

## Supporting Information

### **Stereoselective Preparation of Lipidated Carboxymethyl-proline and Pipecolic acid Derivatives via Coupling of Engineered Crotonases with Alkylmalonyl-CoA Synthetases**

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## I- Materials and methods

Unless otherwise stated, chemicals were from Alfa Aesar (Karlsruhe, Germany), Aldrich (Dorset, UK), Acros chemicals (Loughborough, UK) or Bachem (St. Helens Merseyside, UK), and used without further purification. HPLC grade solvents were purchased from Rathburn (Walkerburn, UK) and used for chemical transformations, work-up and chromatography without further distillation. Dry solvents were from Aldrich (Dorset, UK) or by filtration through columns containing activated aluminum oxide under argon. IPTG was from Melford Laboratories Ltd., electrophoresis grade agarose was from Biorad, and acrylamide/bis-acrylamide stock solution was from Sigma. Bacto Tryptone, Yeast Extract and Bacto Agar for use in culture media were from Oxoid and Difco. Plasmids and enzymes were from Promega, Novagen, New England BioLabs, and Stratagene; unless otherwise stated. Molecular weight markers for SDS-PAGE (Prestained protein marker) were from Invitrogen. 1 kb DNA ladder for DNA electrophoresis was from New England Biolabs. Other materials were from QIAGEN and Roche, unless otherwise stated. Silica gel 60 F254 analytical thin layer chromatography (TLC) plates were obtained from Merck (Darmstadt, Germany) and visualized under UV light, or with potassium permanganate stain. Chromatographic purifications were performed using prepacked SNAP columns on a Biotage SP1 Purification system (Uppsala, Sweden). Yields refer to purified, dried, and spectroscopically pure compounds (except where otherwise stated). FPLC columns and equipment, and small-scale gel filtration columns (PD-10) were from Amersham Biosciences. Spin concentrators for protein concentration were from Amicon. NMR tubes (1 and 2 mm) were from Bruker. Deuterated solvents were from Sigma and Apollo Scientific Ltd.

Water was purified by a Millipore Milli-Q system fitted with a 0.22 µm filter at the outlet. All standard solutions used in molecular biology and microbiology were prepared according to standard procedures<sup>1</sup> using Milli-Q water and were autoclaved or sterilised by filtration, as required. All <sup>1</sup>H and <sup>13</sup>C NMR Spectra were recorded using Bruker AVIII 700 MHz (with <sup>1</sup>H inverse cryoprobe), Bruker AVII 500 MHz with a <sup>13</sup>C cryoprobe, Bruker DRX 500 MHz (with 1 mm inverse microprobe), or Bruker AV 400 MHz. All chemical shifts are given in ppm relative to the solvent peak. Coupling constants (*J*) are reported in Hz to the nearest 0.5 Hz. High Resolution (HR) mass spectrometry data (*m/z*) data were obtained from a Bruker MicroTOF instrument using ESI source and Time of Flight (TOF) analyser. Infrared (IR) spectra were recorded on a Bruker Tensor 27 instrument.

## II- Protein preparation and assays

CarB and ThnE and their variants were prepared and purified as reported<sup>2</sup>.

### CMPS assays

Amino acid aldehydes (**2** and **40**) were obtained by deprotection of the appropriate precursors as reported.<sup>3</sup>

Analytical Wildtype and variant CMPS incubations were performed by sequential addition of the following in a 0.5 mL Eppendorf tube (50 µL total volume):

600 mM Tris.HCl, pH 9.0	35 µL
malonyl-CoA (10 mM)	8 µL
Amino acid semialdehyde in 10% formic acid (15 mM)	5 µL
CMPS <sup>a</sup>	2 µL

<sup>a</sup>The initial concentration of CarB/CarB variants was ~40 mg/mL and that of ThnE/ThnE variants was ~20 mg/mL.

The incubation mixture was then kept at 37 °C for 30 min. To quench the reaction, an equal volume of methanol was added and the mixture cooled on ice for 10 min before centrifugation at 13,000 x g for 3 min. The supernatant was decanted and analysed by LC-MS. Control assays were performed in the same way but with substitution of 50 mM Tris-HCl pH 7.5 for the enzyme-containing solution.

For quantification, the internal standard *p*-aminosalicylic acid was dissolved in the quenching solution of methanol (0.25 mM solution).

For CMPS assays using malonyl-(dethia)CoA analogues, the assays were performed as described above except that a 20 mM solution of the malonyl-(dethia)CoA analogue was used.

### Small scale assay analyses

Products from small scale assays were analysed by LC-MS using either a Primesep 100 column (Sielc, 250 mm x 4.6 mm, 10 µm pore size, for relatively polar products) or an analytical C18 Column (150 mm x 4.6 mm, 5 µm pore size, for relatively nonpolar products) on a Waters 1525µ Binary HPLC Pump system with a Waters 2777 Sample Manager coupled to a Micromass<sup>®</sup> Quattro micro<sup>™</sup> API mass spectrometer (positive electrospray ionization).

The column was equilibrated at 1 mL/min with 5% eluent **B**. After 10 min, a gradient was run to 70% eluent **B** over 20 min. The column was washed with 100% eluent **B** for 10 min before the column was re-equilibrated at 5% eluent **B** for 20 min.

Eluent A:	0.1% HCOOH in H <sub>2</sub> O (v/v)
Eluent B:	0.1% HCOOH in MeCN (v/v)

### **Large scale enzymatic product isolation and preparation for NMR characterization**

Products for NMR analysis were produced by scale-up (10x) of assay conditions and incubation for 1 h at 37 °C, followed by quenching with MeOH (500 µL), centrifugation (13,000 x g) and freeze-drying of the supernatant. The resultant residue was re-suspended in 20 % aqueous methanol (300 µL) and purified using either (i) a Waters Spherisorb column (250 mm x 10 mm, 5 µ, for relatively polar products) pre-equilibrated in 5% aqueous MeOH before a gradient was run to 10-25 % aqueous MeOH (according to the polarity of the product) with 0.1% aqueous formic acid; or (ii) a preparative C18 Column (250 mm x 22 mm, 15 µ, for relatively nonpolar products) pre-equilibrated in 5% aqueous MeCN with 0.1% formic acid before a gradient was run to 100 % MeCN with 0.1% formic acid over 40 min. In some cases, the product was purified twice to obtain decent NMR spectra. Elution was monitored using a Micromass<sup>®</sup> Quattro micro<sup>™</sup> API mass spectrometer (equipped with a Waters 1525µ Binary HPLC Pump system coupled to a Waters 2777 Sample Manager). Fractions with masses corresponding to anticipated products were collected (5-15 mL) and freeze dried. The resultant residue was re-suspended in <sup>2</sup>H<sub>2</sub>O (600 µL), transferred to an Eppendorf vial and freeze-dried. The final residue was re-suspended in <sup>2</sup>H<sub>2</sub>O (12 or 6 µL, according to the NMR system to be used), transferred into a 1 mm NMR tube (Bruker) using a hand centrifuge, and analysed by NMR.

### **Quantification of yields and d.r. of the products of CMPS catalysis.**

Yields of different products of CMPS catalysis (Table 1) were calculated using a combination of LC-MS and <sup>1</sup>H-NMR spectroscopy as follows: (i) The isolated yield obtained with a high yield producing CMPS variant for the product of interest was quantified (in duplicate) from protected L-GHP/L-AASA according to the reported <sup>1</sup>H-NMR method<sup>4</sup> employing [<sup>2</sup>H]<sub>4</sub>-trimethylsilylpropionate as an external or internal standard; (ii) Using this <sup>1</sup>H-NMR quantified yield as a reference, the yields of other CMPSs were determined (mean of two biological repeats) by LC-MS assays using *p*-aminosalicylic acid as an internal standard. The d.r. of the products of CMPS catalysis resulting from incubation of L-GHP/L-AASA (**1/40**) and C-2 alkylmalonyl-CoA (including that produced by Ccr or MatB catalysis) was determined by LC-MS and/or <sup>1</sup>H-NMR analyses.<sup>2</sup>

### Ccr/CMPS coupled assays

Analytical Ccr/CMPS incubations were performed by sequential addition of the following in a 0.5 mL Eppendorf tube:

- Tris-HCL 600 mM pH 9.0	35 µL
- Amino acid semialdehyde (15 mM in 10% formic acid)	5 µL
- Tris-HCL 150 mM pH 7.9	67 µL
- NADPH 80 mM	2.5 µL
- ( <i>E</i> )-Crotonyl-CoA or acryloyl-CoA 10 mM	10 µL
- NaHCO <sub>3</sub> 300mM	11 µL
- Ccr (6.25 µg/µl)	6 µL
- CMPS	3 µL

### MatB/CMPS coupled assays

Analytical MatB/CMPS incubations were performed by sequential addition of the following in a 0.5 mL Eppendorf tube:

- Tris-HCL 600 mM pH 9.0	35 µL
- Amino acid semialdehyde (15 mM in 10% formic acid)	5 µL
- Tris-HCL 600 mM pH 7.9	42.5 µL
- Coenzyme A 10 mM	5.5 µL
- ATP (100 mM in 50 mM Tris-HCL pH 8.0)	1.3 µL
- Malonic acid (derivative, 1M)	2.2 µL
- MgCl <sub>2</sub> 200 mM	4.5 µL
- MatB 1.659 µM	1 µL
- CMPS (~40 mg/ml)	3 µL

The assay mixture was incubated at 37 °C for 2h then treated as described for CMPS assays.

The C-2 alkylmalonic acid derivatives used in the coupled MatB/CMPS assays were obtained either as free acid or as di(m)ethyl ester. In the case of the (m)ethyl ester derivatives, they were subjected to alkaline hydrolysis. The (m)ethyl ester derivative (1 equivalent) and sodium/lithium hydroxide (2.2 equivalents) were dissolved in a round-bottom flask containing 10 ml water. The resulting solution was stirred at room temperature or at 40 °C for 2h then acidified with conc. hydrochloric acid (to pH 2-3) and extracted three times with 30 ml ethyl acetate. The organic layer was then dried using magnesium sulfate and after filtration a quantitative yield of the C-2 alkylmalonic acid was isolated after evaporation in *vacuo*.<sup>5</sup> The identity of the isolated product was confirmed by LC-MS (negative ionization mode, Figure S1) and NMR analyses.

Pantetheine and *N*-acetylcysteamine derivatives were prepared as reported.<sup>5</sup>

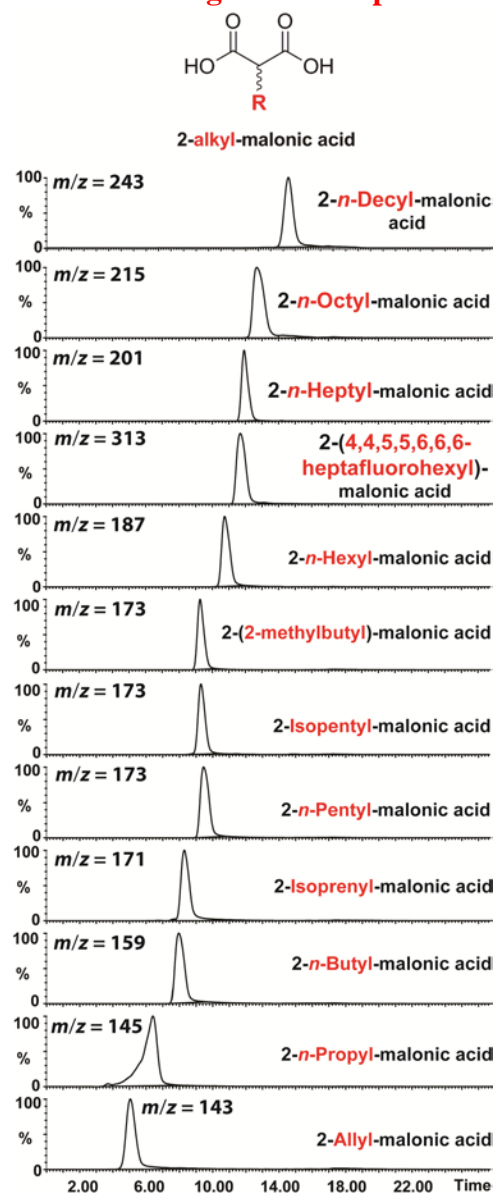
### III- NMR Assignment of products of CMPS catalysis

NMR analyses were recorded at 298 K using a Bruker AVIII 700 MHz spectrometer equipped with a  $^1\text{H}$  TCI-inverse cryoprobe optimised for  $^1\text{H}$  observation (and running TOPSPIN 2 software), unless otherwise stated. Products were analysed by 2D COSY and NOESY (mixing time 800 ms) and stereochemistries were assigned through combined analysis of  $^3J_{\text{HH}}$  coupling constants and NOEs. Chemical shifts are reported in ppm relative to  $\text{D}_2\text{O}$  ( $\delta_{\text{H}}$  4.72); the deuterium signal was used as an internal lock signal and the HDO signal was reduced by presaturation where necessary. For quantification of the carboxymethylproline synthases products of catalysis, trimethylsilane propionic acid sodium salt (TSP) was used as an external or internal standard.

*For the spectroscopic identification of products of CMPSs catalysis, the following general considerations apply:*

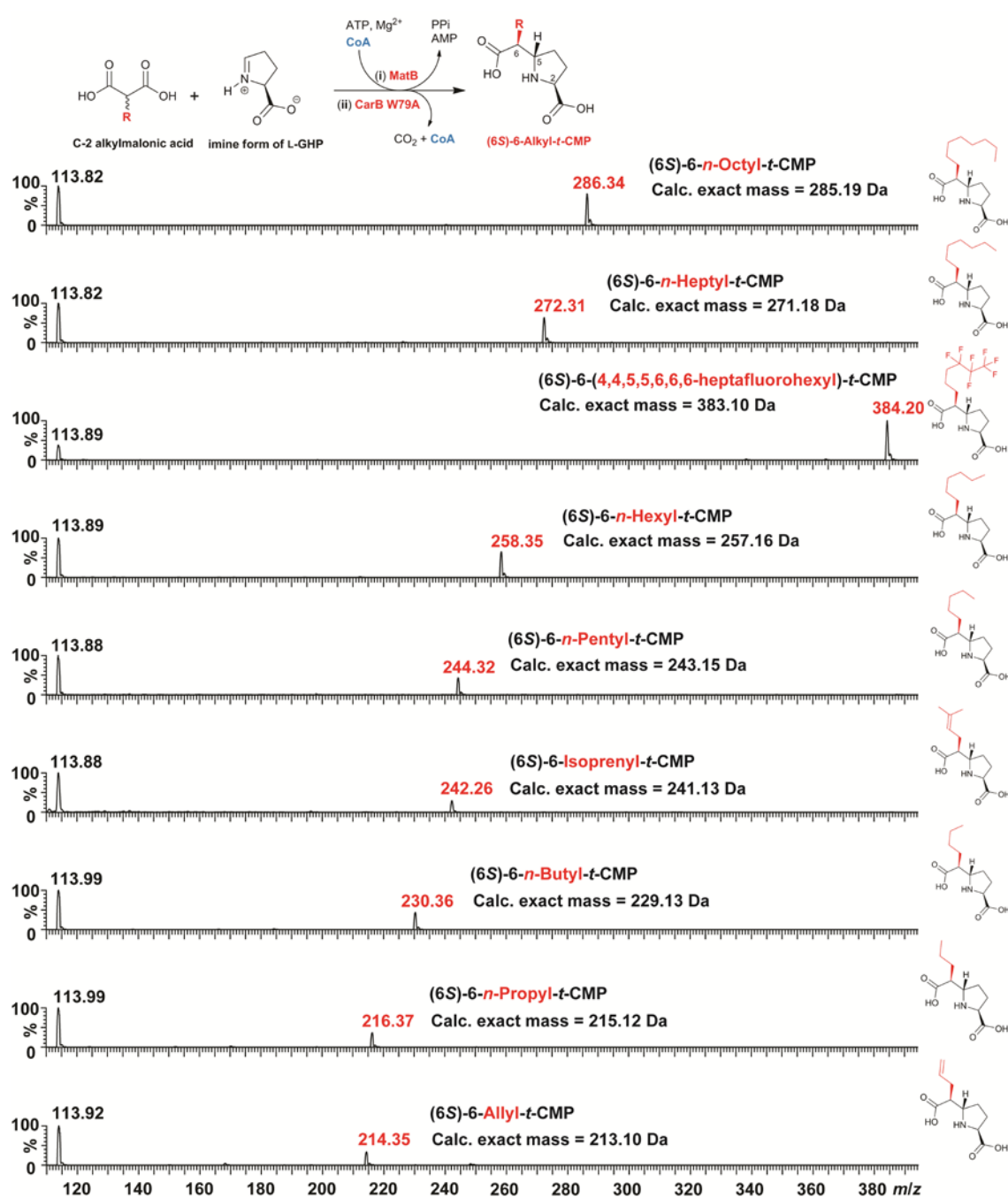
In all cases, the LC-MS analyses (positive or negative ion electrospray ionization) support formation of the assigned product(s) as shown by observation of the molecular ion and the ion arising from decarboxylation of the product. The formation of a ring structure was assigned in part from the  $^1\text{H}$ -NMR chemical shift of the bridgehead proton (H-5 of *t*-CMP/H-6 of *t*-CMPi). All assignments assume that the (*S*)-stereochemistry at C-2 is maintained during the acid-mediated deprotection of amino acid semialdehydes and during product formation. Evidence has been reported confirming that this is the case for the CarB- and ThnE-catalysed conversion of L-GHP (**2**) to (2*S*,5*S*)-carboxymethylproline (**3**).<sup>6,7</sup> For all compounds reported, the assignment of the bridgehead carbon (C-5 of *t*-CMP/C-6 of *t*-CMPi derivatives) as having (*S*)-stereochemistry was in part based on NOE data that showed no correlation between H-2 and the bridgehead proton. The NOE data between other protons within the ring system supported this assignment. For all (major) products of MatB/CMPS catalysis reported in this study, the assignment of C-6 of *t*-CMP/C-7 of *t*-CMPi derivative as having the (*S*)-stereochemistry was based on a combination of coupling constant  $J_{5,6}$  (*t*-CMP)/( $J_{6,7}$  (*t*-CMPi)  $\sim$  6-9 Hz (predicted  $\Phi \sim 130$ -155°) and nOe data that revealed: (i) a moderate nOe correlation between H-5 and H-6 (*t*-CMP)/ H-6 and H-7 (*t*-CMPi) indicating a predominately anticlinal relationship between these two protons; (ii) The observation of a moderate nOe correlation between H-6 and H-4' (*t*-CMP)/ H-7 and H-5' (*t*-CMPi), together with a weak or no correlation between H-6 and H-4 (*t*-CMP)/ H-7 and H-5 (*t*-CMPi); (iii) The observation of a moderate to weak nOe correlation between H-7 and H-4 but not (or weaker) with H-4' (*t*-CMP). For full-annotated spectra of products, see Figures S7 to S23.

#### IV- Chromatograms and spectra of products of coupled MatB/CMPS catalysis



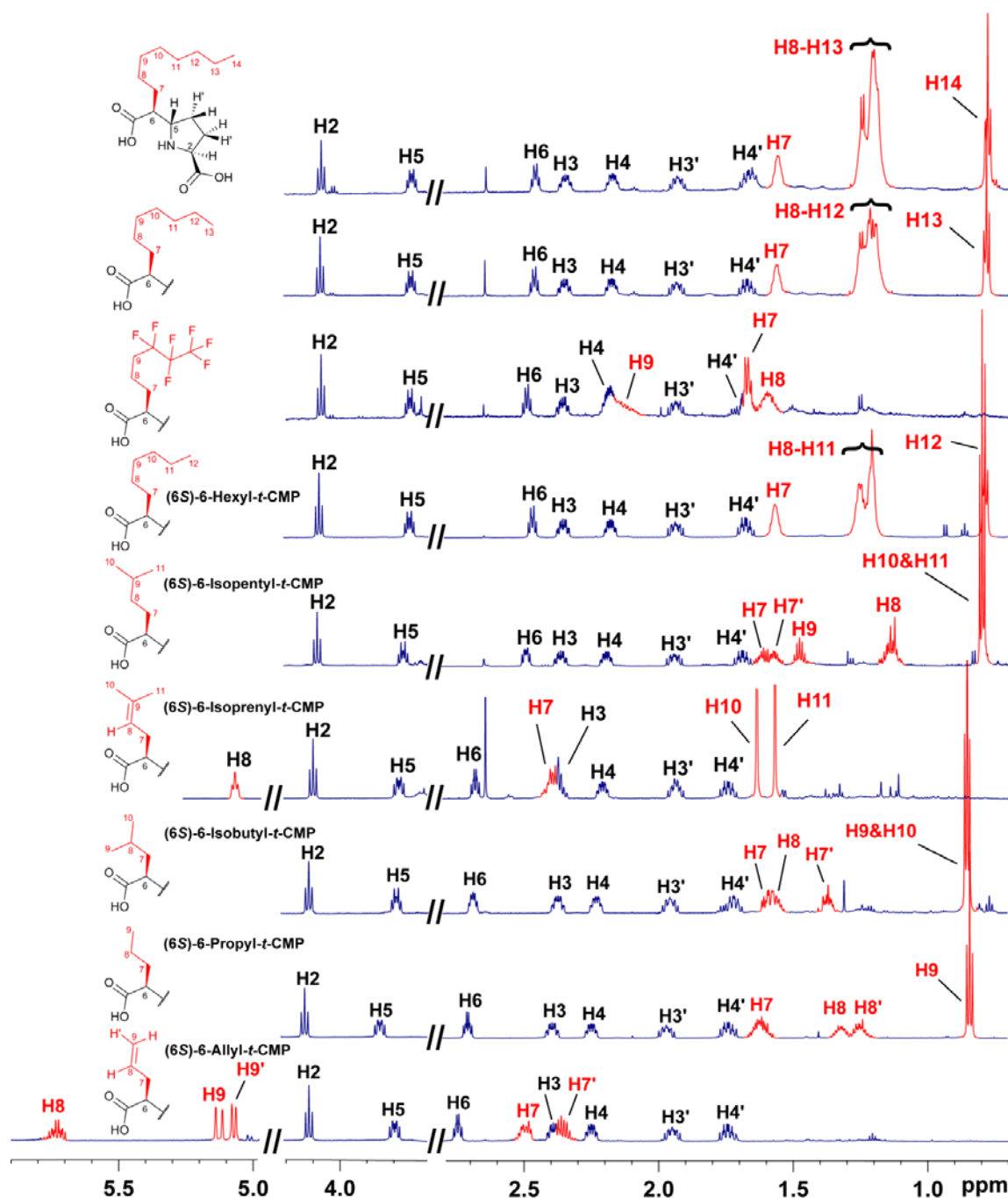
**Fig. S1** Ion extracted LC-MS chromatograms (negative electrospray ionisation) displaying the retention times of the C-2-alkylated malonic acid derivatives prepared and/or used in the coupled MatB/CMPS reactions. Compound numbers from bottom to top correspond to 13, 14, 15, 20, 17, 19, 18, 21, 22, 23, 24, 25 (Table 1).



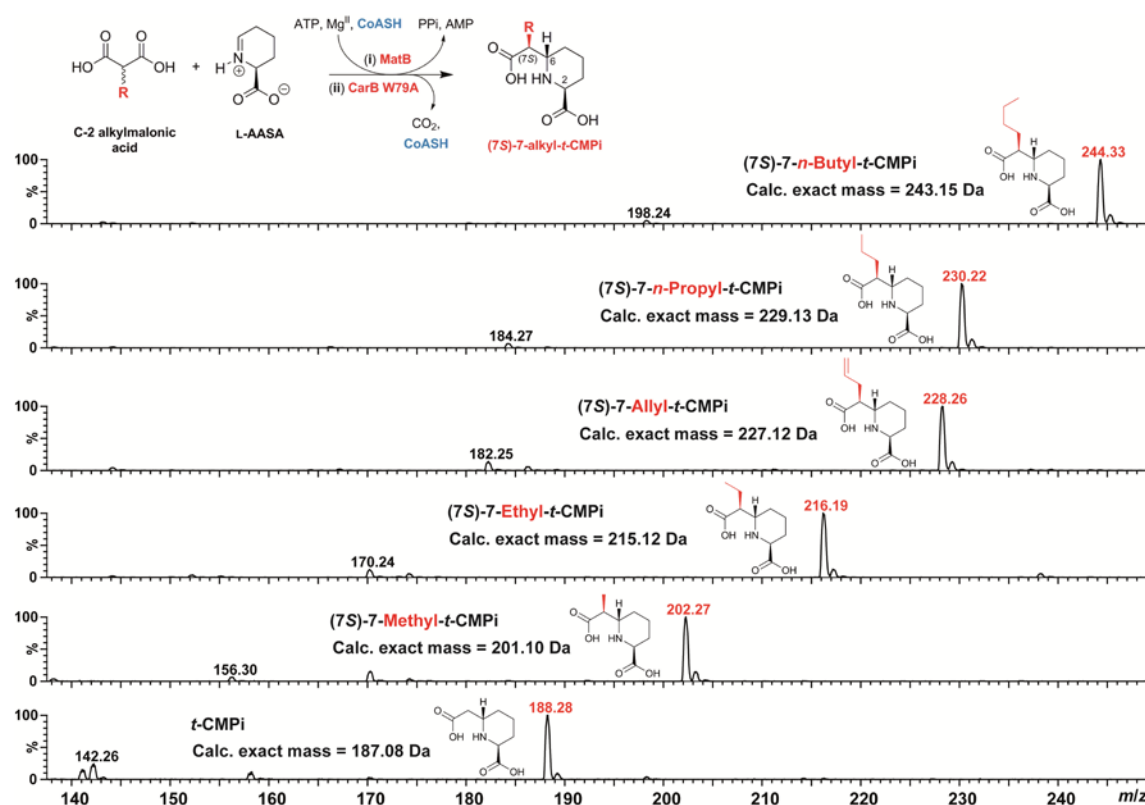


**Fig. S2** The ability of the coupled MatB/CarB W79A-catalysed reactions to generate *t*-CMP derivatives with different C-6 alkyl side chains. The mass spectra (positive ion mode) for the shown products reveal the ability of the coupled enzyme system to produce C-2 alkylated malonyl-CoA derivatives and to react them with L-GHP, in the presence of the cofactors ATP and CoASH, to give the corresponding 6-alkyl-*t*-CMP derivatives. Compound numbers from bottom to top correspond to 28, 29, 30, 35, 32, 36, 37, 38, 39 (Table 1).

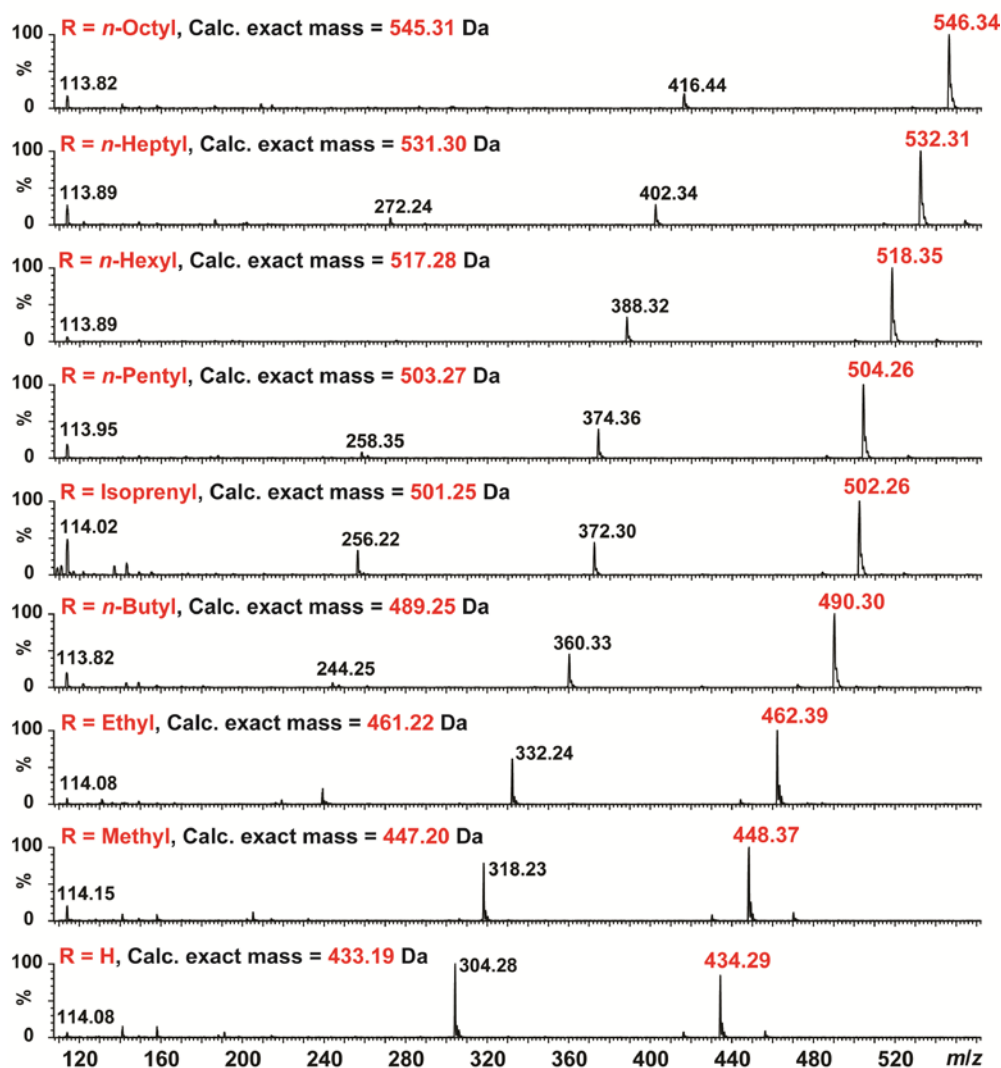




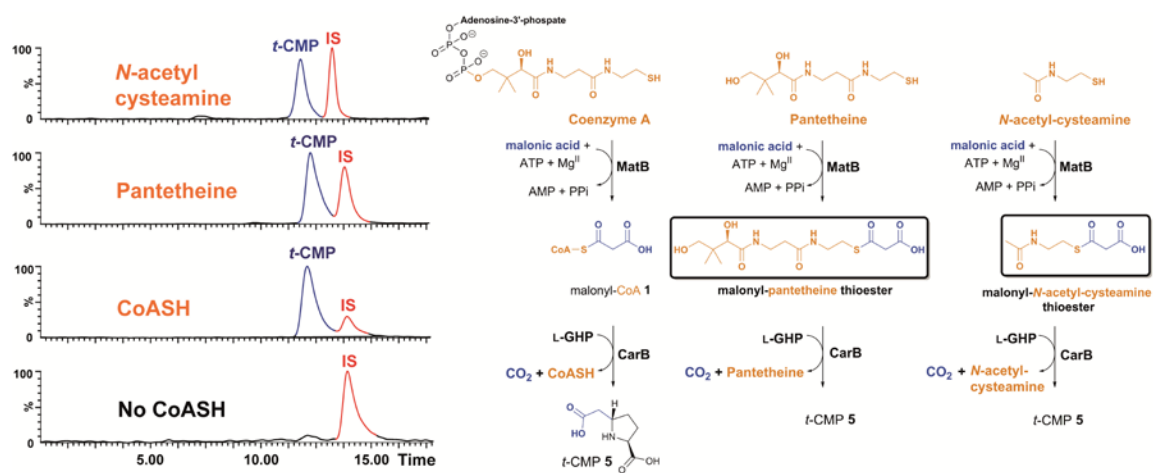
**Fig. S3**  $^1\text{H}$ -NMR spectra of the purified (6*S*)-6-alkyl-*t*-CMP products resulting from the one-pot incubation of the corresponding C-2 alkylated malonic acid derivatives and L-GHP, in the presence of ATP and coenzyme A, as catalysed by the MatB/CarB W79A coupled enzyme system. For detailed characterization of all of the isolated products, see supporting information (Fig. S7 to S23). Compound numbers from bottom to top correspond to 28, 29, 30, 35, 34, 36, 37, 38, 39 (Table 7).



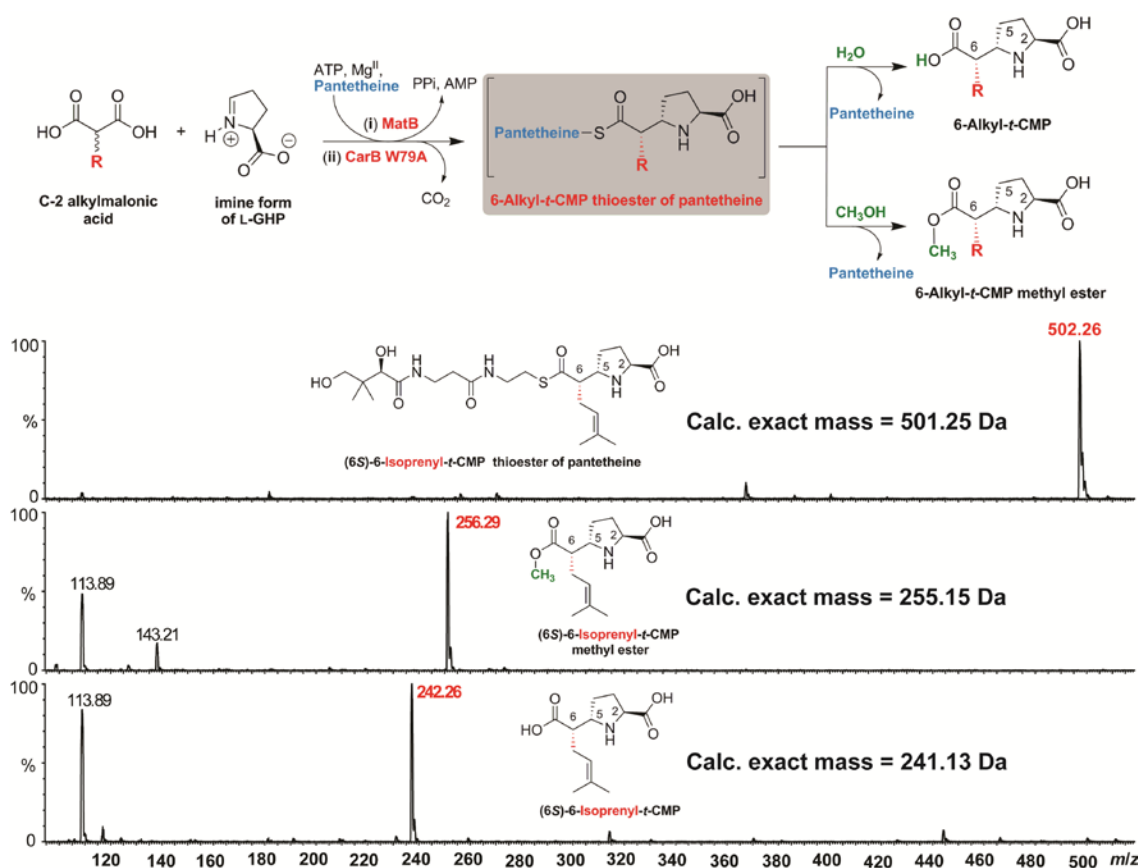
**Fig. S4** The ability of the tandem MatB/CarB W79A-catalysed reactions to generate C-7 alkylated carboxymethylpipercolic acid (*t*-CMPi) derivatives. The mass spectra (positive ion mode) for the shown products reveal the ability of the coupled enzyme system to produce C-2 alkylated malonyl-CoA derivatives and to react them with L-aminoadipate semialdehyde (L-AASA), in the presence of ATP and CoASH, to give the corresponding 7-alkyl-*t*-CMPi derivatives. Compound numbers correspond to **56, 41, 42, 43, 44, 45** (Table 1).



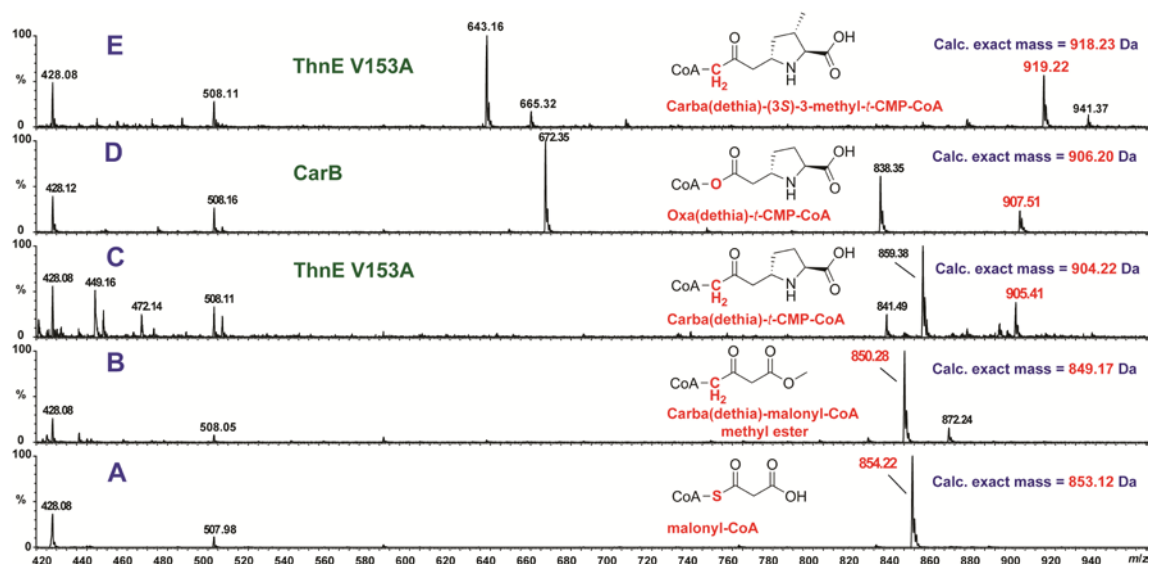
**Fig. S5** CMPSs accept truncated forms of malonyl-CoA **1** as substrates. Mass spectra (positive ion mode) for some of the assigned thioester “products” observed on incubation of various C-2 alkylmalonic acid derivatives with L-GHP, in the presence of ATP and pantetheine (as a CoASH replacement) using MatB/CarB W79A. Note that methanol addition results in methanolysis of the thioester **35**, as demonstrated by studies on the isoprenyl to give the corresponding methyl ester **48** (see Fig. S6).



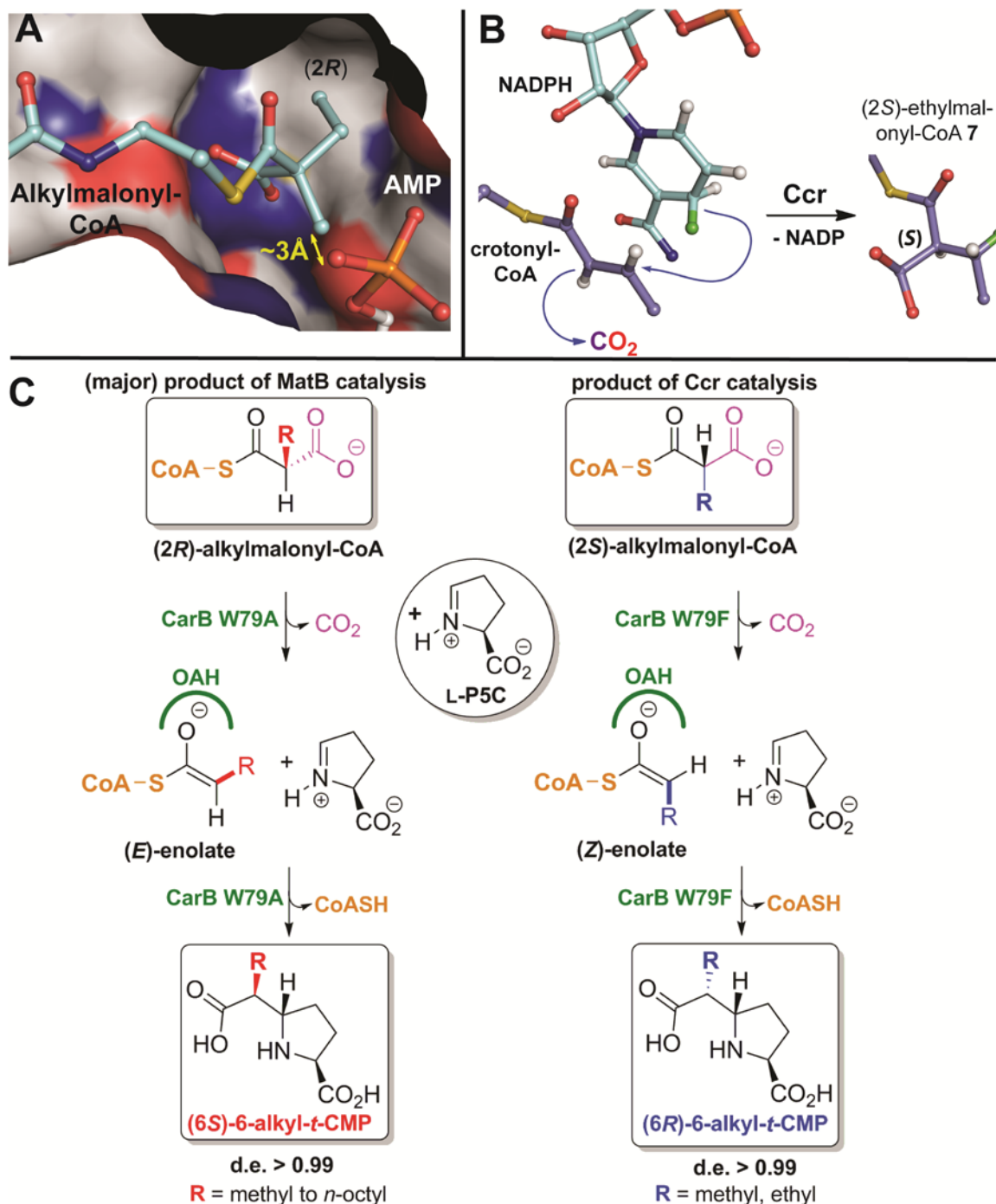
**Fig. S6 Production of *t*-CMP 5 via MatB/CarB catalysis through the intermediacy using truncated malonyl-CoA 1 analogues.** The ion extracted LC-MS chromatograms (positive electrospray ionisation,  $m/z = 174$  and  $154$ , for **5** and the internal standard, respectively) show the production of **5** using malonic acid and L-GHP as substrates in the presence of ATP/Mg<sup>II</sup> and CoASH, pantetheine or *N*-acetyl-cysteamine. Note the higher level of turnover in the case of CoASH implying that the truncated forms of malonyl-CoA are less favoured substrates. The truncated forms of **1** are boxed. *p*-Aminosalicylic acid was used as an internal standard (**IS**, red peak).



**Fig. S7** The mass spectra (positive ion mode) for the products (35, 48 and 49) of incubation of 2-isoprenylmalonic acid, L-GHP, ATP, pantetheine MatB/CarB W79A (preparative scale reaction). The ratio between the three observed products (i.e. (6S)-6-isoprenyl-t-CMP (35), methyl ester (48) and pantetheine thioester (49)) was ~1:1:1.5, respectively.



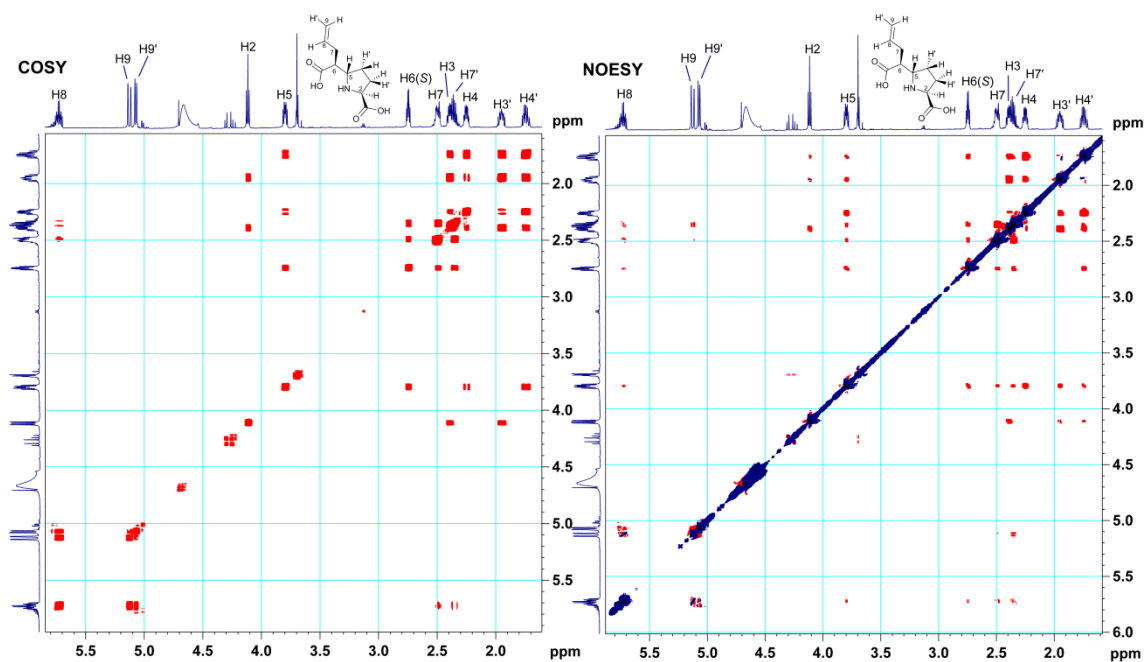
**Fig. S8** CMPSs accept carba- and oxa-(dethia)-malonyl-CoA analogues as substrates. The mass spectra (positive ion mode) for the shown products (**C**, **D** and **E**) reveal the ability of the shown CMPS variants to form carba-(dethia)-*t*-CMP-CoA derivatives (**C** and **E**) and oxa-(dethia)-*t*-CMP-CoA (**D**) from carba-/oxa-(dethia)malonyl-CoA and L-GHP/(3*S*)-3-methyl-L-GHP. **B**: Mass spectrum (positive ion mode) for the methyl ester of carba(dethia)malonyl-CoA, which was used as a precursor to produce carba(dethia)malonyl-CoA *in situ* employing pig liver esterase<sup>8</sup> prior to addition of the CMPS assay components. **A**: Mass spectrum (positive ion mode) for malonyl-CoA (shown for comparison). Compound numbers from bottom to top **1**, **51**, **54**, **55**.



**Fig. S9** Rationales for the stereoselectivity in the synthesis of (6S)- and (6R)-6-alkyl-*t*-CMP derivatives using MatB/CMPS and Ccr/CMPS, respectively. **A** and **B**: Proposed basis for the observed stereoselectivities of the malonyl-CoA synthetase MatB<sup>9</sup> and the crotonyl-CoA carboxylase reductase Ccr<sup>10,11</sup> for the formation of (2R)- and (2S)-alkylmalonyl-CoA, respectively. The model for the Ccr reaction (right) is based on a structure of the 2-octenoyl-CoA carboxylase reductase CinF complexed with NADP and 2-octenoyl-CoA (pdb 4A0S).<sup>12</sup> The model of 2-ethyl-2-methylmalonyl-CoA in the MatB active site (left) is derived from a MatB structure with its products methylmalonyl-CoA and AMP (pdb 3NYQ).<sup>9</sup> Note the steric proximity of the (2S)-methyl group to the AMP-phosphate (~3 Å). Models were generated using Pymol ([www.pymol.org](http://www.pymol.org)); **C**: Proposed outline mechanism for the selective conversion of (2R)- and (2S)-alkylmalonyl-CoA, by MatB and Ccr catalysis, to a specific C-6 epimer of 6-alkyl-*t*-CMP by engineered CMPSs. The CMPS-catalysed decarboxylation of (2R)- or (2S)-alkylmalonyl-CoA is proposed to give rise to the corresponding (E)- and (Z)-enolates, which react with the imine form of L-GHP to give (6S)- and (6R)-6-alkyl-*t*-CMP, respectively.<sup>13</sup>

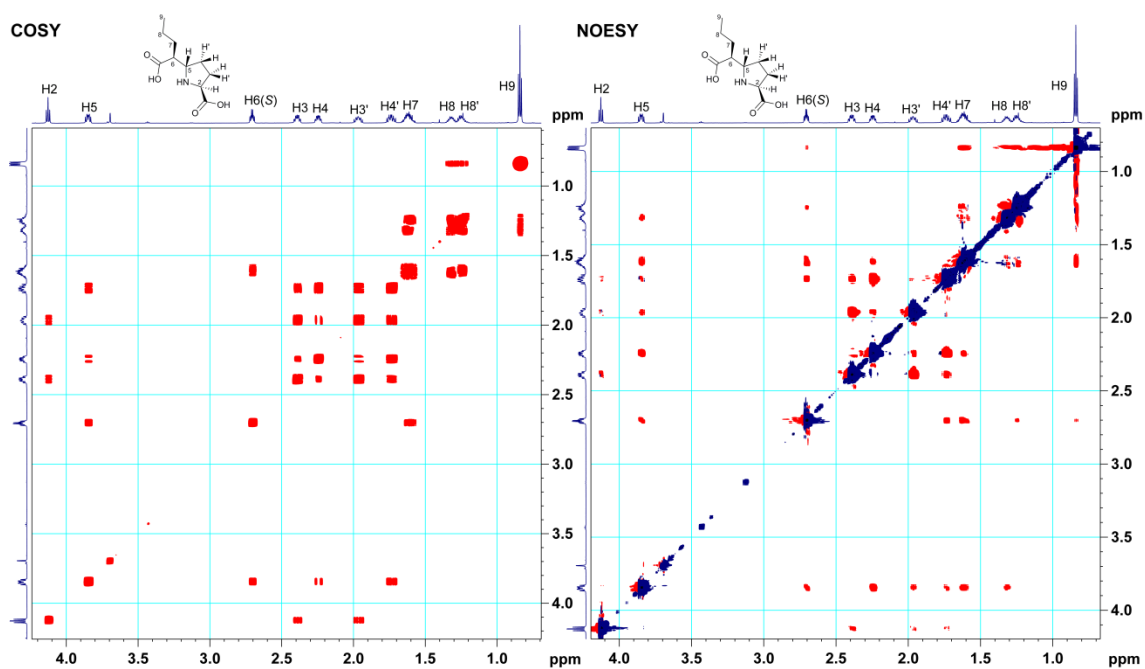


**(6*S*)-6-Allyl-*t*-CMP (43):**



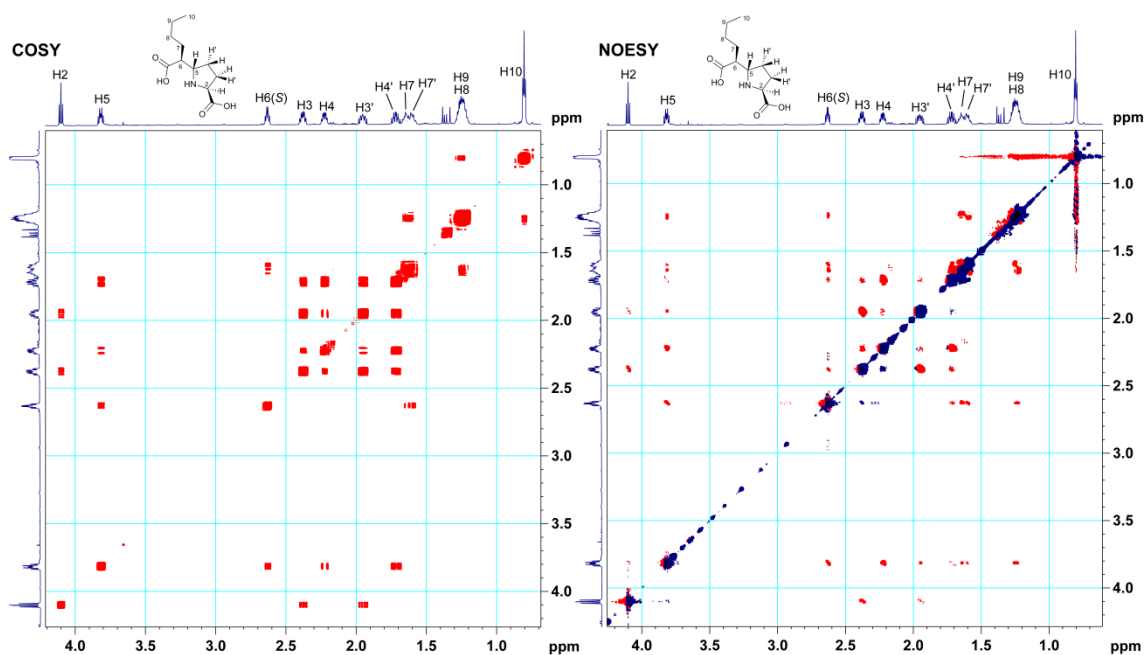
**Fig. S10**  $^1\text{H}$ - $^1\text{H}$  COSY (left) and NOESY (right) spectra for (6*S*)-6-allyl-*t*-CMP (**43**) resulting from the incubation of C-2 allylmalonic acid and L-GHP in the presence of MatB, CarB W79A.

**(6*S*)-6-*n*-Propyl-*t*-CMP (29)**



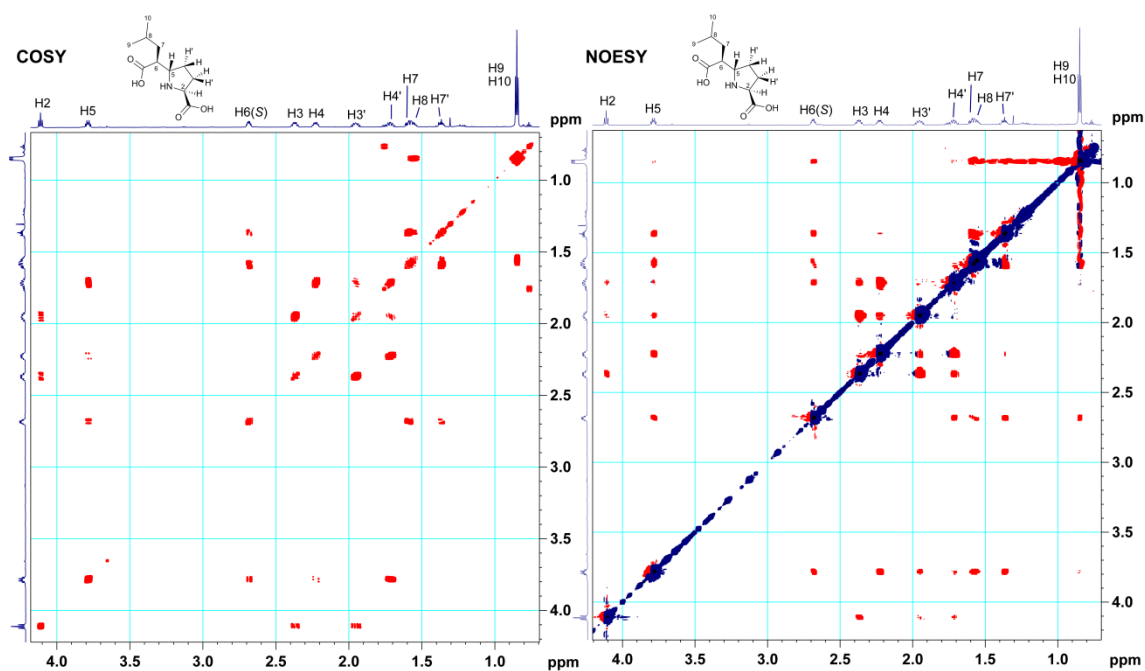
**Fig. S11**  $^1\text{H}$ - $^1\text{H}$  COSY (left) and NOESY (right) spectra for (6*S*)-6-*n*-propyl-*t*-CMP (**29**) resulting from the incubation of C-2 *n*-propylmalonic acid and L-GHP in the presence of MatB, CarB W79A.

**(6*S*)-6-*n*-Butyl-*t*-CMP (30)**



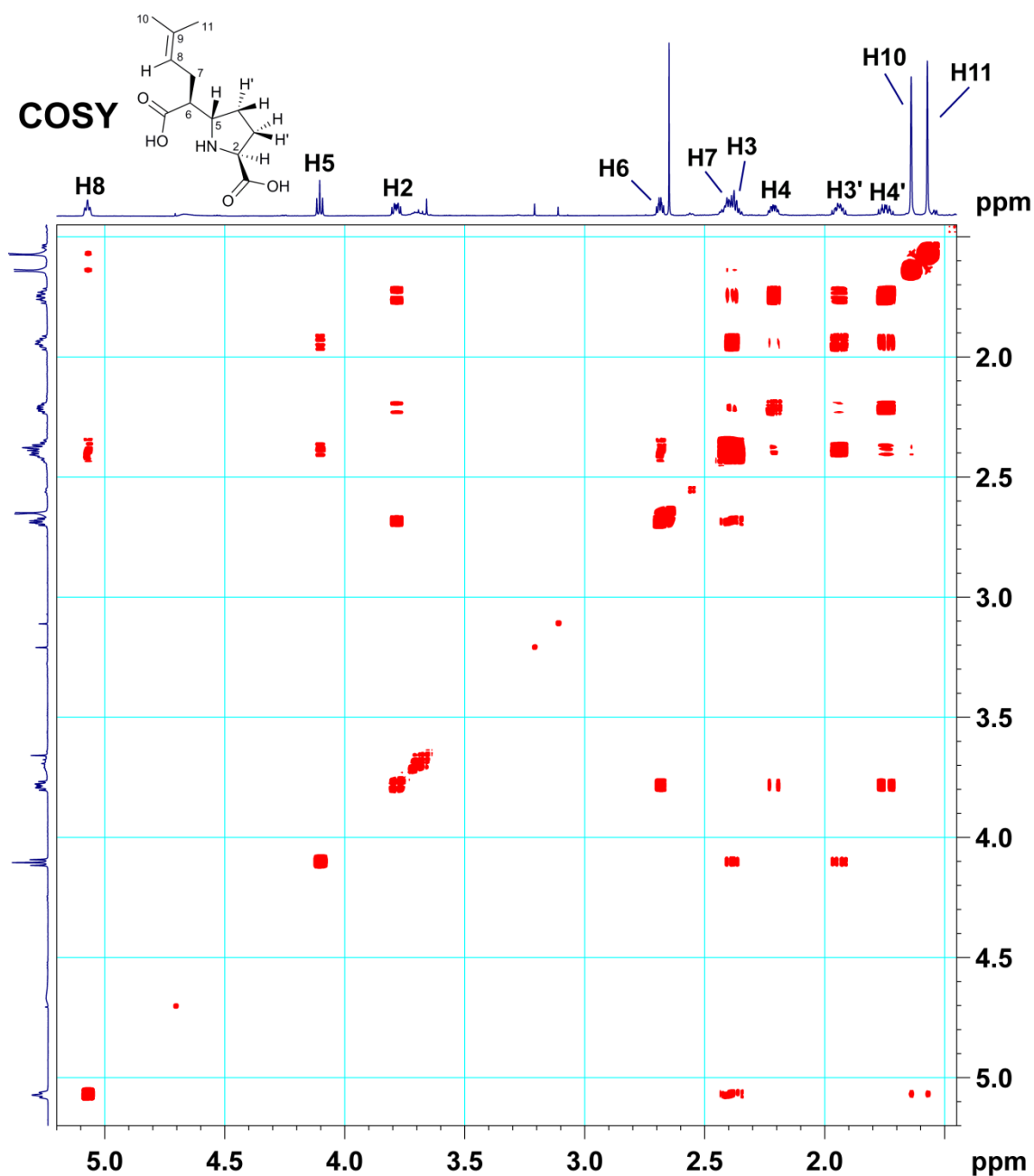
**Fig. S12** <sup>1</sup>H-<sup>1</sup>H COSY (left) and NOESY (right) spectra for (6*S*)-6-*n*-butyl-*t*-CMP (30) resulting from the incubation of C-2 *n*-butylmalonic acid and L-GHP in the presence of MatB, CarB W79A.

**(6*S*)-6-*n*-Isobutyl -*t*-CMP (31)**



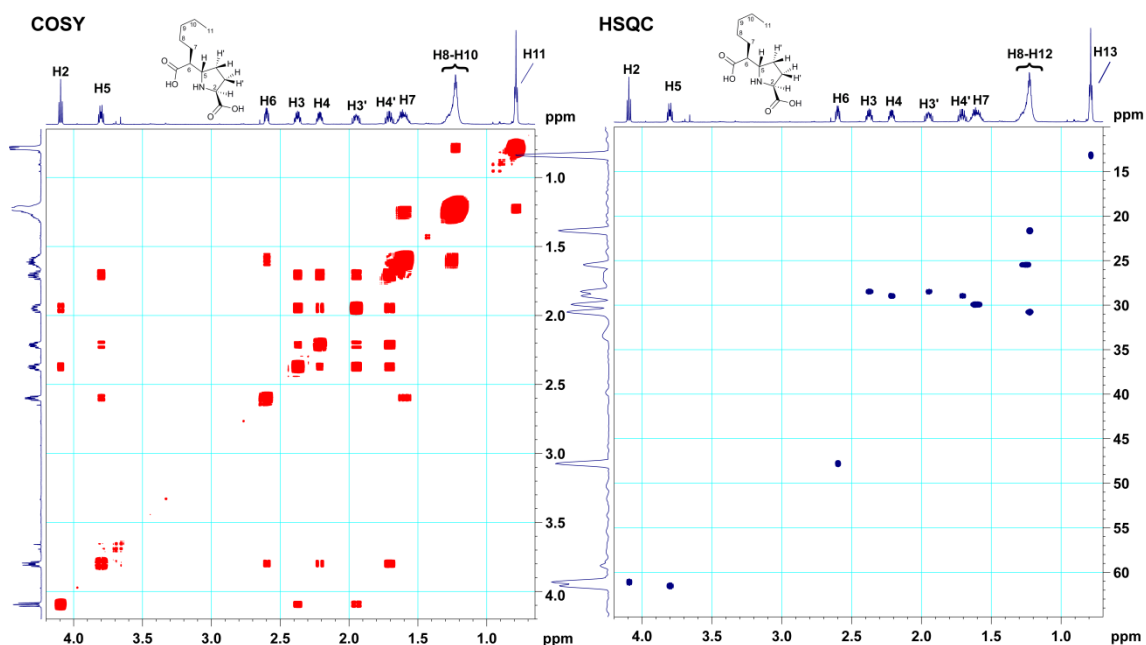
**Fig. S13**  $^1\text{H}$ - $^1\text{H}$  COSY (left) and NOESY (right) spectra for (6*S*)-6-isobutyl-*t*-CMP (**31**) resulting from the incubation of C-2 isobutylmalonic acid and L-GHP in the presence of MatB, CarB W79A.

**(6*S*)-6-Isoprenyl-*t*-CMP (35)**



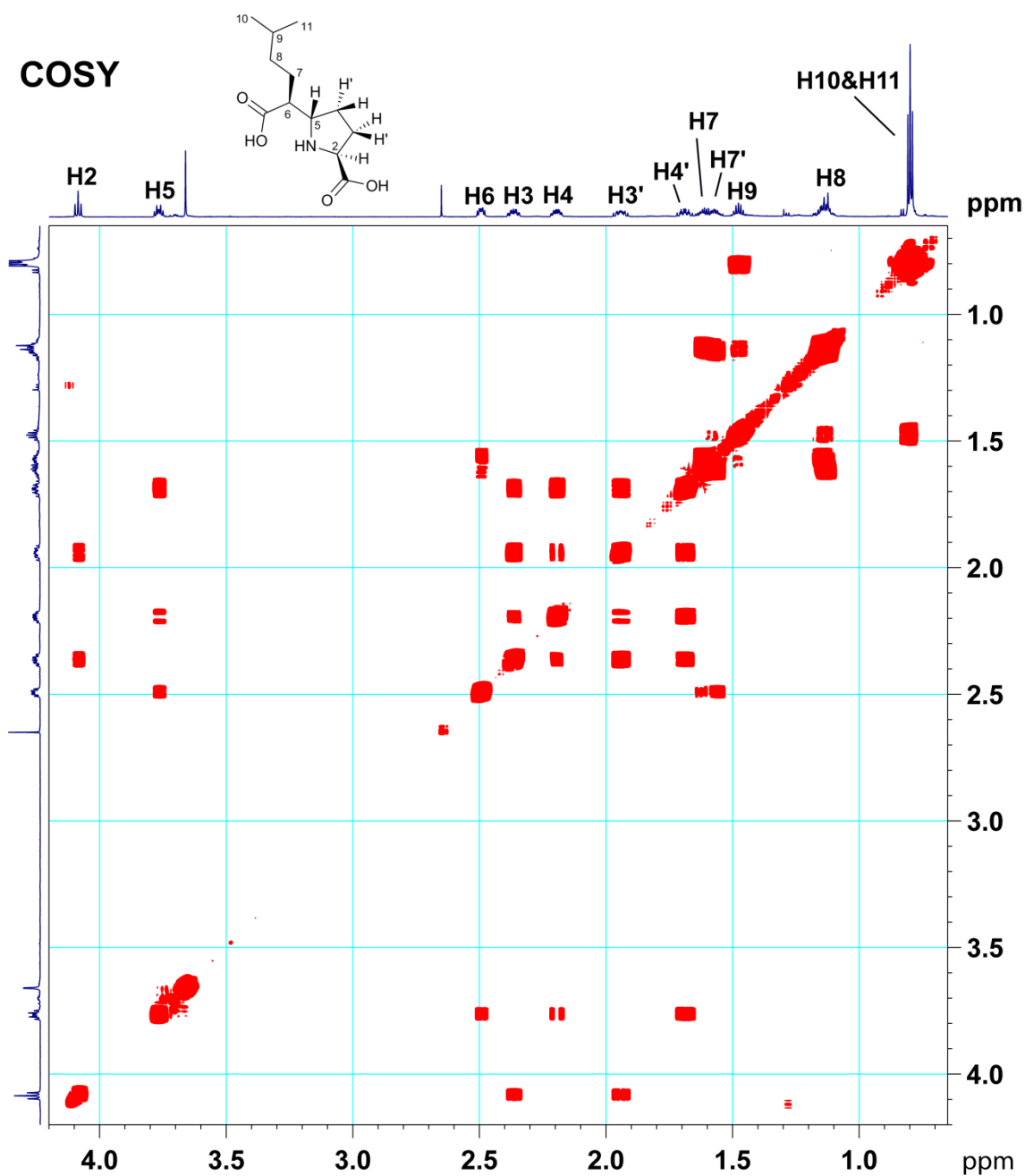
**Fig. S14** <sup>1</sup>H-<sup>1</sup>H COSY spectrum for (6*S*)-6-isoprenyl-*t*-CMP (35) resulting from the incubation of C-2 isoprenylmalonic acid and L-GHP in the presence of MatB, CarB W79A.

**(6*S*)-6-*n*-Pentyl-*t*-CMP (32)**



**Fig. S15** <sup>1</sup>H-<sup>1</sup>H COSY (left) and HSQC (right) spectra for (6*S*)-6-*n*-pentyl-*t*-CMP (32) resulting from the incubation of C-2 *n*-pentylmalonic acid and L-GHP in the presence of MatB, CarB W79A.

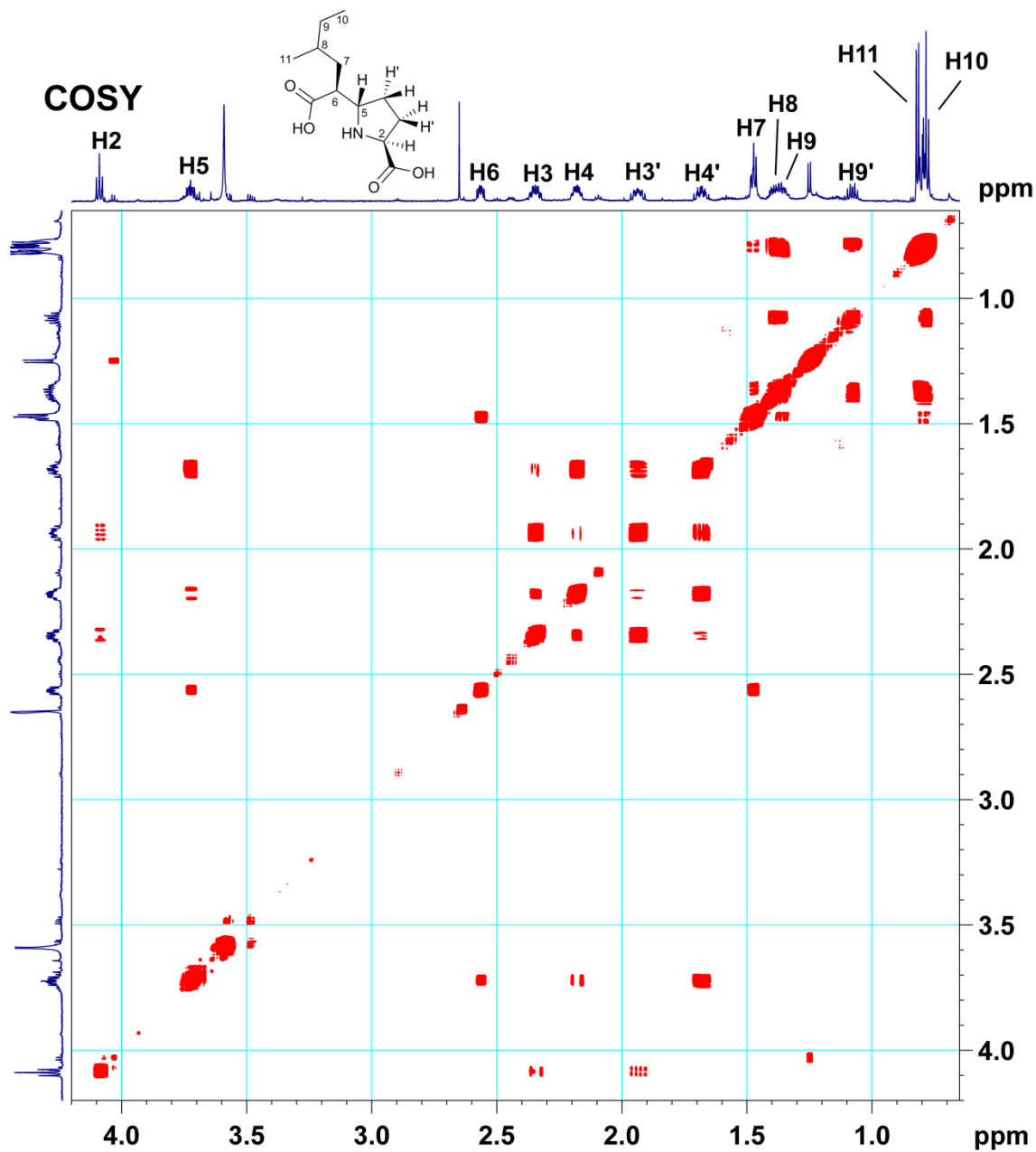
**(6*S*)-6-Isopentyl-*t*-CMP (34)**



**Fig. S16**  $^1\text{H}$ - $^1\text{H}$  COSY spectrum for (6*S*)-6-isopentyl-*t*-CMP (34) resulting from the incubation of C-2 isopentylmalonic acid and L-GHP in the presence of MatB, CarB W79A and other required co-substrates/co-factors.

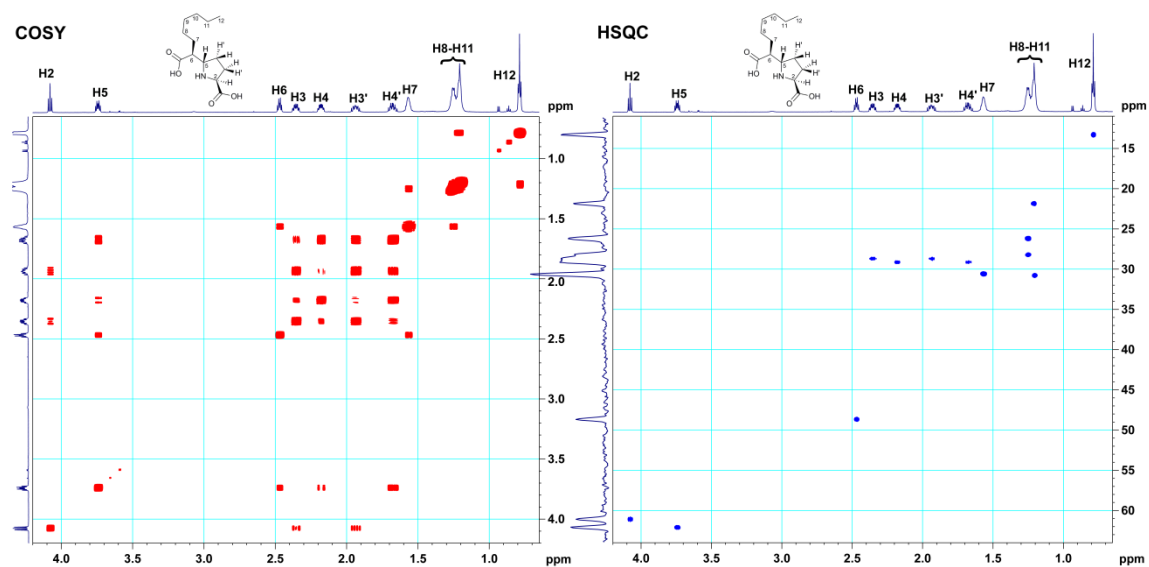


(6*S*)-6-(2-Methylbutyl)-*t*-CMP (33)



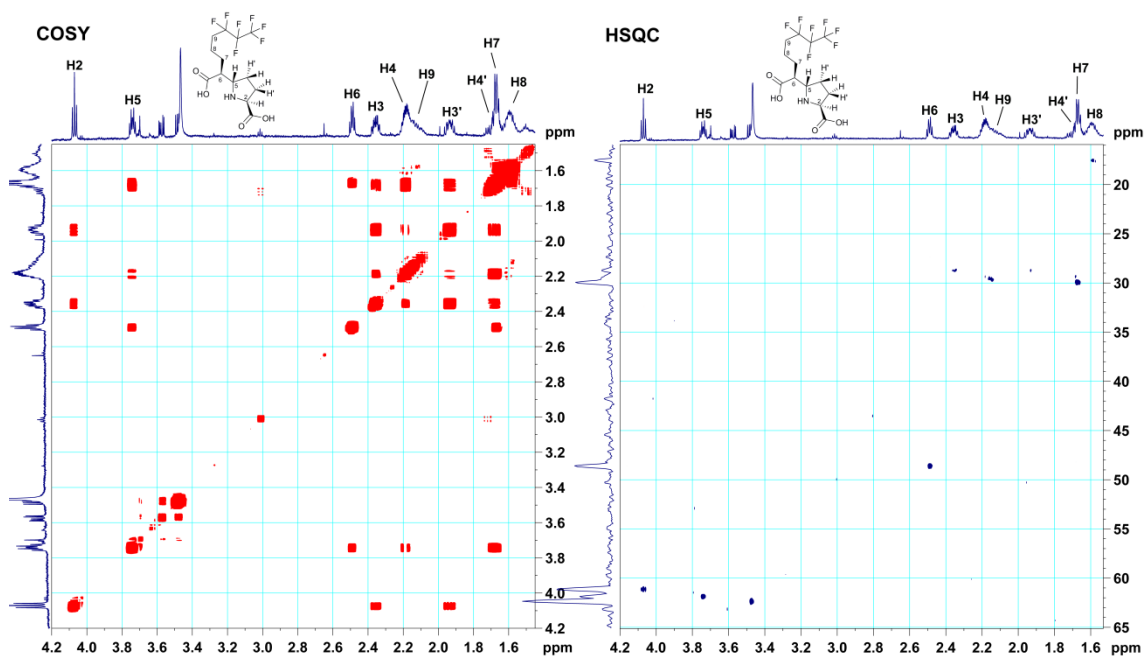
**Fig. S17** <sup>1</sup>H-<sup>1</sup>H COSY spectrum for (6*S*)-6-(2-methylbutyl)-*t*-CMP (33) resulting from the incubation of C-2 (2-methylbutyl)malonic acid and L-GHP in the presence of MatB, CarB W79A.

**(6*S*)-6-*n*-Hexyl-*t*-CMP (36)**



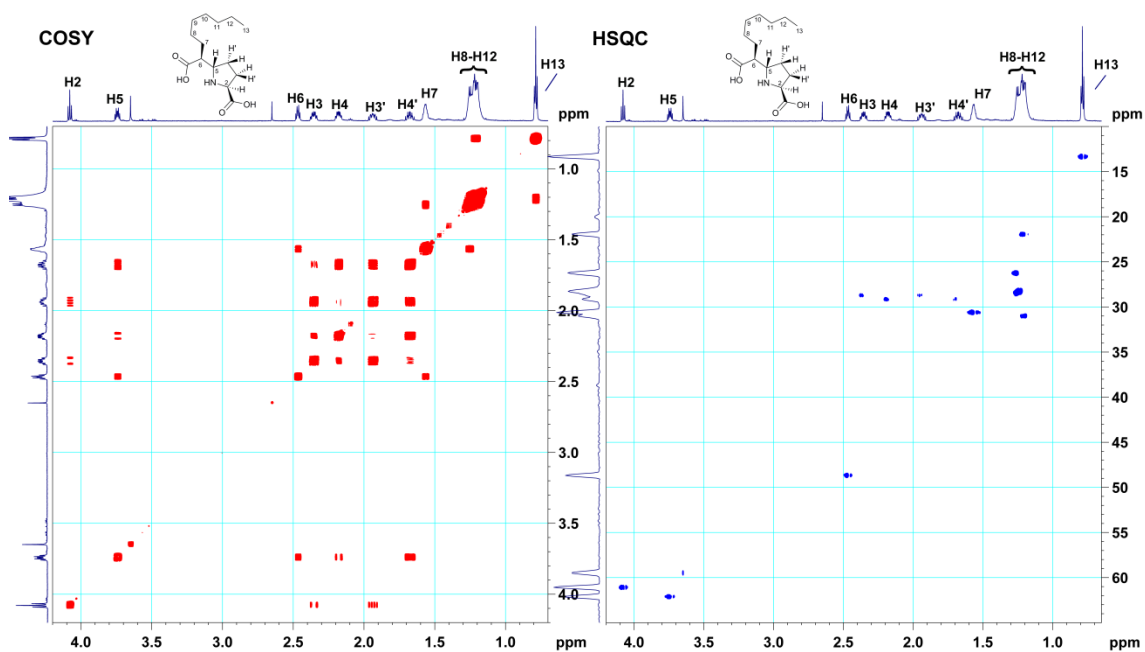
**Fig. S18** <sup>1</sup>H-<sup>1</sup>H COSY (left) and HSQC (right) spectra for (6*S*)-6-*n*-hexyl-*t*-CMP (36) resulting from the incubation of C-2 *n*-hexylmalonic acid and L-GHP in the presence of MatB, CarB W79A.

**(6*S*)-6-(4,4,5,5,6,6,6-Heptafluorohexyl)-*t*-CMP (37)**



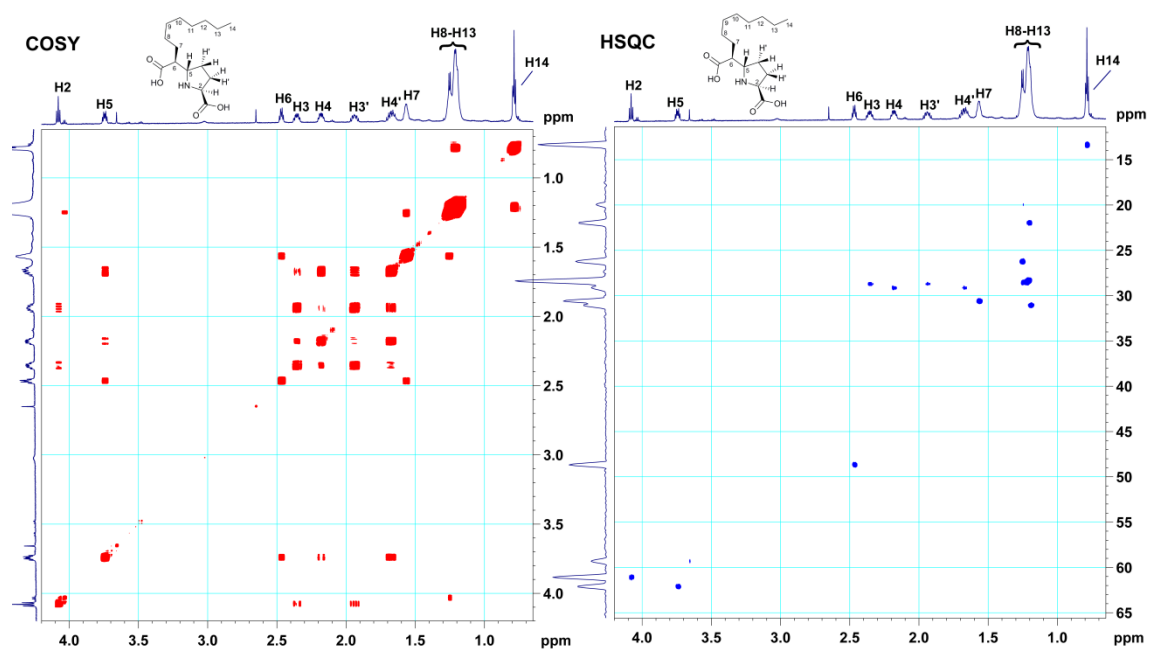
**Fig. S19**  $^1\text{H}$ - $^1\text{H}$  COSY (left) and HSQC (right) spectra for (6*S*)-6-(4,4,5,5,6,6,6-heptafluorohexyl)-*t*-CMP (37) resulting from the incubation of C-2 (4,4,5,5,6,6,6-heptafluorohexyl)-malonic acid and L-GHP in the presence of MatB, CarB W79A.

**(6*S*)-6-*n*-Heptyl-*t*-CMP (38)**



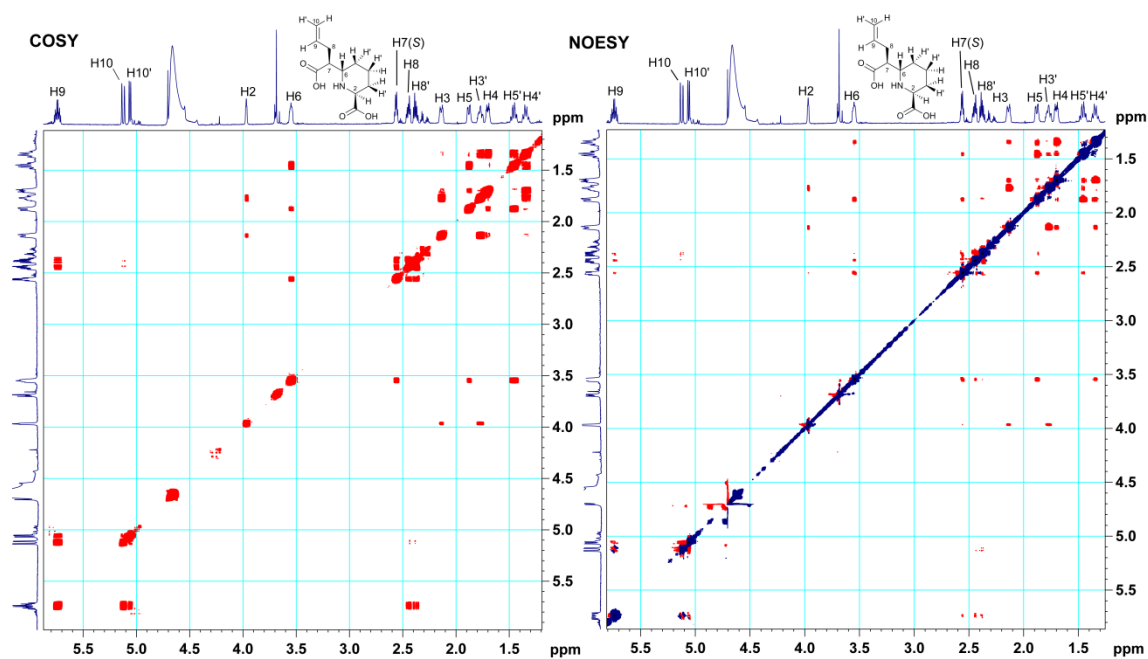
**Fig. S20** <sup>1</sup>H-<sup>1</sup>H COSY (left) and HSQC (right) spectra for (6*S*)-6-*n*-heptyl-*t*-CMP (38) resulting from the incubation of C-2 *n*-heptylmalonic acid and L-GHP in the presence of MatB, CarB W79A.

**(6*S*)-6-*n*-Octyl-*t*-CMP (39)**



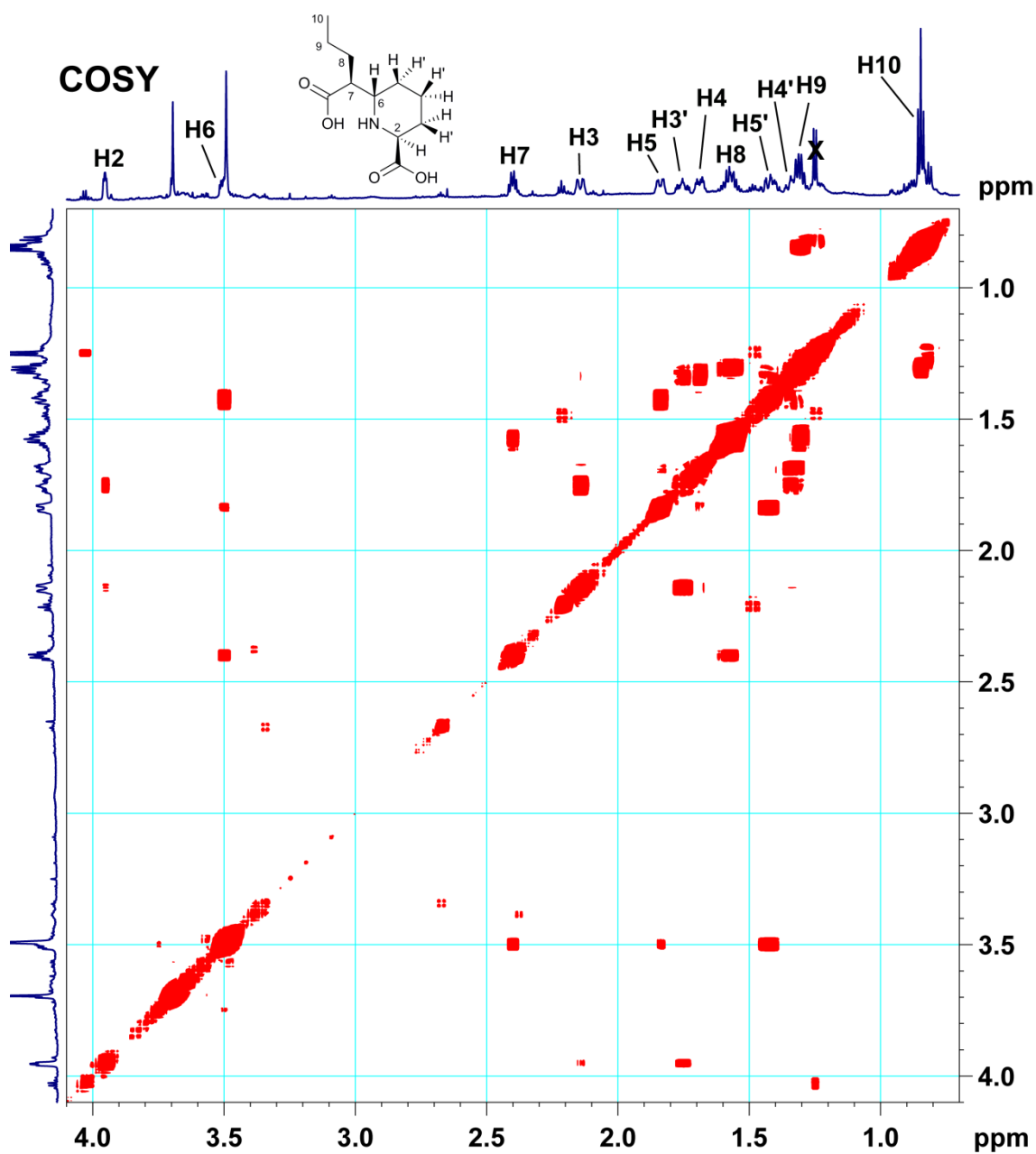
**Fig. S21** <sup>1</sup>H-<sup>1</sup>H COSY (left) and HSQC (right) spectra for (6*S*)-6-*n*-octyl-*t*-CMP (**39**) resulting from the incubation of C-2 *n*-octylmalonic acid and L-GHP in the presence of MatB, CarB W79A.

**(7*S*)-7-Allyl-*t*-CMPi (43)**



**Fig. S22**  $^1\text{H}$ - $^1\text{H}$  COSY (left) and NOESY (right) spectra for (7*S*)-7-allyl-*t*-CMPi (**43**) resulting from the incubation of C-2 allylmalonic acid and L-GHP in the presence of MatB, CarB W79A and other required co-substrates/co-factors.

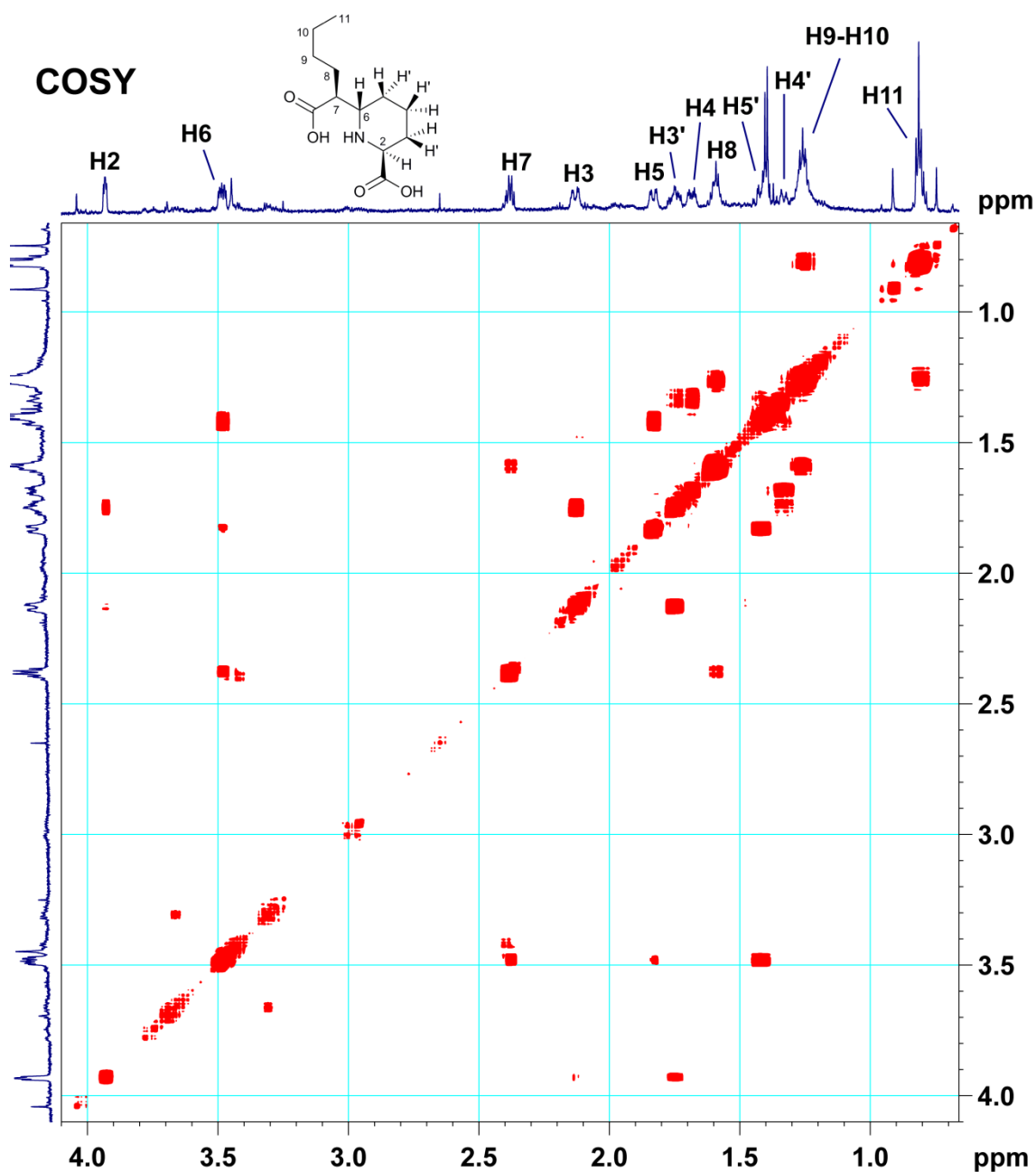
**(7*S*)-7-*n*-Propyl-*t*-CMPi (44)**



**Fig. S23** <sup>1</sup>H-<sup>1</sup>H COSY spectrum for (7*S*)-7-*n*-propyl-*t*-CMPi (44) resulting from the incubation of C-2 *n*-propylmalonic acid and L-GHP in the presence of MatB, CarB W79A.

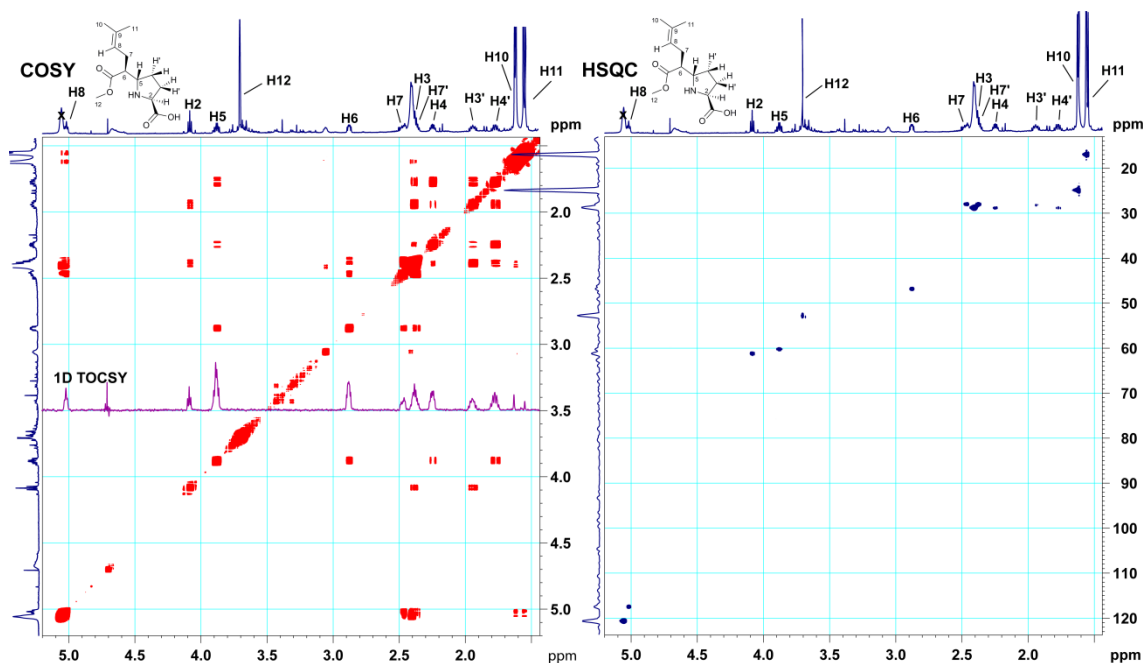


(7*S*)-7-*n*-Butyl-*t*-CMPi (45)

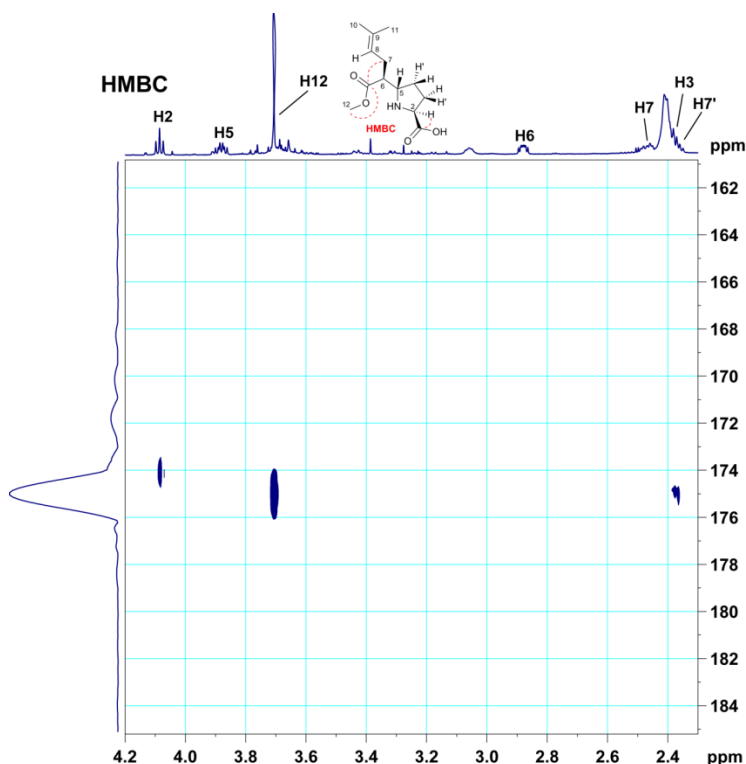


**Fig. S24**  $^1\text{H}$ - $^1\text{H}$  COSY spectrum for (7*S*)-7-*n*-butyl-*t*-CMPi (45) resulting from the incubation of C-2 *n*-butylmalonic acid and L-GHP in the presence of MatB, CarB W79A.

**(2*S*,5*S*)-5-((*S*)-1-Methoxy-5-methyl-1-oxohex-4-en-2-yl)pyrrolidine-2-carboxylic acid (**48**)**



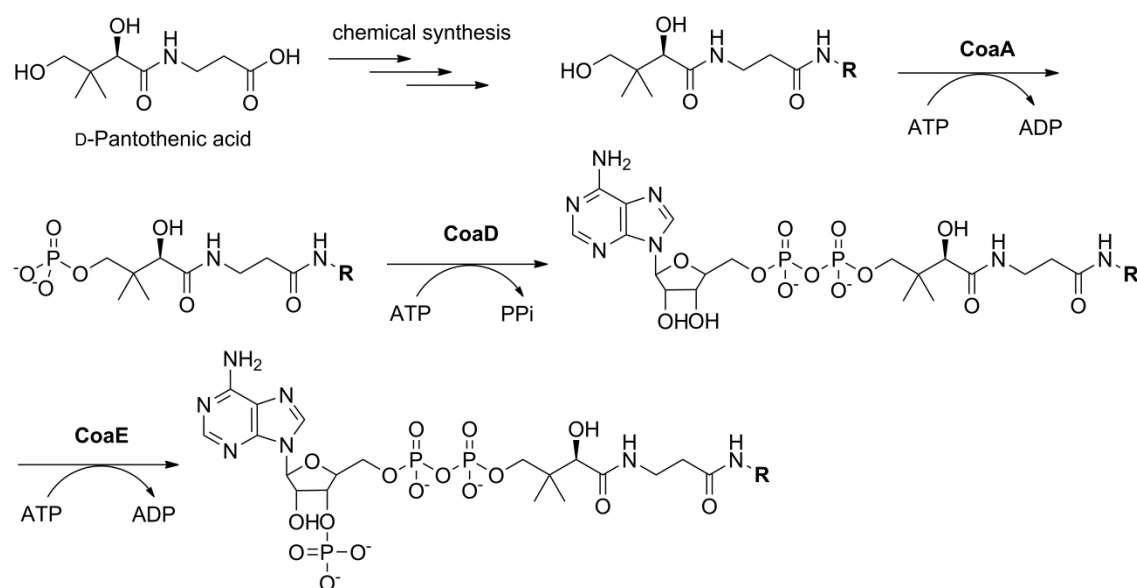
**Fig. S25**  $^1\text{H}$ - $^1\text{H}$  COSY (left) and HSQC (right) and 1D-TOCSY (by selective excitation of H5,  $\tau_m = 150$  ms) spectra for (2*S*,5*S*)-5-((*S*)-1-methoxy-5-methyl-1-oxohex-4-en-2-yl)pyrrolidine-2-carboxylic acid (**48**) resulting from the incubation of C-2 isoprenylmalonic acid and L-GHP in the presence of MatB, CarB W79A, pantetheine (as a replacement for the wildtype substrate coenzyme A).



**Fig. S26** Part of the HMBC spectrum for (2*S*,5*S*)-5-((*S*)-1-methoxy-5-methyl-1-oxohex-4-en-2-yl)pyrrolidine-2-carboxylic acid (**48**) resulting from the incubation of C-2 isoprenylmalonic acid and L-GHP in the presence of MatB, CarB W79A, pantetheine (as a replacement for the wildtype substrate coenzyme A). Note the correlation between the protons at C7 and C12 to carbonyl group of the 5-carboxymethyl substituent revealing the ester on the side chain.

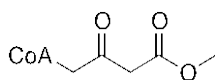
## V- Synthesis of malonyl-(dethia)CoA analogues

**General procedure for the enzymatic conversion of pantetheine analogues into (dethia)CoA analogues:** The enzymatic synthesis of CoA analogues from the corresponding pantetheine analogues was performed by adapting the ‘one-pot’ enzyme cascade catalysis (ECC) procedure from Tosin *et al.*<sup>8</sup> and Wright and coworkers.<sup>14</sup> Pantethenate substrates (10-50  $\mu$ mol) were dissolved in reaction buffer (50 mM HEPES pH 9.0, 25 mM KCl, 10 mM MgCl<sub>2</sub>) together with ATP disodium salt hydrate (8-12 eq.) and the pH was adjusted to 8.0 using 10% aqueous sodium hydroxide solution. The three enzymes (CoaA, CoaD, CoaE, 900  $\mu$ g each) were added at 30 min intervals and the reaction was left at room temperature for 16 h. One assay volume of cold acetonitrile was added and, after vortexing for 1 min, the mixture was left at 0 °C for 15 min before centrifugation (45 min, 13,000 rpm) to remove the precipitated proteins. The supernatant was freeze-dried, dissolved in 0.1% aqueous formic acid (2.0–8.0 mL) and subjected to preparative HPLC.



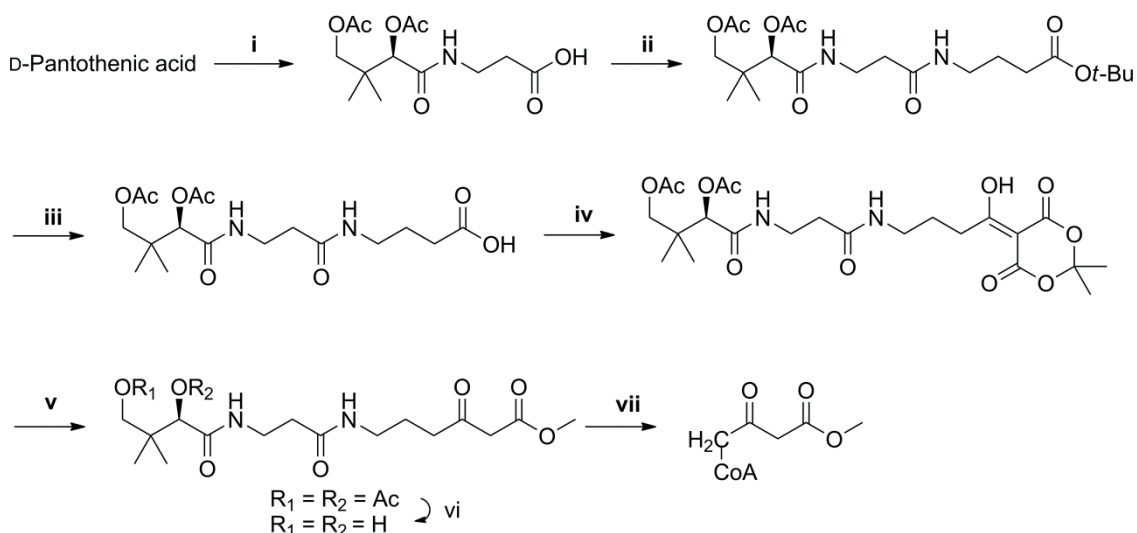
**Scheme S1:** General scheme for the chemoenzymatic synthesis of malonyl-(dethia)CoA analogues. The CoASH biosynthesis enzymes CoaA, CoaD and CoaE can accept pantothenate analogues with different “R” groups.<sup>15,16</sup>

## Malonyl-carba(dethia)CoA methyl ester<sup>8</sup>

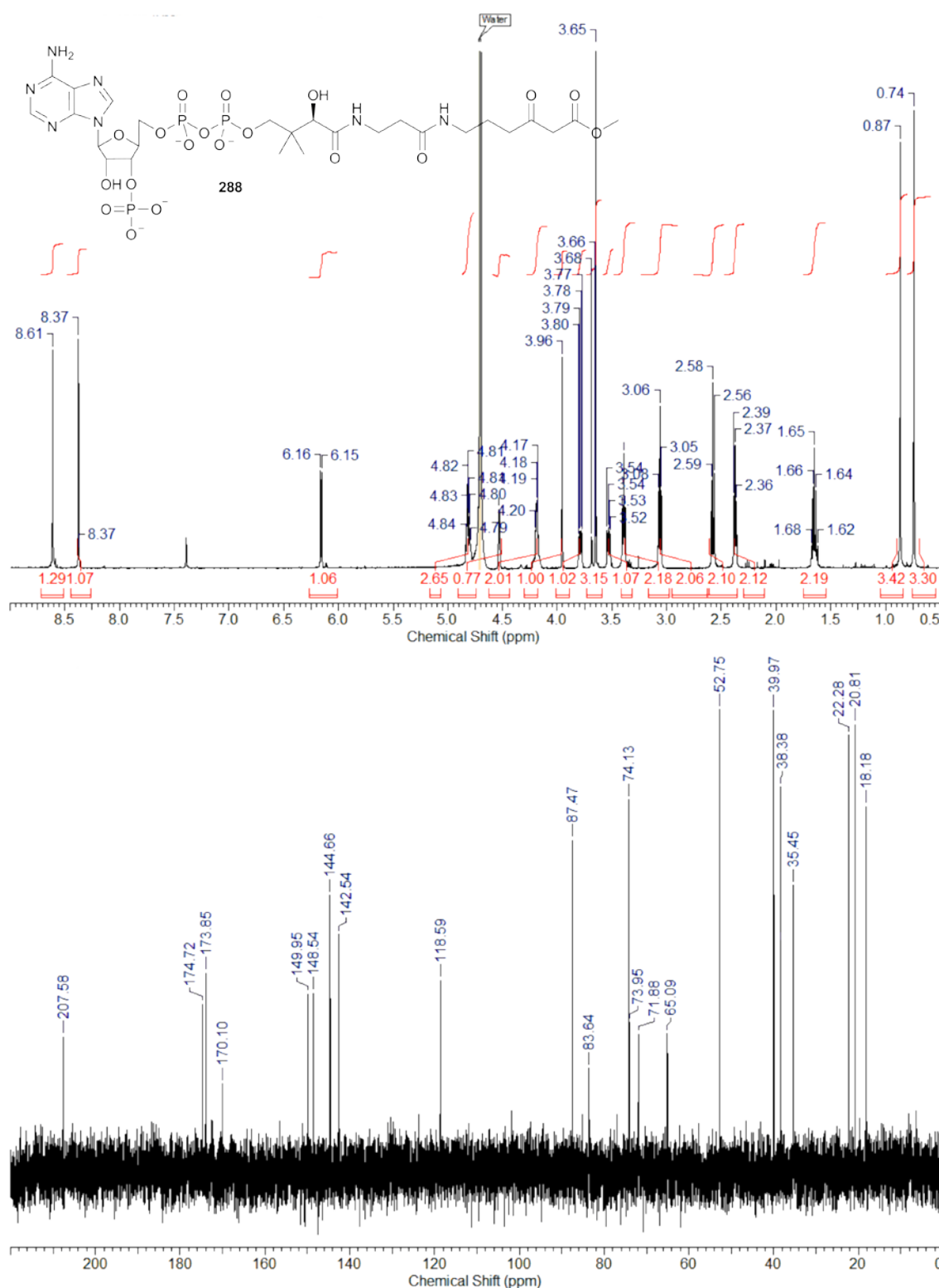


Methyl 6-((*N*-[(*2R*)-2,4-dihydroxy-3,3-dimethylbutanoyl]-β-alanyl)amino)-3-oxohexanoate (10 mg, 27.7 μmol) was incubated under ECC conditions in the presence of CoaA, CoaD and CoaE as described in page 29. Preparative HPLC (2% aqueous acetonitrile, acidified with 0.1% formic acid, over 5 min followed by 2–20% aqueous acetonitrile, acidified with 0.1% formic acid, over 25 min) gave malonyl-carba(dethia)-CoA methyl ester as a white foam (*R* = 17.1 min, 10.6 mg, 45%).

<sup>1</sup>H NMR (500 MHz, D<sub>2</sub>O): δ = 8.66 (*s*, 1H), 8.42 (*s*, 1H), 6.21 (*d*, *J* = 5.5, 1H), 4.86 (*app bs*, 2H), 4.58 (*app bs*, 1H), 4.24 (*app bs*, 2H), 4.00 (*s*, 1H), 3.84 (*dd*, *J* = 10.0, 5.0, 1H), 3.70 (*s*, 3H), 3.58 (*dd*, *J* = 10.0, 5.0, 1H), 3.44 (*t*, *J* = 6.5, 2H), 3.11 (*t*, *J* = 7.0, 2H), 2.62 (*t*, *J* = 7.0, 2H), 2.42 (*t*, *J* = 6.5, 2H), 1.70 (*quin*, *J* = 7.0, 2H), 0.91 (*s*, 3H), 0.79 (*s*, 3H); <sup>13</sup>C NMR (125 MHz, D<sub>2</sub>O): δ = 207.6, 174.8, 173.9, 170.1, 150.0, 148.6, 144.7, 142.6, 118.6, 87.5, 83.6, 74.2, 71.9, 65.1, 52.8, 40.0, 38.4, 35.5, 22.3, 20.8, 18.2; <sup>31</sup>P NMR (202 MHz, D<sub>2</sub>O): δ = 0.9 (*s*, 1P), -9.7 (*app bs*, 1P), -10.3 (*app bs*, 1P); HR ESI-MS: 870.1457 ([*M*+Na-2H]<sup>-</sup>, C<sub>26</sub>H<sub>40</sub>N<sub>7</sub>NaO<sub>19</sub>P<sub>3</sub><sup>-</sup>, calc. 870.1457).

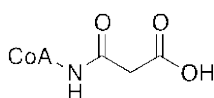


**Scheme S2: Chemoenzymatic synthesis of malonyl-carba (dethia)CoA methyl ester.** (i) Ac<sub>2</sub>O, cat. I<sub>2</sub>, 0 °C–rt, 48 h., 97%; (ii) GABA-*O*-*t*Bu, Et<sub>3</sub>N, HOBt, 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide.HCl, CH<sub>2</sub>Cl<sub>2</sub>, 0 °C–rt, 18 h, 93%; (iii) 10% TFA in CH<sub>2</sub>Cl<sub>2</sub>, 3 h, rt, app. quant.; (iv) Meldrum's acid, DMAP, EDC HCl, CH<sub>2</sub>Cl<sub>2</sub>, 0 °C–rt, 18 h; (v) MeOH, reflux, 18 h, 77% over two steps; (vi) K<sub>2</sub>CO<sub>3</sub>, MeOH, 0 °C, 5 h, 55%; (vii) CoaA, CoaD, CoaE, 50 mM Tris, 20 mM KCl, 10 mM MgCl<sub>2</sub>, ATP, pH 8.0, 16 h, rt, 43–52%.



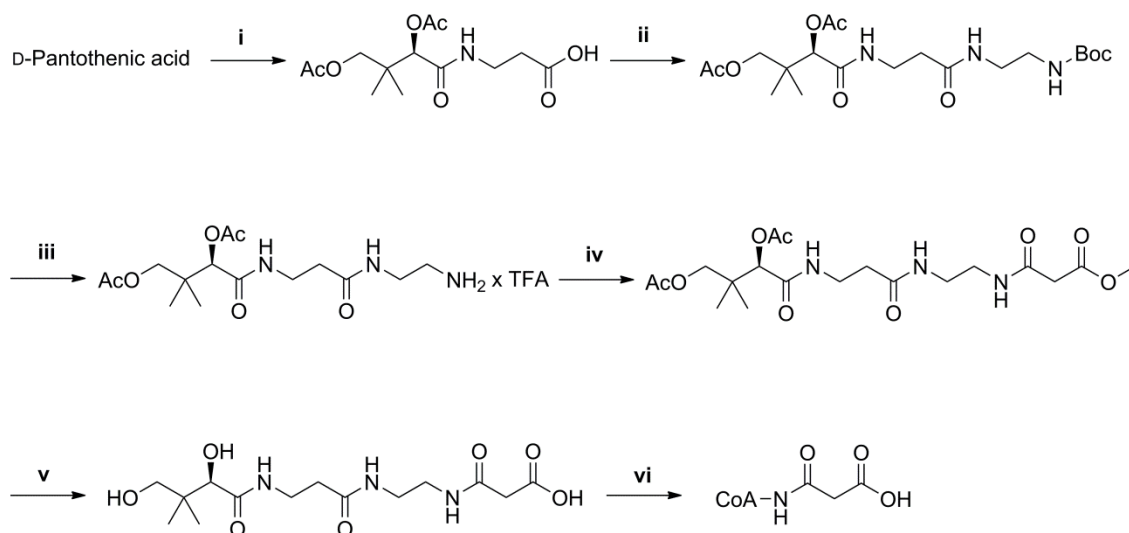
**Fig. S27** <sup>1</sup>H and <sup>13</sup>C NMR spectra (in D<sub>2</sub>O, at 500 MHz and 125 MHz, respectively) for Malonyl-carba(dethia)CoA methyl ester.

## Malonyl-aza(dethia)CoA

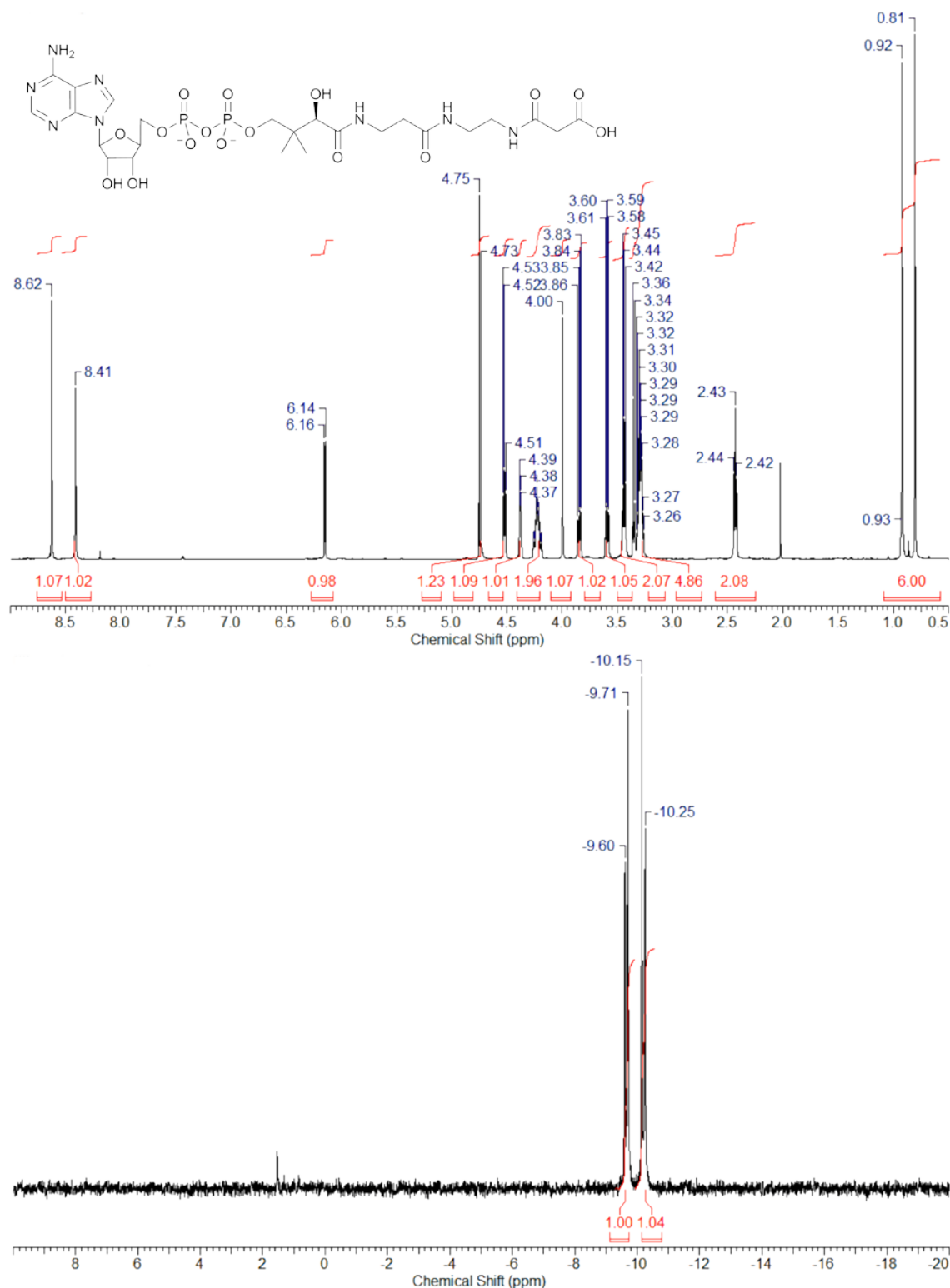


3-([2-((*N* -[(2*R*)-2,4-Dihydroxy-3,3-dimethylbutanoyl]- β-alanyl)amino)ethyl]amino)-3-oxopropanoic acid (12.6 mg, 38.0 μmol) was incubated under ECC conditions in the presence of CoaA, CoaD and CoaE as described in page 29. Preparative HPLC (2% aqueous acetonitrile, acidified with 0.1% formic acid, over 10 min, followed by 2–20% aqueous acetonitrile, acidified with 0.1% formic acid, over 10 min then 20–98% aqueous acetonitrile, acidified with 0.1% formic acid, over 5 min) gave malonyl-aza(dethia)CoA as a white foam (*t*<sub>R</sub> = 19.6 min, 13.4 mg, 42%).

<sup>1</sup>H NMR (500 MHz, D<sub>2</sub>O): δ = 8.62 (*s*, 1H), 8.41 (*s*, 1H), 6.15 (*d*, *J* = 5.5, 1H), 4.89–4.84 (*app bs*, 1H), 4.73 (*app bs*, 1H), 4.58 (*app bs*, 1H), 4.38 (*m*, 1H), 4.28–4.20 (*m*, 2H), 4.00 (*s*, 1H), 3.85 (*dd*, *J* = 10.0, 5.0, 1H), 3.59 (*dd*, *J* = 10.0, 5.0, 1H), 3.44 (*t*, *J* = 6.5, 2H), 3.33–3.26 (*m*, 4H), 2.43 (*t*, *J* = 6.5, 2H), 0.92 (*s*, 3H), 0.81 (*s*, 3H); <sup>31</sup>P NMR (202 MHz, D<sub>2</sub>O): δ = 0.9 (*s*, 1P), - 9.7 (*d*, *J* = 21.0, 1P), - 10.2 (*d*, *J* = 21.0, 1P); HR ESI-MS: 859.1448 ([*M*+*Na*]<sup>+</sup>, C<sub>24</sub>H<sub>39</sub>N<sub>8</sub>NaO<sub>19</sub>P<sub>3</sub><sup>+</sup>, calc. 859.1437).



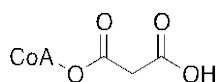
**Scheme S3: Chemoenzymatic synthesis of malonyl-aza (dethia)CoA analogue.** (i) Ac<sub>2</sub>O, cat. I<sub>2</sub>, 0 °C–rt, 48 h., 97%; (ii) *tert*-Butyl (2-aminoethyl)carbamate, Et<sub>3</sub>N, HOBt, EDC HCl, THF, 0 °C–rt, 32 h., 59%; (iii) 10% TFA in CH<sub>2</sub>Cl<sub>2</sub>, 4 h, rt, app. quant.; (iv) Malonate monomethyl ester, DIPEA, HOBt, EDC HCl, THF, 0 °C–rt, 24 h., 22%; (v) K<sub>2</sub>CO<sub>3</sub>, MeOH, rt, 16 h, app. quant.; (vi) CoaA, CoaD, CoaE, 50 mM Tris, 20 mM KCl, 10 mM MgCl<sub>2</sub>, ATP, pH 8.0, 16 h, rt.



**Fig. S28** <sup>1</sup>H and <sup>31</sup>P NMR spectra (in D<sub>2</sub>O at 500 MHz and 202 MHz, respectively) for malonyl-aza (dethia)CoA analogues.

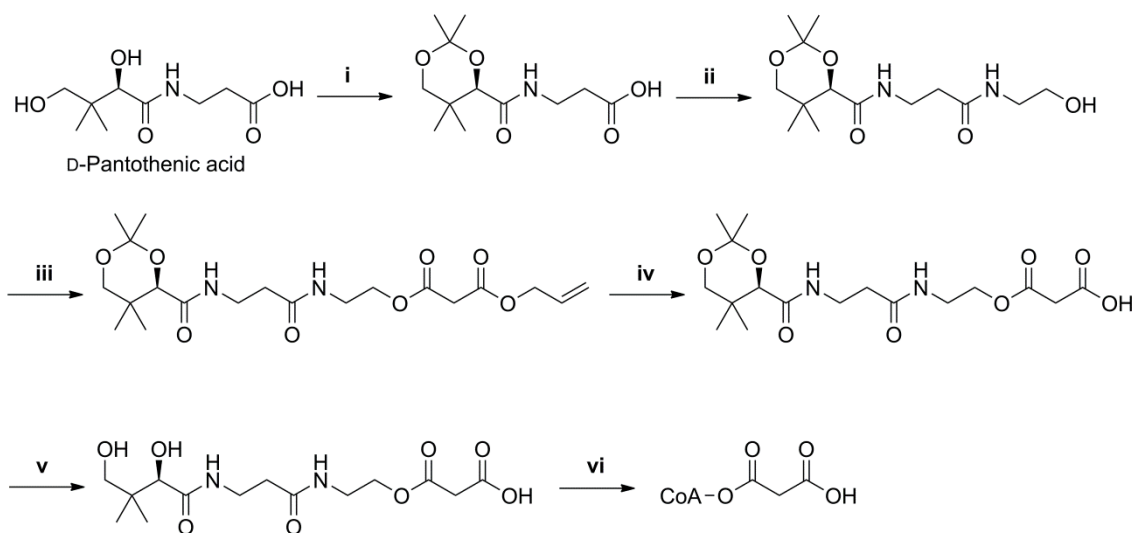


## Malonyl-oxa(dethia)CoA<sup>8</sup>

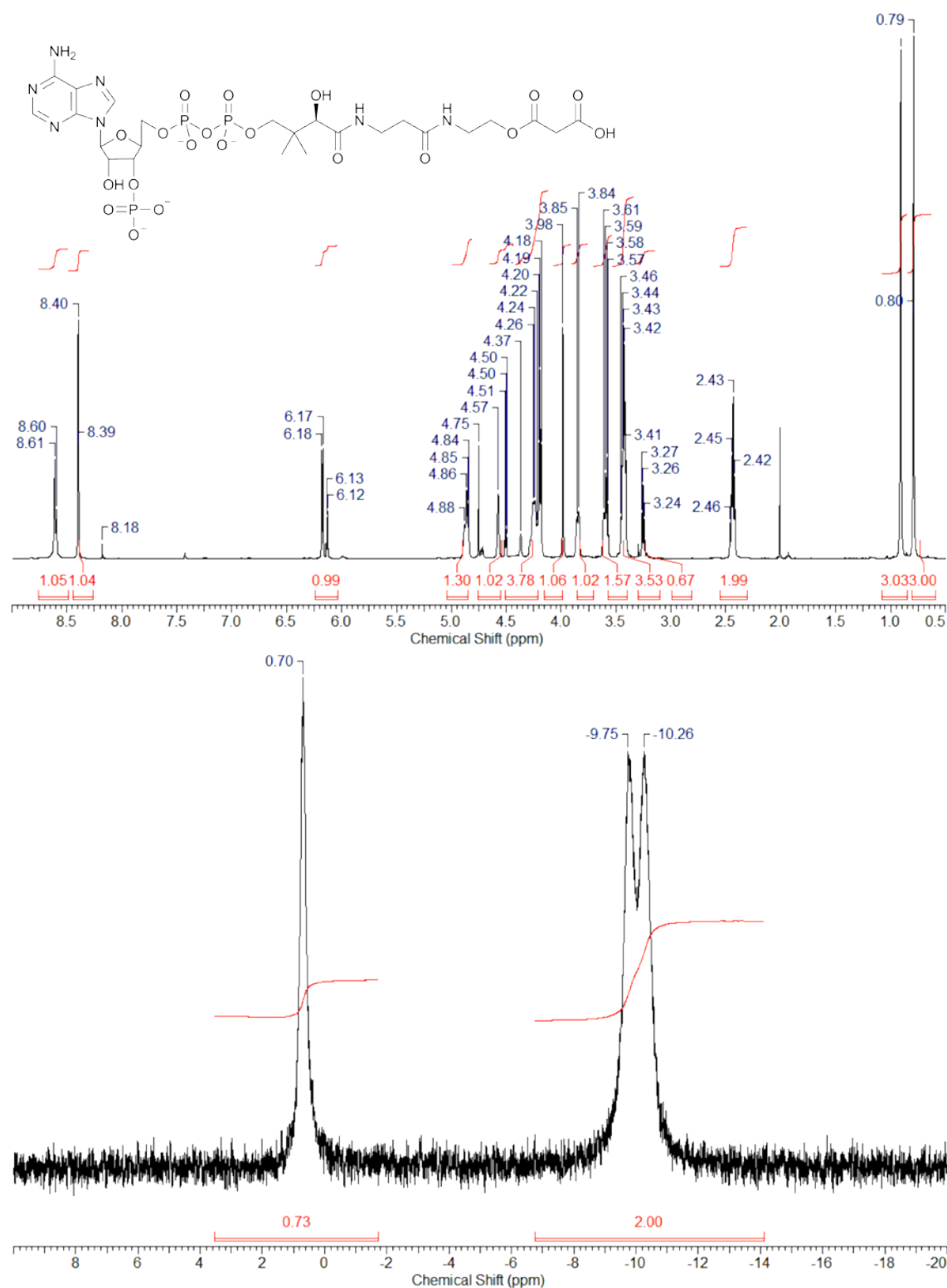


3-[2-((*N*-[(2*R*)-2,4-Dihydroxy-3,3-dimethylbutanoyl]- $\beta$ -alanyl)amino)ethoxy]-3-oxopropanoic acid (9.5 mg, 27.3  $\mu$ mol) was incubated under ECC conditions in the presence of CoaA, CoaD and CoaE as described above. Preparative HPLC (2% aqueous acetonitrile, acidified with 0.1% formic acid, over 5 min, followed by 2–20% aqueous acetonitrile, acidified with 0.1% formic acid, over 15 min and 20–98% aqueous acetonitrile, acidified with 0.1% formic acid, over 5 min) gave malonyl-oxo(dethia)CoA as a white foam ( $t_R$  = 19.6 min, 9.7 mg, 43%).

<sup>1</sup>H NMR (500 MHz, D<sub>2</sub>O):  $\delta$  = 8.67 (*s*, 1H), 8.43 (*s*, 1H), 6.21 (*d*,  $J$  = 5.5, 1H), 4.88–4.85 (*m*, 1H), 4.74 (*s*, 1H), 4.25 (*d*,  $J$  = 7.5, 1H), 4.21 (*t*,  $J$  = 5.5, 2H), 4.01 (*s*, 1H), 3.84 (*dd*,  $J$  = 9.5, 4.5, 1H), 3.58 (*dd*,  $J$  = 9.5, 4.5, 1H), 3.44–3.41 (*m*, 4H), 2.45 (*t*,  $J$  = 6.5, 2H), 0.92 (*s*, 3H), 0.79 (*s*, 3H); <sup>31</sup>P NMR (202 MHz, D<sub>2</sub>O):  $\delta$  = 0.7 (*s*, 1P), - 9.8 (*app bs*, 1P), - 10.3 (*app bs*, 1P); HR ESI-MS: 858.1129([*M*-2H+Na]<sup>+</sup>, C<sub>24</sub>H<sub>36</sub>N<sub>7</sub>NaO<sub>2</sub>P<sub>3</sub><sup>+</sup>, calc. 858.1131).

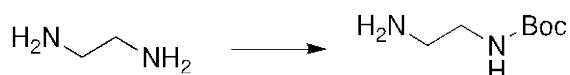


**Scheme S4: Chemoenzymatic synthesis of malonyl-oxo (dethia)CoA analogues.** (i) DMP, *p*-TsOH, rt, 18 h, 81%; (ii) Et<sub>3</sub>N, ethylchloroformate, 2-aminoethanol, CH<sub>2</sub>Cl<sub>2</sub>, 0 °C–rt, 3 h, 55%; (iii) Malonate monoallyl ester, DIPEA, HBTU, THF, 0 °C–rt, 16 h, 30%; (iv) Pd(Ph<sub>3</sub>P)<sub>4</sub>, pyrrolidine, ACN, 0 °C, 3 h, 93%; (v) DOWEX 50WX8-400 (H<sup>+</sup>), H<sub>2</sub>O, rt, 16 h, app. quant.; (vi) CoaA, CoaD, CoaE, 50 mM Tris, 20 mM KCl, 10 mM MgCl<sub>2</sub>, ATP, pH 8.0, 16 h, rt, 43–52%.



**Fig. S29** <sup>1</sup>H and <sup>31</sup>P NMR spectra (in D<sub>2</sub>O at 500 MHz and 202 MHz, respectively) for malonyl-oxo (dethia)CoA analogues.

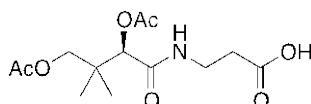
### ***tert*-Butyl (2-aminoethyl)carbamate**



*tert*-Butyl (2-aminoethyl)carbamate was synthesised by adaption of the reported procedures.<sup>17,18</sup> A solution of di-*tert*-butyl dicarbonate (8.73 g, 40 mmol) in 1,4-dioxane (10 mL) was added dropwise over 30 min to a solution of ethane-1,2-diamine (8.1 mL, 3 eq.) in 1,4-dioxane (40 mL). A white precipitate was formed slowly as the reaction was stirred at room temperature. After 4 h, the mixture was filtered and the clear solution was concentrated to yield *tert*-butyl (2-aminoethyl)carbamate as a white low melting point solid (5.79 g, 90%).

mp  $\leq 35^\circ\text{C}$ ; TLC: 3:1  $\text{CH}_2\text{Cl}_2/\text{MeOH}$ ,  $R_f = 0.50$ ; IR (neat)  $\nu/\text{cm}^{-1}$ : 3443 (NH), 3364 (NH), 1704 (OC=O);  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta = 5.11$  (*app bs*, 1H), 3.11 (*app q*,  $J = 5.5$ , 2H), 2.74 (*app t*,  $J = 6.0$ , 2H), 1.39 (*s*, 9H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta = 156.14$ , 79.0, 43.3, 41.7, 28.3; HR ESI-MS : 183.1104 ( $[\text{M}+\text{Na}]^+$ ,  $\text{C}_7\text{H}_{16}\text{N}_2\text{NaO}_2^+$ , calc. 183.1104).

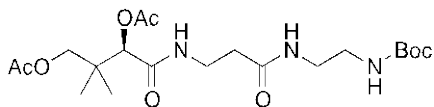
### ***N*-[(2*R*)-2,4-Diacetoxy-3,3-dimethylbutanoyl]- $\beta$ -alanine<sup>8</sup>**



The hemicalcium salt of D-pantothenic acid (4.80 g, 20.0 mmol) was suspended in acetic anhydride (100 mL) with a catalytic amount of iodine (0.30 mg, 1.2 mmol). The reaction mixture was stirred at  $0^\circ\text{C}$  for 2 h, then for 48 h at room temperature. The solvent was evaporated in *vacuo* and the residue was dissolved in dichloromethane (200 mL) before washing with a 1M sodium thiosulfate aqueous solution (100 mL). The phases were separated and the organic layer was dried over magnesium sulfate, filtered and evaporated in *vacuo* to give the crude anhydride as a pale yellow oil. The anhydride was dissolved in a mixture of tetrahydrofuran and water (2:1, 50 mL) and stirred vigorously at room temperature for 16 h. After removal of the solvent, *N*-[(2*R*)-2,4-diacetoxy-3,3-dimethylbutanoyl]- $\beta$ -alanine was obtained as a white solid (5.85 g, 97%).

mp  $82\text{--}84^\circ\text{C}$ ; IR (KBr)  $\nu/\text{cm}^{-1}$ : 3367 (COOH), 1735 (OC=O), 1690 (OC=O), 1652 (NC=O);  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta = 8.74$  (*app bs*, 1H), 6.78 (*app bs*, 1H), 5.29 (*d*,  $J = 4.0$ , 1H), 4.96 (*d*,  $J = 4.0$ , 1H), 4.02 (*dd*,  $J = 11.0$ , 4.0, 1H), 3.82 (*dd*,  $J = 11.0$ , 4.0, 1H), 3.60–3.53 (*m*, 1H), 3.50–3.42 (*m*, 1H), 2.57 (*dd*,  $J = 9.5$ , 5.5, 2H), 2.13 (*d*,  $J = 4.0$ , 3H), 2.06 (*d*,  $J = 4.0$ , 3H), 1.05 (*d*,  $J = 4.0$ , 3H), 1.02 (*d*,  $J = 4.0$ , 3H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta = 176.2$ , 171.1, 169.9, 168.3, 69.2, 53.4, 37.1, 34.5, 33.3, 21.3, 20.8, 20.7, 20.6; HR ESI-MS: 326.1209 ( $[\text{M}+\text{Na}]^+$ ,  $\text{C}_{13}\text{H}_{21}\text{NNaO}_7^+$ , calc. 326.1210).

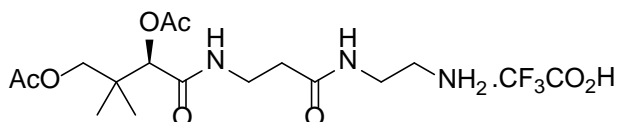
**(14R)-14-Acetoxy-2,2,15,15-tetramethyl-4,9,13-trioxo-3-oxa-5,8,12-triazahehexadecan-16-yl acetate<sup>19</sup>**



*N*-[(2*R*)-2,4-Diacetoxy-3,3-dimethylbutanoyl]- $\beta$ -alanine (1.30 g, 5.0 mmol) was dissolved in anhydrous dichloromethane (25 mL), then *tert*-butyl 2-aminoethylcarbamate (881 mg, 1.1 eq.), triethylamine (1.4 mL, 2 eq.) and hydroxybenzotriazole (745 mg, 1.1 eq.) were added. The resultant solution was cooled to 0 °C and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (1.15 g, 1.2 eq.) was added. The reaction was stirred at 0 °C for 30 min and at room temperature for 18 h. The mixture was then diluted with dichloromethane (50 mL) and the organic phase washed with 0.1 M aqueous hydrochloric acid (30 mL) followed by a sodium hydrogen carbonate solution (30 mL), dried over magnesium sulfate, filtered and evaporated to give (14*R*)-14-acetoxy-2,2,15,15-tetramethyl-4,9,13-trioxo-3-oxa-5,8,12-triazahehexadecan-16-yl acetate as a white solid (1.93 g, 86%).

mp  $\leq$  35 °C; TLC: 19:1 EtOAc/MeOH,  $R_f$  = 0.50; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  = 7.19 (*app bs*, 1H), 6.71 (*app bs*, 1H), 5.33 (*app bs*, 1H), 4.83 (*s*, 1H), 4.02 (*d*,  $J$  = 11.0, 1H), 3.82 (*d*,  $J$  = 11.0, 1H), 3.56-3.48 (*m*, 1H), 3.44-3.40 (*m*, 2H), 3.30-3.18 (*m*, 3H), 2.43-2.29 (*m*, 2H), 2.13 (*s*, 3H), 2.04 (*s*, 3H), 1.40 (*s*, 9H), 1.04 (*s*, 3H), 1.01 (*s*, 3H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>):  $\delta$  = 172.1, 170.9, 170.4, 168.14, 156.8, 79.5, 77.2, 69.2, 40.3, 36.9, 35.5, 35.2, 28.3, 21.3, 20.8, 20.7, 20.4; HR ESI- MS: 468.2313 ([M+Na]<sup>+</sup>, C<sub>20</sub>H<sub>35</sub>N<sub>3</sub>NaO<sub>8</sub><sup>+</sup>, calc. 468.2316).

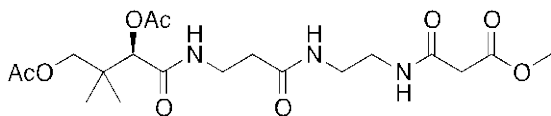
**(2*R*)-4-Acetoxy-1-((3-[(2-aminoethyl)amino]-3-oxopropyl)amino)-3,3-dimethyl-1-oxobutan- 2-yl acetate<sup>14</sup>**



(*R*)-2,2,15,15-Tetramethyl-4,9,13-trioxo-3-oxa-5,8,12-triazahehexadecane-14,16-diyl diacetate (700 mg, 1.73 mmol) was dissolved in dichloromethane (18 mL), then trifluoroacetic acid (2 mL) was added dropwise. The reaction was vigorously stirred at room temperature. The reaction was complete in 4 h, as judged by TLC. The solvent was evaporated *in vacuo*, then trifluoroacetic acid was removed by coevaporation with water in *vacuo*. The trifluoroacetate salt of the title amine was obtained as colourless oil (790 mg, *app. quant.*).

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  = 10.69 (*app bs*, 1H), 7.88 (*app bs*, 2H), 7.48 (*app bs*, 1H), 4.74 (*s*, 1H), 3.96 (*d*,  $J$  = 11.0, 1H), 3.86 (*d*,  $J$  = 11.0, 1H), 3.49 (*app bs*, 2H), 3.18 (*app bs*, 1H), 2.43 (*app bs*, 1H), 2.09 (*s*, 3H), 2.03 (*s*, 3H), 1.00 (*s*, 6H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>):  $\delta$  = 173.9, 171.4, 170.9, 169.5, 161.2, 117.2, 77.6, 69.1, 40.2, 37.3, 37.0, 35.7, 35.3, 20.9, 20.6, 20.4; HR ESI-MS: 346.1968 ([M+Na]<sup>+</sup>, C<sub>15</sub>H<sub>28</sub>N<sub>3</sub>O<sub>6</sub><sup>+</sup>, calc. 346.1973).

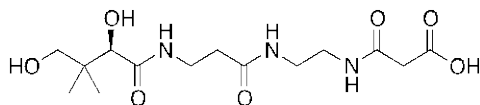
**Methyl 3-([2-((N-[(2*R*)-2,4-diacetoxy-3,3-dimethylbutanoyl]- $\beta$ -alanyl)amino)ethyl]amino)-3-oxopropanoate**



(2*R*)-4-Acetoxy-1-((3-[(2-aminoethyl)amino]-3-oxopropyl)amino)-3,3-dimethyl-1-oxobutan- 2-yl acetate trifluoroacetate salt (300 mg, 0.65 mmol) was dissolved in dried tetrahydrofuran (10 mL) together with 3-methoxy-3-oxopropanoic acid (130mg, 1.4 eq., prepared as reported<sup>20</sup>), diisopropylethylamine (0.225 mL, 2.0 eq.) and hydroxybenzotriazole (123 mg, 1.4 eq.). The solution was cooled to 0 °C and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (190 mg, 1.5 eq.) was added. After stirring for 16 h under these conditions, the solvent was evaporated and then replaced by dichloromethane (30 mL). The organic phase was washed twice with saturated sodium hydrogen carbonate aqueous solution (2 x 20 mL), dried over magnesium sulphate, filtered and evaporated in *vacuo* to yield methyl 3-([2-((N-[(2*R*)-2,4-diacetoxy-3,3-dimethylbutanoyl]- $\beta$ -alanyl)amino)ethyl]amino)-3-oxopropanoate as a colourless oil (62 mg, 22%).

TLC: 9:1 EtOAc/MeOH, *R*<sub>f</sub> = 0.20; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  = 7.42 (*app bs*, 1H), 7.09 (*app bs*, 1H), 6.70 (*app bs*, 1H), 4.82 (*s*, 1H), 4.01 (*d*, *J* = 11.0, 1H), 3.84 (*d*, *J* = 11.0, 1H), 3.73 (*s*, 3H), 3.54-3.34 (*m*, 4H), 3.31 (*s*, 2H), 2.43-2.30 (*m*, 2H), 2.14 (*s*, 3H), 2.05 (*s*, 3H), 1.05 (*s*, 3H), 1.03 (*s*, 3H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>):  $\delta$  = 172.4, 171.0, 170.6, 169.5, 168.2, 166.3, 77.4, 69.2, 53.5, 41.5, 39.7, 39.4, 36.9, 35.6, 35.4, 20.3, 20.8, 20.7, 20.6; HR ESI-MS: 468.1942 ([M+Na]<sup>+</sup>, C<sub>19</sub>H<sub>31</sub>N<sub>3</sub>NaO<sub>9</sub><sup>+</sup>, calc. 468.1953).

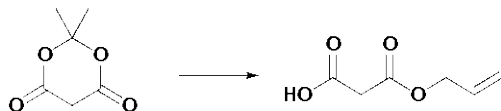
### 3-([2-((*N*-[(2*R*)-2,4-Dihydroxy-3,3-dimethylbutanoyl]- $\beta$ -alanyl)amino)ethyl]amino)-3-oxopropanoic acid



Methyl 3-([2-((*N*-[(2*R*)-2,4-diacetoxy-3,3-dimethylbutanoyl]- $\beta$ -alanyl)amino)ethyl]amino)-3-oxopropanoate (60 mg, 13  $\mu$ mol) was dissolved in methanol (5 mL) and then potassium carbonate (75 mg, 4.0 eq.) was added. The reaction was stirred at room temperature for 18 h, the solvent was evaporated and the residue dissolved in water. The pH was adjusted to 7.0 using 1 M hydrochloric acid solution and the aqueous phase freeze-dried to yield compound as colourless oil. This residue was desalted by preparative HPLC (2% aqueous acetonitrile, acidified with 0.1% formic acid, over 5 min followed by 2–98% aqueous acetonitrile, acidified with 0.1% formic acid, over 30 min) to yield 3-([2-((*N*-[(2*R*)-2,4-dihydroxy-3,3-dimethylbutanoyl]- $\beta$ -alanyl)amino)ethyl]amino)-3-oxopropanoic acid as a colourless oil ( $t_R$  = 15.5 min, 28 mg, 62%).

$^1\text{H}$  NMR (500 MHz,  $\text{D}_2\text{O}$ ):  $\delta$  = 3.94 (*s*, 1H), 3.50–3.42 (*m*, 3H), 3.38–3.27 (*m*, 7H), 2.45 (*t*,  $J$  = 6.5, 2H), 0.88 (*s*, 3H), 0.84 (*s*, 3H);  $^{13}\text{C}$  NMR (125 MHz,  $\text{D}_2\text{O}$ ):  $\delta$  = 175.5, 174.6, 172.2, 169.6, 76.1, 68.7, 42.5, 39.1, 38.9 (2x), 35.8, 35.7, 20.8, 19.4; HR ESI-MS: 346.1626 ( $[\text{M}-\text{H}]^-$ ,  $\text{C}_{14}\text{H}_{24}\text{N}_3\text{O}_7^-$ , calc. 346.1620).

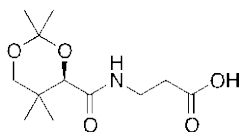
### 3-(Allyloxy)-3-oxopropanoic acid



3-(Allyloxy)-3-oxopropanoic acid was obtained by was obtained by adaption of the previously reported procedure.<sup>21</sup> 2,2-Dimethyl-dioxane-4,6-dione (1.44 g, 10 mmol) and allyl alcohol (0.68 mL, 1 eq.) were stirred under reflux in dried acetonitrile (5 mL) for 24 h under a nitrogen atmosphere. The solvent was then evaporated to give crude 3-(allyloxy)-3-oxopropanoic acid as a colourless liquid (1.44 g, app. quant.).

IR (neat)  $\text{v}/\text{cm}^{-1}$ : 1714 ( $\text{OC}=\text{O}$ );  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  = 11.23 (*app bs*, 1H), 5.96–5.86 (*m*, 1H), 5.37–5.25 (*m*, 2H), 4.66 (*app d*,  $J$  = 6.0, 2H), 3.47 (*s*, 2H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  = 171.9, 166.2, 131.2, 119.0, 66.33, 40.86; HR ESI-MS: 167.0319 ( $[\text{M}+\text{Na}]^+$ ,  $\text{C}_6\text{H}_8\text{NaO}_4^+$ , calc. 167.0315).

### *N*-[(4*R*)-2,2,5,5-tetramethyl-1,3-dioxan-4-yl]carbonyl- $\beta$ -alanine<sup>8,22</sup>

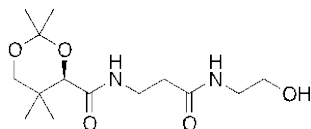


*N*-[(4*R*)-2,2,5,5-Tetramethyl-1,3-dioxan-4-yl]carbonyl)- $\beta$ -alanine was obtained by adaption of the previously reported procedure.<sup>23</sup> The hemicalcium salt of D-pantothenic acid ((*R*)-3- (2,4-dihydroxy-3,3-dimethylbutanamido)propanoic acid, 11.91 g, 50 mmol)

was suspended in 2,2-dimethylpropane (100 mL) and *p*-toluene sulfonic acid (9.51 g, 1 eq.) was added in one portion. The suspension was stirred at room temperature for 16 h, and then the white insoluble solid was filtered off and washed with acetone. The filtrate was evaporated in *vacuo* then the resulting white solid was washed with warm *n*-hexane to yield the hemi-calcium salt of *N*-[(4*R*)-2,2,5,5-tetramethyl-1,3-dioxan-4-yl]carbonyl-β-alanine (10.50 g, 81%).

Mp 90–92 °C (lit. 88–90 °C)<sup>22</sup>; TLC: 4:1 EtOAc/MeOH, *R*<sub>f</sub> = 0.40; IR (KBr)  $\nu/\text{cm}^{-1}$ : 3450 (COOH), 3390 (NH), 1727 (OC=O), 1630 (NC=O); <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  = 7.05 (*app bs*, 1H), 4.11 (*s*, 1H), 3.70 (*d*, *J* = 11.5, 1H), 3.64–3.56 (*m*, 1H), 3.53–3.45 (*m*, 1H), 3.29 (*d*, *J* = 11.5, 1H), 2.62 (*dt*, *J* = 6.0, 2.0, 2H), 1.46 (*s*, 3H), 1.43 (*k*, 3H) 1.04 (*s*, 3H), 0.98 (*s*, 3H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>):  $\delta$  = 170.2, 99.1, 77.1, 71.4, 34.1, 33.9, 33.0, 30.9, 29.4, 22.0, 18.8, 18.7; HR ESI-MS : 282.1322 ([M+Na]<sup>+</sup>, C<sub>12</sub>H<sub>21</sub>NNaO<sub>5</sub><sup>+</sup>, calc. 282.1312).

**(4*R*)-*N*-(3-[(2-Hydroxyethyl)amino]-3-oxopropyl)-2,2,5,5-tetramethyl-1,3-dioxane-4-carboxamide**<sup>8</sup>

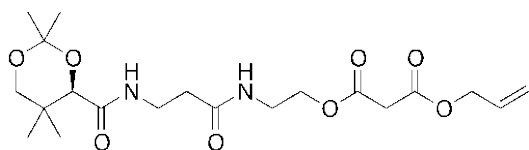


*N*-[(4*R*)-2,2,5,5-Tetramethyl-1,3-dioxan-4-yl]carbonyl)-β-alanine hemi-calcium salt (2.59 g, 10 mmol) was dissolved together with triethylamine (3.0 mL, 2 eq.) in anhydrous dichloromethane (50 mL) at 0 °C. Ethylchloroformate (1.05 mL, 1.1 eq.) was added dropwise and the corresponding anhydride was formed over 30 min before dropwise addition of 2-aminoethanol (1.20 mL, 2 eq.). The reaction was stirred at 0 °C for 30 min followed by 3 h at room temperature. Evaporation of the solvent in *vacuo* gave the crude product which was purified by chromatography on a SNAP-100 column using a gradient of 0–10% methanol in ethyl acetate. Fractions containing the product were pooled and evaporated in *vacuo* to give (4*R*)-*N*-(3-[(2-hydroxyethyl)amino]-3-oxopropyl)-2,2,5,5-tetramethyl-1,3-dioxane-4-carboxamide as a white solid (1.65g, 55%).

Mp 172–175 °C; TLC: 9:1 EtOAc/MeOH, *R*<sub>f</sub> = 0.25; IR (neat)  $\nu/\text{cm}^{-1}$ : 3303 (NH, OH), 1739 (NC=O), 1638 (NC=O); <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  = 7.06 (*t*, *J* = 6.0, 1H), 6.68 (*app bs*, 1H), 4.07 (*s*, 1H), 3.72 (*d*, *J* = 5.0, 1H), 3.69 (*d*, *J* = 11.5, 1H), 3.58 (*dt*, *J* = 11.5, 6.0, 1H), 3.43 (*ddt*, 1H), 3.29 (*d*, *J* = 11.5, 1H), 2.97 (*t*, *J* = 5.5, 1H), 2.49 (*t*, *J* = 6.0, 2H), 1.47 (*s*, 3H), 1.43 (*s*, 3H), 1.04 (*s*, 3H), 0.98 (*s*, 3H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>):  $\delta$  = 171.9, 170.6, 99.1, 77.1, 71.3, 62.0, 42.5, 36.3, 35.0, 32.9, 29.4, 22.1, 18.8, 18.7; HR ESI-MS: 325.1734 ([M+Na]<sup>+</sup>, C<sub>14</sub>H<sub>26</sub>N<sub>2</sub>NaO<sub>5</sub><sup>+</sup>, calc. 325.1726).



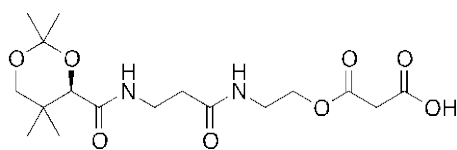
**Allyl 2-[(*N*-[(*4R*)-2,2,5,5-tetramethyl-1,3-dioxan-4-yl]carbonyl)- $\beta$ -alanyl]amino]ethyl malonate<sup>8</sup>**



Malonic acid mono allyl ester (175 mg, 1.2 eq.) and *N,N*-diisopropylethylamine (0.26 mL, 1.5 eq.) were added to a solution of (*4R*)-*N*-(3-[(2-hydroxyethyl)amino]-3-oxopropyl)-2,2,5,5-tetramethyl-1,3-dioxane-4-carboxamide (305 mg, 1.0 mmol) in dried tetrahydrofuran (20 mL). The solution was cooled to 0 °C, *O*-benzotriazole-*N,N,N',N'*-tetramethyl-uronium-hexafluoro-phosphate (HBTU, 455 mg, 1.2 eq.) was then added and the reaction stirred at room temperature for 16 h. The solvent was evaporated in *vacuo* and replaced by ethyl acetate (50 mL). The organic phase was then washed twice with saturated sodium hydrogen carbonate solution (2 x 20 mL) dried over magnesium sulphate, filtered and the filtrate evaporated in *vacuo* to give allyl 2-[(*N*-[(*4R*)-2,2,5,5-tetramethyl-1,3-dioxan-4-yl]carbonyl)- $\beta$ -alanyl]amino]ethyl malonate as a colourless oil (300 mg, 70%).

TLC: 19:1 EtOAc/MeOH,  $R_f$  = 0.55; IR (neat)  $\nu/\text{cm}^{-1}$ : 3413 (NH), 3308 (NH), 1734 (C=O), 1658 (C=O); <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  = 7.05 (*t*,  $J$  = 6.0, 1H), 6.53 (*t*,  $J$  = 5.5, 1H), 5.90 (*ddt*,  $J$  = 17.0, 11.0, 5.5, 1H), 5.33 (*dd*,  $J$  = 17.0, 1.0, 1H), 5.26 (*dd*,  $J$  = 11.0, 1.0, 1H), 4.64 (*dt*,  $J$  = 5.5, 1.0, 2H), 4.23 (*m*, 2H), 4.05 (*s*, 1H), 3.66 (*d*,  $J$  = 11.5, 1H), 3.58-3.44 (*m*, 4H), 3.43 (*s*, 2H), 3.26 (*d*,  $J$  = 11.5, 1H), 2.43 (*t*,  $J$  = 6.0, 2H), 1.44 (*s*, 3H), 1.40 (*s*, 3H), 1.01 (*s*, 3H), 0.95 (*s*, 3H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>):  $\delta$  = 171.3, 170.1, 166.7, 166.3, 131.2, 119.0, 99.0, 71.3, 66.3, 64.1, 41.2, 38.5, 38.1, 35.8, 34.6, 32.8, 29.4, 22.0, 18.8, 18.6; HR ESI-MS: 451.2058 ([M+Na]<sup>+</sup>, C<sub>20</sub>H<sub>32</sub>N<sub>2</sub>NaO<sub>8</sub><sup>+</sup>, calc. 451.2051).

**3-Oxo-3-2-[(*N*-[(*4R*)-2,2,5,5-tetramethyl-1,3-dioxan-4-yl]carbonyl)- $\beta$ -alanyl]amino]ethoxypropanoic acid<sup>8</sup>**

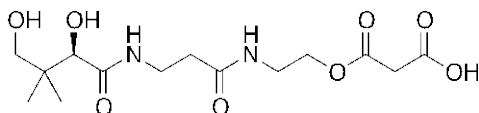


Allyl 2-[(*N*-[(*4R*)-2,2,5,5-tetramethyl-1,3-dioxan-4-yl]carbonyl)- $\beta$ -alanyl]amino]ethyl malonate (295 mg, 0.69 mmol) was dissolved in acetonitrile (10 mL). The resultant solution was cooled to 0 °C, prior to addition of pyrrolidine (25  $\mu$ L, 1 eq.) and tetrakis (triphenylphosphine)-palladium (33 mg, 10% mol/mol). The reaction was found to be complete after 2 h, as judged by TLC, the mixture was then filtered through a pad of Celite<sup>®</sup> 545. The filtrate was evaporated in *vacuo*, then residue was dissolved in ethyl acetate and extracted twice with water. The combined aqueous phases were freeze-dried to give the product as a white solid (247 mg, 78%). For characterization purposes, a small portion was purified by preparative HPLC (2% aqueous acetonitrile, acidified with 0.1% formic acid, over 5 min followed by 2–98% aqueous acetonitrile, acidified with 0.1% formic acid, over 15 min,  $t_R$  = 17.0 min). The remaining crude product was used for the next step without further purification.



$^1\text{H}$  NMR (400 MHz,  $\text{D}_2\text{O}$ ):  $\delta$  = 4.21 (*t*,  $J$  = 5.5, 2H), 3.94 (*s*, 1H), 3.52-3.40 (*m*, 5H), 3.34 (*d*,  $J$  = 11.0, 1H), 2.46 (*t*,  $J$  = 6.5, 2H), 2.18 (*s*, 6H), 0.87 (*s*, 3H), 0.83 (*s*, 3H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{D}_2\text{O}$ ):  $\delta$  = 175.5, 174.6, 174.5, 169.5, 76.1, 68.7, 64.5, 38.9, 38.5, 35.8, 35.6, 30.6, 20.8, 19.4; HR ESI-MS: 411.1730 ( $[\text{M}+\text{Na}]^+$ ,  $\text{C}_{17}\text{H}_{28}\text{N}_2\text{NaO}_8^+$ , calc. 411.1738).

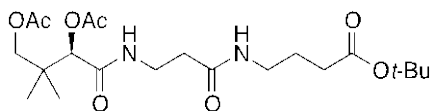
**3-[2-((*N*-[(2*R*)-2,4-Dihydroxy-3,3-dimethylbutanoyl]- $\beta$ -alanyl)amino)ethoxy]-3-oxopropanoic acid<sup>8</sup>**



3-Oxo-3-2-[(*N*-[(4*R*)-2,2,5,5-tetramethyl-1,3-dioxan-4-yl]carbonyl- $\beta$ -alanyl)amino]ethoxypropanoic acid (88 mg, 0.23 mmol) was dissolved in water (10 mL) and DOWEX 50WX8-400 ( $\text{H}^+$ ) resin (100 mg), which had been previously washed with 1 M hydrochloric acid and then with water to pH 7.0. The resulting suspension was stirred at room temperature for 12 h and then filtered. The filtrate was evaporated and the residue was purified by preparative HPLC (2% aqueous acetonitrile, acidified with 0.1% formic acid, over 5 min followed by 2–98% aqueous acetonitrile, acidified with 0.1% formic acid, over 15 min) to give 3-[2-((*N*-[(2*R*)-2,4-dihydroxy-3,3-dimethylbutanoyl]- $\beta$ -alanyl)amino)ethoxy]-3-oxopropanoic acid ( $t_R$  = 14.0 min, 65 mg, 82%).

$^1\text{H}$  NMR (400 MHz,  $\text{D}_2\text{O}$ ):  $\delta$  = 4.21 (*t*,  $J$  = 5.5, 2H), 3.93 (*s*, 1H), 3.52-3.40 (*m*, 5H), 3.34 (*d*,  $J$  = 11.0, 1H), 2.45 (*t*,  $J$  = 6.5, 2H), 0.87 (*s*, 3H), 0.83 (*s*, 3H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{D}_2\text{O}$ ):  $\delta$  = 175.5, 174.5, 171.2, 169.4, 76.1, 68.7, 64.6, 38.9, 38.5, 35.8, 35.6, 20.8, 19.4; HR ESI-MS: 371.1422 ( $[\text{M}+\text{Na}]^+$ ,  $\text{C}_{14}\text{H}_{24}\text{N}_2\text{NaO}_8^+$ , calc. 371.1425).

***tert*-Butyl 4-((*N*-[(2*R*)-2,4-diacetoxy-3,3-dimethylbutanoyl]- $\beta$ -alanyl)amino)butanoate<sup>8</sup>**

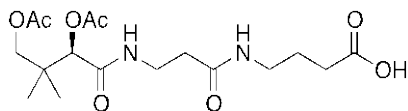


*N*-[(2*R*)-2,4-Diacetoxy-3,3-dimethylbutanoyl]- $\beta$ -alanine (4.65 g, 15.3 mmol), *tert*-butyl- $\delta$ -aminobutanoate hydrochloride (3.3 mg, 1.1 eq.) and 1-hydroxybenzotriazole hydrate (2.27 g, 1.1 eq.) were dissolved in anhydrous dichloromethane (20 mL) before adding triethylamine (4.3 mL, 2 eq.) under a nitrogen atmosphere. The reaction mixture was cooled to 0 °C, then *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (2.85 g, 1.2 eq.) was added. After stirring for 30 min on ice and then at room temperature for 18 h, the mixture was diluted with dichloromethane (50 mL) and washed with a saturated sodium hydrogen carbonate aqueous solution (50 mL) followed by water (50 mL). The organic layer was dried over magnesium sulfate, filtered and evaporated to give *tert*-butyl 4-((*N*-[(2*R*)-2,4-diacetoxy-3,3-dimethylbutanoyl]- $\beta$ -alanyl)amino)butanoate as a white solid (6.33 g, 93%).

mp 50–55 °C; TLC: 9:1  $\text{CH}_2\text{Cl}_2/\text{MeOH}$ ,  $R_f$  = 0.50; IR (KBr)  $\nu/\text{cm}^{-1}$ : 3261 (NH), 3093 (NH), 1736 (NC=O), 1727 (NC=O), 1672 (OC=O), 1643 (OC=O);  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  = 7.06 (*t*,  $J$  = 5.6, 1H), 6.44 (*t*,  $J$  = 5.0, 1H), 4.86 (*s*, 1H), 3.96 (*d*,  $J$  = 11.0, 1H), 3.78 (*d*,  $J$  = 11.0, 1H), 3.56-3.40 (*m*, 2H), 3.25-3.15 (*m*, 2H), 2.31 (*t*,  $J$  = 6.0, 2H),

2.21 (*t*, *J* = 7.0, 2H), 2.09 (*s*, 3H), 2.00 (*s*, 3H), 1.80-1.70 (*m*, 2H), 1.38 (*s*, 9H), 1.00 (*s*, 3H), 0.96 (*s*, 3H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): δ = 172.5, 171.5, 170.8, 169.8, 167.9, 80.4, 76.8, 69.2, 38.8, 36.9, 35.1, 34.8, 32.8, 27.9, 24.5, 21.2, 20.7, 20.6, 20.5; HR ESI-MS: 467.2350 ([M+Na]<sup>+</sup>, C<sub>21</sub>H<sub>36</sub>N<sub>2</sub>NaO<sub>8</sub><sup>+</sup>, calc. 467.2364).

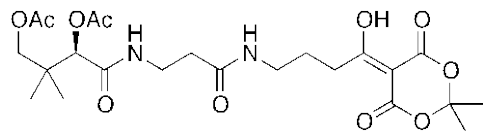
**4-((*N*-[(2*R*)-2,4-Diacetoxy-3,3-dimethylbutanoyl]-β-alanyl)amino)butanoic acid<sup>8</sup>**



*tert*-Butyl 4-((*N*-[(2*R*)-2,4-diacetoxy-3,3-dimethylbutanoyl]-β-alanyl)amino)butanoate (1.5 g, 3.37 mmol) was dissolved in anhydrous dichloromethane (18 mL) and trifluoroacetic acid (2 mL) was added dropwise. After stirring at room temperature for 6 h, the excess of trifluoroacetic acid was co-evaporated several times with toluene and then with dichloromethane to yield the desired acid as a pale orange oil (1.26 g, 96%).

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ = 11.92 (*app bs*, 1H), 7.31 (*app bs*, 1H), 7.05 (*app bs*, 1H), 4.87 (*s*, 1H), 4.01 (*d*, *J* = 11.0, 1H), 3.84 (*d*, *J* = 11.0, 1H), 3.52-3.51 (*m*, 2H), 3.35-3.21 (*m*, 2H), 2.48 (*t*, *J* = 6.0, 2H), 2.39 (*t*, *J* = 7.0, 2H), 2.13 (*s*, 3H), 2.06 (*s*, 3H), 1.89-1.78 (*m*, 2H), 1.04 (*s*, 3H), 1.02 (*s*, 3H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): δ = 176.7, 172.8, 171.1, 170.3, 169.3, 76.7, 69.2, 39.1, 37.1, 35.7, 35.0, 31.3, 24.1, 21.2, 20.7, 20.6, 20.5; HR ESI-MS: 411.1736 ([M+Na]<sup>+</sup>, C<sub>17</sub>H<sub>28</sub>N<sub>2</sub>NaO<sub>8</sub><sup>+</sup>, calc. 411.1738).

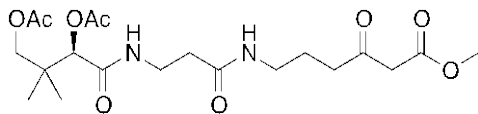
**(2*R*)-4-Acetoxy-1-[(3-([4-(2,2-dimethyl-4,6-dioxo-1,3-dioxan-5-ylidene)-4-hydroxybutyl]amino)-3-oxopropyl)amino]-3,3-dimethyl-1-oxobutan-2-yl acetate<sup>8</sup>**



4-((*N*-[(2*R*)-2,4-Diacetoxy-3,3-dimethylbutanoyl]-β-alanyl)amino)butanoic acid (1.20 g, 3.09 mmol) was dissolved into anhydrous dichloromethane (30 mL) together with 2,2-dimethyl- 1,3-dioxane-4,6-dione 274 (450 mg, 1.01 eq.) and 4-dimethylaminopyridine (450 mg, 1.2 eq.). The mixture was stirred at 0 °C for 10 min. then *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride was added (650 mg, 1.1 eq.). The temperature was slowly raised to room temperature and after 16 h, the reaction was diluted with dichloromethane (50 mL), washed with 0.1 M hydrochloric acid aqueous solution (20 mL), dried over magnesium sulfate, filtered and evaporated in *vacuo* to yield the product as a yellow oil (1.55 g) which was subjected to methanolysis without further purification.

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ = 7.08 (*t*, *J* = 6.0, 1H), 6.29 (*t*, *J* = 5.5, 1H), 4.87 (*s*, 1H), 4.02 (*d*, *J* = 11.0, 1H), 3.84 (*d*, *J* = 11.0, 1H), 3.52 (*apt q*, *J* = 5.5, 2H), 3.44-3.36 (*m*, 2H), 3.29-3.21 (*m*, 2H), 3.16-3.00 (*m*, 2H), 2.46-2.34 (*m*, 2H), 2.14 (*s*, 3H), 2.05 (*s*, 3H), 1.98-1.85 (*m*, 2H), 1.75 (*s*, 3H), 1.74 (*s*, 3H), 1.06 (*s*, 3H), 1.03 (*s*, 3H); HR ESI-MS: 513.2092 ([M-H]<sup>-</sup>, C<sub>23</sub>H<sub>33</sub>N<sub>2</sub>O<sub>11</sub><sup>-</sup>, calc. 513.2090).

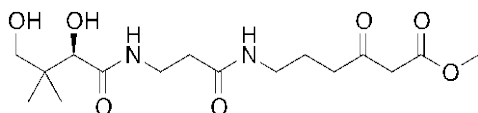
**Methyl 6-((*N*-[(2*R*)-2,4-diacetoxy-3,3-dimethylbutanoyl]-β-alanyl)amino)-3-oxohexanoate<sup>8</sup>**



(2*R*)-4-Acetoxy-1-[(3-([4-(2,2-dimethyl-4,6-dioxo-1,3-dioxan-5-ylidene)-4-hydroxybutyl]amino)-3-oxopropyl)amino]-3,3-dimethyl-1-oxobutan-2-yl acetate (780 mg, 1.5 mmol) was dissolved in dried methanol (20 mL). The resultant solution was stirred under reflux conditions for 18 h. The solvent was then evaporated to give the crude methyl ester as a yellow oil. The residue was purified by silica chromatography (SNAP-50 column, gradient elution using 0–10% methanol in dichloromethane). Methyl 6-((*N*-[(2*R*)-2,4-diacetoxy-3,3-dimethylbutanoyl]-β-alanyl)amino)-3-oxohexanoate was obtained as a colourless oil (520 mg, 77% over 2 steps).

TLC: 9:1 CH<sub>2</sub>Cl<sub>2</sub>/MeOH, *R*<sub>f</sub> = 0.50; IR (KBr)  $\nu$ /cm<sup>-1</sup>: 1735 (NC=O), 1669 (OC=O); <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  = 7.49 (*t*, *J* = 5.5, 1H), 6.07 (*t*, *J* = 5.5, 1H), 4.90 (*s*, 1H), 4.03 (*d*, *J* = 11.0, 1H), 3.84 (*d*, *J* = 11.0, 1H), 3.74 (*s*, 3H), 3.52–3.40 (*m*, 4H), 3.25–3.12 (*m*, 2H), 2.62 (*t*, *J* = 6.5, 2H), 2.36 (*t*, *J* = 5.5, 2H), 2.15 (*s*, 3H), 2.06 (*s*, 3H), 1.80–1.73 (*m*, 2H), 1.06 (*s*, 3H), 1.03 (*s*, 3H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>):  $\delta$  = 202.6, 171.8, 171.0, 170.0, 168.1, 167.8, 77.1, 69.3, 52.4, 48.9, 40.3, 38.7, 37.1, 35.2, 35.1, 23.0, 21.3, 20.9, 20.8, 20.7; HR ESI-MS : 467.2001 ([M+Na]<sup>+</sup>, C<sub>20</sub>H<sub>32</sub>N<sub>2</sub>NaO<sub>9</sub><sup>+</sup>, calc. 467.2000).

**Methyl 6-((*N*-[(2*R*)-2,4-dihydroxy-3,3-dimethylbutanoyl]-β-alanyl)amino)-3-oxohexanoate<sup>8</sup>**



Methyl 6-((*N*-[(2*R*)-2,4-diacetoxy-3,3-dimethylbutanoyl]-β-alanyl)amino)-3-oxohexanoate (520 mg, 1.2 mmol) was dissolved in methanol (20 mL) on ice and potassium carbonate (250 mg, 1.5 eq.) was added prior to stirring for 5 h at 0 °C. The solvent was evaporated and the yellow residue dissolved in water (3 mL) and extracted with ethyl acetate (3 x 10 mL). The combined organic phases were dried over magnesium sulphate, filtered and evaporated to give methyl 6-((*N*-[(2*R*)-2,4-dihydroxy-3,3-dimethylbutanoyl]-β-alanyl)amino)-3-oxohexanoate as a pale yellow oil (230 mg, 55%).

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  = 6.94 (*t*, *J* = 5.5, 1H), 6.70 (*t*, *J* = 5.5, 1H), 4.00 (*s*, 1H), 3.73 (*s*, 3H), 3.60–3.51 (*m*, 2H), 3.49 (*s*, 2H), 3.29–3.17 (*m*, 2H), 2.61 (*t*, *J* = 7.0, 2H), 2.43 (*t*, *J* = 6.0, 2H), 1.83–1.76 (*m*, 2H), 0.98 (*s*, 3H), 0.91 (*s*, 3H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>):  $\delta$  = 202.8, 173.9, 171.7, 168.0, 77.4, 70.7, 52.5, 48.9, 40.3, 39.3, 38.7, 35.8, 35.2, 23.1, 21.4, 20.4; HR ESI-MS : 383.1785 ([M+Na]<sup>+</sup>, C<sub>16</sub>H<sub>28</sub>N<sub>2</sub>NaO<sub>7</sub><sup>+</sup>, calc. 383.1789).

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