Electronic Supplementary Information

Optimised approach to albumin-drug conjugates using monobromomaleimide-C2 linkers

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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 (HSA) was provided by Albumedix (Recombumin® Elite, batch no. AX190201). 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. Borate-buffered saline (BBS) was 25 mM Sodium Borate, 25 mM NaCl and 1 mM EDTA at pH 8.0.

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 ^{o}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 III 600 spectrometer equipped with a DCH cryoprobe. ^{1}H experiments were carried out 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.

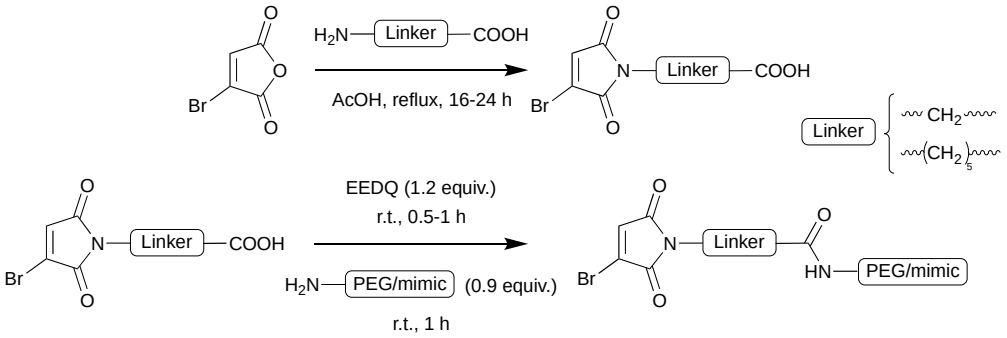
Protein mass spectra were recorded with an LC system connected to an Agilent 6510 Q-TOF spectrometer. A PLRP-s, 1000 Å, 8 µM column was used.

Fluorescence analysis was carried out with a Cary Fluorescence spectrophotometer and recorded with Cary Fluorescence kinetics software. A quartz fluorescence cuvette was used to hold samples. All fluorescence analyses were carried out at room temperature. All samples were baseline corrected.

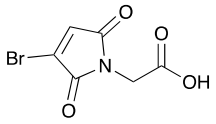
UV analysis of hydrolysis was carried out with either a Cary UV spectrophotometer and recorded with Cary WinUV software, or a Shimadzu UV-2600 spectrophotometer with UVprobe software. A quartz cuvette with a 1 cm path length was used to hold samples. Unless stated, all UV experiments were carried out at room temperature. All samples were baseline corrected.

Synthesis of Compounds

General synthetic scheme for MBM-linker-PEG derivatives:

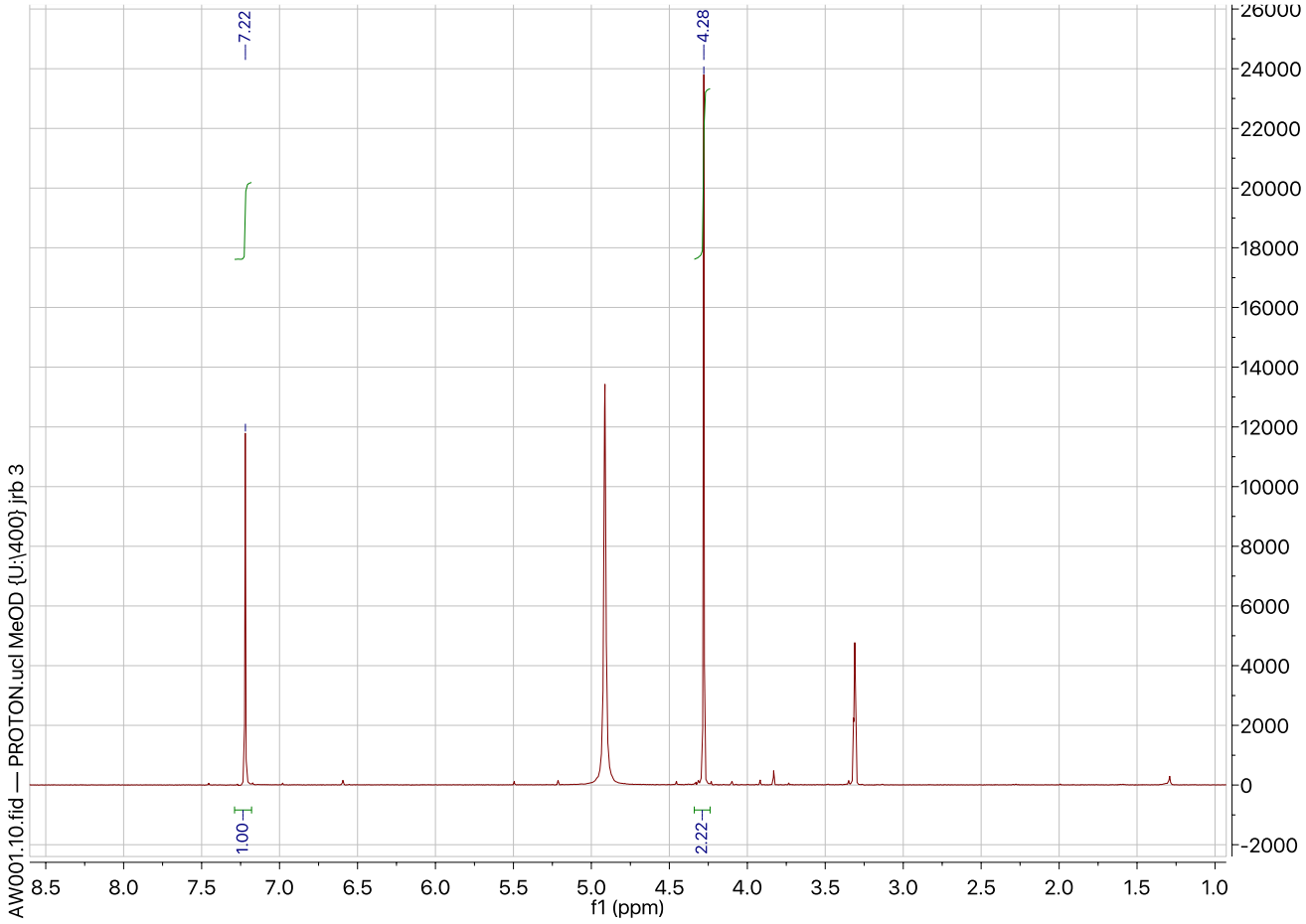


2-(3-bromo-2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)acetic acid **11**



Method adapted from reference.[1] Glycine (203 mg, 2.66 mmol) was added to a stirred solution of bromomaleic anhydride (469 mg, 2.66 mmol) in AcOH (15 mL) and the reaction was stirred at 150 °C for 24 h. The solvent was removed *in vacuo* and the residue purified by column chromatography (3-10% MeOH/DCM) to afford the product as a white solid (481 mg, 2.05 mmol, 77%):

m.p. 154-155°C; ^{1}H NMR (400 MHz, MeOD); δ 7.22 (s, 1H, CBrCH), 4.28 (s, 2H, NCH_2); ^{13}C NMR (75 MHz, MeOD) δ 169.2 (CO), 167.9 (CO), 164.9 (CO), 132.5 (CH), 130.9 (CBr), 38.6 (CH_2); IR (solid) 3090 (m), 2999 (w), 2879 (br), 1704 (s) cm^{-1}; LRMS (ES^-) 232 (100, $[M^{79}Br\text{-}H]^-$), 234 (100, $[M^{81}Br\text{-}H]^-$); HRMS (EI) calcd for $C_6H_4NO_4Br$ $[M^{79}Br\text{-}H]^-$ 231.9245, observed 231.9242.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2.22
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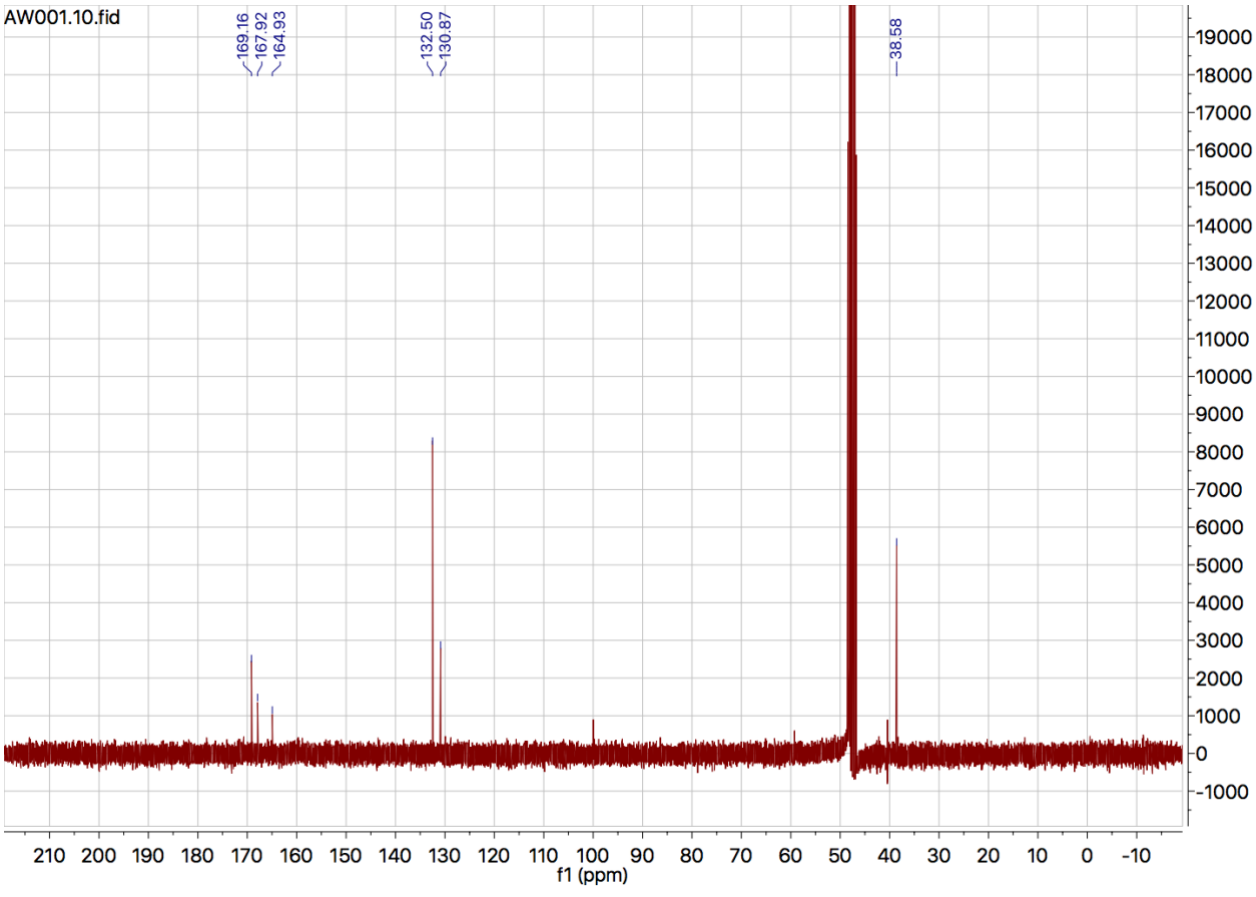
f1 (ppm)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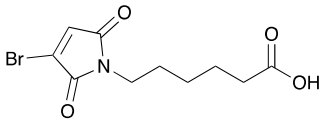
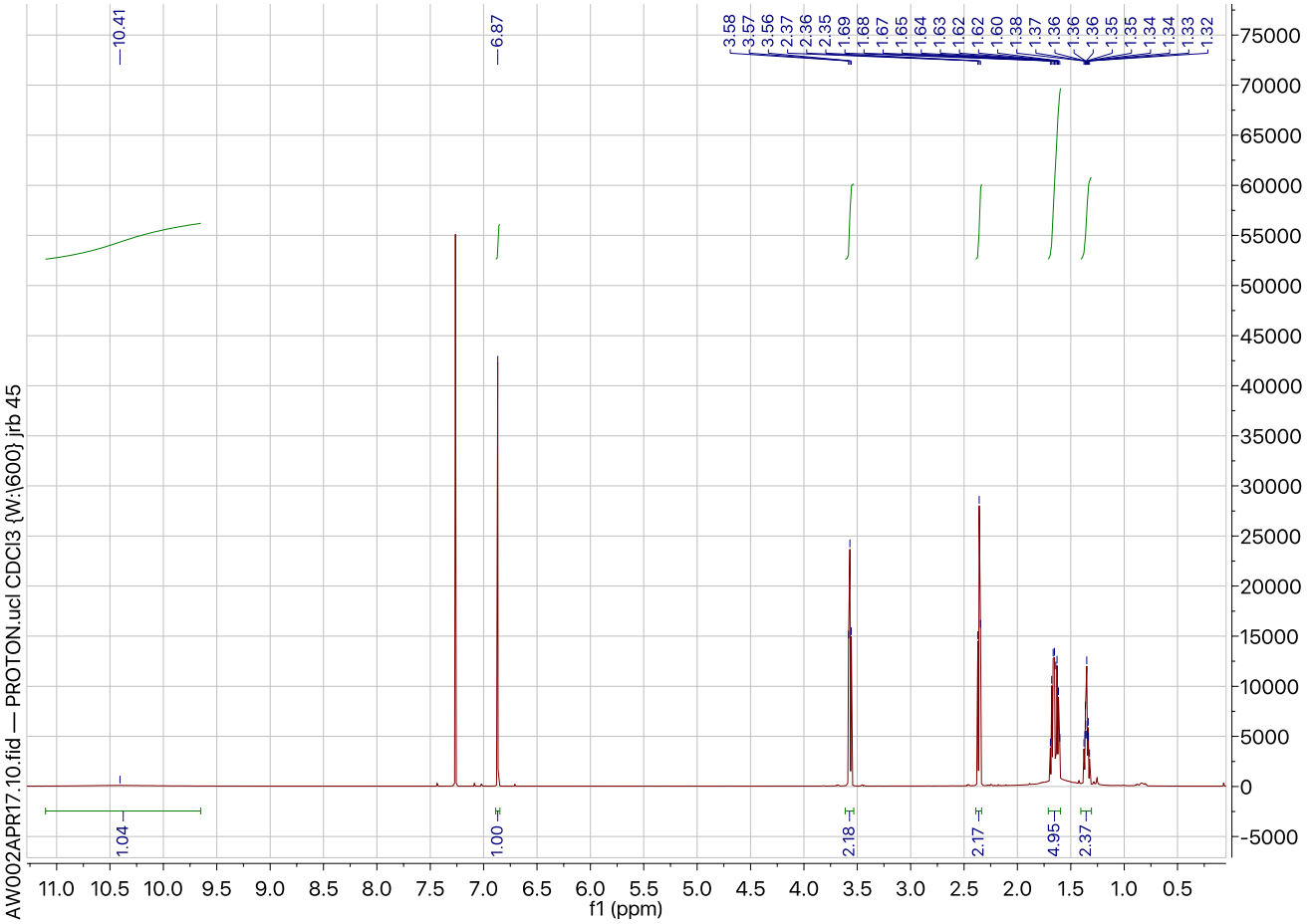
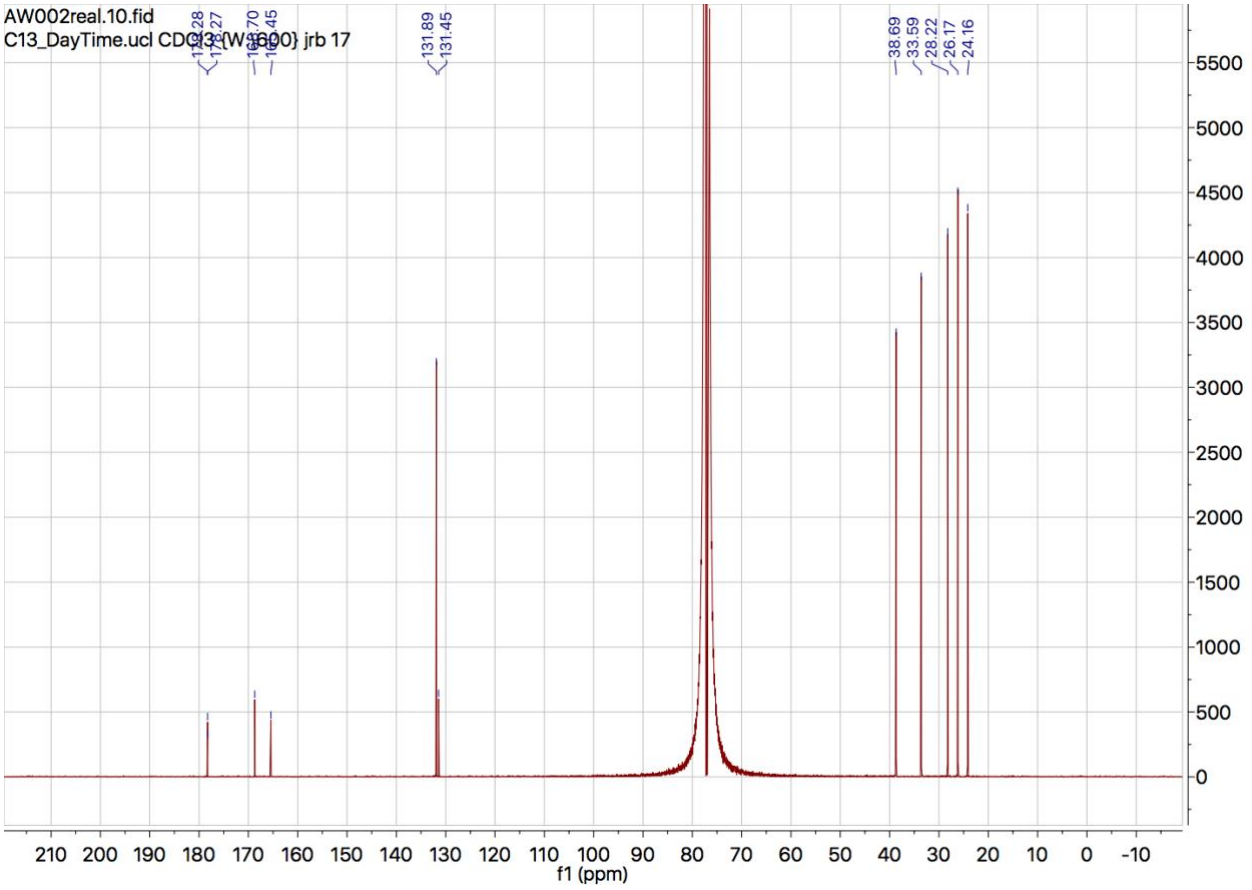
6-(3-bromo-2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoic acid **14**



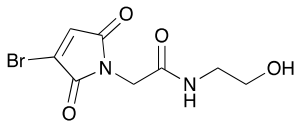
Method adapted from reference.[1]4-aminocaproic acid (394 mg, 3.00 mmol) was added to a stirred solution of bromomaleic anhydride (584 mg, 3.30 mmol) in AcOH (16 mL) and the reaction was stirred at 150 °C for 24 h. The solvent was removed *in vacuo* and the residue purified by column chromatography (3-5% MeOH/DCM) giving the title compound as a white solid (695 mg, 2.39 mmol, 80%):

m.p. 98-99 °C; ^{1}H NMR (600 MHz, $CDCl_3$); δ 10.58 (s, 1H, (COOH), 6.87 (s, 1H, CBrCH), 3.57 (t, *J* = 7.2 Hz, 2H, NCH_2), 2.36 (t, *J* = 7.4 Hz, 2H, CH_2COOH), 1.68 – 1.60 (m, 4H, (*H_2O underneath) $NCH_2CH_2CH_2CH_2$) 1.39-1.31 (m, 2H, $NCH_2CH_2CH_2$); ^{13}C NMR (151 MHz, $CDCl_3$); δ 178.3 (CO), 168.7 (CO), 165.5 (CO), 131.9 (CH), 131.5 (BrC), 38.7 (CH_2), 33.6 (CH_2), 28.2 (CH_2), 26.2 (CH_2), 24.2 (CH_2; IR (solid) 3100 (m), 3090 (m), 2934 (br), 1701 (s) cm^{-1}; LRMS (ES^+) 290 (100, $[M^{79}Br+H]^+$), 292 (95, $[M^{81}Br+H]^+$); HRMS (ES^+) calcd for $C_{10}H_{12}NO_4Br$ $[M^{79}Br+H]^+$ 290.0028, observed 290.0029.



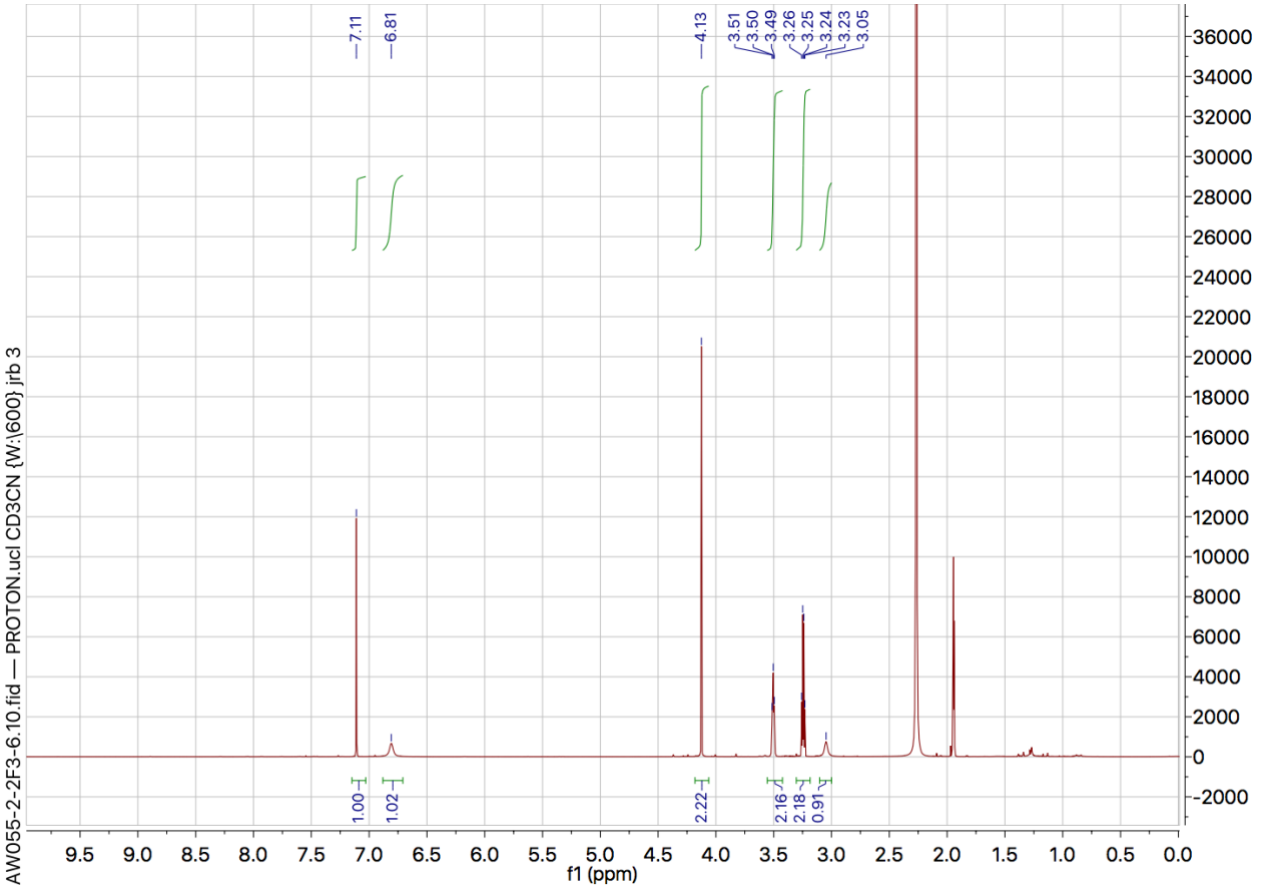


2-(3-bromo-2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-N-(2-hydroxyethyl)acetamide **17**



Method adapted from reference.[2] Bromomaleimide **11** (100 mg, 0.430 mmol) was added to a stirred solution of EEDQ (160 mg, 0.650 mmol) in MeCN (20 mL) and the reaction was stirred at room temperature for 30 min. Ethanolamine (23.0 mg, 0.380 mmol) was then added, followed by DIPEA (53.0 mg, 0.410 mmol) and the reaction was stirred for a further 90 min. The solvent was evaporated *in vacuo* and purification of the residue with column chromatography (50% EtOAc/Petrol then 5-10% MeOH/EtOAc) gave the product as a yellow/orange solid (37.0 mg, 0.130 mmol, 31%):

m.p. 130-131 °C; ^{1}H NMR (600 MHz, CD_3CN); δ 7.11 (s, 1H, CBrCH), 6.81 (s, 1H, NH), 4.13 (s, 2H, NCH_2), 3.50 (t, *J* = 5.5 Hz, 2H, CH_2OH), 3.24 (q, *J* = 5.6 Hz, 2H, $NHCH_2$), 3.05 (s, 1H, OH); ^{13}C NMR (151 MHz, CD_3CN); δ 169.4 (CO), 167.4 (CO), 166.4 (CO), 133.8 (CH), 131.9 (CBr), 61.3 (CH_2), 42.9 (CH_2), 41.7 (CH_2); IR (solid) 3310 (br), 3091 (m), 1720 (s), 1662 (s) cm-1; LRMS (ES^+) 277 (90, $[M^{79}Br+H]^+$), 279 (100, $[M^{81}Br+H]^+$); HRMS (ES^+) calcd for $C_8H_{10}N_2O_4SBr$ $[M^{79}Br+H]^+$ 276.9824 observed 276.9819.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6.81
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3.51
3.50
3.49
3.26
3.25
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3.23
3.05
1.00
1.02
2.22
2.16
2.18
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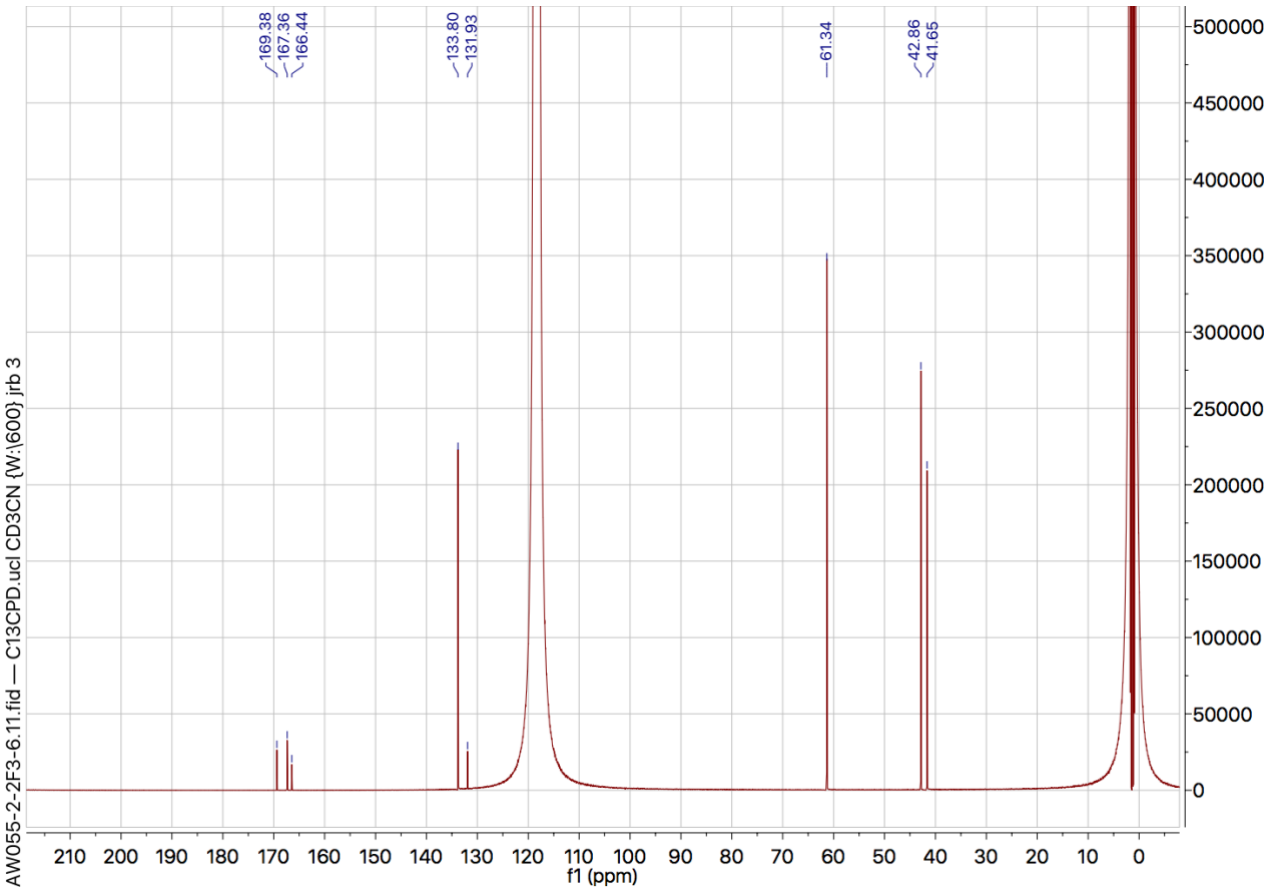
f1 (ppm)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41.65
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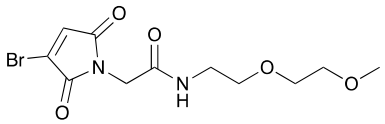
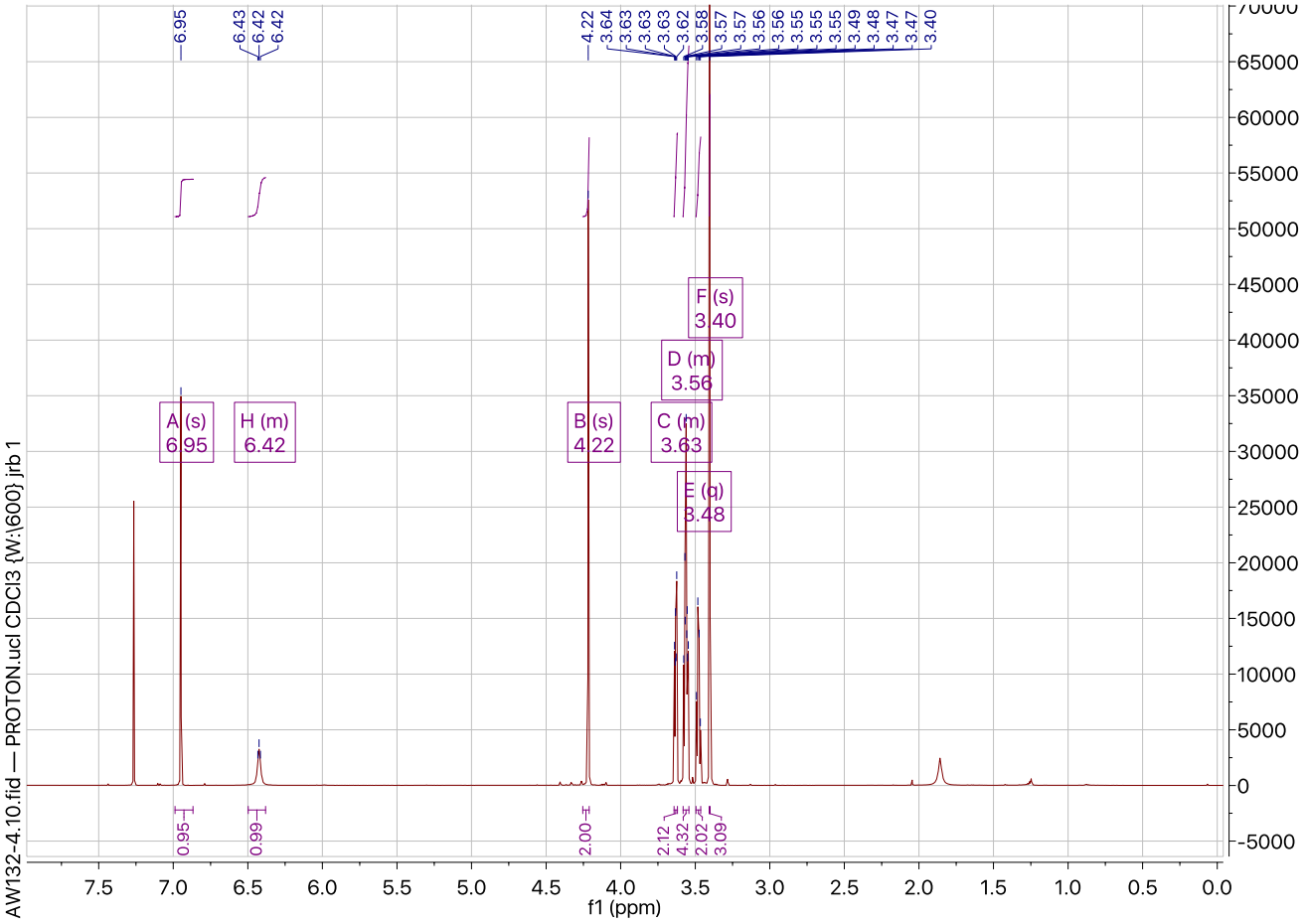
f1 (ppm)

2-(3-bromo-2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-N-(2-(2-methoxyethoxy)ethyl)acetamide **6**



Method adapted from reference.[2] Bromomaleimide **11** (100 mg, 0.427 mmol) was added to a stirred solution of EEDQ (128 mg, 0.513 mmol) in MeCN (10 mL) and the reaction was stirred at room temperature for 1 hr. 2-(2-methoxyethoxy)ethan-1-amine (46.0 mg, 0.385 mmol) was then added, and the reaction was stirred for a further hour before the solvent was removed *in vacuo*. The molecule was purified by column chromatography (0-100% EtOAc in petroleum ether). The solvent was removed *in vacuo* and the product was re-dissolved in EtOAc (10 mL), washed with 10% citric acid (2 x 5 mL), water (1 x 5 mL), Brine (1 x 5 mL) and dried with $MgSO_4$. The product was collected as a clear-brown oil (31.0 mg, 0.0940 mmol, 63%):

^{1}H NMR (600 MHz, $CDCl_3$) δ 6.95 (s, 1H, CH), 6.50 – 6.38 (m, 1H, NH), 4.22 (s, 2H, NCH_2), 3.64 – 3.62 (m, 2H, CH_2), 3.58 – 3.54 (m, 4H, 2 x CH_2), 3.48 (q, *J* = 5.2 Hz, 2H, CH_2), 3.40 (s, 3H, CH_3); ^{13}C NMR (151 MHz, $CDCl_3$) δ 168.1 (CO), 165.5 (CO), 165.1 (CO), 132.3 (CH), 131.8 (CBr), 72.0 (CH_2), 70.3 (CH_2), 69.7 (CH_2), 59.1 (CH_3), 41.1 (CH_2), 39.7 (CH_2); IR (solid) 3312 (w), 2877 (w), 1714 (s), 1669 (s) cm^{-1}; LRMS (NSI) 335 (100, $[M^{79}Br+H]^+$), 337 (100, $[M^{81}Br+H]^+$), 357 (75, $[M^{79}Br+Na]^+$), 359 (75, $[M^{81}Br+Na]^+$), 367 (70, $[M^{79}Br+H+CH_3OH]^+$), 369 (70, $[M^{81}Br+H+CH_3OH]^+$); HRMS (NSI) calcd for $C_{11}H_{15}N_2O_5Br$ $[M^{79}Br+H]^+$ 335.0237, observed 335.0240.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6.42
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3.64
3.63
3.63
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3.58
3.57
3.57
3.56
3.56
3.55
3.55
3.55
3.49
3.48
3.47
3.47
3.40
A (s) 6.95
H (m) 6.42
B (s) 4.22
C (m) 3.63
D (m) 3.56
F (s) 3.40
E (q) 3.48
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0.99
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2.02
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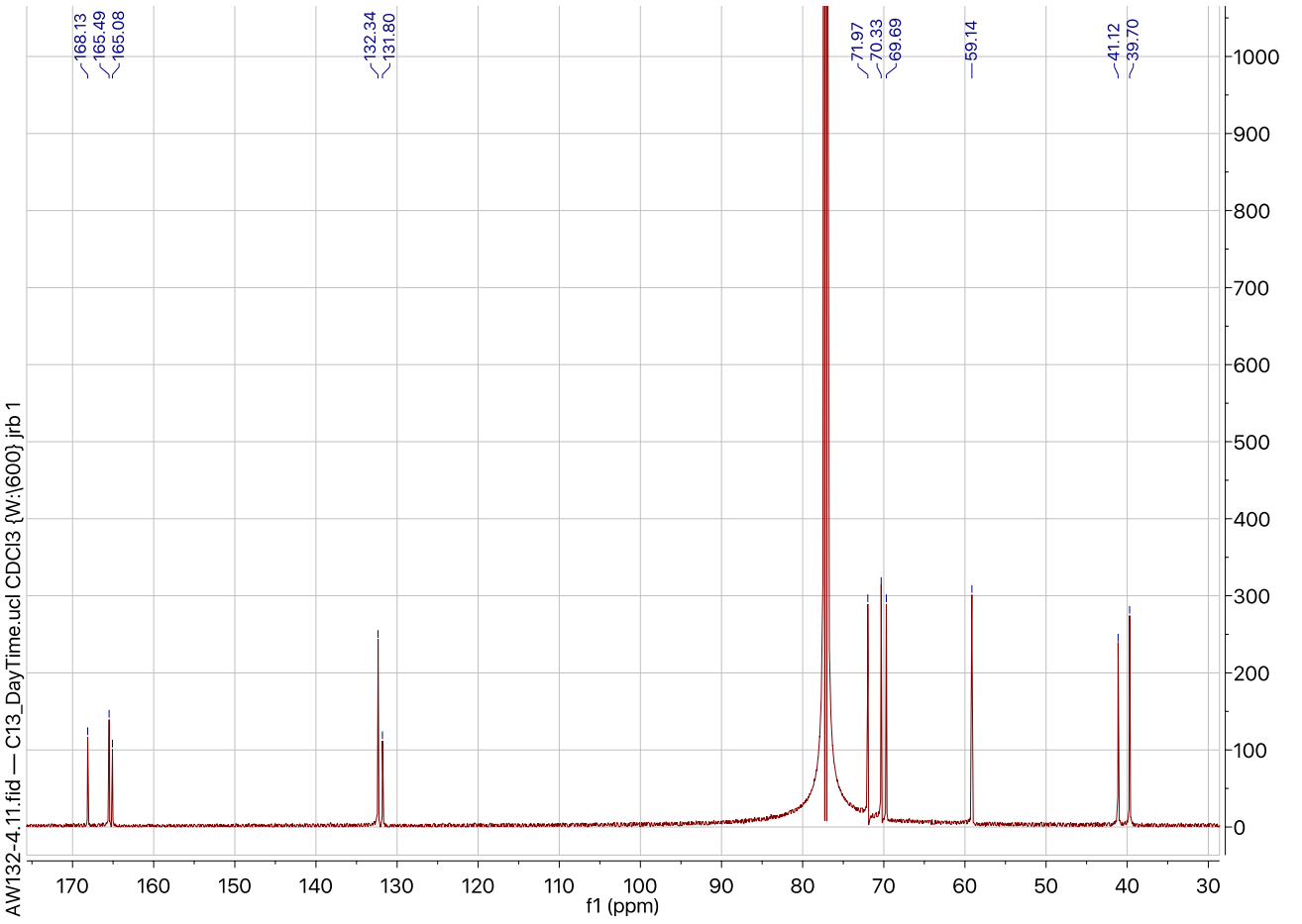
f1 (ppm)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69.69
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39.70
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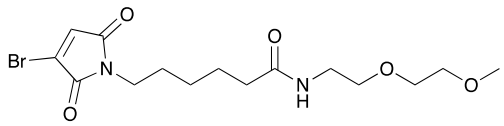
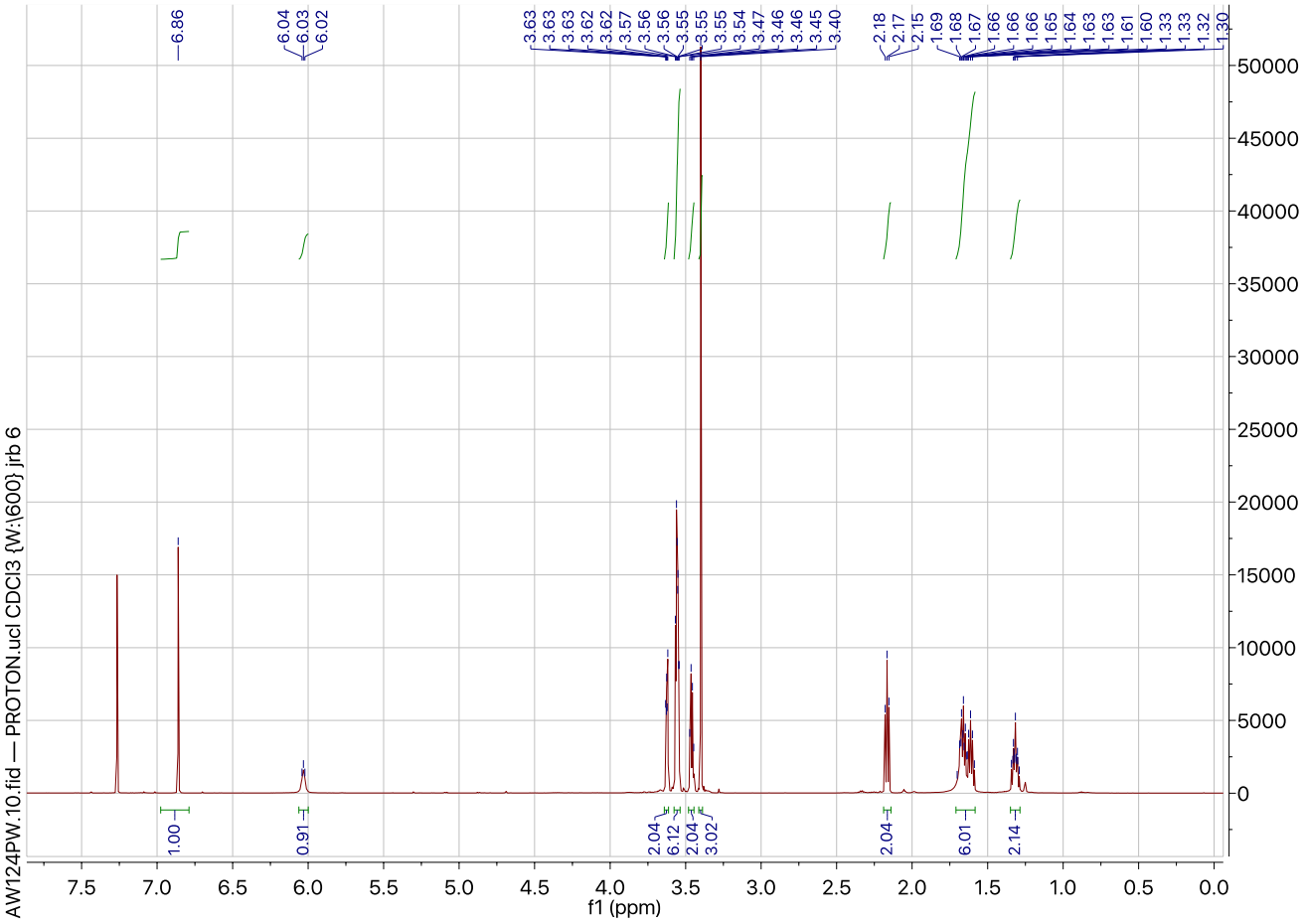
f1 (ppm)

6-(3-bromo-2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-N-(2-(2-methoxyethoxy)ethyl)hexanamide **18**



Method adapted from reference.[2] Bromomaleimide **14** (100 mg, 0.345 mmol) was added to a stirred solution of EEDQ (103 mg, 0.414 mmol) in MeCN (8 mL) and the reaction was stirred at room temperature for 1 hr. 2-(2-methoxyethoxy)ethan-1-amine (369 mg, 0.310 mmol) was then added, and the reaction was stirred for a further hour before the solvent was removed *in vacuo*. The molecule was purified by column chromatography (20-100% EtOAc in petroleum ether, then 0-20% MeOH in EtOAc). The solvent was removed *in vacuo* and the product was re-dissolved in EtOAc (10 mL), washed with 10% citric acid (2 x 5 mL), water (1 x 5 mL), Brine (1 x 5 mL) and dried with $MgSO_4$. The product was collected as a brown oil (7.00 mg, 0.0180 mmol, 5%).

^{1}H NMR (600 MHz, $CDCl_3$) δ 6.86 (s, 1H, CH), 6.04 (d, *J* = 5.9 Hz, 1H, NH), 3.64 – 3.61 (m, 2H, CH_2), 3.57 – 3.54 (m, 6H, 3 x CH_2), 3.46 (q, *J* = 5.3 Hz, 2H, CH_2), 3.40 (s, 3H, CH_3), 2.17 (t, *J* = 7.5 Hz, 2H, CH_2), 1.70 – 1.58 (m, 4H (*H_2O underneath), 2 x CH_2), 1.36 – 1.29 (m, 2H, CH_2); ^{13}C NMR (151 MHz, $CDCl_3$) δ 172.8 (CO), 168.7 (CO), 165.5 (CO), 131.9 (CH), 131.4 (BrC), 72.0 (CH_2), 70.3 (CH_2), 70.1 (CH_2), 59.2 (CH_3), 39.2 (CH_2), 38.8 (CH_2), 36.5 (CH_2), 28.4 (CH_2), 26.4 (CH_2), 25.2 (CH_2); LRMS (ES^+) 391 (95, $[M^{79}Br+H]^+$), 393 (92, $[M^{81}Br+H]^+$), 413 (100, $[M^{79}Br+Na]^+$), 415 (100, $[M^{81}Br+Na]^+$); HRMS (ES^+) calcd for $C_{15}H_{23}N_2O_5Br$ $[M^{79}Br+H]^+$ 391.0863, observed 391.0865.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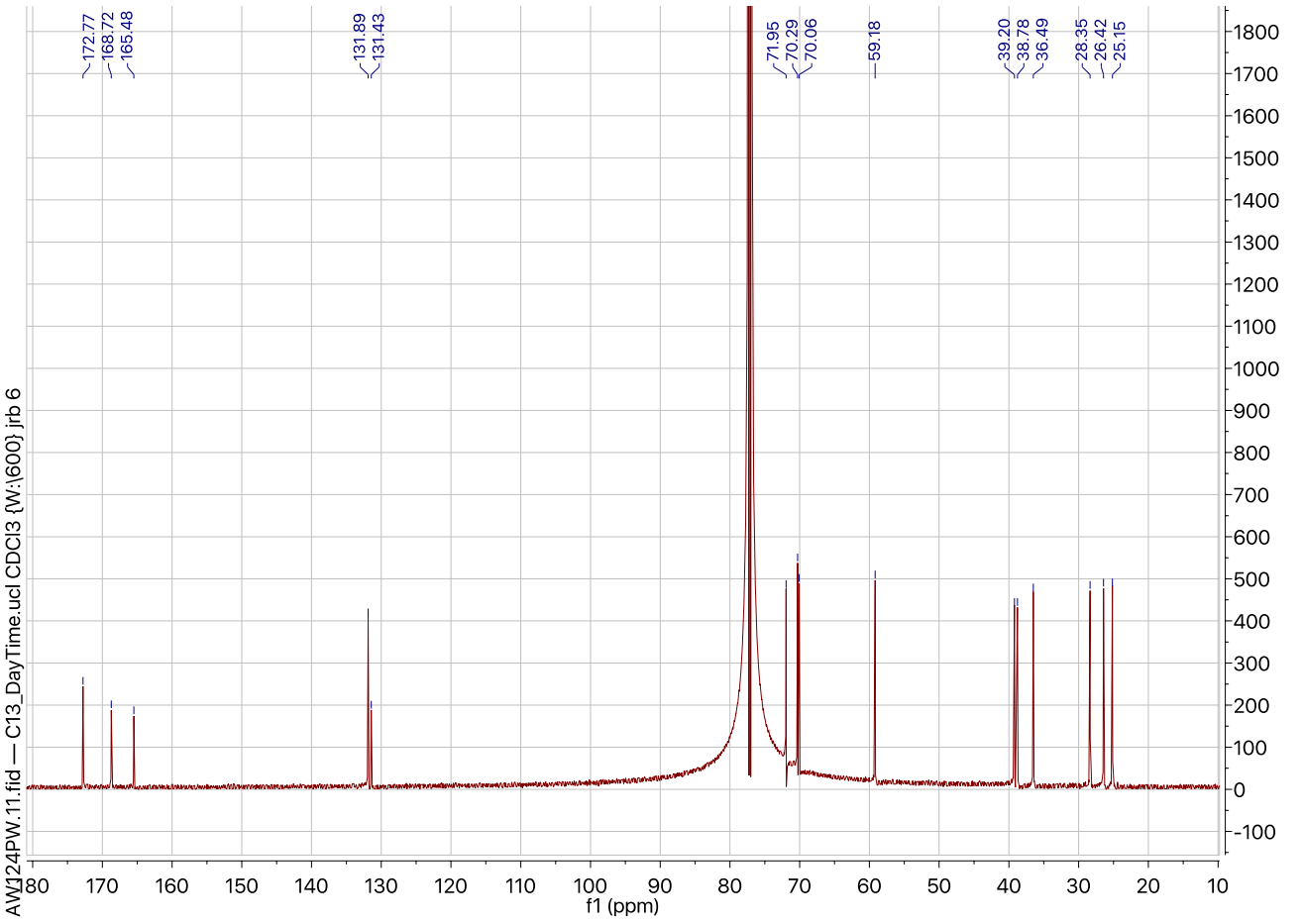
f1 (ppm)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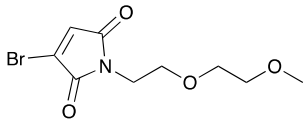
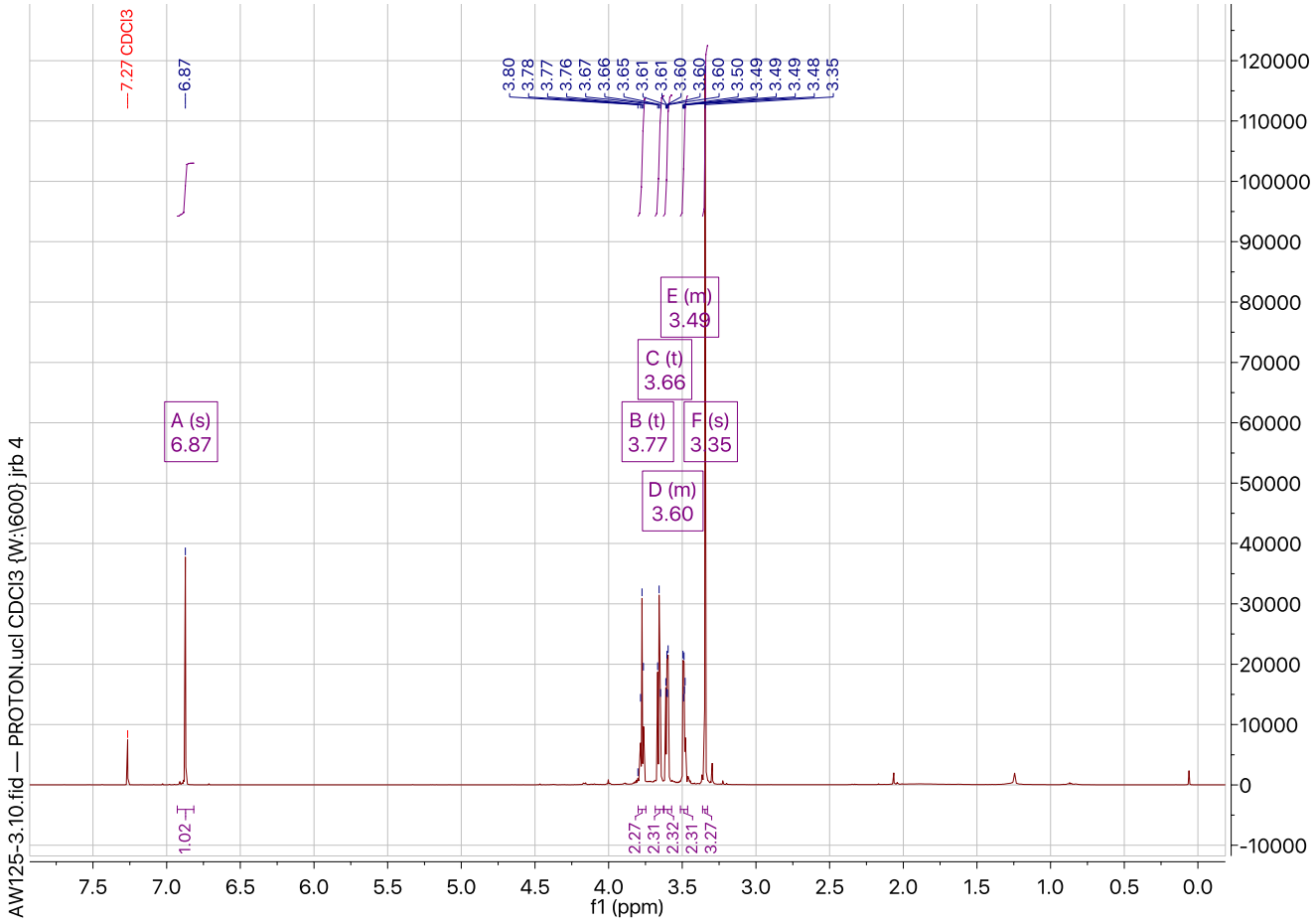
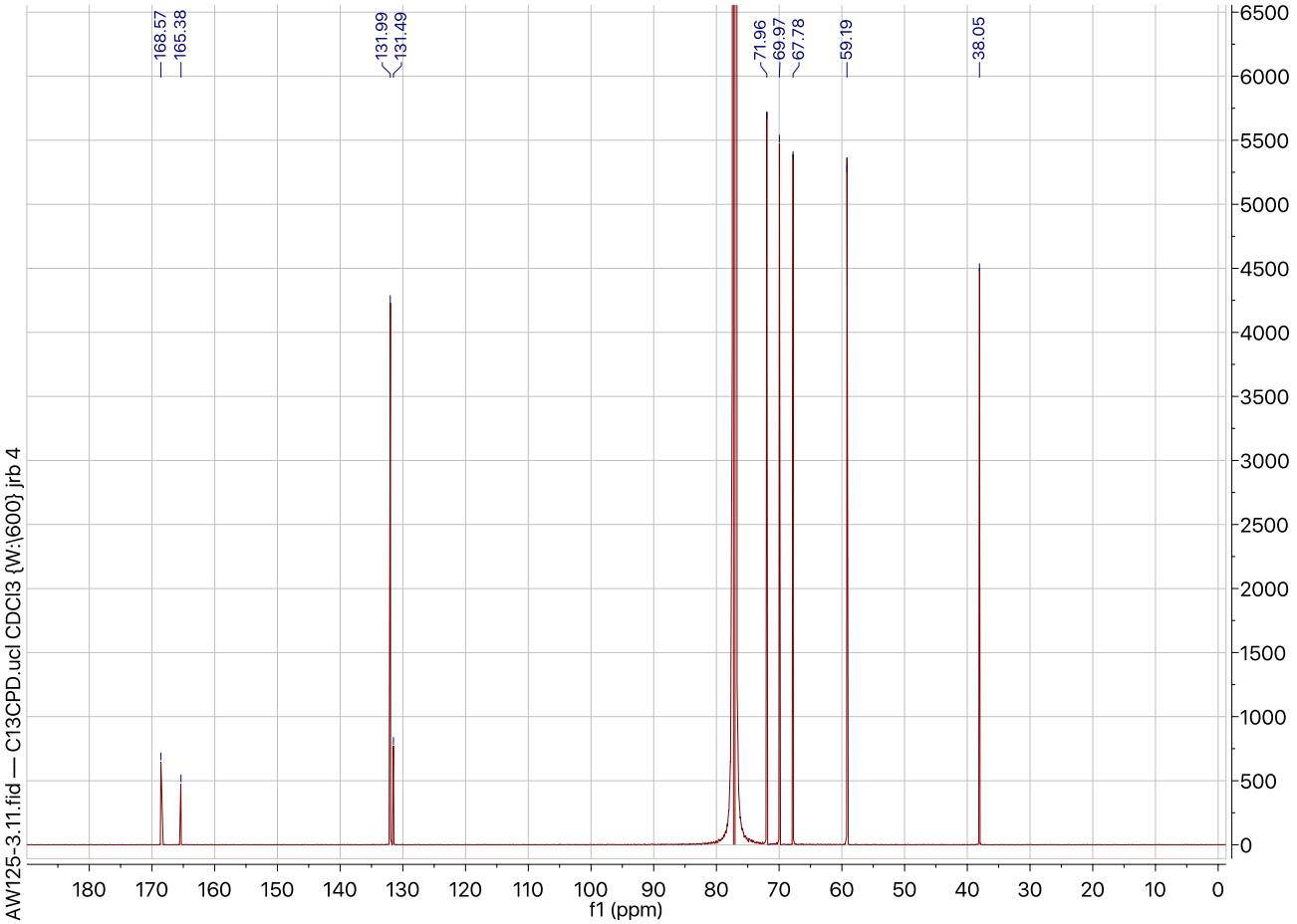
3-bromo-1-(2-(2-methoxyethoxy)ethyl)-1H-pyrrole-2,5-dione **19**



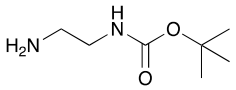
Method adapted from reference.[1] 2-(2-methoxyethoxy)ethan-1-amine (60.0 mg, 0.500 mmol) was added to a stirred solution of bromomaleic anhydride (106 mg, 0.600 mmol) in AcOH (12 mL) and the reaction was stirred at 150 °C for 24 h. The solvent was removed *in vacuo* and the residue purified by column chromatography (0-100% EtOAc in petroleum ether) giving the product as a yellow oil (50.0 mg, 0.181 mmol, 36%):

^{1}H NMR (600 MHz, $CDCl_3$) δ 6.87 (s, 1H, CH), 3.77 (t, *J* = 5.7 Hz, 2H, CH_2), 3.66 (t, *J* = 5.7 Hz, 2H, CH_2), 3.63 – 3.57 (m, 2H, CH_2), 3.51 – 3.46 (m, 2H, CH_2), 3.35 (s, 3H, CH_3).^{13}C NMR (151 MHz, $CDCl_3$) δ 168.6 (CO), 165.4 (CO), 132.0 (CH), 131.5 (BrC), 72.0 (CH_2), 70.0 (CH_2), 67.8 (CH_2), 59.2 (CH_3), 38.1 (CH_2); IR (solid) 3099 (w), 2876 (m), 1709 (s) cm^{-1}; LRMS (ES^+) 278 (100, $[M^{79}Br+H]^+$), 280 (100, $[M^{81}Br+H]^+$), 295 (85, $[M^{79}Br+NH_4]^+$), 297 (85, $[M^{81}Br+NH_4]^+$), 310 (60, $[M^{79}Br+H+CH_3OH]^+$), 312 (60, $[M^{81}Br+H+CH_3OH]^+$), 332 (80, $[M^{79}Br+H+CH_3OH+Na]^+$), 334 (75, $[M^{81}Br+H+CH_3OH+Na]^+$); HRMS (ES^+) calcd for $C_9H_{12}NO_4Br$ $[M^{79}Br+H]^+$ 278.0022, observed 278.0026.



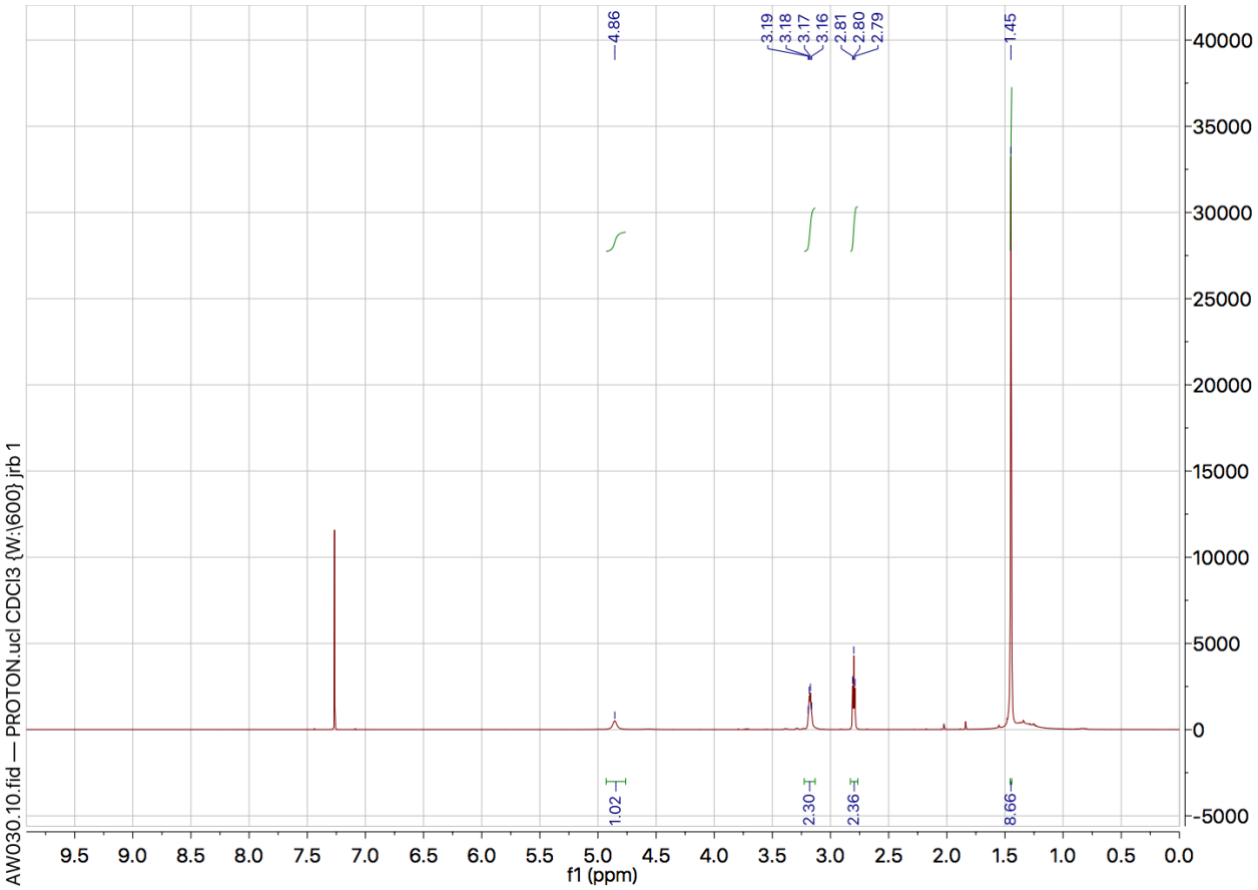

168.57
165.38
131.99
131.49
71.96
69.97
67.78
59.19
38.05
AW125-3.11.fid — C13CPD.ucl CDCl3 {W:\600} jrb 4
f1 (ppm)

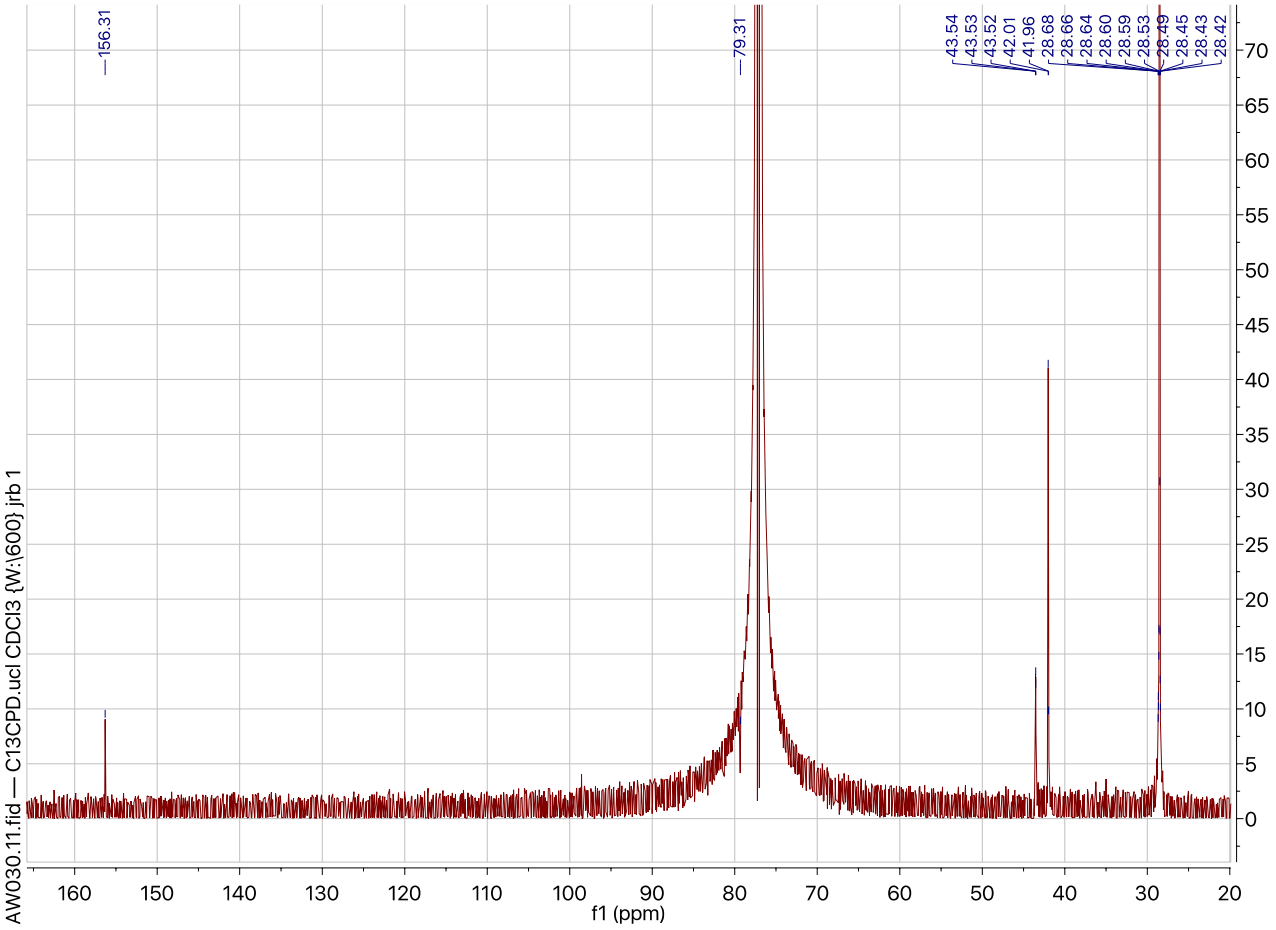
tert-butyl (2-aminoethyl)carbamate[3] **20**



Di-tert-butyl dicarbonate (1.09 g, 5.00 mmol) in dry dioxane (10 mL) was added dropwise at room temperature to a stirred solution of ethylene diamine (2.40 g, 40.0 mmol) in dry dioxane (10 mL) and the reaction was stirred for 22 h at room temperature. The solvent was removed *in vacuo* and water (15 mL) was added. The residue was filtered, and the filtrate extracted with $CHCl_3$ (4 x 10 mL). The organic phase was dried with $MgSO_4$, filtered and dried *in vacuo*. The title compound was isolated as a colourless oil (0.500 g, 3.10 mmol, 62%):

^{1}H NMR (600 MHz, $CDCl_3$); δ 4.86 (s, 1H, NHCO), 3.17 (q, *J* = 6.0 Hz, 2H, C*H*$_2$NH), 2.80 (t, *J* = 5.9 Hz, 2H, NH_2C*H*$_2$), 1.45 (s, 9H, $(CH_3)_3$); ^{13}C NMR (151 MHz, $CDCl_3$); δ 156.3 (CO), 79.3 (C), 43.5 (CH_2), 41.99 (CH_2), 28.5 (CH_3); IR (thin film) cm^{-1}; 3346 (m), 2976 (m), 2932 (m), 1686 (s)




156.31
79.31
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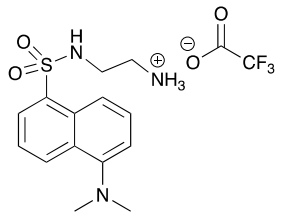
28.49
28.45
28.43
28.42
AW030.11.fid — C13CPD.ucl CDCl3 {W:\600} jrb 1
f1 (ppm)

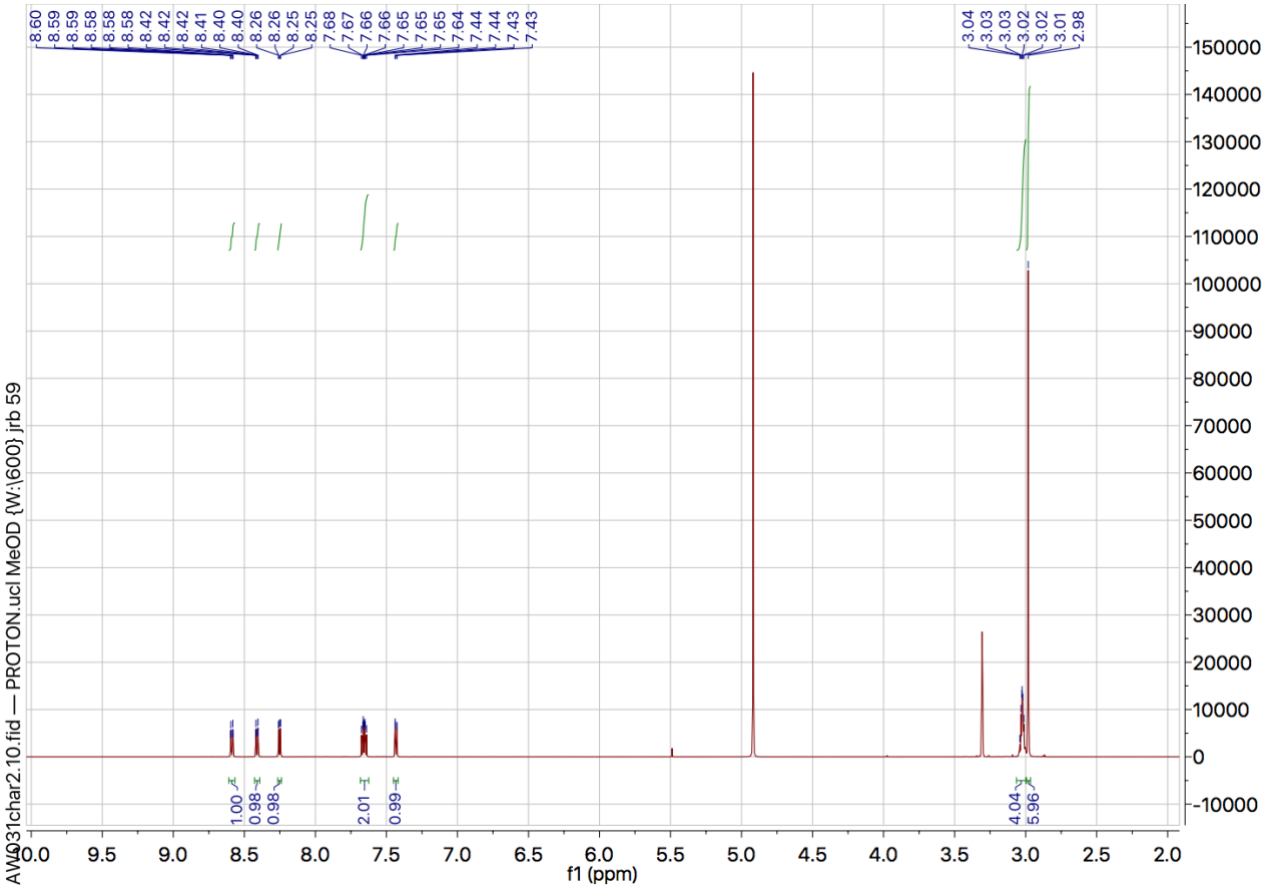
2-((5-(dimethylamino)naphthalene)-1-sulfonamido)ethan-1-aminium 2,2,2-trifluoroacetate[4] **15**



To a flask containing mono-BOC-protected ethylenediamine (0.510 g, 3.18 mmol) in dry DCM (150 mL) was added a solution of dansyl chloride (1.01 g, 3.73 mmol) and triethylamine (0.871 g, 8.60 mmol) in dry DCM (150 mL). The reaction mixture was then stirred at room temperature for 4 h. The solvent was removed *in vacuo* and the crude product purified with flash chromatography (solvent system of 20-30% EtOAc/DCM).

TFA (40 mL) was then added to the product, and the reaction was stirred for 2 h at room temperature. Addition of DCM (100 mL) to the residue at 0 ^{o}C resulted in the precipitation of a solid after 4 h which was washed with diethyl ether (3 x 50 mL) to afford the title compound as a yellow gum (0.921 g, 2.26 mmol, 71%):

^{1}H NMR (600 MHz, MeOD); δ 8.59 (d, *J* = 8.6 Hz, 1H, ArH), 8.41 (d, *J* = 8.7 Hz, 1H, ArH), 8.25 (dd, *J* = 7.3, 1.2 Hz, 1H, ArH), 7.66 (dd, *J* = 8.5, 5.8 Hz, 1H, ArH), 7.65 (dd, *J* = 8.5, 5.4 Hz, 1H, ArH), 7.43 (dd, *J* = 7.6, 0.9 Hz, 1H, ArH), 3.07 – 2.99 (m, 4H, CH_2), 2.98 (s, 6H, 2 x CH_3); ^{13}C NMR (151 MHz, MeOD); δ 151.4 (ArC), 136.1 (ArC), 130.9 (ArC), 130.8 (ArC), 130.7 (ArC), 130.7 (ArC), 129.4 (ArC), 124.9 (ArC), 121.4 (ArC), 117.3 (ArC), 46.1 (CH_3), 41.2 (CH_2), 40.8 (CH_2); IR (solid) cm^{-1}; 3069 (m), 2892 (m), 1666 (s); LRMS (ES^{+}) 294 (100, $[M]^{+}$); HRMS (ES^{+}) calcd for $C_{14}H_{20}N_3O_2S$ $[M]^{+}$ 294.1276, observed 294.1269.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3.04
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2.98
1.00
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0.99
4.04
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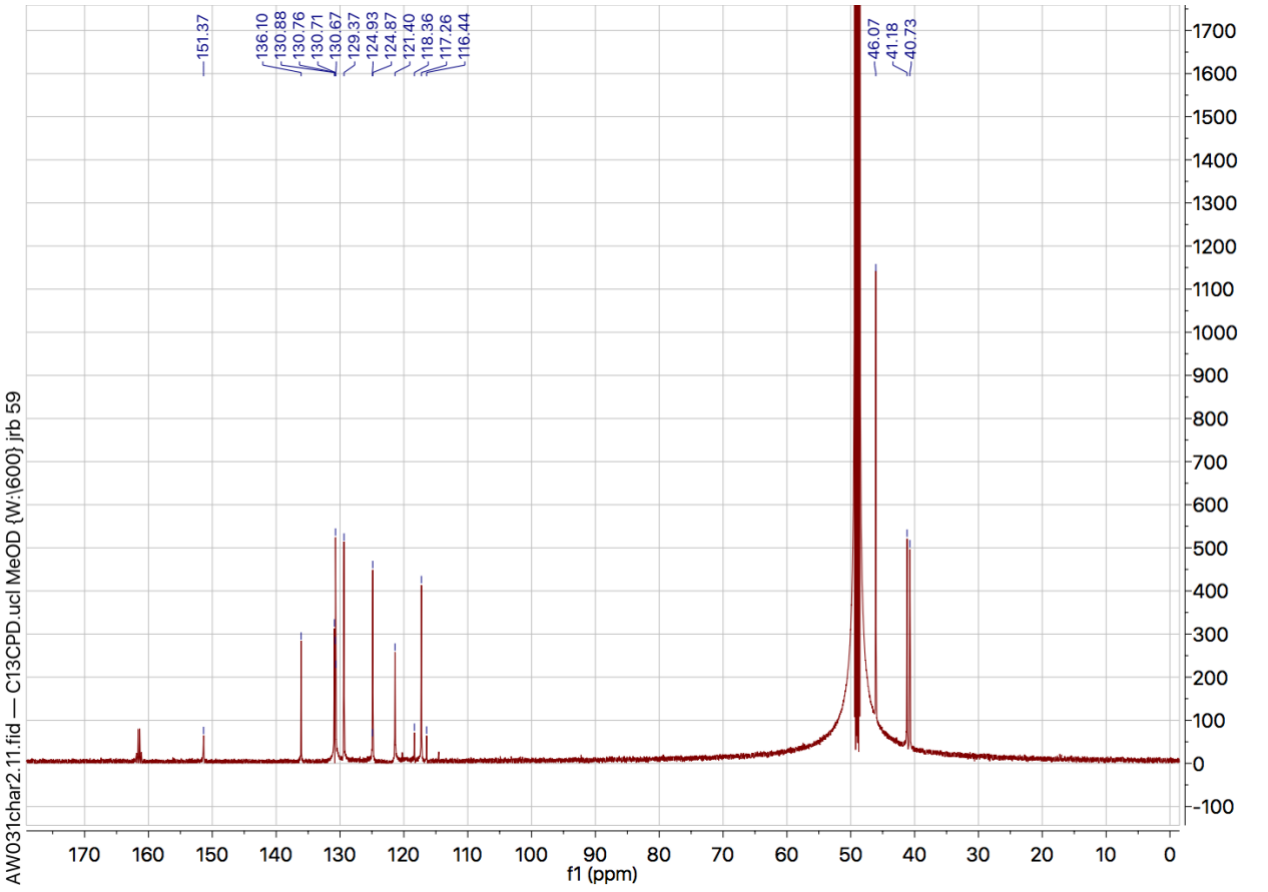
f1 (ppm)


151.37
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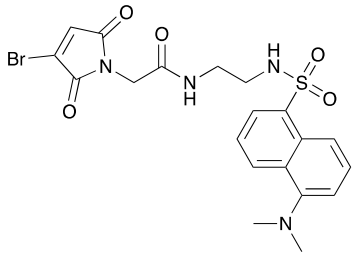
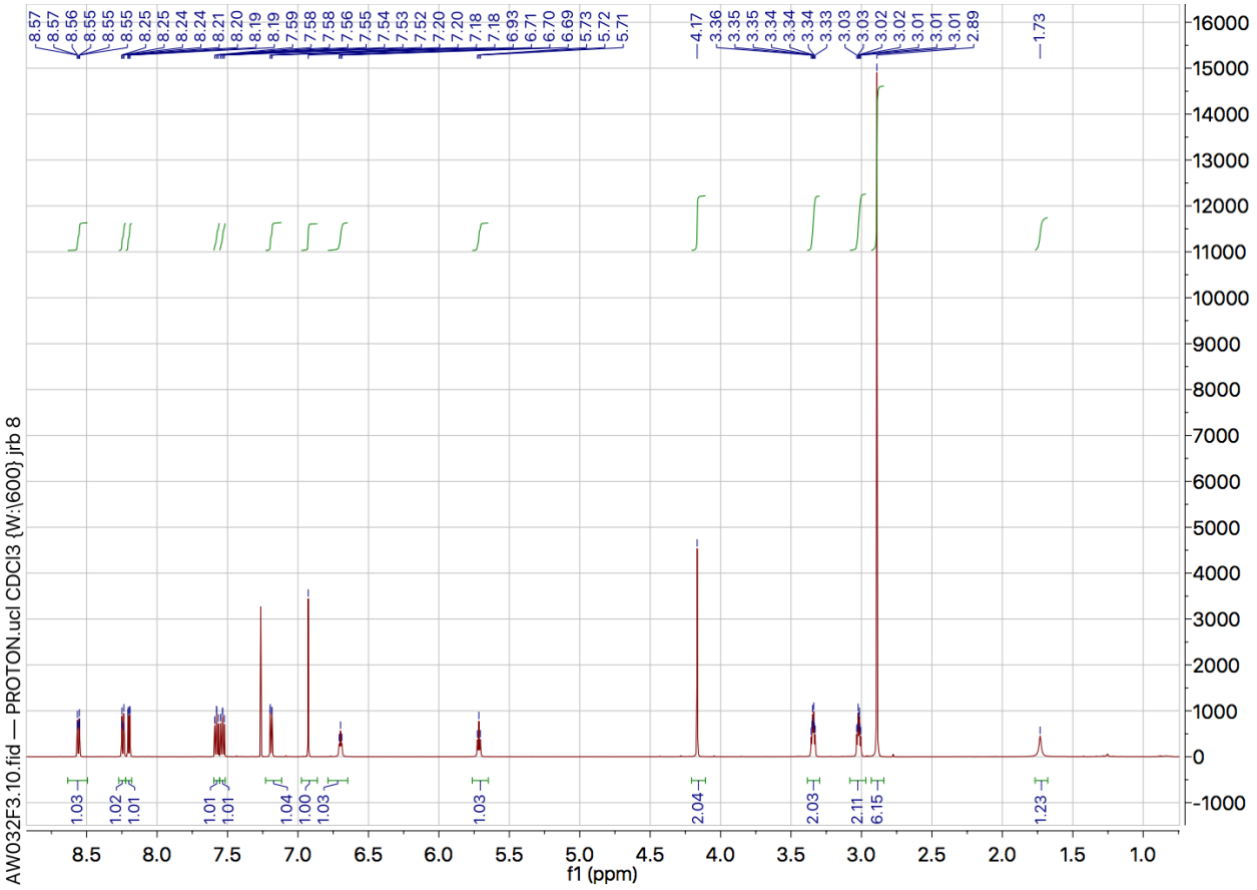
121.40
118.36
117.26
116.44
46.07
41.18
40.73
AW031char2.11.fid — C13CPD.ucl MeOD {W:\600} jrb 59
f1 (ppm)

2-(3-bromo-2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-N-(2-((5-(dimethylamino)naphthalene)-1-sulfonamido)ethyl)acetamide **3**



Method adapted from reference.[2] Bromomaleimide **11** (45 mg, 0.18 mmol) was added to a solution of EEDQ (72 mg, 0.28 mmol) in MeCN (15 mL) and the reaction was stirred at room temperature for 30 min. Meanwhile, the corresponding dansylamine **15** (50 mg, 0.17 mmol) was dissolved in MeCN (5 mL) and DIPEA (25 mg, 0.20 mmol) was added and the mixture stirred for 5 min. The dansylamine solution was then added to the initial reaction mixture and stirred for a further hour. The solvent was removed *in vacuo* and the residue purified by column chromatography (10-20% EtOAc/Petrol). The title compound was isolated as a yellow oil (27 mg, 0.05 mmol, 31%):

^{1}H NMR (600 MHz, $CDCl_3$); δ 8.56 (d, *J* = 8.5 Hz, 1H, ArH), 8.24 (d, *J* = 8.6 Hz, 1H, ArH), 8.20 (dd, J = 7.3, 1.3 Hz, 1H, ArH), 7.58 (dd, *J* = 8.6, 7.6 Hz, 1H, ArH), 7.54 (dd, *J* = 8.5, 7.3 Hz, 1H, ArH), 7.19 (dd, *J* = 7.6, 0.8 Hz, 1H, ArH), 6.93 (s, 1H, CBrCH), 6.70 (t, *J* = 5.6 Hz, 1H, NH), 5.72 (t, *J* = 6.3 Hz, 1H, NH), 4.17 (s, 2H, NCH_2), 3.34 (app. q, *J* = 5.6 Hz, 2H, NHC*H*$_2$), 3.02 (app. q, *J* = 6.1 Hz, 2H, C*H*$_2$NH), 2.89 (s, 6H, $N(CH_3)_2$); ^{13}C NMR (crude) (151 MHz, $CDCl_3$); δ 168.2 (CO), 166.5 (CO), 165.2 (CO), 152.3 (ArC), 134.0 (ArC), 132.4 (ArC), 131.8 (CH), 131.0 (ArC), 130.0 (BrC), 129.9 (ArC), 129.5 (ArC), 128.9 (ArC), 123.4 (ArC), 118.5 (ArC), 115.5 (ArC), 45.5 (CH_3), 42.5 (CH_2), 41.2 (CH_2), 40.0 (CH_2); IR (thin film) 3110 (m), 2988 (m), 2943 (m), 1707 (s) cm^{-1}; LRMS (ES^+) 509 (99, $[M^{79}Br+H]^+$), 511 (100, $[M^{81}Br+H]^+$); HRMS (ES^+) calcd for $C_{20}H_{21}N_4O_5SBr$ $[M^{79}Br+H]^+$ 509.0494, observed 509.0493.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1.04
1.00
1.03
1.03
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2.03
2.11
6.15
1.23
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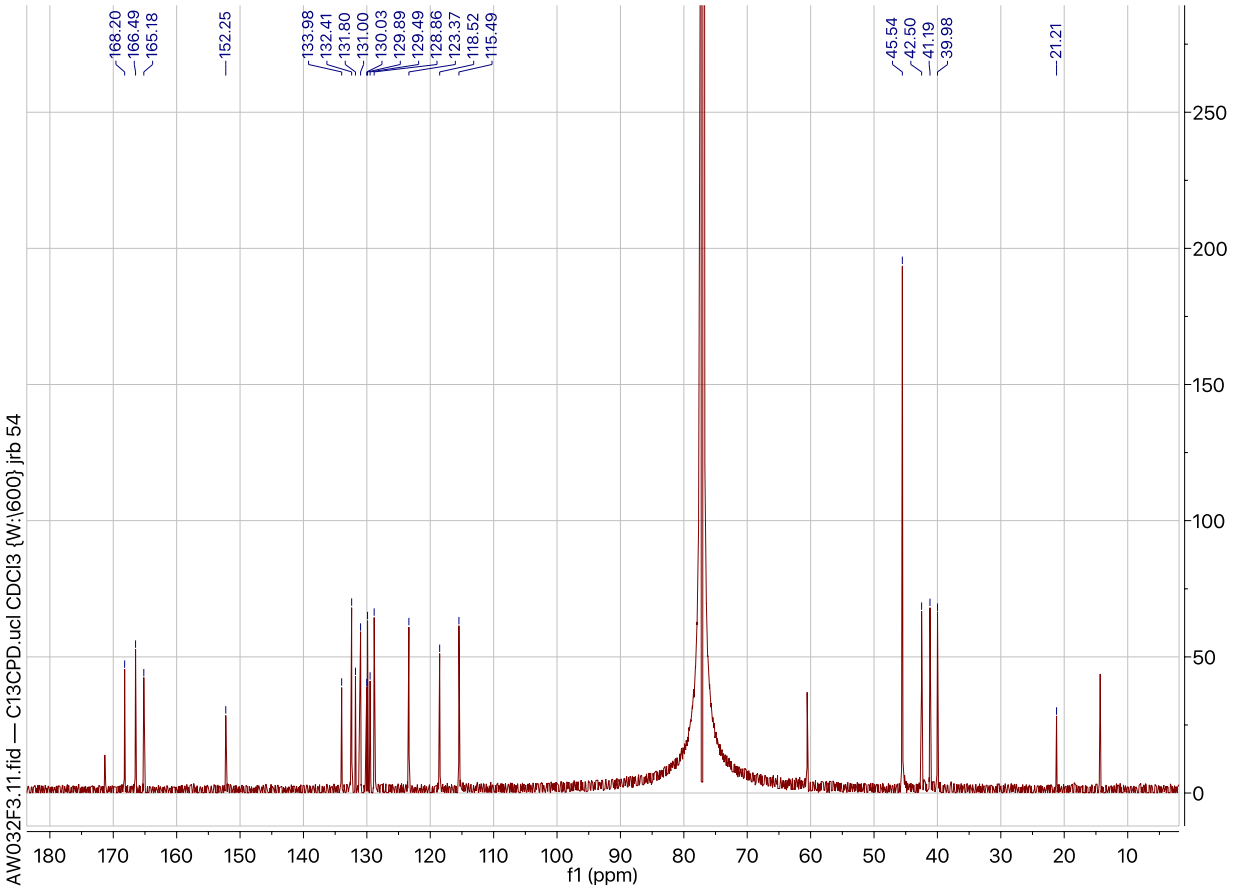
f1 (ppm)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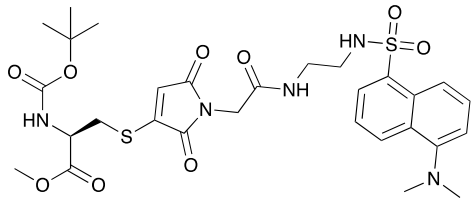
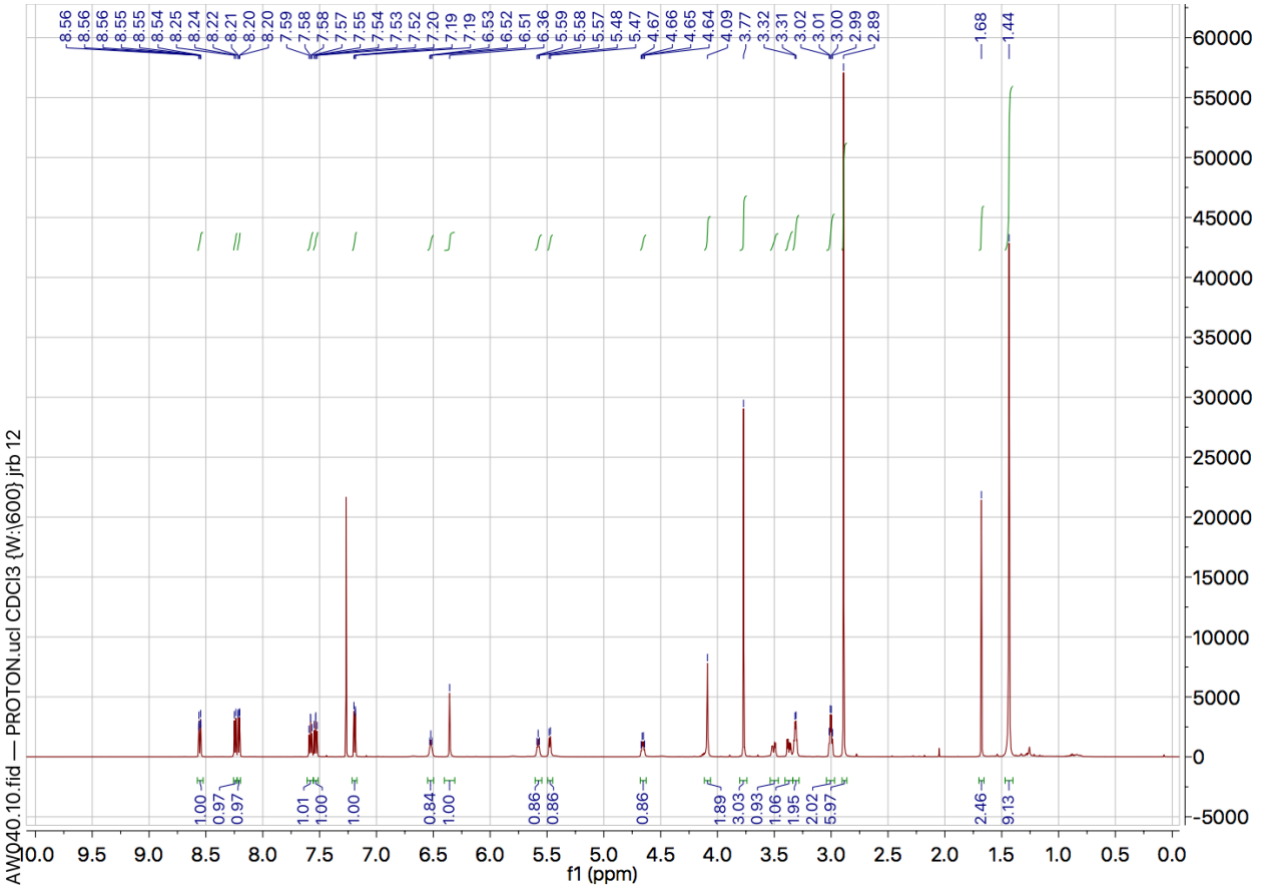
165.18
152.25
133.98
132.41
131.80
131.00
130.03
129.89
129.49
128.86
123.37
118.52
115.49
45.54
42.50
41.19
39.98
21.21
AW032F3.11.fid — C13CPD.ucl CDCl3 {W:\600} jrb 54
f1 (ppm)

methyl N-(tert-butoxycarbonyl)-S-(1-(2-((2-((5-(dimethylamino)naphthalene)-1-sulfonamido)ethyl)amino)-2-oxoethyl)-2,5-dioxo-2,5-dihydro-1H-pyrrol-3-yl)cysteinate **5**



Bromomaleimide **3** (50 mg, 0.098 mmol) was added to a solution of N-(tert-Butoxycarbonyl)-L-cysteine methyl ester (19 mg, 0.081 mmol) and sodium acetate (10 mg, 0.12 mmol) in MeOH (50 mL) and the reaction was stirred at room temperature for 30 min. The solvent was removed *in vacuo* and the residue purified by column chromatography (40-50% EtOAc/Petrol). The product was isolated as an oil (38 mg, 0.057 mmol, 70%):

^{1}H NMR (600 MHz, $CDCl_3$) δ 8.55 (dt, *J* = 8.5, 1.1 Hz, 1H, ArH), 8.24 (d, *J* = 8.6 Hz, 1H, ArH), 8.21 (dd, *J* = 7.3, 1.3 Hz, 1H, ArH), 7.58 (dd, *J* = 8.6, 7.5 Hz, 1H, ArH), 7.53 (dd, *J* = 8.5, 7.3 Hz, 1H, ArH), 7.19 (d, *J* = 7.5 Hz, 1H, ArH), 6.52 (t, *J* = 5.8 Hz, 1H, NH), 6.36 (s, 1H, CH), 5.58 (t, *J* = 6.3 Hz, 1H, NH), 5.48 (d, *J* = 7.3 Hz, 1H, NH), 4.68 – 4.64 (m, 1H, NHC*H*), 4.09 (s, 2H, NHCH_2), 3.77 (s, 3H, OCH_3), 3.51 (dd, *J* = 13.7, 5.2 Hz, 1H, SC*H*H), 3.37 (dd, *J* = 13.7, 5.6 Hz, 1H, SCH*H*), 3.31 (q, *J* = 5.6 Hz, 2H, NHCH_2), 3.00 (q, *J* = 6.0 Hz, 2H, CH_2NHS), 2.89 (s, 6H, $N(CH_3)_2$), 1.68 (s, 3H, CH_3), 1.44 (s, 9H, $(CH_3)_3$); ^{13}C NMR (151 MHz, $CDCl_3$); δ 170.3 (CO), 168.9 (CO), 167.5 (CO), 166.9 (CO), 155.2 (CO), 152.2 (SC), 150.6 (ArC), 134.1 (ArC), 130.9 (ArC), 130.0 (ArC), 129.9 (ArC), 129.5 (ArC), 128.8 (ArC), 123.4 (ArC), 119.1 (CH), 118.6 (ArC), 115.5 (ArC), 81.0 (C), 53.3 (CH), 52.6 (CH_3), 45.5 (CH_3), 42.5 (CH_2), 40.9 (CH_2), 39.9 (CH_2), 34.0 (CH_2), 28.4 (CH_3); IR (solid); 3302 (m), 2976 (m), 2931 (m), 1711 (s); LRMS (ES^+) 664 (100, $[M]^+$); HRMS (ES^+) calcd for $C_{29}H_{38}N_5O_9S_2$ $[M]^+$ 664.2111, observed 664.2056.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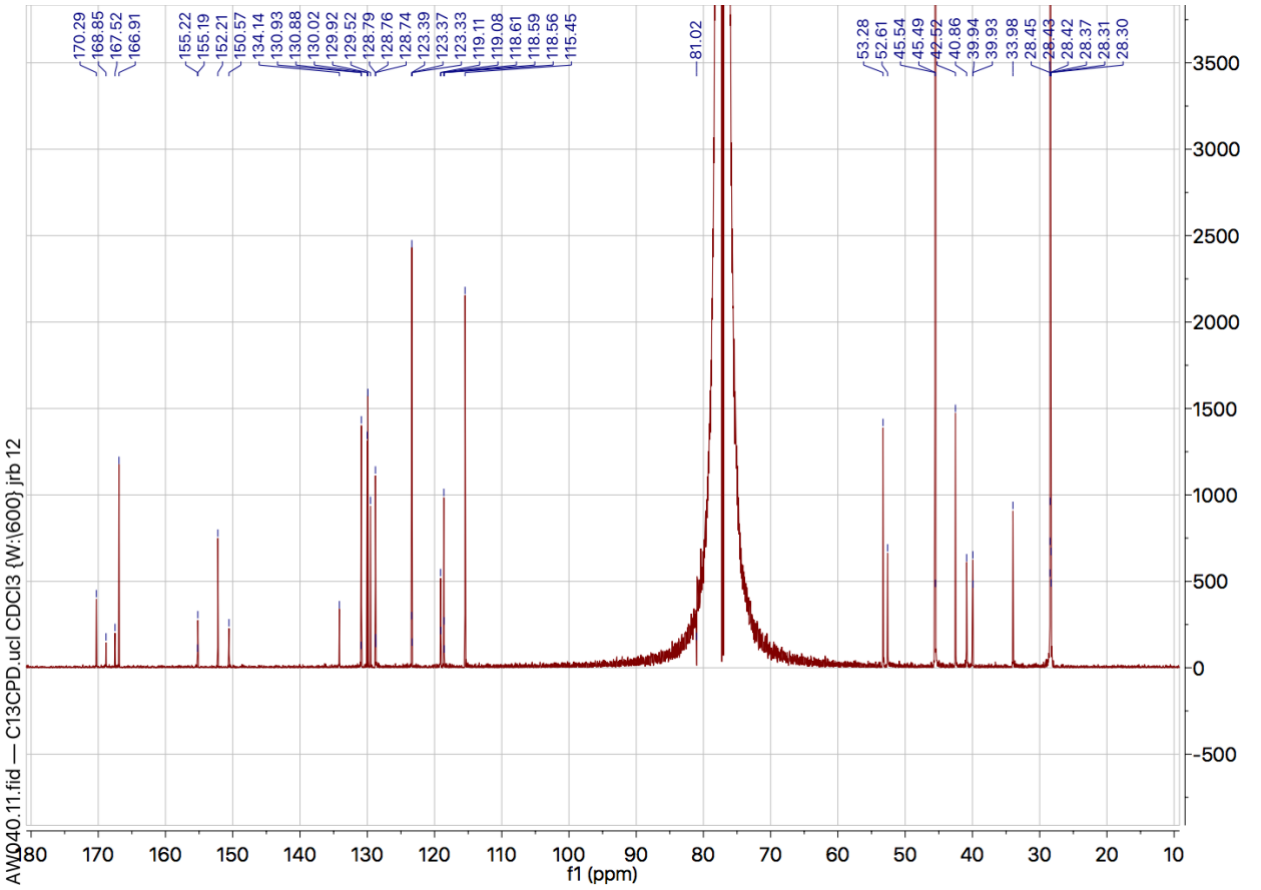
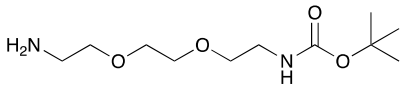
f1 (ppm)

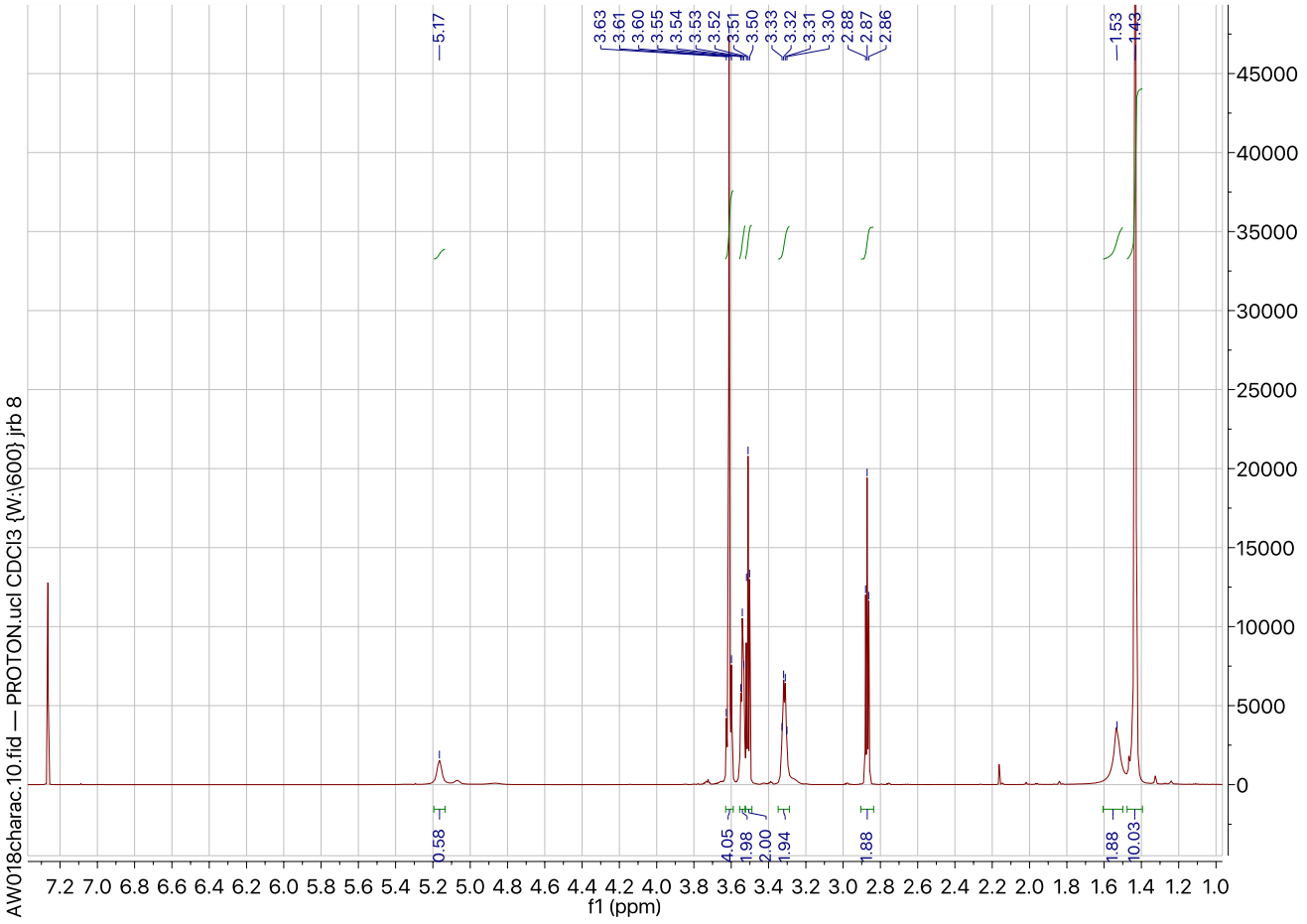

AW040.11.fid — C13CPD.ucl CDCl3 {W:\600} jrb 12
f1 (ppm)

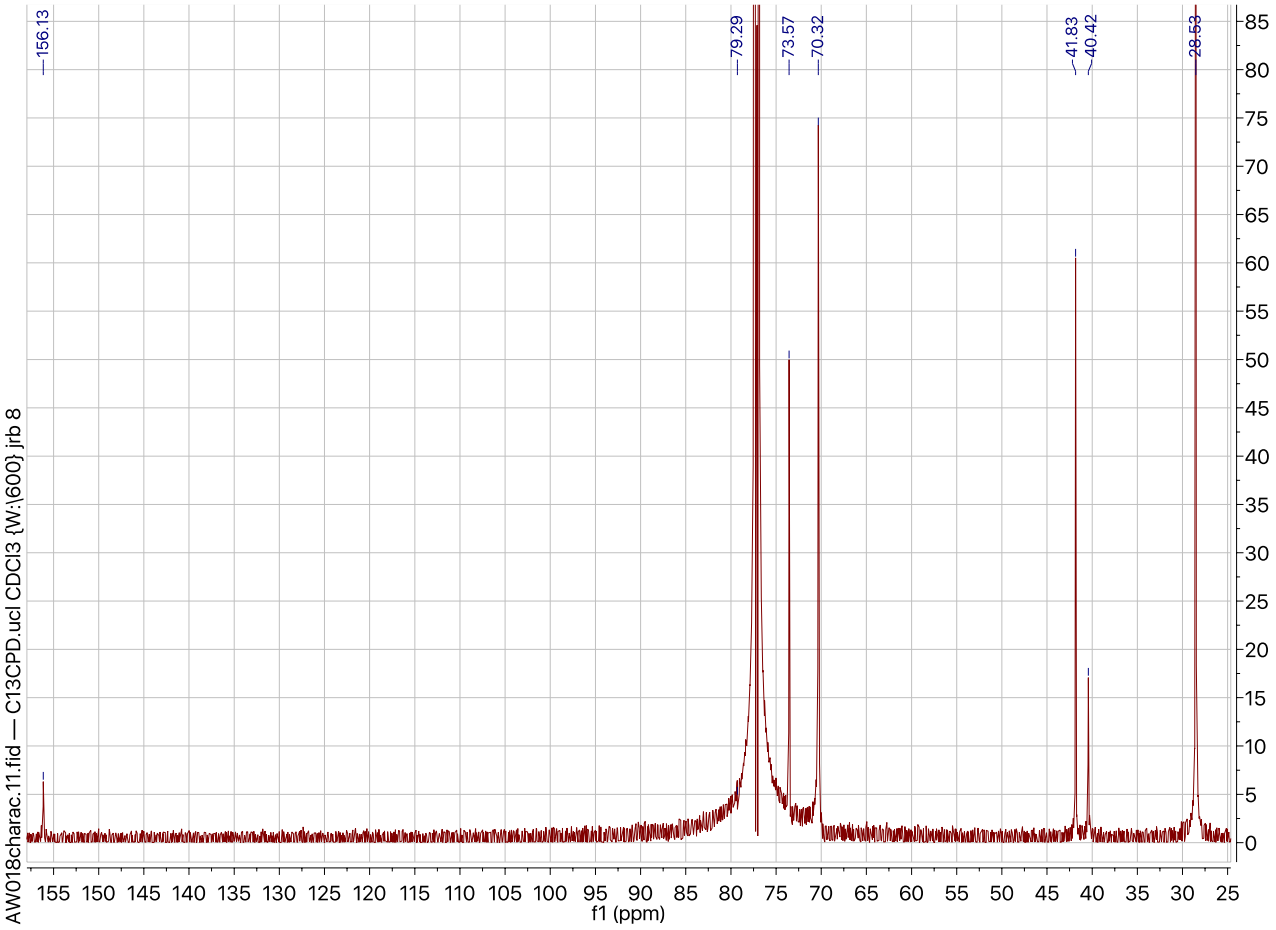
tert-butyl (2-(2-(2-aminoethoxy)ethoxy)ethyl)carbamate **21**



Method adapted from reference.[3] Di-tert-butyl dicarbonate (1.11 g, 5.09 mmol) in dioxane (7.5 mL) was added dropwise at room temperature over 90 min to a stirred solution of 2,2′-(Ethylenedioxy)bis(ethylamine) (4.79 g, 32.3 mmol) in dioxane (20 mL) and the reaction was stirred for a further 90 min at room temperature. The solvent was removed *in vacuo* and water (15 mL) was added. The oily residue was filtered, and the filtrate extracted with $CHCl_3$ (4 x 25 mL). The organic phase was dried with $MgSO_4$, filtered and dried *in vacuo* to give the title compound as a colourless oil (1.11 g, 4.47 mmol, 87% yield):

^{1}H NMR (600 MHz, $CDCl_3$); δ 5.17 (s, 1H, NH), 3.63 – 3.60 (m, 4H, 2 x CH_2), 3.55 (t, *J* = 5.2 Hz, 2H, CH_2), 3.52 (t, *J* = 5.2 Hz, 2H, CH_2), 3.33 (q, *J* = 5.3 Hz, 2H, CH_2), 2.87 (t, *J* = 5.2 Hz, 2H, CH_2), 1.53 (s, 2H, NH_2), 1.43 (s, 9H, $(CH_3)_3$); ^{13}C NMR (151 MHz, $CDCl_3$); δ 156.1 (CO), 79.3 (C), 73.6 (CH_2), 70.3 (CH_2), 41.8 (CH_2), 40.4 (CH_2), 28.5 (CH_3); IR (thin film) cm^{-1}; 3340 (w), 2975 (m), 2930 (m) 1696 (s); LRMS (ES$^+$) 249 (100, $[M+H]^+$); HRMS (ES$^+$) calcd for $C_{11}H_{24}N_2O_4$ $[M+H]^+$ 249.1914, observed 249.1816.

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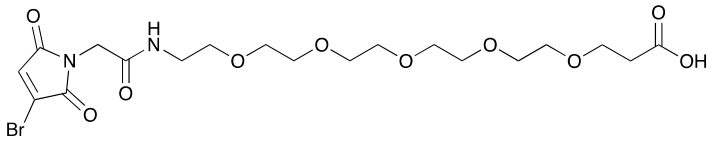
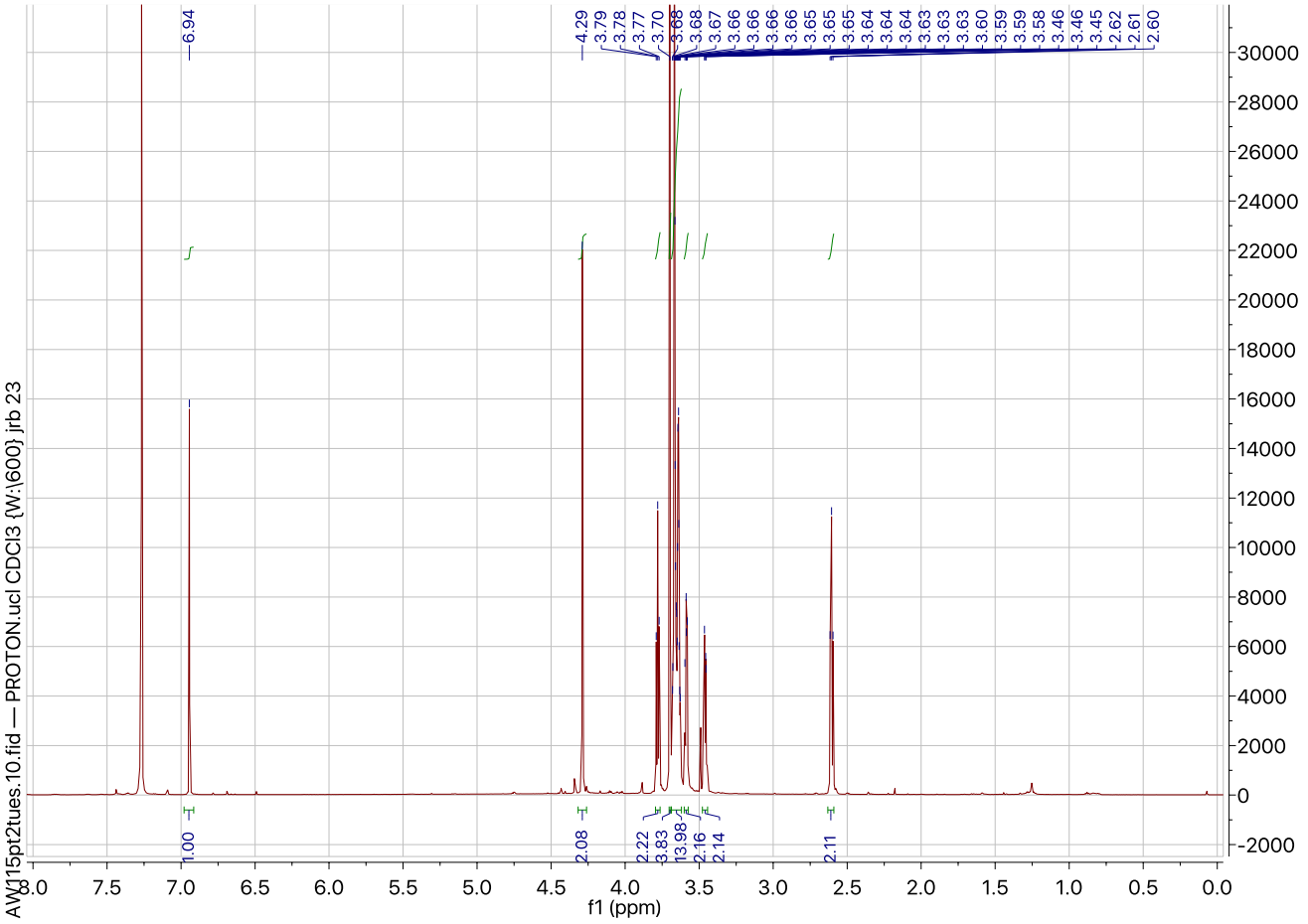
41.83
40.42
28.53
AW018charac.11.fid — C13CPD.ucl CDCl3 {W:\600} jrb 8
f1 (ppm)

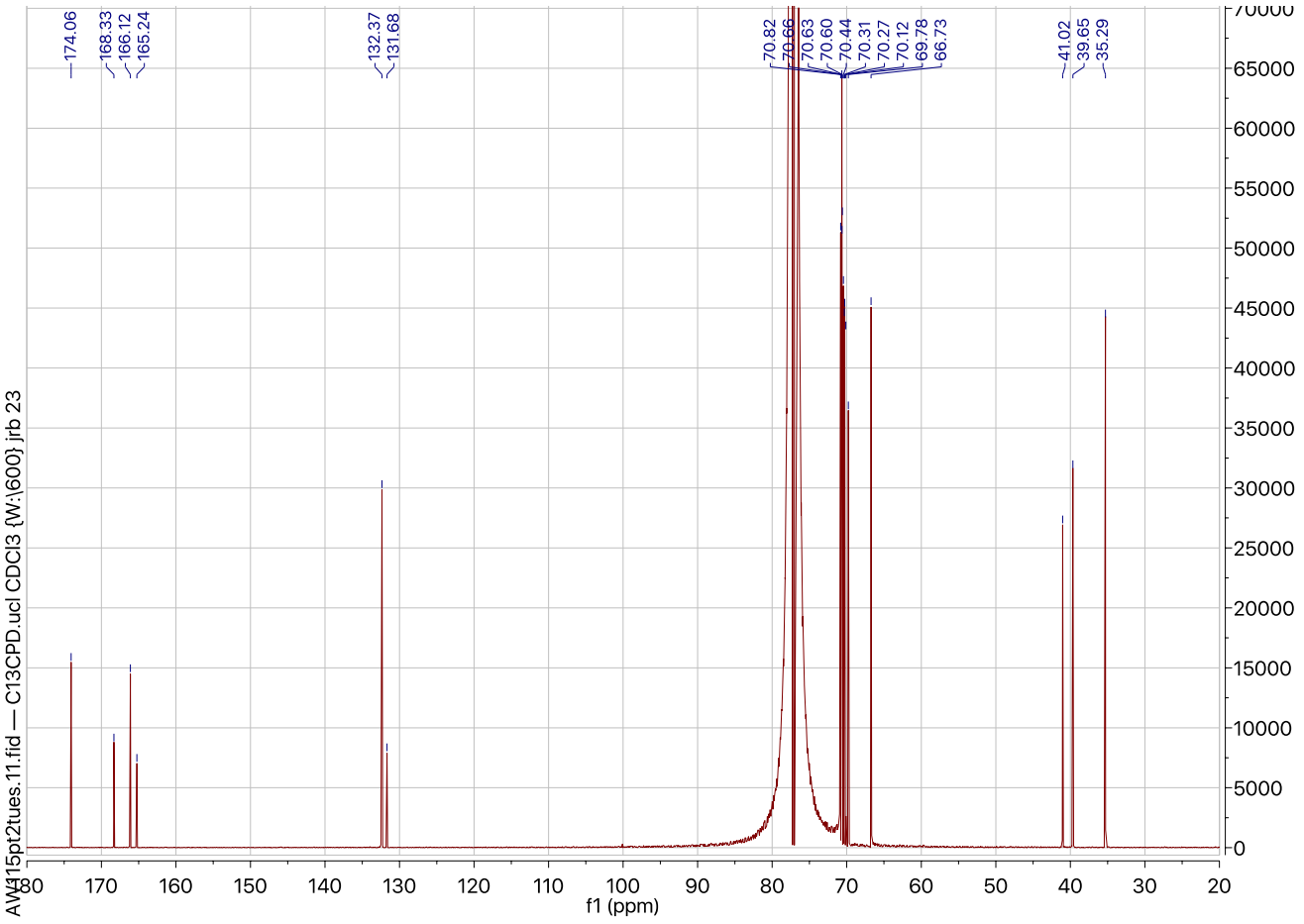
1-(3-bromo-2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)-2-oxo-6,9,12,15,18-pentaoxa-3-azahenicosan-21-oic acid **16**



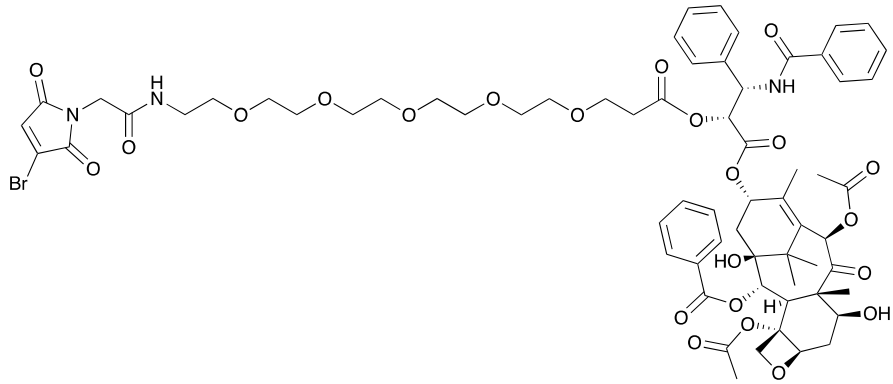
Method adapted from reference.[2] EEDQ (0.909 g, 3.65 mmol) was added to a stirred solution of bromomaleimide **11** (0.711 g, 3.04 mmol) in MeCN (35 mL). The reaction was stirred at room temperature for 1 h, and *tert*-butyl 1-amino-3,6,9,12,15-pentaoxaoctadecan-18-oate was added (1.00 g, 2.74 mmol). The reaction was stirred for a further 30 min, and the solvent was removed *in vacuo*. The crude product was dissolved in EtOAc (100 mL) and washed with 10% citric acid solution (3 x 20 mL), water (2 x 20 mL), brine (2 x 20 mL) and dried with $MgSO_4$. The organic layer was concentrated *in vacuo* and the crude product purified with flash chromatography (0-10% MeOH in DCM). The product was then stirred in TFA (5 mL) for 5 h at room temperature. The resulting mixture was concentrated *in vacuo* and the product was obtained as a dark brown, highly viscous oil (0.984 g, 1.88 mmol, 68%).

^{1}H NMR (600 MHz, $CDCl_3$) δ 6.94 (s, 1H, CH), 4.29 (s, 2H, NCH_2), 3.78 (t, *J* = 5.8 Hz, 2H, CH_2), 3.70 (s, 4H, 2 x CH_2), 3.69 – 3.62 (m, 12H, 6 x CH_2), 3.59 (dd, *J* = 5.5, 4.3 Hz, 2H, CH_2), 3.46 (q, *J* = 5.3 Hz, 2H, CH_2), 2.61 (t, *J* = 5.9 Hz, 2H, CH_2);^{13}C NMR (151 MHz, $CDCl_3$) δ 174.1 (CO), 168.3 (CO), 166.1 (CO), 165.2 (CO), 132.4 (CH), 131.7 (CBr), 70.8 (CH_2), 70.7 (CH_2), 70.6 (CH_2), 70.6 (CH_2), 70.4 (CH_2), 70.3 (CH_2), 70.3 (CH_2), 70.1 (CH_2), 69.8 (CH_2), 66.7 (CH_2), 41.0 (CH_2), 39.7 (CH_2), 35.3 (CH_2); IR (thin film) 2873 (br), 1717 (s), 1685 (m) cm^{-1}; LRMS (ES^+) 542 (98, $[M^{79}Br+NH_4]^+$), 544 (100, $[M^{81}Br+NH_4]^+$), 574 (40, $[M^{79}Br+H+CH_3OH+NH_4]^+$), 576 (40, $[M^{81}Br+ H+CH_3OH+NH_4]^+$); HRMS ($ES^+$) calcd for $C_{19}H_{29}N_2O_{10}Br$ $[M^{79}Br+H]^+$ 525.1073, observed 525.1078.


6.94
4.29
3.79
3.78
3.77
3.70
3.68
3.67
3.66
3.65
3.64
3.63
3.60
3.59
3.58
3.46
3.45
2.62
2.61
2.60
1.00
2.08
2.22
3.83
13.98
2.16
2.14
2.11
AW115pt2tues.10.fid — PROTON.ucl CDCl3 {W:\600} jrb 23
f1 (ppm)


174.06
168.33
166.12
165.24
132.37
131.68
70.82
70.66
70.63
70.60
70.44
70.31
70.27
70.12
69.78
66.73
41.02
39.65
35.29
AW115pt2tues.11.fid — C13CPD.ucl CDCl3 {W:\600} jrb 23
f1 (ppm)

MBM-C2-PEG[5]-PTX **12**



Owing to the cytotoxic nature of the compound, this final compound was synthesised by *Recipharm AB* in the following manner:

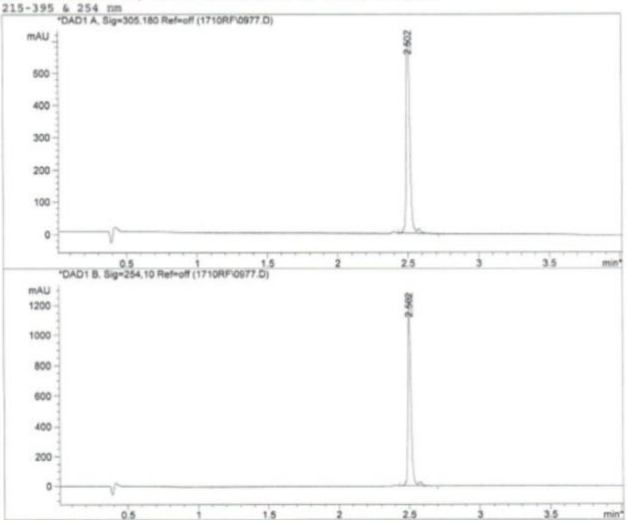
To a solution of Bromomaleimide **16** (0.990 g, 1.88 mmol) in DCM (100 mL) at 0 ºC, DCC (0.390 g, 1.88 mmol) was added. The mixture was stirred at 0 ºC for 15 min and then at room temperature for another 15 min. Paclitaxel (1.60 g, 1.88 mmol) was then added at 0 ºC followed by DMAP (23.0 mg, 0.190 mmol). The mixture was allowed to slowly reach room temperature overnight. The solution was filtered to remove the majority of the dicyclohexylurea impurity and then concentrated to a light brown solid. The material was purified using preparative HPLC and gave the target molecule (853 mg, 0.627 mmol, 33%) as a white solid.

Purity of 98% confirmed by HPLC, mass spectrometry and ^{1}H NMR analysis (LC trace shows single product):

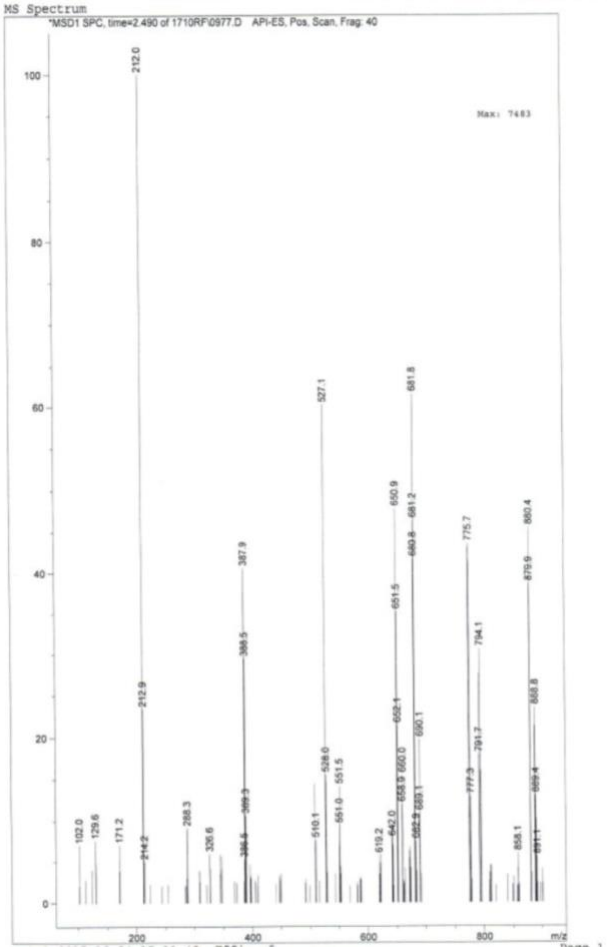
Data File C:\HPCHEM\1\DATA\1710RF\0977.D Sample Name: ALB2MW7

pure batch 1

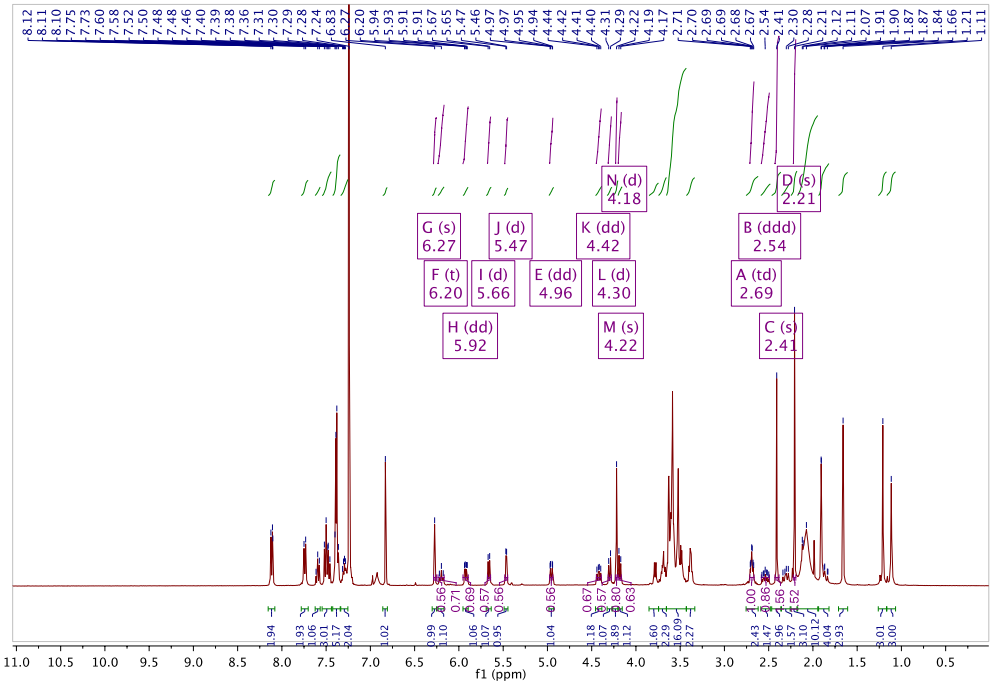
```
Injection Date  : 2017-10-24 15:06:42          Seq. Line :  3
Sample Name     : ALB2MW7                      Location : Vial 21
Acq. Operator   : Effie_rf                     Inj :  1
Acq. Instrument : Instrument 1                 Inj Volume : Inj prog
Sequence File   : C:\HPCHEM\1\SEQUENCE\USETHIS.S
Method          : C:\HPCHEM\1\METHODS\STANDARD\ST1097A3.M
Last changed    : 2017-09-13 17:40:17  by Effie_rf
STANDARD METHOD FOR REGISTRATION INTO CHEMSPEC
ACE 3 C8 50x3.0 mm, 10-97% acetonitrile in 3 min, 1 ml/min
215-395 & 254 nm
```



MS Spectrum

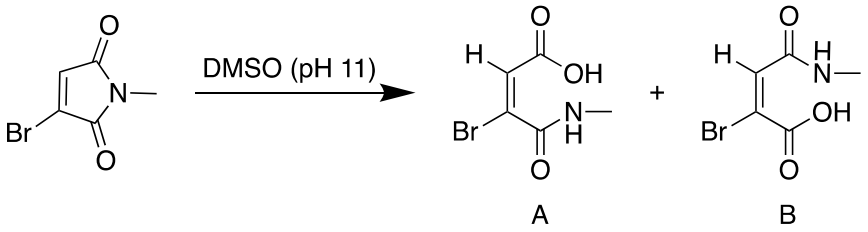


MS Data shows half mass ion for compound: 681.8 Da; Expected full mass ion: 1361 Da



Regioselectivity of Hydrolysis:

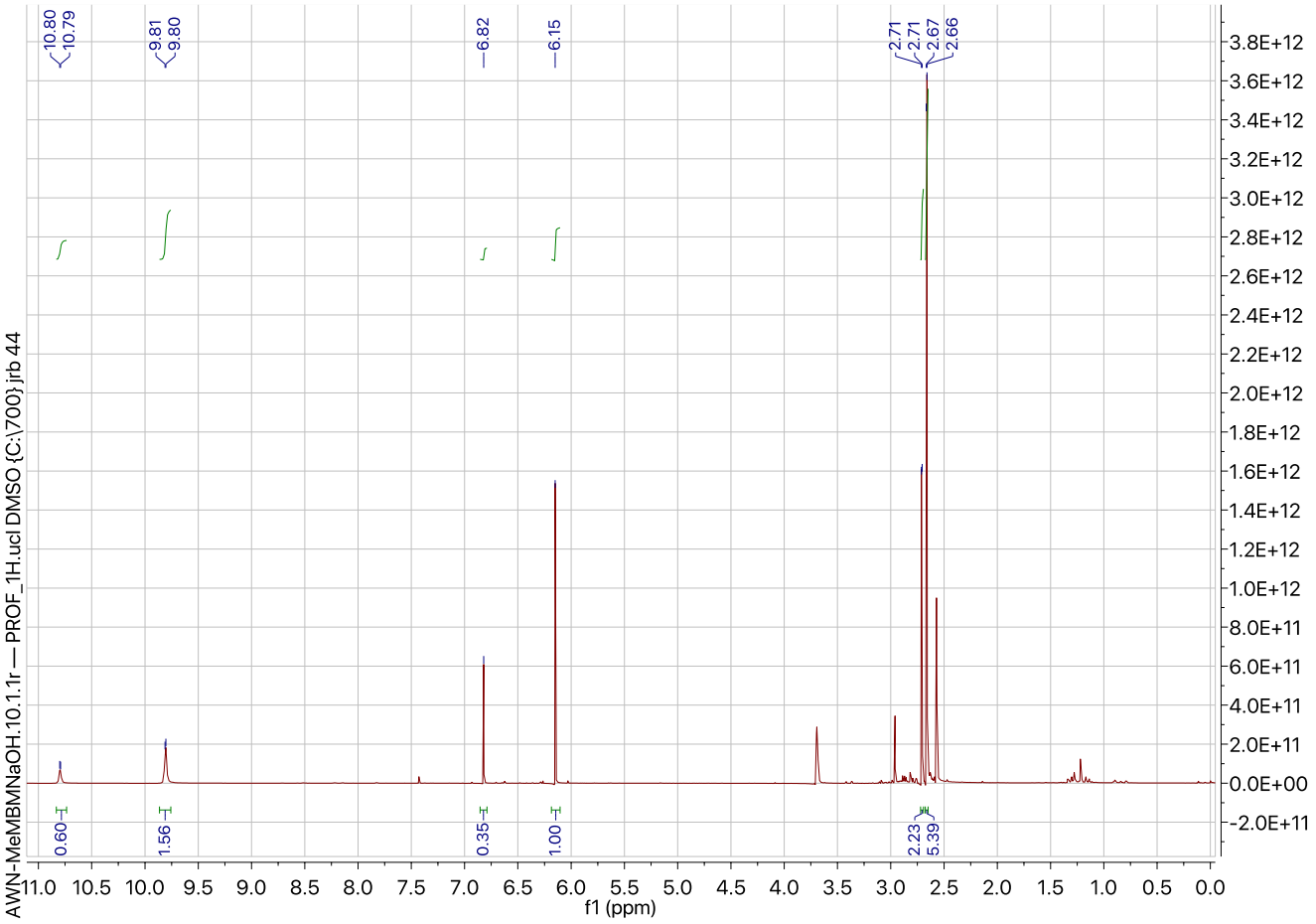
Hydrolysis of N-methylmonobromomaleimide

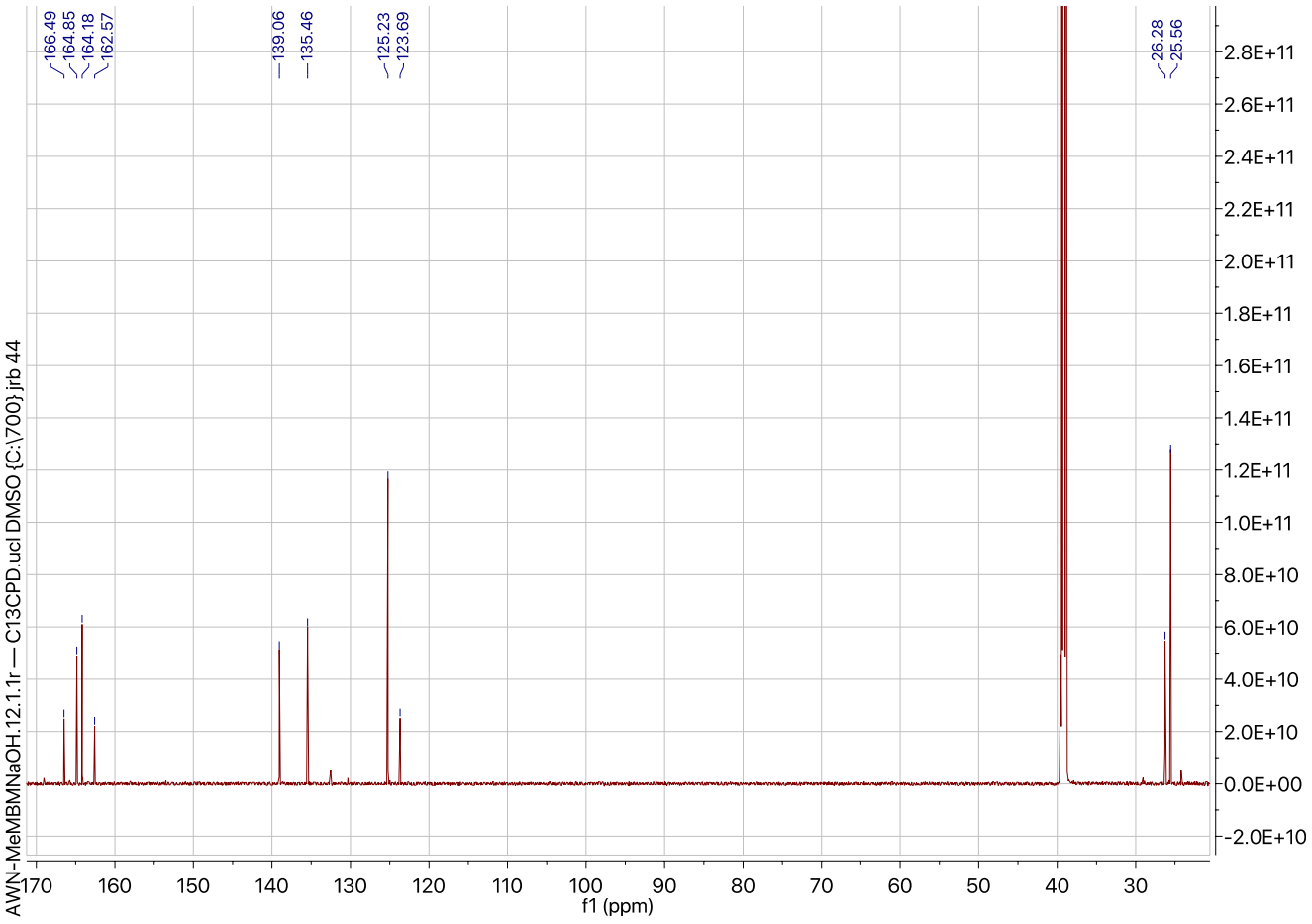


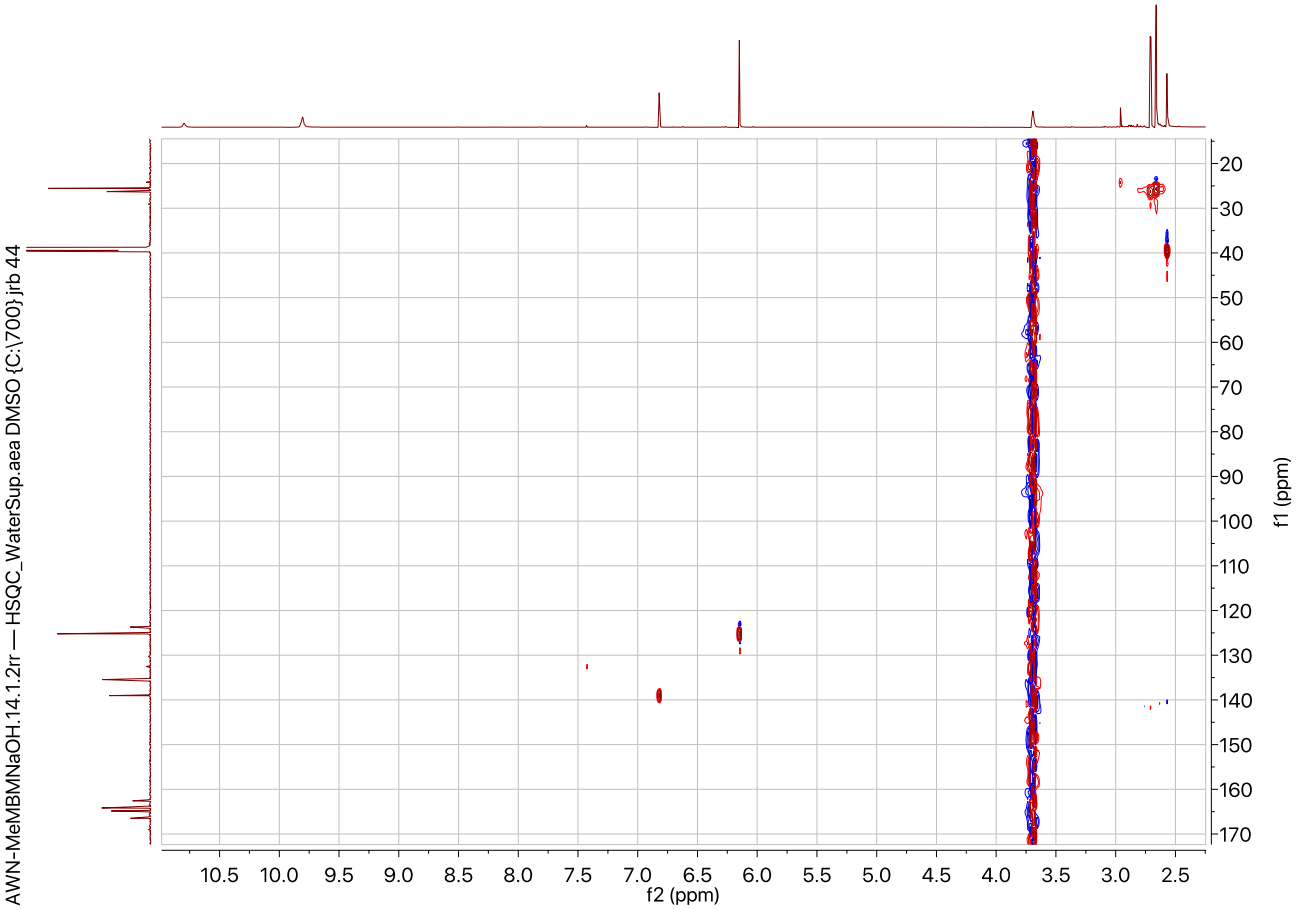
N-Methyl monobromomaleimide (6.00 mg, 0.0316 mmol) was dissolved in DMSO (800 µL). NaOH (1M) was added dropwise to bring the solution to pH 11. The solution was analysed by NMR. Proton and carbon correlation was elucidated by HMBC and HSQC. The ratio of the major and minor hydrolyte regioisomers was worked out as an average of the integrations of distinct non-labile proton environments (hydrolytes A and B observed in ratio 28:72).

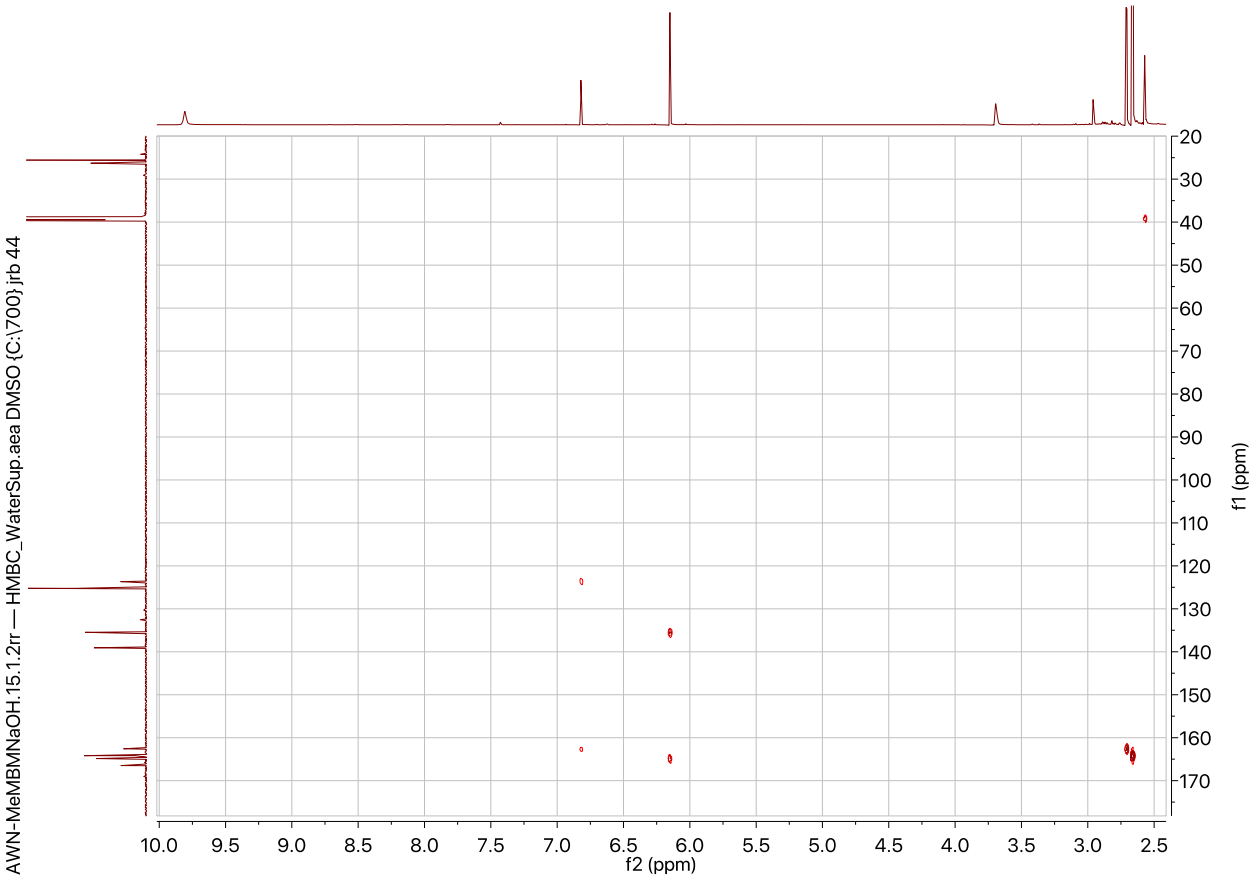
^{1}H NMR (700 MHz, DMSO) δ 10.80 (s, 1H, NH^{A}), 9.81 (d, *J* = 5.5 Hz, 1H, NH^{B}), 6.82 (s, 1H, H^{A}), 6.15 (s, 1H, H^{B}), 2.71 (d, *J* = 4.6 Hz, 3H, CH_3^{A}), 2.66 (d, *J* = 4.7 Hz, 3H, CH_3^{B}).

^{13}C NMR (176 MHz, DMSO) δ 166.5 ($COOH^{A}$), 164.8 ($COOH^{B}$), 164.2 ($CONH^{B}$), 162.6 ($CONH^{A}$), 139.1 ($C{=}CH^{A}$), 135.5 (CBr^{B}), 125.2 ($C{=}CH^{B}$), 123.7 (CBr^{A}), 26.3 (CH_3^{A}), 25.6 (CH_3^{B}).

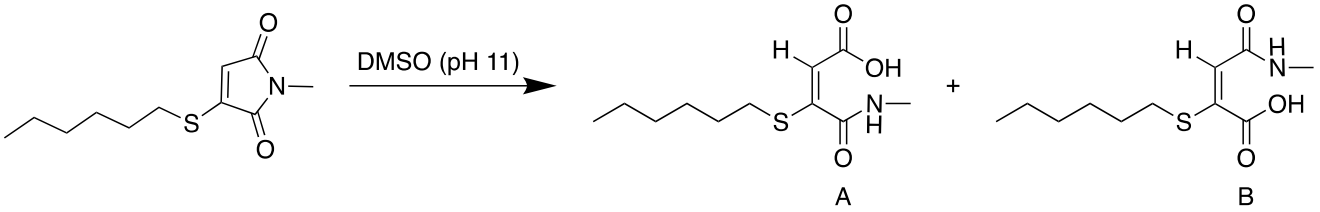

10.80
10.79
9.81
9.80
6.82
6.15
2.71
2.71
2.67
2.66
0.60
1.56
0.35
1.00
2.23
5.39
AWN-MeMBMNaOH.10.1.1r — PROF_1H.ucl DMSO {C:\700} jrb 44
f1 (ppm)


166.49
164.85
164.18
162.57
139.06
135.46
125.23
123.69
26.28
25.56
AWN-MeMBMNaOH.12.1.1r — C13CPD.ucl DMSO {C:\700} jrb 44
f1 (ppm)


AWN-MeMBMNaOH.14.1.2rr — HSQC_WaterSup.aea DMSO {C:\700} jrb 44
f2 (ppm)
f1 (ppm)


AWN-MeMBMNaOH.15.1.2rr — HMBC_WaterSup.aea DMSO {C:\700} jrb 44
f2 (ppm)
f1 (ppm)

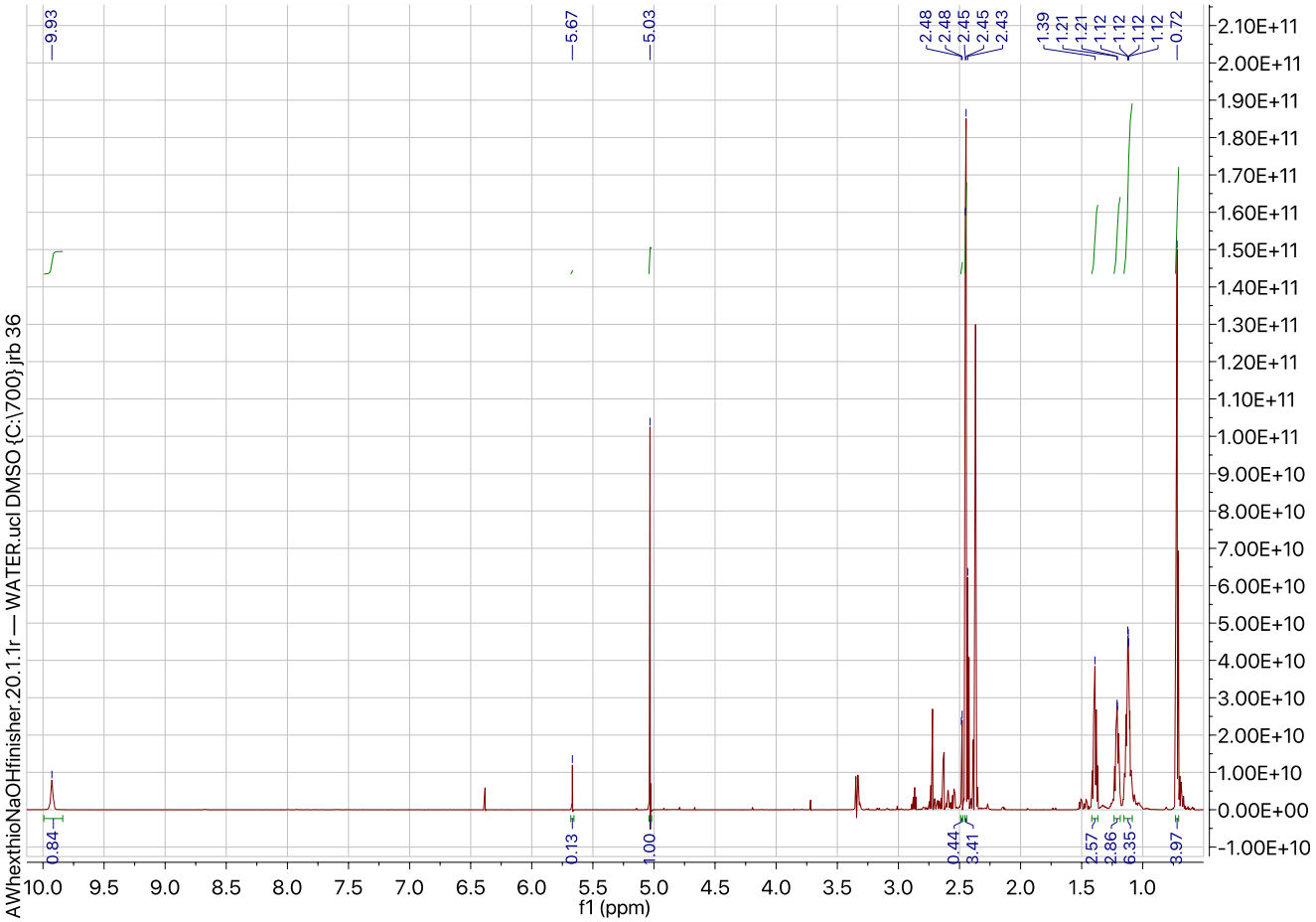
Hydrolysis of 3-(hexylthio)-1-methyl-1*H*-pyrrole-2,5-dione

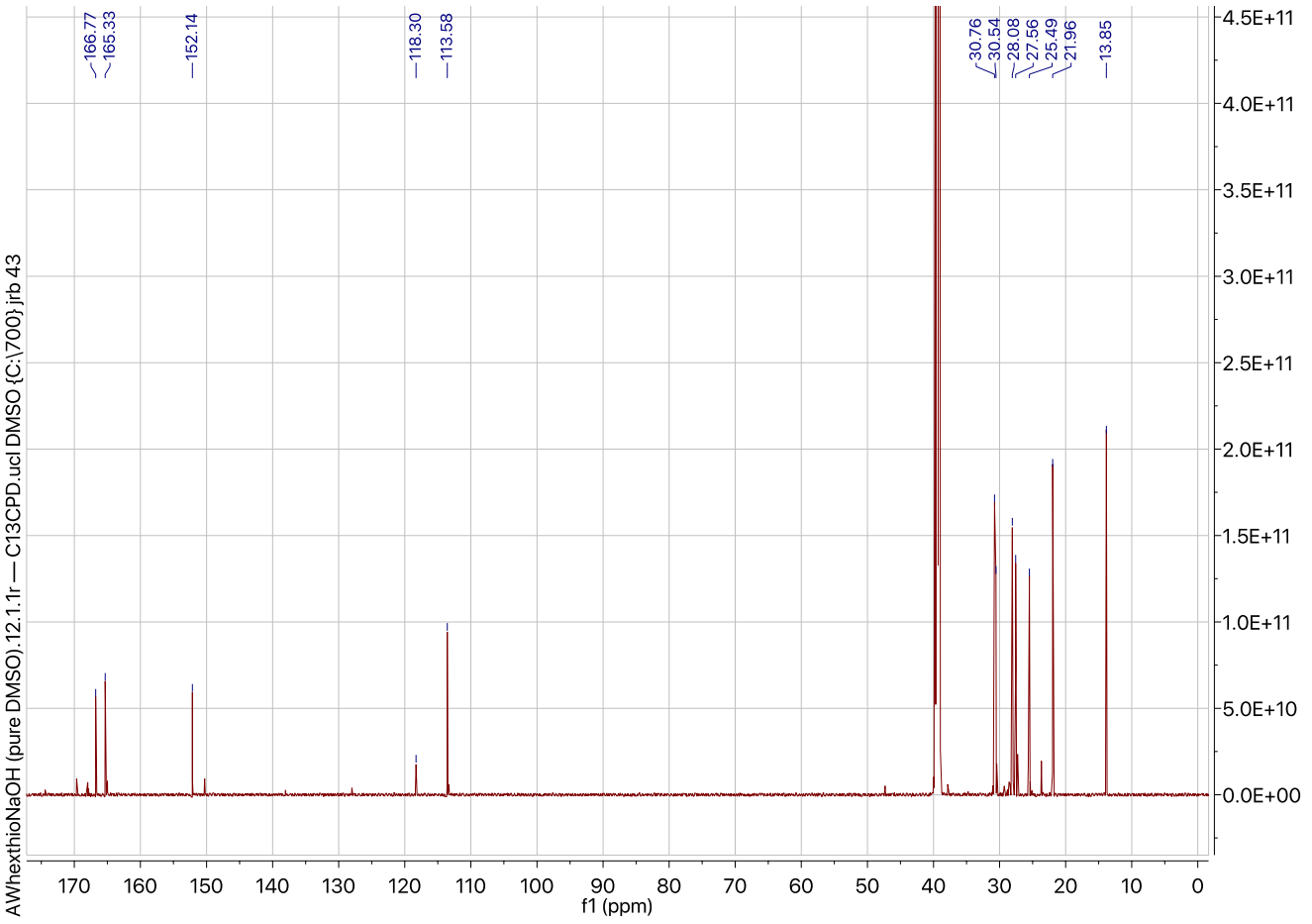


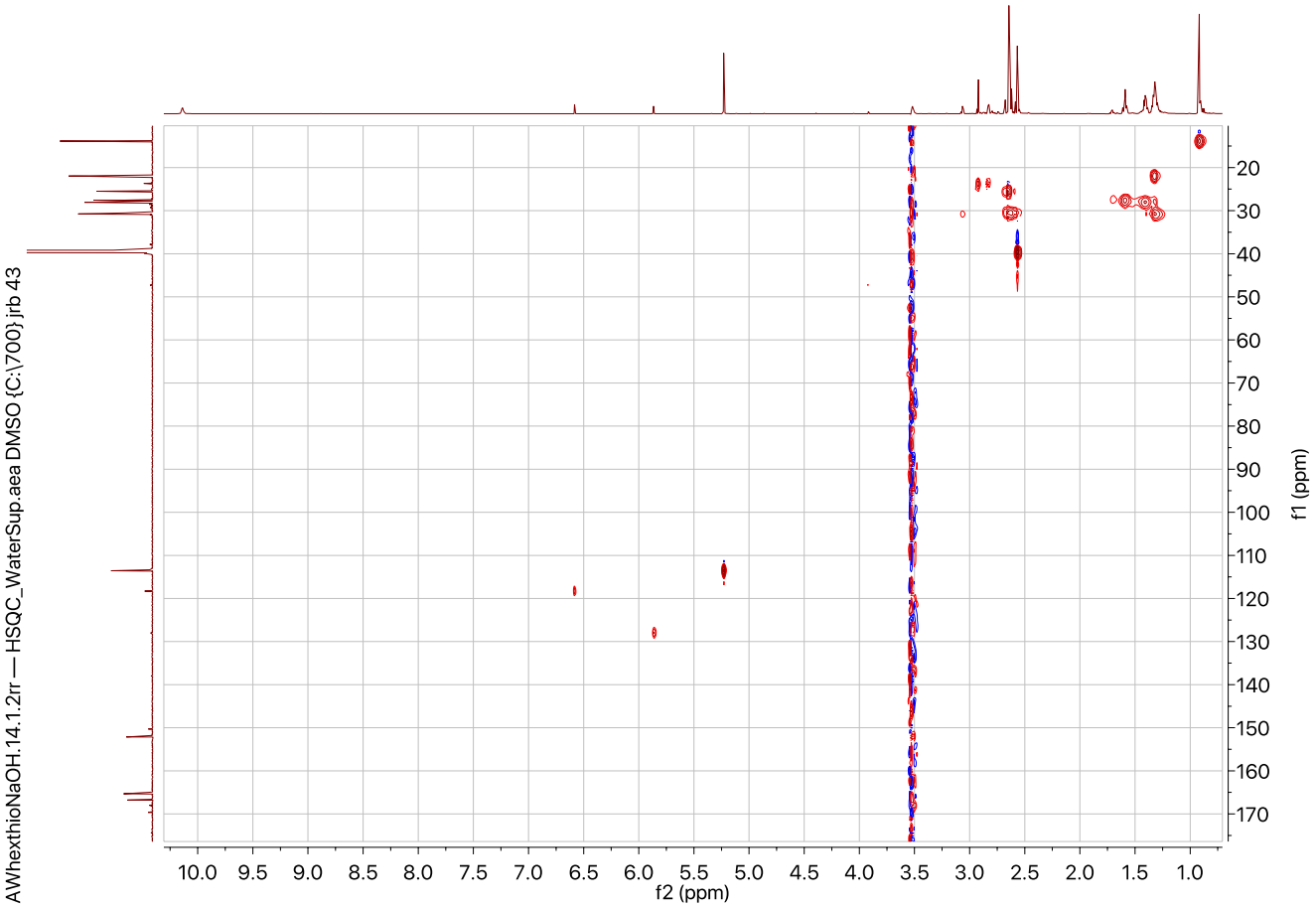
3-(hexylthio)-1-methyl-1*H*-pyrrole-2,5-dione (6.00 mg, 0.0264 mmol) was dissolved in DMSO (800 µL). NaOH (1M) was added dropwise to bring the solution to pH 11. The solution was analysed by NMR. Proton and carbon correlation was elucidated by HMBC and HSQC. The ratio of the major and minor hydrolyte regioisomers was worked out as an average of the integrations of distinct non-labile proton environments (hydrolytes A and B observed in ratio 12:88).

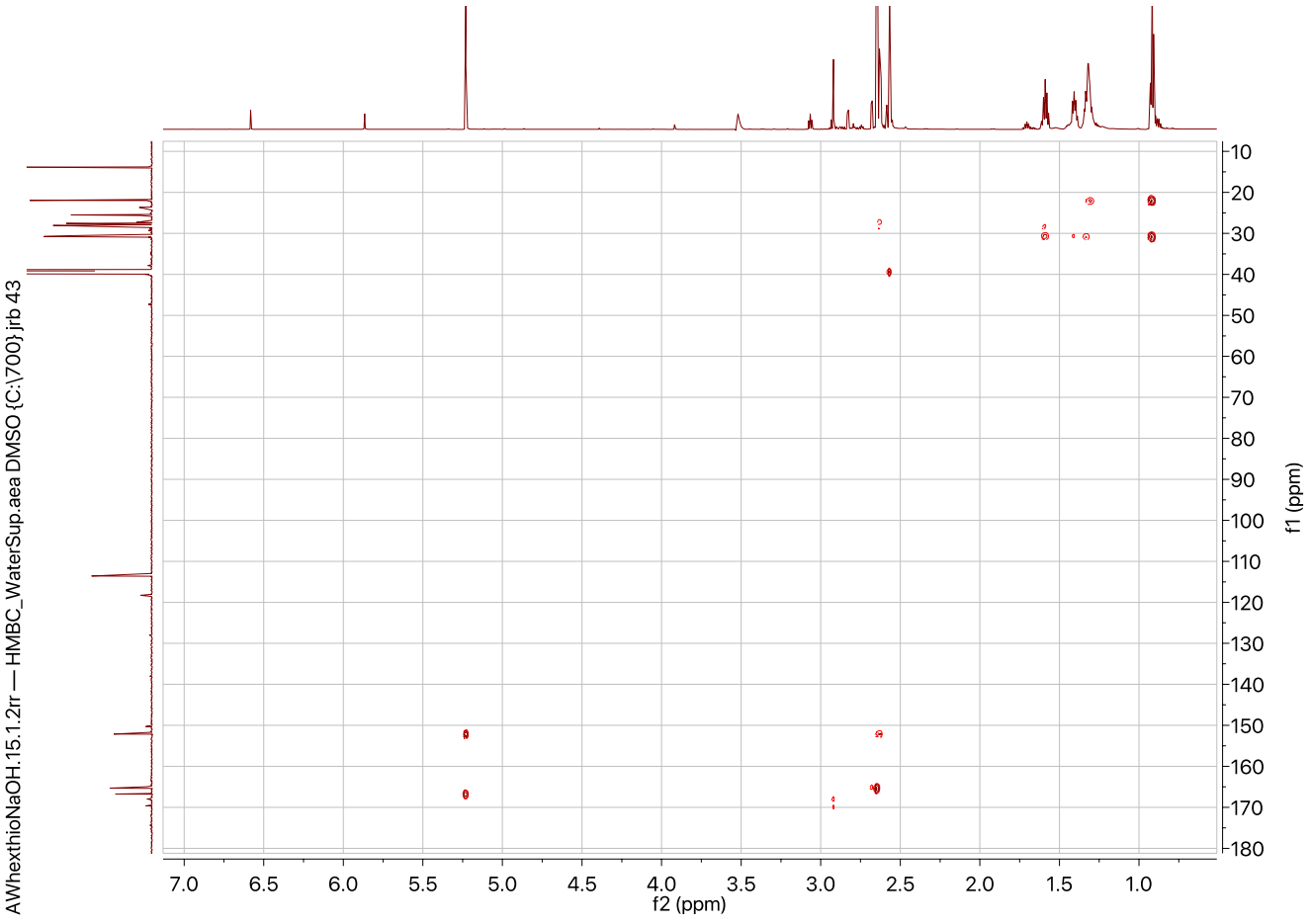
^{1}H NMR (700 MHz, DMSO) δ 9.93 (s, 1H, NH^{B}), 5.67 (s, 1H, $C{=}CH^{A}$), 5.03 (s, 1H, $C{=}CH^{B}$), 2.48 (d, *J* = 4.5 Hz, 3H, $CH_3{}^{A}$), 2.45 (d, *J* = 4.6 Hz, 3H, $CH_3{}^{B}$), 2.43 (t, *J* = 7.4 Hz, 2H, $SCH_2{}^{B}$), 1.39 (p, *J* = 7.8 Hz, 2H, $CH_2{}^{B}$), 1.24 – 1.18 (m, 2H, $CH_2{}^{B}$), 1.16 – 1.06 (m, 4H, 2 x $CH_2{}^{B}$), 0.72 (t, *J* = 7.0 Hz, 3H, $CH_3{}^{B}$). Not all environments visible by NMR for minor isomer.

^{13}C NMR (176 MHz, DMSO) δ 166.8 ($COOH^{B}$), 165.3 ($CONH^{B}$), 152.1 (SC^{B}), 118.3 ($C{=}CH^{A}$), 113.6 ($C{=}CH^{B}$), 30.8 ($CH_2{}^{B}$), 30.5 ($CH_2{}^{B}$), 28.1 ($CH_2{}^{B}$), 27.6 ($CH_2{}^{B}$), 25.5 ($NCH_3{}^{B}$), 22.0 ($CH_2{}^{B}$), 13.8 ($CH_3{}^{B}$). Not all environments visible by NMR for minor isomer.


9.93
5.67
5.03
2.48
2.48
2.45
2.45
2.43
1.39
1.21
1.21
1.12
1.12
1.12
1.12
0.72
0.84
0.13
1.00
0.44
3.41
2.57
2.86
6.35
3.97
AWhexthioNaOHfinisher.20.1.1r — WATER.ucl DMSO {C:\700} jrb 36
f1 (ppm)


166.77
165.33
152.14
118.30
113.58
30.76
30.54
28.08
27.56
25.49
21.96
13.85
AWhexthioNaOH (pure DMSO).12.1.1r — C13CPD.ucl DMSO {C:\700} jrb 43
f1 (ppm)


AWhexthioNaOH.14.1.2rr — HSQC_WaterSup.aea DMSO {C:\700} jrb 43
f2 (ppm)
f1 (ppm)


AWhexthioNaOH.15.1.2rr — HMBC_WaterSup.aea DMSO {C:\700} jrb 43
f2 (ppm)
f1 (ppm)

Protein Mass Spectra

Spectra were obtained at either UCL or at Albumedix. Where MS data has been obtained at Albumedix, this has been indicated in the text.

UCL MS Protocol:

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. Three LC-MS methods were used with the same LC column and mobile phases A (5% MeCN in aqueous 0.1% formic acid) and B (95% MeCN, 5% water, 0.1% formic acid).

The first LC-MS method used the following conditions (method **A**):- 15% B for 2 min increase to 32%B in 1 min (isocratic for 1 min at 32%B) increase to 35%B in 10 min, following by increase to 95%B over 4 min (isocratic for 2 min at 95%B), at 22min of LC-MS run 15%B for another 3 min to condition the LC column for the next run. The flow rate was 0.3 mL/min. The column temperature was set to 60°C. The electrospray source of the 6510 QTOF was operated in positive mode, gas temperature at 350 °C, gas flow 10 L/min, nebuliser at 30 psi, Vcap 3500, fragmentor at 350 skimmer1 at 65, AMU 250. The mass range was set to 100-3,100 Da. The data was acquired in profile mode.

In the second LC-MS method, LC gradient was as follows (method **B**):- 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. The electrospray sources operated with gas temperature 325 °C, gas flow 5 L/min, nebuliser at 20 psi, Vcap at 4,000, fragmentor at 175, Skimmer 65, AMU 140. The mass range was 100 to 3,000 Da.

In the third LC-MS method, the LC column was kept at 30 °C (method **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.

The QTOF mass spectrometer operated using positive electrospray ionisation (ESI) scanning at the *m/z* range from 100 to 3100 Da and acquiring profile data. The electrospray source operated with a capillary voltage of 4000, fragmentor at 175, skimmer at 65 and octopole RF peak at 750. Nitrogen was used as the nebuliser and desolvation gas at a flow of 5 L/min. The

acquisition rate was 1 spectra/sec and acquisition time 1000 msec/spectrum. Lockspray was used during analysis to maintain mass accuracy. The data was processed using MassHunter software (version B.07.00) and deconvoluted using maximum entropy deconvolution algorithm over the entire peak area in the total ion chromatogram (TIC).

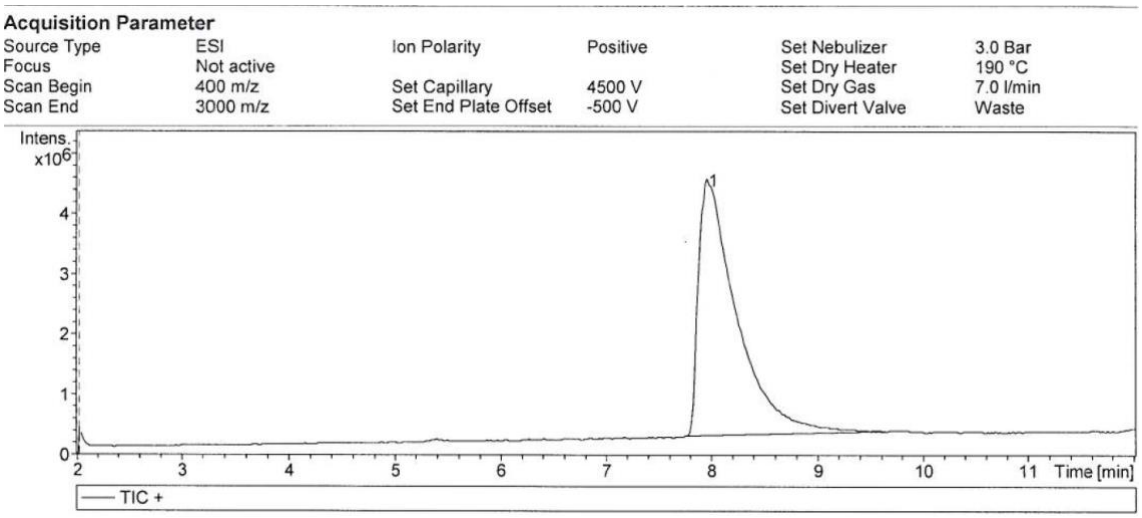
The entire peak area in the TIC was used to analyse the chromatograms, to give a true reflection of the conjugation efficiency. The exact timings can be found on the non-deconvoluted and deconvoluted data.

Albumedix MS protocol:

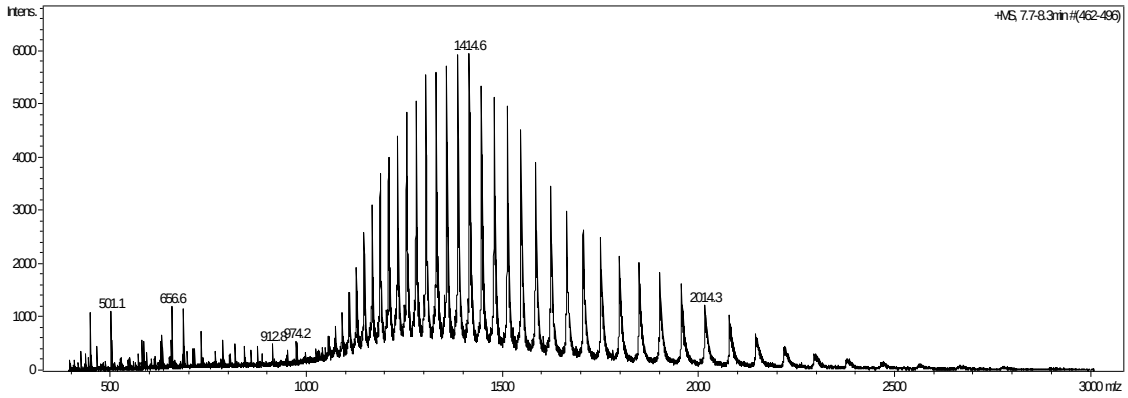
Liquid chromatography was performed on protein samples using a Waters Acquity (Waters) with a BEH 50 × 2.1 mm ACQUITY BEH 1.7 µm C4 column (Waters) employing a 15 min 0-70% Acetonitrile (Rathburn) / water analytical gradient with 0.1% formic acid at a flow rate of 0.4 mL/min. Eluted proteins were directly introduced to a Bruker MicrOTOF II mass spectrometer (Bruker Daltonics) *via* an ESI source. MS mode: ES+. Scan range (*m/z*): 400–3000. Data obtained in continuum mode. The electrospray source of the MS was operated with a capillary voltage of 4.5 kV and a capillary exit voltage of 160 V. Nitrogen was used as the nebulizer and dry gas at a 3.0 bar pressure for the nebulizer gas and 7.0 L/min for the dry gas. Ion series were generated by integration of the total ion chromatogram (TIC) over the 8–9 min range. Total mass spectra for protein samples were reconstructed from the ion series using the Compass DataAnalysis software using a baseline subtraction of 0.5 and a Gaussian smoothing with a width of 0.076 Da before deconvolution. All instrument control and sample tables were controlled using BioPharma Compass™ (Bruker Daltonics).

Native HSA Spectra:

(a)



(b)



(c)

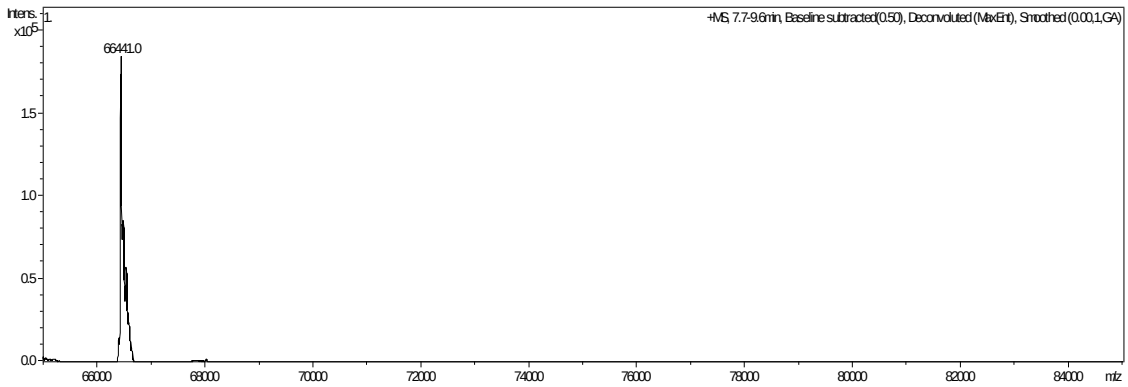
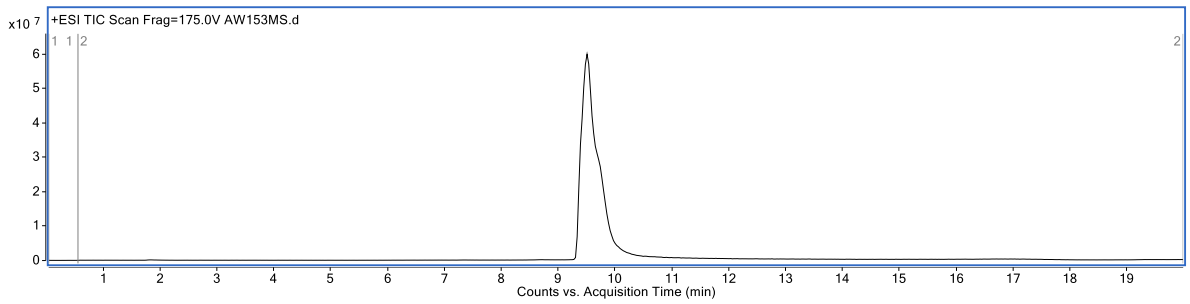


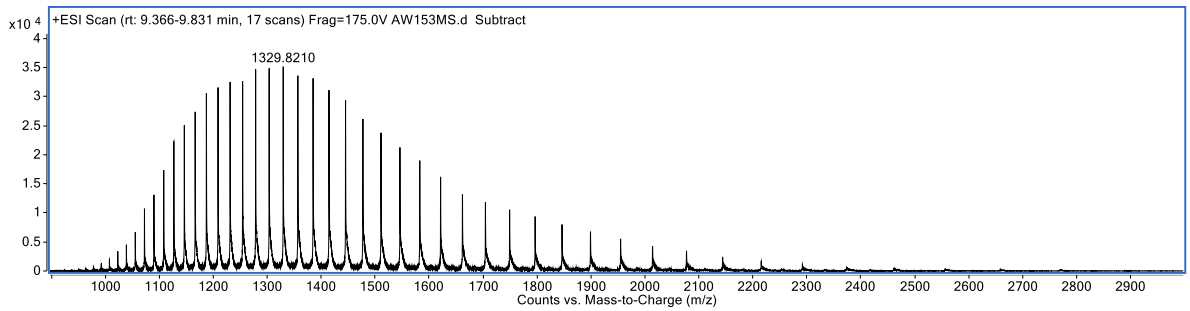
Figure S1A: (a) TIC chromatogram (b) non-deconvoluted data (c) deconvoluted data for native HSA. Expected Mass: 66439 Da. Observed Mass: 66431 Da. MS data obtained at Albumedix.

Native HSA Spectra:

(a)



(b)



(c)

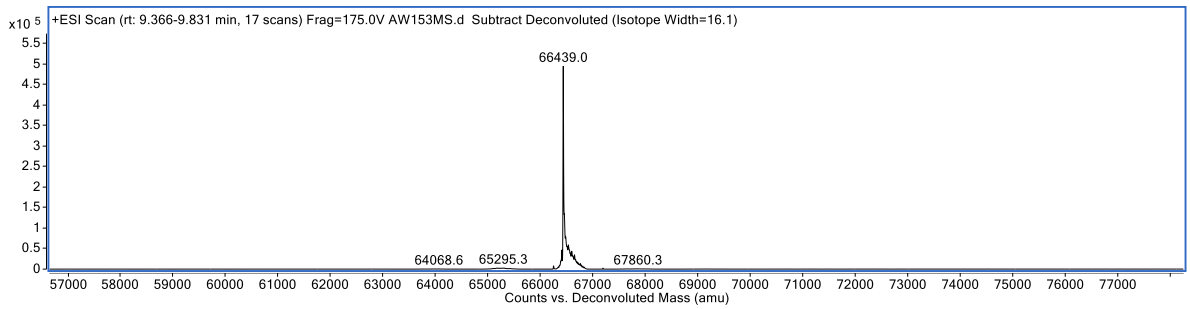
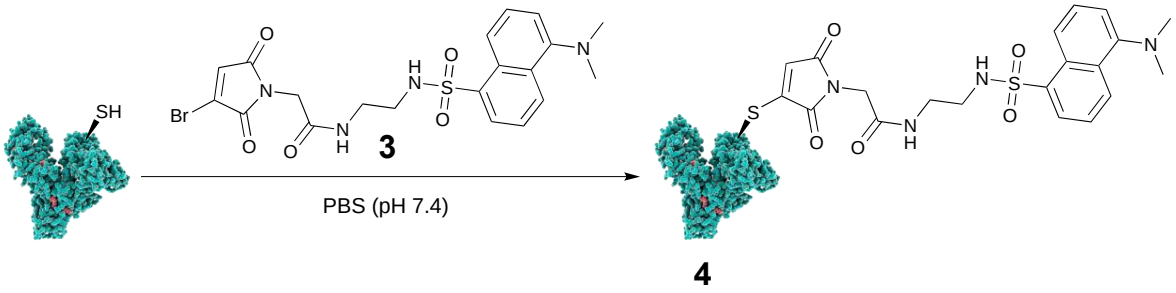


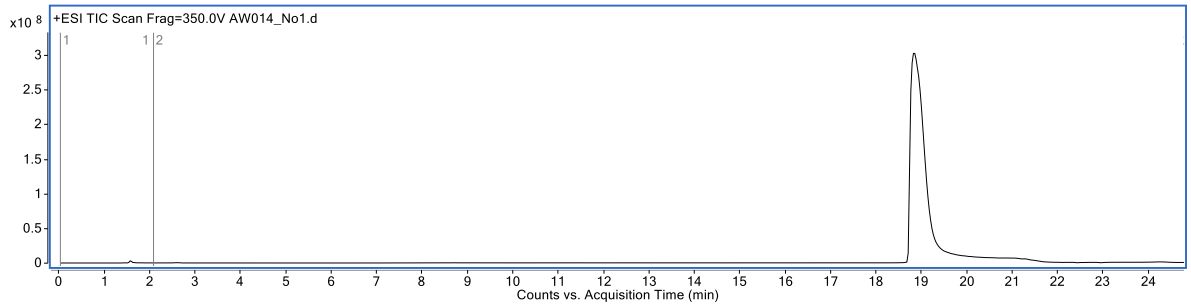
Figure S1B: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for native HSA. Expected Mass: 66439 Da. Observed Mass: 66439 Da. Data obtained with MS method **B**.

Bromomaleimide **3** bioconjugation to HSA

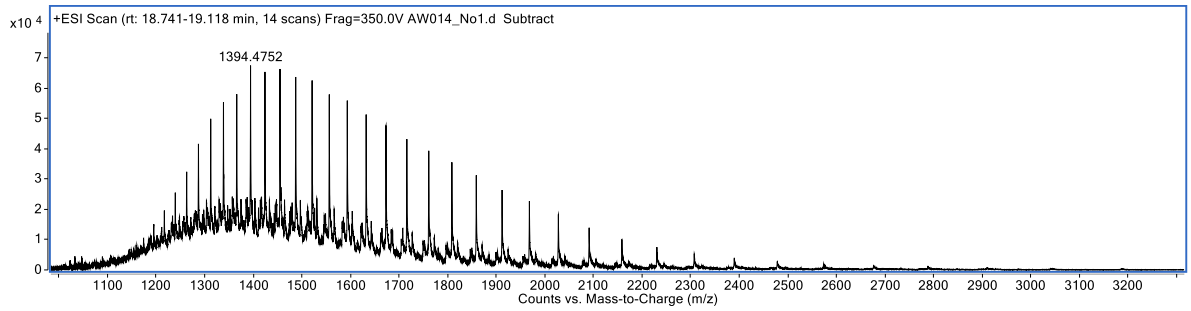


Bromomaleimide **3** (16.3 µL, 12.5 mM, 1.5 equiv. in DMF) was added to HSA (750 µL, 181 µM) in PBS buffer (12 mM phosphates, pH 7.4) and left at room temperature. After 10 minutes, the excess linker was removed *via* ultrafiltration (10 kDa MWCO, Amicon® Ultra-4 Centrifugal Filter Units). Conjugates were analysed by LCMS. Native HSA: 66,439 Da (expected 66,439); Cysteine modification: 66,885 Da (expected: 66,868 Da for hydrolysed maleimide due to time taken to acquire data); Double modification: 67,314 Da (expected: 67315 Da).

(a)



(b)



(c)

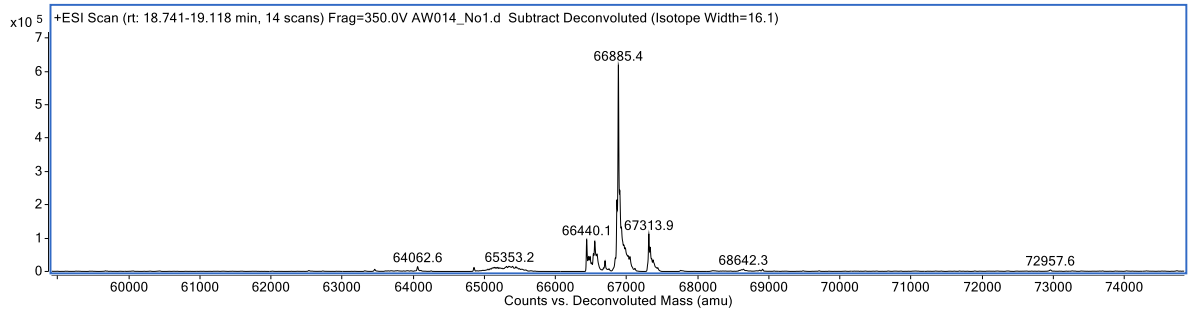
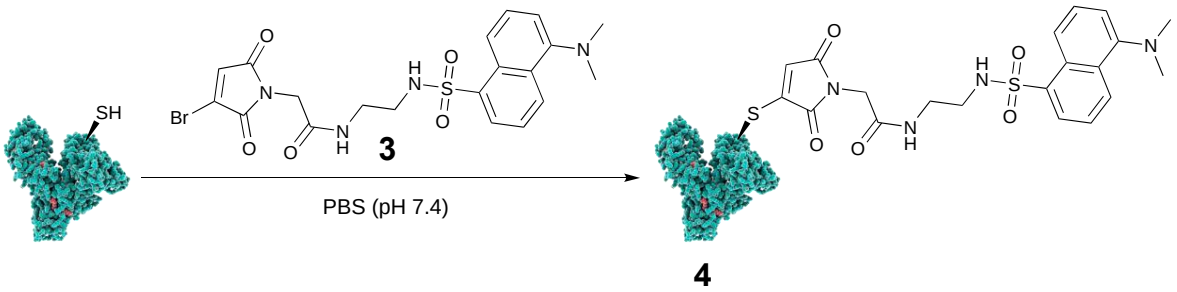


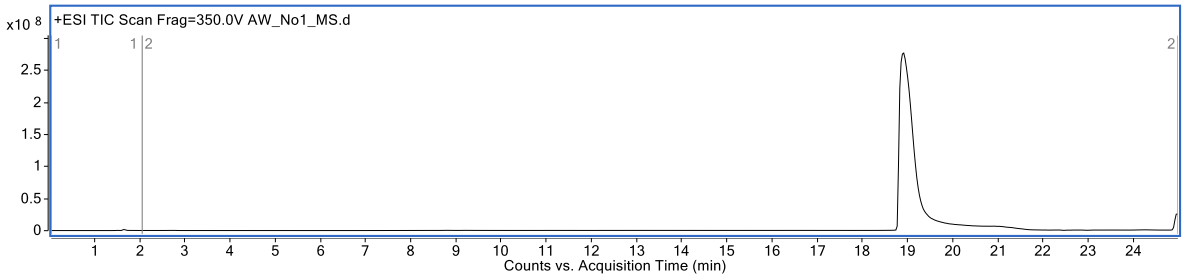
Figure S2: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for conjugate **4**. Data obtained with MS method **A**.

Bromomaleimide **3** bioconjugation to HSA (protocol limiting lysine modification) - used for fluorescence hydrolysis study in manuscript (Fig.1)

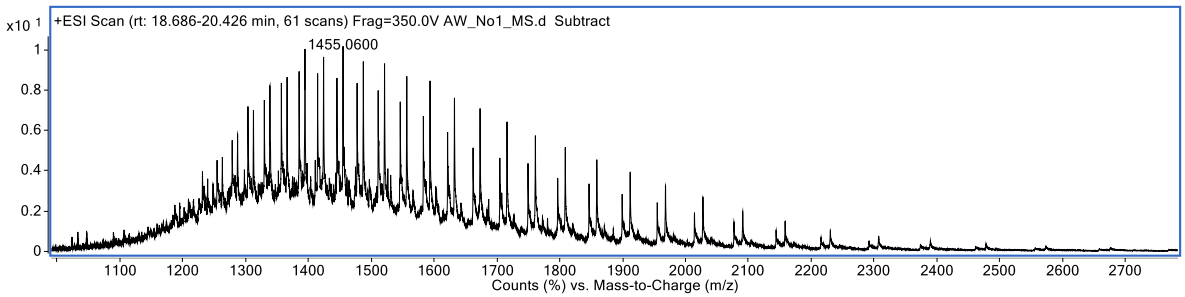


Bromomaleimide **3** (0.24 µL, 50 mM, 0.9 equiv. in DMF) was added to HSA (300 µL, 44 µM) in PBS buffer (12 mM phosphates, pH 7.4) and left at room temperature. After 10 minutes, the excess linker was removed *via* ultrafiltration (10 kDa MWCO, Amicon® Ultra-4 Centrifugal Filter Units). Conjugates were analysed by LCMS. Native HSA: 66,439 Da (expected 66,439); Cysteine Modification: 66,886 Da due to hydrolysis (+18 Da) during time taken to acquire data (expected: 66,868 Da); Double modification: 67,315 Da (expected: 67315 Da).

(a)



(b)



(c)

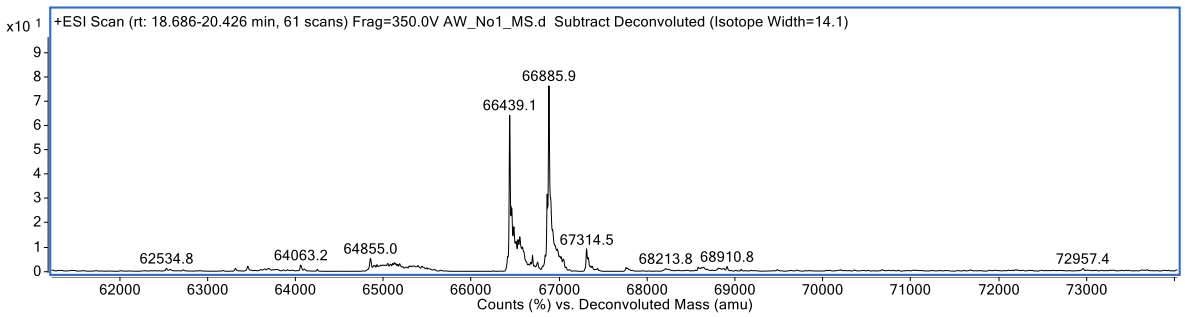
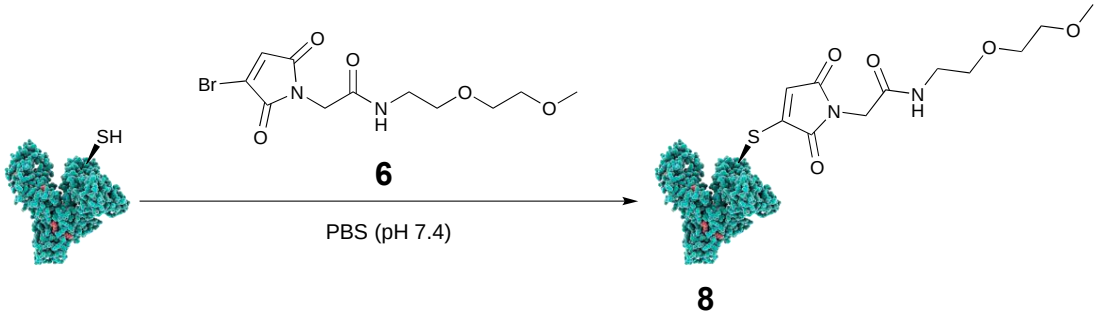


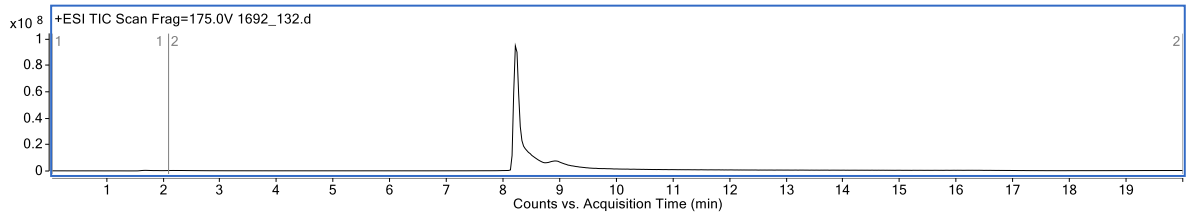
Figure S3: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for conjugate **4** (protocol limiting lysine addition). Data obtained with MS method **A**.

Bromomaleimide **6** bioconjugation to HSA

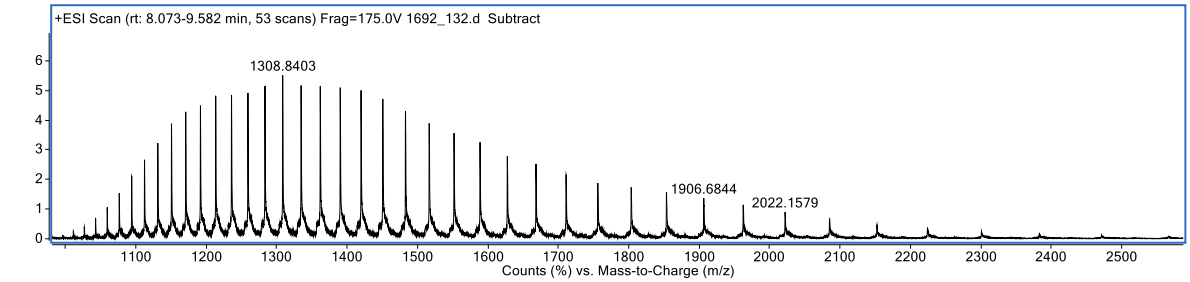


Bromomaleimide reagent **6** (3.3 µL, 10 mM, 2 equiv. in DMF) was added to HSA (165 µM, 100 µL) in PBS buffer (12 mM phosphates, pH 7.4) and left at room temperature. After 10 minutes, the excess linker was removed *via* ultrafiltration (10 kDa MWCO, Amicon® Ultra-4 Centrifugal Filter Units). Conjugates were analysed by LCMS. Cysteine Modification: 66,694 (expected: 66,694 Da).

(a)



(b)



(c)

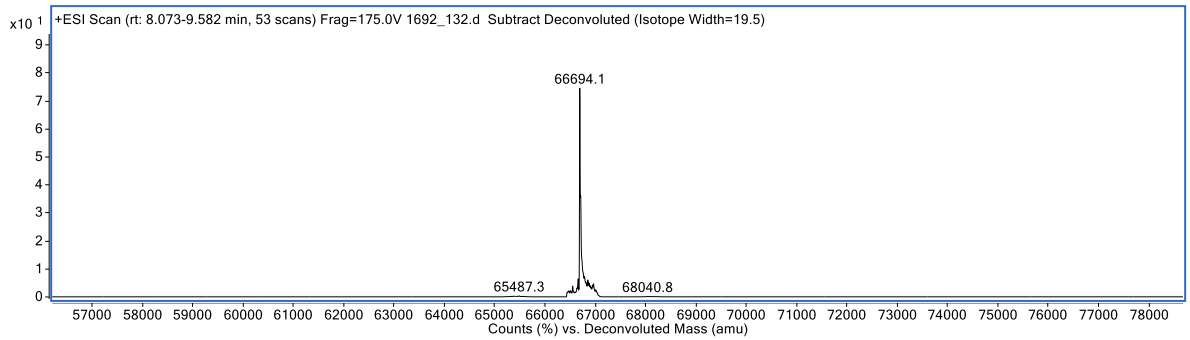
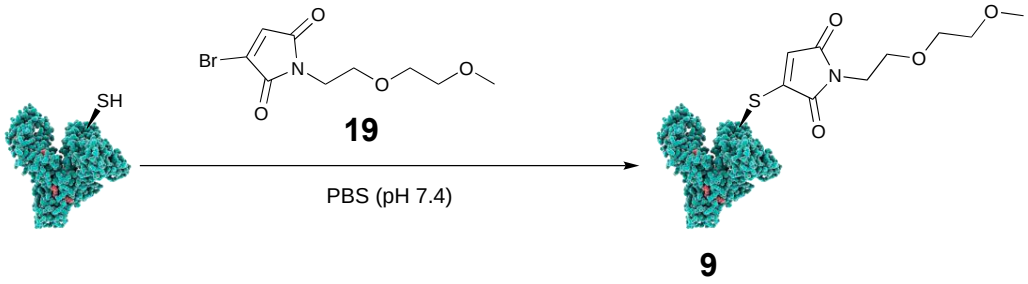


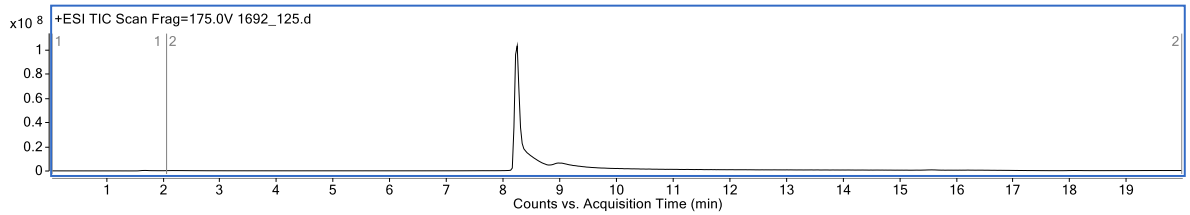
Figure S4: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for conjugate **8.** Data obtained with MS method **C**.

Bromomaleimide **19** bioconjugation to HSA

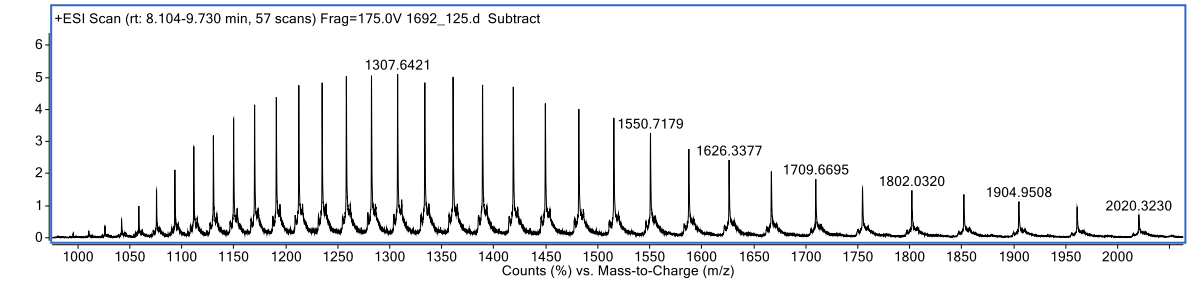


Bromomaleimide reagent (3.3 µL, 10 mM, 2 equiv. in DMF) was added to HSA (165 µM, 100 µL) in PBS buffer (12 mM phosphates, pH 7.4) and left at room temperature. After 10 minutes, the excess linker was removed *via* ultrafiltration (10 kDa MWCO, Amicon® Ultra-4 Centrifugal Filter Units). Conjugates were analysed by LCMS. Cysteine Modification: 66,639 (expected: 66,637 Da).

(a)



(b)



(c)

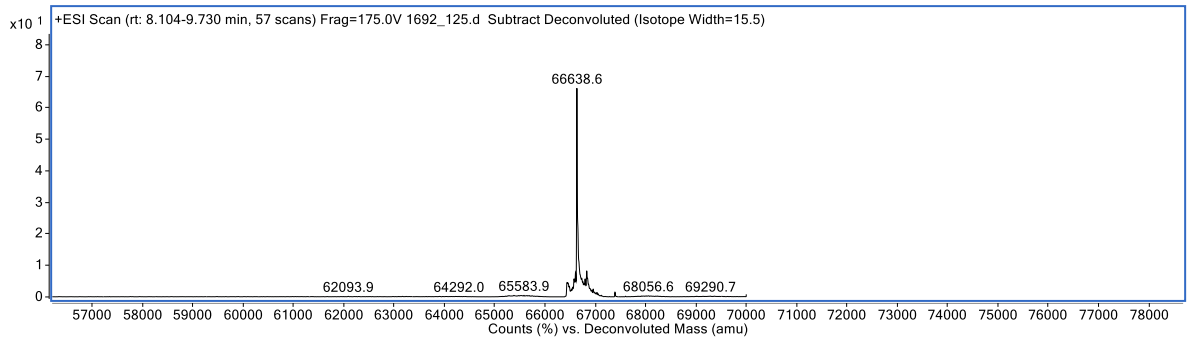
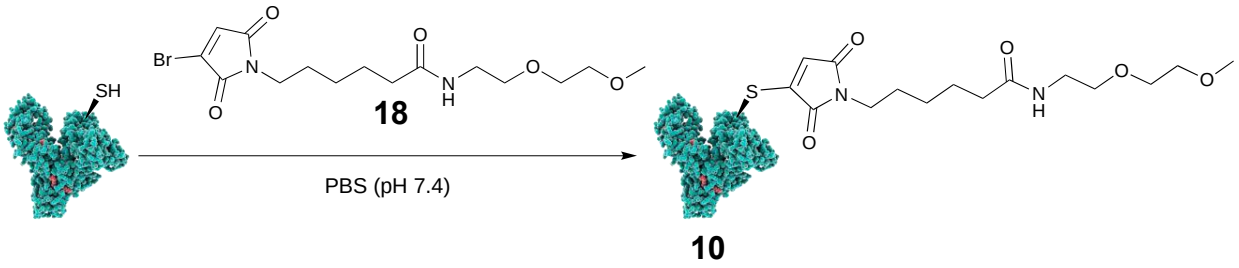


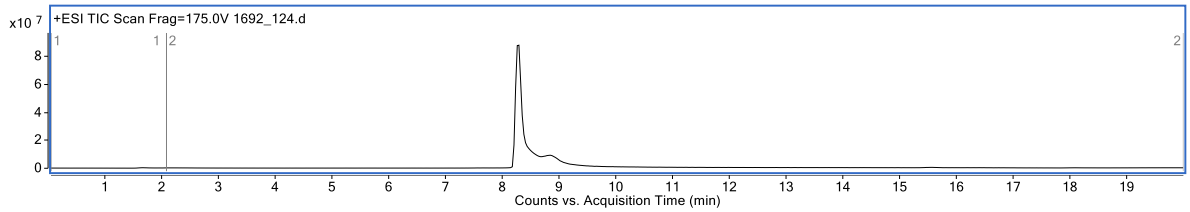
Figure S5: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for conjugate **10.** Data obtained with MS method **C**.

Bromomaleimide **18** bioconjugation to HSA

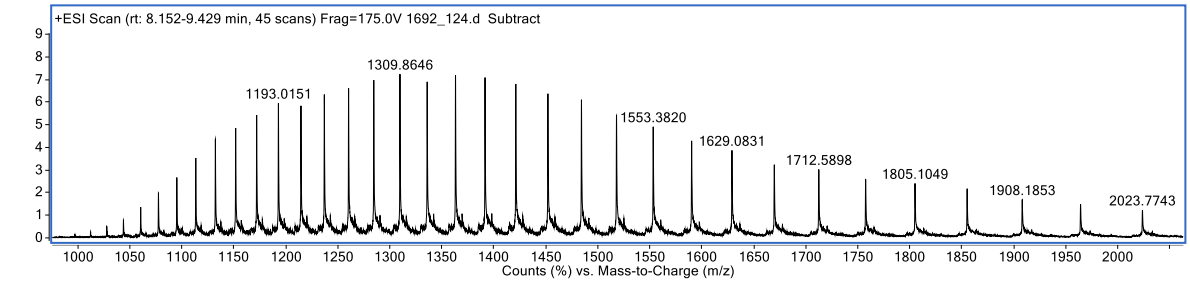


Bromomaleimide reagent (3.3 µL, 10 mM in DMF) was added to HSA (165 µM, 100 µL) in PBS buffer (12 mM phosphates, pH 7.4) and left at room temperature. After 10 minutes, the excess linker was removed *via* ultrafiltration (10 kDa MWCO, Amicon® Ultra-4 Centrifugal Filter Units). Conjugates were analysed by LCMS. Cysteine Modification: 66,750 (expected: 66,750 Da).

(a)



(b)



(c)

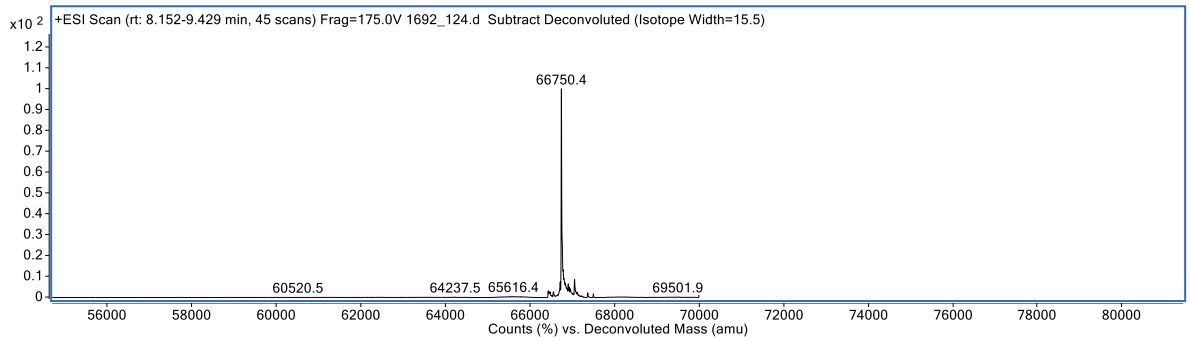
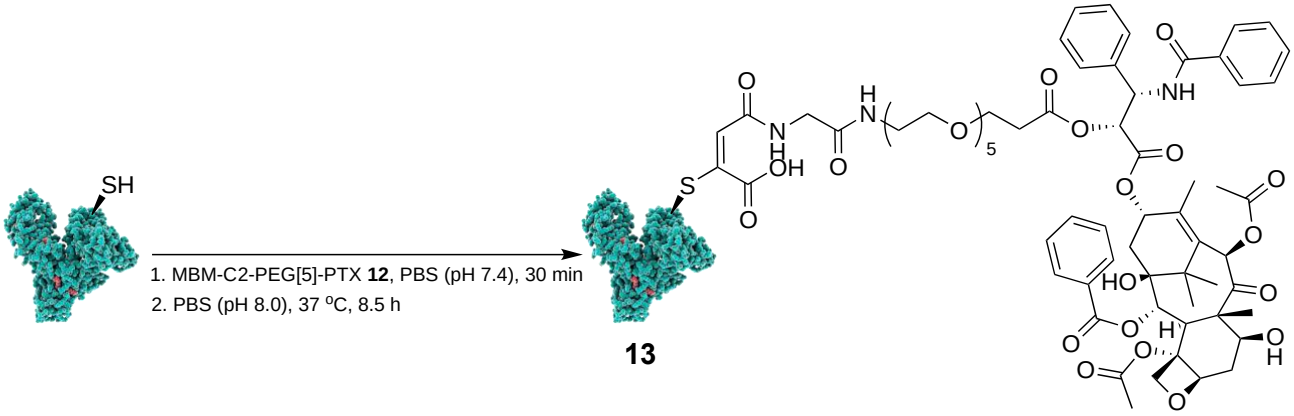


Figure S6: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for conjugate **9.** Data obtained with MS method **C**.

MBM-C2-PEG[5]-PTX **12** bioconjugation to HSA, followed by pH 8, 37 ^{o}C hydrolysis for 8.5 h



Bromomaleimide **12** (0.45 mL, 10 mM, 1.5 equiv. in DMF) was added to HSA (300 µM, 10 mL) in PBS (40 mM, pH 7.4). The reaction was stirred at room temperature for 30 minutes. The conjugate was purified by HIC purification (conjugation and purification protocol carried out at Recipharm).

Procedure for HIC purification:

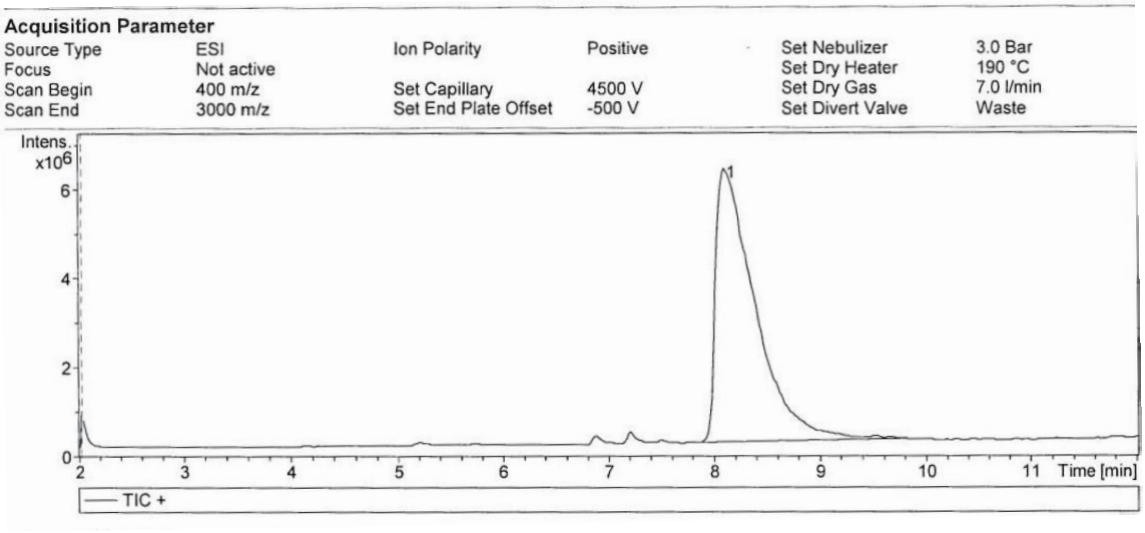
The HSA conjugate sample was diluted with equal volumes of ammonium sulphate (1.5 M) and sodium octanoate (10 mM). The pH was corrected to 7.0 to give a resulting solution of ammonium sulphate (750 mM), sodium phosphate (20mM) and sodium octanoate (5mM).

A GE Healthcare HiTrap Butyl FF column was equilibrated with ammonium sulphate (750 mM), sodium phosphate (20 mM), sodium octanoate (5 mM) at pH 7.0 for 1 CV (at 5 mL/min for 5 mL column). The sample was loaded at a slow flow rate (0.2 CV – 1 mL/min for 5 mL column). The column was washed with equilibration buffer (5 CV) at a slow flowrate. The pure HSA conjugate was eluted with an isocratic step in 5 CV of PBS pH 7.0 at 1 mL/min – peak collection was done from the first peak of fractions with notable 280 nm signal.

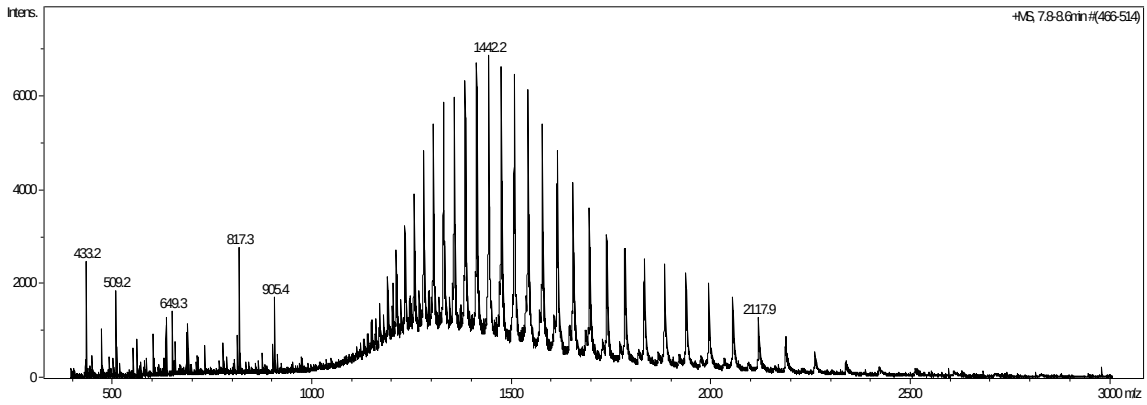
Hydrolysis protocol:

The resulting conjugate (250 µL, 100 µM) was then diluted 20-fold into pH 8.0 PBS. The pH was corrected to 8.0 with NaOH (20 mM) and the sample was left at 37 ^{o}C for 8.5 h before conjugates were analysed by LCMS (hydrolysis protocol and LCMS analysis carried out at Albumedix). Cysteine Modification: 67,737 Da (expected: 66,738 Da).

(a)



(b)



(c)

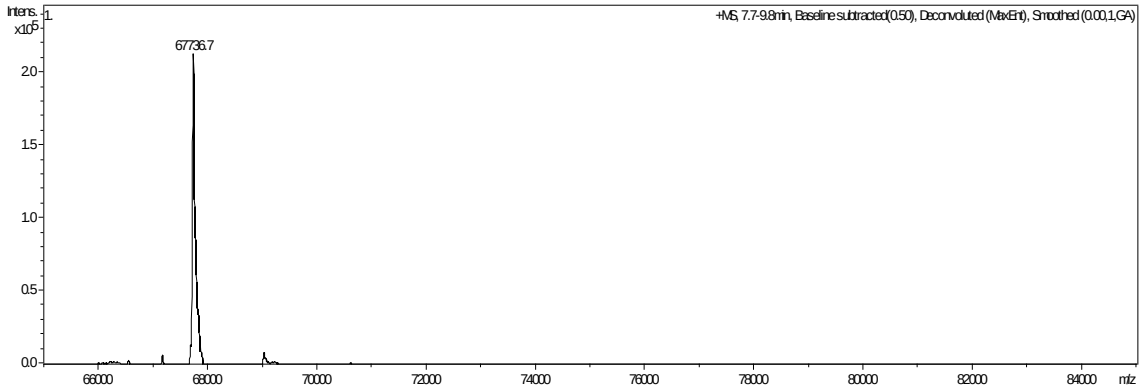
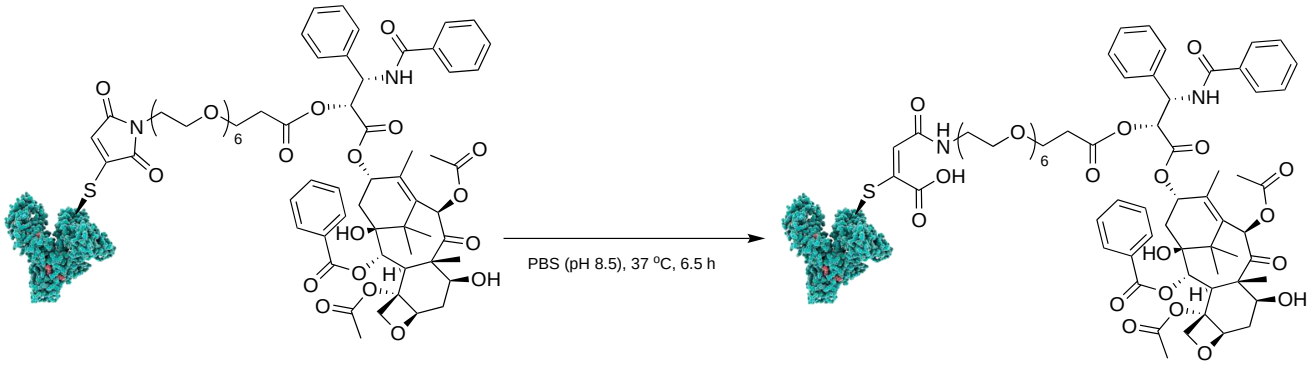


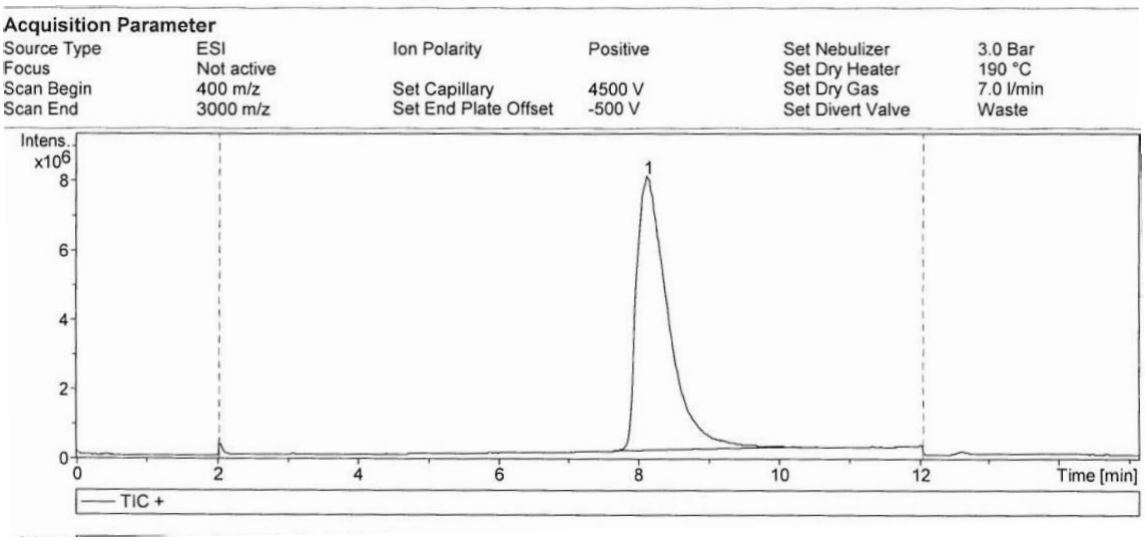
Figure S7: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for conjugate **13**. MS Data obtained at Albumedix.

MBM-PEG[6]-PTX conjugate hydrolysed at pH 8.5, 37 ^{o}C for 6.5 h

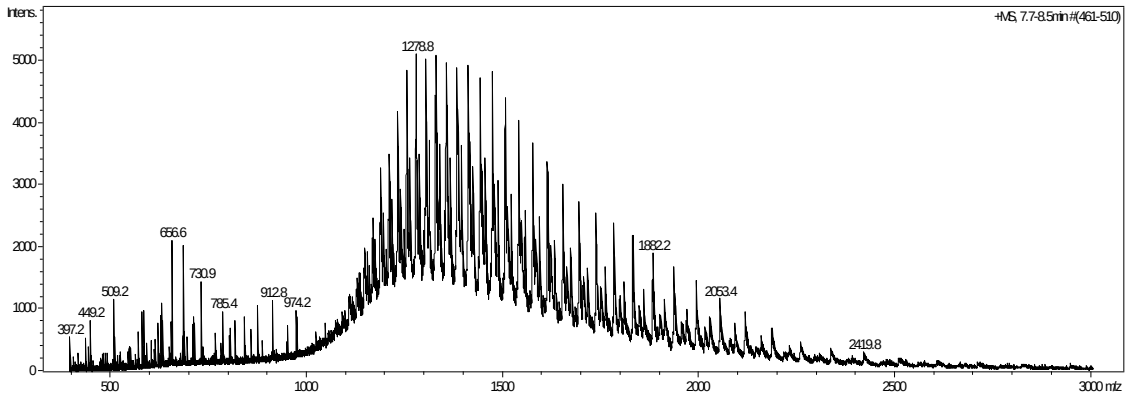


The conjugate (250 µL, 100 µM, kindly provided by Albumedix) was then diluted 20-fold into pH 8.5 PBS. The pH was corrected to pH 8.5 with NaOH (20 mM) and the sample was left at 37 ^{o}C for 8.5 h to hydrolyse before conjugates were analysed by LCMS (hydrolysis protocol and LCMS analysis carried out at Albumedix). Cysteine modification: 67,722 Da (expected: 66,725 Da); degraded conjugate (loss of PTX) 66,886 Da (expected: 66,889 Da); native HSA: 66,442 Da (expected: 66,439 Da)

(a)



(b)



(c)

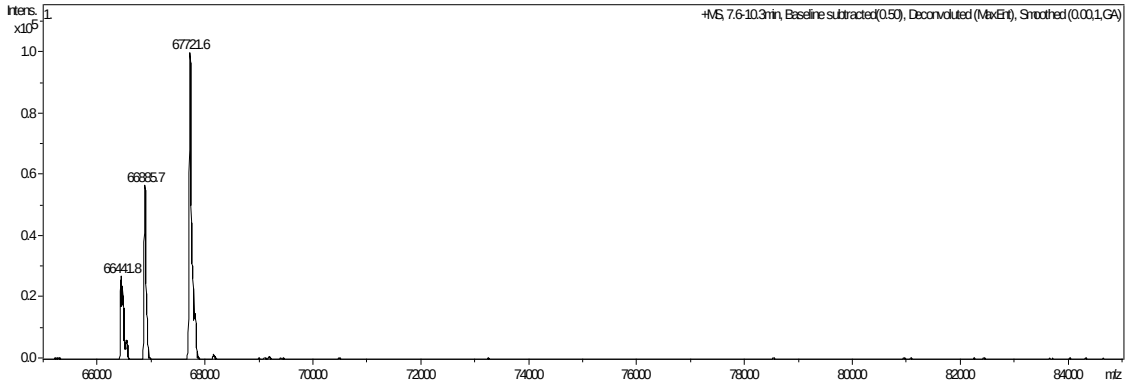


Figure S8: (a) TIC chromatogram; (b) non-deconvoluted data (c) deconvoluted data for MBM-PEG[6]-PTX conjugate**,** post hydrolysis**.** MS Data obtained at Albumedix.

Kinetic Data

Note on Analysis:

All hydrolysis experiments were carried out in aqueous buffers at pH 8.0 with the hydroxide ion at a stable concentration to classify the reaction as pseudo-first order. For pseudo-first order reactions, the half-life (the time taken for the concentration of the reactant to decrease by half) can be calculated as follows:

$$[A]_{1/2} = \frac{1}{2}[A]_0$$

where $[A]_0$ is the initial concentration of the starting material. After the duration of one half-life, $t=t_{1/2}$:

$$\frac{[A]_{1/2}}{[A]_0} = \frac{1}{2} = e^{-kt_{1/2}}$$

$$\ln 0.5 = -kt_{1/2}$$

We know that (- ln 0.5 = ln 2), therefore:

$$t_{1/2} = \frac{\ln 2}{k} \approx \frac{0.693}{k}$$

Method for Fluorescence:

In order to work out k, the following was carried out:

Given that the concentration of the product is proportional to its fluorescence (at a given wavelength) we can use the initial and final fluorescence values (assuming that all product derives from starting material) that are obtained from the fluorimeter to work out the concentration of the starting material and plot the natural log of these values against time.

The negative of the gradient of the graph was then defined as k.

The half-life value was obtained and then used to work out the $t_{99\%}$ (time taken for 99% hydrolysis):

$$t_{1/2} \times 7 = t_{99\%}$$

Method for Absorbance:

In order to work out k, the following was carried out:

Given that the concentration of the starting material is proportional to its absorbance (at a given wavelength) we can use the absorbance values that are obtained from the UV spectrophotometer and plot the natural log of these values against time. The negative of the gradient of the graph was then defined as k.

The half-life value was obtained and then used to work out the $t_{99\%}$ (time taken for 99% hydrolysis):

$$t_{1/2} \times 7 = t_{99\%}$$

Fluorescence Data

Dansyl fluorophore is quenched by maleimide through Photoinduced electron transfer (PET). Upon hydrolysis, fluorescence "turns on".[5] Dansylamine group excitation wavelength of 337 nm, emission wavelength of 559 nm.

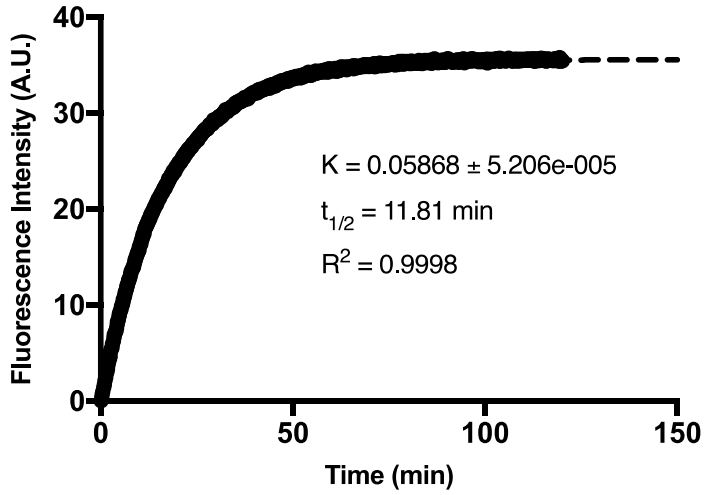
Baseline removed and data fitted with Prism Graphpad 7.0's non-linear regression software. Mode: One phase exponential association.

Molecule	k'	$t_{1/2}$	$t_{99\%}$ (min)
Maleimide reagent **3**[a]	0.05868	11.8	82.7
Cysteine Model **5**[b]	0.01401	49.5	346.4
Conjugate **4**[a]	0.0006819	1017.0	7119.0

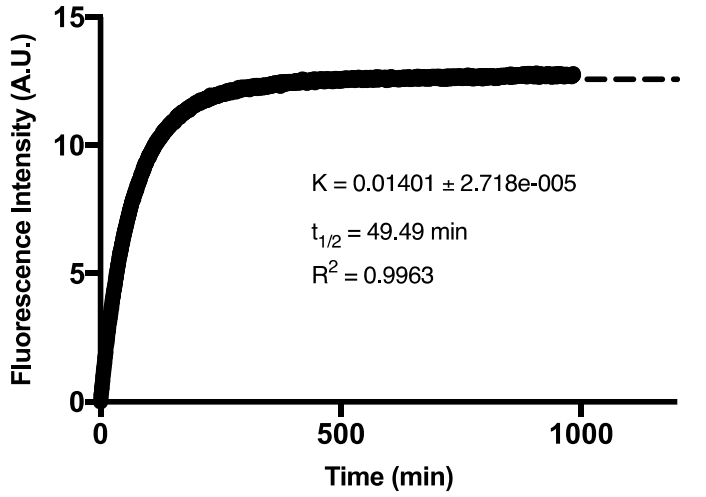
Reactions carried out in 1% DMF in BBS (pH 8.0) at RT. [a] carried out at 90 μM. [b] carried out at 56 μM. $t_{99\%}$ (time taken for 99% completion) defined as the time taken for 7 half-lives.

N.B. Extrapolation of curve in Prism (using least squares fit) for conjugate has allowed calculation of a $t_{99\%}$ that is greater than experiment acquisition time.

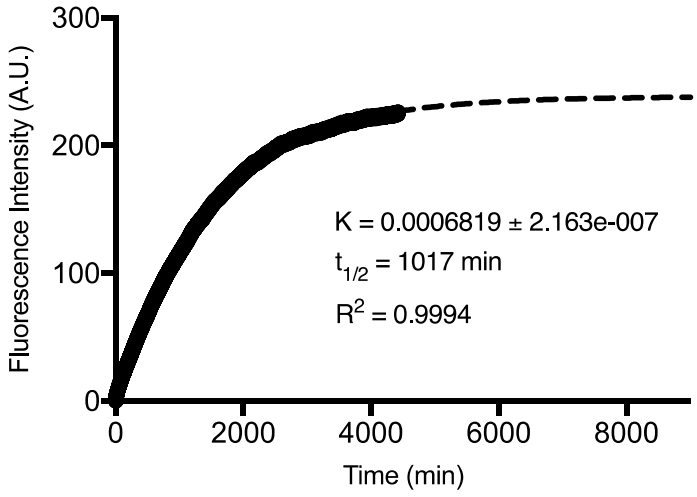
Reagent **3** Hydrolysis (pH 8, RT)

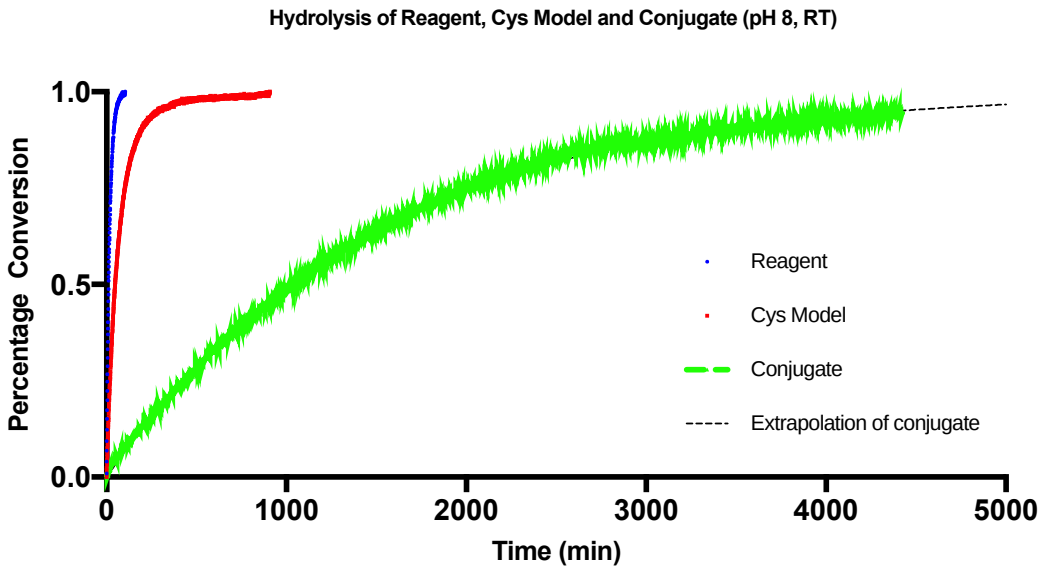


Cys Model **5** Hydrolysis (pH 8, RT)



Conjugate **4** hydrolysis (pH 8, RT)

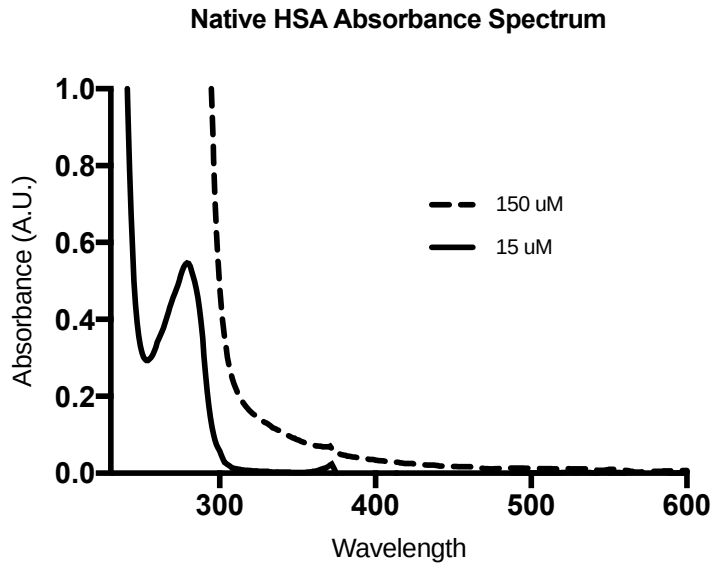




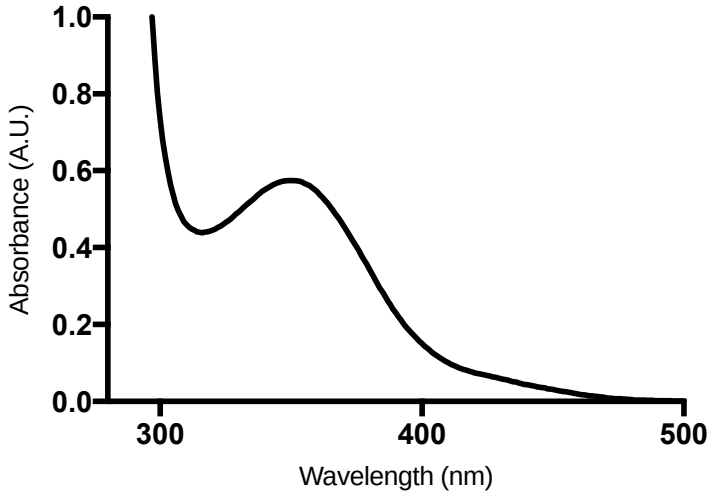
UV Data

Note on UV Absorbance of Monobromomaleimides

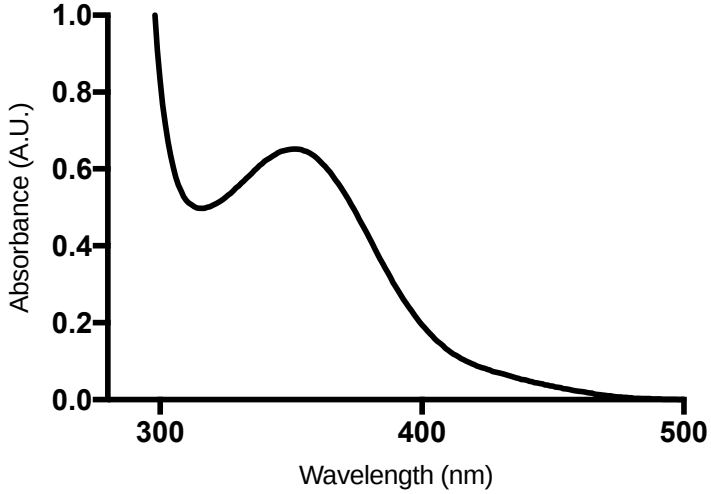
Upon conjugation to a thiol the monobromomaleimide absorbance increases significantly (λ_{max} = 350 nm). The graphs below show the UV spectra of native HSA, and the characteristic absorbance at 350 nm of monothiomaleimides:



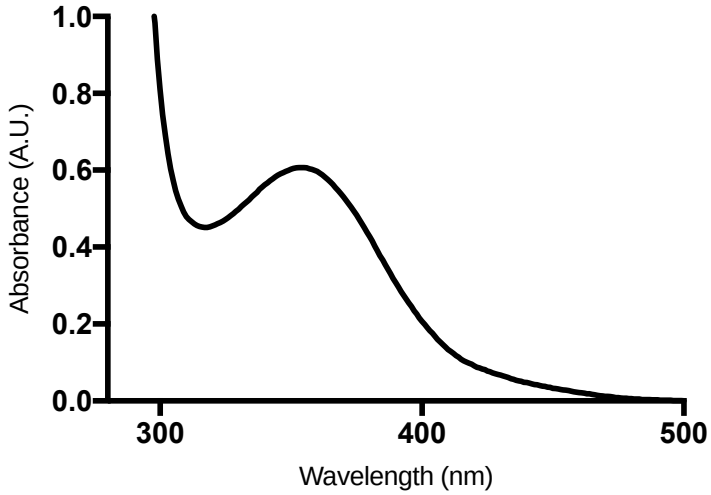
C-2 Model Conjugate **8**



N-PEG Model Conjugate **9**



C-6 Model Conjugate **10**

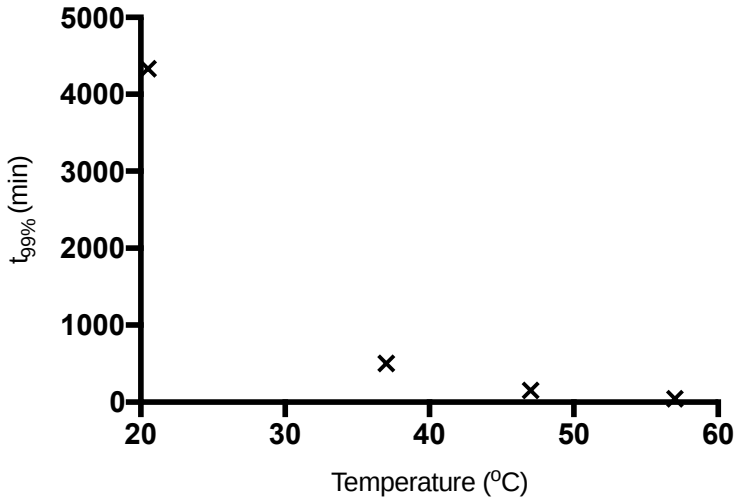


UV Hydrolysis Data

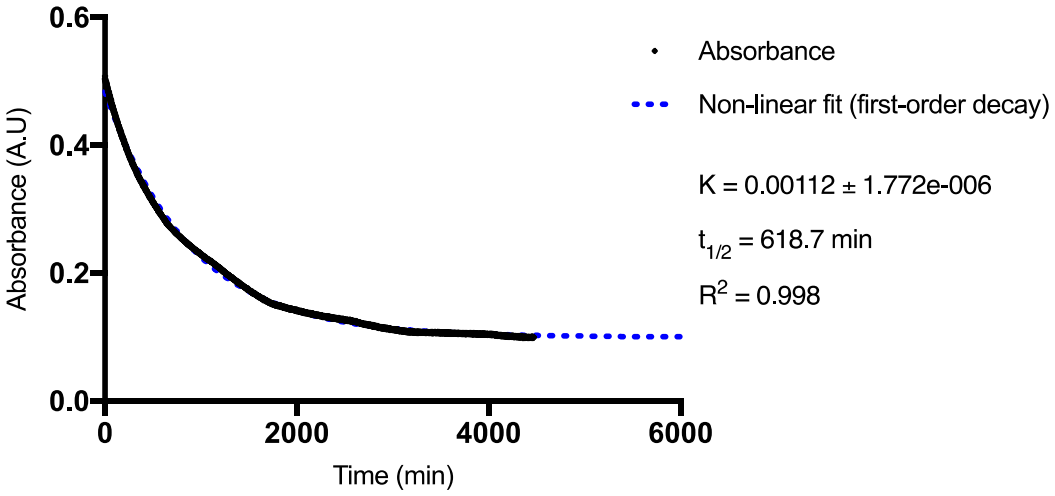
Bromomaleimide reagent (5.4 µL, 10 mM in DMF) was added to HSA (180 µM, 200 µL) in PBS buffer (12 mM phosphates, pH 7.4) and left at room temperature. After 10 minutes, the excess linker was removed *via* ultrafiltration (10 kDa MWCO, Amicon® Ultra-4 Centrifugal Filter Units), and buffer exchanged into BBS (25 mM, pH 8.0, 1 mM EDTA). The conjugates were then made up to approx. 90 µM and pipetted into a quartz cuvette. The characteristic thiomaleimide absorbance was monitored (350 nm with baseline correction) over time.

Entry	Molecule	Temp (°C)	k' (min^{-1})	$t_{1/2}$ (min)	$t_{99\%}$ (min)
1	7	20.5	0.001120	619	4333
2	7	37.0	0.009594	72	504
3	7	47.0	0.031920	22	154
4	7	57.0	0.118400	6	42
5	8	37.0	0.011070	63	441
6	9	37.0	0.001098	631	4417
7	10	37.0	0.000437	1586	11102

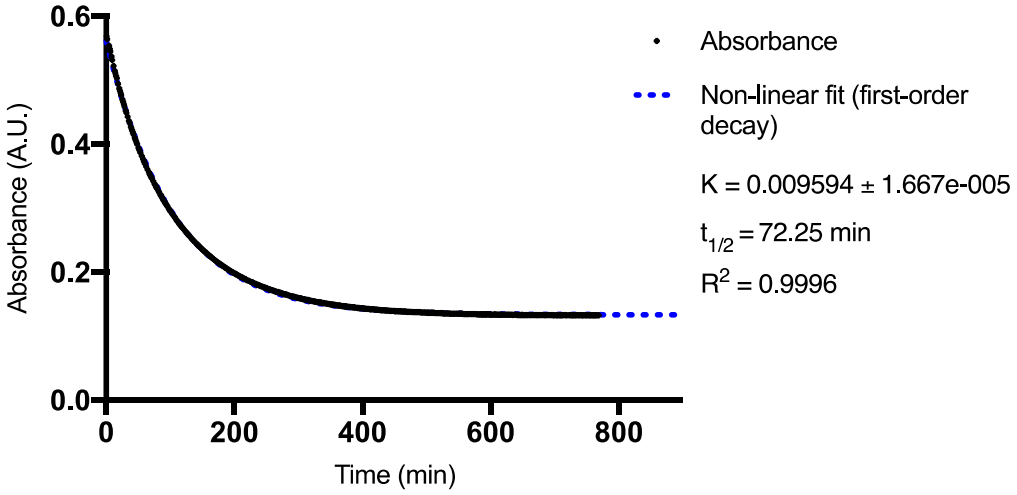
Conjugate **7** Hydrolysis Temperature Dependence



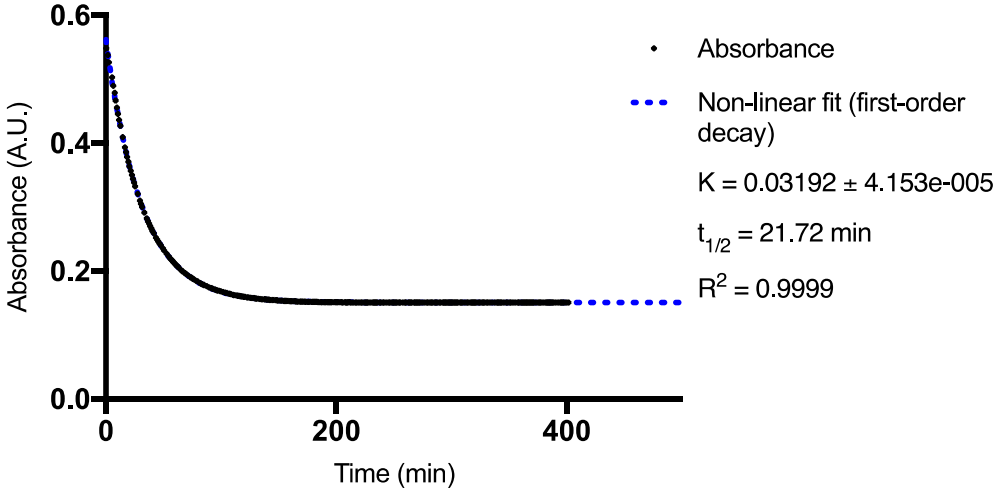
Hydrolysis of C2-ethanolamine Conjugate **7** at pH 8, 20.5 °C



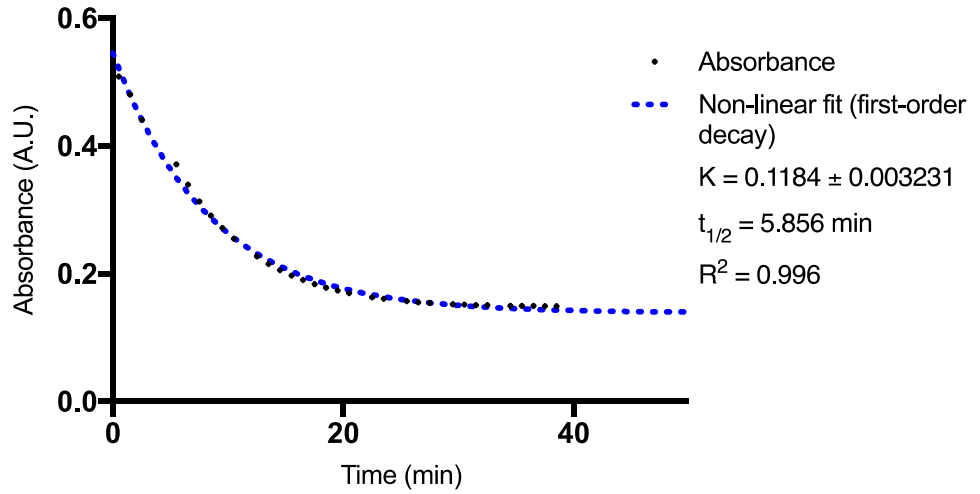
Hydrolysis of C2-ethanolamine Conjugate **7** at pH 8, 37 °C



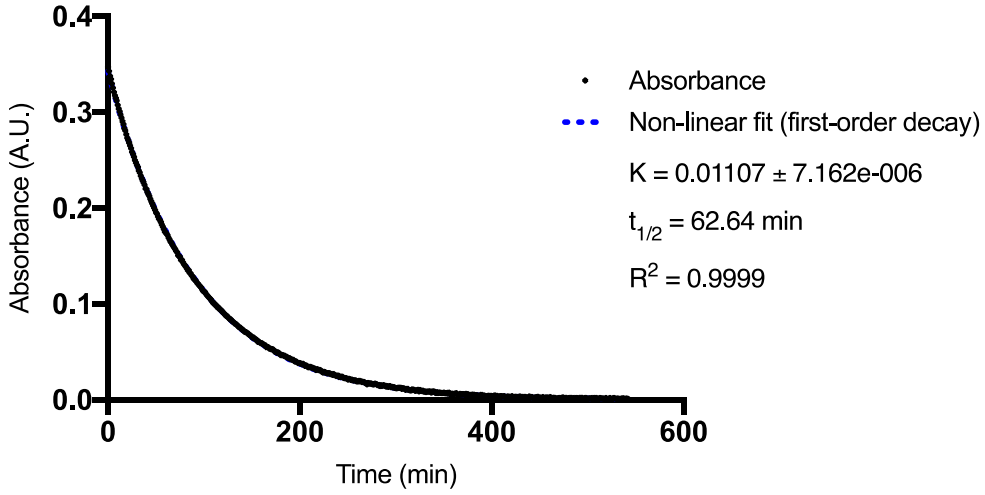
Hydrolysis of C2-ethanolamine Conjugate **7** at pH 8, 47 °C



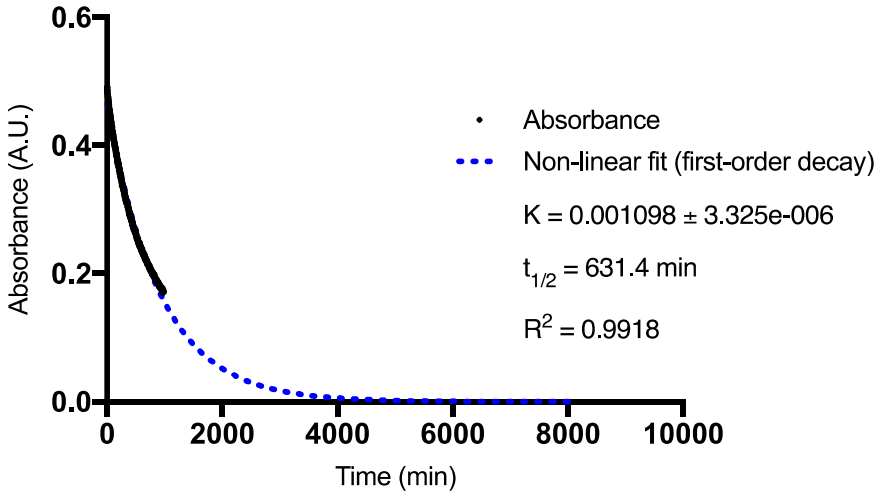
Hydrolysis of C2-ethanolamine Conjugate **7** at pH 8, 57 °C

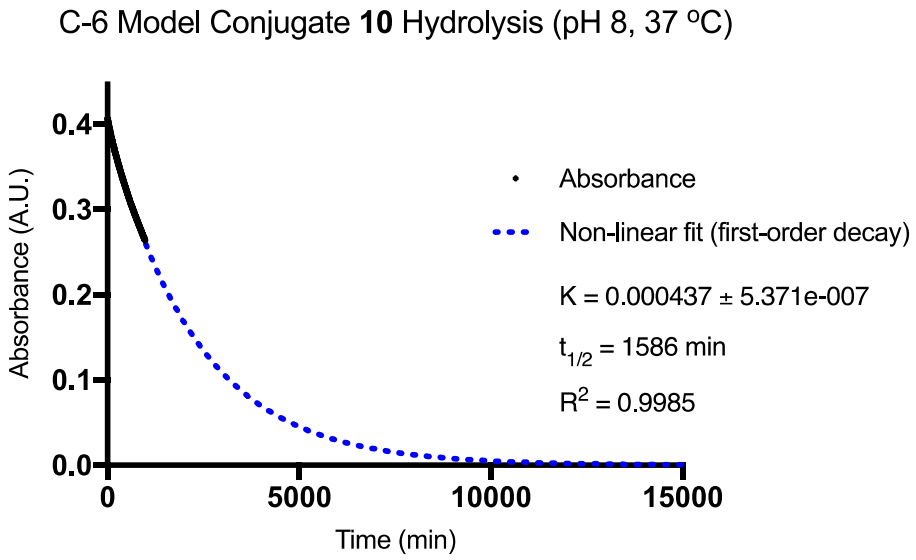


C-2 Model Conjugate **8** Hydrolysis (pH 8, 37 °C)



N-PEG Model Conjugate **9** Hydrolysis (pH 8, 37 °C)

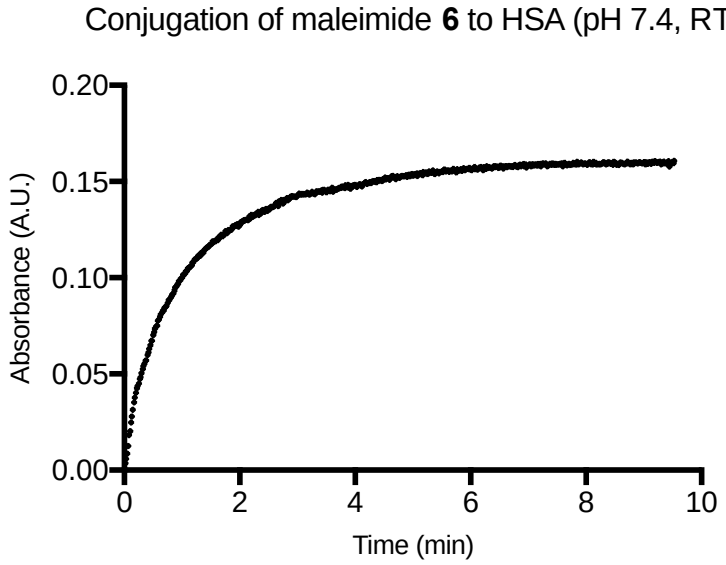



C-6 Model Conjugate 10 Hydrolysis (pH 8, 37 ºC)
Absorbance (A.U.)
0.4
0.3
0.2
0.1
0.0
0
5000
10000
15000
Time (min)
Absorbance
Non-linear fit (first-order decay)
K = 0.000437 ± 5.371e-007
t1/2 = 1586 min
R2 = 0.9985

UV Conjugation Data

Protocol:

Bromomaleimide **6** (5.4 µL, 10 mM in DMF) was added to HSA (180 µM, 200 µL) in PBS buffer (12 mM phosphates, pH 7.4) and pipetted into a cuvette at room temperature. The characteristic thiomaleimide absorbance was monitored (350 nm with baseline correction) over time using the UV spectrophotometer in kinetics mode.



MTT Assay

Human MDA-MB-231-luc-D3H2LN cells (Caliper Life Sciences) were seeded in 96-well plates (10,000 cells/well) using cell media (D5796, Sigma-Aldrich) containing 10% FBS, 1% NEAA and 1% PenStrep. Cells were cultured for 24 hours before addition of either free paclitaxel or HSA-paclitaxel diluted in cell media at 5 nM, 10 nM, 50 nM, 100 nM, 500 nM and 1000 nM paclitaxel concentration for 24 hours, 48 hours or 72 hours. The media was then removed and 200 µL fresh media with 0.5 mg/ml MTT (methylthiazolyldiphenyltetrazolium bromide, Sigma-Aldrich) was added to each well. Cells were then incubated for 4 hours at 37°C to allow formation of purple formazan crystals. Growth medium was removed and 100 µL DMSO (dimethyl sulfoxide, Sigma-Aldrich) was added to each well. Plates were incubated for 5 minutes while shaking at room temperature and the absorbance then measured at 580 nm using a CLARIOstar (BMG Labtech, Germany). MTT data were normalised to an untreated cell control.

Release of PTX from Conjugate in Cellular Media

Conjugate **13** (at a concentration of 0.30 mg/mL, 4.43 µM) was incubated in cell media (D5796, Sigma-Aldrich) containing 1% NEAA and 1% PenStrep. The media was incubated at 37 ^{o}C and analysed by LCMS after 48 h. Intact conjugate: 67,737 Da (expected: 66,738 Da); cleaved conjugate (loss of PTX at ester) 66,901 Da (expected: 66,902 Da).

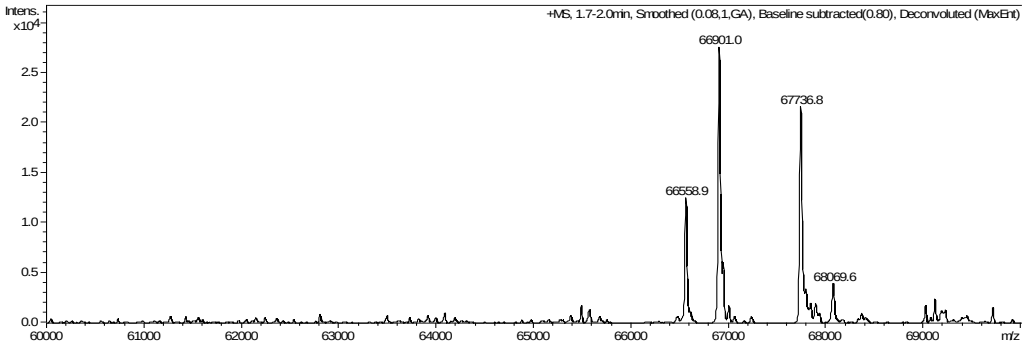


Figure S9: Deconvoluted MS data for conjugate **13** after 48 h at pH 7.4 and 37 ^{o}C**.** MS Data obtained at Albumedix.

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