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I*n Vitro* Kinetic Study of the Squalestatin Tetraketide Synthase Dehydratase Reveals the Stereochemical Course of a Fungal Highly Reducing Polyketide Synthase

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Electronic Supplementary Information

1. Cloning, Expression and Purification of SQTKS DH Domain

PCR was used to amplify a region corresponding to residues 989-1312 of *phpks1*. 1 μL (100 ng) of linearised pOPINF vector was incubated with 1 μL of the purified PCR product and 8 μL water was added and the solution added to dry InFusion enzyme mix (Clontech) and incubated at 42 ˚C for 30 min. The reaction was diluted by the addition of TE buffer (40 μL) and and then transformed into *E. coli* XL-1 Blue. The cells were plated onto LB-agar containing carbenicillin (50 $\mu g \cdot ml^{-1}$), X-Gal (0.02 %) and IPTG (0.5 mM) and incubated overnight at 37 ˚C. Colonies were screened by blue-white screening. Two white colonies were picked and grown in 1.5 mL LB + carbenicillin (50 $\mu g \cdot ml^{-1}$) overnight at 37 ˚C. Cells were harvested by centrifugation and plasmids were isolated using a Qiagen Bio-Robot 8000 and confirmed by PCR analysis. One confirmed clone was designated as pOPINF-DH.

E. coli BL21(DE3) was transformed with pOPINF-DH and selected on agar plates containing carbenicillin (50 $\mu g \cdot ml^{-1}$). A single colony was used to inoculate a 50 mL sterile LB starter culture medium containing carbenicillin (50 $\mu g \cdot ml^{-1}$). This was grown to stationary phase overnight at 30 °C with shaking. This starter culture was used to inoculate medium supplemented with carbenicillin (50 $\mu g \cdot ml^{-1}$) in 500 mL flasks each containing 200 mL of LB medium. The flasks were incubated at 37 °C with shaking for 4 h. The temperature of the culture was then adjusted to 16 °C. The cultures were induced with 0.1 mM IPTG and incubated with shaking for a further 4 h, before harvest by centrifugation. The cell pellets were suspended in binding buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM imidazole, 10 % glycerol, 100 mM L-Arginine and 100 mM L-Glutamic acid) containing protease inhibitor cocktail (Sigma) and were refrigerated overnight before being purified the following day.

The cell pellet from *E. coli* cultures was suspended in binding buffer (35-50 mL for each litre of culture). The cell suspension was sonicated in a glass beaker at 0 ˚C at full power for 30 second bursts with 30 seconds rest between bursts for a total of 6 minutes. The lysate was clarified by centrifugation. A 25 mL his-trap (GE healthcare) column attached to an FPLC instrument, and charged with 100 mM $NiSO_4$, was equilibrated with 200 mL binding buffer. The clarified lysate was then loaded onto the column using an FPLC super loop. The column was then washed with enough binding buffer for the UV trace to settle. The bound protein was eluted over a gradient of 0-100 % elution buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 400 mM imidazole, 10 % glycerol, 100 mM L-Arginine and 100 mM L-Glutamic acid) in 100 mL. 5 mL fractions were collected. Fractions for which a peak in the UV280 absorbance trace was observed were analysed by SDS-PAGE to determine the purity and size of the eluted protein.

Fractions containing DH protein were combined and loaded onto a A HiPrepTM 26/10 desalting column which had previously been equilibrated with 1.5 column volumes of desalting buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 % glycerol 100 mM L-Arginine and 100 mM L-Glutamic acid). Up to 10 mL of protein (for 100 mL HiPrepTM 26/10 column) was injected and eluted over one column volume.

Fractions containing DH protein were concentrated using a Vivaspin 20 centrifugal concentrator with a 10 kDa cutoff. A superdex S300 column was equilibrated with 1.5 column volumes of desalting buffer. Up to 2 mL of protein (for 120 mL S300 column) was injected and eluted over one column volume. Fractions containing DH protein were combined and used for *in vitro* kinetic analysis.

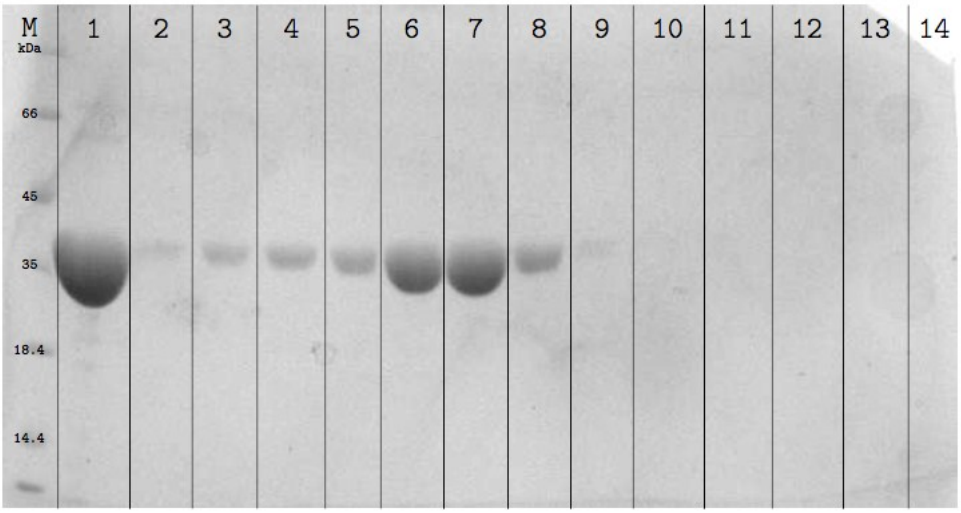


Figure S1.1 Purification of DH protein. SDS-PAGE analysis of concentrated Ni^{2+}-affinity-purified SQTKS DH (lane 1) and fractions eluted from Superdex S300 column (lanes 2-14). Lane M, molecular weight markers as indicated.

Tryptic Digestion and Analysis of Peptides

Proteins were analysed by in-solution digestion or in-gel digestion. For in-gel digestion, a band of interest was excised from a freshly-run SDS-PAGE gel and destained in 50 % EtOH. The gel pieces were washed with 200 µL 100 mM ammonium bicarbonate, then dehydrated using acetonitrile. Disulphide linkages were reduced by the addition of 30 µL 50 mM DTT. The gel pieces were incubated at room temperature for 30 min, then free cysteine residues were capped by the addition of 30 µL 100 mM iodoacetamide solution. The gel pieces were dehydrated by the addition of acetonitrile, then washed with 200 µL 100 mM ammonium bicarbonate. The dehydration and washing procedure was repeated twice more to remove small molecule contaminants, and the gel pieces were finally dehydrated. 1 µg of Promega modified sequencing grade trypsin was dissolved in 50 mM ammonium bicarbonate and added to the gel pieces. These were incubated overnight at 37 ˚C to ensure complete digestion. Peptides were extracted from the gel pieces by the addition of 50 µL 50 % acetonitrile, 5 % acetic acid. The tube was sonicated in a water bath for 10 min and the supernatant was removed and transferred to a fresh tube. This process was repeated twice more to complete the extraction of peptides from the gel. The pooled supernatant was evaporated in a vacuum centrifuge at 37 ˚C to dryness and resuspended in 10 µL 2 % acetic acid.

1 µL of the peptide solution was spotted onto a MALDI sample plate, mixed with 1 µL saturated α-cyano-4-hydroxycinnamic acid (α-CHCA) solution and allowed to dry. Peptides were analysed using a MALDI-TOF spectrometer operating in positive ion reflectron mode. The MASCOT server was used to interrogate the NCBI protein database to find potential matching proteins based on masses of tryptic fragments.

For in-solution digestion of proteins, 100 mM Tris-HCl pH 8.0 was used as a buffer. DTT solution was added to 100 µL of a protein solution to a final concentration of 5 mM, and the mixture was incubated at room temperature for 30 min. Iodoacetamide was added to a final concentration of 10 mM and the mixture was incubated at room temperature for 30 min. 1 µg Promega modified sequencing grade trypsin was added and the mixture was incubated overnight at 37 ˚C. 20 µL samples were analysed by LC-MS using the conditions given below.

Biophysical Characterisation

The protein's identity was confirmed by mass spectrometry. An ESI+ spectrum was collected between *m/z* = 550 - 1450 and deconvoluted using the Max-Ent function of Waters MassLynx Software. The observed mass of the protein (38008) matched the expected mass of the protein without the N-terminal methionine residue (38002 Da)

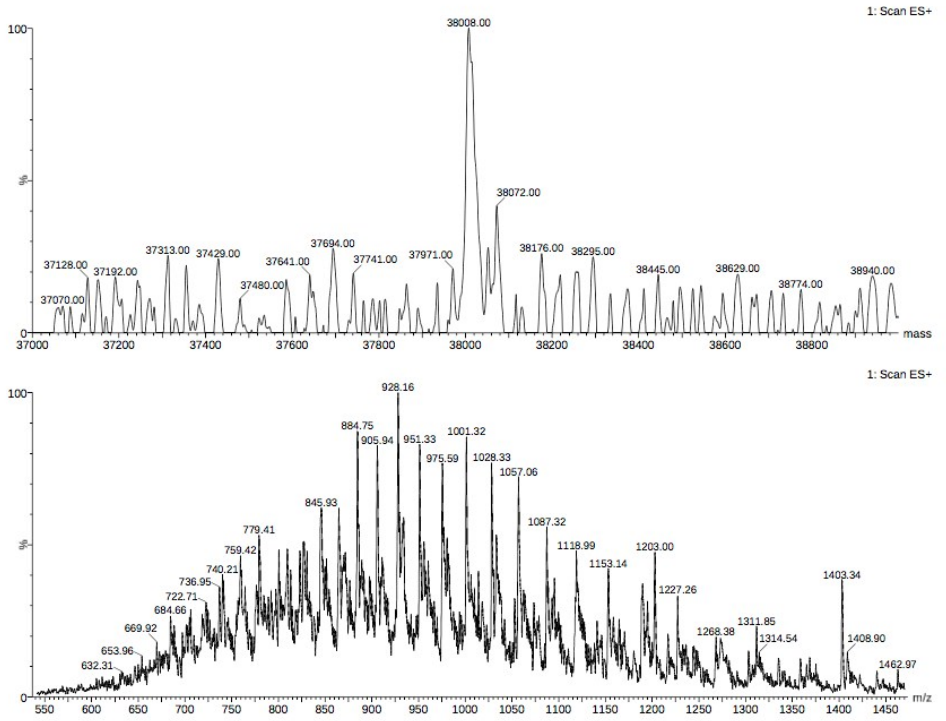
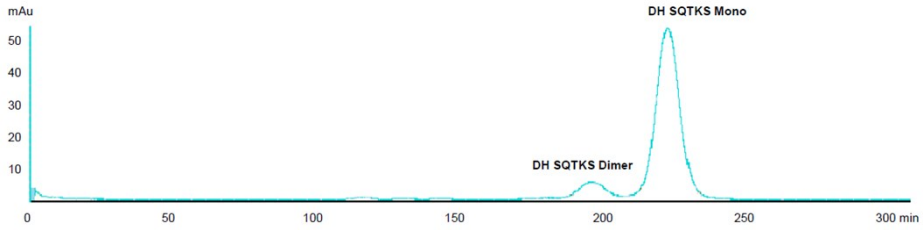
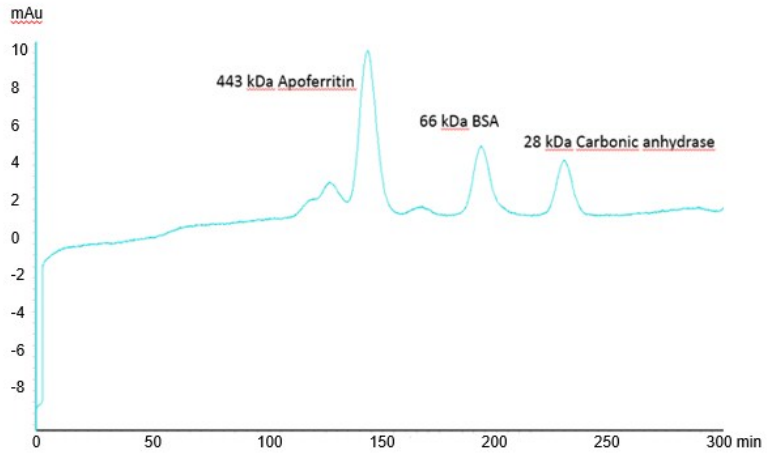


Figure S1.2. ESI+ spectum of denatured DH protein (bottom) and deconvoluted spectrum (top).

Gel filtration analysis





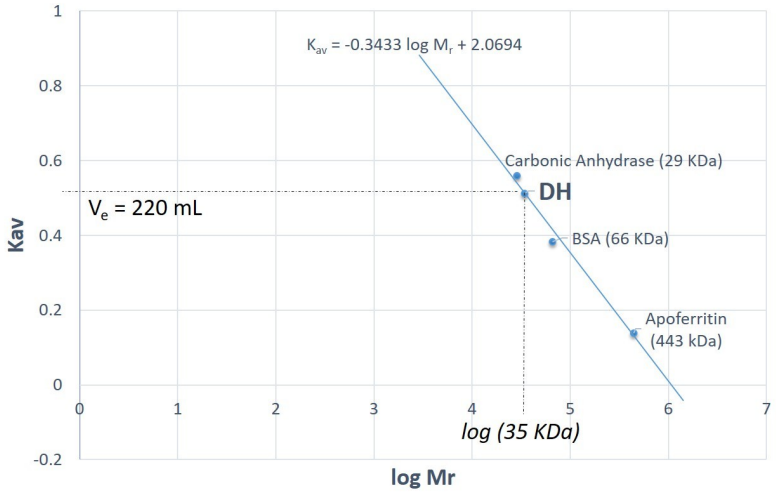


Figure S1.3. Results of gel filtration analysis of isolated DH domain.

Calibration of GE HiLoad 26/600 Superdex 200 Column (geometric column volume, V_c 320 mL) was performed in 50 mM Tris, pH 8.0, 150 mM NaCl, 20 % glycerol eluted at 1 mL/min. The column was calibrated with carbonic anhydrase, bovine serum albumin, and apoferritin (Sigma), using dextran blue to calculate the void volume (V_0 = 114.8 mL). The partition coefficient $K_{av} = (V_e-V_0)/(V_c-V_0)$ where V_e = Elution volume. The measured size of the DH was 35 KDa which agrees well with the calculated MW of 38 KDa for the monomeric species.

2. Substrate Synthesis

All reagents were purchased from commercial suppliers and used without further purification, unless otherwise stated. Common anhydrous solvents were dried by passing through a modified Grubbs system of alumina columns, manufactured by Anhydrous Engineering. DMF was purchased as an anhydrous solvent. Triethylamine and diisopropylamine were distilled over calcium hydride and stored under an inert nitrogen atmosphere. All moisture or air sensitive reactions were carried out in flame dried glassware under a positive pressure of dry nitrogen using standard Schlenk-line techniques. After aqueous work-up of reaction mixtures, organic solutions were routinely dried with anhydrous magnesium sulphate. Column chromatography was performed using Merck Kieselgel 60 silica gel, eluting with the solvent system stated. Columns were eluted under pressure and the fractions analysed by TLC. Fractions with the same R_f were pooled and concentrated *in vacuo*.

^{1}H and ^{13}C NMR spectra were recorded at 25 °C using Varian 400-MR or Jeol ECS 400 spectrometers. Spectra are referenced to $CDCl_3$ at 7.26 ppm for proton and 77.2 ppm for carbon. Coupling constants are given to 1 decimal place and are in Hertz (Hz). The abbreviations used to denote ^{1}H NMR multiplicities are as follows; s, singlet; d doublet; dd, doublet of doublets; dq, doublet of quartets; t, triplet; q, quartet; qd, quartet of doublets, m, multiplet; br, broad. Abbreviations used to describe ^{1}H NMR assignment are as follows; Ar, aryl. Signal assignment was aided by analysis of COSY, HMBC and HMQC where necessary.

Thin layer chromatography was carried out on Merck glass-backed TLC plates coated with 0.2 mm silica gel or on Merck DC-Alufolien Kieselgel 60 F254 aluminium-backed silica plates TLC plates. Plates were visualised with 254 nm UV light and developed with either a $KMnO_4$ or *p*-anisaldehyde solution and developed with a heat gun, where appropriate. Infrared (IR) spectroscopy was recorded on a Perkin Elmer Spectrum 100 FT-IR spectrometer with an ATR diamond cell irradiating between 4000 cm^{-1} and 600 cm^{-1}. Melting points were determined using an electrothermal melting point apparatus and are uncorrected. Optical rotations were determined with the sodium D line (λ= 589 nm) using a Perkin Elmer 241 MC polarimeter. $[\alpha]_D$ values are quoted in units $10^{-1}\cdot deg\cdot cm^2\cdot g^{-1}$. Mass spectra (both high and low resolution) were recorded on a Bruker Daltonics Apek 4e 7.0T FT-MS mass spectrometer and were obtained by electrospray ionisation (ESI) or chemical ionisation (CI).

Ethyl 2*R*,3*R*-3-(*tert*-butyldimethylsilyloxy)-2-methylbutyrate 2*R*,3*R*-6[1]

Diisopropylamine (12.2 mL, 87.1 mmol) was dissolved in anhydrous THF (80 mL) under nitrogen and cooled to -78 °C. *n*-Butyl lithium (2.36 M, 36.8 mL, 87.1 mmol) was added dropwise and the mixture stirred at -78 °C for 1 h. A solution of ethyl (3*R*)-3-hydroxybutyrate **5** (5.01 g, 37.9 mmol) in anhydrous THF (20 mL) was added dropwise. The cold bath was removed and the mixture stirred at RT for 20 min, after which it was cooled back to -78 °C and methyl iodide (1.4 eq., 3.30 mL, 53.0 mmol) was added dropwise. The reaction was stirred at 0 °C for 3 h, then quenched with 6 M aq. HCl (60 mL). The layers were separated and the aqueous layer was extracted with Et_2O (4 x 40 mL). The organic phases were combined, dried ($MgSO_4$), filtered and concentrated *in vacuo*.

The resulting residue was dissolved in anhydrous CH_2Cl_2 (100 mL) under nitrogen. Pyridine (6.40 mL, 79.1 mmol) was added. The mixture was cooled to 0 °C and *tert*-butyldimethylsilyl triflate (10.4 mL, 45.3 mmol) was added dropwise. The reaction was stirred at RT for 1 h, after which it was quenched with sat. aq. $NaHCO_3$, (20 mL). The layers were separated and the aqueous layer was extracted with CH_2Cl_2 (2 x 100 mL). The organic phases were combined, dried ($MgSO_4$), filtered and concentrated *in vacuo*. Purification of the crude residue by flash chromatography (5% EtOAc in petroleum ether) gave the title compound 2*R*,3*R*-**6** (8.6 g, 32.9 mmol, 86% over two steps) as a colourless oil. $[\alpha]_D$ - 36.0 *(c* 1.0, $CHCl_3$), [lit. $[\alpha]_D$ -31.5 *(c* 1.9, $CHCl_3$)][2]; δ_H (400 MHz, $CDCl_3$); 0.02 (3H, s, $SiCH_3$), 0.05 (3H, s, $SiCH_3$), 0.85 (9H, s,$Si(CH_3)_3$), 1.07 (3H, d, *J* = 7.1, 2-CH_3), 1.10 (3H, d, *J* = 6.3, 3-CH_3), 1.25 (3H, t, *J* = 7.0, OCH_2CH_3), 2.47 (1H, m, 2-H), 4.01 (1H, dq, *J* = 6.3, 6.2, 3-H), 4.10 (2H, q, *J* = 7.0, OCH_2); δ_C (100 MHz, $CDCl_3$); -4.9 (Si-CH_3), -4.2 (Si-CH_3), 12.8 (2-CH_3), 14.3 (OCH_2CH_3), 18.1 (Si-$C(CH_3)_3$) 20.7 (3-CH_3), 25.9 (Si-C(CH_3), 48.3 (2-CH), 60.3 (OCH_2), 70.3 (3-CH), 175.3 (CO).

Methyl 2*S*,3*S*-3-(*tert*-butyldimethylsilyloxy)-2-methybutyrate 2*S*,3*S*-6[4]

Diisopropylamine (5.0 mL, 35.7 mmol) was dissolved in anhydrous THF (30 mL) under nitrogen and cooled to -78 °C. *n*-Butyl lithium (2.15 M, 16.5 mL, 35.5 mmol) was added dropwise and the mixture stirred at -78 °C for 1 hour. A solution of methyl 3*S*-3-hydroxybutyrate (Sigma Aldrich, 2.00 g, 16.9 mmol) in anhydrous THF (12 mL) was added dropwise. The cold bath was removed and the mixture stirred at room temperature for 20 minutes, after which it was cooled back to -78 °C and methyl iodide (1.30 mL, 20.9 mmol) was added dropwise. The reaction was stirred at 0 °C for 3 hours, then quenched

with 6 M aq. HCl (20 mL). The layers were separated and the aqueous layer was extracted with Et_2O (4 x 20 mL). The organic layers were combined, dried ($MgSO_4$), filtered and concentrated *in vacu*o.

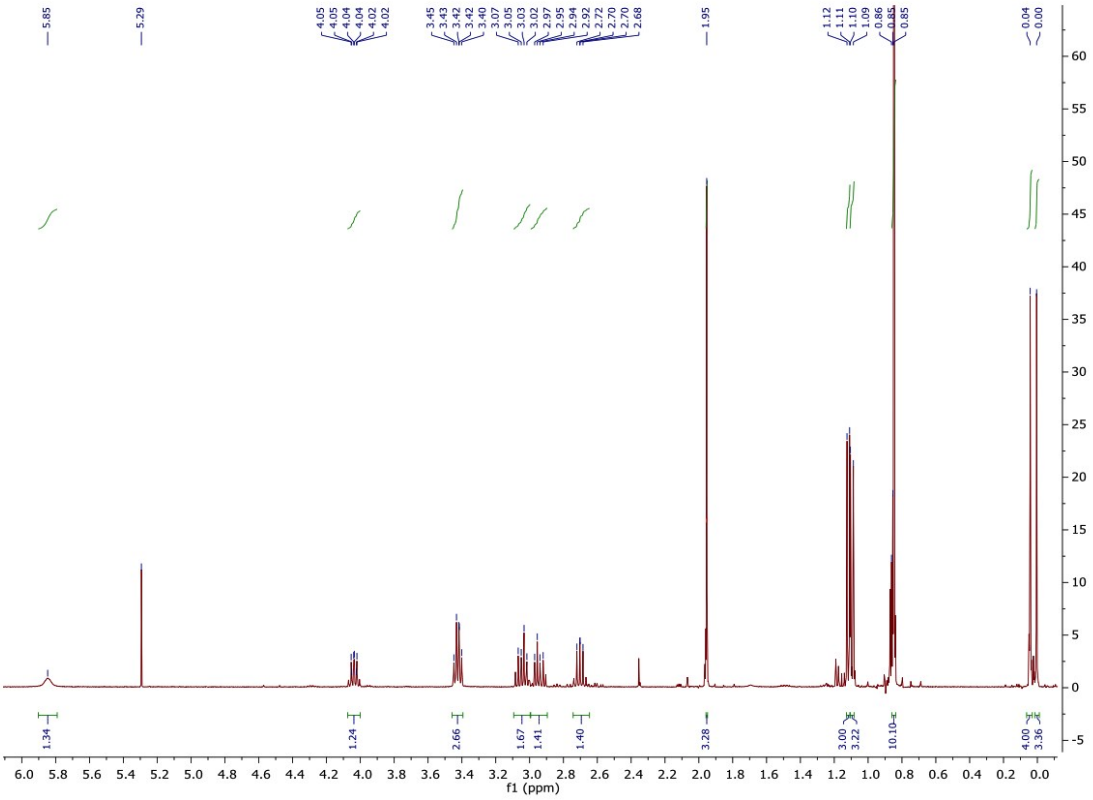
The resulting residue was dissolved in anhydrous CH_2Cl_2 (40 mL) under nitrogen. Pyridine (2.90 mL, 35.9 mmol) was added. The mixture was cooled to 0 °C and *tert*-butyldimethylsilyl triflate (4.70 mL, 20.3 mmol) was added dropwise. The reaction was stirred at room temperature for 1 hour, after which it was quenched with sat. aq. $NaHCO_3$, (20 mL). The layers were separated and the aqueous layer was extracted with CH_2Cl_2 (2 x 40 mL). The organic layers were combined, dried ($MgSO_4$), filtered and concentrated *in vacuo*. Purification of the crude residue by flash chromatography (5% EtOAc, in petroleum ether) gave the title compound (2.50 g, 10.3 mmol, 63% over two steps) as a pale yellow oil. [□]$_D$ +37.3 *(c* 1.0, $CHCl_3$), [lit. [□]$_D$ +37.1 *(c* 1.43, $CHCl_3$)].[4] δ_H (400 MHz, $CDCl_3$); 0.01 (3H, s, Si-CH_3), 0.04 (3H, s, Si-CH_3), 0.85 (9H, s, Si-C(CH_3)$_3$), 1.06 (3H, d, *J* = 7.1, 2-CH_3), 1.10 (3H, d, *J* = 6.2, 3-CH_3), 2.49 (1H, m, 2-CH), 3.65 (3H, s, CO-CH_3), 3.99 (1H, dq, *J* = 7.4, 6.2, 3-CH). δ_C (100 MHz, $CDCl_3$); -5.0 (Si-CH_3), -4.2 (Si-CH_3), 12.9 (2-CH_3), 18.0 (Si-*C*(CH_3)), 20.7 (3-CH_3), 25.8 (Si-C(*C*H_3)$_3$), 48.3 (2-CH), 51.5 (O-CH_3), 70.3 (3-CH); ν_{max}(neat) / cm^{-1}: 2953 (CH), 2930 (CH), 2857 (CH), 1740 (C=O), 1252 (CO), 833 (Si-CH_3).

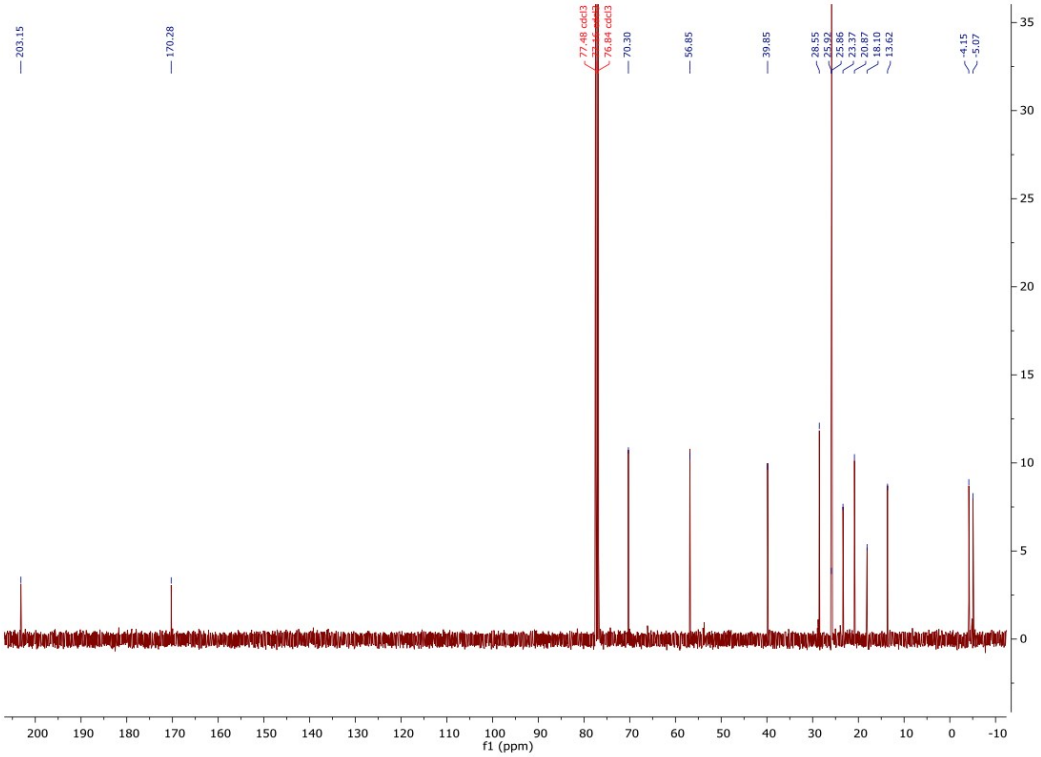
2*R*,3*R*-3-(*tert*-butyldimethylsilyloxy)-2-methylbutyric acid *N*-acetylcysteamine thiolester 2*R*,3*R*-7

To ester 2*R*,3*R*-**6** (1.50 g, 5.76 mmol) in THF (5 mL), MeOH (12 mL) and lithium hydroxide (414 mg, 17.2 mmol) in water (2 mL) were added. The reaction mixture was heated to 60 °C overnight. The reaction was diluted with water (8 mL) and extracted with Et_2O (2 x 15 mL). The aqueous layer was acidified to pH 3.0 with H_2SO_4 (6 M) and further extracted with EtOAc (3 x 15 mL). The organic layers were combined, dried ($MgSO_4$), filtered and concentrated *in vacuo* to give a yellow oil. Purification by flash chromatography (SiO_2, 10% EtOAc/ Petrol/ 0.5% acetic acid, visualised with $KMnO_4$) afforded the acid.

To a solution of acid (402 mg, 1.73 mmol) in anhydrous CH_2Cl_2 (25 mL) at 0 °C under nitrogen was added EDCI (405 mg, 2.60 mmol) and DMAP (23.0 mg, 0.19 mmol). After stirring at 0 °C for 15 minutes, HSNAC[3] (210 mg, 1.76 mmol) was added in anhydrous CH_2Cl_2 (10 mL). The mixture was allowed to warm to room temperature and stirred overnight. The reaction was quenched with water (15 mL) and the layers separated. The aqueous phase was extracted with CH_2Cl_2 (3 x 25 mL). The organic layers were combined, dried ($MgSO_4$), filtered and concentrated *in vacuo*. Purification by flash chromatography gave the title compound (360 mg, 1.08 mmol, 62%) as a pale yellow oil. [α]$_D$ -69.4 *(c* 1.0, $CHCl_3$); δ_H (400 MHz, $CDCl_3$); 0.01 (3H, s, Si-CH_3), 0.05 (3H, s, Si-CH_3), 0.85 (9H, s, Si-C(CH_3)$_3$), 1.10 (3H, d, *J* = 7.0, 2-CH_3), 1.12

(3H, d, *J* = 6.1, 3-CH_3), 1.96 (3H, s, CO-CH_3), 2.71 (1H, m, 2-CH), 3.00 (2H, m, S-CH_2), 3.43 (2H, app. q, *J* = 6.2, N-CH_2), 4.03 (1H, m, 3-CH); δ_C (100 MHz, $CDCl_3$); -5.1 (Si-CH_3), -4.2 (Si-CH_3), 13.6 (2-CH_3), 18.1 (Si*C*$(CH_3)_3$), 20.9 (3-CH_3), 23.4 (CO-CH_3), 25.9 (SiC(*C*$H_3)_3$), 28.5 (S-CH_2), 39.9 (N-CH_2), 56.9 (2-CH), 70.3 (3-CH), 170.0 (N-CO), 203.2 (S-CO); ν_{max} (neat) / cm^{-1}: 3285 (NH), 2955 (CH), 2929 (CH), 2884 (CH), 2586 (CH), 1687 (CO), 1653 (CO); *m/z* (ESI) 356.17 $[M]Na^+$; Found (ESI) 356.1686 ($C_{15}H_{31}NaNO_3SSi$ requires 356.1692).



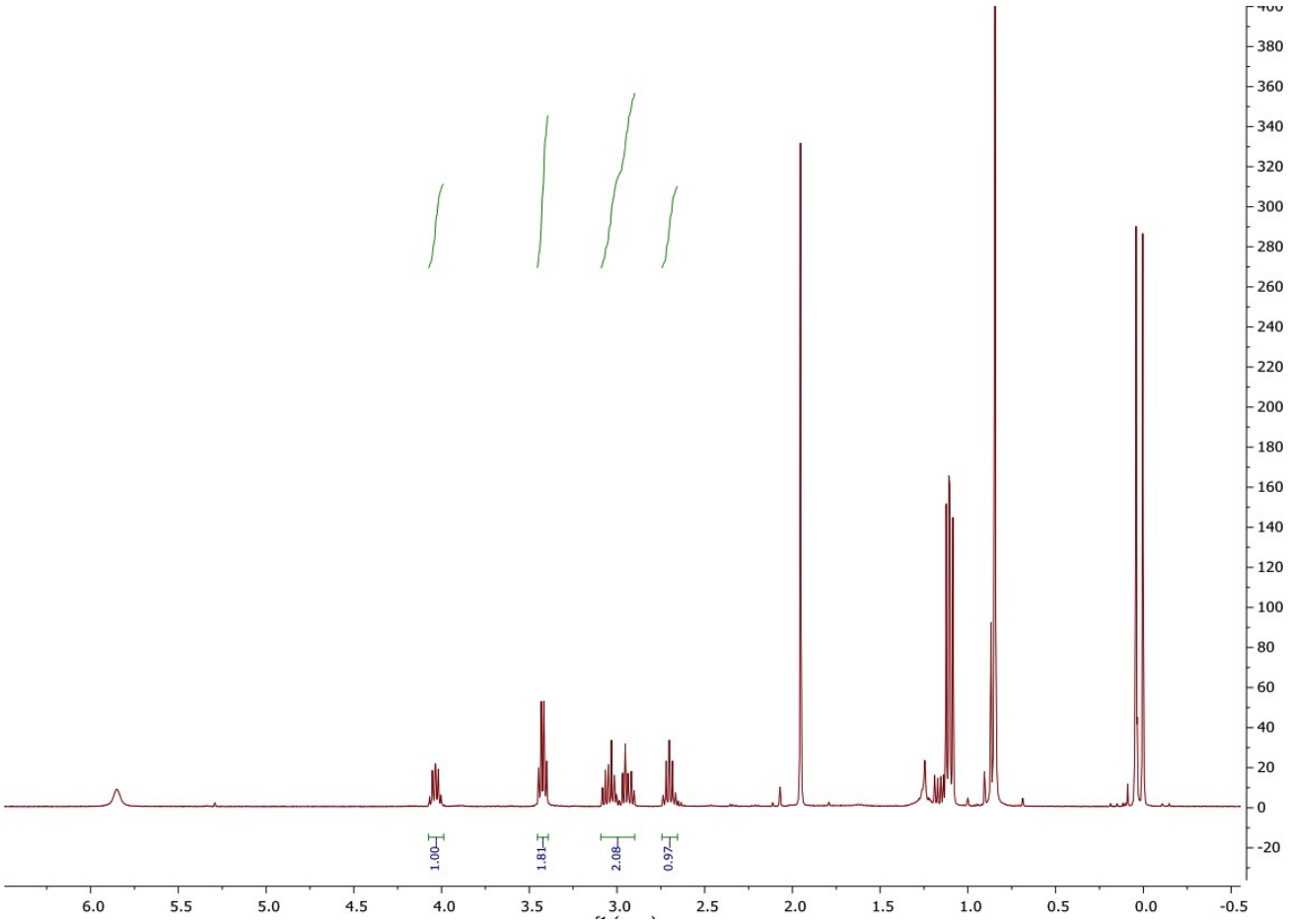


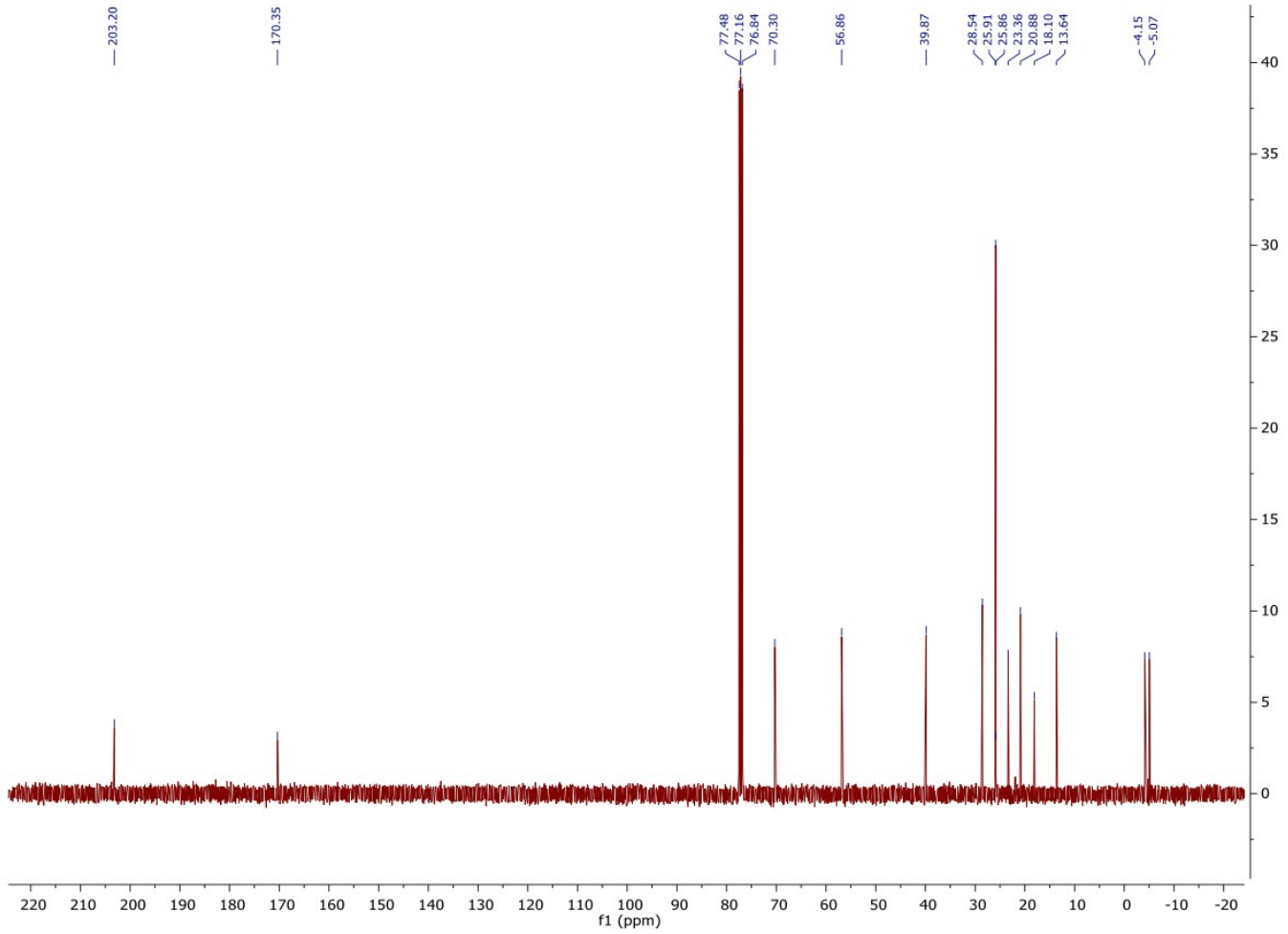
2*S*,3*S*-3-(*tert*-butyldimethylsilyloxy)-2-methylbutyric acid *N*-acetylcysteamine thiolester 2*S*,3*S*-7

Ester 2*S*,3*S*-**6**[4,5,6] (1.00 g, 4.06 mmol) was dissolved in THF (5 mL) and MeOH (8 mL), and lithium hydroxide (291 mg, 12.2 mmol) in water (1.5 mL) was added. The reaction mixture was heated to 60 °C overnight. The reaction was cooled and diluted with water (7 mL) and extracted with Et_2O (2 x 10 mL). The aqueous layer was acidified to pH 3.0 with H_2SO_4 (6 M) and further extracted with EtOAc (3 x 15 mL). The organic layers were combined, dried ($MgSO_4$), filtered and concentrated *in vacuo* to give a yellow oil. Purification by flash chromatography (SiO_2, 10% EtOAc/ petroleum ether, 0.5% acetic acid visualised with $KMnO_4$) afforded the acid (416 g, 1.79 mmol, 44%) as a pale yellow oil.

The acid (225 mg, 0.97 mmol) was dissovled in anhydrous CH_2Cl_2 (10 mL) at 0 °C under nitrogen. EDCI (245 mg, 1.28 mmol) and DMAP (1.0 mg, 0.08 mmol) were added. After stirring at 0 °C for 15 minutes, HSNAC[3] (152 mg, 1.28 mmol) was added in dry CH_2Cl_2 (10 mL). The mixture was allowed to warm to room temperature and stirred overnight. The reaction was quenched with water (5 mL) and the layers separated. The aqueous phase was extracted with CH_2Cl_2 (3 x 15mL). The organics were combined, dried ($MgSO_4$), filtered and concentrated *in vacuo*. Purification by flash chromatography (SiO_2, 3% MeOH/ CH_2Cl_2 visualised with $KMnO_4$) gave the title compound 2*S*,3*S*-**7** (169 mg, 0.51 mmol,

52%) as a pale yellow oil. $[\alpha]_D$ +56.9 *(c* 1.0, $CHCl_3$); δ_H (400 MHz, $CDCl_3$); 0.01 (3H, s, Si-CH_3), 0.05 (3H, s, Si-CH_3), 0.85 (9H, s, Si-C(CH_3)$_3$), 1.10 (3H, d, *J* = 7.0, 2-CH_3), 1.12 (3H, d, *J* = 6.1, 3-CH_3), 1.96 (3H, s, CO-CH_3), 2.71 (1H, m, 2-CH), 3.00 (2H, m, S-CH_2), 3.43 (2H, app. q, *J* = 6.2, N-CH_2), 4.03 (1H, m, 3-CH); δ_C (100 MHz, $CDCl_3$); -5.1 (Si-CH_3), -4.2 (Si-CH_3), 13.6 (2-CH_3), 18.1 (Si*C*(CH_3)$_3$), 20.9 (3-CH_3), 23.4 (CO-CH_3), 25.9 (SiC(*C*H_3)$_3$), 28.5 (S-CH_2), 39.9 (N-CH_2), 56.9 (2-CH), 70.3 (3-CH), 170.0 (N-CO), 203.2 (S-CO); ν_{max} (neat) / cm^{-1}: 3285 (NH), 2955 (CH), 2929 (CH), 2884 (CH), 2586 (CH), 1687 (CO), 1653 (CO); *m/z* (ESI) 356.17 $[M]Na^+$; Found (ESI) 356.1683 ($C_{15}H_{31}NaNO_3SSi$ requires 356.1692).





2*R*,3*R*-3-hydroxy-2-methylbutyric acid *N*-acetylcysteamine thiolester 2*R*,3*R*-8[7]

2*R*,3*R*-**7** (150 mg, 0.45 mmol) was dissolved in THF (1.3 mL), water (1.3 mL) and acetic acid (4 mL) and stirred at room temperature for 5 days. The reaction was diluted with water (25 mL) and the aqueous layer washed with CH_2Cl_2 (25 mL). The aqueous layer was concentrated to give the title compound **2*R*,3*R*-8** (78.0 mg, 0.36 mmol, 79%) as a colourless oil. $[\alpha]_D$ -32.8 *(c* 0.33, $CHCl_3$); δ_H (400 MHz, $CDCl_3$); 1.19, (3H, d, *J* = 7.1, 2-CH_2), 1.23 (3H, d, *J* = 6.3, 3-CH_2), 1.96 (3H, s, CO-CH_3), 2.69 (1H, m, 2-CH), 3.05 (2H, m, S-CH_2), 3.45 (2H, m, N-CH_2), 3.94 (1H, m, 3-CH), 5.88 (1H, br s, NH); δ_C (100 MHz, $CDCl_3$); 14.9 (2-CH_2), 21.2 (3-CH_3), 23.3 (CO-CH_3), 28.7 (S-CH_2) 39.5 (N-CH_2), 56.1 (2-CH), 70.1 (3-CH), 170.7 (NC=O), 204.0 (SC=O); ν_{max} (neat) / cm^{-1}: 3287 (NH), 3134 (OH), 2973 (CH), 2932 (CH), 1654 (NC=O), 1548 (SC=O); 1438 (NH); *m/z* (ESI) 242.08 $[M]Na^+$; Found (ESI) 242.0822 ($C_9H_{17}NaNO_3S$ requires 242.0827).

2*S*,3*S*-3-hydroxy-2-methylbutyric acid *N*-acetylcysteamine thiolester 2*S*,3*S*-8[7]

2*S*,3*S*-**7** (100 mg, 0.30 mmol) was dissolved in THF (0.9 mL), water (0.9 mL) and acetic acid (2.6 mL) and stirred at room temperature for 5 days. The reaction was diluted with water (25 mL) and the aqueous layer washed with CH_2Cl_2 (25 mL). The aqueous layer was concentrated to give the title compound **2*S*,3*S*-8** (37 mg, 0.16 mmol, 56%) as a colourless oil. $[\alpha]_D$ +36.8 *(c* 0.64, $CHCl_3$); δ_H (100 MHz, $CDCl_3$); 1.17 (3H, d, *J* = 7.1, 2-CH_3), 1.23 (3H, d, *J* = 6.3, 3-CH_3), 1.95 (3H, s, CO-CH_3), 2.68 (1H, m, 2-CH), 3.02 (2H, m, S-CH_2), 3.43 (2H, m, N-CH_2), 3.94 (1H, m, 3-CH), 6.15 (1H, br s, NH); δ_C (100 MHz, $CDCl_3$); 15.0 (2-CH_3), 21.2 (3-CH_3), 23.3 (NCO-CH_3), 28.7 (S-CH_2), 39.5 (N-CH_2), 56.1 (2-CH), 70.1 (3-CH), 170.8 (NC=O), 204.1 (SC=O); ν_{max}(neat) / cm^{-1}:3287 (NH), 3134 (OH), 2973 (CH), 2931 (CH), 1653 (NC=O), 1549 (SC=O), 1449 (NH); *m/z* (ESI) 242.08 $[M]Na^+$; Found (ESI) 242.0819 ($C_9H_{17}NaNO_3S$ requires 242.0827).

4'*R*-*N*-[(2*R*,3*S*)-3-Hydroxy-2-methylbutyryl]-4'-benzyloxazolidin-2-one 2*R*,3*S*,4'*R*-10[8,9]

4*R*-4-Benzyl-3-propionyloxazolidin-2-one[10] 4*R*-**9** (860 mg, 3.69 mmol) was dissolved in anhydrous CH_2Cl_2 (10 mL) and cooled to -78 °C under N_2. Dibutyl boron triflate (1 M in CH_2Cl_2, 7.4 mL, 7.58 mmol) was added dropwise over 1 hour. After 30 minutes triethylamine (1.28 mL, 9.23 mmol) was then added dropwise and the solution stirred for 1 hour at -78 °C, then 100 minutes at 0 °C. The solution was cooled to -78 °C and a solution of acetaldehyde (2.4 mL, 42.9 mmol) in dry CH_2Cl_2 (4 mL) also at -78 °C was added slowly. After stirring for 2 hours at -78 °C and then 100 minutes at 0 °C, 1 M phosphate buffer (pH 7.0, 28 mL) and MeOH (28 mL) was added, the resulting mixture was stirred vigorously in an ice bath as 30% H_2O_2 (14 mL) was added dropwise. After 30 minutes at 10 - 15 °C, the solvent was removed *in vacuo* and the resulting residue was partitioned between saturated $NaHCO_3$ aq. (20 mL) and CH_2Cl_2 (20 mL). The aqueous layer was extracted with CH_2Cl_2 (2 x 8 mL) and the combined organic layers were dried ($MgSO_4$), filtered, and concentrated *in vacuo* to give a crude pale yellow oil. The crude material was purified by flash chromatography (SiO_2, 20% EtOAc in petroleum ether, visualized with $KMnO_4$) to give the aldol adduct as a colourless oil (693 mg, 2.50 mmol, 68% yield). $[\alpha]_D$ -47.2 *(c* 1.0, $CHCl_3$), [lit. $[\alpha]_D$ -54.8 *(c* 0.96, $CHCl_3$)].[9] δ_H (400 MHz, $CDCl_3$): 1.21 (3H, d, *J* = 6.4 , 2-CH_3), 1.27 (3H, d, *J* = 7.1 , 3-CH_3), 2.79 (1H, dd, *J* = 13.4, 9.4 , 6'-CH_2), 3.25 (1H, dd, *J* = 13.4, 3.4 , 6'-CH_2), 3.74 (1H, qd, *J* = 7.1, 3.0, 2- CH), 4.21 (3H, m, 5'-CH & 3-CH), 4.69 (1H, m, 4'-CH), 7.20-7.35 (5H, m, Ar-H); ν_{max} (neat) / cm^{-1}: 3509 (OH), 2977 (CH), 2926 (CH), 1772 (C=O), 1690 (NC=O), 1349 (C-N), 1208 (COC).

4'*S*-*N*-[(2*S*,3*R*)-3-Hydroxy-2-methylbutyryl]-4'-benzyloxazolidin-2-one 2*S*,3*R*,4'*S*-10[16,11]

4*S*-4-Benzyl-3-propionyloxazolidin-2-one[10] 4*S*-**9** (850 mg, 3.64 mmol) was dissolved in anhydrous CH_2Cl_2 (10 mL) and cooled to -78 °C under N_2. Dibutyl boron triflate (1 M in CH_2Cl_2, 7.30 mL, 7.30 mmol) was added dropwise over 1 hour. After 10 minutes triethylamine (1.27 mL, 9.11 mmol) was added dropwise and the solution stirred for 1 hour at -78 °C, then 100 minutes at 0 °C. The solution was cooled to -78 °C and a solution of acetaldehyde (10 eq, 2.05 mL, 36.5 mmol) in anhydrous CH_2Cl_2 (1.0 mL) also at -78 °C was added slowly. After stirring for 2 hours at -78 °C and then 100 minutes at 0 °C, phosphate buffer (pH 7.1, 28 mL) and MeOH (28 mL) was added, the resulting mixture was stirred vigorously in an ice bath as 30% H_2O_2 (14 mL) was added dropwise. After 30 minutes at 10 - 15 °C, the solvent was removed *in vacuo* and the resulting residue was partitioned between sat. aq. $NaHCO_3$ and CH_2Cl_2 (8 mL). The aqueous layer was extracted with CH_2Cl_2 (2 x 8 mL) and the combined organic layers were washed with sat. aq. $NaHCO_3$ (8 mL), dried ($MgSO_4$), filtered, and concentrated *in vacu*o to give a pale yellow oil. The crude material was purified by flash chromatography (SiO_2, 20% to 40% EtOAc /petroleum ether, visualized with $KMnO_4$) to give the the aldol adduct as a colourless oil (602 mg, 2.17 mmol, 59%). $[\alpha]_D$ +48.8 *(c* 1.0, $CHCl_3$), [lit. $[\alpha]_D$ +51.1 *(c* 1.0, $CHCl_3$)];[11] δ_H (400 MHz, $CDCl_3$); 1.19 (3H, d, *J* = 6.4 , 2-CH_3), 1.27 (3H, d, *J* = 7.0 , 3-CH_3), 2.79 (1H, dd, *J* = 13.4, 9.4, CH_2Ph), 2.89 (1H, br s, OH), 3.26 (1H, dd, *J* = 13.4, 3.4, CH_2Ph), 3.75 (1H, dq, *J* = 7.1, 2.9 , 2-CH), 4.21 (3H, m, 3'-CH_2 & 3-CH), 4.71 (1H, m, 4'-CH), 7.32, (5H, m, Ar-H); δ_C (100 MHz, $CDCl_3$) 10.6 (3-CH_3), 19.8 (2-CH_3), 38.0 (5'-CH_2), 43.3 (2-CH), 55.2 (4'-CH), 66.3 (3'-CH), 67.8 (3-CH), 127.3 (Ar-C), 129.1 (Ar- C), 129.6 (Ar-C), 135.1 (Ar-C), 153.3 (1'-CO), 177.5 (1-CO); ν_{max} (neat) / cm^{-1}: 3439 (OH), 2987 (CH), 1775 (CO), 1688 (CO), 889 (Ar-CH).

4'*R*-*N*-[(2*R*,3*S*)-3-(*tert*-butyldimethylsilyloxy)-2-methylbutyryl]-4-benzyloxazolidin-2-one 2*R*,3*S*,4'*R*-11[16]

Aldol adduct 2*R*,3*S*,4'*R*-**10** (693 mg, 2.50 mmol) was dissolved in anhydrous CH_2Cl_2 (20 mL) under N_2. TBDMSCl (753 mg, 5.00 mmol), imidazole (678 mg, 9.96 mmol) and DMAP (3 mg, 0.02 mmol) were added and the reaction mixture stirred overnight. The reaction was quenched with sat. aq. $NaHCO_3$ (10 mL) and extracted with CH_2Cl_2 (3 x 25 mL). The organic extracts were combined, dried ($MgSO_4$), filtered and concentrated *in vacuo*. Purification by flash chromatography (SiO_2, 10% EtOAc in petroleum ether, visualised with $KMnO_4$) gave the product (729 mg, 1.86 mmol, 75%) as a colourless crystalline solid. $[\alpha]_D$

-43.1 *(c* 1.0, $CHCl_3$), Enantiomer [lit. $\alpha]_D$ +49.2 *(c* 1.0, $CHCl_3$)];[16] δ_H (400 MHz, $CDCl_3$); 0.02 (3H, s, Si-CH_3), 0.05 (3H, s, Si-CH_3), 0.88 (9H, s, SiC-CH_3), 1.19 (3H, d, *J* = 6.1, 2-CH_3), 1.22 (3H, d, J = 6.8 , 3-CH_3), 2.77 (1H, dd, *J* = 13.4, 9.6 , 6'-CH_2), 3.29 (1H, dd, *J* = 13.4, 3.24 , 6'-CH_2), 3.83 (1H, dq, *J* = 6.7, 5.9 , 2-CH), 4.09 (1H, m, 3-CH), 4.17 (2H, m, 5'-CH_2), 4.62 (1H, m, 4'-CH), 7.20-7.36 (5H, m, Ar-H); δ_C (100 MHz, $CDCl_3$): -4.9 ($SiCH_3$), -4.4 ($SiCH_3$), 12.6 (2-CH_3), 18.1 (Si*C*(CH_3)$_3$), 21.7 (3-CH_3), 25.8 (SiC(CH_3)$_3$), 37.8 (C-6'), 45.0 (C-2), 55.7 (C-4'), 66.0 (C-5'), 69.7 (3-CH), 127.4 (Ar-C), 129.0 (Ar-C), 129.5 (Ar-C), 135.4 (O-CO), 153.0 (C-2'), 175.4 (1-CO).

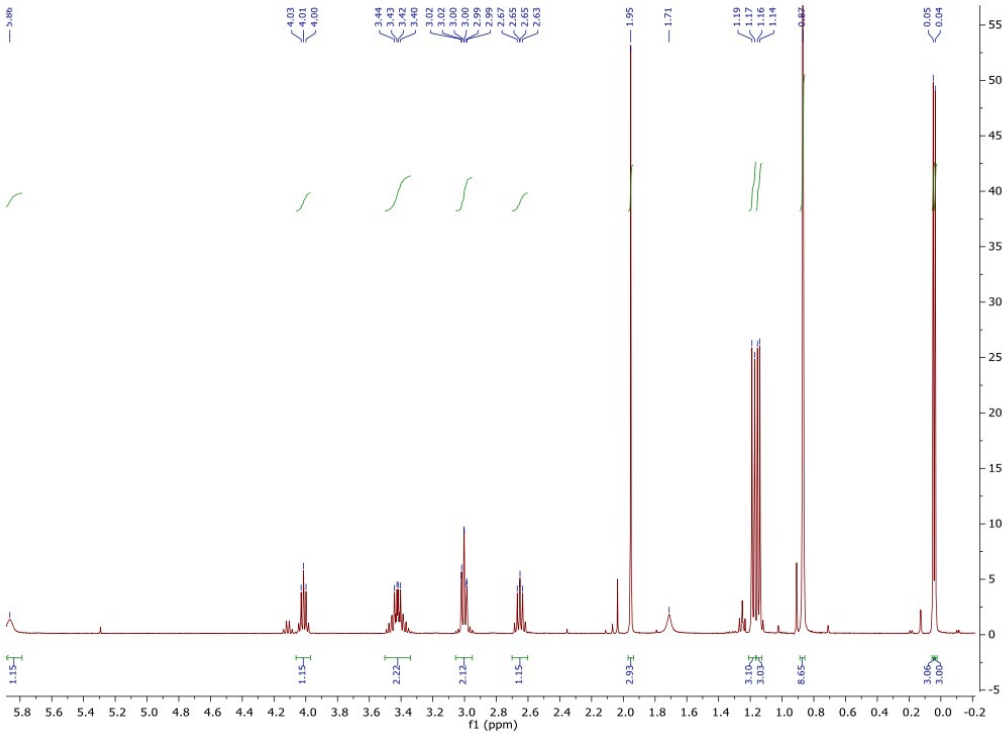
4'*S*-*N*-[2*S*,3*R*-3-(*tert*-butyldimethylsilyloxy)-2-methylbutyryl]-4'-benzyloxazolidin-2-one 2*S*,3*R*,4'*S*-11[16,12]

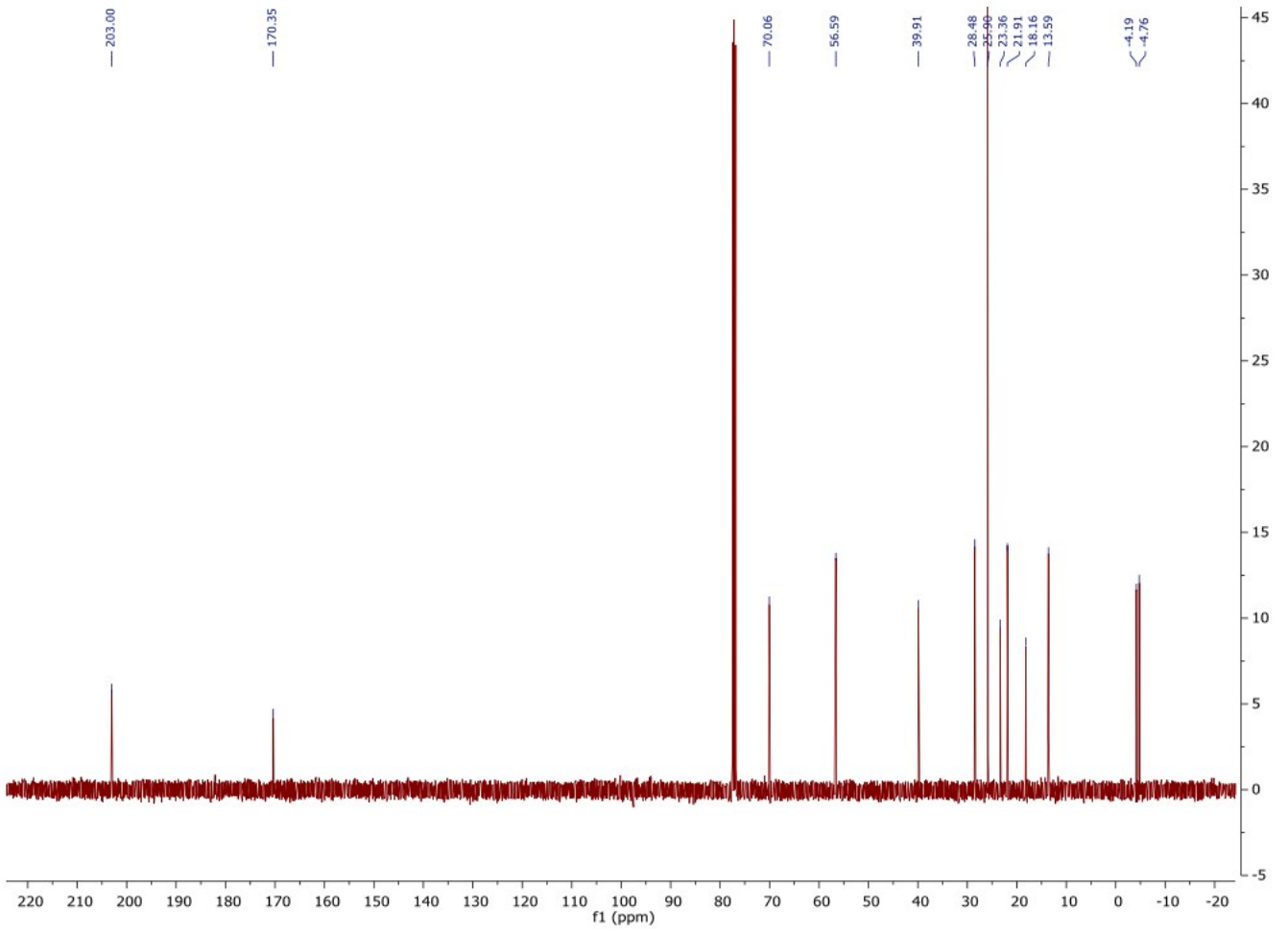
Aldol adduct 2*S*,3*R*,4'*S*-**10** (1.00 g, 3.61 mmol) was dissolved in anhydrous CH_2Cl_2 (30 mL) under N_2. TBDMSCl (1.10 g, 7.30 mmol), imidazole (0.98 g, 14.4 mmol) and DMAP (3 mg, 0.02 mmol) were added and stirred overnight. The reaction was quenched with sat. aq. $NaHCO_3$ (34 mL), extracted with CH_2Cl_2. The organic extracts were dried ($MgSO_4$), filtered and concentrated. Purification by flash chromatography (SiO_2, 10% EtOAc in petroleum ether visualised with $KMnO_4$) gave the product (532 mg, 1.36 mmol, 38%) as a colourless crystalline solid. $[\alpha]_D$ +51.8 *(c* 1.0, $CHCl_3$), [lit. $[\alpha]_D$ +49.2 *(c* 1.0, $CHCl_3$)];[16] δ_H (400 MHz, $CDCl_3$): 0.02 (3H, s, $SiCH_3$), 0.05 (3H, s, $SiCH_3$), 0.87 (9H, s, SiC(CH_3)$_3$), 1.18 (3H, d, *J* = 6.1, 3-CH_3), 1.22 (3H, d, *J* = 6.8, 2-CH_3), 2.77 (1H, dd, *J* = 13.3, 9.7, CH_2Ph), 3.29 (1H, dd, *J* = 13.3, 3.3, CH_2Ph), 3.83 (1H, qd, *J* = 6.8, 5.7, 2-H), 4.08 (1H, m, N-CH), 4.16 (2H, m, 5'-H_2), 4.63 (1H, m, 4'-H), 7.21-7.36 (5H, m, Ar-H); ν_{max} (neat)/cm^{-1}: 2955 (CH), 2929 (CH), 2885 (CH), 2856 (CH), 1778 (CO), 1694 (CO), 809 (Ar C).

2*S*,3*R*-3-(*tert*-butyldimethylsilyloxy)-2-methylbutanoyl-*N*-acetylcysteamine thiolester 2*S*,3*R*-12

A solution of 2*S*,3*R*,4'*S*-**11** (377 mg, 0.96 mmol) dissolved in THF/H_2O (20 mL/6 mL) was cooled to 0 °C. Hydrogen peroxide (6.85 mmol, 0.70 mL 30% *w/v* H_2O_2) and LiOH (61 mg 2.55 mmol) were added. The reaction was warmed to room temperature. After 4 hours the excess peroxide was quenched using aq. $Na_2S_2O_3$ (1.5 M) and the THF removed *in vacuo*. The resulting slurry was acidified to pH 2.0 using 2M HCl and extracted with CH_2Cl_2 (3 x 20 mL). The organic extracts were combined, washed with brine (10 mL), dried, filtered and concentrated *in vacuo*. Purification of the crude material by flash chromatography (SiO_2, 10 EtOAc/ petroleum ether/ 0.1% Acetic acid, visualised with $KMnO_4$) afforded the acid (181 mg, 0.78 mmol, 81%) as a colourless oil. $[\alpha]_D$ +20.6 *(c* 1.0, $CHCl_3$), [lit. $[\alpha]_D$ +25 *(c* 0.51, $CHCl_3$)].[13]

To a solution of the acid (100 mg, 0.43 mmol) in anhydrous CH_2Cl_2 (6 mL) at 0 °C under nitrogen was added EDCI (93.0 mg, 0.60 mmol) and DMAP (5 mg, 0.04 mmol). After stirring at 0 °C for 15 minutes, HSNAC[3] (71 mg, 0.60 mmol) was added in anhydrous CH_2Cl_2 (2.5 mL). The mixture was allowed to warm to room temperature and stirred overnight. The reaction was quenched with water (7 mL) and the layers separated. The aqueous phase was extracted with CH_2Cl_2 (3 x 15 mL). The organics were combined, dried ($MgSO_4$), filtered and concentrated *in vacuo*. Purification by flash chromatography (SiO_2, 3% MeOH/ CH_2Cl_2 visualised with $KMnO_4$) gives the title compound (67 mg, 0.20 mmol, 46%) as a colourless oil. $[\alpha]_D$ +15.6 (*c* 1.0, $CHCl_3$); δ_H (400 MHz, $CDCl_3$); 0.04 (3H, s, Si-CH_3), 0.05 (3H, s, Si-CH_3), 0.87 (9H, s, SiC(CH_3), 1.15 (3H, d, *J* = 6.1 , 2-CH_3), 1.18 (3H, d, *J* = 6.9 , 3-CH_3), 1.95 (3H, s, CO-CH_3), 2.65 (1H, m, 2-CH), 3.0 (2H, m, S-CH_2), 3.42 (2H, m, N-CH_2), 4.01 (1H, m, 3-CH), 5.86 (1H, br s, NH); δ_C (100 MHz, $CDCl_3$); -4.9 (Si-CH_3), -4.3 (Si-CH_3), 13.4 (2-CH_3), 18.0 (Si*C*(CH_3)$_3$), 21.8 (3-CH_3), 23.2 (NHCOCH_3), 25.7 (SiC(*C*H_3)$_3$), 28.3 (S-CH_2), 39.8 (NH-CH_2), 56.4 (2-CH), 69.9 (3-CH), 170.2 (NHCO), 202.8 (SCO); ν_{max} (neat) / cm^{-1}: 3279 (NH), 2955 (CH), 2929 (CH), 2886 (CH), 2856 (CH), 1682 (CO), 1654 (CO), 1550 (NH), 1374 (CH), 1360 (CH); *m/z* (ESI) 356.17 $[M]Na^+$; Found (ESI) 356.1671 ($C_{15}H_{31}NNaO_3SSi$ requires 356.1692).



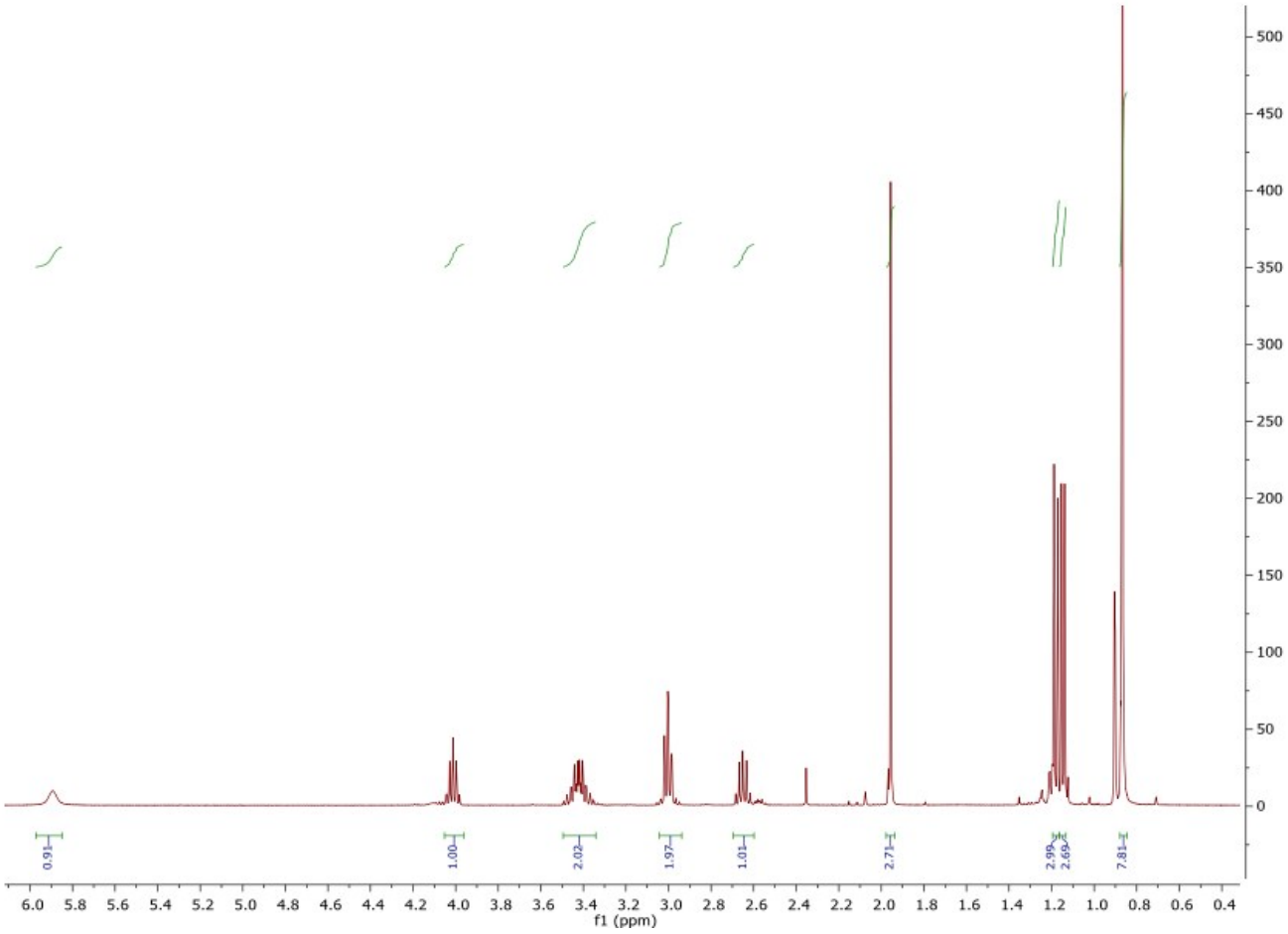


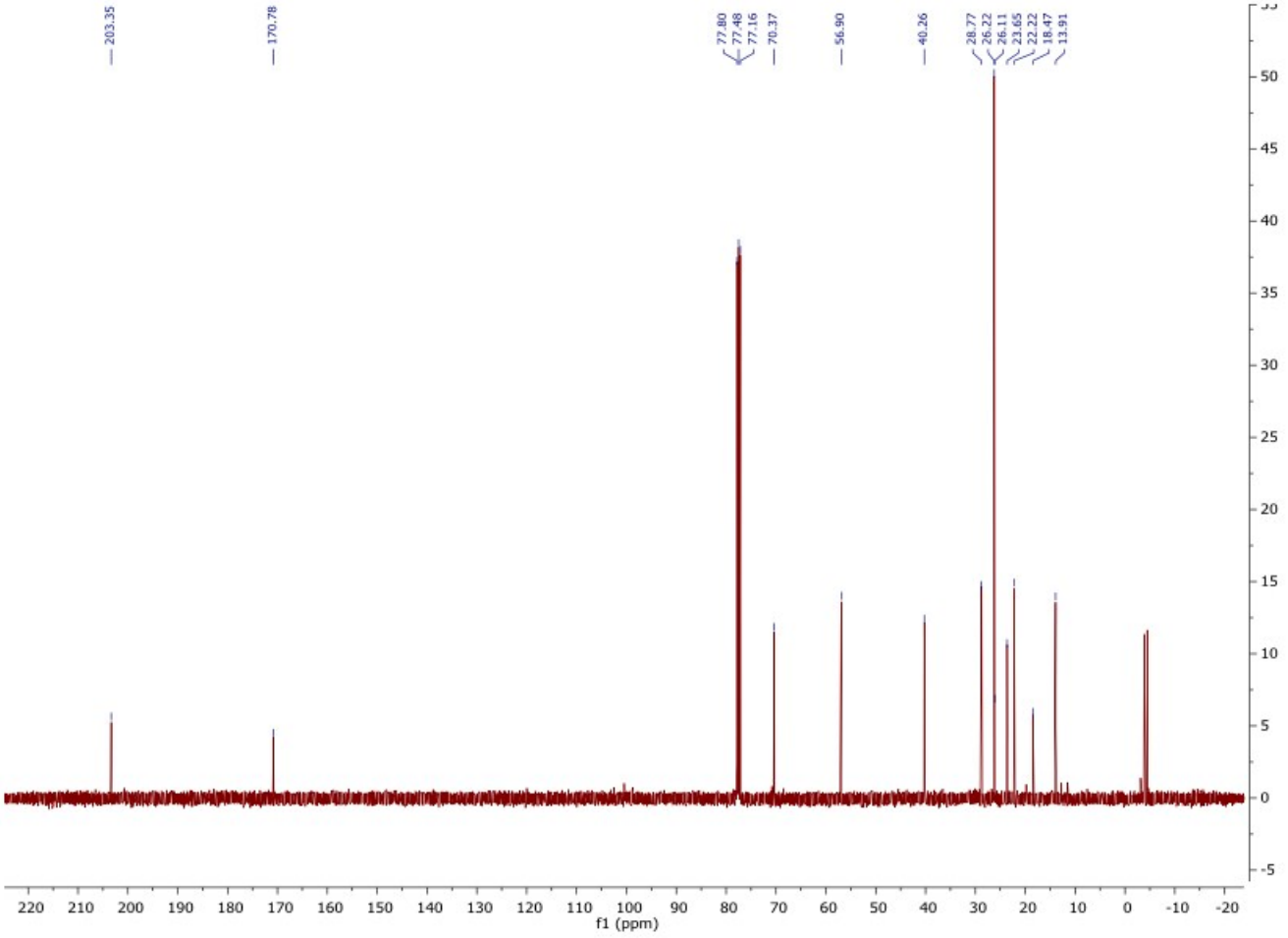
2*R*,3*S*-3-(*tert*-butyldimethylsilyloxy)-2-methylbutanoyl-*N*-acetylcysteamine thiolester 2*R*,3*S*-12

A solution of 2*R*,3*S*,4'*R*-**11** (718 mg, 1.83 mmol) dissolved in THF/H_2O (60 mL / 9 mL) was cooled to 0 °C. Hydrogen peroxide (10.2 mmol, 1.04 mL, 30% *w/v* H_2O_2) and LiOH (153 mg, 6.39 mmol) was added. The reaction was warmed to room temperature. After 4 hours the excess peroxides were quenched using aq. $Na_2S_2O_3$ (1.5 M) and the THF removed *in vacuo*. The resulting slurry was acidified to pH 2.0 using 2M HCl and extracted with CH_2Cl_2 (3 x 30 mL). The organic layers were combined, washed with brine (30 mL), dried ($MgSO_4$), filtered and concentrated *in vacuo*. Purification of the crude material by flash chromatography (SiO_2, 10% EtOAc / petroleum ether / 0.1% Acetic acid, visualised with $KMnO_4$) afforded the acid (230 mg, 0.99 mmol, 54%) as a colourless oil. $[\alpha]_D$ -35.3 (*c* 1.0, $CHCl_3$), [lit. $[\alpha]_D$ -22.3 (*c* 1.07, $CHCl_3$)].[14]

The acid (185 mg, 0.80 mmol) was dissolved in anhydrous CH_2Cl_2 (12 mL) at 0 °C under nitrogen. EDCI (1.0 eq, 173 mg, 1.11 mmol), and DMAP (1.0 eq, 48 mg, 0.39 mmol) were added. After stirring at 0

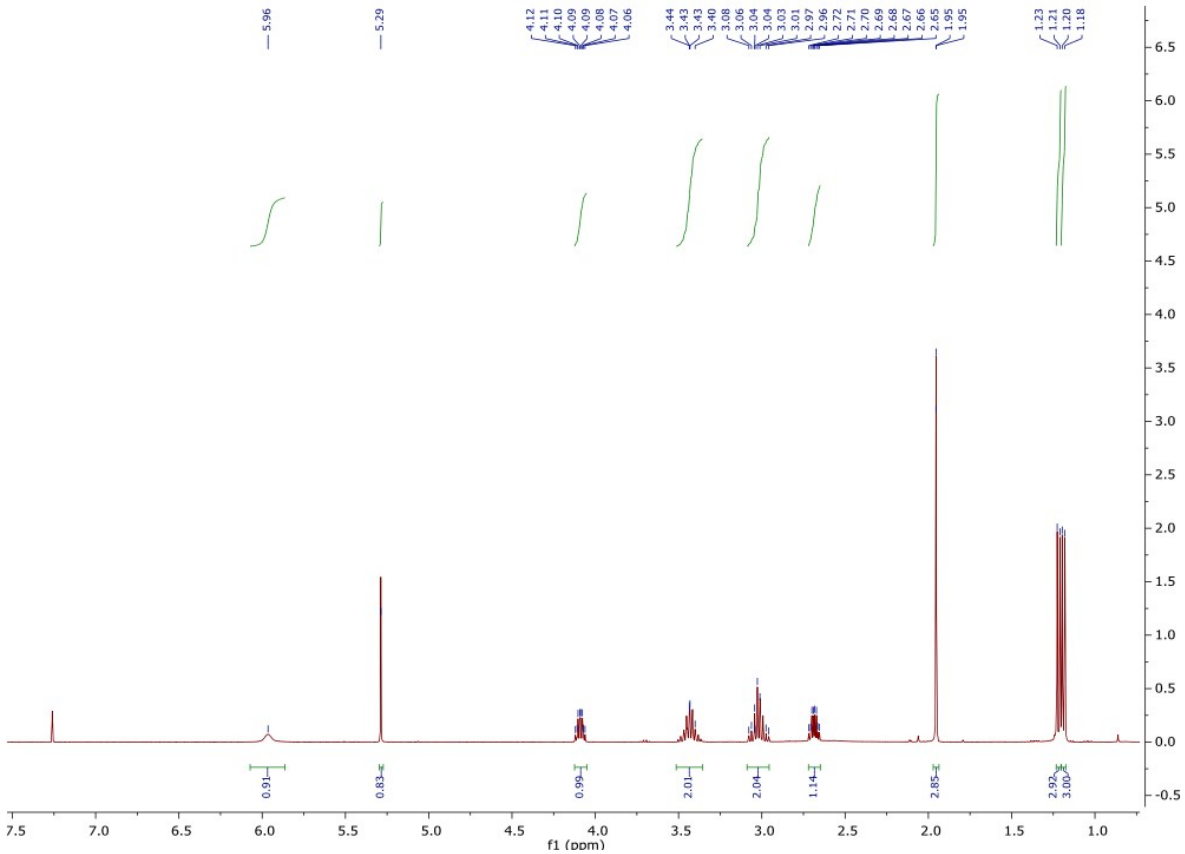
°C for 15 minutes, HSNAC[3] (133 mg, 1.12 mmol) in anhydrous CH_2Cl_2 (10 mL) was added. The mixture was allowed to warm to room temperature and stirred overnight. The reaction was quenched with water (15 mL) and the layers separated. The aqueous phase was extracted with CH_2Cl_2 (3 x 25 mL). The organics were combined, dried ($MgSO_4$), filtered and concentrated *in vacuo*. Purification by flash chromatography gave the title compound (193 mg, 0.58 mmol, 72%). $[\alpha]_D$ -11.0 *(c* 1.0, $CHCl_3$); δ_H (400 MHz, $CDCl_3$); 0.04 (3H, s, $SiCH_3$), 0.06 (3H, s, $SiCH_3$), 0.88 (9H, s, $SiC\text{-}(CH_3)_3$), 1.15 (3H, d, *J* = 6.2 , 2-CH_3), 1.19 (3H, d, *J* = 6.8 , 3-CH_3), 2.65 (1H, m, 2-CH), 3.01 (2H, m, S-CH_2), 3.43 (2H, m, N-CH_2), 4.02 (1H, m, 3-CH), 5.80 (1H, brs, NH); δ_C (100 MHz, $CDCl_3$); -5.1 (Si-CH_3), -4.2 (Si-CH_3), 13.6 (2-CH_3), 18.1 ($SiC(CH_3)_3$), 20.9 (3-CH_3), 23.4 (CO-CH_3), 25.9 ($SiC(CH_3)_3$), 28.5 (S-CH_2), 39.8 (N-CH_2), 56.9 (2-CH), 70.3 (3-CH), 170.4 (NC=O), 203.2 (SC=O); ν_{max} (neat) / cm^{-1}: 3286 (NH), 2955 (CH), 2930 (CH), 2857 (CH), 1683 (C=O), 1656 (C=O), 1551 (NH), 836 ($SiCH_3$); *m/z* (ESI) 356.17 $[M]Na^+$; Found (ESI) 356.1689 ($C_{15}H_{31}NNaO_3SSi$ requires 356.1692).





2*S*,3*R*-3-Hydroxy-2-methylbutanoyl-*N*-acetylcysteamine thiolester 2*S*,3*R*-13[12,14]

2*S*,3*R*-**12** (119 mg, 0.36 mmol) was dissolved in THF (0.9 mL), water (0.9 mL) and acetic acid (2.6 mL) and stirred at room temperature for 5 days. The reaction was diluted with water (25 mL) and the aqueous layer washed with CH_2Cl_2 (25 mL). The aqueous layer was concentrated to give the title compound (37 mg, 0.17 mmol, 48%) as a colourless oil. $[\alpha]_D$ +3.0 *(c* 1.0, $CHCl_3$); δ_H (400 MHz, $CDCl_3$): 1.19 (3H, d, *J* = 6.3 , 3-CH_3), 1.22 (3H, d, *J* = 7.1 , 2-CH_3), 1.95 (3H, s, CO-CH_3), 2.69 (1H, qd, *J* = 7.1, 4.0 , 2-CH), 3.04 (2H, m, S-CH_2), 3.43 (2H, m, NH-CH_2), 4.09 (1H, qd, *J* = 6.3, 4.0, 3-CH), 5.96 (1H, br s, NH); δ_C (100 MHz, $CDCl_3$): 11.7 (2-CH_3), 20.3 (3-CH_3), 23.3 (CO-CH_3), 28.7 (S-CH_2), 39.5 (N-CH_2), 54.7 (2-CH), 68.5 (3-CH), 170.7 (NC=O), 204.1 (SC=O); ν_{max} (neat) / cm^{-1}: 3288 (NH), 3135 (OH), 2930 (CH), 1655 (NCO), 1548 (SCO); *m/z* (ESI) 242.08 $[M]Na^+$; Found (ESI) 242.0816 ($C_9H_{17}NaNO_3S$ requires 242.0827).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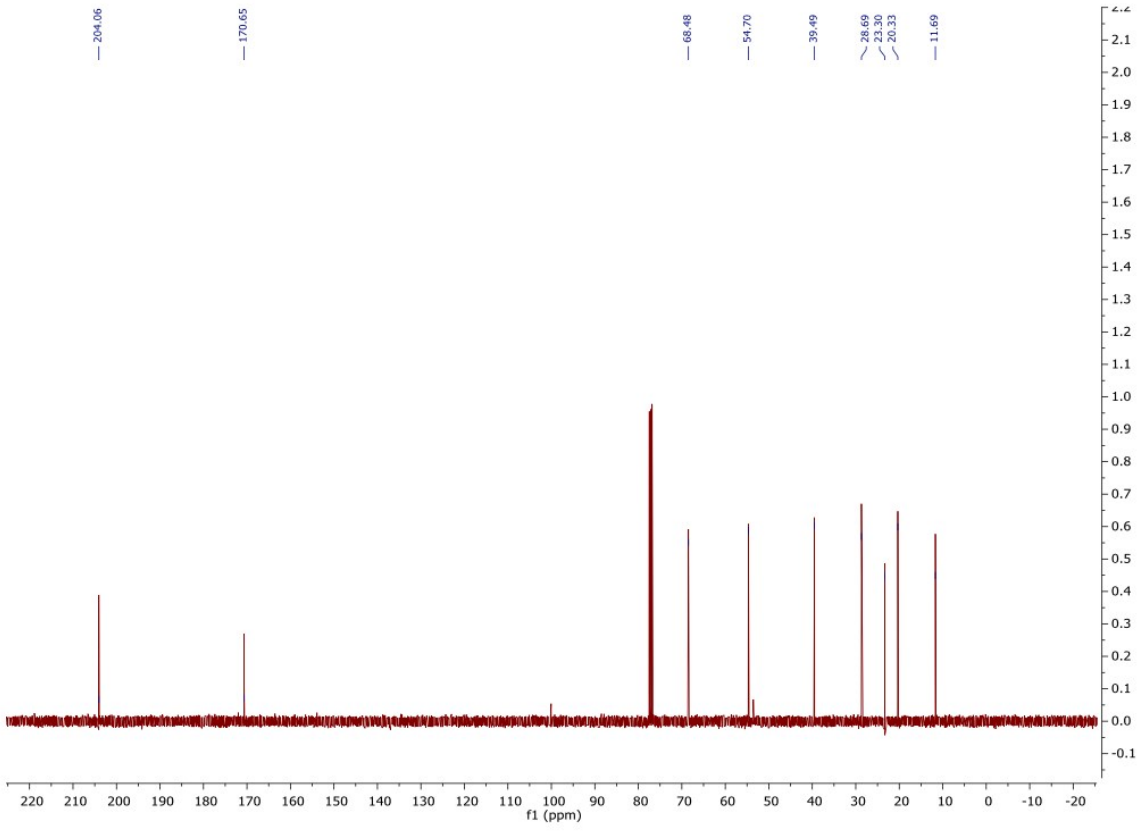
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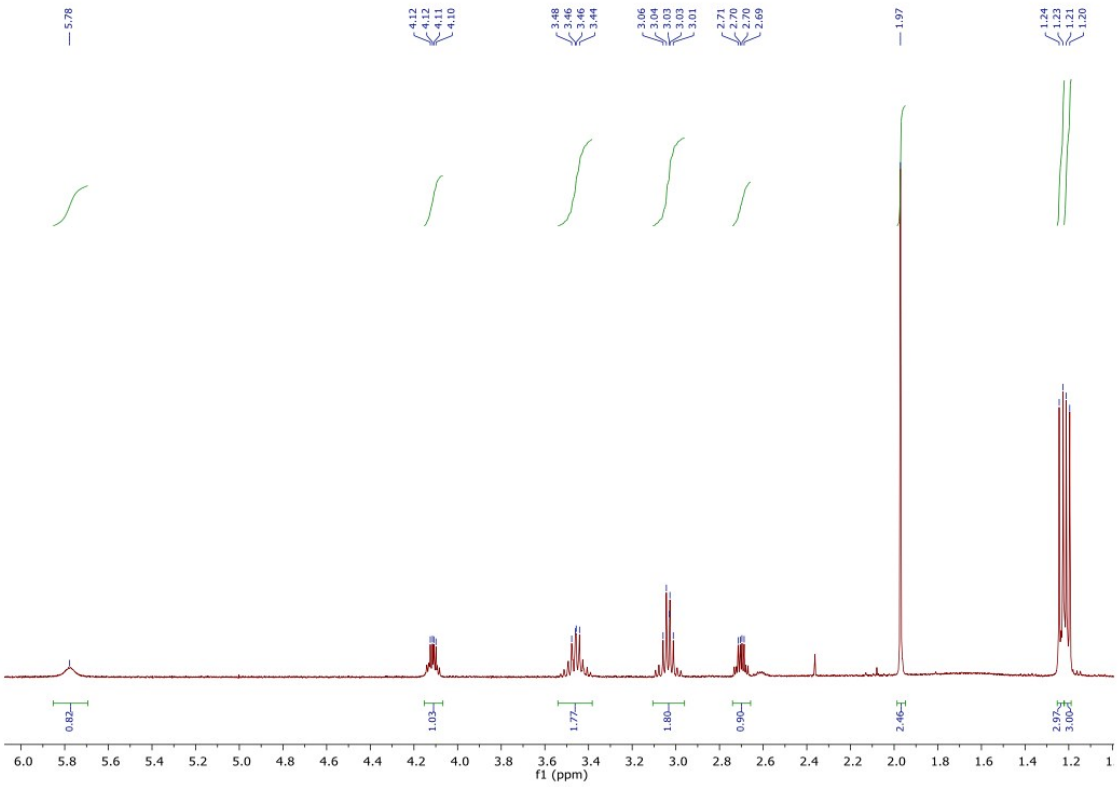
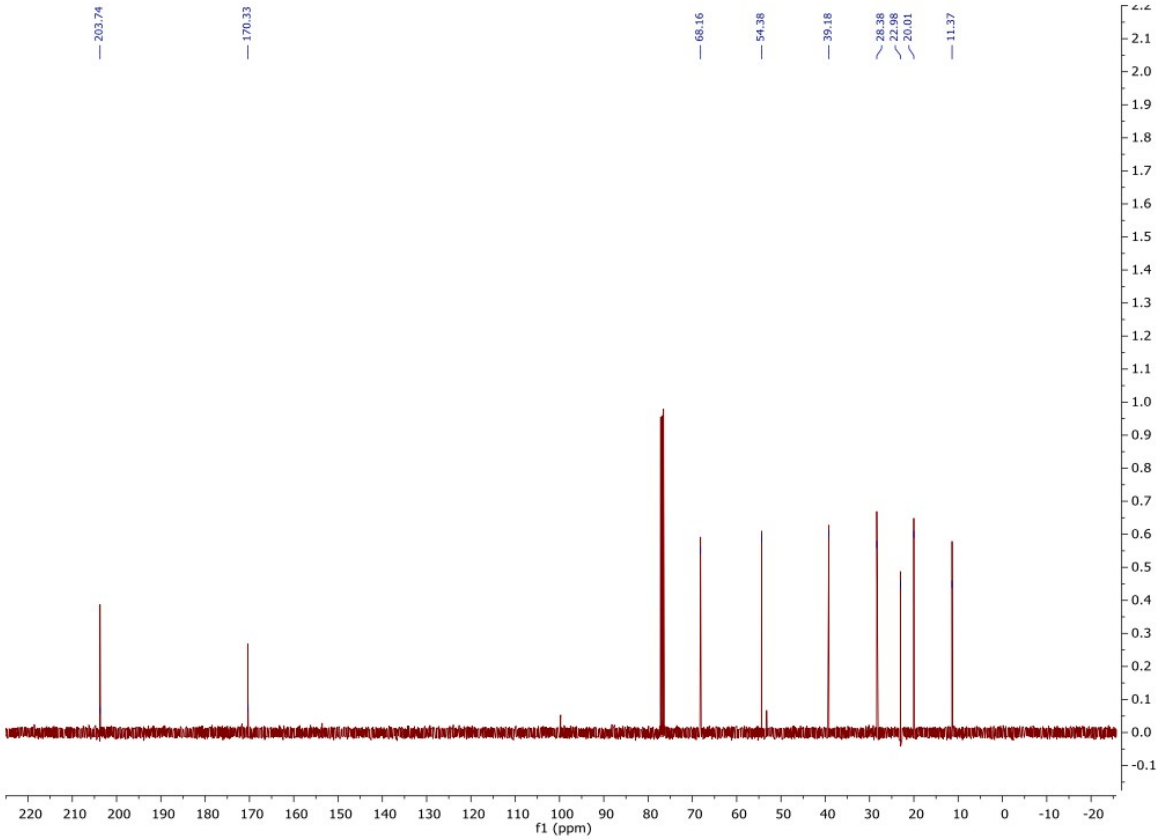
f1 (ppm)

2*R*,3*S*-3-Hydroxy-2-methylbutanoyl-*N*-acetylcysteamine thiolester 2*R*,3*S*-13

2*R*,3*S*-**12** (180 mg, 0.54 mmol) was dissolved in THF (1.5 mL), water (1.5 mL) and acetic acid (5 mL) and stirred at room temperature for 3 days. The reaction was diluted with water (15 mL) and the aqueous layer washed with CH_2Cl_2 (15 mL). The aqueous layer was concentrated to give the title compound (62.0 mg, 0.28 mmol, 52%) as a colourless oil. $[\alpha]_D$ -4.9 *(c* 1.0, $CHCl_3$); δ_H (400 MHz, $CDCl_3$); 1.20 (3H, d, *J* = 6.4 , 3-CH_3), 1.23 (3H, d, *J* = 7.1 , 2-CH_3), 1.97 (3H, s, CO-CH_3), 2.70 (1H, qd, *J* = 7.1, 4.0, 2-CH), 3.04 (2H, m, S-CH_2), 3.46 (2H, m, N-CH_2), 4.11 (1H, qd, *J* = 6.4, 4.0, 3-CH), 5.78 (1H, br s, NH); δ_C (100 MHz, $CDCl_3$); 11.4 (2-CH_3), 20.1 (3-CH_3), 23.0 (CO-CH_3), 28.4 (S-CH_2), 39.2 (N-CH_2), 54.4 (2-CH), 68.2 (3-CH), 170.3 (N-CO), 203.7 (S-CO); ν_{max} (neat) / cm^{-1}: 3299 (NH), 3134 (OH), 2974 (CH), 2934 (CH), 1653 (C=O), 1549 (C=O), 1451 (NH); *m/z* (ESI) 242.08 $[M]Na^+$; Found (ESI) 242.0813 ($C_9H_{17}NaNO_3S$ requires 242.0827).





Ethyl 3*R*-3-(tert-butyldimethylsilyloxy)butyrate 3*R*-14[15,16]

Pyridine (1.20 mL, 14.8 mmol) was added to ethyl (3*R*)-3-hydroxybutyrate **4** (1.00 g, 7.57 mmol) dissolved in CH_2Cl_2 (20 mL) and cooled to 0 °C. *tert*-Butyldimethylsilyl triflate (2.4 mL, 10.5 mmol) was added dropwise. The mixture allowed to warm to room temperature and stirred overnight, then quenched with sat. aq. $NaHCO_3$ (20 mL). The layers were separated and the aqueous layer extracted with CH_2Cl_2 (3 x 20 mL). The combined organic layers were dried ($MgSO_4$), filtered and concentrated *in vacuo*. Purification by flash chromatography (SiO_2, 5% EtOAc/ petroleum ether visualised with $KMnO_4$) gives the title compound (1.15 g, 4.67 mmol, 62%) as a colourless oil. $[\alpha]_D$ -23.0 *(c* 1.0, $CHCl_3$), [lit. $[\alpha]_D$ -27.5 *(c* 1.02, $CHCl_3$)];[17] δ_H (400 MHz, $CDCl_3$); 0.03 (3H, s, Si-CH_3), 0.05 (3H, s, Si-CH_3), 0.85 (9H, s, Si-$C(CH_3)_3$), 1.18 (3H, d, *J* = 6.1, 3-CH_3), 1.25 (3H, t, *J* = 7.1, CH_2CH_3), 2.35 (1H, dd, *J* = 14.3, 5.4, 2-CH_2), 2.46 (1H, dd, *J* = 14.3, 7.7, 2-CH_2), 3.44 (2H, m, O-CH_2), 4.27 (1H, m, 3-CH); ν_{max} (neat) / cm^{-1}: 2957 (CH), 2930 (CH), 2896 (CH), 2857 (CH), 1736 (CO).

Methyl 3*S*-3-(tert-butyldimethylsilyloxy)butyrate 3*S*-14[18,19,20]

Pyridine (4.10 mL, 50.7 mmol) was added to (3*S*)-methyl-3-hydroxybutyrate 3*S*-**4** (1.00 g, 8.47 mmol) dissolved in CH_2Cl_2 (20 mL) and cooled to 0 °C. *tert*-Butyldimethylsilyl triflate (2.4 mL, 10.5 mmol) was added dropwise. The mixture allowed to warm to room temperature and stirred for 1 hour and quenched with sat. aq. $NaHCO_3$ (20 mL). The layers were separated and the aqueous layer extracted with CH_2Cl_2 (3 x 20 mL). The combined organic layers were dried ($MgSO_4$), filtered and concentrated *in vacuo*. Purification by flash chromatography (SiO_2, 5% EtOAc/ petroleum ether visualised with $KMnO_4$) gives the title compound (1.38 g, 5.94 mmol, 70%) as a pale yellow oil. $[\alpha]_D$ +29.6 *(c* 1.0, $CHCl_3$), [lit. $[\alpha]_D$ +30.3 *(c* 1.0, $CHCl_3$)];[21] δ_H (400 MHz, $CDCl_3$); 0.02 (3H, s, Si-CH_3), 0.04 (3H, s, Si-CH_3), 0.84 (9H, s, Si-$C(CH_3)_3$), 1.17 (3H, d, *J* = 6.1, 3-CH_3), 2.35 (1H, dd, *J* = 14.5, 5.8, 2-CH_2), 2.45 (1H, dd, *J* = 14.5, 7.7, 2-CH_3), 3.64 (3H, s, O-CH_3), 4.26 (1H, m, 3-CH); ν_{max} (neat) / cm^{-1}: 2956 (CH), 2930 (CH), 2858 (CH). 1710 (C=O), 1001 (CO), 827 ($SiCH_3$).

3*R*-3-(*tert*-butyldimethylsilyloxy)butyric acid *N*-acetylcysteamine thiolester 3*R*-15[22]

3*R*-**14** (517 mg, 2.10 mmol) was dissolved in THF (2.5 mL), MeOH (4 mL) and lithium hydroxide (271 mg, 11.3 mmol) in water (0.75 mL) was added. The reaction mixture was heated to 60 °C overnight. The reaction was diluted with water (5 mL) and extracted with Et_2O (2 x 5 mL). The aqueous layer was acidified to pH 3.0 with H_2SO_4 (6 M) and further extracted with EtOAc (3 x 7 mL). The organic layers were combined, dried ($MgSO_4$), filtered and concentrated *in vacuo* to give a pale yellow oil. Purification by flash chromatography (SiO_2, 10% EtOAc/ petroleum ether/ 0.5% acetic acid visualised with $KMnO_4$) afforded the acid (299 mg, 1.37 mmol, 65%) as a colourless oil. $[\alpha]_D$ -12.0 *(c* 1.0, $CHCl_3$), [lit. $[\alpha]_D$ -10.8 *(c* 0.25, $CHCl_3$)].[23]

The acid (200 mg, 0.92 mmol) was dissolved in anhydrous CH_2Cl_2 (12 mL) at 0 °C under nitrogen. EDCI (213 mg, 1.37 mmol) and DMAP (16 mg, 0.13 mmol) were added. After stirring at 0 °C for 15 minutes, HSNAC[3] (163 mg, 1.37 mmol) was added in anhydrous CH_2Cl_2 (5 mL). The mixture was allowed to warm to room temperature and stirred overnight. The reaction was quenched with water (5 mL) and the layers separated. The aqueous phase was extracted with CH_2Cl_2 (3 x 15 mL). The organics were combined, dried ($MgSO_4$), filtered and concentrated *in vacuo*. Purification by flash chromatography (SiO_2, 3% MeOH/ CH_2Cl_2 visualised with $KMnO_4$) gives the title compound **3*R*-15** (174 mg, 0.54 mmol,

56%) as a colourless oil. $[\alpha]_D$ -26.8 *(c* 1.0, $CHCl_3$); δ_H (400 MHz, $CDCl_3$); 0.02 (3H, s, Si-CH_3), 0.05 (3H, s, Si-CH_3), 0.85 (9H, Si-C(CH_3)$_3$), 1.18 (3H, d, *J* = 6.1, 3-CH_3), 1.96 (3H, s, CO-CH_3), 2.59 (1H, dd, *J* = 5.1, 14.7, 2-CH_2), 2.75 (1H, dd, *J* = 7.8, 14.4, 2-CH_2), 3.01 (2H, m, S-CH_2), 3.43 (2H, m, N-CH_2), 4.29 (1H, m, 3-CH), 5.91 (1H, br s, NH); δ_C (100 MHz, $CDCl_3$): -4.9 ($SiCH_3$), -4.8 ($SiCH_3$), 18.1 (Si*C*(CH_3)$_3$), 23.6 (3-CH_3), 23.9 ($COCH_3$) 25.9 (SiC(*C*H_3)$_3$), 28.9 (SCH_2), 39.8 (NCH_2), 53.9 (2-CH_2), 66.1 (3-CH), 170.5 (NCO), 196.5(SCO); ν_{max} (neat) / cm^{-1}: 3288 (NH), 2965 (CH), 2929 (CH), 2895 (CH), 2856 (CH), 1688 (CO), 1656;); *m/z* (ESI) 342.15 $[M]Na^+$; Found (ESI) 342.1530 ($C_{14}H_{29}NNaO_3SSi$ requires 342.1535).

3*S*-3-(*tert*-butyldimethylsilyloxy)butyric acid *N*-acetylcysteamine thiolester 3*S*-15[23]

3*S*-**14** (1.0 g, 4.31 mmol) was dissolved in THF (5 mL), MeOH (8 mL) and lithium hydroxide (542 mg, 12.9 mmol) in water (1.5 mL) was added. The reaction mixture was heated to 60 °C overnight. The reaction was diluted with water (7 mL) and extracted with Et_2O (2 x 10 mL). The aqueous layer was acidified to pH 3.0 with H_2SO_4 (3 M) and further extracted with EtOAc (3 x 15 mL). The organic layers were combined, dried ($MgSO_4$), filtered and concentrated *in vacuo* to give a pale yellow oil. Purification by flash chromatography (SiO_2, 10% EtOAc/ petroleum ether/ 0.5% acetic acid visualised with $KMnO_4$) afforded the acid (299 mg, 1.37 mmol, 32%) as a colourless oil. $[\alpha]_D$ +11.7 *(c* 1.0, $CHCl_3$), [lit. $[\alpha]_D$ +11.9 *(c* 1.29 , $CHCl_3$)].[24]

The acid (300 mg, 1.37 mmol) was dissolved in dry CH_2Cl_2 (19 mL) at 0 °C under nitrogen. EDCI (376 mg, 2.42 mmol) and DMAP (17 mg, 0.14 mmol) were added. After stirring at 0 °C for 15 minutes, HSNAC[3] (228 mg, 1.91 mmol) was added in anhydrous CH_2Cl_2 (7.5 mL). The mixture was allowed to warm to room temperature and stirred overnight. The reaction was quenched with water (15 mL) and the layers separated. The aqueous phase was extracted with CH_2Cl_2 (3 x 25 mL). The organics were combined, dried ($MgSO_4$), filtered and concentrated *in vacuo*. Purification by flash chromatography (SiO_2, 3% MeOH/ CH_2Cl_2 visualised with $KMnO_4$) gives the title compound **3*S*-15** (174 mg, 0.54 mmol, 40%) as a colourless oil. $[\alpha]_D$ +101.5 *(c* 1.0, $CHCl_3$); δ_H (400 MHz, $CDCl_3$); 0.01 (3H, s, $SiCH_3$), 0.04 (3H, s, $SiCH_3$), 0.84 (9H, s, SiC(CH_3)$_3$), 1.17 (3H, d, *J* = 6.1, 3-CH_3), 1.95 (3H, s, $COCH_3$), 2.58 (1H, dd, *J* = 5.0, 14.4, 2-CH_2), 2.74 (1H, dd, *J* = 7.4, 14.4, 2-CH_2), 3.01 (2H, m, S-CH_2), 3.41 (2H, m, N-CH_2), 4.28 (1H, m, 3-CH), 5.84 (1H, br s, NH); δ_C (100 MHz, $CDCl_3$): -4.9 ($SiCH_3$), -4.8 ($SiCH_3$), 18.1 (Si*C*(CH_3)$_3$), 23.4 (3-CH_3), 23.8 ($NHCOCH_3$) 25.9 (SiC(*C*H_3)$_3$), 28.8 (SCH_2), 39.8 (NCH_2), 53.9 (2-CH_2), 66.1 (3-CH), 170.5 (NCO), 196.1(SCO); ν_{max} (neat) / cm^{-1}: 3281 (NH), 2955 (CH), 2929 (CH), 2887 (CH), 2856 (CH), 1689 (CO), 1652 (CO), 1550

(NH), 1375 (CH), 1361 (CH); *m/z* (ESI) 342.15 $[M]Na^+$; Found (ESI) 342.1545 ($C_{14}H_{29}NNaO_3SSi$ requires 342.1535).

3*R*-3-Hydroxy-butyric acid *N*-acetylcysteamine thiolester 3*R*-16[25]

3*R*-**15** (100 mg, 0.31 mmol) was dissolved in THF (0.9 mL), water (0.9 mL) and acetic acid (2.6 mL) and stirred at room temperature for 5 days. The reaction was diluted with water (25 mL) and the aqueous layer washed with CH_2Cl_2 (25 mL). The aqueous layer was concentrated to give the title compound **3*R*-16** (28 mg, 0.14 mmol, 44%) as a colourless oil. $[\alpha]_D$ -23.2 *(c* 1.0, $CHCl_3$); δ_H (400 MHz, $CDCl_3$); 1.23 (3H, d, *J* = 6.3, 3-CH_3), 1.96 (3H, s, $COCH_3$), 2.72 (2H, m, 2-CH_2), 3.03 (2H, m, S-CH_2), 3.43 (2H, m, N-CH_2), 4.24 (1H, m, 3-CH), 6.03 (1H, br s, NH); δ_C (100 MHz, $CDCl_3$); 22.9 (3-CH_3), 23.7 (CO-CH_3), 28.9 (S-CH_2), 39.4 (N-CH_2), 52.6 (2-CH_2), 65.1 (3-CH), 170.8 (NCO), 199.4 (SCO); ν_{max} (neat) / $cm^{-1:}$ 3288 (NH), 3074 (OH), 2967 (CH), 2920 (CH), 1654 (C=O), 1550 (NH); *m/z* (ESI) 228.07 $[M]Na^+$; Found (ESI) 228.0668 ($C_8H_{15}NNaO_3S$ requires 228.0670).

3*S*-3-Hydroxy-butyric acid *N*-acetylcysteamine thiolester 3*S*-16[24,26]

3*S*-**15** (119 mg, 0.37 mmol) was dissolved in THF (0.9 mL), water (0.9 mL) and acetic acid (2.6 mL) and stirred at room temperature for 3 days. The reaction was diluted with water (15 mL) and the aqueous layer washed with CH_2Cl_2 (15 mL). The aqueous layer was concentrated to give the title compound **3*S*-16** (70 mg, 0.34 mmol, 63%) as a colourless oil. $[\alpha]_D$ +27.9 *(c* 1.0, $CHCl_3$); δ_H (400 MHz, $CDCl_3$); 1.24 (3H, d, *J* = 6.3, 3-CH_3), 1.97 (3H, s, $COCH_3$), 2.73 (2H, m, 2-CH_2), 3.04 (2H, m, S-CH_2), 3.45 (2H, m, N-CH_2), 4.26 (1H, m, 3-CH), 5.81 (1H, br s, NH); δ_C (100 MHz, $CDCl_3$); 22.8 ($COCH_3$), 23.2 (3-CH_3), 28.9 (S-CH_2), 39.3 (N-CH_2), 52.6 (2-CH_2), 65.1 (3-CH), 170.7 (NHC=O), 199.4 (SC=O); ν_{max} (neat) / cm^{-1}: 3290 (OH), 3086 (NH), 2931 (CH), 1652 (CO), 1548 (NH), 1431 (CH), 1074 (CN); *m/z* (ESI) 228.07 $[M]Na^+$; Found (ESI) 228.0669 ($C_8H_{15}NNaO_3S$ requires 226.0670).

3. Assay Procedures and Kinetic Analysis

DH Kinetic Assays

DH enzyme assays were performed in a final volume of 100 μL and contained the following:

Component	Volume / μL	Final concentration
1 M Tris-HCl pH 8.0 containing L-arginine 2 M L-glutamic acid 2 M	5	50 mM 100 mM 100 mM
50 % Glycerol	40	20 %
Substrate mimic (100 mM)	1 - 10	1 - 10 mM
DH (10 mg·mL^{-1})	40	100 μM
H_2O	to final volume of 100	-

The mixture was incubated at 30 °C for 1 hour. 20 μL aliquots were taken at set time points and mixed with 60 μL of acetonitrile and 20 μl H_2O to precipitate the enzyme and halt the reaction. The suspension was centrifuged in a microcentrifuge to remove the precipitated protein. The supernatant was removed and analysed by LC-MS as described below.

DH Inhibition Assays

Inhibition assays were performed in a volume of 100 μL and contained the following:

Component	Volume / μL	Final concentration
1 M Tris-HCl pH 8.0 containing L-arginine 2 M L-glutamic acid 2 M	5	50 mM 100 mM 100 mM
50 % Glycerol	40	20 %
Substrate (2*R*,3*R*-**8**)	5	2.0 mM
Inhibitor (2*S*,3*S*-**8**) or (2*R*,3*S*-**13**) or (2*S*,3*R*-**13**) (40 mM)	5	2.0 mM
DH (5 mg·mL^{-1})	40	50 μM
H_2O	5	-

The mixture was incubated at 30 °C for 1 hour. 20 μL aliquots were taken at time points and mixed with

60 μL of acetonitrile and 20 μl H_2O to precipitate the enzyme and halt the reaction. The suspension was centrifuged in a microcentrifuge to remove the precipitated protein. The supernatant was removed and analysed by LC-MS as described below.

4. LCMS analysis

Chromatography was performed using a Waters 2695 HPLC system and Phenomenex Kinetex C_{18} reverse-phase column (150 x 2.5 mm, 100 Å with a flow rate of 1 mL·min^{-1}. Solvents (HPLC grade) were **A**: H_2O + 0.05% formic acid and **B**: CH_3CN + 0.05% formic acid. A gradient elution was performed as follows over 10 minutes:

Time / minutes	**% B**
0.00	10
6.00	58
6.50	90
7.50	90
8.50	10
10.00	10

Single Ion Response (SIR) of certain mass ions were monitored over different time points as follows:

Event	***m/z* / Da**	**Time / minutes**
MS Scan	100 - 600 ES^-	0.00 - 10.00
SIR (internal standard)	196.4	1.40 - 3.40
SIR (Substrate) $[M]Na^+$	242.2	2.00 - 4.00
SIR (Product) $[M]Na^+$	224.2	4.00 - 6.00

Compounds were quantified by injection of standard amounts of substrate and product and integration of their SIR peaks. Typically, 20 μL of sample was injected, containing *ca* 20 nmol substrate or product and detection was by Waters 2998 PDA and Waters Quatro micro ESI MS. MS was performed in ESI^+ and ESI^- modes. Typical MS conditions were as follows: capillary voltage 3.3 kV; cone voltage 30 V; desolvation gas flow 600 L·min^{-1}; cone gas flow 50 L·min^{-1}.

4.1 Kinetic Data

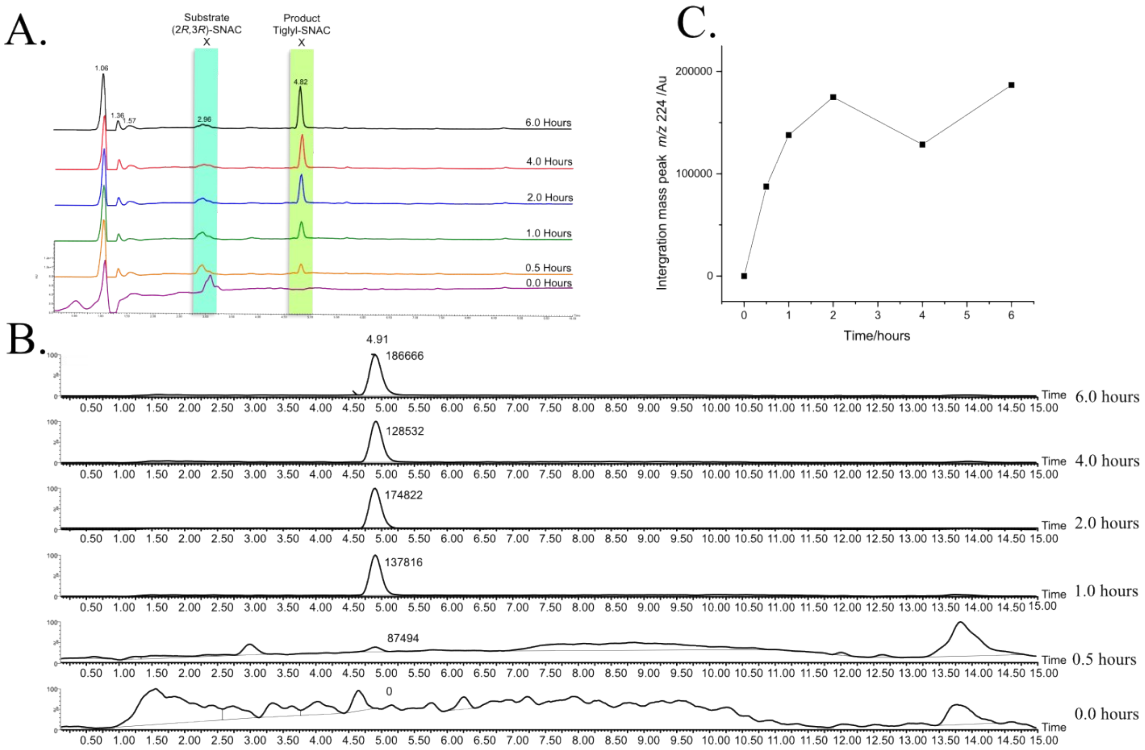


Figure S4.1: **A**, Overlay of LCMS UV spectra during time course assay, the increase in the product **17** UV peak can clearly be seeing over time; **B**, ESI$^+$ trace of **17**, *m/z* 224 [M]Na$^+$; **C**, Timecourse of ESI$^+$ peak area of **17** *m/z* 224 [M] Na$^+$ in the presence of DH enzyme.

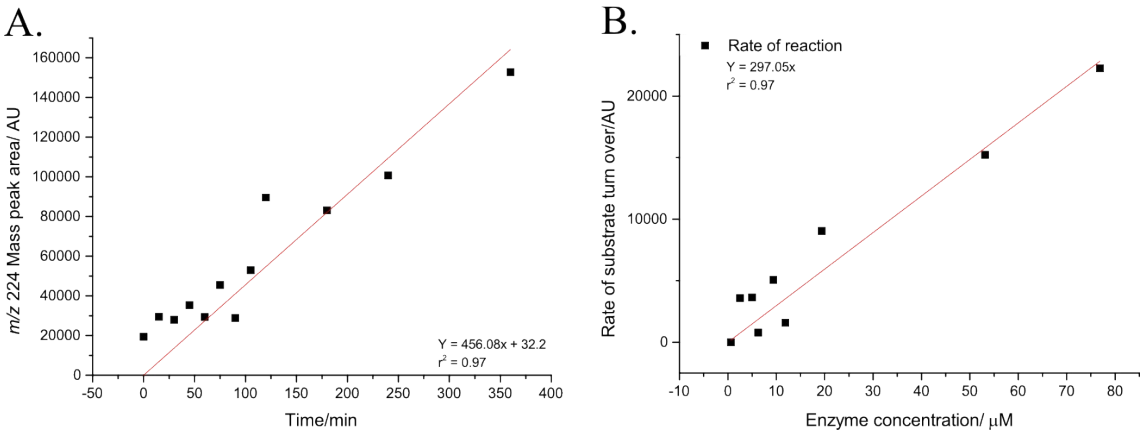


Figure S4.2: **A**, Example graph of rate determination at a protein concentration of 9.38 μM; **B**, Rate of reaction plotted against enzyme concentration.

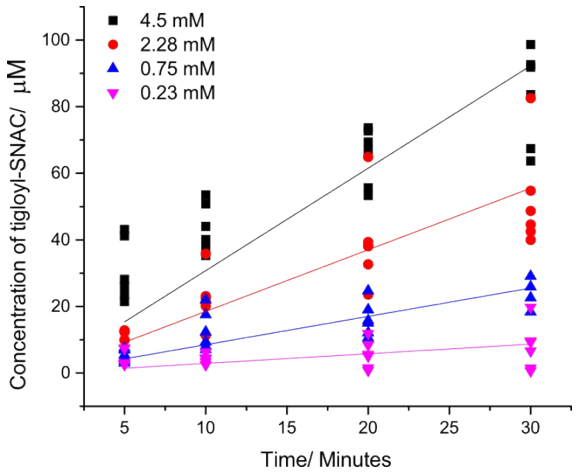


Figure S4.3: Selected data from the investigation into the change on concentration of product **17** over time at various concentrations 2*R*,3*R*-**8** (black, 4.5 mM, red, 2.28 mM, blue, 0.75 M, pink, 0.23 mM).

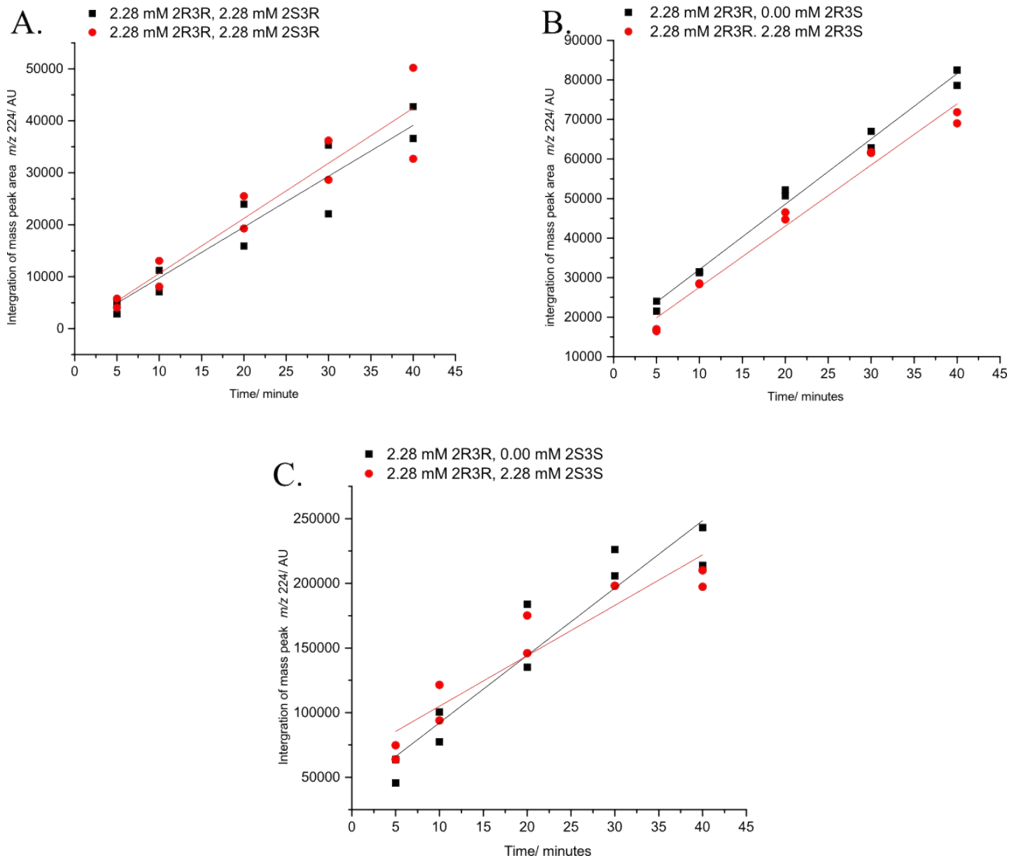


Figure S4.4: **A**, Inhibition studies of active substrate 2*R*,3*R*-**8** vs 2*S*,3*R*-**13** (2.28 mM) with SQTKS DH (132 µM), peak area of product **17**, ion mass 224 [M]Na^+; **B**, Inhibition studies of active substrate 2*R*,3*R*-**8** vs 2*R*,3*S*-**13** (2.28 mM) with SQTKS DH (132 µM),peak area of product **17**, ion mass 224 [M]Na^+; **C**, Inhibition studies of active substrate 2*R*,3*R*-**8** vs 2*S*,3*S*-**8** (2.28 mM) with SQTKS DH (132 µM),peak area of product **17**, ion mass 224 [M]Na^+.

5. Protein Modelling and Substrate Docking

5.1 Generation of a DH model structure

A model of the DH was built using the SwissModel server[27] using the CurF dehydratase (pdb id 3kg6) as the template[28] which resulted in a model with a GMQE score of 0.53. The CurF-SQTKS model was aligned with the crystal structure of CurFDH (PyMol, Figure). The RMSD of the SQTKS DH protein model and the CurFDH crystal structure (SuperPose web server) was 1.44 Å, which is well within an acceptable range.[29]

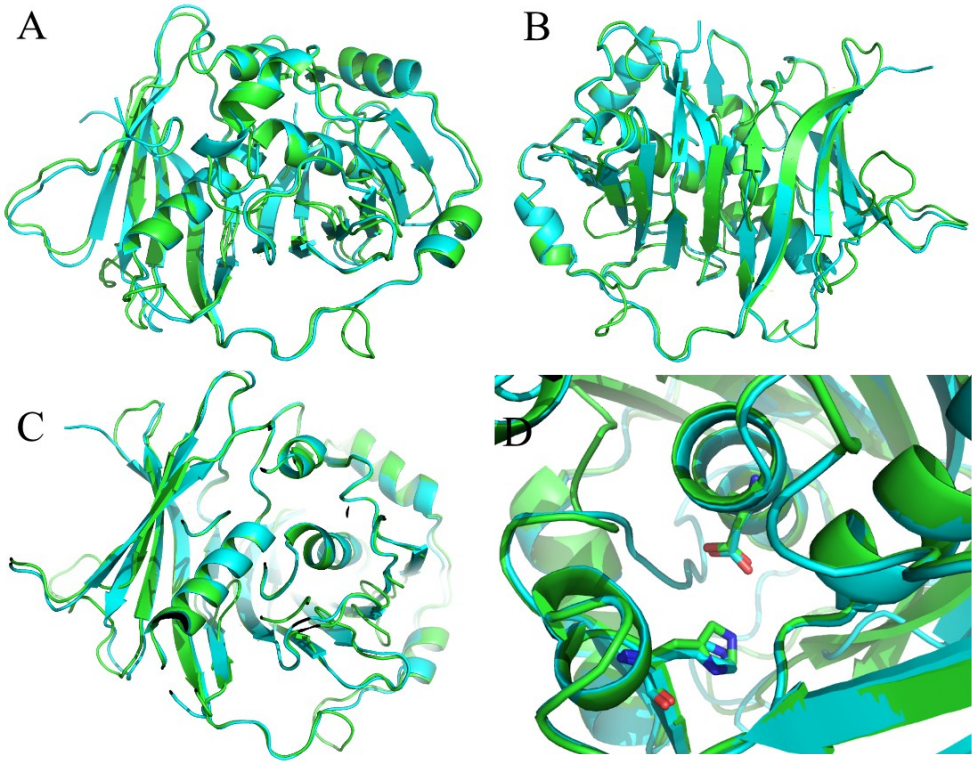


Figure S5.1.1: **A,** Front view of CurF-SQTKS model (green) aligned with CurFDH crystal structure (Blue); **B**, Front view of CurF-SQTKS model (green) aligned with CurFDH crystal structure (Blue); **C**, Alignment of α-helices in CurF-SQTKS model aligned with CurFDH crystal structure; **D**, Active site catalytic amino acids alignment.

The QMEAN local scores for the CurF-SQTKS model range from 0 to 9 (Figure S5.1.2). The regions with low scores (0 - 2) correlate to the core structures of the DH domain around the active site. Higher QMEAN scores (3 - 9) are located on the periphery of the model, away from the active site (Figure S5.1.2). The Anolea parameter (atomic empirical force potential) was used to assess the packing quality of the model (Figure S5.1.2). Negative (green)

regions show favourable packing environments, while positive (red) regions show unfavourable packing arrangements. The area of favourable packing corresponds with the regions with low QMEAN results.

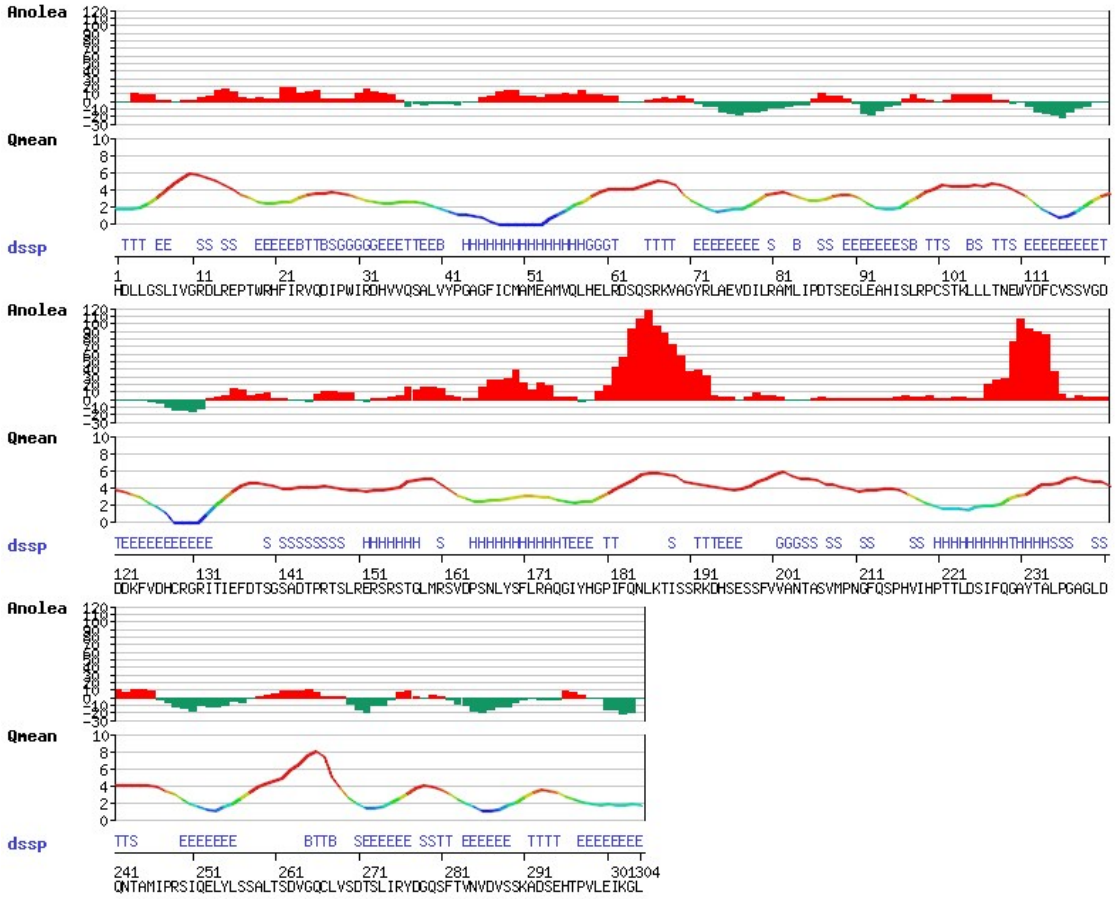


Figure S5.1.2: QMEAN local results for the CurF-SQTKS model. Low QMEAN values indicate a regions of potentially higher reliability. High values indicate regions with a higher probability of inaccuracies. Anolea (atomic empirical force potential) results assess the packing quality of the model Negative (green) regions show favourable packing environments, while positive (red) regions show unfavourable packing arrangements. dssp: H, α-helix; B, β-bridge; E, strand; G, Helix-3; I, Helix-5; T, turn; S, bend.

The residue error diagram (Swiss-Model, Figure S5.1.3) indicates that areas of lower accuracy (red) are located on the periphery of the protein, while the core displays lower structural eror (yellow / green / blue) .

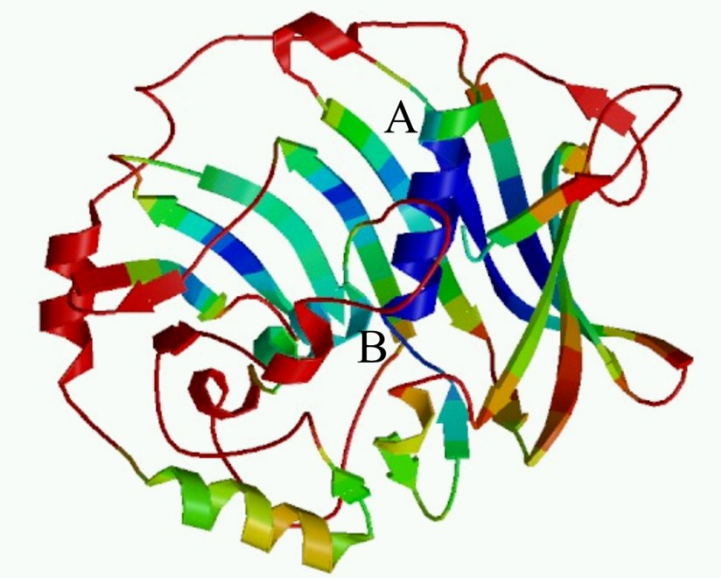


Figure S5.1.3: Residue error diagram, blue correlate to regions of higher reliability and red correlated to regions of lower reliability.

```
SQTKS          ------------------NKAHR-------QRVHPPHDLLGSLIVG-RDLREPTWRHFIR
3KG6CurF       ----------------------------------SNAHPLLGEKINLAGIEDQHRFQSYIG
3EL6Ery4       MGSSHHHHHHSSGLVPRGSHMHRPADVSALGVRGAEHPLLLAAVDVPGHGG-AVFTGRLS
                                                  * **   :          :   :

                          *   +  + + +
SQTKS          VQDIPWIRDHVVQSALVYPGAGFICMAMEAMVQLHELRDSQSRKVAGYRLAEVDILRAML
3KG6CurF       AESPGYLNHHQVFGKVLFPSTGYLEIAASAGKSLFT-------SQEQVVVSDVDILQSLV
3EL6Ery4       TDEQPWLAEHVVGGRTLVPGSVLVDLALAAGEDVGL-------PV----LEELVLQRPLV
               .:.   ::  .* * .  :  *.:  :  :*  *  .:                :  ::  :  :  ::

SQTKS          IPDTSEGLEAHISLRPCSTKLLLTNEWYDFCVSSV-----GDDDKFVDHCRGRTTIEFDT
3KG6CurF       IPETEIKTVQTVVS-------FAENNSYKFEIFSPSEGENQQTPQWVLHAQGKIYTEPTR
3EL6Ery4       LAGAGALLRMSVGA-------PDESGRRTIDVHAAEDVADLADAQWSQHATGTLAQGVA-
               :  :          :              .    :  :  :           ::   *.  * :

SQTKS          SGSADTPRTSLRERSRSTGLMRSVDPSNLYSFLRAQGIYHGPIFQNLKTISSRKDHSESS
3KG6CurF       NSQAKID-----LEKYQAECSQAIEIEEHYREYRSKGIDYGSSFQGIKQLWKGQGKALGE
3EL6Ery4       --AGPRD-----TEQWPPEDAVRIPLDDHYDGLAEQGYEYGPSFQALRAAWRKDDSVYAE
                  .          ..           :  .:  *    :*  :*  **  ::       .  ..

                                        *  +
SQTKS          FVVANTASVMPNGFQSPHVIHPTTLDSIFQGAYTALPGAGLDQNTAMIPRSIQELYLSSA
3KG6CurF       MAFPEELTAQL----ADYQLHPALLDAAFQIVSYAIPHTETDK--IYLPVGVEKFKLYRQ
3EL6Ery4       VSI----AADE----EGYAFHPVLLDAVAQTLSLGALGEPG-G--GKLP-----------
               . .     :.          :  :**.  **:  *    .              :*

SQTKS          LTSDVGQCLVSDTSLIRYDGQSFTVNVD--------VSSKADSEHTPVLEIKGLRNQSVG
3KG6CurF       TISQ-----VWAIAEIRQTNLTA-------------NIFLVDNQGTVLVELEGLRVK---
3EL6Ery4       ---F-----AWNTVTLHASGATSVRVVATPAGADAMALRVTDPAGHLVATVDSLVVRSTG
                        .      ::     :                      .*     :  :..*  :

SQTKS          QMA-------
3KG6CurF       ----VTEP--
3EL6Ery4       EKWEQPEPRG
```

Figure S5.1.4: Sequence alignment of SQTKS-DH, CurF-DH and Ery-DH4 using Clustal Omega: yellow, identical amino acids; green strongly similar; blue, weakly similar. Catalytic amino acids marked *. Conserved amino acids involved in the hydrogen bonding network marked +.

The protein sequences of SQTKS-DH, CurF-DH and Ery-DH4,[30] were aligned using Clustal Omega (Figure S5.1.4). H37 and D210 of CurF-DH are known to be the active site residues. Ery-DH4 has been show to possess a hydrogen bonding network (G73, L76, P78, S80 and Q235) in addition to the catalytic amino acids (Ery-DH4: H69, D231).[7] A number of these amino acids are also highly conserved in CurF-DH (P46, L44, Q214) and SQTKS-DH (P53, V51, Q239), it is highly likely that these amino acids will also form a hydrogen bonding network in SQTKS-DH. Inspection of the aligned structures (Figure S5.1.5) shows the active site residues are also spatially preserved.

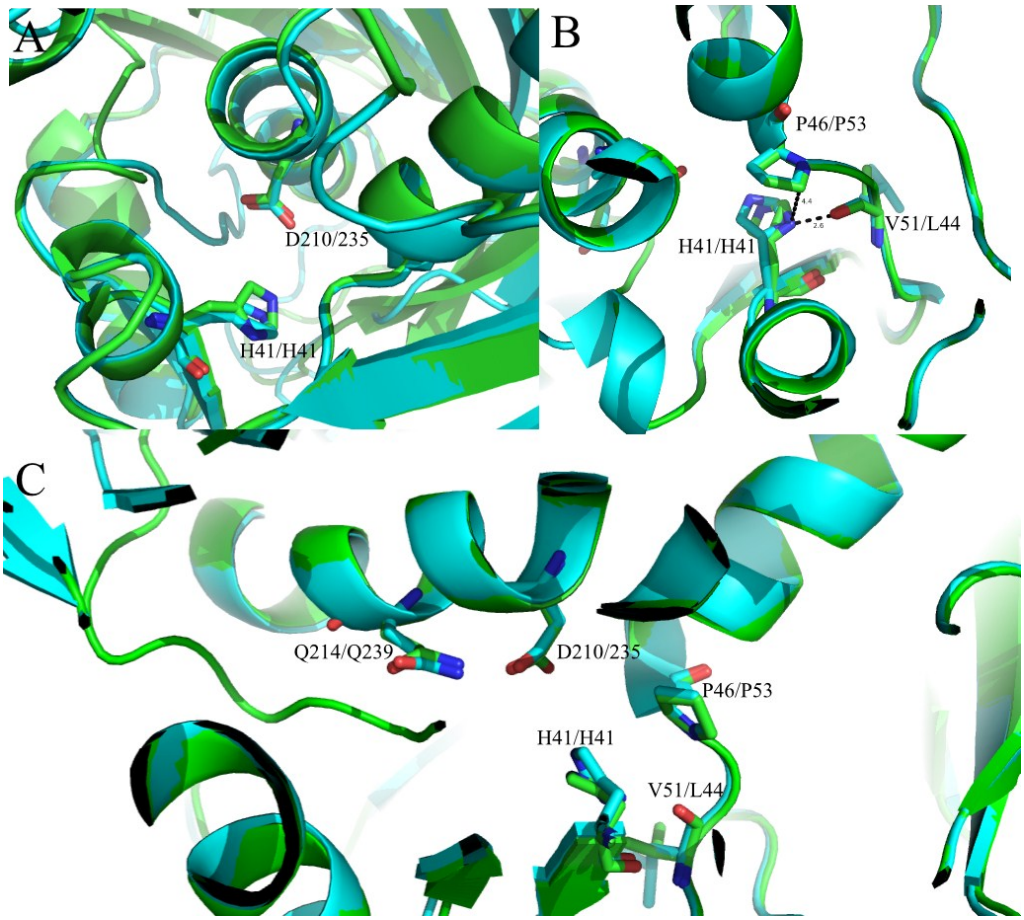


Figure S5.1.5: **A**, Active site amino acids in CurF (H41/D210) and CurF-SQTKS model (H41/D235); **B**, Lysine/valine stabilisation of H41 in acitve site; **C**, Amino acids which from the hydrogen bonding network and help position the catalytic amino acid.

5.2 Substrate docking.

The 2*R*,3*R* SNAC substrate **8** was manually docked in the active site using PyMol,[31] and the assemby was minimised using the YASARA minimisation server (http://www.yasara.org/minimizationserver.htm).[32] The resulting structure was analysed using PyMol. The docked model is attached to the electronic supplemetarty information as a pdb file.

6. References

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