Electronic Supplementary Information

Targeted covalent inhibitors of MDM2 using electrophile-bearing stapled peptides

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Contents

1. Supplementary figures

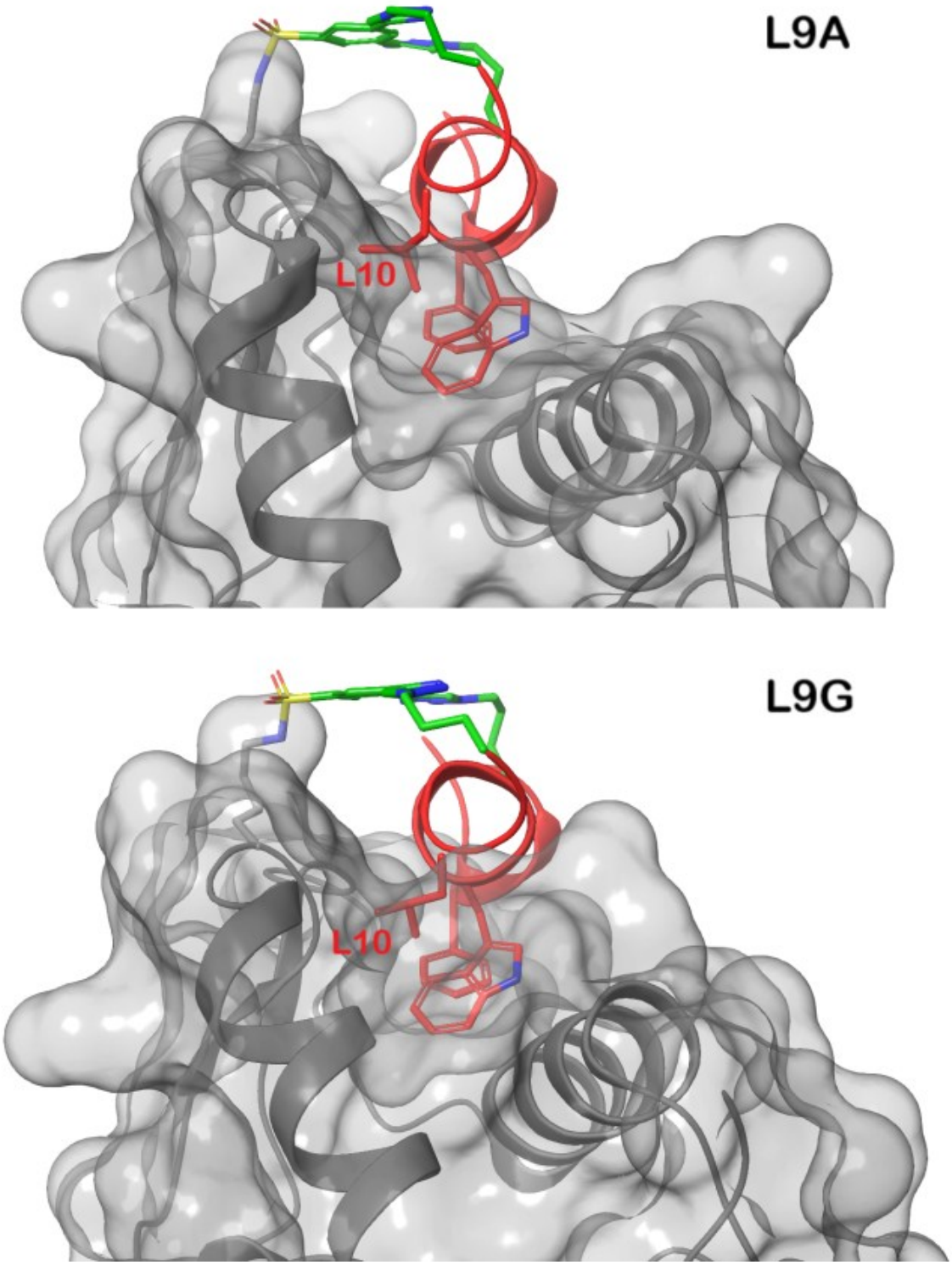
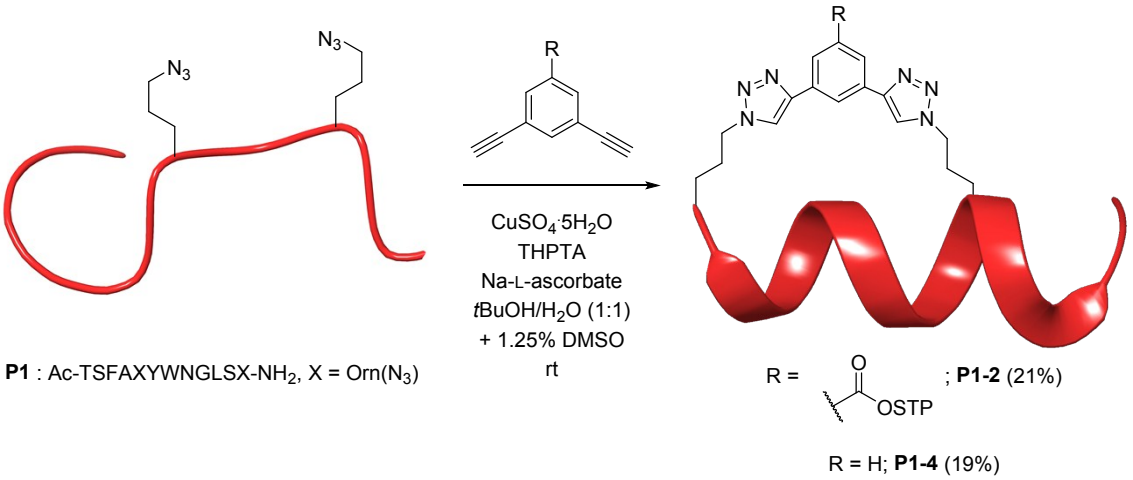
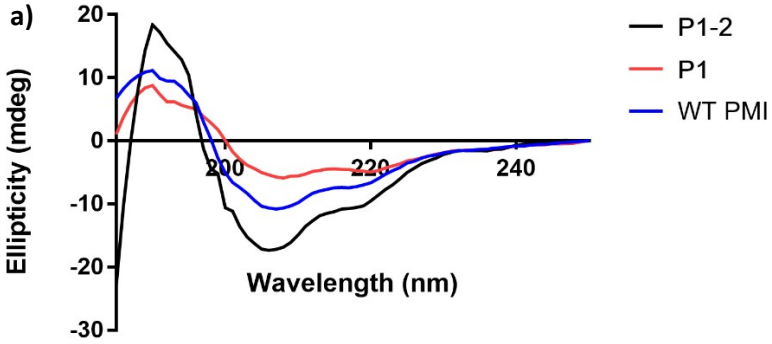


Fig. S1 Molecular dynamics simulation of PMI stapled peptide (red) covalently linked with MDM2 (grey) via sulfonamide linkage. L9A mutation resulted in Leu10, an important hotspot,[1] pulling away from the pocket whereas L9G did not.



Scheme S1 Two-component CuAAC peptide stapling of **P1** with staples **2** and **4**



b)

Peptide	%helicity
WT PMI	15
P1	12
P1-2	22

Fig. S2 a) Circular dichroism spectra for peptides **P1**, **P1-2**, and wild-type (WT) PMI. The graph shows higher helicity for the stapled peptide **P1-2** compared to the linear diazidopeptide **P1** and WT PMI. b) % helicity for the three peptides. Stapling increased the helicity of the peptide compared to both wild-type and the linear diazidopeptide.

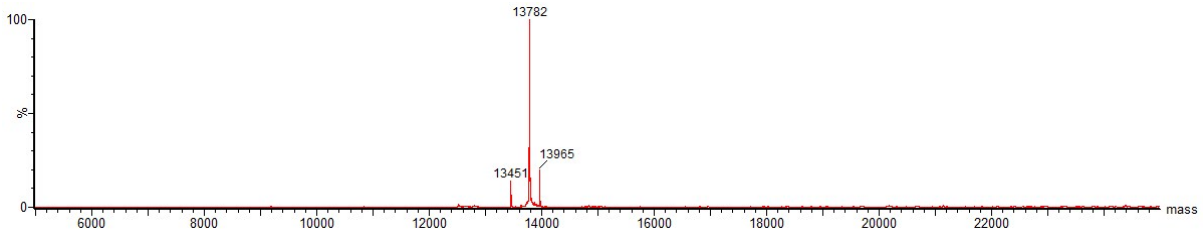


Fig. S3 ESI-MS spectrum of MDM2 incubated with **P1-4**, the control peptide, which possessed no electrophilic moieties resulted in no change in the mass of MDM2. Condition: **P1-4** (25 μM) was incubated with MDM2 (25 μM) in PBS buffer (+10% DMSO) at 37 °C for 1 h. $[MDM2 + H]^+$ = 13782 Da.

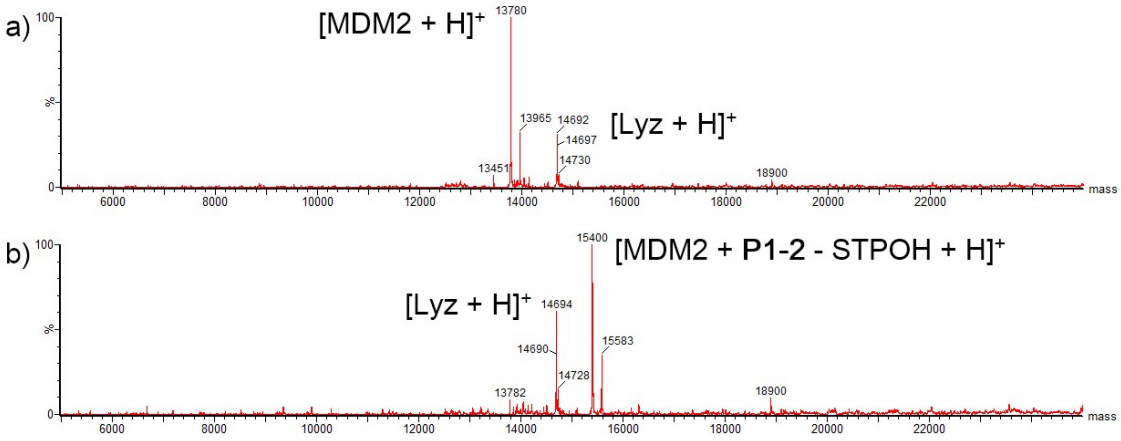


Fig. S4 STP ester-functionalised stapled peptide was selective towards binding MDM2 covalently. a) ESI-MS spectrum of MDM2 and lysozyme (Lyz). $[Lyz + H]^+$ = 14694 Da. b) **P1-2** (25 μM) was incubated with MDM2 (25 μM) and lysozyme (25 μM) in PBS buffer (+10% DMSO) at 37 °C for 1 h. ESI-MS spectrum of the reaction showed the exclusive attack on MDM2 with no modified lysozyme observed.

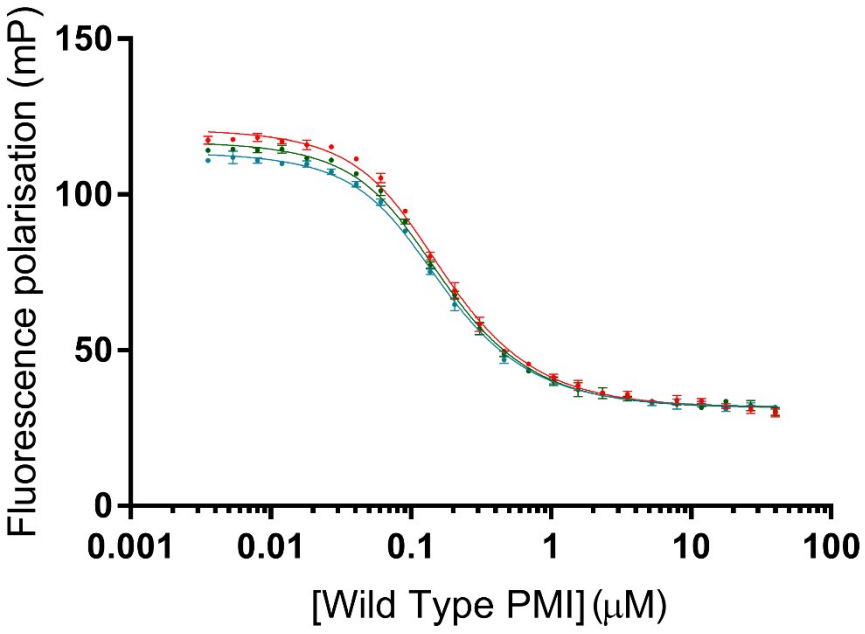


Fig. S5 Competitive FP assay of wild-type PMI showing no change in affinity over time. Each curve represents one time point: 9 min (red), 41 min (green), and 120 min (blue).

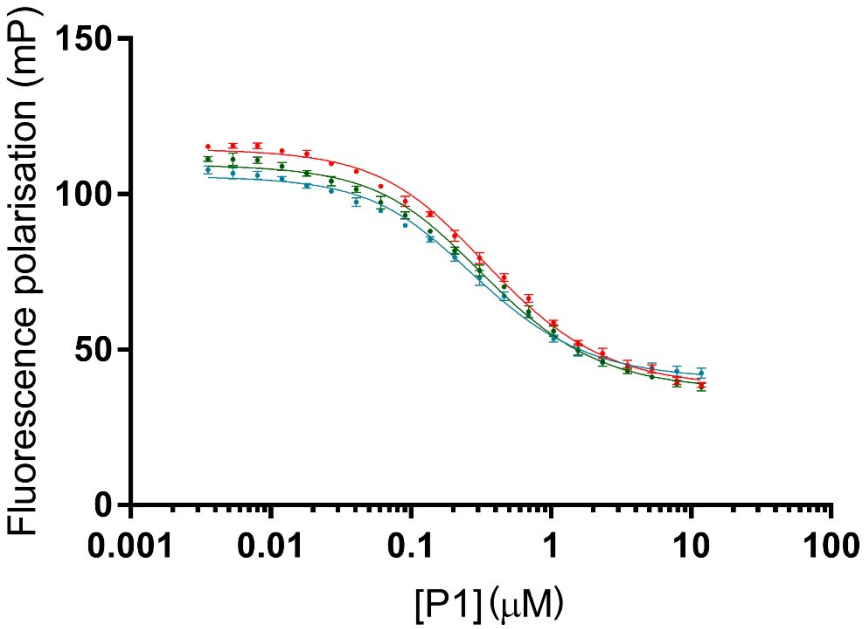


Fig. S6 Competitive FP assay of **P1** showing no change in affinity over time. Each curve represents one time point: 9 min (red), 41 min (green), and 120 min (blue).

2. Molecular modelling

Initial molecular models of the covalent *i*, *i*+7 stapled peptide were created using the Builder module in PyMOL.[2]

Preparation of structures

The structure of MDM2 in complex with a stapled peptide (PDB code 5AFG[3]) was used as the initial structure for molecular dynamics (MD) simulations. The stapled peptide (LTFXEYWAQLXS, in which X are the stapled residues) was mutated to the PMI peptide (TSFAEYWNLLSP) using the LEaP module of AMBER 14.[4] The point mutations E69A and K70A in MDM2 were reversed. PMI was modified into stapled peptides **P1-1** and **P1-2** (Ac-TSFAXYWNGLSX-NH_2) by replacing residues 5 and 12 with a two-component triazole staple, formed by a double-click reaction between two azido-ornithine residues and 3,5-diethynylbenzenesulfonyl fluoride, and between two azido-ornithine residues and 4-((3,5-diethynylbenzoyl)oxy)-2,3,5,6-tetrafluorobenzenesulfonate, respectively. Leu9 was mutated to Gly in these stapled peptides to avoid steric clashing of the side chain with the staple. The respective sulfonamide and ester covalent complexes of these stapled peptides with Lys94 of MDM2 were further generated. MDM2 was capped at its N- and C-termini by acetyl and N-methyl groups respectively while the peptides were capped at their N- and C-termini by acetyl and amide groups respectively. PDB2PQR[5] was used to determine the protonation states of residues. Each MDM2 complex was solvated with TIP3P water molecules[6] in a periodic truncated octahedron box, such that its walls were at least 9 Å away from the complex, and neutralised with sodium ions.

Molecular dynamics

Energy minimisations and MD simulations were performed with the sander and PMEMD modules of AMBER 14[4] respectively. Three independent MD simulations were carried out on each of the complexes using the ff14SB[7] and generalised AMBER force fields (GAFF).[8] Atomic charges for the stapled residues were derived using the R.E.D. Server,[9] which fits restrained electrostatic potential (RESP) charges[10] to a molecular electrostatic potential (MEP) computed by the Gaussian 09 program[11] at the HF/6-31G* theory level. All bonds involving hydrogen atoms were constrained by the SHAKE algorithm,[12] allowing for a time step of 2 fs. Nonbonded interactions were truncated at 9 Å, while the particle mesh Ewald method[13] was used to account for long range electrostatic interactions under periodic boundary conditions. Weak harmonic positional restraints with a force constant of 2.0 kcal mol^{-1} $Å^{-2}$ were placed on the protein and peptide non-hydrogen atoms during the minimisation and equilibration steps. Energy minimisation was carried out using the steepest descent algorithm for 500 steps, followed by the conjugate gradient algorithm for another 500 steps. The systems were then heated gradually to 300 K over 50 ps at constant volume before equilibration at a constant pressure of 1 atm for another 50 ps. Subsequent unrestrained equilibration (2 ns) and production (100 ns) runs were carried out at 300 K using a Langevin thermostat[14] with a collision frequency of 2 ps^{-1}, and 1 atm using a Berendsen barostat[15] with a pressure relaxation time of 2 ps.

3. Synthesis

3.1. General details

General methods: All the reactions were carried out with freshly distilled solvents, unless otherwise stated.

Solvents: Dichloromethane, ethyl acetate, methanol and toluene were distilled from calcium hydride. Tetrahydrofuran was dried over sodium wire and distilled from a mixture of lithium aluminium hydride and calcium hydride with triphenylmethane as an indicator. Diethyl ether was distilled from a mixture of lithium aluminium hydride and calcium hydride.

Reagents: All chemicals were obtained from commercial sources and used without further purification. The chemicals were handled in accordance with COSHH regulations.

Yield: Refer to chromatographically and spectroscopically pure compounds unless otherwise stated.

Flash column chromatography: Carried out on silica gel (Merck Kieselgel 60 F254 230-400 mesh) with distilled solvents under a positive pressure of nitrogen.

Analytical thin layer chromatography (TLC): Carried out on glass plates coated with silica (0.2 mm, Merck Kieselgel 60 F254). Visualisation was done using UV irradiation (254 nm and 365 nm) or staining with potassium permanganate, ninhydrin, or vanillin dips made using standard procedures. Retention factors (R_f) are quoted to the nearest 0.01.

Preparative thin layer chromatography: Carried out on glass plates coated with silica (1 mm, Merck Kieselgel 60 F254). Visualisation was done using UV fluorescence (254 nm and 365 nm).

General nuclear magnetic resonance spectroscopy: Processed using TopSpin v. 3.5 (Bruker). An aryl, quaternary, or two or more possible assignments were given when signals could not be distinguished by any means. Measured coupling constants are reported for mutually coupled signals; coupling constants are labelled apparent (app) in the absence of an observed mutual coupling, or multiplet (m) when none can be determined.

Proton magnetic resonance (^{1}H-NMR) spectroscopy: Recorded using an internal deuterium lock (at 298 K unless stated otherwise) on Bruker DPX (400 MHz; 1H-13C DUL probe) or Bruker Avance III HD (400 MHz; Smart probe) spectrometers. Proton assignments are supported by ^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC or ^{1}H-^{13}C HMBC spectra, or by analogy. Chemical shifts (δ_H) are quoted in ppm to the nearest 0.01 ppm and are referenced to the residual non-deuterated solvent peak. Discernible coupling constants (J) for mutually coupled protons are reported as measured values in Hz, rounded to the nearest 0.1 Hz. Data are reported as: chemical shift, multiplicity (br, broad; s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; or a combination thereof), coupling constants, and number of nuclei.

Carbon nuclear magnetic resonance (^{13}C-NMR) spectroscopy: Recorded using an internal deuterium lock (at 298 K unless stated otherwise) on Bruker DPX (101 MHz) or Bruker Avance III HD (101 MHz) spectrometers with broadband proton decoupling. Carbon spectra assignments are supported by DEPT editing, ^{1}H-^{13}C HSQC or ^{1}H-^{13}C HMBC spectra, or by analogy. Chemical shifts (δ_C) are quoted in ppm to the nearest 0.1 ppm and are referenced to the deuterated solvent peak. Coupling constants between carbon and other nuclei (X) over n bonds ($^nJ_{C\text{-}X}$) are reported as measured values in Hz,

rounded to the nearest 0.1 Hz. Data are reported as: chemical shift, multiplicity (if not a singlet), and coupling constants.

Fluorine nuclear magnetic resonance (^{19}F-NMR) spectroscopy: Recorded on Bruker Avance III (376 MHz; QNP Cryoprobe) or Bruker Avance III HD (376 MHz; Smart probe) spectrometers. Chemical shifts (δ_F) are quoted in ppm to the nearest 0.1 ppm, from $CFCl_3$, and are uncorrected. Data are reported as: chemical shift, multiplicity (if not a singlet), and coupling constants.

High-resolution mass spectrometry (HRMS): Recorded using a Waters LCT Premier Time of Flight (ToF) mass spectrometer, the ThermoFinnigan Orbitrap Classic mass spectrometer, or Waters Vion IMS Qtof mass spectrometer. ASAP refers to atmospheric solids analysis probe. Reported mass values are within the error limits of ±5 ppm mass units.

Liquid chromatography-mass spectrometry (LCMS): Chromatographs were recorded using a Waters ACQUITY H-Class UPLC with an ESCi Multi-Mode Ionisation Waters SQ Detector 2 spectrometer using MassLynx 4.1 software; ESI refers to the electrospray ionisation technique; LC system: solvent A: 2 mM NH_4OAc in water/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

Infrared spectroscopy: Recorded neat on a Perkin Elmer Spectrum One FT-IR spectrometer fitted with an attenuated total reflectance (ATR) sampling accessory. Selected absorption maxima ($\tilde{\nu}_{max}$) are quoted in wavenumbers (cm^{-1}) with the following abbreviations: w, weak; m, medium; s, strong; vs, very strong; br, broad.

High-performance liquid chromatography (HPLC): Analytical chromatographs were obtained on an Agilent 1260 Infinity using a Supercosil ABZ+PLUS column (150 mm × 4.6 mm, 3 µm) eluting with a linear gradient system (solvent A: 0.05% (v/v) TFA in water, solvent B: 0.05% (v/v) TFA in MeCN) over 15 min at a flow rate of mL/min. Semi-preparative HPLC was carried out on an Agilent 1260 Infinity using a Supercosil ABZ+PLUS column (250 mm × 21.2 mm, 5 µm) eluting with a linear gradient system (solvent A: 0.1% (v/v) TFA in water, solvent B: 0.05% (v/v) TFA in MeCN) over 20 min at a flow rate of 20 mL/min. HPLC was monitored by UV absorbance at 220 and 254 nm.

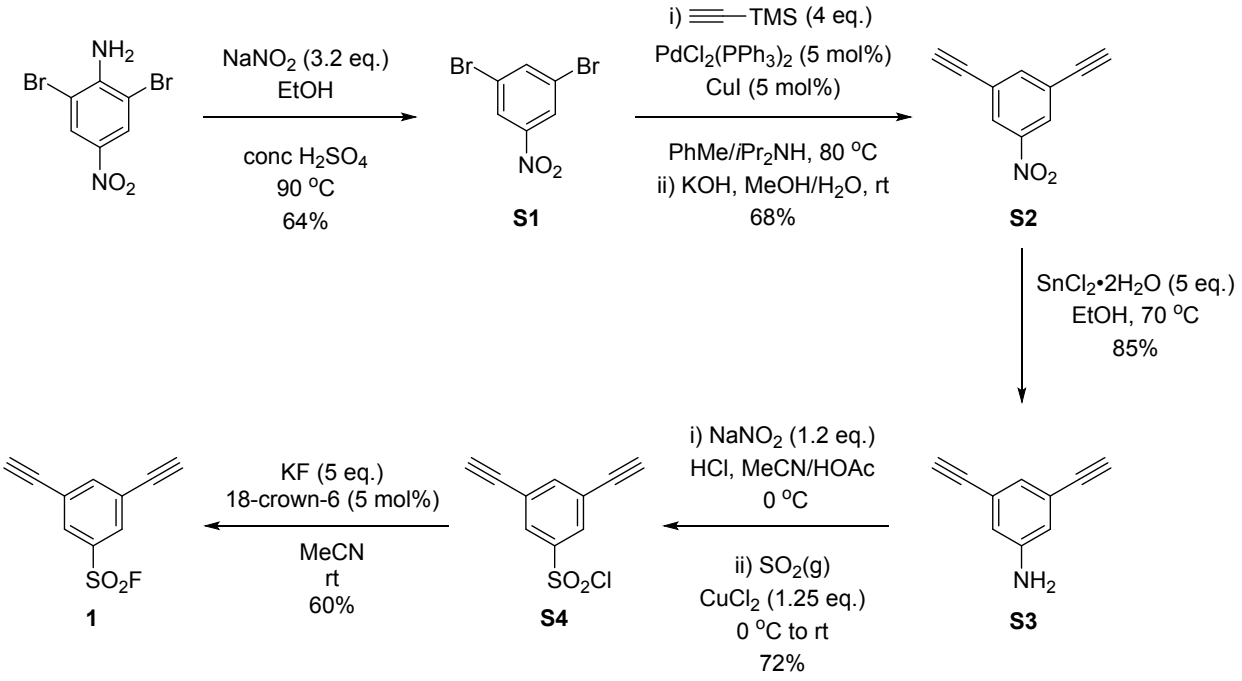
Melting points (mp): Measured using a Büchi melting point B545 apparatus and are uncorrected.

Elemental analysis: Performed by the University of Cambridge Microanalytical Laboratory in the Department of Chemistry and are quoted to the nearest 0.01% for all elements.

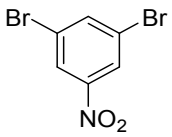
3.2. Synthetic procedures

3.2.1. Staple syntheses

3.2.1.1. Synthesis of staple 1



3,5-dibromonitrobenzene (S1)



To an ice-cooled solution of 2,6-dibromo-4-nitroaniline (11.8 g, 40.0 mmol) in ethanol (250 mL), was added drop-wise concentrated H_2SO_4 (21.3 mL, 0.383 mol) over 30 min. The reaction mixture was heated to 60 °C and $NaNO_2$ (8.83 g, 0.128 mol) was added portion-wise. The reaction was then heated to reflux at 90 °C for 3 h. The mixture was allowed to cool to room temperature before being poured into ice-water. The resulting brown solid was filtered and washed with water. The crude product was purified by column chromatography (eluting gradient hexane:CH_2Cl_2 from 40:1 to 6:1) to give 3,5-dibromonitrobenzene as a pale yellow solid (7.19 g, 64%).

IR (ATR) $\tilde{\nu}_{max}$/cm^{-1}: 3076 (s), 1802 (w), 1763 (w), 1528 (s), 1334 (s)

^{1}H NMR (400 MHz, $CDCl_3$): δ_H/ppm = 8.33 (d, *J* = 1.7 Hz, 2H), 8.01 (t, *J* = 1.7 Hz, 1H)

^{13}C NMR (101 MHz, $CDCl_3$): δ_C/ppm = 149.2, 140.2, 125.7, 123.6

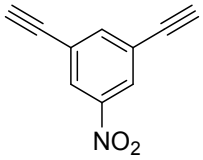
Mp 104-106 °C (lit.[16] 104 °C)

R_f = 0.25 (hexane:CH_2Cl_2 = 6:1)

HRMS (ASAP$^+$): *m/z* $[M]^+$ calcd for $C_6H_3{}^{79}Br_2NO_2$: 278.8531; found: 278.8526 (Δ -1.8 ppm)

Prepared according to Görl *et al*.[17] Spectroscopic data were in accordance with the literature. [17]

1,3-diethynyl-5-nitrobenzene (S2)



3,5-dibromonitrobenzene **S1** (1.18 g, 4.20 mmol), $Pd(PPh_3)Cl_2$ (884 mg, 1.26 mmol), CuI (40.0 mg, 0.210 mmol) and PPh_3 (110 mg, 0.419 mmol) were dissolved in iPr_2NH (4 mL) and toluene (12 mL) under a N_2 atmosphere. The mixture was stirred at room temperature for 5 min before (trimethylsilyl)acetylene (2.37 mL, 16.8 mmol) was added. The reaction mixture was stirred at 80 °C for 17 h. It was then allowed to cool to room temperature and a solution of KOH (943 mg, 16.8 mmol) in water (0.7 mL) and MeOH (2.8 mL) was added in one portion. The mixture was stirred for another 3 h, quenched with saturated aqueous NH_4Cl solution (80 mL) and then extracted with CH_2Cl_2 (3 × 70 mL). The combined organic phases were filtered through Celite®, washed with aqueous HCl (2 M, 70 mL), water (70 mL), and brine (70 mL), dried over $MgSO_4$ and the solvent was removed *in vacuo*. The crude product was purified by column chromatography (eluting gradient hexane:CH_2Cl_2 from 40:1 to 6:1) to give 1,3-diethynyl-5-nitrobenzene as a grey solid (488 mg, 68%).

IR (ATR) $\tilde{\nu}_{max}$/cm^{-1}: 3287 (m), 3269 (s), 3075 (w), 2112 (m), 1738 (s), 1535 (vs), 1352 (vs)

^{1}H NMR (400 MHz, $CDCl_3$): δ_H/ppm = 8.29 (d, *J* = 1.4 Hz, 2H), 7.88 (t, *J* = 1.4 Hz, 1H), 3.24 (s, 2H)

^{13}C NMR (101 MHz, $CDCl_3$): δ_C/ppm = 148.1, 140.9, 127.0, 124.4, 80.9, 80.4

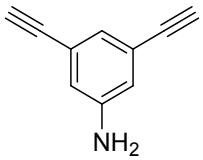
Mp 115-117 °C (lit.[18] 113.5-115 °C)

R_f = 0.13 (hexane:CH_2Cl_2 = 6:1)

HRMS (ASAP$^+$): *m/z* $[M]^+$ calcd for $C_{10}H_5NO_2$: 171.0320; found: 171.0319 (Δ -0.6 ppm)

Procedure adapted from Severin *et al*.[19] Spectroscopic data were in accordance with the literature.[19]

3,5-diethynylaniline (S3)



Under a N_2 atmosphere, a solution of 1,3-diethynyl-5-nitrobenzene **S2** (771 mg, 4.50 mmol) and $SnCl_2{\cdot}2H_2O$ (5.08 g, 22.5 mmol) in EtOH (45 mL) was heated to 70 °C for 1.5 h. The reaction mixture was allowed to cool to room temperature and quenched with addition of aqueous NaOH solution (2 M) to pH 9. The aqueous phase was extracted with Et_2O (3 × 50 mL), and the combined organic phases were dried over Na_2SO_4 and solvent removed *in vacuo* to give 3,5-diethynylaniline as a brown solid (538 mg, 85%). This compound was used in the next step without further purification.

IR (ATR) $\tilde{\nu}_{max}$/cm^{-1}: 3414 (m), 3331 (m), 3278 (s), 2110 (w), 1737 (s), 1587 (vs)

^{1}H NMR (400 MHz, $CDCl_3$): δ_H/ppm = 7.03 (t, *J* = 1.3 Hz, 1H), 6.78 (d, *J* = 1.3 Hz, 2H), 3.72 (br s, 2H), 3.03 (s, 2H)

^{13}C NMR (101 MHz, $CDCl_3$): δ_C/ppm = 146.4, 126.2, 123.2, 118.9, 83.0, 77.3

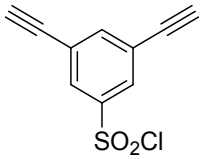
Mp 56-57 °C (lit.[20] 125-127 °C)

R_f = 0.15 (hexane:EtOAc = 5:1)

HRMS (ASAP$^+$): *m/z* $[M]^+$ calcd for $C_{10}H_7N$: 141.0578; found: 141.0576 (Δ -1.4 ppm)

Spectroscopic data were in accordance with the literature.[21]

3,5-diethynylbenzenesulfonyl chloride (S4)



Acetic acid (2.80 mL) and concentrated aqueous HCl (1.40 mL) were added to an ice-cooled solution of 3,5-diethynylaniline **S3** (494 mg, 3.50 mmol) in MeCN (28 mL). A solution of $NaNO_2$ (289 mg, 4.20 mmol) in water (525 µL) was added over 10 minutes at 0 °C. After stirring for further 20 minutes, SO_2 was bubbled in over 40 minutes at 0 °C. A solution of $CuCl_2$ (592 mg, 4.40 mmol) in water (875 µL) was added and the mixture was allowed to warm to room temperature and stirred for further 16 h. The solvent was removed *in vacuo* and the residue re-dissolved in water (20 mL) and CH_2Cl_2 (30 mL). The two phases were separated; the aqueous phase was extracted with CH_2Cl_2 (3 × 25 mL). The combined organic phases were dried over Na_2SO_4, filtered and the solvent removed *in vacuo*. The red-brown oily residue was purified by column chromatography (eluting gradient hexane:CH_2Cl_2 from 40:1 to 5:1) to give 3,5-diethynylbenzenesulfonyl chloride as a beige solid (566 mg, 72%).

IR (ATR) $\tilde{\nu}_{max}$/cm^{-1}: 3292 (s), 3268 (s), 3072 (m), 2111 (w), 1590 (m), 1434 (s), 1375 (vs), 1172 (vs)

^{1}H NMR (400 MHz, $CDCl_3$): δ_H/ppm = 8.09 (d, *J* = 1.4 Hz, 2H), 7.89 (t, *J* = 1.4 Hz, 1H), 3.30 (s, 2H)

^{13}C NMR (101 MHz, $CDCl_3$): δ_C/ppm = 144.9, 141.4, 130.1, 124.9, 81.6, 80.1

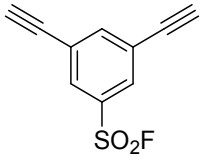
Mp 69-71 °C

R_f = 0.27 (hexane:CH_2Cl_2 = 5:1)

HRMS (ASAP$^+$): *m/z* $[M]^+$ calcd for $C_{10}H_5ClO_2S$: 223.9699; found: 223.9694 (Δ -2.2 ppm)

Elemental analysis: calcd for $C_{10}H_5ClO_2S$: C 53.46%, H 2.24%, Cl 15.78%, O 14.24%, S 14.27%; found: C 53.38% H 2.04%, Cl 14.90%, S 12.14%

3,5-diethynylbenzenesulfonyl fluoride (1)



Potassium fluoride (87.2 mg, 1.50 mmol) and 18-crown-6 (3.96 mg, 0.0150 mmol) were added to a solution of 3,5-diethynylbenzenesulfonyl chloride **S4** (67.4 mg, 0.300 mmol) in MeCN (1.5 mL), under a N_2 atmosphere. The reaction mixture was stirred at room temperature for 3 days and the solvent removed *in vacuo*. EtOAc (10 mL) was added to the residue and the organic phase washed with water (3 × 5 mL), and brine (5 mL), dried over Na_2SO_4, filtered, and solvent removed *in vacuo* to give 3,5-diethynylbenzenesulfonyl fluoride as a white solid (37.3 mg, 60%).

IR (ATR) $\tilde{\nu}_{max}$/cm^{-1}: 3287 (s), 2111 (w), 1592 (w), 1434 (s), 1407 (s), 1202 (vs)

^{1}H NMR (400 MHz, $CDCl_3$): δ_H/ppm = 8.06 (d, *J* = 1.5 Hz, 2H), 7.92 (t, *J* = 1.4 Hz, 1H), 3.29 (s, 2H)

^{13}C NMR (101 MHz, $CDCl_3$): δ_C/ppm = 141.8, 134.2 (d, $^2J_{C\text{-}F}$ = 26.2 Hz), 131.5, 124.9, 81.6, 80.0

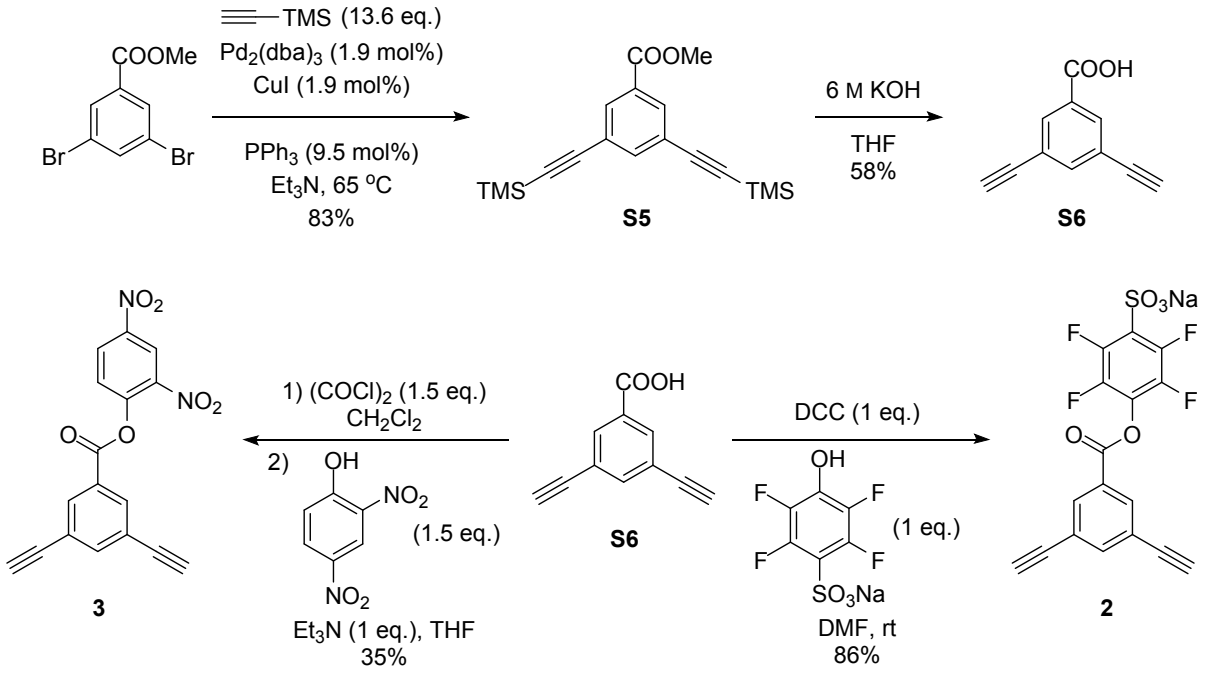
^{19}F NMR (376 MHz, $CDCl_3$): δ_F/ppm = 65.8

Mp 77-79 °C

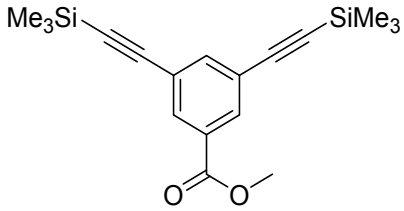
R_f = 0.20 (hexane:CH_2Cl_2 = 5:1)

HRMS (ASAP$^+$): *m/z* $[M]^+$ calcd for $C_{10}H_5FO_2S$: 207.9994; found: 207.9992 (Δ -1.0 ppm)

3.2.1.2. Synthesis of staples 2 and 3



Methyl 3,5-bis((trimethylsilyl)ethynyl)benzoate (S5)



Methyl 3,5-dibromobenzoate (1.52 g, 5.17 mmol), $Pd_2(dba)_3$ (89.7 mg, 0.0980 mmol), CuI (19.1 mg, 0.100 mmol) and PPh_3 (129 mg, 0.492 mmol) were added to Et_3N (25 mL), followed by trimethylsilylacetylene (9.94 mL, 70.3 mmol). The reaction mixture was stirred at 65 °C for 20 h, then concentrated *in vacuo*. The residue was re-dissolved in CH_2Cl_2 (30 mL), filtered through Celite®, then solvent removed *in vacuo*. The crude product was purified by flash column chromatography (hexane:EtOAc = 99:1) to give the title compound as a yellow solid (1.41 g, 83%)

IR (ATR) $\tilde{\nu}_{max}$/cm^{-1}: 2957 (m), 2900 (w), 2157 (m), 1729 (s), 1588 (m)

^{1}H NMR (400 MHz, $CDCl_3$): δ_H/ppm = 8.05 (d, *J* = 1.6 Hz, 2H), 7.74 (t, *J* = 1.6 Hz, 1H), 3.93 (s, 3H), 0.25 (s, 18H)

^{13}C NMR (101 MHz, $CDCl_3$): δ_C/ppm = 165.8, 139.2, 132.8, 130.7, 124.0, 103.1, 96.3, 52.5, -0.1

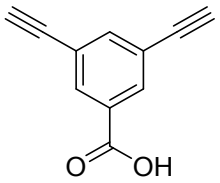
Mp 73-76 °C (lit.[22] 71-73 °C)

R_f = 0.31 (hexane:EtOAc = 20:1)

HRMS (ASAP$^+$): *m/z* $[M+H]^+$ calcd for $C_{18}H_{25}O_2Si_2$: 329.1393; found: 329.1383 (Δ -3.0 ppm)

Procedure adapted from Lau *et al*. [22] Spectroscopic data were in accordance with the literature. [22]

3,5-diethynylbenzoic acid (S6)



Aqueous KOH (2.50 mL, 15.0 mmol, 6.00 M) was added to a solution of methyl 3,5-bis((trimethylsilyl)ethynyl)benzoate **S5** (850 mg, 2.59 mmol) in THF (15 mL). The reaction mixture was stirred at rt for 18 h, and the volatiles were removed *in vacuo*. The remaining aqueous mixture was acidified with aqueous HCl (40 mL, 2 M) and extracted with Et_2O (3 × 40 mL). The combined organic phases were dried over $MgSO_4$ and the solvent removed *in vacuo*. The crude product was recrystallised from $CHCl_3$ to give 3,5-diethynylbenzoic acid as a deep beige fine powder (255 mg, 58%)

IR (ATR) $\tilde{\nu}_{max}$/cm^{-1}: 3285 (m), 2600 (br m), 1682 (vs), 1588 (s)

^{1}H NMR (400 MHz, $CDCl_3$): δ_H/ppm = 8.19 (d, *J* = 1.6 Hz, 2H), 7.82 (t, *J* = 1.6 Hz, 1H), 3.17 (s, 2H)

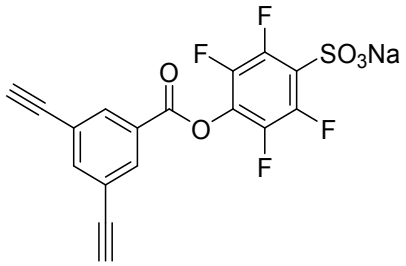
^{13}C NMR (101 MHz, $CDCl_3$): δ_C/ppm = 169.3, 140.2, 133.9, 130.0, 123.4, 81.5, 79.3

Mp 173 °C dec (lit. 173 °C[23]; 250 °C dec[22])

HRMS (ASAP$^+$): *m/z* $[M+H]^+$ calcd for $C_{11}H_7O_2$: 171.0446; found: 171.0448 (Δ 1.2 ppm)

Procedure adapted from Lau *et al*. [22] Spectroscopic data were in accordance with the literature. [22]

Sodium 4-((3,5-diethynylbenzoyl)oxy)-2,3,5,6-tetrafluorobenzenesulfonate (2)



To the solution of sodium 2,3,5,6-tetrafluoro-4-hydroxybenzenesulfonate (134 mg, 0.500 mmol) and 3,5-diethynylbenzoic acid (85.1 mg, 0.500 mmol) in DMF (1.25 mL) was added DCC (103 mg, 0.500 mmol), under a N_2 atmosphere. The reaction mixture was stirred at room temperature for 21 h, then stirred at 0 °C for 1 h, filtered and washed with DMF (80 µL). The product was triturated from the filtrate using Et_2O then filtered to give sodium 4-((3,5-diethynylbenzoyl)oxy)-2,3,5,6-tetrafluorobenzenesulfonate as a beige powder (181 mg, 86%).

IR (ATR) $\tilde{\nu}_{max}$/cm^{-1}: 3282 (m), 1756 (s), 1646 (m), 1480 (vs), 1184 (vs)

^{1}H NMR (500 MHz, DMSO-d_6): δ_H/ppm = 8.20 (d, *J* = 1.5 Hz, 2H), 8.00 (t, *J* = 1.5 Hz, 1H), 4.50 (s, 2H)

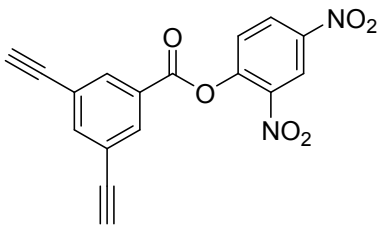
^{13}C NMR (126 MHz, DMSO-d_6): δ_C/ppm = 160.8, 143.0 (d, $^1J_{C\text{-}F}$ = 249.3 Hz), 140.4, 140.0 (dd, *J* = 250.9, 17.6 Hz), 133.3, 127.9 (t, $^2J_{C\text{-}F}$ = 14.5 Hz), 127.6, 125.0 (t, $^2J_{C\text{-}F}$ = 18.9 Hz), 123.7, 83.7, 80.8

^{19}F NMR (376 MHz, DMSO-d_6): δ_F/ppm = -140.0 (dd, *J* = 25.5, 9.7 Hz), -154.4 (dd, *J* = 25.5, 9.8 Hz)

Mp 150 °C (dec)

HRMS (ESI$^-$): *m/z* $[M-Na]^-$ calcd for $C_{17}H_5F_4O_5S$: 396.9799; found: 396.9795 (Δ -1.2 ppm)

2,4-dinitrophenyl 3,5-diethynylbenzoate (3)



Under a N_2 atmosphere, DMF (20 µL) was added to a suspension of 3,5-diethynylbenzoic acid (85.1 mg, 0.500 mmol) in CH_2Cl_2 (2.5 mL) and the mixture was then cooled to 0 °C. Oxalyl chloride (64.3 µL, 0.750 mmol) was added and the reaction mixture stirred at rt for 5 h. The solvent was removed *in vacuo* to give the crude acid chloride.

The crude acid chloride was re-dissolved in THF (1 mL) and added to a solution of 2,4-dinitrophenol (138 mg, 0.750 mmol) and Et_3N (69.7 µL, 0.500 mmol) in THF (1 mL) at 0 °C. The reaction mixture was stirred at rt for 21 h, then quenched with the addition of saturated $NaHCO_3$ (5 mL). The mixture was extracted with Et_2O (3 × 10 mL). The combined organic phases were dried over $MgSO_4$, filtered then the solvent removed *in vacuo*. The crude product was purified by column chromatography (eluting gradient hexane:EtOAc from 40:1 to 5:1) to give 2,4-dinitrophenyl 3,5-diethynylbenzoate as an off-white powder (59.2 mg, 35%).

IR (ATR) $\tilde{\nu}_{max}$/cm^{-1}: 3285 (m), 3082 (w), 1750 (s), 1611 (m), 1532 (vs), 1348 (vs)

^{1}H NMR (500 MHz, $CDCl_3$): δ_H/ppm = 9.05 (s, 1H), 8.60 (d, *J* = 8.9 Hz, 1H), 8.26 (s, 2H), 7.90 (s, 1H), 7.65 (d, *J* = 8.9 Hz, 1H), 3.22 (s, 2H)

^{13}C NMR (126 MHz, $CDCl_3$): δ_C/ppm = 162.2, 148.6, 145.5, 141.8, 141.1, 134.2, 129.3, 128.4, 126.8, 123.9, 122.1, 81.1, 80.0

Mp 155 °C (dec)

R_f = 0.21 (hexane:EtOAc = 5:1)

LCMS (ESI$^+$): *m/z* $[M+NH_4]^+$ calcd for $C_{17}H_{12}N_3O_6$: 354.1; found: 354.5

3.2.2. Linear peptide syntheses

Azido amino acid Fmoc-Orn(N_3)-OH was synthesised as previously reported.[22]

Peptide synthesis was carried out on solid-phase using Fmoc-protecting group strategy on a CEM LibertyBlue Automated Microwave Peptide Synthesizer using Rink Amide MBHA LL resin (0.33 mmol/g) (Merck Millipore). Fmoc-protected amino acids were made up as a solution of 0.2 M in DMF to give 5 equivalents relative to resin loading. Oxyma Pure was made up as a 1 M solution in DMF to give 5 equivalents relative to resin and *N*,*N*′-diisopropylcarbodiimide as a 1 M solution in DMF to give 10 equivalents relative to the resin. All amino acid couplings were double coupled and heated to 90 °C for two mins, except for Fmoc-Orn(N_3)-OH which was single coupled and heated to 90 °C for 270 seconds for each coupling. Fmoc deprotection was carried out using 20 % (v/v) piperidine in DMF and heated to 90 °C for 60 seconds twice. The Ac capping of the *N*-terminus was carried out using 20 % (v/v) Ac_2O in DMF and heated to 40 °C for 10 minutes.

3.2.3. Peptide stapling

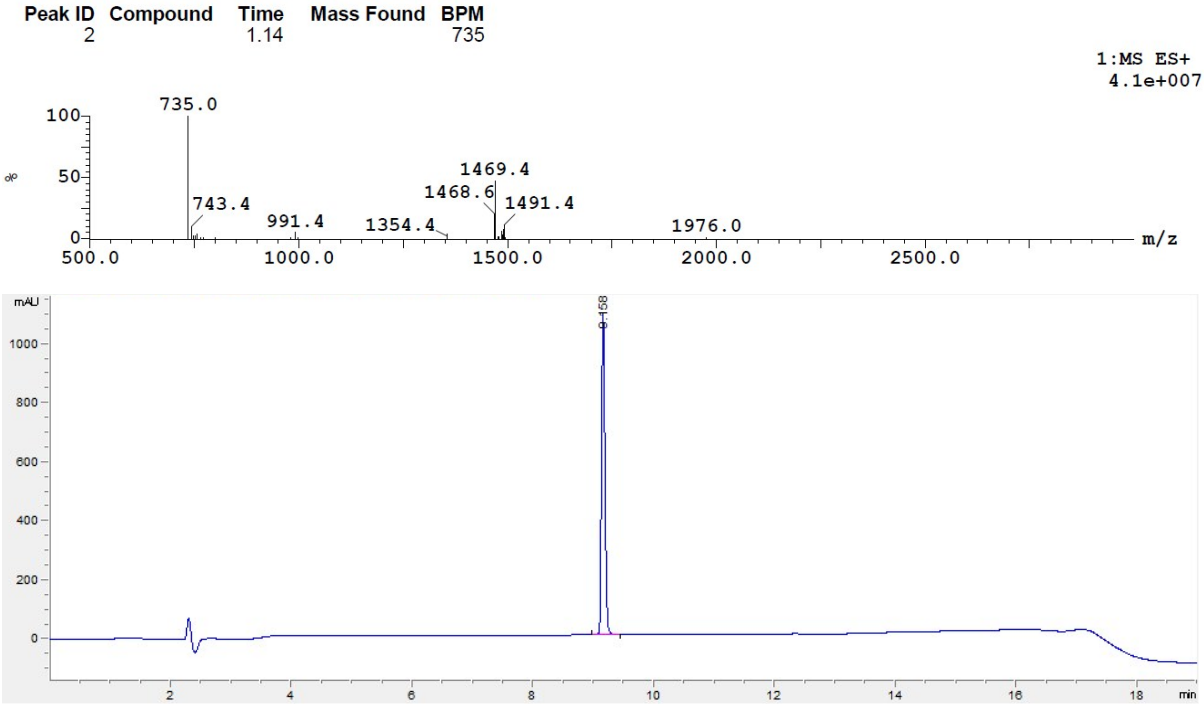
Under a N_2 atmosphere, a solution of **P1** in DMSO (1 eq., 0.4 mg/µL) was added to a degassed *t*BuOH/water (1:1) making the concentration of **P1** to 1 mg/mL. The dialkynyl linker (1.1 eq.) was then added. Sodium L-ascorbate (3 eq.) was added to a degassed aqueous solution of $CuSO_4$•$5H_2O$ (1 eq.) and tris(3-hydroxypropyltriazolylmethyl)amine (1 eq.) under nitrogen. The copper solution was added to the reaction mixture, stirred at rt, and monitored by HPLC until its completion. The mixture was lyophilised and then purified by semi-preparative HPLC.

3.2.4. LCMS data and analytical HPLC traces for peptides

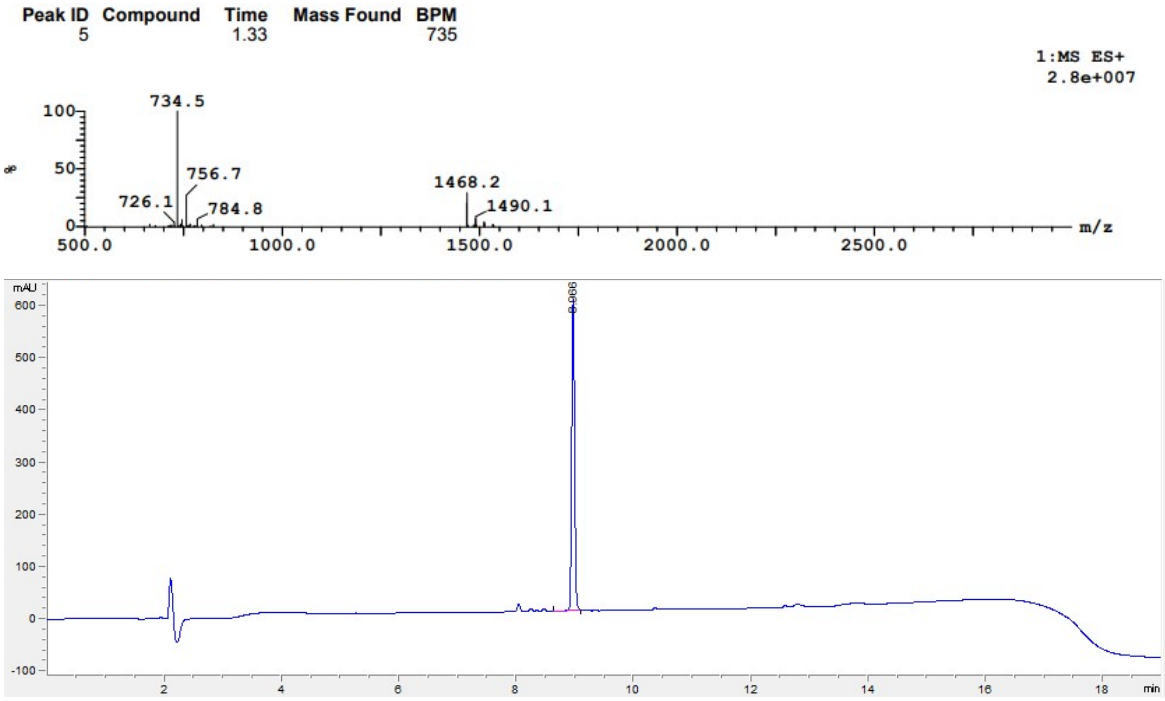
Peptide	Sequence*	Exact Mass	*m/z* found	*m/z* calcd	Species	Purity†	t_R (min)‡
WT PMI	Ac-TSFAEYWNNLSP-NH_2	1467.70	735.0	734.9	$[M+2H]^{2+}$	99%	9.16
P1	Ac-TSFAXYWNGLSX-NH_2	1465.69	734.5	733.9	$[M+2H]^{2+}$	93%	8.97
P1-2		1885.66	933.5	932.9	$[M-Na+3H]^{2+}$	98%	8.71
P1-4		1591.73	797.6	796.9	$[M+2H]^{2+}$	96%	8.76

*Shown for linear peptides only. X = Orn(N_3) †Determined by analytical HPLC. ‡Obtained from analytical HPLC runs with 5-95 gradient (an x-y gradient signifies the amount of solvent B being x % at the start and y% at the end of the run, respectively.).

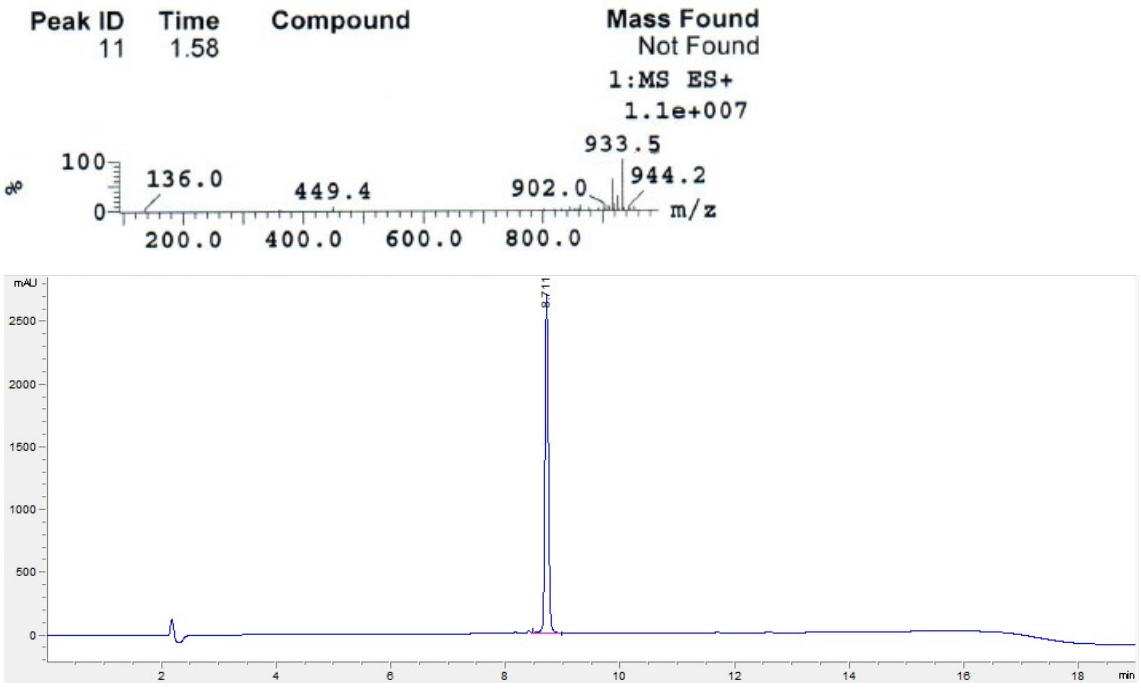
WT PMI



P1


Peak ID Compound Time Mass Found BPM
5 1.33 735
1:MS ES+
2.8e+007
734.5
756.7
726.1
784.8
1468.2
1490.1
m/z
500.0
1000.0
1500.0
2000.0
2500.0
mAU
8.066
min

P1-2


Peak ID Time Compound Mass Found
11 1.58 Not Found
1:MS ES+
1.1e+007
933.5
944.2
136.0
449.4
902.0
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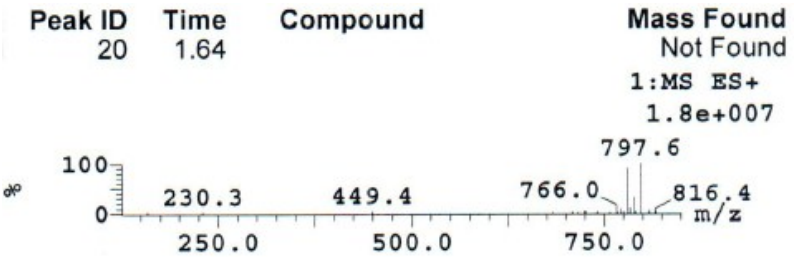
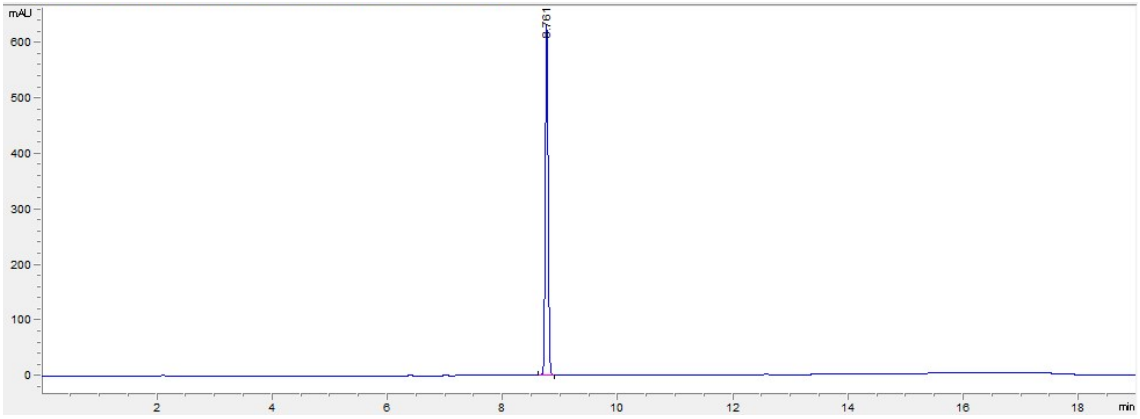
600.0
800.0
mAU
8.711
min

P1-4





3.2.5. Circular dichroism

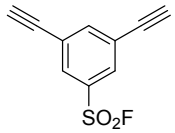
CD spectra of selected peptides were recorded on an AVIV 410 circular dichroism spectropolarimeter using a 1 mm path length quartz cuvette. CD measurements were performed at 298 K over a range of 185-260 nm using a response time of 0.5 s, 1 nm pitch and 0.5 nm bandwidth. Peptides were dissolved in 1:1 MQ water/acetonitrile to a final concentration of 100 µM. The recorded spectra represent a smoothed average of three scans, zero-corrected at 260 nm. The %helicity was calculated from mean residue ellipticity ($[\theta]_{222}$) and compared to the theoretical maximum value (θ_{222}^{∞} = -39,500 deg cm^2 dmol^{-1}) according to the following equation:[24]

$$\%helicity = \frac{[\theta]_{222}}{\theta_{222}^{\infty}\left[1 - \frac{ik}{n}\right]}$$

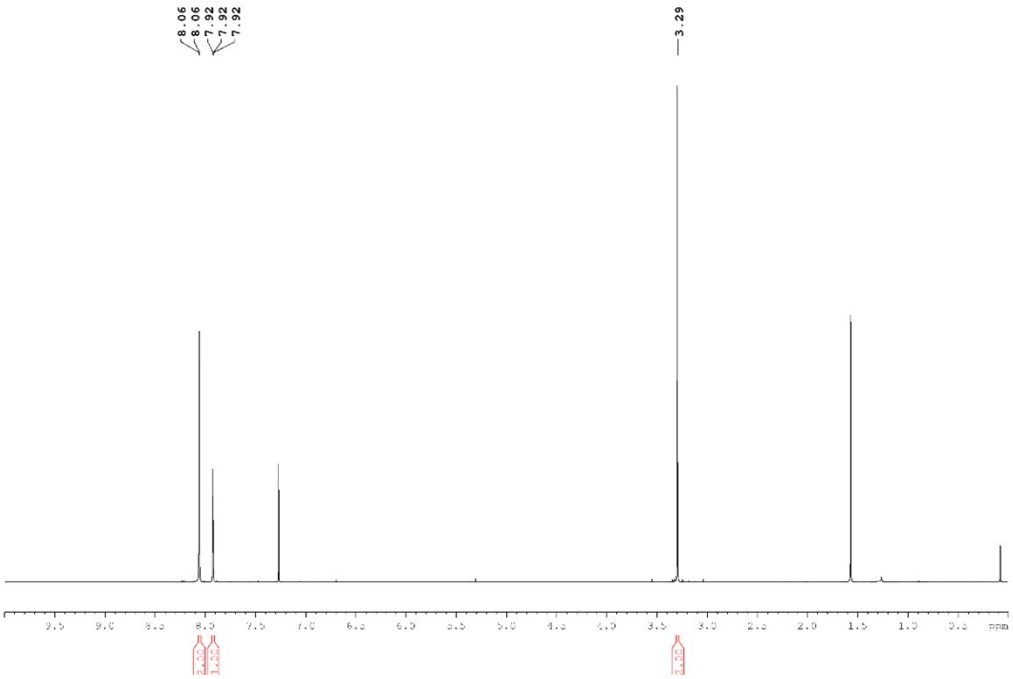
where *i* is the number of helices in the sample, *k* is the wavelength specific constant (2.57 at 222 nm), and *n* is the number of amino acid residues in the peptide.

4. ^{1}H, ^{13}C and ^{19}F NMR spectra

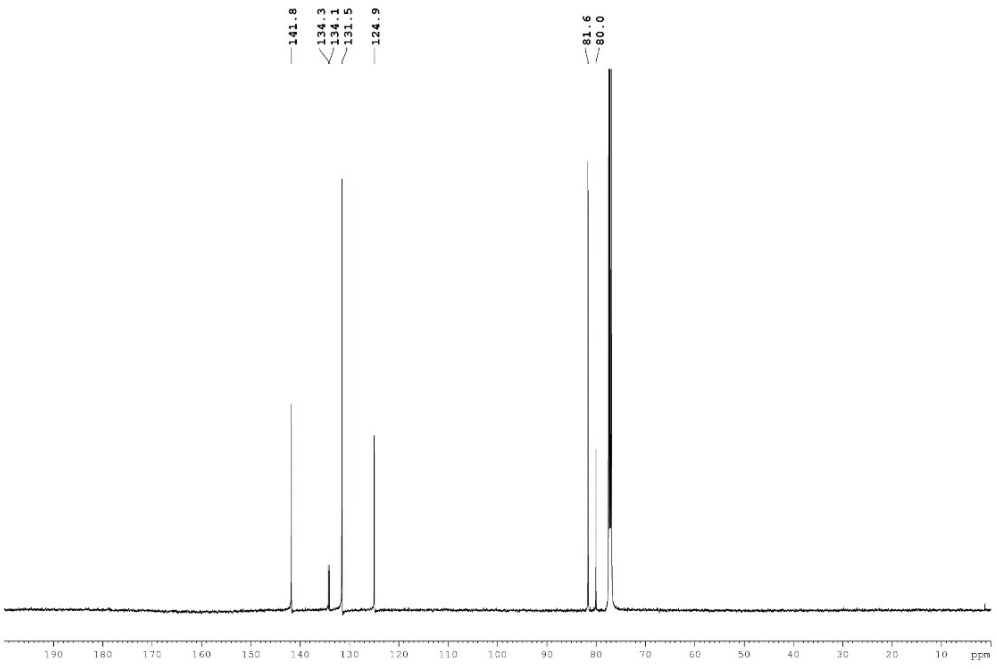
3,5-diethynylbenzenesulfonyl fluoride (1)



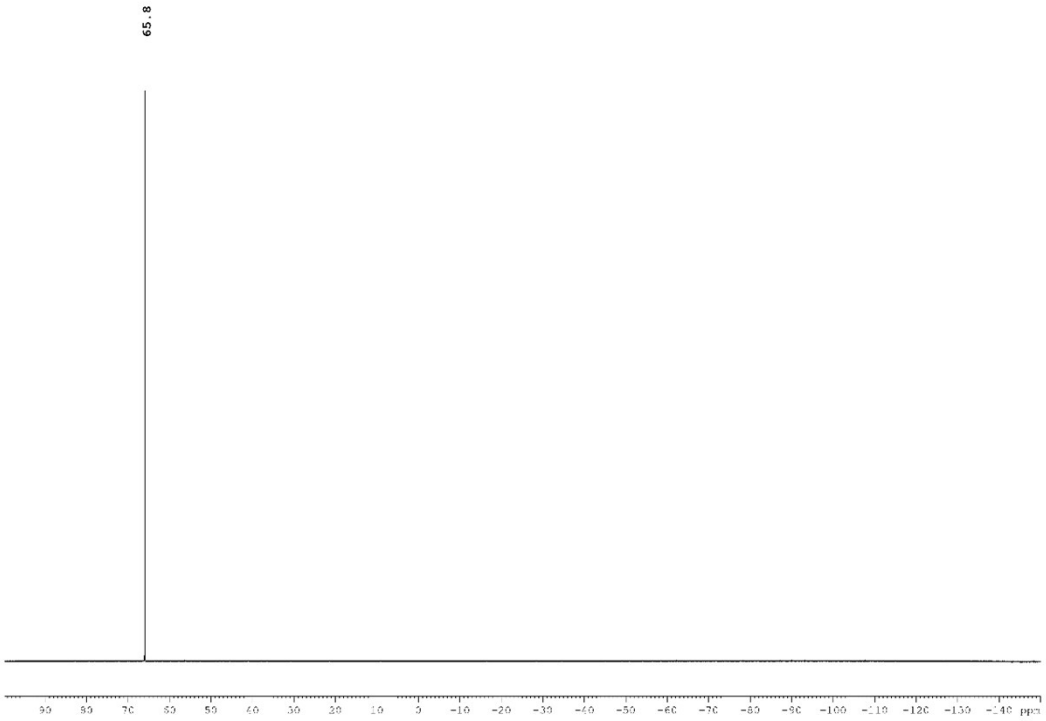
^{1}H NMR



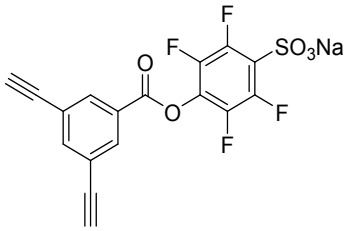
^{13}C NMR



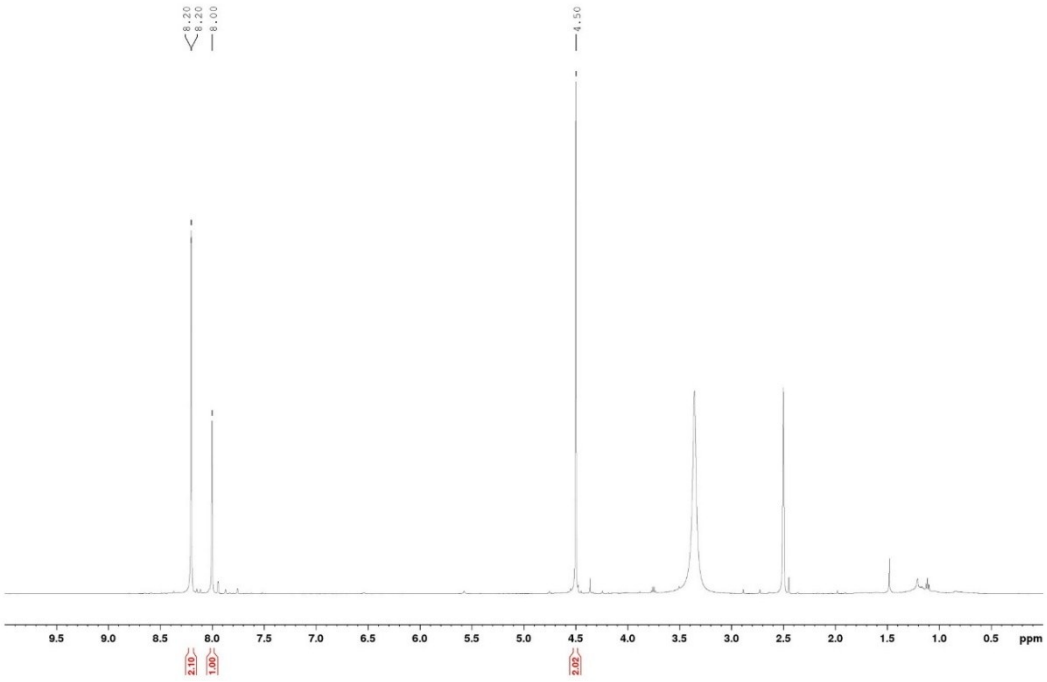
^{19}F NMR


65.8
95
90
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
-110
-120
-130
-140
ppm

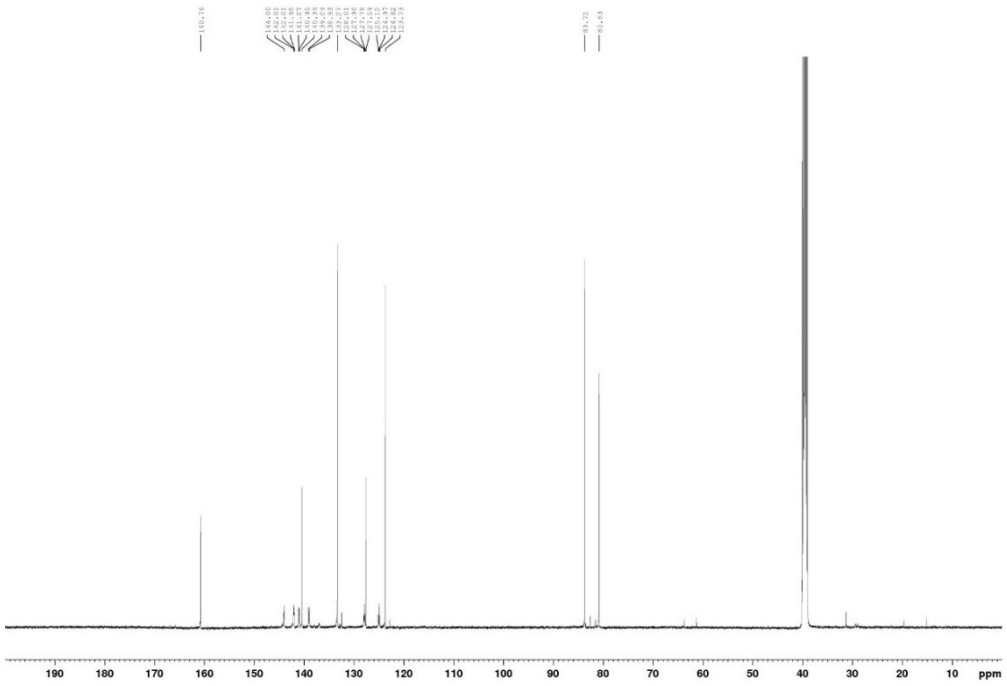
Sodium 4-((3,5-diethynylbenzoyl)oxy)-2,3,5,6-tetrafluorobenzenesulfonate (2)



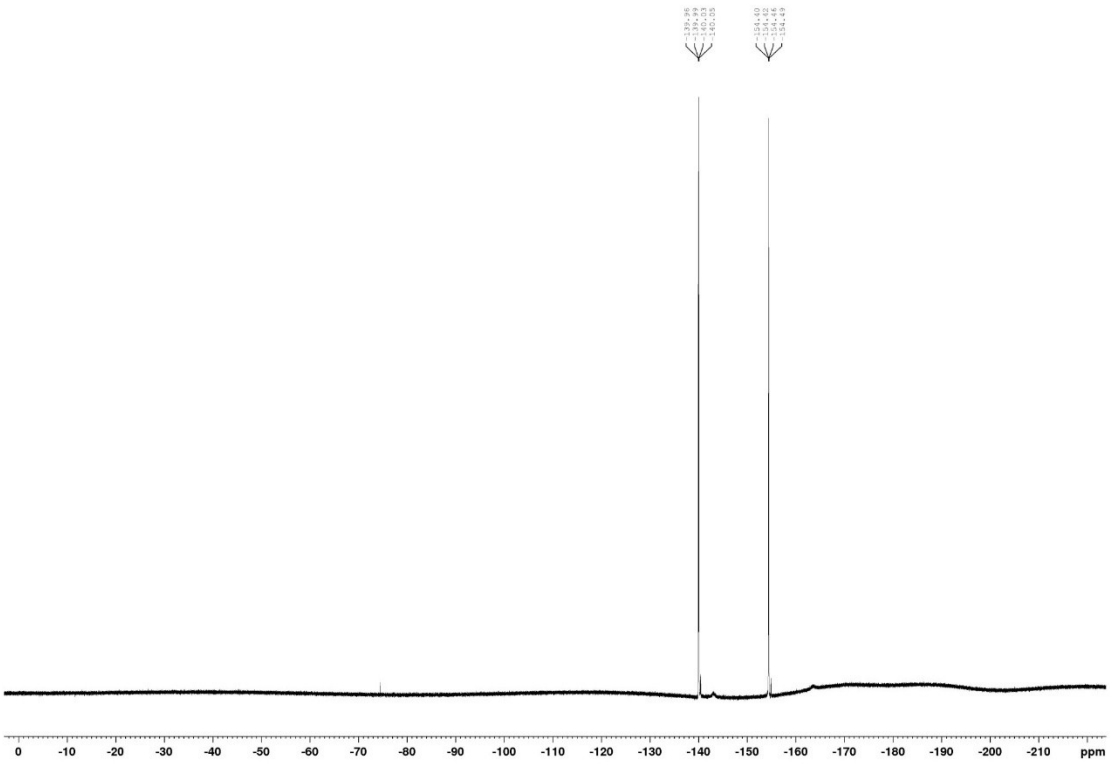
^{1}H NMR



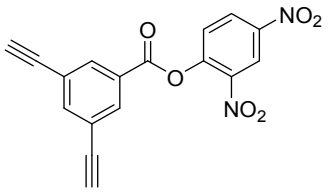
^{13}C NMR



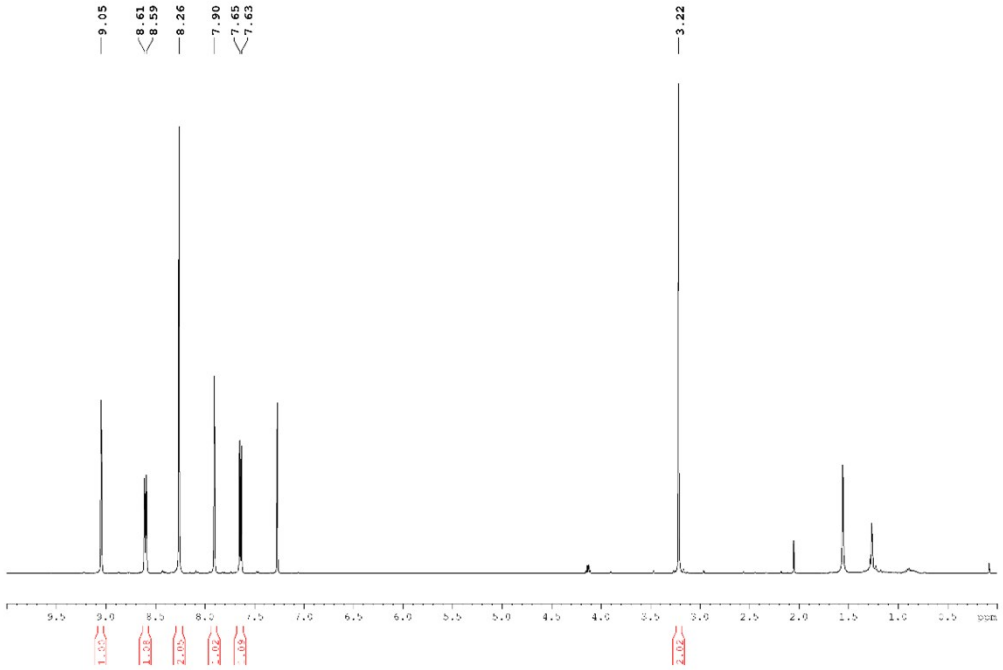
^{19}F NMR


0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
-110
-120
-130
-140
-150
-160
-170
-180
-190
-200
-210
ppm

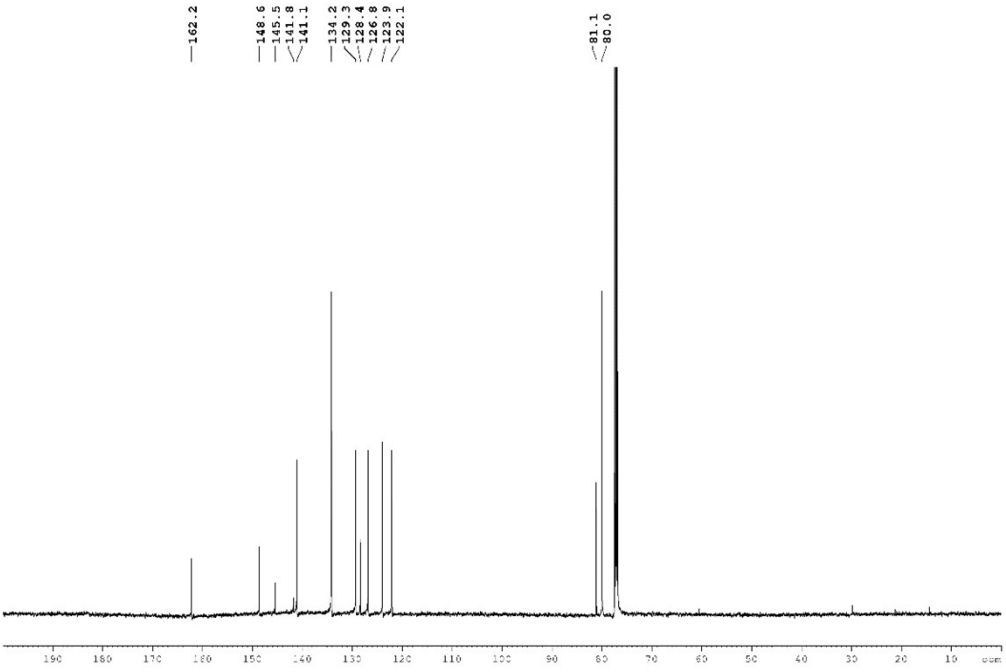
2,4-dinitrophenyl 3,5-diethynylbenzoate (3)



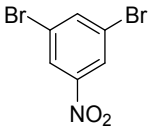
^{1}H NMR



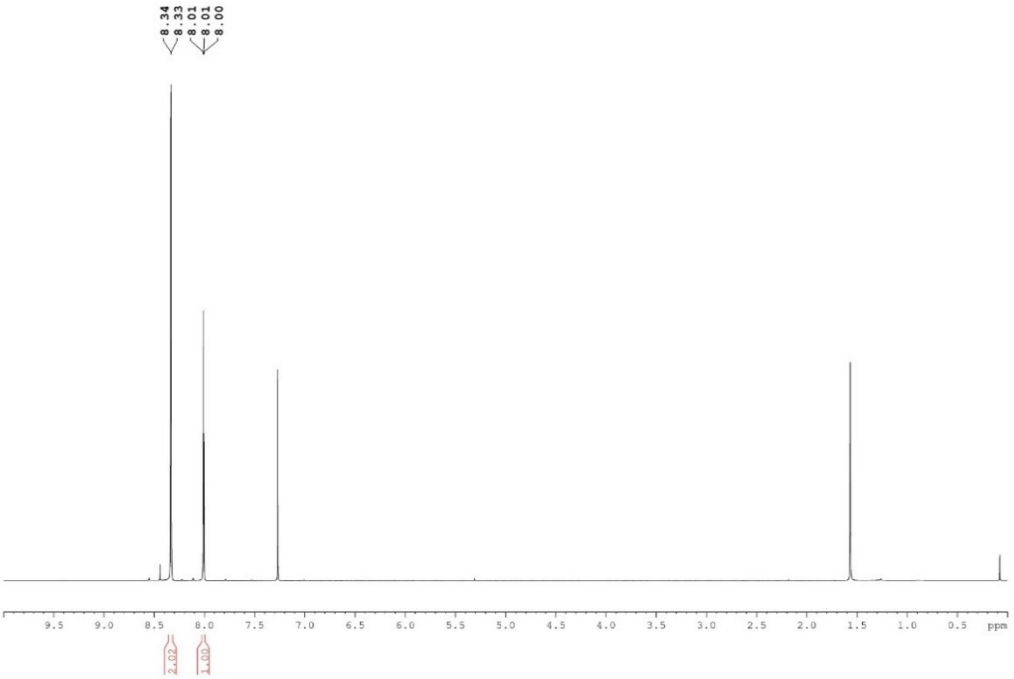
^{13}C NMR



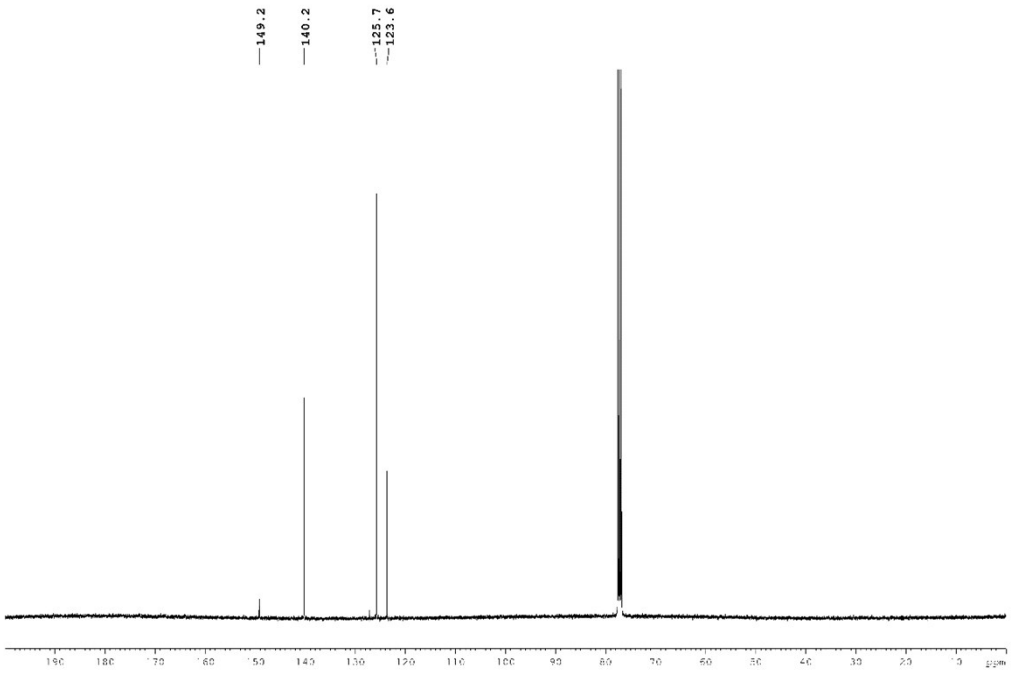
3,5-dibromonitrobenzene (S1)



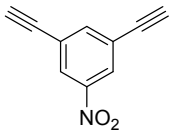
^{1}H NMR



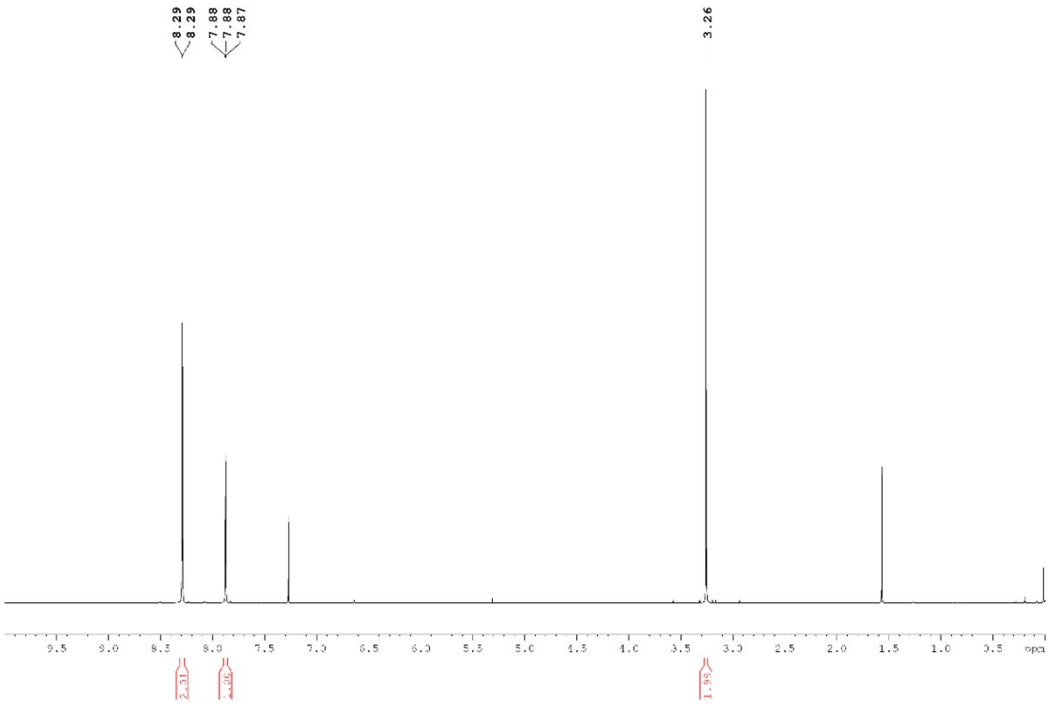
^{13}C NMR



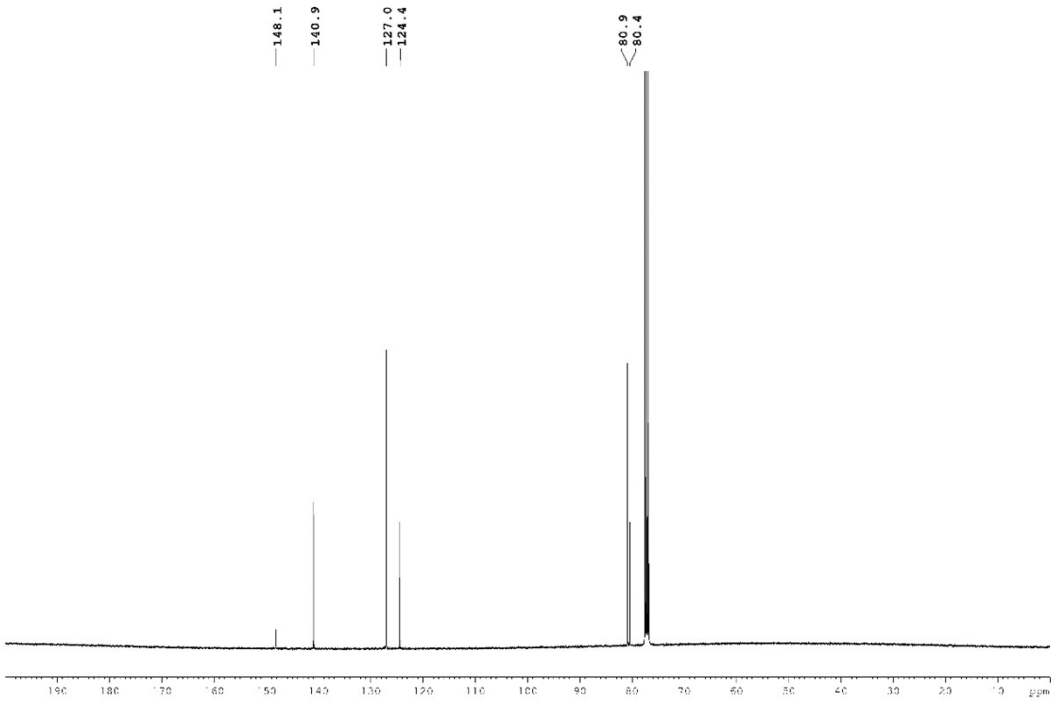
1,3-diethynyl-5-nitrobenzene (S2)



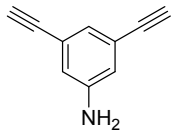
^{1}H NMR



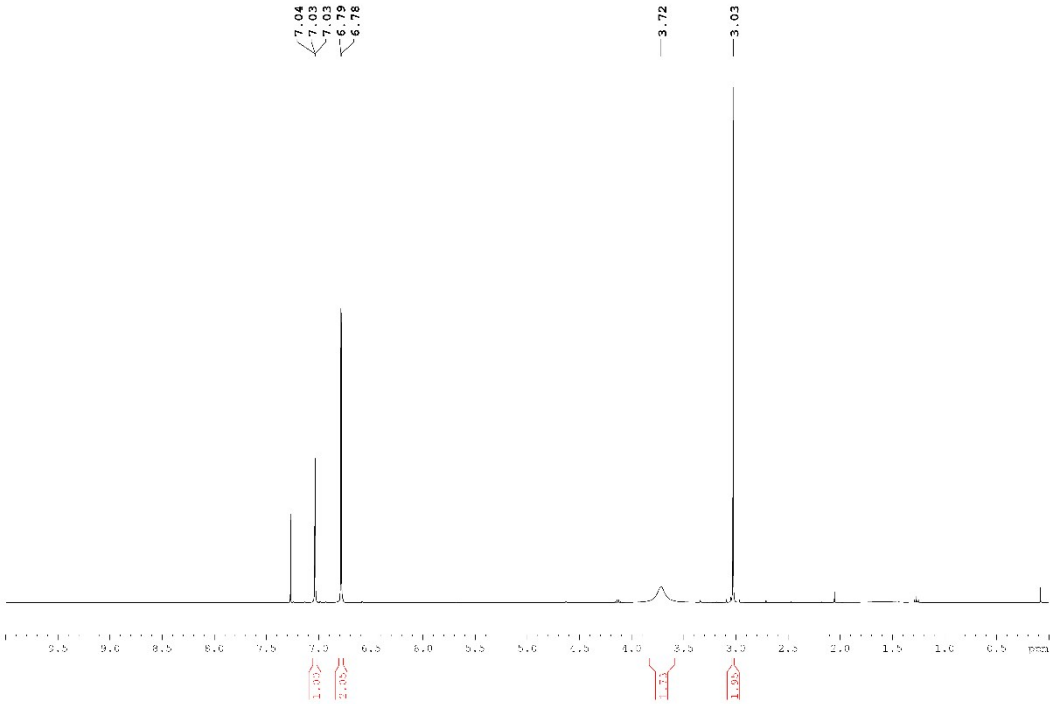
^{13}C NMR



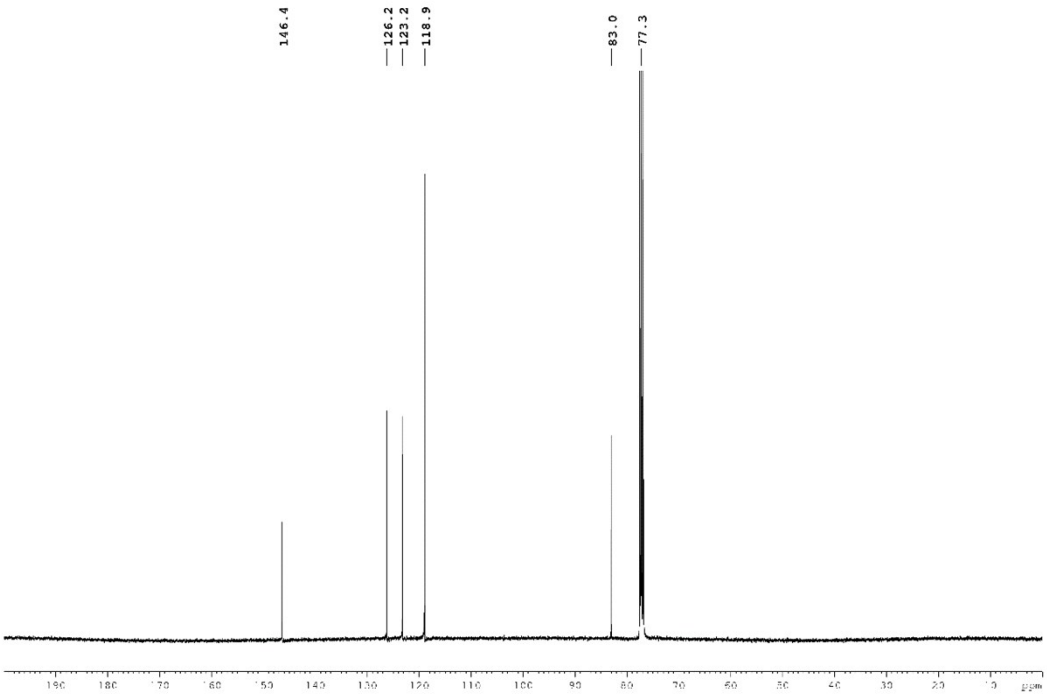
3,5-diethynylaniline (S3)



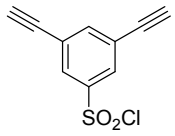
^{1}H NMR



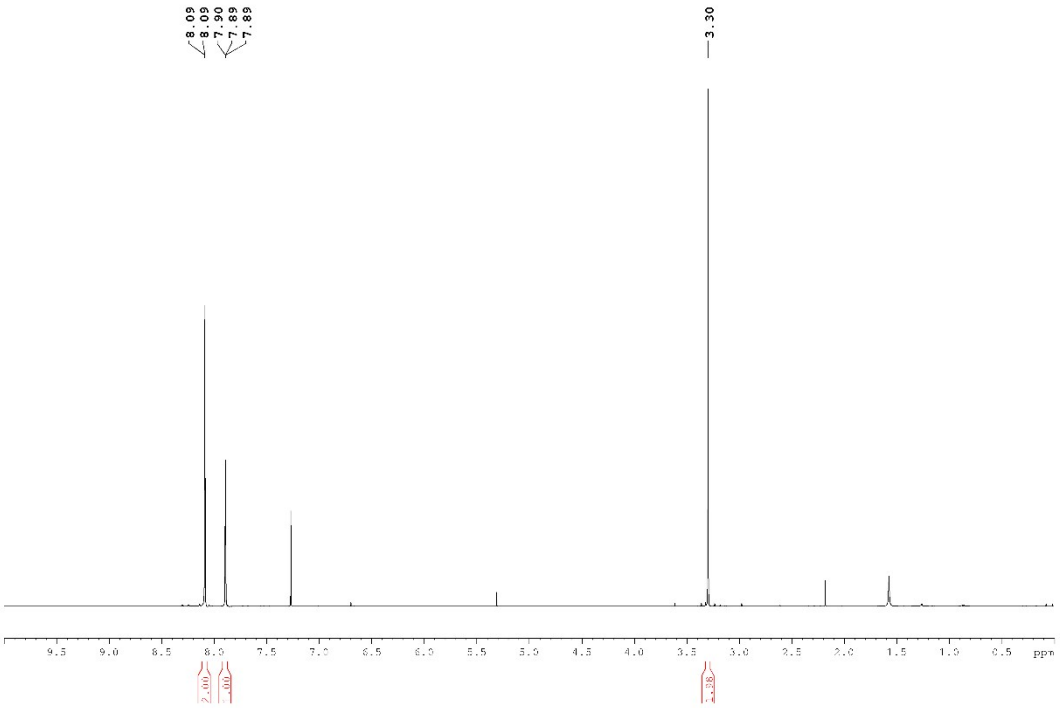
^{13}C NMR



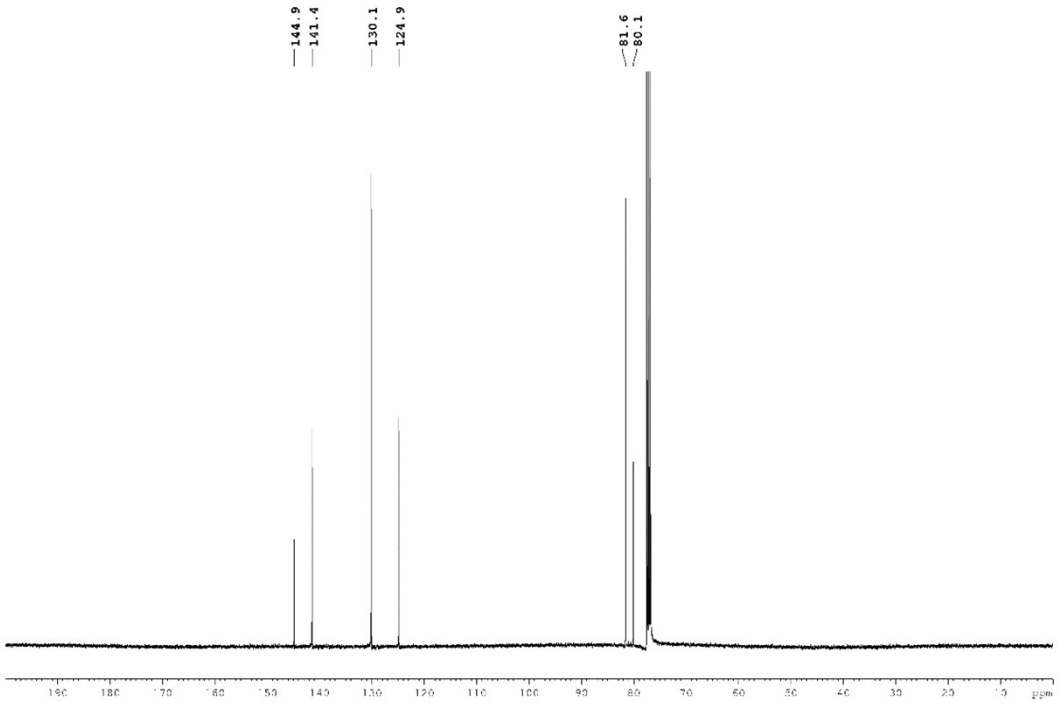
3,5-diethynylbenzenesulfonyl chloride (S4)



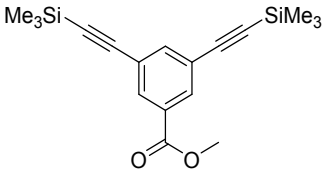
^{1}H NMR



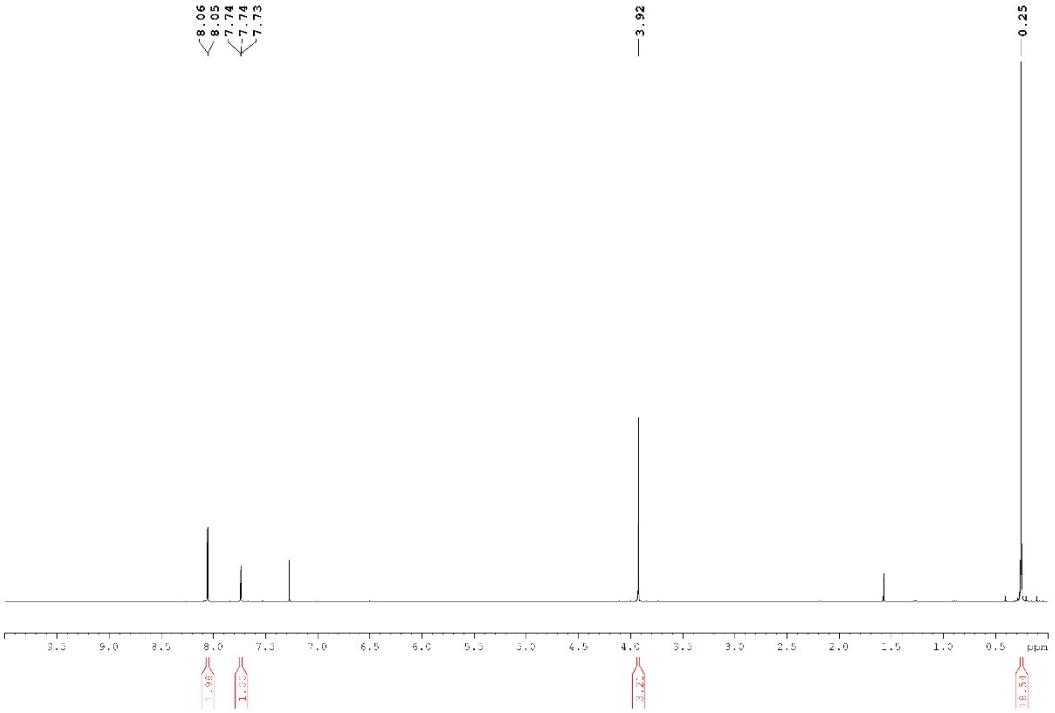
^{13}C NMR



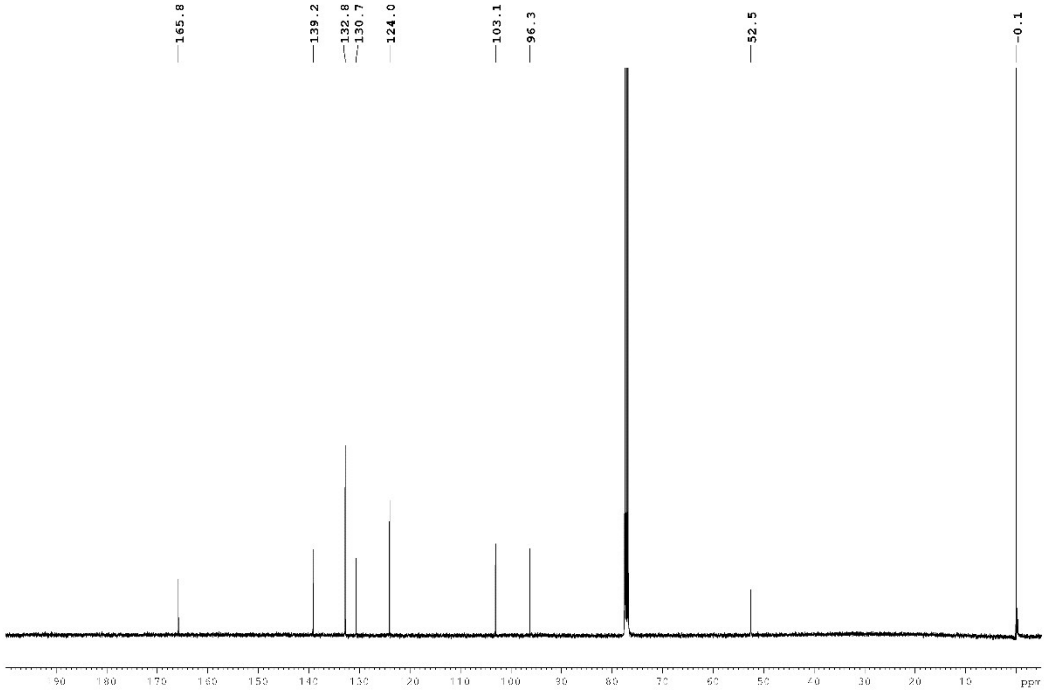
Methyl 3,5-bis((trimethylsilyl)ethynyl)benzoate (S5)



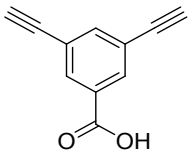
^{1}H NMR



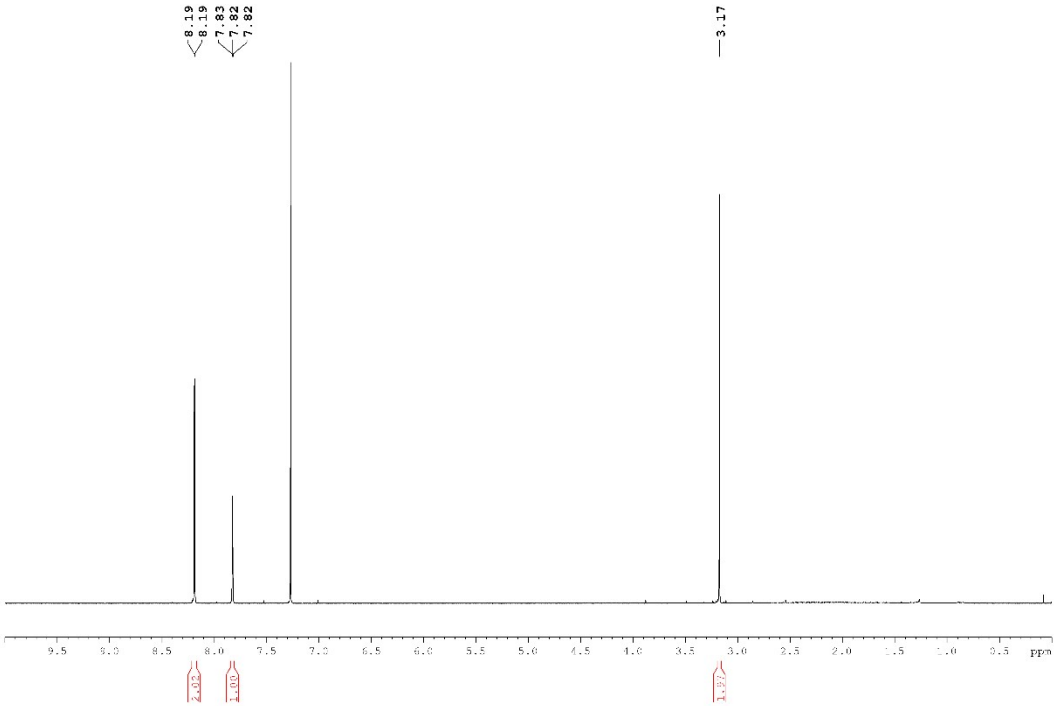
^{13}C NMR



3,5-diethynylbenzoic acid (S6)



^{1}H NMR



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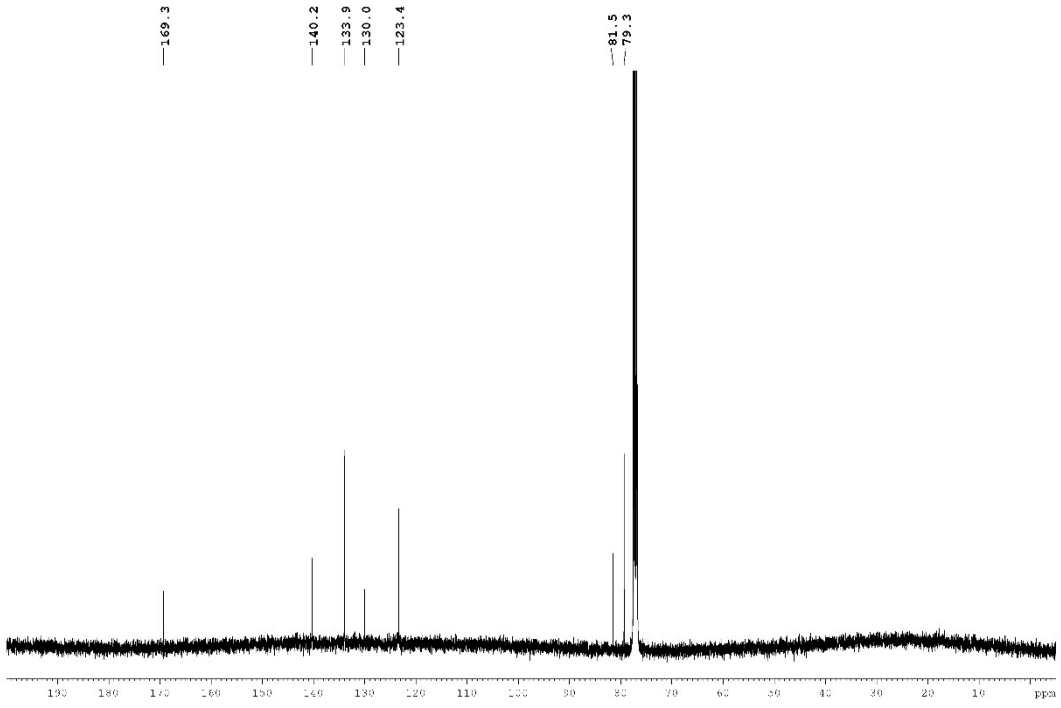


5. Stability and reactivity test protocols

5.1. Stability tests in CuAAC peptide stapling condition

Sodium L-ascorbate (3 eq.) was added to a degassed aqueous solution of $CuSO_4 \bullet 5H_2O$ (1 eq.) and tris(3-hydroxypropyltriazolylmethyl)amine (1 eq.) under nitrogen. The mixture was then added to a solution of a dialkynyl linker (1.1 eq., 0.73 mM) and caffeine (0.3 eq.) in degassed *t*BuOH/water (1:1) at rt under nitrogen. The reactions were monitored by analytical HPLC by comparison of integrations with the internal standard (caffeine) peak at 254 nm.

5.2. Stability tests in aqueous media for linkers

At 37 °C, PBS (1 mL) was added caffeine solution in MeCN (0.75 mM, 200 µL) and linker solution in 3:2 MeCN:PBS (2.5 mM, 800 µL). The reactions were monitored by analytical HPLC by comparison of integrations with the internal standard (caffeine) peak at 254 nm.

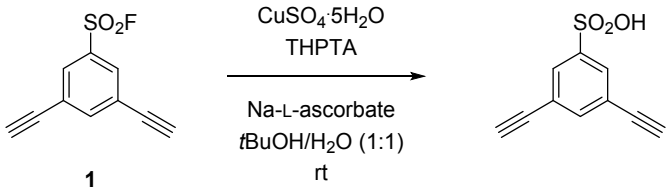
5.3. Reactivity tests with lysine for linkers

At 37 °C, a stock solution of *N*α-Ac-Lys-OH in PBS (4 mM, 1 mL) was added caffeine solution in MeCN (0.75 mM, 200 µL) and linker solution in 3:2 MeCN:PBS (2.5 mM, 800 µL). The reactions were monitored by analytical HPLC by comparison of integrations with the internal standard (caffeine) peak at 254 nm.

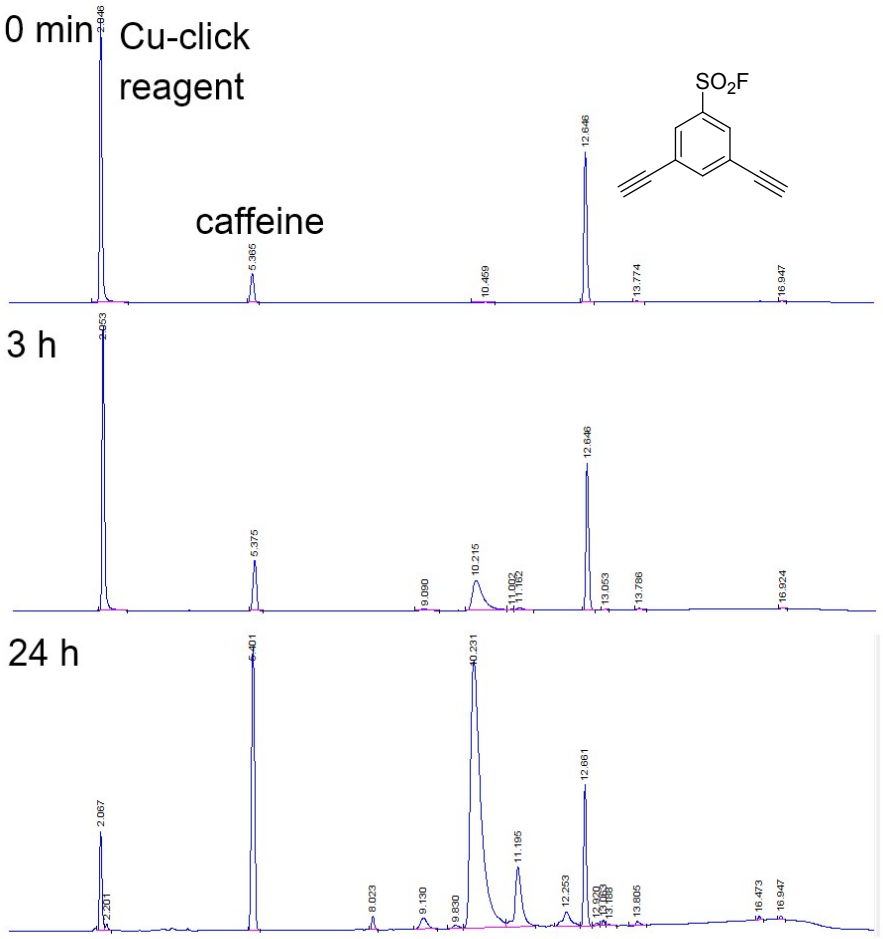
5.4. HPLC traces from stability and reactivity traces

Stability tests in CuAAC peptide stapling condition

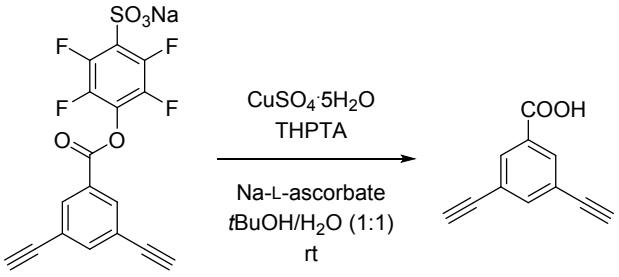
Compound **1**



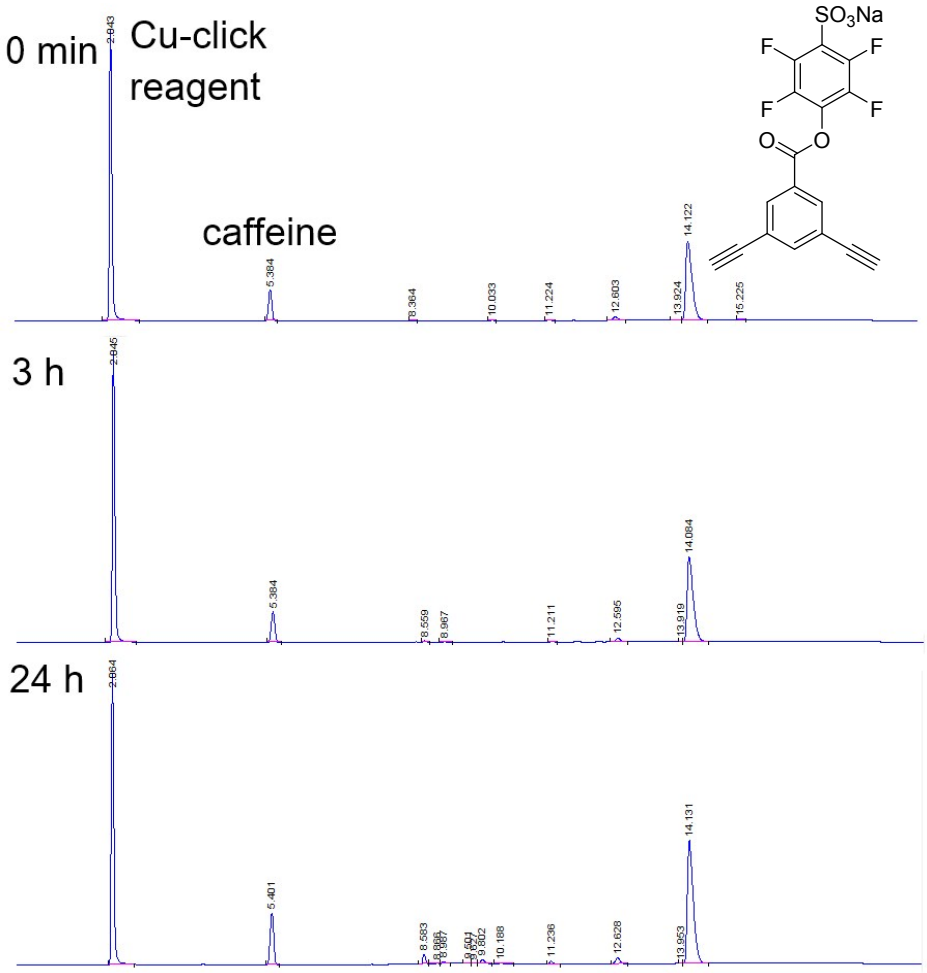
1



Compound **2**

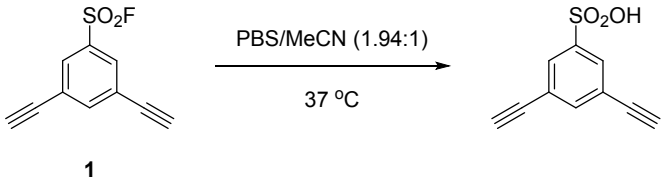


2 **S6**

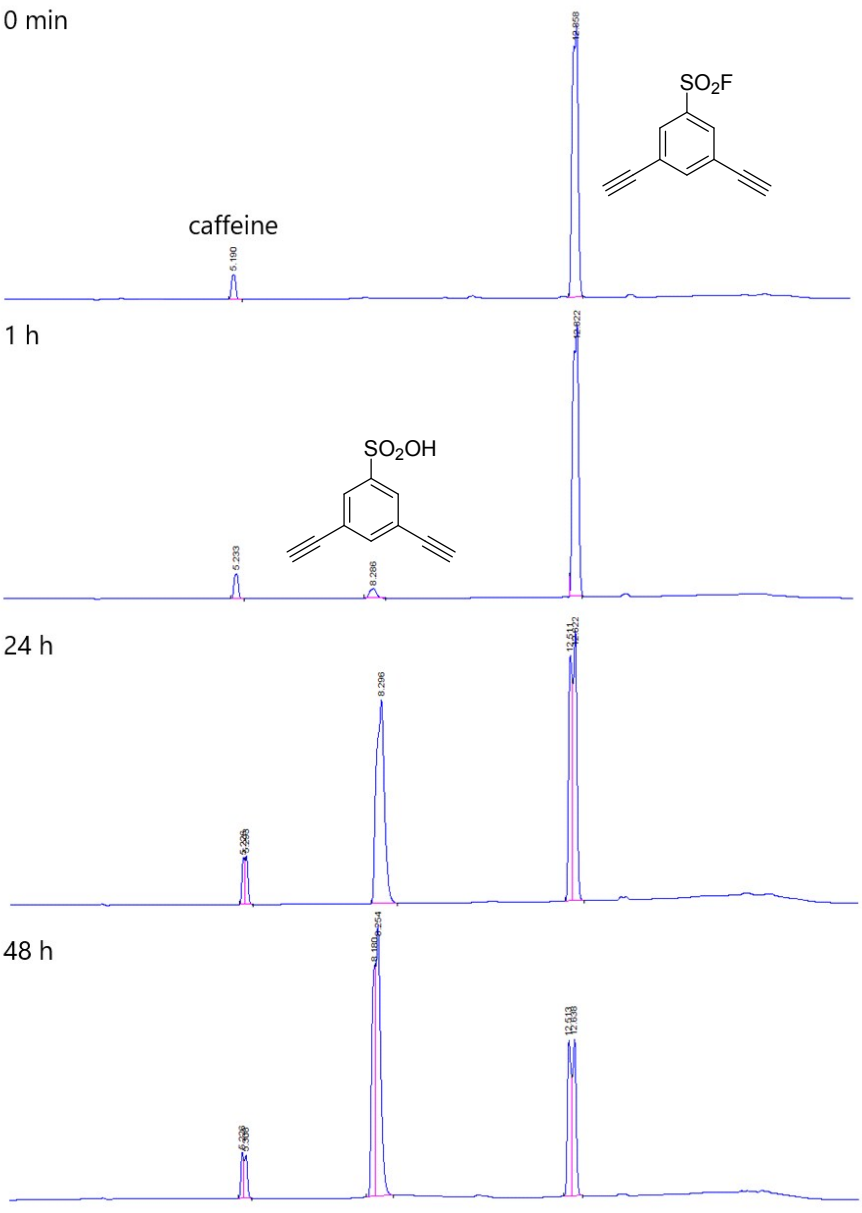


Stability tests in aqueous media

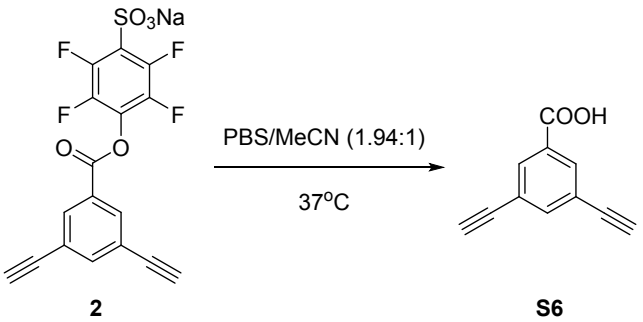
Compound **1**



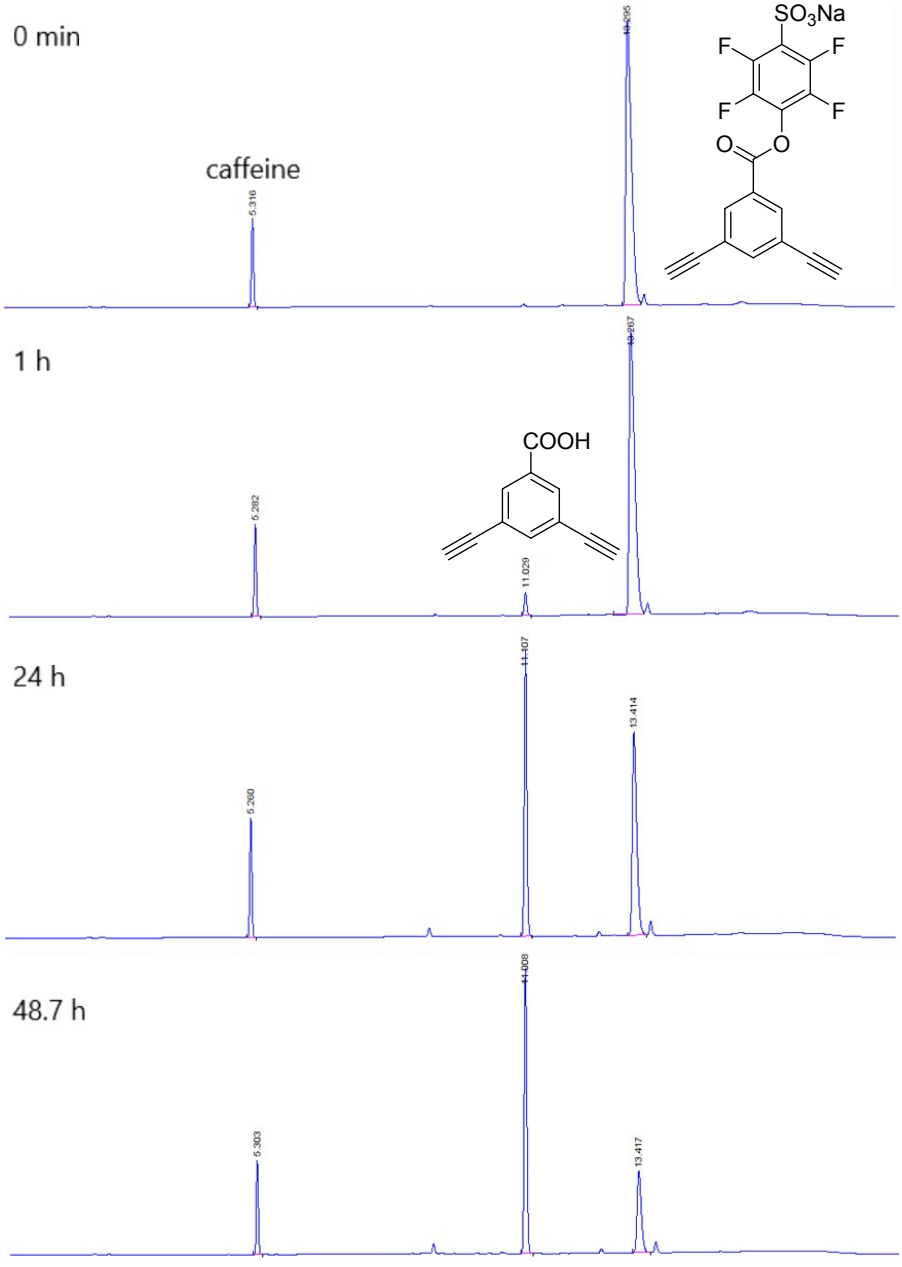
1



Compound **2**

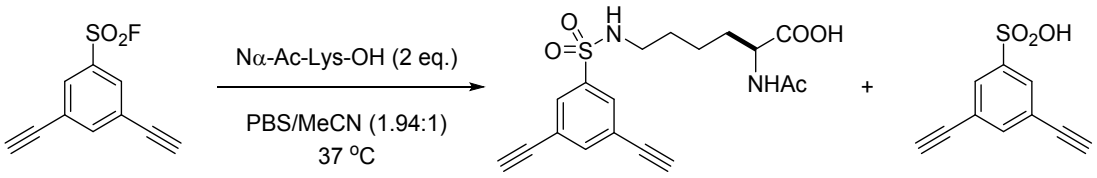


2 **S6**

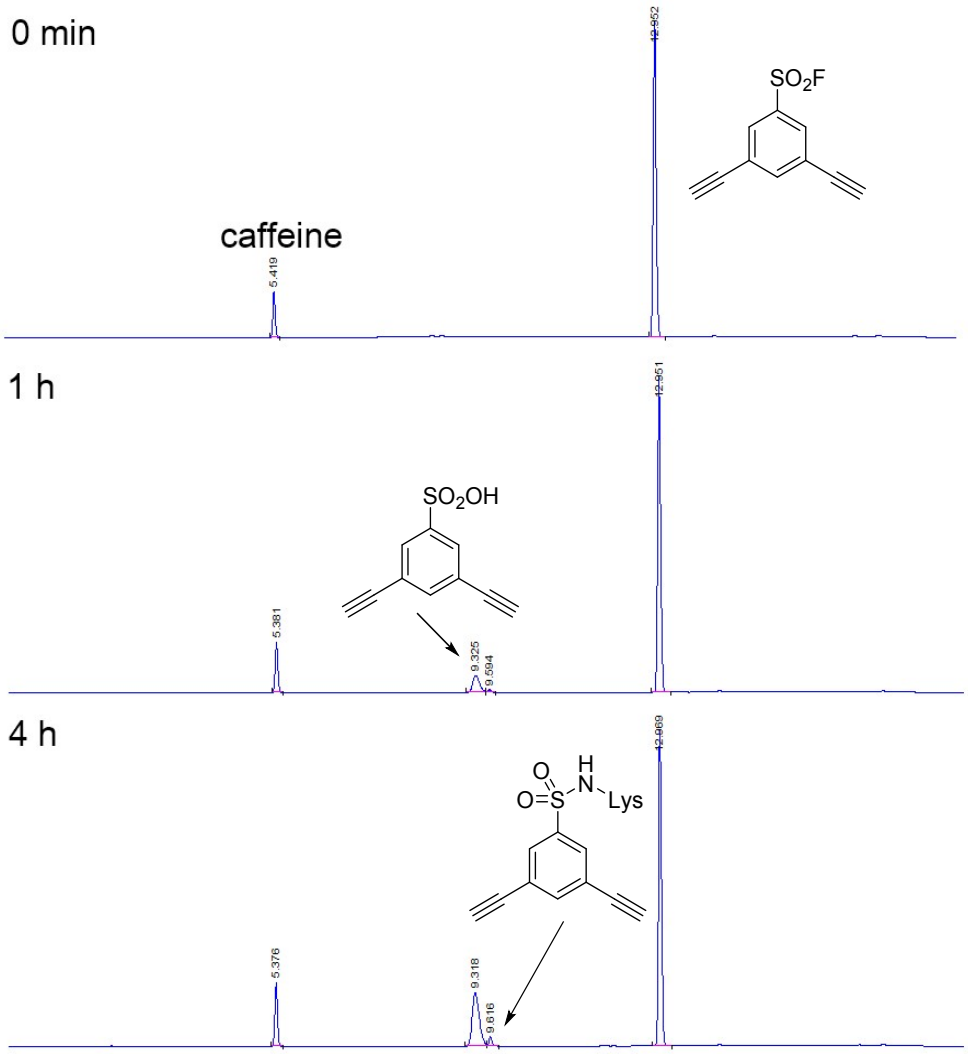


Reactivity tests with Lys

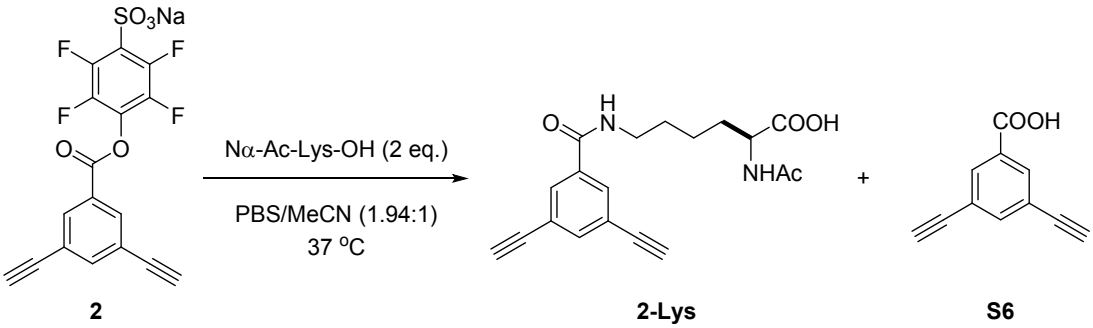
Compound **1**



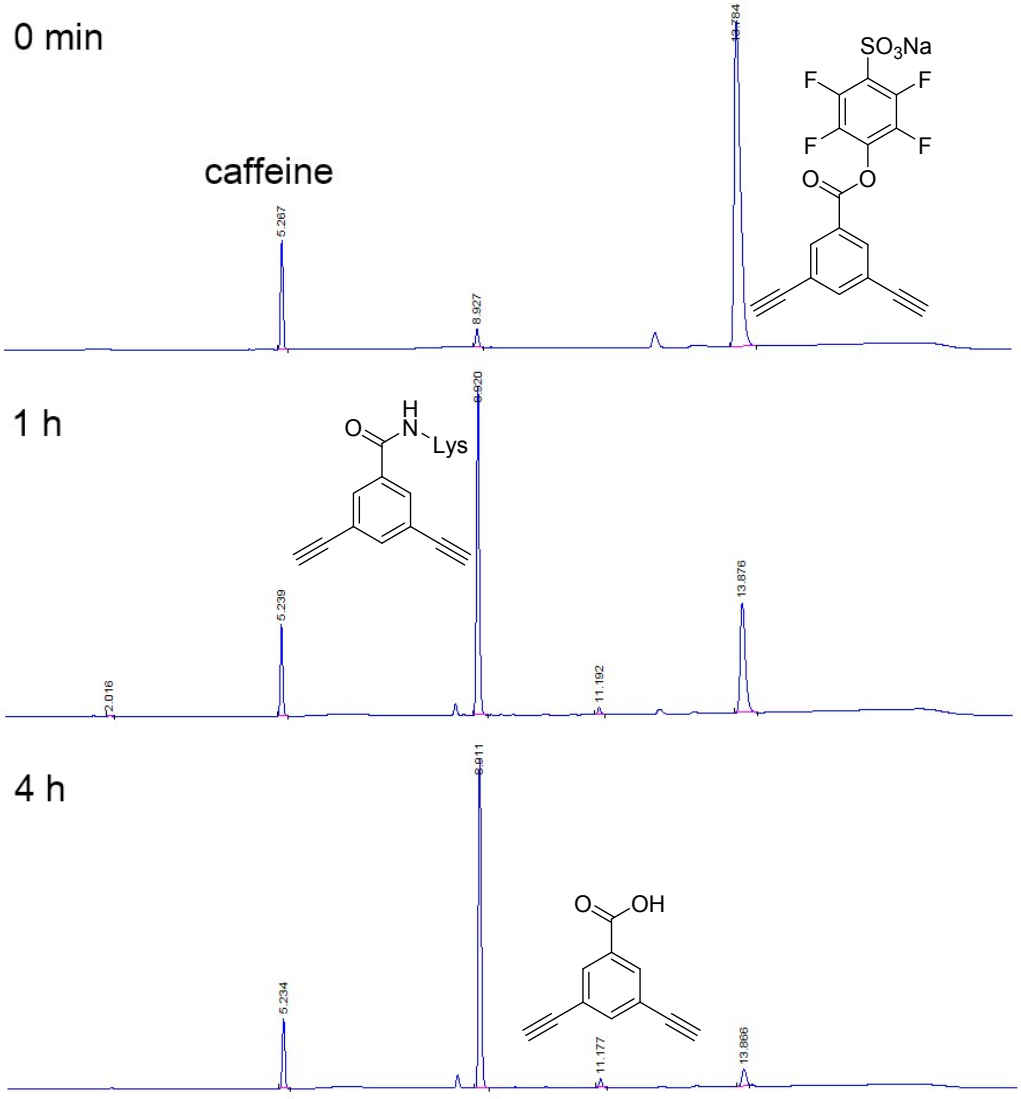
1



Compound **2**



2 **2-Lys** **S6**



6. Biological experiments

6.1. Expression and purification of MDM2 (6-125)

Coding region of MDM2, residues 6-125 were cloned into a pRSET vector with an N-terminal 6His-tag and TEV protease cleavage site via BamHI & KpnI restriction sites. Plasmid was transformed into C41 cells and expressed in 2xYT medium supplemented with 100 μg/mL ampicillin. Cultures were grown in baffled 2 L flasks to an OD600 = 0.6, prior to induction with 0.3 mM IPTG at 18 °C for around 16 hours. Cells were harvested by centrifugation at 7000 × g at 4 °C and were resuspended in 50 mM Tris-HCl, 300 mM NaCl, 15 mM imidazole, 1 mM DTT, SigmaFAST EDTA-free protease inhibitor cocktail tablet (Sigma) and DNaseI (Sigma), pH 8.0. Resuspended cells were lysed using a C5-emulsiflex (Avestin). Lysate was then clarified by centrifugation at 45,000 × g at 4°C. His-tagged proteins were bound to a 5 mL HisTrap Excel column using an AKTA PURE chromatography system and washed with 20 CV of 50 mM Tris-HCl, 300 mM NaCl, 15 mM imidazole, 1 mM DTT, pH 8.0. Proteins were eluted with 5 CV of 50 mM Tris-HCl, 300 mM NaCl, 300 mM imidazole, 1 mM DTT, pH 8.0, directly into a pre-equilibrated 26/10 desalting column of 20 mM Tris-HCl, 180 mM NaCl, 1 mM DTT, pH 8.0. Eluent fractions containing 6His-tagged MDM2 were pooled and 6His-TEV protease (S219V) was added to cleave overnight at 4°C. Cleaved proteins were then run over a 5 mL HisTrap Excel column, collecting the flow through. Proteins were diluted in 20 mM Bis-Tris, 1 mM DTT, pH 6.5 to approximately 20 mM NaCl. MonoS 10/100 GL column was then equilibrated in 20 mM Bis-Tris, 30 mM NaCl, 1 mM DTT, pH 6.5 before loading protein samples onto the column. Proteins were then eluted in a linear gradient with 20 mM Bis-Tris, 1 M NaCl, 1 mM DTT, pH 6.5 over 20 CV. Elution fractions were run on a 15% SDS PAGE gel to assess purity (>90%), before concentrating using a Vivaspin 20, 5K MWCO centrifugal concentrator (Sartorius Stedim). Proteins were snap frozen in liquid nitrogen and stored at -80°C for long term storage.

6.2. Covalent cross-linking determination

MDM2 protein stock solution (195 μM) was diluted in PBS buffer to 55.6 μM. PBS (5 μL) was added to the diluted MDM2 solution (5 μL) in a 500 μL-centrifuge tube. DMSO (0.556 μL) and the peptide solution (0.556 μL, 500 μM in DMSO) were then added successively and the mixture incubated at 37 °C for 1 h. The negative control used DMSO in place of peptide solution. Lysozyme solution (55.6 μM) was used instead of PBS for selectivity assay. The mass of the protein was detected by LCMS as described below.

LCMS was performed on a Xevo G2-S TOF mass spectrometer coupled to an Acquity UPLC system using an Acquity UPLC BEH300 C4 column (1.7 μm, 2.1 × 50 mm). Water with 0.1% formic acid (solvent A), and 95% acetonitrile and 5% water with 0.1% formic acid (solvent B), were used as the mobile phase at a flow rate of 0.2 mL min^{-1}. The gradient was programmed as follows: 95% A for 0.93 min, then a gradient to 100% B over 4.28 min, then 100% B for 1.04 minutes, then a gradient to 95% A over 1.04 min. The electrospray source was operated with a capillary voltage of 2.0 kV and a cone voltage of 40 V. Nitrogen was used as the desolvation gas at a total flow of 850 L h^{-1}. Total mass spectra were reconstructed from the ion series using the MaxEnt algorithm pre-installed on MassLynx software (v. 4.1 from Waters) according to the manufacturer's instructions.

6.3. MDM2 competitive fluorescence polarisation assay

Competitive fluorescence polarisation was carried out using MDM2 and a 5-TAMRA labelled FP tracer peptide in a similar fashion as previously described.[22,25] The dissociation constant was previously described by Lau *et al*.[22] Stock solutions of peptide inhibitors in DMSO (10 mM) were diluted in assay buffer (1 × PBS + 0.05% (v/v) Tween-20 + 3% (v/v) DMSO) to a top concentration of 80 µM (final assay concentration of 40 µM). 1.5-fold serial dilutions were performed by multichannel pipette, in the assay plate (OptiPlate™ -384 F, Perkin Elmer) to produce a 24-point titration with a volume of 20 µL. An equal volume of MDM2 and fluorescent tracer peptide were then added to each well, by multichannel pipette for a final concentration of 95 nM and 50 nM respectively. All titrations were conducted in triplicate. Fluorescence polarisation was measured using a BMG ClarioStar plate reader using an excitation filter at 540 nm and an emission filter at 590 nm, with a 20 nm bandwidth. Plates were read at time points indicated. Graphs were plotted using GraphPad Prism 7.0 and analysed using the equations below.[26] The equations described by Wang describe competitive binding, where neither protein nor ligand are depleted. However, in the case of a covalent ligand (**P1-2**), both the target protein and covalent ligand are depleted in a stoichiometric manner over the course of the incubation, resulting in a deviation from the model fit over time. Nonetheless, the system can still illustrate the time-dependent nature of a covalent ligand's interaction with the target protein and thus we quote an apparent dissociation constant, $K_{d,app}$.

Here, r = anisotropy measured, r_0 = anisotropy of free peptide, r_b = anisotropy of MDM2:5-TAMRA peptide complex, K_{d1} = dissociation constant of 5-TAMRA peptide to MDM2, K_{d2} = (apparent) dissociation constant of non-labelled ligand to MDM2, $[P]_t$ = MDM2 concentration, $[L]_t$ = non-labelled ligand concentration and $[L]_{st}$ = 5-TAMRA peptide concentration.

$$r = r_0 + (r_b + r_0) \times \frac{2\sqrt{(d^2 - 3e)}\cos(\theta/3) - 9}{3K_{d1} + 2\sqrt{(d^2 - 3e)}\cos(\theta/3) - d}$$

$$d = K_{d1} + K_{d2} + [L]_{st} + [L]_t - [P]_t$$

$$e = K_{d1}([L]_t - [P]_t) + K_{d2}([L]_{st} - [P]_t) + K_{d1}K_{d2}$$

$$\theta = \cos^{-1}\left(\frac{-2d^3 + 9de - 27f}{2\sqrt{(d^2 - 3e)^3}}\right)$$

$$f = -K_{d1}K_{d2}[P]_t$$

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