Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Site-Selective Peptide Functionalisation Mediated via Vinyl-Triazine Linchpins

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Table Contents

1. General information

All solvents and reagents were used as received, purchased from commercial suppliers, and used without further purification, unless otherwise stated. Ethyl acetate, methanol, dichloromethane, and acetonitrile were distilled from calcium hydride. PE refers to petroleum ether fraction between 40 – 60 °C upon distillation.

Non-aqueous reactions were conducted under a stream of nitrogen using oven-dried glassware. Temperatures of 0 °C were maintained using an ice-water bath. Room temperature (r.t.) refers to ambient temperature.

Yields refer to spectroscopically and chromatographically pure compounds unless otherwise stated. Reactions were monitored by thin layer chromatography (TLC) or liquid chromatography mass spectroscopy (LCMS). TLC was performed using glass plates pre-coated with Merck silica gel 60 F254 and visualised by quenching of UV fluorescence (λ_{max} = 254 nm) or by staining with potassium permanganate. Retention factors (R_f) are quoted to 0.01. Flash column chromatography was carried out using slurry-packed Merck 9385 Kieselgel 60 SiO_2 (230–400 mesh) under positive pressure.

UV-LCMS was carried out using a Waters ACQUITY H-Class UPLC with an ESCi Multi-Mode Ionisation Waters SQ Detector 2 spectrometer using MassLynx 4.2 software; ESI refers to the electrospray ionisation technique; LC system: solvent A: 2 mM NH_4OAc in H_2O/MeCN (95:5); solvent B: MeCN; solvent C: 2% formic acid; column: ACQUITY UPLC® CSH C18 (2.1 mm × 50 mm, 1.7 µm, 130 Å) at 40 °C; gradient: 5 – 95 % B with constant 5 % C over 1 min at flow rate of 0.6 mL/min; detector: PDA eλ Detector 220 – 800 nm, interval 1.2 nm.

Preparative HPLC was carried out on an Agilent 1260 Infinity using a Supelcosil ABZ+PLUS column (250 mm x 21.2 mm, 5 µm) eluting with a linear gradient system with mobile phases (A) 0.1% TFA in water (v/v) and (B) 0.05% TFA in MeCN (v/v) over 20 min at a flow rate of 20 mL/min.

Analytical HPLC was carried out using an Agilent 1260 Infinity system with a reversed-phase Supelcosil™ ABZ+PLUS column (150 mm × 4.6 mm, 3 µm) eluting with a linear gradient system (solvent A: 0.05% (v/v) TFA in H_2O, solvent B: 0.05% (v/v) TFA in MeCN) over 18 minutes, at a flow rate of 1 mL/min. Analytical HPLC was monitored by UV absorbance at 254 and 280 nm.

^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker DPX-400 (400 MHz, 101 MHz), Bruker Avance 400 QNP (400 MHz, 101 MHz) and Bruker Avance 500 Cryo Ultrashield (500 MHz, 126 MHz). For the purposes of kinetic studies, peak intensities over time were determined by analysis using Bruker Topspin and Bruker Dynamics Centre. In ^{1}H NMR, chemical shifts (δ) are reported in parts per million (ppm) and the spectra are calibrated to the resonance due to incomplete deuteration of the NMR solvent ($CDCl_3$: 7.26 ppm, s; DMSO-d_6: 2.50 ppm, qn). Coupling constants (J) are reported in Hertz (Hz) to the nearest 0.1 Hz. NMR data are reported as follows: chemical shift, multiplicity (app = apparent; s =

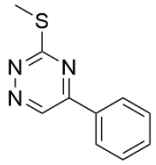
singlet; d = doublet; t = triplet; q = quartet; qn = quintet; m = multiplet; or as a combination of these, e.g. dd, dt etc.), integration, coupling constant(s) and assignment. All ^{13}C NMR spectra were recorded on the same spectrometers with complete proton decoupling. In ^{13}C NMR, chemical shifts (δ) are reported in ppm with the solvent resonance as the internal standard ($CDCl_3$: 77.16 ppm, t; DMSO-d_6: 39.52 ppm, sept). Structural assignment of resonances was performed with the help of DEPT135 and 2D NMR gradient experiments (COSY, multiplicity edited $^{1}H–^{13}C$ HSQC, $^{1}H–^{13}C$ HMBC) or by analogy to fully interpreted spectra of related compounds.

Infrared (IR) spectra were recorded neat on a Perkin-Elmer Spectrum One FT-IR spectrometer with internal referencing. Selected absorption maxima (ν_{max}) are reported in wavenumbers (cm^{-1}) with the following abbreviations: w = weak; m = medium; s = strong; br = broad.

High resolution mass spectrometry (HRMS) measurements were recorded with a Micromass Q-TOF mass spectrometer or a Waters LCT Premier Time of Flight mass spectrometer. Mass values are reported within the error limits of ±5 ppm mass units.

2. Experimental

3-(Methylthio)-5-phenyl-1,2,4-triazine (2a)

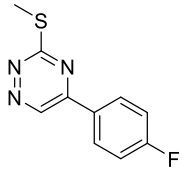


To a solution of *S*-methylthiosemicarbazide hydroiodide **1** (1 g, 4.29 mmol) in ethanol (40 mL) was added phenyl glyoxal hydrate (633 mg, 4.76 mmol) and $NaHCO_3$ (901 mg, 10.7 mmol). The reaction mixture was then stirred at reflux for 5 hours. The reaction mixture was concentrated *in vacuo* diluted with water and extracted with CH_2Cl_2 (3 × 20 mL), dried over $MgSO_4$, and concentrated *in vacuo*. Crude material was purified *via* column chromatography (SiO_2, pure PE – 1:5 EtOAc:PE) to afford **2a** as a yellow solid (550 mg, 2.71 mmol, 63% yield).

^{1}H NMR (500 MHz, $CDCl_3$): δ 9.34 (s, 1H), 8.16 – 8.13 (m, 2H), 7.61 – 7.52 (m, 3H), 2.72 (s, 3H) ppm.

All spectra were consistent with previously published data.[1]

5-(4-Fluorophenyl)-3-(methylthio)-1,2,4-triazine (2b)

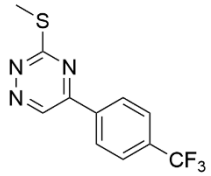


To a stirred solution of *S*-methylthiosemicarbazide hydroiodide **1** (1g, 4.29 mmol) in ethanol (40 mL) was added 2-(4-Fluorophenyl)-2-oxoacetaldehyde hydrate (760 mg, 5 mmol) and $NaHCO_3$ (1.07 g, 12.7 mmol) slowly. The reaction mixture was stirred at reflux for 4 hours. The reaction mixture was concentrated *in vacuo*, diluted with water (30 mL), and extracted with CH_2Cl_2 (3 × 20 mL), dried over $MgSO_4$, and concentrated *in vacuo*. Crude material was purified *via* column chromatography (SiO_2, 1:7 EtOAc:PE) to afford **2b** as a yellow solid (651 mg, 2.94 mmol, 69% yield).

^{1}H NMR (500 MHz, $CDCl_3$): δ 9.34 (s, 1H), 8.23 – 8.15 (m, 2H), 7.28 – 7.20 (m, 2H), 2.72 (s, 3H) ppm.

All spectra were consistent with previously published data.[2]

5-(4-(Trifluoromethyl)phenyl)-3-(methylthio)-1,2,4-triazine (2c)



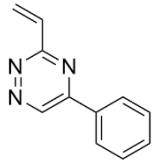
To a stirred solution of *S*-methylthiosemicarbazide hydroiodide **1** (699 mg, 3.00 mmol) in ethanol (50.0 mL) was added 4-(Trifluoromethyl) phenylglyoxal hydrate (606 mg, 3.00 mmol) and $NaHCO_3$ (756 mg, 9.00 mmol). The reaction mixture was stirred at reflux for 15 hours. The reaction mixture was concentrated *in vacuo*, diluted with water, and extracted with CH_2Cl_2 (3 × 20 mL), dried over $NaSO_4$,

and concentrated *in vacuo*. Crude material was purified *via* column chromatography (SiO_2, 1:15 EtOAc:PE) to afford **2c** as a yellow solid (440mg, 54% yield).

^{1}H NMR (400 MHz, $CDCl_3$) δ 9.41 (s, 1H), 8.27 (d, *J* = 8.2 Hz, 2H), 7.82 (d, *J* = 8.2 Hz, 2H), 2.75 (s, 3H) ppm.

All spectra were consistent with previously published data.[2]

5-Phenyl-3-vinyl-1,2,4-triazine (3a)

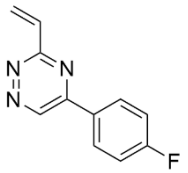


Nitrogen-flushed round-bottom flask was charged with **2a** (145 mg, 0.713 mmol) and copper(I) thiophene-2-carboxylate (408 mg, 2.14 mmol). Then was added 1,4-dioxane (10 mL) and 3,3,4,4-tetramethyl-1-vinylborolane-2,5-dione (266 µL, 1.57 mmol). Nitrogen was bubbled through the stirred reaction mixture at r.t. for 10 minutes. $Pd(PPh_3)_4$ (47 mg, 0.0406 mmol) was added to the reaction mixture, which was then stirred at 95 °C for 4 hours. The reaction mixture was concentrated *in vacuo* and then purified *via* flash chromatography (SiO_2, 1:5 EtOAc:*n*-hexane) to afford **3a** as a yellow solid (42 mg, 0.229 mmol, 32% yield).

^{1}H NMR (500 MHz, CD_2Cl_2) δ 9.54 (s, 1H), 8.26 – 8.21 (m, 2H), 7.64 – 7.55 (m, 3H), 7.14 (dd, *J* = 17.4, 10.7 Hz, 1H), 6.90 (d, *J* = 1.6 Hz, 1H), 6.86 (d, *J* = 1.6 Hz, 1H), 5.93 (dd, *J* = 10.7, 1.6 Hz, 7H) ppm.

All spectra were consistent with previously published data.[3]

5-(4-Fluorophenyl)-3-vinyl-1,2,4-triazine (3b)

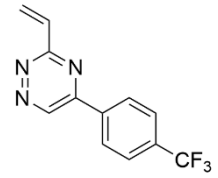


Nitrogen-flushed round-bottom flask was charged with **2b** (300 mg, 1.36 mmol) and copper(I) thiophene-2-carboxylate (776 mg, 4.07 mmol). Then was added 1,4-dioxane (5 mL) and potassium vinyltrifluoroborate (400 mg, 2.99 mmol). Nitrogen was bubbled through the stirred reaction mixture at r.t. for 10 minutes. $Pd(PPh_3)_4$ (160 mg, 0.138 mmol) was added to the reaction mixture, which was then stirred at 95 °C for 3 hours. The reaction mixture was concentrated *in vacuo* and then purified *via* flash chromatography (SiO_2, 1:5 EtOAc:*n*-hexane) to afford **3b** as a yellow solid (140 mg, 0.696 mmol, 51% yield).

^{1}H NMR (500 MHz, $CDCl_3$): δ 9.50 (s, 1H), 8.27 – 8.22 (m, 2H), 7.29 – 7.23 (m, 2H), 7.14 (dd, *J* = 17.4, 10.7 Hz, 1H), 6.89 (dd, *J* = 17.4, 1.5 Hz, 1H), 5.94 (dd, *J* = 10.6, 1.5 Hz, 1H) ppm.

All spectra were consistent with previously published data.[2]

5-(4-(Trifluoromethyl)phenyl)-3-vinyl-1,2,4-triazine (3c)

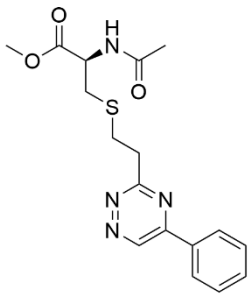


Round-bottom flask was charged with **2c** (271 mg, 1.00 mmol), potassium vinyltrifluoroborate (294 mg, 2.20 mmol), copper(I) thiophene-2-carboxylate (540 mg, 3.00 mmol) and $Pd(PPh_3)_4$ (55 mg, 5 mol%). The reaction vessel was evacuated and purged with nitrogen. Then was added degassed 1,4-dioxane (5 mL). The reaction mixture was stirred at 95 °C for 4 hours. The reaction mixture was concentrated *in vacuo* and then purified *via* flash chromatography (SiO_2, 1:8 EtOAc:PE) to afford **3c** as a yellow solid (151 mg, 0.601 mmol, 60 % yield).

^{1}H NMR (500 MHz, $CDCl_3$): δ 9.57 (s, 1H), 8.33 (d, *J* = 8.1 Hz, 2H), 7.84 (d, *J* = 8.1 Hz, 2H), 7.17 (dd, *J* = 17.4, 10.7 Hz, 1H), 6.92 (dd, *J* = 17.4, 1.4 Hz, 1H), 5.98 (dd, *J* = 10.6, 1.4 Hz, 1H) ppm.

All spectra were consistent with previously published data.[2]

Methyl *N*-acetyl-*S*-(2-(5-phenyl-1,2,4-triazin-3-yl)ethyl)-L-cysteinate (4a)



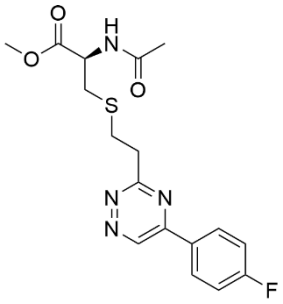
To a solution of **3a** (20 mg, 0.109 mmol) in 3:7 MeCN:NaP_i (50 mM, pH 8; 11mL) was added *N*-acetyl cysteine methyl ester (38 mg, 0.220 mmol). The reaction mixture was stirred at 37 °C for 5 hours. The reaction mixture was concentrated *in vacuo*. The remaining aqueous was extracted with CH_2Cl_2. The organics were combined, dried over $MgSO_4$, concentrated *in vacuo* and the crude was purified *via* C18 chromotography (0–100% MeCN in H_2O) to afford **4a** as a yellow solid (23 mg, 65 µmol, 59% yield).

^{1}H NMR (500 MHz, $CDCl_3$) δ 9.58 (s, 1H), 8.21 – 8.15 (m, 2H), 7.65 – 7.54 (m, 3H), 6.63 (d, *J* = 7.1 Hz, 1H), 4.90 (dt, *J* = 7.8, 4.9 Hz, 1H), 3.75 (s, 3H), 3.45 (app t, *J* = 7.2 Hz, 2H), 3.17 (app t, *J* = 7.6 Hz, 1H), 3.13 (dd, *J* = 14.1, 5.2 Hz, 1H), 3.06 (dd, *J* = 13.9, 4.7 Hz, 1H), 2.07 (s, 3H) ppm.

^{13}C NMR {H} (126 MHz, $CDCl_3$) δ 171.3, 170.1, 167.7, 155.5, 144.5, 133.4, 132.7, 129.5, 127.7, 52.7, 52.0, 37.0, 34.3, 30.6, 23.2 ppm.

HRMS (ESI) calcd. for $C_{17}H_{21}N_4O_3S$ *m/z* 361.1334 $[M+H]^+$, found 361.1340.

Methyl *N*-acetyl-*S*-(2-(5-(4-fluorophenyl)-1,2,4-triazin-3-yl)ethyl)-L-cysteinate (4b)



To a solution of **3b** (20 mg, 0.99 mmol) in 3:7 MeCN:NaP_i (50 mM, pH 8; 10 mL) was added *N*-acetyl cysteine methyl ester (35 mg, 1.98 mmol). The reaction mixture was stirred at 37 °C for 6 hours. The reaction mixture was concentrated *in vacuo*. The remaining aqueous was extracted with CH_2Cl_2 (3 × 10 mL). The organics were dried ($MgSO_4$), concentrated *in vacuo* and the crude was purified *via* C18 chromotography (0–100% MeCN in H_2O) to afford **4b** as a yellow solid (29 mg, 78 µmol, 79% yield).

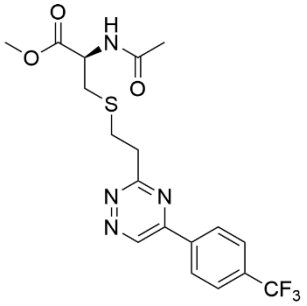
^{1}H NMR (500 MHz, $CDCl_3$) δ 9.55 (s, 1H), 8.30 – 8.11 (m, 2H), 7.26 (t, *J* = 8.5 Hz, 2H), 6.63 (d, *J* = 7.2 Hz, 1H), 4.89 (dt, *J* = 7.8, 4.9 Hz, 1H), 3.75 (s, 3H), 3.44 (app t, *J* = 7.2 Hz, 2H), 3.16 (app t, *J* = 7.3 Hz, 2H), 3.12 (dd, *J* = 13.9, 5.1 Hz, 1H), 3.06 (dd, *J* = 13.9, 4.8 Hz, 1H), 2.07 (s, 3H).

^{13}C NMR {H} (101 MHz, $CDCl_3$) δ 171.4, 170.1, 167.7, 165.7 (d, $J_{C\text{-}F}$ = 254.7 Hz), 154.4, 144.2, 130.1 (d, $J_{C\text{-}F}$ = 9.1 Hz), 129.6 (d, $J_{C\text{-}F}$ = 3.2 Hz), 116.8 (d, $J_{C\text{-}F}$ = 22.1 Hz), 52.8, 52.0, 37.1, 34.4, 30.6, 23.2 ppm.

^{19}F NMR {H} (376 MHz, $CDCl_3$) δ -106.86 ppm.

HRMS (ESI) calcd. for $C_{17}H_{20}FN_4O_3S$ *m/z* 379.1240 $[M+H]^+$, found 379.1250.

Methyl *N*-acetyl-*S*-(2-(5-(4-fluorophenyl)-1,2,4-triazin-3-yl)ethyl)-L-cysteinate (4c)



To a solution of **3c** (105 mg, 417 µmol) in 1:1 MeCN:NaP_i (50 mM, pH 8; 4 mL) was added *N*-acetyl cysteine methyl ester (148 mg, 836 µmol). The reaction mixture was stirred at 37 °C for 1.5 hours. The reaction mixture was concentrated *in vacuo.* The remaining aqueous was extracted with CH_2Cl_2 (3 x 10 mL). The organics were combined, dried over $MgSO_4$, concentrated *in vacuo* and the crude purified *via* flash chromatography (SiO_2, 0 – 60% EtOAc:PE) to afford **4c** as a yellow solid (120 mg, 280 µmol, 67% yield).

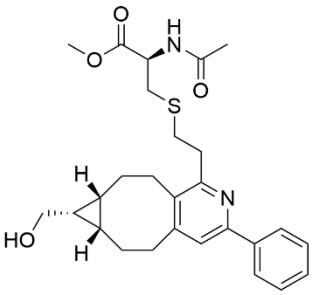
^{1}H NMR ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.62 (s, 1H), 8.30 (d, *J* = 8.2 Hz, 2H), 7.84 (d, *J* = 8.3 Hz, 2H), 6.56 (d, *J* = 7.3 Hz, 1H), 4.89 (dt, *J* = 7.7, 4.9 Hz, 1H), 3.75 (s, 3H), 3.49 (app t, *J* = 7.2 Hz, 2H), 3.18 (app t, *J* = 7.1 Hz, 2H), 3.12 (dd, *J* = 14.0, 5.0 Hz, 1H), 3.06 (dd, *J* = 13.9, 4.9 Hz, 1H), 2.07 (s, 3H) ppm.

^{13}C NMR {H} (101 MHz, $CDCl_3$) δ 171.4, 170.1, 154.1, 144.6, 137.0, 134.4 (app t, $J_{C\text{-}F}$ = 32.8 Hz), 128.2, 126.5 (q, J = 3.7 Hz), 125.1, 52.9, 52.1, 37.1, 34.5, 30.6, 23.3 ppm.

^{19}F NMR {H} (376 MHz, $CDCl_3$) δ -63.11 ppm.

HRMS (ESI) calcd. for $C_{17}H_{20}F_3N_4O_3S$ *m/z* 429.1233 $[M+H]^+$, found 429.1208.

BCN-triazine conjugate (5a)



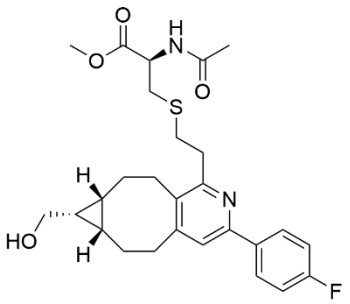
To a solution of **4a** (20 mg, 55.5 µmol) in NaP_i (50 mM, pH 8) and MeCN (1:1, 0.3 mL) was added ((1*R*,8*S*,9*S*)-bicyclo[6.1.0]non-4-yn-9-yl)methanol **6** (16 mg, 111 µmol). The reaction mixture was stirred at 37°C for 3 hours. The reaction mixture was concentrated *in vacuo* to remove MeCN. The resultant aqueous was extracted with CH_2Cl_2 and the organics combined, dried over $MgSO_4$, and concentrated *in vacuo*. The crude was purified *via* C18 chromatography (25–75% MeCN in H_2O) to afford **5a** as a clear gum (18 mg, 37.3 µmol, 67% yield).

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.99 (dt, J = 8.2, 1.7 Hz, 2H), 7.47 – 7.40 (m, 2H), 7.39 – 7.33 (m, 2H), 6.54 (s, 1H), 4.86 (dtd, J = 7.7, 5.1, 2.5 Hz, 1H), 3.81 – 3.61 (m, 5H), 3.24 – 2.90 (m, 8H), 2.89 – 2.75 (m, 2H), 2.41 – 2.30 (m, 1H), 2.30 – 2.18 (m, 1H), 1.99 (app d, J = 3.4 Hz, 3H), 1.94 – 1.75 (m, 1H), 1.70 – 1.57 (m, 1H), 1.57 – 1.38 (m, 2H), 1.17 – 1.00 (m, 1H), 1.02 – 0.75 (m, 2H) ppm.

^{13}C NMR {H} (126 MHz, $CDCl_3$) δ 171.5, 170.1, 170.1, 156.7, 153.9, 152.2, 139.5, 133.9, 128.7, 128.6, 126.8, 120.8, 59.6, 52.8, 52.8, 52.2, 52.2, 35.2, 34.8, 34.7, 34.3, 32.2, 26.4, 23.2, 23.2 ppm.

HRMS (ESI) calcd. for $C_{27}H_{34}N_2O_4S$ *m/z* 482.2239 $[M+H]^+$, found 482.2247.

BCN-triazine conjugate (5b)



To a solution of **4b** (31mg, 81.9 µmol) in NaP_i (50 mM, pH 8) and MeCN (1:1, 1 mL) was added ((1*R*,8*S*,9*S*)-bicyclo[6.1.0]non-4-yn-9-yl)methanol **6** (14.8 mg, 98.5 µmol). The reaction mixture was stirred at 37°C for 2 hours. The reaction mixture was concentrated *in vacuo* to remove the MeCN. The resultant aqueous was extracted with CH_2Cl_2 and the organics combined, dried ($MgSO_4$) and

concentrated *in vacuo*. The crude was purified *via* C18 chromatography (25–75% MeCN in H_2O) to afford **5b** as a clear gum (29 mg, 57.9 µmol, 71% yield).

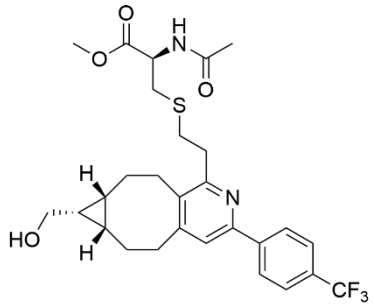
^{1}H NMR (500 MHz, $CDCl_3$) δ 7.98 (dd, *J* = 8.5, 5.6 Hz, 2H), 7.30 (s, 1H), 7.11 (t, *J* = 8.6 Hz, 2H), 6.51 (d, *J* = 4.2 Hz, 1H), 4.85 (dt, *J* = 9.9, 3.7 Hz, 1H), 3.76 – 3.54 (m, 5H), 3.21 – 2.90 (m, 8H), 2.82 (dt, *J* = 14.8, 7.8 Hz, 2H), 2.28 (ddd, *J* = 14.5, 12.3, 4.9 Hz, 2H), 1.99 (d, *J* = 2.8 Hz, 2H), 1.75 – 1.58 (m, 1H), 1.56 – 1.42 (m, 2H), 1.20 – 1.01 (m, 1H), 0.99 – 0.67 (m, 2H) ppm.

^{13}C NMR {H} (126 MHz, $CDCl_3$) δ 171.4, 170.0, 164.2, 162.2, 156.6, 152.8, 152.1, 135.6 (d, $J_{C\text{-}F}$ = 3.0 Hz), 128.7, 128.4 (d, $J_{C\text{-}F}$ = 8.2 Hz), 120.3, 115.4 (d, $J_{C\text{-}F}$ = 21.5 Hz), 59.5, 52.7, 52.7, 52.1, 52.0, 35.1, 34.7, 34.6, 34.2, 32.0, 26.3, 24.0, 23.1, 22.0 ppm.

^{19}F NMR {H} (471 MHz, $CDCl_3$) δ -113.90 ppm.

HRMS (ESI) calcd. for $C_{27}H_{34}FN_2O_4S$ *m/z* 501.2223 $[M+H]^+$, found 501.2224.

BCN-triazine conjugate (5c)



To a solution of **4c** (43 mg, 100 µmol) in NaP_i (50 mM, pH 8) and MeCN (1:1, 4 mL) was added ((1*R*,8*S*,9*S*)-bicyclo[6.1.0]non-4-yn-9-yl)methanol **6** (18 mg, 120 µmol). The reaction mixture was stirred at 37°C for 3 hours then addition alkyne (9mg, 60 µmol) was added and the reaction mixture stirred overnight. The reaction mixture was concentrated *in vacuo* to remove the methanol. The resultant aqueous was concentrated *in vacuo* then crude purified *via* C18 chromatography (25–75% MeCN in H_2O) to afford **5c** as a clear gum (42mg, 76.3 µmol, 76% yield).

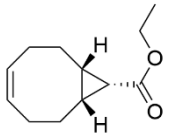
^{1}H NMR (500 MHz, $CDCl_3$) δ 8.12 (d, *J* = 8.2 Hz, 2H), 7.69 (d, *J* = 8.3 Hz, 2H), 7.39 (s, 1H), 6.47 (d, *J* = 4.6 Hz, 1H), 4.86 (dtd, *J* = 7.6, 5.1, 2.6 Hz, 1H), 3.84 – 3.54 (m, 5H), 3.23 – 2.93 (m, 8H), 2.85 (dt, *J* = 17.6, 9.0 Hz, 2H), 2.47 – 2.20 (m, 2H), 2.00 (app d, 3H) 1.84 – 1.69 (m, 1H), 1.68 – 1.57 (m, 1H), 1.55 – 1.49 (m, 2H), 1.17 – 1.03 (m, 1H), 1.00 – 0.74 (m, 2H).

^{13}C NMR {H} (126 MHz, $CDCl_3$) δ 171.4, 170.0 (app d, *J* = 1.2 Hz), 157.0, 152.17, 142.8, 130.3 (q, $J_{C\text{-}F}$ = 32.2 Hz), 124.3(q, $J_{C\text{-}F}$ = 277.2 Hz), 120.9 (s, $J_{C\text{-}F}$ = 19.1 Hz), 59.5, 52.7, 52.7, 52.0, 52.0, 35.1, 34.7, 34.7, 34.2, 31.9, 26.4, 24.0, 23.1, 22.1 ppm.

^{19}F NMR {H} (471 MHz, $CDCl_3$) δ -62.46 ppm.

HRMS (ESI) calcd. for $C_{27}H_{34}F_3N_2O_4S$ *m/z* 551.2191 $[M+H]^+$, found 551.2190.

Ethyl (1*R*,8*S*,9*S*) -bicyclo[6.1.0]non-4-ene-9-carboxylate (SM1)



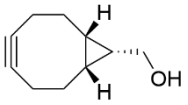
To $Rh_2(OAc)_4$ (118 mg, 0.27 mmol) was added 1,5-cyclooctadiene (50 mL, 408 mmol) and the reaction mixture was stirred vigorously. Ethyl diazoacetate (13wt% in CH_2Cl_2, 4.54 mL, 43.0 mmol)

was added over 17 hours. After addition was complete the reaction mixture was purified *via* flash chromatography (SiO_2, 1:99 diethyl ether:*n*-hexane) to afford the **SM1** as a clear oil (2.65 g, 13.4 mmol, 32% yield).

^{1}H NMR (500 MHz, $CDCl_3$): δ 5.57 (ddt, *J* = 5.4, 3.6, 0.9 Hz, 2H), 4.08 (q, *J* = 7.1 Hz, 2H), 2.53 – 2.42 (m, 2H), 2.24 – 2.10 (m, 2H), 2.08 – 1.96 (m, 2H), 1.86 – 1.73 (m, 2H), 1.67 (d, *J* = 17.6 Hz, 1H), 1.42 – 1.30 (m, 2H), 1.23 (t, *J* = 7.1 Hz, 3H) ppm.

All spectra were consistent with previously published data.[4]

((1*R*,8*S*,9*S*)-bicyclo[6.1.0]non-4-yn-9-yl)methanol (6)

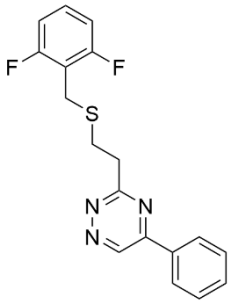


To a solution of $LiAlH_4$ (4 M in ether, 2.8 mmol) in Et_2O (11 mL) was added dropwise at 0 °C a solution of **SM1** (582 mg, 3 mmol) in Et_2O (10 mL). This suspension was stirred for 15 minutes at ambient temperature. The reaction mixture was cooled to 0 °C and additional $LiAlH_4$ in THF (4 M in ether, 2 mL, 0.8 mmol) was added and the suspension stirred for 30 minutes, then cooled down to 0 °C, and water was added carefully until the grey solid had turned into white. The reaction mixture was dried over Na_2SO_4 then the solid was filtered off and washed thoroughly with Et_2O (50 mL). Without further purification the alcohol was dissolved in CH_2Cl_2 (22 mL) and at 0 °C a solution of Br_2 (166 µL, 3.23 mmol) in CH_2Cl_2 (2 mL) was added dropwise until the yellow colour persisted. The reaction mixture was quenched with aq. 10% $Na_2S_2O_3$ (5 mL) and extracted with CH_2Cl_2 (2×20 mL). The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. Without further purification the dibromide was dissolved in THF (10 mL) and a solution of KO^{t}Bu (5 mL, 2 M in THF, 10 mmol) was added dropwise at 0 °C. Then the solution was refluxed for 2.5 hours. The mixture was quenched with sat. NH_4Cl (20 mL) and extracted with CH_2Cl_2 (3 × 20 mL). The organics were combined, dried over Na_2SO_4 and concentrated *in vacuo*. The crude material was purified by flash chromatography (SiO_2, 1:2 EtOAc:PE) to afford **6** as a white solid (130 mg, 0.87 mmol, 29% yield).

^{1}H NMR (500 MHz, $CDCl_3$): δ 3.73 (d, *J* = 7.9 Hz, 2H), 2.36 – 2.12 (m, 6H), 1.66 – 1.53 (m, 2H), 1.34 (tt, *J* = 9.1, 7.9 Hz, 2H), 0.99 – 0.88 (m, 2H) ppm.

All spectra were consistent with previously published data.[4]

3-(2-((2,6-difluorobenzyl)thio)ethyl)-5-phenyl-1,2,4-triazine (7a)



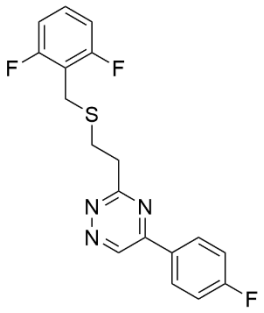
To a solution of **3a** (20 mg, 0.11 mmol) in 1:1 MeCN:NaP_i (50 mM, pH 8;11 mL) was added (2,6-difluorophenyl)methanethiol (44 mg, 0.27 mmol). The reaction mixture was stirred at 37 °C for 15 hours. The reaction mixture was concentrated *in vacuo* and the crude material purified by flash chromatography (SiO_2, 1:5 EtOAc:*n*-hexane) to afford **7a** as a yellow oil (31 mg, 0.09 mmol, 84% yield).

^{1}H NMR ^{1}H NMR (400 MHz, CD_2Cl_2) δ 9.57 (s, 1H), 8.21 – 8.16 (m, 2H), 7.66 – 7.53 (m, 3H), 7.23 (tt, *J* = 8.4, 6.5 Hz, 1H), 6.97 – 6.86 (m, 2H), 3.86 (s, 2H), 3.47 (t, *J* = 7.4 Hz, 2H), 3.16 (t, *J* = 7.4 Hz, 2H) ppm.

^{13}C NMR {H} (101 MHz, CD_2Cl_2): δ 168.2 (C_8), 161.6 (d, $^{2}J_{C\text{-}F}$ = 248.2, C_3), 155.4 (C_{10}), 144.8 (C_9), 134.0 (C_{11}), 132.7 (C_{14}), 129.7 (C_{12}), 129.1 (t, $J_{C\text{-}F}$ = 10.3 Hz, C_1), 127.9 (C_{13}), 115.7 (t, $J_{C\text{-}F}$ = 19.4 Hz, C_4), 111.6 (dd, $J_{C\text{-}F}$ = 25.4, 12.4 Hz C_2), 37.3 (C_7), 30.1 (C_6), 22.9 (C_5) ppm.

HRMS (ESI) calcd. for $C_{18}H_{16}F_2N_3S$ *m/z* 344.1045 $[M+H]^+$, found 344.1045.

3-(2-((2,6-difluorobenzyl)thio)ethyl)-5-(4-fluorophenyl)-1,2,4-triazine(7b)



To a solution of **3b** (20 mg, 0.09 mmol) in 1:1 MeCN:NaP_i (50 mM, pH 8; 5 mL) was added (2,6-difluorophenyl)methanethiol (32 mg, 0.2 mmol). The reaction mixture was stirred at 37°C for 17 hours. The reaction mixture was concentrated *in vacuo* and the crude material purified by flash chromatography (SiO_2, pure *n*-hexane – 1:5 EtOAc:*n*-hexane) to afford **7b** as a yellow oil (23.2 mg, 69.6 µmol, 70% yield).

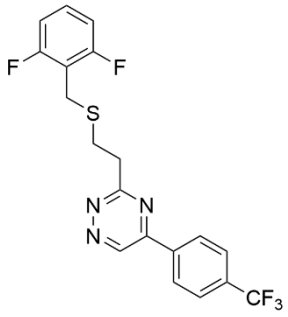
^{1}H NMR (700 MHz, $CDCl_3$) δ 9.54 (s, 1H), 8.25 – 8.17 (m, 2H), 7.29 – 7.17 (m, 3H), 6.92 – 6.86 (m, 2H), 3.85 (s, 2H), 3.50 (t, *J* = 7.4 Hz, 2H), 3.16 (t, *J* = 7.4 Hz, 2H) ppm.

^{13}C NMR {H} (176 MHz, $CDCl_3$) δ 168.04, 166.39, 164.95, 161.31 (dd, $^{2}J_{C\text{-}F}$ = 248.8, 7.9 Hz), 154.21, 144.07, 130.03 (d, $J_{C\text{-}F}$ = 9.0 Hz), 129.79 (d, $J_{C\text{-}F}$ = 3.1 Hz), 128.75 (t, $J_{C\text{-}F}$ = 10.3 Hz), 116.78 (d, $J_{C\text{-}F}$ = 22.0 Hz), 115.37, 111.46 (dd, $J_{C\text{-}F}$ = 21.1, 4.5 Hz), 36.97, 29.80, 22.73 (t, $J_{C\text{-}F}$ = 2.9 Hz) ppm.

^{19}F NMR {H} (376 MHz, $CDCl_3$) δ -106.20, -114.88 ppm.

HRMS (ESI) calcd. for $C_{18}H_{15}F_3N_3S$ *m/z* 362.0991 $[M+H]^+$, found 362.0939.

3-(2-((2,6-difluorobenzyl)thio)ethyl)-5-(4-(trifluoromethyl)phenyl)-1,2,4-triazine (7c)



To a solution of **3c** (20 mg, 79.6 µmol) in 1:1 MeCN:NaP_i (50 mM, pH 8; 5 mL) was added (2,6-difluorophenyl)methanethiol (25 mg, 156 µmol). The reaction mixture was stirred at 37°C for 19 hours. The reaction mixture was concentrated *in vacuo* and the crude material purified by flash chromatography (SiO_2, pure PE – 1:5 EtOAc:PE) to afford **7c** as a yellow oil (48 mg, 117 µmol, 75%).

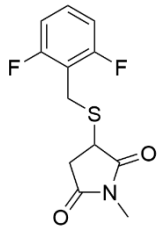
^{1}H NMR (700 MHz, $CDCl_3$) δ 9.61 (s, 1H), 8.29 (d, J = 8.2 Hz, 2H), 7.83 (d, J = 8.3 Hz, 2H), 7.20 (tt, J = 8.4, 6.5 Hz, 1H), 6.92 – 6.83 (m, 2H), 3.85 (s, 2H), 3.54 (t, J = 7.4 Hz, 2H), 3.17 (t, J = 7.4 Hz, 2H) ppm.

^{13}C NMR {H} (176 MHz, $CDCl_3$): δ 167.39, 160.33 (dd, $J_{C\text{-}F}$ = 248.7, 7.9 Hz), 152.89, 143.40, 136.12 (app d, $J_{C\text{-}F}$ = 1.3 Hz), 133.11 (q, $J_{C\text{-}F}$ = 32.9 Hz), 127.81 (t, $J_{C\text{-}F}$ = 10.3 Hz), 127.16, 125.45 (q, $J_{C\text{-}F}$ = 3.7 Hz), 122.75 (q, $J_{C\text{-}F}$ = 272.6 Hz), 114.34 (t, $J_{C\text{-}F}$ = 19.2 Hz), 110.49 (dd, $J_{C\text{-}F}$ = 21.1, 4.5 Hz), 35.96, 28.79, 21.75 (t, $J_{C\text{-}F}$ = 2.9 Hz) ppm.

^{19}F NMR {H} (376 MHz, $CDCl_3$) δ -63.09, -114.90 ppm.

HRMS (ESI) calcd. for $C_{18}H_{15}F_5N_3S$ *m/z* 412.0907 $[M+H]^+$, found 412.0920.

3-((2,6-difluorobenzyl)thio)-1-methylpyrrolidine-2,5-dione(8)



A solution of 1-methyl-1H-pyrrole-2,5-dione (100 mg, 0.901 mmol) in 1:1 MeCN:NaP_i (50 mM, pH 8; 5 mL) was added (2,6-difluorophenyl)methanethiol (288 mg, 1.80 mmol). The reaction mixture was stirred at r.t. for 15 hours. The reaction mixture was concentrated *in vacuo* and the resulting aqueous extracted with CH_2Cl_2(25 mL × 3). The organics were combined, dried over $MgSO_4$ and concentrated *in vacuo*. The resultant crude was purified *via* flash column chromatography (SiO_2, pure PE – 1:5 EtOAc:PE) to afford **8** (233 mg, 0.86 mmol, 95%) as a light-yellow oil.

^{1}H NMR (500 MHz, CDCl3) δ 7.31 – 7.19 (m, 1H), 6.98 – 6.84 (m, 2H), 4.25 (d, J = 13.6 Hz, 1H), 3.99 (d, J = 13.6 Hz, 1H), 3.80 (dd, J = 9.2, 4.1 Hz, 1H), 3.11 (dd, J = 18.8, 9.2 Hz, 1H), 3.02 (s, 3H), 2.50 (dd, J = 18.7, 4.1 Hz, 1H) ppm.

All spectra were consistent with previously published data.[5]

3. References

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(4) Dommerholt, J.; Schmidt, S.; Temming, R.; Hendriks, L. J. A.; Rutjes, F. P. J. T.; van Hest, J. C. M.; Lefeber, D. J.; Friedl, P.; van Delft, F. L. Readily Accessible Bicyclononynes for Bioorthogonal Labeling and Three-Dimensional Imaging of Living Cells. *Angew. Chem. Int. Ed.* **2010**, *49* (49), 9422–9425.

(5) Seki, H.; Walsh, S. J.; Bargh, J. D.; Parker, J. S.; Carroll, J.; Spring, D. R. Rapid and Robust Cysteine Bioconjugation with Vinylheteroarenes. *Chem. Sci.* **2021**, *12* (26), 9060–9068.

4. Determination of Cysteine Bioconjugation Rate Constant

4.1. General procedure

To a solution of linker **3a, b** or **c** [0.21 mL, 7.00μmol, 33.3 mM in MeCN-d_3] at 37 °C was added a solution of sodium 3-(trimethylsilyl)propane-1-sulfonate [0.49 mL, 7.00μmol, 14.3 mM in NaP_i (pH 8, 50 mM in D_2O)]. The reaction mixture was transferred to and NMR tube and used to calibrate the NMR machine at 37 °C with the sample spinning. To the reaction mixture was added a solution of Ac-Cys-OMe [0.7 mL, 10 mM in 3:7 MeCN-d_3/ NaP_i (pH 8, 50 mM in D_2O)] and mixed by vigorous shaking. An NMR was taken, and further 49 measurements taken every ~ 135 seconds ~~for~~. The vinyl peaks at between 8.0 and 6.5 ppm were integrated to determine the concentration of substrates **3a-c**.

The following equations were used to determine second order rate constants k_2.

$$\frac{dE}{dt} = -k_2[Nu][E]$$

$$When\ [Nu] = [E], [E]dt = -k_2[E]^2$$

$$Integration\ gives\ \frac{1}{[E]_t} = k_2 t + \frac{1}{[E]_{t=0}}$$

Thus, the second order rate constant k_2 can be determined by plotting $y = \frac{1}{[E]_t}$ and x = t; the gradient is equal to k_2.

Table S1. Second order rate constants of vinyl heteroarene reaction with Ac-Cys-OMe.

Linker	Second order rate constant ($M^{-1}s^{-1}$)
3a	0.0774 ± 0.0003
3b	0.0490 ± 0.0057
3c	0.237±0.136

4.2. Cysteine reactivity with linkers (3a-c)

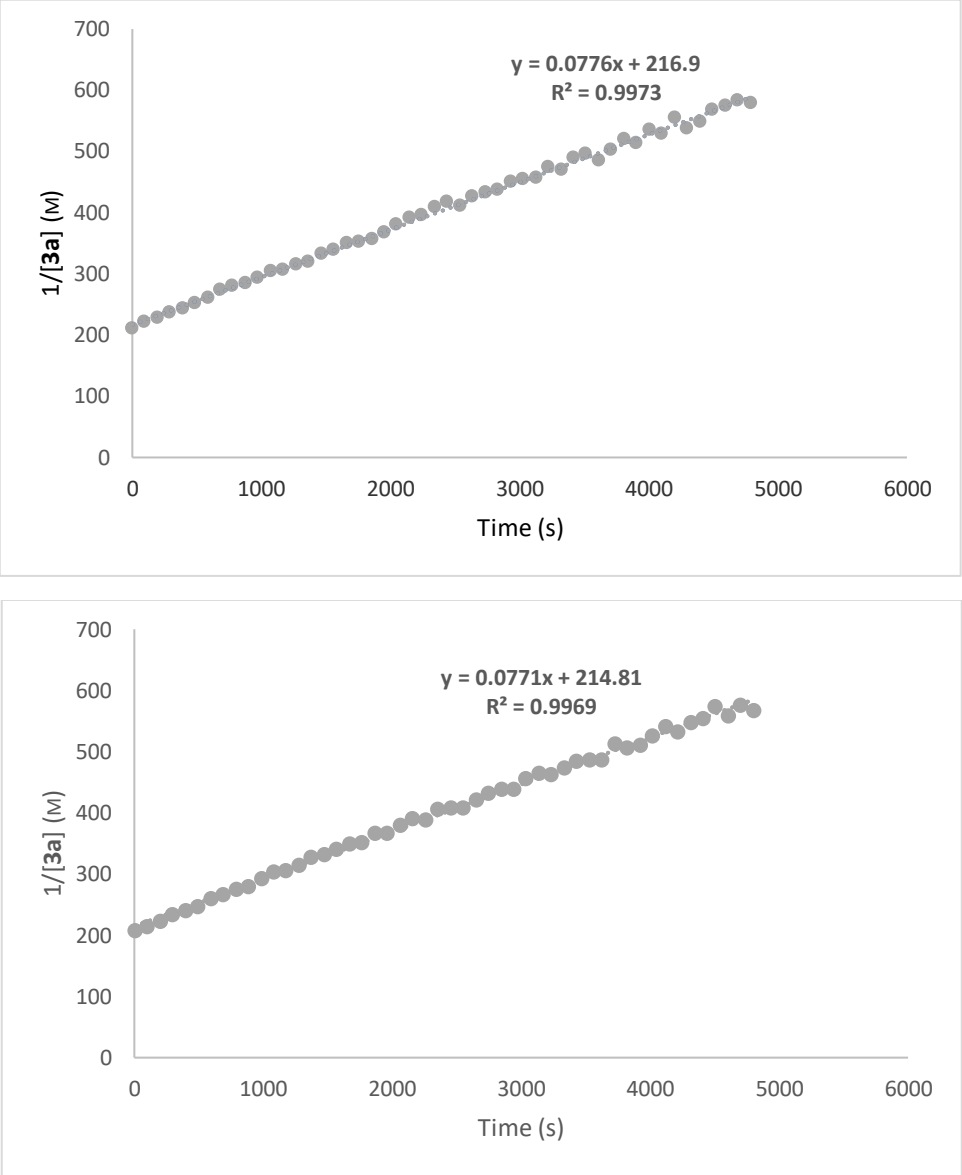
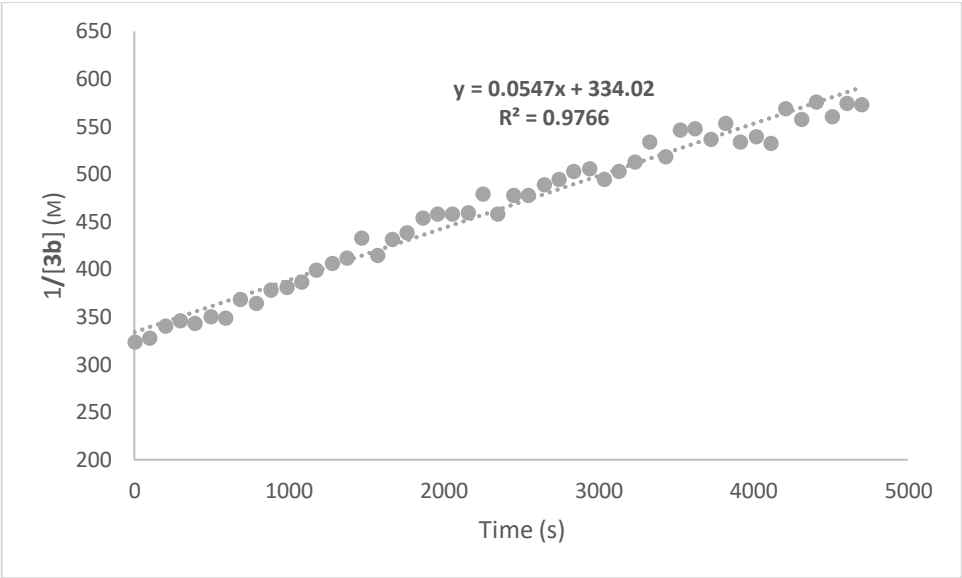


Figure S1: Kinetic data used to calculate second order rate constants for the reaction of Ac-Cys-OMe with vinyl-triazine **3a** in 3:7 MeCN-d_3/NaP$_i$ (pH 8, 50 mM in D_2O). Two measurements were taken (plots *a* and *b*) and averaged.



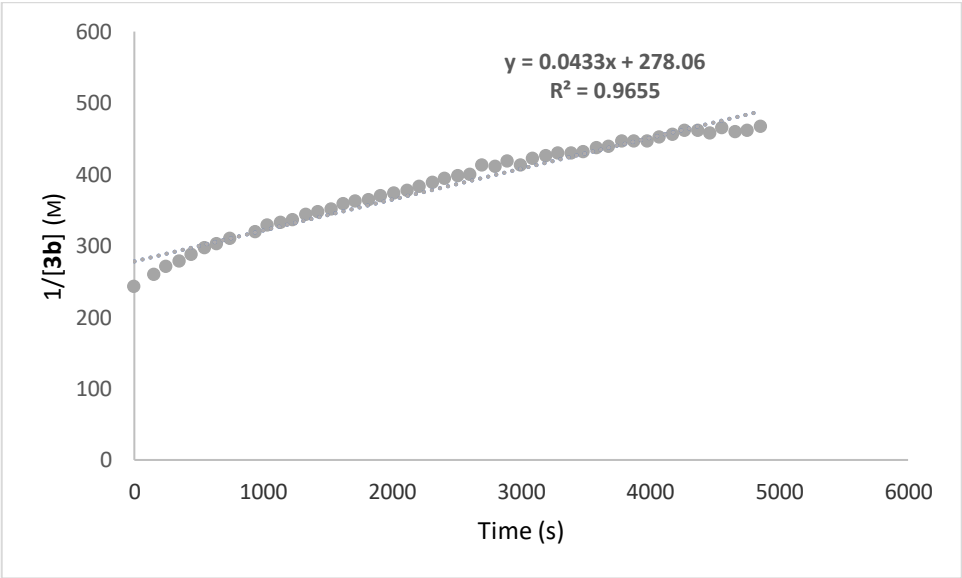


Figure S2: Kinetic data used to calculate second order rate constants for the reaction of Ac-Cys-OMe with vinyl-triazine **3b** in 3:7 MeCN-d_3/NaP$_i$ (pH 8, 50 mM in D_2O). Two measurements were taken (plots *a* and *b*) and averaged.

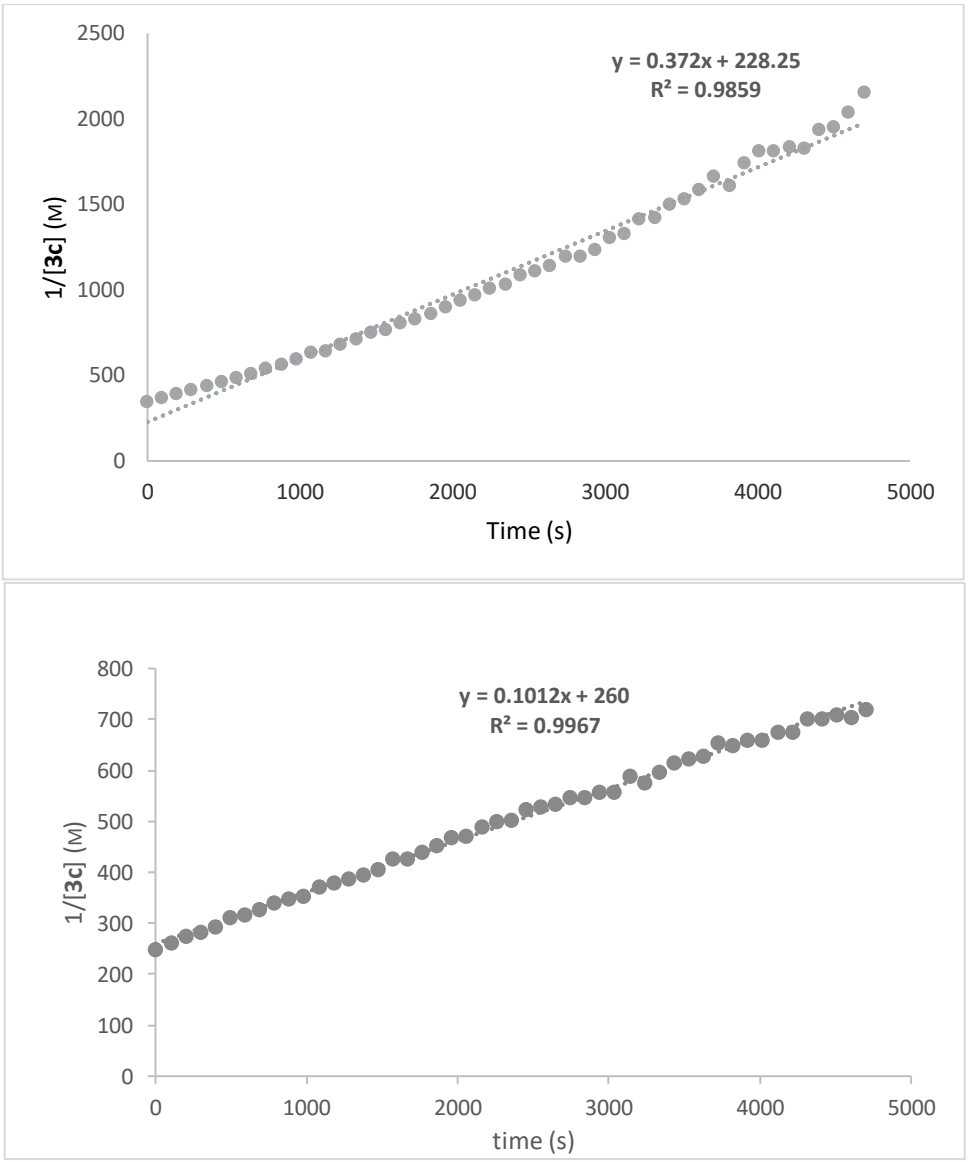


Figure S3: Kinetic data used to calculate second order rate constants for the reaction of Ac-Cys-OMe with vinyl-triazine **3c** in 3:7 MeCN-d_3/NaP$_i$ (pH 8, 50 mM in D_2O). Two measurements were taken (plots *a* and *b*) and averaged.

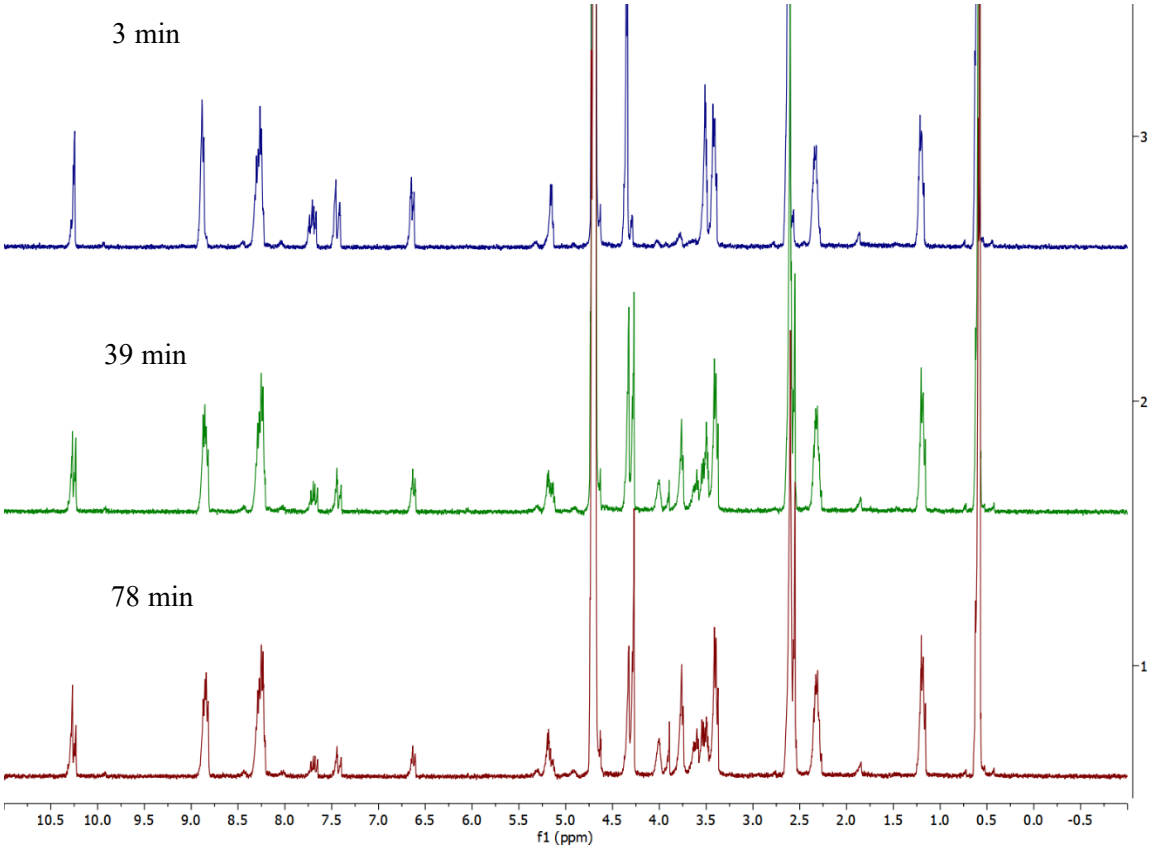


Figure S4: Representative ^{1}H NMR spectra of the reaction of triazine **3a** with Ac-Cys-OMe in 3:7 MeCN-d_3/NaP$_i$ (pH 8, 50 mM in D_2O).

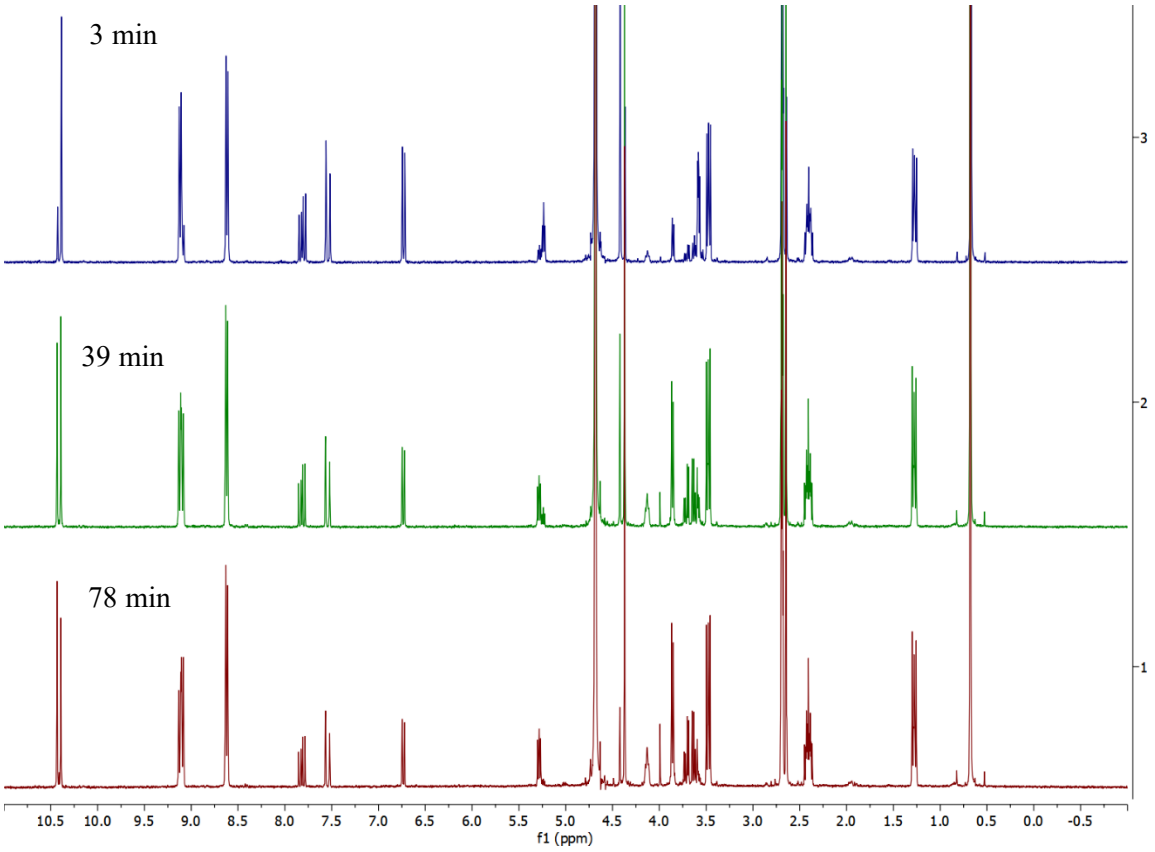


Figure S5: Representative ^{1}H NMR spectra of the reaction of triazine **3b** with Ac-Cys-OMe in 3:7 MeCN-d_3/NaP$_i$ (pH 8, 50 mM in D_2O).

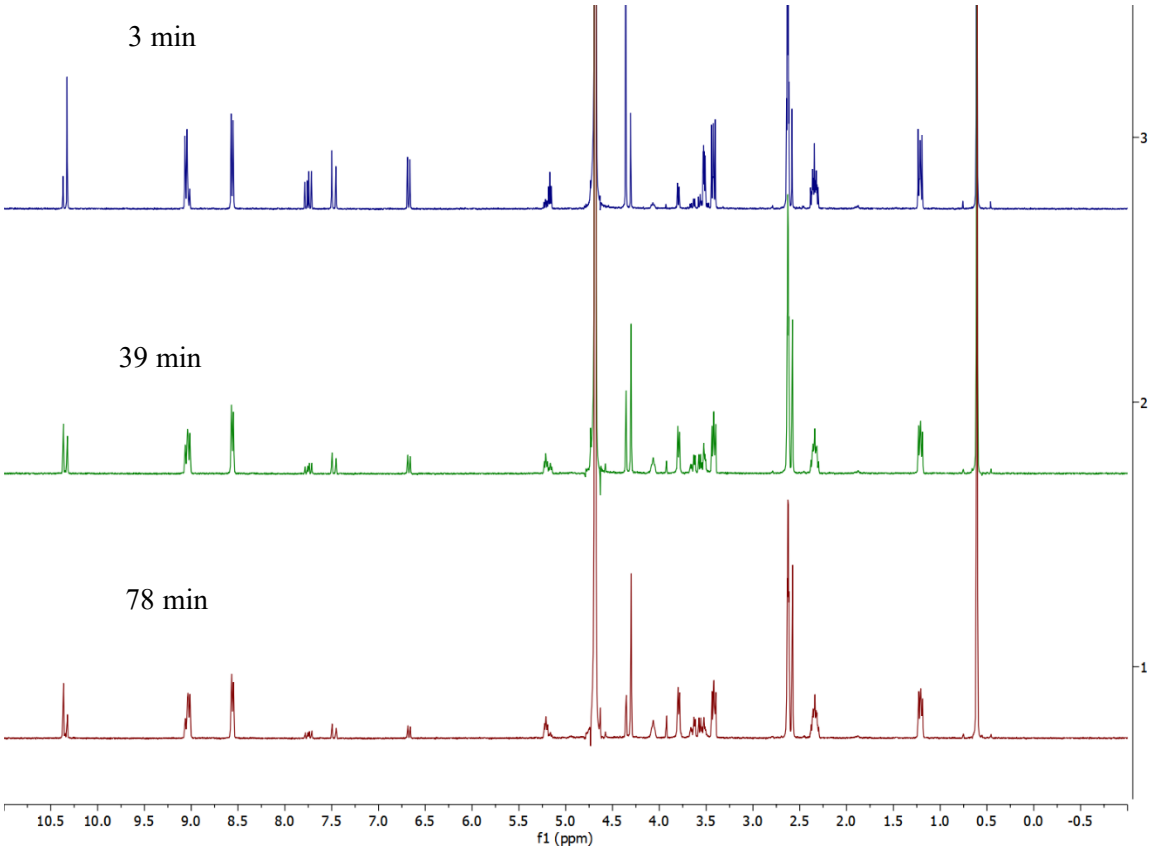


Figure S6: Representative ^{1}H NMR spectra of the reaction of triazine **3c** with Ac-Cys-OMe in 3:7 MeCN-d_3/NaP$_i$ (pH 8, 50 mM in D_2O).

5. Determination of IEEDA Rate Constants

5.1. General procedure

To a solution of linker-Ac-Cys-OMe **4a**, **b** or **c** [0.21 mL, 7.00μmol, 33.3 mM in MeCN-d_3] at 37 °C was added a solution of sodium 3-(trimethylsilyl)propane-1-sulfonate [0.49 mL, 7.00 μmol, 14.3 mM in NaP_i (pH 8, 50 mM in D_2O)]. The reaction mixture was transferred to and NMR tube and used to calibrate the NMR machine at 37 °C with the sample spinning. To the reaction mixture was added a solution of (1*R*,8*S*,9*S*)-bicyclo[6.1.0]non-4-yn-9-ylmethanol **6** [0.7 mL, 10 mM in 3:7 MeCN-d_3/NaPi (pH8, 50 mM)] and mixed by vigorous shaking. An NMR was taken, and further 49 measurements taken every ~ 135 seconds ~~for~~. The compound peak between 9.0 ppm and 7.5 ppm were integrated to determine the concentration of substrates **4a-c.**

Table S2. Second order rate constants of Linker-*N*-acyl-Cys-OMe with BCN.

Linker	Second order rate constant ($M^{-1}s^{-1}$)
4a	0.0243 ± 0.0044
4b	0.0375 ± 0.0008
4c	0.0164 ±0.0021

5.2. BCN reactivity with linker-Ac-Cys-Ome (4a-c)

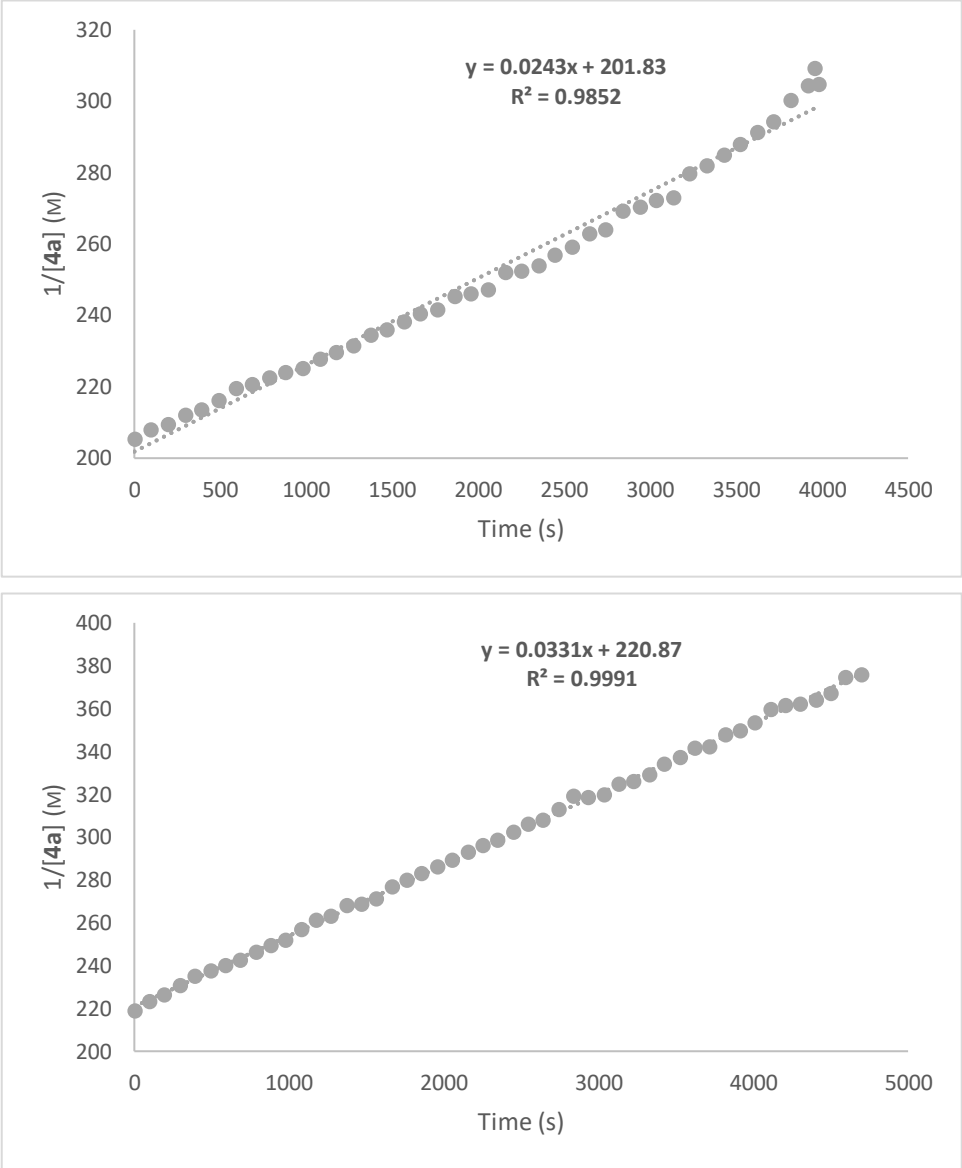


Figure S7: Kinetic data used to calculate second order rate constants for the reaction of vinyl-triazine **4a** with **6** in 3:7 MeCN-d_3/NaP$_i$ (pH 8, 50 mM in D_2O). Two measurements were taken (plots *a* and *b*) and averaged.

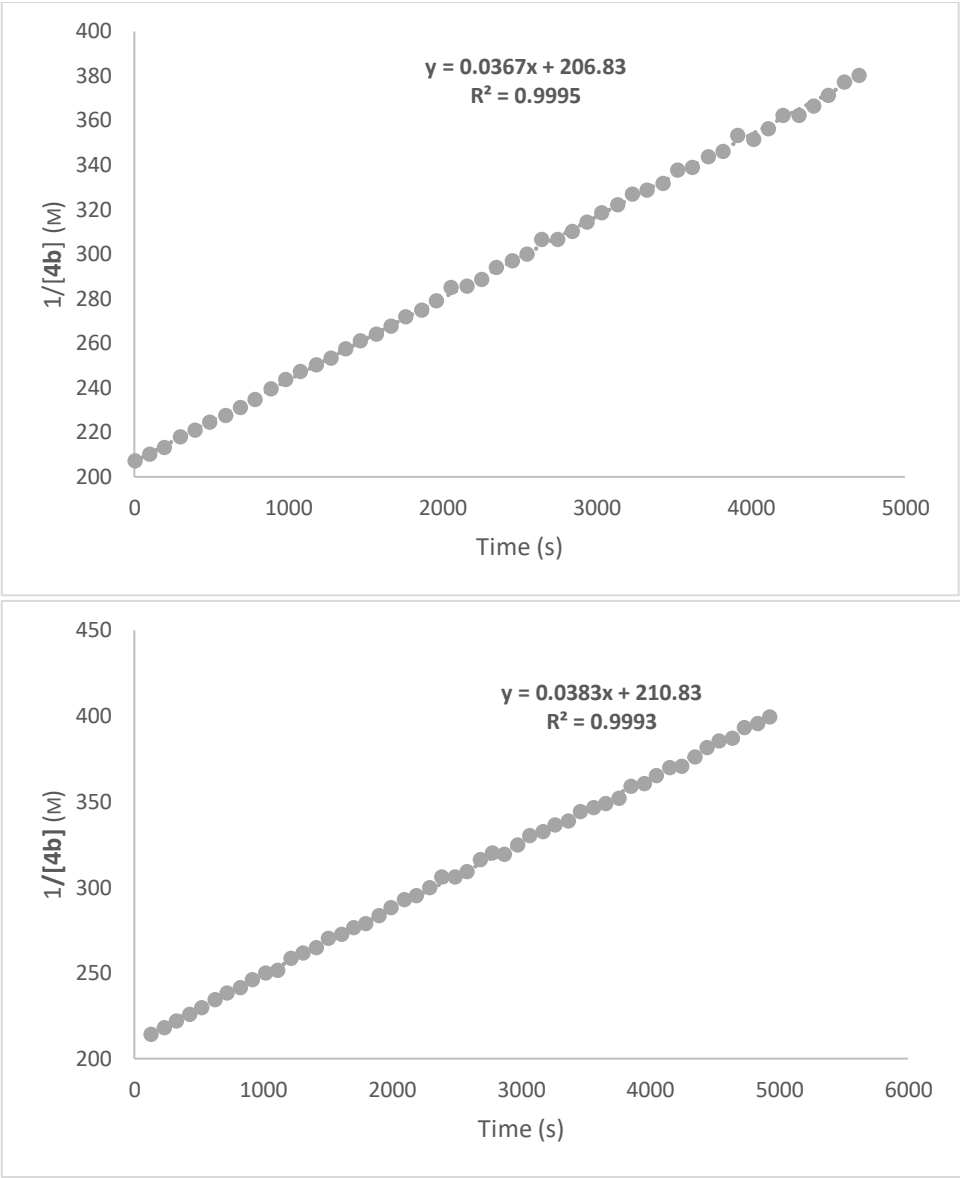
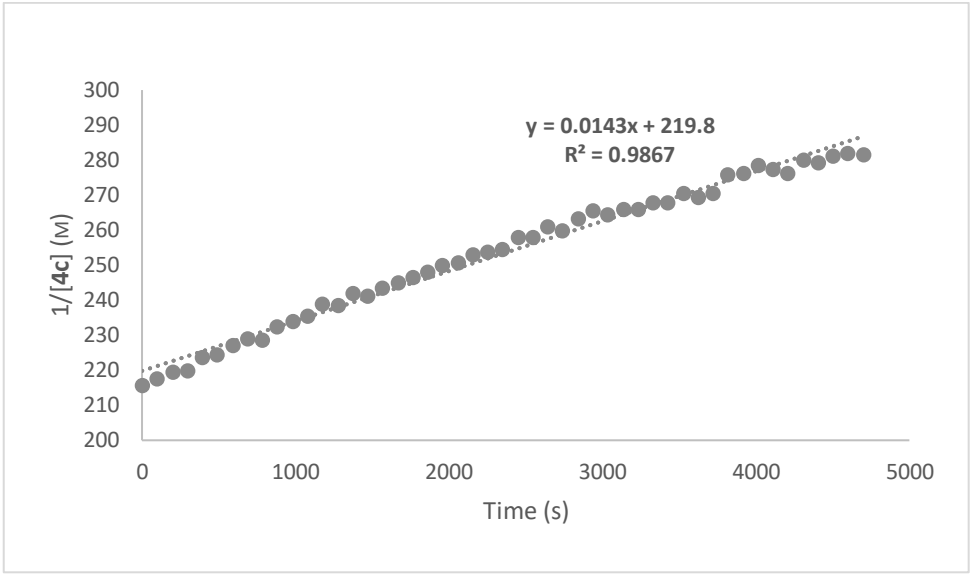


Figure S8: Kinetic data used to calculate second order rate constants for the reaction of vinyl-triazine **4b** with **6** in 3:7 MeCN-d_3/NaP$_i$ (pH 8, 50 mM in D_2O). Two measurements were taken (plots *a* and *b*) and averaged.



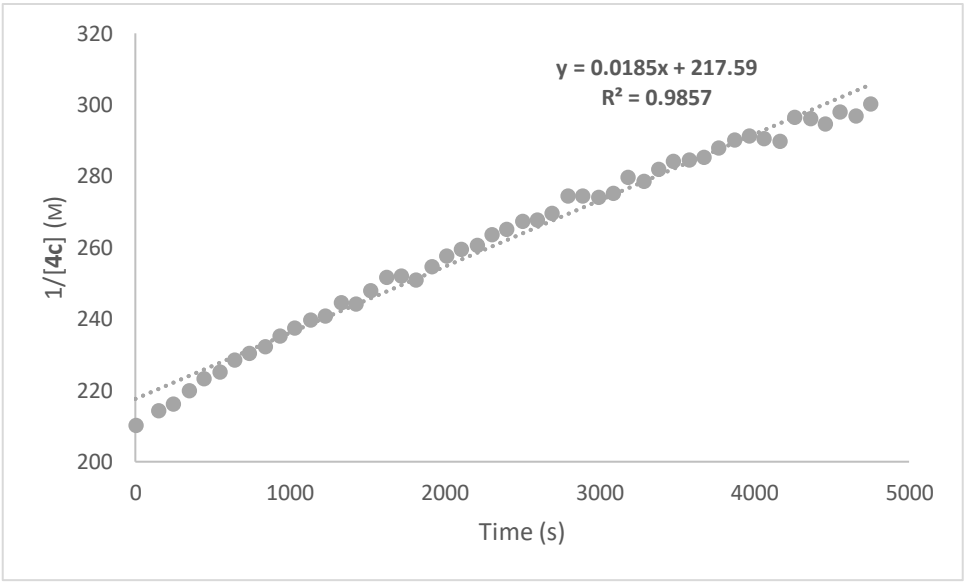


Figure S9: Kinetic data used to calculate second order rate constants for the reaction of vinyl-triazine **4c** with **6** in 3:7 MeCN-d_3/NaP$_i$ (pH 8, 50 mM in D_2O). Two measurements were taken (plots *a* and *b*) and averaged.

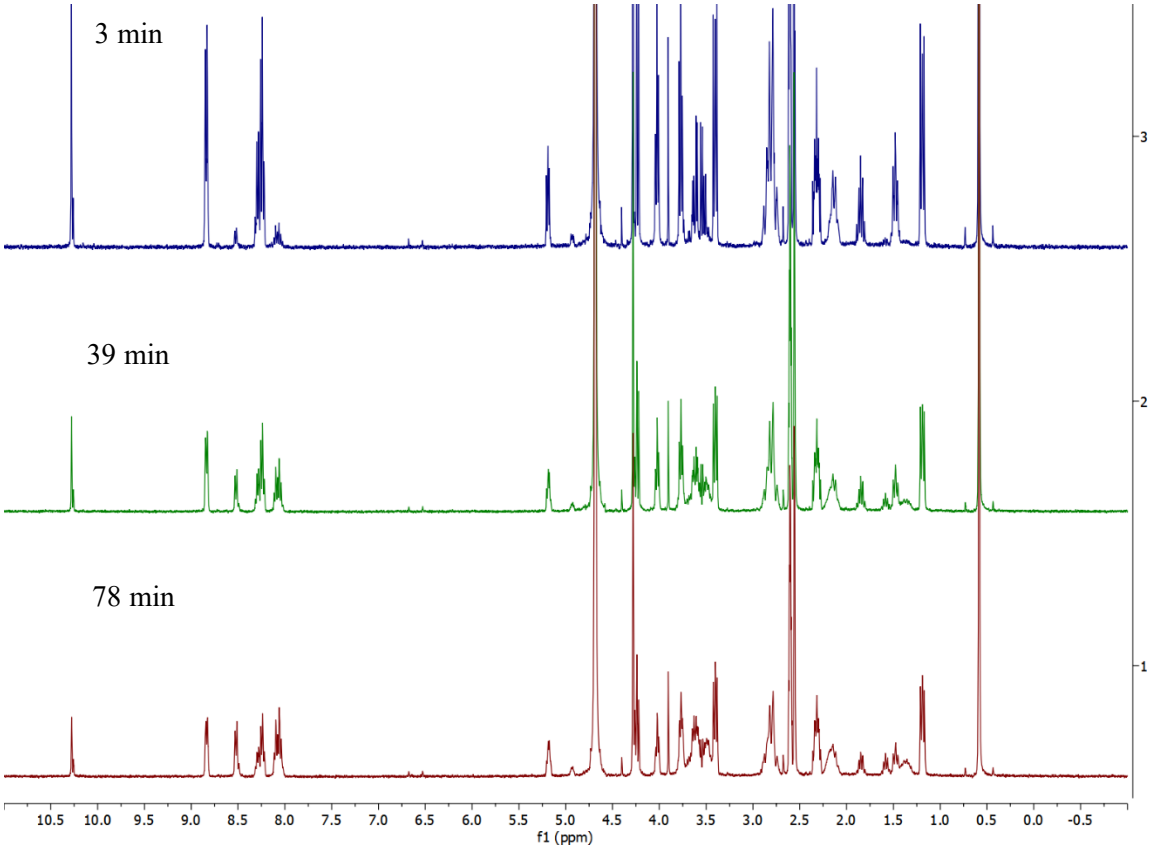


Figure S10: Representative ^{1}H NMR spectra of the reaction of vinyl-triazine **4a** with **6** in 3:7 MeCN-d_3 /NaP$_i$ (pH 8, 50 mM in D_2O).

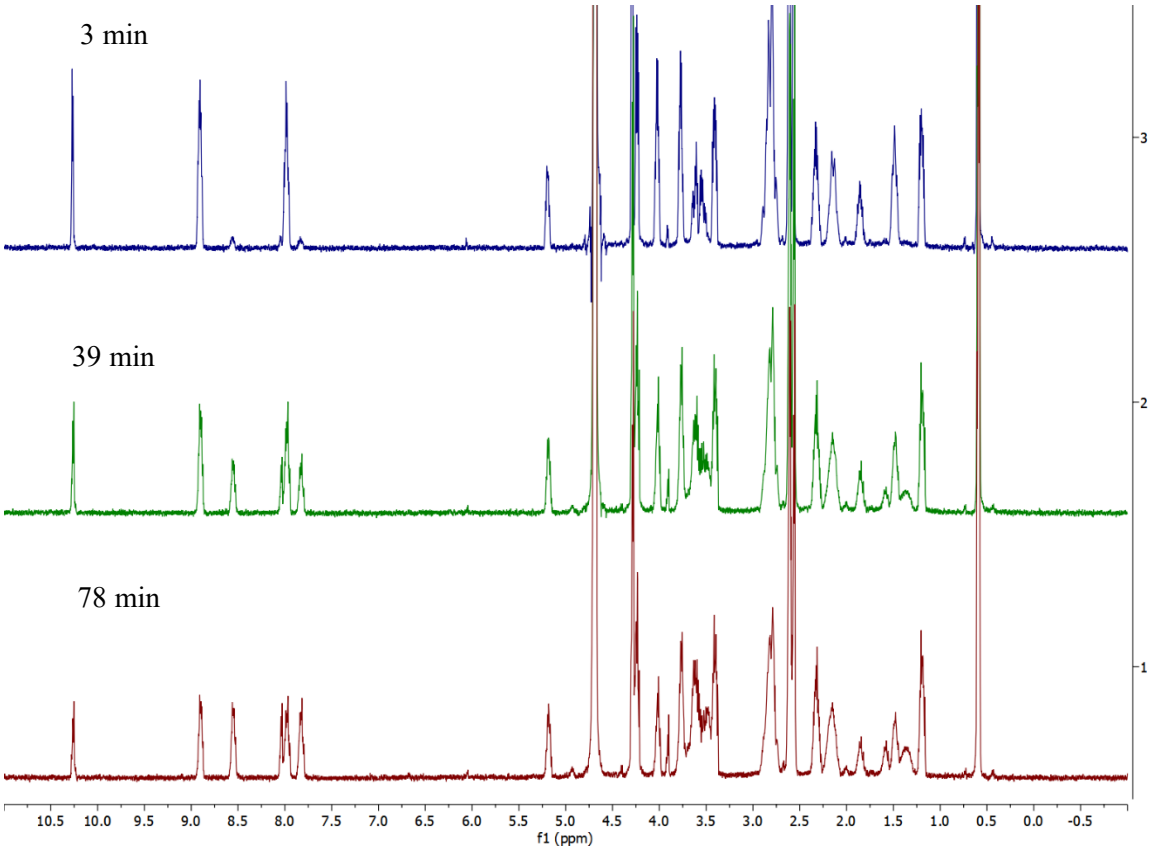


Figure S11: Representative ^{1}H NMR spectra of the reaction of vinyl-triazine **4b** with **6** in 3:7 MeCN-d_3/NaP$_i$ (pH 8, 50 mM in D_2O).

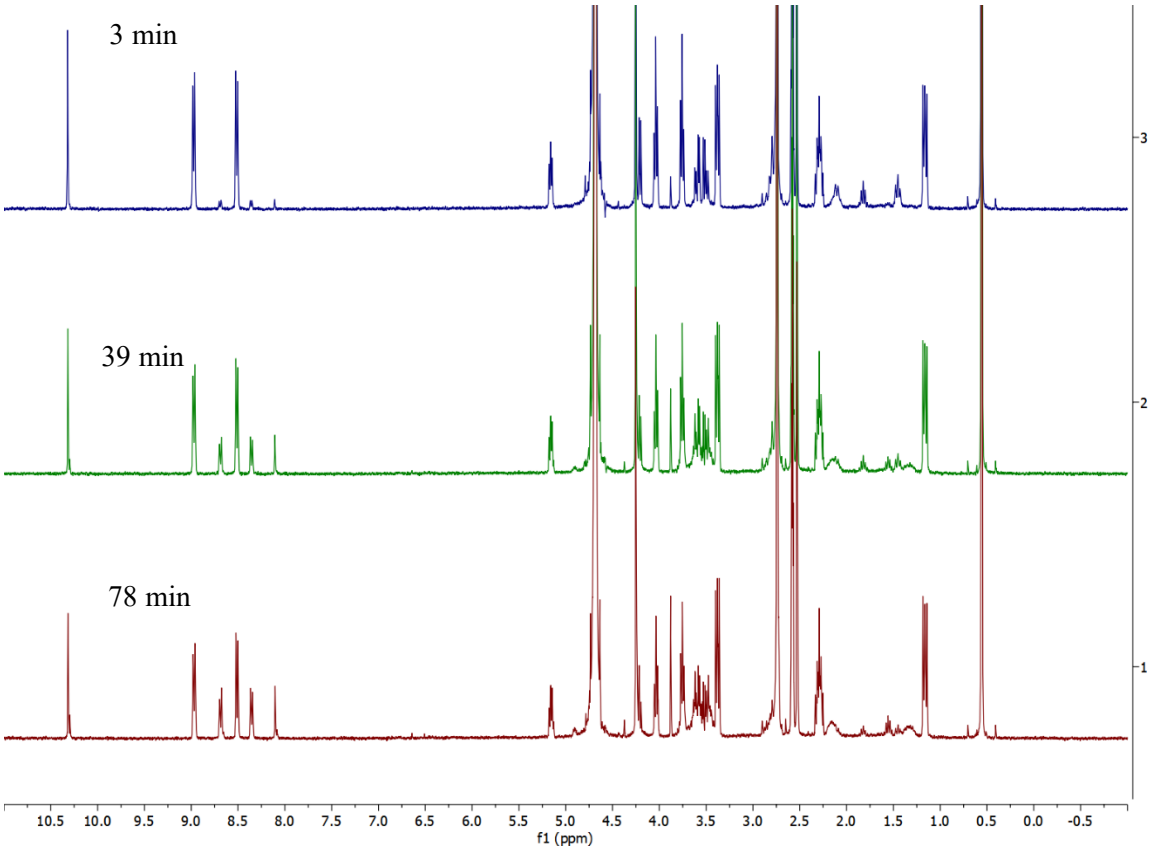


Figure S12: Representative ^{1}H NMR spectra of the reaction of vinyl-triazine **4c** with **6** in 3:7 MeCN-d_3/NaP$_i$ (pH 8, 50 mM in D_2O).

6. Nucleophilic Amino Acid Reactivity

6.1. General procedure

To a solution of linker **3c** [596 µL, 10 mM in MeCN] was added a solution of a nucleophilic amino acid [596 µL, 10 mM in NaP*i* (pH 8 50 mM)]. The reaction mixture was then stirred at 37 °C for 24 hours. HPLC analysis was used to identify if a reaction had occurred, and UV-LCMS analysis was used to identify the UV peaks.

6.2. HPLC Traces for the Reaction Between 3c and Amino Acids

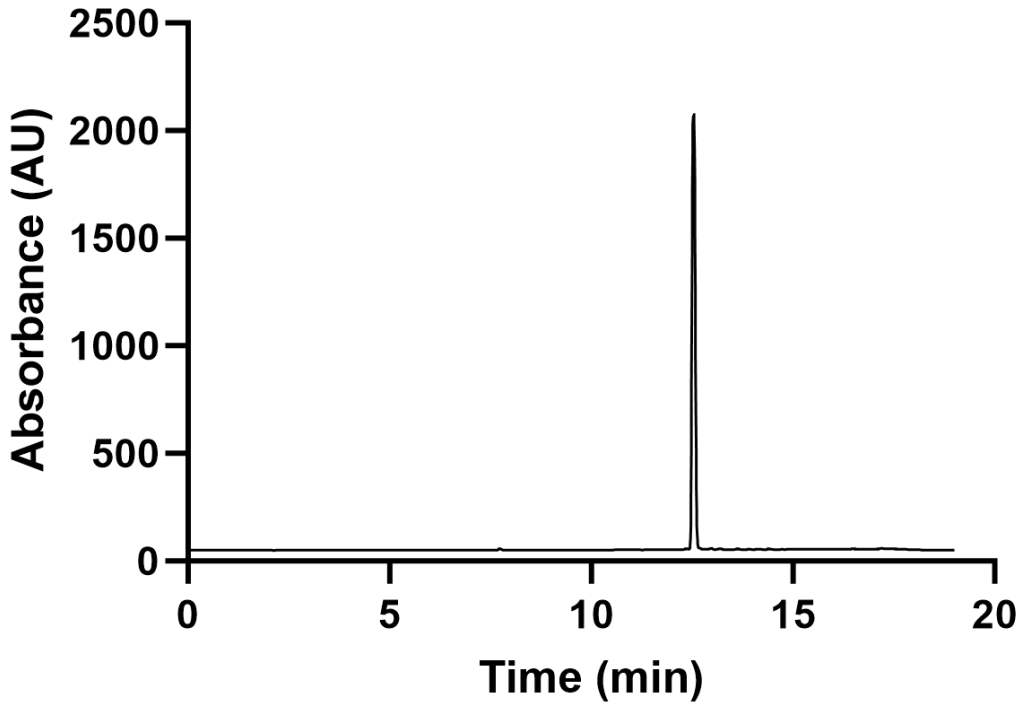


Figure S13: HPLC trace of reaction between **3c** and methyl (*tert*-butoxycarbonyl)-D-serinate.

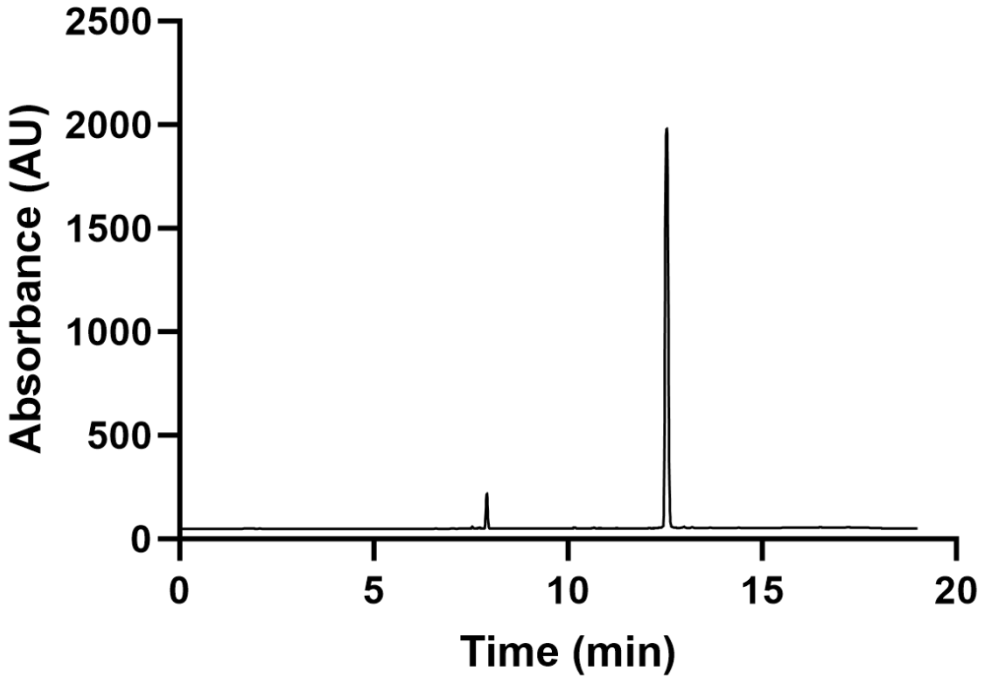


Figure S14: HPLC trace of reaction between **3c** and methyl acetyl-D-lysinate hydrochloride. Minimal reactivity was observed after 24 hours.

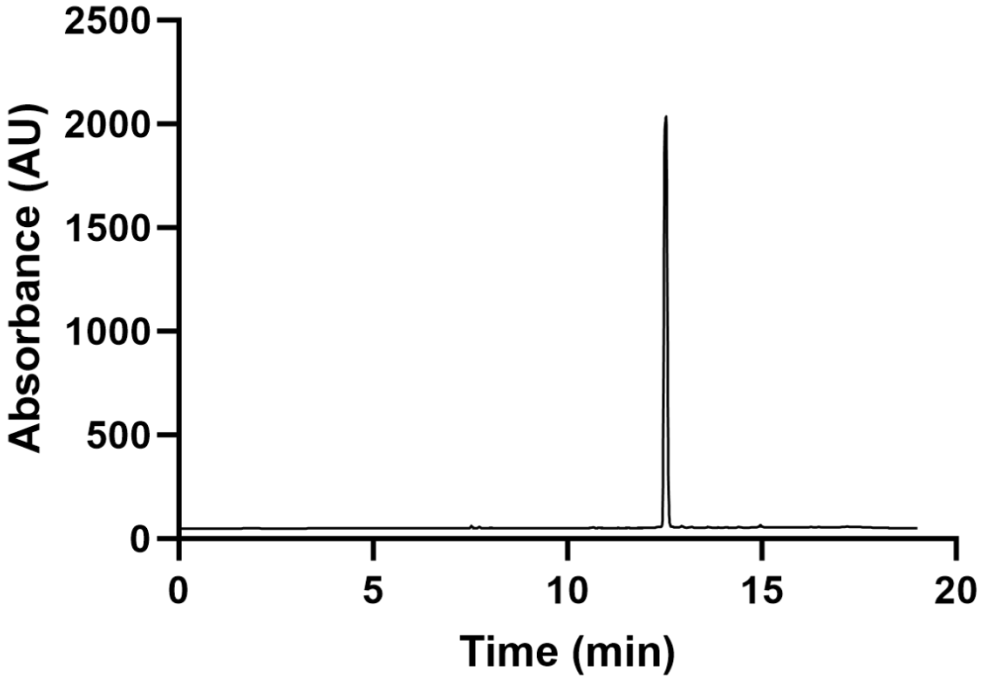


Figure S15: HPLC trace of reaction between **3c** and methyl methyl D-threoninate hydrochloride.

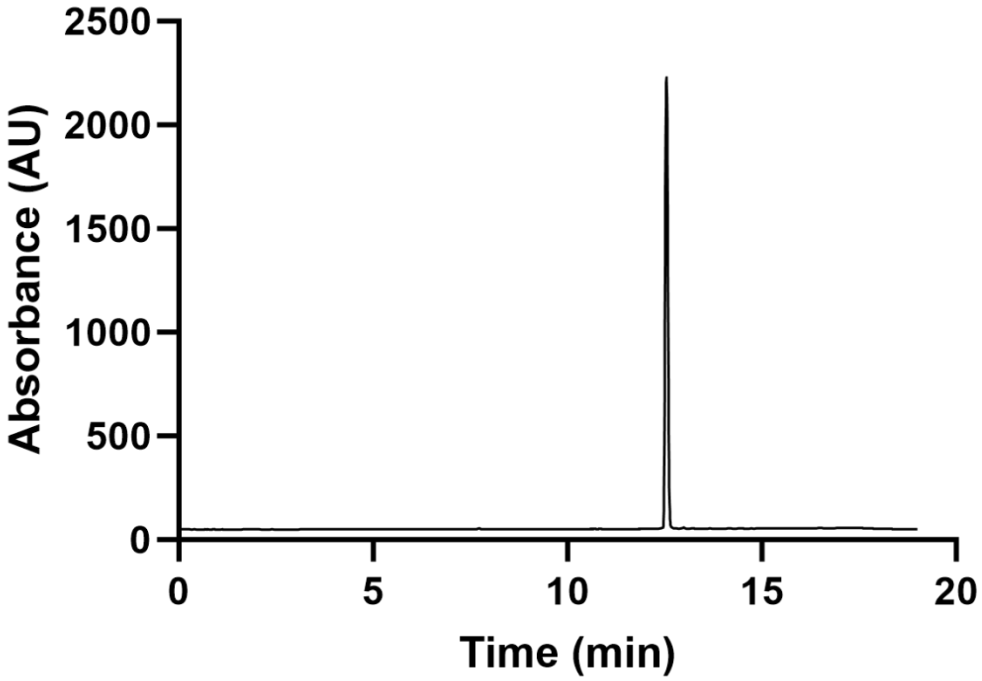
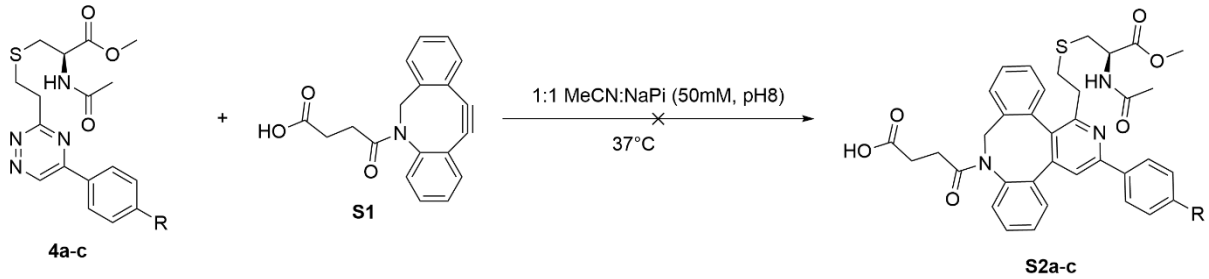


Figure S16: HPLC trace of reaction between **3c** and methyl acetyl-D-asparagine.

7. Determination of IEEDA selectivity

7.1. DBCO reactivity with linker-Ac-Cys-OMe general procedure



Scheme S1: The failed reaction between linker-Ac-Cys-OMe **4a-c** with DBCO **S1**.

To a solution of **4a**, **b** or **c** (10 mM in 1:1 MeCN-d_3/ NaP$_i$ (pH 8, 50 mM); 100 μL) was added a solution of **S1** (10 mM in 1:1 MeCN-d_3/ NaP$_i$ (pH 8, 50 mM); 100 μL). The reaction mixture was stirred at 37 °C for 24 hours. HPLC and UV-LCMS analysis was performed after 24 hours and indicated the desired reaction had not occurred.

7.2. DBCO reactivity with linker-Ac-Cys-OMe HPLC UV traces

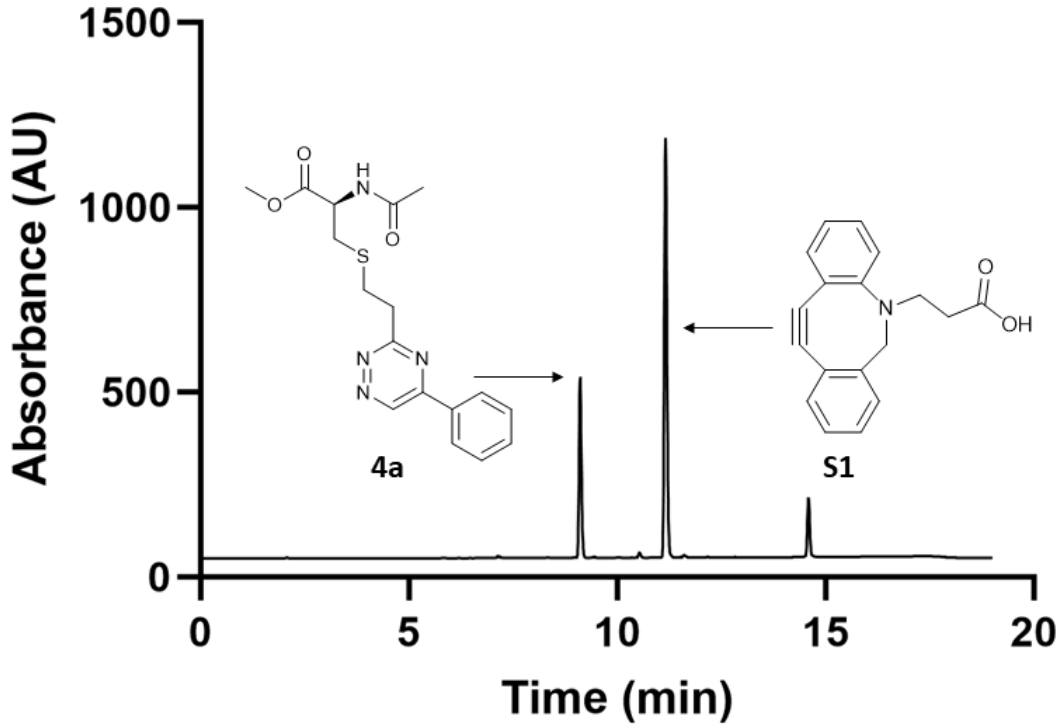


Figure S17: HPLC trace of reaction between **4a** and **S1** after 24 hours.

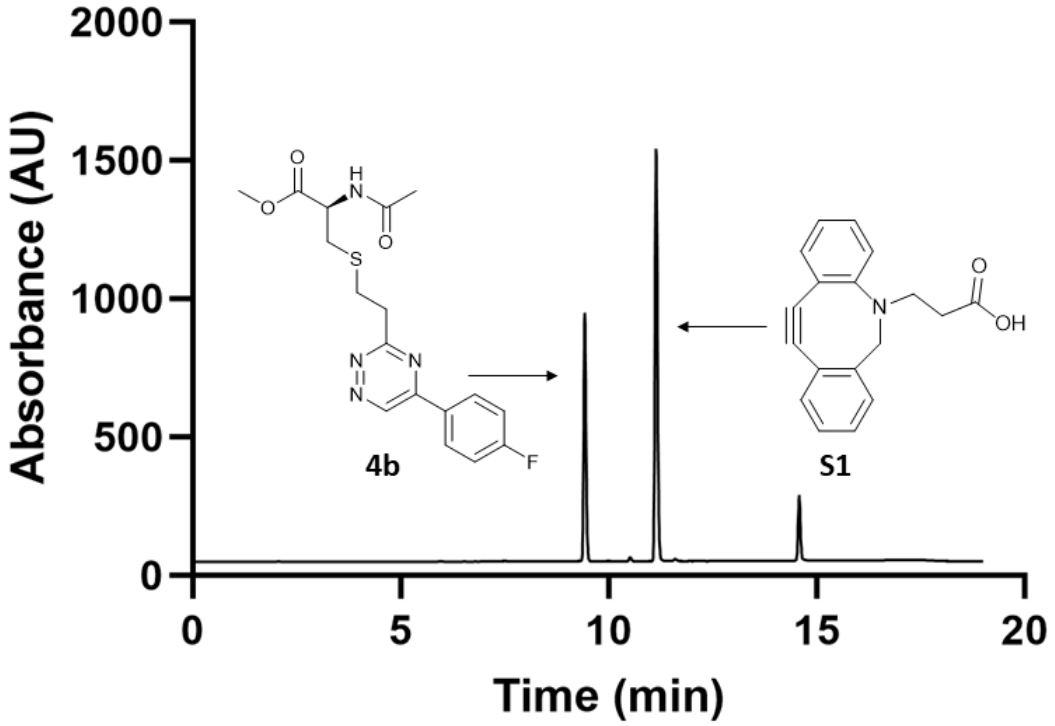


Figure S18: HPLC trace of reaction between **4a** and **S1** after 24 hours.

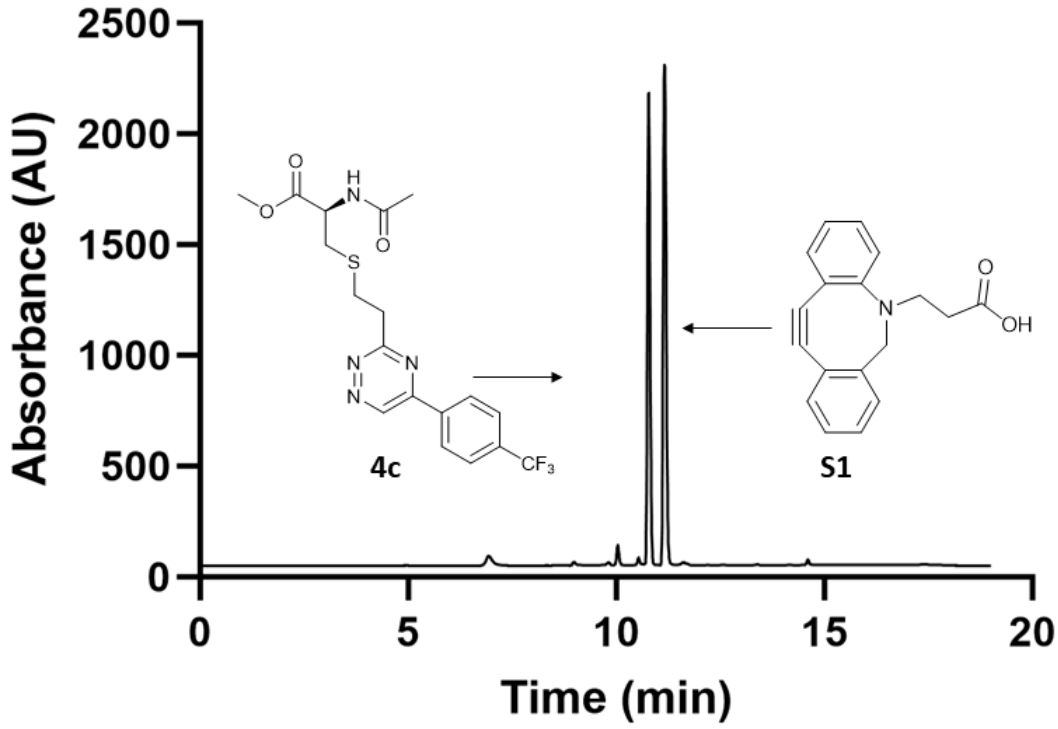


Figure S19: HPLC trace of reaction between **4c** and **S1** after 24 hours.

8. Thioether-Triazine Stability Studies

8.1. General procedure

A solution of linker-difluorobenzyl mercaptan conjugate **7**–**8** (5 mM), 1-thioglycerol (50 mM), and sodium trifluoroacetate (5 mM) in 1:1 MeCN-d_3/NaP$_i$ (pH 7.4, 50 mM in H_2O) was prepared. This solution was transferred to an NMR tube and incubated at 37 °C for 10 days. NMRs were taken each day and the integration of the internal standard set to 100. Then the integration for the two fluorine atoms originating difluorobenzyl mercaptan were recorded.

Table S3. Integration values for the stability investigation of **7a**.

Day	Integration		Average starting material remaining (%)
	Repeat 1	Repeat 2	
0	52.80	49.42	100
1	n/a	n/a	n/a
2	52.3	46.39	96
3	53.56	46.73	98
4	53.54	46.64	98
5	53.49	46.9	98
6	53.48	47.62	99
7	52.80	48.30	99
8	53.61	49.70	101
9	53.68	49.55	101
10	53.38	50.66	102

Table S4. Integration values for the stability investigation of **7b**.

Day	Integration		Average starting material remaining (%)
	Repeat 1	Repeat 2	
0	25.66	25.75	100
1	26.19	25.43	100
2	25.99	25.8	101
3	n/a	n/a	n/a
4	n/a	n/a	n/a
5	25.68	25.24	99
6	26.15	25.65	101
7	n/a	n/a	n/a
8	25.71	25.66	100
9	25.48	25.31	99
10	24.59	24.14	95

Table S5. Integration values for the stability investigation of **7c**.

Day	Integration		Average starting material remaining (%)
	Repeat 1	Repeat 2	
0	67.83	67.57	100
1	n/a	n/a	n/a
2	64.57	67.24	98
3	n/a	n/a	n/a
4	n/a	n/a	n/a
5	n/a	n/a	n/a
6	n/a	n/a	n/a
7	n/a	n/a	n/a
8	65.66	66.81	99
9	66.05	67.38	99
10	65.86	66.75	98

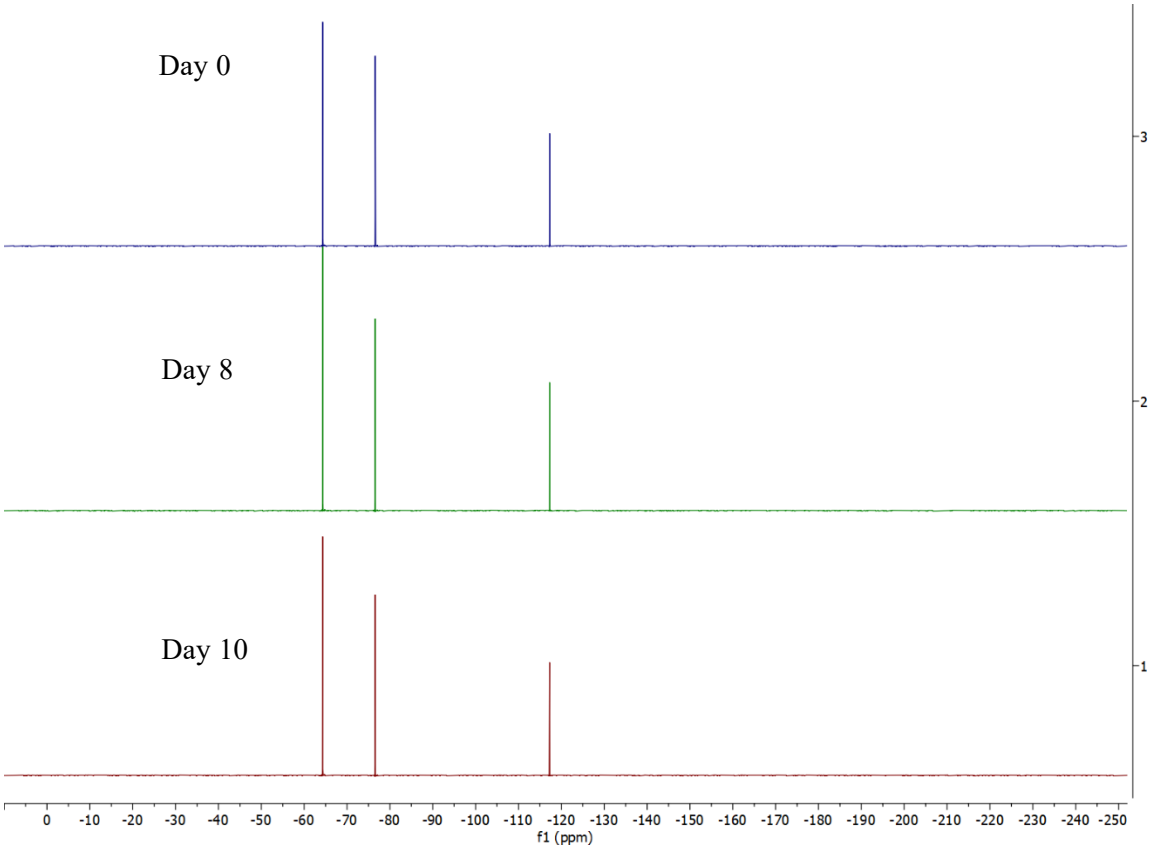


Figure S20: Representative ^{19}F NMR spectra for the stability investigation of **7c** in 3:7 MeCN-d_3/NaP$_i$ (pH 7.4, 50 mM in H_2O).

Table S6. Integration values for the stability investigation of **8**.

Day	Integration		Average starting material remaining (%)
	Repeat 1	Repeat 2	
0	83.17	93.45	100
1	n/a	n/a	n/a
2	65.29	73.75	79
3	57.15	64.71	69
4	51.53	57.62	62
5	46.44	50.31	55
6	40.19	44.71	48
7	36.10	40.81	44
8	31.90	37.21	39
9	29.82	34.66	36
10	27.35	32.04	34

9. Peptide Synthesis

Automated peptide synthesis was carried out on a CEM Liberty Automated Microwave Peptide Synthesiser using 2-chlorotrityl chloride resin pre-modified with first amino acid (H-Gly-2-Cl-Trt, loading 0.66 mmol/g) or on a Rink amide resin (loading 0.4 mmol/g). The scale for each resin-bound peptide was 0.25 mmol. All automatic peptide couplings were performed with Fmoc-protected amino acids (0.2M), Oxyma pure (1M) and DIC (1M) as coupling reagents in DMF. Fmoc deprotection was achieved with a solution of 20 % piperidine in DMF at 23 °C for 2 × 10 min. After automated peptide synthesis were all resin-bound peptides transferred to plastic syringes each equipped with a porous disk, washed with CH_2Cl_2 (5×10 mL), DMF (2×10 mL) and CH_2Cl_2 again (2×10 mL) and stored dried in a freezer.

Upon peptide synthesis, UV-LCMS analysis was performed as follows: analytical sample of a resin (~5 mg) was transferred into a plastic Eppendorf tube, treated with a cleavage cocktail (CH_2Cl_2/TFA 1:1, 1 mL, v/v) and shaken for 1–3 hours at ambient temperature. For peptides containing trityl protecting group, 100 μL of TIPS was added to a cleavage cocktail with TFA as a quencher. The cleavage cocktails were then evaporated under a stream of nitrogen, cleaved compounds extracted into DMSO, filtered, and submitted to UV-LCMS analysis to determine purity of prepared peptides.

For manual solid-phase synthesis plastic syringes were used, each equipped with a porous disk. All external reagents were dissolved in a glass beaker in an appropriate solvent and added to the resin at once. The volume of used solvent was 1 mL per 100 mg of resin, unless stated otherwise. All manual solid-phase reactions were performed at ambient temperature. For manual washing, resin slurry was shaken with the fresh solvent for at least 1 min before changing the solvent. Manual Fmoc deprotection has been performed with 5 % DBU in CH_2Cl_2 (1 mL per 100 g of resin) at ambient temperature for 20 minutes.

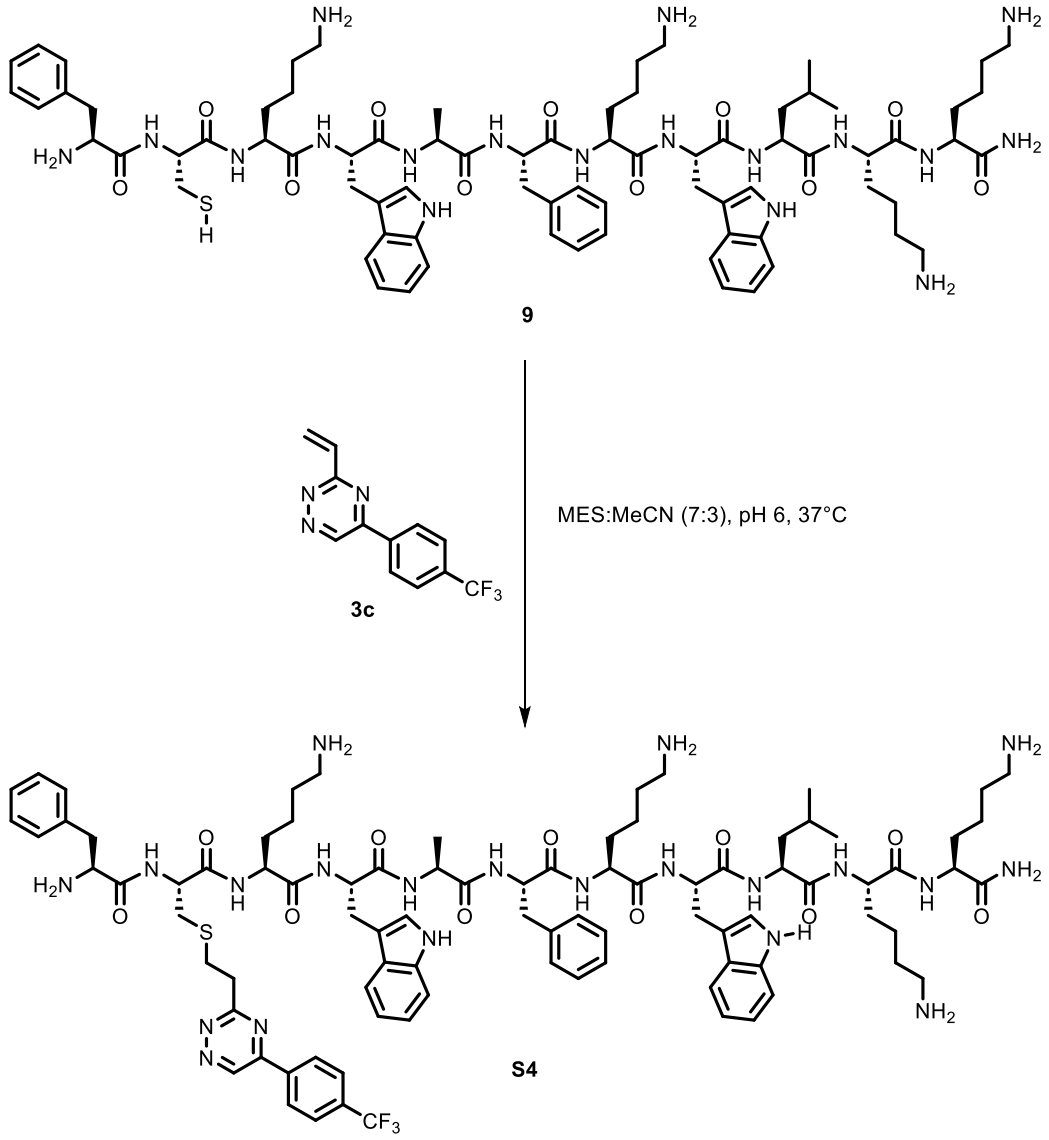
For the full cleavage of peptides from the resin prior to HPLC purification was used cleavage cocktail TFA/H_2O/TIPS (95:2.5:2.5) and the cleavage time has been prolonged to 4 hours. The cocktail was then transferred into 1 mL Eppendorf, spun on a centrifuge and then the cleavage cocktails have been evaporated under stream of nitrogen and crude products have been suspended in DMSO prior to HPLC purification.

General procedure for N-terminal acetylation:

Fmoc deprotected resin (200 mg) has been washed with CH_2Cl_2 (3×10 mL). DMAP (0.015M) was dissolved in CH_2Cl_2 (2 mL) and acetic anhydride (0.15M) has been added subsequently. The reaction mixture was added to a plastic syringe with the resin at once and the slurry has been shaken for 16 hours at ambient temperature. Resin has been washed with CH_2Cl_2 (3×10 mL), DMF (3×15 mL) and CH_2Cl_2 (5×10 mL), small portion has been cleaved and analysed by UV-LCMS.

10. Peptide Modification

10.1. Modification of 9

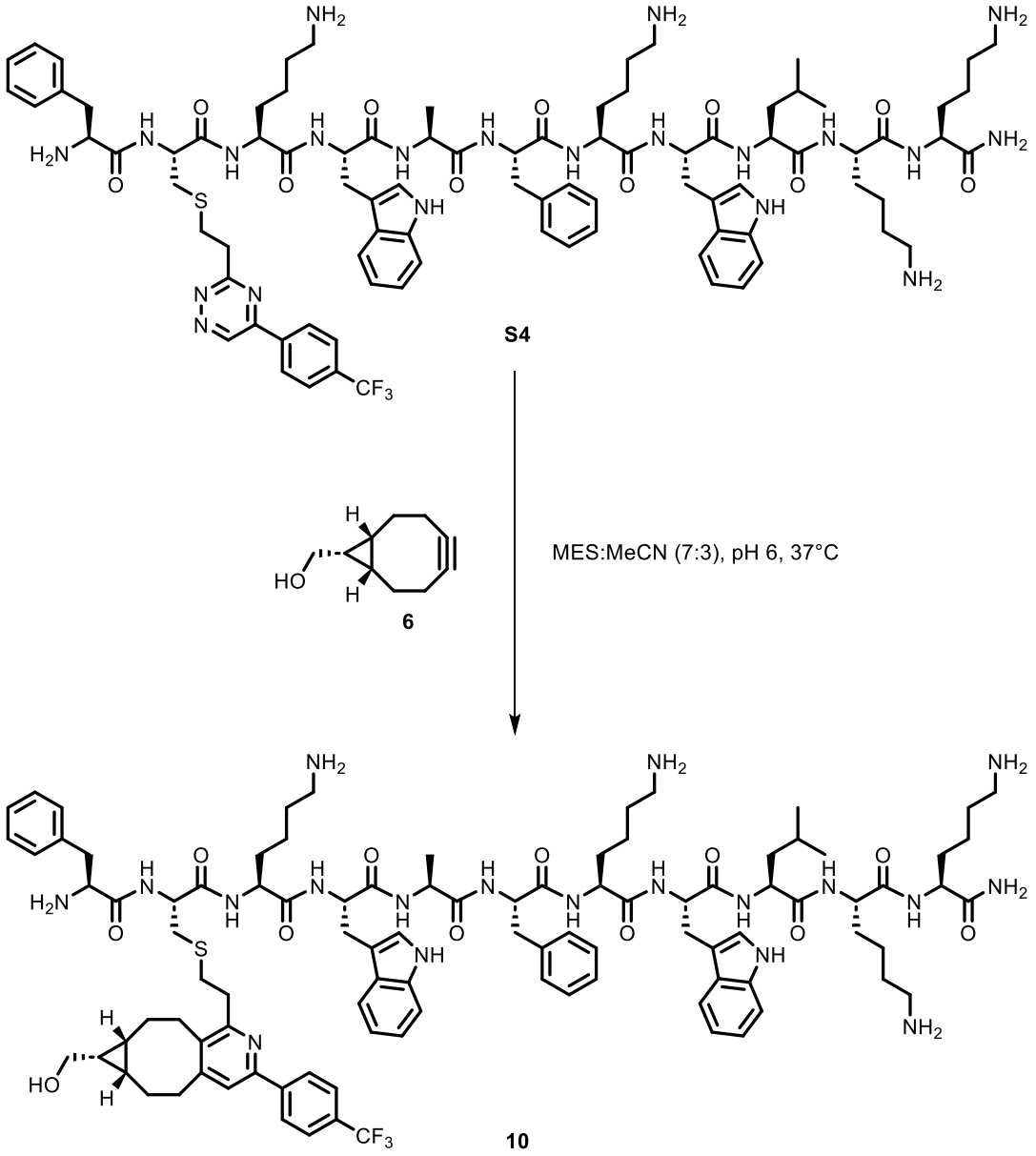


Scheme S2: Reaction of **9** with 1 equiv. of **3c** to afford **S4**.

To a stirred solution of **9** (10mM, 1.35 mL) in 3:7 MeCN:MES (pH6, 50mM) at 37 °C was added 944 µL of MES (pH6, 50mM) followed by a solution of **3c** (406 µL, 33 mM) in MeCN. The reaction mixture was stirred at 37 °C for 18 hours. The resultant mixture was purified *via* preparative LCMS (0–35% MeCN (0.1% formic acid):H_2O (0.1% formic acid)) to afford the desired product **S3** as the formic acid salt and a white fluffy solid (>90% conversion *via* HPLC, 12.3 mg, 46% yield).

LRMS (ESI) calcd. for $C_{88}H_{121}F_3N_{21}O_{11}S$ *m/z* 1736.92 $[M+H]^{3+}$, found 1736.44.

10.2. Modification of S4

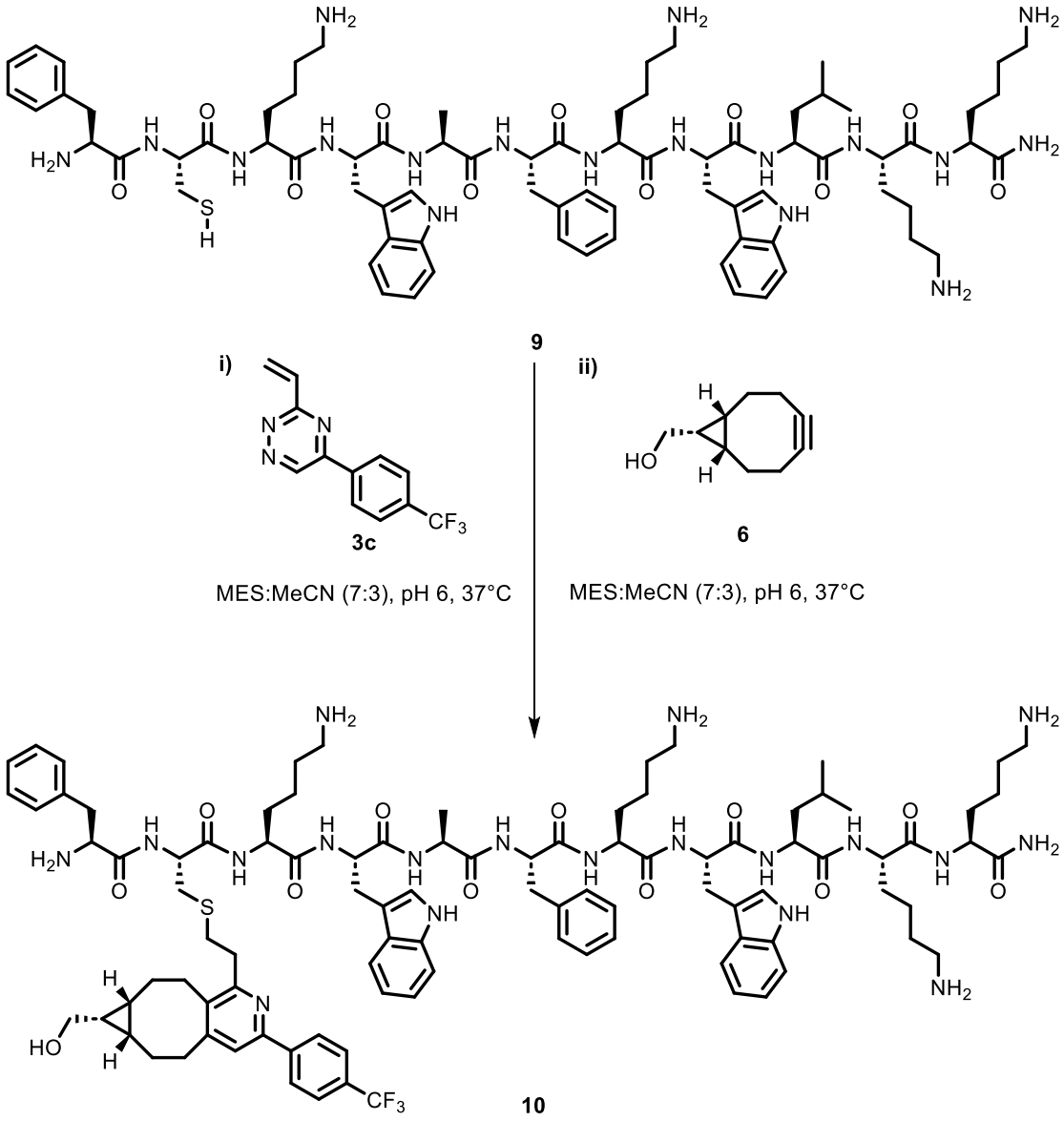


Scheme S3: Reaction of **S4** with 1 equiv. of **6** to afford **10**.

To a stirred solution of **S4** (10mM, 0.422 mL) in 3:7 MeCN:MES (pH6, 50mM) at 37 °C was added a solution of **6** (0.844 mL, 10mM) in 3:7 MeCN:MES (pH6, 50mM). The reaction mixture was stirred at 37 °C for 5 hours. The resultant mixture was purified via preparative LCMS (0-35% MeCN (0.1% formic acid):H_2O (0.1% formic acid) to afford the desired product **10** as the formic acid salt and a white fluffy solid (>95 % conversion *via* HPLC, 11.7 mg, 76% yield).

LRMS (ESI) calcd. for $C_{98}H_{131}F_3N_{19}O_{12}S$ *m/z* 1854.99 $[M-H]^-$, found 1855.78.

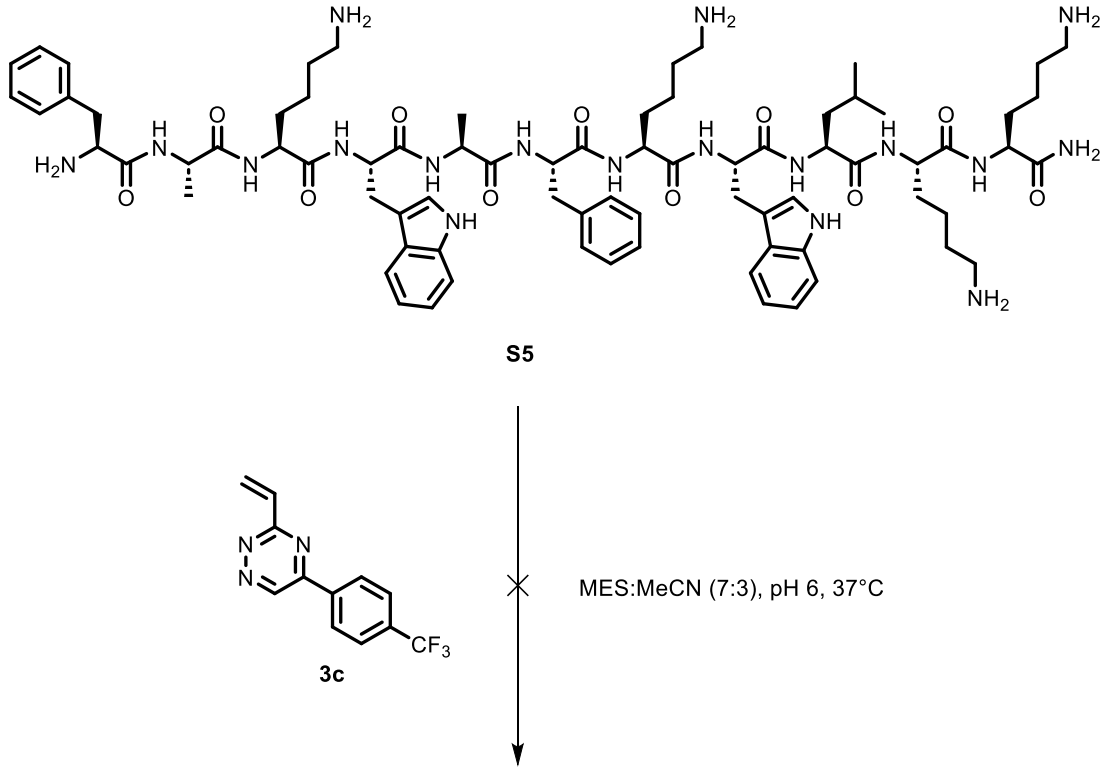
10.3. One-Pot Modification of 9



Scheme S4: One-pot modification of **9** with **3c** and **6**.

To a stirred solution of **9** (10 mM, 504 µL) in 3:7 MeCN:MES (pH 6, 50 mM) at 37 °C was added 353 µL of MES (pH 6, 50 mM) followed by a solution of **3c** (152 µL, 33 mM) in MeCN. The reaction mixture was stirred at 37 °C for 25 hours. Then was added a **6** (3.1 mg, 0.02 mmol). The reaction mixture was stirred at 37 °C for 17 hours. The resultant mixture was purified *via* preparative LCMS (0-95% MeCN (0.1% formic acid):H_2O (0.1% formic acid)) to afford the desired product **10** as the formic acid salt and a white fluffy solid (2.80 mg, 30% yield).

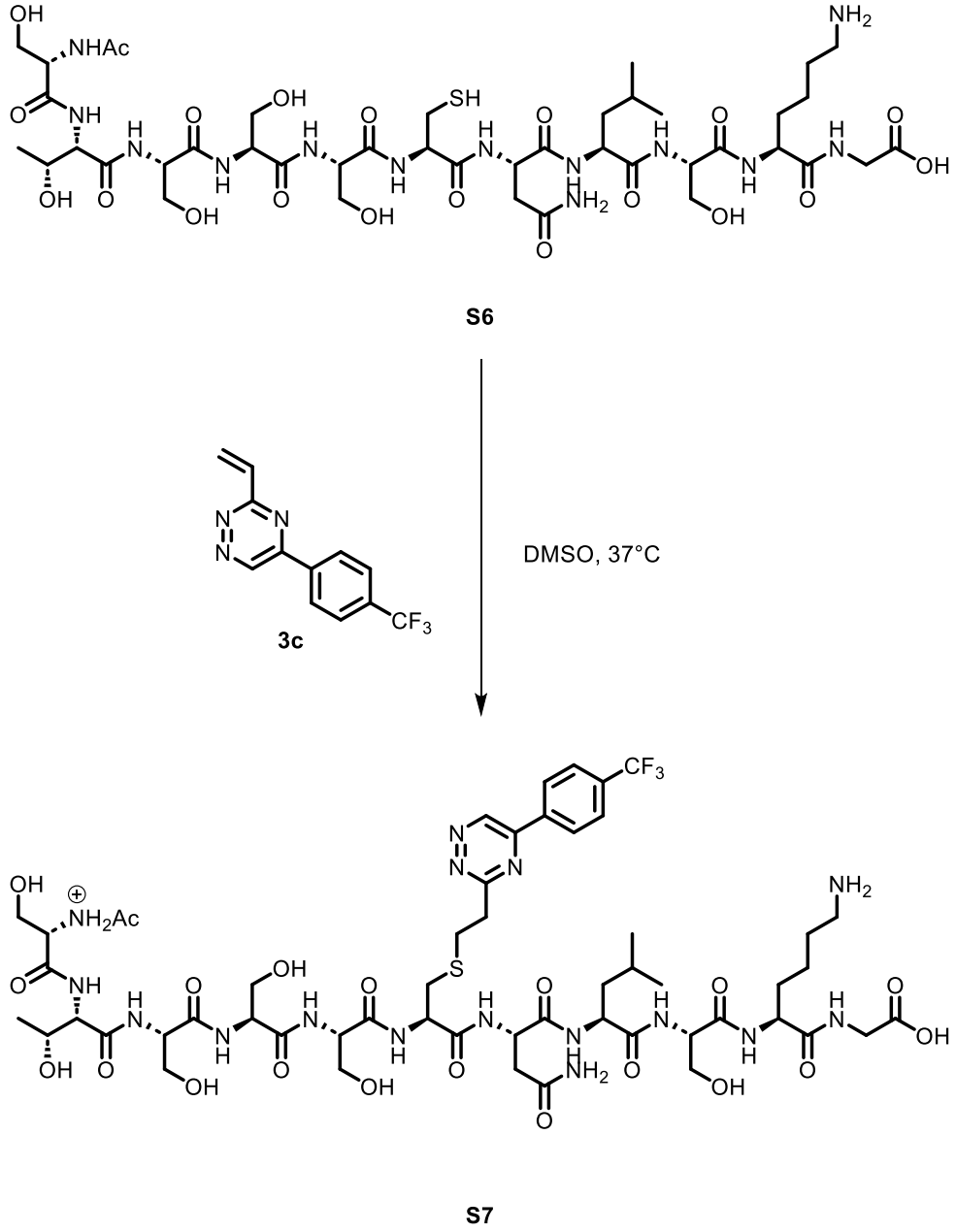
10.4. Attempted Modification of S5



Scheme S5: The attempted modification of **S5** with **3c**.

To a stirred solution of **S5** (10 mM, 386 µL) in 3:7 MeCN:MES (pH 6, 50 mM) at 37 °C was added 270 µL of MES (pH 6, 50 mM) followed by a solution of **3c** (116 µL, 33 mM) in MeCN. The reaction mixture was stirred at 37 °C for 210 hours.

10.5. Modification of S6

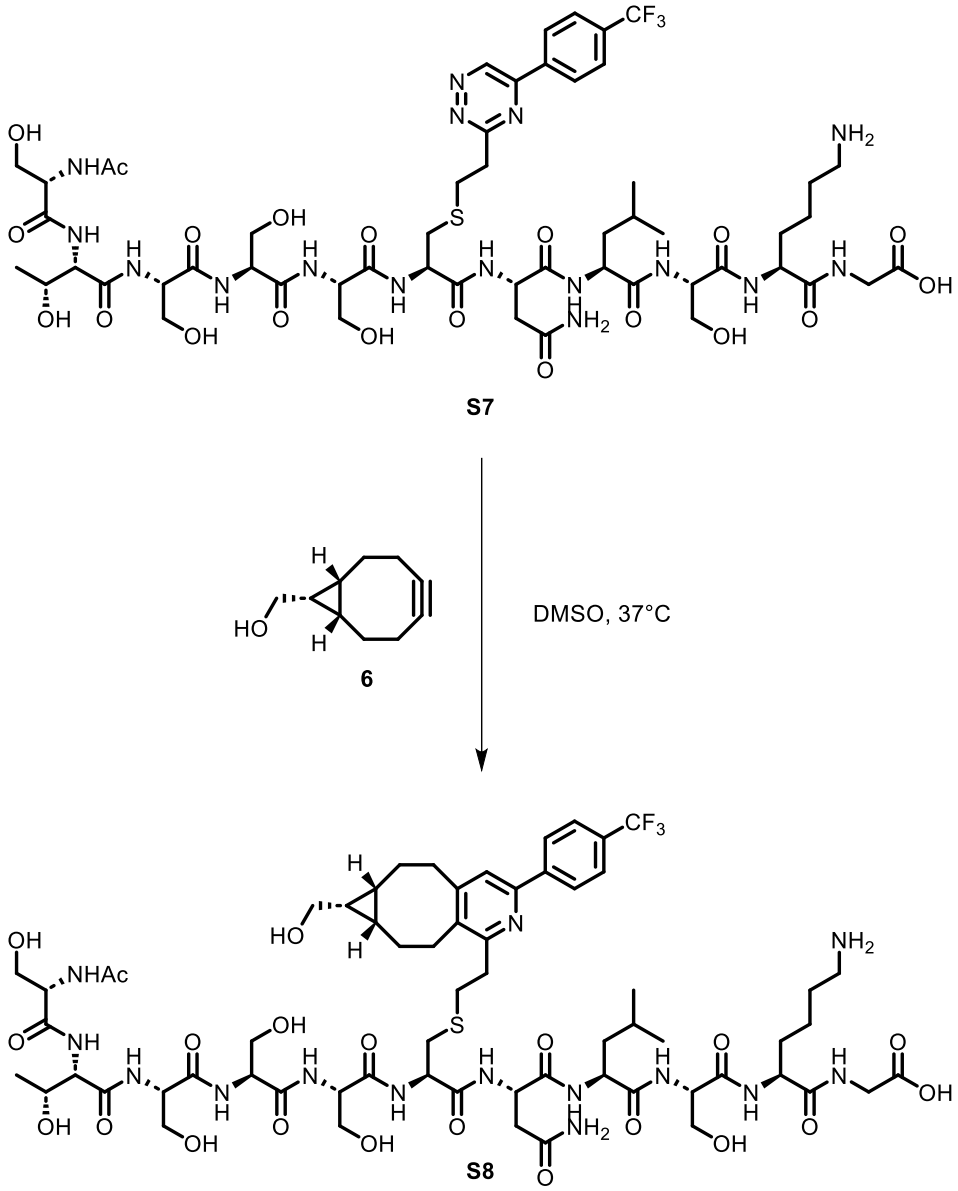


Scheme S6: The modification of **S6** with **3c**.

To **S6** (5.00 mg, 4.50 µmol) and **3c** (6.00 mg, 23.8µmol) was added 4.5mL of DMSO. The reaction mixture was stirred at 37 °C for 22 hours. The resultant mixture was purified via preparative LCMS (0-95% MeCN (0.1% formic acid):H_2O (0.1% formic acid) to afford the desired product **10** as the formic acid salt and a white fluffy solid (2.1 mg, 34 % yield).

LRMS (ESI) calcd. for $C_{54}H_{81}F_3N_{16}O_{20}S$ *m/z* 1362.55 $[M-H]^-$, found 1388.83.

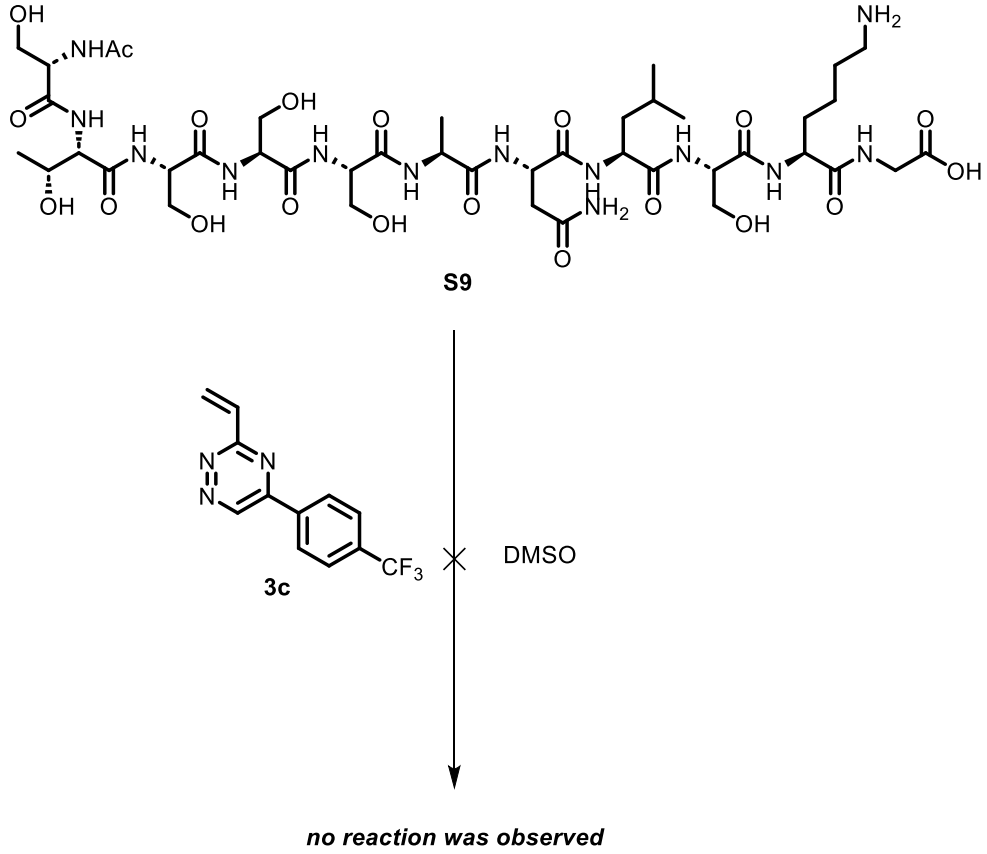
10.6. Modification of S7



Scheme S7: The modification of **S7** with **6**.

To **S7** (1.9 mg, 1.39 µmol) was added a solution of **6** (5 mM, 1.4 mL) in DMSO. The reaction mixture was stirred at 37 °C for 19 hours. A crude HPLC shows consumption of the **S7** to **S8**, however **S8** was not isolated.

10.7. Attempted Modification of S9



Scheme S8: Attempted modification of **S9** with **3c**.

To a solution of **S9** in DMSO (2 mM, 0.4 mL) was added a solution of **3c** in DMSO (2 mM, 0.4 mL). The reaction mixture was stirred at 37 °C for 24 hours. No reaction was observed.

10.8. HPLC traces of peptides

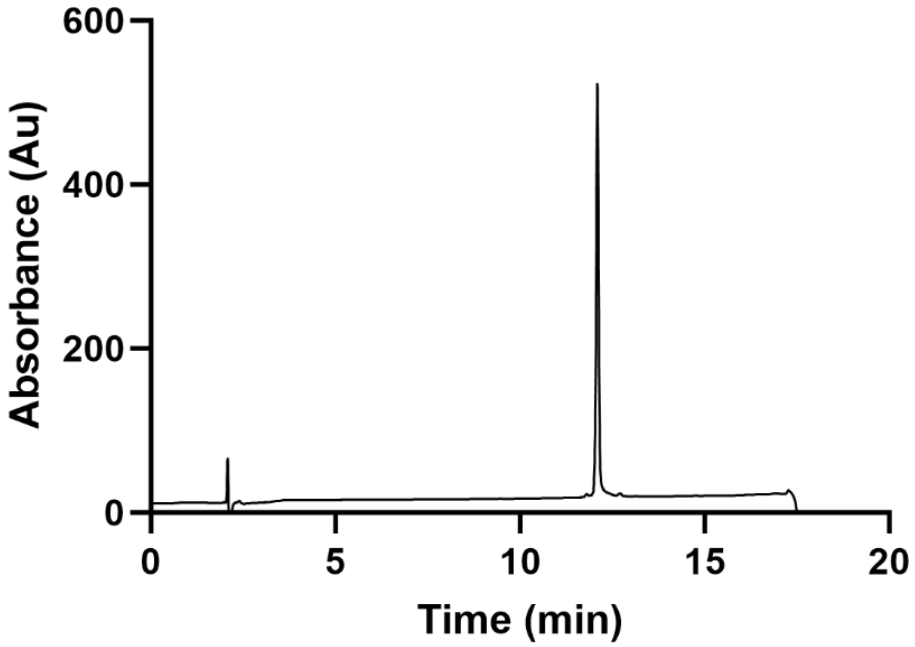


Figure S21: HPLC trace of **9** at a gradient of a gradient of 4-40% MeCN (0.05% TFA):H_2O (0.05% formic acid).

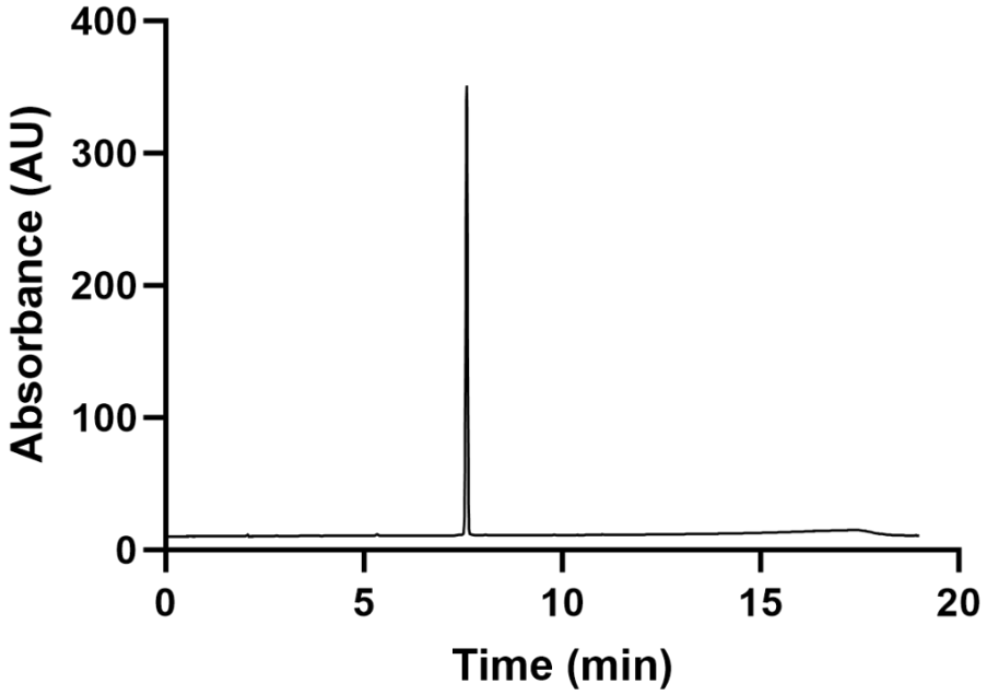


Figure S22: HPLC traces of **S4** at gradient 0-95% MeCN (0.05% TFA):H_2O (0.05% formic acid).

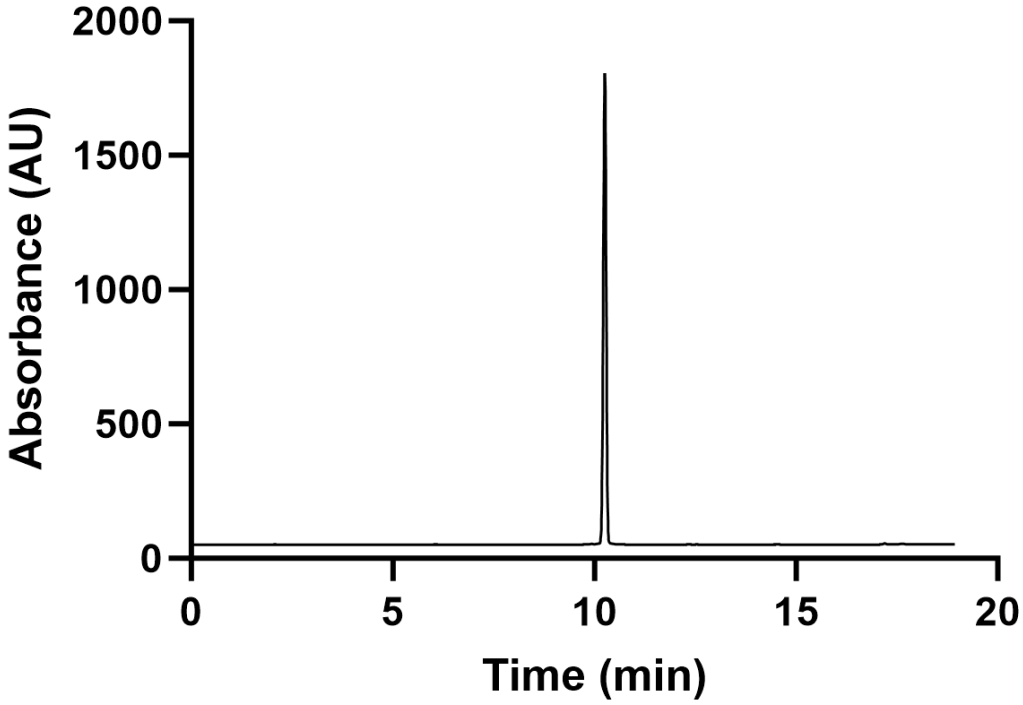


Figure S23: HPLC traces of **S4** at gradients 20-60% MeCN (0.05% formic acid):H_2O (0.05% formic acid) indicating >95% purity.

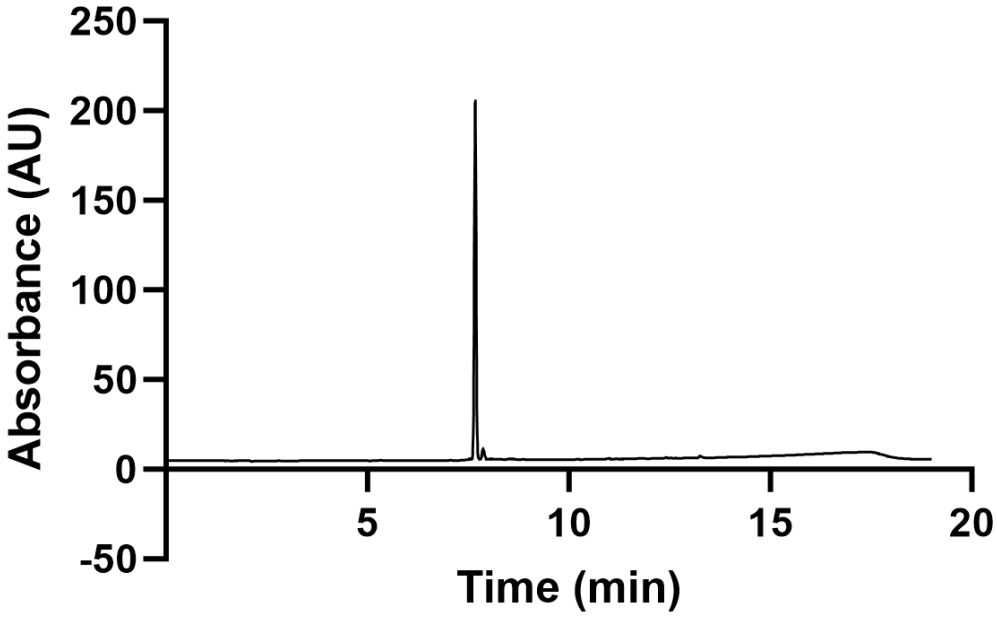


Figure S24: HPLC traces of **10** at gradients 0-95% MeCN (0.05% TFA):H_2O (0.05% formic acid).

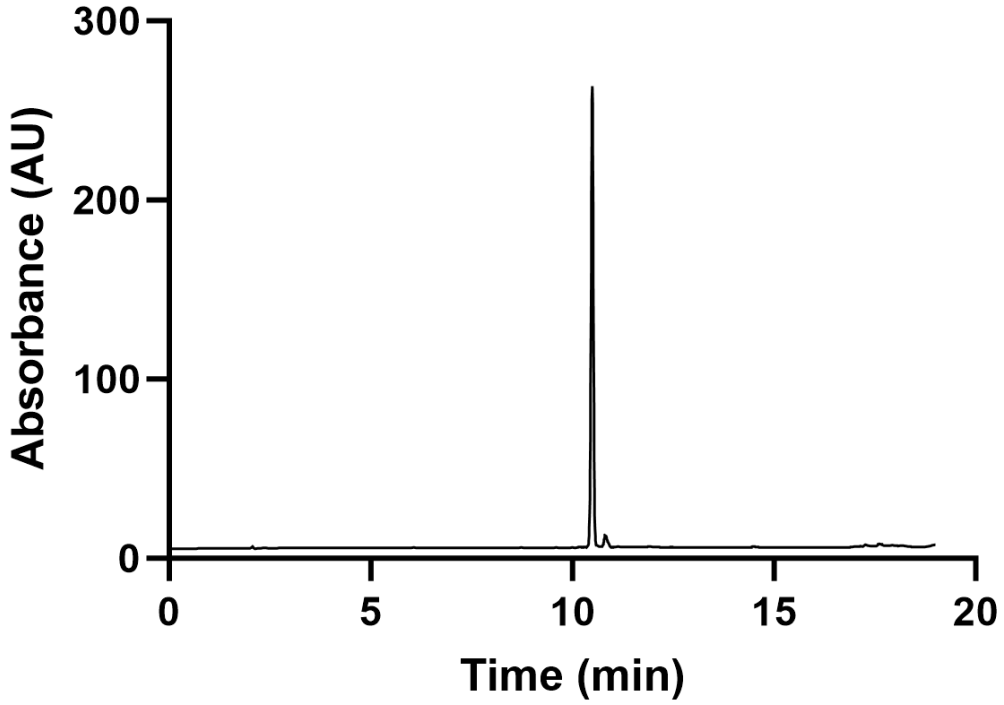


Figure S25: HPLC traces of **10** at gradients 20-60% MeCN (0.05% formic acid):H_2O (0.05% formic acid) indicating >95% purity.

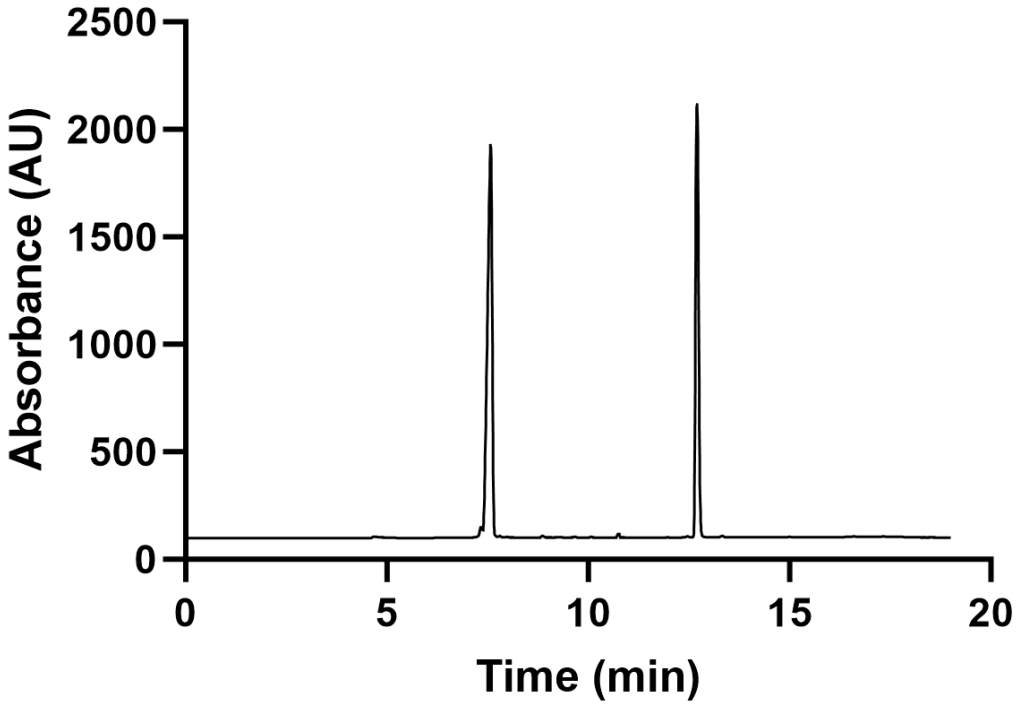


Figure S26: HPLC traces for the attempted modification of **S5** with **3c** at time 0 min.

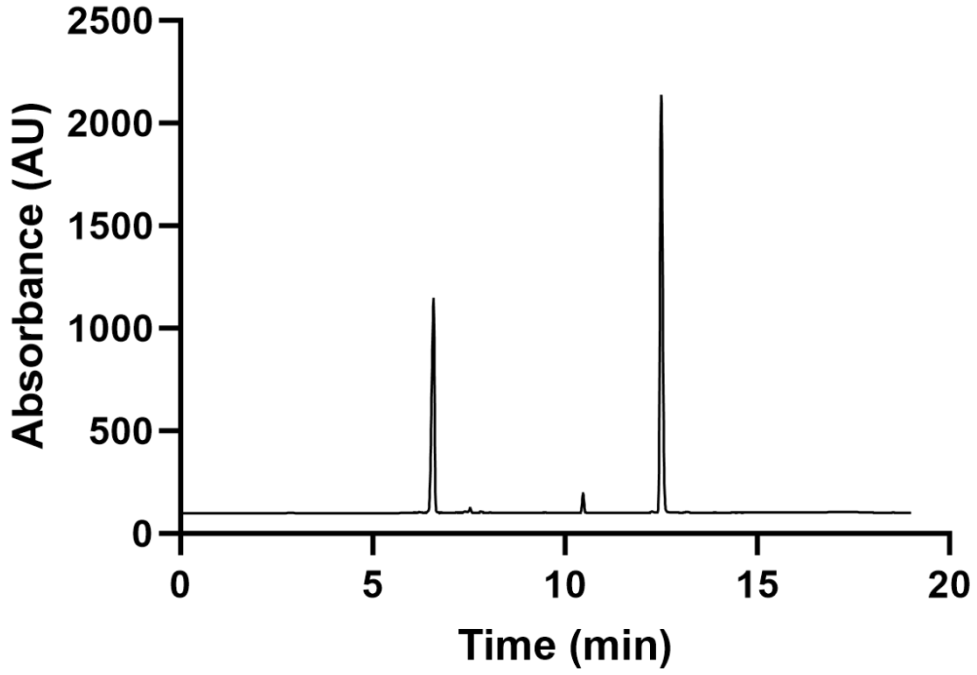


Figure S27: HPLC traces for the attempted modification of **S5** with **3c** at time 16 hours.

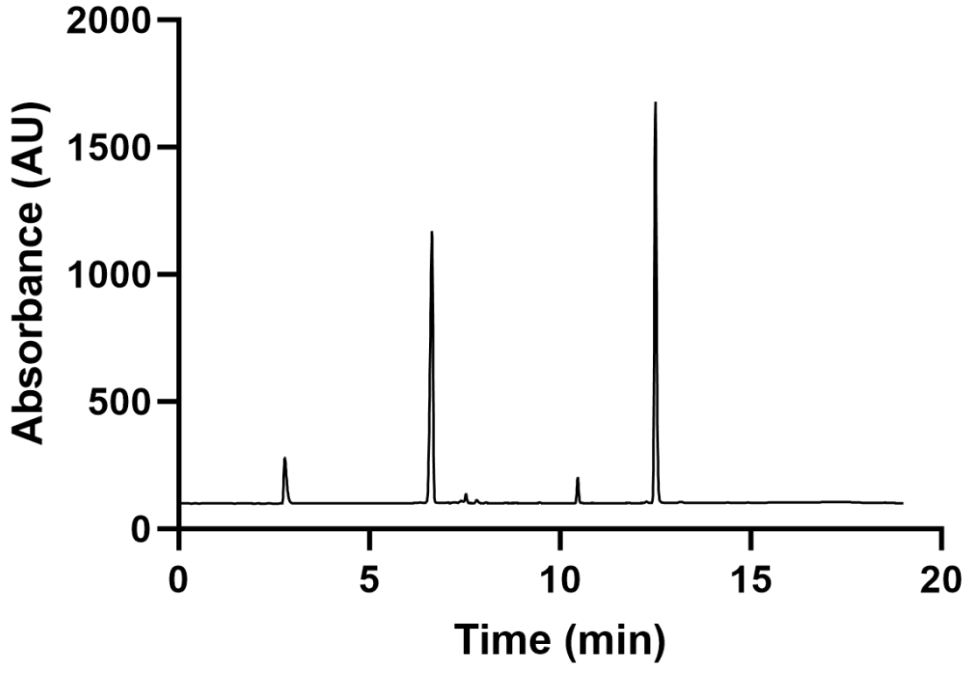


Figure S28: HPLC traces for the attempted modification of **S5** with **3c** at time 24 hours.

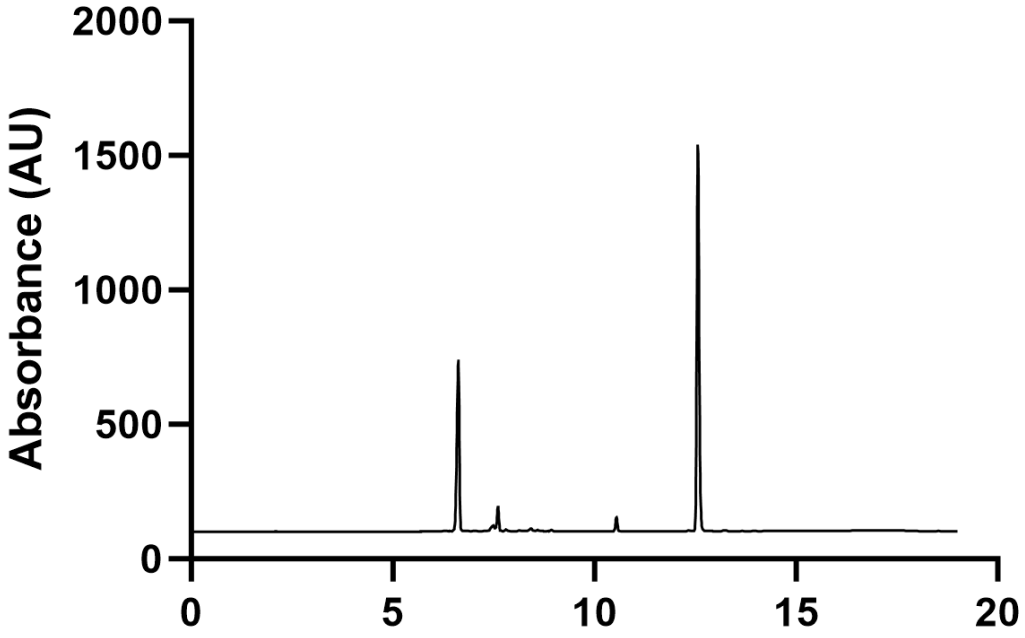


Figure S29: HPLC traces for the attempted modification of **S5** with **3c** at time 210 hours (a week).

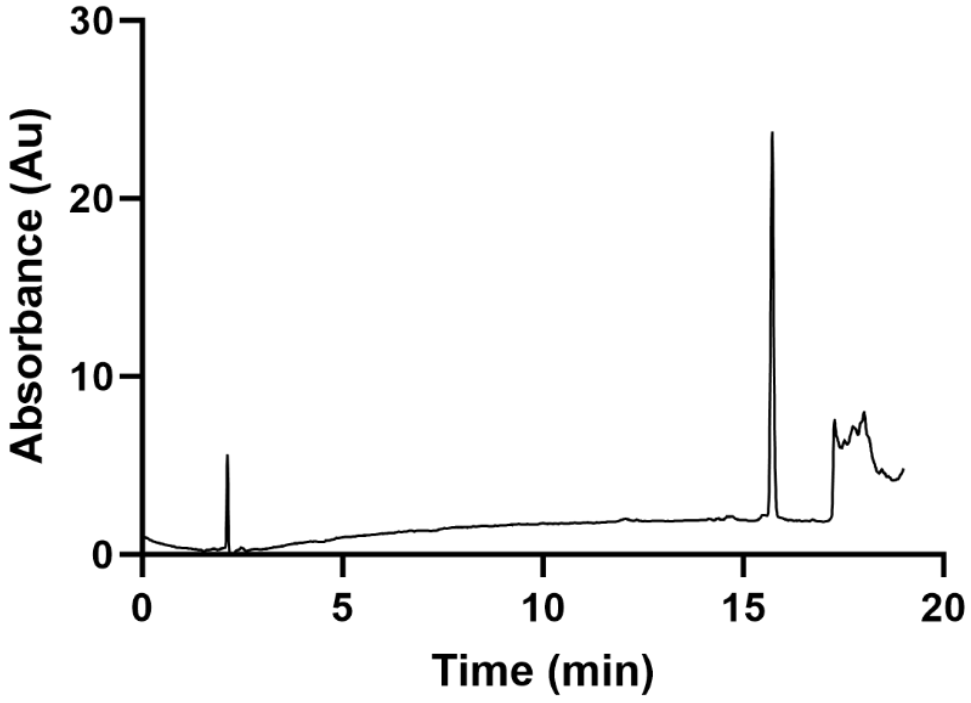


Figure S30: HPLC trace of **S7** at a gradient of 5-35% MeCN (0.05% TFA):H_2O (0.05% formic acid).

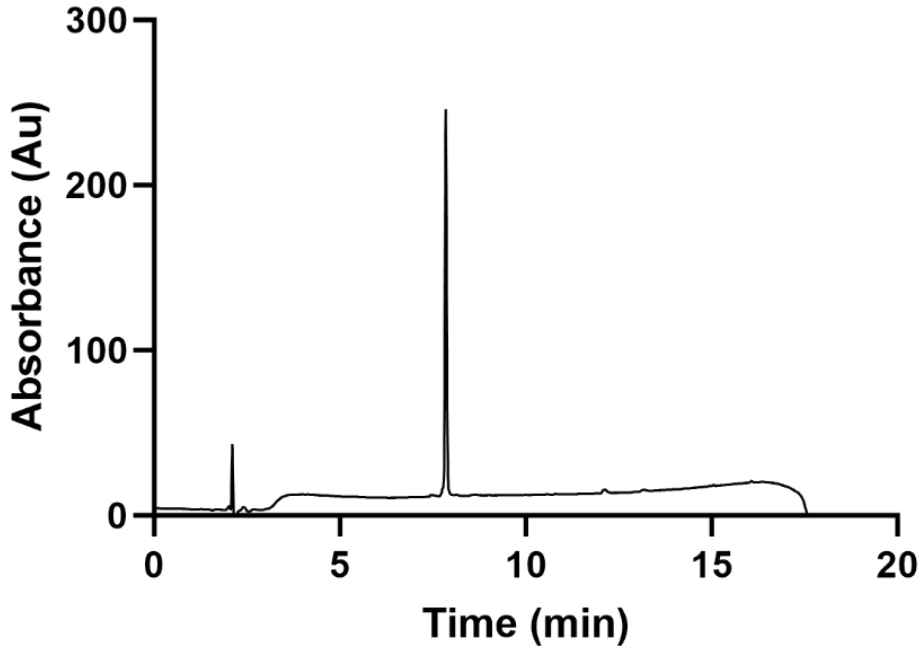


Figure S31: HPLC trace of **S7** at a gradients of 5-95% MeCN (0.05% TFA):H_2O (0.05% formic acid).

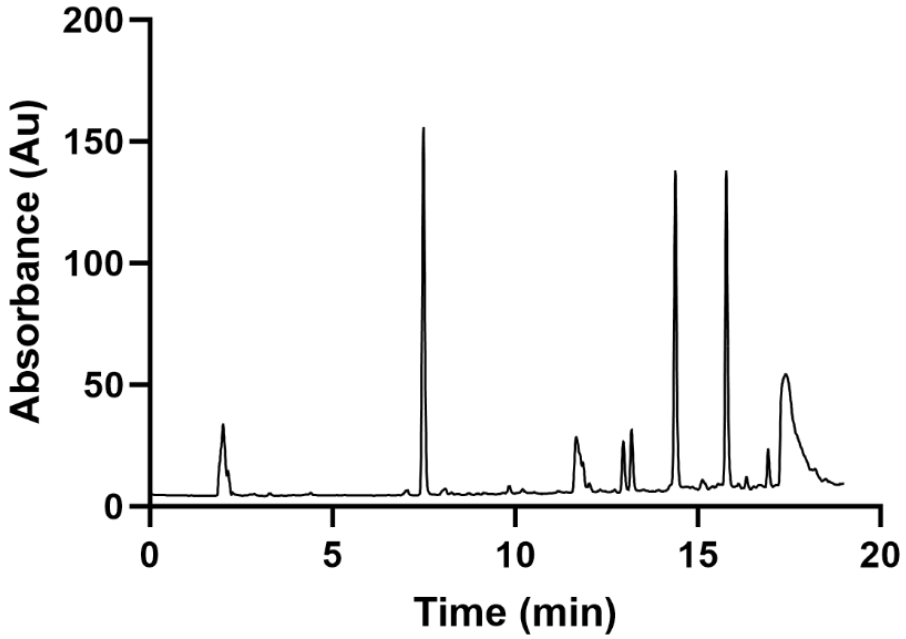


Figure S32. Crude HPLC trace of the reaction between **S7** and **6** at 19 hours at a gradient of 5-35% MeCN (0.05% TFA):H_2O (0.05% formic acid).

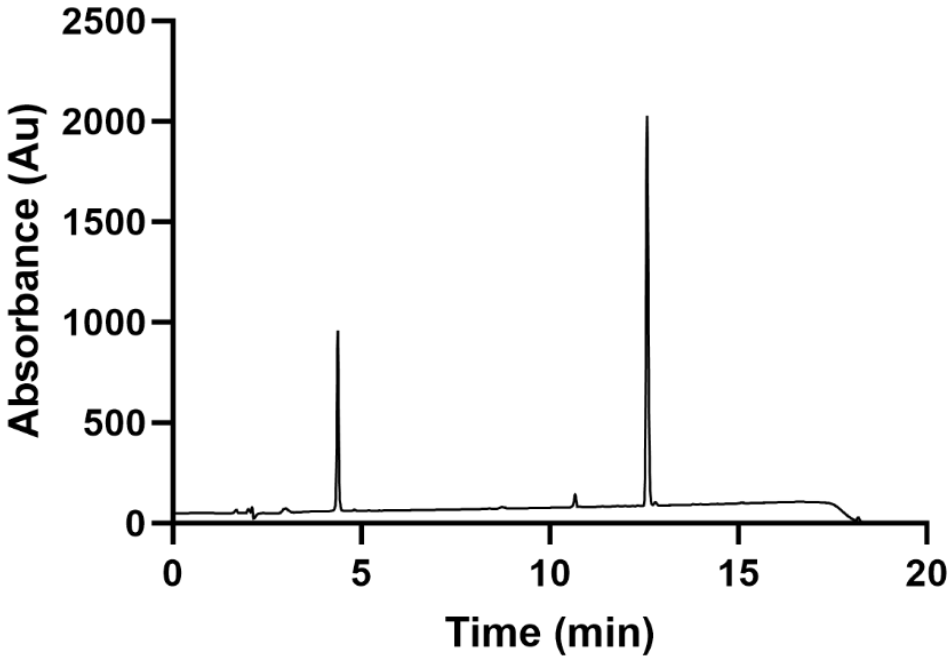


Figure S33. The HPLC trace of the reaction between **S9** and **3c** after 24hr.

10.9. Mass Spectrometry of Peptides

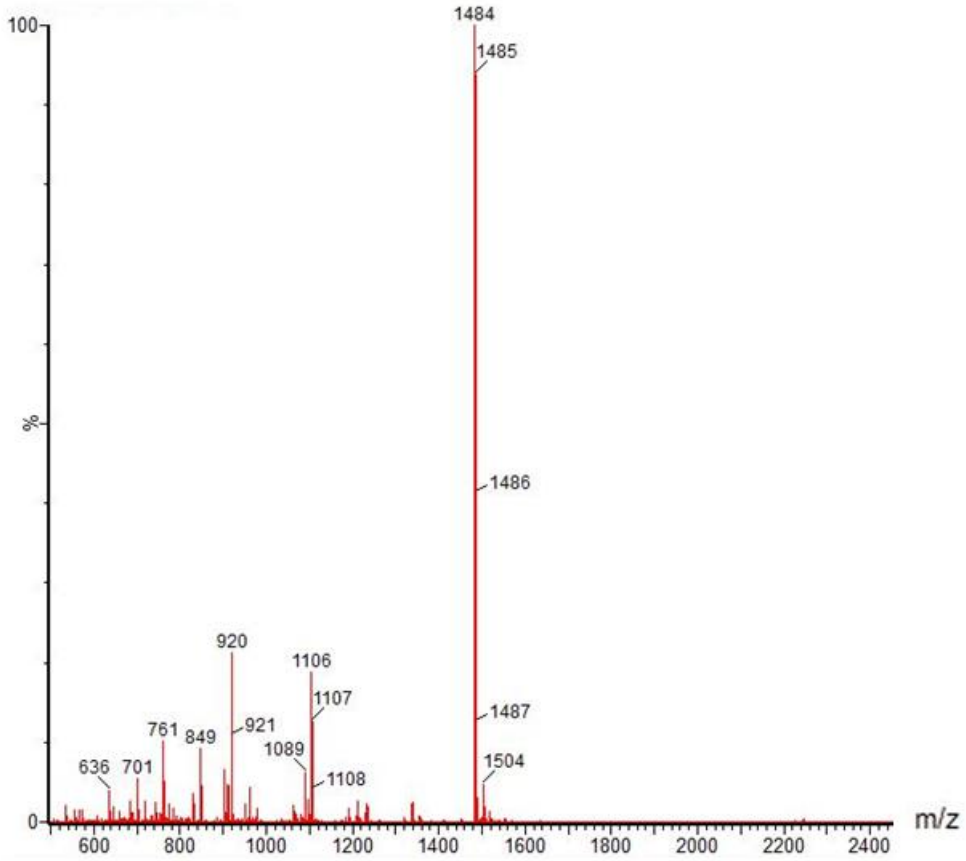


Figure S34. The mass spectrum of **9**.

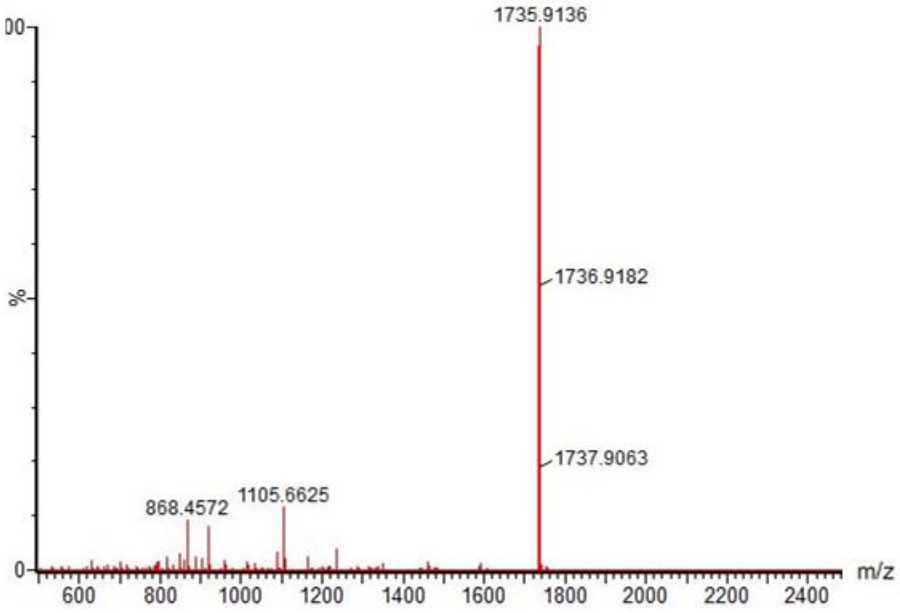


Figure S35. The mass spectrum of **S4**.

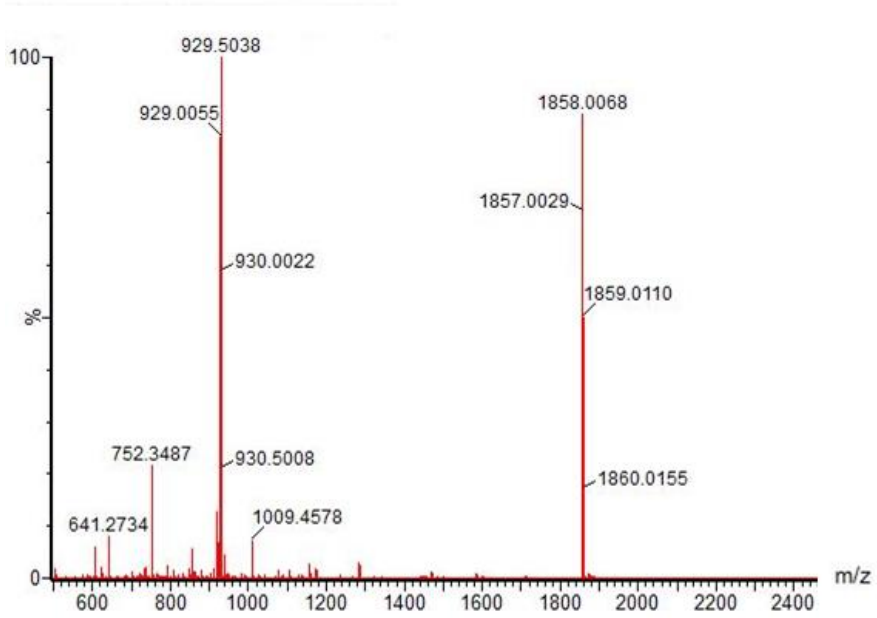


Figure S36. The mass sectrum of **10**.

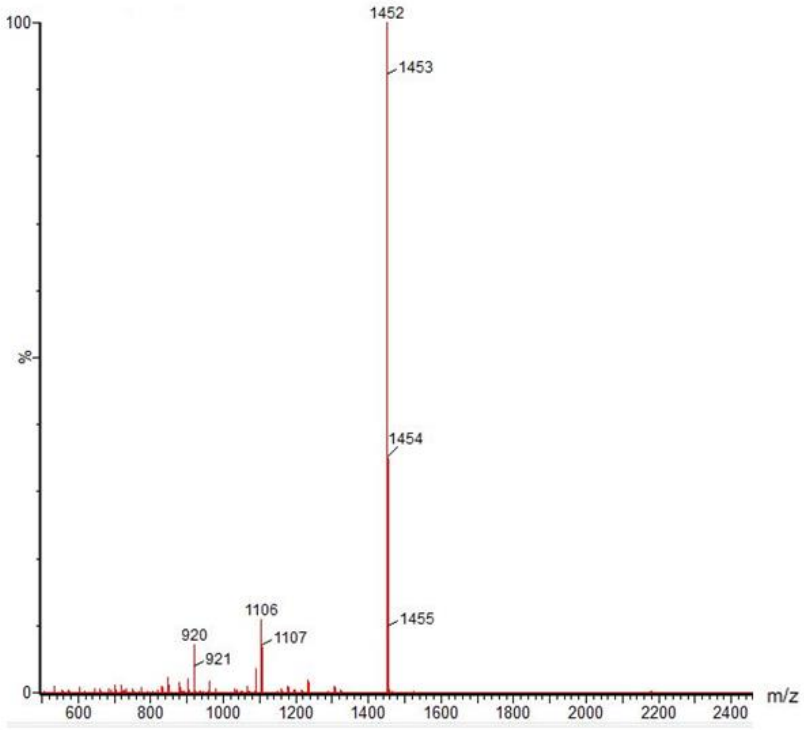


Figure S37: The mass spectrum of **S5.**

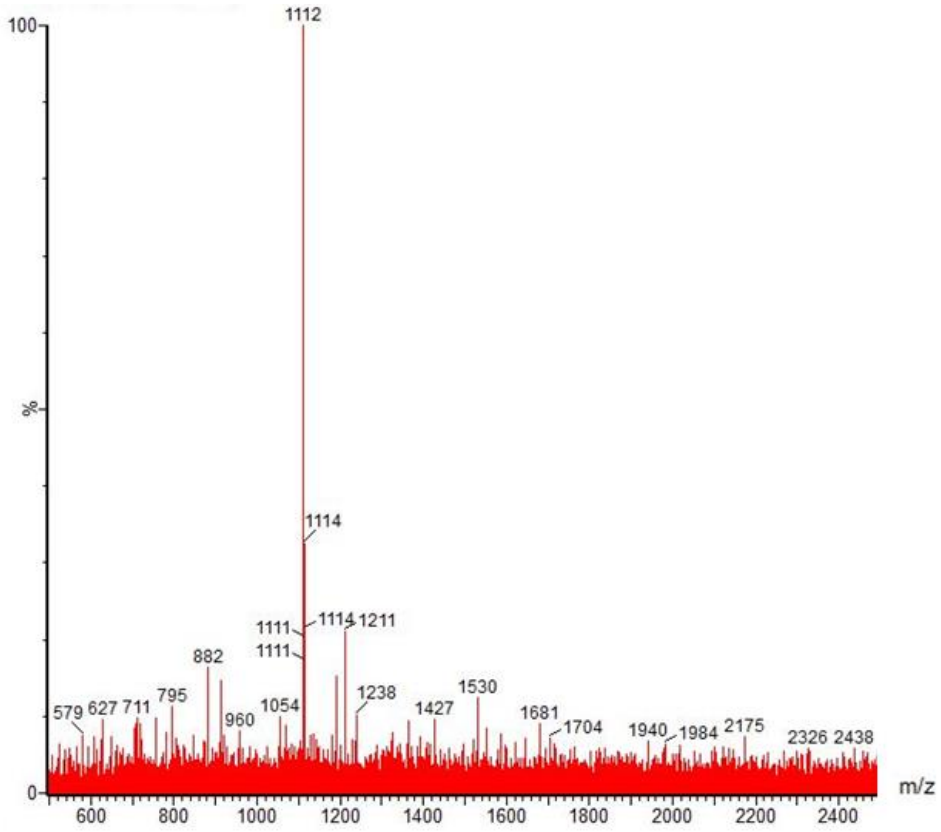


Figure S38: The mass spectrum of **S6**

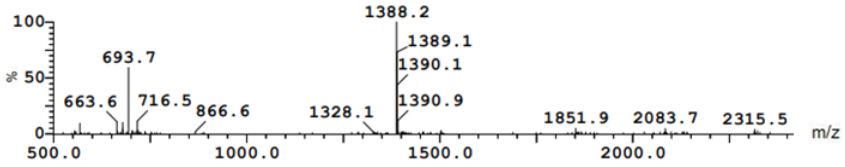


Figure S39: The mass spectrum of **S7**.

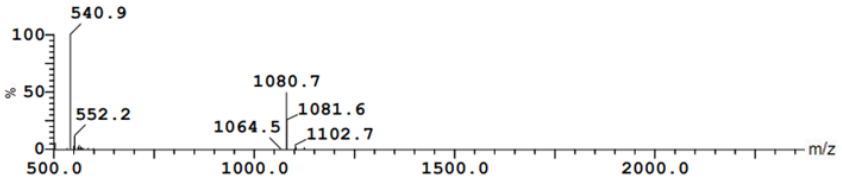
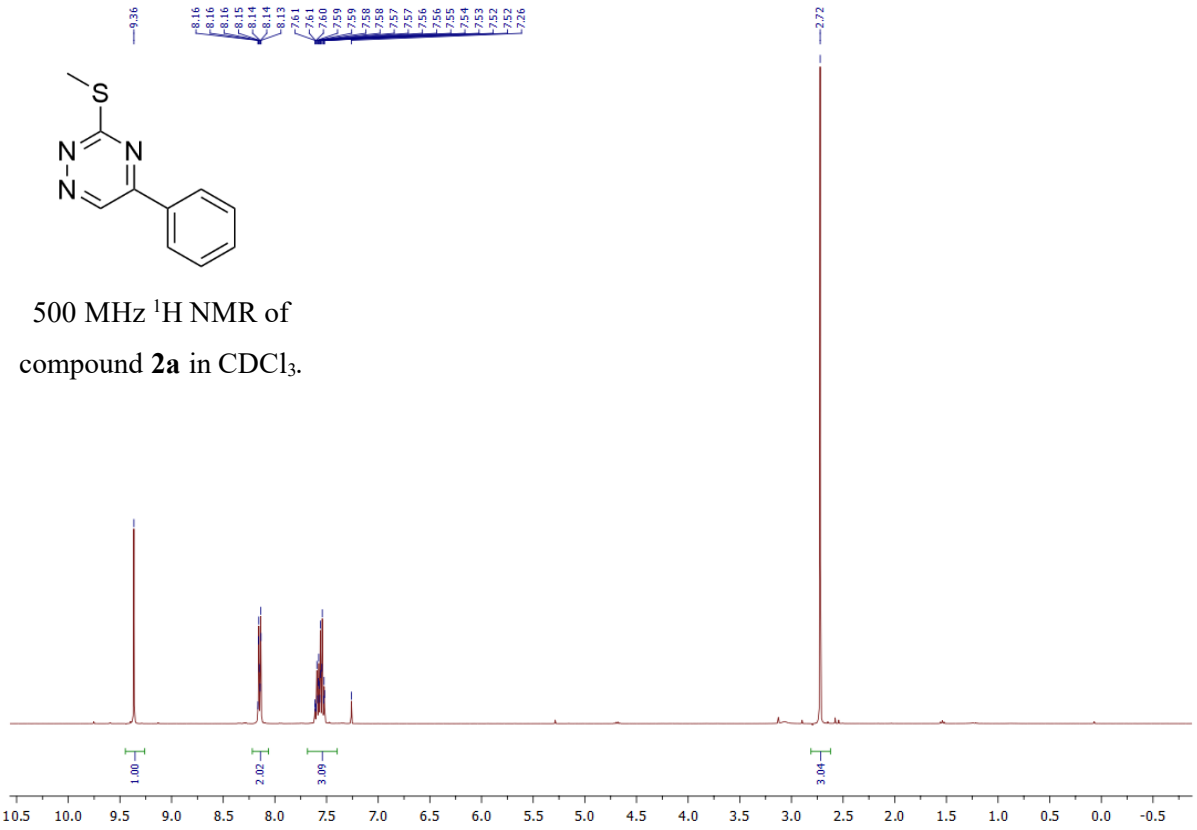


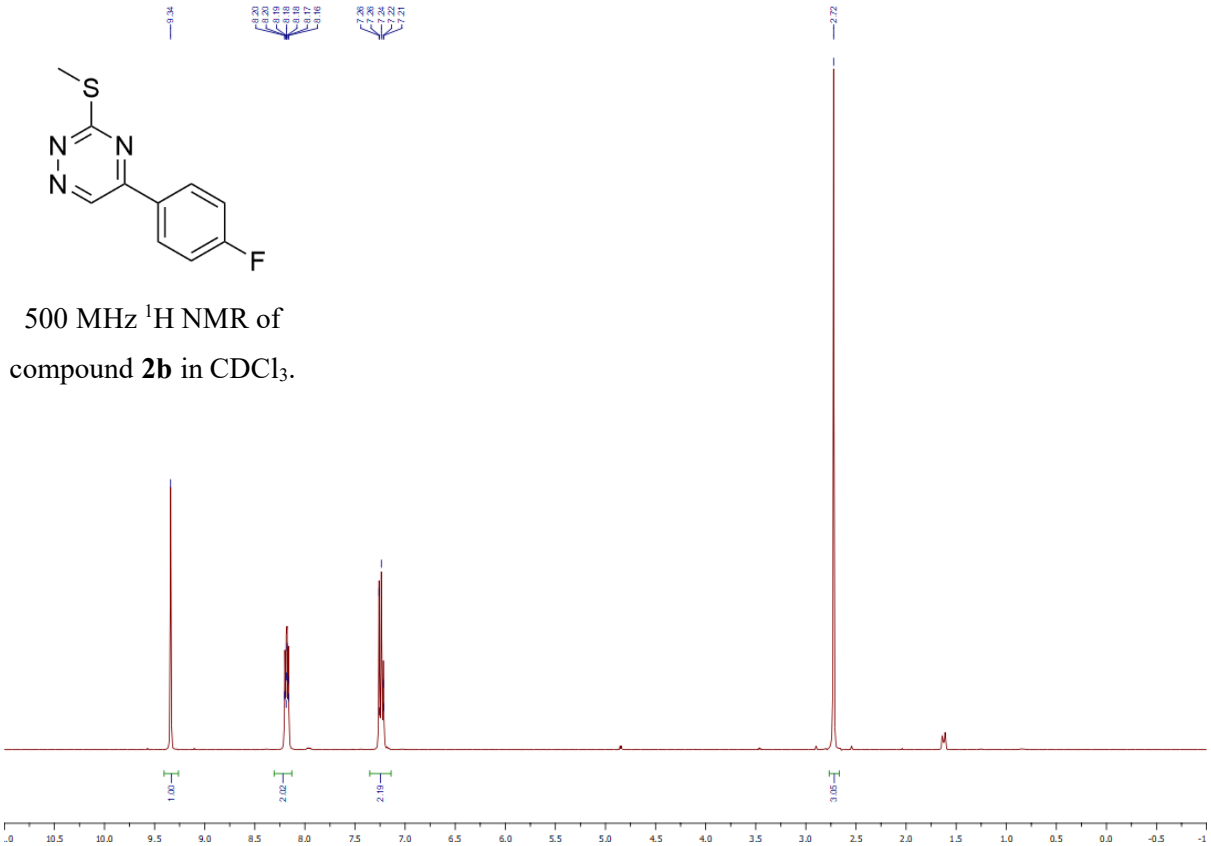
Figure S40: The mass spectrum of **S9**

11. NMR

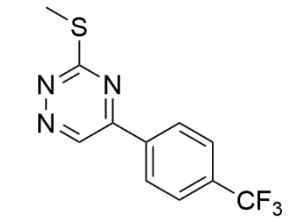
11.1 NMRs of compounds



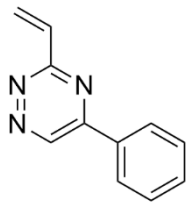
500 MHz ^{1}H NMR of compound **2a** in $CDCl_3$.



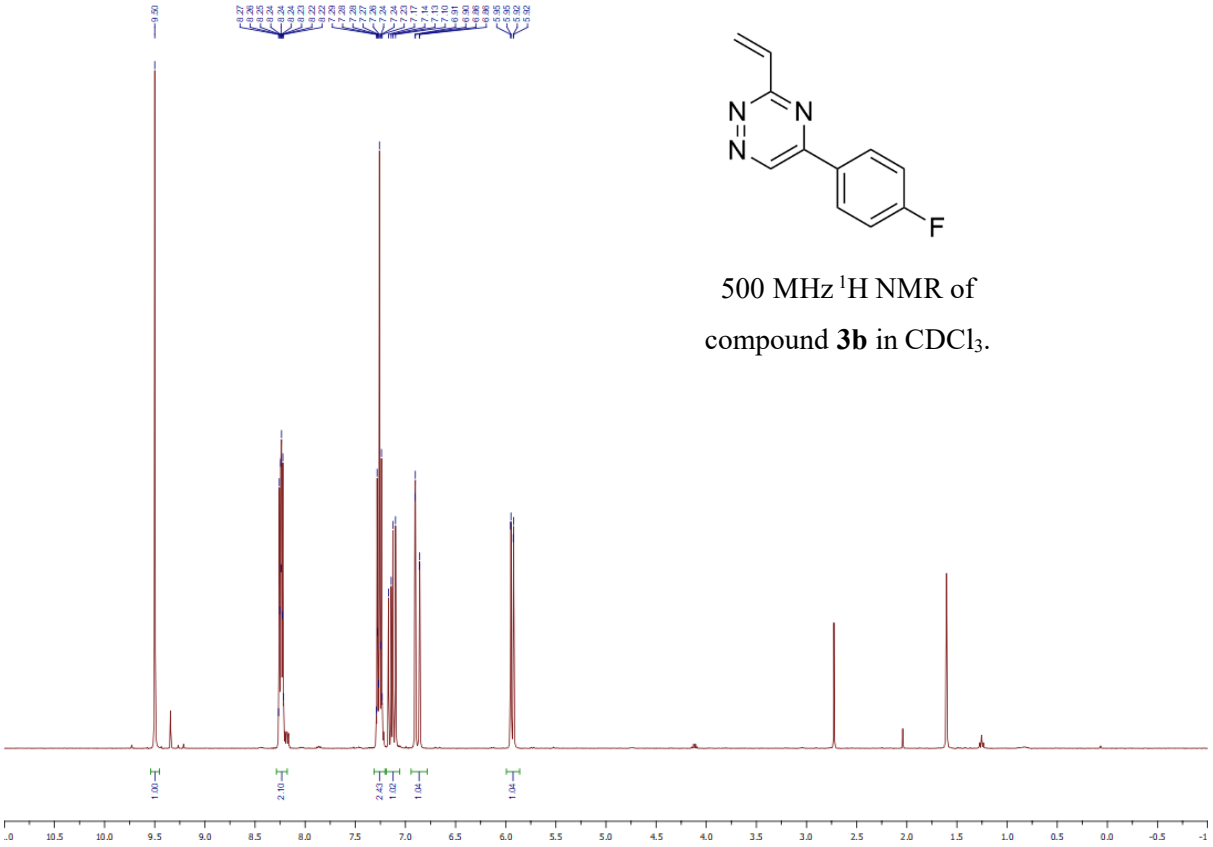
500 MHz ^{1}H NMR of compound **2b** in $CDCl_3$.



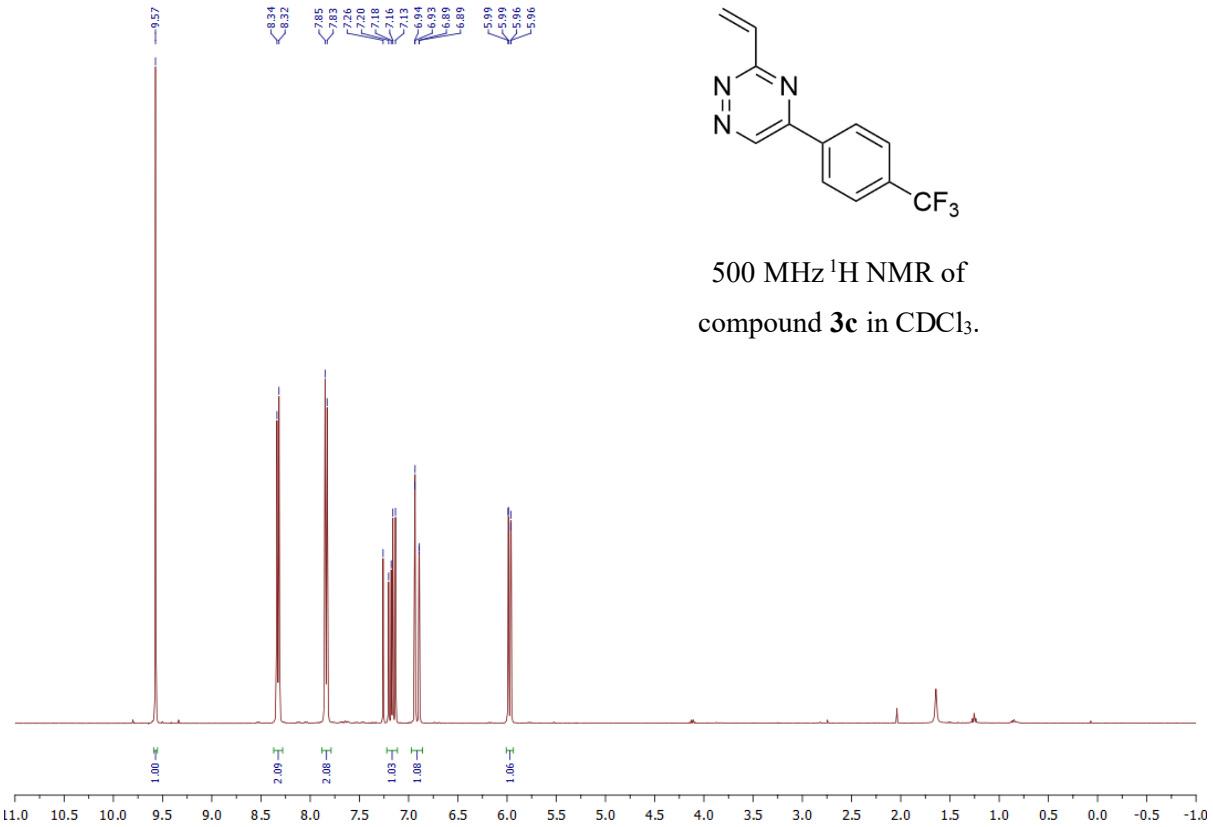
400 MHz ^{1}H NMR of compound **2c** in $CDCl_3$.



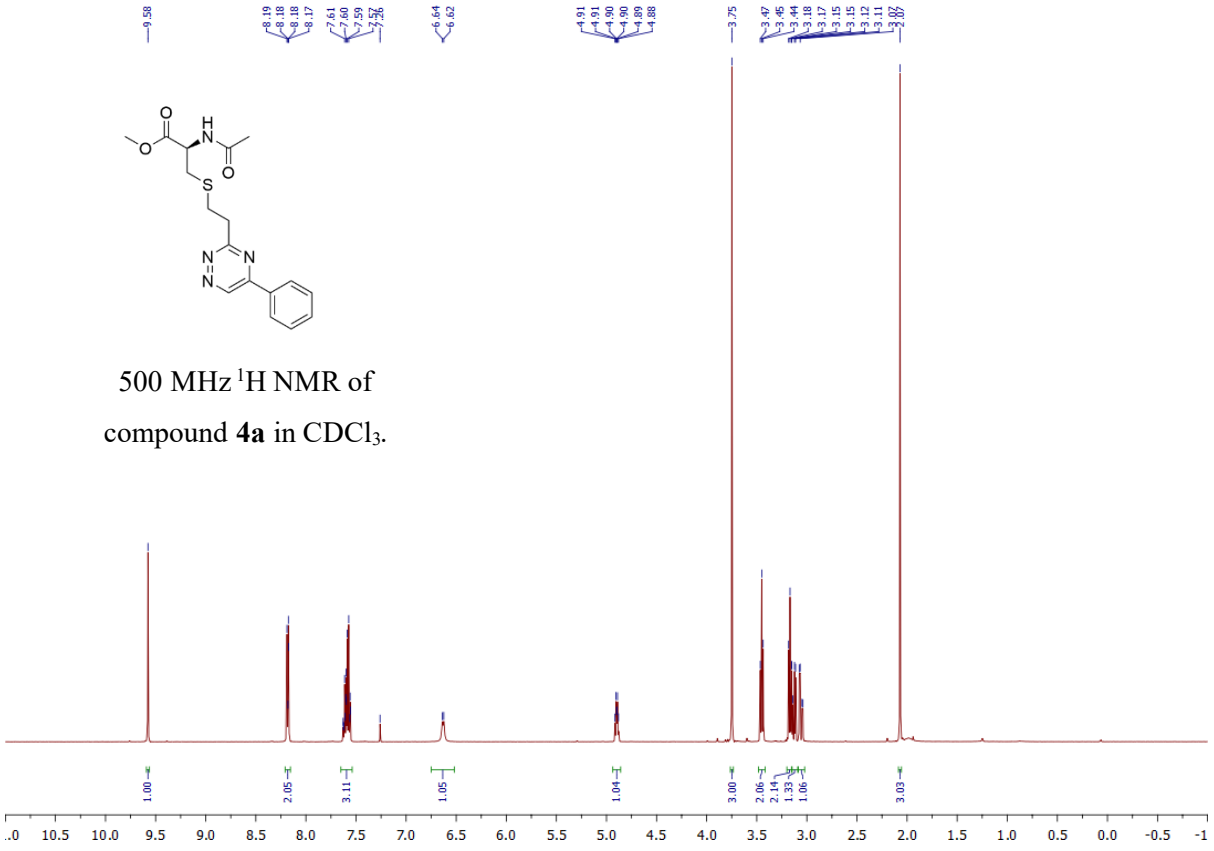
500 MHz ^{1}H NMR of compound **3a** in CD_2Cl_2.



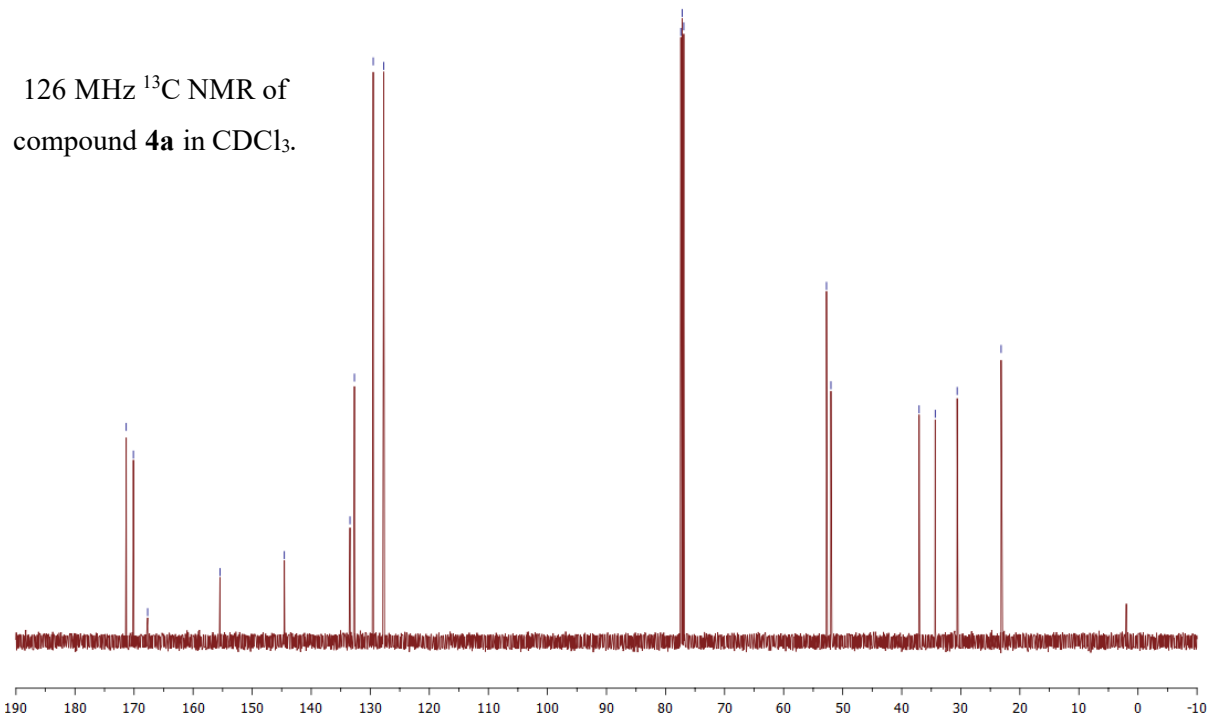
500 MHz ^{1}H NMR of compound **3b** in $CDCl_3$.



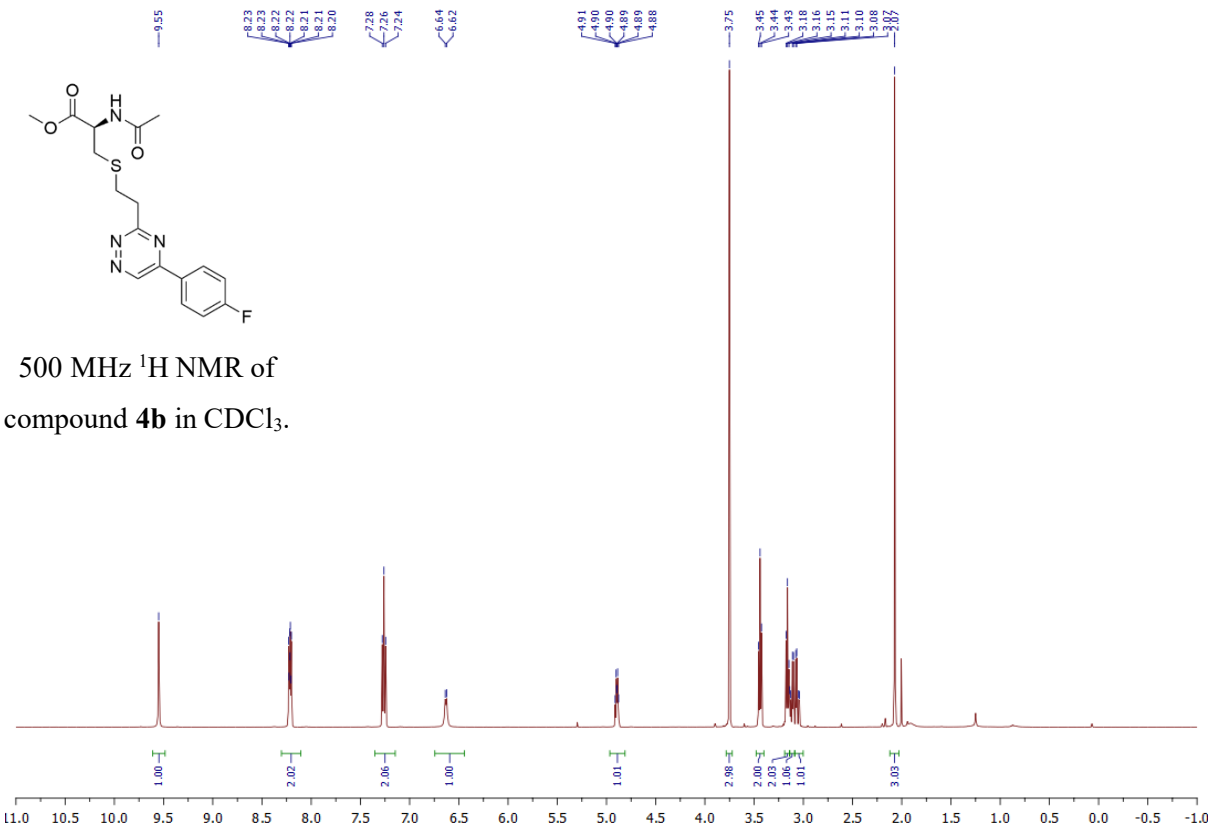
500 MHz ^{1}H NMR of compound **3c** in $CDCl_3$.



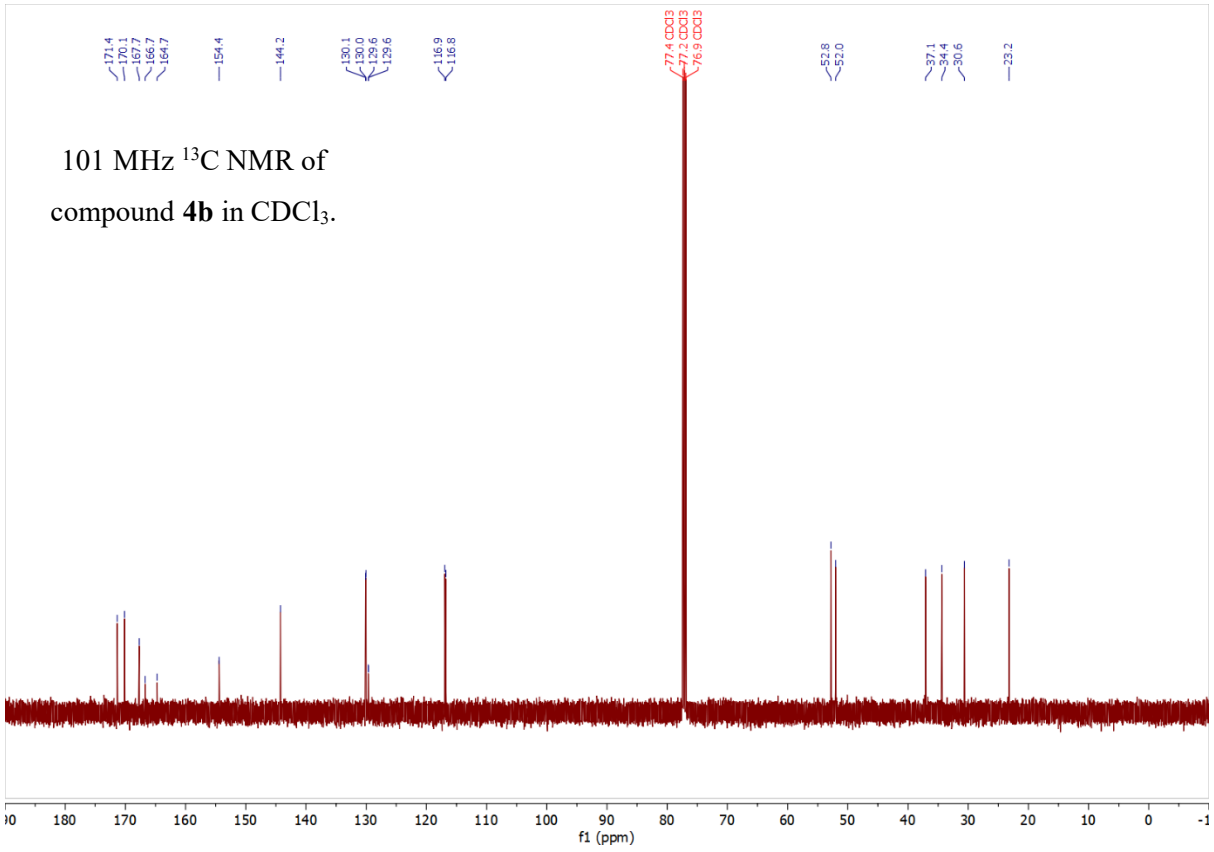
500 MHz ^{1}H NMR of compound **4a** in $CDCl_3$.



126 MHz ^{13}C NMR of compound **4a** in $CDCl_3$.

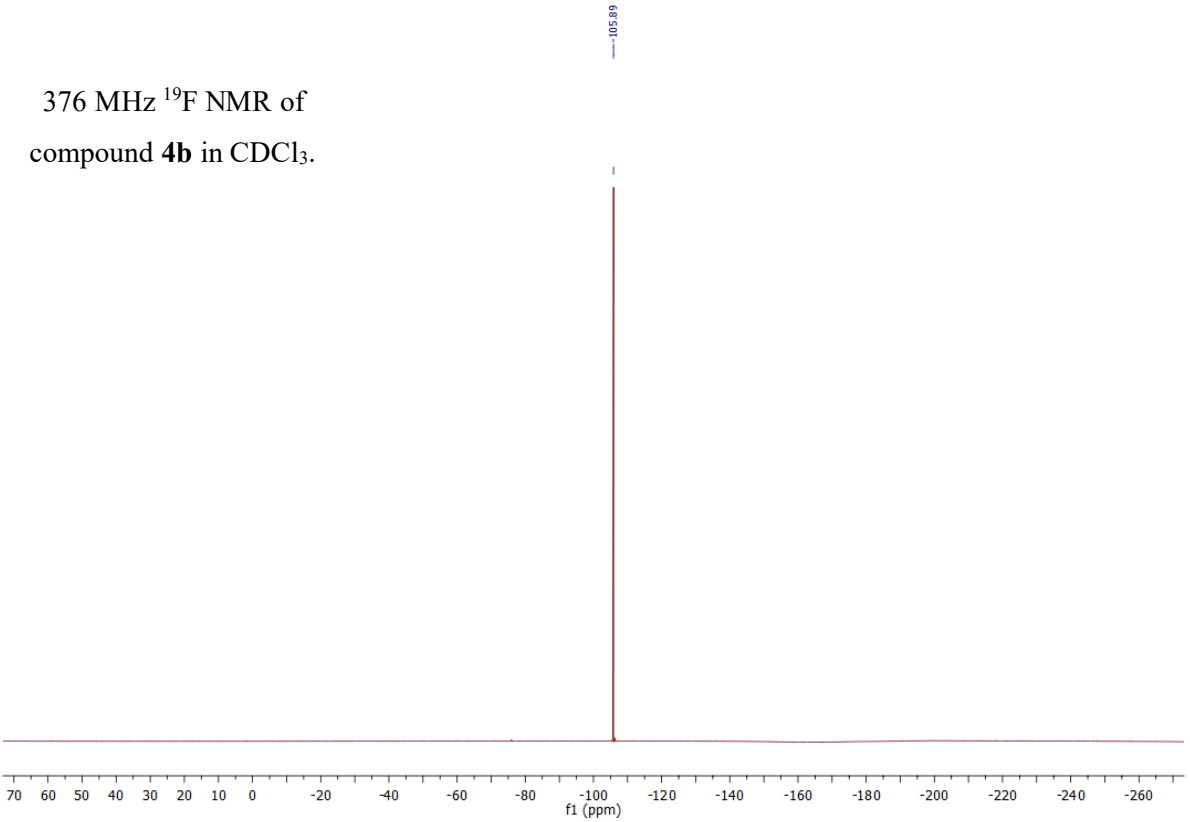


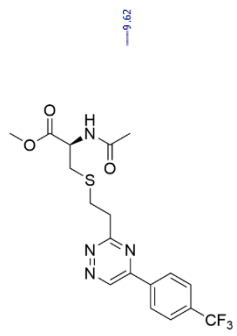
500 MHz ^{1}H NMR of compound **4b** in $CDCl_3$.



101 MHz ^{13}C NMR of compound **4b** in $CDCl_3$.

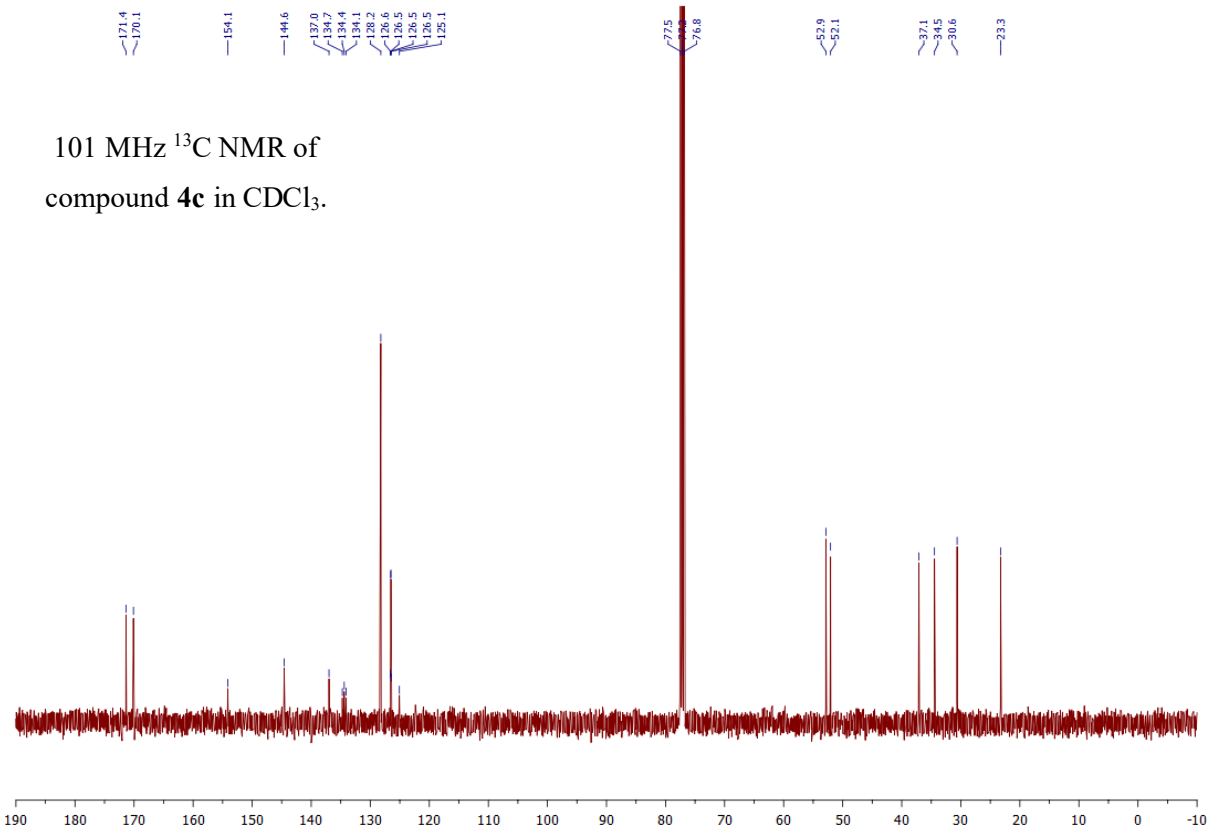
376 MHz ^{19}F NMR of compound **4b** in $CDCl_3$.



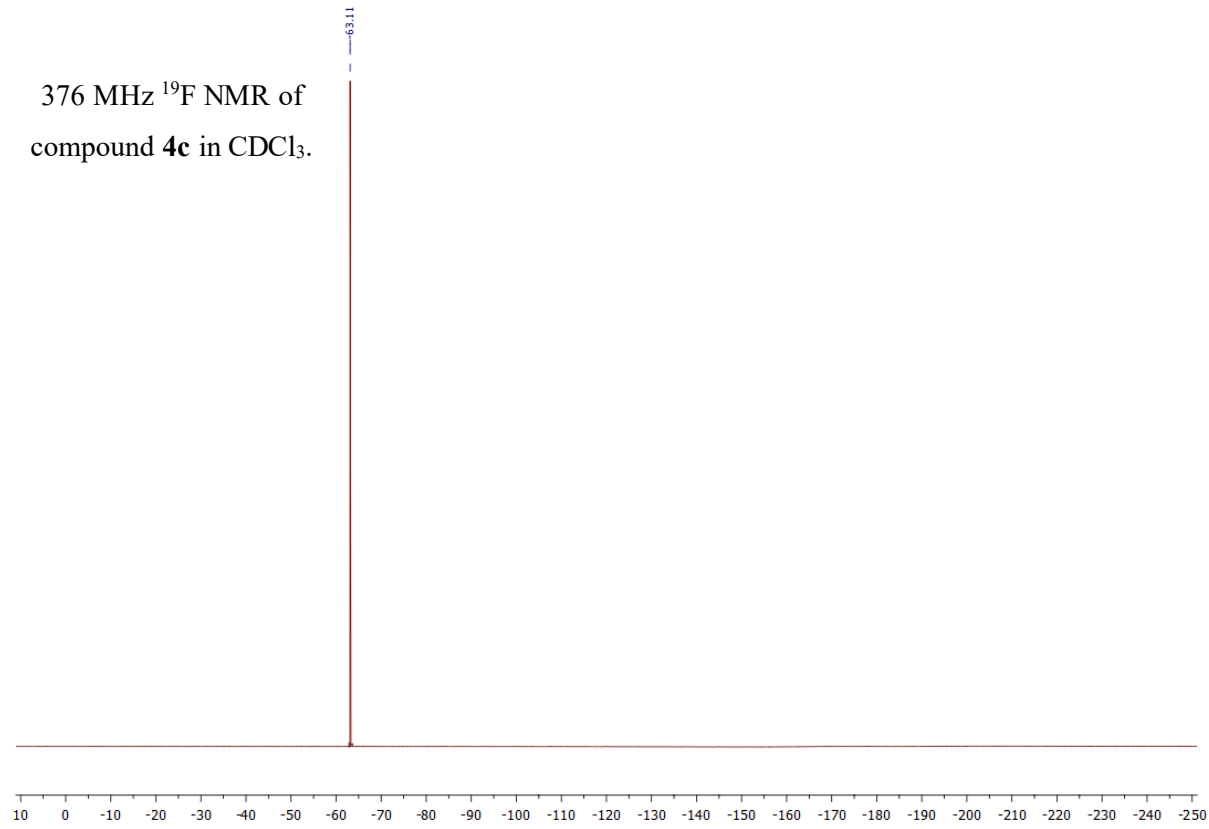


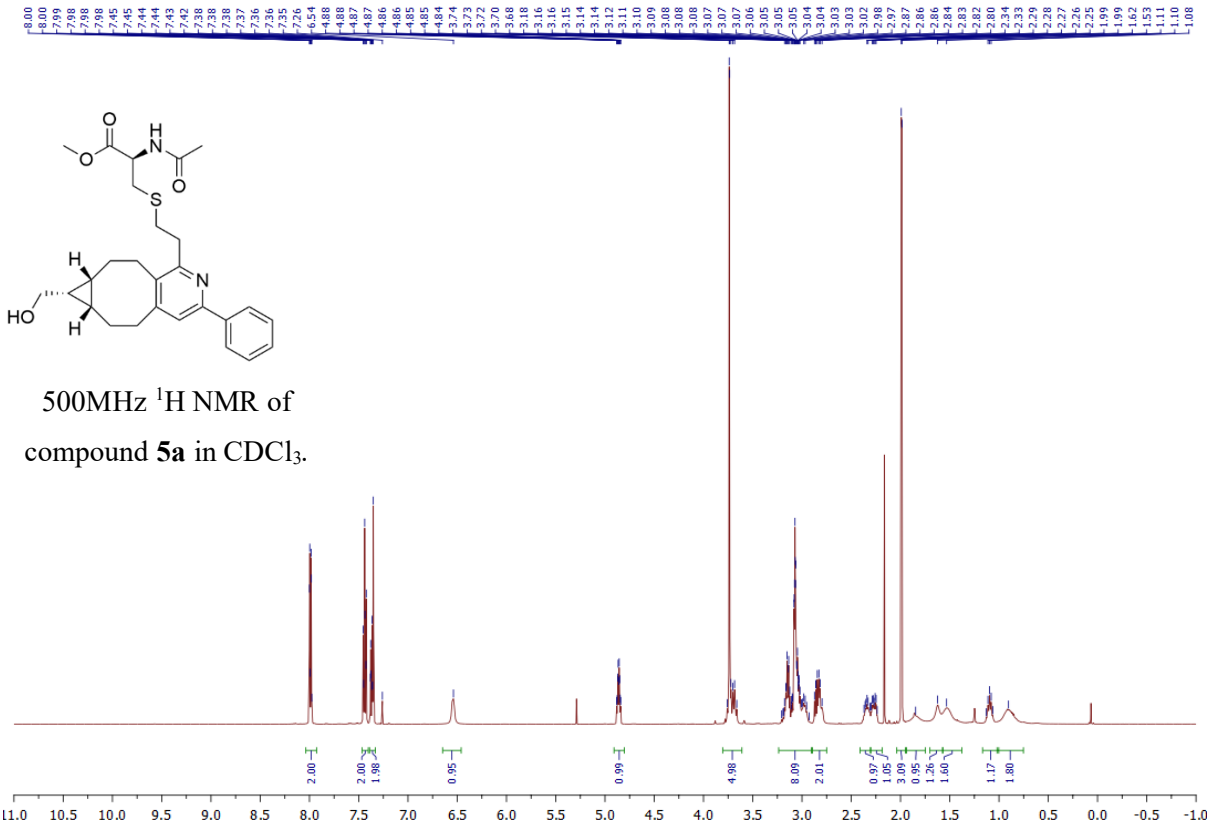
400MHz ^{1}H NMR of compound **4c** in $CDCl_3$.

101 MHz ^{13}C NMR of compound **4c** in $CDCl_3$.

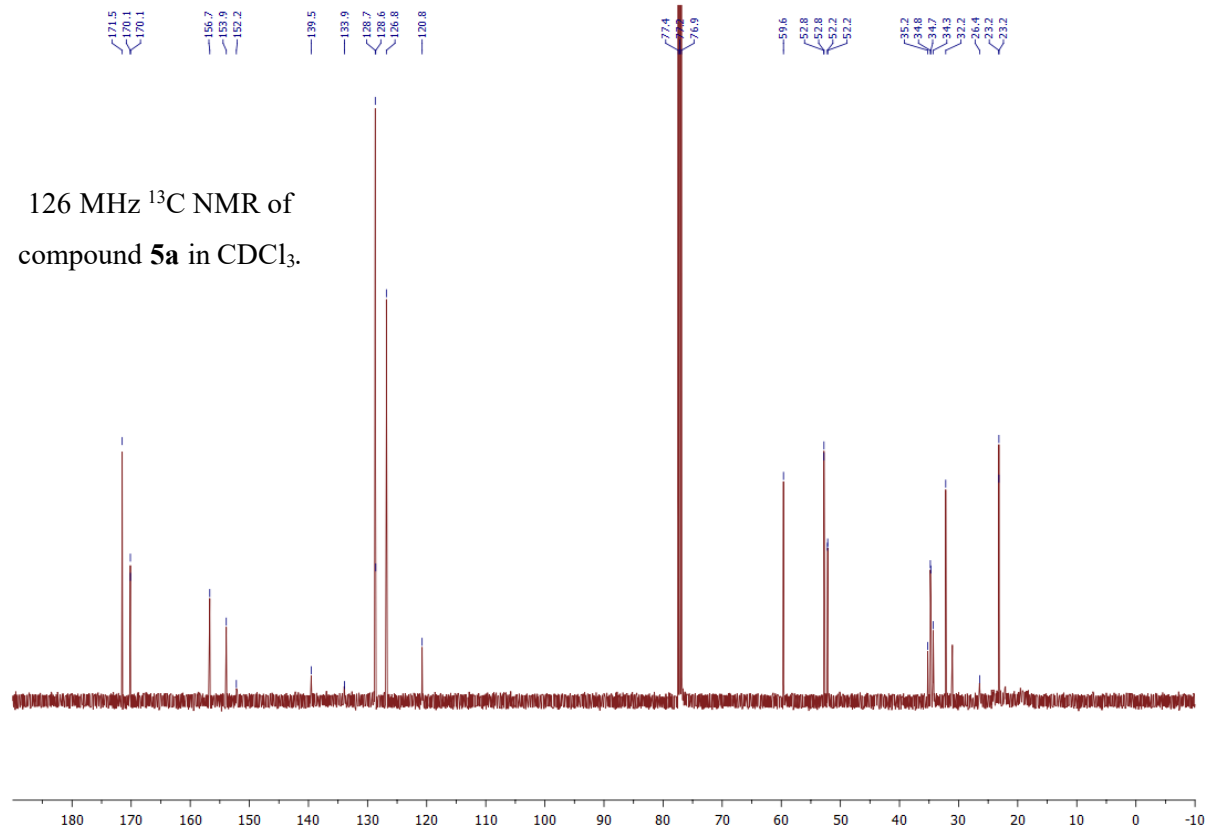


376 MHz ^{19}F NMR of compound **4c** in $CDCl_3$.

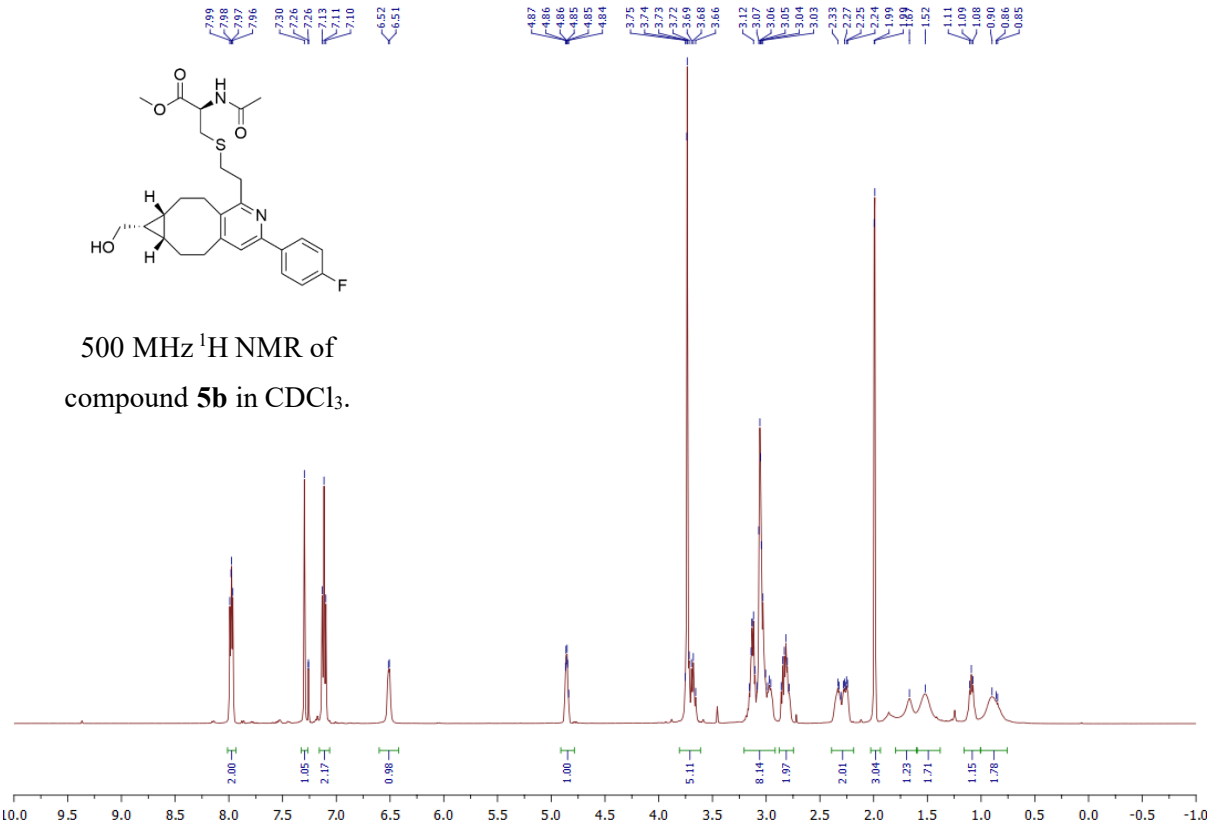




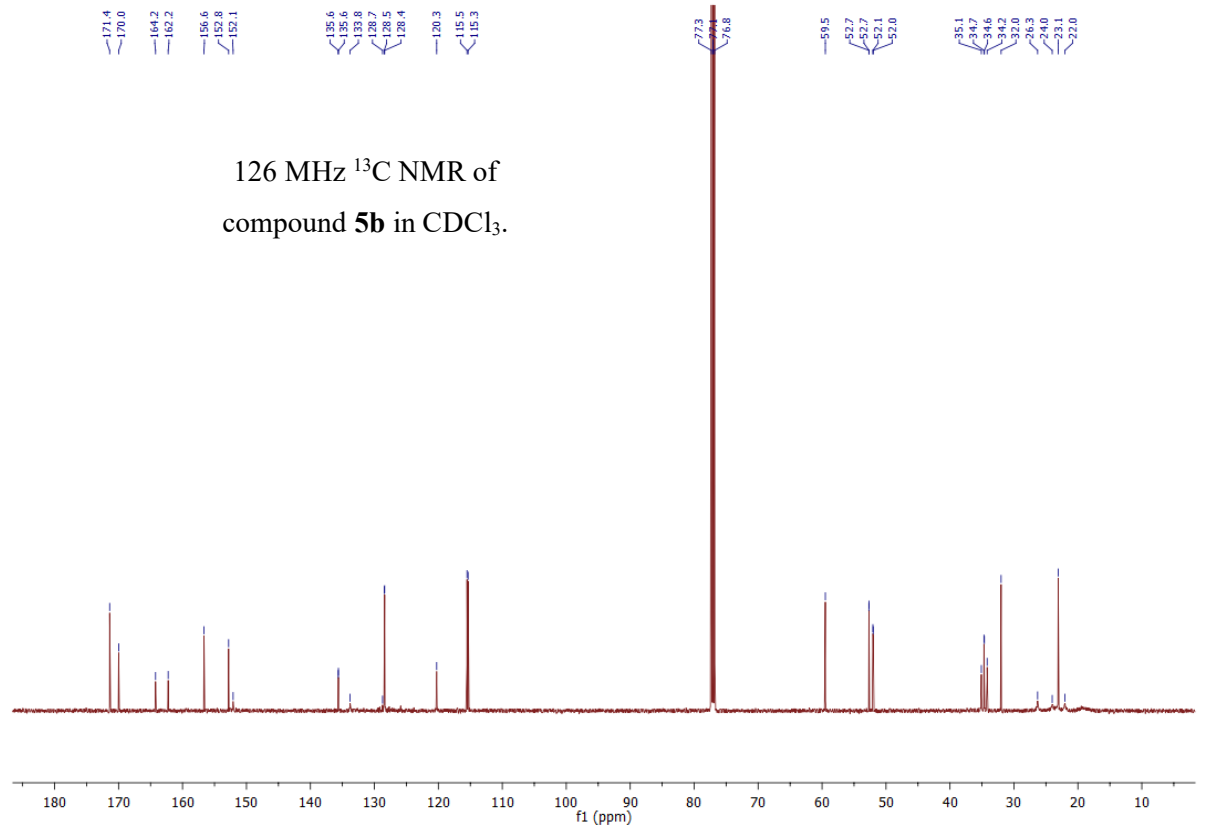
500MHz ^{1}H NMR of compound **5a** in $CDCl_3$.



126 MHz ^{13}C NMR of compound **5a** in $CDCl_3$.

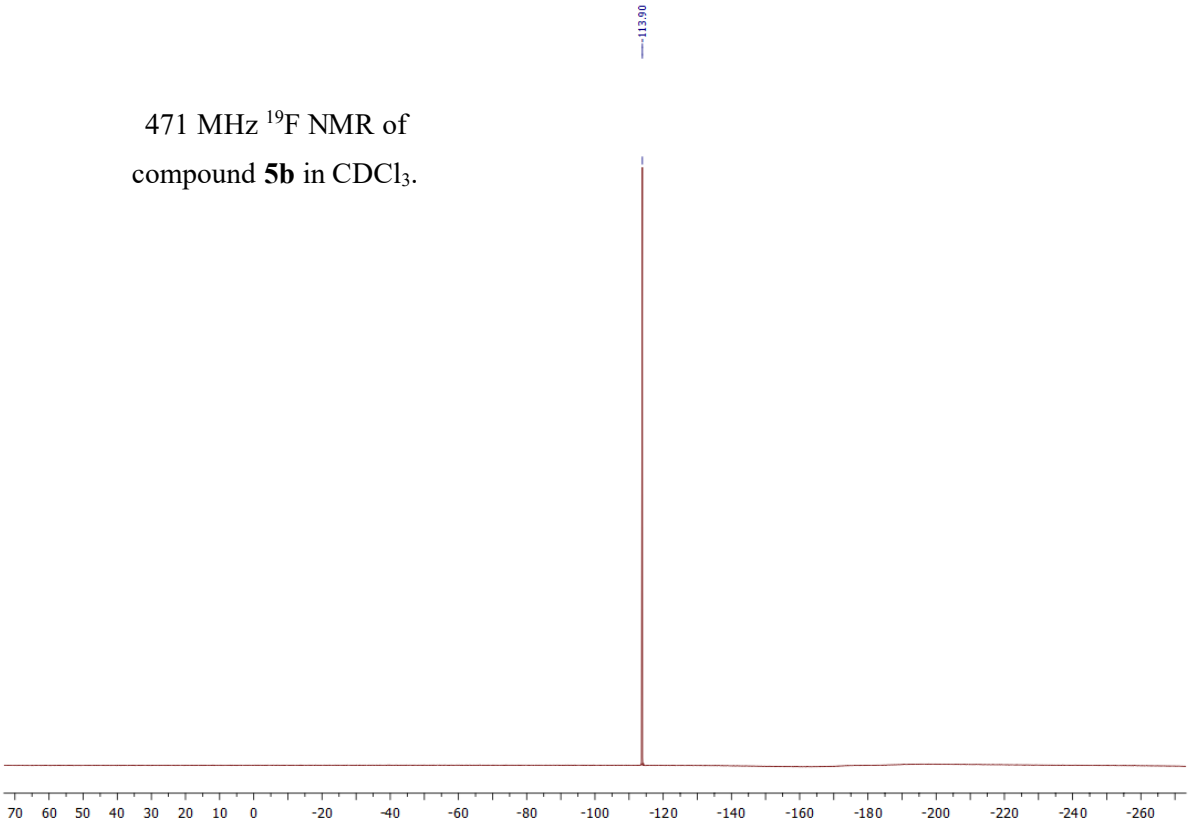


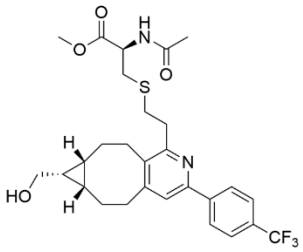
500 MHz ^{1}H NMR of compound **5b** in $CDCl_3$.



126 MHz ^{13}C NMR of compound **5b** in $CDCl_3$.

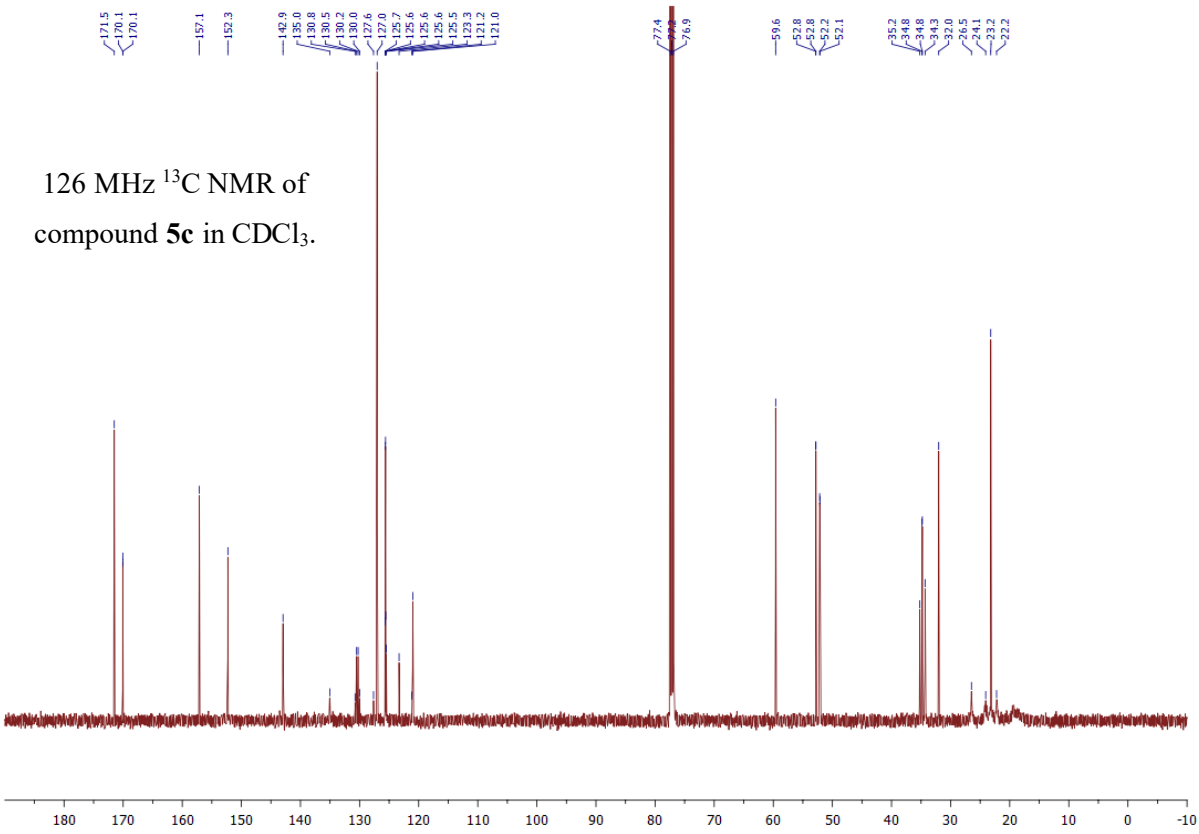
471 MHz ^{19}F NMR of compound **5b** in $CDCl_3$.



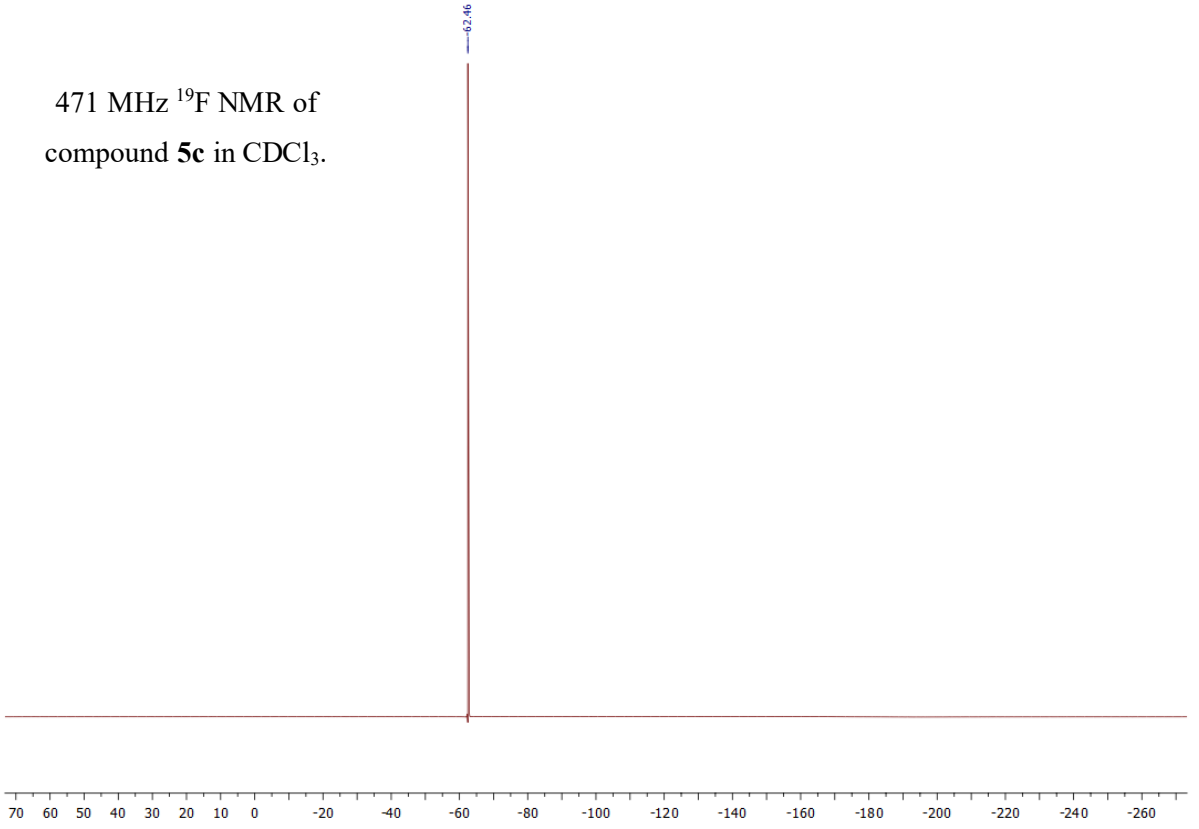


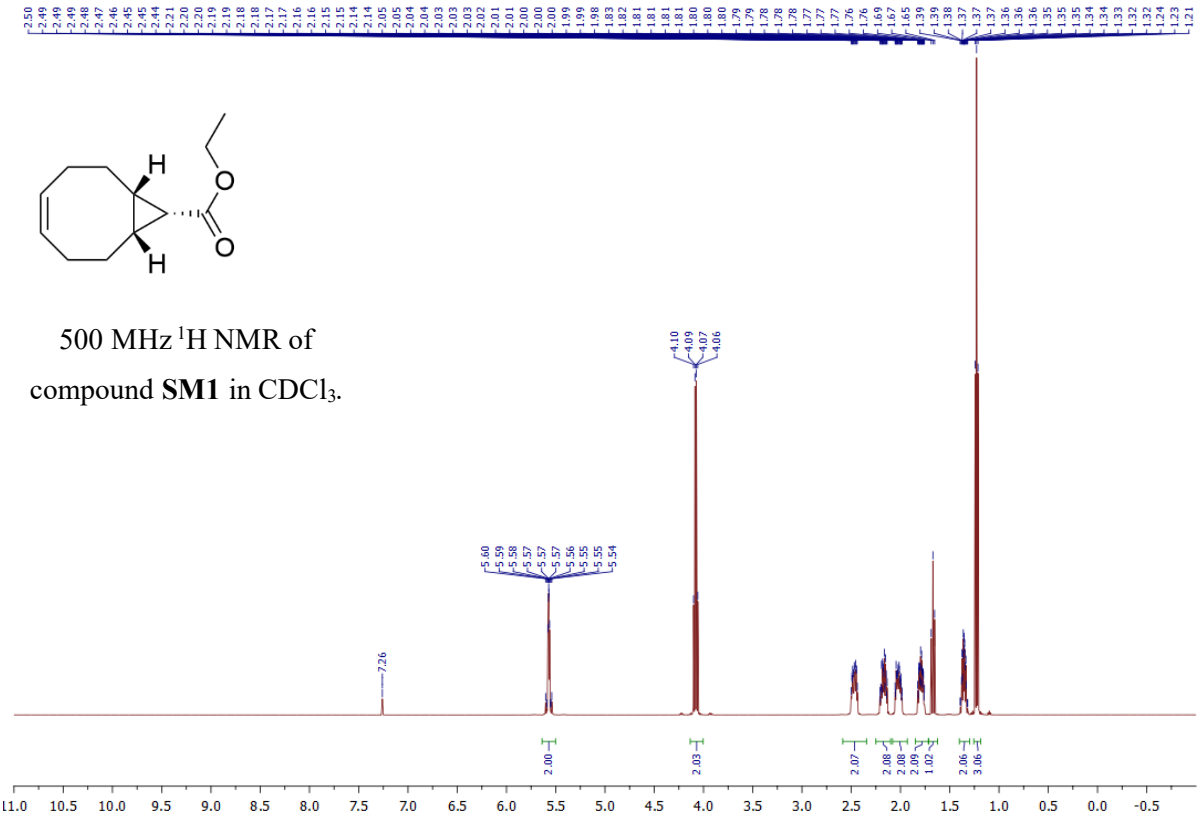
500 MHz ^{1}H NMR of compound **5c** in $CDCl_3$.

126 MHz ^{13}C NMR of compound **5c** in $CDCl_3$.

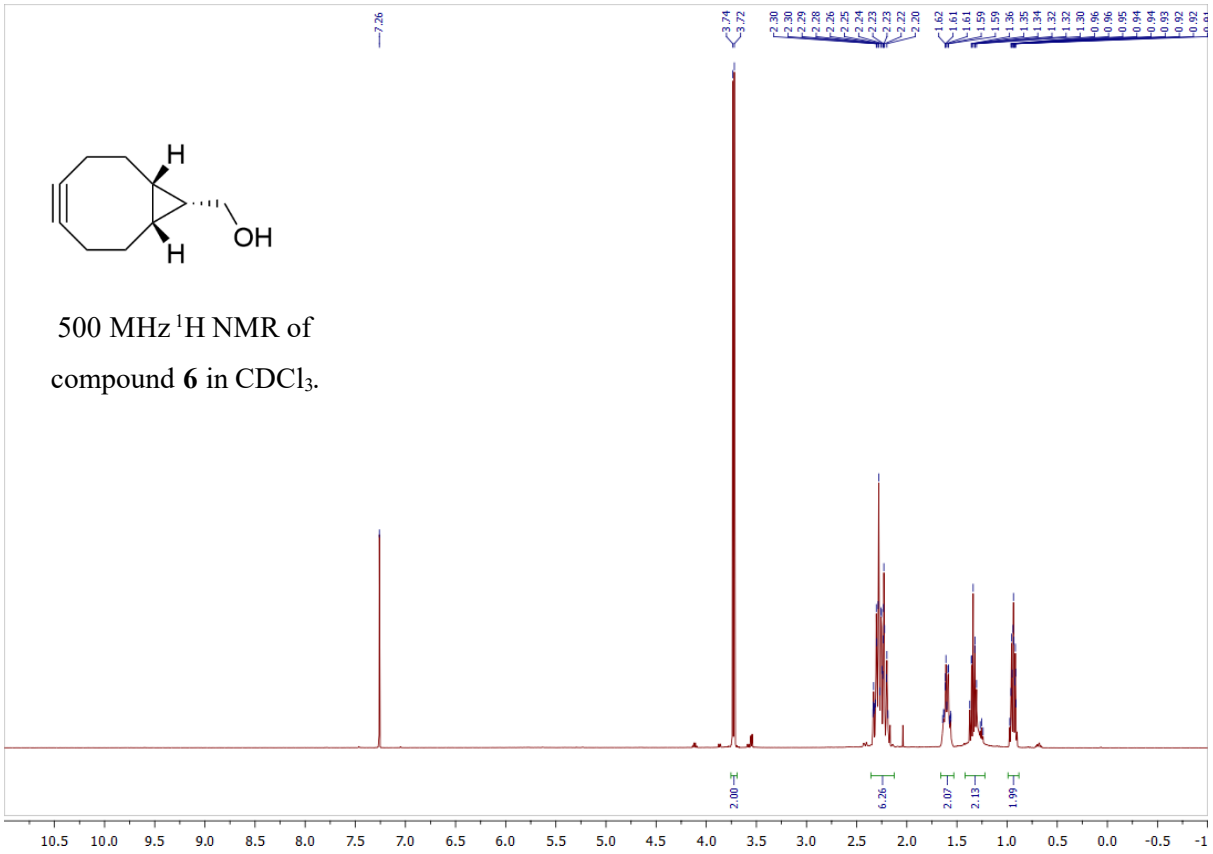


471 MHz ^{19}F NMR of compound **5c** in $CDCl_3$.

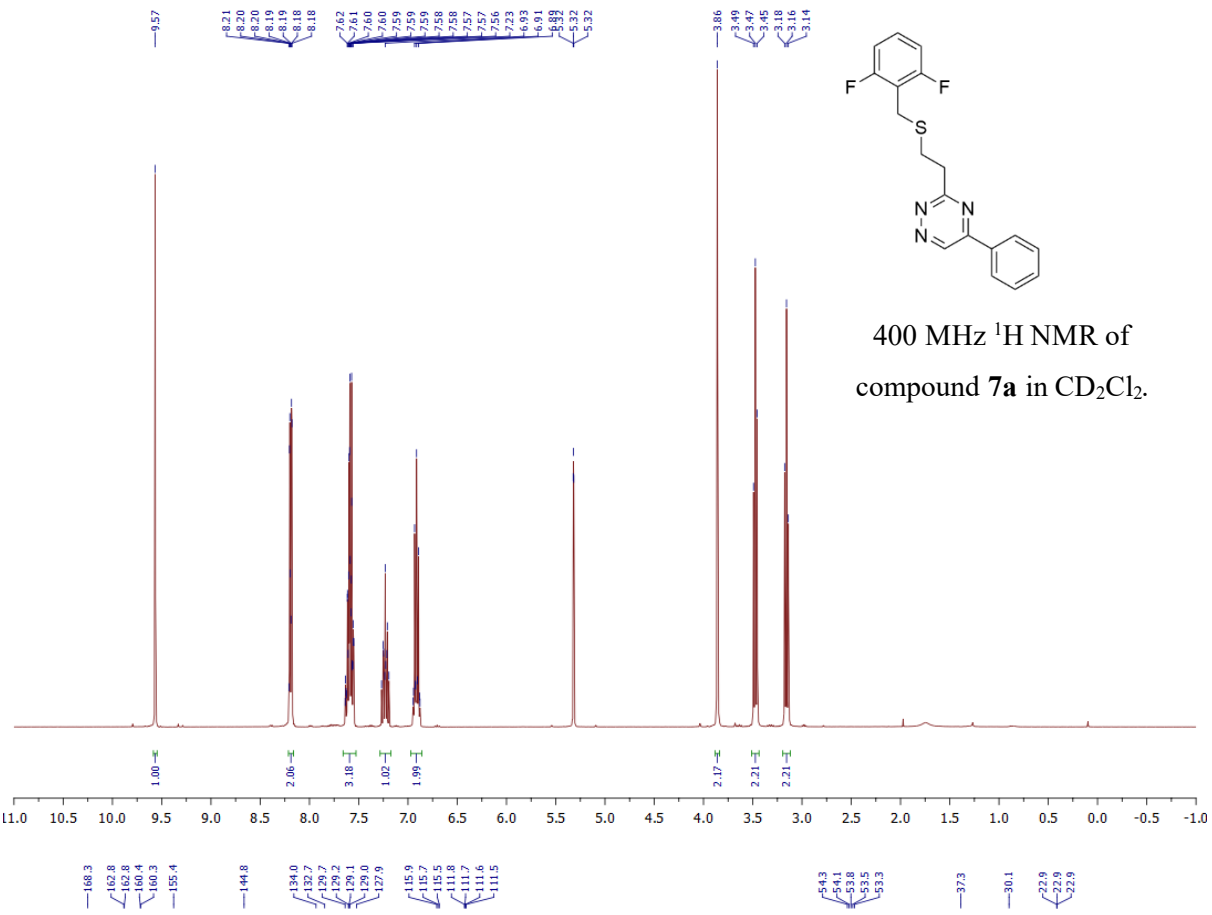




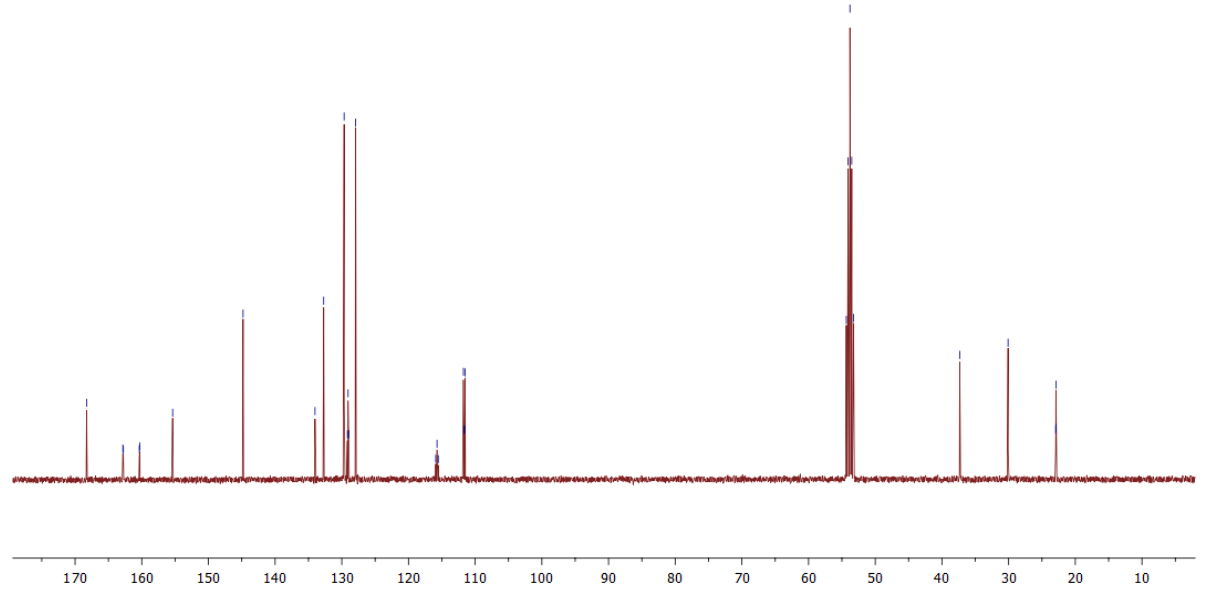
500 MHz ^{1}H NMR of compound **SM1** in $CDCl_3$.



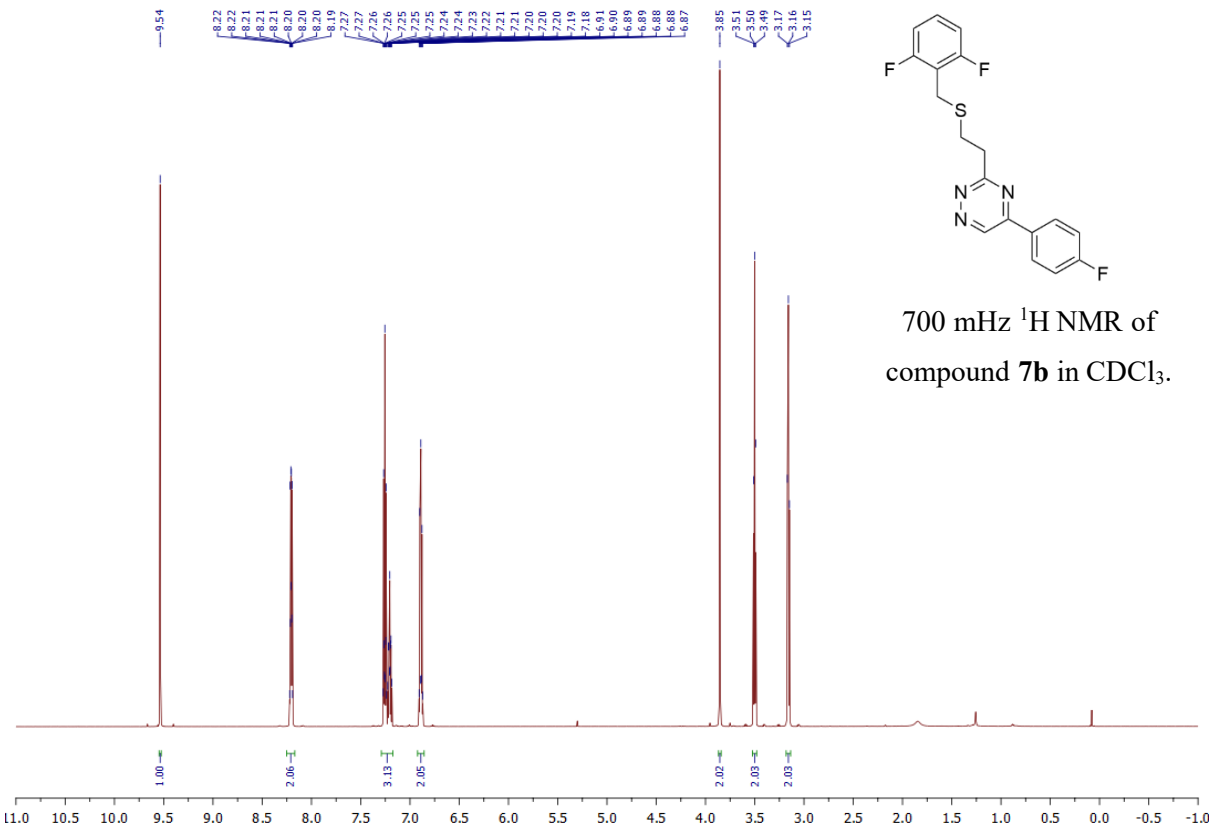
500 MHz ^{1}H NMR of compound **6** in $CDCl_3$.



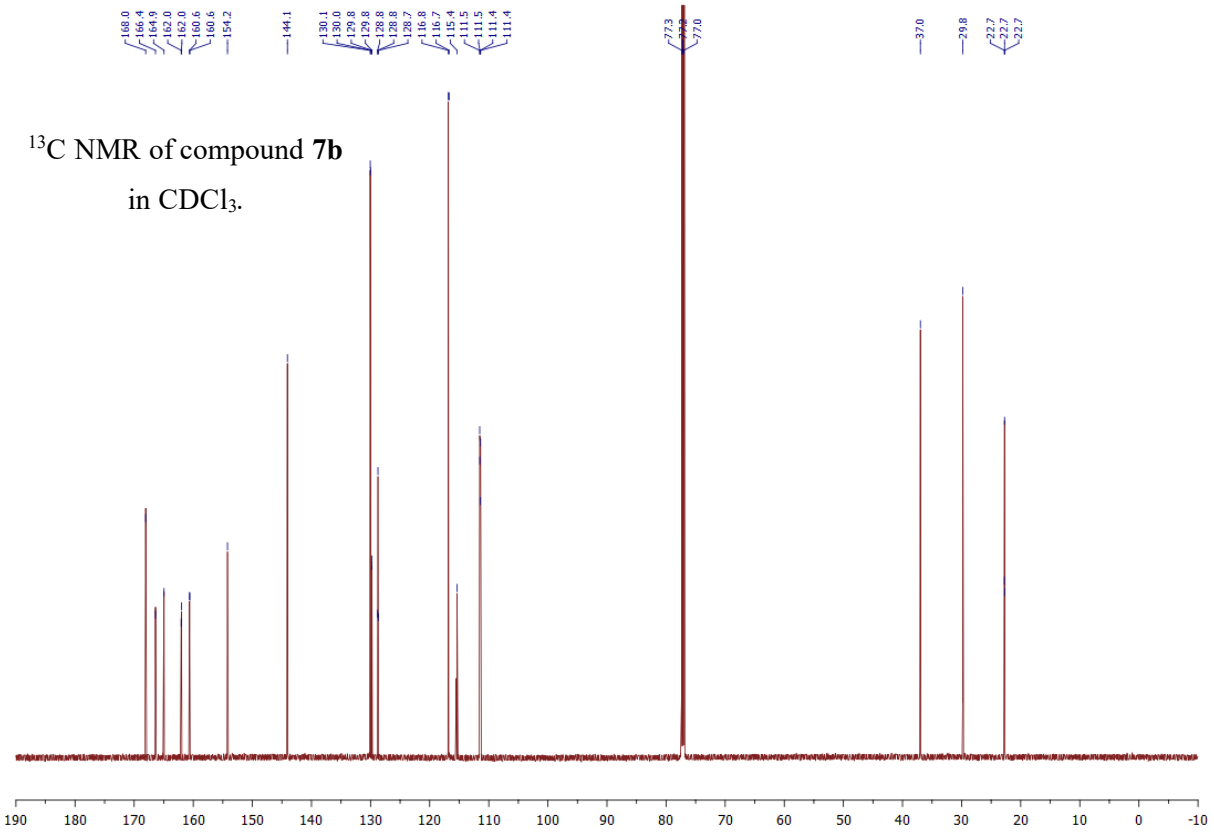
400 MHz ^{1}H NMR of compound **7a** in CD_2Cl_2.



101 MHz ^{13}C NMR of compound **7a** in CD_2Cl_2.

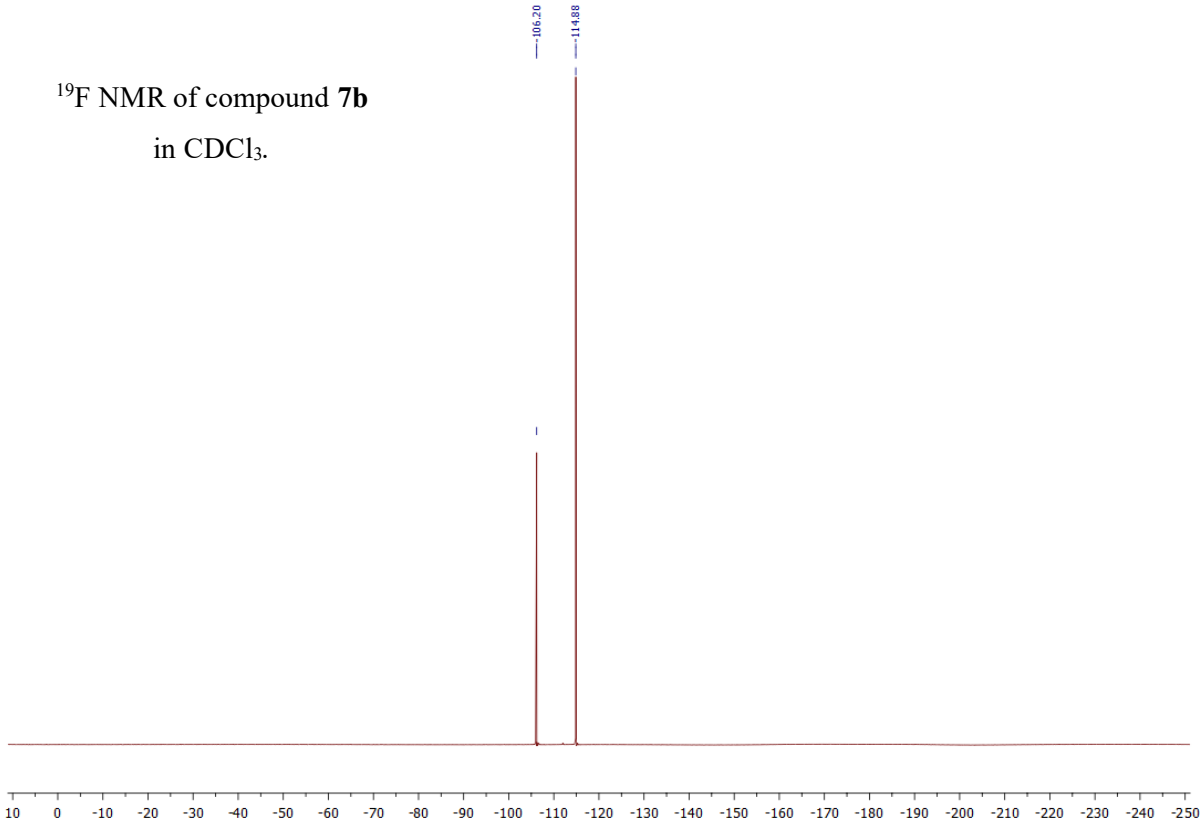


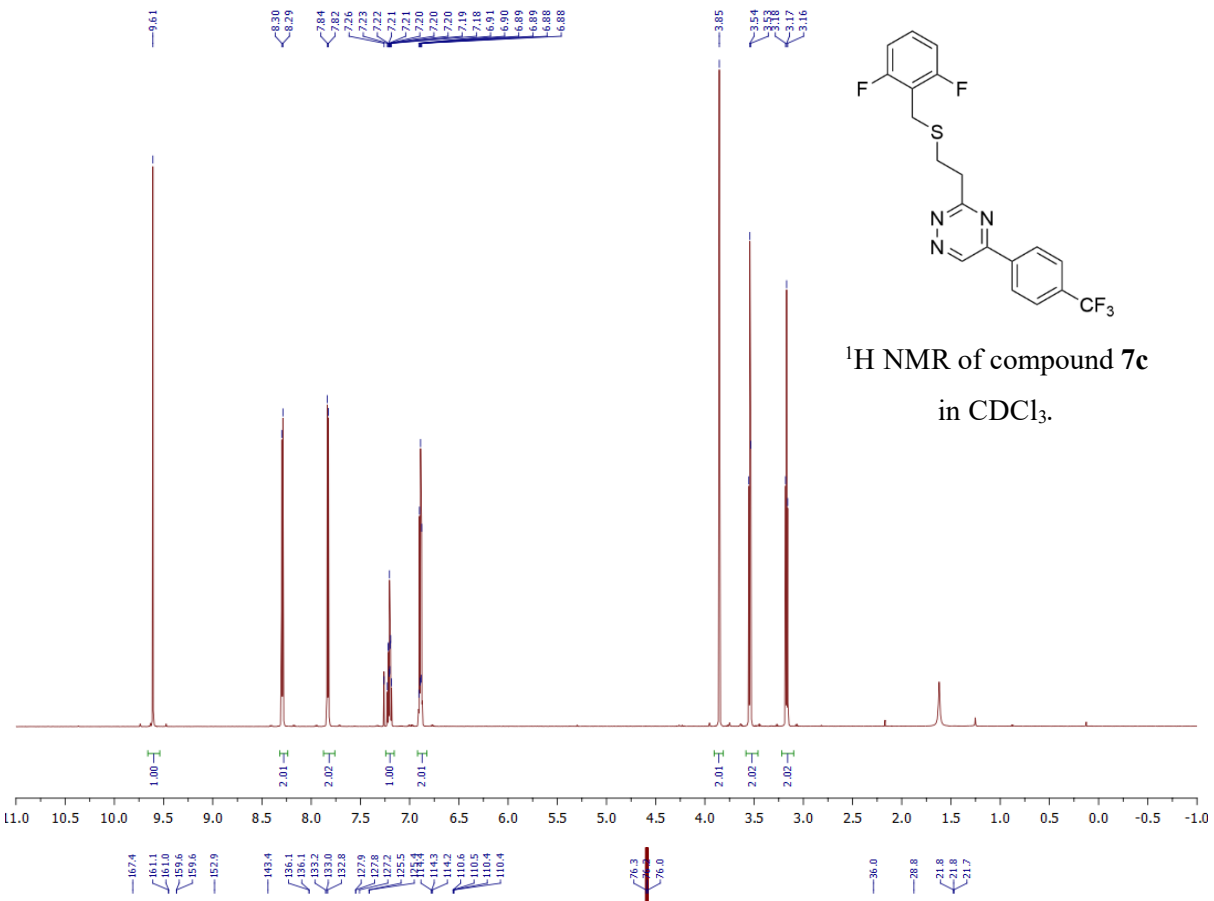
700 mHz ^{1}H NMR of compound **7b** in $CDCl_3$.



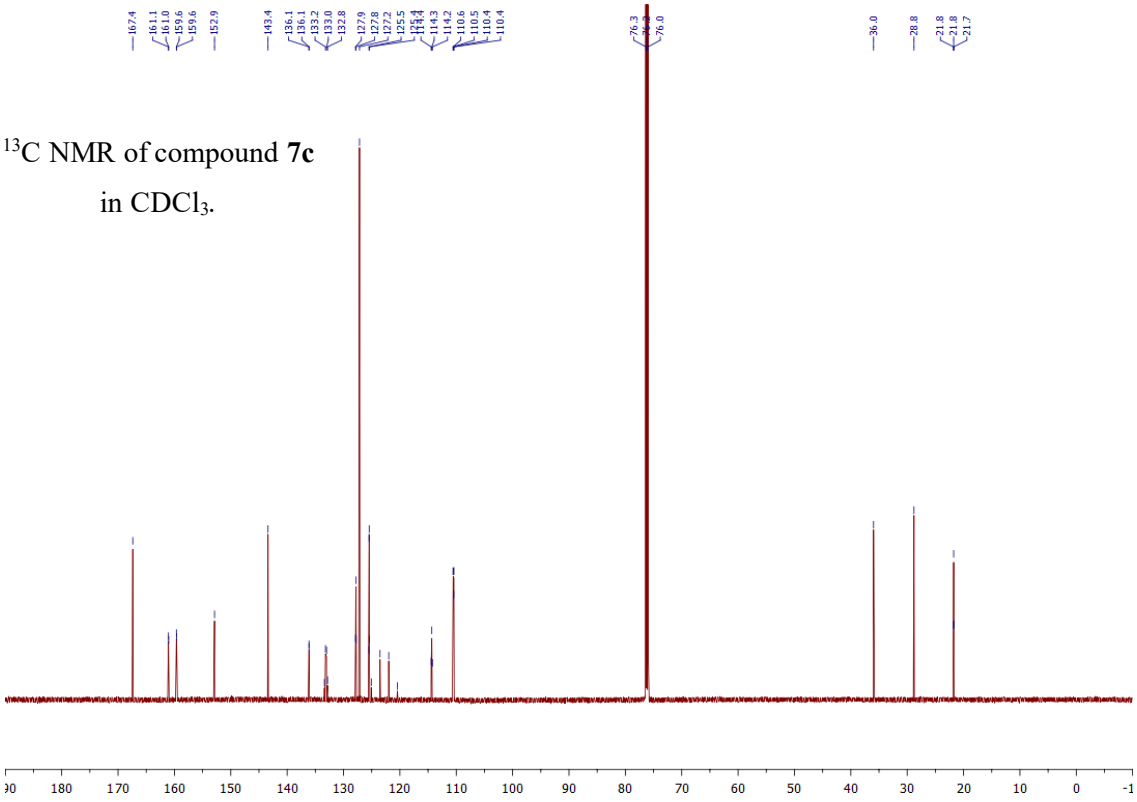
^{13}C NMR of compound **7b** in $CDCl_3$.

^{19}F NMR of compound **7b** in $CDCl_3$.



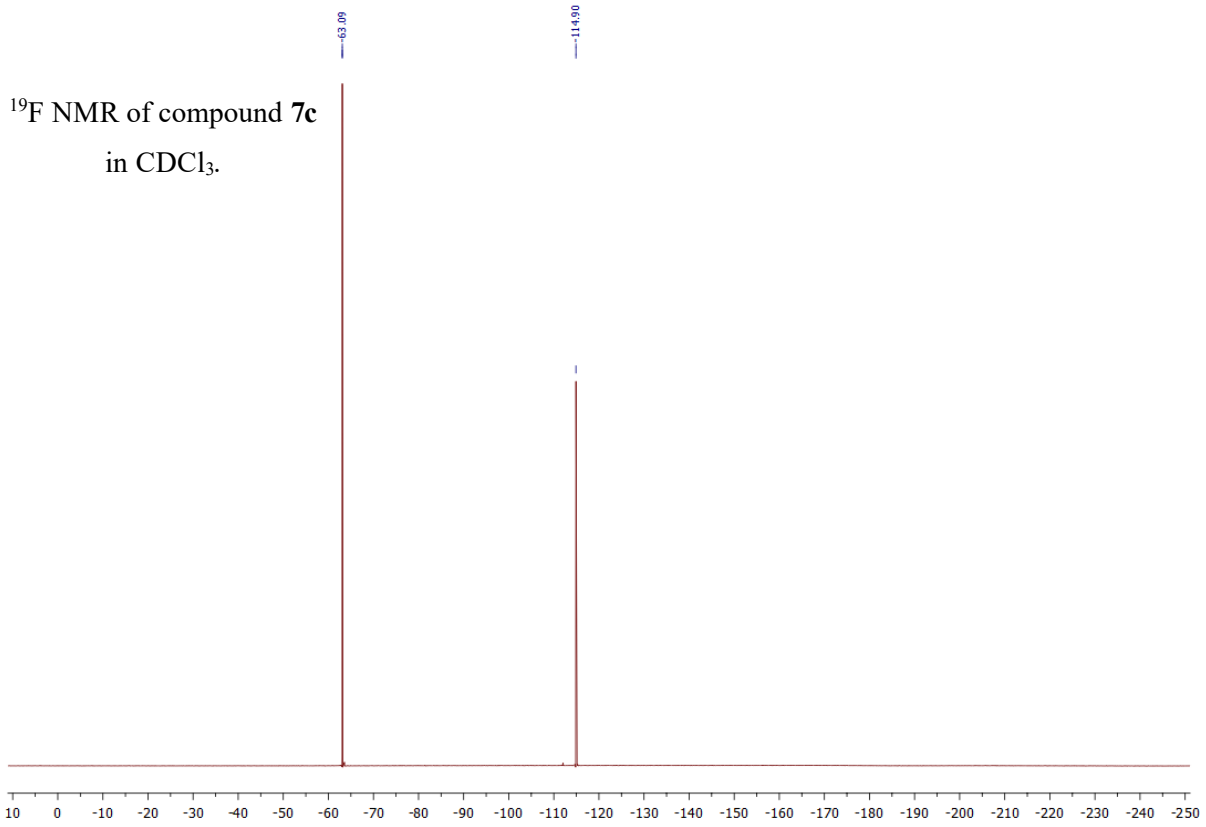


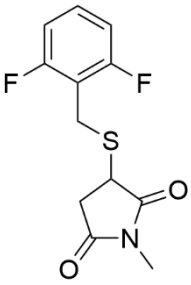
^{1}H NMR of compound **7c** in $CDCl_3$.



^{13}C NMR of compound **7c** in $CDCl_3$.

^{19}F NMR of compound **7c** in $CDCl_3$.





500 MHz ^{1}H NMR of compound **8** in $CDCl_3$.

11.2. Example NMRs from Stability Studies of 7a-c and 8:

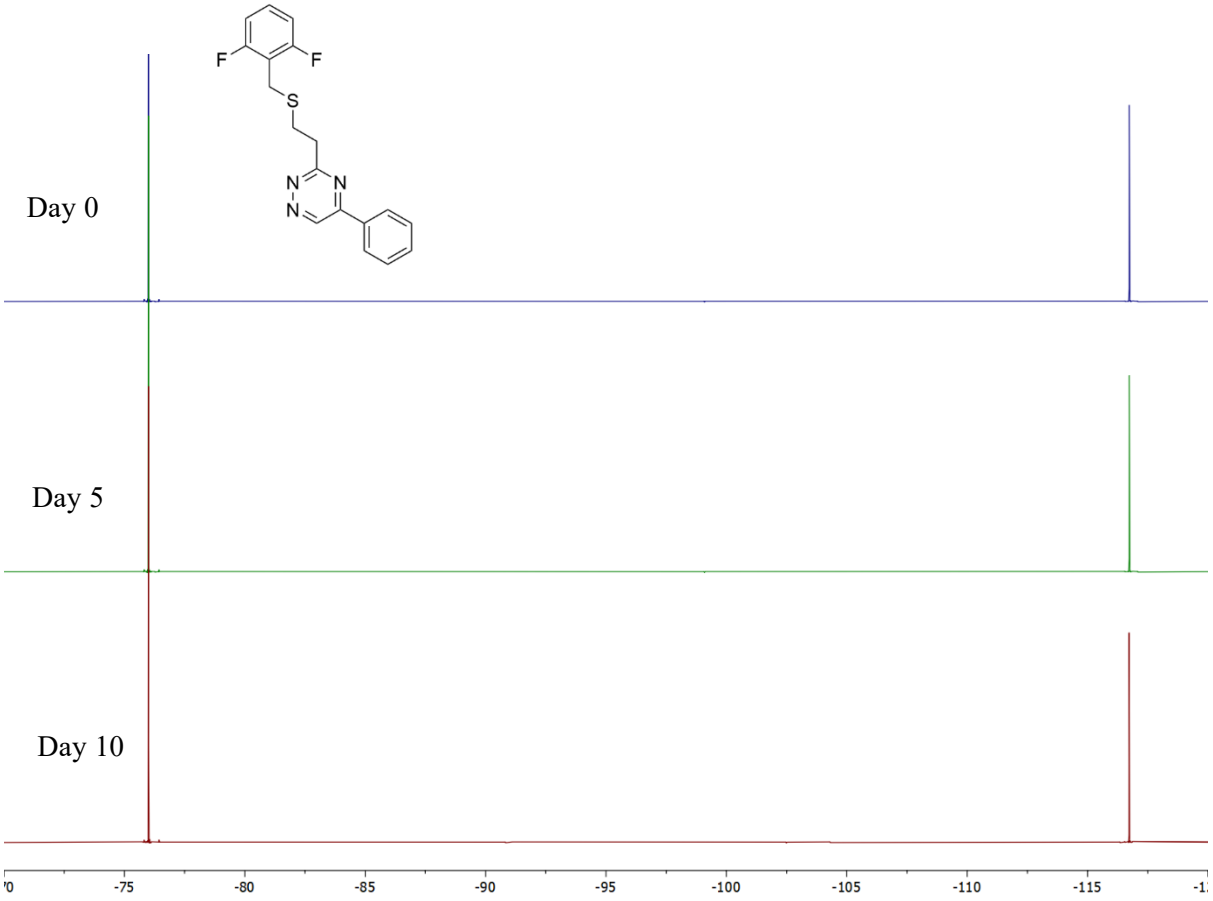


Figure S41: Example ^{19}F NMRs from the stability study of **7a**.

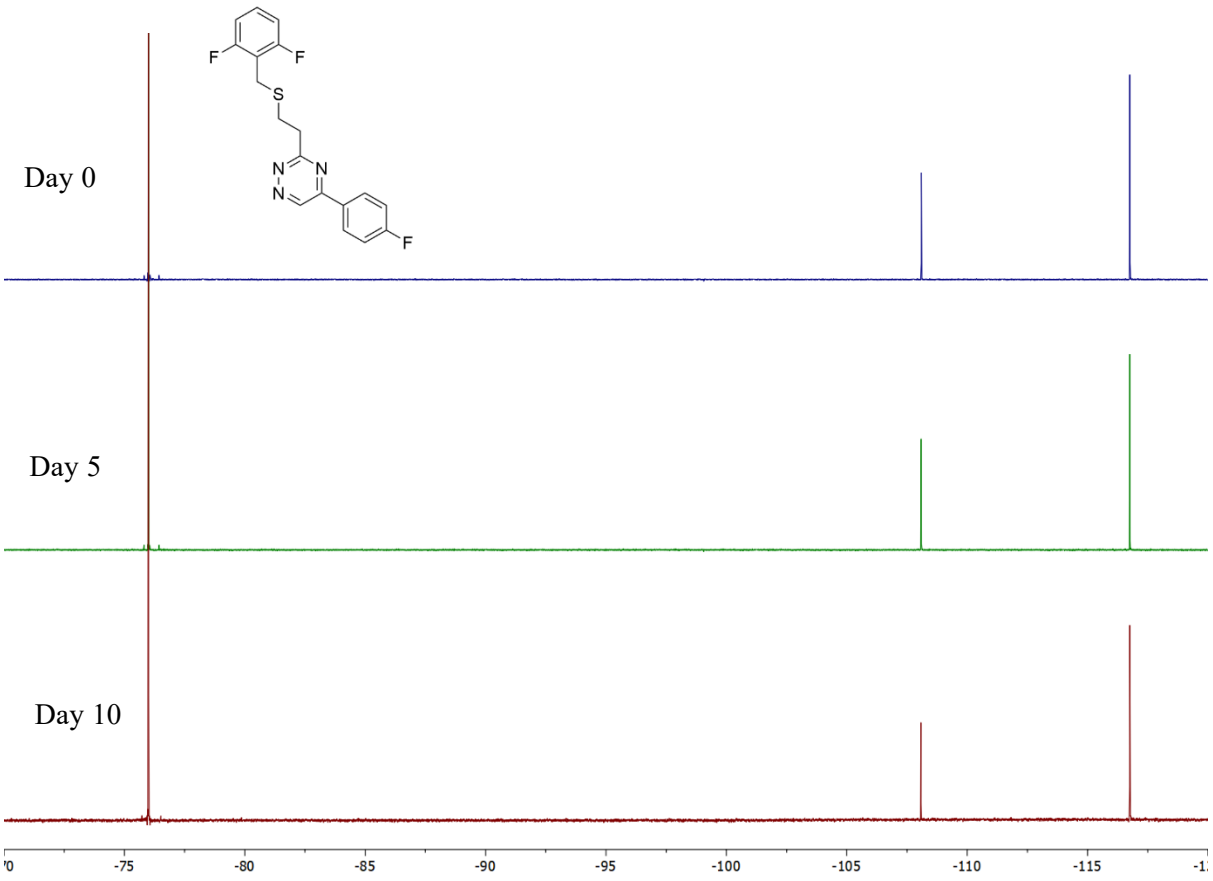


Figure S42: Example ^{19}F NMRs from the stability study of **7b**.

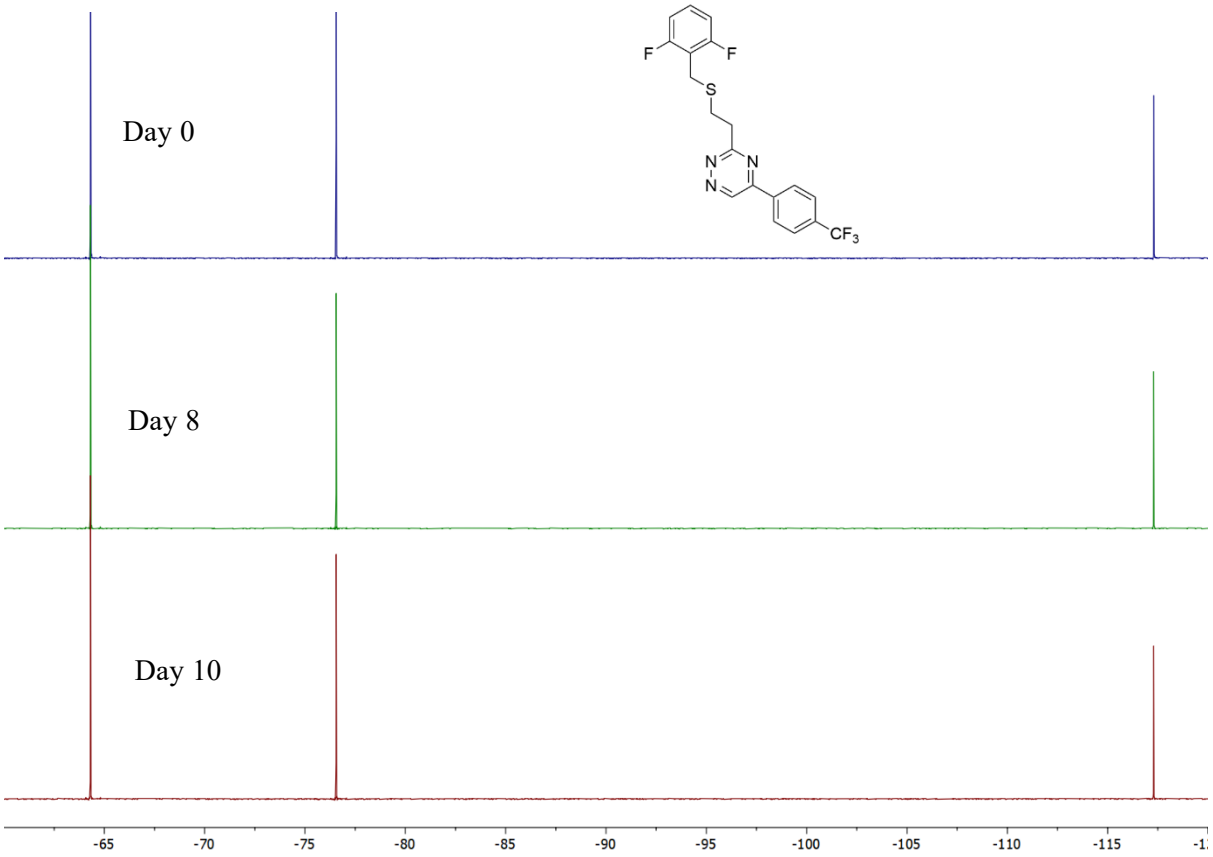


Figure S43: Example ^{19}F NMRs from the stability study of **7c**.

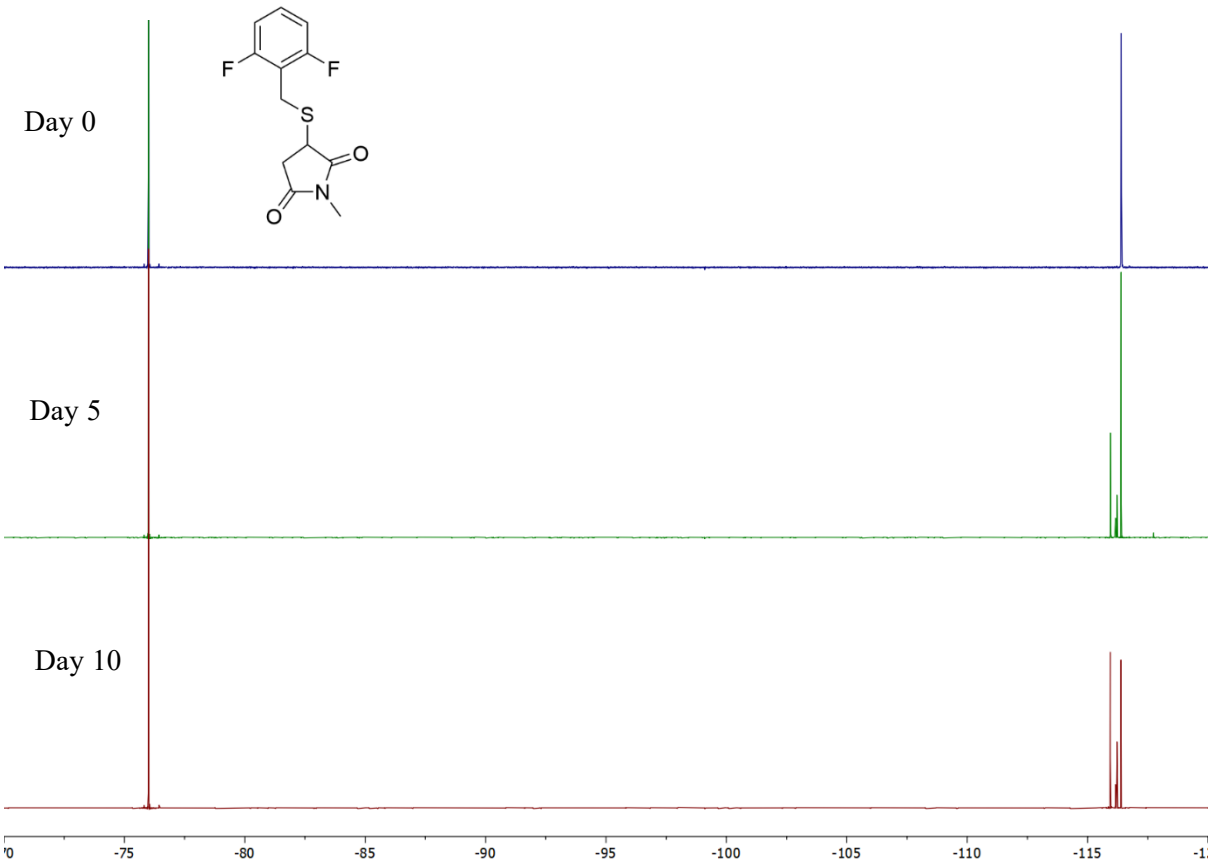


Figure S44: Example ^{19}F NMRs from the stability study of **8**.