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Electronic Supplementary Information

One-Pot Thiol-Amine Bioconjugation to Maleimides; Simultaneous Stabilisation and Dual Functionalisation

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General Remarks

Materials:

All commercially available chemicals were acquired from either Sigma-Aldrich or Alfa-Aesar and used without further purification. Recombinant Human Serum Albumin (WT HSA) was provided by Albumedix (Recombumin® Elite, batch no. AX190201). Albumin mutant C34A + K93C (mHSA) was kindly provided by Albumedix, having been expressed according to literature.[1] All buffer solutions were prepared with doubly deionised water, degassed with argon and filtered before use. Phosphate-buffered saline (PBS) was 137 mM NaCl and 12 mM sodium phosphates at pH 7.4 unless otherwise stated. Phosphate buffer (PB) was 100 mM unless otherwise stated. DBM-azide **19** was synthesised according to literature.[2]

Methods:

All organic syntheses were carried out at atmospheric pressure, under argon. All bioconjugation reactions were carried out at atmospheric pressure in 1.5 ml Eppendorf tubes. Room temperature is defined as 20-22 °C.

Centrifugation was carried out with an Eppendorf 5415 R centrifuge, at 12.6 RPM.

Reactions were analysed by thin layer chromatography (TLC), with Merck KGaA silica gel 60 F_{254} TLC plates. TLC plates were visualised with a UVLS-26 EL series UV lamp, at 254 or 365 nm. Ninhydrin, Dragendorff's reagent, potassium permanganate and iodine were used as stains.

Flash chromatography was carried out on a Biotage Isolera One 3.0, using Graceresolv or Biotage normal phase columns. Where dry loading was used, the sample was adsorbed onto Geduran SI 60 silica gel (40-63 μM).

^{1}H and ^{13}C NMR analysis was carried out on a Bruker Avance Neo 700 equipped with a 5 mm helium-cooled broadband cryoprobe or a Bruker Avance III 600 spectrometer equipped with a DCH cryoprobe. ^{1}H experiments were carried out at 700 or 600 MHz and ^{13}C experiments at 151 MHz. Unless otherwise stated all NMR experiments were carried out at room temperature. All NMR analysis was carried out in the deuterated solvent system stated.

Infra-red spectra were recorded on a Bruker Platinum ALPHA FT-IR spectrometer operating in attenuated total reflection (ATR) mode. Absorptions are characterized as s (sharp), br (broad), m (medium), w (weak).

Melting points were recorded for all solids at room temperature with a Gallenkamp digital Melting-point apparatus 5A 6797. All melting points are uncorrected.

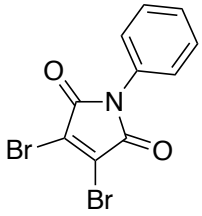
High and low resolution (HRMS/LRMS) mass spectra acquired with electrospray ionisation (ES/ES+/ES-) modes were obtained at UCL with Agilent 6510 QTOF. High and low resolution (HRMS/LRMS) mass spectra acquired with nanospray (NSI) ionisation modes were obtained at The National Mass Spectrometry Facility Swansea on a Thermo Scientific LTQ Orbitrap XL.

Protein LCMS was performed with an LC system connected to an Agilent 6510 Q-TOF spectrometer. A PLRP-s, 1000 Å, 8 μM column was used. Peptide LCMS was performed on a Waters Acquity UPLC connected to Waters Acquity Single Quad Detector (SQD). A Hypersil Gold C4, 1.9 μm, 2.1 μm × 50 μm column was used.

UV analysis was carried out with either a Cary UV spectrophotometer and recorded with Cary WinUV software, or a Shimadzu UV-2600 spectrophotometer with UVprobe software. A quartz cuvette with a 1 cm path length was used to hold samples. Unless stated, all UV scan experiments were carried out at room temperature. All samples were baseline corrected. UV Kinetics experiments were carried out with Cary Kinetics software and unless stated all kinetics experiments were carried out at 25 ^{o}C: absorbance data at 375 and 415 nm were obtained, with data timepoints at 415 nm obtained at 2.6 seconds after the respective timepoint at 375 nm. NB: This small time difference was not taken into account in the kinetics graph for GFP. Data points acquired when amine was being added to the cuvette (outside the machine) have been omitted. For clarity, the 375 nm data was plotted until a plateau was observed, upon which point the 415 nm data was plotted to show the progress of amine addition. The 415 nm plot was then stopped when a plateau was observed. Where experiments have been left in the UV machine for longer (due to running overnight experiments, for example) before MS analysis, this has been indicated in the text.

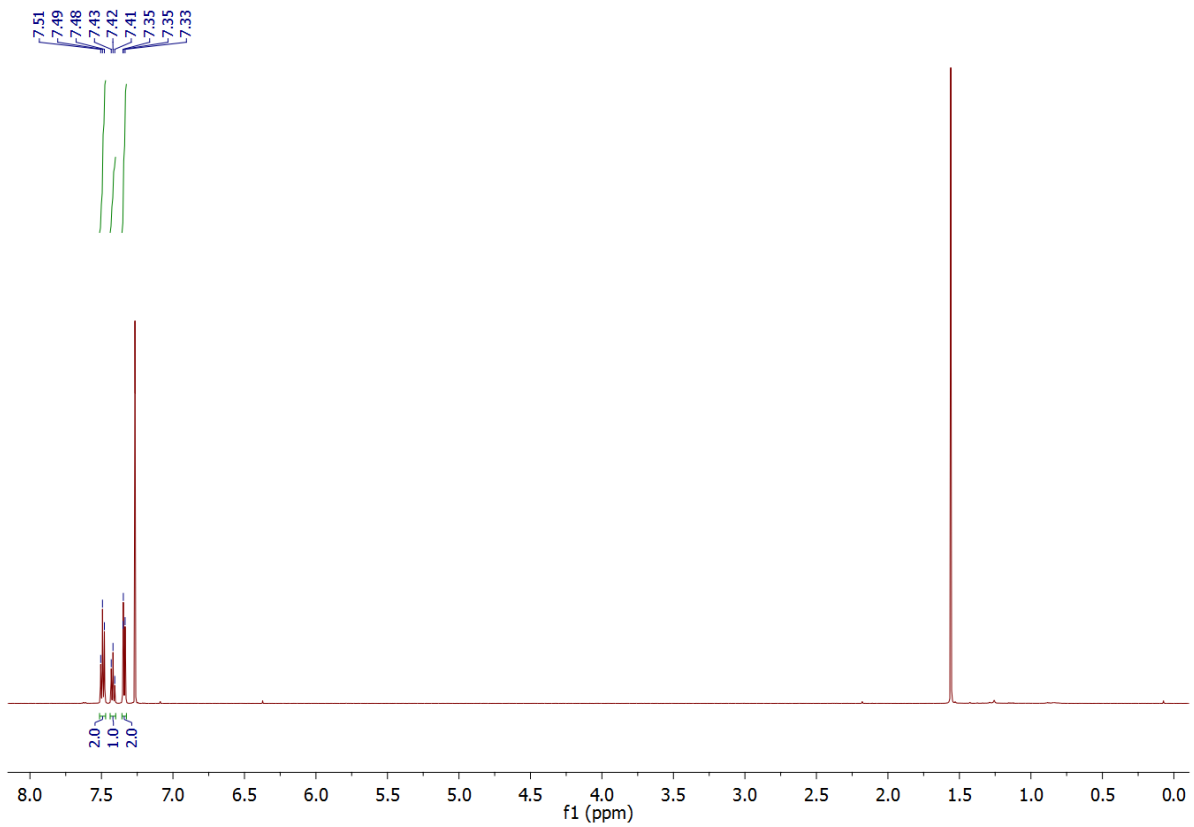
Synthesis of Compounds

Synthesis of 3,4-dibromo-1-phenyl-1*H*-pyrrole-2,5-dione[3] (**27**)



To *N*-phenylmaleimide (0.430 g, 2.50 mmol) and sodium acetate (0.615 g, 7.50 mmol) dissolved in acetic acid (25 mL), bromine (0.28 mL, 5.50 mmol) was added dropwise and the resultant solution heated under reflux for 30 minutes (120 °C). The solvent was removed *in vacuo* and the remaining solid dissolved in EtOAc (90 mL) and washed with 20% aqueous $Na_2S_2O_3$ (40 mL × 3), saturated $NaHCO_3$ (40 mL × 2) and brine (50 mL). The organic phase was dried over $MgSO_4$ and the solvent removed *in vacuo* to yield the crude product. Purification by column chromatography (1-20% EtOAc/petroleum ether) yielded the product as a faint yellow powder (0.722 g, 2.18 mmol, 87%).

m.p. 161.0-164.0 °C (lit. 162-163 °C)[3]; ^{1}H NMR (600 MHz, $CDCl_3$); δ 7.49 (t, *J* = 7.8 Hz, 2H, CH), 7.42 (t, *J* = 7.5 Hz, 1H, CH), 7.34 (d, *J* = 7.2 Hz, 2H, CH); ^{13}C NMR (151 MHz, $CDCl_3$); δ 163.0 (CO), 131.1 (C), 130.0 (C), 129.5 (CH), 128.8 (CH), 126.2 (CH); IR (solid); 1740 (s), 2950 (w) cm^{-1}.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7.0
6.5
6.0
5.5
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2.0
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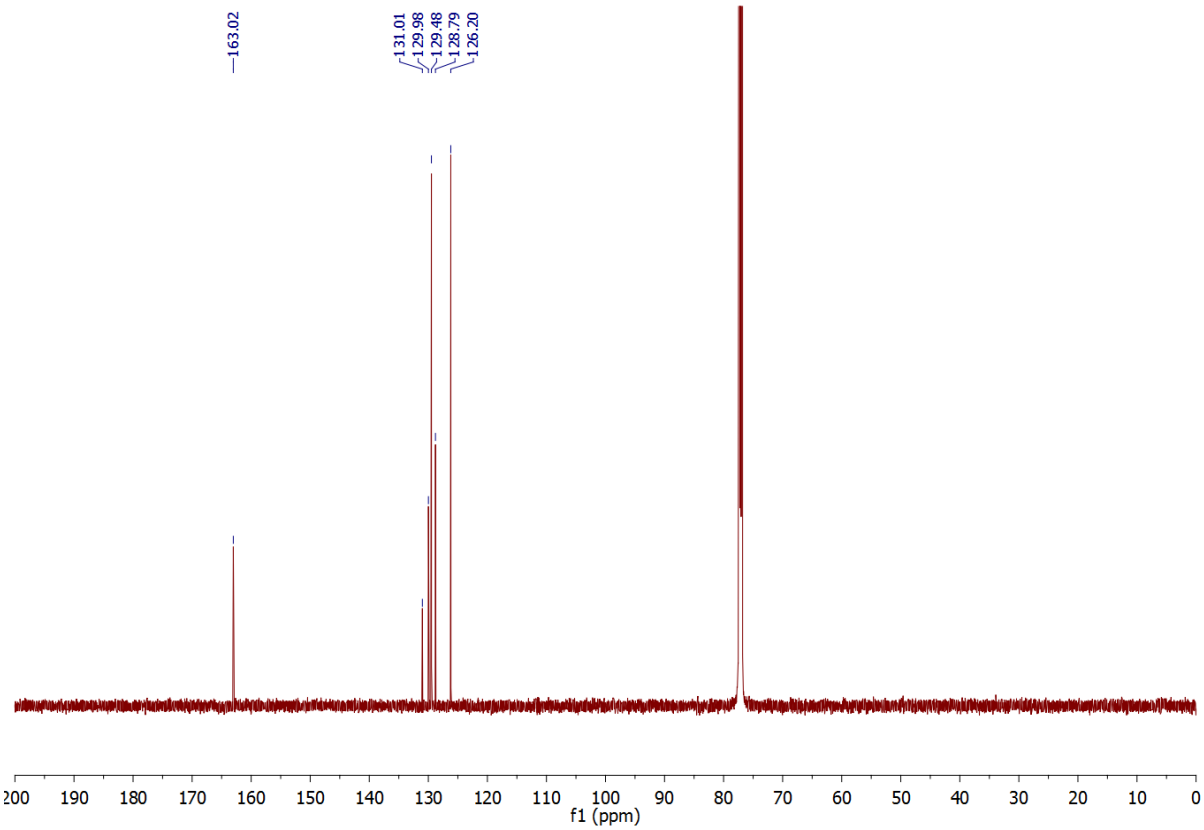
1.0
0.5
0.0
f1 (ppm)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110
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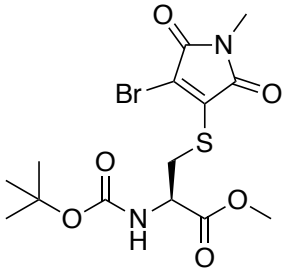
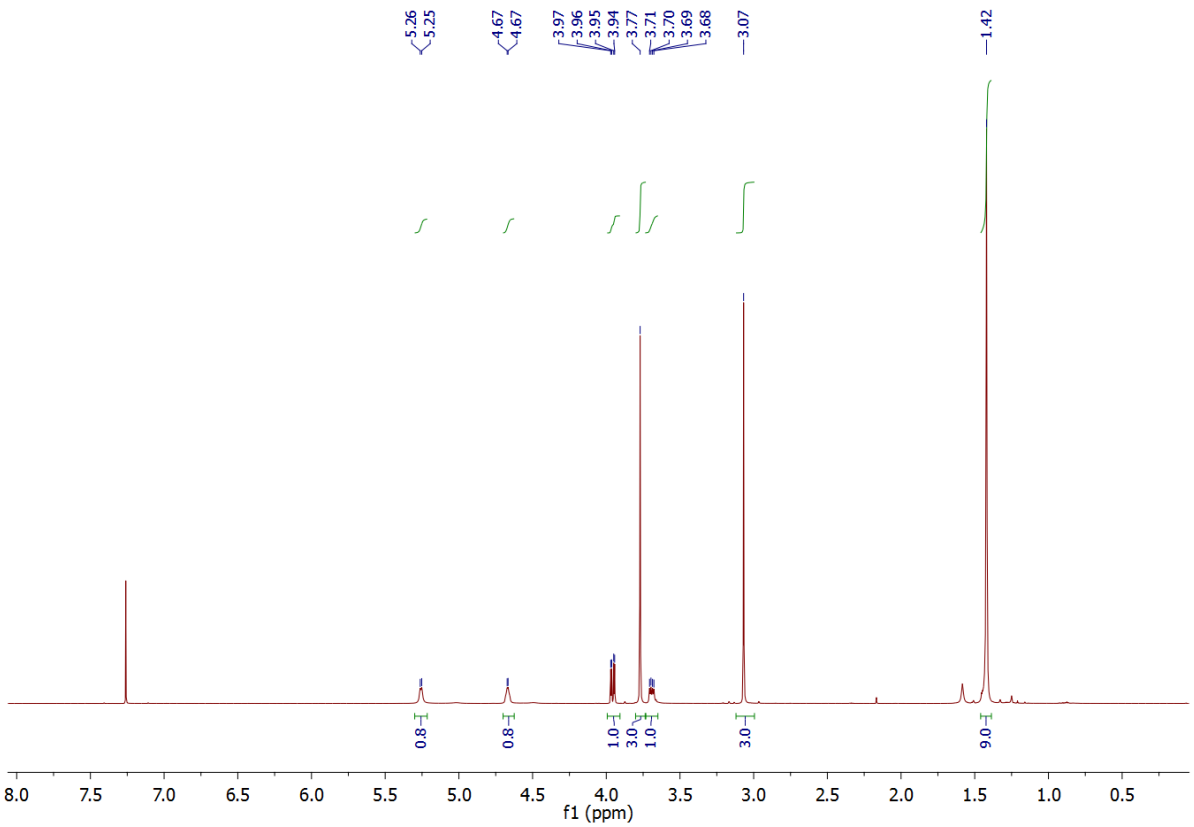
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

Synthesis of methyl S-(4-bromo-1-methyl-2,5-dioxo-2,5-dihydro-1H-pyrrol-3-yl)-*N*-(tert-butoxycarbonyl)-L-cysteinate (**6**)



To 3,4-dibromo-1-methyl-1H-pyrrole-2,5-dione (0.343 g, 1.27 mmol) and sodium acetate (0.055 g, 0.669 mmol) dissolved in methanol (100 mL), methyl (*tert*-butoxycarbonyl)-*L*-cysteinate (0.150 g, 0.637 mmol) dissolved in methanol (20 mL) was added dropwise over 90 minutes and the resultant solution stirred at room temperature for 30 minutes. The solvent was removed *in vacuo* to yield the crude product. Purification by column chromatography (0-100% EtOAc/petroleum ether) yielded the product as a yellow oil that crystallised upon standing (0.222 g, 0.524 mmol, 82%).

m.p. 126.0-129.0 °C; ^{1}H NMR (600 MHz, $CDCl_3$); δ 5.26 (d, *J* = 7.2 Hz, 1H, CONH), 4.67 (d, *J* = 4.4 Hz, 1H, HNC*H*CO), 3.95 (dd, *J* = 14.1, 4.5 Hz, 1H, SC*H*H), 3.77 (s, 3H, OCH_3), 3.69 (dd, *J* = 14.0, 6.7 Hz, 1H, SCH*H*), 3.07 (s, 3H, NCH_3), 1.42 (s, 9H, $C(CH_3)_3$); ^{13}C NMR (151 MHz, $CDCl_3$); δ 170.6 (CO), 166.2 (CO), 164.1 (CO), 155.0 (CO), 142.2 (CS), 120.3 (BrC), 80.7 (C), 53.9 (CH), 53.0 (CH_3), 33.4 (CH_2), 28.4 (CH_3), 25.1 (CH_3); IR (solid); 1740 (s), 2950 (w), 3350 (m) cm^{-1}; LRMS (ES$^+$) 323.0 (60, [M^{79}Br+H-Boc]$^+$), 325.0 (60, [M^{81}Br+H-Boc]$^+$) 423.0 (65, [M^{79}Br+H]$^+$), 425.0 (60, [M^{81}Br+H]$^+$); HRMS (EI) calcd for $C_{14}H_{20}N_2O_6SBr$ [M^{79}Br+H]$^+$ 423.0226, observed 423.0222.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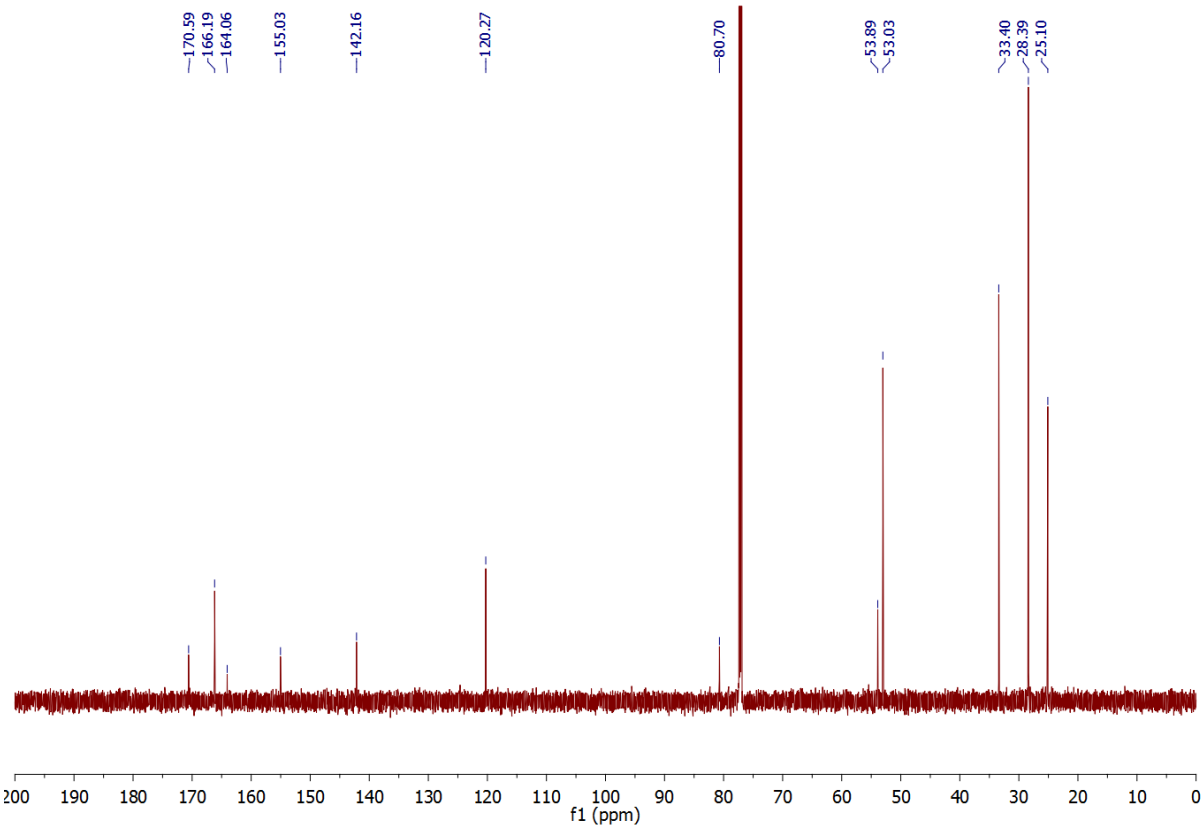
f1 (ppm)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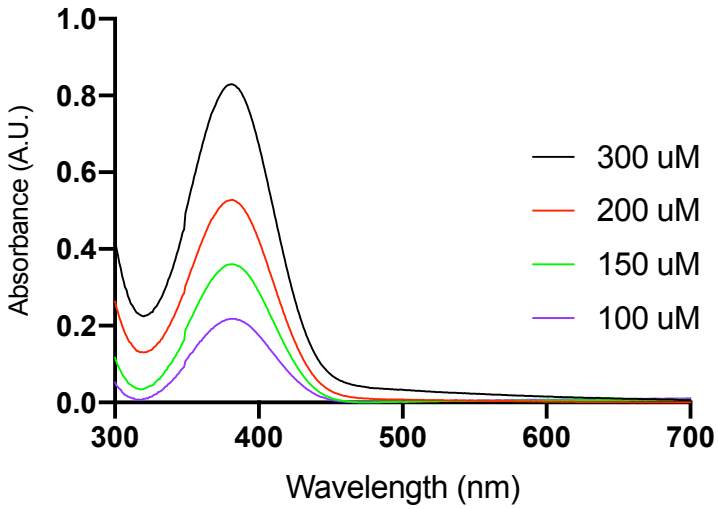
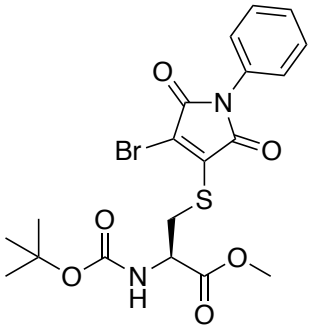
f1 (ppm)



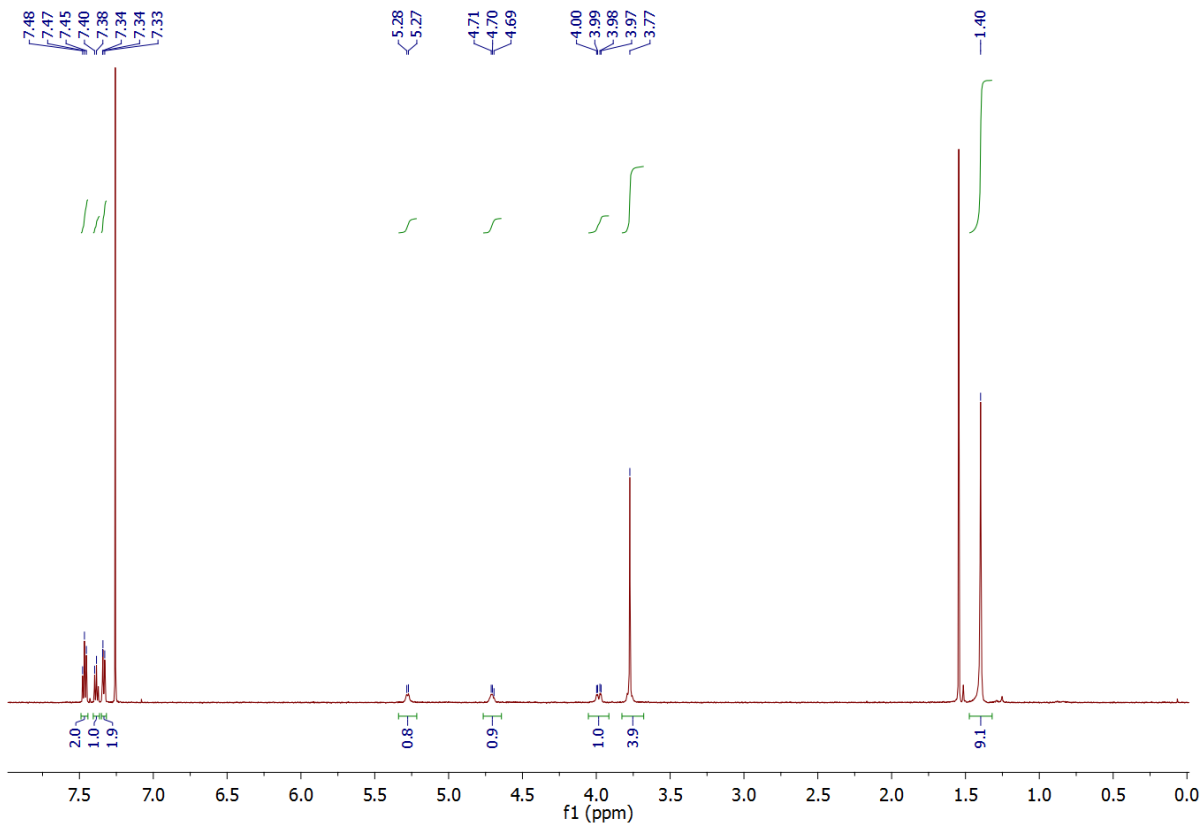
Absorbance spectra recorded in 10% THF in PBS (pH 7.4).

Synthesis of S-(4-bromo-2,5-dioxo-1-phenyl-2,5-dihydro-1*H*-pyrrol-3-yl)-*N*-(*tert*-butoxycarbonyl)-*L*-cysteinate (**5**)



To 3,4-dibromo-1-phenyl-1*H*-pyrrole-2,5-dione (0.294 g, 0.888 mmol) and sodium acetate (0.048 g, 0.583 mmol) dissolved in methanol (75 mL), methyl (*tert*-butoxycarbonyl)-*L*-cysteinate (0.131 g, 0.555 mmol) dissolved in methanol (20 mL) was added dropwise over 90 minutes. The resultant solution was stirred for a further 30 minutes. A colour change from pale yellow to yellow was observed. The solvent was removed *in vacuo* and the crude product purified by column chromatography (0-100% EtOAc/petroleum ether) to yield the product as a yellow powder (0.220 g, 0.453 mmol, 82%);

m.p. 139.0-141.0 °C; ^{1}H NMR (600 MHz, $CDCl_3$); δ 7.47 (t, *J* = 9.0 Hz, 2H, ArH), 7.39 (t, *J* = 9.0 Hz, 1H, ArH), 7.34 (d, *J* = 6.0 Hz, 2H, ArH), 5.28 (d, *J* = 7.8 Hz, 1H, NH), 4.70 (dd, *J* = 11.7, 7.2 Hz, 1H, HNC*H*CO), 3.98 (dd, *J* = 14.1 Hz, 4.2 Hz, 1H, SC*H*HCH), 3.77 (br s, 4H, OCH_3/SCH*H*CH), 1.40 (s, 9H, $C(CH_3)_3$); ^{13}C NMR (151 MHz, $CDCl_3$); δ 170.6 (CO), 165.1 (CO), 162.8 (CO), 155.1 (CO), 142.4 (CS), 131.2 (ArC), 129.3 (ArC), 128.5 (ArC), 126.3 (ArC), 120.6 (BrC), 80.8 (C), 53.9 (CH), 53.1 (CH_3), 33.4 (CH_2), 28.3 (CH_3); IR (solid); 1735 (s), 2900 (w), 3400 (m) cm^{-1}; LRMS (ES$^+$) 485.0 (100, [M^{79}Br+H]$^+$), 487.0 (100, [M^{81}Br+H]$^+$); HRMS (EI) calcd for $C_{19}H_{22}N_2O_6SBr$ [M^{79}Br+H]$^+$ 485.0376, observed 485.0363.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4.00
3.99
3.98
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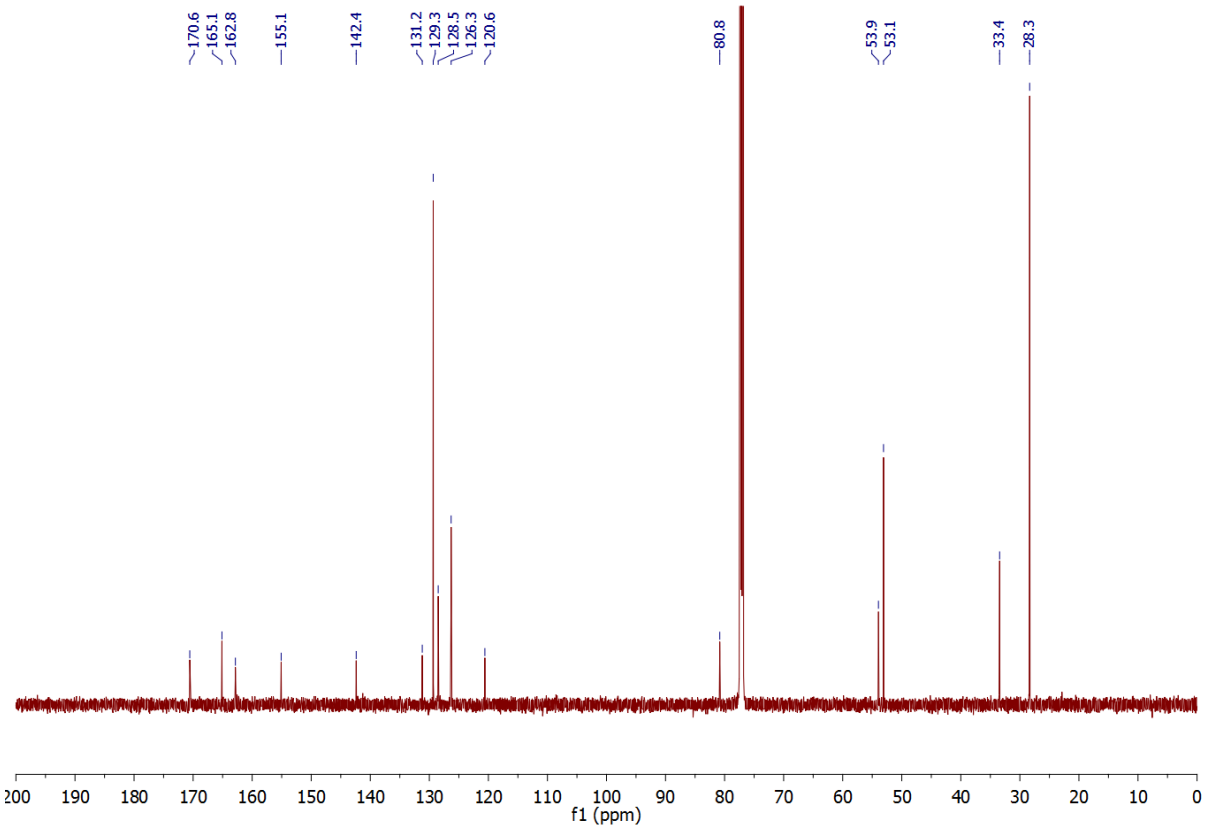
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f1 (ppm)

Amine Reactions in Organic Solvent

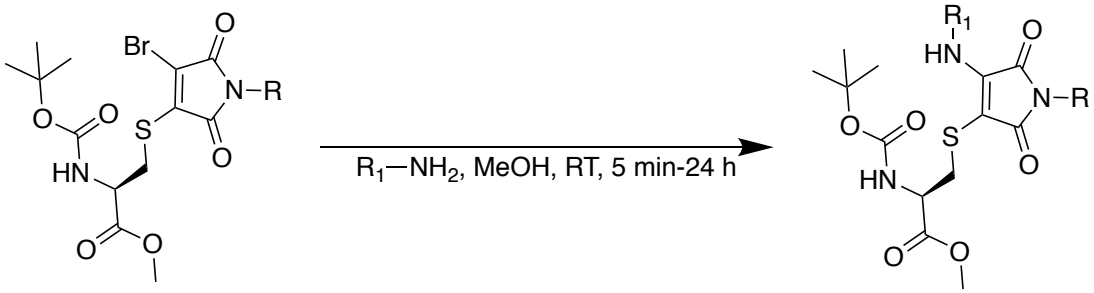
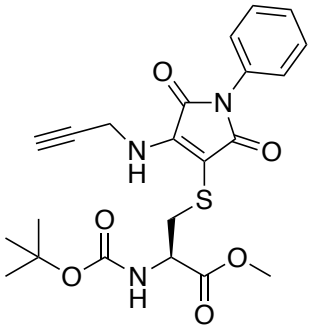


Table S1: Amine reactions in methanol.

R	R^1-(Amine)	Equiv. of amine	Time†	Yield
Ph	Propargylamine	2	24 h*	53%
Ph	Piperidine	2	20 min	98%
Ph	Piperidine	5	10 min	99%
Ph	Pyrrolidine	2	15 min	96%
Ph	Pyrrolidine	5	5 min	99%
Me	Pyrrolidine	2	10 min	92%
Me	Aniline	2	24 h	82%
Me	*p*-anisidine	2	24 h	90%
Me	*N*-Me *p*-anisidine	2	24 h	88%

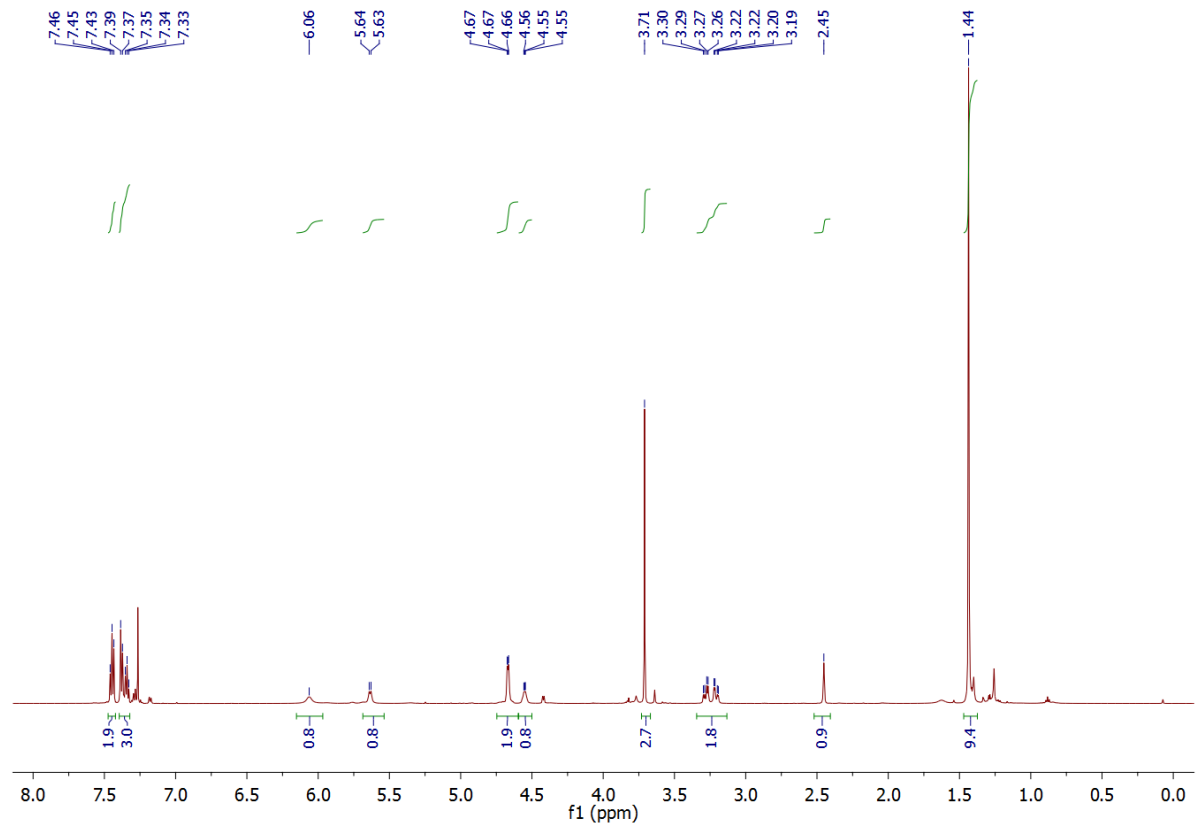
All R=Me reactions are 21 mg **6** in 24 mL (2.1 mM) final volume, R=Ph reactions are all different conc. (2.1-7.8 mM of **5**). † Reaction times measured by TLC. * TLC analysis not carried out before 24 h.

Synthesis of methyl *N*-(*tert*-butoxycarbonyl)-S-(2,5-dioxo-1-phenyl-4-(prop-2-yn-1-ylamino)-2,5-dihydro-1*H*-pyrrol-3-yl)-*L*-cysteinate (**28**)



To S-(4-bromo-2,5-dioxo-1-phenyl-2,5-dihydro-1*H*-pyrrol-3-yl)-*N*-(*tert*-butoxycarbonyl)-*L*-cysteinate (0.036 g, 0.074 mmol) dissolved in methanol (10 mL), 3-amino-1-propyne (0.010 mL, 0.15 mmol) was added dropwise and the resultant solution stirred at room temperature for 24 hours. The solvent was removed *in vacuo* to yield the crude product. Purification by column chromatography (0-40% EtOAc/petroleum ether) yielded the product as a yellow oil (0.018 g, 0.039 mmol, 53%).

^{1}H NMR (600 MHz, $CDCl_3$); δ 7.45 (t, *J* = 7.8 Hz, 2H, ArH), 7.38 (d, *J* = 7.7 Hz, 2H, ArH), 7.34 (t, *J* = 7.3 Hz, 1H, ArH), 6.06 (br s, 1H, NH), 5.63 (d, *J* = 7.6 Hz, 1H, NH), 4.67-4.66 (m, 2H, C*H*$_2$CCH), 4.56-4.55 (m, 1H, NHC*H*CO), 3.71 (s, 3H, OCH_3) 3.28 (dd, *J* = 14.0, 5.7 Hz, 1H, SC*H*HCH), 3.21 (dd, *J* = 14.0, 4.2 Hz, 1H, SCH*H*CH), 2.45 (s, 1H, CH_2CCH), 1.44 (s, 9H, $C(CH_3)_3$); ^{13}C NMR (151 MHz, $CDCl_3$); δ 171.3 (CO), 169.7 (CO), 164.7 (CO), 155.4 (CO), 131.7 (C), 129.6 (C), 129.1 (C), 128.8 (C), 127.6 (C), 125.8 (C), 80.4 (C), 79.4 (C), 74.0 (C), 53.6 (C), 52.7 (CH_3), 38.0 (CH_2), 33.7 (CH_2), 28.4 (CH_3); IR (thin film); 1600 (s), 2960 (w), 3300 (m) cm^{-1}; LRMS (ES$^+$) 482.1 (100, [M+Na]$^+$), 460.2 (95, [M+H]$^+$); HRMS (ES$^+$) calcd for $C_{22}H_{26}N_3O_6S$ [M+H]$^+$ 460.1542, observed 460.1540.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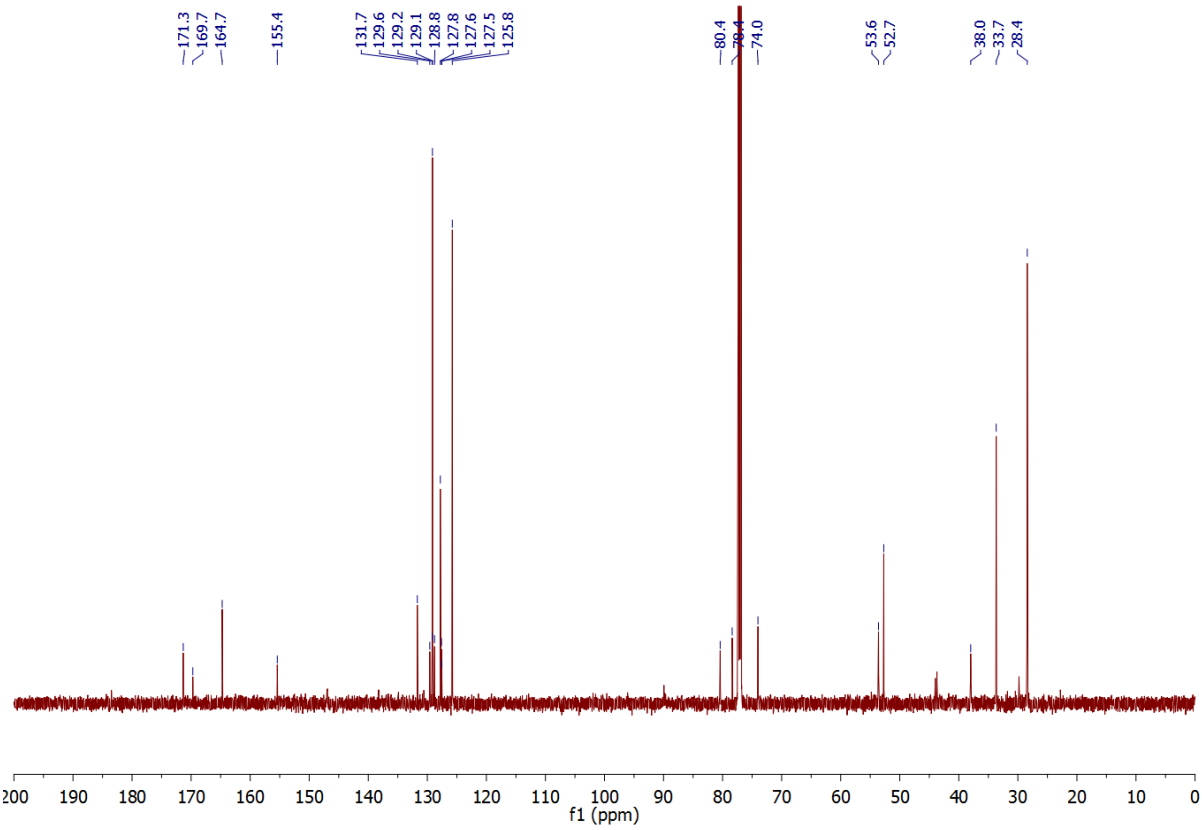
f1 (ppm)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74.0
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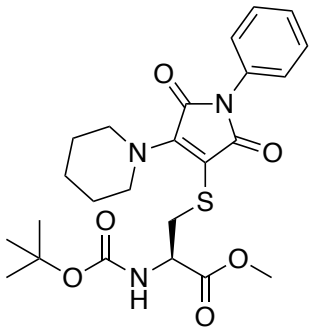
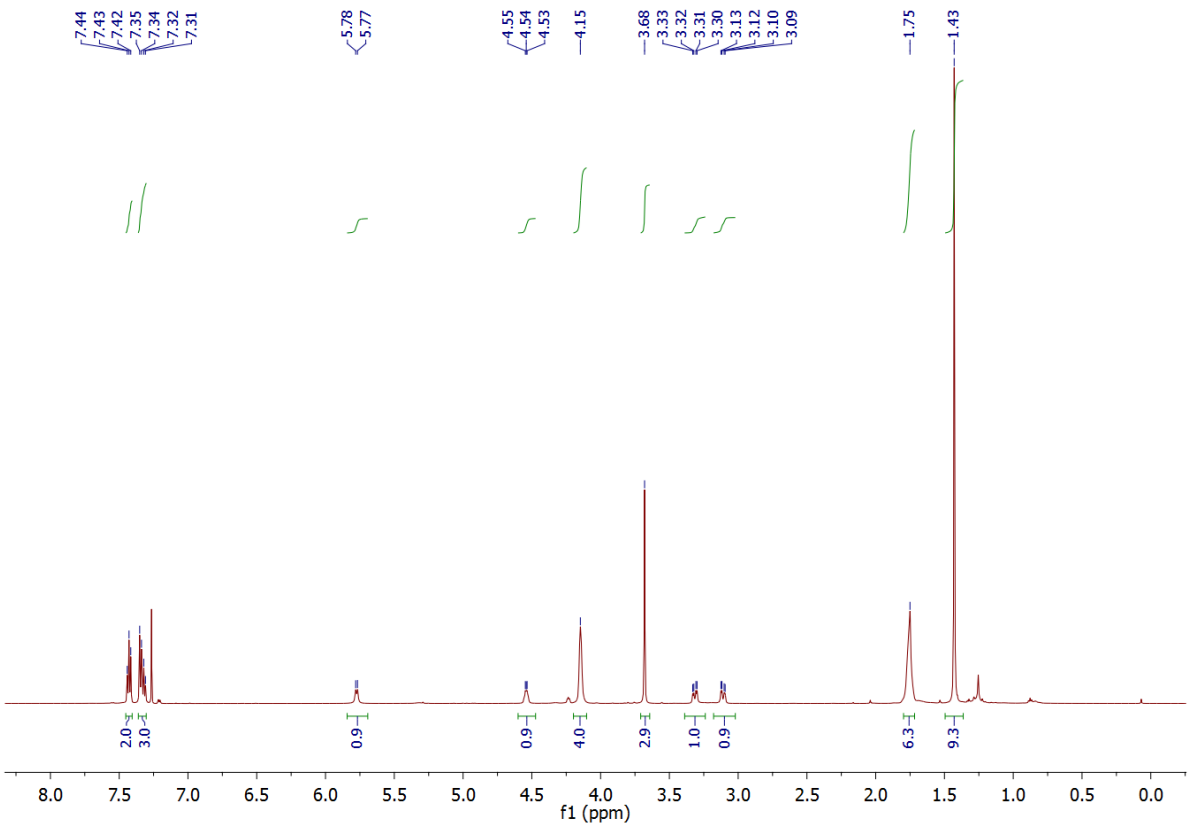
38.0
33.7
28.4
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

Synthesis of methyl *N*-(*tert*-butoxycarbonyl)-S-(2,5-dioxo-1-phenyl-4-(piperidin-1-yl)-2,5-dihydro-1*H*-pyrrol-3-yl)-*L*-cysteinate (**7**)



To S-(4-bromo-2,5-dioxo-1-phenyl-2,5-dihydro-1*H*-pyrrol-3-yl)-*N*-(*tert*-butoxycarbonyl)-*L*-cysteinate (0.024 g, 0.050 mmol) dissolved in methanol (15 mL), piperidine (0.001 mL, 0.100 mmol) dissolved in methanol (4 mL) was added dropwise and the resultant solution stirred at room temperature for 20 minutes. The solvent was removed *in vacuo* to yield the crude product. Purification by column chromatography (0-40% EtOAc/petroleum ether) yielded the product as an orange oil (0.024 g, 0.049 mmol, 98%). *N.B. Same protocol followed for 5 equiv. experiment shown in table S1.*

^{1}H NMR (600 MHz, $CDCl_3$); δ 7.43 (t, *J* = 7.8 Hz, 2H, ArH), 7.35-7.31 (m, 3H, ArH), 5.77 (d, *J* = 7.9 Hz, 1H, NH), 4.54 (dt, *J* = 8.4, 4.8 Hz, 1H, NHC*H*CO), 4.15 (br s, 4H, $N(CH_2)_2$), 3.68 (s, 3H, OCH_3), 3.31 (dd, *J* = 13.9, 4.9 Hz, 1H, SC*H*HCH), 3.11 (dd, *J* = 13.9, 4.4 Hz, 1H, SCH*H*CH), 1.76-1.74 (m, 6H, $N(CH_2)_2(CH_2)_3$), 1.43 (s, 9H, $C(CH_3)_3$); ^{13}C NMR (151 MHz, $CDCl_3$); δ 171.5 (CO), 169.4 (CO), 165.1 (CO), 155.4 (CO), 149.0 (CN), 131.9 (ArC), 129.0 (ArC), 127.6 (ArC), 126.3 (ArC), 90.4 (CS), 80.2 (C), 53.3 (CH_3), 52.6 (CH), 50.3 (CH_2), 37.7 (CH_2), 28.4 (CH_3), 26.8 (CH_2), 24.2 (CH); IR (thin film); 1650 (s), 2980 (m), 3250 (w) cm^{-1}; LRMS (ES$^+$) 490.1 (70, $[M+H]^+$); HRMS (ES$^+$) calcd for $C_{24}H_{32}N_3O_6S$ $[M+H]^+$ 490.1934, observed 490.1479.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5.77
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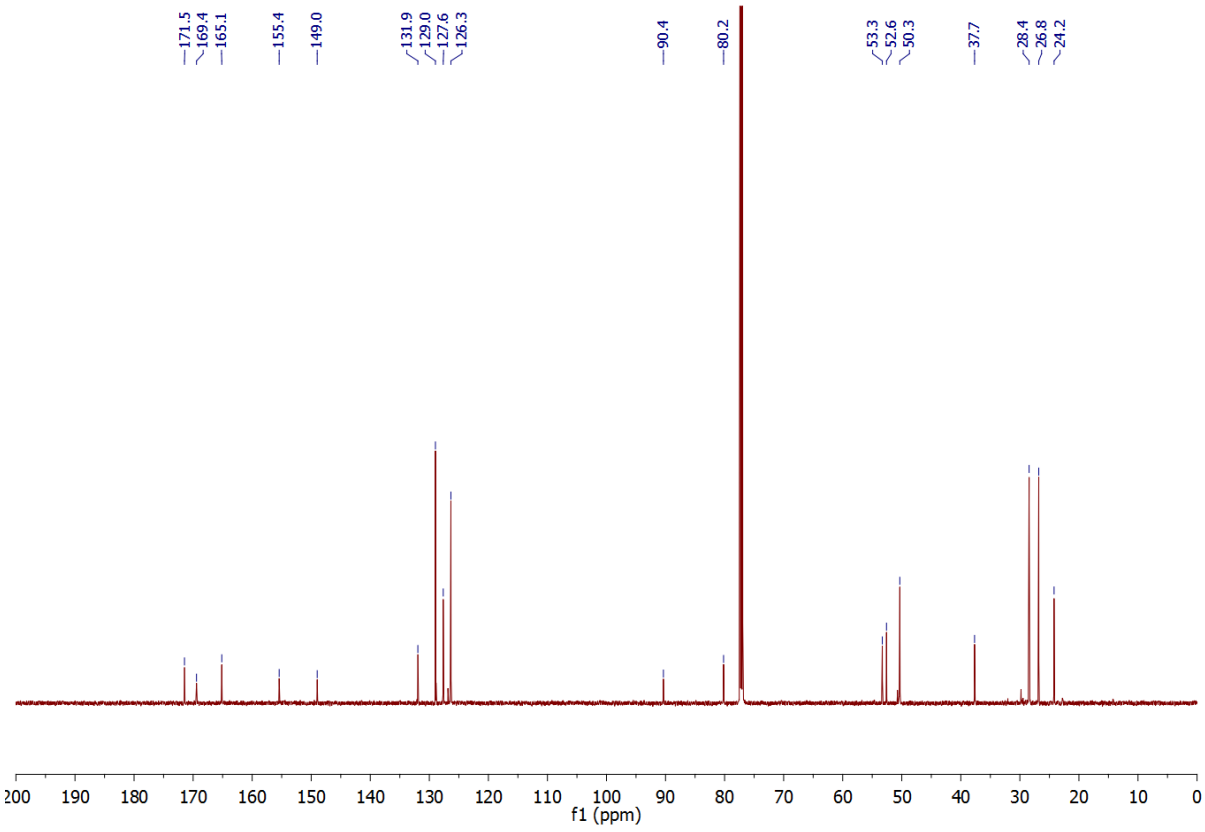
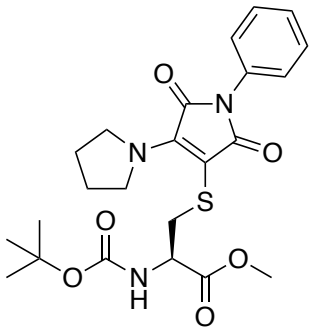
3.68
3.33
3.32
3.31
3.30
3.13
3.12
3.10
3.09
1.75
1.43
2.0
3.0
0.9
0.9
4.0
2.9
1.0
0.9
6.3
9.3
8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)

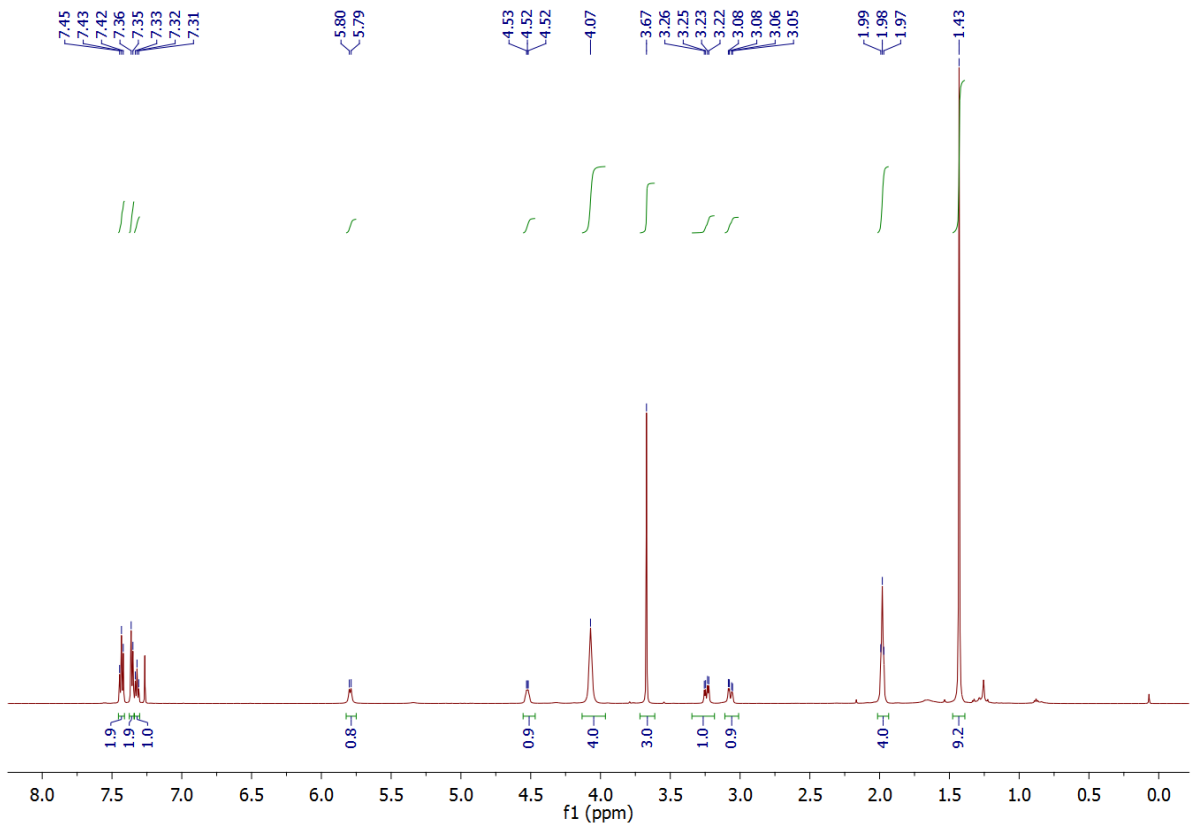

171.5
169.4
165.1
155.4
149.0
131.9
129.0
127.6
126.3
90.4
80.2
53.3
52.6
50.3
37.7
28.4
26.8
24.2
200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

Synthesis of methyl *N*-(*tert*-butoxycarbonyl)-S-(2,5-dioxo-1-phenyl-4-(pyrrolidin-1-yl)-2,5-dihydro-1*H*-pyrrol-3-yl)-*L*-cysteinate (**8**)



To S-(4-bromo-2,5-dioxo-1-phenyl-2,5-dihydro-1*H*-pyrrol-3-yl)-*N*-(*tert*-butoxycarbonyl)-*L*-cysteinate (0.024 g, 0.050 mmol) dissolved in methanol (20 mL), pyrrolidine (0.007 g, 0.100 mmol) dissolved in methanol (4 mL) was added dropwise and the resultant solution stirred for 15 minutes at room temperature. The solvent was removed *in vacuo* to yield the crude product. Purification by column chromatography (0-40% EtOAc/petroleum ether) yielded the product as an orange oil that crystallised upon standing (0.023 g, 0.048 mmol, 96%). *N.B. Same protocol followed for 5 equiv. experiment shown in table S1.*

m.p. 127.0-131.0 °C; ^{1}H NMR (600 MHz, $CDCl_3$); δ 7.43 (t, *J* = 7.8 Hz, 2H, ArH), 7.36 (d, *J* = 7.6 Hz, 2H, ArH), 7.32 (t, *J* = 7.4 Hz, 1H, ArH), 5.79 (d, *J* = 7.9 Hz, 1H, NH), 4.52 (dt, *J* = 8.4, 4.6 Hz, 1H, NHC*H*CO), 4.07 (br s, 4H, $N(CH_2)_2$), 3.67 (s, 3H, OCH_3), 3.24 (dd, *J* = 13.9, 5.5 Hz, 1H, SC*H*HCH), 3.07 (dd, *J* = 13.9, 4.2 Hz, 1H, SCH*H*CH), 1.98 (t, *J* = 6.5 Hz, 4H, $N(CH_2)_2(CH_2)_2$), 1.43 (s, 9H, $C(CH_3)_3$); ^{13}C NMR (151 MHz, $CDCl_3$); δ 171.6 (CO), 170.1 (CO), 164.6 (CO), 155.5 (CO), 147.7 (CN), 132.1 (ArC), 129.0 (ArC), 127.6 (ArC), 126.2 (ArC), 87.8 (CS), 80.1 (C), 53.3 (CH_2), 52.5 (CH), 51.8 (CH_3), 39.0 (CH_2), 28.4 (CH_3), 25.3 (CH_2); IR (solid); 1550 (s), 2900 (w), 3440 (w) cm^{-1}; LRMS (ES^+) 476.2 (100, $[M+H]^+$); HRMS (ES^+) calcd for $C_{23}H_{30}N_3O_6S$ $[M+H]^+$ 476.1855, observed 476.1854.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7.43
7.42
7.36
7.35
7.33
7.32
7.31
5.80
5.79
4.53
4.52
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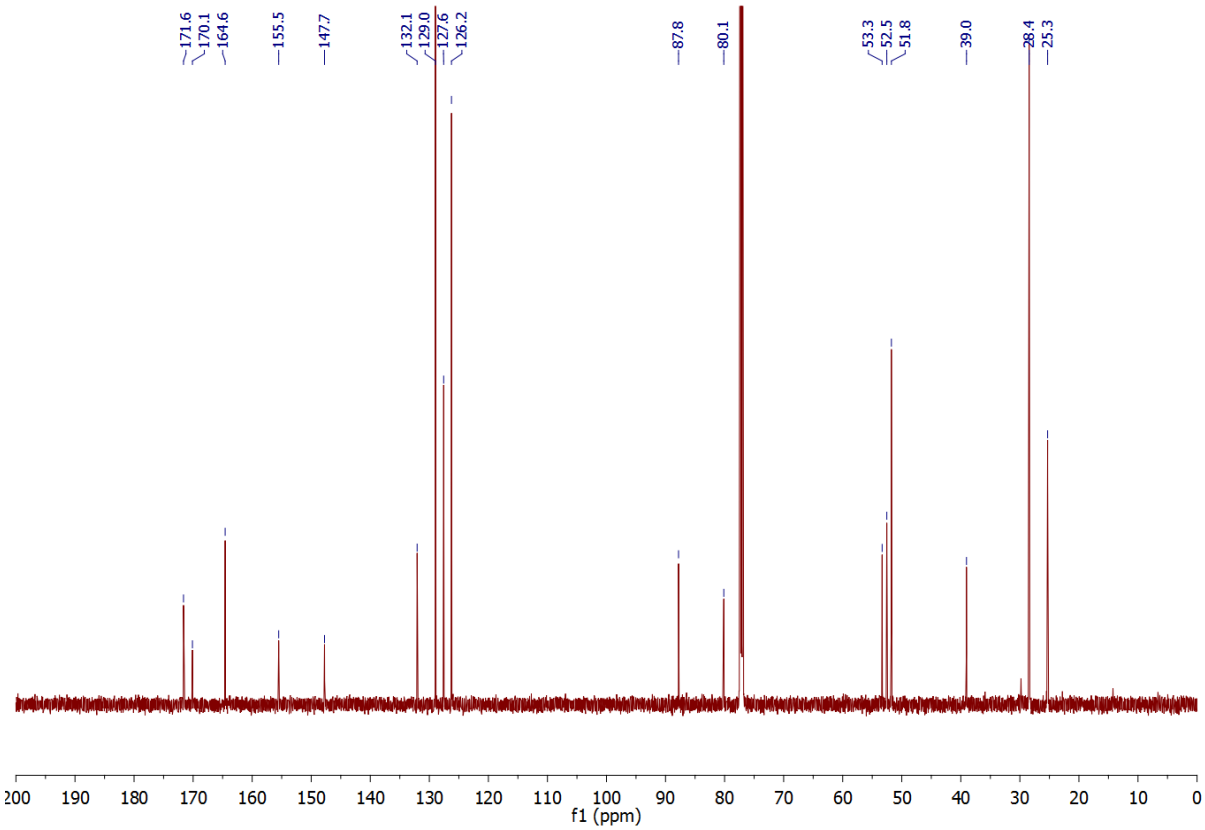
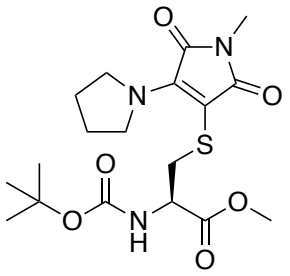
4.07
3.67
3.26
3.25
3.23
3.22
3.08
3.08
3.06
3.05
1.99
1.98
1.97
1.43
1.9
1.9
1.0
0.8
0.9
4.0
3.0
1.0
0.9
4.0
9.2
8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)

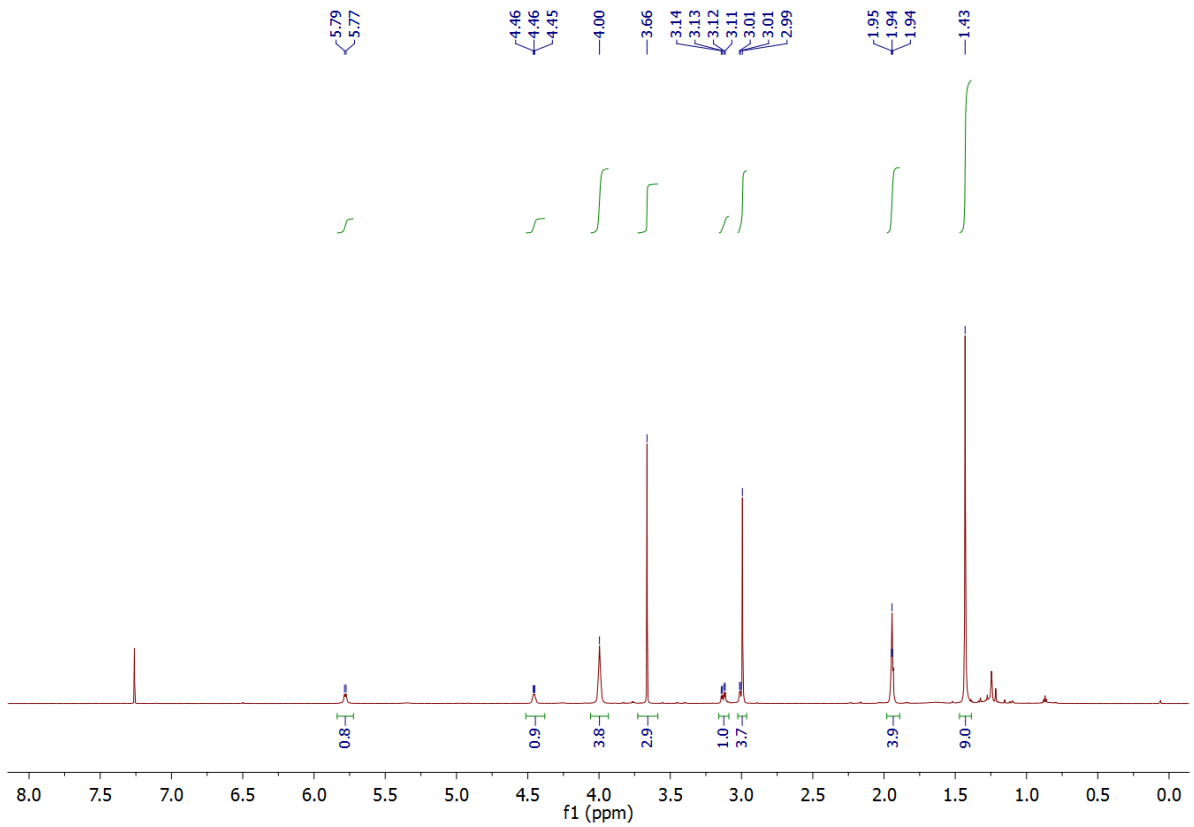

171.6
170.1
164.6
155.5
147.7
132.1
129.0
127.6
126.2
87.8
80.1
53.3
52.5
51.8
39.0
28.4
25.3
200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

Synthesis of methyl *N*-(*tert*-butoxycarbonyl)-S-(2,5-dioxo-1-methyl-4-(pyrrolidin-1-yl)-2,5-dihydro-1*H*-pyrrol-3-yl)-*L*-cysteinate (**9**)



To methyl 3-((4-bromo-1-methyl-2,5-dioxo-2,5-dihydro-1H-pyrrol-3-yl)thio)-2-((tert-butoxycarbonyl)amino)propanoate (0.021 g, 0.050 mmol) dissolved in methanol (20 mL) pyrrolidine (0.0083 mL, 0.100 mmol) dissolved in methanol (4 mL) was added dropwise for 10 min and the resultant solution stirred for 10 min at room temperature. The solvent was removed *in vacuo* to yield the crude product. Purification by column chromatography (0-40% EtOAc/Cyclohexane) yielded the product as an orange oil (0.019 g, 0.046 mmol, 92%).

^{1}H NMR (600 MHz, $CDCl_3$); δ 5.78 (d, *J* = 7.6 Hz, 1H, NH), 4.46-4.45 (m, 1H, NHC*H*CO), 4.00 (br s, 4H, $N(CH_2)_2$), 3.66 (s, 3H, OCH_3), 3.13 (dd, *J* = 13.9, 5.5 Hz, 1H, SC*H*HCH), 3.01-2.99 (m, 4H, SCH*H*/NCH_3), 1.95-1.94 (m, 4H, $N(CH_2)_2(CH_2)_2$), 1.43 (s, 9H, $C(CH_3)_3$); ^{13}C NMR (151 MHz, $CDCl_3$); δ 171.6 (CO), 171.5 (CO), 165.8 (CO), 155.5 (CO), 148.3 (CN), 87.1 (CS), 80.1 (C), 53.4 (CH_3), 52.5 (CH), 51.5 (CH_2), 39.3 (CH_2), 28.5 (CH_3), 25.3 (CH_2), 24.2 (CH_3); IR (thin film); 1600 (s), 2950 (w), 3350 (m) cm^{-1}; LRMS (ES^+) 414.2 (100, $[M+H]^+$); HRMS (ES+) calcd for $C_{18}H_{28}N_3O_6S$ $[M+H]^+$ 414.1699, observed 414.1705.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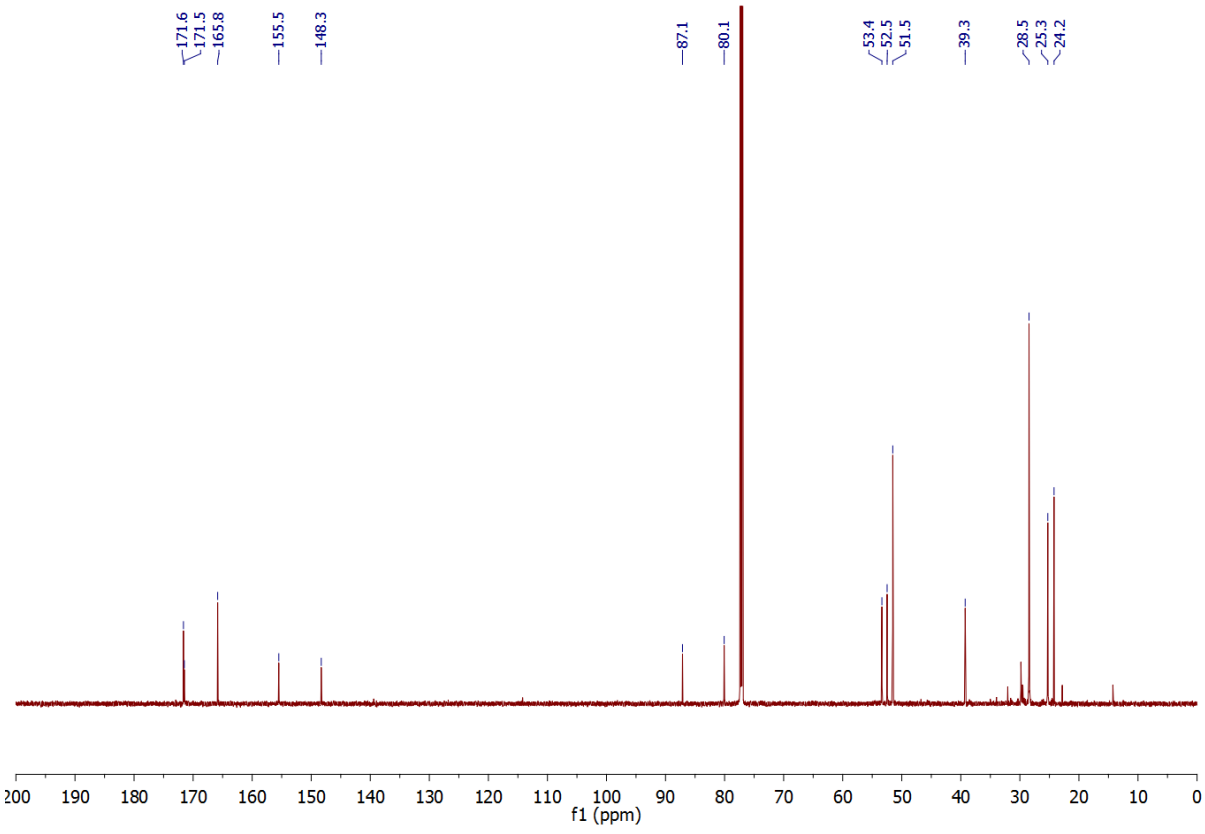
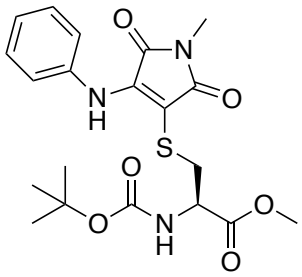
3.14
3.13
3.12
3.11
3.01
3.01
2.99
1.95
1.94
1.94
1.43
0.8
0.9
3.8
2.9
1.0
3.7
3.9
9.0
8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)

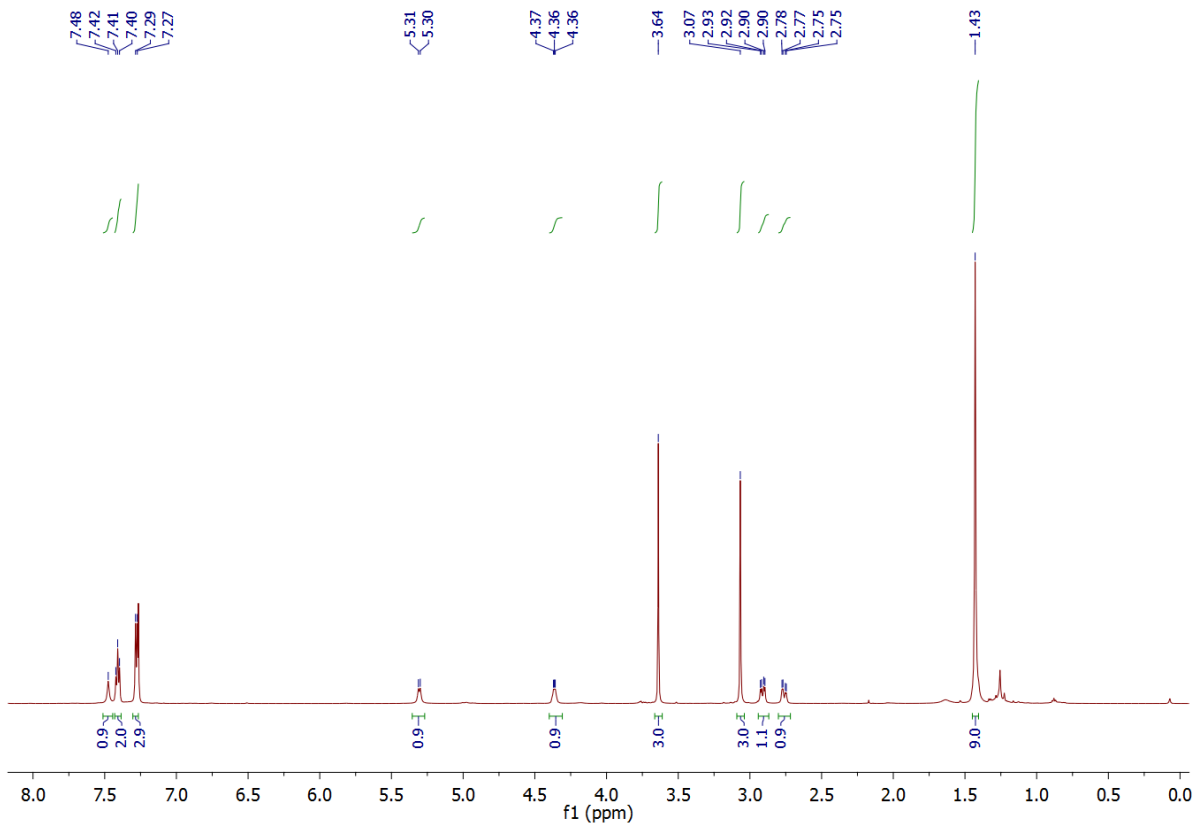

171.6
171.5
165.8
155.5
148.3
87.1
80.1
53.4
52.5
51.5
39.3
28.5
25.3
24.2
200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

Synthesis of methyl *N*-(tert-butoxycarbonyl)-S-(1-methyl-2,5-dioxo-4-(phenylamino)-2,5-dihydro-1H-pyrrol-3-yl)-L-cysteinate (**10**)



To methyl S-(4-bromo-1-methyl-2,5-dioxo-2,5-dihydro-1H-pyrrol-3-yl)-*N*-(tert-butoxycarbonyl)-L-cysteinate (0.021 g, 0.050 mmol) dissolved in methanol (20 mL) aniline (0.009 mL, 0.100 mmol) dissolved in methanol (4 mL) was added dropwise and the resultant solution stirred for 24 hours at room temperature. The solvent was removed *in vacuo* to yield the crude product. Purification by column chromatography (0-40% EtOAc/petroleum ether) yielded the product as an orange oil (0.018 g, 0.041 mmol, 82%).

^{1}H NMR (600 MHz, $CDCl_3$); δ 7.48 (br s, 1H, ArNH), 7.41 (t, *J* = 7.7 Hz, 2H, ArH), 7.29-7.27 (m, 2H, ArH), 5.30 (d, *J* = 7.8 Hz, 1H, NH), 4.36 (dt, *J* = 9.6, 4.8 Hz, 1H, NHC*H*CO,), 3.64 (s, 3H, OCH_3), 3.07 (s, 3H, NCH_3), 2.91 (dd, *J* = 13.9, 5.1 Hz, 1H SC*H*HCH), 2.76 (dd, *J* = 13.9, 4.1 Hz, 1H, SCH*H*CH), 1.43 (s, 9H, $C(CH_3)_3$); ^{13}C NMR (151 MHz, $CDCl_3$); δ 171.1 (CO), 170.9 (CO), 166.7 (CO), 155.2 (CO), 144.3 (C), 135.9 (C), 128.8 (C), 126.6 (C), 124.3 (C), 92.4 (CS), 80.2 (C), 53.2 (CH), 52.5 (CH_3), 37.1 (CH_2), 28.4 (CH_3), 24.5 (CH_3); IR (thin film); 1600 (s), 2950 (w), 3400 (w) cm^{-1}; LRMS (ES^+) 458.2 (100, $[M+Na]^+$); HRMS (ES^+) calcd for $C_{20}H_{26}N_3O_6S$ $[M+Na]^+$ 458.1464, observed 458.1508.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7.40
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7.27
5.31
5.30
4.37
4.36
4.36
3.64
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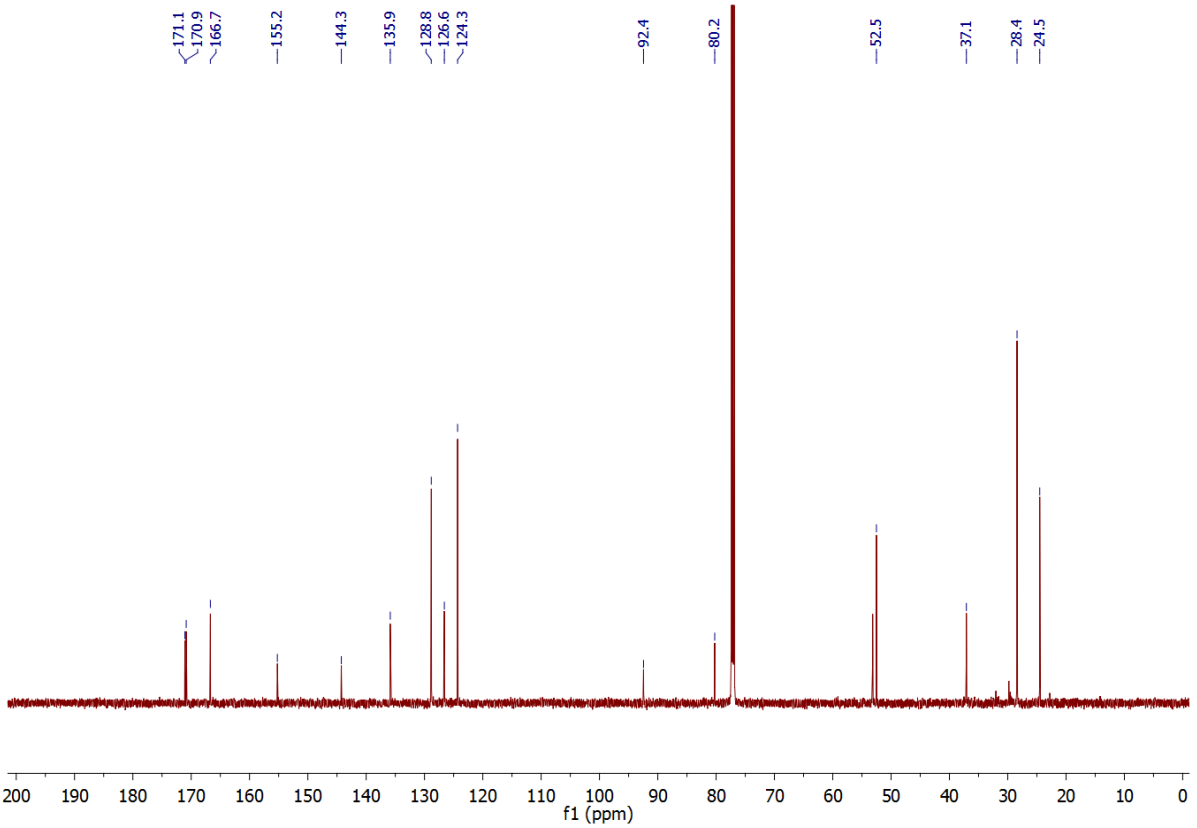
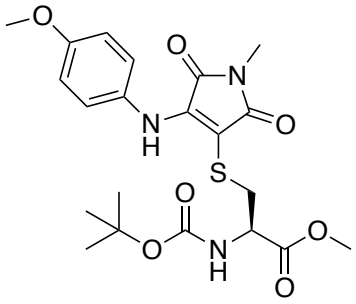
2.93
2.92
2.90
2.90
2.78
2.77
2.75
2.75
1.43
0.9
2.0
2.9
0.9
0.9
3.0
3.0
1.1
0.9
9.0
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)

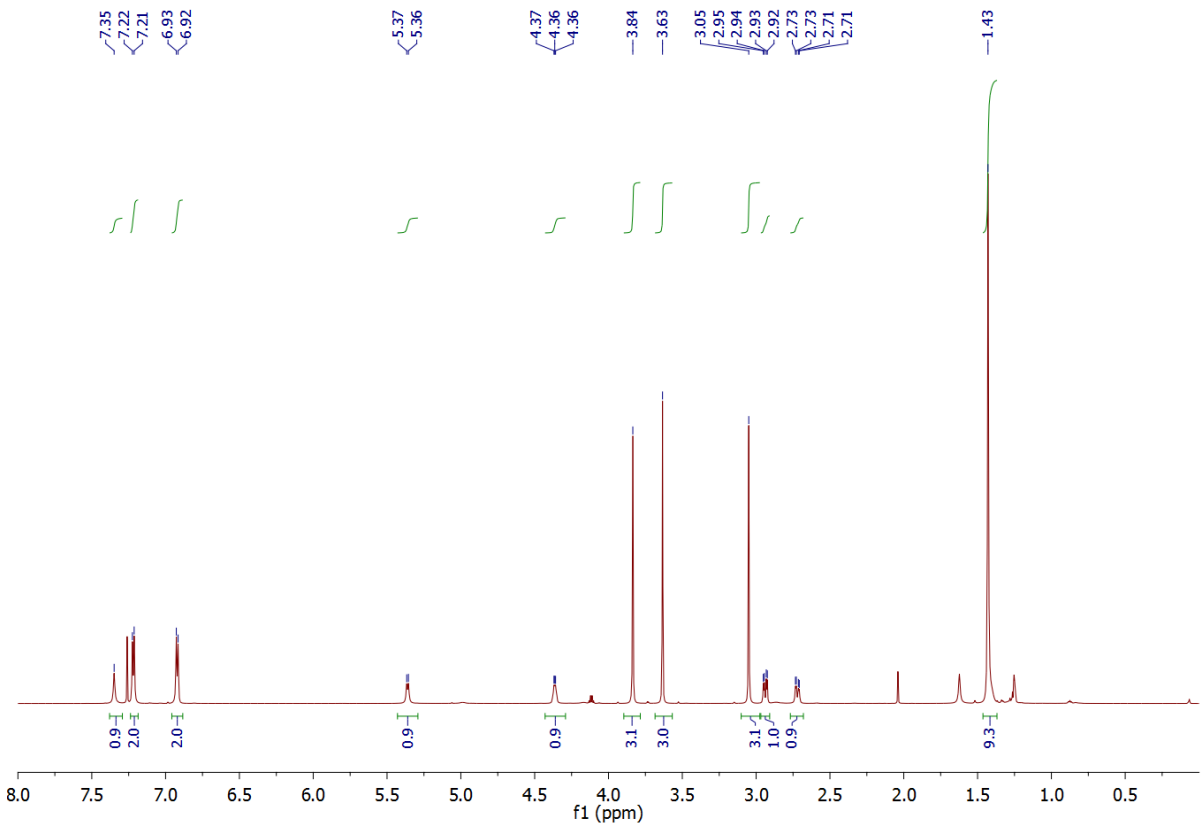

171.1
170.9
166.7
155.2
144.3
135.9
128.8
126.6
124.3
92.4
80.2
52.5
37.1
28.4
24.5
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

Synthesis of methyl *N*-(*tert*-butoxycarbonyl)-*S*-(4-((4-methoxyphenyl)amino)-1-methyl-2,5-dioxo-2,5-dihydro-1*H*-pyrrol-3-yl)-*L*-cysteinate (**11**)



To methyl S - (4 - bromo - 1 - methyl - 2,5 - dioxo - 2,5 - dihydro - 1H - pyrrol - 3 - yl) - N - (tert - butoxycarbonyl) - L - cysteinate (0.021 g, 0.050 mmol) dissolved in methanol (20 mL), *p*-anisidine (0.012 g, 0.100 mmol) dissolved in methanol (4 mL) was added dropwise and the resultant solution stirred for 24 hours at room temperature. The solvent was removed *in vacuo* to yield the crude product, which was then dissolved in EtOAc (30mL) and washed with 10% citric acid solution (2 x 10 mL), water (1 x 10 mL) and dried over $MgSO_4$. The mixture was filtered and the solvent again removed *in vacuo* before purification by column chromatography (0-50% EtOAc /hexane) yielded the product as a yellow oil (0.021 g, 0.045 mmol, 90%);

^{1}H NMR (700 MHz, $CDCl_3$); δ 7.35 (s, 1H, ArNH), 7.22 (d, *J* = 8.3 Hz, 2H, ArH), 6.92 (d, *J* = 8.4 Hz, 2H, ArH), 5.36 (d, *J* = 8.4 Hz, 1H, NH), 4.36 (dt, *J* = 9.4, 5.0 Hz, 1H, CH), 3.84 (s, 3H, $ArOCH_3$), 3.63 (d, *J* = 1.1 Hz, 3H, $COOCH_3$), 3.05 (s, 3H, NCH_3), 2.94 (dd, *J* = 14.1, 5.0 Hz, 1H, SC*H*H), 2.72 (dd, *J* = 14.1, 4.7 Hz, 1H, SCH*H*), 1.43 (s, 9H, $(CH_3)_3$); ^{13}C NMR (176 MHz, $CDCl_3$); δ 171.2 (CO), 171.1 (CO), 166.7 (CO), 158.6 (CO), 155.3 (C), 145.2 (C), 128.7 (C), 126.5 (C), 114.0 (C), 90.6 (SC), 80.2 (C), 55.6 (CH), 53.2 (CH_3), 52.5 (CH_3), 37.2 (CH_2), 28.4 (CH_3), 24.5 (CH_3); IR (thin film); 1679 (s), 1703 (s), 1752 (s), 3340 (m), 3374 (m) cm^{-1}; LRMS (ES^+) 466.2 (100, $[M+H]^+$); HRMS (ES^+) calcd for $C_{21}H_{28}N_3O_7S$ $[M+H]^+$ 466.1648, observed 466.1632. ε_{415} 4250 M^{-1}.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7.21
6.93
6.92
5.37
5.36
4.37
4.36
4.36
3.84
3.63
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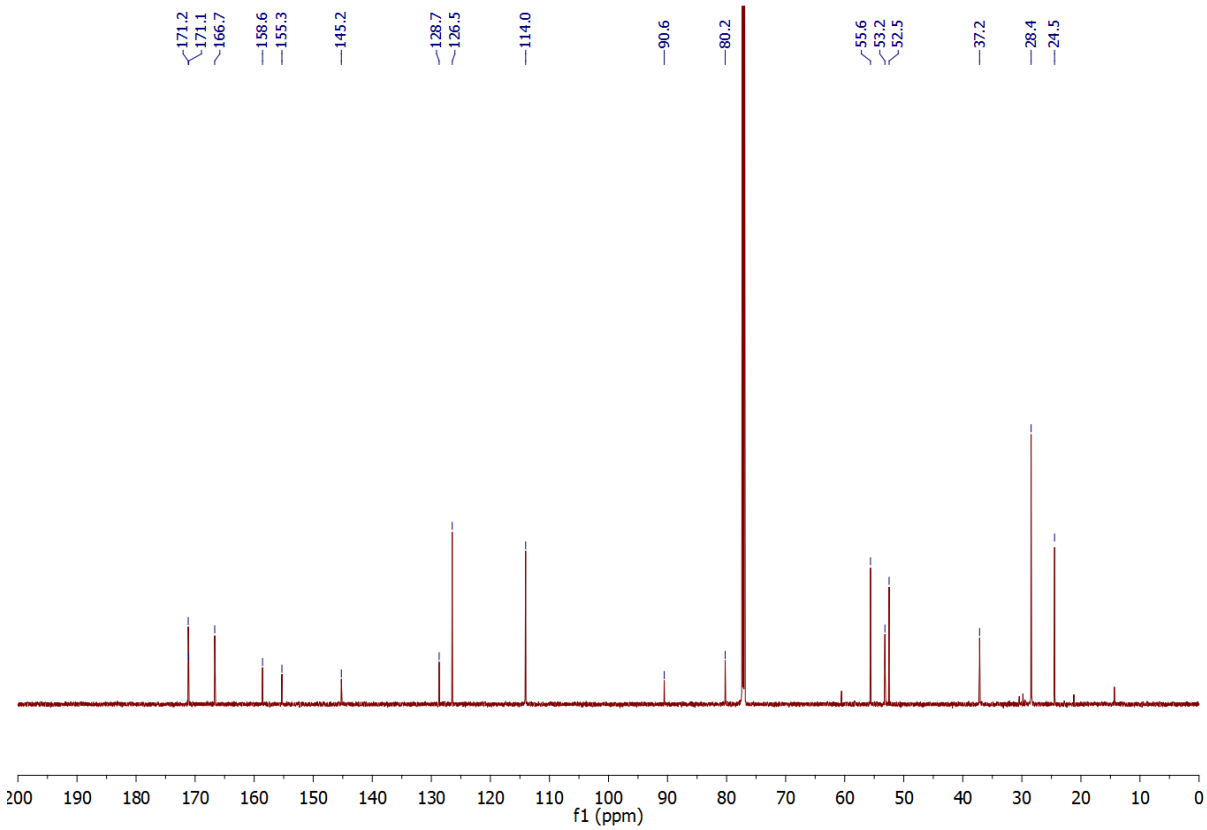
2.95
2.94
2.93
2.92
2.73
2.73
2.71
2.71
1.43
0.9
2.0
2.0
0.9
0.9
3.1
3.0
3.1
1.0
0.9
9.3
f1 (ppm)


171.2
171.1
166.7
158.6
155.3
145.2
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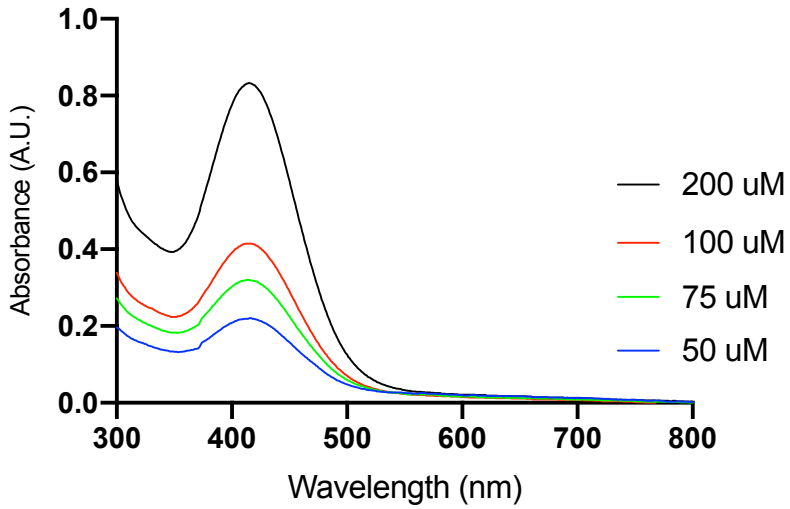
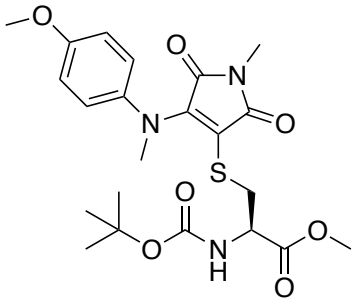
126.5
114.0
90.6
80.2
55.6
53.2
52.5
37.2
28.4
24.5
f1 (ppm)



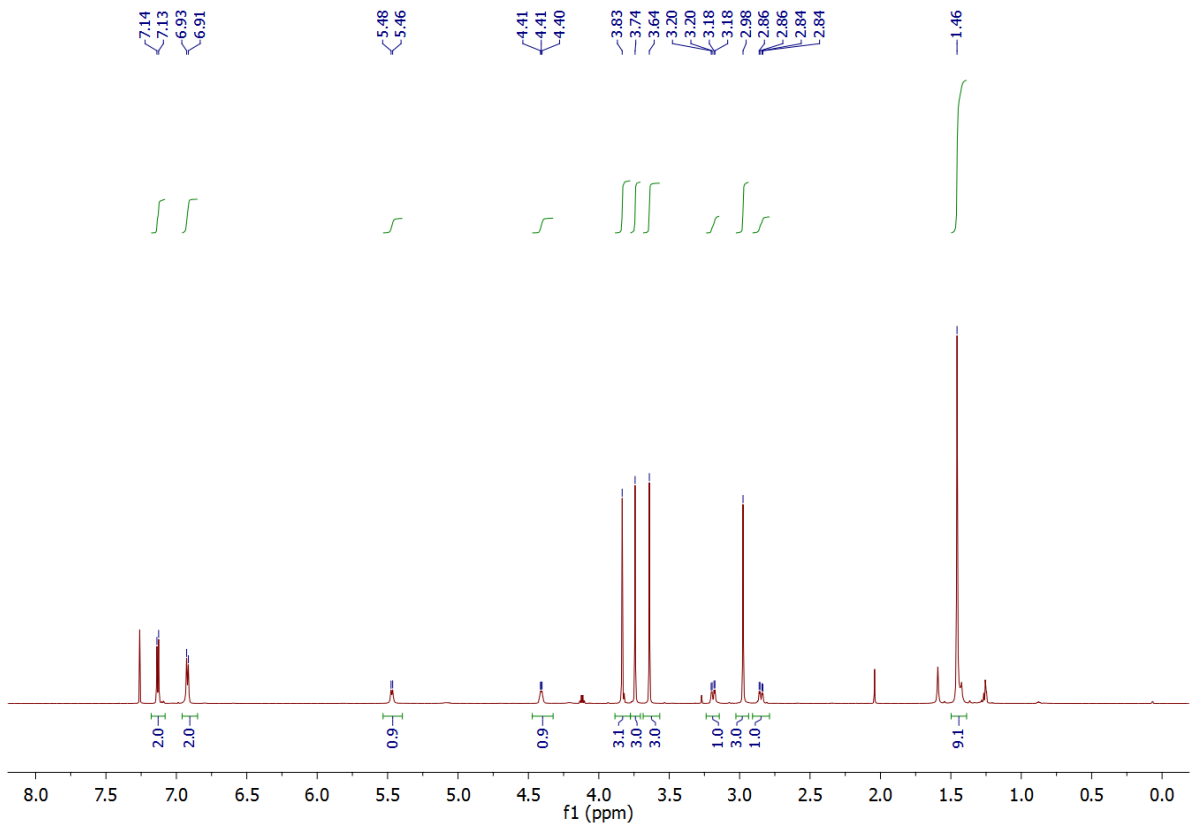
Absorbance spectra recorded in 10% THF in PBS (pH 7.4).

Synthesis of methyl *N*-(*tert*-butoxycarbonyl)-*S*-(4-((4-methoxyphenyl)(methyl)amino)-1-methyl-2,5-dioxo-2,5-dihydro-1*H*-pyrrol-3-yl)-*L*-cysteinate (**12**)



To methyl S - (4 - bromo - 1 - methyl - 2,5 - dioxo - 2,5 - dihydro - 1H - pyrrol - 3 - yl) - N - (tert - butoxycarbonyl) - L - cysteinate (0.021 g, 0.050 mmol) dissolved in methanol (20 mL), *N*-methyl *p*-anisidine (0.014 g, 0.100 mmol) dissolved in methanol (4 mL) was added dropwise and the resultant solution stirred for 24 hours at room temperature. The solvent was removed *in vacuo* to yield the crude product, which was then dissolved in EtOAc (30 mL) and washed with 10% citric acid solution (2 x 10 mL), water (1 x 10 mL) and dried over $MgSO_4$. The mixture was filtered and the solvent again removed *in vacuo* before purification by column chromatography (0-50% EtOAc/hexane) yielded the product as an orange oil (0.021 g, 0.044 mmol, 88%);

^{1}H NMR (700 MHz, $CDCl_3$); δ 7.13 (d, *J* = 8.4 Hz, 2H, ArH), 6.92 (d, *J* = 8.4 Hz, 2H, ArH), 5.47 (d, *J* = 8.4 Hz, 1H, NH), 4.41 (dt, *J* = 9.2, 4.8 Hz, 1H, CH), 3.83 (s, 3H, $ArOCH_3$), 3.74 (s, 3H, $ArNCH_3$), 3.64 (s, 3H, $COOCH_3$), 3.19 (dd, *J* = 14.1, 4.9 Hz, 1H, SC*H*H), 2.98 (s, 3H, NCH_3), 2.85 (dd, *J* = 14.2, 4.7 Hz, 1H, SCH*H*), 1.46 (s, 9H, $(CH_3)_3$); ^{13}C NMR (176 MHz, $CDCl_3$) δ 171.5 (CO), 170.1 (CO), 166.1 (CO), 158.8 (CO), 155.4 (C), 148.9 (C), 139.0 (C), 126.8 (C), 114.7 (C), 95.0 (SC), 80.2 (C), 55.6 (CH), 53.1 (CH_3), 52.4 (CH_3), 43.3 (CH_3), 36.9 (CH_2), 28.5 (CH_3), 24.2 (CH_3); IR (thin film); 1697 (s), 1747 (s), 2932 (br), 2999 (w), 3366 (m) cm^{-1}; LRMS (ES$^+$) 480.2 (100, $[M+H]^+$); HRMS (ES+) calcd for $C_{22}H_{30}N_3O_7S$ $[M+H]^+$ 480.1804, observed 480.1798.


7.14
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6.91
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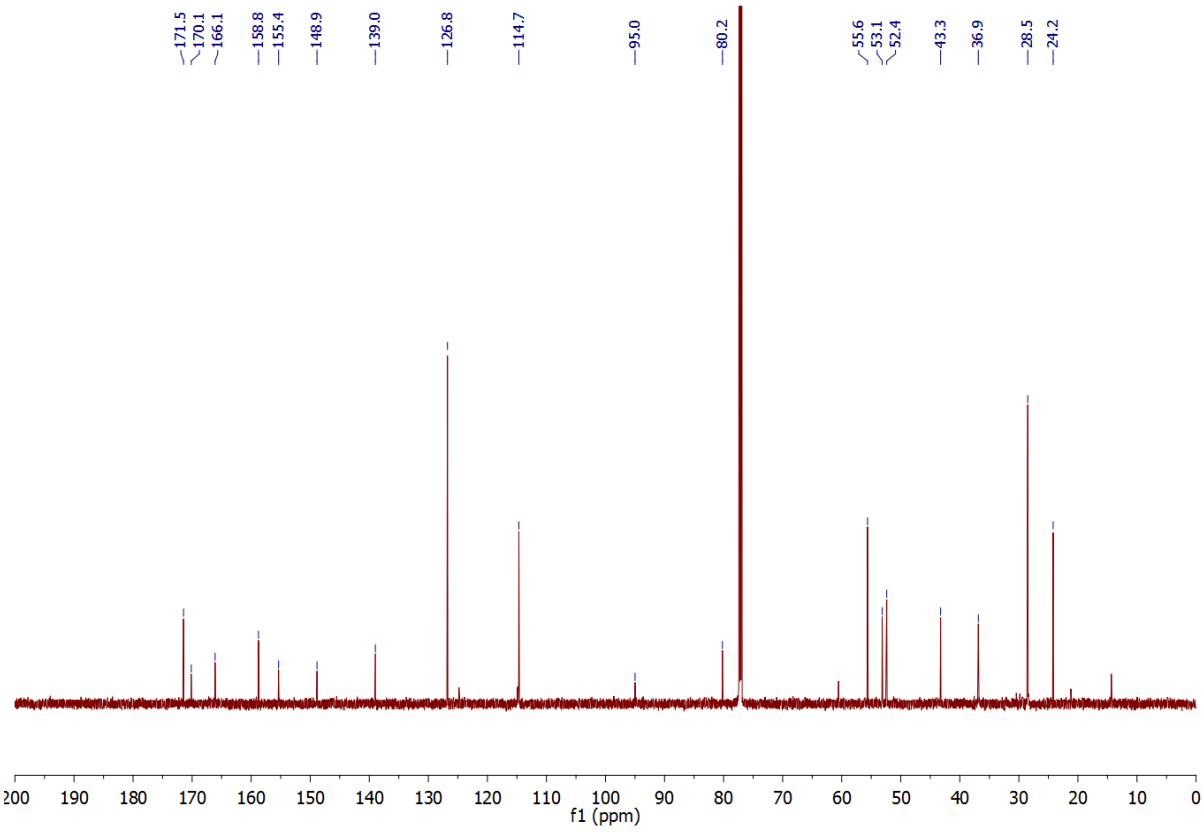
4.41
4.40
3.83
3.74
3.64
3.20
3.20
3.18
3.18
2.98
2.86
2.86
2.84
2.84
1.46
2.0
2.0
0.9
0.9
3.1
3.0
3.0
1.0
3.0
1.0
9.1
8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)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155.4
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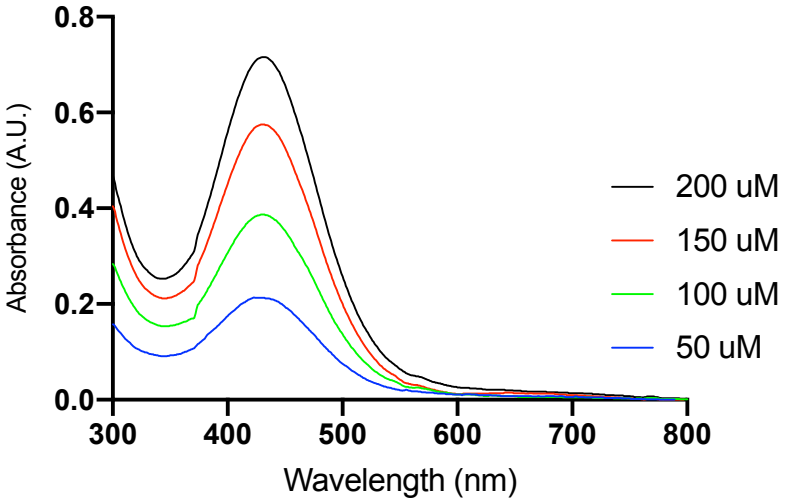
126.8
114.7
95.0
80.2
55.6
53.1
52.4
43.3
36.9
28.5
24.2
200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)



Absorbance spectra recorded in 10% THF in PBS (pH 7.4).

Amine Reactions in Buffer

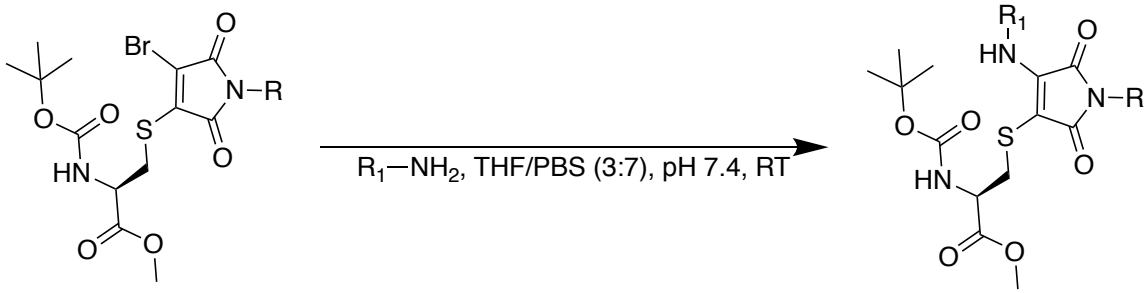
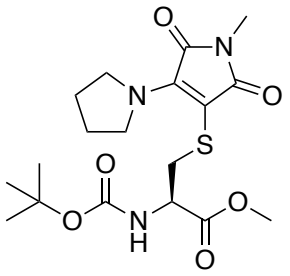


Table S2: Amine reactions in THF/PBS buffer.

R	R[1]-(Amine)	Equiv. of amine	Yield
Me	Pyrrolidine	100	72%
Me	Aniline	100	96%
Me	*p*-anisidine	10	90%
Me	*p*-anisidine	100	86%
Me	*N*-Me *p*-anisidine	10	76%

All reactions are 21 mg **6** in 50 mL (1 mM) final volume. PBS (pH 7.4, 25 mM phosphates) was used.

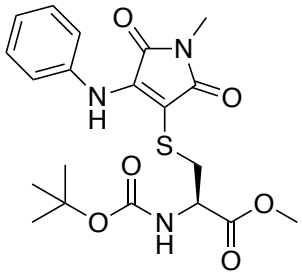
Synthesis of methyl *N*-(*tert*-butoxycarbonyl)-S-(2,5-dioxo-1-methyl-4-(pyrrolidin-1-yl)-2,5-dihydro-1*H*-pyrrol-3-yl)-*L*-cysteinate under buffered conditions (**9**)



Methyl S-(4-bromo-1-methyl-2,5-dioxo-2,5-dihydro-1H-pyrrol-3-yl)-*N*-(tert-butoxycarbonyl)-L-cysteinate (0.021 g, 0.050 mmol) was dissolved in tetrahydrofuran (15 mL). Pyrrolidine (0.411 mL, 5.000 mmol) was dissolved in PBS (35 mL, 25 mM phosphates) and the pH adjusted to 7.4 using HCl (1 M). The resultant amine solution was quickly added to the initial solution and stirred at room temperature for 1 hour. The solution was extracted with $CHCl_3$ (3 × 30 mL) and dried over $MgSO_4$. The solvent was removed *in vacuo* to yield the crude product. Purification by column chromatography (0-40% EtOAc/petroleum ether) yielded the product as an orange oil (0.015 g, 0.036 mmol, 72%).

Analytical data matches that of the organic solvent method to compound (vide supra).

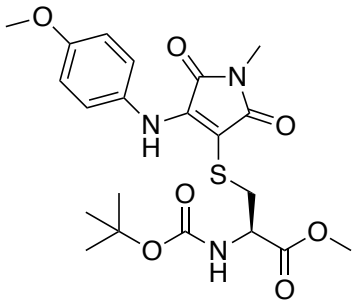
Synthesis of methyl *N*-(tert-butoxycarbonyl)-S-(1-methyl-2,5-dioxo-4-(phenylamino)-2,5-dihydro-1H-pyrrol-3-yl)-L-cysteinate under buffered conditions (**10**)



Methyl S-(4-bromo-1-methyl-2,5-dioxo-2,5-dihydro-1H-pyrrol-3-yl)-*N*-(tert-butoxycarbonyl)-L-cysteinate (0.021 g, 0.050 mmol) was dissolved in tetrahydrofuran (5 mL). Aniline (0.466 g, 0.457 µL, 5.000 mmol) in THF (10 mL) was dissolved in PBS (35 mL, 25 mM phosphates) and the pH adjusted to 7.4 using HCl (1 M). The resultant amine solution was quickly added to the initial solution and stirred at room temperature overnight. Brine (10 mL) was added to the solution, and the organic product was extracted with $CHCl_3$ (3 × 30 mL) and dried over $MgSO_4$. The solvent was removed *in vacuo* to yield the crude product. Purification by column chromatography (0-40% EtOAc/petroleum ether) yielded the product as an orange oil (0.021 g, 0.048 mmol, 96%).

Analytical data matches that of the organic solvent method to compound (vide supra).

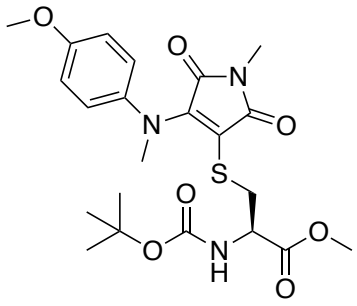
Synthesis of methyl *N*-(*tert*-butoxycarbonyl)-*S*-(4-((4-methoxyphenyl)amino)-1-methyl-2,5-dioxo-2,5-dihydro-1*H*-pyrrol-3-yl)-*L*-cysteinate under buffered conditions (**11**)



Methyl *S*-(4-bromo-1-methyl-2,5-dioxo-2,5-dihydro-1*H*-pyrrol-3-yl)-*N*-(*tert*-butoxycarbonyl)-*L*-cysteinate (0.021 g, 0.050 mmol) was dissolved in tetrahydrofuran (5 mL). *p*-anisidine (0.062 g, 0.500 mmol) in THF (10 mL) was dissolved in PBS (35 mL, 25 mM phosphates) and the pH adjusted to 7.4 using HCl (1 M). The resultant amine solution was quickly added to the initial solution and stirred at room temperature for 1 hour. Brine (10 mL) was added to the solution, and the organic product was extracted with $CHCl_3$ (3 × 30 mL) and dried over $MgSO_4$. The solvent was removed *in vacuo* to yield the crude product, which was then dissolved in EtOAc (30mL) and washed with 10% citric acid solution (2 x 10 mL), water (1 x 10 mL) and dried over $MgSO_4$. The mixture was filtered and the solvent again removed *in vacuo* before purification by column chromatography (0-50% EtOAc /hexane) yielded the product as a yellow oil (0.021 g, 0.045 mmol, 90%). *N.B. Same protocol followed for 100 equiv. experiment shown in table S2.*

Analytical data matches that of the organic solvent method to compound (vide supra).

Synthesis of methyl *N*-(*tert*-butoxycarbonyl)-*S*-(4-((4-methoxyphenyl)(methyl)amino)-1-methyl-2,5-dioxo-2,5-dihydro-1*H*-pyrrol-3-yl)-*L*-cysteinate under buffered conditions (**12**)

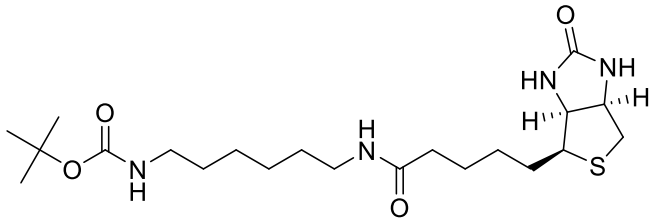


Methyl *S*-(4-bromo-1-methyl-2,5-dioxo-2,5-dihydro-1*H*-pyrrol-3-yl)-*N*-(*tert*-butoxycarbonyl)-*L*-cysteinate (0.021 g, 0.050 mmol) was dissolved in tetrahydrofuran (5 mL). *N*-methyl *p*-anisidine (0.067 g, 0.500 mmol) in THF (10 mL) was dissolved in PBS (35 mL, 25 mM phosphates) and the pH adjusted to 7.4 using HCl (1 M). The resultant amine solution was quickly added to the initial solution and stirred at room temperature for 1 hour. Brine (10 mL) was added to the solution, and the organic product was extracted with $CHCl_3$ (3 × 30 mL) and dried over $MgSO_4$. The solvent was removed *in vacuo* to yield the crude product, which was then dissolved in EtOAc (30mL) and washed with 10% citric acid solution (2 x 10 mL), water (1 x 10 mL) and dried over $MgSO_4$. The mixture was filtered and the solvent again removed *in vacuo* before purification by column chromatography (0-40% EtOAc/hexane) yielded the product as an orange oil (0.018 g, 0.038 mmol, 76%).

Analytical data matches that of the organic solvent method to compound (vide supra).

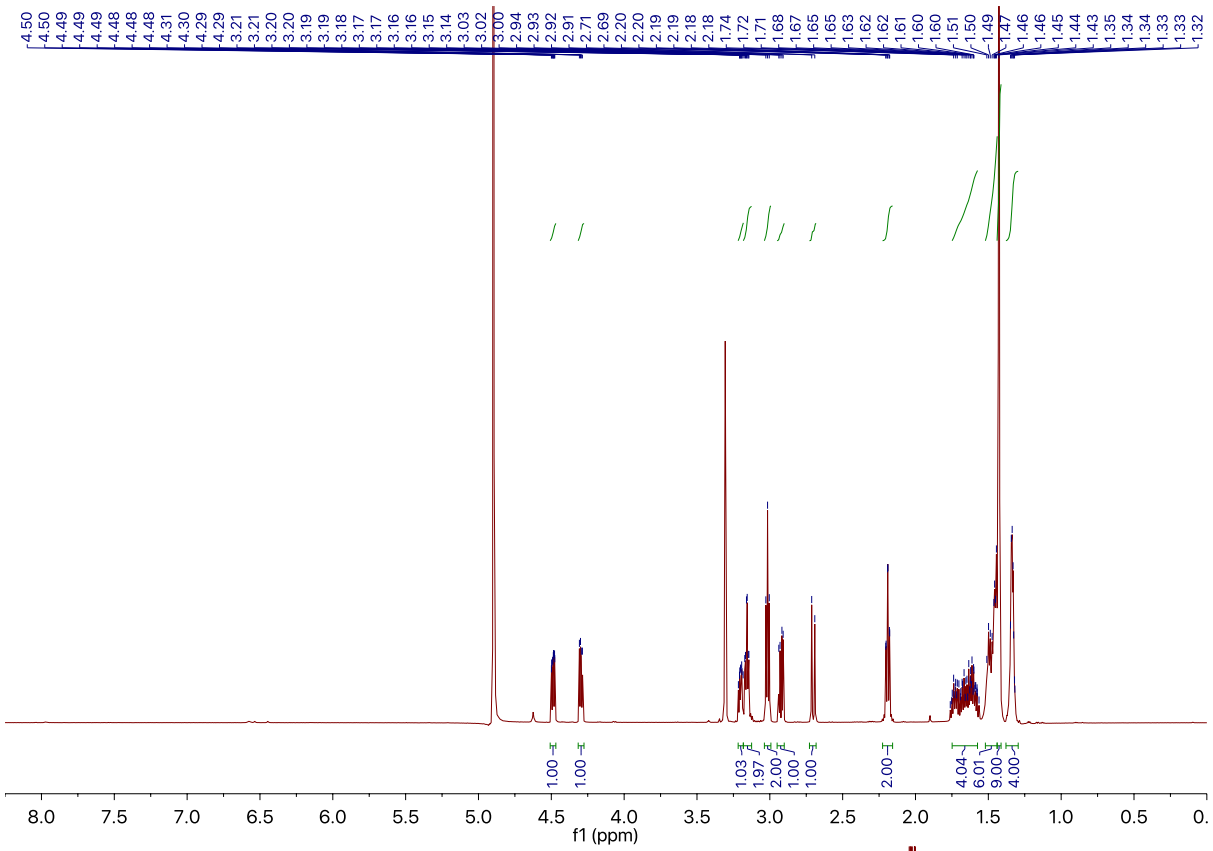
Synthesis of functionalised DBMs

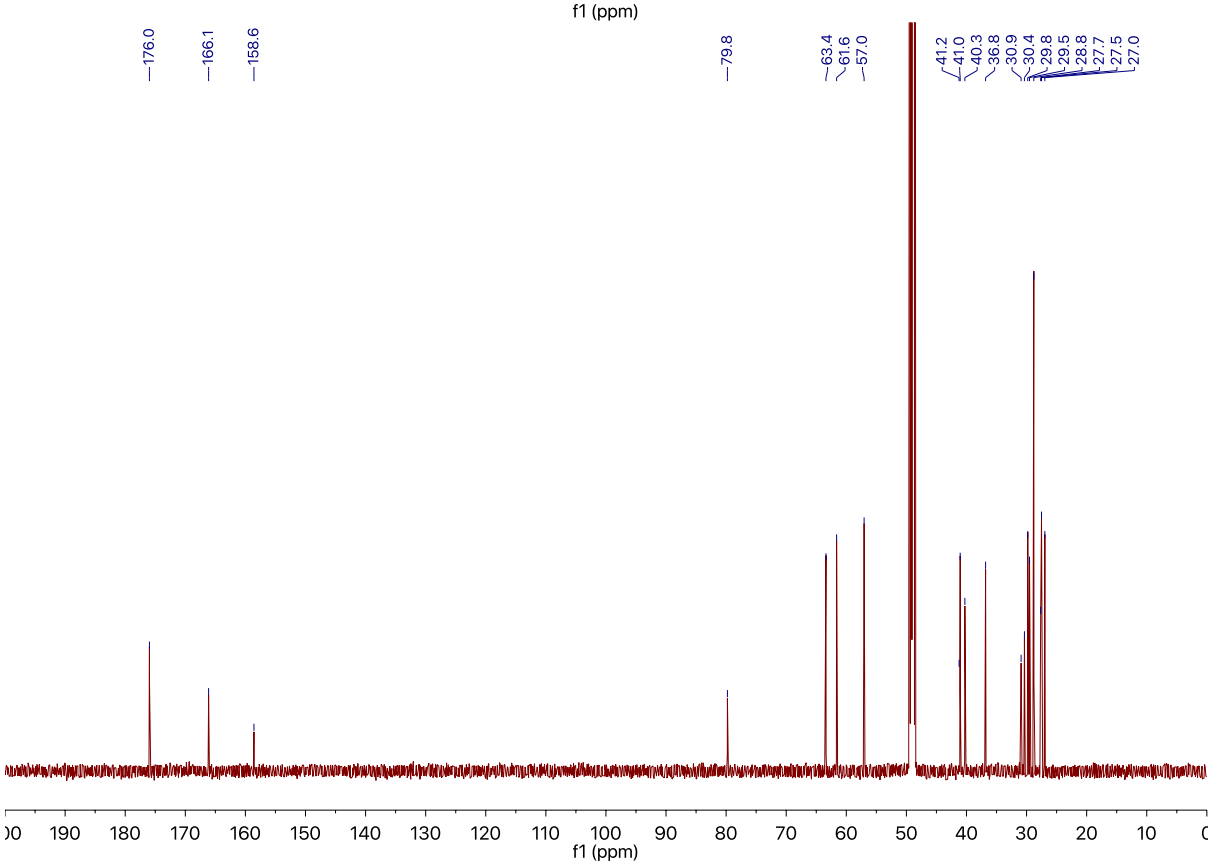
Synthesis of tert-butyl (6-(5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido) hexyl)carbamate (**29**)



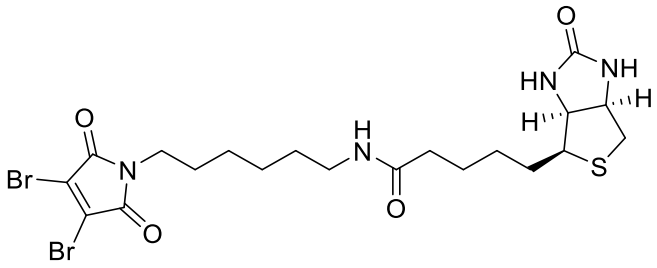
D-Biotin (221 mg, 0.905 mmol), HBTU (360 mg, 0.949 mmol) and DIPEA (236 µL, 1.36 mmol) were dissolved in DMF (5 mL) and stirred for 20 min at RT. *N*-Boc-1,6-hexanediamine (205 mg, 1.16 mmol) in DMF (2 mL) was then added and the resultant mixture was stirred for 24 h at RT. After this period, the solvent was removed *in vacuo*. Purification by column chromatography (0-10% MeOH/DCM) afforded the target compound as a white crystalline solid (396 mg, 0.895 mmol, 99%).

m.p. 184.0-186.0 ^{o}C; ^{1}H NMR (600 MHz, CD_3OD); δ 4.49 (dd, *J* = 7.8, 4.6 Hz, 1H, SCH_2CH), 4.30 (dd, *J* = 7.9, 4.5 Hz, 1H, SCHC*H*), 3.22-3.18 (m, 1H, SCH), 3.16 (td, *J* = 6.9, 2.8 Hz, 2H, $CH_2NHC(O)CH_2$), 3.02 (t, *J* = 7.0 Hz, 2H, $OC(O)NHCH_2$), 2.92 (dd, *J* = 12.7, 5.0 Hz, 1H, SC*H*H), 2.70 (d, *J* =12.7 Hz, 1H, SCH*H*), 2.19 (t, *J* = 7.2 Hz, 2H, $NHC(O)CH_2$), 1.76-1.56 (m, 4H, $SCHCH_2$ and $SCHCH_2CH_2CH_2$), 1.52-1.44 (m, 6H, $OC(O)NHCH_2CH_2$, $CH_2C(O)NHCH_2CH_2$ and $SCHCH_2CH_2$), 1.43 (s, 9H, $(C(CH_3)_3)$, 1.35-1.32 (m, 4H, $OC(O)NHCH_2CH_2CH_2$ and $CH_2C(O)NHCH_2CH_2CH_2$); ^{13}C NMR (150 MHz, CD_3OD); δ 176.0 (CO), 166.1 (CO), 158.6 (CO), 79.8 (C), 63.4 (CH), 61.6 (CH), 57.0 (CH), 41.2 (CH_2), 41.0 (CH_2), 40.2 (CH_2), 36.8 (CH_2), 30.9 (CH_2), 30.4 (CH_2), 29.8 (CH_2), 29.5 (CH_2), 28.8 (CH_3), 27.7 (CH_2), 27.5 (CH_2), 27.0 (CH_2); IR (solid); 1516 (s), 1621 (m), 1687 (s), 2932 (w), 3252 (w, br), 3386 (w) cm^{-1}; LRMS (ESI) *m/z* 465.2 ($[M+Na]^+$, 17), 443.3 ($[M+H]^+$, 100), 343.2 (45); HRMS (ESI) calcd for $C_{21}H_{39}N_4OS$ $[M+H]^+$ 443.2687, observed 443.2675.


4.50 4.50 4.49 4.49 4.49 4.48 4.48 4.48 4.31 4.30 4.29 4.29 3.21 3.21 3.20 3.20 3.19 3.19 3.18 3.17 3.17 3.16 3.16 3.15 3.14 3.03 3.02 3.00 2.94 2.93 2.92 2.91 2.71 2.69 2.20 2.20 2.19 2.19 2.18 2.18 1.74 1.72 1.71 1.68 1.67 1.65 1.65 1.63 1.62 1.62 1.61 1.60 1.60 1.51 1.50 1.49 1.47 1.46 1.46 1.45 1.44 1.43 1.35 1.34 1.34 1.33 1.33 1.32
1.00 1.00 1.03 1.97 2.00 1.00 2.00 4.04 6.01 9.00 4.00
8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.
f1 (ppm)

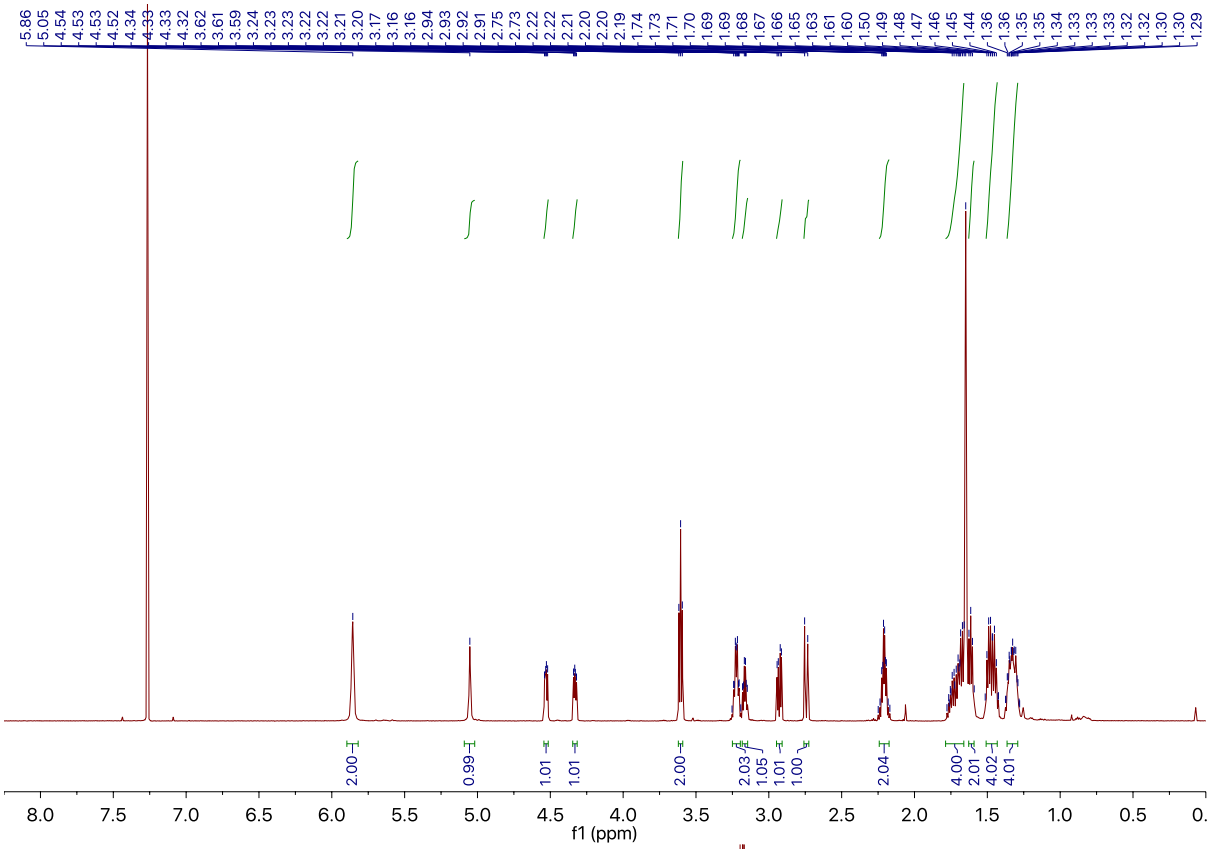

176.0 166.1 158.6 79.8 63.4 61.6 57.0 41.2 41.0 40.3 36.8 30.9 30.4 29.8 29.5 28.8 27.7 27.5 27.0
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10
f1 (ppm)

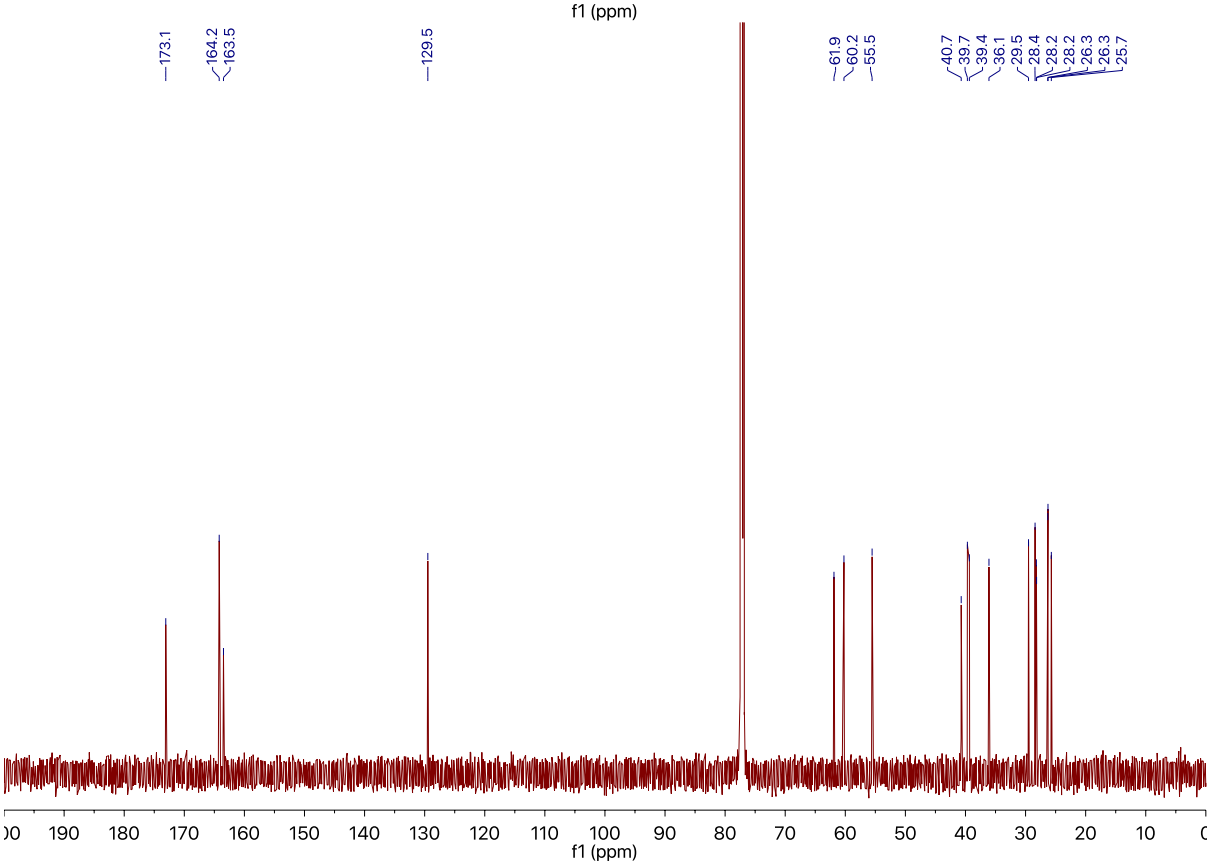
Synthesis of *N*-(6-(3,4-dibromo-2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexyl)-5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide (**21**)



Compound **29** (180 mg, 0.407 mmol) was dissolved in TFA (8 mL). After 18 h at RT, the solvent was removed *in vacuo*. The crude residue was dissolved in AcOH (18 mL) and dibromomaleic acid (112 mg, 0.409 mmol) was added. The reaction was heated to reflux for 3 h. All volatile material was then removed *in vacuo*. Purification by column chromatography (0-12% MeOH/DCM) afforded the product as a pale yellow solid (143 mg, 0.247 mmol, 61%).

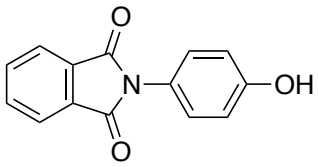
m.p. 158.0-160.0 ^{o}C; ^{1}H NMR (600 MHz, CD_3Cl); δ 5.86 (br s, 2H, N*H*C(O)N*H*), 5.05 (br s, 1H, NHC(O)), 4.53 (dd, *J* = 7.5, 5.1 Hz, 1H, SCH_2CH), 4.33 (dd, *J* = 7.3, 4.9 Hz, 1H, SCHC*H*), 3.61 (t, *J* = 7.2 Hz, 2H, NCH_2), 3.25-3.19 (m, 2H, $NHCH_2$), 3.18-3.15 (m, 1H, SCH), 2.93 (dd, *J* = 12.9, 5.0 Hz, 1H, SC*H*H), 2.74 (d, *J* = 12.8 Hz, 1H, SCH*H*), 2.25-2.17 (m, 2H, $NHC(O)CH_2$), 1.78-1.66 (m, 4H, $SCHCH_2$ and $C(O)CH_2CH_2$), 1.63-1.59 (m, 2H, NCH_2CH_2), 1.51-1.42 (m, 4H, $NHCH_2CH_2$ and $SCHCH_2CH_2$), 1.37-1.28 (m, 4H, $NCH_2CH_2CH_2$ and $NHCH_2CH_2CH_2$); ^{13}C NMR (150 MHz, $CDCl_3$); δ 173.1 (CO), 164.2 (CO), 163.5 (CO), 129.5 (CBr), 61.9 (CH), 60.2 (CH), 55.5 (CH), 40.7 (CH_2), 39.7 (CH_2), 39.4 (CH_2), 36.1 (CH_2), 29.5 (CH_2), 28.4 (CH_2), 28.2 (CH_2), 28.2 (CH_2), 26.3 (CH_2), 26.3 (CH_2), 25.7 (CH_2); IR (solid); 1544 (m), 1641 (m), 1693 (s), 1715 (s), 2859 (w), 2931 (w), 3289 (w, br) cm^{-1}; LRMS (ESI) m/z 605 ([^{81,81}M+Na]$^{+}$, 8), 603 ([^{81,79}M+Na]$^{+}$, 16), 601 ([^{79,79}M+Na]$^{+}$, 8), 583 ([^{81,81}M+H]$^{+}$, 54), 581 ([^{81,79}M+H]$^{+}$, 100), 579 ([^{79,79}M+Na]$^{+}$, 49); HRMS (ESI) calcd for $C_{20}H_{29}{}^{81,79}Br_2N_4O_4S$ [M+H]$^{+}$ 581.0251, observed 581.0242.


5.86 5.05 4.54 4.53 4.53 4.52 4.34 4.33 4.33 4.32 3.62 3.61 3.59 3.24 3.23 3.23 3.22 3.22 3.21 3.20 3.17 3.16 3.16 2.94 2.93 2.92 2.91 2.75 2.73 2.22 2.22 2.21 2.20 2.20 2.19 1.74 1.73 1.71 1.70 1.69 1.69 1.68 1.67 1.66 1.65 1.63 1.61 1.60 1.50 1.49 1.48 1.47 1.46 1.45 1.44 1.36 1.36 1.35 1.35 1.34 1.33 1.33 1.32 1.32 1.30 1.30 1.29
2.00 0.99 1.01 1.01 2.00 2.03 1.05 1.01 1.00 2.04 4.00 2.01 4.02 4.01
8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.
f1 (ppm)


173.1 164.2 163.5 129.5 61.9 60.2 55.5 40.7 39.7 39.4 36.1 29.5 28.4 28.2 28.2 26.3 26.3 25.7
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10
f1 (ppm)

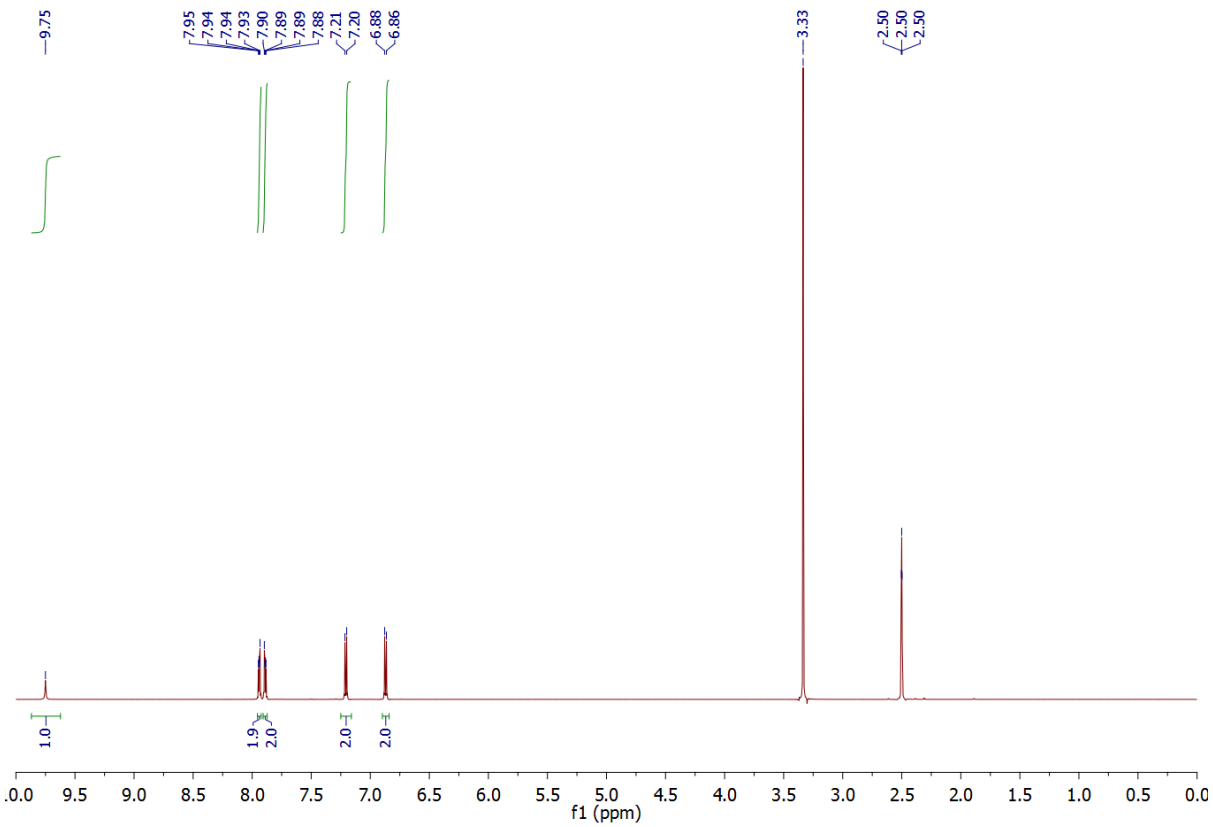
Synthesis of *p*-Anisidine Analogues

Synthesis of 2-(4-hydroxyphenyl)isoindoline-1,3-dione[4] (**30**)



Synthesised according to reference. *p*-Aminophenol (0.275 g, 2.52 mmol was added to phthalic anhydride (0.373 g, 2.52 mmol) dissolved in acetic acid (10 mL). The mixture was stirred and refluxed for 16 h. After cooling to room temperature, the precipitate was filtered and washed with methanol (10 mL). Removal of solvent by evaporation afforded a violet solid (0.391 g, 1.63 mmol, 65%).

^{1}H NMR (600 MHz, DMSO); δ 9.75 (s, 1H, OH), 7.95-7.93 (m, 2H, ArH), 7.90-7.88 (m, 2H, ArH), 7.21 (dt, *J* = 8.9, 2.8 Hz, 2H, ArH), 6.87 (dt, *J* = 8.9, 2.8 Hz, 2H, ArH); ^{13}C NMR (151 MHz, DMSO); δ 167.4 (CO), 157.3 (ArC), 134.6 (ArC), 131.6 (ArC), 128.8 (ArC), 123.3 (ArC), 122.9 (ArC), 115.4 (ArC).


9.75
7.95
7.94
7.94
7.93
7.90
7.89
7.89
7.88
7.21
7.20
6.88
6.86
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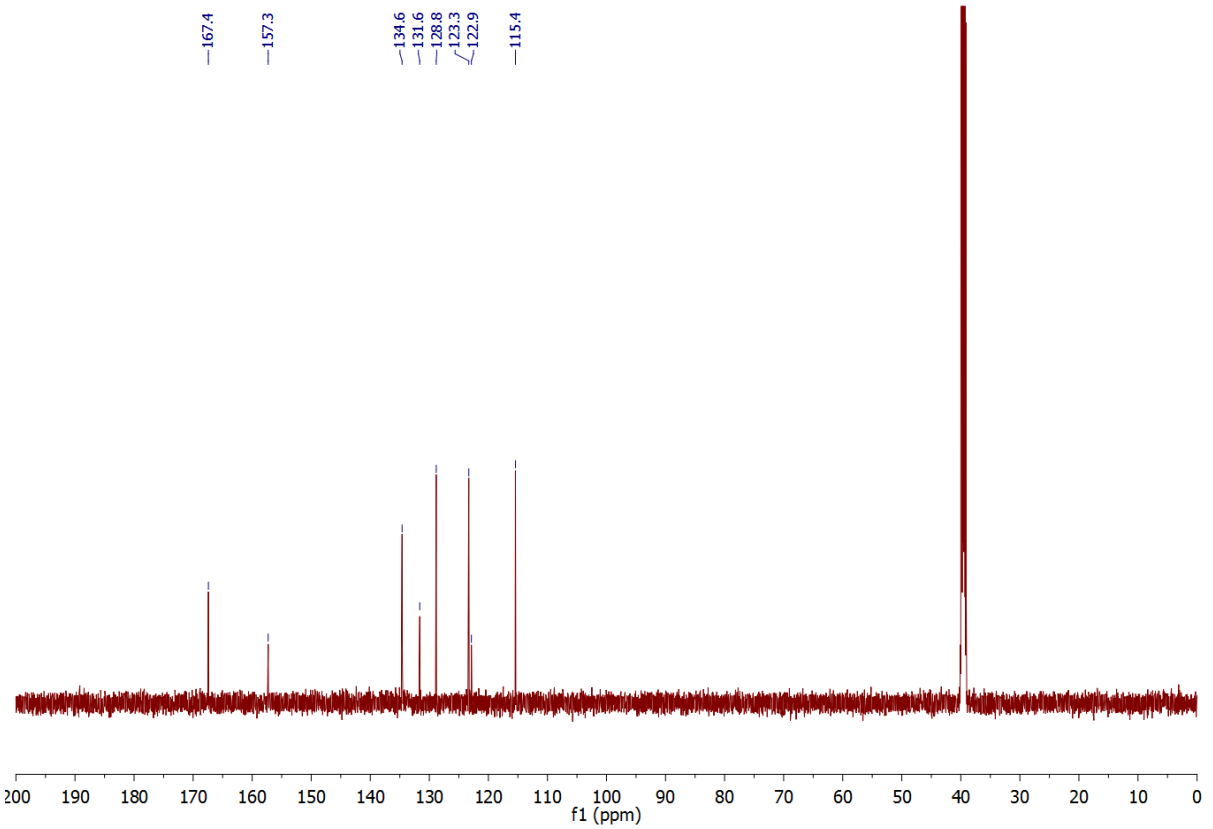
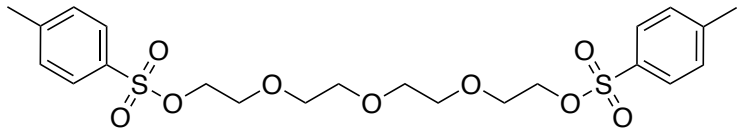
2.50
2.50
2.50
1.0
1.9
2.0
2.0
2.0
f1 (ppm)

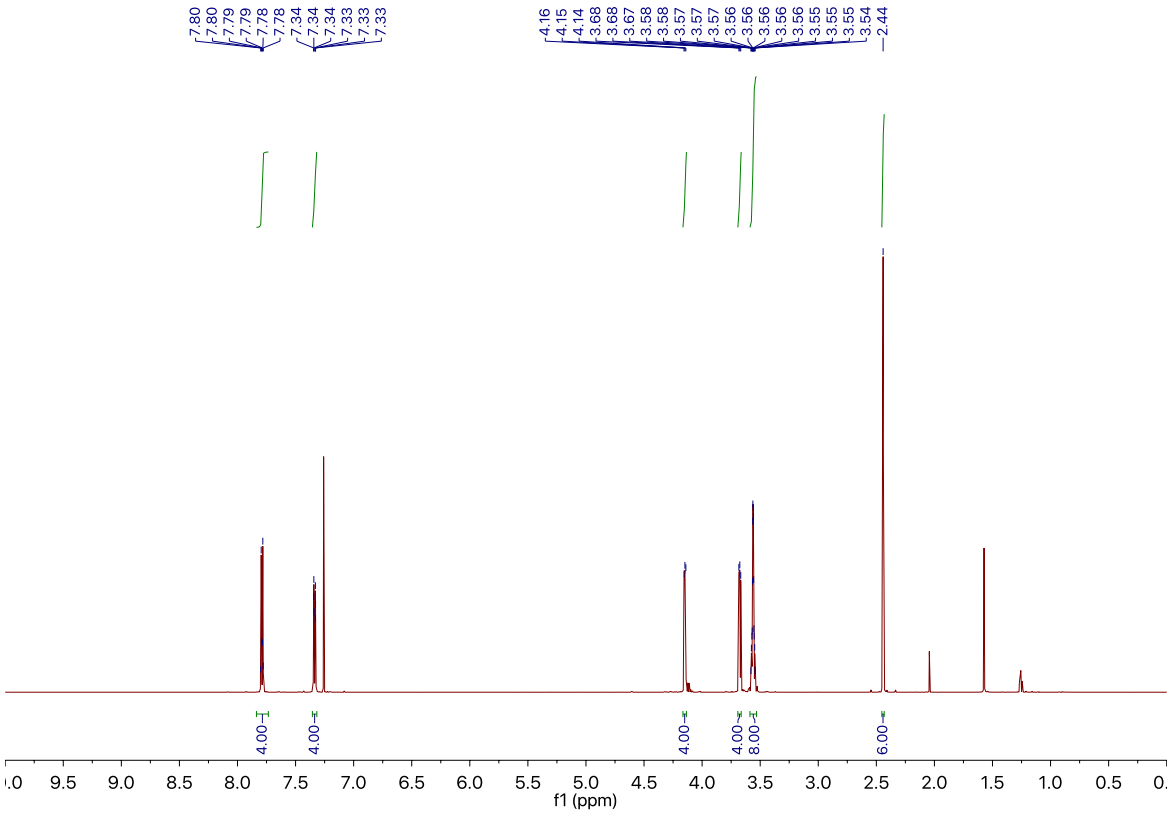

167.4
157.3
134.6
131.6
128.8
123.3
122.9
115.4
f1 (ppm)

Synthesis of ((oxybis(ethane-2,1-diyl))bis(oxy))bis(ethane-2,1-diyl) bis(4-methylbenzenesulfonate)[5] (**31**)



Tosyl chloride (2.33 g, 12.23 mmol) dissolved in DCM (5 mL) was added in two portions, 30 min apart, to a stirred solution of tetra ethylene glycol (1.08 g, 5.56 mmol) in pyridine (3.6 mL) at 0 °C. The mixture was stirred for 6 h at 0 °C. Ice water (30 mL) was added to the reaction mixture and the target molecule was extracted with EtOAc (3 x 30 mL). The organic layer was washed with HCl (2M, 2 x 13 mL). The organic layer was then dried with $MgSO_4$, filtered and the solvent removed on the rotary evaporator to afford a colourless oil (1.74 g, 3.46 mmol, 62%).

^{1}H NMR (600 MHz, $CDCl_3$); δ 7.79 (dt, *J* = 8.4, 2.0 Hz, 4H, ArH), 7.34-7.33 (m, 4H, ArH), 4.16-4.14 (m, 4H, CH_2OS), 3.68-3.67 (m, 4H, OCH_2), 3.58-3.54 (m, 8H, 4 x OCH_2), 2.44 (s, 6H, 2 x CH_3); ^{13}C NMR (151 MHz, $CDCl_3$); δ 145.0 (ArC), 133.0 (ArC), 130.0 (ArC), 128.1 (ArC), 70.9 (CH_2), 70.7 (CH_2), 69.4 (CH_2), 68.8 (CH_2), 21.8 (CH_3).


7.80
7.80
7.79
7.79
7.78
7.78
7.34
7.34
7.33
7.33
7.33
4.16
4.15
4.14
3.68
3.67
3.58
3.58
3.57
3.57
3.57
3.56
3.56
3.56
3.56
3.56
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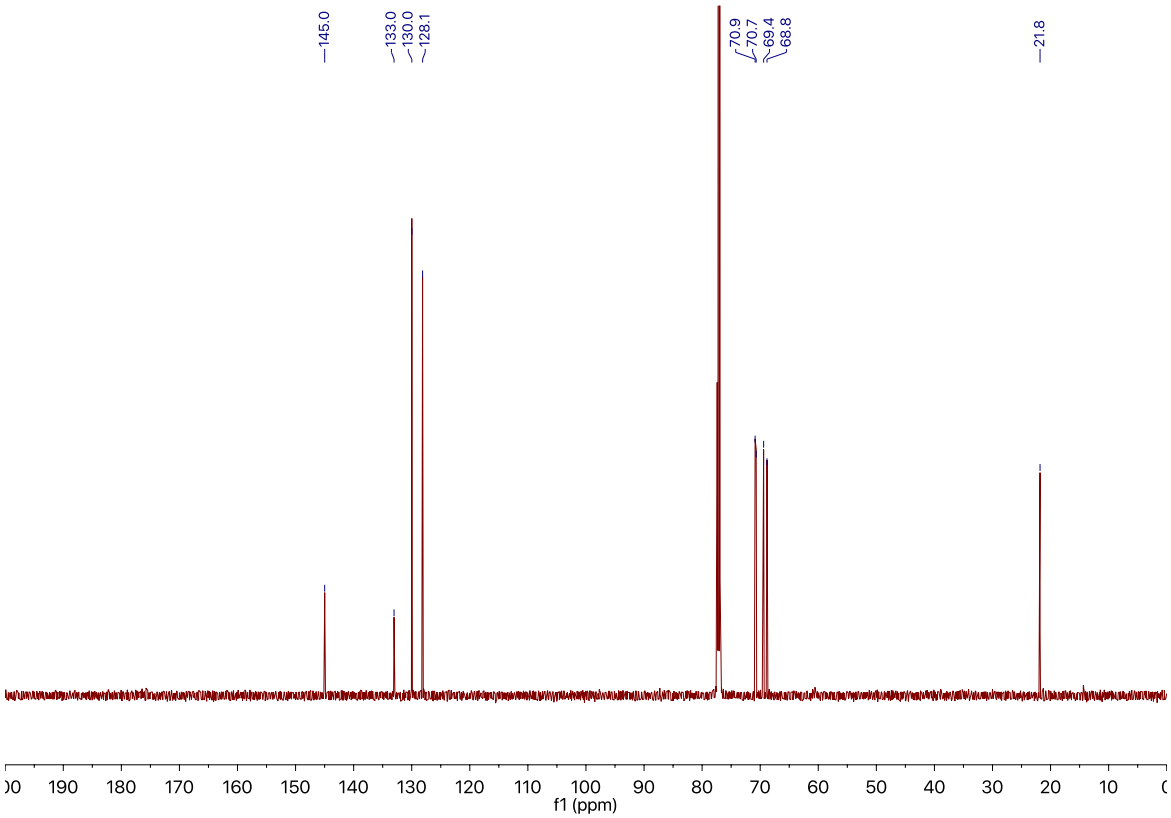
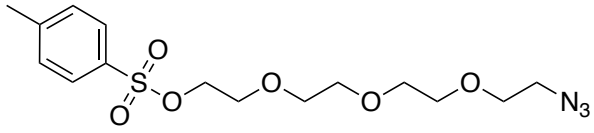
2.44
4.00
4.00
4.00
4.00
8.00
6.00
f1 (ppm)

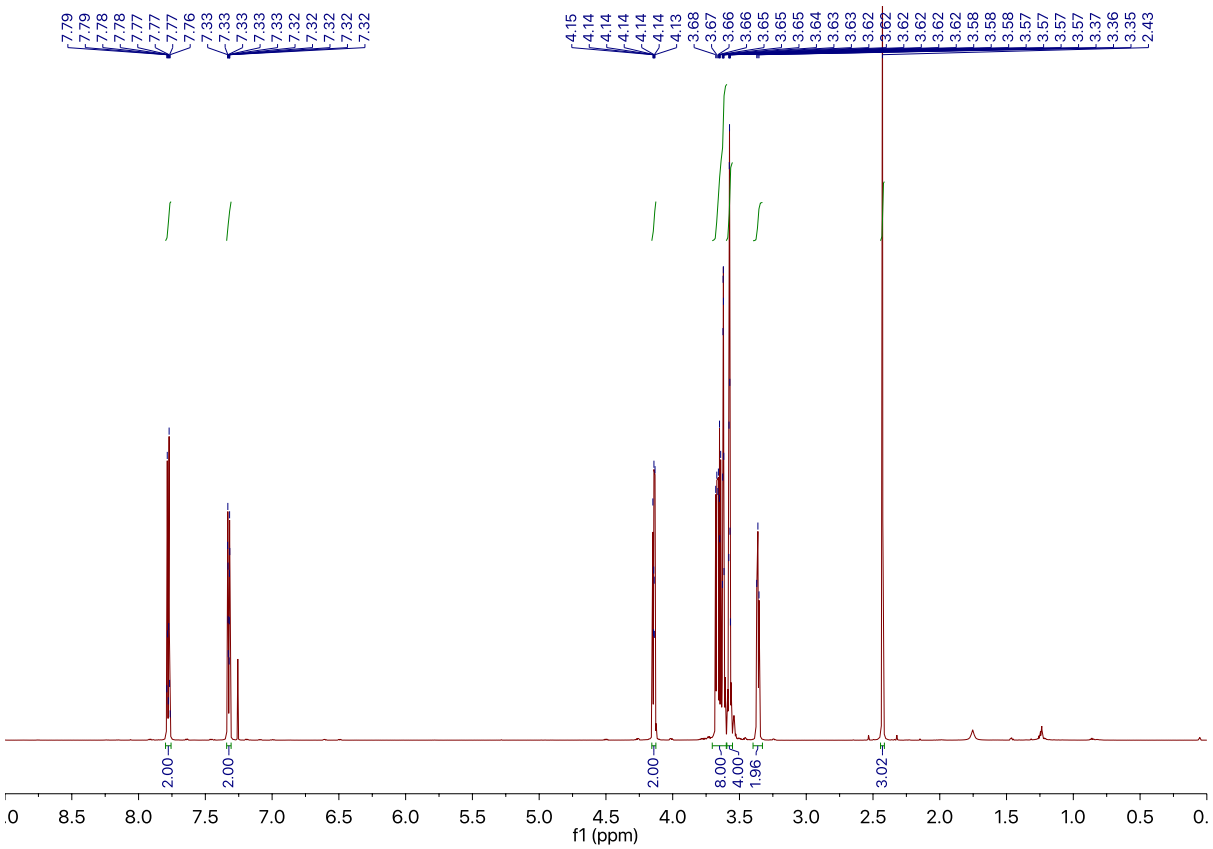

145.0
133.0
130.0
128.1
70.9
70.7
69.4
68.8
21.8
f1 (ppm)

Synthesis of 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl 4-methylbenzenesulfonate[6] (**32**)



Synthesised according to reference. To a solution of tetraethylene glycol di-*para*-toluene sulfonate (0.240 g, 0.480 mmol) in ethanol (5 mL) was added sodium azide (0.033 g, 0.510 mmol). The resulting solution was heated at 80 °C for 16 h. The reaction was then poured into ice water (15 mL) and the product was extracted with ethyl acetate (3 x 15 mL). The organic extracts were washed with water (15 mL), brine (15 mL) and dried with $MgSO_4$. The resulting mixture was filtered, the solvent removed on the rotary evaporator and the crude product purified by column chromatography (25-80% EtOAc/petroleum ether) to yield a light brown oil (0.063 g, 0.169 mmol, 35%).

^{1}H NMR (600 MHz, $CDCl_3$); δ 7.79-7.76 (m, 2H, ArH), 7.33-7.32 (m, 2H, ArH), 4.15-4.13 (m, 2H, $SOCH_2$), 3.68-3.62 (m, 8H, OCH_2), 3.58-3.57 (m, 4H, OCH_2), 3.36 (t, 2H, *J* = 5.3 Hz, CH_2N_3), 2.43 (s, 3H, CH_3); ^{13}C NMR (151 MHz, $CDCl_3$); δ 144.9 (ArC), 133.1 (ArC), 129.9 (ArC), 128.1 (ArC), 70.9 (CH_2), 70.8 (CH_2), 70.8 (CH_2), 70.7 (CH_2), 70.2 (CH_2), 69.4 (CH_2), 68.8 (CH_2), 50.8 (CH_2), 21.8 (CH_3); IR (thin film); 2100 (m), 2869 (w) cm^{-1}; LRMS (ES$^+$) 391.2 (100, $[M+NH_4]^+$); HRMS (ES$^+$) calcd for $C_{15}H_{24}N_3O_6S$ $[M+NH_4]^+$ 391.1651, observed 391.1606.


7.79
7.79
7.78
7.78
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7.77
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7.76
7.33
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7.32
7.32
7.32
4.15
4.14
4.14
4.14
4.14
4.13
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3.64
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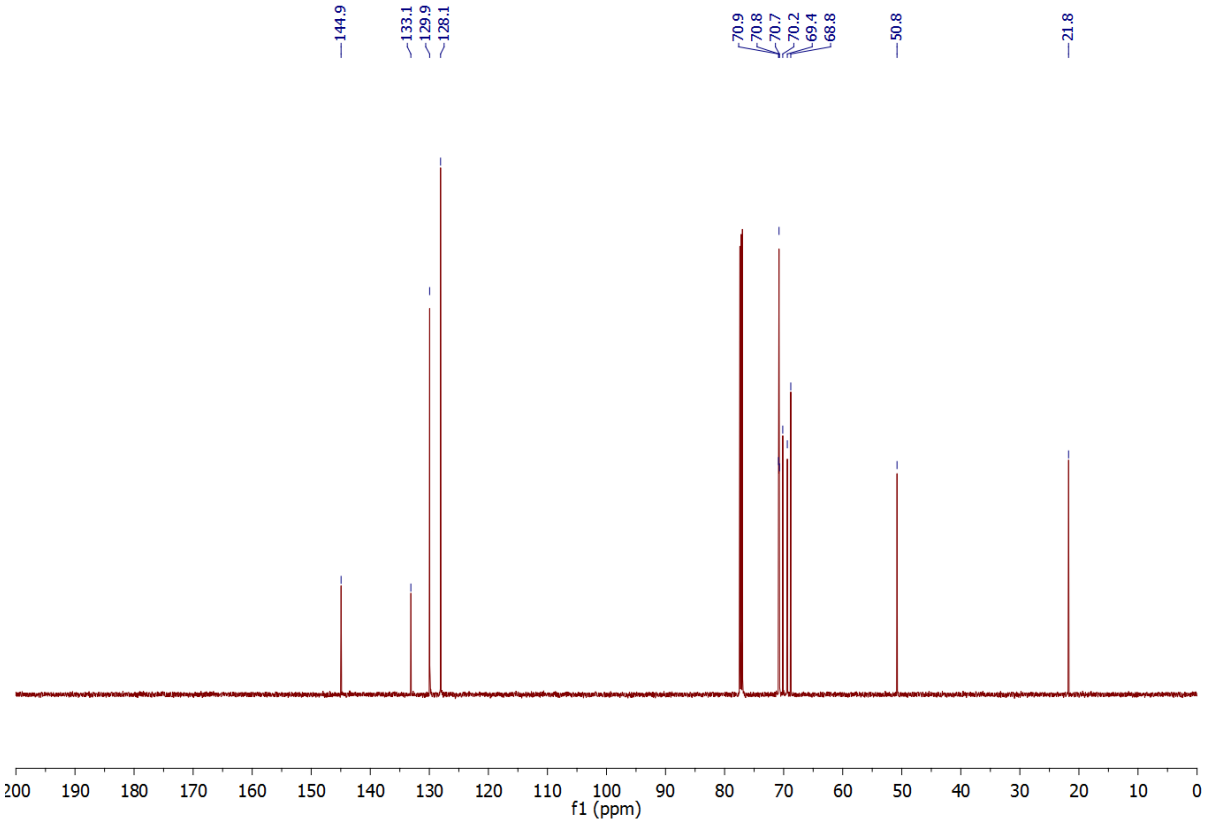
8.00
4.00
1.96
3.02
f1 (ppm)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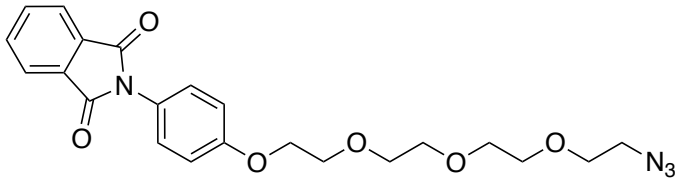
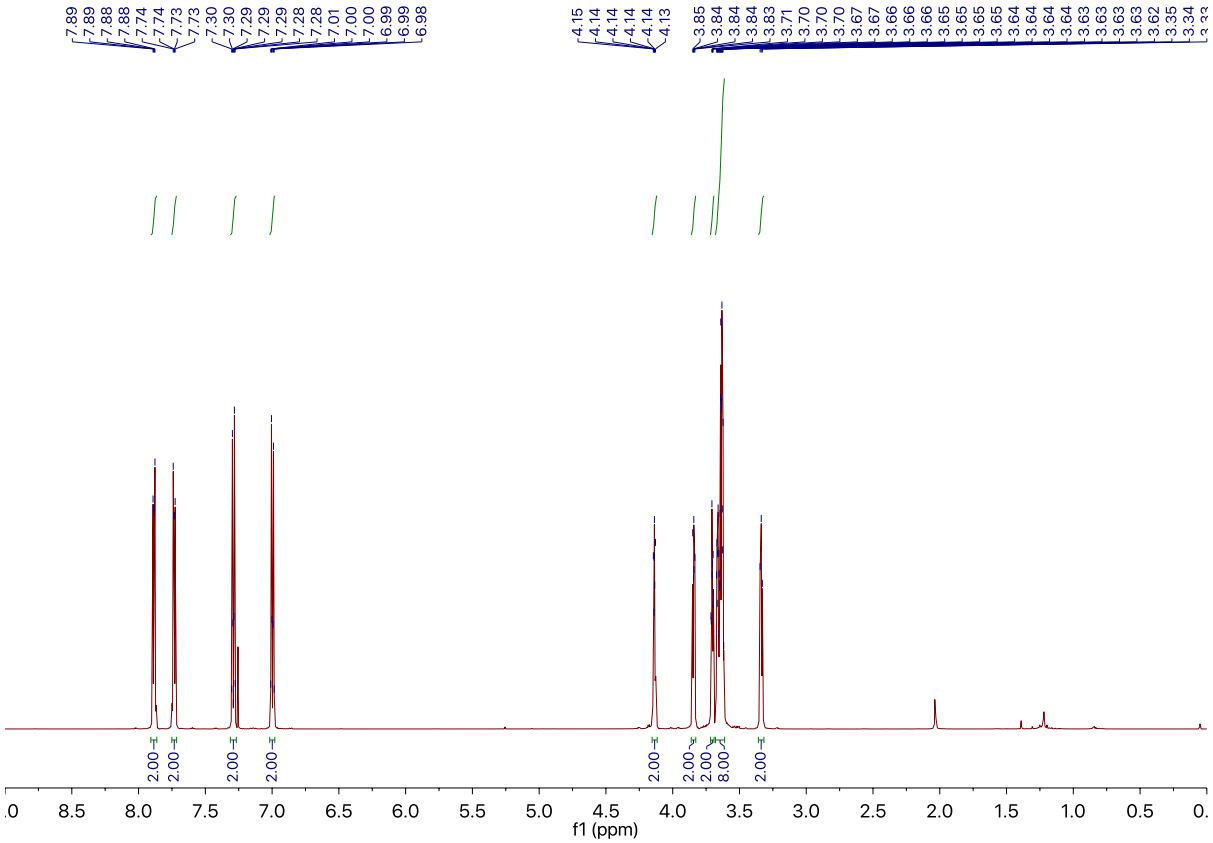
133.1
129.9
128.1
70.9
70.8
70.7
70.2
69.4
68.8
50.8
21.8
f1 (ppm)

Synthesis of 2-(4-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)phenyl)isoindoline-1,3-dione (**33**)



Phthalimide **30** (0.189 g, 0.791 mmol) was added to potassium *tert*-butoxide (725 µL, 1M in THF) in DMF (20 mL) and left to stir at room temperature for 5 min. Azide **32** (0.245 g, 0.659 mmol) was then added, and the reaction mixture was heated at 90 °C for 16 h. Water (100 mL) was added to the reaction, and the organic phase was extracted with EtOAc (3 x 30 mL). The organic extracts were washed with saturated lithium chloride solution (2 x 30 mL) and then dried with $MgSO_4$. The solvent was removed on the rotary evaporator and the crude product was purified by column chromatography (25-80% EtOAc/petroleum ether). The product was collected as a clear yellow oil (0.202 g, 0.459 mmol, 70%).

^{1}H NMR (600 MHz, $CDCl_3$); δ 7.89-7.88 (m, 2H, ArH), 7.74-7.73 (m, 2H, ArH), 7.30-7.28 (m, 2H, ArH), 7.01-6.98 (m, 2H, ArH), 4.15-4.13 (m, 2H, OCH_2), 3.85-3.83 (m, 2H, OCH_2), 3.71-3.70 (m, 2H, OCH_2), 3.67-3.62 (m, 8H, 4 x OCH_2), 3.35-3.33 (m, 2H, CH_2N_3); ^{13}C NMR (151 MHz, $CDCl_3$); δ 167.6 (CO), 158.6 (ArC), 134.5 (ArC), 131.9 (ArC), 128.0 (ArC), 124.6 (ArC), 123.8 (ArC), 115.2 (ArC), 71.0 (CH_2), 70.8 (CH_2), 70.8 (CH_2), 70.8 (CH_2), 70.1 (CH_2), 69.7 (CH_2), 67.9 (CH_2), 50.8 (CH_2); IR (thin film); 1704 (s), 2112 (m), 2864 (w) cm^{-1}; LRMS (ES^+) 441.2 (100, $[M+H]^+$); HRMS (ES^+) calcd for $C_{22}H_{25}N_4O_6$ $[M+H]^+$ 441.1747, observed 441.1762.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7.89
7.88
7.88
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7.74
7.73
7.73
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7.28
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7.01
7.00
7.00
6.99
6.98
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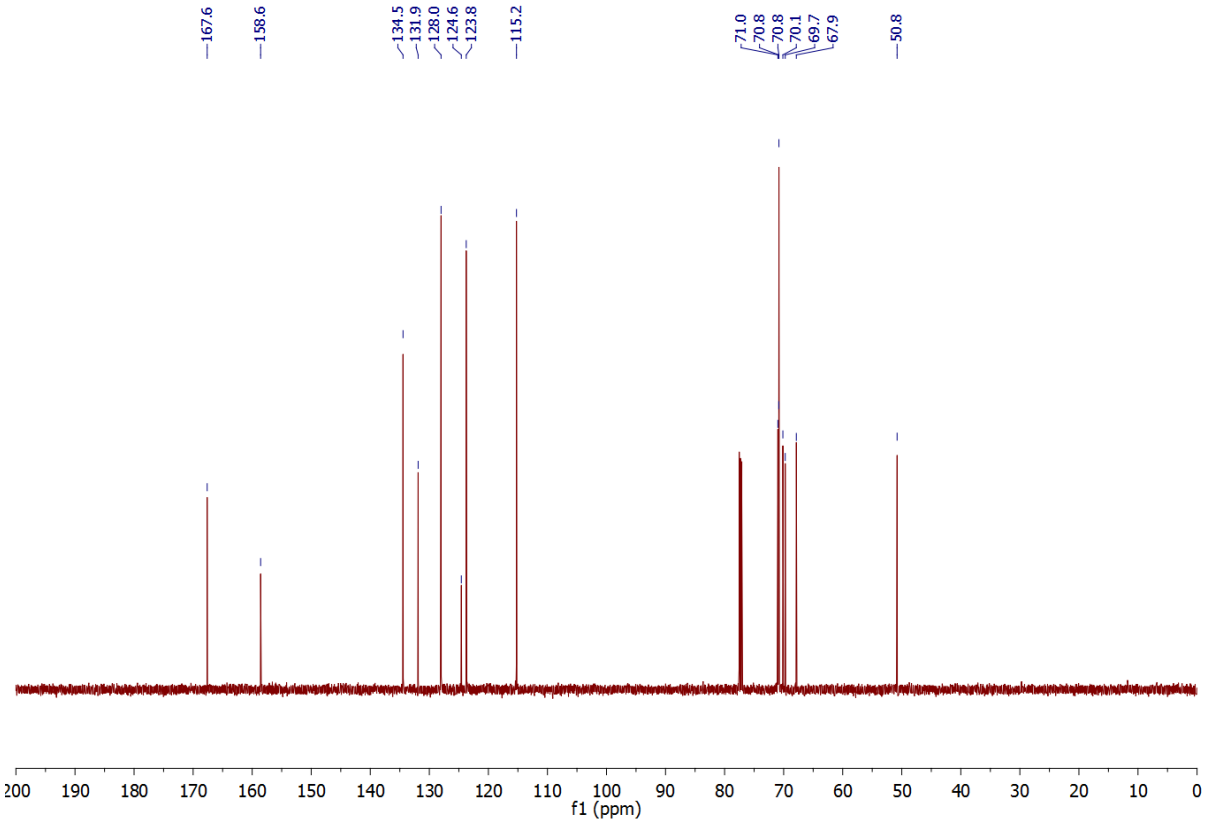
2.00
8.00
2.00
f1 (ppm)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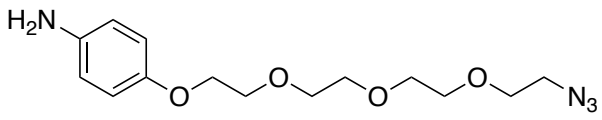
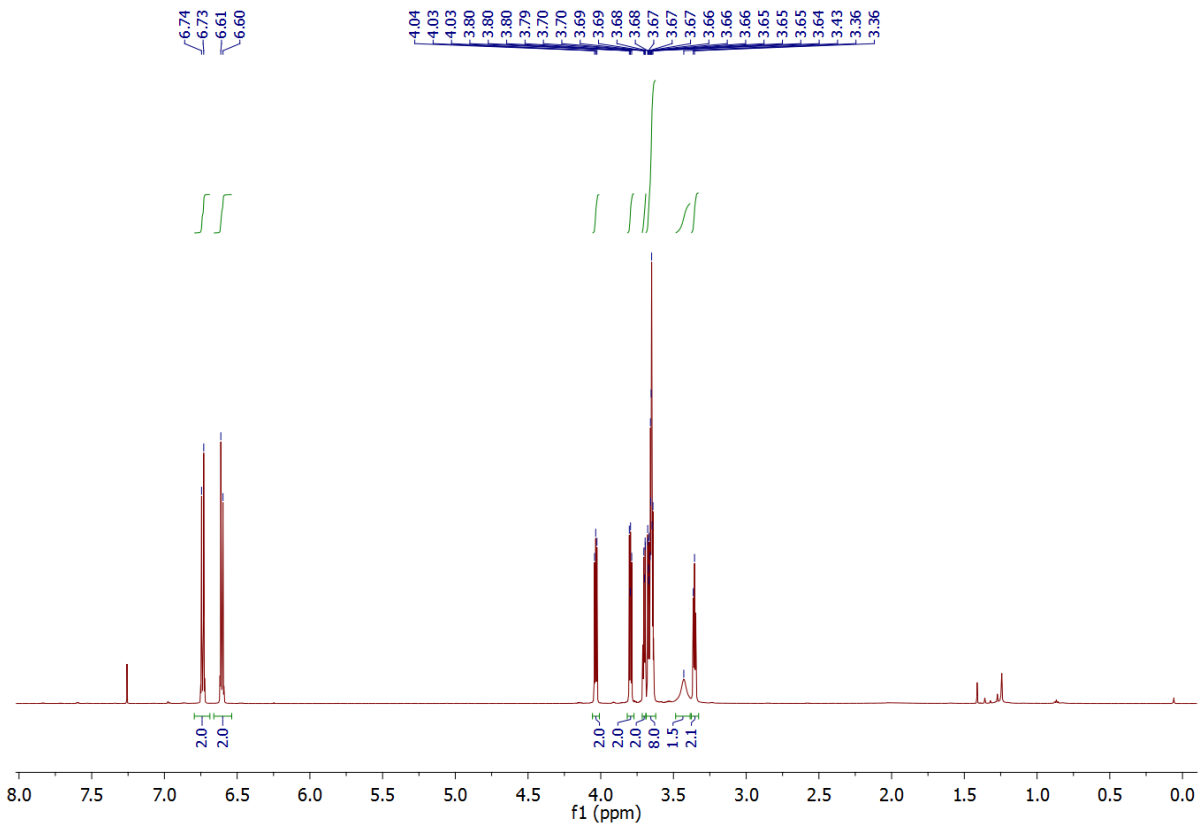
158.6
134.5
131.9
128.0
124.6
123.8
115.2
71.0
70.8
70.8
70.1
69.7
67.9
50.8
f1 (ppm)

Synthesis of 4-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)aniline[7] (**22**)



To phthalimide **33** (0.141 g, 0.320 mmol) in THF (10 mL) was added hydrazine monohydrate (330 µL, 5.74 mmol, 60% solution). The reaction was stirred at room temperature for 2 h before water (30 mL) was added. The crude product was extracted with EtOAc (3 x 30 mL), washed with brine (20 mL), dried with $MgSO_4$, filtered and the solvent removed on the rotary evaporator. The crude product was purified by column chromatography (20-100% EtOAc/petroleum ether) to yield the product as a brown oil (0.073 g, 0.233 mmol, 73%).

^{1}H NMR (600 MHz, $CDCl_3$); δ 6.74-6.73 (m, 2H, ArH), 6.61-6.60 (m, 2H, ArH), 4.04-4.03 (m, 2H, OCH_2), 3.80-3.79 (m, 2H, OCH_2), 3.70-3.69 (m, 2H, OCH_2), 3.68-3.64 (m, 8H, 4 x OCH_2), 3.43 (br s, 2H, NH_2), 3.36 (t, *J* = 5.1 Hz, 2H, CH_2N_3); ^{13}C NMR (151 MHz, $CDCl_3$); δ 152.0 (ArC), 140.4 (ArC), 116.4 (ArC), 116.0 (ArC), 70.9 (CH_2), 70.8 (CH_2), 70.8 (CH_2), 70.8 (CH_2), 70.1 (CH_2), 70.0 (CH_2), 68.3 (CH_2), 50.8 (CH_2); IR (thin film); 2098 (m), 2869 (m), 3356 (w), 3430 (w) cm^{-1}; LRMS (ES$^+$) 311.2 (100, [M+H]$^+$); HRMS (ES$^+$) calcd for $C_{15}H_{19}N_8$ [M+H]$^+$ 311.1733, observed 311.1719.


6.74
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6.60
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4.03
4.03
3.80
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3.80
3.79
3.70
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3.69
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3.65
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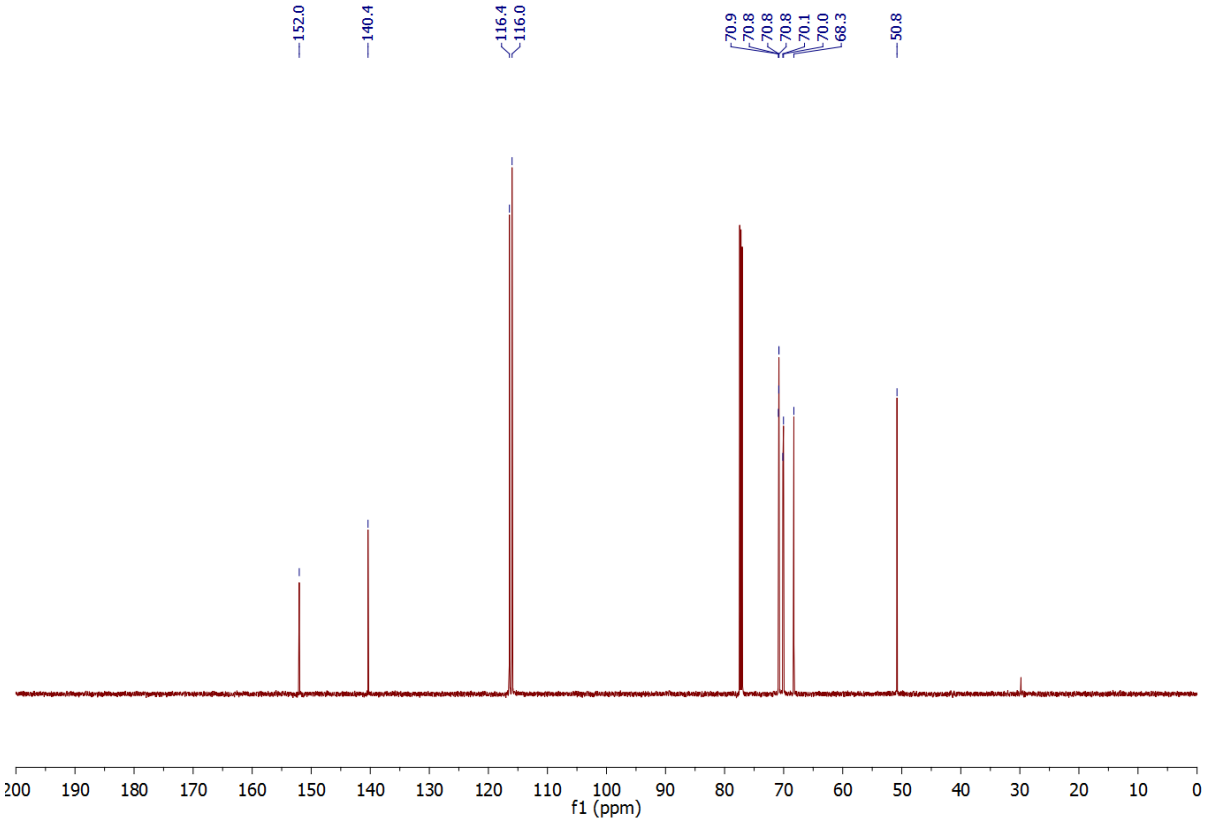
3.64
3.43
3.36
3.36
2.0
2.0
2.0
2.0
2.0
8.0
1.5
2.1
8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)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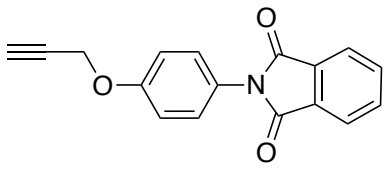
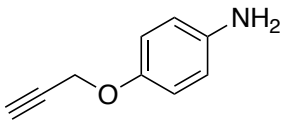
140.4
116.4
116.0
70.9
70.8
70.8
70.8
70.1
70.0
68.3
50.8
200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

Synthesis of 2-(4-(prop-2-yn-1-yloxy)phenyl)isoindoline-1,3-dione[8] (**34**)



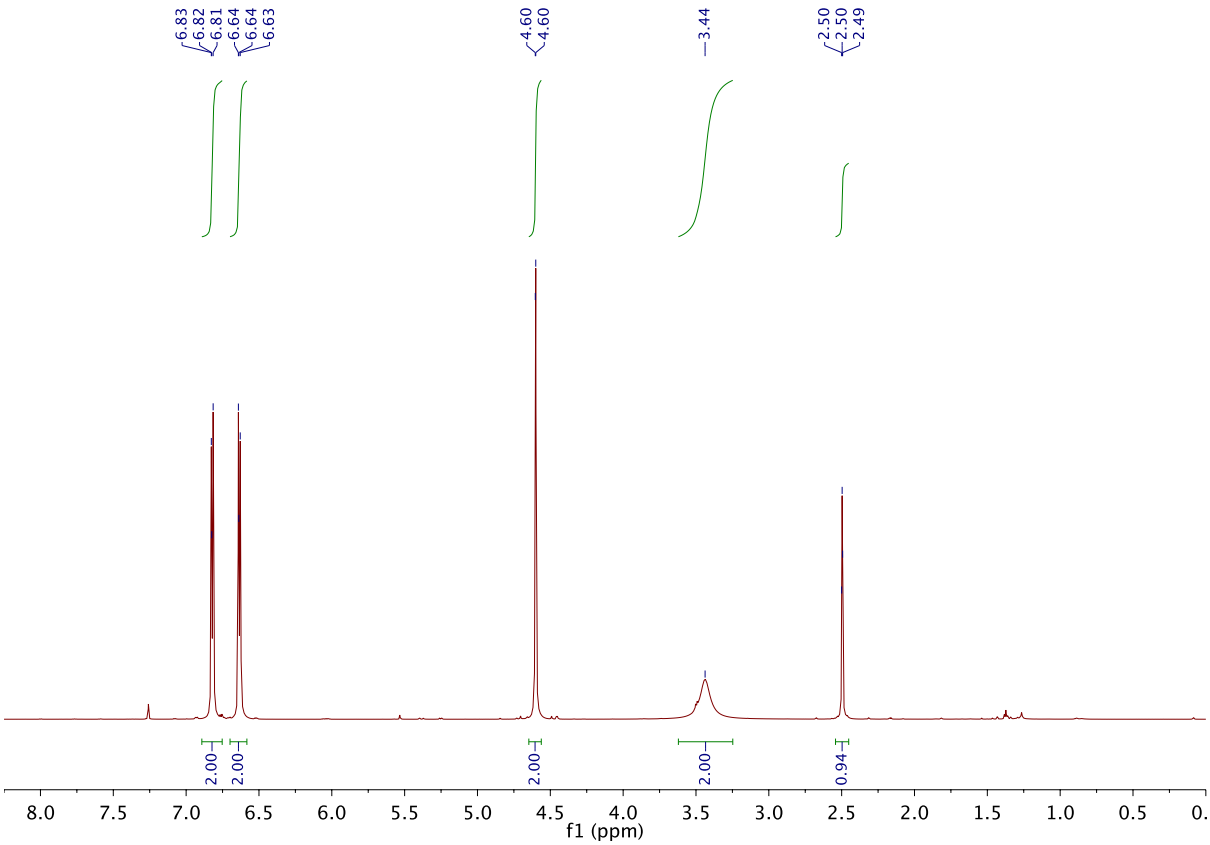
To phthalimide **30** (0.300 g, 1.254 mmol, 1.2 eq.) dissolved in 20 mL of DMF was added potassium tert-butoxide (1.15 mL, 1.149 mmol, 1.1 eq.). The mixture was stirred for 10 min before propargyl bromide (0.215 g, 1.045 mmol, 80% in toluene) was added to the reaction mixture and the resultant solution was refluxed and monitored by TLC. After 3 h, the reaction was quenched with water (100 mL). The organic phase was extracted with EtOAc (3 x 25 mL). Removal of solvent by evaporation afforded the crude product as a brown solid (0.386 g) which was used in the next step without further purification.

Synthesis of 4-(prop-2-yn-1-yloxy)aniline[9] (**18**)



To the crude **34** (0.386 g) in THF (10 mL) was added hydrazine monohydrate (0.67 mL, 13.94 mmol, >10 eq.). The reaction mixture was stirred at rt and monitored by TLC. The reaction was quenched with water (30 mL) after 2 h. The organic phase was extracted with EtOAc (3 x 60 mL) and washed with brine (60 mL). The organic phase was dried over ($MgSO_4$), filtered and concentrated *in vacuo*. The crude was purified by column chromatography (20-50% EtOAc/petroleum ether) to afford the product as a dark oil (114.3 mg, 0.777 mmol, 74% over 2 steps).

^{1}H NMR (700 MHz, $CDCl_3$); δ 6.82 (d, *J* = 8.8 Hz, 2H, ArH), 6.64 (d, *J* = 8.8 Hz, 2H, ArH), 4.60 (d, *J* = 2.4, 2H, CH_2), 3.44 (s, 2H, NH_2), 2.49 (t, *J* = 2.4, 1H, HCC); ^{13}C NMR (151MHz, $CDCl_3$); δ 150.8 (ArC), 141.1 (ArC), 116.6 (ArC), 116.4 (ArC), 79.3 (C), 75.3 (CH), 56.9 (CH_2); IR (thin film); 1203 (m); 1504 (m), 3280 (w), 3427-3353 (w) cm^{-1}; LRMS (ESI) 148.1 (100%, $[M+H]^+$); HRMS (ES^+) calcd for C_9H_9NO $[M+H]^+$ 148.0684, observed 148.0757.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6.63
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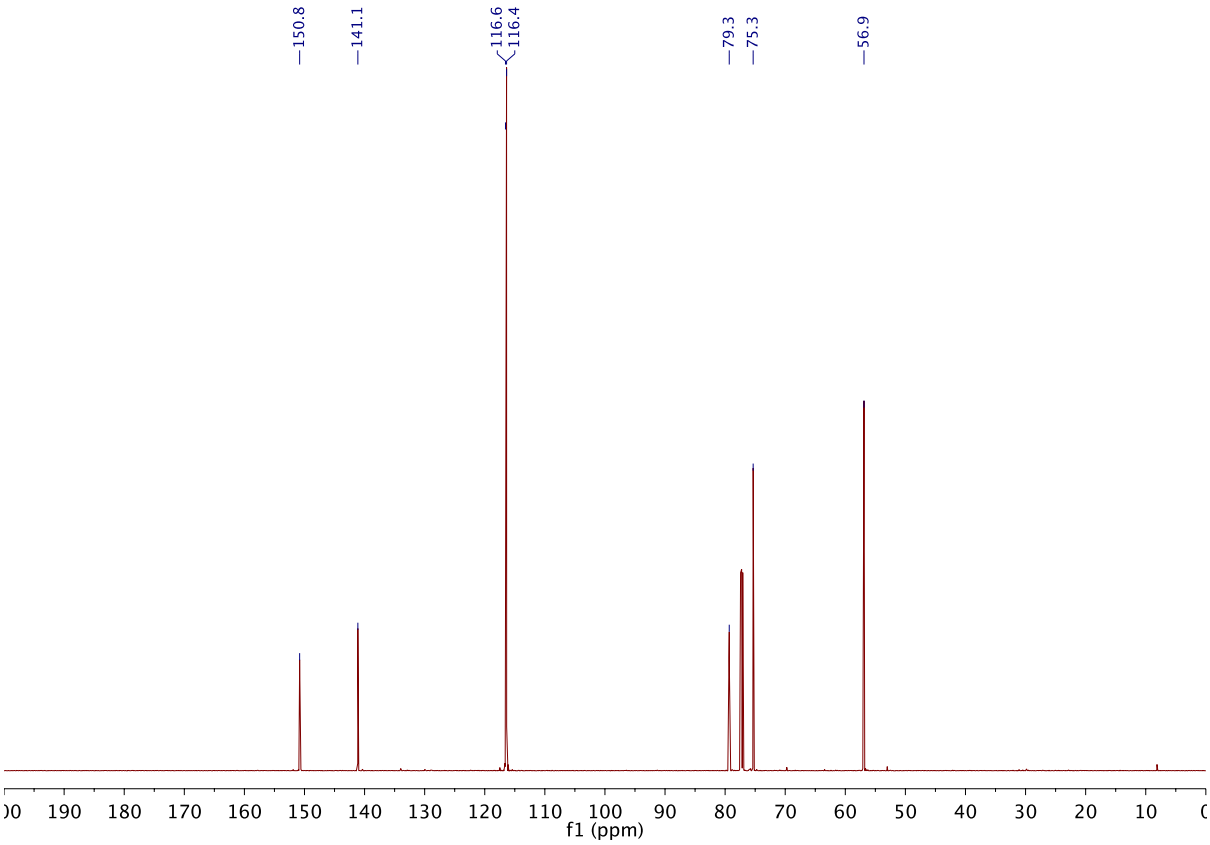
2.00
2.00
2.00
0.94
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
f1 (ppm)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75.3
56.9
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180
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160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

NMR Reactions

NMR reactions comparing amine reactivity with bromothiomaleimide **6**:

Methyl *S*-(4-bromo-1-methyl-2,5-dioxo-2,5-dihydro-1*H*-pyrrol-3-yl)-*N*-(*tert*-butoxycarbonyl)-*L*-cysteinate (0.002 g, 0.0047 mmol) was dissolved in CD_3CN (0.280 mL) in an NMR tube. Amine dissolved in phosphate buffer with 1% CD_3CN (2 eq., 0.700 mL, 13.5 mM amine, 100 mM phosphates, pH 7.4) was added to the organic solution (the resulting pH was then measured to be pH 7.8) and data acquisition carried out over the course of 24 h. Air inside a pasteur pipette was saturated with TMS vapour. The vapour was then bubbled through the NMR tube before the reaction was vortexed to ensure homogeneity of the reaction mixture. The reaction was left at room temperature and monitored by NMR. The ratio of the integrals of the starting material and product peaks could be calculated and then the progress of the reaction monitored until completion.

Table S3: Summary of NMR timecourse study.

R	Amine	Equiv.	Reaction Time (for 99%)	Efficiency
Me	Pyrrolidine	2	>900 min	Side products formed
Me	Aniline	2	786 min	Quantitative conversion
Me	*p*-Anisidine	2	185 min	Quantitative conversion
Me	*N*-Me *p*-anisidine	2	186 min	Quantitative conversion

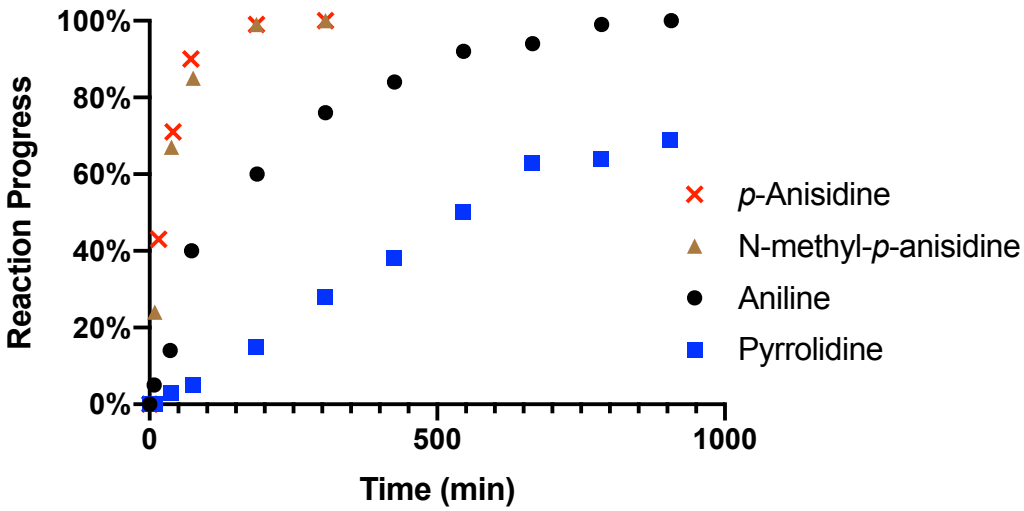


Figure S1: Timecourse NMR study carried out in phosphate buffer solution at pH 7.8. Peak integration used to measure reaction progress.

NMR reactions comparing *p*-anisidine with analogues **18** and **22** in reaction with bromothiomaleimide **6**.

Amine (2 eq.) was dissolved in CD_3CN (250 µL) and phosphate buffer (700 µL, 100 mM, pH 6.75) was added to the methyl 3-((4-bromo-1-methyl-2,5-dioxo-2,5-dihydro-1H-pyrrol-3-yl)thio)-2-((tert-butoxycarbonyl)amino)propanoate (0.0025 g, 0.0059 mmol, 1 eq.) dissolved in CD_3CN (250 µL) in a NMR tube. The pH of the final solution was measured at 7.4. The reaction was monitored by NMR. The ratio of the integrals of the starting material and product peaks could be calculated and then the progress of the reaction monitored until completion.

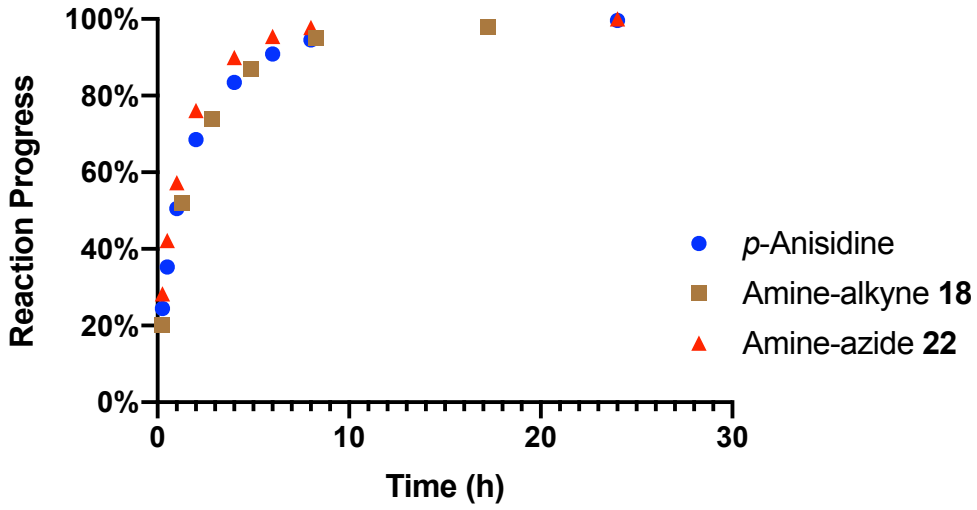


Figure S2: Timecourse NMR study carried out in phosphate buffer solution at pH 7.4. Peak integration used to measure reaction progress.

Protein and Peptide Mass Spectra

Protein samples:

HSA samples were buffer swapped into H_2O before being diluted to 4 µM for MS analysis. For MS analysis, capillary liquid chromatography mass spectrometry (CapLC-MS) was performed on an Agilent 1100/1200 LC system connected to a 6510A QTOF mass spectrometer (Agilent, UK). 10 µL of sample (4 µm) was injected onto an Agilent PLRP-S (150 mm x 2.1 mm, 1000 Å, 8 µm) column. Two LC-MS methods were used with the same LC column and mobile phases A (H_2O with 0.1% formic acid) and B (MeCN, 0.1% formic acid).

Method A:

The LC column was heated to 60 °C. The gradient elution was as follows: 25% B (isocratic for 1 min) increase to 99% B in 16 min and stay at 99% B for 2 min following a sharp decrease to 25% B in 0.1 min and isocratic for 1.9 min at 25% B. The flow rate was 0.25 mL/min. Agilent 6510 QTOF mass spectrometer was operated in a positive polarity mode, coupled with an ESI ion source. The ion source parameters were set up with a VCap of 4000V, a gas temperature at 325 °C, a dry gas flow rate at 5 L/min and a nebulizer of 20 psig. MS Tof was acquired under conditions of a fragmentor at 175 V, a skimmer at 65 V, QuadAMU at 140, an octopole RF peak at 750 and an acquisition rate of 1 spectra/s in profile mode, within a scan range between 100 and 3100 m/z. The .d data was then analysed by deconvoluting the spectrum to a zero charge mass spectra using a maximum entropy deconvolution algorithm within the MassHunter software version B.07.00.

Method B:

The LC column was heated to 60 °C. The gradient elution was as follows: 25% B for 1 min followed by increase to 99% B over 16 min. After 2 min, 99% B was decreased to 25% over 0.1 min and maintained at 25% B for 1.9 min. The flow rate was 0.3 mL/min. Agilent 6510 QTOF mass spectrometer was operated in a positive polarity mode, coupled with an ESI ion source. The ion source parameters were set up with a VCap of 4000V, a gas temperature at 325 °C, a dry gas flow rate at 5 L/min and a nebulizer of 20 psig. MS Tof was acquired under conditions of a fragmentor at 175 V,

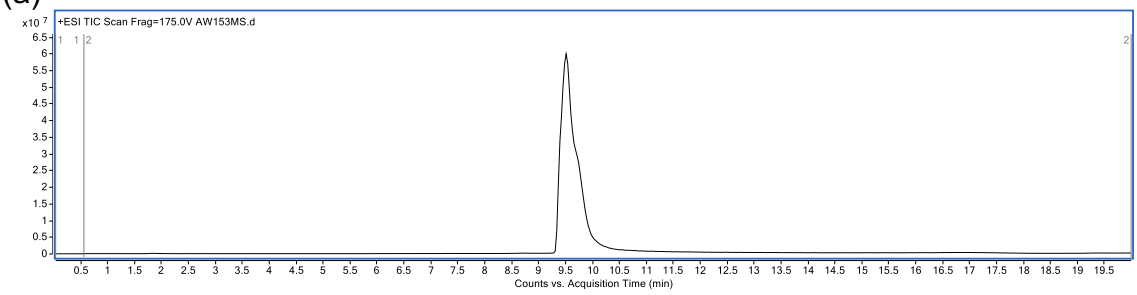
a skimmer at 65 V, QuadAMU at 140, an octopole RF peak at 750 and an acquisition rate of 1 spectra/s in profile mode, within a scan range between 100 and 3100 m/z. The .d data was then analysed by deconvoluting the spectrum to a zero charge mass spectra using a maximum entropy deconvolution algorithm within the MassHunter software version B.07.00.

Peptide Samples:

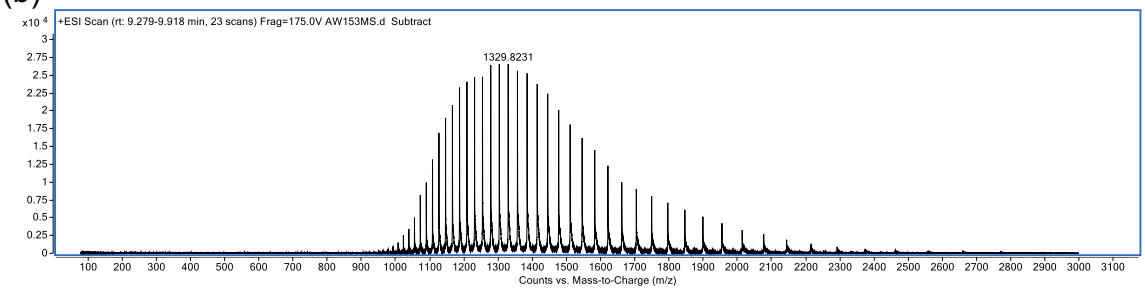
LCMS was performed on samples using a Waters Acquity uPLC connected to Waters Acquity Single Quad Detector (SQD). All samples were run with the following parameters. Column: Hypersil Gold C4, 1.9 µm, 2.1 µm × 50 µm. Wavelength: 254 nm. Mobile Phase: 95:5 Water (0.1% Formic Acid): MeCN (0.1% Formic Acid) Gradient over 4 min (to 5:95 Water (0.1% Formic Acid): MeCN (0.1% Formic Acid). Flow Rate: 0.6 mL/min. MS Mode: ES+. Scan Range: m/z = 250 – 2000. Scan time: 0.25 s. Data obtained in continuum mode. The electrospray source of the MS was operated with a capillary voltage of 3.5 kV and a cone voltage of 50 V. Nitrogen was used as the nebulizer and desolvation gas at a total flow of 600 L/h. Ion series were generated by integration of the total ion chromatogram (TIC) over the appropriate range. Total mass spectra for protein samples were reconstructed from the ion series using the MaxEnt 1 algorithm pre-installed on MassLynx software.

Native WT HSA

(a)



(b)



(c)

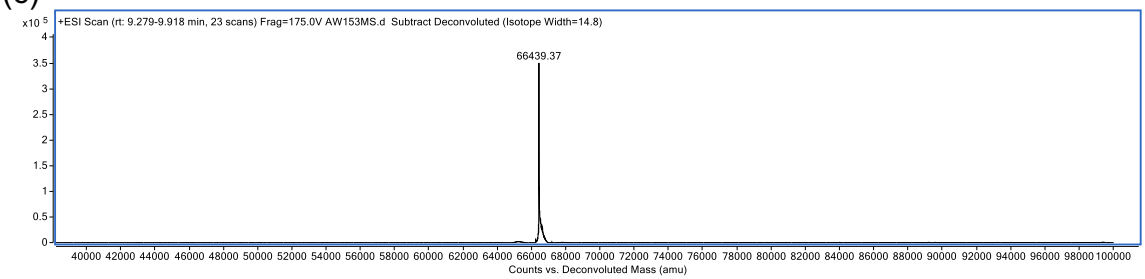
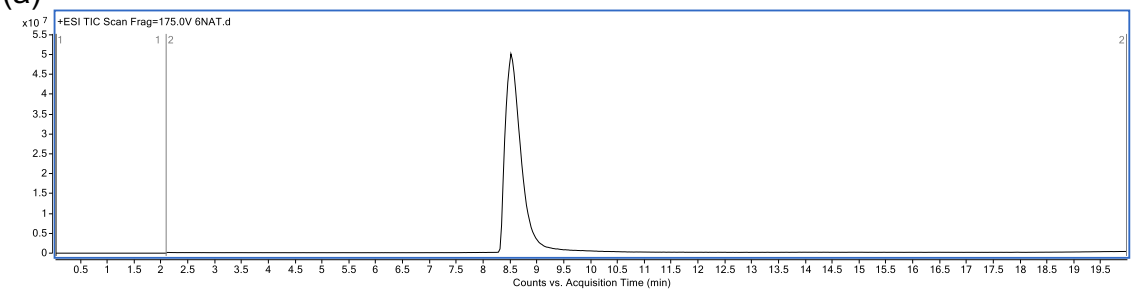


Figure S3: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for native WT HSA. Expected mass: 66439 Da. Observed mass: 66439 Da. Data obtained with MS method A.

Native mHSA

(a)



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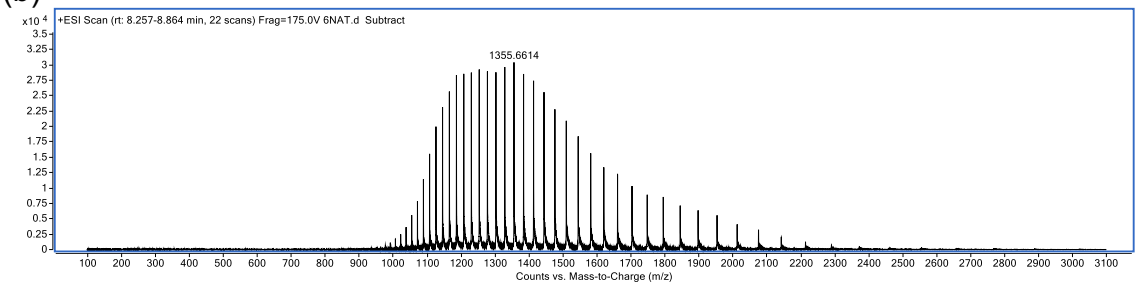

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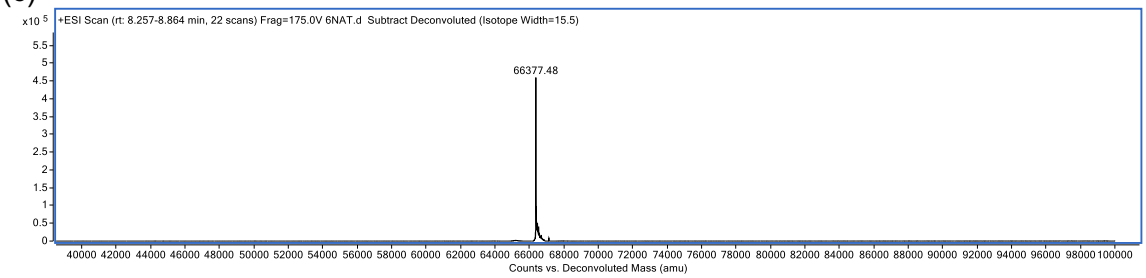


Figure S4: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for mHSA. Expected mass: 66381 Da. Observed mass: 66377 Da. Data obtained with MS method B.

Expression and Reduction of GFP: Cysteine Mutant Green Fluorescent Protein (GFPS147C)

GFPS147C was expressed in competent E. coli BL21(DE3) cells as described previously.[10] Due to the presence of a small amount of dimer species, GFPS147C was then reduced prior to conjugation and characterised as below:

TCEP (31.3 µL, 20 mM in deionised water, 25 equiv.) was added to a solution of GFPS147C (500 µL, 50 µM) in PBS (pH 7.4, 5 mM EDTA) and the solution was incubated at 37 °C for 90 min. Excess reagents were removed *via* ultrafiltration (10 kDa MWCO, Amicon® Ultra-4 Centrifugal Filter Units) into PBS (pH 7.4, 5 mM EDTA) for further experiments. Samples were desalted (7 kDa MWCO, ZebaSpin®) prior to LCMS analysis. Concentration was determined photometrically using ε_{280} = 20,500 M^{-1} cm^{-1}.

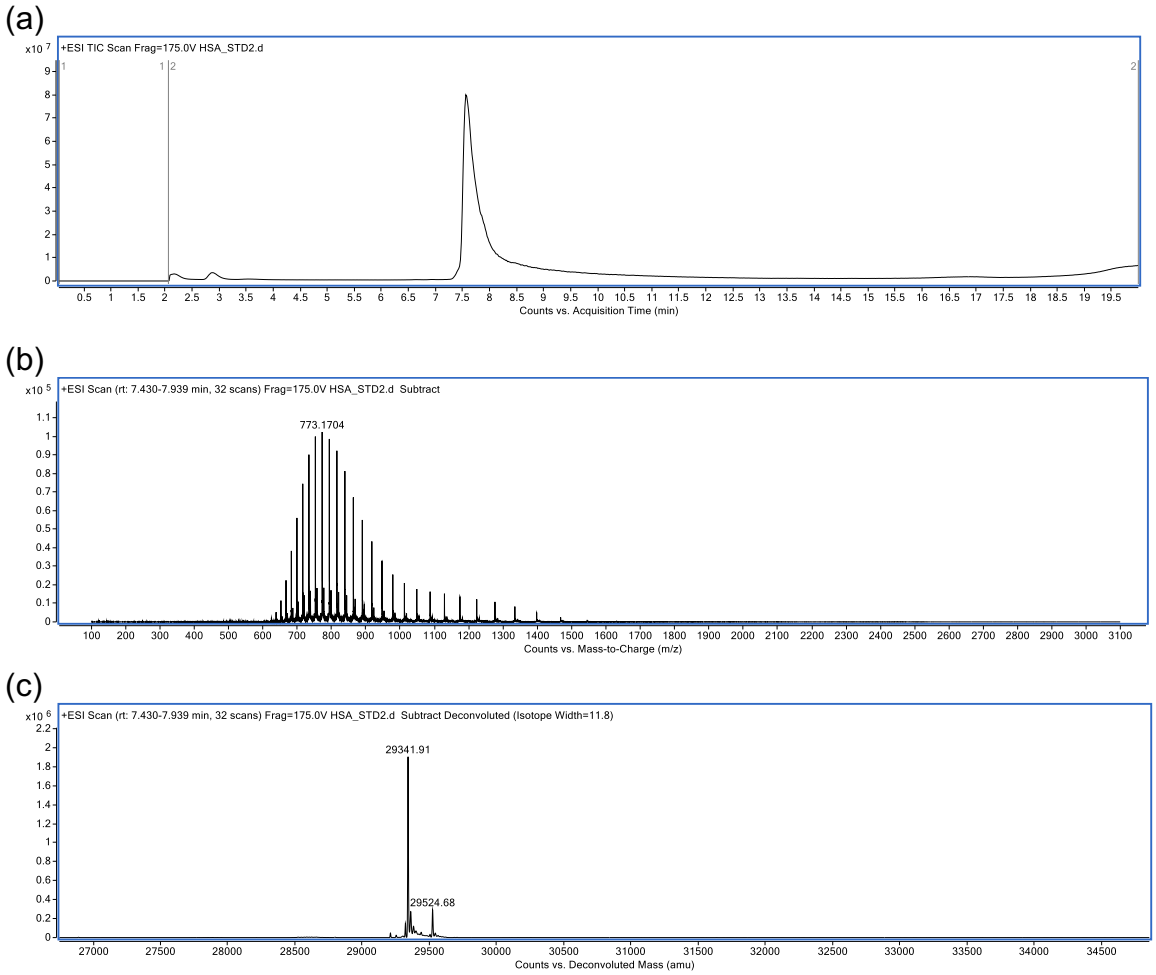
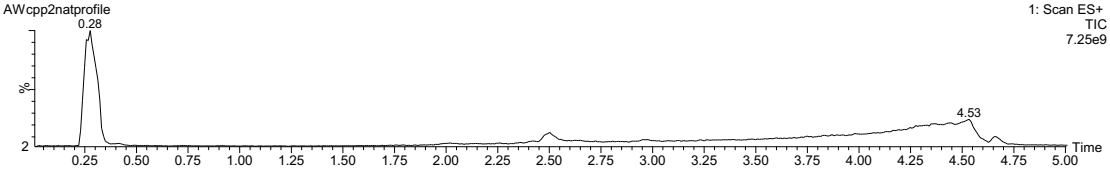


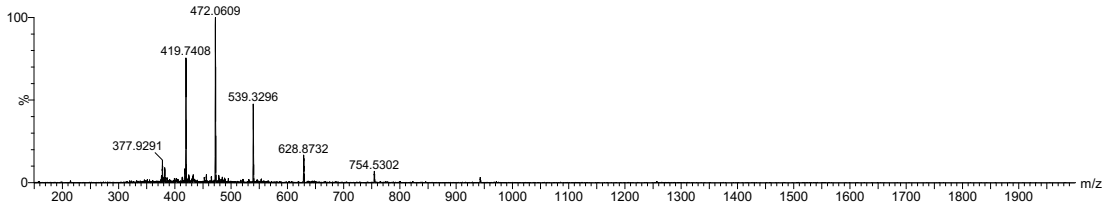
Figure S5: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for reduced GFP. Observed mass: 29342 Da and 29525 Da. Data obtained with MS method B.

Native CPP

(a)



(b)



(c)

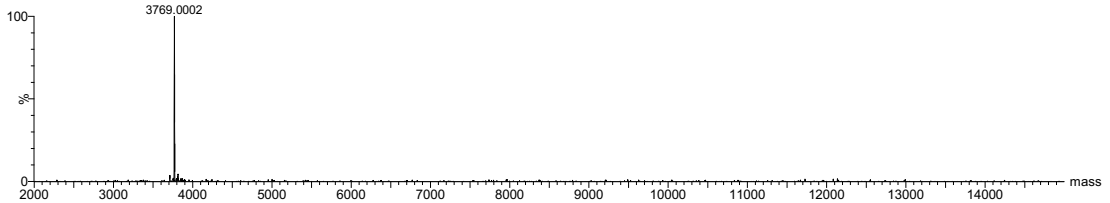
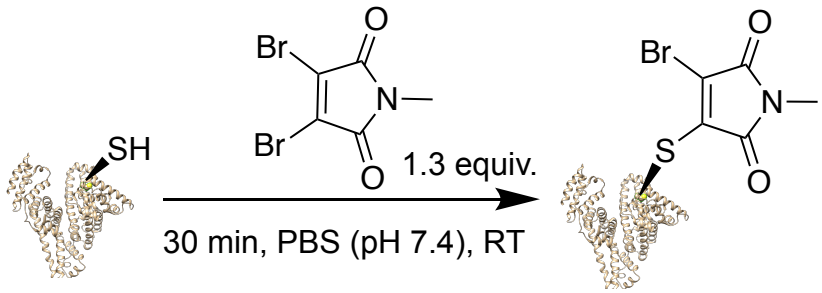


Figure S6: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for native CPP in H_2O. Expected mass: 3765 Da. Observed mass: 3769 Da. Data obtained with peptide LCMS method.

WT HSA, *N*-Me DBM (**14**)



N-Me DBM **13** (1.3 µL, 10 mM, 1.3 equiv. in DMSO) was added to HSA (100 µM, 100 µL) in PBS buffer (12 mM phosphates, pH 7.4) at RT. After 30 minutes, excess small molecule was removed (7 kDa MWCO, ZebaSpin®). Conjugates were then analysed by LCMS.

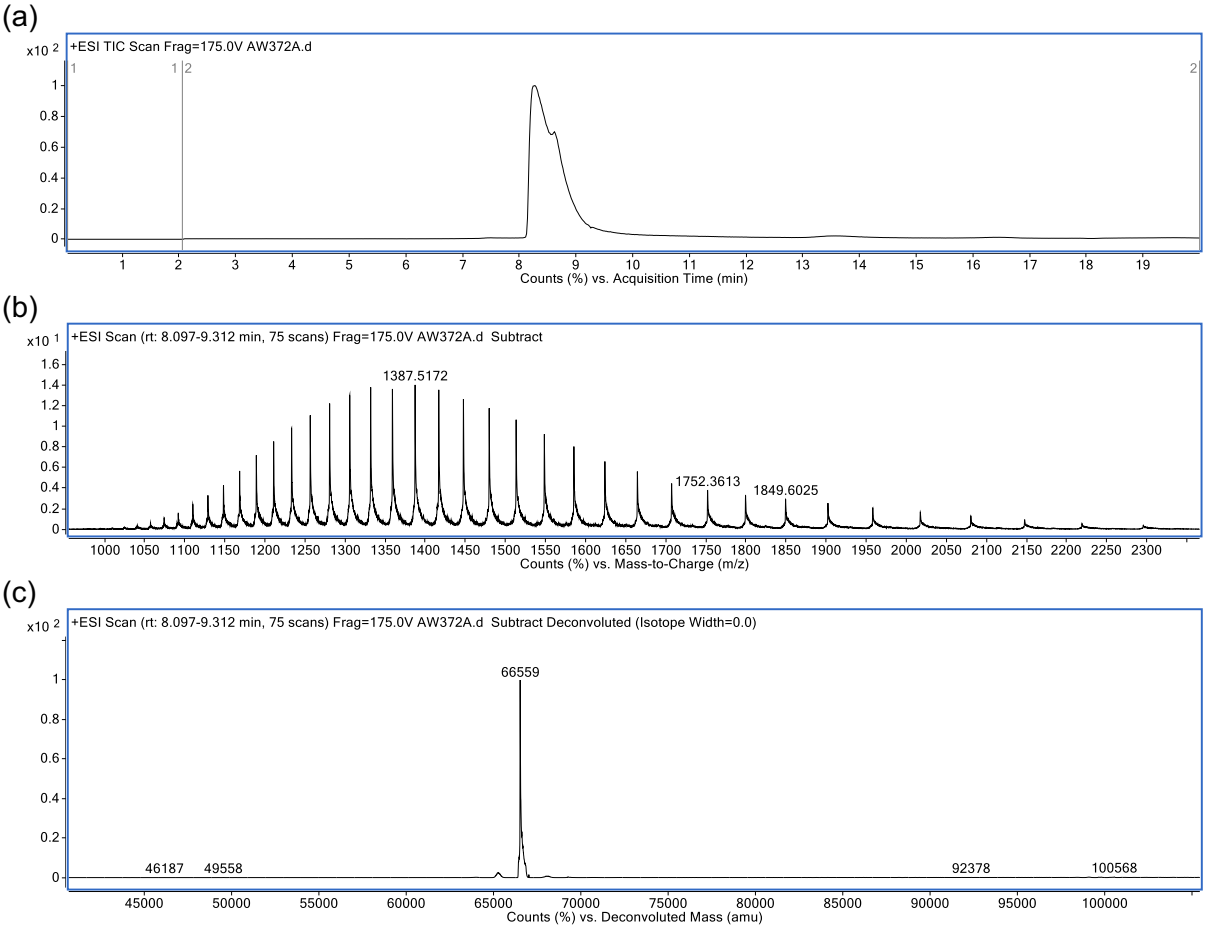
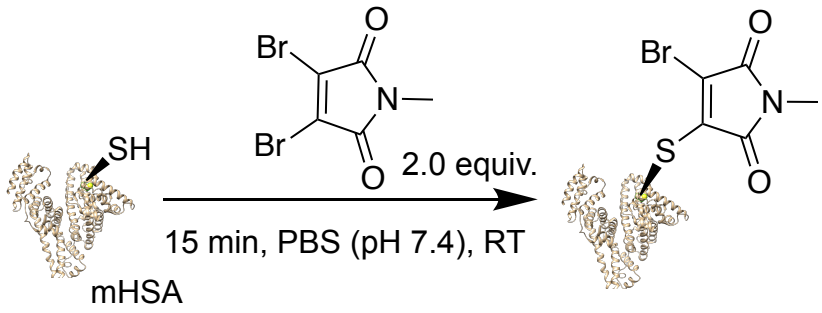


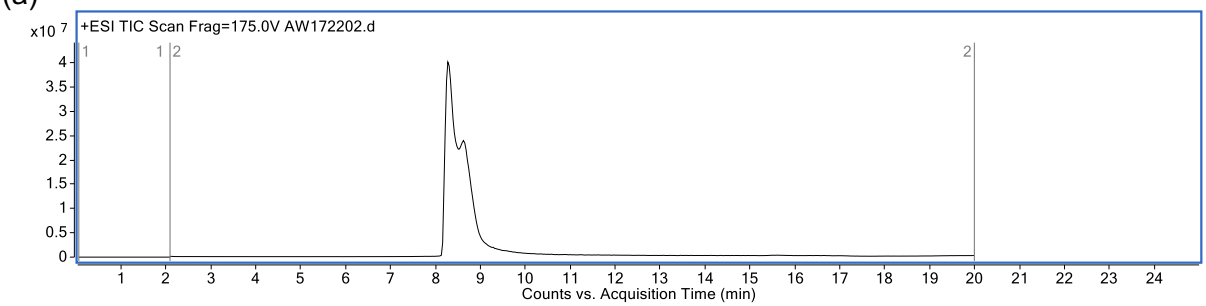
*Figure S7: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for N-Me DBM **13** conjugated to WT HSA. Expected mass: 66548 Da (loss of Br). Observed mass: 66559 Da (loss of Br + partial hydrolysis). Data obtained with MS method B.*

mHSA, *N*-Me DBM (**15**)

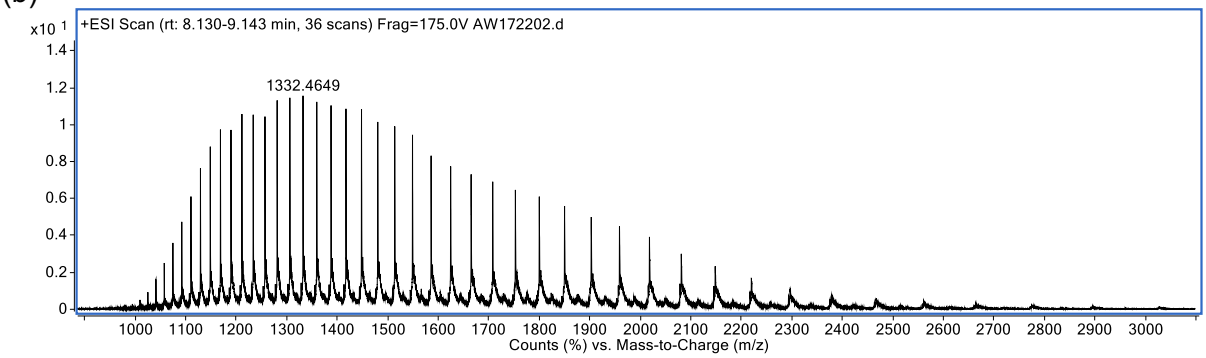


N-Me DBM **13** (3.3 µL, 10 mM, 2.0 equiv. in DMSO) was added to HSA (165 µM, 100 µL) in PBS buffer (12 mM phosphates, pH 7.4) at RT. After 15 minutes, excess small molecule was removed *via* ultrafiltration (10 kDa MWCO, Amicon® Ultra-4 Centrifugal Filter Units). Conjugates were then analysed by LCMS.

(a)



(b)



(c)

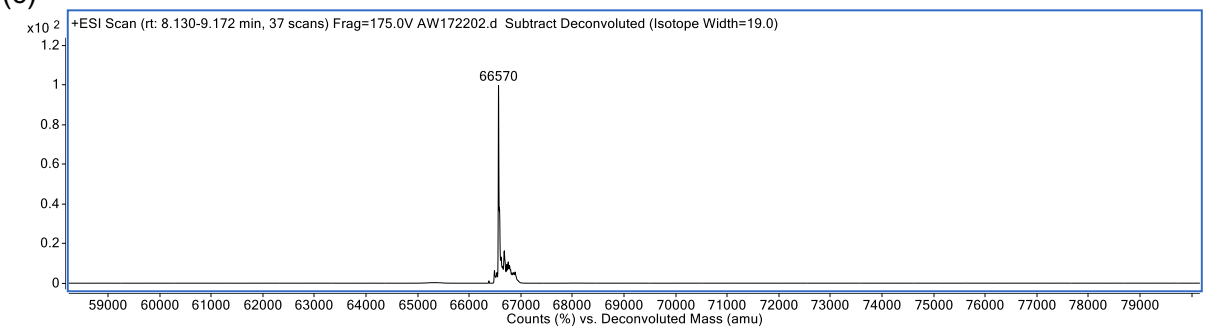
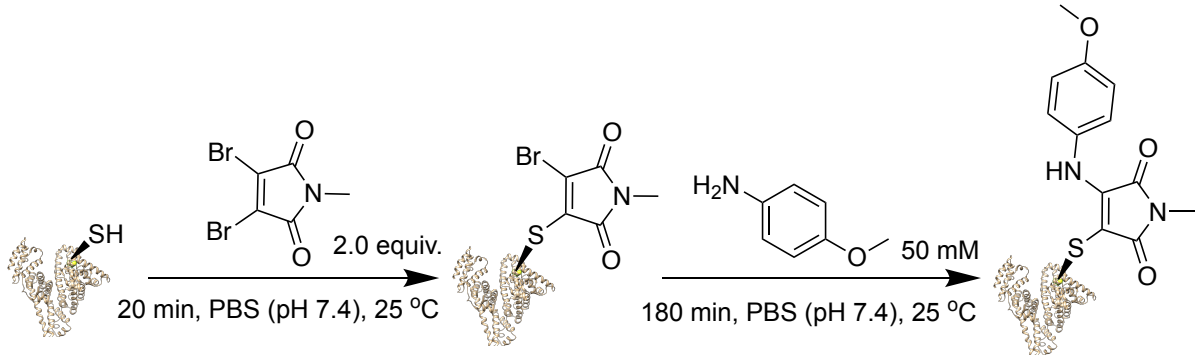


*Figure S8: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for N-Me DBM **13** conjugated to mHSA. Expected mass: 66570 Da. Observed mass: 66570 Da. Data obtained with MS method B.*

WT HSA, *N*-Me DBM, *p*-Anisidine (**16**)



N-Me DBM **13** (3.6 µL, 10 mM, 2.0 equiv. in DMSO) was added to WT HSA (100 µM, 180 µL) in PBS buffer (12 mM phosphates, pH 7.4) in a quartz cuvette and monitored by spectrophotometer at 25 °C. After the increase in absorbance at 375 nm had stopped (20 minutes), *p*-anisidine (2.9 µL, 3.2 M in DMSO) was added, and the reaction was returned to the spectrophotometer at 25 °C. The absorbance increase at 415 nm had stopped after a further 180 minutes (UV analysis was continued until minute 946), excess small molecule was removed (7 kDa MWCO, ZebaSpin®). Conjugates were then analysed by LCMS.

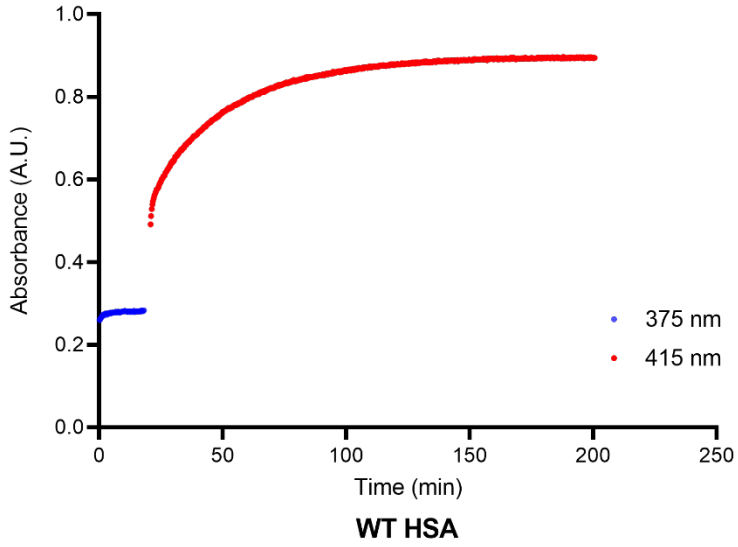
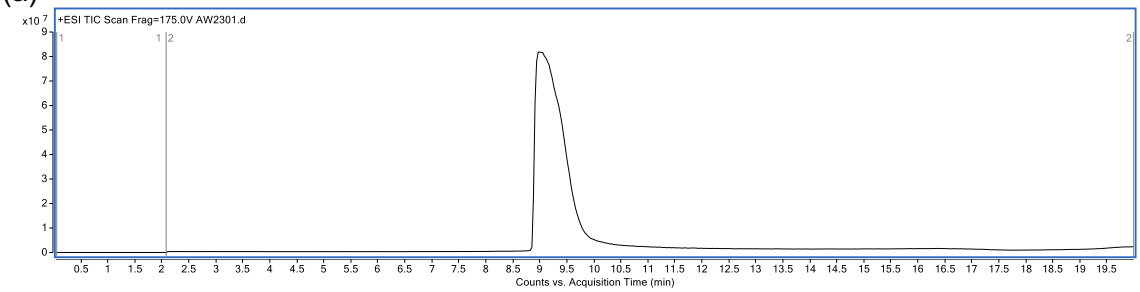
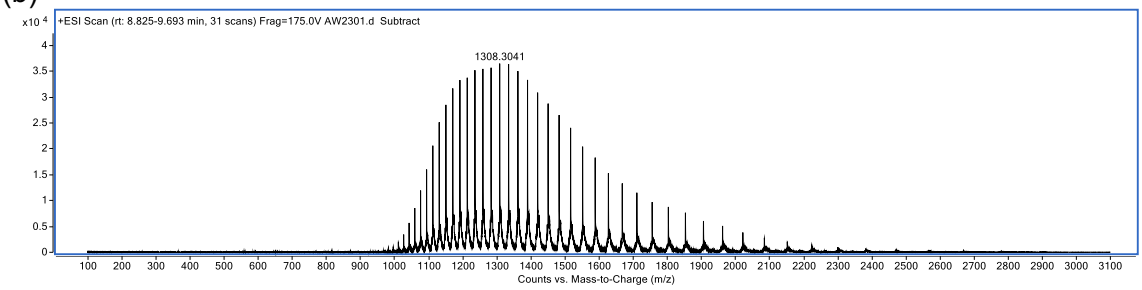


*Figure S9: UV trace of DBM **13** conjugation to WT HSA, followed by addition of p-anisidine. N.B. 200 min of data is plotted here (UV data acquired until 946 min).*

(a)



(b)



(c)

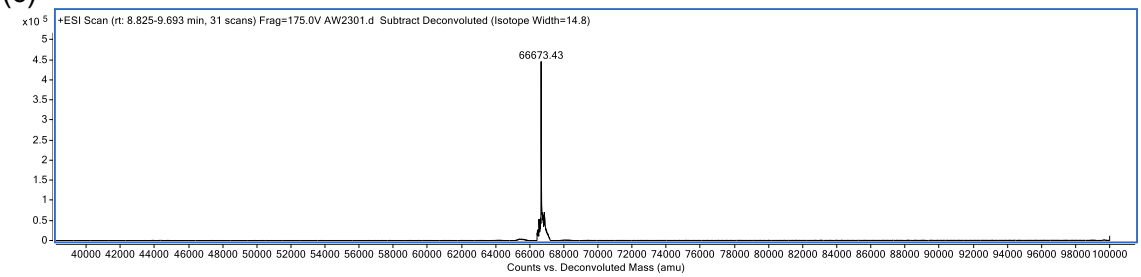
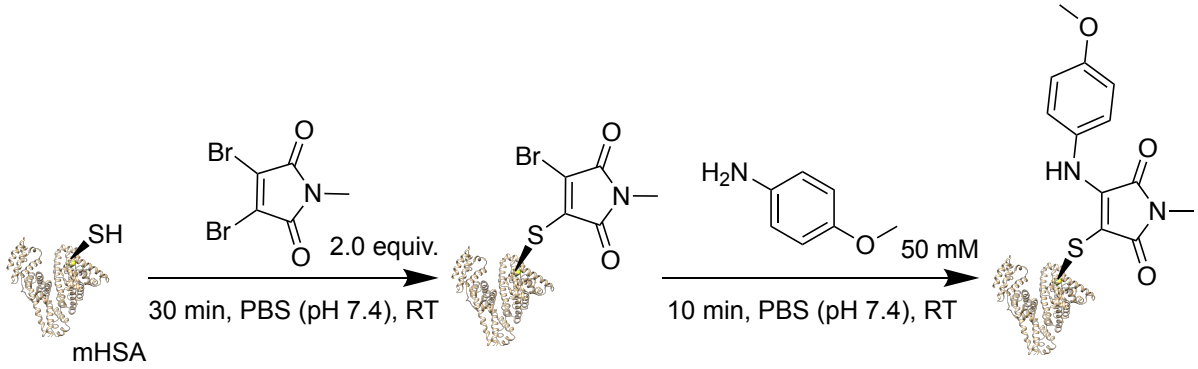


*Figure S10: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for N-Me DBM **13** conjugated to WT HSA with subsequent addition of p-anisidine. Expected mass: 66671 Da. Observed mass: 66673 Da. Data obtained with MS method B.*

mHSA, *N*-Me DBM, *p*-Anisidine (**17**)



N-Me DBM **13** (3.6 µL, 10 mM, 2.0 equiv. in DMSO) was added to mHSA (100 µM, 180 µL) in PBS buffer (12 mM phosphates, pH 7.4) in a quartz cuvette and monitored by spectrophotometer at ambient temperature (23 °C). After the increase in absorbance at 375 nm had stopped (30 minutes), *p*-anisidine (2.9 µL, 3.2 M in DMSO) was added, and the reaction was returned to the spectrophotometer at 23 °C. The absorbance increase at 415 nm had stopped after a further 10 minutes (UV analysis was continued until minute 45), excess small molecule was removed (7 kDa MWCO, ZebaSpin®). Conjugates were then analysed by LCMS.

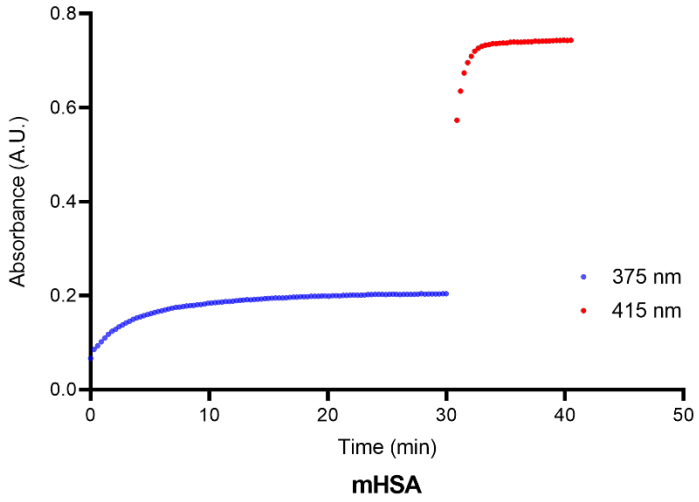
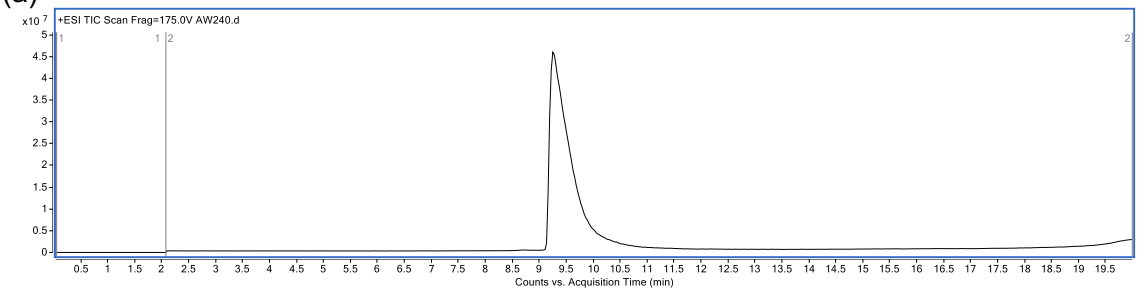
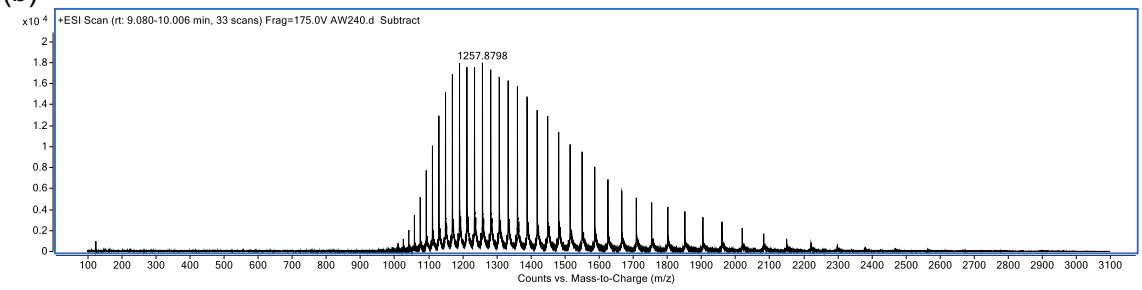


*Figure S11: UV trace of DBM **13** conjugation to mHSA, followed by addition of p-anisidine. N.B. 40 min of data is plotted here (UV data acquired until 45 min).*

(a)



(b)



(c)

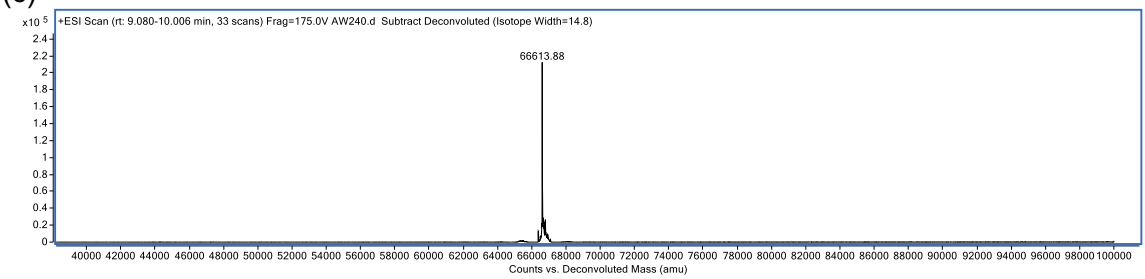
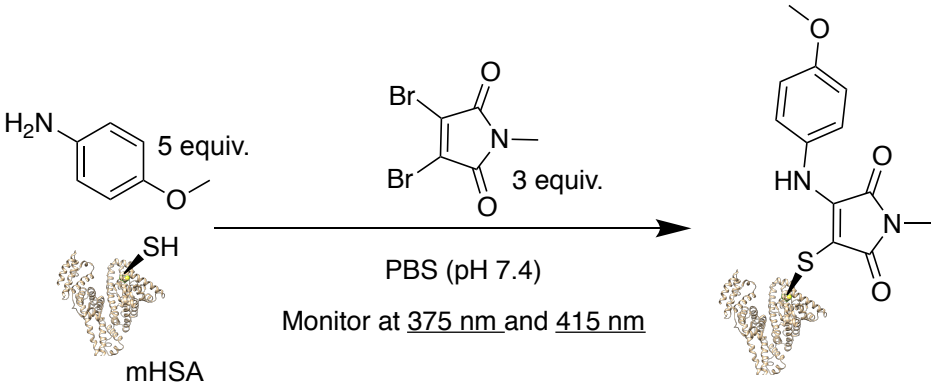


*Figure S12: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for N-Me DBM **13** conjugated to mHSA with subsequent addition of p-anisidine. Expected mass: 66612 Da. Observed mass: 66614 Da. Data obtained with MS method B.*

mHSA, *N*-Me DBM, *p*-Anisidine (*in situ*) (**17**)



p-Anisidine (2.81 µL, 32 mM, 5.0 equiv. in DMSO) was added to mHSA (100 µM, 180 µL) in PBS buffer (12 mM phosphates, pH 7.4). *N*-Me DBM **13** (5.4 µL, 10 mM, 3.0 equiv. in DMSO) was added and the reaction left at room temperature overnight. Excess small molecule was removed (7 kDa MWCO, ZebaSpin®). Conjugates were analysed by LCMS.

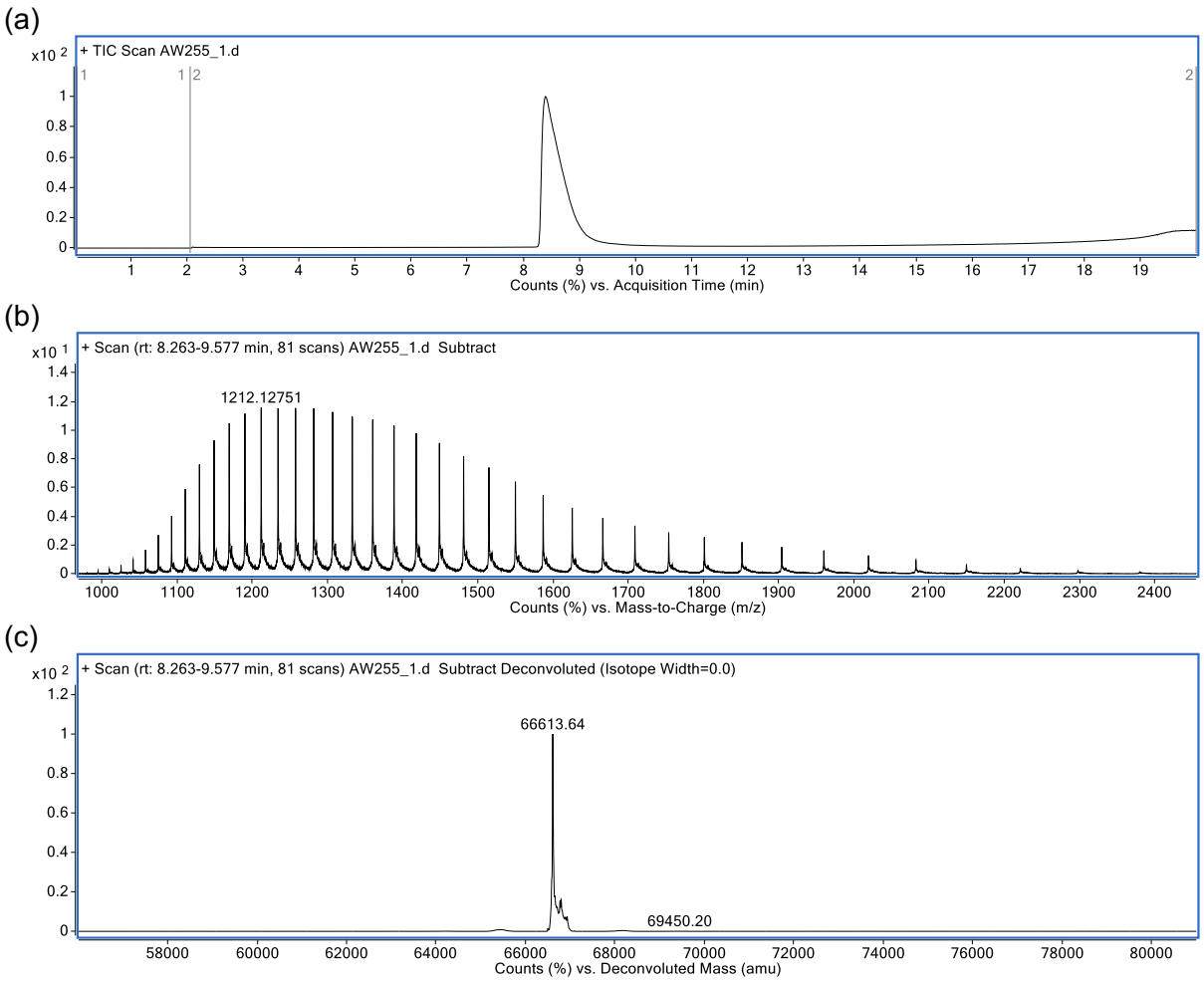
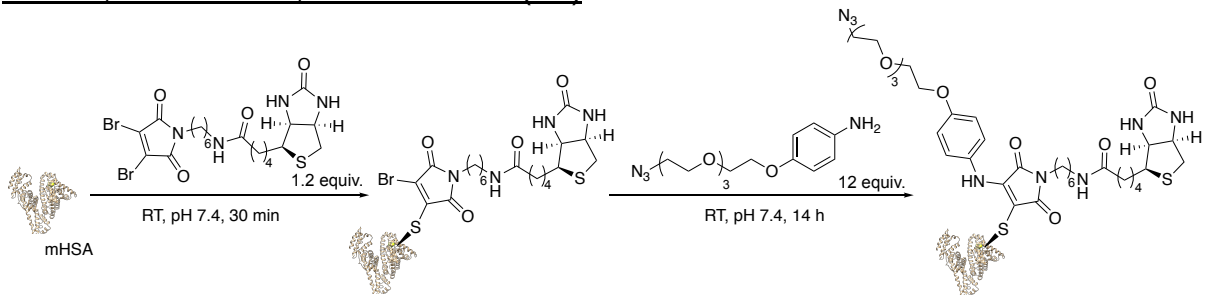


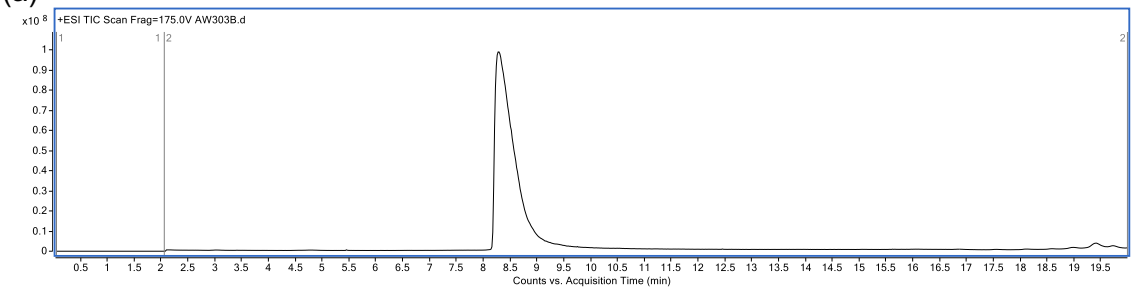
*Figure S13: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for in situ N-Me DBM **13** conjugated to mHSA with p-anisidine. Expected mass: 66612 Da. Observed mass: 66614 Da. Data obtained with MS method B.*

mHSA, Biotin DBM, Amine-Azide (**23**)

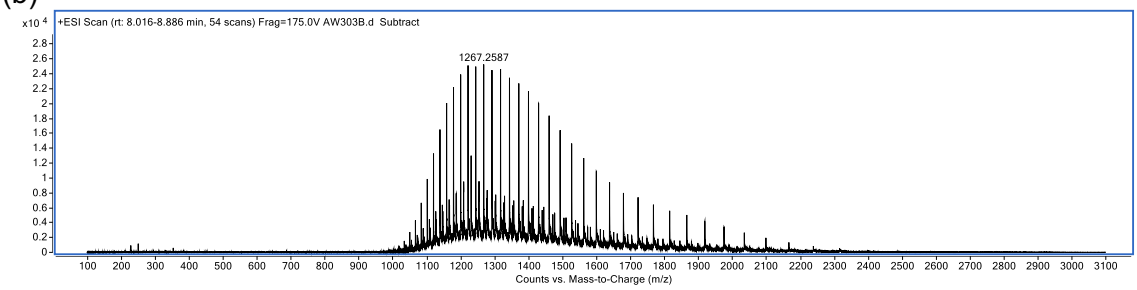


DBM **21** (0.9 µL, 10 mM, 1.2 equiv. in DMSO) was added to mHSA (100 µM, 75 µL) in PBS buffer (12 mM phosphates, pH 7.4) and left at room temperature. After 30 minutes, amine-azide **22** (0.28 µL, 0.32 M, 12.0 equiv. in DMSO) was added, and the reaction was left at RT for 14 h, before excess small molecule was removed (7 kDa MWCO, ZebaSpin®). Conjugates were then analysed by LCMS.

(a)



(b)



(c)

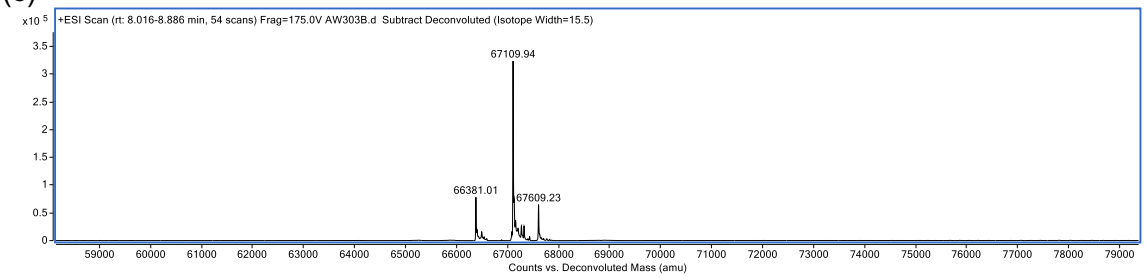
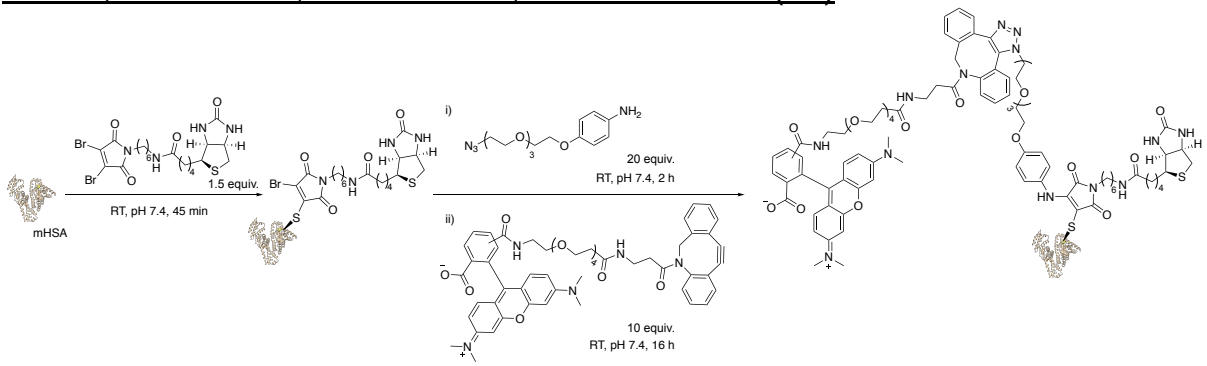


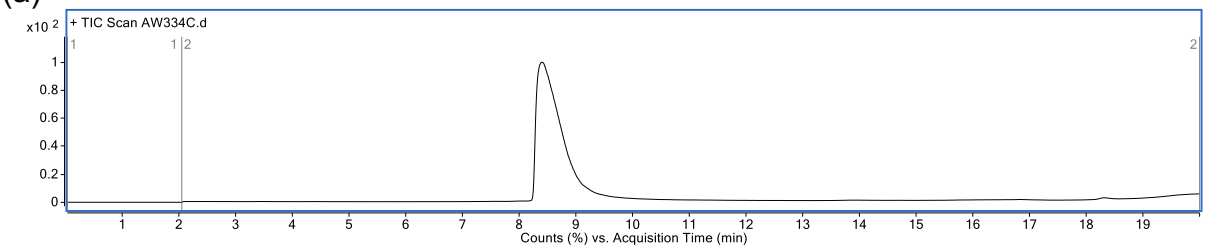
*Figure S14: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for biotin DBM **21** conjugated to mHSA with subsequent addition of amine-azide **22**. Expected mass: 67111 Da. Observed mass: 67110 Da. Native protein expected mass: 66381 Da. Observed mass: 66381 Da. Non-specific modification expected mass: 67611 Da. Observed mass: 67609 Da. Data obtained with MS method B.*

mHSA, Biotin DBM, Amine-Azide, TAMRA-DBCO (**24**)

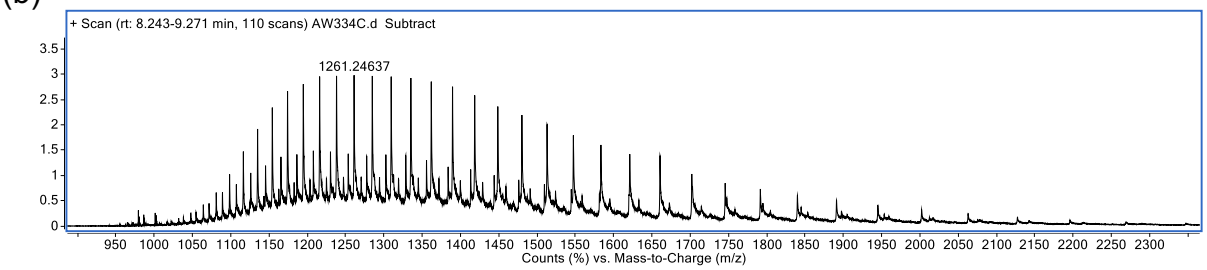


DBM **21** (1.5 µL, 10 mM, 1.5 equiv. in DMSO) was added to mHSA (100 µM, 100 µL) in PBS buffer (12 mM phosphates, pH 7.4) and left at room temperature. After 45 minutes, amine-azide **22** (0.20 µL, 1.0 M, 20 equiv. in DMSO) was added, and the reaction was left at RT for 2 h, before excess small molecule was removed (7 kDa MWCO, ZebaSpin®). TAMRA-DBCO (2.0 µL, 10 mM, 10 equiv. in DMF) was added to 20 µL of the resultant solution, and left at room temperature for 16 h, before excess small molecule was removed (7 kDa MWCO, ZebaSpin®). Conjugates were then analysed by LCMS.

(a)



(b)



(c)

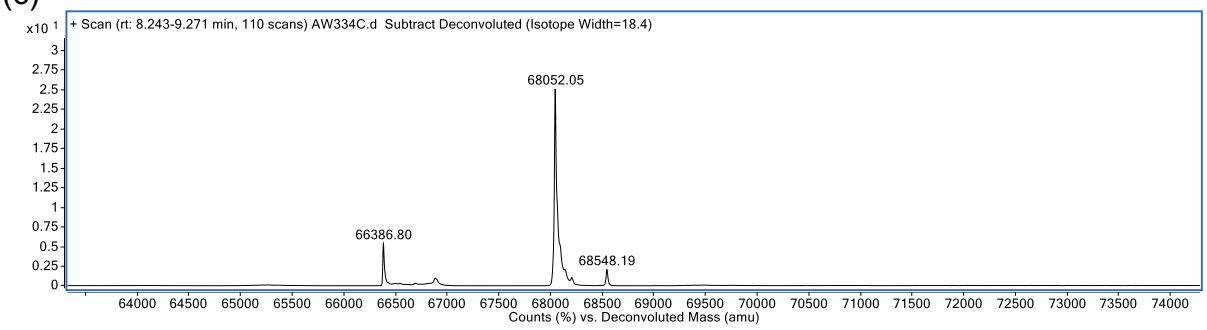
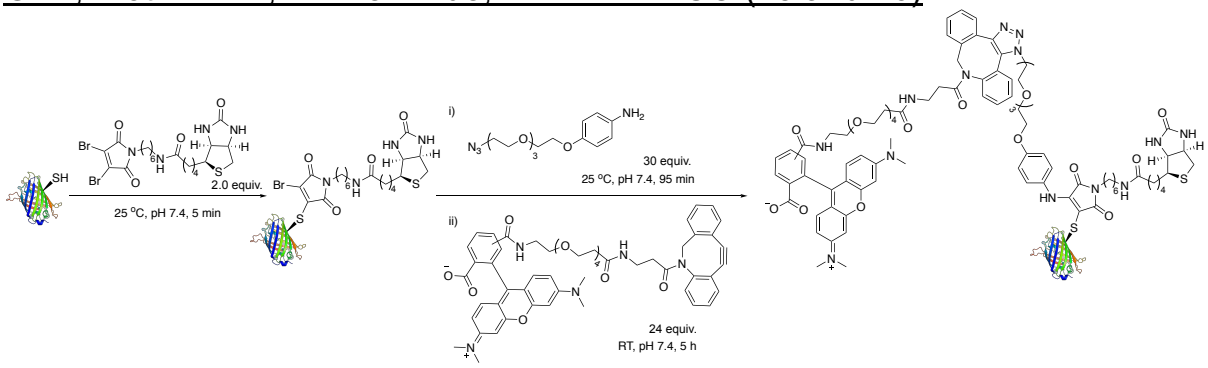


*Figure S15: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for biotin DBM **21** conjugated to mHSA with subsequent addition of amine-azide **22** and TAMRA-DBCO. Expected mass: 68047 Da. Observed mass: 68052 Da. Native protein expected mass: 66381 Da. Observed mass: 66387 Da. Non-specific modification expected mass: 68547 Da. Observed mass: 68548 Da. Data obtained with MS method B.*

GFP, Biotin DBM, Amine-Azide, TAMRA-DBCO (**25** and **26**)



DBM **21** (1.44 µL, 10 mM, 2.0 equiv. in DMSO) was added to reduced GFP (40 µM, 180 µL) in PBS buffer (12 mM phosphates, pH 7.4, 5 mM EDTA) in a quartz cuvette and monitored by spectrophotometer at 25 °C. After the increase in absorbance at 375 nm had stopped (5 minutes), amine-azide **22** (2.16 µL, 0.1 M, 30 equiv. in DMSO) was added, and the reaction was returned to the spectrophotometer at 25 °C. When the absorbance increase at 415 nm had stopped (a further 95 minutes), excess small molecule was removed (7 kDa MWCO, ZebaSpin®), and the intermediate amino-thio conjugate was analysed by LCMS. The resultant solution was then diluted to 32 µM with PBS buffer (12 mM phosphates, pH 7.4). TAMRA-DBCO (10.0 µL, 10 mM, 24 equiv. in DMF) was added to 130 µL of the resultant solution, and left at room temperature for 5 h, before excess small molecule was removed (7 kDa MWCO, ZebaSpin®). Conjugates were then analysed by LCMS.

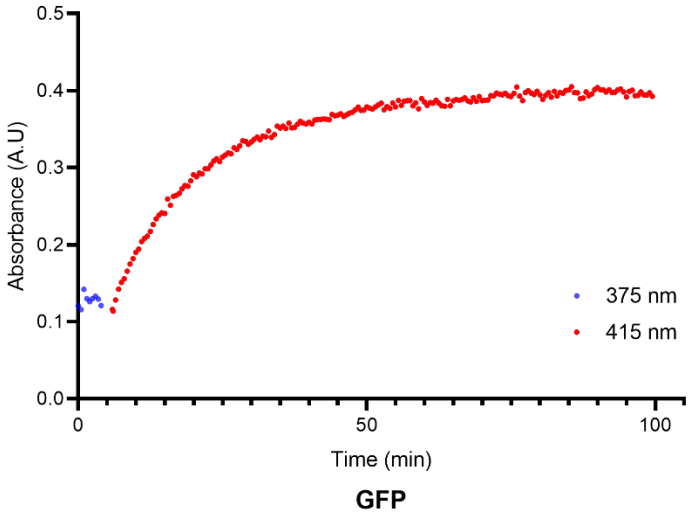


*Figure S16: UV trace of DBM **21** conjugation to GFP, followed by addition of amine azide **22**.*

Intermediate amino-thio conjugate (**25**):

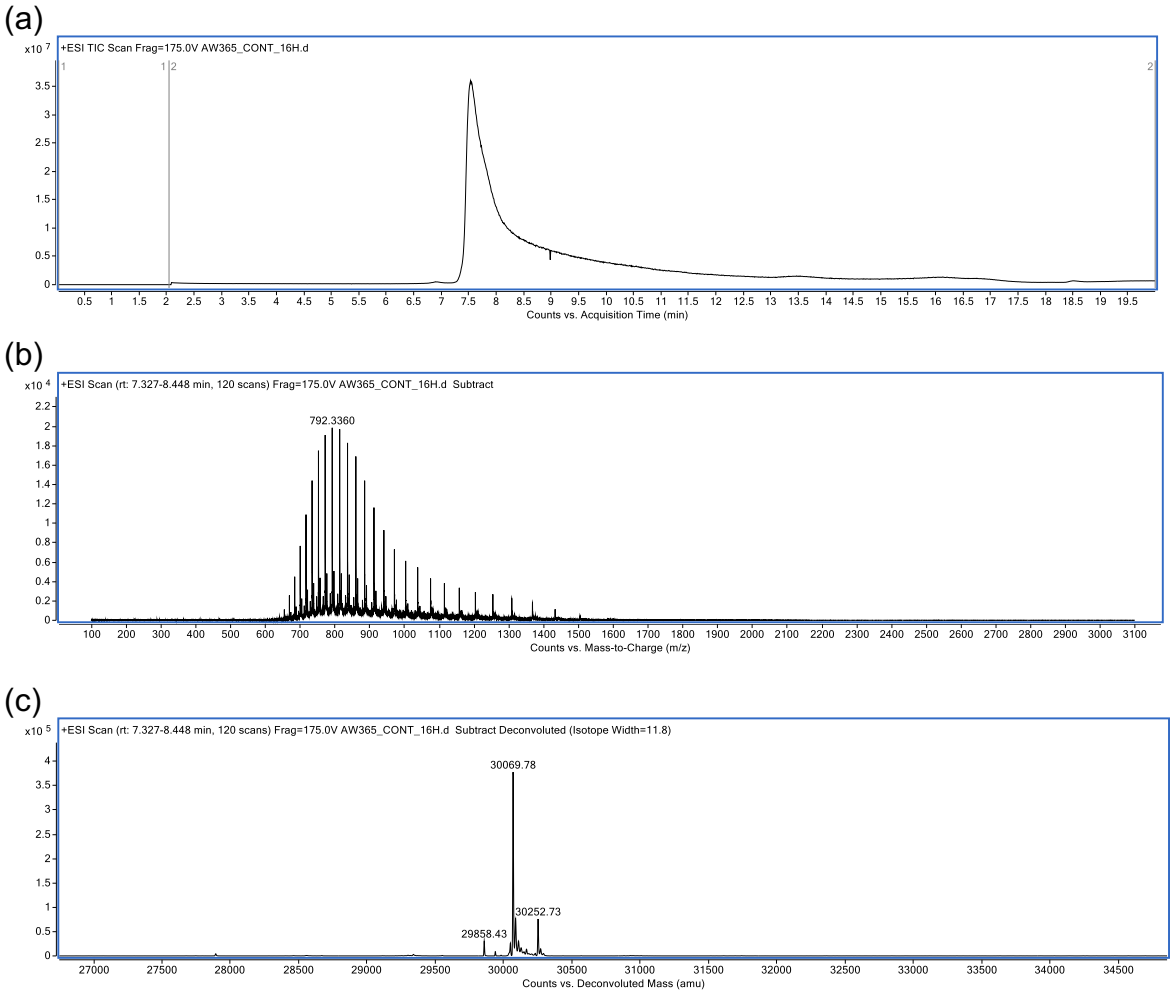


*Figure S17: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for biotin DBM **21** conjugated to GFP with subsequent addition of amine-azide **22**. Expected mass: 30072 Da and 30254 Da. Observed mass: 30070 Da and 30253 Da. Data obtained with MS method B.*

Final conjugate (**26**):

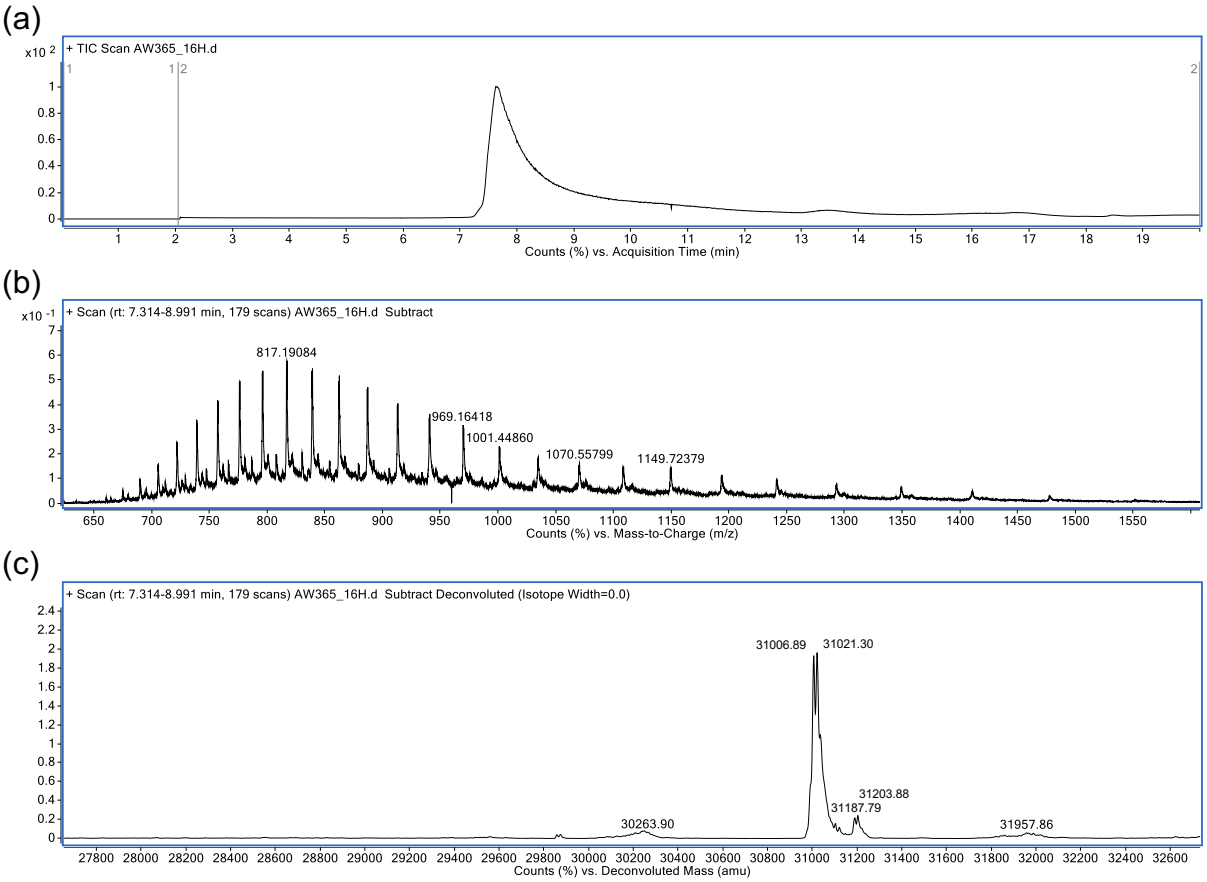
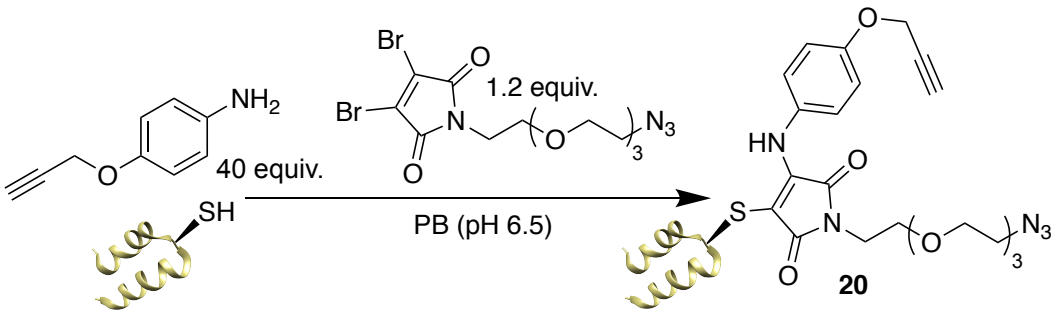


*Figure S18: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for biotin DBM **21** conjugated to GFP with subsequent addition of amine-azide **22** and TAMRA-DBCO. Expected mass: 31008 Da and 31190 Da. Observed mass: 31007 Da and 31188 Da.Oxidation observed at 31021 and 31204 Da, this is known to occur with methionine-rich proteins[11] and crucially is not observed in the amino-thio intermediate. Data obtained with MS method B.*

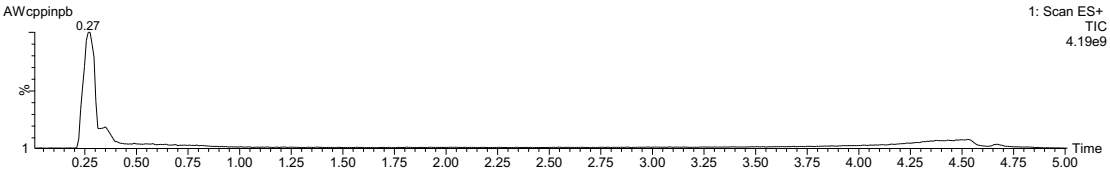
CPP, Azide DBM, Amine-Alkyne (*in situ*) (**20**)



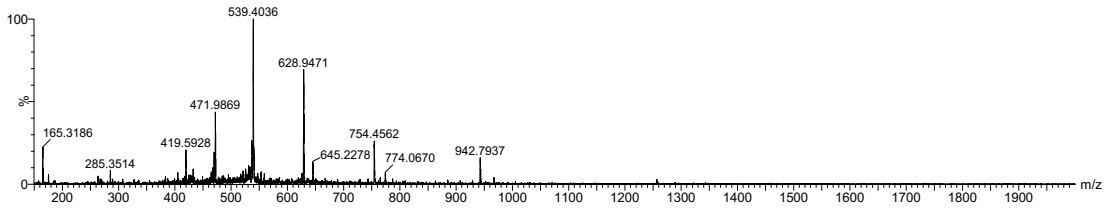
Amine-alkyne **18** (10 µL, 100 mM, 40 equiv. in DMSO) was added to CPP (0.9 mM, 25 µL) in PB buffer (10 mM phosphates, pH 6.5). DBM **19** (3 µL, 10 mM, 1.2 equiv. in DMSO) was added and the reaction left at 37 °C for 30 min. The crude reaction mixture was analysed by LCMS. Purification by HPLC was carried out before conjugates were analysed again by LCMS.

Native peptide (in 10 mM PB):

(a)



(b) 0.20-0.40 min of LC trace:



(c)

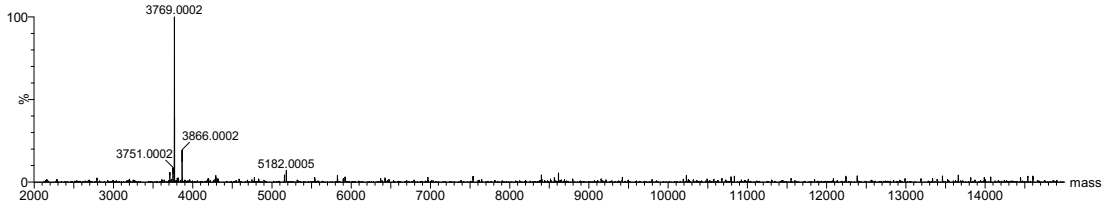
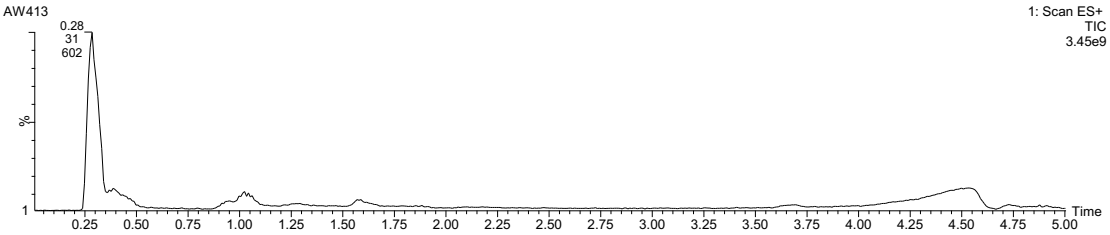


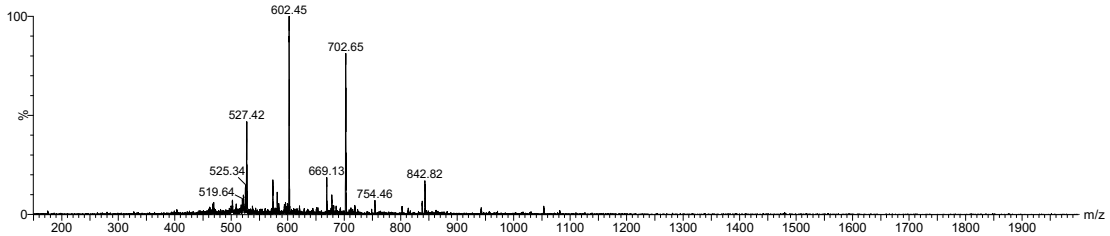
Figure S19: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for native CPP in PB. Expected mass: 3765 Da. Observed mass: 3769 Da. Data obtained with peptide LCMS method.

LC of reaction mixture (in 10 mM PB):

(a)



(b) 0.25-0.35 min of LC trace:



(c)

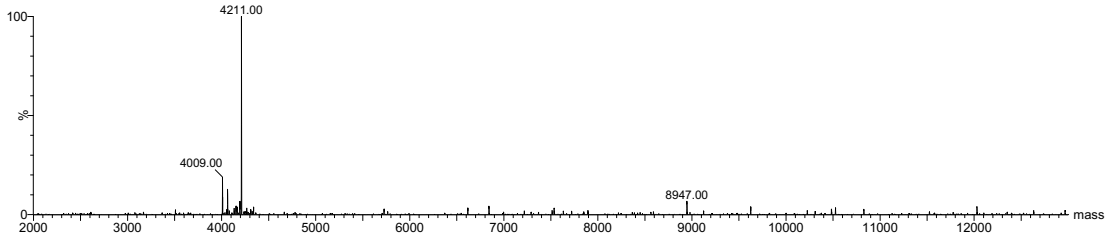
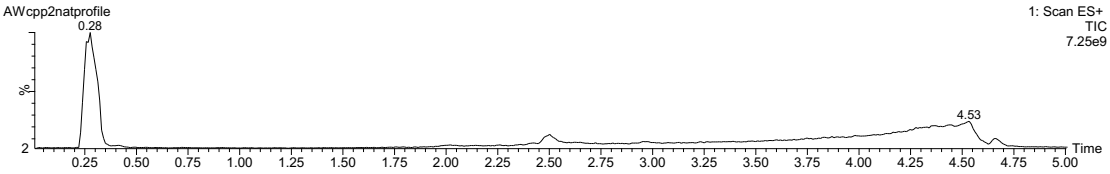


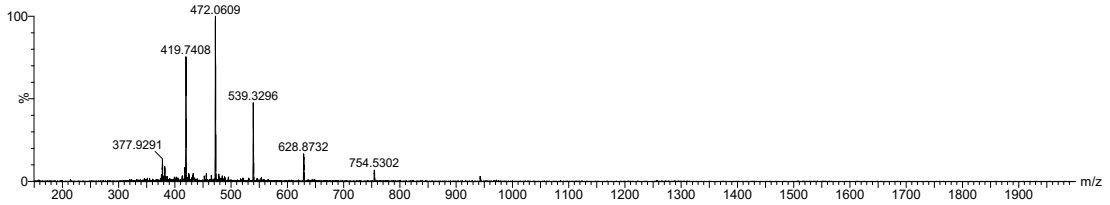
*Figure S20: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for crude in situ DBM **19** conjugated to CPP with amine alkyne **18**. Expected mass: 4208 Da. Observed mass: 4211 Da. Data obtained with peptide LCMS method.*

Native peptide (in H_2O):

(a)



(b) 0.25-0.30 min of LC trace:



(c)

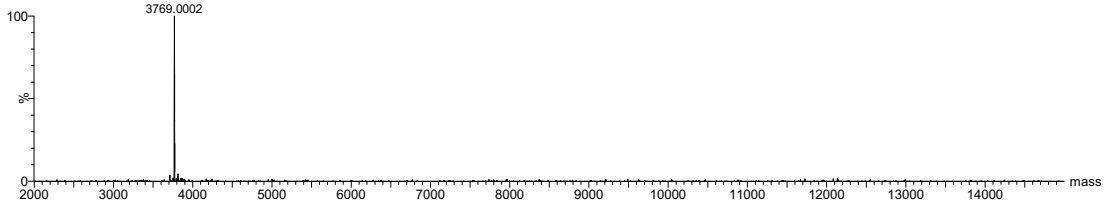
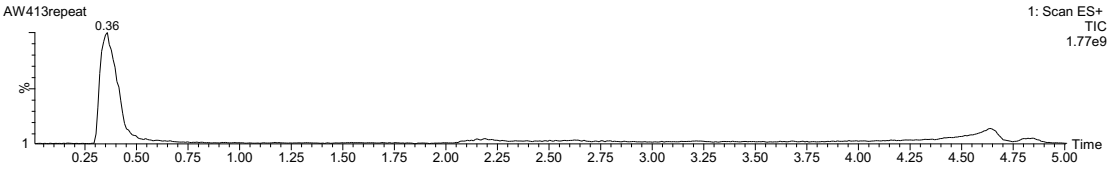


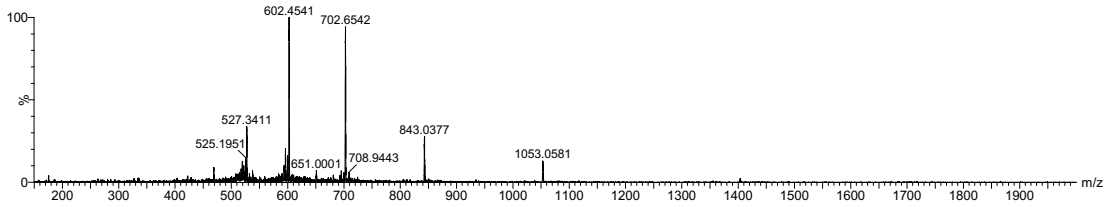
Figure S21: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for native CPP in H_2O. Expected mass: 3765 Da. Observed mass: 3769 Da. Data obtained with peptide LCMS method.

Post HPLC (in H_2O) (**20**):

(a)



(b) 0.30-0.50 min of LC trace:



(c)

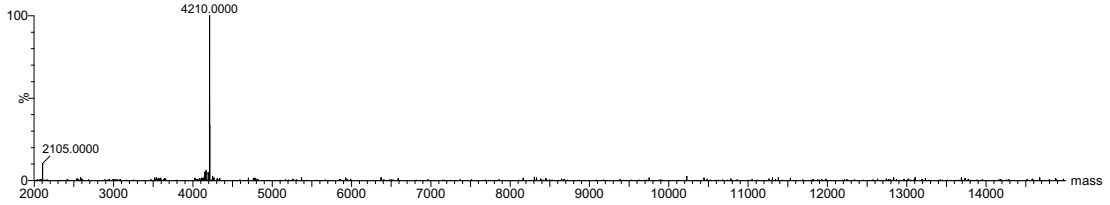
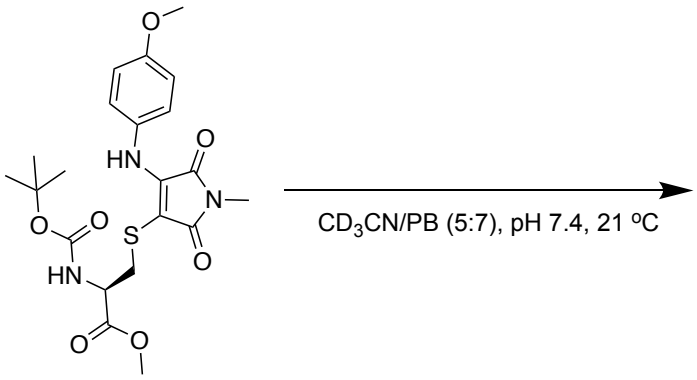


*Figure S22: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for conjugate **20**. Expected mass: 4208 Da. Observed mass: 4210 Da. Data obtained with peptide LCMS method.*

Stability Experiments

Small Molecule Study

Buffer stability (pH 7.4)



Aminothiomaleimide **11** (4.30 µmol, 500 µL, 8.60 mM in CD_3CN) was added to PB (700 µL, 100 mM phosphates). The pH was corrected to 7.4 and the solution was incubated at 21 ºC for 6 days. The solution was analysed by NMR. The *N*-Me peak at 2.98 ppm was monitored carefully to observe if a reaction occurred. No reaction was observed.

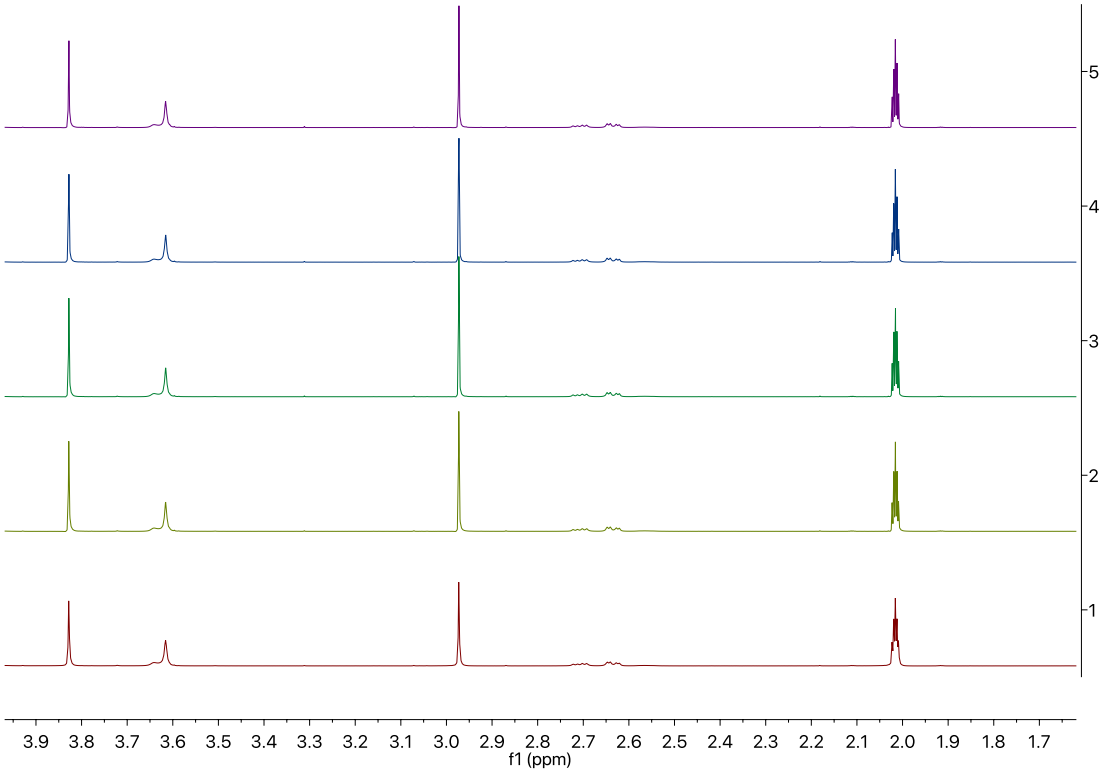
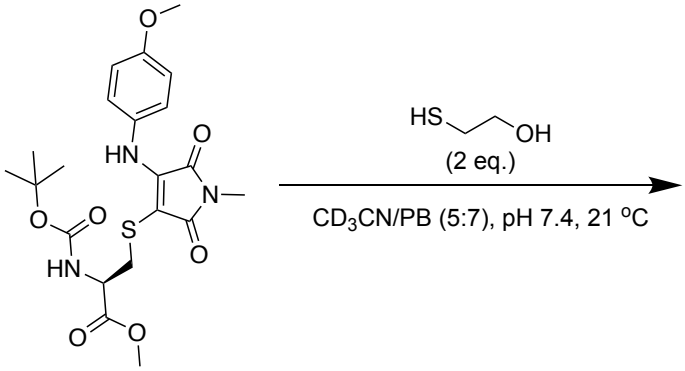


*Figure S23: Time course NMR study of **11** in buffer at pH 7.4.*

NMR spectra number	Timepoint
1	T=0
2	24 h
3	70 h
4	76 h
5	147 h

Thiol stability



Aminothiomaleimide **11** (4.30 µmol, 500 µL, 8.60 mM in CD_3CN) was added to 2-mercaptoethanol (8.60 µmol, 700 µL, 12.28 mM in PB (100 mM phosphates), 2 equiv.). The pH was corrected to 7.4 and the solution was incubated at 21 °C for 7 days. The solution was analysed by NMR. The *N*-me peak at 2.98 ppm was monitored carefully to observe if a reaction occurred. No reaction was observed.

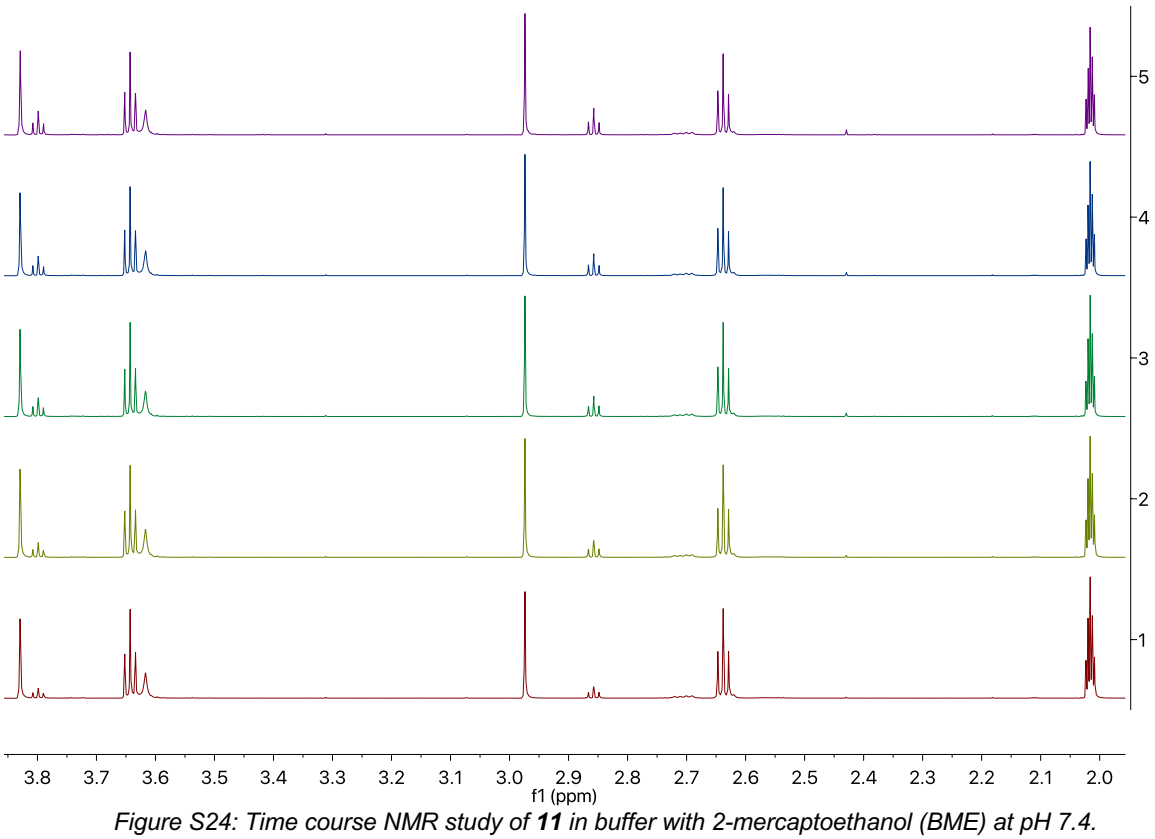


*Figure S24: Time course NMR study of **11** in buffer with 2-mercaptoethanol (BME) at pH 7.4.*

NMR spectra number	Timepoint
1	19 h
2	43 h
3	66 h
4	72 h
5	7 days

Control vs T5 NMR

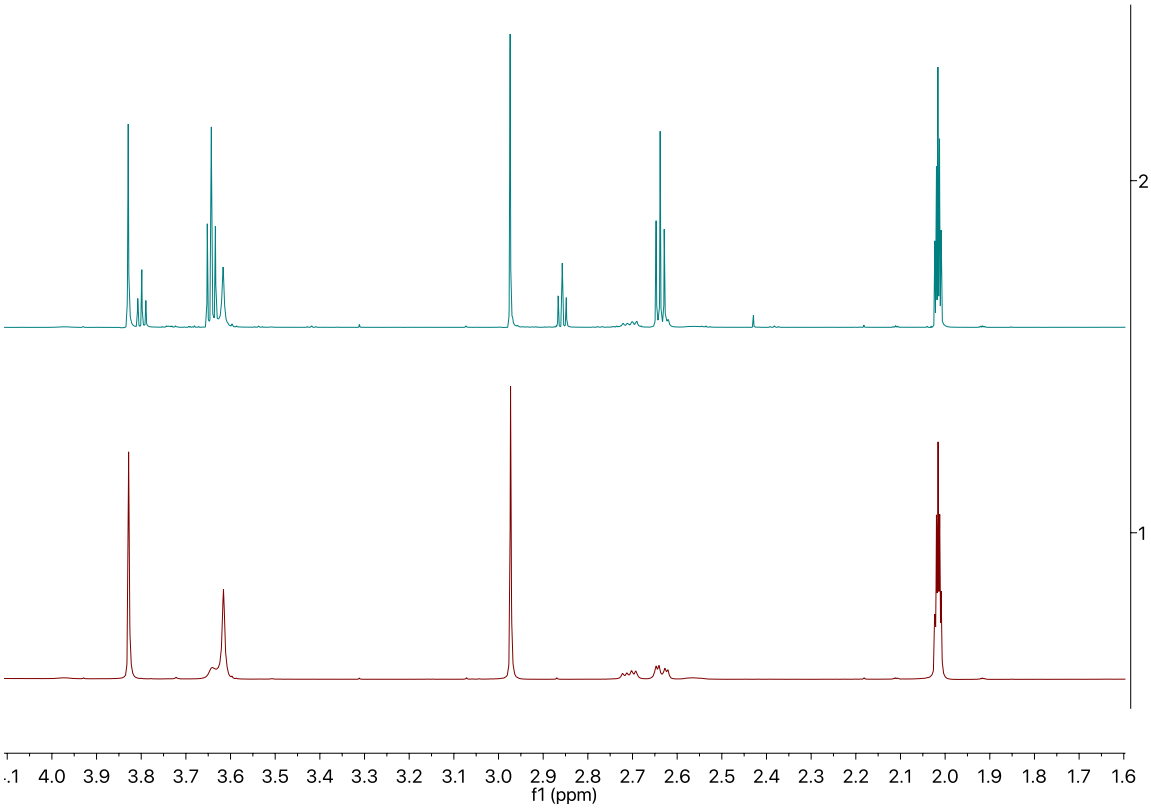
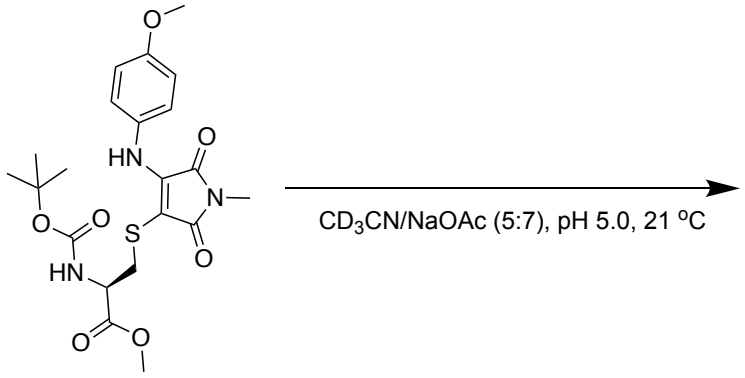


Figure S25: Stacked NMR spectra to show control vs 7 day timepoint after BME addition.

NMR spectra number	Timepoint
1	Control (No BME added)
2	7 days

Buffer stability (pH 5)



Aminothiomaleimide **11** (2.15 µmol, 500 µL, 4.30 mM in CD_3CN) was added to NaOAc buffer (700 µL, 100 mM). The pH was corrected to 5.0 and the solution was incubated at 21 °C for 8 days. The solution was analysed by NMR. The *N*-Me peak at 2.97 ppm was monitored carefully to observe if a reaction occurred. No reaction was observed.

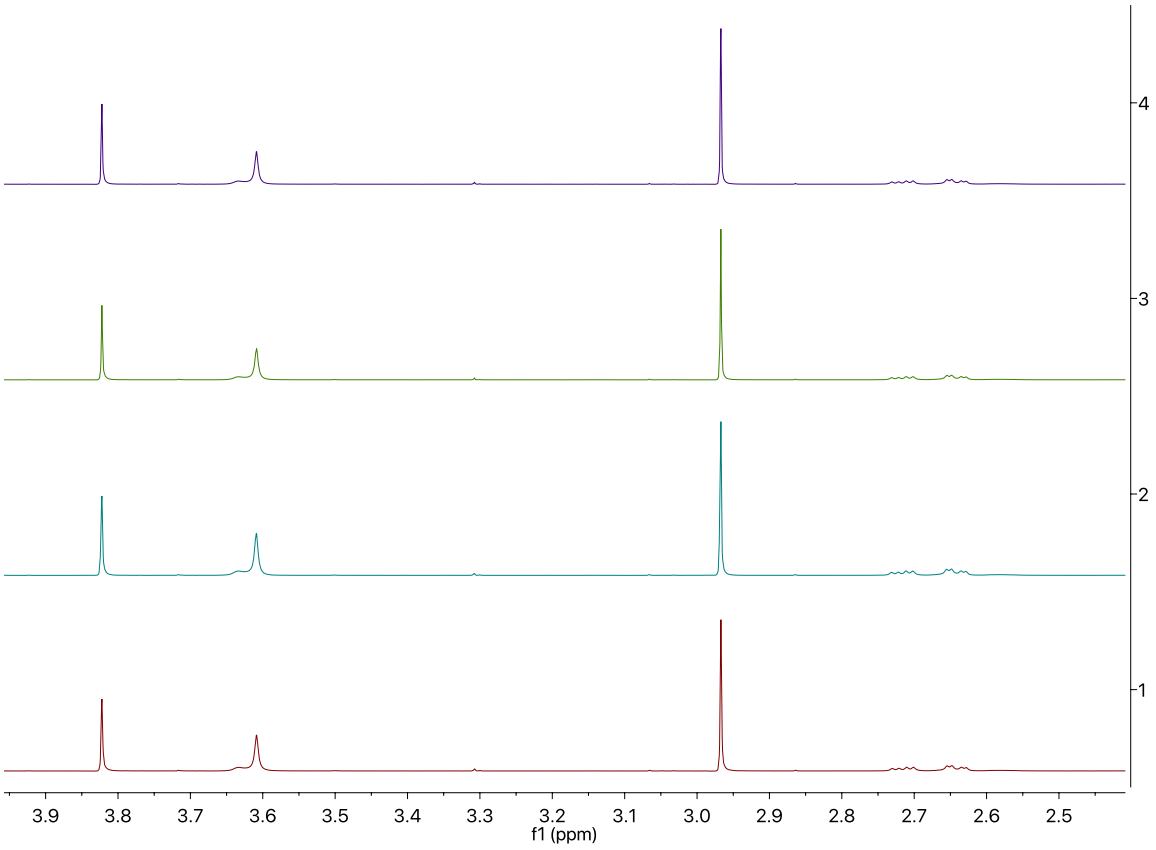
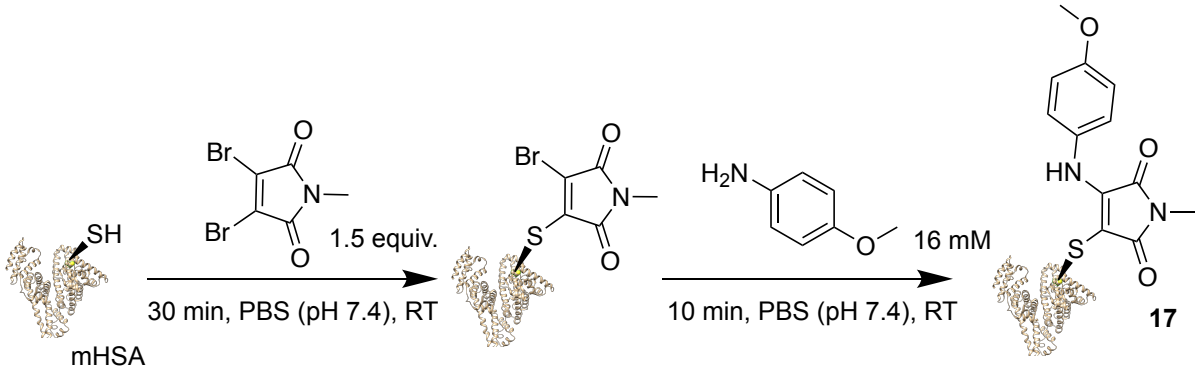


*Figure S26: Time course NMR study of **11** in buffer at pH 5.0.*

NMR spectra number	Timepoint
1	T=0
2	2 days
3	5 days
4	8 days

Thiol Stability of Conjugate **17**



Bromomaleimide reagent **13** (0.9 µL, 10 mM, 1.5 equiv. in DMSO) was added to mHSA (96 µM, 60 µL) in PBS buffer (12 mM phosphates, pH 7.4) and left at room temperature. After 30 minutes, *p*-anisidine (1 µL, 1 M in DMSO) was added, and the reaction left at room temperature for a further 10 minutes. Excess small molecule was removed (7 kDa MWCO, ZebaSpin®). Conjugates were analysed by LCMS before use in stability assays described below.

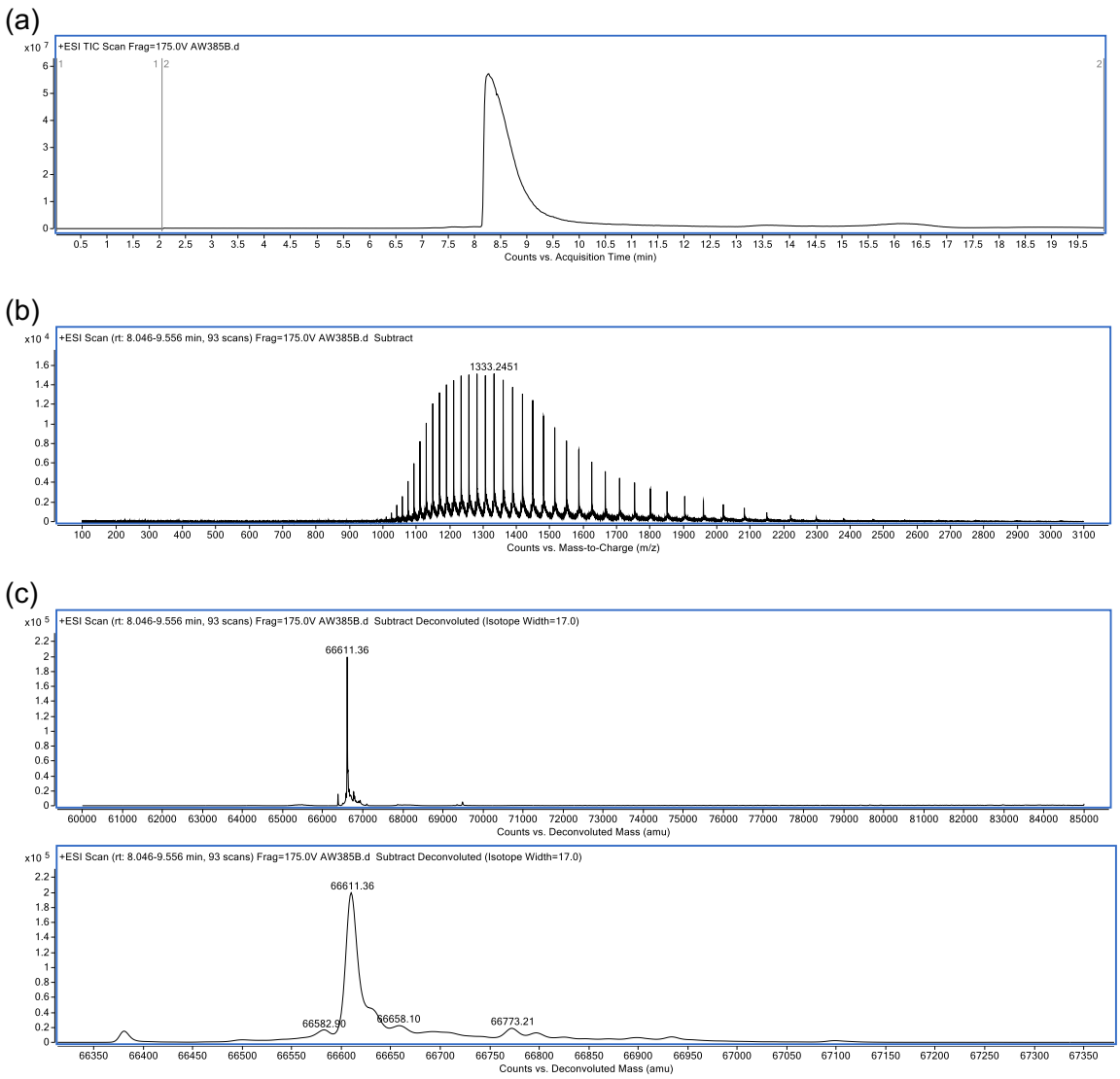
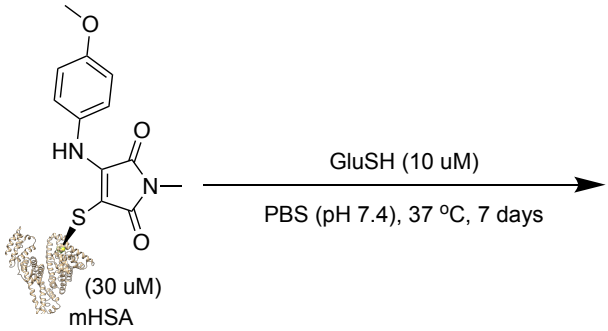


*Figure S27: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for N-Me DBM **13** conjugated to mHSA with subsequent addition of p-anisidine. Expected mass: 66612 Da. Observed mass: 66611 Da. Data obtained with MS method B.*

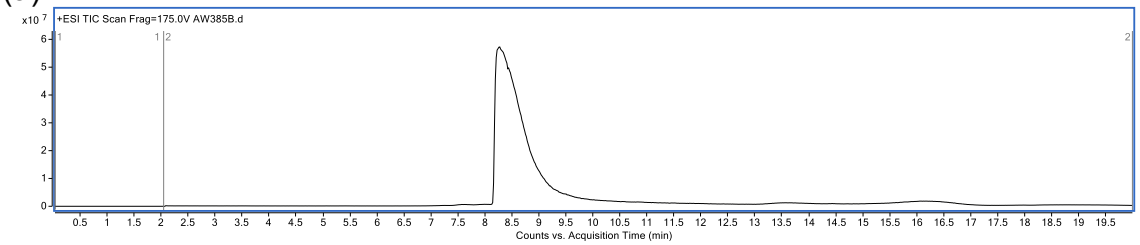
Extracellular Thiol Concentration and pH



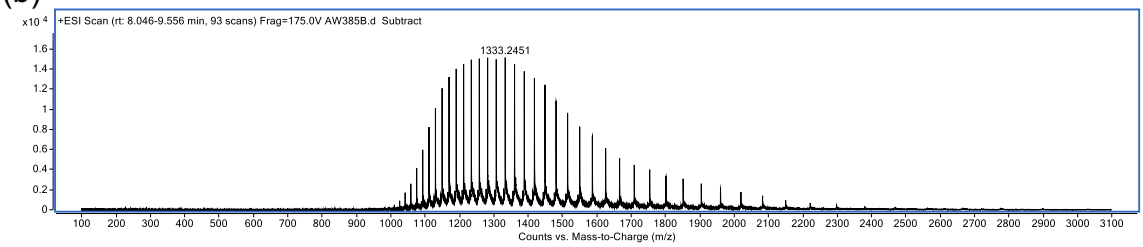
Conjugate **17** (30 μM) was incubated in a solution of GluSH (10 μM in PBS) at pH 7.4 at 37 °C. Timepoints were taken over 7 days and analysed by LCMS. The T0-T4 timepoint data is shown below. Expected conjugate mass: 66,612 Da.

T0 – Before GluSH addition

(a)



(b)



(c)

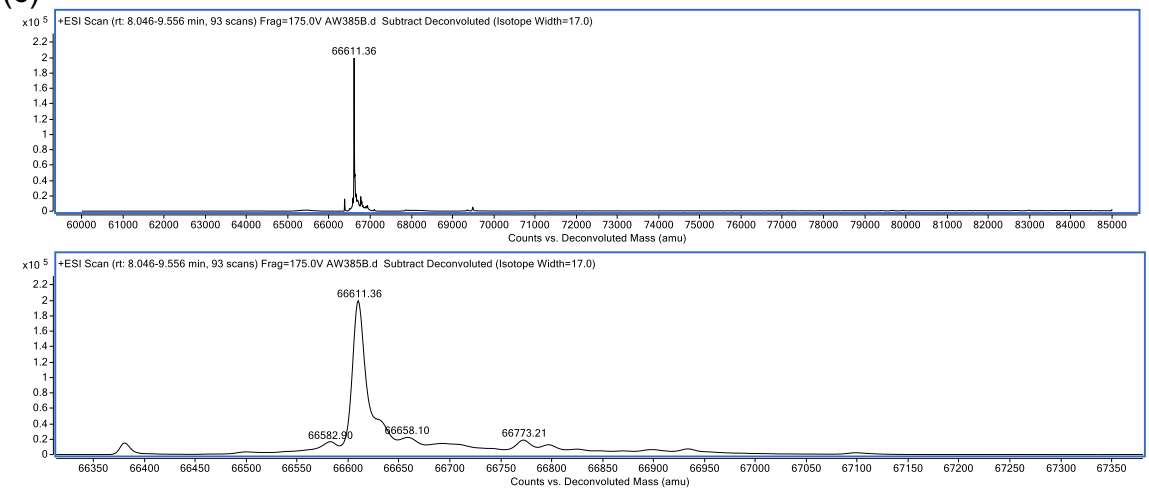
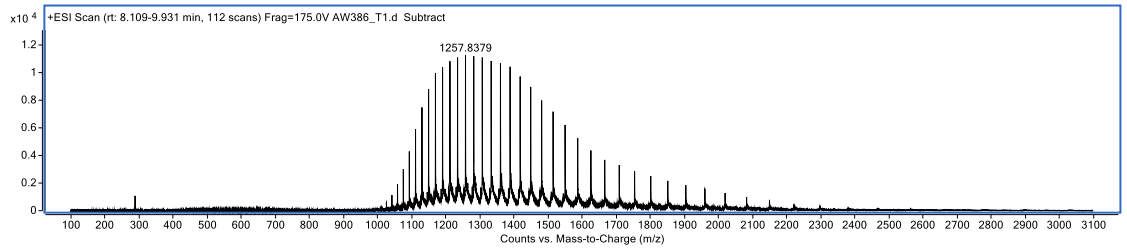
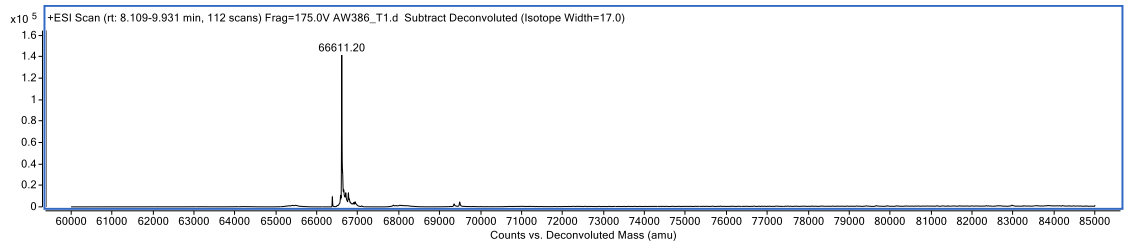
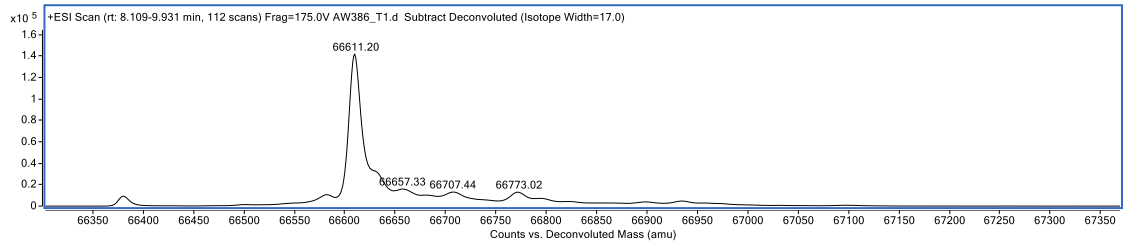


*Figure S28: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for conjugate **17**. Expected mass: 66612 Da. Observed mass: 66611 Da. Data obtained with MS method B.*

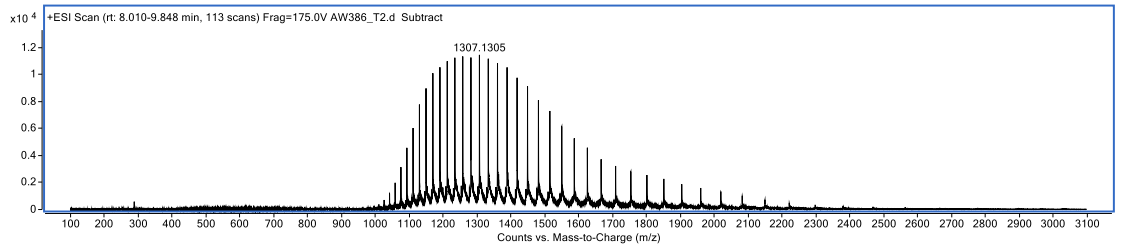
T1 – 24 h post GluSH addition

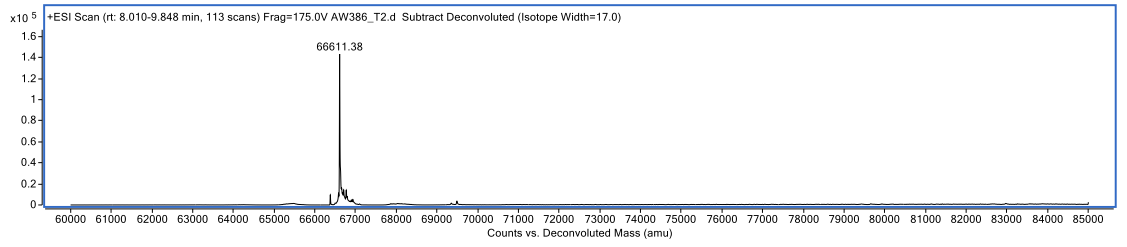


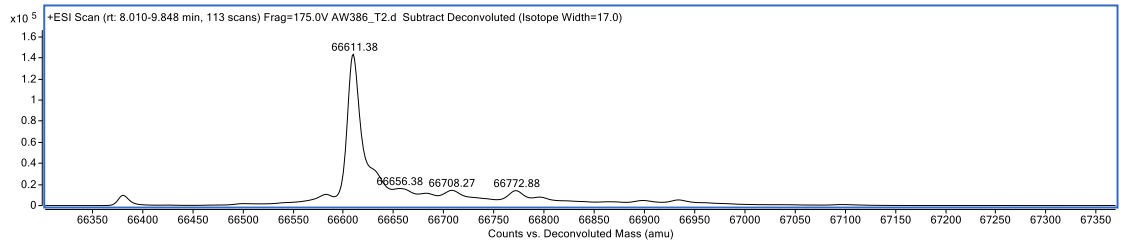




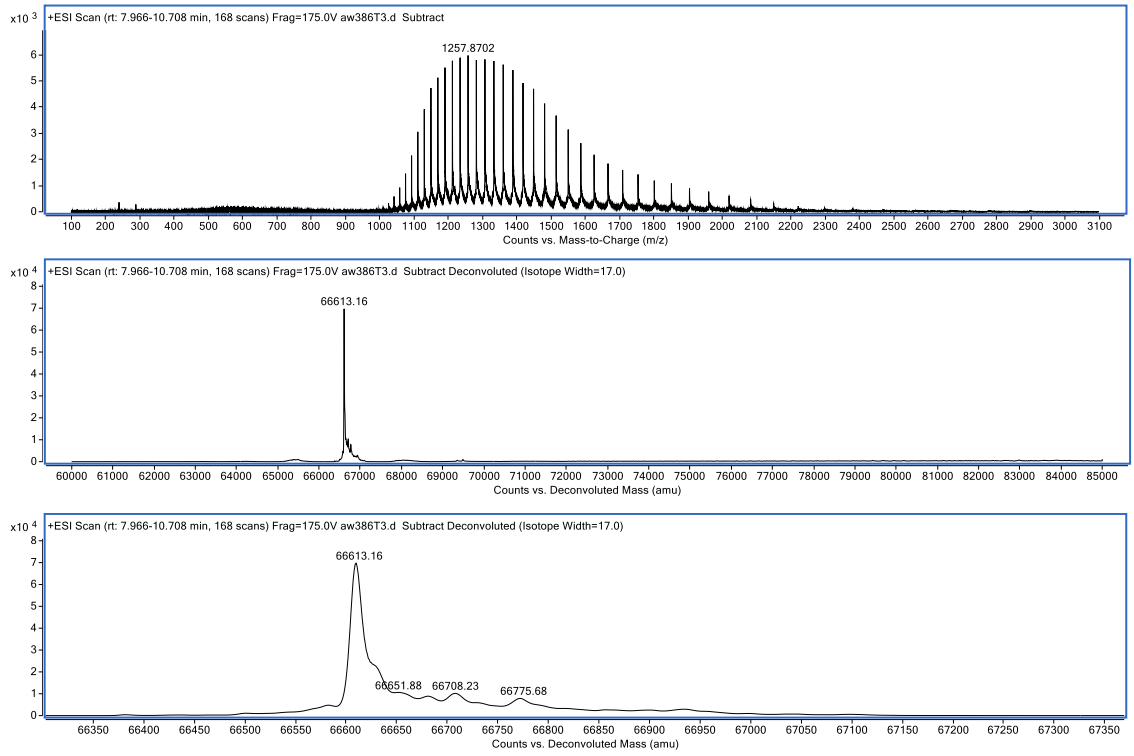
T2 – 48 h post GluSH addition







T3 – 6 days post GluSH addition



T4 – 7 days post GluSH addition

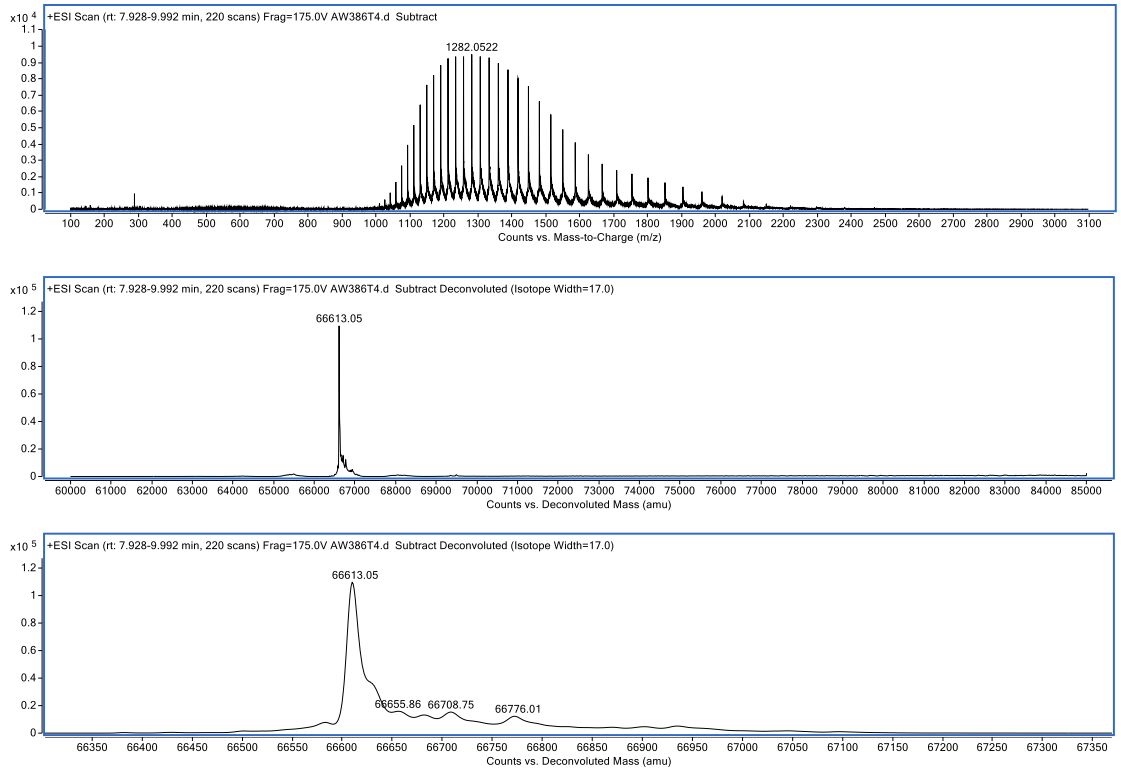
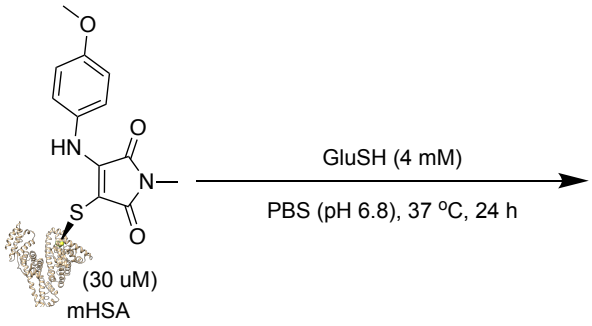


*Figure S29: (a) non-deconvoluted data and (b) deconvoluted data for incubation of conjugate **17** with GSH (10 μM, pH 7.4, 37 °C, 7 days). Expected mass: 66612 Da. Observed mass: 66613 Da. Data obtained with MS method B.*

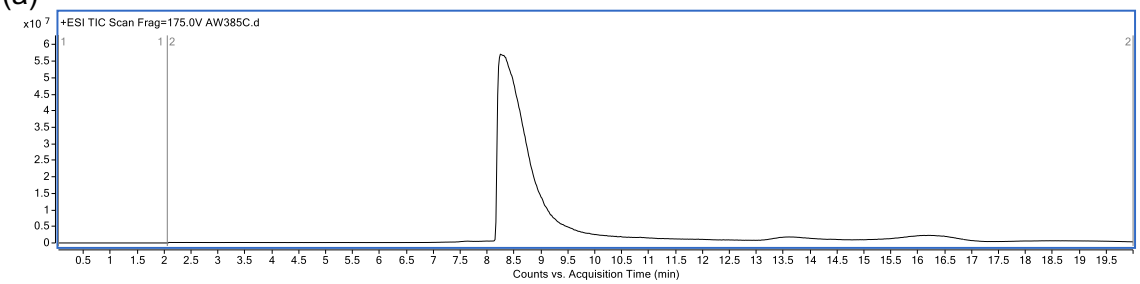
Intracellular Thiol Concentration and pH



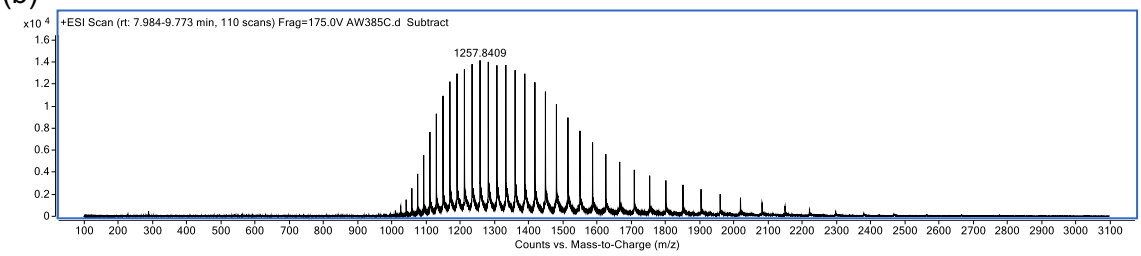
Conjugate **17** (30 μM) was incubated in a solution of GluSH (4 mM in PBS) at pH 6.8 at 37 °C. Timepoints were taken over 24 h and analysed by LCMS. The T0-T2 timepoint data is shown below. Expected conjugate mass: 66,612 Da.

T0 – Before GluSH addition

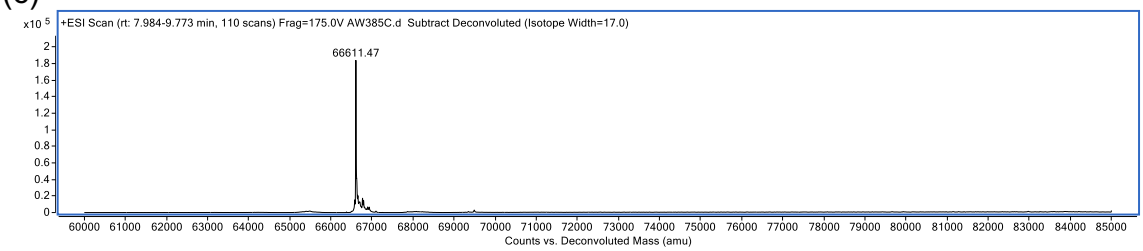
(a)



(b)



(c)



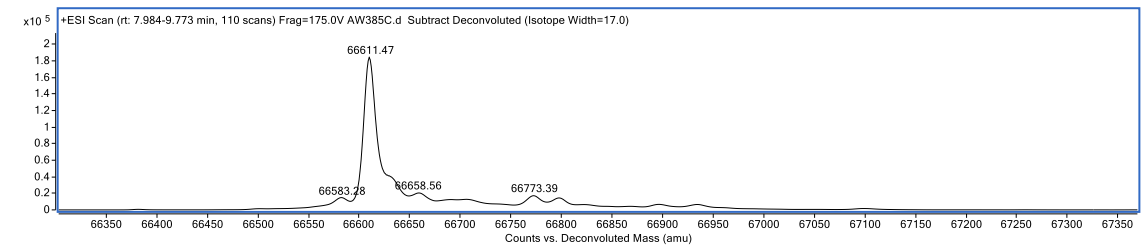
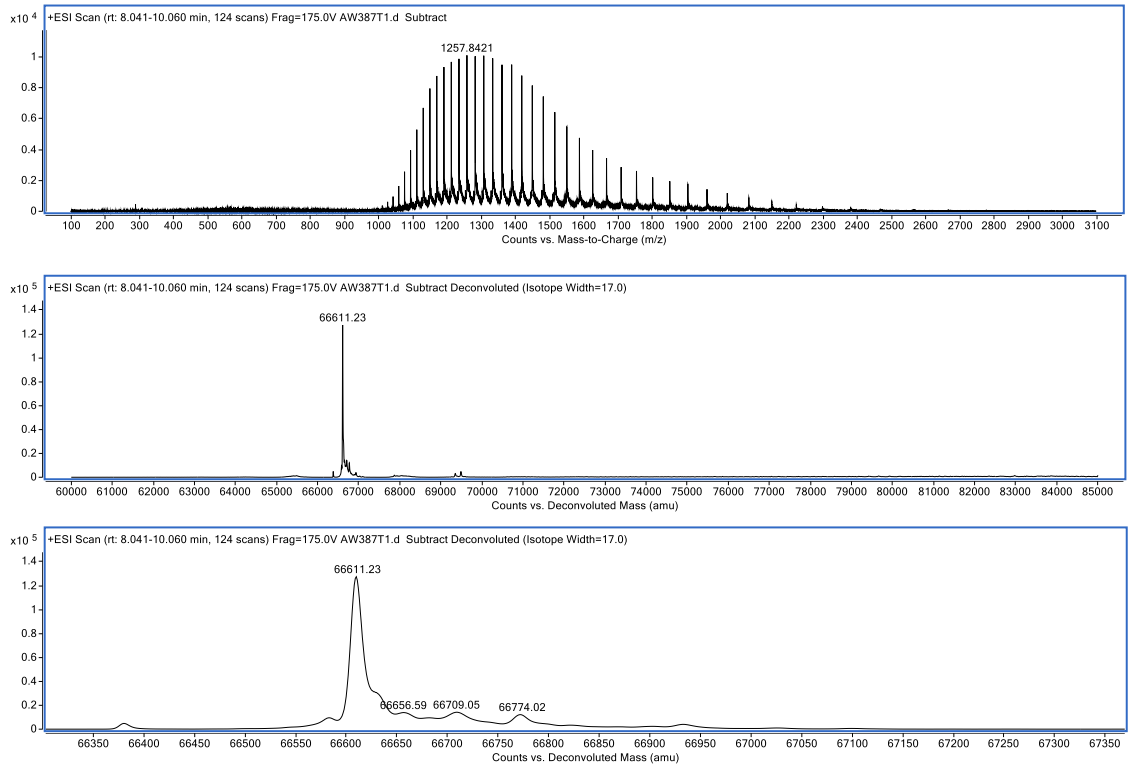


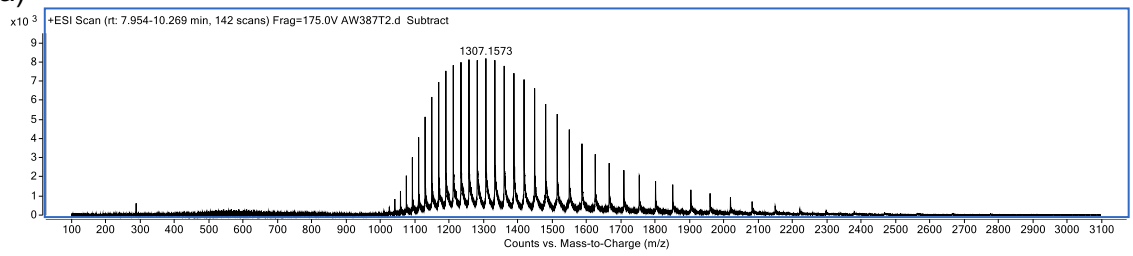
*Figure S30: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for conjugate **17**. Expected mass: 66612 Da. Observed mass: 66611 Da. Data obtained with MS method B.*

T1 – 3 h post GluSH addition



T2 – 24 h post GluSH addition

(a)



(b)

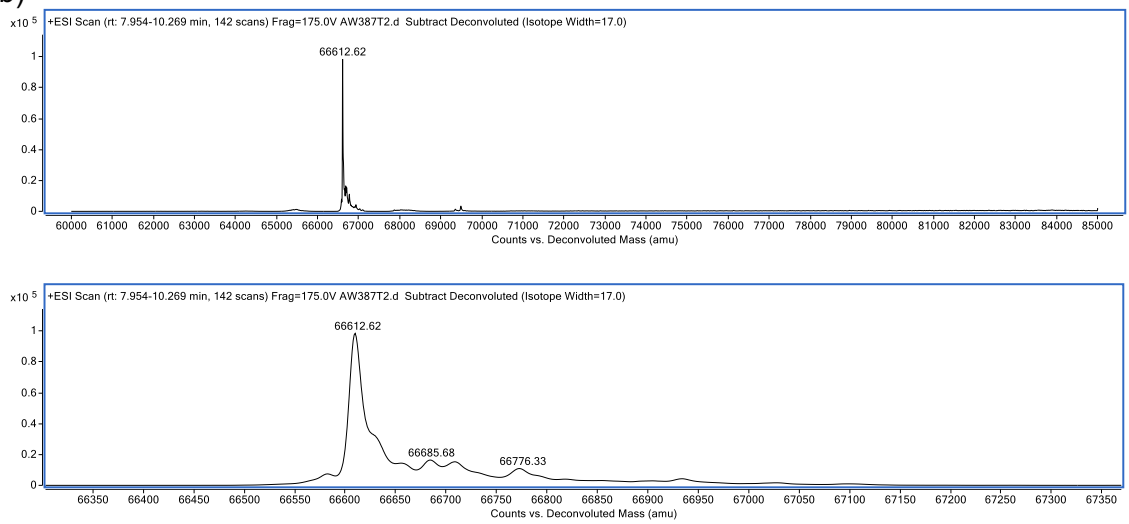
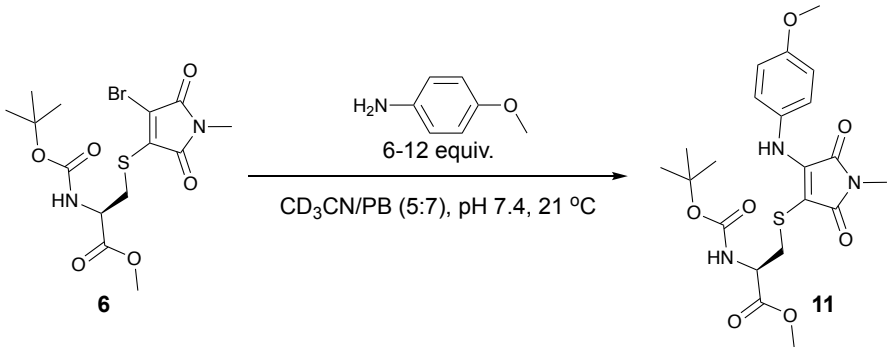


*Figure S31: (a) non-deconvoluted data and (b) deconvoluted data for incubation of conjugate **17** with GSH (4 mM, pH 6.8, 37 °C, 24 h). Expected mass: 66612 Da. Observed mass: 66613 Da. Data obtained with MS method B. Small peak at 66686 Da could be tentatively assigned as HSA-GSH dimer although this is unconfirmed.*

Second Order Kinetics of Amine Addition

Small Molecule Analysis

The pseudo first-order rate constant for amine addition to the bromothiomaleimide was obtained by reacting **6** with an excess of *p*-anisidine under buffered conditions. Four different experiments were carried out with 6, 8, 10 and 12 equiv. of *p*-anisidine. The progress of the reaction was monitored by NMR through integration of the starting material and product *N*-methyl groups. No other products were observed by NMR. This data was fitted using Graphpad Prism's first-order exponential association mode to give a pseudo first-order rate constant (k_{obs}) (Figure S32). By plotting k_{obs} against *p*-anisidine concentration, the second order rate constant could be obtained (Figure S33).



General protocol:

Bromothiomaleimide **6** (0.002 g, 0.0047 mmol) was dissolved in CD_3CN (250 µL). PB (700 µL, 100 mM phosphates, pH 6.75) was added to *p*-anisidine (6, 8, 10 or 12 equiv.) dissolved in CD_3CN (250 µL). The *p*-anisidine solution was then added to the solution of **6** and mixed thoroughly (the pH of the final solution was measured at 7.4) before NMR analysis.

Data:

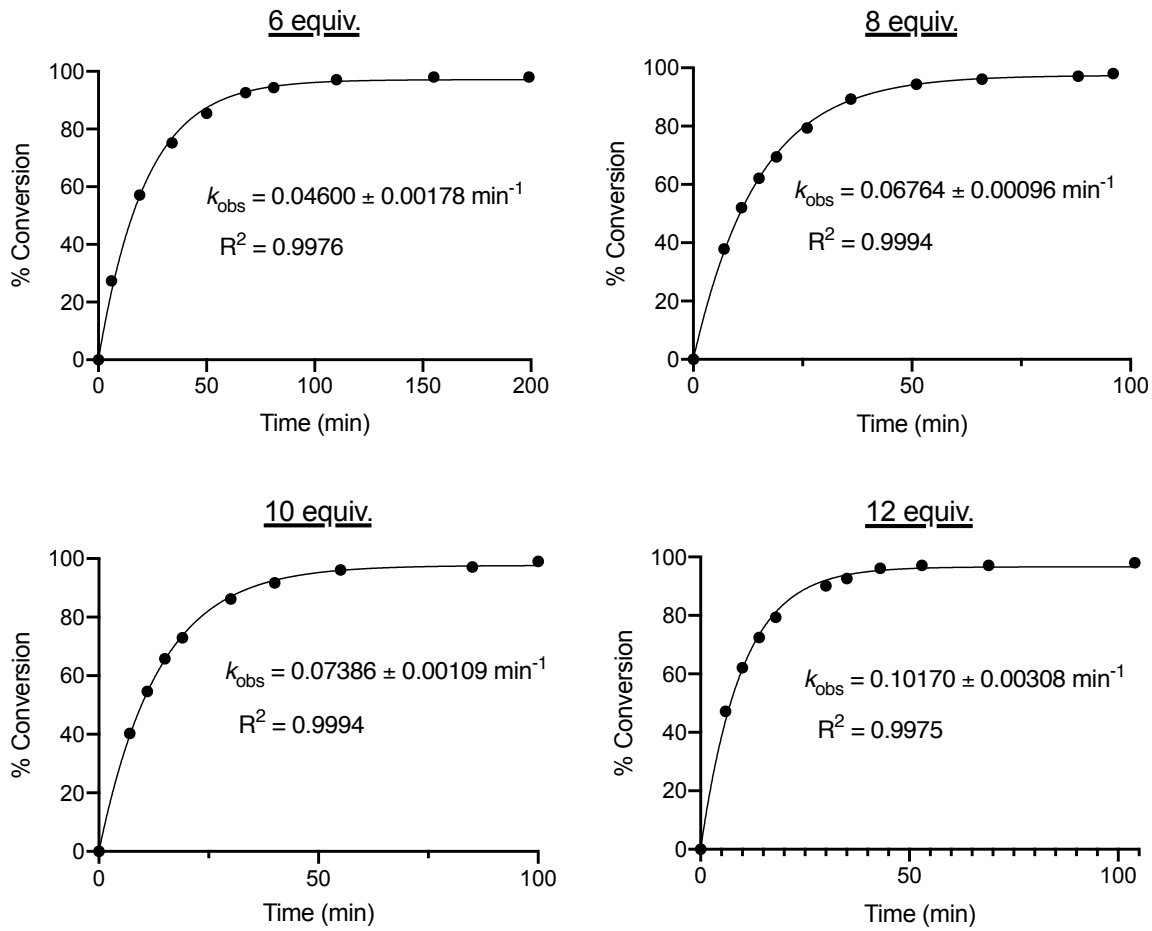


*Figure S32: NMR timecourse reactions between p-anisidine (in excess) and bromothiomaleimide **6** under buffered conditions (pH 7.4, 21 °C). The data point at the origin has been added afterwards for clarity.*

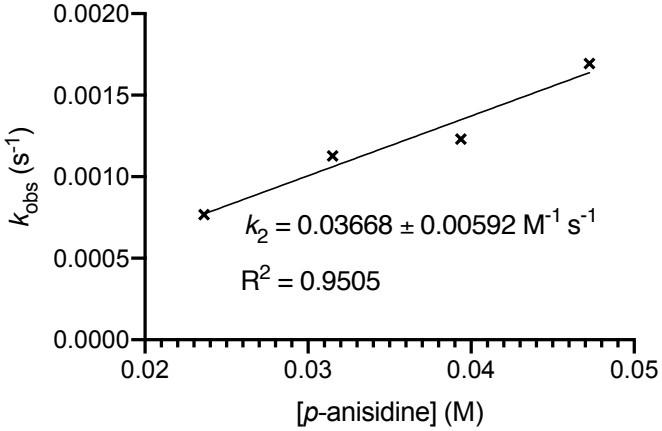


Figure S33: Plot of k_{obs} vs [p-anisidine]. The slope of the best-fit line gives the second order rate constant k_2. N.B. For this graph k_{obs} values from Fig. S32 were converted from min^{-1} to s^{-1}.

Example NMR data (12 equiv. experiment)

Below is NMR data (of alternate timepoints) from the reaction of **6** with 12 equiv. of *p*-anisidine. It has been included to show the reader the peak progression of the *N*-methyl peaks in the starting material and product. The ratio of integrals between these two peaks was used to calculate the progress of the reaction, in the manner of the references.[12,13]

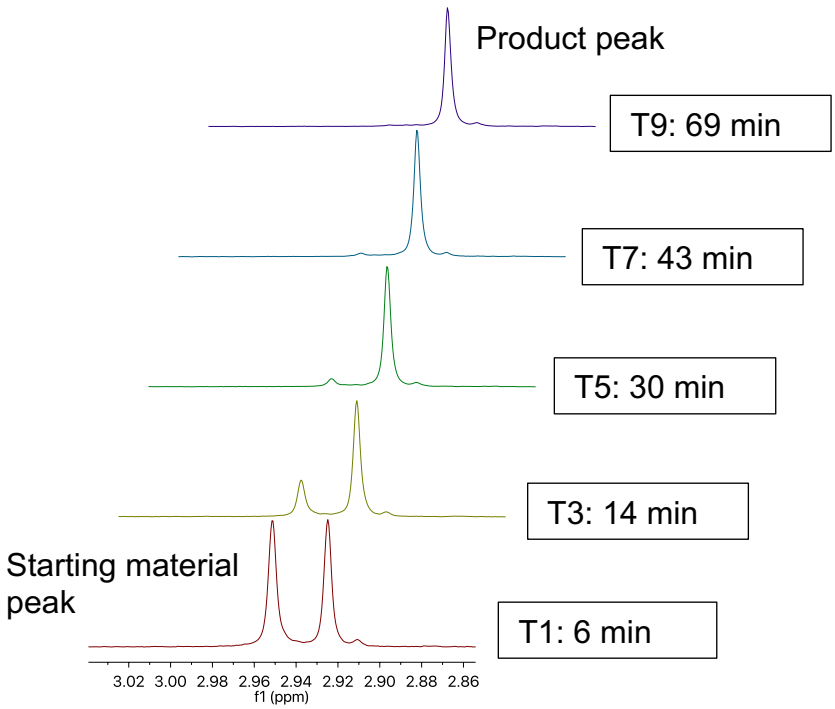
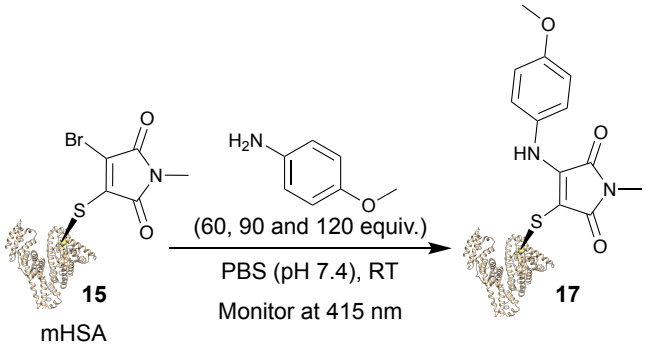


*Figure S34: Alternate NMR Timepoints for the 12 equiv. reaction with **6** showing the development of the N-Me peaks corresponding to the starting material and product.*

mHSA Protein Analysis

Firstly, the pseudo first-order rate constant for amine addition to the bromothiomaleimide **15** was obtained by reacting the **15** with an excess of *p*-anisidine under buffered conditions. Three different experiments were carried out with 60, 90 and 120 equiv. of *p*-anisidine. The progress of the reaction was monitored by UV through the increase in absorbance at 415 nm. This data was fitted using Graphpad Prism's first-order exponential association mode to give a pseudo first-order rate constant (k_{obs}) (Figure S35). By plotting k_{obs} against *p*-anisidine concentration, the second order rate constant k_2 could be obtained (Figure S36).



General protocol:

N-Me DBM **13** (5.31 µL, 1.0 equiv., 10 mM in DMSO) was added to mHSA (531 µL, 100 uM) in PBS (pH 7.4, 10 mM phosphates). The reaction mixture was left at room temperature for 30 min.

Aliquots of the freshly prepared conjugate **15** (160 µL, 99 µM) in PBS (pH 7.4, 10 mM phosphates) were removed from the fridge. DMSO was then added to the 60 and 90 equiv. reactions (4.8 µL and 2.4 µL for the 60 and 90 equiv. reactions respectively to ensure the final concentration of the protein remained the same for all reactions), before *p*-anisidine (0.2 M in DMSO, 120, 90 and 60 equiv.) was added. The reaction was mixed thoroughly and then added to the spectrophotometer. The absorbance at 415 nm was quickly zeroed and then the absorbance increase was monitored over time.

Data:

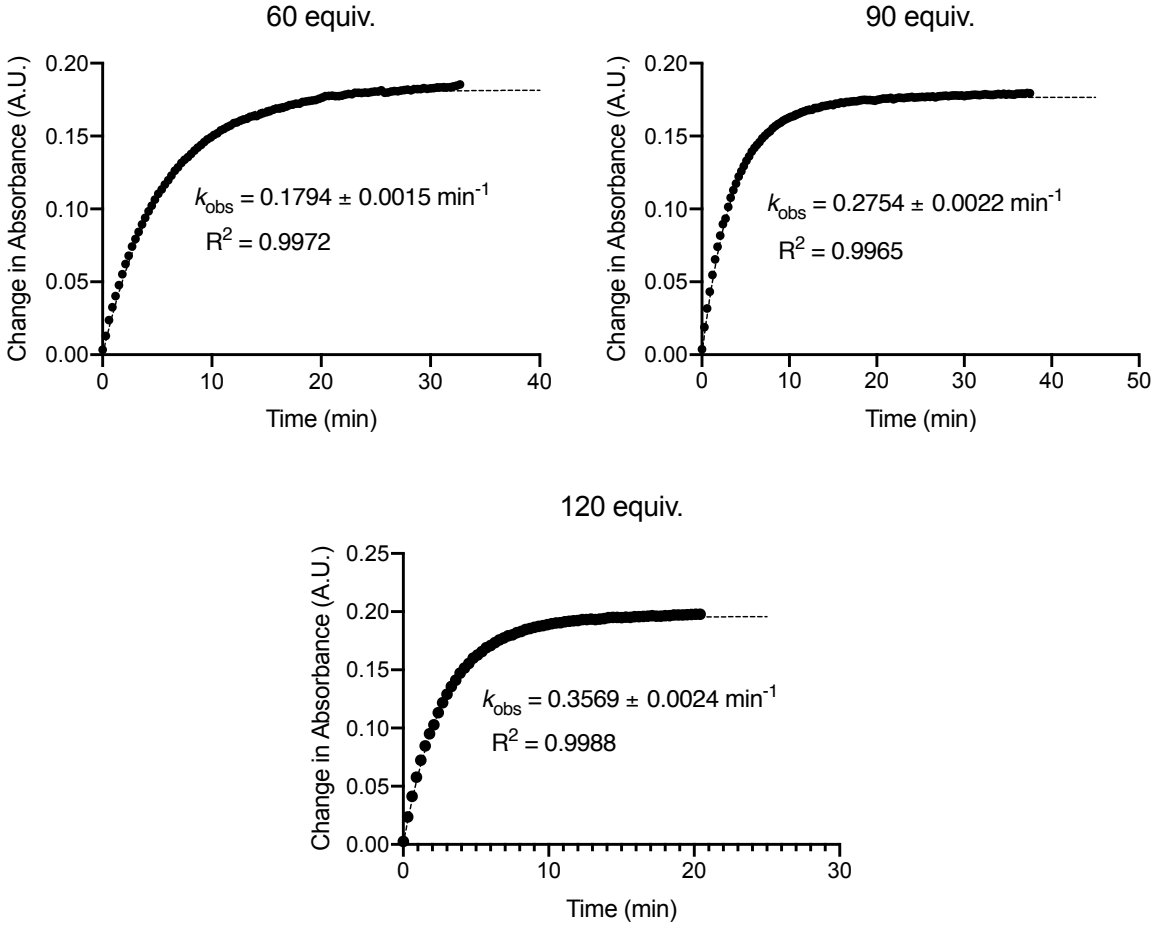


*Figure S35: UV kinetics data for the increase in absorbance at 415 nm upon addition of 60, 90 and 120 equiv. of p-anisidine to intermediate mHSA conjugate **15**.*

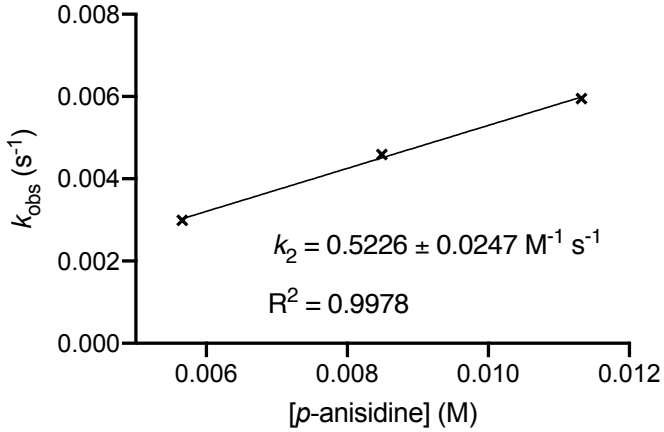


Figure S36: Plot of k_{obs} vs [p-anisidine]. The slope of the best-fit line gives the second order rate constant k_2. N.B. For this graph k_{obs} values from Fig. S35 were converted from min^{-1} to s^{-1}.

Additional Pseudo-first Order Data

The data from experiments shown in Figure S9 and Figure S16 (for WT-HSA and GFP reactions respectively) were used to plot the increase in absorbance at 415 nm, upon addition of amine, against time. Due to the large excess of amine in these reactions, the concentration could be treated as constant and thus a pseudo first-order analysis could be carried out. This data was fitted using Graphpad Prism's first-order exponential association mode to give a pseudo first-order rate constant (k_{obs}). Goodness of fit is shown as R^2. It should be noted that a functionalised DBM and amine were used in the GFP experiment in Figure S38.

WT-HSA

Conditions: *N*-Me DBM **13** (3.6 µL, 10 mM, 2.0 equiv. in DMSO) was added to WT HSA (100 µM, 180 µL) in PBS buffer (12 mM phosphates, pH 7.4) in a quartz cuvette and monitored by spectrophotometer at 25 °C. After the increase in absorbance at 375 nm had stopped (20 minutes), *p*-anisidine (2.9 µL, 3.2 M in DMSO) was added, and the reaction was returned to the spectrophotometer at 25 °C. The absorbance increase at 415 nm had stopped after a further 180 minutes (UV analysis was continued until minute 946), excess small molecule was removed (7 kDa MWCO, ZebaSpin®). Conjugates were then analysed by LCMS.

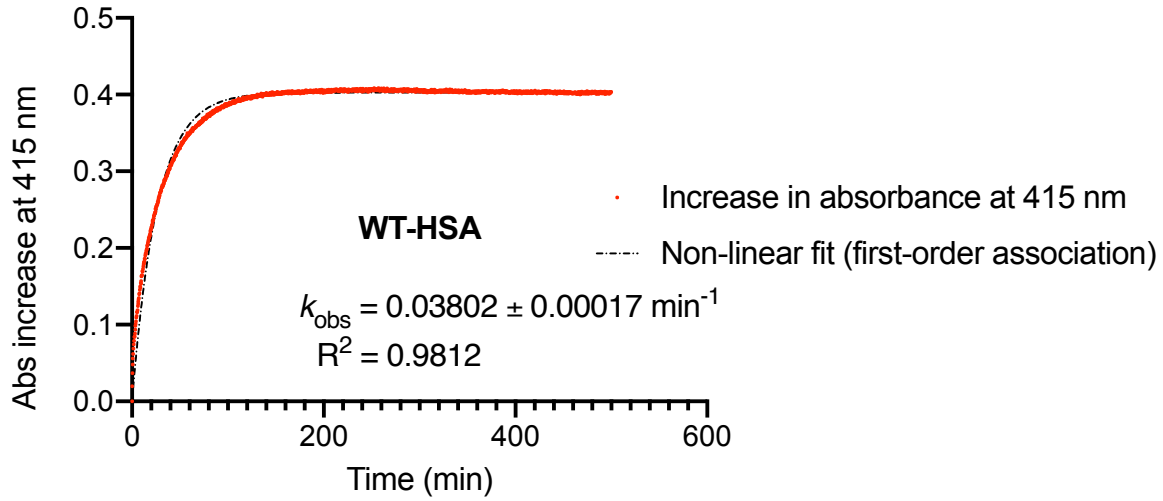


Figure S37: The increase in absorbance at 415 nm (after amine addition) plotted against time for WT-HSA, using data from the experiment shown Figure S9. Pseudo first-order rate constant and goodness of fit also shown. N.B. 500 min of data is plotted here, although the amine addition was monitored for a further 426 min.

GFP

Conditions: DBM **21** (1.44 µL, 10 mM, 2.0 equiv. in DMSO) was added to reduced GFP (40 µM, 180 µL) in PBS buffer (12 mM phosphates, pH 7.4, 5 mM EDTA) in a quartz cuvette and monitored by spectrophotometer at 25 °C. After the increase in absorbance at 375 nm had stopped (5 minutes), amine-azide **22** (2.16 µL, 0.1 M, 30 equiv. in DMSO) was added, and the reaction was returned to the spectrophotometer at 25 °C. When the absorbance increase at 415 nm had stopped (a further 95 minutes), excess small molecule was removed (7 kDa MWCO, ZebaSpin®), and the amino-thio conjugate was analysed by LCMS.

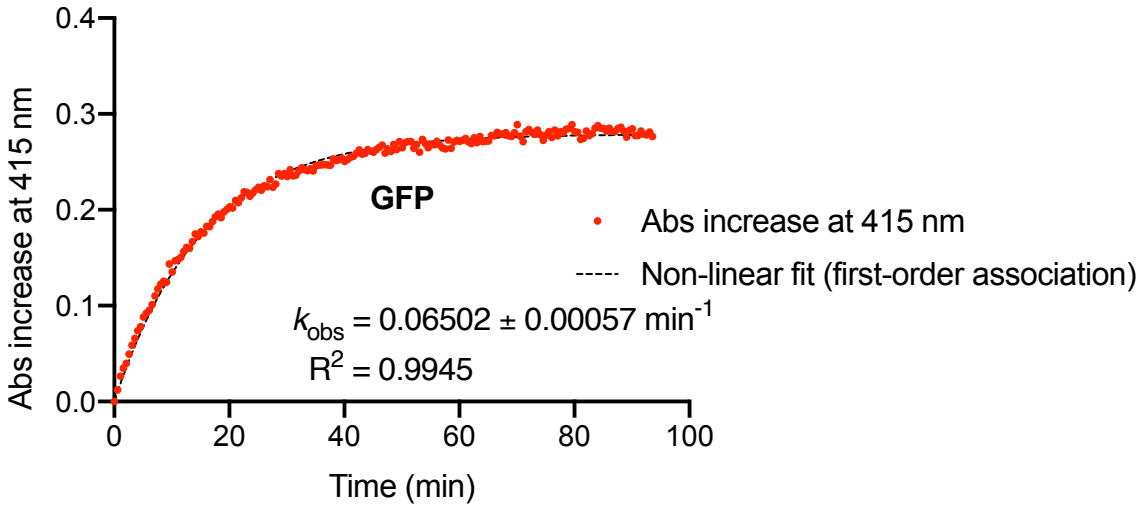


Figure S38: The increase in absorbance at 415 nm (after amine addition) plotted against time for mHSA, using data from the experiment shown in Figure S16. Pseudo first-order rate constant and goodness of fit also shown.

References

1 K. K. Schelde, K. Nicholls, F. Dagnæs-Hansen, K. Bunting, H. Rawsthorne, B. Andersen, C. J. A. Finnis, M. Williamson, J. Cameron and K. A. Howard, *J. Biol. Chem.*, 2019, **294**, 3735–3743.

2 L. Castañeda, Z. V. F. Wright, C. Marculescu, T. M. Tran, V. Chudasama, A. Maruani, E. A. Hull, J. P. M. Nunes, R. J. Fitzmaurice, M. E. B. Smith, L. H. Jones, S. Caddick and J. R. Baker, *Tetrahedron Lett.*, 2013, **54**, 3493–3495.

3 M. Dubernet, V. Caubert, J. Guillard and M. C. Viaud-Massuard, *Tetrahedron*, 2005, **61**, 4585–4593.

4 K. Zhang, J. Liu, S. Ohashi, X. Liu, Z. Han and H. Ishida, *J. Polym. Sci. Part A Polym. Chem.*, 2015, **53**, 1330–1338.

5 K. M. Bonger, R. J. B. H. N. van den Berg, L. H. Heitman, A. P. IJzerman, J. Oosterom, C. M. Timmers, H. S. Overkleeft and G. A. van der Marel, *Bioorganic Med. Chem.*, 2007, **15**, 4841–4856.

6 K. Dao, R. R. Sawant, J. A. Hendricks, V. Ronga, V. P. Torchilin and R. N. Hanson, 2012, **23**, 785–795.

7 G. Burgy, T. Tahtouh, E. Durieu, B. Foll-Josselin, E. Limanton, L. Meijer, F. Carreaux and J. P. Bazureau, *Eur. J. Med. Chem.*, 2013, **62**, 728–737.

8 L. Boddu, V. Potlapati and N. J. P. Subhashini, *J. Heterocycl. Chem.*, 2019, **56**, 3197–3205.

9 W. Zhang, Z. Li, M. Zhou, F. Wu, X. Hou, H. Luo, H. Liu, X. Han, G. Yan, Z. Ding and R. Li, *Bioorganic Med. Chem. Lett.*, 2014, **24**, 799–807.

10 P. Moody, M. E. B. Smith, C. P. Ryan, V. Chudasama, J. R. Baker, J. Molloy and S. Caddick, *ChemBioChem*, 2012, **13**, 39–41.

11 R. L. Levine, J. Moskovitz and E. R. Stadtman, *IUBMB Life*, 2000, **50**, 301–307.

12 J. M. Baskin, J. A. Prescher, S. T. Laughlin, N. J. Agard, P. V. Chang, I. A. Miller, A. Lo, J. A. Codelli and C. R. Bertozzi, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 16793–16797.

13 X. Ning, J. Guo, M. A. Wolfert and G. J. Boons, *Angew. Chem. Int. Ed.*, 2008, **47**, 2253–2255.