESI-HRMS** (m/z): $[M]^+$ calcd. for $C_{32}H_{43}N_4O^+$, 499.3431; found, 499.3401.

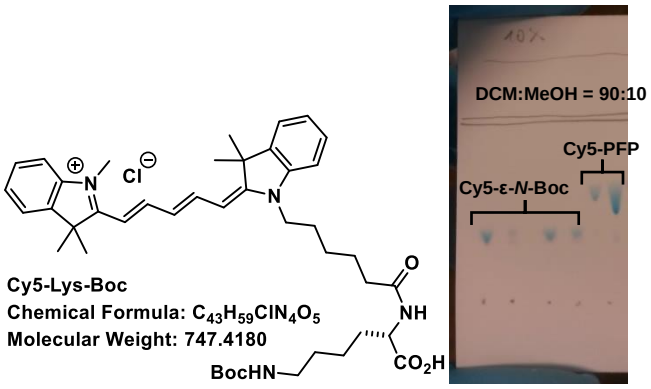
Cy5-PFP





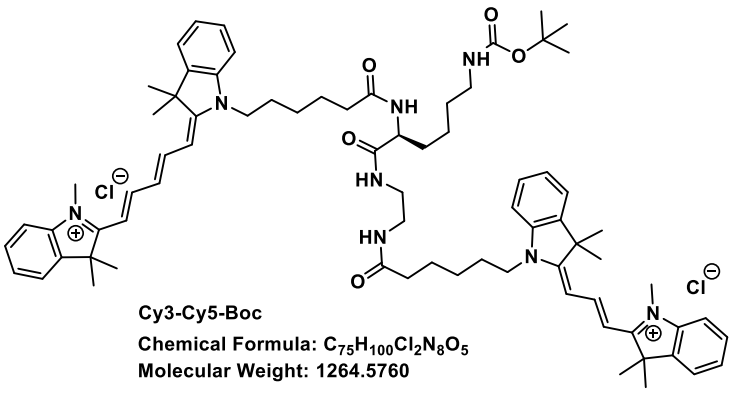
According to a procedure from Silnikov,[9] di-*iso*-propylcarbodiimide (DIC, 20 μL, 16 mg, 129 μmol, 3.7 eq) and pentafluorophenol (27 mg, 147 μmol, 4.2 eq) were added to a solution of **Cy5** (18 mg, 35 μmol, 1.0 eq)[2] in CH_2Cl_2 (5.0 mL) at room temperature. After 1-2 h, TLC indicated full conversion (CH_2Cl_2:MeOH = 90:10, disappearance of blue starting material spot, and appearance of blue product spot). The reaction mixture was directly applied to a silica gel column for purification by flash column chromatography (SiO_2, CH_2Cl_2 → CH_2Cl_2:i-PrOH = 98:2 → CH_2Cl_2:i-PrOH = 95:5 → CH_2Cl_2:i-PrOH = 90:10). The title compound **Cy5-PFP** (22 mg, 32 μmol, 91% yield) was obtained as blue film. **^{1}H NMR** (500 MHz, $CDCl_3$): δ = 8.20 (t, *J* = 12.6 Hz, 2H), 7.34 (t, *J* = 6.9 Hz, 4H), 7.20 (td, *J* = 7.4, 1.6 Hz, 2H), 7.07 (dd, *J* = 19.3, 8.0 Hz, 2H), 6.84 (t, J = 12.4 Hz, 1H), 6.44 (d, *J* = 13.5 Hz, 1H), 6.38 (d, *J* = 13.6 Hz, 1H), 4.11 (t, *J* = 7.2 Hz, 2H), 3.69 (s, 3H), 2.72 (t, J = 7.2 Hz, 2H), 1.91–1.84 (m, 4H), 1.76 (s, 6H), 1.72 (s, 6H), 1.68–1.58 (m, 2H) ppm; **^{13}C NMR** (125 MHz, $CDCl_3$): δ = 173.7, 173.0, 169.5, 154.4, 143.0, 142.2, 141.5, 141.1, 128.7, 128.6, 127.0, 125.2, 125.1, 122.5, 122.3, 110.5, 110.4, 104.4, 104.1, 49.44, 49.38, 44.2, 33.1, 32.1, 28.2, 28.0, 27.2, 26.2, 24.5 ppm; **^{19}F NMR** (471 MHz, $CDCl_3$): δ = −152.7 (dd, J = 22.2, 4.5 Hz), −158.1 (t, J = 21.7 Hz), −162.4 (dt, J = 22.3, 11.1 Hz) ppm; **ESI-HRMS** (m/z): $[M]^+$ calcd. for $C_{38}H_{38}N_2O_2F_5^+$, 649.2853; found, 649.2852.

Cy5-ε-*N*-Boc-(L)-lysine



ε-*N*-Boc-(L)-lysine (23 mg, 89 µmol, 3.1 eq) and di-*iso*-propylethylamine (40 µL, 220 µmol, 7.9 eq) were added to a solution of **Cy5-PFP** (22 mg, 28 µmol, 1.0 eq) in CH_2Cl_2 (2.0 mL) at room temperature. After 24 h, TLC indicated full conversion (CH_2Cl_2:MeOH = 90:10, disappearance of blue starting material spot, and appearance of blue product spot). The reaction mixture was directly applied to a silica gel column for purification by flash column chromatography (SiO_2, CH_2Cl_2 → CH_2Cl_2:MeOH = 95:5 → CH_2Cl_2:MeOH = 90:10 → CH_2Cl_2:MeOH = 80:20 → CH_2Cl_2:MeOH = 60:40). The title compound **Cy5-Lys-Boc** (17 mg, 23 µmol, 82% yield) was obtained as blue film. **^{1}H NMR** (500 MHz, DMSO-d_6): δ = 8.33 (t, *J* = 13.1 Hz, 2H), 7.87 (s, 1H), 7.60 (d, *J* = 7.4 Hz, 2H), 7.43–7.33 (m, 4H), 7.23 (td, *J* = 7.3, 2.1 Hz, 2H), 6.74 (d, *J* = 5.1 Hz, 1H), 6.57 (t, *J* = 12.3 Hz, 1H), 6.30 (d, *J* = 13.8 Hz, 1H), 6.26 (d, *J* = 13.9 Hz, 1H), 4.16–4.05 (m, 2H), 3.59 (s, 3H), 2.91–2.81 (m, 3H), 2.11 (dd, *J* = 7.1, 5.1 Hz, 2H), 1.74–1.60 (m, 14H), 1.54 (dt, *J* = 14.5, 7.2 Hz, 2H), 1.40–1.28 (m, 14H), 1.27–1.20 (m, 3H) ppm; **^{13}C NMR** (125 MHz, DMSO-d_6): δ = 173.2, 172.5, 171.8, 155.5, 154.0, 142.8, 142.0, 141.1, 141.0, 128.4, 128.3, 125.5, 124.7, 124.6, 122.4, 122.3, 111.0, 103.3, 103.1, 77.3, 48.8, 43.3, 39.6, 34.9, 31.2, 31.1, 29.2, 28.3, 27.2, 27.0, 26.6, 25.7, 24.9, 22.8 ppm; **ESI-HRMS** (m/z): $[M]^+$ calcd. for $C_{43}H_{59}N_4O_5^+$, 711.4485; found, 711.4489.

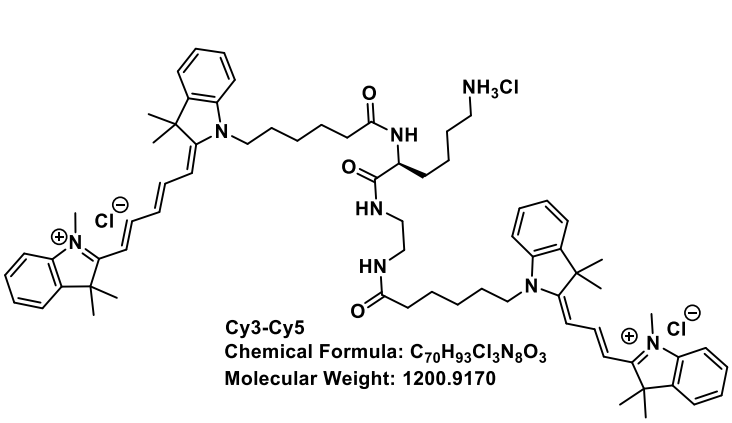
Cy3-Cy5-Boc

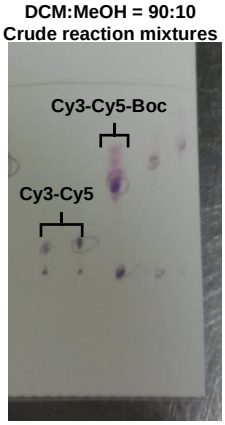


Cy5-ε-*N*-Boc-(L)-lysine (15 mg, 20 µmol, 1.1 eq), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (12 mg, 60 µmol, 3.3 eq), 1-hydroxybenzotriazol hydrate (2 mg, 13 µmol, 0.7 eq), pyridine (50 µL, 620 µmol, 34 eq), and di-*iso*-propylethylamine (250 µL, 1.4 mmol, 78 eq) were added to a solution of **Cy3-En-HCl** (10 mg, 18 µmol, 1.0 eq) in DMF (3.0 mL) at room temperature. After 42 h, LCMS analysis (*Agilent Infinity 1260* LC system, employing *Agilent Zorbax Eclipse Plus* column, C18, 3.5 µm particle size, 95 Å pore size, 4.6 mm x 100 mm; method: 40 °C, flow rate = 0.5 mL · min^{-1}, H_2O:MeCN, 0.1% HCO_2H, 95:5, 5 min hold, gradient to 5:95 over 20 min, 2.5 min hold) indicated full conversion. The volatiles were removed under reduced pressure, the residue was dissolved in

H_2O:MeCN 70:30, filtered with a syringe filter, and the filtrate was subjected to three rounds of RP-HPLC (*Phenomex Gemini* C18 column, 10 µm particle size, 110 Å pore size, 100 mm × 250 mm; Method: flow rate = 3.0 mL · min^{-1}, H_2O:MeCN, 0.1% HCOOH, 85:15, gradient to 65:35 over 30 min, gradient to 57:43 over 28 min, gradient to 0:100 over 1 min, RT = 42.9 min). Lyophilization of the pure product fractions afforded the title compound **Cy3-Cy5-Boc** (11 mg, 9 µmol, 50% yield) as purple powder. **ESI-HRMS** (m/z): $[M]^{2+}$ calcd. for $C_{75}H_{100}N_8O_5^{2+}$, 596.3903; found, 596.3906.

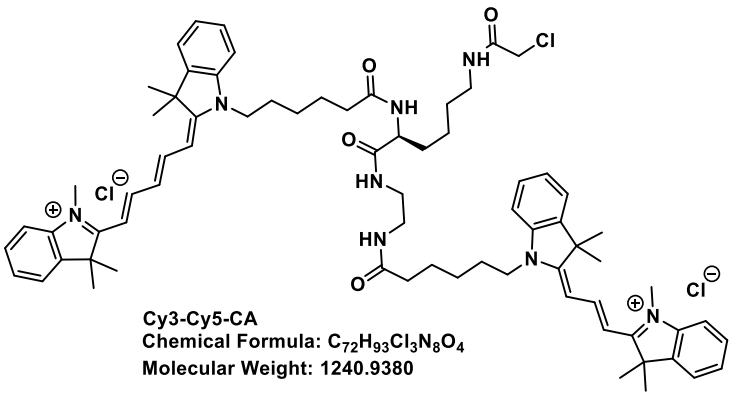
Cy3-Cy5





Cy3-Cy5-Boc (8 mg, 6.6 µmol, 1.0 eq) was dissolved in CH_2Cl_2 (5.0mL) and cooled to 0 °C using an ice bath. A solution of hydrogen chloride in 1,4-dioxane (4 M, 1.0 mL, 4.0 mmol, 610 eq) was added dropwise under vigorous stirring. The ice bath was removed after complete addition. After 1 h, TLC indicated full conversion (CH_2Cl_2:MeOH = 80:20, disappearance of purple starting material spot and appearance of purple product spot). The solvents were removed under reduced pressure to yield **Cy3-Cy5** as purple solid and used without further purification. Analytically pure compound was obtained after RP-HPLC (*Phenomex Gemini* C18 column, 10 µm particle size, 110 Å pore size, 10.0 mm × 250 mm; Method: flow rate = 2.0 mL · min^{-1}, H_2O:MeCN, 0.1% HCOOH, 95:5 for 5 min, gradient to 20:80 over 55 min, RT = 35.2 min). **ESI-HRMS** (m/z): $[M+H]^{3+}$ calcd. for $C_{70}H_{93}ClN_8O_3^{2+}$, 364.5785; found, 364.5778.

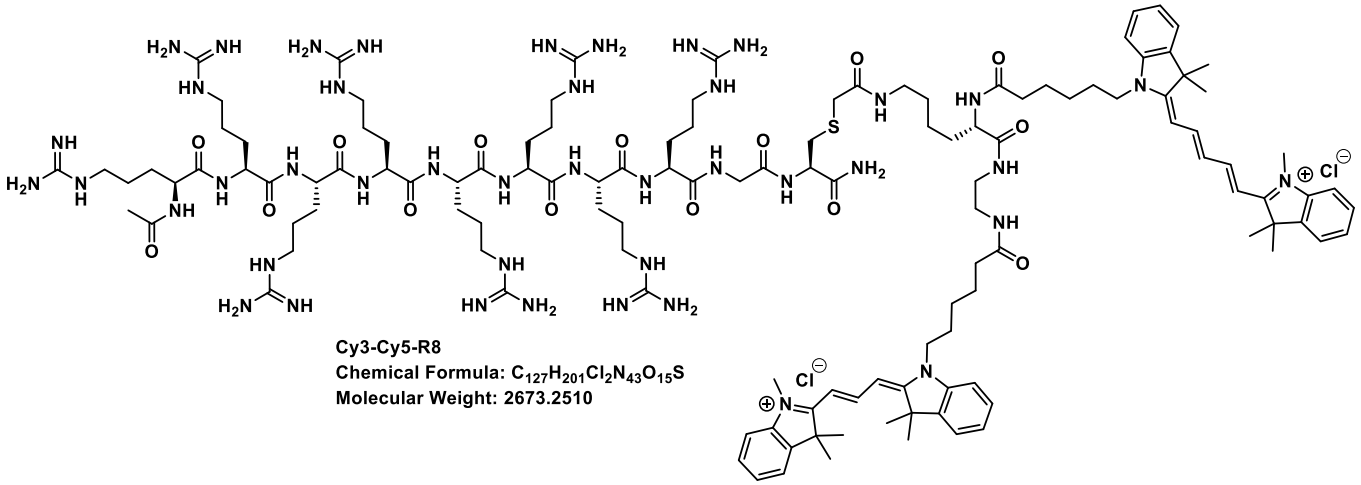
Cy3-Cy5-CA



Cy3-Cy5 (1.4 mg, 1.2 µmol, 1.0 eq) was suspended in CH_2Cl_2 (3.0 mL) and cooled to 0 °C using an ice bath. Di-*iso*-propylethylamine (DIPEA, 11 µL, 65 µmol, 54 eq) and a solution of chloroacetyl chloride in CH_2Cl_2 (1.0 mL, 0.2 mg/mL ≡ 0.2 mg, 2.4 µmol, 2.0 eq) were added dropwise under fast stirring. The ice bath was removed 30 min after completed addition. After additional 30 min, LCMS analysis (*Agilent Infinity 1260* LC system, employing *Agilent Zorbax Eclipse Plus* column, C18, 3.5 µm particle size, 95 Å pore size, 4.6 mm x 100 mm; method: 40 °C, flow rate = 0.5 mL · min^{-1}, H_2O:MeCN, 0.1% HCO_2H, 95:5,

5 min hold, gradient to 5:95 over 20 min, 2.5 min hold) indicated full conversion. The solvents were removed under fine vacuum at 30 °C. Prolonged reaction times and further workup led to partial or full decomposition of the crude product. Hence, the crude **Cy3-Cy5-CA** was used in further reactions. An analytically pure sample was obtained by flash column chromatography (SiO_2, CH_2Cl_2 → CH_2Cl_2:MeOH = 95:5 → CH_2Cl_2:MeOH = 90:10 → CH_2Cl_2:MeOH = 80:20). **LCMS** (m/z): $[M]^{2+}$ calcd. for $C_{72}H_{93}ClN_8O_4^{2+}$, 584.4; found, 584.5.

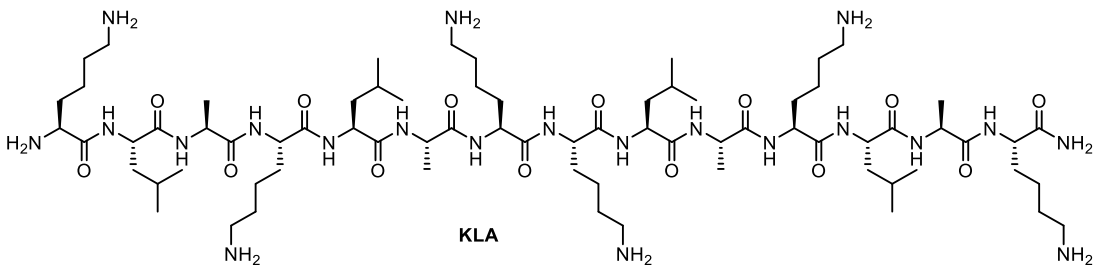
Cy3-Cy5-R8



Crude **Cy3-Cy5-CA** from the step before was dissolved in DMF (2.0 mL). Di-*iso*-propylethylamine (DIPEA, 50 µL, 287 µmol, 239 eq) and **Ac-R$_8$GC-NH$_2$** (5.0 mg, 3.4 µmol, 2.8 eq) were added. The solution was heated to 40 °C. After 48 h, LCMS analysis (*Agilent Infinity 1260* LC system, employing *Agilent Zorbax Eclipse Plus* column, C18, 3.5 µm particle size, 95 Å pore size, 4.6 mm x 100 mm; method: 40 °C, flow rate = 0.5 mL · min^{-1}, H_2O:MeCN, 0.1% HCO_2H, 95:5, 5 min hold, gradient to 5:95 over 20 min, 2.5 min hold) indicated no further conversion. The solvents were removed under reduced pressure, the residue was dissolved in H_2O:MeCN 70:30, filtered with a syringe filter, the filtrate was purified by RP-HPLC (*Phenomex Gemini* C18 column, 10 µm particle size, 110 Å pore size, 10.0 mm × 250 mm; Method: flow rate = 3.0 mL · min^{-1}, H_2O:MeCN, 0.1% HCOOH, 95:5, 5 min hold, gradient to 40:60 over 50 min, gradient to 20:80 over 5 min, RT = 26.5 min). Fractions containing the product were lyophilized to afford the title compound **Cy3Cy5-R8** (0.6 mg, 0.2 µmol, 17% yield over two steps) as purple solid. **ESI-HRMS** (m/z): $[M+2H]^{4+}$ calcd. for $C_{127}H_{203}N_{43}O_{15}S_4^{2+}$, 650.9044; found, 650.9064.

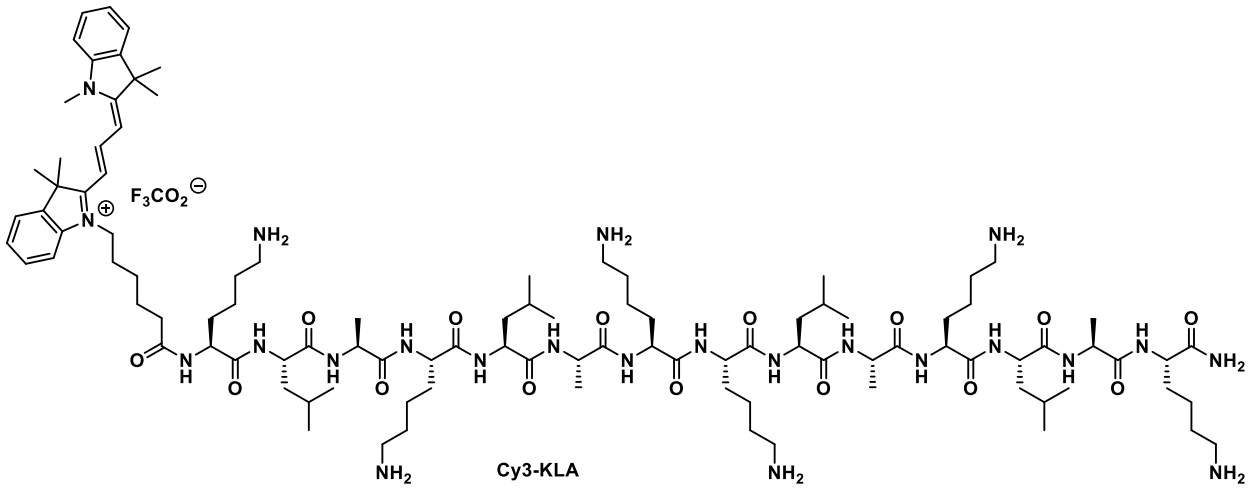
2.2 Synthesis of KLA Conjugates

KLA



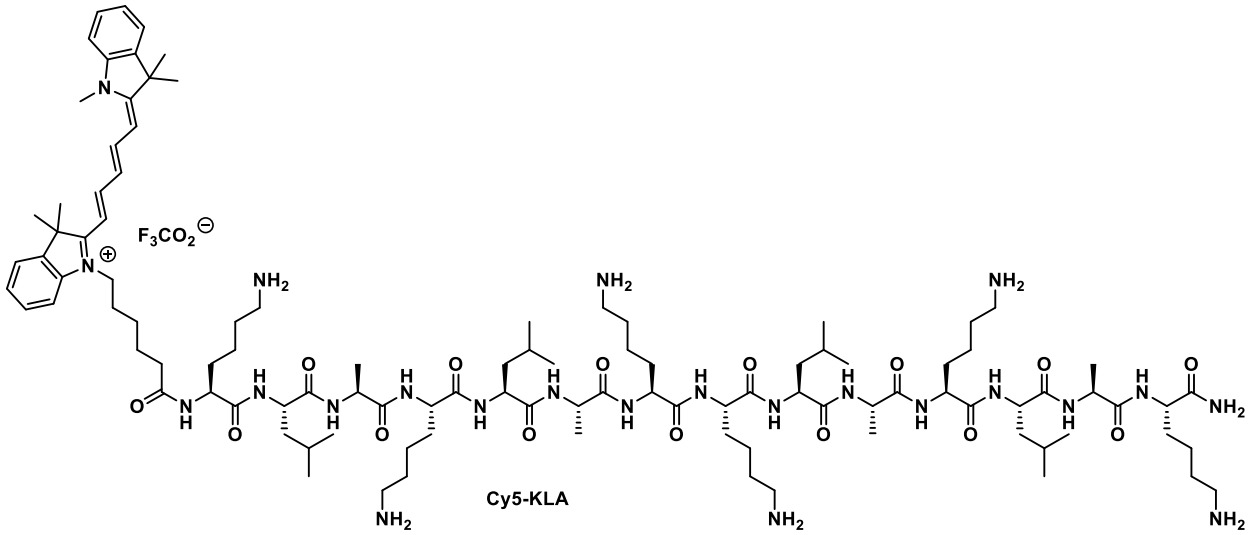
The peptide chain was synthesized by SPPS on Rink Amide MBHA resin, previously swollen in DCM/DMF (1:1, 10 mL) for 90 min. Each amino acid coupling step was carried out with Fmoc-protected amino acid (2 eq), HBTU (2 eq), HOBt (2 eq) and DIPEA (8 eq) in 5 mL of DMF for 45 min. After each coupling step, the respective Fmoc protecting group was removed by two incubations with 20% piperidine:DMF (v/v) for 5 min each. After the final Fmoc deprotection, cleavage was performed in TFA/Water/TIS 95:2.5:2.5 solution for 2 h. The resin was removed by filtration and the filtrate was concentrated by N_2 flow. The crude product was precipitated with cold diethyl ether, centrifuged (4,000 g for 10 min) and the supernatant was discarded. The precipitate was dissolved in water and purified by RP-HPLC (*Phenomex Gemini* C18 column, 10 µm particle size, 110 Å pore size, 10.0 mm × 250 mm; Method: flow rate = 5.0 mL · min^{-1}, H_2O:MeCN, 0.1% CF_3COOH, 100:0 for 5 min, gradient to 7:3 over 40 min, RT = 37.7 min). Lyophilization of the pure product fractions afforded compound **KLA** as white powder (41% yield, 0.05 mmol scale). **ESI-HRMS** (m/z): $[M+H]^+$ calcd.for $C_{72}H_{139}N_{21}O_{14}$ 1523.0883, found 1523.0891.

Cy3-KLA



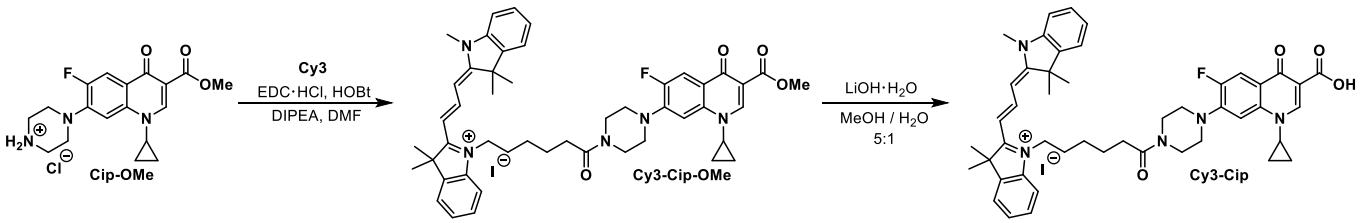
The peptide chain was synthesized by SPPS on Rink Amide MBHA resin, previously swollen in DCM/DMF (1:1, 10 mL) for 90 min. Each amino acid coupling step was carried out with Fmoc-protected amino acid (2 eq), HBTU (2 eq), HOBt (2 eq) and DIPEA (8 eq) in DMF (5 mL) for 45 min. After each coupling step, the respective Fmoc protecting group was removed by two incubations with 20% piperidine:DMF (v/v) for 5 min each. After the final Fmoc deprotection, $Cy3^+I^-$ was coupled using the standard method on solid phase (3 eq of $Cy3^+I^-$, 3 eq of HBTU, 3 eq of HOBt and 8 eq of DIPEA, 72 h). Cleavage was performed in TFA/Water/TIS 95:2.5:2.5 solution for 2 h. The resin was removed by filtration and the filtrate was concentrated by N_2 flow. The crude product was precipitated with cold diethyl ether, centrifuged (4,000 g for 10 min) and the supernatant was discarded. The precipitate was dissolved in water and purified by RP-HPLC (*Phenomex Gemini* C18 column, 10 µm particle size, 110 Å pore size, 10.0 mm × 250 mm; Method: flow rate = 5.0 mL · min^{-1}, H_2O:MeCN, 0.1% CF_3COOH, 9:1 gradient to 56:44 over 34 min, RT = 30.9 min). Lyophilization of the pure product fractions afforded compound **Cy3-KLA** as red powder (33% yield, 0.05 mmol scale). **ESI-HRMS** (m/z): $[M+H]^{2+}$ calcd.for $C_{102}H_{174}N_{23}O_{15}^+$ 981.6816, found 981.6830.

Cy5-KLA



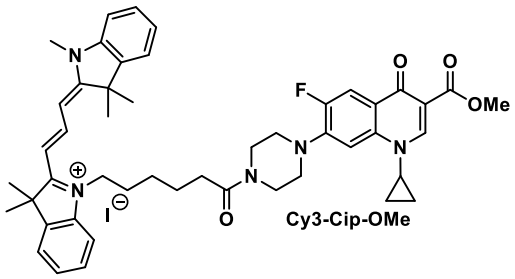
The peptide chain was synthesized by SPPS on Rink Amide MBHA resin, previously swollen in DCM/DMF (1:1, 10 mL) for 90 min. Each amino acid coupling step was carried out with Fmoc-protected amino acid (2 eq), HBTU (2 eq), HOBt (2 eq) and DIPEA (8 eq) in of DMF (5 mL) for 45 min. After each coupling step, the respective Fmoc protecting group was removed by two incubations with 20% piperidine:DMF (v/v) for 5 min each. After the final Fmoc deprotection, **Cy5** was coupled using the standard method on solid phase (3 eq of **Cy5**, 3 eq of HBTU, 3 eq of HOBt and 8 eq of DIPEA, 72 h). Cleavage was performed in TFA/Water/TIS 95:2.5:2.5 solution for 2 h. The resin was removed by filtration and the filtrate was concentrated by N_2 flow. The crude product was precipitated with cold diethyl ether, centrifuged (4,000 g for 10 min) and the supernatant was discarded. The precipitate was dissolved in water and purified by RP-HPLC (*Phenomex Gemini* C18 column, 10 µm particle size, 110 Å pore size, 10.0 mm × 250 mm; Method: flow rate = 5.0 mL · min^{-1}, H_2O:MeCN, 0.1% CF_3COOH, 4:1 for 5 min, gradient to 2:3 over 40 min, RT = 26.9 min). Lyophilization of the pure product fractions afforded compound **Cy5-KLA** as blue powder (30% yield, 0.05 mmol scale). **HRMS:** $[M+H]^{2+}$ calcd.for $C_{104}H_{176}N_{23}O_{15}^{+}$ 994.1892, found 994.1909.

2.3 Synthesis of Cy3-Cip



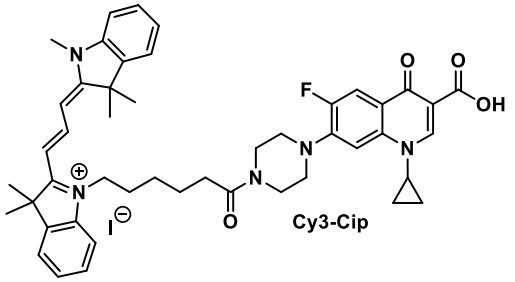
Scheme S2: Synthetic scheme of **Cy3-Cip**. DIPEA: di-*iso*-propylethylamine; EDC: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HOBt: 1-hydroxybenzotriazole; DMF: dimethylformamide.

Cy3-Cip-OMe



To a stirred solution of **Cy3** (500 mg, 0.86 mmol, 1.0 eq), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (656 mg, 3.42 mmol, 4.0 eq), 1-hydroxybenzotriazole hydrate (462 mg, 3.42 mmol, 4.0 eq), and di-*iso*-propylethylamine (1.80 mL, 10.2 mmol, 12.0 eq) in DMF (10 mL), was added a solution of **Cip-OMe**[4] in DMF (10 mL). After addition, the mixture was stirred at room temperature until LCMS analysis (*Bruker* LCMS, general method) indicated full conversion (24 h). The mixture was concentrated under vacuum to remove DMF and purified directly by flash column chromatography (SiO_2, DCM : MeOH = 20 : 1 → 5 : 1) to afford the target product **Cy3-Cip-OMe** (700 mg, 0.69 mmol, 79 %). **^{1}H NMR** (500 MHz, DMSO-d_6): δ = 8.43 (s, 1H), 8.35 (t, *J* = 13.5 Hz, 1H), 7.73 (d, *J* = 13.0 Hz, 1H), 7.63 (t, *J* = 7.0 Hz, 2H), 7.48–7.39 (m, 5H), 7.29 (q, *J* = 7.5 Hz, 2H), 6.55 (dd, *J* = 13.0 Hz, *J* = 4.5 Hz, 2H), 4.14 (t, *J* = 7.0 Hz, 2H), 3.7 (s, 3H), 3.66 (s, 3H), 3.66–3.61 (m, 4H), 3.38 (q, *J* = 7.0 Hz, 1H), 3.19 (d, *J* = 18.5 Hz, 4H), 2.39 (t, *J* = 7.0 Hz, 2H), 1.80-1.74 (m, 2H), 1.70 (s, 6H), 1.68 (s, 6H), 1.64-1.58 (m, 2H), 1.48-1.42 (m, 2H), 1.25 (d, *J* = 6.5 Hz, 2H), 1.10-1.07 (m, 2H) ppm; **^{13}C NMR** (126 MHz, DMSO-d_6): δ = 174.4, 173.7, 171.5, 170.6, 164.9, 152.4 (d, *J* = 247.2 Hz), 149.7, 148.3, 143.6, 143.5, 142.6, 141.9, 140.6, 140.5, 138.0, 128.6, 128.5, 125.2, 125.1, 122.6, 122.4, 122.1, 122.0, 111.5, 109.0, 106.5, 103.0, 102.4. 64.9, 51.3, 49.9, 49.4, 48.84, 48.83, 44.7, 43.7, 40.7, 40.1, 34.8, 31.9, 31.4, 27.5, 27.2, 26.9, 25.9, 24.6, 15.2, 7.5 ppm; **HRMS:** calcd.for $C_{48}H_{55}N_5O_4F$ $[M]^+$ 784.4238, found 784.4244.

Cy3-Cip

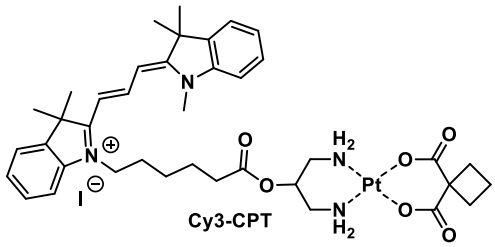


To a stirred solution of **Cy3-Cip-OMe** (225 mg, 0.25 mmol, 1.0 eq) in methanol (5 mL) and water (0.2 mL), was added lithium hydroxide monohydrate (31.0 mg, 0.74 mmol, 3.0 eq). The mixture was stirred at room temperature until LCMS analysis (*Bruker* LCMS system, general method) indicated full conversion (15 h). After removal of MeOH under reduced pressure, dichloromethane (100 mL) was added and washed with deionized water (50 mL). The organic phases were separated and then concentrated under reduced pressure. The obtained residual dark red oil was dissolved in acetonitrile (1 mL) and the product precipitated by the addition of cold diethyl ether (50 mL). After centrifuging (4000 rpm for 10 minutes), the supernatant was removed. Drying by freeze dryer to afford product **Cy3-Cip** as red powder (143 mg, 0.16 mmol, 64 %). **^{1}H NMR** (500 MHz, DMSO-d_6): δ = 8.61 (br, 1H), 8.30 (t, *J* = 13.0 Hz, 1H), 7.73 (br, 1H), 760 (t, *J* = 6.5 Hz, 2H), 7.46-7.33 (m, 5H), 7.27-7.24 (m, 2H), 6.60 (d, *J* = 12.5 Hz, 2H), 4.15 (br, 2H), 3.65 (s, 3H), 3.60 (br, 4H), 3.38 (q, *J* = 6.5 Hz, 1H), 3.17 (s, 1H), 3.15-3.12 (m, 4H), 2.33 (br, 2H), 1.74 (br, 2H), 1.66 (s, 6H), 1.64 (s, 6H), 1.57-1.55 (m, 2H), 1.41 (br, 2H), 1.26-1.24 (m, 2H), 0.97 (br, 2H)

ppm; **^{13}C NMR** (126 MHz, DMSO-d_6): δ = 174.3, 173.5, 170.6, 166.0, 152.1 (d, *J* = 246.3 Hz), 149.6, 143.2, 143.1, 142.6, 141.9, 140.54, 140.48, 138.2, 128.6, 128.5, 125.2, 125.0, 122.5, 122.3, 121.6, 117.4, 111.5, 111.3, 111.2, 105.7, 103.5, 102.6, 99.5, 64.9, 49.9, 49.5, 48.6, 48.1, 44.7, 43.7, 40.7, 40.1, 34.2, 31.9, 31.4, 27.4, 27.2, 26.9, 25.8, 24.6, 15.2, 7.5 ppm; **HRMS:** calcd. for $C_{47}H_{53}N_5O_4F$ $[M]^+$ 770.4086, found 770.4082.

2.4 Synthesis of Cy3-CPT

Cy3-CPT

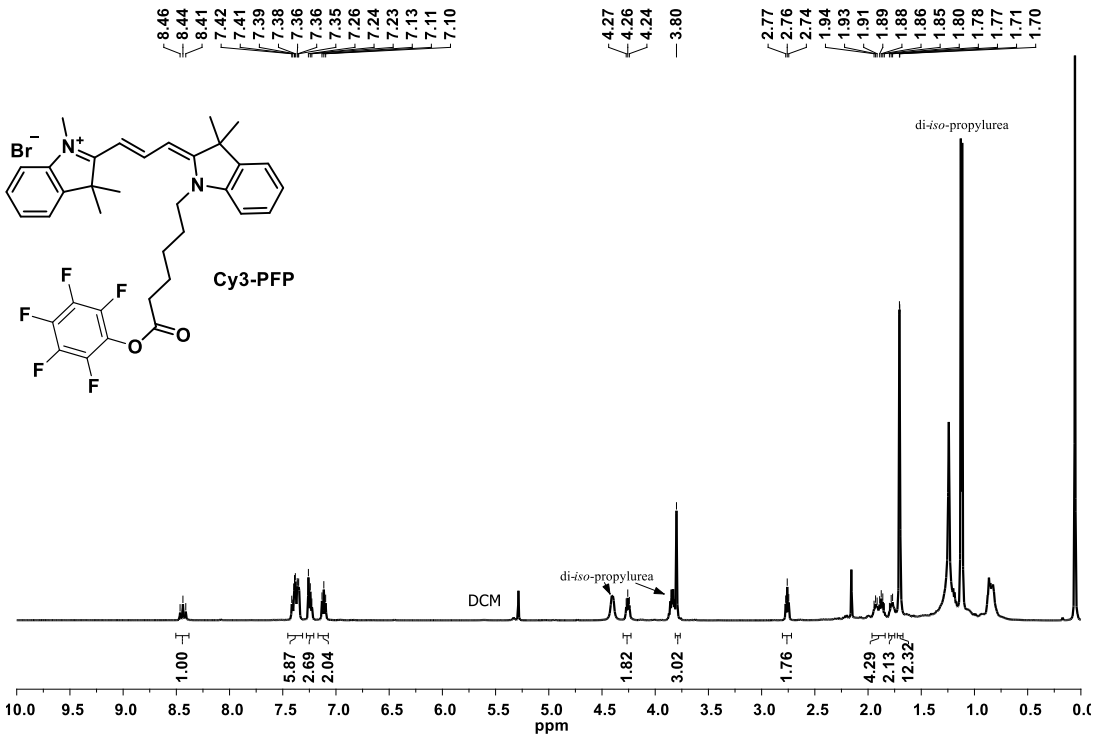


To a stirred solution of **Cy3** (189 mg, 0.32 mmol, 1.0 eq), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (184 mg, 0.96 mmol, 3.0 eq), 1-hydroxybenzotriazole hydrate (130 mg, 0.96 mmol, 3.0 eq), and di-*iso*-propylethylamine (244 μL, 1.28 mmol, 4.0 eq) in DMF (6 mL), was added ***cis*-CBDCA-(HO-pda)-Pt** (645 mg, 0.51 mmol, 1.6 eq).[5-7] The mixture was stirred at room temperature until LCMS analysis (*Agilent Infinity 1260* LC system, employing *Agilent Zorbax Eclipse Plus* column, C18, 3.5 μm particle size, 95 Å pore size, 4.6 mm × 100 mm; Method: 40 °C, flow rate = 1 mL · min^{-1}, H_2O:MeCN, 0.1% HCO_2H, 99:1, 5 min hold, gradient to 5:95 over 20 min, 1 min hold, gradient to 99:1 over 0.5 min, 1.5 min hold) indicated full conversion (46 h). The mixture was concentrated under vacuum to remove DMF. The residual dark red oil was dissolved in acetonitrile (3 mL) and the product precipitated by addition of cold diethyl ether (50 mL). After centrifuging (4000 rpm for 10 minutes), the supernatant was discarded, and the precipitate was purified by the same procedure for another 2 times to afford a red solid. The solid was then washed with deionized water (50 mL) and dried via freeze dryer to afford **Cy3-CPT** (143 mg, 0.14 mmol, 45%). **^{1}H NMR** (500 MHz, DMSO-d_6): δ = 8.34 (t, *J* = 13.5 Hz, 1H), 7.64 (d, *J* = 7.5 Hz, 2H), 7.47-7.40 (m, 4H), 7.32-7.27 (m, 2H), 6.47 (dd, *J* = 13.5 Hz, *J* = 9.5 Hz, 2H), 5.57 (br, 2H), 5.09 (br, 2H), 4.86 (br, 1H), 4.12 (t, *J* = 7.0 Hz, 2H), 3.66 (s, 3H), 3.32 (br, 2H), 2.69 (t, *J* = 7.5 Hz, 2H), 2.62 (t, *J* = 7.5 Hz, 2H), 2.38 (t, *J* = 7.0 Hz, 2H), 1.80-1.74 (m, 2H), 1.70 (s, 12H), 1.66-1.60 (m, 4H), 1.60-1.53 (2H), 1.47-1.39 (m, 2H) ppm; **^{13}C NMR** (126 MHz, DMSO-d_6): δ = 177.4, 174.5, 173.6, 171.8, 149.7, 142.6, 141.9, 140.6, 140.5, 128.7, 128.6, 125.3, 125.1, 122.5, 122.4, 111.6, 111.4, 102.9, 102.3, 69.2, 55.6, 48.9 (d, *J* = 6.0 Hz), 44.6, 43.7, 33.2, 31.5, 30.7, 30.1, 28.9, 27.5, 27.3, 26.7, 25.7, 24.7, 23.8, 15.0 ppm; **HRMS:** calcd. for $C_{39}H_{51}N_4O_6{}^{194}Pt$ $[M]^+$ 865.3435, found 865.3428.

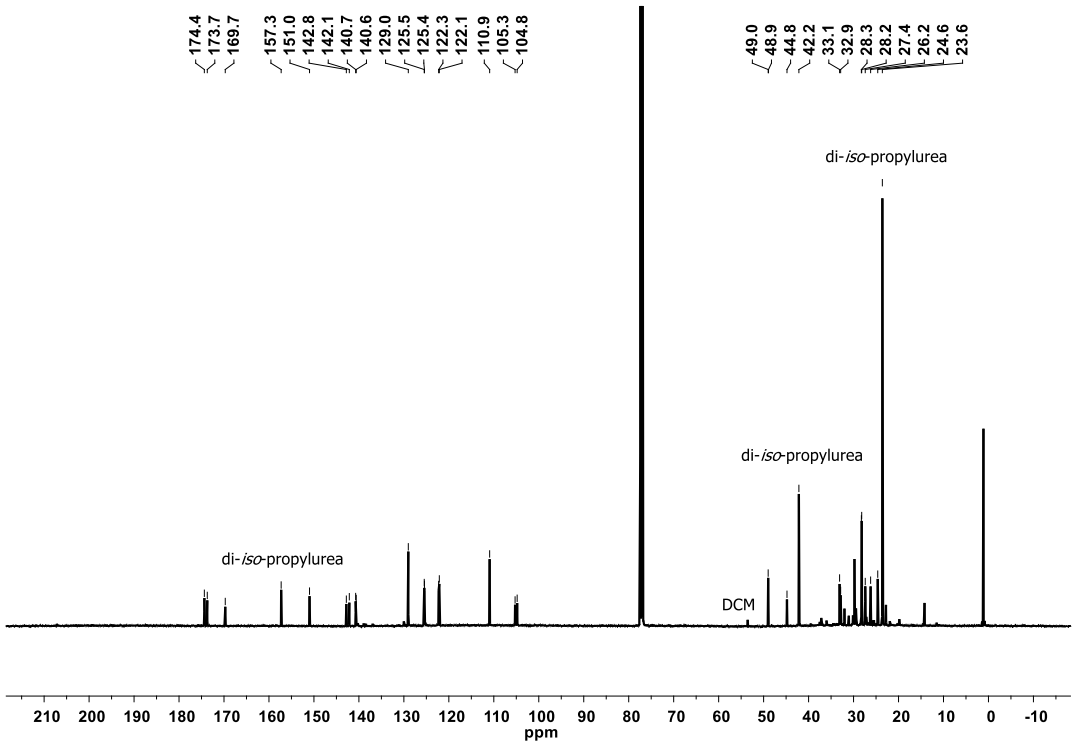
3. Analytical Data

Cy3-PFP

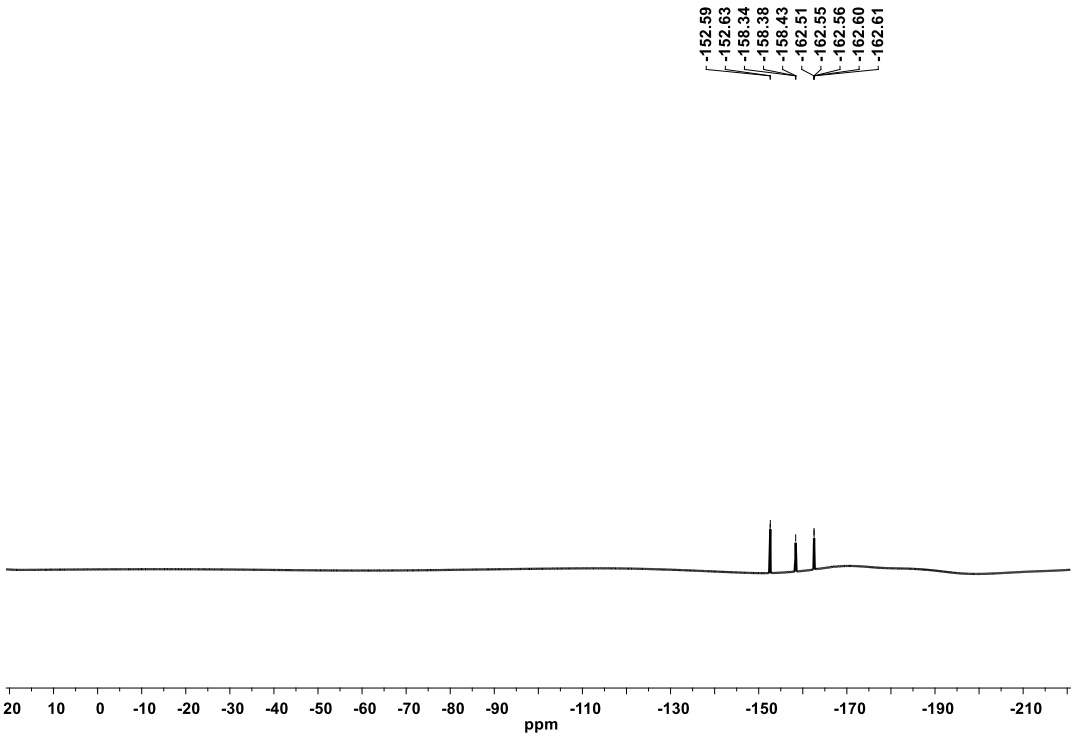
^{1}H-NMR



^{13}C-NMR

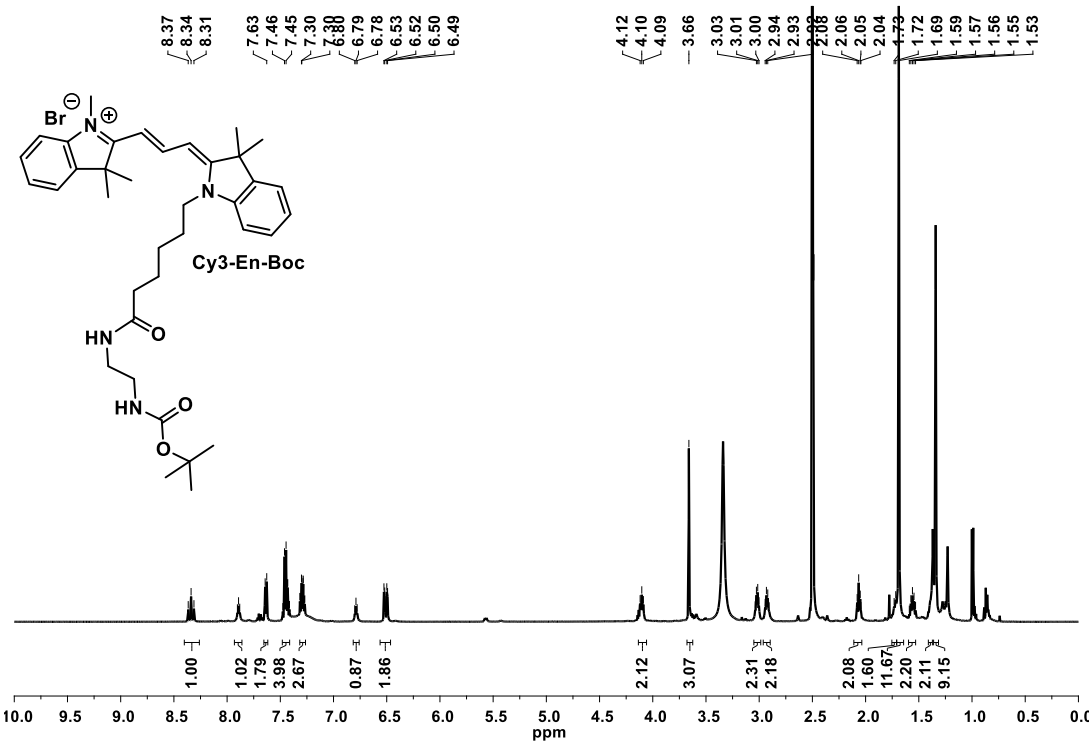


^{19}F-NMR


-152.59
-152.63
-158.34
-158.38
-158.43
-162.51
-162.55
-162.56
-162.60
-162.61
20 10 0 -10 -20 -30 -40 -50 -60 -70 -80 -90 -110 -130 -150 -170 -190 -210
ppm

Cy3-mono-*N*-Boc-ethylenediamine

^{1}H-NMR



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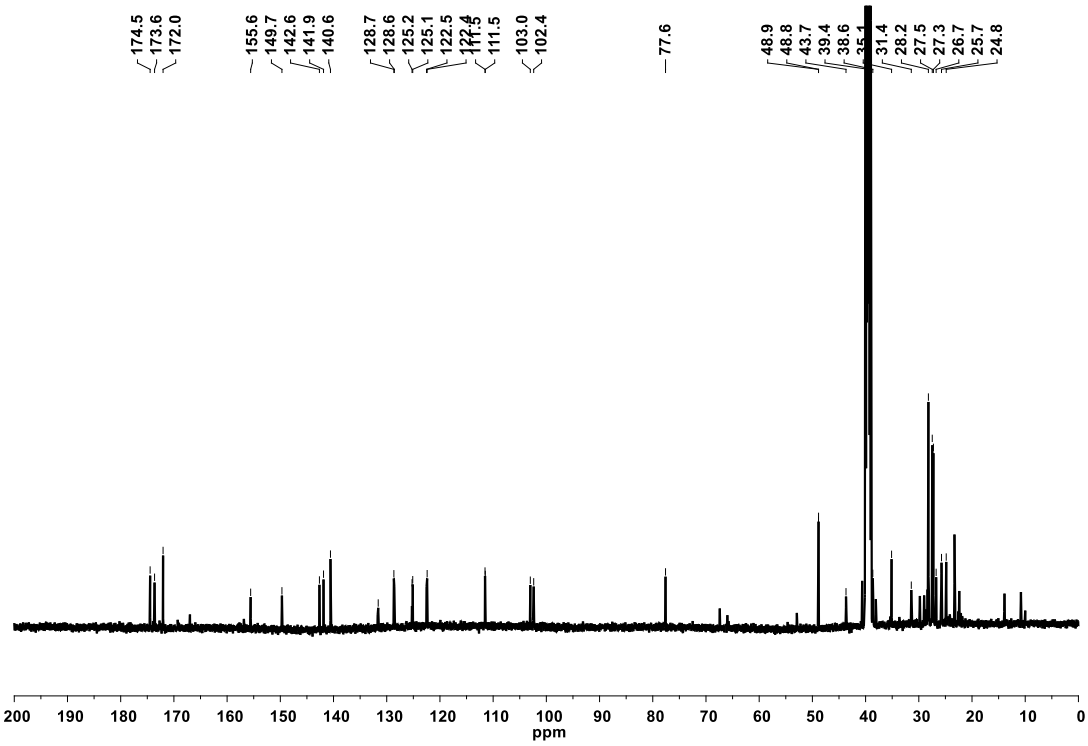
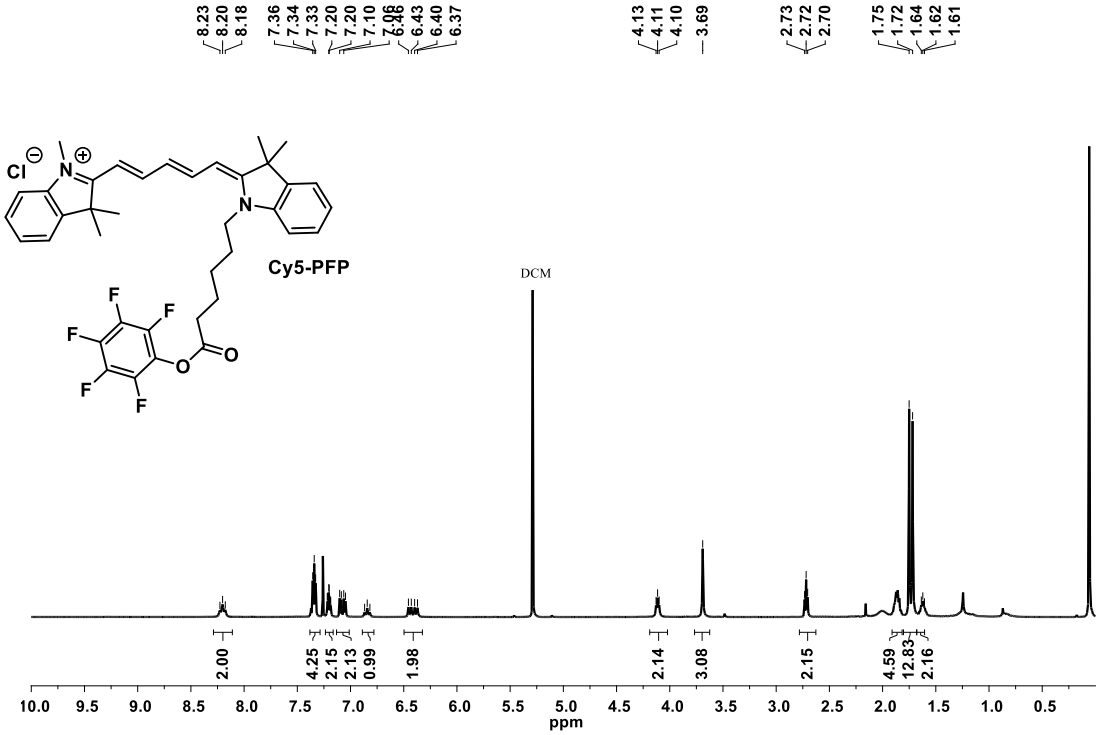
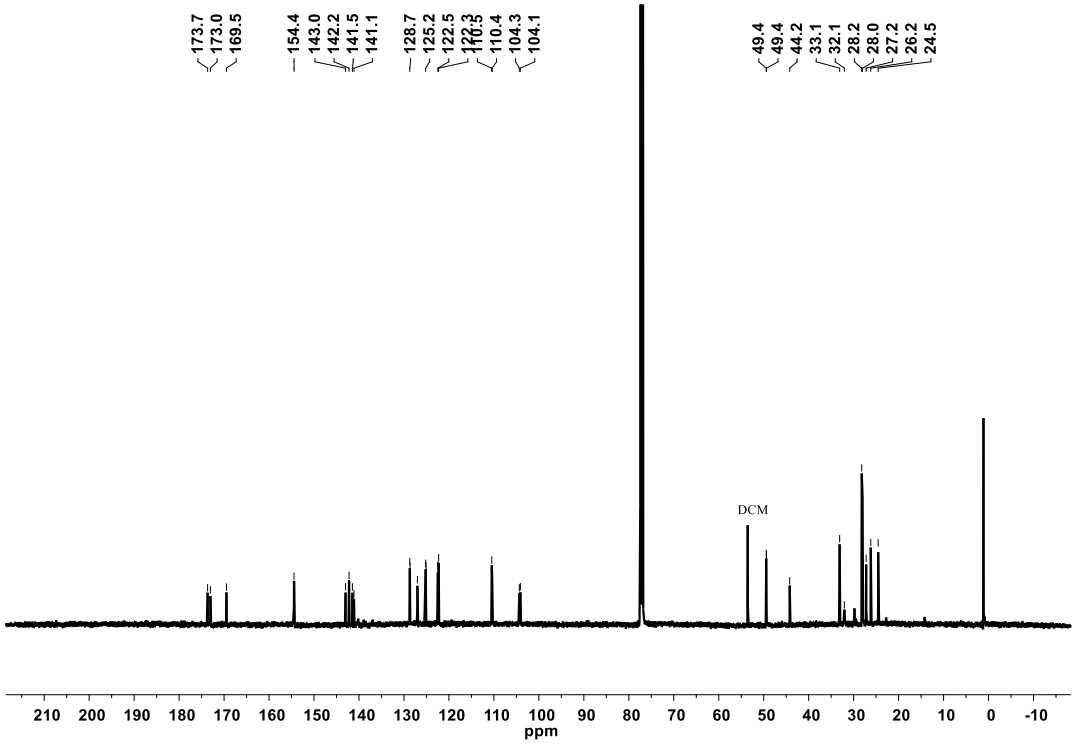


Cy5-PFP

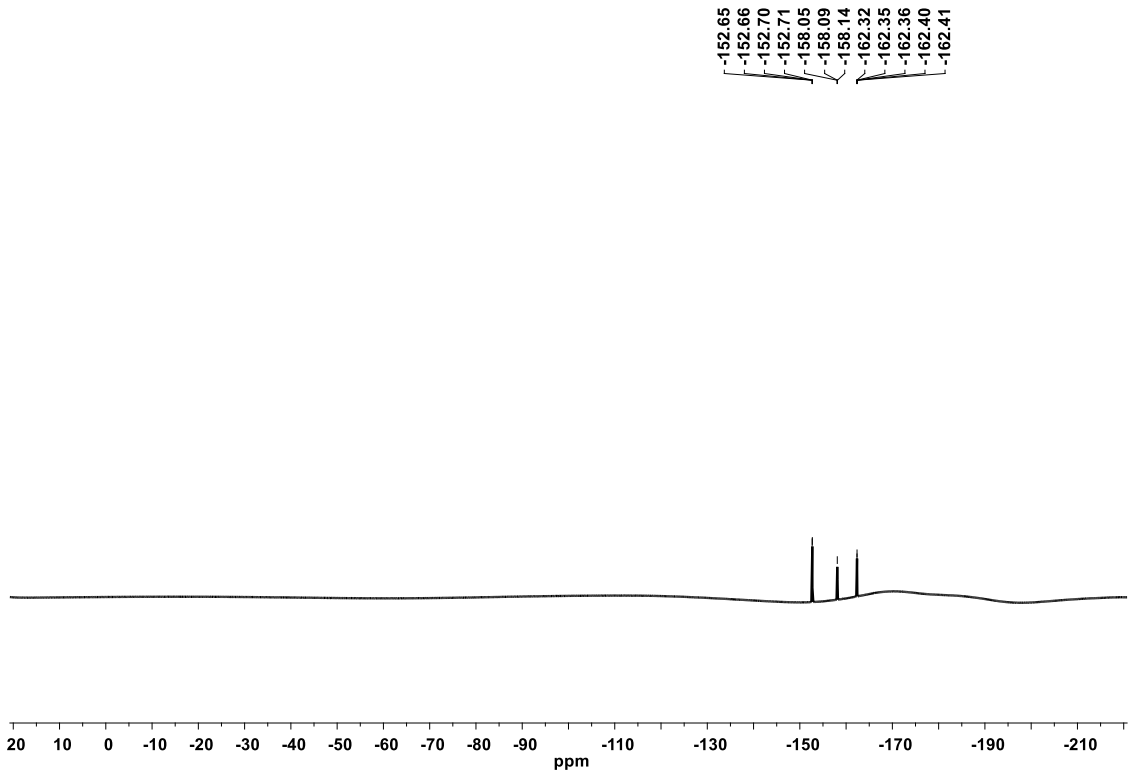
^{1}H-NMR



^{13}C-NMR



^{19}F-NMR


-152.65
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-158.05
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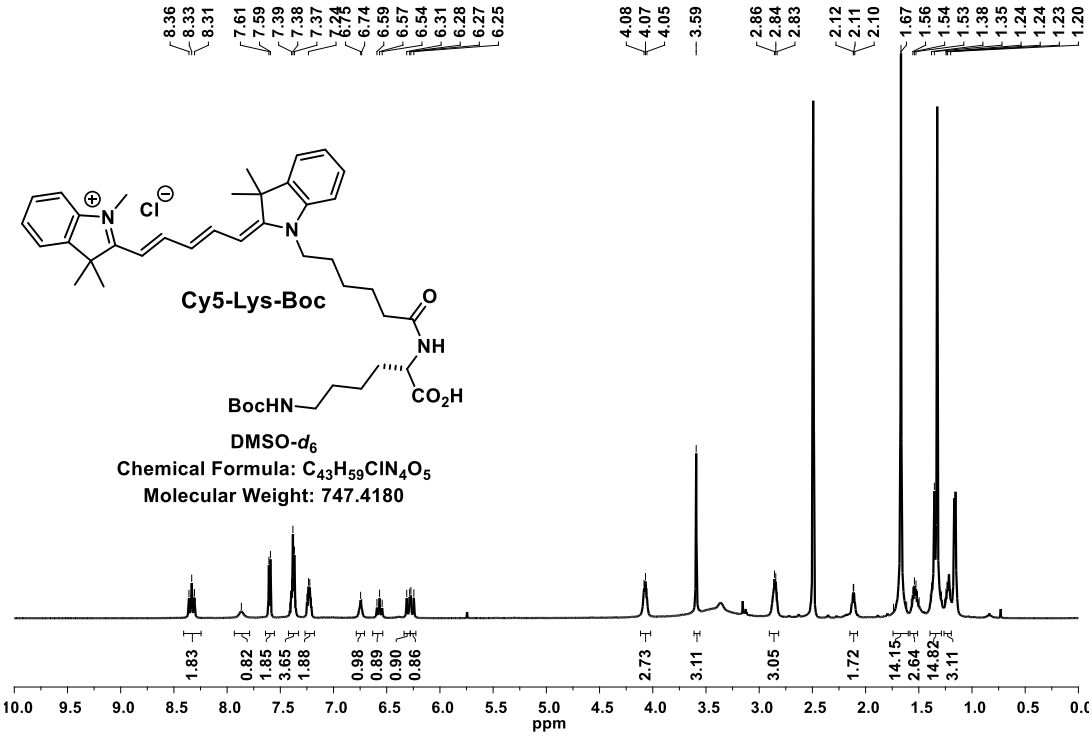
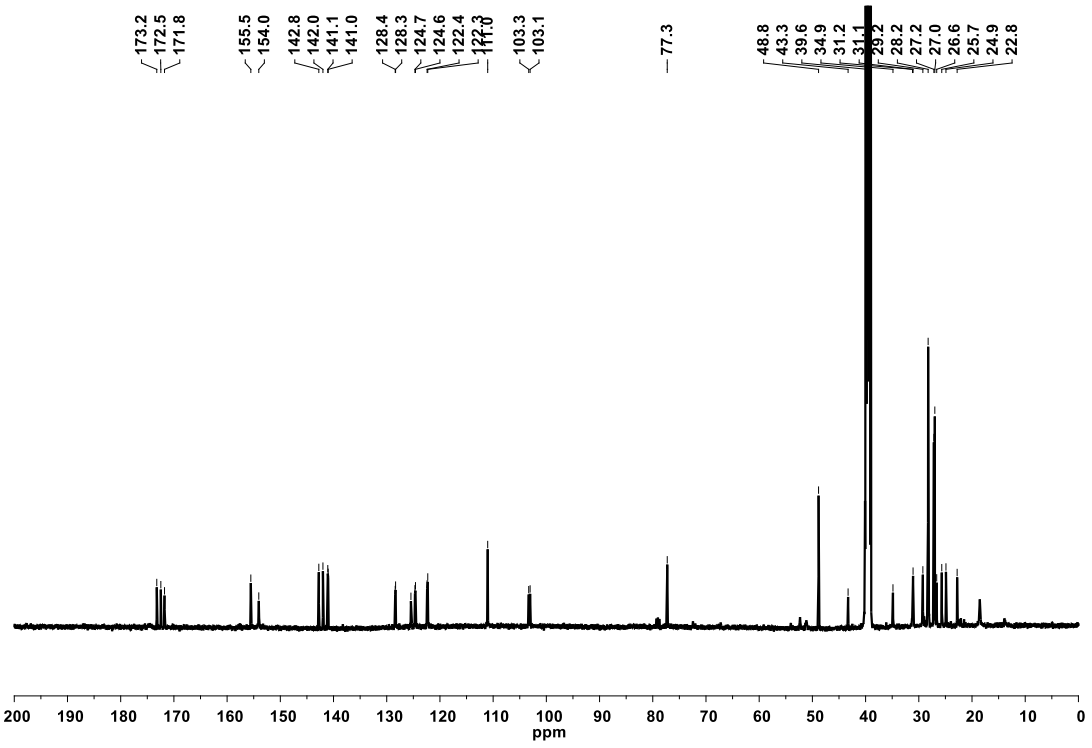
-158.14
-162.32
-162.35
-162.36
-162.40
-162.41
20
10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-110
-130
-150
-170
-190
-210
ppm

Cy5-ε-*N*-Boc-(L)-lysine

^{1}H-NMR

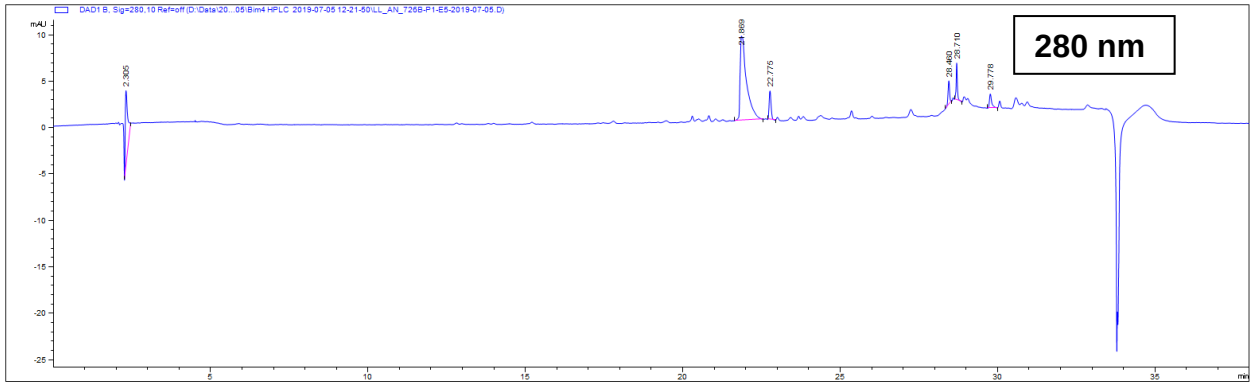


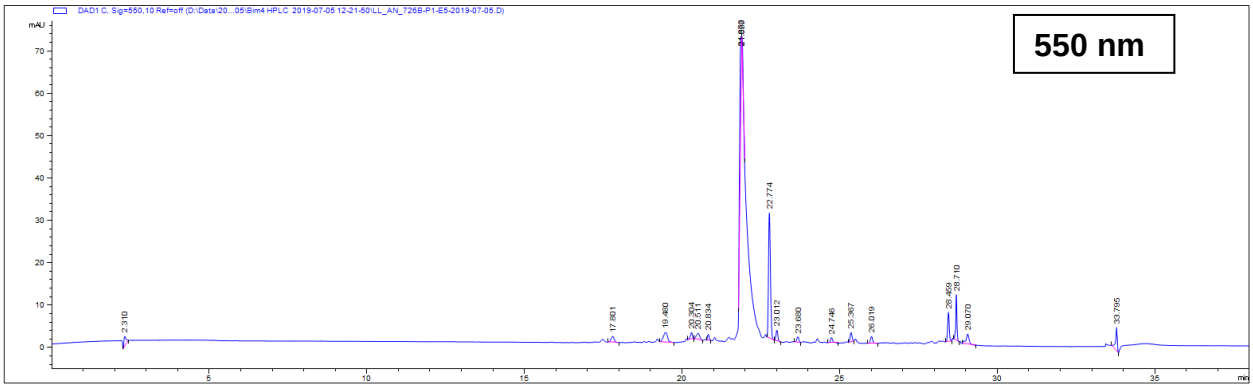
^{13}C-NMR

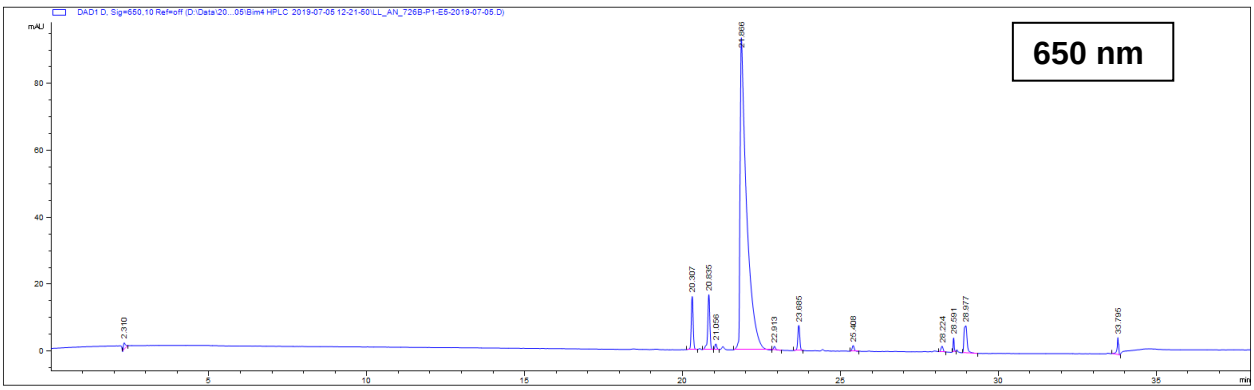


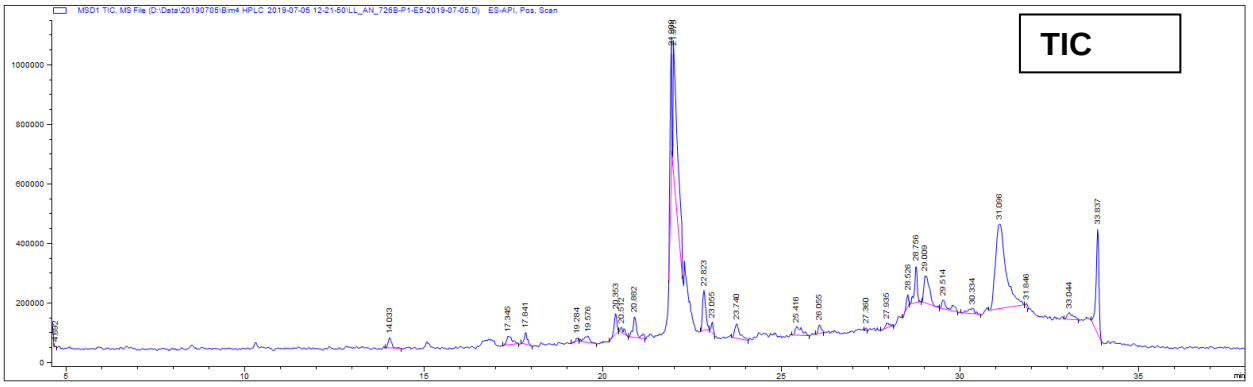
Cy3-Cy5-Boc

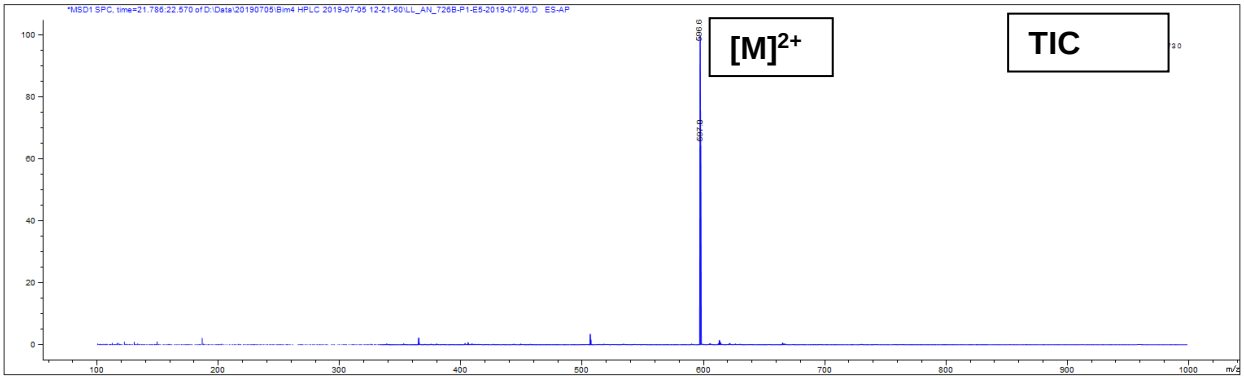
LCMS







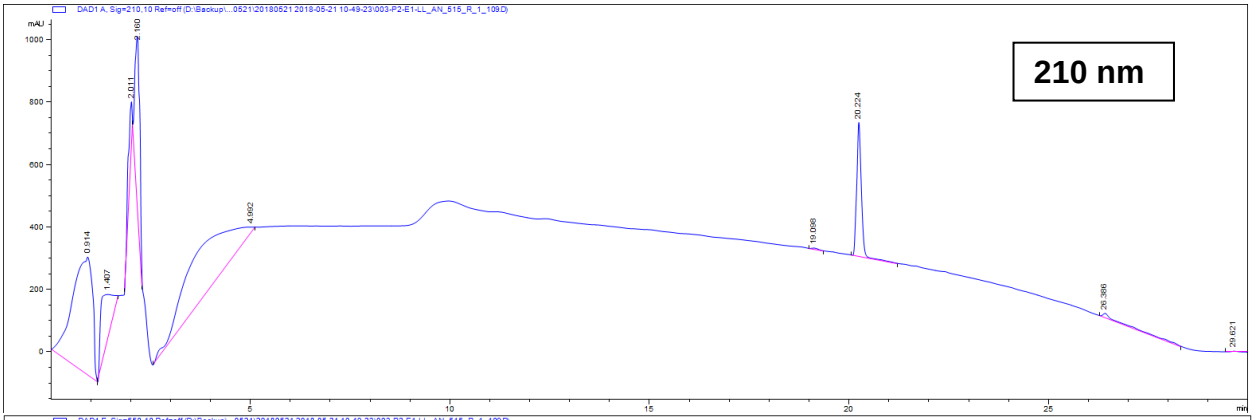


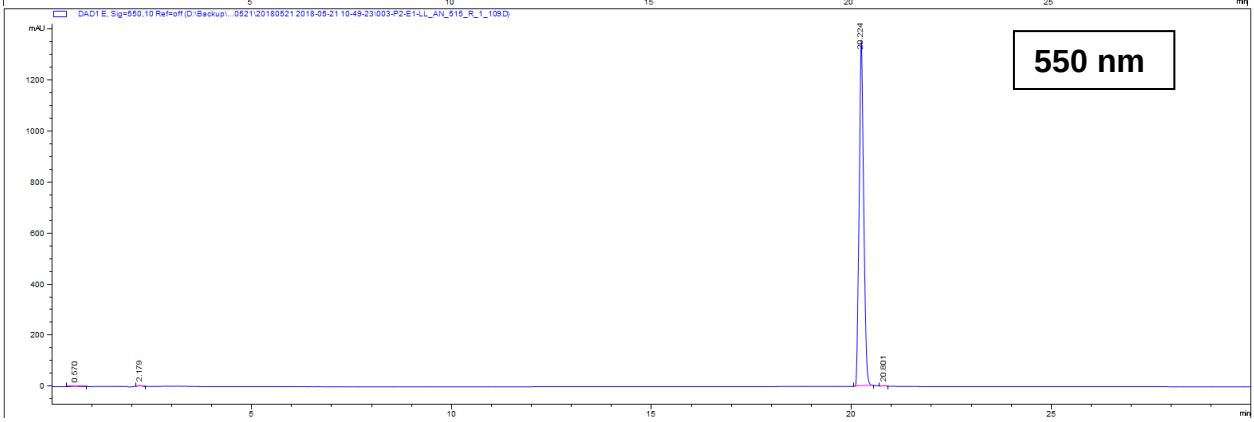


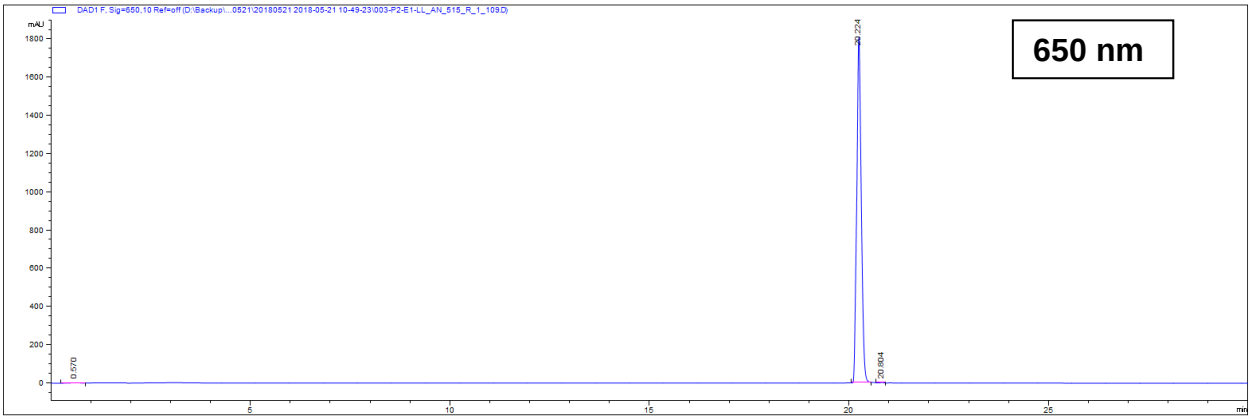
210 nm, 550 nm, 650 nm and Total Ion Current (TIC) chromatograms recorded on the *Agilent Infinity 1260* LC system, employing *Agilent Poroshell 120 EC-C18* column, C18, 3.5 µm particle size, 95 Å pore size, 4.6 mm x 100 mm; method: 40 °C, flow rate = 0.5 mL · min^{-1}, H_2O:MeCN, 0.1% HCO_2H, 85:15, 1 min hold, gradient to 20:80 over 24 min, gradient to 5:95 over 0.1 min, 4.9 min hold.

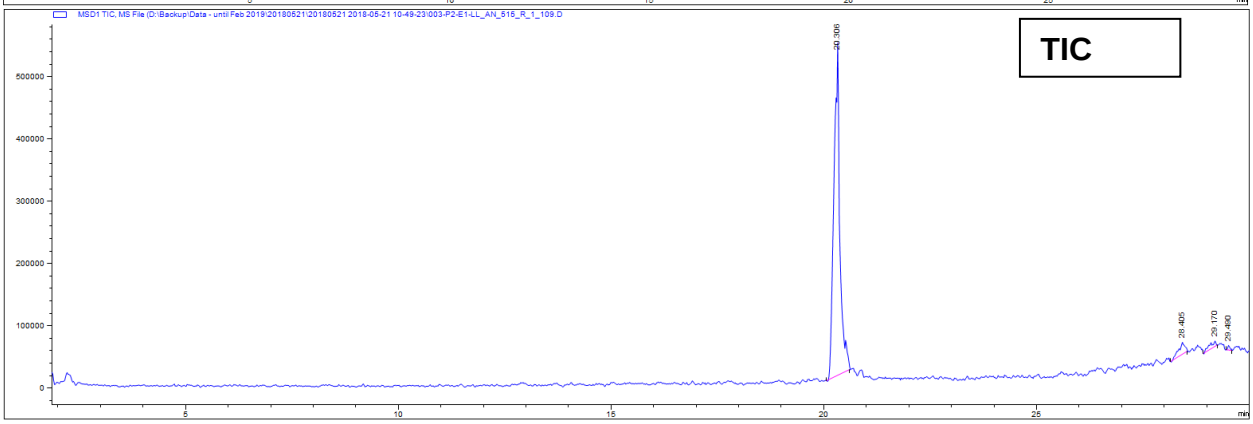
Cy3-Cy5

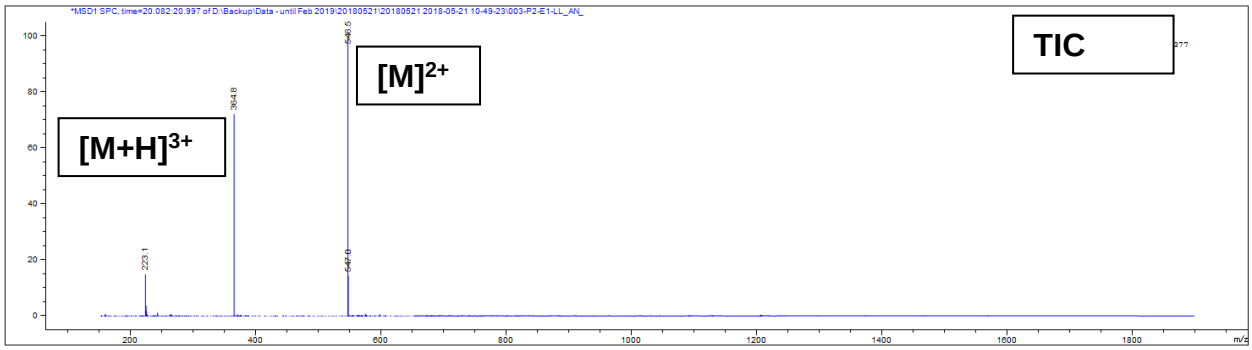
LCMS







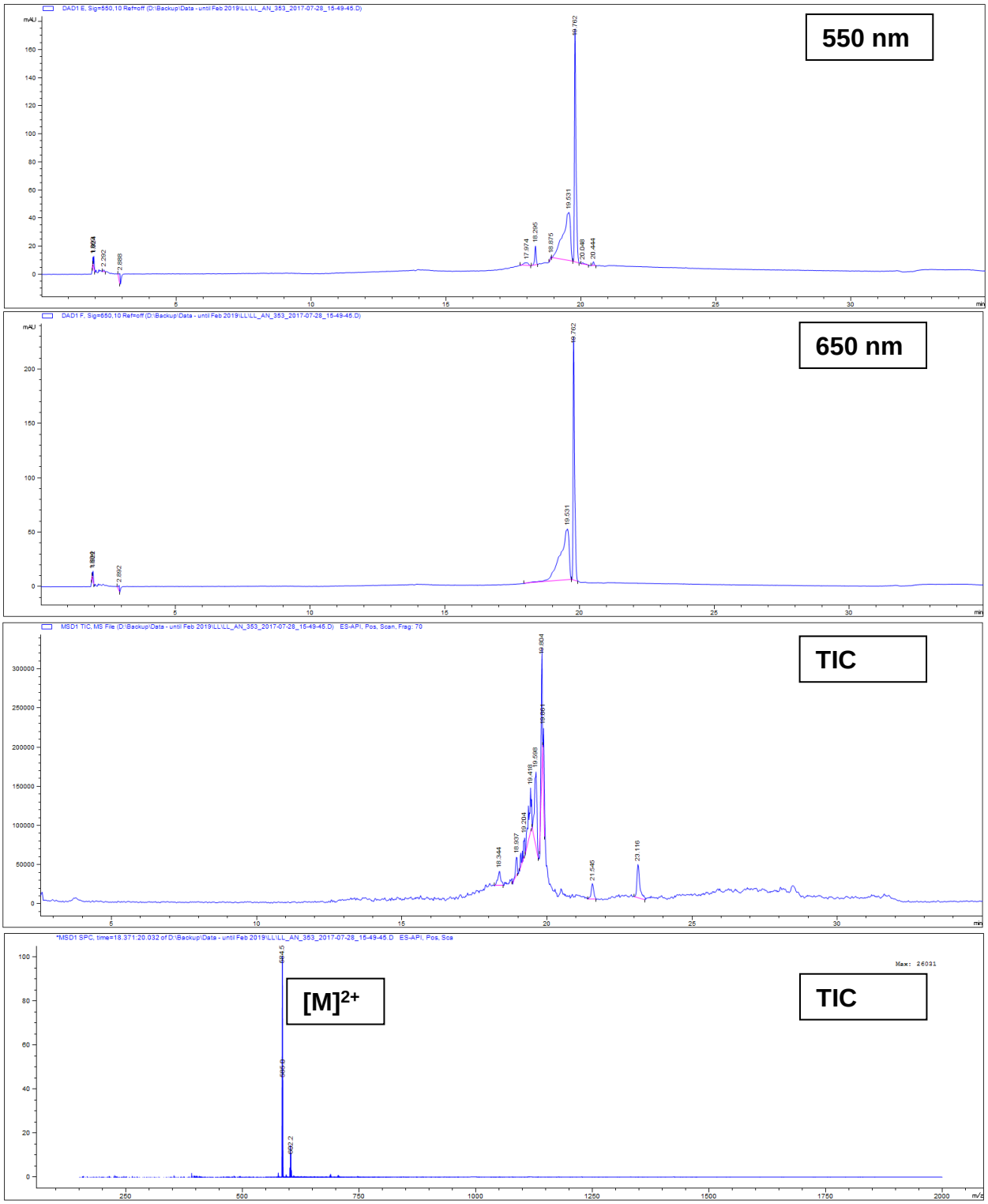




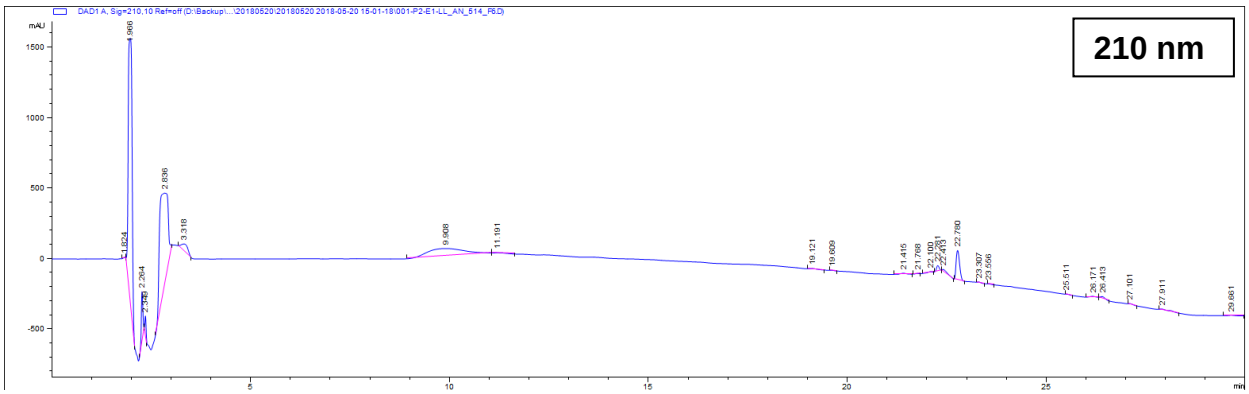
210 nm, 550 nm, 650 nm and Total Ion Current (TIC) chromatograms recorded on the *Agilent Infinity 1260* LC system, employing *Agilent Zorbax Eclipse Plus* column, C18, 3.5 µm particle size, 95 Å pore size, 4.6 mm x 100 mm; method: 40 °C, flow rate = 0.5 mL · min^{-1}, H_2O:MeCN, 0.1% HCO_2H, 95:5, 5 min hold, gradient to 5:95 over 20 min, 2.5 min hold.

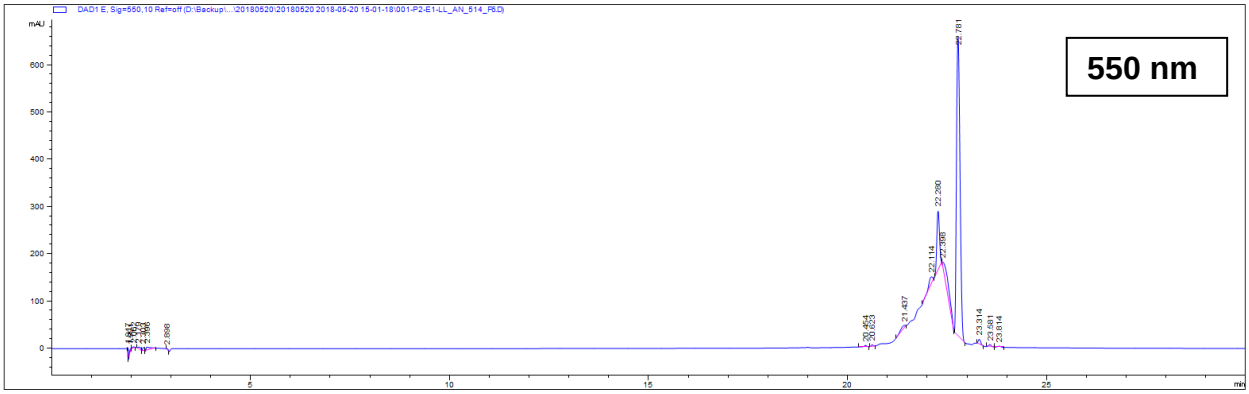
Cy3-Cy5-CA

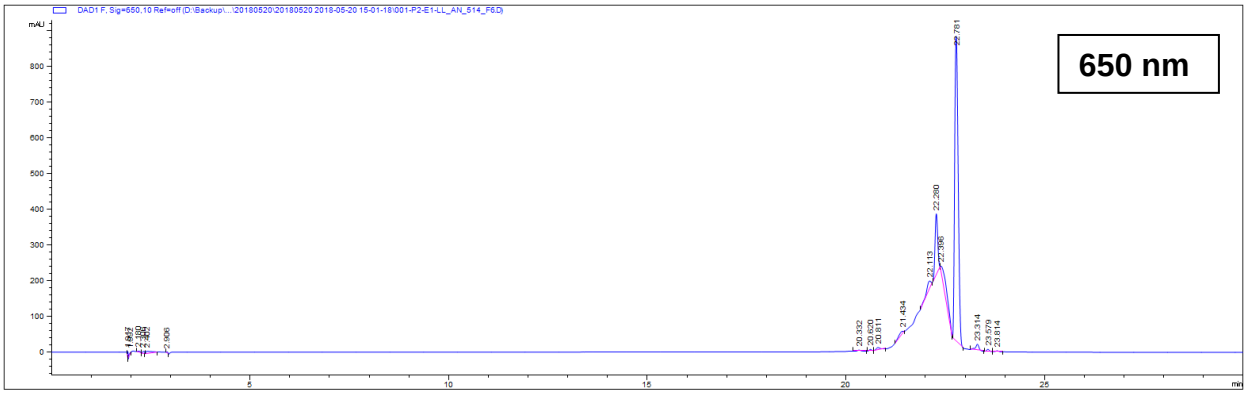
LCMS

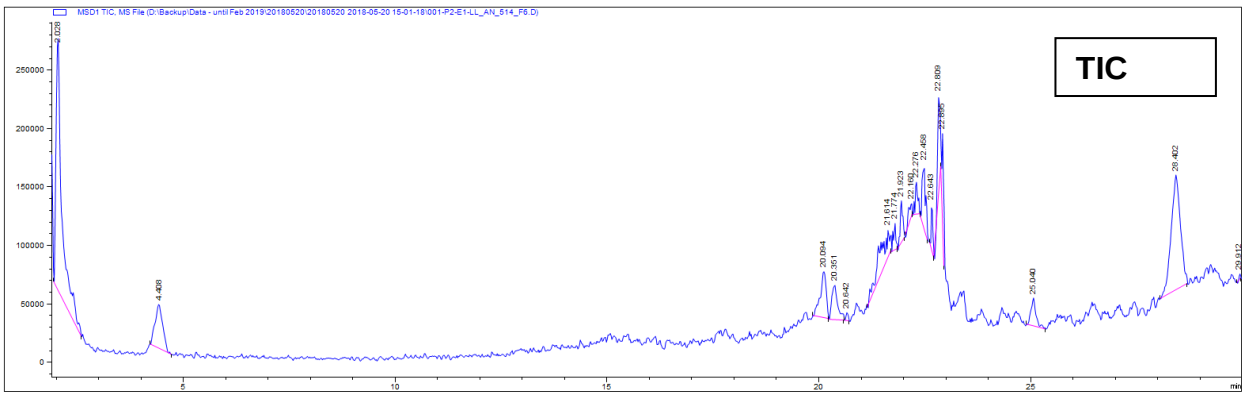


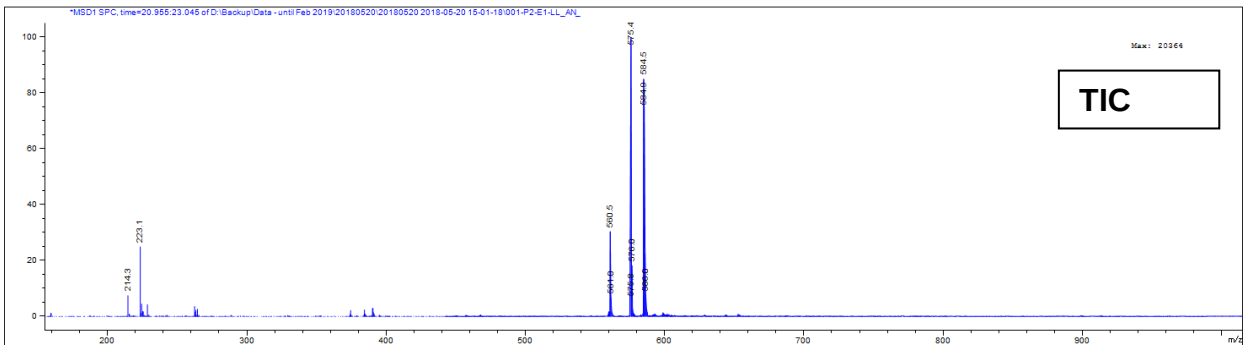
550 nm, 650 nm and Total Ion Current (TIC) chromatograms recorded on the *Agilent Infinity 1260* LC system, employing *Agilent Zorbax Eclipse Plus* column, C18, 3.5 µm particle size, 95 Å pore size, 4.6 mm x 100 mm; method: 40 °C, flow rate = 0.5 mL · min^{-1}, H_2O:MeCN, 0.1% HCO_2H, 95:5, 5 min hold, gradient to 5:95 over 20 min, 2.5 min hold. Purified sample **Cy3-Cy5-CA**.


210 nm


550 nm


650 nm

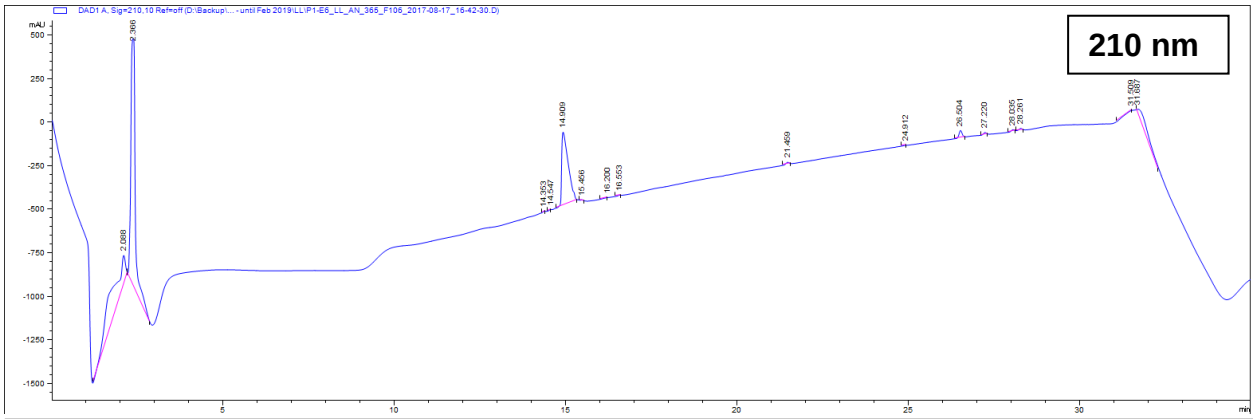

TIC

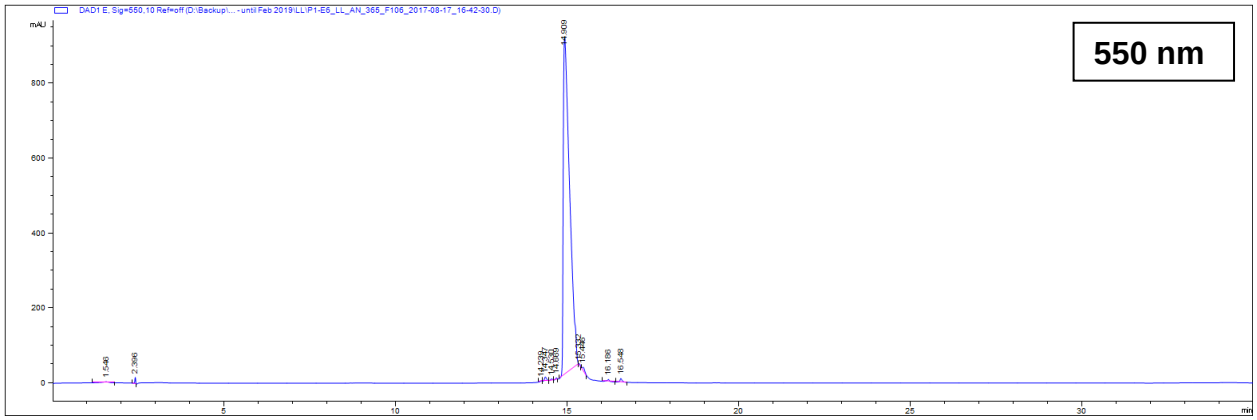

TIC

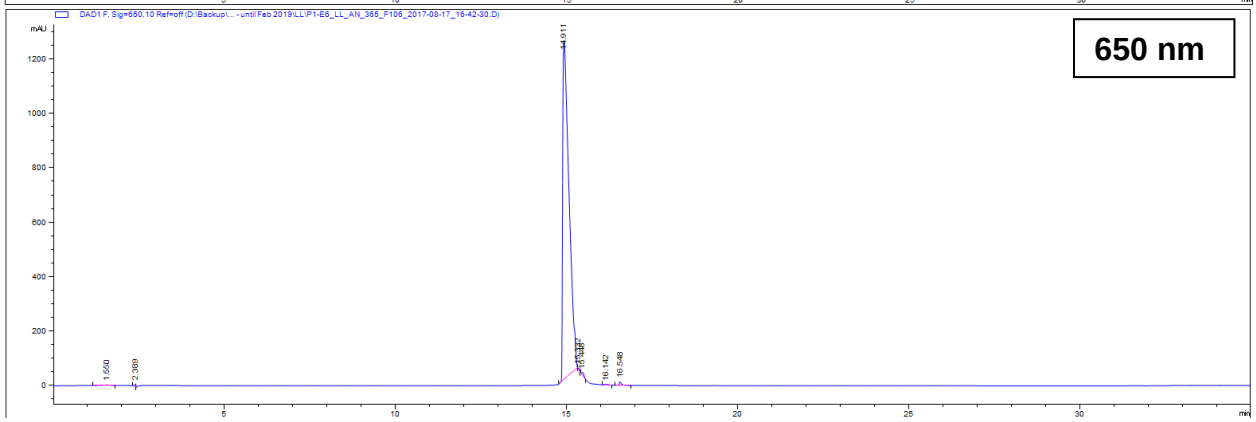
210 nm, 550 nm, 650 nm and Total Ion Current (TIC) chromatograms recorded on the *Agilent Infinity 1260* LC system, employing *Agilent Zorbax Eclipse Plus* column, C18, 3.5 µm particle size, 95 Å pore size, 4.6 mm x 100 mm; method: 40 °C, flow rate = 0.5 mL · min^{-1}, H_2O:MeCN, 0.1% HCO_2H, 95:5, 5 min hold, gradient to 5:95 over 20 min, 2.5 min hold. Crude sample **Cy3-Cy5-CA**.

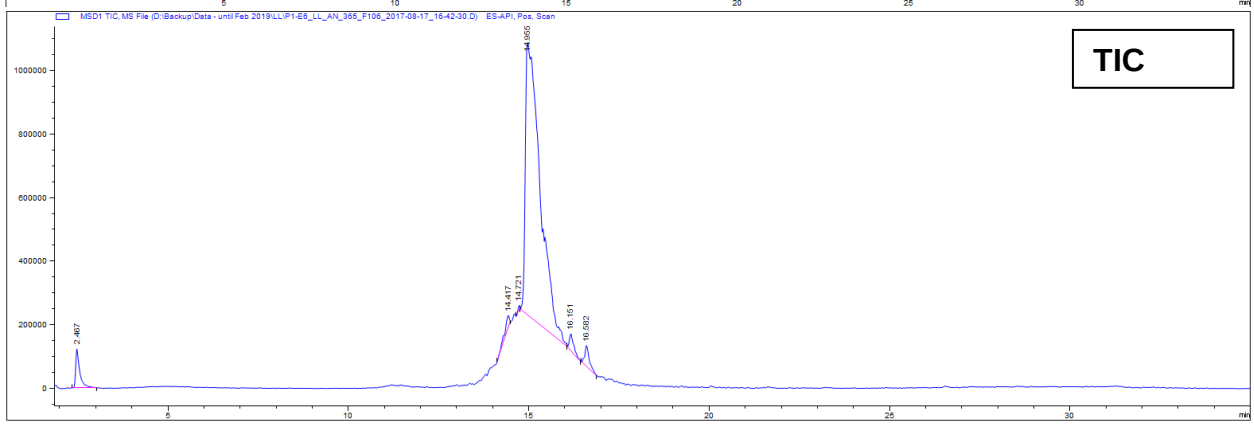
Cy3-Cy5-R8

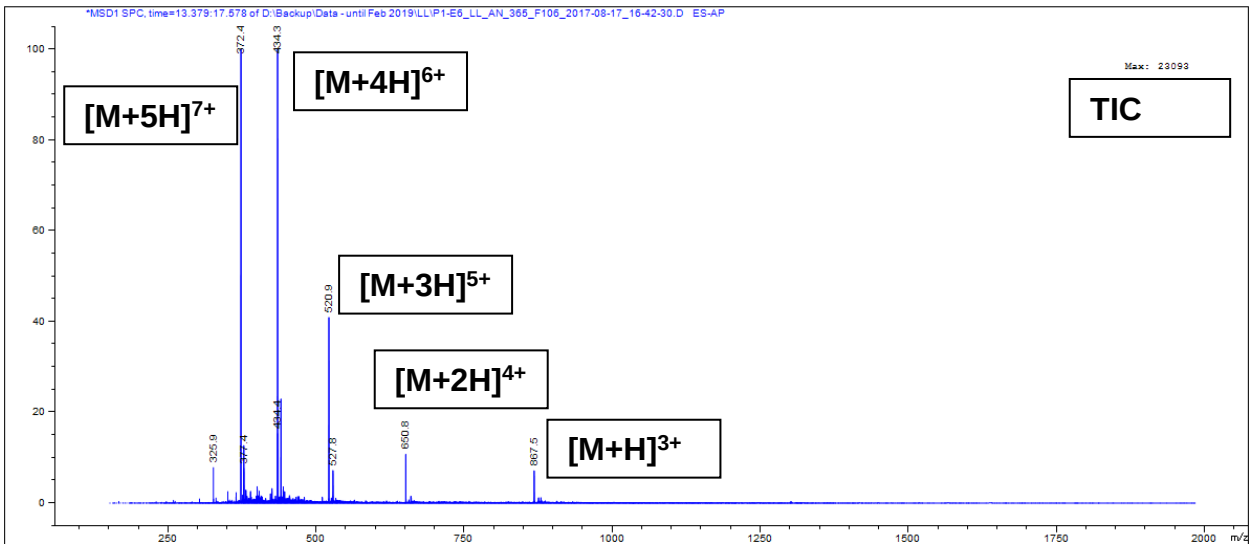
LCMS







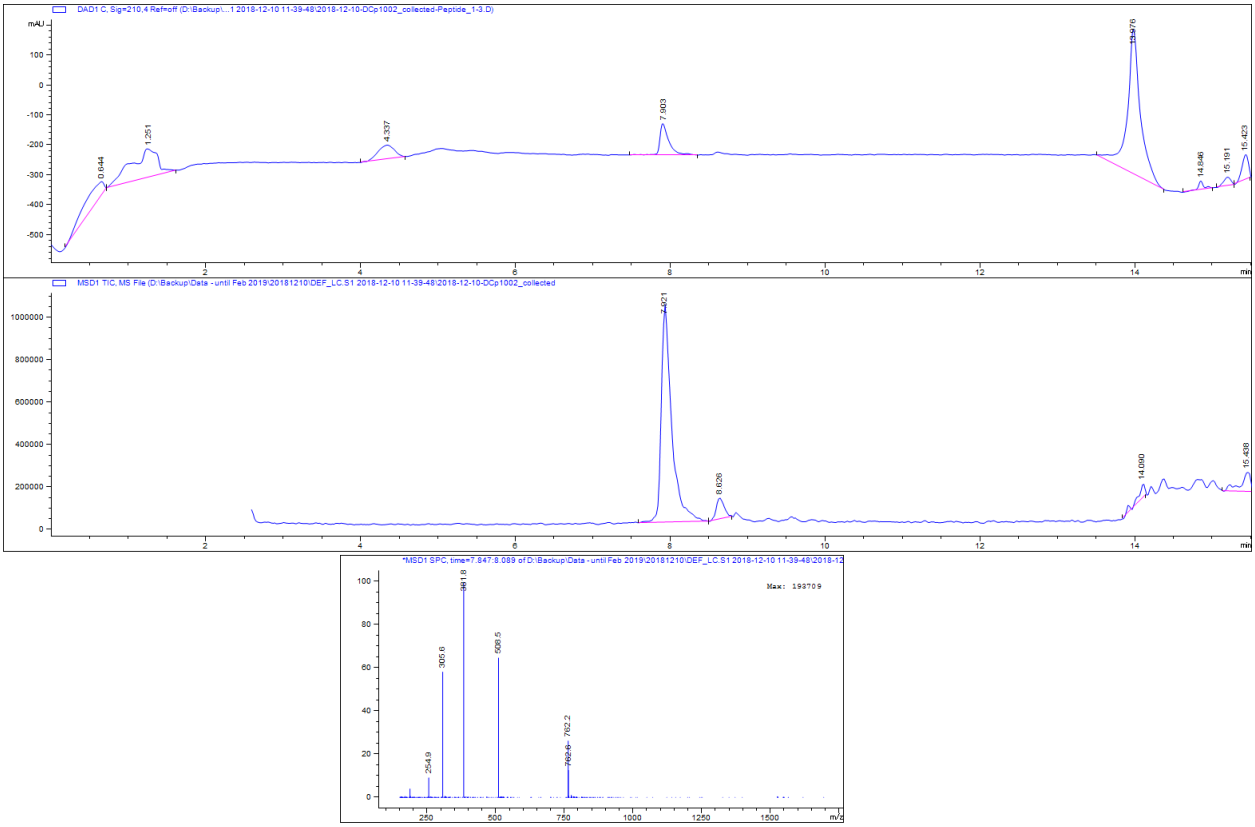




550 nm, 650 nm and Total Ion Current (TIC) chromatograms recorded on the *Agilent Infinity 1260* LC system, employing *Agilent Zorbax Eclipse Plus* column, C18, 3.5 µm particle size, 95 Å pore size, 4.6 mm x 100 mm; Method: 40 °C, flow rate = 0.5 mL · min^{-1}, H_2O:MeCN, 0.1% HCO_2H, 95:5, 5 min hold, gradient to 5:95 over 20 min, 2.5 min hold.

KLA

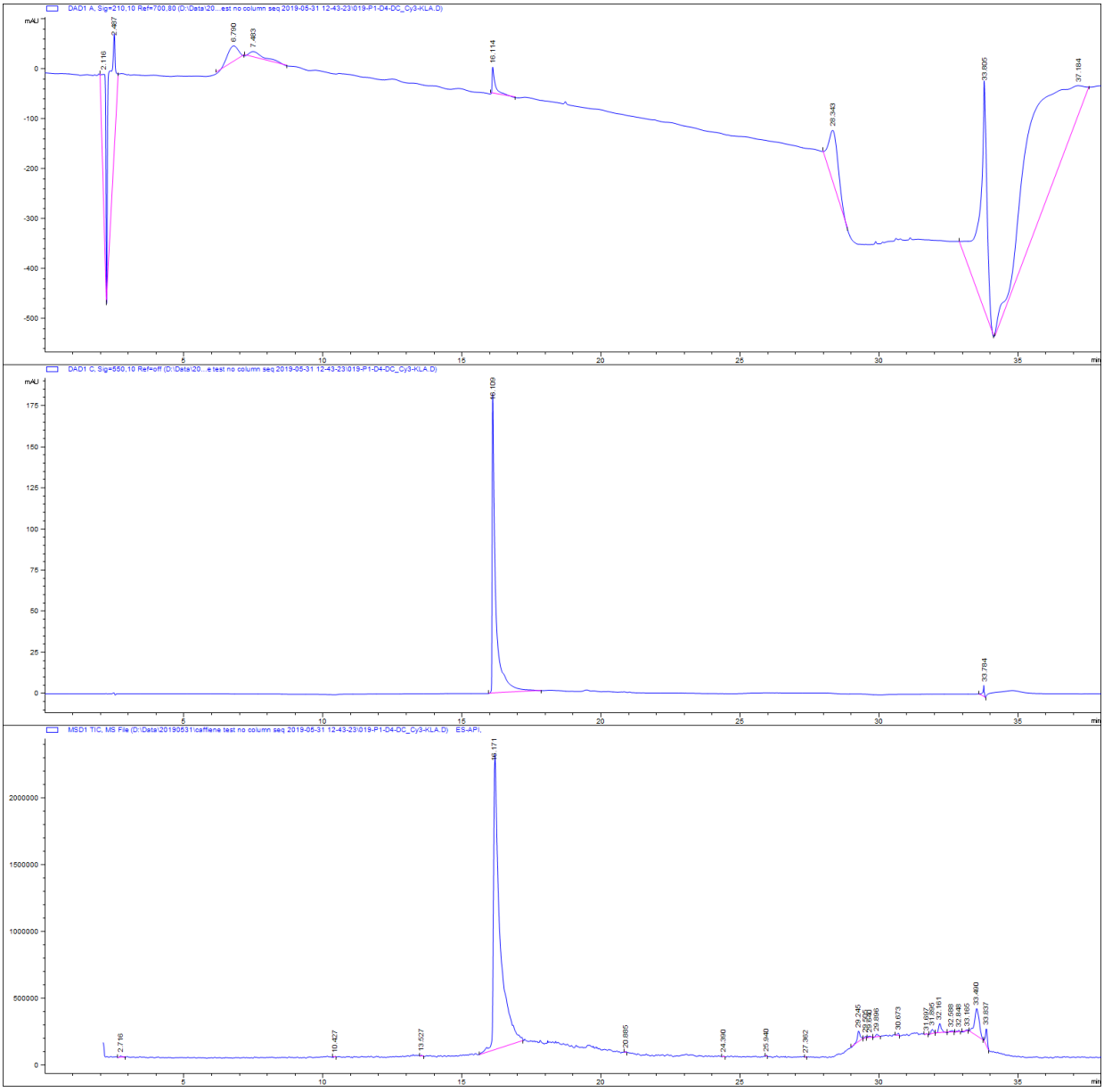
LCMS

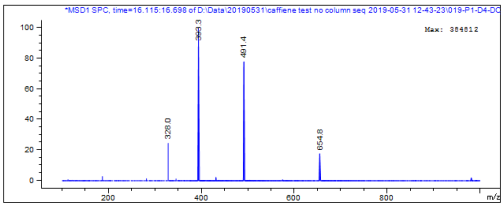


210 nm, 550 nm and Total Ion Current (TIC) chromatograms recorded on the *Agilent Infinity 1260* LC system, employing *Agilent Zorbax Eclipse Plus column,* C18, 3.5 µm particle size, 95 Å pore size, 4.6 mm × 100 mm; Method = 40 °C, flow rate = 0.5 mL · min^{-1}, H_2O:MeCN, 0.1% HCOOH, 99:1, 2 min hold, gradient to 35:65 over 22 min.

Cy3-KLA

LCMS

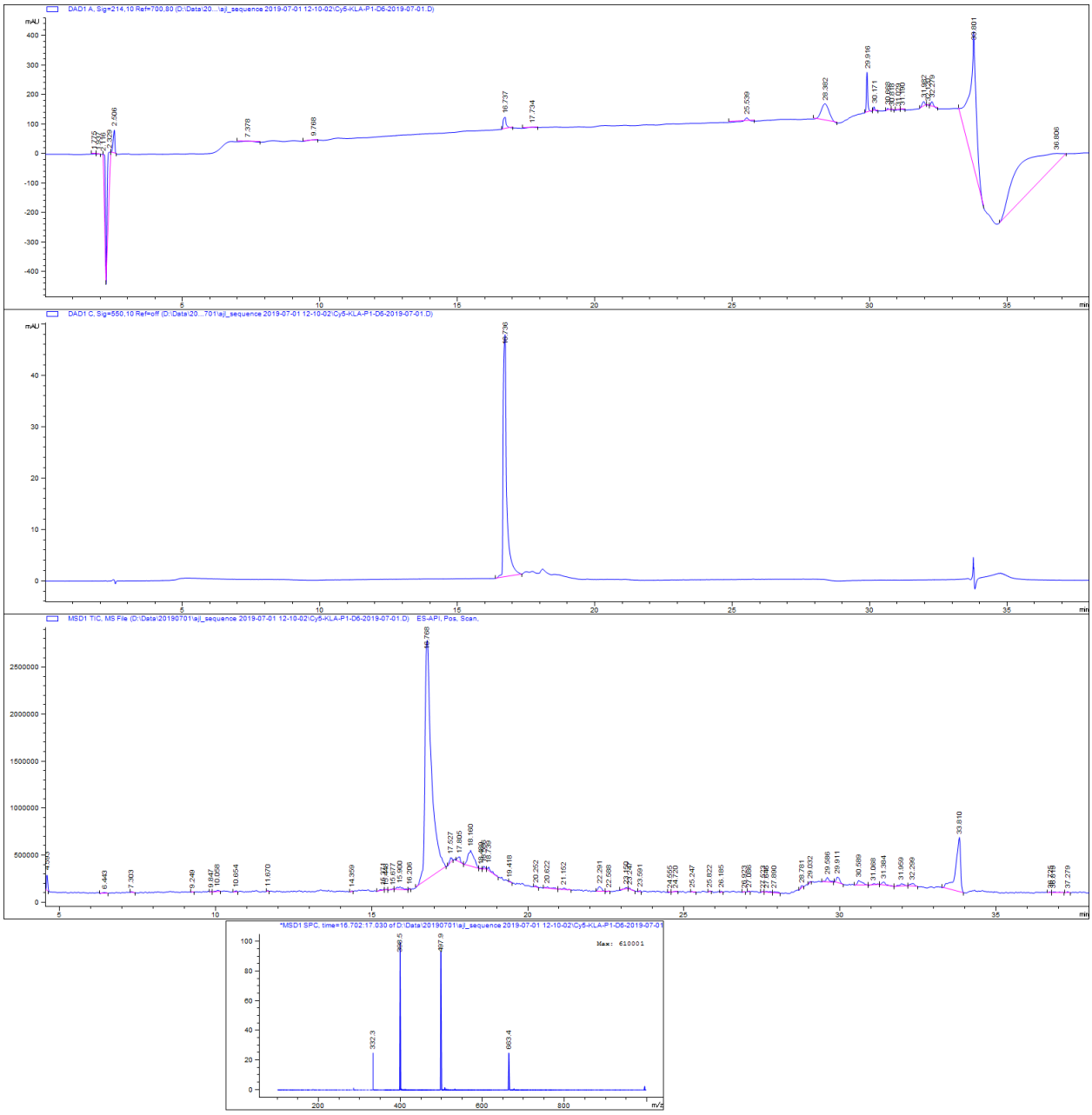




210 nm, 550 nm and Total Ion Current (TIC) chromatograms recorded on the *Agilent Infinity 1260* LC system, employing *Agilent Poroshell 120 EC-C18* column, 4 µm particle size, 120 Å pore size, 4.6 mm × 100 mm; Method = 40 °C, flow rate = 0.5 mL · min^{-1}, H2O:MeCN, 0.1% HCOOH, 95:5, 2 min hold, gradient to 40:60 over 23 min.

Cy5-KLA

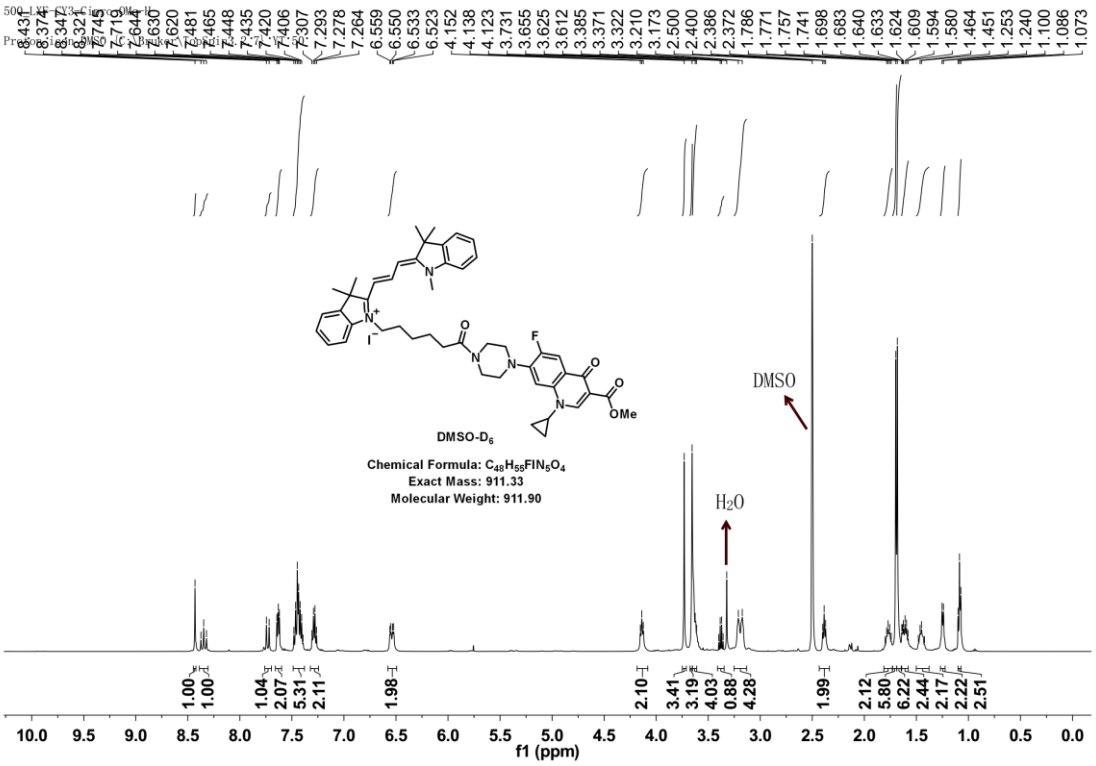
LCMS



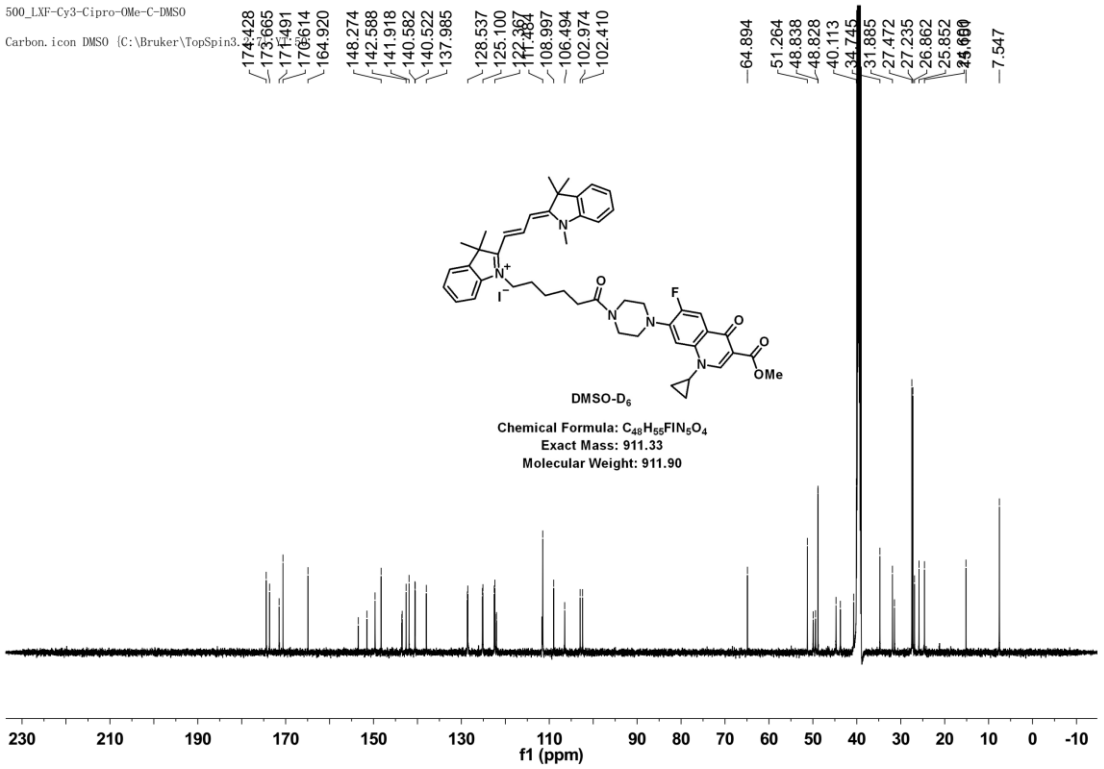
210 nm, 550 nm and Total Ion Current (TIC) chromatograms recorded on the *Agilent Infinity 1260* LC system, employing *Agilent Poroshell 120 EC-C18* column, 4 µm particle size, 120 Å pore size, 4.6 mm × 100 mm; Method = 40 °C, flow rate = 0.5 mL · min^{-1}, H2O:MeCN, 0.1% HCOOH, 95:5, 2 min hold, gradient to 40:60 over 23 min.

Cy3-Cip-OMe

^{1}H-NMR

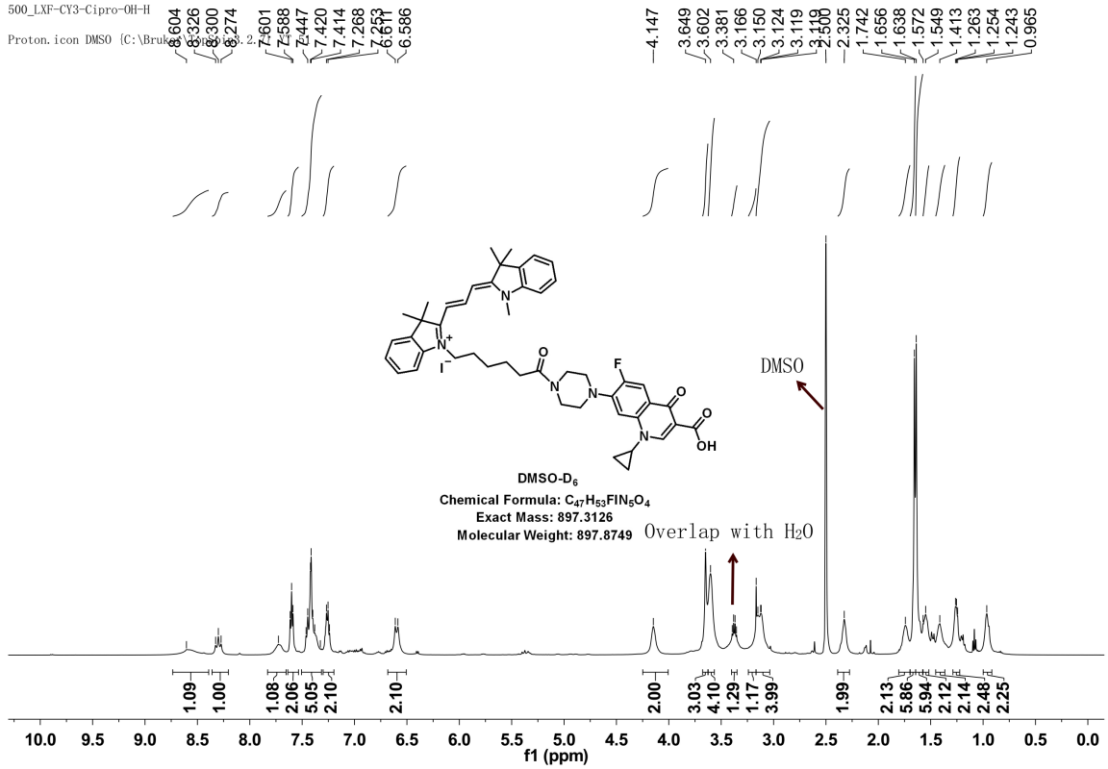


^{13}C-NMR

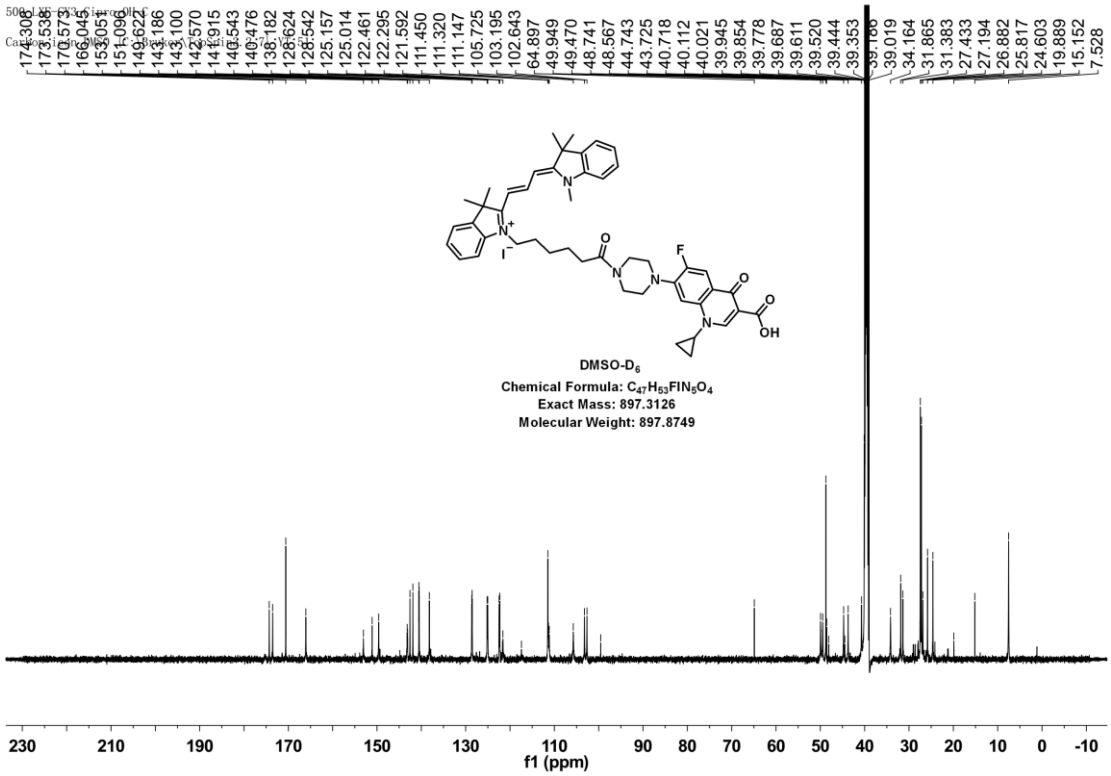


Cy3-Cip

^{1}H-NMR

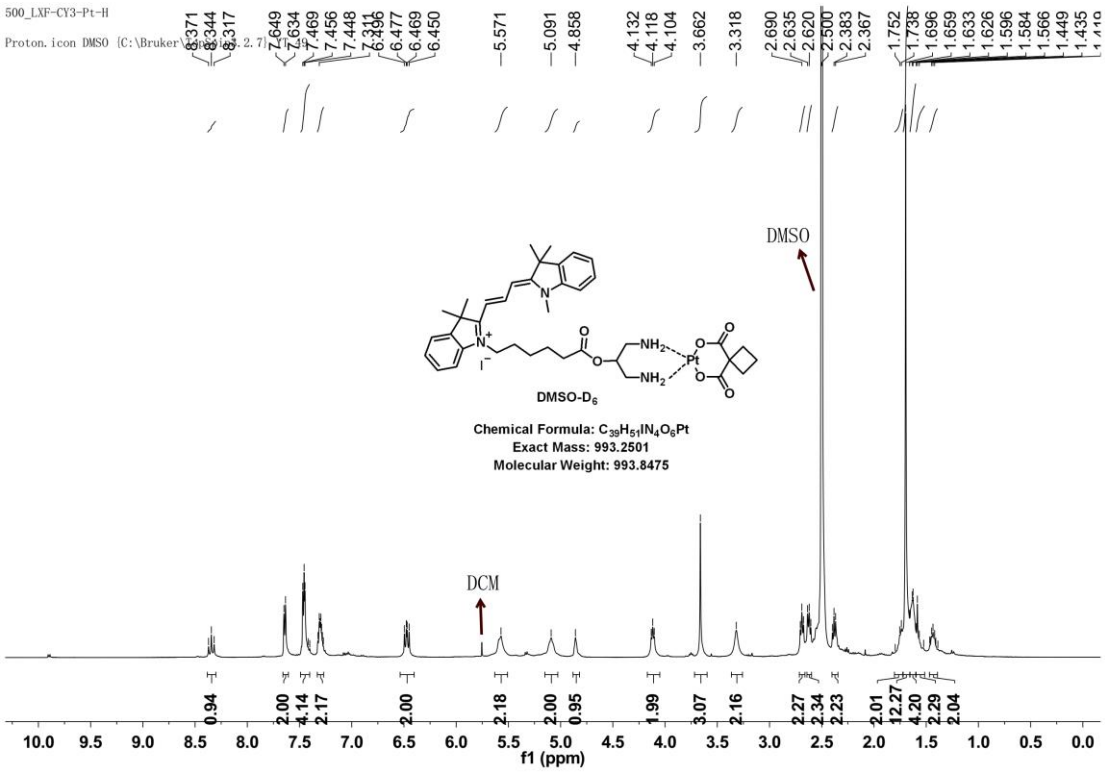


^{13}C-NMR

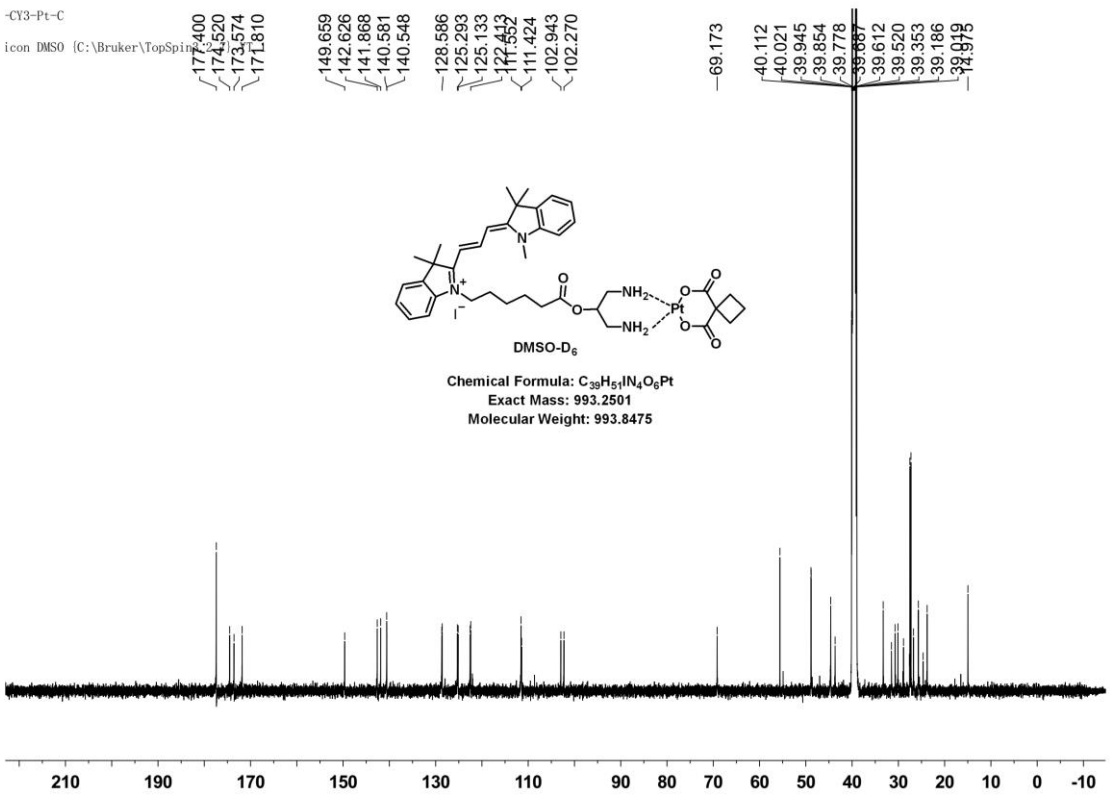


Cy3-CPT

^{1}H-NMR



^{13}C-NM



3. Biological Experiments

Determination of dye-containing molecule concentration. Construct concentrations were calculated from the Beer–Lambert law [$\boldsymbol{A} = \varepsilon\, c\, l$] where A is the absorbance value, l is the path length and ε is the extinction coefficient. For **Cy3**-containing constructs, concentration was determined using ε of **Cy3** (150,000 $L{\cdot}mol^{-1}{\cdot}cm^{-1}$ at the excitation wavelength of 555 nm), and for the dual dyes using ε of **Cy5** (250,000 $L{\cdot}mol^{-1}{\cdot}cm^{-1}$ at the excitation wavelength of 646 nm).[10] Measurements were conducted using a ThermoFisher NanoDrop ND-ONE-W spectrophotometer. Absorbance values were measured in 10 mm path-length cuvettes (Fisher Scientific, #11847832).

Cell culture. HeLa, HEK, 10T1/2, MCF7 and KB cells were routinely tested for mycoplasma infection. HeLa, HEK, 10T1/2 and MCF7 cells were maintained in T75 flasks at 37 °C in a 5% CO_2 atmosphere in DMEM (Fisher Scientific, #11574516) supplemented with 10% (v/v) FBS (Fisher Scientific, #11573397). KB cells were maintained in T75 flasks at 37 °C in a 5% CO_2 atmosphere in RPMI 1640 (Fisher Scientific, #27016021) supplemented with 10% (v/v) FBS (Fisher Scientific, #11573397). Cells were maintained at a sub-confluent monolayer, and split at 80-85% confluency. For splitting, cells were washed with PBS, trypsinized in 1 mL of trypsin (Fisher Scientific, #11560626) and 200 µL of the 1000 µL trypsin cell suspension is re-suspended in 12 mL fresh DMEM containing 10% (v/v) FBS in a new T75 flask.

Confocal microscopy. HeLa cells were seeded at a density of 3×10^5 cells per imaging dish (MatTek, #P35G-1.5-10-C) and grown at 37 °C in a 5% CO_2 atmosphere in DMEM (ThermoFisher, #61965026) supplemented with 10% (v/v) FBS (ThermoFisher, #10270106) for 24 h. Cells were washed twice with 1X PBS (pH 7.4) and once with the imaging medium, *i.e.* serum free RPMI (ThermoFisher, #32404014) containing 1% (w/v) BSA (Fisher Scientific, #11493823) prior to undertaking the relevant staining procedures.

For nuclear staining, cells were incubated with Hoechst 33258 (10 mg/mL; Invitrogen, #H3569) at 10 µg/mL in the imaging medium at 37 °C for 5 min. For mitochondrial staining, cells were washed with PBS and incubated with MitoTracker® Green (Invitrogen, #M7514) at 20 nM in imaging medium at 37 °C for 30 min and washed twice with PBS before imaging in imaging media. For the constructs, concentrations and procedures are detailed in each protocol listed.

Cell analysis was conducted using a Leica TCS SP5 confocal laser-scanning microscope using 488 nm Argon laser, and 543 nm, 633 nm HeNe lasers. Images were collected using a 63 x 1.4 NA objective at 2.5 times zoom. Images were 16bit depth, 98.6 x 98.6 microns with 5.192 pixels per micron resolution gathered through a 95.5 µm pinhole. Excitation/emission used for Hoechst 33258, MitoTracker® Green, **Cy3**, and **Cy5** were 405/480 nm, 488/520 nm, 543/575 nm, and 633/680 nm respectively. Line-by-line generated images were acquired via sequential scanning between the fluorophores. For each representative image displayed, 5 to 10 distinct fields of view were imaged. All imaging was performed on live cells unless otherwise stated.

Cell uptake experiments at 37 °C. Cells were treated with the compound at the indicative concentration for 1 h at 37 °C in imaging medium with or without 10% FBS (for serum dependent uptake). Media was removed, cells washed twice with PBS and once with serum free RPMI. For nuclear stain, cells were incubated with Hoechst nuclear stain (Fisher Scientific, #10778843) following the procedure previously described, and then imaged. When nuclear stain wasn't used, cells were washed after compound treatment three times with PBS and incubated in serum free RPMI prior to imaging.

Cell uptake experiments at 4 °C. Cells were chilled to 4 °C for 5 minutes before washing with chilled PBS and 10 minutes treatment with the constructs at indicated concentrations at 4 °C. They were washed with chilled PBS and incubated in pre-chilled RPMI for imaging.

Pre/post fixed cell imaging. Cells were washed with PBS and incubated with MitoTracker® (20 nM) for 20 minutes, washed with PBS and then incubated with **Cy3** (10 µM) in serum free DMEM for a further 20 minutes. Cells were washed with PBS and incubated in serum free DMEM when imaged on the microscope, prior to fixation. Fixation was conducted via media removal, cells washed with PBS and

then incubated at the room temperature with 3% paraformaldehyde in PBS for 20 minutes. Cells were further washed with PBS and incubated with 10 mM NH_4Cl for 5 minutes prior to imaging again on the confocal microscope.

Depolarization experiments. Cells were washed with PBS, and **Cy3** (10 µM) was added and incubated for 10 minutes in 10% FBS DMEM. Cells were washed and incubated in 500 µL of the aforementioned DMEM for imaging 0 minutes, then a further 500 µL of carbonyl cyanide m-chlorophenyl hydrazine in 10% FBS DMEM (CCCP, Fisher Scientific, 10175140) at 40 µM was added to the cells (final concentration in well of 20 µM CCCP, 10% FBS). Cells were imaged at 0, 2 and 6 minutes of CCCP treatment. CCCP media was then removed and cells washed 3 times with PBS prior to incubation in aforementioned 10%FBS DMEM. Cells were incubated at 37°C 5% CO_2 atmosphere, and imaged at 10 minutes and 60 minutes after CCCP removal.

Imaging Cy3 conjugates. Media was removed, and cells washed three times with PBS prior to staining with MitoTacker at 20 nM in serum free DMEM for 20 minutes. Cells were washed again twice with PBS and incubated with Hoechst stain (at concentrations previously described) in serum free DMEM for 5 minutes. Cells were washed twice with PBS and treated with the appropriate conjugates at 10 µM for 10 minutes under tissue culture conditions prior to washing with PBS and imaging.

Image analysis. All images were processed equally and analyzed using the software program Fiji (version 1.0) as part of the ImageJ suite. For co-localization graphs, guides of 45-52 µm were drawn on the image and profile plots generated for each channel along the guide from left to right. Data sets generated from this were collected and processed in Microsoft Excel. Pearson's coefficient values were calculated with Fiji using the "Coloc 2" function. Ten images were analyzed to calculate the mean Pearson's coefficient.

Cell viability assays. Cells were seeded at a density of 2×10^4 cells per well in a Corning 96-well plate (Fisher Scientific, #10357891) and grown at 37 °C in a 5% CO_2 atmosphere in DMEM (for HeLa, HEK, 10T1/2 and MCF7) or RPMI (for KB) supplemented with 10% (v/v) FBS for 24 h. Constructs were diluted into DMEM (for HeLa HEK, 10T1/2 and MCF7) or RPMI (for KB) supplemented with 10% FBS to the appropriate concentration, and cells in each well were incubated with 100 µL of the solution. After 24 h at 37 °C, 20 µL of CellTiter-Blue® (Promega, #G8080) was added to each well. The plate was incubated for another 4 h at 37 °C before analysis on a Perkin Elmer Victor X plate reader (excitation 531 nm; emission 595 nm). Each data point is calculated from three biological replicates (*i.e.* cells split from three different passages), and each biological replicate is calculated from three technical replicates (*i.e.* cells split from the same passage). Values from cell-only (*i.e.* non-treated) wells with cell titer blue in each biological replicate were set as 100% viability. For treatments containing **Cy3**, blanks were generated with cell-free wells containing the compounds and not adding cell titer blue. The fluorescent reading for these wells depicted background Cy3 fluorescence, and values deducted from the treatment readings.

Statistical analysis. For all statistical analysis, data were obtained from three independent experiments (N = 3), and significance values were calculated using a paired Student's t-test.

4. References

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