Supporting Information

Stereoselective Production of Dimethyl-Substituted Carbapenams via Engineered Carbapenem Biosynthesis Enzymes

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Contents

I- Materials and Methods

Chemicals were purchased from Alfa Aesar (Karlsruhe, Germany), Aldrich (Dorset, UK), Acros chemicals (Loughborough, UK), Tokyo Chemical Industry UK Ltd, 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. IPTG was from Melford Laboratories Ltd., electrophoresis grade agarose was from Bioline, 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. Oligonucleotide primers were from Sigma-Genosys. 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. Yields refer to purified and dried 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[1] using Milli-Q water and were autoclaved or sterilised by filtration, as required.

All NMR Spectra were recorded using a Bruker AVIII 700 MHz machine (with a ^{1}H inverse cryoprobe). 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. Prediction of dihedral angle (Φ) between vicinal hydrogen atoms was conducted through MestRe-J software employing the "HLA (Chemical groups)" equation.[2]

Low resolution ESI mass spectrometry was performed on a Micromass® Quattro micro™ API mass spectrometer operating in positive or negative ionisation mode. High resolution (HR) ESI mass spectrometry was carried out on a Bruker μTOF spectrometer.

II- Protein Preparation

Recombinant CarB, CarB variants,[3] ThnE and ThnE variants,[3] and CarA[4] were prepared and purified (>95% by SDS-PAGE analysis) as reported. The following Table lists the CMPS variants used in this study:

CarB Variants	ThnE Variants
Wildtype CarB	Wildtype ThnE
CarB W79F	ThnE V153M
CarB W79A	ThnE V153L
CarB W79F/M108A	ThnE V153A
CarB M108A	ThnE V153I
CarB M108L	ThnE W124F
CarB M108I	ThnE W124F/V153M
CarB Q111N	
CarB H229A	

Table S1: CMPS variants employed in this study.

III- Enzyme Assays

III-A CMPS assays

Amino acid aldehydes were obtained by deprotection of the appropriate precursors as reported.[5] Analytical Wildtype and variant CMPS incubations were performed by sequential addition of the following to a 0.5 mL Eppendorf tube (50 µL total volume):

		Final Concentration
600 mM Tris.HCl, pH 9.0	35 µL	420 mM
malonyl-CoA (or derivative thereof, 10 mM)	8 µL	1.60 mM
Amino acid semialdehyde in 10% formic acid (15 mM)	5 µL	1.50 mM
CMPS[a]	2/5 µL	~19-21 µM[a]

[a]The initial concentration of CarB/CarB variants was ~40 mg/mL (~0.48 mM) and that of ThnE/ThnE variants was ~20 mg/mL (~0.21 mM).

The incubation mixture was then kept at 37 °C for 30 min. To quench the reaction, an equal volume of methanol was added; the mixture was then 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 manner 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).

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) on a Waters 1525µ Binary HPLC Pump system with a Waters 2777 Sample Manager coupled to a Micromass® Quattro micro™ API mass spectrometer (ESI+). The column was equilibrated at 1 mL/min with 5% eluent **B**. After 5 min, a gradient

was run to 70% eluent **B** over 20 min. The column was washed with 100% eluent **B** for 5 min before the column was re-equilibrated at 5% eluent **B** for 10 min (overall run time is 40 min per assay).

Eluent **A**:	0.05% HCOOH in H_2O (v/v)
Eluent **B**:	0.05% 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 or both of (**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® Quattro micro™ API mass spectrometer (ESI+) equipped with a Waters 1525µ Binary HPLC Pump system coupled to a Waters 2777 Sample Manager). Fractions with *m*/*z* corresponding to $[M+1]^+$ of the anticipated products were collected (5-15 mL) and freeze dried. The resultant residue was re-suspended in ^2H_2O (600 µL), transferred to an Eppendorf vial and refreeze-dried. The final residue was re-suspended in ^2H_2O (13 µL for a 1 mm NMR tube) or 75 µL (for a 2 mm NMR tubes) and transferred into an NMR tube (Bruker) using a hand centrifuge, and analysed by NMR spectroscopy.

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

Yields of different products of CMPS catalysis were calculated using a combination of LC-MS and ^{1}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 (or derivative thereof) according to the reported ^{1}H-NMR method[6] employing $[^2H]_4$-trimethylsilylpropionate as an external or internal standard; (**ii**) Using this ^{1}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 reported products of (coupled) CMPS catalysis was determined by LC-MS and/or ^{1}H-NMR analyses.[3]

III-B CarA assays

CarA analytical scale incubations were performed as reported.[4] For large-scale incubations: the product of two preparative scale CMPS assays, after purification and freeze-drying, were incubated with the components of CarA assay (x3) for 1 h at 37 °C. The reaction mixture was then quenched with equivalent volume of acetonitrile, incubated on ice for 10 min, centrifuged (13,000 x *g*) and the supernatant was purified using a preparative C18 Column (250 mm x 22 mm, 15 µ) 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. Elution was monitored using a Micromass® Quattro micro™ API mass spectrometer (ESI-). Fractions with *m*/*z* corresponding to $[M-1]^-$ of the anticipated product were collected (~10 mL), 0.1 N sodium bicarbonate was added to pH 7.0, and then the neutralized fraction was freeze-dried. The resultant residue was re-suspended in D_2O (500 µL), transferred to an Eppendorf vial and freeze-dried. The final residue was re-suspended in D_2O (75 µL), transferred into a 2 mm NMR tube using a hand centrifuge, and analysed by NMR using a Bruker AVIII 700 with ^{1}H inverse cryoprobe.

IV- Assignment of reported enzyme-catalysed products

NMR analyses were recorded at 298 K using a Bruker AVIII 700 MHz spectrometer equipped with a ^{1}H TCI-inverse cryoprobe optimised for ^{1}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 $^{3}J_{HH}$ coupling constants and NOEs. Selective 1D TOCSY experiments were conducted employing DIPSI2 mixing scheme. Chemical shifts are reported in ppm relative to D_2O (δ_H 4.72); the deuterium signal was used as an internal lock signal and the HDO signal was reduced by presaturation wherever necessary. For quantification of the carboxymethylproline synthases products of catalysis, trimethylsilane propionic acid sodium salt (TSP) was used as an external 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) supported the formation of the product as shown by observation of the molecular ion, the ion arising from decarboxylation of the product, and the ion resulting from the loss of the side chain at C-5. The formation of a ring structure was assigned in part from the ^{1}H-NMR chemical shift of the bridgehead proton (H-5). 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 to (2*S*,5*S*)-carboxymethylproline (*t*-CMP).[19,25,29] For all compounds reported, the assignment of the bridgehead carbon (C-5) as having (*S*)-stereochemistry was in part based on NOESY 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. The assignment of C-6 of *t*-CMP derivatives substituted at this carbon as having (*R*) or (*S*)-stereochemistry was based on a combination of coupling constant $J_{5,6}$ (*t*-CMP) and NOESY data. In some cases, the assignment of stereochemistry at C-6 was further confirmed by conversion of the product into the corresponding β-lactam derivative by CarA catalysis.

V- Spectra of products of (coupled) CMPS catalysis

Stereochemical assignment of 2,6-dimethyl-*t*-CMP epimers resulting from incubation of 2-methyl-GHP,[7] methylmalonyl-CoA, and CMPSs

Note: HRMS (ESI-TOF) *m/z*: $[M + H]^+$ Calcd for $C_9H_{16}O_4N$ 202.1074; Found 202.1076.

For both epimers of 2,6-dimethyl-*t*-CMP produced by CarB H229A catalysis, the *trans*-relationship between H-5 and the methyl group at C-2 of the products was assigned on the basis of lack of observation of nOe correlations between these two entities (Figs. S1 and S2).

- The stereochemistry at C-6 of **the first epimer to elute** was assigned as (6*S*) based on the following observations (Fig. S1):

❖ A $J_{5,6}$ value of ~7.0 Hz (predicted averaged Φ ~ 141°) together with a moderate nOe correlation between H-5 and H-6 indicating a predominantly anticlinal arrangement of these two protons.

❖ A moderate nOe correlations between the methyl group at C-6 and both protons at C-4 (H-4 > H-4'), as well as a moderate nOe between H-6 and H-4'.

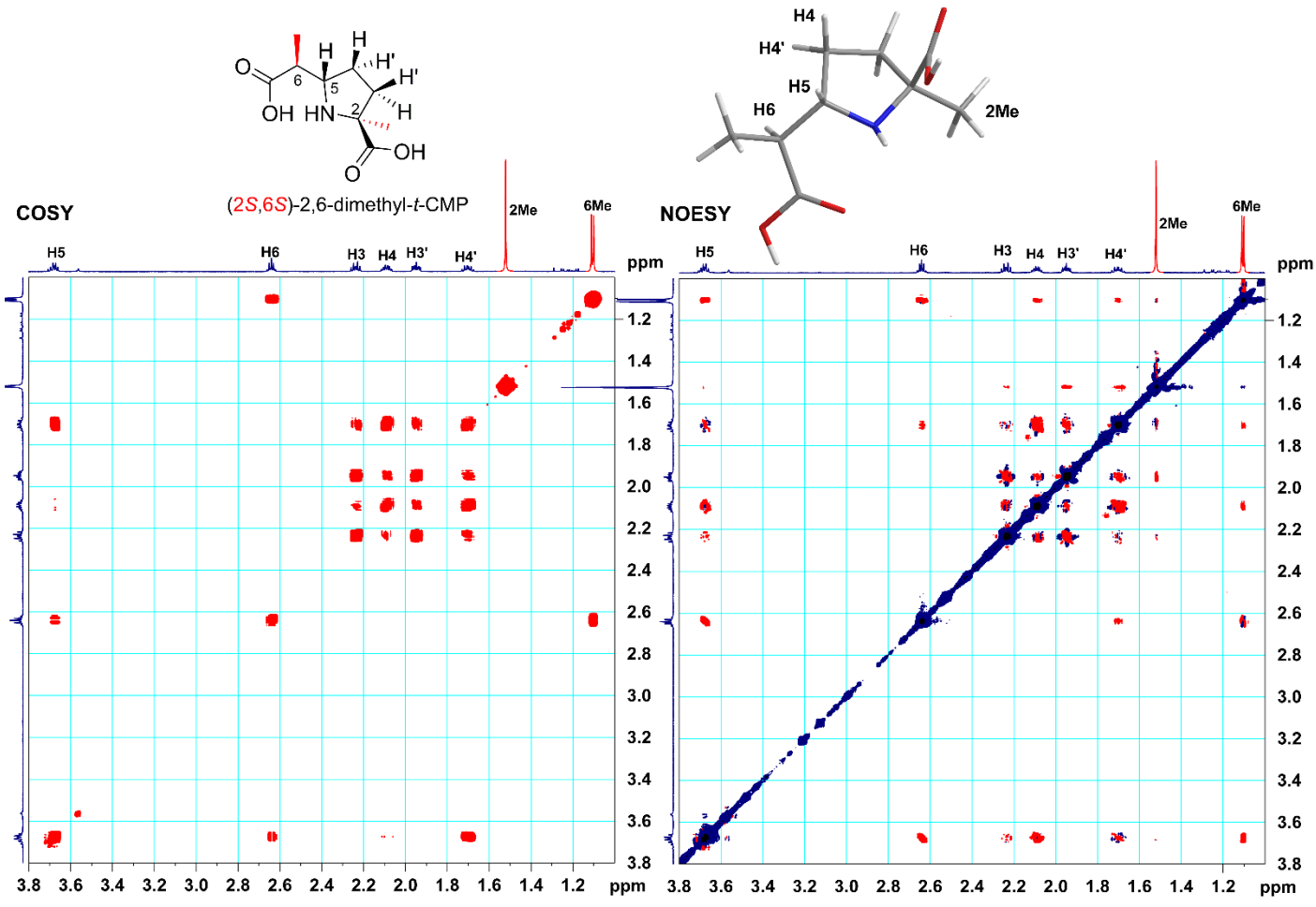

Figure S1: ^{1}H-^{1}H COSY and NOESY spectra for (2*S*,6*S*)-2,6-dimethyl-*t*-CMP produced from incubation of racemic 2-methyl-GHP and C-2 epimeric methylmalonyl-CoA by CarB H229A catalysis. The appended energy minimized 3D model, generated by ChemBio3D, is based on the coupling constant and 2D NOESY spectral data.

- The stereochemistry at C-6 of **the second epimer to elute** was assigned as (6*R*) based on the following observations (Fig. S2):

❖ A $J_{5,6}$ value of ~7.0 Hz (predicted averaged Φ ~ 136º) together with a moderate nOe correlation between H-5 and H-6 indicating a predominantly anticlinal arrangement of these two protons.

❖ A moderate nOe observed between H-6 and H-4', together with almost no observed nOe between the methyl group at C-6 and the protons at C-4.

❖ The assignment of stereochemistry at C-6 was further confirmed by conversion of the product into the corresponding β-lactam derivative by CarA catalysis (Fig. S15).

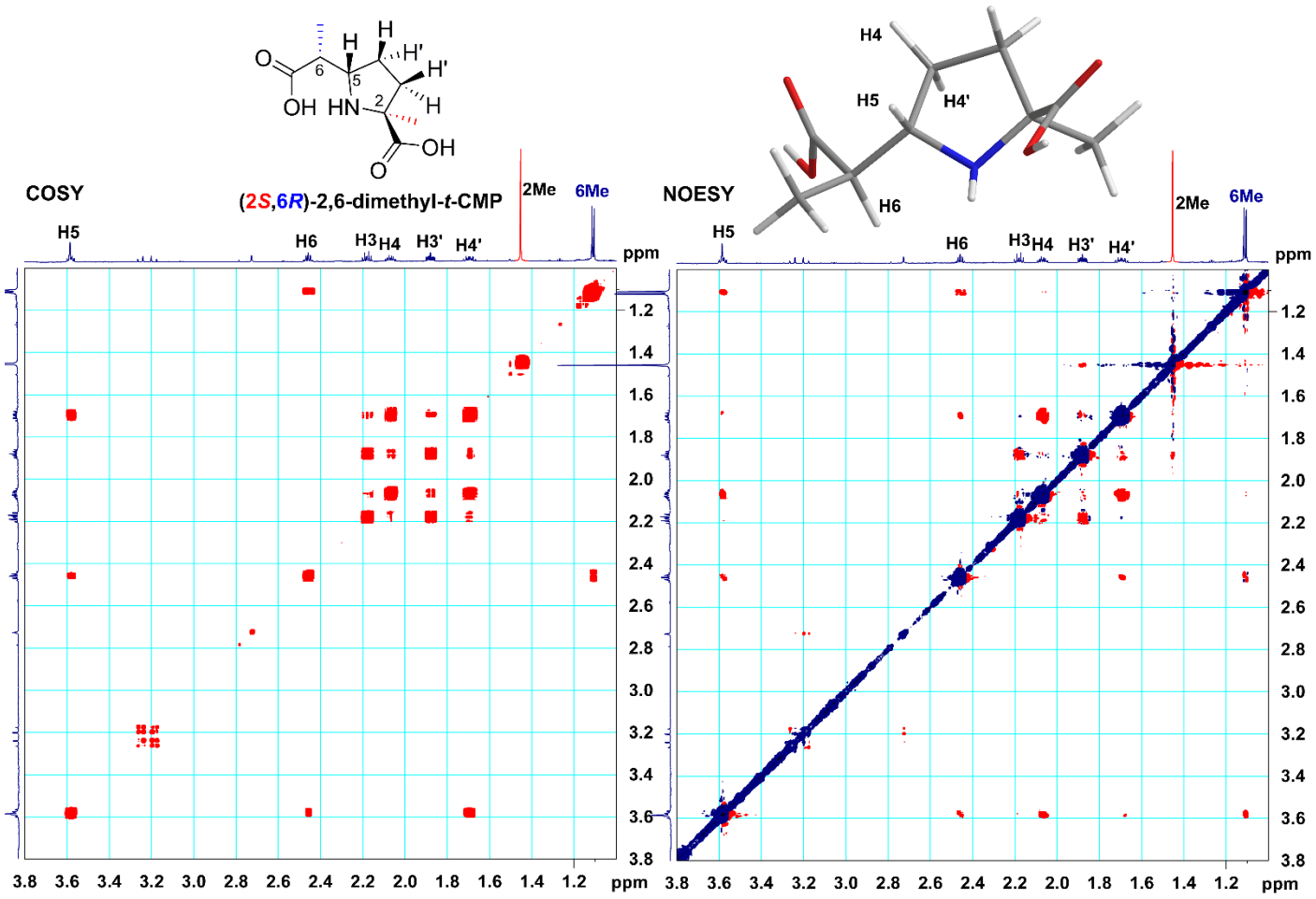

Figure S2: ^{1}H-^{1}H COSY and NOESY spectra for (2*S*,6*R*)-2,6-dimethyl-*t*-CMP produced from incubation of racemic 2-methyl-GHP and C-2 epimeric methylmalonyl-CoA by CarB H229A catalysis. The appended energy minimized 3D model, generated by ChemBio3D, is based on the coupling constant and 2D NOESY spectral data.

Stereochemical assignment of 3,6-dimethyl-*t*-CMP epimers resulting from incubation of 3-methyl-GHP derivatives,[7] methylmalonyl-CoA, and CMPSs

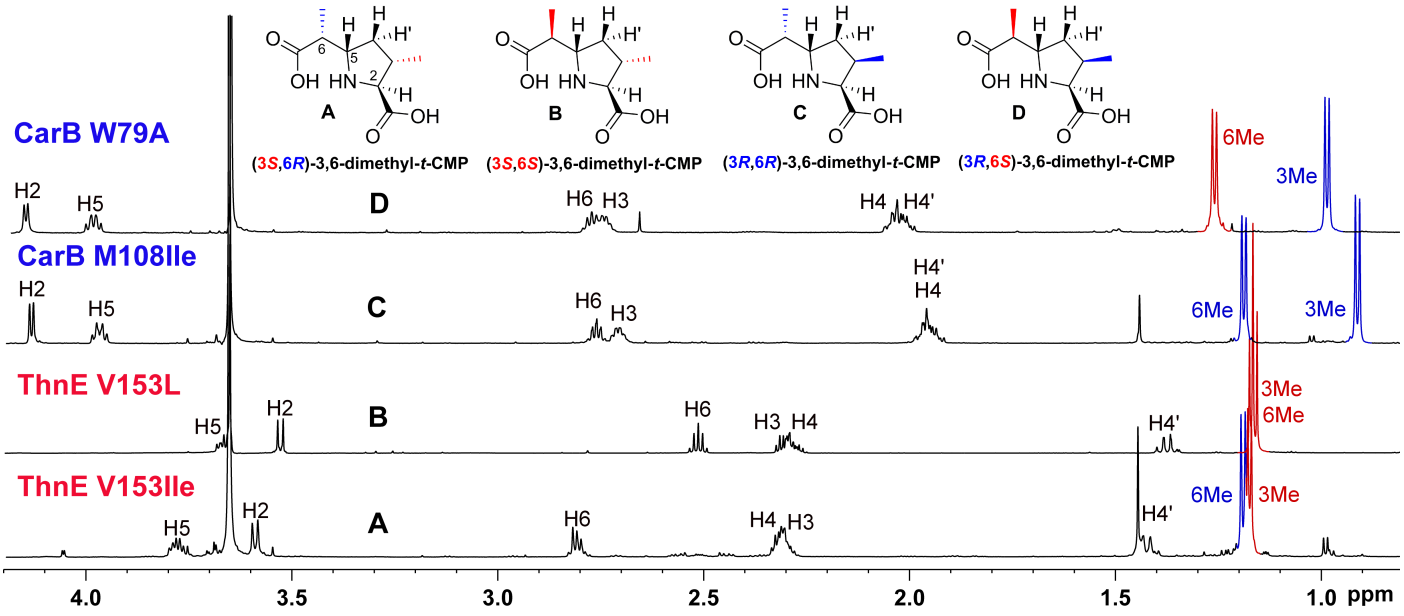

Figure S3. ^{1}**H NMR spectra for the purified C-6 epimers of (3*S*)-3,6-dimethyl-*t*-CMP (A and B) and those of (3*R*)-3,6-dimethyl-*t*-CMP (C and D) resulting from incubation of the C-2 epimeric methylmalonyl-CoA and the racemic (2*S*,3*S*)-3-methyl-GHP or (2*S*,3*R*)-3-methyl-GHP, respectively, via the catalysis of the shown CMPS variants.**

Stereochemical assignment of 3,6-dimethyl-*t*-CMP epimers resulting from incubation of racemic (2*S*,3*S*)-3-methyl-GHP,[7] methylmalonyl-CoA, and CMPSs

Note: HRMS (ESI-TOF) *m/z*: $[M + H]^+$ Calcd for $C_9H_{16}O_4N$ 202.1074; Found 202.1077.

- **For the first eluting epimer** (the major epimer of ThnE V153I catalysis), assuming the stereochemistry at C-2 is (*S*) based on reports that D-GHP is not a substrate for either CarB or ThnE,[8-9] the 2D NOESY data reveal the stereochemistry at C-3 as (*S*) on the basis of the following observations:
  - A moderate nOe correlation between H-2 and the methyl group at C-3 together with the absence of any correlation between H-2 and H-3.
- The stereochemistry at C-6 was assigned as (6*R*) based on the following observations (Fig. S4):
  - A $J_{5,6}$ value of 6.8 Hz (predicted averaged Φ ~ 135º) together with a very weak nOe between H-5 and H-6 indicating a predominantly anticlinal arrangement of these two protons.
  - A moderate nOe correlation between H-5 and the methyl group at C-6, together with the (near) absence of any correlation between the two protons at C-4 and the methyl group at C-6.
  - A very weak nOe correlation between H-6 and H-4'.
  - The assignment of stereochemistry at C-6 was further confirmed by conversion of the product into the corresponding β-lactam derivative by CarA catalysis (Fig. S18).

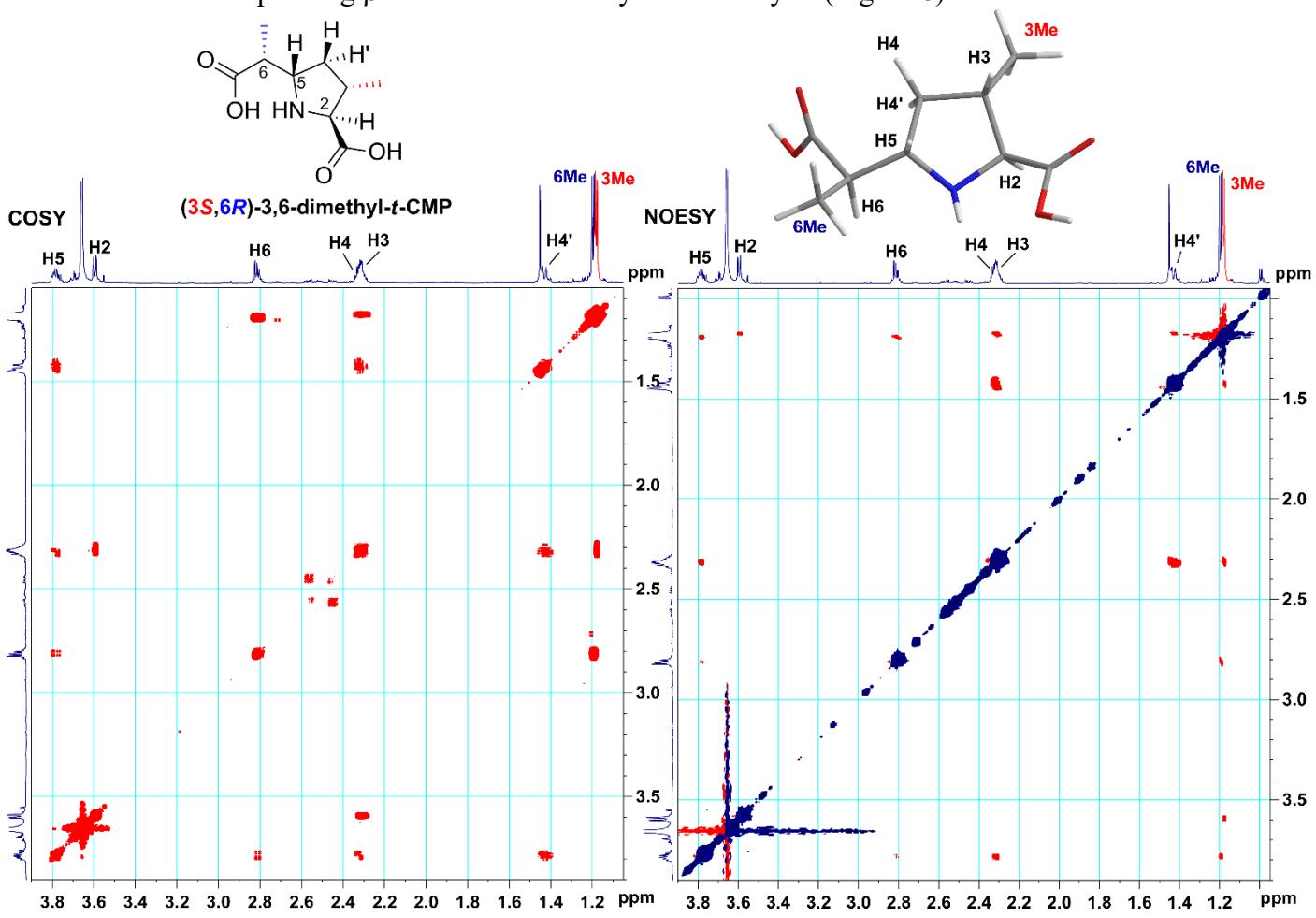

Figure S4: ^{1}H-^{1}H COSY and NOESY spectra for the (3*S*,6*R*)-3,6-dimethyl-*t*-CMP epimer produced from incubation of the racemic pair containing (2*S*,3*S*)-3-methyl-GHP and methylmalonyl-CoA by ThnE V153I catalysis. The appended energy minimized 3D model, generated by ChemBio3D, is based on the coupling constant and 2D NOESY spectral data.

- **For the late eluting epimer (the major epimer of ThnE V153A catalysis), the 2D NOESY data reveal the stereochemistry at C-3 as (*S*) on the basis of the following observations:**
  - ❖ A moderate nOe correlation between H-2 and the methyl group at C-3 together with a very weak correlation between H-2 and H-3.
  - ❖ The absence of an nOe correlation between H-5 and the methyl group at C-3.
- The stereochemistry at C-6 was assigned as (6*S*) based on the following observations:
  - ❖ A $J_{5,6}$ value of 7.3 Hz (predicted averaged Φ ~ 143º) together with a very weak nOe between H-5 and H-6 indicate a predominantly anticlinal arrangement of these two protons.
  - ❖ Moderate nOe correlations between the methyl group at C-6 with both of H-5 and H-4.
  - ❖ A weak to moderate nOe between H-6 and H-4' and a weak correlation between H-6 and H-4.
  - ❖ The assignment of stereochemistry at C-6 was further confirmed by conversion of the product into the corresponding β-lactam derivative by CarA catalysis (Fig. S19).

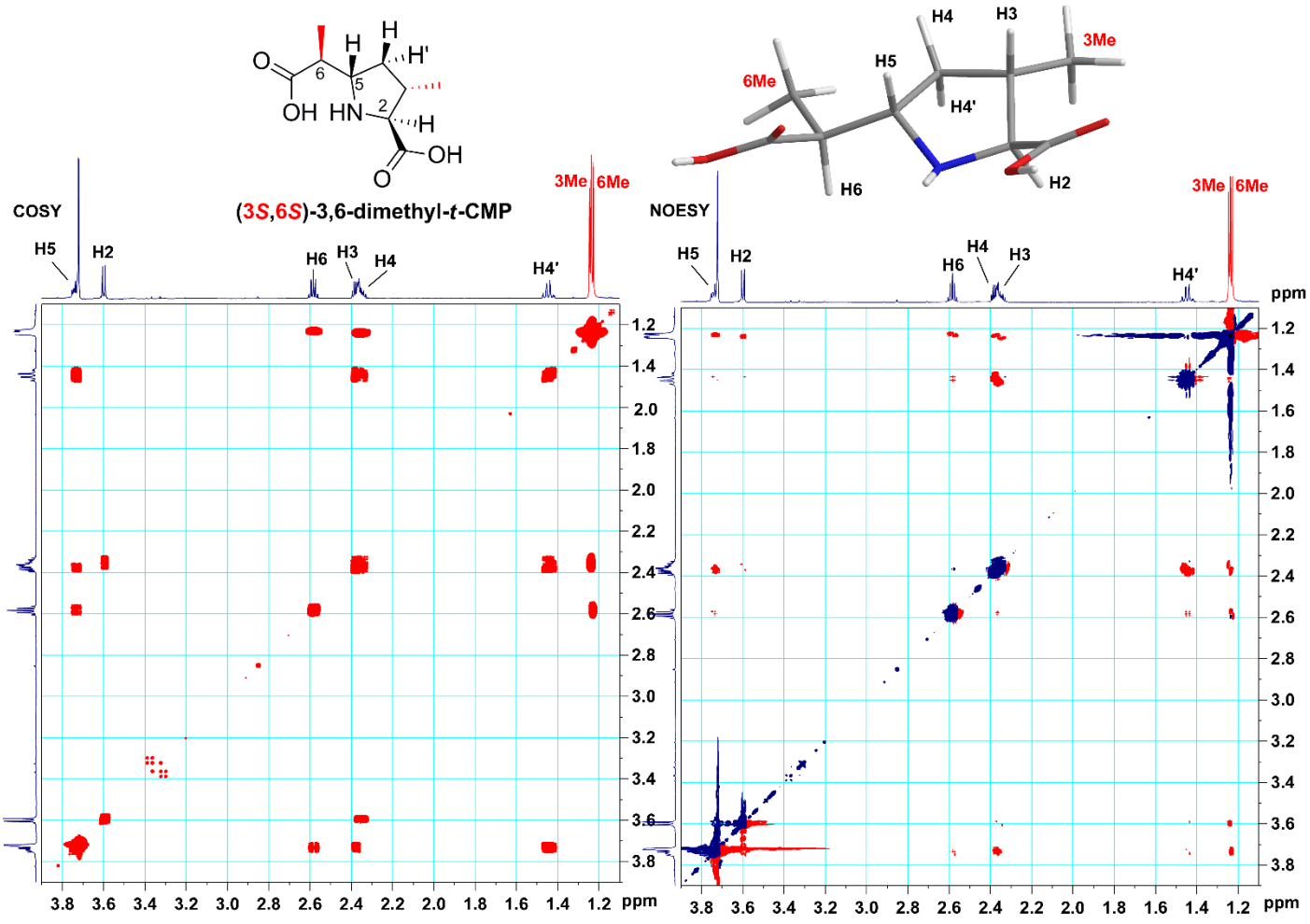

Figure S5: ^{1}H-^{1}H COSY and NOESY spectra for the (3*S*,6*S*)-3,6-dimethyl-*t*-CMP epimer produced from incubation of the racemic pair containing (2*S*,3*S*)-3-methyl-GHP and methylmalonyl-CoA by ThnE V153L catalysis. The appended energy minimized 3D model, generated by ChemBio3D, is based on the coupling constant and 2D NOESY spectral data.

Entry	Variant	(3*S*,6*R*): (3*S*,6*S*)	Overall % isolated yield	% Yield of the methyl ester
1	Wildtype CarB	69:31	45	
2	Wildtype ThnE	89:11	16	
3	ThnE V153I	93:07	23	
4	ThnE W124F	57:43	13	21
5	ThnE W124F/V153M	13:87	16	20
6	ThnE H274A	68:32	6	19
7	ThnE V153L	14:86	49	
8	ThnE V153M	15:85	47	
9	ThnE V153A	10:90	45	

Table S2: The ratio of C-6 epimers and the % overall yield (isolated) of (3*S*)-3,6-dimethyl-*t*-CMP epimers resulting from incubation of the racemic pair containing (2*S*,3*S*)-3-methyl-GHP,[7] methylmalonyl-CoA, and CMPSs, determined as reported.[6] The most selective variants (d.e. ≥ 0.8) are shaded. CMPS-catalysed reactions that resulted in the formation of substantial amount (>5%) of the methyl ester of 3,6-dimethyl-*t*-CMP (Fig. S6) are reported.

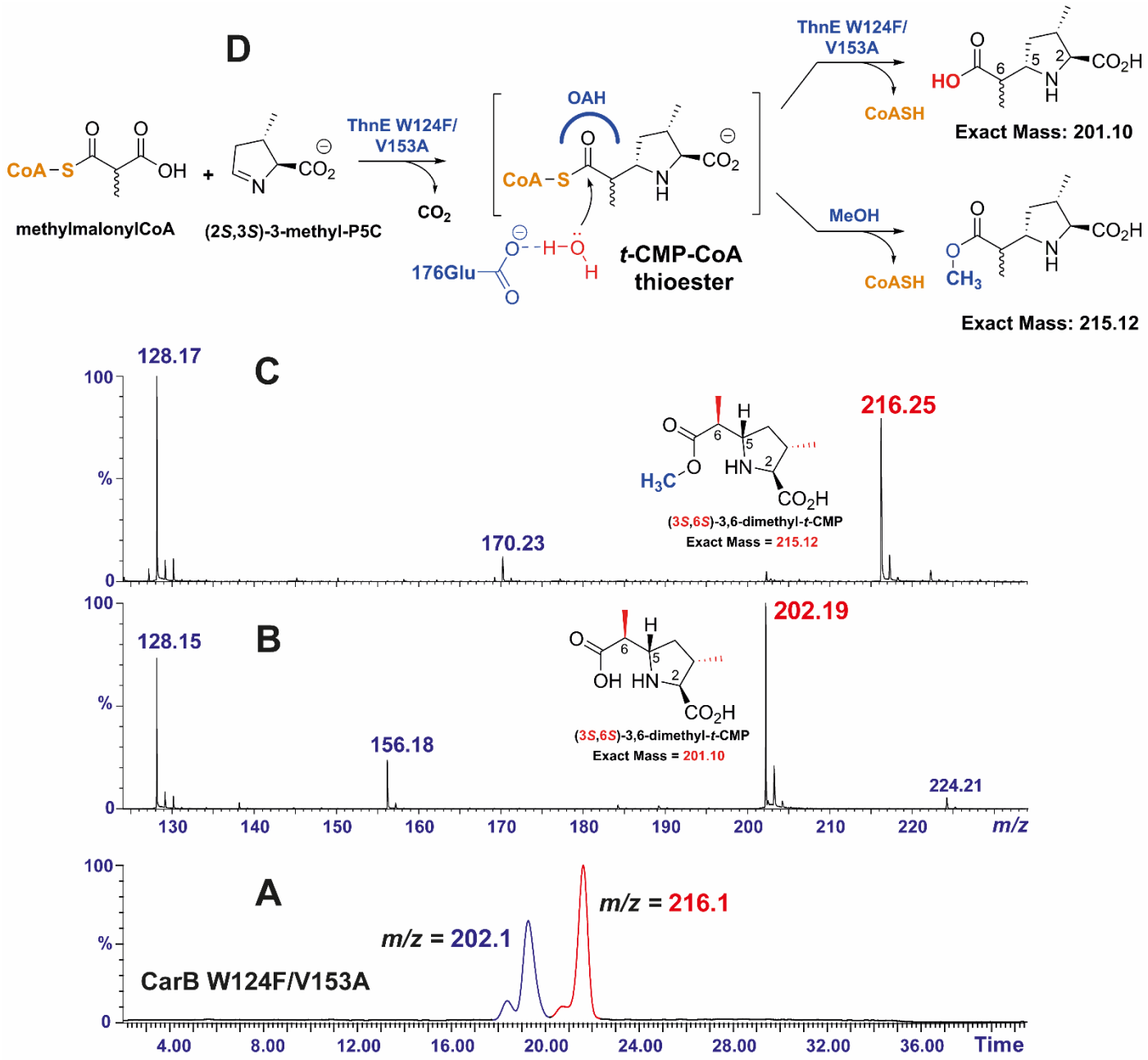

Figure S6: Formation of 3,6-dimethyl-*t*-CMP methyl ester following the methanolic quenching of the incubation of (2*S*,3*S*)-3-methyl-GHP and methylmalonyl-CoA as catalysed by ThnE W124F/V153M catalysis. A: Ion-extracted LC-MS chromatogram (ESI+) showing the co-production of the two C-6 epimers of (*3S*)-3,6-dimethyl-*t*-CMP (blue peaks, *m/z* = 202.1) and the corresponding methyl esters (red peaks, *m/z* = 216.1); **B** and **C**: Mass spectra (ESI+) of the major epimer of ThnE W124F/V153M catalysis, (*3S*,6*S*)-3,6-dimethyl-*t*-CMP (from major peak with *m/z* = 202.1), and its methyl ester (from major peak with *m/z* = 216.1), respectively. In both **B** and **C**, note the characteristic fragment at *m/z* ~ 128.2 (corresponding to loss of the C-5 side chain) and the fragment at *m/z* ~ 156.2 (**B**)/170.2 (**C**), corresponding to decarboxylation of the parent *t*-CMP derivative; **D**: Mechanism proposed for formation of the methyl ester from the thioester of the *t*-CMP-CoA intermediate.[10]

Stereochemical assignment of 3,6-dimethyl-*t*-CMP epimers resulting from incubation of racemic (2*S*,3*R*)-3-methyl-GHP, methylmalonyl-CoA, and CMPSs

Note: HRMS (ESI-TOF) *m/z*: $[M + H]^+$ Calcd for $C_9H_{16}O_4N$ 202.1074; Found 202.1076.

- **For the first eluting epimer** (the major epimer of CarB M108I catalysis), assuming the stereochemistry at C-2 is (*S*) based on reports that D-GHP is not a substrate for either CarB or ThnE,[8-9] the 2D NOESY data imply the stereochemistry at C-3 as (*R*) on the basis of the following observations:
  - ❖ A moderate nOe correlation between H-2 and H-3 together with the absence of any correlation between H-2 and the methyl group at C-3.
  - ❖ The presence of a moderate nOe correlation between H-5 and the methyl group at C-3.
- The stereochemistry at C-6 was assigned as (6*R*) based on the following observations:
  - ❖ A $J_{5,6}$ value of 7.2 Hz (predicted averaged Φ ~ 138º) together with a weak to moderate nOe between H-5 and H-6 indicate a predominantly anticlinal arrangement of these two protons.
  - ❖ The (near complete) absence of nOe correlations between H-6 and both protons at C-4.
  - ❖ The presence of a weak to moderate nOe correlation between the methyl group at C-6 and H-4'.
  - ❖ The assignment of stereochemistry at C-6 was further confirmed by conversion of the product into the corresponding β-lactam derivative by CarA catalysis (Fig. S21).

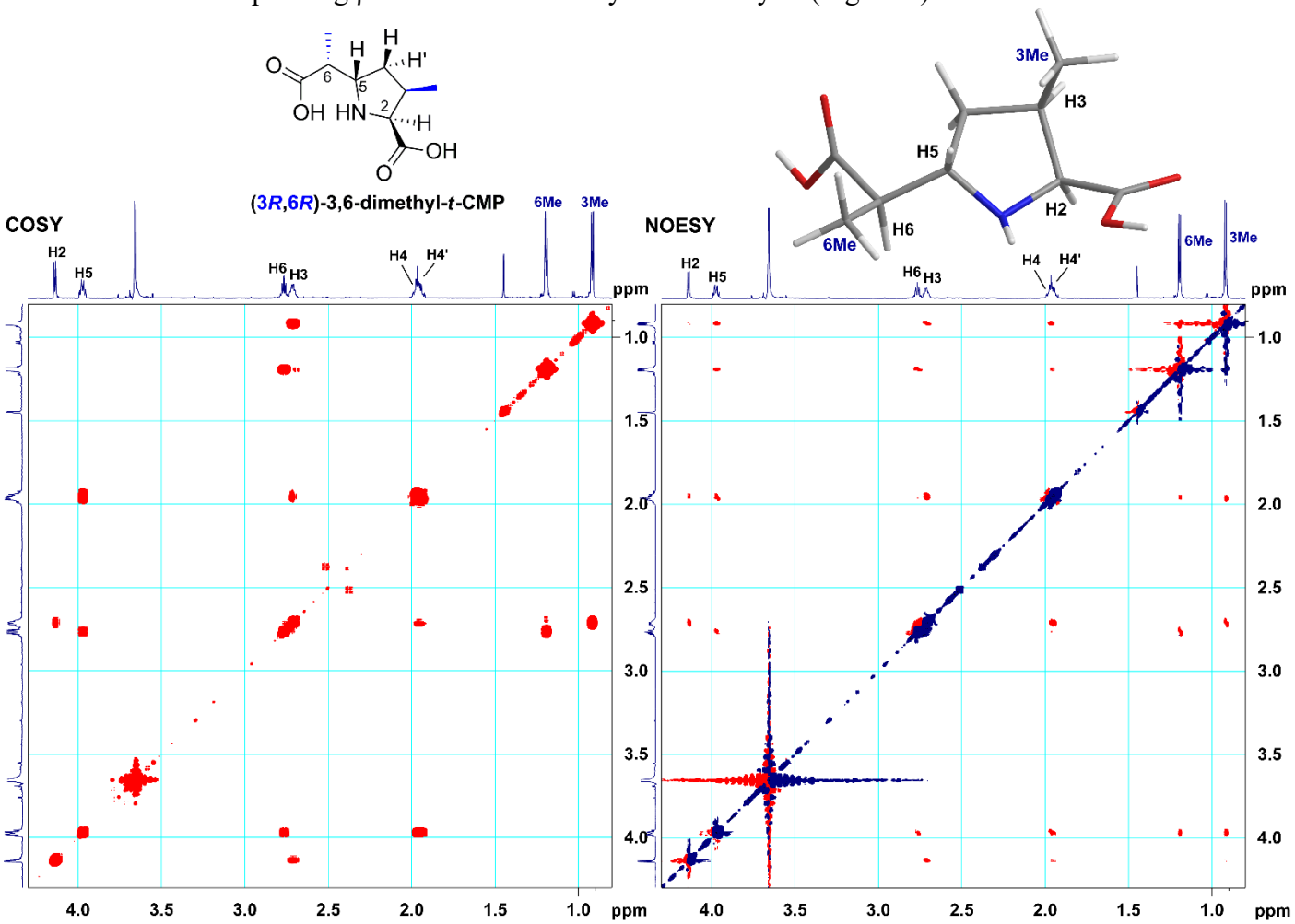

Figure S7: ^{1}H-^{1}H COSY and NOESY spectra for the (3*R*,6*R*)-3,6-dimethyl-*t*-CMP epimer produced from incubation of the racemic pair containing (2*S*,3*R*)-3-methyl-GHP and methylmalonyl-CoA by CarB M108I catalysis. The appended energy minimized 3D model, generated by ChemBio3D, is based on the coupling constant and 2D NOESY spectral data.

For the later eluting epimer (the major product of CarB W79A catalysis), the 2D NOESY data reveal the stereochemistry at C-3 as (*R*) on the basis of the following observations:

- ❖ A moderate nOe correlation between H-2 and H-3 together with absence of any correlation between H-2 and the methyl group at C-3.
- ❖ Presence of a moderate nOe between H-5 and the methyl group at C-3.

- The stereochemistry at C-6 was assigned as (6*S*) based on the following observations:
  - ❖ A $J_{5,6}$ value of 8.9 Hz (predicted averaged Φ ~ 154º) together with a weak nOe between H-5 and H-6 indicate a predominantly antiperiplanar arrangement of these two protons.
  - ❖ The moderate nOe correlations between the methyl group at C-6 and both protons at C-4 (H-4 > H-4') as well as between the methyl group at C-6 and H-5.
  - ❖ The moderate nOe between H-6 and H-4'.
  - ❖ The assignment of stereochemistry at C-6 was further confirmed by conversion of the product into the corresponding β-lactam derivative by CarA catalysis (Fig. S22).

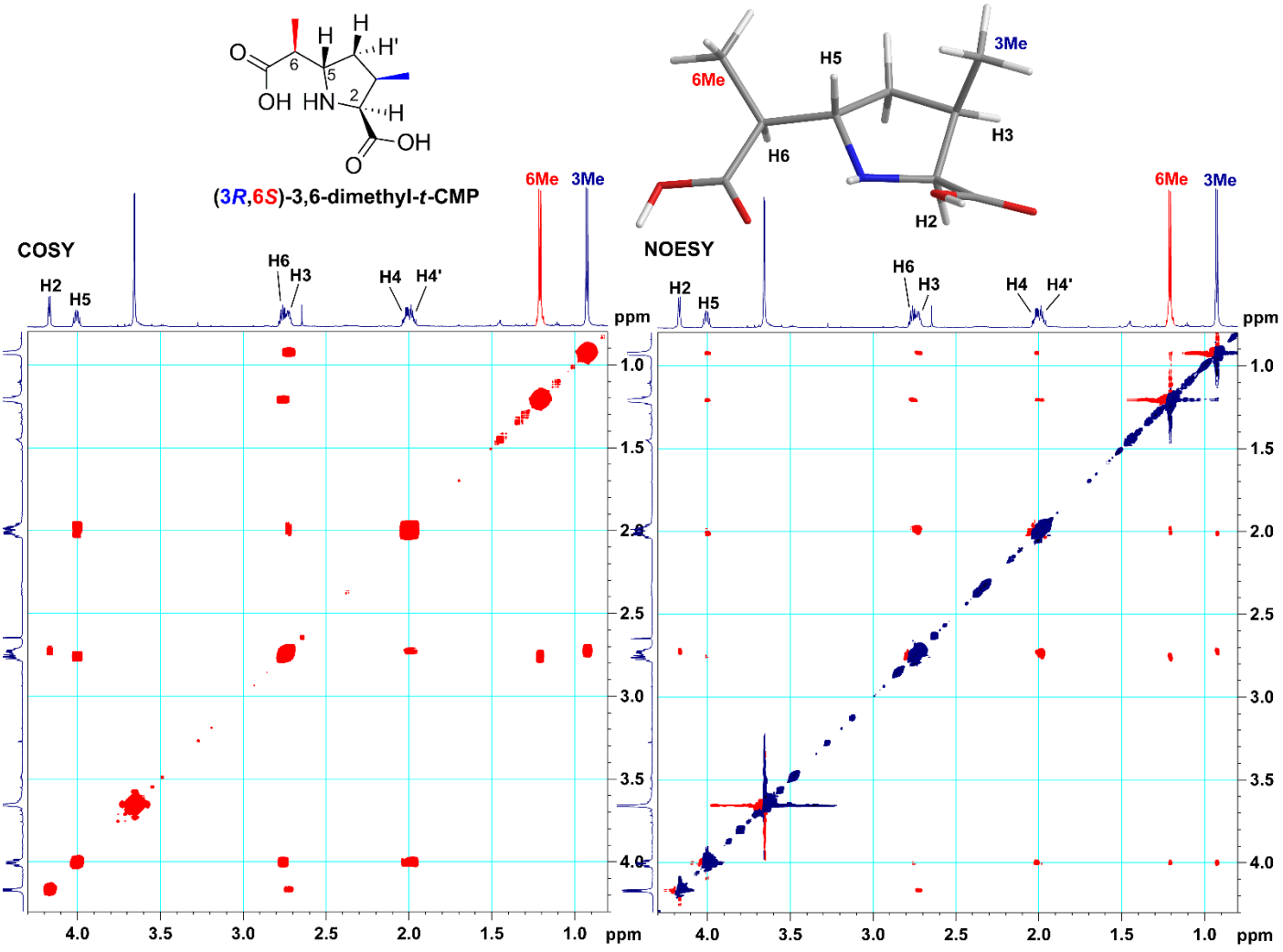

Figure S8: ^{1}H-^{1}H COSY and NOESY spectra for the (3*R*,6*S*)-3,6-dimethyl-*t*-CMP epimer produced from incubation of the racemic pair containing (2*S*,3*R*)-3-methyl-GHP and methylmalonyl-CoA by CarB W79A catalysis. The appended energy minimized 3D model, generated by ChemBio3D, is based on the coupling constant and 2D NOESY spectral data.

Entry	Variant	(3*R*,6*R*): (3*R*,6*S*)	Overall % isolated yield
1	CarB M108L	33:67	51
2	CarB M108A	18:82	32
3	CarB M108I	53:47	54
4	CarB H229A	34:66	39
5	CarB W79F	12:88	56
6	CarB W79F/M108A	07:93	52
7	CarB W79A	03:97	45
8	ThnE V153L	04:96	46
9	ThnE V153M	06:94	37

Table S3: The ratio of C-6 epimers and the % overall yield (isolated) of (3*R*)-3,6-dimethyl-*t*-CMP epimers resulting from incubation of racemic (2*S*,3*R*)-3-methyl-GHP,[7] methylmalonyl-CoA, and CMPSs, determined as reported.[6] The most selective variants (d.e. ≥ 0.8) are shaded.

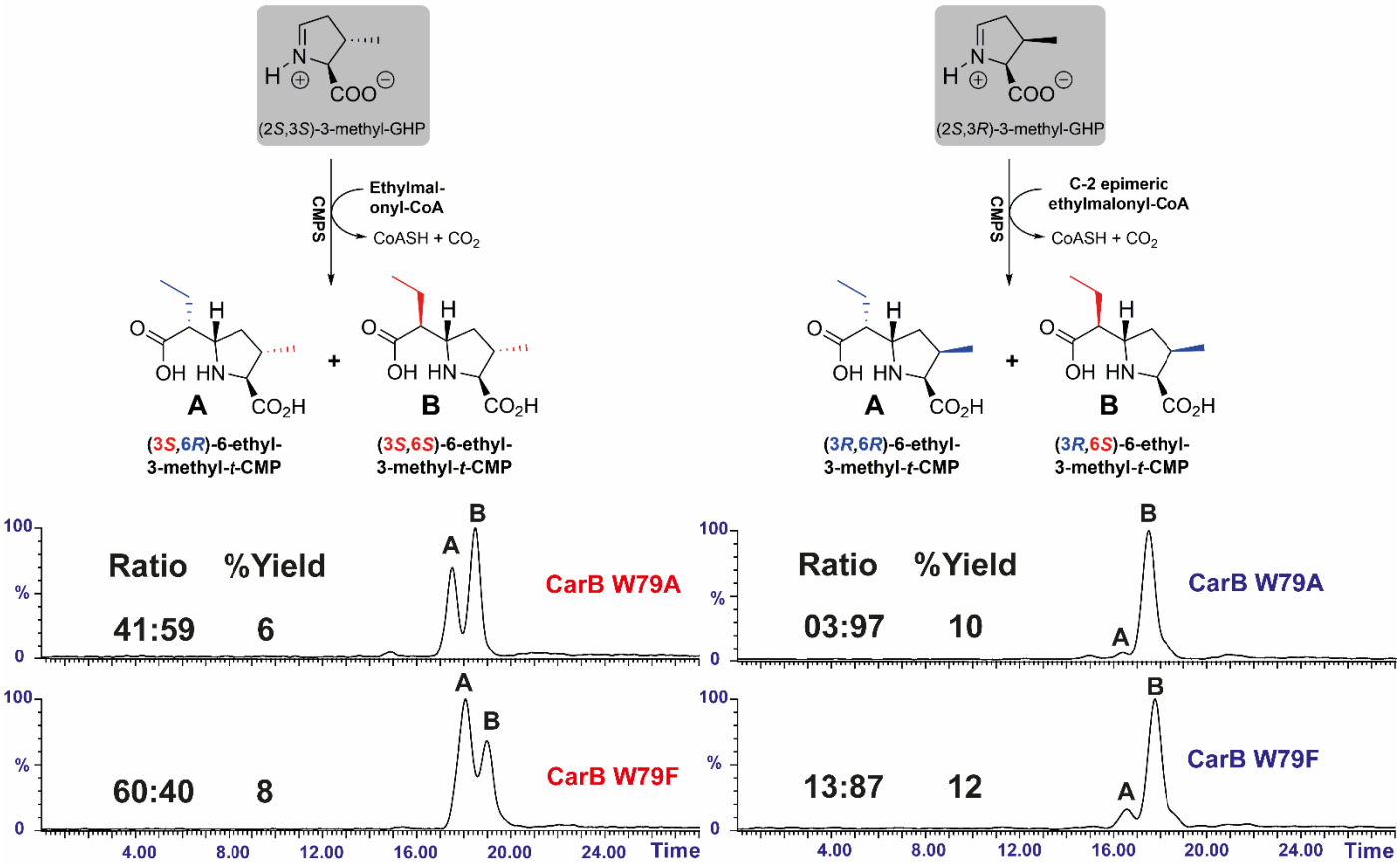

Figure S9: Preparation of 6-ethyl-3-methyl-*t*-CMP diastereomers by CMPS catalysis from the incubation of C-2 epimeric ethylmalonyl-CoA and the racemic pair of (2*S*,3*S*)-3-methyl-GHP/(2*S*,3*R*)-3-methyl-GHP, respectively, under standard conditions. The ion-extracted LC-MS chromatograms (ESI+, *m/z* = 216.1) display the yields and diastereomeric ratios for the two C-6 epimers of (3*S*)- 6-ethyl-3-methyl-*t*-CMP (left) and (3*R*)- 6-ethyl-3-methyl-*t*-CMP (right). In each case, assignment of the stereochemistry at C-6 of the early eluting epimer as (*R*) was based on comparison of the retention time of the early eluting epimer to that of the single epimer (with (6*R*)-stereochemistry) resulting from the coupled Ccr/CarB W79F, as reported,[3,10] (for example, see Fig. S10).

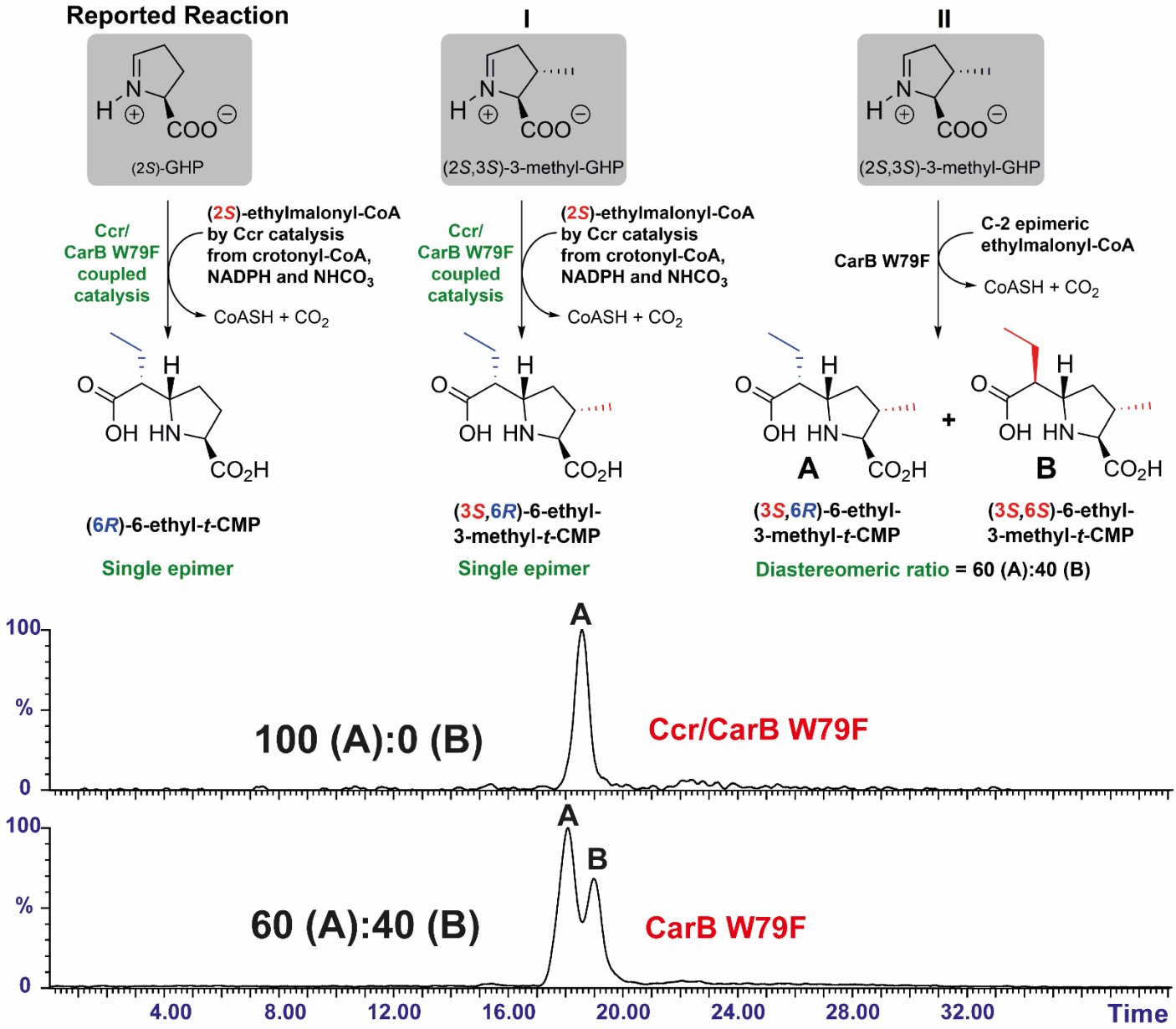

Figure S10: Preparation of (3*S*,6*R*)-6-ethyl-3-methyl-*t*-CMP by coupled Ccr/CarB W79F catalysis. The ion-extracted LC-MS chromatograms (ESI+, *m/z* = 216.1) display the diastereomeric ratios for the two C-6 epimers of (3*S*)-6-ethyl-3-methyl-*t*-CMP when C-2 epimeric ethylmalonyl-CoA is used as a co-substrate (bottom chromatogram and reaction scheme **II**) and when (2*S*)-ethylmalonyl-CoA[11] is *in situ* prepared as a product of Ccr catalysis (top chromatogram and reaction scheme **I**).

Stereochemical assignment of the 5,6-dimethyl-*t*-CMP epimer resulting from incubation of racemic 5-methyl-GHP, methylmalonyl-CoA, and CMPSs

Note: HRMS (ESI-TOF) *m*/*z*: $[M + H]^+$ Calcd for $C_9H_{16}O_4N$ 202.1074; Found 202.1075.

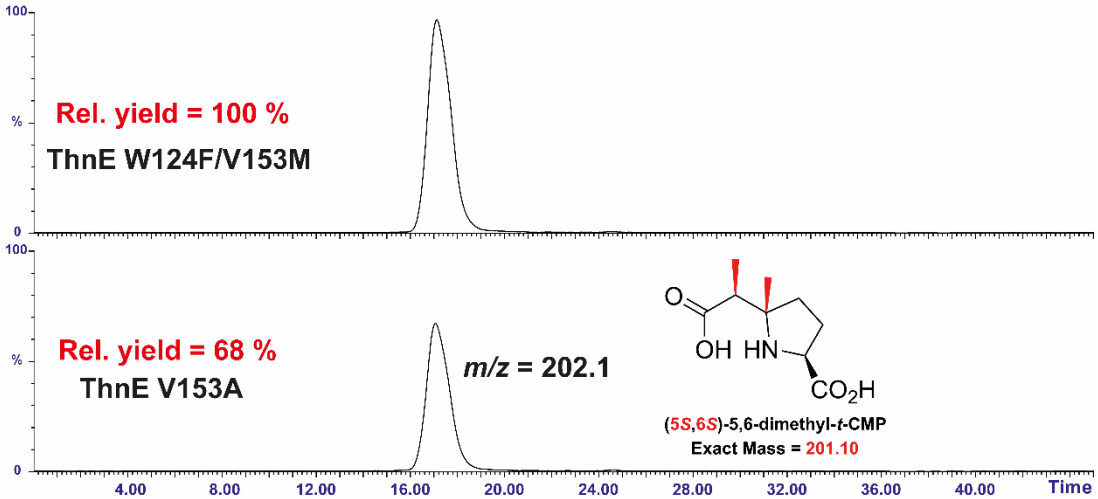

Figure S11: **Ion-extracted LC-MS chromatograms (positive ion electrospray ionization) displaying the relative yields for the production of (5*S*,6*S*)-5,6-dimethyl-*t*-CMP as catalysed by the shown CMPSs from racemic 5-methyl-GHP and C-2 epimeric methylmalonyl-CoA**. *p*-Aminosalicylic acid was used as an internal standard (not shown for clarity).

- The stereochemistry at C-6 of the single epimer observed (Fig. S12) was assigned as (6*S*) based on the following observations:

❖ A weak nOe between H-6 and the methyl group at C-5 (together with a weak to moderate nOe correlation between the methyl groups at C-5 and C-6) indicate a predominantly anticlinal arrangement of H-6 and the methyl group at C-5.

❖ A weak nOe between H-2 and both H4' and H6 indicate that they are on the same face of the ring system.

❖ The moderate nOe correlations between the methyl group at C-6 and both protons at C-4 (H4>H4'), as well as the moderate nOe correlations between H-6 and at least one of the protons at C-4 (H4').

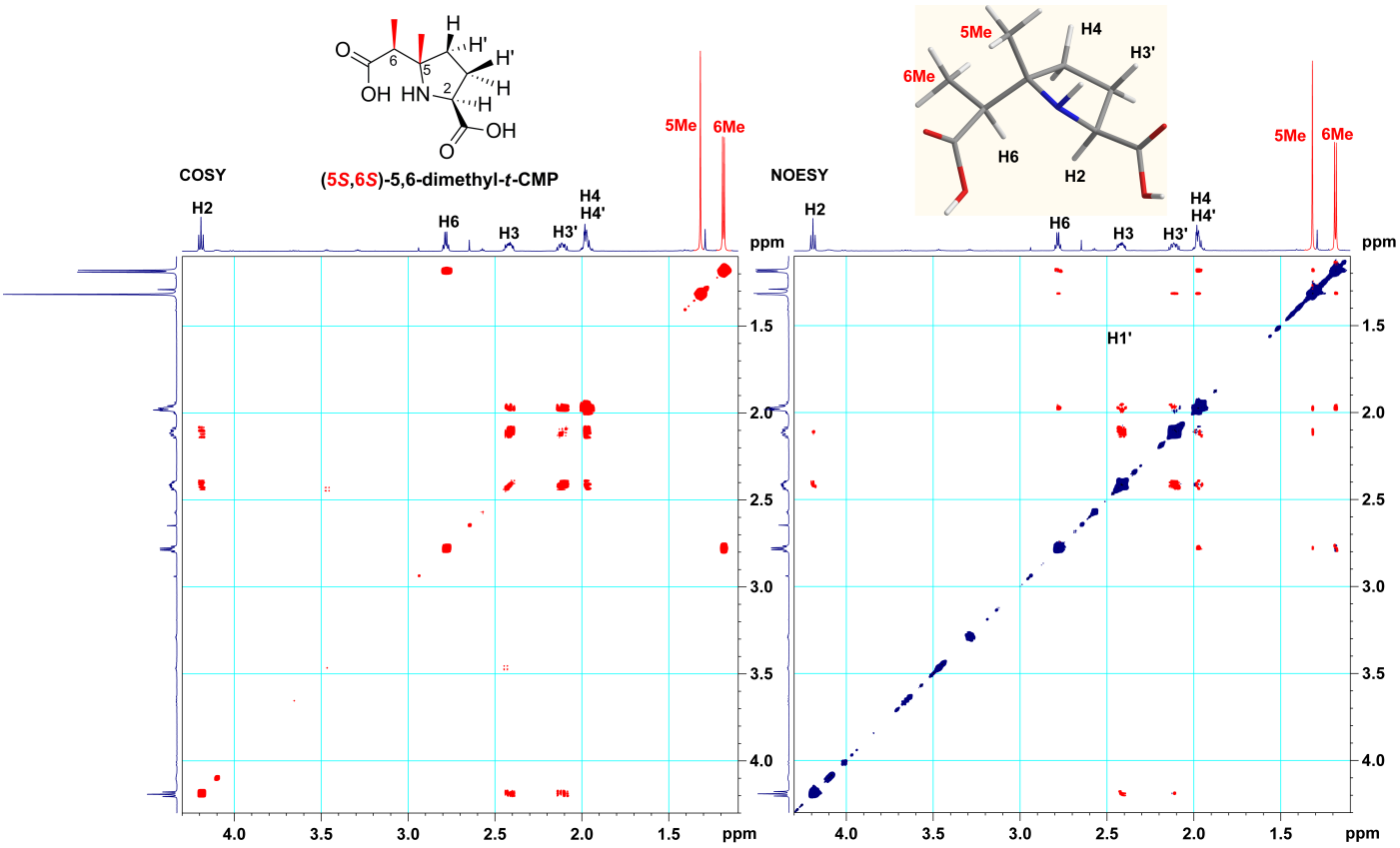

Figure S12: ^{1}H-^{1}H COSY and NOESY spectra for the (5*S*,6*S*)-5,6-dimethyl-*t*-CMP epimer produced from incubation of the racemic 5-methyl-GHP and C-2 epimeric methylmalonyl-CoA by ThnE W124F/V153M catalysis. An appended energy minimized 3D model, generated by ChemBio3D, is based on 2D NOESY spectral data. Note that a NOESY analysis of a slightly less pure sample afforded reasonable resolution of the H4/H4' protons, which assisted with the definition of the (6*S*)-stereochemistry.

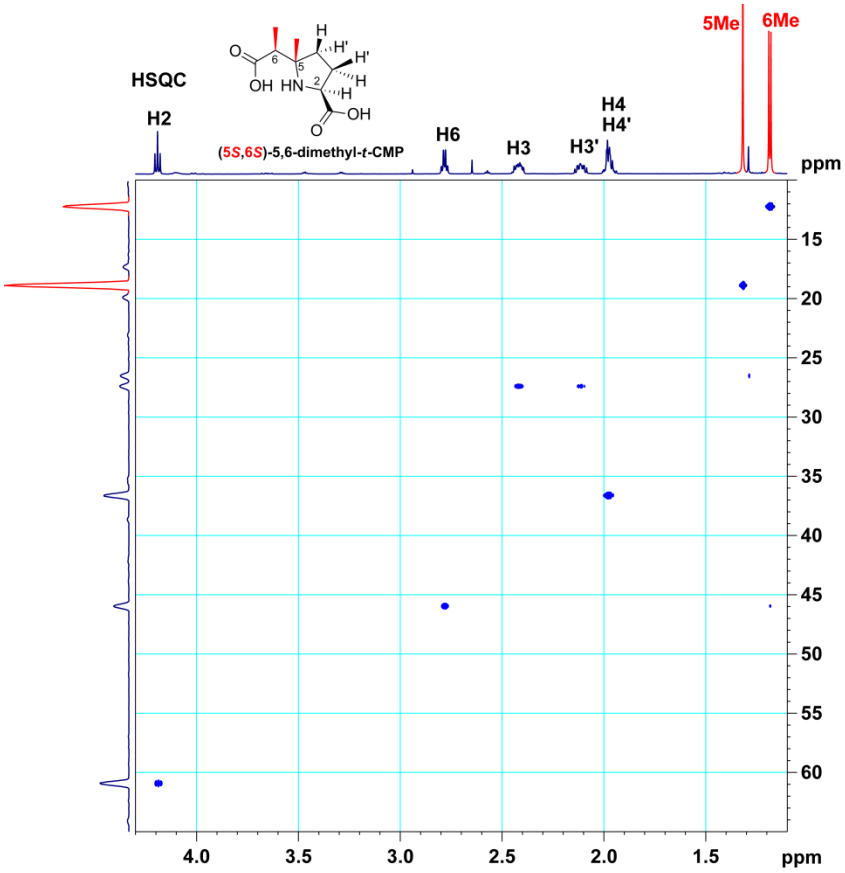

Figure S13: ^{1}H-^{13}C HSQC spectrum for the (5*S*,6*S*)-5,6-dimethyl-*t*-CMP epimer produced from incubation of the racemic 5-methyl-GHP and C-2 epimeric methylmalonyl-CoA by ThnE W124F/V153M catalysis.

Assignment of the β-lactam derivative resulting from incubation of (6*R*)-2,6-dimethyl-*t*-CMP, ATP/Mg^{II}, and CarA

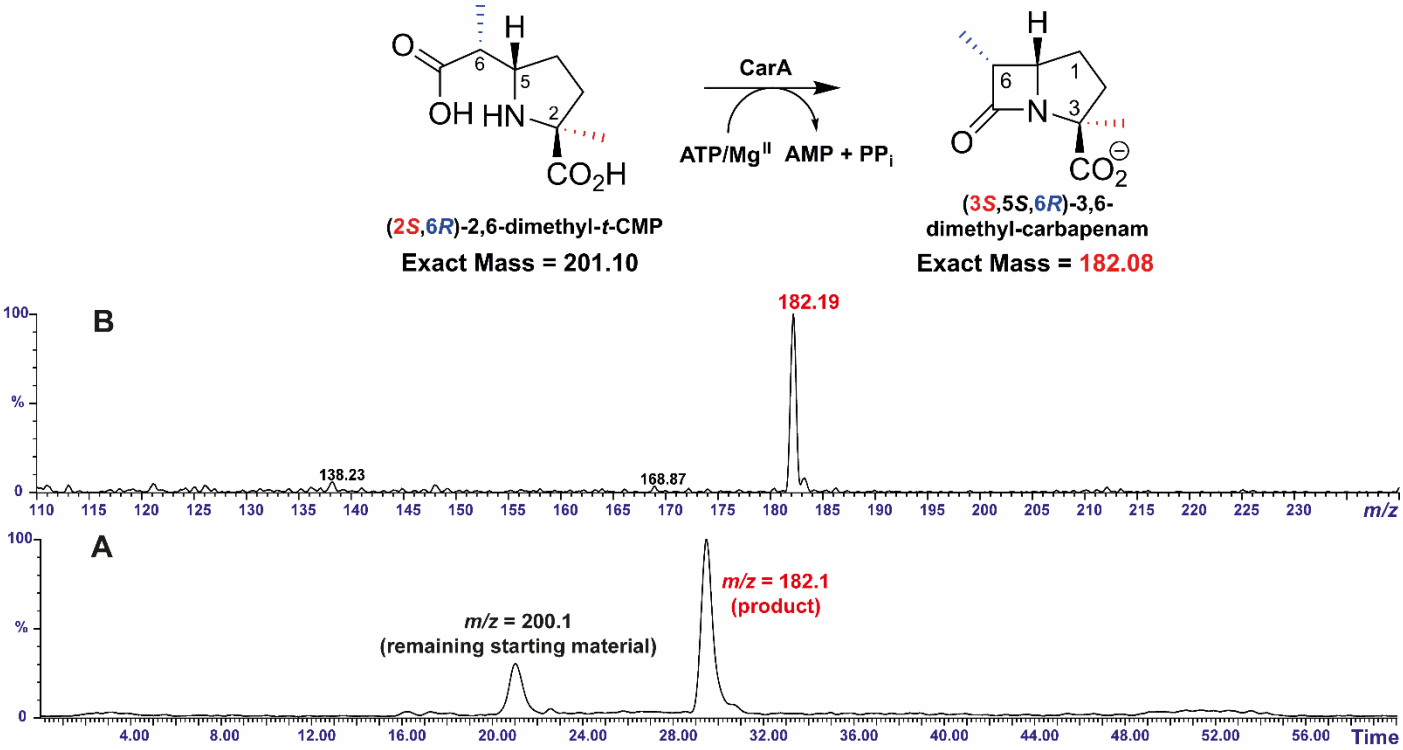

Figure S14: Formation of (3*S*,5*S*,6*R*)-3,6-dimethylcarbapenam-3-carboxylic acid from (2*S*,6*R*)-2,6-dimethyl-*t*-CMP by Car A catalysis, under standard conditions. A: Ion extracted LC-MS chromatogram (negative ion mode) for the CarA catalysed conversion, displaying the carbapenam product (*m/z* = 182.1) and the remaining staring material of (2*S*,6*R*)-2,6-dimethyl-*t*-CMP (*m/z* = 200.1); **B**: MS spectrum (negative ion electrospray ionization) of the carbapenam product. Note that the (2*S*,6*S*)-2,6-dimethyl-*t*-CMP **epimer** is not a substrate for CarA, as confirmed by LC-MS analyses.

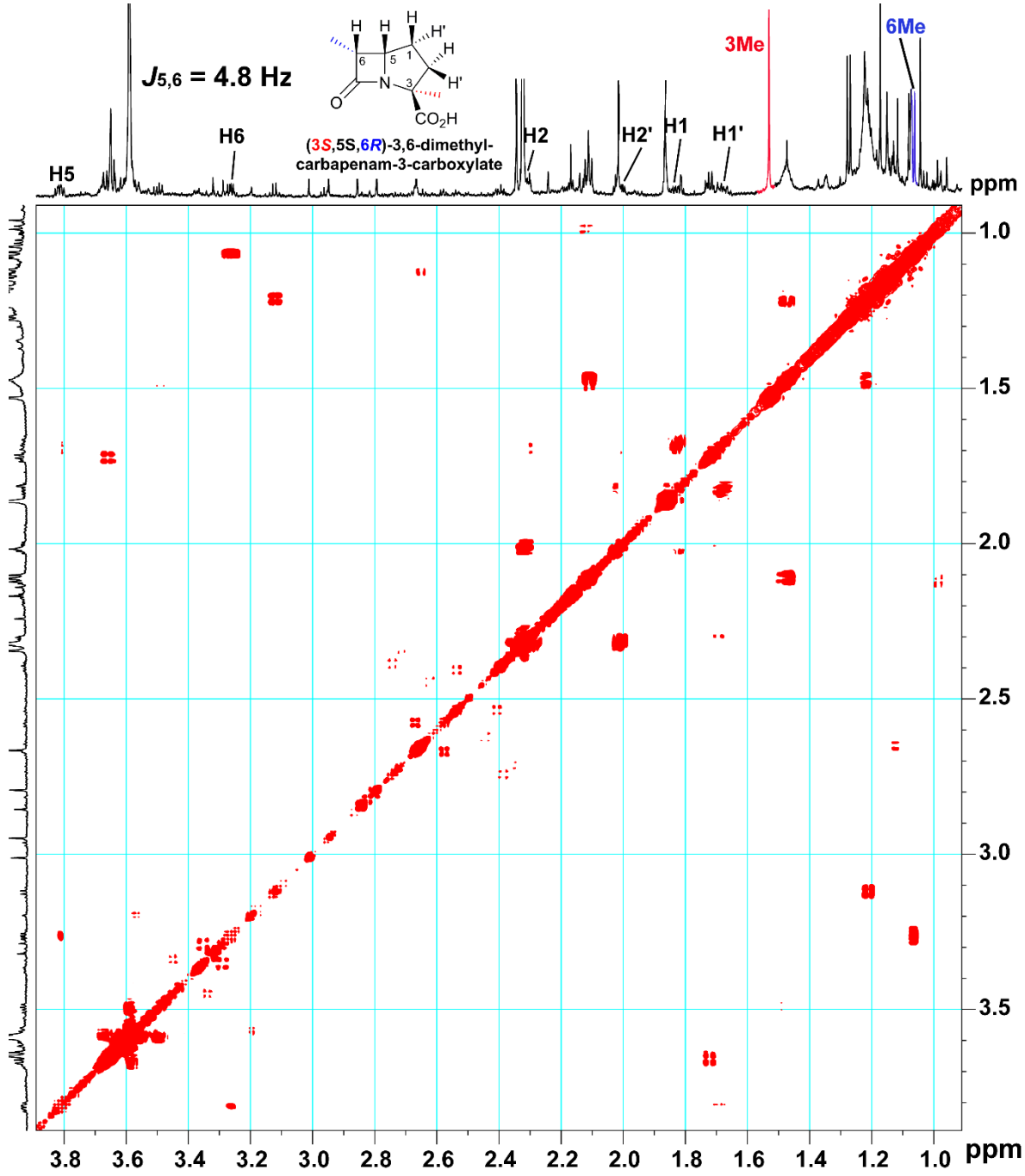

Figure S15: ^{1}H-^{1}H COSY spectrum for the (3*S*,5*S*,6*R*)-3,6-dimethylcarbapenam-3-carboxylic acid produced by incubation of (2*S*,6*R*)-2,6-dimethyl-*t*-CMP with ATP/Mg^{II} as catalysed by CarA. The stereochemistry at C-6 was assigned/further confirmed as (*R*) on the basis of the $J_{5,6}$ = 4.8 Hz (typical for β-lactams with H5 and H6 in a *cis*-relationship).[12]

Assignment of the β-lactam derivatives resulting from incubation of 3,6-dimethyl-*t*-CMP diastereomers and ATP/Mg^{II} as catalysed by CarA

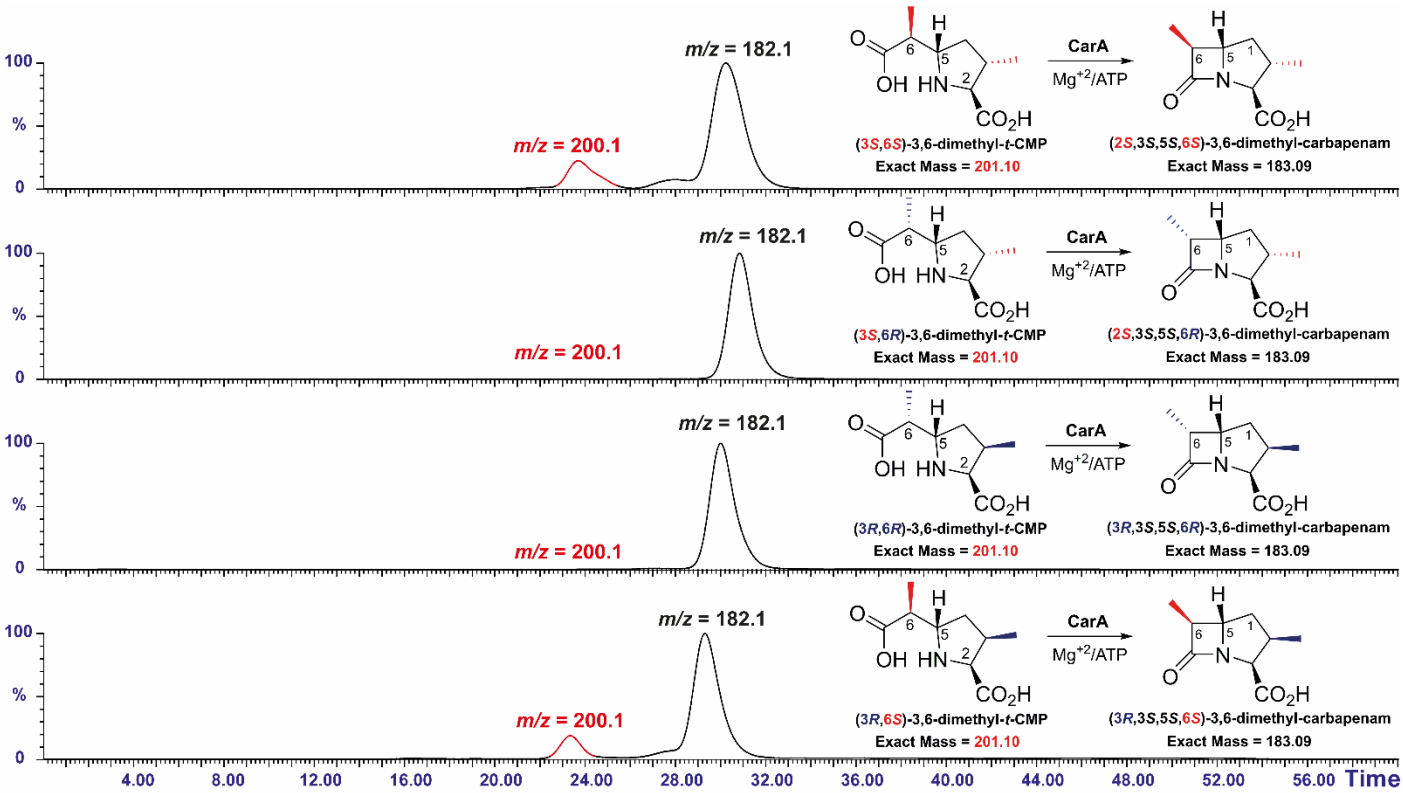

Figure S16: Efficient production of the four diastereomers of 2,6-dimethylcarbapenam-3-carboxylic acid from the corresponding 3,6-dimethyl-*t*-CMP diastereomers by Car A catalysis, under standard conditions. The ion extracted LC-MS chromatograms (negative ion mode) of the CarA catalysed conversions display the carbapenam product (*m/z* = 182.1) and the remaining staring material, if any, of 3,6-dimethyl-*t*-CMP (*m/z* = 200.1). Note that the (6*S*)-3,6-dimethyl-*t*-CMP diastereomers are less favoured as substrates for CarA compared to the (6*R*)-diastereomers.

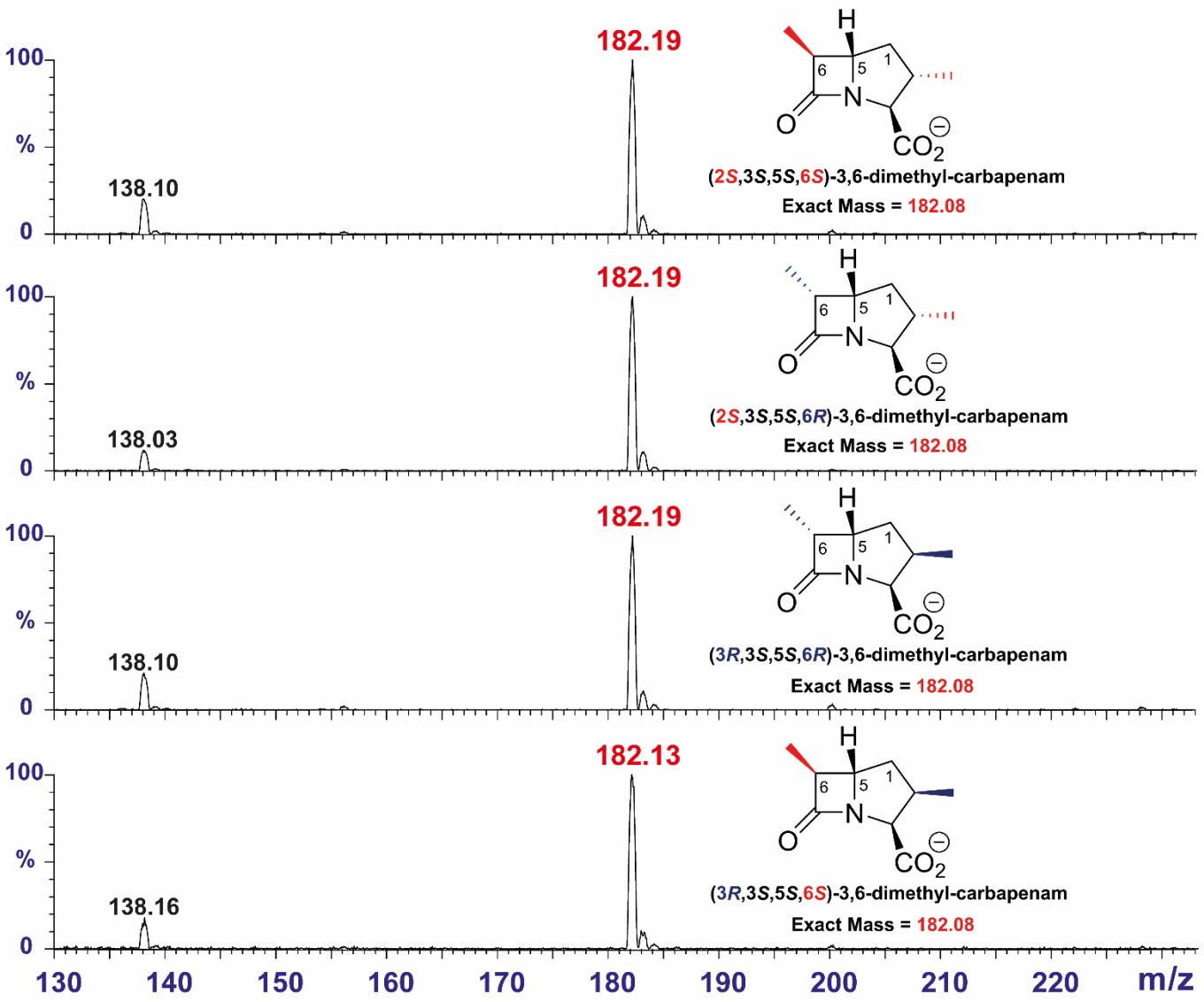

Figure S17: MS spectra (negative ion electrospray ionization) of the four shown diastereomers of 2,6-dimethylcarbapenam-3-carboxylic acid produced from the corresponding 3,6-dimethyl-*t*-CMP diastereomers by Car A catalysis, under standard conditions. Note the characteristic fragment at m/z = 138.1, corresponding to the decarboxylation of the parent carbapenem [M-44]$^-$.

Assignment of the β-lactam derivative resulting from incubation of (3*S*,6*R*)-3,6-dimethyl-*t*-CMP, ATP/Mg^{II}, and CarA

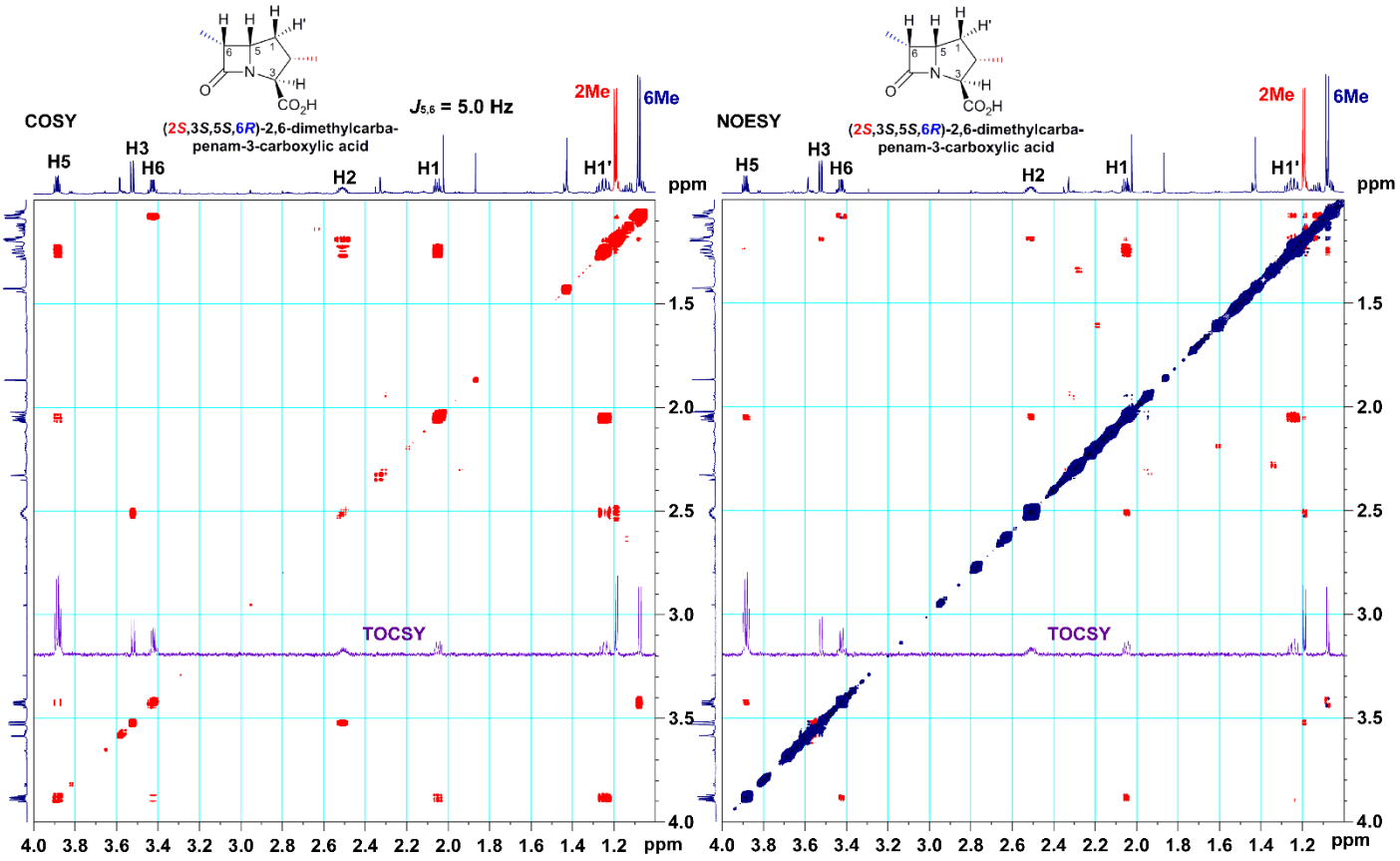

Figure S18: 2D COSY and NOESY spectra (with 1D TOCSY traces superimposed) for the (2*S*,3*S*,5*S*,6*R*)-2,6-dimethylcarbapenam-3-carboxylic acid produced by incubation of (3*S*,6*R*)-3,6-dimethyl-*t*-CMP with ATP/Mg^{II} as catalysed by CarA. The stereochemistry at C-6 was assigned/confirmed as (*R*) on the basis of the $J_{5,6}$ = 5.0 Hz (typical for β-lactams with H5 and H6 in a *cis*-relationship).[12] Note that the above NOESY spectrum is, to our knowledge, the only of its kind reported so far for a carbapenam prepared either from an isolated enzyme or from a natural source.

Assignment of the β-lactam derivative resulting from incubation of (3*S*,6*S*)-3,6-dimethyl-*t*-CMP, ATP/Mg^{II}, and CarA

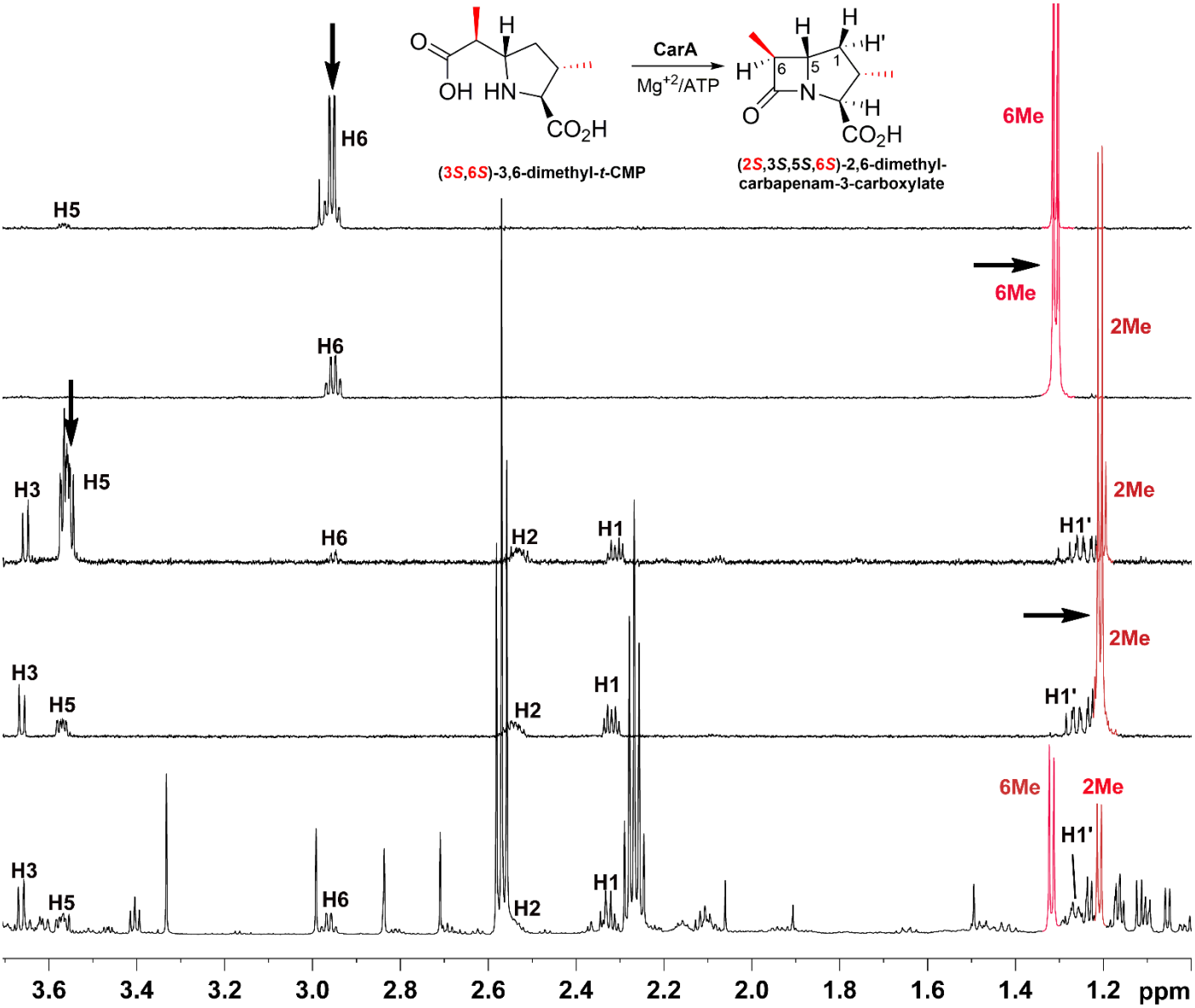

Figure S19: ^{1}H and 1D TOCSY spectra for the (2*S*,3*S*,5*S*,6*S*)-2,6-dimethylcarbapenam-3-carboxylic acid produced by incubation of (3*S*,6*S*)-3,6-dimethyl-*t*-CMP with ATP/Mg^{II} as catalysed by CarA. The proton(s) selectively excited for the TOCSY experiments are arrowed.

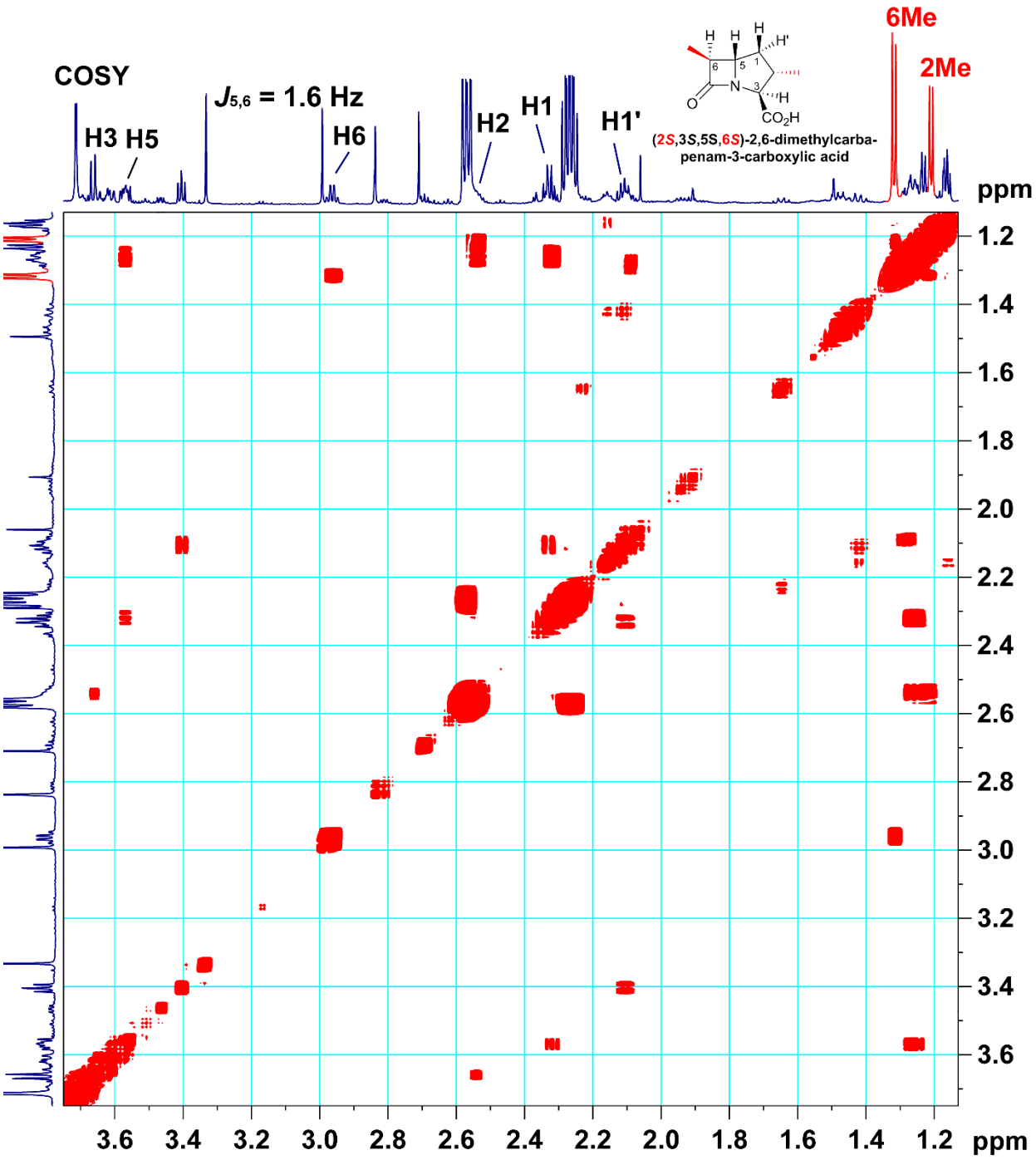

Figure S20: ^{1}H-^{1}H COSY spectrum for the (2*S*,3*S*,5*S*,6*S*)-2,6-dimethylcarbapenam-3-carboxylatic acid produced by incubation of (3*S*,6*S*)-3,6-dimethyl-*t*-CMP with ATP/Mg^{II} as catalysed by CarA. The stereochemistry at C-6 was assigned/confirmed as (*S*) on the basis of the $J_{5,6}$ = 1.6 Hz (typical for β-lactams with H5 and H6 in a *trans*-relationship).[12]

Assignment of the β-lactam derivative resulting from incubation of (3*R*,6*R*)-3,6-dimethyl-*t*-CMP, ATP/Mg^{II}, and CarA

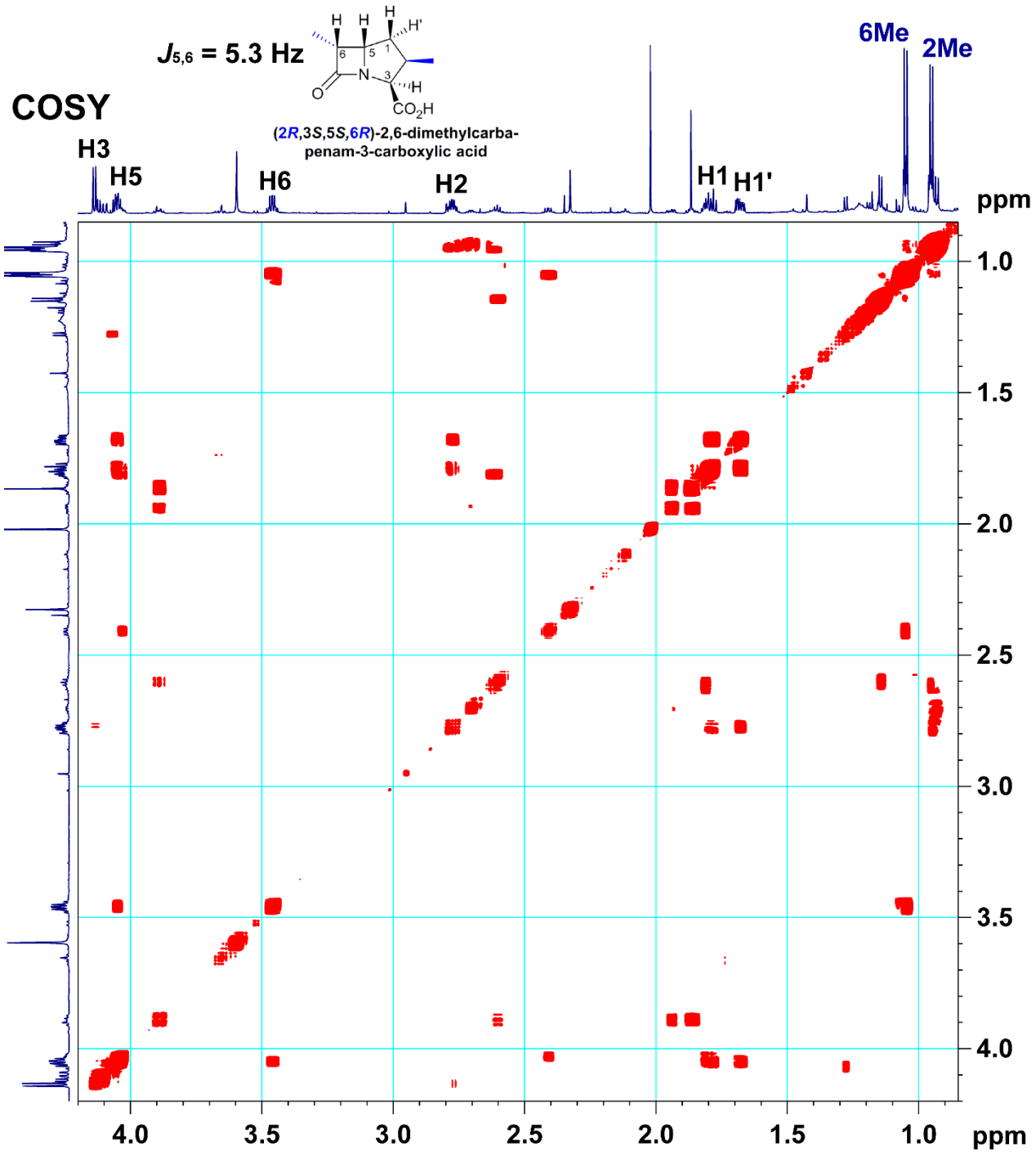

Figure S21: ^{1}H-^{1}H COSY spectrum for the (2*R*,3*S*,5*S*,6*R*)-2,6-dimethylcarbapenam-3-carboxylic acid produced by incubation of (3*R*,6*R*)-3,6-dimethyl-*t*-CMP with ATP/Mg^{II} as catalysed by CarA. The stereochemistry at C-6 was assigned/confirmed as (*R*) on the basis of the $J_{5,6}$ = 5.3 Hz (typical for β-lactams with H5 and H6 in a *cis*-relationship).[12]

Assignment of the β-lactam derivative resulting from incubation of (3*R*,6*S*)-3,6-dimethyl-*t*-CMP, ATP/Mg^{II}, and CarA

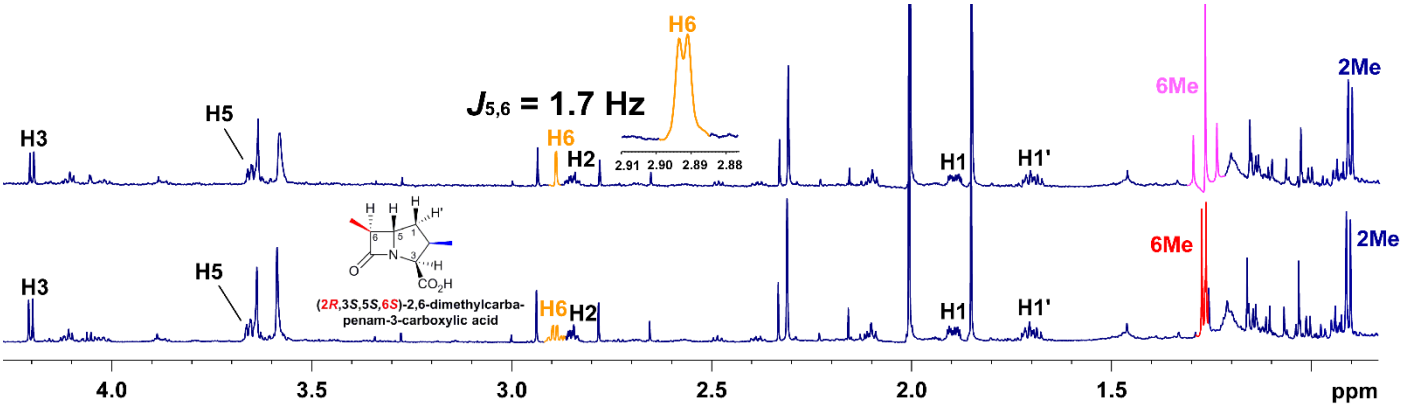

Figure S22: ^{1}H-NMR spectra for the (2*R*,3*S*,5*S*,6*S*)-2,6-dimethylcarbapenam-3-carboxylic acid produced by incubation of (3*R*,6*S*)-3,6-dimethyl-*t*-CMP and ATP/Mg^{II} as catalysed by CarA. Note the collapse of the H-6 multiplet, in the top spectrum, into a doublet (expanded), upon selective irradiation (decoupling) of the methyl group at C-6 (in pink). The stereochemistry at C-6 was assigned/confirmed as (*S*) on the basis of the $J_{5,6}$ = 1.7 Hz (typical for β-lactams with H5 and H6 in a *trans*-relationship).[12]

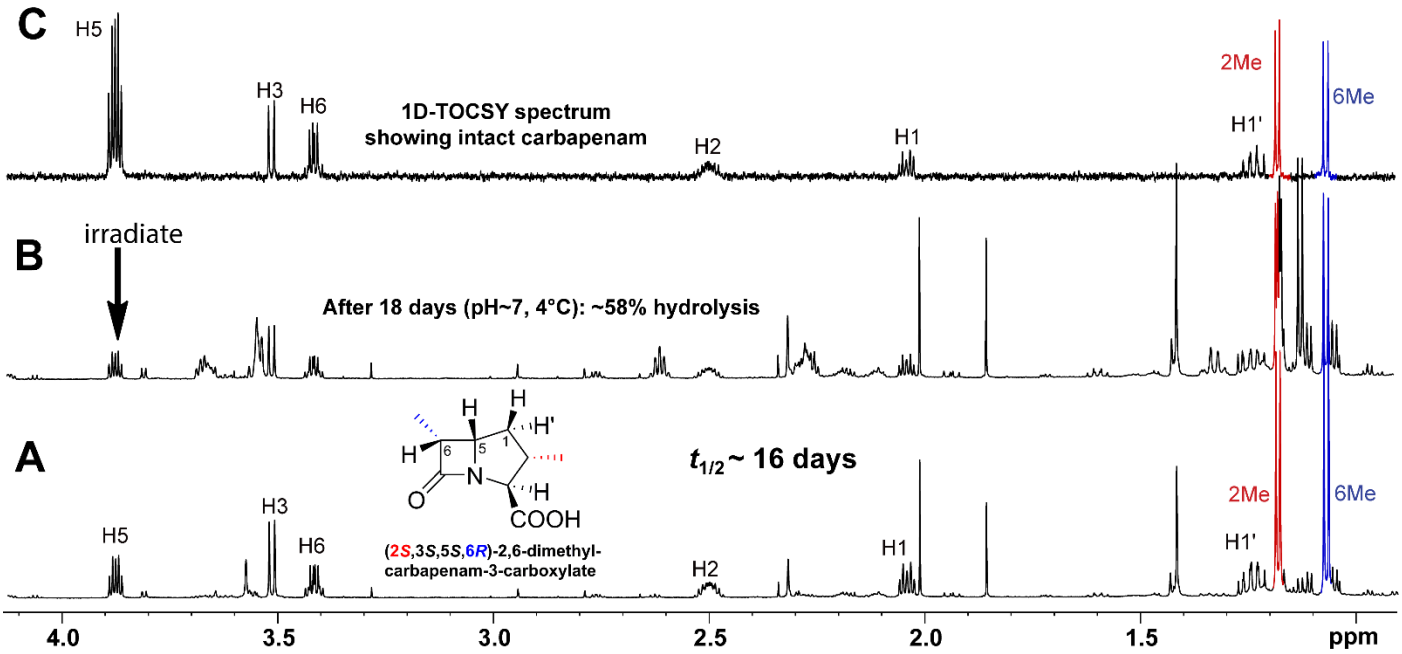

Figure S23: Example for stability to hydrolysis of the disubstituted-carbapenams prepared in this study by CarA catalysis. Following LC-MS purification under slightly acidic conditions (0.05% Aq. formic acid), the collected fraction (*m/z* = 182.1) was neutralized with 1N sodium bicarbonate, frozen in liquid nitrogen, then lyophilized overnight, and subjected to NMR analyses (**A**). The sample was subsequently kept (inside a 2 mm NMR tube) at 4°C and its stability to hydrolysis was monitored over time by ^{1}H NMR analyses (**B**). The 1D TOCSY spectrum **C**, obtained by selective excitation of H-5 (spectrum **B**) reveals the existence of the intact (2*S*,3*S*,5*S*,6*R*)-2,6-dimethyl-carbapenam-3-carboxylate.

VI- References

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