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Control of β-branching in Kalimantacin Biosynthesis: Application of Direct Observe ^{13}C NMR to Polyketide Programming.

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Figure S1 HCS cassettes and modular architecture of exo-β-methylene branch incorporations. Examples of polyketides containing β-branches from *cis*-AT (**) and *trans*-AT PKS. The green dot shows the position of incorporation of acetate C-2 introduced by the HCS cassette. The pink ECH_2 annotation refers to a modular configuration of the domain. β-Branching pathway in the related *cis*-AT PKS compounds curacin[1] and jamaicamide[2]: following ECH_1-catalysed dehydration, the pathways diverge to form a chlorinated *endo*-β-methyl (curacin) that is subsequently cyclised to a cyclopropane ring, and an *exo*-β-methylene (jamaicamide) catalysed by ECH_2 domains CurF and JamJ respectively. The shared modular architecture of pederin[3] and the related compounds onnamide, diaphorin,[4] nosperin[5] and psymberin[6] are shown. The ECH di-domain in the pederin-related compounds and phormidolide possess an *N*-terminal domain (underlined) that is often truncated and possibly non-functional.[7] Domain length is annotated for ECH domains except DipP for which the length was not determined in the literature and sequence alignment was inconclusive for boundary assignment. Number of ACPs arranged as mono or tandem domains is shown in brackets. Epoxidation of an *exo*-β-methylene by a flavin-dependent monooxygenase results in epoxide moieties in spliceostatin[8] and thailanstatin.[9] Phormidolide contains five β-branches introduced by one *trans*-acting HCS cassette and tailoring ECH domains located in PhmE and PhmI at the point of *exo*-β-methylene incorporation. β-branching ACP_A are labelled (*).

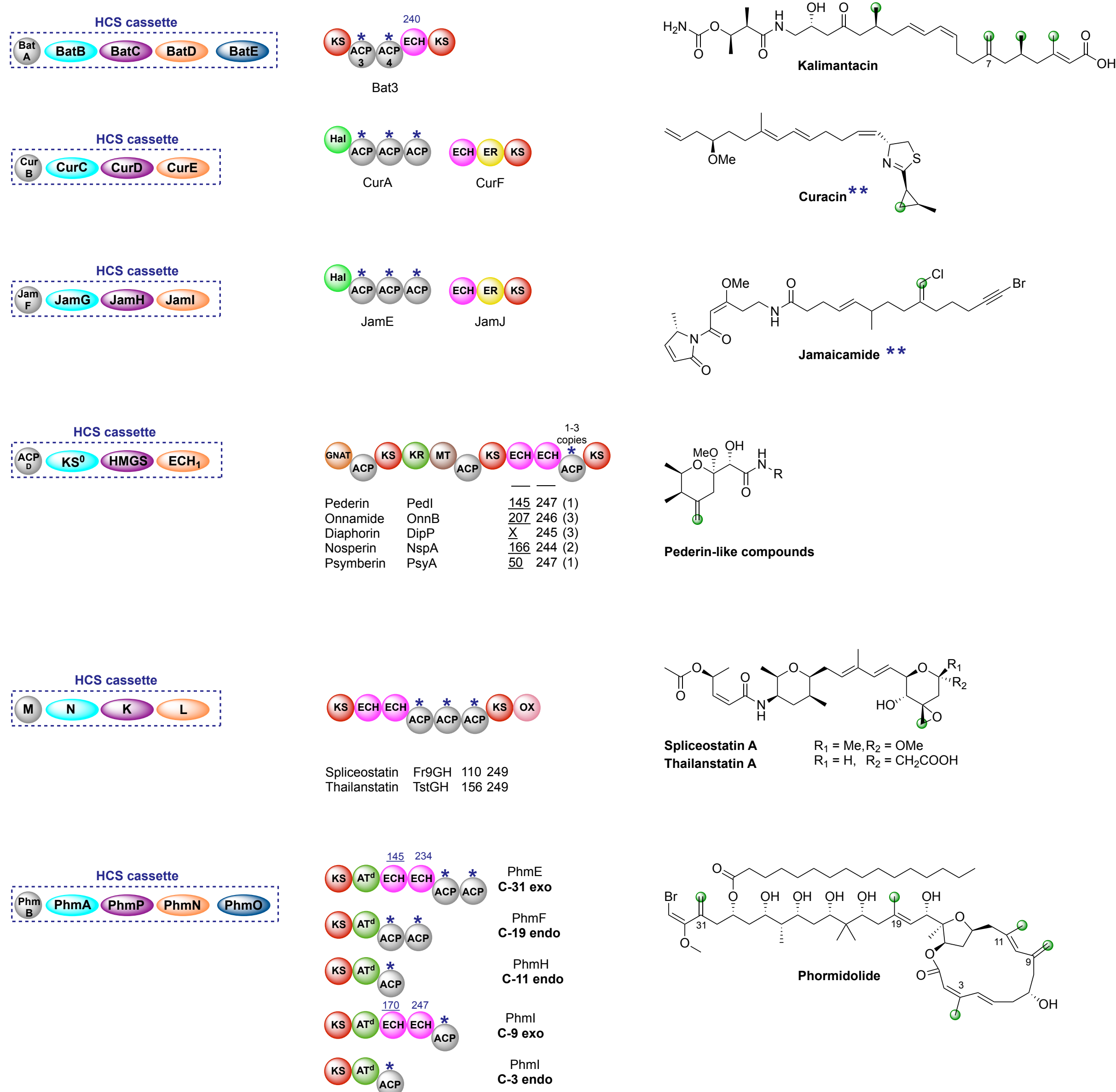


Figure S2. **Characterisation of the HCS cassette (BatA, C, D, E) and tailoring enzymes (BatK, mECH) by SDS-PAGE, ESI-MS and SEC.**

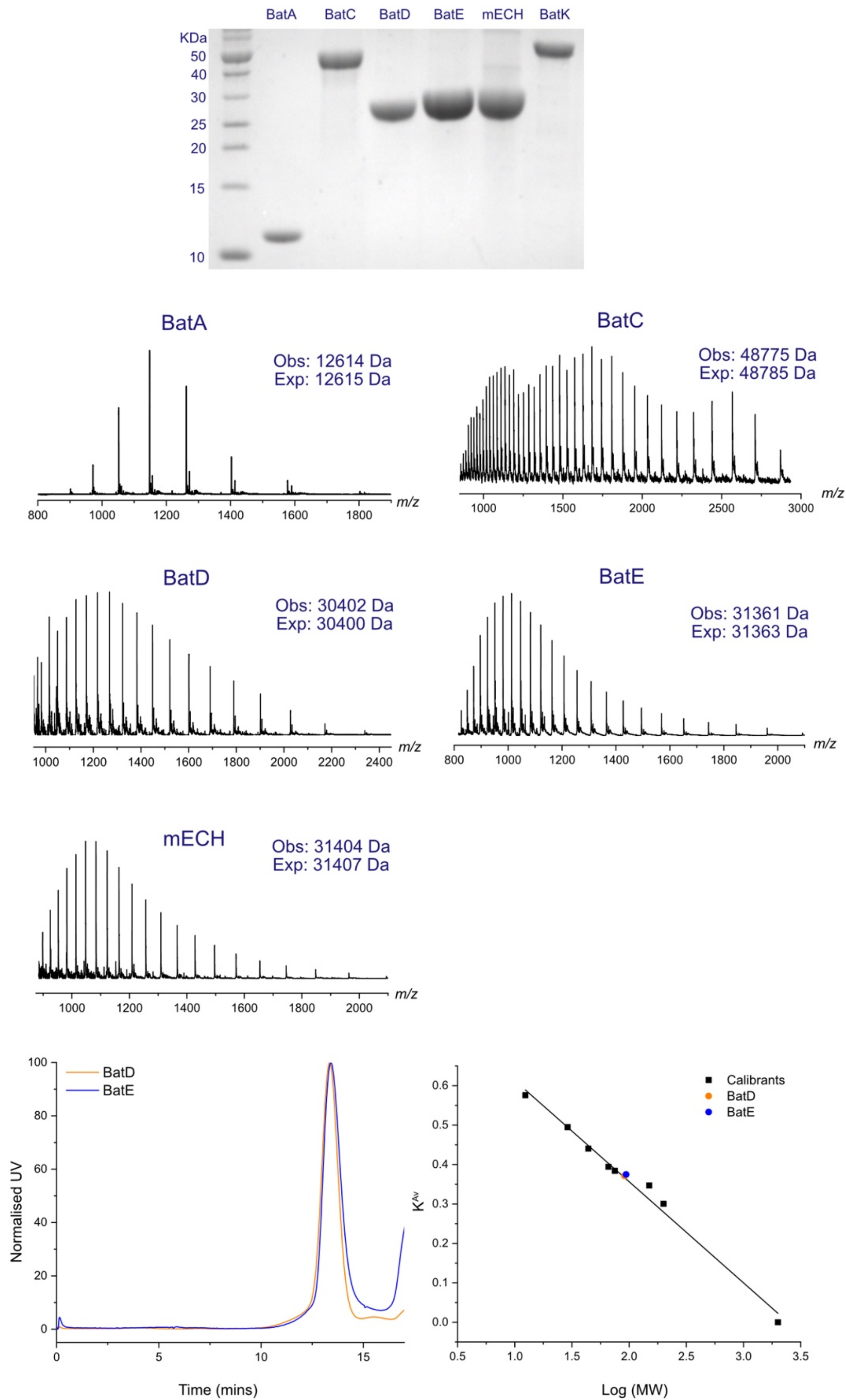


Figure S3. Characterisation of modular ACPs from Bat3 and a di-domain construct of ACP4-mECH (4M) by SDS-PAGE and ESI-MS.

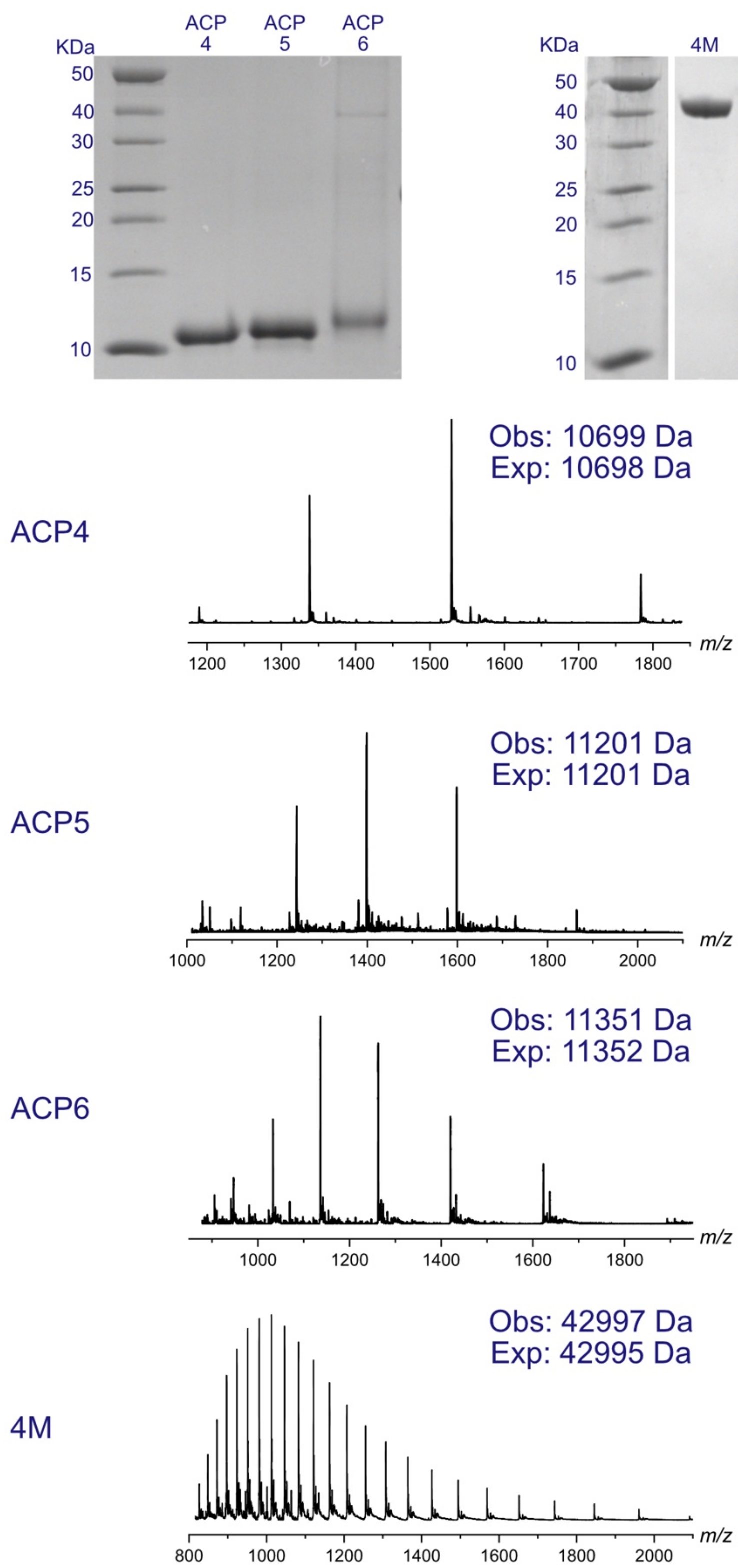


Figure S4. **^{1}H and ^{13}C spectra and MS ejection assay of [^{13}C]-Ac-BatA and [^{13}C]-Ac-ACP4. A**. ^{1}H spectrum of [^{13}C]-Ac-BatA contains upfield shifted methyl peaks around 0.0-0.5 ppm which indicate protein folding. **B**. ^{13}C spectrum of [^{13}C]-Ac-BatA gave a single signal (32.75 ppm) and MS analysis by Ppant ejection (304.2 Da) confirmed the 1 Da increase in mass relative to unlabelled Ac-BatA (303.2 Da). **C**. ^{1}H spectrum of [^{13}C]-Ac-ACP4. **D**. ^{13}C spectrum of [^{13}C]-Ac-ACP4 gives a single signal at 32.75 ppm that, along with MS analysis by Ppant ejection (304.2 Da), confirmed the loading of [^{13}C]-labelled acetate on to ACP4.

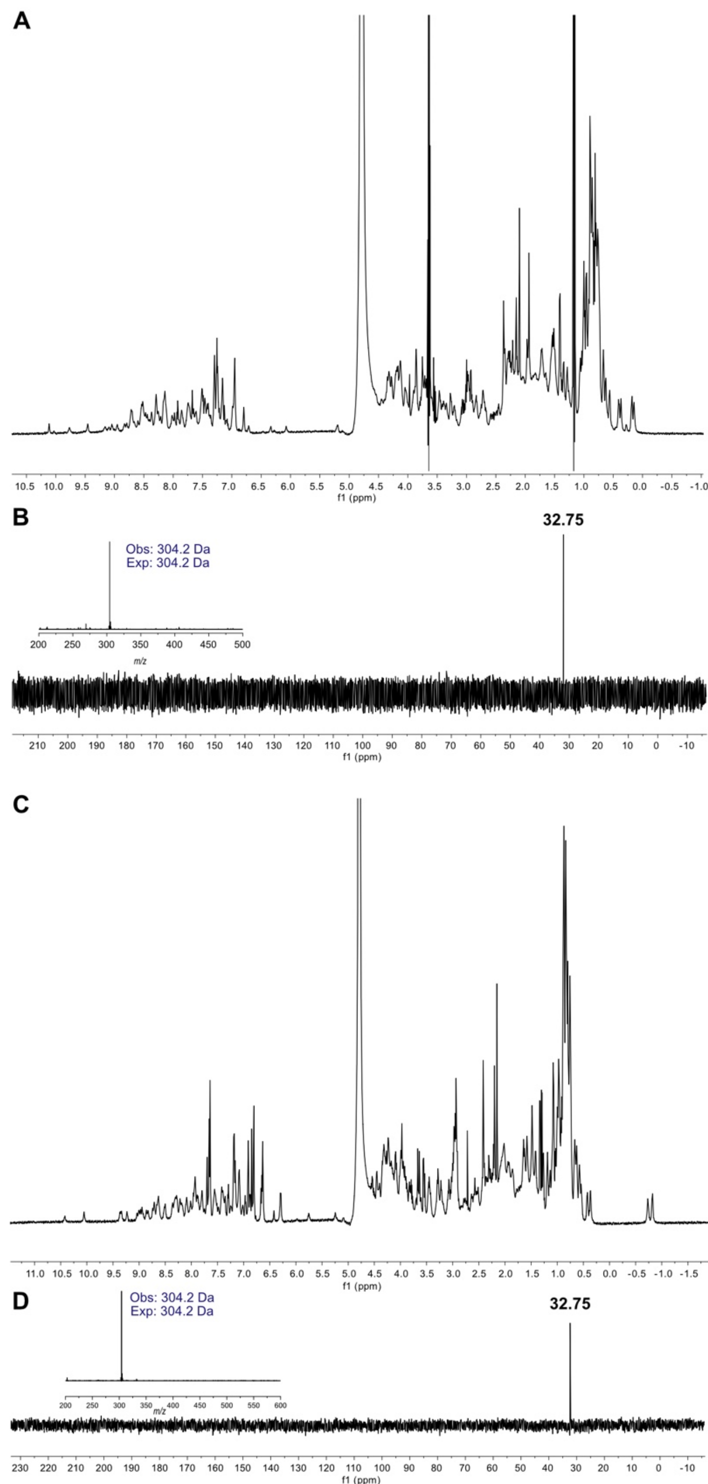
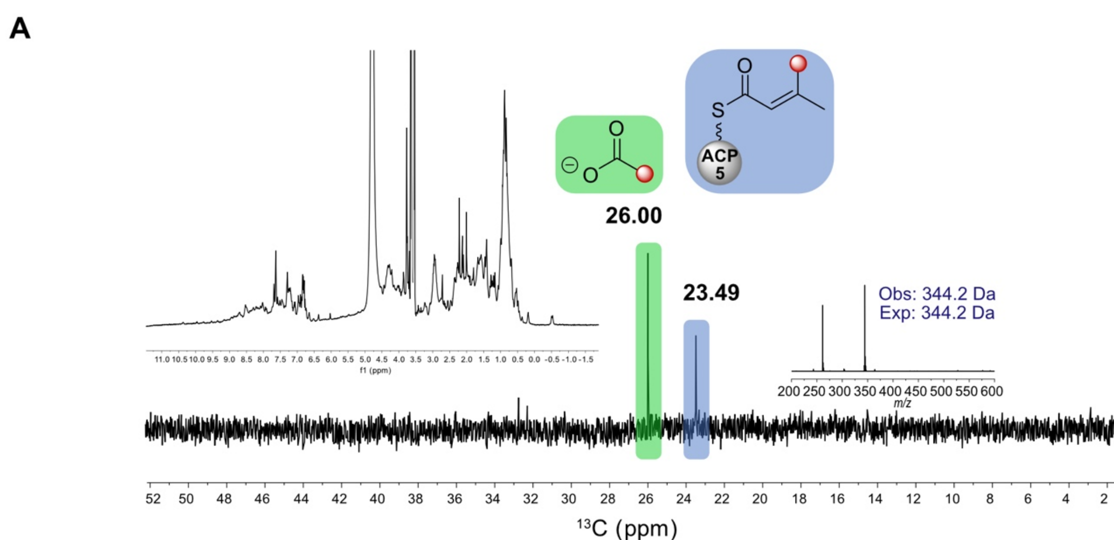


Figure S5. **NMR and MS-ejection assay of endo-β-methyl branch formation on ACP5. A.** Characterisation of an *endo*-β-methyl branch attached to ACP5 from the reaction Acac-ACP5 + [^{13}C]-Ac-BatA + BatC + BatD + BatE. ^{13}C DEPT-edited spectrum and MS analysis by Ppant ejection assay confirming the identity of the *endo*-β-methyl at 23.49 ppm and a mass of 344.2 Da by Ppant ejection. Insert: ^{1}H spectrum showing upfield shifted methyl peaks indictive of folding. **B.** ^{1}H-^{13}C HSQC spectrum of the reaction Acac-ACP5 + [^{13}C]-Ac-BatA + BatC + BatD + BatE. Correlations for the [^{13}C]-*endo*-β-methyl product (23.49 ppm/1.95 ppm) and [^{13}C]-acetate (26.00 ppm/ 1.91 ppm) were observed and these were consistent with the chemical shifts for ACP4.

A



B

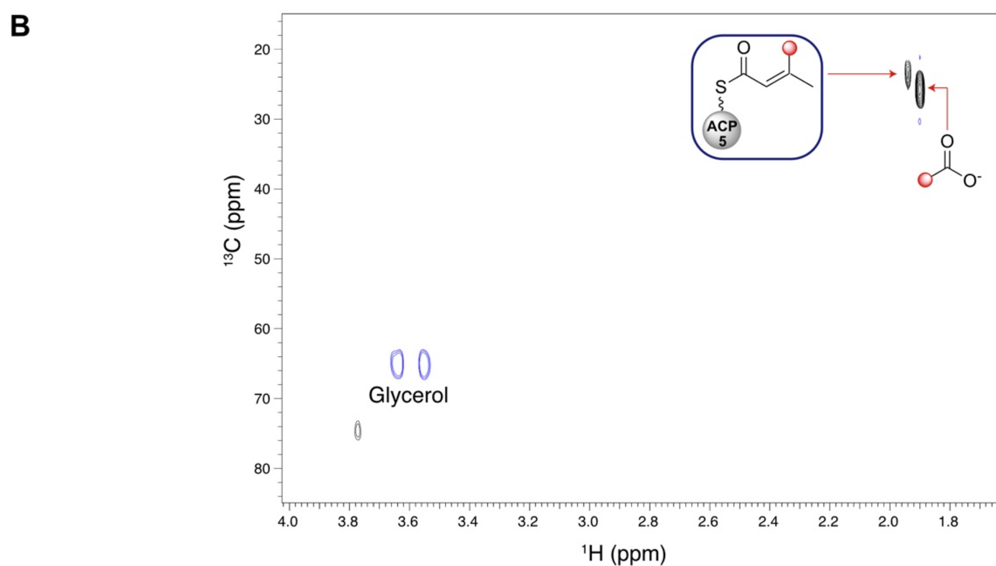


Figure S6. **2D ¹H TOCSY and NOESY analysis of BatD/E catalysed conversion of HMG-pantetheine 5. A**. Stacked plot of ¹H NMR spectra showing the slow conversion of HMG-pantetheine **5** to enoyl-pantetheine **6** catalysed by BatD and BatE. The peak at 6.18 ppm (red) corresponds to the alkene proton of MG-pantetheine **9** and the peak at 6.17 ppm the alkene proton of enoyl-pantetheine **6**. **B**. Two-dimensional TOCSY spectrum showing the correlations from the alkene protons of the MG-pantetheine **9** (6.18 ppm) and enoyl-pantetheine **6** (6.17 ppm). **C**. Two-dimensional NMR assignment of MG-pantetheine **9**. Left panel: TOCSY correlations from the alkene proton at 6.18 ppm to the CH_3 at 2.14 ppm and a CH_2 at 3.07 ppm. Right panel: NOESY spectrum showing a correlation from the alkene proton at 6.18 ppm only to the CH_2 at 3.07 ppm, confirming the *cis*-arrangement (*E*-alkene).

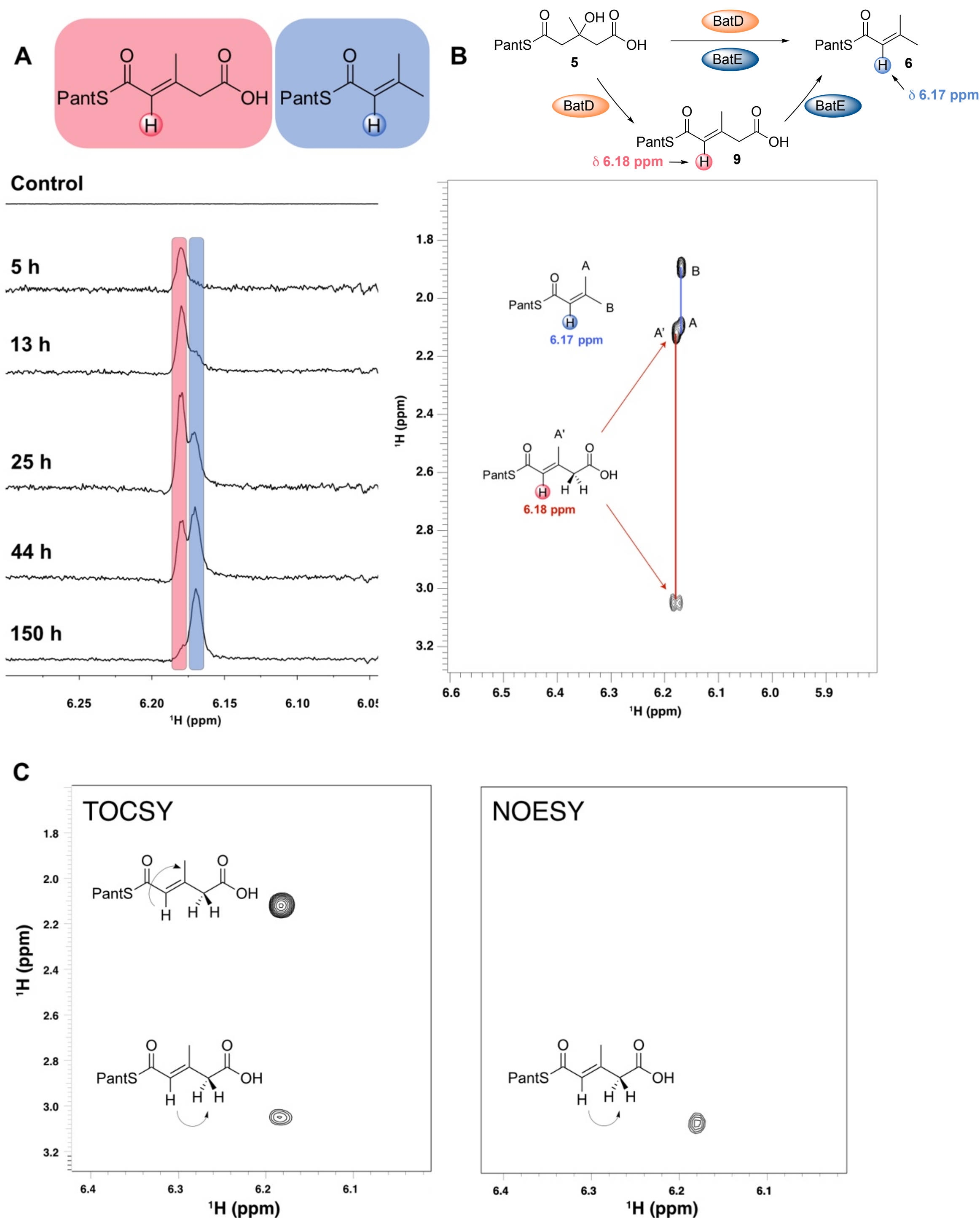


Figure S7. Structural characterisation of mECH and 4M. A. Analytical gel filtration of mECH (pink 13.42 mins) and 4M (green) run on a calibrated S200 column. **B.** Fitting of the retention time for mECH and 4M from the analytical gel filtration. The retention time suggests that 4M is dimeric in solution but the mECH domain is poorly fit to either a monomeric or dimeric form, most likely related to lack of folding/monodispersity. **C.** Circular dichroism spectrum for mECH (pink) does not indicate the expected α-helical dip at 208 nm and 222 nm or the β-strand dip at 218 nm, whereas 4M contains dips at 208 nm and 222 nm consistent with an α-helical protein although the separate contributions of ACP4 versus mECH to the curve cannot be quantified. **D.** ^{1}H-^{15}N HSQC spectrum for 4M shows a greater dispersion of peaks compared to mECH. **E.** Overlay of ^{1}H-^{15}N HSQC spectra for 4M (black) and ACP4 (red) shows overlap of the ACP peaks as well as additional peaks arising from either the linker region or mECH domain.

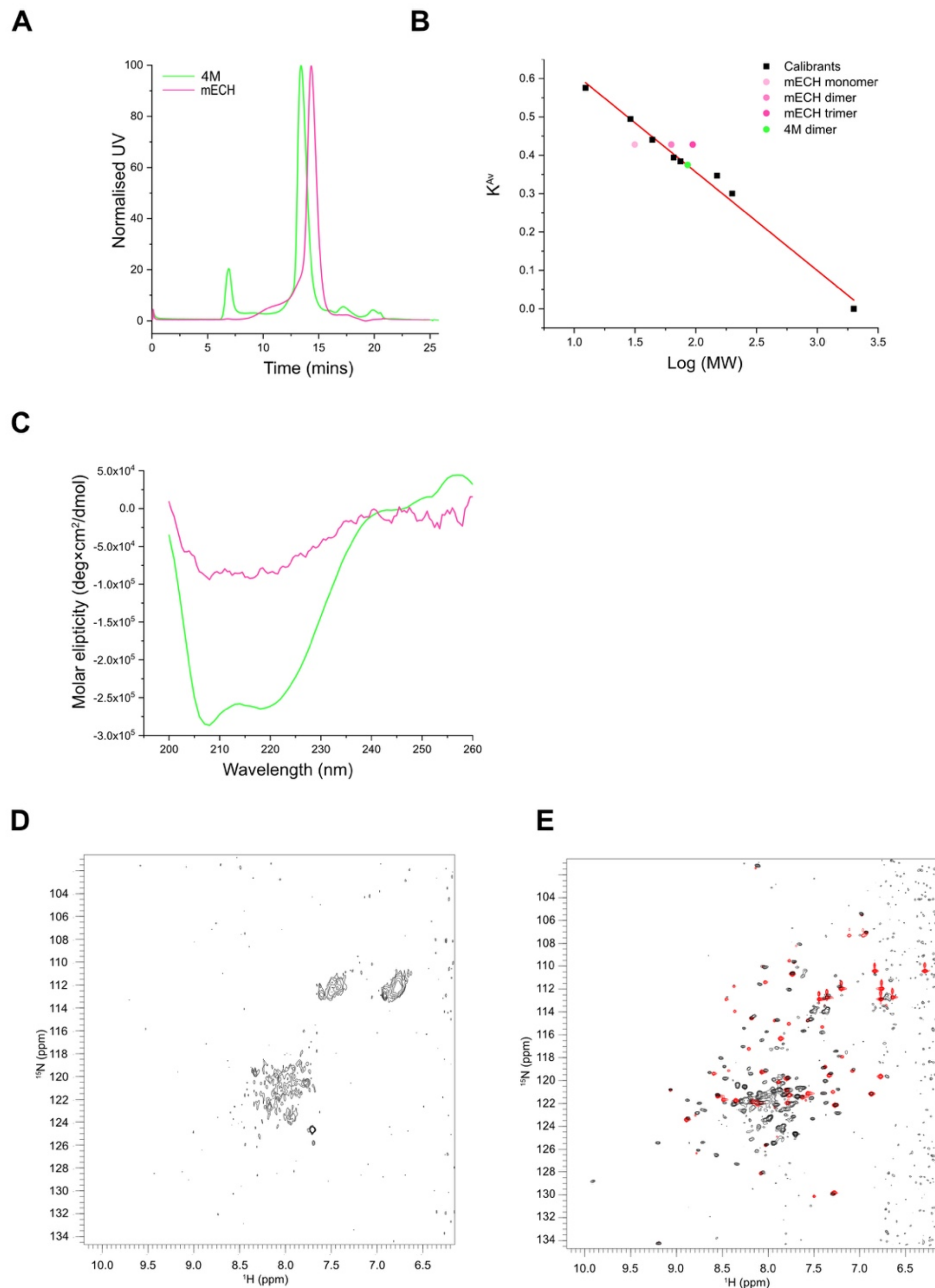


Figure S8. ^{1}H and ^{13}C spectra and MS-eject of [^{13}C]-Ac-4M. A. ^{1}H spectrum of [^{13}C]-Ac-4M. **B.** ^{13}C DEPT spectrum of [^{13}C]-Ac-4M gave a peak at 32.75 ppm along with MS analysis by Ppant ejection (304.2 Da). The peaks at 65.29ppm and 74.87 ppm correspond to glycerol.

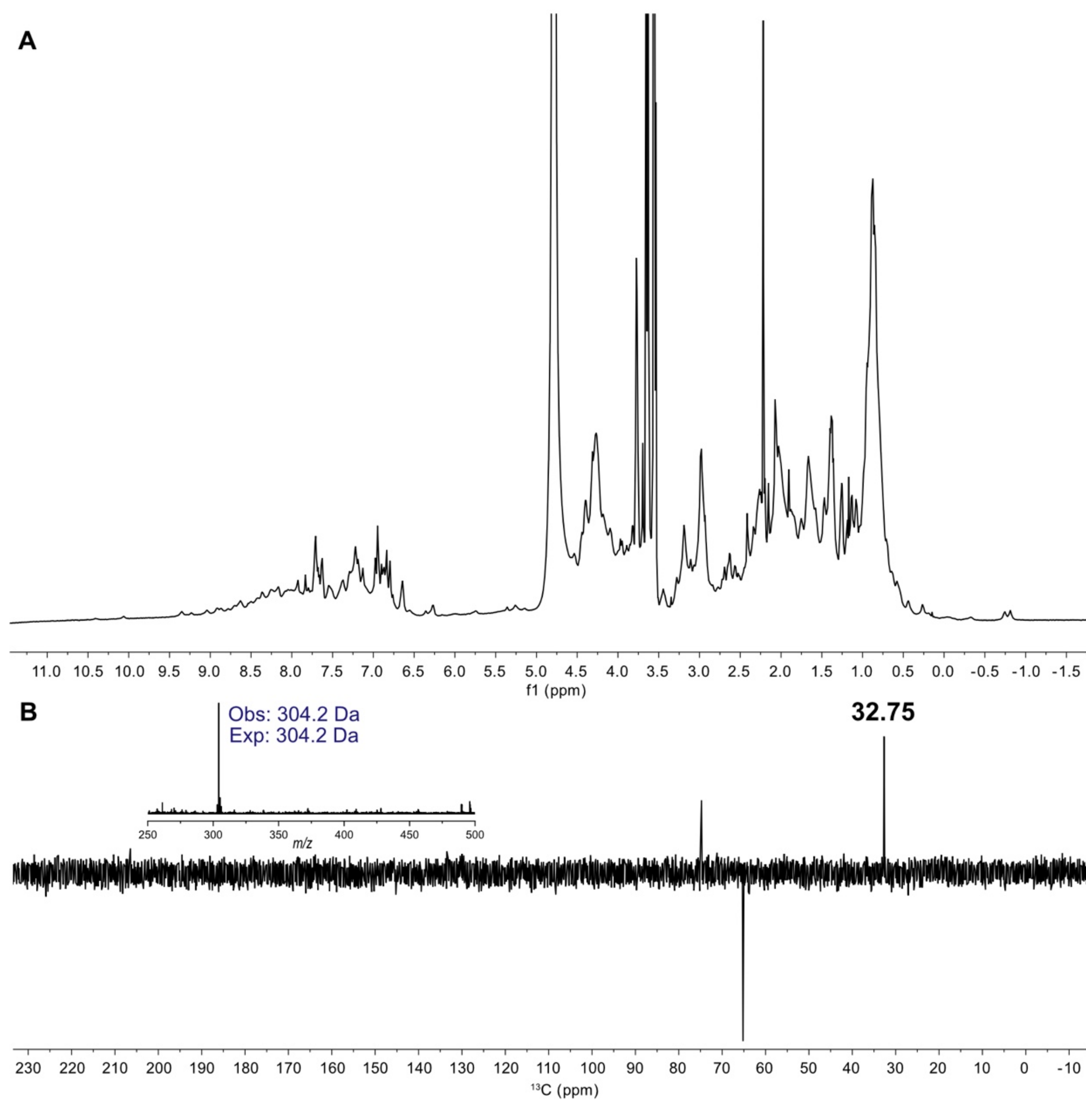
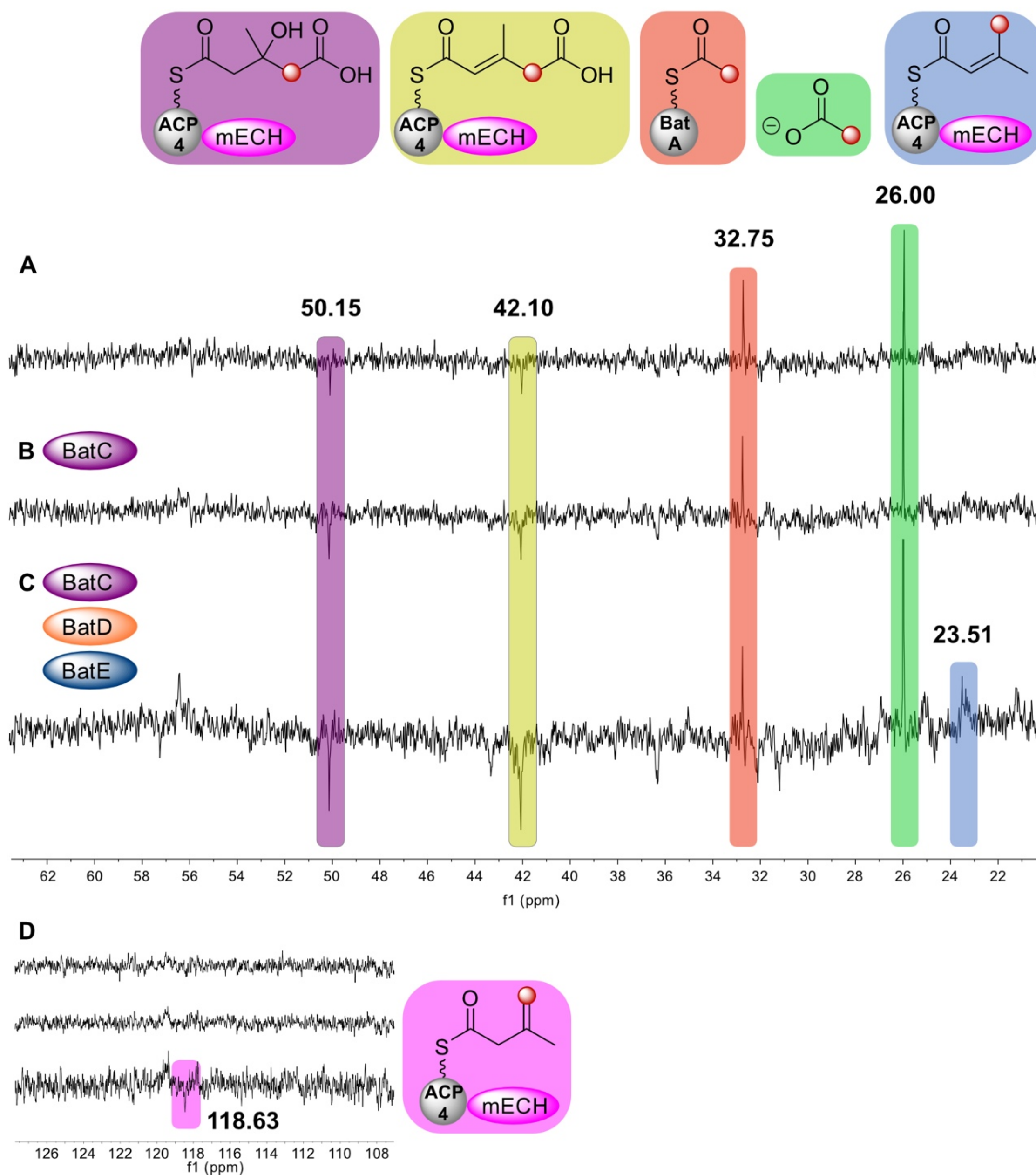
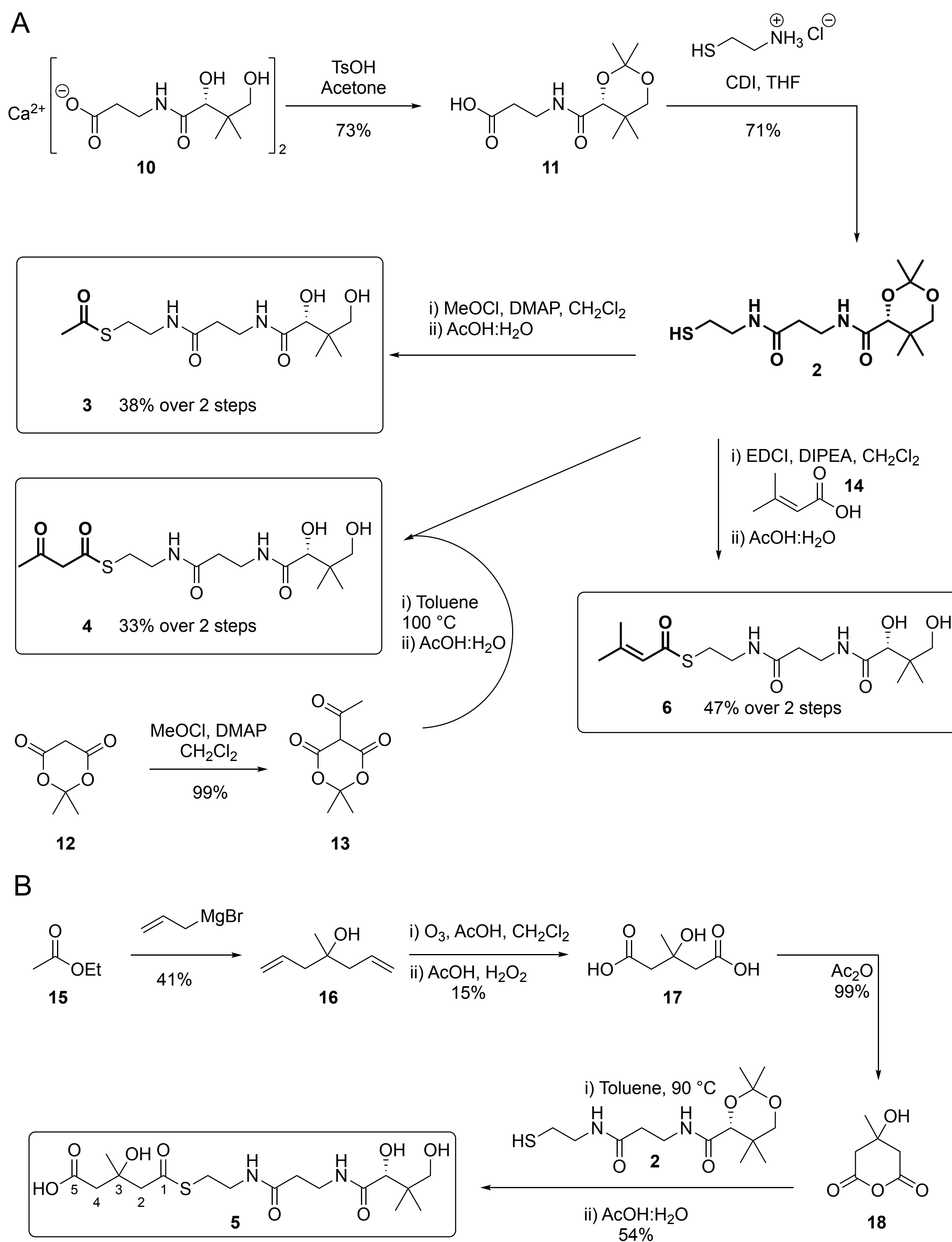


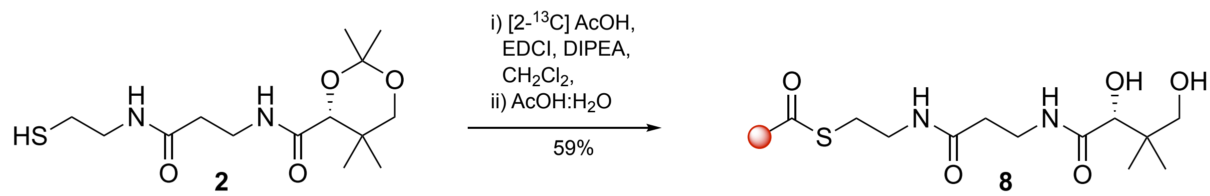
Figure S9. ^{13}C spectra and MS-eject of [^{13}C]-Ac-BatA, AcAc-4M with BatC, BatD and BatE. Stack of ^{13}C DEPT spectra of the β-branching pathway for the di-domain 4M to give an *endo*-β-methyl (23.51 ppm) and *exo*-β-methylene branch (118.63 ppm) from the reaction Acac-4M + [^{13}C]-Ac-BatA + BatC + BatD + BatE. A) 4 h, B) 8 h, C) 12 h, D) Zoom of spectra around 108-126 ppm showing the *exo*-β-methylene forming after 12 h. The persistence of the dehydrated intermediate at 42.10 ppm suggests poor efficiency of the reaction.



Scheme S1 A. Synthesis of key protected pantetheine intermediate **2** and further functionalisation to give acyl (**3, 6**) and β-ketothioester (**4**) pantetheines required for *in vitro* reconstitution of the β-branching pathway. **B**. Synthesis of HMG-pantetheine **5**.



Scheme S2. Synthesis of [^{13}C]-Ac-pantetheine **8.**



Materials and Methods.

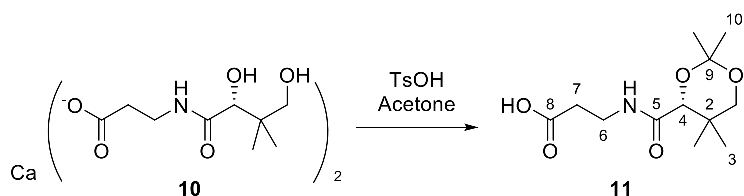
General experimental for chemical synthesis

All air and moisture sensitive reactions were carried out under an N_2 atmosphere using anhydrous solvents, obtained by passing solvent through a column of anhydrous alumina using equipment from Anhydrous Engineering and stored in a Straus flask fitted with a J. Youngs valve before use. Commercially available reagents were used without further purification. Flash column chromatography was performed with silica gel (technical grade, 40-63 μM particle size). Analytical thin layer chromatography (TLC) was performed on Merck, aluminium backed 60 F_{254} silica plates. TLC plates were visualised by UV fluorescence (UV254 lamp), basic $KMnO_4$ solution or an ethanolic solution of phosphomolybdic acid.

Nuclear magnetic resonance (NMR) spectra were recorded at ambient temperature in $CDCl_3$ (unless stated otherwise) on one of the following spectrometers; Varian 400-MR, Varian VNMRS500, Bruker Advance III HD 500 Cryo, Bruker VNMRS600 Cryo, Bruker 700 micro-cryo. ^{1}H NMR are reported in the format: chemical shift (ppm), integration, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad, dd = doublet of doublets, ddd = doublet of doublets of doublets, dt = doublet of triplets, qd = quartet of doublets), coupling constant (*J*, in Hertz), assignment. Infrared spectra were recorded on a Perkin Elmer Spectrum One Fourier Transform Infrared Spectrometer (FT-IR) with signal intensities reported as follows: br = broad, w = weak, m = medium, s= strong. Optical rotations were measured on a Bellingham and Stanley Ltd ADP220 polarimeter and melting points were determined on an Electrothermal IA6301 melting point apparatus. Small molecule high resolution mass spectrometry (HRMS) was performed on a Bruker microTOF spectrometer using electrospray ionisation (ESI).

1.1 Compound Characterisation

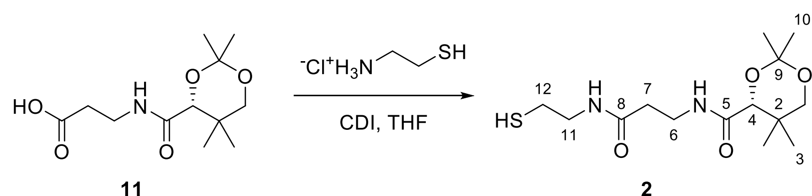
(*R*)-3-(2,2,5,5-tetramethyl-1,3-dioxane-4-carboxamido)propionic acid 11



To flame dried molecular sieves under a nitrogen atmosphere was added acetone (250 mL). After stirring for 20 min, D-pantothenic acid hemicalcium salt **10** (5.00 g, 21.0 mmol) and *para-*

toluenesulfonic acid (4.79 g, 25.2 mmol) were added and the thick white slurry was stirred at room temperature for 16 h. The reaction mixture was filtered through a bed of Celite, washed with acetone (2 x 50 mL) and the solvent removed under reduced pressure. The residue was dissolved in EtOAc (100 mL) and washed with half saturated brine (3 x 100 mL) and dried over $MgSO_4$. The crude product was triturated with *n*-hexane furnishing **11** as a white solid (3.96 g, 73 %). $[\alpha]_D^{24}$ +62.0 (*c* 1.0, $CHCl_3$). δ_H (400 MHz, $CDCl_3$) 10.50 (1H, br s, OH), 7.07 (1H, t, *J* 6.0, NH), 4.11 (1H, s, 4-H), 3.67 (1H, d, *J* 12.0, 1-*H*H), 3.63-3.53 (1H, m, 6-*H*H), 3.51-3.42 (1H, m, 6-H*H*), 3.27 (1H, d, *J* 12.0, 1-H*H*), 2.59 (2H, t, *J* 6.0, 7-H_2), 1.43 (3H, s, 10-H_3), 1.41 (3H, s, 10-H_3), 1.01 (3H, s, 3-H_3), 0.95 (3H, s, 3-H_3). δ_C (100 MHz, $CDCl_3$) 176.5 (C-8), 170.3 (C-5), 99.2 (C-9), 77.1 (C-4), 71.5 (C-1), 34.2 (C-6), 33.9 (C-7), 33.0 (C-2), 29.5 (C-10), 22.1 (C-3), 18.9 (C-3), 18.8 (C-10). ν_{max} (neat) / cm^{-1} 3388 (br, w), 2957 (w), 1723 (s), 1194 (s), 1097 (s). *m/z* (ESI^+) calc. for [$C_{12}H_{21}NO_5Na$] 282.1312 found 282.1309. The data are in accordance with the literature.[10]

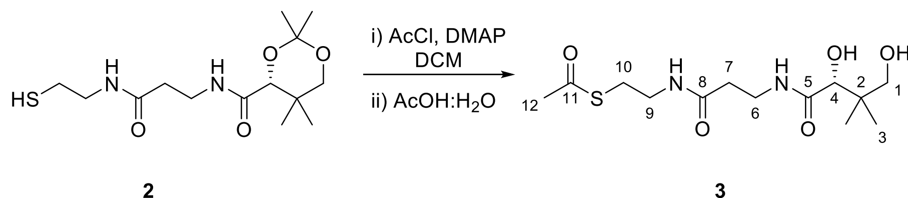
Pantetheine dimethyl ketal 2



To acid **11** (2.00 g, 7.71 mmol) in dry THF (40 mL) under a nitrogen atmosphere was added CDI (1.87 g, 11.6 mmol). The reaction mixture was stirred at room temperature for 30 min then cysteamine hydrochloride (1.31 g, 11.6 mmol) was added and stirred for 16 h. The reaction was quenched with sat. aq. NH_4Cl (30 mL) the phases separated, and the aqueous phase extracted with CH_2Cl_2 (2 x 40 mL). The combined organic phases were washed with brine (20 mL), dried over $MgSO_4$ and concentrated *in vacuo*. The colourless oil was purified by column chromatography (80-100% EtOAc:petrol) furnishing **2** as a white solid (1.73 g, 71%). m.p. 99-101 °C. $[\alpha]_D^{24}$ +48.0 (*c* 1.0, $CHCl_3$). Lit[184] $[\alpha]_D^{24}$ +33.4 (*c* 1.0, MeOH). δ_H (400 MHz, $CDCl_3$) 7.01 (1H, br s, NH), 6.42 (1H, br s, NH), 4.06 (1H, s, 4-H), 3.66 (1H, d, *J* 12.0, 1-*H*H), 3.61-3.49 (2H, m, 6-H_2), 3.48-3.33 (2H, m, 11-H_2), 3.26 (1H, d, *J* 12.0, 1-H*H*), 2.64 (2H, q, *J* 7.0, 12-H_2), 2.45 (2H, t, *J* 6.0, 7-H_2), 1.44 (3H, s, 10-H_3), 1.40 (3H, s, 10-H_3), 1.36 (1H, t, *J* 8.5, SH), 1.01 (3H, s, 3-H_3), 0.95 (3H, s, 3-H_3). δ_C (100 MHz, $CDCl_3$) 171.1 (C-8). 170.2 (C-5), 99.1 (C-9), 77.1 (C-4), 71.4 (C-1), 42.4 (C-11), 36.1 (C-7), 34.8 (C-6), 32.9 (C-2), 29.5 (C-10), 24.5 (C-12), 22.1 (C-3), 18.9 (C-3), 18.7 (C-10). ν_{max} (neat) / cm^{-1} 3300 (br, w), 2938 (w), 2550

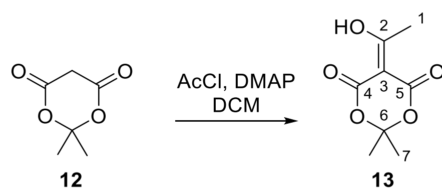
(w), 1650 (s), 1524 (s), 1196 (m), 1096 (s). *m/z* (ESI^+) calc. for [$C_{14}H_{26}N_2O_4SNa$] 341.1505 found 341.1507. The data are in accordance with the literature.[10-11]

***S*-Acetyl-pantetheine 3**



To thiol **2** (0.15 g, 0.47 mmol) in dry CH_2Cl_2 (10 mL) at 0 °C under a nitrogen atmosphere was added acetyl chloride (0.10 mL, 1.41 mmol) and DMAP (0.12 g, 0.94 mmol). The reaction mixture was allowed to warm to room temperature and stirred for 16 h, then quenched with 1 M HCl (15 mL). The aqueous phase was extracted with CH_2Cl_2 (2 x 20 mL), the combined organic phases were washed with brine (15 mL), dried over $MgSO_4$ and concentrated *in vacuo*. The crude oil was purified by column chromatography (5% MeOH:CH_2Cl_2) furnishing a colourless oil (80 mg, 47%). To the protected pantetheine (70 mg, 0.19 mmol) was added 2:1 AcOH: H_2O (8 mL), the reaction mixture stirred at room temperature for 5 h then concentrated under reduced pressure. The crude oil was purified by column chromatography (5-7% MeOH:CH_2Cl_2) furnishing **3** as a colourless oil (50 mg, 81%). $[\alpha]_D^{24}$ +22.0 (*c* 1.0, $CHCl_3$). δ_H (500 MHz, CD_3OD) 3.90 (1H, s, 4-H), 3.55-3.48 (2H, m, 6-H_2), 3.47-3.40 (2H, m, 1-H_2), 3.40-3.34 (2H, m, 9-H_2), 3.01 (2H, t, *J* 7.0, 10-H_2), 2.42 (2H, t, *J* 7.0, 7-H_2), 2.34 (3H, s, 12-H_3), 0.93 (6H, s, $(CH_3)_2$). δ_C (125 MHz, CD_3OD) 197.0 (C-11), 176.0 (C-5), 173.9 (C-8), 77.3 (C-4), 70.3 (C-1), 40.4 (C-2), 40.0 (C-9), 36.4 (C-7), 36.3 (C-6), 30.5 (C-12), 29.5 (C-10), 21.3 (C-3), 20.9 (C-3). ν_{max} (neat) / cm^{-1} 3298 (br), 2874 (br, w), 1644 (s), 1530 (s). *m/z* (ESI^+) calc. for [$C_{13}H_{24}N_2O_5SNa$] 343.1298 found 343.1293. The data are in accordance with the literature.[10]

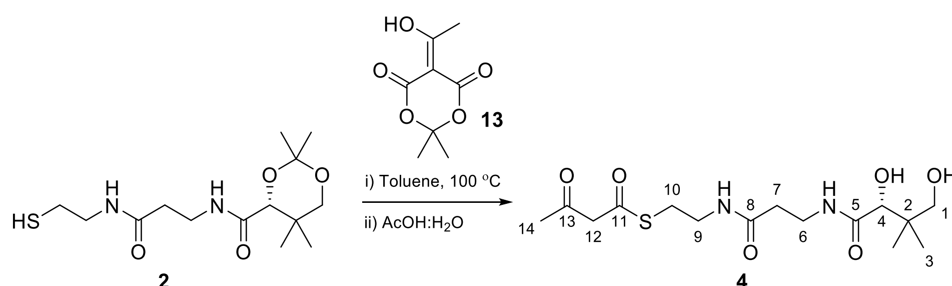
Acetyl Meldrum's Acid 13



To Meldrum's acid **12** (0.40 g, 2.78 mmol) in dry CH_2Cl_2 (8 mL) at 0 °C under a nitrogen atmosphere was added acetyl chloride (0.24 mL, 3.33 mmol) and DMAP (0.68 g, 5.56 mmol).

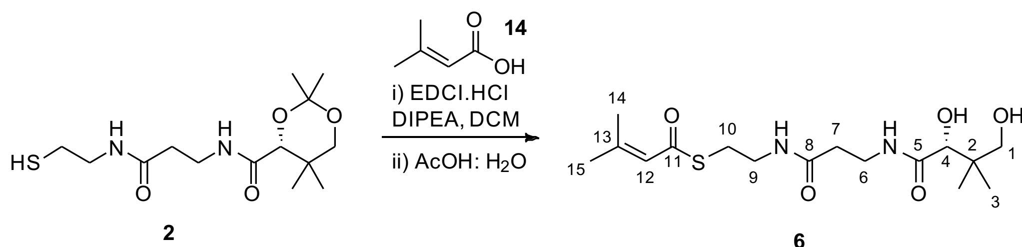
The reaction mixture was allowed to warm to room temperature and stirred for 1 h, then quenched by the addition of 1 M HCl (15 mL). The aqueous phase was extracted with CH_2Cl_2 [12]concentrated *in vacuo* furnishing **13** as an orange solid (510 mg, 99%) that did not require further purification. δ_H (400 MHz, $CDCl_3$) 15.13 (1H, s, OH), 2.66 (3H, s, 1-H_3), 1.74 (6H, s, $(CH_3)_2$). δ_C (100 MHz, $CDCl_3$) 194.6 (C-2), 170.2 (CO), 160.5 (CO), 104.9 (C-6), 91.9 (C-3), 26.8 (C-7), 23.5 (C-1). The data are in accordance with the literature.[12]

***S*-Acetoacetyl-pantetheine 4**



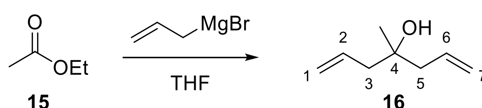
To thiol **2** (0.10 g, 0.31 mmol) and Meldrum's acid **13** (0.12 g, 0.63 mmol) under a nitrogen atmosphere was added dry toluene (4 mL). The reaction mixture was heated to reflux and stirred for 16 h. Following cooling to room temperature the solvent was removed under reduced pressure and the crude oil was purified by column chromatography (0-10% MeOH:CH_2Cl_2) furnishing a colourless oil (96 mg). To the protected pantetheine (80 mg, 0.20 mmol) was added 2:1 AcOH:H_2O (8 mL) and the reaction mixture stirred at room temperature for 5 h. The solvent was removed under reduced pressure and the crude oil was purified by column chromatography (5% MeOH:CH_2Cl_2) furnishing **4** as a colourless oil (51 mg, 54%). $[\alpha]_D^{24}$ +20.0 (*c* 1.0, MeOH). δ_H (500 MHz, CD_3OD) 3.90 (1H, s, 4-H), 3.54-3.48 (2H, m, 6-H_2), 3.48-3.41 (2H, m, 1-H_2), 3.40-3.35 (2H, m, 9-H_2), 3.07 (2H, t, *J* 6.5, 10-H_2), 2.43 (2H, t, *J* 6.5, 7-H_2), 2.24 (2.2H, s, 14-H_3 keto), 1.94 (0.6H, s, 14-H_3 enol), 0.93 (6H, s, $(CH_3)_2$). δ_C (125 MHz, CD_3OD) 202.4 (C-13), 193.7 (C-11), 176.0 (C-5), 174.0 (C-8), 77.3 (C-4), 70.4 (C-1), 40.4 (C-2), 39.8 (C-9), 36.4 (C-7), 36.3 (C-6), 30.3 (C-14 keto), 29.7 (C-10), 21.3 (C-3), 20.9 (C-3), 20.8 (C-14 enol). ν_{max} (neat) / cm^{-1} 3300 (br), 2931 (br, w), 1719 (w), 1644 (s), 125 (m). *m/z* (ESI^+) calc. for $[C_{15}H_{26}N_2O_6SNa]$ 385.1409 found 385.1400. The data are in accordance with the literature.[10]

***S*-(13-Methylbut-12-enoyl)-pantetheine 6**



To thiol **2** (0.15 g, 0.47 mmol), acid 3,3-dimethylacrylic acid **14** (57 mg, 0.57 mmol) and EDCI (0.14 g, 0.71 mmol) under a nitrogen atmosphere was added CH_2Cl_2 (5 mL) and DIPEA (0.16 mL, 0.94 mmol). The reaction mixture was stirred at room temperature for 16 h then concentrated under reduced pressure. The crude oil was purified by column chromatography (EtOAc) furnishing a colourless oil (95 mg, 51%). To the protected pantetheine (80 mg, 0.20 mmol) was added 2:1 AcOH: H_2O (8 mL), the reaction mixture stirred at room temperature for 5 h then concentrated under reduced pressure. The crude oil was purified by column chromatography (5-7% MeOH:CH_2Cl_2) furnishing **6** as a colourless oil (66 mg, 92%). $[\alpha]_D^{24}$ +24.0 (*c* 1.0, MeOH). δ_H (400 MHz, CD_3OD) 6.04 (1H, s, 12-H), 3.88 (1H, s, 4-H), 3.52-3.44 (2H, m, 6-H_2), 3.44-3.39 (2H, m, 1-H_2), 3.37-3.31 (2H, m, 9-H_2), 3.01 (2H, t, *J* 7.0, 10-H_2), 2.40 (2H, t, *J* 7.0, 7-H_2), 2.14 (3H, s, 14-H_3), 1.89 (3H, s, 15-H_3), 0.91 (6H, s, $(CH_3)_2$). δ_C (100 MHz, CD_3OD) 190.0 (C-11), 176.0 (C-5), 173.9 (C-8), 155.8 (C-13), 123.9 (C-12), 77.3 (C-4), 70.4 (C-1), 40.4 (C-2), 40.3 (C-9), 36.4 (C-7), 36.3 (C-6), 29.0 (C-10), 27.2 (C-15), 21.3 (C-3), 21.2 (C-14), 20.9 (C-3). ν_{max} (neat) / cm^{-1} 3296 (br, w), 2930 (w), 1629 (s), 1527 (s). *m/z* (ESI^+) calc. for $[C_{16}H_{28}N_2O_5SNa]$ 383.1617 found 383.1623.

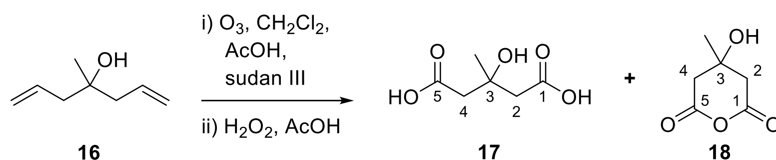
4-Methylhepta-1,6-dien-4-ol 16



To freshly distilled ethyl acetate **15** (1.80 g, 20.5 mmol) in dry THF (10 mL) at 0 °C under a nitrogen atmosphere was added allylmagnesium bromide (45 mL, 45 mmol, 1.0 M in Et_2O). The reaction mixture was allowed to warm to room temperature, stirred for 16 h then quenched with 1 M HCl (30 mL). The aqueous phase was extracted with Et_2O (3 x 30 mL), the combined organic phases were then dried over $MgSO_4$ and concentrated *in vacuo*. The crude yellow oil was purified by column chromatography (10% EtOAc:petrol) furnishing **16** as a colourless oil (1.05 g, 41%). δ_H (400 MHz, $CDCl_3$) 5.87 (2H, ddt, *J* 17.5, 10.5, 7.5, 2-H and 6-H), 5.17-5.07 (4H, m, 1-H_2 and 7-H_2), 2.22 (4H, m, 3-H_2 and 5-H_2), 1.17 (3H, s, CH_3). δ_C (100 MHz, $CDCl_3$)

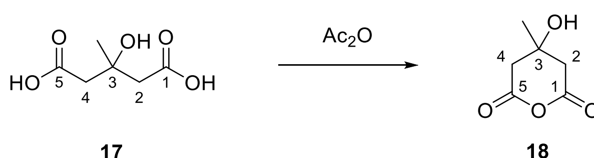
134.0 (C-2 and C-6), 118.9 (C-1 and C-7), 71.8 (C-4), 46.3 (C-3 and C-5), 26.8 (CH_3). *m/z* (ESI^+) calc. for [$C_8H_{14}O$] 149.1 found 149.1. The data are in accordance with the literature.[13]

3-Hydroxy-3-methylpentanedioic acid 17 and HMG-anhydride 18



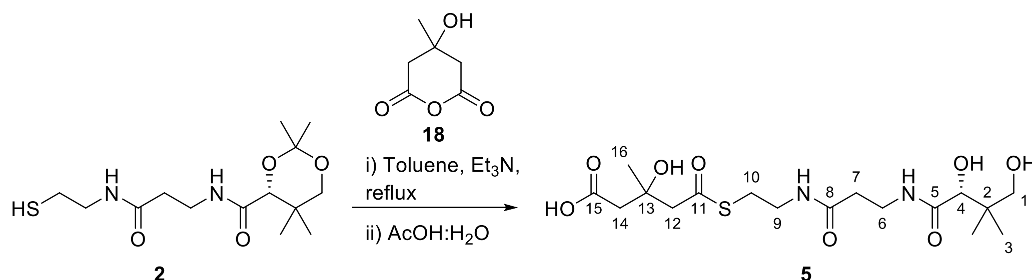
Alcohol **16** (0.50 g, 3.96 mmol) was dissolved in CH_2Cl_2 (10 mL) and AcOH (1 mL) at -78 °C and the tip of a spatula of sudan-III dye was added. Ozone was passed through the solution until the red colour dissipated then the reaction mixture was allowed to warm to room temperature. The solvent was removed under a stream of nitrogen. To the crude oil was added AcOH (20 mL) and 30% solution of H_2O_2 (13 mL), the reaction mixture was heated to reflux and stirred for 16 h. After cooling to 0 °C, the reaction was quenched with sat. aq. Na_2SO_3 (20 mL), extracted with EtOAc (2 x 20 mL), the combined organic extracts washed with brine (15 mL), dried over $MgSO_4$ and concentrated *in vacuo*. Diacid **17** was isolated as a white solid that didn't require any further purification (97 mg, 15%). δ_H (400 MHz, $(CD_3)_2CO$) 2.69 (4H, s, 2-H_2 and 4-H_2), 1.38 (3H, s, CH_3). δ_C (100 MHz, $(CD_3)_2CO$) 173.2 (CO), 70.1 (C-3), 45.2 (C-2 and C-4), 27.6 (CH_3). *m/z* (ESI^+) calc. for [$C_6H_{10}O_5Na$] 185.0420 found 185.0414.

The combined aqueous extracts were concentrated *in vacuo*, and to the resulting white solid at 0 °C was added Ac_2O (20 mL). The reaction mixture was allowed to warm to room temperature and stirred for 16 h. The subsequent red reaction mixture was concentrated under reduced pressure, the residue dissolved in CH_2Cl_2 and washed with brine (10 mL), dried over $MgSO_4$ and concentrated *in vacuo* furnishing **18** (102 mg, 15%) as a red solid. δ_H (400 MHz, $(CD_3)_2CO$) 4.65 (1H, br s, OH), 3.01 (2H, d, *J* 16.0, 2-*H*H and 4-*H*H), 2.86 (2H, d, *J* 16.0, 2-H*H* and 4-H*H*), 1.43 (3H, s, CH_3). δ_C (100 MHz, $(CD_3)_2CO$) 167.4 (C-1 and C-5), 67.8 (C-3), 44.3 (C-2 and C-4), 27.9 (CH_3). *m/z* (ESI^+) calc. for [$C_6H_8O_4Na$] 167.0315 found 167.0310. The data are in accordance with the literature.[13]



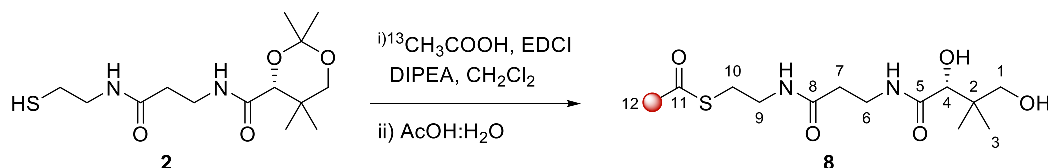
Diacid **17** (55 mg, 0.34 mmol) and Ac_2O (2 mL) were stirred at room temperature for 16 h. The reaction mixture was concentrated under reduced pressure furnishing **18** as a grey solid (48 mg, 99%). Spectroscopic data were in accordance with the data above.

S-(3-Hydroxy-3-methylglutaryl)-pantetheine 5



To thiol **2** (30 mg, 92.5 μmol) and anhydride **18** (20 mg, 139 μmol) under a nitrogen atmosphere was added dry toluene (6 mL) and Et_3N (5 drops). The reaction mixture was stirred at room temperature for 1 h then the solvent removed under reduced pressure. AcOH (4 mL) and H_2O (2 mL) were added and the reaction mixture was stirred for 5 h, then the solvent removed under reduced pressure. The crude red oil was purified by column chromatography (10% MeOH:CH_2Cl_2 + 0.1% AcOH) furnishing **5** as a colourless oil (21 mg, 54%). δ_H (400 MHz, CD_3OD) 3.88 (1H, s, 4-H), 3.53-3.46 (2H, m, 6-H_2), 3.46-3.40 (2H, m, 1-H_2), 3.38-3.33 (2H, m, 9-H_2), 3.01 (2H, t, *J* 6.5, 10-H_2), 2.95 (2H, d, *J* 3.0, 14-H_2), 2.61 (2H, s, 12-H_2), 2.40 (2H, t, *J* 6.5, 7-H_2), 1.35 (3H, s, 16-H_3), 0.91 (6H, s, $(CH_3)_2$). δ_C (125 MHz, CD_3OD) 198.3 (C-11), 176.1 (C-5), 175.2 (C-15), 173.9 (C-8), 77.3 (C-4), 71.1 (C-13), 70.4 (C-1), 55.0 (C-14), 46.1 (C-12), 40.4 (C-2), 39.9 (C-9), 36.4 (C-7), 36.3 (C-6), 29.5 (C-10), 27.8 (C-16), 21.3 (C-3). 20.9 (C-3). *m/z* (ESI^-) calc. for $[C_{17}H_{29}N_2O_8S]$ 421.1650 found 421.1653.

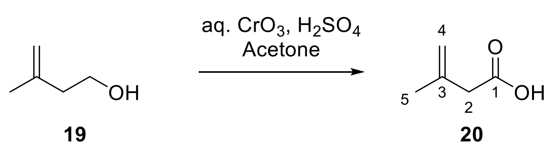
S-[^{13}C]-Acetyl pantetheine 8



To thiol **2** (0.10 g, 0.34 mmol) in CH_2Cl_2 under a nitrogen atmosphere was added [2-^{13}C]-acetic acid (25 μL, 0.41 mmol), EDCI (90 mg, 0.50 mmol) and DIPEA (120 μL, 0.68 mmol.). The reaction mixture was stirred for 16 h, then the solvent removed under reduced pressure. The crude colourless oil was purified by column chromatography (50-80% EtOAc:hexane) furnishing a colourless oil (75 mg, 60%). To the oil (50 mg, 0.14 mmol) was added acetic acid

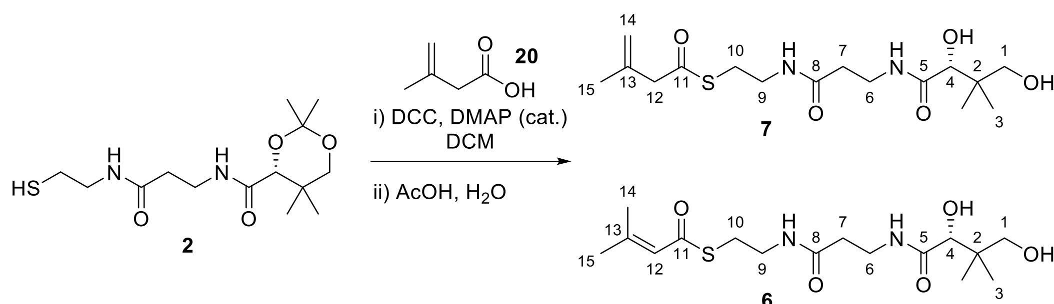
(6 mL) and water (3 mL), the solution was stirred at room temperature for 3 h then concentrated *in vacuo*. The crude oil was purified by column chromatography (80% EtOAc:hexane) furnishing **8** as a colourless oil (44 mg, 98%). δ_H (500 MHz, CD_3OD) 3.90 (1H, s, 4-H), 3.55-3.48 (2H, m, 6-H_2), 3.47-3.40 (2H, m, 1-H_2), 3.40-3.34 (2H, m, 9-H_2), 3.01 (2H, t, *J* 6.5, 10-H_2), 2.42 (2H, t, *J* 6.5, 7-H_2), 2.34 (3H, d, *J* 130 Hz, 12-H_3), 0.93 (6H, s, $(CH_3)_2$). δ_C (125 MHz, CD_3OD) 197.0 (d, *J* 48.0 Hz, C-11), 176.0 (C-5), 173.9 (C-8), 77.3 (C-4), 70.3 (C-1), 40.4 (C-2), 40.0 (C-9), 36.4 (C-7), 36.3 (C-6), 30.5 (enhanced C-12), 29.5 (C-10), 21.3 (C-3), 20.9 (C-3). *m/z* (ESI^+) calc. for $[^{13}C_1C_{12}H_{24}N_2O_5SNa]$ 344.1332 found 344.1345.

3-Methylbut-3-enoic acid 20



To 3-methylbut-3-en-1-ol **19** (1.00 mL, 9.90 mmol) in acetone (50 mL) at 0 °C was added Jones reagent (5.20 mL, 2.67 M, 13.9 mmol) dropwise and the mixture stirred for 1 h. To the solution was added Et_2O (30 mL) and 2 M NaOH (40 mL). The aqueous layer was then acidified to pH 2 with 1 M HCl and extracted with Et_2O (3 x 40 mL). The combined organic layers were washed with brine (2 x 20 mL), dried over $MgSO_4$ and the solvent removed *in vacuo* furnishing **20** as a pale-yellow oil (560 mg, 56%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.33 (1H, br s, COO*H*), 4.95 (1H, s, 4-*H*H), 4.88 (1H, s, 4-H*H*), 3.08 (2H, s, 2-H_2), 1.83 (3H, s, 5-H_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.6 (C-1), 138.1 (C-3), 115.5 (C-4), 43.2 (C-2), 22.5 (C-5). The data are in accordance with the literature.[14]

***S*-(13-Methylbut-13-enoyl) pantetheine 7 / *S*-(13-Methylbut-12-enoyl) pantetheine 6**



To thiol **2** (50 mg, 0.16 mmol) in DCM (3 mL) at room temperature under a nitrogen atmosphere was added 3-methyl-3-butenoic acid **20** (16 μL, 17.7 mg, 0.16 mmol), N,N'-dicyclohexylcarbodiimide (29.2 μL, 39 mg, 0.19 mmol) and DMAP (4.8 mg, 39 μmol). The

reaction mixture stirred for 2 h then the solution was filtered and washed with 1 M HCl (5 mL), sat. aq. $NaHCO_3$ (5 mL) and brine (10 mL). The combined organic phases were dried over $MgSO_4$ and concentrated *in vacuo*. The crude oil was purified by column chromatography (60% EtOAc:petrol) furnishing a colourless oil (40 mg, 57%) as a 1:1 mixture of *endo* and *exo* isomers. The mixture of isomers (5 mg, 12 μmol) was stirred in H_2O (2 mL) and AcOH (2 mL) at room temperature for 3 h. The solvent was removed *in vacuo* and the crude oil purified by column chromatography (70-100% EtOAc:petrol) furnishing **7** and **6** as a colourless oil (4 mg, 92%) as a 60:40 mixture of exo and endo isomers. ^{1}H NMR (600 MHz, 50 mM sodium phosphate, 100 mM NaCl, pH 8.0, 10% D_2O) δ 8.18 (1H, br s, NH), 8.05 (1H, br s, NH), 6.17 (1H, s, 12-H (endo)), 5.01 (1H, s, 14-*H*H (exo)), 3.98 (1H, s, 4-H (exo)), 3.97 (1H, s, 4-H (endo)), 3.52 – 3.44 (3H, obscured multiplet, 1-*H*H, 6-H_2), 3.41 – 3.36 (5H, obscured multiplet, 1-H*H*, 9-H_2 12-H_2 (exo)), 3.05 (2H, t, *J* 6.5, 10-H_2), 2.46 (overlapping t, *J* 6.5, 7-H_2 (endo and exo)), 2.11 (3H, s, 14-H_3 (endo)), 1.90 (3H, s, 15-H_3 (endo)), 1.75 (3H, s, 15-H_3 (exo)), 0.90 (3H, s, 3-H_3), 0.87 (3H, s, 3-H_3). 14-H*H* obscured by solvent suppression. ^{13}C NMR (150 MHz, D_2O) δ 204.7 (C-11 (exo)), 195.4 (C-11 (endo)), 177.8 (C-5), 176.7 (C-8), 160.2 (C-13 (endo)), 141.9 (C-13 (exo)), 124.9 (C-12 (endo)), 118.4 (C-14 (exo)), 78.5 (C-4), 71.2 (C-1), 54.7 (C-12 (exo)), 41.6 (C-9 (endo)), 41.3 (C-9 (exo)), 41.3 (C-2), 38.3 (C-6), 38.2 (C-7 (exo)), 38.0 (C-7 (endo)), 30.9 (C-10 (exo)), 30.6 (C-10 (endo)), 29.2 (C-15 (endo)), 24.2 (C-15 (exo)), 23.5 (C-14 (endo)), 23.2 (C-3), 21.8 (C-3); ν_{max} (thin film)/cm^{-1} 3290 (br), 2940 (m), 2800 (m), 1648 (s), 1536 (s), 1047 (s); *m/z* (ESI+) HRMS: Calculated for $C_{16}H_{29}N_2O_5S$ [M+H$^+$] 361.1792, found 361.1790.

1.2 Materials and Methods

Reagents were purchased from Sigma Aldrich, Thermo Fisher or Merck Millipore. *E. coli* competent cells were purchased from New England Biolabs (T7 Express and 5-α) or Merck Millipore (Novagen BL21 (DE3)). All enzymes used were purchased from Thermo Fisher Scientific.

Plasmid Preparation

The genes encoding ACP4, ACP5, ACP6, 4M and BatK were amplified from *P. fluorescens* BCCM_ID9359 using KAPA HiFi HotStart ReadyMixPCR kit (KAPABIOSYSTEMS). The PCR product was purified using a PureLink PCR Purification kit (Thermo Fisher) and cloned into a pre-linearised (KpnI and HindIII) POPINF vector using Infusion (Takarabio). The nucleotide sequence for BatA, BatD, BatE and mECH were codon optimised for *E. coli*, then synthesised and subcloned into a pET151/D-TOPO vector by Thermo Fisher. BatC was kindly donated by Dr. Paul Race. MupN was expressed and purified as described previously.[15] CoaA/CoaD/CoaE were prepared as described previously.[10]

Primer	Sequence
ACP4_For	aagttctgtttcagggcccg**AGCCAGGTTAAAGAAGGTC**
ACP4_Rev	atggtctagaaagcttta**TGCCAGCTCGCTTTCTTTAA**
ACP5_For	aagttctgtttcagggcccg**TCTGTTGCTGACTTGCGAATGGAGTTGG**
ACP5_Rev	atggtctagaaagcttta**TCAACAAATGGCTCGCCAACACTTTGATAG**
ACP6_For	aagttctgtttcagggcccg**GATCTCTCAAAGCTGGAGGCAGAGCTCTGT**
ACP6_Rev	atggtctagaaagcttta**CCGCCAGGAACAGAGTGAAGCGCTTGAG**
4M_For	aagttctgtttcagggcccg**ATGAGTCAGGTCAAAGAGGGGTTGCGAAGA**
4M_Rev	atggtctagaaagcttta**TTAATGGTCATGCAGCGCGTCCAGCC**
BatK_For	aagttctgtttcagggcccg**ATGATGCCTATGGCTACCATTACGGC**
BatK_Rev	atggtctagaaagcttta**TTACGCCAGATAACCCGACATGGACACAAA**

General Expression Procedure

E. coli BL21 (DE3) transformed with plasmid DNA were grown on LB agar supplemented with carbenicillin at 37 °C overnight. A single colony was picked and added to 50 mL LB/carb and grown at 37 °C for 16 h. 2 mL of this seed broth was used to inoculate flasks containing 200 mL LB/carb medium and incubated at 37 °C until an OD_{600} of 1.0 was reached. The culture was then induced with isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated at 16 °C.

The cells were harvested by centrifugation at 6,000 rpm and re-suspended in column buffer A (50 mM Tris, 500 mM NaCl, 10% glycerol, pH 8.0). The cells were lysed by sonication, clarified by centrifugation and the soluble fraction applied to a HisTrap HP 5 mL Ni column (GE Life Sciences). Protein was eluted *via* a linear gradient from 5 to 100% column buffer B (50 mM Tris, 100 mM NaCl, 10% glycerol, 0.8 M imidazole, pH 8.0) and collected in 1 mL fractions. Fractions were analysed by SDS-PAGE and those containing the protein of interest were pooled, buffer exchanged in to reaction buffer (50 mM Tris, 100 mM NaCl, pH 8.0) using a HiPrep 26/10 desalting column (GE Life Sciences) or further purified by Superdex S75 size exclusion chromatography (SEC) using reaction buffer. Protein was concentrated using a Vivapsin concentrator (GE Life Sciences) and stored at 4 °C, or supplemented with 10% glycerol, aliquoted, flash frozen and stored at -80 °C.

Expression conditions for each protein are summarised below.

Protein	Plasmid	IPTG (µM)	Temperature/Time
BatA	BatA_pET151	250	16 °C/16 h
BatC	BatC_POPINF	250	16 °C/16 h
BatD	BatD_pET151	250	16 °C/16 h
BatE	BatE_pET151	250	16 °C/16 h
mECH	mECH_pET151	250	16 °C/16 h
BatK	BatK_POPINF	250	16 °C/16 h
ACP4	ACP4_POPINF	1000	16 °C/5 h
ACP5	ACP5_POPINF	250	16 °C/10 h
ACP6	ACP6_POPINF	250	16 °C/10 h
4M	4M_POPINF	1000	16 °C/5 h

M9 expression *via* media transfer

A single colony was picked and cultured in 50 mL LB medium supplemented with carbenicillin (100 µg/mL) for 16 h at 37 °C. 2 mL of pre-culture was added to 200 mL LB/carb and incubated at 37 °C until OD_{600} 1.0 was reached. The cells were harvested by centrifugation (6000 rpm for 10 mins) and washed twice with sterile M9 medium. They were then resuspended in M9 and used to inoculate 100 mL M9 supplemented with carbenicillin (100 µg/mL), $^{15}NH_4Cl$ (1 g/L), glucose (2 g/L) and metal mix (100 µL) and incubated at 16 °C for 1 h. The culture was induced with IPTG (250 µM) and incubated at 16 °C. The protein was purified as per the unlabelled material.

One-pot pantetheine upgrade and loading of ACPs

Adapting literature procedures,[10, 16] to apo-ACP in reaction buffer was added each reagent in series and the reaction mixture was incubated at room temperature until complete loading was observed. Reaction progress was monitored by ESI-MS and typical reaction times were 1 h (100 µL reactions) or 1-3 h (1 mL or larger reactions). Once full loading was achieved, excess reagents were removed using a HiPrep 26/10 desalting column (GE Life Sciences) or Zeba column (Thermo Fisher) equilibrated with reaction buffer. Loaded ACPs were concentrated using Sartorius Vivaspin20 with a 3 or 10 kDa molecular weight cut-off.

Reagent	Stock	Small-scale Volume (µL)	Large-scale Volume (µL)	Final Concentration
ACP		84	840	50-100 µM
$MgCl_2$	1 M	1	10	10 mM
Pantetheine	100 mM	1	10	1 mM
CoA mix (A, D, E)	33 µM each	3	30	1 µM each
MupN	15 µM	10	100	1.5 µM
ATP	100 mM	1	10	1 mM

ESI-MS sample preparation

Samples were desalted for ESI-MS analysis using a C_4 ZipTip™ (Millipore). The Ziptip was activated with two 10 µL aspirations of solution A (50% acetonitrile in water) and equilibrated with five 10 µL aspirations of solution B (0.1% TFA in water). A 10 µL sample was then loaded onto the ZipTip with 15-20 aspirations, returning the sample to the same tube between aspirations. The protein was desalted with ten 10 µL washes with solution C (5% MeOH, 0.1% TFA in water) and discarded to waste. Finally, the sample was eluted with 10 µL of solution D (70% acetonitrile, 0.1% TFA in water).

Denatured samples were analysed on a Synapt G2-Si (Waters) fitted with a TriVersa NanoMate® (Advion) using the following parameters: sample cone 10 V, capillary voltage 1.5 kV, transfer collision energy 5 V. The source was set to positive mode and spectra were acquired over 600-3000 m/z and analysed using MassLynx™ 4.1 software. The spectra were smoothed and background subtraction applied. Deconvolution of the charge states was undertaken using MagTran 1.03. For Ppant ejection assays, an appropriate charge state was

isolated using the MSMS functionality. The trap collision energy was increased until fragmentation was observed (typically between 5 V and 30 V) and spectra were collected from 200-2000 *m/z*.

β-branching assay by MS

BatA was loaded with Ac-pantetheine **3** or [^{13}C]-Ac-pantetheine **8** and the modular ACP (ACP4, 5 or 6) was loaded with Acac-pantetheine **4** (100 µL total volume per reaction). Once full loading was achieved, the sample was desalted into reaction buffer using a Zeba column (Thermo Fisher). Ac-BatA (50 µM) and Acac-ACP (50 µM) were mixed and BatC (5 µM) added to the 100 µL reaction, which was incubated at room temperature for 1 h. A 10 µL sample was taken for MS analysis and 5 µM of the required catalytic domains (BatD/BatE/mECH) was added. The reaction was incubated at room temperature for 1 h and analysed by MS (10 µL aliquot).

BatK reduction

The modular ACP (~100 µM) was loaded with enoyl-pantetheine **6** and monitored by MS until complete loading was achieved (~1 h). BatK (5 µM) and NADH (1 mM) were added and the reaction mixture incubated at room temperature for 1 h. MS analysis by Ppant ejection was used to determine the outcome of the reaction.

β-branching assay by NMR

Apo-BatA was loaded with [^{13}C]-Ac-pantethine **8**, monitored by MS until complete loading was achieved (typically 1 to 3 h). The loaded ACP was desalted into NMR buffer using a HiPrep 26/10 desalting column and concentrated using Vivaspin (GE Life Sciences) or Amicon (Merck) spin concentrators. [^{13}C]-Ac-BatA was stored in aliquots at -80 °C and single aliquots thawed on ice when required. Modular ACPs were freshly loaded with Acac-pantethine **4**, desalted and concentrated as described, however, these ACPs would be used immediately due to hydrolytic instability of the β-keto thioester.

To form [^{13}C]-HMG-ACP, Acac-ACP, [^{13}C]-Ac-BatA and BatC were incubated at room temperature for 1 h. 10% D_2O was added to the sample, and the mixture transferred to an NMR tube for analysis. To assess ECH function, [^{13}C]-HMG-ACP was formed over 1 h, then BatD alone or BatD and BatE were added to the reaction. After incubation for 1 h, 10% D_2O was added and the sample transferred to an NMR rube.

Optimisation meant the concentration of modular ACP, BatA and the catalytic enzymes varied with each experiment according shown below. This was determined by protein expression, stock concentrations and dilution during NMR sample preparation.

ACP_A	ACP_A (µM)	ACP_D	ACP_D (µM)	ACP_A:ACP_D	BatC (µM)	BatD (µM)	BatE (µM)
[13C]-Ac-BatA	345						
[13C]-Ac-ACP4	340						
Acac-ACP4	215	[13C]-Ac-BatA	198	1.1	7		
Acac-ACP4	342	[13C]-Ac-BatA	145	2.4	5.4	26.8	
Acac-ACP4	267	[13C]-Ac-BatA	196	1.4	7	5	5
Acac-ACP5	156	[13C]-Ac-BatA	168	0.9	13.5	10.2	9.8
[13C]-Ac-4M	150						
Acac-4M	202	[13C]-Ac-BatA	150	1.3	13		
Acac-4M	226	[13C]-Ac-BatA	164	1.4	5.4	24.2	
Acac-4M	261	[13C]-Ac-BatA	272	1.0	10.9	13.5	11.8

NMR parameters

All protein NMR experiments were acquired on a Varian VNMRS 600 cryo spectrometer at 20 °C and spectra referenced using 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS). ^{1}H spectra recorded in 10% D_2O used a DPFGSE for water suppression and a sweep width of 8000 Hz with 32000 points.[17] Standard experiments as implemented in ChemPack (VnmrJ v2.3) were used for ^{13}C, DEPT, HSQC, HMBC, TOCSY and NOESY data collection.

Circular Dichroism

Samples were prepared in 50 mM sodium phosphate (pH 8.0) and the concentration adjusted to 10 µM. Spectra were averaged over 5 scans recorded at 5 °C using a 1 cm quartz cuvette and a JASCO J-1500 Spectrophotometer. Background subtraction and analysis took place using the Spectra Manager™ suite (JASCO).

Analytical S200 Gel Filtration

500 µL of protein was injected onto a Superdex 200 column (GE Healthcare) equilibrated with 25 mM Tris, 150 mM NaCl, pH7.5 at 0.5 mL/min. The column was pre-calibrated with blue dextran (2000 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), conalbumin (75 kDa), bovine serum albumin (66 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa) and

cytochrome C (12.4 kDa) from a Gel Filtration Calibration Kit (GE Healthcare). By plotting the gel-phase distribution coefficient (Kav) against the logarithm of the molecular weight (Log MW) and fitting with a straight line (y = -0.256x + 0.870, R^2 = 0.9876) a calibration curve may be obtained. The elution volume of an analyte may be used to estimate its molecular weight.

$$\mathrm{Kav} = \frac{\mathrm{Ve} - \mathrm{Vo}}{\mathrm{Vc} - \mathrm{Vo}}$$

Kav = gel-phase distribution coefficient, Ve = elution volume, Vo = column void volume = 7.08 mL (based on blue dextran), Vc = geometric column volume = 24 mL.

NMR Spectra

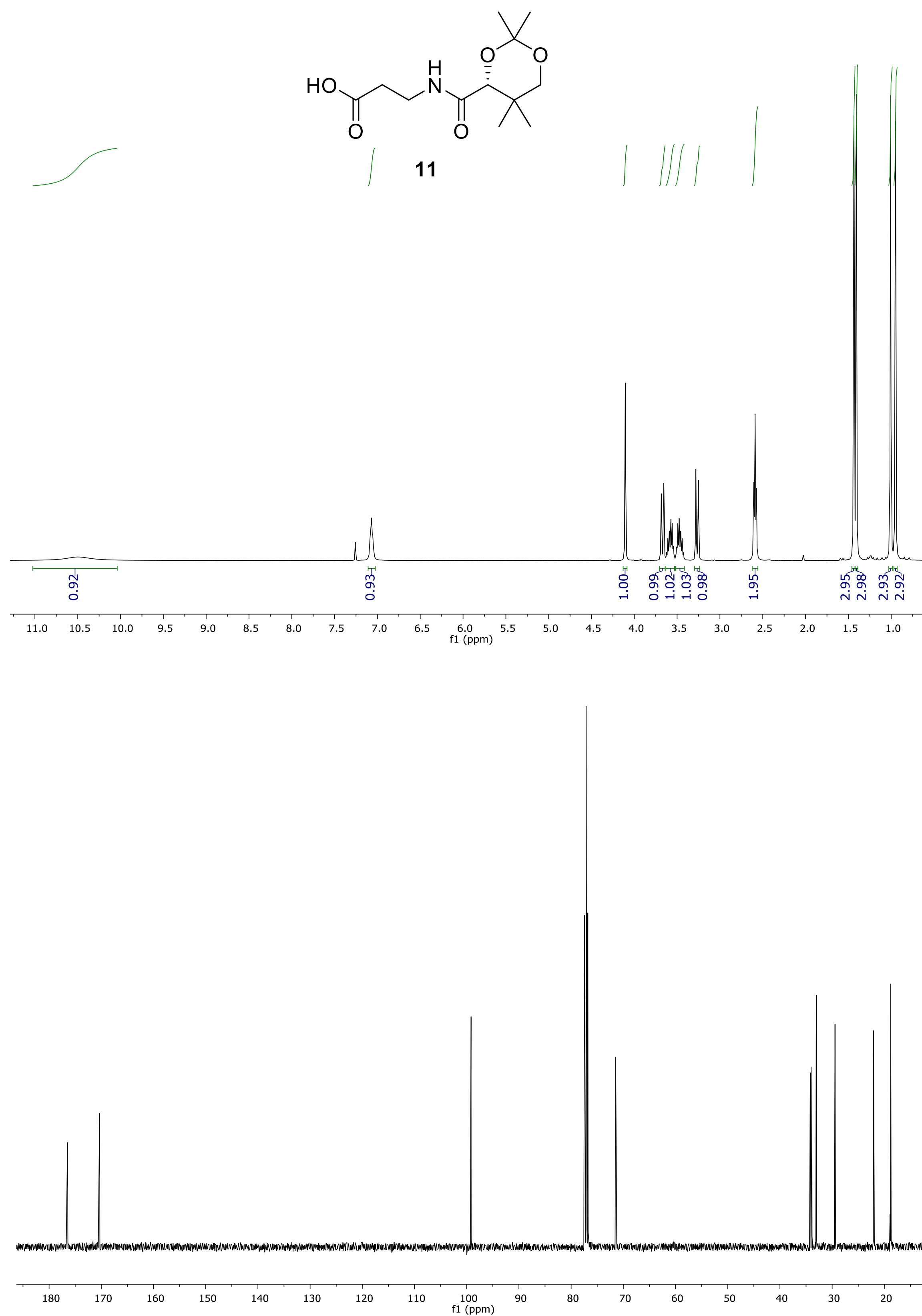


Figure S10. ^{1}H (top) and ^{13}C (bottom) NMR spectra of **11**

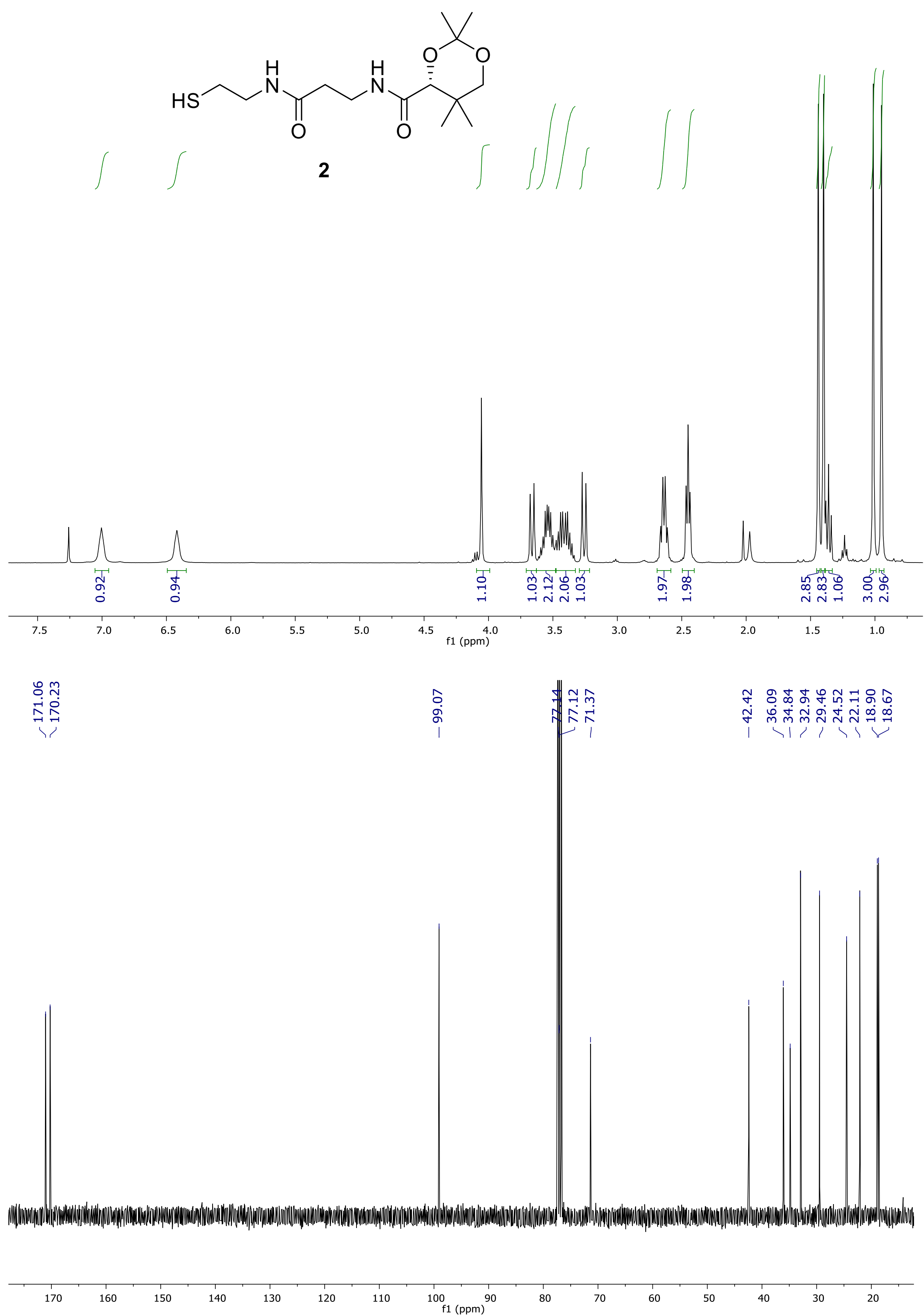


Figure S11. ^{1}H (top) and ^{13}C (bottom) NMR spectra of **2.**

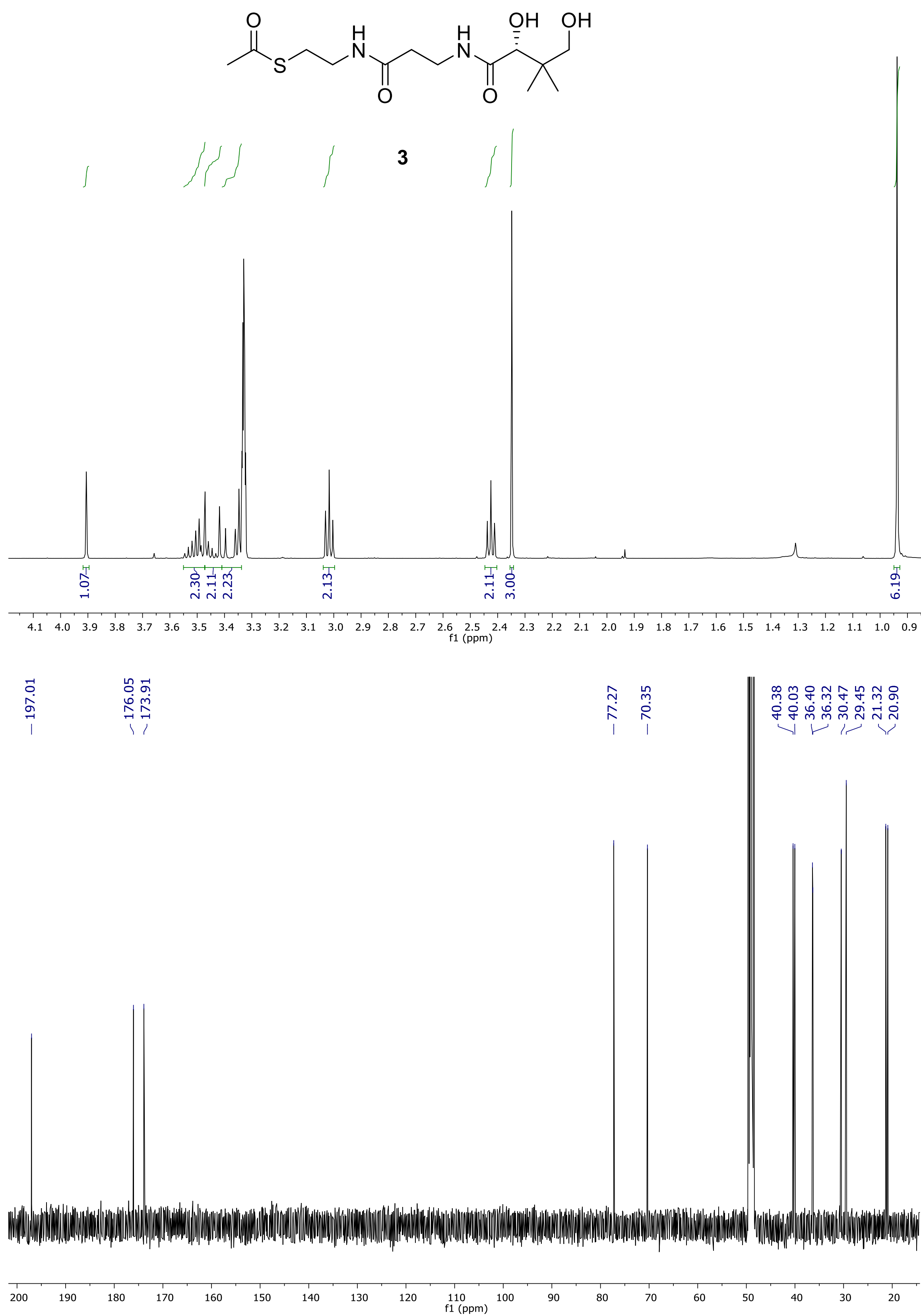


Figure S12. ^{1}H (top) and ^{13}C (bottom) NMR spectra of **3.**

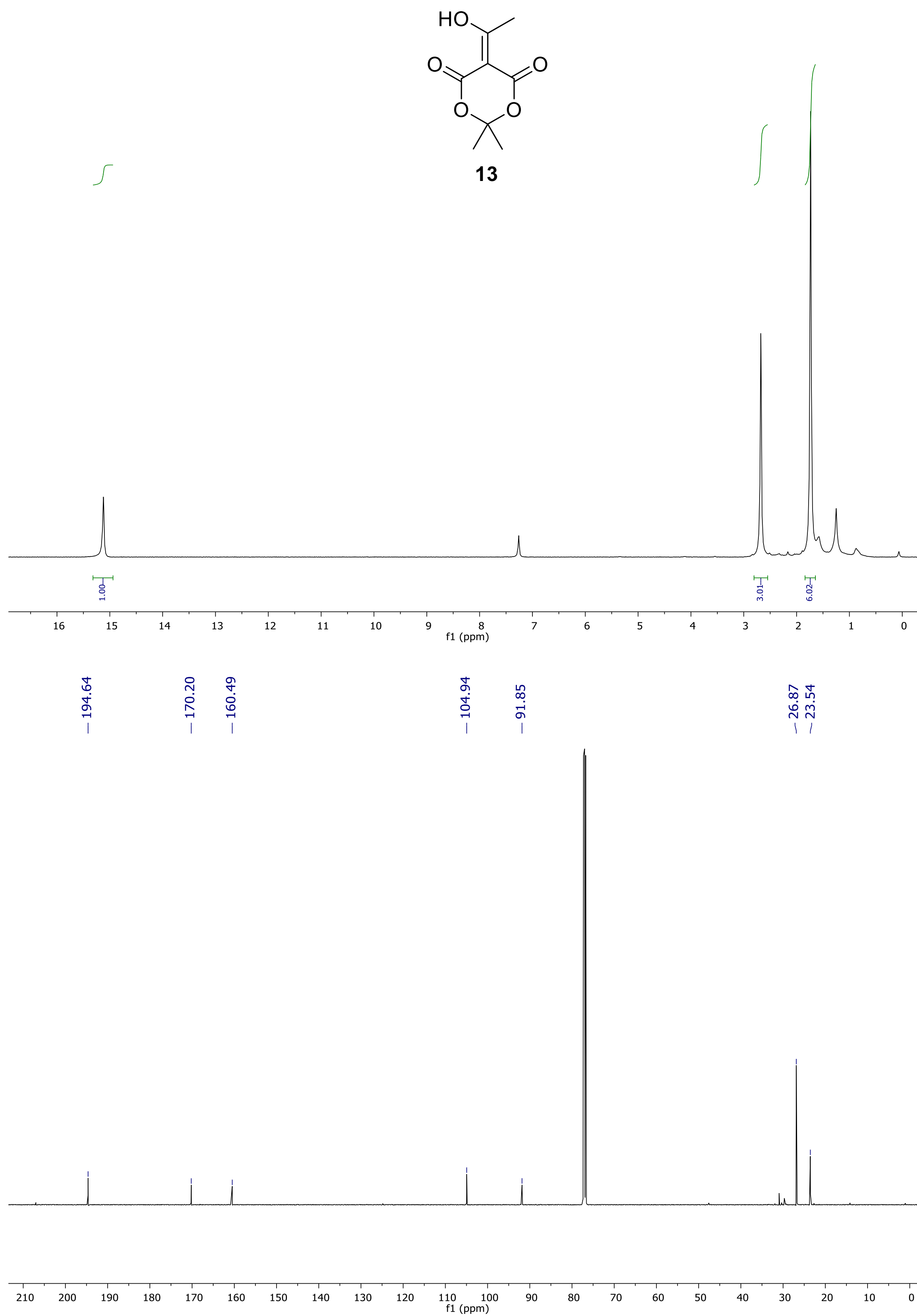


Figure S13. ^{1}H (top) and ^{13}C (bottom) NMR spectra of **13.**

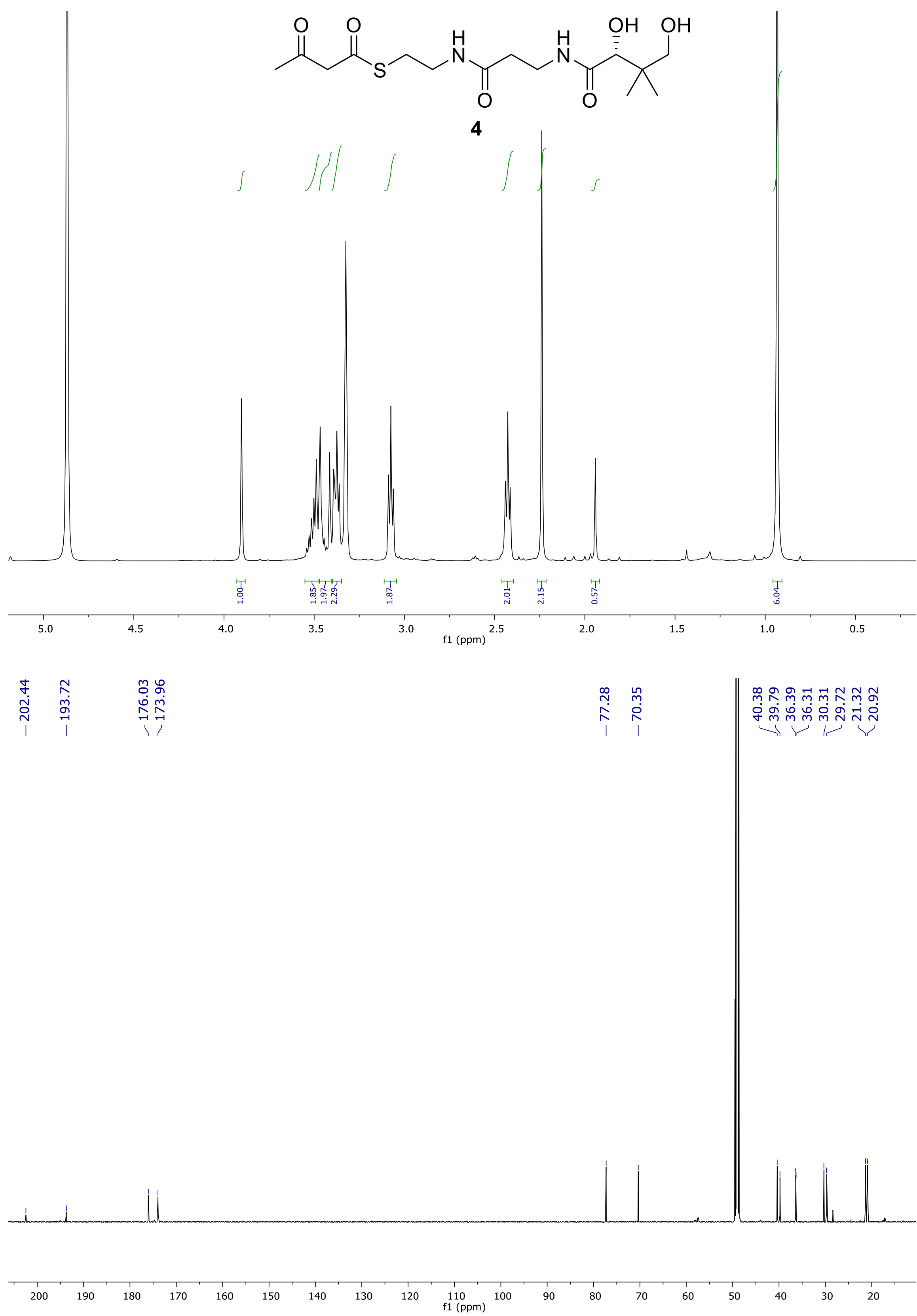


Figure S14. ^{1}H (top) and ^{13}C (bottom) NMR spectra of **4.**

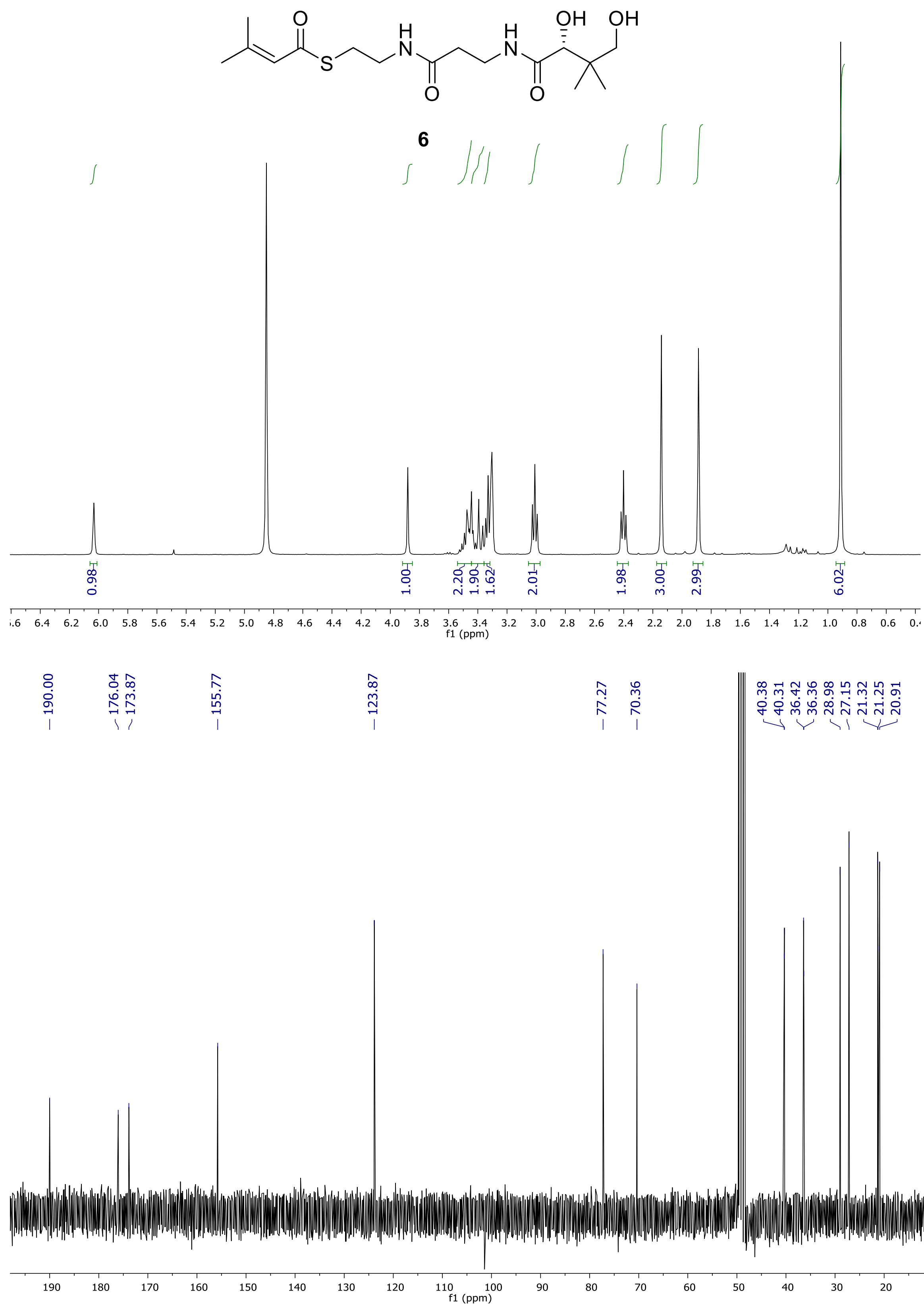


Figure S15. ^{1}H (top) and ^{13}C (bottom) NMR spectra of **6.**

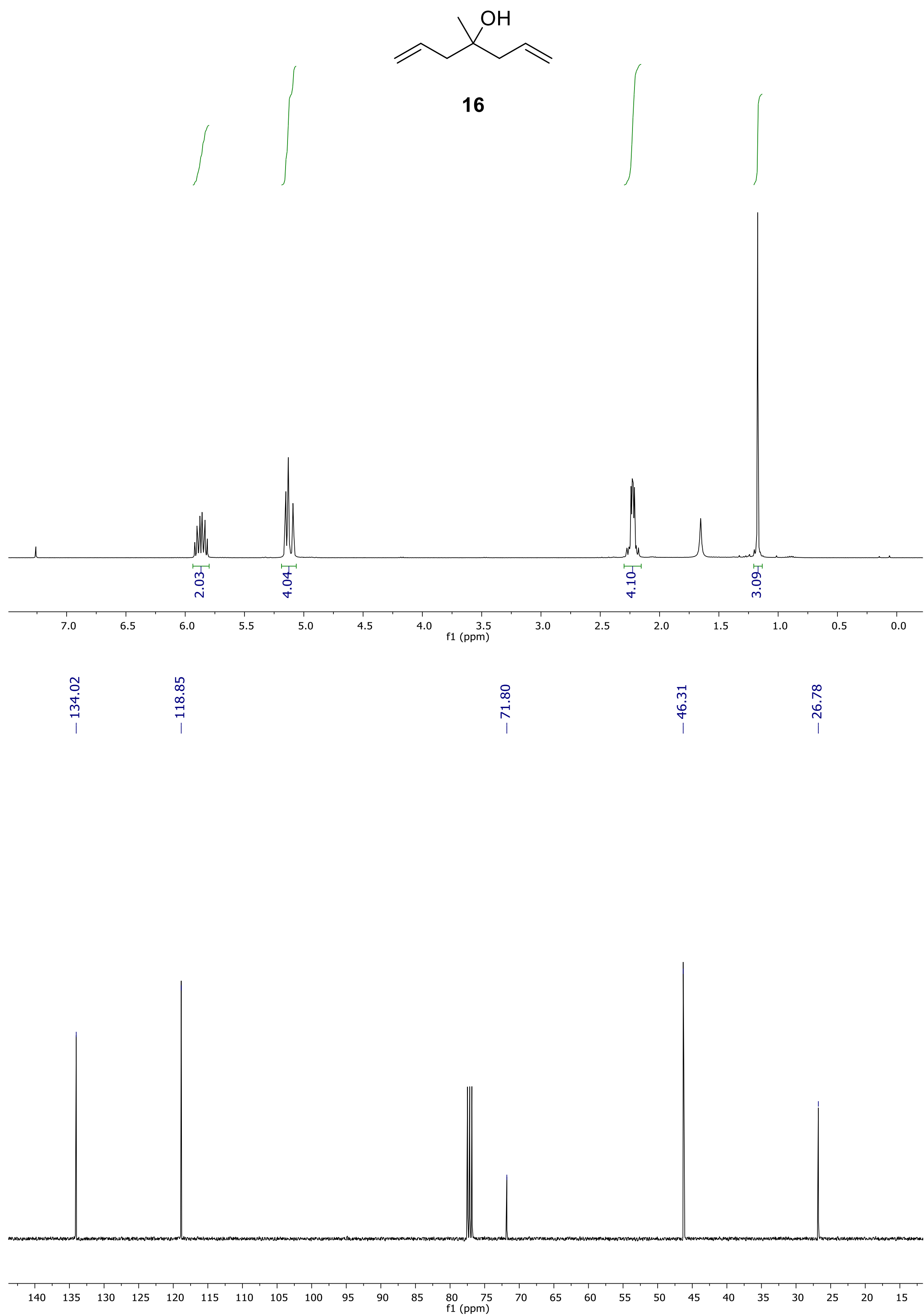
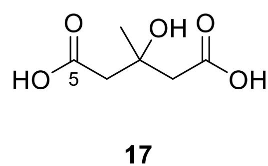
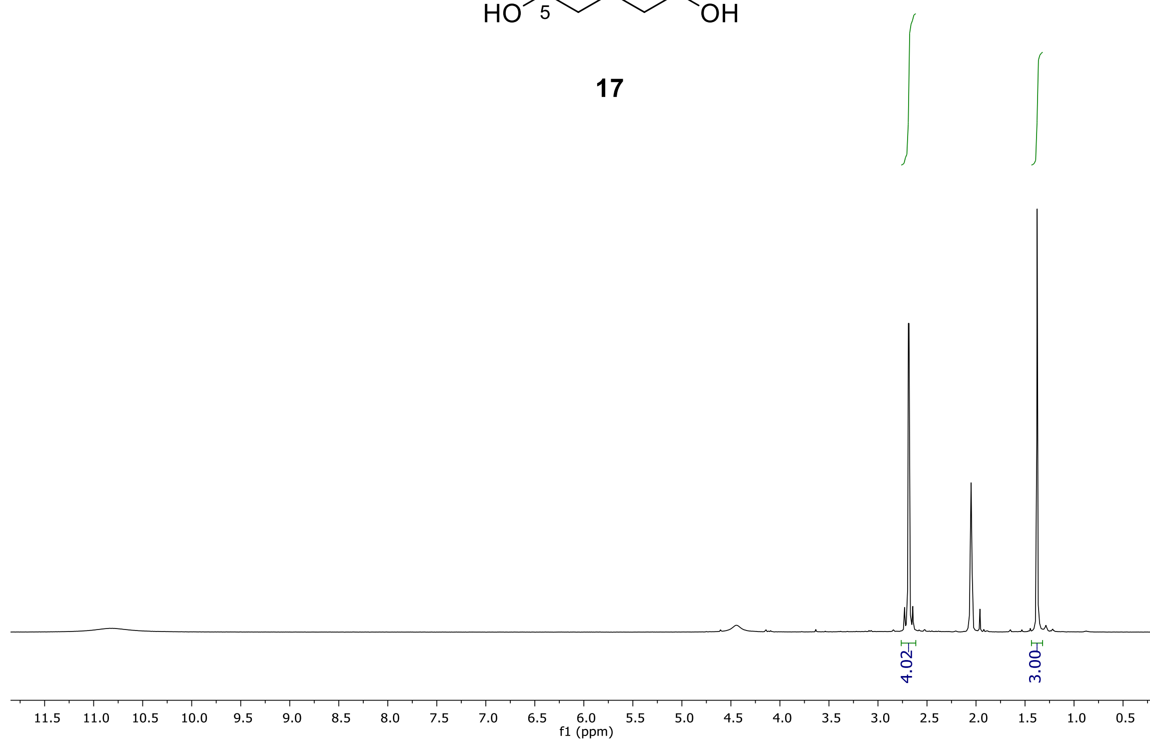


Figure S16. ^{1}H (top) and ^{13}C (bottom) NMR spectra of **16.**





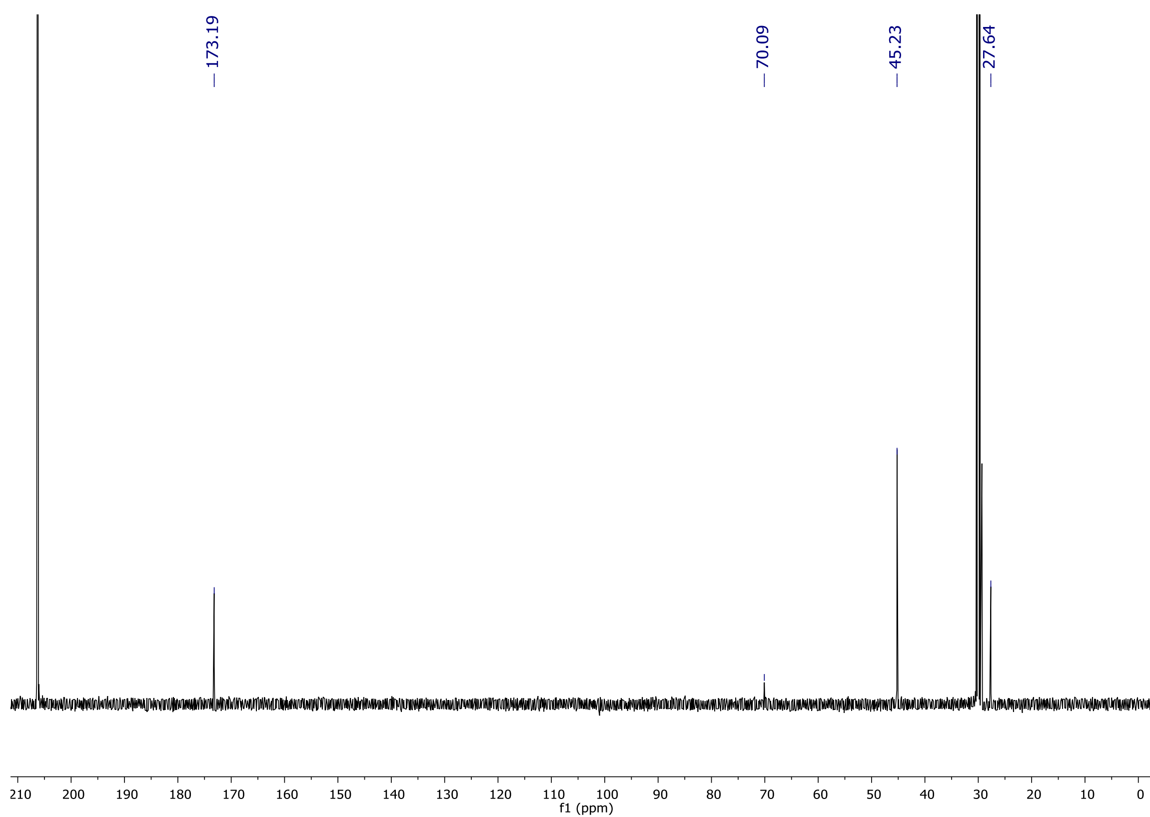


Figure S17. ^{1}H (top) and ^{13}C (bottom) NMR spectra of **17.**

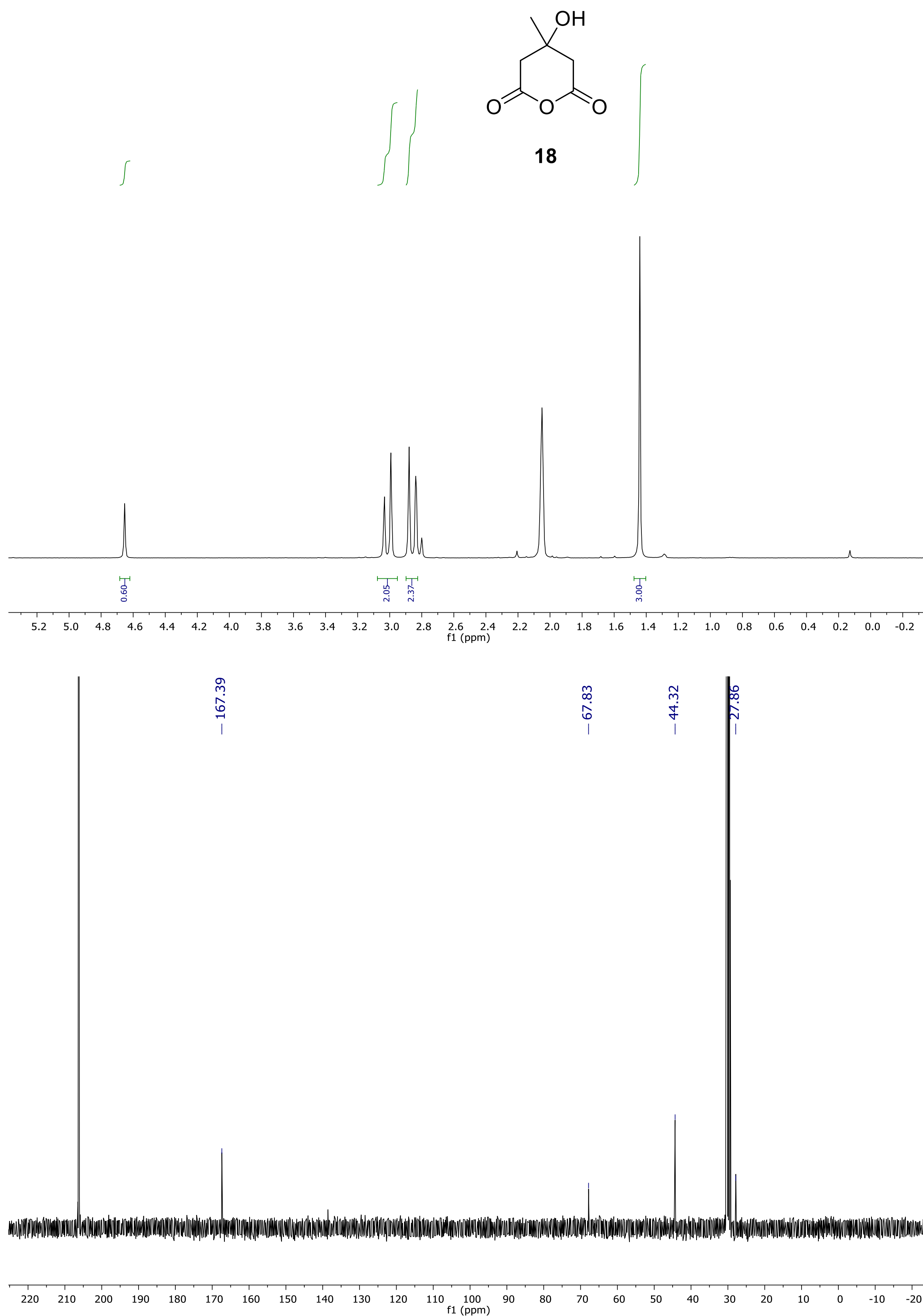


Figure S18. ^{1}H (top) and ^{13}C (bottom) NMR spectra of **18.**

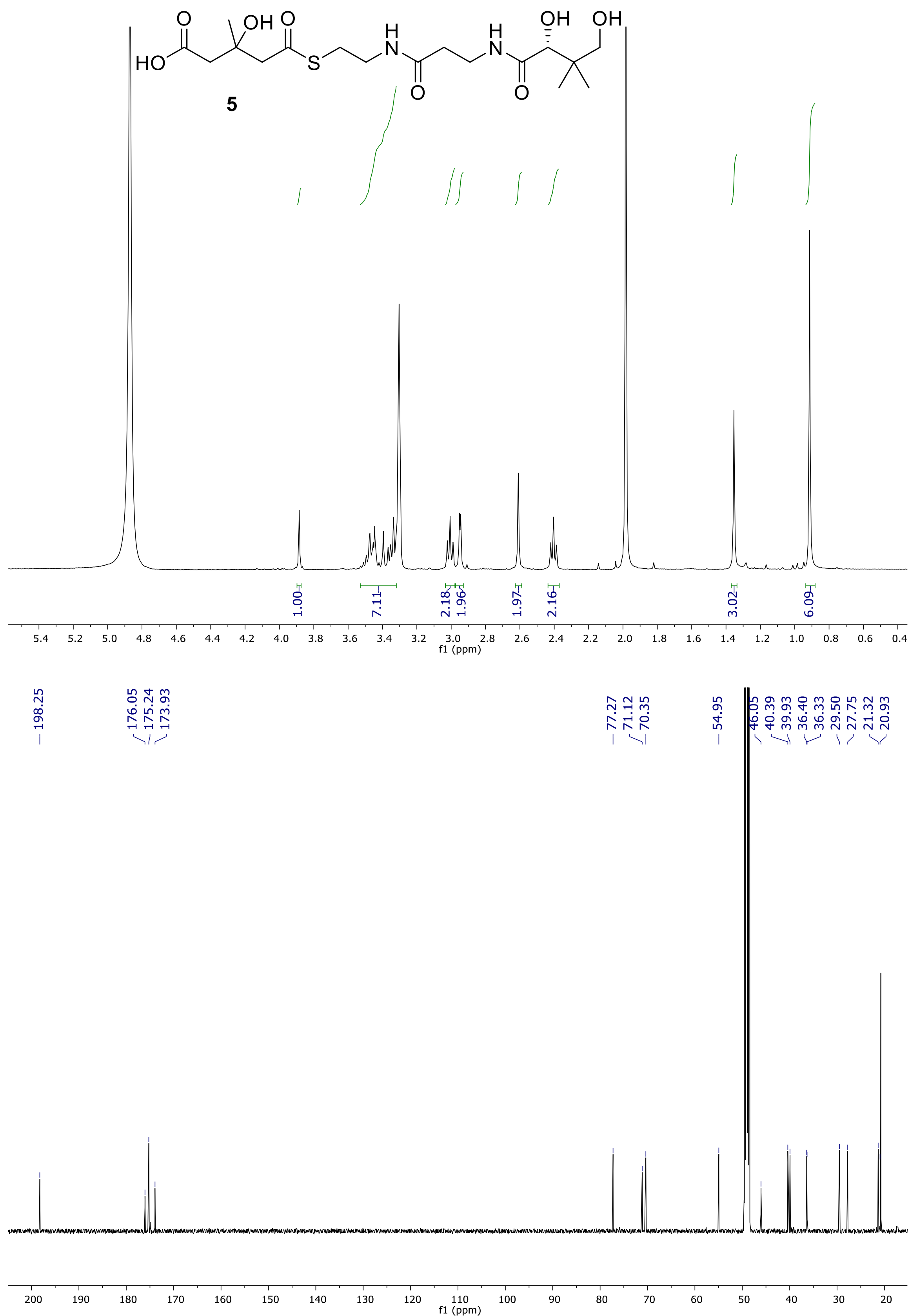


Figure S19. ^{1}H (top) and ^{13}C (bottom) NMR spectra of **5.**

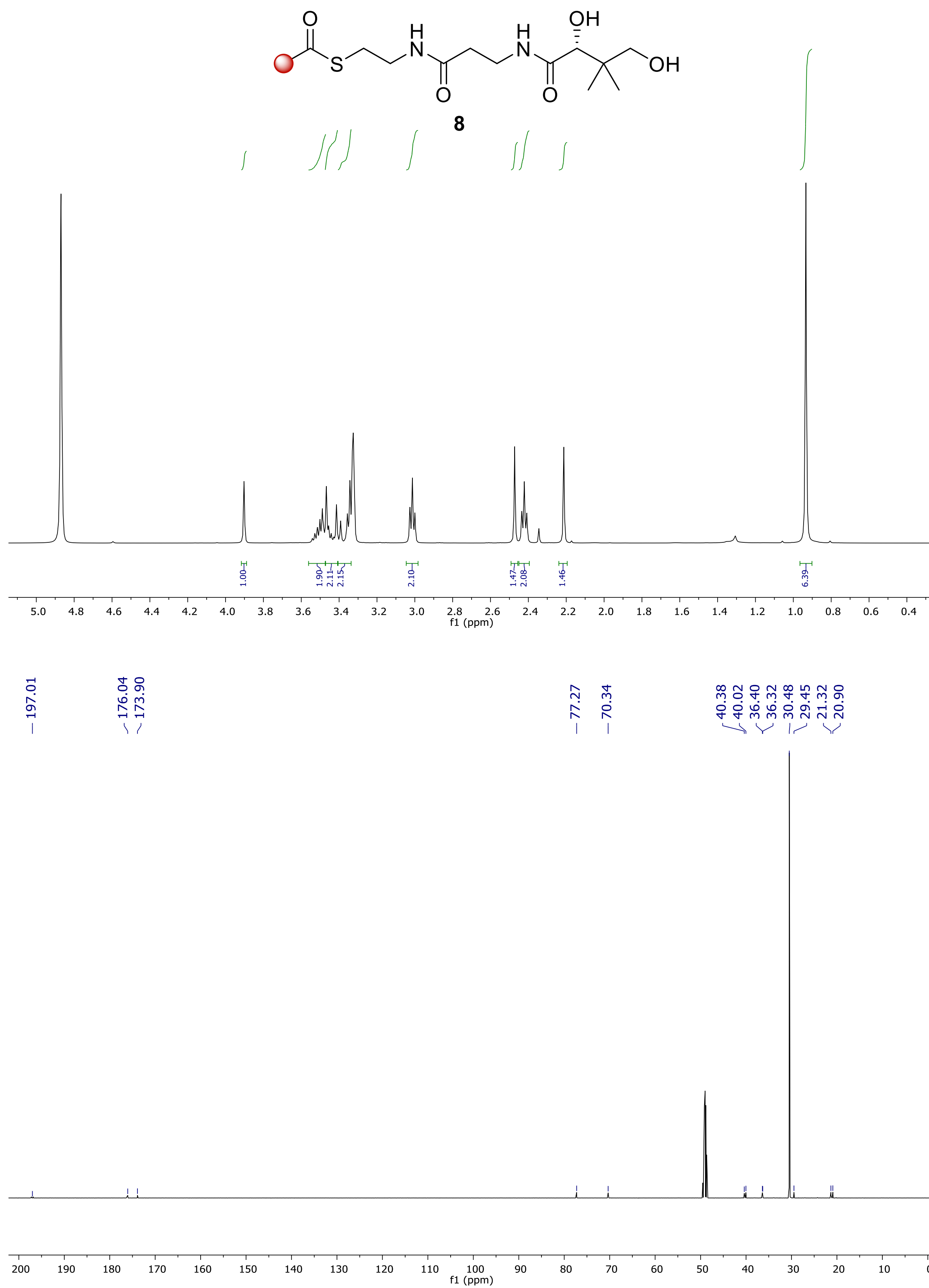


Figure S20. ^{1}H (top) and ^{13}C (bottom) NMR spectra of **8.**

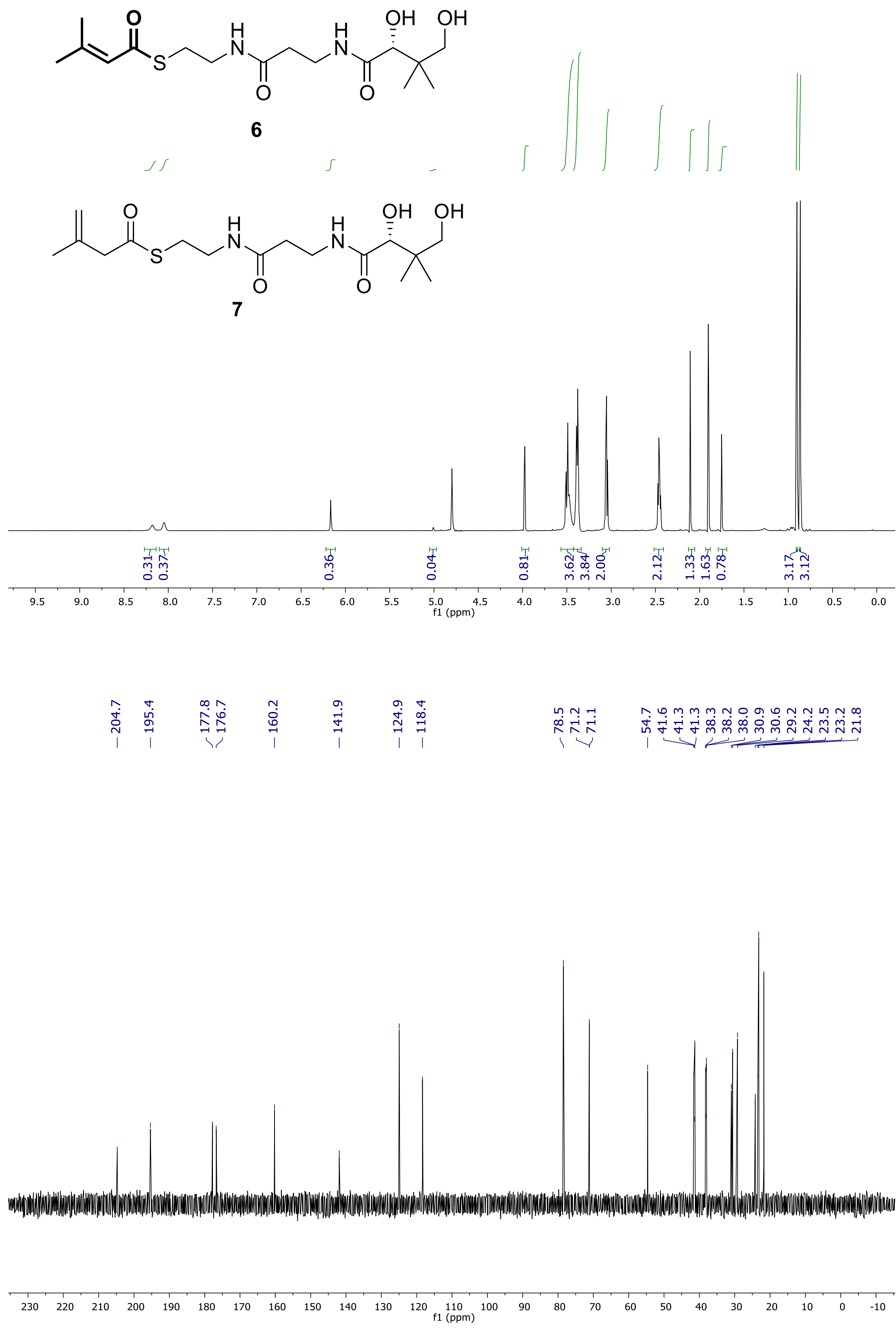


Figure S21. ^{1}H (top) and ^{13}C (bottom) NMR spectra of **6**/**7**.

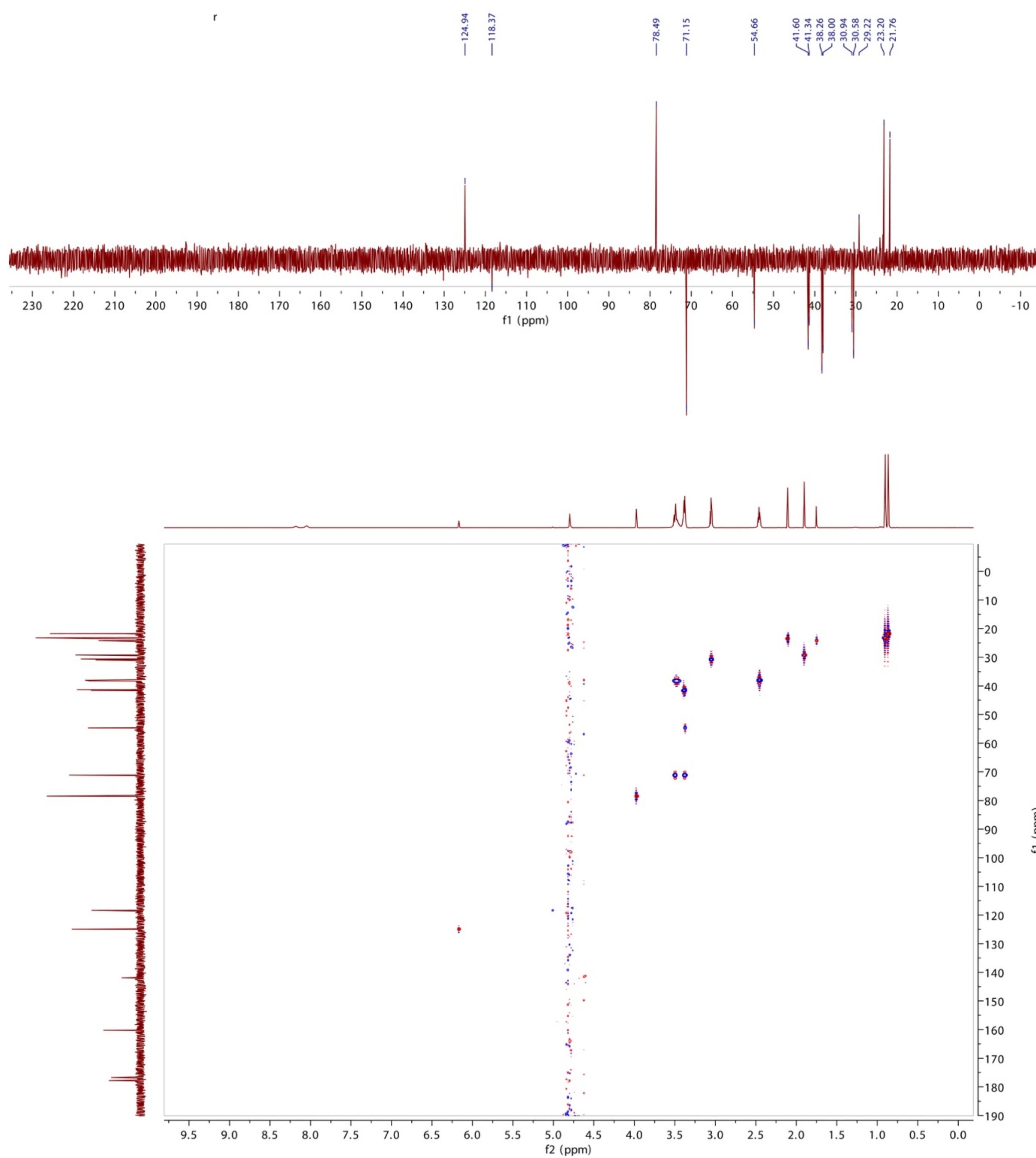


Figure S22. DEPT ^{13}C (top) and ^{1}H-^{13}C HSQC NMR spectra of **6**/**7.** A weak correlation of from the ^{1}H to the ^{13}C of the exomethylene carbon is visible close to the residual water peak.

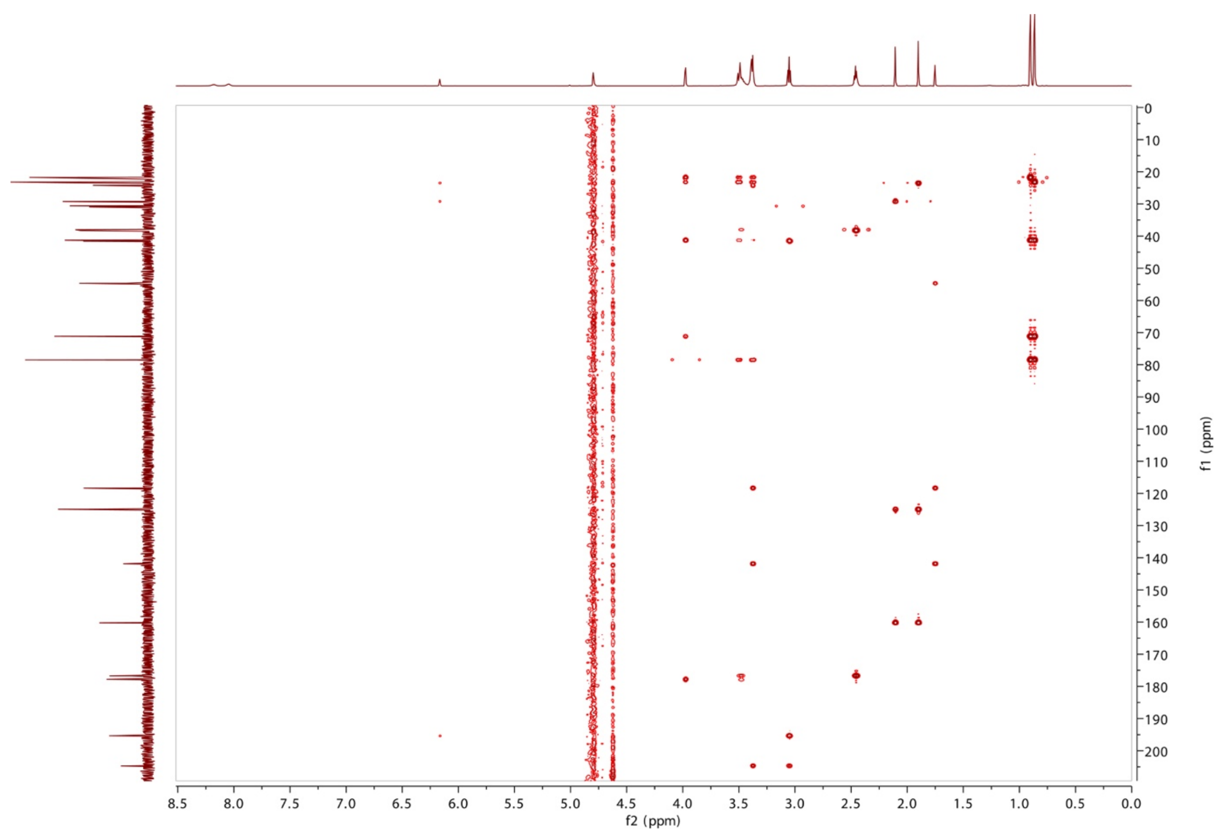


Figure S23. HMBC NMR spectrum of **6**/**7.**

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