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Supporting Information

DOI: 10.1002/cbic.200800652

Supporting Information

for

Evidence that Thienamycin Biosynthesis Proceeds via C-5 Epimerization: ThnE Catalyzes the Formation of (2*S*,5*S*)-*trans*-Carboxymethylproline

Refaat B. Hamed, Edward T. Batchelar, Jasmin Mecinovic, Timothy D. W. Claridge, and Christopher J. Schofield*

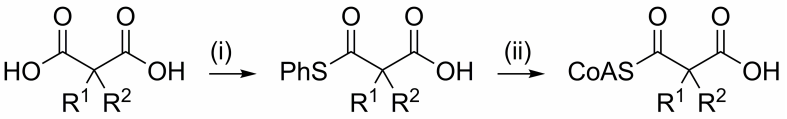
Chemicals were obtained from Sigma Aldrich., unless otherwise stated. L- and D-GSA were prepared as reported.[1] DNA manipulations were carried out according to standard protocols.[2] Protein concentrations were determined using a Nanodrop spectrophotometer ND-1000 (Thermo Scientific) at 280 nm, after protein extinction coefficients and molecular weights were estimated using Expasy's ProtParam tool.[3]

Synthesis and purification of C-2 alkylated malonyl-CoA derivatives: C-2 alkylated malonyl-CoA derivatives were prepared from coenzyme A and the corresponding malonic acid as reported[4] and purified (if required) by HPLC using a Phenomenex C18 Luna column (250 mm x 10mm, 5µ). Solvent reservoir A contained 100 mM ammonium formate pH 5.0 and reservoir B contained methanol. The column was pre-equilibrated in 5 % B at 2mL/min. After 7 min, a gradient was run to 95 % B over 15 min. These conditions were maintained for 17 min before returning to 5 % B over 1 min and the column re-equilibrated for 10 min. Dimethylmalonyl- and isopropylmalonyl–CoA eluted at 26 and 30 min, respectively. For the NMR data of dimethylmalonyl-CoA, see Batchelar *et al.*[5]

Ethylmalonyl–CoA ^{1}H NMR (^2H_2O, referenced to the ^{2}HOH peak at 4.70 ppm): d 0.66 (s, 3H, b), 0.80 (m, 6H, b' and j), 1.72 (t, 2H, i), 2.34 (t, 2H, e), 2.94 (m, 2H, g), 3.25 (m, 2H, f), 3.36 (m, 2H, d), 3.47 (m, lH, a), 3.75 (m, lH, a), 3.93 (s, lH, c), 4.16 (bs, 2H, 5'), 4.51 (bs, 1H, 4'), 6.10 (d, lH, 1'), 8.18 (s, lH, 2), 8.46 (s, lH, 8). H2' and H3' were obscured by the HO^{2}H signal. H$_h$ was exchanged with ^2H_2O. The nomen-

clature used to describe the protons on the CoA moiety has been described previously by Patel and Walt.[6]

Isopropylmalonyl–CoA ^{1}H NMR ($^{2}H_2O$, referenced to the ^{2}HOH peak at 4.70 ppm): d 0.59 (s, 3H, b), 0.72 (d, 3H, j), 0.74 (s, 3H, b'), 0.77 (d, 3H, k), 2.13 (m, 1H, i), 2.28 (m, 2H, e), 2.88 (m, 2H, g), 3.11 (s,1H, h), 3.18 (m, 2H, f), 3.30 (m, 2H, d), 3.40 (m, lH, a), 3.69 (m, lH, a), 3.87 (s, lH, c), 4.09 (bs, 2H, 5'), 4.51 (bs, 1H, 4'), 6.03 (d, lH, 1'), 8.12 (s, lH, 2), 8.41 (s, lH, 8). H2' and H3' were obscured by the ^{2}HOH signal.



Ethylmalonyl-CoA: R^1= ethyl, R^2= H
Dimethylmalonyl-CoA: R^1= methyl, R^2= methyl
Isopropylmalonyl-CoA: R^1= isopropyl, R^2= H

Scheme S1. Synthesis of C-2 alkylated malonyl-CoA derivatives:[4] i) C-2 alkylated malonic acid, PhSH, N,N'-dicyclohexylcarbodiimide, dimethylformamide; ii) coenzyme A, $NaHCO_3$ (aq), pH 8.0.

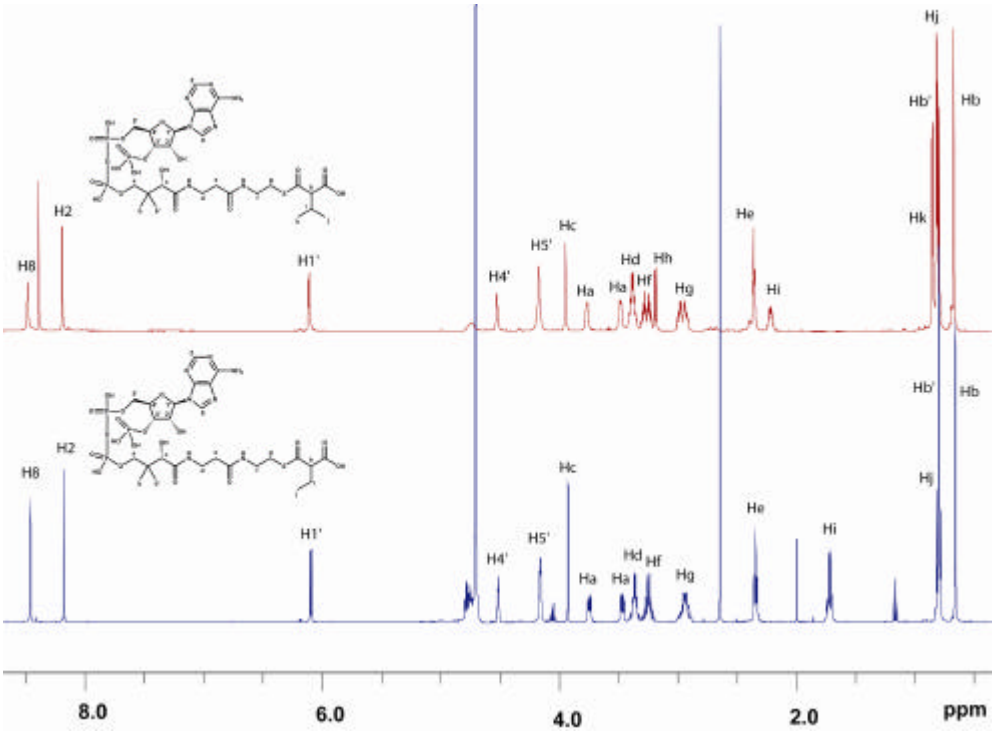


Figure S1. ^{1}H NMR of purified ethylmalonyl-CoA (bottom) and isopropylmalonyl-CoA (top, with water suppression). The signals corresponding to protons from H2' and H3' are obscured by

the residual ^{2}HOH signal (4.7 ppm). H_h in the bottom spectrum was exchanged with $^{2}H_2O$. The signal at ~ 8.2 ppm in the top spectrum corresponds to formic acid.

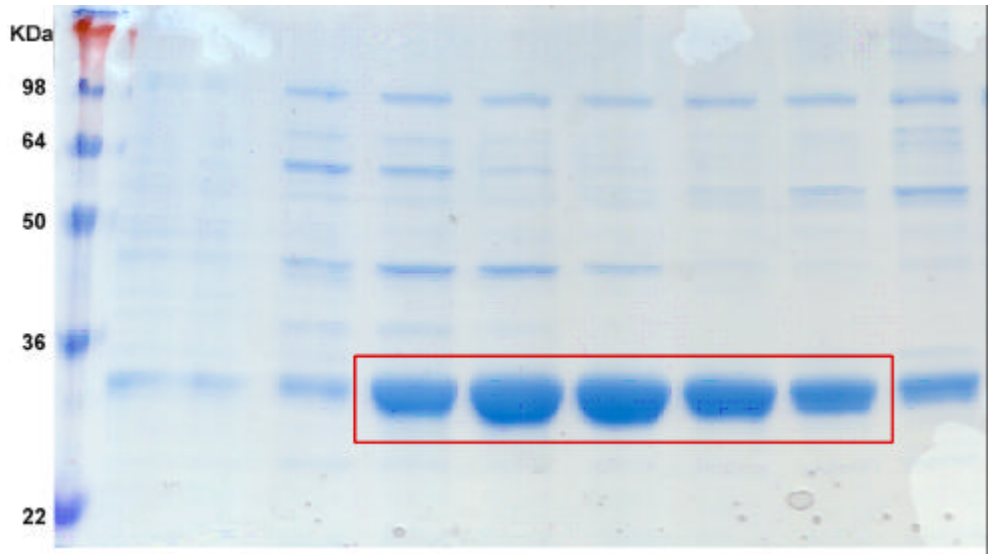


Figure S2: SDS-PAGE analysis of purified recombinant ThnE?2-46 produced in *E. coli*. The prestained molecular weight markers (Invitrogen) are on the left hand side of the gel. The bands corresponding to ThnE?2-46 are boxed in red.

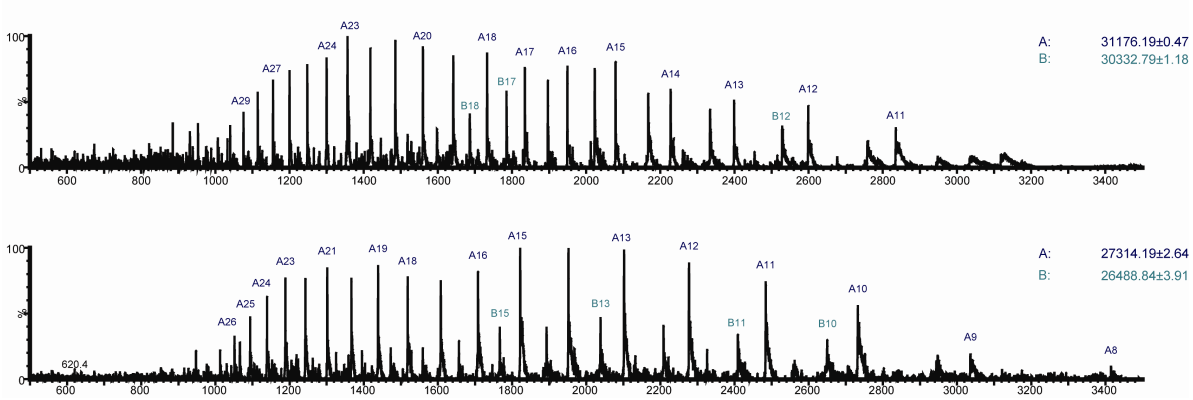


Figure S3: ESI-MS analyses of wt ThnE (top) and ThnE?2-46 (bottom) under denaturing conditions showing the monomeric proteins. For monomeric ThnE: calculated *m/z* 32,289.5 Da, observed *m/z* 31,176.2 Da, which is consistent with cleavage of the first 11 amino acid residues from the *N*-terminus and with the result of Edman degradation analysis (ThnE?1-11 *m/z* 31,175.2 Da). For monomeric ThnE?2-46: calculated *m/z* 27,313.1 Da, observed *m/z* 27,314.2 Da.

Electrospray Ionisation Mass Spectrometry (ESI-MS): For ESI-MS under non-denaturing conditions, wild type (wt) ThnE and ThnE?2-46 were desalted using a Bio-Spin 6 Column (Bio-Rad) into 15 mM ammonium acetate (pH 7.5). The protein stock solution was diluted with the same buffer to a final concentration of 100 µM. The protein was diluted to a final concentration of 15 µM prior to ESI-MS analysis. For denaturing experiments, protein (100 µM) was diluted with 0.5 % formic acid to a final concentration of 2 µM. Data were acquired on a Quadrupole Time Of Flight (Q-TOF) mass spectrometer (Q-TOF micro, Micromass, Altrincham, U. K.) interfaced with a Nanomate (Advion Biosciences, Ithaca, N. Y., U. S. A.) with a chip voltage of 1.70 kV and a delivery pressure 0.25 psi (1 psi = 6.81 kPa). The sample cone voltage was typically 80 V with a source temperature of 40 °C and with an acquisition/scan time of 10 s/1 s. Calibration and sample acquisition were performed in positive ion mode in the range of 500–5,000 *m/z*. The pressure at the interface between the atmospheric source and the high vacuum region was fixed at 6.60 mbar. External instrument calibration was achieved using sodium iodide. Data were processed employing Masslynx 4.0 (Waters).

Quantitative Gel Filtration: The oligomerization states of wt ThnE and ThnE?2-46 were analyzed by size exclusion chromatography. Calibration for size exclusion chromatography (Superdex 200 HR) was carried out using cytochrome c (12 kDa), chymotrypsin (25 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), and blue dextran (2000 kDa) (Gel Filtration Calibration Kit, Amersham Biosciences). An elution volume parameter (K_{av}) was calculated for each of the calibration proteins and a calibration curve constructed. By calculating K_{av} for wt ThnE and ThnE?2-46, the native molecular weights were estimated 88.5 kDa and 78.9 kDa respectively.

ThnE assays: ThnE incubations were performed by sequential addition of the following: to a solution of Tris•HCl pH 9.0 (600mM, 35 µL), CoA derivative (10mM, 8 µL), GSA/P5C in 10% formic acid (15mM, 5 µL) and wt ThnE/ThnE?2-46 (2mM, 2 µL), followed by incubation at 37 °C (10 mins). An equal volume of methanol was then added and the mixture cooled on ice (10 mins) before centrifugation at 13 000 rpm (10 mins). The supernatant was decanted and analyzed by liquid chromatography/

time of flight mass spectrometry (LC/TOFMS). Control assays were performed as before but with substitution of Tris•HCl pH 7.5 (50mM) for ThnE.

Products from small scale assays were analyzed using a Waters LCT Classic mass spectrometer equipped with a 2790 sample/solvent manager with a Primesep 100 column (Sielc), using a gradient from 5 % aqueous CH_3CN + 0.1 % formic acid (*v/v*) to 100 % CH_3CN + 0.1 % formic acid (*v/v*).

Products for NMR analysis were produced by scale-up of assay conditions (10 x), followed by quenching with MeOH (500 µL), centrifugation (13,000 rpm) and lyophilization of the supernatant. The resultant residue was re-suspended in 15 % aqueous methanol (200 µL) and purified using a mixed mode Waters Spherisorb column (250 mm x 10 mm, 5 µ) pre-equilibrated in 5 % aqueous MeOH. A gradient was run to 10 % aqueous MeOH with 0.1 % formic acid. Elution was monitored using a Waters ZMD mass spectrometer equipped with 2700 sample manager and 600 controller. Fractions with masses corresponding to anticipated products were collected (~10 mL) and lyophilized. The resultant residue was re-suspended in ^2H_2O (700 µL), transferred to an Eppendorf vial and lyophilized. The final residue was resuspended in ^2H_2O (6 or 12 µL, according to probe), transferred into a 1 mm NMR tube, and analyzed by NMR using a Bruker AVII 500 MHz spectrometer fitted with a 1 mm TXI inverse microprobe or a Bruker AVIII 700 MHz spectrometer with a 5 mm TCI inverse cryoprobe. Products were analysed by 2D COSY and NOESY (mixing time 800 ms) and stereochemistries were assigned through combined analysis of $^3J_{HH}$ coupling constants and NOEs.

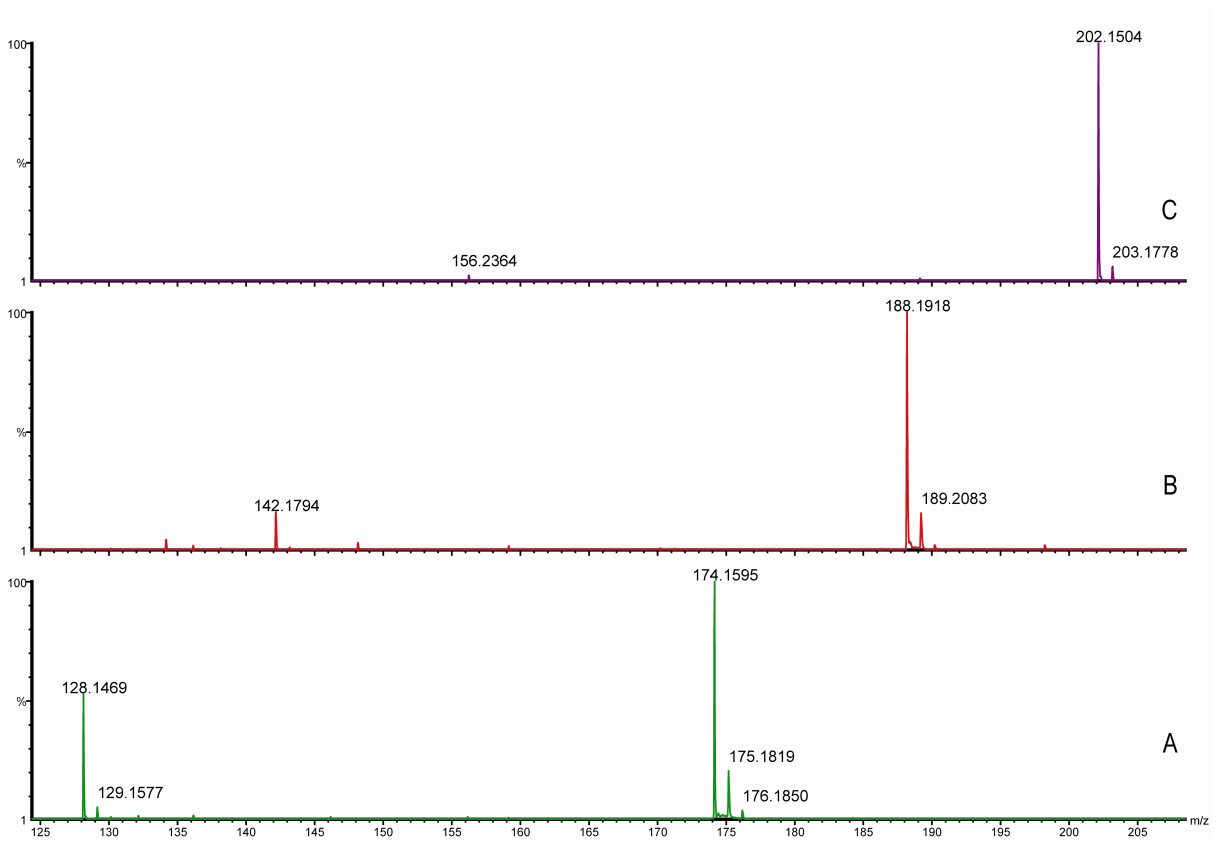


Figure S4: LC/TOFMS analysis of enzymatically produced CMP derivatives. **A**: ThnE produced *t*-CMP $[M+H]^+$ = 174 Da, **B**: ThnE produced 6-methyl-*t*-CMP $[M+H]^+$ = 188 Da and **C**: CarB produced 6-ethyl-*t*-CMP $[M+H]^+$ = 202 Da.

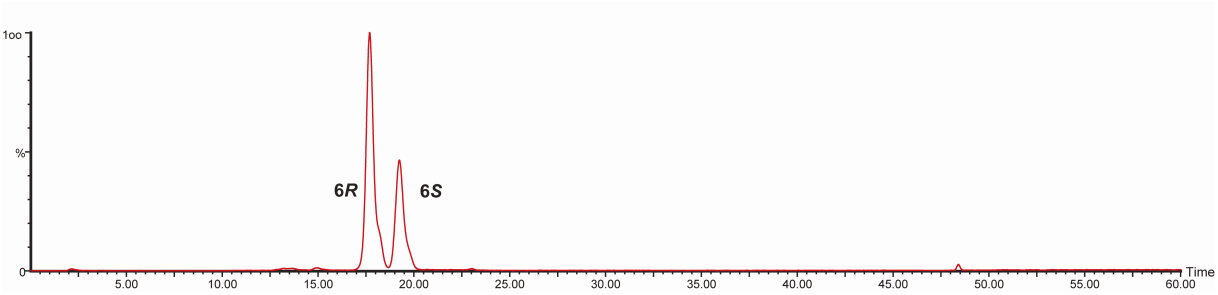


Figure S5: LC/TOFMS analysis of the two C-6 epimers of 6-ethyl-*t*-CMP $[M+H]^+$ = 202 Da produced by CarB. Note the 2:1 ratio between the 6*R*:6*S* epimers. The stereochemistry at C-6 was defined by NMR analyses (see below).

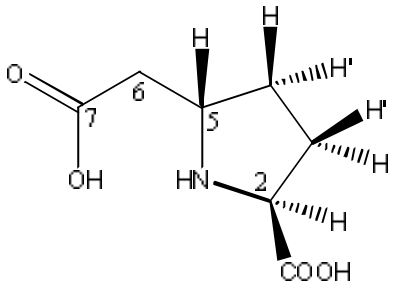
Assignment of the structures of the catalytic products of ThnE and ThnE?2-46

1- Product of incubation of malonyl-CoA and L-GHP in the presence of CarB/ThnE:

Table S1: ^{1}H NMR data for (2*S*,5*S*)-carboxymethylproline (*t*-CMP) isolated from CarB or ThnE catalyzed reaction (700MHz, $^{2}H_2O$). The "centre of gravity" of the chemical shift for multiplets is reported.

Proton no.	δ_H compound of *t*-CMP
H-2	4.20 (br t, *J* = 8.5 Hz)
H-5	3.95 (m)
H-6	2.77 (2H, m)
H-3	2.35 (m)
H-4	2.18 (m)
H-3'	1.97 (m)
H-4'	1.70 (m)

Incubating malonyl-CoA with L-GHP in the presence of CarB/ThnE resulted in the production of CMP as confirmed by LC-MS; *m/z* 174 Da $[M+H]^+$ and by NMR analyses (Figures 2, S6-7). Authentic (2*S*,5*S*)- and (2*S*,5*R*)-CMP (*t*-CMP and *c*-CMP respectively) were prepared by synthesis.[7] The spectral data for purified CMP obtained from CarB/ThnE incubations were near identical to authentic *t*-CMP.



(2*S*,5*S*)-carboxymethylproline

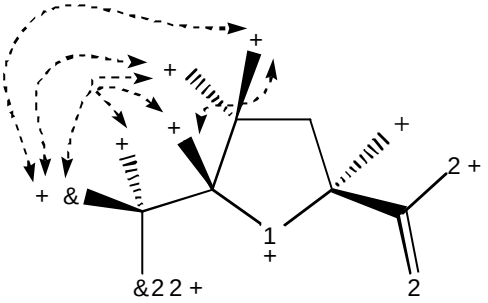
2- Product of incubation of methylmalonyl-CoA and L-GHP in the presence of CarB/ ThnE:

Table S2: ^{1}H NMR and 2D NOESY data for the two C-6 epimers of 6-methyl-*t*-CMP (700MHz, $^{2}H_2O$). The NOE data refers to the significant NOE observed between the protons in column 1 and those indicated in columns 3 and 5.

Proton no.	d_H of 6*S* epimer	NOE	d_H of 6*R* epimer	NOE
H-2	4.23 (br.t, *J* = 8.5 Hz)		4.20 (br.t, *J* = 8.5 Hz)	
H-5	3.82 (m)	4, 8	3.85 (m)	4, 6, 8
H-6	2.80 (dq, *J* = 9.8, 7.2 Hz)	4' (w), 8	2.90 (dq, *J* = 6.8, 7.0 Hz)	4' (w), 5, 8
H-3	2.42 (m)		2.41 (m)	
H-4	2.25 (m)	5, 8	2.22 (m)	5
H-3'	2.01 (m)		2.00 (m)	
H-4'	1.78 (m)	6 (w), 8	1.78 (m)	6 (w)
H-8	1.23 (d, *J* = 7.2 Hz)	5, 6, 4, 4'	1.24 (d, *J* = 7.0 Hz)	5, 6

Incubating methylmalonyl-CoA with L-GHP in the presence of CarB or ThnE resulted in the production of a compound with a mass corresponding to the anticipated 6-methyl-CMP: *m/z* 188 Da $[M+H]^+$. ^{1}H- and 2D NMR analyses of the purified product revealed the existence of the two epimers of 6-methyl-t-CMP in different ratios for CarB and ThnE. The stereochemistry at C-6 of the minor epimer produced by ThnE catalysis was assigned as (*S*) based on the following observations:

- The $J_{5,6}$ value of 9.8 Hz (predicted averaged F ~ 170º) together with a weak NOE observed between H-5 and H-6 indicating a predominantly *anti* conformation for these two protons.
- The observation of an NOE between H-6 and H-4', together with the observation of an NOE between the C-6 methyl group and both C-4 protons.



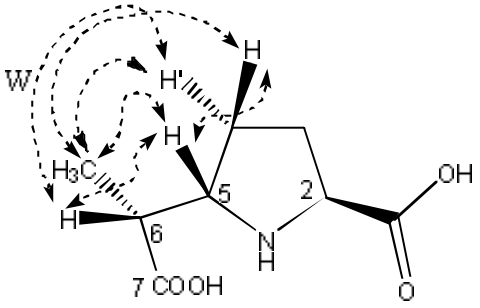
6 6 6 PHMK OMK03

NB: The dashed arrows represent the observed NOEs.

The stereochemistry at C-6 of the major epimer produced by ThnE catalysis was identified as (*R*) based on the following observations:

The $J_{5,6}$ value of 6.8 Hz (predicted averaged *F* ~ 35º) together with the strong NOE observed between H-5 and H-6, indicating a predominantly *gauche* arrangement between these two protons.

The observed NOE between the C-6 methyl group to both protons at C-4 (H-4' > H-4), as well as the weak NOE observed between H-6 and H-4'.



(2*S*,5*S*,6*R*)-6-methyl-*t*-CMP

3- Product of incubation of ethylmalonyl-CoA and L-GHP in the presence of CarB:

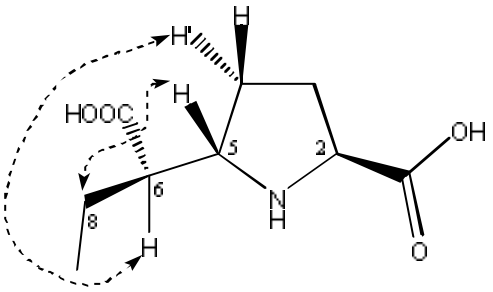
Table S3: ^{1}H NMR data for the two epimers of 6-ethyl-*t*-CMP (500 and 700MHz, $^{2}H_2O$).

Proton no.	d_H of 6*R* epimer	d_H of 6*S* epimer
H-2	4.11 (br. t, *J* = 8.2 Hz)	4.01 (br. t, *J* = 8.3 Hz)
H-5	3.75 (m)	3.69 (m)

H-6	2.52 (m)	2.35 (m)
H-3	2.37 (m)	2.30 (m)
H-4	2.15 (m)	2.12 (m)
H-3'	1.97 (m)	1.88 (m)
H-4'	1.73 (m)	1.60 (m)
H-8	1.63 (m)	1.58 (m)
H-8'		1.50 (m)
H-9	0.89 (t, *J* = 7.3 Hz)	0.80 (t, *J* = 7.5 Hz)

Incubating ethylmalonyl-CoA with L-GHP in the presence of CarB resulted in the production of a compound with a mass corresponding to the anticipated 6-ethyl-CMP: *m/z* 202 Da $[M+H]^+$. The yield of purified 6-ethyl-CMP produced by CarB was not sufficient to obtain good quality 2D NMR analyses and therefore analyses were carried out for the two fractions of 6-ethyl-CMP produced by a variant of CarB. ^{1}H- and 2D NMR analyses of the two fractions revealed the existence of the two epimers of 6-ethyl-*t*-CMP. The stereochemistry at C-6 for fraction 1 of purified 6-ethyl-*t*-CMP was assigned as (*R*) based on the following observations:

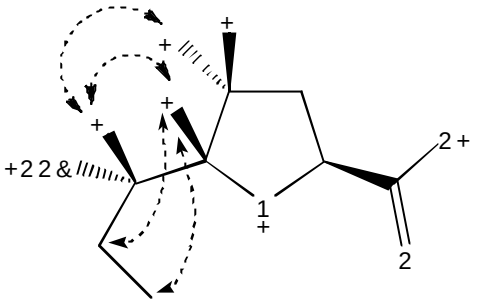
- The $J_{5,6}$ value of 9.2 Hz together with the weak NOE between H-5 and H-6 indicating a predominantly *anti* arrangement of these two protons.
- The observed NOE between H-5 and H-8 and H-8', together with absence of any observed NOE between C-8 protons to any of C-4 protons.



(2*S*,5*S*,6*R*)-6-ethyl-*t*-CMP

The stereochemistry at C-6 for fraction 2 of purified 6-ethyl-*t*-CMP was assigned as (*S*) based on the following observations:

- The $J_{5,6}$ value of 6.3 Hz together with the strong NOE between H-5 and H-6 indicating a predominantly *gauche* arrangement of these two protons.
- The strong NOE observed between H-5 and H-8, H-8', coupled to a strong NOE observed between H-5 and C-9 protons together with weak NOE between H-6 and H-4'.



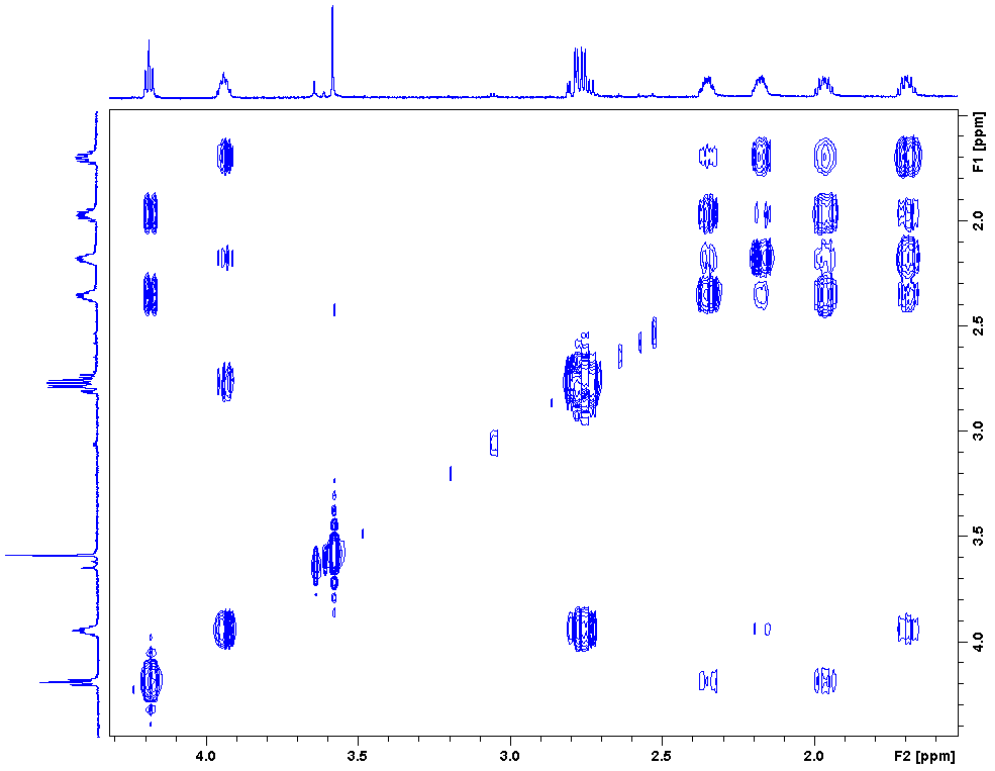


Figure S6: 2D COSY of *t*-CMP produced by CarB.

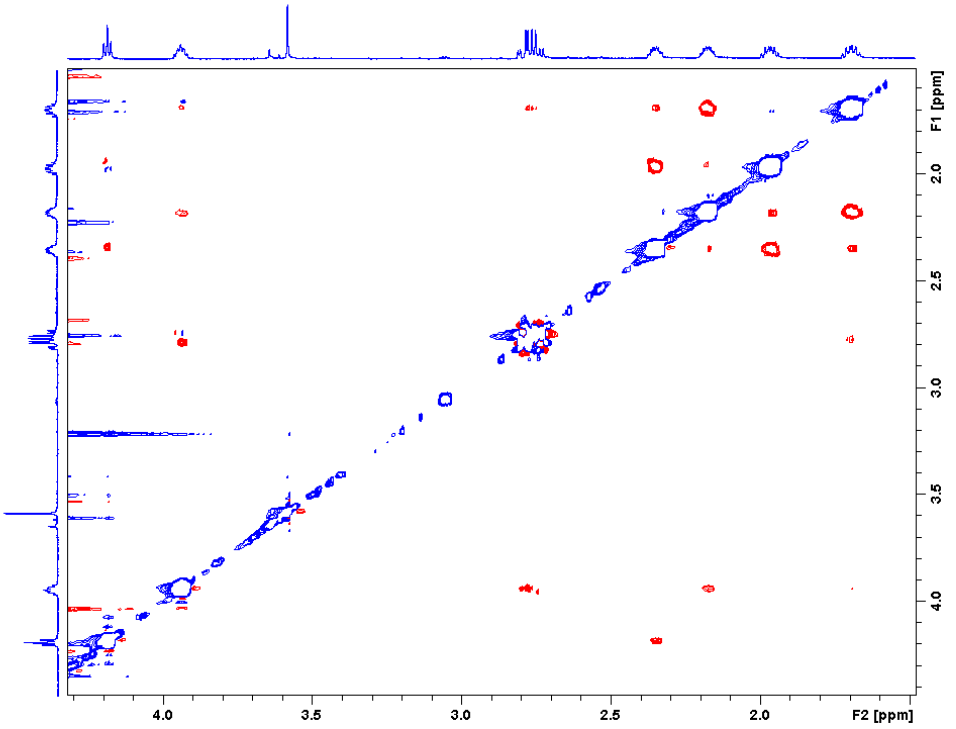


Figure S7: 2D NOESY of *t*-CMP produced by CarB.

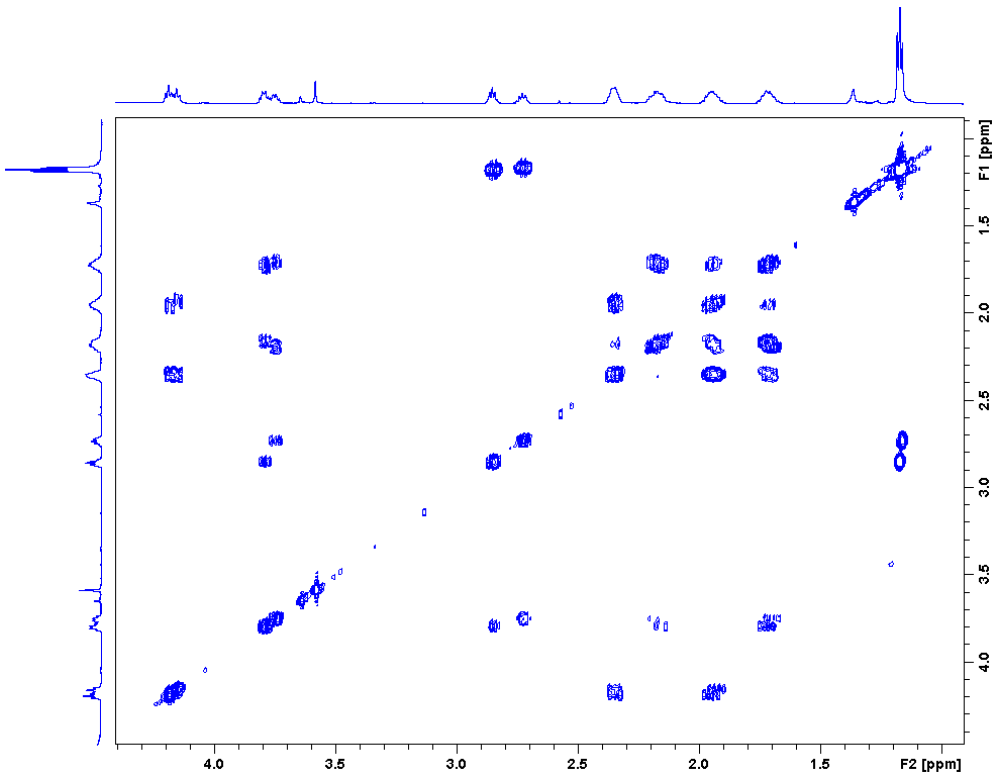


Figure S8: 2D COSY of the two epimers of 6-methyl-*t*-CMP produced by CarB.

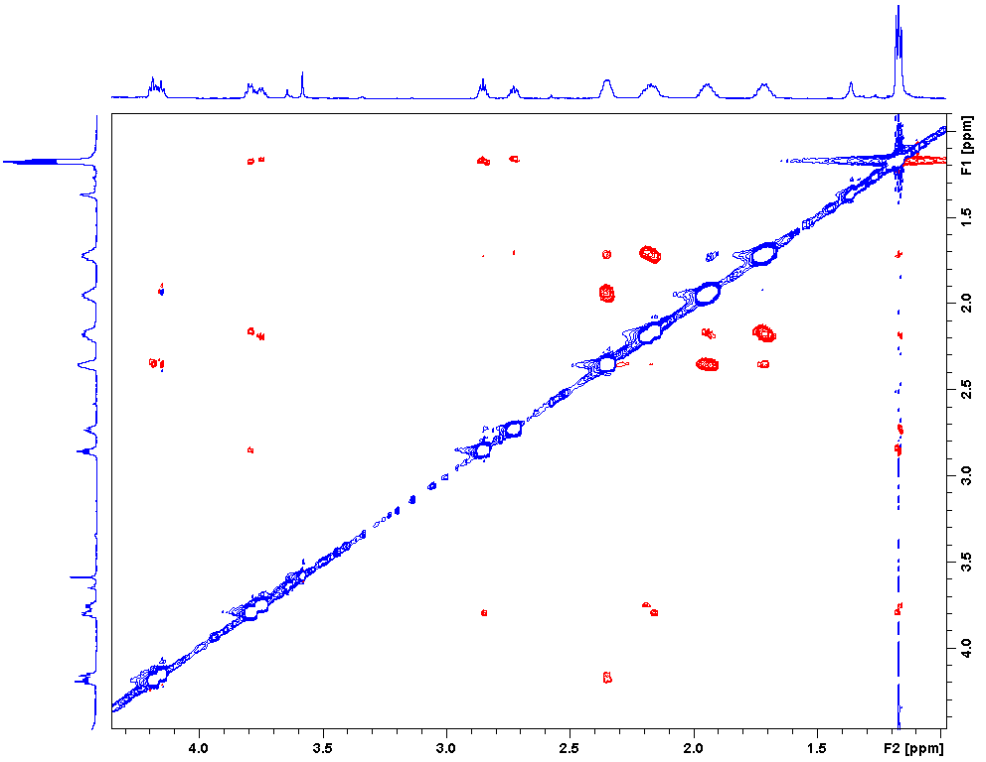


Figure S9: 2D NOESY of the two epimers of 6-methyl-*t*-CMP produced by CarB.

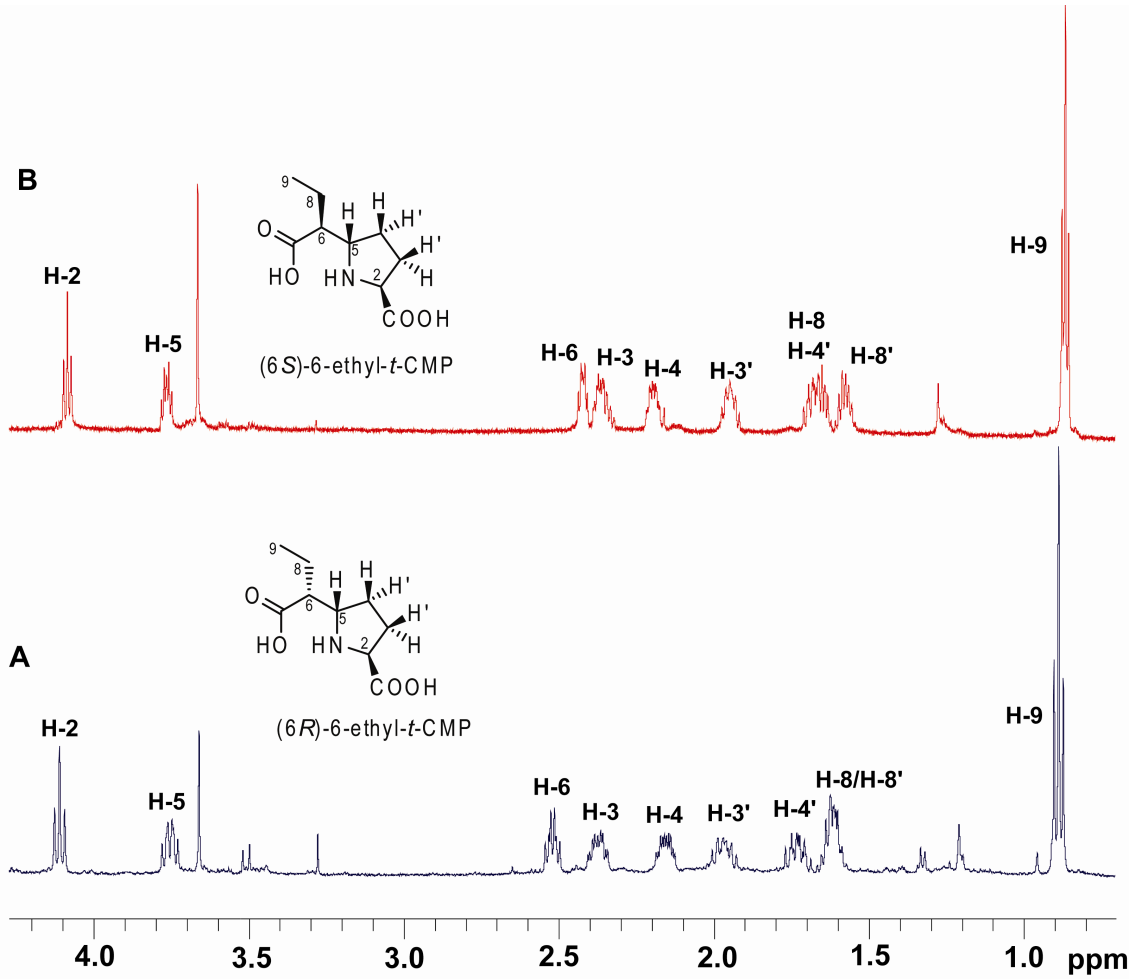


Figure S10: ^{1}H NMR spectra for the two epimers of 6-ethyl-*t*-CMP produced by a CarB variant. Spectra A and B represent the 6*R* and 6*S* epimers of 6-ethyl-*t*-CMP, respectively.

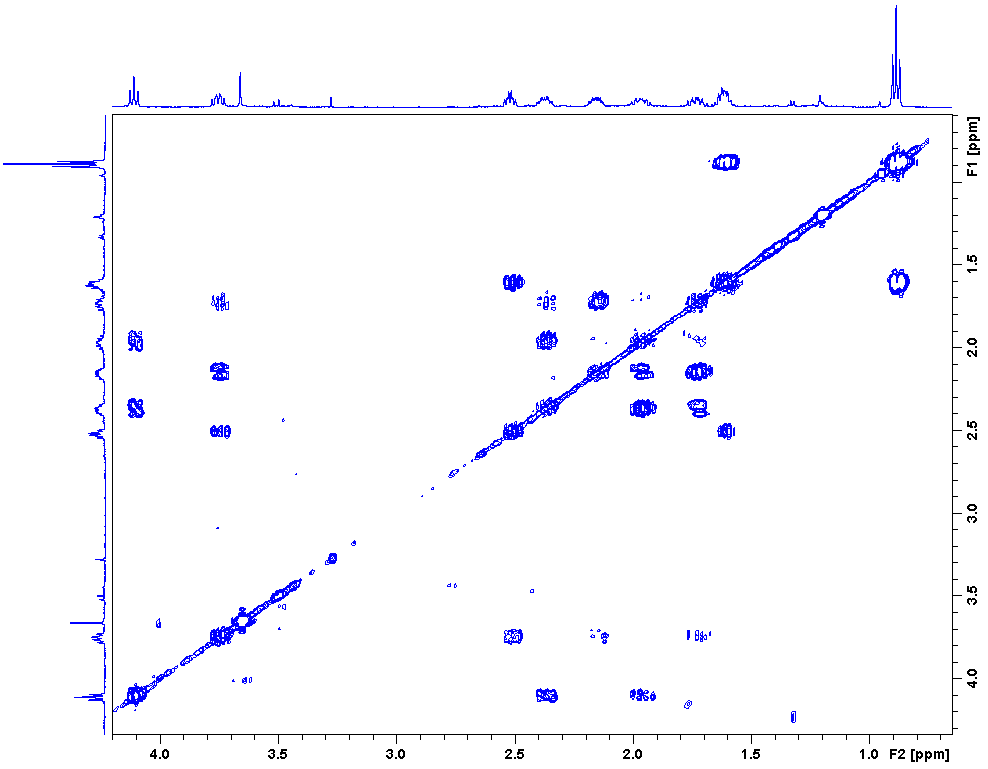


Figure S11: 2D COSY of the (6*R*)-6-ethyl-*t*-CMP produced by a CarB variant.

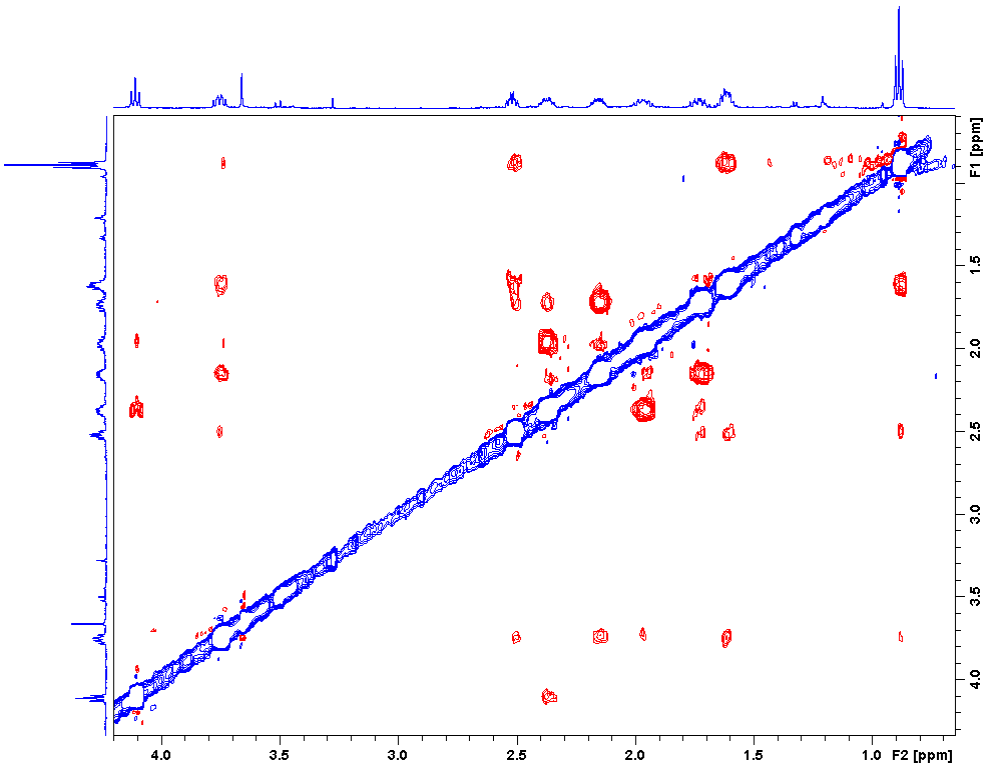


Figure S12: 2D NOESY of the (6*R*)-6-ethyl-*t*-CMP produced by a CarB variant.

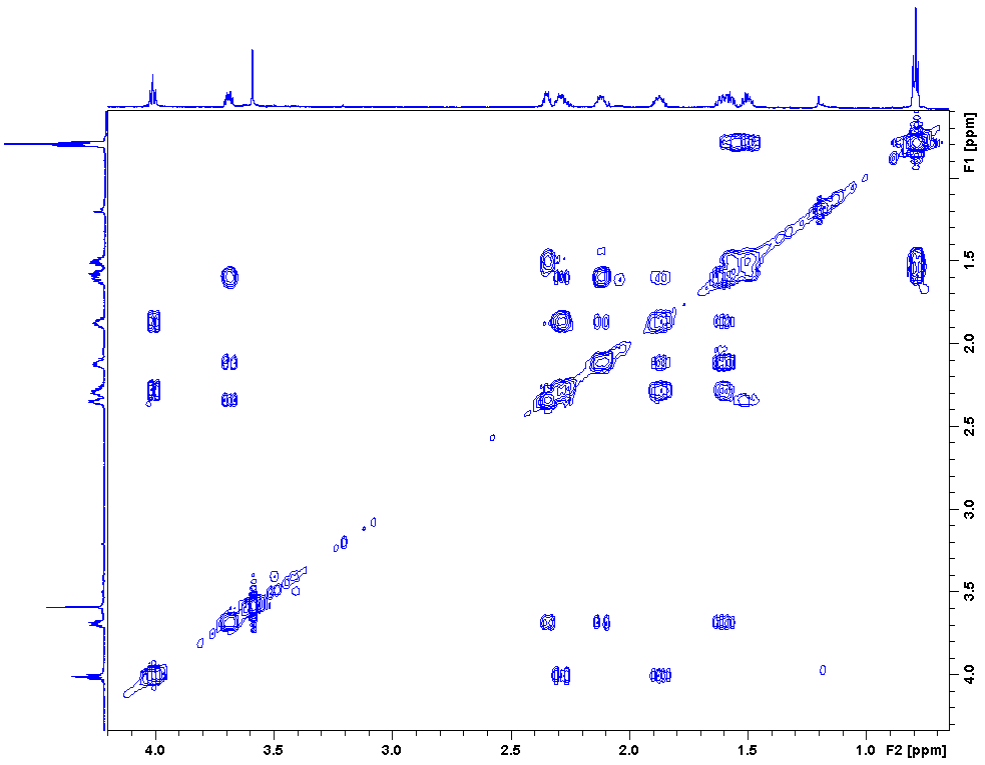


Figure S13: 2D COSY of the (6*S*)-6-ethyl-*t*-CMP produced by a CarB variant.

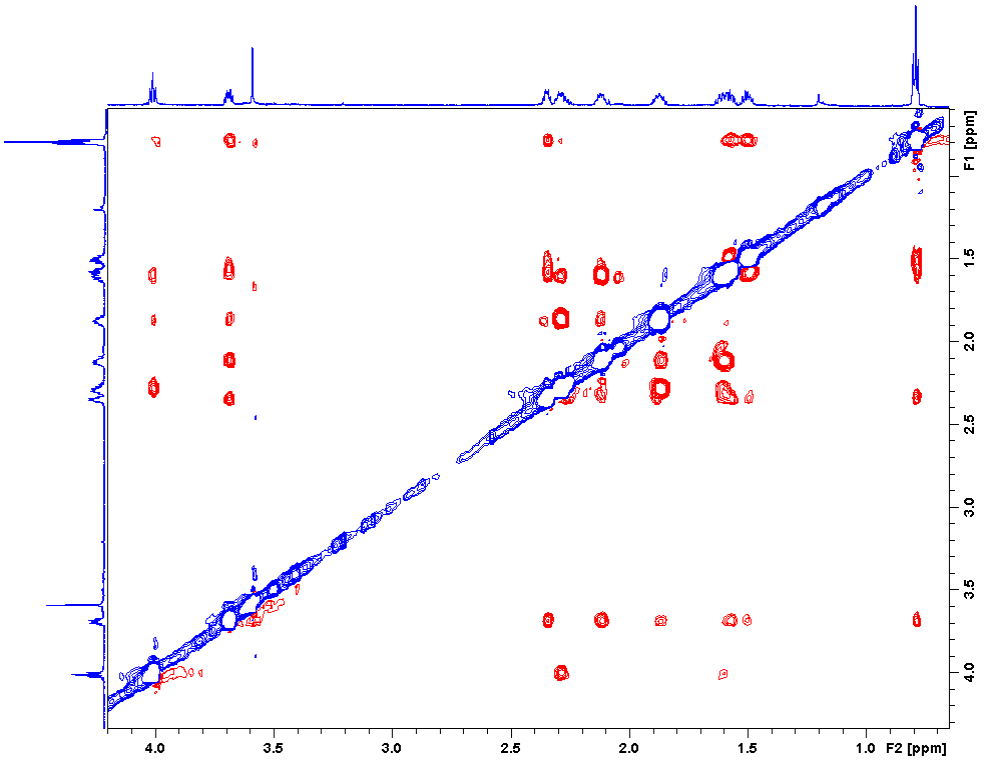


Figure S14: 2D NOESY of the (6*S*)-6-ethyl-*t*-CMP produced by a CarB variant.

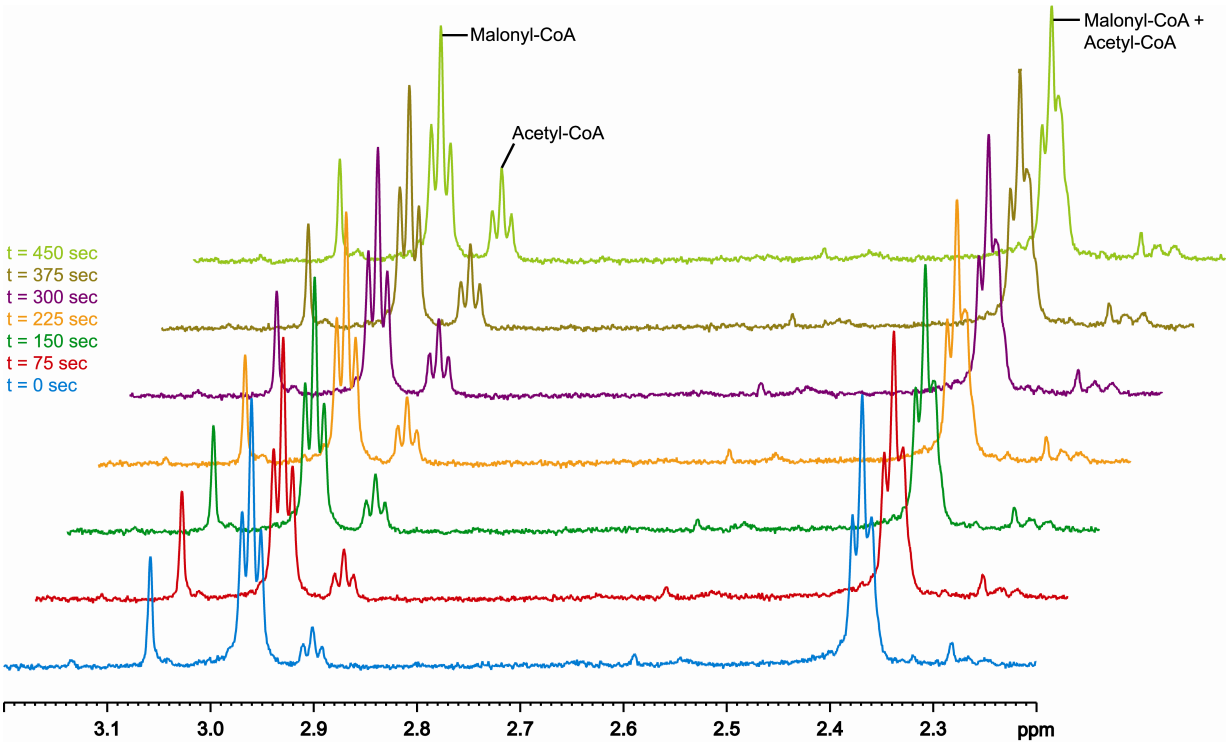


Figure S15: ^{1}H NMR spectra showing the ThnE catalyzed decarboxylation of malonyl-CoA into acteyl-CoA, in real time, at 37°C. Malonyl-CoA decarboxylation was followed by measuring the decrease in intensity of the triplet at d_H ~2.97 ppm. Acetyl-CoA was monitored by the increase in intensity of the triplet at ~2.90 ppm. The time t = 0 indicates the time at which data acquisition was started following sample preparation and insertion in the probe.

Real-time NMR monitoring of wt ThnE and ThnE?2-46 -catalyzed reactions: ^{1}H NMR monitoring and specific activity determination for wt ThnE and ThnE?2-46 catalyzed reactions were performed using a Bruker AVIII 700 MHz spectrometer (with inverse TCI cryoprobe optimized for ^{1}H observation, running TOPSPIN 2 software) with conditions similar to those used for ThnE assays except that 100mM CoA stocks in H_2O were diluted to the appropriate concentration with $^{2}H_2O$, 600mM [$^{2}H_{11}$]-Tris•^{2}HCl (adjusted to p^{2}H 9.0 with ^{2}HCl) in $^{2}H_2O$ buffer was used; assays were scaled-up to a final volume of 75µL, transferred to a 2 mm NMR tube (Bruker) and analyzed by using 8 consecutive experiments each of either 16 or 32 scans, lasting 75 or 150 s respectively. The sample temperature was maintained at 310 K throughout the run. The spectra were integrated using absolute intensity scaling to monitor changes in the signals of interest.

Table S4. Specific activities measured by comparing changes in the ratio of substrate to product by NMR over time and reported in mmol mg^{-1} s^{-1} for ThnE and ThnE?2-46.

Enzyme / Reaction	ThnE		ThnE?2-46	
	- L-GHP	+ L-GHP	- L-GHP	+ L-GHP
Malonyl-CoA to acetyl-CoA	6.065 ± 0.135	3.461 ± 0.172	4.418 ± 0.428	5.594 ± 0.476
Methylmalonyl-CoA to propionyl-CoA	0.733 ± 0.118	0.363 ± 0.027	0.761 ± 0.138	0.347 ± 0.016
Acetyl-CoA to coenzyme A	0.155 ± 0.001	0.062 ± 0.015	0.100 ± 0.015	0.058 ± 0.007
Propionyl-CoA to coenzyme A	0.609 ± 0.119	0.141 ± 0.004	0.715 ± 0.121	0.069 ± 0.003

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