

Supporting Information

Bioorthogonal Ligation-Activated Fluorogenic FRET Dyads

Evelin Albitz, Dóra Kern, Attila Kormos, Márton Bojtár, György Török, Adrienn Biró, Ágnes Szatmári, Krisztina Németh,* and Péter Kele**

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1. General

All starting materials were obtained from commercial suppliers (Sigma-Aldrich, Fluka, Merck, Alfa Aesar, Reanal, Molar Chemicals, Fluorochem) and used without further purification.

Analytical thin-layer chromatography (TLC) was performed on silica gel 60 F₂₅₄ precoated aluminum TLC plates from Merck. Flash column chromatography was performed on a *Teledyne ISCO COMBI Flash Nextgen 300+* automated flash chromatographer with silica gel (25-40 μm) from Zeochem or RediSep[®]R_f C18 High Performance GOLD column. Microwave experiments were performed on *AntonPaar Monowave 400* microwave reactor using sealed tubes and for each experiment, fast heating to 100 °C and maintaining constant temperature for one hour.

NMR spectra were recorded on a *Varian Inova 500 MHz* and *Varian Inova 300 MHz* spectrometer. Chemical shifts (δ) are given in parts per million (ppm) using solvent signals as the reference. Coupling constants (J) are reported in Hertz (Hz).

Analytical RP-HPLC-UV/Vis-MS experiments were performed on a *SHIMADZU LCMS-2020* system by using a Phenomenex Kinetex EVO C18 column (50×2.10 mm I.D.) with 2.6 μm silica (100 Å pore size) as a stationary phase with a photodiode array UV/Vis ($\lambda=190\text{-}800$ (0 min 0% B; 2.0 min 100% B; 2.5 min 100% B; 3.0 min 0% B; 4.0 min 0% B) with eluents A (95% H₂O, 5% MeCN, and 0.1% HCOOH) and B (95% MeCN, 5% H₂O, and 0.1% HCOOH) and an ESI-MS detector. Linear gradient elution was used at a flow rate of 1.0 mL min⁻¹ at 40°C. The samples were dissolved in MeCN - H₂O mixture.

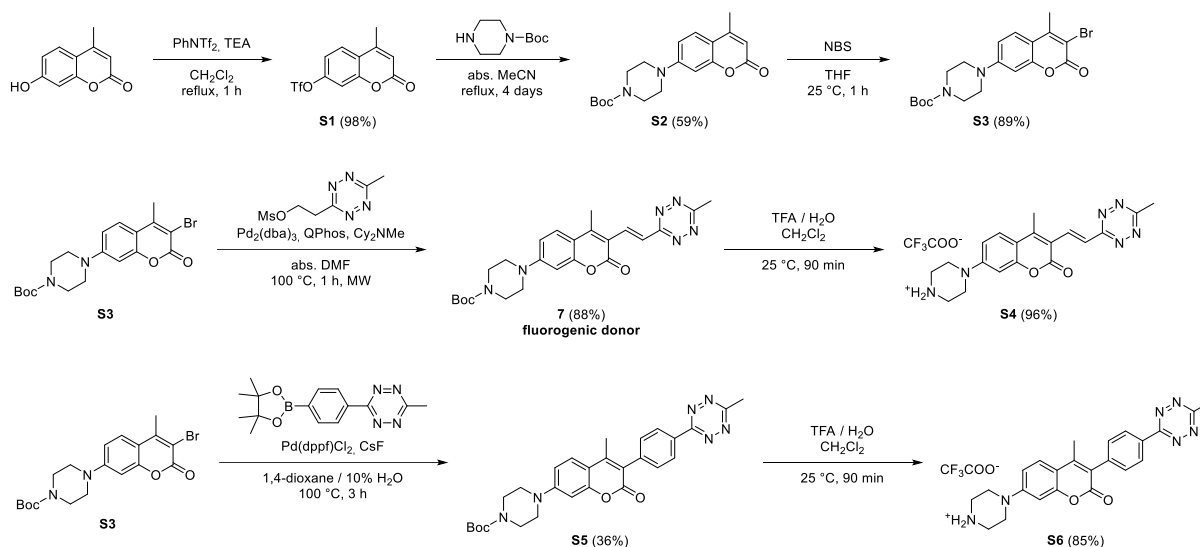
Semipreparative HPLC was performed on a *Wufeng Chrom LC100 HPLC* system using a Gemini C18 column (150 × 21 mm I.D.) with 5 μm silica (110 pore size) as a stationary phase.

Spectroscopic measurements were performed on a *Jasco FP 8300* spectrofluorometer and a *JASCO v750* spectrophotometer in all-sodium PBS (pH=7.4, containing 0.1% SDS) at r. t. Quartz cuvettes with path length of 1 cm were used.

The exact masses were determined with an *Agilent 6230* time-of-flight mass spectrometer.

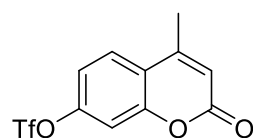
2. Synthesis

2.1. Synthesis of coumarin donor units



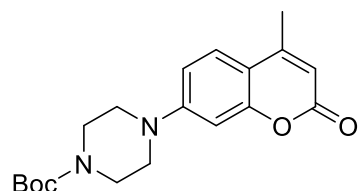
Scheme 1. Synthesis of tetrazine-substituted coumarin **S4** and **S6**.

4-Methyl-2-oxo-2H-chromen-7-yl trifluoromethanesulfonate (**S1**)¹



A mixture of 4-methylumbelliferone (1.76 g, 10 mmol, 1.0 equiv), PhNTf₂ (3.75 g, 10.5 mmol, 1.05 equiv) and triethylamine (4.15 mL, 30 mmol, 3.0 equiv) was refluxed in 50 mL dichloromethane (stabilized with amylene) for 1 hour. The reaction was cooled to room temperature, diluted with 50 mL dichloromethane and extracted with 3x 30 mL sat. NaHCO₃ solution. The organic phase was dried over anhydrous MgSO₄, filtered and concentrated in vacuo. The crude product was purified by flash chromatography on silica gel (hexane-ethyl acetate 0 to 60%) to yield 3.01 g (98%) of white solid. ¹H NMR (500 MHz, CDCl₃) δ 7.69 (d, *J* = 8.7 Hz, 1H), 7.26 (d, *J* = 2.5 Hz, 1H), 7.23 (dd, *J* = 8.7, 2.5 Hz, 1H), 6.34 (s, 1H), 2.45 (s, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 159.6, 154.3, 151.4, 150.9, 126.5, 120.2, 118.8 (q, *J* = 320 Hz) 117.5, 116.1, 110.7, 18.8. LCMS: *m/z* calcd. for [C₁₁H₈F₃O₅S]⁺: 309; found 309 [M+H]⁺.

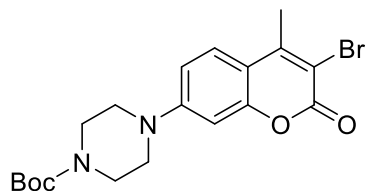
Tert-butyl 4-(4-methyl-2-oxo-2H-chromen-7-yl)piperazine-1-carboxylate (**S2**)²



A mixture of coumarin **S1** (1 g, 3.2 mmol, 1.0 equiv), *tert*-butyl piperazine-1-carboxylate (3.02 g, 16.2 mmol, 5.0 equiv) and 15 mL anhydrous acetonitrile was purged with N₂ for 15 minutes. The reaction was refluxed for 4 days in a pressure tube. Then, the reaction mixture was concentrated in vacuo, dissolved in 50 mL dichloromethane, washed with 2x 20 mL 2M Na₂CO₃ solution and with 1x 20 mL brine. The organic phase was dried over anhydrous MgSO₄, filtered and concentrated in vacuo. The crude product was purified by flash chromatography on silica gel (hexane-ethyl acetate 0 to 50%) to yield 655 mg (59%) of off-white solid. ¹H NMR (500 MHz, CDCl₃) δ 7.43

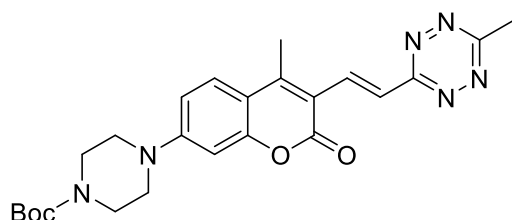
(d, $J = 8.8$ Hz, 1H), 6.80 (dd, $J = 8.9, 2.5$ Hz, 1H), 6.69 (d, $J = 2.5$ Hz, 1H), 6.04 (s, 1H), 3.59 (t, $J = 5.2$ Hz, 4H), 3.29 (t, $J = 5.1$ Hz, 4H), 2.35 (s, 3H), 1.48 (s, 9H). ^{13}C NMR (126 MHz, CDCl_3) δ 161.6, 155.4, 154.6, 153.4, 152.5, 125.4, 111.9, 111.6, 111.0, 101.6, 80.2, 47.6, 28.4, 18.4. LCMS: m/z calcd. for $[\text{C}_{19}\text{H}_{25}\text{N}_2\text{O}_4]^+$: 344; found 344 $[\text{M}+\text{H}]^+$.

Tert-butyl 4-(3-bromo-4-methyl-2-oxo-2H-chromen-7-yl)piperazine-1-carboxylate (S3)



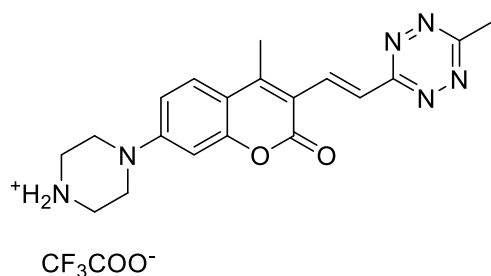
Coumarin **S2** (300 mg, 0.871 mmol, 1.0 equiv) was dissolved in 10 mL tetrahydrofuran, the mixture was cooled to 0 °C and *N*-bromo succinimide (155 mg, 0.871 mmol, 1.0 equiv) was added. The reaction mixture was stirred at room temperature for 1 hour. The solvent was evaporated, and the crude product was purified by flash chromatography on silica gel (hexane-ethyl acetate 0 to 50%) to yield 324 mg (89%) of yellow solid. ^1H NMR (500 MHz, CDCl_3) δ 7.46 (d, $J = 9.0$ Hz, 1H), 6.81 (dd, $J = 9.0, 2.5$ Hz, 1H), 6.66 (d, $J = 2.6$ Hz, 1H), 3.59 (t, $J = 5.3$ Hz, 4H), 3.31 (t, $J = 5.2$ Hz, 4H), 2.53 (s, 3H), 1.48 (s, 9H). ^{13}C NMR (126 MHz, CDCl_3) δ 157.7, 154.7, 153.9, 153.4, 151.2, 126.0, 112.0, 111.6, 108.3, 101.1, 80.4, 47.5, 28.5, 19.3. HRMS: m/z calcd. for $[\text{C}_{19}\text{H}_{24}\text{BrN}_2\text{O}_4]^+$: 423.0919; found 423.0925 $[\text{M}+\text{H}]^+$ and m/z calcd. for $[\text{C}_{19}\text{H}_{23}\text{BrN}_2\text{O}_4\text{Na}]^+$: 445.0739; found 445.0738 $[\text{M}+\text{Na}]^+$.

Tert-butyl (E)-4-(4-methyl-3-(2-(6-methyl-1,2,4,5-tetrazin-3-yl)vinyl)-2-oxo-2H-chromen-7-yl)piperazine-1-carboxylate (7, fluorogenic donor)



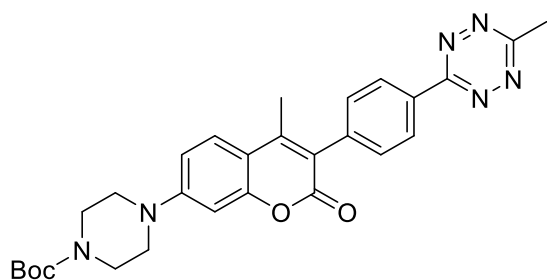
A mixture of bromo coumarin **S3** (120 mg, 0.283 mmol, 1.0 equiv), 2-(6-methyl-1,2,4,5-tetrazin-3-yl)ethyl methanesulfonate³ (185 mg, 0.849 mmol, 3.0 equiv), Pd_2dba_3 (26 mg, 0.0283 mmol, 0.1 equiv), QPhos (20 mg, 0.0283 mmol, 0.1 equiv), *N,N*-dicyclohexylmethylamine (300 μL , 1.415 mmol, 5.0 equiv) and 4 mL anhydrous dimethylformamide was purged with N_2 for 15 minutes. The reaction mixture was heated in a microwave reactor at 100 °C for 1 hour. The solvent was evaporated in vacuo and the crude product was purified by flash chromatography on silica gel (dichloromethane-methanol 0 to 2%) to yield 116 mg (88%) of orange solid. ^1H NMR (500 MHz, CDCl_3) δ 8.44 (d, $J = 15.9$ Hz, 1H), 8.28 (d, $J = 15.8$ Hz, 1H), 7.60 (d, $J = 9.0$ Hz, 1H), 6.84 (dd, $J = 9.0, 2.5$ Hz, 1H), 6.69 (d, $J = 2.5$ Hz, 1H), 3.61 (t, $J = 5.2$ Hz, 4H), 3.38 (t, $J = 5.3$ Hz, 4H), 3.03 (s, 3H), 2.64 (s, 3H), 1.49 (s, 9H). ^{13}C NMR (126 MHz, CDCl_3) δ 166.2, 165.5, 159.8, 155.0, 154.7, 153.8, 151.9, 132.5, 126.9, 125.4, 115.8, 112.0, 111.8, 100.7, 80.5, 47.3, 28.6, 21.3, 15.2. HRMS: m/z calcd. for $[\text{C}_{24}\text{H}_{29}\text{N}_6\text{O}_4]^+$: 465.2250; found 465.2251 $[\text{M}+\text{H}]^+$ and m/z calcd. for $[\text{C}_{24}\text{H}_{28}\text{N}_6\text{O}_4\text{Na}]^+$: 487.2070; found 487.2074 $[\text{M}+\text{Na}]^+$.

(E)-4-(4-methyl-3-(2-(6-methyl-1,2,4,5-tetrazin-3-yl)vinyl)-2-oxo-2H-chromen-7-yl)piperazin-1-ium
2,2,2-trifluoroacetate (**S4**)



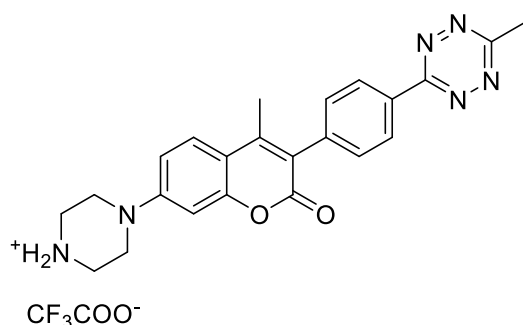
Coumarin **5** (100 mg, 0.215 mmol, 1.0 equiv) was dissolved in 5 mL dichloromethane, the solution was cooled to 0 °C and a mixture of 5 mL dichloromethane, 2 mL trifluoroacetic acid and 0.1 mL H₂O was added dropwise. The reaction was stirred at room temperature for 90 minutes. The solvent was evaporated, and 10 mL cold diethyl ether was added to the residue. The product was filtered and dried to yield 75 mg (96%) red solid. ¹H NMR (500 MHz, DMSO-d₆) δ 9.08 (s, 2H), 8.30 (d, *J* = 15.9 Hz, 1H), 8.02 (d, *J* = 15.8 Hz, 1H), 7.82 (d, *J* = 9.1 Hz, 1H), 7.06 (dd, *J* = 9.1, 2.6 Hz, 1H), 6.93 (d, *J* = 2.5 Hz, 1H), 3.65 (t, *J* = 5.3 Hz, 4H), 3.25 (t, *J* = 5.2 Hz, 4H), 2.94 (s, 3H), 2.64 (s, 3H). ¹³C NMR (126 MHz, DMSO-d₆) δ 165.9, 164.4, 158.9, 154.2, 153.1, 152.9, 131.9, 127.7, 124.0, 114.2, 111.8, 111.3, 100.0, 43.8, 42.3, 20.8, 14.8. HRMS: *m/z* calcd. for [C₁₉H₂₁N₆O₂]⁺: 365.1726; found 365.1723 [M]⁺.

Tert-butyl 4-(4-methyl-3-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-2-oxo-2H-chromen-7-yl)piperazine-1-carboxylate (**S5**)



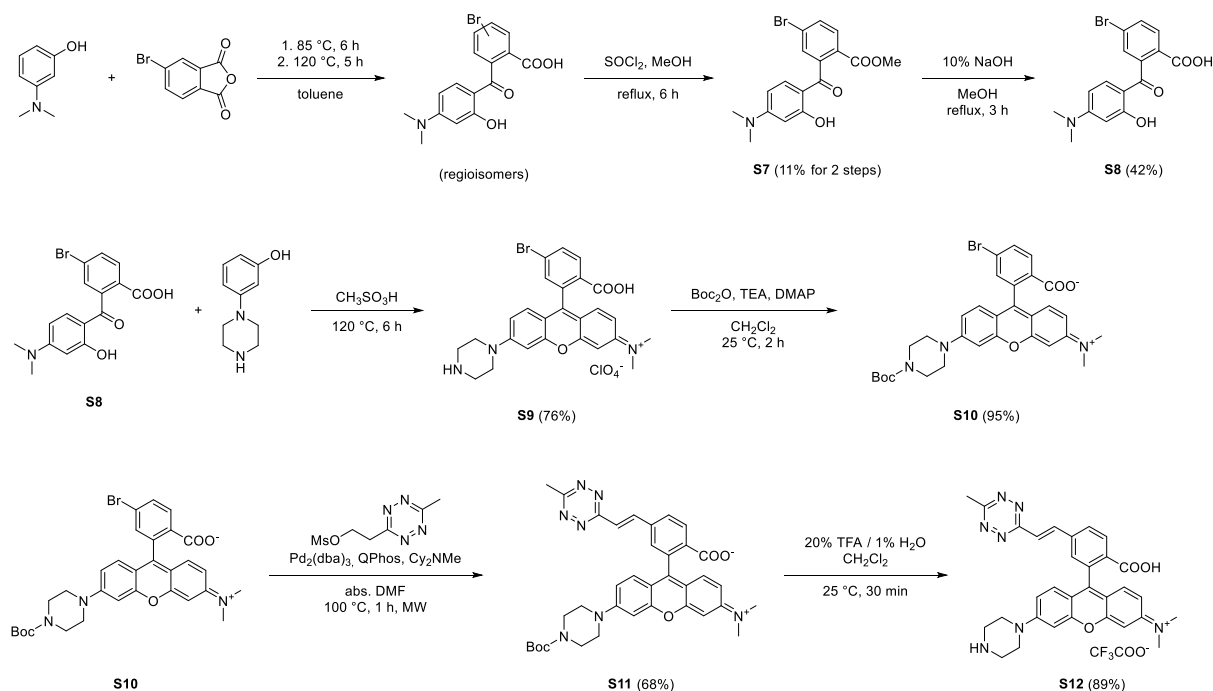
A mixture of bromo coumarin **S3** (50 mg, 0.118 mmol, 1.0 equiv), 3-methyl-6-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)-1,2,4,5-tetrazine⁴ (42 mg, 0.142 mmol, 1.2 equiv), Pd(dppf)Cl₂ (8 mg, 0.0118 mmol, 0.1 equiv) and CsF (53 mg, 0.354 mmol, 3.0 equiv) was suspended in 3 mL 1,4-dioxane and 0.3 mL H₂O. The reaction mixture was stirred at 100 °C for 3 hours. The solvent was evaporated, and the crude product was purified by flash chromatography on silica gel (hexane-ethyl acetate 0 to 100%) to yield 22 mg (36%) of red solid. ¹H NMR (500 MHz, CDCl₃) δ 8.66 (d, *J* = 8.4 Hz, 2H), 7.56 – 7.53 (m, 3H), 6.87 (dd, *J* = 8.9, 2.5 Hz, 1H), 6.77 (d, *J* = 2.4 Hz, 1H), 3.62 (t, *J* = 5.3 Hz, 4H), 3.34 (t, *J* = 5.3 Hz, 4H), 3.11 (s, 3H), 2.32 (s, 3H), 1.50 (s, 9H). ¹³C NMR (126 MHz, CDCl₃) δ 167.4, 164.1, 161.3, 154.8, 154.7, 153.4, 148.5, 139.7, 131.6, 131.4, 128.0, 126.2, 122.5, 112.3, 112.0, 101.5, 80.4, 47.8, 28.6, 21.3, 16.6. HRMS: *m/z* calcd. for [C₂₈H₃₁N₆O₄]⁺: 515.2407; found 515.2399 [M+H]⁺ and *m/z* calcd. for [C₂₈H₃₀N₆O₄Na]⁺: 537.2226; found 537.2220 [M+Na]⁺.

4-(4-Methyl-3-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-2-oxo-2H-chromen-7-yl)piperazin-1-ium
2,2,2-trifluoroacetate (**S6**)



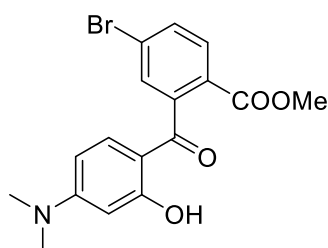
Coumarin **S5** (17 mg, 0.033 mmol, 1.0 equiv) was dissolved in 1 mL dichloromethane, the solution was cooled to 0 °C and a mixture of 1 mL dichloromethane, 400 μ L trifluoroacetic acid and 20 μ L H₂O was added dropwise. The reaction was stirred at room temperature for 60 minutes. The solvent was evaporated, and 5 mL cold diethyl ether was added to the residue. The product was filtered and dried to yield 14.5 mg (85%) red solid. ¹H NMR (500 MHz, DMSO-d₆) δ 9.00 (s, 2H), 8.53 (d, *J* = 8.0 Hz, 2H), 7.74 (d, *J* = 8.9 Hz, 1H), 7.61 (d, *J* = 8.1 Hz, 2H), 7.09 (dd, *J* = 8.9, 2.6 Hz, 1H), 7.00 (d, *J* = 2.5 Hz, 1H), 3.61 (t, *J* = 5.1 Hz, 4H), 3.27 (t, *J* = 5.2 Hz, 4H), 3.02 (s, 3H), 2.30 (s, 3H). ¹³C NMR (126 MHz, DMSO-d₆) δ 167.1, 163.1, 160.1, 154.0, 152.3, 148.6, 139.1, 131.5, 131.1, 127.0, 126.9, 121.3, 111.8, 111.6, 100.8, 44.2, 42.3, 20.8, 16.3. HRMS: *m/z* calcd. for [C₂₃H₂₃N₆O₂]⁺: 415.1882; found 415.1883 [M]⁺.

2.2. Synthesis of rhodamine donor unit



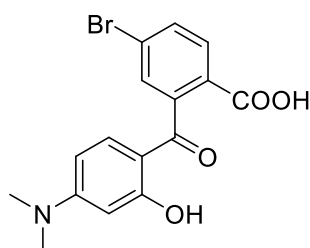
Scheme 2. Synthesis of tetrazine-substituted rhodamine **S12**.

*Methyl 4-bromo-2-(4-(dimethylamino)-2-hydroxybenzoyl)benzoate (S7)*⁵



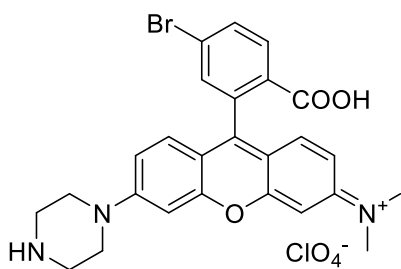
5-Bromophthalic anhydride (4.5 g, 20 mmol, 1.0 equiv) and 3-dimethylaminophenol (3 g, 22 mmol, 1.1 equiv) were dissolved in 50 mL toluene. The solution was stirred at 85 °C for 6 hours, then 5 hours at 120 °C. After cooling, the solvent was removed, and the residue was dissolved in 50 mL methanol. Thionyl chloride (2.3 mL, 30 mmol, 1.5 equiv) was added dropwise while keeping the temperature at 0 °C. The mixture was refluxed for 6 hours. Then, the solvent was removed, the residue was extracted with CH₂Cl₂ and water. The organic phase was dried over anhydrous MgSO₄, filtered and evaporated under reduced pressure. The pink crude product was purified by silica flash chromatography (hexane-CH₂Cl₂ 0 to 10%) to yield a 1:1 mixture of regioisomers as yellow oil. The required 4-bromo isomer was obtained by recrystallization from toluene to yield 816 mg (11%) yellow crystals. ¹H NMR (500 MHz, DMSO) δ 12.24 (s, 1H), 7.90 (d, *J* = 8.4 Hz, 1H), 7.86 (dd, *J* = 8.4, 2.0 Hz, 1H), 7.68 (d, *J* = 2.0 Hz, 1H), 6.84 (d, *J* = 9.1 Hz, 1H), 6.23 (d, *J* = 9.1, 2.5 Hz, 1H), 6.12 (d, *J* = 2.5 Hz, 1H), 3.66 (s, 3H), 3.01 (s, 6H). ¹³C NMR (126 MHz, DMSO) δ 195.7, 165.1, 164.2, 155.9, 142.0, 133.7, 132.6, 131.8, 130.4, 127.4, 126.4, 109.2, 104.6, 97.0, 52.4, 39.6. LCMS: *m/z* calcd. for [C₁₇H₁₇BrNO₄]⁺: 378, found: 378 [M+H]⁺.

*4-Bromo-2-(4-(dimethylamino)-2-hydroxybenzoyl)benzoic acid (S8)*⁵



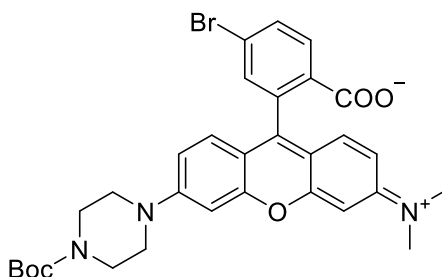
Ester **S7** (800 mg, 2.16 mmol, 1.0 equiv) was dissolved in 20 mL methanol. 10 % NaOH solution (1.25 equiv) was added, then the mixture was refluxed for 3 hours. After cooling the methanol was removed, and the residue was dissolved in 15 mL distilled water. The pH of the solution was set to 1.5 with 5 % H₂SO₄. The precipitated crystals were filtered, washed with cold water, and dried under vacuum to yield 315 mg (42%) yellow crystals. ¹H NMR (500 MHz, DMSO) δ 12.30 (s, 1H), 7.89 (d, *J* = 8.4 Hz, 1H), 7.81 (dd, *J* = 8.3, 2.0 Hz, 1H), 7.59 (d, *J* = 1.9 Hz, 1H), 6.82 (d, *J* = 9.1 Hz, 1H), 6.22 (dd, *J* = 9.1, 2.5 Hz, 1H), 6.09 (d, *J* = 2.4 Hz, 1H), 3.00 (s, 6H). ¹³C NMR (126 MHz, DMSO) δ 196.5, 166.2, 164.3, 155.9, 142.0, 133.9, 132.5, 132.1, 130.2, 129.0, 125.9, 109.6, 104.6, 97.1, 39.6. LCMS: *m/z* calcd. for [C₁₆H₁₅BrNO₄]⁺: 364, found: 364 [M+H]⁺.

N-(9-(5-bromo-2-carboxyphenyl)-6-(piperazin-1-yl)-3*H*-xanthen-3-ylidene)-*N*-methylmethanaminium perchlorate (**S9**)



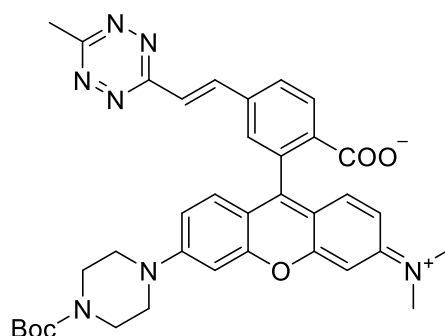
3-Hydroxyphenylpiperazin (245 mg, 1.37 mmol, 1.0 equiv) and compound **S8** (500 mg, 1.37 mmol, 1.0 equiv) were dissolved in 5 mL methanesulfonic acid. The mixture was heated at 120 °C overnight. After cooling to room temperature, the solution was poured on 50 mL iced water, then 10 mL perchloric acid (74 %) was added dropwise. The resulting precipitate was filtered, washed with cold, diluted perchloric acid, and dried under vacuum to yield 633 mg (76%) red crystals. ¹H NMR (500 MHz, CD₃OD) δ 8.24 (d, *J* = 8.5 Hz, 1H), 7.99 (dd, *J* = 8.5, 2.1 Hz, 1H), 7.68 (d, *J* = 2.0 Hz, 1H), 7.31 (d, *J* = 2.0 Hz, 1H), 7.26 – 7.22 (m, 3H), 7.19 (dd, *J* = 9.6, 2.4 Hz, 1H), 7.05 (d, *J* = 2.4 Hz, 1H), 3.97 (t, *J* = 5.3 Hz, 4H), 3.45 (t, *J* = 5.3 Hz, 4H), 3.37 (s, 6H). ¹³C (126 MHz, CD₃OD) δ 167.2, 160.4, 160.0, 159.8, 158.9, 157.7, 137.0, 134.8, 134.2, 134.1, 132.4, 132.0, 131.3, 128.6, 117.1, 116.3, 115.8, 115.7, 100.0, 97.6, 45.1, 44.2, 41.3. HRMS: *m/z* calcd. for [C₂₆H₂₅BrN₃O₃]⁺: 506.1079, found: 506.1072 [M]⁺.

4-Bromo-2-(6-(4-(*tert*-butoxycarbonyl)piperazin-1-yl)-3-(dimethyliminio)-3*H*-xanthen-9-yl)benzoate (**S10**)



Rhodamine **S9** (200 mg, 0.39 mmol, 1 equiv) was dissolved in 5 mL CH₂Cl₂. After the addition of triethylamine (58 μL, 0.410 mmol, 1.05 equiv), DMAP (10 mg, 0.08 mmol, 0.2 equiv) and di-*tert*-butyl dicarbonate (258 mg, 1.18 mmol, 3 equiv) the solution was stirred at room temperature for 2 hours. The solvent was removed, the obtained red crude product was purified with preparative-HPLC (H₂O : MeCN starting from 95 : 5 to 0 : 100) to yield 227 mg (95%) red powder. ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.11 (d, *J* = 8.4 Hz, 1H), 7.90 (dd, *J* = 8.4, 1.9 Hz, 1H), 7.54 (d, *J* = 2.0 Hz, 1H), 7.20 – 7.10 (m, 4H), 7.04 (dd, *J* = 9.5, 2.4 Hz, 1H), 6.92 (d, *J* = 2.4 Hz, 1H), 3.71 (t, *J* = 5.4 Hz, 4H), 3.63 (t, *J* = 5.2 Hz, 4H), 3.29 (s, 6H), 1.49 (s, 9H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 162.7, 158.5, 158.3, 157.4, 154.3, 139.6, 133.8, 133.6, 132.8, 132.5, 132.5, 132.0, 131.9, 130.7, 130.6, 129.2, 129.2, 129.1, 129.0, 112.2, 107.4, 79.6, 46.2, 28.5, 9.0. HRMS: *m/z* calcd. for [C₃₁H₃₃BrN₃O₅]⁺: 606.1604, found: 606.1607 [M]⁺.

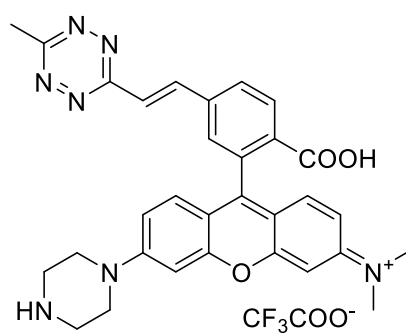
(E)-2-(6-(4-(*tert*-butoxycarbonyl)piperazin-1-yl)-3-(dimethyliminio)-3*H*-xanthen-9-yl)-4-(2-(6-methyl-1,2,4,5-tetrazin-3-yl)vinyl)benzoate (**S11**)



A mixture of bromo rhodamine derivative **S10** (40 mg, 0.066 mmol, 1 equiv), 2-(6-methyl-1,2,4,5-tetrazin-3-yl)ethyl methanesulfonate³ (57 mg, 0.26 mmol, 4 equiv), *N,N*-dicyclohexylmethylamine (57 μ L, 0.26 mmol, 4 equiv), Pd₂(dba)₃ (12 mg, 0.013 mmol, 0.2 equiv) and QPhos (9.4 mg, 0.013 mmol, 0.2 equiv) were dissolved in 4 mL anhydrous dimethylformamide in a 10 mL sealed microwave pressure tube with a magnetic stir bar. The mixture was heated in a microwave reactor at 100 °C for 1 hour. The solvent was evaporated under reduced pressure and the crude product was purified with preparative-HPLC (H₂O : MeCN starting from 95 : 5 to 0 : 100) to yield 29 mg (68%) pink powder.

¹H NMR (500 MHz, DMSO-d₆) δ 8.27 (d, *J* = 16.3 Hz, 1H), 8.20 (dd, *J* = 8.1, 0.8 Hz, 1H), 8.03 (d, *J* = 8.0 Hz, 1H), 7.84 (d, *J* = 1.2 Hz, 1H), 7.82 (d, *J* = 16.4 Hz, 1H), 6.78 (d, *J* = 2.3 Hz, 1H), 6.74 (dd, *J* = 8.9, 2.4 Hz, 1H), 6.63 (d, *J* = 8.9 Hz, 1H), 6.59 (d, *J* = 9.5 Hz, 1H), 6.54 – 6.48 (m, 2H), 3.45 (t, *J* = 4.9 Hz, 4H), 3.21 (t, *J* = 4.9 Hz, 4H), 2.95 (s, 6H), 2.94 (s, 3H), 1.41 (s, 9H). ¹³C NMR (75 MHz, DMSO-d₆) δ 168.3, 166.3, 163.9, 153.8, 153.4, 152.4, 152.1, 152.0, 141.9, 137.9, 129.9, 129.9, 128.5, 128.5, 127.1, 125.0, 124.9, 123.3, 111.9, 109.1, 108.8, 105.7, 101.4, 98.0, 83.9, 79.0, 47.3, 28.0, 20.9. HRMS: *m/z* calcd. for [C₃₆H₃₈N₇O₅]⁺: 648.2934, found: 648.2927 [M]⁺.

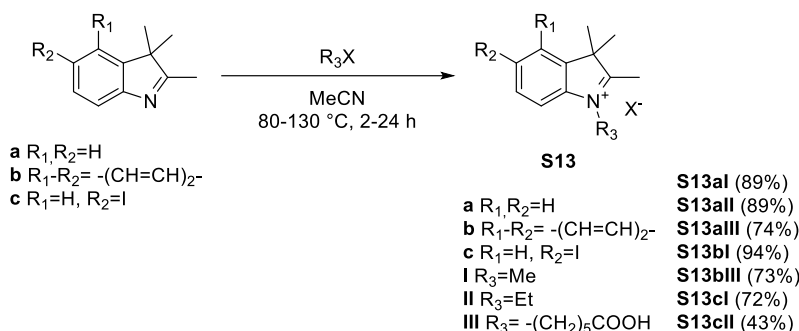
(E)-*N*-(9-(2-carboxy-5-(2-(6-methyl-1,2,4,5-tetrazin-3-yl)vinyl)phenyl)-6-(piperazin-1-yl)-3*H*-xanthen-3-ylidene)-*N*-methylmethanaminium 2,2,2-trifluoroacetate (**S12**)



Rhodamine **S11** (23 mg, 0.036 mmol, 1 equiv) was dissolved in a mixture of 6 mL CH₂Cl₂, 1.2 mL trifluoroacetic acid and 60 μ L water, then the mixture was stirred at room temperature for 30 minutes. After the solvent was removed, the crude product was purified with preparative-HPLC (H₂O (0.1% TFA) - MeCN (0.1% TFA) starting from 95 : 5 to 0 : 100) to yield 21 mg (89%) pink powder.

¹H NMR (500 MHz, CD₃OD) δ 8.42 (d, *J* = 8.3 Hz, 1H), 8.39 (d, *J* = 16.4 Hz, 1H), 8.22 (dd, *J* = 8.5, 1.4 Hz, 1H), 7.82 (d, *J* = 1.2 Hz, 1H), 7.76 (d, *J* = 16.3 Hz, 1H), 7.33 – 7.28 (m, 3H), 7.24 (dd, *J* = 9.4, 2.5 Hz, 1H), 7.18 (dd, *J* = 9.6, 2.6 Hz, 1H), 7.04 (d, *J* = 2.3 Hz, 1H), 3.97 – 3.93 (m, 4H), 3.46 – 3.42 (m, 4H), 3.37 (s, 6H), 3.02 (s, 3H). ¹³C NMR (126 MHz, CD₃OD, based on HSQC and HMBC) δ 172.3, 167.9, 165.3, 159.5, 159.4, 157.2, 140.7, 138.6, 136.2, 132.9, 132.6, 132.1, 130.3, 130.2, 125.9, 116.6, 115.4, 115.3, 99.8, 97.2, 44.8, 43.8, 40.9, 20.8. HRMS: *m/z* calcd. for [C₃₁H₃₀N₇O₃]⁺: 548.2410, found: 548.2411 [M]⁺.

2.3. Synthesis of indolium salts

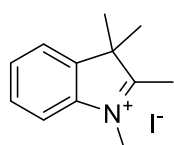


Scheme 3. Synthesis of indolium salts.

General procedure of S13 derivatives

2,3,3-trimethyl-3*H*-indole, 1,1,2-trimethyl-1*H*-benzo[*e*]indole or 5-iodo-2,3,3-trimethyl-3*H*-indole was dissolved in acetonitrile. Iodomethane or iodoethane or 6-bromohexanoic acid was added, then the mixture was transferred to a pressure tube and heated for 2-16 hours. After cooling to room temperature, ethyl acetate was added and the resulting precipitate was filtered, washed with cold ethyl acetate or diethyl ether and dried. The crude product was used without further purification.

1,2,3,3-Tetramethyl-3*H*-indol-1-ium iodide (**S13aI**)⁶



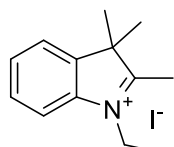
2,3,3-trimethyl-3*H*-indole (5.0 g, 31.4 mmol, 1.0 equiv), iodomethane (5.86 mL, 94.2 mmol, 3.0 equiv).

Reaction conditions: 80 °C, 2 h.

Beige powder (8.43 g, 89%).

¹H NMR (500 MHz, DMSO-*d*₆) δ 7.94 – 7.90 (m, 1H), 7.85 – 7.82 (m, $J = 5.3, 2.9$ Hz, 1H), 7.65 – 7.58 (m, $J = 7.7$ Hz, 2H), 3.99 (s, 3H), 2.79 (s, 3H), 1.54 (s, 6H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 195.9, 142.0, 141.5, 129.2, 128.7, 123.2, 115.0, 53.9, 34.8, 21.7, 14.3. LCMS: *m/z* calcd. for [C₁₂H₁₆N]⁺: 174; found 174 [M]⁺.

1-Ethyl-2,3,3-trimethyl-3*H*-indol-1-ium iodide (**S13aII**)⁷



2,3,3-trimethyl-3*H*-indole (5.0 g, 31,4 mmol, 1.0 equiv), iodoethane (7.5 mL, 94.2 mmol, 3.0 equiv).

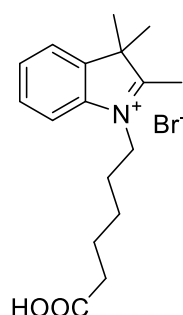
Reaction conditions: 80 °C, 16 h.

Pale pink powder (8.84 g, 89%).

¹H NMR (500 MHz, DMSO-*d*₆) δ 8.01 – 7.96 (m, 1H), 7.88 – 7.83 (m, 1H), 7.65 – 7.59 (m, $J = 6.3, 2.8$ Hz, 2H), 4.51 (q, $J = 7.4$ Hz, 2H), 2.86 (s, 3H), 1.54 (s, 6H), 1.45 (t, $J = 7.3$ Hz, 3H). ¹³C NMR (126

MHz, DMSO-d₆) δ 196.0, 141.9, 140.6, 129.3, 128.9, 123.5, 115.2, 54.1, 43.1, 21.8, 13.9, 12.6. LCMS: m/z calcd. for [C₁₃H₁₈N]⁺: 188; found 188 [M]⁺.

*1-(5-Carboxypentyl)-2,3,3-trimethyl-3H-indol-1-ium bromide (S13aIII)*⁶



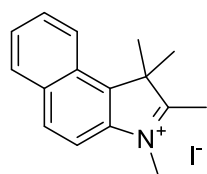
2,3,3-trimethyl-3H-indole (1.23 g, 7.7 mmol, 1.0 equiv), 6-bromohexanoic acid (3.0 g, 15.4 mmol, 2.0 equiv).

Reaction conditions: 130 °C, 16 h.

Pale purple powder (2.01 g, 74%).

¹H NMR (500 MHz, DMSO-d₆) δ 8.00 – 7.96 (m, 1H), 7.87 – 7.82 (m, *J* = 5.8, 2.6 Hz, 1H), 7.65 – 7.60 (m, *J* = 5.3, 3.2 Hz, 2H), 4.46 (t, *J* = 7.6 Hz, 2H), 2.85 (s, 3H), 2.23 (t, *J* = 7.2 Hz, 2H), 1.89 – 1.82 (m, 2H), 1.60 – 1.51 (m, 8H), 1.47 – 1.40 (m, *J* = 15.0, 7.7 Hz, 2H). ¹³C NMR (126 MHz, DMSO-d₆) δ 197.0, 174.7, 142.3, 141.5, 129.9, 129.4, 124.0, 116.0, 54.7, 48.0, 33.8, 27.4, 25.9, 24.5, 22.5, 14.6. LCMS: m/z calcd. for [C₁₇H₂₄NO₂]⁺: 274; found 274 [M]⁺.

*1,1,2,3-Tetramethyl-1H-benzo[e]indol-3-ium iodide (S13bI)*⁸



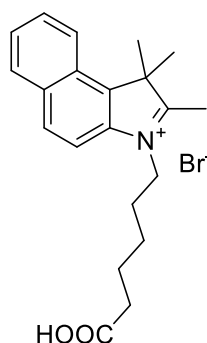
1,1,2-trimethyl-1H-benzo[e]indole (1.0 g, 4.778 mmol, 1.0 equiv), iodomethane (595 μ L, 9.556 mmol, 2.0 equiv).

Reaction conditions: 80 °C, 16 h.

Yellow powder (1.6 g, 94%).

¹H NMR (500 MHz, DMSO-d₆) δ 8.36 (dd, *J* = 8.3, 1.2 Hz, 1H), 8.29 (d, *J* = 8.9 Hz, 1H), 8.21 (d, *J* = 8.2 Hz, 1H), 8.10 (d, *J* = 8.9 Hz, 1H), 7.79 (ddd, *J* = 8.3, 6.9, 1.3 Hz, 1H), 7.72 (ddd, *J* = 8.1, 6.9, 1.2 Hz, 1H), 4.10 (s, 3H), 2.88 (s, 3H), 1.76 (s, 6H). ¹³C NMR (126 MHz, DMSO-d₆) δ 195.8, 139.4, 136.5, 133.0, 130.4, 129.7, 128.3, 127.1, 123.3, 113.1, 55.2, 35.1, 21.2, 14.0. MS: m/z calcd. for [C₁₆H₁₈N]⁺: 224; found: 224 [M]⁺.

*3-(5-Carboxypentyl)-1,1,2-trimethyl-1H-benzo[e]indol-3-ium bromide (S13bIII)*⁸



1,1,2-trimethyl-1H-benzo[e]indole (1.0 g, 4.778 mmol, 1.0 equiv), 6-bromohexanoic acid (1.86 g, 9.556 mmol, 2.0 equiv).

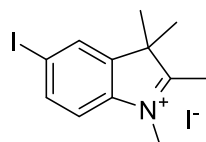
Reaction conditions: 80 °C, 16 h.

Green powder (1.4 g, 73%).

¹H NMR (500 MHz, DMSO-d₆) δ 8.37 (d, *J* = 8.4 Hz, 1H), 8.29 (d, *J* = 8.9 Hz, 1H), 8.22 (d, *J* = 8.2 Hz, 1H), 8.16 (d, *J* = 8.9 Hz, 1H), 7.79 (ddd, *J* = 8.3, 6.9, 1.4 Hz, 1H), 7.73 (ddd, *J* = 8.1, 6.9, 1.2 Hz, 1H), 4.59 (t, *J* = 7.7 Hz, 2H), 2.96 (s, 3H), 2.24 (t, *J* = 7.2 Hz, 2H), 1.91 (p, *J* = 7.8 Hz, 2H), 1.77 (s, 6H), 1.58 (p, *J* = 7.3 Hz, 2H), 1.47 (p, *J* = 7.5 Hz, 2H). ¹³C NMR (126 MHz, DMSO-d₆) δ 196.3, 174.2, 138.4, 136.9, 133.0, 130.6, 129.7, 128.3, 127.2,

127.2, 123.4, 113.3, 55.5, 47.7, 33.3, 27.1, 25.4, 24.0, 21.6, 13.8. MS: m/z calcd. for $[C_{21}H_{26}NO_2]^+$: 324; found: 324 $[M]^+$.

*5-Iodo-1,2,3,3-tetramethyl-3H-indol-1-ium iodide (S13cI)*⁹



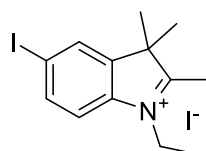
5-iodo-2,3,3-trimethyl-3H-indole [7] (2.0 g, 7.0 mmol, 1.0 equiv), iodomethane (1.3 mL, 21.0 mmol, 3.0 equiv).

Reaction conditions: 80 °C, 1.5 h.

Pale brown powder (2.15 g, 72%).

¹H NMR (500 MHz, DMSO-d₆) δ 8.28 (s, 1H), 8.00 (d, *J* = 8.3 Hz, 1H), 7.72 (d, *J* = 8.5 Hz, 1H), 3.94 (s, 3H), 2.74 (s, 3H), 1.52 (s, 7H). ¹³C NMR (126 MHz, DMSO-d₆) δ 196.0, 143.7, 141.9, 137.4, 132.1, 117.0, 95.9, 54.0, 34.7, 21.4, 14.1. LCMS: m/z calcd. for $[C_{12}H_{15}IN]^+$: 300; found 300 $[M]^+$.

*1-Ethyl-5-iodo-2,3,3-trimethyl-3H-indol-1-ium iodide (S13cII)*¹⁰



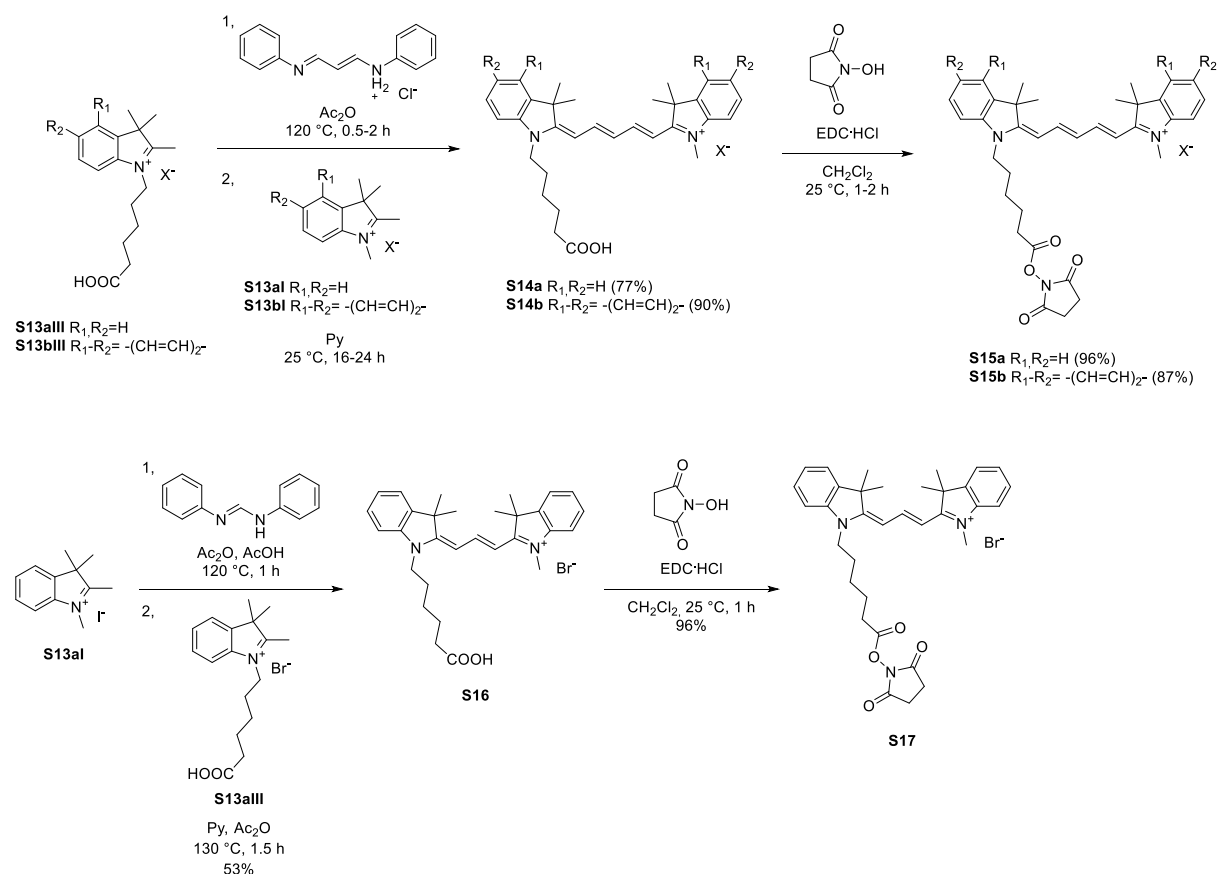
5-iodo-2,3,3-trimethyl-3H-indole [7] (530 mg, 1.86 mmol, 1.0 equiv), iodoethane (1.5 mL, 18.5 mmol, 10.0 equiv).

Reaction conditions: 80 °C, 16 h.

Beige powder (536 mg, 43%).

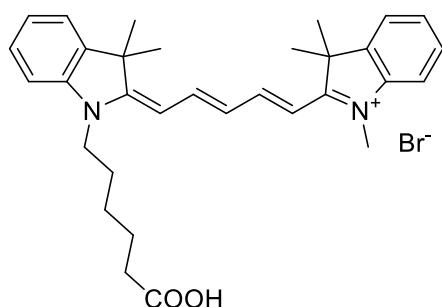
¹H NMR (500 MHz, DMSO-d₆) δ 8.30 (s, 1H), 7.99 (d, *J* = 8.5 Hz, 1H), 7.79 (d, *J* = 8.4 Hz, 1H), 4.47 (q, *J* = 7.3 Hz, 2H), 2.81 (s, 3H), 1.53 (s, 6H), 1.42 (t, *J* = 7.3 Hz, 3H). ¹³C NMR (126 MHz, DMSO-d₆) δ 196.1, 144.1, 140.5, 137.5, 132.4, 117.2, 96.1, 54.2, 43.1, 21.6, 13.8, 12.5. LCMS: m/z calcd. for $[C_{13}H_{17}IN]^+$: 314; found 314 $[M]^+$.

2.4. Synthesis of cyanine acceptor units



Scheme 4. Synthesis of cyanine **S15a**, **S15b** and **S17**.

2-(-5-(1-(5-carboxypentyl)-3,3-dimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-1,3,3-trimethyl-3H-indol-1-ium bromide (**S14a**)⁶



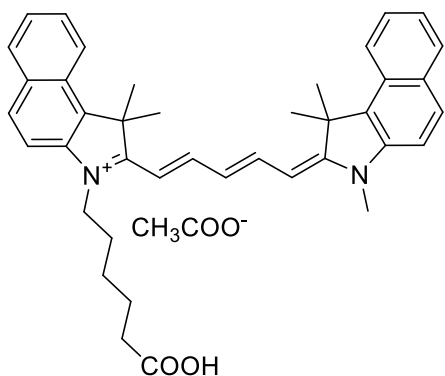
Compound **S13aIII** (200 mg, 0.565 mmol, 1.0 equiv) and *N*-(3-(phenylimino)prop-1-en-1-yl)benzenaminium chloride (174 mg, 0.678 mmol, 1.2 equiv) was dissolved in 1 mL acetic anhydride and stirred at 120 °C for 1 hour. After cooling to room temperature, **S13aI** (236 mg, 0.791 mmol, 1.4 equiv) and 1 mL pyridine was added to the mixture and stirred at room temperature for 16 hours. The solvents were removed in vacuo,

the crude product was purified by flash chromatography on silica gel (dichloromethane-methanol 0 to 5%) to yield 244 mg (77%) of blue crystals.

¹H NMR (500 MHz, CD₃OD) δ 8.24 (t, $J = 13.1$ Hz, 2H), 7.48 (d, $J = 7.4$ Hz, 2H), 7.41 (t, $J = 7.7$ Hz, 2H), 7.29 (d, $J = 7.9$ Hz, 2H), 7.26 (t, $J = 7.4$ Hz, 2H), 6.64 (t, $J = 12.4$ Hz, 1H), 6.29 (d, $J = 13.6$ Hz, 1H), 6.28 (d, $J = 13.7$ Hz, 1H), 5.50 – 4.07 (m, 15H), 3.63 (s, 3H), 2.28 (t, $J = 7.2$ Hz, 2H), 1.83 (p, $J = 7.6$ Hz, 2H), 1.74 – 1.65 (m, 14H), 1.50 (p, $J = 7.7, 7.1$ Hz, 2H). ¹³C NMR (126 MHz, CD₃OD) δ 177.5, 173.9, 173.3, 154.1, 142.9, 142.2, 141.3, 141.1, 128.3, 128.3, 125.3, 124.8, 122.0, 121.8, 110.6, 110.4,

103.0, 102.9, 49.2, 49.1, 43.5, 34.6, 30.1, 26.8, 26.6, 26.4, 26.1, 24.8. LCMS: m/z calcd. for $[C_{32}H_{39}N_2O_2]^+$: 483; found 483 $[M]^+$.

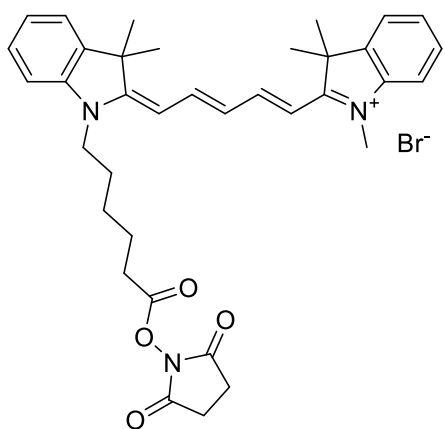
*3-(5-Carboxypentyl)-1,1-dimethyl-2-(5-(1,1,3-trimethyl-1,3-dihydro-2H-benzo[e]indol-2-ylidene)penta-1,3-dien-1-yl)-1H-benzo[e]indol-3-ium acetate (S14b)*⁸



Compound **S13bIII** (400 mg, 1.23 mmol, 1.0 equiv) and *N*-(3-(phenylimino)prop-1-en-1-yl)benzenaminium chloride (330 mg, 1.48 mmol, 1.2 equiv) was dissolved in 10 mL acetic anhydride, and heated at 120 °C for 30 minutes. After cooling to room temperature, **S13bI** (387 mg, 1.73 mmol, 1.4 equiv) and 10 mL pyridine was added. The resulting mixture was stirred overnight at room temperature. The solvent was evaporated under reduced pressure, the blue crude product was

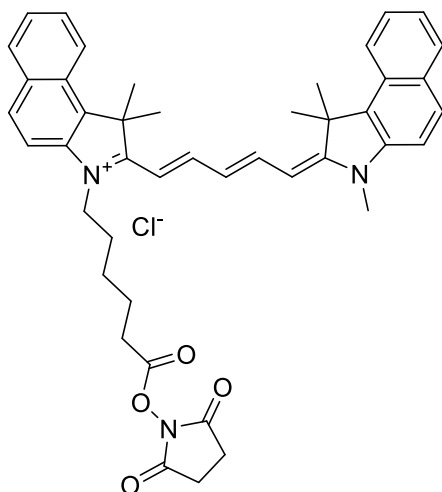
purified by silica flash chromatography (CH_2Cl_2 -methanol 0 to 10%) to yield 712 mg (90%) blue crystals. ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.44 (t, J = 13.1 Hz, 2H), 8.25 (m, 2H), 8.12 – 8.04 (m, 4H), 7.74 (t, J = 9.0 Hz, 2H), 7.68 (t, J = 7.7 Hz, 2H), 7.51 (t, J = 7.5 Hz, 2H), 6.63 (t, J = 12.3 Hz, 1H), 6.35 (m, 2H), 4.23 (t, J = 7.4 Hz, 2H), 3.74 (s, 3H), 2.23 – 2.18 (m, 2H), 2.11 (s, 3H), 1.97 (s, 6H), 1.96 (s, 6H), 1.76 (p, J = 7.7 Hz, 2H), 1.57 (p, J = 7.1 Hz, 2H), 1.43 (p, J = 7.7 Hz, 2H). ¹³C NMR (126 MHz, CD₃OD) δ 177.2, 176.6, 175.9, 173.9, 154.4, 154.3, 141.6, 141.0, 135.1, 134.9, 133.5, 133.4, 131.7, 131.6, 131.1, 129.5, 129.4, 128.7, 126.6, 126.3, 126.1, 124.4, 123.3, 123.0, 112.1, 111.9, 108.9, 104.1, 103.9, 52.4, 52.4, 45.0, 38.3, 34.6, 28.4, 27.7, 27.6, 27.3, 25.7, 21.3. LCMS: m/z calcd. for $[C_{40}H_{43}N_2O_2]^+$: 584; found: 584 $[M]^+$.

*2-(5-(1-(6-((2,5-dioxopyrrolidin-1-yl)oxy)-6-oxohexyl)-3,3-dimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-1,3,3-trimethyl-3H-indol-1-ium bromide (S15a)*⁶



A mixture of cyanine **S14a** (57 mg, 0.1 mmol, 1.0 equiv), *N*-hydroxysuccinimide (23 mg, 0.2 mmol, 2.0 equiv) and EDC·HCl (38 mg, 0.2 mmol, 2.0 equiv) in 5 mL dichloromethane was stirred at room temperature for 2 hours. Then, diluted with 20 mL dichloromethane, extracted with 30 mL 1 M HCl solution and 30 mL brine. The organic phase was dried over anhydrous MgSO₄, filtered and concentrated in vacuo to yield 65 mg (98%) of blue crystals. The product was used without any further purification. LCMS: m/z calcd. for $[C_{36}H_{42}N_3O_4]^+$: 580; found 580 $[M]^+$.

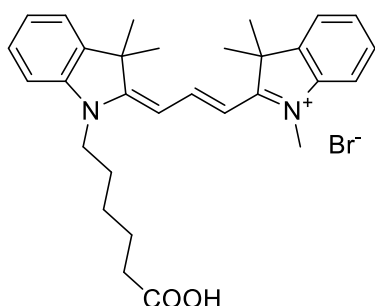
3-(6-((2,5-Dioxopyrrolidin-1-yl)oxy)-6-oxohexyl)-1,1-dimethyl-2-(5-(1,1,3-trimethyl-1,3-dihydro-2H-benzo[e]indol-2-ylidene)penta-1,3-dien-1-yl)-1H-benzo[e]indol-3-ium chloride (S15b)



Cyanine **S14b** (109 mg, 0.17 mmol, 1 equiv), *N*-hydroxysuccinimide (39 mg, 0.34 mmol, 2 equiv) and EDC·HCl (65 mg, 0.34 mmol, 2 equiv) were dissolved in 5 mL dichloromethane and the mixture was stirred at room temperature for 2 hours. The solution was diluted with dichloromethane, water and 1M HCl (50 mL each), the phases were separated, and the aqueous phase was extracted 2 times with dichloromethane. The combined organic phase was dried over anhydrous MgSO₄ and concentrated in vacuo to yield 106 mg (87%) blue crystals. The product was used without any

further purification. LCMS: *m/z* calcd. for [C₄₄H₄₆N₃O₄]⁺: 680; found 680 [M]⁺.

2-(3-(1-(5-carboxypentyl)-3,3-dimethylindolin-2-ylidene)prop-1-en-1-yl)-1,3,3-trimethyl-3H-indol-1-ium bromide (S16)⁶

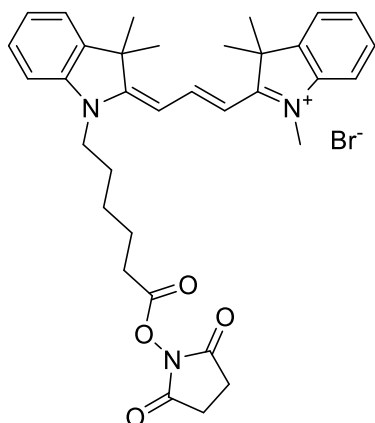


Compound **S13aI** (500 mg, 1.66 mmol, 1.5 equiv) and *N,N'*-diphenylformimidamide (652 mg, 3.32 mmol, 3.0 equiv) was reacted in a mixture of 2.5 mL acetic acid and 2.5 mL acetic anhydride at 120 °C for 1 hour. The reaction mixture was cooled to room temperature, 20 mL ethyl acetate was added, the precipitate was filtered, washed with ethyl acetate and dried. This intermediate hemi-cyanine was dissolved in 5 mL pyridine, **S13aIII** (392 mg, 1.11 mmol, 1.0 equiv)

and 0.5 mL acetic anhydride was added and stirred at 130 °C for 90 minutes. The solvent was evaporated, and the residue was purified by flash chromatography on silica gel (dichloromethane-methanol 0 to 10%) to yield 314 mg (53%) of pink crystals.

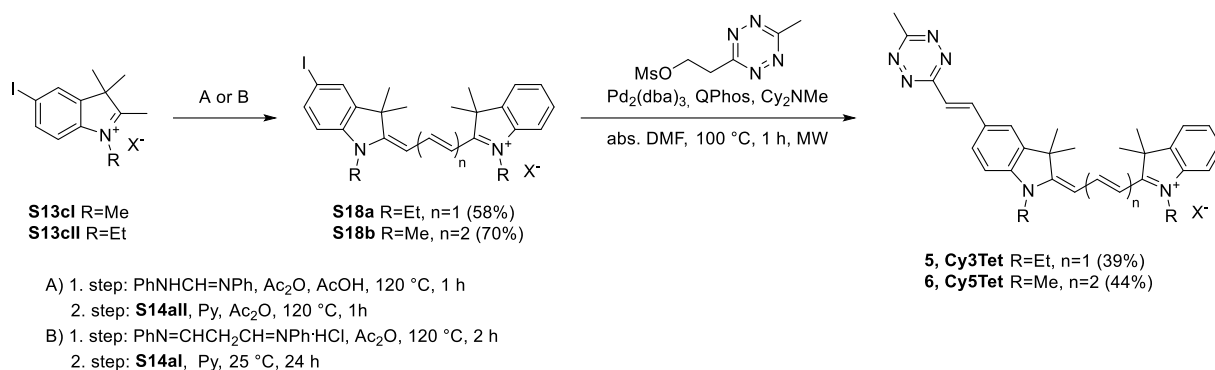
¹H NMR (500 MHz, CDCl₃) δ 8.38 (t, *J* = 13.5 Hz, 1H), 7.39 – 7.34 (m, 4H), 7.25 – 7.21 (m, 2H), 7.17 – 7.11 (m, 2H), 6.73 (d, *J* = 13.4 Hz, 1H), 6.63 (d, *J* = 13.4 Hz, 1H), 4.09 (t, *J* = 7.7 Hz, 2H), 3.72 (s, 3H), 2.41 (t, *J* = 7.1 Hz, 2H), 1.84 (p, *J* = 7.7 Hz, 2H), 1.76 (p, *J* = 7.2 Hz, 2H), 1.70 (s, 6H), 1.70 (s, 6H), 1.57 (p, *J* = 7.5 Hz, 2H). ¹³C NMR (126 MHz, CDCl₃) δ 177.0, 174.7, 174.0, 150.7, 142.8, 142.0, 140.7, 140.6, 129.1, 129.0, 125.59, 125.56, 122.3, 122.2, 111.13, 111.10, 104.4, 103.8, 49.2, 49.2, 44.6, 35.3, 32.1, 28.3, 28.2, 26.7, 26.3, 24.6. LCMS: *m/z* calcd. for [C₃₀H₃₇N₂O₂]⁺: 457; found 457 [M]⁺.

2-(3-(1-(6-((2,5-dioxopyrrolidin-1-yl)oxy)-6-oxohexyl)-3,3-dimethylindolin-2-ylidene)prop-1-en-1-yl)-1,3,3-trimethyl-3H-indol-1-ium bromide (**S17**)⁶



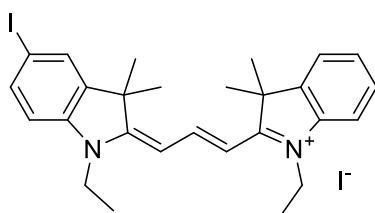
A mixture of cyanine **S16** (108 mg, 0.2 mmol, 1.0 equiv), *N*-hydroxysuccinimide (46 mg, 0.4 mmol, 2.0 equiv) and EDC·HCl (77 mg, 0.4 mmol, 2.0 equiv) in 10 mL dichloromethane was stirred at room temperature for 1 hours. Then, diluted with 40 mL dichloromethane, extracted with 40 mL 1 M HCl solution and 40 mL brine. The organic phase was dried over anhydrous MgSO₄, filtered and concentrated in vacuo to yield 122 mg (96%) of pink crystals. The product was used without any further purification. LCMS: *m/z* calcd. for [C₃₄H₄₀N₃O₄]⁺: 554; found 554 [M]⁺.

2.5. Synthesis of fluorogenic cyanines



Scheme 5. Synthesis of fluorogenic cyanine **S19a** and **S19b**.

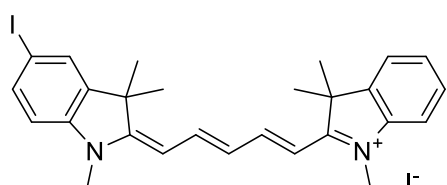
1-Ethyl-2-(3-(1-ethyl-5-iodo-3,3-dimethylindolin-2-ylidene)prop-1-en-1-yl)-3,3-dimethyl-3H-indol-1-ium iodide (**S18a**)



Compound **S13cII** (500 mg, 1.13 mmol, 2.0 equiv) and *N,N'*-diphenylformimidamide (444 mg, 2.26 mmol, 4.0 equiv) was reacted in a mixture of 2 mL acetic acid and 2 mL acetic anhydride at 120 °C for 1 hour. The reaction mixture was cooled to room temperature, 20 mL ethyl acetate was added, the precipitate was filtered and washed with ethyl acetate and dried. This intermediate hemi-cyanine was dissolved in 5 mL pyridine, **S13aII** (186 mg, 0.590 mmol, 1.0 equiv) and 0.5 mL acetic anhydride was added and stirred at 120 °C for 1 hour. The solvent was evaporated, and the residue was purified by flash chromatography on silica gel (dichloromethane-methanol 0 to 5%) to yield 218 mg (58%) of pink crystals. ¹H NMR (500 MHz, CDCl₃) δ 8.41 (t, *J* = 13.3 Hz, 1H), 7.69 (d, *J* = 8.2 Hz, 1H), 7.63 (s, 1H), 7.47 (d, *J* = 13.5 Hz, 1H), 7.44 – 7.35 (m, 3H), 7.28 (t, *J* = 7.4 Hz, 1H), 7.18 (d, *J* = 7.9 Hz, 1H), 6.91 (d, *J* = 8.3 Hz, 1H), 4.37 (q, *J* = 7.2 Hz, 2H), 4.28 (q, *J* = 7.2 Hz, 2H), 1.71 (s, 6H), 1.69 (s, 6H), 1.51 (t, *J* = 7.1 Hz, 3H), 1.47 (t, *J*

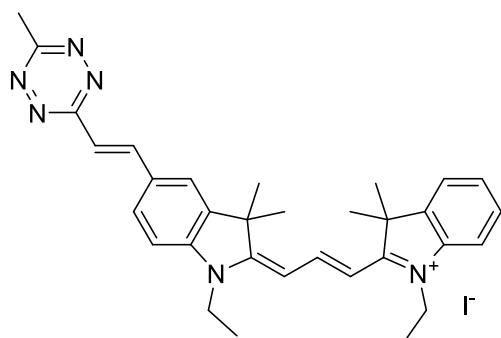
= 7.1 Hz, 3H). ^{13}C NMR (126 MHz, CDCl_3) δ 174.1, 171.8, 150.9, 142.9, 141.8, 141.7, 141.0, 137.8, 131.3, 129.1, 125.7, 122.3, 112.5, 111.2, 105.5, 104.4, 88.2, 49.2, 48.6, 40.6, 40.2, 28.2, 28.1, 13.2, 12.9. LCMS: m/z calcd. for $[\text{C}_{27}\text{H}_{32}\text{IN}_2]^+$: 511; found 511 $[\text{M}]^+$. HRMS: m/z calcd. for $[\text{C}_{27}\text{H}_{32}\text{IN}_2]^+$: 511.1610; found 511.1613 $[\text{M}]^+$.

*2-(5-(5-iodo-1,3,3-trimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-1,3,3-trimethyl-3H-indol-1-ium iodide (S18b)*⁸



Compound **S13cI** (700 mg, 1.64 mmol, 1.0 equiv) and *N*-(3-(phenylimino)prop-1-en-1-yl)benzenaminium chloride (510 mg, 2.29 mmol, 1.2 equiv) was dissolved in 5 mL acetic anhydride and stirred at 120 °C for 2 hours. Then, it was cooled to room temperature, **S13aI** (592 mg, 1.97 mmol, 1.2 equiv) and 5 mL pyridine was added to the mixture and stirred at room temperature for 24 hours. The solvents were removed in vacuo, the crude product was purified by flash chromatography on silica gel (dichloromethane-methanol 0 to 2%) to yield 876 mg (70%) of blue crystals. ^1H NMR (500 MHz, CD_3OD) δ 8.27 (t, J = 13.1 Hz, 1H), 8.20 (t, J = 13.1 Hz, 1H), 7.78 (d, J = 1.4 Hz, 1H), 7.71 (dd, J = 8.3, 1.5 Hz, 1H), 7.52 (d, J = 7.4 Hz, 1H), 7.44 (t, J = 7.7 Hz, 1H), 7.35 (d, J = 8.0 Hz, 1H), 7.31 (t, J = 7.5 Hz, 1H), 7.05 (d, J = 8.3 Hz, 1H), 6.63 (t, J = 12.4 Hz, 1H), 6.35 (d, J = 13.9 Hz, 1H), 6.18 (d, J = 13.5 Hz, 1H), 3.67 (s, 3H), 3.55 (s, 3H), 1.73 (s, 6H), 1.70 (s, 6H). ^{13}C NMR (126 MHz, CD_3OD) δ 175.2, 171.9, 154.9, 153.4, 143.2, 143.0, 142.7, 141.4, 137.2, 131.0, 128.4, 125.6, 125.4, 121.9, 111.9, 110.9, 104.1, 102.3, 86.9, 49.5, 48.6, 30.4, 29.9, 26.4, 26.3. LCMS: m/z calcd. for $[\text{C}_{27}\text{H}_{30}\text{IN}_2]^+$: 509; found 509 $[\text{M}]^+$.

1-Ethyl-2-(3-(1-ethyl-3,3-dimethyl-5-((E)-2-(6-methyl-1,2,4,5-tetrazin-3-yl)vinyl)indolin-2-ylidene)prop-1-en-1-yl)-3,3-dimethyl-3H-indol-1-ium iodide (5, Cy3Tet)



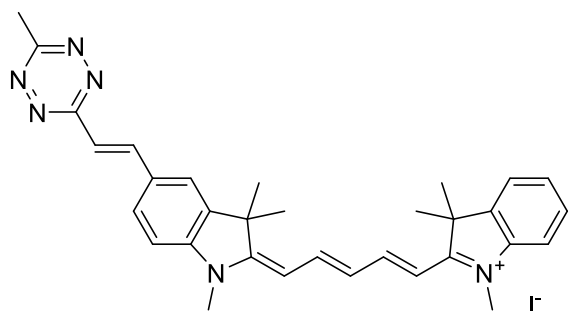
A mixture of cyanine **S18a** (30 mg, 0.0470 mmol, 1.0 equiv), 2-(6-methyl-1,2,4,5-tetrazin-3-yl)ethyl methanesulfonate [3] (31 mg, 0.141 mmol, 3.0 equiv), Pd_2dba_3 (4.3 mg, 0.0047 mmol, 0.1 equiv), QPhos (3.3 mg, 0.0047 mmol, 0.1 equiv), *N,N*-dicyclohexylmethylamine (50 μL , 0.236 mmol, 5.0 equiv) and 1 mL anhydrous dimethylformamide was purged with N_2 for 15 minutes. The reaction mixture was heated in a microwave reactor at 100 °C for 1 hour. The solvent was evaporated in vacuo and the crude product was purified by flash chromatography on silica gel (dichloromethane-methanol 0 to 5%) and preparative HPLC (water-acetonitrile starting from 95 : 5 to 0 : 100) to yield 12 mg (39%) of pink crystals.

^1H NMR (500 MHz, CDCl_3) δ 8.44 (t, J = 13.3 Hz, 1H), 8.32 (d, J = 16.2 Hz, 1H), 7.68 (dd, J = 8.1, 1.7 Hz, 1H), 7.66 (d, J = 1.6 Hz, 1H), 7.47 – 7.38 (m, 4H, contained in this multiplet: 7.44 (d, J = 16.3 Hz,

1H)), 7.35 (d, $J = 13.1$ Hz, 1H), 7.29 (t, $J = 7.5$ Hz, 1H), 7.19 (d, $J = 7.9$ Hz, 1H), 7.16 (d, $J = 8.2$ Hz, 1H), 4.39 (q, $J = 7.2$ Hz, 2H), 4.33 (q, $J = 7.2$ Hz, 2H), 3.06 (s, 3H), 1.76 (s, 6H), 1.74 (s, 6H), 1.52 (apparent q, $J = 6.7$ Hz, 6H, containing: 1.53 (t, $J = 6.7$ Hz, 3H) and 1.52 (t, $J = 6.7$ Hz, 3H)).

^{13}C NMR (126 MHz, CDCl_3) δ 174.4, 172.3, 166.4, 164.9, 151.2, 143.8, 141.8, 141.7, 141.2, 139.9, 132.2, 130.1, 129.2, 125.9, 122.3, 121.1, 120.2, 111.4, 111.0, 106.5, 105.4, 54.0, 49.4, 48.6, 40.6, 40.3, 29.5, 28.5, 28.2, 21.3, 13.2, 12.9. HRMS: m/z calcd. for $[\text{C}_{32}\text{H}_{37}\text{N}_6]^+$: 505.308; found 505.3082 $[\text{M}]^+$.

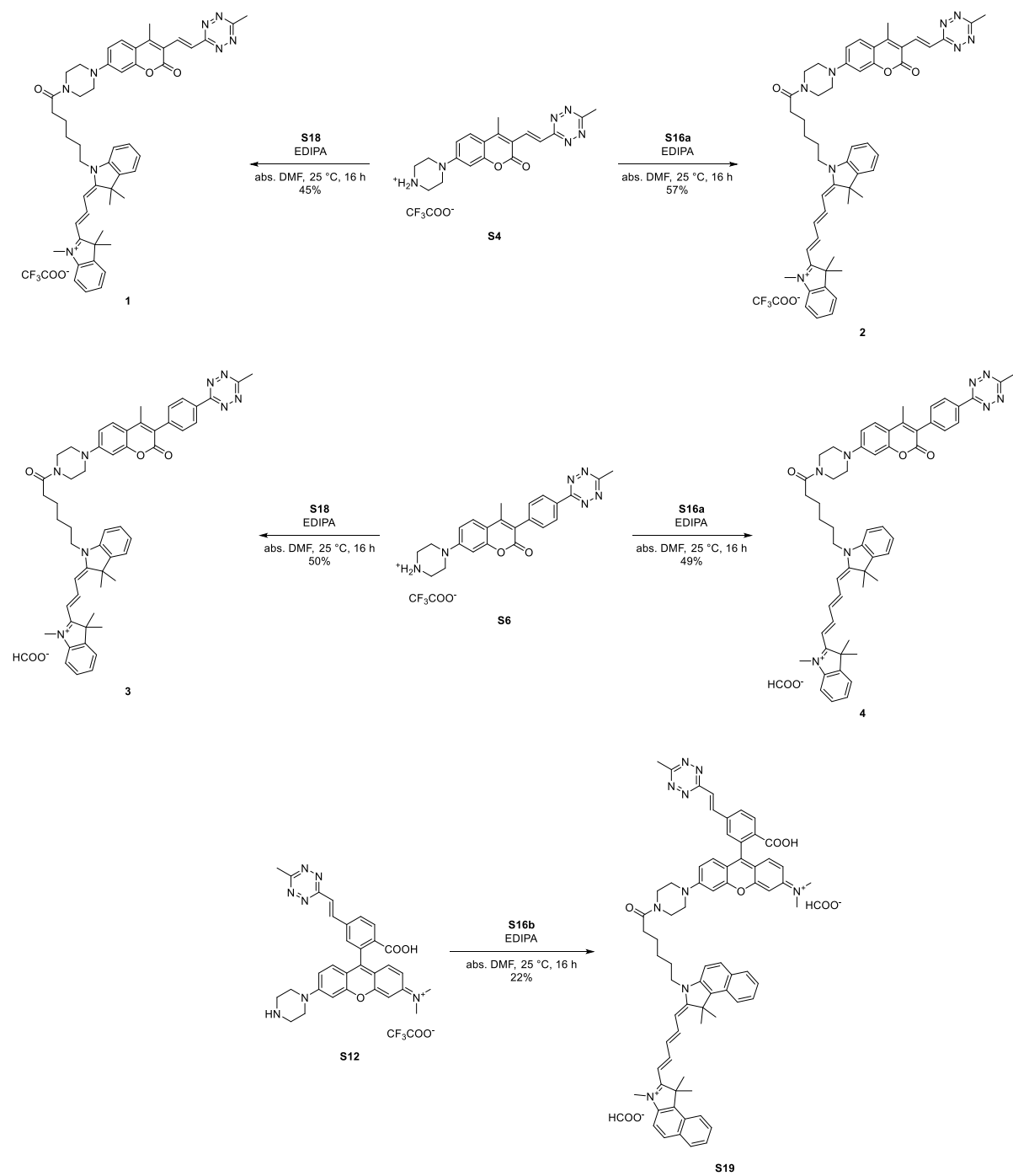
1,3,3-Trimethyl-2-(5-(1,3,3-trimethyl-5-((E)-2-(6-methyl-1,2,4,5-tetrazin-3-yl)vinyl)indolin-2-ylidene)penta-1,3-dien-1-yl)-3H-indol-1-ium iodide (6, Cy5Tet)



A mixture of cyanine **S18b** (30 mg, 0.0471 mmol, 1.0 equiv), 2-(6-methyl-1,2,4,5-tetrazin-3-yl)ethyl methanesulfonate [3] (31 mg, 0.141 mmol, 3.0 equiv), Pd_2dba_3 (4.3 mg, 0.0047 mmol, 0.1 equiv), QPhos (3.3 mg, 0.0047 mmol, 0.1 equiv), *N,N*-dicyclohexylmethylamine (50 μL , 0.236 mmol, 5.0 equiv) and 1 mL anhydrous dimethylformamide was

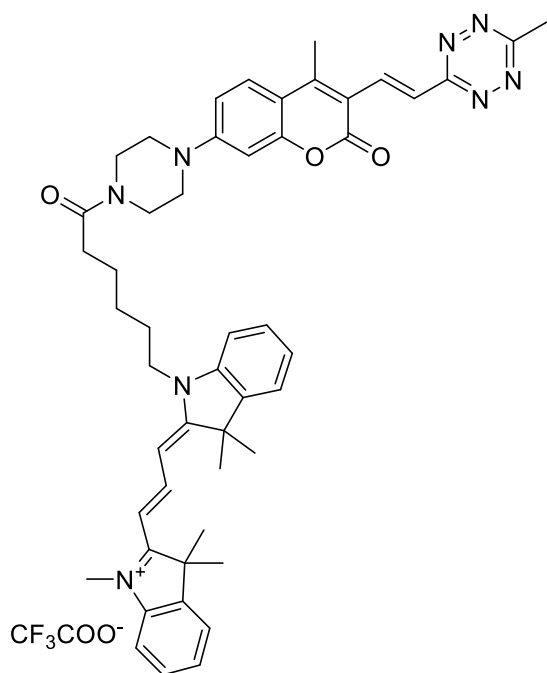
purged with N_2 for 15 minutes. The reaction mixture was heated in a microwave reactor at 100 $^\circ\text{C}$ for 1 hour. The solvent was evaporated in vacuo and the crude product was purified by flash chromatography on silica gel (dichloromethane-methanol 0 to 10%) and preparative HPLC (water–acetonitrile starting from 95 : 5 to 0 : 100) to yield 13 mg (44%) of blue crystals. ^1H NMR (500 MHz, CDCl_3) δ 8.31 (d, $J = 16.2$ Hz, 1H), 8.03 (t, $J = 12.9$ Hz, 1H), 7.97 (t, $J = 12.9$ Hz, 1H), 7.67 – 7.63 (m, 2H), 7.44 – 7.38 (m, 3H, contained in this multiplet: 7.42 (d, $J = 16.2$ Hz, 1H)), 7.29 (t, $J = 7.5$ Hz, 1H), 7.18 (d, $J = 7.9$ Hz, 1H), 7.09 (d, $J = 8.6$ Hz, 1H), 6.72 (t, $J = 12.3$ Hz, 1H), 6.41 (d, $J = 13.7$ Hz, 1H), 6.19 (d, $J = 13.2$ Hz, 1H), 3.72 (s, 3H), 3.60 (s, 3H), 3.06 (s, 3H), 1.74 (s, 6H), 1.71 (s, 6H). ^{13}C NMR (126 MHz, CDCl_3) δ 175.3, 171.7, 166.4, 164.9, 154.9, 152.9, 144.8, 142.7, 141.7, 141.4, 140.1, 131.9, 129.9, 129.0, 127.3, 126.2, 122.4, 121.1, 120.0, 117.4, 115.1, 111.2, 110.4, 105.7, 103.9, 49.9, 48.7, 28.3, 27.9, 21.3. HRMS: m/z calcd. for $[\text{C}_{32}\text{H}_{35}\text{N}_6]^+$: 503.2923; found 503.2932 $[\text{M}]^+$.

2.6. Synthesis of fluorogenic dyads



Scheme 6. Synthesis of fluorogenic dyads

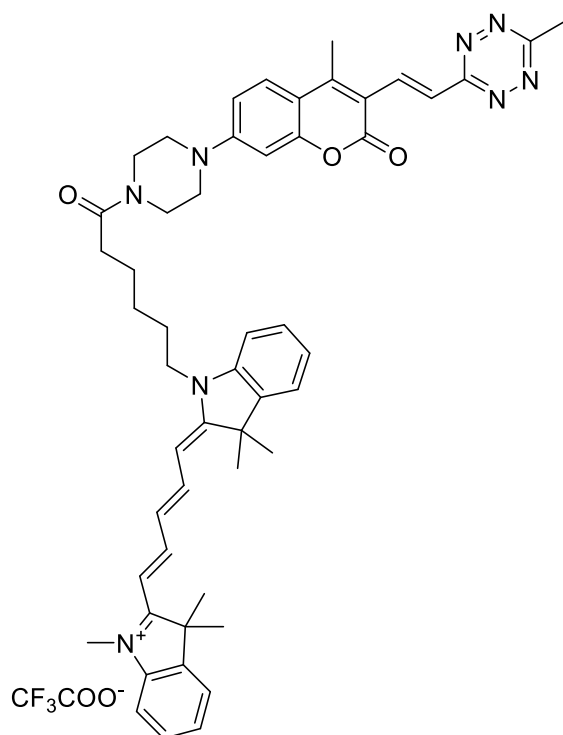
2-((*E*)-3-((*E*)-3,3-dimethyl-1-(6-(4-(4-methyl-3-((*E*)-2-(6-methyl-1,2,4,5-tetrazin-3-yl)vinyl)-2-oxo-2H-chromen-7-yl)piperazin-1-yl)-6-oxohexyl)indolin-2-ylidene)prop-1-en-1-yl)-1,3,3-trimethyl-3H-indol-1-ium 2,2,2-trifluoroacetate (**1**)



Cyanine **S17** (20 mg, 0.032 mmol, 1.0 equiv), coumarin **S4** (17.5 mg, 0.038 mmol, 1.2 equiv) and *N,N*-diisopropylethylamine (13.7 μ L, 0.079 mmol, 2.5 equiv) was reacted in 3 mL anhydrous dimethylformamide at room temperature for 16 hours. The solvent was evaporated, and the residue was purified by preparative HPLC (H₂O (0.1% TFA) – MeCN (0.1% TFA) starting from 95 : 5 to 0 : 100) to yield 13 mg (45%) pink crystals. ¹H NMR (500 MHz, CD₃OD) δ 8.51 (t, *J* = 13.5 Hz, 1H), 8.38 (d, *J* = 15.9 Hz, 1H), 8.08 (d, *J* = 15.8 Hz, 1H), 7.65 (d, *J* = 9.1 Hz, 1H), 7.54 (d, *J* = 7.4 Hz, 1H), 7.47 (d, *J* = 7.7 Hz, 1H), 7.43 (t, *J* = 7.7 Hz, 1H), 7.38 – 7.34 (m, 2H), 7.29 (t, *J* = 7.4 Hz, 1H), 7.26 – 7.22 (m, 2H), 6.92 (dd, *J* = 9.0,

2.5 Hz, 1H), 6.66 (d, *J* = 2.5 Hz, 1H), 6.44 (d, *J* = 13.4 Hz, 1H), 6.40 (d, *J* = 13.4 Hz, 1H), 4.18 (t, *J* = 7.2 Hz, 2H), 3.74 – 3.69 (m, 4H), 3.62 (s, 3H), 3.44 – 3.37 (m, 4H), 2.98 (s, 3H), 2.58 (s, 3H), 2.49 (t, *J* = 7.0 Hz, 2H), 1.90 (p, *J* = 7.4 Hz, 2H), 1.78 – 1.73 (m, 8H), 1.71 (s, 6H), 1.51 (p, *J* = 7.8, 7.3 Hz, 2H). ¹³C NMR (126 MHz, CD₃OD) δ 176.6, 176.2, 174., 167.7, 166.5, 161.5, 155.9, 155.0, 154.6, 152.1, 143.8, 143.4, 142.2, 142.0, 133.5, 130.0, 129.9, 128.4, 126.8, 126.7, 125.6, 123.5, 123.3, 115.8, 112.8, 112.7, 112.6, 112.2, 103.7, 103.6, 100.6, 50.7, 50.5, 48.1, 47.8, 46.3, 45.2, 42.4, 33.1, 31.8, 28.4, 28.4, 28.2, 27.3, 26.1, 21.1, 15.1. HRMS: *m/z* calcd. for [C₄₉H₅₅N₈O₃]⁺: 803.4397; found 803.4416 [M]⁺.

2-((1*E*,3*E*)-5-((*E*)-3,3-dimethyl-1-(6-(4-(4-methyl-3-((*E*)-2-(6-methyl-1,2,4,5-tetrazin-3-yl)vinyl)-2-oxo-2*H*-chromen-7-yl)piperazin-1-yl)-6-oxohexyl)indolin-2-ylidene)penta-1,3-dien-1-yl)-1,3,3-trimethyl-3*H*-indol-1-ium 2,2,2-trifluoroacetate (**2**)

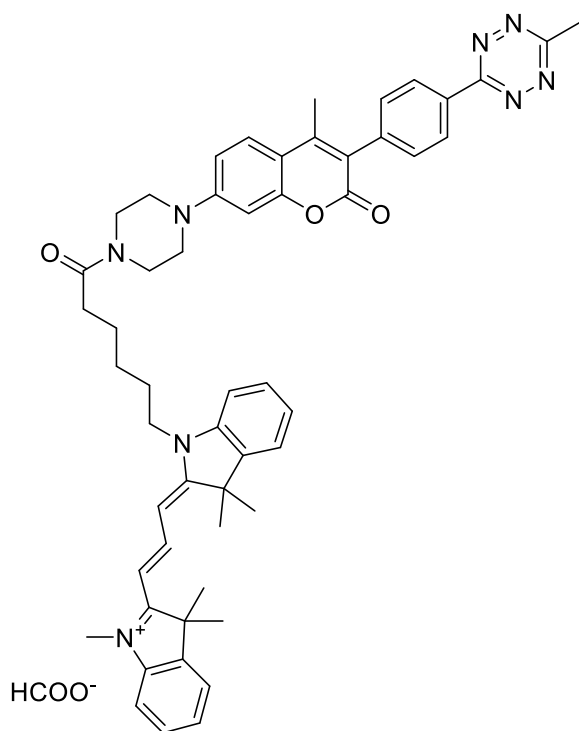


Cyanine **S15a** (11 mg, 0.017 mmol, 1.0 equiv), coumarin **S4** (9.2 mg, 0.020 mmol, 1.2 equiv) and *N,N*-diisopropylethylamine (7.3 μ L, 0.042 mmol, 2.5 equiv) was reacted in 1.5 mL anhydrous dimethylformamide at room temperature for 16 hours. The solvent was evaporated, and the residue was purified by preparative HPLC (H₂O (0.1% TFA) – MeCN (0.1% TFA) starting from 95 : 5 to 0 : 100) to yield 9 mg (57%) green crystals. ¹H NMR (500 MHz, CD₃OD) δ 8.37 (d, *J* = 15.9 Hz, 1H), 8.2 2 (t, *J* = 12.9 Hz, 1H), 8.17 (t, *J* = 13.1 Hz, 1H), 8.08 (d, *J* = 15.9 Hz, 1H), 7.69 (d, *J* = 9.1 Hz, 1H), 7.49 (dd, *J* = 7.5, 1.1 Hz, 1H), 7.42 – 7.39 (m, 2H), 7.34 – 7.29 (m, 2H), 7.25 (t, *J* = 7.4 Hz, 1H), 7.22 – 7.15 (m, 2H), 6.97 (dd, *J* = 9.1, 2.6 Hz, 1H), 6.72 (d, *J* = 2.5 Hz,

1H), 6.59 (t, *J* = 12.4 Hz, 1H), 6.30 (d, *J* = 13.7 Hz, 1H), 6.18 (d, *J* = 13.7 Hz, 1H), 4.15 (t, *J* = 6.9 Hz, 2H), 3.74 – 3.70 (m, 4H), 3.54 (s, 3H), 3.46 – 3.42 (m, 4H), 3.00 (s, 3H), 2.55 (s, 3H), 2.48 (t, *J* = 7.1 Hz, 2H), 1.88 (p, *J* = 7.0 Hz, 2H), 1.76 – 1.70 (m, 8H), 1.61 (s, 6H), 1.51 – 1.44 (m, 2H).

¹³C NMR (126 MHz, CD₃OD) δ 143.0, 166.5, 161.5, 156.0, 155.3, 155.1, 154.5, 144.0, 143.8, 143.5, 142.7, 142.4, 133.5, 131.2, 129.8, 129.7, 128.4, 126.3, 126.2, 125.7, 123.4, 123.2, 115.8, 114.0, 112.9, 112.7, 112.2, 111.8, 111.5, 104.4, 104.3, 100.7, 96.3, 93.2, 50.6, 50.4, 48.1, 47.8, 46.3, 44.7, 42.5, 33.0, 31.5, 28.3, 28.0, 27.8, 27.2, 26.0, 21.1, 15.0. HRMS: *m/z* calcd. for [C₅₁H₅₇N₈O₃]⁺: 829.4554; found 829.4567 [M]⁺.

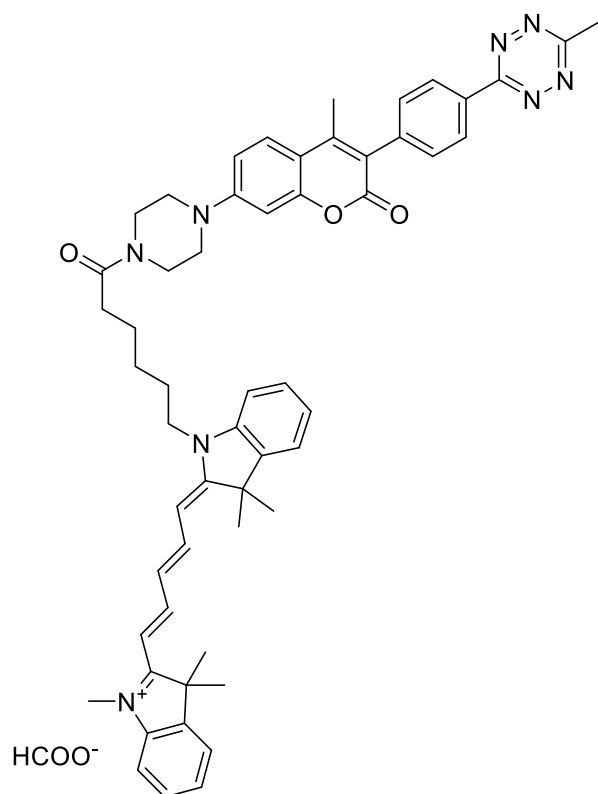
2-((*E*)-3-((*E*)-3,3-dimethyl-1-(6-(4-(4-methyl-3-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-2-oxo-2*H*-chromen-7-yl)piperazin-1-yl)-6-oxohexyl)indolin-2-ylidene)prop-1-en-1-yl)-1,3,3-trimethyl-3*H*-indol-1-ium formate (**3**)



Cyanine **S17** (7.2 mg, 0.0114 mmol, 1.0 equiv), coumarin **S6** (7.0 mg, 0.0137 mmol, 1.2 equiv) and *N,N*-diisopropylethylamine (5.0 μ L, 0.0285 mmol, 2.5 equiv) was reacted in 1 mL anhydrous dimethylformamide at room temperature for 16 hours. The solvent was evaporated, and the residue was purified by preparative HPLC (H_2O (0.1% HCOOH) – MeCN (0.1% HCOOH) starting from 95 : 5 to 0 : 100) to yield 5.5 mg (51%) pink crystals. ^1H NMR (500 MHz, CD_3OD) δ 8.64 (d, $J = 8.3$ Hz, 2H), 8.54 (t, $J = 13.4$ Hz, 1H), 7.64 (d, $J = 9.1$ Hz, 1H), 7.57 (d, $J = 8.2$ Hz, 2H), 7.55 (d, $J = 7.4$ Hz, 1H), 7.50 (d, $J = 7.4$ Hz, 1H), 7.46 – 7.39 (m, 2H), 7.37 (d, $J = 8.0$ Hz, 1H), 7.32 – 7.26 (m, 3H), 7.00 (dd, $J = 9.0, 2.5$ Hz, 1H), 6.79 (d, $J = 2.4$ Hz, 1H),

6.47 (d, $J = 13.4$ Hz, 1H), 6.43 (d, $J = 13.4$ Hz, 1H), 4.19 (t, $J = 7.2$ Hz, 2H), 3.79 – 3.70 (m, 4H), 3.65 (s, 3H), 3.45 – 3.35 (m, 4H), 3.06 (s, 3H), 2.50 (t, $J = 7.1$ Hz, 2H), 2.30 (s, 3H), 1.90 (p, $J = 7.3$ Hz, 2H), 1.79 – 1.71 (m, 14H), 1.52 (p, $J = 7.8$ Hz, 2H). ^{13}C NMR (126 MHz, CD_3OD) δ 176.6, 176.3, 174.0, 168.9, 165.2, 163.3, 155.8, 154.6, 152.1, 151.1, 150.4, 143.9, 143.4, 142.2, 142.0, 140.8, 133.0, 132.7, 130.0, 129.9, 128.7, 127.8, 126.8, 126.7, 123.5, 123.3, 122.8, 113.2, 113.0, 112.6, 112.2, 103.7, 103.7, 101.5, 50.7, 50.6, 48.3, 46.5, 45.2, 42.5, 33.1, 31.8, 30.7, 28.4, 28.4, 28.2, 27.4, 26.2, 21.1, 16.7. HRMS: m/z calcd. for $[\text{C}_{53}\text{H}_{57}\text{N}_8\text{O}_3]^+$: 853.4554; found 853.4567 $[\text{M}]^+$.

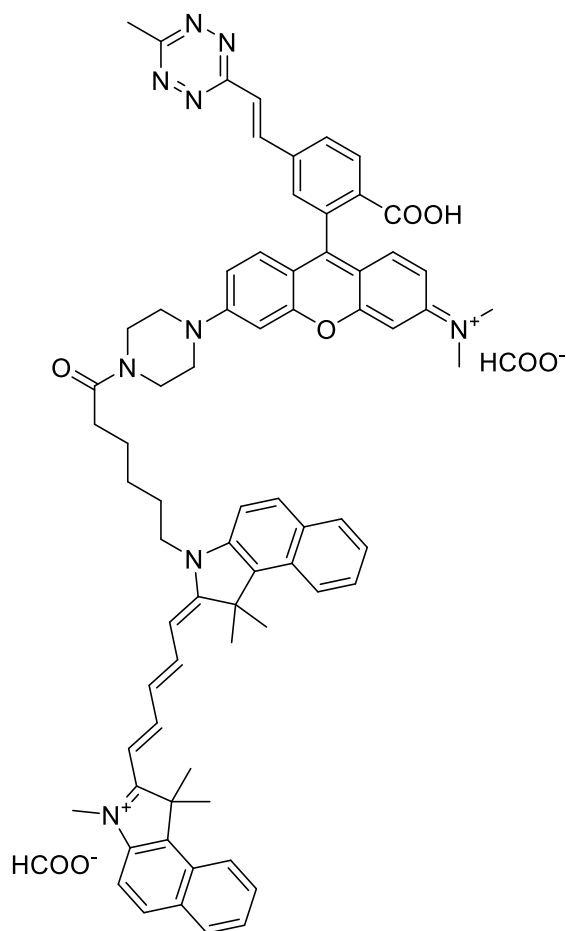
2-((1*E*,3*E*)-5-((*E*)-3,3-dimethyl-1-(6-(4-(4-methyl-3-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-2-oxo-2*H*-chromen-7-yl)piperazin-1-yl)-6-oxohexyl)indolin-2-ylidene)penta-1,3-dien-1-yl)-1,3,3-trimethyl-3*H*-indol-1-ium formate (**4**)



Cyanine **S15a** (7.5 mg, 0.0114 mmol, 1.0 equiv), coumarin **S6** (7.0 mg, 0.0137 mmol, 1.2 equiv) and *N,N*-diisopropylethylamine (5.0 μ L, 0.0285 mmol, 2.5 equiv) was reacted in 1 mL anhydrous dimethylformamide at room temperature for 16 hours. The solvent was evaporated, and the residue was purified by preparative HPLC (H_2O (0.1% HCOOH) – MeCN (0.1% HCOOH) starting from 95 : 5 to 0 : 100) to yield 5.5 mg (49%) blue crystals. ^1H NMR (500 MHz, CD_3OD) δ 8.61 (d, J = 8.3 Hz, 2H), 8.24 (t, J = 11.8 Hz, 1H), 8.19 (t, J = 12.0 Hz, 1H), 7.63 (d, J = 9.0 Hz, 1H), 7.51 – 7.43 (m, 4H), 7.42 – 7.36 (m, 2H), 7.31 (d, J = 7.9 Hz, 1H), 7.28 – 7.22 (m, 3H), 7.00 (dd, J = 9.0, 2.5 Hz, 1H), 6.80 (d, J = 2.5 Hz, 1H), 6.62 (t, J = 12.4 Hz, 1H), 6.32 (d, J = 13.7 Hz, 1H), 6.23 (d, J =

13.7 Hz, 1H), 4.15 (t, J = 7.0 Hz, 2H), 3.76 – 3.70 (m, 4H), 3.58 (s, 3H), 3.43 – 3.38 (m, 4H), 3.06 (s, 3H), 2.49 (t, J = 7.1 Hz, 2H), 2.24 (s, 3H), 1.88 (p, J = 7.1 Hz, 2H), 1.78 – 1.71 (m, 8H), 1.64 (s, 6H), 1.53 – 1.46 (m, 2H). ^{13}C NMR (126 MHz, CD_3OD) δ 175.3, 175.0, 174.8, 174.0, 168.8, 165.3, 165.2, 163.2, 155.8, 154.7, 154.5, 151.1, 144.1, 143.6, 142.7, 142.5, 140.7, 132.9, 132.7, 129.8, 129.7, 128.6, 128.6, 127.7, 126.28, 126.27, 123.4, 123.2, 122.6, 113.0, 112.2, 111.9, 104.4, 101.5, 50.6, 50.5, 48.2, 46.5, 44.7, 42.6, 33.1, 31.6, 28.6, 28.3, 28.0, 27.8, 27.3, 26.1, 21.1, 16.6. HRMS: m/z calcd. for $[\text{C}_{55}\text{H}_{59}\text{N}_8\text{O}_3]^+$: 879.471; found 879.4719 $[\text{M}]^+$.

2-((1*E*,3*E*,5*E*)-5-(3-(6-(4-(9-(2-carboxy-5-((*E*)-2-(6-methyl-1,2,4,5-tetrazin-3-yl)vinyl)phenyl)-3-(dimethyliminio)-3*H*-xanthen-6-yl)piperazin-1-yl)-6-oxohexyl)-1,1-dimethyl-1,3-dihydro-2*H*-benzo[*e*]indol-2-ylidene)penta-1,3-dien-1-yl)-1,1,3-trimethyl-1*H*-benzo[*e*]indol-3-ium formate (**S19**)



Cyanine **S15b** (13 mg, 0.018 mmol, 1 equiv), rhodamine **S12** (12 mg, 0.018 mmol, 1 equiv) and *N,N*-diisopropylethylamine (7.8 μ L, 0.045 mmol, 2.5 equiv) was dissolved in 4 mL anhydrous dimethylformamide. The mixture was stirred at room temperature for 4 hours. After the completion of the reaction the solvent was removed, and the crude product was purified with preparative-HPLC (H₂O (0.1% TFA) – MeCN (0.1% TFA) starting from 95 : 5 to 0 : 100).

Purple powder (5.3 mg, 22 %).

¹H NMR (500 MHz, CD₃OD) δ 8.42 – 8.30 (m, 4H), 8.25 (d, *J* = 8.5 Hz, 2H), 8.19 (dd, *J* = 8.3, 1.8 Hz, 1H), 8.01 (d, *J* = 8.8 Hz, 1H), 7.96 (d, *J* = 8.4 Hz, 1H), 7.91 (d, *J* = 8.3 Hz, 1H), 7.79 (d, *J* = 8.7 Hz, 1H), 7.72 (d, *J* = 16.3 Hz, 1H), 7.69 – 7.59 (m, 4H), 7.51 – 7.44 (m, 3H), 7.23 (d, *J* = 9.3 Hz, 1H), 7.20 (d, *J* = 9.6 Hz, 1H), 7.14 (dd, *J* = 9.7, 2.5 Hz, 1H), 7.05 (dd, *J* = 9.5, 2.5 Hz, 1H), 7.00 (d, *J* = 2.5 Hz, 1H),

6.66 (t, *J* = 12.3 Hz, 1H), 6.39 (d, *J* = 13.7 Hz, 1H), 6.24 (d, *J* = 13.6 Hz, 1H), 4.29 (t, *J* = 6.9 Hz, 2H), 3.76 (t, *J* = 5.2 Hz, 4H), 3.72 – 3.66 (m, 4H), 3.54 (s, 3H), 3.18 (s, 6H), 3.01 (s, 3H), 2.48 (t, *J* = 7.0 Hz, 2H), 2.06 – 2.02 (m, 12H), 1.99 – 1.94 (m, 2H), 1.80 – 1.72 (m, 2H), 1.55 – 1.47 (m, 2H). ¹³C NMR (126 MHz, CD₃OD) δ 175.1, 172.7, 166.8, 164.3, 157.6, 140.2, 139.5, 139.3, 137.6, 133.3, 132.2, 132.0, 131.9, 131.6, 131.4, 130.9, 130.8, 130.3, 130.1, 129.7, 129.7, 129.1, 128.9, 128.0, 127.9, 127.4, 127.4, 126.7, 124.9, 124.9, 124.8, 124.6, 124.5, 121.9, 121.9, 121.2, 114.0, 113.8, 113.7, 111.2, 111.1, 110.8, 110.5, 106.8, 103.2, 102.8, 102.4, 101.9, 101.3, 97.9, 97.3, 95.8, 51.1, 50.9, 46.5, 46.0, 44.4, 43.4, 39.5, 31.6, 30.4, 29.3, 29.3, 29.0, 28.1, 27.2, 26.2, 22.3. HRMS: *m/z* calcd. for [C₇₁H₇₀N₉O₄]⁺: 1112.5551, found: 1112.5560 [M]⁺.

3. Spectroscopic characterization

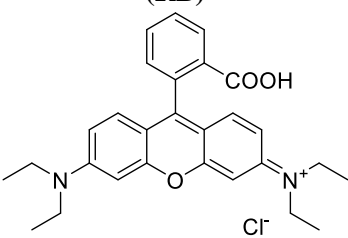
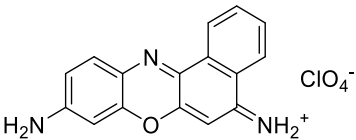
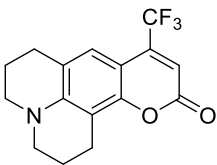
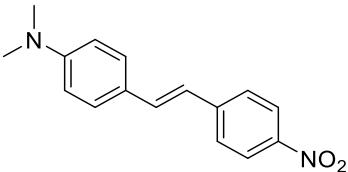
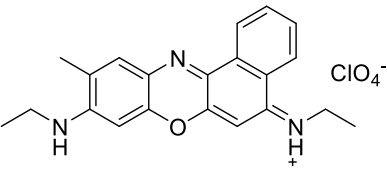
Photophysical measurements were performed on a *JASCO FP 8300* spectrofluorometer and a *JASCO v750* spectrophotometer. A stock solution in DMSO was prepared from the solid dyes (0.5-1 mM). All experiments were conducted in all-sodium PBS (pH=7.4, containing 0.1% SDS), in order to prevent aggregation.

All of the dyes (1 mM) were reacted with (1*R*,8*S*,9*S*)-Bicyclo6.1.0non-4-yn-9-ylmethanol (BCN) in DMSO at room temperature. The completion of the reaction was verified by HPLC-MS.

Excitation and emission spectra were recorded using 1 μ M concentration of the compounds while absorbance spectra were recorded using 5 μ M concentration (DMSO content was kept under 1% in each case). The excitation and emission wavelength are given for each spectrum. Turn-on values were calculated dividing start and end fluorescence intensity values of time course measurements at the emission maxima of the products.

Quantum yields determined using standards can be found in *Table 1*.

Table S1. Standards for quantum yield determinations

<p>Rhodamine B (RB)</p>  <p>$\Phi=0.65$ (basic EtOH)¹¹ used for 1, 3, 5 and their BCN conjugates.</p>	<p>Cresly violet perchlorate (CV)</p>  <p>$\Phi=0.54$ (MeOH)¹² used for 2, 4, 6 and their BCN conjugates.</p>	<p>Coumarin 153 (C153)</p>  <p>$\Phi=0.546$ (EtOH)¹³ used for 1, 3, 7 and their BCN conjugates.</p>
<p>4-dimethylamino-4'-nitrostilbene (DANS)</p>  <p>$\Phi=0.32$ (1,4-dioxane)¹⁴ used for 2, 4 and their BCN conjugates.</p>	<p>Oxazine 170 perchlorate (Ox170)</p>  <p>$\Phi=0.579$ (EtOH)¹³ used for S19 and its BCN conjugate.</p>	

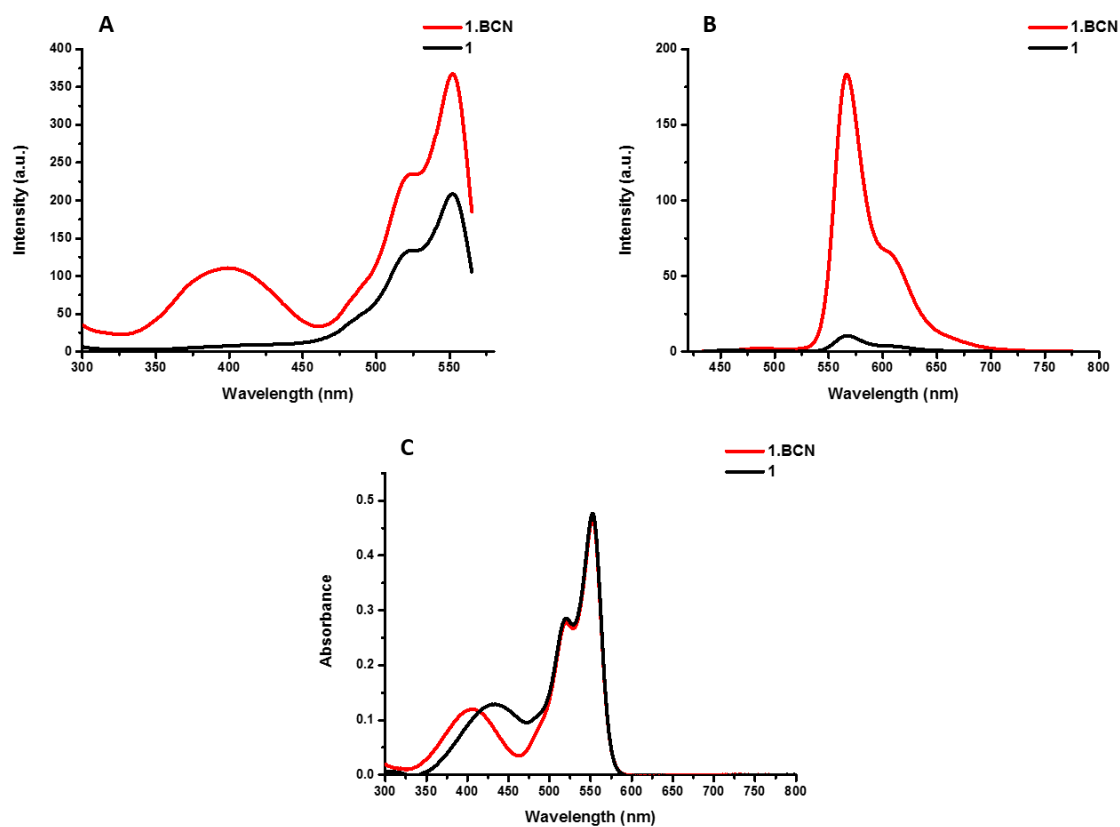


Figure S1. A) Excitation spectra (λ_{em} : 580 nm), B) emission spectra (λ_{exc} : 405 nm) and C) absorbance spectra of fluorogenic cassette **1** and **1.BCN**.

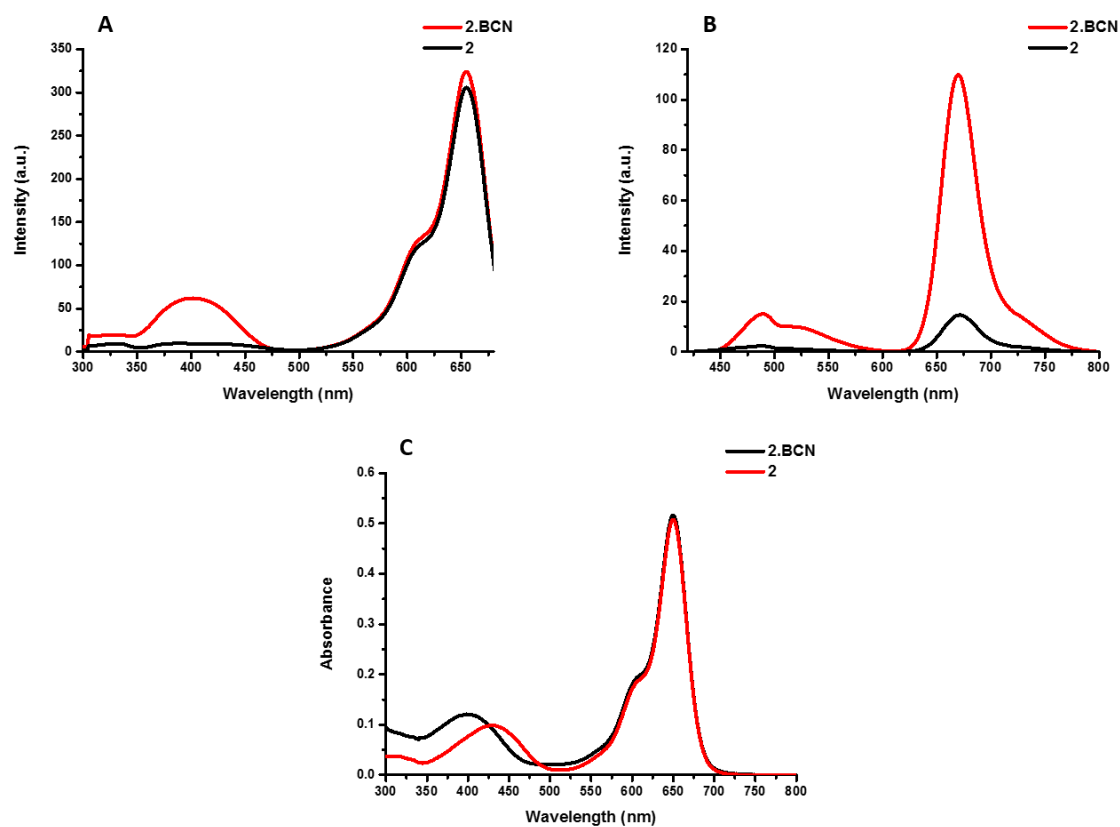


Figure S2. A) Excitation spectra (λ_{em} : 690 nm), B) emission spectra (λ_{exc} : 405 nm) and C) absorbance spectra of fluorogenic cassette **2** and **2.BCN**.

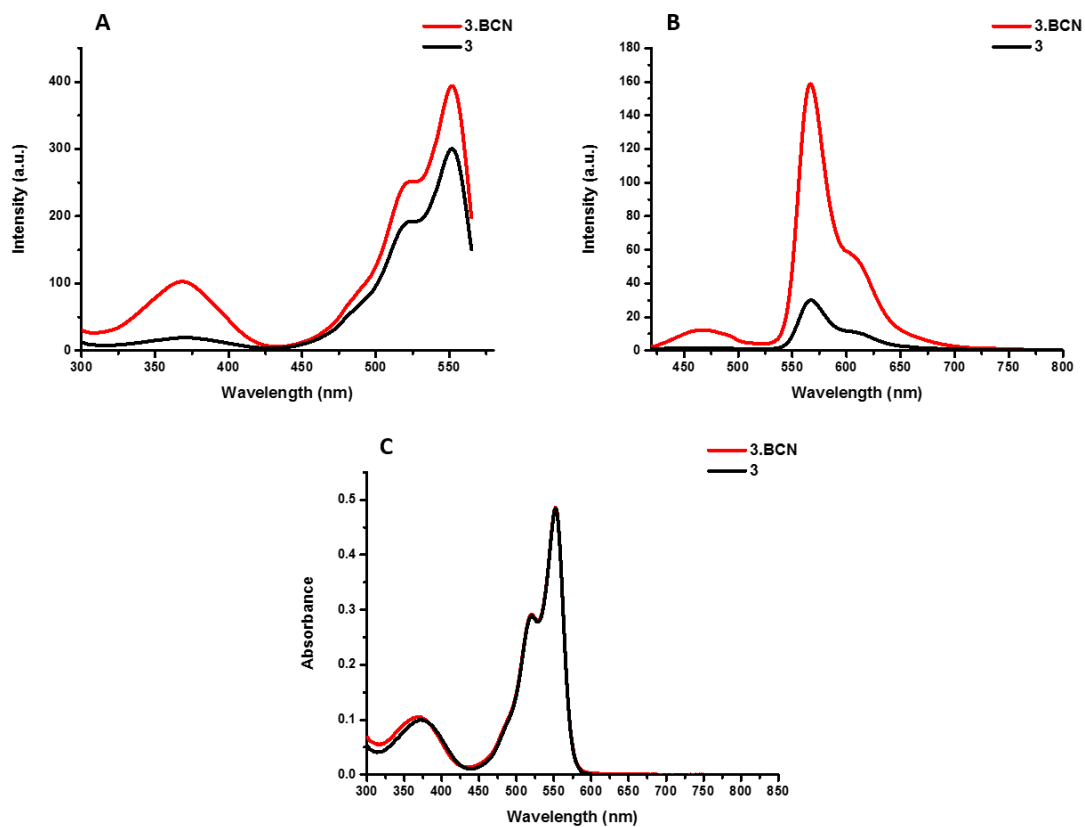


Figure S3. A) Excitation spectra (λ_{em} : 580 nm), B) emission spectra (λ_{exc} : 370 nm) and C) absorbance spectra of fluorogenic cassette **3** and **3.BCN**.

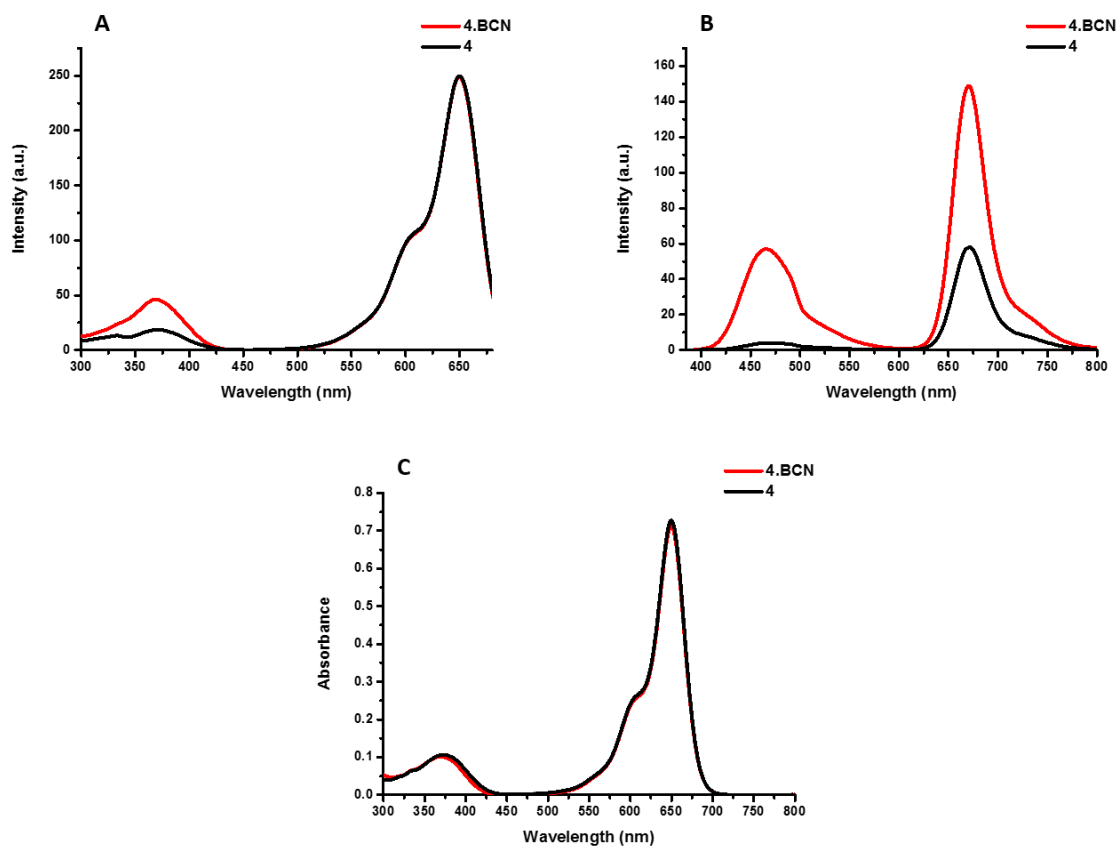


Figure S4. A) Excitation spectra (λ_{em} : 700 nm), B) emission spectra (λ_{exc} : 370 nm) and C) absorbance spectra of fluorogenic cassette **4** and **4.BCN**.

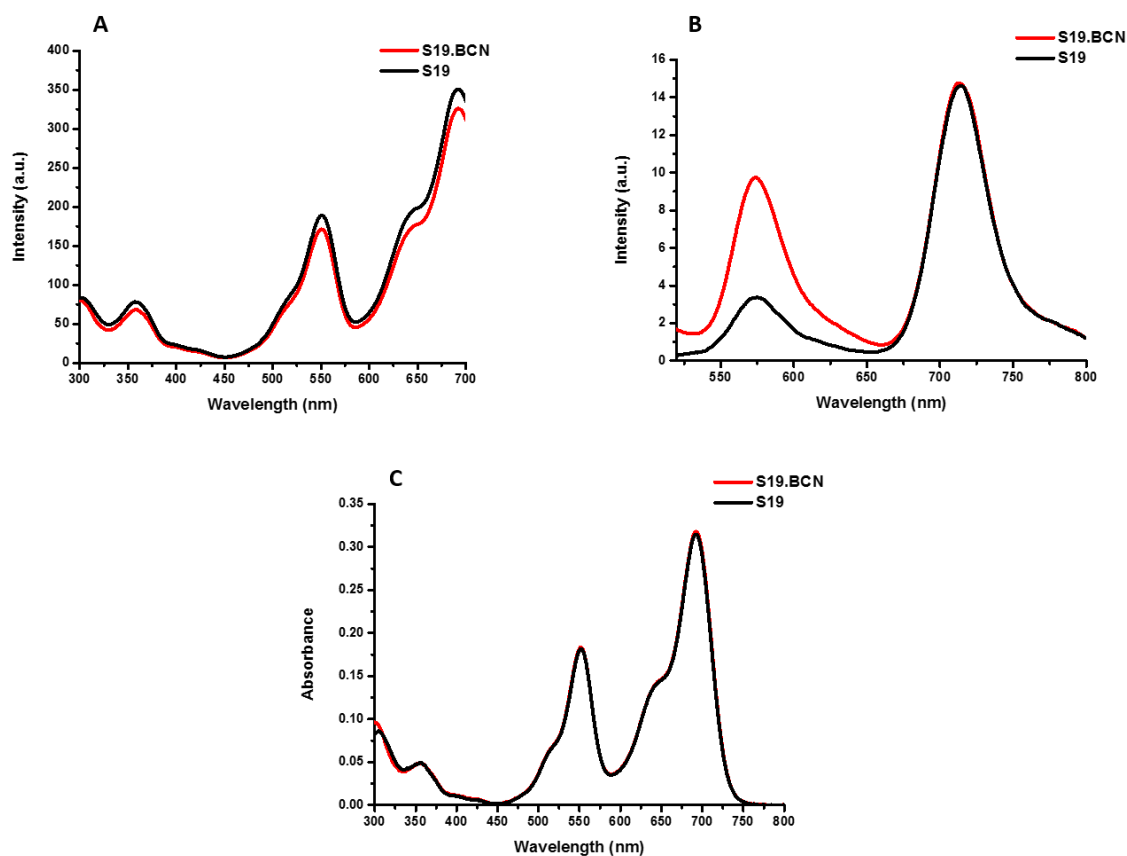


Figure S5. A) Excitation spectra (λ_{em} : 720 nm), B) emission spectra (λ_{exc} : 500 nm) and C) absorbance spectra of fluorogenic cassette **S19** and **S19.BCN**.

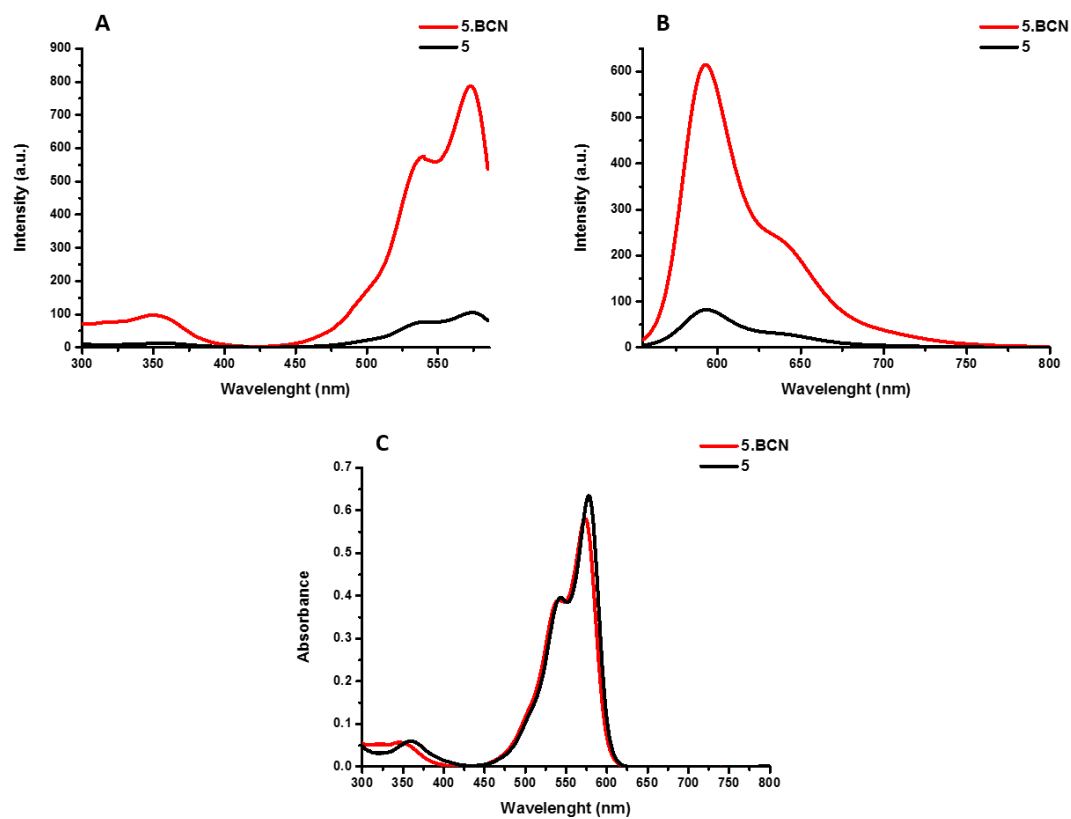


Figure S6 A) Excitation spectra (λ_{em} : 600 nm), B) emission spectra (λ_{exc} : 540 nm) and C) absorbance spectra of fluorogenic cyanine **5** and **5.BCN**.

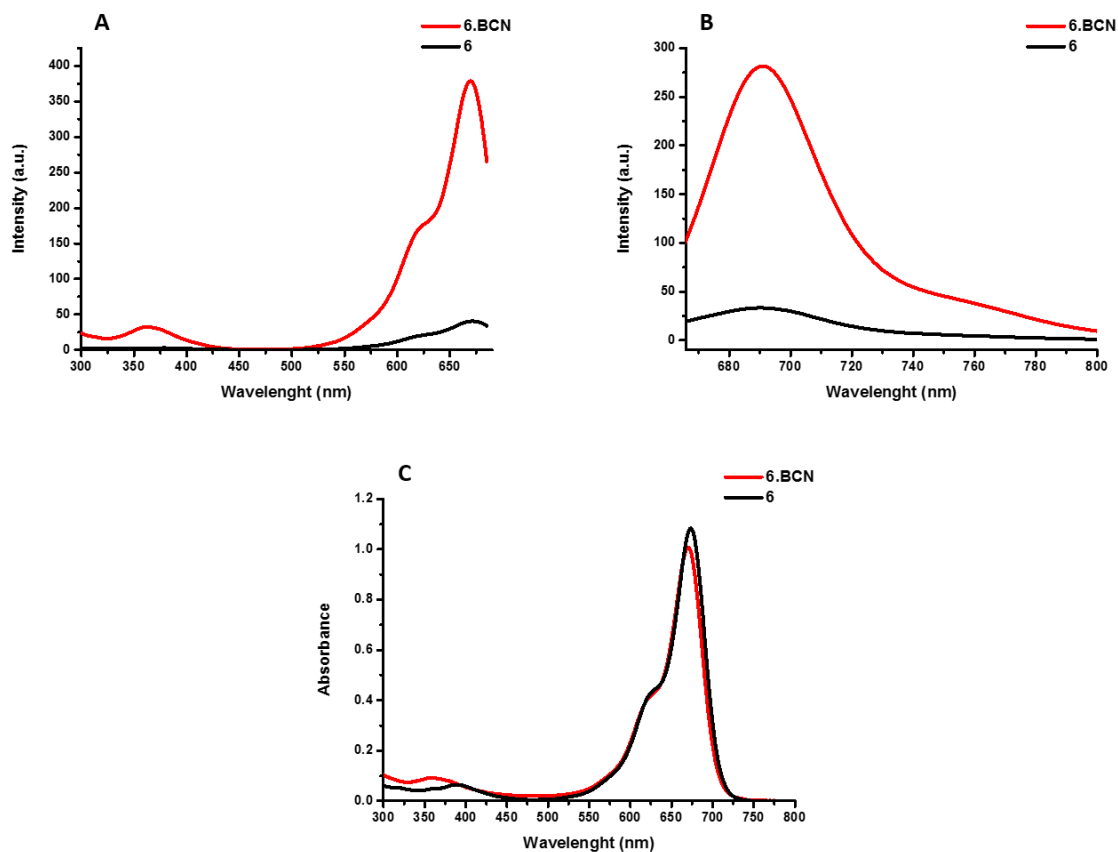


Figure S7. A) Excitation spectra (λ_{em} : 700 nm), B) emission spectra (λ_{exc} : 620 nm) and C) absorbance spectra of fluorogenic cyanine **6** and **6.BCN**.

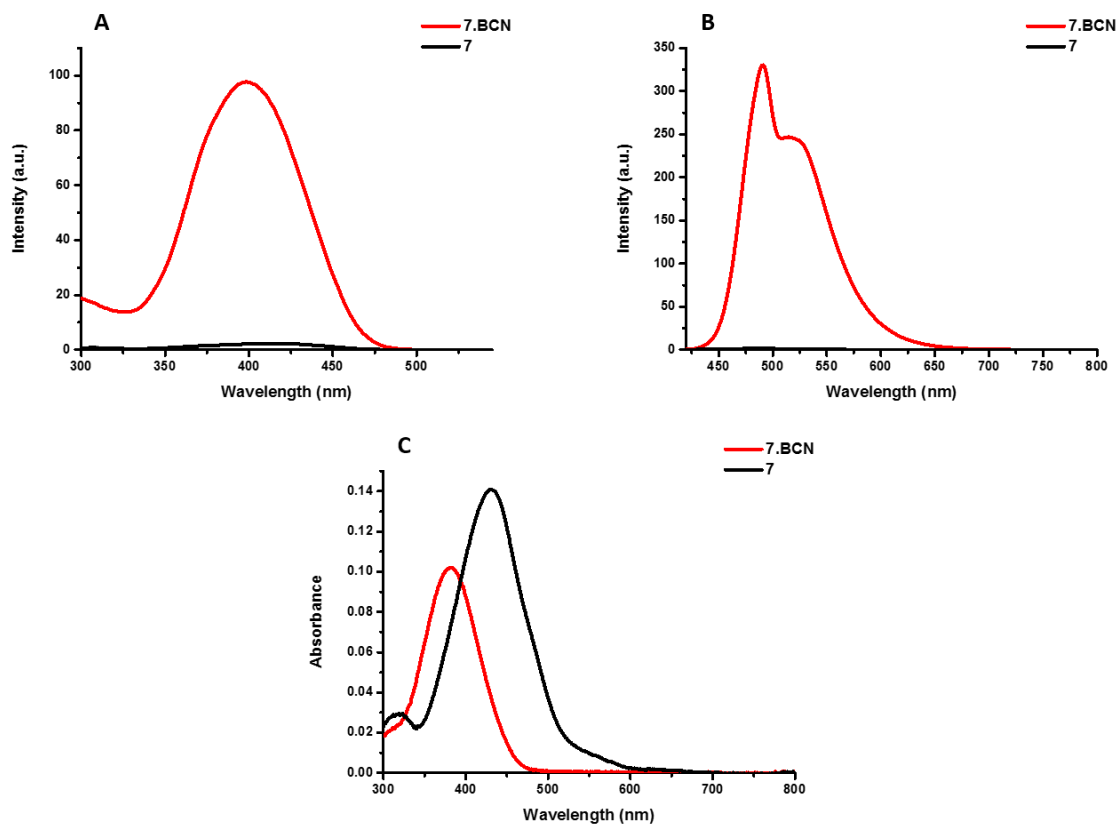


Figure S8. A) Excitation spectra (λ_{em} : 560 nm), B) emission spectra (λ_{exc} : 405 nm) and C) absorbance spectra of fluorogenic donor unit **7** and **7.BCN**.

Table S2. Main photophysical data of Cassettes **1-4** and **S20**, Cyanines **5, 6** and their BCN conjugates and fluorogenic donor **7** and its BCN conjugate.

	$\lambda_{\text{abs,max}}$ (nm)	$\lambda_{\text{em,max}}$ (nm)	ϵ^{a} ($\text{M}^{-1}\text{cm}^{-1}$)	$\Phi_{\text{A}}^{\text{b,d}}$ (%)	$\Phi_{\text{D}}^{\text{c,d}}$ (%)	$\Phi_{\text{A}}/\Phi_{\text{D}}$	$\Phi_{\text{BCN}}/\Phi_{\text{Tet}}$	$I_{\text{BCN}}/I_{\text{BCN}}$
1	552 434	566 ^e	95012 24945	8.0 ^{RB}	1.0 ^{C153}	7.78		
1.BCN	552 406	566 ^e	93374 22527	15.3 ^{RB}	14.5 ^{C153}	1.05	14.5 ^e	15.9 ^e
2	650 428	668 ^e	104313 20509	16.4 ^{CV}	2.0 ^{DANS}	8.23		
2.BCN	650 403	668 ^e	104296 23282	9.5 ^{CV}	12.4 ^{DANS}	0.76	6.2 ^e	7.2 ^e
3	552 371	566 ^f	97731 20073	8.3 ^{RB}	2.0 ^{C153}	4,15		
3.BCN	552 367	566 ^f	96659 20504	12.0 ^{RB}	14.2 ^{C153}	0,85	7.1 ^f	5.2 ^f
4	650 372	669 ^f	145584 21083	22.5 ^{CV}	3.3 ^{DANS}	6.72		
4.BCN	650 370	669 ^f	142729 20256	24.3 ^{CV}	16.8 ^{DANS}	1.45	5.1 ^f	2.5 ^f
S19	692 552	714 ^g	68800 40900	12.9 ^{OX170}	9.6 ^{OX170}	1.34		
S19.BCN	692 552	713 ^g	55400 40900	14.8 ^{OX170}	8.6 ^{OX170}	1.72	0.9 ^g	1.0 ^g
5	578	593 ^h	126958	2.9 ^{RB}	–	–	6.0 ^h	7.9 ^h
5.BCN	573	592 ^h	117289	17.3 ^{RB}	–	–		
6	673	691 ⁱ	218564	1.0 ^{CV}	–	–	7.8 ^{i,j}	9.6 ^j
6.BCN	670	691 ⁱ	204769	7.8 ^{CV}	–	–		
7	430	490 ^e	32008	–	0.1 ^{C153}	–	395 ^e	167 ^e
7.BCN	382	491 ^e	21108	–	39.5 ^{C153}	–		

^a determined at $\lambda_{\text{abs,max}}$ (corresponding row); ^b excited at acceptor absorbance maximum; ^c excited at donor absorbance maximum; ^d abbreviation of used standard dyes (see *Table 1*); ^e excitation at 405 nm; ^f excitation at 370 nm; ^g excitation at 650 nm; ^h excitation at 520 nm; ⁱ excitation at 600 nm; ^j Caused by aggregation. Biological studies revealed c.a. 2-3 values.

3.1. FRET efficiency

FRET efficiency was calculated from the photobleaching half-lifetimes using the following equation¹⁵:

$$E = 1 - \frac{\tau_{\text{D}}}{\tau_{\text{DA}}}$$

where τ_{D} is the photobleaching half-lifetime of the donor in the absence of an acceptor (compound **7**) and τ_{DA} is the photobleaching half-lifetime of the donor in the presence of an acceptor (compounds **1** and **2**).

Table S3. FRET efficiency

	FRET efficiency
1.BCN	0.89
2.BCN	0.71

3.2. Aggregation measurements

A stock solution in DMSO was prepared from dyes **1**, **2**, **5** and **6** (1 mM). All of the dyes (1 mM) were reacted with BCN in DMSO at room temperature. The completion of the reaction was verified by HPLC-MS.

The absorbance and emission spectra were recorded at 1 μ M dye concentration in different media:

- 0, 40, 60, 80, 100% PBS (pH=7.4) in MeCN
- 0, 40, 60, 80, 100% all-sodium PBS-SDS (pH=7.4, containing 0.1% SDS) in MeCN
- DMEM medium (without phenol red with 10% FBS)

(DMSO content was kept under 1% in each case). The emission spectra were corrected with the absorbance value measured at the excitation wavelength and the turn-on values were calculated at the emission maxima of the products.

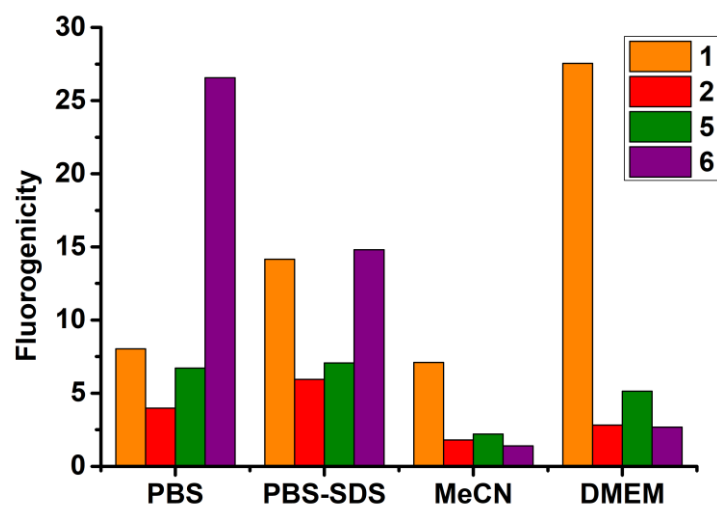


Figure S9. Fluorogenicity in different media

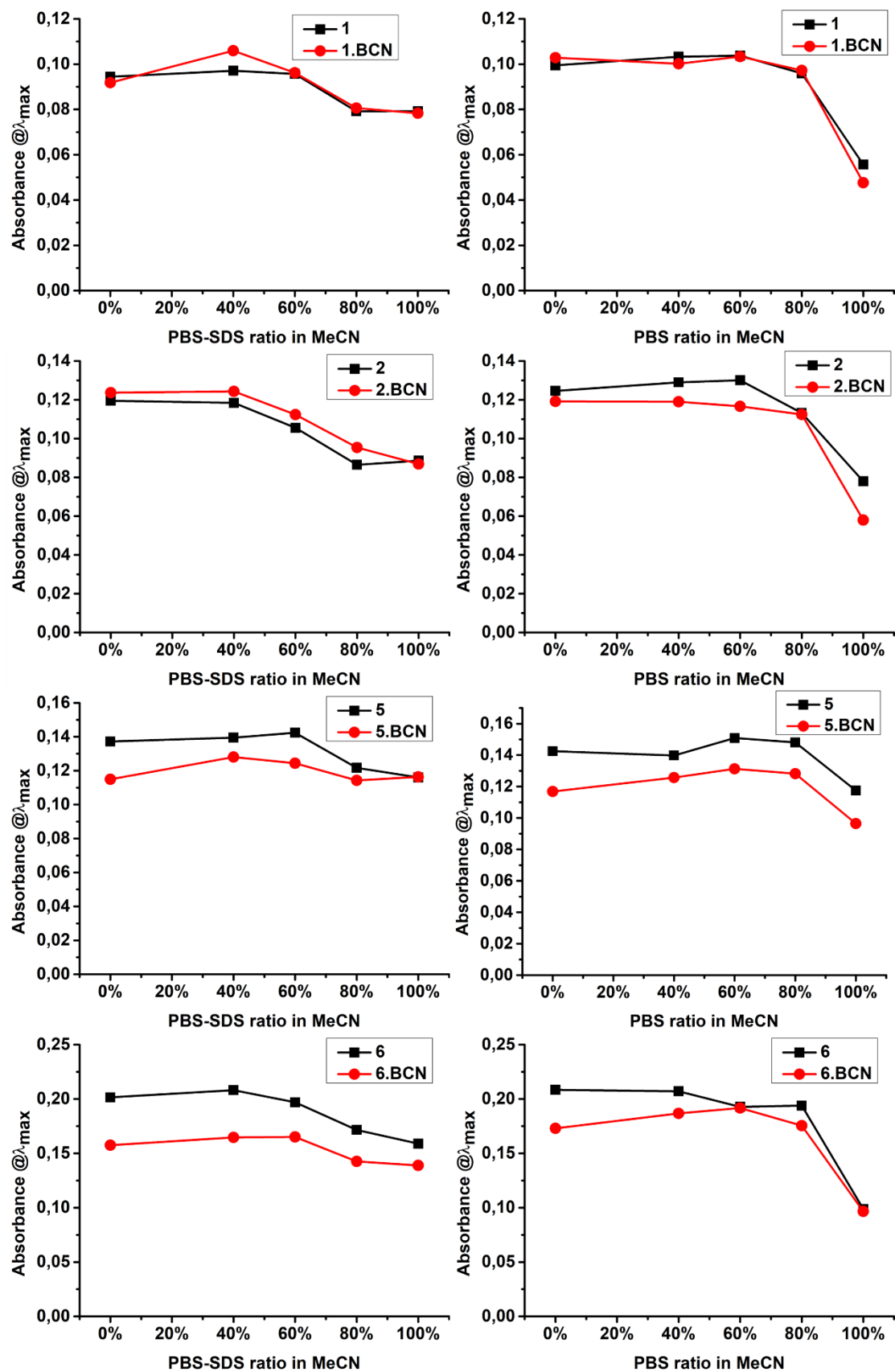


Figure S10. Absorbance values at λ_{max} of dyes 1, 2, 5, 6 and their BCN conjugates in different PBS/MeCN or PBS-SDS/MeCN mixtures.

4. Live cell labeling

4.1. Cell culture

HEK293T (ATCC CRL-3216), and COS-7 (Sigma 87021302) cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco 41965-039), whereas HepG2 (ATCC HB-8065) cells were cultured in DMEM/F12 (Gibco 11320-033) medium. Both media were supplemented with 10% FBS (Gibco 10500-064), 1% penicillin-streptomycin (Gibco 15140-122), and 1% Glutamax (Gibco 35050-061) and additionally, DMEM with further 1% sodium pyruvate (Life Technologies, Gibco 11360-070). The cells were cultured at 37°C in a 5% CO₂ atmosphere and passaged - using trypsin (0,05% for HEK293T and COS-7: Gibco 25300-054; and 0,25% for HepG2 cells: Gibco 25200-056) - every 3–4 days up to 20 passages.

4.2. Effect of dyes on cell viability

A viability test was carried out to assess the toxicity of dyes **1**, **2**, **5**, **6** and **7** and their Halo-BCN conjugated forms on HEK293T cells. Therefore, the dyes in the concentration of 2 mM were reacted bioorthogonally with Halo-BCN in 5 times excess in DMSO for 24 hours in the dark. The completion of the reactions was verified by HPLC-MS. Cells were transferred into a 48-well plate (Thermo Fisher Scientific, 130187) (30,000 cells/well) and incubated for 20 – 24 h at 37 °C in a 5% CO₂ atmosphere. Cells were treated with compounds **1**, **2**, **5**, **6** and **7** as well as Halo-BCN, Halo-**1**, Halo-**2**, Halo-**5**, Halo-**6** and Halo-**7** in the concentration range of 0.3 μM-10 μM for 90 min followed by slight washing and a 4 h incubation period (conciliated with the duration of live cell labeling and imaging) at 37 °C in 5% CO₂ atmosphere. The effect of the dyes on the viability was compared to the impact of the known toxic drug DOX (doxorubicin hydrochloride, Fluorochem, 021790). After the incubation period, supernatants were replaced with 0.5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (in complete DMEM) and incubated for 120 min at 37 °C in the dark. The insoluble formazan crystals were dissolved in 250 μl DMSO. Absorbance was detected at 540 nm using a Biotek Synergy 2 Cytation 3 imaging plate reader with Gen5 software version 3.08 (Biotek Winooski, VT, USA). Viability was expressed as percentage (n=3) of the readings of untreated control cells.

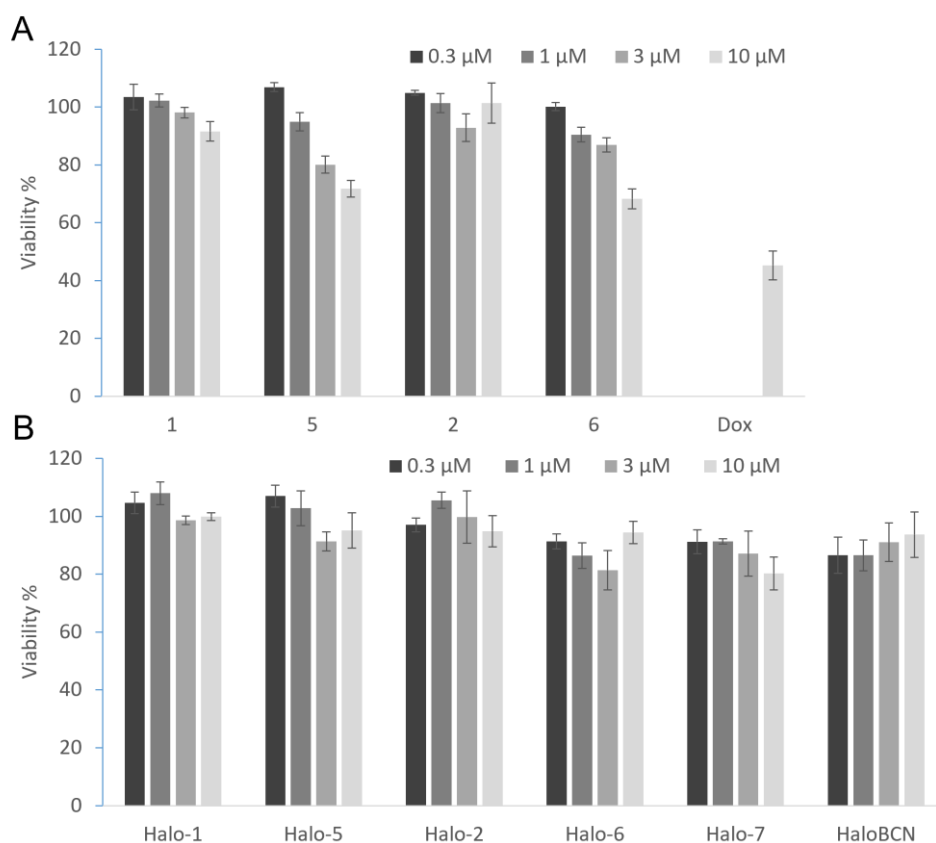


Figure S11. Viability of HEK293T cells upon treatment of dyes **1, 2, 5, 6** and **7** (A) as well as Halo-BCN, Halo-1, Halo-2, Halo-5, Halo-6 and Halo-7 (B) (0.3 - 10 μ M) in the percentage of untreated controls and comparing to cytotoxic doxorubicin (DOX, 10 μ M) (n=3).

4.3. Live-cell labeling

HEK293T (40,000 cell/well) or COS-7 (15,000 cell/well) cells were transferred into μ -Slide 8 well plates (Ibidi 80827) and were incubated for 40 h at 37°C in a 5% CO₂ atmosphere. In the case of HEK293T cells Ibidi plates were pretreated with 0.01 mg/mL Poly-L-lysine (Sigma P5899) for 4 hours at room temperature and washed afterwards. Bioorthogonally reactive chemical reporter BCN was administered in HaloTag substrate or in non-canonical amino acid (ncAA). Therefore, cells were transfected with 0.25 μ g Lamin-HaloTag¹⁶ or Lamp1-HaloTag plasmid using Lipofectamine3000 (ThermoScientific L3000-008) transfection agent in OptiMEM medium (Gibco 31985-062) for four hours according to the manufacturer's protocol. Similar procedure was carried out for ncAA installation. Cells were incubated with 0.25 μ g Lamin-GFP^{39TAG} or Lamin-L^{TAG}-miRFP in combination with 0.25 μ g tRNA^{PyI}/NES-PyIRS^{AF} plasmid (obtained from EMBL within Material Transfer Agreement)¹⁷ in the presence of 250 μ M BCN^{endo}-lysine (Sichem SC-8014) during the transfection. Subsequently, the supernatant was replaced with ncAA free medium for overnight. One day after transfection, cells were labeled with the fluorescent dye **1, 2, 5, 6** at a concentration of 1-6 μ M (in indicator free complete DMEM (Gibco 21063-029) for 90 min at 37°C in the dark. In case of HaloTag fusion protein Halo-BCN substrate (3 μ M; 60 min¹⁶) was added to the cells before the fluorescent labeling step. Afterwards, one

of the two following protocols was used: in the “no-wash” protocol cells were imaged after the labeling step; in the “wash” protocol a two-hour washing step – with indicator-free complete DMEM – was interpolated followed by fixation (4% PFA for 10 min at 25 °C) and quick washing – twice – with PBS prior to imaging.

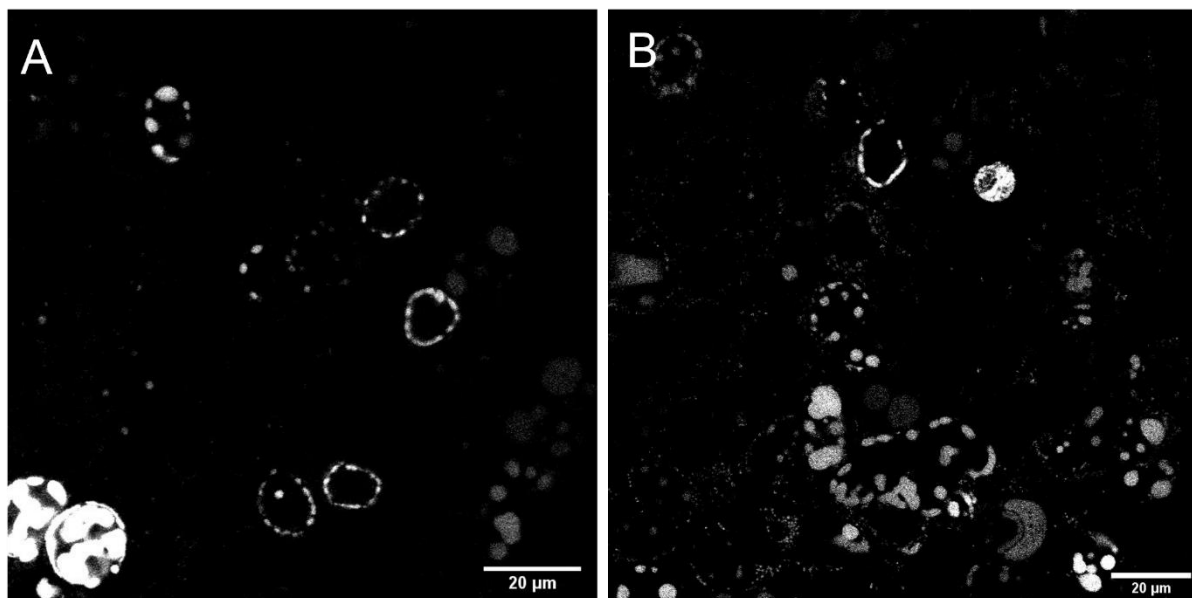


Figure S12. Confocal microscopy images of Lamin-HaloTag expressing HEK293T cells treated with Halo-BCN (3 μ M) and probes **1** (A), **2** (B) (6 μ M) without washing. Scale bar: 20 μ m. Spectral detection: A) (dye **1**): λ_{exc} : 405 nm / λ_{em} : 560-800 nm; B) (dye **2**): λ_{exc} : 405 nm / λ_{em} : 560-800 nm.

4.4. Construction of vectors for bioorthogonal labeling

All restriction endonucleases were purchased from NEB (New England Biolabs, MA, USA), when not stated otherwise.

4.4.1. *Lamp1-HaloTag* construct

The insert encoding the Lamp1 protein was created by restriction with HindIII and BamHI enzymes using the pcDNA3-Lyso-TORCAR (a gift from Jin Zhang; Addgene plasmid #64929; <http://n2t.net/addgene:64929>; RRID:Addgene_64929).¹⁸ The Lamp1 insert was then ligated into TOMM20-GFP^{TAG} plasmid vector backbone (created previously from plasmids: TOMM20-N-mCherry plasmid (#55146) and GFP^{39TAG} (vector obtained from EMBL (Edward Lemke’s lab) within Material Transfer Agreement¹⁹)) exchanging TOMM20 sequence to Lamp1 resulted in the Lamp1-GFP^{39TAG} construct. Lamp1-HaloTag construct was created by the ligation of the products of the simultaneous restriction of LaminA-HaloTag (vector backbone)¹⁶ and Lamp1-GFP^{39TAG} (insert) with NheI and BamHI enzymes.

4.4.2. Lamin-GFP^{TAG} construct

LaminA protein fragment was generated with PCR and cutted by NheI and BamHI enzymes using the source plasmid mCherry-LaminA-C-18 a gift from Michael Davidson (Addgene plasmid # 55068; <http://n2t.net/addgene:55068>; RRID:Addgene_55068) as a template and TOMM20-GFP^{TAG} as vector backbone.

Primers:

Lamin, NheI, FW:

5'- TTATT GCTAGC GCCACCATG GTAGAGACCCCGTCCCAGCG

Lamin, BamHI, Rev:

CCCCAGAACTGCAGCATCATGG GCGGTGG GGATCC ATTAAT-3'

4.4.3. Lamin-L^{TAG}-miRFP construct

As a first step a linker fragment (L) bearing an Amber stop code (TAG) was inserted into the plasmid pmiRFP670-N1 (a kindly gift from Vladislav Verkhusha (Addgene plasmid # 79987; <http://n2t.net/addgene:79987>; RRID:Addgene_79987)²⁰ with the restriction of XhoI és HindIII endonucleases.

Oligomer sequences of the linker:

Linker^{TAG}, XhoI, FW:

5'-TCGAGGCTGAAGATGATGTGGAAGGCGGTAGCGGGGATCCATCAC-
CATGGCACTTAGCAATTAGCCA-3'

Linker^{TAG}, HindIII, Rev:

5'-AGCTTGGCTAATTGCTAGTGCCATGGTGATGGATCCCCGCTACCGCCT-
TCCACATCATCTTCAGCC-3'

The PCR product LaminA from plasmid mCherry-LaminA-C-18 (Addgene #55068) was ligated into the Linker^{TAG}-miRFP backbone vector plasmid cutted with NheI és XhoI enzymes.

Primers:

Lamin, NheI, FW:

5'-TTATT GCTAGC GCCACCATGGTAGAGACCCCGTCCCAGCG-3'

Lamin, XhoI, Rev:

5'- CCCCAGAACTGCAGCATCATGCTCGAG ATTAAT-3'

4.5. Immunolabeling

4.5.1. Immunostaining of TOMM20 for STED imaging

COS-7 cells were seeded onto μ -Slide 8 well plates (Ibidi 80827) at 15,000 cells/well density and grown for 48 h at 37 °C (5% CO₂) in complete DMEM medium. Immunofluorescence labeling was carried out as follows. The samples were gently washed (3 times) with pre-warmed Dulbecco's modified phosphate-

buffered saline (DPBS) and then fixed and permeabilized with 4% formaldehyde (Sigma F8775) and 0.1% Triton X-100 (Serva 37238) in DPBS for 5 min at room temperature. After several washing steps, the cells were incubated in blocking buffer for 1 h at room temperature (DPBS containing 2% bovine serum albumin (Sigma A4503), 1% fish gelatin (Sigma G7765), 0.1% Triton X-100, and 5% goat serum (Gibco 16210-064)). Then the samples were incubated for overnight at 4°C temperature with anti-TOMM20 antibody (1:250, ab186734, Abcam) diluted in blocking buffer. After washing with DPBS, the cells were incubated for 1 h at room temperature with the secondary antibody (goat anti-rabbit IgG (Jackson ImmunoResearch, 111-005-003)) conjugated with fluorescent dyes (**1**, **2**, **5** and **6**) at a final concentration of 10 µg/ml in blocking buffer. After careful washing with DPBS samples were imaged with confocal and STED microscopy.

4.5.2. Multicolor fluorescence labeling

For multicolor labeling, HaloTag modification and immunostaining was combined. HepG2 cells at 50,000 cells/well density were seeded onto µ-Slide 8 well plates (Ibidi 80827) in DMEM/F12 complete medium and grown for 48 h at 37 °C (5% CO₂). For multicolor labeling cells were transfected with 0.25 µg Lamin-HaloTag¹⁶ or Lamp-HaloTag. The next day cells were treated with 3 µM Halo-7 for 90 min prior to fixation and immunostaining.

Immunofluorescence labeling was carried out as described above (part SI 4.5.1) with the exception that the samples here were incubated with the mixture of anti-keratin-19 (1:250, ab194399, Abcam) and anti-TOMM20 antibodies (1:250, ab186734, Abcam) followed by labeling with mixture of secondary antibodies (goat anti-rabbit IgG and goat anti-mouse IgG samples (Jackson ImmunoResearch, 111-005-003 and 115-005-003, respectively)) conjugated with probes **1** and **2**.

Preparation of Halo-7: Coumarin **7** (5 mg, 0.011 mmol, 1 equiv), BCN-HaloTag¹⁶ (5 mg, 0.012 mmol, 1.1 equiv) and 3 mL DMSO were stirred at room temperature for 2 hours. Then, the mixture was purified by preparative-HPLC (H₂O – MeCN; starting from 95 : 5 to 0 : 100 v/v%) to yield 5 mg (54%) Halo-7. The product purity was checked with LC-MS.

4.5.3. Fluorescent modification of the secondary antibody

As a first step buffer exchange (to 110 mM NaHCO₃/ Na₂CO₃, pH 9.0) of goat anti-rabbit IgG and goat anti-mouse IgG samples (Jackson ImmunoResearch, 111-005-003 and 115-005-003, respectively) was carried out using Sephadex G25 “Fine” desalting gel (Pharmacia Fine Chemicals, Sweden) with SpinPrep column technology (Sigma, St Louis, MO, USA). (1*R*,8*S*,9*s*)-bicyclo[6.1.0]non-4-yn-9-ylmethyl *N*-succinimidyl ester (NHS-BCN; Sigma–Aldrich, cat no.: 744867) was added to IgG (2.3 mg/ml; 15 µM) at a concentration of 100 µM at room temperature for 20 mins. Afterward, in a one-pot reaction, dyes were added at 150 µM concentration for an additional 20 mins. The excess reagents were

removed by using the desalting G25 spin column described above. Reaction yields, IgG concentration, and purity were checked by the capillary electrophoresis method.

4.5.4. Capillary electrophoresis

The background electrolyte (BGE) components phosphoric acid and triethyl amine acid were purchased from Sigma (St. Louis, MO, USA)/ Merck GmbH (Darmstadt, Germany).

Capillary electrophoresis was performed with an Agilent Capillary Electrophoresis 3D^{CE} system (Agilent Technologies, Waldbronn, Germany) applying DB-WAX capillary (Agilent Technologies, Waldbronn, Germany) with total and effective length of 33.5 cm and 25 cm, respectively, and an internal diameter of 50 μm (Agilent Technologies, Santa Clara, CA, USA). On-line absorption at 200 nm and 405, 535 nm or 599 nm was monitored for the protein and the fluorescent dyes, respectively, with a DAD UV-Vis detector. The capillary was kept at constant temperature of at 25 $^{\circ}\text{C}$. Between the measurements the capillary was rinsed subsequently with distilled water then with BGE (100 mM phosphate and triethyl amine (pH 2.5)) for 3 minutes each. Samples were injected with a pressure of 5×10^3 Pa for 6 sec. Runs were performed in positive-polarity mode with 20 kV.

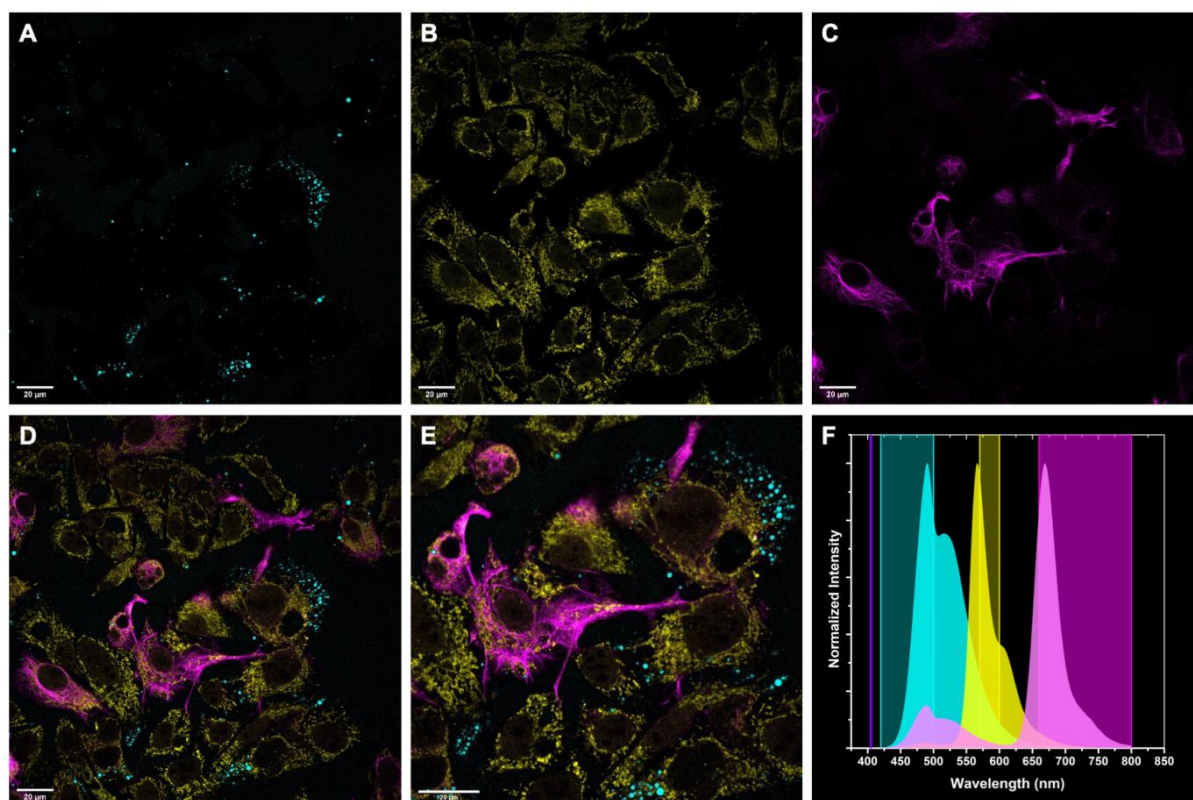


Figure S13. Multicolor imaging of HepG2 cells. Lamp1-HaloTag was reacted with half-cassette **7 (Halo-7)**, A); λ_{exc} : 405 nm / λ_{em} : 420-500 nm;), while TOMM20 B); (dye **1**) λ_{exc} : 405 nm / λ_{em} : 560-600 nm;) and CK-19 (C); (dye **2**) λ_{exc} : 405 nm / λ_{em} : 650-800 nm;) were stained with **1** or **2** labeled antibodies. D): Overlay of A+B+C. E magnified part of D. Scale bar: 20 μm . F: Emission spectra of dyes **7** (cyan), **1** (yellow) and **2** (magenta) using for multicolor labeling. Excitation wavelength (405 nm) and detection windows for the dyes are presented by the colored areas.

4.6. Photobleaching studies

To compare the photostability of the probes **1**, **2** and **7** HEK293T cells were transfected with Lamin-HaloTag plasmid and cells were labelled with Halo-**7** (3 μ M, 90 min) or Halo-BCN (3 μ M 90 min) in combination with compound **1** and **2** (6 μ M, 90 min) and fixed (for details cf. part SI 4.3). Photobleaching characteristics were assessed with a Zeiss LSM 710 microscope, applying a Zeiss PlanApo 40x (NA 1.4) oil immersion objective using the 405 nm laser for excitation. All imaging parameters (the investigated area, magnification, laser intensity, the pixel number and dwell time etc.) were kept constant. The only exception was the emission wavelength, i.e., 415 nm – 520 nm range was set for compound **7**, and 540 nm - 800 nm for **1** and **2**. Bleaching kinetics were acquired for 80 cycles (1250 sec) and averages of relative fluorescence intensities of 9-14 regions of interest were compared.

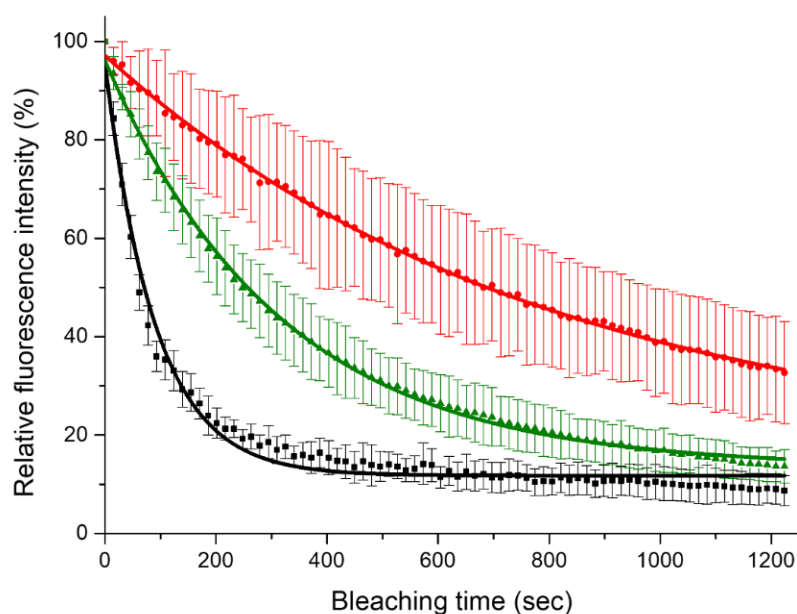


Figure S14. Photostability of dyes given as photobleaching half-lifetime ($t_{1/2}$ (τ) in sec). Regions of interest of confocal microscopy images of Lamin-HaloTag expressing HEK293T cells treated with Halo-**7** (black) or Halo-BCN (3 μ M) combined with probes **1** (red), **2** (green) were evaluated. Using 405 nm for excitation and 415-520 nm (for **7**) or 540-800 nm (for **1** and **2**) for emission detection window revealed 62.5 ± 1.9 s ($n=9$), 546.7 ± 11.6 s ($n=14$) and 218.3 ± 2.7 s ($n=13$) photobleaching half-lifetime for **7**, **1** and **2**, respectively.

4.7. Confocal and STED imaging and analysis

Confocal images were acquired on a Zeiss LSM 710 microscope, applying a Zeiss PlanApo 40x (NA 1.4) oil immersion objective using the 405 nm, 488 nm, 543 nm and 633 nm lasers for excitation. Spectral detection parameters using PMT detector were: GFP exc.: 488 nm/ em. range: 495-600 nm; miRFP exc.: 633 nm/ em. range: 645-800 nm; **compound 7**: exc.: 405 nm/ em. range: 420-500 nm; **compound 1**: exc.: 405 nm/ em. range: 560-600 nm or 540-800 or 570-600 (depending on the parallel labeling); **compound 2**: exc.: 405 nm/ em. range: 620-800 nm or 540-800 or 650-800 (depending on the

parallel labeling); **Compound 5**: 543 nm/ em. range: 560-800 nm; **Compound 6**: 633 nm/ em. range: 645-800 nm.

For demonstrating multicolor labeling channel subtraction approach was used in order to obviate the effects of spectral overlapping and difference in brightness due to different labeling density.

The channel extraction process (Fiji for ImageJ software²¹) was as follows for Lamin-HaloTag multicolor labeling (Manuscript Figure 5):

Channel dye 7: $\text{Signal}_{\text{Dye } 7} = \text{Signal}_{\text{Dye } 7} (m)$; Color balance min: 30/ max: 255

Channel dye 1: $\text{Signal}_{\text{Dye } 1} = \text{Signal}_{\text{Dye } 1} (m) - \text{Signal}_{\text{Dye } 7} (m)$; Color balance min: 5/ max: 100

Channel dye 2: $\text{Signal}_{\text{Dye } 2} = \text{Signal}_{\text{Dye } 2} (m) - \text{Signal}_{\text{Dye } 1} (m)$; Color balance min: 5/ max: 100

(m)= signal measured, not modified

The channel extraction process was as follows for Lamp-HaloTag multicolor labeling (Supporting Information Figure S13):

Channel dye 7: $\text{Signal}_{\text{Dye } 7} = \text{Signal}_{\text{Dye } 7} (m) - \text{Signal}_{\text{Dye } 1} (m) - \text{Signal}_{\text{Dye } 2} (m)$;

Color balance min: 0/ max: 30

Channel dye 1: $\text{Signal}_{\text{Dye } 1} = \text{Signal}_{\text{Dye } 1} (m)$; Color balance min: 10/ max: 120

Channel dye 2: $\text{Signal}_{\text{Dye } 2} = \text{Signal}_{\text{Dye } 2} (m) - \text{Signal}_{\text{Dye } 1} (m)$; Color balance min: 10/ max: 120

(m)= signal measured, not modified

STED images with 775 nm depletion were acquired using an Abberior Expert Line microscope built on a Nikon Ti2 frame. The system is equipped with 405, 488, 560 and 640 nm pulsed excitation lasers and a 775 nm (1.2 W) pulsed laser for depletion. Images were acquired by using a Nikon CFI PL APO 100x/1.45 oil immersion objective with spectral detection (**compound 1**: exc.: 405 nm/ em. range: 560-755 nm; **compound 2**: exc.: 405 nm/ em. range: 600-755 nm; **Compound 5**: exc.: 560 nm/ em. range: 574-755 nm; **Compound 6**: exc.: 640 nm/ em. range: 650-755 nm) using avalanche photodiode detectors (APD) in photon counting mode.

Applying the Huygens Professional software (SVI), we performed deconvolution for image restoration on the recorded STED images. The deconvolution was based on theoretical point spread function (PSF). Images were analyzed using ImageJ software (NIH)²¹. We selected the results of some representative line analysis for demonstration. Non-linear Gaussian curve was fitted to the normalized fluorescence intensity values by using Origin Pro 9 software. For characterizing the resolution efficiency, the full width at half maximum values (FWHM) were given.

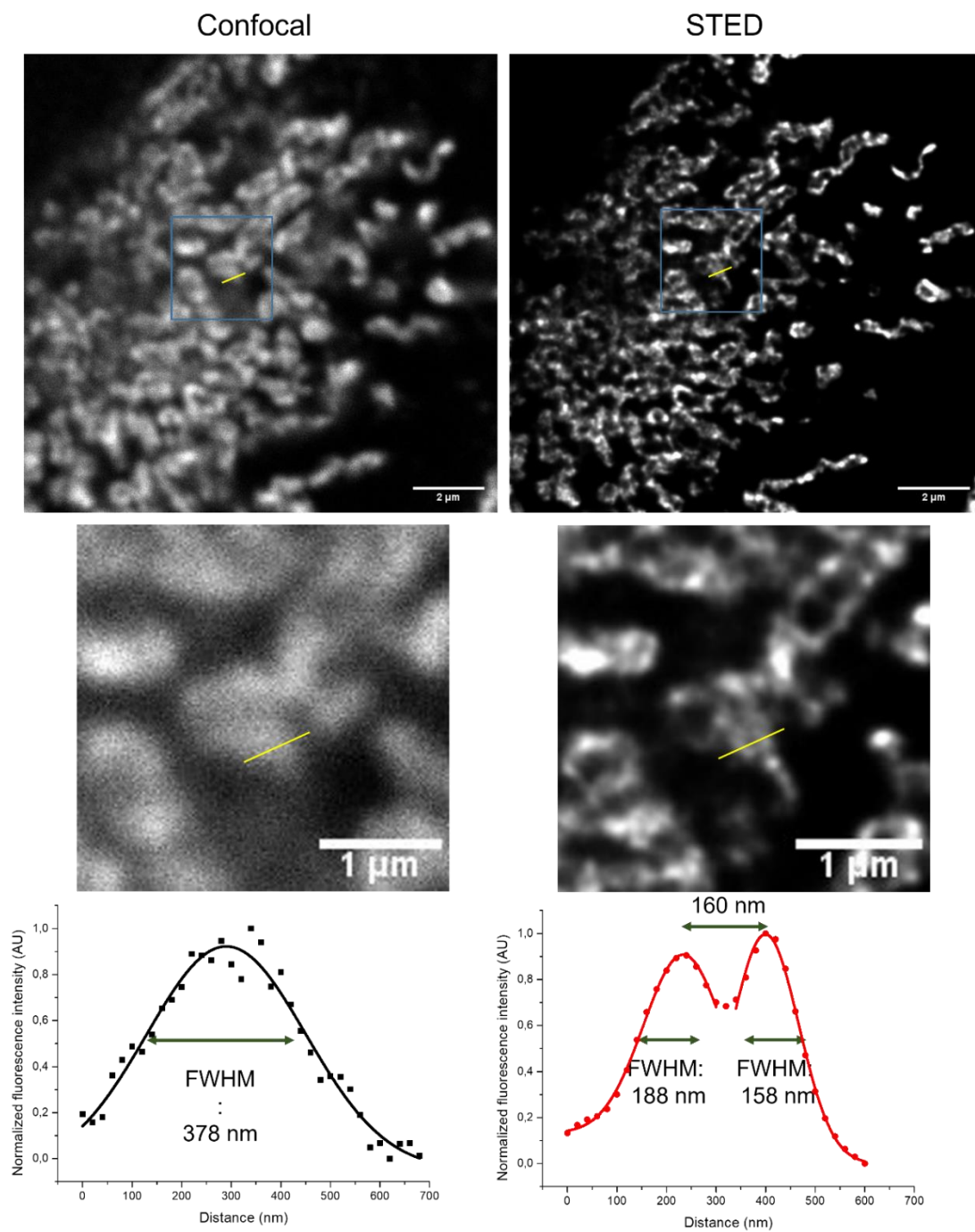
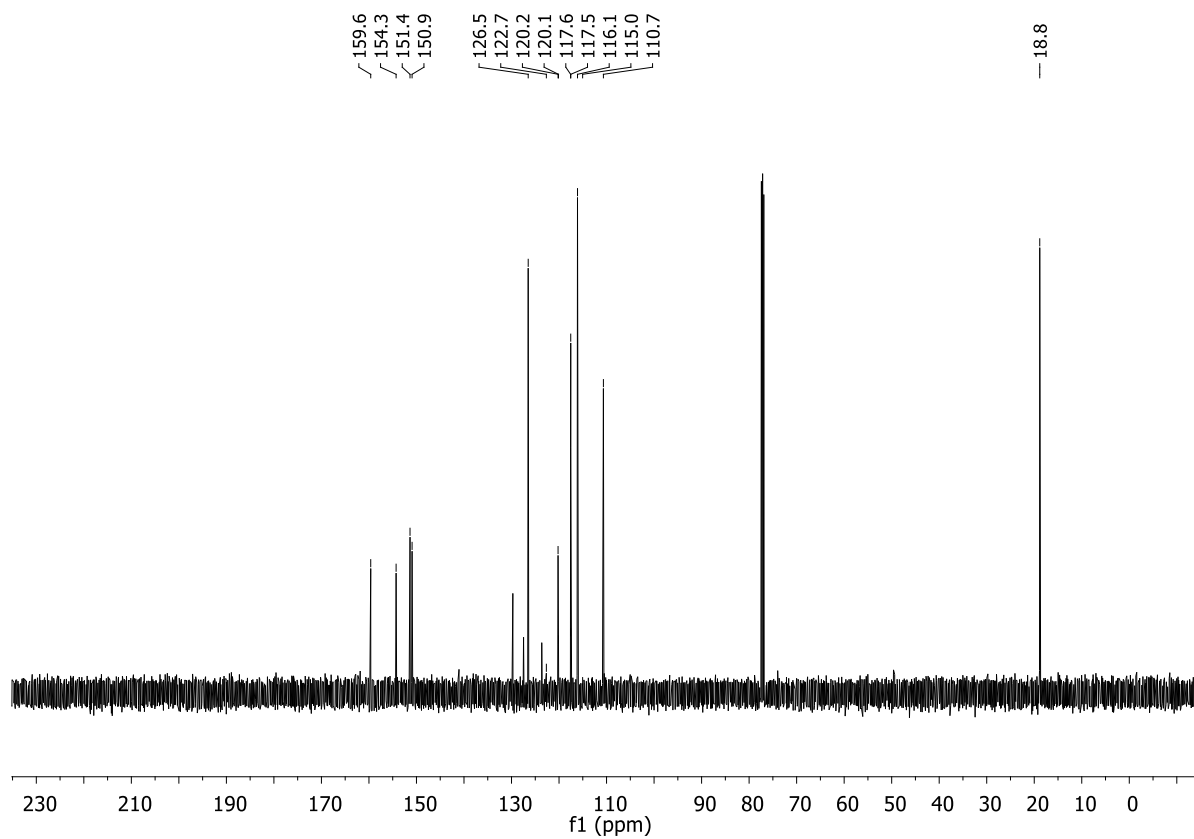
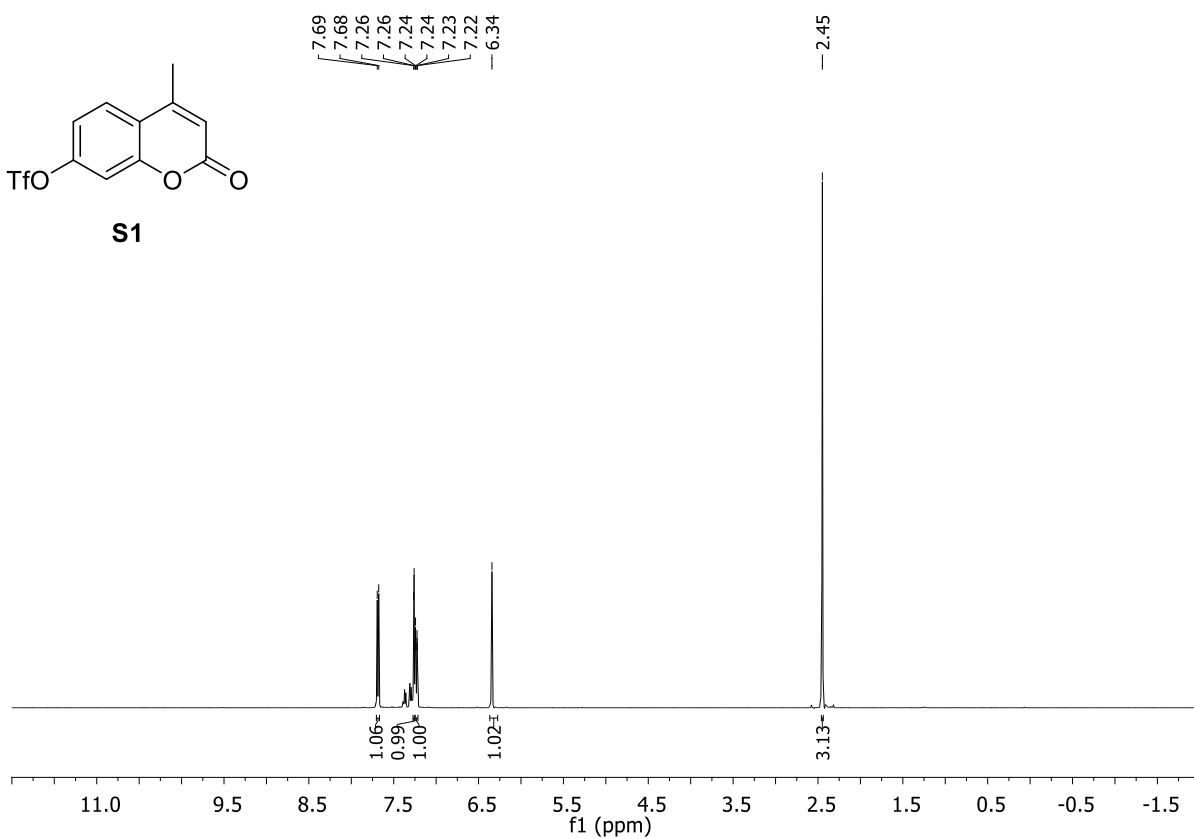
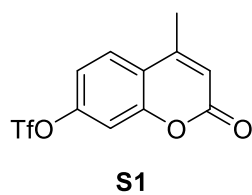


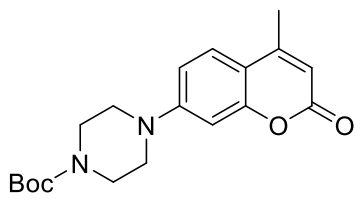
Figure S15. Confocal and STED images of COS-7 cells treated with anti-TOMM20 antibodies labeled with **5** (λ_{exc} : 560 nm / λ_{em} : 574-755 nm; λ_{STED} = 775 nm (pulsed)). Scale bar: 5 μm and in magnified pictures: 1 μm. FWHM: Full width at half maxima.

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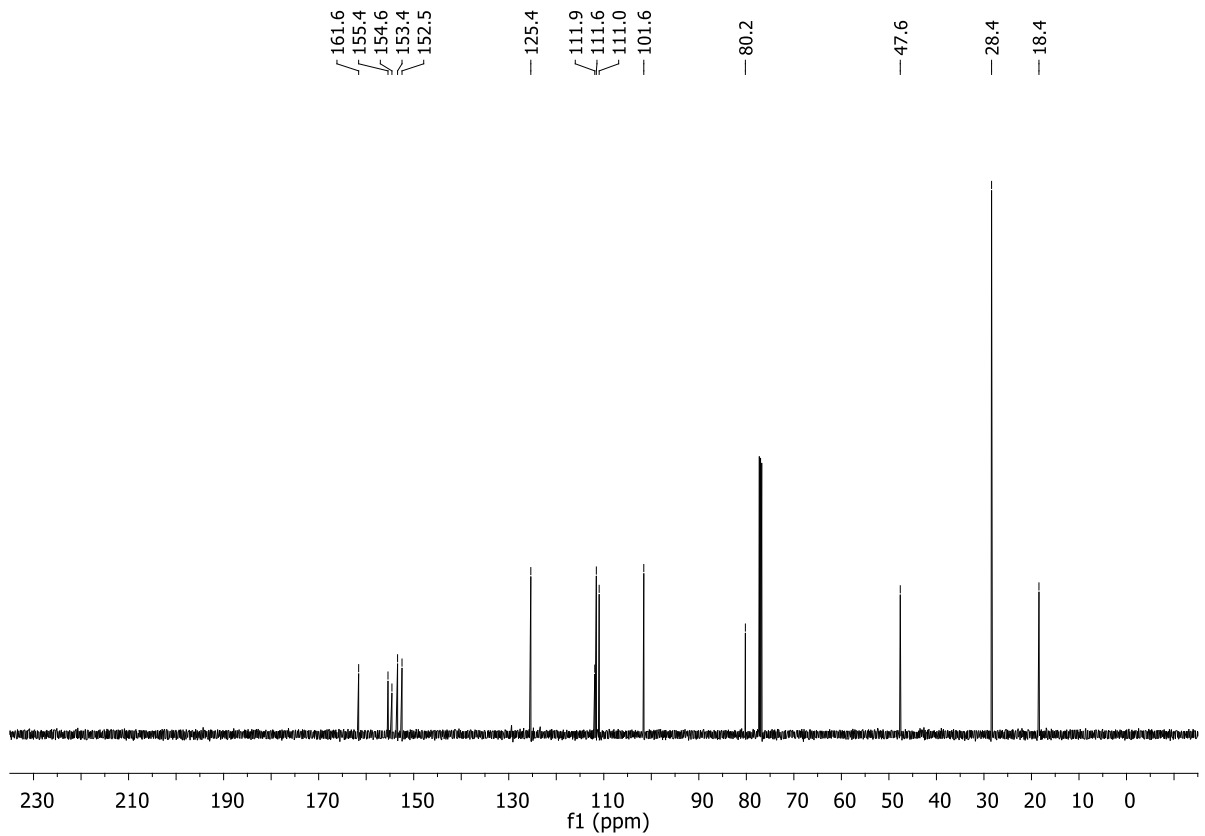
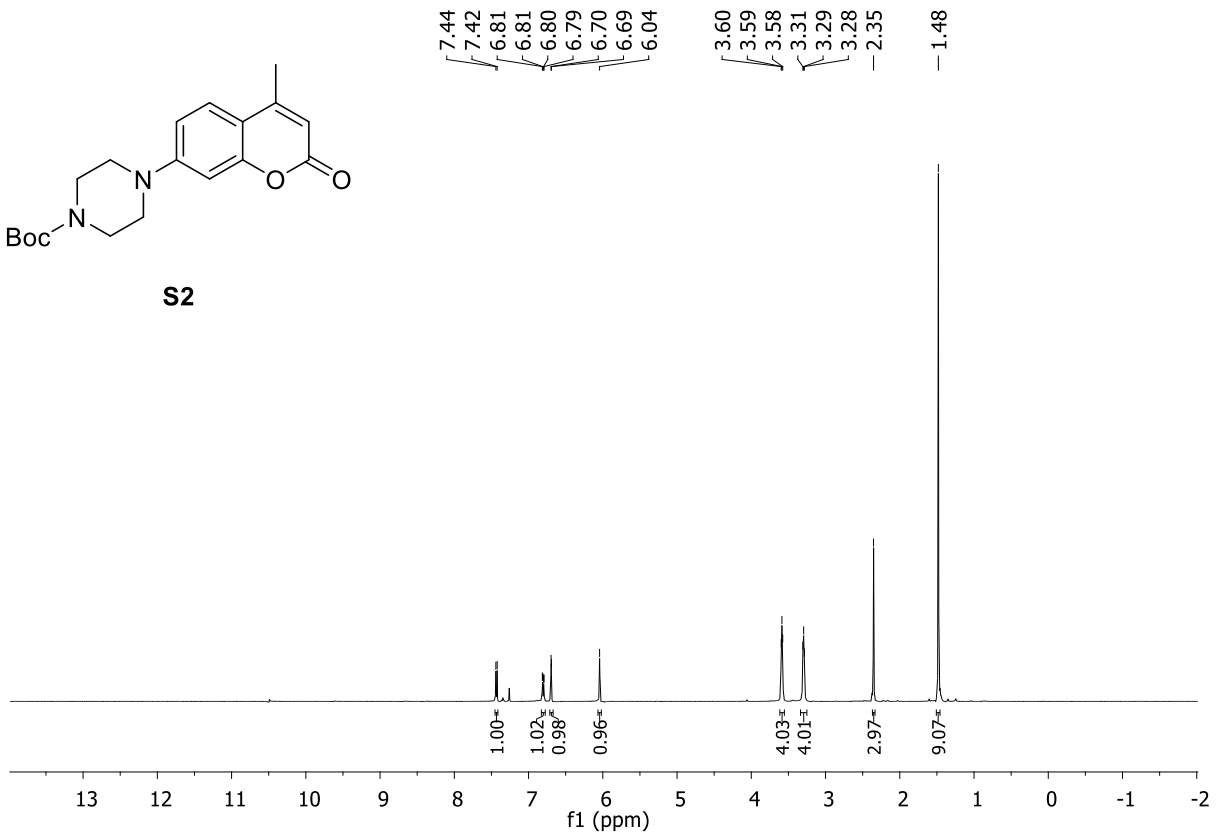
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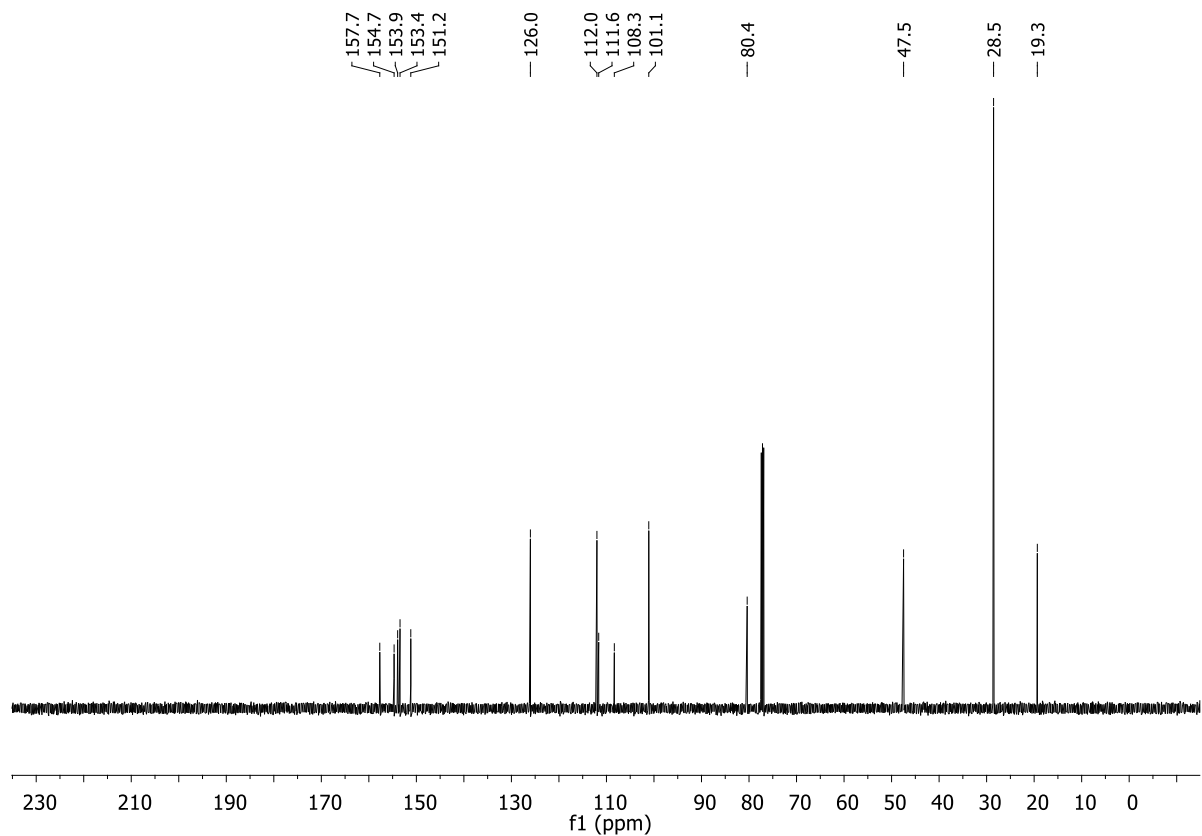
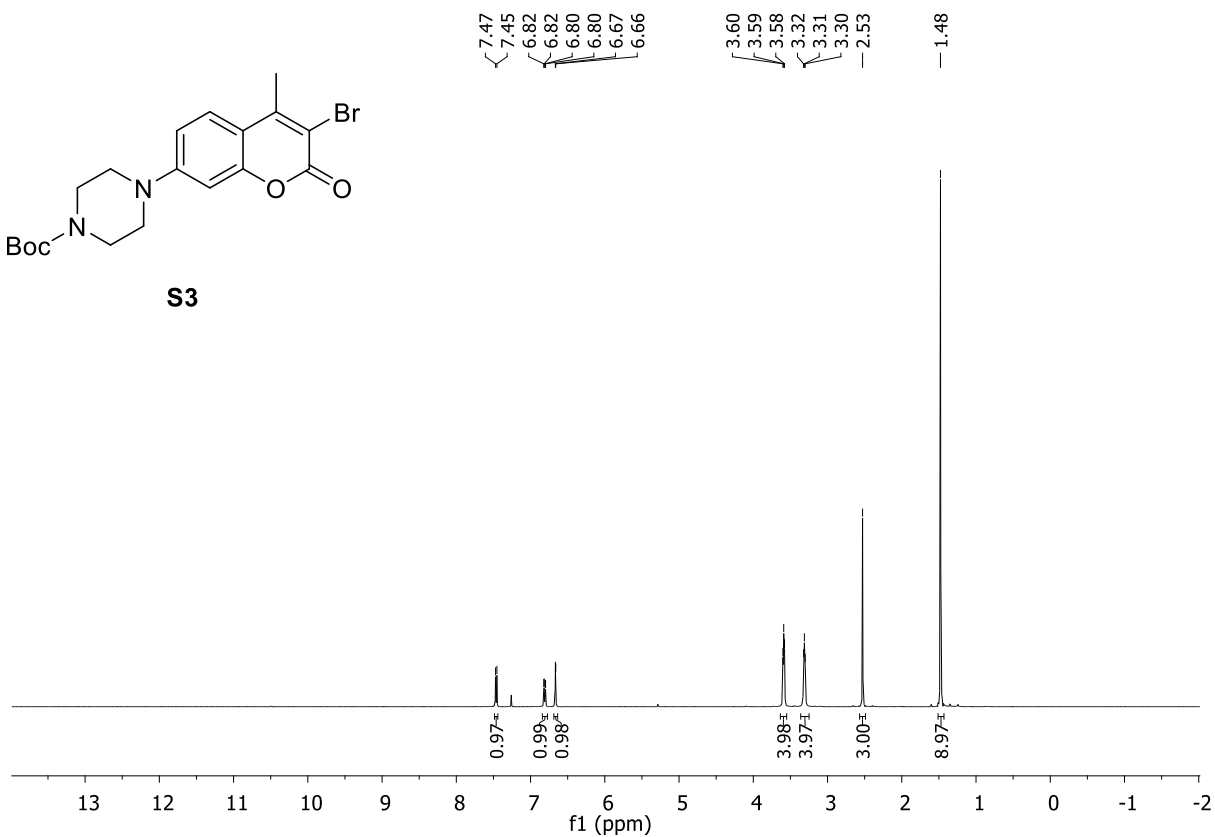
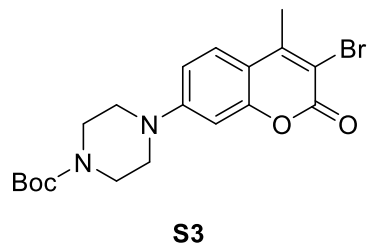
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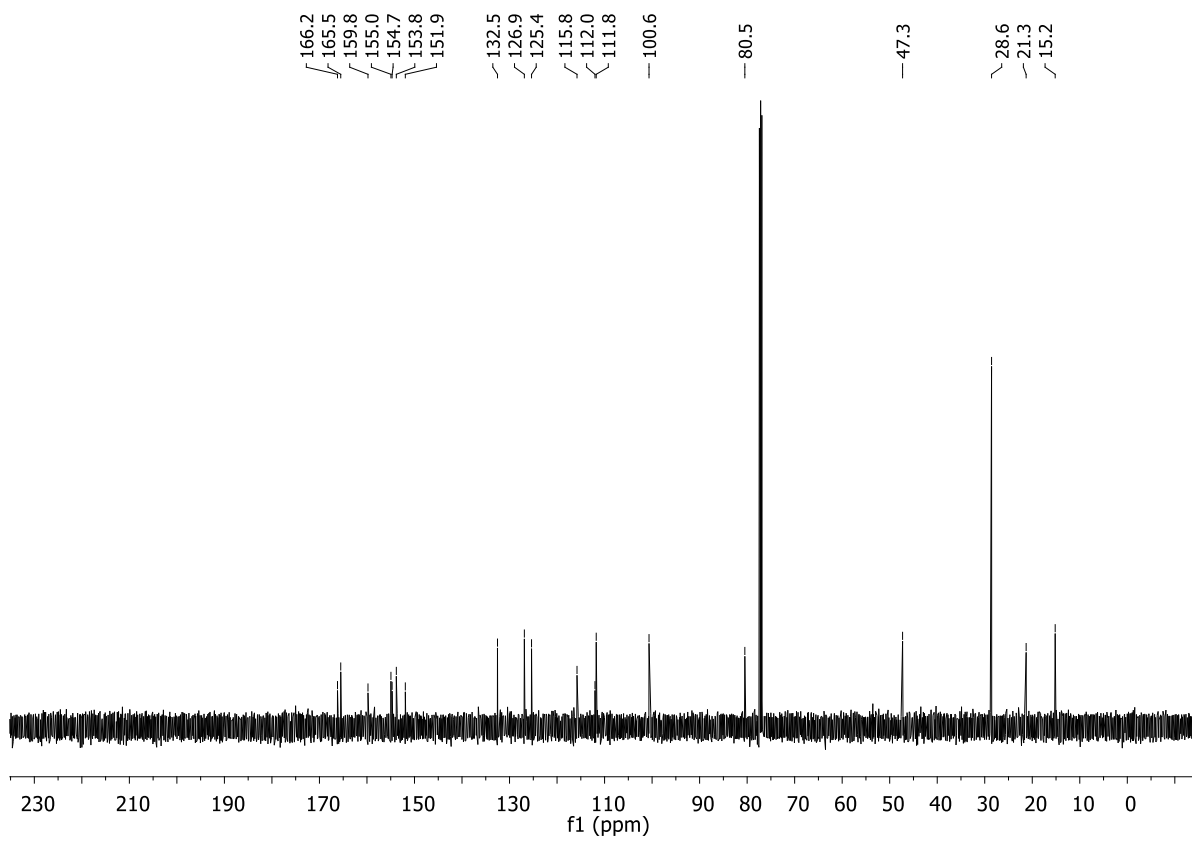
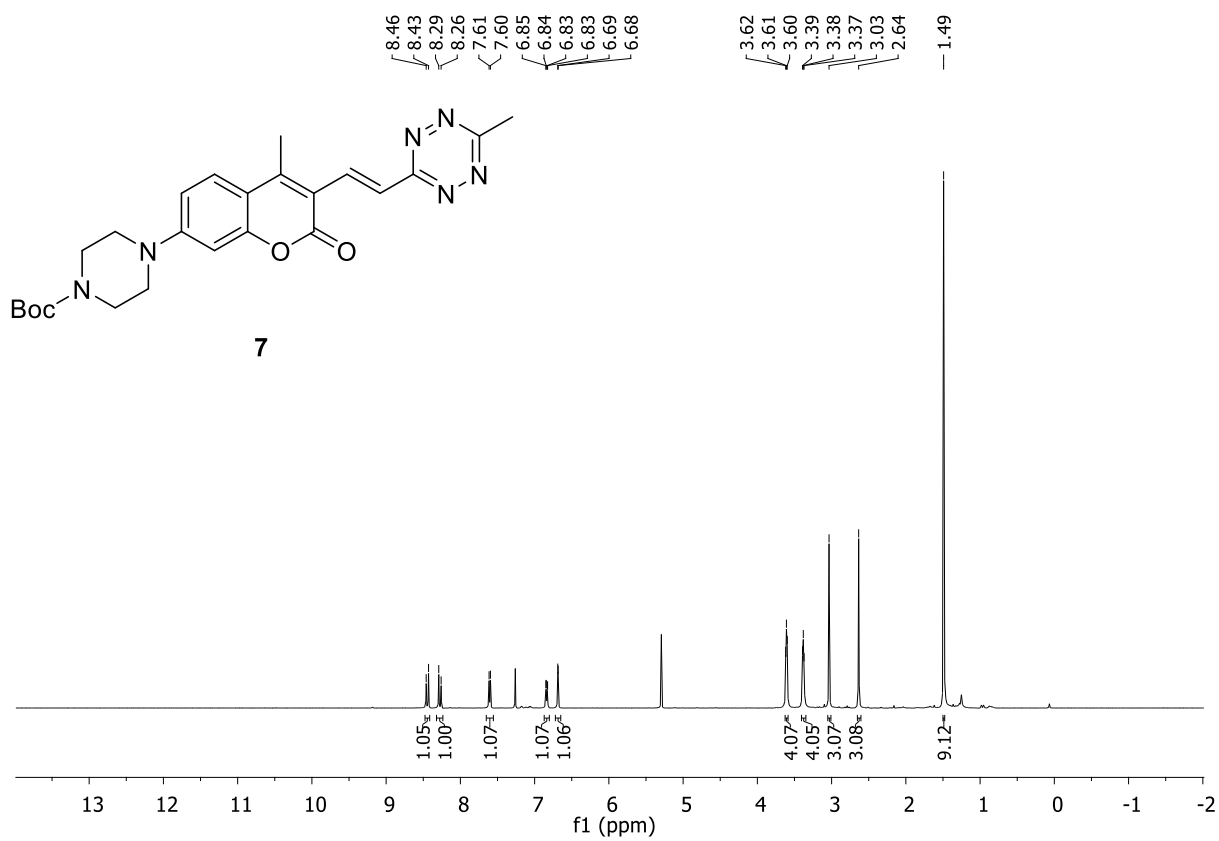


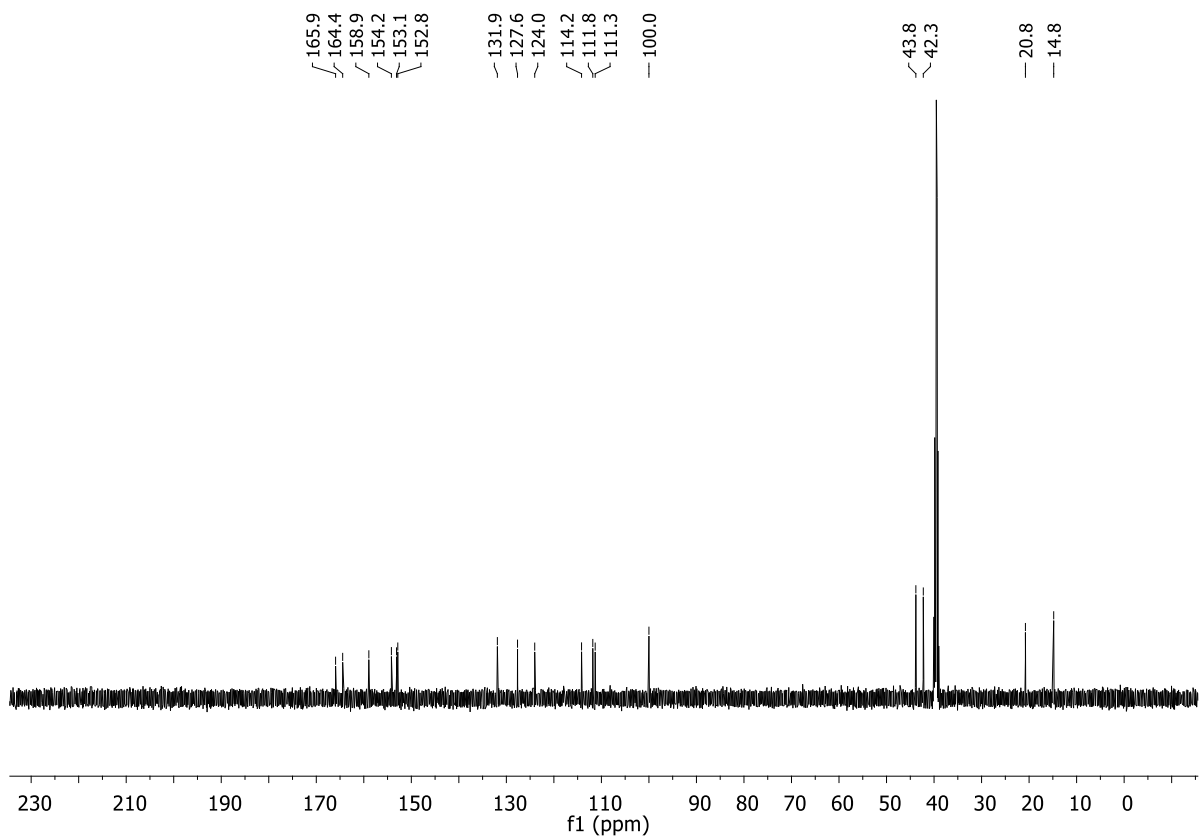
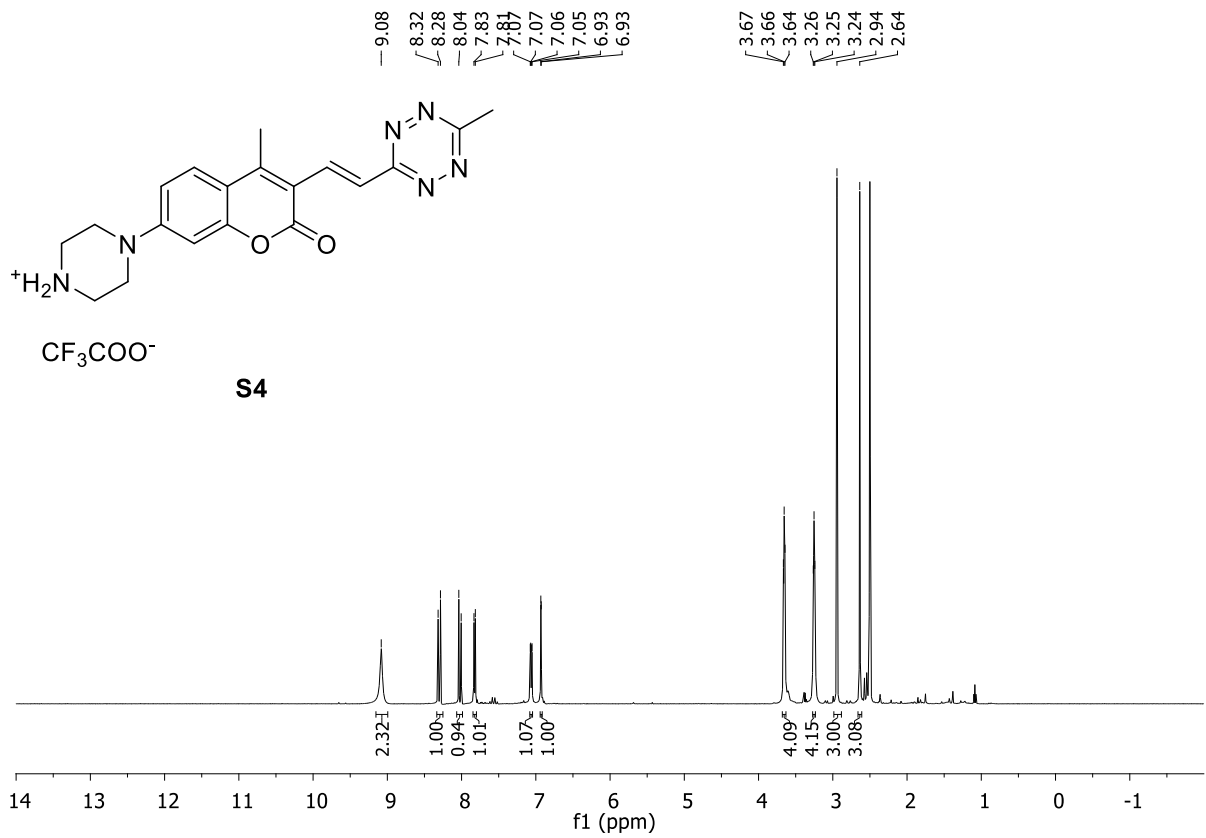


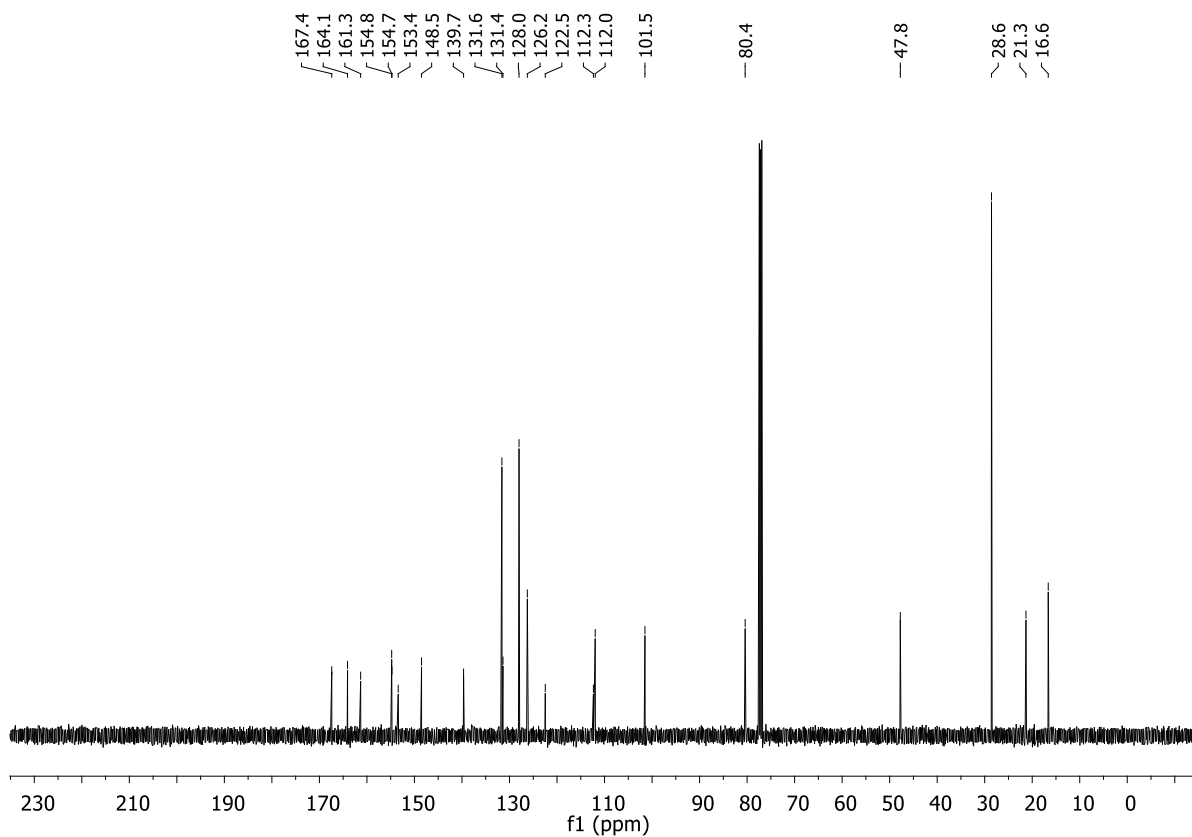
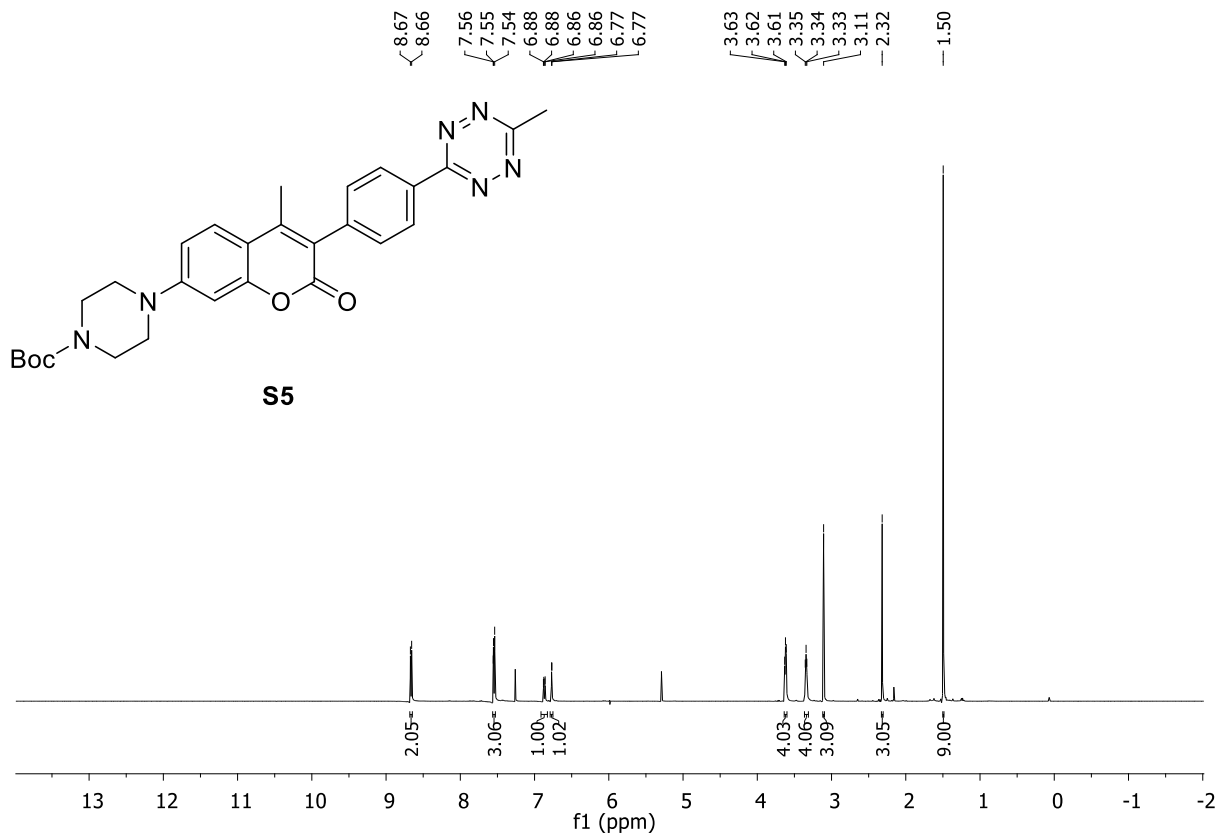
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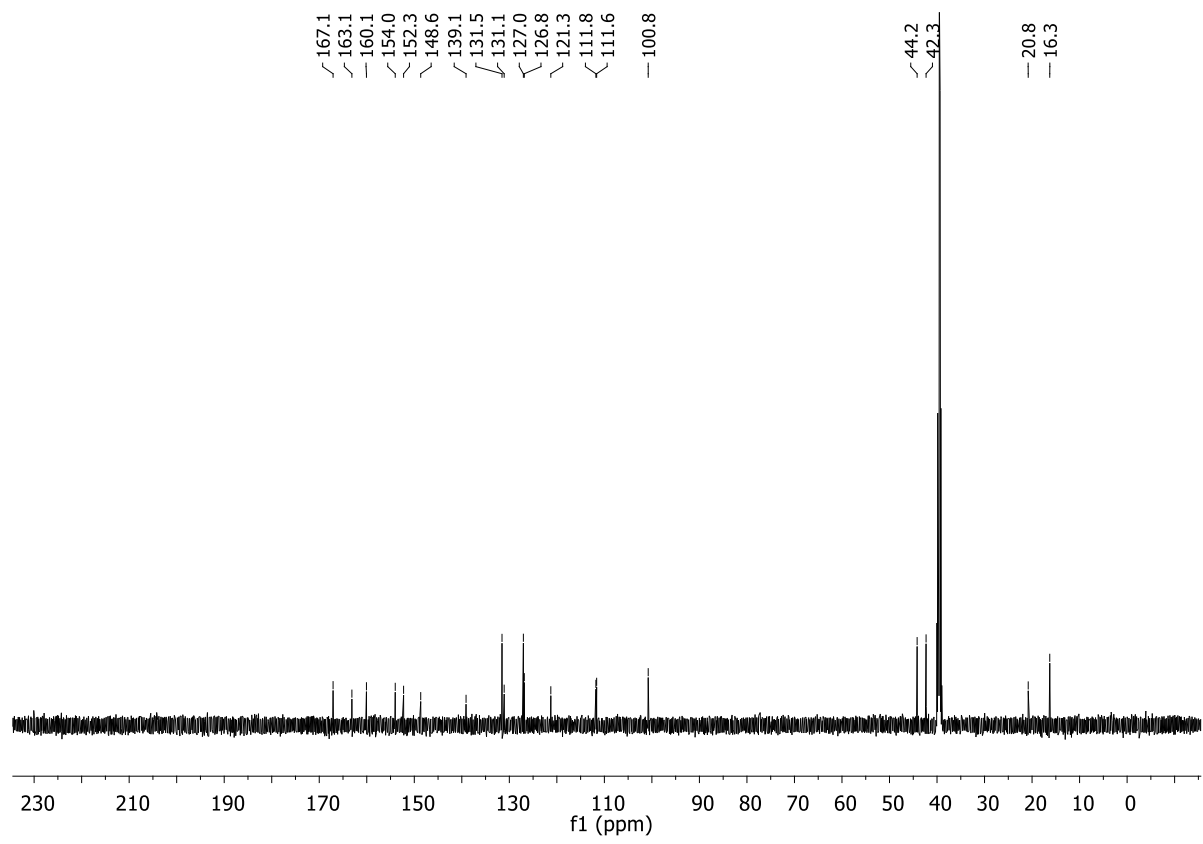
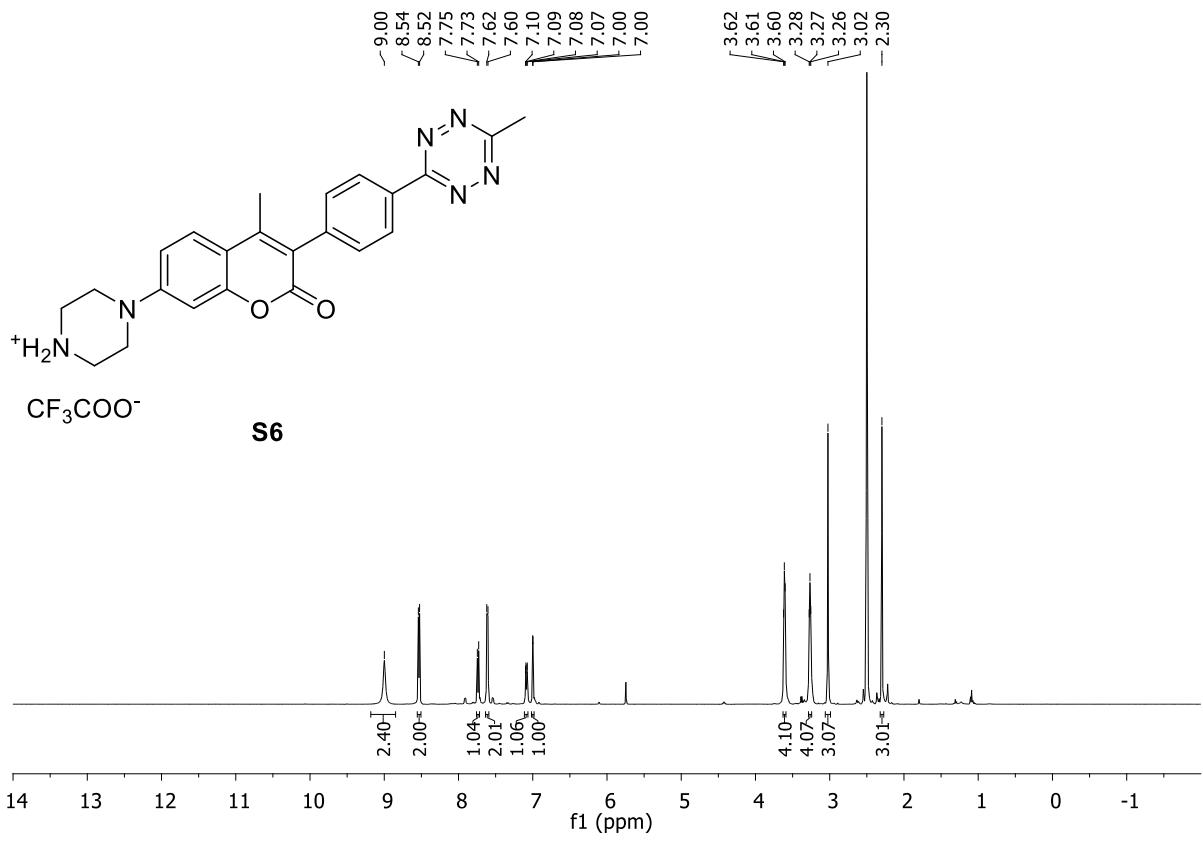


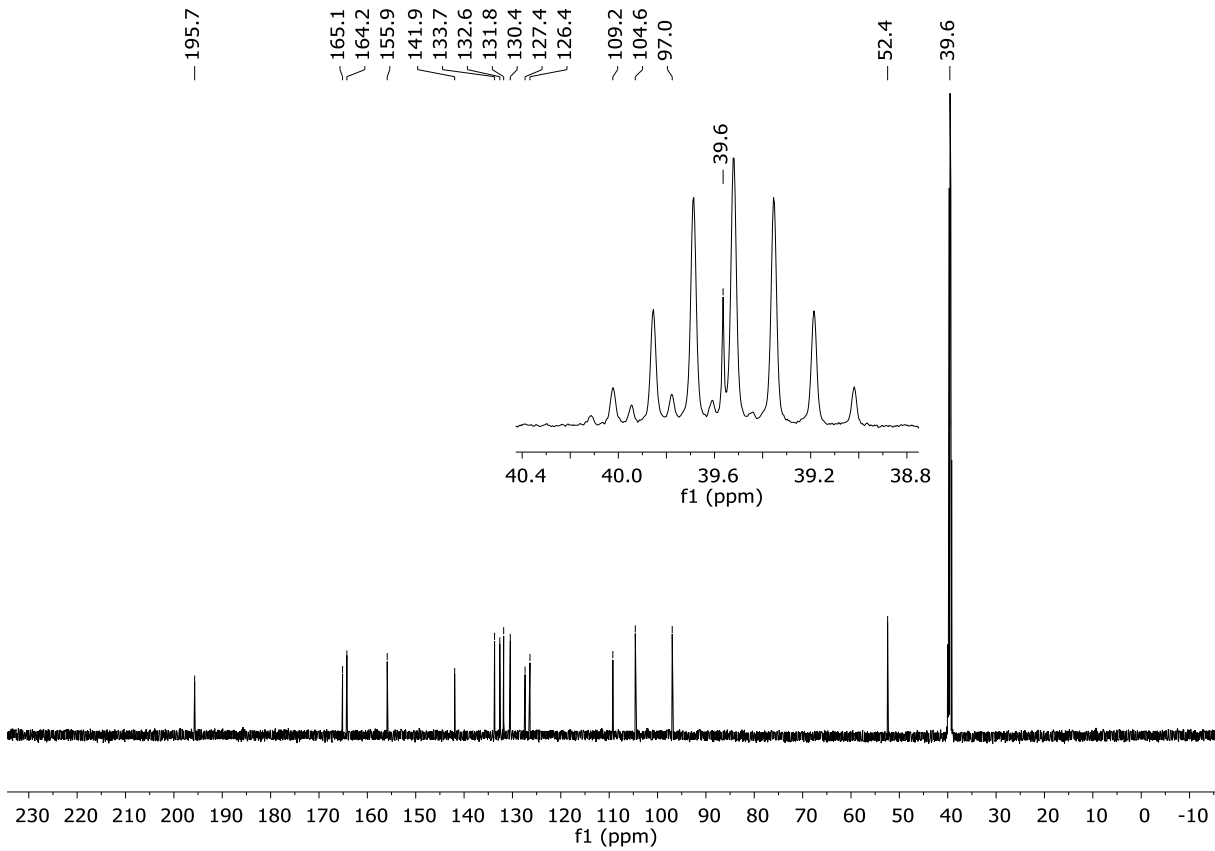
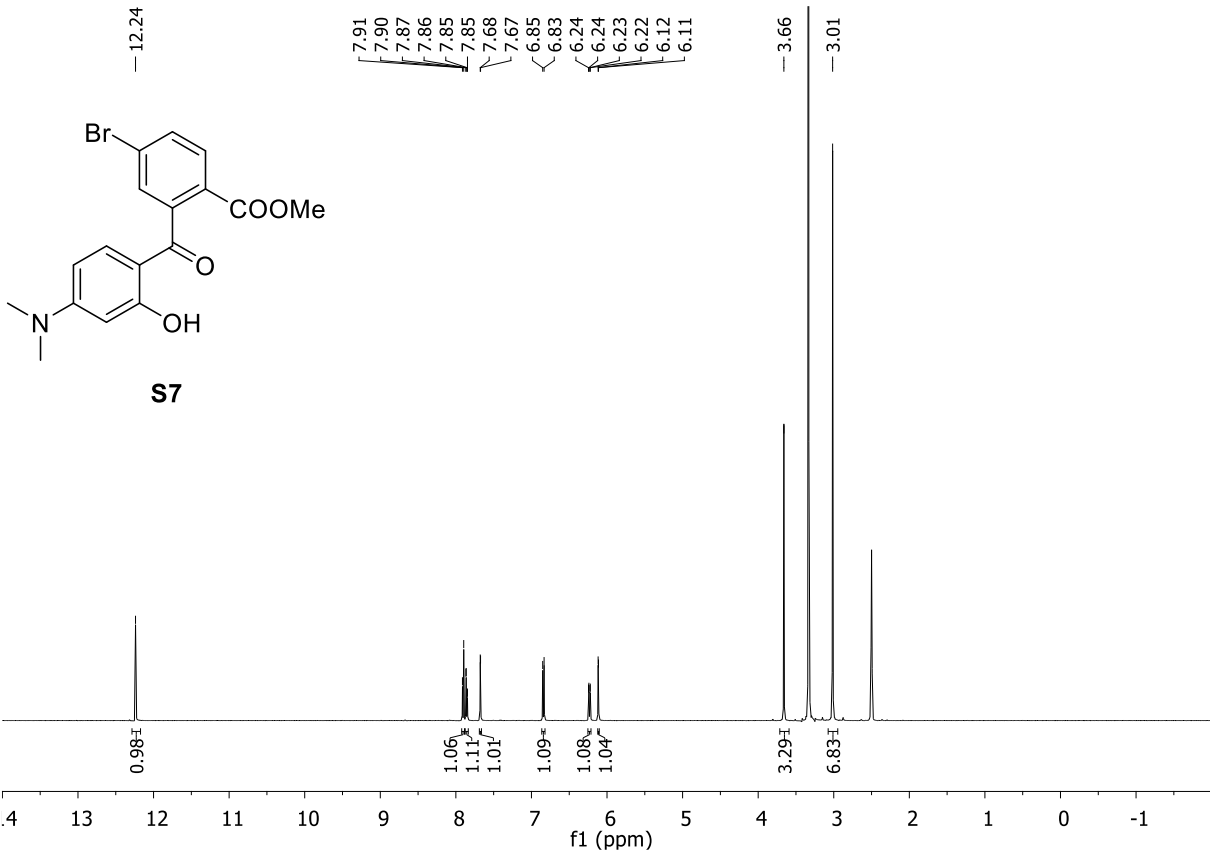


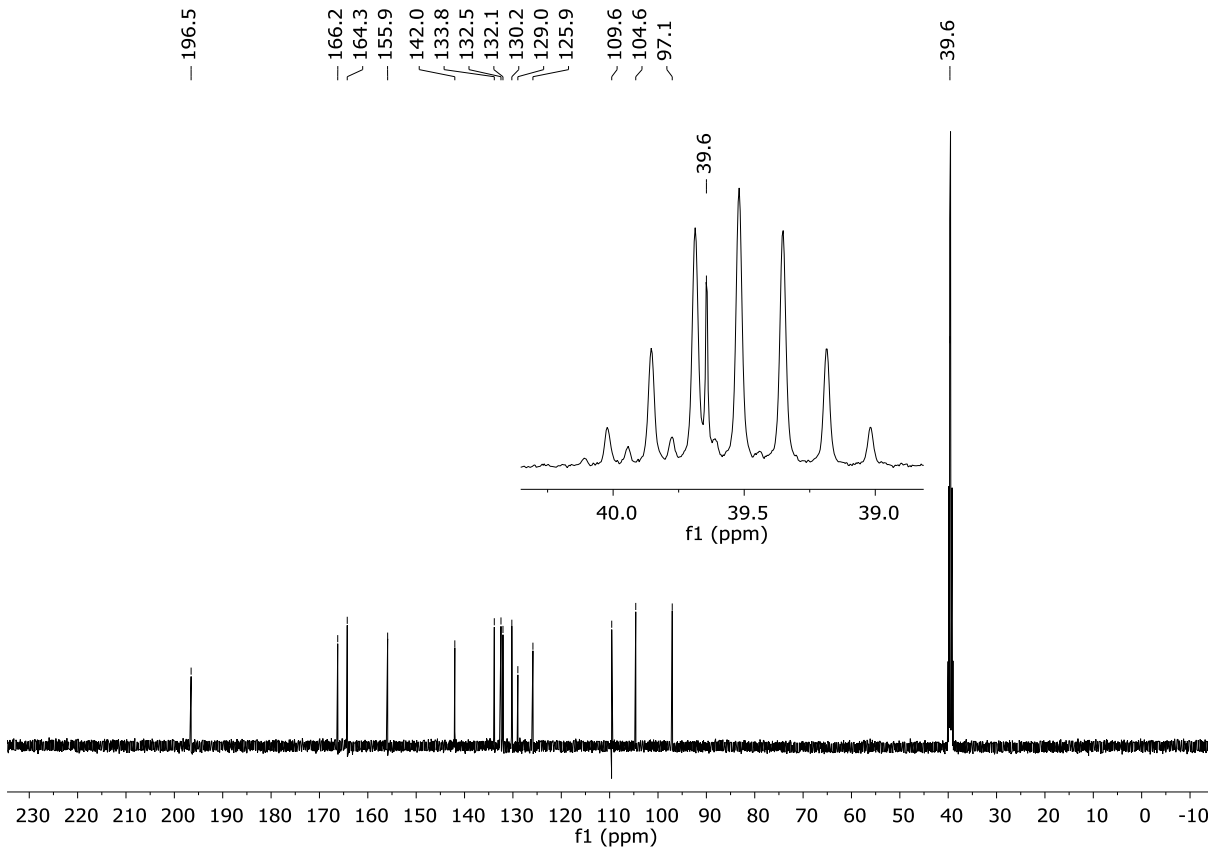
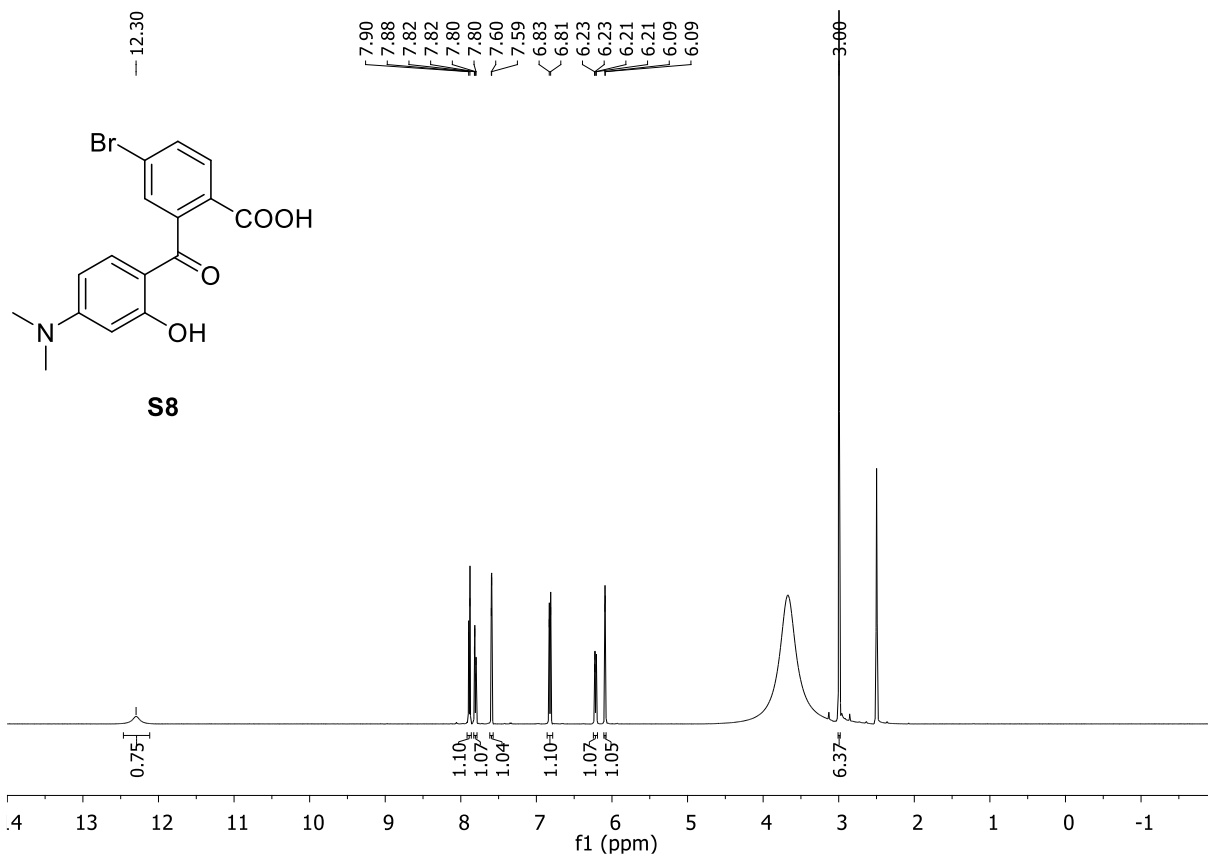


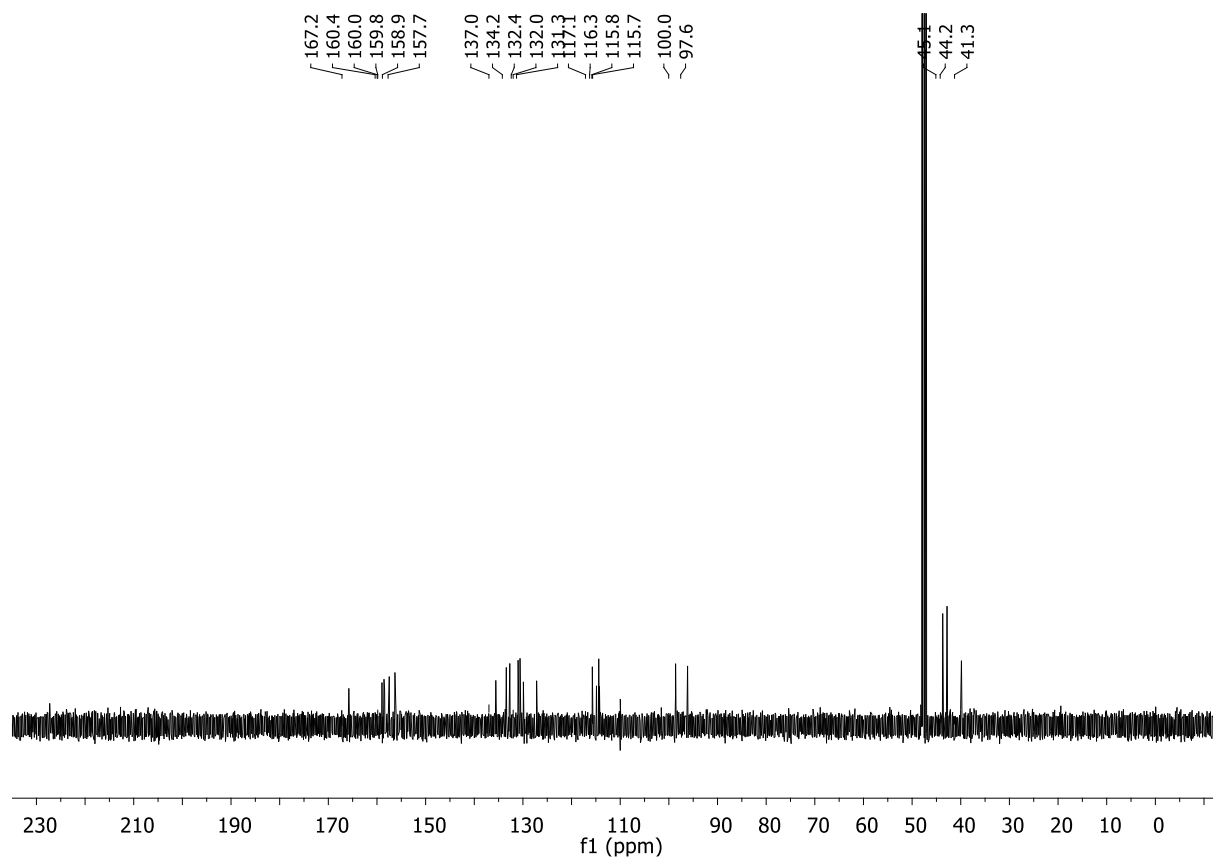
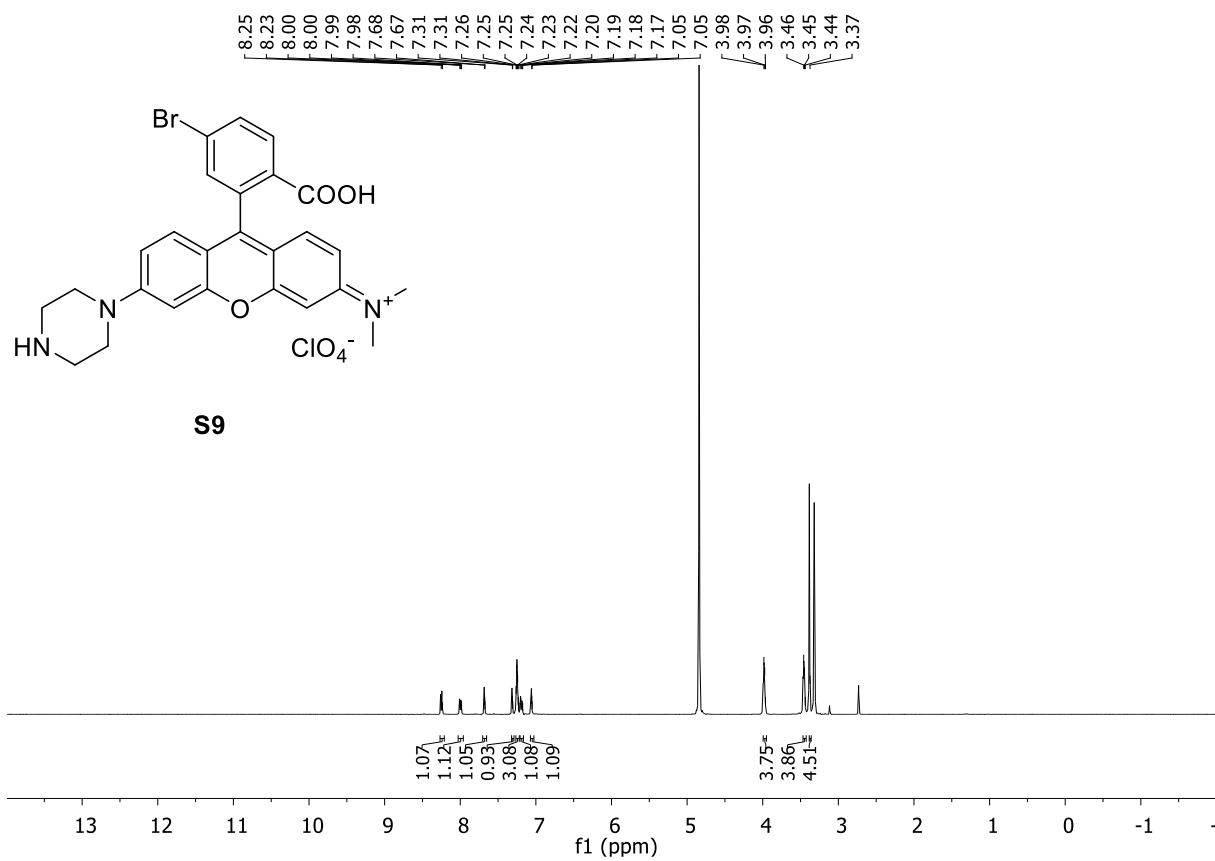


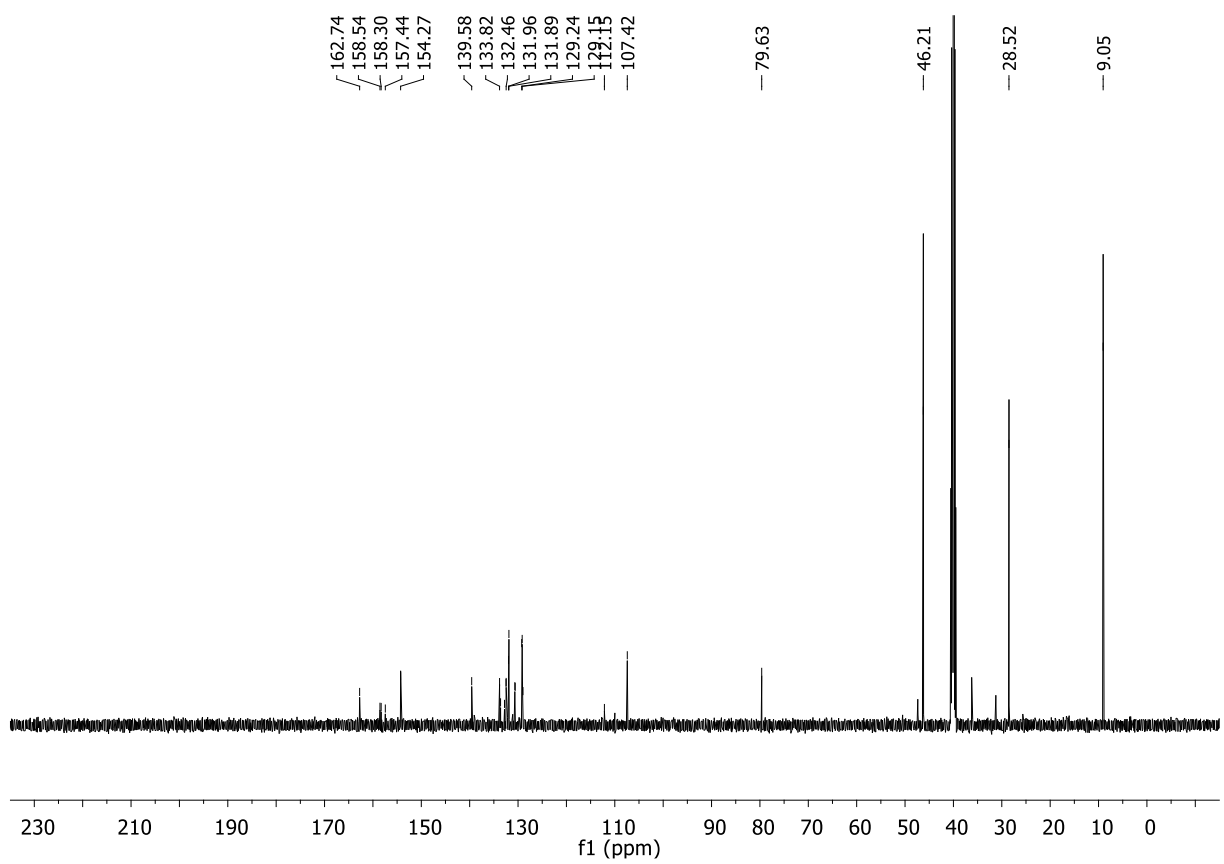
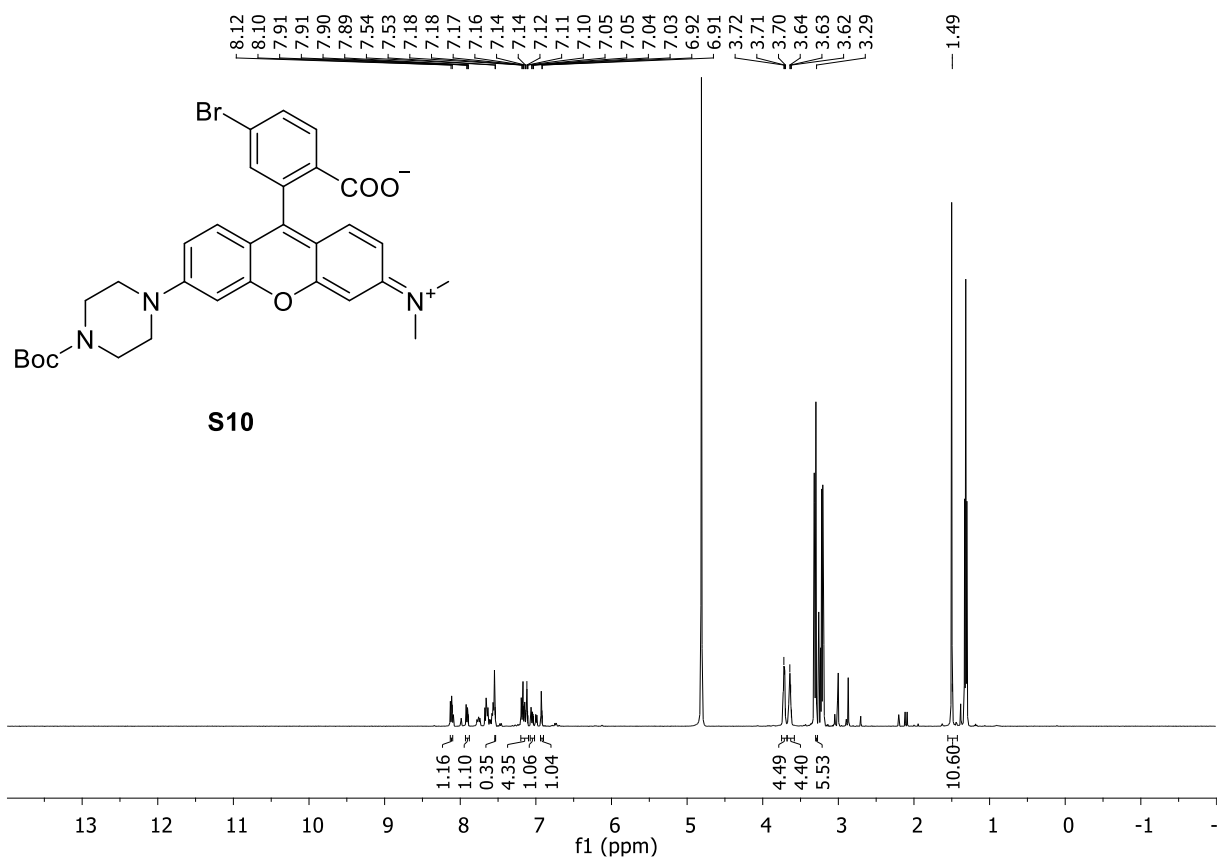


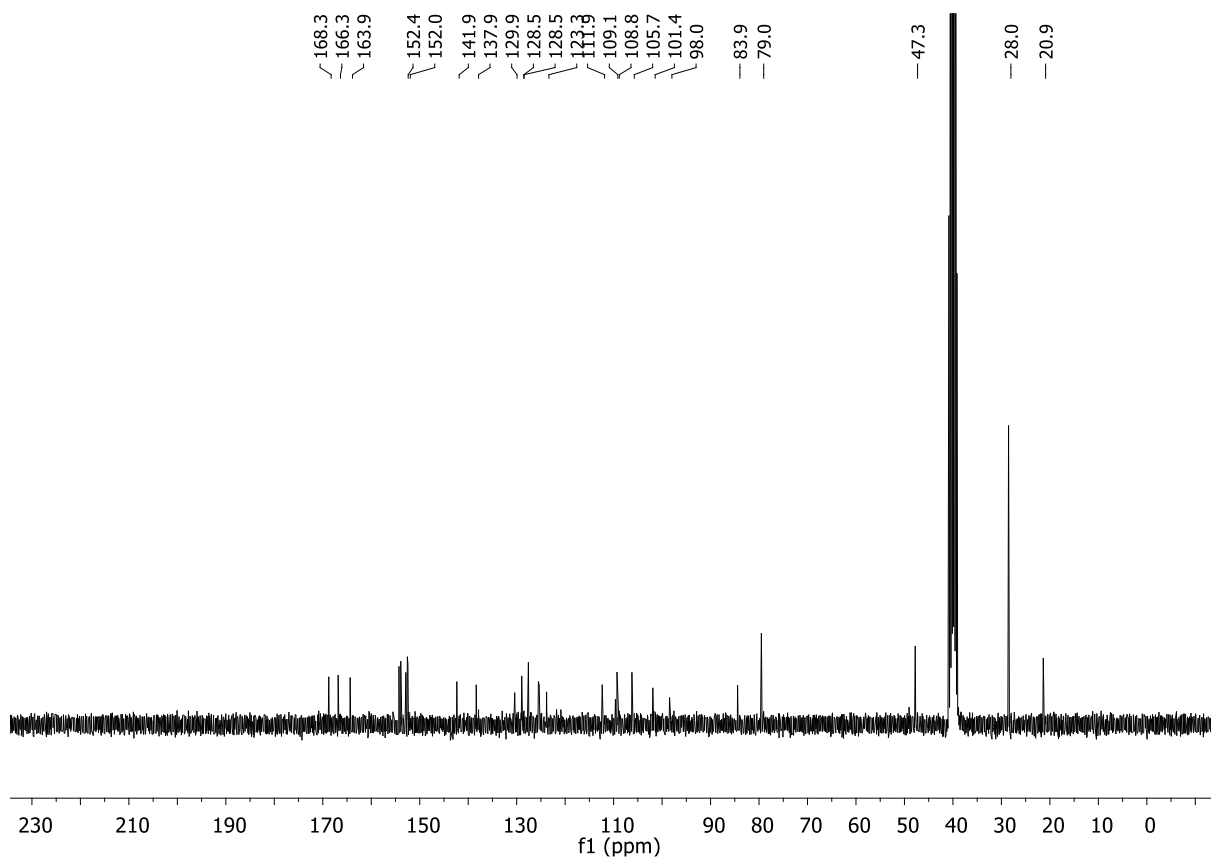
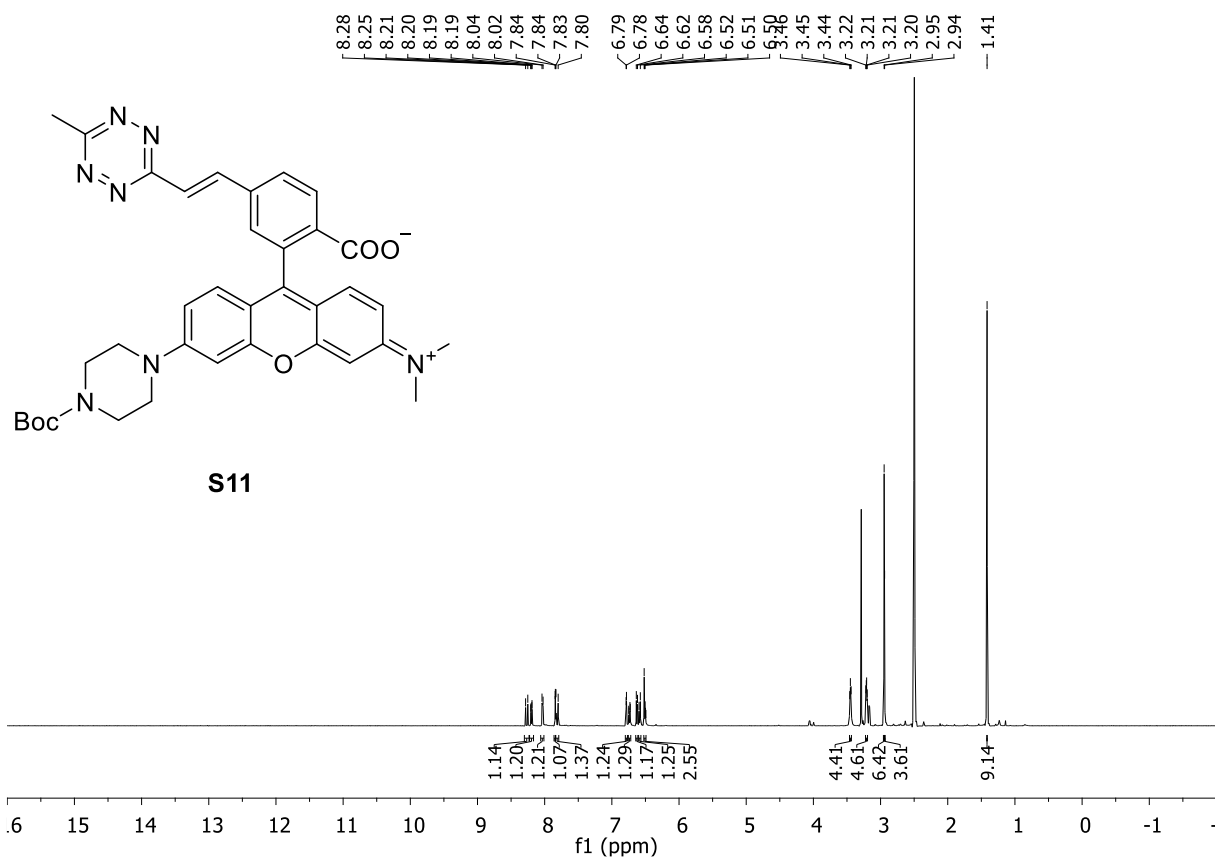


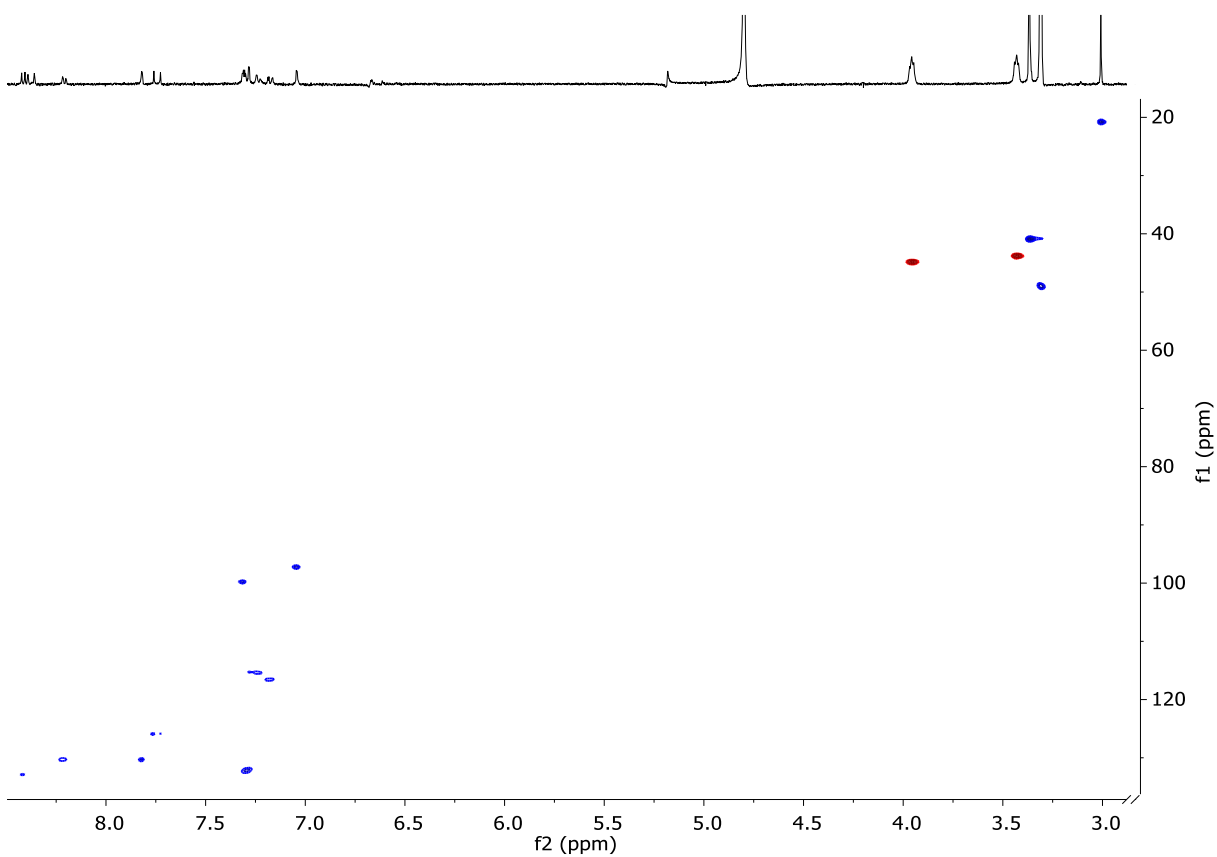
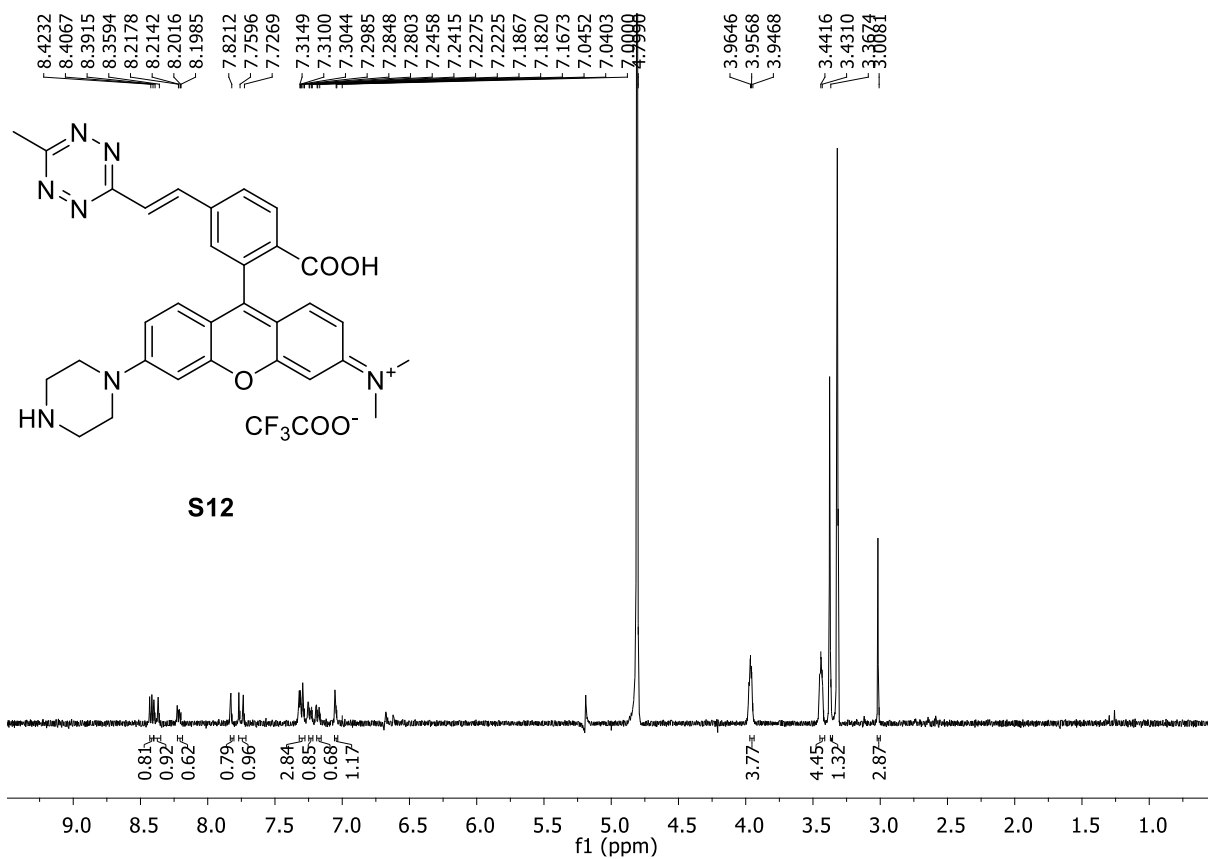


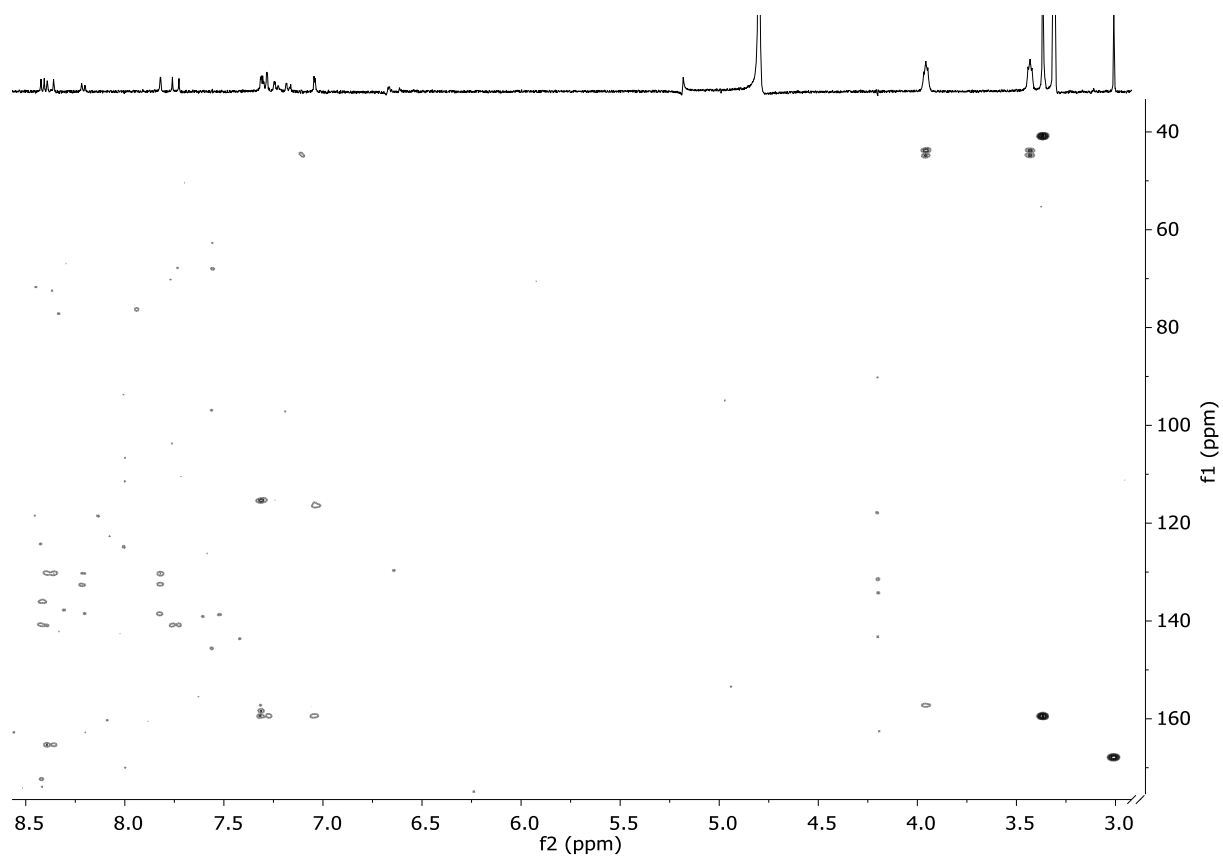


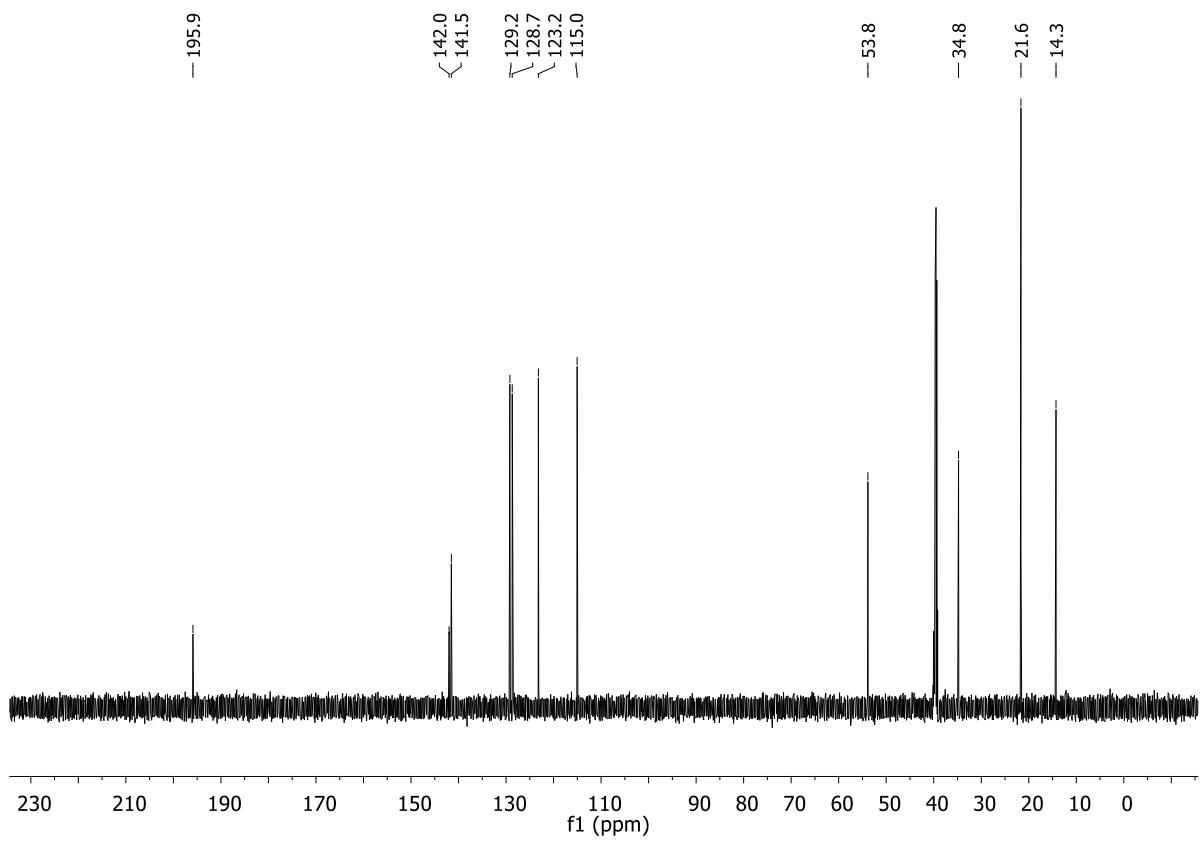
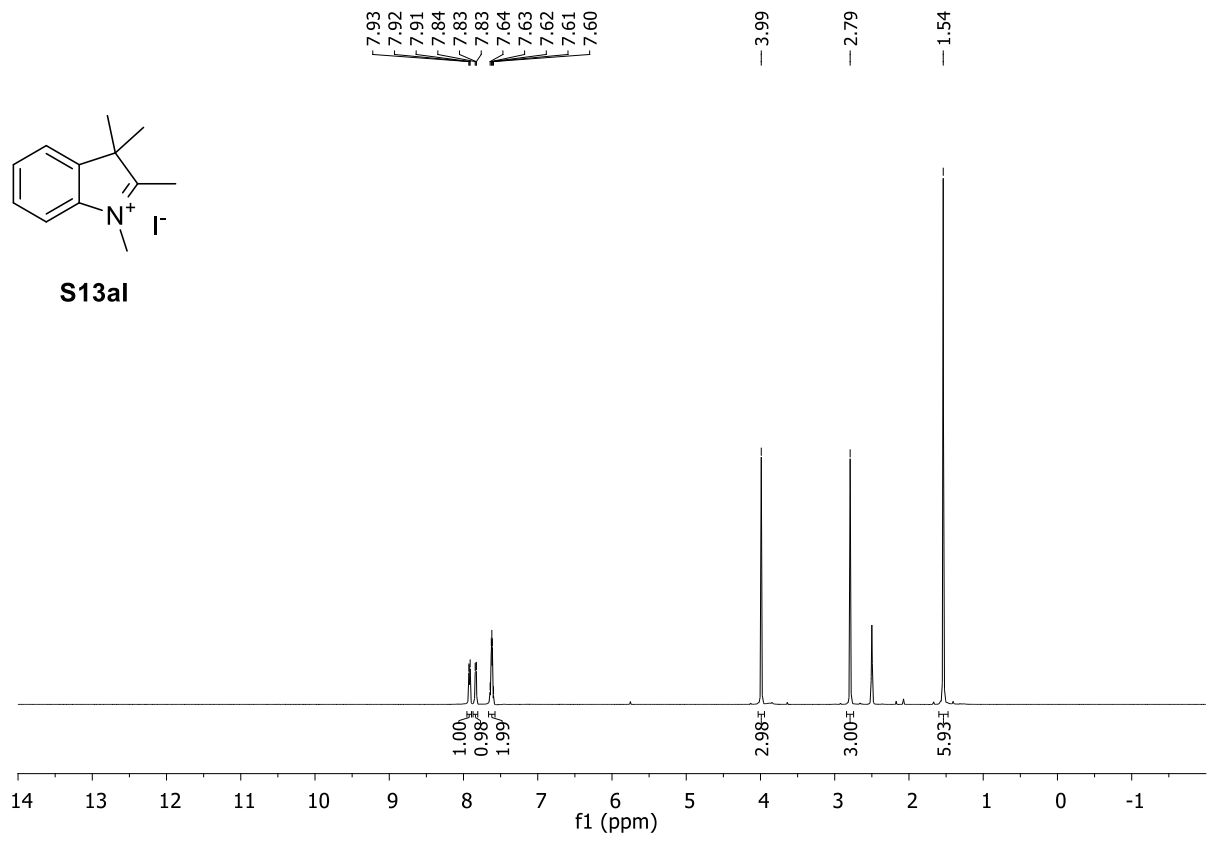
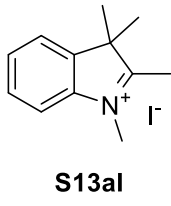


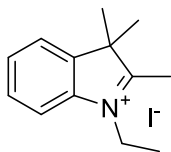




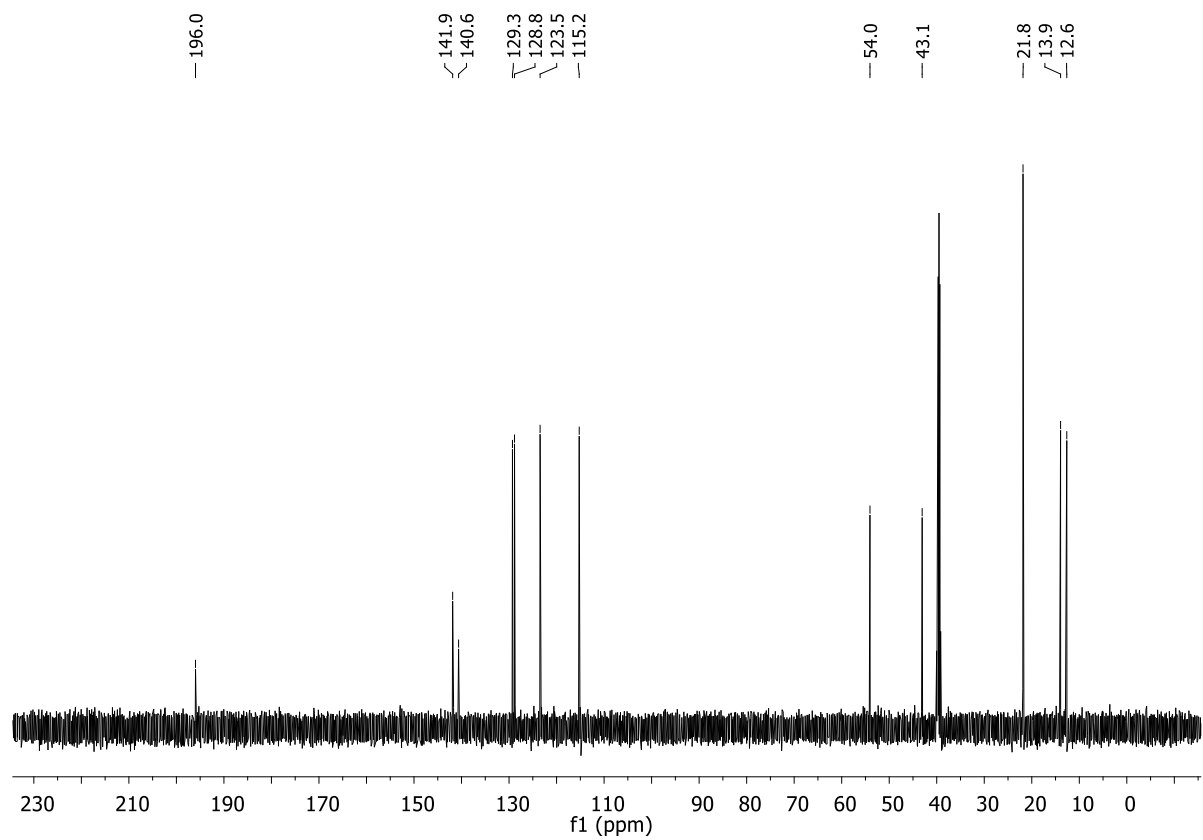
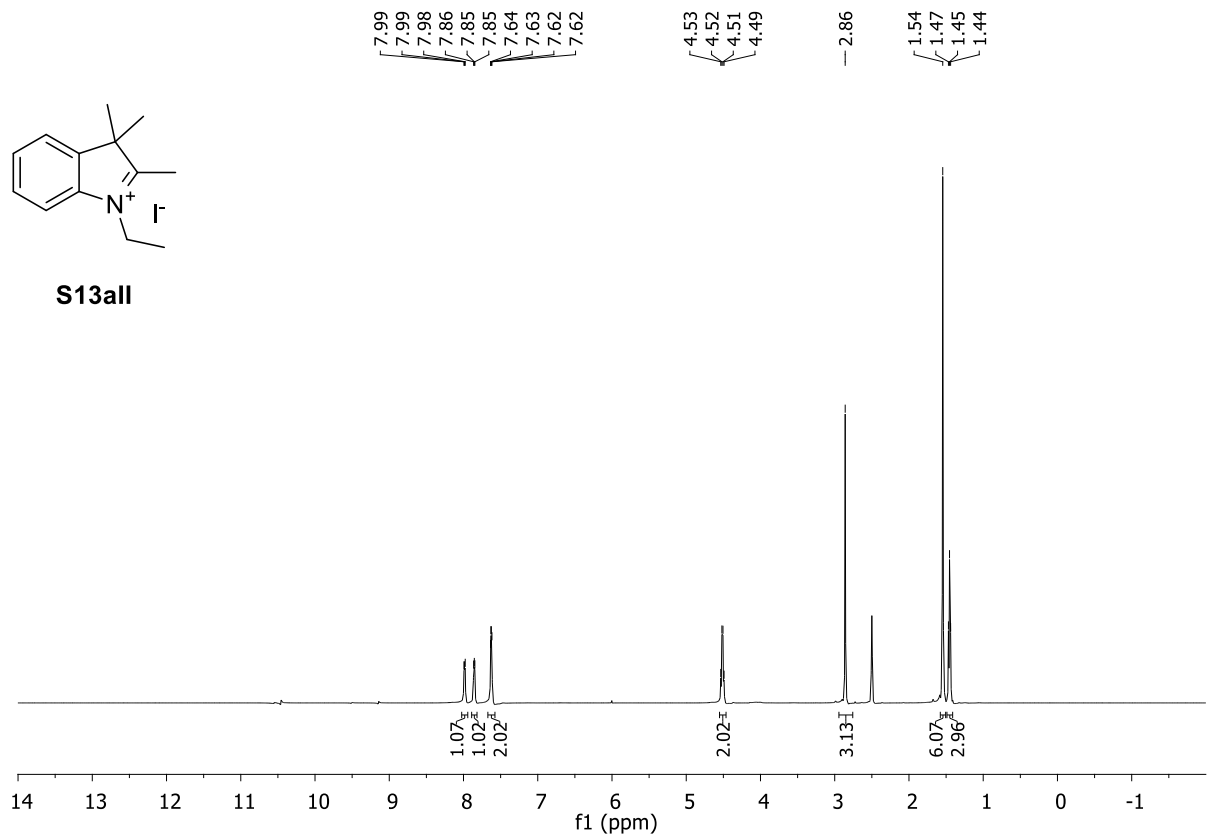


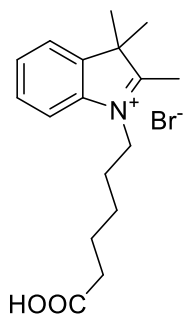




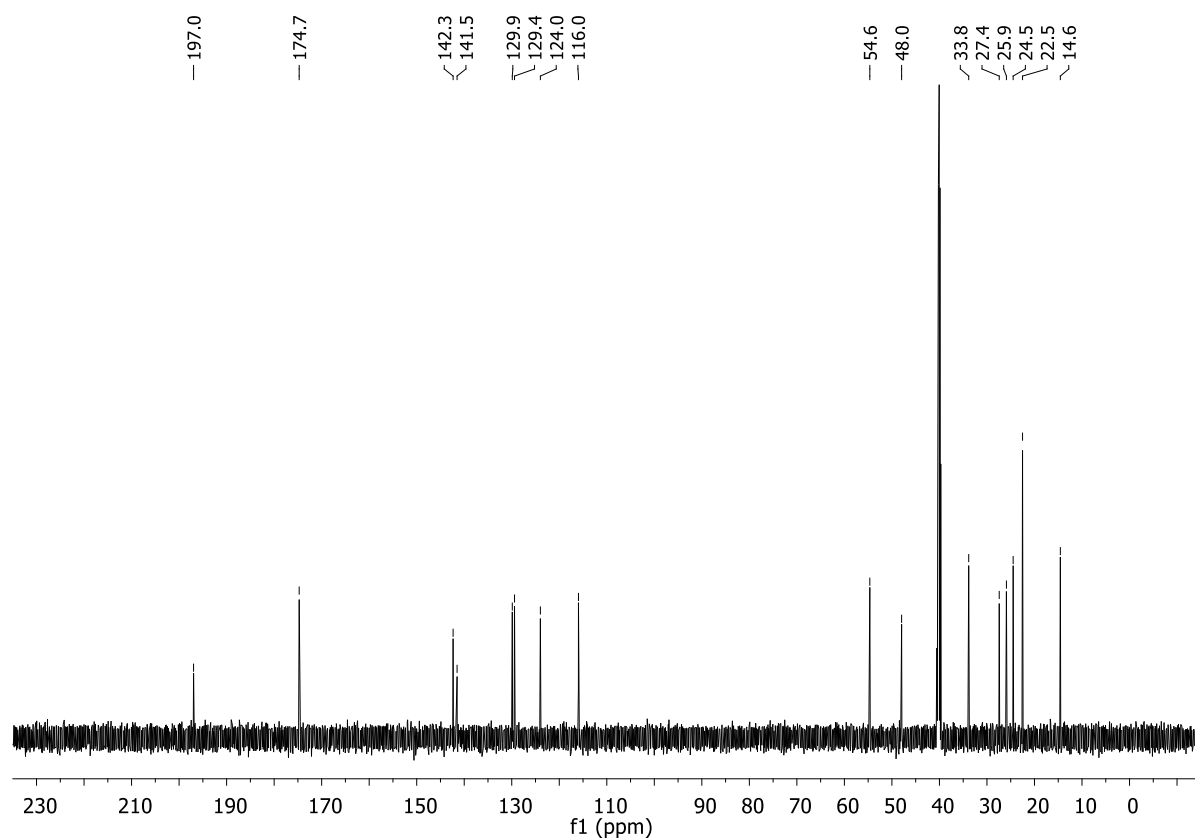
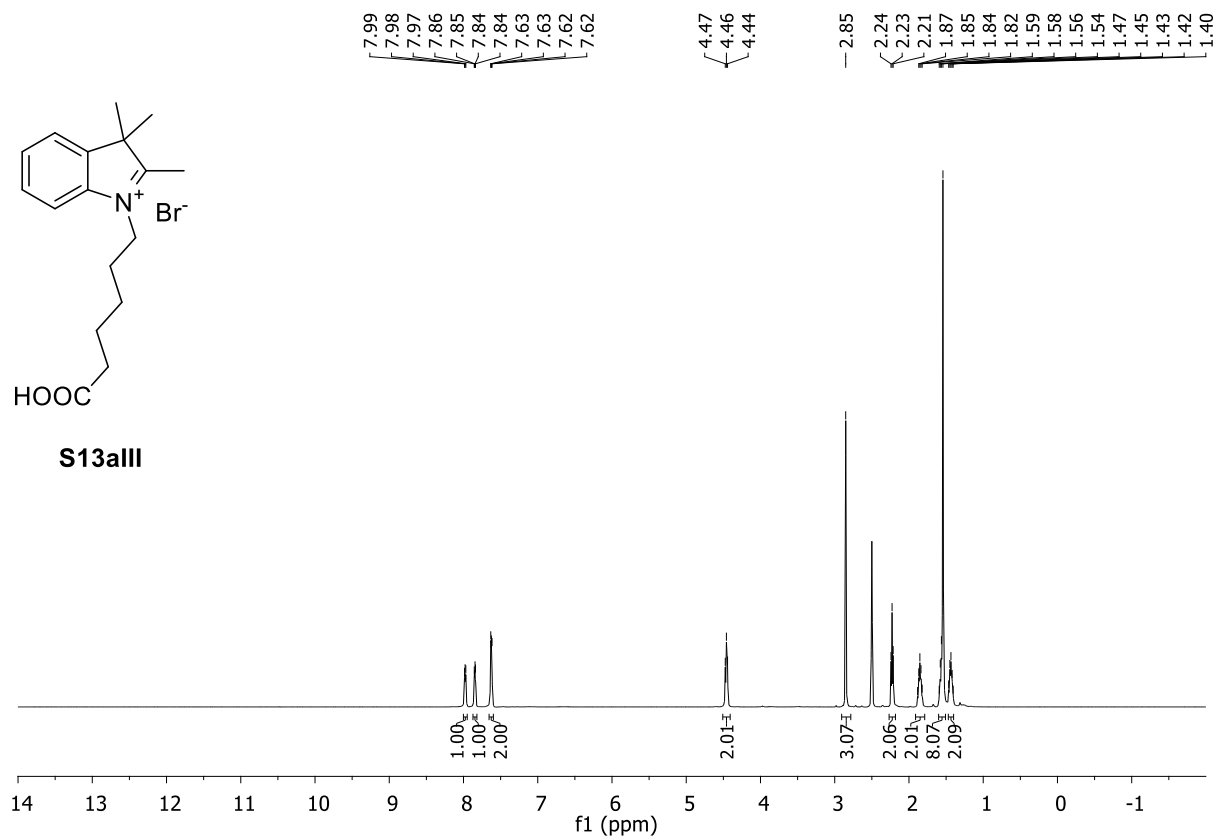


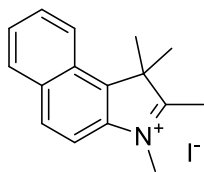
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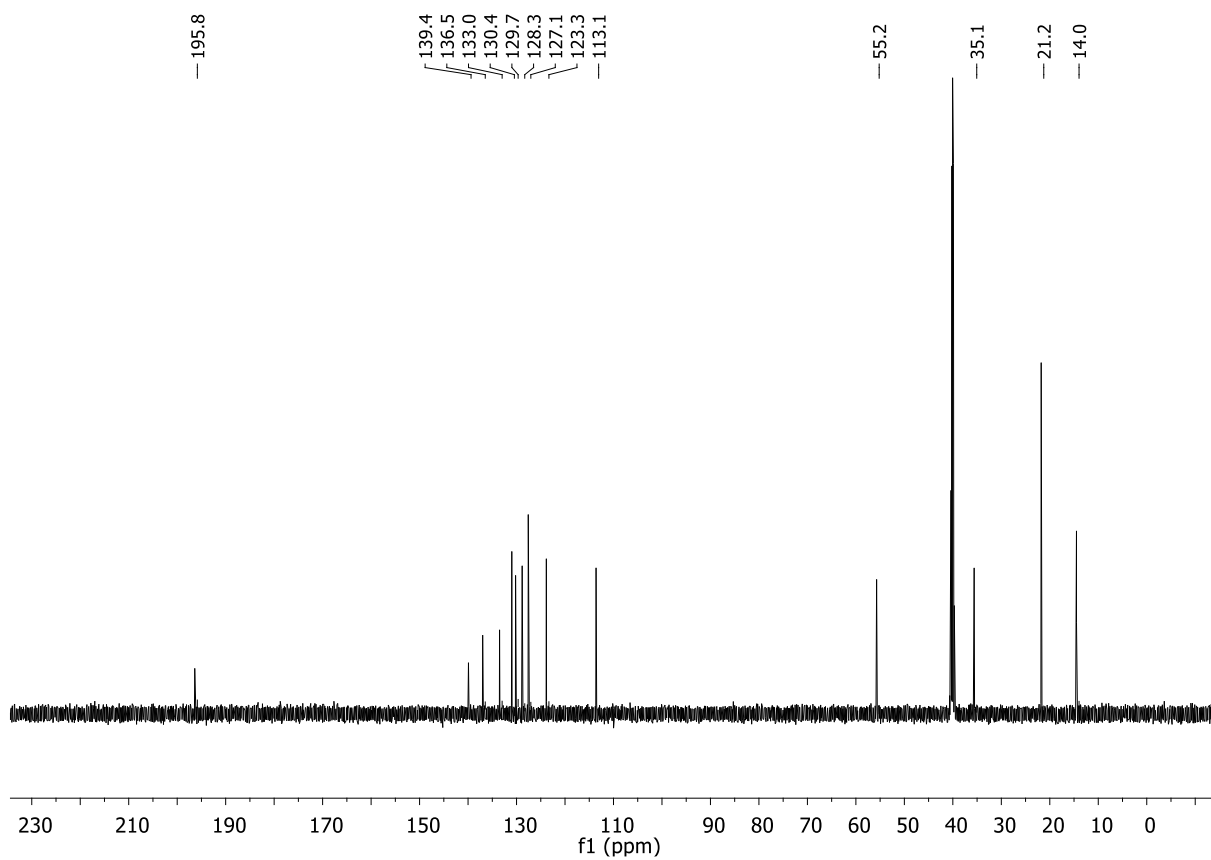
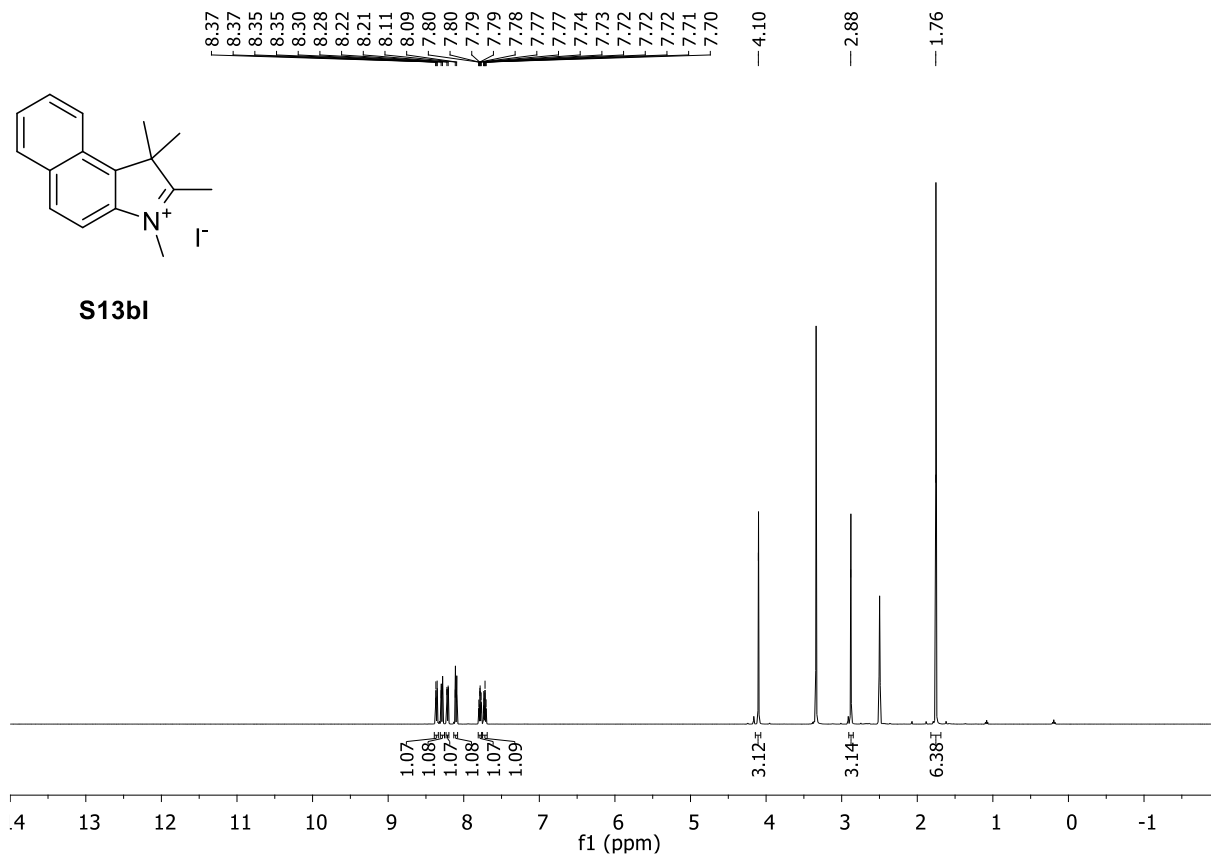


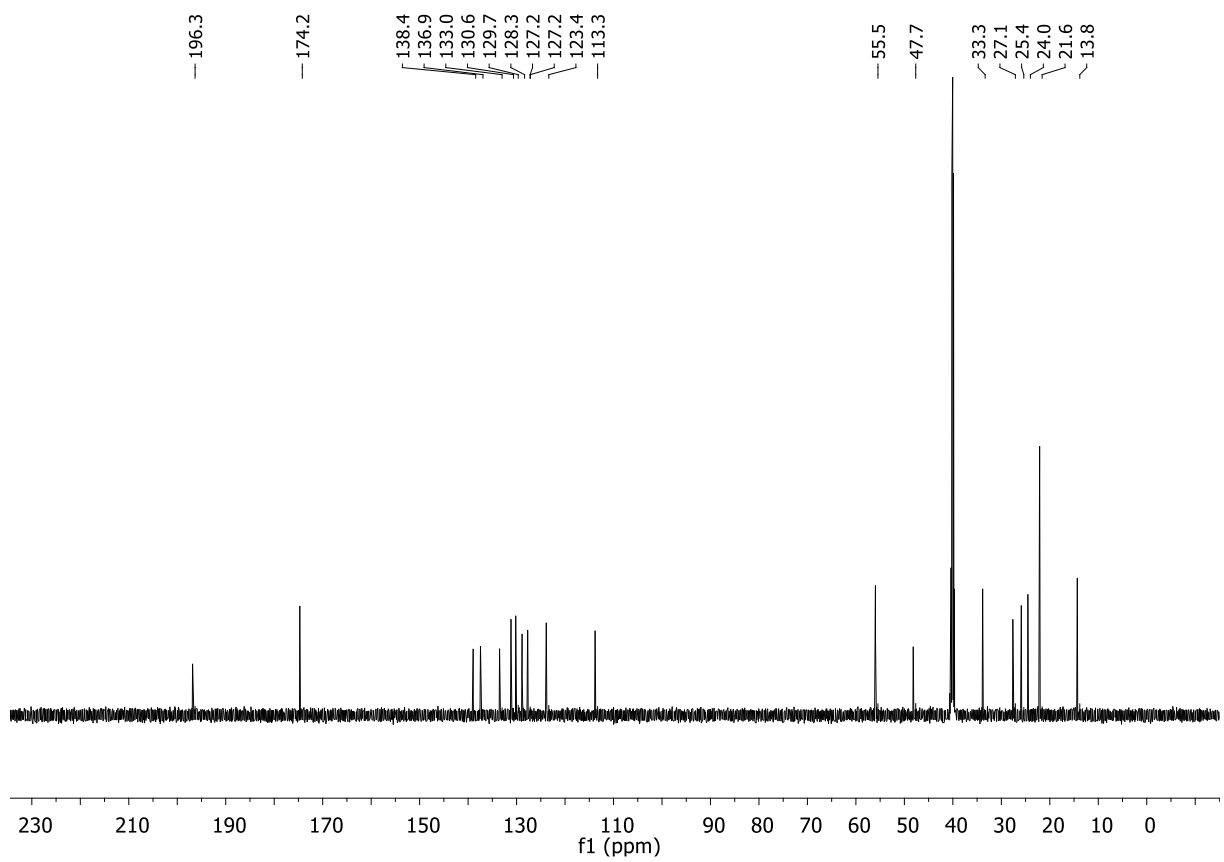
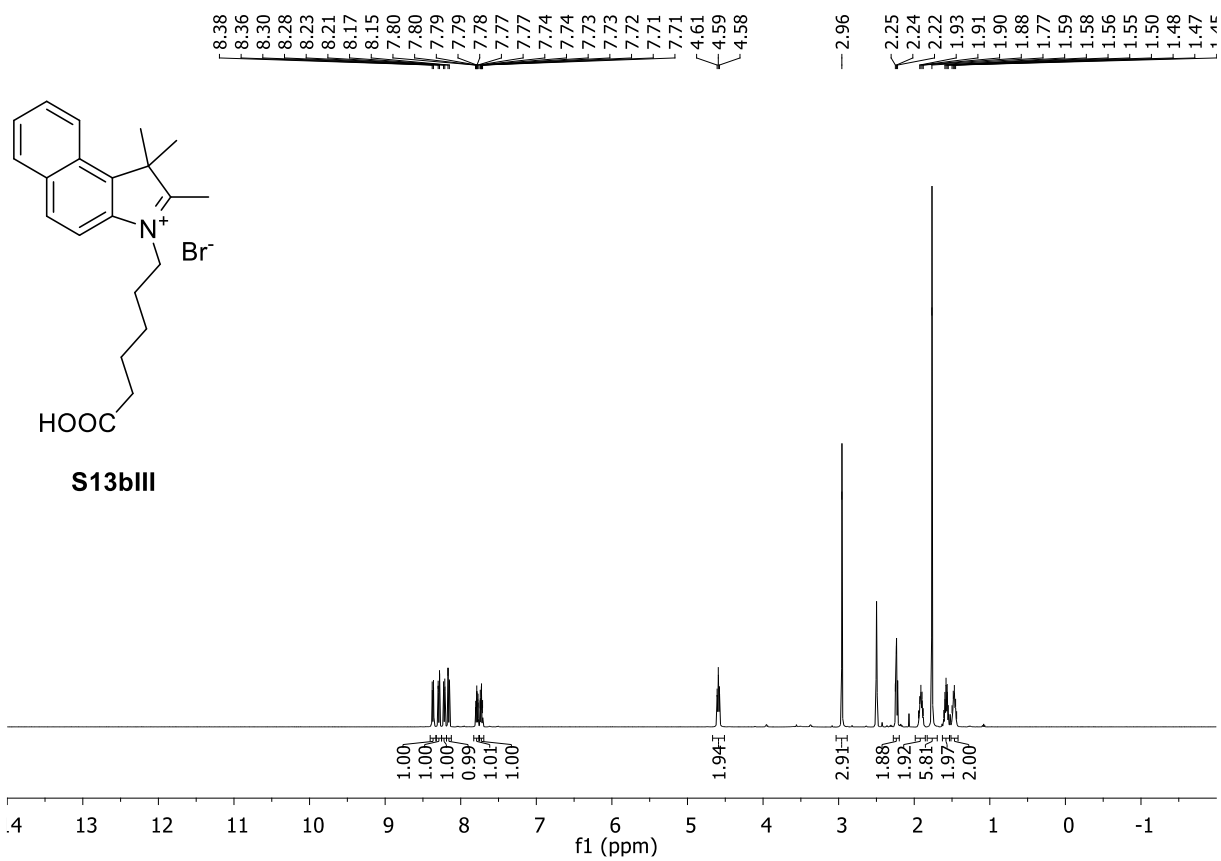
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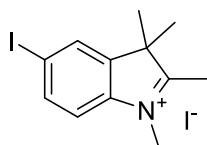




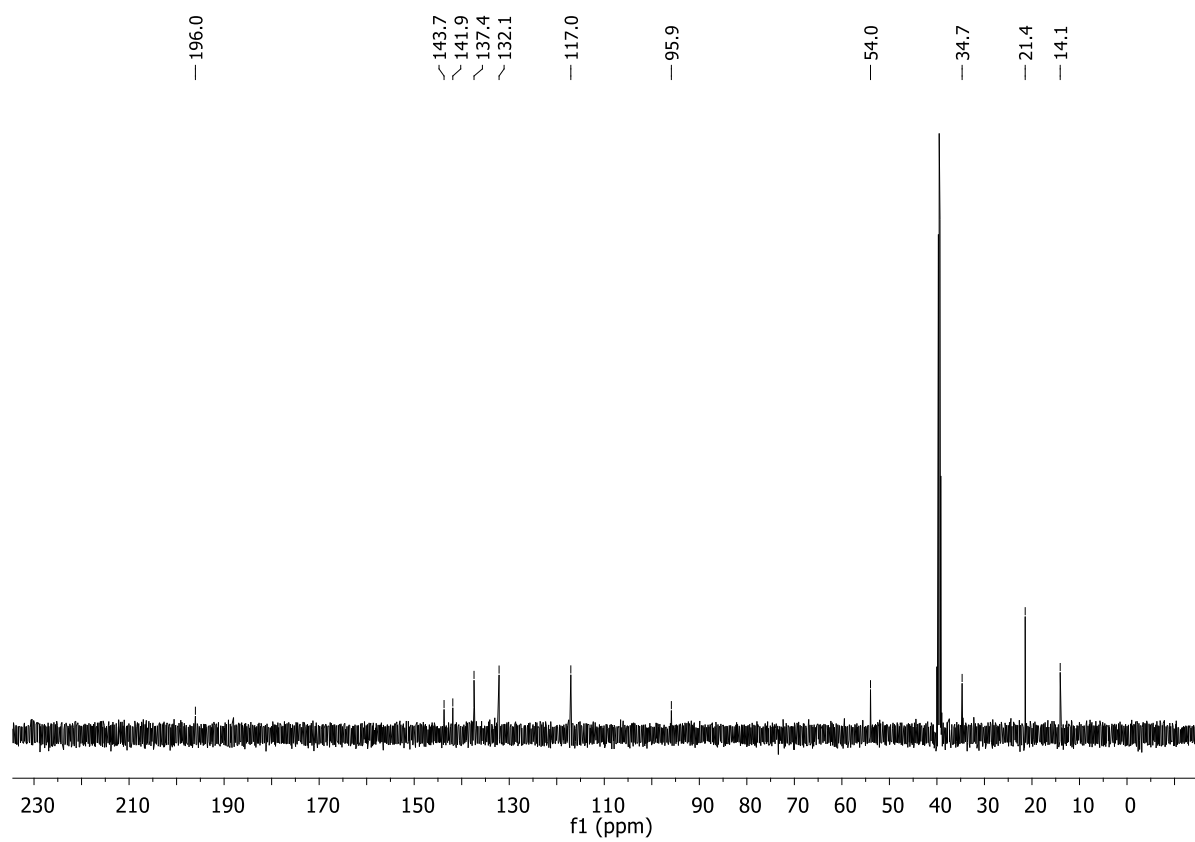
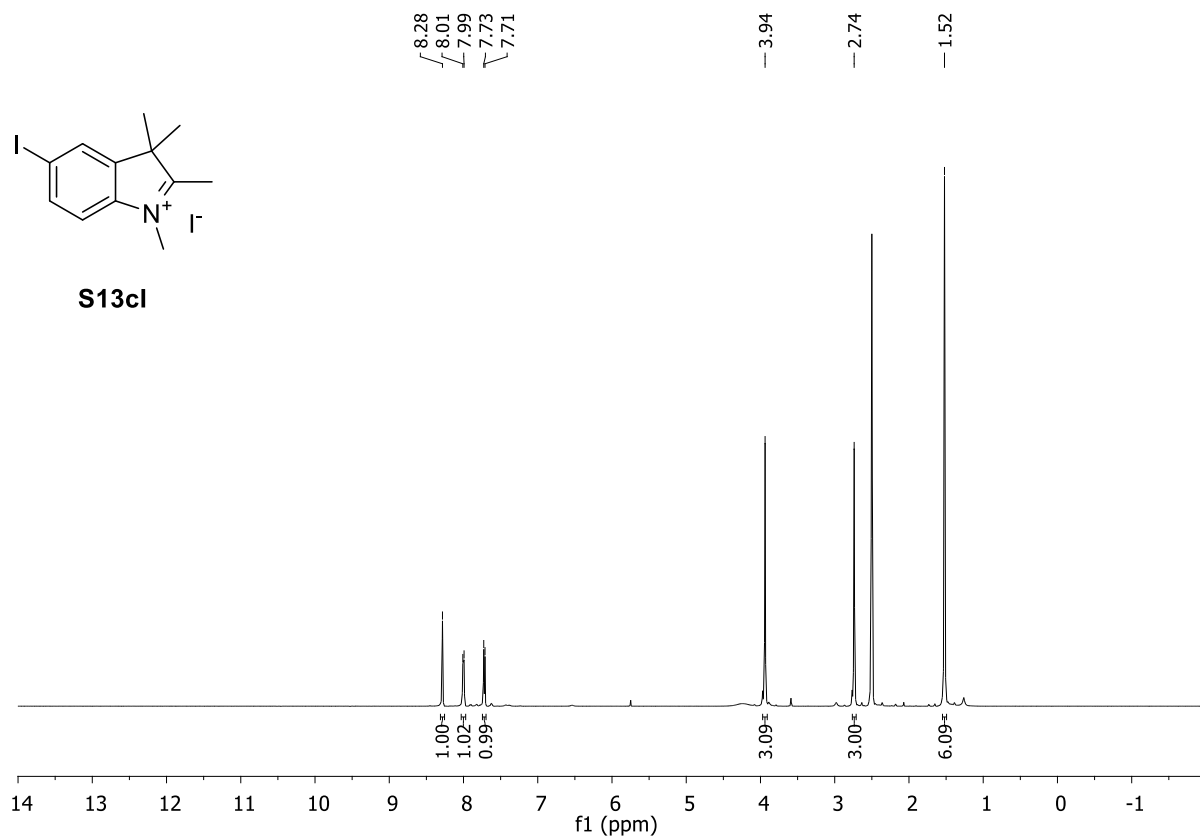
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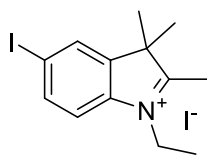






S13cl





S13cII

