Supporting Information (SI)

Evaluation of bioorthogonally applicable tetrazine Cy3-probes for fluorogenic labeling schemes

Evelin Albitz[a,b], Krisztina Németh[a], Gergely Knorr*[,a] and Péter Kele*[,a]

[a] *Chemical Biology Research Group, Institute of Organic Chemistry, Research Centre for Natural Sciences. Magyar tudósok krt. 2. H-1117, Budapest, Hungary. e-mail:kele.peter@ttk.hu*

[b] *Hevesy György PhD School of Chemistry, Eötvös Loránd University. Pázmány Péter sétány 1/a. H-1117, Budapest, Hungary.*

Contents

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 F_{254} precoated aluminium 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 (λ=190-800 (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% H_2O, 5% MeCN, and 0.1% HCOOH) and B (95% MeCN, 5% H_2O, 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 - H_2O mixture.

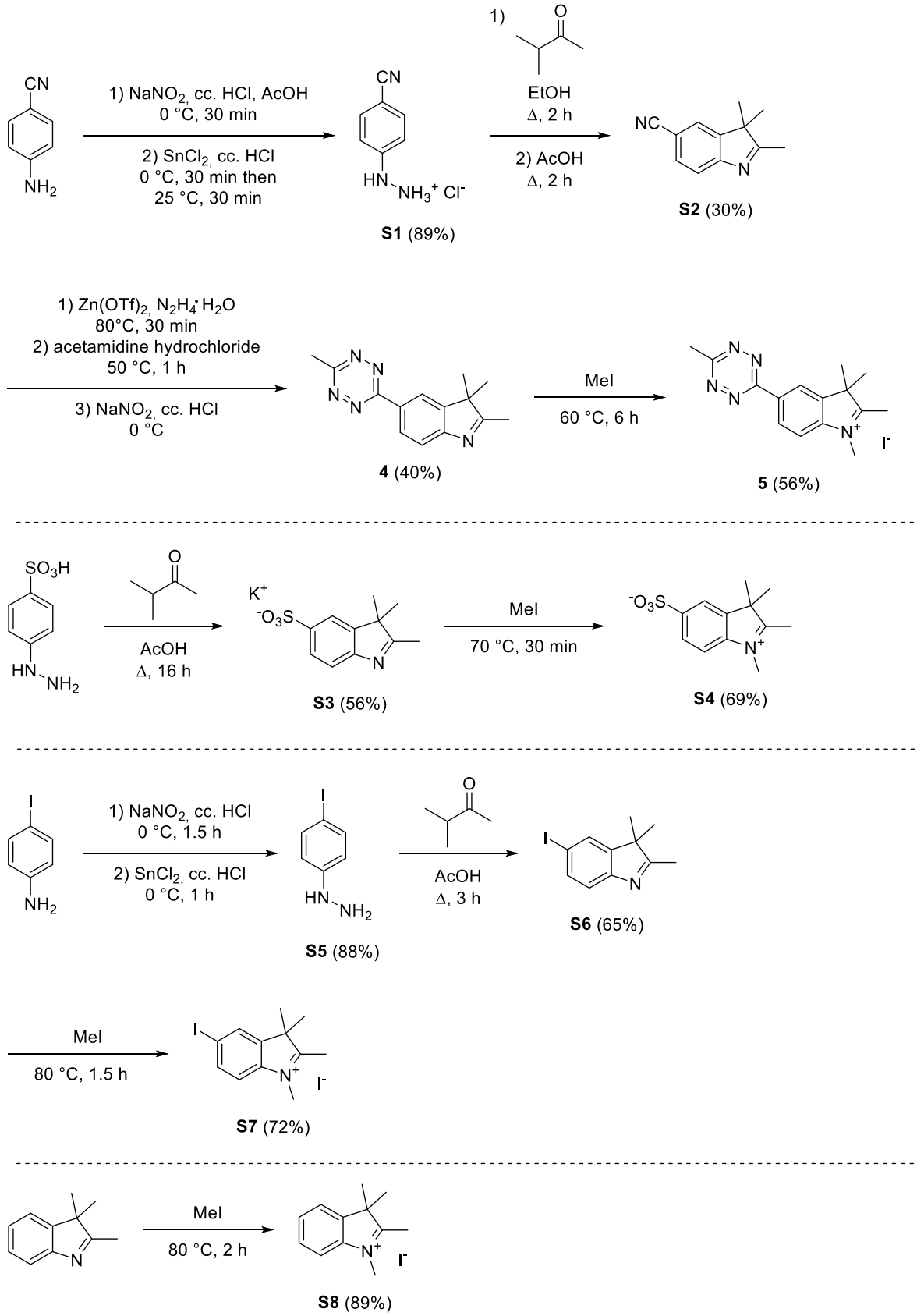
Semipreparative HPLC was performed on a *Wufeng Chrom LC100 HPLC* system using a Gemini C18 column (150 × 21 mm I.D.) with 5 μm silica (110 pore size) as a stationary phase.

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.

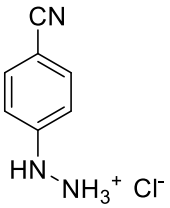
2. Synthesis

2.1. Synthesis of indolium salts



Scheme 1. Synthesis of indolium salt **5**, **S4**, **S7** and **S8**.

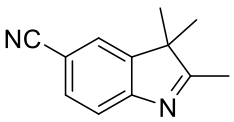
2-(4-Cyanophenyl)hydrazin-1-ium chloride **(S1)**



Compound **S1** was synthesized according to a literature procedure.[1]

4-aminobenzonitrile (3.77 g, 31.9 mmol, 1.0 equiv) was dissolved in a mixture of acetic acid (15 mL) and concentrated HCl (32 mL). This solution was cooled to 0 °C then a solution of $NaNO_2$ (2.64 g, 38.3 mmol, 1.2 equiv) in 10 mL H_2O was added keeping the temperature between 0-5 °C. The resulting brown solution was stirred at 0 °C for 30 minutes. To the mixture, a solution of $SnCl_2 \cdot 2H_2O$ (15.84 g, 70.2 mmol, 2.2 equiv) in 32 mL of concentrated HCl was added dropwise keeping the temperature between 0-5 °C. The reaction mixture was stirred at 0 °C for 30 minutes, then at room temperature for 30 minutes. The resulting precipitate was filtered and washed with water, small amount of methanol and diethyl ether. Then the product was dried under vacuum to yield 4.81 g (89%) of tan solid. ^{1}H NMR (500 MHz, DMSO-d6) δ 10.64 (bs, 3H), 9.15 (bs, 1H), 7.70 (d, *J* = 8.8 Hz, 2H), 7.06 (d, *J* = 8.8 Hz, 2H). ^{13}C NMR (126 MHz, DMSO-d6) δ 149.5, 133.3, 119.3, 113.8, 102.0. LCMS: m/z calcd. for $[C_7H_8N_3]^+$: 134; found: 134 $[M]^+$.

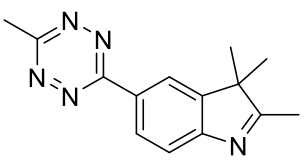
2,3,3-Trimethyl-3H-indole-5-carbonitrile **(S2)**



Compound **S2** was synthesized according to a literature procedure with modification.[2]

Hydrazine compound **S1** (2.8 g, 16.5 mmol, 1.0 equiv) and 3-methyl-2-butanone (5.3 mL, 49.5 mmol, 3.0 equiv) were dissolved in 28 mL ethanol and refluxed under nitrogen for 2 hours. Then the solvent and the excess of 3-methyl-2-butanone were evaporated under vacuum. The residue was dissolved in 28 mL acetic acid and refluxed for 2 hours. After that the acetic acid was removed under vacuum and the crude product was purified by flash chromatography on silica gel (hexane – ethyl acetate 0 to 100%) to yield 900 mg (30%) of dark orange solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.59 (dd, *J* = 8.0, 1.3 Hz, 1H), 7.56 (d, *J* = 7.9 Hz, 1H), 7.52 (d, *J* = 1.4 Hz, 1H), 2.30 (s, 3H), 1.30 (s, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 192.4, 157.3, 146.7, 132.7, 125.2, 120.7, 119.4, 108.6, 54.3, 22.8, 15.7. LCMS: m/z calcd. for $[C_{12}H_{13}N_2]^+$: 185; found: 185 $[M]^+$.

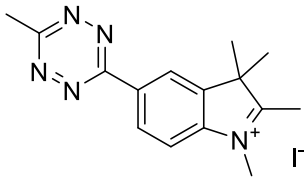
2,3,3-Trimethyl-5-(6-methyl-1,2,4,5-tetrazin-3-yl)-3H-indole **(4)**[3]



Indole **S2** (1.00 g, 5.4 mmol, 1.0 equiv) and $Zn(OTf)_2$ (0.986 g, 2.72 mmol, 0.5 equiv) were dissolved in hydrazine monohydrate (13.5 mL, 271.5 mmol, 50 equiv) and stirred at 80 °C for 30 minutes. Then, the reaction mixture was cooled to 50 °C and acetamidine hydrochloride (5.13 g, 54.30 mmol, 10.0 equiv) was added portionwise for 1 hour. After cooling to 0 °C, the mixture was diluted with 50 mL ethyl acetate and 50 mL water, then $NaNO_2$ (3.70 g, 54.3 mmol, 10.0 equiv) was added. With vigorous stirring AcOH was added dropwise to set the pH=3. The two phases were separated, and the aqueous phase was extracted with ethyl acetate three times. The combined organic

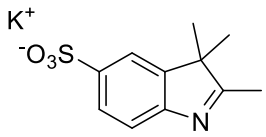
phases were extracted with cc. $NaHCO_3$ solution three times, dried over $MgSO_4$, and evaporated under vacuum. The crude product was purified by flash chromatography on silica gel (dichloromethane-methanol 0 to 10%) to yield 550 mg (40%) of pink solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.60 (dd, *J* = 8.1, 1.7 Hz, 1H), 8.52 (d, *J* = 1.3 Hz, 1H), 7.71 (d, *J* = 8.1 Hz, 1H), 3.08 (s, 3H), 2.35 (s, 3H), 1.40 (s, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 191.6, 167.0, 164.4, 157.8, 146.9, 128.8, 128.5, 121.1, 120.8, 54.3, 23.1, 21.2, 15.9. HRMS: m/z calcd. for $[C_{14}H_{16}N_5]^+$: 254.1400; found: 254.1402 $[M+H]^+$.

1,2,3,3-Tetramethyl-5-(6-methyl-1,2,4,5-tetrazin-3-yl)-3H-indol-1-ium iodide **(5)**



In a pressure tube indole **4** (200 mg, 0.790 mmol, 1 equiv) was dissolved in methyl iodide (1475 µL, 23.690 mmol, 30 equiv) and reacted at 60 °C for 6 hours. After cooling to room temperature, ethyl acetate was added and the resulting precipitate was filtered, washed with cold ethyl acetate and dried to yield 223 mg (71%) of dark red solid. The product was used without further purification. ^{1}H NMR (500 MHz, DMSO-d6) δ 8.91 (s, 1H), 8.72 (d, *J* = 8.2 Hz, 1H), 8.20 (d, *J* = 8.3 Hz, 1H), 4.06 (s, 3H), 3.04 (s, 3H), 2.86 (s, 3H), 1.65 (s, 6H). ^{13}C NMR (126 MHz, DMSO-d6) δ 199.0, 167.8, 163.3, 145.5, 143.2, 133.4, 128.9, 122.8, 116.7, 55.0, 35.6, 22.1, 21.4, 15.1. HRMS: m/z calcd. for $[C_{15}H_{18}N_5]^+$: 268.1556; found: 268.1560 $[M]^+$.

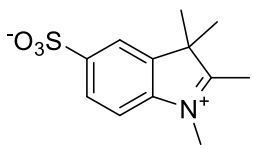
Potassium 2,3,3-trimethyl-3H-indole-5-sulfonate **(S3)**[4]



Compound **S3** was synthesized according to a literature procedure.

4-Hydrazineylbenzenesulfonic acid (5.0 g, 25.0 mmol, 1.0 equiv) and 3-methyl-2-butanone (2.7 mL, 25.0 mmol, 1.0 equiv) were dissolved in 50 mL acetic acid and refluxed under nitrogen for 16 hours. After the acetic acid was removed under vacuum, the residue was dissolved in 25 mL methanol, then 25 mL saturated KOH in isopropanol was slowly added. The resulting precipitation was filtered, washed with isopropanol and dried under vacuum to yield 3.85 g (56%) yellow solid. The crude product was used without further purification. ^{1}H NMR (500 MHz, DMSO-d6) δ 7.63 (d, *J* = 1.2 Hz, 1H), 7.55 (dd, *J* = 7.9, 1.6 Hz, 1H), 7.34 (d, *J* = 7.9 Hz, 1H), 2.21 (s, 3H), 1.25 (s, 6H). ^{13}C NMR (126 MHz, DMSO-d6) δ 188.7, 153.6, 145.2, 145.1, 125.1, 119.1, 118.1, 53.2, 22.5, 15.1. LCMS: m/z calcd. for $[C_{11}H_{14}NO_3S]^+$: 240; found: 240 $[M^-+2H]^+$.

1,2,3,3-Tetramethyl-3H-indol-1-ium-5-sulfonate **(S4)**[5]

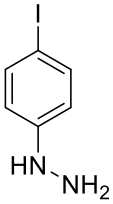


Compound **S4** was synthesized according to a literature procedure with modifications.

In a pressure tube indole **S3** (1.00 g, 3.79 mmol, 1 equiv) was dissolved in (2.36 mL, 37.79 mmol, 10 equiv) methyl iodide and reacted at 70 °C for 30 minutes. After cooling to room temperature, ethyl acetate was added and the resulting precipitate was filtered, washed with cold ethyl acetate and dried to yield 664 mg (69%) The crude product was used

without further purification. ^{1}H NMR (500 MHz, DMSO-d6) δ 7.97 (s, 1H), 7.81 (d, *J* = 8.3 Hz, 1H), 7.77 (d, *J* = 8.3 Hz, 1H), 3.95 (s, 3H), 2.74 (s, 3H), 1.51 (s, 6H). ^{13}C NMR (126 MHz, DMSO-d6) δ 196.8, 149.4, 141.9, 141.2, 126.2, 120.5, 114.5, 54.0, 34.7, 21.6, 14.0. LCMS: m/z calcd. for $[C_{12}H_{16}NO_3S]^+$: 254; found: 254 $[M+H]^+$.

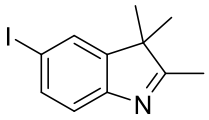
2-(4-Iodophenyl)hydrazin-1-ium chloride **(S5)**[4]



Compound **S5** was synthesized according to a literature procedure.

4-Iodoaniline (10.0 g, 45.7 mmol, 1.00 equiv) was dissolved in 50 mL concentrated HCl. This solution was cooled to 0 °C then a solution of $NaNO_2$ (3.24 g, 46.6 mmol, 1.02 equiv) in 75 mL H_2O was added keeping the temperature between 0-5 °C. The solution was stirred at 0 °C for 1.5 hours. To the mixture, a solution of $SnCl_2 \cdot 2H_2O$ (25.00 g, 109.7 mmol, 2.4 equiv) in 35 mL of concentrated HCl was added dropwise keeping the temperature between 0-5 °C. The reaction mixture was stirred at 0 °C for 1 hour. The resulting precipitate was filtered, dissolved in 1M NaOH solution and extracted with dichloromethane. The combined organic phase was dried over $MgSO_4$ and evaporated under vacuum to yield 9.43 g (88%) of brown solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.48 (d, *J* = 8.7 Hz, 2H), 6.61 (d, *J* = 8.7 Hz, 2H), 5.18 (bs, 1H), 3.55 (bs, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 150.9, 137.9, 114.4, 80.4. LCMS: m/z calcd. for $[C_6H_8IN_2]^+$: 235; found: 235 $[M+H]^+$.

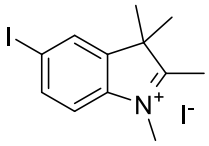
5-Iodo-2,3,3-trimethyl-3H-indole **(S6)**[4]



Compound **S6** was synthesized according to a literature procedure.

Hydrazine compound **S5** (7.0 g, 30.0 mmol, 1.0 equiv) and 3-methyl-2-butanone (3.7 mL, 36.0 mmol, 1.2 equiv) were dissolved in 200 mL acetic acid and refluxed under nitrogen for 3 hours. After the acetic acid was removed under vacuum, the residue was purified by flash chromatography on silica gel (hexane – ethyl acetate 0 to 30%) to yield 5.4 g (65%) of brown oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.61 (dd, *J* = 8.0, 1.7 Hz, 1H), 7.58 (d, *J* = 1.6 Hz, 1H), 7.28 (d, *J* = 8.0 Hz, 1H), 2.25 (s, 3H), 1.28 (s, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 188.4, 153.6, 148.3, 136.8, 130.8, 122.0, 90.0, 54.2, 23.1, 15.5. LCMS: m/z calcd. for $[C_{11}H_{13}N_1]^+$: 286; found: 286 $[M+H]^+$.

5-Iodo-1,2,3,3-tetramethyl-3H-indol-1-ium iodide **(S7)**[6]

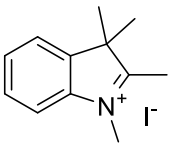


Compound **S7** was synthesized according to a literature procedure with modifications.

In a pressure tube indole **S6** (2.00 g, 7.0 mmol, 1.0 equiv) was dissolved in (1.3 mL, 21.0 mmol, 3.0 equiv) methyl iodide and reacted at 80 °C for 1.5 hours. After cooling to room temperature, ethyl acetate was added and the resulting precipitate was filtered, washed with cold ethyl acetate and dried to yield 2.15 g (72%) pale brown powder. The crude product was used without further purification. ^{1}H 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, 6H). ^{13}C NMR (126 MHz, DMSO-d6) δ 196.0, 143.7, 141.8, 137.4, 132.1, 117.0, 96.0, 54.0, 34.8, 21.4, 14.2. LCMS: m/z calcd. for $[C_{12}H_{15}IN]^+$: 300; found: 300 $[M]^+$.

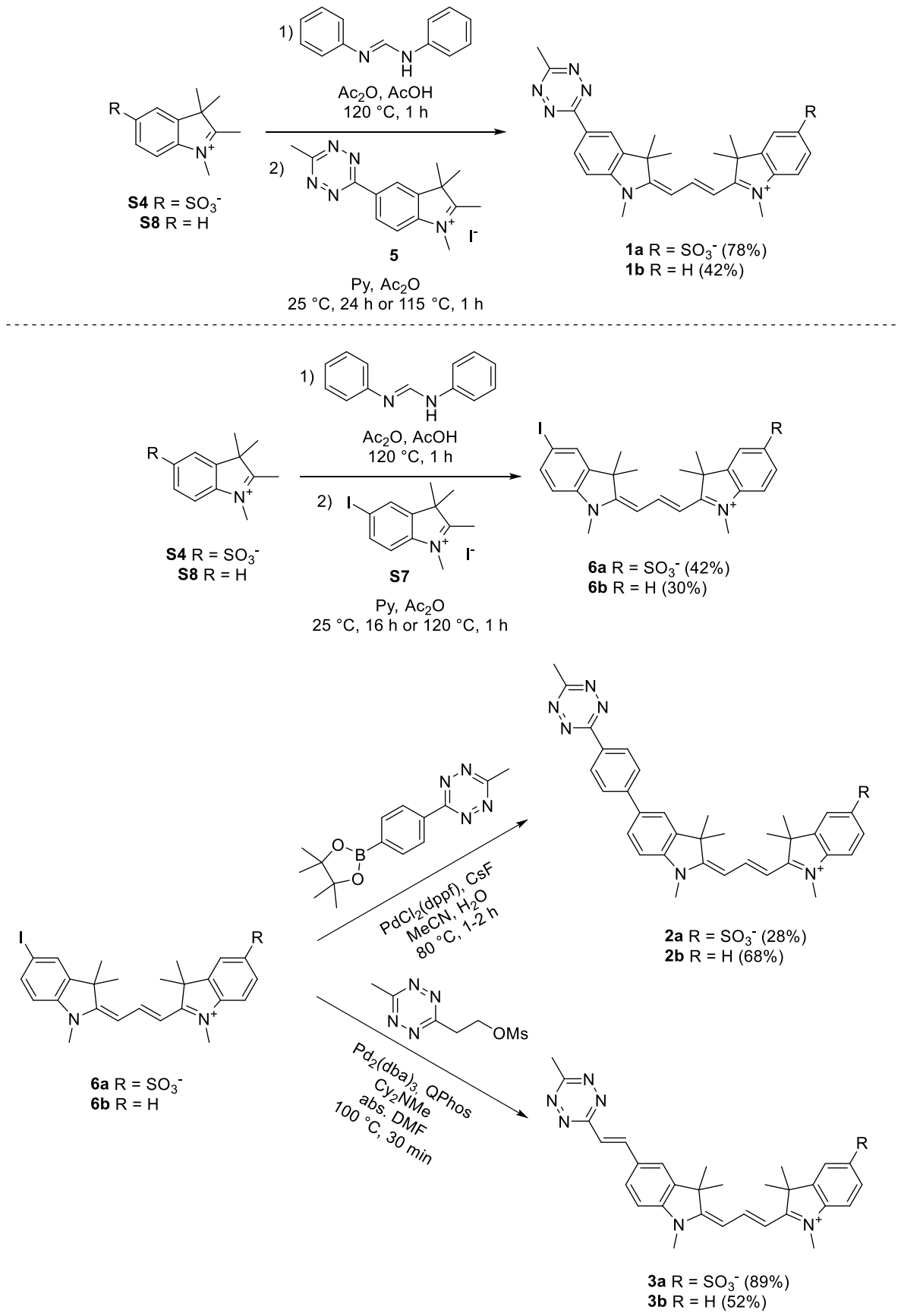
1,2,3,3-Tetramethyl-3H-indol-1-ium iodide **(S8)**[7]



Compound **S8** was synthesized according to a literature procedure with modifications.

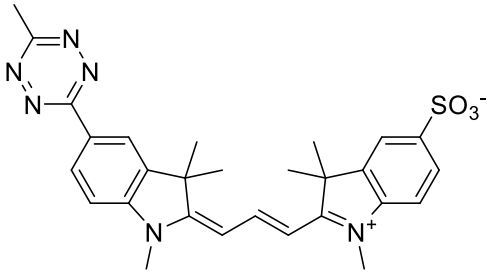
In a pressure tube 2,3,3-trimethyl-3*H*-indole (5.0 g, 31.4 mmol, 1.0 equiv) was dissolved in methyl iodide (5.86 mL, 94.2 mmol, 3.0 equiv). and reacted at 80°C for 2 hours. After cooling to room temperature, ethyl acetate was added and the resulting precipitate was filtered, washed with cold ethyl acetate and dried to yield 8.43 g (89%) beige powder. The crude product was used without further purification. ^{1}H NMR (500 MHz, DMSO-d6) δ 7.92 (dd, *J* = 5.9, 2.4 Hz, 1H), 7.83 (dd, *J* = 5.3, 2.8 Hz, 1H), 7.65 – 7.58 (m, 2H), 3.99 (s, 3H), 2.79 (s, 3H), 1.54 (s, 6H). ^{13}C NMR (126 MHz, DMSO-d6) 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 $[C_{12}H_{16}N]^+$: 174; found 174 $[M]^+$.

2.2. Synthesis of fluorogenic cyanines



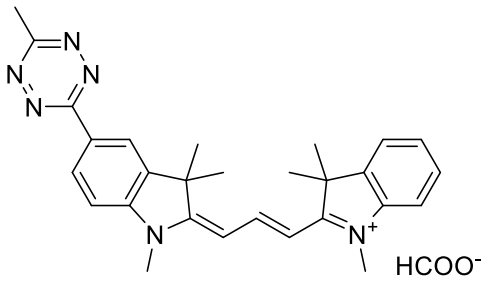
Scheme 2. Synthesis of fluorogenic cyanine **1a-b**, **2a-b** and **3a-b**.

1,3,3-Trimethyl-2-(3-(1,3,3-trimethyl-5-(6-methyl-1,2,4,5-tetrazin-3-yl)indolin-2-ylidene)prop-1-en-1-yl)-3H-indol-1-ium-5-sulfonate **(1a)**



Compound **S4** (48 mg, 0.190 mmol, 2.5 equiv) and *N*,*N*'-diphenylformamidine (60 mg, 0.304 mmol, 4.0 equiv) was reacted in a mixture of 500 µL acetic acid and 500 µL acetic anhydride at 120 °C for 1 hour. The reaction mixture was cooled to room temperature, 10 mL ethyl acetate was added, the precipitate was filtered and washed with ethyl acetate and dried. This intermediate hemi-cyanine was dissolved in 3 mL pyridine, Compound **5** (30 mg, 0.076 mmol, 1.0 equiv) and 500 µL acetic anhydride was added and stirred at 25 °C for 24 hours. Then 10 mL ethyl acetate was added, the precipitate was filtered and washed with ethyl acetate. The crude product was purified by flash chromatography on silica gel (dichloromethane-methanol 0 to 20%) to yield 31 mg (78%) of purple crystals. Before spectroscopic measurements the product was further purified by preparative HPLC (water–acetonitrile starting from 95 : 5 to 0 : 100). ^{1}H NMR (500 MHz, CD_3OD) δ 8.77 (d, *J* = 1.2 Hz, 1H), 8.75 (dd, *J* = 8.4, 1.6 Hz, 1H), 8.70 (t, *J* = 13.5 Hz, 1H), 8.05 (d, *J* = 1.4 Hz, 1H), 8.01 (dd, *J* = 8.3, 1.6 Hz, 2H), 7.65 (d, *J* = 8.4 Hz, 1H), 7.51 (d, *J* = 8.3 Hz, 1H), 6.62 (d, *J* = 13.6 Hz, 1H), 6.58 (d, *J* = 13.3 Hz, 1H), 3.82 (s, 3H), 3.82 (s, 3H), 3.13 (s, 3H), 1.94 (s, 6H), 1.89 (s, 6H). ^{13}C NMR (126 MHz, CD_3OD) δ 178.3, 176.9, 168.6, 165.0, 153.0, 145.2, 144.6, 143.1, 142.3, 130.6, 130.2, 130.2, 128.3, 122.6, 121.4, 112.8, 112.3, 105.5, 104.6, 51.0, 50.5, 32.2, 31.9, 28.3, 28.0, 21.1. HRMS: m/z calcd. for $[C_{28}H_{31}N_6O_3S]^+$: 531.2172; found: 531.2177 $[M+H]^+$.

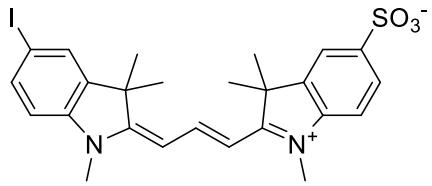
1,3,3-trimethyl-2-(3-(1,3,3-trimethyl-5-(6-methyl-1,2,4,5-tetrazin-3-yl)indolin-2-ylidene)prop-1-en-1-yl)-3H-indol-1-ium formate **(1b)**



Compound **S8** (48 mg, 0.158 mmol, 2.5 equiv) and *N*,*N*'-diphenylformamidine (50 mg, 0.253 mmol, 4.0 equiv) was reacted in a mixture of 500 µL acetic acid and 500 µL acetic anhydride at 120 °C for 1 hour. The reaction mixture was cooled to room temperature, 10 mL ethyl acetate was added, the precipitate was filtered and washed with ethyl acetate and dried. This intermediate hemi-cyanine was dissolved in 1 mL pyridine, compound **5** (25 mg, 0.0633 mmol, 1.0 equiv) and 100 µL acetic anhydride was added and stirred at 115 °C for 1 hour. After cooling to room temperature, the solvents were evaporated under vacuum. The residue was purified by flash chromatography on silica gel (dichloromethane-methanol 0 to 20%). Before spectroscopic measurements the product was further purified by preparative HPLC (water–acetonitrile containing 0.1% HCOOH starting from 95 : 5 to 0 : 100) to yield 13.2 mg (42%) of purple crystals. ^{1}H NMR (500 MHz, CD_3OD) δ 8.76 – 8.73 (m, 2H), 8.68 (t, *J* = 13.5 Hz, 1H), 7.66 (d, *J* = 7.4 Hz, 1H),

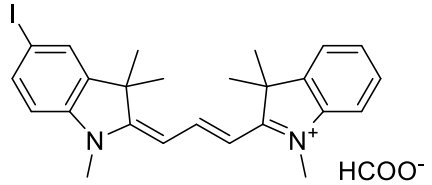
7.61 (d, *J* = 8.9 Hz, 1H), 7.56 (d, *J* = 7.9 Hz, 1H), 7.51 (d, *J* = 7.8 Hz, 1H), 7.45 (t, *J* = 7.4 Hz, 1H), 6.63 (d, *J* = 13.7 Hz, 1H), 6.52 (d, *J* = 13.2 Hz, 1H), 3.83 (s, 3H), 3.72 (s, 3H), 3.12 (s, 3H), 1.94 (s, 6H), 1.87 (s, 6H). ^{13}C NMR (126 MHz, CD_3OD) δ 178.2, 175.9, 168.6, 165.0, 152.5, 143.9, 142.9, 142.5, 130.2, 130.2, 130.1, 127.6, 123.5, 122.6, 112.9, 112.5, 105.5, 103.8, 73.7, 71.5, 62.2, 51.2, 32.2, 28.4, 28.0, 21.0. HRMS: m/z calcd. for $[C_{28}H_{31}N_6]^+$: 451.2610; found: 451.2612 $[M+H]^+$.

2-(3-(5-Iodo-1,3,3-trimethylindolin-2-ylidene)prop-1-en-1-yl)-1,3,3-trimethyl-3H-indol-1-ium-5-sulfonate **(6a)**



Compound **S4** (400 mg, 1.58 mmol, 2.0 equiv) and *N*,*N*'-diphenylformamidine (620 mg, 3.16 mmol, 4.0 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, 50 mL ethyl acetate was added, the precipitate was filtered and washed with ethyl acetate and dried. This intermediate hemi-cyanine was dissolved in 10 mL pyridine, compound **S7** (258 mg, 0.79 mmol, 1.0 equiv) and 1 mL acetic anhydride was added and stirred at 25 °C for 16 hours. Then 50 mL ethyl acetate was added, the precipitate was filtered and washed with ethyl acetate. The crude product was purified by flash chromatography on silica gel (dichloromethane-methanol 0 to 20%) to yield 187 mg (42%) of purple crystals. ^{1}H NMR (500 MHz, DMSO-d6) δ 8.32 (t, *J* = 13.5 Hz, 1H), 8.01 (d, *J* = 1.6 Hz, 1H), 7.81 (d, *J* = 1.4 Hz, 1H), 7.77 (dd, *J* = 8.3, 1.6 Hz, 1H), 7.69 (dd, *J* = 8.2, 1.5 Hz, 1H), 7.41 (d, *J* = 8.3 Hz, 1H), 7.27 (d, *J* = 8.4 Hz, 1H), 3.66 (s, 3H), 3.60 (s, 3H), 1.69 (s, 6H), 1.68 (s, 6H). ^{13}C NMR (126 MHz, DMSO-d6) δ 175.1, 173.4, 149.5, 146.0, 142.8, 142.6, 142.5, 140.0, 137.0, 131.0, 126.1, 119.7, 113.5, 110.7, 103.5, 102.6, 89.2, 49.0, 48.7, 31.6, 31.3, 27.1. HRMS: m/z calcd. for $[C_{25}H_{28}IN_2O_3S]^+$: 563.0859; found: 563.0845 $[M+H]^+$.

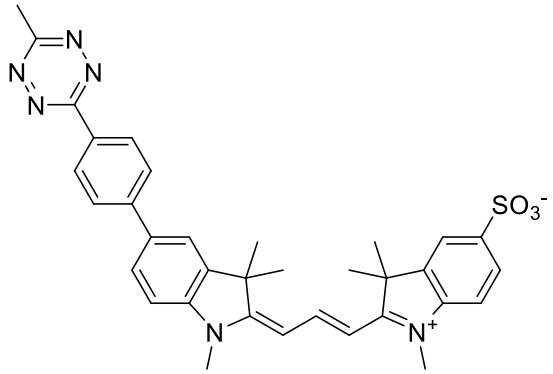
2-(3-(5-iodo-1,3,3-trimethylindolin-2-ylidene)prop-1-en-1-yl)-1,3,3-trimethyl-3H-indol-1-ium formate **(6b)**[8]



Compound **S8** (700 mg, 2.33 mmol, 1.55 equiv) and *N*,*N*'-diphenylformamidine (913 mg, 4.65 mmol, 3.1 equiv) was reacted in a mixture of 3 mL acetic acid and 3 mL acetic anhydride at 120 °C for 1 hour. The reaction mixture was cooled to room temperature, 50 mL ethyl acetate was added, the precipitate was filtered and washed with ethyl acetate and dried. This intermediate hemi-cyanine was dissolved in 10 mL pyridine, compound **S7** (640 mg, 1.50 mmol, 1.0 equiv) and 1.5 mL acetic anhydride was added and stirred at 120 °C for 1 hour. Then the solvents were evaporated under vacuum. The residue was purified by flash chromatography on silica gel (dichloromethane-methanol 0 to 2%) to yield 279 mg (30%) of purple crystals. Before NMR measurements the product was further

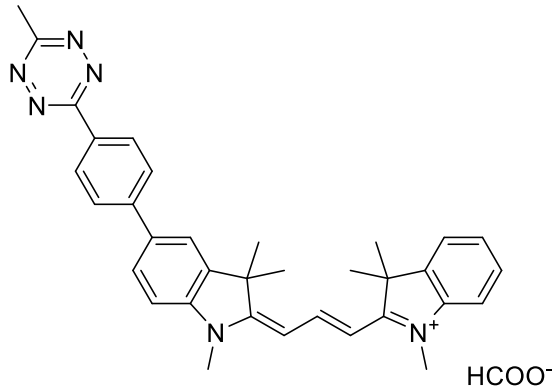
purified by preparative HPLC (water–acetonitrile containing 0.1% HCOOH starting from 95 : 5 to 0 : 100). ^{1}H NMR (500 MHz, DMSO-d6) δ 8.31 (t, *J* = 13.5 Hz, 1H), 8.15 (s, 1H), 8.03 (d, *J* = 1.4 Hz, 1H), 7.78 (dd, *J* = 8.3, 1.5 Hz, 1H), 7.65 (d, *J* = 7.4 Hz, 1H), 7.50 (d, *J* = 7.8 Hz, 1H), 7.46 (t, *J* = 7.6 Hz, 1H), 7.32 (t, *J* = 7.3 Hz, 1H), 7.27 (d, *J* = 8.4 Hz, 1H), 6.49 (d, *J* = 13.6 Hz, 1H), 6.40 (d, *J* = 13.4 Hz, 1H), 3.67 (s, 3H), 3.59 (s, 3H), 1.68 (s, 6H), 1.67 (s, 6H). ^{13}C NMR (126 MHz, DMSO-d6) δ 174.9, 173.2, 163.0, 149.5, 142.8, 142.6, 142.6, 140.7, 137.0, 131.0, 128.6, 125.5, 122.4, 113.4, 111.7, 103.4, 102.4, 89.1, 49.0, 48.7, 31.5, 31.3, 27.2, 27.1. HRMS: m/z calcd. for $[C_{25}H_{28}IN_2]^+$: 483.1297; found: 483.1288 $[M]^+$.

1,3,3-Trimethyl-2-(3-(1,3,3-trimethyl-5-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)indolin-2-ylidene)prop-1-en-1-yl)-3H-indol-1-ium-5-sulfonate **(2a)**



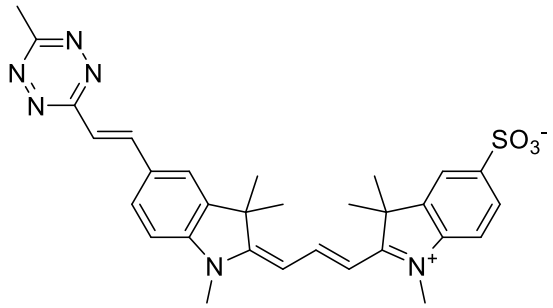
A mixture of cyanine **6a** (30 mg, 0.053 mmol, 1.0 equiv), 3-methyl-6-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)-1,2,4,5-tetrazine[9,10] (32 mg, 0.107 mmol, 2.0 equiv), $Pd(dppf)Cl_2$ (4 mg, 0.0055 mmol, 0.1 equiv) and CsF (80 mg, 0.5267 mmol, 10.0 equiv) was suspended in 1000 µL acetonitrile and 100 µL H_2O. The reaction mixture was stirred at 80 °C for 2 hours. The solvent was evaporated, and the crude product was purified by flash chromatography on silica gel (dichloromethane – methanol 0 to 20%) to yield 9 mg (28%) of purple crystals. Before spectroscopic measurements the product was further purified by preparative HPLC (water–acetonitrile starting from 95 : 5 to 0 : 100). ^{1}H NMR (500 MHz, DMSO-d6) δ 8.58 (d, *J* = 8.4 Hz, 2H), 8.38 (t, *J* = 13.5 Hz, 1H), 8.14 (s, 1H), 8.08 (d, *J* = 8.4 Hz, 2H), 7.92 (d, *J* = 8.2 Hz, 1H), 7.82 (s, 1H), 7.70 (d, *J* = 8.2 Hz, 1H), 7.59 (d, *J* = 8.4 Hz, 1H), 7.41 (d, *J* = 8.2 Hz, 1H), 6.48 (d, *J* = 13.5 Hz, 1H), 3.70 (s, *J* = 14.8 Hz, 3H), 3.67 (s, 3H), 3.02 (s, 3H), 1.78 (s, 6H), 1.72 (s, 6H). ^{13}C NMR (126 MHz, DMSO-d6) δ 174.9, 174.2, 171.1, 167.0, 163.1, 149.4, 146.0, 144.8, 143.2, 142.9, 142.5, 141.5, 139.9, 135.8, 130.7, 127.9, 127.5, 127.3, 126.1, 121.0, 119.8, 111.9, 110.6, 103.3, 103.1, 48.9, 31.6, 27.3, 27.2, 20.8. HRMS: m/z calcd. for $[C_{34}H_{35}N_6O_3S]^+$: 607.2485; found: 607.2498 $[M+H]^+$.

1,3,3-trimethyl-2-(3-(1,3,3-trimethyl-5-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)indolin-2-ylidene)prop-1-en-1-yl)-3H-indol-1-ium formate **(2b)**



A mixture of cyanine **6b** (50 mg, 0.0819 mmol, 1.0 equiv), 3-methyl-6-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)-1,2,4,5-tetrazine[9,10] (49 mg, 0.1638 mmol, 2.0 equiv), $Pd(dppf)Cl_2$ (6 mg, 0.0082 mmol, 0.1 equiv) and CsF (62 mg, 0.4095 mmol, 5.0 equiv) was suspended in 2000 µL acetonitrile and 200 µL H_2O. The reaction mixture was stirred at 80 °C for 1 hours. The solvent was evaporated, and the crude product was purified by flash chromatography on silica gel (dichloromethane – methanol 0 to 20%). Before spectroscopic measurements the product was further purified by preparative HPLC (water–acetonitrile containing 0.1% HCOOH starting from 95 : 5 to 0 : 100) to yield 32 mg (68%) of purple crystals. ^{1}H NMR (500 MHz, DMSO-d6) δ 8.58 (d, *J* = 8.5 Hz, 2H), 8.37 (t, *J* = 13.5 Hz, 1H), 8.14 (d, *J* = 1.6 Hz, 1H), 8.08 (d, *J* = 8.5 Hz, 2H), 7.92 (dd, *J* = 8.4, 1.7 Hz, 1H), 7.65 (d, *J* = 7.3 Hz, 1H), 7.59 (d, *J* = 8.4 Hz, 1H), 7.52 – 7.43 (m, *J* = 8.1 Hz, 2H), 7.32 (td, *J* = 7.4, 1.5 Hz, 1H), 6.48 (apparent t containing: 6.50 (d, *J* = 13.7 Hz, 1H), 6.47 (d, *J* = 13.6 Hz, 1H)), 3.69 (s, 3H), 3.68 (s, 3H), 3.02 (s, 3H), 1.78 (s, 6H), 1.71 (s, 6H). ^{13}C NMR (126 MHz, DMSO-d6) δ 174.7, 174.1, 167.0, 163.0, 149.4, 143.2, 143.0, 142.6, 141.4, 140.6, 135.7, 130.7, 128.5, 127.9, 127.5, 127.3, 125.3, 122.4, 121.0, 111.8, 111.6, 103.2, 102.9, 49.0, 48.8, 31.5, 27.3, 27.2, 20.8.
HRMS: m/z calcd. for $[C_{34}H_{35}N_6]^+$: 527.2923; found: 527.2922$[M+H]^+$.

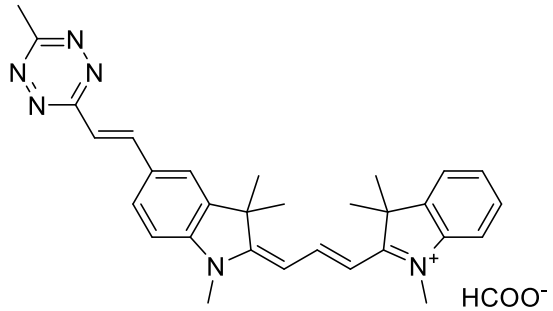
1,3,3-Trimethyl-2-(3-(1,3,3-trimethyl-5-((E)-2-(6-methyl-1,2,4,5-tetrazin-3-yl)vinyl)indolin-2-ylidene)prop-1-en-1-yl)-3H-indol-1-ium-5-sulfonate **(3a)**



A mixture of cyanine **6a** (30 mg, 0.053 mmol, 1.0 equiv), 2-(6-methyl-1,2,4,5-tetrazin-3-yl)ethyl methanesulfonate[11] (35 mg, 0.162 mmol, 3.0 equiv), $Pd_2(dba)_3$ (2.4 mg, 0.0026 mmol, 0.05 equiv), QPhos (1.9 mg, 0.0026 mmol, 0.05 equiv), *N*,*N*-dicyclohexylmethylamine (35 µL, 0.162 mmol, 3.0 equiv) and 2 mL anhydrous dimethylformamide was purged with N_2 for 15 minutes. The reaction mixture was stirred at 100 °C for 30 minutes. The solvent was evaporated in vacuo and the crude product was purified by flash chromatography on silica gel (dichloromethane-methanol 0 to 20%) to yield 26 mg (89%) of purple crystals. Before spectroscopic measurements the product was further purified by preparative HPLC (water–acetonitrile starting from 95 : 5 to 0 : 100). ^{1}H NMR (500 MHz, DMSO-d6) δ 8.37 (t, *J* = 13.5 Hz, 1H), 8.31 – 8.27 (m, 2H), 7.91 (dd, *J* = 8.2, 0.8 Hz, 1H), 7.83 (d, *J* = 1.0 Hz, 1H), 7.72 (d, *J* = 16.2 Hz, 1H), 7.70 (dd, *J* = 8.1,

1.3 Hz, 1H), 7.51 (d, *J* = 8.3 Hz, 1H), 7.43 (d, *J* = 8.3 Hz, 1H), 6.51 (d, *J* = 13.6 Hz, 1H), 6.46 (d, *J* = 13.3 Hz, 1H), 3.69 (s, 3H), 3.66 (s, 3H), 2.96 (s, 3H), 1.76 (s, 6H), 1.72 (s, 6H). ^{13}C NMR (126 MHz, DMSO-d6) δ 175.4, 173.8, 170.8, 165.9, 164.3, 149.5, 146.2, 144.2, 142.4, 141.3, 140.1, 139.2, 132.0, 130.2, 126.1, 121.4, 120.1, 119.8, 111.5, 110.8, 103.9, 103.2, 49.1, 48.5, 31.7, 27.3, 27.1, 20.8. HRMS: m/z calcd. for $[C_{30}H_{33}N_6O_3S]^+$: 557.2329; found: 557.2335 $[M+H]^+$.

1,3,3-trimethyl-2-(3-(1,3,3-trimethyl-5-((E)-2-(6-methyl-1,2,4,5-tetrazin-3-yl)vinyl)indolin-2-ylidene)prop-1-en-1-yl)-3H-indol-1-ium formate **(3b)**



A mixture of cyanine **6b** (30 mg, 0.0492 mmol, 1.0 equiv), 2-(6-methyl-1,2,4,5-tetrazin-3-yl)ethyl methanesulfonate[11] (32 mg, 0.1475 mmol, 3.0 equiv), $Pd_2(dba)_3$ (2.3 mg, 0.0025 mmol, 0.05 equiv), QPhos (2.0 mg, 0.0025 mmol, 0.05 equiv), *N*,*N*-dicyclohexylmethylamine (32 µL, 0.1475 mmol, 3.0 equiv) and 1 mL anhydrous dimethylformamide was purged with N_2 for 15 minutes. The reaction mixture was stirred at 100 °C for 30 minutes. The solvent was evaporated in vacuo and the crude product was purified by flash chromatography on silica gel (dichloromethane-methanol 0 to 15%). Before spectroscopic measurements the product was further purified by preparative HPLC (water–acetonitrile starting from 95 : 5 to 0 : 100) to yield 13.4 mg (52%) of purple crystals. ^{1}H NMR (500 MHz, CD_3OD) δ 8.54 (t, *J* = 13.5 Hz, 1H), 8.31 (d, *J* = 16.2 Hz, 1H), 8.00 (s, 1H), 7.79 (d, *J* = 7.9 Hz, 1H), 7.60 – 7.52 (m containing: 7.57 (d, *J* = 16.1 Hz, 1H), 7.54 (d, *J* = 7.0 Hz, 1H)), 7.44 (d, *J* = 7.5 Hz, 1H), 7.38 (d, *J* = 3.6 Hz, 1H), 7.36 (d, *J* = 4.1 Hz, 1H), 7.32 (t, *J* = 7.4 Hz, 1H), 6.47 (d, *J* = 13.6 Hz, 1H), 6.39 (d, *J* = 13.3 Hz, 1H), 3.69 (s, 3H), 3.65 (s, 3H), 2.96 (s, 3H), 1.80 (s, 6H), 1.75 (s, 6H). ^{13}C NMR (126 MHz, CD_3OD) δ 177.5, 176.0, 167.7, 166.1, 159.7, 152.2, 145.7, 144.0, 142.9, 142.3, 140.9, 134.2, 131.1, 130.0, 127.2, 123.5, 122.5, 121.5, 112.7, 112.4, 104.9, 103.9, 50.9, 50.2, 32.0, 28.3, 28.0, 21.1. HRMS: m/z calcd. for $[C_{30}H_{33}N_6]^+$: 477.2767; found: 477.2762 $[M+H]^+$.

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 (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 in DMSO) 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 μM concentration of the compounds while absorbance spectra were recorded using 5 μM concentration (DMSO content was kept under 1% in each case). The excitation and emission wavelength are given for each spectrum. Quantum yields were determined using Rhodamine B standard.

Table S1 – Main photophysical data of cyanine dyes and their BCN conjugates. [a]

	$\lambda_{abs,max}$ (nm)	$\lambda_{em,max}$ (nm)	ε[b] ($M^{-1}cm^{-1}$)	Φ[c] (%)	Bx10^4 (εxϕ)	Φ_{BCN}/Φ_{Tet}	I_{BCN}/I_{Tet}[d]	B_{BCN}/B_{Tet}
1a	565	578	115171	1.63	18.8	13.5	21.0	12.4
1a.BCN	558	571	105965	21.92	232.3			
1b	561	575	81559	1.13	9.2	13.4	19.3	12.8
1b.BCN	555	570	77844	15.18	118.2			
2a	568	586	140493	3.19	44.8	4.3	4.3	4.0
2a.BCN	567	586	132191	13.56	179.3			
2b	567	587	132180	2.94	38.9	5.5	5.8	5.3
2b.BCN	565	585	128072	16.22	207.7			
3a	578	593	162848	0.97	15.8	10.5	11.9	9.0
3a.BCN	575	596	139938	10.19	142.6			
3b	577	584	114960	2.91	33.5	4.7	6.1	4.4
3b.BCN	572	592	107227	13.80	148.0			

[a] in all sodium PBS (pH=7.4, containing 0.1% SDS); [b] determined at $\lambda_{abs,max}$; [c] Relative to rhodamine B (ϕ=0.65); [d] determined at $\lambda_{em,max}$ of the BCN conjugate.

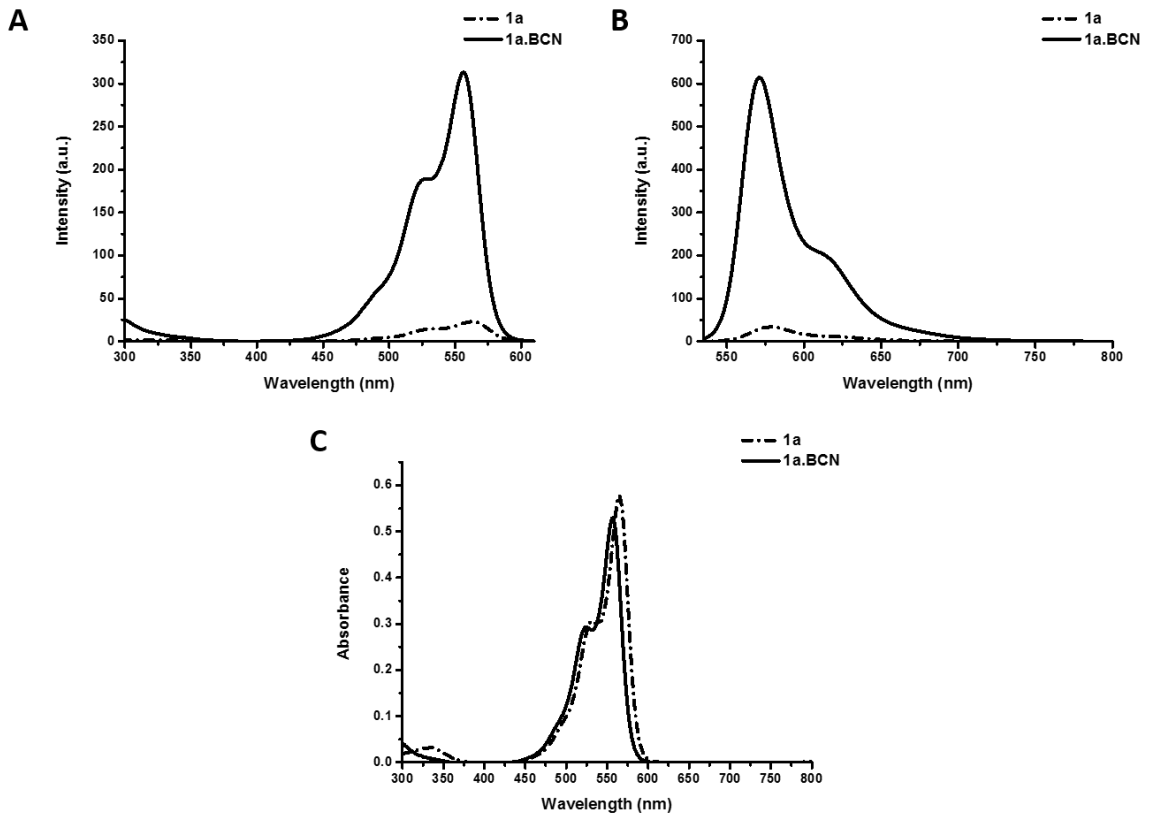


Figure S1. A) Excitation spectra (λ_{em}: 620 nm), B) emission spectra (λ_{exc}: 520 nm) and C) absorbance spectra of fluorogenic cyanine **1a** and **1a.BCN**.

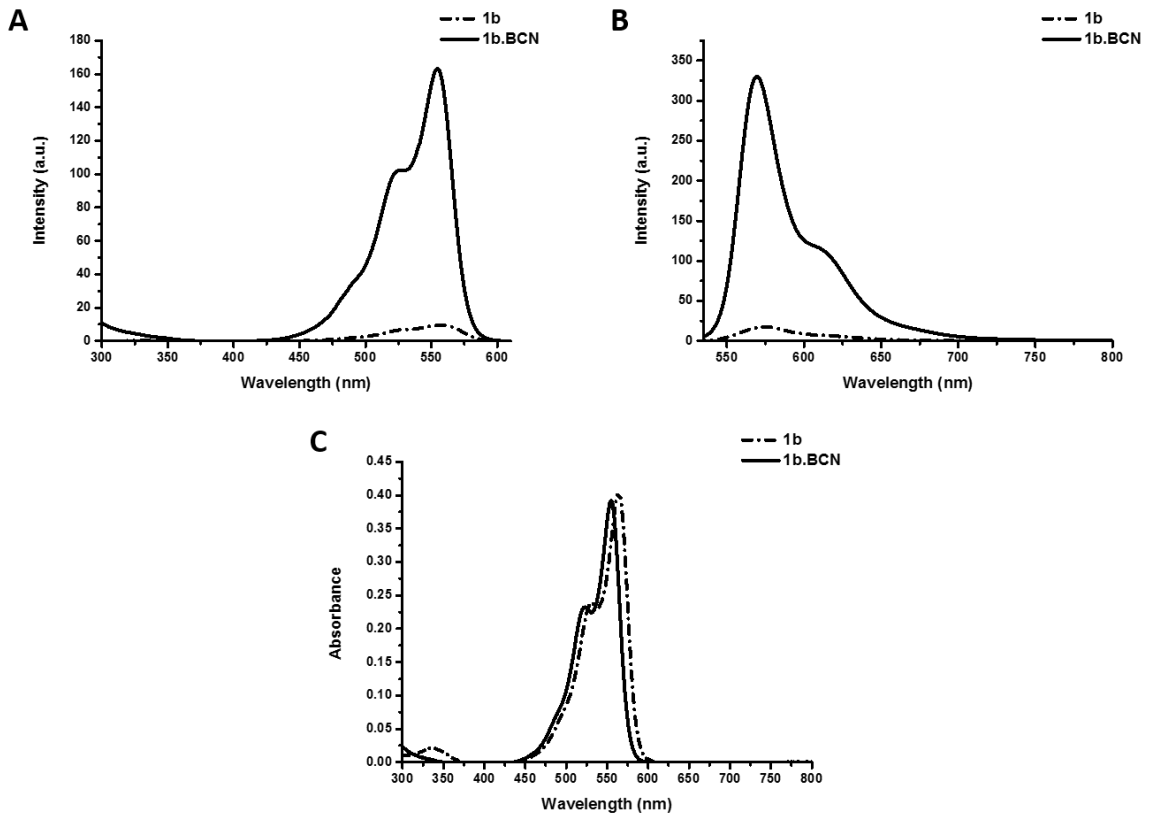


Figure S2. A) Excitation spectra (λ_{em}: 620 nm), B) emission spectra (λ_{exc}: 520 nm) and C) absorbance spectra of fluorogenic cyanine **1b** and **1b.BCN**.

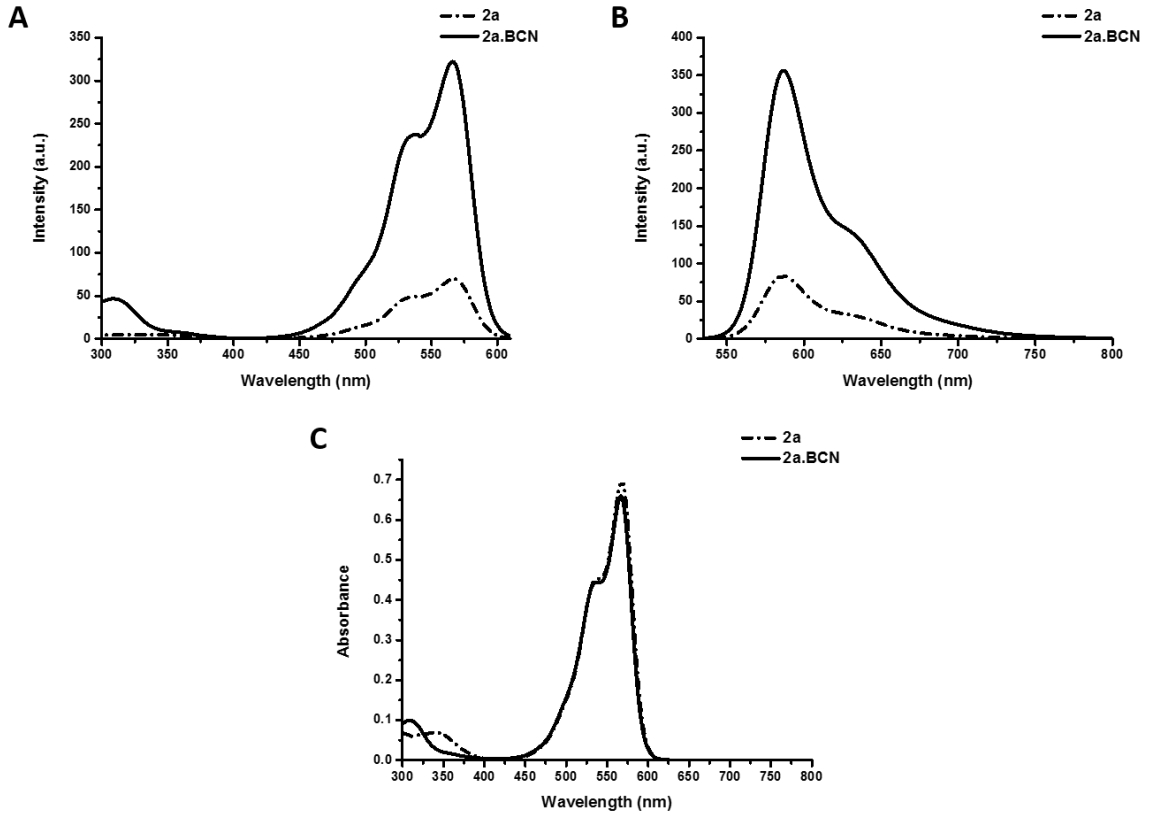


Figure S3. A) Excitation spectra (λ_{em}: 620 nm), B) emission spectra (λ_{exc}: 520 nm) and C) absorbance spectra of fluorogenic cyanine **2a** and **2a.BCN**.

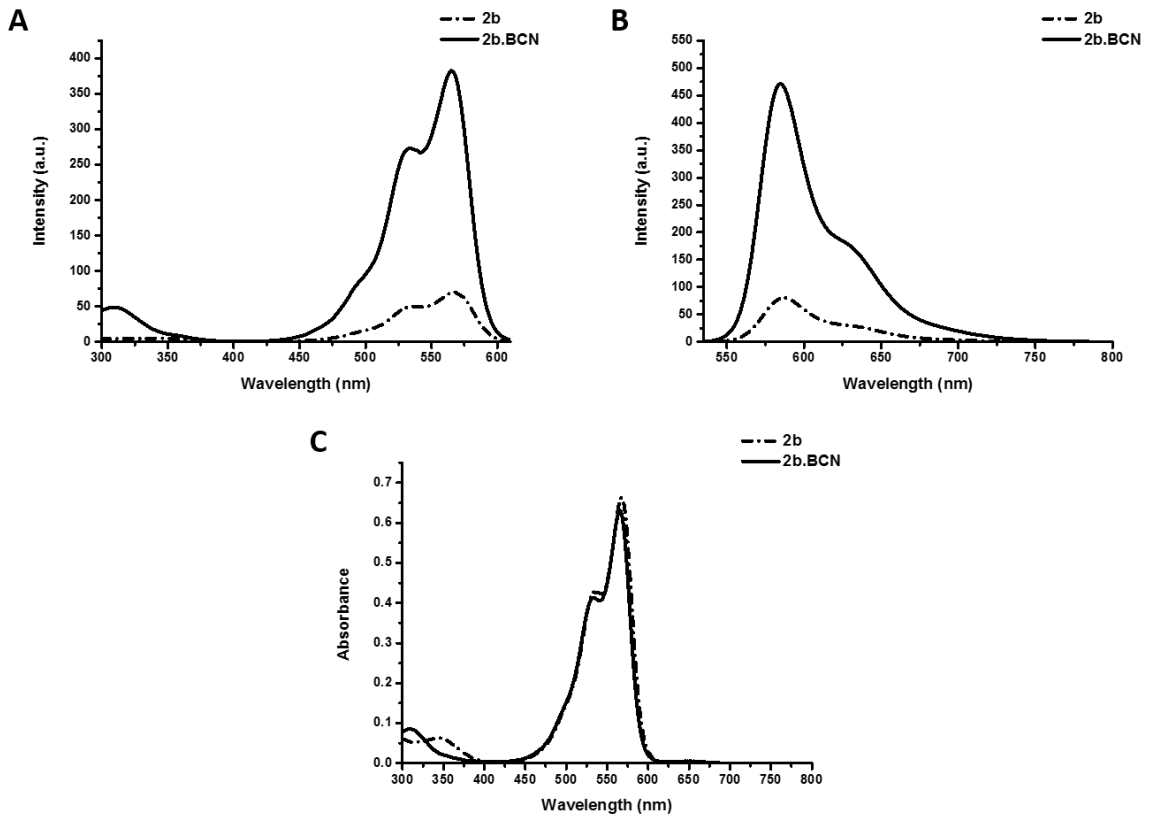


Figure S4. A) Excitation spectra (λ_{em}: 620 nm), B) emission spectra (λ_{exc}: 520 nm) and C) absorbance spectra of fluorogenic cyanine **2b** and **2b.BCN**.

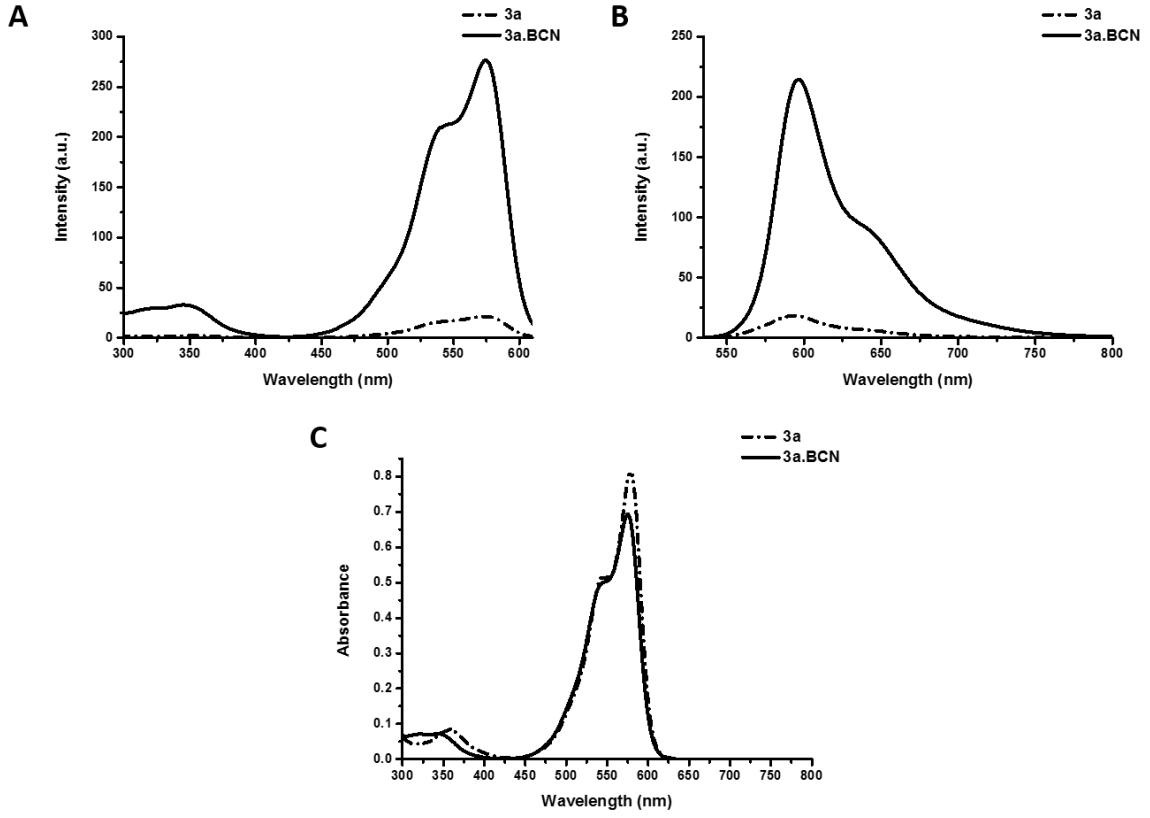


Figure S5. A) Excitation spectra (λ_{em}: 620 nm), B) emission spectra (λ_{exc}: 520 nm) and C) absorbance spectra of fluorogenic cyanine **3a** and **3a.BCN**.

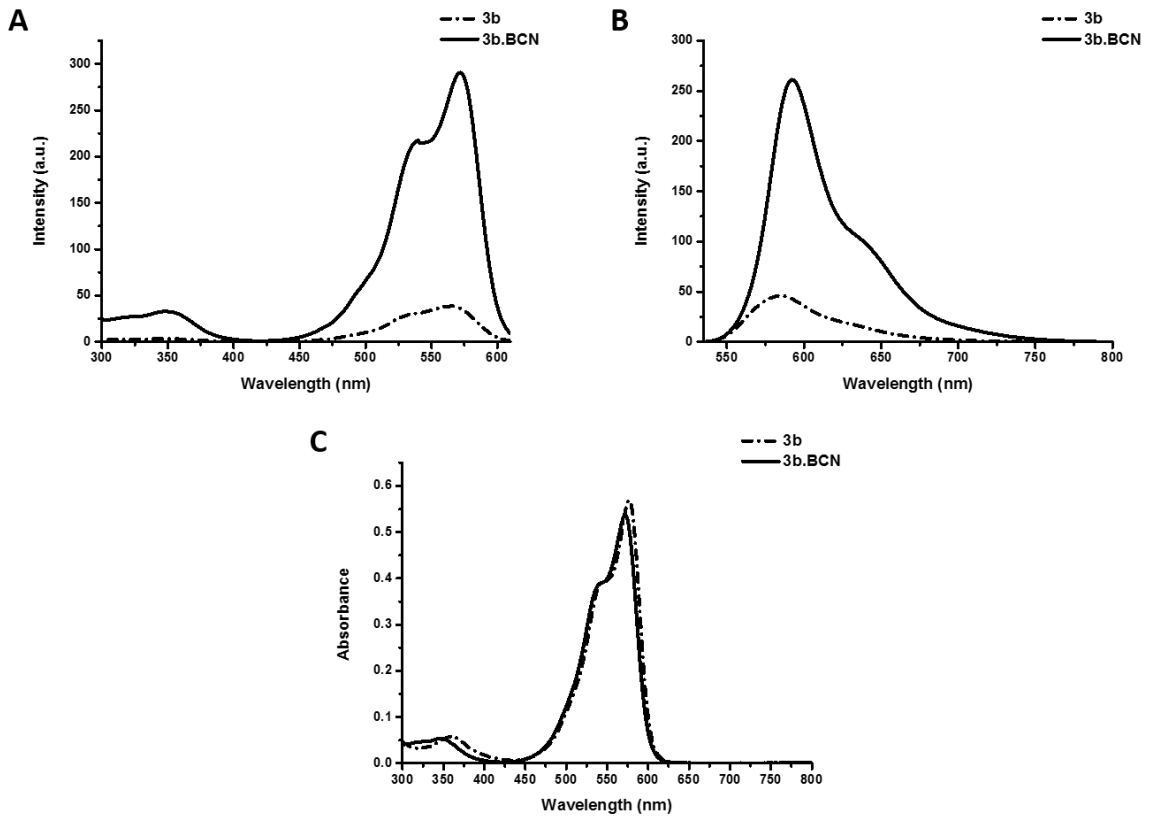


Figure S6. A) Excitation spectra (λ_{em}: 620 nm), B) emission spectra (λ_{exc}: 520 nm) and C) absorbance spectra of fluorogenic cyanine **3b** and **3b.BCN**.

4. Protein labelling

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) supplemented with 10% FBS (Gibco 10500-064), 1% penicillin-streptomycin (Gibco 15140-122), 1% sodium pyruvate (Life Technologies, Gibco 11360-070) and 1% Glutamax (Gibco 35050-061). The cells were cultured at 37°C in a 5% CO_2 atmosphere and passaged - using trypsin (0.05%; Gibco 25300-054) - every 3–4 days up to 20 passages.

4.2. Bioorthogonal labelling of live cells

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% CO_2 atmosphere. In the case of HEK293T cells Ibidi plates were pre-treated 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 μg Lamin-HaloTag[12] plasmid using JetPrime (Polyplus 114-07) transfection agent for four hours according to the manufacturer's protocol. Similar procedure was carried out for ncAA installation. Cells were incubated with 0.25 μg $IR^{K676TAG}$-GFP [obtained from EMBL within Material Transfer Agreement[13] or 0.25 μg $Vimentin^{N116TAG}$-GFP[14] in combination with 0.25 μg $tRNA^{Pyl}$/NES-$PylRS^{AF}$ plasmid (obtained from EMBL within Material Transfer Agreement)[15] in the presence of 250 μM 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 labelled with the fluorescent dyes **1a, 1b, 3a and 3b** at a concentration of 1-3 μM (in complete DMEM (Gibco 21063-029) for 90 min at 37°C in the dark. In case of HaloTag fusion protein Halo-BCN substrate (3 μM; 60 min)[12] was added to the cells before the fluorescent labelling step. Afterwards, a two-hour washing step – with complete DMEM – was interpolated followed by fixation (4% PFA for 10 min at 25°C) and quick washing – twice – with PBS prior to imaging.

5. Photobleaching studies

To test the photostability of the probes **1a**, **1b**, **3a** and **3b** HEK293T cells were transfected with $IR^{676BCN\text{-}Lys}$-GFP plasmid and cells were labelled with **1a** or **3a** or with Lamin-HaloTag plasmid and labelled consecutively in two steps first with Halo-BCN (3 μM, 60 min) and then **1b, 3b** (3 μM, 90 min) and afterwards washed and fixed (for details cf. part *SI 4.2*). Photostability characteristics were assessed with a Zeiss LSM 710 microscope, applying a Zeiss PlanApo 40x (NA 1.4) oil immersion objective using the 543 nm laser for excitation. All imaging parameters (the investigated area 212.5x212.5 μm; magnification 40x, the pixel number 1024x1024; and dwell time 1.85 μsec/pixel etc.) were kept constant. The detection wavelength range was set to 560 nm – 800 nm. Fluorescence intensity changes were acquired for 160 cycles (frames) (40 minutes) and averages of relative fluorescence intensities (in the percentage of the initial values) of 6-13 regions of interest were calculated.

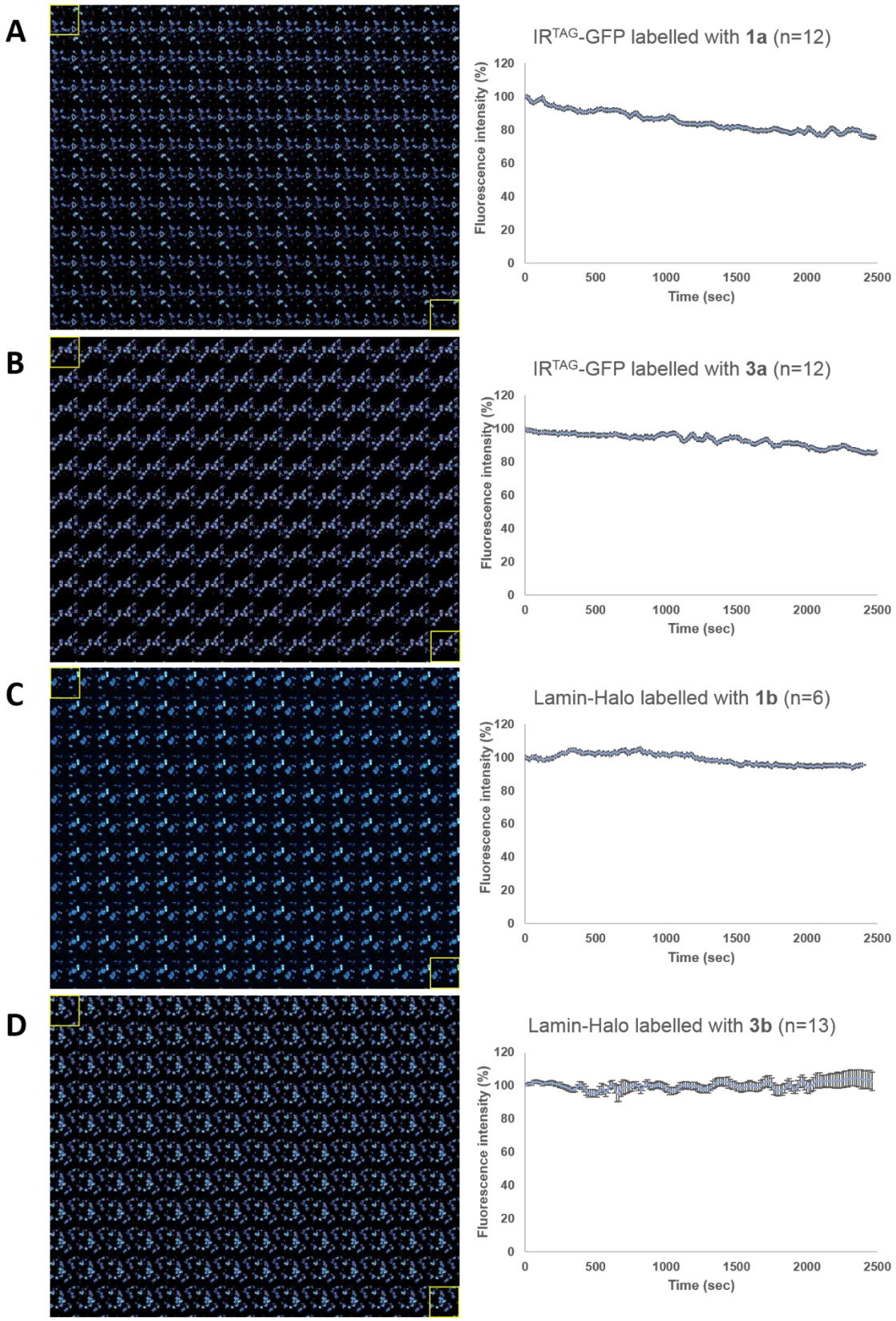


Figure S7. - Photostability of dyes **1a**, **1b, 3a** and **3b.** 160 cycles of confocal microscopy images of $IR^{676BCNK}$-GFP expressing HEK293T cells treated with **1a** and **3a** (A, B) or Lamin-HaloTag expressing HEK293T cells treated with Halo-BCN (3 μM) in combination with probes **1b** and **3b** (3 μM) (C, D) were acquired. Fluorescence intensity changes (using 534 nm for excitation and 555-800 nm for emission detection window) of regions of interest (n=12; n=12; n=6; n=13 for **1a, 3a, 1b, 3b**, respectively) were evaluated. Left panel represented the consecutive images 1-160 (initial and terminal images highlighted with a yellow box); right panel shows the average fluorescent intensity of ROIs.

6. Effect of probes on cell viability

A viability test was carried out to assess the toxicity of **1a, 1b, 3a** and **3b** probes on HEK-293T cells. Cells (30,000 cells/well) were transferred into a 48-well plate (Thermo Fisher Scientific, 130187) - coated for 4h with 0.01 mg/ml Poly-L-lysine (Sigma P5899) - and were incubated for 48 h at 37 °C in a 5% CO_2 atmosphere. Cells were treated with dyes in the concentration of 0.3, 1 and 3 µM for 90 minutes at 37 °C in the dark, according to the applied incubation concentrations and periods for sample treatment for microscopy analyses. This step was followed by a 2 h incubation period (conciliated with the duration of washing step of live cell labelling) with 0.5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; (Sigma-Aldrich, M5655) solution (in complete DMEM) at 37 °C in 5% CO_2 atmosphere 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 3.11 (Biotek Winooski, VT, USA). Linearity of the viability test was validated with the cell number dependence of MTT absorbance (*Figure S8*). Viability was expressed as percentage (n=3) of the readings of untreated control cells. For comparison we treated the cells with the cytotoxic SN-38 irinotecan analogue topoisomerase inhibitor drug (100 µM) (Fluorochem, 046379) (*Figure S9*).

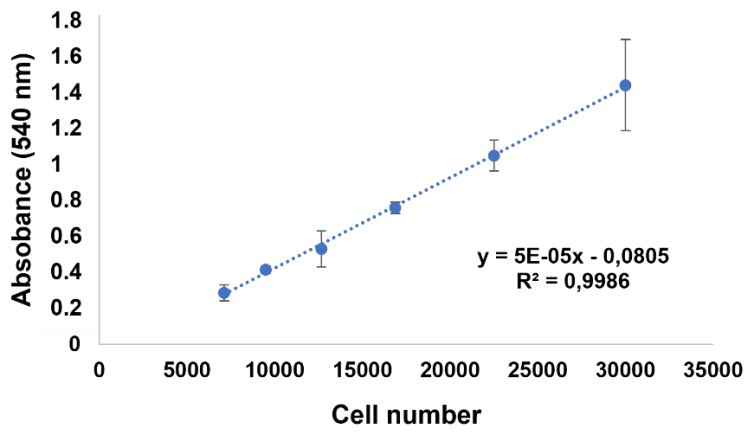


Figure S8. – Linearity of viability test. Absorbance of MTT (540 nm) at the function of cell number (n=3).

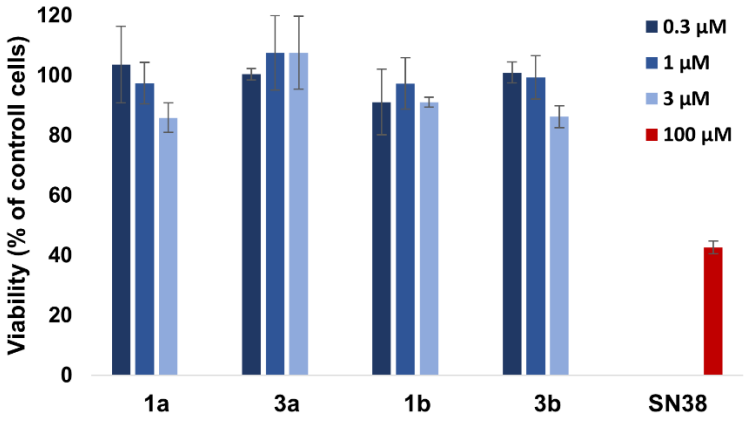


Figure S9. – Viability of HEK293T cells upon treatment of dyes **1a-b** and **3a-b** (0.3 – 3 µM) in the percentage of untreated controls and comparing to cytotoxic SN38 (100 µM) (n=3).

7. Confocal and STED imaging and analysis

Confocal and STED images were acquired on a Leica TCS SP8 STED 3X microscope using the 488 nm for excitation of the reporter fluorescent protein and 552 nm laser for the probes and 660 nm STED (1.5 W, continuous wave) laser for depletion. The images were taken using a Leica HC PL APO 100x/1.40 oil immersion objective using the Leica HyD detector. Spectral detection parameters were: exc.: 488 nm/ em. range: 500-550 nm for GFP; exc.: 552 nm/ em. range: 565-650 nm for compounds **1a, 1b, 3a, 3b**.

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 analysed using Leica Application Suite X and Fiji for ImageJ software (NIH). 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.

8. Quantum mechanical calculations

Quantum mechanical calculations were performed with Gaussian16W. The ground state structures were optimized with density functional theory (DFT), using the B3LYP functional and 6-31+G(d) basis set with polarizable continuum model (PCM) in water as solvent. HOMO and LUMO levels, as well as oscillator strength were calculated with the optimized ground state geometry using time dependent (TD) DFT calculations on the B3LYP/6-31+G(d) level.

Table S2 – TD-DFT Calculated data for the first three excited states for molecules **1a**, **1b**, **3a**, **3b** (B3LYP/6-31+G(d))

	Transition	E (eV)	*f*	CI expansion coefficient
1a	HOMO-1→LUMO	2.1773	0.0046	0.70428
	HOMO→LUMO	2.2996	0.0174	0.70018
	HOMO→LUMO+1	2.4993	1.9932	0.70176
1b	HOMO-1→LUMO	2.1769	0.0046	0.70587
	HOMO→LUMO	2.2758	0.0123	0.70094
	HOMO→LUMO+1	2.5179	1.8856	0.70231
3a	HOMO-2→LUMO	2.2074	0.0052	0.64732
	HOMO-2→LUMO+1			-0.28683
	HOMO-1→LUMO	2.3021	0.2043	-0.11004
	HOMO→LUMO			0.68829
	HOMO→LUMO+1			-0.10251
	HOMO→LUMO	2.3980	2.0710	0.10721
	HOMO→LUMO+1			0.69368
3b	HOMO-2→LUMO	2.2066	0.0051	0.65069
	HOMO-2→LUMO+1			-0.27915
	HOMO-1→LUMO	2.2559	0.1141	-0.10443
	HOMO→LUMO			0.68230
	HOMO→LUMO+1			-0.14332
	HOMO→LUMO	2.3804	1.8536	0.14637
	HOMO→LUMO+1			0.68604

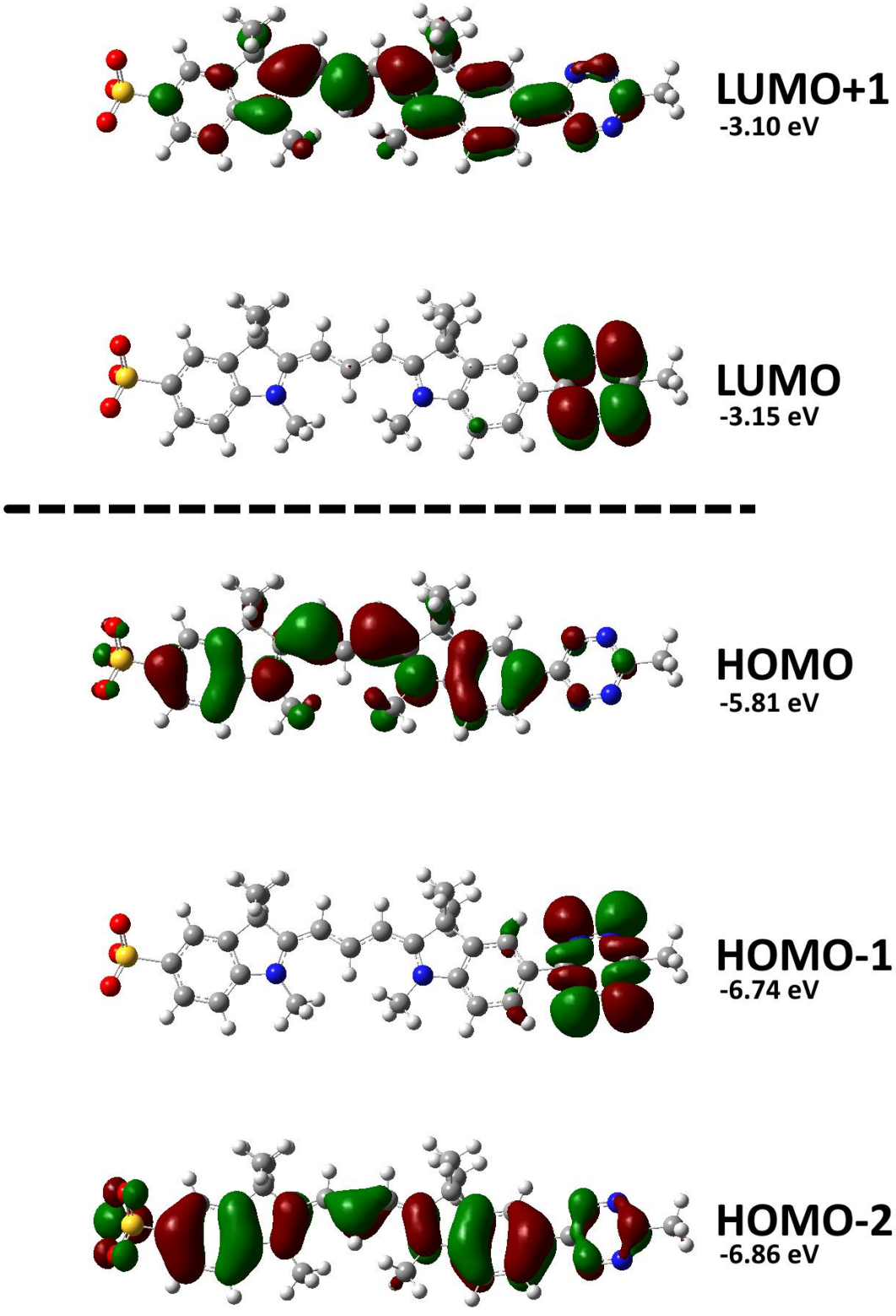


Figure S10. – Orbital density distribution of **1a**.

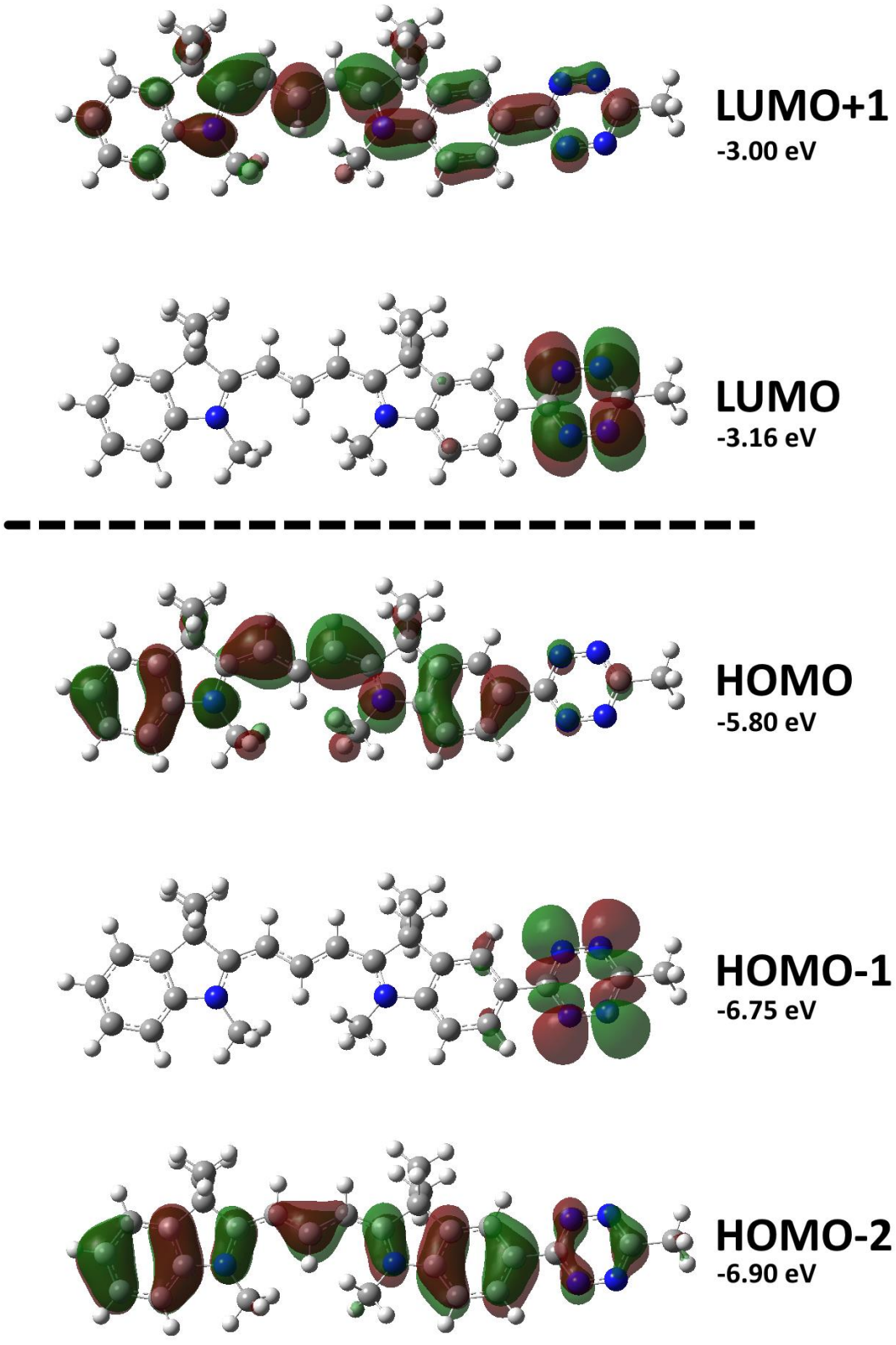


Figure S11. – Orbital density distribution of **1b**.

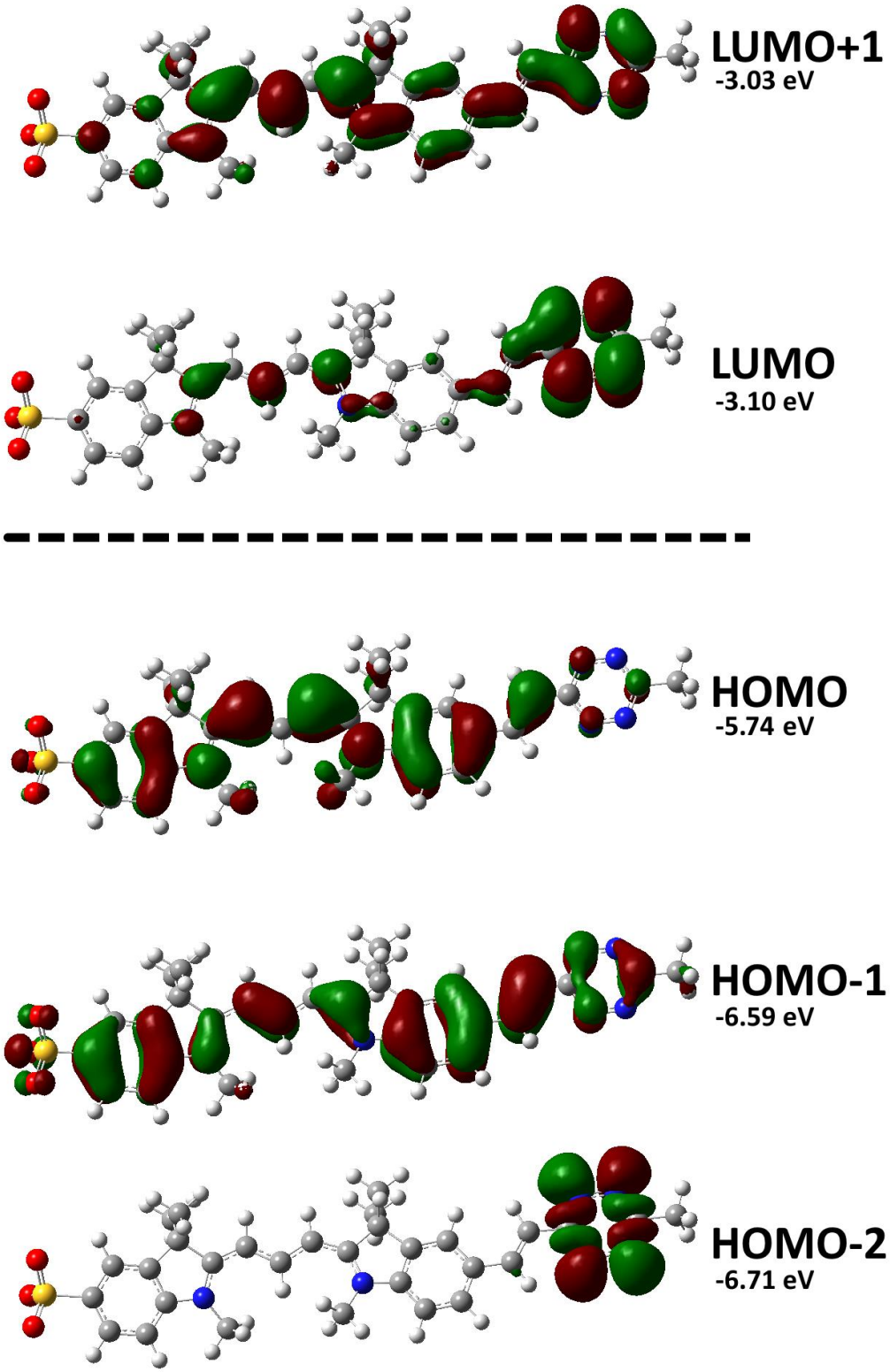


Figure S12. – Orbital density distribution of **3a**.

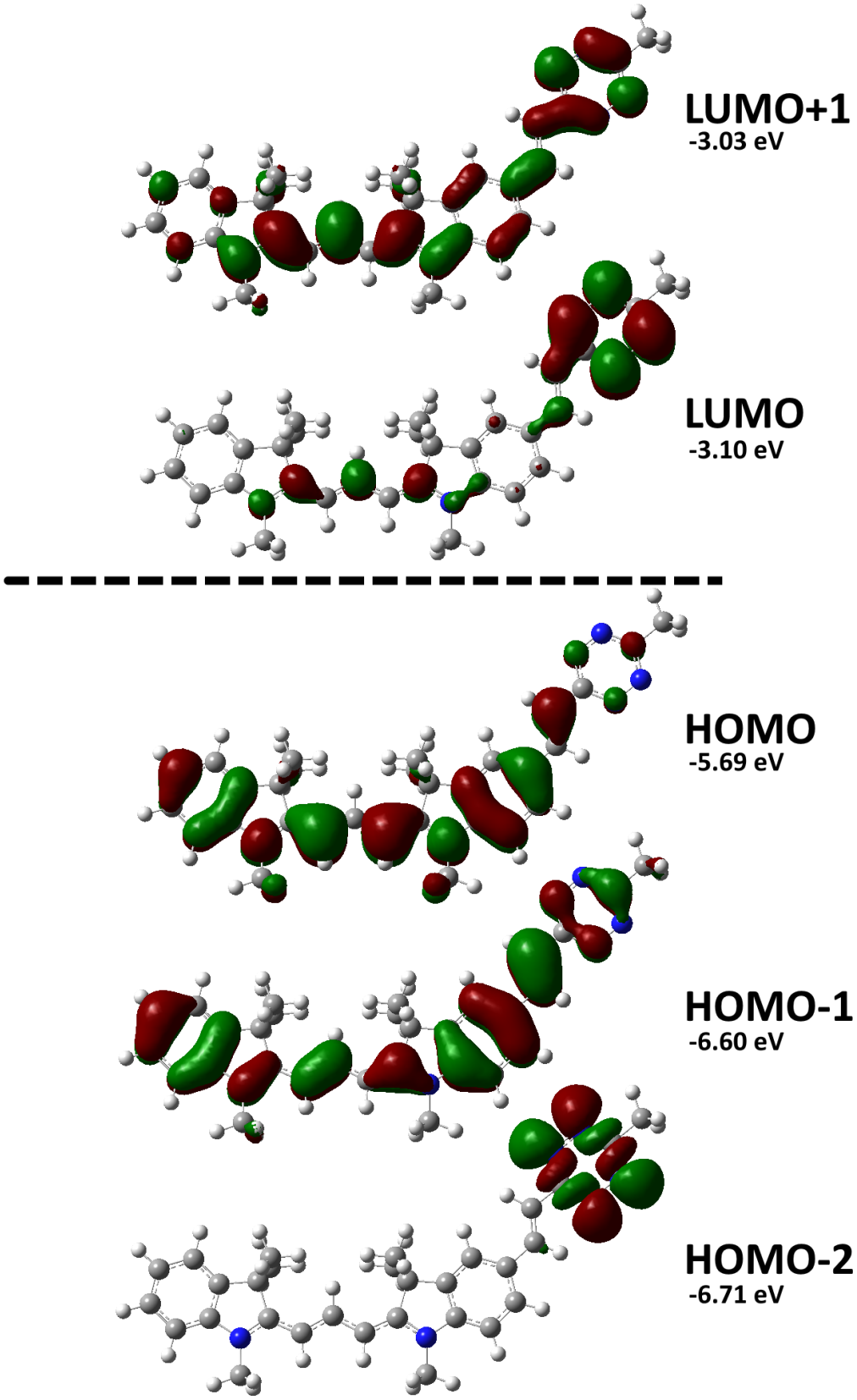


Figure S13. – Orbital density distribution of **3b**.

References

(1) N. M. Gallagher, J. J. Bauer, M. Pink, S. Rajca, A. Rajca, *J. Am. Chem. Soc.* 2016, **138**, 9377–9380.

(2) G. M. Shivashimpi, S. S. Pandey, R. Watanabe, N. Fujikawa, Y. Ogomi, Y. Yamaguchi, S. Hayase, *Tetrahedron Lett.* 2012, **53**, 5437–5440.

(3) L. Chen, F. Li, M. Nandi, L. Huang, Z. Chen, J. Wei, W. Chi, X. Liu, J. Yang, *Dyes Pigm.* 2020, **177**, 108313.

(4) G. Knorr, E. Kozma, J. M. Schaart, K. Németh, Gy. Török, P. Kele, *Bioconjugate Chem.* 2018, **29**, 1312–1318.

(5) S. Van der Wal, J. Kuil, A. R. P. M. Valentijn, F. W. B. Van Leeuwen, *Dyes Pigm.* 2016, **132**, 7–19.

(6) E. Albitz, D. Kern, A. Kormos, M. Bojtár, Gy. Török, A. Biró, Á. Szatmári, K. Németh, P. Kele, *Angew. Chem. Int. Ed.* 2022, **61**, e202111855.

(7) M. V. Kvach, A. V. Ustinov, I. A. Stepanova, A. D. Malakhov, M. V. Skorobogatyi, V. V. Shmanai, V. A. Korshun, *Eur. J. Org. Chem.* 2008, **12**, 2107–2117.

(8) A. Fegan, P. S. Shirude, S. Balasubramanian, *Chem. Commun.* 2008, **2**, 2004–2006.

(9) G. Knorr, E. Kozma, A. Herner, E. A. Lemke, P. Kele, *Chem. - Eur. J.* 2016, **22**, 8972–8979.

(10) E. Németh, G. Knorr, K. Németh, P. Kele, *Biomolecules* 2020, **10**, 397.

(11) H. Wu, J. Yang, J. Šečkutė, N. K. Devaraj, *Angew. Chem.* 2014, **126**, 5915–5919.

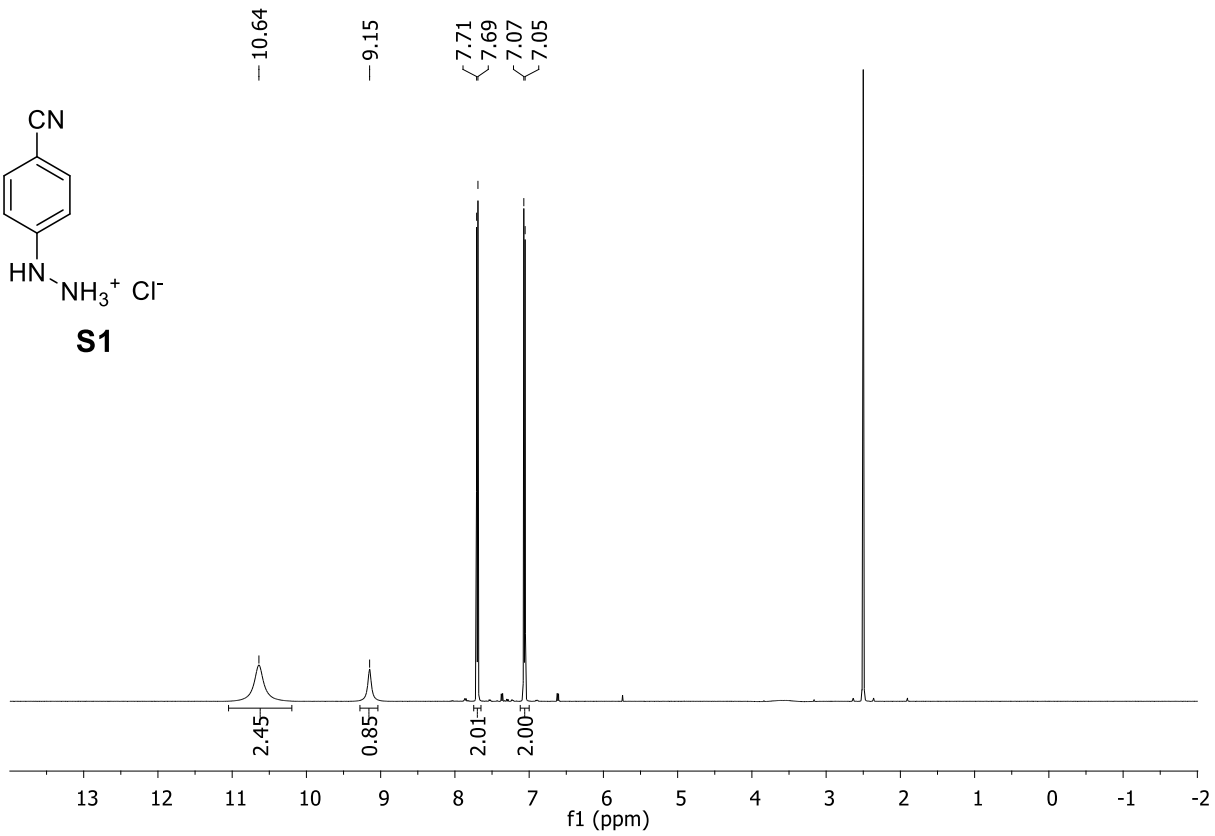
(12) Á. Szatmári, G. B. Cserép, T. Á. Molnár, B. Söveges, A. Biró, Gy. Várady, E. Szabó, K. Németh, P. Kele, *Molecules* 2021, **26**, 4988.

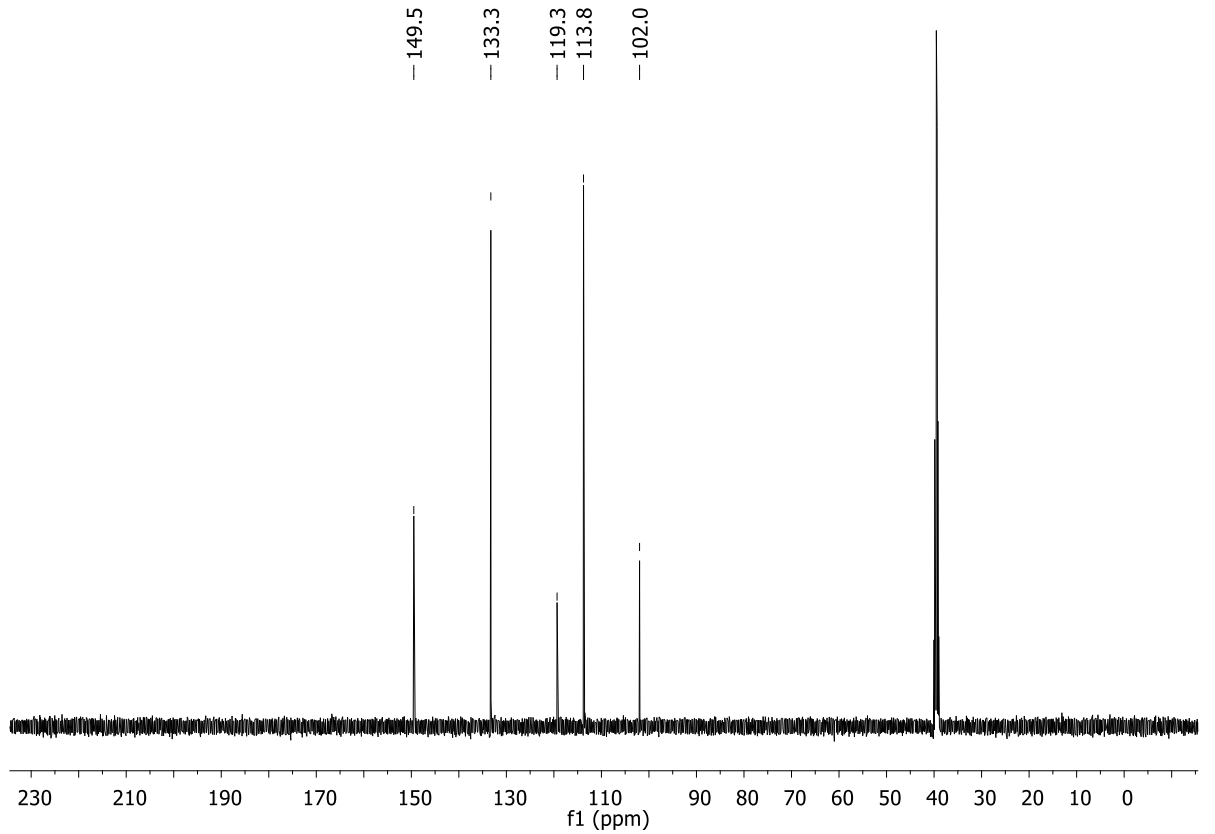
(13) I. Nikic, J. H. Kang, G. E. Girona, I. V. Aramburu, E. A. Lemke, *Nat. Protoc.* 2015, **10**, 780–791.

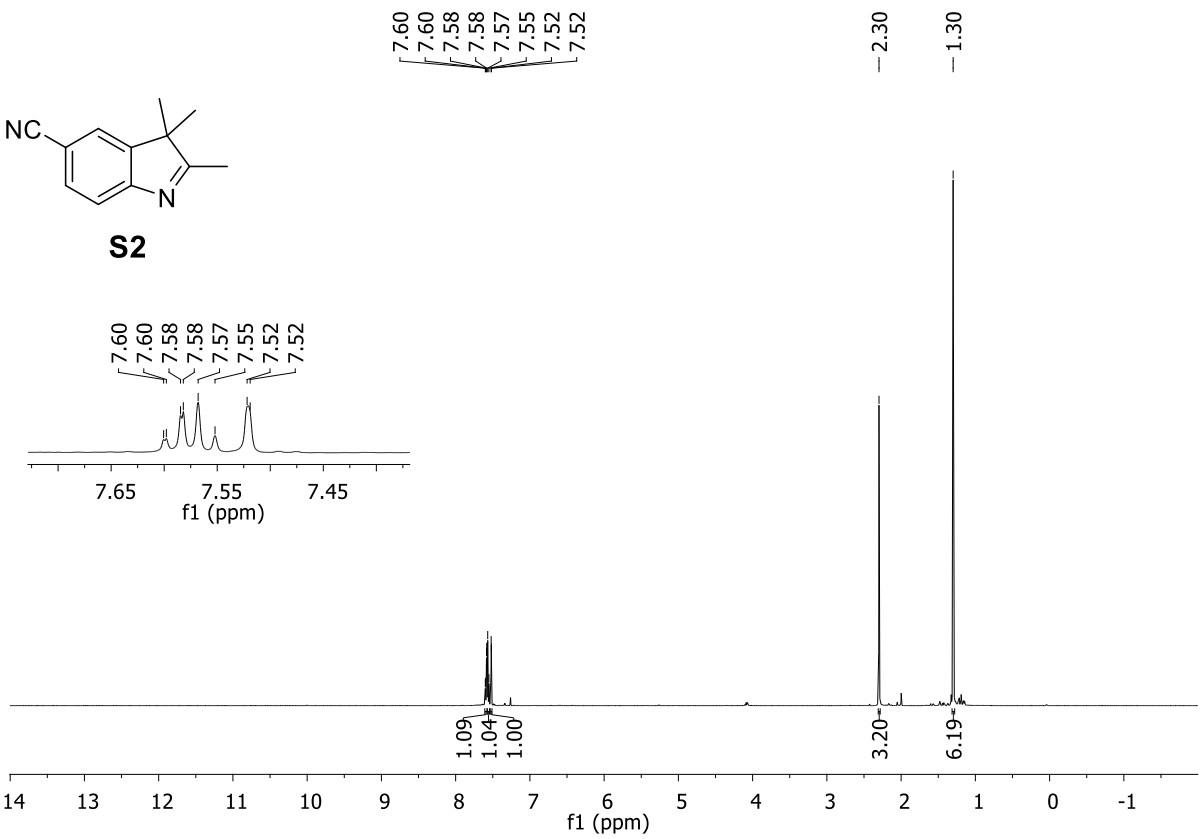
(14) A. Egyed, A. Kormos, B. Söveges, K. Németh, P. Kele, *Bioorg. Med. Chem.* 2020, **28**, 115218.

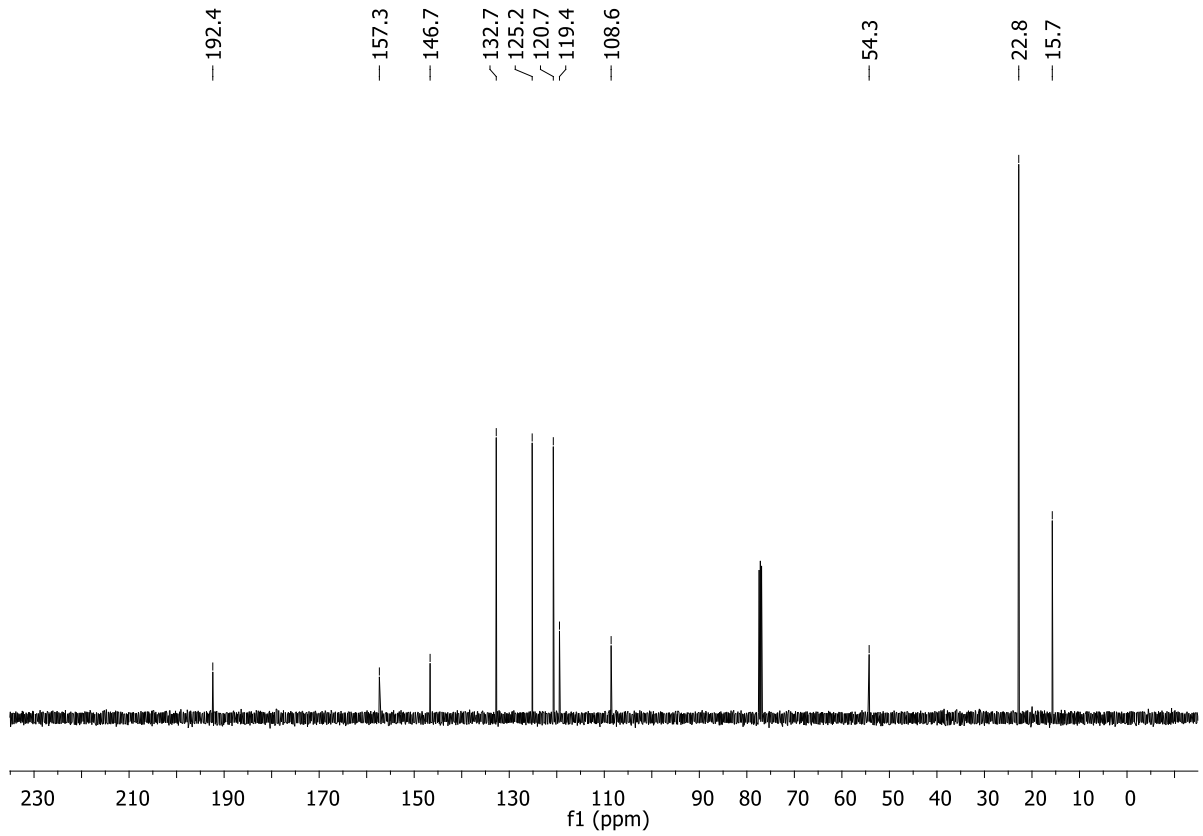
(15) I. Nikić, G. Estrada Girona, J. H. Kang, G. Paci, S. Mikhaleva, C. Koehler, N. V. Shymanska, C. Ventura Santos, D. Spitz, E. A. Lemke, *Angew. Chem. Int. Ed.* 2016, **55**, 16172–16176.

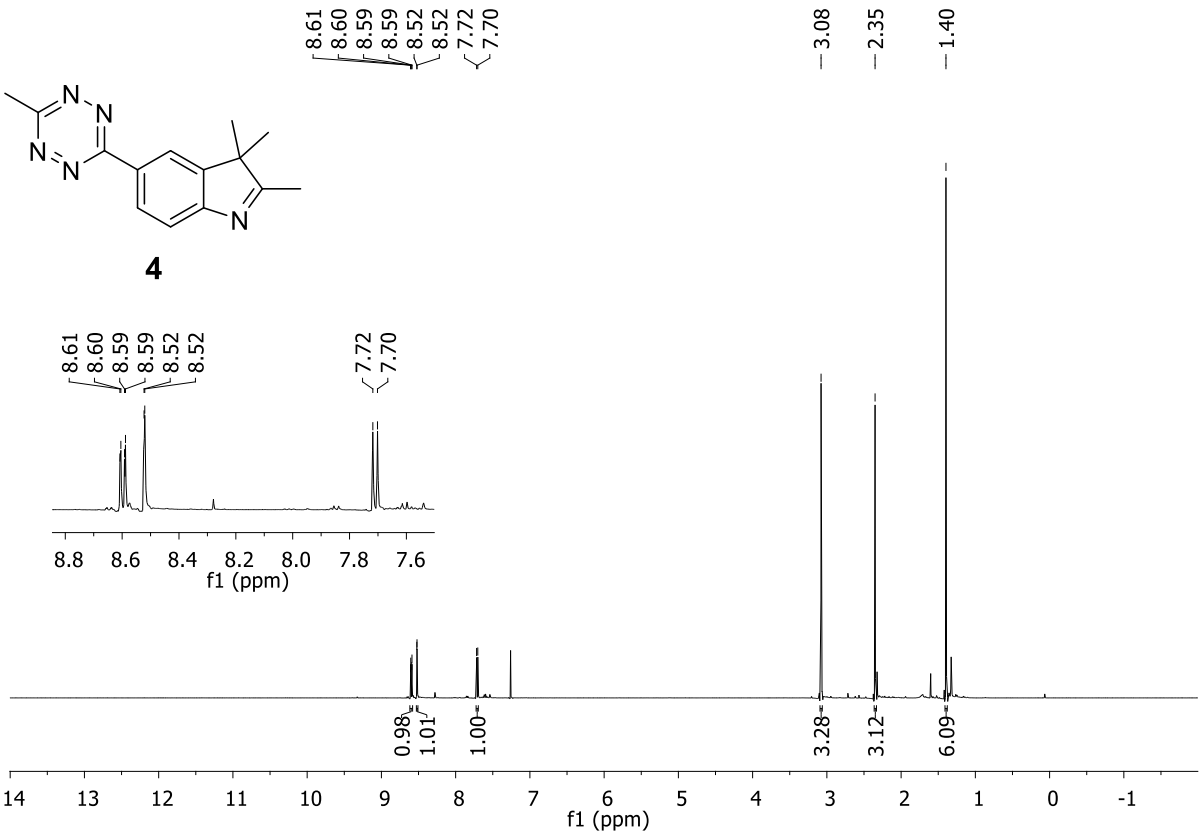
NMR spectra

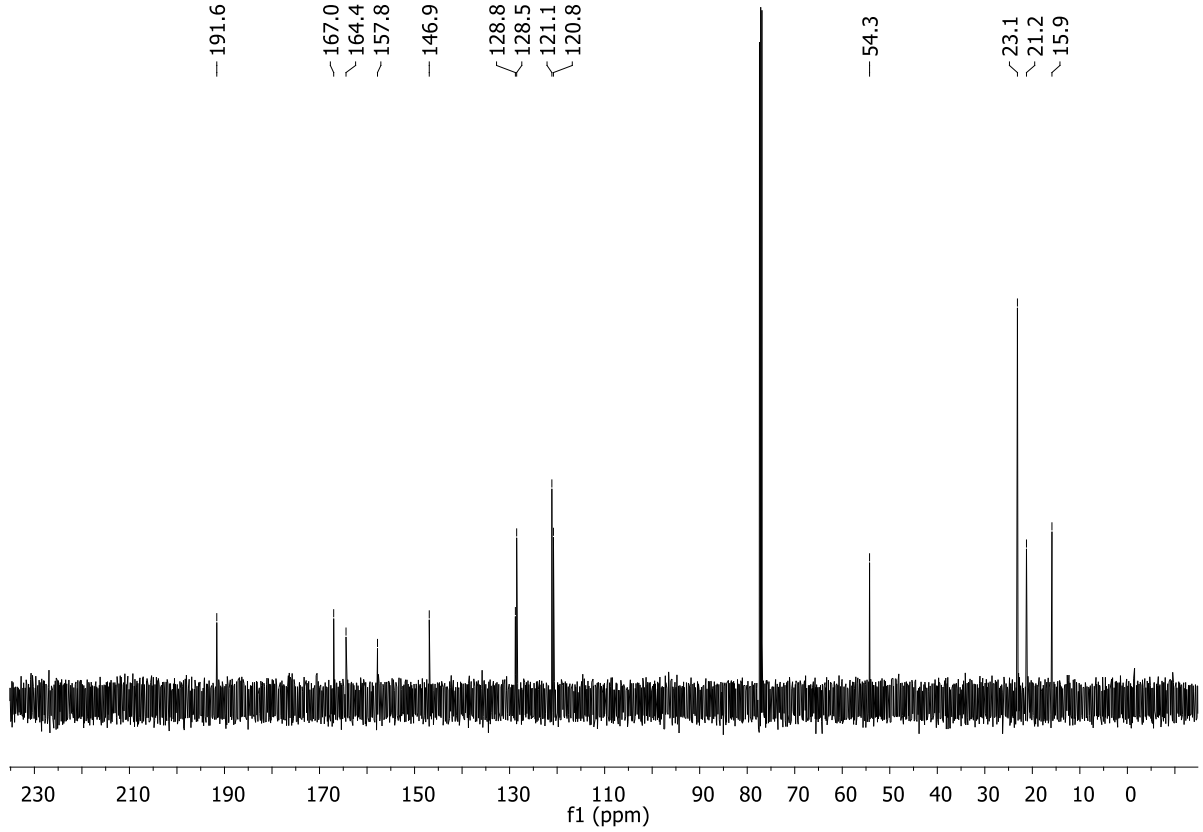

10.64
9.15
7.71
7.69
7.07
7.05
CN
HN
NH3+ Cl-
S1
2.45
0.85
2.01
2.00
13 12 11 10 9 8 7 6 5 4 3 2 1 0 -1 -2
f1 (ppm)

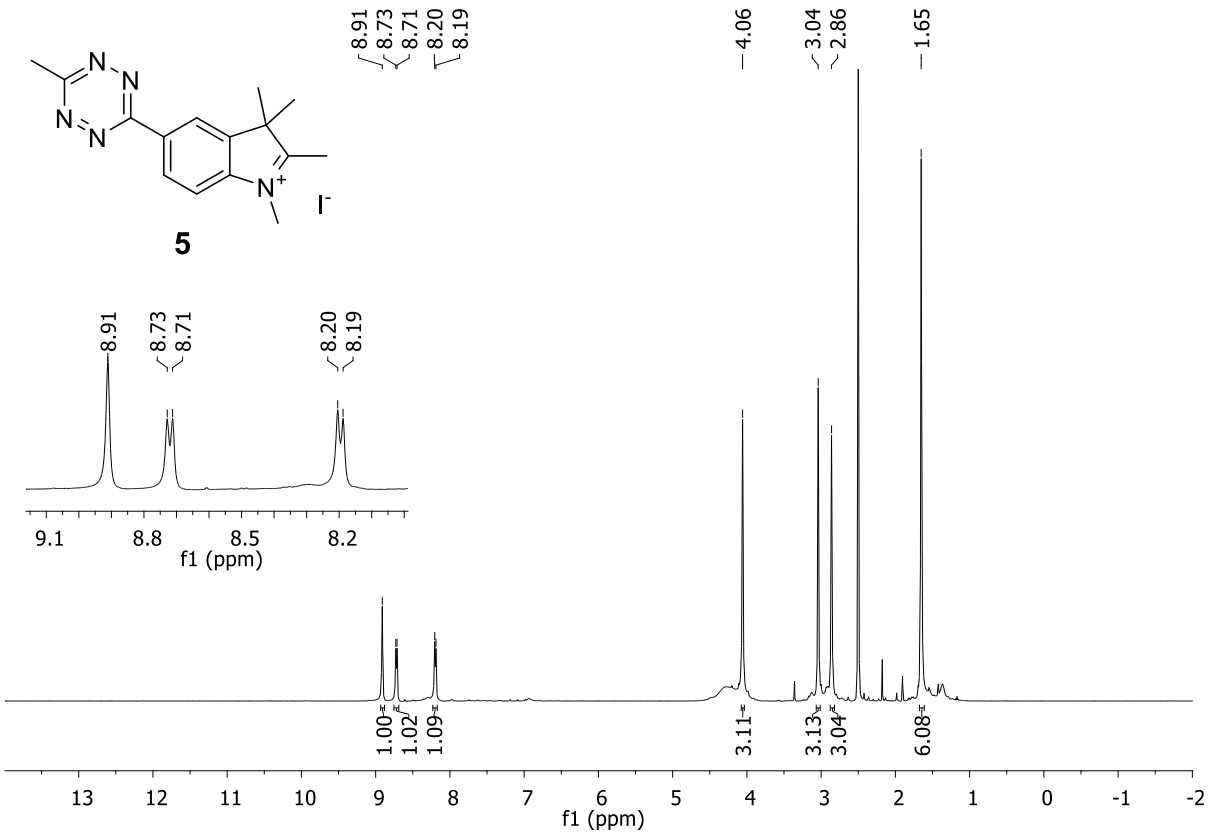

149.5
133.3
119.3
113.8
102.0
230 210 190 170 150 130 110 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

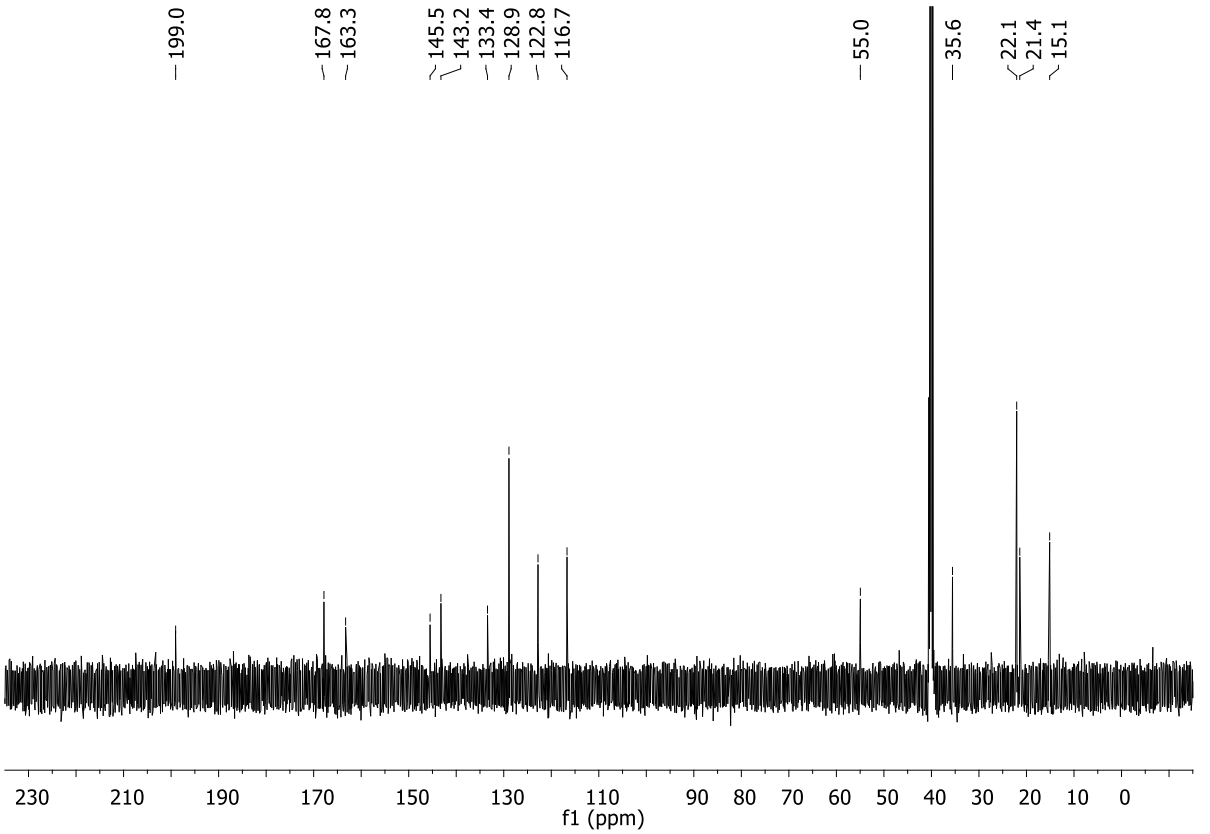

7.60
7.60
7.58
7.58
7.57
7.55
7.52
7.52
2.30
1.30
NC
N
S2
7.65
7.55
7.45
f1 (ppm)
1.09
1.04
1.00
3.20
6.19
14 13 12 11 10 9 8 7 6 5 4 3 2 1 0 -1
f1 (ppm)

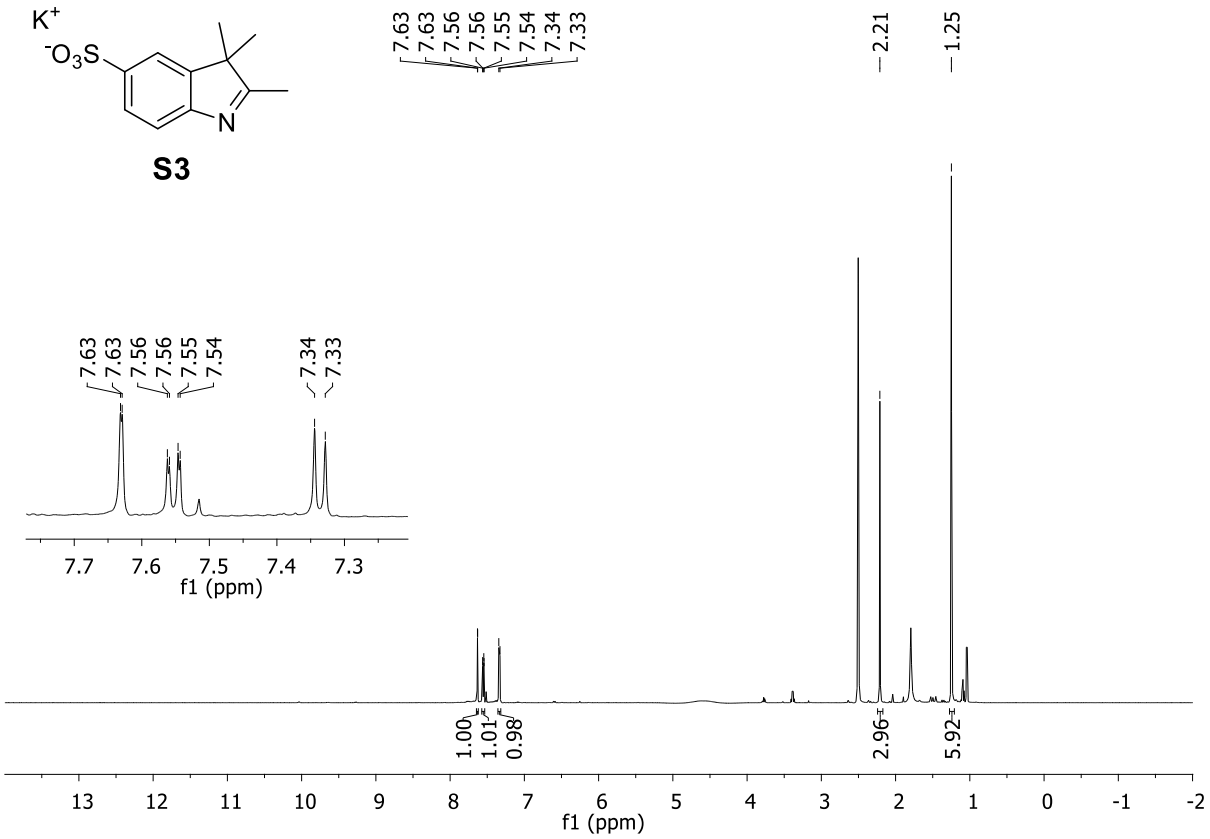

192.4
157.3
146.7
132.7
125.2
120.7
119.4
108.6
54.3
22.8
15.7
230 210 190 170 150 130 110 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

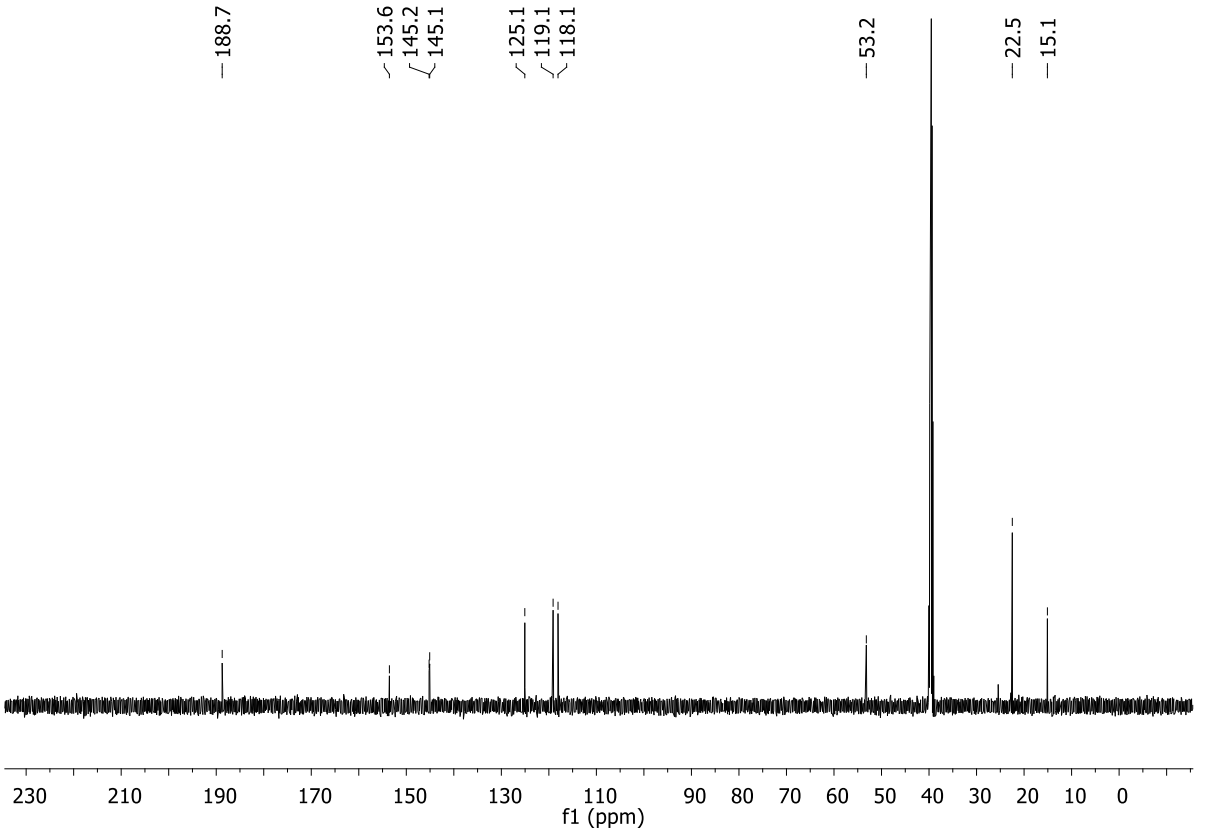

8.61
8.60
8.59
8.59
8.52
8.52
7.72
7.70
3.08
2.35
1.40
4
8.8 8.6 8.4 8.2 8.0 7.8 7.6
f1 (ppm)
0.98
1.01
1.00
3.28
3.12
6.09
14 13 12 11 10 9 8 7 6 5 4 3 2 1 0 -1
f1 (ppm)

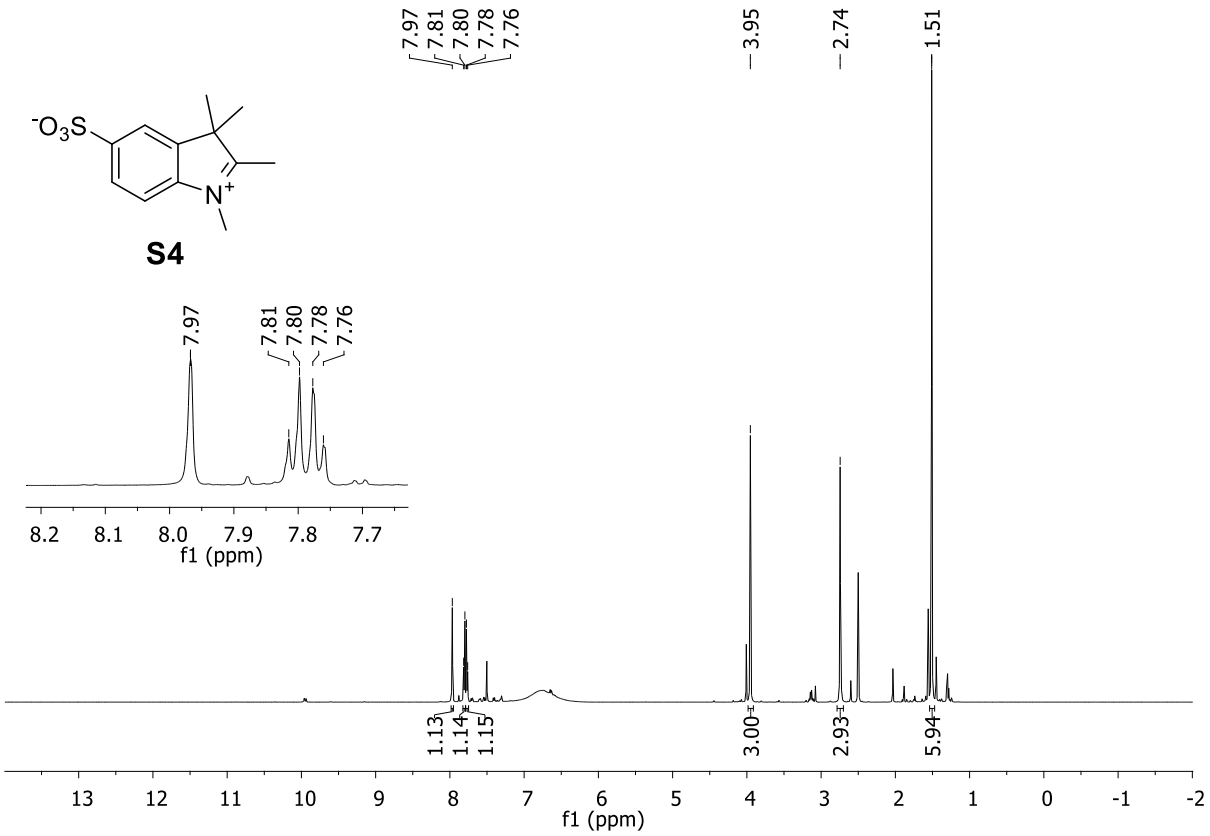

191.6
167.0
164.4
157.8
146.9
128.8
128.5
121.1
120.8
54.3
23.1
21.2
15.9
230 210 190 170 150 130 110 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

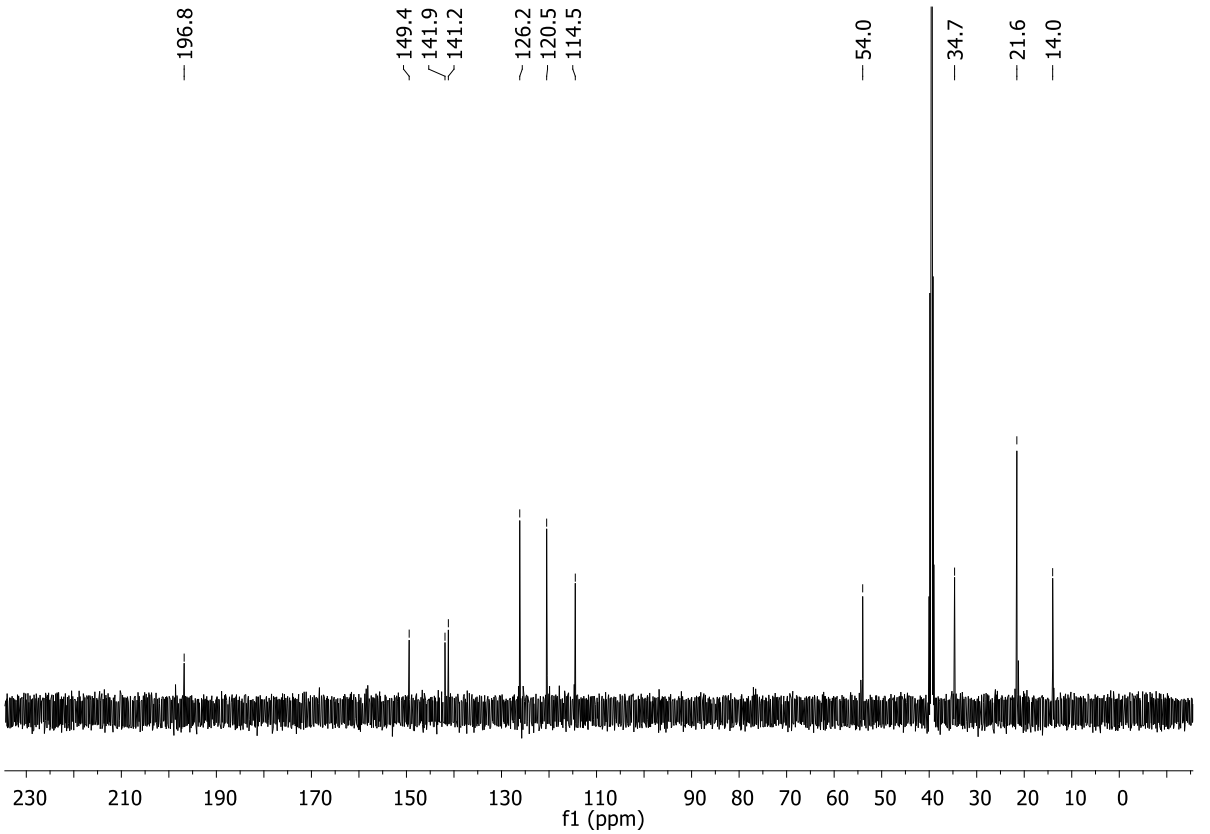

8.91
8.73
8.71
8.20
8.19
4.06
3.04
2.86
1.65
I-
5
9.1
8.8
8.5
8.2
f1 (ppm)
1.00
1.02
1.09
3.11
3.13
3.04
6.08
13 12 11 10 9 8 7 6 5 4 3 2 1 0 -1 -2
f1 (ppm)

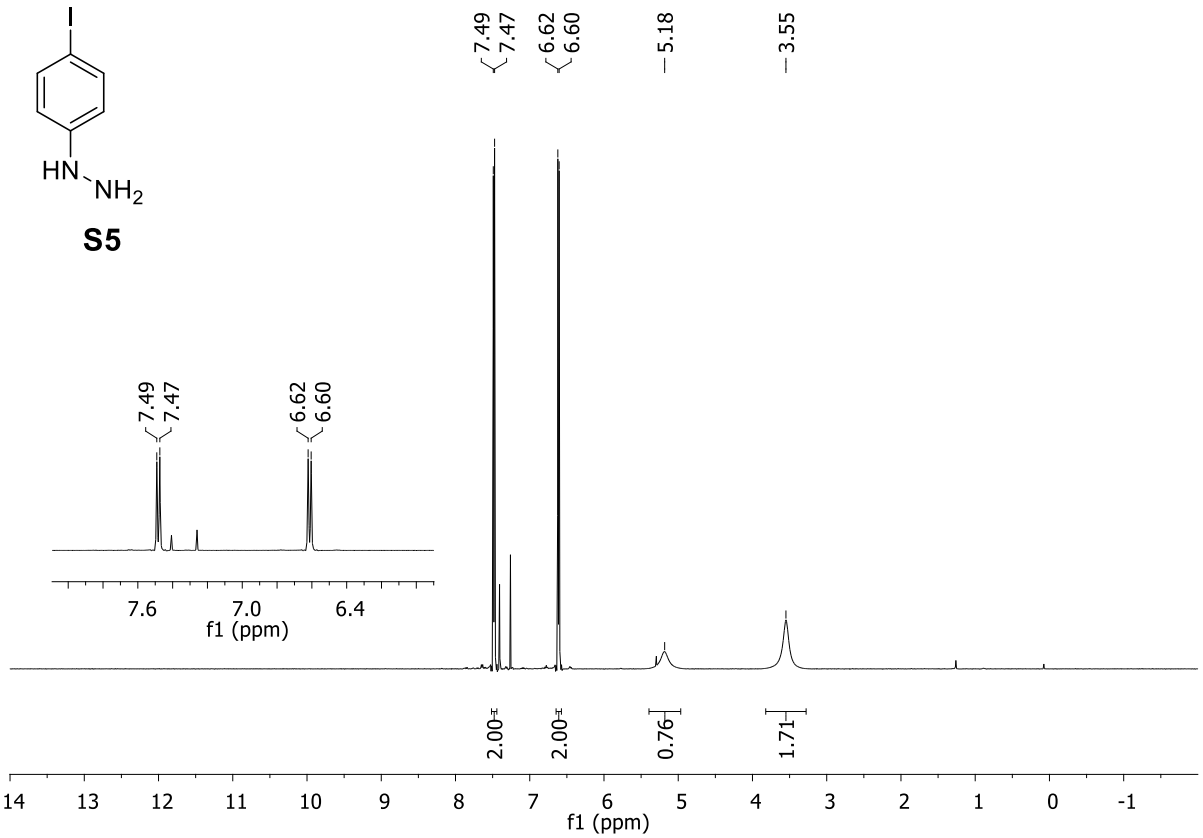

199.0
167.8
163.3
145.5
143.2
133.4
128.9
122.8
116.7
55.0
35.6
22.1
21.4
15.1
230 210 190 170 150 130 110 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

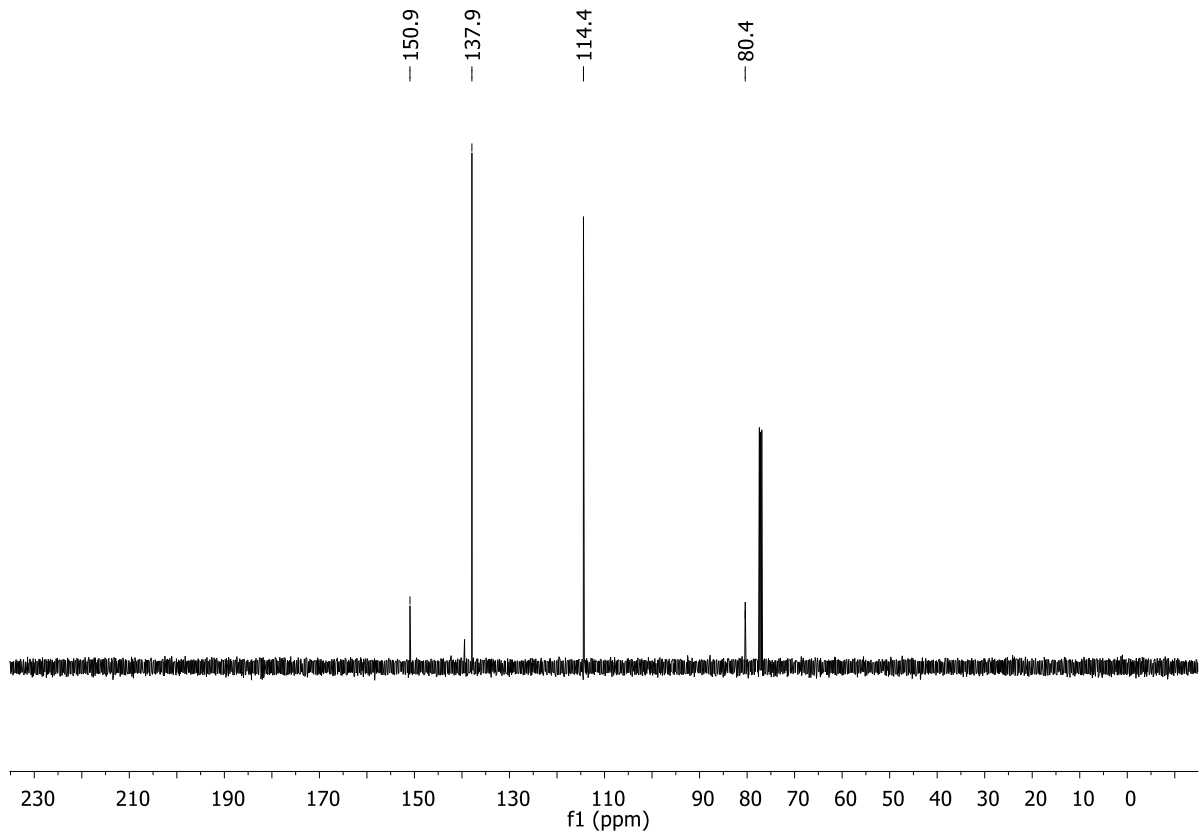

K+
-O3S
N
S3
7.63
7.63
7.56
7.56
7.55
7.54
7.34
7.33
2.21
1.25
1.00
1.01
0.98
2.96
5.92
f1 (ppm)

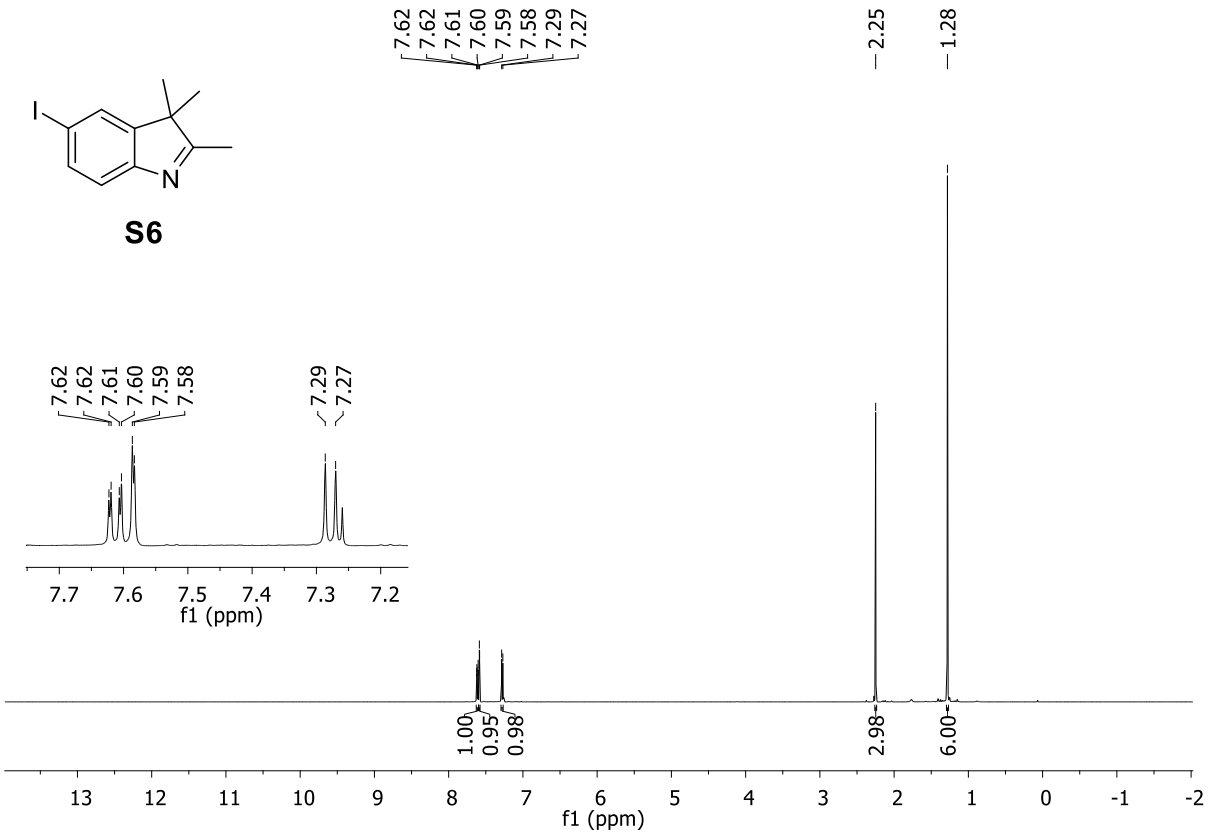

188.7
153.6
145.2
145.1
125.1
119.1
118.1
53.2
22.5
15.1
f1 (ppm)

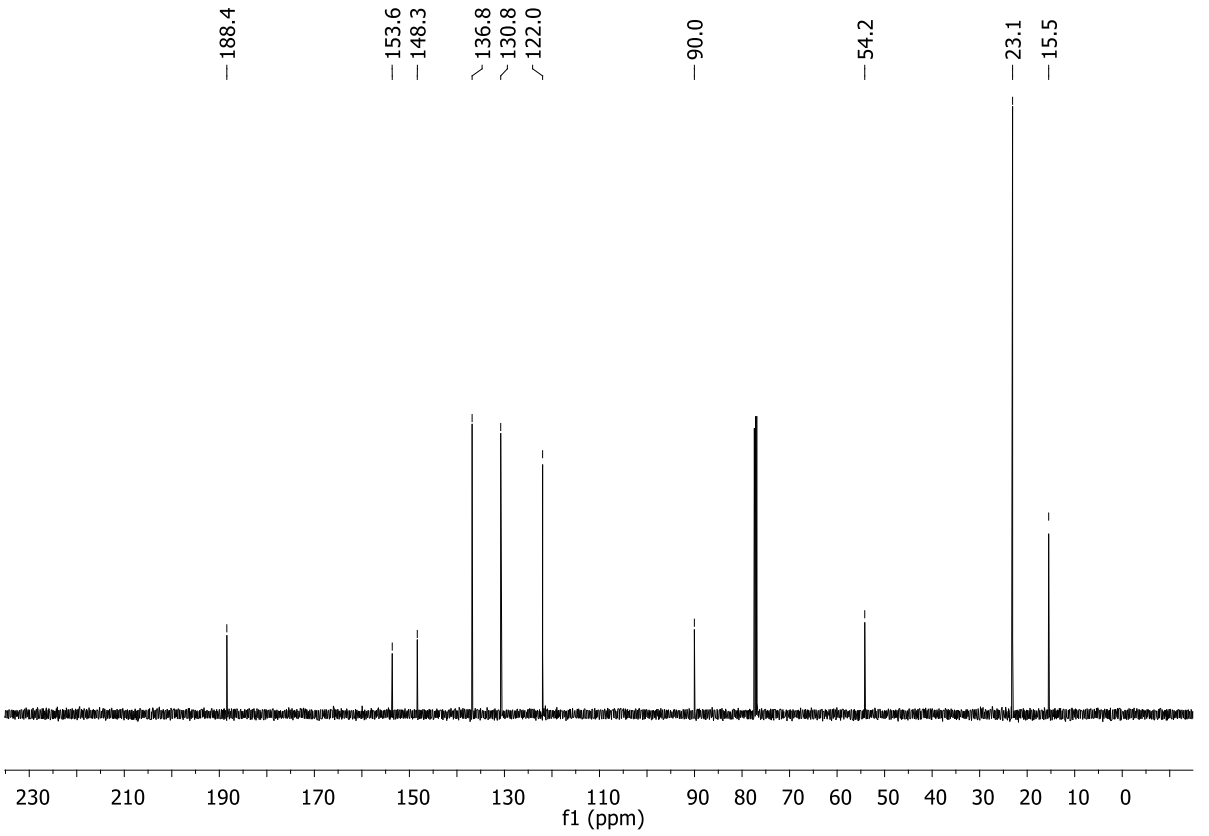

7.97
7.81
7.80
7.78
7.76
3.95
2.74
1.51
S4
1.13
1.14
1.15
3.00
2.93
5.94
f1 (ppm)

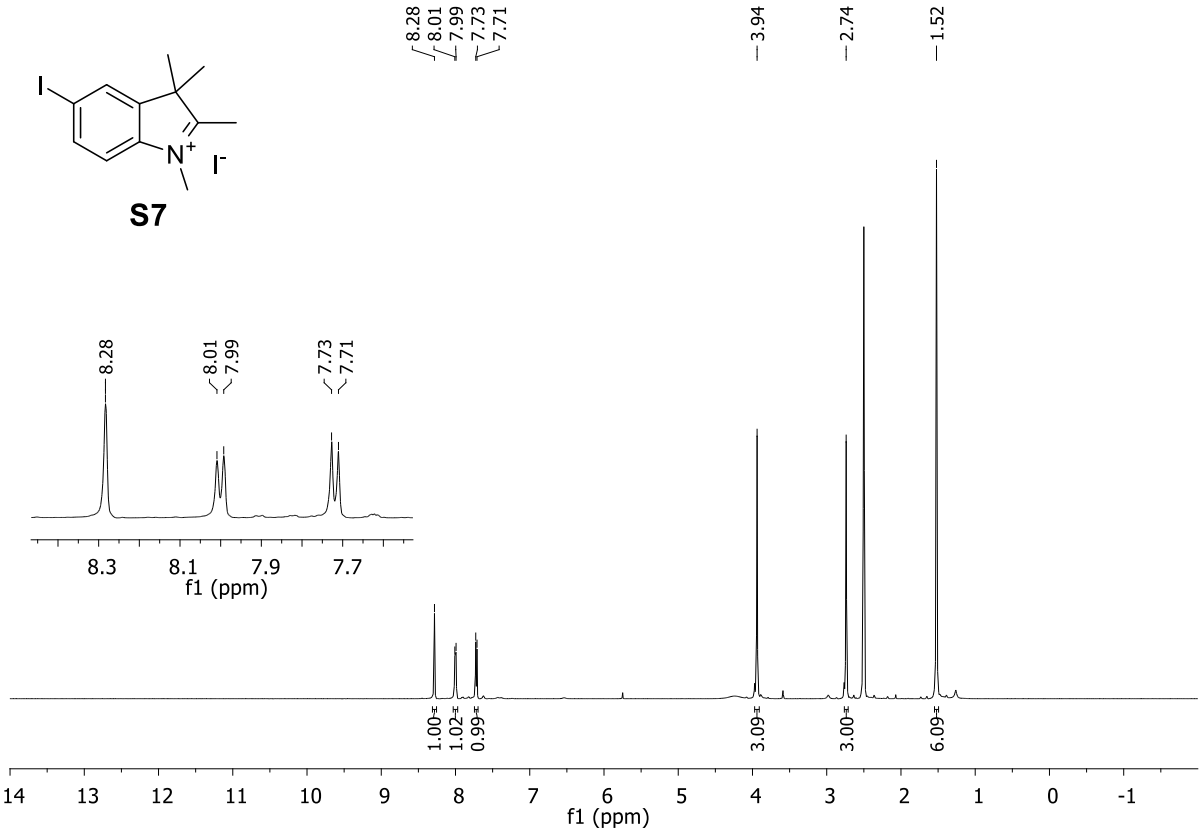

196.8
149.4
141.9
141.2
126.2
120.5
114.5
54.0
34.7
21.6
14.0
f1 (ppm)

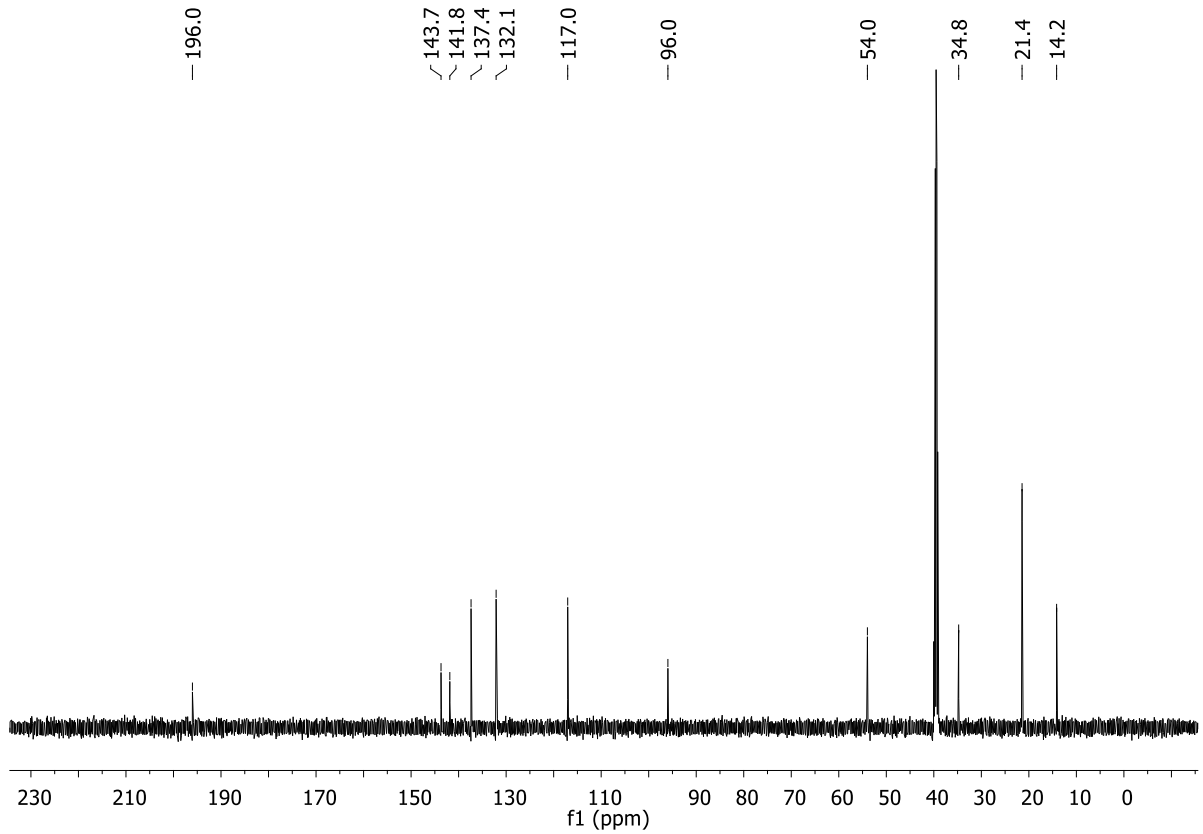

HN
NH2
S5
7.49
7.47
6.62
6.60
5.18
3.55
2.00
2.00
0.76
1.71
f1 (ppm)

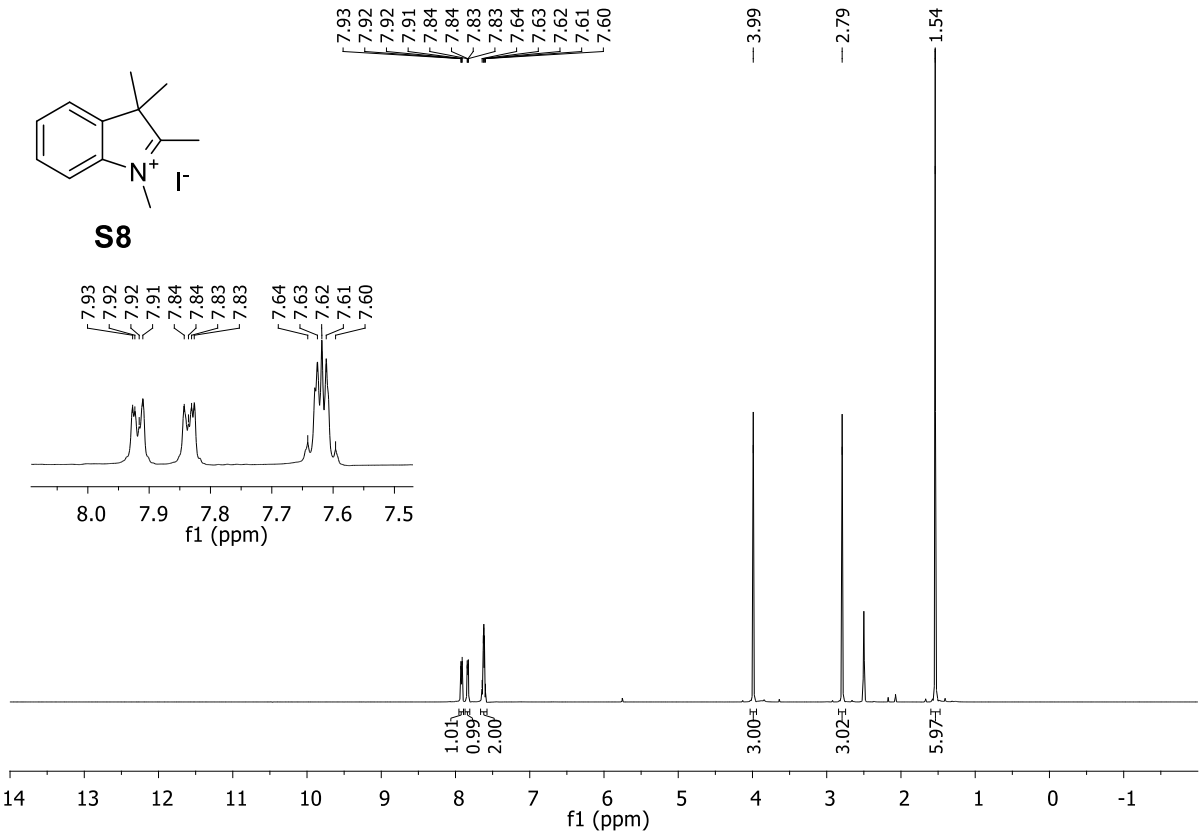

150.9
137.9
114.4
80.4
f1 (ppm)

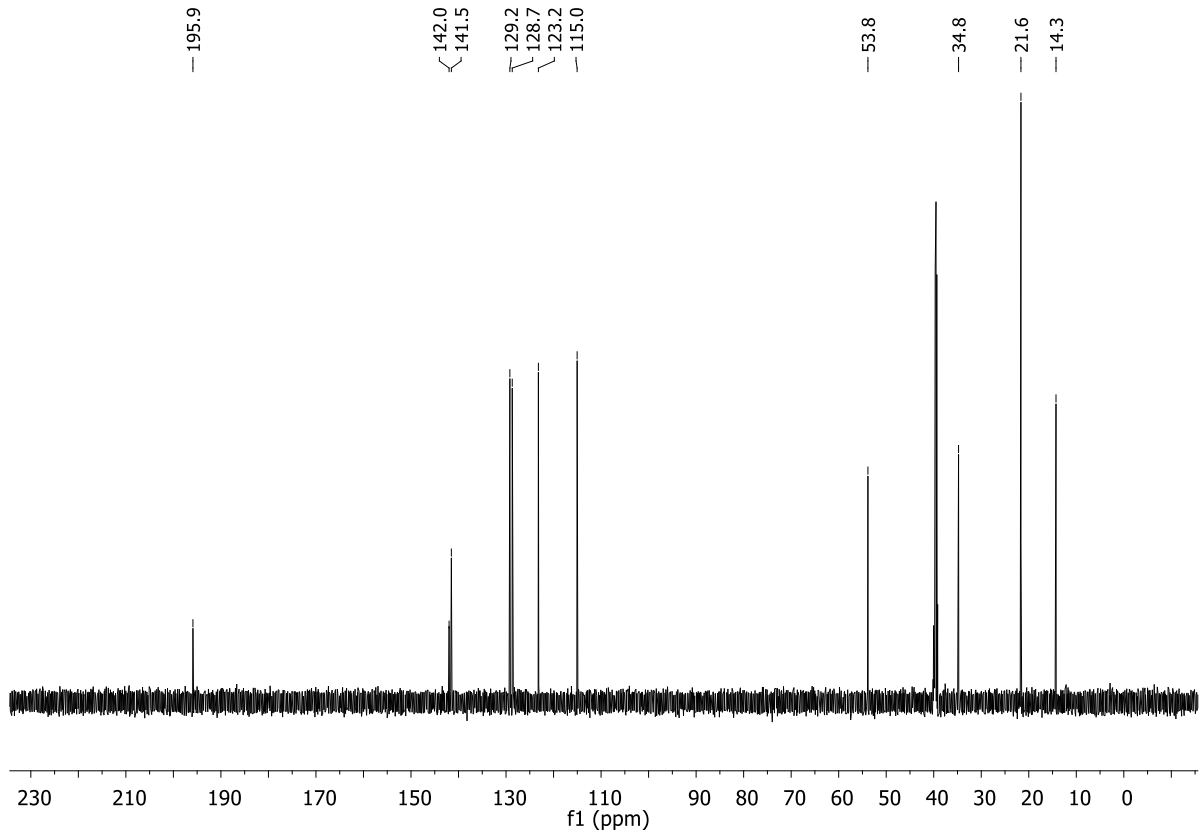

7.62
7.62
7.61
7.60
7.59
7.58
7.29
7.27
2.25
1.28
I
N
S6
7.62
7.62
7.61
7.60
7.59
7.58
7.29
7.27
7.7
7.6
7.5
7.4
7.3
7.2
f1 (ppm)
1.00
0.95
0.98
2.98
6.00
13
12
11
10
9
8
7
6
5
4
3
2
1
0
-1
-2
f1 (ppm)

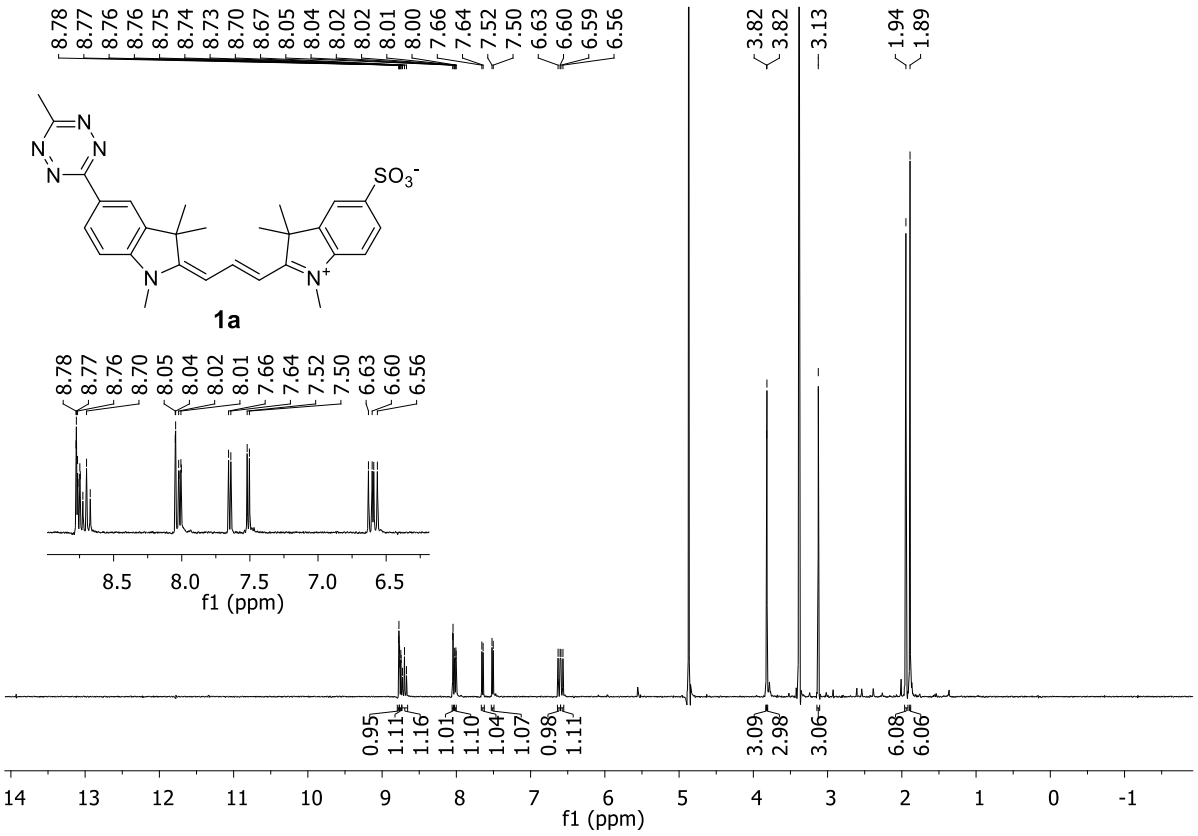

188.4
153.6
148.3
136.8
130.8
122.0
90.0
54.2
23.1
15.5
230
210
190
170
150
130
110
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

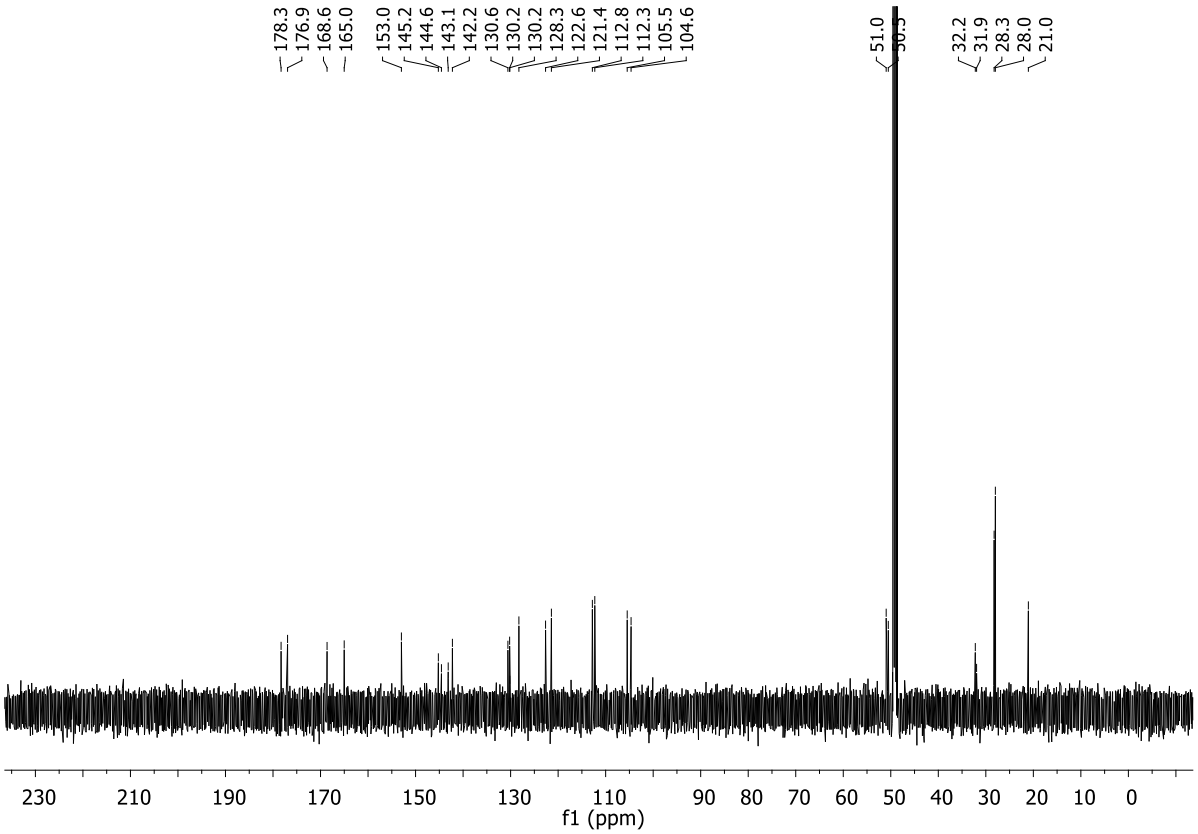

S7
8.28
8.01
7.99
7.73
7.71
3.94
2.74
1.52
1.00
1.02
0.99
3.09
3.00
6.09
f1 (ppm)

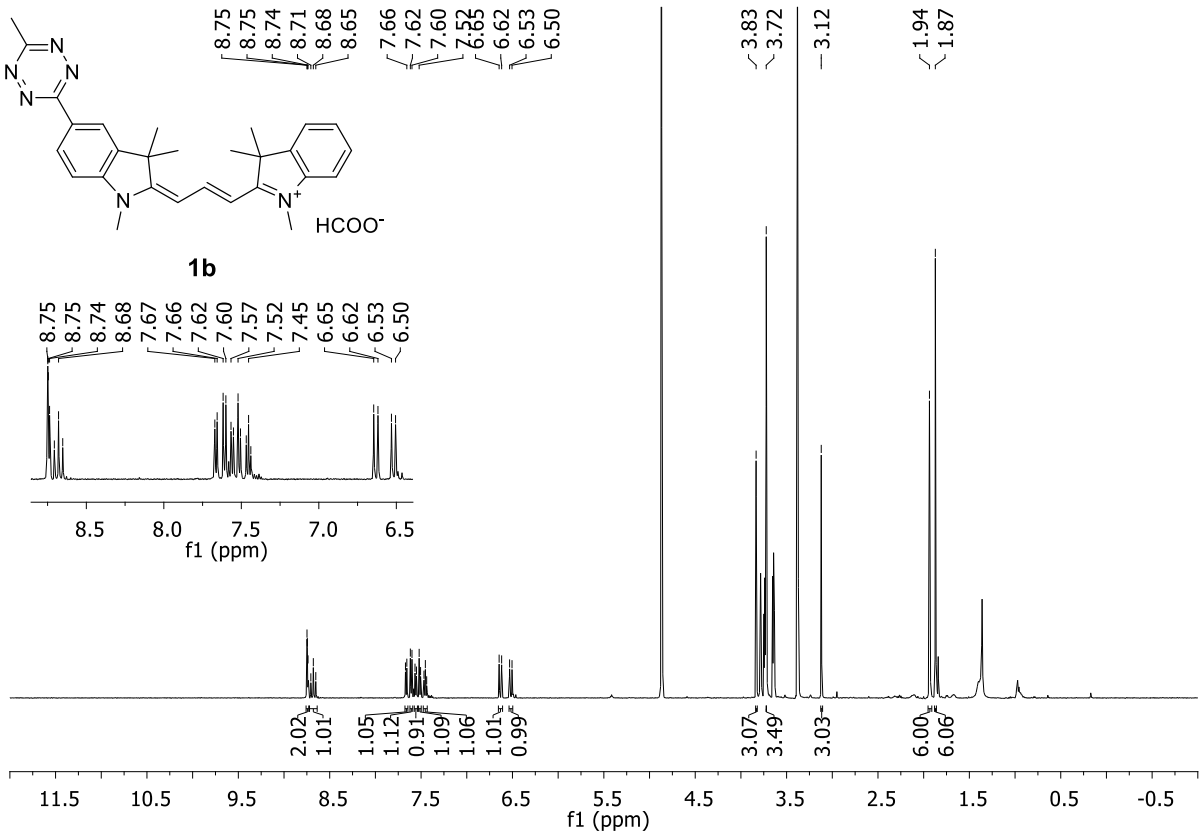

196.0
143.7
141.8
137.4
132.1
117.0
96.0
54.0
34.8
21.4
14.2
f1 (ppm)

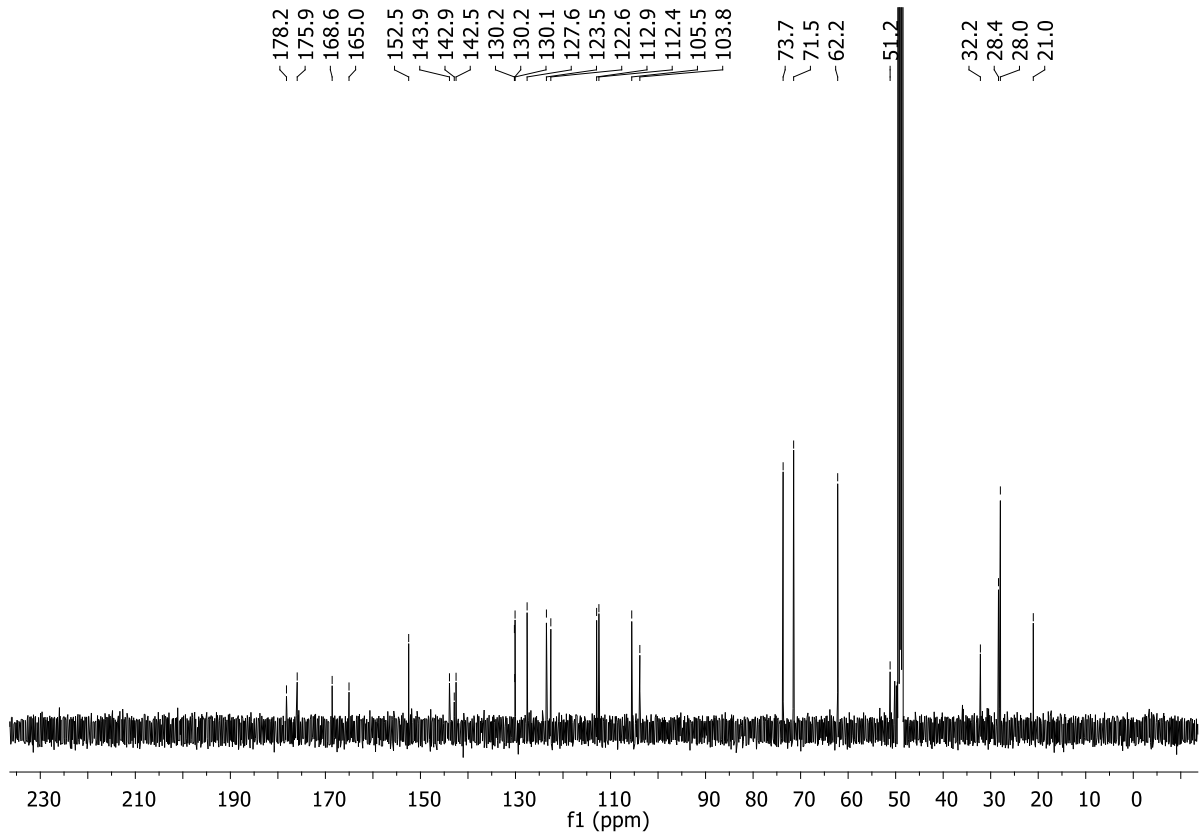

7.93
7.92
7.92
7.91
7.84
7.84
7.83
7.83
7.64
7.63
7.62
7.61
7.60
3.99
2.79
1.54
N+
I-
S8
1.01
0.99
2.00
3.00
3.02
5.97
f1 (ppm)

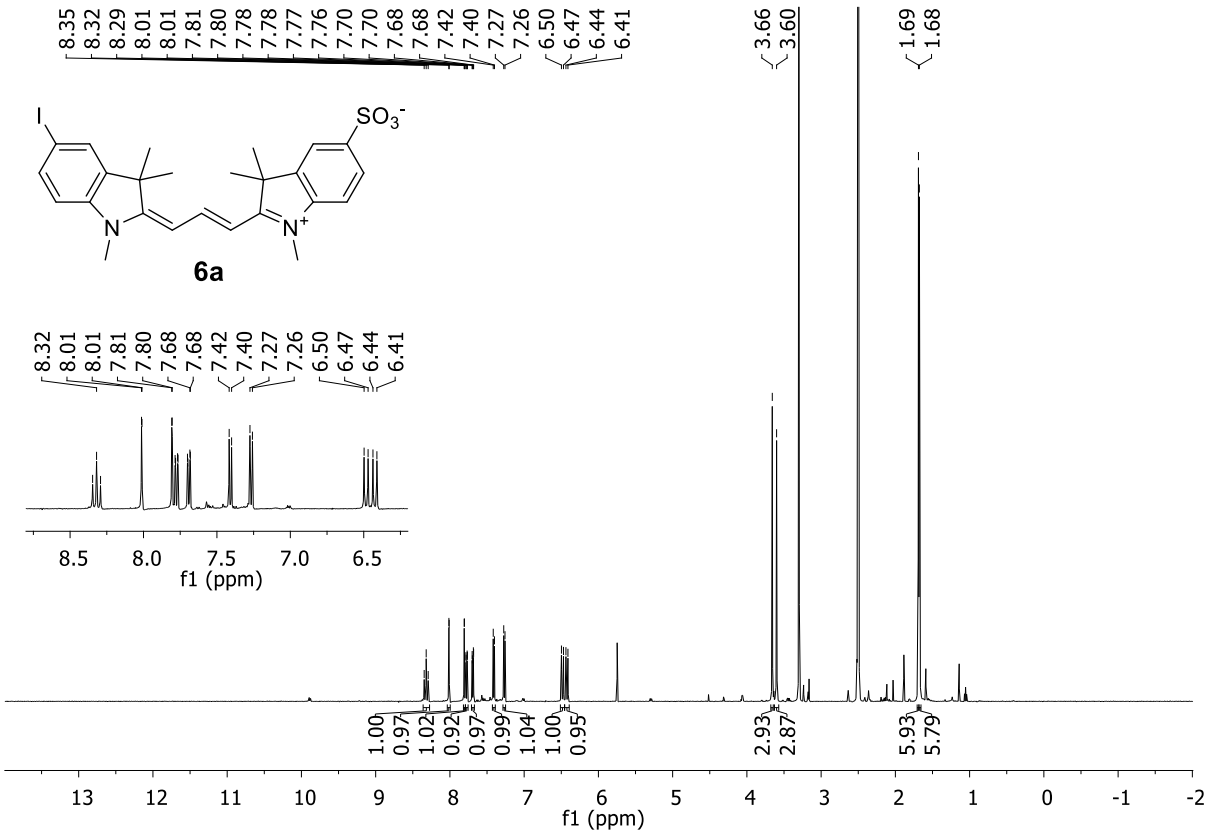

195.9
142.0
141.5
129.2
128.7
123.2
115.0
53.8
34.8
21.6
14.3
f1 (ppm)

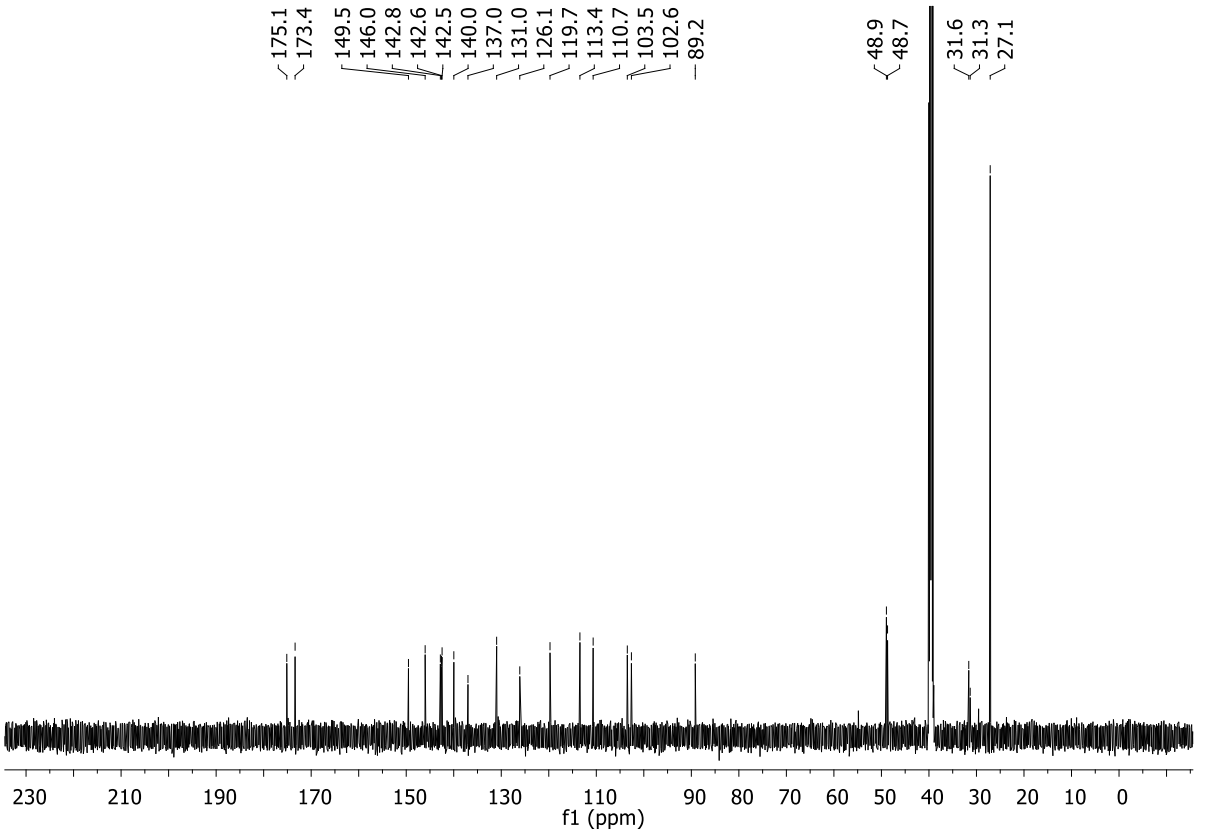

8.78 8.77 8.76 8.76 8.75 8.74 8.73 8.70 8.67 8.05 8.04 8.02 8.02 8.01 8.00 7.66 7.64 7.52 7.50 6.63 6.60 6.59 6.56
3.82 3.82 3.13 1.94 1.89
SO3-
1a
8.78 8.77 8.76 8.70 8.05 8.04 8.02 8.01 7.66 7.64 7.52 7.50 6.63 6.60 6.56
8.5 8.0 7.5 7.0 6.5
f1 (ppm)
0.95 1.11 1.16 1.01 1.10 1.04 1.07 0.98 1.11 3.09 2.98 3.06 6.08 6.06
14 13 12 11 10 9 8 7 6 5 4 3 2 1 0 -1
f1 (ppm)

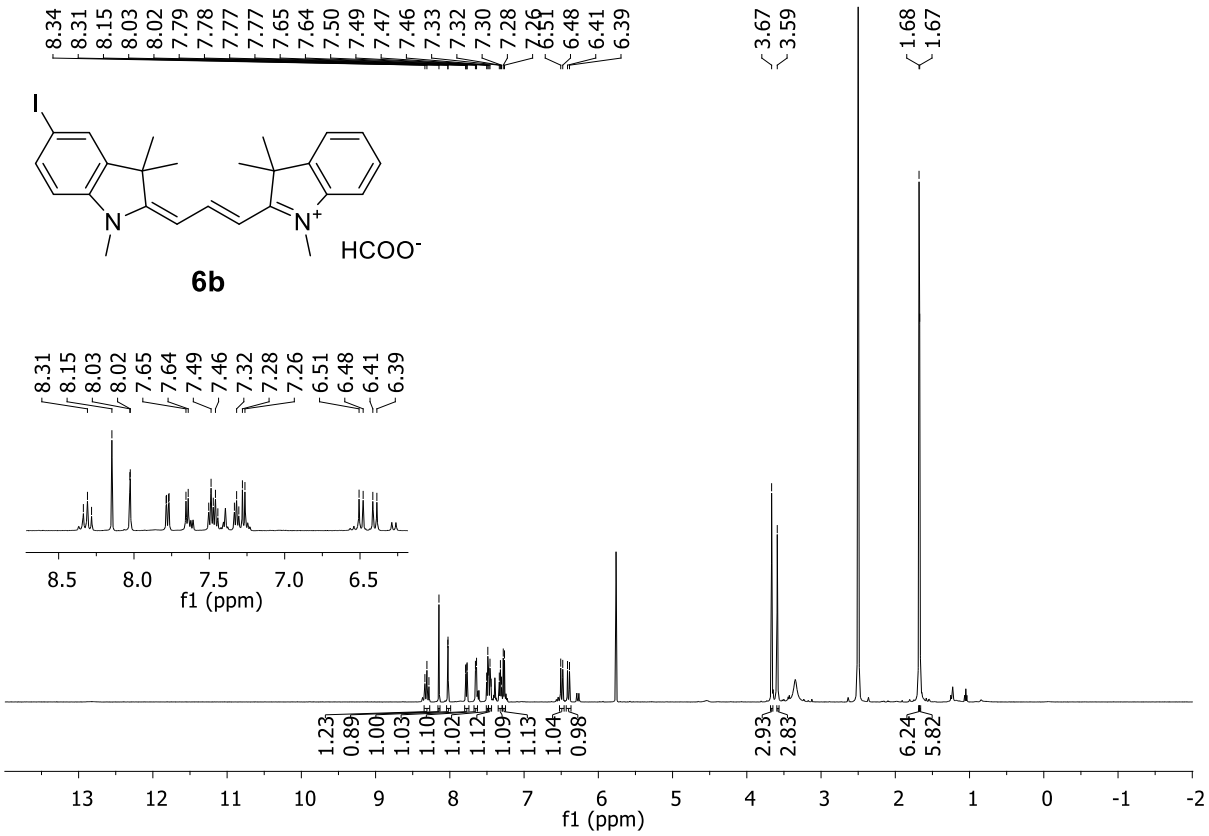

178.3 176.9 168.6 165.0 153.0 145.2 144.6 143.1 142.2 130.6 130.2 130.2 128.3 122.6 121.4 112.8 112.3 105.5 104.6
51.0 50.5 32.2 31.9 28.3 28.0 21.0
230 210 190 170 150 130 110 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

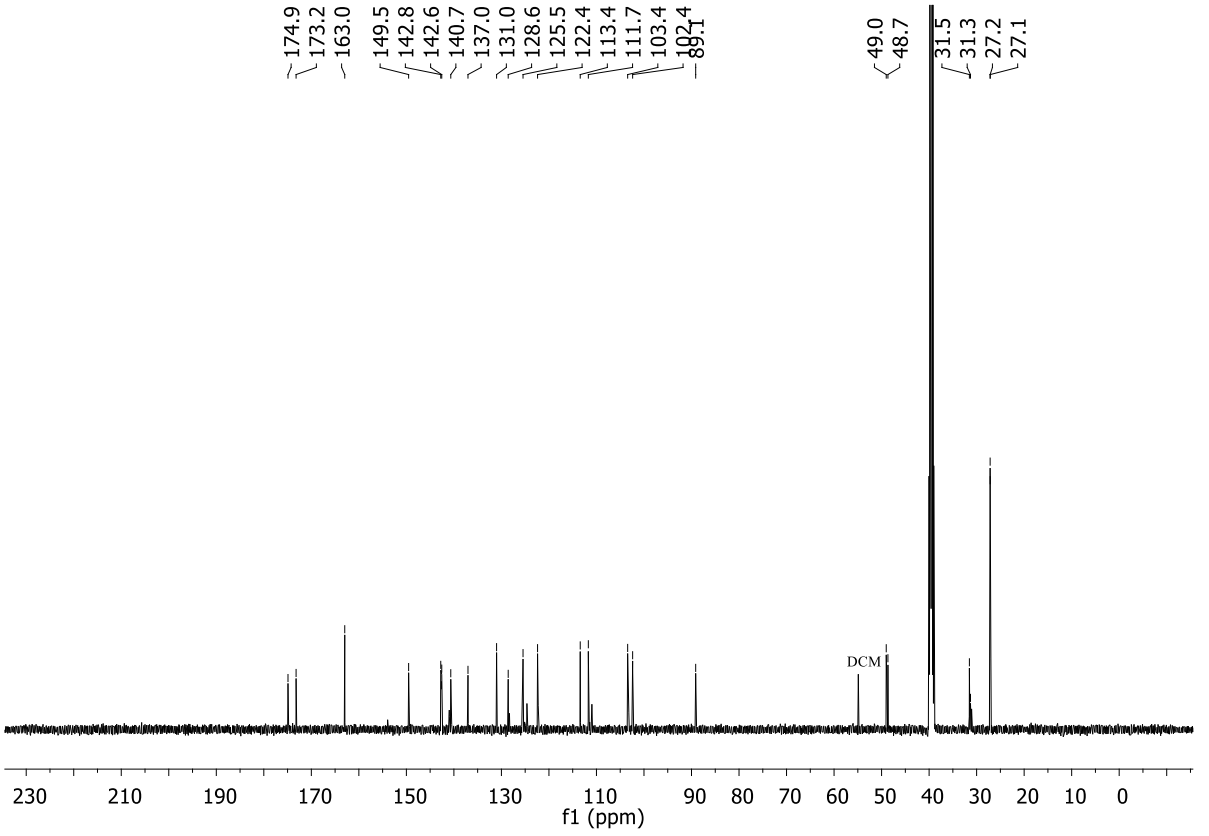

8.75
8.75
8.74
8.71
8.68
8.65
7.66
7.62
7.60
7.52
6.65
6.62
6.53
6.50
3.83
3.72
3.12
1.94
1.87
HCOO-
1b
8.75
8.75
8.74
8.68
7.67
7.66
7.62
7.60
7.57
7.52
7.45
6.65
6.62
6.53
6.50
8.5
8.0
7.5
7.0
6.5
f1 (ppm)
2.02
1.01
1.05
1.12
0.91
1.09
1.06
1.01
0.99
3.07
3.49
3.03
6.00
6.06
11.5
10.5
9.5
8.5
7.5
6.5
5.5
4.5
3.5
2.5
1.5
0.5
-0.5
f1 (ppm)

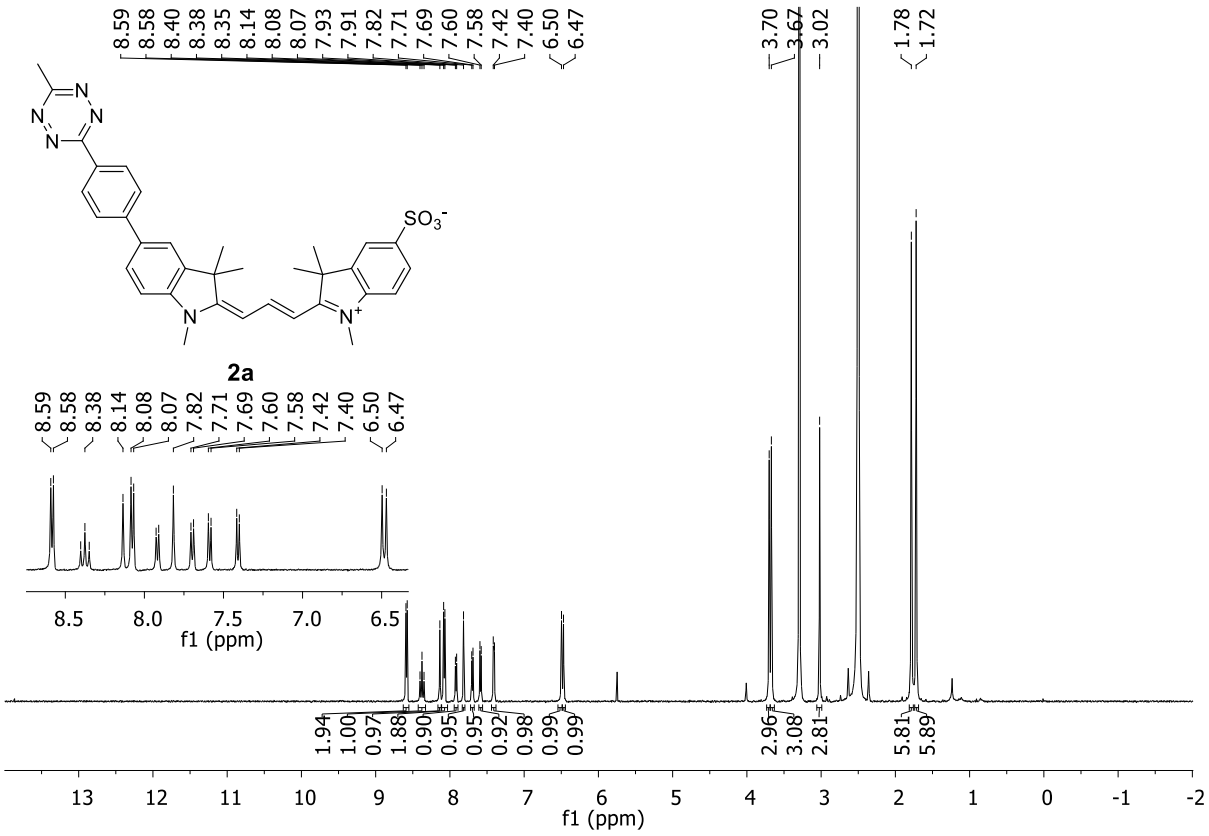

178.2
175.9
168.6
165.0
152.5
143.9
142.9
142.5
130.2
130.2
130.1
127.6
123.5
122.6
112.9
112.4
105.5
103.8
73.7
71.5
62.2
51.2
32.2
28.4
28.0
21.0
230
210
190
170
150
130
110
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

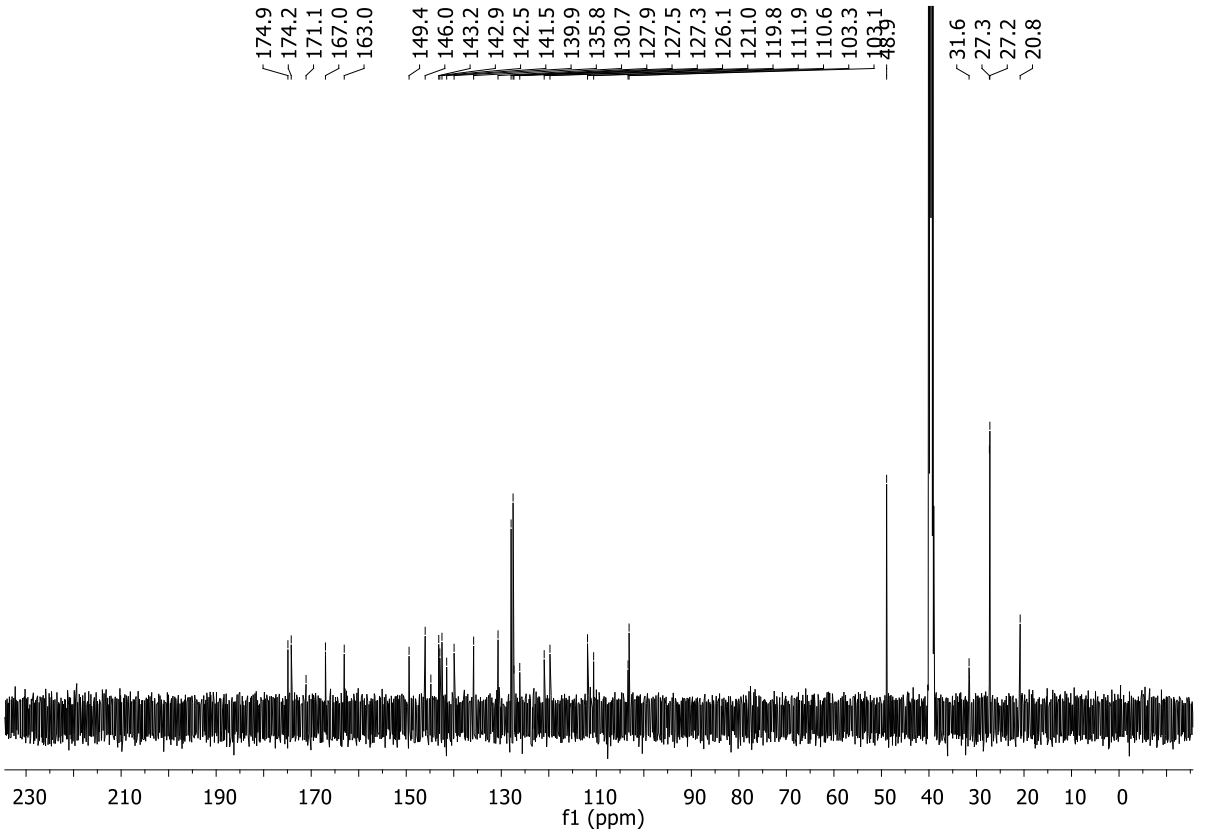

8.35 8.32 8.29 8.01 8.01 7.81 7.80 7.78 7.78 7.77 7.76 7.70 7.70 7.68 7.68 7.42 7.40 7.27 7.26 6.50 6.47 6.44 6.41
3.66 3.60
1.69 1.68
I
SO3-
N
N+
6a
8.32 8.01 8.01 7.81 7.80 7.68 7.68 7.42 7.40 7.27 7.26 6.50 6.47 6.44 6.41
8.5 8.0 7.5 7.0 6.5
f1 (ppm)
1.00 0.97 1.02 0.92 0.97 0.99 1.04 1.00 0.95 2.93 2.87 5.93 5.79
13 12 11 10 9 8 7 6 5 4 3 2 1 0 -1 -2
f1 (ppm)

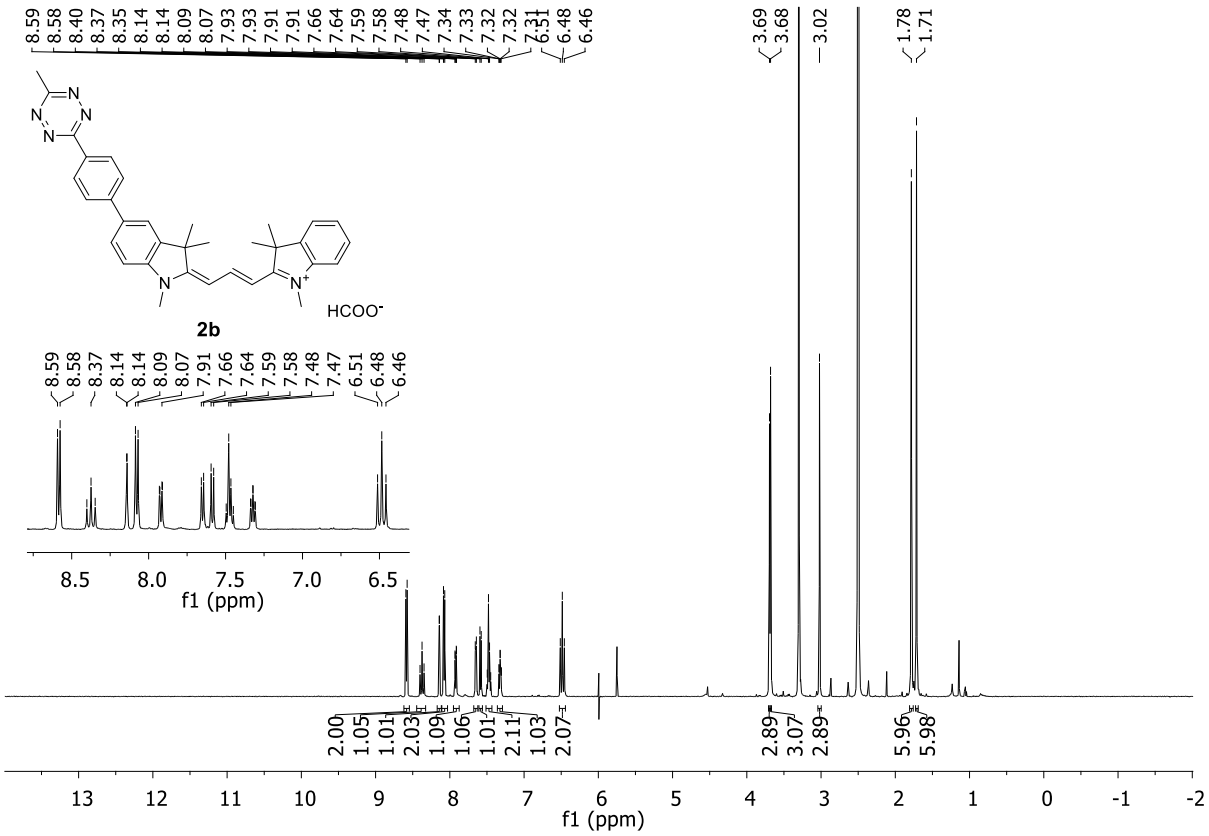

175.1 173.4 149.5 146.0 142.8 142.6 142.5 140.0 137.0 131.0 126.1 119.7 113.4 110.7 103.5 102.6 89.2
48.9 48.7 31.6 31.3 27.1
230 210 190 170 150 130 110 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

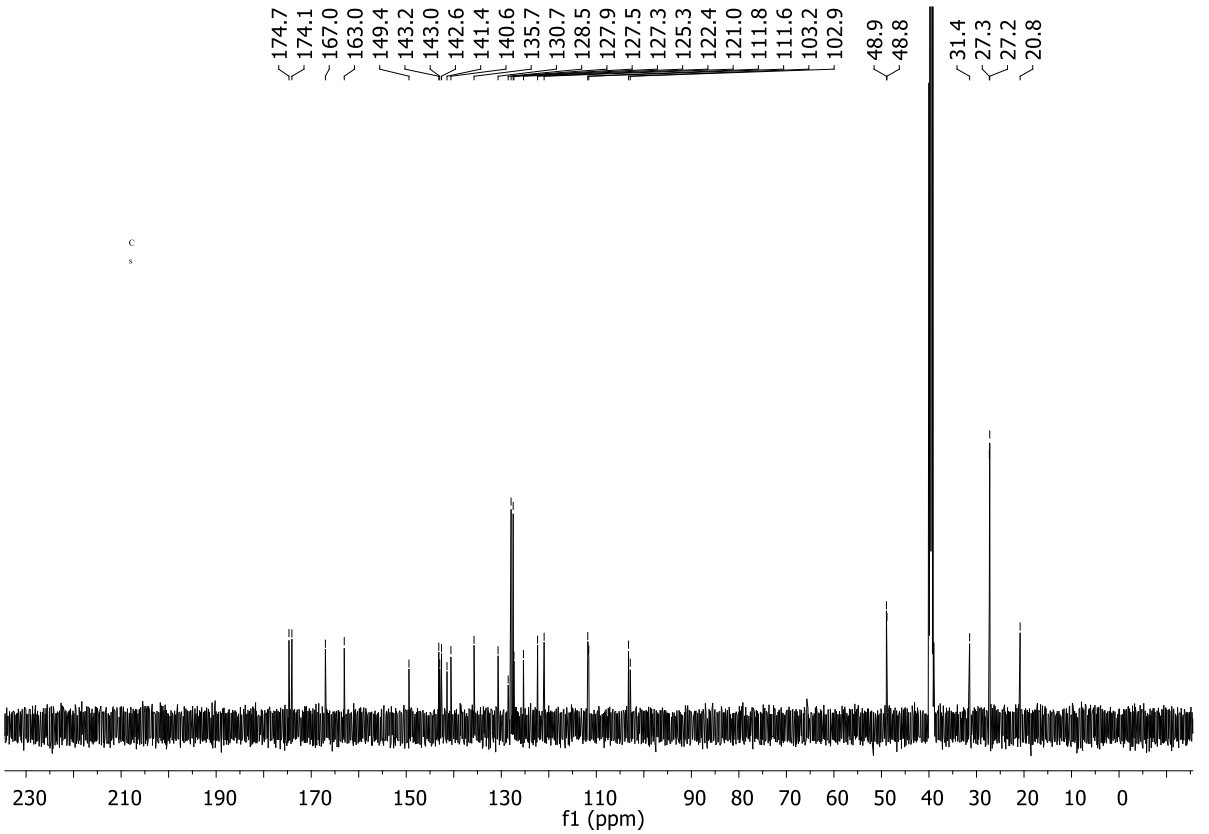

8.34
8.31
8.15
8.03
8.02
7.79
7.78
7.77
7.77
7.65
7.64
7.50
7.49
7.47
7.46
7.33
7.32
7.30
7.28
7.26
6.51
6.48
6.41
6.39
3.67
3.59
1.68
1.67
I
N
N+
HCOO-
6b
8.31
8.15
8.03
8.02
7.65
7.64
7.49
7.46
7.32
7.28
7.26
6.51
6.48
6.41
6.39
8.5
8.0
7.5
7.0
6.5
f1 (ppm)
1.23
0.89
1.00
1.03
1.10
1.02
1.12
1.09
1.13
1.04
0.98
2.93
2.83
6.24
5.82
13
12
11
10
9
8
7
6
5
4
3
2
1
0
-1
-2
f1 (ppm)

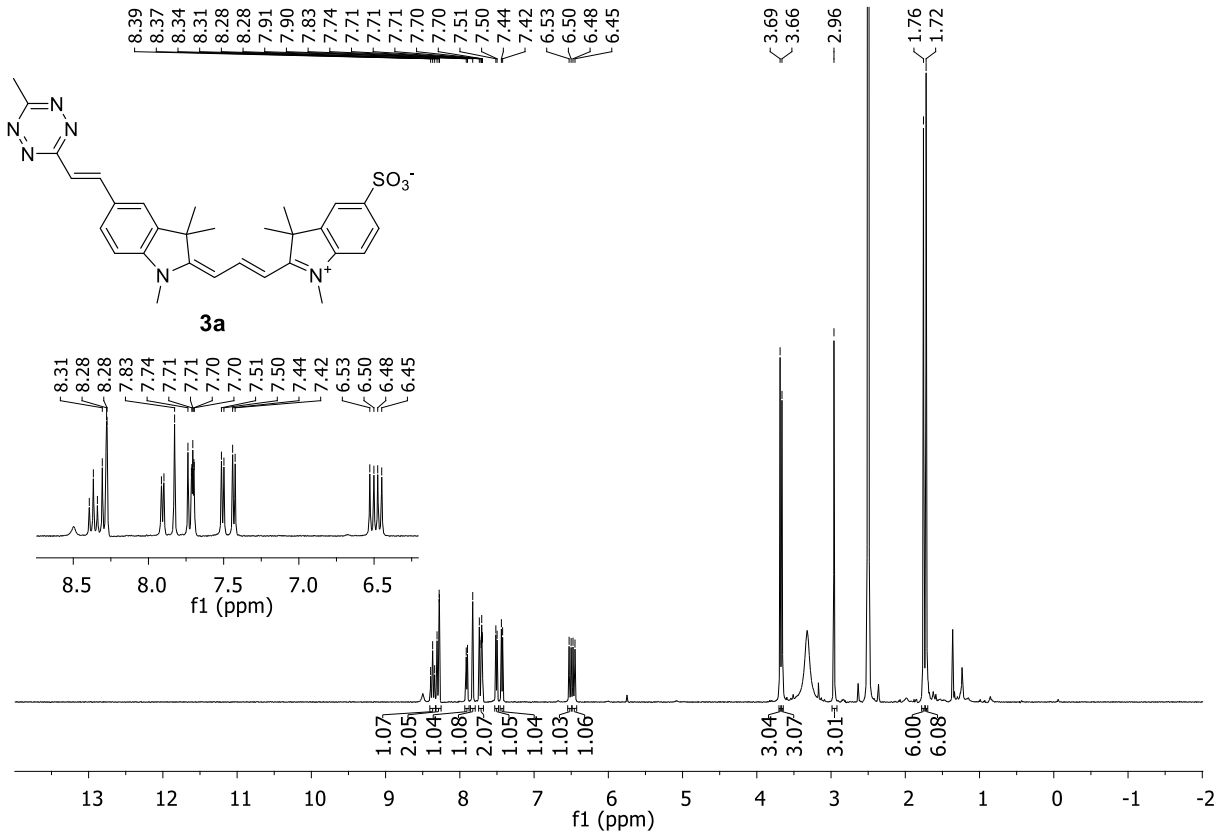

174.9
173.2
163.0
149.5
142.8
142.6
140.7
137.0
131.0
128.6
125.5
122.4
113.4
111.7
103.4
102.4
89.1
49.0
48.7
31.5
31.3
27.2
27.1
DCM
230
210
190
170
150
130
110
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

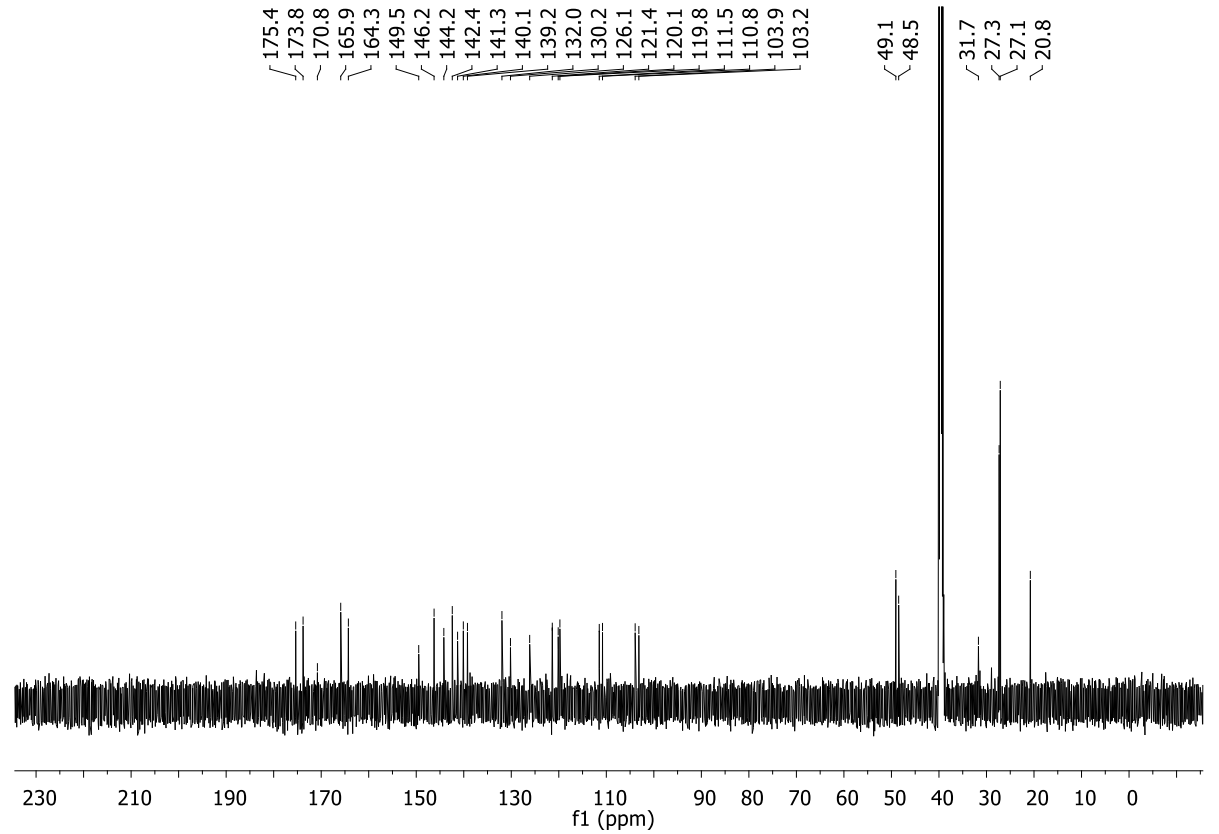

8.59
8.58
8.40
8.38
8.35
8.14
8.08
8.07
7.93
7.91
7.82
7.71
7.69
7.60
7.58
7.42
7.40
6.50
6.47
3.70
3.67
3.02
1.78
1.72
SO3-
N+
2a
f1 (ppm)
1.94
1.00
0.97
1.88
0.90
0.95
0.95
0.92
0.98
0.99
0.99
2.96
3.08
2.81
5.81
5.89

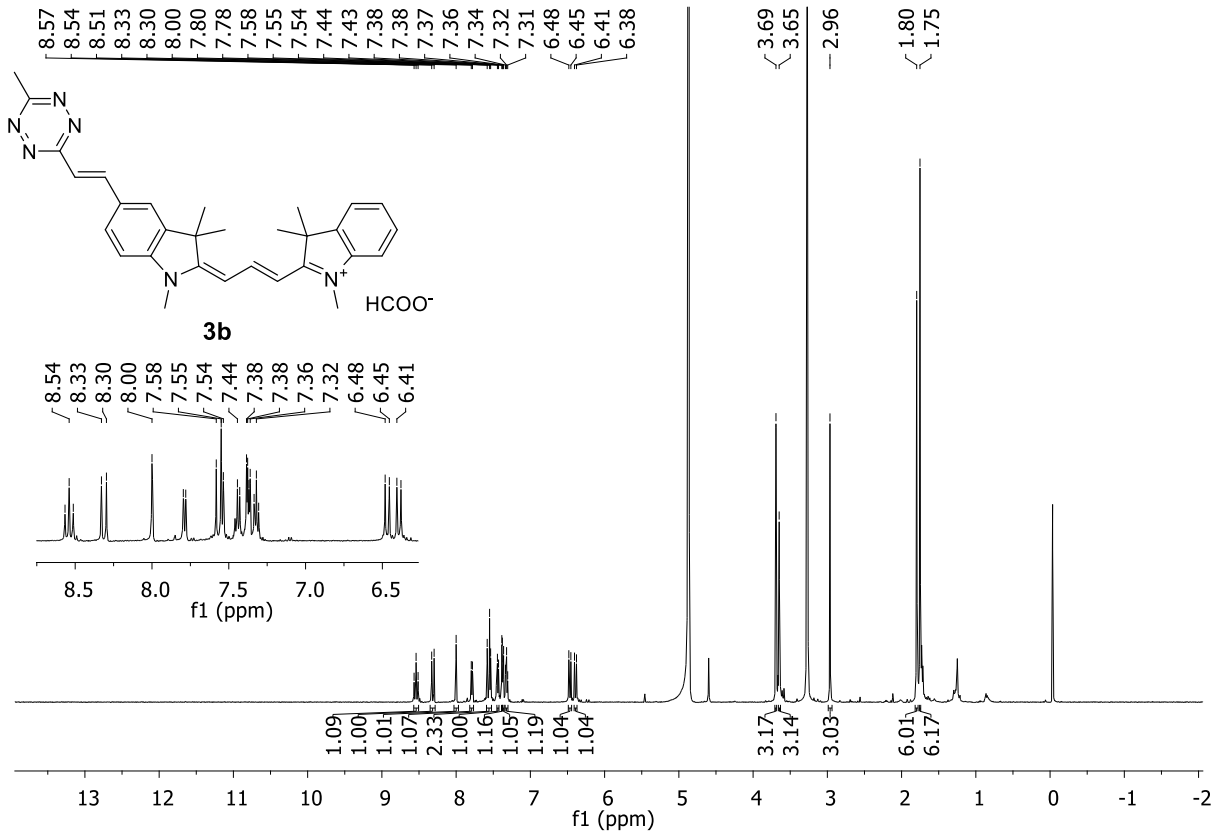

174.9
174.2
171.1
167.0
163.0
149.4
146.0
143.2
142.9
142.5
141.5
139.9
135.8
130.7
127.9
127.5
127.3
126.1
121.0
119.8
111.9
110.6
103.3
103.1
48.9
31.6
27.3
27.2
20.8
f1 (ppm)

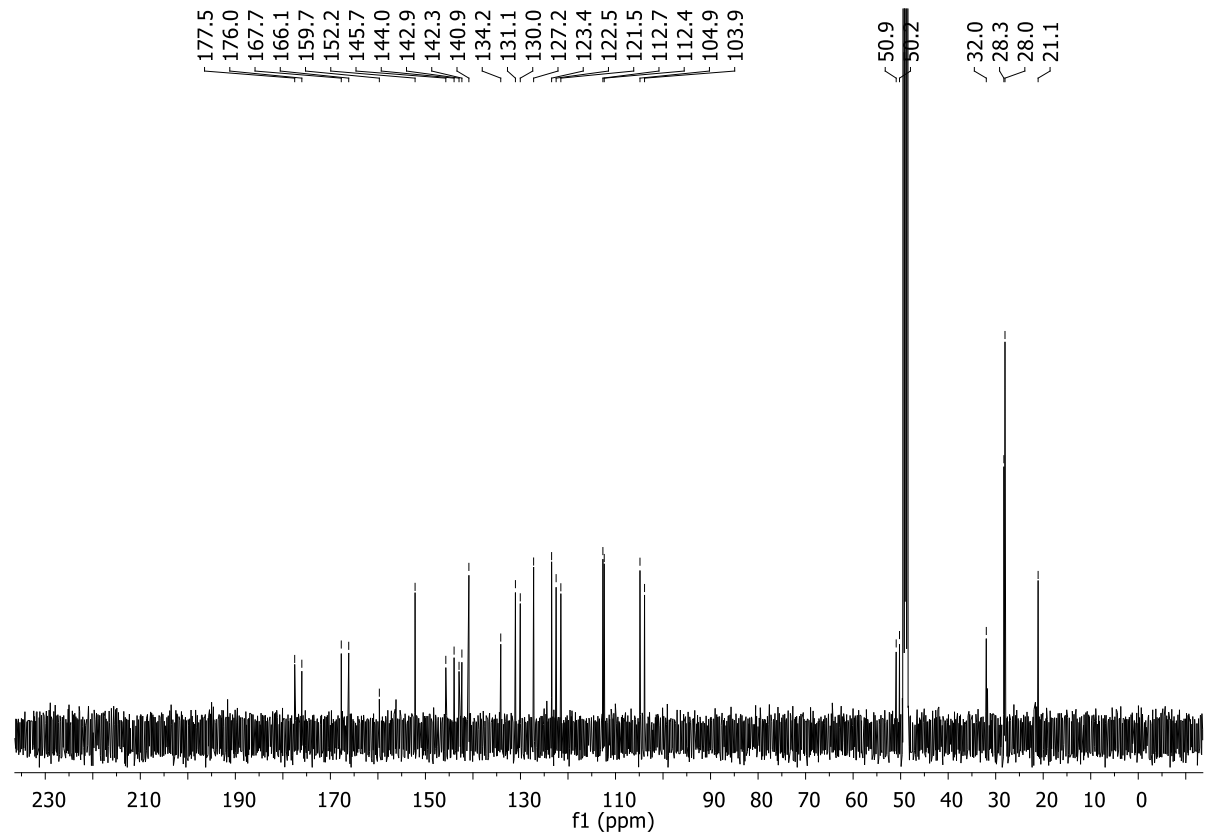

8.59 8.58 8.40 8.37 8.35 8.14 8.14 8.09 8.07 7.93 7.93 7.91 7.91 7.66 7.64 7.59 7.58 7.48 7.47 7.34 7.33 7.32 7.32 7.31 6.51 6.48 6.46
3.69 3.68 3.02 1.78 1.71
HCOO-
2b
8.59 8.58 8.37 8.14 8.14 8.09 8.07 7.91 7.66 7.64 7.59 7.58 7.48 7.47 6.51 6.48 6.46
8.5 8.0 7.5 7.0 6.5
f1 (ppm)
2.00 1.05 1.01 2.03 1.09 1.06 1.01 2.11 1.03 2.07 2.89 3.07 2.89 5.96 5.98
13 12 11 10 9 8 7 6 5 4 3 2 1 0 -1 -2
f1 (ppm)


174.7 174.1 167.0 163.0 149.4 143.2 143.0 142.6 141.4 140.6 135.7 130.7 128.5 127.9 127.5 127.3 125.3 122.4 121.0 111.8 111.6 103.2 102.9 48.9 48.8 31.4 27.3 27.2 20.8
230 210 190 170 150 130 110 90 80 70 60 50 40 30 20 10 0
f1 (ppm)


8.39
8.37
8.34
8.31
8.28
8.28
7.91
7.90
7.83
7.74
7.71
7.71
7.71
7.70
7.70
7.51
7.50
7.44
7.42
6.53
6.50
6.48
6.45
3.69
3.66
2.96
1.76
1.72
SO3-
N
N+
3a
8.31
8.28
8.28
7.83
7.74
7.71
7.71
7.70
7.70
7.51
7.50
7.44
7.42
6.53
6.50
6.48
6.45
8.5
8.0
7.5
7.0
6.5
f1 (ppm)
1.07
2.05
1.04
1.08
2.07
1.05
1.04
1.03
1.06
3.04
3.07
3.01
6.00
6.08
13
12
11
10
9
8
7
6
5
4
3
2
1
0
-1
-2
f1 (ppm)


175.4
173.8
170.8
165.9
164.3
149.5
146.2
144.2
142.4
141.3
140.1
139.2
132.0
130.2
126.1
121.4
120.1
119.8
111.5
110.8
103.9
103.2
49.1
48.5
31.7
27.3
27.1
20.8
230
210
190
170
150
130
110
90
80
70
60
50
40
30
20
10
0
f1 (ppm)


8.57 8.54 8.51 8.33 8.30 8.00 7.80 7.78 7.58 7.55 7.54 7.44 7.43 7.38 7.38 7.37 7.36 7.34 7.32 7.31 6.48 6.45 6.41 6.38
3.69 3.65 2.96 1.80 1.75
HCOO-
3b
8.54 8.33 8.30 8.00 7.58 7.55 7.54 7.44 7.38 7.38 7.36 7.32 6.48 6.45 6.41
8.5 8.0 7.5 7.0 6.5
f1 (ppm)
1.09 1.00 1.01 1.07 2.33 1.00 1.16 1.05 1.19 1.04 1.04 3.17 3.14 3.03 6.01 6.17
13 12 11 10 9 8 7 6 5 4 3 2 1 0 -1 -2
f1 (ppm)


177.5 176.0 167.7 166.1 159.7 152.2 145.7 144.0 142.9 142.3 140.9 134.2 131.1 130.0 127.2 123.4 122.5 121.5 112.7 112.4 104.9 103.9
50.9 50.2 32.0 28.3 28.0 21.1
230 210 190 170 150 130 110 90 80 70 60 50 40 30 20 10 0
f1 (ppm)