NSPs: chromogenic linkers for fast, selective, and irreversible cysteine modification

Supporting Information

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Contents

Material and Methods

Chemicals were purchased from Sigma Aldrich, MedChemExpress, Fluorochem, Novabiochem or Bachem and used as received unless otherwise noted.

NMR experiments were performed at 25 °C on Bruker Avance III NMR spectrometers operating at 600 MHz, 500 MHz or 400 MHz proton frequency. NMR spectrometers were equipped with inverse dual channel, broadband probe heads with z-gradients. ^{13}C shifts were determined by 1D or 2D (HMBC and HMQC/HSQC) NMR experiments. Spectra were referenced to residual non-deuterated ($CHCl_3$) or partially deuterated (DMSO-d_5, CHD_2OD) solvent peaks of the NMR solvent (^1H NMR) or the signal of the deuterated solvent (^{13}C NMR).

ESI-HRMS experiments were conducted with a Bruker maXis 4G or a Bruker maXis II. For details of HRMS analysis of modified proteins see respective section.

LC-MS analyzes were performed with Shimadzu LC-MS 2020 systems. The machines were additionally equipped with PDA detectors. Column: Agilent Zorbax RR Eclipse, XDB-C18, 4.6 × 75 mm, 3.5 µm. Solvents were A (water : acetonitrile : formic acid = 97 : 3 : 0.1) and B (acetonitrile : water : formic acid = 95 : 5 : 0.1). The flow rate was set to 1 mL/min and the temperature to 40 °C. Method 1: 0 min – 0% B; 1 min – 5% B; 9 min – 95% B; 11 min – 95% B. Method 2: 0 min – 0% B; 18 min – 70% B; 20 min 95% B; 22 min – 95% B. The LC-MS method for the determination of plasma stability of **3a**, **3f**, **3g** is specified in the respective section of the Supporting Information.

Preparative HPLC separations were carried out with a Water Prep LC 4000 System; column: Dr. Maisch, Reprospher 100 C18-DE, 40 × 150 mm, 5 µm, 100Å. (A) water/acetonitrile/TFA = 97 : 3 : 0.1; (B) acetonitrile/TFA = 99.9 : 0.1. Flow rates were between 10 and 20 mL/min. Isolated yields for compounds purified by preparative HPLC do not consider possible residual TFA after lyophilization.

Peptides used in synthesis were prepared with a Liberty Blue peptide synthesizer from CEM using standard methods and DIC and Oxyma as coupling agents.

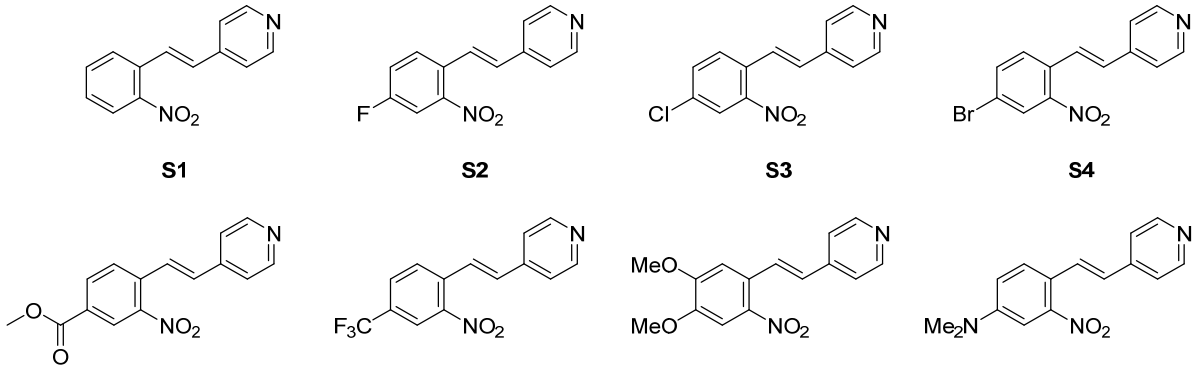
NH_4HCO_3-buffer (100 mM, pH 8.0) was degassed by bubbling with N_2 for 30 minutes before use.

Reactions at 37 °C were carried out in HPLC vials and agitated in a Thermomixer (Eppendorf, Thermomixer C, equipped with a cryo block and a ThermoTop) at 650-700 rpm if not indicated otherwise.

Synthesis

Preparation of 2-nitrostyryl pyridinium ions

Preparation of 2-nitrostyryl pyridines



S1 **S2** **S3** **S4**

S5 **S6** **S7** **S8**

Nitrostyryl pyridines were prepared according to a published procedure where **S1** and **S7** were already described.[1]

General procedure 1:
A mixture of the corresponding 2-nitrobenzaldehyde (3.0 mmol) and 4-picoline (0.97 mL, 0.93 g, 10 mmol) in acetic anhydride (3 mL) was heated at 140 °C oil bath temperature for 24 hours. After cooling, the mixture was diluted with CH_2Cl_2 (50 mL) and the organic phase was washed with sat. aq. $NaHCO_3$ solution (50 mL). After drying with Na_2SO_4 and the removal of volatiles, the crude product was purified by column chromatography over silica (cyclohexane : AcOEt = 100 : 0 to 55 : 45). The products were obtained as yellow solids.

S2: 616 mg, 84%. HRMS (m/z): (ESI+) calcd $[M+H]^+$: 245.0721, found: 245.0724. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.64 – 8.59 (m, 2H), 7.75 (ddd, *J* = 13.6, 8.1, 2.7 Hz, 3H), 7.42 – 7.35 (m, 3H), 6.92 (d, *J* = 16.1 Hz, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 162.9, 160.9, 150.5, 143.7, 131.3, 131.3, 130.5, 130.5, 128.7, 128.6, 127.6, 127.6, 121.3, 121.2, 121.1, 112.8, 112.6.

S3: 656 mg, 84%. HRMS (m/z): (ESI+) calcd $[M+H]^+$: 261.0425, found: 261.0428. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.67 – 8.61 (m, 1H), 8.03 (d, *J* = 2.2 Hz, 1H), 7.75 (d, *J* = 16.2 Hz, 1H), 7.71 (d, *J* = 8.5 Hz, 1H), 7.62 (ddd, *J* = 8.5, 2.1, 0.6 Hz, 1H), 7.43 – 7.38 (m, 1H), 6.98 (d, *J* = 16.1 Hz, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 150.3, 148.3, 143.8, 135.0, 133.7, 131.7, 130.7, 129.8, 127.6, 125.3, 121.5.

S4: 723 mg, 79%. HRMS (m/z): (ESI+) calcd. $[M+H]^+$: 304.9921, found: 304.9922. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.63 – 8.57 (m, 2H), 8.26 (d, *J* = 2.0 Hz, 1H), 8.00 (dd, *J* = 8.5, 2.0 Hz, 1H), 7.94 (d, *J* = 8.5 Hz,

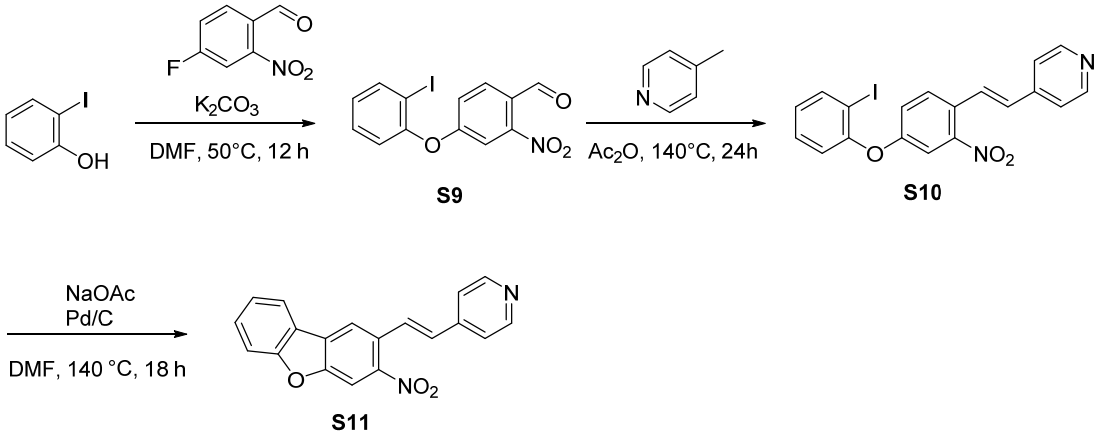
1H), 7.65 (d, *J* = 16.2 Hz, 1H), 7.60 – 7.54 (m, 2H), 7.35 (d, *J* = 16.2 Hz, 1H). ^{13}C NMR (101 MHz, DMSO) δ 150.3, 148.5, 143.3, 136.3, 131.4, 130.2, 127.2, 1226.7, 121.3, 121.3.

S5: 631 mg, 74%. HRMS (m/z): (ESI+) calcd. $[M+H]^+$: 285.0870, found: 285.0870. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.69 – 8.62 (m, 3H), 8.28 (ddd, *J* = 8.1, 1.7, 0.6 Hz, 1H), 7.85 (d, *J* = 8.2 Hz, 1H), 7.81 (dd, *J* = 16.2, 0.7 Hz, 1H), 7.45 – 7.40 (m, 2H), 7.09 (d, *J* = 16.1 Hz, 1H), 3.99 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 164.8, 150.4, 148.1, 143.6, 136.0, 133.9, 133.0, 131.1, 128.9, 127.7, 126.4, 121.6, 53.0.

S6: 684 mg, 77%. HRMS (m/z): (ESI+) calcd. $[M+H]^+$: 295.0689, found: 295.0691. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.69 – 8.63 (m, 1H), 8.31 (d, *J* = 1.6 Hz, 1H), 7.90 (s, 1H), 7.82 (d, *J* = 16.1 Hz, 1H), 7.45 – 7.40 (m, 1H), 7.07 (d, *J* = 16.2 Hz, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 150.5, 147.9, 143.3, 135.6, 135.6, 133.4, 131.6, 131.4, 130.0, 129.9, 129.7, 127.3, 122.6, 122.6, 121.5.

S8: 488 mg, 60%. HRMS (m/z): (ESI+) calcd.: $[M+H]^+$: 270.1237, found: 270.1241. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.55 – 8.49 (m, 2H), 7.63 (d, *J* = 16.1 Hz, 1H), 7.58 (d, *J* = 8.8 Hz, 1H), 7.34 – 7.28 (m, 2H), 7.13 (d, *J* = 2.8 Hz, 1H), 6.85 (dd, *J* = 8.9, 2.8 Hz, 1H), 6.79 (d, *J* = 16.1 Hz, 1H), 3.02 (s, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 150.3, 150.2, 149.5, 144.7, 128.8, 128.2, 126.5, 120.9, 118.3, 116.2, 106.8, 40.2.

S11: The preparation of the diarylether **S9** and the cyclization of **S10** to the dibenzofuran **S11** was performed in analogy to a published procedures.[2]

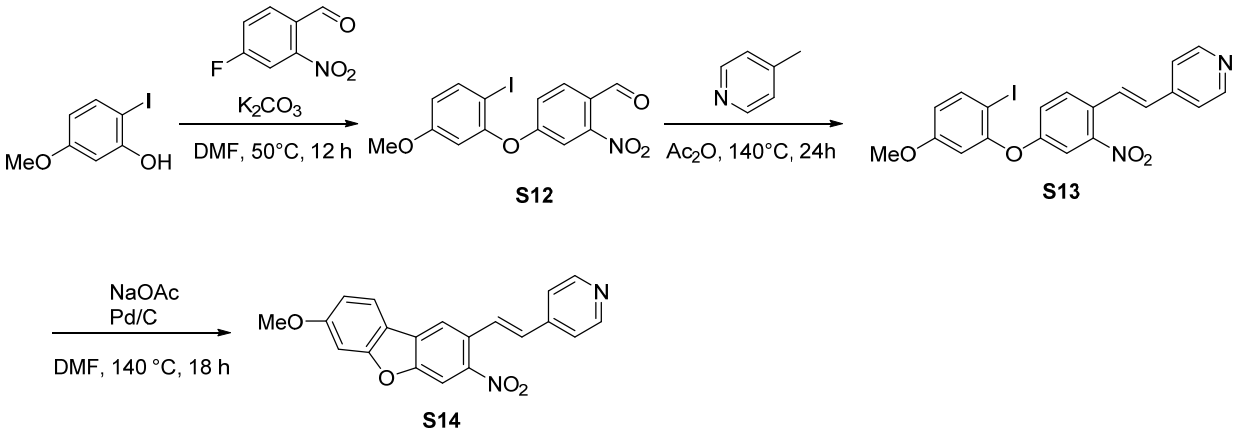


4-Fluoro-2nitrobenzaldehyde (0.51 g, 3.0 mmol) was dissolved in DMF (10 mL). First 2-iodophenol (0.66 g, 3.0 mmol) and then K_2CO_3 (0.83 g, 6.0 mmol) were added and the reaction was purged with N_2 and stirred at room temperature for 6 h and then at 50 °C for 12 h. When TLC indicated completion, the mixture was allowed to cool to room temperature, poured into aqueous NH_4Cl (20 mL) and extracted with EtOAc (3 x 20 mL). The organic phase was washed with H_2O (40 mL), brine (40 mL), and dried with $MgSO_4$. After removal of volatiles under reduced pressure the crude product **S9** was obtained as a yellow solid that was directly used in the next step.

S10 was prepared according to *general procedure 1*: 677 mg, 51% over two steps and used directly in the next step.

S11: A 25-mL round bottom flask was charged with **S10** (0.44 g, 1.0 mmol). To the vial was added sodium acetate (0.25 g, 3.0 mmol), and 10% Pd/C (0.11 g) and the vial was purged with nitrogen before adding dry DMF (6 mL). The reaction was stirred at 140 °C for 18 h. The reaction mixture was diluted with EtOAc (30 mL) and passed through a pad of silica to remove Pd/C particles. The filtrate was washed with brine (3 ×), dried over Na_2SO_4 and volatiles removed under reduced pressure. The crude material was purified by column chromatography over silica (cyclohexane : EtOAc = 100:0 - 55:45). The product **S11** was obtained as a yellow solid (247 mg, 78%). HRMS (m/z): (ESI+) calcd. $[M+H]^+$: 317.0921, found: 317.0925. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.67 – 8.61 (m, 2H), 8.27 (s, 1H), 8.22 (s, 1H), 8.13 – 8.02 (m, 1H), 7.90 (d, *J* = 16.0 Hz, 1H), 7.72 – 7.57 (m, 2H), 7.51 – 7.42 (m, 3H), 7.02 (d, *J* = 16.0 Hz, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 158.6, 154.8, 150.4, 146.6, 144.2, 130.1, 129.9, 129.6, 129.4, 128.1, 124.1, 122.5, 121.9, 121.3, 120.3, 112.6, 109.2.

S14: **S12** and **S14** were prepared in analogy to published procedures and as described for **S9** and **S11** above.



S12: 4-fluoro-2nitrobenzaldehyde (0.51 g, 3.0 mmol) was dissolved in DMF (10 mL). 2-iodo-5-methoxyphenol (0.75 g, 3.0 mmol) and K_2CO_3 (0.83 g, 6.0 mmol) were then sequentially added and the reaction was purged with N_2 and stirred at room temperature for 6 h and then 50 °C for 12 h in an oil bath. The reaction progress was followed by TLC. After the reaction was deemed complete, the mixture was cooled to room temperature and poured into aqueous NH_4Cl (20 mL) and extracted with EtOAc (3 × 20 mL). The organic phase was washed with H_2O (40 mL), brine (40 mL), dried with anhydrous $MgSO_4$ and evaporated to dryness. The crude material was purified by column chromatography over silica (cyclohexane : EtOAc = 100:0 - 20:80) and **S12** was obtained as a yellow solid (1.05 g, 88 %). ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.32 (s, 1H), 7.99 (d, *J* = 8.6 Hz, 1H), 7.80 – 7.74 (m, 1H), 7.50 (d, *J* = 2.4 Hz, 1H), 7.22 (ddd, *J* = 8.7, 2.5, 0.7 Hz, 1H), 6.72 – 6.66 (m, 2H), 3.80 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 187.0, 161.9, 161.6, 154.4, 151.7, 140.6, 131.9, 125.2, 121.3, 114.5, 112.4, 108.5, 78.1, 55.9.

S13: was prepared according to *general procedure 1* on a 2 mmol scale in 3 mL acetic anhydride and obtained as a yellow solid (815 mg, 86%). HRMS (m/z): (ESI+) calcd. $[M+H]^+$: 475.0149, found:

475.0154. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.64 – 8.59 (m, 2H), 7.79 – 7.68 (m, 3H), 7.51 (d, *J* = 2.6 Hz, 1H), 7.41 – 7.35 (m, 2H), 7.21 (dd, *J* = 8.9, 2.6 Hz, 1H), 6.92 (d, *J* = 16.1 Hz, 1H), 6.68 – 6.60 (m, 2H), 3.78 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 161.7, 157.6, 155.3, 150.4, 148.8, 144.0, 140.4, 130.3, 130.02, 127.9, 126.6, 122.4, 121.3, 113.6, 113.3, 107.8, 78.0, 55.8.

S14: Prepared as described for **S11** above on a 1.0 mmol scale. Yellow solid (298 mg, 86%). HRMS (m/z): (ESI+) calcd. $[M+H]^+$: 347.1026, found: 347.1031. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.74 – 8.64 (m, 2H), 8.62 (s, 1H), 8.43 (s, 1H), 8.19 (d, *J* = 8.6 Hz, 1H), 7.92 (d, *J* = 16.2 Hz, 1H), 7.70 – 7.64 (m, 2H), 7.42 (d, *J* = 2.3 Hz, 1H), 7.33 (d, *J* = 16.1 Hz, 1H), 7.13 (dd, *J* = 8.6, 2.2 Hz, 1H), 3.91 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 161.6, 159.6, 154.0, 149.2, 145.5, 130.9, 130.0, 129.3, 129.0, 127.6, 123.1, 121.4, 119.9, 114.8, 113.0, 108.5, 96.8, 56.0.

Quaternization of 2-nitrostyryl pyridines

General procedure 2:

To a solution of the 2-nitrostyryl pyridine (1.0 mmol) in dry CH_3CN (5 mL) was added methyl iodide (0.13 mL; 0.28 g, 2.0 mmol) and the mixture stirred overnight at room temperature. After concentration under reduced pressure the solid product was separated by filtration and washed with diethylether. The products were obtained as yellow solids after drying under reduced pressure in quantitative yields.

1a: HRMS (m/z): (ESI+) calcd. $[M]^+$: 241.0972, found: 241.0975. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.93 (d, *J* = 6.3 Hz, 2H), 8.28 (d, *J* = 6.5 Hz, 2H), 8.15 (d, *J* = 16.2 Hz, 1H), 8.11 (dd, *J* = 8.2, 1.3 Hz, 1H), 8.02 (dd, *J* = 7.9, 1.4 Hz, 1H), 7.87 (td, *J* = 7.6, 1.3 Hz, 1H), 7.71 (td, *J* = 7.8, 1.4 Hz, 1H), 7.52 (d, *J* = 16.2 Hz, 1H), 4.30 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 151.5, 148.4, 145.5, 135.2, 134.0, 130.9, 130.3, 129.1, 127.6, 124.9, 124.2, 47.2.

1b: HRMS (m/z): (ESI+) calcd. $[M]^+$: 259.0877, found: 259.0880. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.92 (d, *J* = 6.3 Hz, 1H), 8.26 (d, *J* = 6.3 Hz, 1H), 8.09 (d, *J* = 9.7 Hz, 2H), 7.82 (d, *J* = 8.9 Hz, 1H), 7.50 (d, *J* = 16.1 Hz, 1H), 4.29 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 151.4, 145.6, 134.0, 131.3, 131.2, 127.8, 126.8, 124.2, 121.5, 121.3, 112.7, 112.5, 47.2.

1c: HRMS (m/z): (ESI+) calcd. $[M]^+$: 275.0582, found: 275.0584. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.97 – 8.91 (m, 2H), 8.35 – 8.25 (m, 2H), 8.23 (d, *J* = 2.1 Hz, 1H), 8.11 – 8.03 (m, 2H), 7.97 (dd, *J* = 8.6, 2.2 Hz, 1H), 7.56 (d, *J* = 16.2 Hz, 1H), 4.30 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 151.2, 148.8, 145.6, 134.7, 133.7, 133.7, 130.5, 128.9, 128.3, 124.7, 124.3, 47.3.

1d: HRMS (m/z): (ESI+) calcd. $[M]^+$: 319.0077, found: 319.0084. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.93 (d, *J* = 6.4 Hz, 2H), 8.33 (d, *J* = 2.0 Hz, 1H), 8.27 (d, *J* = 6.8 Hz, 2H), 8.09 (dd, *J* = 8.4, 2.1 Hz, 1H), 8.05 (d, *J* =

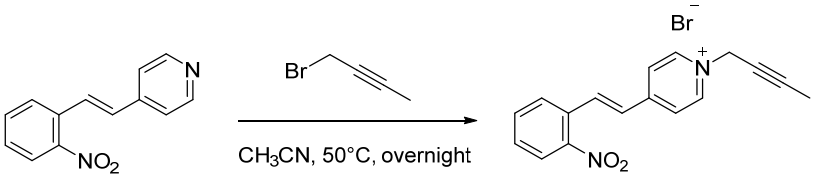
16.2 Hz, 1H), 7.97 (d, *J* = 8.5 Hz, 1H), 7.57 (d, *J* = 16.2 Hz, 1H), 4.29 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 151.2, 148.9, 145.6, 136.6, 133.8, 130.6, 129.2, 128.3, 127.5, 124.3, 122.9, 47.3.

1e: HRMS (m/z): (ESI+) calcd. $[M]^+$: 299.1026, found: 299.1028. ^{1}H NMR (500 MHz, DMSO-d6) δ 8.96 (d, *J* = 6.6 Hz, 2H), 8.52 (d, *J* = 1.8 Hz, 1H), 8.35 (dd, *J* = 8.2, 1.8 Hz, 1H), 8.30 (d, *J* = 6.9 Hz, 2H), 8.18 (d, *J* = 13.2 Hz, 1H), 8.16 (d, *J* = 5.1 Hz, 1H), 7.63 (d, *J* = 16.2 Hz, 1H), 4.31 (s, 3H), 3.94 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 164.2, 151.0, 148.3, 145.7, 134.4, 134.1, 133.7, 131.2, 129.9, 129.5, 125.4, 124.5, 52.9, 47.3.

1f: HRMS (m/z): (ESI+) calcd. $[M]^+$: 309.0845, found: 309.0850. ^{1}H NMR (500 MHz, MeOD-d_4) δ 8.89 – 8.84 (m, 2H), 8.43 (d, *J* = 1.8 Hz, 1H), 8.31 – 8.22 (m, 3H), 8.21 (d, *J* = 8.2 Hz, 1H), 8.12 (dd, *J* = 8.3, 1.9 Hz, 1H), 7.55 (d, *J* = 16.2 Hz, 1H), 4.40 (s, 3H). ^{13}C NMR (126 MHz, MeOD-d_4) δ 153.7, 149.8, 146.8, 136.3, 135.9, 135.8, 133.3, 133.0, 131.9, 131.3, 131.3, 131.3, 130.4, 126.2, 125.4, 123.4, 123.4, 123.3, 123.3, 48.4.

1g: HRMS (m/z): (ESI+) calcd. $[M]^+$: 301.1183, found: 301.1186. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.90 (d, *J* = 6.8 Hz, 2H), 8.23 (d, *J* = 6.9 Hz, 2H), 8.18 (d, *J* = 16.1 Hz, 1H), 7.68 (s, 1H), 7.51 (d, *J* = 16.1 Hz, 1H), 7.46 (s, 1H), 4.28 (s, 3H), 4.00 (s, 3H), 3.92 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 152.9, 151.9, 149.7, 145.4, 141.5, 135.7, 126.4, 124.7, 124.0, 110.2, 108.0, 56.6, 56.3, 47.1.

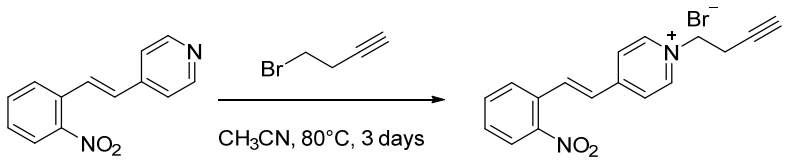
1h was prepared in analogy to *general procedure 2* with 1-bromo-2-butyne as the electrophile and under heating.



S1 **1h**

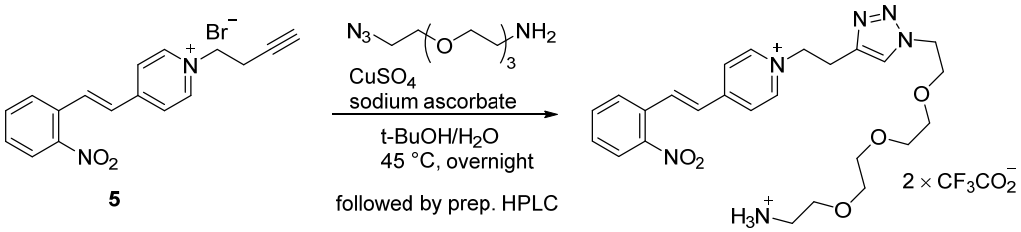
To a solution of **S1** (0.11 g, 0.50 mmol) in dry CH_3CN (15 mL) was added 1-bromo-2-butyne (0.13 g, 1.0 mmol) and the resulting mixture was stirred at 50 °C overnight. After concentration under reduced pressure the solid product was separated by filtration and washed with Et_2O. **1h** was obtained as a yellow solid after drying under reduced pressure (180 mg, 99%). HRMS (m/z): (ESI+) calcd. $[M]^+$: 279.1128, found: 279.1130. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.10 (d, *J* = 6.4 Hz, 2H), 8.34 (d, *J* = 6.4 Hz, 2H), 8.19 (d, *J* = 16.2 Hz, 1H), 8.11 (d, *J* = 8.1 Hz, 1H), 8.04 (d, *J* = 7.8 Hz, 1H), 7.87 (t, *J* = 7.6 Hz, 1H), 7.72 (t, *J* = 7.8 Hz, 1H), 7.59 (d, *J* = 16.2 Hz, 1H), 5.54 (d, *J* = 3.2 Hz, 2H), 1.95 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 152.7, 148.4, 144.3, 135.8, 134.0, 131.0, 130.2, 129.1, 127.6, 124.9, 124.7, 87.1, 71.1, 49.6, 3.3.

5 was prepared in analogy to *general procedure 2* with 4-bromo-1-butyne as the electrophile and under heating.



S1 **5**

To a solution of **S1** (0.11 g, 0.50 mmol) in dry CH_3CN (15 mL) was added 4-bromo-1-butyne (0.13 g, 1.0 mmol) and the mixture was heated to 80 °C for 3 days. After concentration under reduced pressure the solid product was separated by filtration and washed with Et_2O. **5** was obtained as a yellow solid after drying under reduced pressure (180 mg, 99%). HRMS (m/z): (ESI+) calcd. $[M]^+$: 279.1128, found: 279.1129. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.06 (d, *J* = 6.8 Hz, 1H), 8.35 (d, *J* = 6.9 Hz, 1H), 8.20 (d, *J* = 16.1 Hz, 1H), 8.12 (dd, *J* = 8.2, 1.3 Hz, 1H), 8.02 (dd, *J* = 8.0, 1.4 Hz, 1H), 7.87 (td, *J* = 7.7, 1.3 Hz, 1H), 7.72 (td, *J* = 7.8, 1.4 Hz, 1H), 7.53 (d, *J* = 16.1 Hz, 1H), 4.70 (t, *J* = 6.6 Hz, 1H), 3.09 (t, *J* = 2.6 Hz, 1H), 2.97 (td, *J* = 6.7, 2.7 Hz, 1H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 161.8, 157.9, 154.5, 145.3, 143.5, 140.4, 139.8, 138.6, 137.1, 134.4, 133.9, 88.7, 84.6, 67.4, 29.8.



1i

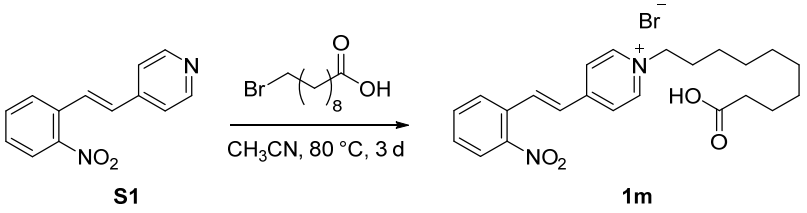
1i: To a suspension of 1-amino-11-azido-3,6,9-trioxaundecane (47 µL, 52 mg, 0.24 mmol) and **5** (54 mg, 0.15 mmol) in water / tert-butanol (1 mL : 1.8 mL) was added aq. sodium ascorbate (1.0 M, 0.15 mL) and aq. $CuSO_4$ (1.0 M, 30 µL) and the resulting mixture was stirred overnight at 45 °C. Subsequently volatiles were removed under reduced pressure and the resulting residue was purified by reversed phase HPLC. The product **1i** was obtained after lyophilization. Lyophilization was repeated 3 more times under addition of water (5 mL). The product was obtained as a red oil (72 mg, 69%). HRMS (m/z): (ESI+) calcd. $[M]^+$: 497.2507, found: 497.2509. ^{1}H NMR (500 MHz, CD_3OD) δ 8.77 (d, *J* = 7.0 Hz, 2H), 8.23 (d, *J* = 16.1 Hz, 1H), 8.20 – 8.16 (m, 2H), 8.12 (dd, *J* = 8.2, 1.3 Hz, 1H), 7.96 (dd, *J* = 8.1, 1.5 Hz, 1H), 7.87 (s, 1H), 7.81 (td, *J* = 7.7, 1.3 Hz, 1H), 7.68 (ddd, *J* = 8.6, 7.5, 1.4 Hz, 1H), 7.39 (d, *J* = 16.1 Hz, 1H), 4.90 (t, *J* = 6.8 Hz, 2H), 4.58 – 4.53 (m, 2H), 3.87 (dd, *J* = 5.6, 4.7 Hz, 2H), 3.72 – 3.53 (m, 12H), 3.46 (t, *J* = 6.8 Hz, 2H), 3.12 (t, *J* = 5.1 Hz, 2H). ^{13}C NMR (126 MHz, CD_3OD, signals arising from TFA are not listed) δ 154.9, 150.0, 145.9, 143.1, 138.3, 135.0, 132.0, 132.0, 130.3, 128.3, 126.1, 126.0, 125.3, 71.5, 71.4, 71.3, 71.2, 70.4, 67.9, 61.3, 51.3, 40.6, 27.9.

1j: HRMS (m/z): (ESI+) calcd. $[M]^+$: 284.1394, found: 284.1396. ^{1}H NMR (600 MHz, CD_3OD) δ 8.66 (d, *J* = 6.9 Hz, 2H), 8.09 – 8.02 (m, 3H), 7.89 (d, *J* = 9.0 Hz, 1H), 7.26 (d, *J* = 16.0 Hz, 1H), 7.23 (d, *J* = 2.7 Hz, 1H), 7.07 (dd, *J* = 9.0, 2.8 Hz, 1H), 4.29 (s, 3H), 3.12 (s, 6H). ^{13}C NMR (151 MHz, MeOD) δ 155.2, 153.2, 152.3, 146.0, 137.4, 130.4, 124.7, 122.9, 116.9, 116.8, 107.5, 47.6, 40.2.

1k was prepared in analogy to *general procedure 2* under heating. Methyl iodide (63 µL, 0.14 g, 1.0 mmol) was added to a solution of **S11** (0.16 g, 0.51 mmol) in dry CH_3CN (5 mL). The resulting mixture was stirred at 50 °C for 2 days. After concentration under reduced pressure the solid product was separated by filtration and washed with Et_2O. **1k** was obtained as a yellow solid after drying under reduced pressure (229 mg, 98 %). HRMS (m/z): (ESI+) calcd. $[M]^+$: 331.1077, found: 331.1082. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.94 (d, *J* = 6.8 Hz, 2H), 8.87 (s, 1H), 8.59 (s, 1H), 8.35 (dt, *J* = 7.7, 1.1 Hz, 1H), 8.31 (d, *J* = 6.8 Hz, 2H), 8.28 (d, *J* = 16.0 Hz, 1H), 7.90 – 7.85 (m, 1H), 7.72 (ddd, *J* = 8.5, 7.3, 1.4 Hz, 1H), 7.63 – 7.54 (m, 2H), 4.30 (s, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 157.8, 154.6, 151.7, 147.2, 145.6, 136.2, 130.2, 128.6, 126.8, 126.3, 124.5, 124.1, 122.6, 121.9, 121.7, 112.4, 109.3, 47.2.

1l was prepared in analogy to *general procedure 2* under heating. Methyl iodide (63 µL, 0.14 g, 1.0 mmol) was added to a solution of **S14** (0.17 g, 0.50 mmol) in dry CH_3CN (5 mL). The resulting mixture was stirred at 50 °C for 2 days. After concentration under reduced pressure the solid product was separated by filtration and washed with Et_2O. **1l** was obtained as a yellow solid after drying under reduced pressure (244 mg, > 99 %). HRMS (m/z): (ESI+) calcd. $[M]^+$: 361.1183, found: 361.1189. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.95 – 8.90 (m, 2H), 8.69 (s, 1H), 8.53 (s, 1H), 8.33 – 8.26 (m, 3H), 8.20 (d, *J* = 8.6 Hz, 1H), 7.54 (dd, *J* = 16.1, 3.3 Hz, 1H), 7.47 (t, *J* = 2.3 Hz, 1H), 7.16 (dd, *J* = 8.6, 2.2 Hz, 1H), 4.30 (s, 3H), 3.92 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 161.8, 159.7, 154.6, 151.7, 145.8, 145.6, 136.6, 129.0, 126.7, 126.6, 124.0, 123.1, 120.4, 114.7, 113.2, 108.9, 96.9, 56.1, 47.2.

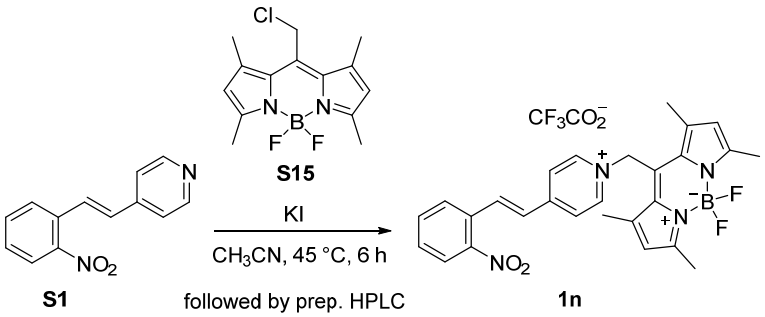
1m was prepared in analogy to *general procedure 2* with 10-bromodecanoic acid as the electrophile under heating. A solution of 10-bromodecanoic acid (0.19 g, 0.75 mmol) and **S1** (0.11g, 0.50 mmol) in dry CH_3CN (5 mL) was heated to 80 °C for 3 days. After concentration under reduced pressure the solid product was separated by filtration and washed with Et_2O. **1m** was obtained as a yellow solid after drying under reduced pressure (239 mg, > 99 %).



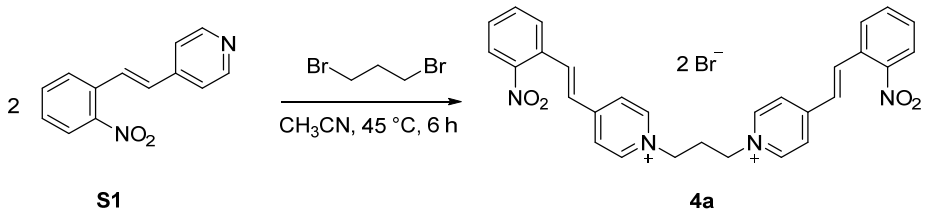
S1 1m

HRMS (m/z): (ESI+) calcd. $[M]^+$: 397.2122, found: 397.2122. ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.96 (s, 1H), 9.05 (d, *J* = 6.9 Hz, 2H), 8.31 (d, *J* = 6.9 Hz, 2H), 8.18 (d, *J* = 16.2 Hz, 1H), 8.11 (d, *J* = 8.2 Hz, 1H), 8.01 (d, *J* = 8.0 Hz, 1H), 7.87 (t, *J* = 7.4 Hz, 1H), 7.75 – 7.68 (m, 1H), 7.54 (d, *J* = 16.1 Hz, 1H), 4.55 (dd, *J* = 9.2, 5.6

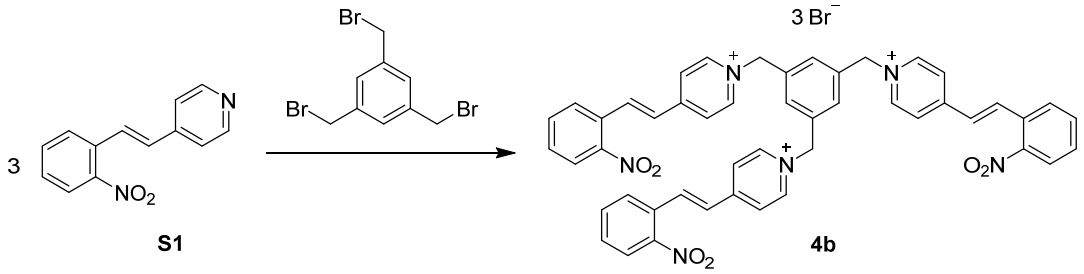
Hz, 2H), 2.18 (t, *J* = 7.4 Hz, 2H), 1.92 (q, *J* = 7.5, 5.6 Hz, 2H), 1.51 – 1.44 (m, 2H), 1.29 (s, 4H), 1.25 (s, 6H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 174.5, 151.8, 148.4, 144.7, 135.4, 134.0, 130.9, 130.3, 129.1, 127.7, 124.9, 124.6, 60.0, 33.7, 30.6, 28.6, 28.6, 28.5, 28.3, 25.4, 24.5.



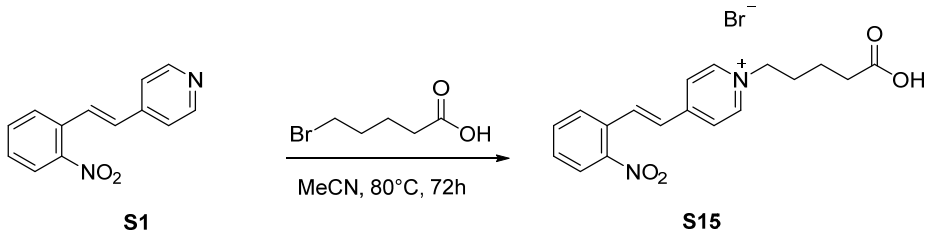
1n: **S1** (0.11 g, 0.50 mmol) and **S15** (74 mg, 0.25 mmol) were dissolved in dry CH_3CN (15 mL). A spatula tip of KI was added and the mixture heated to 45 °C for 6 hours. The mixture was purified by preparative HPLC to obtain **1n** as a red solid (62 mg, 42 %). HRMS (m/z): (ESI+) calcd. $[M]^+$: 487.2116, found: 487.2120. LC-MS method 1, retention time: 4.7 min. ^{1}H NMR (600 MHz, CD_3OD) δ 8.92 (d, *J* = 6.8 Hz, 2H), 8.31 – 8.24 (m, 3H), 8.13 (dd, *J* = 8.2, 1.2 Hz, 1H), 7.96 (dd, *J* = 7.9, 1.5 Hz, 1H), 7.81 (td, *J* = 7.7, 1.3 Hz, 1H), 7.68 (ddd, *J* = 8.5, 7.5, 1.4 Hz, 1H), 7.41 (d, *J* = 16.1 Hz, 1H), 6.29 (s, 2H), 6.13 (s, 2H), 2.54 (s, 6H), 2.23 (s, 6H). ^{13}C NMR (151 MHz, CD_3OD, signals arising from TFA are not listed) δ 160.2, 155.9, 150.0, 144.6, 143.4, 139.2, 135.0, 134.0, 132.1, 131.9, 130.4, 128.1, 127.9, 126.9, 126.1, 124.6, 55.7, 15.6, 14.9.



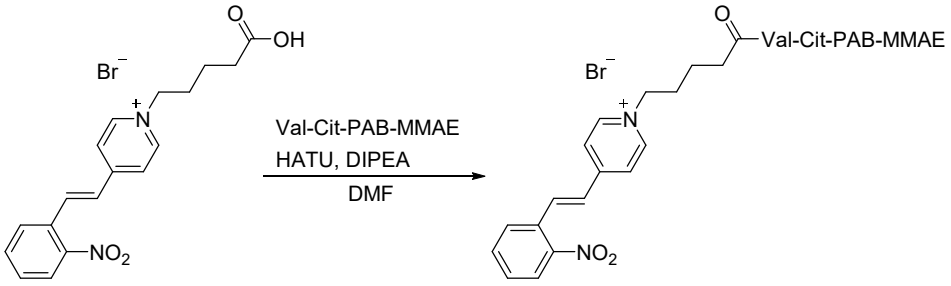
4a: 1,3-Dibromo-propane (40 mg, 0.20 mmol) and **S1** (0.11 g, 0.50 mmol) were dissolved in dry CH_3CN (15 mL) and the mixture was heated to 80 °C for 3 days. After concentration under reduced pressure the solid product was separated by filtration and washed with Et_2O. **4a** was obtained as a yellow solid after drying under reduced pressure (128 mg, 98 %). HRMS (m/z): (ESI+) calcd. $[M]^{2+}$: 247.0972, found: 247.0973. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.08 (d, *J* = 6.9 Hz, 4H), 8.36 (d, *J* = 6.9 Hz, 4H), 8.21 (d, *J* = 16.1 Hz, 2H), 8.12 (dd, *J* = 8.2, 1.3 Hz, 2H), 8.01 (dd, *J* = 7.9, 1.4 Hz, 2H), 7.87 (td, *J* = 7.6, 1.2 Hz, 2H), 7.72 (td, *J* = 7.8, 1.3 Hz, 2H), 7.56 (d, *J* = 16.1 Hz, 2H), 4.70 (t, *J* = 7.2 Hz, 4H), 2.71 – 2.65 (m, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 152.2, 148.4, 145.0, 135.7, 134.0, 130.97, 130.3, 129.1, 127.6, 124.9, 124.7, 56.8, 31.4.



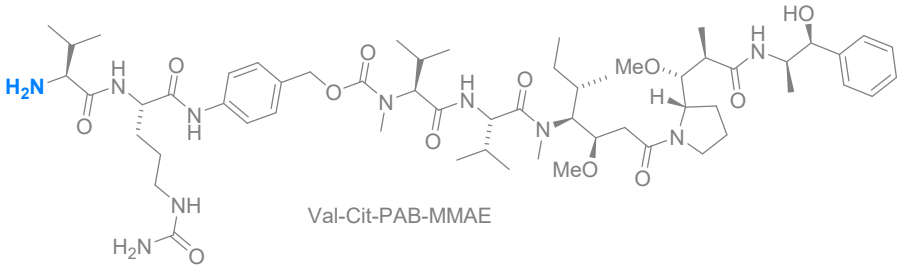
4b: 1,3,5-Tris(bromomethyl)benzene (36 mg, 0.10 mmol) and **S1** (90 mg, 0.40 mmol) were dissolved in dry CH_3CN (5 mL) and the mixture was heated to 80 °C overnight. After concentration under reduced pressure the solid product was separated by filtration and washed with Et_2O. **4b** was obtained as a yellow solid after drying under reduced pressure (98 mg, 95 %). HRMS (m/z): (ESI+) calcd. $[M]^{3+}$: 265.0972, found: 265.0971. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.20 (d, *J* = 6.4 Hz, 6H), 8.37 (d, *J* = 6.3 Hz, 6H), 8.21 (d, *J* = 16.1 Hz, 3H), 8.10 (d, *J* = 8.1 Hz, 3H), 8.00 (d, *J* = 7.8 Hz, 3H), 7.85 (t, *J* = 7.6 Hz, 3H), 7.76 – 7.67 (m, 6H), 7.58 (dd, *J* = 16.1, 2.4 Hz, 3H), 5.88 (d, *J* = 3.1 Hz, 6H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 152.5, 148.4, 145.1, 136.1, 136.0, 134.0, 131.0, 130.2, 129.5, 129.1, 127.6, 124.9, 61.7.



S15: 5-Bromovaleric acid (0.18 g, 1.0 mmol) and **S1** (0.11 g, 0.50 mmol) were dissolved in dry CH_3CN (15 mL) and the mixture stirred at 80 °C for 3 days. After concentration under reduced pressure the solid product was separated by filtration and washed with Et_2O. **S15** was obtained as a yellow solid after drying under reduced pressure (203 mg, >99 %). HRMS (m/z): (ESI+) calcd. $[M]^+$: 327.1339, found: 327.1340. ^{1}H NMR (500 MHz, CD_3OD) δ 8.91 (d, *J* = 6.9 Hz, 2H), 8.28 – 8.20 (m, 3H), 8.12 (dd, *J* = 8.2, 1.3 Hz, 1H), 7.98 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.81 (td, *J* = 7.6, 1.3 Hz, 1H), 7.68 (ddd, *J* = 8.5, 7.5, 1.4 Hz, 1H), 7.43 (dd, *J* = 16.1, 1.4 Hz, 1H), 4.61 (t, *J* = 7.4 Hz, 2H), 2.42 (t, *J* = 7.2 Hz, 2H), 2.13 – 2.03 (m, 2H), 1.73 – 1.64 (m, 2H). ^{13}C NMR (126 MHz, CD_3OD) δ 176.6, 154.7, 150.0, 145.7, 138.0, 135.0, 132.1, 131.9, 130.4, 128.3, 126.1, 126.1, 61.9, 33.8, 31.6, 22.4.

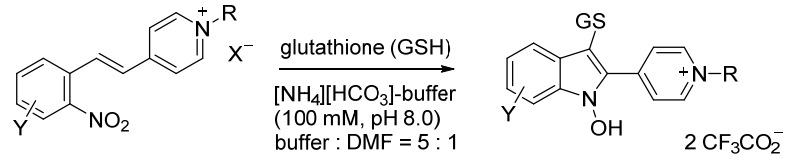


S15 **NSP-MMAE**



NSP-MMAE: **S15** (4.9 mg, 12 µmol), HATU (5.8 mg, 15 µmol), DIPEA (5.3 µl, 30 µmol), and Val-Cit-PAB-MMAE (12 mg, 10 µmol) were dissolved in DMF (4 mL) and stirred at room temperature for 2 hours. After purification by preparative HPLC **NSP-MMAE** was obtained after lyophilization. Lyophilization was repeated 3 more times under addition of water (3 mL). Yellow solid (4.2 mg, 27%). HRMS (m/z): (ESI+) calcd. $[M]^+$: 1431.8286, found: 1431.8312.

Preparation of glutathione conjugates



1a-i, k, l, m, n **3a-i, k, l, m, n**

General procedure 3 (for the reaction of **1a**-**f**, **h**, **i**):

Glutathione (8.9 mg, 29 µmol) was dissolved in NH_4HCO_3-buffer (5 mL, 100 mM, pH 8.0, pH adjusted with 5 M NaOH), and a solution of the 2-nitrostyrylpyridinium derivative (23-25 µmol) in DMF (1 mL) was added. The mixture was stirred at room temperature for 1 hour and subsequently injected into the preparative HPLC to isolate the product.

General procedure 4 (for the reaction of **1g**, **k**, **l**, **m**, **n**):

Glutathione (8.9 mg, 29 µmol) was dissolved in NH_4HCO_3-buffer (5 mL, 100 mM, pH 8.0), and a solution of the 2-nitrostyrylpyridinium derivative (12-15 µmol) in DMF (1 mL, MeOH for **1n**) was added. The mixture was stirred at room temperature for 4 hours and subsequently injected into the preparative HPLC to isolate the product.

3a: *General procedure 3* (24 µmol scale, 8.8 mg **1a**). Yellow solid (14 mg, 77%). HRMS (m/z): (ESI+) calcd. $[M]^+$: 530.1704, found: 530.1713. LC-MS method 1, T_R = 3.7 min. ^{1}H NMR (500 MHz, CD_3OD) δ 8.90 (d, *J* = 7.0 Hz, 2H), 8.62 (d, *J* = 6.9 Hz, 2H), 7.86 (d, *J* = 8.1 Hz, 1H), 7.60 (d, *J* = 8.3 Hz, 1H), 7.45 (ddd, *J* = 8.2, 6.9, 1.0 Hz, 1H), 7.29 (ddd, *J* = 8.0, 6.9, 1.0 Hz, 1H), 4.43 (s, 3H), 4.20 (dd, *J* = 8.7, 5.0 Hz, 1H), 3.96 (t, *J* = 6.5 Hz, 1H), 3.79 – 3.65 (m, 2H), 3.18 (dd, *J* = 13.7, 5.0 Hz, 1H), 2.97 (dd, *J* = 13.8, 8.8 Hz, 1H), 2.46 (dt, *J* = 15.3, 7.5 Hz, 1H), 2.36 (dt, *J* = 15.9, 6.9 Hz, 1H), 2.10 (qd, *J* = 7.3, 5.7 Hz, 2H). ^{13}C NMR (126 MHz, CD_3OD, signals arising from TFA are not listed) δ 174.1, 172.5, 172.5, 171.6, 146.4, 145.8, 138.0, 134.5, 128.5, 127.7, 127.4, 123.3, 121.4, 111.0, 106.8, 54.6, 53.4, 48.2, 41.6, 38.9, 32.3, 27.0.

3b: *General procedure 3* (24 µmol scale, 9.3 mg **1b**). Yellow solid (15 mg, 81%). HRMS (m/z): (ESI+) calcd. $[M]^+$:548.1610, found: 548.1609. LC-MS method 1, T_R = 3.8 min. ^{1}H NMR (500 MHz, CD_3OD) δ 8.90 (d, *J* = 6.5 Hz, 2H), 8.60 (d, *J* = 5.1 Hz, 2H), 7.85 (ddd, *J* = 8.9, 5.1, 1.3 Hz, 1H), 7.30 (dd, *J* = 9.0, 2.0 Hz, 1H), 7.08 (tt, *J* = 9.0, 2.2 Hz, 1H), 4.43 (s, 3H), 4.19 (ddd, *J* = 8.6, 5.0, 3.3 Hz, 1H), 3.99 (td, *J* = 6.4, 2.0 Hz, 1H), 3.83 – 3.68 (m, 2H), 3.17 (dd, *J* = 13.7, 5.0 Hz, 1H), 2.96 (dd, *J* = 13.8, 8.9 Hz, 1H), 2.48 (dt, *J* = 15.2, 7.3 Hz, 1H), 2.40 (dt, *J* = 16.5, 7.0 Hz, 1H), 2.12 (hept, *J* = 7.3 Hz, 2H). ^{13}C NMR (126 MHz, CD_3OD, signals arising from TFA are not listed) δ 174.1, 172.5, 172.4, 171.5, 164.6, 162.6, 145.9, 138.2, 138.1, 135.2, 128.3, 124.3, 123.4, 123.3, 112.6, 112.4, 107.3, 97.0, 96.8, 54.5, 53.4, 48.3, 41.7, 39.0, 32.3, 27.0.

3c: *General procedure 3* (24 µmol scale, 9.7 mg **1c**). Yellow solid (15 mg, 79%). HRMS (m/z): (ESI+) calcd. $[M]^+$: 564.1314, found: 564.1310. LC-MS method 1, T_R = 4.0 min. ^{1}H NMR (500 MHz, MeOD) δ 8.92 (d, *J* = 6.6 Hz, 2H), 8.60 (d, *J* = 6.6 Hz, 2H), 7.82 (d, *J* = 8.6 Hz, 1H), 7.61 (d, *J* = 1.8 Hz, 1H), 7.26 (dd, *J* = 8.6, 1.8 Hz, 1H), 4.44 (s, 3H), 4.17 (dd, *J* = 8.9, 4.9 Hz, 1H), 3.98 (t, *J* = 6.4 Hz, 1H), 3.82 – 3.68 (m, 2H), 3.16 (dd, *J* = 13.8, 5.0 Hz, 1H), 2.94 (dd, *J* = 13.7, 8.9 Hz, 1H), 2.48 (dt, *J* = 15.3, 7.5 Hz, 1H), 2.39 (dt, *J* = 15.8, 6.9 Hz, 1H), 2.12 (hept, *J* = 7.4 Hz, 2H). ^{13}C NMR (126 MHz, MeOD, signals arising from TFA are not listed) δ 174.1, 172.5, 172.4, 171.6, 146.0, 138.0, 135.3, 133.3, 128.6, 126.2, 124.0, 122.9, 110.7, 106.9, 54.5, 53.4, 48.3, 41.7, 39.0, 32.3, 27.0.

3d: *General procedure 3* (25 µmol scale, 11 mg **1d**). Yellow solid (14 mg, 70%). HRMS (m/z): (ESI+) calcd. $[M]^+$: 608.0809, found: 608.0806. LC-MS method 1, T_R = 4.1 min. ^{1}H NMR (500 MHz, MeOH) δ 8.92 (d, *J* = 6.4 Hz, 2H), 8.60 (d, *J* = 7.0 Hz, 2H), 7.78 (s, 1H), 7.77 (dd, *J* = 6.6, 0.6 Hz, 1H), 7.42 – 7.37 (m, 1H), 4.44 (s, 3H), 4.18 (dd, *J* = 8.9, 5.0 Hz, 1H), 3.98 (t, *J* = 6.4 Hz, 1H), 3.81 – 3.68 (m, 2H), 3.15 (dd, *J* = 13.7, 5.0 Hz, 1H), 2.94 (dd, *J* = 13.7, 8.9 Hz, 1H), 2.48 (dt, *J* = 15.9, 7.5 Hz, 1H), 2.39 (ddd, *J* = 15.9, 7.5, 6.5 Hz, 1H), 2.20 – 2.05 (m, 2H). ^{13}C NMR (126 MHz, MeOD, signals arising from TFA are not listed) δ 174.1, 172.5, 172.4, 171.6, 146.0, 146.0, 138.3, 135.1, 128.6, 126.6, 126.5, 123.1, 120.9, 113.7, 106.9, 54.5, 53.4, 48.4, 41.6, 39.0, 32.3, 27.0.

3e: *General procedure 3* (23 µmol scale, 10 mg **1e**). Yellow solid (16 mg, 82%). HRMS (m/z): (ESI+) calcd. [M]+: 588.1759, found: 588.1755. LC-MS method 1, T_R = 3.8 min. ^{1}H NMR (500 MHz, CD_3OD) δ 8.97 (d, *J*

= 6.6 Hz, 2H), 8.62 (d, *J* = 6.7 Hz, 2H), 8.27 (d, *J* = 1.1 Hz, 1H), 7.95 – 7.86 (m, 2H), 4.47 (s, 3H), 4.18 (dd, *J* = 8.9, 4.9 Hz, 1H), 3.97 (d, *J* = 12.0 Hz, 4H), 3.82 – 3.61 (m, 2H), 3.16 (dd, *J* = 13.8, 4.9 Hz, 1H), 2.95 (dd, *J* = 13.8, 8.9 Hz, 1H), 2.47 (dt, *J* = 15.2, 7.4 Hz, 1H), 2.39 (dt, *J* = 15.9, 7.0 Hz, 1H), 2.12 (dp, *J* = 18.0, 7.3 Hz, 2H). ^{13}C NMR (126 MHz, CD_3OD, signals arising from TFA are not listed) δ 174.1, 172.5, 172.4, 171.5, 168.6, 146.2, 145.8, 137.3, 136.8, 130.7, 129.1, 128.6, 123.5, 121.4, 113.1, 106.2, 54.6, 53.4, 52.8, 48.5, 41.7, 39.1, 32.3, 27.0.

3f: *General procedure 3* (23 µmol scale, 10 mg **1f**). Yellow solid (14 mg, 71%). HRMS (m/z): (ESI+) calcd. $[M]^+$: 598.1578, found: 598.1582. LC-MS method 1, T_R = 4.2 min. ^{1}H NMR (500 MHz, CD_3OD) δ 8.98 (d, *J* = 6.9 Hz, 2H), 8.63 (d, *J* = 6.9 Hz, 2H), 8.04 (dt, *J* = 8.5, 0.8 Hz, 1H), 7.92 (dt, *J* = 1.7, 0.8 Hz, 1H), 7.53 (dd, *J* = 8.6, 1.6 Hz, 1H), 4.47 (s, 3H), 4.18 (dd, *J* = 8.8, 5.0 Hz, 1H), 3.99 (t, *J* = 6.4 Hz, 1H), 3.82 – 3.61 (m, 2H), 3.16 (dd, *J* = 13.7, 5.0 Hz, 1H), 2.95 (dd, *J* = 13.8, 8.8 Hz, 1H), 2.49 (dt, *J* = 15.9, 7.5 Hz, 1H), 2.41 (ddd, *J* = 15.9, 7.4, 6.5 Hz, 1H), 2.21 – 2.05 (m, 2H). ^{13}C NMR (151 MHz, CD_3OD, signals arising from TFA are not listed) δ 174.1, 172.5, 172.4, 171.5, 146.2, 145.7, 137.1, 136.4, 129.7, 129.1, 128.8, 128.6, 127.0, 125.2, 122.6, 119.3, 119.3, 108.6, 108.6, 106.2, 54.5, 53.3, 48.5, 41.6, 39.1, 32.3, 27.0.

3g: *General procedure 4* (12 µmol scale, 5.1 mg **1f**). Yellow solid (8.1 mg, 83%). HRMS (m/z): (ESI+) calcd. $[M]^+$: 590.1915, found: 590.1912. LC-MS method 1, T_R = 3.7 min. ^{1}H NMR (600 MHz, CD_3OD) δ 8.78 (d, *J* = 7.0 Hz, 2H), 8.61 (d, *J* = 7.1 Hz, 2H), 7.25 (s, 1H), 7.07 (s, 1H), 4.37 (s, 3H), 4.26 (dd, *J* = 8.9, 4.8 Hz, 1H), 3.96 (s, 3H), 3.94 (s, 3H), 3.91 – 3.85 (m, 1H), 3.81 – 3.70 (m, 2H), 3.18 (dd, *J* = 13.8, 5.0 Hz, 1H), 3.01 – 2.91 (m, 1H), 2.48 (dt, *J* = 15.3, 7.4 Hz, 1H), 2.37 (dt, *J* = 15.7, 6.9 Hz, 1H), 2.17 – 2.06 (m, 2H). ^{13}C NMR (151 MHz, CD_3OD, signals arising from TFA are not listed) δ 174.1, 172.5, 171.5, 152.8, 149.0, 146.2, 145.4, 133.8, 132.1, 127.0, 121.6, 107.3, 101.9, 92.9, 56.9, 56.7, 54.8, 53.4, 47.8, 41.6, 39.0, 32.3, 27.0.

3h: *General procedure 3* (24 µmol scale, 8.6 mg **1h**). Yellow solid (15 mg, 79%). HRMS (m/z): (ESI+) calcd. $[M]^+$: 568.1860, found: 568.1853. LC-MS method 1, T_R = 3.9 min. ^{1}H NMR (500 MHz, CD_3OD) δ 9.07 (d, *J* = 7.1 Hz, 2H), 8.69 (d, *J* = 7.1 Hz, 2H), 7.87 (d, *J* = 8.2 Hz, 1H), 7.61 (d, *J* = 8.4 Hz, 1H), 7.46 (ddd, *J* = 8.2, 7.0, 1.1 Hz, 1H), 7.29 (ddd, *J* = 8.0, 6.9, 1.0 Hz, 1H), 5.52 (d, *J* = 2.5 Hz, 2H), 4.25 – 4.18 (m, 1H), 3.95 (td, *J* = 6.5, 2.2 Hz, 1H), 3.73 (d, *J* = 1.6 Hz, 2H), 3.20 (dd, *J* = 13.7, 5.2 Hz, 1H), 2.99 (dd, *J* = 13.7, 8.6 Hz, 1H), 2.46 (dd, *J* = 15.7, 7.6 Hz, 1H), 2.34 (dt, *J* = 15.9, 6.8 Hz, 1H), 2.16 – 2.03 (m, 2H), 2.01 (t, *J* = 2.5 Hz, 3H). ^{13}C NMR (126 MHz, CD_3OD, signals arising from TFA are not listed) δ 174.1, 172.5, 172.4, 171.6, 147.3, 144.3, 138.2, 134.3, 128.6, 127.7, 127.6, 123.4, 121.5, 111.0, 107.3, 89.5, 70.5, 54.6, 53.4, 51.4, 41.6, 38.9, 32.3, 27.0, 3.3.

3i: *General procedure 3* (23 µmol scale, 17 mg **1i**). Orange solid (22 mg, 81%). HRMS (m/z): (ESI+) calcd. $[M]^+$: 786.3239, found: 786.3240. LC-MS method 1, T_R = 3.4 min. ^{1}H NMR (500 MHz, CD_3OD) δ 8.90 – 8.82 (m, 2H), 8.65 – 8.58 (m, 2H), 7.92 (d, *J* = 2.0 Hz, 1H), 7.88 – 7.83 (m, 1H), 7.60 (dd, *J* = 8.0, 1.8 Hz, 1H), 7.48 – 7.41 (m, 1H), 7.32 – 7.25 (m, 1H), 4.97 (t, *J* = 6.8 Hz, 2H), 4.56 (td, *J* = 5.2, 1.9 Hz, 2H), 4.24 (ddd, *J* = 8.7, 5.0, 2.0 Hz, 1H), 3.98 (td, *J* = 6.5, 2.0 Hz, 1H), 3.92 – 3.82 (m, 2H), 3.74 (d, *J* = 2.4 Hz, 2H),

3.72 – 3.64 (m, 2H), 3.66 – 3.57 (m, 8H), 3.54 – 3.49 (m, 2H), 3.23 – 3.15 (m, 1H), 3.12 (d, *J* = 4.5 Hz, 2H), 3.00 (ddd, *J* = 13.8, 8.8, 2.0 Hz, 1H), 2.60 – 2.43 (m, 1H), 2.39 (qd, *J* = 9.0, 8.0, 1.9 Hz, 1H), 2.20 – 2.04 (m, 2H). ^{13}C NMR (126 MHz, CD_3OD, signals arising from TFA are not listed) δ 174.1, 172.6, 172.4, 171.6, 146.9, 145.1, 143.2, 138.1, 134.2, 128.4, 127.8, 127.5, 125.4, 123.4, 121.5, 111.0, 107.3, 71.4, 71.4, 71.3, 71.2, 70.4, 67.8, 61.4, 54.8, 53.4, 51.4, 41.7, 40.6, 39.0, 32.3, 27.9, 27.0.

3k: *General procedure 4* (12 µmol scale, 5.5 mg **1k**). Yellow solid (7.7 mg, 76%). HRMS (m/z): (ESI+) calcd. $[M]^+$: 620.1810, found: 620.1808. LC-MS method 1, T_R = 4.4 min. ^{1}H NMR (600 MHz, CD_3OD) δ 8.90 (d, *J* = 6.9 Hz, 2H), 8.63 (d, *J* = 6.9 Hz, 2H), 8.48 (d, *J* = 0.9 Hz, 1H), 8.16 – 8.12 (m, 1H), 7.70 (d, *J* = 0.8 Hz, 1H), 7.57 (d, *J* = 8.1 Hz, 1H), 7.49 (ddd, *J* = 8.3, 7.3, 1.3 Hz, 1H), 7.39 (td, *J* = 7.5, 1.0 Hz, 1H), 4.43 (s, 3H), 4.26 (dd, *J* = 8.8, 5.0 Hz, 1H), 3.93 (t, *J* = 6.5 Hz, 1H), 3.83 – 3.67 (m, 2H), 3.28 (dd, *J* = 13.8, 5.0 Hz, 1H), 3.06 (dd, *J* = 13.8, 8.8 Hz, 1H), 2.48 (dt, *J* = 15.3, 7.5 Hz, 1H), 2.39 (dt, *J* = 15.8, 6.9 Hz, 1H), 2.15 – 2.02 (m, 2H). ^{13}C NMR (151 MHz, CD_3OD, signals arising from TFA are not listed) δ 174.1, 172.5, 172.5, 171.7, 158.5, 157.6, 146.1, 145.8, 138.6, 135.4, 128.6, 128.2, 125.4, 125.0, 124.2, 123.2, 121.9, 113.0, 112.3, 107.7, 92.6, 54.7, 53.5, 48.2, 41.7, 39.0, 32.4, 27.0.

3l: *General procedure 4* (12 µmol scale, 5.9 mg **1l**). Yellow solid (8.0 mg, 77%). HRMS (m/z): (ESI+) calcd. $[M]^+$: 650.1915, found: 650.1903. LC-MS method 1, T_R = 4.5 min. ^{1}H NMR (600 MHz, CD_3OD) δ 8.89 (d, *J* = 6.8 Hz, 2H), 8.64 (d, *J* = 6.9 Hz, 2H), 8.34 (d, *J* = 0.8 Hz, 1H), 7.98 (d, *J* = 8.5 Hz, 1H), 7.65 (d, *J* = 0.8 Hz, 1H), 7.16 (d, *J* = 2.2 Hz, 1H), 6.99 (dd, *J* = 8.5, 2.2 Hz, 1H), 4.43 (s, 3H), 4.27 (dd, *J* = 8.7, 5.2 Hz, 1H), 3.98 – 3.91 (m, 1H), 3.91 (s, 3H), 3.80 – 3.69 (m, 2H), 3.26 (dd, *J* = 13.8, 5.2 Hz, 1H), 3.05 (dd, *J* = 13.8, 8.7 Hz, 1H), 2.54 – 2.45 (m, 1H), 2.40 (dt, *J* = 15.9, 6.9 Hz, 1H), 2.09 (dp, *J* = 21.5, 7.2 Hz, 1H).

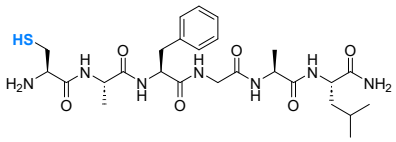
3m: *General procedure 4* (12 µmol scale, 5.7 mg **1m**). Yellow solid (7.4 mg, 67%). HRMS (m/z): (ESI+) calcd. $[M]^+$: 686.2854, found: 686.2852. LC-MS method 1, T_R = 4.4 min. ^{1}H NMR (500 MHz, CD_3OD) δ 8.98 (d, *J* = 7.1 Hz, 2H), 8.65 (d, *J* = 6.9 Hz, 2H), 7.86 (d, *J* = 8.1 Hz, 1H), 7.60 (d, *J* = 8.4 Hz, 1H), 7.45 (ddd, *J* = 8.3, 7.0, 1.0 Hz, 1H), 7.28 (ddd, *J* = 8.0, 7.0, 0.9 Hz, 1H), 4.67 – 4.60 (m, 2H), 4.25 (dd, *J* = 8.6, 5.0 Hz, 1H), 3.95 (t, *J* = 6.5 Hz, 1H), 3.73 (d, *J* = 1.3 Hz, 2H), 3.20 (dd, *J* = 13.7, 5.0 Hz, 1H), 2.99 (dd, *J* = 13.7, 8.7 Hz, 1H), 2.45 (dt, *J* = 15.9, 7.4 Hz, 1H), 2.38 – 2.31 (m, 1H), 2.28 (t, *J* = 7.4 Hz, 2H), 2.21 – 2.02 (m, 4H), 1.63 – 1.55 (m, 2H), 1.51 – 1.40 (m, 4H), 1.36 (d, *J* = 1.5 Hz, 6H). ^{13}C NMR (126 MHz, CD_3OD, signals arising from TFA are not listed) δ 177.7, 174.1, 172.5, 172.4, 171.6, 146.6, 144.9, 138.1, 134.3, 128.7, 127.7, 127.4, 123.3, 121.5, 111.0, 107.0, 62.4, 54.7, 53.5, 41.7, 38.9, 34.9, 32.4, 32.3, 30.3, 30.2, 30.1, 30.0, 27.2, 27.0, 26.0.

3n: *General procedure 4*. DMF was replaced with MeOH. (15 µmol scale, 9.2 mg **1n**). Red solid (6.4 mg, 53%). HRMS (m/z): (ESI+) calcd. $[M]^+$: 776.2844, found: 776.2849. LC-MS method 1, T_R = 4.8 min. ^{1}H NMR (600 MHz, CD_3OD) δ 9.01 (d, *J* = 7.0 Hz, 2H), 8.77 (d, *J* = 7.0 Hz, 2H), 7.85 (d, *J* = 8.0 Hz, 1H), 7.60 (d, *J* = 8.3 Hz, 1H), 7.46 (ddd, *J* = 8.2, 7.0, 1.1 Hz, 1H), 7.28 (ddd, *J* = 8.0, 6.9, 1.0 Hz, 1H), 6.31 (s, 2H), 6.19 (s, 1H), 4.27 (dd, *J* = 8.5, 4.9 Hz, 1H), 3.91 (td, *J* = 6.6, 2.4 Hz, 1H), 3.71 (s, 2H), 3.20 (dd, *J* = 13.8, 4.8 Hz, 1H),

3.01 (dd, *J* = 13.8, 8.5 Hz, 1H), 2.55 (s, 6H), 2.40 (dt, *J* = 15.3, 7.5 Hz, 1H), 2.26 – 2.32 (m, 7H), 2.05 (qd, *J* = 7.1, 4.0 Hz, 2H). ^{13}C NMR (151 MHz, CD_3OD, signals arising from TFA are not listed) δ 173.9, 172.5, 172.3, 171.7, 160.2, 147.7, 143.8, 143.5, 138.3, 134.1, 133.7, 129.1, 128.0, 127.9, 127.9, 124.6, 123.5, 121.6, 111.1, 108.3, 55.7, 54.8, 53.6, 41.6, 39.0, 32.4, 27.1, 15.8, 15.8, 14.9.

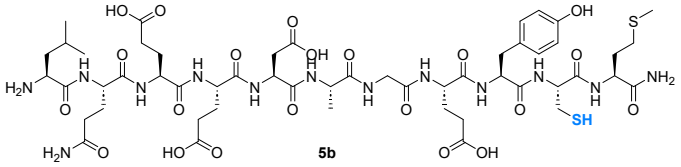
Preparation of other linear, cyclic and bicylic peptide conjugates

Peptides used in synthesis were prepared with a Liberty Blue peptide synthesizer from CEM using standard methods and DIC and Oxyma as coupling agents.



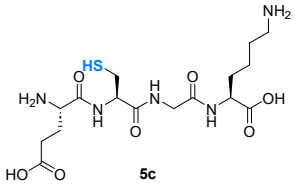
5a

cys-ala-phe-gly-ala-leu-NH_2



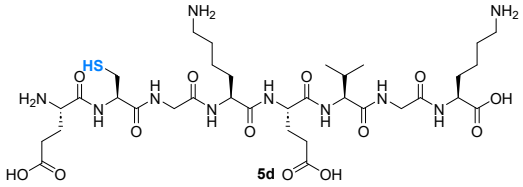
5b

gly-leu-gln-glu-glu-asp-ala-gly-glu-tyr-cys-met-NH_2



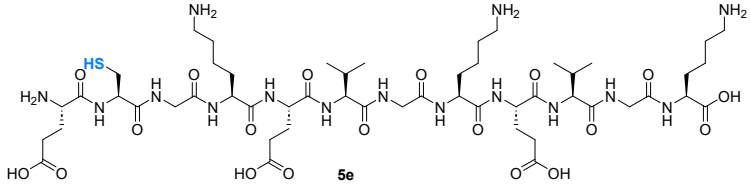
5c

glu-cys-gly-lys



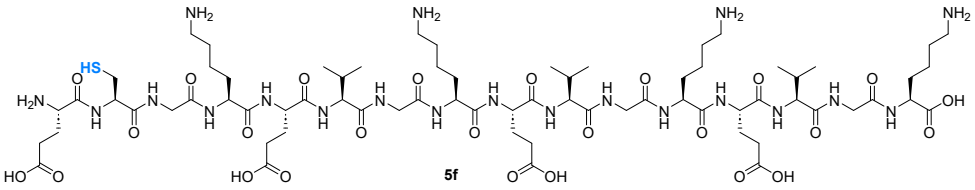
5d

glu-cys-gly-lys-glu-val-gly-lys



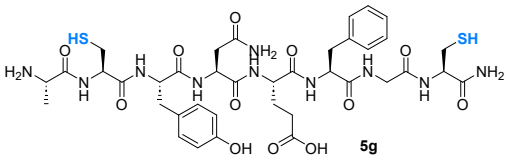
5e

glu-cys-gly-lys-glu-val-gly-lys-glu-val-gly-lys



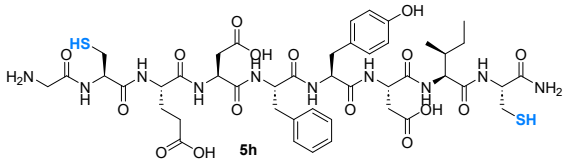
5f

glu-cys-gly-lys-glu-val-gly-lys-glu-val-gly-lys-glu-val-gly-lys



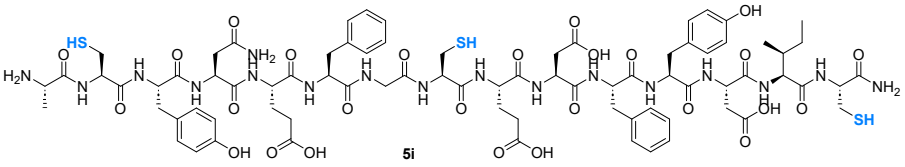
5g

ala-cys-tyr-asn-glu-phe-gly-cys-NH_2



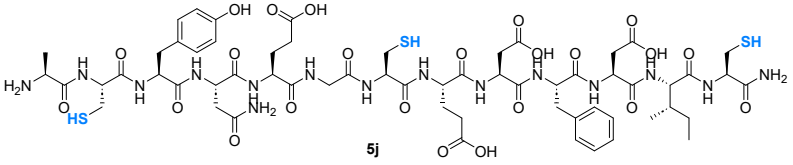
5h

gly-cys-glu-asp-phe-tyr-asp-ile-cys-NH_2



5i

ala-cys-tyr-asn-glu-phe-gly-cys-glu-asp-phe-tyr-asp-ile-cys-NH_2



5j

ala-cys-tyr-asn-glu-gly-cys-glu-asp-phe-asp-ile-cys-NH_2

General procedure 5 (for the preparation of **6a**-**d)**:

The peptide (30 µmol) was dissolved in NH_4HCO_4-buffer (9.0 mL, pH 8.0) and **1a** (7.4 mg, 20 µmol) in DMF (1 mL) was added and the mixture stirred for 1 hour at room temperature and subsequently injected into the preparative HPLC for product isolation.

General procedure 6 (for the preparation of **6e**,**f**):

The peptide (15 µmol) was dissolved in NH_4HCO_4-buffer (9.0 mL, pH 8.0) and **1a** (3.7 mg, 10 µmol) in DMF (1 mL) was added and the mixture stirred for 2 hour at room temperature and subsequently injected into the preparative HPLC for product isolation.

General procedure 7 (for the prepartion of **cycle1**, **2**):

The peptide (10 µmol) was dissolved in NH_4HCO_4-buffer (9.0 mL, pH 8.0) and **4a** (6.5 mg, 10 µmol) in DMF (1 mL) was added and the mixture stirred for 1 hour at room temperature and subsequently injected into the preparative HPLC for product isolation.

General procedure 8 (for the preparation of **bicycle1**, **2**):

The peptide (6 µmol) was dissolved in NH_4HCO_4-buffer (9.0 mL, pH 8.0) and **4b** (6.2 mg, 6.0 µmol) in DMF (1 mL) was added at 0 °C and the mixture stirred for 1 hour at room temperature and subsequently injected into the preparative HPLC for product isolation.

6a: *General procedure 5*. 17 mg **5a**. Yellow solid (16 mg, 80%). HRMS (m/z): (ESI+) calcd. $[M+H]^+$: 802.3705, found: 802.3715. LC-MS method 1, T_R = 4.1 min. ^{1}H NMR (600 MHz, CD_3OD) δ 8.92 (d, *J* = 6.9 Hz, 2H), 8.60 (d, *J* = 6.9 Hz, 2H), 7.95 (dt, *J* = 8.2, 0.9 Hz, 1H), 7.62 – 7.58 (m, 1H), 7.46 (ddd, *J* = 8.2, 7.0, 1.1 Hz, 1H), 7.32 (ddd, *J* = 8.0, 7.0, 1.0 Hz, 1H), 7.25 – 7.19 (m, 4H), 7.18 – 7.11 (m, 1H), 4.44 (s, 3H), 4.40 (dd, *J* = 8.8, 6.1 Hz, 1H), 4.34 (dd, *J* = 10.5, 4.4 Hz, 1H), 4.23 (q, *J* = 7.3 Hz, 1H), 4.19 (q, *J* = 7.1 Hz, 1H), 3.95 – 3.86 (m, 2H), 3.63 (d, *J* = 16.5 Hz, 1H), 3.18 (dd, *J* = 13.8, 4.6 Hz, 1H), 3.15 – 3.07 (m, 2H), 2.98 (dd, *J* = 13.9, 8.8 Hz, 1H), 1.35 (d, *J* = 7.2 Hz, 3H), 1.22 (d, *J* = 7.1 Hz, 3H), 0.95 (d, *J* = 6.3 Hz, 3H), 0.90 (d, *J* = 6.4 Hz, 3H). ^{13}C NMR (151 MHz, MeOD, signals arising from TFA are not listed) δ 177.7, 175.3, 174.3, 174.1, 172.0, 167.9, 146.1, 146.0, 138.4, 137.7, 134.4, 130.3, 129.5, 128.5, 127.9, 127.7, 127.5, 123.6, 121.2, 111.0, 104.7, 56.9, 53.8, 52.9, 51.4, 50.6, 48.4, 43.6, 41.4, 39.4, 38.1, 26.0, 23.6, 21.6, 17.8, 17.7.

6b: *General procedure 5*. 40 mg **5b**. Yellow solid (26 mg, 75%). HRMS (m/z): (ESI+) calcd. $[M+2H]^{2+}$: 783.3054, found: 783.3065. LC-MS method 1, T_R = 4.0 min. ^{1}H NMR (600 MHz, CD_3OD) δ 8.91 (d, *J* = 6.6 Hz, 2H), 8.54 (d, *J* = 6.9 Hz, 2H), 7.90 (d, *J* = 8.1 Hz, 1H), 7.60 (d, *J* = 8.3 Hz, 1H), 7.45 (ddd, *J* = 8.1, 6.9, 1.0 Hz, 1H), 7.29 (ddd, *J* = 7.9, 6.9, 0.9 Hz, 1H), 7.10 (d, *J* = 8.5 Hz, 2H), 6.71 (d, *J* = 8.5 Hz, 2H), 4.70 (dd, *J* = 7.5, 5.6 Hz, 1H), 4.46 (s, 3H), 4.42 (t, *J* = 7.1 Hz, 1H), 4.39 – 4.23 (m, 6H), 3.95 – 3.85 (m, 3H), 3.82 (dq, *J* = 10.9, 3.6 Hz, 1H), 3.72 (dt, *J* = 16.9, 3.5 Hz, 1H), 3.54 (dd, *J* = 16.8, 5.0 Hz, 1H), 3.19 – 3.14 (m, 1H), 3.04 (ddd, *J* = 14.3, 10.1, 4.8 Hz, 2H), 2.96 (dd, *J* = 16.9, 6.3 Hz, 1H), 2.85 (dd, *J* = 16.9, 7.0 Hz, 1H), 2.55 – 2.28 (m, 10H), 2.17 – 2.07 (m, 5H), 2.05 – 1.91 (m, 8H), 1.85 (dq, *J* = 9.6, 4.6 Hz, 1H), 1.78 – 1.62 (m, 3H), 1.44 (d, *J* = 7.2 Hz, 3H), 0.99 (dd, *J* = 9.4, 6.0 Hz, 6H). ^{13}C NMR (151 MHz, CD_3OD, signals arising from TFA are

not listed) δ 177.7, 176.7, 176.5, 176.5, 176.3, 176.1, 174.7, 174.5, 174.4, 174.2, 173.9, 173.5, 173.3, 172.9, 172.4, 172.3, 170.7, 157.4, 145.9, 138.2, 135.3, 131.3, 129.1, 128.6, 127.4, 127.3, 123.3, 121.5, 116.4, 111.0, 106.1, 58.0, 57.6, 57.5, 57.3, 57.2, 55.2, 54.7, 54.6, 54.3, 53.7, 52.9, 51.9, 51.5, 48.4, 44.3, 43.8, 41.6, 38.3, 36.8, 36.5, 32.3, 32.3, 31.2, 31.1, 31.1, 31.1, 28.6, 28.0, 27.8, 27.5, 25.4, 23.1, 22.0, 17.5, 17.4, 17.4, 17.3, 17.2, 17.0, 15.2.

6c: *General procedure 5*. 16 mg **5c.** Yellow solid (15 mg, 78%). HRMS (m/z): (ESI+) calcd. $[M+H]^+$: 658.2654, found: 658.2656. LC-MS method 2, T_R = 4.7 min. ^{1}H NMR (600 MHz, CD_3OD) δ 8.92 (d, *J* = 6.9 Hz, 2H), 8.62 (d, *J* = 6.9 Hz, 2H), 7.89 (d, *J* = 8.1 Hz, 1H), 7.60 (d, *J* = 8.3 Hz, 1H), 7.45 (ddd, *J* = 8.2, 7.0, 1.0 Hz, 1H), 7.29 (ddd, *J* = 8.1, 7.0, 1.0 Hz, 1H), 4.44 (s, 3H), 4.40 (dd, *J* = 9.1, 4.9 Hz, 1H), 4.29 (dd, *J* = 7.6, 6.1 Hz, 1H), 3.96 (t, *J* = 6.3 Hz, 1H), 3.76 – 3.65 (m, 2H), 3.22 (dd, *J* = 13.5, 6.1 Hz, 1H), 3.14 (dd, *J* = 13.5, 7.6 Hz, 1H), 2.97 – 2.85 (m, 2H), 2.57 – 2.44 (m, 2H), 2.17 – 2.02 (m, 2H), 1.95 – 1.86 (m, 1H), 1.78 – 1.65 (m, 1H), 1.68 – 1.60 (m, 2H), 1.51 – 1.39 (m, 2H). ^{13}C NMR (151 MHz, CD_3OD, signals arising from TFA are not listed) δ 175.8, 175.2, 171.7, 170.9, 170.1, 146.3, 146.0, 138.0, 134.3, 128.5, 127.6, 127.3, 123.3, 121.4, 111.0, 106.7, 55.1, 53.6, 53.2, 48.3, 43.2, 40.5, 38.8, 32.1, 30.1, 27.9, 27.7, 23.7.

6d: *General procedure 5*. 26 mg **5d**. Yellow solid (22 mg, 75%). HRMS (m/z): (ESI+) calcd. $[M+H]^+$: 1071.4928, found: 1071.4936. LC-MS method 2, T_R = 4.6 min. ^{1}H NMR (600 MHz, CD_3OD) δ 8.92 (d, *J* = 6.8 Hz, 2H), 8.61 (d, *J* = 6.9 Hz, 2H), 7.89 (d, *J* = 8.1 Hz, 1H), 7.60 (d, *J* = 8.3 Hz, 1H), 7.45 (dd, *J* = 8.3, 7.0 Hz, 1H), 7.29 (ddd, *J* = 8.1, 6.9, 1.0 Hz, 1H), 4.49 – 4.45 (m, 1H), 4.44 (s, 3H), 4.41 – 4.34 (m, 1H), 4.30 (td, *J* = 7.4, 6.2, 3.5 Hz, 1H), 4.08 (d, *J* = 7.0 Hz, 1H), 4.00 (td, *J* = 6.3, 1.9 Hz, 1H), 3.93 (d, *J* = 16.7 Hz, 1H), 3.90 – 3.84 (m, 1H), 3.74 (d, *J* = 16.6 Hz, 1H), 3.64 – 3.55 (m, 1H), 3.22 (dd, *J* = 13.6, 6.2 Hz, 1H), 3.19 – 3.12 (m, 1H), 2.92 (dt, *J* = 10.2, 7.4 Hz, 4H), 2.60 – 2.48 (m, 2H), 2.46 – 2.31 (m, 2H), 2.19 – 2.02 (m, 4H), 1.93 (tdd, *J* = 8.6, 5.4, 2.5 Hz, 2H), 1.87 – 1.81 (m, 1H), 1.77 (dq, *J* = 9.7, 5.0, 4.3 Hz, 1H), 1.67 (ddt, *J* = 26.9, 15.3, 8.2 Hz, 5H), 1.53 – 1.39 (m, 4H), 1.06 – 0.92 (m, 7H). ^{13}C NMR (151 MHz, CD_3OD, signals arising from TFA are not listed) δ 175.9, 171.4, 146.3, 146.0, 128.5, 127.4, 123.3, 121.4, 117.1, 111.0, 60.8, 55.2, 54.2, 53.6, 53.0, 49.6, 49.4, 49.3, 49.1, 49.0, 48.9, 48.7, 48.6, 48.3, 43.7, 43.4, 40.6, 40.5, 32.1, 32.0, 31.6, 31.2, 30.1, 28.0, 28.9, 27.7, 23.7, 23.7, 19.7, 18.9.

6e: *General procedure 6*. 19 mg **5e.** Yellow solid (15 mg, 76%). HRMS (m/z): (ESI+) calcd. $[M+2H]^{2+}$: 742.8638, found: 742.8647. LC-MS method 2, T_R = 4.7 min.

6f: *General procedure 6*. 25 mg **5f.** Yellow solid (19 mg, 76%). HRMS (m/z): (ESI+) calcd. $[M+2H]^{2+}$: 949.4775, found: 949.4775. LC-MS method 1, T_R = 3.2 min.

6g: *General procedure 5*. 4.9 mg sodium 2-mercaptoethanesulfonate was reacted with 7.4 mg **1a.** Yellow solid (5.8 mg, 79%). HRMS (m/z): (ESI+) calcd. $[M+H]^+$: 365.0624, found: 365.0619. LC-MS method 1, T_R = 4.2 min. ^{1}H NMR (600 MHz, DMSO-d_6) δ 11.92 (s, 1H), 9.05 (d, *J* = 6.9 Hz, 2H), 8.52 (d, *J* = 6.9 Hz, 2H), 7.82 (d, *J* = 8.0 Hz, 1H), 7.63 (d, *J* = 8.3 Hz, 1H), 7.46 (ddd, *J* = 8.2, 6.9, 1.1 Hz, 1H), 7.30 (ddd, *J* = 8.1, 6.9, 1.0 Hz, 1H), 4.36 (s, 3H), 2.89 – 2.83 (m, 2H), 2.38 – 2.32 (m, 2H). ^{13}C NMR (151 MHz, DMSO-d_6, signals

arising from TFA are not listed) δ 145.1, 143.6, 135.8, 133.8, 126.8, 125.9, 125.8, 122.0, 120.2, 110.1, 104.6, 51.6, 47.6, 32.3.

cycle 1: General method 7. 9.0 mg **5g**. Yellow solid (10 mg, 60%). HRMS (m/z): (ESI+) calcd. $[M+2H]^{2+}$: 681.2470, found: 681.2481. LC-MS method 1, T_R = 3.9 min. ^{1}H NMR (600 MHz, CD_3OD) δ 9.03 (dd, *J* = 6.9, 5.1 Hz, 4H), 8.69 (d, *J* = 7.0 Hz, 2H), 8.59 (d, *J* = 7.0 Hz, 2H), 7.91 (dt, *J* = 8.2, 1.0 Hz, 1H), 7.83 (dt, *J* = 8.2, 1.0 Hz, 1H), 7.63 – 7.59 (m, 1H), 7.57 (dt, *J* = 8.4, 0.9 Hz, 1H), 7.47 (ddd, *J* = 8.2, 7.0, 1.1 Hz, 1H), 7.43 (ddd, *J* = 8.2, 7.0, 1.0 Hz, 1H), 7.31 (ddd, *J* = 8.0, 7.0, 1.0 Hz, 1H), 7.29 – 7.23 (m, 5H), 7.19 (tt, *J* = 5.8, 2.1 Hz, 1H), 7.02 (d, *J* = 8.5 Hz, 2H), 6.68 (d, *J* = 8.5 Hz, 2H), 4.98 – 4.93 (m, 4H), 4.68 – 4.55 (m, 2H), 4.41 (dd, *J* = 9.0, 5.2 Hz, 1H), 4.32 (dd, *J* = 8.4, 6.4 Hz, 1H), 3.91 – 3.84 (m, 2H), 3.80 (q, *J* = 7.1 Hz, 1H), 3.73 (d, *J* = 16.4 Hz, 1H), 3.66 (d, *J* = 16.4 Hz, 1H), 3.18 – 3.13 (m, 2H), 3.06 – 2.91 (m, 7H), 2.80 (dd, *J* = 14.3, 9.1 Hz, 1H), 2.75 (t, *J* = 6.3 Hz, 2H), 2.12 – 1.96 (m, 2H), 1.83 – 1.69 (m, 2H), 1.34 (d, *J* = 7.1 Hz, 3H).

cycle 2: General method 7. 11 mg **5h**. Yellow solid (11 mg, 61%). HRMS (m/z): (ESI+) calcd. $[M+2H]^{2+}$: 760.2759, found: 760.2756. LC-MS method 1, T_R = 4.3 min. ^{1}H NMR (600 MHz, CD_3OD) δ 9.08 (d, *J* = 6.9 Hz, 2H), 9.06 (d, *J* = 6.9 Hz, 2H), 8.76 (d, *J* = 6.6 Hz, 2H), 8.66 (d, *J* = 7.0 Hz, 2H), 7.89 (d, *J* = 8.1 Hz, 1H), 7.84 (dt, *J* = 8.2, 1.0 Hz, 1H), 7.62 (dt, *J* = 8.4, 0.9 Hz, 1H), 7.47 (ddd, *J* = 8.2, 7.0, 1.1 Hz, 1H), 7.42 (d, *J* = 8.3 Hz, 1H), 7.40 – 7.36 (m, 1H), 7.32 (ddd, *J* = 8.0, 6.9, 1.0 Hz, 1H), 7.27 (ddd, *J* = 8.0, 6.8, 1.1 Hz, 1H), 7.12 – 7.06 (m, 3H), 7.03 (t, *J* = 7.5 Hz, 2H), 6.76 – 6.65 (m, 4H), 4.85 (q, *J* = 6.6 Hz, 4H), 4.78 (td, *J* = 12.6, 12.1, 5.4 Hz, 1H), 4.49 (dd, *J* = 8.1, 5.6 Hz, 1H), 4.30 (s, 1H), 4.25 – 4.13 (m, 3H), 4.07 (dd, *J* = 8.6, 5.0 Hz, 1H), 3.99 (dd, *J* = 10.0, 3.4 Hz, 1H), 3.85 (d, *J* = 16.1 Hz, 1H), 3.74 (d, *J* = 16.1 Hz, 1H), 3.14 – 2.89 (m, 9H), 2.80 (dd, *J* = 17.0, 6.9 Hz, 2H), 2.68 (dd, *J* = 16.4, 5.6 Hz, 1H), 2.58 – 2.46 (m, 2H), 2.39 (q, *J* = 7.9, 7.5 Hz, 2H), 1.97 (td, *J* = 16.2, 15.7, 8.3 Hz, 2H), 1.89 (dq, *J* = 14.5, 7.2 Hz, 1H), 1.53 (ddd, *J* = 13.4, 7.5, 3.3 Hz, 1H), 1.35 – 1.22 (m, 1H), 0.98 – 0.87 (m, 6H).

bicycle 1: General method 8. 11mg **5i**. Yellow solid (4.3 mg, 25%). HRMS (m/z): (ESI+) calcd. $[M+3H]^{3+}$: 843.6340, found: 843.6345. LC-MS method 1, T_R = 4.4 min.

bicycle 2: General method 8. 8.9 mg **5j**. Yellow solid (4.4 mg, 28%). HRMS (m/z): (ESI+) calcd. $[M+3H]^{3+}$: 740.2568, found: 740.2585. Note: the calculated mass refers to an isotopologue with a calculated relative abundance of 85.6%.

The yields for the linear peptides with a single cysteine **6a**-**f** were ≥ 75%, while only 60% and 61%, resepectively were obtained for **cycle 1** and **cycle 2**. The isolated yields dropped further for **bicycle 1** and **bicycle 2** with 25% and 28%, respectively.

We believe the reason resides in the competitive formation of oligomers as judged by formation of yellow precipitates. This effect was particularly pronounced for peptides with three cysteines for the formation of bicycles. Since these oligomers are not sufficiently soluble they were not be detected by LC-MS. As an additional complication a mixture of oligomers is likely to form, which might also contain disulfide bridges.

To illustrate this behavior we conducted analytical scale reactions (1 mL scale) for the formation of **6f**, **cycle 2** and **bicycle 2** under the conditions described for the preparative reactions above. While all LC-MS chromatogram traces showed one main peak, traces of the peptides cyclized via a disulfide bridge were detected for in the reaction to form **cycle 2** and **bicycle 2**. This suggests that the main side products are insoluble oligomers, which is also indicated by the turbity of the solutions in these cases (Figure S1).

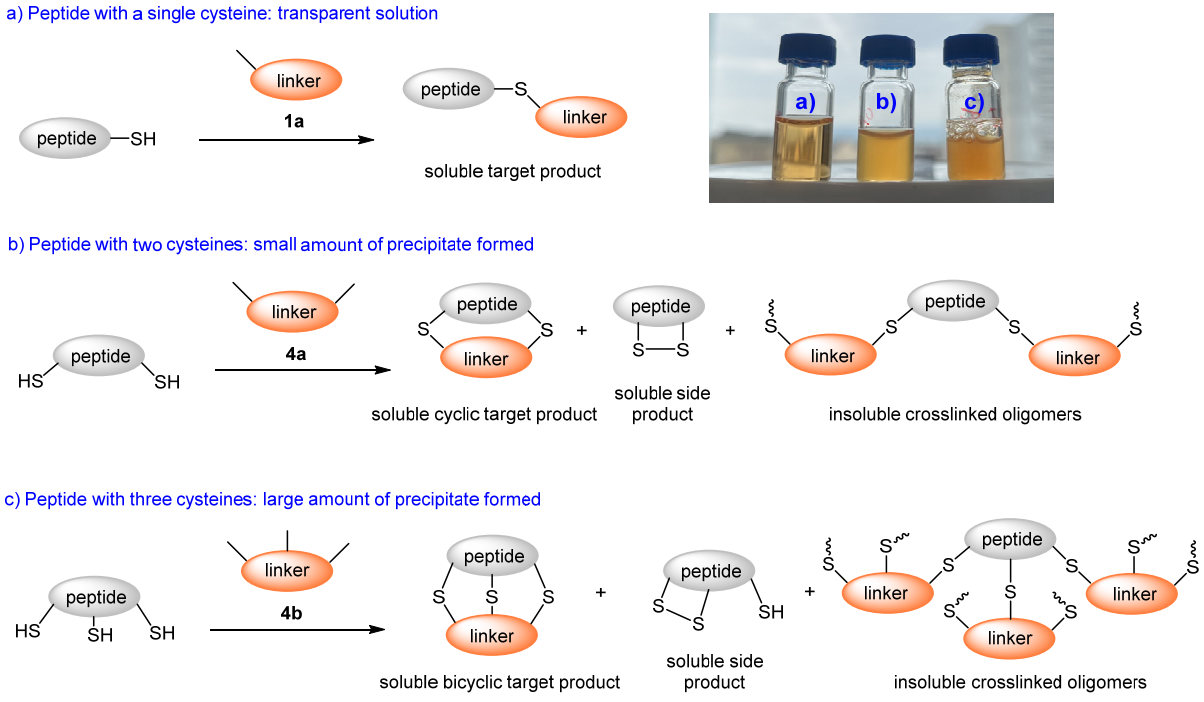


Figure S1. Rational for the lower yield in the formation of cyclic and bicyclic peptides. The inset photo shows reaction mixtures for the formation of linear peptide **6f** (a), **cycle 2** (b) and **bicycle 2** (c). The different degree of precipitation can be clearly seen. The fill-height of the vials differs because the photo was taken after material was removed for LC-MS analysis.

6f: The peptide **5f** (1.5 µmol) was dissolved in NH_4HCO_4-buffer (0.90 mL, pH 8.0) and **1a** (1.0 µmol) in DMF (0.10 mL) was added and the mixture stirred for 2 hours at 37 °C and analyzed by LC-MS (Figure S2).

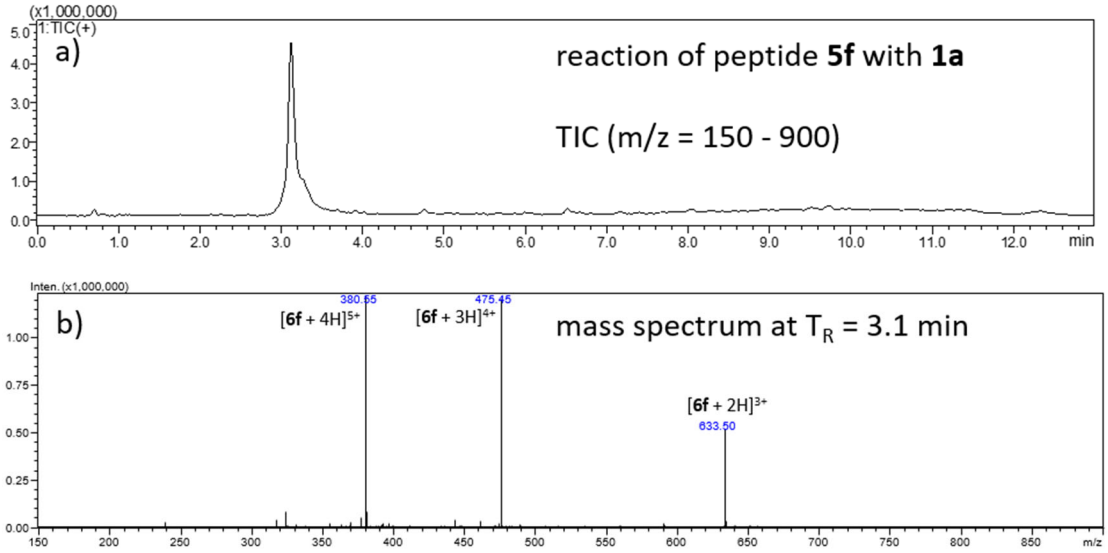


Figure S2. a) LC-MS chromatogram of the reaction of **5f** with **1a** showing the TIC in the m/z range from 150 – 900. b) Mass spectrum at retention time T_R = 3.1 min.

cycle 2: The peptide **5h** (1.0 µmol) was dissolved in NH_4HCO_4-buffer (0.90 mL, pH 8.0) and **4a** (1.0 µmol) in DMF (0.10 mL) was added and the mixture stirred for 1 hour at room temperature and subsequently analyzed by LC-MS (Figure S3).

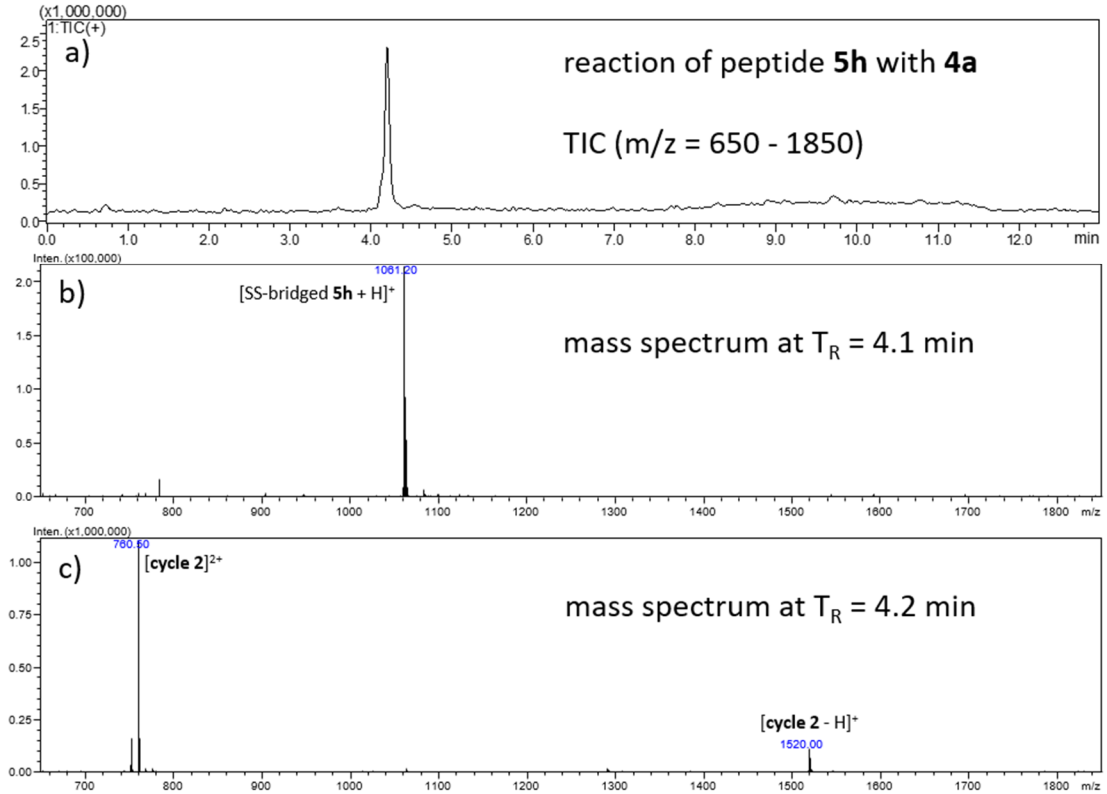


Figure S3. a) LC-MS chromatogram of the reaction of **5h** with **4a** showing the TIC in the range of 650-1850. b) Mass spectrum at retention time T_R = 4.1 min. c) Mass spectrum at T_R = 4.2 min.

bicycle 2: The peptide **5j** (0.60 µmol) was dissolved in NH_4HCO_4-buffer (0.90 mL, pH 8.0) and **4b** (0.60 µmol) in DMF (0.10 mL) was added at 0 °C and the mixture stirred for 1 hour at room temperature and analyzed by LC-MS (Figure S4).

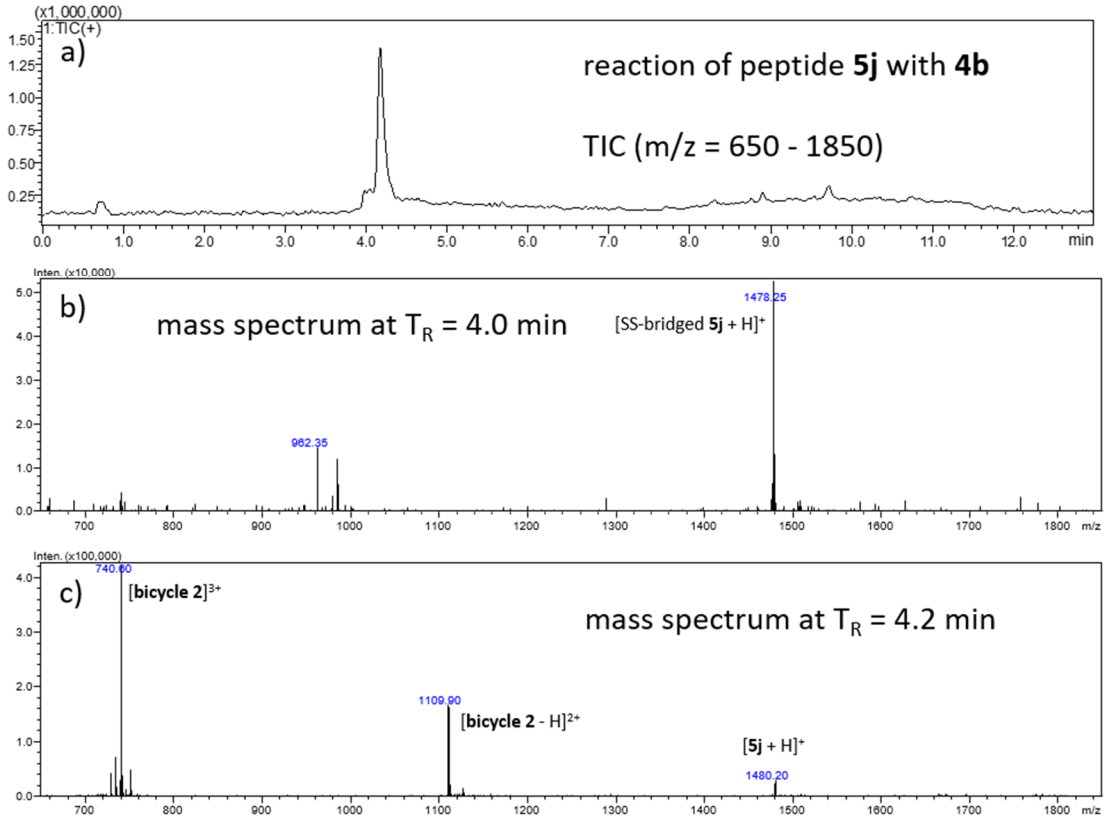


Figure S4. a) LC-MS chromatogram of the reaction of **5j** with **4b** showing the TIC in the range of 650-1850. b) Mass spectrum at retention time T_R = 4.0 min. c) Mass spectrum at T_R = 4.2 min.

Attempted reaction with lysine as an alternative nucleophile

In order to test if lysine can act as a nucleophile under the conditions employed for cysteine conjugation, a buffered solution of **1a** and lysine was monitored by LC-MS.

The following stock solutions were prepared:

NH_4HCO_3 buffer (100 mM, pH 8.0), pH adjusted with 5M NaOH.

Stock solution A: Lysine (8.0 mM) in NH_4HCO_3-buffer.

Stock solution B: **1a** (40 mM) in DMF.

Stock solution A (50 µL) was added into NH_4HCO_3 buffer (445 µL), followed by stock solution B (5.0 µL). The mixture was agitated at 37 °C and then monitored by LCMS at 4 h and 24 h, no product was observed by LC-MS. Final concentrations: 0.80 mM lysine, 40 µM **1a**. No lysine conjugate was observed.

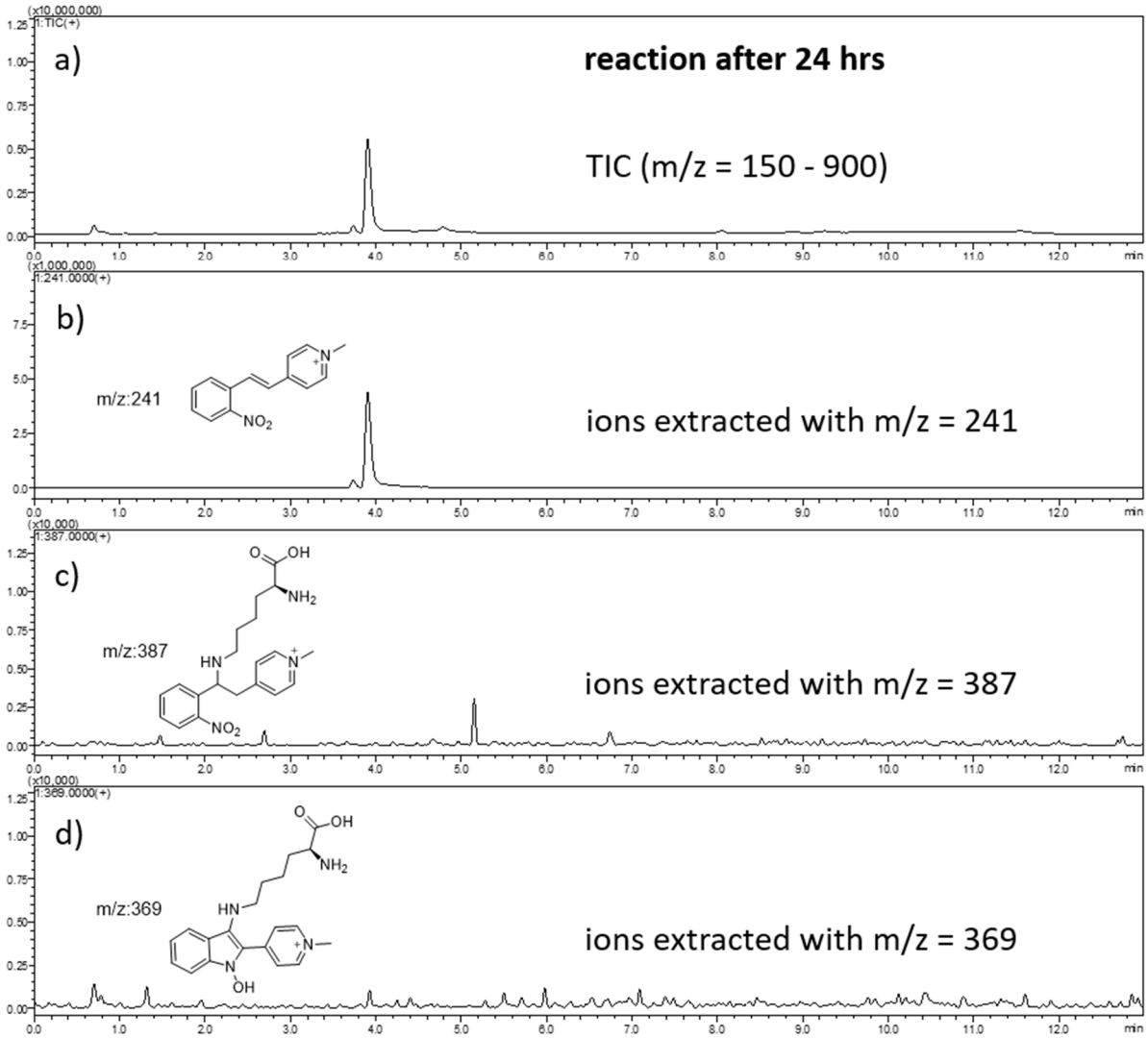


Figure S5. LC-MS chromatogram of the reaction mixture containing lysine (0.80 mM) and 1a (40 µM) in NH_4HCO_3-buffer (100 mM, pH 8.0) at 37 °C after 24 hours. a) TIC in the mass range 150 – 900 Th; b) ions extracted with m/z = 241 Th; c) ions extracted with m/z = 387 Th; d) ions extracted with m/ z = 369 Th.

Determination of extinction coefficients, UV-VIS yields and reaction rates

Note: Extinction coefficients were determined in 100 mM NH_4HCO_3-buffer (pH 8.0) without the addition of organic solvents. Solutions for the determination of UV-VIS yields and reaction rates contained 1% of DMF. We compared the absorption spectra of **3a**,**f**,**g** with and without 1% DMF and observed only a negligible difference in the relevant wavelength region.

Determination of extinction coefficients

The glutathione conjugates **3a**-**i**,**k**-**l** were isolated by preparative HPLC in good purity according to ^{1}H, ^{13}C NMR, LC-MS and LC-UV analysis. Commonly encountered residual TFA and water after lyophilization would however lead to an underestimation of the extinction coefficients if determined directly. The electrophiles **1a**-**m** on the other hand do do not require preparative HPLC for their isolation and the concentration can be set accurately by weighing. The transformations **1a**-**f**,**h**,**i**,**m**,**n** → **3a**-**f**,**h**,**i**,**m**,**n** are accompanied by a change in the UV-spectra revealing a sharp isosbestic points. The ratio of absorbance between isosbestic point and absorption maximum of **3** is concentration independent and can be reliably determined in the UV spectra of isolated compounds **3a**-**i**,**k**-**m**.

The knowledge of the concentration of solutions of starting materials **1**, the determination of the isosbestic point for the reactions **1** → **3**, and the determination of the ratio of the absorbance at the isosbestic point and the absorption maximum of **3** in the isolated compounds **3** allow therefore the determination of $\varepsilon_{\lambda max}$ for compounds **3**.

For example the isosbestic point for the transformation **1a** → **3a** appears at a wavelength of 355 nm. At a concentration of 40 µM ($c_{0\_\mathbf{1a}}$) an absorption of 0.485 is measured and allows to calculate an extinction coefficient $\varepsilon_{iso\_1a\rightarrow3a}$ of $1.2 \cdot 10^4\ M^{-1}cm^{-1}$ (equation 1).

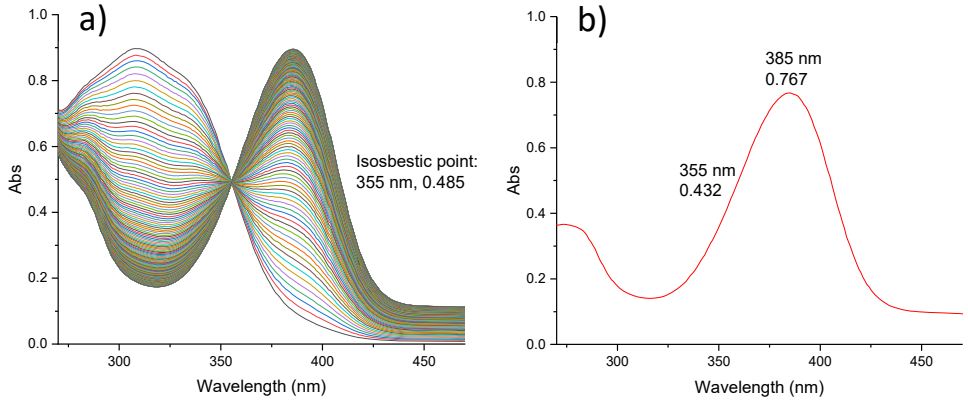


Figure S6. a) UV spectra recorded every 3s for the transformation of **1a** → **3a** with an initial concentration of **1a** of 40 µM and 4.0 mM GSH, in NH_4HCO_3-buffer (100 mM, pH 8.0.), 1% DMF at 20 °C. b) UV spectrum of isolated **3a** in NH_4HCO_3-buffer (100 mM, pH 8.0). The absorption values at the isosbestic point are indicated in both panels. λ_{max} is additionally shown in b).

With the ratio of absorption between $\lambda_{max\_\mathbf{3a}}$ (385 nm) and $\lambda_{iso\_1a\rightarrow3a}$ (355 nm), determined with the isolated compound **3a**, the extinction coefficient for **3a** at λ_{max} can be calculated (equations 2 and 3).

$$\varepsilon_{3a(355nm)} = \varepsilon_{1a(355nm)} = \frac{\boldsymbol{A}_{355nm}}{C_0 d} = \frac{0.485}{40\ \mu\mathrm{M}*1\ \mathrm{cm}} = 12125\ \mathrm{M}^{-1}\mathrm{cm}^{-1} \quad [1]$$

$$\varepsilon_{3a(385nm)}/\varepsilon_{3a(355nm)} = \frac{\boldsymbol{A}_{385nm}}{\boldsymbol{A}_{355nm}} = \frac{0.767}{0.432} \quad [2]$$

$$\varepsilon_{3a(385nm)} = \varepsilon_{3a(355nm)} \cdot \frac{0.767}{0.432} = 2.2 \times 10^4\ \mathrm{M}^{-1}\mathrm{cm}^{-1} \quad [3]$$

This method was also applied for **3g**,**k**,**l** although the isosbestic point was not as sharply defined for the corresponding transformations. See further below.

Determination of UV-VIS yields

The following stock solutions were prepared:

NH_4HCO_3 buffer (100 mM, pH 8.0), pH adjusted with 5M NaOH.

Stock solution A: glutathione (48 mM) in NH_4HCO_3-buffer.

Stock solution B: **1a**-**m** (4.0 mM) in DMF.

General procedure 9 (for **1a**-**f**,**h**,**i**): Stock solution A (10 µL) was added into NH_4HCO_3 buffer (890 µL), followed by stock solution B (100 µL). The mixture was agitated at 37 °C for 1 hour;

General procedure 10 (for **1g**,**k**,**l**,**m**): Stock solution A (10 µL) was added into NH_4HCO_3 buffer (940 µL), followed by stock solution B (50 µL). The mixture was agitated at 37 °C for 1 hour.

Quantitative conversion was observed by LC-MS (TIC, m/z = 150-900) and LC-UV (200-600 nm) after 1 hour except for **1g** and **1l**. After 4 hours the reactions with **1g** and **1l** had not reached full conversion according to LC-MS analysis. Consequently, their yields were measured by UV-VIS measurements instead. For the UV-VIS measurement (250 – 650 nm) 200 µL of the reaction mixture was diluted with 800 µL buffer.

Conversion for **1g** → **3g** and **1l** → **3l** was determined by applying the following calculation:

$$A = A_{sub} + A_{pro} = \varepsilon_{sub} c_{sub} + \varepsilon_{pro} c_{pro} = \varepsilon_{sub}(c_0 - c_{pro}) + \varepsilon_{pro} c_{pro} \quad [4]$$

$$c_{pro} = (\mathrm{A} - \varepsilon_{sub} c_0)/(\varepsilon_{pro} - \varepsilon_{sub}) \quad [5]$$

$$yield = c_{pro}/c_0 \quad [6]$$

3g: λ_{max} = 422nm, ε_{1g} = 4000 M^{-1} cm^{-1}, ε_{3g} = 22300 M^{-1} cm^{-1}. A = 0.82

3l: λ_{max} = 413nm, ε_{1l} = 4700 M^{-1} cm^{-1}, ε_{3l} = 21200 M^{-1} cm^{-1}. A = 0.78

$Yield_{3g}$ = 90%, $Yield_{3l}$ = 90%.

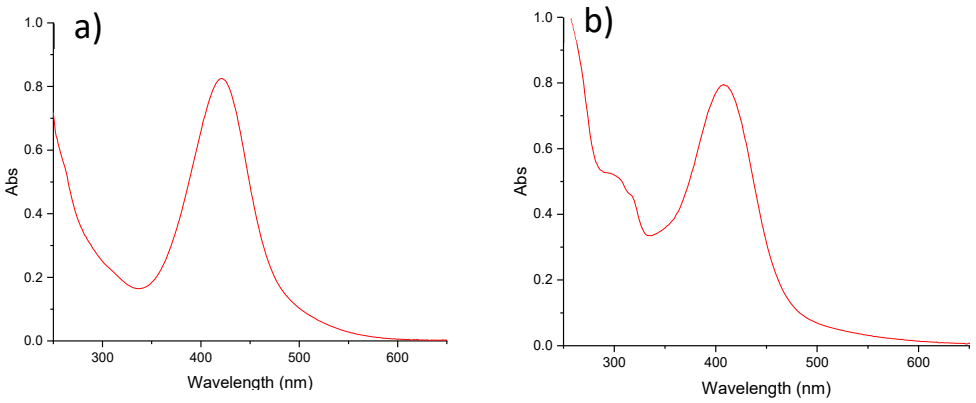


Figure S7. UV spectra of reaction mixtures after 4 hours a) **3g** b) **3l**.

Determination of reaction rates

Reaction rates were determined by recording UV spectra of the reactions **1** → **3** every 3 seconds from 270 nm to 470 nm over 10 minutes. Pseudo-first-order-conditions were ensured by employing a large excess of glutathione (100 eq.).

The reactions were conducted in quartz glass UV cuvettes with **1a**-**f**,**h**,**i**,**m** (40 µM), and glutathione (4.0 mM) in degassed NH_4HCO_3 buffer (100 mM, pH 8.0) in a volume of 1 mL with 1% DMF at 20 °C.

The product concentrations were calculated with the following formulas:

$$A = A_{sub} + A_{pro} = \varepsilon_{sub} c_{sub} + \varepsilon_{pro} c_{pro} = \varepsilon_{sub}(c_0 - c_{pro}) + \varepsilon_{pro} c_{pro} \quad [7]$$

$$c_{pro} = (A - \varepsilon_{sub} c_0)/(\varepsilon_{pro} - \varepsilon_{sub}) \quad [8]$$

Second order rate constant were obtained by applying a linear fit to the first seven datapoints in a plot of product concentration vs. time and the following calculation:

$$reaction\ rate = slope = \frac{dc_3}{dt} = k c_1 c_{GSH} \quad [9]$$

$$k = \frac{slope}{c_1 c_{GSH}} = \frac{slope}{40\ \mu M \cdot 4\ mM} \quad [10]$$

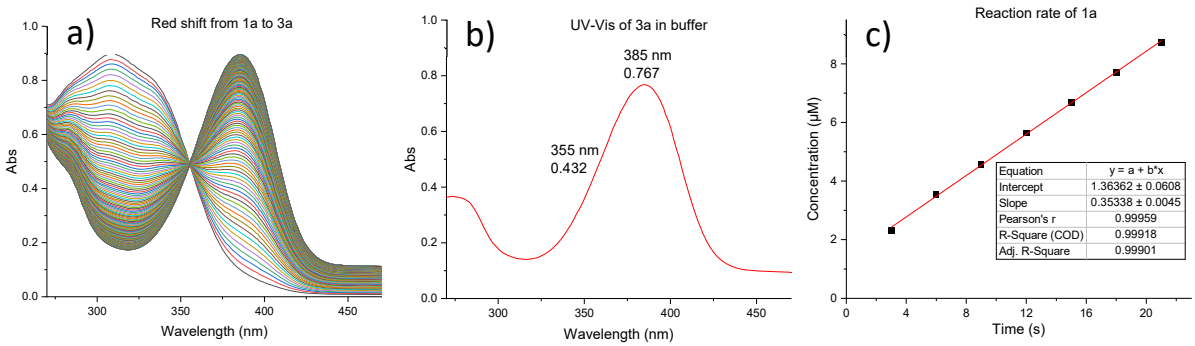


Figure S8. a) UV spectra recorded every 3s for the transformation of **1a** → **3a** with an initial concentration of **1a** of 40 µM and 4.0 mM GSH, in NH_4HCO_3-buffer (100 mM, pH 8.0.), 1% DMF at 20 °C. b) UV spectrum of isolated **3a** in NH_4HCO_3-buffer (100 mM, pH 8.0). Absorption at λ_{max} and λ_{iso} are indicated. c) Determination of the slope in a plot of product concentration (**3a**) vs. time.

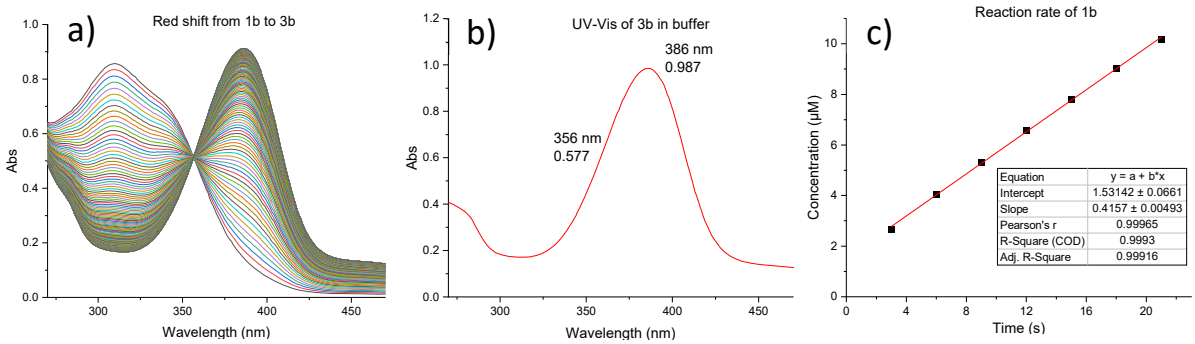


Figure S9. a) UV spectra recorded every 3s for the transformation of **1b** → **3b** with an initial concentration of **1b** of 40 µM and 4.0 mM GSH, in NH_4HCO_3-buffer (100 mM, pH 8.0.), 1% DMF at 20 °C. b) UV spectrum of isolated **3b** in NH_4HCO_3-buffer (100 mM, pH 8.0). Absorption at λ_{max} and λ_{iso} are indicated. c) Determination of the slope in a plot of product concentration (**3b**) vs. time.

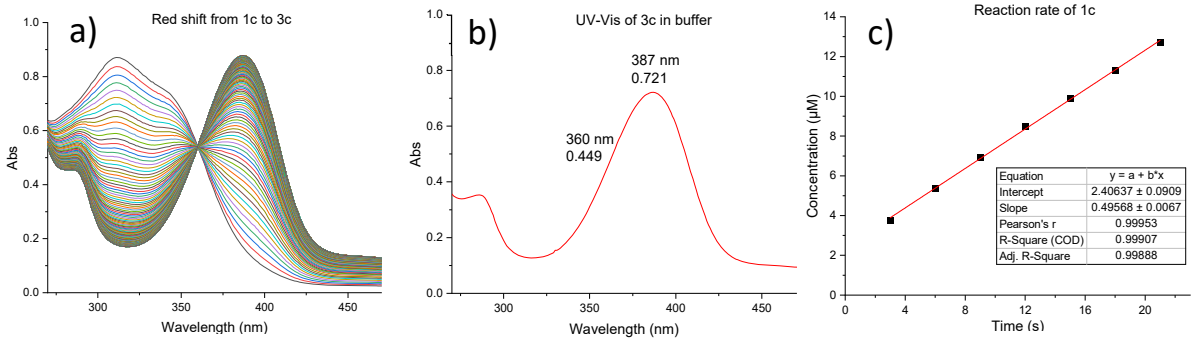


Figure S10. a) UV spectra recorded every 3s for the transformation of **1c** → **3c** with an initial concentration of **1c** of 40 µM and 4.0 mM GSH, in NH_4HCO_3-buffer (100 mM, pH 8.0.), 1% DMF at 20 °C. b) UV spectrum of isolated **3c** in NH_4HCO_3-buffer (100 mM, pH 8.0). Absorption at λ_{max} and λ_{iso} are indicated. c) Determination of the slope in a plot of product concentration (**3c**) vs. time.

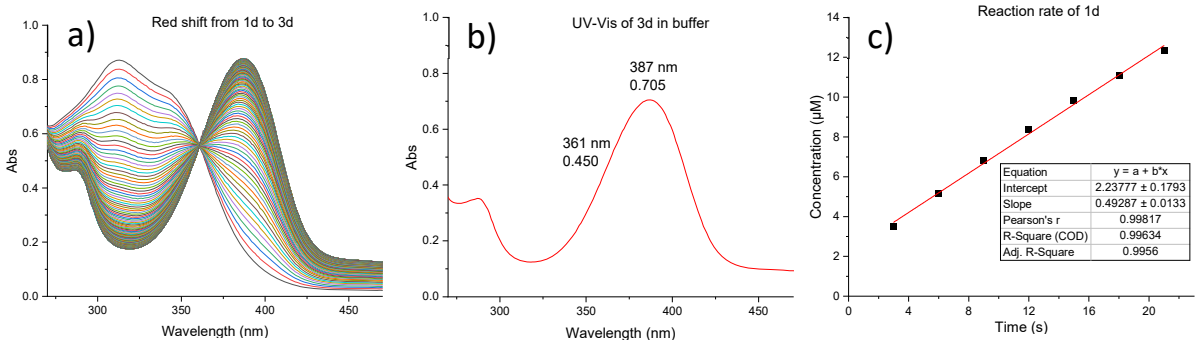


Figure S11. a) UV spectra recorded every 3s for the transformation of **1d** → **3d** with an initial concentration of **1d** of 40 µM and 4.0 mM GSH, in NH_4HCO_3-buffer (100 mM, pH 8.0.), 1% DMF at 20 °C. b) UV spectrum of isolated **3d** in NH_4HCO_3-buffer (100 mM, pH 8.0). Absorption at λ_{max} and λ_{iso} are indicated. c) Determination of the slope in a plot of product concentration (**3d**) vs. time.

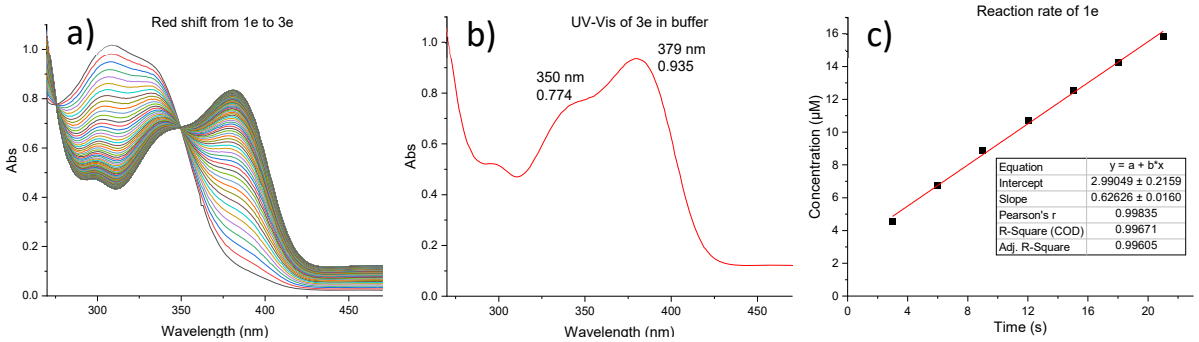


Figure S12. a) UV spectra recorded every 3s for the transformation of **1e** → **3e** with an initial concentration of **1e** of 40 µM and 4.0 mM GSH, in NH_4HCO_3-buffer (100 mM, pH 8.0.), 1% DMF at 20 °C. b) UV spectrum of isolated **3e** in NH_4HCO_3-buffer (100 mM, pH 8.0). Absorption at λ_{max} and λ_{iso} are indicated. c) Determination of the slope in a plot of product concentration (**3e**) vs. time.

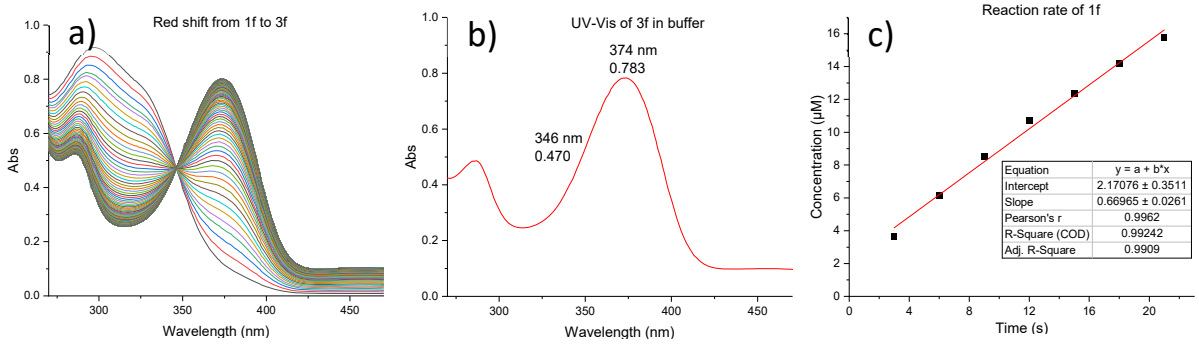


Figure S13. a) UV spectra recorded every 3s for the transformation of **1f** → **3f** with an initial concentration of **1f** of 40 µM and 4.0 mM GSH, in NH_4HCO_3-buffer (100 mM, pH 8.0.), 1% DMF at 20 °C. b) UV spectrum of isolated **3f** in NH_4HCO_3-buffer (100 mM, pH 8.0). Absorption at λ_{max} and λ_{iso} are indicated. c) Determination of the slope in a plot of product concentration (**3f**) vs. time.

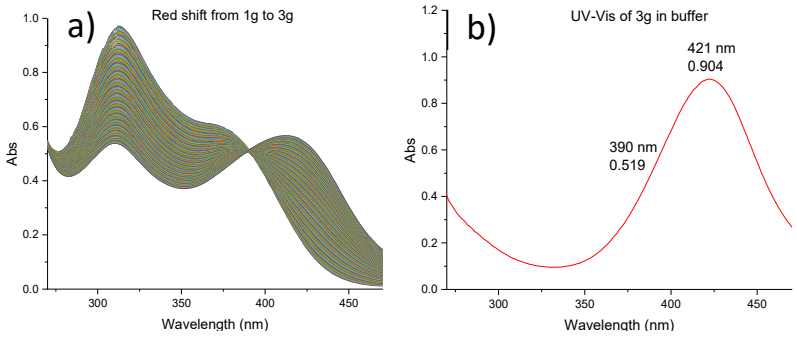


Figure S14. a) UV spectra recorded every 3s for the transformation of **1g** → **3g** with an initial concentration of **1g** of 40 µM and 4.0 mM GSH, in NH_4HCO_3-buffer (100 mM, pH 8.0.), 1% DMF at 20 °C. b) UV spectrum of isolated **3g** in NH_4HCO_3-buffer (100 mM, pH 8.0). Absorption at λ_{max} and λ_{iso} are indicated.

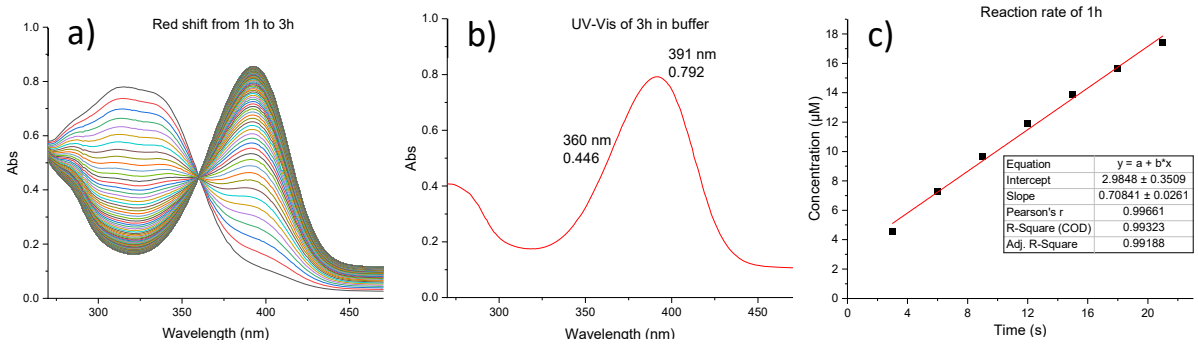


Figure S15. a) UV spectra recorded every 3s for the transformation of **1h** → **3h** with an initial concentration of **1h** of 40 µM and 4.0 mM GSH, in NH_4HCO_3-buffer (100 mM, pH 8.0.), 1% DMF at 20 °C. b) UV spectrum of isolated **3h** in NH_4HCO_3-buffer (100 mM, pH 8.0). Absorption at λ_{max} and λ_{iso} are indicated. c) Determination of the slope in a plot of product concentration (**3h**) vs. time.

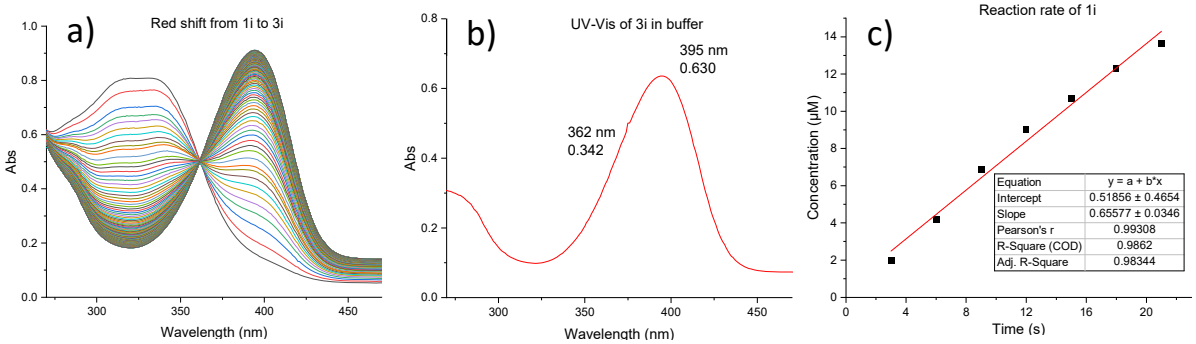


Figure S16. a) UV spectra recorded every 3s for the transformation of **1i** → **3i** with an initial concentration of **1i** of 40 µM and 4.0 mM GSH, in NH_4HCO_3-buffer (100 mM, pH 8.0.), 1% DMF at 20 °C. b) UV spectrum of isolated **3i** in NH_4HCO_3-buffer (100 mM, pH 8.0). Absorption at λ_{max} and λ_{iso} are indicated. c) Determination of the slope in a plot of product concentration (**3i**) vs. time.

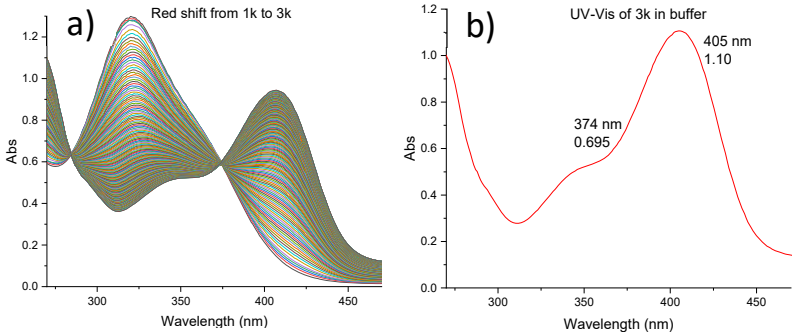


Figure S17. a) UV spectra recorded every 3s for the transformation of **1k** → **3k** with an initial concentration of **1k** of 40 µM and 4.0 mM GSH, in NH_4HCO_3-buffer (100 mM, pH 8.0.), 1% DMF at 20 °C. b) UV spectrum of isolated **3k** in NH_4HCO_3-buffer (100 mM, pH 8.0). Absorption at λ_{max} and λ_{iso} are indicated.

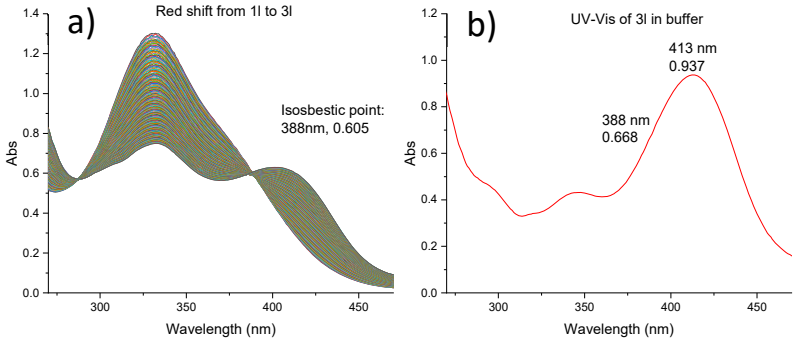


Figure S18. a) UV spectra recorded every 3s for the transformation of **1l** → **3l** with an initial concentration of **1l** of 40 µM and 4.0 mM GSH, in NH_4HCO_3-buffer (100 mM, pH 8.0.), 1% DMF at 20 °C. b) UV spectrum of isolated **3l** in NH_4HCO_3-buffer (100 mM, pH 8.0). Absorption at λ_{max} and λ_{iso} are indicated.

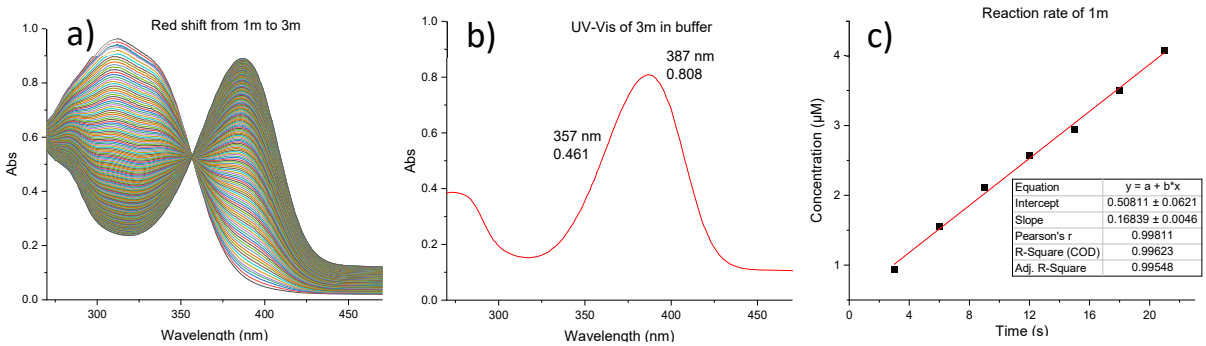


Figure S19. a) UV spectra recorded every 3s for the transformation of **1m** → **3m** with an initial concentration of **1m** of 40 µM and 4.0 mM GSH, in NH_4HCO_3-buffer (100 mM, pH 8.0.), 1% DMF at 20 °C. b) UV spectrum of isolated **3m** in NH_4HCO_3-buffer (100 mM, pH 8.0). Absorption at λ_{max} and λ_{iso} are indicated. c) Determination of the slope in a plot of product concentration (**3m**) vs. time.

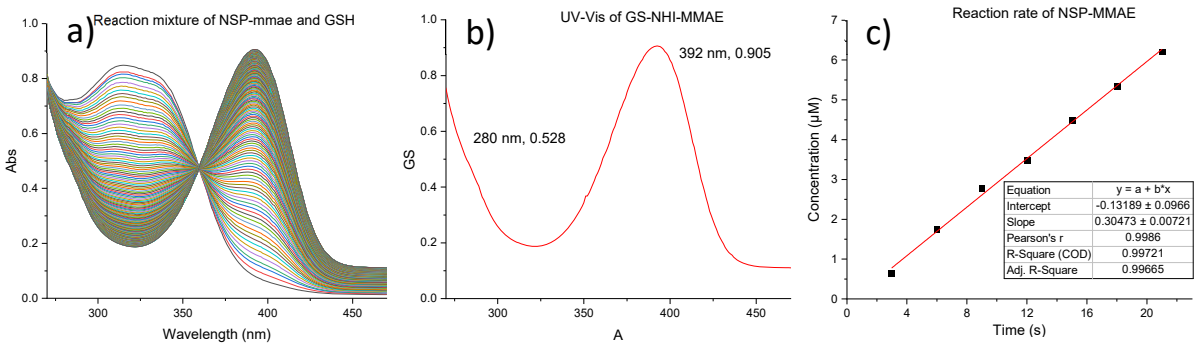


Figure S20. a) UV spectra recorded every 3s for the transformation of **NSP-MMAE** → **GS-NHI-MMAE** with an initial concentration of **NSP-MMAE** of 40 µM and 4.0 mM GSH, in NH_4HCO_3-buffer (100 mM, pH 8.0.), 1% DMF at 20 °C. b) UV spectrum of isolated **GS-NHI-MMAE** in NH_4HCO_3-buffer (100 mM, pH 8.0). Absorption at λ_{max} and λ_{iso} are indicated. c) Determination of the slope in a plot of product concentration (**GS-NHI-MMAE**) vs. time.

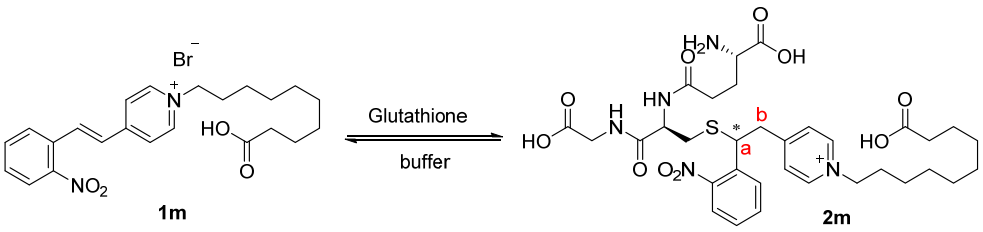
Figure S21. a) UV spectra recorded every 3s for the transformation of **1a** → **3-TCEP** with an initial concentration of **1a** of 40 µM and 4.0 mM TCEP, in NH_4HCO_3-buffer (100 mM, pH 8.0.), 1% DMF at 20 °C. b) Determination of the slope in a plot of product concentration (**3-TCEP**) vs. time.

Table S1: Reaction rate constants for NSP-electrophiles with GSH and TCEP (for **1a**) at 20 °C.

reaction	rate constant ($M^{-1}s^{-1}$)
1a + GSH → **3a**	2.2
1b + GSH → **3b**	2.6
1c + GSH → **3c**	3.1
1d + GSH → **3d**	3.1
1e + GSH → **3e**	3.9
1f + GSH → **3f**	4.2
1h + GSH → **3h**	4.4
1i + GSH → **3i**	4.1
1m + GSH → **3m**	1.1
NSP-mmae + GSH → **GS-NHI-MMAE**	1.9
1a + **TCEP** → **3-TCEP**	1.4

Observation and isolation of intermediate addition products

Under low initial concentrations of **1** (0.4 mM), the intermediate product **2** in the reaction with GSH (1.2 eq.) could not be observed by LC-MS for any of the derivatives of **1**. Weak signals for the addition product **2** could however be detected by LC-MS in two cases (**1g** → **3g** and **1m** → **3m**) when the reactions were run at a higher initial concentration of **1** (4.0 mM **1**, 48 mM GSH). Very weak signals were also observed for **2k** and **2l** when the reactions were run at higher concentrations. For the reaction **1m** → **2m** → **3m** the intermediate **2m** could be isolated and the reversibility of the addition **1m** + GSH → **2m** was probed (see further below).



To probe the concentration dependence, solutions of **1m** (0.2 mM, 4.0 mM and 8.0 mM, respectively) were allowed to react with GSH (1.2 equiv.) in a buffer : DMF = 4 : 1 mixture at 25 °C for 1 hour. Buffer = 100 mM NH_4HCO_3, pH 8.0.

For LC-MS analysis the samples were diluted as follows:

Reaction with c_0 of **1m** = 0.20 mM: 100 µL of the reaction mixture were diluted with 400 µL MeOH.

Reaction with c_0 of **1m** = 4.0 mM: 10 µL of the reaction mixture were diluted with 990 µL MeOH.

Reaction with c_0 of **1m** = 8.0 mM: 5 µL of the reaction mixture were diluted with 995 µL MeOH.

Identical volumes were injected for the analyses (5 µL, Figure S22).

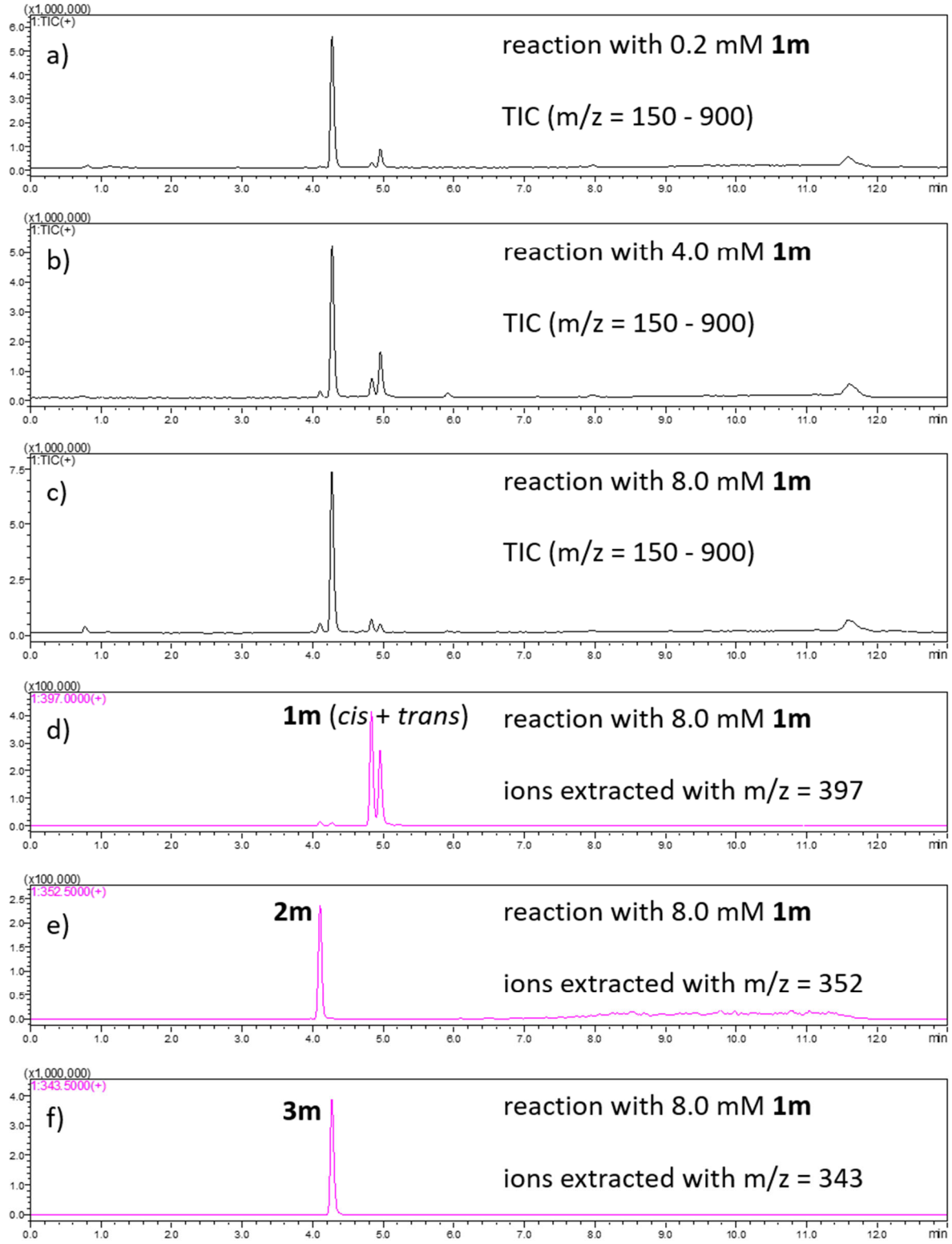


Figure S22. LC-MS chromatograms of the reaction of **1m** with 1.2 eq. of GSH after 1 hour reaction time at 25 °C at different initial concentrations of **1m**. a) TIC for reaction with 0.2 mM **1m**. b) TIC for reaction with 4.0 mM **1m**. c) TIC for reaction with 8.0 mM **1m**. d) Extracted ion chromatogram for m/z = 397 which corresponds to $[M+H]^+$ of **1m**. e) Extracted ion chromatogram for m/z = 397 which corresponds to $[M+2H]^{2+}$ of **2m**. f) Extracted ion chromatogram for m/z = 397 which corresponds to $[M+2H]^{2+}$ of **3m**. The diastereomers of **2m** are not resolved.

For the isolation of **2m** the following reaction was conducted:

Glutathione (14 mg, 48 µmol) was dissolved in NH_4HCO_3 buffer (8 mL, pH 8.0, 100 mM), 4-(2-nitrostyryl)pyridinium (19 mg, 40 µmol) in DMF (2 mL) was added. The mixture was stirred at room temperature for 4 hours before the product was isolated by preparative HPLC.

2m was isolated as a mixture of diastereomers. HRMS (m/z): (ESI+) calcd. $[M]^+$: 704.2960, found: 704.2949. The structure was confirmed by 2D-NMR analysis.

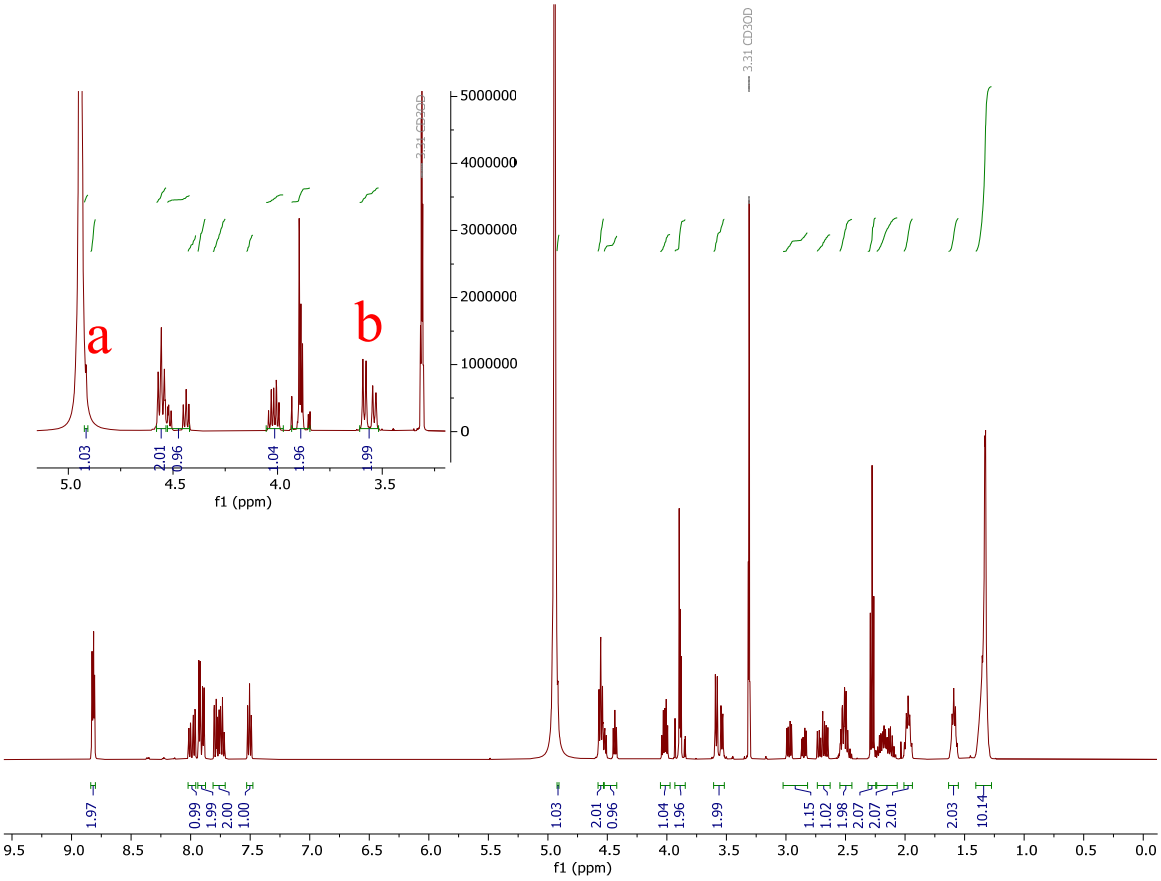


Figure S23. 1H NMR (500 MHz, CD_3OD) of **2m**.

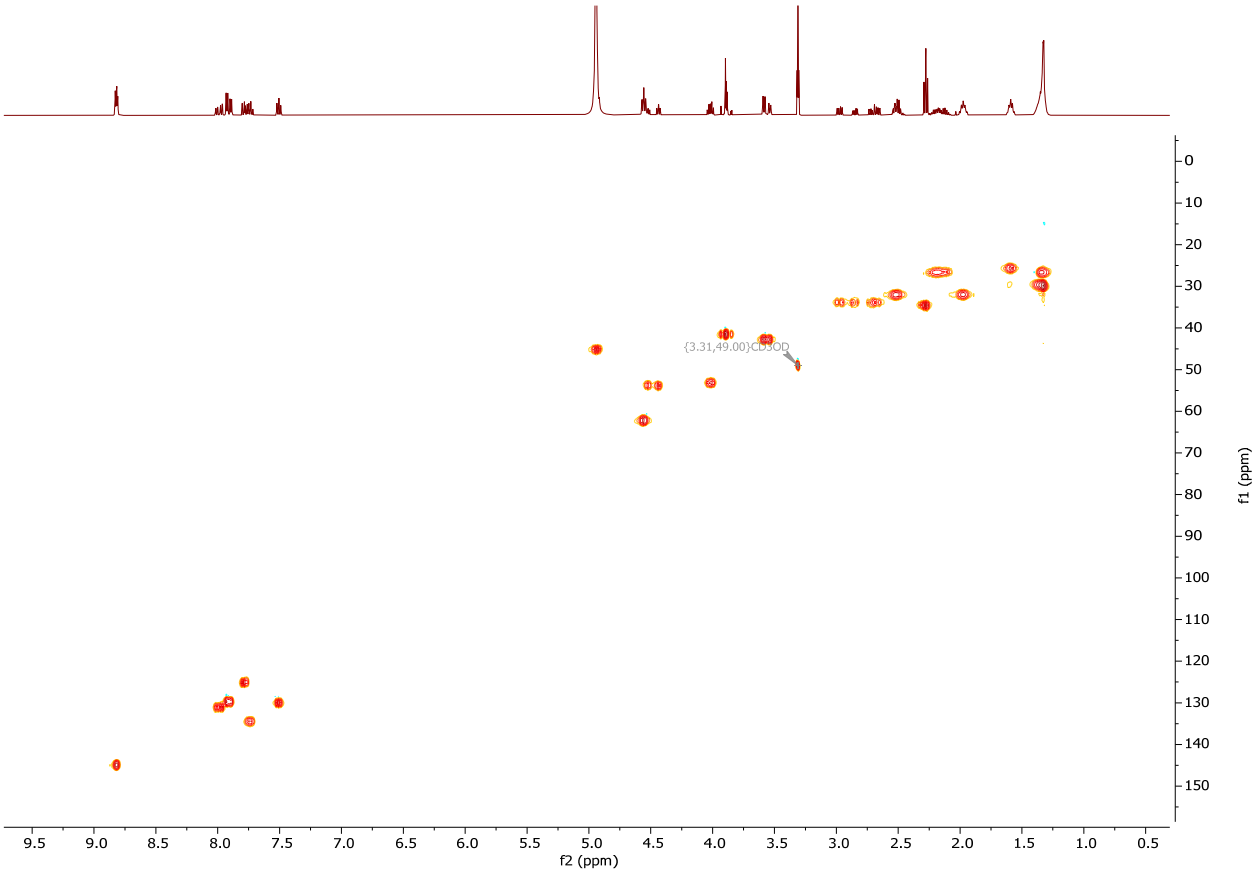


Figure S24. HMQC of **2m**.

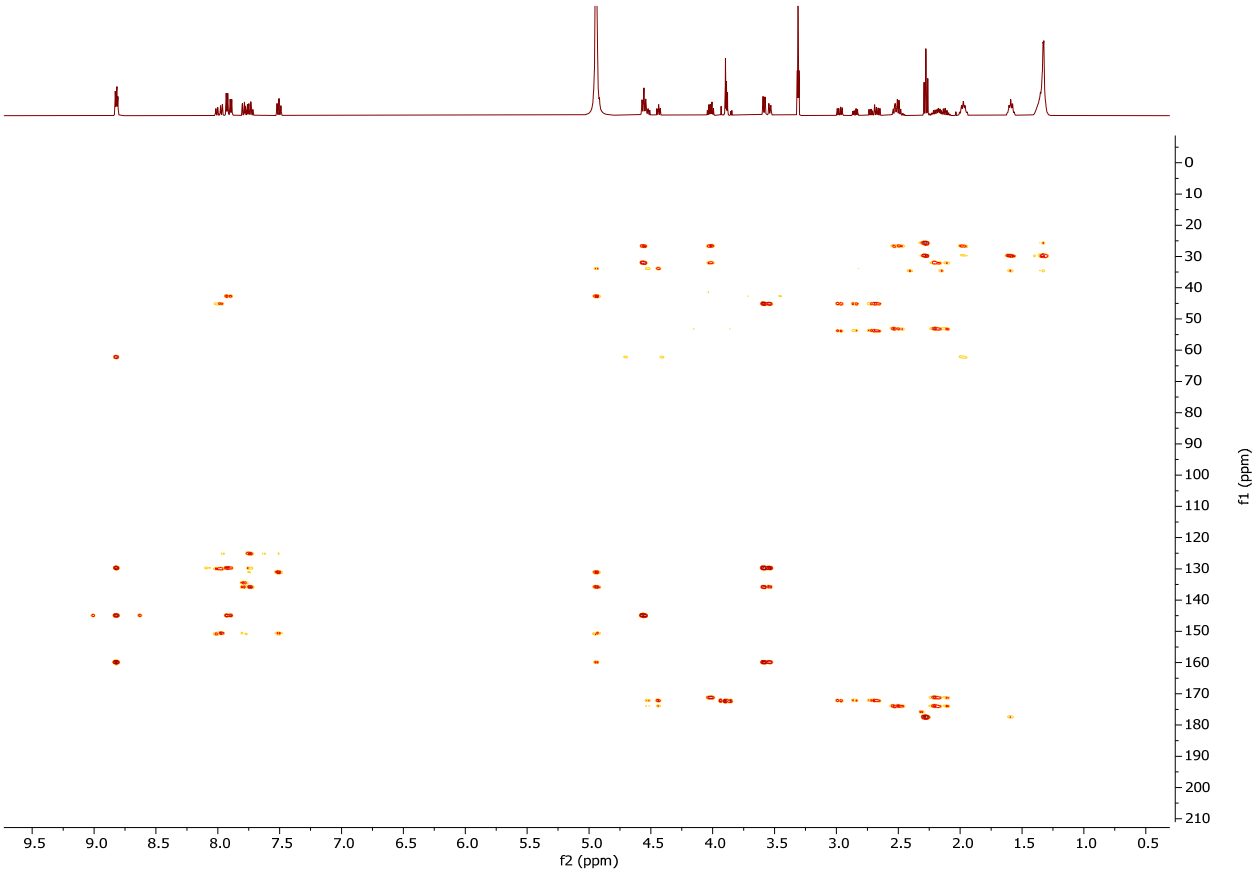


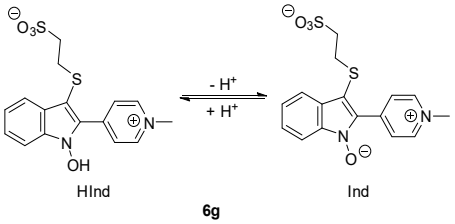
Figure S25. HMBC of **2m**.

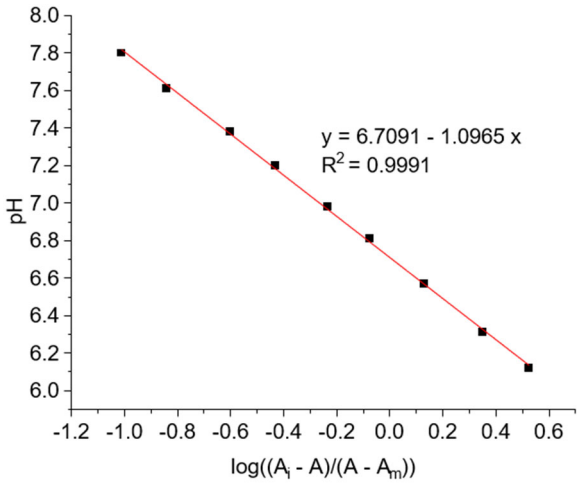
To test the reversibility of the addition of glutathione to **1m** the following test were conducted:

A) A solution of **2m** (0.40 mM) in NH_4HCO_3-buffer (100 mM, pH 8.0) was agitated in a Thermomixer at 37 °C for 4 hours. In the LC-MS analysis **1m**, **2m** and **3m** were observed (see Figure 1e in the main text).

B) A solution of **2m** (0.40 mM) and cysteine (4.0 mM) in NH_4HCO_3-buffer (100 mM, pH 8.0) was agitated at 37 °C for 4 hours. In the LC-MS analysis **2m(cys)**, **2m**, **3m(cys), 3m** and **1m** were observed (see Figure 1f in the main text).

pK_a-Determination of **6g**





The pK_a of **6g** was determined by UV-VIS titration. 40 µM unbuffered solutions of **6g** were adjusted to pH 2.0 or 12.0 by addition of diluted HCl or NaOH solution, respectively and the UV spectra of the resulting solutions recorded in 1 cm quartz cuvettes. These samples served to obtain the spectra of the fully protonated or deprotonated species, respectively. 382 nm was selected as the wavelength for the UV-VIS titration and corresponds to the absorption maximum of the deprotonated species. Datapoints with intermediate pH-values were obtained by adjusting the pH of buffered solutions (buffer: NaPi, 100 mM, pH 7.00) of **6g** (40 µM) with diluted HCl or NaOH respectively. A plot according to formula 11 results in a straight line that intersects the ordinate at the pK_a-value.

$$pH = pK_a + \log \frac{A_{Ind}-A}{A-A_{HInd}} \qquad [11]$$

Table S2. UV-VIS titration of **6g**

pH	A_{382nm}	$\frac{A_{Ind}-A}{A-A_{HInd}}$	$\log \frac{A_{Ind}-A}{A-A_{HInd}}$
2.00	0.56019 (A_{HInd})		
6.12	0.62064	3.33118	0.5226
6.31	0.64117	2.23322	0.34893
6.57	0.67155	1.35109	0.13068
6.81	0.70223	0.84336	-0.07399
6.98	0.72547	0.58411	-0.2335
7.20	0.75123	0.37052	-0.43119
7.38	0.7695	0.25092	-0.60046
7.61	0.78892	0.14469	-0.83955
7.80	0.79873	0.09763	-1.01043
12.00	0.82201 (A_{Ind})		

Modification of proteins

HRMS-analyzes of the modified proteins were conducted on a Bruker maXis II coupled to a Shimadzu UPLC (Nexera X2). Column: Phenomenex Jupiter C4 300A, 50 mm × 2 mm, 5 µm. Solvents were A (water + 0.1% v/v formic acid), B (CH_3CN + 0.1% v/v formic acid). The flow was set to 0.3 mL/minute and the temperature to 30 °C. 0 min – 10% B, 2min – 10% B, 9 min – 55%B, 11 min – 55% B, 13 min - 95% B, 15 min – 95% B, 17 min – 10% B, 20 min – 10% B.

Modification of reduced aprotinin

The three disulfide bonds in aprotinin were reduced by treatment with TCEP.

Sequence of aprotinin: RPDFCLEPPYTGPCKARIIRYFYNAKAGLCQTFVYGGCRAKRNNFKSAEDCMRTCGGA

Buffer solution: NH_4HCO_3-buffer (100 mM, pH 8.0), degassed by bubbling with nitrogen for 30 minutes.
Stock solution A: **1a** in DMF (10 mM).
Stock solution B: TCEP·HCl (8.0 mM) in NH_4HCO_3-buffer.
Stock solution C: Aprotinin (0.20 mM, sigma order number 10820) in NH_4HCO_3-buffer.

Stock solution C (0.20 mL, 40 nmol) and stock solution B (30 µL, 0.24 µmol) were added to NH_4HCO_3-buffer (690 µL), and the mixture was agitated for 3 hours at 37 °C. Subsequently, stock solution A was added (80 µL, 0.80 µmol) and the mixture stirred for another 3 hours before it was analyzed by HRMS.

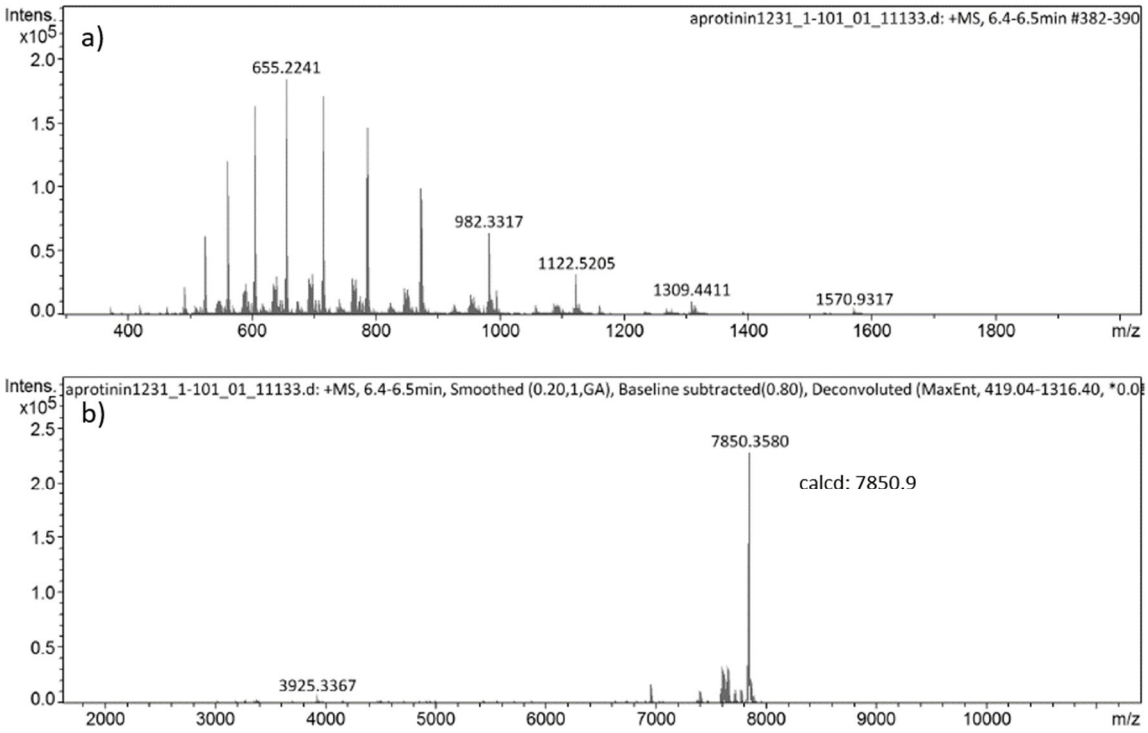
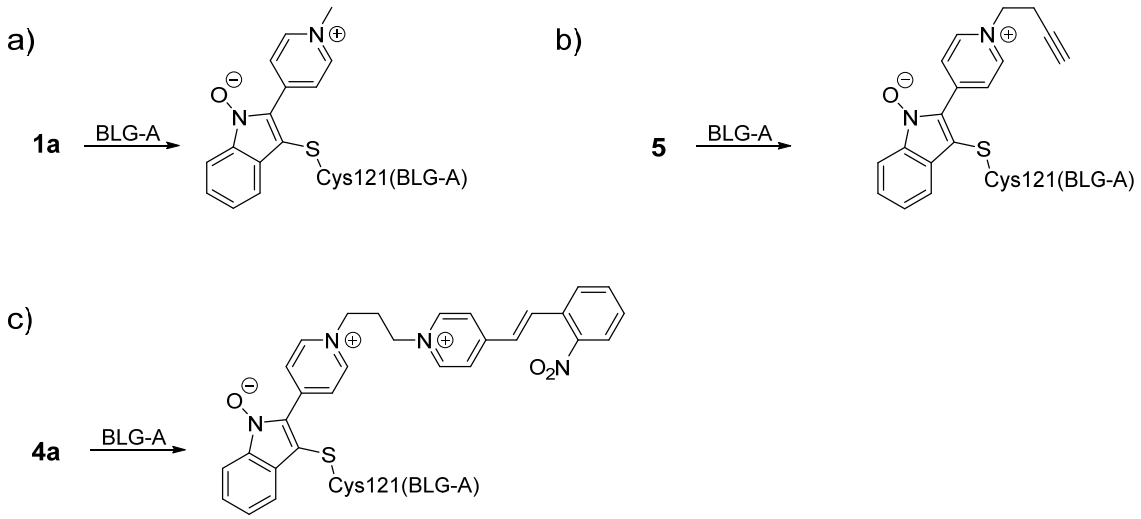


Figure S26. a) High resolution mass spectrum of 6-fold modified aprotinin. b) deconvoluted mass spectrum.

Modification of native β-Lactoglobulin A

Sequence of β-Lactoglobulin A: LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEEL-KPTPEGDLEILLQKWENDECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLVCQCLVRT-PEVDDEALEKFDKALKALPMHIRLSFNPTQLEEQCHI

β-Lactoglobulin A (BLG-A) was modified in separate reactions (a, b,c in Scheme below) with either **1a**, **5** or **4a**.



Buffer solution: NH_4HCO_3-buffer (100 mM, pH 8.0), degassed by bubbling with nitrogen for 30 minutes.

Stock solution A1: **1a** in DMF (10 mM).

Stock solution A2: **5** in DMF (10 mM).

Stock solution A3: **4a** in DMF (10 mM).

Stock solution B: β-Lactoglobulin A (0.40 mM, sigma order number L7880) in NH_4HCO_3-buffer (0.40 mM).

a) Stock solution B (50 µL, 20 nmol) was added to NH_4HCO_3-buffer (930 µL), followed by stock solution A1 (20 µL, 0.20 µmol). The mixture was agitated for 2 hours at 37 °C before analysis by HRMS.

b) Stock solution B (50 µL, 20 nmol) was added to NH_4HCO_3-buffer (930 µL), followed by stock solution A2 (20 µL, 0.20 µmol). The mixture was agitated for 2 hours at 37 °C before ananlysis by HRMS.

c) Stock solution B (50 µL, 20 nmol) was added to NH_4HCO_3-buffer (930 µL), followed by stock solution A3 (10 µL, 0.10 µmol). The mxiture was agitated for 2 hours at 37 °C before analysis by HRMS.

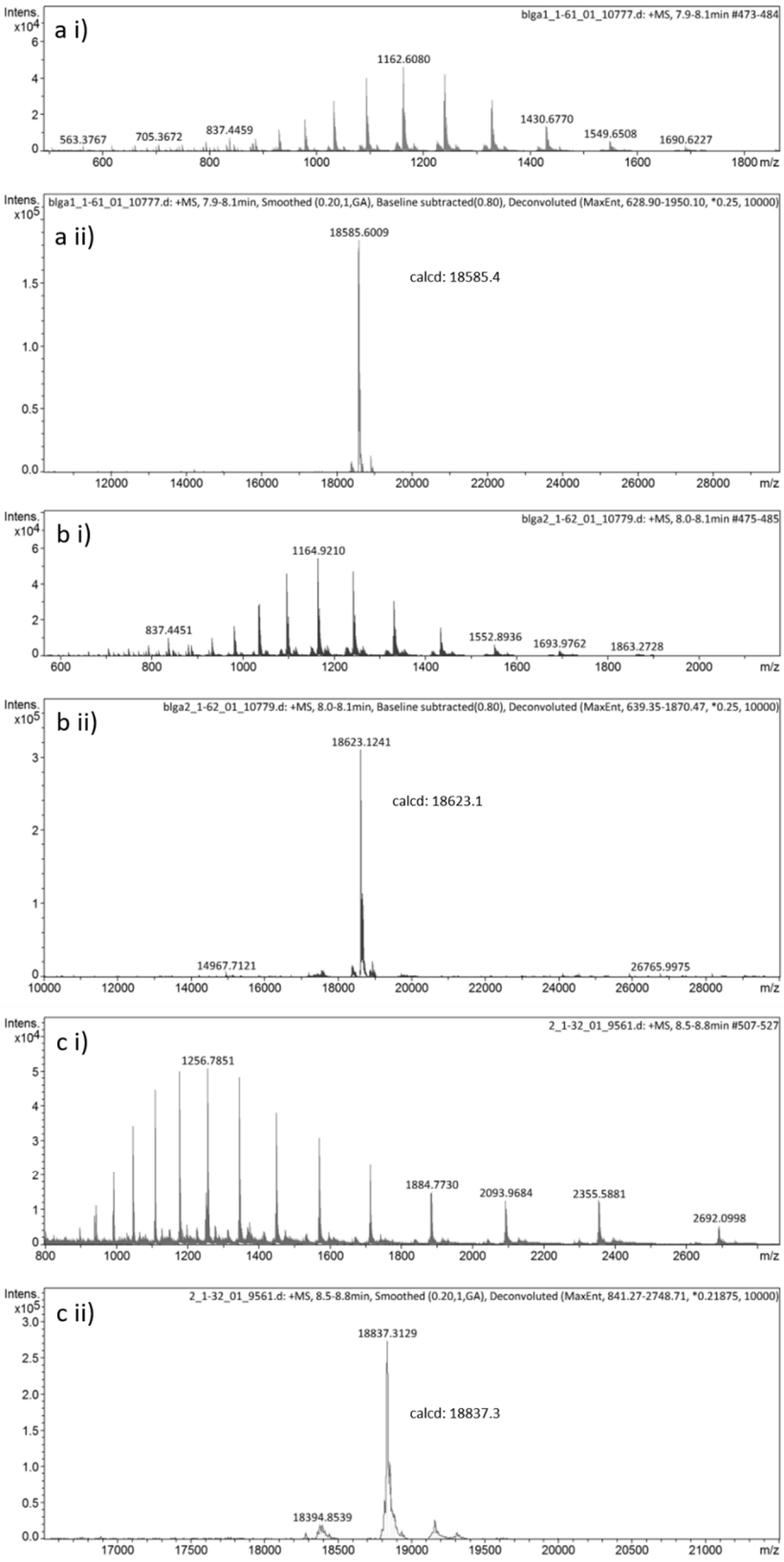


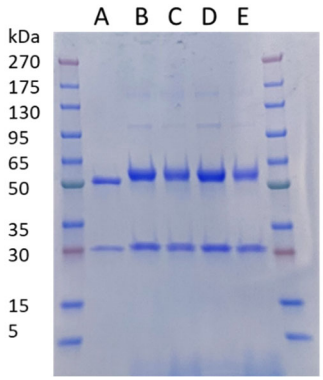
Figure S27. a i) High resolution mass spectrum of BLG-A modified with **1a**; a ii) deconvoluted mass spectrum of BLG-A modified with **1a**. b i) High resolution mass spectrum of BLG-A modified with **1a**; b ii) deconvoluted mass spectrum of BLG-A modified with **5**. c i) High resolution mass spectrum of BLG-A modified with **1a**; c ii) deconvoluted mass spectrum of BLG-A modified with **4a**.

Buffer solution: EDTA (0.50 mM), Tris·HCl (25 mM), NaCl (25 mM), pH 8.0 (adjusted with NaOH). The buffer was degassed by bubbling with N_2 for 30 min before the experiment.
Stock solution A: **NSP-MMAE** in DMF (1.0 mM).
Stock solution B: TCEP·HCl in reaction buffer (4.0 mM).
Stock solution C: Trastuzumab (40 µM, MedChemExpress order number HY-P9907) in reaction buffer.

Reaction for HRMS-analysis: Stock solution B (10 µL, 40 nmol) and solution C (100 µL, 0.0040 µmol) were added to the buffer solution (810 µL). After agitation for 90 minutes at 37 °C, stock solution A (80 µL, 0.080 µmol) was added and agitation continued for 3 hours at 37 °C. The mixture was dialysed against NH_4HCO_3 buffer (50 mM, pH 8.0) for 3 days (buffer exchange after 12 hours and 36 hours) at room temperature (Pur-A-Lyzer™, Maxi 6000, capacity 0.1-3 mL, dialysis tubes, MWCO 6-8 kDa).

Reactions for SDS-PAGE analyses:
A: stock solution B (1.0 µL, 4.0 nmol) and stock solution C (10 µL, 0.40 nmol) were added to the buffer solution (89 µL). The mixture was agitated for 90 minutes at 37 °C.
B: stock solution B (1.0 µL, 4.0 nmol) and stock solution C (10 µL, 0.40 nmol) were added to the buffer solution (89 µL). The mixture was agitated for 90 minutes at 37 °C. Stock solution A (8.0 µL, 8.0 nmol) was added and agitation continued for 3 hours at 37 °C.
C: stock solution B (1.0 µL, 4.0 nmol) and stock solution C (10 µL, 0.40 nmol) were added to the buffer solution (89 µL). The mixture was agitated for 90 minutes at 37 °C. Stock solution A (8.0 µL, 8.0 nmol) was added and agitation continued for 12 hours at 37 °C.
D: stock solution B (1.0 µL, 4.0 nmol) and stock solution C (10 µL, 0.40 nmol) were added to the buffer solution (73 µL). The mixture was agitated for 3 hours at 37 °C. Stock solution A (16 µL, 16 nmol) was added and agitation continued for 3 hours at 37 °C.
E: stock solution B (1.0 µL, 4.0 nmol) and stock solution C (10 µL, 0.40 nmol) were added to the buffer solution (73 µL). The mixture was agitated for 3 hours at 37 °C. Stock solution A (16 µL, 16 nmol) was added and agitation continued for 12 hours at 37 °C.

SDS-PAGE samples were prepared by mixing 50 µL (A, B, C, D, E) samples with 6× SDS-PAGE loading dye (10 µL) in 1.5 mL polypropylene tubes. The samples were then incubated at 95 °C for 10 minutes. After a brief centrifugation, 10 µL sample was loaded into gradient polyacrylamide SDS-gel (4 to 15%, *Mini-PROTEAN TGX Gels, BIO-RAD*). The gel was run at 150 V for 45 min and stained with Coomassie brilliant blue G/R-250 dye and destained with destaining solution.



Recipe for 6× SDS loading dye (50 ml)

- 15 mL 1M Tris·HCl (pH 6.8, pH adjusted with NaOH)
- 6 g SDS
- a small amount of Bromophenol blue for color
- 30 mL Glycerol
- 2 mL Betamercaptoethanol
- Water was added to a total volume of 50 mL

Recipe for Coomassie brilliant blue staining dye

- Coomassie Blue R250 1g and Coomassie Blue G250 1g were first dissolved in 300 mL ethanol.
- 50 mL acetic acid were added
- Water was added to a total volume of 1 L.

Recipe for destaining solution

- 10 % acetic acid
- 20 % ethanol
- 70 % water

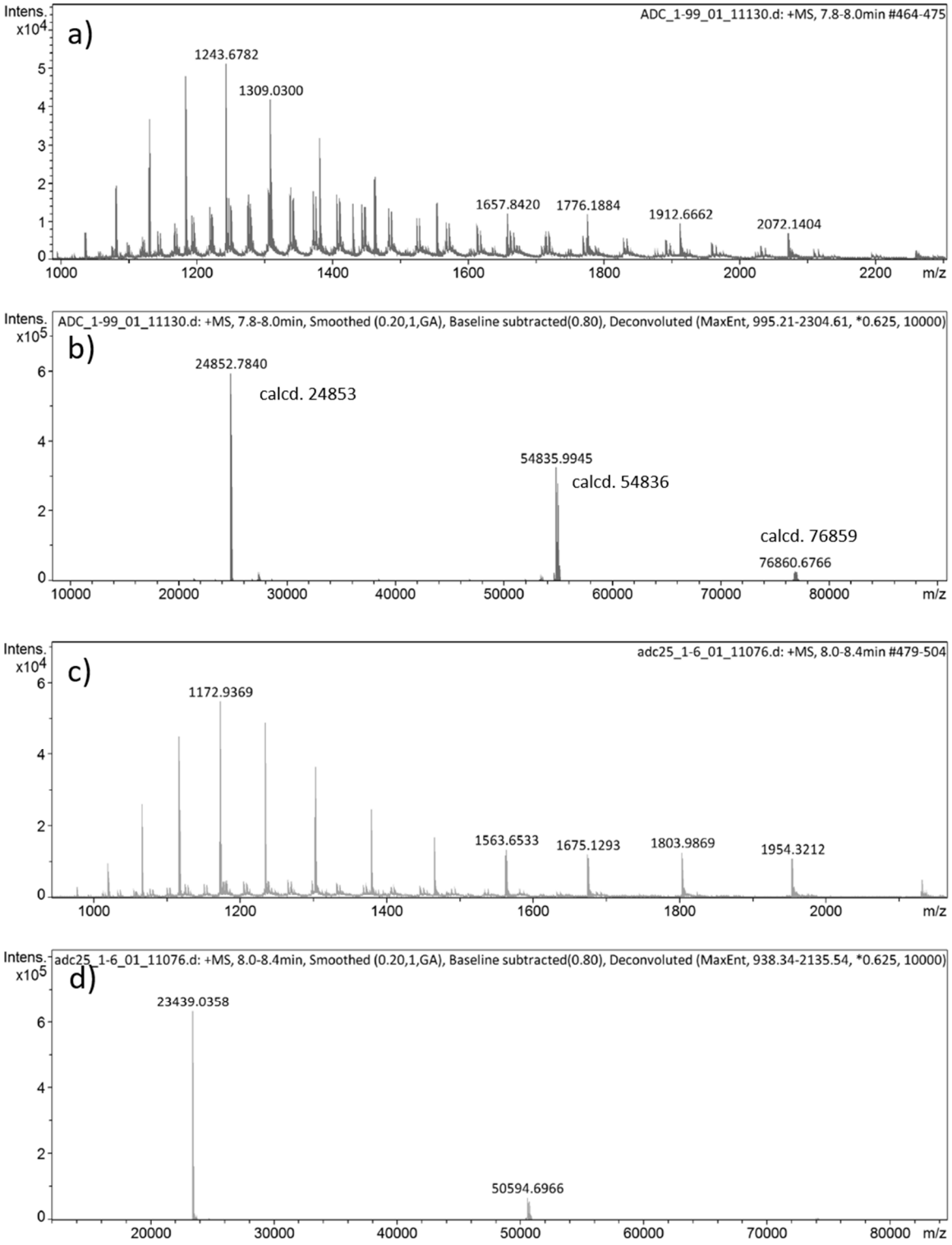


Figure S28. a) High resolution mass spectrum of trastuzumab modified with **NSP-MMAE**; b) deconvoluted mass spectrum of trastuzumab modified with **NSP-MMAE**. c) High resolution mass spectrum of unmodified trastuzumab d) deconvoluted mass spectrum of unmodified trastuzumab.

Determination of drug-to-antibody ratio

The drug-to-antibody ratio (DAR) was determined by UV-VIS spectroscopy in the dialyzed antibody preparation according to formula 11.

$$\mathrm{DAR} = \frac{A_{392(\mathrm{ADC})}/\varepsilon_{392(\mathrm{cDLC})}}{(A_{280(\mathrm{ADC})} - \frac{A_{280(\mathrm{cDLC})}}{A_{392(\mathrm{cDLC})}} A_{392(\mathrm{ADC})})/\varepsilon_{280(\mathrm{AB})}} = 7.3 \qquad [11]$$

with

$A_{392(\mathrm{ADC})}$ = absorption of the Antibody-Drug-Conjugate at 392 nm

$\varepsilon_{392(\mathrm{cDLC})}$= extinction coefficient of the cyclized-Drug-Linker-Conjugate (see further below)

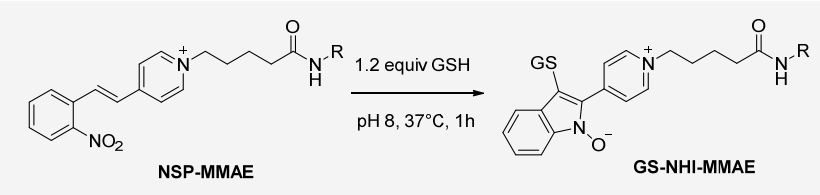
$A_{280(\mathrm{ADC})}$ = absorption of the Antibody-Drug-Conjugate at 280 nm

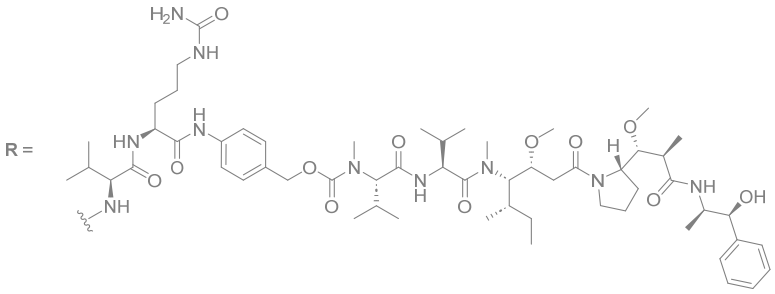
$A_{280(\mathrm{cDLC})}$ = absorption of the cyclized-Drug-Linker-Conjugate at 280 nm

$A_{392(\mathrm{cDLC})}$ = absorption of the cyclized-Drug-Linker-Conjugate at 392 nm

$\varepsilon_{280(\mathrm{AB})}$ = extinction coefficient of the unmodified AntiBody[3] = 2.25 × 10^5 M^{-1} cm^{-1}

The cyclized-Drug-Linker-Conjugate (**GS-NHI-MMAE**) was prepared by reacting glutathione with **NSP-MMAE**





Glutathione (0.24 mM) and NSP-MMAE (0.20 mM) were allowed to react in NH_4HCO_3-buffer (100 mM, pH 8.0) at 37 °C under agitation for 1 hour when LC-MS analysis indicated full conversion. The raction mixture (0.20 mL) was then diluted into NH_4HCO_3-buffer (0.80 mL, 100 mM, pH 8.0) to measure the UV-VIS spectrum of the conjugate at a concentration of 40 µM.

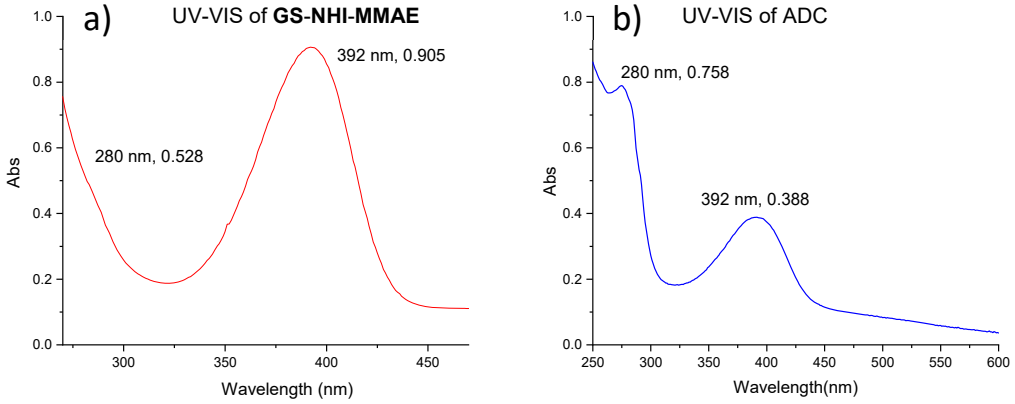


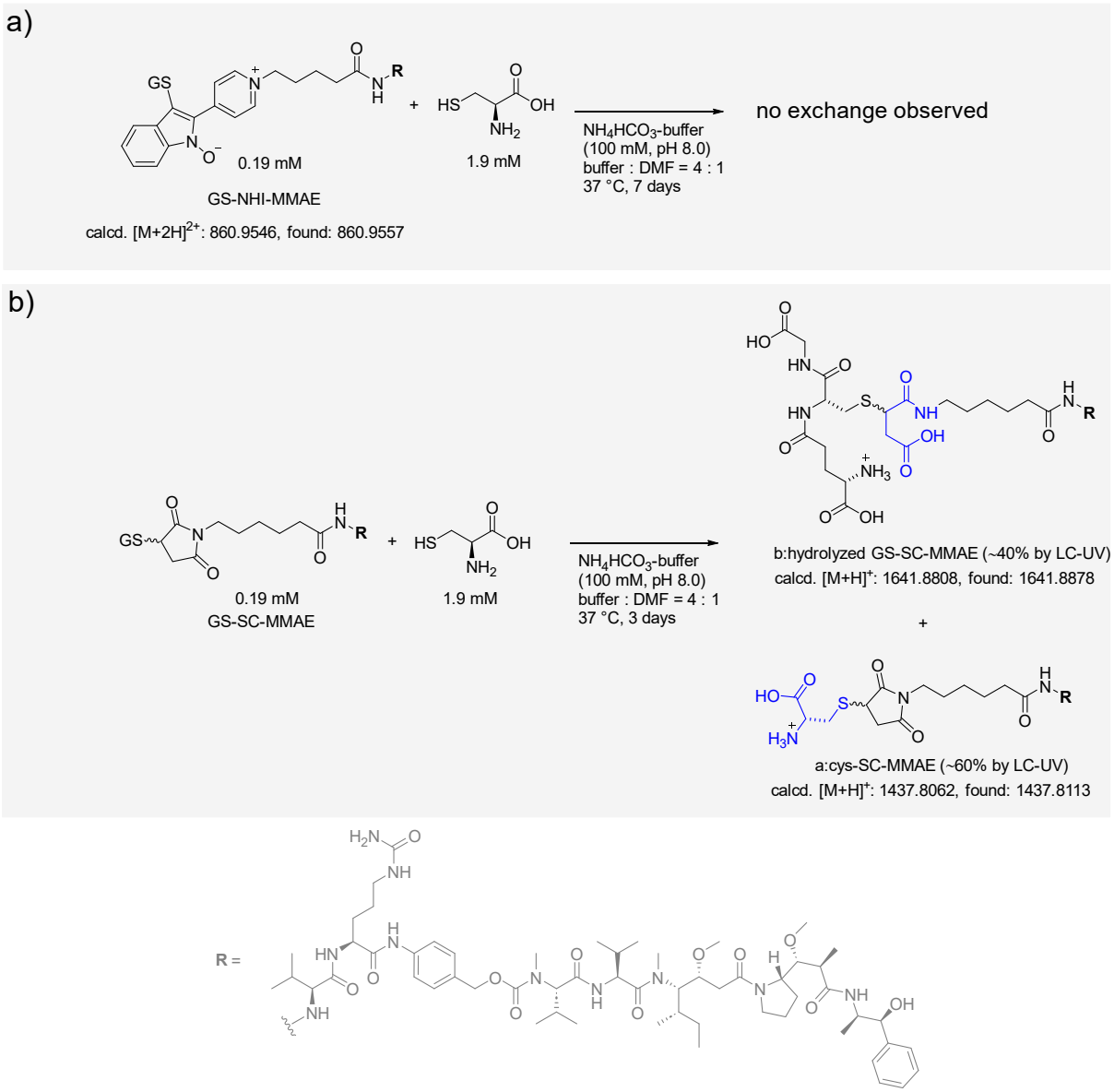
Figure S29. a) UV-VIS spectrum of cyclized-Drug-Linker-Conjugate at 40 µM concentration. b) UV-VIS spectrum of the antibody-drug-conjugate after dialysis. The measured absorption values at 280 and 392 nm are indicated.

Stability comparison for GS-NHI-MMAE with a succinimide analogue

The following stock solutions were prepared for the formation of GS-NHI-MMAE and GS-SC-MMAE and the subsequent stability tests.

Buffer solution: NH_4HCO_3-buffer (100 mM pH 8.0), degassed by bubbling with nitrogen for 30 minutes
Stock solution A: glutathione (50 mM) in NH_4HCO_3-buffer
Stock solutions B1: NSP-MMAE (1.0 mM) in DMF
Stock solution B2: MI-Val-Cit-PAB-MMAE (1.0 mM, CAS: 646502-53-6) in DMF;
Stock solution C: cysteine (50 mM) in NH_4HCO_3-buffer

a) Stock solution A (4.8 µL, 0.24 µmol) and stocksolution B1 (200 µL, 0.20 µmol) were added into 795.2 µl buffer solution and the mixture agitated at 37 °C for 1 hour when quantitative formation of GS-NHI-MMAE was confirmed by LC-MS. Stock solution C (40 µL) was added and agitation continued at 37 °C. Samples were taken after 1, 3, 5, and 7 days and analyzed by LC-MS.
b) Stock solution A (4.8 µL, 0.24 µmol) and stocksolution B2 (200 µL, 0.20 µmol) were added into 795.2 µl buffer solution and the mixture agitated at 37 °C for 1 hour when quantitative formation of GS-SC-MMAE was confirmed by LC-MS. Stock solution C (40 µL) was added and agitation continued at 37 °C. Samples were taken after 2 hours, 1 day, days and 3 days, and analyzed by LC-MS.



Scheme S1. Experiments to probe thiol exchange a) the glutathione conjugate (GS-NHI-MMAE) of NSP-MMAE is exposed to cysteine for 7 days at 37 °C, pH 8.0; only minor decomposition and no thiol exchange is observed. b) In contrast, the corresponding glutathione conjugate (GS-SC-MMAE) of the commercial drug linker MI-Val-Cit-PAB-MMAE readily exchanges with cysteine and also shows competing hydrolysis. The solutions contain additionally ca. 0.2 mM GSH before the addition of cysteine. Note: the regioselectivity of hydrolysis has not been investigated.

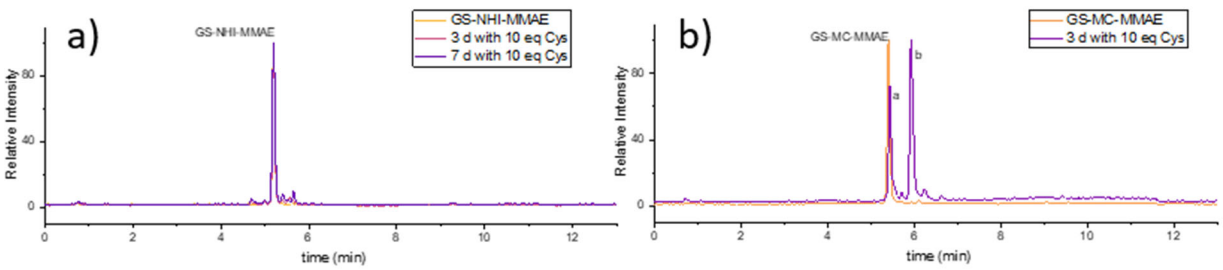


Figure S30. overlaid LC-MS chromatograms (TIC m/z 650 – 1850) for reaction a) before the addition of cysteine and after 3 days and 7 days after the addition of cysteine; and b) before the addition of cysteine and 3 days after the addition of cysteine.

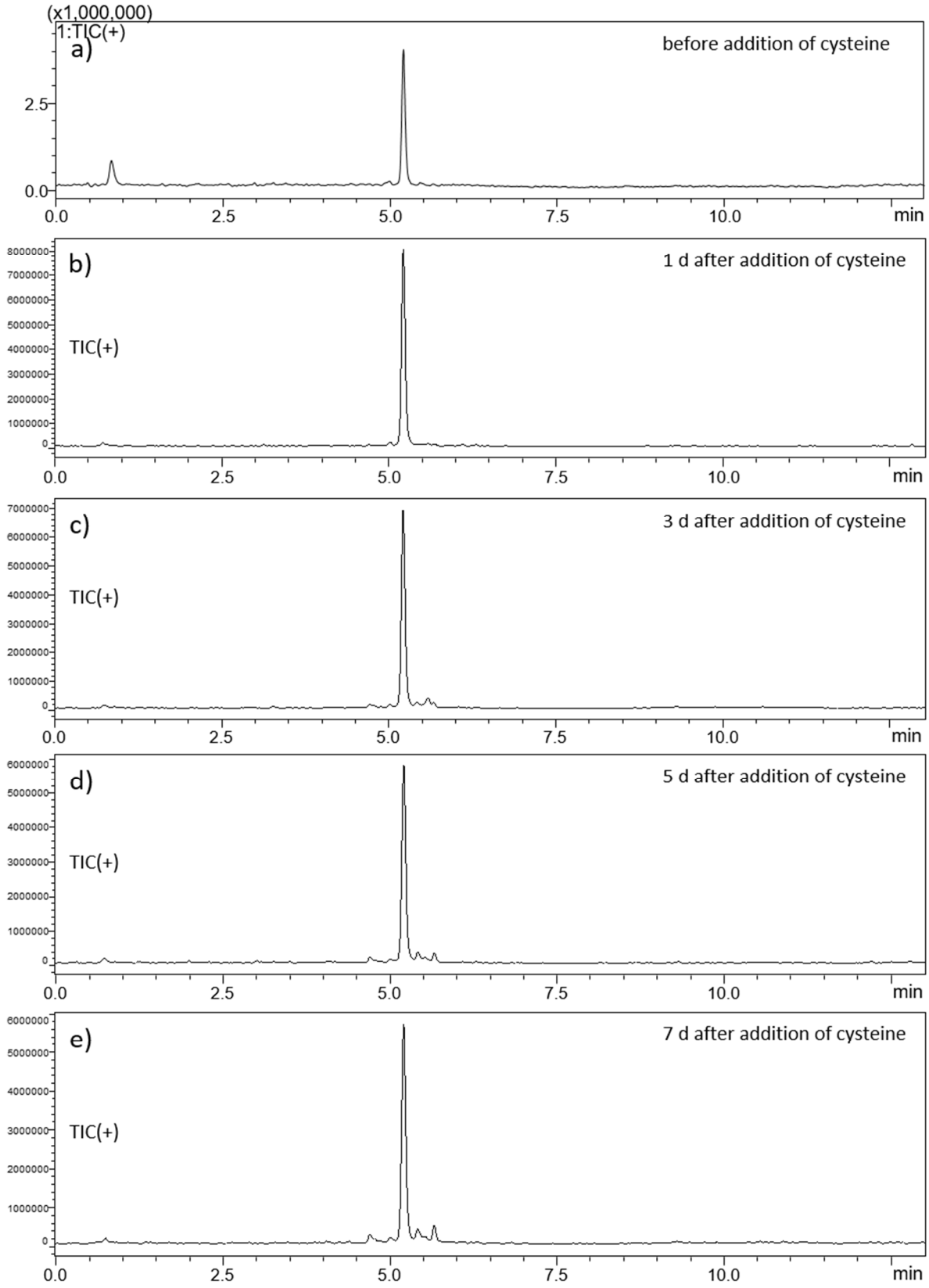


Figure S31. LC-MS chromatograms (TIC a) m/z 150-900 b)-e) m/z 650 – 1850) of a) the reaction of NSP-MMAE with glutathione after 1 hour; b) 1 day c) 3 days d) 5 days e) 7 days after addition of cysteine to the reaction mixture. Note that labels and tick marks for the time axis for b)-e) have been copied from a).

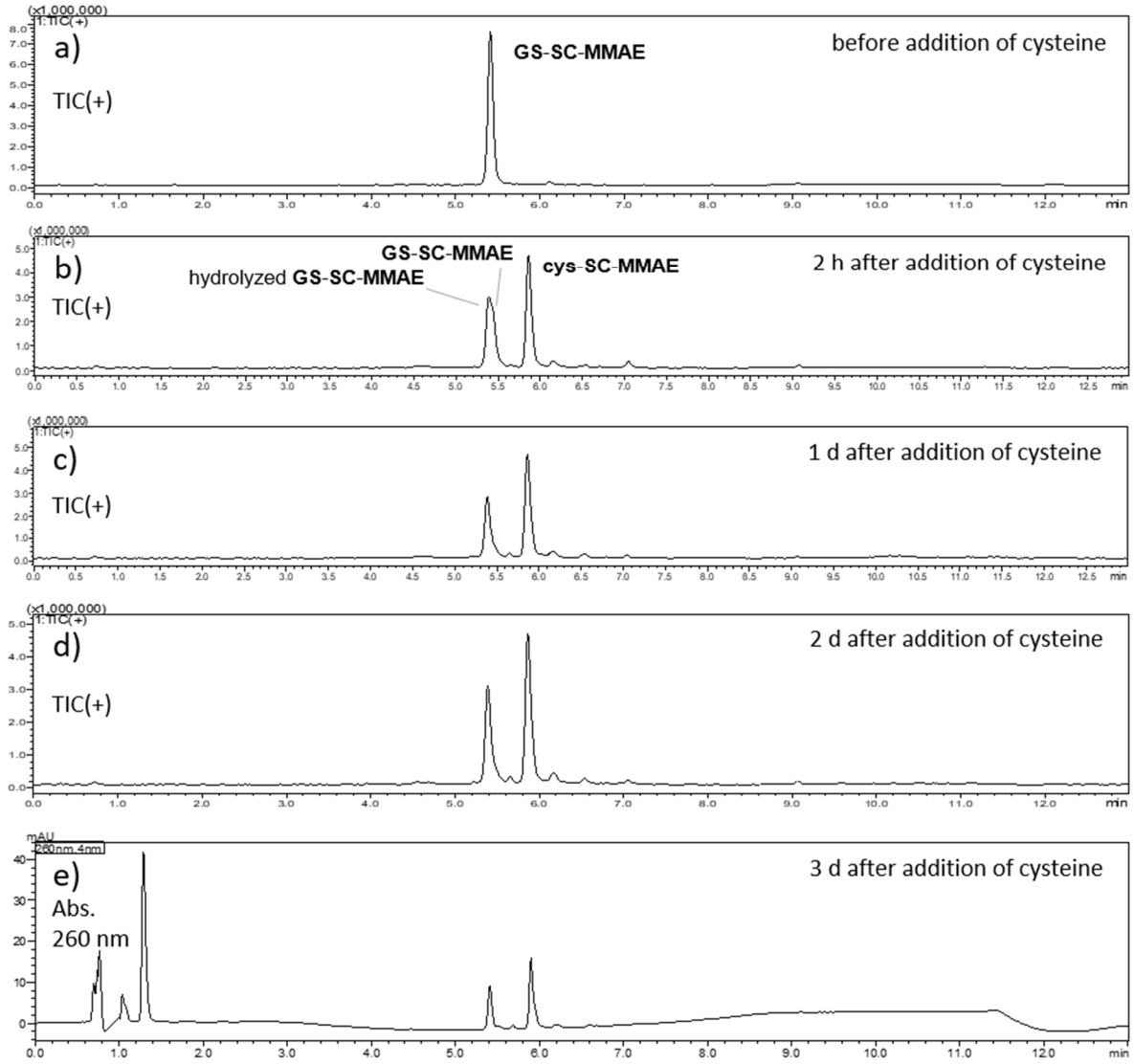
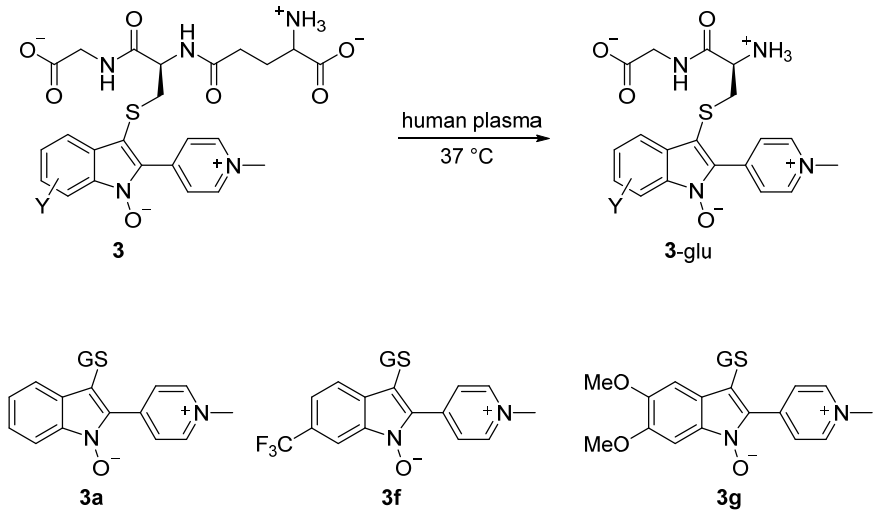


Figure S32. LC-MS chromatograms (TIC m/z 650 – 1850, a,b,c,d) and LC-UV chromatogram (260 nm, e) of a) the reaction of MI-Val-Cit-PAB-MMAE with glutathione after 1 hour; b) 2 hours, c) 1 day, d) 2 days, e) 3 days after the addition of cysteine to the reaction mixture.

Stock solutions of the purified TFA-salts of **3a**, **3f**, **3g**, respectively (40 mM in DMF, 18 µL for **3a**, 20 µL for **3f**, 20 µL for **3g**, respectively) were each mixed with human plasma (982 µL for **3a**, 980 µL for **3f**, 980 µL for **3g**, pooled human plasma (blood-derived) K2 EDTA, Loxo GmbH) to obtain three individual samples in glass 1.8 mL HPLC vials. The samples were agitated in a Thermomixer (650 – 700 rpm, 37 °C). For analysis 10 µL of the sample were diluted with 90 µL deionized water at the indicated time points and analyzed by LC-MS. The injection volume was 4 µL.

In all three samples, the formation of the respective species where a glutamate residue had been cleaved off was observed (Scheme S2). In accordance with the m/z value and the absorption spectrum, these species still contained the intact N-hydroxyindole pyridinium chromophore.



Scheme S2. Hydrolysis of the glutathione moiety of 3a,f,g in human plasma. An arbitrary protonation state has been drawn.

The stability of the N-hydroxyindole pyridinium unit in human plasma was determined by comparing the peak area of identified species after 48 hours, i.e., **3** and **3**-glu, to the area of **3** at time zero in the HPLC chromatogram (Table S3, Figure S33).

Table S3. Peak area of conjugates **3a**, **3f**, **3g** and of the identified fragments **3a**-glu, **3f**-glu, **3g**-glu at various time points upon incubation in human plasma.

time [hrs]	peak area [arbitrary units], (%)[a]					
	360 nm		344 nm		415 nm	
	3a	**3a**-glu	**3f**	**3f**-glu	**3g**	**3g**-glu
0	219581 (100%)	0 (0%)	239238 (100%)	0 (0%)	287091 (100%)	0 (0%)
48	41148 (19%)	107041 (49%)	87206 (36%)	134670 (56%)	186243 (65%)	34422 (12%)

a) % of peak area relative to peak area of **3a**, **3f**, **3g**, at t = 0 hours, respectively.

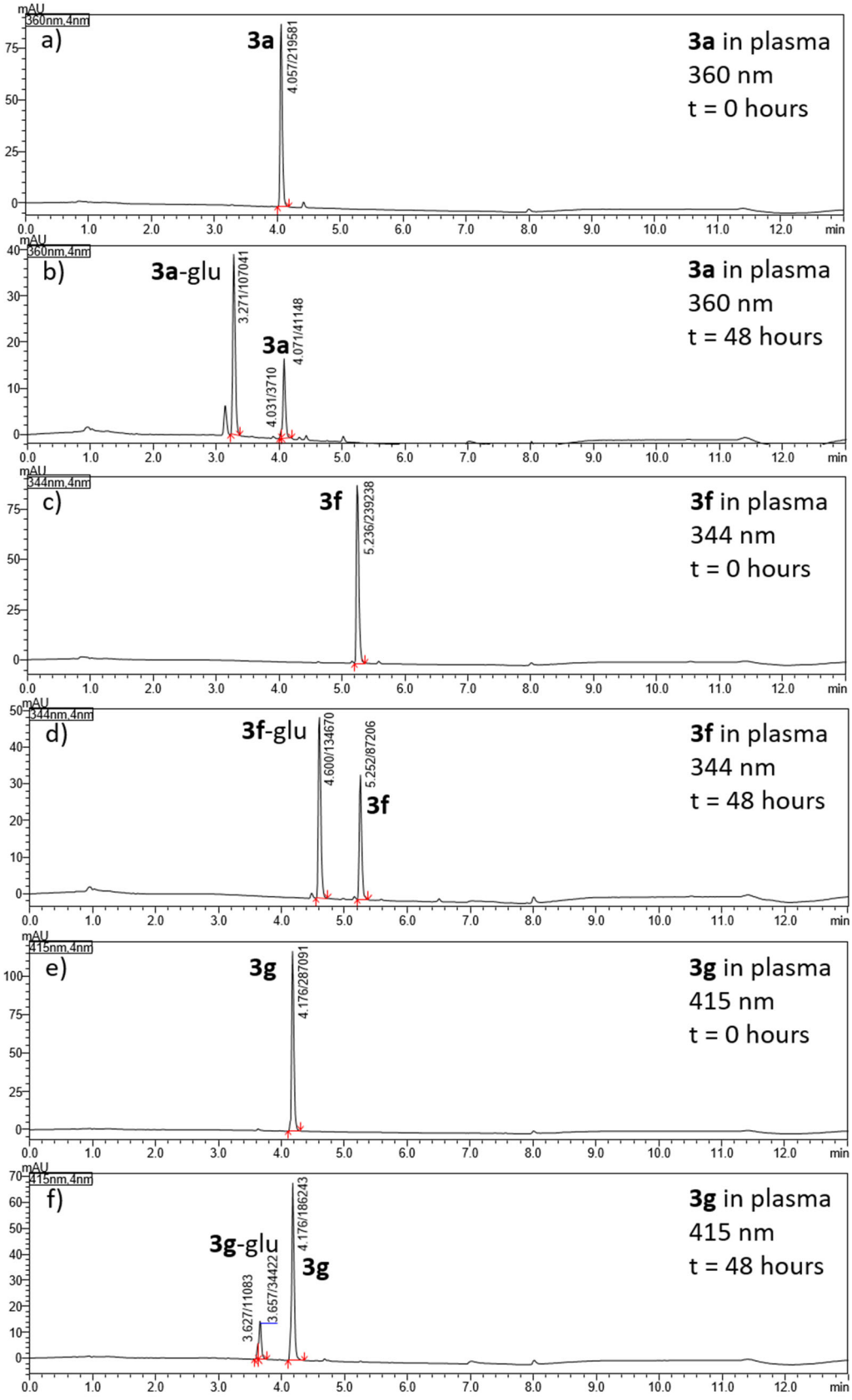


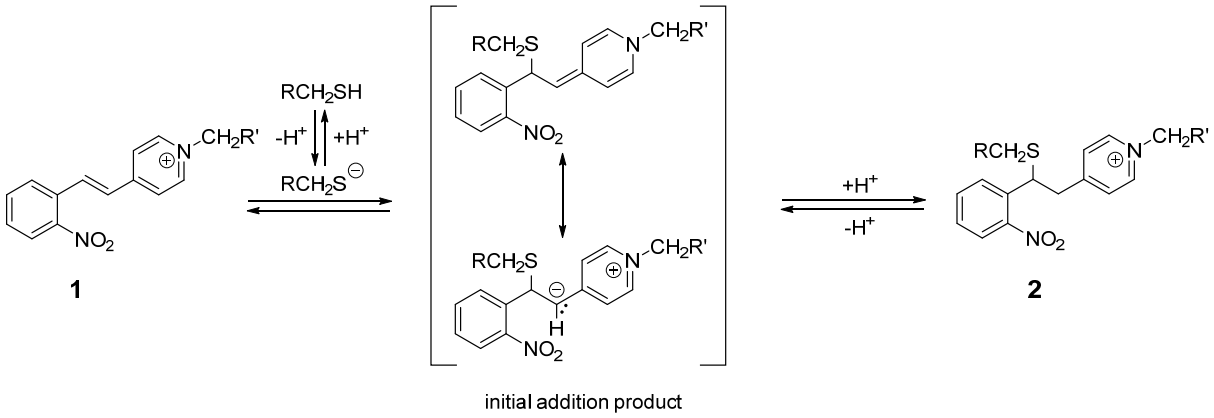
Figure S33. UV-traces of HPLC chromatograms of **3a**,**g**,**f** in human plasma at time = 0 hours and 48 hours. a) **3a**, 0 hours, detection wavelength 360 nm; b) **3a**, 48 hours; c) **3f**, 0 hours, detection wavelength 344 nm; d) **3f**, 48 hours; e) **3g**, 0 hours, detection wavelength 415 nm; f) **3e**, 48 hours.

Mechanistic considerations

While we have not carried out a mechanistic investigation of the reaction, we include here a short discussion of tentative mechanistic pathways and results from the literature for the interested reader.

The reactivity of nitroarenes has been discussed in several review articles.[4-8]

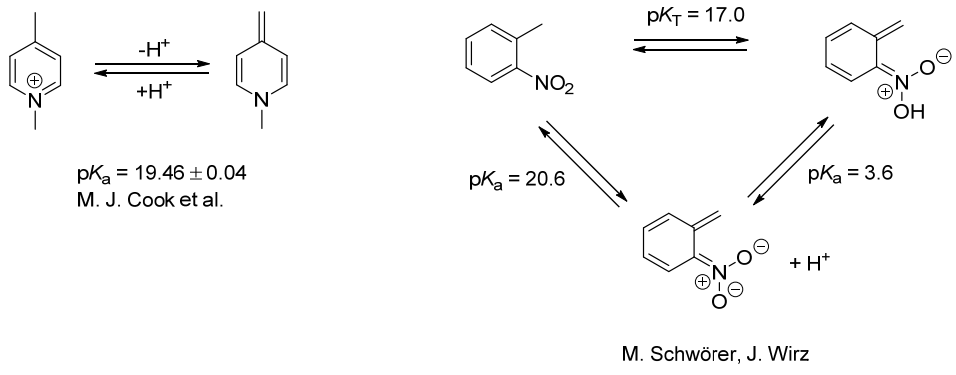
The click-and-lock reaction presented in the present paper is conducted at pH 8.0 in 0.1 M $[NH_4][HCO_3]$. Since the nucleophilicity of the thiolate anion is much higher than the nucleophilicity of the thiol we assume that the thiolate anion is the reactive nucleophile.[9]



Scheme S3. Attack of the thiolate nucleophile at Michael acceptor 1 and subsequent protonation of the product.

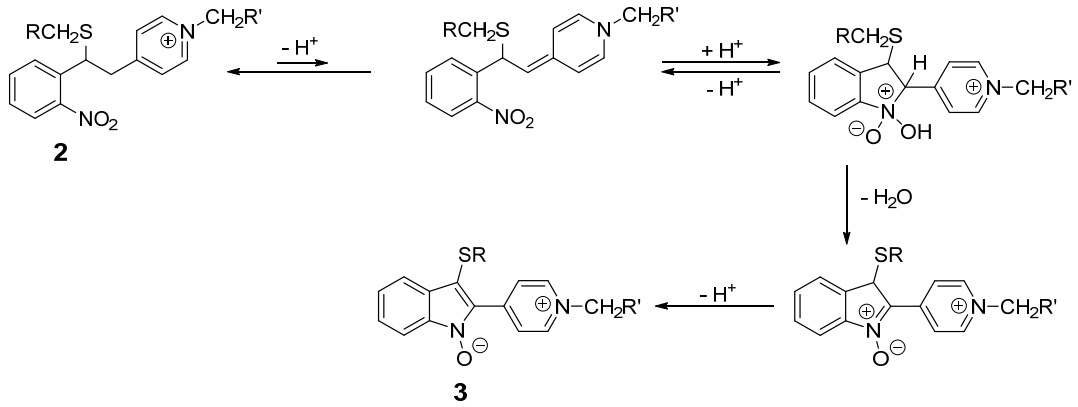
The initial addition product (Scheme S3) could directly enter the cyclization reaction or be protonated to form **2**. In the case of substrate **1m** (R' = $-(CH_2)_8CO_2H$) we could isolate and characterize **2**. When **2m** was exposed to the reaction conditions the cyclization to the corresponding hydroxyindole **3** was observed (see Figure 1e in the main text).

2 has two weakly acidic benzylic positions. The pK_a-values of the truncated substructures have been reported (Scheme S4).[10, 11] The sulfur substituent in **2** will likely provide an additional moderate contribution to the acidification of the 2-nitrotoluene substructure.[12]



Scheme S4. Reported pK_a-values for the truncated C-H acidic motifs in **2**, the equilibrium constant for the tautomeric equilibrium of 2-nitrotoluene and the pK_a of the tautomer of 2-nitrotoluene.[10, 11]

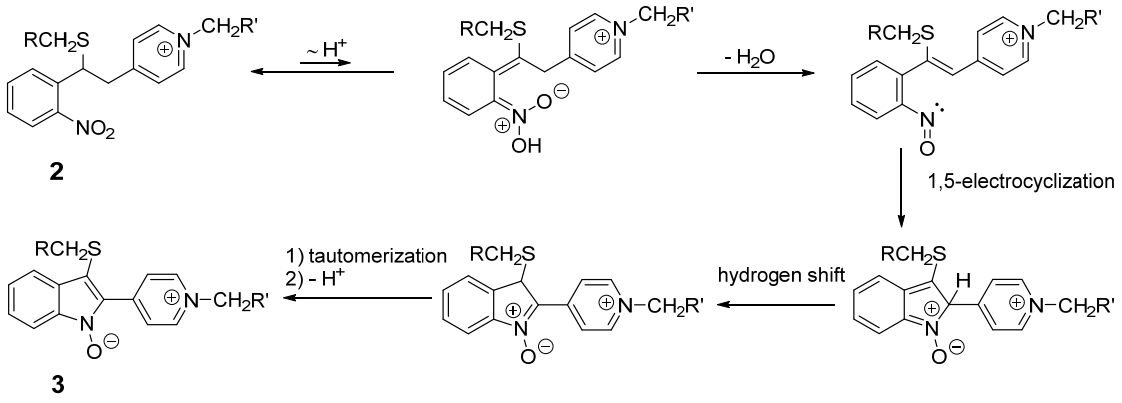
The initial addition product shown in Scheme S3 could directly attack the nitro group (Scheme S5). Although direct attack of the nitro group has been reported for intramolecular reactions[5-8] and also for intermolecular reactions,[13-15] the nitro group is generally considered a poor electrophile. Nitrobenzene is furthermore an exceedingly weak base, so that an activation of the nitro group in a significant equilibrium ratio can not be expected.[16] Reactions of nitroarenes with nucleophiles, such as Grignard reagents, can also proceed via an initial electron transfer process.[4]



Scheme S5. Nucleophilic attack of the nitro-group at nitrogen.

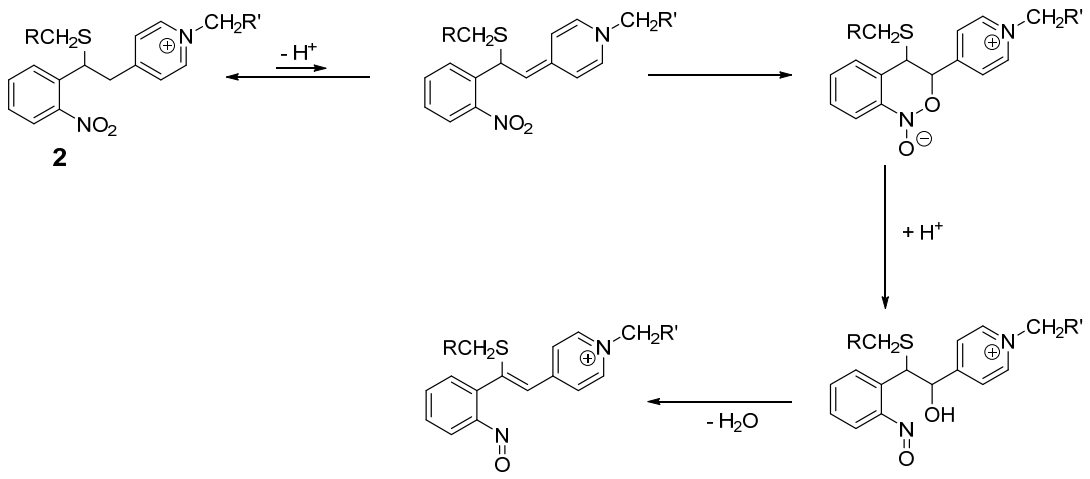
An established mechanism for the formation of N-hydroxy indoles proceeds through the cyclization of 2-nitrosostyrenes.[17, 18] This mechanism has been invoked in the cyclization of 2-nitrostyrenes and related compounds under various reductive conditions.[19-22]

However, few examples of dehydrative redox isomerizations that convert 2-ethyl-nitrobenzene derivatives to 2-nitrosostyrenes have been reported as far as we know. Wróbel and Mąkosza investigated the hydroxyindole synthesis under basic conditions from 1-ethyl-2-nitrobenzene derivatives that carried an electron withdrawing group in the benzylic position.[23] They proposed the formation of a 2-nitrosostyrene intermediate which cyclizes to form the product. Although the pK_a-values of our two C-H acidic positions are expected to be significantly higher than those in the systems reported by Mąkosza and Wróbel, they likely do not differ dramatically between each other (Scheme S4) and deprotonation might occur at either position (Scheme S6).



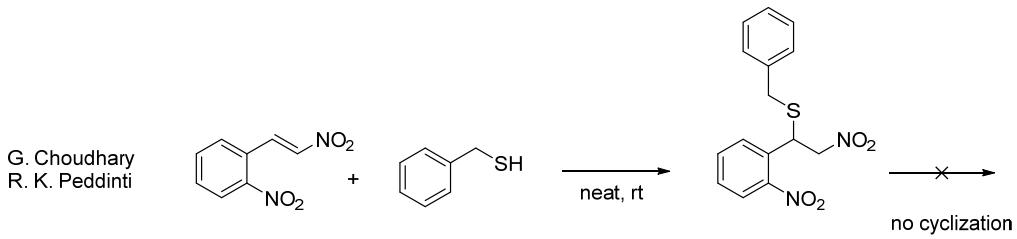
Scheme S6. Formation of a 2-nitrosostyrene intermediate and subsequent cyclization.

An alternative pathway that might enable the dehydrative isomerization to a 2-nitrosostyrene derivative can be found in analogy to a proposal invoked by Yokoshima and coworkers for their synthesis of Aurachins.[24] Here the reaction of the nucleophilic carbon center would be directed at the oxygen of the nitro group. The resulting cyclic structure could ring-open and subsequently eliminate water to form the nitrosostyrene intermediate (Scheme S7).



Scheme S7. Alternative pathway for the formation of an 2-nitrosostyrene intermediate.

Furthermore it is noteworthy that no cyclization was observed following the addition of benzyl mercaptane to 1-nitro-2-(2-nitrovinyl)benzene by Choudhary and Peddinti in their study of nitrogen and sulfur nucleophiles to (nitrovinyl)arenes under neat conditions (Scheme S8).[25]



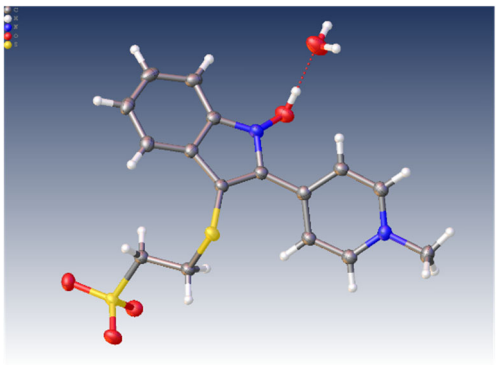
Scheme S8. Michael addition without cyclization reported by Choudhary and Peddinti.

Finally, the maybe most familiar mechanism for the tautomerization of the 2-nitrotoluene moiety is photochemical.[11, 26] We can exclude a significant photochemical contribution to product formation because we did not irradiate the solutions.

We can, however, not exclude the involvement of radical intermediates.

Single crystal X-ray diffraction data and experimental

6g (5.0 mg) was added to a water/acetone-mixture (5 mL, water/acetone = 4 : 1). The mixture was heated to 50 °C under stirring and filtered while warm. Upon cooling to room temperature the filtrate was collected and stored in the fridge at 4 °C. Single yellow block-shaped crystals formed upon standing.



Experimental.[27, 28 29, 30 31] Single yellow block-shaped crystals. A suitable crystal of **6g_150K** with dimensions 0.20 × 0.17 × 0.10 mm^3 was selected and mounted on a mylar loop in perfluoroether oil on a STOE STADIVARI diffractometer and kept at a steady T = 150 K during data collection. The structure was solved with the **ShelXT** 2018/2 solution program using dual methods and by using **Olex2** 1.5 as the graphical interface. The model was then refined with **ShelXL** 2018/3 using full matrix least squares minimisation on $\boldsymbol{F^2}$.

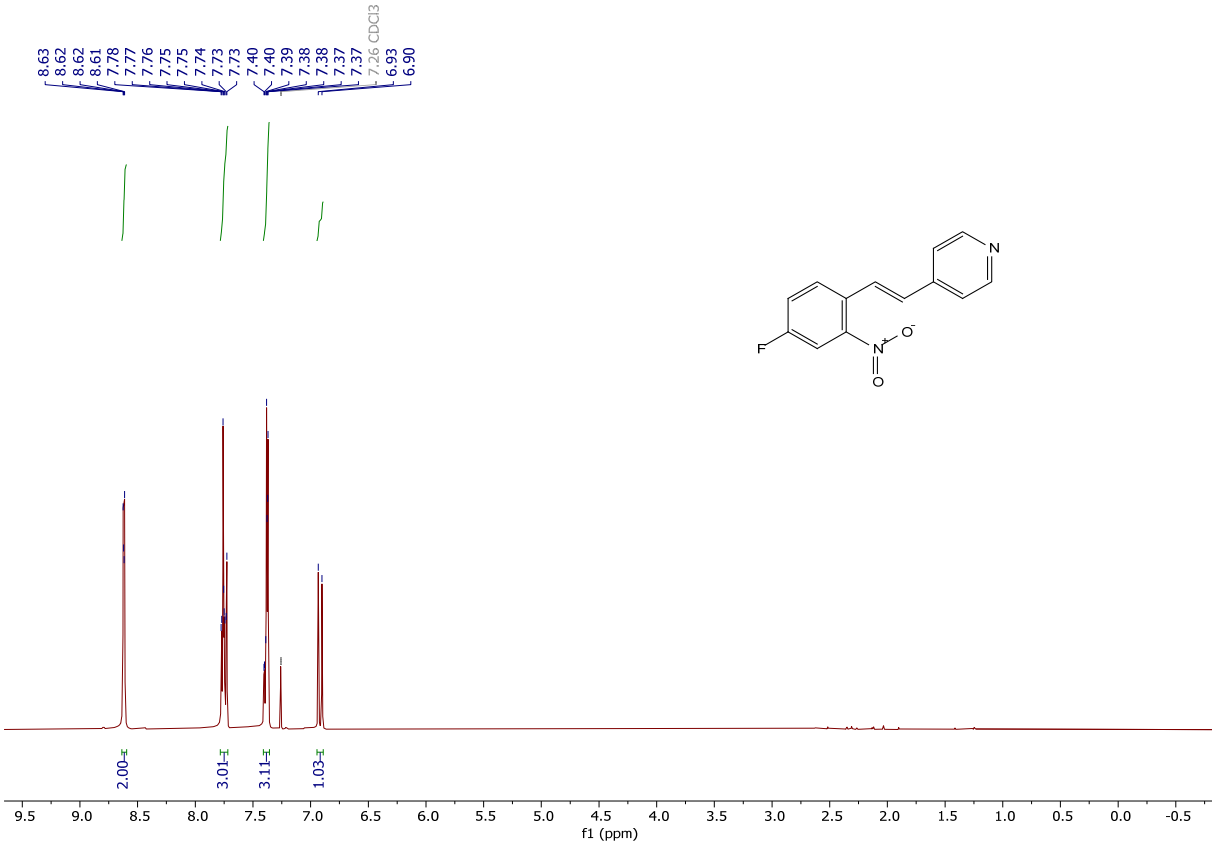
Crystal Data. $C_{16}H_{18}N_2O_5S_2$, M_r = 382.44, triclinic, P-1 (No. 2), a = 8.2197(2) Å, b = 9.7011(2) Å, c = 11.5746(2) Å, α = 113.735(2) °, β = 92.388(2) °, γ = 99.774(2) °, V = 826.42(3) $Å^3$, T = 150 K, Z = 2, Z' = 1, μ(Cu K_α) = 3.208, 22220 reflections measured, 3201 unique (R_{int} = 0.0147) which were used in all calculations. The final wR_2 was 0.1010 (all data) and R_1 was 0.0379 (I≥2 σ(I)).

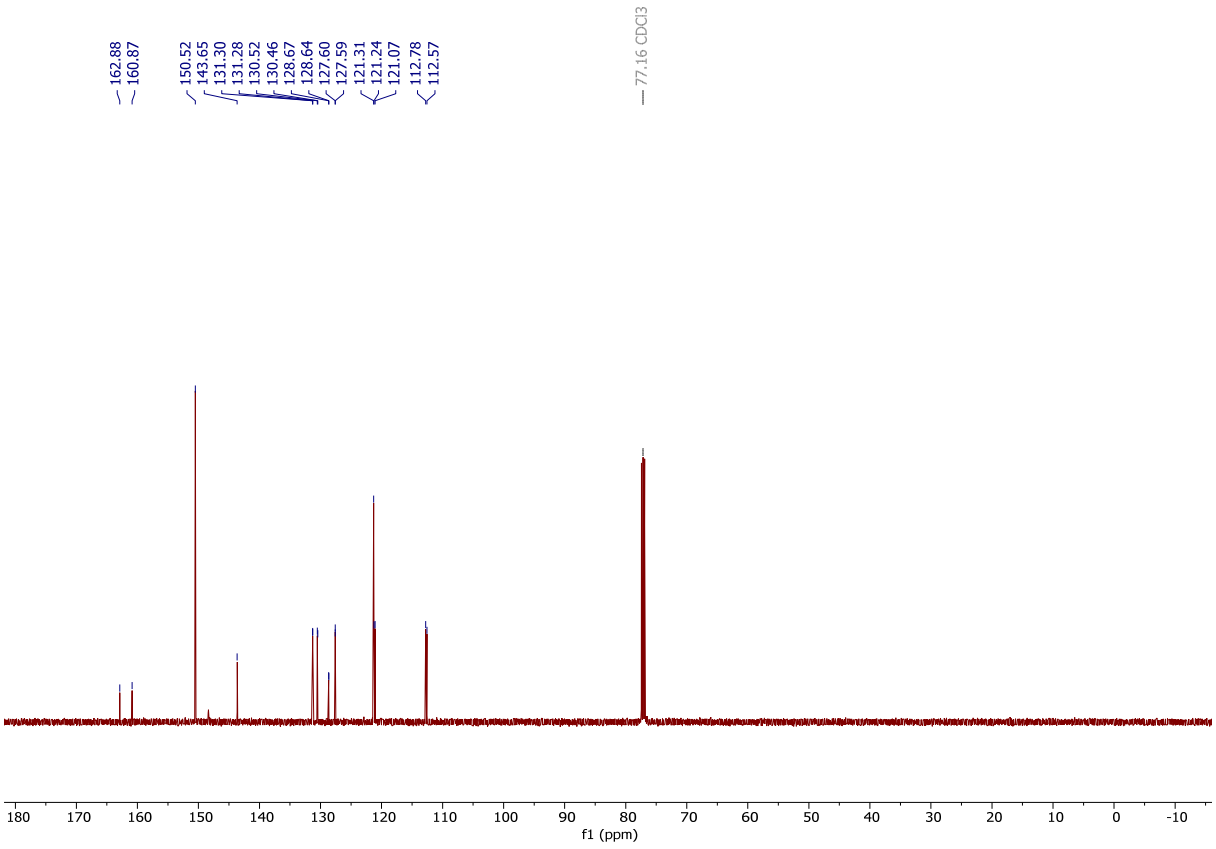
CCDC deposition number: 2278235

Compound	**6g_150K**
Formula	$C_{16}H_{18}N_2O_5S_2$
$D_{calc.}$/ g cm^{-3}	1.537
μ/mm^{-1}	3.208
Formula Weight	382.44
Colour	yellow
Shape	block-shaped
Size/mm^3	0.20×0.17×0.10
T/K	150
Crystal System	triclinic
Space Group	P-1
a/Å	8.2197(2)
b/Å	9.7011(2)
c/Å	11.5746(2)
α/°	113.735(2)
β/°	92.388(2)
γ/°	99.774(2)
V/Å^3	826.42(3)
Z	2
Z'	1
Wavelength/Å	1.54186
Radiation type	Cu Kα
Θ_{min}/°	5.076
Θ_{max}/°	72.830
Measured Refl's.	22220
Indep't Refl's	3201
Refl's I≥2 σ(I)	3191
R_{int}	0.0147
Parameters	232
Restraints	0
Largest Peak	0.397
Deepest Hole	-0.427
GooF	1.085
wR_2 (all data)	0.1010
wR_2	0.1009
R_1 (all data)	0.0381
R_1	0.0379

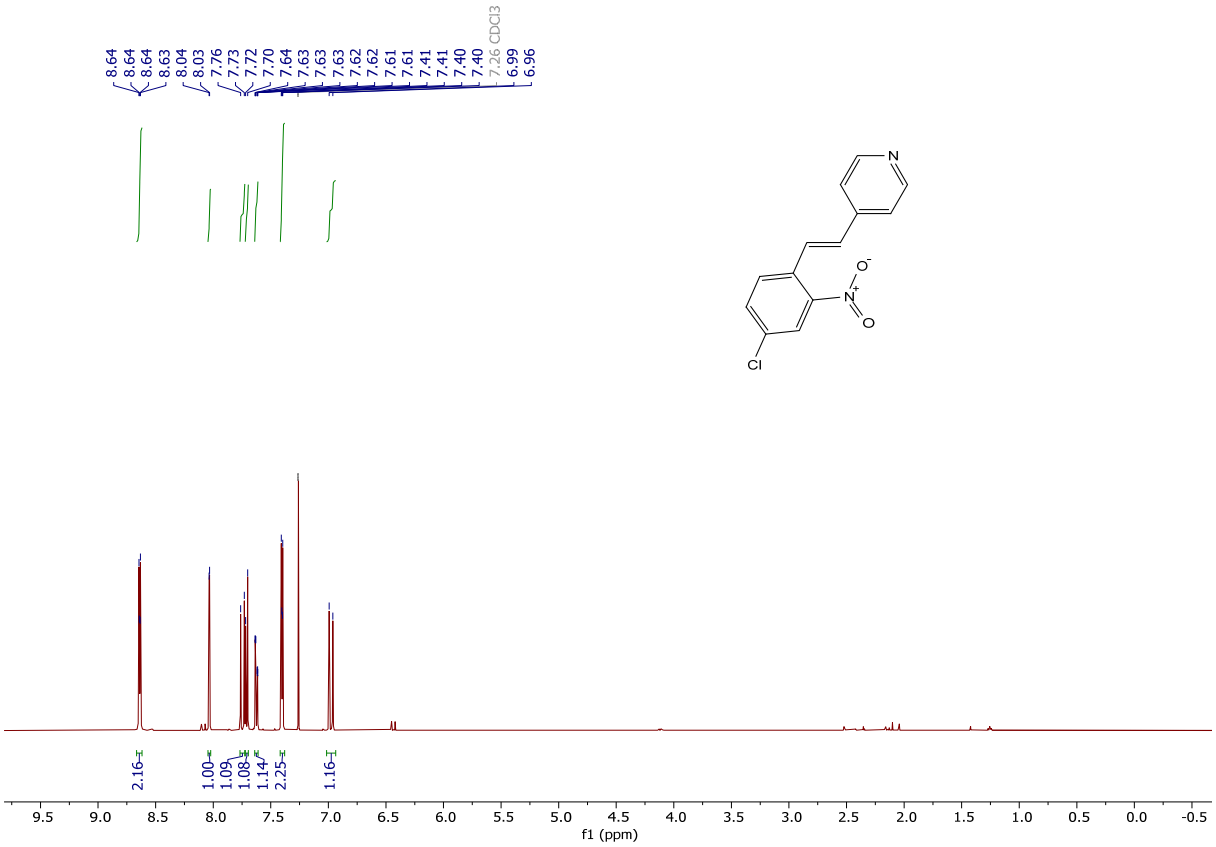
NMR spectra

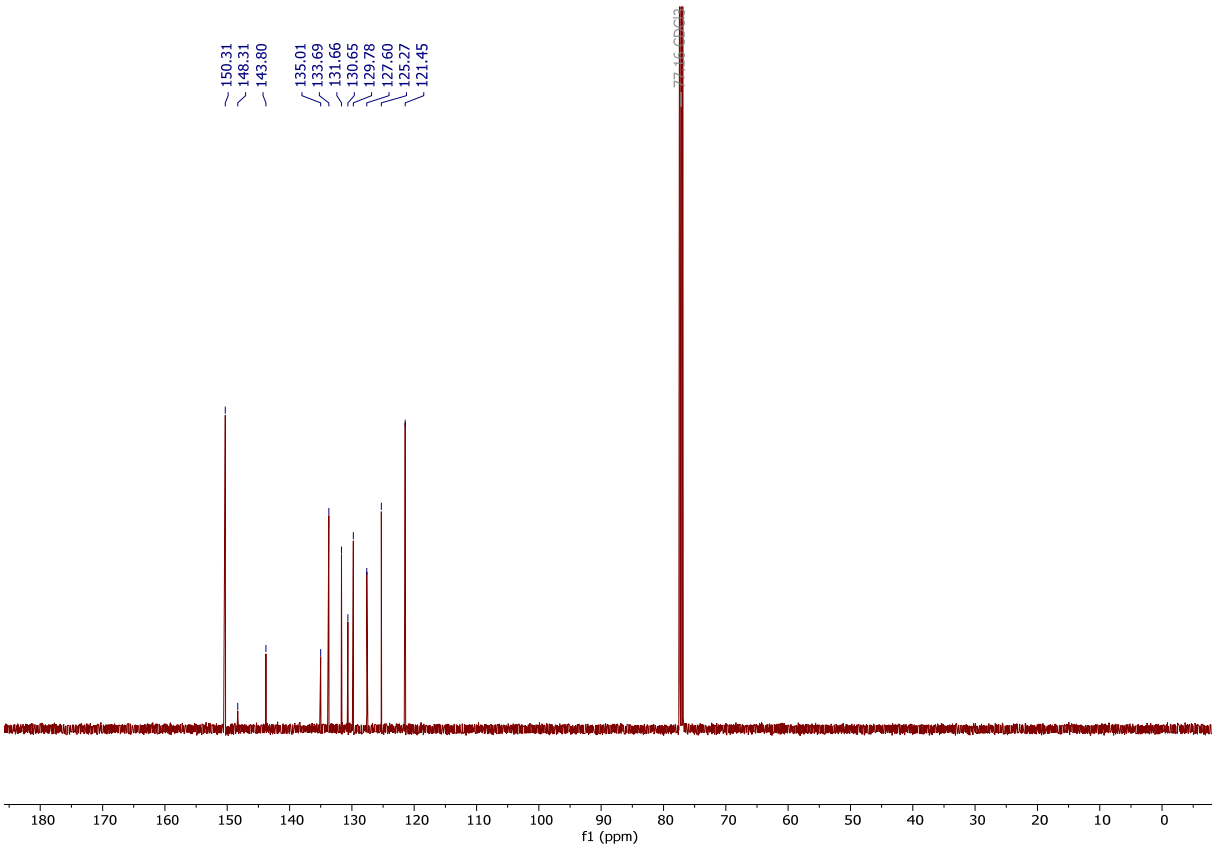
^{1}H and ^{13}C NMR ($CDCl_3$): **S2**



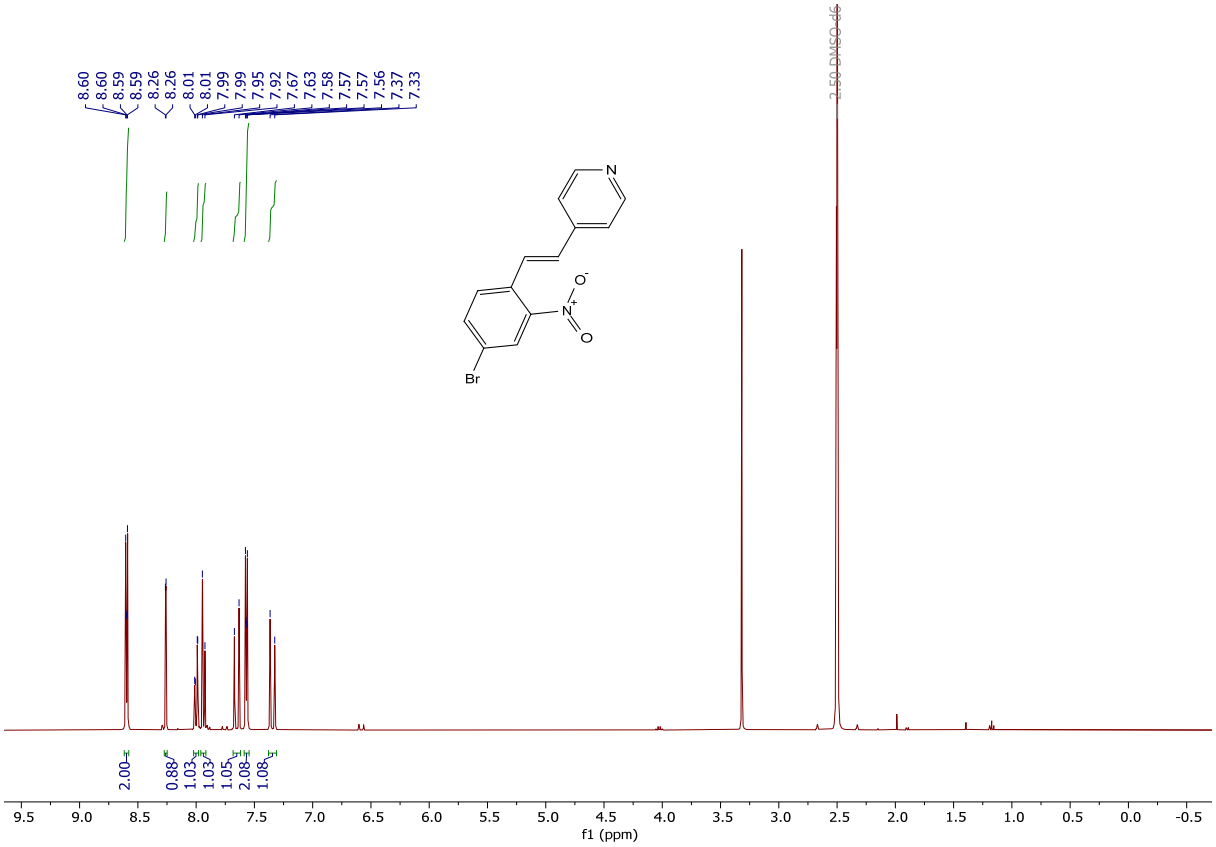


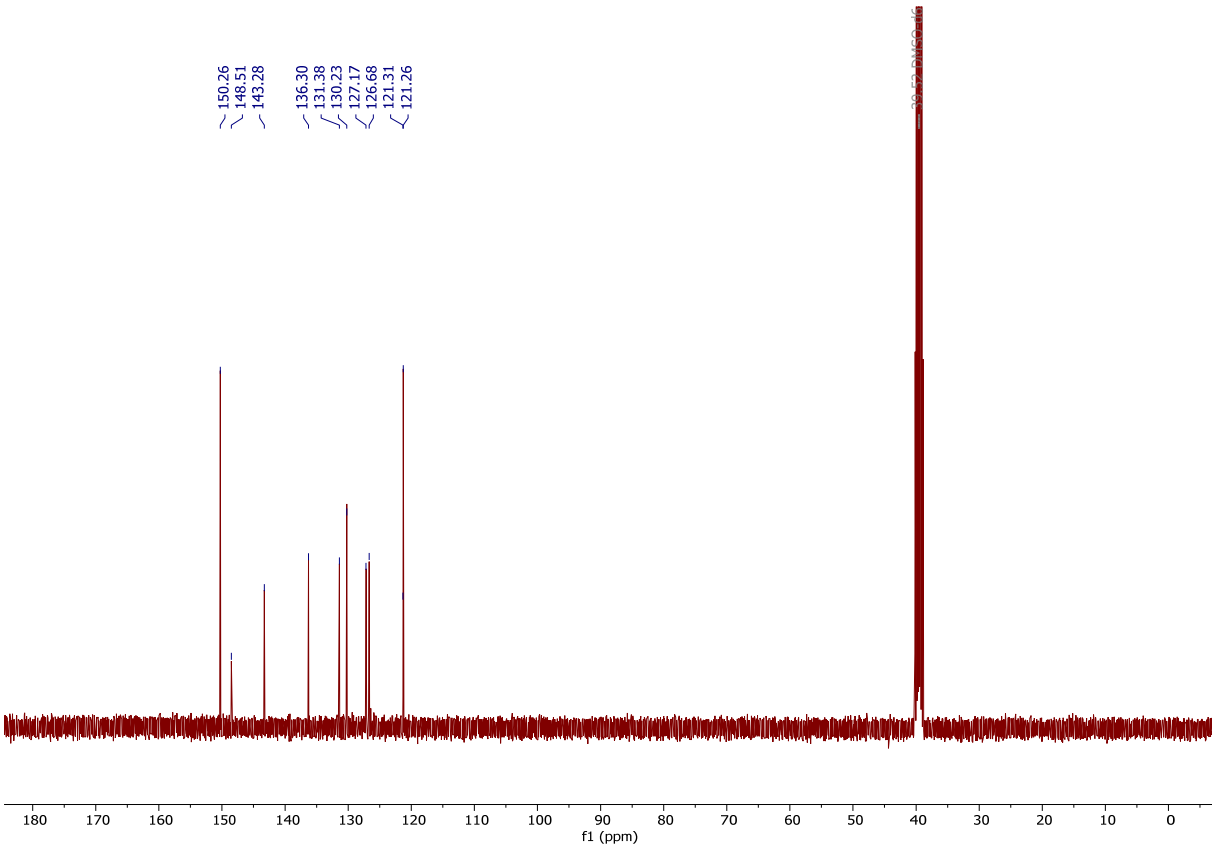
^{1}H and ^{13}C NMR ($CDCl_3$): **S3**



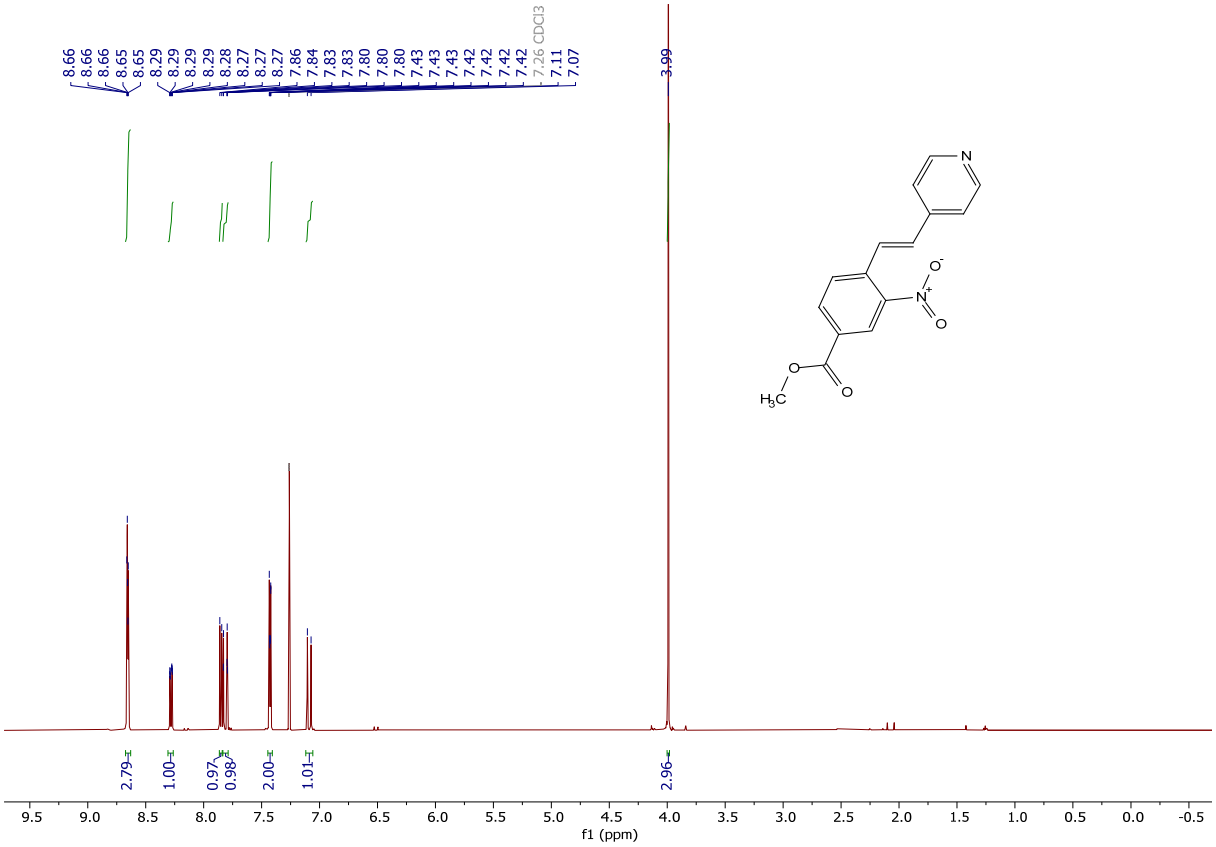


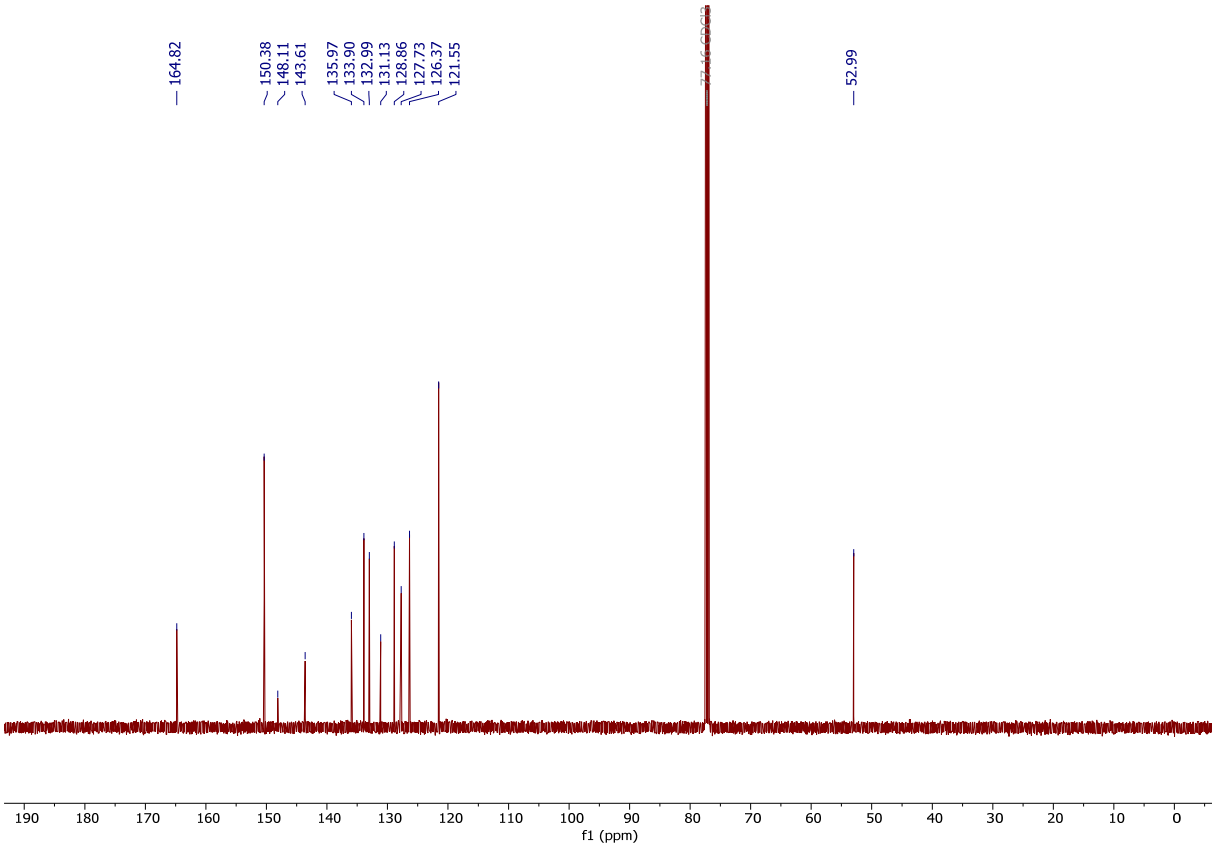
^{1}H and ^{13}C NMR (DMSO-d_6): **S4**



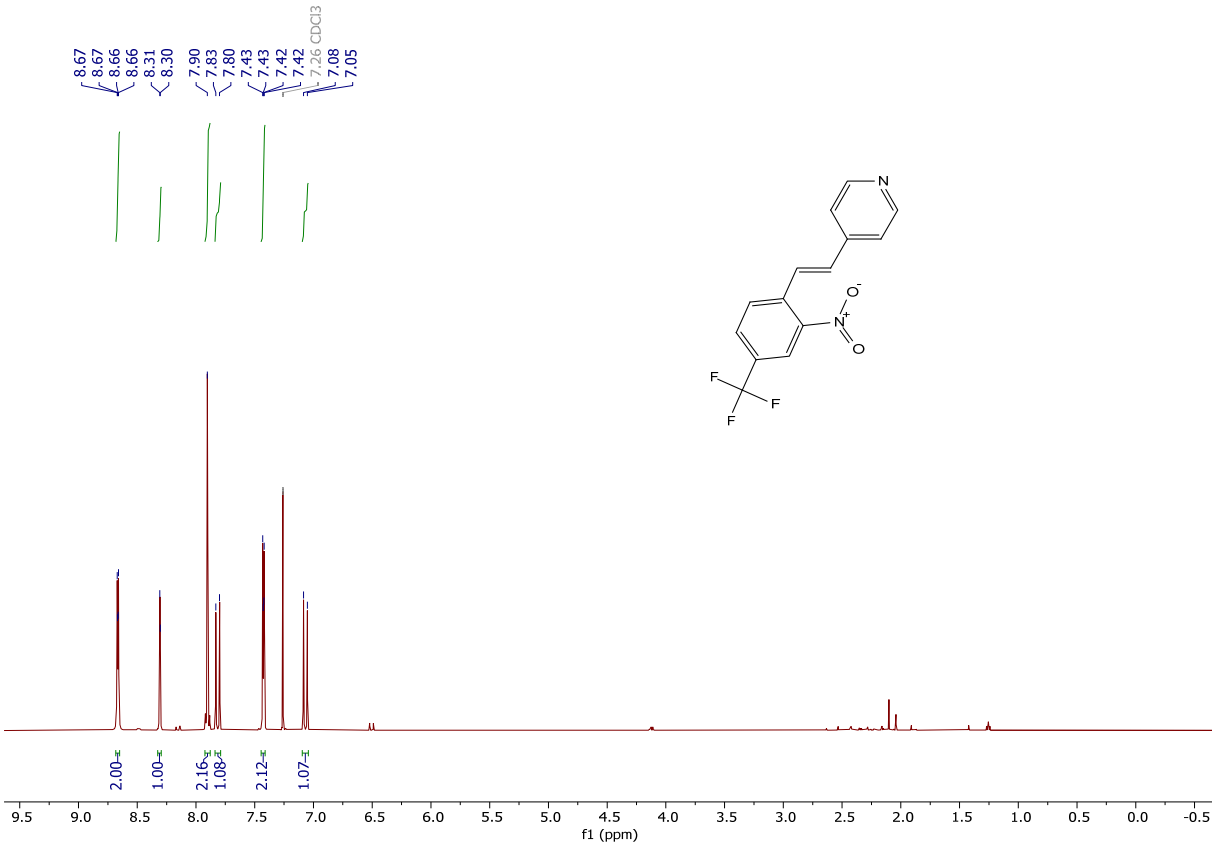


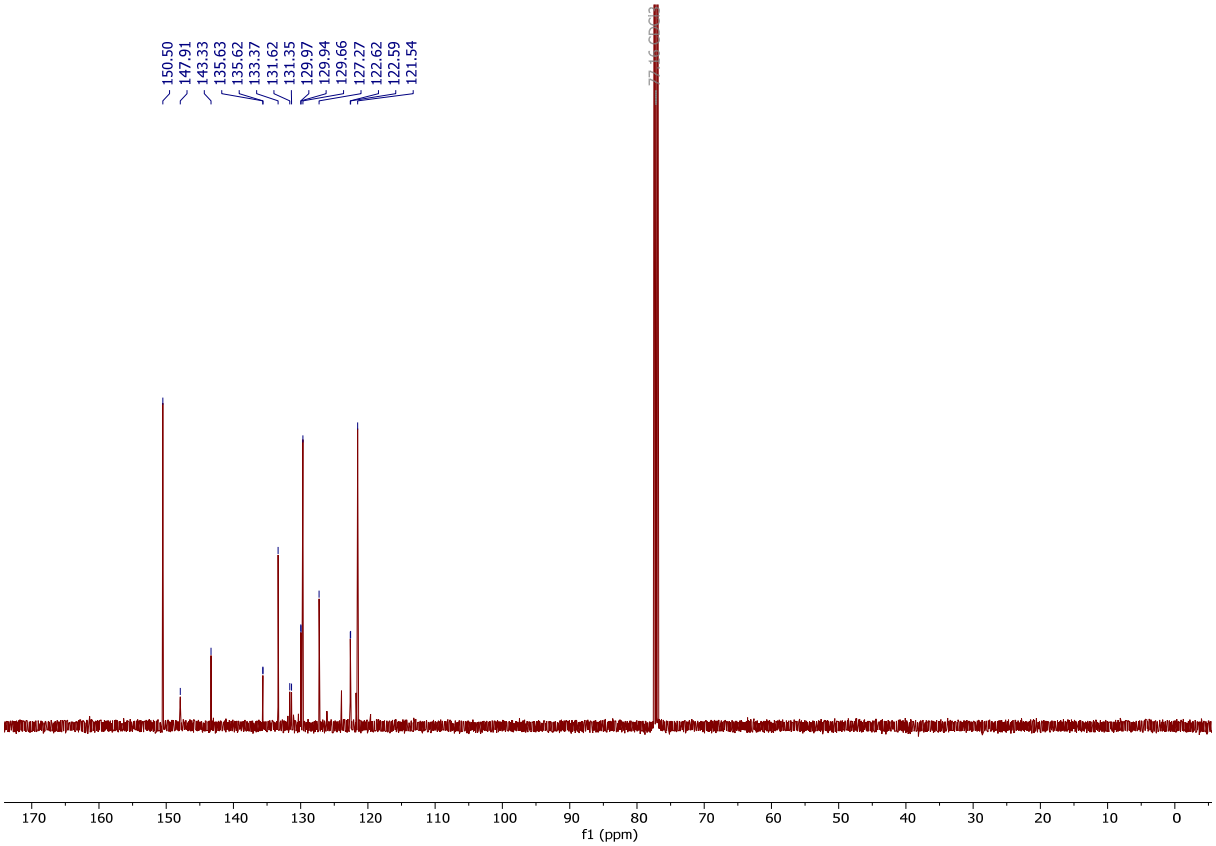
^{1}H and ^{13}C NMR ($CDCl_3$): **S5**



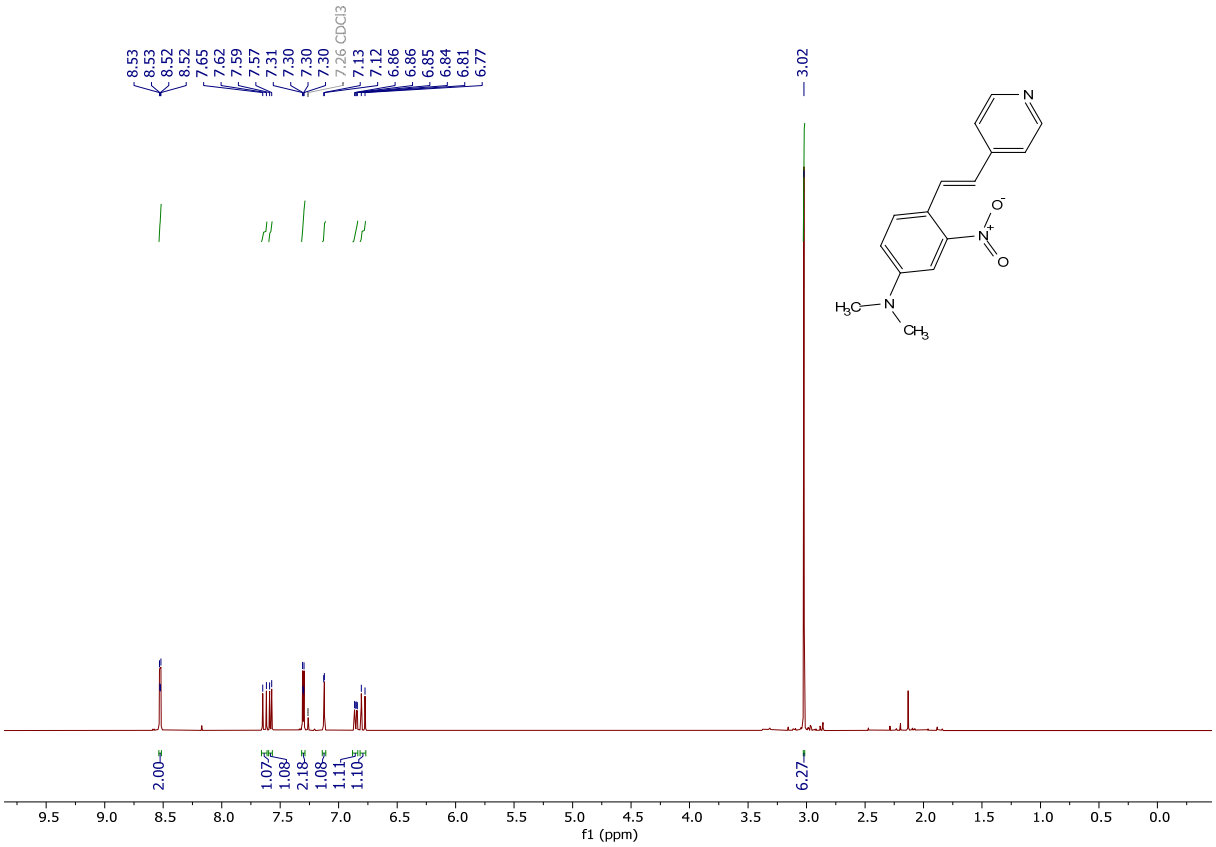


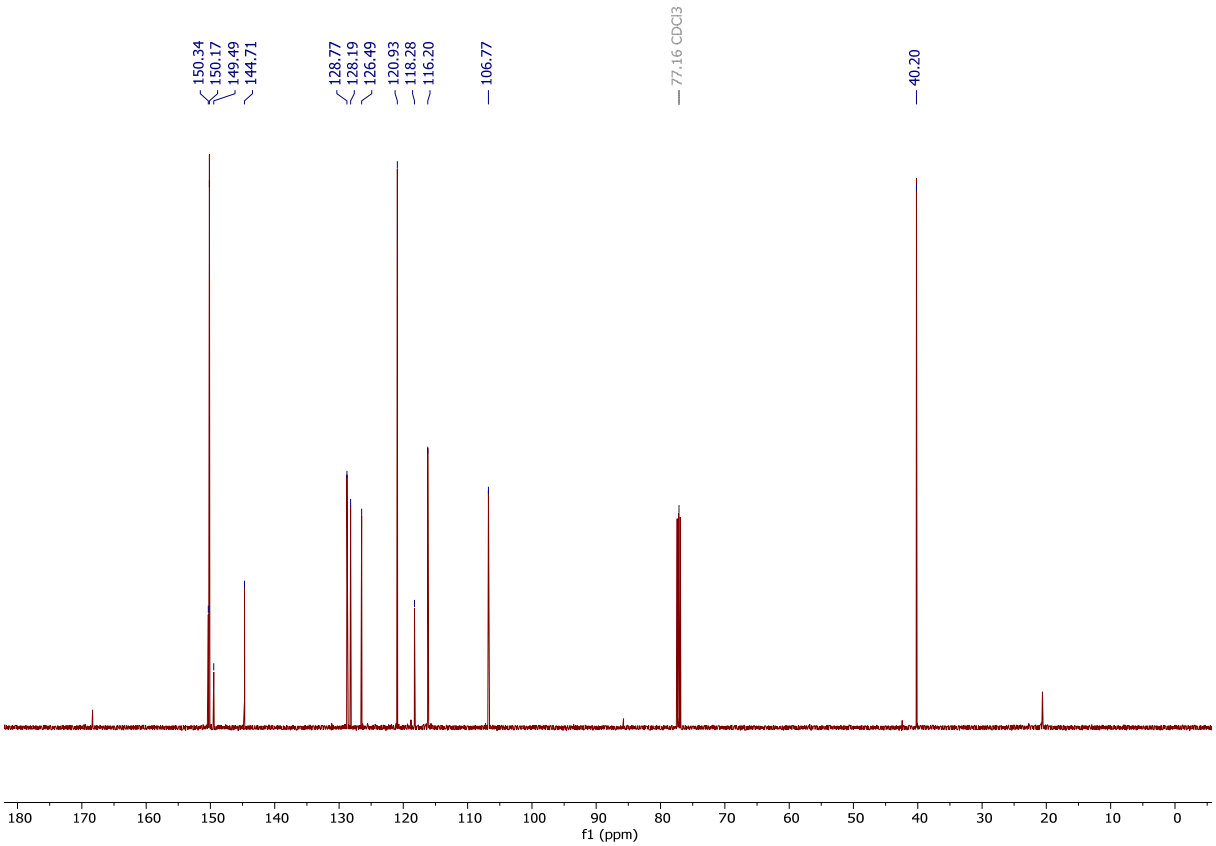
^{1}H and ^{13}C NMR ($CDCl_3$): **S6**



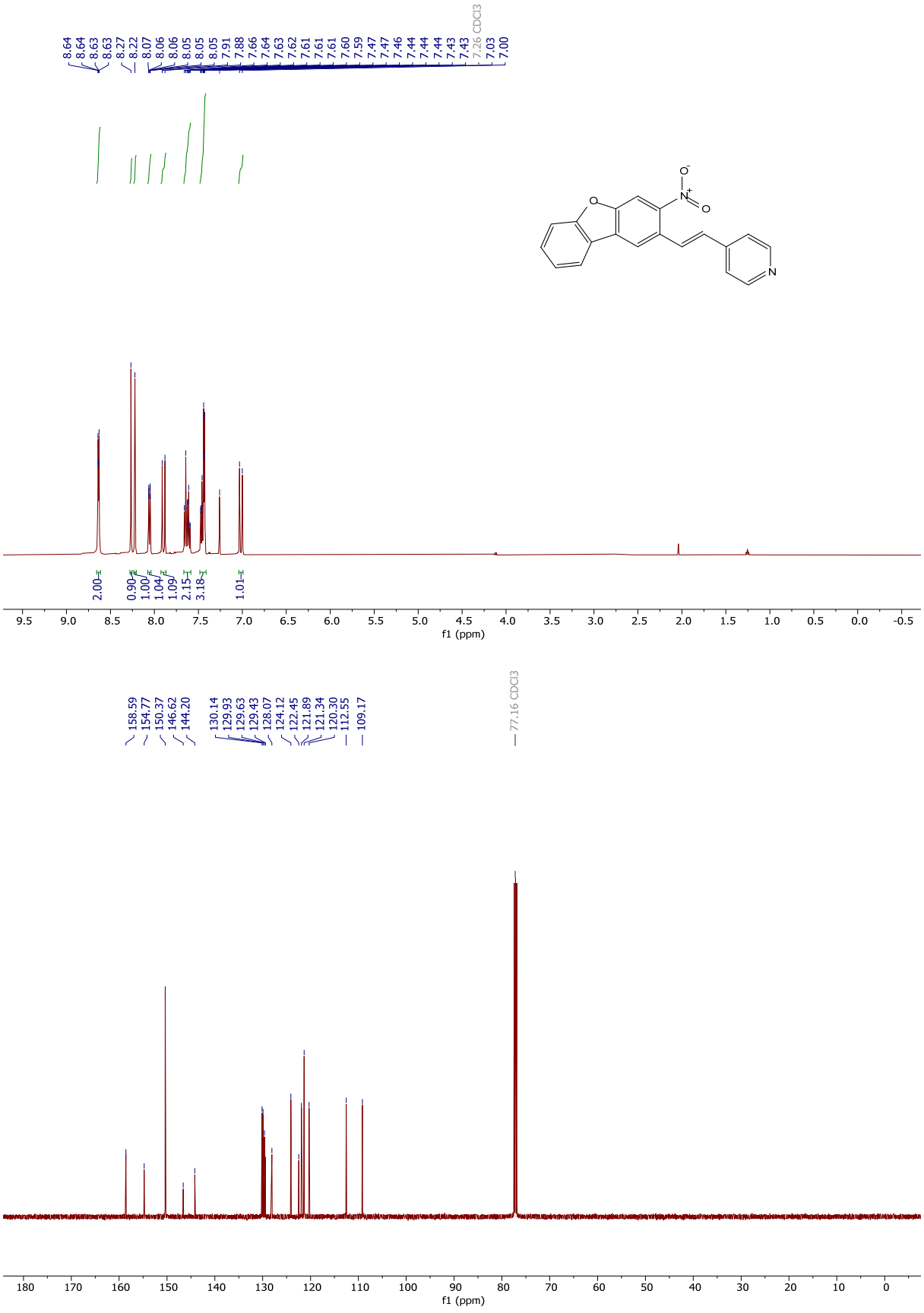


^{1}H and ^{13}C NMR ($CDCl_3$): **S8**

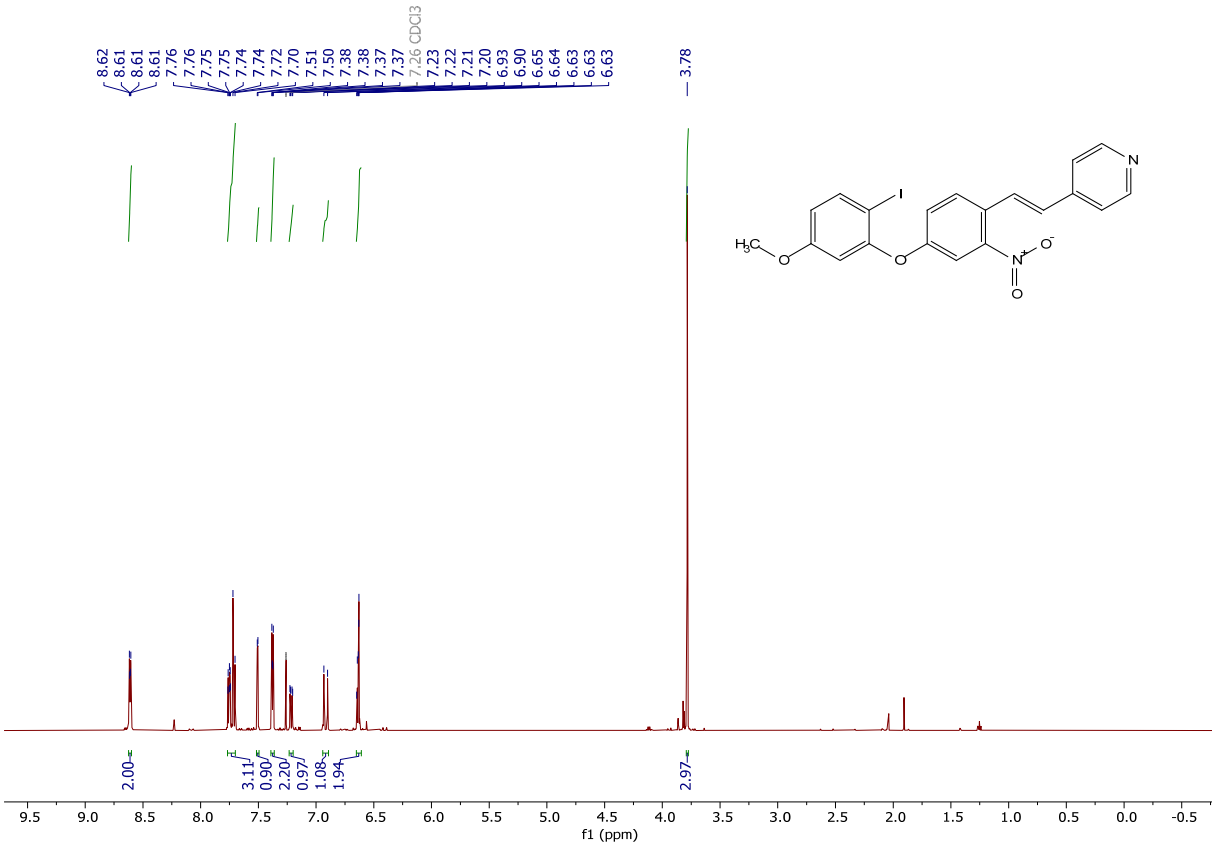


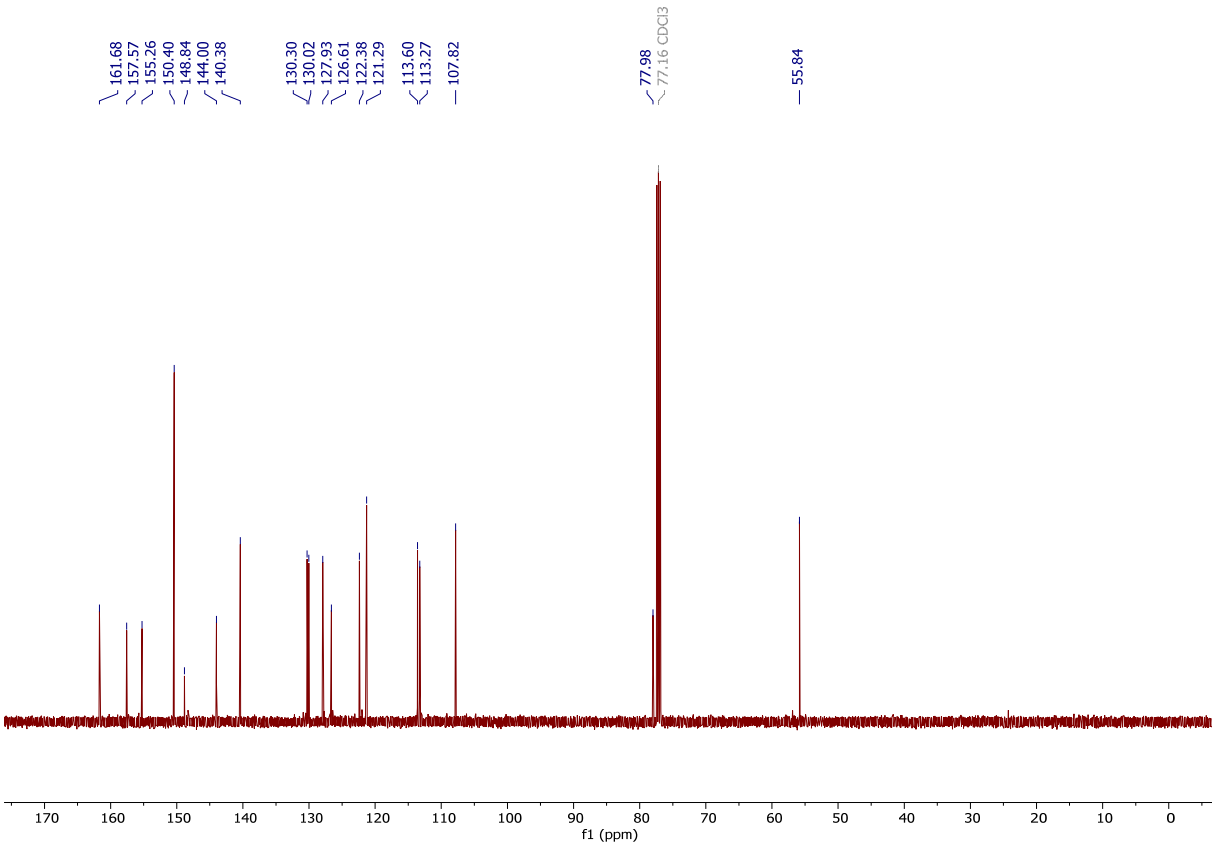


^{1}H and ^{13}C NMR ($CDCl_3$): **S11**

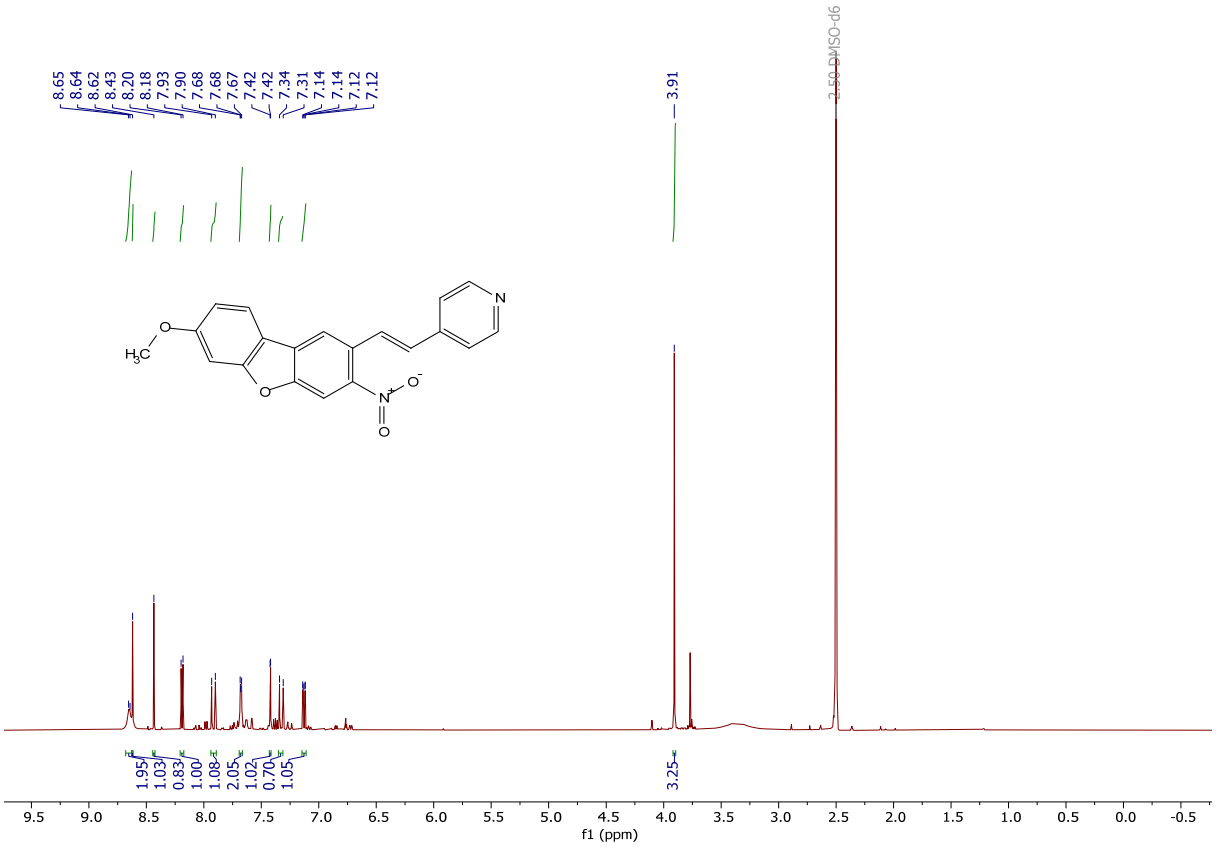


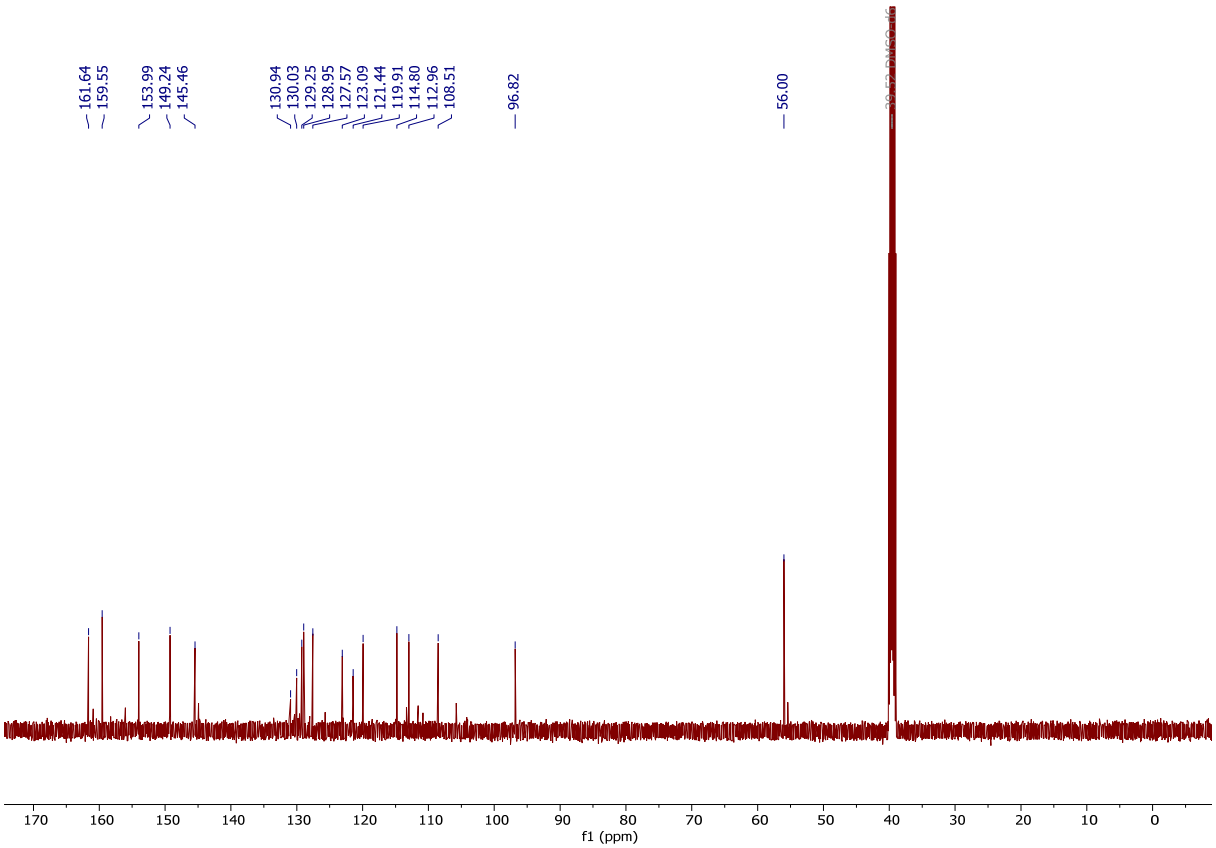
^{1}H and ^{13}C NMR ($CDCl_3$): **S13**



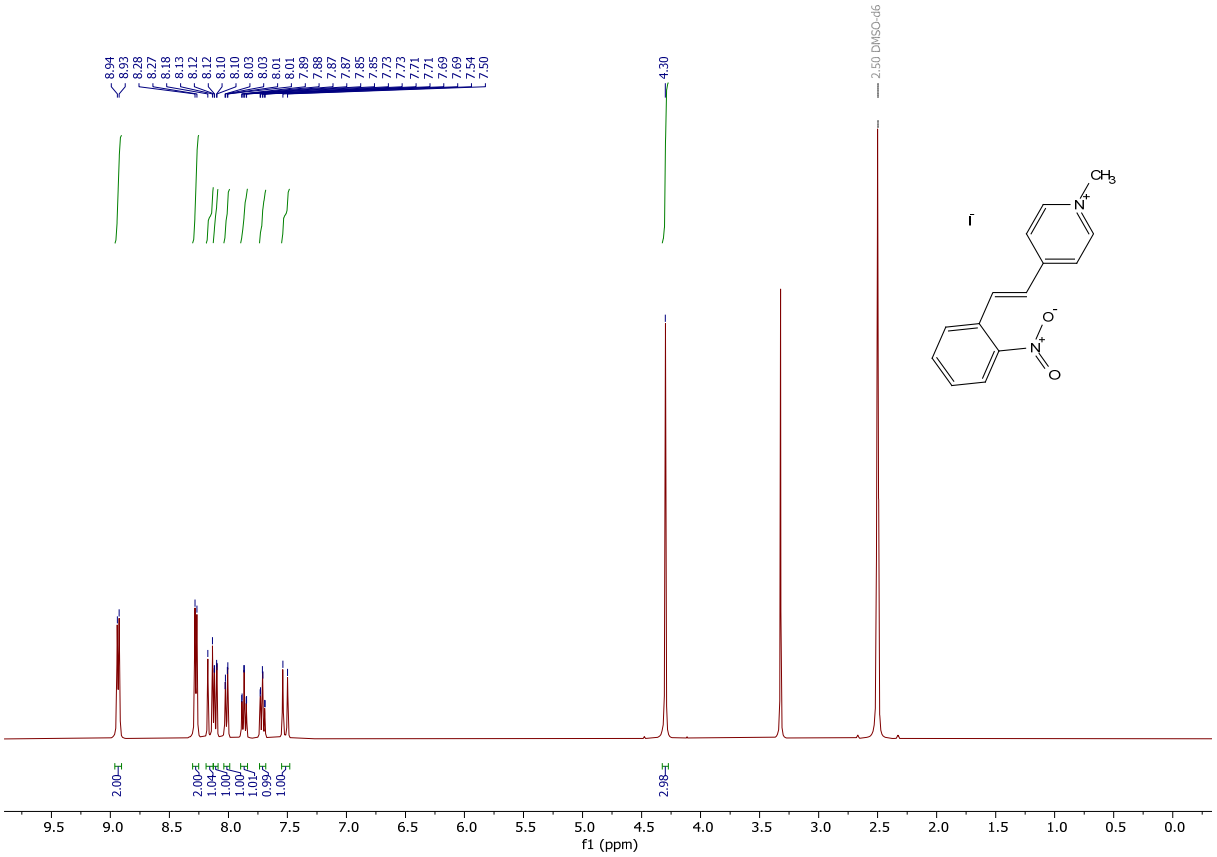


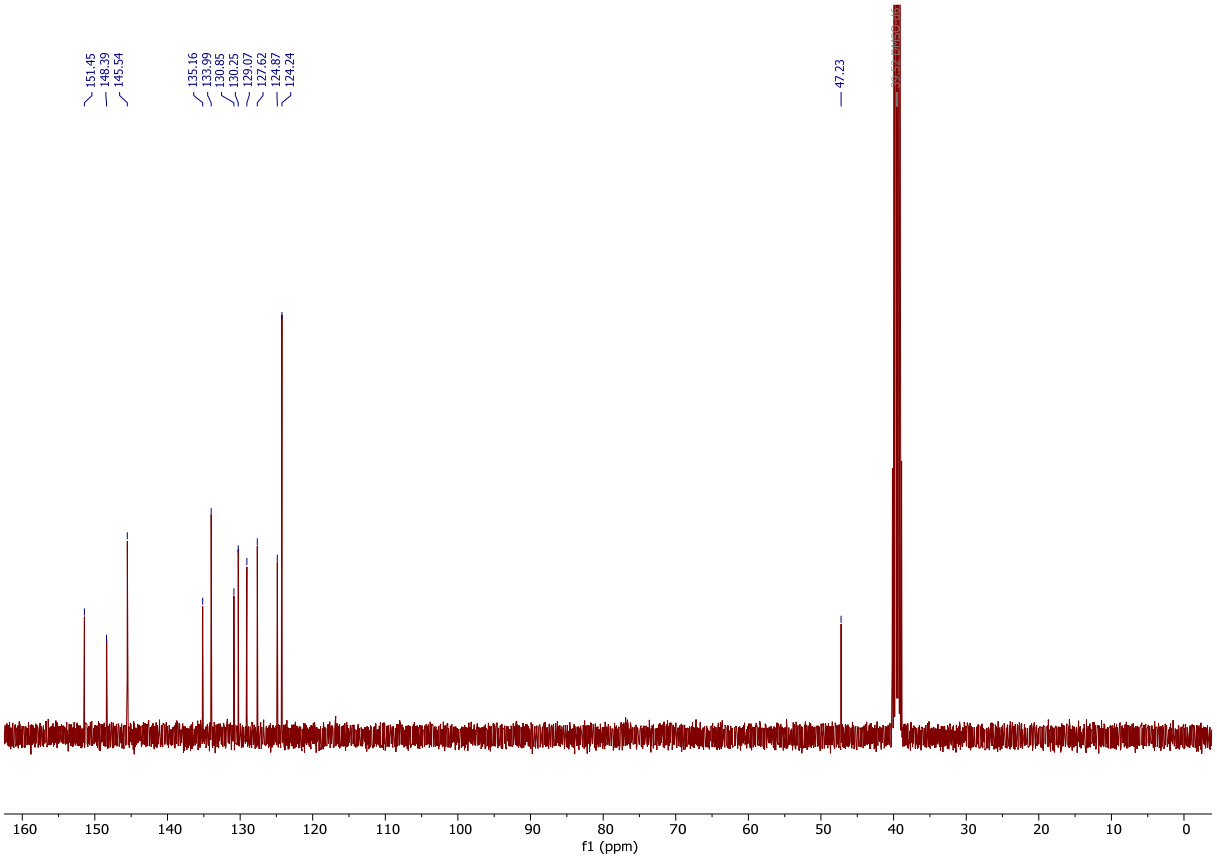
^{1}H and ^{13}C NMR (DMSO-d_6): **S14**



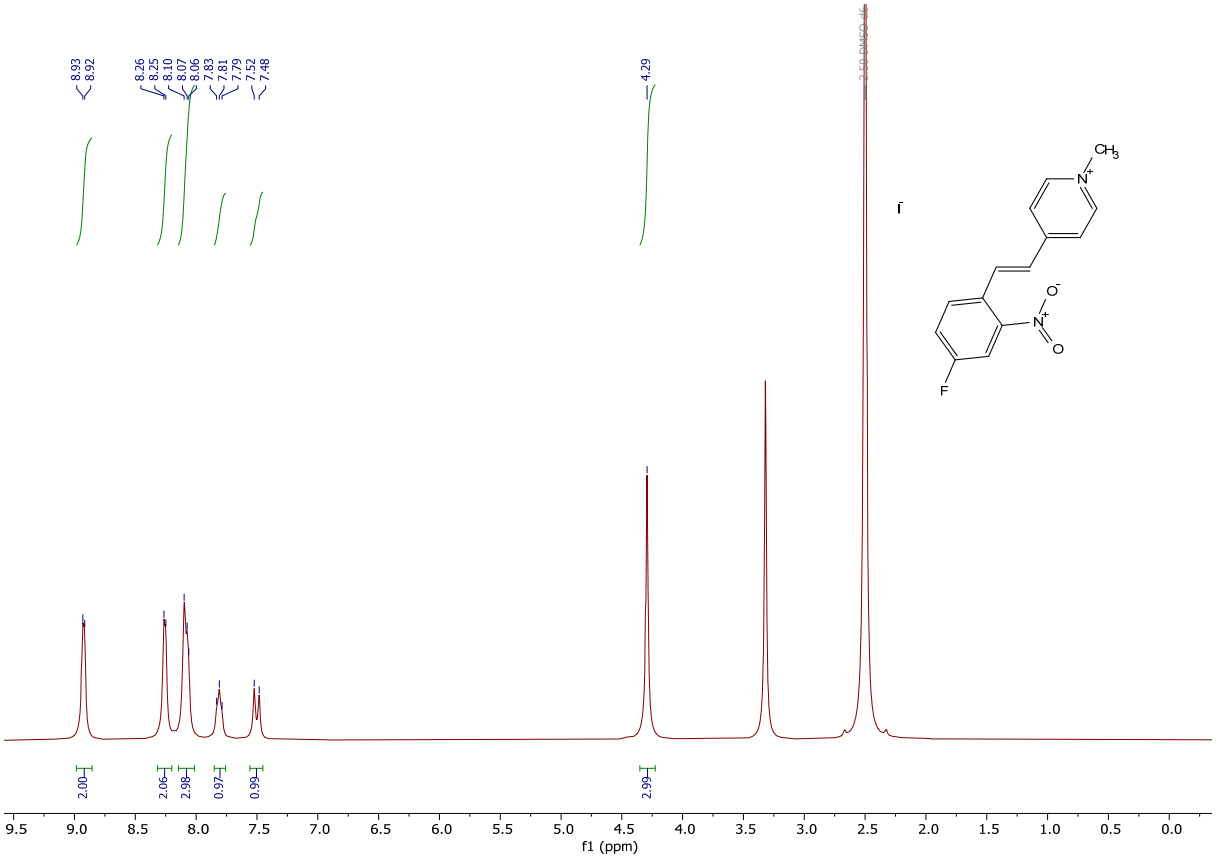


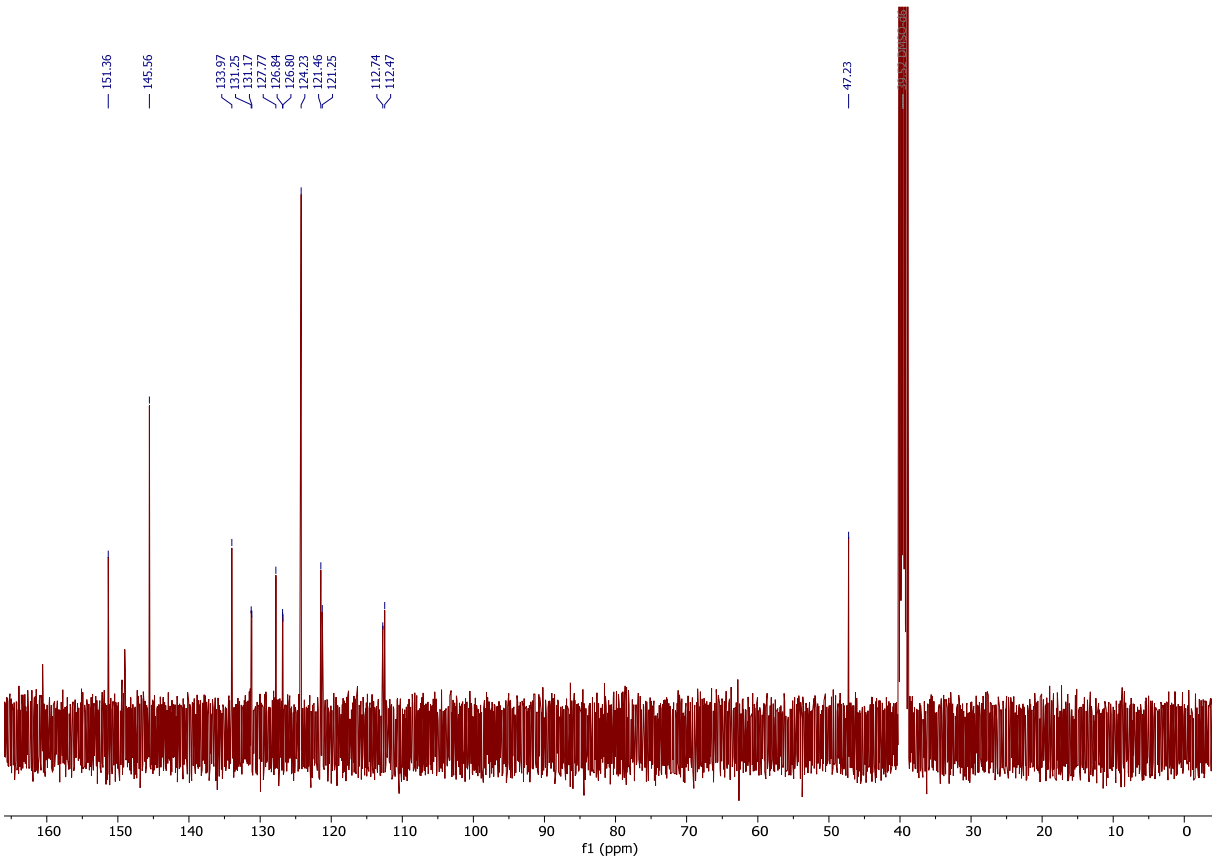
^{1}H and ^{13}C NMR (DMSO-d_6): **1a**



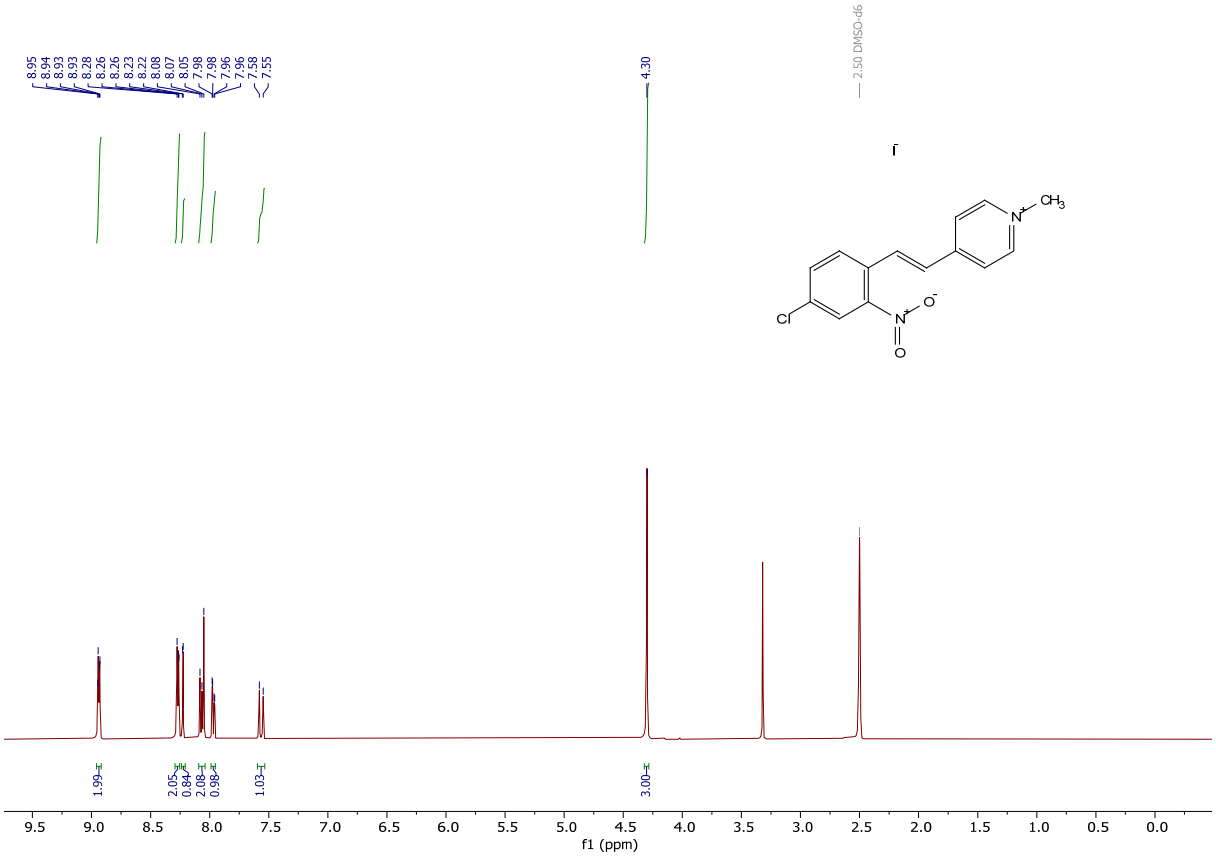


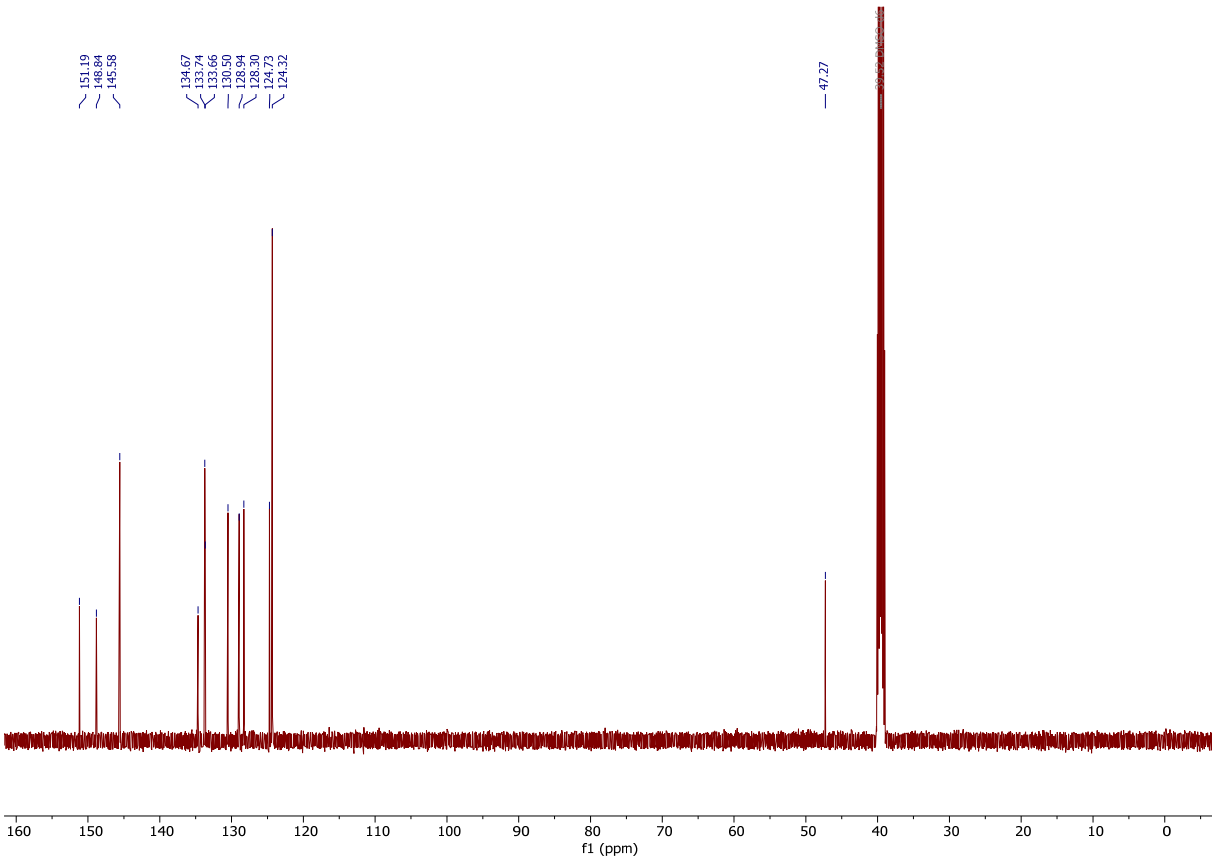
^{1}H and ^{13}C NMR (DMSO-d_6): **1b**



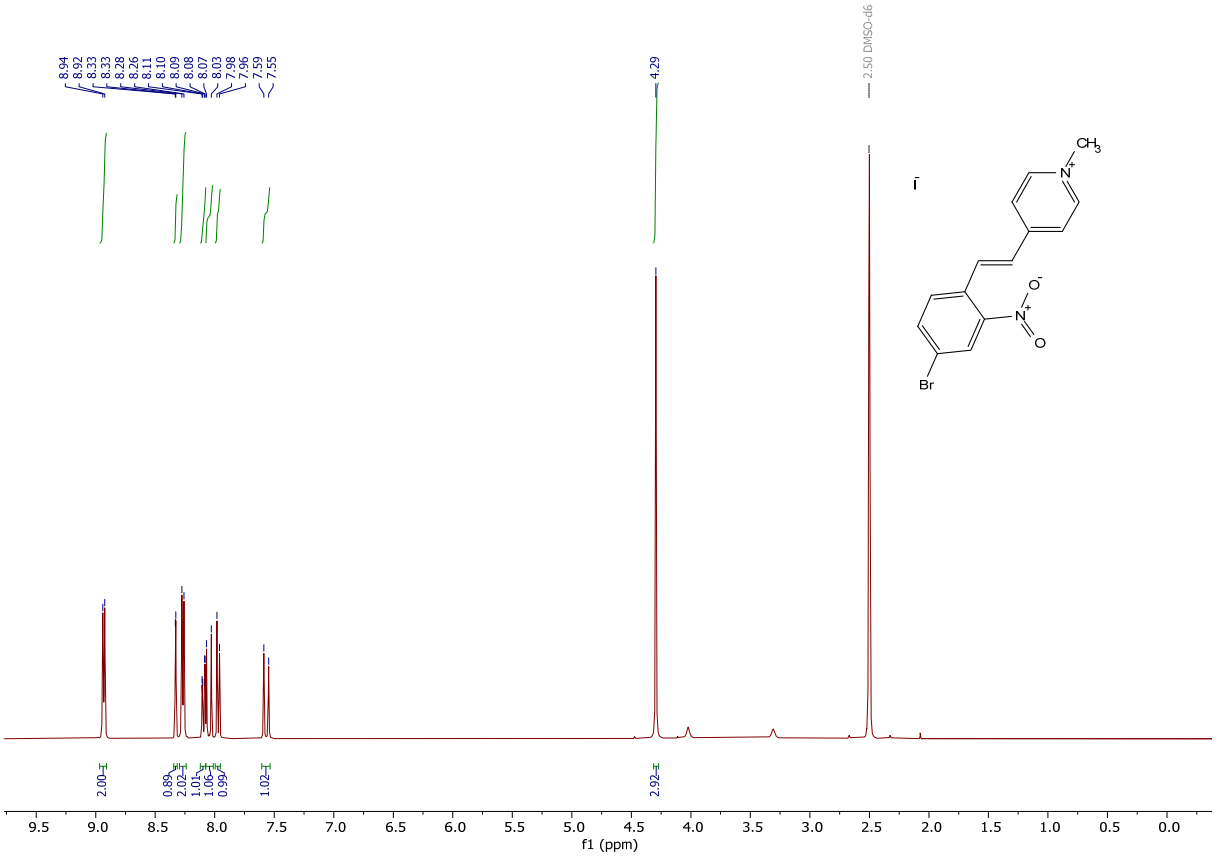


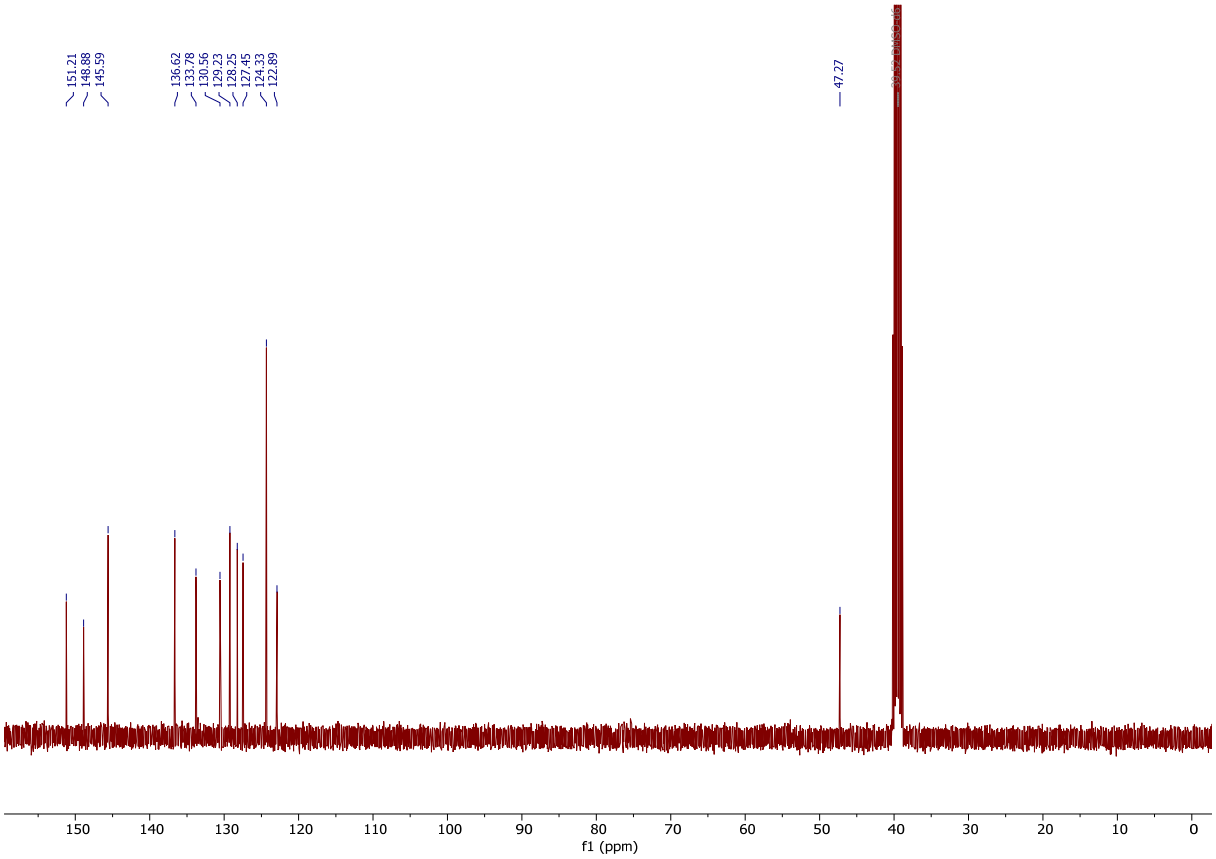
^{1}H and ^{13}C NMR (DMSO-d_6): **1c**



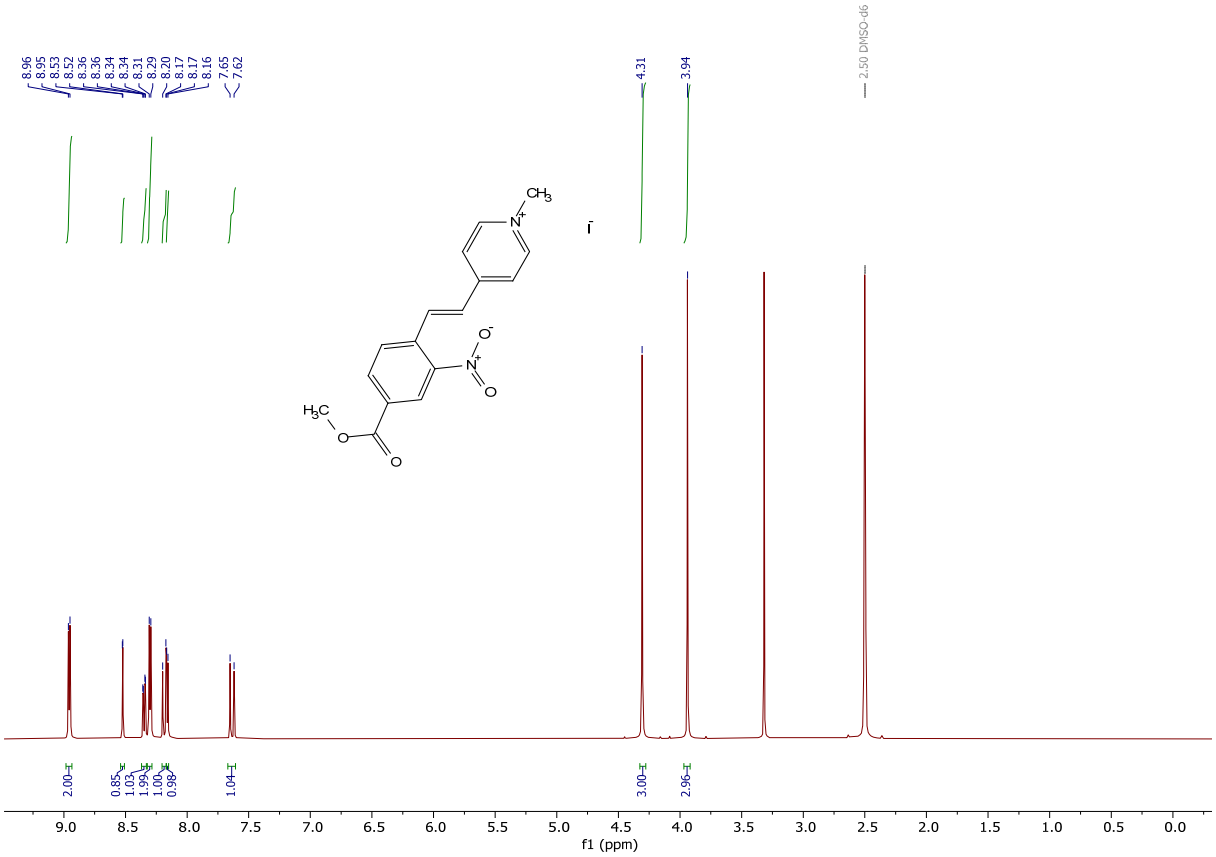


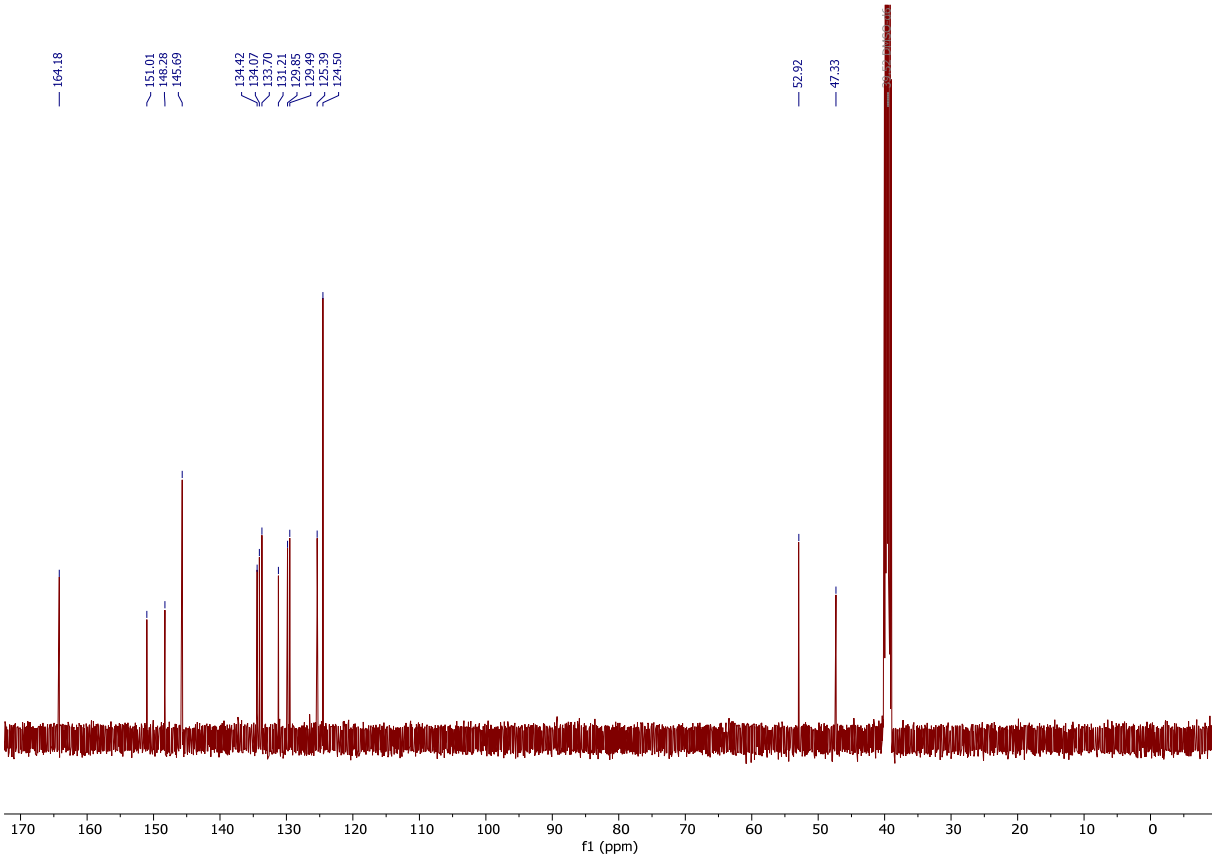
^{1}H and ^{13}C NMR (DMSO-d_6): **1d**



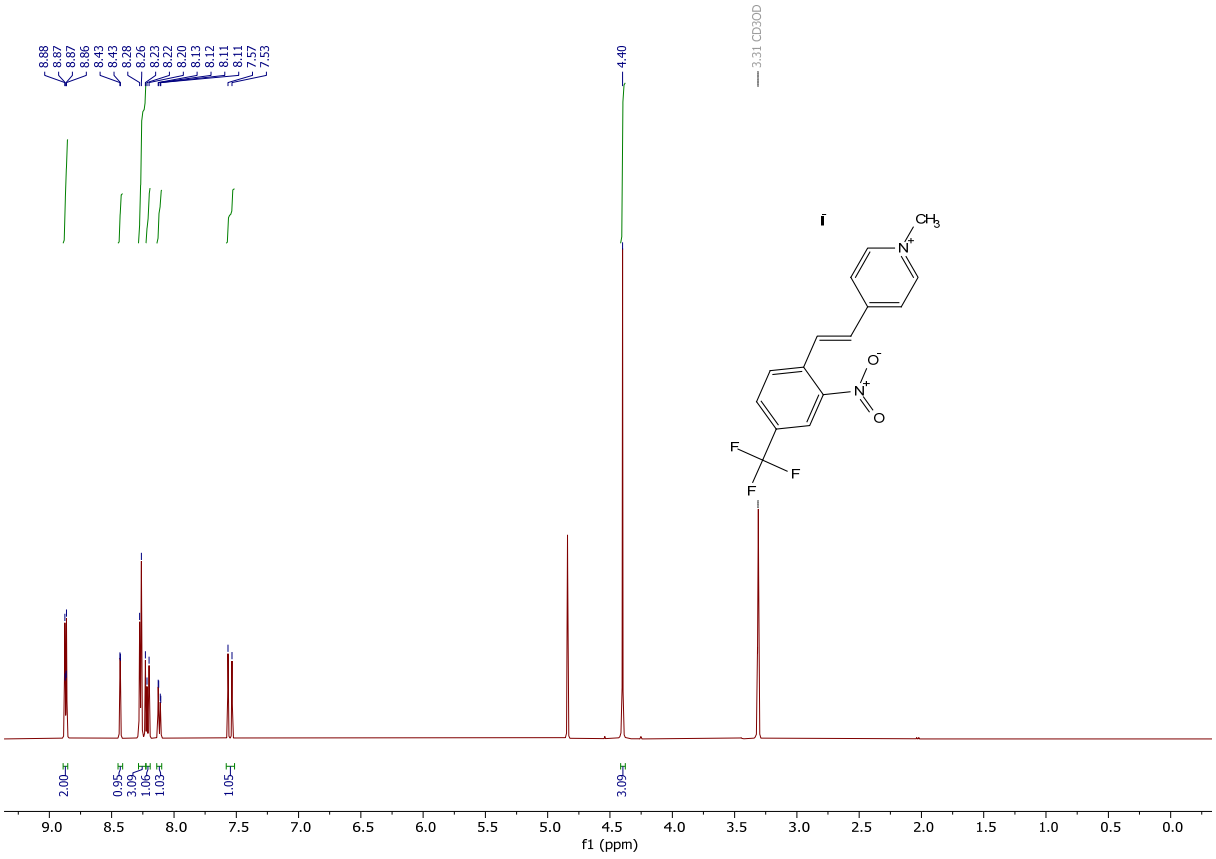


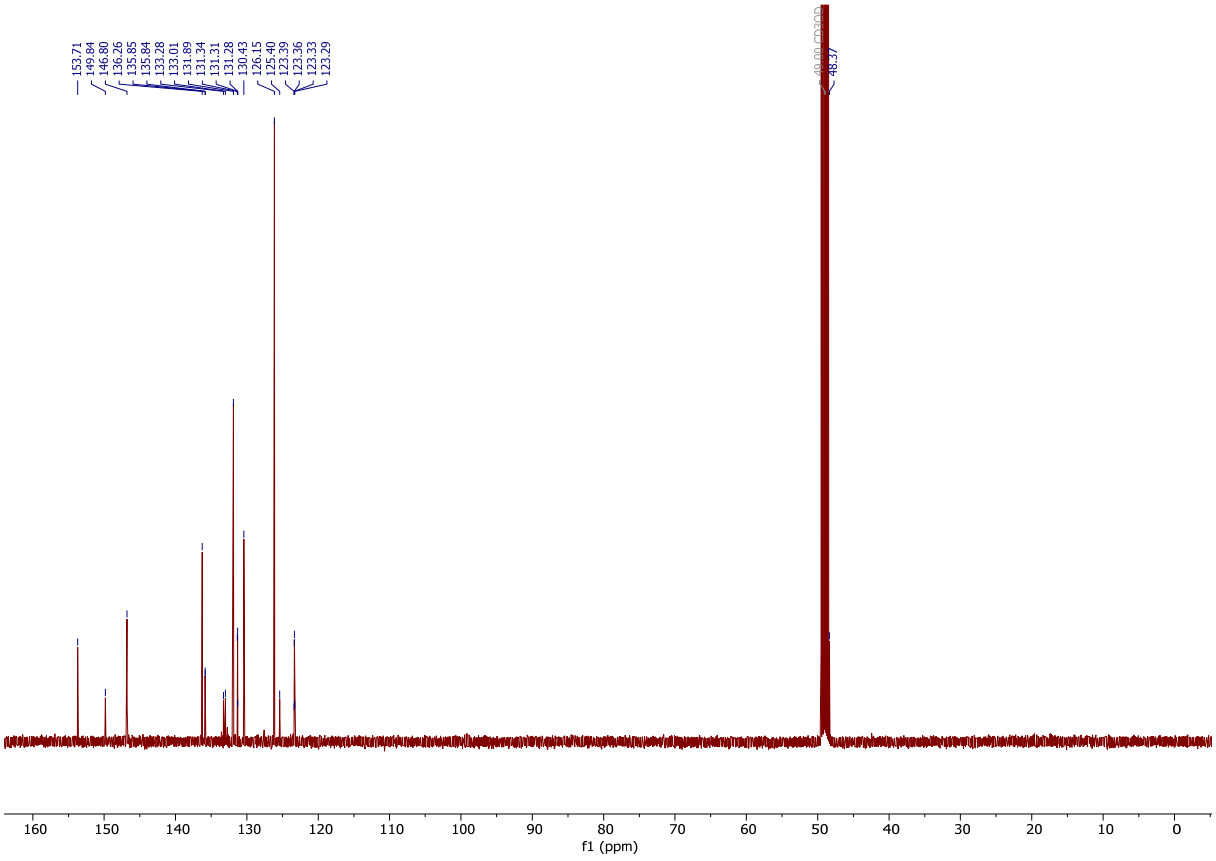
^{1}H and ^{13}C NMR (DMSO-d_6): **1e**



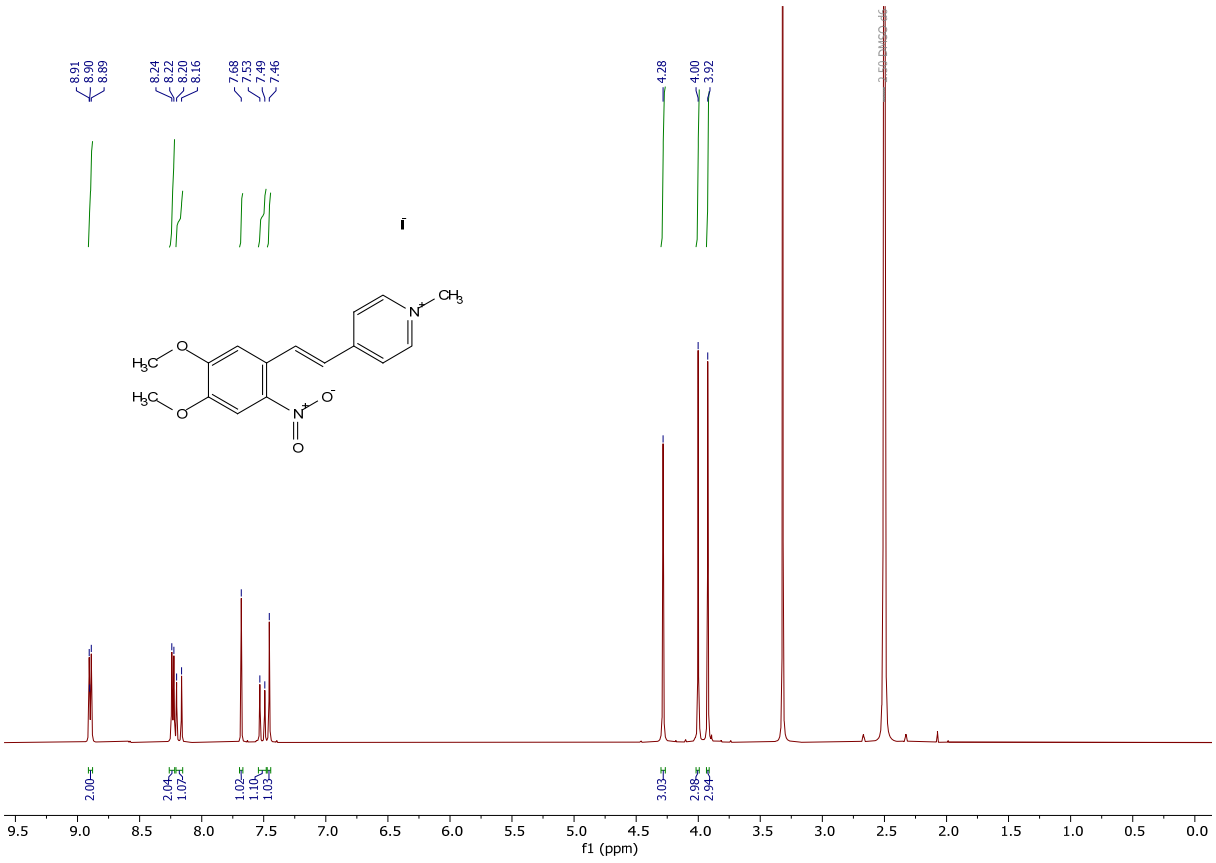


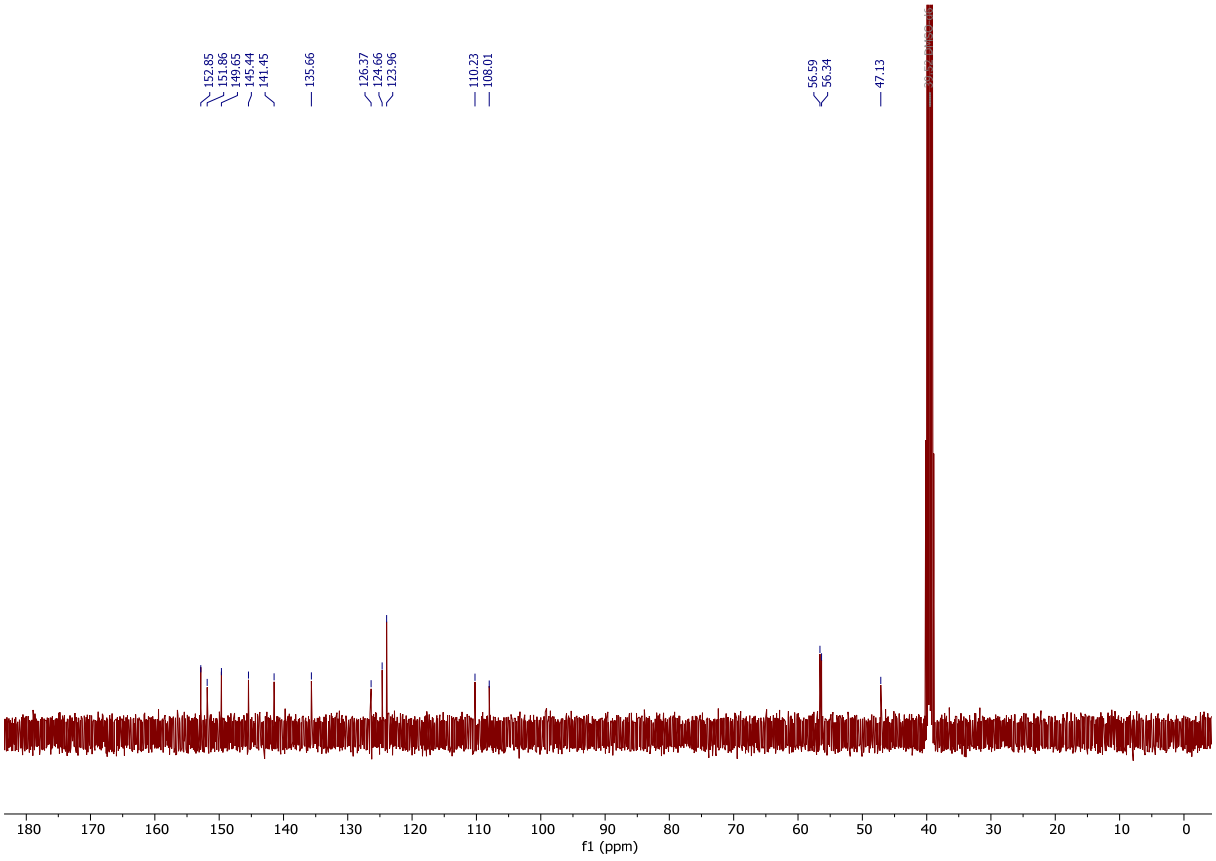
^{1}H and ^{13}C NMR (CD_3OD): **1f**



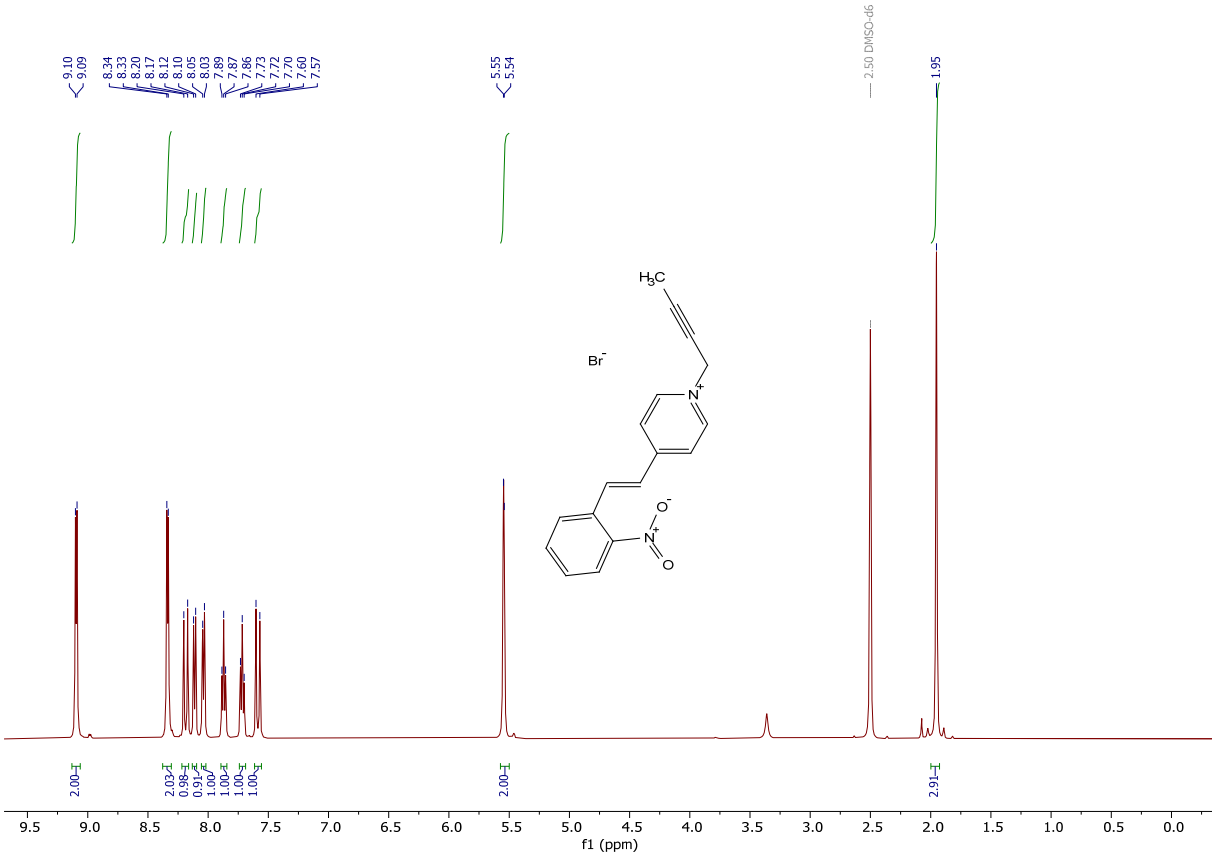


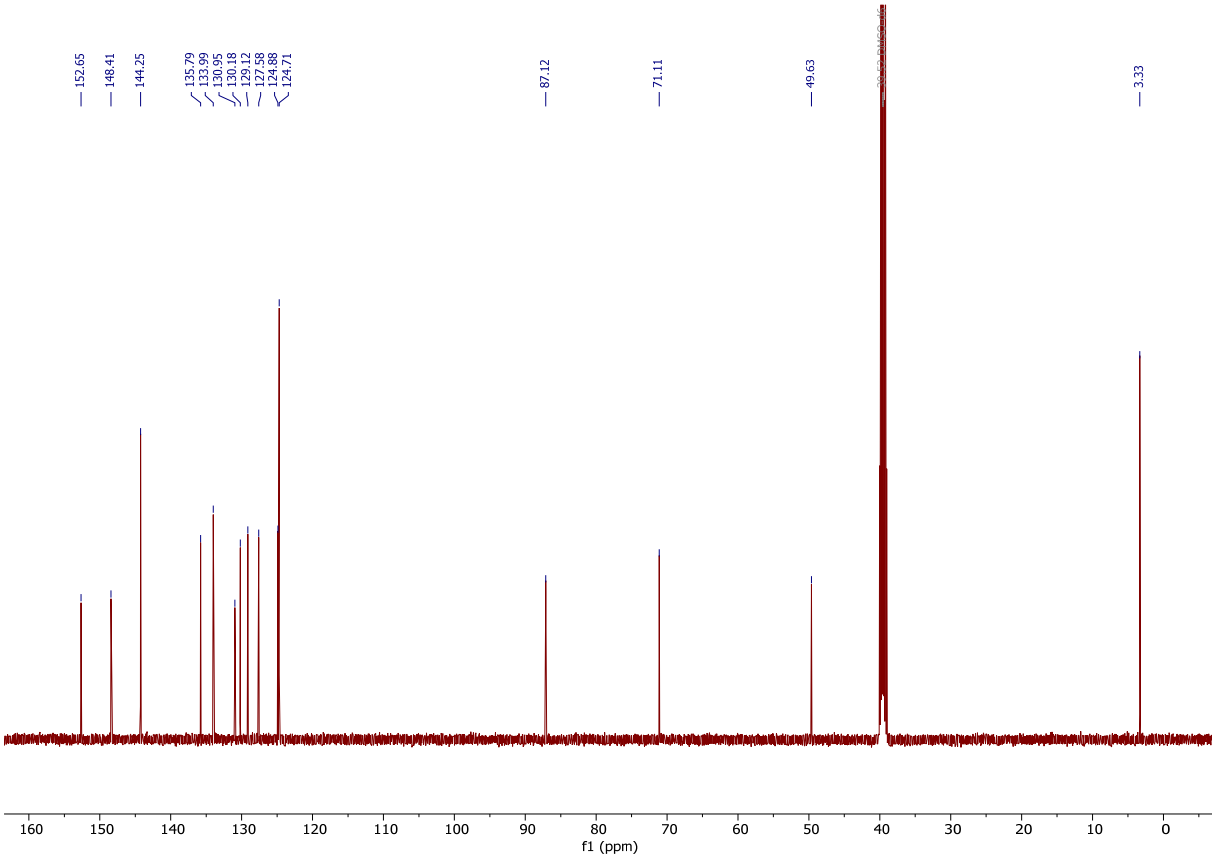
^{1}H and ^{13}C NMR (DMSO-d_6): **1g**



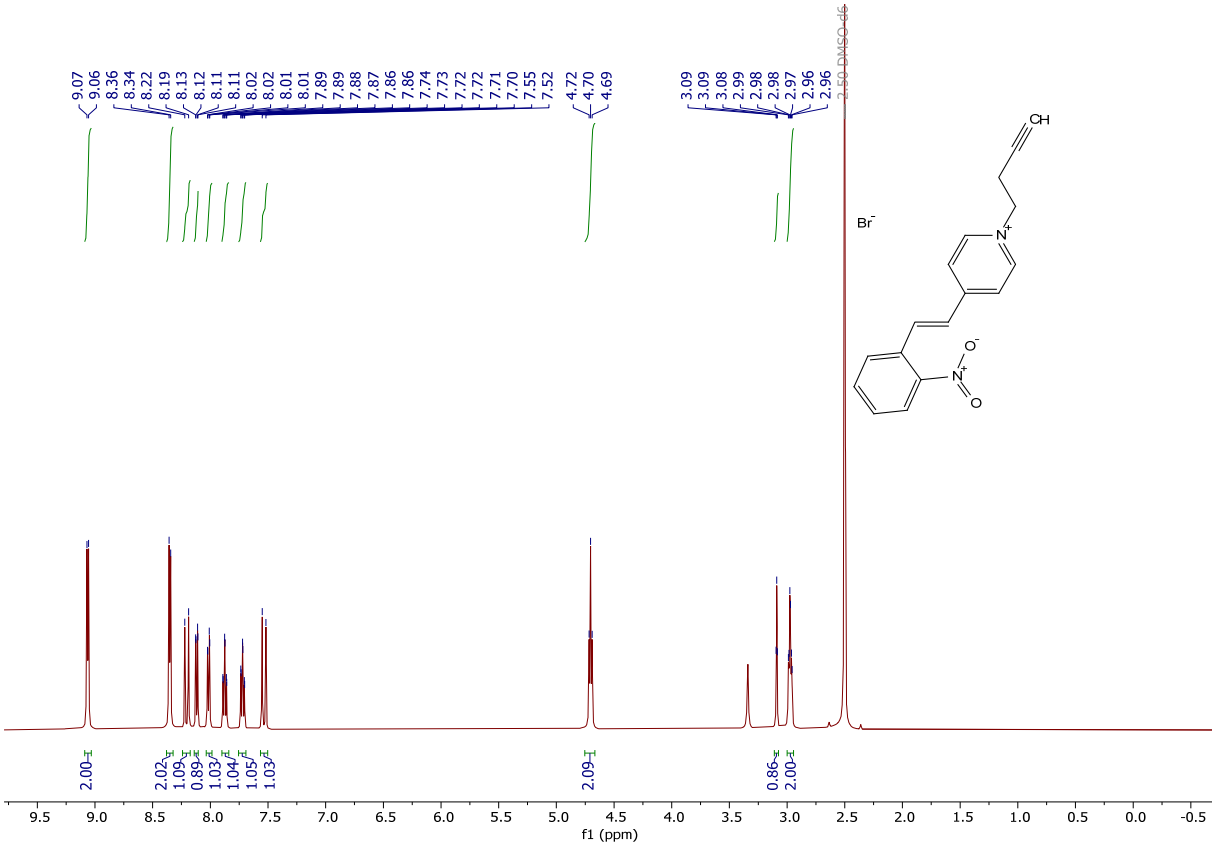


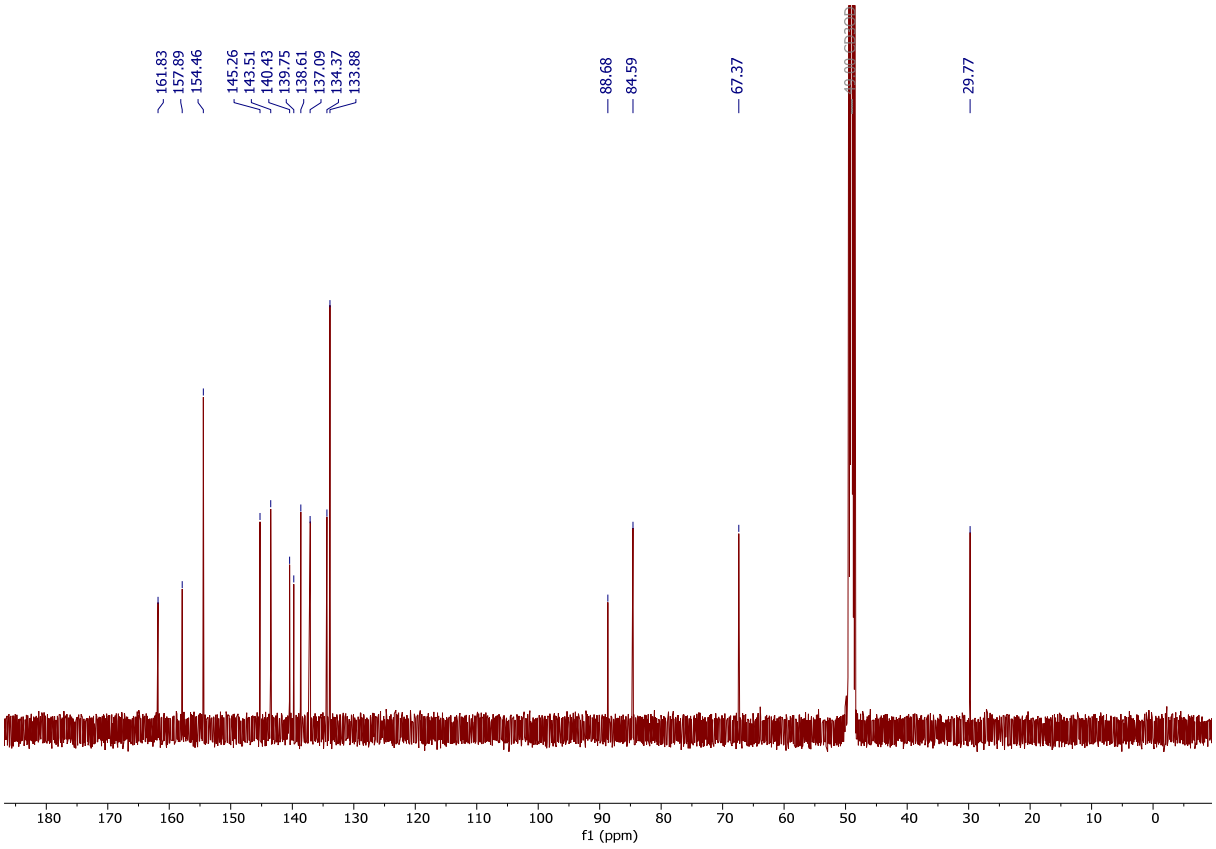
^{1}H and ^{13}C NMR (DMSO-d_6): **1h**



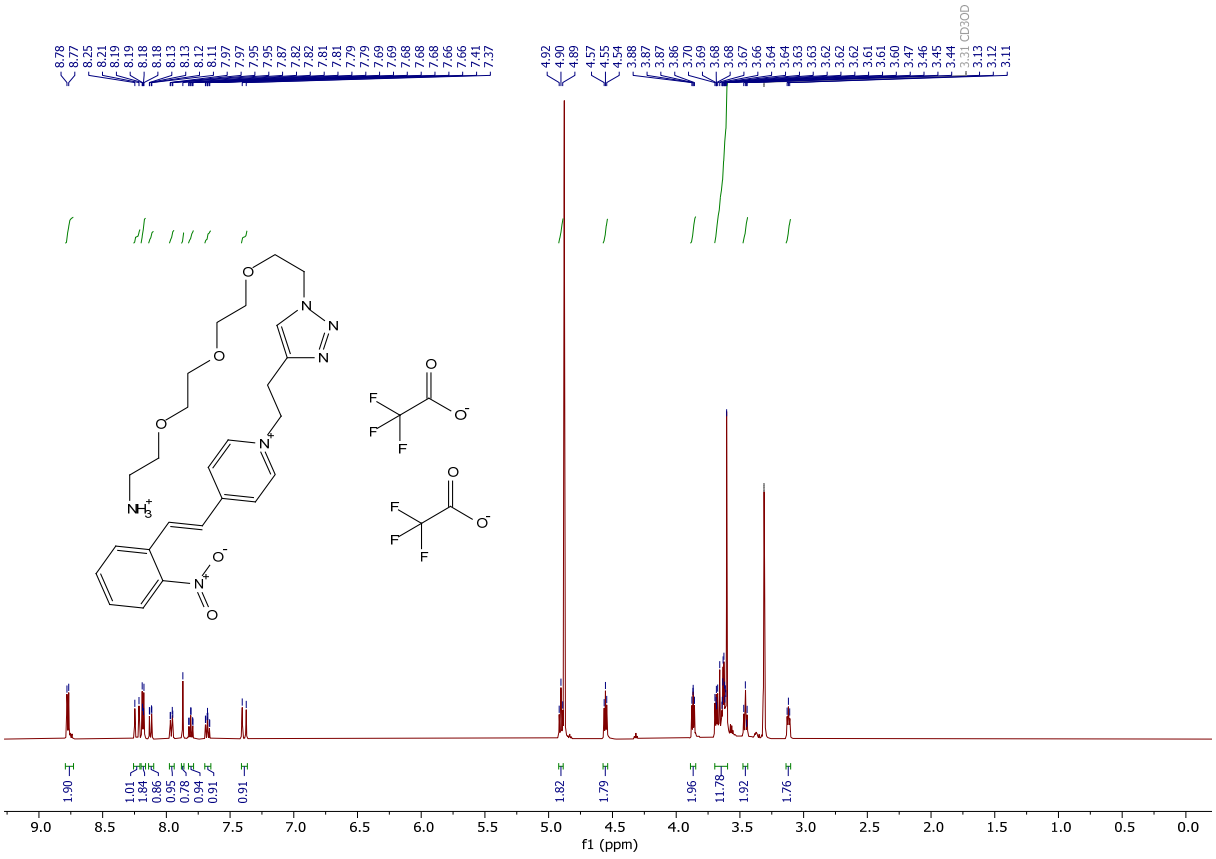


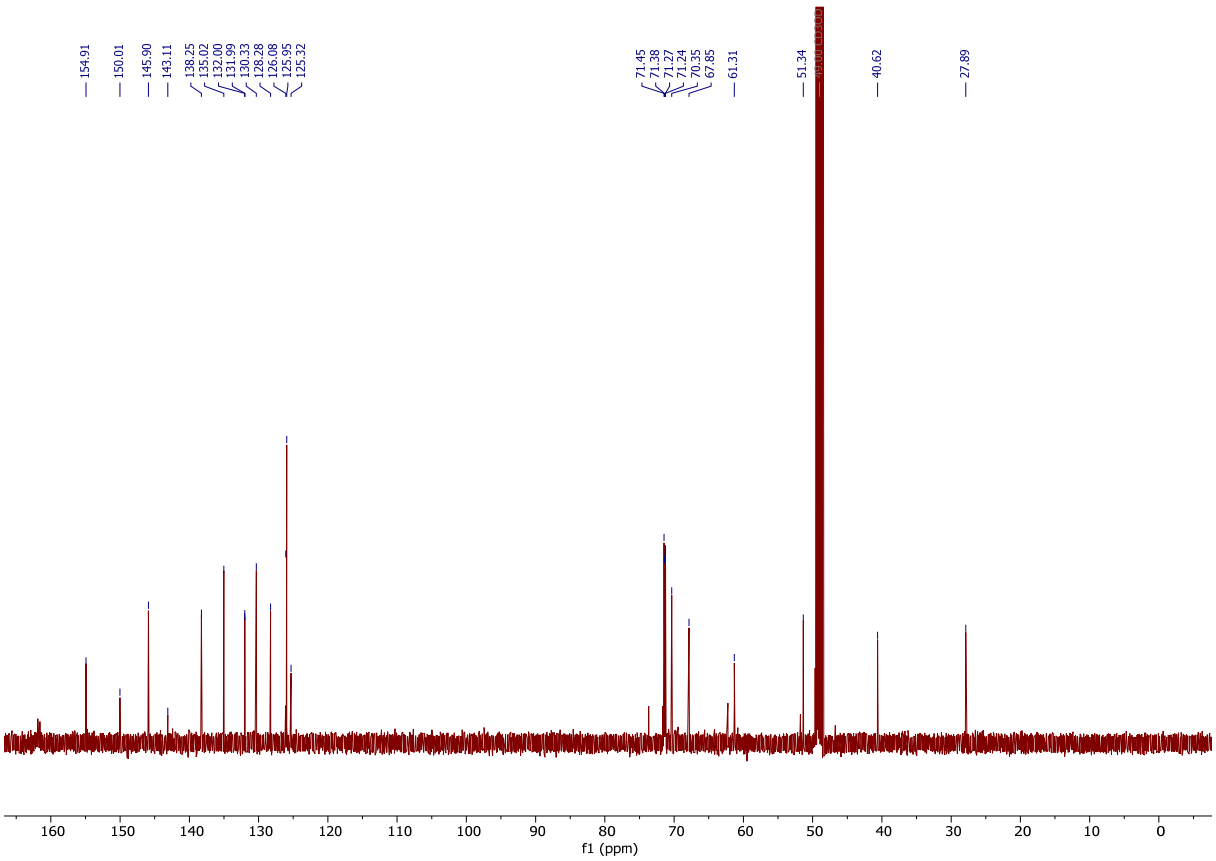
^{1}H and ^{13}C NMR (DMSO-d_6): **5**



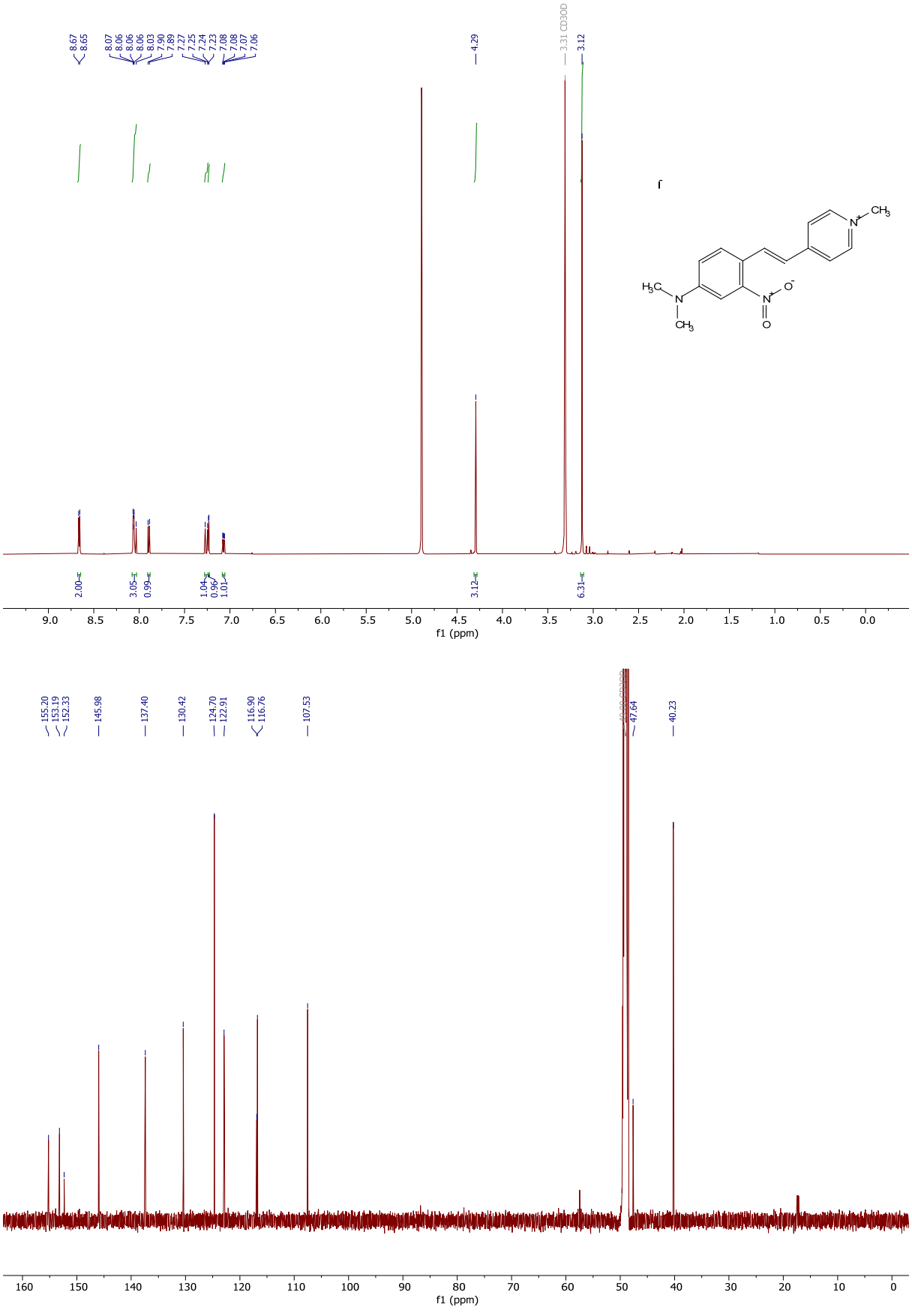


^{1}H and ^{13}C NMR (CD_3OD): **1i**

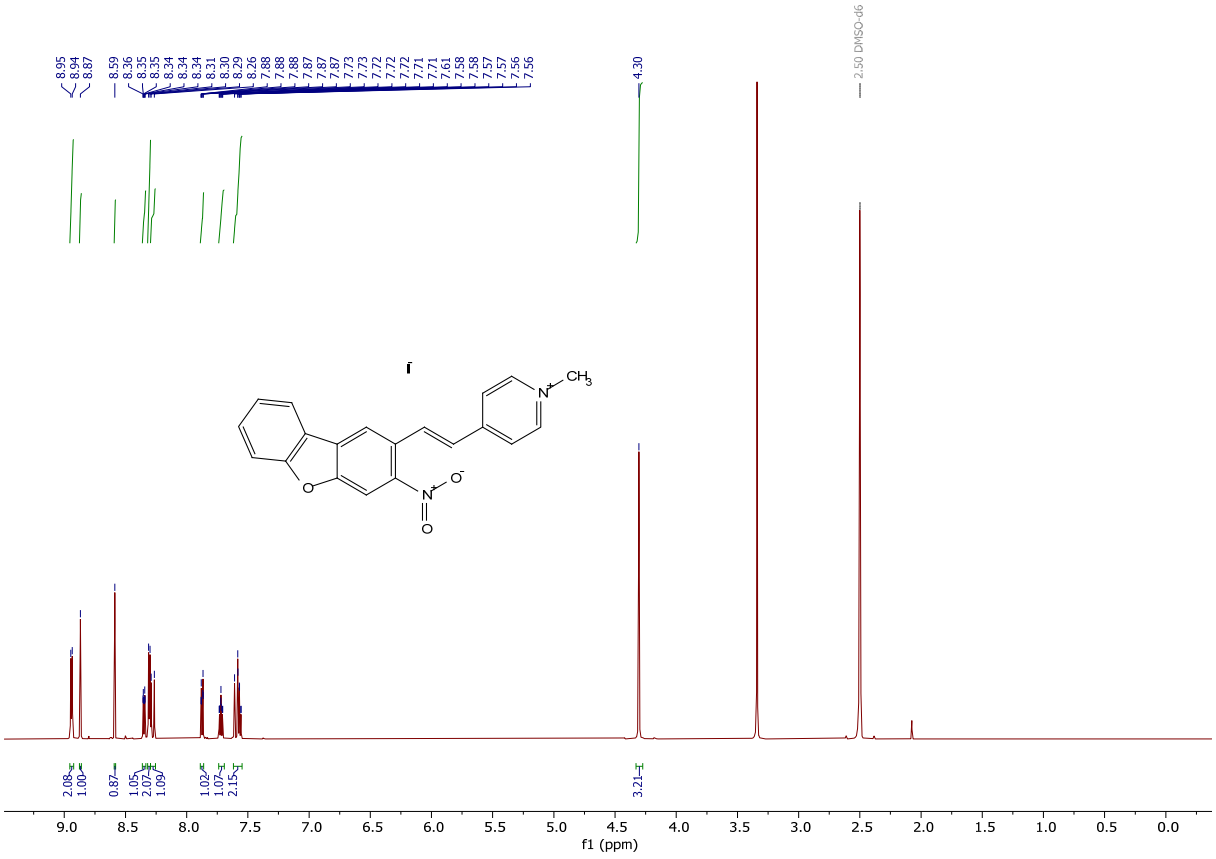


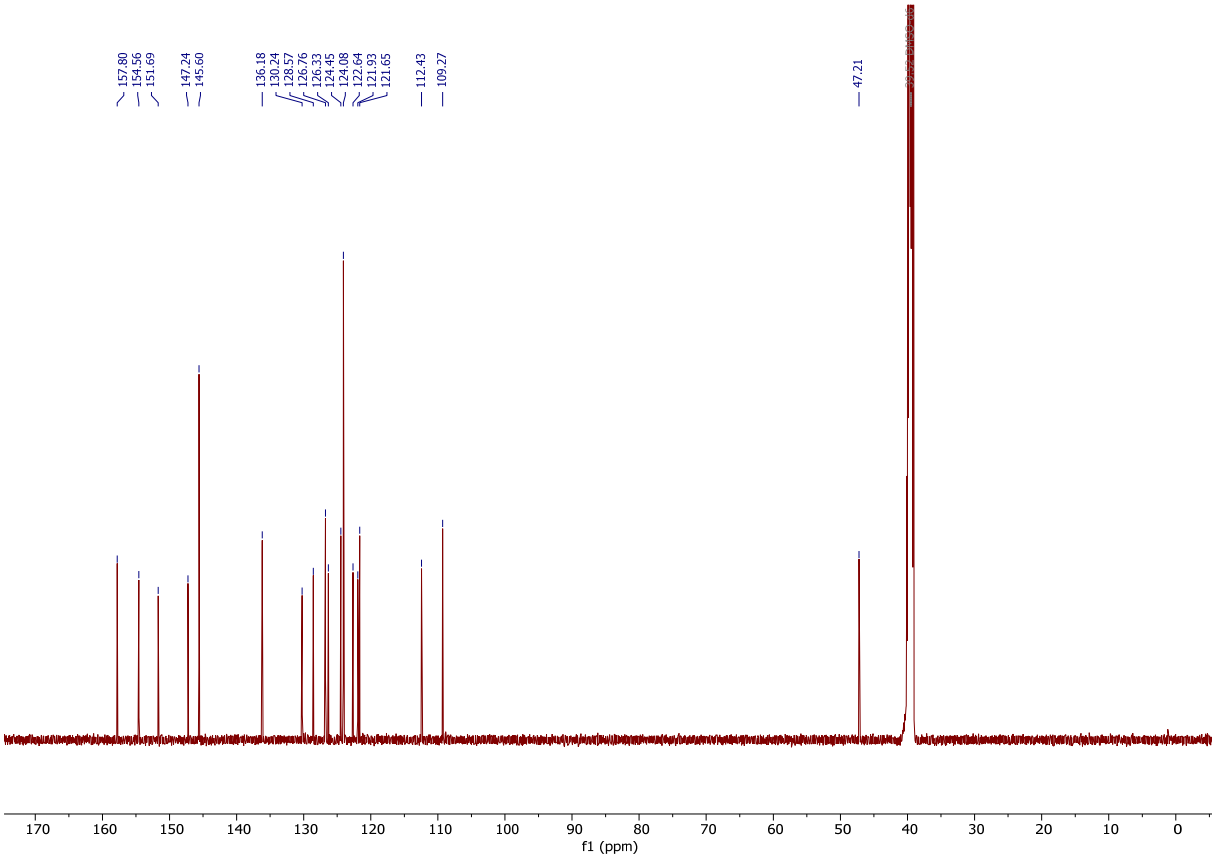


^{1}H and ^{13}C NMR (CD_3OD): **1j**

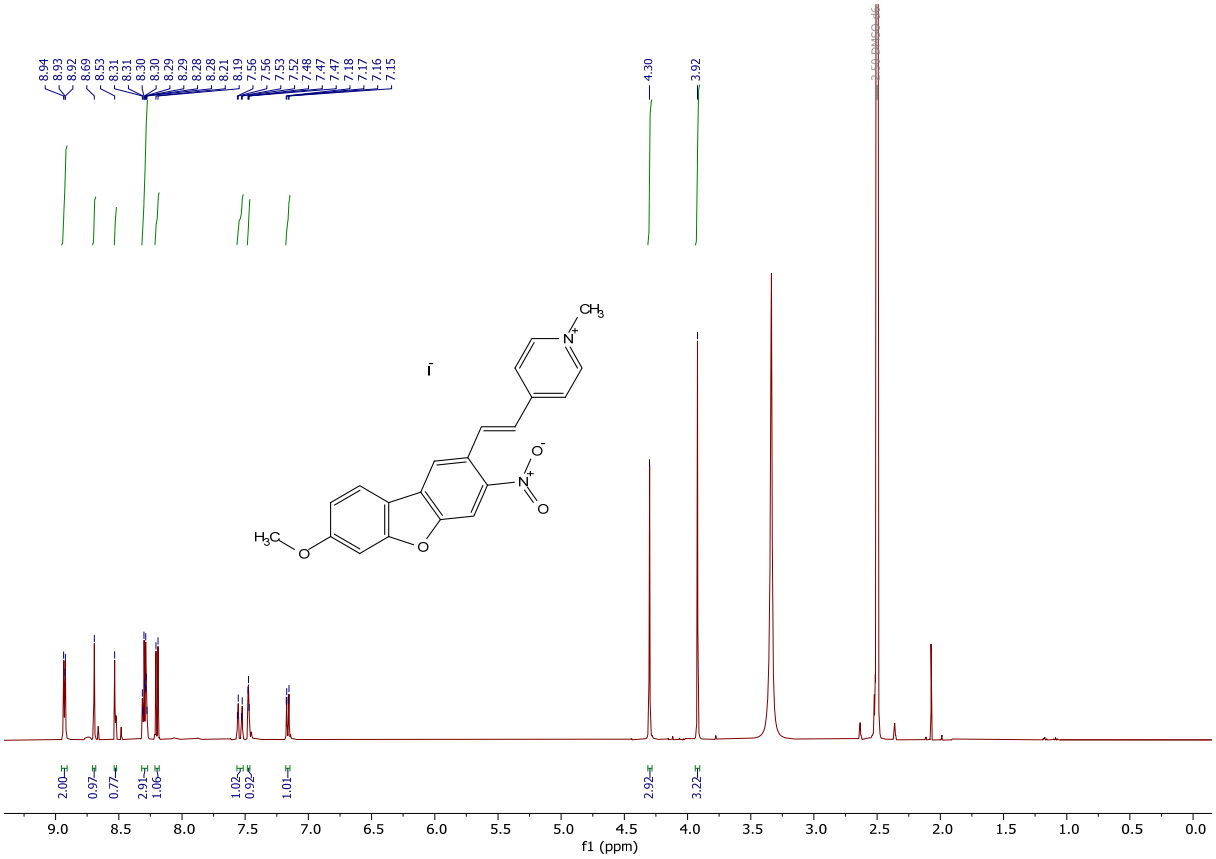


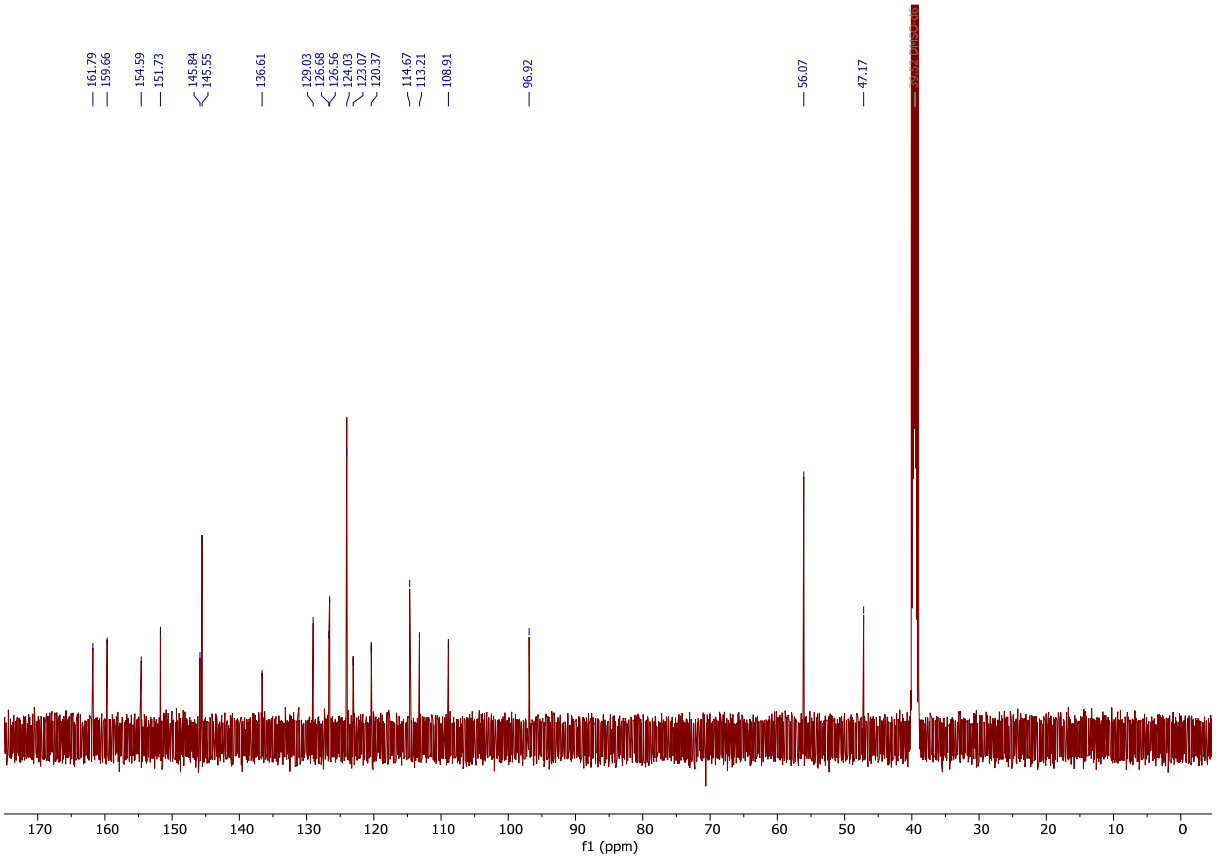
^{1}H and ^{13}C NMR (DMSO-d_6): **1k**



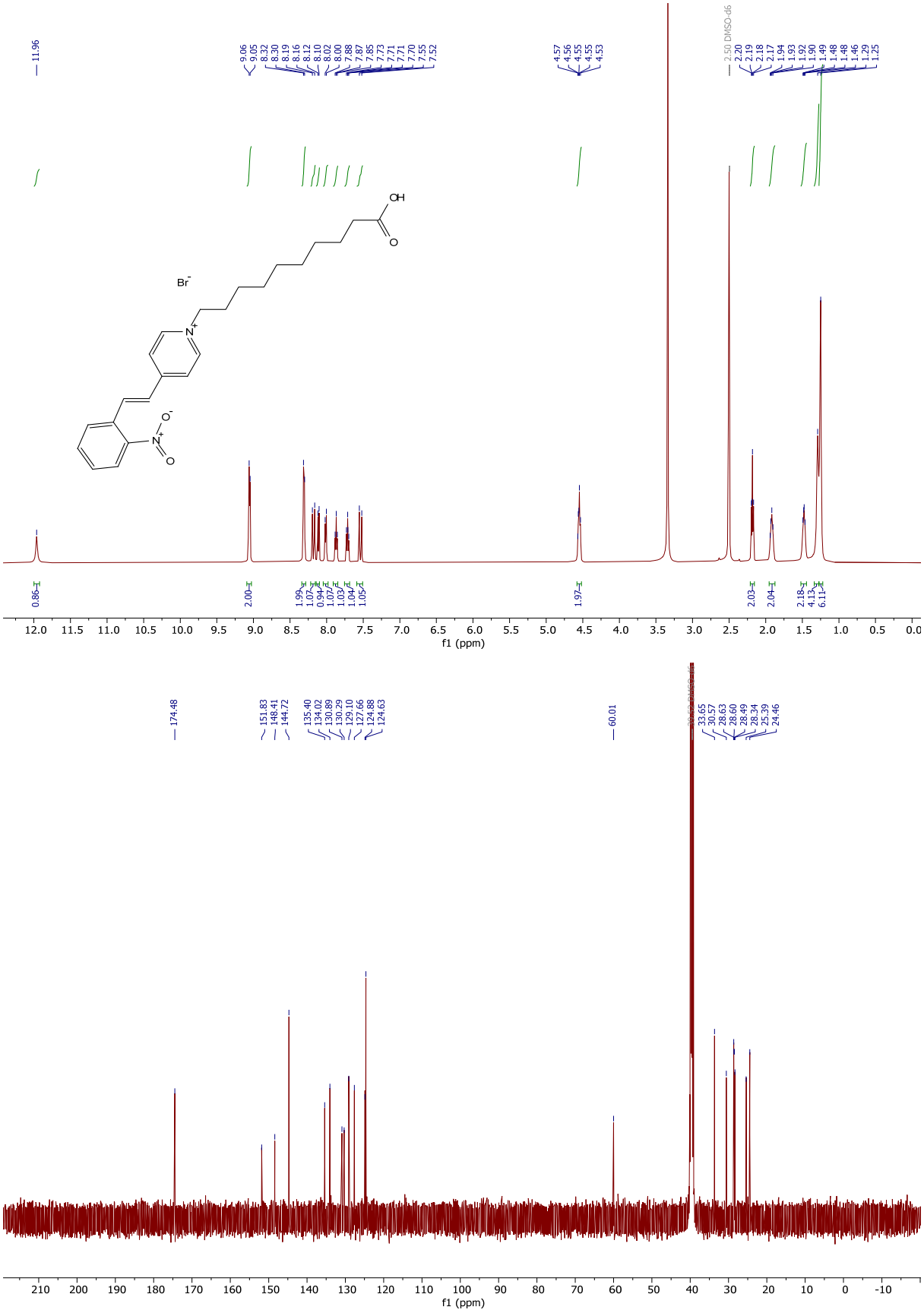


^{1}H and ^{13}C NMR (DMSO-d_6): **1l**

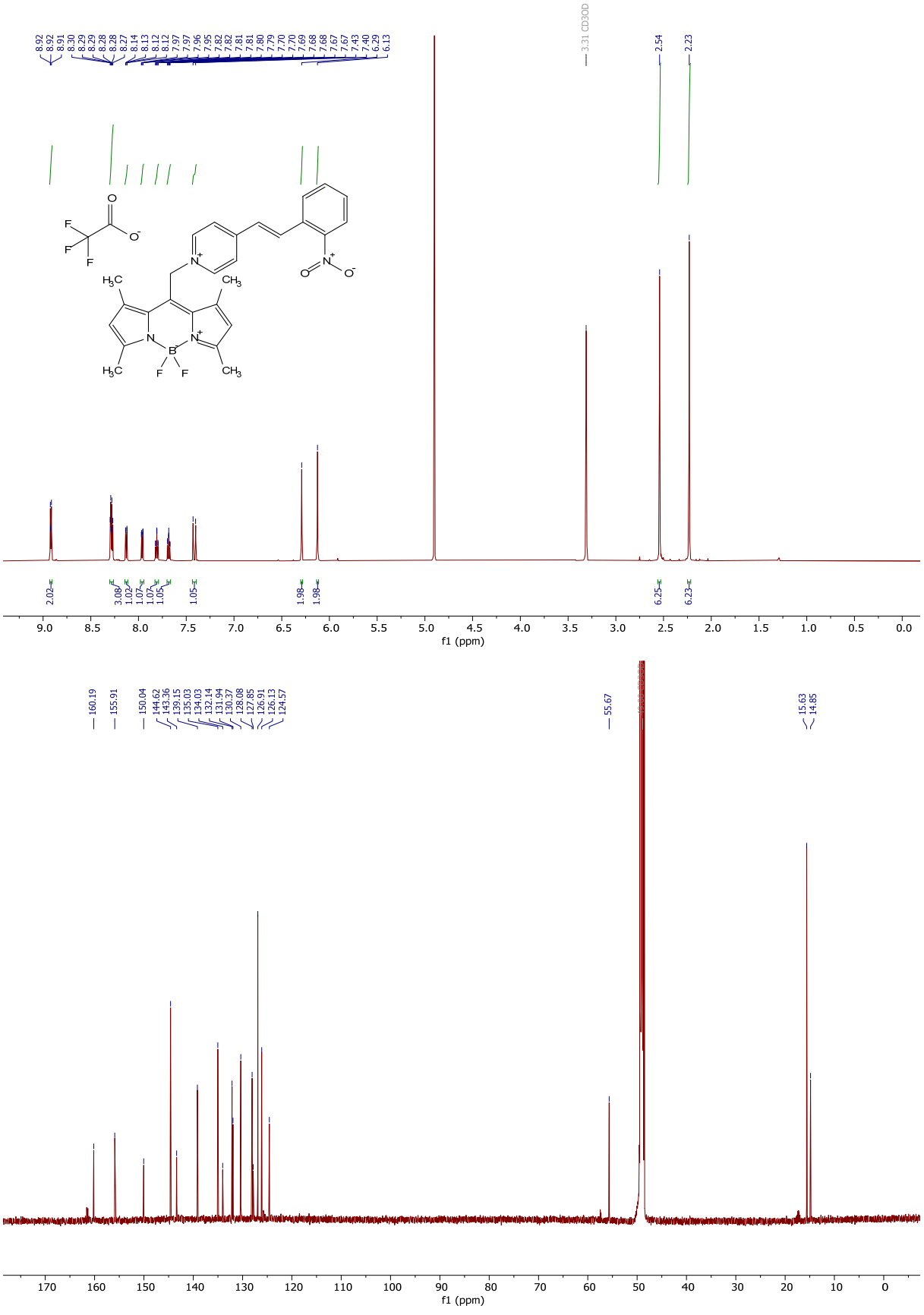




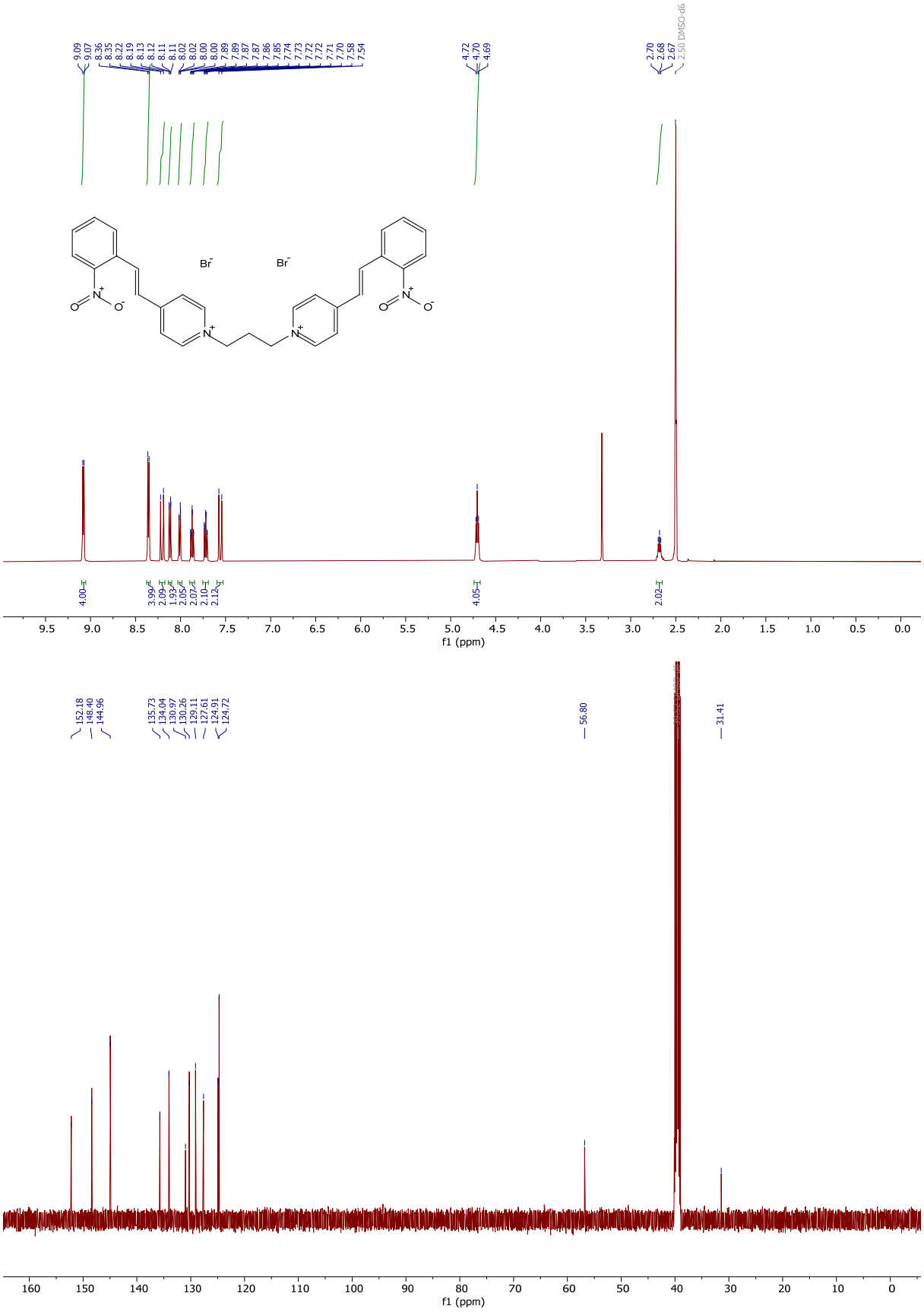
^{1}H and ^{13}C NMR (DMSO-d_6): **1m**



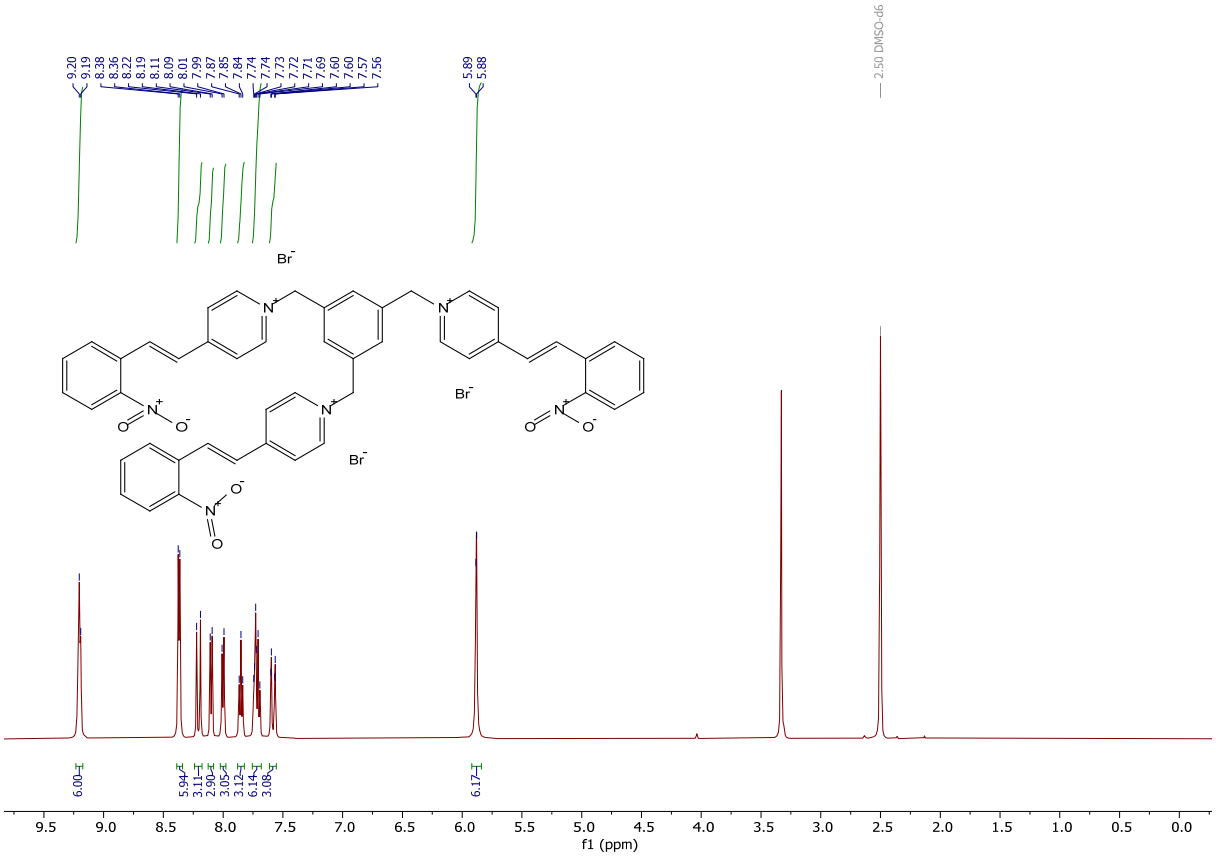
^{1}H and ^{13}C NMR (CD_3OD): **1n**

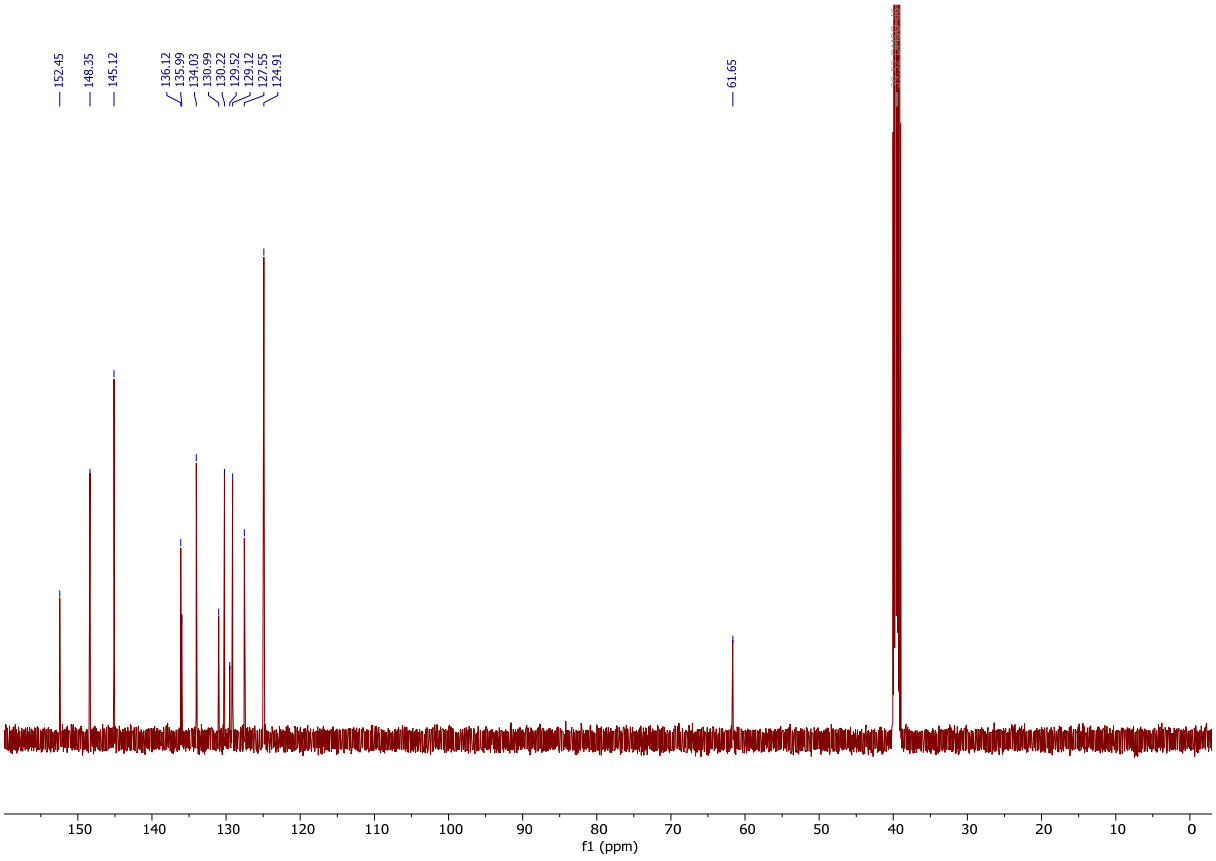


^{1}H and ^{13}C NMR (DMSO-d_6): **4a**

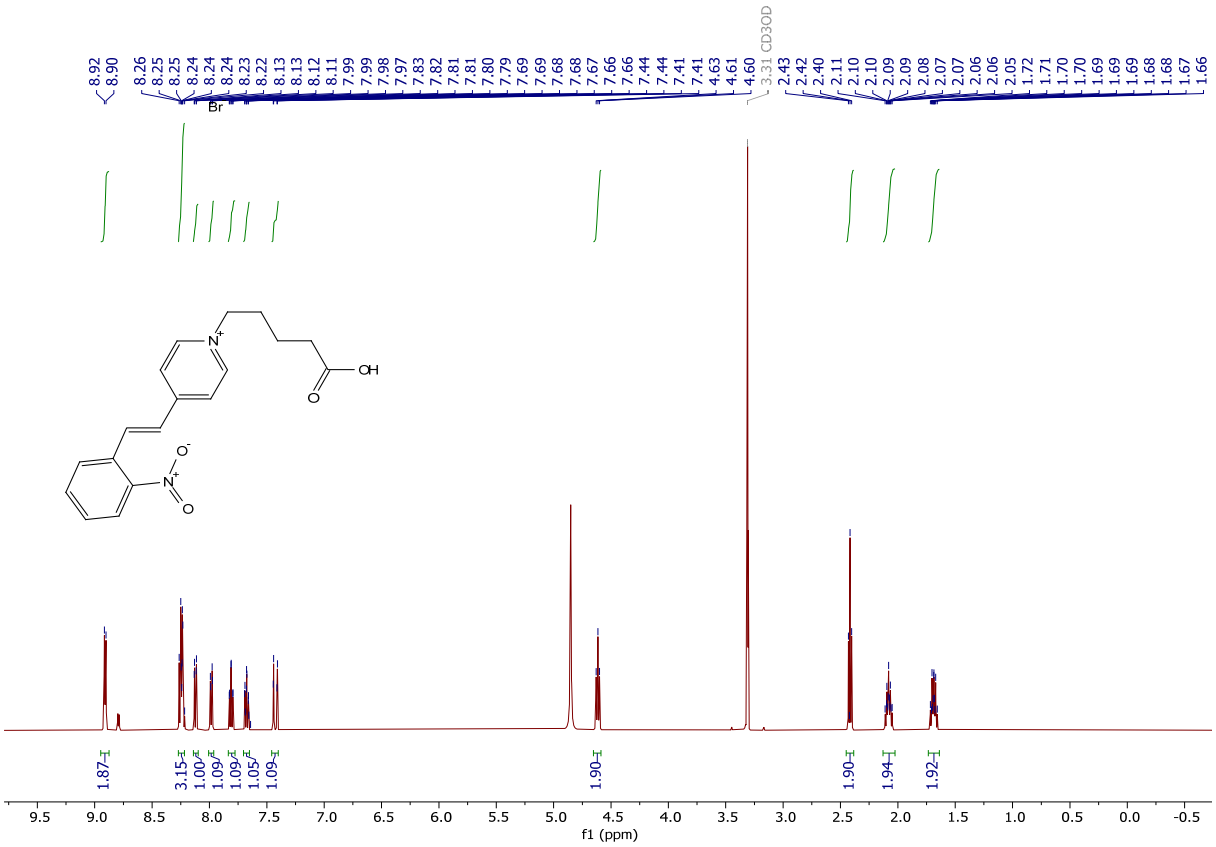


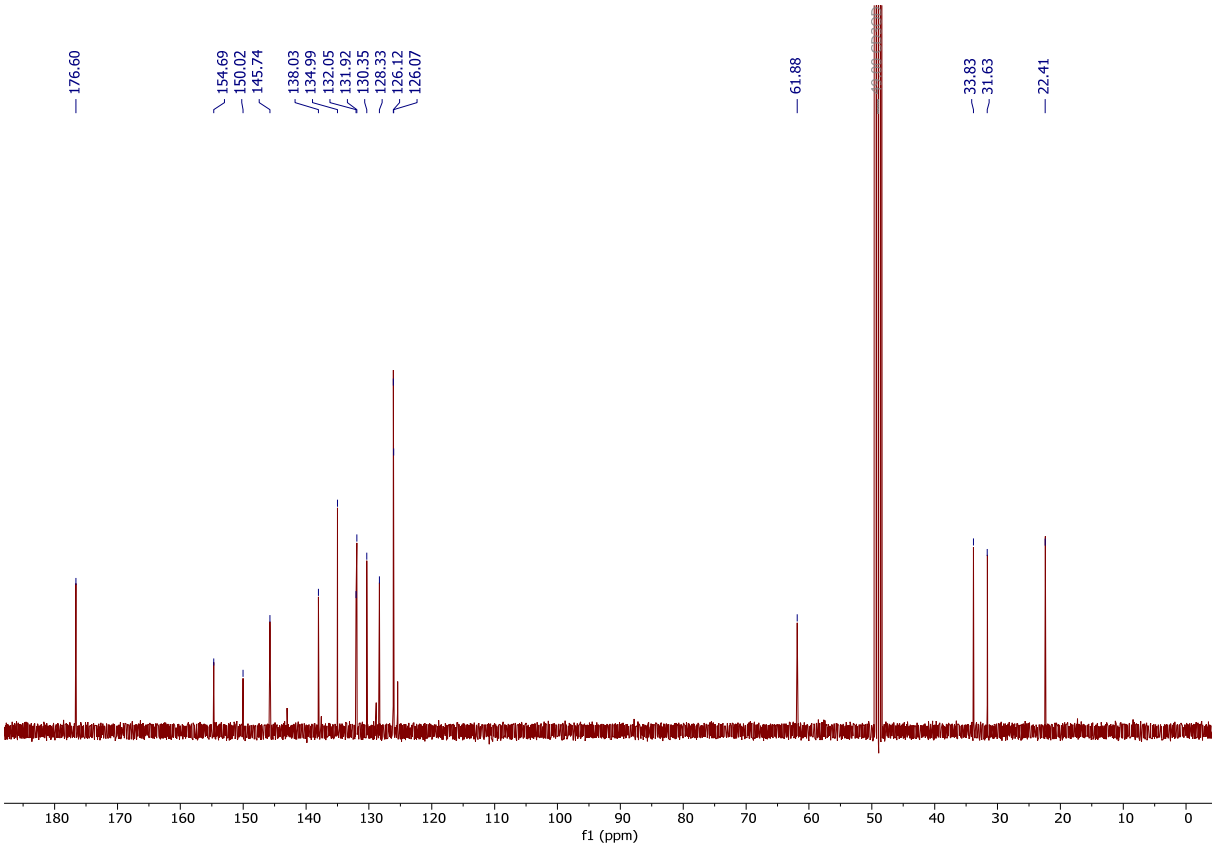
^{1}H and ^{13}C NMR (DMSO-d_6): **4b**



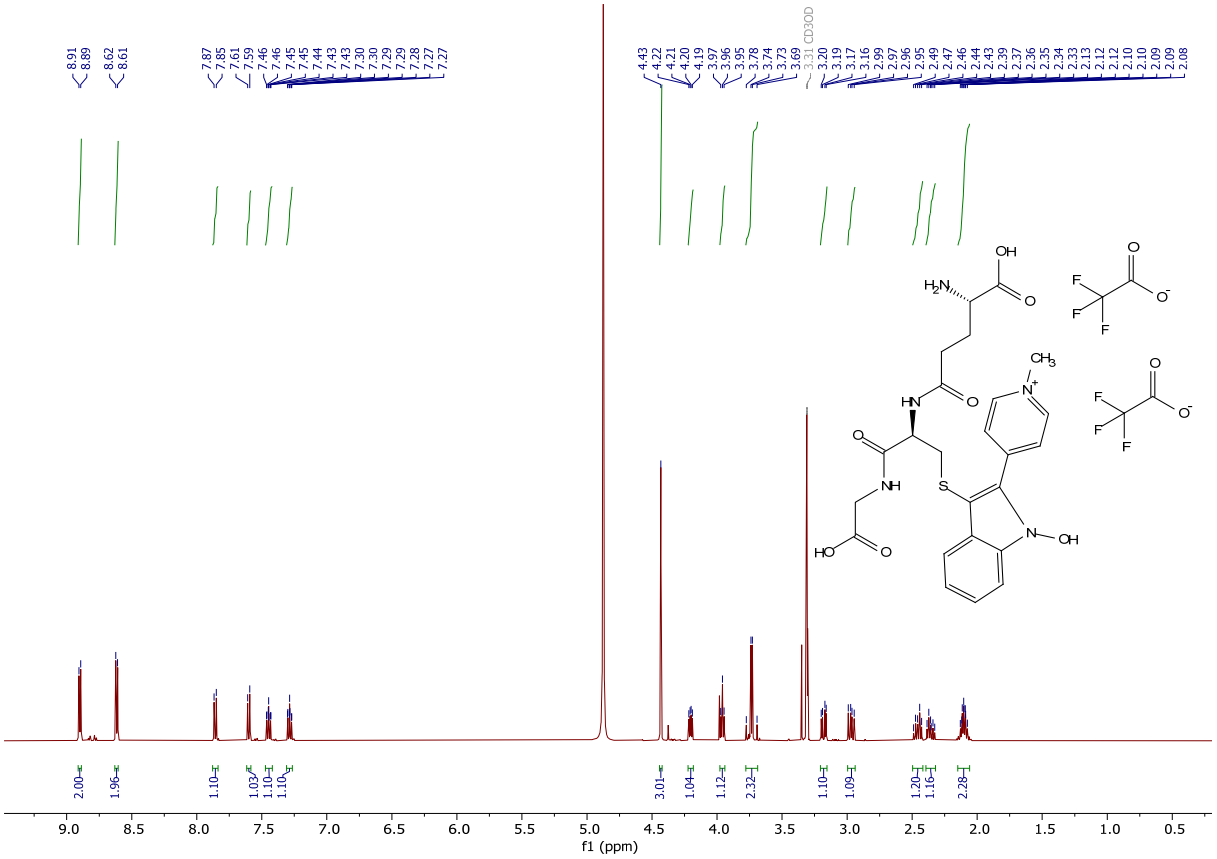


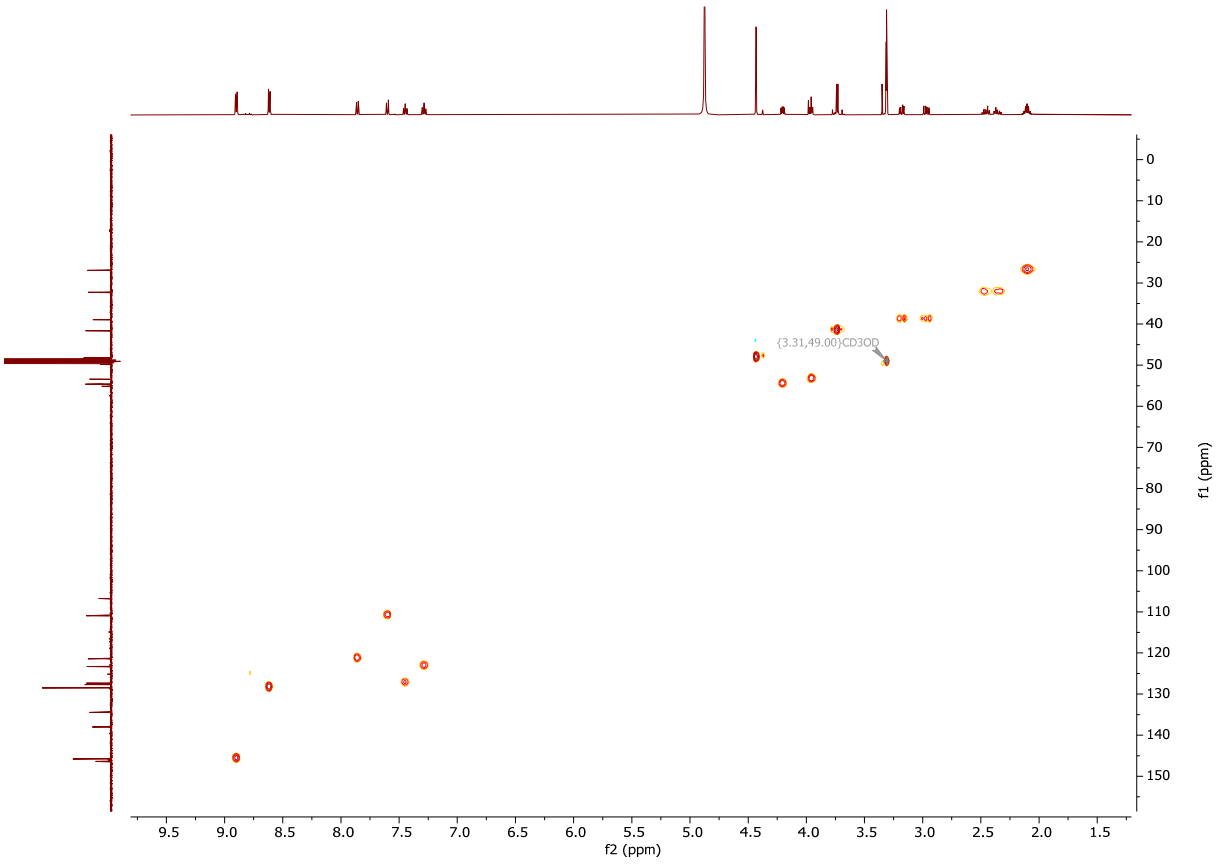
^{1}H and ^{13}C NMR (CD_3OD): **S14**

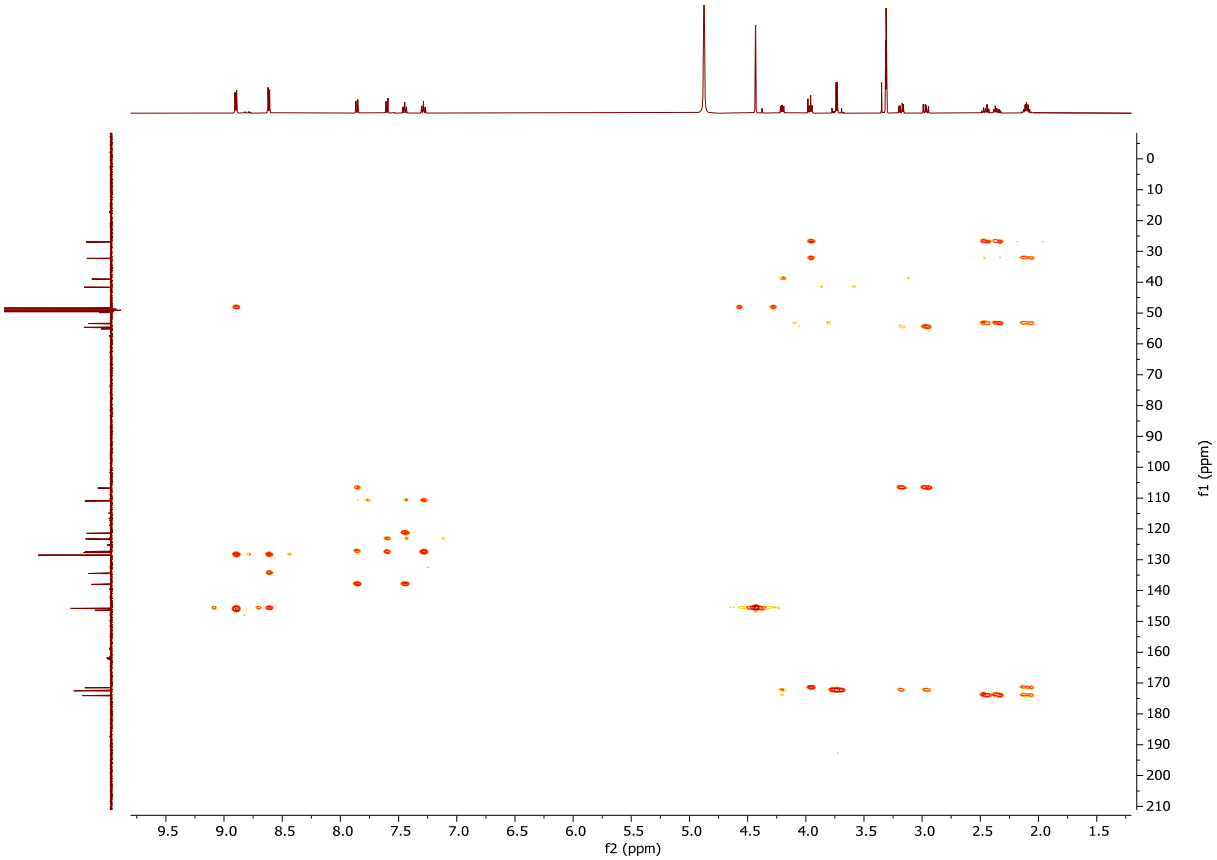


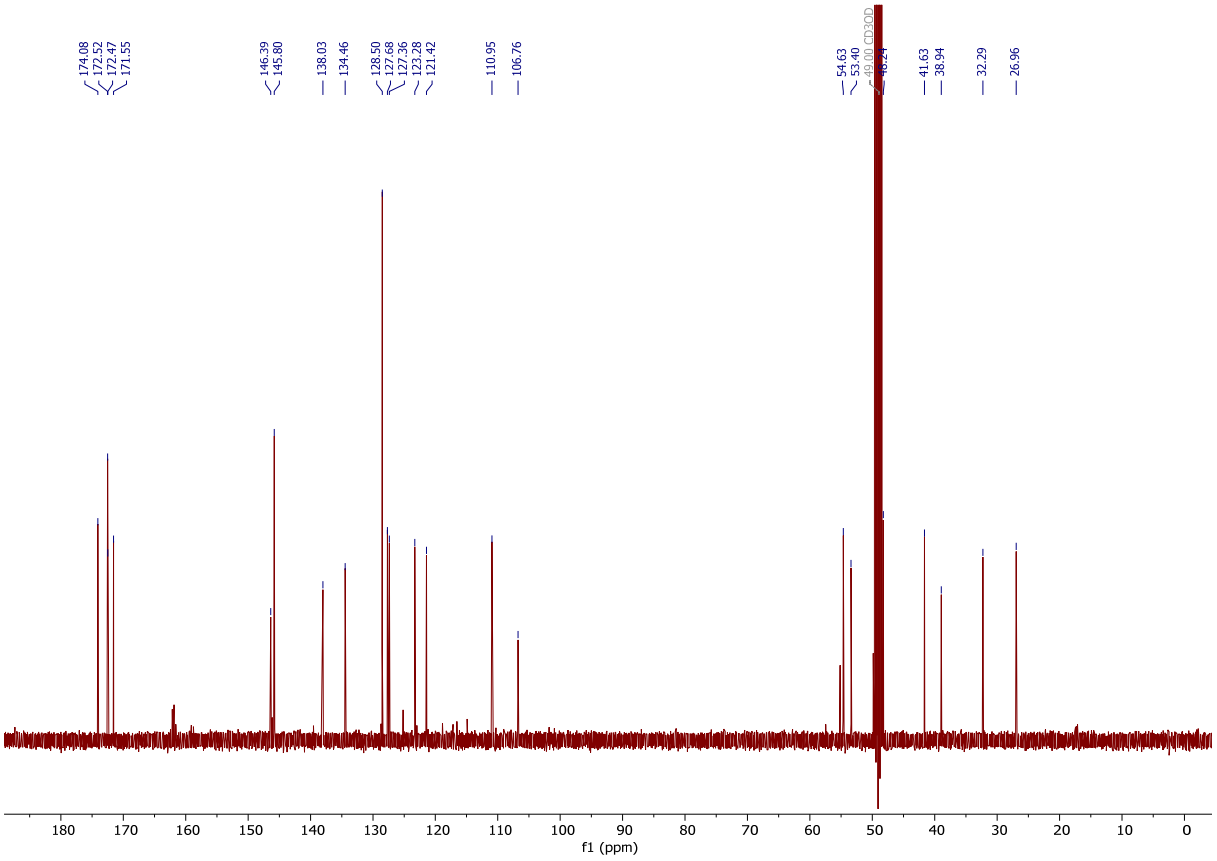


^{1}H, HMQC, HMBC and ^{13}C NMR (CD_3OD): **3a**

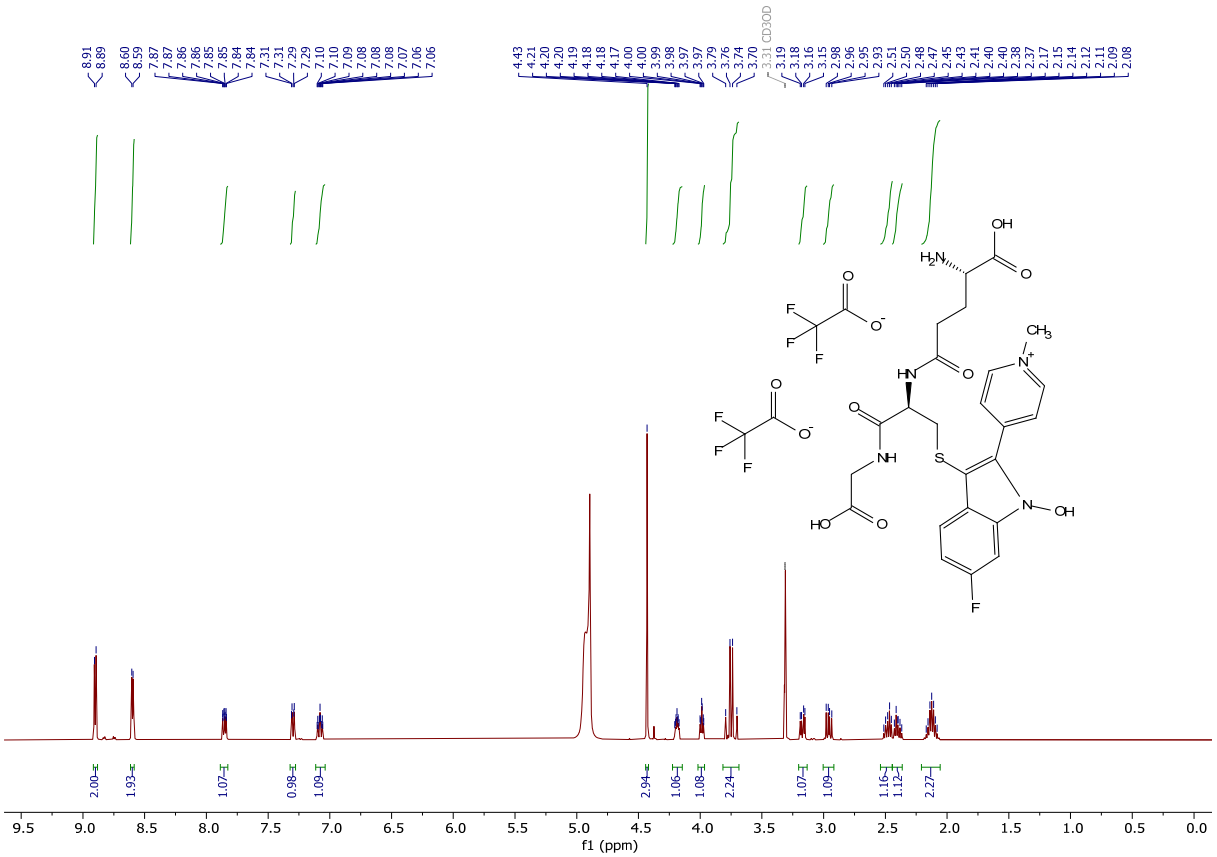


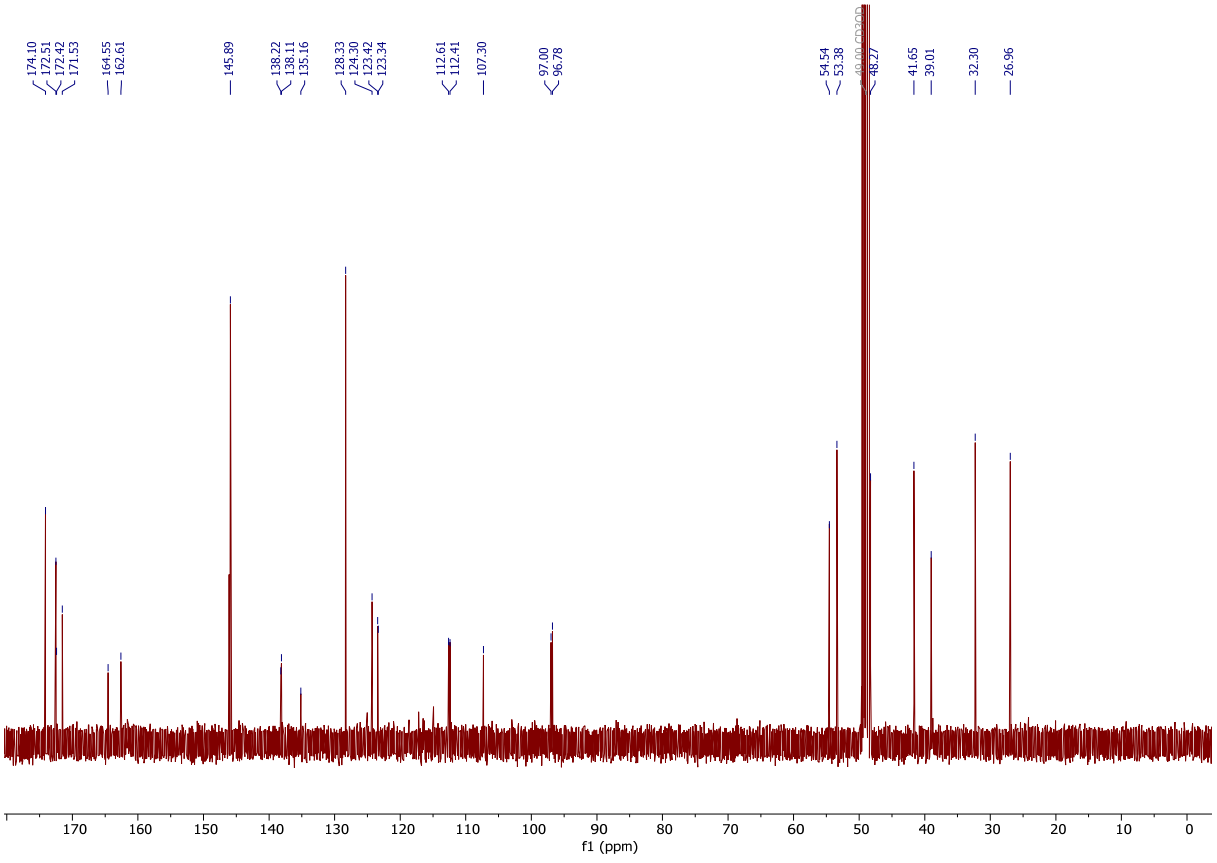



f2 (ppm)
f1 (ppm)

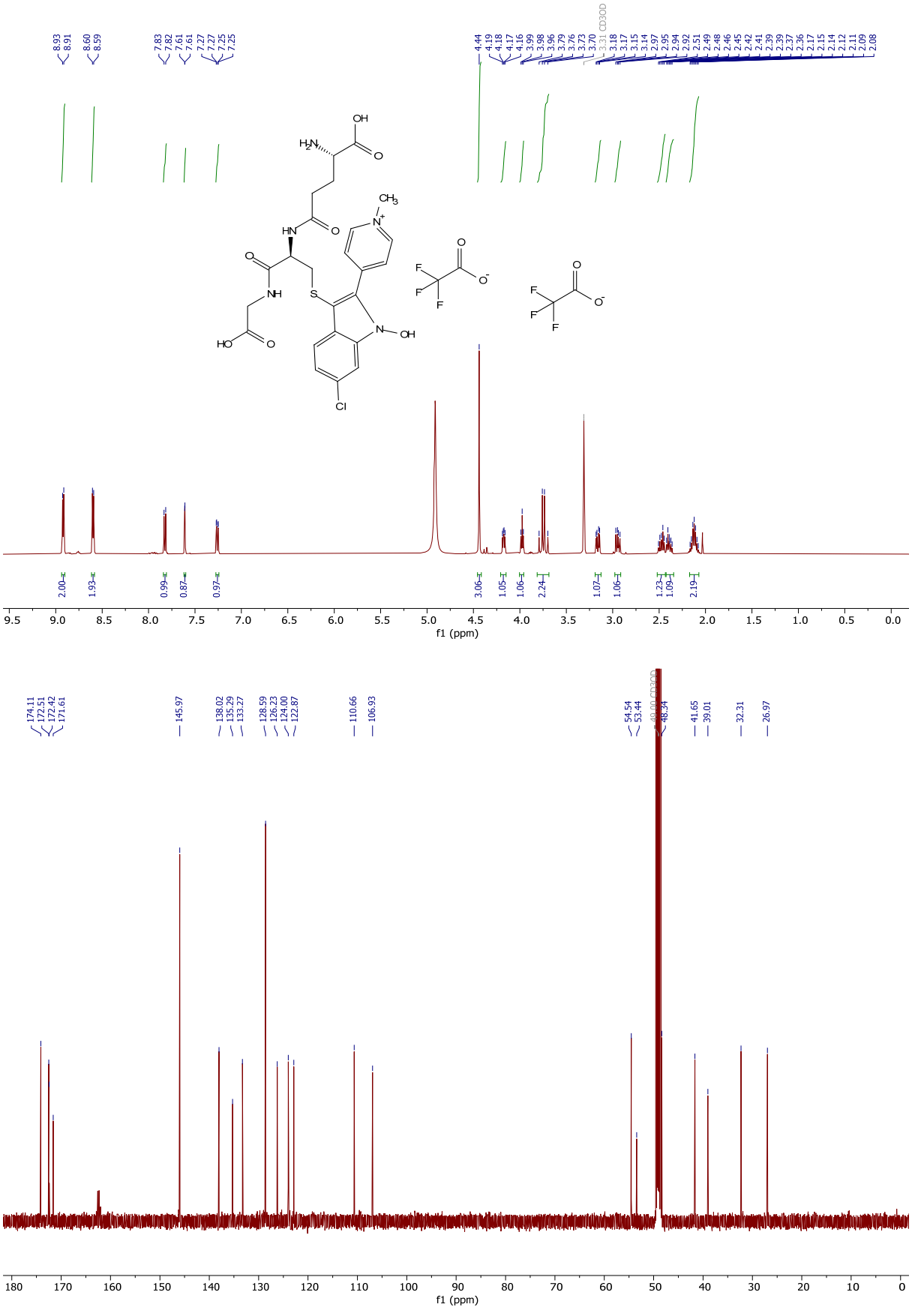

174.08
172.52
172.47
171.55
146.39
145.80
138.03
134.46
128.50
127.68
127.36
123.28
121.42
110.95
106.76
54.63
53.40
49.00 CD3OD
48.24
41.63
38.94
32.29
26.96
f1 (ppm)

^{1}H and ^{13}C NMR (CD_3OD): **3b**

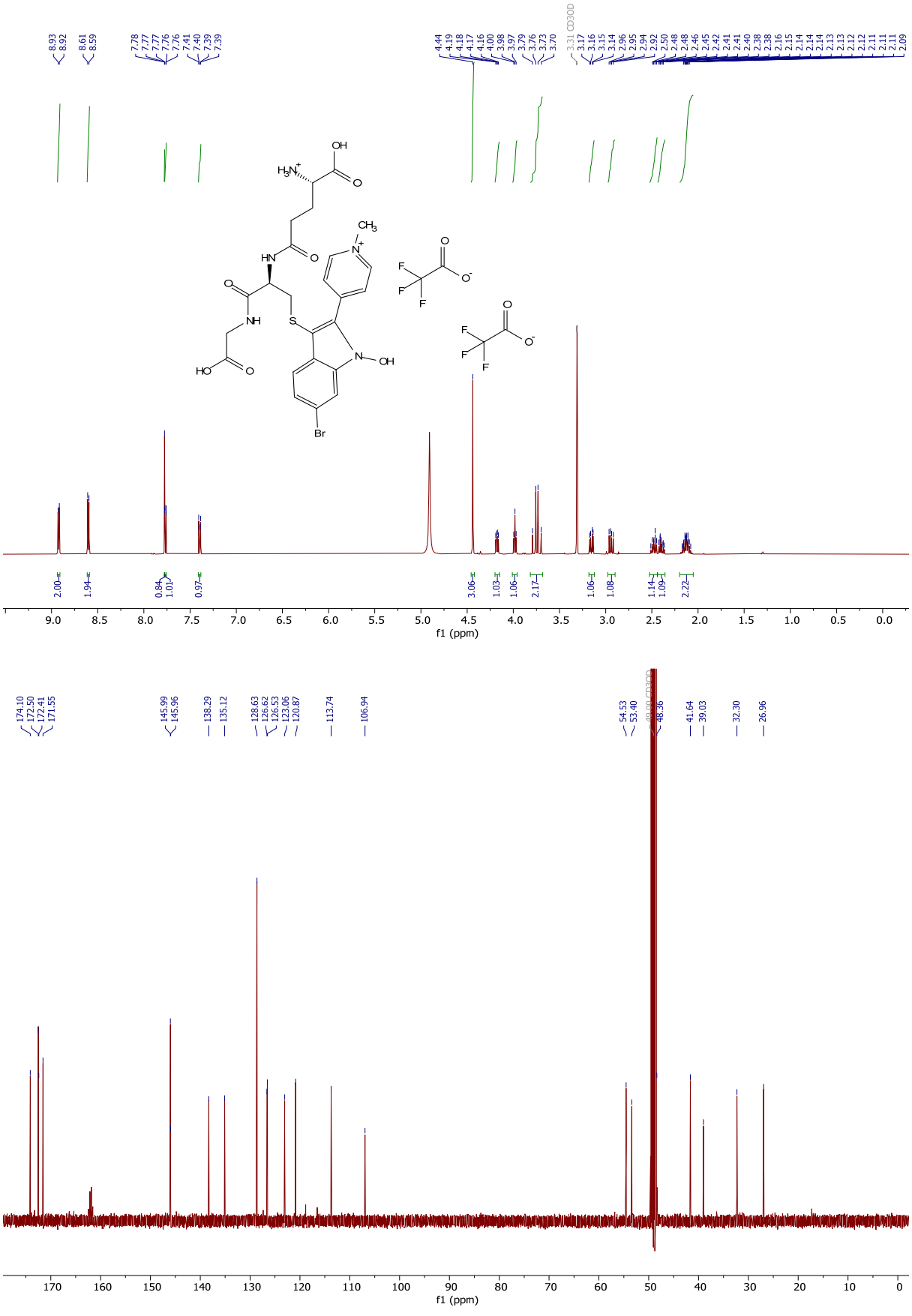




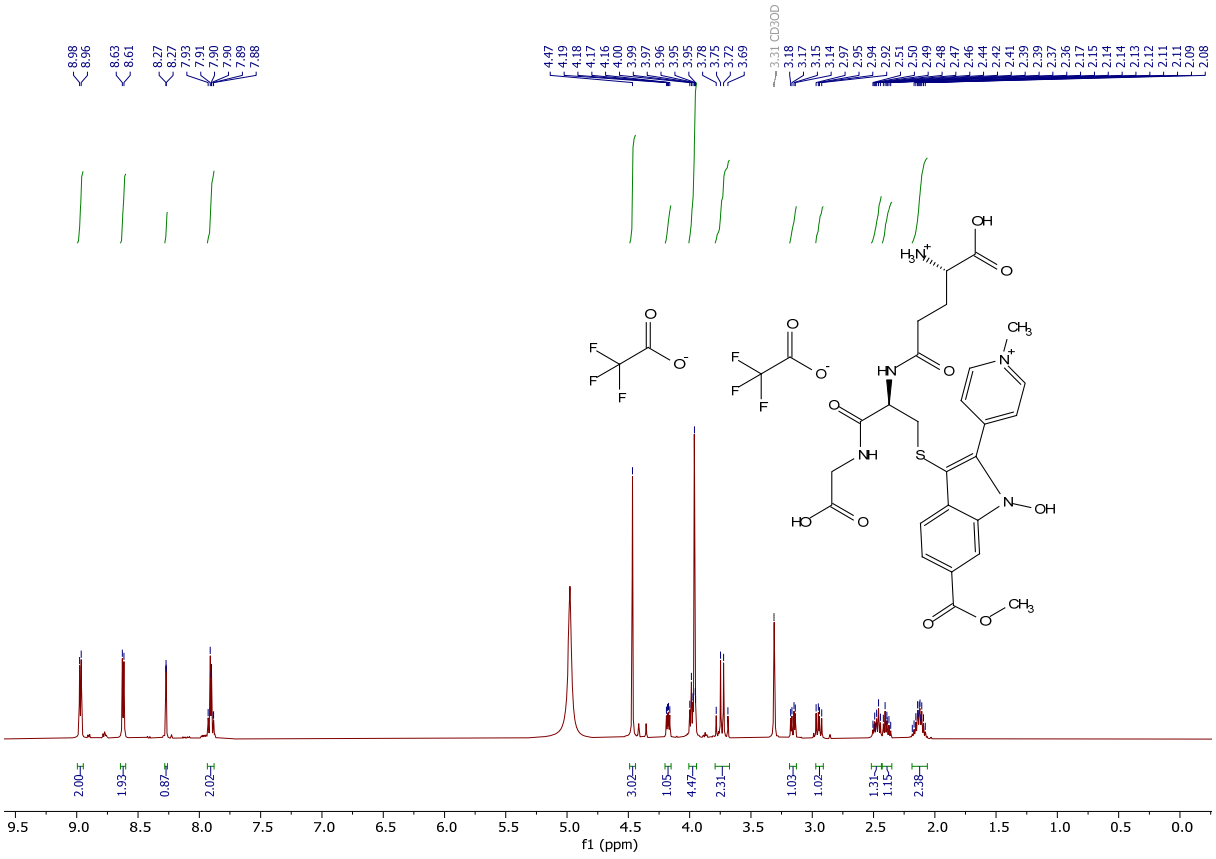
^{1}H and ^{13}C NMR (CD_3OD): **3c**

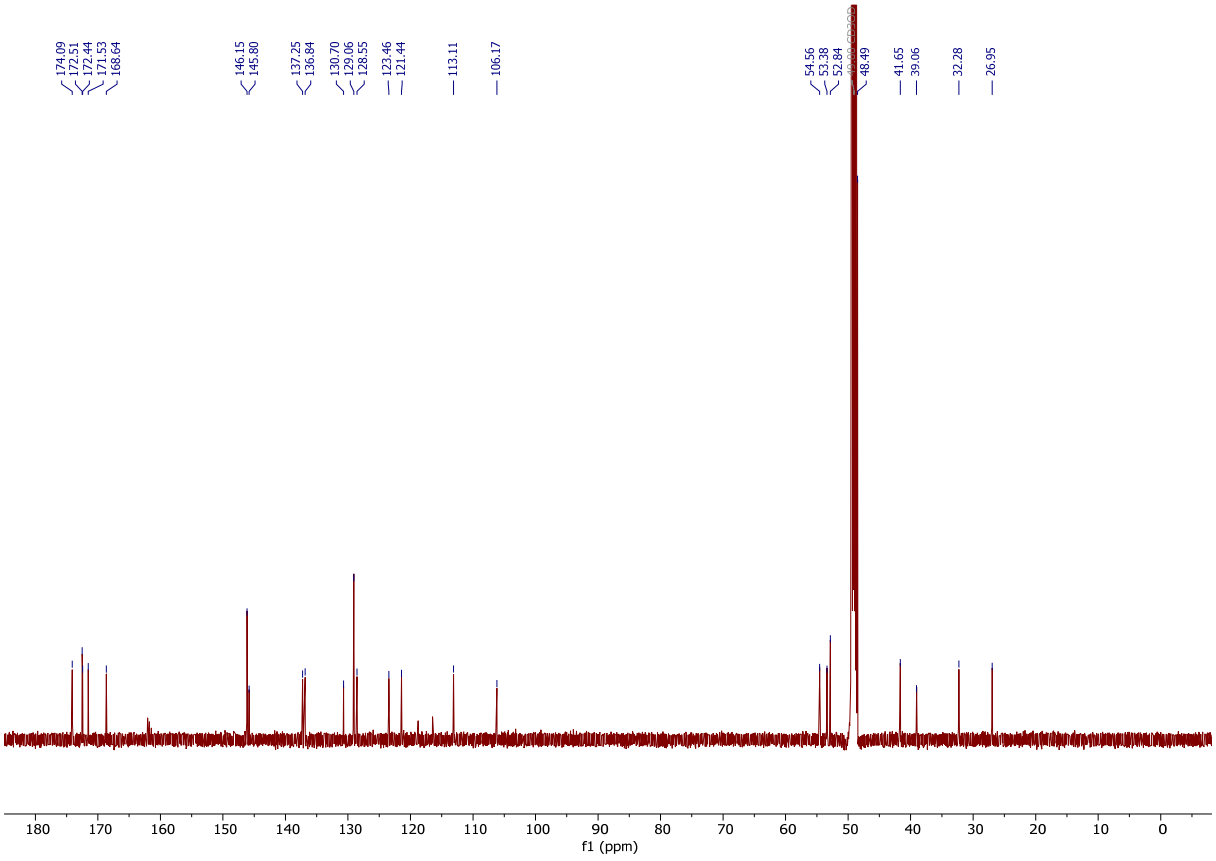


^{1}H and ^{13}C NMR (CD_3OD): **3d**

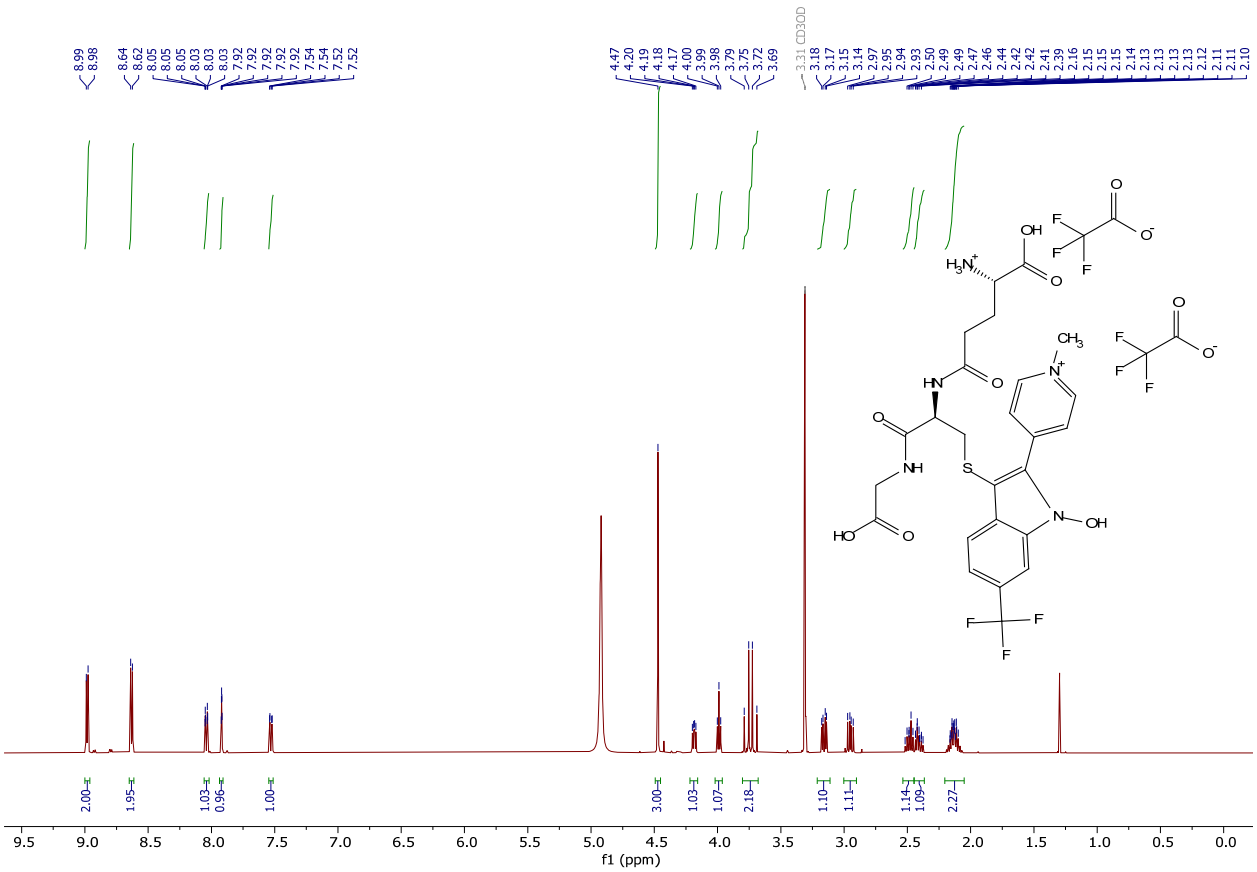


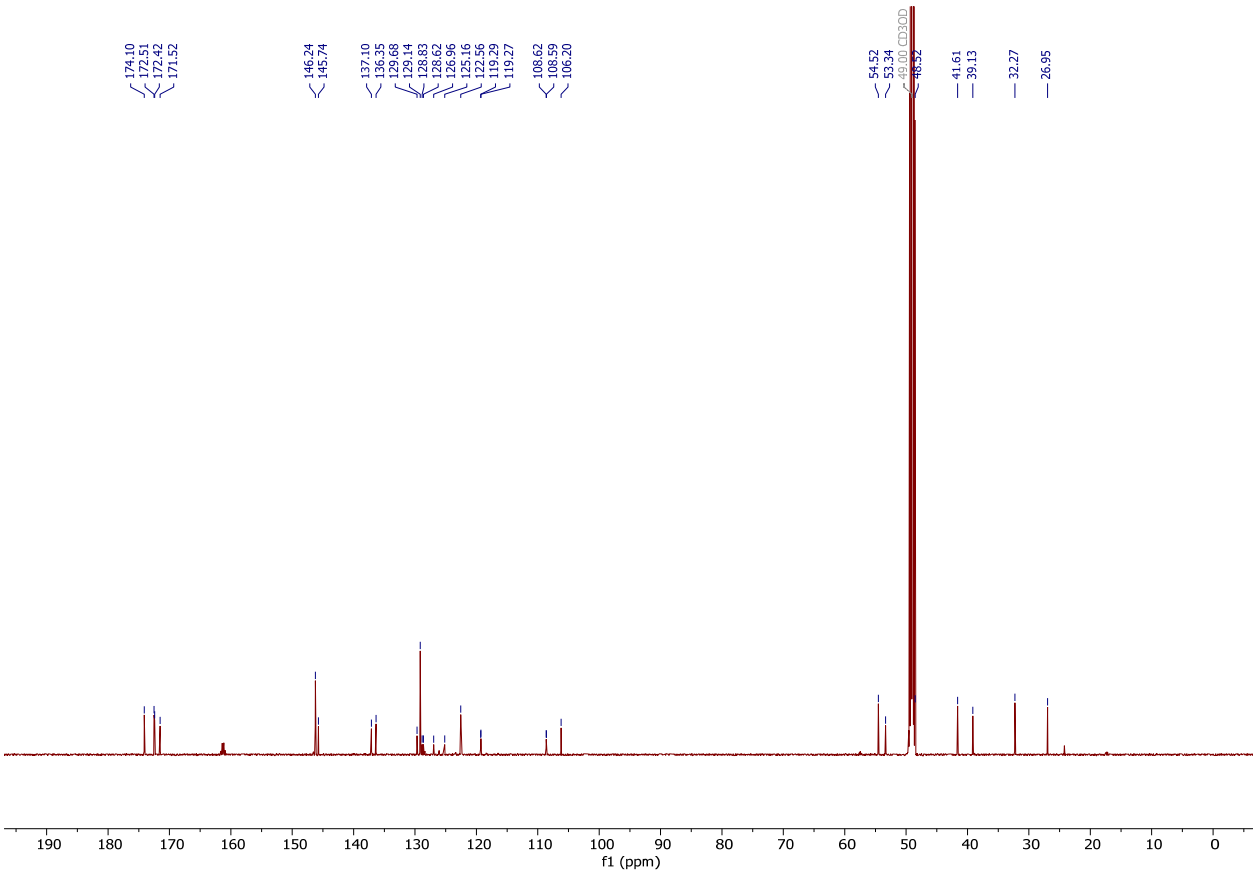
^{1}H and ^{13}C NMR (CD_3OD): **3e**



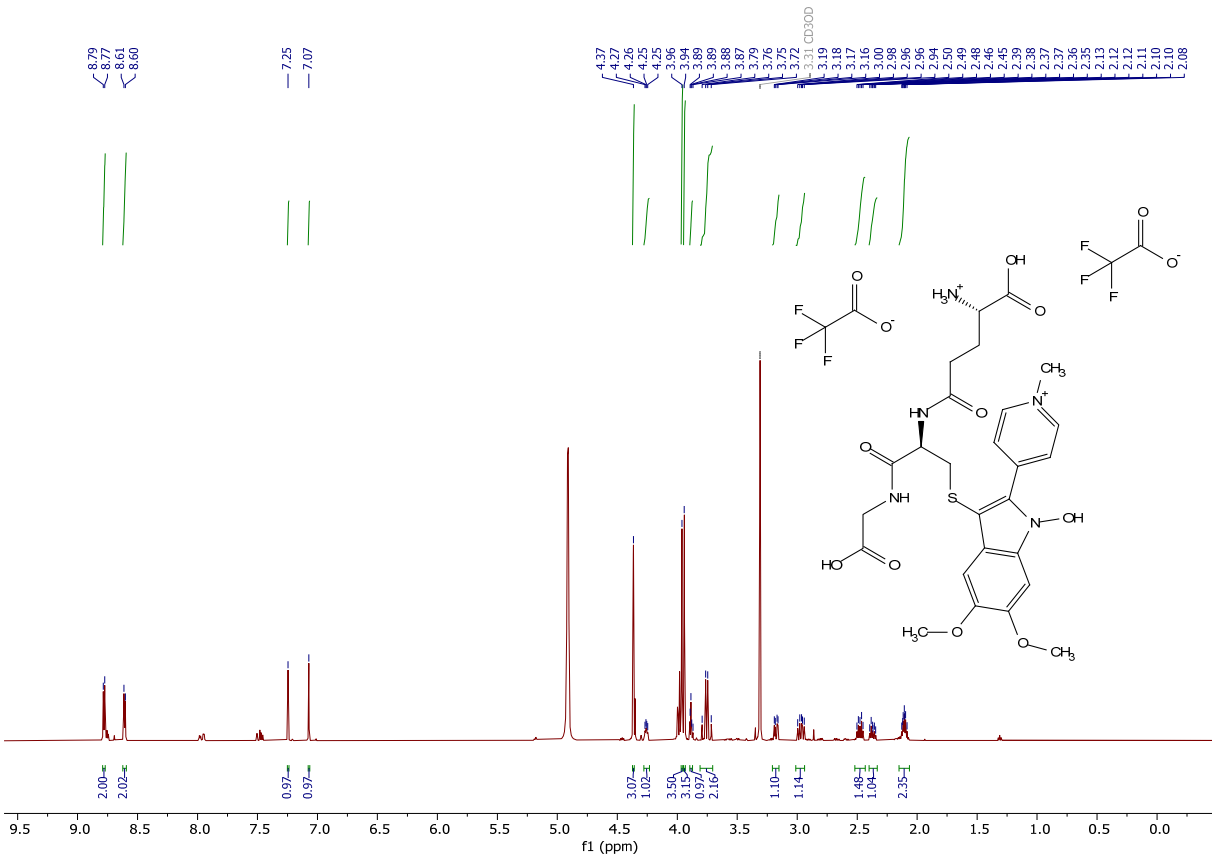


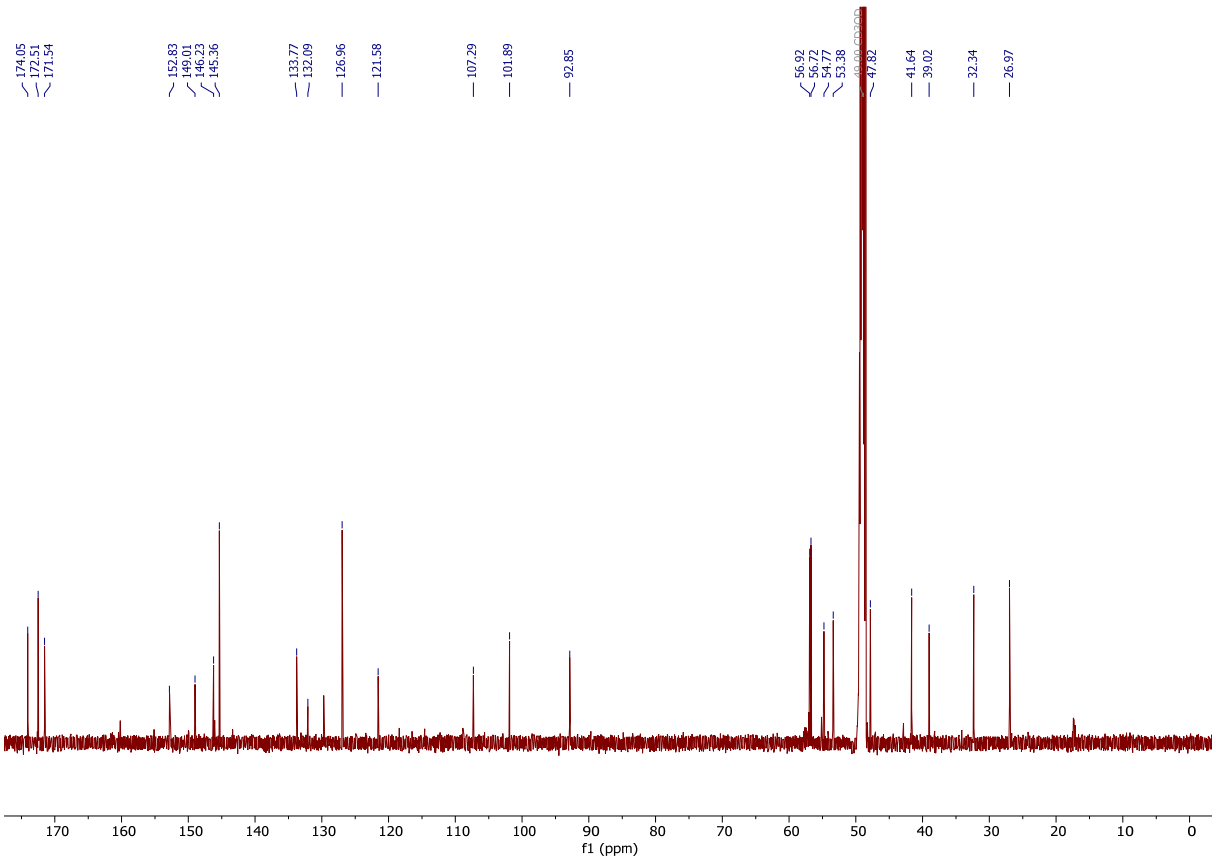
^{1}H and ^{13}C NMR (CD_3OD): **3f**



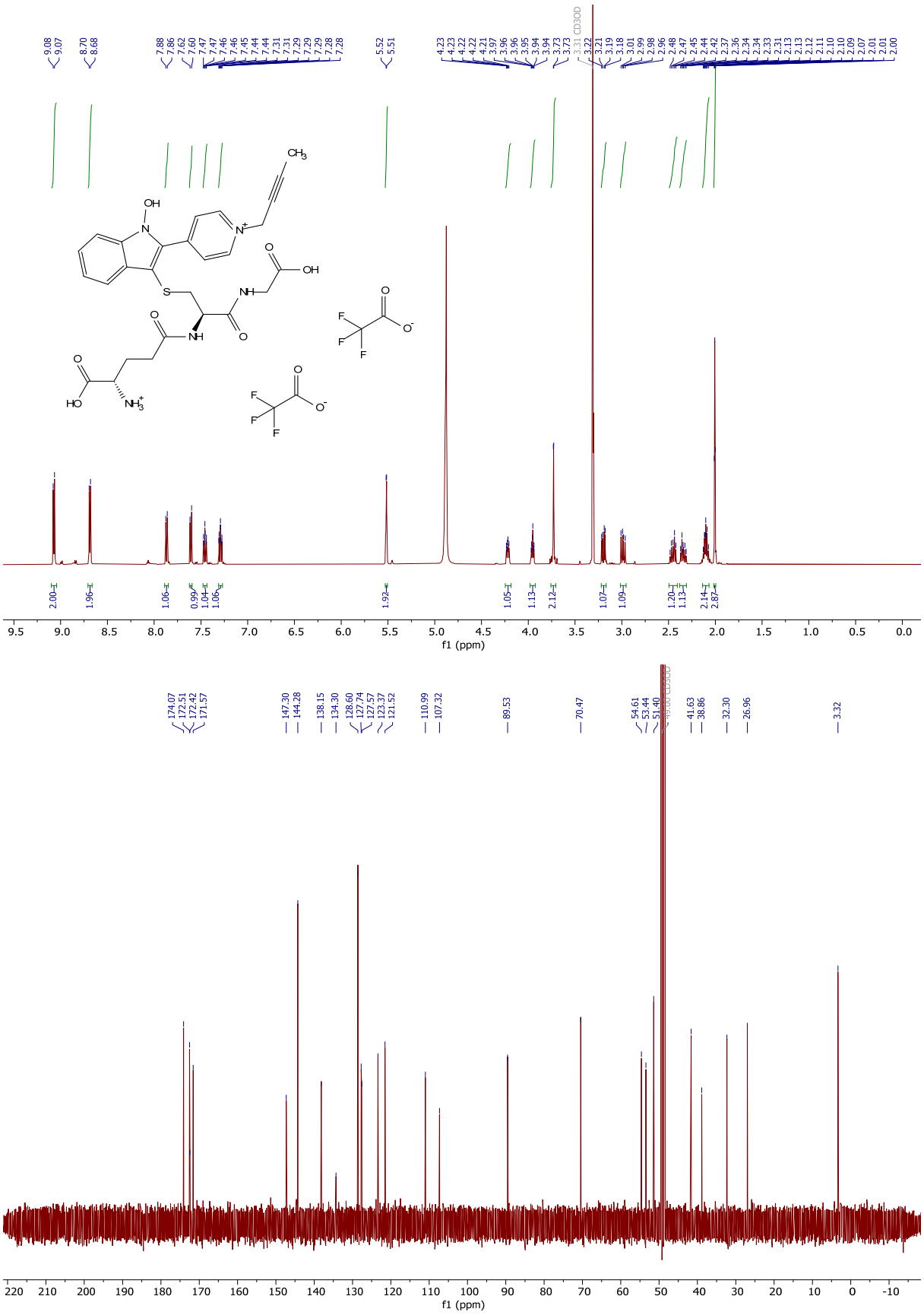


^{1}H and ^{13}C NMR (CD_3OD): **3g**

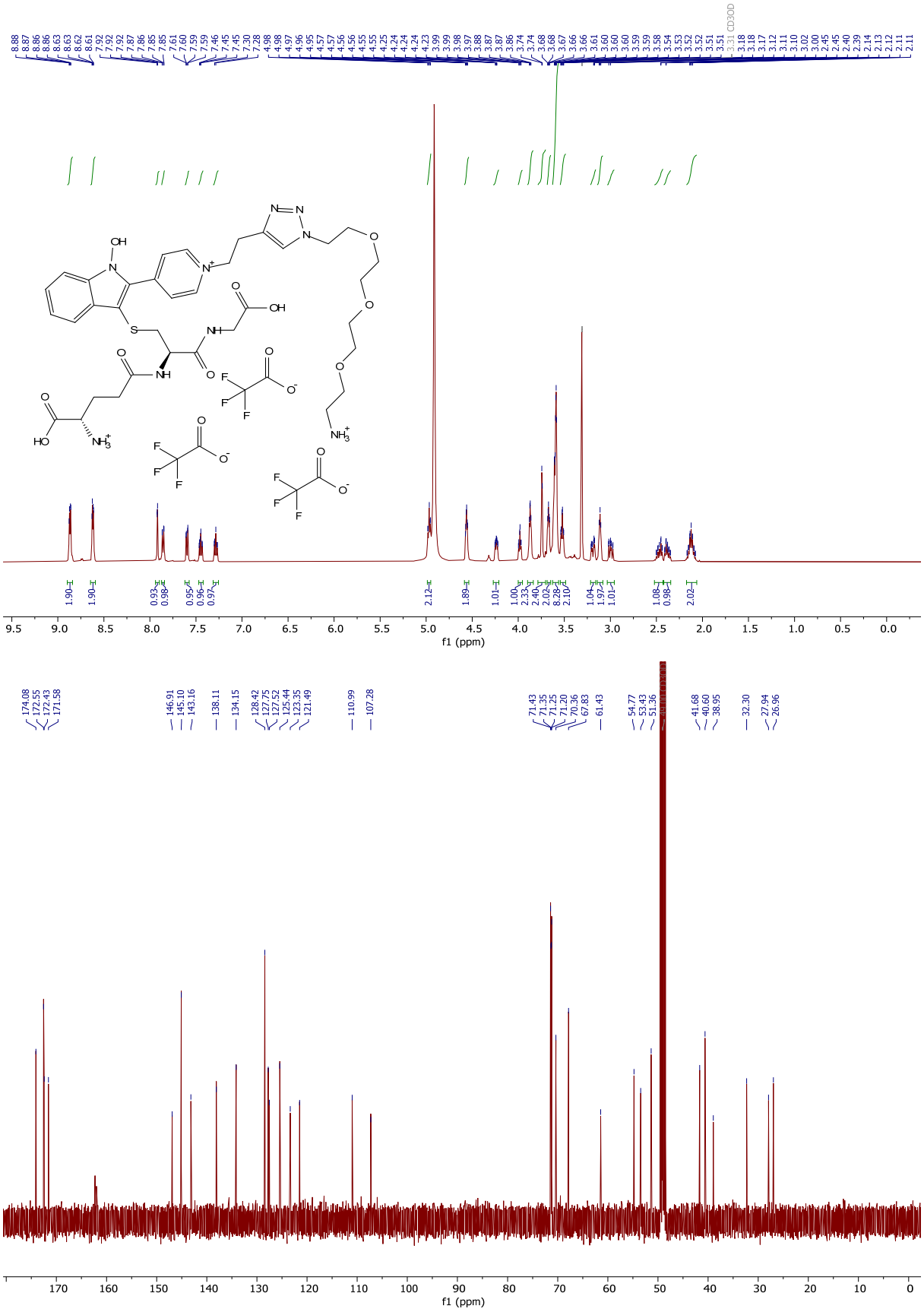




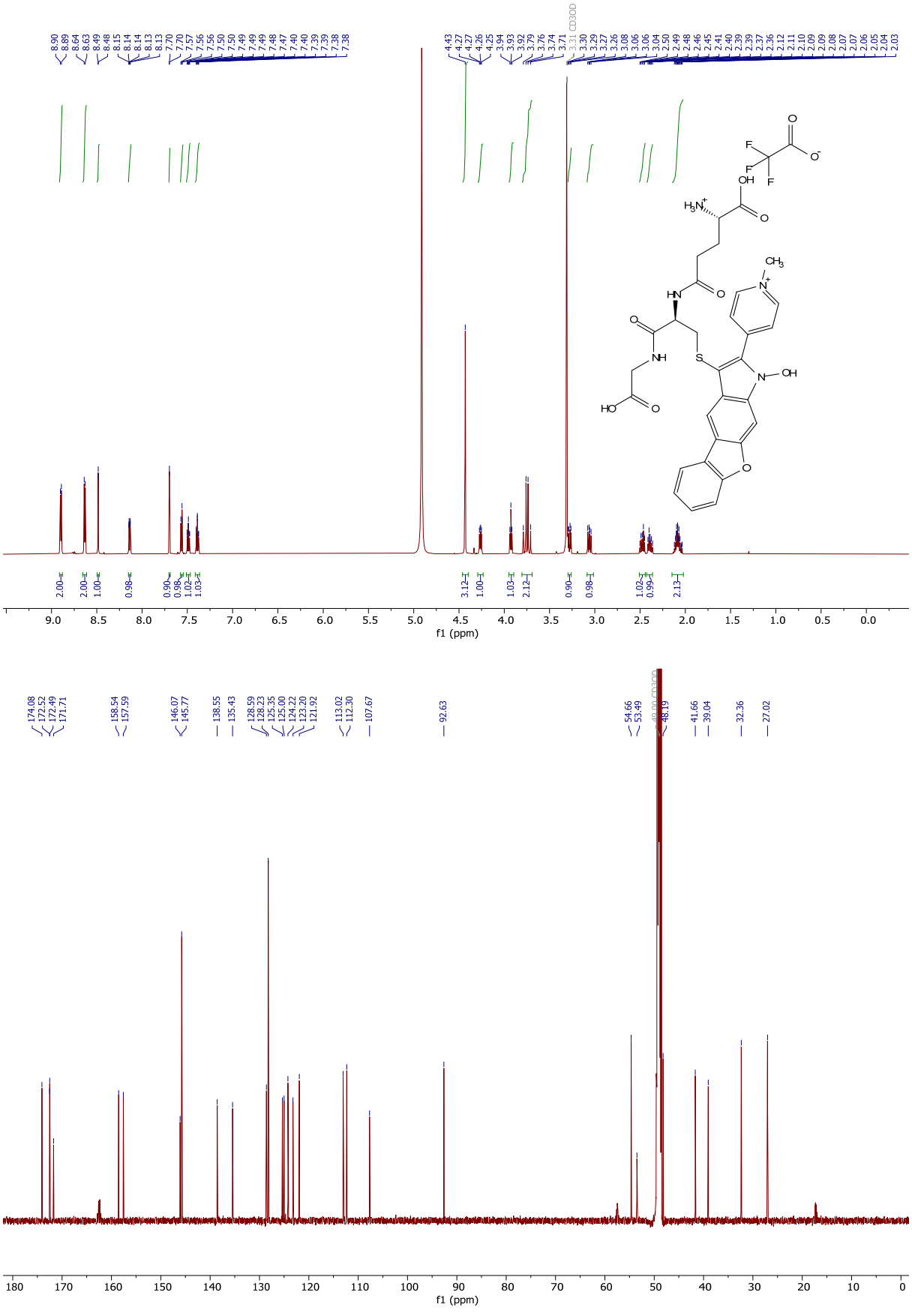
^{1}H and ^{13}C NMR (CD_3OD): **3h**



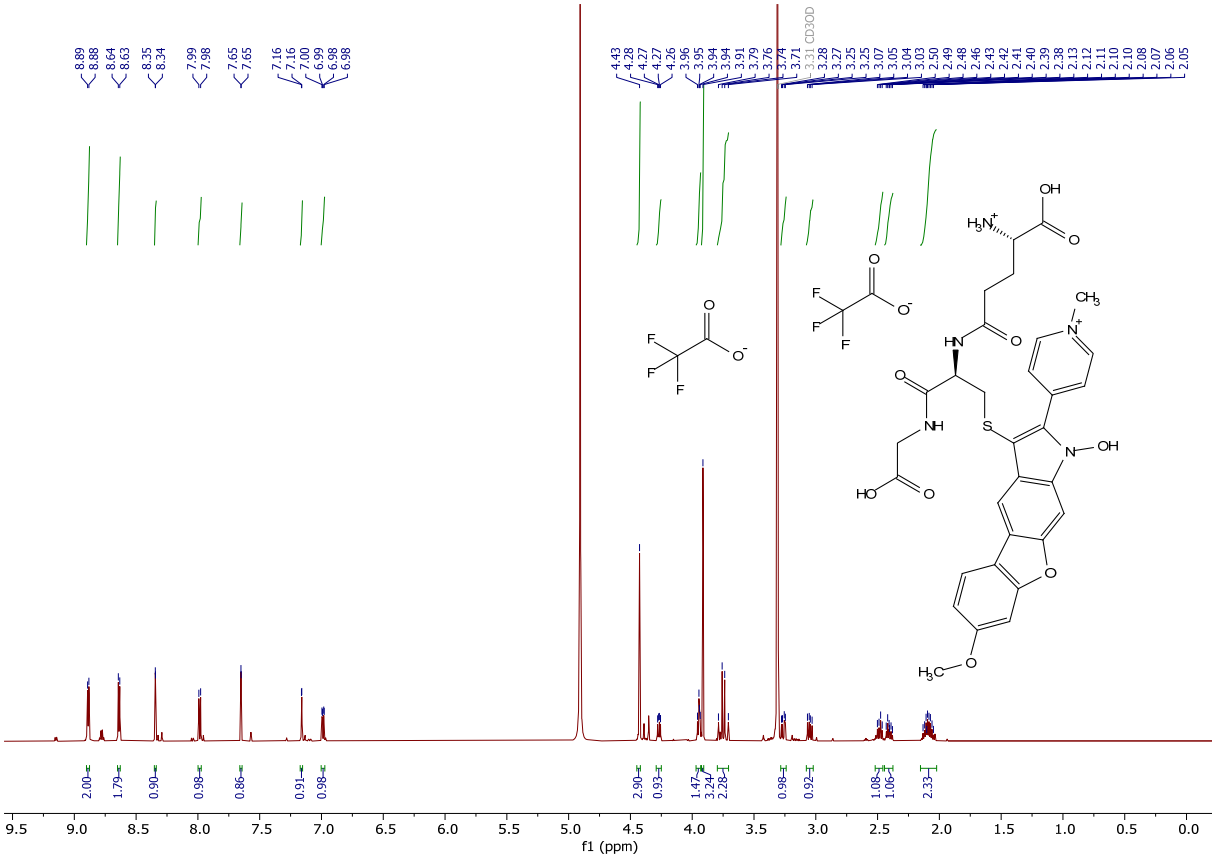
^{1}H and ^{13}C NMR (CD_3OD): **3i**

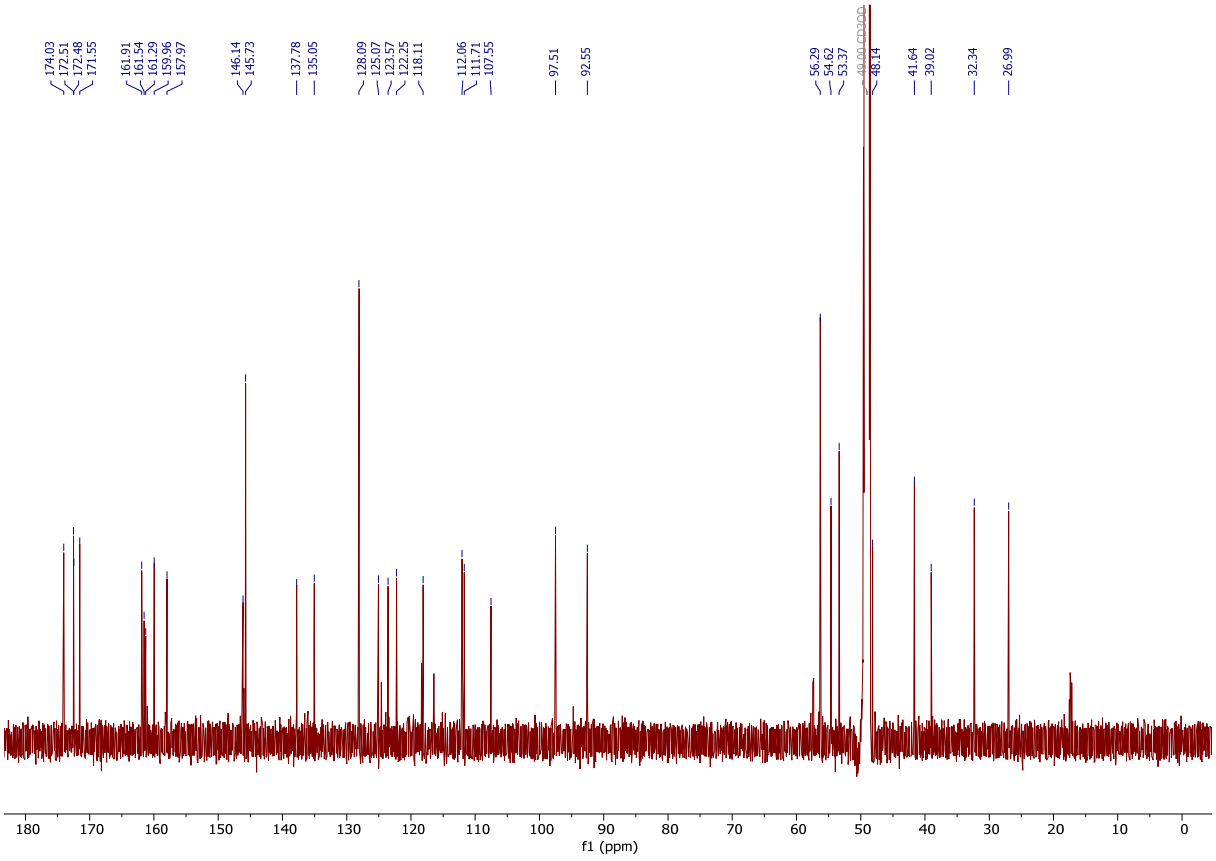


^{1}H and ^{13}C NMR (CD_3OD): **3k**

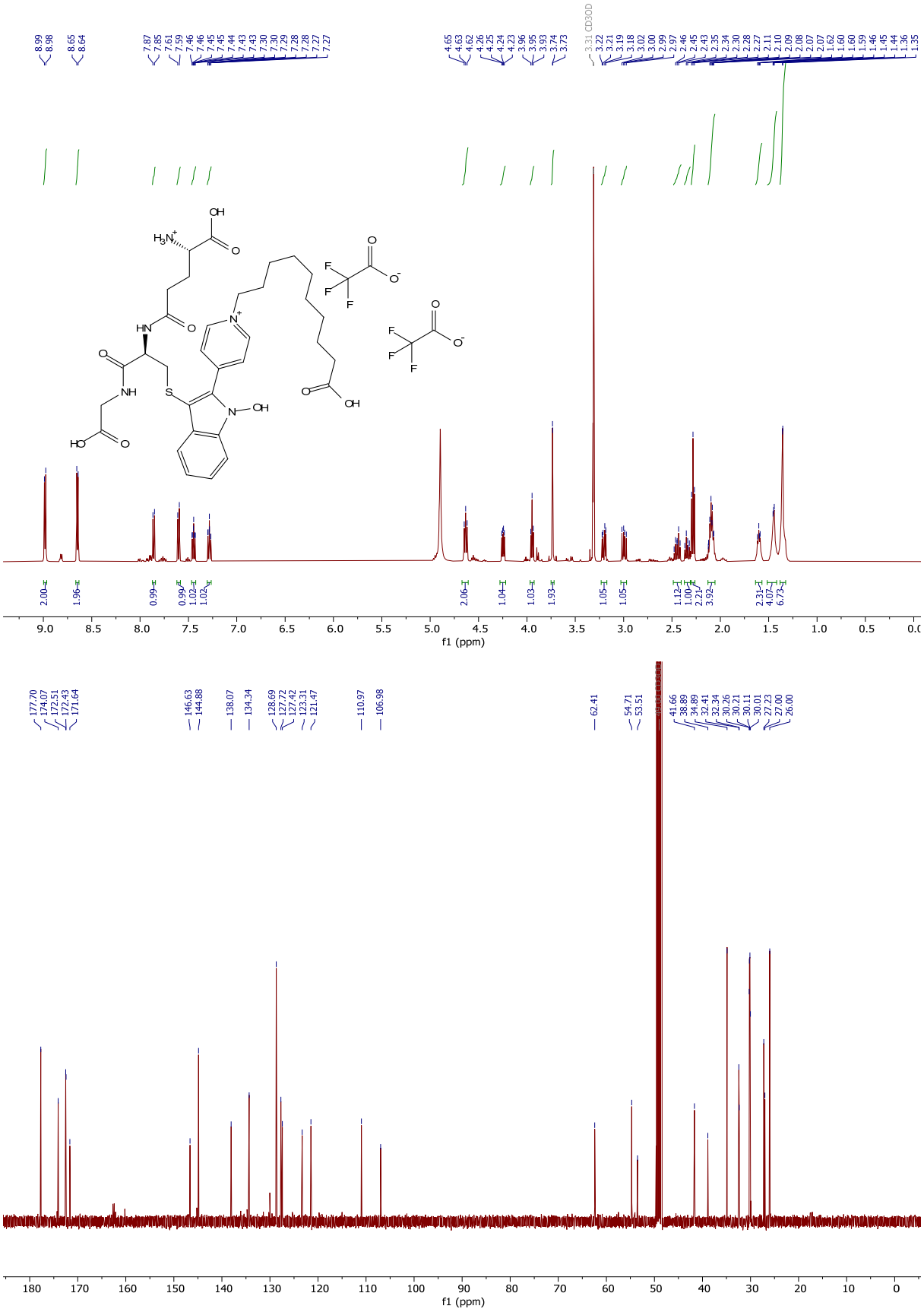


^{1}H and ^{13}C NMR (CD_3OD): **3l**

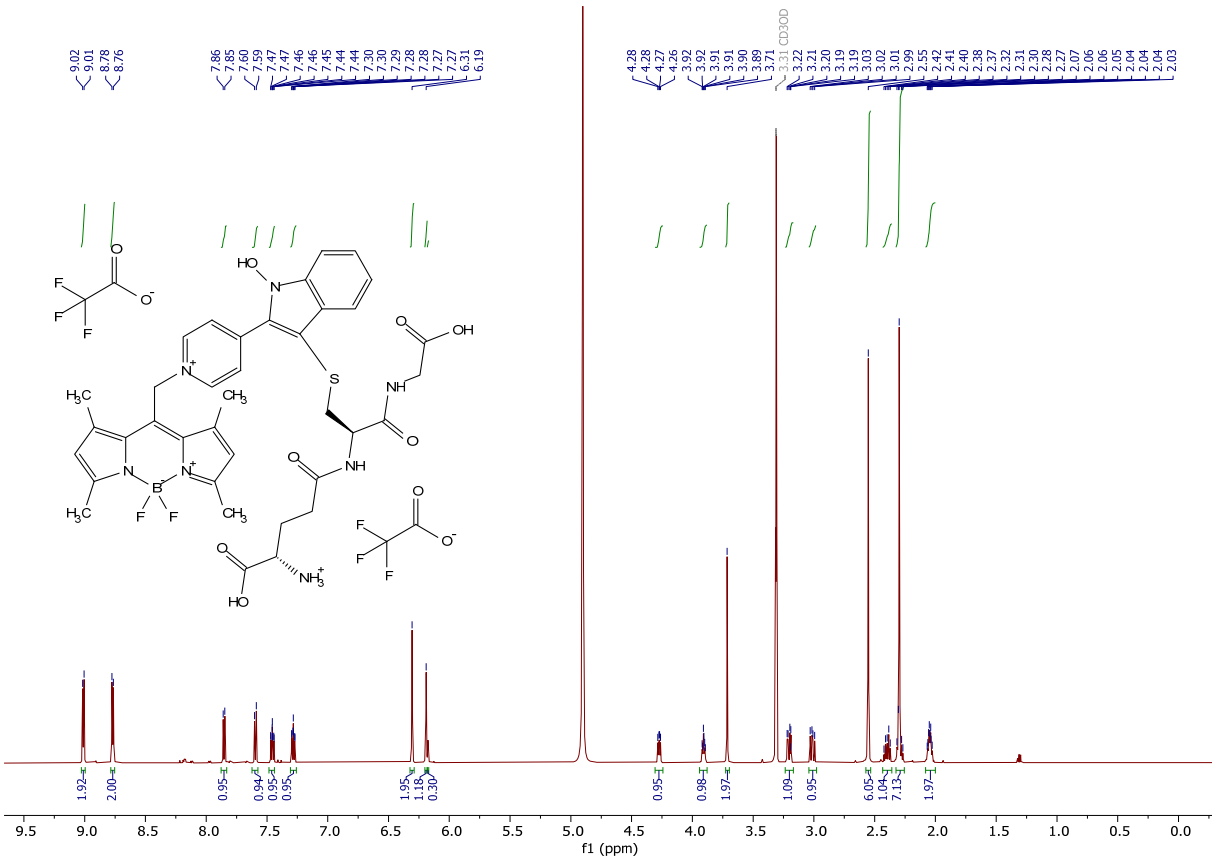


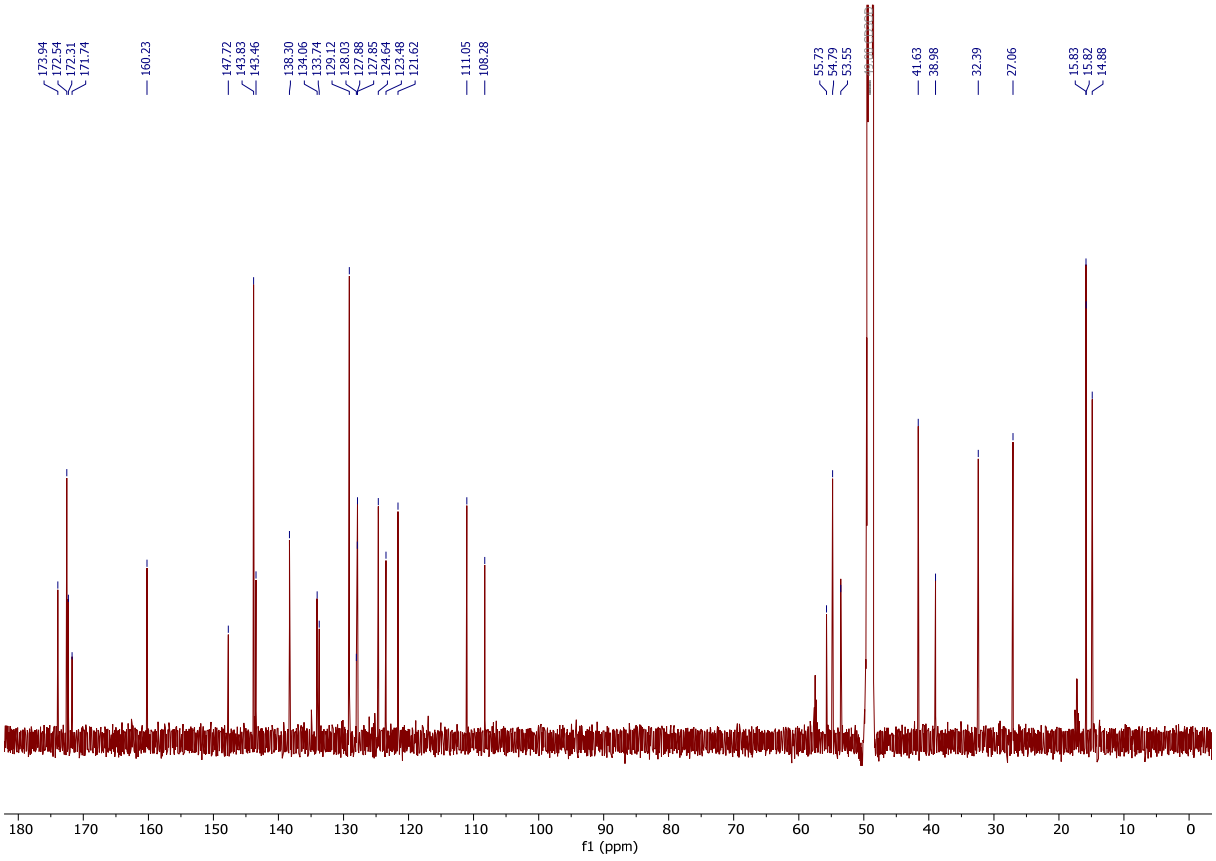


^{1}H and ^{13}C NMR (CD_3OD): **3m**

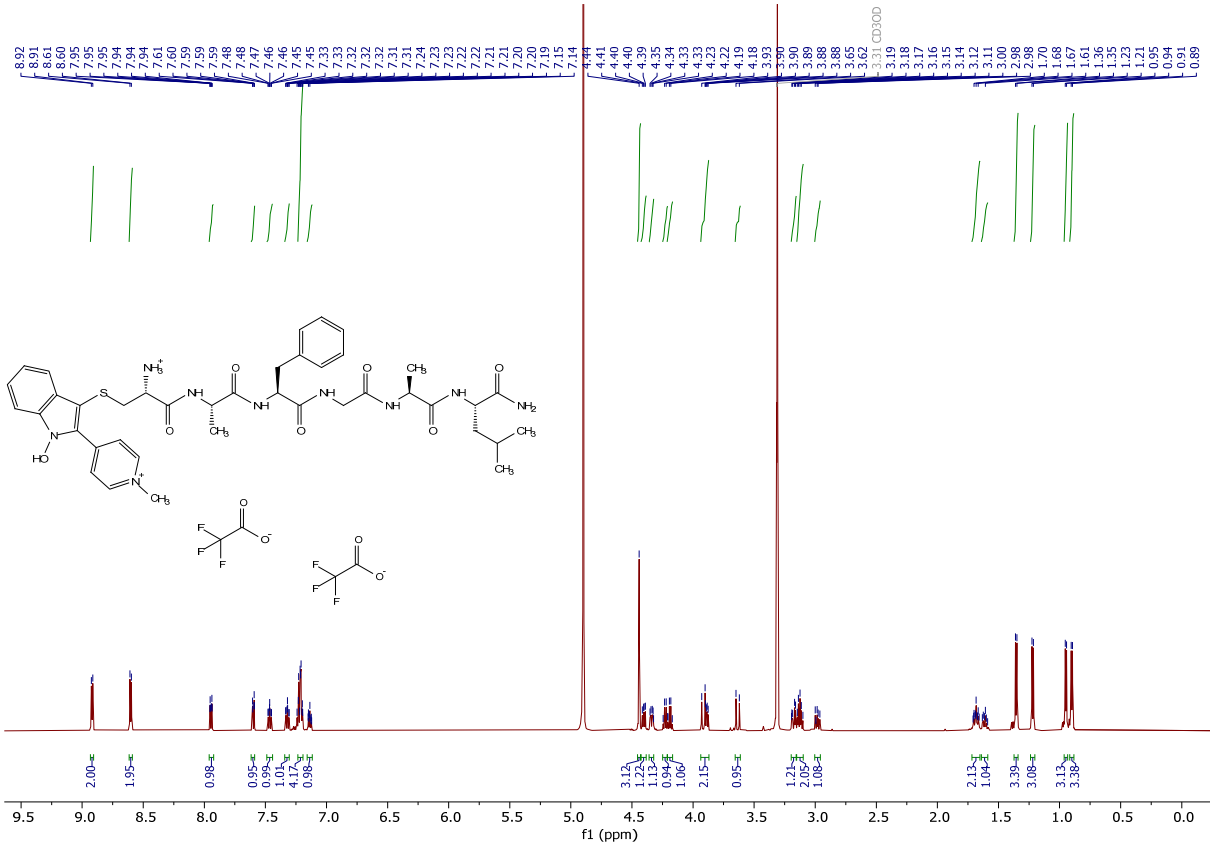


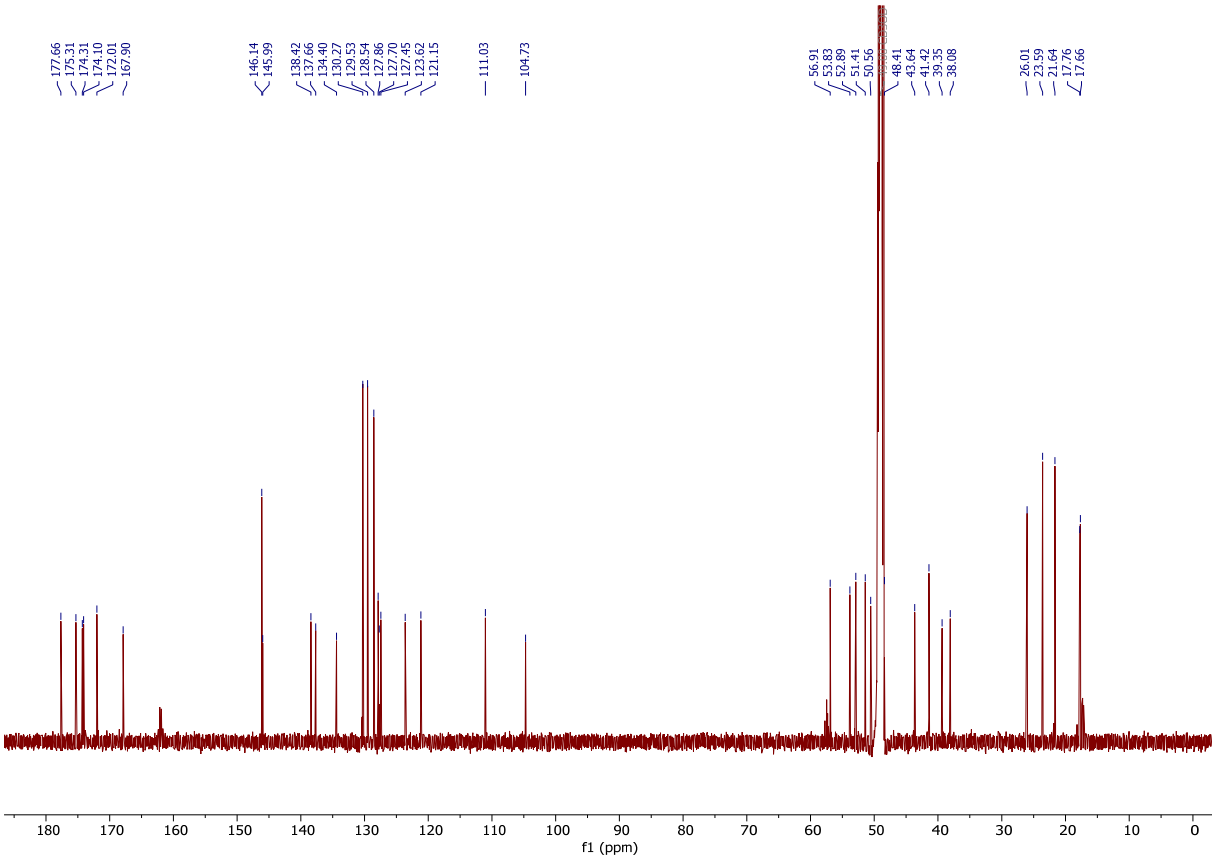
^{1}H and ^{13}C NMR (CD_3OD): **3n**



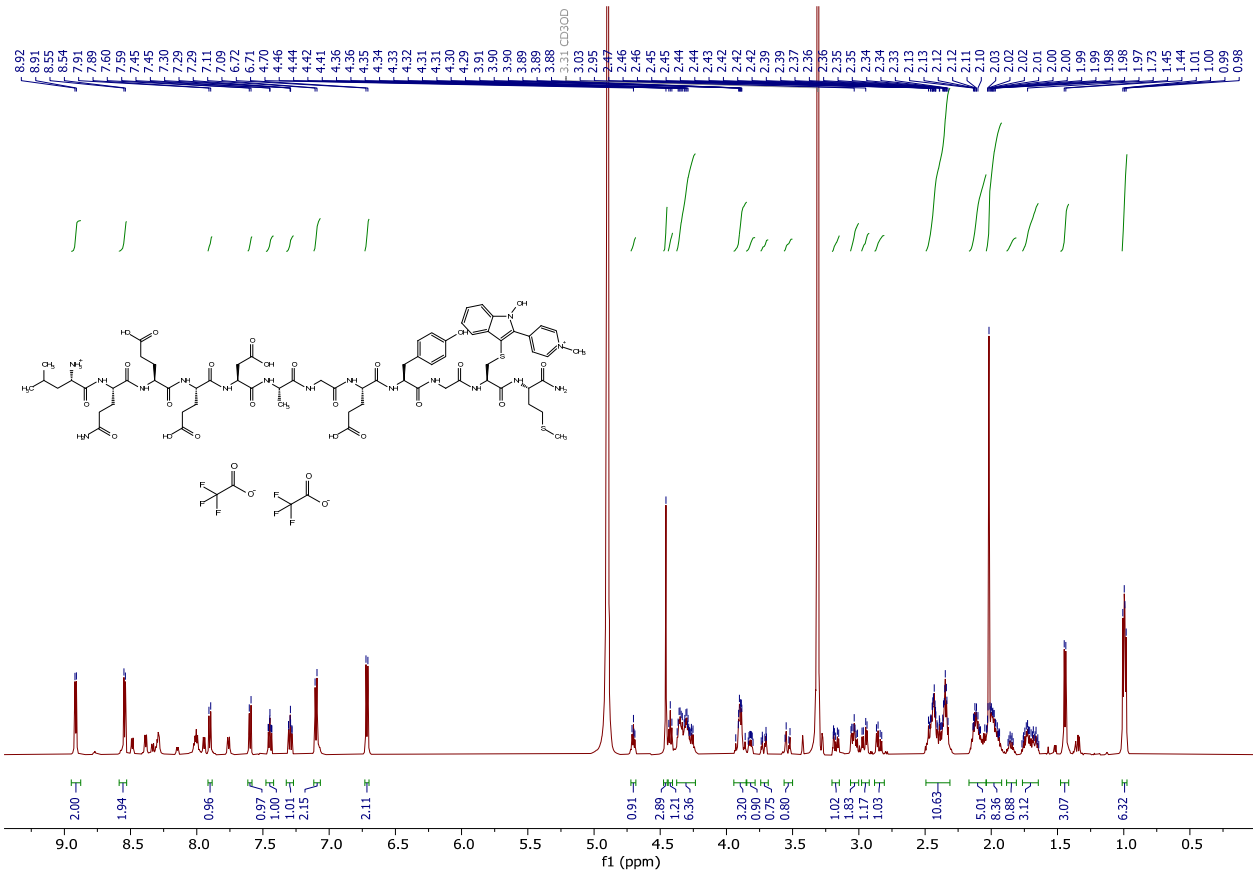


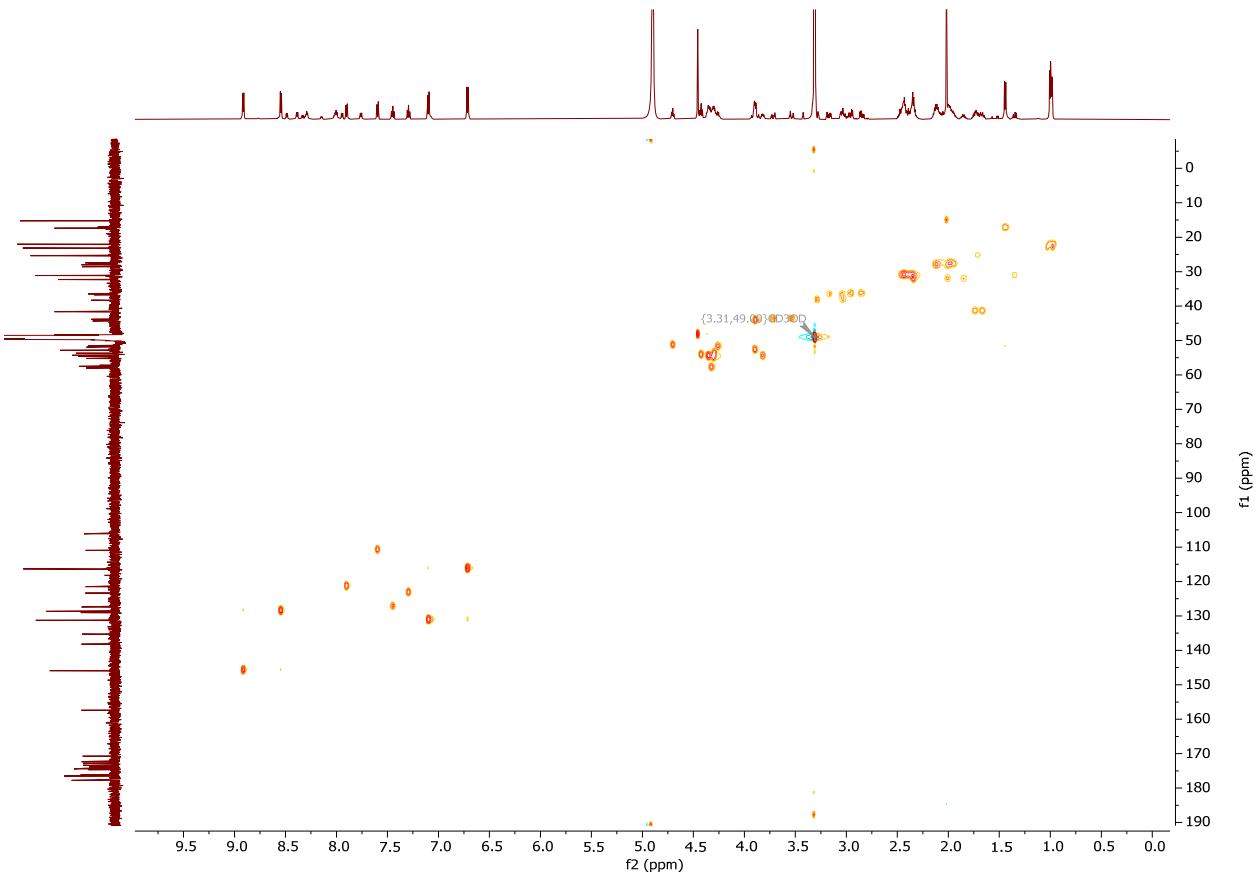
^{1}H and ^{13}C NMR (CD_3OD): **6a**

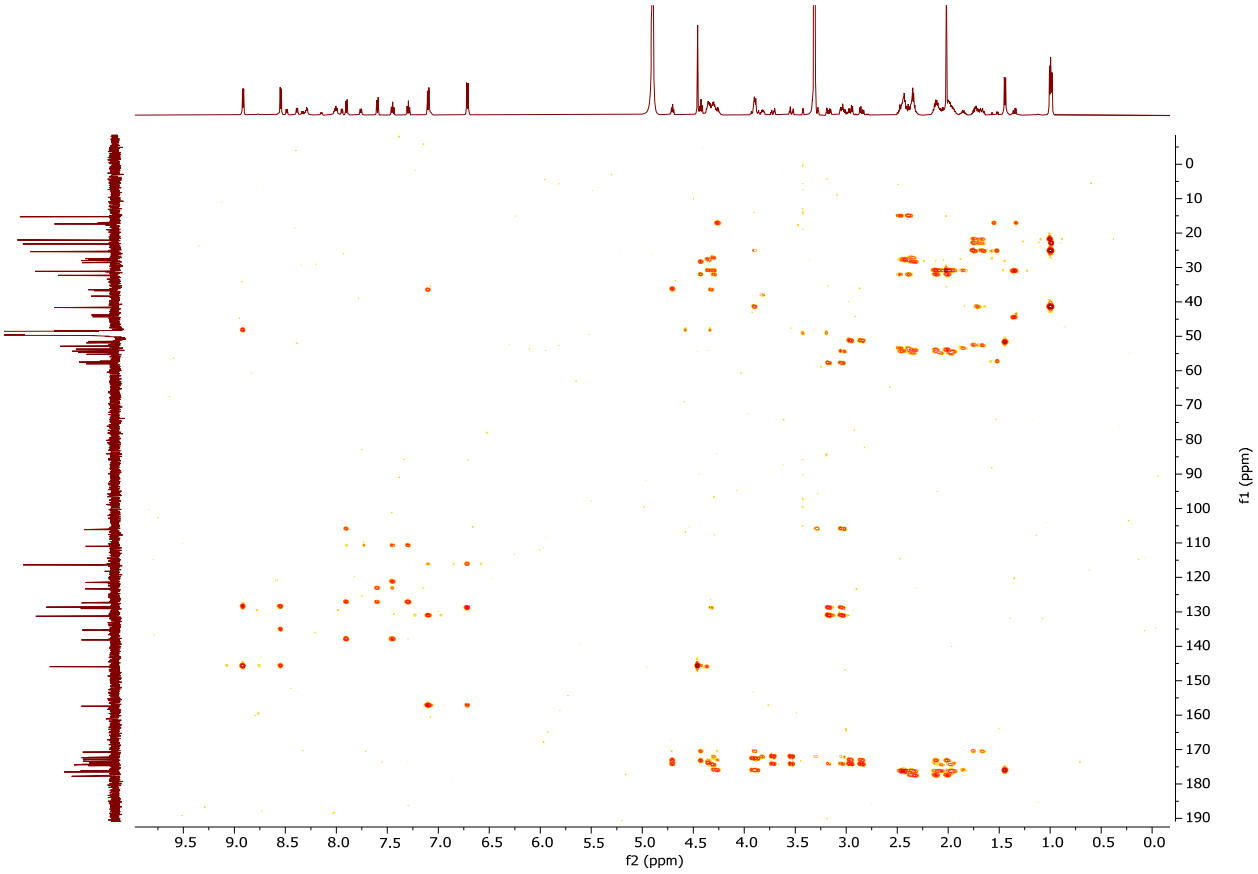


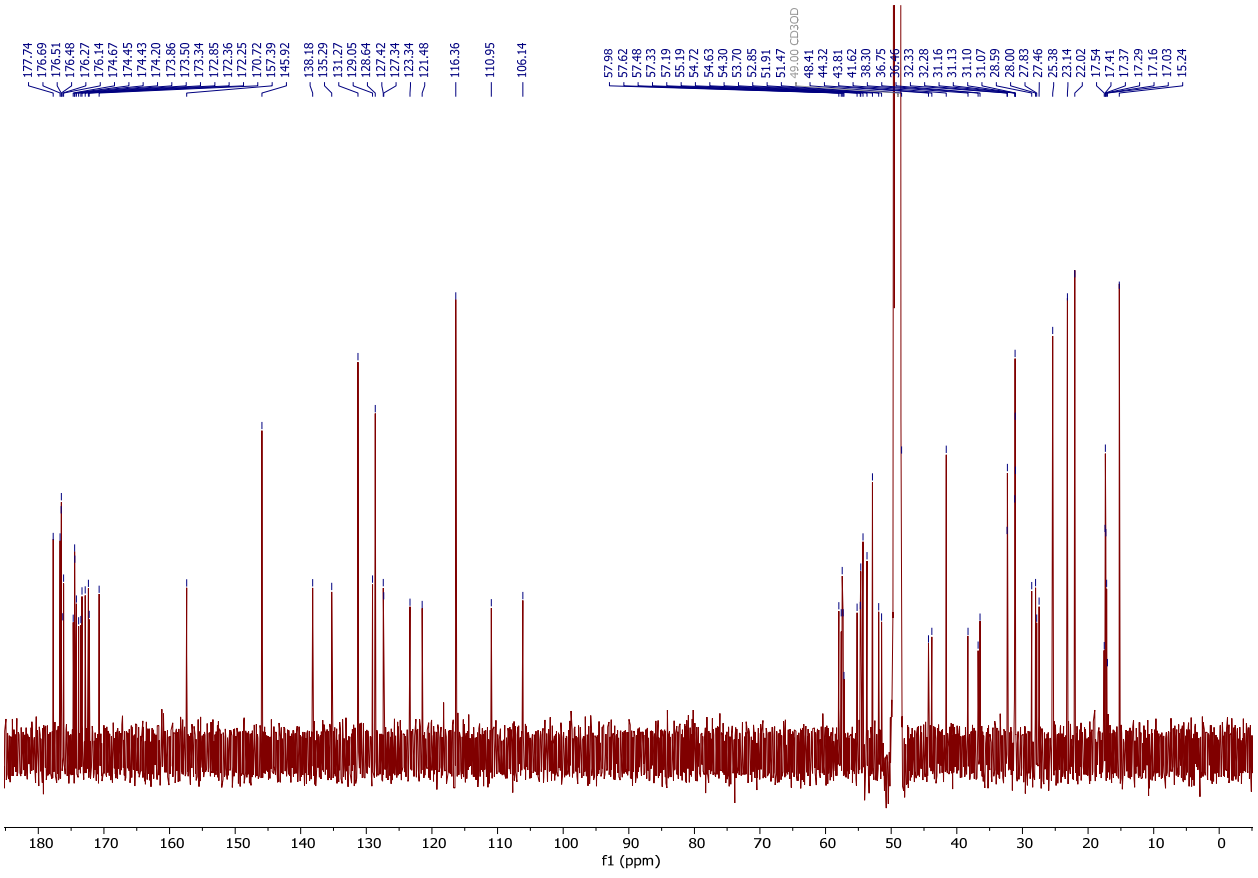


^{1}H, HSQC, HMBC and ^{13}C NMR (CD_3OD): **6b**

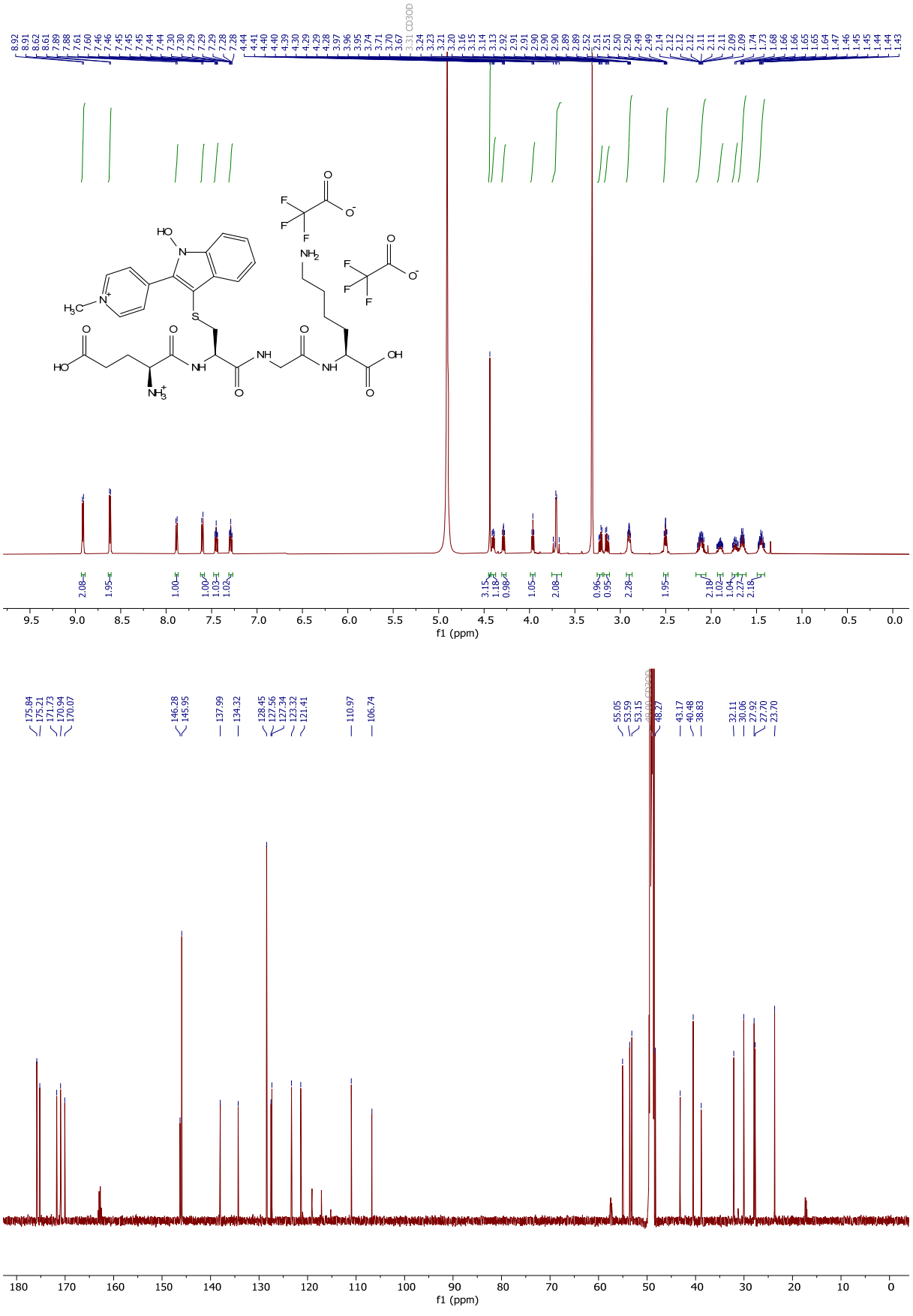




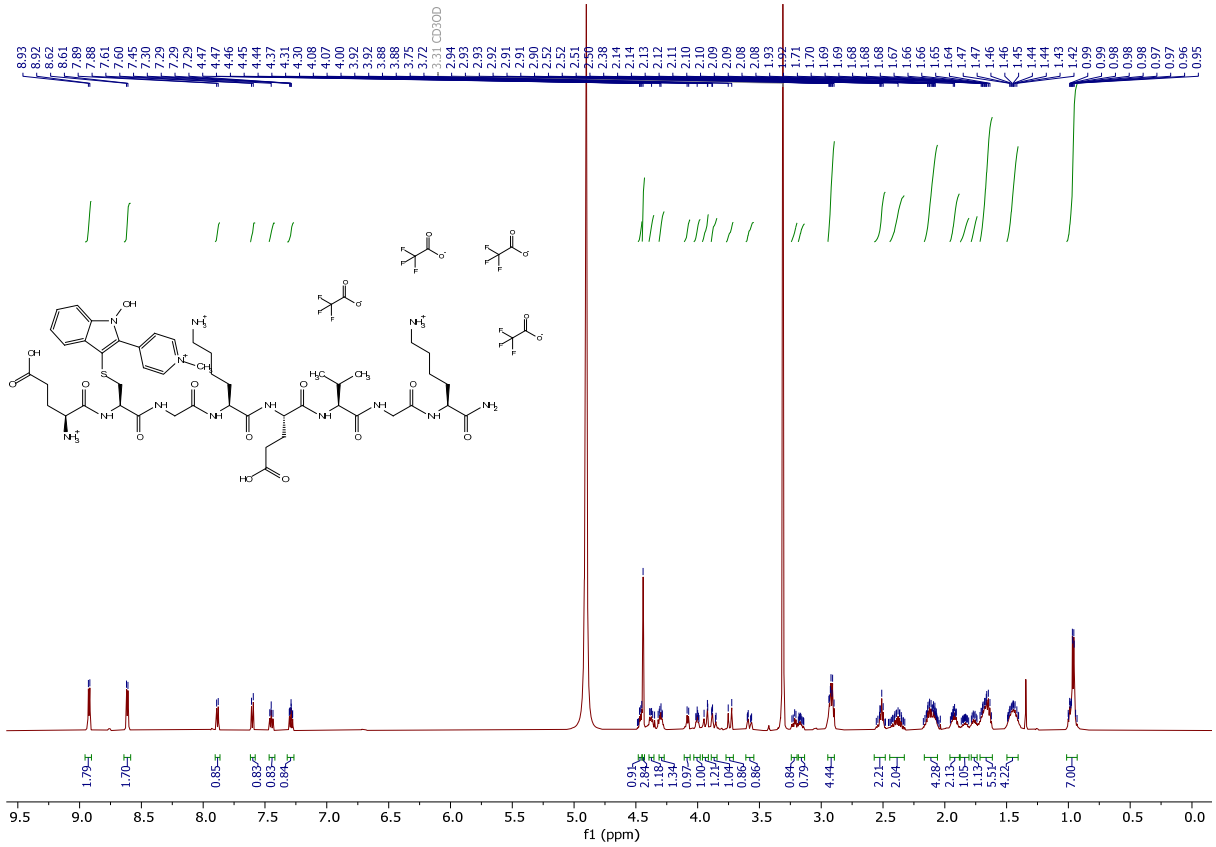

f2 (ppm)
f1 (ppm)

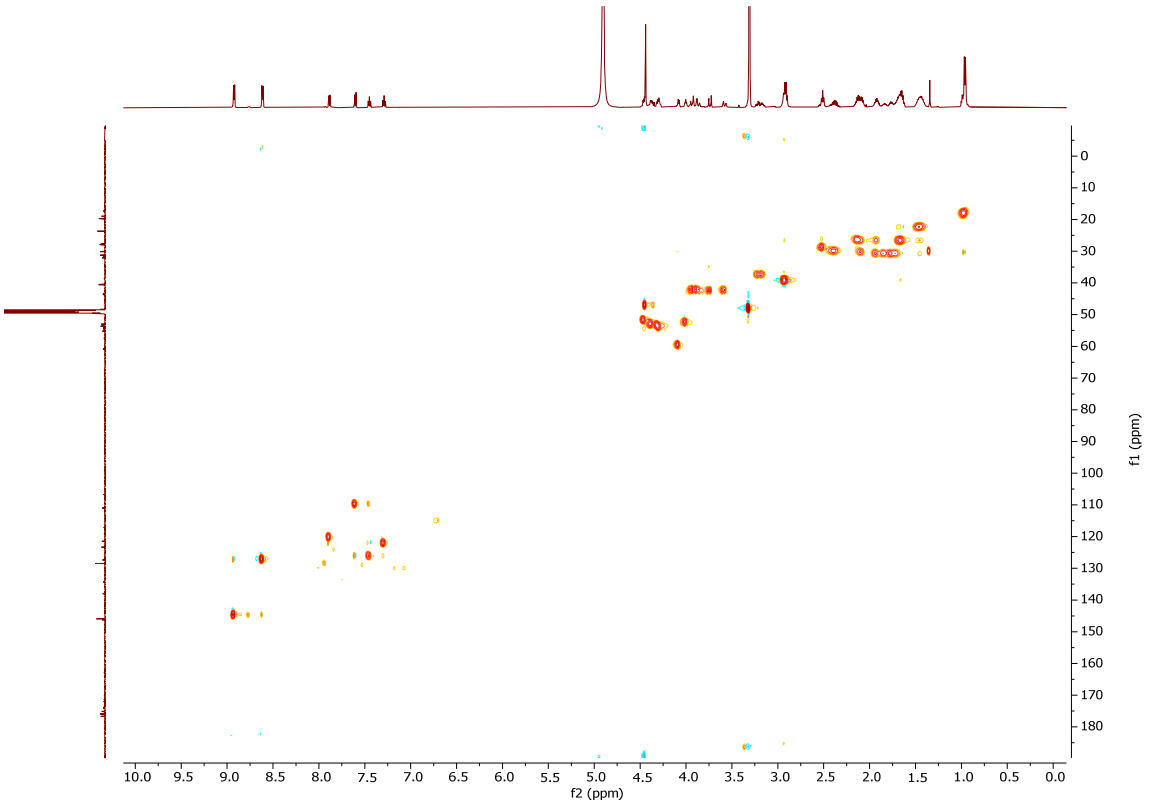

177.74
176.69
176.51
176.48
176.27
176.14
174.67
174.45
174.43
174.20
173.86
173.50
173.34
172.85
172.36
172.25
170.72
157.39
145.92
138.18
135.29
131.27
129.05
128.64
127.42
127.34
123.34
121.48
116.36
110.95
106.14
57.98
57.62
57.48
57.33
57.19
55.19
54.72
54.63
54.30
53.70
52.85
51.91
51.47
49.07
CD3OD
48.41
44.32
43.81
41.62
38.30
36.75
36.46
32.33
32.28
31.16
31.13
31.10
31.07
28.59
28.00
27.83
27.46
25.38
23.14
22.02
17.54
17.41
17.37
17.29
17.16
17.03
15.24
f1 (ppm)

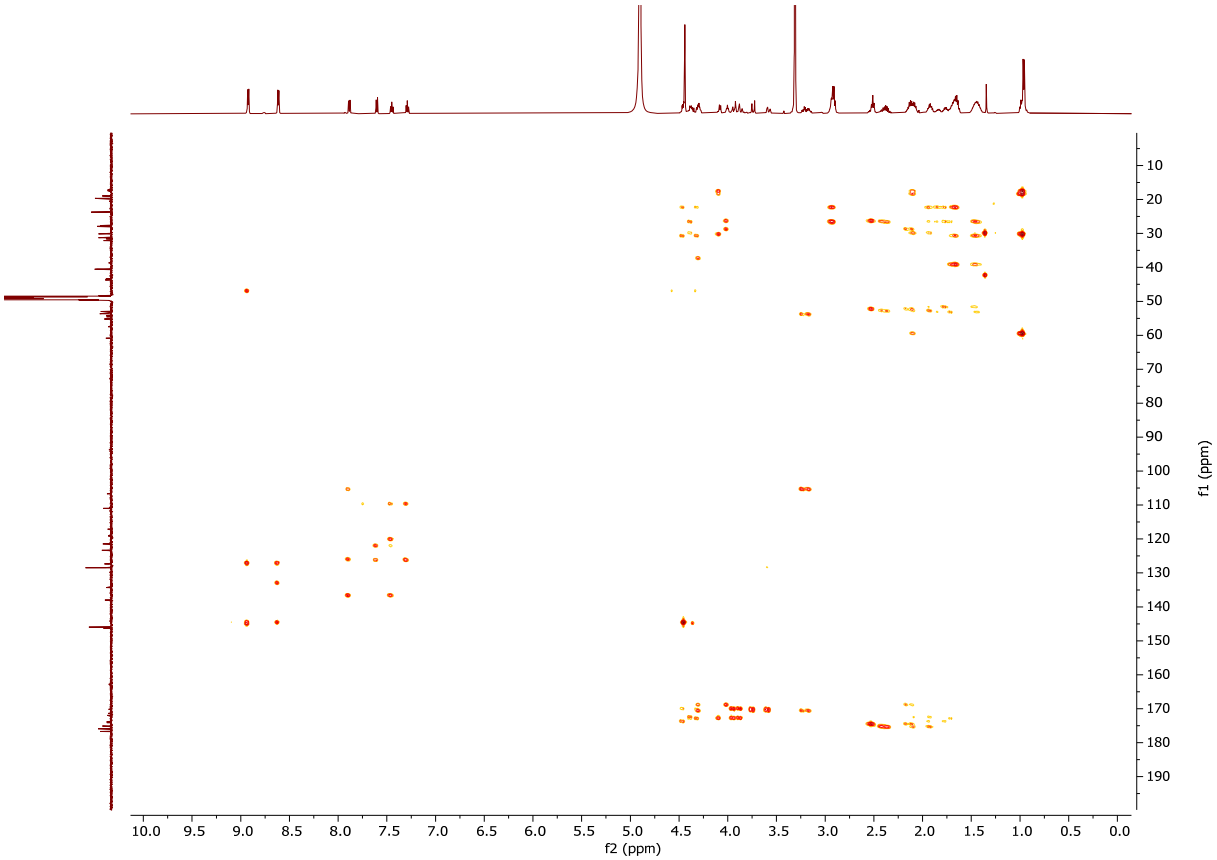
^{1}H and ^{13}C NMR (CD_3OD): **6c**

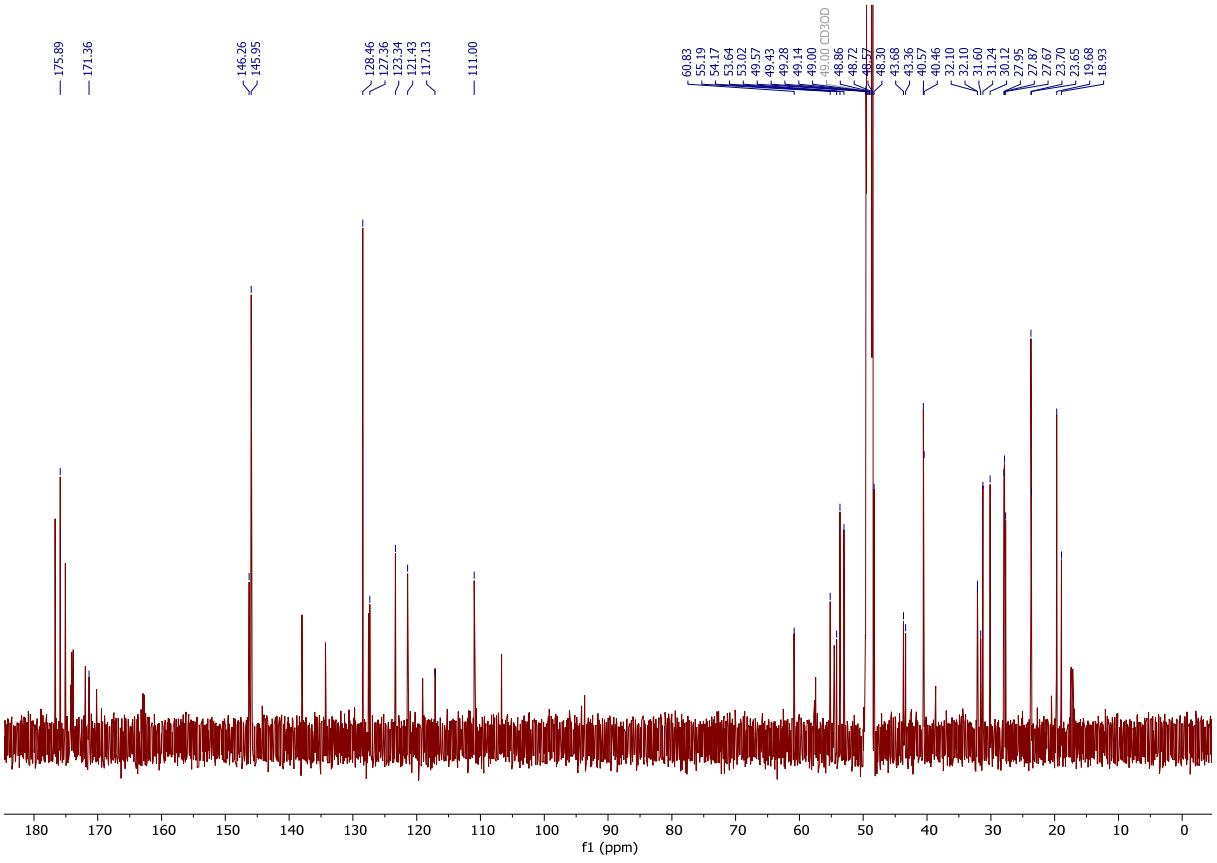


^{1}H, HSQC, HMBC and ^{13}C NMR (CD_3OD): **6d**

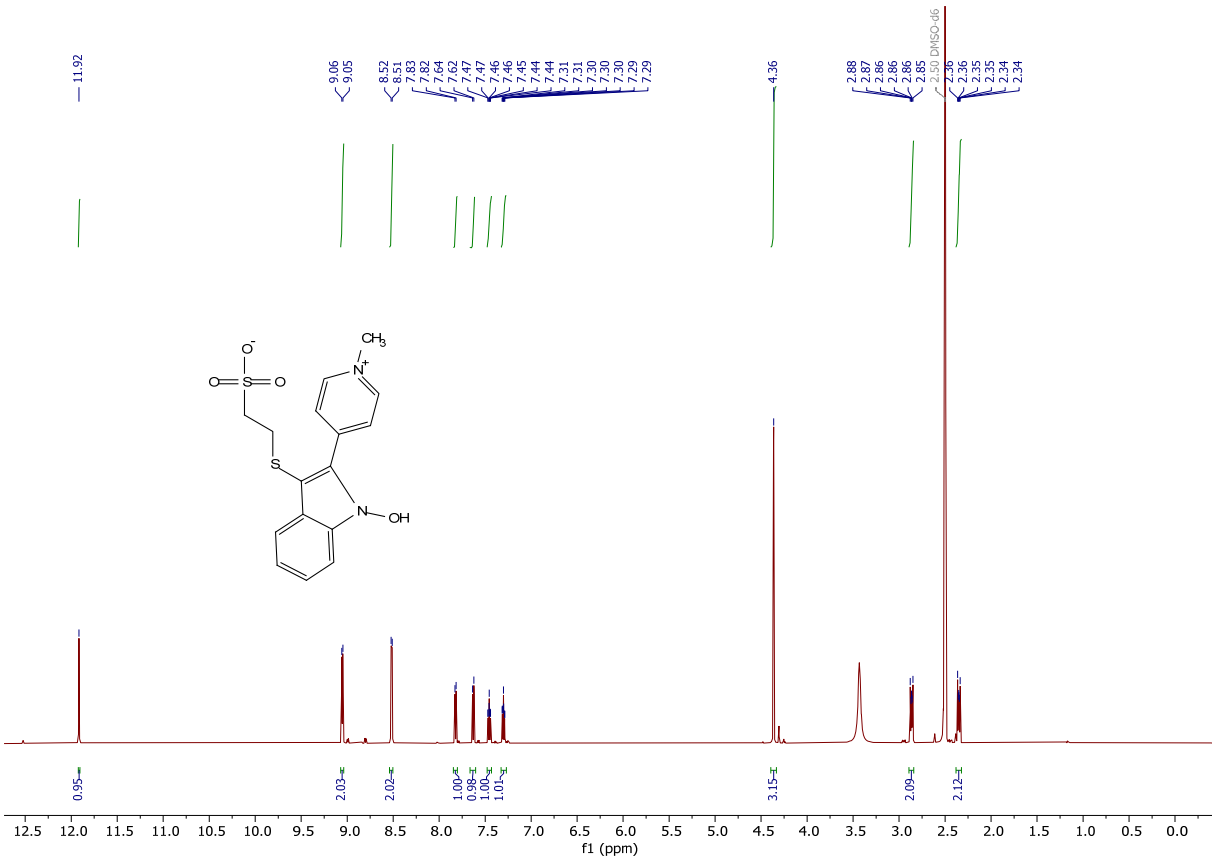


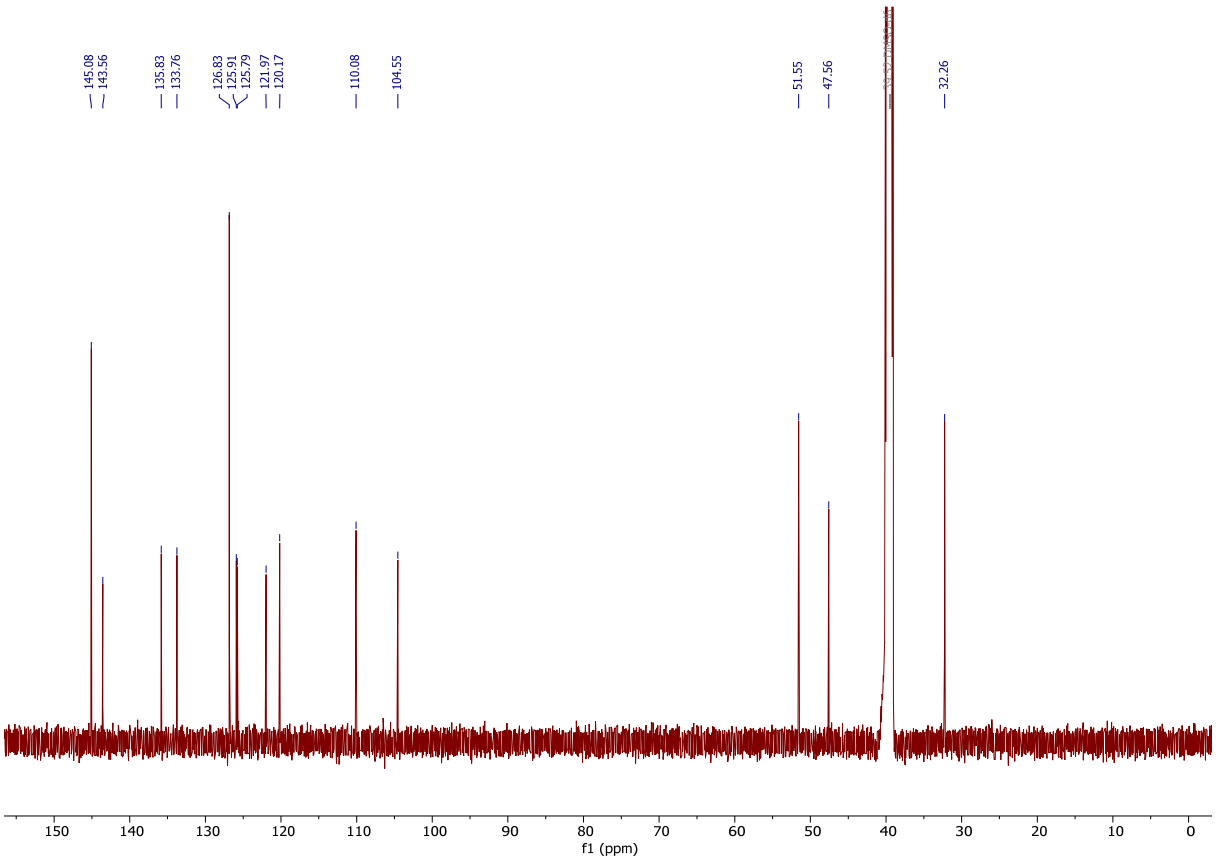



f2 (ppm)
f1 (ppm)

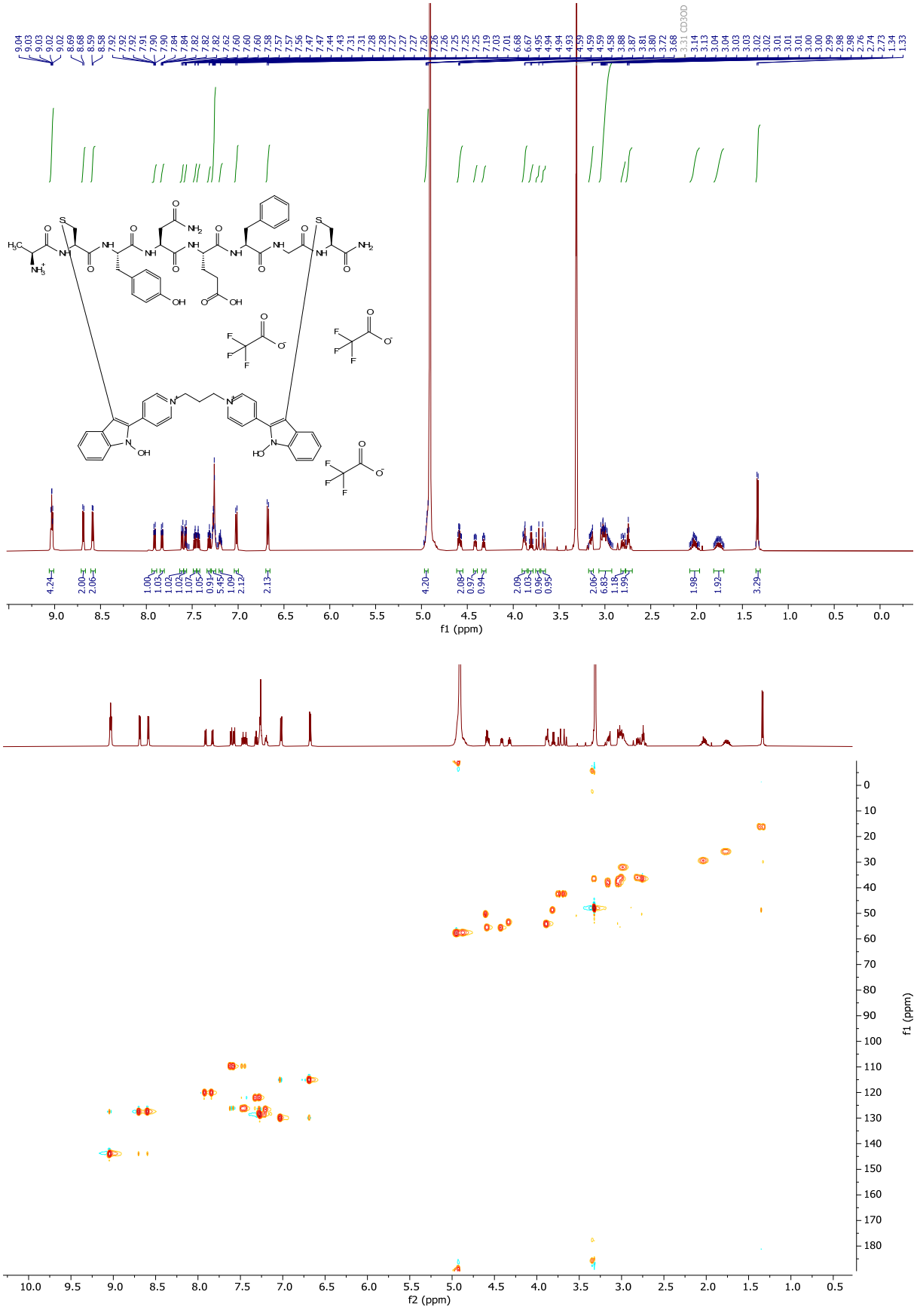

175.89
171.36
146.26
145.95
128.46
127.36
123.34
121.43
117.13
111.00
60.83
55.19
54.17
53.64
53.02
49.57
49.43
49.28
49.14
49.00
CD3OD
48.86
48.72
48.57
48.30
43.68
43.36
40.57
40.46
32.10
31.60
31.24
30.12
27.95
27.87
27.67
23.70
23.65
19.68
18.93
f1 (ppm)

^{1}H and ^{13}C NMR (DMSO-d6): **6g**



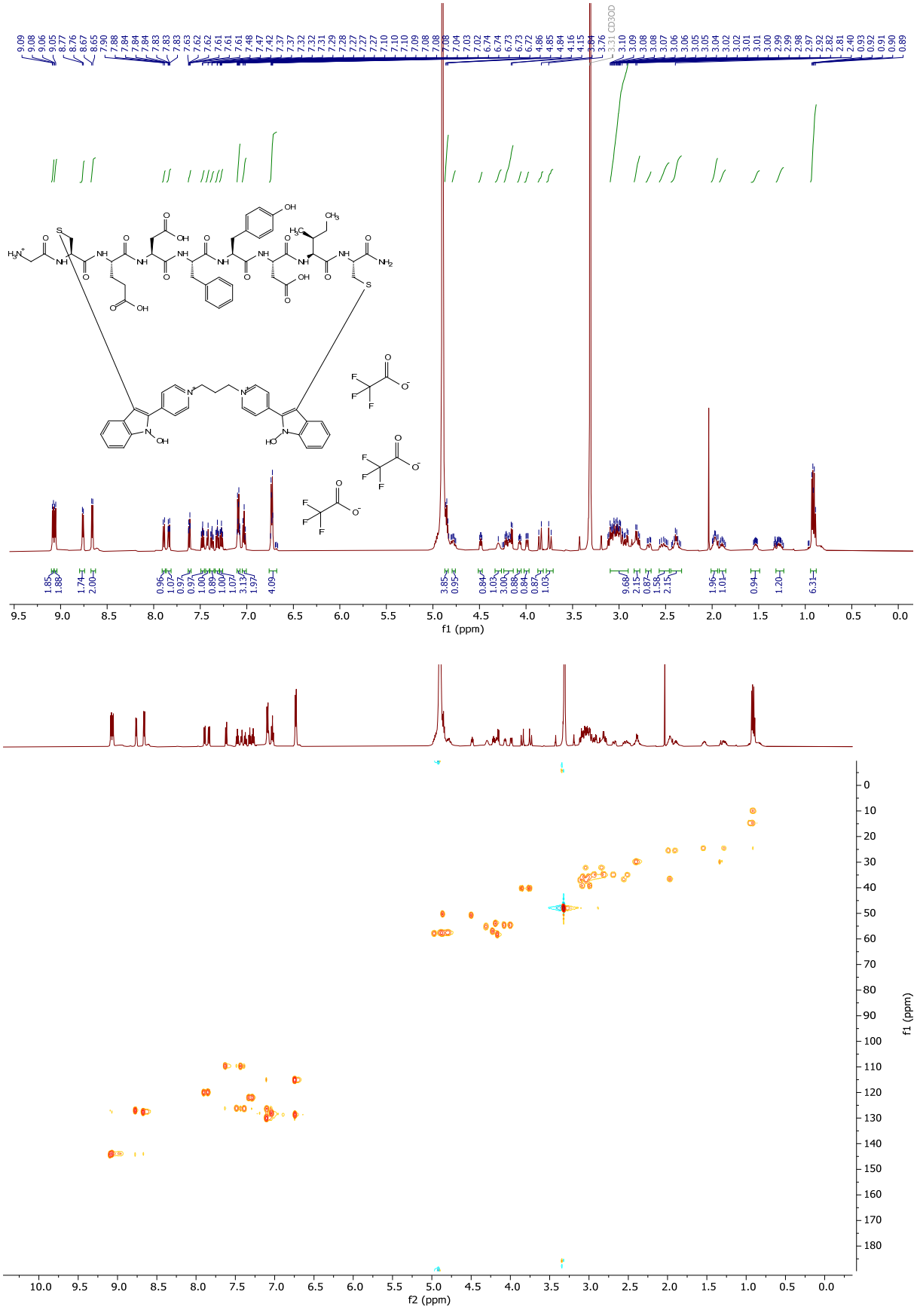


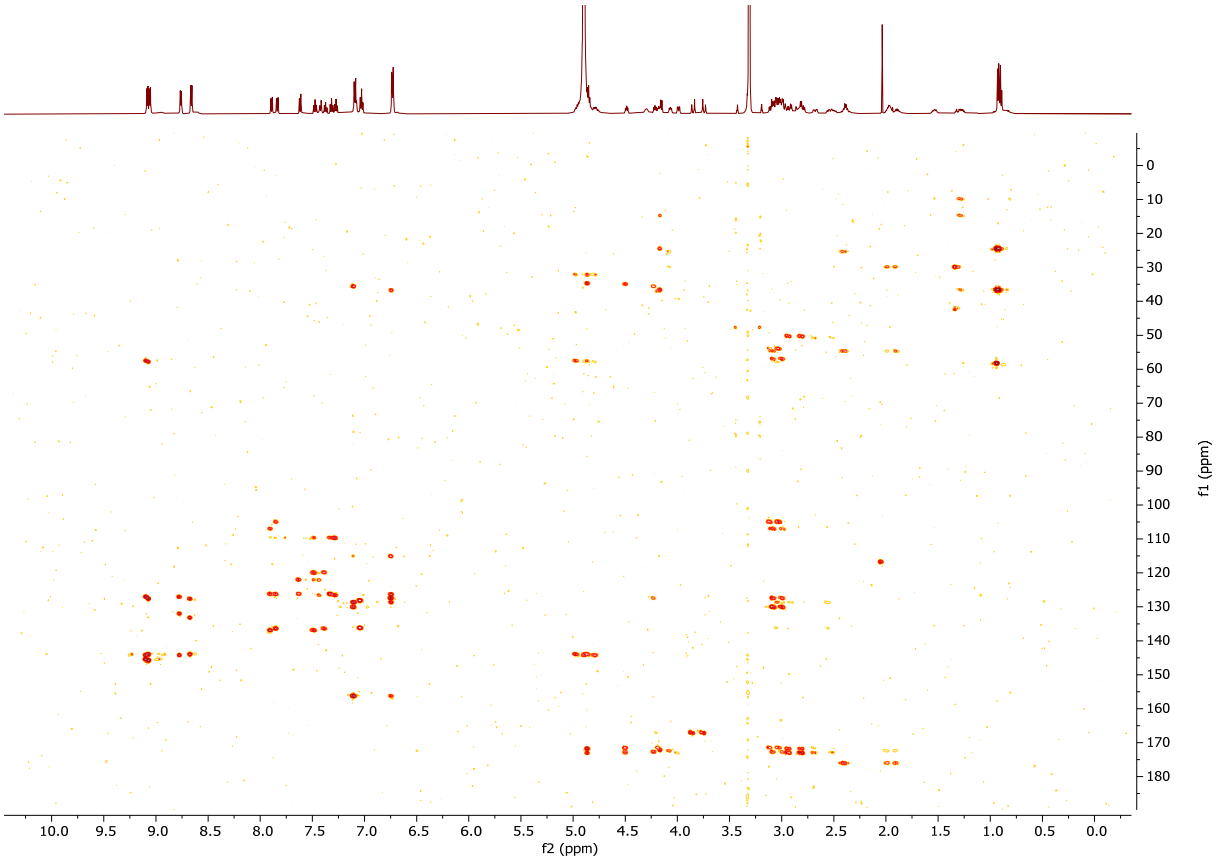
^{1}H, HSQC and HMBC (CD_3OD): **cycle1**




0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
f1 (ppm)
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
f2 (ppm)

^{1}H, HSQC and HMBC (CD_3OD): **cycle2**




0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
f1 (ppm)
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f2 (ppm)

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