

***Engineering Carboxymethylproline Synthases towards
the Biosynthetic Production of Carbapenem
Antibiotics.***

A thesis submitted to the Board of the Faculty of Physical Sciences of the University of Oxford in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

José Rubén Gómez Castellanos, *MSc*

Hertford College, Oxford

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Ad Maiorem Dei Gloriam

I would like to dedicate this work to my parents, Adriana and Rubén, to all the love and effort that they always have put on my growth and development. Thank you.

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Abstract

Mechanistic and biocatalytic studies of two carboxymethylproline synthases (CMPSs), CarB and ThnE, members of the crotonase superfamily of enzymes, both in isolation and in conjunction with the activity of the crotonyl-CoA carboxylase/reductase (Ccr) the malonyl-CoA synthetase (MatB) and the methylmalonyl-CoA epimerase (MCE) are presented.

Protein engineering studies on carboxymethylproline synthases aimed at enabling stereoselective C–C bond formation leading to *N*-heterocycles *via* control of trisubstituted enolate intermediates were carried out. Active site substitutions, including at the oxyanion binding site, enabled the production of substituted *N*-heterocycles in high diastereomeric excesses *via* stereocontrolled enolate formation and reaction.

The biocatalytic promiscuity of malonyl-CoA ligase and the stereoselectivity of crotonyl-CoA carboxylase/reductase were successfully coupled to the selective tri-substituted enolate forming capacity of engineered carboxymethylproline synthases for the preparation of functionalized 5- and 6-membered *N*-heterocycles substituted with a variety of alkyl side chains at the C-5/C-6 positions at high diastereomeric excess. The effect of methylmalonyl-CoA epimerase on the diastereoselectivity of the carboxymethylproline synthase-catalysed enolated alkylation was also demonstrated.

The results illustrate the utility of the crotonase superfamily of enzymes for stereoselective biocatalysis and demonstrate the power of coupled enzyme systems to enhance diastereoselectivity and to expand the range of accepted substrates.

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Collaborations

NMR analyses were performed by Dr. Refaat B. Hamed and Dr. Timothy D. W. Claridge, University of Oxford.

CMPS were designed and prepared in collaboration with Dr. Refaat B. Hamed and Mr. Daniel Harding, University of Oxford.

The protected forms of the amino acid aldehydes used here were prepared by Prof. Dr. Christian Ducho, Dr. Jasmin Mecinovic, Dr. John Sorensen and Dr. Luc Henry, while at the University of Oxford.

(2*S*,5*S*,6*R*)-6-ethylcarbapenam methyl ester was prepared by Dr. Armin Thalhammer, while at the University of Oxford.

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The *matB/pET28b* and *mcee/pET28b* plasmids were a kind gift from Prof. Adrian Keatinge-Clay, The University of Texas at Austin.

Human methylmalonyl-CoA epimerase was a generous gift by Dr. Wyatt W. Yue, University of Oxford.

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Table of abbreviations

(v/v)	volume to volume ratio
(w/v)	weight to volume ratio
2OG	2-oxoglutarate
AASA	α -aminoadipate semialdehyde
7-ACA	7-aminocephalosporanic acid
6-APA	6-aminopenicillanic acid
BLAs	β -lactam antibiotics
bp	base pair
°C	degrees centigrade
CarA	carbapenam synthetase
CarB	carboxymethylproline synthase
CarC	carbapenem synthase
CAS	clavamate synthase
Ccr	crotonyl-CoA carboxylase/reductase
CMP	5-(carboxymethyl)-proline
CMPS	<i>t</i> -(carboxymethyl)-proline synthase
CMPi	6-(carboxymethyl)-pipecolic acid
CoA	coenzyme A
COSY	correlation spectroscopy
CoASH	the free form of Coenzyme A
δ	chemical shift
d.e.	diastereomeric excess
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid

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d.r.	diastereomeric ratio
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ESI-MS	electrospray-ionisation mass spectrometry
FPLC	fast protein liquid chromatography
GSA	glutamate semialdehyde
HPLC	high performance liquid chromatography
Hz	hertz
IS	internal standard
<i>J</i>	coupling constant in Hz
IPTG	isopropyl- β -D-thiogalactoside
kb	kilobases
kDa	kilodalton
LB	Luria-Bertani medium
LC-MS	liquid chromatography mass spectrometry
MatB	malonyl-CoA ligase
MCE	methylmalonyl-CoA epimerase
MW	molecular weight
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
NOESY	nuclear Overhauser effect spectroscopy
OAH	oxyanion hole
OD ₆₀₀	optical density at 600 nm (in absorption units)
PAGE	polyacrylamide gel electrophoresis
PBPs	penicillin binding proteins

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PCR	polymerase chain reaction
ppm	parts per million
RNA	ribonucleic acid
r.p.m.	revolutions per minute
SDS	sodium dodecylsulfate
<i>t</i>	<i>trans</i>
TEMED	<i>N,N,N',N'</i> -tetramethylethylene diamine
ThnE	the CMPS in <i>Streptomyces cattleya</i>
Tris	2-amino-2-(hydroxymethyl)propane-1,3-diol
TOCSY	total correlation spectroscopy
UV-VIS	ultraviolet-visible

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Chapter 1. Introduction.

The 20th century has been witness to one of the most preeminent stories of success in human therapeutics for the treatment of infections: the discovery and development of antibiotics and antibacterial agents. The introduction of antibiotics helped drop the death rates from infectious disease by an astonishing 95%, from 797 per hundred thousand in 1900 to 36 per hundred thousand in 1980.¹

The golden age of antibiotic discovery in the 20th century was relatively short lived. The two decades from 1940 to 1960 saw the discovery of most of the major classes of natural antibiotics. The isolation of microbial metabolites from Nature was stimulated by Sir Alexander Fleming's serendipitous discovery of a penicillin-producing fungus and was closely followed by systematic search of antibacterial-producing microorganisms by pioneers such as René Dubos and Selman Waksman. The sulfa drugs were introduced in the 1930s and have been in continuous use for 70 years. The first versions of the quinolone synthetic drugs were introduced in 1962.¹

To date, the family of β -lactam antibiotics remain the most important class of antibiotics used in the clinic, with more than 60 per cent of the global market.² However, β -lactam (and other classes of) antibiotic research by major pharmaceutical companies has decreased over the past two decades, mainly due to potential revenues from new molecules not being sufficient to justify the high costs of development.³ The threat of rising antimicrobial resistance,⁴ made worst by a critical shortage of new antibacterial against multidrug-resistant strains^{5, 6} is stimulating renewed interest in antibiotics development.⁷⁻¹⁰

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Discovery and development of β -lactam antibiotics.

The observations of Scottish microbiologist and pharmacologist Sir Alexander Fleming, in 1928, on the antibiotic properties of the extracts from the mould *Penicillium notatum* sparked the start of a golden era of natural antimicrobial drugs.¹¹ Fleming's discovery was complemented by the work of Australian pharmacologist and pathologist Professor Howard, Lord Florey, German biochemist Sir Ernst Boris Chain, British biochemist Sir Edward Abraham and British biologist and biochemist Norman Heatley, who subsequently worked on the isolation and characterization of penicillin. In 1945, Fleming, Florey and Chain shared the Nobel Prize for Physiology or Medicine. The isolation of penicillin and the description of antibiotic activity were followed by the characterization of its structure by Dorothy Crowfoot Hodgkin in 1949,¹² using the techniques she had developed in X-ray crystallography and confirming the structure proposed by Chain.¹³

The large-scale production of penicillin G from *Penicillium chrysogenum* was achieved in 1941 by a group lead by Professor Kenneth B. Raper at the US Department of Agriculture Northern Regional Research Laboratory.^{14, 15} This was followed by the observation that the addition of phenylacetic acid to the fermentation media greatly enhanced its yield; this, in turn, provoked attempts to prepare novel penicillins by supplementing the fermentation process with appropriate precursors. Penicillin V, the result of introducing a phenoxyethyl moiety, was the most successful result of this strategy, showing a greater suitability for oral administration (Figure 1.1).¹⁶

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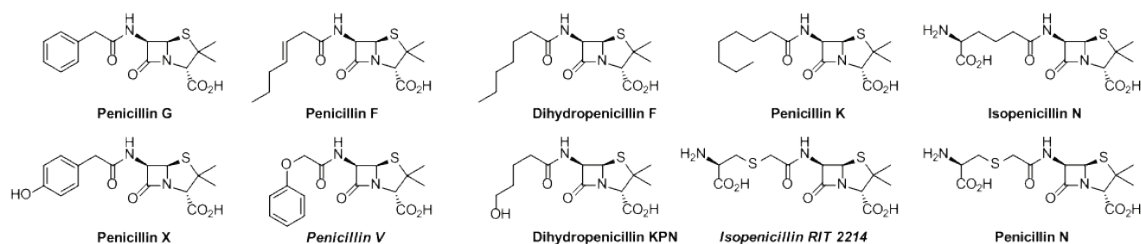


Figure 1.1. Fermentation-derived penicillins. Compounds in *italics* are obtained as the result of specific precursor addition to the fermentation medium: phenoxyacetic acid for penicillin V¹⁷ and L-S-carboxymethylcysteine for RIT 2214.¹⁸ Most of the shown penicillins (except penicillins N and KPN) are produced by *P. chrysogenum* and *P. notatum* in varying ratios depending on the strain and culture conditions. Penicillin F, a major product of *P. notatum*, is designated so to identify it as the penicillin discovered by Sir Alexander Fleming.¹⁹ Penicillin G is a major product of *P. chrysogenum* grown on corn-steep liquor. Penicillin KPN is produced by strains of the genus *Paecilomyces*.²⁰

Along with penicillins, other naturally occurring β -lactam antibiotics include the cephalosporins, the clavams, the monobactams and the carbapenems (Figure 1.2). Although some β -lactam antibiotics have other targets, all clinically used β -lactam antibiotics target transpeptidase enzymes, also known as penicillin binding proteins, which are involved in bacterial cell wall biosynthesis, *via* the formation of stable acyl-enzyme complexes. These complexes result in the inhibition of the transpeptidase activity; hence in impaired peptidoglycan biosynthesis and consequently cytolysis of the bacterium.²¹

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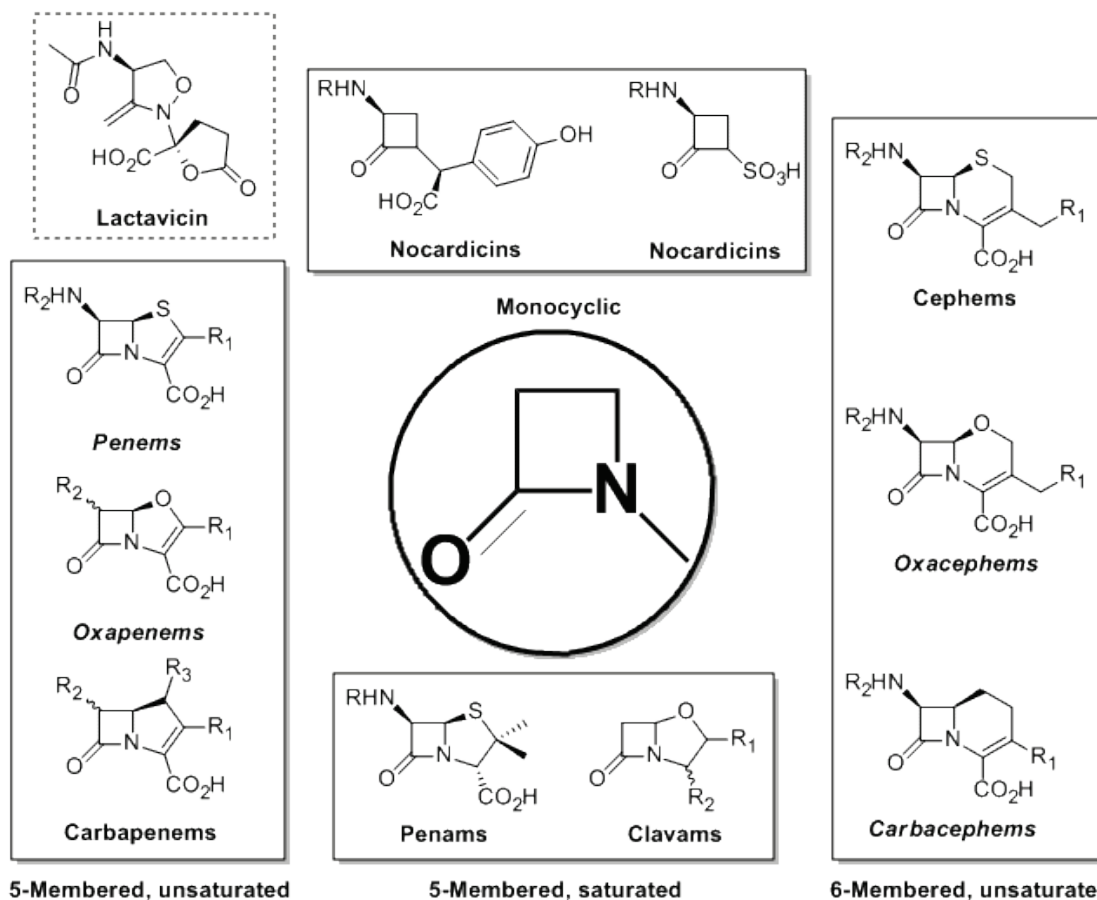


Figure 1.2. Major subfamilies of β -lactams. The names of synthetic β -lactam antibiotic subfamilies are in *italics*. Lactavicin (dashed box) is a naturally-occurring γ -lactam antibiotic which inhibits penicillin binding proteins. Clavulanic acid (a clavam with (5*R*)-stereochemistry) is a serine β -lactamase inhibitor; clavams (with the 5*S*-stereochemistry) have antibacterial and antifungal activities (the latter not *via* the inhibition of penicillin binding proteins).

Soon after the early clinical trials of penicillin in the 1940's, resistance to β -lactam antibiotics was identified.²² The three most important mechanisms of resistance are drug inactivation by β -lactamases,²³ mutation of penicillin binding proteins to block inhibitor binding,²⁴ and ejection of β -lactams by efflux pumps.²⁵ The largest family of identified β -lactamases likely evolved from penicillin-binding proteins and shares a similar nucleophilic catalytic residue for its binding activity.²⁶ β -Lactam antibiotics show different degrees of stability towards β -lactamases, with the cephalosporins and the carbapenems showing increased stability, when generally compared to the penicillins.²⁷

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The naturally occurring β -lactam compounds clavulanic acid,²⁸ sulbactam²⁹ and tazobactam³⁰ are widely used β -lactamase inhibitors (Figure 1.3). Because these compounds themselves are not potent enough antibiotics, they are usually prescribed in combination with a β -lactam antibiotic, *i.e.*, amoxicillin and clavulanic acid are marketed as co-amoxiclav, ampicillin and sulbactam are available under the trade-name Unasyn® (Pfizer), and piperacillin and tazobactam are marketed as Zosyn® (Pfizer).

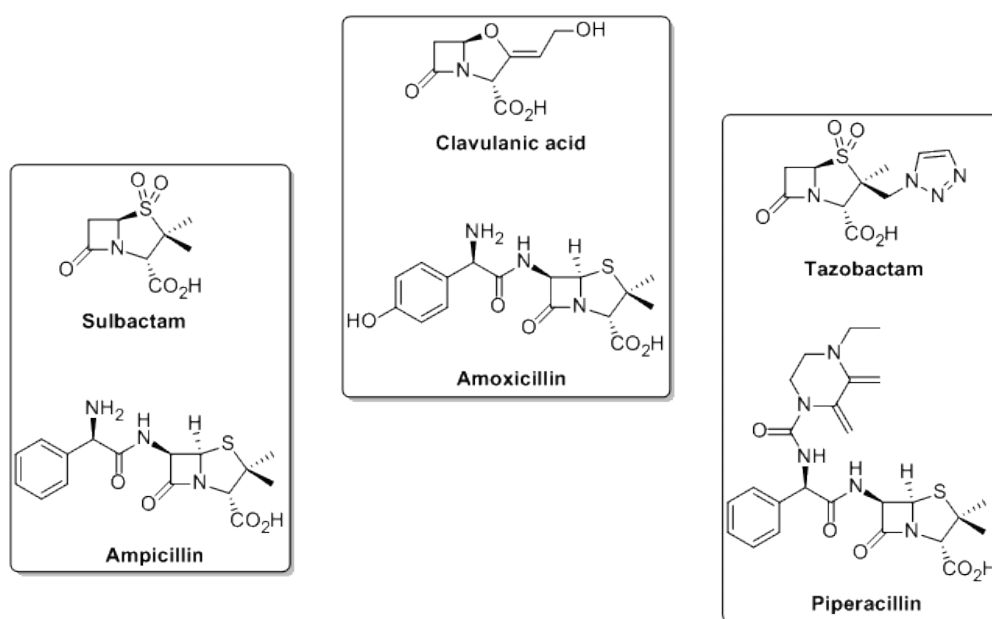


Figure 1.3. Structures of some β -lactamase inhibitors. Clavulanic acid, sulbactam and tazobactam are used clinically in combination with β -lactam antibiotics to protect the latter against the effect of β -lactamases.

An important step in the development of clinically useful penicillins was the discovery that different penicillins could be obtained by the use of different growth media and strains. The combination of corn-steep liquor, as growth media, the serendipitous discovery of the high yielding strain *P. chrysogenum*, and the use of aerated deep fermentation had a historically important role in the development of penicillins, starting with Penicillin V, as previously discussed. The development of procedures to produce penicillins with neutral hydrophobic side chains (*via*

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supplementation of the fermentation media with the appropriate precursors) was also important in enabling the purification of penicillins by extraction from said media.^{17, 31} Although this work is a pioneering example of metabolic engineering, the type of penicillins that could be produced following this technique was rather limited. A crucial further development was the procedure for the production of 6-aminopenicillanic acid.³² Extracted from hydrophobic penicillins, 6-aminopenicillanic acid can be acylated by penicillin acylases³³ to yield semi-synthetic penicillins with a diversity of side chains, e.g., piperacillin, amoxicillin and ampicillin (Figure 1.4A).

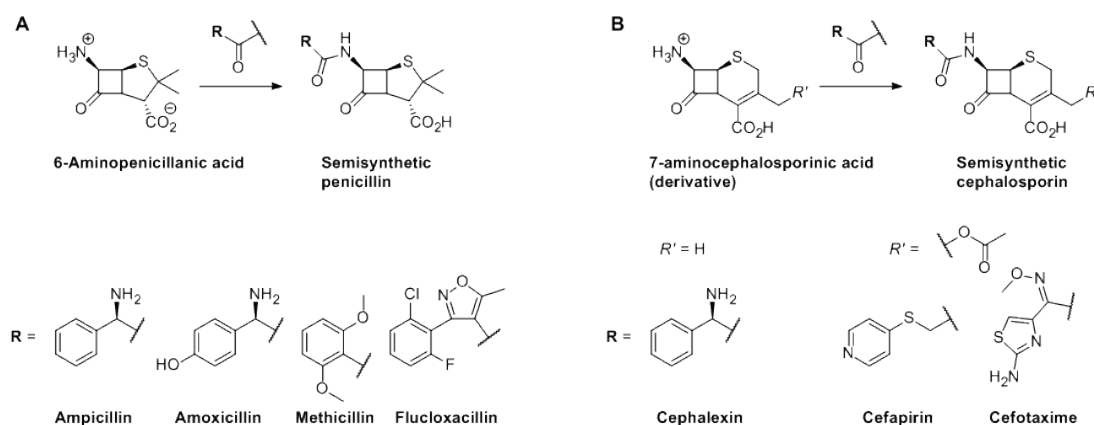


Figure 1.4. Examples of semisynthetic penicillins (A) and cephalosporins (B) derived from 6-aminopenicillanic acid and 7-aminocephalosporinic acid (derivative), respectively.

The need to address penicillin resistance, plus enhancing the activity spectrum of the molecules, initiated a search for new β -lactam antibiotics, which resulted in the isolation of cephalosporin C from *Acremonium chrysogenum*.³⁴ Cephalosporin C (Figure 1.5) shows some resistance to Class A β -lactamases. Procedures have been developed for the production of 7-aminocephalosporanic acid to be used in a similar fashion to 6-aminopenicillanic acid, but this has been hindered by the fact that it is not possible to efficiently produce cephalosporins with hydrophobic side chains simply by the addition of hydrophobic acetic acid derivatives to the fermentation media (Figure 1.4 B). One successful approach to this issue has been the development of chemical methods for the

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ring expansion of penicillins with hydrophobic side chains to yield cephalosporins;³⁵ enzymatic or chemical deacylation is then used to hydrolyse the side chain at C-7 of the resultant cephalosporin. Compared to penicillins, cephalosporins have additional sites for modification (*e.g.*, at the C-3' position), which allows for further modification. For example, esterases have been identified that efficiently catalyse the hydrolysis of the C-3' ester of cephalosporin C to give an allylic alcohol which can be subsequently modified. Thousands of cephalosporin derivatives, modified at C-7 and/or C-3', have been produced semi-synthetically following these strategies, many of those reaching the clinic Figure 1.5.³⁵⁻³⁷

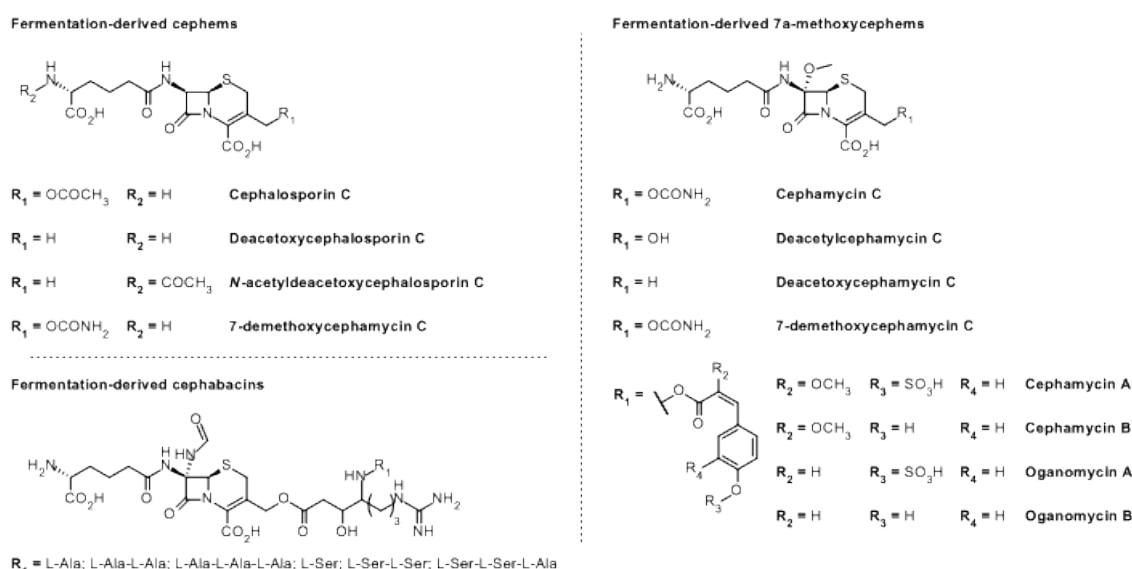


Figure 1.5. Some cephalosporins isolated from natural sources. The first cephalosporin to be isolated was Cephalosporin C from strains of the fungi *Acetamonium* sp.³⁸ 7 α -methoxycephems were isolated from *Streptomyces* sp.³⁹⁻⁴² The cephabacins are produced by some gram-negative bacteria, *e.g.* *Xanthomonas lactamgena*⁴³⁻⁴⁶ and *Lysobacter lactamgenus*.^{43, 44}

In the late 1970's, new strategies involving the use of supersensitive strains of bacteria were used to screen massive numbers of samples of soil microorganisms for β -lactam antibiotics.^{47, 48} The new screens, coupled to advances in chromatography, led to

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the discovery of three new subclasses of β -lactam antibiotics, including the clavams, the monobactams and the carbapenems.

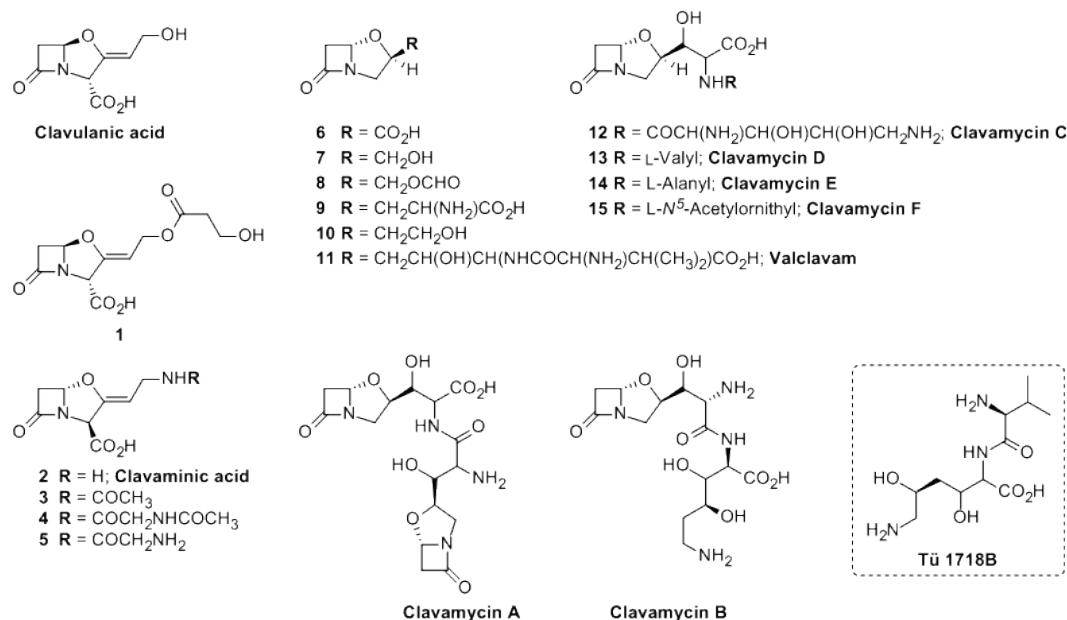


Figure 1.6. Clavams isolated from natural sources.⁴⁹ In addition to clavulanic acid and its β -hydroxypropionyl derivative 1 (with (5*R*)-stereochemistry), *Streptomyces clavuligerus* produces clavams 6-9 (with (5*S*)-stereochemistry⁵⁰). Clavulanic acid has also been from *S. jumonjinensis* and *S. katsurahamanus*. The N-acyl derivatives 3-5 of clavaminic acid (2) accumulate in a *S. clavuligerus* mutant blocked in clavulanic acid production. The hydroxyethyl clavam (10), valclavam (11), and Tü 1718B (boxed) were isolated from *S. antibioticus* Tü 1718B which does not produce clavulanic acid; Tü 1718B is a degradation product of 11.^{51, 52} The structure of valclavam is related to that of clavamycins A-F which are produced by *S. hygroscopicus*.

Clavulanic acid (Figure 1.6) was the first member of the clavam subfamily to be isolated. It was found to be a potent inhibitor of Class A β -lactamases isolated from *Streptomyces clavuligerus*, but is a relatively weak antibiotic.²⁸ The clavams are principally distinguished from penicillins by the replacement of the sulfur atom in the five-member ring fused to the β -lactam with an oxygen atom.

The monobactams (a 3-aminobactamic acid derivative, Figure 1.7 A) were the first naturally-occurring monocyclic β -lactams to be identified, and along with the nocardicins (3-aminocardicinic acid derivatives, Figure 1.7 B), represent the two most

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populated sub-families of monocyclic β -lactams, both being inhibitors of bacterial cell wall synthesis.⁵³ Isolated from *Nocardia uniformis* subsp. *tsuyamanensis*,⁵⁴ Nocardicin A presents a weak to moderate activity against Gram-negative and Gram-positive bacteria, and some resistance to β -lactamases, though, to date the nocardicins have not found clinical applications due to their poor antibacterial properties. Further development has yielded aztreonam (Azactam®, Bristol-Myers Squibb, Figure 1.7 A), which is effective against Gram-negative bacteria and unusually resistant to hydrolysis by β -lactamases.⁵⁵ In addition, there are the tabtoxins (structurally unique, with an unsubstituted nitrogen on the β -lactam core (Figure 1.7 C), and the “conjugate” β -lactams (where the β -lactam nucleus is *N*-linked to a terpenoid, Figure 1.7 D); the occurrence of conjugate β -lactams is notable as it marks the apparent ability of higher plants to produce β -lactams.

Thienamycin, named after its novel β -thioenamine chromophore,⁵⁶ was the first carbapenem to be isolated and characterized, in 1976 from *Streptomyces cattleya*.^{57, 58} The carbapenems are distinguished from penicillins (penams) by the replacement of the sulphur atom in the five-member ring with a methylene group, and by desaturation between C-2 and C-3^{59, 60} (Figure 1.2). Thienamycin was found to be one of the most potent broad-spectrum antibacterial agents that have been isolated from natural sources.⁶¹ Thienamycin has good activity against many Gram-positive and Gram-negative bacteria, shows stability to some β -lactamases, and exhibits some β -lactamase inhibitory properties. Despite its efficacy, its chemical and metabolic instability have prevented its widespread use in the clinic. Instead, derivatives of thienamycin have been developed synthetically and are now widely used as antibiotics.

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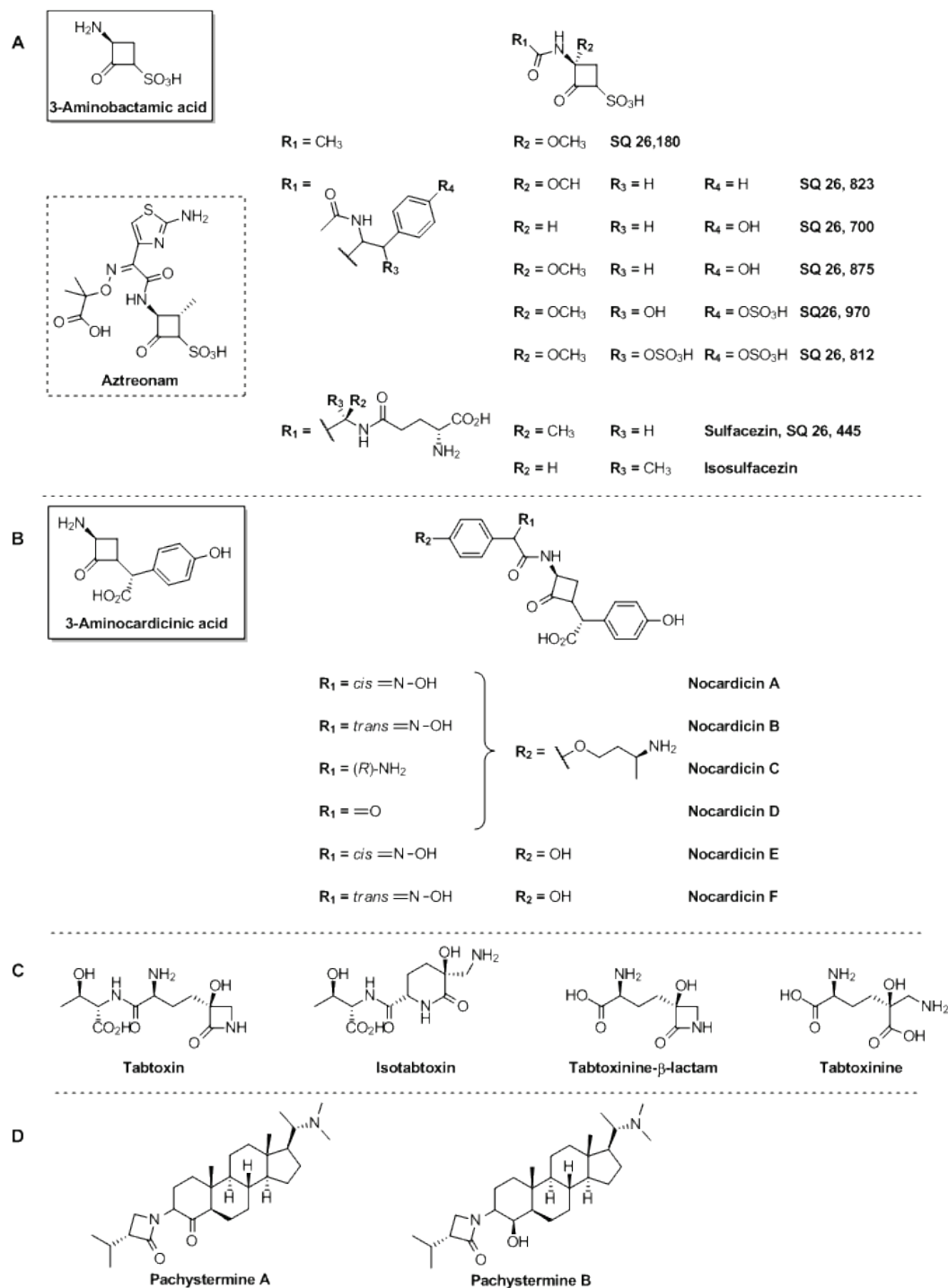


Figure 1.7. Monocyclic β -lactams isolated from natural sources: the monobactams (A); the nocardicins (B); the tabtoxins (C); and the conjugate β -lactams (D). The nuclei for monobactams and nocardicins are boxed. Sulfazecin and its epimer are produced by *Pseudomonas acidophila* and *P. mesoacidophila*, respectively.^{62, 63} Sulfazecin has also been isolated from *Glucobacter* and *Acetobacter*.^{64, 65} SQ 26,180 has been isolated from *Chromobacter violaceum* ATCC 31532.^{65, 66} Nocardicins A-G were isolated from *Nocardia uniformis*.^{54, 67-70} Isotabtoxin is a stable product of tabtoxin rearrangement; tabtoxinine is the hydrolysis product of tabtoxinine- β -lactam.

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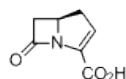
Carbapenem antibiotics.

Since the pioneering work at Merck Research Laboratories that resulted in the discovery and isolation of thienamycin, more than 45 naturally occurring carbapenems have been described, including the olivanic acids, *epi*-thienamycins, carpetimycins, asprenomycins, pluracidomycins, and carbapenems of the PS- and OA-6129 series⁷¹ (Figure 1.8). The majority of these carbapenems have been isolated from the fermentation broths of *Streptomyces* spp.; an exception is the simplest carbapenam/em isolated from the fermentation broths of members of the Enterobacteriaceae family, e.g. the plant pathogen *Pectobacterium carotovorum* (formerly *Erwinia carotavora*),⁷² and the carbapenem AB-110-D, the only known carbapenem with a (Z) geometry at C-2, isolated from the actinomycete *Kitasatosporia papulosa*.⁷³

The basic structure of carbapenems differs from that of penicillins only by the substitution of a carbon atom for sulfur at position 1 and the presence of an unsaturated bond between carbon atoms 2 and 3 in the secondary ring. It is the side chains attached to this basic two-ring structure that differentiate the carbapenems from each other.¹⁰² Particularly, the side chain at C-2 has been important in overcoming the instability of the original cysteaminy side chain of thienamycin, leading to the development of the first synthetic carbapenem, the *N*-formimidoyl derivative imipenem.¹⁰³ Imipenem bears the more basic amidine moiety, protonated at physiological pH, and thus unable to perform the nucleophilic attack responsible for the hydrolysis of the β -lactam ring. Unfortunately, imipenem, along with panipenem,¹⁰⁴ an ethanimidoylpyrrolidinyl derivative, are hydrolyzed by dehydropeptidase I at renal brush border cells which required the development of additional compounds to avoid this. As a result, imipenem is co-administered with cilastatin, and panipenem is so with betamipron.¹⁰³⁻¹⁰⁵ The

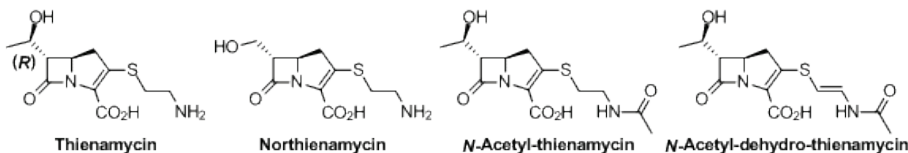
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The simplest carbapenem

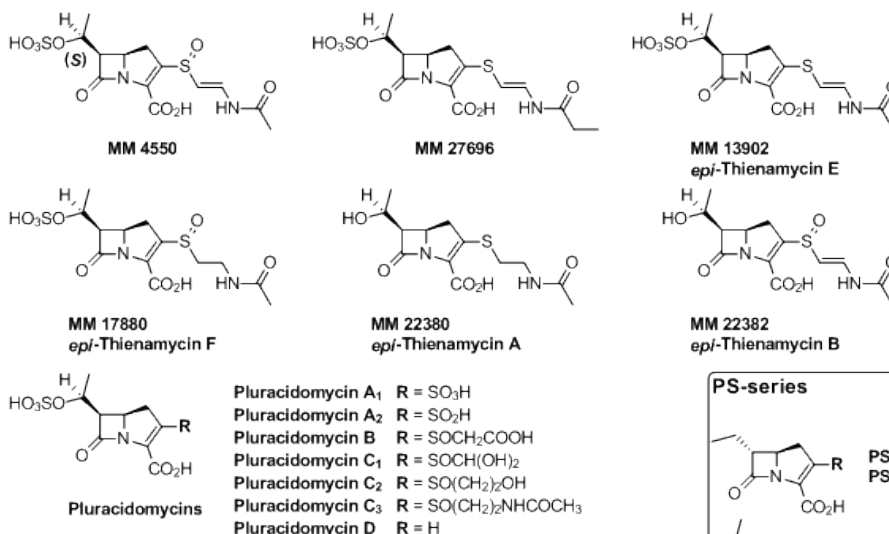


(5R)-1-carbapen-2-em-3-carboxylic acid

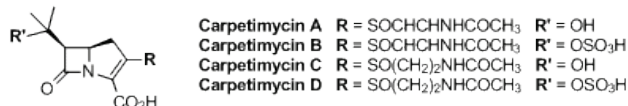
Thienamycin (-like) structures



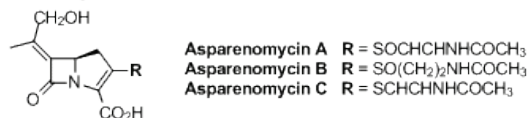
Olivanic acids and Pluracidomycins



Carpetimycins



Asparenomycins



Carbapenams

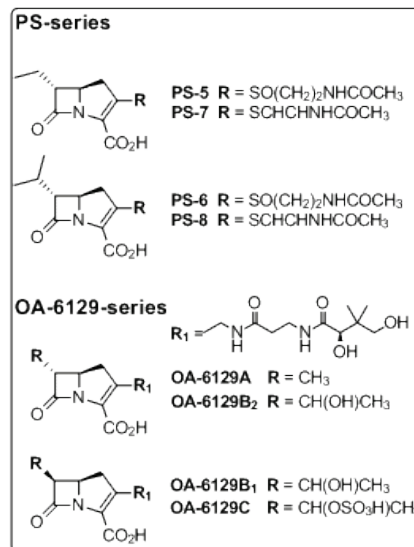
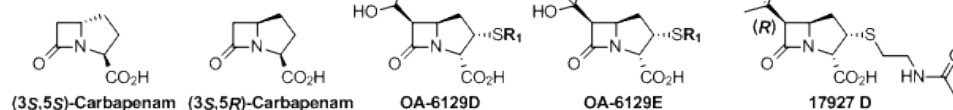


Figure 1.8. Carbapen(em/am)s from natural sources.⁷⁴ Some stereochemical assignments are provisional and some of the sulfoxide stereochemistries are unknown. Thienamycin, northienamycin, N-acetyl-thienamycin and N-acetyl-dehydro-thienamycin were all isolated from *S. cattleya* (wild-type or mutant).^{57, 58, 75, 76} Epi-thienamycins A-F were isolated from *S. flavogriseus*^{77, 78} and *S. olivaceus*.⁷⁹⁻⁸³ The olivanic acids MM 4550^{79, 82, 83} and MM 27696⁸⁴ were isolated from *S. olivaceus*. The pluracidomycins,

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characterised by containing two or three acidic moieties in their structure, were isolated from *S. pluracidomyceticus*;⁸⁵⁻⁸⁷ in addition, the culture co-produced *epi*-thienamycin A, *epi*-thienamycin F and MM 4550.⁸⁷ The PS-series of carbapenems were isolated from *S. cremus* subsp. *auratilis*.⁸⁸⁻⁹⁰ Carpetimycins were isolated from *Streptomyces* sp. KC-664⁹¹⁻⁹⁴ Asparenomycins were isolated from *S. tokumonensis*, *S. argentealus* and another *Streptomyces* sp.^{95, 96} The OA-6129-series of carbapenems, characterised by a pantetheinyl moiety at C-2, were isolated from *Streptomyces* sp. OA-6129 and of a *S. fulvoviridis* mutant.⁹⁷⁻¹⁰⁰ The carbapenam 17927 D (or its (S)- enantiomer) was isolated from *S. fulvoviridis*, and exhibited no antimicrobial activity.¹⁰¹

addition of a dehydropeptidase I inhibitor also prevents nephrotoxicity when imipenem or panipenem are administered alone.

Further research revealed that stability to human renal dehydropeptidase I could be achieved by the introduction of a 1- β -methyl substituent into the carbapenem structure.¹⁰⁶ The combination of this feature with a precise stereochemistry in the pyrrolidine group at C-2' led to the development of meropenem.¹⁰⁷ This new analogue was less susceptible to hydrolysis by dehydropeptidase I, and showed an enhanced potential against microbial infections of the central nervous system,¹⁰⁸ which clinical experience had shown was contraindicated with imipenem/cilastatin.^{109, 110}

Along the journey to the discovery of more stable carbapenems with a broader antibacterial spectrum, other compounds currently available (*i.e.* ertapenem, biapenem and doripenem, Figure 1.9 B) were developed, and several other novel structures were also identified, many of them similar to the carbapenems currently available in the clinic *i.e.*, containing a 1- β -methyl and a pyrrolidine ring moiety at C-2 (*e.g.* tebipenem, lenapenem, tomopenem and faropenem, Figure 1.9 C). These novel carbapenems also include antipseudomonal carbapenems, anti-methicillin-resistant *Staphylococcus aureus* carbapenems (*i.e.* cationic and dithiocatiocarbamate carbapenems), orally available

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carbapenems, trinem carbapenems, a dual quinolonyl carbapenem, amongst others¹¹¹ (Figure 1.9 C).

Several chemical approaches have been developed for the synthesis of carbapenems, since fermentation was not an efficient method of production.¹¹²⁻¹¹⁷ These synthetic approaches rely mainly on the derivatization of the intermediate (3*R*,4*R*)-4-acetoxy-[(1*R*)-*tert*-butyldimethylsilyloxyethyl]-azetidin-2-one *via* elimination/addition at C-4 and subsequent cyclisation to form the 5-membered carbocyclic ring¹¹⁸ (Figure 1.9 D).

Biosynthesis of carbapenems.

An important difference between the penicillin and cephalosporin development stories and that of the carbapenems is that in the later case, along with the clavams and monobactams, the semi-synthetic approach has been much less productive. The situation with carbapenems is particularly frustrating, since extensive efforts have not yielded increased fermentation titres of carbapenems to useful large-scale levels. As a result, total synthesis methods had to be developed to mass-produce clinically useful carbapenems. Whilst the routes are remarkably efficient,^{56, 121} the available synthetic methodology limits the range of accessible compounds; one particular limitation being the reliance on (3*R*,4*R*)-4-acetoxy-3-[1-(*tert*-butyldimethylsilyloxy)ethyl]azetidin-2-one as a synthetic intermediate. The problem of efficient and inexpensive biotechnological production of carbapenems has stimulated efforts to understand and manipulate their biosynthetic pathways. As with related pioneering studies into the biosynthesis of penicillin and cephalosporin, this work may help to meet current demand for new and existing antibiotics to be produced effectively and in a sustainable manner.

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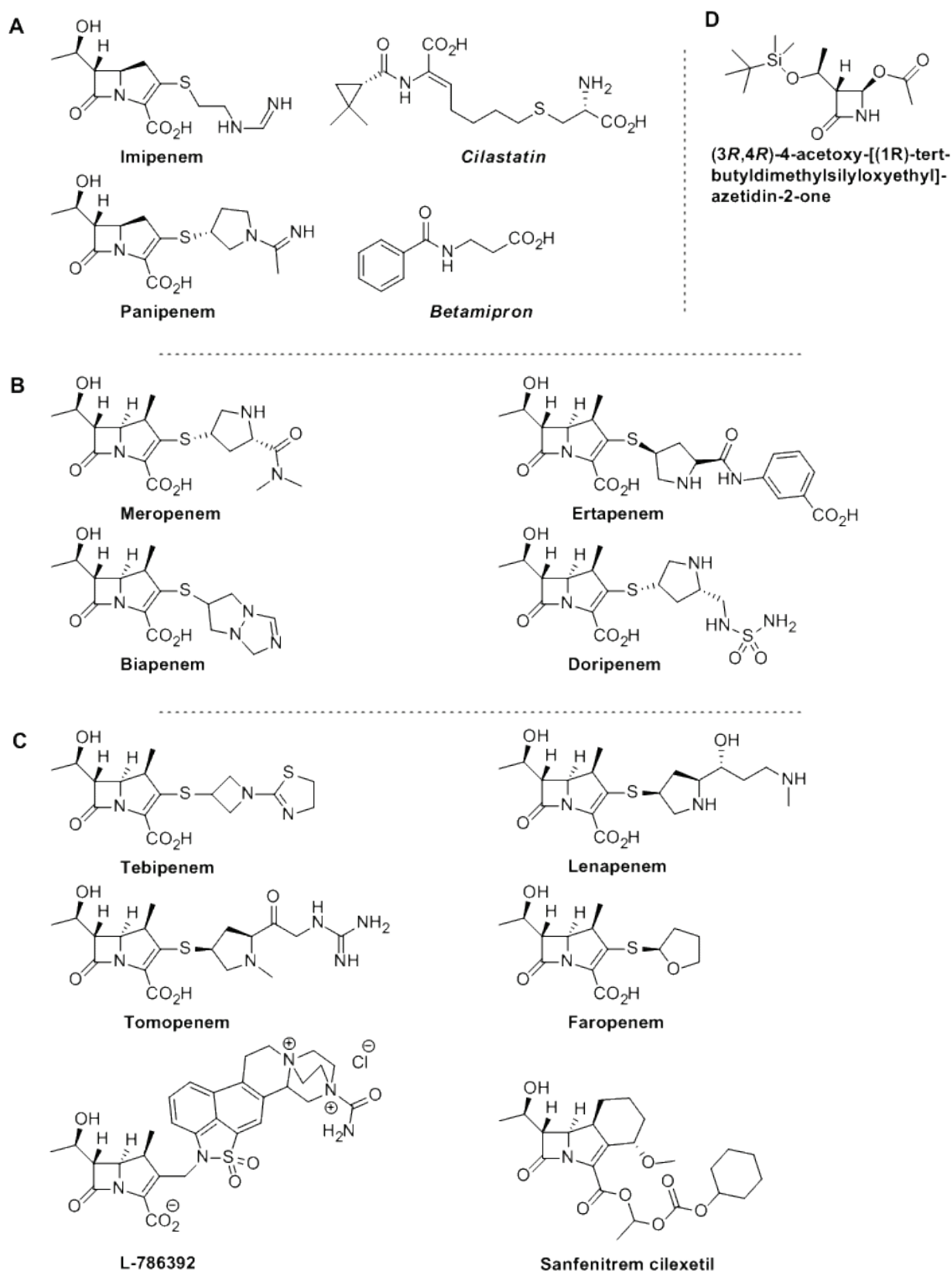


Figure 1.9. Commercially-available and under-research carbapenems.^{106, 111, 119, 120} The first generation of synthetic carbapenems (**A**) required the co-administration of an inhibitor of dehydropeptidase-I (*in bold italics*). Subsequent molecules addressed this issue by the introduction of a 1- β -methyl moiety, along with pyrrolidine ring moieties at C2 for improved half-lives (**B**). Some molecules still in the development pipeline (**C**). The most common intermediate in the synthesis of clinically used carbapenems is (3R,4R)-4-acetoxy-[(1R)-tert-butylidimethylsilyloxyethyl]-azetidin-2-one (**D**).

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The isolation and characterisation of carbapen-2-em-3-carboxylate, the simplest carbapenem known in nature, was an important step towards the understanding of the biosynthesis of carbapenems, due to the complexity of the pathways leading to the C-2 and C-6 functionalised molecules. Pioneering labelling studies in the 1980's (Figure 1.10) have established that labelled acetate and glutamate are incorporated into the β -lactam carbons and the fused pyrroline ring of both carbapenem-2-em-3-carboxylate and thienamycin, respectively.^{122, 123} Furthermore, labelled cysteine and methionine have been reported to be incorporated into the side chains of thienamycin at C-2 and C-6, respectively.¹²²

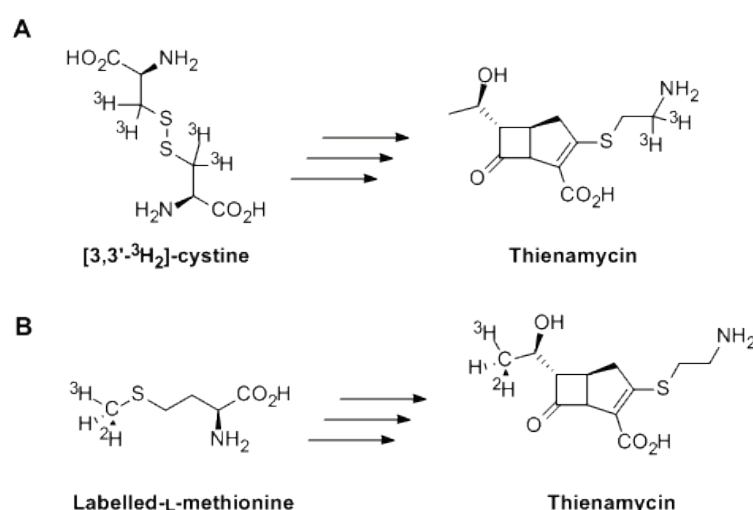


Figure 1.10. Summary of labelling studies on the side chains at C-2 (A) and C-6 (B) of thienamycin in *Streptomyces cattleya*. Also, feeding L-[methyl-¹³C,²H₃]methionine to *S. cattleya* resulted in retention of all three deuterium atoms at ¹³C-9 and retention of one deuterium atom at ¹³C-8.^{74, 122, 124}

Carbapen-2-em-3-carboxylate (Figure 1.8) was first isolated from *Pectobacterium carotavorum* and *Serratia* sp in 1982;¹²⁵ subsequently, it was found out to be a product of the insect pathogen *Photobacterium luminiscens*.¹²⁶ The isolation, sequencing and initial assignment of the *P. carotavorum* biosynthetic cluster for carbapen-2-em-3-carboxylate have revealed that nine proteins are encoded by the genes *carA-H* and *carR* (Figure 1.11 and Table 1.1).^{127, 128} After some uncertainty with regard to the

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stereochemistry of the carbapenam intermediates,¹²⁹⁻¹³² it is now well-established that the only three enzymes absolutely required for the biosynthesis of carbapen-2-em-3-carboxylate are the products of the genes *carA*, *carB* and *carC*.^{128, 133} The product of *carR* has been identified as a DNA binding LuxR homologue transcriptional regulator that functions in the presence of *N*-3(oxohexanoyl-L-homoserine lactone (OHHL).¹³⁴ The CarR:OHHL complex binds to the *carA* promoter and activates the expression of the *carA-H* cluster. The products of *carF* and *carG* are proposed to be involved in intrinsic resistance to carbapen-2-em-3-carboxylate via an uncharacterised mechanism.¹²⁸ The product of *carI* is involved in a quorum-sensing system involving the synthesis of OHHL.^{135, 136} The putative products of *carD* and *carE*, a proline dehydrogenase and a 2Fe-2S ferredoxin respectively, are likely involved in the oxidation of proline¹²⁷ to give an equilibrating mixture of isomers (L-glutamate semi-aldehyde, L-GSA/L-5-hydroxyproline, L-5HP/L-pyrroline-5-carboxylate, L-P5C), collectively abbreviated L-GHP, which is a co-substrate for the carboxymethylproline synthase CarB. A member of the crotonase superfamily of enzymes, CarB catalyses the formation of (2*S*,5*S*)-carboxymethylproline (*t*-CMP) from malonyl-CoA and L-GHP (L-P5C).^{137, 138} The ATP-dependent carbapenam synthetase CarA then catalyses the β -lactam ring closure to yield the (2*S*,5*S*)-carbapenam nucleus.¹³⁹ Finally, the carbapenam synthase CarC, a 2OG-dependent oxygenase, catalyses the highly unusual C-5 epimerisation of the carbapenam nucleus to (2*S*,5*R*)-carbapenam which then undergoes desaturation, catalysed also by CarC, to afford the active antibiotic carbapen-2-em-3-carboxylate.^{140, 141}

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Table 1.1. Genes constituting the reported (5*R*)-carbapen-2-em-3-carboxylate gene cluster in *Pectobacterium carotovorum* and the (predicted) roles of the (putative) proteins they (may) encode for. Proteins that have been assigned they biochemical function are in **bold**. AA, predicted number of amino acid residues.

Gene	AA	(Proposed) function of encoded protein
<i>carR</i>	244	Transcriptional regulator
<i>carA</i>	503	β-Lactam synthetase
<i>carB</i>	250	Carboxymethylproline synthase
<i>carC</i>	273	Carbapenem synthase
<i>carD</i>	376	Proline dehydrogenase
<i>carE</i>	92	2Fe-2S ferredoxin
<i>carF</i>	288	Protein involved in resistance
<i>carG</i>	177	Protein involved in resistance
<i>carH</i>	184	Unknown

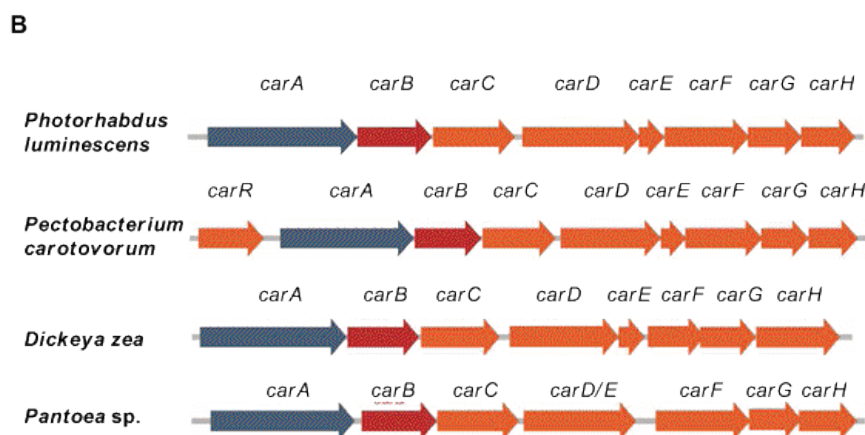
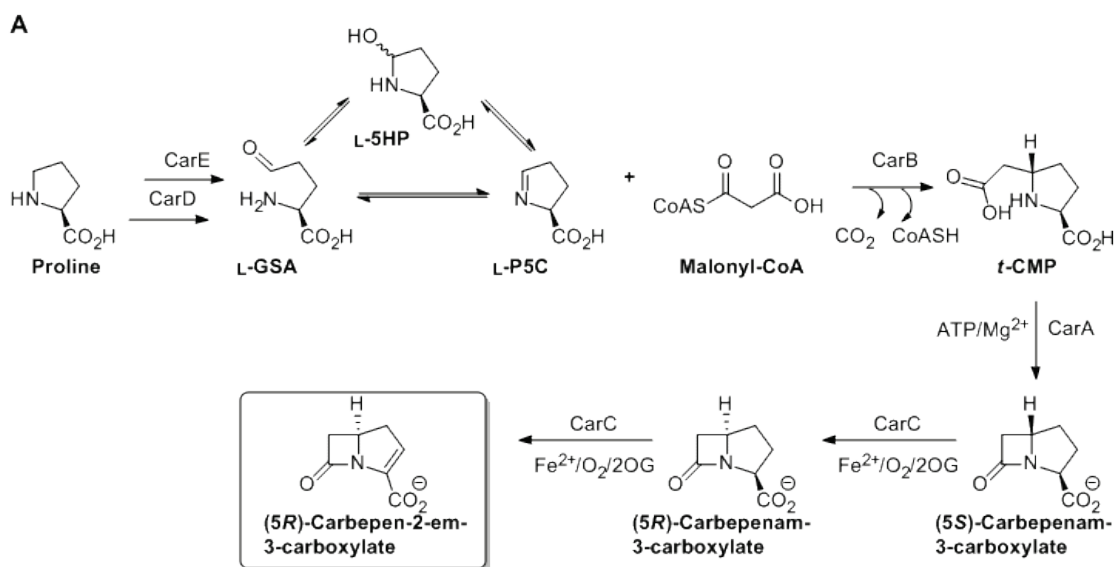


Figure 1.11. The biosynthetic pathway leading to the simplest carbapenem, (5*R*)-carbapen-2-em-3-carboxylate, in *Pectobacterium carotovorum* (A) and the gene clusters of producing organisms (verified and putative, B). L-GSA, L-glutamate semi-aldehyde; L-5HP, L-5-hydroxyproline; L-P5C, L-

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pyrroline-5-carboxylate; *t*-CMP, *trans*-carboxymethylproline. The production of (5*R*)-carbapen-2-em-3-carboxylate by *Dickeya zea* and *Pantoea* sp. is yet to be assessed. Note that in the case of *Pantoea* sp., *carD* and *CarE* exist as a single gene, unlike other producers of (5*R*)-carbapen-2-em-3-carboxylate where separate genes exist for *CarD* and *CarE*. See Table 1.1 for the proposed role of the encoded proteins.

The *carB* gene in *P. carotovorum*, and its homologue in *S. cattleya*, *thnE*, encode for 250- and 294-residue proteins, respectively. Both have been expressed in recombinant *E. coli*, purified and characterized as carboxymethylproline synthases (CMPSs).^{137, 138, 142-144} CarB and ThnE are unique members of the crotonase superfamily of enzymes^{145, 146} because they catalyse three different reactions within the same active site, namely malonyl-CoA decarboxylation, C-C bond formation and thioester hydrolysis. The crotonase superfamily of enzymes catalyses a diversity of reactions that have precedent in the “carbonyl” chemistry of organic synthesis.¹⁴⁵

Whilst the CarB-catalysed formation of *t*-CMP is considered the “committed step” (*i.e.* the effectively irreversible enzymatic reaction that occurs at a branch during the biosynthesis of a molecule¹⁴⁷) in the biosynthesis of carbapen-2-em-3-carboxylate in *P. carotovorum*,¹⁴⁰ the role of ThnE in the early stages of thienamycin biosynthesis in *S. cattleya* (Figure 1.22 and Table 1.2) is likely to provide *t*-CMP or a derivative of it.^{142, 144, 148} In addition to its ability to catalyse the formation of *t*-CMP (from malonyl-CoA and L-GHP), ThnE catalyses the *in vitro* conversion of methylmalonyl-CoA to (6*R*)-6-methyl-*t*-CMP as the major product, 60% diastereomeric excess, compared to 10% in the case of CarB, suggesting the possibility that the C-6 alkyl group might be introduced at an earlier stage in the biosynthesis of thienamycin, as discussed later on. However, the lack of *in vitro* conversion of ethylmalonyl-CoA to 6-ethyl-*t*-CMP by ThnE suggests subsequent further methylation and hydroxylation steps would be required if ThnE is responsible for the final C-6 stereochemistry in thienamycin.¹⁴⁴

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Figure 1.12. Partial Sequence alignment for known and putative CMPSs catalysing the formation of t-CMP in carbapenem biosynthesis. The oxyanion hole forming residues are in pink and the catalytically important glutamate residue¹⁴⁹ in orange. The figure was generated using Clustal W¹⁵⁰ and Genedoc.¹⁵¹ α -Helices (cyan cylinders) and β -strands represent the assigned secondary structure of CarB.¹⁴³

CarB crystallises as a homotrimer, dimer of trimers (hexamer) and a trimer of trimers (nonamer). However, both CarB and ThnE present themselves predominantly as trimers in solution.^{143, 144} Proteins of the crotonase superfamily often share low sequence similarities, though their overall architecture is defined by a common fold formed by repeated $\beta\beta\alpha$ -motifs that assemble into two approximately perpendicular β -sheets surrounded by α -helices¹⁴⁵ (Figure 1.13). The catalytic machinery of almost all crotonase superfamily enzyme is able to stabilize an enolate intermediate *via* an “oxyanion hole” comprising two backbone NH groups.¹⁴⁵ In the cases of CarB and ThnE, the active site residues forming the canonical oxyanion hole are proposed to be Met108_{CarB}/Val153_{ThnE} and Gly62_{CarB}/Gly107_{ThnE} (Figure 1.12, Figure 1.13B).^{143, 144} The CarB:acetyl-CoA complex reveals the CoA thioester derivative bound in a characteristic U-shaped conformation (Figure 1.13 B) and a conserved tunnel binds the pantetheine part for coenzyme A leading to the binding pocket of L-GHP. The L-GHP co-substrate (in its L-P5C form) is predicted to be anchored, *via* its carboxylate group, into the bottom of the active site pocket of CarB by residues Val140 (NH), Gly141 (NH), Gln111 (N ϵ 2), His229 (N ϵ 2), as described in Fig (Figure 1.13 C).¹⁴³ The Trp79 residue forms part of the hydrophobic face of the active site of CarB (Figure 1.13 C) and proved to be important in the engineering studies on it, as described in this

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thesis.^{152, 153} The conserved side chain of Glu131 is positioned suitably for hydrolysis of the *t*-CMP-CoA thioester intermediate^{143, 149} (Figure 1.13 B).

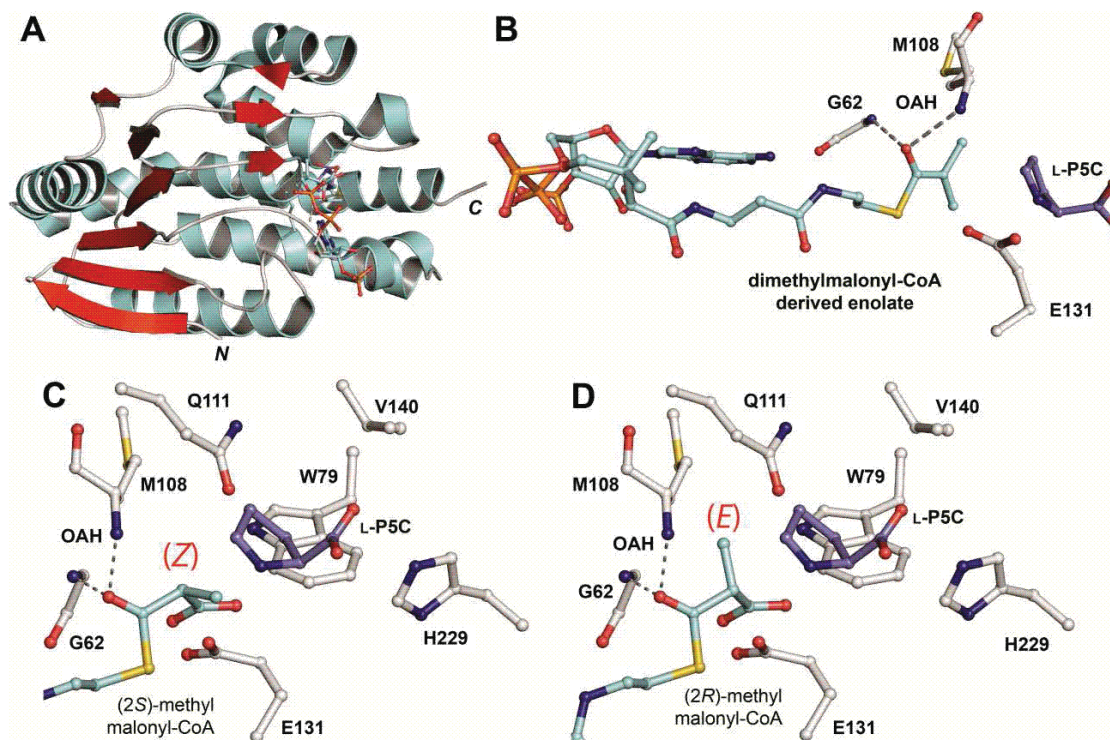


Figure 1.13. Structural views of the carboxymethylproline synthase CarB.¹⁴³ A: A CarB monomer (PDB 2A81) displaying the repeated $\beta\beta\alpha$ -motif which is characteristic of the crotonase superfamily;¹⁴⁵ B: The substrate binding pocket with dimethylmalonyl-CoA derived enolate and L-pyrroline-5-carboxylate (L-P5C) modelled into. Note the characteristic U-shaped conformation of CoA, the location of the thioester carbonyl in the oxyanion hole, and the side chain of Glu131 is suitably positioned ($\sim 4\text{\AA}$ from the thioester carbonyl) to activate a water molecule for hydrolysis of a *t*-CMP-CoA intermediate (path 3, Figure 1.14); C and D: Models of the C-2 epimers of methylmalonyl-CoA (*2S*-epimer, C; *2R* epimer, D) in the substrate binding pocket. Note that, in both cases, an orthogonal relationship between the methylmalonyl-CoA carboxylate and the oxyanion hole-stabilized carbonyl is maintained, as required for the stereoelectronically favoured decarboxylation. The enolate (*E/Z*) that would be generated following decarboxylation is shown in brackets, in each case. Figure from reference ¹⁵⁴.

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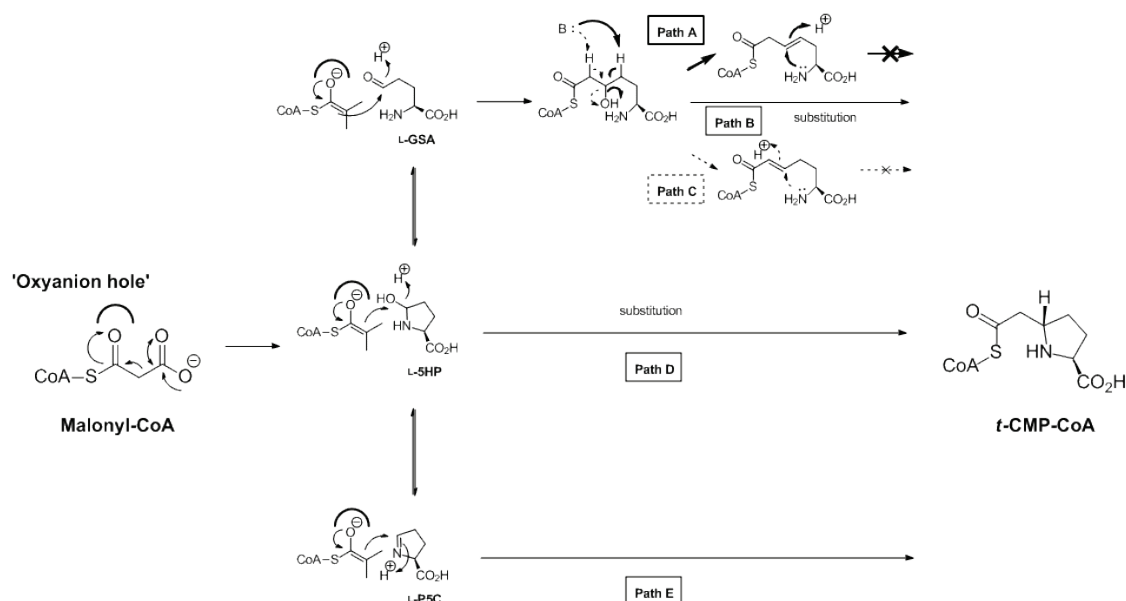


Figure 1.14. Possible C-C bond formation reactions leading to the formation of *t*-CMP-CoA intermediate as catalysed by CMPSs. The most likely path is Path E.¹⁴⁹

CarB and ThnE catalysis is proposed to proceed *via* decarboxylation of malonyl-CoA to give an oxyanion-hole-stabilised enolate (Figure 1.13 B). C-C bond formation is then proposed to proceed *via* reaction of the formed enolate with potentially any of the three forms of L-GHP (Figure 1.14): (i) with L-P5C (path E); (ii) with L-5HP in an S_N2 reaction (path D); or, (iii) with L-GSA in an aldol reaction followed by a substitution reaction (path B), the elimination of a water molecule across C₄-C₅ (path A), or across C₅-C₆ and conjugate addition (path C). The ability of CarB to catalyse the formation of 6,6-dimethyl- $^{[2]H}_6$ -*t*-CMP from $^{[2]H}_6$ -L-GHP and dimethylmalonyl-CoA eliminates paths A and C.¹⁴⁹ Path B cannot be entirely eliminated but seems unlikely as it involves a substitution reaction at the satirically hindered “neopentyl” position (C-5). The enolate reaction directly with L-5HP (path D) is unlikely for the following reasons: (i) An S_N2 -type mechanism would have to occur on just one of the two epimeric hemiaminals of L-5HP to eventually produce *t*-CMP exclusively; (ii) Both of the C-5 hemiaminal epimers of L-5HP can readily eliminate water to form protonated L-P5C likely giving a lower energy reaction path (*i.e.* path E).¹⁴⁹ A further, if indirect, evidence

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for this mechanisms, is the observation that the hindered dimethylmalonyl-CoA derived enolate reacts, because the S_N1 iminium ion route (path E) is less sterically demanding than an S_N2 route (paths B or D). Furthermore, the imine form of L-GHP (L-P5C) is the predominant one ($> 95\%$ by $^1\text{H-NMR}$) at pH 7.7, the final pH of the CarB assay mixture.^{155, 156} Analysis of the structure of CarB coupled to the observation that *t*-CMP is the only detectable diastereoisomer, implies that the nucleophilic attack of the enolate on the anion occurs stereospecifically on the *re* face of L-P5C to furnish the intermediate *t*-CMP-CoA (Figure 1.13 B).^{138, 149}

For hydrolysis of the *t*-CMP-CoA intermediate, three mechanisms have been proposed (Figure 1.15).¹⁴⁹ A mechanism that involves a ketene intermediate (path 1), an anhydride intermediate (path 2), or through direct attack of a water molecule (activated by Glu131) onto the carbonyl of the thioester of *t*-CMP-CoA (path 3). The ketene mechanism is relevant because of the propensity of crotonases to form enolates,^{145, 157} and the likelihood of an enolate being involved in the C-C bond formation in CarB. However, the successful CarB-catalysed conversion of dimethylmalonyl-CoA to 6,6-dimethyl-*t*-CMP rules out the ketene intermediate mechanism (path 1).^{149, 158} Incubation in buffered H_2^{18}O led to the incorporation of a single ^{18}O into the 6,6-dimethyl-*t*-CMP product; trypsin digest analysis on CarB from the same incubation revealed no ^{18}O incorporation from solvent into the side chains of Glu131; this eliminates path 2B and reveals that CarB thioester hydrolysis occurs by a different mechanism than that proposed for the crotonase 3-hydroxyisobutyryl-CoA hydrolase.¹⁵⁷ Path 2A cannot be entirely ruled out, but the available evidence points against it because this path requires hydrolysis adjacent to a sterically hindered quaternary atom carbon.¹⁴⁹ Therefore, the CarB catalysed thioester hydrolysis is proposed to occur *via* direct attack of a water

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molecule (activated by Glu131) on the carbonyl carbon of the *t*-CMP-CoA intermediate (path 3A).¹⁴⁹

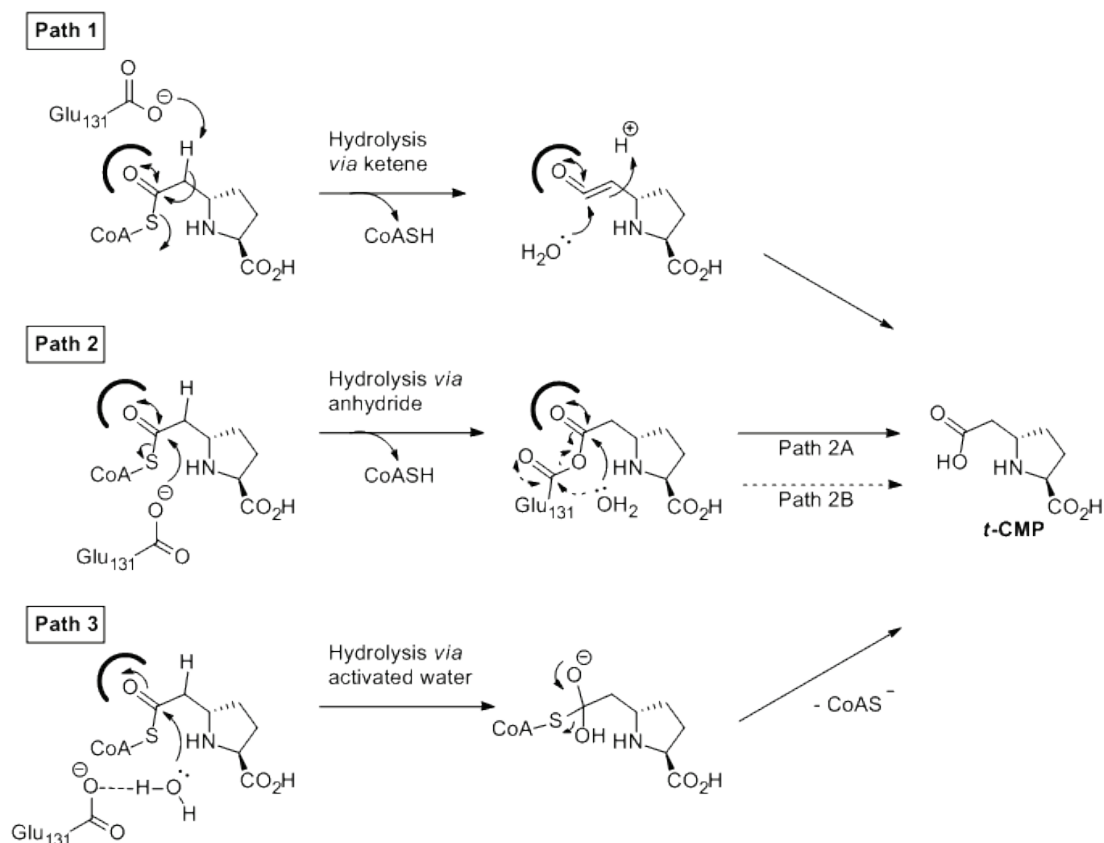


Figure 1.15. Possible thioester hydrolysis mechanisms for CarB catalysis to produce 6-alkyl-*t*-CMP derivatives. Path 3 is the most likely mechanism.¹⁴⁹

Previous engineering studies on CarB/ThnE. CarB and ThnE are proposed to act as a “stereochemical gateway” in carbapenem biosynthesis; only L-GHP has been observed to be transformed to *t*-CMP with concomitant CoASH production; D-GHP stimulates uncoupled turnover with CoASH production but no C-C bond formation activity has been detected.^{143, 144, 159} Research in our group initiated structure- and homology-guided CMPS engineering studies towards the deepening of our understanding of CMPS mechanism of catalysis as well to produce new CMPS variants that could catalyse reactions inaccessible to wild-type enzymes.

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The CarB E131 A/Q/C/S variants were unable to catalyse the production of *t*-CMP or *t*-CMP-CoA from L-GHP and malonyl-CoA, but were shown to catalyse the decarboxylation of malonyl-CoA to acetyl-CoA.¹⁴⁹ In contrast, the more conservative CarB E131D variant is able to catalyse the formation of *t*-CMP, albeit at a low yield. These results revealed Glu131 to be an important residue in the active site for both C-C bond formation and thioester hydrolysis, but not important for decarboxylation.¹⁴⁹

Analogues of L-GHP (L-P5C) with different chain lengths (*i.e.* L-aminoadipate semialdehyde and L-aminopimelate semialdehyde) were accepted by wild-type and engineered CMPSs to produce the corresponding 6- and 7-membered-*N*-heterocycles stereoselectively (Figure 1.16).¹⁶⁰

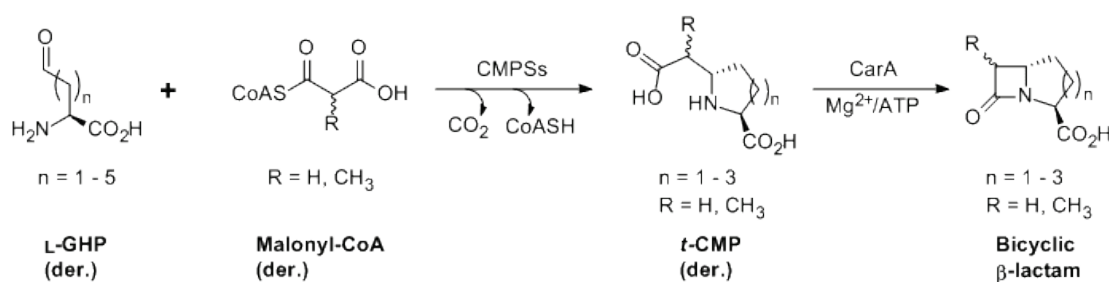


Figure 1.16. Chemoenzymatic synthesis of functionalised 5-, 6- and 7-membered carboxymethyl-functionalised *N*-heterocycles catalysed by carboxymethylproline synthases (CMPSs). L-glutamate semialdehyde (L-GHP) and derivatives: $n = 1$, L-aspartate semialdehyde; $n = 2$, L-glutamate semialdehyde; $n = 3$, L-aminoadipate semialdehyde; $n = 4$, L-aminopimelate semialdehyde; $n = 5$ L-aminosuberate semialdehyde. Besides *t*-CMP ($n = 1$ $R = \text{H}$) and (6*R*/*S*)-6-methyl-*t*-CMP ($n = 1$ $R = \text{CH}_3$), only (2*S*,6*S*)-6-(carboxymethyl)-pipecolic acid ($n = 2$, $R = \text{H}$), 7-methyl-6-(carboxymethyl)-pipecolic acid ($n = 2$, $R = \text{CH}_3$; 7*R*:7*S*, 2:1), (2*S*,7*S*)-7-(carboxymethyl)-azepane-2-carboxylic acid ($n = 3$ $R = \text{H}$) and 8-methyl-7-(carboxymethyl)-azepane-2-carboxylic acid ($n = 3$ $R = \text{CH}_3$; undetermined C-8 stereochemistry) were observed. CMPSs = CarB wt, ThnE wt, CarB H229A. The carboxymethyl-functionalised *N*-heterocycles yielded the corresponding bicyclic β -lactam derivatives under the action of the β -lactam synthetase, CarA (see below).¹⁶⁰

Overall, the results on CMPS engineering studies emphasise the applicability of crotonases in biocatalysis and suggest that they may provide a useful platform technology for catalyzing synthetically challenging reactions, *e.g.* to catalyse the

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stereoselective generation of an enolate of choice and its reaction with varied electrophiles.

The carbapenam synthetase CarA (and its homologue in thienamycin biosynthesis, ThnM)

The *P. carotovorum* carbapenam synthetase CarA,¹³⁹ and its homologue in the thienamycin biosynthesis gene cluster in *S. cattleya*, ThnM,¹⁶¹ catalyse the β -lactam ring formation of (2*S*,5*S*)-carbapenam from *t*-CMP (Figure 1.11 and Figure 1.22). Carbapenam synthetases are closely related β -lactam synthetase, the second enzyme in the clavam biosynthesis pathway responsible for the cyclisation of *N*²-(2-carboethyl)-arginine to give the monocyclic β -lactam deoxyguanidino-proclavamate.¹⁴⁰

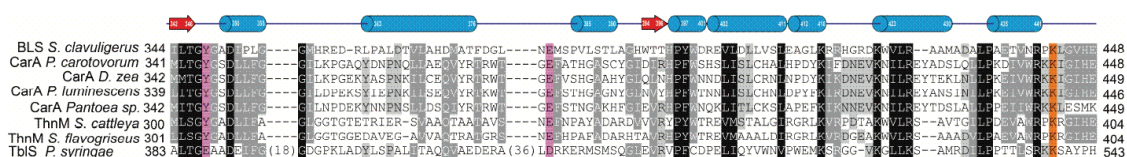


Figure 1.17. Partial sequence alignment for known and putative β -lactam synthetases involved in the biosynthesis of clavams, carbapenems and monobactams. Catalytically important residues are highlighted. The highlighted lysine residue is proposed to assist in ring cyclisation *via* stabilisation of the proposed “tetrahedral” intermediate.¹⁶² The highlighted tyrosine-glutamic acid dyad is proposed to deprotonate the amine involved in intramolecular β -lactam formation.¹⁶³ α -Helices (cyan cylinders) and β -strands (red arrows) represent the assigned secondary structure of β -lactam synthetase (PDB 1MB9).¹⁶⁴

Both β -lactam synthetase and CarA/ThnM are related to asparagine synthetase type A, which catalyses the ATP-dependent transfer of ammonia from glutamine to aspartate to give asparagines and glutamate (Figure 1.17 and Figure 1.18). Crystal structures have been reported for all of them,^{139, 164-166} except for ThnM (Figure 1.18).

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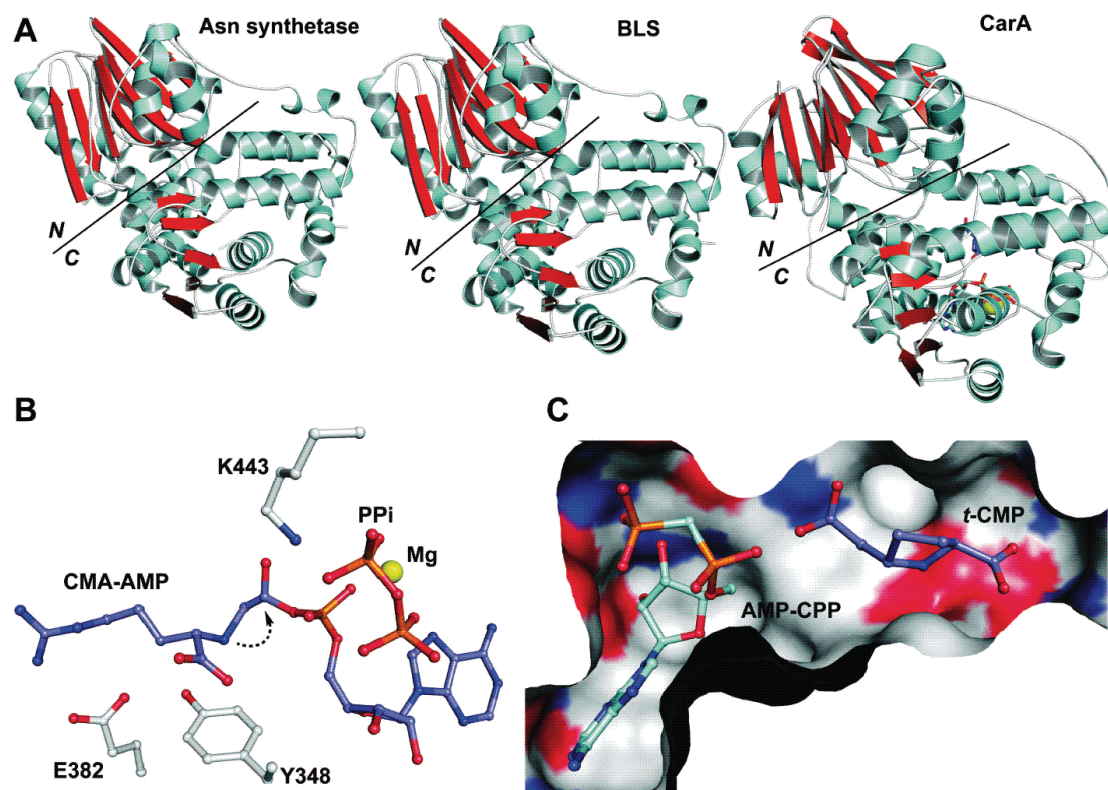


Figure 1.18. Crystal structures of β -lactam synthetases. A. Views from the structures of the β -lactam synthetases CarA and β -lactam synthetase (PDB 1Q19 and 1MB9, respectively) in comparison to that of asparagine synthetase (PDB 1CT9)¹⁶⁵ showing the *N*- and *C*-terminal domains; B. View from a crystal structure of β -lactam synthetase (PDB 1MBZ) showing the acyl-adenylate N^2 -(2-carboxymethyl)arginine-AMP trapped species generated by the reaction ATP with the substrate analogue N^2 -(2-carboxymethyl)arginine.¹⁶⁴ The latter is one carbon shorter than the natural substrate; thus, N^2 -(2-carboxymethyl)arginine-AMP does not undergo cyclisation to give the highly strained 3-membered ring. C. View from a crystal structure of CarA (PDB 1Q19)¹³⁹ in complex with the substrate (2*S*,5*S*)-5-carboxymethylproline (*t*-CMP) and an ATP analogue, α,β -methyleneadenosine-5'-triphosphate (AMP-CPP) with *t*-CMP positioned in an, apparently, "productive" conformation for adenylation and subsequent β -lactam formation. Figures from reference 154.

Crystallographic analyses of asparagine synthetase type A¹⁶⁵ versus the β -lactam synthetases¹⁶⁶ reveal that the latter have maintained the characteristic two domain fold of the former: an *N*-terminal nucleophile glutaminase domain responsible for the production of ammonia, and a *C*-terminal synthetase domain which catalyses the formation of β -aspartyl-AMP to form asparagine. However, as β -lactam ring formation is an intramolecular process, the β -lactam synthetases do not require the release of

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ammonia from glutamine, rendering the glutaminase reaction of the *N*-terminal domain redundant. This is consistent with the results obtained from the replacement of the catalytically-important, nucleophilic Cys1 residue of asparagine synthetase A with Phe1 in β -lactam synthetase, together with the presence of nine additional *N*-terminal residues which occupy the corresponding glutamine binding pocket in asparagine synthetase A.¹⁶⁶ In CarA, the Cys1 of asparagine synthetase A is replaced by the nucleophilic Ser1 which occupies a similar position; however, other important residues for glutamine binding are missing, reflecting the lack of a glutamine binding pocket.¹³⁹ The *C*-terminal domain of all three enzymes the substrate carboxyl group is activated *via* ATP-mediated adenylation at a conserved active site, which is followed by nucleophilic-nitrogen attack (Figure 1.17).

Whilst both β -lactam synthetase and asparagine synthetase A crystallise as dimers, CarA crystallises as a tetramer.¹³⁹ For the three proteins the *N*-terminal domain consists of two antiparallel β -sheets that form a sandwich, flanked on each side by short α -helices; also, the *C*-terminal domain comprises anything from 11-14 α -helices surrounding a 5-stranded parallel β -sheet; and, finally, their respective active sites are located in a cleft in the *C*-terminal domain formed by four β -strands and five α -helices.¹⁶⁴

The mechanism of CarA

The mechanism of CarA involves the ordered binding of ATP/Mg^{II} and *t*-CMP to the apoenzyme. The reaction proceeds then *via* the formation of an acyl-adenylate intermediate, followed by intramolecular 4-*exo*-trig-cyclisation to give a β -lactam *via* a tetrahedral intermediate (Figure 1.19), with pyrophosphate being the final product to be

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released.^{139, 164, 166, 167} The substrate for CarA (and likely ThnM), *t*-CMP undergoes catalysed cyclisation to yield the (2*S*,5*S*)-carbapenam nucleus.^{139, 140, 161}

CarA has been shown to catalyse the ring closure of at least three of the four possible diastereomers of *t*-CMP¹⁶⁸ (to yield the corresponding carbapenam), *t*-carboxyethylproline (to the corresponding γ -lactam), and some α -amino-diacids (*e.g.* glutaric acid, α -aminoadipic acid, α -aminopimelic acid to the corresponding lactams).¹⁶⁷ Recently, the ability of CarA to form β -lactam bicyclic systems other than carbapenams from 6- and 7-membered ring analogues of *t*-CMP (*i.e.* (2*S*,6*S*)-6-carboxymethyl-pipecolic acid (*t*-CMPi), and (2*S*,7*S*)-7-carboxymethyl-azepane-2-carboxylic acid, respectively) has been demonstrated; CarA has also been demonstrated to exercise stereoselective bias; in the case of a 2:1 mixture of (7*R*)-7-methyl-*t*-CMPi and (7*S*)-7-methyl-*t*-CMPi, where selective conversion of the former to give (4*S*,6*S*,7*R*)-7-methyl-carbacepham was observed (Figure 1.16).¹⁶⁰

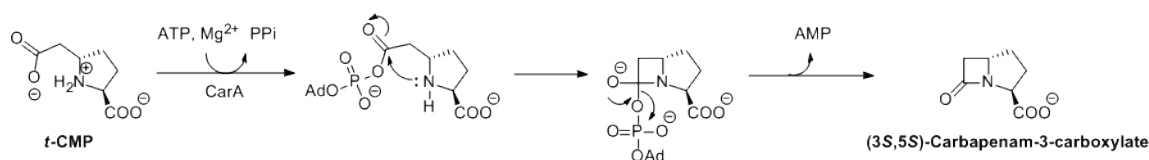


Figure 1.19. The proposed mechanism for CarA (involving intramolecular nucleophilic attack).¹⁶⁵

The close structural and mechanistic relation between asparagine synthetase A, β -lactam synthetase and CarA/ThnM, coupled to their different substrates specificities, suggests that it may have been relatively for the later three to have evolved initially from the structural platform of asparagine synthetase A; whether they evolved separately from asparagine synthetase A or from one another is unclear yet.

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Carbapenem synthase (CarC)

The 2-oxoglutarate-dependent (2OG) oxygenase CarC catalyses the C-5 epimerisation of (2*S*,5*S*)- to (2*S*,5*R*)-carbapenam, followed by desaturation between C-2 and C-3 of the latter to give carbapen-2-em-3-carboxylate (Figure 1.11).^{130, 141} While the CarC-catalysed desaturation has precedent in reactions catalysed by other 2OG oxygenases, including clavaminic acid synthase (in clavulanic acid biosynthesis)^{169, 170} and enzymes in flavonoid biosynthesis,¹⁷¹ its catalysed epimerisation activity is unique and provides an additional example of how structural diversity can be achieved *via* epimerisation.¹⁴⁰

CarC has been reported to accept all four possible isomers of its carbapenam substrate.¹⁶⁸ The relaxed substrate specificities of CarA and CarC, coupled to the tight substrate specificity of CarB by accepting only L-GHP and not D-GHP, imply that it is CarB which controls the stereochemical course of carbapen-2-em-3-carboxylate biosynthetic pathway.

CarC structure and mechanism

Structurally, CarC is closely similar to other 2OG oxygenases; monomeric CarC contains the distorted double stranded β -helix core that supports HXD/E...H motif that binds Fe^{II} to which 2OG coordinates in a bidentate manner (Figure 1.20 B).¹⁴¹ The crystal structure of CarC in complex with *N*-acetyl-proline (as an analogue to (3*S*,5*S*)-carbapenam) have provided an insight into its substrate binding (Figure 1.20 A). Two orientations of (3*S*,5*S*)-carbapenam in the active site of CarC are proposed: orientation **I** positions C-3 and C-2 close to the iron centre with C-5 exposed to the solvent medium (Figure 1.20 B); and, orientation **II** which positions C-5 close to Fe^{2+} .¹⁴¹

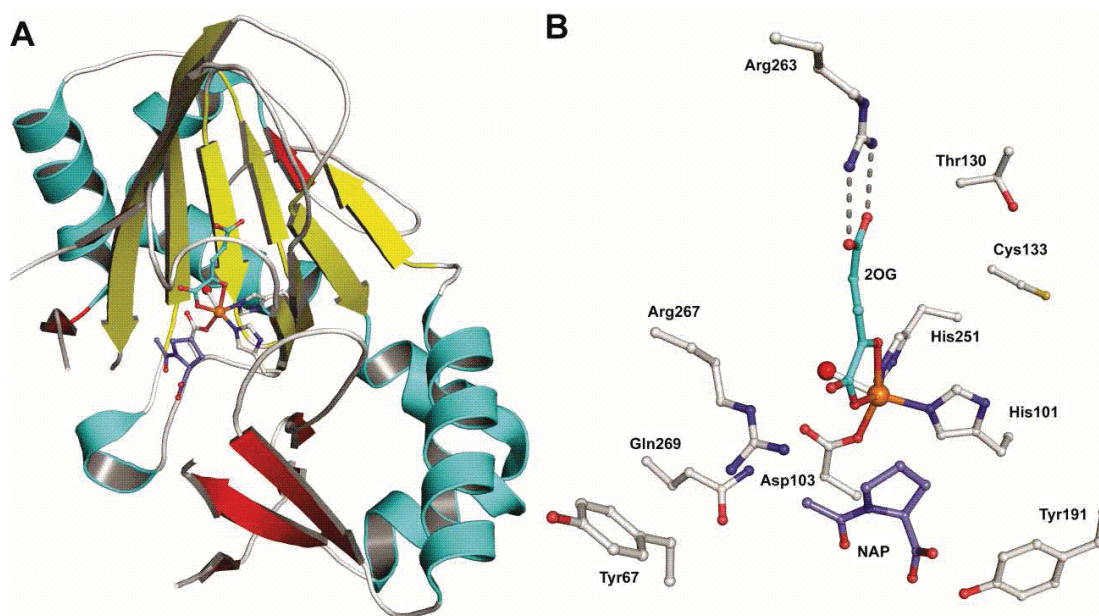


Figure 1.20. Structural views of the carbapenem synthase, CarC.¹⁴¹ A. A CarC monomer (PDB 1NX8) displaying the conserved double stranded β -helix core (yellow strand) that supports ligands bound to a single Fe^{2+} to which 2OG complexes in a bidentate manner. B. The active site of CarC (monomer B) showing the binding sites for iron, the prime substrate analogue (*N*-acetyl-L-proline) and 2OG. Figures from reference ¹⁵⁴.

All the mechanisms that have been proposed for the catalytic activity of CarC involve a radical intermediate (Figure 1.21). Labelling studies have shown loss of hydrogen at C-5 during the reaction.¹³⁰ *Ab initio* calculations on the radicals derived by hydrogen abstraction from C-1, C-2, C-3, C-5 and C-6 of (3*S*,5*S*)-carbapenam reveal that the C-3 radical is the lowest in energy.¹⁷² Loss of hydrogen from C-3 is the most likely scenario in orientation **I**; C-5 epimerization could occur through β -scission of the radical at C-3 to eventually give (5*R*)-carbapenam-3-carboxylate (Figure 1.21). However, in the cases of the radicals at C-3, C-1, C-2 and C-6, the large barrier associated with ring opening and the unfavourable entropy ring closure imply that significant enzyme catalysis would be required. On the other hand, in the case of the radical at C-5 (orientation **II**), epimerisation on it is predicted to be exothermic, and in agreement with labelling studies.¹³⁰ If a ferryl intermediate was to both abstract the C-5 hydrogen and directly re-hydrogenate the same position, re-orientation of the substrate

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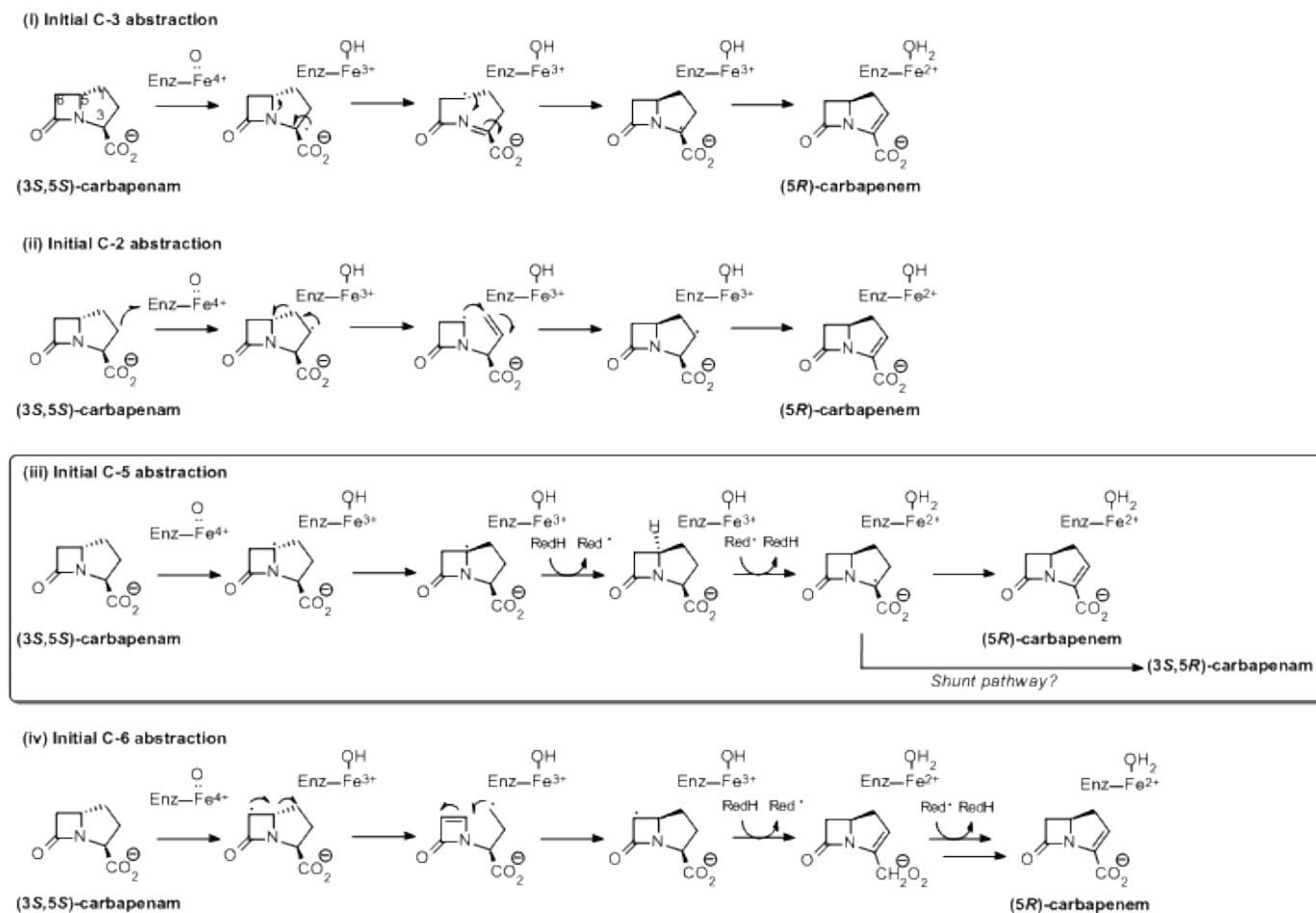


Figure 1.21. Proposed possibilities for the CarC-catalysed epimerisation/desaturation process.¹⁴¹ The 5-*endo*-trig (or 4-*exo*-trig) radical cyclisation in (i) and (ii) have synthetic precedent,¹⁷⁶ including with an amine substrate.¹⁷⁷ Note the possible intermediation of datively-stabilised radicals. Epimerisation *via* hydrogen abstraction at C-1 (as is initial electron loss from nitrogen) in a process analogous to (i) is also a possibility, but modelling studies suggest this is less likely. The mechanism proceeding *via* initial C-5 abstraction (iii) is considered the most likely.

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would be required; conformational changes upon substrate binding have been observed for 2OG oxygenases.^{173, 174} Modelling studies also suggest that (3*S*,5*R*)-carbapenam could be accommodated in the active site,^{172, 175} but there is no clear driving force for such a conformational change in case of CarC catalysis.

Biosynthesis of thienamycin.

The first labelling studies^{74, 122, 124} conducted on cultures of *S. cattleya*, and the isolation of many C-2 and C-6 functionalised carbapenems opened the path for proposals on the nature of the biosynthetic route of thienamycin. The identification of the thienamycin biosynthesis cluster,¹⁷⁸ as well as the sequencing of the genome of *S. cattleya*,¹⁷⁹ have allowed research to take a more delineated genetic and biochemical approach.

The thienamycin gene cluster in S. cattleya

The identified gene cluster comprises more than twenty genes (Figure 1.22 and Table 1.2). Analysis of the cluster reveals homology between some of these and a number of the genes in the cluster for carbapen-2-em-3-carboxylate in *P. carotovorum*: *thnE* and *thnM* have been identified as homologues of *carB* and *carA*, respectively; though no corresponding homologue has been identified for *carC*.¹⁷⁸ ThnE catalyses the same reaction as CarB (*i.e.* the formation of *t*-CMP from L-GHP and malonyl-CoA)^{144, 161} and ThnM catalyses the ATP-dependant β -lactam ring formation, just as CarA does.¹⁶¹ The products of *thnG* and *thnQ*, encoding for 2OG oxygenases, were anticipated to be involved in C-5 epimerisation and/or desaturation between C-2 and C-3, similarly to CarC in carbapen-2-em-3-carboxylate. However, recent reports have revealed that neither ThnG nor ThnQ are able to catalyse the reactions that CarC does;

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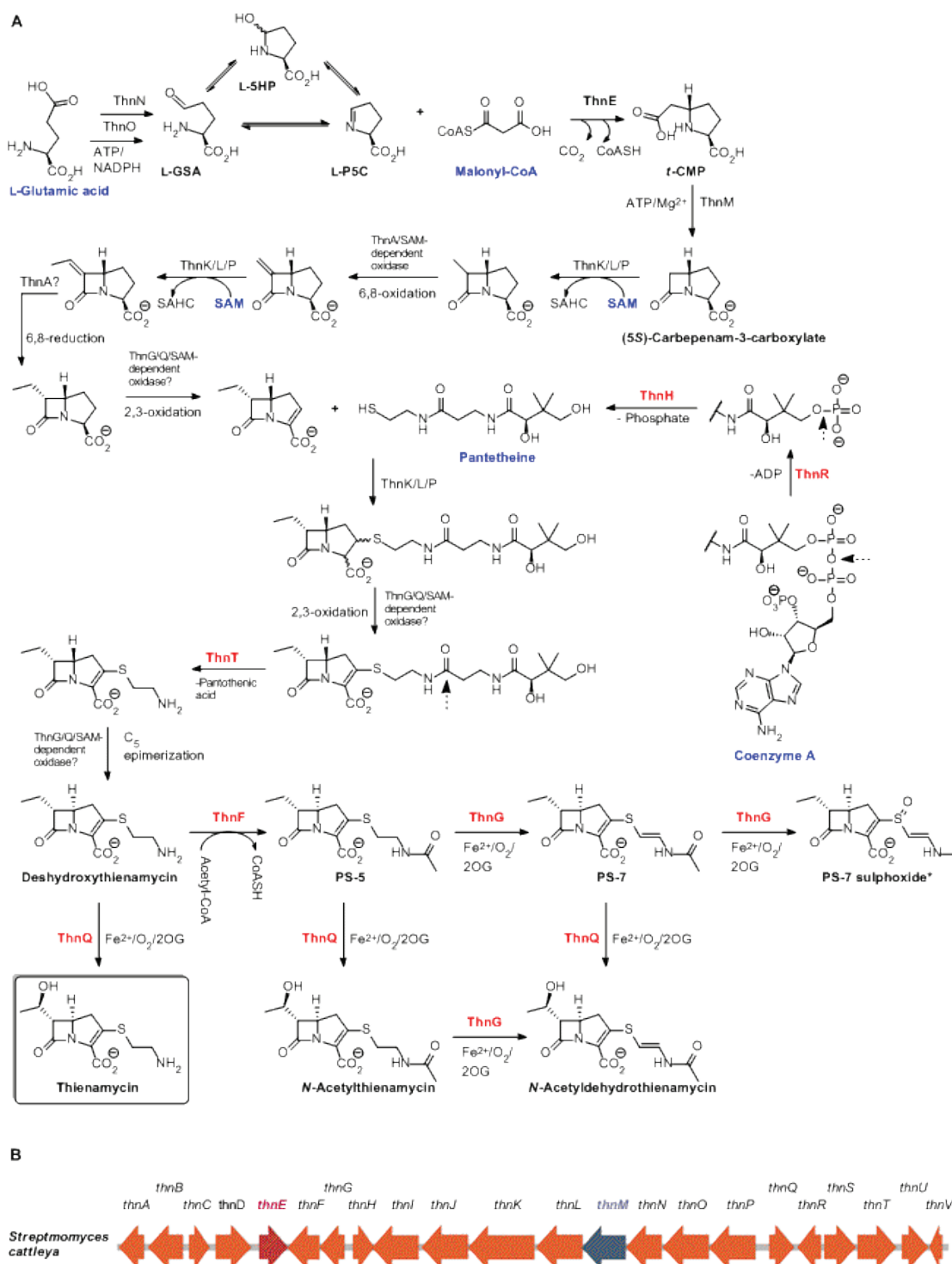


Figure 1.22. Proposed biosynthetic pathway leading to thienamycin (A) and the reported thienamycin gene cluster in *Streptomyces cattleya* (B). SAHC: S-adenosylhomocysteine. The enzymes catalysing the experimentally-reported steps are in red. The precursors for the skeleton of thienamycin are in blue. *PS-7 sulphoxide was detected only in *in vitro* assays with ThnG. See Table for (proposed) roles of the proteins.

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instead they have been showed to be involved in oxidising the C-2 and C-6 side chains of thienamycin, respectively.¹⁸⁰

The transcriptional activator ThnI is essential for thienamycin biosynthesis and regulates the expression of nine genes in the cluster (*thnH*, *thnJ*, *thnK-thnQ*).¹⁸¹ ThnU activates cephamycin C biosynthesis genes which are not part of the thienamycin gene cluster, raising the question as to the exact physical boundaries of the thienamycin gene cluster.¹⁸¹

Table 1.2. The genes constituting the reported thienamycin gene cluster and the (predicted) roles of the (putative) proteins that they encode for. The predicted number of amino acid residues (AA) for each of the proteins is shown. Proteins with assigned biochemical function are in bold.

Gene	AA	(Proposed) Function of encoded protein
<i>thnA</i>	259	Oxidoreductase, similar to 3-oxoacyl-[acyl-carrier-protein] reductase
<i>thnB</i>	340	Lactone-dependent transcriptional regulator
<i>thnC</i>	209	Lactone-efflux transmembrane protein
<i>thnD</i>	363	Alcohol dehydrogenase
<i>thnE</i>	294	ThnE (crotonase); <i>t</i>-CMP formation
<i>thnF</i>	327	ThnF (N-acetyltransferase); N-acetylthienamycin formation
<i>thnG</i>	263	ThnG (2OG oxygenase); oxidation of C-2 side chain
<i>thnH</i>	224	ThnH; cleavage of 4-phosphopantetheine to produce pantetheine
<i>thnI</i>	476	Transcriptional activator, essential for thienamycin biosynthesis
<i>thnJ</i>	483	Transport protein involved in thienamycin secretion
<i>thnK</i>	681	Methyltransferase
<i>thnL</i>	474	Methyltransferase
<i>thnM</i>	458	ThnM; β-lactam synthetase
<i>thnN</i>	367	Carboxylate reductase component (similar to <i>griC</i>)
<i>thnO</i>	472	Carboxylate reductase component (similar to <i>griD</i>)
<i>thnP</i>	484	Methyltransferase
<i>thnQ</i>	259	ThnQ (2OG oxygenase); hydroxylation of C-6 side chain
<i>thnR</i>	240	ThnR; cleavage of CoASH to produce 4-phosphopantetheine
<i>thnS</i>	329	β -Lactamase; probably involved in resistance mechanisms
<i>thnT</i>	399	ThnT; pantetheine hydrolase
<i>thnU</i>	268	Transcriptional activator for cephamycin C biosynthesis gene cluster
<i>thnV</i>	137	Cysteine transferase

Assembly of the bicyclic core of thienamycin (ThnN, ThnO, ThnE, ThnM)

ThnN and ThnO show homology to GriC and GriD (Figure 1.23) from the grinoxone biosynthesis pathway in *Streptomyces griseus*;¹⁸² ThnN shows 33% identity

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and 49% similarity to GriC and ThnO shows 29% identity and 39% similarity to GriD. GriC and GriD have been proposed to reduce 3-amino-4-hydroxybenzoic acid into 3-amino-4-hydroxybenzaldehyde; GriC shows homology to AMP-binding proteins and GriD shows homology to NAD(P)⁺-dependent aldehyde dehydrogenases, and, together, both enzymes constitute an ATP- and NAD(P)⁺-dependent carboxylic acid reductase system. Therefore, both ThnN and ThnO are likely to be involved in catalyzing the reduction of glutamic acid (or a derivative of) to L-GHP to provide the pyrroline-3-carboxylate part of thienamycin. The β -lactam protons originate from malonyl-CoA (or a derivative of); thus, ThnE can catalyse the conversion of L-GHP and malonyl-CoA into *t*-CMP,^{144, 161} which is a substrate for the ATP-dependent synthetase ThnM to produce (3*S*,5*S*)-carbapenem (Figure 1.22 A).¹⁶¹ As mentioned earlier, it is reported that the two 2OG oxygenases identified in the thienamycin gene cluster, ThnG and ThnQ, cannot convert (3*S*,5*S*)-carbapenam to carbapen-2-em-3-carboxylate;¹⁸⁰ therefore the nature of the epimerization of C-5 and desaturation between C-2 and C-3 is still unclear.

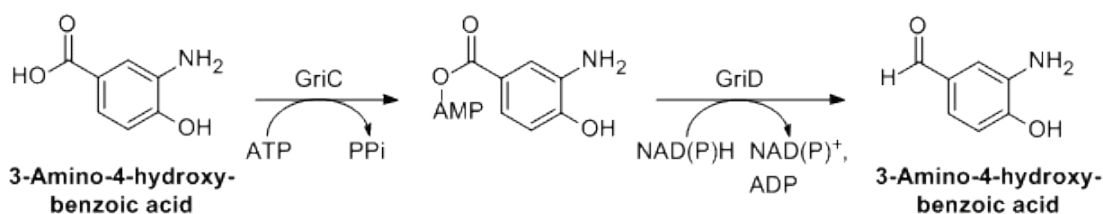


Figure 1.23. The possible role of the reductase GriC/GriD in the biosynthesis of grinoxone on *Streptomyces griseus*.¹⁸²

The C-6 hydroxyethyl side chain (putative roles of ThnL, ThnK, and ThnP)

The earliest labelling studies on thienamycin suggest that the two carbons of the C-6 hydroxyethyl side chain are introduced through separate methyl transfers from methionine (Figure 1.10).^{74, 122, 124} It has been proposed that at least two of the three putative radical *S*-adenosylmethionine-dependent superfamily enzymes (*i.e.* ThnK,

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ThnL and ThnP) are involved in the methyl-transfer catalysis to afford the C-6 ethyl side chain in thienamycin.^{148, 178} Since *S*-adenosylmethionine-dependent enzymes have been demonstrated to catalyse a remarkably diverse set of reactions, employing the oxidizing power of the generated 5'-deoxyadenosyl radical, it will not be surprising that ThnK, ThnL or ThnP may catalyse reactions beyond the predicted methyl-transfer in thienamycin biosynthesis, such as the C-5 epimerisation or the desaturation between C-2 and C-3, as well as the ligation of the cysteaminy side chain to C-2. Insertional inactivation experiments on *thnL* and *thnP* resulted in thienamycin non-producing strains; in the specific case of *thnP* the accumulation of a compound with a mass corresponding to that of carbapenam-3-carboxylate.¹⁴⁸ These results, if confirmed by the characterization of the accumulated metabolite, suggest the intermediation of carbapenam-3-carboxylate in thienamycin biosynthesis, as in the case of carbapen-2-em-3-carboxylate, and also suggests that ThnP catalysis precedes that of ThnL.

The C-6 hydroxyethyl side chain; hydroxylation of the C-6 (m)ethyl side chain by ThnQ.

Experiments using recombinant ThnQ have been shown to catalyse the stereoselective hydroxylation on the C-6 ethyl side chain of the metabolites PS-7 and PS-5 to yield, *N*-acetyl-dehydro-thienamycin and *N*-acetyl-thienamycin, respectively (Figure 1.22).¹⁸⁰

The C-2 cysteaminy side chain (ThnR, ThnH, ThnT, ThnF and ThnG)

The side chain at C-2 of many carbapenems is generally a derivative of cysteamine or dehydrocysteamine, with the sulfur atom occasionally found as the sulfoxide. As noted in Figure 1.8, members of the pluracidomycin group have been found to have more oxidized or truncated C-2 substituents, while those of the OA-6129 subfamily are

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characterized by a pantetheinyl moiety at C-2. Several naturally occurring carbapenems contain a pantetheinyl side chain at C-2 (Figure 1.8), which have been proposed to be precursors to the analogous 2-cysteaminylcarbapenems.⁹⁹ The mutation of a strain of *S. fulvoviridis*, which mainly produces the *N*-acetyl-2-cysteaminylcarbapenems PS-5, and *epi*-thienamycins A, C and F has resulted in a strain that produces instead the 2-pantetheinylcarbapenems OA-6129A, B1, B2 and C (Figure 1.24 A);⁹⁹ the mutant strain also lacks the acylase activity which removes the *N*-pantothenyl side chain of OA-6129A to give NS-5 (Figure 1.24 B).⁹⁹ A933 acylase, the acylase in *S. fulvoviridis* involved in the exchange of the pantothenyl moiety of AO-6129 carbapenems with acetyl-CoA, has been partly purified and characterised.^{183, 184} Other activities characterised for A933 acylase include the depantothenylation of OA-6129A to NS-5 (Figure 1.24 B);⁹⁹ the acylation of 2-cysteaminylcarbapenems and 6-aminopenicillanic with acyl-CoA;¹⁸³ and the deacylation of *N*-acetyl-L-amino acids, but not that of *N*-acetyl-D-amino acids,¹⁸⁴ although, the enzyme is unable to catalyse the deacylation of PS-5 to NS-5.¹⁸³ Similar acylase activity has been detected in *S. cattleya*, though this acylase has been reported as labile and not amenable to purification, but with a similar pattern of specificity to that of *S. fulvoviridis*.¹⁸³

The enzymology involved in the biosynthesis of the C-2 side chain has been further explored with the concerted roles of ThnR, ThnH and ThnT to incrementally cleave CoA to 4-phosphopantetheine.¹⁸⁵ ThnR, a CoA pyrophosphatase, shows homology to the Nudix hydrolase family.¹⁸⁶ ThnH, which hydrolyses the phosphate of 4-phosphopantetheine, shows weak homology to the haloacid dehalogenase superfamily of hydrolases.¹⁸⁷ ThnT, an Ntn-related enzyme,¹⁸⁸ catalyses the depantothenylation of both *cis*- and *trans*-2-pantetheinylcarbapenams into the 2-cysteaminyl analogues¹⁸⁵

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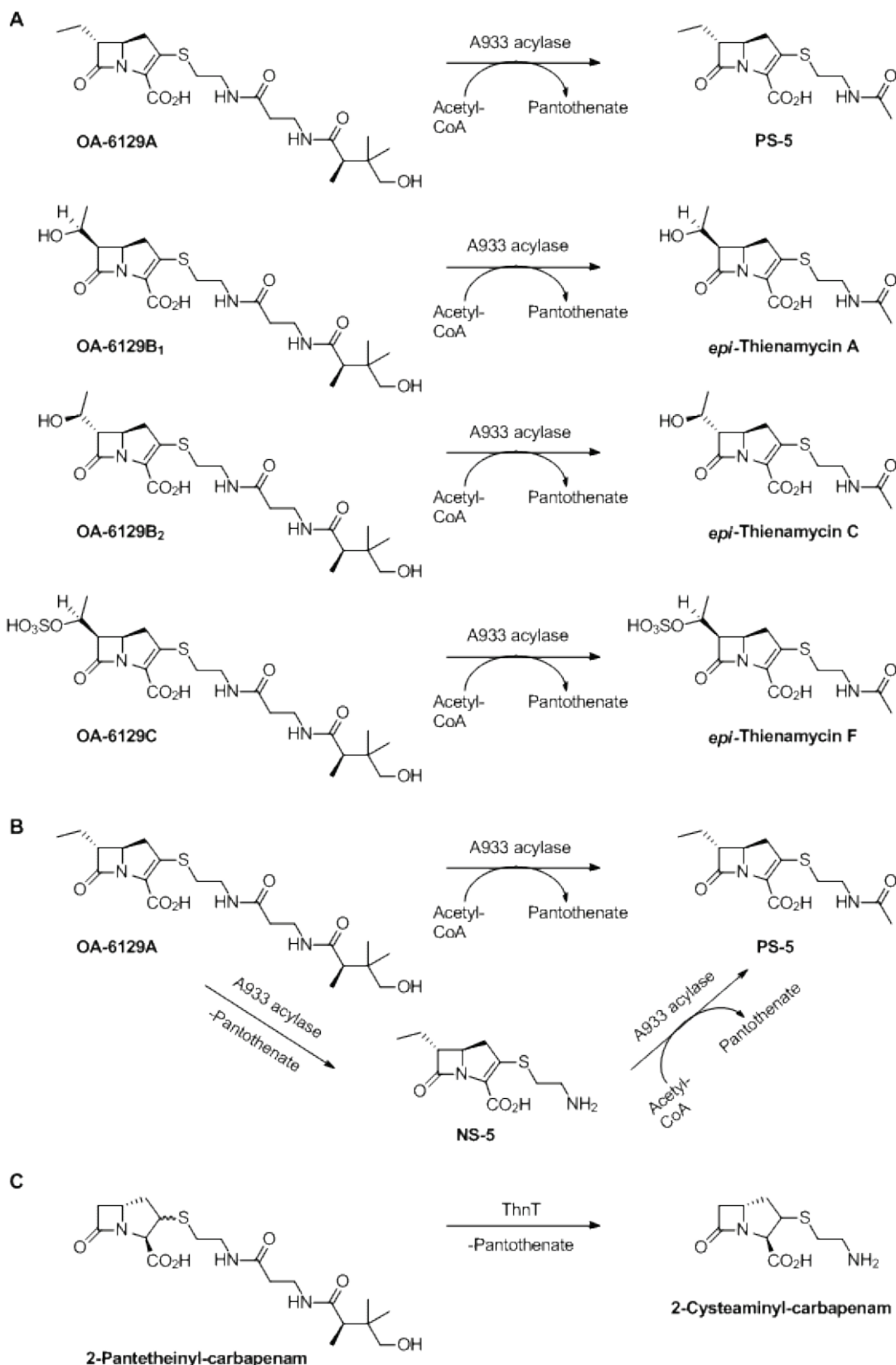


Figure 1.24. Transacylase catalysed reactions with substituted carbapenems. A and B. A933 (*Streptomyces fulvoviridis*) catalysed reactions.^{99, 183, 184} C. ThnT (*S. cattleya*) catalysed reaction.¹⁸⁵

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(Figure 1.24 C), suggesting that ThnT might be the homologue of the A933 acylase. Recently, insertional-inactivation experiments on *thnR* and *thnT*, yielded strain mutants capable of thienamycin production, which would put in doubt their proposed roles in the biosynthesis of thienamycin.¹⁴⁸ Furthermore, the mechanism and timing of ligation of CoA (or a derivative of) into the bicyclic carbapenam(em) remain to be determined.

Radio labelling studies on resting cells of *S. cattleya* resulted in high levels of incorporation of radiolabelled cysteine into the C-2 cysteaminy side chain of thienamycin; in contrast, [³⁵S]-cystamine and [³⁵S]-pantethine are poorly incorporated,¹²² implying that they are not direct precursors of the cysteaminy side chain. Feeding a mixture of [3,3'-³H₂]-cystine and [³⁵S]-cystine to *S. cattleya* resulted in the production of thienamycin with the same ³H:³⁵S ratio as the starting mixture (Figure 1.10),¹²² consistent with the proposal that dehydrothienamycin is not an intermediate in thienamycin biosynthesis. Additionally, radiolabelled β-alanine has been shown to be specifically incorporated into the C-2 pantetheiny side chain of the OA-6129 series compounds, but labelled pantothenate was not taken up.¹⁸⁴ On the basis of these findings and the preliminary biochemical studies on ThnR, ThnH and ThnT it would seem feasible that β-alanine is taken up converted first into (phospho)pantothenate, subsequently condensing with cysteine, finally decarboxylating to yield (phospho)pantetheine and, eventually, form coenzyme A.

The conversion of thienamycin to *N*-acetylthienamycin has been predicted to be catalysed by ThnF, a putative acetyl-transferase. Showing weak homology to the GNAT superfamily, ThnF, has been shown to catalyse the *N*-acetylation of a model compound containing cysteamine in the presence of acetyl-CoA as a co-substrate (Figure 1.25).¹⁸⁵

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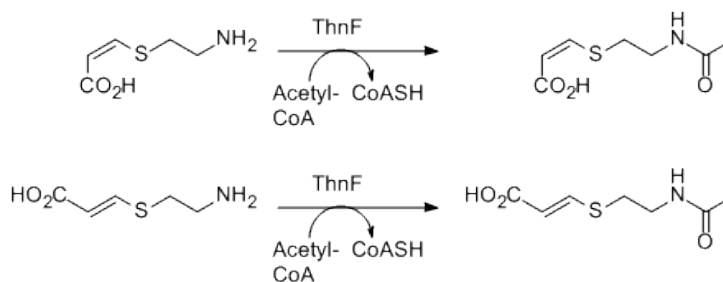


Figure 1.25. ThnF catalysed reaction with a substrate analogue.¹⁸⁵ Note that the putative substrate for ThnF is thienamycin.

The 2OG oxygenase ThnG catalyses the oxidation of the *N*-acetyl-2-cysteaminyl side chain of *N*-acetylthienamycin to produce *N*-acetyl-dehydrothienamycin (desaturation reaction); and, PS-5 to produce PS-7 and PS-7 sulfoxide (desaturation followed by sulfoxidation reaction, Figure 1.22 A).¹⁸⁰ Insertional inactivation of *thnG* resulted in a mutant strain that showed a 2.5 fold increase in thienamycin production, and accumulation of a metabolite with a mass corresponding to that of 2,3-dihydrothienamycin in the mutant strain.¹⁴⁸ Overall, these results indicate that ThnG is not essential for thienamycin biosynthesis, but contributes to the diversity found in the C-2 side chain.

Aims and Objectives.

The aims of the work of this thesis:

1. To carry out engineering studies on the carboxymethylproline synthases, CMPSs, CarB and ThnE with the aim of producing variants capable of controlling enolate formation and reactivity of the substrates *t*-carboxymethylproline, *t*-CMP (and *t*-carboxymethylpipecolic acid, *t*-CMPi), and (alkyl)malonyl-CoA;
2. To explore the possibility of coupling the biocatalytic promiscuity of the wild-type malonyl-CoA ligase MatB, and the diastereospecificity of the crotonyl-CoA

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carboxylase/reductase Ccr to the selective trisubstituted enolate forming capacity of engineered CMPS; as well as to demonstrate the effect of methylmalonyl-CoA epimerase, MCE, on the diastereoselectivity of the crotonase-catalysed enolated alkylation.

The objectives of the work described in this thesis were as follows:

1. To obtain functionalized *t*-CMP derivatives alkylated at C-2, C-3, C-4, C-5 or C-6, from appropriately substituted amino acid aldehydes and alkylmalonyl-CoA, in high diastereomeric excess;
2. To obtain bi-functionalized *t*-CMP derivatives methylated at C-4 and C-6, in high diastereomeric excess;
3. To couple the trisubstituted enolate- and C–C bond-forming capacities of engineered carboxymethylproline synthases CMPSs to the biocatalytic promiscuity of the malonyl-CoA synthetase MatB to enable the one-pot stereoselective preparation of 5- and 6-membered *N*-heterocycles functionalized with a range of alkyl-substituted carboxymethyl side chains, starting from alkyl-substituted malonic acids and aminoacid semialdehydes.

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Chapter 2. Engineering Studies on Carboxymethylproline Synthases towards a Stereoselective C-C Bond Formation through Altered Enolate Reactivity.

As described in the introduction, previous work in our group has shown that CarB/ThnE catalysis proceeds *via* decarboxylation of malonyl-CoA to give an enzyme bound enolate stabilized by an oxyanion hole, which reacts with the imine form of L-GHP (or a derivative of) in a C-C bond forming reaction, and demonstrated the substrate promiscuity of CarB/ThnE towards the acceptance of C-2-alkylmalonyl and amino acid aldehydes with different chain lengths.¹⁻⁴

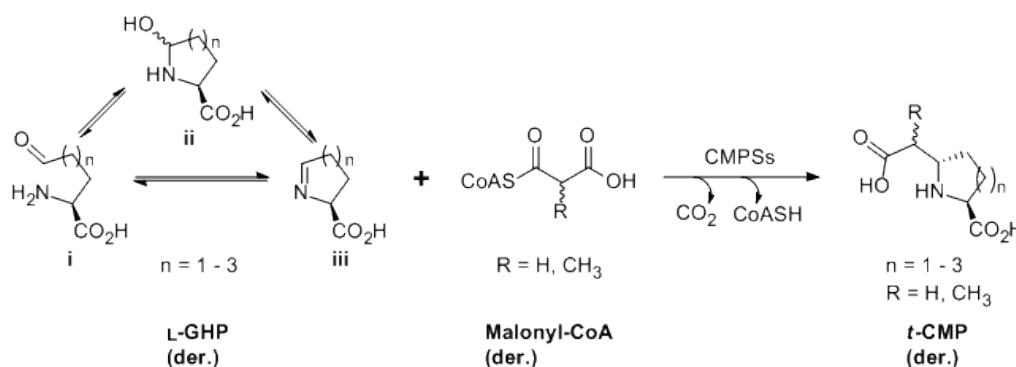


Figure 2.1. The reaction catalysed by carboxymethylproline synthases (CMPSs). CMPSs (CarB and ThnE) catalysis is proposed to proceed *via* decarboxylation of (alkyl)malonyl-CoA to yield an enolate; C-C bond formation is then proposed to proceed *via* reaction of the formed enolate with the imine form (iii) of L-GHP.

Nature employs a number of strategies to construct C-C bonds.^{5, 6} The nucleophilic attack of a resonance-stabilized carbanion onto an electron-deficient species is of relevance to CMPS catalysis; CMPSs, as members of the crotonase superfamily employ enolate intermediates in their mechanisms of action.⁷ Many of these reactions (that have precedent in the carbonyl chemistry of organic synthesis) feature the stabilization of the enolate intermediate *via* two backbone NH groups (in most, but not all, cases)

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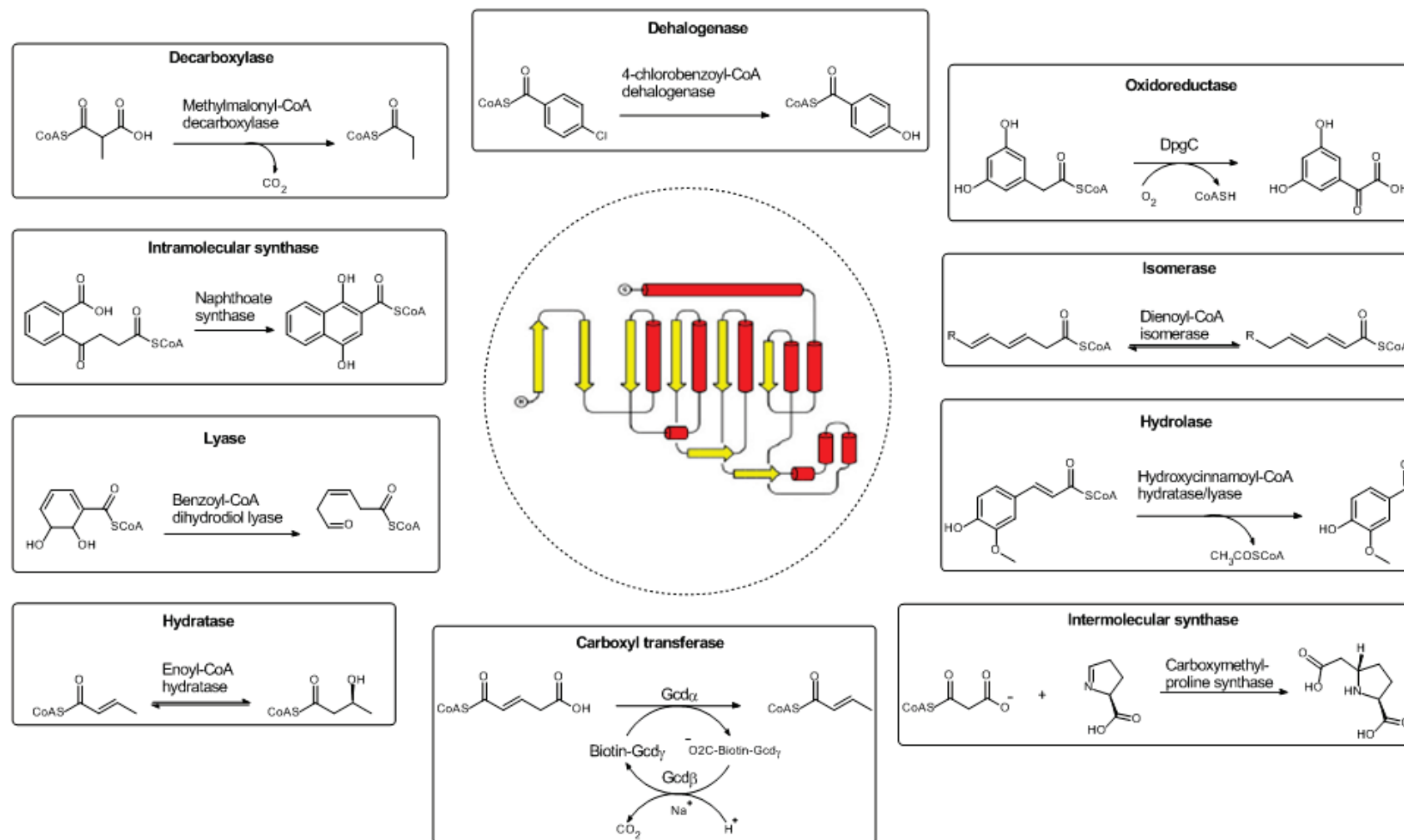


Figure 2.2. The crotonase superfamily of enzymes.⁷ Members of the crotonase superfamily are classified according to the type of reaction they catalyse. Note the similarity between the chemistry of the crotonase superfamily and that of synthetic “carbonyl” chemistry (modified from 7).

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analogous to, and therefore referred to as, the oxyanion hole of serine proteases.^{8,9} This feature in the crotonase superfamily demonstrates how a common polypeptide fold can be used as a scaffold to catalyse a wide variety of reactions.¹

In spite of its versatility, widespread occurrence and biological significance of crotonase superfamily enzymes, there have only been limited studies on the application of crotonases in biocatalysis,^{4, 10} or their engineering.^{11, 12} The alkylation of enolates is an important reaction in organic chemistry, yet it has been left largely unexplored in biocatalysis, particularly with respect to the crotonase superfamily (which operates mainly on mechanisms that proceed *via* an enolate intermediates). The exception is the use of catalytic antibodies that allow control of reactions with exquisite selectivity,¹³ and the engineering of various enzyme families, in particular aldolases, for stereoselective C-C bond formation reactions.¹⁴⁻¹⁶ However, it has not been possible as of now to design a platform to efficiently render the catalytic formation of C-C bonds of choice *via* the controlled formation of the (*E*)-or (*Z*)-enolates. Therefore, it was decided to carry out engineering studies on our available CMPSs (CarB and ThnE) with the aim of producing variants capable of controlling enolate formation and/or reactivity.

Selection of residues for substitution and screening of the generated variants for the production of 6-methyl-t-CMP epimers.

It is likely that a pre-requisite for the stereoselective formation of a C-C bond *via* an α -substituted enolate intermediate under kinetic control is the selective formation of (*E*)- or (*Z*)-enolates. The sequences of known and putative CMPSs (CarB from *P. carotovorum*, ThnE from *S. cattleya*, and CpmB from *Photorhabdus luminiscens*³) were

¹ For a comprehensive review on the crotonase superfamily, the reader is referred to: R. B. Hamed, E. T. Batchelar, I. J. Clifton and C. J. Schofield, *Cell. Mol. Life Sci.*, 2008, **65**, 2507-2527.

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examined in light of the reported crystal structure for CarB¹ (Figure 1.12) to identify candidate residues for substitution that may enable the stereoselective formation of (*E*)- or (*Z*)-enolates from C-2 epimeric methylmalonyl-CoA to allow its conversion to the (*6R*)- or (*6S*)-epimers of 6-methyl-*t*-CMP in the presence of L-GHP.

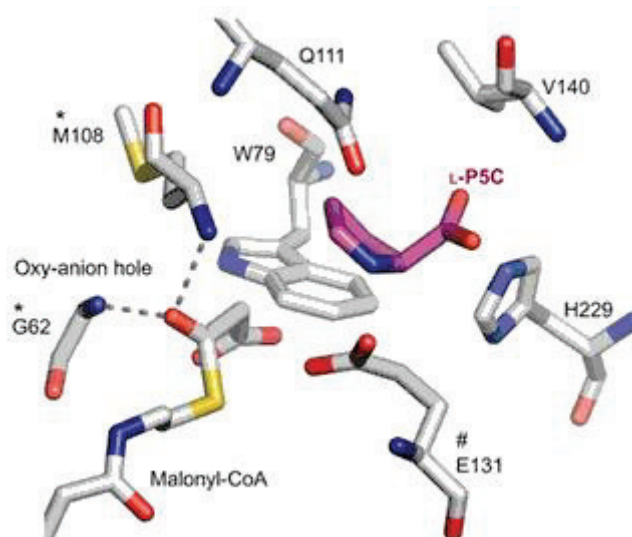


Figure 2.3. View from CarB crystal structure with malonyl-CoA and L-P5C modelled in the active site.¹ Note the orthogonal relationship between the terminal carboxylate of malonyl-CoA and the carbonyl stabilized by the oxyanion hole as prerequisite for the stereoelectronically favoured decarboxylation (figure prepared by Dr. R. Hamed and adapted from 17).

Although crystal structures of CarB/ThnE in complex with L-GHP are not available, knowledge that the enolate intermediate is probably bound to the oxyanion hole formed by the backbone amides of Met108_{CarB}/Val153_{ThnE} and Gly62_{CarB}/Gly107_{ThnE}^{1, 3} in a similar fashion to many members of the crotonase superfamily,⁷ enabled docking studies that identified residues likely to interact with the substrates. This analysis yielded three residues targeted for modification: the oxyanion hole-forming residue Met 108, substituted by a valine residue as observed for the analogous residue in ThnE, Val153 (Figure 1.12); and, Gln111 and Trp79 due to their proposed role in L-GHP binding and to form part of the hydrophobic face of the active site, respectively (Figure 2.3).

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The CarB and ThnE variants were prepared according to the QuickChange® Site-Directed Mutagenesis Protocol (Stratagene; for particulars, see Methods), from a *pET24a/carB* construct (CarB mutants) and a *pET24a/thnEV153V* construct (ThnE mutants; it was found that the silent mutation at residue Val153 of ThnE resulted in enhancement of the protein production levels in *E. coli*³) as templates. The CarB¹⁸ and ThnE³ variants were produced following the reported methodologies (See Methods for a detailed description). The variants produced for the work described onwards can be seen in Table 2.1 and Figure 2.4.

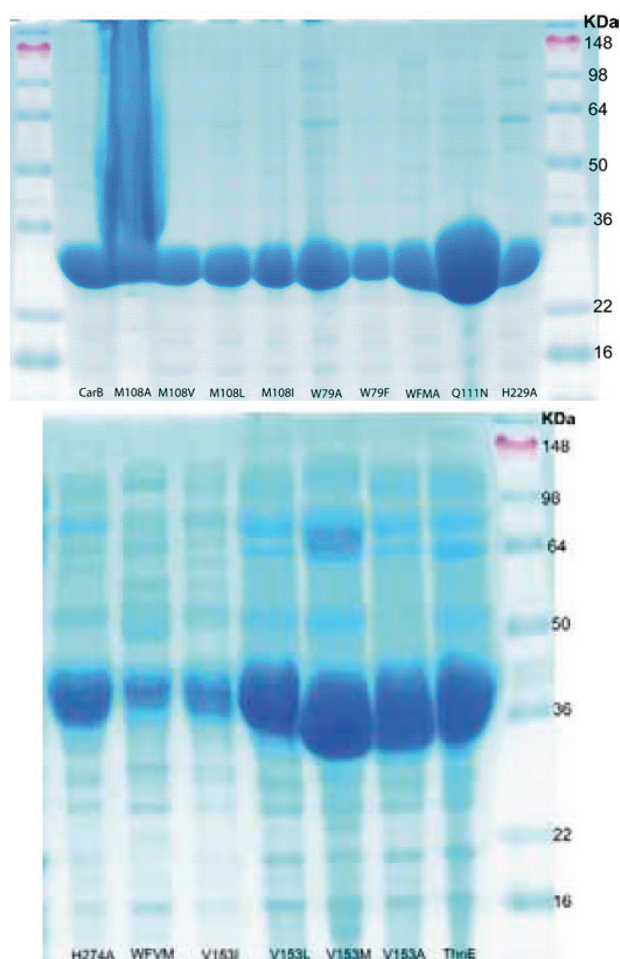


Figure 2.4. SDS-PAGE analysis of the purified CarB (above) and ThnE (below), wt and variants. The expected molecular weight of CarB is 27.58 KDa; that of ThnE is 31.18 KDa. The molecular weight markers (Invitrogen) are flanking on both sides.

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Table 2.1. The full list of CMPSs prepared and utilized along this study.

CarB (<i>P. carotovorum</i>)	ThnE (<i>S. cattleya</i>)
CarB wt.	ThnE wt
CarB M108V	ThnE V153M
CarB M108A	ThnE V153A
CarB M108L	ThnE V153L
CarB M108I	ThnE V153I
CarB W79F	ThnE W124F
CarB W79A	
CarB H 229A	ThnE H274A
CarB W79F M108V	ThnE W124F V153M
CarB W79F M108A	
CarB W79A M108V	
CarB Q111A	
CarB Q111N	
CarB Q111A M108V	
CarB Q111A M108I	
CarB Q111N M108V	
CarB Q111N M108VI	

The catalytic activity of the mutants was determined by the assay previously developed in our group and employing liquid chromatography coupled to mass spectrometry (LC-MS) using a PrimeSep 100® column (SiELC) with a reverse-phase gradient of acetonitrile in water and a quadrupole mass spectrometer in positive electrospray ionization mode, as detailed in Methods. Screening of CarB M108V, Q111A, and W79F for the formation of 6-methyl-*t*-CMP from L-GHP and (2*R/S*)-2-methylmalonyl-CoA revealed two products at $m/z = 188$ $[M+H]^+$, indicative of the formation of the C-6 epimers of 6-methyl-*t*-CMP in different ratios (Table 2.1) under standard incubation conditions. For each of these variants, the reactions were scaled up (see Methods), and the diastereomeric ratios (d.r) of the two epimers (Figure 2.5) and stereochemical assignments (Figure 2.7 and Figure 2.8) were determined by NMR analyses. The results demonstrate that substitutions of key residues in the active site can make a substantial difference to the stereochemical outcome of CMPS catalysis.

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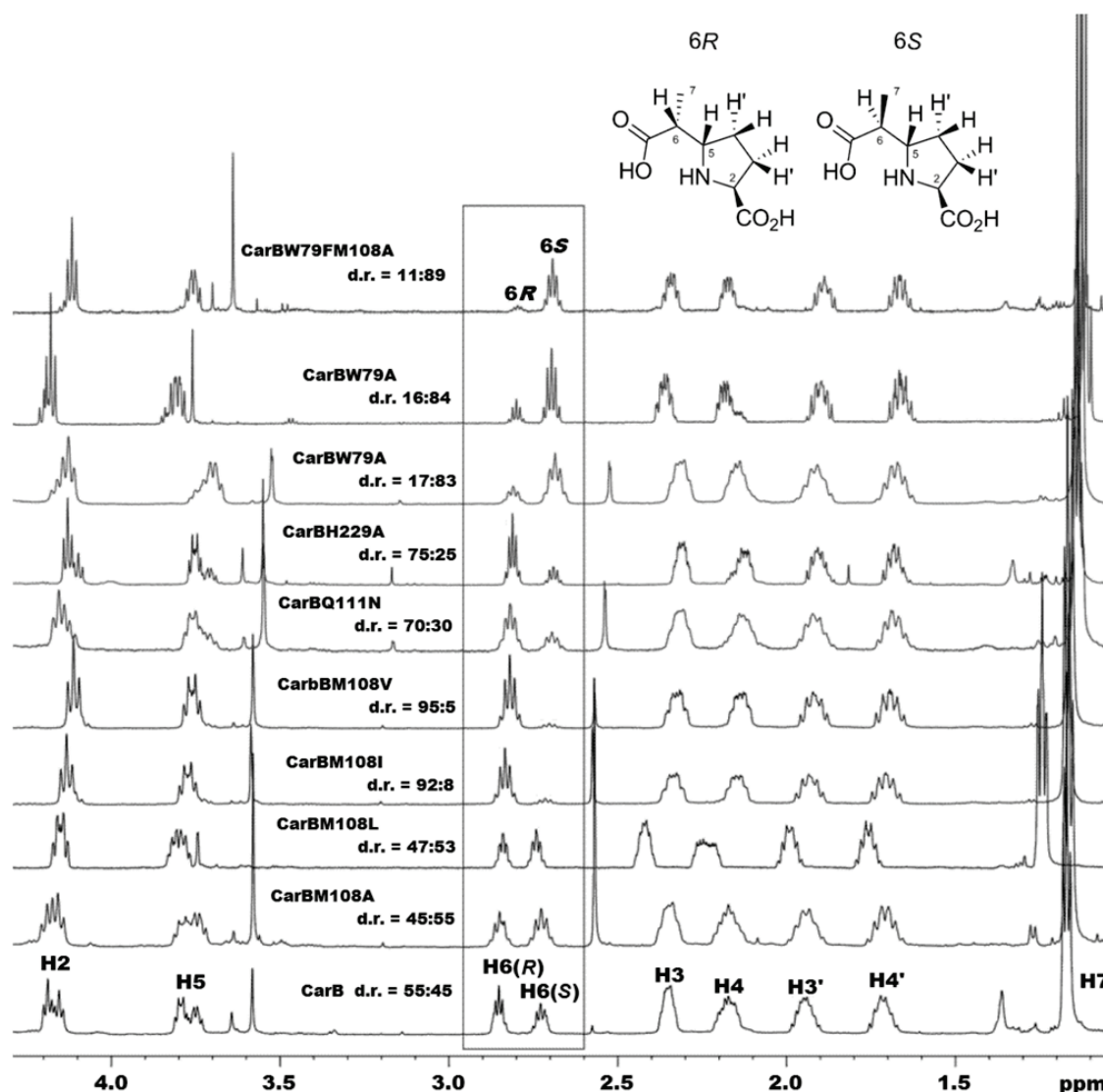


Figure 2.5. ^1H -NMR spectra for the two C-6 epimers of 6-methyl-*t*-CMPs produced by CarB and its variants. Standard incubation conditions from (2*R*/*S*)-2-methylmalonyl-CoA and L-GHP. For each variant the diastereomeric excess (d.e.) was calculated from the difference between the two H-6 integrals. NMR analysis performed by Dr. R. Hamed.

The results for the CarB M108V and CarB W79F variants were the most encouraging, because the d.r. values obtained (95:5 and 17:83, 6*R*:6*S*) were substantially different from those of wild-type CarB (55:45, 6*R*:6*S*) and pointed to the possibility of high stereoselective formation of either (6*R*)- or (6*S*)-6-methyl-*t*-CMP (*i.e.* (2*S*,5*S*,6*R*)- or (2*S*,5*S*,6*S*)-6-methyl-CMP).

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The initial results led to a second round of substitutions targeting residues Met108_{CarB}, and Trp79_{CarB}. The variants that resulted from this were: CarB M108I, CarB M108L, CarB M108A, CarB W79A, the double mutants CarB W79F/M108A, CarB W79F/M108V, as well as CarB H229A. All these variants formed the two C-6 epimers of 6-methyl-*t*-CMP at different ratios (Table 2.2 and Figure 2.5). The isolated yields of the C-6 epimers of 6-methyl-*t*-CMP were determined by a combination of LC-MS and NMR, as described in Methods (the same method was used subsequently to quantify all other substituted *N*-heterocycles produced by CMPS catalysis). Although some of the isolated yields were lower than those for wild-type CarB, in some cases (*e.g.* CarB W79F and CarB M108I) high stereoselectivity was accompanied by a comparable or higher yield of 6-methyl-*t*-CMP when compared to the wild-type enzymes. These results are indicative that engineering of the active site of CarB can substantially vary the d.r. of the product from the 55:45 (6*R*:6*S*) of the wild-type enzyme towards a diastereomeric excess of either (2*S*,5*S*,6*R*)- 6-methyl-CMP (*e.g.* CarB M108V and CarB M108I), or (2*S*,5*S*,6*S*)-6-methyl-CMP (*e.g.* CarB M108A/W79F).

Table 2.2. The results for 6-methyl-*t*-CMP catalysed by CarB and variants. CarB M108V and CarB M108I showed the best diastereoselectivity and yield towards the (*R*)-epimer.

Catalyst	Ratio (<i>R:S</i>)	% Yield
CarB wt	55:45	34
CarB M108V	95:5	14
CarB M108A	45:55	43
CarB M108L	47:53	49
CarB M108I	92:8	24
CarB W79F	17:83	55
CarB W79A	16:84	34
CarB H 229A	75:25	51
CarB W79F M108V	56:44	26
CarB W79F M108A	11:89	19

Substitutions on wild-type ThnE were then planned and carried out (Table 2.3). The work yielded the following variants and ratios (6*R*:6*S*) of 6-methyl-*t*-CMP: ThnE V153I

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(78:22), ThnE V153M (13:87), ThnE V153L (10:90), ThnE V153A (9:91), ThnE W124F (55:45) and ThnE V153M/W79F (13:87) (Figure 2.6). These results demonstrate that ThnE is also amenable to residue manipulation to alter stereoselectivity, in a similar fashion to CarB W79F and M108I. The yield of the stereoselective variant ThnE V153M (Table 2.3) was ~12 times higher than that of the wild-type enzyme, revealing that substitution can improve yield as well.

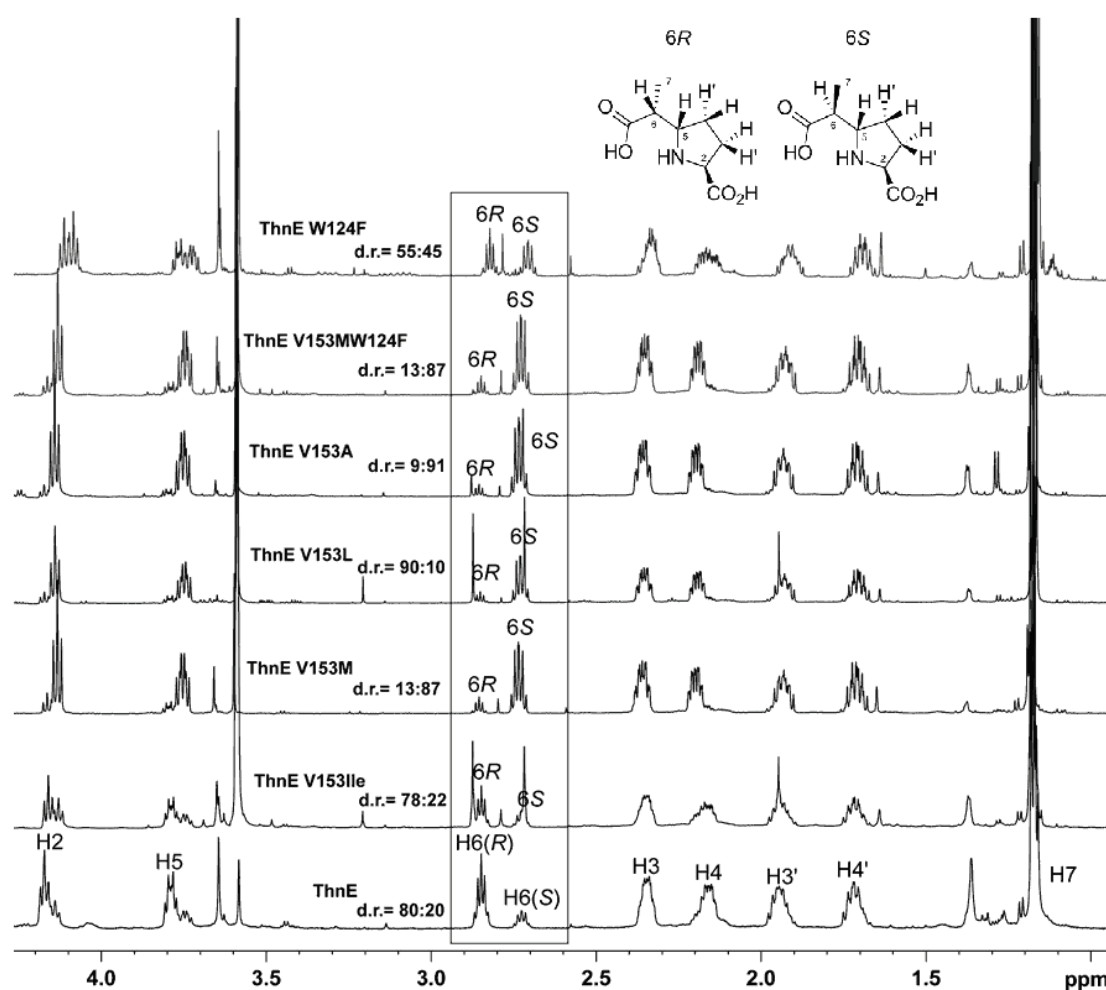


Figure 2.6. ¹H-NMR spectra for the two C-6 epimers of 6-methyl-t-CMPs produced by ThnE and its variants. Standard incubation conditions from (2*R*/*S*)-2-methylmalonyl-CoA and L-GHP. For each variant the diastereomeric excess (d.e.) was calculated from the difference between the two H-6 integrals. NMR analysis performed by Dr. R. Hamed.

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Table 2.3. The results for 6-methyl-*t*-CMP catalysed by ThnE and variants. ThnE V153A and ThnE V153L showed the best diastereoselectivity and yield towards the (*S*)-epimer, as opposed to the results of the CarB mutants.

Catalyst	Ratio (<i>R:S</i>)	% Yield
ThnE wt	80:20	4
ThnE V153M	13:87	13
ThnE V153A	9:91	25
ThnE V153L	10:90	39
ThnE V153I	78:22	8
ThnE W124F	55:45	3
ThnE W124F V153M	13:87	13

Confirming the stereochemical assignment of the two C-6 epimers of 6-methyl-*t*-CMP.

In order to assign the stereochemistry of the two epimers of 6-methyl-*t*-CMP confidently, purified samples of each epimer, as produced by the two optimal stereoselective CMPSs (*i.e.*, CarB M108V and ThnE V153A for (6*R*)-, and (6*S*)-6-methyl-*t*-CMP, respectively) were prepared for NMR analysis, employing a two-step HPLC procedure (see Methods). The purified epimers (Figure 2.7) were then subjected to 2D NOESY analysis (Figure 2.8).

The stereochemistry at C-6 of the major epimer produced by ThnE V153A catalysis was assigned as (*S*) based on the value of 8.1 Hz for $J_{5,6}$ (predicted average $\Phi \sim 150^\circ$), together with a moderate NOE correlation observed between H-5 and H-6, that indicate a neat *anti* conformation for these two protons; and, the observation of an NOE correlation between H-6 and H-4', together with the observation of NOE correlations between the methyl group at C-6 and both C-4 protons (H-4 > H-4'; Figure 2.8 A).

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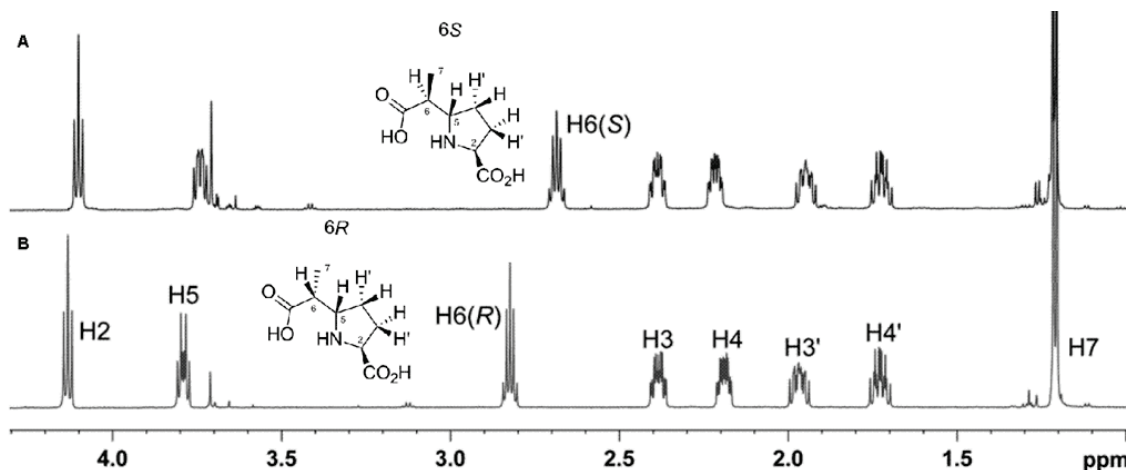


Figure 2.7. ¹H-NMR spectra for the purified (6S)- (A) and (6R)-6-methyl-*t*-CMP (B) from CarB M108V and ThnE V153A respectively. NMR analysis performed by Dr. R. Hamed.

The stereochemistry at C-6 of the major epimer produced by CarB M108V catalysis was assigned as (*R*) based on the value of 6.8 Hz for $J_{5,6}$ (predicted average $\Phi \sim 135^\circ$), together with a strong NOE correlation observed between H-5 and H-6, that indicate a predominantly *gauche* conformation for these two protons; and, the observation of an NOE correlation between the methyl group at C-6 and both C-4 protons ($H-4 < H-4'$), as well as the weak NOE observed between H-6 and H-4', together with weak NOE correlation observed between the methyl group at C-6 and H-2 (Figure 2.8 B).

Screening variants for the production of 6-ethyl-*t*-CMP epimers.

The results for the formation of 6-methyl-*t*-CMP encouraged us to investigate whether the CMPS variants in hand could catalyse the stereoselective formation of trisubstituted enolates with an ethyl group by testing the reaction of L-GHP with (2*R*/2*S*)-2-ethylmalonyl-CoA³ (Figure 2.9). Previous work in our group had already pointed to the capacity of wild-type CarB, but not ThnE, to accept 2-ethylmalonyl-CoA as a substrate for the formation of the two C-6 epimers of 6-ethyl-*t*-CMP.³ Unfortunately, none of the ThnE variants in hand were able to produce isolable quantities of 6-ethyl-*t*-CMP, though some of the CarB variants catalysed the production

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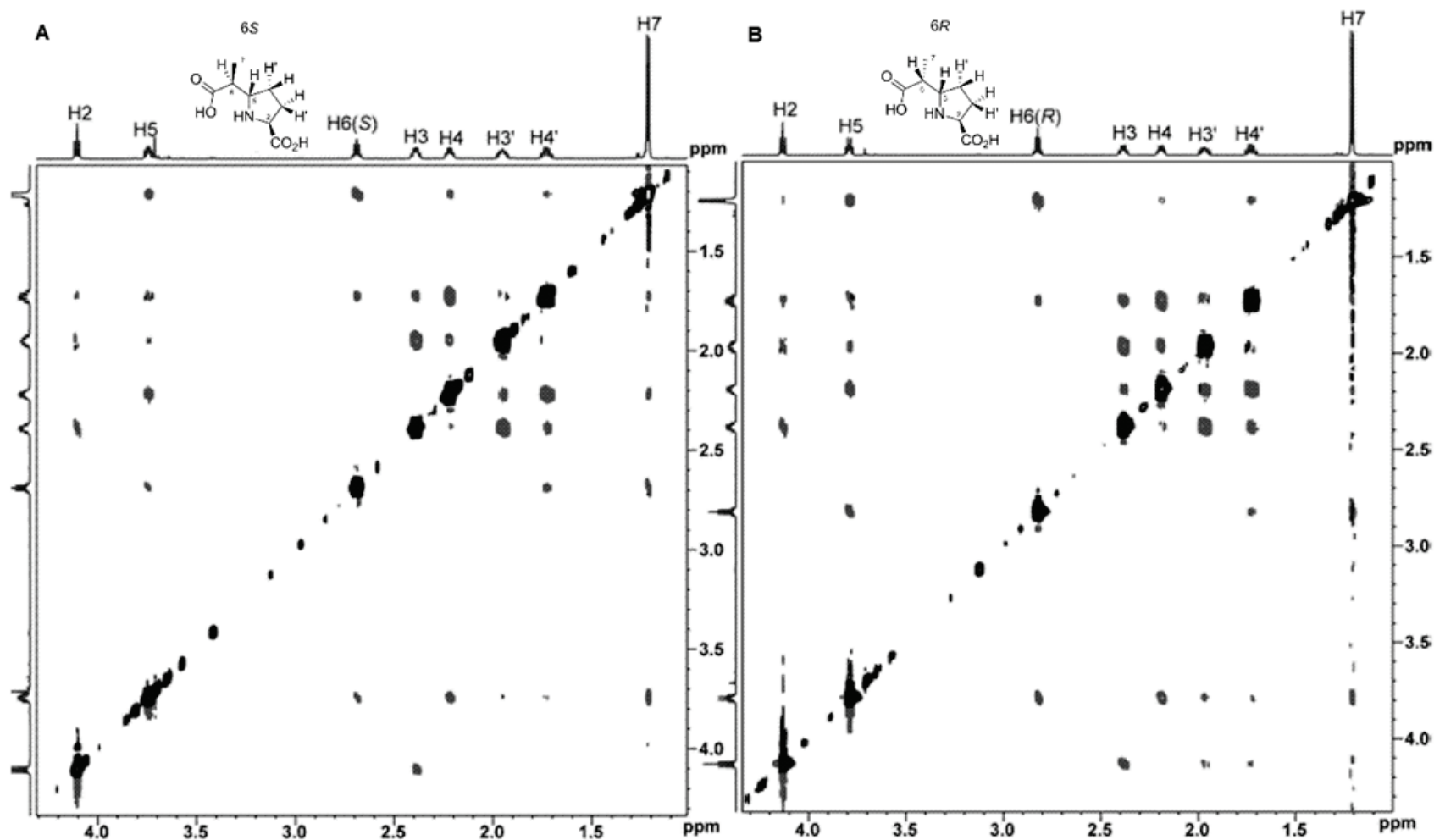


Figure 2.8. ^1H - ^1H NOESY spectra for the purified (6R)- (A) and (6S)-6-methyl-*t*-CMP (B) from CarB M108V and ThnE V153A respectively. NMR analysis performed by Dr. R. Hamed.

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of the two C6 epimers in reasonable yields and variable ratios, as revealed by the two signals at $m/z = 202$ $[M+H]^+$ observed by LC-MS (Figure 2.10 and Table 2.4).

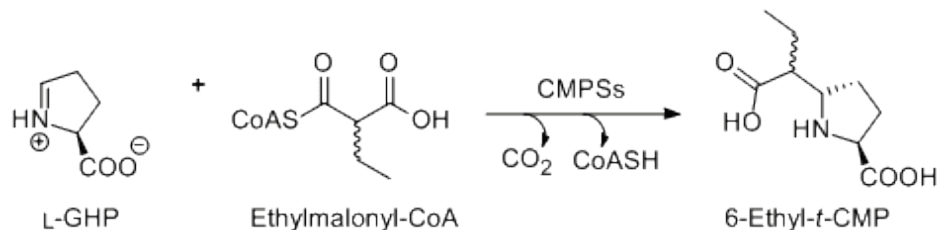


Figure 2.9. The product of CMPSs catalyzes for L-GHP and ethylmalonyl-CoA.

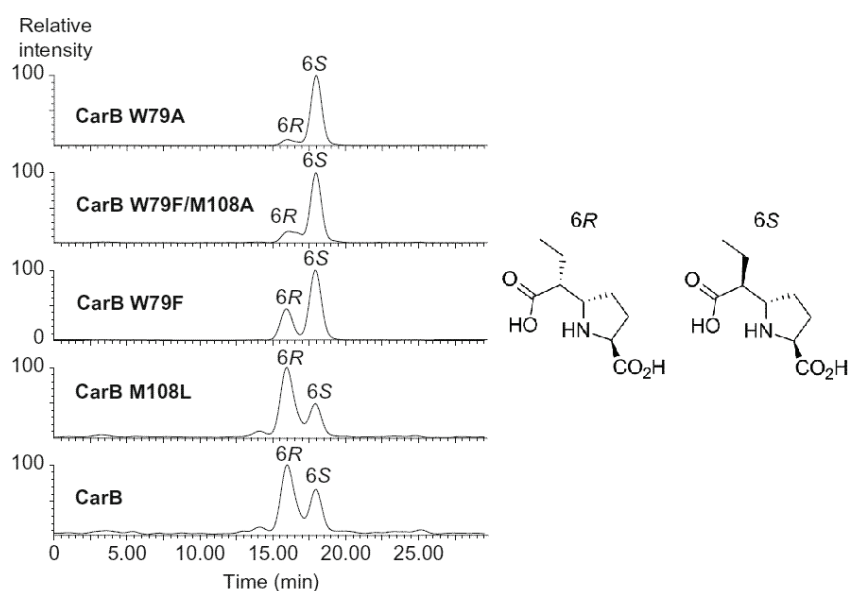


Figure 2.10. LC-MS chromatogram showing the production of the two epimers of 6-ethyl-*t*-CMP by the indicated CarB variants.

Table 2.4. The results for 6-ethyl-*t*-CMP catalysed by CarB.

Catalyst	Ratio (<i>R</i> : <i>S</i>)	% Yield
CarB wt	65:35	4
CarB M108L	72:28	5
CarB W79F	32:68	44
CarB W79A	12:88	36
CarB W79F M108V	34:66	12
CarB W79F M108A	17:83	12

Subsequent scale-up of the reaction for one of the highest yielding variants (*i.e.* CarB W79F), purification of the products and NMR analyses (Figure 2.11) of the isolated epimers enabled the assignment of the stereochemistry at C-6 of the epimer

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with shorter retention time as (6*R*)- (*i.e.* (2*S*,5*S*,6*R*)-6-ethyl-CMP) and that of the epimer with the longest retention time as (6*S*)- (*i.e.* (2*S*,5*S*,6*S*)-6-ethyl-CMP) (Figure 2.12 and Figure 2.13).

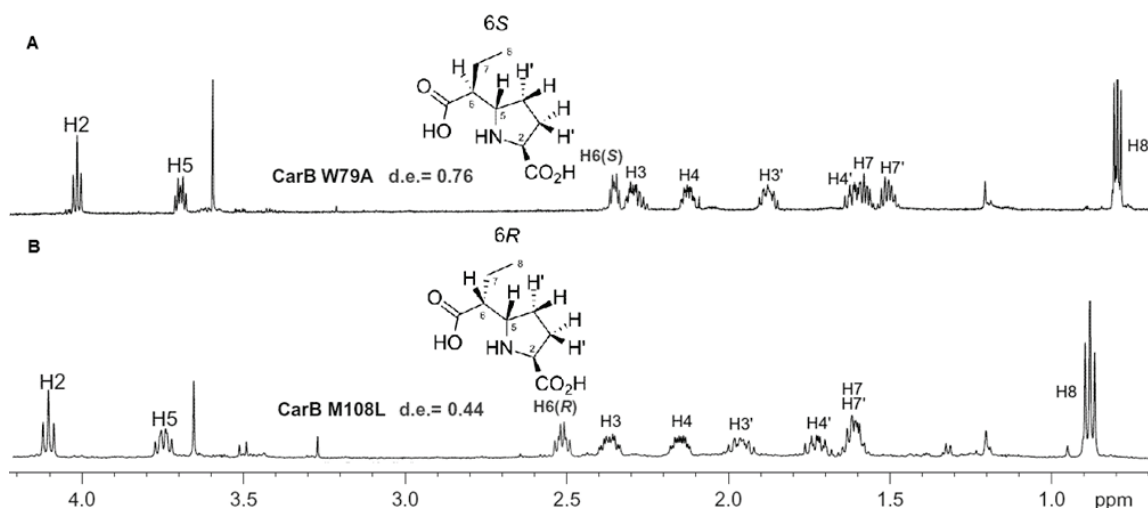


Figure 2.11. ¹H-NMR spectra for the purified (6*S*)- (A) and (6*R*)-6-ethyl-*t*-CMP (B) from CarB M108L and CarB W79A respectively. NMR analysis performed by Dr. R. Hamed.

The stereochemistry at C-6 of the epimer with the shortest retention time was assigned as (*R*) based on the value of 9.2 Hz for $J_{5,6}$ (predicted average $\Phi \sim 152^\circ$) together with a weak NOE correlation observed between H-5 and H-6, that indicate a predominantly *anti* conformation for these two protons; and, the observation of an NOE correlation between H-5 and H-8/H-8', together with the absence of any observed NOE correlations between H-8/H-8' to any C-4 protons (Figure 2.12).

The stereochemistry at C-6 of the second epimer was assigned as (*S*) based on the value of 6.3 Hz for $J_{5,6}$ (predicted average $\Phi \sim 34^\circ$), together with a strong NOE correlation observed between H-5 and H-6, that indicate a predominantly *gauche* conformation for these two protons; and, the observation of an NOE correlation between H-5 and H-8/H-8', coupled to a strong NOE correlation observed between H-5

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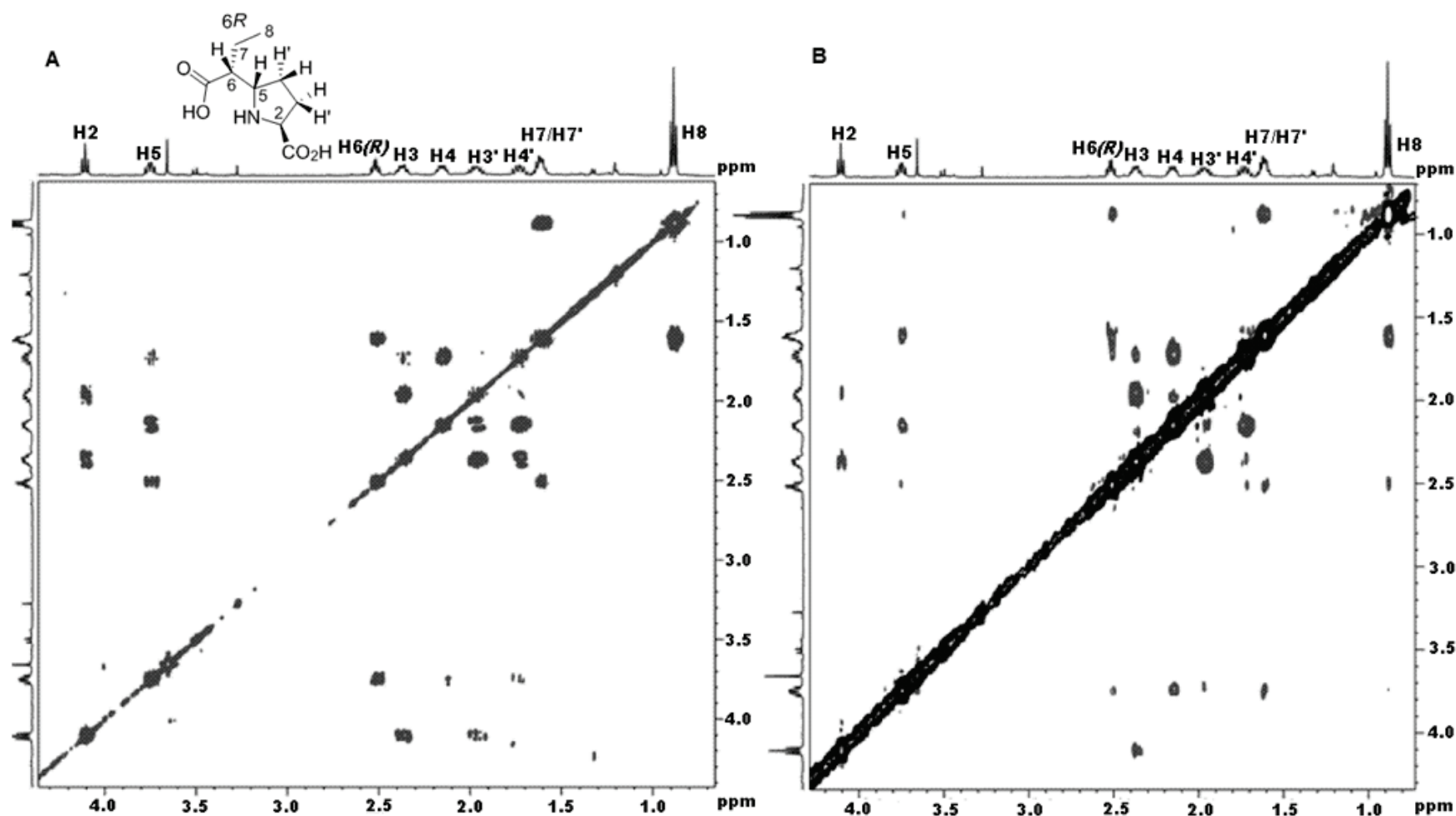


Figure 2.12. ¹H-¹H COSY (A) and NOESY (B) spectra for purified (6R)-ethyl-*t*-CMP produced from (2R/S)-2-ethylmalonyl-CoA and L-GHP by CarB W79F catalysis. NMR analysis performed by Dr. R. Hamed.

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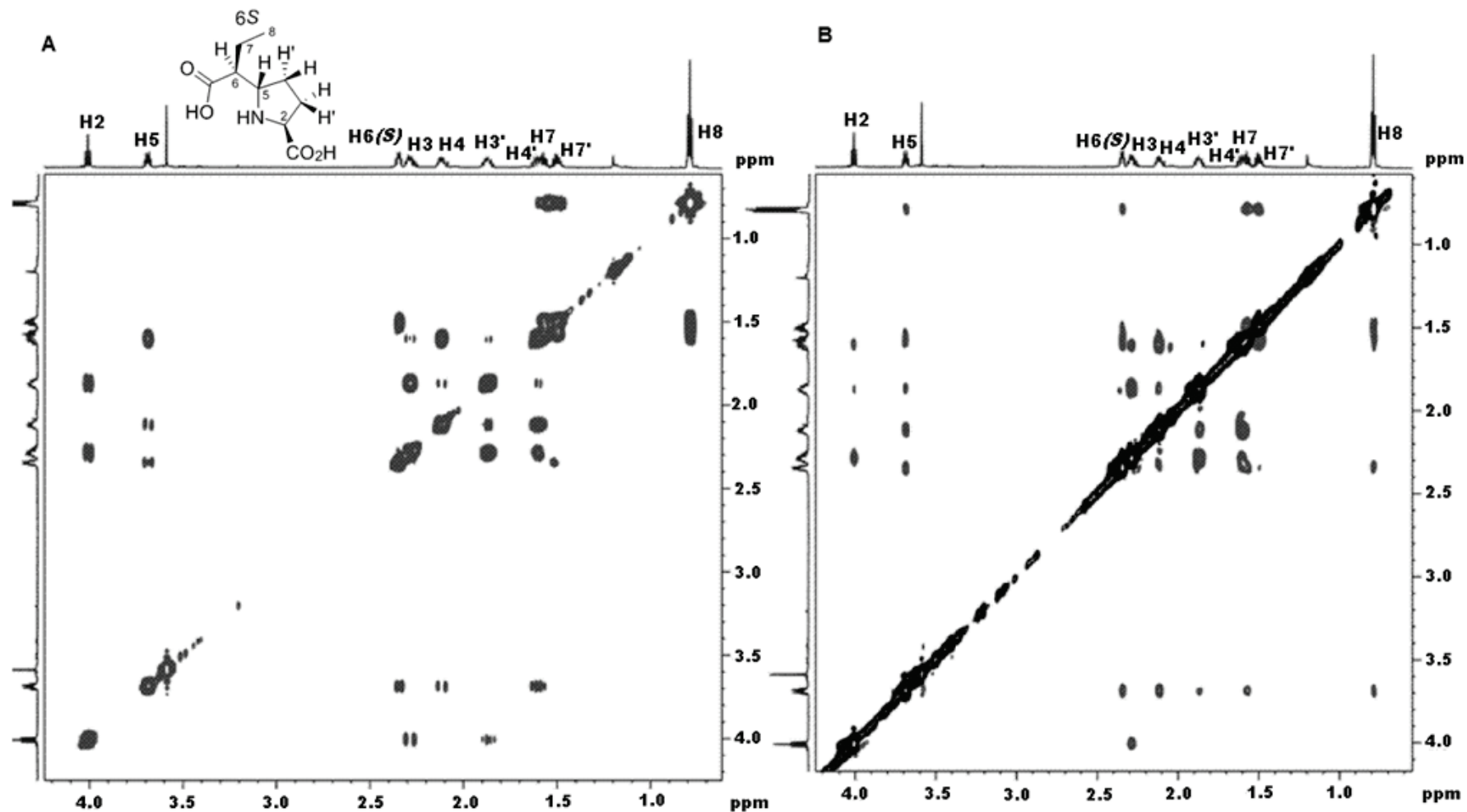


Figure 2.13. ^1H - ^1H COSY (A) and NOESY (B) spectra for purified (6*S*)-ethyl-*t*-CMP produced from (2*R/S*)-2-ethylmalonyl-CoA and L-GHP by CarB W79F catalysis. NMR analysis performed by Dr. R. Hamed.

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and C-9 protons together with a weak NOE correlation between H-6 and H-4' (Figure 2.13).

To confirm the stereochemical assignment at C-6 of the two epimers of 6-ethyl-*t*-CMP, the (2*S*,5*S*,6*R*)-6-ethylcarbapenam methyl ester was synthesized from protected (2*S*)-glutamate by Mr. A Thalhammer, and its structure was confirmed by NMR analyses. Enzymatic ester and β -lactam hydrolysis of it yielded (6*R*)-6-ethyl-*t*-CMP, which showed the same retention time (Figure 2.14) as the product assigned the (6*R*)-geometry prepared by CarB W79F. Chromatography of (6*R*)-6-ethyl-*t*-CMP produced by hydrolysis of (2*S*,5*S*,6*R*)-6-ethylcarbapenam methyl ester resulted in the enrichment of the peak corresponding to (6*R*)-6-ethyl-*t*-CMP (*i.e.* (2*S*,5*S*,6*R*)-6-ethyl-CMP) produced by CarB W79F catalysis (Figure 2.14).

The d.r. values of the two 6-ethyl-*t*-CMP epimers (6*R*:6*S*) produced by CMPS catalysis were determined by LC-MS analysis (Figure 2.10, Table 2.4). CarB M108V and CarB M108I produced the best diastereomeric excess (80:20) albeit with very low yields of < 2%. With (2*R*/2*S*)-2-ethylmalonyl-CoA as a substrate, CarB W79F and CarB W79A maintained the same bias towards the (6*S*)-6-alkyl-*t*-CMP epimer observed with methylmalonyl-CoA. Notably, higher yields of 6-ethyl-*t*-CMP than wild-type CarB (ca. 10 timer higher; Table 2.4) were observed. Although the observed stereoselectivities for the production of 6-ethyl-*t*-CMP epimers were lower than those for the 6-methyl-*t*-CMP epimers, these results demonstrate that *t*-CMP derivatives with different C6 alkyl substituents can be produced by CMPSs catalysis.

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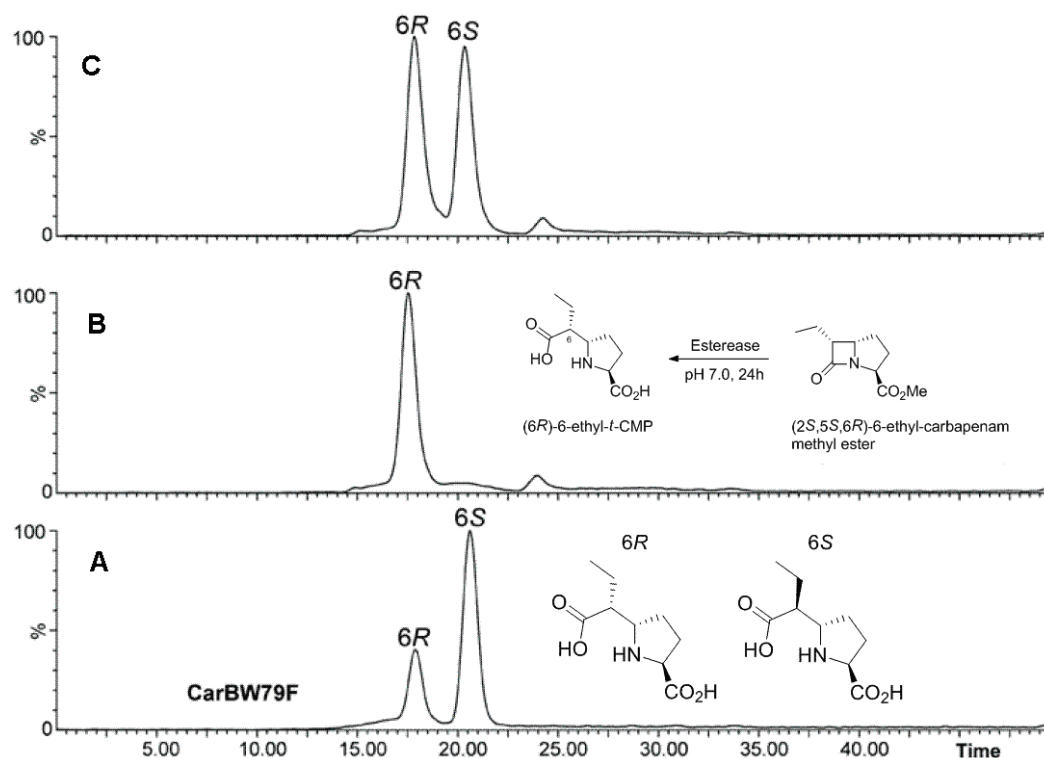


Figure 2.14. Stereochemical assignment of the two epimers of 6-ethyl-*t*-CMP produced by CMPSs catalysis. A. LC-MS for the two epimers of 6-ethyl-*t*-CMP produced from incubation of (2*R*/*S*)-2-ethyl-*t*-CMP and L-GHP by CarB W79F catalysis; B. LC-MS for the hydrolysis product of (2*S*,5*S*,6*R*)-6-ethylcarbapenam methyl ester; C. Co-chromatography of the two epimers of 6-ethyl-*t*-CMP produced by CarB W79F catalysis together with the hydrolysis product of (2*S*,5*S*,6*R*)-6-ethylcarbapenam methyl ester. The peak corresponding to (6*R*)-6-ethyl-*t*-CMP is enriched.

Screening of variants for the production of 7-methyl- and 7-ethyl-*t*-CMPi epimers.

As described in Chapter 1, previous work in our group has reported the capability of CarB to catalyse the production of *N*-heterocycles with ring sizes different than five (*i.e.* 6- and 7-membered rings from L-aminoadipate semialdehyde and L-aminopimelate semialdehyde respectively, Figure 1.16).⁴ Therefore, it was investigated whether CMPS variants could catalyze the stereoselective formation of the C-7 epimers of (2*S*,6*S*)-7-methyl-carboxymethylpipecolic acid (7-methyl-*t*-CMPi) from L-aminoadipate semialdehyde (L-AASA) and (2*R*/*S*)-2-methylmalonyl-CoA. As with the formation of 6-ethyl-*t*-CMP, the ThnE variants that were tested did not yield detectable levels of 7-

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methyl-*t*-CMPi, though some of the CarB variants did as revealed by the presence of signals at $m/z = 202$ $[M+H]^+$ after LC-MS analysis of the catalysis samples (Figure 2.15). The d.r. of the two epimers ($7R:7S$) was calculated by analytical LC-MS analysis (Table 2.5).

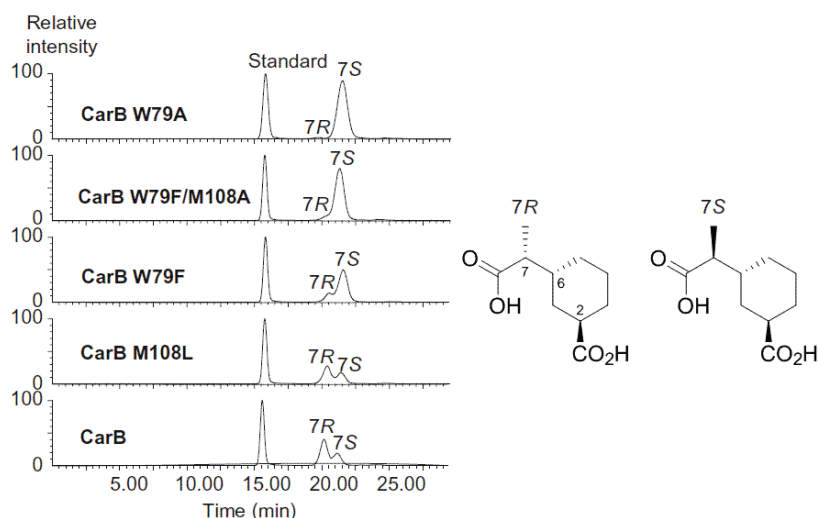


Figure 2.15. LC-MS chromatograms showing the production of the two epimers of 7-methyl-*t*-CMPi by the indicated variants of CarB. *p*-aminosalicylic acid was used as an internal Standard ($t_R = 14.85$ min).

NMR analyses (Figure 2.16) of the major epimer produced by the best catalyst, CarB W79A, allowed establishing the C-7 stereochemistry as (*S*) based on the $J_{6,7}$ value of 8.1 Hz as well as the weak NOE between H-6 and H-7 (compared to that between H-6 and the C-7 methyl group) indicating a predominantly *anti* relationship of the two protons; and the NOE between H-7 and H-5' > H-5 as well as that between the methyl group on C-7 and H-5 > H-5'.⁴ The very high d.r. in this case (< 1:99, $7R:7S$) may reflect an increase in the steric demand during the formation of the six-member ring product when compared to the five-member ring one.

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Table 2.5. The results for 7-methyl-*t*-CMP catalysed by CarB and variants. CarB W79A and CarB W79F M108A show the best diastereoselectivity and yield.

Catalyst	Ratio (<i>R</i> : <i>S</i>)	% Yield
CarB wt	66:34	7
CarB M108L	60:40	5
CarB W79F	17:83	9
CarB W79A	1:99	11
CarB W79F M108V	24:76	10
CarB W79F M108A	5:95	12

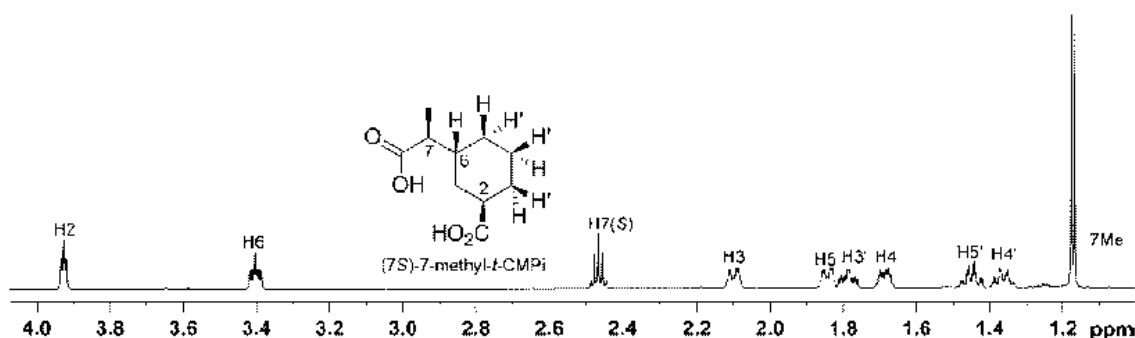


Figure 2.16. ¹H-NMR spectrum for the (7*S*)-7-methyl-*t*-CMPi produced by incubation of (2*R*/*S*)-2-methylmalonyl-CoA and L-AASA in the presence of CarB W79A. NMR analysis performed by Dr. R. Hamed.

The CarB W79-variants were the only ones that catalysed the production of the two C-7 epimers of 7-ethyl-*t*-CMPi from L-AASA and (2*R*/*S*)-2-ethylmalonyl-CoA as judged by the presence of signals at $m/z = 216$ [$M+H^+$] under LC-MS analysis (Figure 2.18 and Table 2.6). The d.r of the two epimers (6*R*:6*S*) for the Trp79 were: CarB W79F (13:87), CarB W79F M108A (9:91), and CarB W79A (1:99). The yield of 7-ethyl-*t*-CMPi produced by CarB W79A catalysis was ~ 4 times higher than that of CarB W79F or CarB W79F M108A catalysis.

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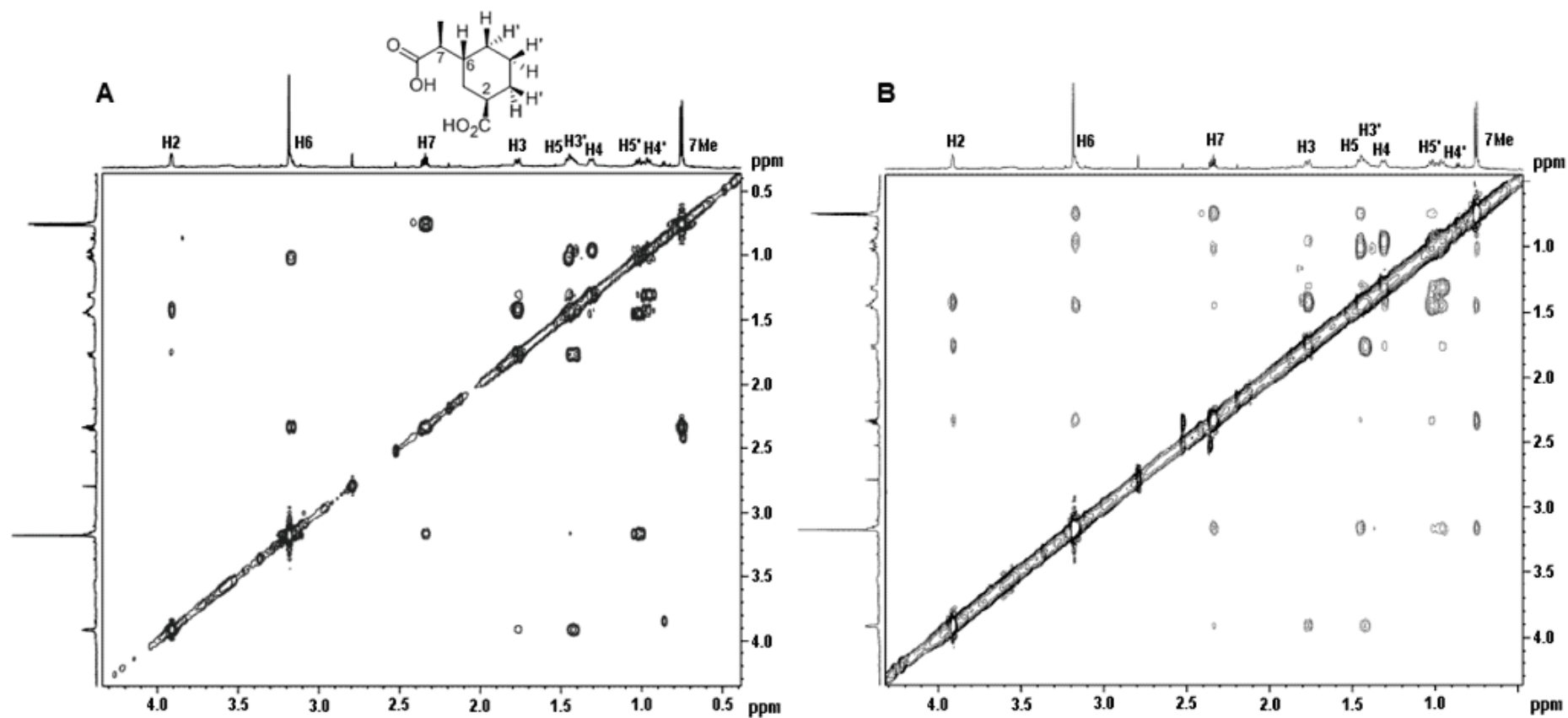


Figure 2.17. 2D COSY (A) and 2D NOESY (B) spectra for the (7S)-7-methyl-*t*-CMPi produced by incubation of (2R/S)-2-methylmalonyl-CoA and L-AASA in the presence of CarB wt.⁴ NMR analysis performed by Dr. R. Hamed.

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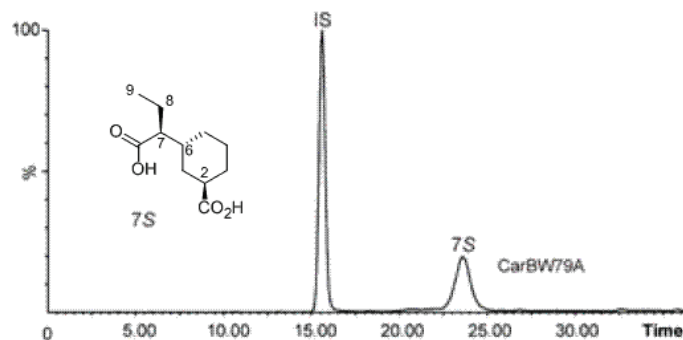


Figure 2.18. LC-MS chromatogram showing the production of a major single epimer of (7*S*)-7-ethyl-*t*-CMPi by CarB W79A catalysis. *p*-Aminosalicylic acid was used as an internal standard (IS).

Table 2.6. The results for 7-ethyl-*t*-CMP catalysed-production by CarB and variants. As with 7-methyl-*t*-CMP, CarB W79A and CarB W79F M108A show the best diastereoselectivity and yield.

Catalyst	Ratio (<i>R</i> : <i>S</i>)	% Yield
CarB W79F	13:87	4
CarB W79A	< 1:99	6
CarB W79F M108V	19:81	1
CarB W79F M108A	9:91	6

NMR analyses (Figure 2.19) of the purified major epimer produced by CarB W79A catalysis enabled assignment of the stereochemistry at C-7 as 7*S* based on a value of 5.8 Hz for $J_{6,7}$ (predicted averaged $\Phi = 132^\circ$), together with NOE correlations observed between H-6 and both H-7 and H-8 indicating a predominately *gauche* arrangement for H-6 and H-7; and, the observation of an NOE correlation between H-7 and H-5 > H-5', together with the observation of NOE correlations between the methyl group at C-9 and H-6. (7*S*)-7-ethyl-*t*-CMPi is proposed to adopt a chair conformation based on the coupling constant values and the observed NOESY correlations.

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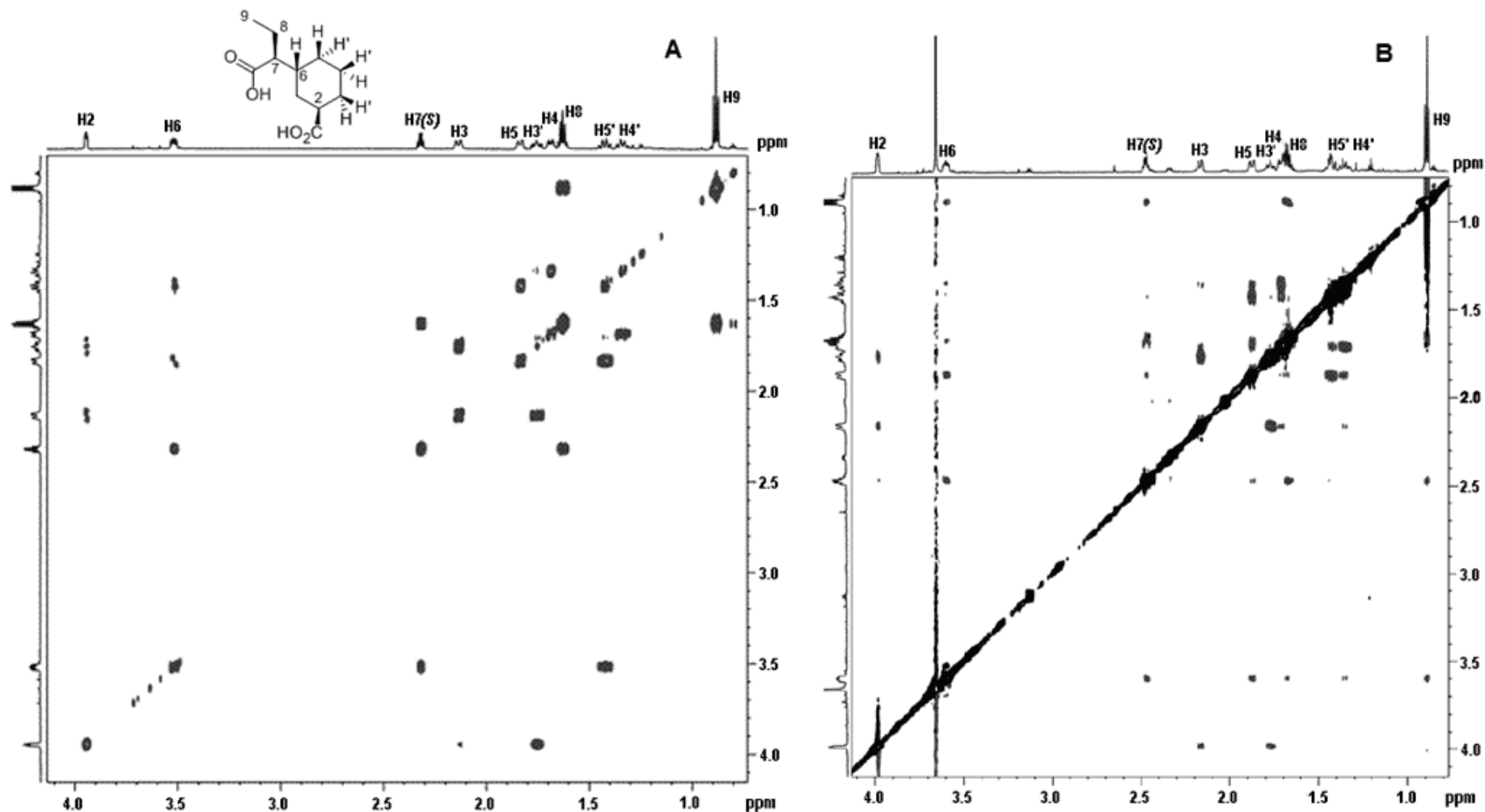


Figure 2.19. ^1H - ^1H COSY (A) and ^1H - ^1H NOESY (B) spectra for the (7S)-7-ethyl-L-CMPi produced by the incubation of (2R/S)-2-ethylmalonyl-CoA and L-AASA by CarB W79A catalysis.

Effect of pH and temperature on diastereoselectivity.

There is an interest in studying the kinetics of the catalytic reaction of CMPS towards understanding its mechanism. The investigation of the effects of conditions on stereoselectivity of the catalysed reactions using three CMPSs was studied, using variants two of which are selective for the preparation of (6*R*)-6-methyl-*t*-CMP (CarB and CarB M108V) and one is so for (6*S*)-6-methyl-*t*-CMP (ThnE V153A). Time-course analyses (Figure 2.20) at different temperatures (37 and 4 °C) and pH values (6.9, 7.3, 7.7 and 8.0) revealed that, in each case, the observed diastereoselectivity towards the major epimer produced was enhanced both at early time points and at lower pH or temperature values. These results suggest, particularly at the early points of the reaction, that a factor in determining the overall diastereoselectivity of the reactions is the rate of equilibration of the C-2 epimers of alkylmalonyl-CoA, which occurs because of the acidity of the C-2 malonyl hydrogen. For biocatalytic purposes, it may be possible that a combination of active-site engineering with pH and/or temperature may even lead to a higher control of the diastereoselectivity of the reaction (See Chapter 4 for use of coupled enzymes to enhance diastereoselectivity).

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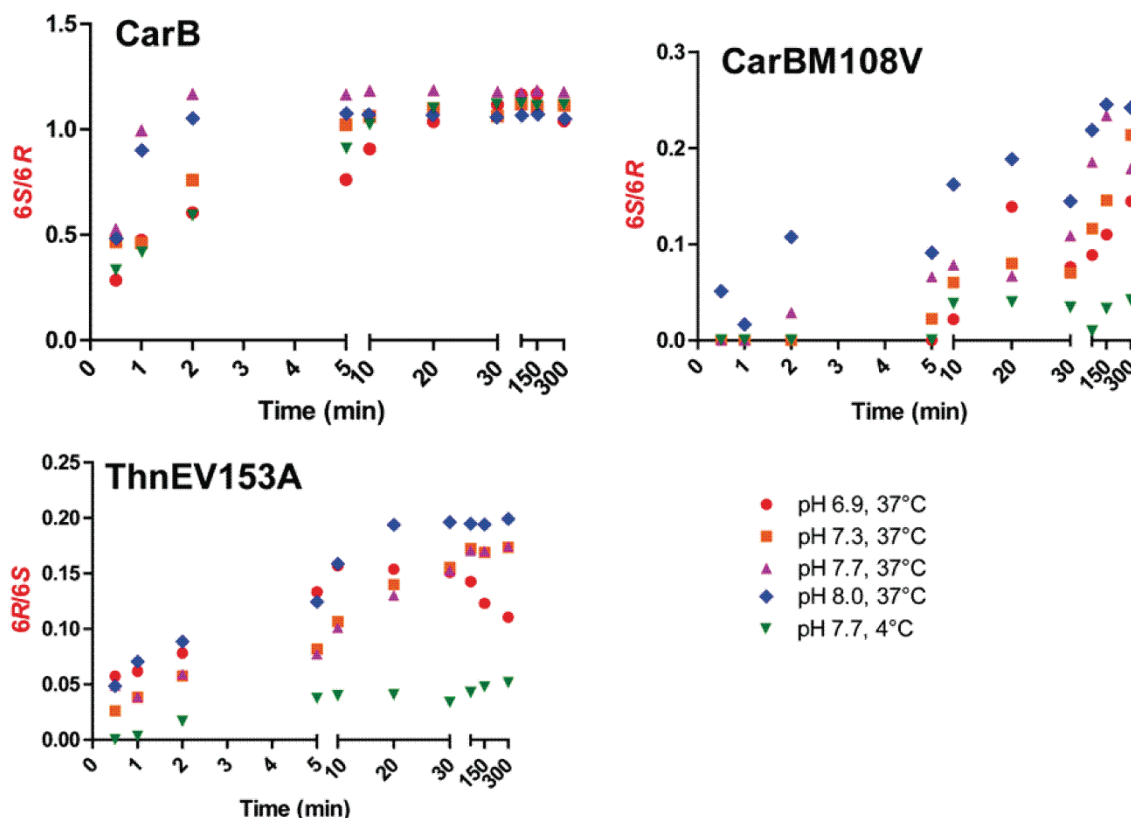


Figure 2.20. Kinetic time course showing the effect of pH and temperature on the diastereoselectivity of the reaction of (2*R*/*S*)-2-methylmalonyl-CoA and L-GHP as catalysed by CMPSs (CarB, CarB M108V and ThnE V153A). MOPSO and TRIS buffers were used to adjust to the final pH. Time courses were carried out at the pH values of 6.9, 7.3, 7.7 and 8.0 while maintaining the temperature at 37 °C, and at temperatures of 37 and 4 °C while maintaining the pH at 7.7. All data points represent the mean of two independent experiments.

Discussion.

Overall, the results demonstrate the ability of CMPSs to stereoselectively control the formation of trisubstituted enolates and their subsequent reaction with electrophiles (*e.g.* L-GHP and L-AASA) to give products in high diastereomeric excess. The substitution of residues Met108_{CarB}/Val153_{ThnE} and Trp79_{CarB}/Trp124_{ThnE} has been found to significantly affect the observed d.r. of products of CMPSs catalysis on alkylated malonyl-CoA derivatives, away from the ~ 55:45 (6*R*:6*S*) ratio observed for wild-type CarB and 6-methyl-*t*-CMP; as with CarB, single-residue substitutions on ThnE were sufficient to substantially alter the d.r. of the observed products.

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Variations on the active site of CMPSs have also altered the yield of the products obtained. For example, substitution of Trp79_{CarB} for a Phe or Ala residue resulted in a substantial (~ 10-fold) enhancement in the overall yield of 6-ethyl-*t*-CMP (Table 2.4). On the other hand, variations in incubation conditions were found to further enhance d.r. values towards the major epimer observed (Figure 2.20), probably due to an effect on the rate of epimerization of the proton at C-2 of the alkylmalonyl-CoA substrates.

At present, the precise chemical basis behind the diastereoselectivity exhibited by engineered CMPSs is not clear; the introduced substitutions may influence the binding of alkylmalonyl-CoA, the rate of decarboxylation, or the reactivity of L-P5C to the enolate intermediate. The necessary close spatial relationship of the binding site for (alkyl)malonyl-CoA and L-P5C implies that some of the targeted residues are probably involved in both the decarboxylation and the alkylation steps. Nonetheless, an important number of structure-activity relationships have been identified as a result of this research. One notable case is that CMPSs variants with a β -branched residue at position 108_{CarB}/153_{ThnE} (*i.e.* Val or Ile) on the oxyanion hole favour the formation of products with the (6*R*)-stereochemistry (Table 2.2 and Table 2.3); furthermore, for some ThnE variants, substitution of the wild-type Val153 for a non- β -branched residue (*i.e.* Ala, Leu, Met) results in a shift towards the (6*S*)-stereochemistry (Table 2.3). Another important structure activity relation is that variants at Trp79_{CarB} generally favoured the formation of epimers with (6*S*)/(7*S*)-stereochemistry (Table 2.2Table 2.6); this stereoselectivity was further enhanced by introducing an Ala at residue 108_{CarB} (to yield a double mutant), demonstrating the beneficial effects of combining substitutions and that variation of more than one residue can be useful for altering the d.r. of the products.

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Given that the crotonase superfamily catalyses a wide range of enolate-based chemistry^{7, 19} involving reactions with a number of electrophiles including oxygen,²⁰ the results continue to suggest that CMPSs may be a useful platform technology for engineering studies aimed at performing enolate reactions of choice.

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Chapter 3. Carboxymethylproline Synthases Catalysis Enables Flexible Production of Functionalized Prolines and Carbapenams.

The biocatalytic versatility of wild-type and variants of the CMPSs, CarB and ThnE, was further explored in the preparation of functionalized *t*-CMP derivatives methylated at C-2, C-3, C-4 or C-5, building upon the results obtained from the manipulation of CMPSs towards the functionalization of C-6/C-7 in the 6- and 7-membered carboxymethyl-*N*-heterocycles obtained.

Preparation of C-4-methylated t-CMP derivatives.

As described in the introduction, methylation at C-4 of *t*-CMP (equivalent to C-1 in carbapenems) is pharmacologically important because older carbapenem antibiotics (*i.e.* imipenem and panipenem) are subject to dehydropeptidase-I degradation in the brush border of renal tubules.¹ This required the administration of an dehydropeptidase-I inhibitor such as cilastatin or, in the case of panipenem, betamipron, an organic anion tubular transport inhibitor to prevent uptake into renal tubes.² Later carbapenems, including meropenem, ertapenem, biapenem and doripenem are stable to dehydropeptidase-I degradation because of the presence of a 1- β methyl substituent on the carbapenem nucleus (Figure 1.9).³⁻⁵

In order to test the ability of wild-type and selected mutants of both CMPSs in hand to accept *t*-CMP with mono- or dimethyl substituents at C-4, the *N*-Boc-*tert*-butyl ester protected forms of (4*S*)-4-methyl-L-GHP, (4*R*)-4-methyl-L-GHP and 4,4-dimethyl-L-GHP were synthesized from pyroglutamate⁶ by Dr. C. Ducho. The unprotected forms were obtained by standard acid hydrolysis (see Methods). Upon incubation with CMPSs

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and malonyl-CoA, 4,4-dimethyl-L-GHP was converted to a single product with varying yields as observed under LC-MS analysis (standard conditions, $[M+H] = 202$, Figure 3.1, Table 3.1)

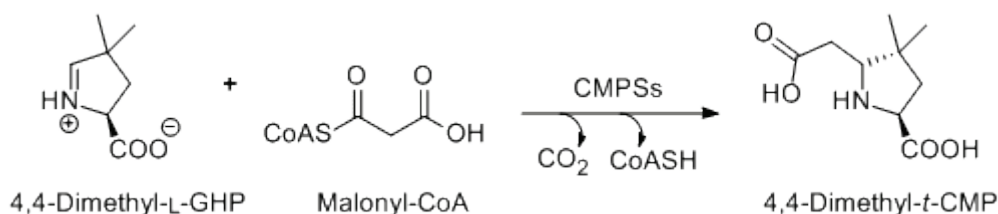
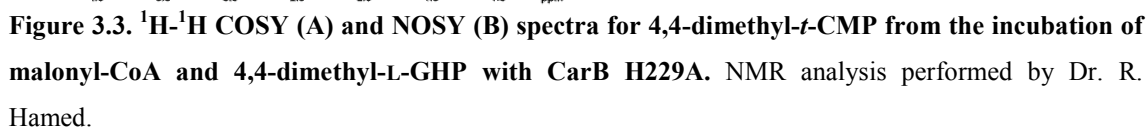
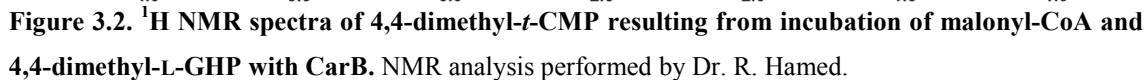


Figure 3.1 The product of CMPSs catalysis for 4,4-dimethyl-L-GHP and malonyl-CoA.

Table 3.1. The percent (%) overall yield (isolated) of 4,4-dimethyl- CMP, produced by CMPSs. Determined as reported.⁷

Variant	% isolated yield of 4,4'-dimethyl- <i>t</i> -CMP
CarB	48
CarB M108A	36
CarB M108L	36
CarB M108V	12
CarB M108I	10
CarB Q111N	48
CarB H229A	41
CarB W79F	48
CarB W79F M108A	46
CarB W79A	46
ThnE	32
ThnE V153A	47
ThnE V153L	17
ThnE V153M	15
ThnE V153I	17
ThnE W124F	2
ThnE W24F V153M	2
ThnE H274A	12

This product was assigned as 4,4-dimethyl-*t*-CMP on the basis of LC-MS and NMR analysis (Figure 3.2 and Figure 3.3), assuming, as before, that the (*S*)-stereochemistry at C-2 is maintained after deprotection of the semialdehydes, that the stereochemistry at the bridgehead C-5 is (*S*) since there is no NOE correlation observed between the proton at the bridgehead and H-2, and on coupling constant values for the H-6 side chain protons of $J_{5,6} = 3.3, 17.7$ Hz and $J_{5,6'} = 10.7, 17.7$ Hz.



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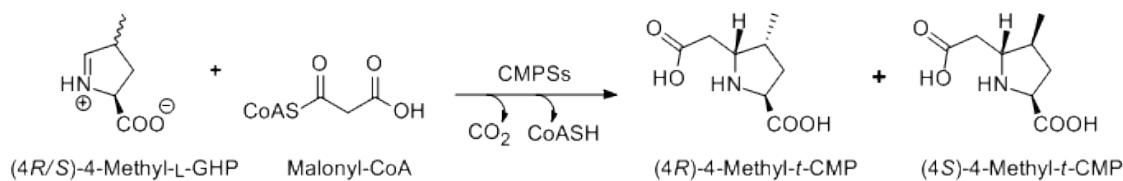


Figure 3.4. The products of CMPSSs catalysis for 4-methyl-L-GHP and malonyl-CoA.

Evidence that C-4 epimerization occurred during the acid-mediated deprotection came from the hydrolysis of *N*-Boc-L-GHP *tert*-butyl ester in $^2\text{HCOO}^2\text{H}/^2\text{H}_2\text{O}$ which led to the incorporation of two deuterium atoms at C-4 of L-GHP.⁸ Based on previous observations that CMPSSs select from equilibrating mixtures of alkylmalonyl-CoA derivatives, the possibility of stereoselective production of 4-methyl-*t*-CMP from a mixture of 4-methyl-L-GHP epimers was investigated. A set of CarB variants were then screened by analytical LC-MS for the formation of 4(*S*)- and 4(*R*)-4-methyl-*t*-CMP from C-4 epimeric 4-methyl-L-GHP and malonyl-CoA under standard conditions. All the CarB and ThnE variants tested were able to catalyse the formation of the two C-4 epimers with varying yields and diastereomeric ratios (Table 3.2). For most of the CMPSSs, the reactions were scaled up and the diastereomeric ratios of the two epimers confirmed by ^1H -NMR analysis (Figure 3.5).

Table 3.2. The ratio of C-4 epimers and the % overall yield (isolated) of 4-methyl-*t*-CMP produced by CMPSSs catalysis. CarB M108V, CarB M108I, ThnE, ThnE V153L, ThnE V153I, ThnE W124F, ThnE W124F V153M and ThnE H274A showed the best diastereoselectivity (d.r. $\geq 90:10$). Determined as reported.⁷

Variant	Diastereomeric ratio (4 <i>S</i> :4 <i>R</i>)	Combined yield
CarB	51:49	31
CarB M108A	54:46	37
CarB M108L	56:44	26
CarB M108V	93:07	17
CarB M108I	90:10	18
CarB Q111N	70:30	27
CarB H229A	65:35	36
CarB W79F	61:39	34
CarB W79F M108A	73:27	11
CarB W79A	66:34	5
ThnE	92:08	44
ThnE V153A	74:26	47

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ThnE V153L	93:07	46
ThnE V153M	89:11	43
ThnE V153I	98:02	43
ThnE W124F	97:03	5
ThnE W124F V153M	92:08	9
ThnE H274A	99:01	39

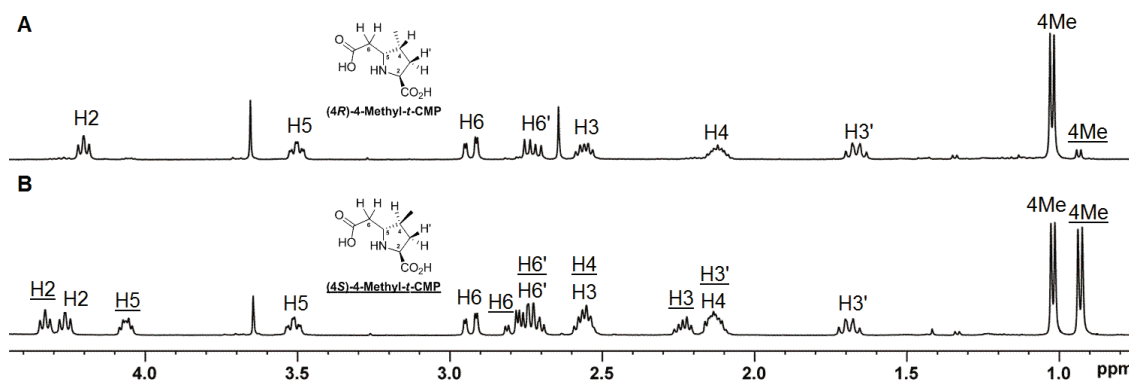


Figure 3.5. ¹H NMR spectra of (4*R*)- and (4*S*)-4-methyl-*t*-CMP resulting from the incubation of CarB M108V (A) and wild-type CarB (B). The signals for (4*S*)-4methyl-*t*-CMP are underlined; the ratios between (4*R*)- and (4*S*)- isomers in A are 7:93, and in B 49:51. NMR analysis performed by Dr. R. Hamed.

The stereochemistry at C-4 of the major epimer produced by the selective CarB M108V catalysis was assigned as (*S*) based on the observation of a strong NOE between H-5 and the methyl group at C-4, together with a weak NOE between H-2 and H-4 (Figure 3.6) The stereochemistry at C-4 of the epimer was assigned (from a mixture of the two diastereomers produced by CarB H229A catalysis, Figure 3.7) as (*R*) based on the lack of observation of an NOE between H-5 and the C-4 methyl protons.

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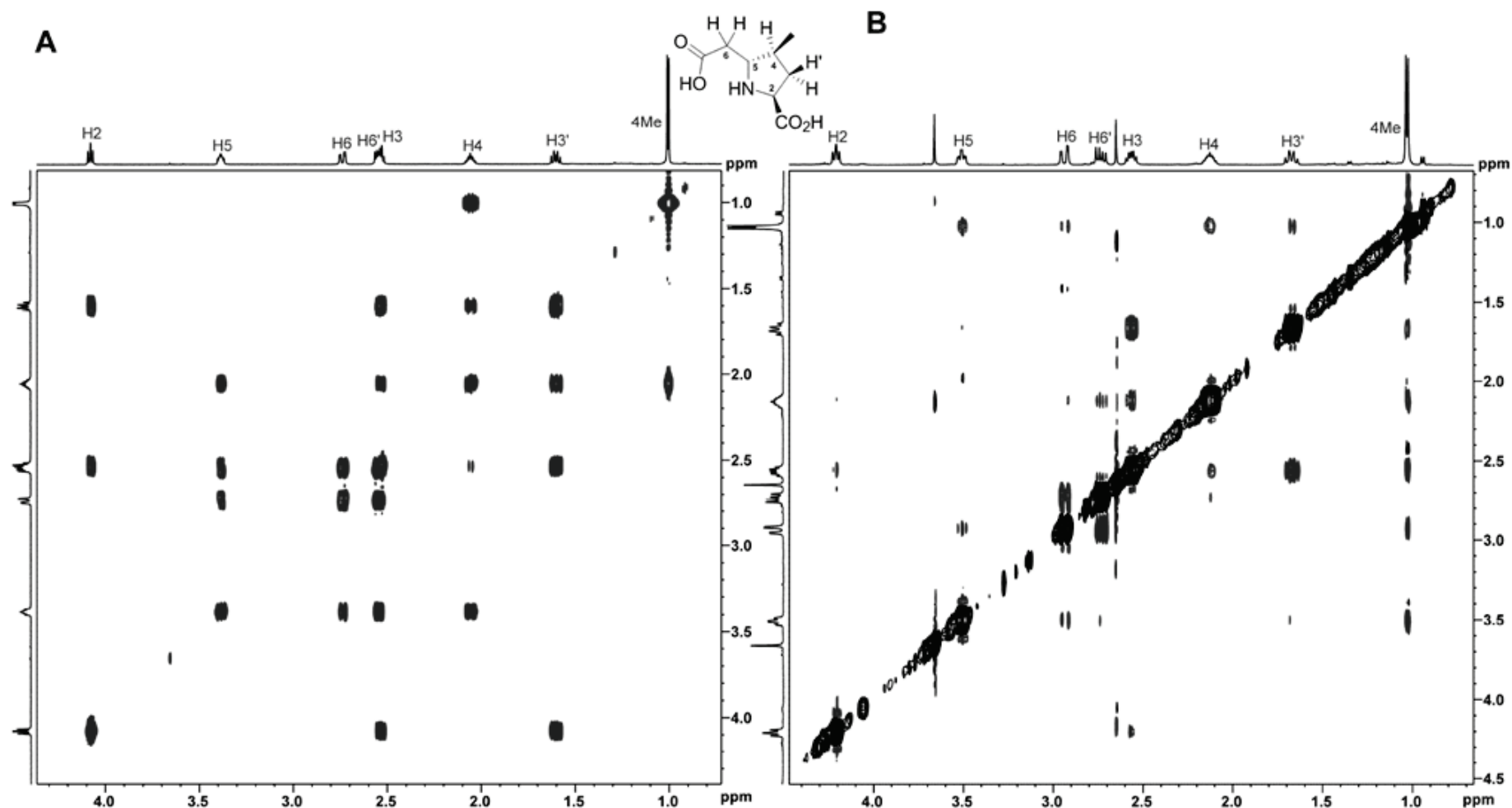


Figure 3.6. ^1H - ^1H COSY (700 MHz, A) and NOESY (500 MHz with a 1mm microprobe, B) spectra for 4(S)-4-methyl-L-CMP produced from incubation of 4(R/S)-4-methyl-L-GHP and malonylCoA by CarB M108V and ThnE H274A catalysis respectively. NMR analysis performed by Dr. R. Hamed.

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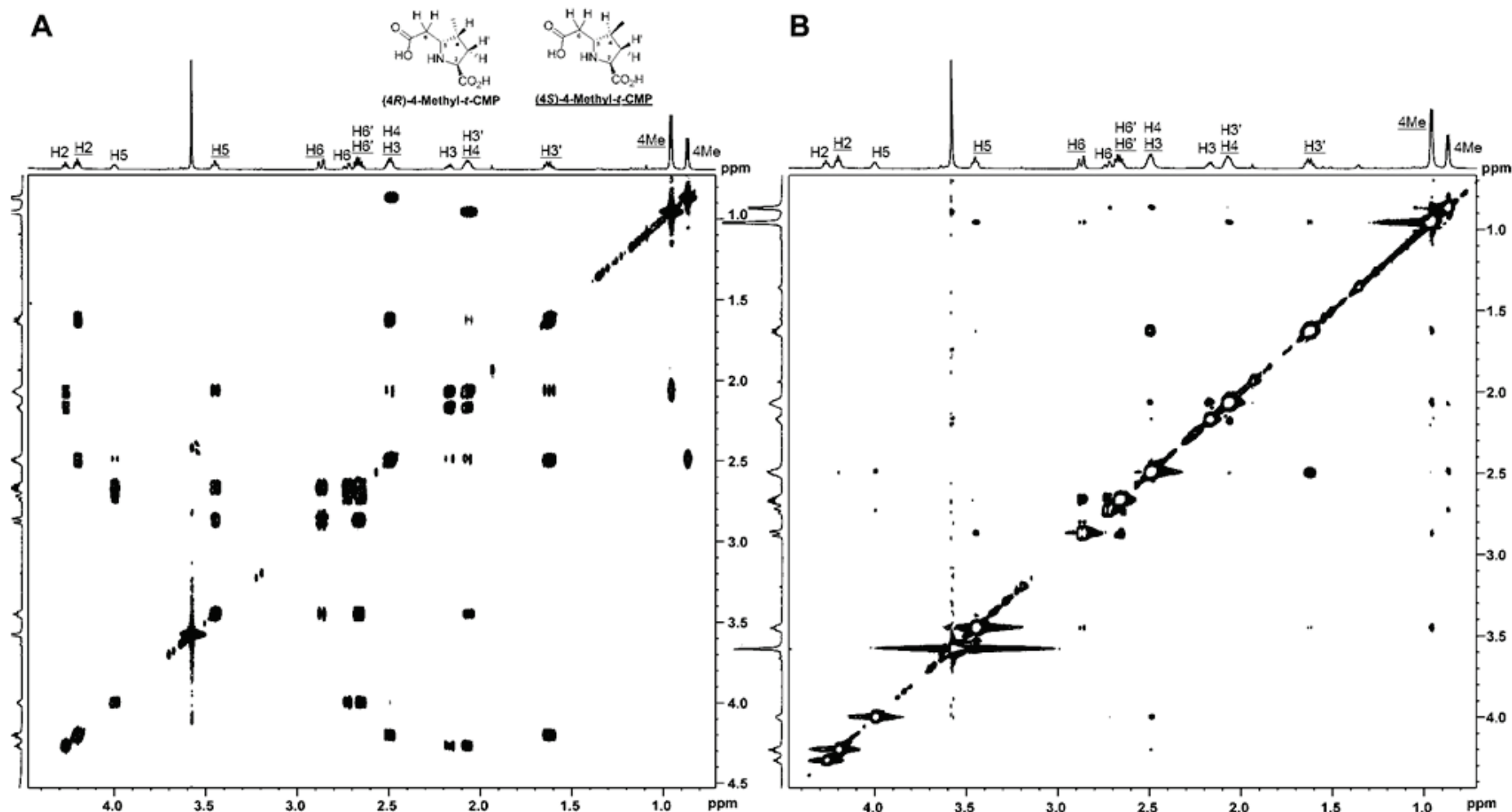


Figure 3.7. ^1H - ^1H COSY (700 MHz, left) and NOESY (500 MHz with a 1mm microprobe, right) spectra for 4(S)- and 4(R)-4-methyl-L-CMP produced from 4(R/S)-4-methyl-L-GHP and malonylCoA by CarB H229A catalysis. NMR analysis performed by Dr. R. Hamed.

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It is notable that, in opposition to the C-6 substituted *t*-CMP compounds, all CMPSs (including ThnE, Figure 3.8) with a branched β -residue (*e.g.* Val or Ile) at positions 108_{CarB}/153_{ThnE} produced (4*S*)-4-methyl-*t*-CMP as the major C-4 epimer (ThnE V153I and ThnE H274A producing a d.r. > 98), further demonstrating that subtle changes in the active site can have a considerable effect on the stereochemical outcome of the reaction. Furthermore, the yield of 4,4-dimethyl-*t*-CMP (Table 3.1) by CMPSs that favoured the formation of (4*S*)-4-methyl-*t*-CMP was considerably lower than that of CarB, which produced the (4*S*)- and (4*R*)-epimers in a near 1:1 ratio. The yield of 4,4-dimethyl-*t*-CMP by CarB was approximately four to five times higher than that of CarB M108V(I). A possible explanation for these selectivities would be a steric clash between the methyl substituent on (4*R*)-4-methyl-L-GHP and the β -branched amino acids at residue 108_{CarB} seen when those CarB variants were used.

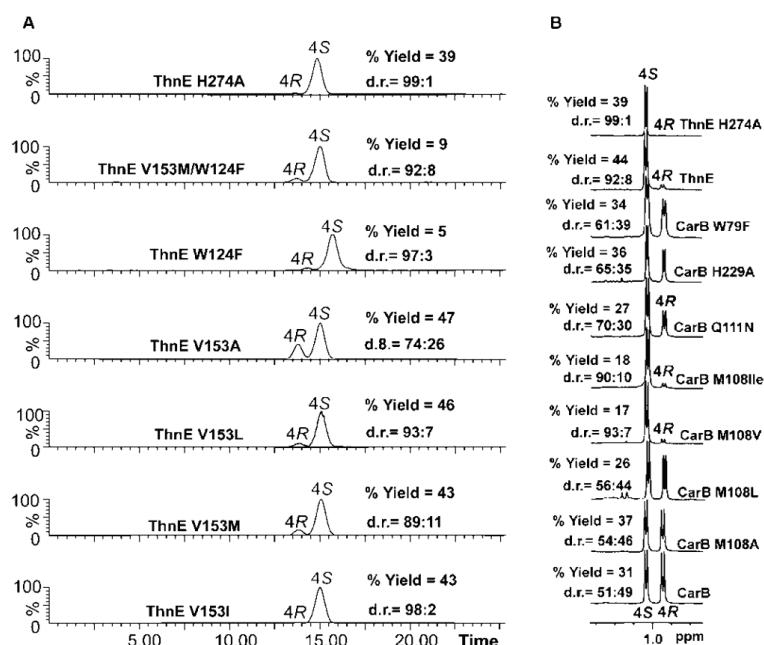


Figure 3.8. Ion extracted LC-MS chromatograms (positive electrospray ionization, A) and part of the ¹H NMR spectra (B) showing the d.r. of the C-4 epimers of 4-methyl-*t*-CMP produced from incubation of malonylCoA and (4*S*/*R*)-4-methyl-L-GHP by the shown CMPSs. Time is in minutes. NMR analysis performed by Dr. R. Hamed.

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Preparation of C-3-methylated-*t*-CMP derivatives.

All carbapenems known to date are substituted at position C-3 with complex moieties (a cysteaminyll moiety in the initial case of thienamycin, the *N*-formimidoyl derivative in imipenem, and a substituted thiopyrrolidine moiety for the most recently developed carbapenems), while those found in the equivalent position on penicillins tend to be (di)methyl substitutions.^{3, 9, 10} Therefore, it is of pharmacological interest to construct CMP-derivatives substituted at C-3. Both epimers of 3-methyl-L-GHP were obtained from commercially available L-threonine-*O*-*t*-Bu ester in seven steps (23% overall yield) by Dr. J. Mecinovic (design and synthesis) and Mr. L Henry (synthesis). Final deprotection of compounds yielded mixtures in d.r. excess of (3*S*)-3-methyl-L-GHP and (3*R*)-3-methyl-L-GHP.

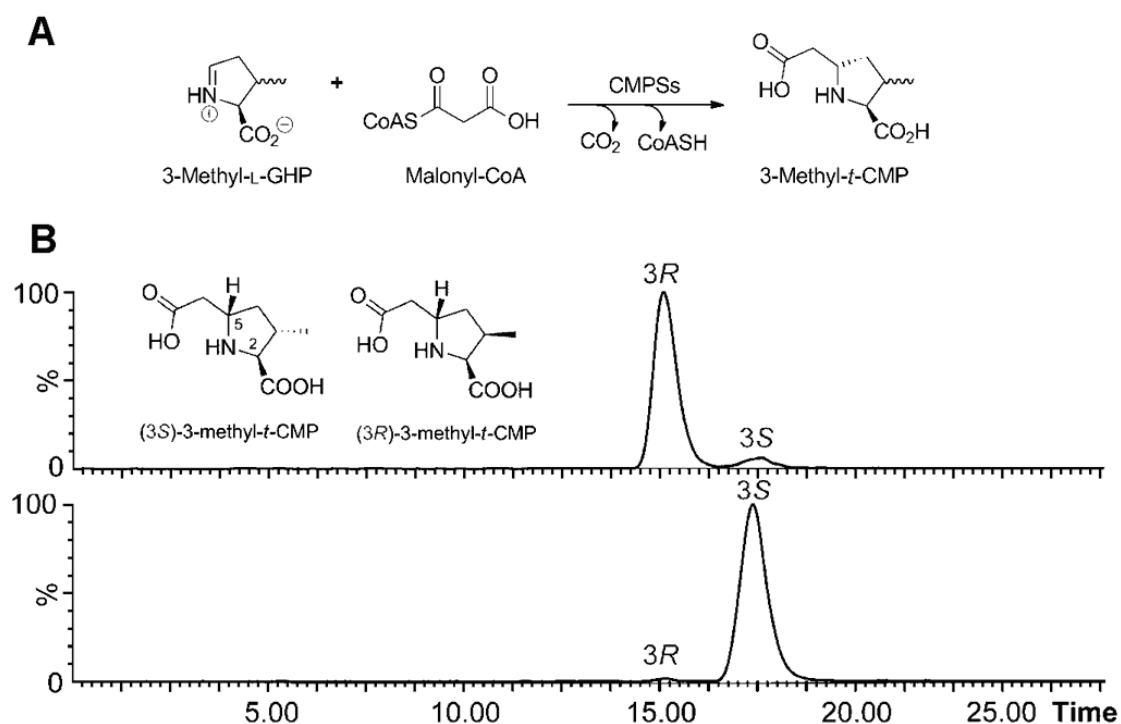


Figure 3.9. The products of CMPSs catalysis for 3-methyl-L-GHP and malonyl-CoA (A) and ion-extracted LC-MS chromatograms (positive electrospray ionization) showing the production of the two C-3 epimers of 3-methyl-*t*-CMP by wild-type CarB (B). The top chromatogram shows formation of (3*R*)-3-methyl-*t*-CMP as the major product from incubation of (3*R*)-3-methyl-L-GHP (~ 95% purity)

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and malonyl-CoA. The bottom chromatogram shows the formation of (3*S*)-3-methyl-*t*-CMP, as the major product, from incubation of (3*S*)-3-methyl-L-GHP (~ 95% purity) and malonyl-CoA. Time is in minutes.

The incubation of (3*S*)-3-methyl-L-GHP and malonyl-CoA with CMPSs variants resulted in the observation of a major chromatographic peak after LC-MS analysis at the anticipated mass of $m/z = 188$ $[M+H]^+$ (Figure 3.9). Scale up and purification of this product (catalysed by CarB, it being the catalyst affording the highest yield, see

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Table 3.3) led its isolation for further analysis and characterization by NMR to assign its C-3 stereochemistry. As before, assuming the stereochemistry at C-2 is (*S*),¹¹,¹² 2D NOESY analysis allowed for the assignment of this product as (*3S*)-3-methyl-*t*-CMP (Figure 3.10 and Figure 3.11). Likewise, incubation of (*3R*)-3-methyl-L-GHP and malonyl-CoA in the presence of CarB provided a product with a $m/z = 188$ $[M+H]^+$ (Figure 3.9) that was characterized as (*3R*)-3-methyl-*t*-CMP (Figure 3.10 and Figure 3.12,

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Table 3.3). These results demonstrate that CMPSs can catalyze the formation of C-3-substituted-*t*-CMP derivatives without loss of stereochemistry.

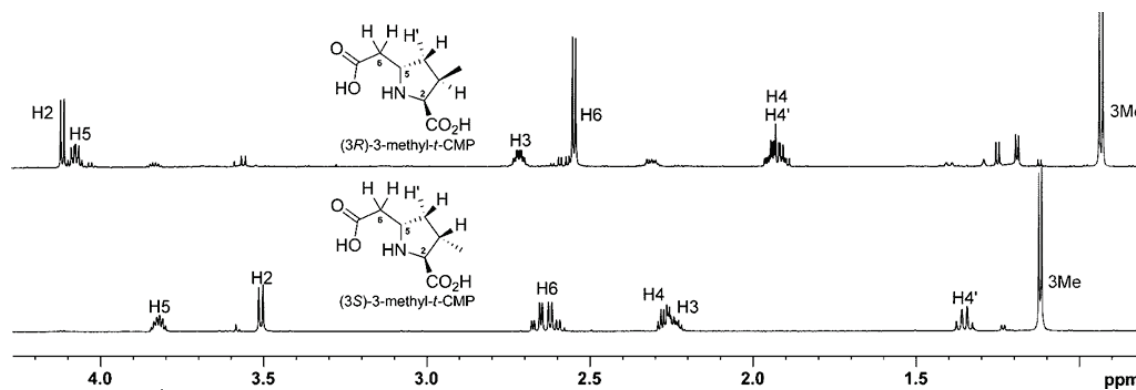


Figure 3.10. ¹H NMR spectra for (3*R*)- and (3*S*)-3-methyl-*t*-CMP by catalysis of wild-type CarB. NMR analysis performed by Dr. R. Hamed.

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Table 3.3. The percent overall yield (% , isolated) of (3*S*)-3-methyl-*t*-CMP and (3*R*)-3-methyl-*t*-CMP by CMPSSs. Determined as reported.⁷

Variant	% yield of (3 <i>S</i>)-3-methyl- <i>t</i> -CMP	% yield of (3 <i>R</i>)-3-methyl- <i>t</i> -CMP
CarB	16	12
CarB M108A	13	7
CarB M108L	15	4
CarB M108V	12	6
CarB M108I	14	5
CarB Q111N	10	4
CarB H229A	13	4
CarB W79F	15	4
CarB W79F M108A	12	4
CarB W79A	6	4
ThnE	9	2
ThnE V153A	11	4
ThnE V153L	12	4
ThnE V153M	12	4
ThnE V153I	9	4
ThnE W124F	2	2
ThnE W124F V153M	3	3
ThnE H274A	5	4

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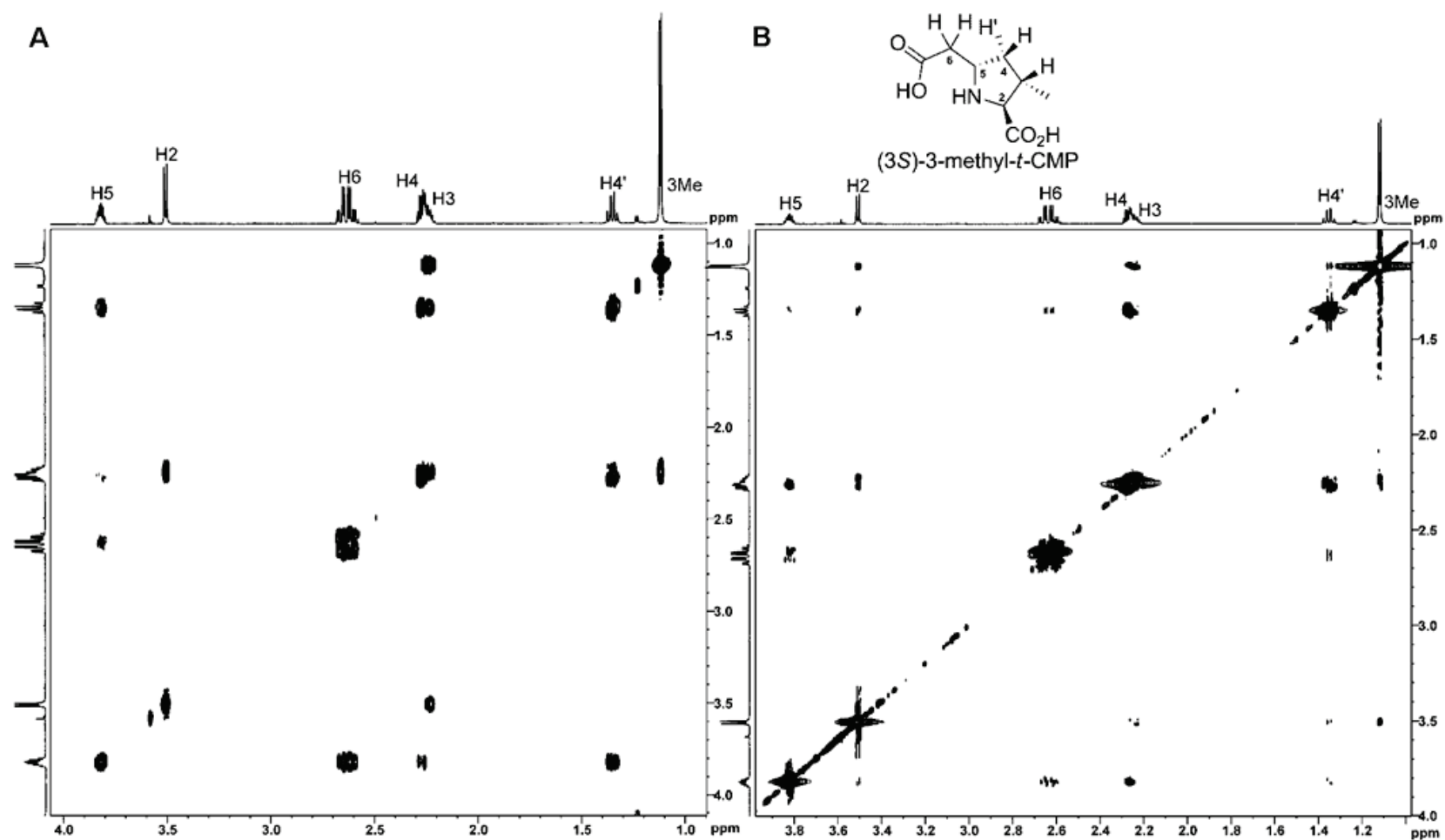


Figure 3.11. ^1H - ^1H COSY (A) and NOESY (B) spectra for (3S)-3-methyl-t-CMP produced from incubation of the pair containing (3S)-3-methyl-L-GHP and malonyl-CoA by CarB catalysis. NMR analysis performed by Dr. R. Hamed.

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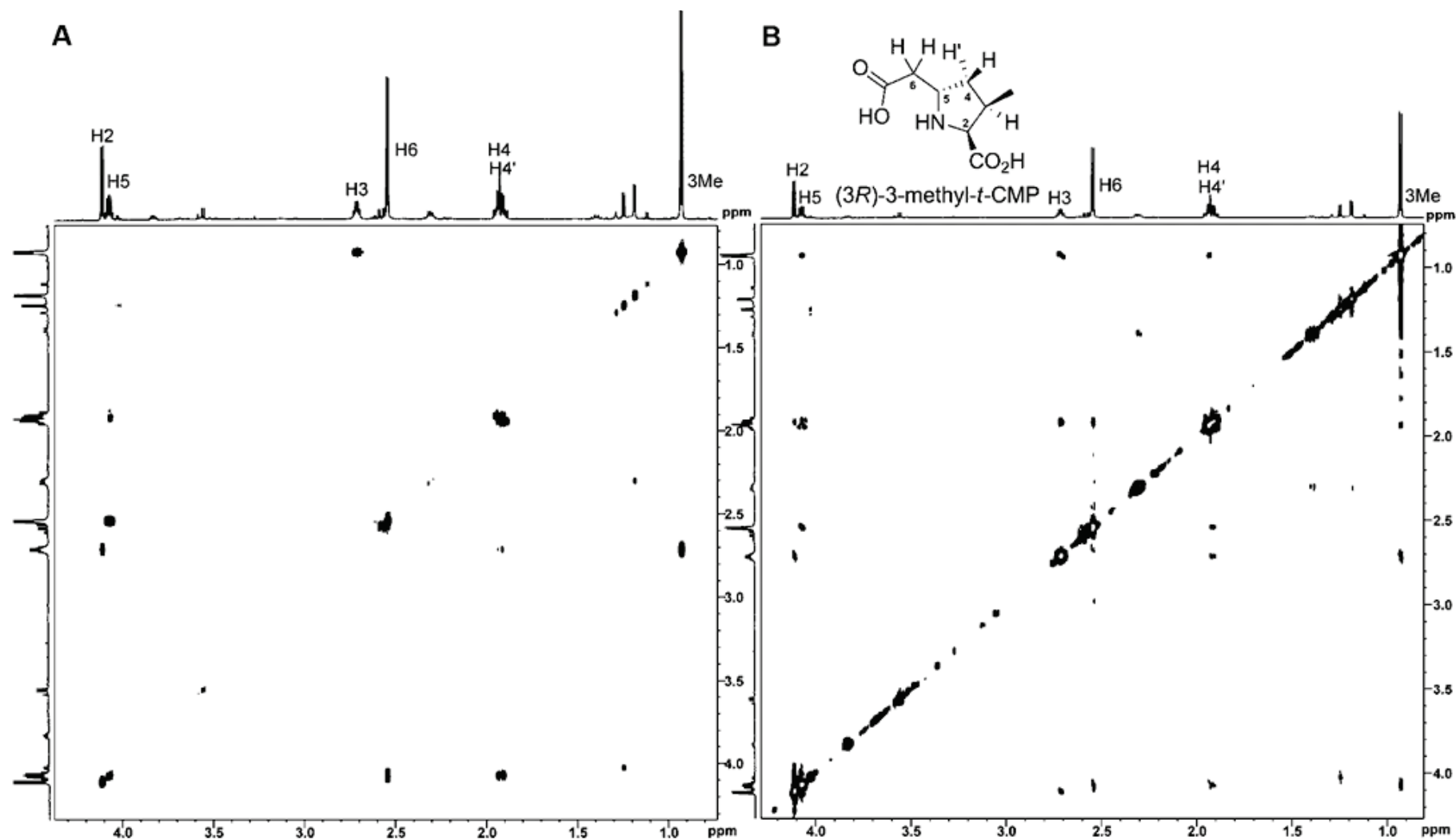


Figure 3.12. ¹H-¹H COSY (A) and NOESY (B) spectra for (3R)-3-methyl-*t*-CMP produced from incubation of the pair containing (3R)-3-methyl-L-GHP and malonyl-CoA by CarB catalysis. NMR analysis performed by Dr. R. Hamed.

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Preparation of C-2 and C-5-methylated-t-CMP derivatives; formation of quaternary centres by biocatalysis.

The construction of quaternary stereocentres has been identified as a significant challenge in the synthesis of natural products. Also, as noted previously the stability and antibacterial activity of carbapenems is increased by the introduction of substituents in positions C-1, C-2 and C-6. Therefore, it was investigated whether the array of CMPSs prepared so far could catalyze the production of heterocycles with asymmetric quaternary centres at positions C-2 and C-5.

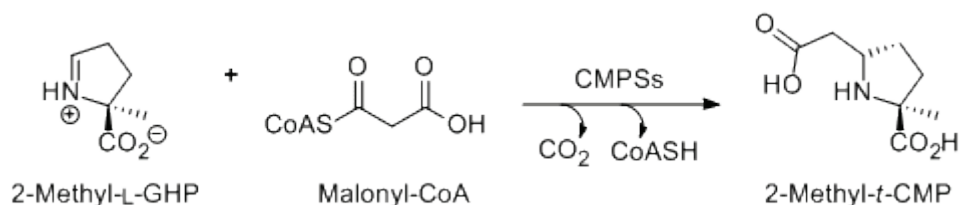


Figure 3.13. The product of CMPSs catalysis for 2-methyl-L-GHP and malonyl-CoA.

(2*R/S*)-2-methyl-GHP was synthesized in 6 steps (29% overall yield) by Dr. C. Ducho. The catalytic capability of the available CMPSs was tested with 2-methyl-L-GHP and malonyl-CoA. Products of catalysis were analysed by LC-MS, revealing a product with a $m/z = 188$ $[M+H]^+$. CarB H229A was observed to be the highest-yielding variant (Figure 3.14 and Table 3.4).

Table 3.4. The percent overall yield (% isolated) of 2-methyl-t-CMP produced by the best CMPSs.

Variant	% isolated yield of 2-methyl-t-CMP
CarB	3
CarB H229A	16
ThnE	≥ 1

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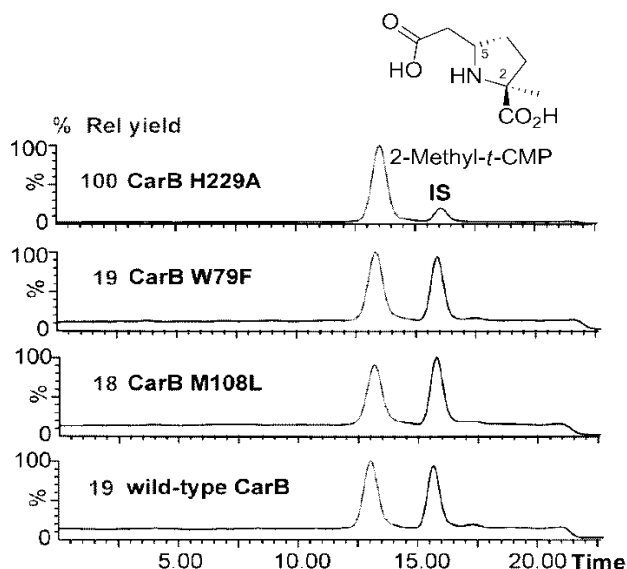


Figure 3.14. Ion-extracted LC-MS chromatograms (positive electrospray ionization) displaying the relative yields for the formation of (2*S*)-2-methyl-*t*-CMP from racemic 2-methyl-GHP and malonyl-CoA catalyzed by the shown CMPSs. ThnE V153L, ThnE V153M, ThnE H274A, and ThnE V153A also produce (2*S*)-2-methyl-*t*-CMP, and the relative yields are 17%, 13%, 12%, and 7%, respectively. *p*-Aminosalicylic acid is used as an internal standard (IS, t_R ca. 15:00 min). Time is in minutes.

Scale-up and purification of this reaction, followed by 2D NOESY NMR analysis revealed a *trans*-relationship between H-5 and the methyl group at C-2 of the product (Figure 3.15). Therefore, the product of CarB H229A catalysis, assigned as (2*S*)-2-methyl-*t*-CMP, demonstrates the capability of CMPSs to catalyze the formation of *t*-CMP derivatives with a quaternary centre at C-2, while at the same time eliminates the proton-abstraction mechanism at C-2 as an option for these substrates.

The high yield in the case of CarB H229A can be attributed initially to an increased active site volume that enables productive binding of the quaternary C-2 stereocentre (Figure 3.16). CarB H229A provides access to two *t*-CMP analogues, (2*S*)-2-methyl-*t*-CMP and (2*S*,7*S*)-7-(carboxymethyl)azepane-2-carboxylic acid,⁷ both not efficiently produced by any other of the CMPSs available. The common factor between the starting materials of the two products (2-methyl-L-GHP and L-aminopimelate semialdehyde respectively) is their increased steric demand compared to the natural substrate L-GHP.

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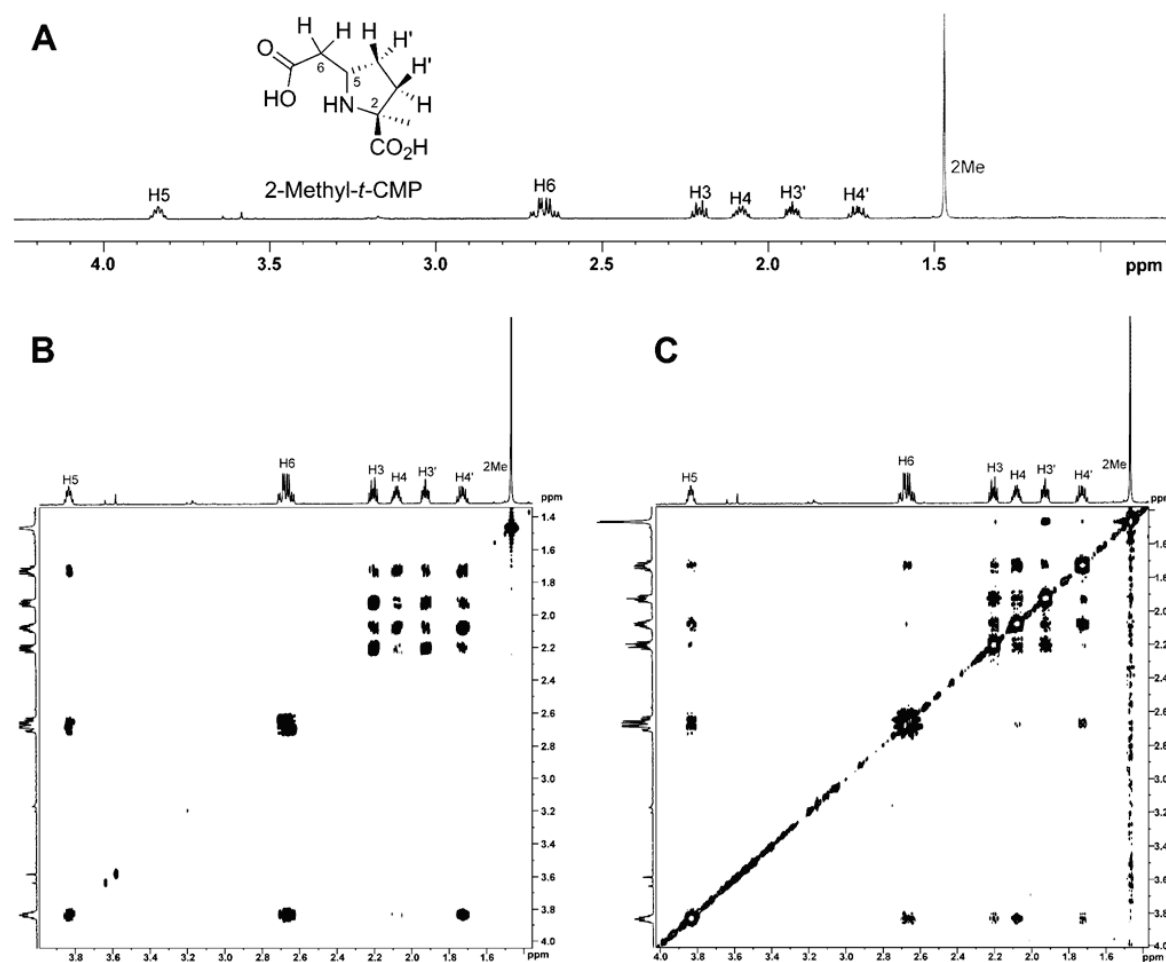


Figure 3.15. ^1H NMR (A) ^1H - ^1H COSY (B) and NOESY (C) spectra of 2-methyl-*t*-CMP derivatives resulting from incubation of malonyl-CoA and 2-methyl-L-GHP in the presence of CarB H229A under standard conditions. Assuming the stereochemistry at C-2 is (*S*), based on the reports that wild-type CarB and ThnE only accepts L-GHP as a substrate,^{11, 12} the stereochemistry at C-5 must be (*S*) based on the absence of any detectable NOE correlation between H-5 and the methyl group at C-2, together with other NOE correlations between protons at C-3 and C-4. NMR analysis performed by Dr. R. Hamed.

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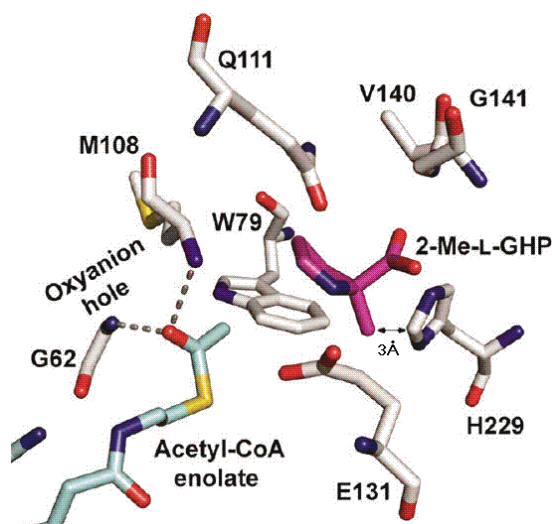


Figure 3.16. A view derived from a CarB crystal structure with the acetyl-CoA enolate and the imine form of 2-methyl-L-GHP modelled into the active site. His 229 is predicted to be one of the closest residues to the imine form of 2-methyl-L-GHP ($\sim 3\text{ \AA}$ from the τ -nitrogen of His 229 to the methyl group at C-2). Figure prepared by Dr. R. Hamed.

Finally, to test the production of C-5 methylated *t*-CMP, 5-methyl-L-GHP was synthesized by Dr. C. Ducho. Screening of the CMPS variants led to the identification of ThnE V153A as the one with the highest relative yield (Figure 3.18).

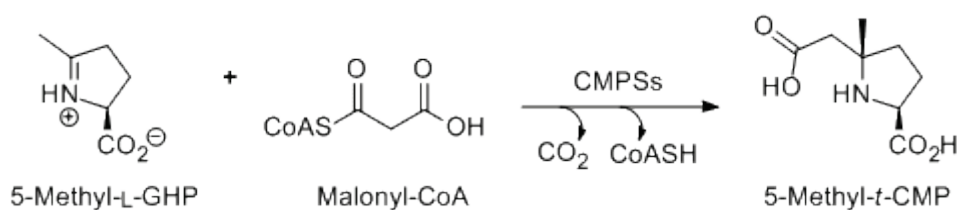


Figure 3.17. The product of CMPSs catalysis for 5-methyl-L-GHP and malonyl-CoA.

With CarB and ThnE yields for the formation of 5-methyl-*t*-CMP were null or very low respectively, possibly due to steric hindrance.¹³

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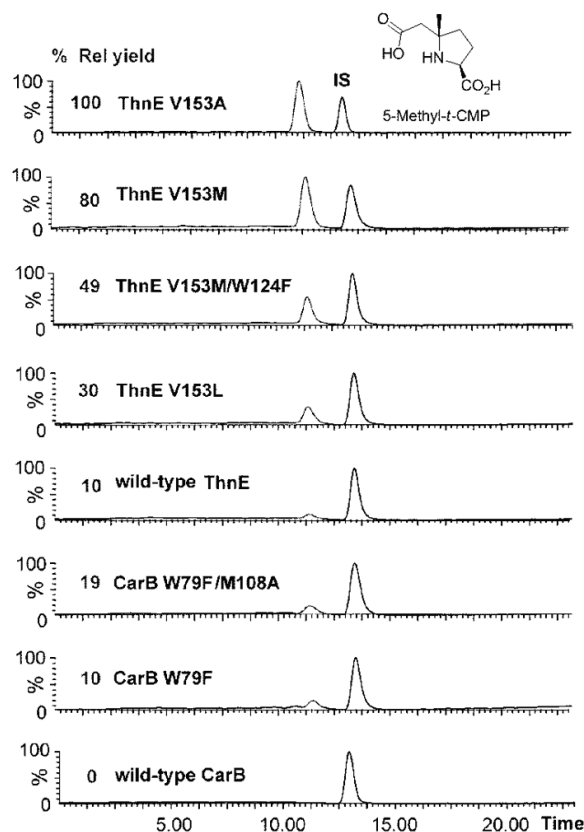


Figure 3.18. Ion extracted LC-MS chromatograms (positive electrospray ionization) showing the relative yields for the formation of (5*S*)-5-methyl-*t*-CMP resulting from the incubation of 5-methyl-L-GHP under CMPSs catalysis. ThnE H274A and ThnE W124F also produce (5*S*)-5-methyl-*t*-CMP, to 7% and 3% relative yield, respectively. *p*-Aminosalicylic acid is shown as an internal standard (IS) eluting consistently around 13:00 min.

The yield of ThnE V153A variant was ~ 10 times higher than that of the wild-type revealing that substitution can alter the yield substantially. Scale-up and purification of the ThnE V153A product of catalysis led to the isolation of 5-methyl-*t*-CMP, characterized by the presence of an AB quartet for the diastereotopic H-6 protons (δ_{H} 2.47, 2.53, $J = 16$ Hz) in its ^1H NMR spectrum (Figure 3.19 A). 2D NOESY analyses (Figure 3.19) reveal a *trans*-relationship between H-2 and the methyl group at C-5, identifying the product as (5*S*)-5-methyl-*t*-CMP, based on the absence of any detectable NOE correlation between H-3' and the methyl group at C-5 (Figure 3.19). It is notable that CMPS variants can accommodate such an increase in the steric bulk adjacent to the site of C-C bond formation.

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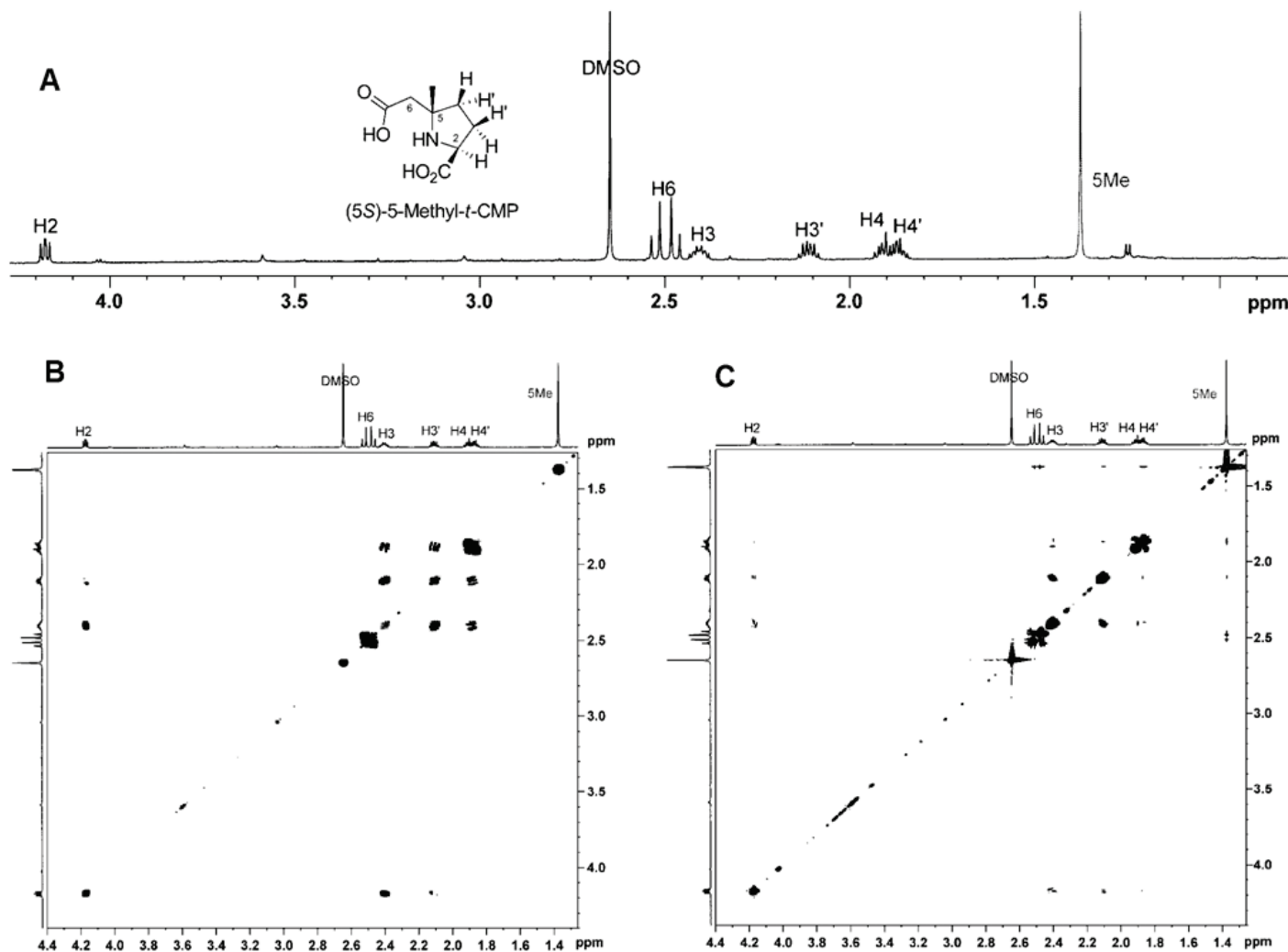


Figure 3.19. ^1H NMR (A), ^1H - ^1H COSY (B) and NOESY (C) spectra for (5S)-5-methyl-*t*-CMP produced from incubation of 5-methyl-L-GHP and malonyl-CoA by ThnE V153A catalysis. NMR analysis performed by Dr. R. Hamed.

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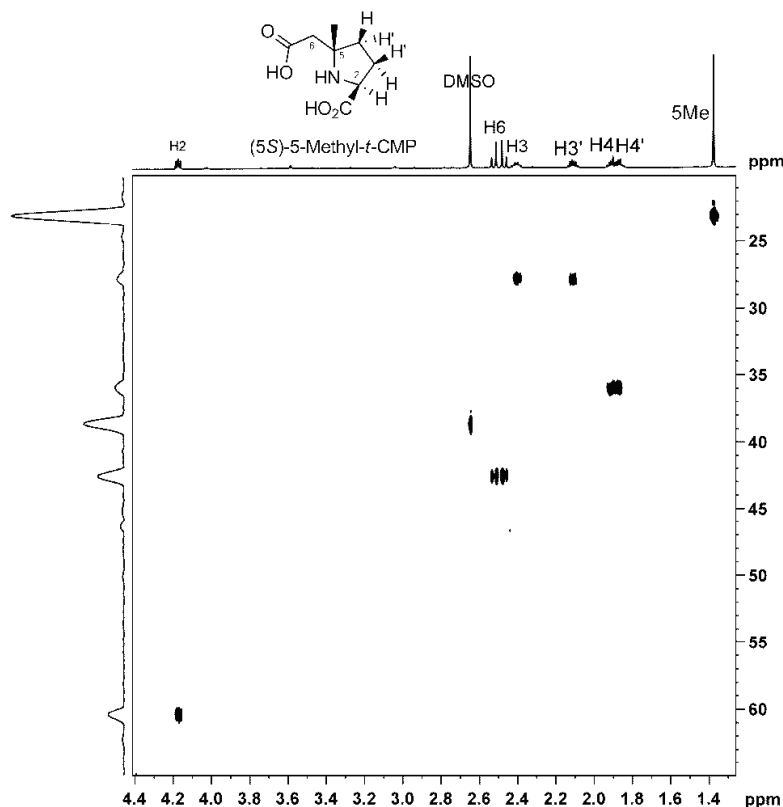


Figure 3.20. ^1H - ^{13}C HSQC spectra for (5*S*)-5-methyl-*t*-CMP produced from incubation of 5-methyl-L-GHP and malonyl-CoA by ThnE V153A catalysis. NMR analysis performed by Dr. R. Hamed.

Conversion of the prepared *t*-CMP derivatives into bicyclic β -lactams.

The seven carboxymethyl-functionalized *N*-heterocycles generated by CMPSs catalysis were tested as substrates for the β -lactam ring-forming enzyme carbapenam synthetase (CarA) from *P. carotovorum*.^{14, 15} CarA catalyzed the conversion of all tested *t*-CMP derivatives to the respective bicyclic β -lactam derivatives as demonstrated by LC-MS analysis (Figure 3.23).

In the case of the CarA reaction of a $\sim 1:1$ mixture of the C-4 epimers (4*S*)- and (4*R*)-4-methyl-*t*-CMP produced by CarB catalysis, CarA exhibited higher diastereoselective bias towards the (4*S*)-epimer as a substrate (d.r. of products = 24:76

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(1*R*)-:(1*S*)-1-methylcarbapenam as determined by LC-MS under standard conditions (Figure 3.21)

The major product of CarA catalysis, (1*S*)-1-methylcarbapenam has the same stereochemistry at C-4 as the commercially available carbapenems showing an improved stability against dehydropeptidases.¹⁶

6,6-Dimethyl-*t*-CMP, a product of CMPS catalysis previously described in our group,¹⁷ was also found to be a substrate of CarA, producing the expected 6,6-dimethyl carbapenam product (scheme 3vii Figure 3.23). Both dimethylated product described here 4,4- and 6,6-dimethyl carbapenams were scaled up and purified and their structures were confirmed by NMR analyses (Figure 3.24, Figure 3.25 and Figure 3.26).

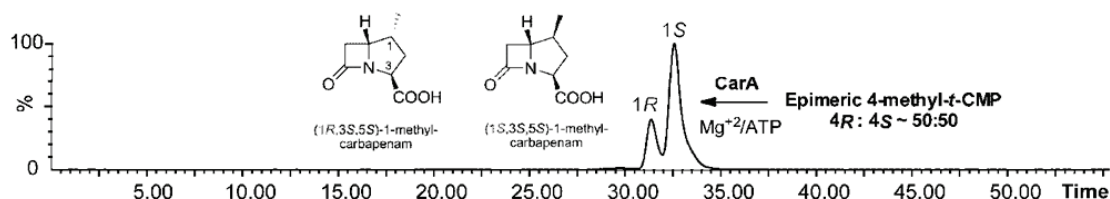


Figure 3.21. Selectivity of CarA for the production of (1*S*)-1-methyl-carbapenam. Ion extracted chromatogram ($m/z = 168$, negative electrospray ionization) showing the CarA-catalysed conversion of an epimeric mixture of (4*R/S*)-4-methyl-*t*-CMP (1:1) into the corresponding 1-methyl-carbapenams (1*R*:1*S*, 24:76). Time is in minutes.

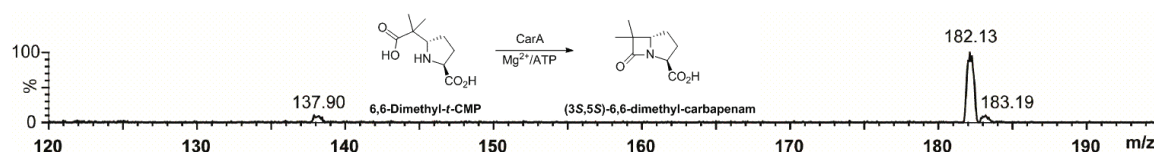


Figure 3.22. Mass spectrum (negative electrospray ionization) for (3*S*,5*S*)-6,6-dimethyl carbapenam product of catalysis of CarA and 6,6-dimethyl-*t*-CMP.

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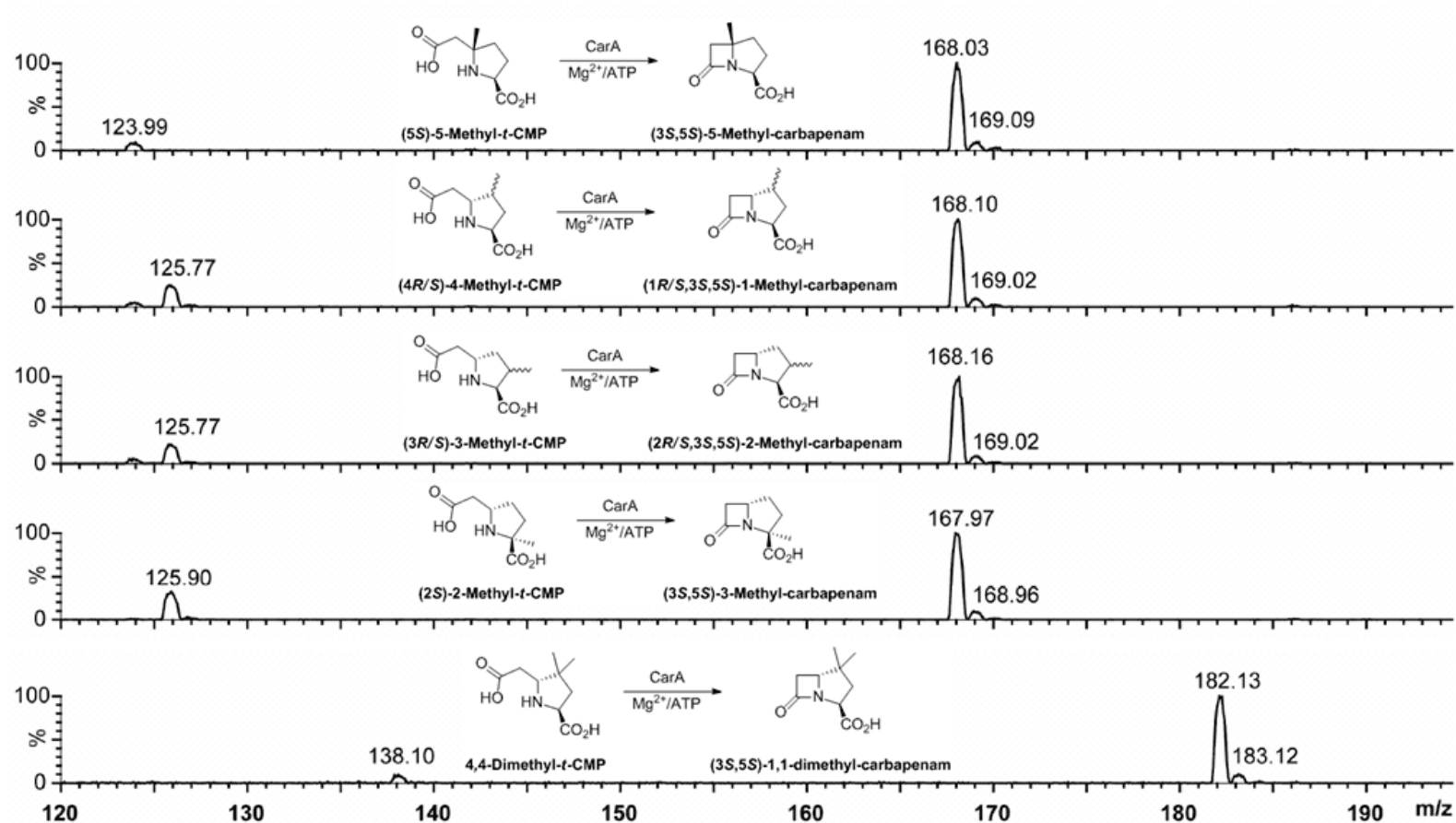


Figure 3.23. Mass spectra (negative electrospray ionization) for the substituted-carbapenams produced by CarA-catalysis from the corresponding substituted-*t*-CMPs.

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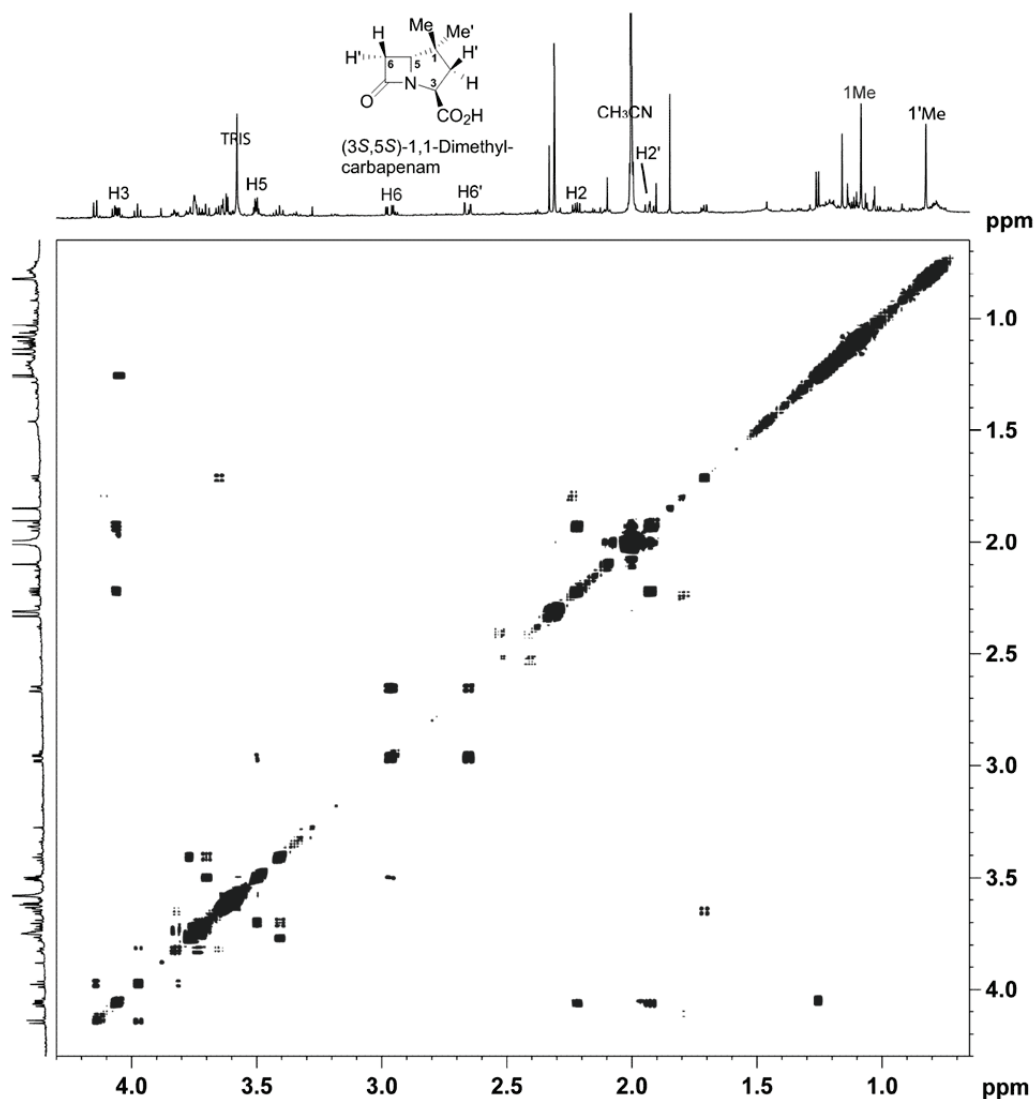


Figure 3.24. ^1H - ^1H COSY of (3S,5S)-1,1-dimethyl-carbapenam produced from 4,4-dimethyl-*t*-CMP by CarA catalysis. The assignment of the β -lactam protons is based on the coupling constant value to H-5 where $J_{5,6} = 4.7$ Hz (consistent with a *cis*- relationship) and $J_{5,6'} = 1.7$ Hz (consistent with a *trans*-relationship). NMR analysis performed by Dr. R. Hamed.

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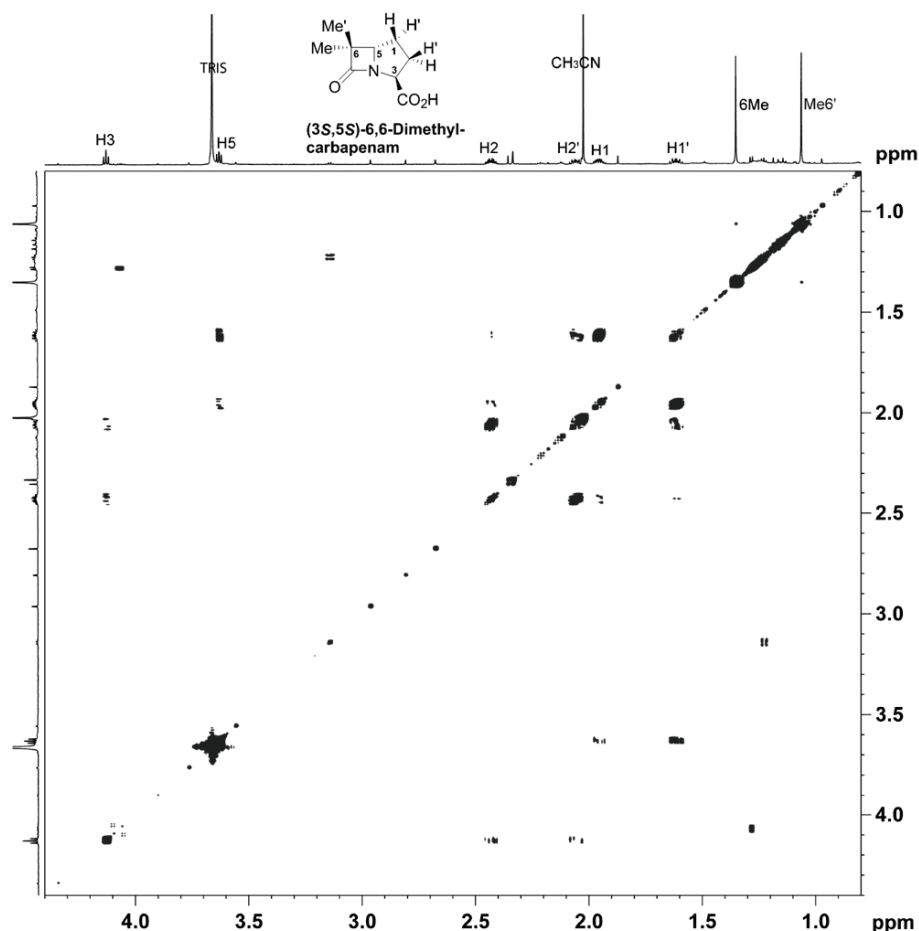


Figure 3.25. ^1H - ^1H COSY for (3*S*,5*S*)-6,6-dimethyl-carbapenam produced from 6,6-dimethyl-*t*-CMP by CarA catalysis. NMR analysis performed by Dr. R. Hamed.

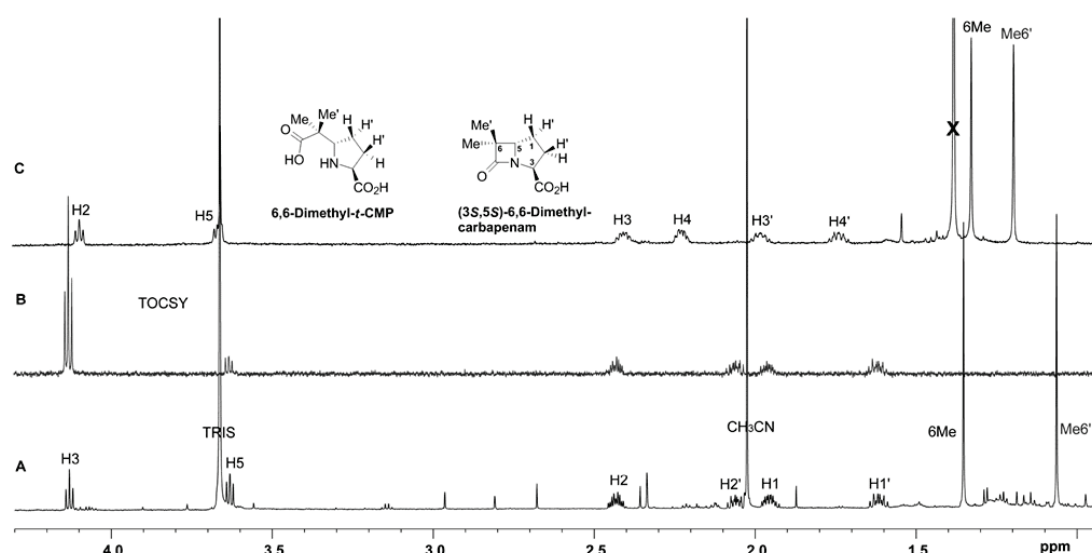


Figure 3.26. Formation of stable carbapenams from disubstituted-*t*-CMP derivatives by CarA catalysis. A: ^1H NMR spectrum of (3*S*,5*S*)-6,6-dimethyl-carbapenam; B: 1D TOCSY ($\tau_m = 150$ ms) for 6,6-dimethyl-*t*-CMP obtained by selective excitation of H-3; C: ^1H NMR spectrum of 6,6-dimethyl-*t*-CMP produced by incubation of L-GHP and dimethylmalonyl-CoA by CarB catalysis. NMR analysis performed by Dr. R. Hamed.

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Although the yields obtained for the methylated bicyclical carbapenams varied, these results demonstrate the capability of CarA for converting substrate analogues of *t*-CMP and it is likely that yields and diastereoselectivities may be improved by further residue manipulation in the active site. These results further expand the scope of substrates for CarA which has been shown to accept at least three of the four possible isomers of CMP.¹⁴

NMR studies on the bicyclical products of CarA catalysis have revealed that functionalization with two methyl groups at C-6 or C-4 has a significant stabilizing effect on the carbapenams with respect to the rate of β -lactam hydrolysis: the $t_{1/2}$ for (3*S*,5*S*)-6,6-dimethyl-carbapenam was ~ 2 weeks, and that of (3*S*,5*S*)-1,1-dimethyl-carbapenam was 2 days (as determined by ¹H NMR analyses at 4 °C and pH 7) as compared to the half-life of carbapenam. Although studies were not carried out on the stability of 5-methylcarbapenam prepared by enzymatic catalysis, 5-methylcarbapenam prepared by total synthesis has been shown to pose a higher degree of stability against hydrolysis (*e.g.* by renal dehydropeptidase I) compared to the analogous 5-unsubstituted carbapenams though possessing low antibiotic activity.^{17, 18} Thus CMPS/CarA may be used to prepare bicyclic β -lactams with improved stabilities.

Conclusions.

The results presented here demonstrate the capacity of CMPSs for the preparation of *t*-CMP derivatives functionalized at all four carbon positions of the proline ring, including the sterically demanding C-2 and C-5 positions. The use of active site variants enabled the generation of CMPS-catalysts operating with much higher yields than wild-type enzymes (*e.g.* C-2 C-5). Certain variants are also capable of the stereoselective product formation including *via* preferential conversion of one diastereomer from an

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equilibrating mixture of (4*S/R*)-4-methyl-L-GHP, likely *via* a dynamic kinetic resolution process (which will be further studied in Chapter 5 of this dissertation). It is notable that single residue substitutions in the wild-type CarB/ThnE active sites can lead to such a substantial improvement in yield and/or diastereoselectivity, and it is plausible that further improvements can be achieved if CMPSs are the subject of commercially focused work. Such studies would also likely involve the use of modified enzymes in cell cultures, due to the costs of coenzyme A derivatives. Despite these limitations, CMPS catalysis *in vitro* has proved to be a most useful way for the preparation of functionalized prolines which are suitable for further modification as exemplified by the production of functionalized bicyclical β -lactams from the *t*-CMP products catalysed by CarA. Such compounds are of interest with respect to inhibition of nucleophilic enzymes and are not readily accessible *via* synthesis, a limitation that has hindered studies on their biological activities and chemical properties.

Proline analogues, including some of the ones described here, are constituents of numerous natural products,¹⁹ such as siderochelin A,²⁰ bottromycin,²¹ roseotoxin B,^{22, 23} kainic acid,²⁴⁻²⁶ and lactacystin.^{27, 28} Proline derivatives are also used as pharmaceuticals, such as captopril and saxagliptin, and in biomedical research; *e.g.*, α -substituted prolines are used as templates in structure-function relationship studies directed towards elucidation of biologically active conformations.^{29, 30} In organic synthesis, proline and its analogues are also used as catalysts for asymmetric synthesis.³¹

Furthermore, β -lactams are finding new therapeutic applications in addition to their established use as antibiotics, such as protease inhibitors³² (*e.g.* elastase), cholesterol absorption inhibitors³³ (*e.g.*, ezetimibe, a monobactam³⁴), as a delivery vehicle for

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anticancer drugs,³² and to regulate levels of the excitatory neurotransmitter glutamate.³⁵ Therefore, these results have an added importance in the effort to expand our access to such novel β -lactam molecules.

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Chapter 4. Dual Enzyme-controlled Enolate formation and Reactivity. The Combined use of Carboxymethylproline Synthases and Crotonyl-CoA Carboxylase/Reductase, Malonyl-CoA Synthetase, or Methylmalonyl-CoA Epimerase.

In Chapter 2, evidence was presented that the CMPS variants CarB M108V and CarB M108I displayed high selectivity towards the (6*R*)-epimer of 6-methyl-*t*-CMP (95:5 and 92:8, 6*R*:6*S*, respectively; Table 2.2). Conversely the CMPS variants ThnE V153A and ThnE V153L revealed selectivity towards the (6*S*)-epimer (9:91 and 10:90, 6*R*:6*S*, respectively; Table 2.3).

Although these ratios represent significant improvements on those achieved with wild-type CarB and ThnE, if the ratios could be further improved, it could enhance the possibility of them being used in an industrial scale. One way to achieve this might be by using a dynamic kinetic resolution process. Dynamic kinetic resolution¹⁻⁶ occurs when two stereoisomers in a mixture are able to epimerize easily (resulting, in essence, in a racemic, or epimeric, starting material mix at all points during the reaction), react with different reaction rates, resulting in an enantioenriched sample of the less reactive enantiomer. This allows the species with the lower activation barrier to form in a theoretical yield of up to 100%. This contrasts with a standard kinetic resolution, which necessarily has a maximum yield of 50%. For this reason, dynamic kinetic resolution has had practical applications to catalysis, as long as sufficient levels of efficiency are

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reached, as defined by a high selectivityⁱ ($E > 50$),⁷⁻⁹ and a racemisation/epimerization constant (k_{rac}) greater than the resolution constant (k_A).⁹ For the reaction catalysed by CMPSs, alkylmalonyl-CoA would be such a starting material for dynamic kinetic resolution. As described in Chapter 2, the kinetics of the epimerization of methyl- and ethylmalonyl-CoA greatly affect the rate of the formation of the preferred product of catalysis. If the epimerization rate of alkylmalonyl-CoA products could be better controlled, it may enable a kinetic dynamic resolution process to enhance the diastereoselectivity of CMPSs (Figure 4.1).

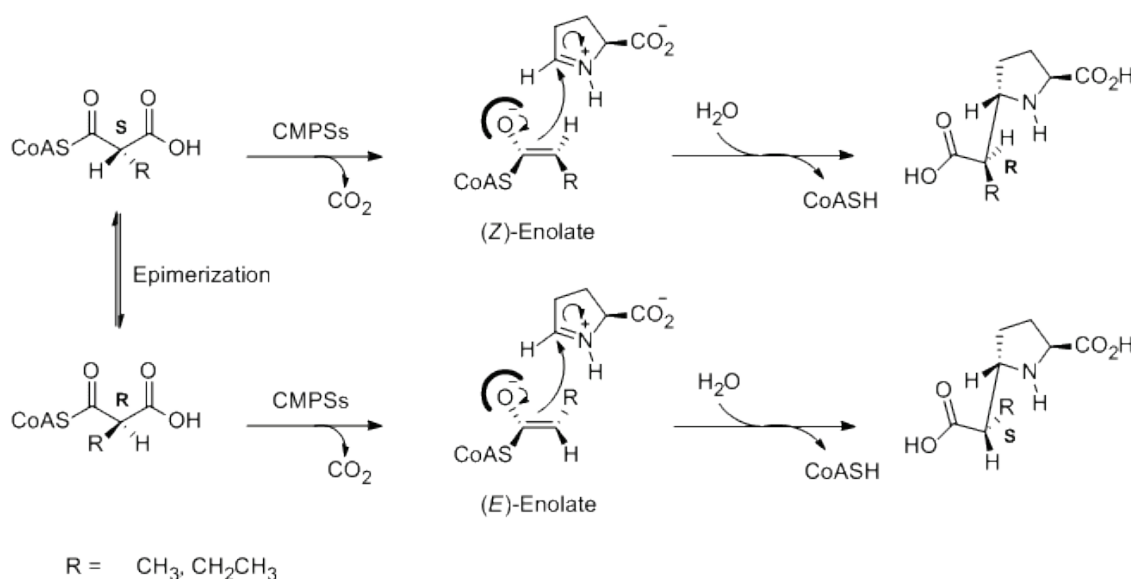


Figure 4.1. Proposed mechanism for the CMPS-catalyzed formation and reaction of enolates. The decarboxylation of a specific alkylmalonyl-CoA enantiomer is proposed to give rise to either the (*E*)- or (*Z*)-enolates, which later react with the imine form of L-GHP/L-AASA to give a specific diastereomer of the carboxymethyl-substituted *N*-heterocycle. Altering the rate of epimerization of the alkylmalonyl-CoA compound would affect the yield of the given diastereomer, in a dynamic kinetic resolution process. The possibility of limited conversion of (2*R*)-methylmalonyl-CoA to the (*Z*)-enolate, and (2*S*)-2-methylmalonyl-CoA to the (*E*)-enolate cannot be eliminated.

Methylmalonyl-CoA epimerase (MCE) is an enzyme of widespread importance in nature. MCE is responsible for the conversion of (2*S*)-methylmalonyl-CoA into (2*R*)-

ⁱ The dimensionless *E* value describes the selectivity of a resolution and remains constant throughout the reaction.

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methylmalonyl-CoA (Figure 4.2). Vertebrates employ MCE for the catabolism of compounds *via* propionyl-CoA (*e.g.*, metabolites of valine, isoleucine, methionine, threonine, thymine and cholesterol).¹⁰ In humans, defects of MCE can lead to methylmalonic aciduria, a rare but severe autosomal, recessive, genetic disease.¹¹⁻¹³ Among bacteria, MCE is involved in propionate fermentation, autotrophic CO₂ fixation *via* the 3-hydroxypropionate pathway, the regeneration of glyoxylate, and the biosynthesis of polyketide antibiotics.¹⁴⁻²⁰

The activity of MCE is involved in the conversion of C₃-units (propionyl-CoA) into substrates of the tricarboxylic pathway (Figure 4.2). (2*S*)-Methylmalonyl-CoA is a metabolite of some branched chain amino acids, as well as of thymine and cholesterol. All these degradative pathways proceed *via* propionyl-CoA which is in turn converted to (2*S*)-methylmalonyl-CoA by propionyl-CoA carboxylase; the latter is then isomerised into (2*R*)-methylmalonyl-CoA by the action of MCE.²¹

Human MCE (the product from the *MCEE* gene¹³) was kindly provided by Dr. W. Yue at the Nuffield Department of Medicine, University of Oxford, in 20 mg/mL aliquots in 10mM HEPES-NaOH, pH 7.5 buffer supplemented with 500 mM NaCl and 5% glycerol (v/v). The *mce/pET28b* construct (from *Streptomyces coelicolor*) was a generous gift from Prof. A. Keating-Clay at the University of Texas at Austin; bacterial MCE was produced following a published procedure²² and buffered under the same conditions as human (see Methods). The effects of both MCEs on CMPSs catalysis were investigated. It was predicted that co-incubation of MCE with CMPSs would enhance the diastereoselectivity of the CMPS variant in use, by affecting the rate of epimerization of methylmalonyl-CoA in a dynamic kinetic resolution process (Figure 4.3).

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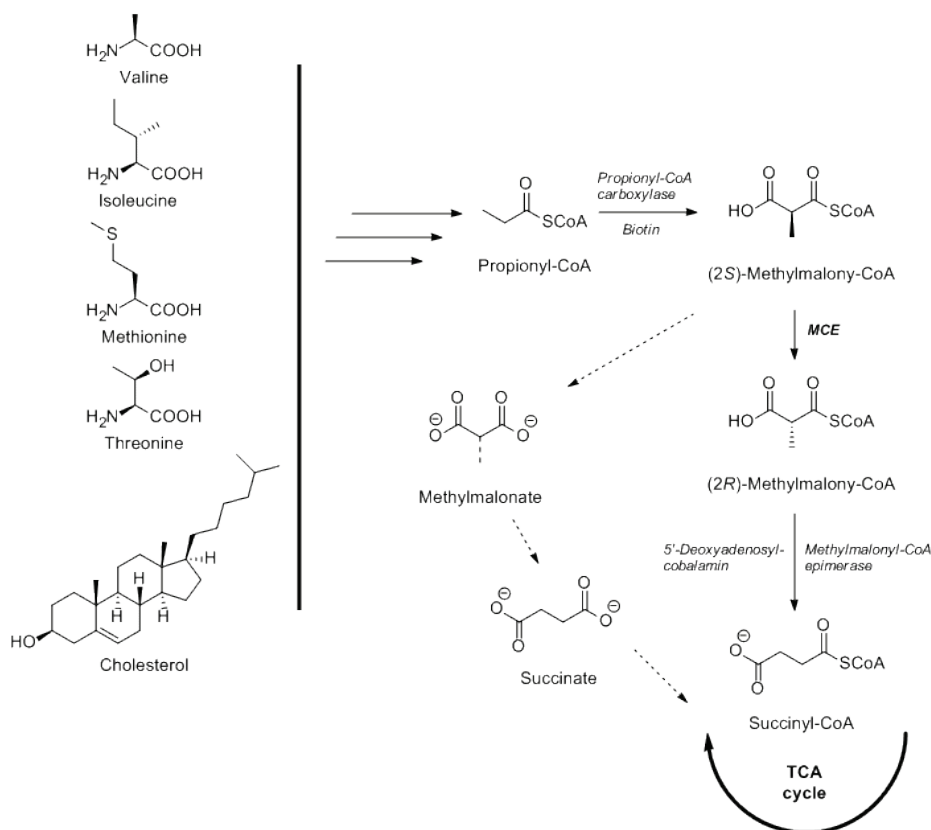


Figure 4.2. Reactions involved in the conversion of C₃-units into substrates of the tricarboxylic (TCA) cycle.²¹ The hatched line represents a hypothetical pathway. Figure adapted from 21.

A standard CMPS assay was supplemented with 1.32 μmol of bacterial or human MCE, in co-incubation with CMPSs or with an MCE pre-incubation time of 30 min (see Methods). CarB, CarB M108V(I) and ThnE V153A were the CMPSs variants tested. The co-incubation of human or bacterial MCE with CMPSs resulted in an enhancement of the diastereoselectivity of the variants that had the best yield of 6-methyl-*t*-CMP, with CarB M108V producing $\sim 99:1$ (6*R*:6*S*), compared to 95:5 (6*R*:6*S*) without MCE, as shown by LC-MS and NMR analyses (Figure 4.3). ThnE V153A produced 4:96 (6*R*:6*S*), compared to 9:91 (6*R*:6*S*) without MCE, as shown by LC-MS and NMR analyses (Figure 4.3). These results also strengthen the proposal that the stereochemistry at C-2 of the alkylmalonyl-CoA substrate governs that of C-6 in the 6-alkyl-*t*-CMP product.

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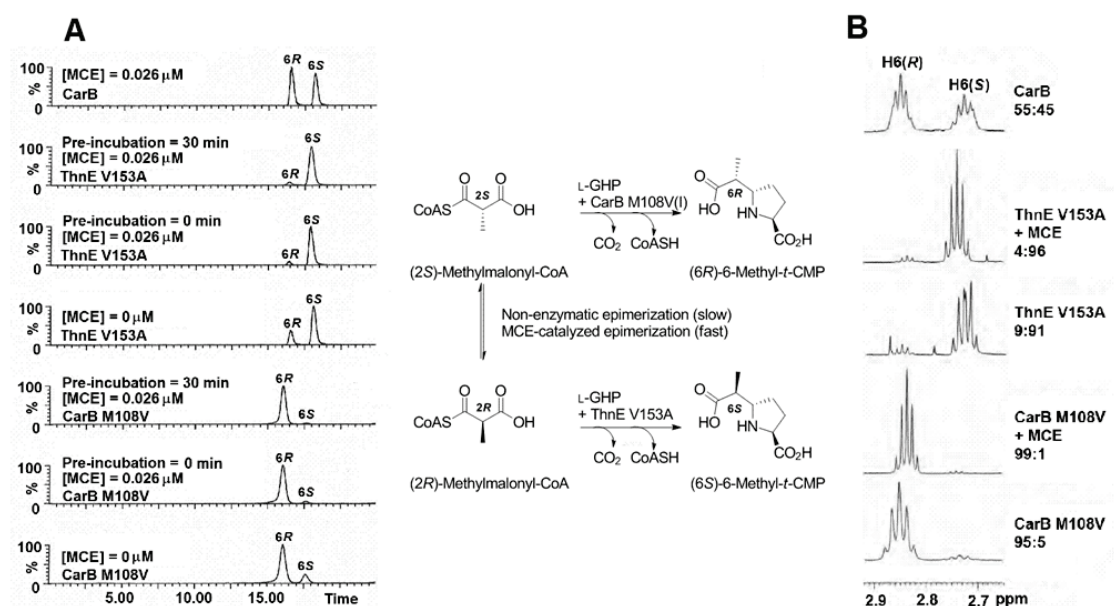


Figure 4.3. Enzymatic epimerization of methylmalonyl-CoA by MCE vs. non-enzymatic epimerization. A. Ion extracted LC-MS chromatograms. B. Part of the ^1H NMR spectra showing the effect of MCE against the ratio of production of (6R)- and (6S)-6-methyl-t-CMP by CarB, CarB M108V and ThnE V153A. Note the enhancement in the diastereomeric excess upon addition of MCE to the CMPS reaction.

Coupling of crotonyl-CoA carboxylase/reductase or malonyl-CoA ligase with CMPSs catalyses to enhance the diastereoselectivity of (6R)- or (6S)-6-alkylmalonyl-CoA derivatives, respectively.

Another possible way for the stereocontrol of the product of CMPS catalysis is to control the stereochemistry of the alkylmalonyl-CoA substrate. Two enzymes were investigated that could aid in this respect: crotonyl-CoA carboxylase reductase (Ccr) and malonyl-CoA ligase (MatB)

Ccr, a member of the medium-chain dehydrogenase/reductase superfamily, is a key enzyme in the ethylmalonyl-CoA pathway.²³ The ethylmalonyl-CoA pathway has been identified as an alternative to the glyoxylate cycle as the path towards the synthesis of cell constituents from C_2 -units.^{23, 24} In the ethylmalonyl-CoA pathway Ccr employs (*E*)-

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crotonyl-CoA, reduced nicotinamide adenine dinucleotide phosphate (NADPH) as reductant, and either CO_2 or HCO_3^- as a carboxylating agent to yield (2*S*)-2-ethylmalonyl-CoA and NADP^+ (Figure 4.4); Ccr has also been observed to reduce (*E*)-crotonyl-CoA to butyryl-CoA.²³⁻²⁵ The type of reaction that Ccr catalyses, *i.e.* the reductive carboxylation of an enoyl-thioester, is, to date, a unique biochemical reaction, that may have an important role in providing (m)ethylmalonyl-CoA for the biosynthesis of a number of metabolites in different organisms.^{23, 25}

In Chapter 2, evidence was presented that ThnE catalyses the conversion of methylmalonyl-CoA to give an 80:20 ratio of the (6*R*):(6*S*)-epimers, respectively, of C-6 methyl-*t*-CMP. This result is of interest because the stereochemistry at C-6 of the major epimer is the same as that at C-6 in thienamycin. However, and at least under standard conditions, recombinant ThnE does not accept ethylmalonyl-CoA, as a co-substrate to L-GHP, to produce 6-ethyl-*t*-CMP. Nonetheless, one possibility in the biosynthesis of thienamycin, and associated natural products, is that the C-6 ethyl side chain is introduced in a single step catalysed by ThnE or a homologue, from L-GHP and 2-ethylmalonyl-CoA. Intrigued by this possibility, engineered CMPS variants were designed, produced and coupled *in vitro* to Ccr from *Rhodobacter sphaeroides* [a natural producer of (2*S*)-ethylmalonyl-CoA] to investigate the possibility of producing (6*R*)-6-ethyl-*t*-CMP in a good yield, as well as to further investigate the control of diastereoselectivity of CMPS-catalysed reactions.

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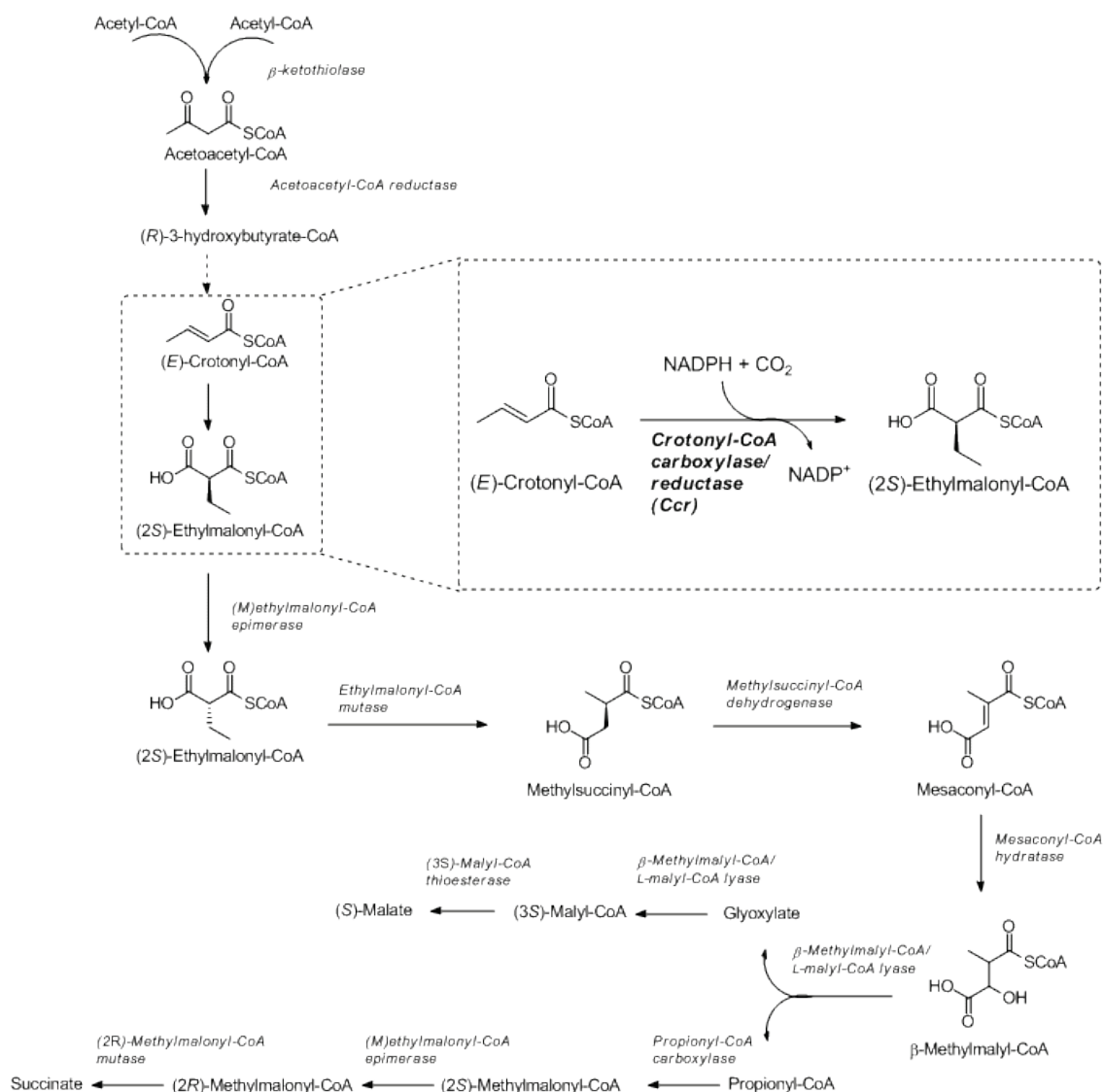


Figure 4.4. The ethylmalonyl-CoA pathway as defined in isocitrate lyase-negative organism *R. spheroides*.^{23, 24, 26, 27} Crotonyl-CoA carboxylase/reductase (Ccr) was identified as a key enzyme of the ethylmalonyl-CoA pathway. The bifunctional β -methylmalyl-CoA/malyl-CoA lyase catalyses the cleavage of β -methylmalyl-CoA as well as the condensation of acetyl-CoA and glyoxylate to form malyl-CoA. The dotted line indicates a step that has not yet been defined. The formation of crotonyl-CoA is followed by reactions that are apparently unique to this pathway; crotonyl-CoA carboxylase/reductase (Ccr), (m)ethylmalonyl-CoA epimerase, ethylmalonyl-CoA mutase and (2S)-methylsuccinyl-CoA dehydrogenase catalyse the transformation of crotonyl-CoA to give (2S)-mesoacetyl-CoA.

MatB is a synthetase that catalyses the formation of malonyl-CoA from malonic acid and coenzyme A, *via* ATP-mediated activation (Figure 4.5).²² MatB is an adenylate-forming enzyme that is reported to be best classified with acyl-CoA synthetases, and which is homologous to enzymes such as acyl-CoA synthetases, 4-

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chlorobenzoyl-CoA ligase, the adenylation domains of non-ribosomal peptides synthetases, and firefly luciferases.²²

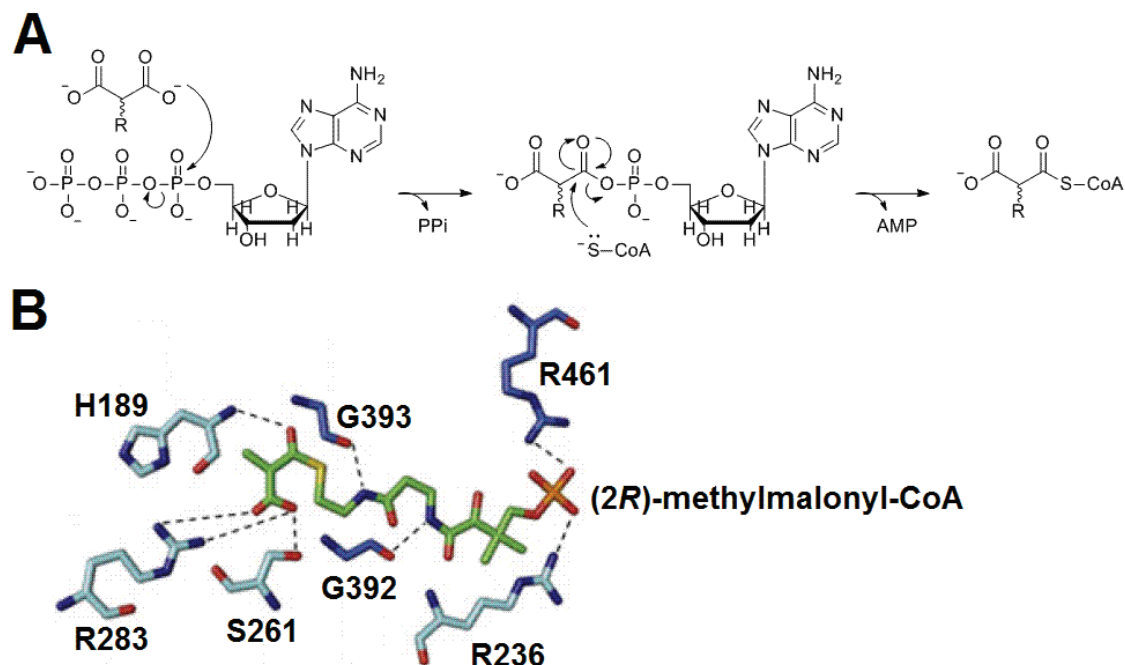


Figure 4.5. The two-step reaction mechanism of MatB (*S. coelicolor*).²² A. In the reaction forming the adenylate, the ATP α -phosphate is attacked by a malonate derivative carboxylate to form the adenylate intermediate and pyrophosphate; in the thioester-forming reaction, CoASH displaces AMP to produce the extender alkylmalonyl-CoA and AMP. B. Representation of interactions observed between (2R)-methylmalonyl-CoA and surrounding MatB residues (Figure adapted from reference 22).

It has been reported that MatB from *Streptomyces coelicolor* can synthesize the polyketide extender units malonyl-CoA, methylmalonyl-CoA, ethylmalonyl-CoA, methoxymalonyl-CoA, and hydroxymalonyl-CoA, as well as the equivalent pantetheine- and *N*-acetylcysteamine-linked extender units, demonstrating that MatB is not only promiscuous toward malonyl derivatives, but also thiol acceptors.²² Therefore, the use of MatB to produce malonyl-CoA derivatives to act as substrates for CMPS was investigated.

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Coupling of Ccr and CMPSs catalyses.

The *ccr/pET13* construct was a generous gift from Prof. B. Alber and Dr. T. Erb, from which Ccr was obtained following the published methodology,²³ described in Methods and used to prepare (2*S*)-2-ethylmalonyl-CoA, which was directly incubated with L-GHP and CMPSs under the standard conditions (Figure 4.6; see Methods). CarB W79F and CarB W79A were chosen for the Ccr co-incubation taking into consideration the yields they showed when synthetic (racemic) ethylmalonyl-CoA was used, as described in Chapter 2.

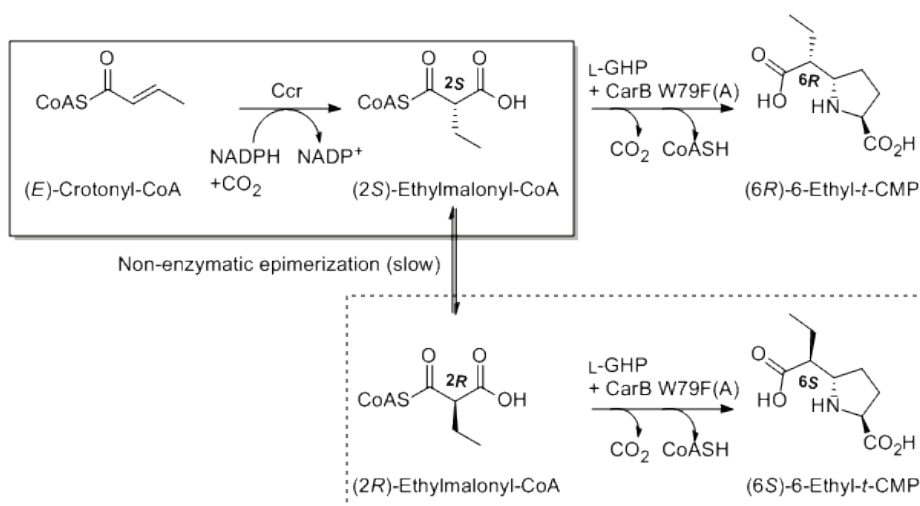


Figure 4.6. Stereoselective formation of (6*R*)-6-ethyl-*t*-CMP from (*E*)-crotonyl-CoA and L-GHP is observed in Ccr-CarB W79F(A) coupled assays. Both (6*R*)- and (6*S*)-6-ethyl-*t*-CMP (dashed box) are observed when the Ccr incubation time is sufficiently long (before the addition of the CMPS, boxed), probably due to non-enzymatic epimerization of the ethylmalonyl-CoA produced by Ccr catalysis.

In contrast to the result observed after the incubation of CarB W79F with (2*R/S*)-2-ethylmalonyl-CoA, where a 32:68 ratio of (6*R*:6*S*)-6-ethyl-*t*-CMP was observed (see Chapter 2), with the Ccr-CarB W79F(A) coupled reactions a single peak ($m/z = 202$ [$M+H$]⁺) corresponding to (6*R*)-6-ethyl-*t*-CMP was observed (Figure 4.7).

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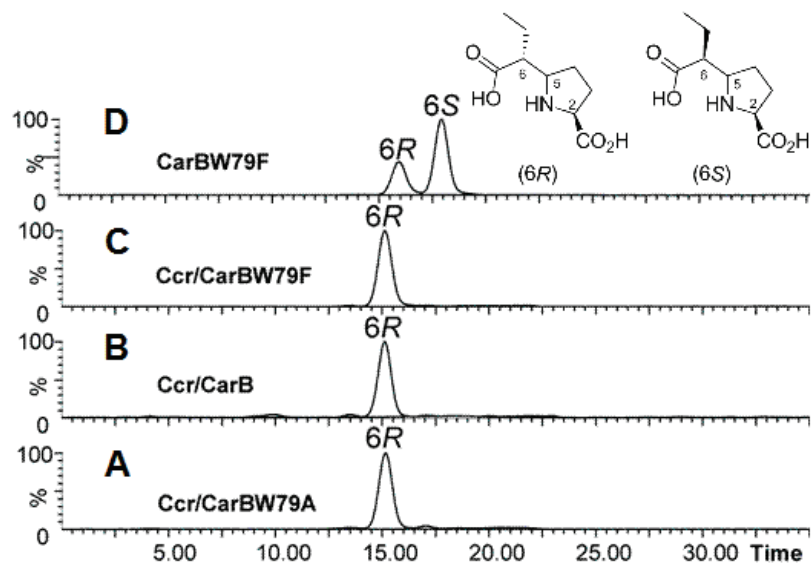


Figure 4.7. CMPS incubation with Ccr-produced (2S)-2-ethylmalonyl-CoA and (2R/S)-2-ethylmalonyl-CoA. A, B and C: stereoselective production of (6R)-6-ethyl-*t*-CMP from incubation of Ccr and the corresponding CarB variant with L-GHP. D: production of a mixture of C-6 epimers of 6-ethyl-*t*-CMP from incubation of (2R/S)-2-ethylmalonyl-CoA and L-GHP by CarB W79F catalysis

To better understand the nature of the coupled Ccr-CMPS reactions, experiments in which the incubation times with Ccr (before addition of CarB W79F) were varied were carried out (Figure 4.9), over a time course of 5 hours. The results of these experiments revealed that extending the incubation time of Ccr prior to the addition of CarB W79F, partly restored the bias towards the (6S)-6-ethyl-*t*-CMP epimer that is observed as the major product when the reaction is performed in the absence of Ccr with (2R/S)-2-ethylmalonyl-CoA as the substrate (see Figure 2.10). Coupling Ccr catalysis with that of CarB wild-type, CarB M108L and CarB W79F M108A, under standard co-incubation conditions, resulted in the stereoselective formation of (6R)-6-ethyl-*t*-CMP as revealed by LC-MS analysis (Figure 4.7) The most likely explanation for these observations is that the (2S) epimer of ethylmalonyl-CoA, as the initial product of Ccr catalysis,²⁴ gives rise to (6R)-6-ethyl-*t*-CMP; however, upon non-enzymatic epimerization under standard assay conditions (Figure 4.7) the (2R)-epimer of ethylmalonyl-CoA gives rise to (6S)-ethyl-*t*-CMP (Figure 4.1)

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Unlike the incubation of ethylmalonyl-CoA produced by Ccr catalysis and L-GHP with CMPSs, which resulted in the formation of (6*R*)-6-ethyl-*t*-CMP (Figure 4.7 A-C), it was found that incubation of ethylmalonyl-CoA from Ccr with L-AASA and CarB W79A (the highest yielding CMPS variant producing 6-ethyl-*t*-CMP) did not result in production of detectable 7-ethyl-*t*-CMPi under standard conditions. This result is consistent with the outcome of the reaction of CarB W79A and (2*R/S*)-2-ethylmalonyl-CoA and L-AASA that produced solely (7*S*)-7-ethyl-*t*-CMPi (Figure 2.15). Taken together, the Ccr-CMPS coupled reactions support a mechanism in which a specific alkylmalonyl-CoA C₂-epimer is converted to a specific enolate at the active site of CarB/ThnE. This adds support to the structure-activity relationships previously described for CMPSs, which, at least in part, is proposed to be determined by a steric clash between the alkyl-substituent on the (*E*)-enolate and selected residues in the active site (Figure 4.8).

The variations in incubation conditions can further enhance the d.r. values towards the major epimer observed (Figure 4.6), probably (at least in part) due to differences in the rate of C-2 alkylmalonyl-CoA epimerization. These results reveal the potential of CMPS enzymes for dynamic kinetic discrimination based on the acid/base-catalysed equilibration of C-2 epimeric alkylmalonyl-CoA substrates. Consistent with this proposal, the use of a single substrate epimer, that is, (2*S*)-ethylmalonyl-CoA, produced by Ccr catalysis, can significantly alter the stereochemical outcome of the CMPS-catalysed reaction (Figure 4.7).

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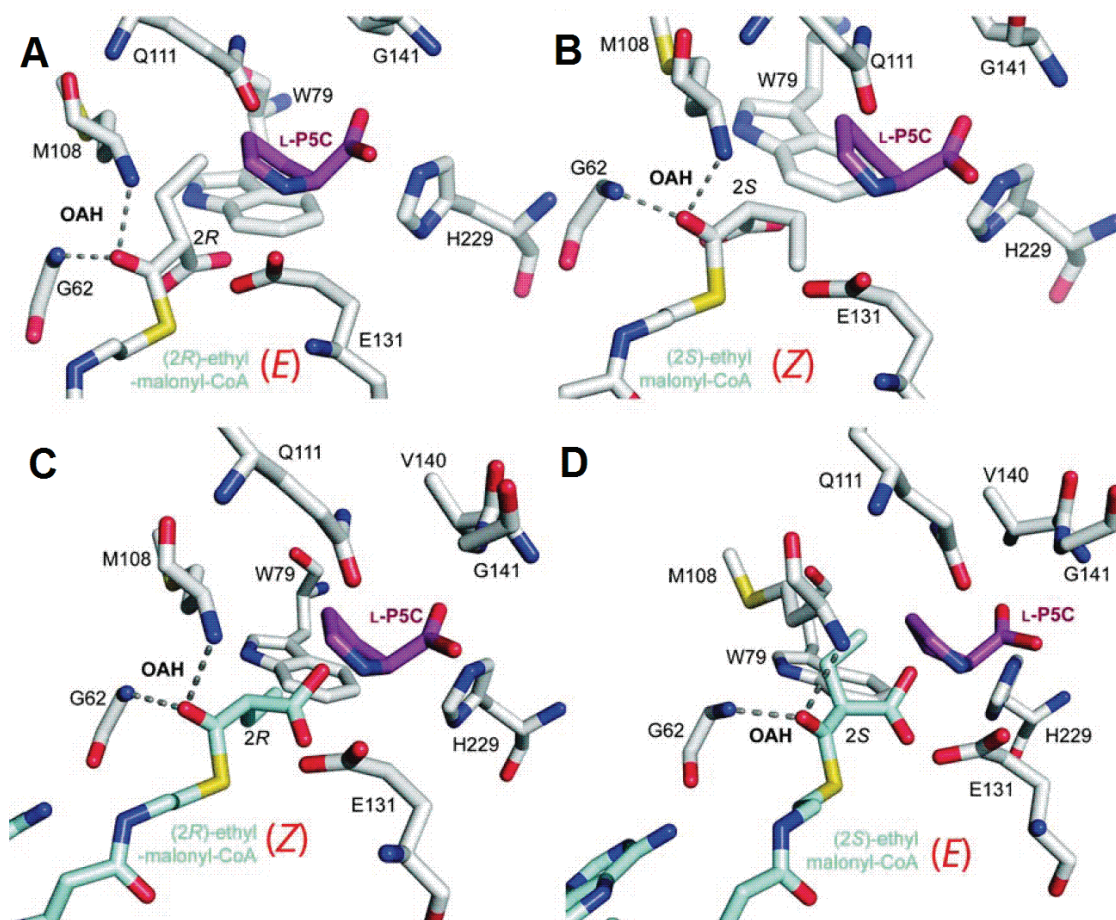


Figure 4.8. View from a CarB crystal structure with the two C-2 epimers of ethylmalonyl-CoA (*2R* epimer, A and C; *2S* epimer, B and D) modelled into the active site. The orientation is such that the malonyl-CoA carboxylate group either points away from residue Glu-131 (A and B) or towards it (C and D). The enolate form (*E/Z*) which would be generated following the stereoelectronically favoured decarboxylation is shown in each case. Note that, in all cases, an orthogonal relationship between the ethylmalonyl-CoA carboxylate and the carbonyl stabilized by the oxyanion hole (OAH) is maintained as required for the stereo-electronically favoured decarboxylation (Figure prepared by Dr. R Hamed and adapted from ²⁸).

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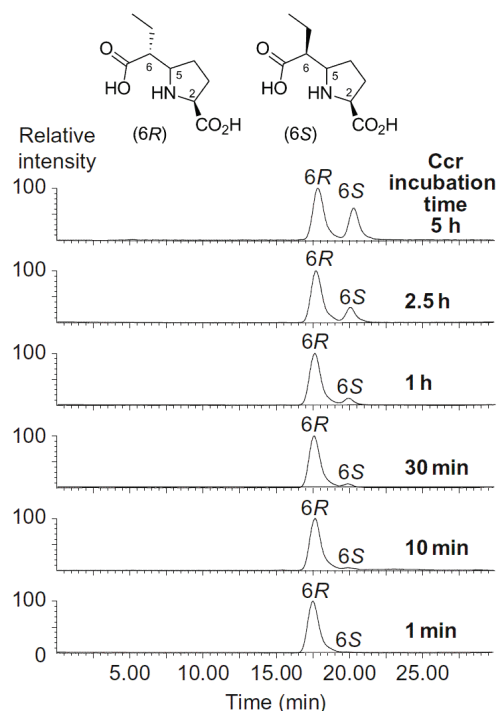


Figure 4.9. LC-MS chromatograms for the products of Ccr-CarB W79F coupled reactions. The Ccr assay was carried out for the indicated time before addition of CarB W79F. Note that the longer the time of the initial Ccr reaction, the higher the amount of (6S)-6-ethyl-t-CMP produced after subsequent CarB W79F incubation.

These results might be exploited in practical applications using CMPS variants in cells, as they imply that variations in, for example, the ratios of the coupled enzymes may be used to control the diastereoselective formation of the desired product.

Use of MCE to modify the diastereoselectivity of the coupled Ccr/CMPS reaction.

Coupling the reaction of Ccr (reported to produce (2S)-ethylmalonyl-CoA) to CMPSs and MCE catalysis reveals an effect equivalent to that seen when extending the pre-incubation time of Ccr before the addition of CarB W79F(A); *i.e.* a reversal to the preferred stereoselectivity of the CarB W79F(A) mutants, the (6S)-6-ethyl-t-CMP product, due to the epimerization of the (2S)-methylmalonyl-CoA substrate to the (2R)-epimer (see Chapter 2, Fig 2.10). As seen in Chapter 2, for the coupled Ccr-CarBW79F(A) reaction the sole observed product is the (6R)-6-ethyl-t-CMP product.

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This is due to the fact that, in spite of (2*R*)-ethylmalonyl-CoA being the preferred substrate of the CarB W79F(A) variants,ⁱⁱ (2*S*)-ethylmalonyl-CoA is still readily accepted by the CMPS in question, apparently more rapidly so than the time it would take for (2*S*)-ethylmalonyl-CoA to non-enzymatically epimerize to yield the preferred substrate. When (substantial) epimerization occurs, either slowly/non-enzymatically or rapidly by the action of MCE (Figure 4.10), the preferred substrate (2*R*)-ethylmalonyl-CoA becomes available and preferentially reacts, enhancing the yield of (6*S*)-6-ethyl-*t*-CMP.

ⁱⁱ In Chapter 2 it is established that the Trp79_{CarB} variants optimally yield 6-ethyl-*t*-CMP; therefore, they were selected for the Ccr-coupled assays.

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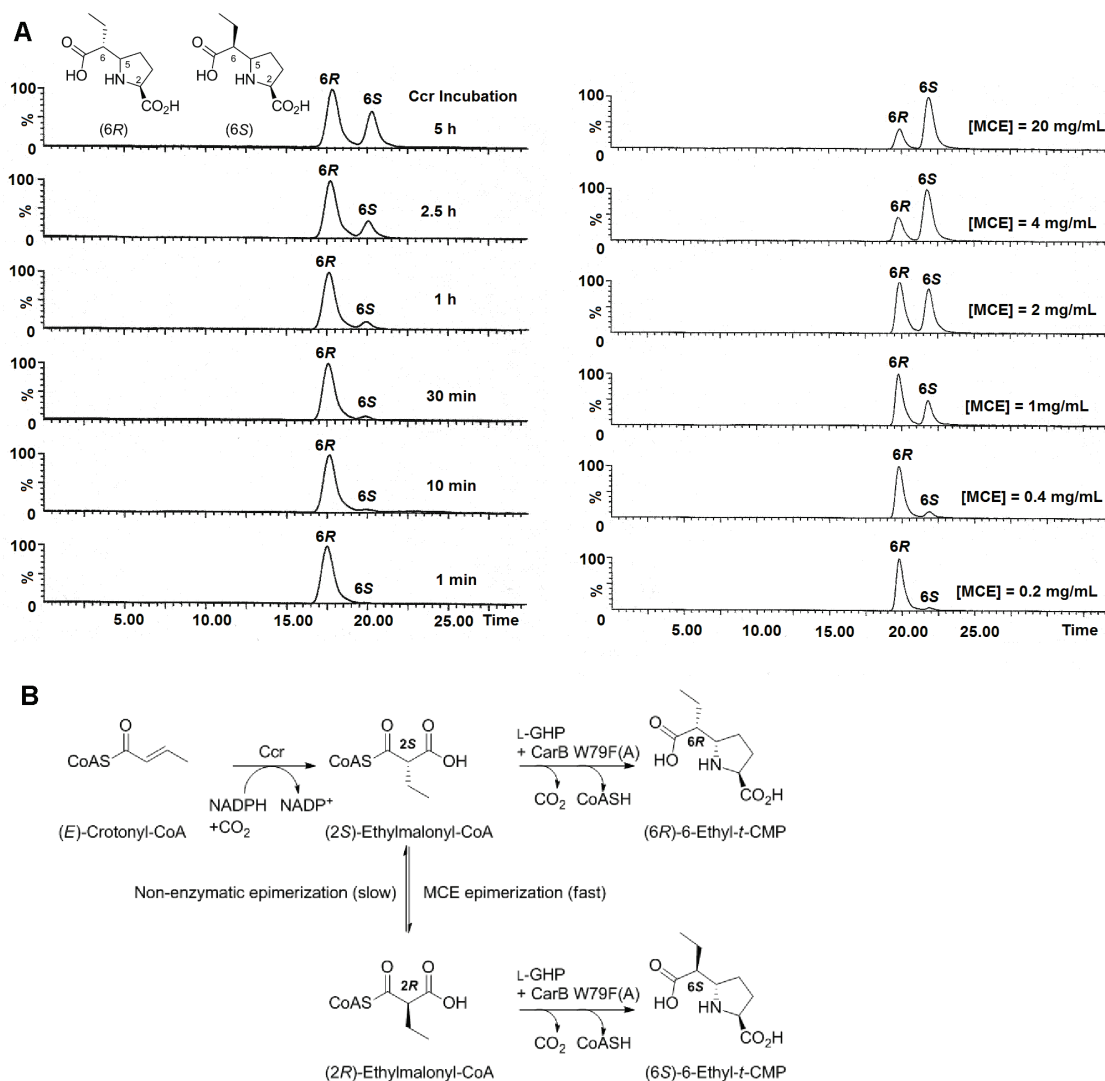


Figure 4.10. Ion extracted LC-MS chromatograms (positive ion mode, **A**) demonstrating the effect of MCE on the diastereoselectivity of the Ccr/CMPS reaction, **B**. The left panel of **A** demonstrates the stereoselectivity obtained with the noted Ccr incubation time prior to the CarB reaction; the longer the incubation prior to CarB incubation, the more non-enzymatic epimerization occurs, as seen by the increase in the concentration of the signal for the 6*S*- stereoisomer of (6*R*)-*t*-CMP. The right panel of **A** indicates the effect of the concentration of MCE in the epimerization of ethylmalonyl-CoA and resulting product after 1 minute of CarB reaction; higher concentrations of MCE are equal to extended time on non-enzymatic epimerization. The effect of the slow, non-enzymatic epimerization, *versus* the faster enzymatic epimerization caused by MCE is explained in **B**. Time is in minutes.

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Use of malonyl-CoA ligase for the formation of malonyl-thioesters of coenzyme A, pantetheine and N-acetyl-cysteamine, and their use as potential substrates for CMPSs catalysis.

The *matB/pET28* construct was a kind gift from Prof. A. Keatinge-Clay. MatB was produced and purified following the published procedure²² (see Methods). The standard CMPS assay (with wild-type CarB) was supplemented with purified MatB (47.4 μ M) to test for the production of *t*-CMP from malonic acid, coenzyme A and L-GHP (see the Methodology for a description of the enzymatic assay conditions). After two hours of incubation at 37 °C (equivalent to overnight reaction time at room temperature) a single peak at $m/z = 174$ $[M + H]^+$ (corresponding to *t*-CMP) was observed after LC-MS analysis under standard conditions (Figure 4.11). In a similar fashion as that reported for the final module and thioesterase from 6-deoxyerythronolide B synthase,²² the coupled MatB/CarB reaction was also capable of synthesizing *t*-CMP from the pantetheine- and N-acetylcysteinemalonyl derivatives under the standardized CMPS/MatB conditions, albeit in lower yields (Figure 4.11)

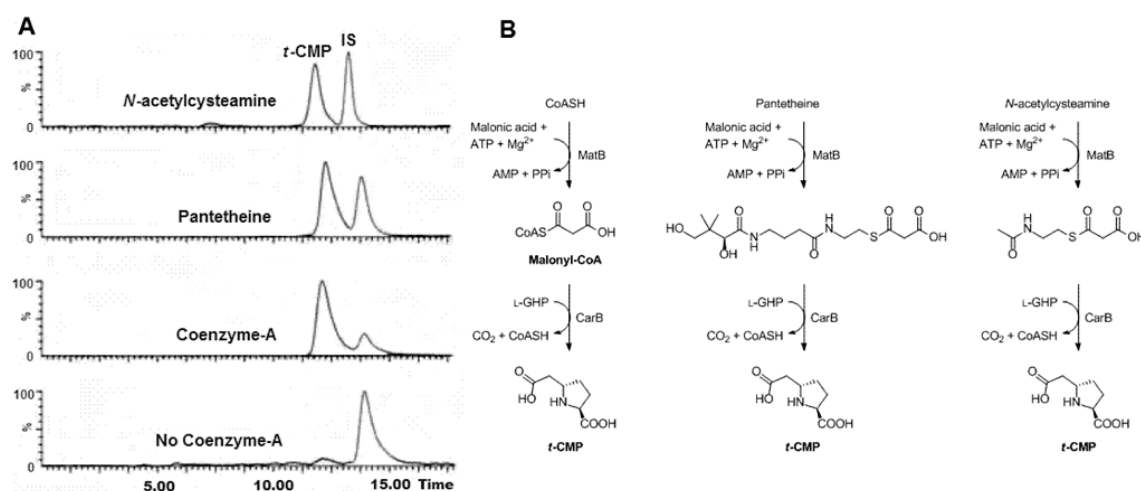


Figure 4.11. Extracted ion chromatograms (A) showing the production of *t*-CMP from coupled MatB/CarB reactions (B). The substrates are malonic acid, L-GHP, and coenzyme A, pantetheine or N-acetyl cysteamine (B). Note the higher level of turnover in the case of coenzyme A, as compared to

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pantetheine and *N*-acetyl cysteamine, implying that the truncated forms of malonyl-CoA are less favourable substrates for CarB. *p*-Aminosalicylic acid was used as an internal standard (IS, ca. 14.8 minutes). Time is in minutes.

Use of MatB for the selective formation of (2*R*)-alkyl-substituted derivatives of malonyl-CoA and their use for the selective production of (6*S*)- and (7*S*)-alkyl-substituted *t*-CMP and *t*-CMPi, respectively, as catalysed by CMPSs.

The results obtained with the coupled assays of MatB and CarB promoted investigations to pursue the possibility of expanding the current library of available CoA-derivative compounds, which had been limited by the commercial availability of alkylmalonyl-CoA compounds, by using the MatB reaction and commercially available malonic acid derivatives to yield alkylmalonyl-CoA. Compounds that were commercially available and which were used for this purpose were: methylmalonic acid, dimethylmalonic acid, ethylmalonic acid, allylmalonic acid, propylmalonic acid, isopropylmalonic acid, ethylmethylmalonic acid, butylmalonic acid, isobutylmalonic acid, pentylmalonic acid and hexylmalonic acid (Figure 4.12).

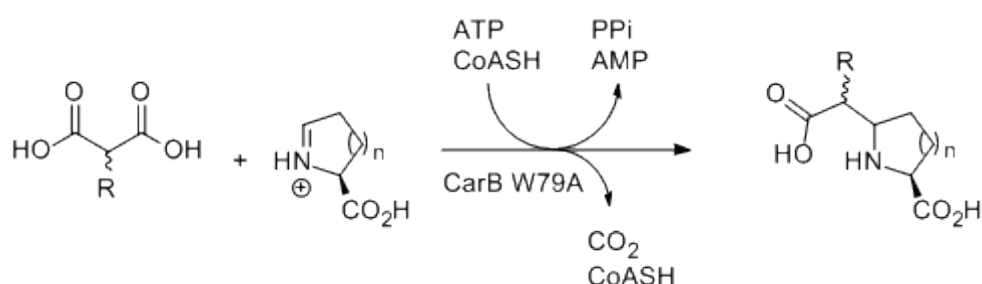


Figure 4.12. The coupled catalytic activity of MatB and CarB W79A yields C-6/C-7 functionalized *t*-CMP/*t*-CMPi products.

LC-MS analyses revealed that the coupled system of MatB and CMPS (CarB W79F(A) variants were used because of their capacity to better accommodate bulky substituents at C-6 of *t*-CMP, see Chapter 2), under standard conditions (see Methods) is capable of yielding the full range of *t*-CMP products from the alkyl-malonic acid and

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L-GHP substrates, as made evident by the appearance of a single peak at the expected m/z (see Figure 4.13). Similar results were obtained when L-GHP was substituted with L-AASA as a substrate; products ranging from *t*-CMPi and 7-methyl-*t*-CMPi to 7-butyl-*t*-CMPi were obtained, including the unsaturated product 7-allyl-*t*-CMPi (Figure 4.14).

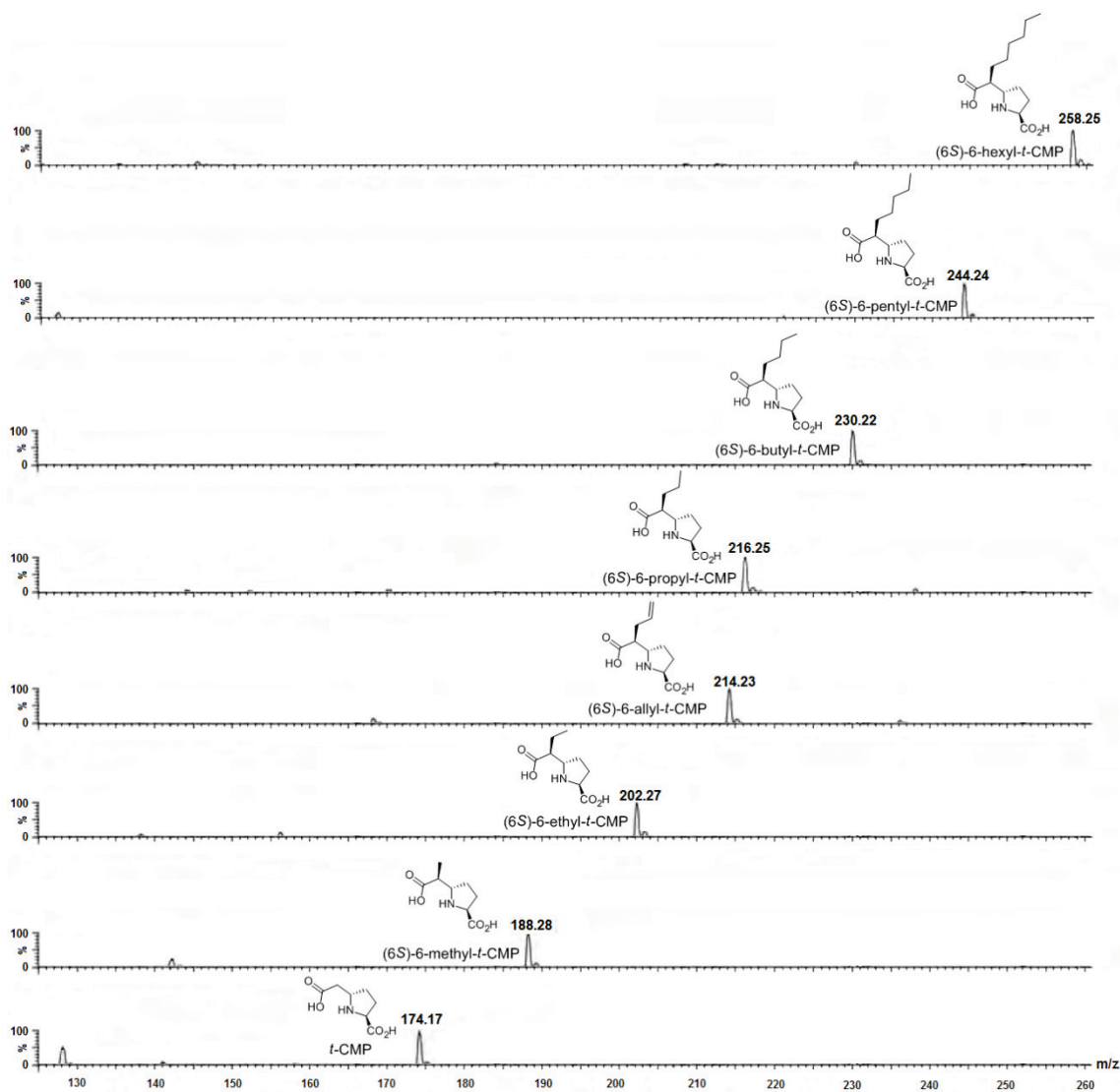


Figure 4.13. Mass spectra (positive ion mode) for the products of catalysis of the MatB/CarB W79A coupled assays with diverse alkylmalonyl-CoA substrates and L-GHP. The m/z of the expected compounds is indicated.

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Table 4.1. Products resulting from the incubation of (2*R*)-alkylmalonic acids and L-GHP with MatB and CarBW79A. d.r. Refers to the C-6 stereochemistry.

Product	d.r. (<i>R:S</i>)	Yield (%)
<i>t</i> -CMP	NA	34
6-Methyl- <i>t</i> -CMP	15:85	4
6-Ethyl- <i>t</i> -CMP	5:95	14
6-Allyl- <i>t</i> -CMP	0:100	21
6-Propyl- <i>t</i> -CMP	0:100	21
6-Butyl- <i>t</i> -CMP	0:100	24
6-isoButyl- <i>t</i> -CMP	0:100	24
6-Pentyl- <i>t</i> -CMP	0:100	49
6-Hexyl- <i>t</i> -CMP	0:100	43

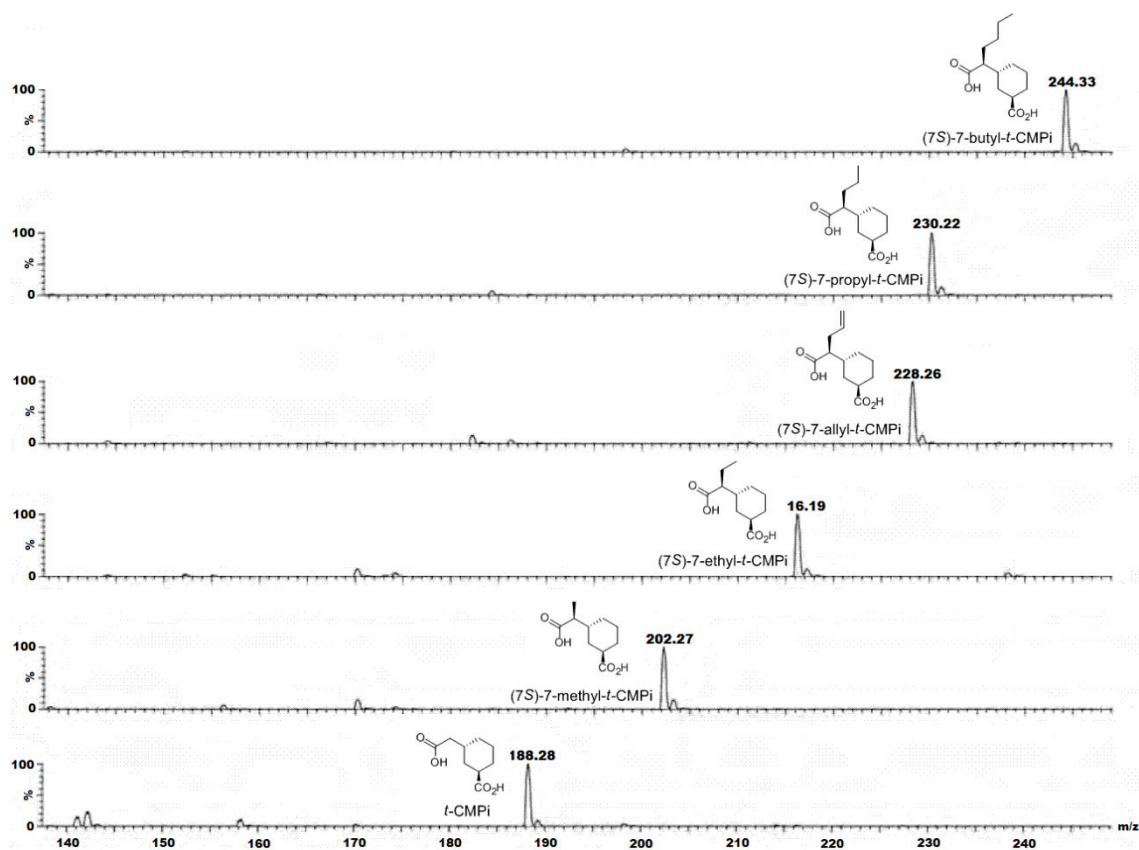


Figure 4.14. Mass spectra (positive ion mode) for the products of catalysis of the MatB/CarB W79A coupled assays with diverse alkylmalonyl-CoA substrates and L-AASA. The m/z of the expected compounds is indicated.

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Table 4.2. The products resulting from the incubation of (2*R*)-alkylmalonic acids and L-AASA with MatB and CarBW79A. d.r. Refers to the C-7 stereochemistry.

Product	d.r. (<i>R:S</i>)	Yield (%)
<i>t</i> -CMPi	NA	19
7-Methyl- <i>t</i> -CMPi	5:95	26
7-Ethyl- <i>t</i> -CMPi	1:99	34
7-Allyl- <i>t</i> -CMPi	0:100	51
7-Propyl- <i>t</i> -CMPi	0:100	8
7-Butyl- <i>t</i> -CMPi	0:100	48

Discussion.

The biocatalytic promiscuity of the wild-type malonyl-CoA ligase MatB, and the diastereospecificity of the crotonyl-CoA carboxylase/reductase Ccr have been successfully coupled to the selective trisubstituted enolate forming capacity of engineered CMPS for the preparation of functionalized 5- and 6-membered *N*-heterocycles substituted with a variety of alkyl side chains at the C-6/C-7 positions in high diastereomeric excess. The effect of methylmalonyl-CoA epimerase, MCE, on the diastereoselectivity of the crotonase-catalysed enolated alkylation is also demonstrated. The results further illustrate the utility of crotonase superfamily enzymes for stereoselective biocatalysis and demonstrate the power of coupled enzyme systems to enhance diastereoselectivity and to expand substrate acceptance range. Overall, the results also point towards a possible enzymatic system for obtaining pharmaceutically improved C-6 substituted carbapenem antibiotics.

Further variability in the products could be introduced by the substitution of L-GHP by any of the methyl-L-GHP variants that have been previously discussed (see Chapter 3), or by exploring the further diversity of malonic acid derivatives available. Preliminary data indicates very good results can be obtained for the production of (4*S*,6*S*)-4-methyl-6-ethyl-*t*-CMP; these results could be further enhanced by the action of MCE to produce (4*S*,6*R*)-4-methyl-6-ethyl-*t*-CMP as a scaffold towards the

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production of clinically useful carbapenem antibiotics. It was also found that the use of tartronic acid (2-hydroxypropanedioic acid) as a substrate for MatB, in conjunction with CarB W79A and L-GHP, gives good yields of 4-methyl-6-hydroxyl-*t*-CMP. The hydroxyl moiety at C-6 could be use to introduce further diversity and may ultimately help in the design of hybrid antibiotics with the characteristics of both carbapenems and penicillins. It is possible that application of other protein engineering approaches will also enable enhancement in stereoselectivity and/or expansion in the range of electrophiles accepted in CMPS-catalysed reactions.

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Chapter 5. Enzymatic-controlled Formation of (4*S*,6*R*)-4-Methyl-6-alkyl-*t*-CMP via Mutual Dynamic Kinetic Resolution by CMPSs or Ccr/MatB/CMPS coupled systems.

As discussed in Chapter 1, the asymmetric structure of the clinically used carbapenems dictates their pharmacodynamics and pharmacokinetic profile (Figure 5.1). Various chemical approaches have been developed for the chemical synthesis of carbapenems, because fermentation has not proved to be a sufficiently enough efficient method for their production.¹⁻⁶ To date, commercially used synthetic approaches rely on derivatization of the intermediate (3*R*,4*R*)-4-acetoxy-[(1*R*)-*tert*-butyldimethylsilyloxyethyl]-azetidin-2-one, typically *via* elimination/addition at C-4 and subsequent cyclization to form the 5-membered carbocyclic ring⁷ (Figure 5.1).

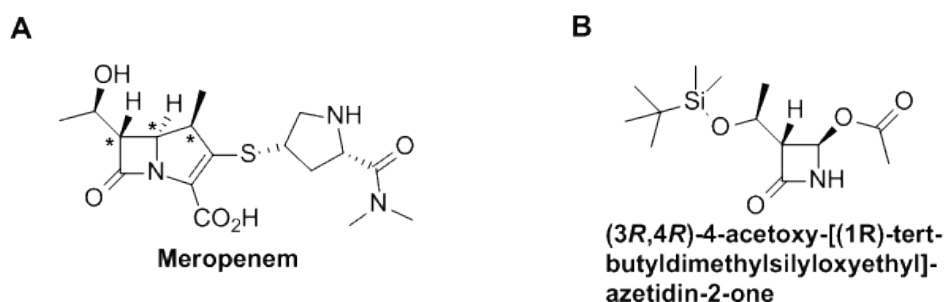


Figure 5.1. The structure of the first carbapenem antibiotic to exploit the inhibitory activity of the 1 β -methyl group on the dehydropeptidase I, meropenem (A); and the structure of the synthetic intermediate (3*R*,4*R*)-4-acetoxy-[(1*R*)-*tert*-butyldimethylsilyloxyethyl]-azetidin-2-one (B).

Processes for the resolution of diastereomers can be divided into several groups including direct crystallization of enantiomers, crystallization of diastereomeric salts, chromatographic methods and kinetic resolution (either by esterification/acylation or hydrolysis). For kinetic resolution,⁸⁻¹³ enzymes are probably the most versatile reagent

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identified to date. Many fine chemicals producers employ enzymes including proteases, lyases, esterases, acylases and amidases for kinetic resolution. Alternatively, racemisation can be achieved *in situ* by dynamic kinetic resolution using a synthetic catalyst or racemases, and many others. The currently acceptable enantiomeric excess for commercial purposes is normally considered to be 99%, although the future target that many companies have set is greater than this. Given that there is an ever increasing need for chiral compounds including alcohols, diols, amines, amides, α -amino acids, β -amino acids, amino alcohols, carboxylic acids and esters, there is a clear need for new methods for their production.^{14, 15}

Mutual kinetic resolution involves reactions between two sets of racemic compounds, the outcome of which is dictated by kinetic processes. For a kinetic resolution to be described as mutual, the interaction between two sets of racemates is required for results to occur. As described in Chapter 4, the reaction of CMPS with alkylmalonyl-CoA substrates behaves in a dynamic kinetic resolution fashion; if one takes into consideration the epimerization that 4-methyl-L-GHP suffers while being deprotected (Chapter 3) resulting in an epimeric mixture, the combination of the two substrates (4-methyl-L-GHP and an alkylmalonyl-CoA) would give us a mutual dynamic kinetic resolution process; this concept will be explored in this Chapter. The work described here explores the possibility of enhancing the diastereoselectivity at C-6 by use of Ccr and MatB, while controlling the diastereoselectivity at C-4 with a CMPS variant.

The catalytic activity of CMPSs utilising methylmalonyl-CoA and 4-methyl-L-GHP substrates.

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In Chapters 2 and 3, the results of the engineering of CMPSs on methylmalonyl-CoA and 4-methyl-L-GHP substrates to obtain (6*R*)-6-methyl-*t*-CMP and (4*S*)-4-methyl-*t*-CMP in a diastereoselective manner have been described. These compounds are of interest in efforts to obtain carbapenem molecules by means of biocatalysis or fermentation, since both enantiomers have the appropriate stereochemistry at C-6 and C-4, respectively, as observed in clinically used carbapenems. Downstream processing of these products could be directed towards a carbapenam nucleus (as catalysed by β -lactam synthetases, *i.e.* ThnM or CarA), followed by desaturation/epimerization to yield the carbapenem nucleus (as catalysed by a carbapenem synthetase, *e.g.* CarC, or by an as yet unknown mechanism, likely in thienamycin biosynthesis; see Chapter 1)

In Chapters 2 and 3, it was reported that the best biocatalysts obtained for the production of (6*R*)-6-methyl-*t*-CMP and (4*S*)-4-methyl-*t*-CMP were the Met108_{CarB} variants (namely CarBM108V and CarBM108I (95:5 and 92:8 6*R*:6*S*, and 93:07 and 90:10, respectively; Tables 2.2 and 3.2). In Chapter 3, it was also reported that double mutations in the active site of CMPSs can further enhance the diastereoselectivity achieved by individual modifications; *e.g.*, for the production of (4*S*)-4-methyl-*t*-CMP, the CarBW79F108A variant achieves a 73:27 (4*S*:4*R*) ratio, compared to 61:39 (4*S*:4*R*) for CarBW79F and 54:46 (4*S*:4*R*) for CarBM108A.

With these results in mind, it was envisioned that the assembly of (6*R*,4*S*)-4,6-dimethyl-*t*-CMP from methylmalonyl-CoA and 4-methyl-L-GHP was feasible through the catalytic activity of modified CMPSs. (6*R*,4*S*)-4,6-Dimethyl-*t*-CMP could act as a scaffold for the semi-synthesis of substituted carbapenems with potentially improved chemical and enzymatic stability profiles. It is important to remember that both starting materials were available as racemic mixtures. This combination was also of interest to

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demonstrate the use of CMPSs catalysis in three contiguous stereocentres for the formation of (4*S*,5*S*,6*R*)-4,6-dimethyl-CMP) at the same time had not been tested.

Based on the observation that CMPSs can catalyse the formation of the two C-4 epimers of 4-methyl-*t*-CMP and the two C-6 epimers of 6-methyl-*t*-CMP (CMPS biocatalysis exhibits different ratios), Dr. R. Hamed anticipated¹⁶ that the four possible epimers of 4,6-dimethyl-*t*-CMP could be formed from the incubation of 4-methyl-L-GHP, methylmalonyl-CoA and CarB (CarB exhibits a stereoselectivity ~ 1:1 when the C-4 and C-6 methyl epimers are produced separately from the appropriate precursors; Tables 2.2 and 3.2). In reality, LC-MS analysis reported in Dr. R. Hamed's doctoral dissertation¹⁶ revealed that only three chromatographically-distinct products with the anticipated $m/z = 202$ $[M+H]^+$ were produced. Subsequent scale-up and LC-MS purification allowed for NMR analyses to determine the stereochemistry at C-4 and C-6 of the products.¹⁶

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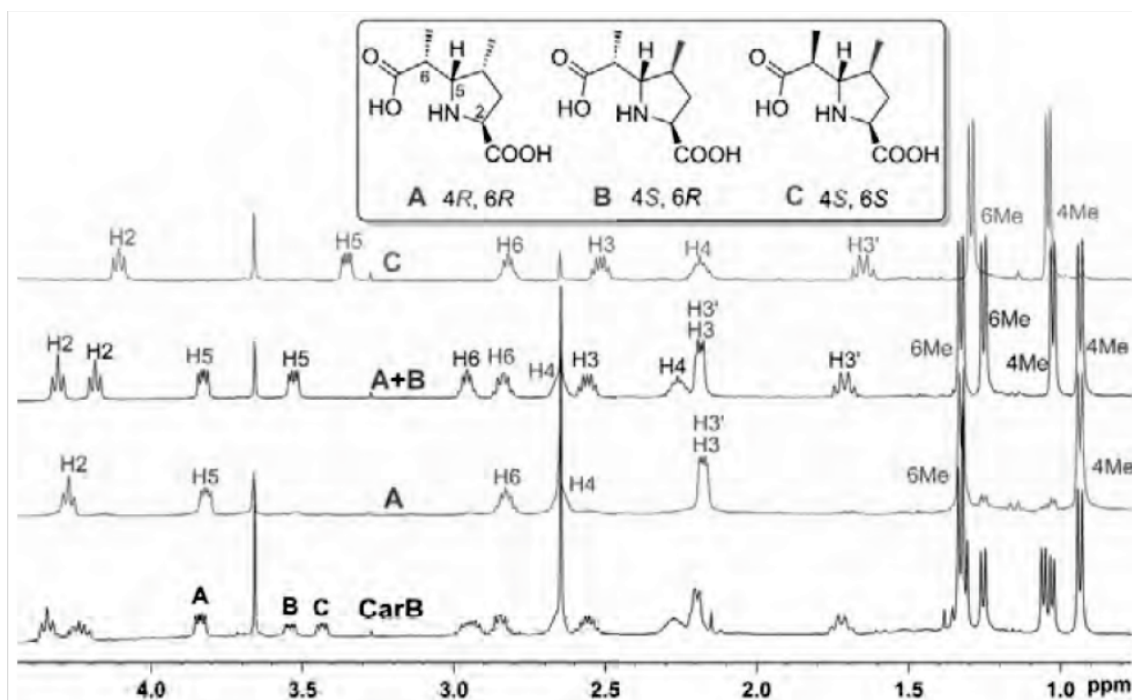


Figure 5.2. ^1H -NMR spectra for the three detected the (4*R*,6*R*);(4*S*,6*R*);(4*S*,6*S*)-4,6-dimethyl-*L*-CMP (A, B and C, respectively) products of catalysis from methylmalonyl-CoA and 4-methyl-*L*-GHP by CarB. Figure prepared by Dr. R. Hamed.¹⁶

The compound with the shortest retention time (Figure 5.2 A) was assigned an (*R*)-stereochemistry at C-4 based on the observation of a strong NOE between H-2 and the C-4 methyl group, together with the absence of an NOE between the methyl group C-4 and H-5. The stereochemistry at C-6 was assigned as (*R*) on the basis of a $J_{5,6}$ value of 10 Hz (predicted $\phi \sim 170^\circ$) in addition to a weak NOE between H-5 and H-6 indicating an antiperiplanar arrangement for these two protons; and, a strong NOE correlation observed between H-6 and C-4 methyl group, as well as the absence of any NOE between the C-6 methyl group to H-4 or the methyl group at C-4 (Figure 5.3).

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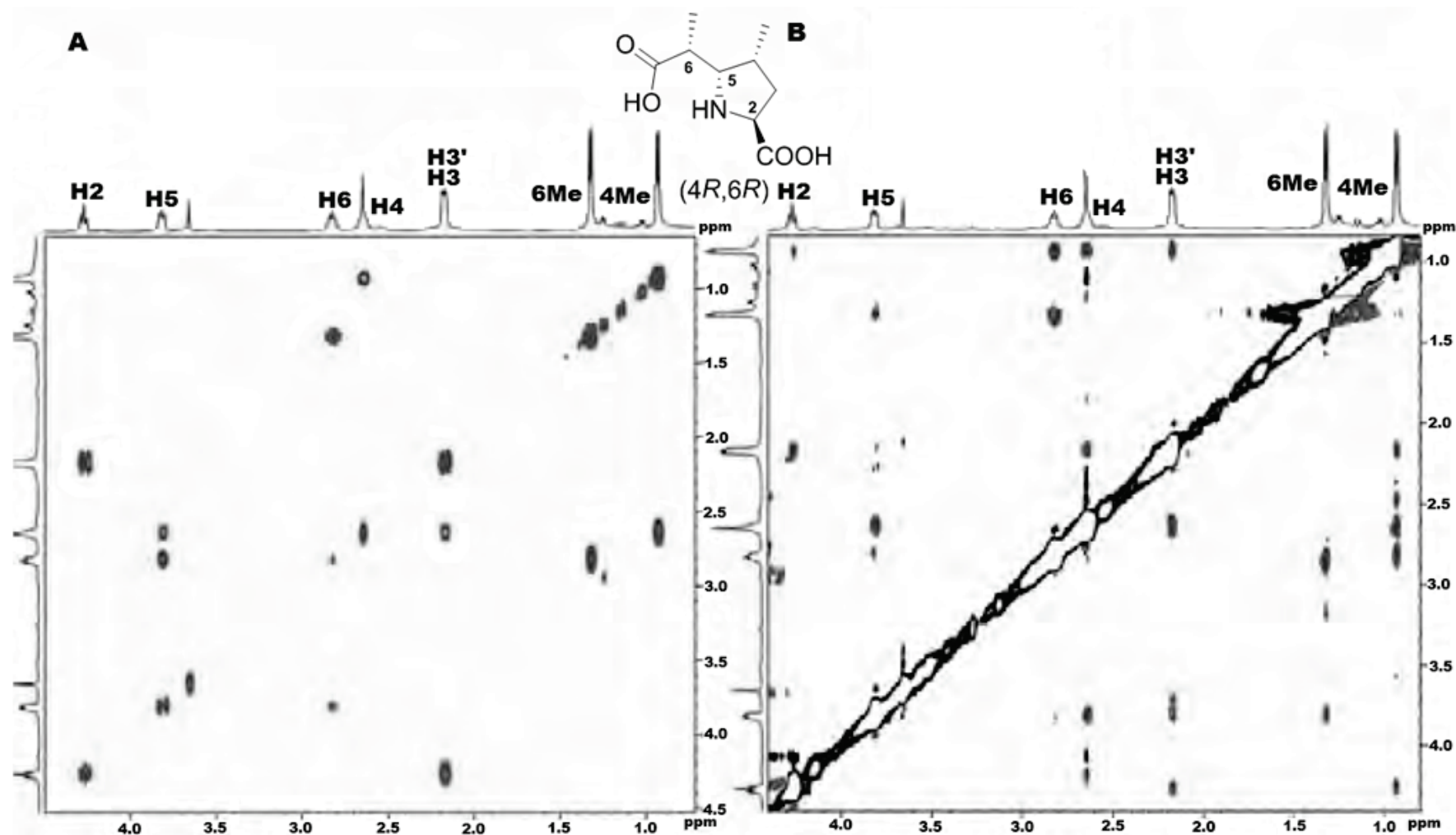


Figure 5.3. ^1H - ^1H COSY (A) and NOESY (B) spectra for $(4R,6R)$ -4,6-dimethyl-*L*-CMP produced from 4-methyl-*L*-GHP and methylmalonyl-CoA by CarB. Figure produced by Dr. R. Hamed.¹⁶

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The compound with the second shortest retention time (Figure 5.2 B) was assigned an (*S*)-stereochemistry at C-4 based on the observation of a strong NOE between H-5 and the C-4 methyl group, together with the observation of a weak NOE between H-5 and H-4. The stereochemistry at C-6 was assigned as (*S*) based on the observations of a $J_{5,6}$ value of 4.8 Hz (predicted $\phi \sim 40^\circ$), together with a strong NOE between H-5 and H-6 indicating a predominately *gauche* relationship between these two protons; a strong NOE correlations between H-6 and both H-4 and the methyl group at C-4; and, the observation of an NOE between the C-6 methyl group to both H-4 and the C-4 methyl group (Figure 5.4)

The compound with the longest retention time (Figure 5.2 C) was assigned an (*S*)-stereochemistry at C-4 based on the observation of a strong NOE between H-5 and the C-4 methyl group, together with the observation of a weak NOE between H-5 and H-4. The stereochemistry at C-6 was assigned as (*R*) based on a $J_{5,6}$ value of 4.8 Hz (predicted $\phi \sim 50^\circ$), together with a strong NOE between H-5 and H-6 indicating a predominately *gauche* relationship between these two protons; and, the observation of a weak NOE between H-6 and the methyl group at C-4, together with the absence of an NOE between H-6 and H-4, and the lack of NOE correlations between the C-6 methyl and H-4 and the C-4 methyl group (Figure 5.5)

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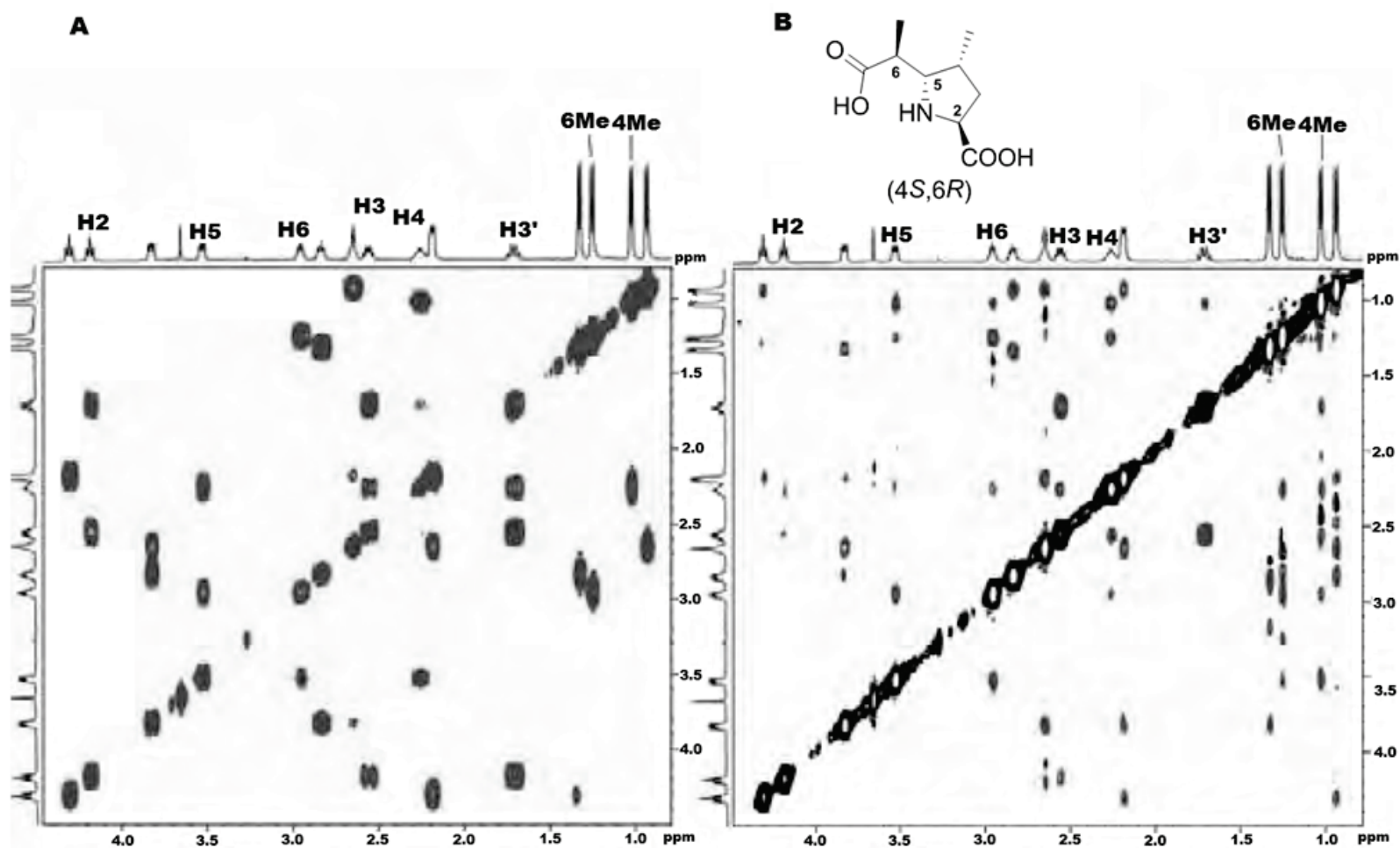


Figure 5.4. ^1H - ^1H COSY (A) and NOESY (B) spectra for a mixture of (4R,6R)-4,6-dimethyl-*t*-CMP and (4S,6R)-4,6-dimethyl-*t*-CMP produced from 4-methyl-L-GHP and methylmalonyl-CoA by CarB. Signals without label correspond to those of (4R,6R)-4,6-dimethyl-*t*-CMP (Figure 5.3), and those labelled correspond to (4S,6R)-4,6-dimethyl-*t*-CMP. Figure prepared with help from Dr. R. Hamed.¹⁶

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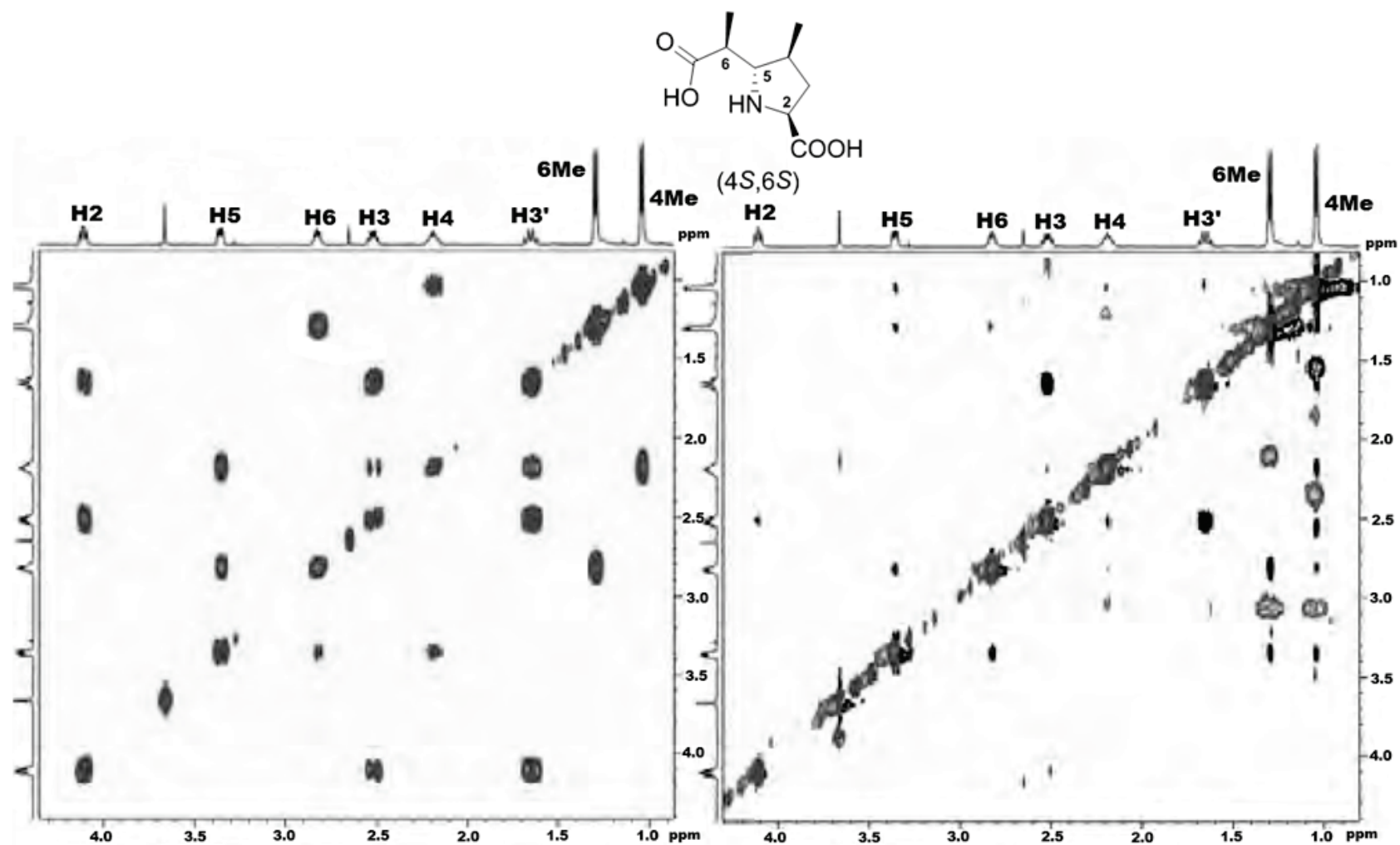


Figure 5.5. ¹H-¹H COSY and NOESY spectra for (4S,6S)-4,6-dimethyl-L-CMP (C) produced from 4-methyl-L-GHP and methylmalonyl-CoA by CarB. Figure produced by Dr. R. Hamed.¹⁶

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Screening of available CarB variants, by analytical LC-MS, for formation of 4,6-dimethyl-*t*-CMP from 4-methyl-L-GHP and methylmalonyl-CoA revealed that CarB variants were able to catalyze the formation of the same three epimers of 4,6-dimethyl-*t*-CMP, in varying ratios (Table 5.1). Because of the lack of base-line resolution, the reaction was scaled up and the diastereomeric ratios, of the three observed epimers, were determined by ¹H-NMR spectroscopy, after purification and collection of the three compounds as a single fraction (Figure 5.6). The resolved signals corresponding to the H-5 protons of the three epimers of 4,6-dimethyl-CMP were used to determine the ratio of the epimers (4*R*:6*R*; 4*S*:6*R*; 4*S*:6*S*) as follows (Figure 7.11): wild-type CarB (50:25:25), CarB M108A (34:33:33), CarB M108L (43:31:26), CarB M108V (17:75:8), CarB M108Ile (19:69:12), CarB Q111N (20:60:20), CarB W79F (43:10:47), and CarB H229A (37:30:33). In all cases, no unambiguous evidence for the formation of the (4*R*,6*S*)-4,6-dimethyl-*t*-CMP diastereomer was observed.¹⁶

In contrast, screening of ThnE/ThnE-based variants by analytical LC-MS for the formation of 4,6-dimethyl-CMP from 4-methyl-L-GHP and methylmalonyl-CoA revealed that ThnE and the ThnE variants did not form detectable quantities of 4,6-dimethyl-*t*-CMP.¹⁶

Table 5.1. The CMPSs variants used in the first round of experiments towards the production of 4,6-dimethyl-*t*-CMP and the yields of the diastereomers that were obtained.¹⁶

Catalyst	(4 <i>R</i> ,6 <i>R</i>)-	(4 <i>S</i> ,6 <i>R</i>)-	(4 <i>S</i> ,6 <i>S</i>)-
	4,6-dimethyl- <i>t</i> -CMP		
CarB	50	25	25
CarBM108A	34	33	33
CarBM108L	43	31	26
CarBM108V	17	75	8
CarBM108I	19	69	12
CarBQ111N	20	60	20
CarBH229A	37	30	33
CarBW79F	43	10	47

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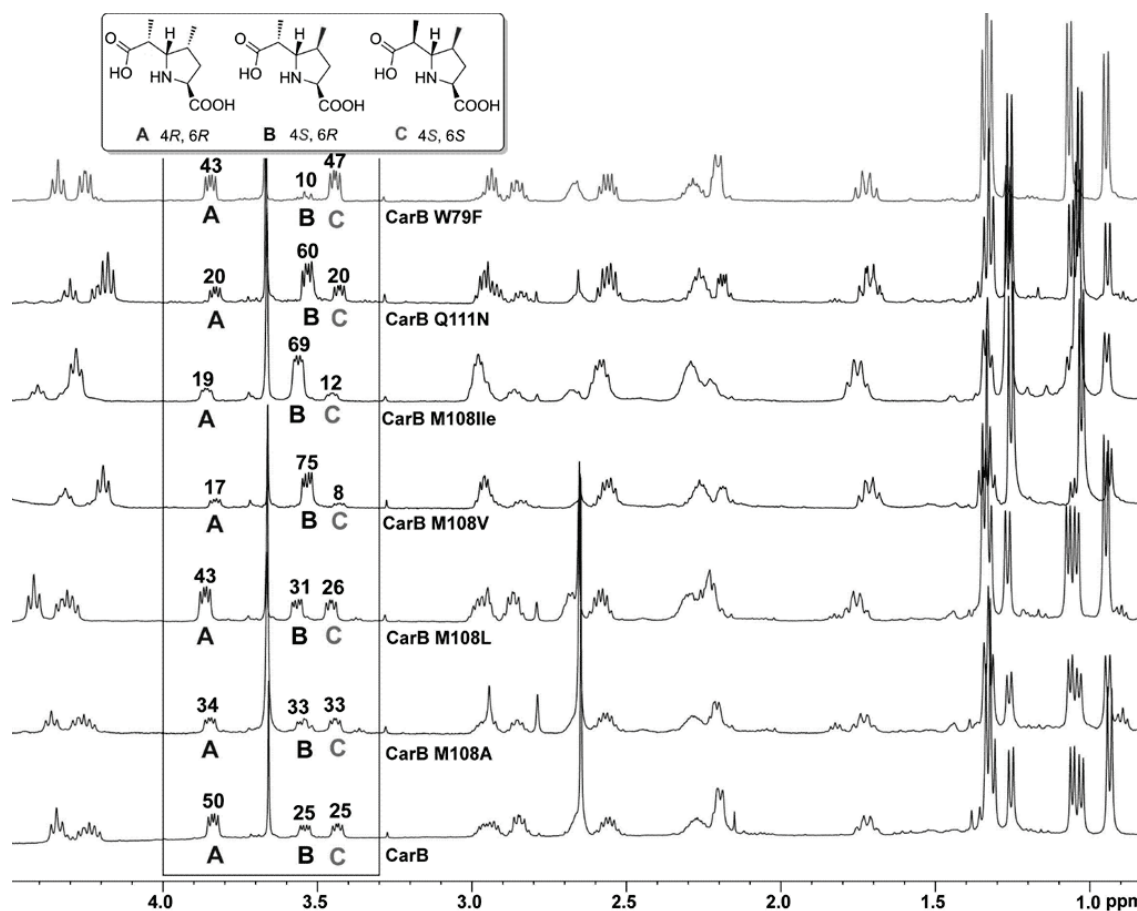


Figure 5.6. Determination of the diastereoselectivity ratios of the (4*R*,6*R*);(4*S*,6*R*);(4*S*,6*S*)-4,6-dimethyl-*t*-CMP (A, B and C, respectively) products of catalysis from methylmalonyl-CoA and 4-methyl-L-GHP by the shown CMPSs. Figure prepared by Dr. R Hamed.¹⁶

Furthering the enantioselectivity of CMPSs on methylmalonyl-CoA and 4-methyl-L-GHP.

The results from the first round of catalysis pointed towards two important residues in the active site to control the diastereoselectivity in the formation of 4,6-dimethyl-*t*-CMP: Met108_{CarB} and Gln111_{CarB}. A second round of mutagenesis aimed to exploit changes in these two residues by individual variations (particularly with Gln111_{CarB}) and their joint effect with the production of double mutants (Table 5.2). LC-MS analysis of the products of catalysis of the new CMPS variants that were prepared revealed, as a proof-of-principle, that the stereoselective production of (4*S*,6*R*)-4,6-dimethyl-*t*-CMP is significantly enhanced by the introduction of substitutions at Met108_{CarB} and

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Gln111_{CarB}. These variants, in general, have more space in relation to C-4 and C-6 positions, while at the same time favouring formation of the (Z)-enolate from methylmalonyl-CoA (See Chapter 2). Compared to wild-type CarB which exhibited a ratio of 25:25:50 (4*S*,6*R*:4*S*,6*S*:4*R*:6*R*)-4,6-dimethyl-*t*-CMP, the CMPS variants from the second round of mutagenesis exhibited ratios of (4*S*,6*R*:4*R*:6*R*)-4,6-dimethyl-*t*-CMP as follows: CarB Q111A (93:7), CarB Q111L (82:18), CarB M108I Q111N (99:1), CarB M108V Q111N (97:3), CarB M108V Q111A (92:2), CarB M108I Q111A (94:6), CarB W79F M108V (75:25), CarB W79F M108A (75:25), CarB W79A M108V (92:8) and CarB M108V H229A (95:5). CarB M108I Q111N presented the highest stereoselectivity of all CMPSs tested, albeit in significantly lower yields than CarB wild-type; the low yields prevented scale up for NMR testing to analyse if any traces at all of the (4*S*,6*S*)-4,6-dimethyl-*t*-CMP product were present.

Table 5.2. The observed enhancements on the production of (4*S*,6*R*)-4,6-dimethyl-*t*-CMP diastereomer by the catalysis of double mutants of CarB compared to the wild-type, as revealed by LC-MS analysis.

Catalyst	(4 <i>S</i> ,6 <i>R</i>)-4,6-dimethyl- <i>t</i> -CMP
CarB wild-type	25
CarB Q111A	93
CarB Q111L	82
CarB M108I Q111N	99
CarB M108V Q111N	97
CarB M108V Q111A	92
CarB M108I Q111A	94
CarB M108V H229A	95
CarB W79F M108V	75
CarB W79F M108A	75
CarB W79A M108V	92

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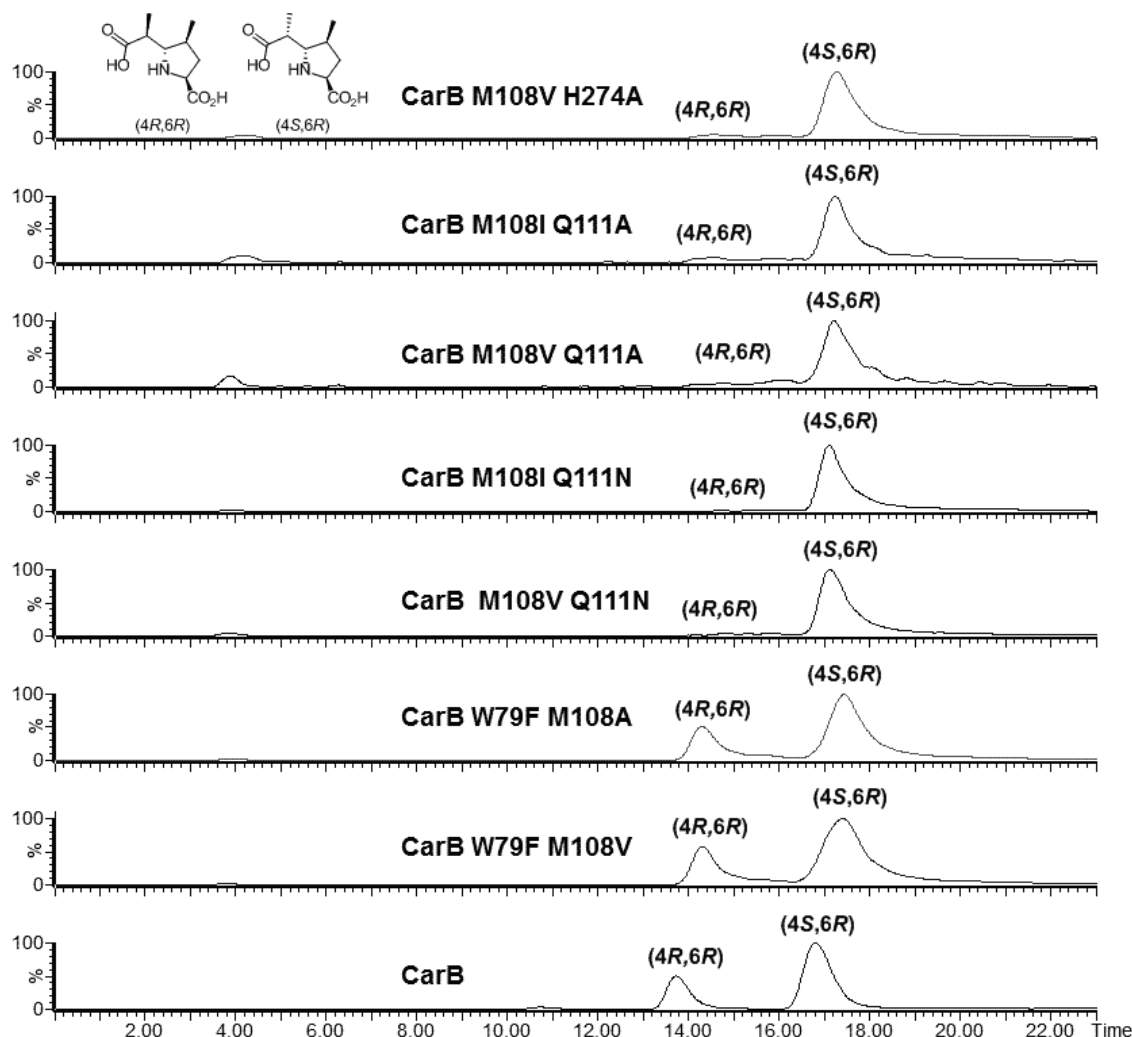


Figure 5.7. Determination of the diastereoselectivity ratios of the (4R,6R)- and (4S,6R)-4,6-dimethyl-*t*-CMP products of catalysis from methylmalonyl-CoA and 4-methyl-L-GHP by the shown CarB double-mutants. CarB M108I Q111N exhibits the highest selectivity compared to CarB wild-type. No NMR data is available yet because of the low yields of the reaction; the possibility of traces of the (4S,6S)-diastereomer cannot be ruled out for this variant.

Improving the diastereoselectivity of CMPS towards the production of (4S,6R)- and (4S,6S)-4-methyl-6-ethyl-*t*-CMP with the aid of Ccr and MatB, respectively.

As described in Chapter 4, the Ccr/CMPS and MatB/CMPS coupled reactions have been shown to produce the (6R)- and (6S)-epimers of 6-ethyl-*t*-CMP in a diastereoselective manner. With this knowledge in hand and the results described in the previous sections for the diastereomers of 4,6-dimethyl-*t*-CMP, it was proposed to

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demonstrate that the coupled reactions of Ccr/CMPS and MatB/CMPS with 4-methyl-L-GHP could yield the expected (4*S*,6*R*)- and (4*S*,6*S*)-epimers of 4-methyl-6-ethyl-*t*-CMP.

LC-MS analyses point towards the use of CarB W79A as the highest yielding variant when used either with Ccr or MatB to produce (4*S*,6*R*)- or (4*S*,6*S*)-4,6-dimethyl-*t*-CMP (for the individual conditions of the Ccr/CMPS or MatB/CMPS reactions, see Methods). The stereochemistry of the products of catalysis from the Ccr/CarB W79A and MatB/CarB W79A coupled reactions with 4-methyl-L-GHP as the substrate for the CMPS were deduced from the elucidation of the structures of the three diastereomers obtained from the CarB W79F catalysis of epimeric methylmalonyl and 4-methyl-L-GHP, as reported in Dr. R. Hamed doctoral dissertation.¹⁶ Similarly to the formation of 4,6-dimethyl-*t*-CMP, only three chromatographically-distinct peaks with the anticipated $m/z = 216$ $[M+H]^+$ were observed by LC-MS under standard conditions. Subsequent scale-up and LC-MS purification led to the isolation of > 85% (by ¹H-NMR) pure fractions of each of the three species observed in the analytical assays. The three fractions isolated were subjected to NMR analyses to determine their stereochemistries at C-4 and C-6, which as will be immediately explained, correspond to that of the equivalent fractions of the 4,6-dimethyl-*t*-CMP products

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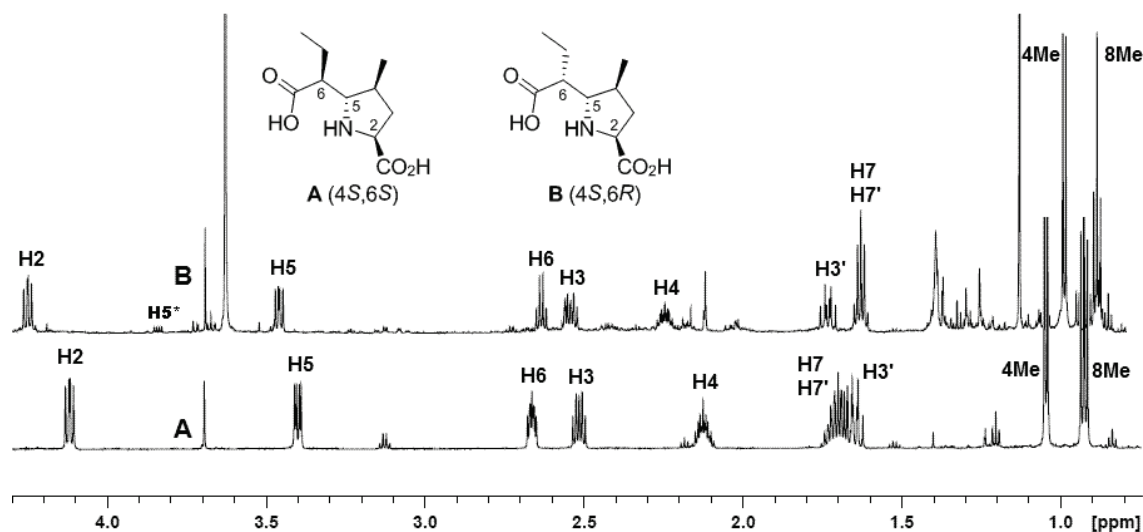


Figure 5.8. ^1H -NMR spectra for the products of catalysis from the MatB/CarB W79A (A) and Ccr/CarB W79A (B) reactions with 4-methyl-L-GHP as the CMPS substrate. The product of catalysis of Ccr has been demonstrated to be (2*S*)-ethylmalonyl-CoA, while for MatB it has been demonstrated to be (2*R*)-ethylmalonyl-CoA. The MatB/CarB W79A reaction seems to yield the (4*S*,6*S*)-product in a diastereospecific fashion, while the Ccr/CarB W79A reaction yields the (4*S*,6*R*)-product in a diastereoselective fashion (88:12, (4*S*,6*R*): (4*R*,6*R*)) as demonstrated by the signal H5* in B). NMR produced by Dr. R. Hamed.

The compound with the shortest retention time was assigned the (*R*)-stereochemistry at C-4 based on the observation of a strong NOE between H-2 and the C-4 methyl group, together with the absence of any NOE between the methyl group at C-4 and H-5. The stereochemistry at C-6 was assigned as (*R*)- on the basis of the lack of NOE between H-5 and H-6, together with the value of $J_{5,6} \sim 11$ Hz implying a predominately *anti* relationship between the two protons; and a strong NOE between H-5 and H-7 as well as 8Me together with the absence of any detectable NOE between H-4 and H-7, nor between 4Me to H-7 (Figure 5.9).

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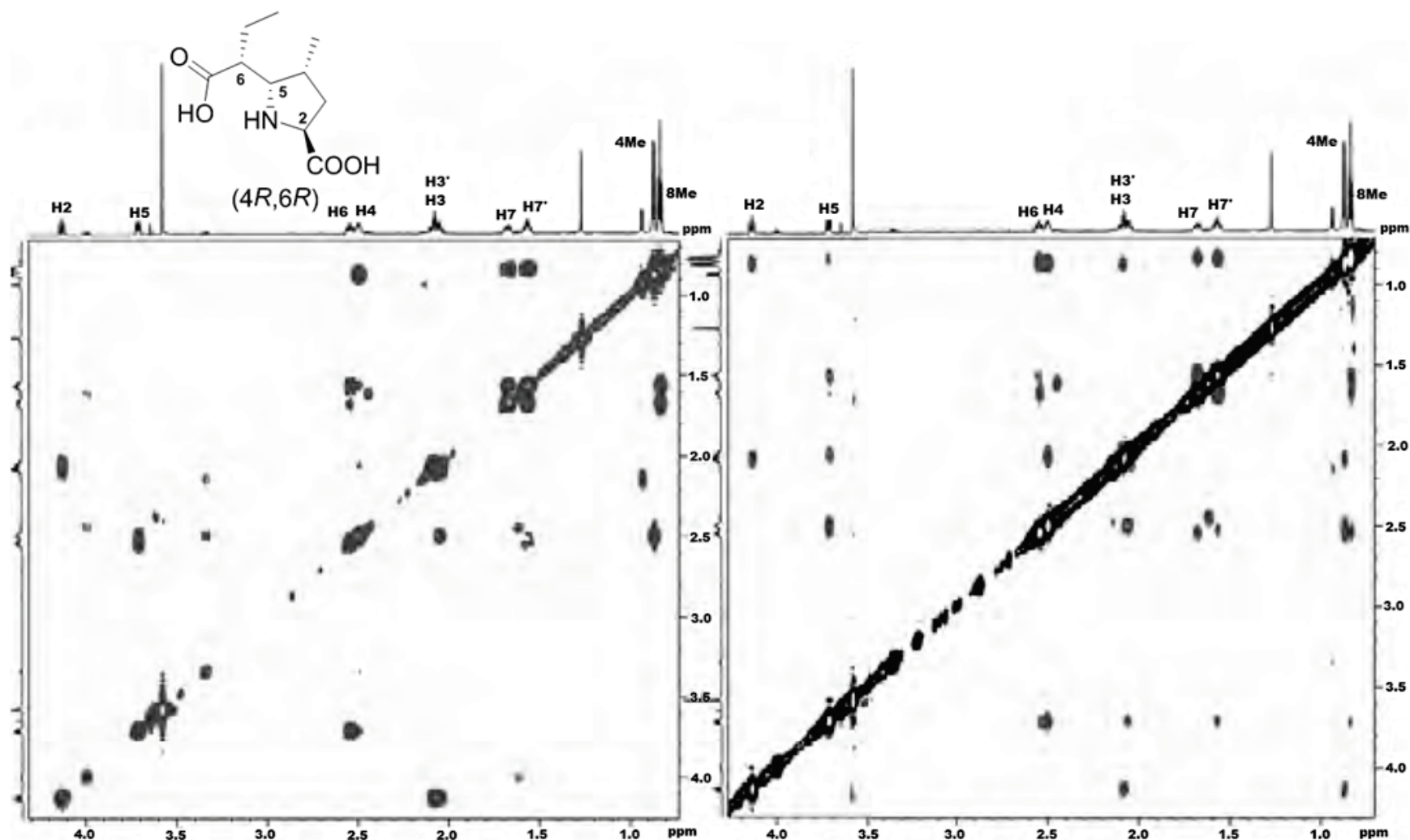


Figure 5.9. ^1H - ^1H COSY and NOESY spectra for (4R,6R)-4-methyl-6-ethyl-L-CMP produced from epimeric 4-methyl-L-GHP and epimeric ethylmalonyl-CoA by CarB W79F catalysis. Figure produced by Dr. R. Hamed.¹⁶

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The compound with the second shortest retention time was assigned the (*S*)- stereochemistry at C-4, based on the observation of a strong NOE correlation between H-5 and the C-4 methyl group, coupled to the NOE correlation between H-4 and H-2. The stereochemistry at C-6 was assigned as (*R*)- on the basis of a strong NOE between H-5 and H-6, together with the value of $J_{5,6} \sim 7.3$ Hz implying a predominately *gauche* relationship between the two protons; and a strong NOE between H-5 and H-7/H-7', as well as a weak NOE between H-5 and 8Me together with weak NOEs between H-4 and both 4Me and H-7/H-7' (Figure 5.10).

The compound with the longest retention time was assigned the (*S*)- stereochemistry at C-4, based on the observation of a strong NOE correlation between the methyl group at C-4 and H-5. Assigning the stereochemistry at C-6 was hindered by the fact that the H-6 and H-3 protons showed the same chemical shift. However, taking into consideration the assignment of the stereochemistry at C-6 of the previous diastereomers, and only 4 possibilities can be anticipated, one can conclude the stereochemistry at C-6 to be (*S*) (Figure 5.11).

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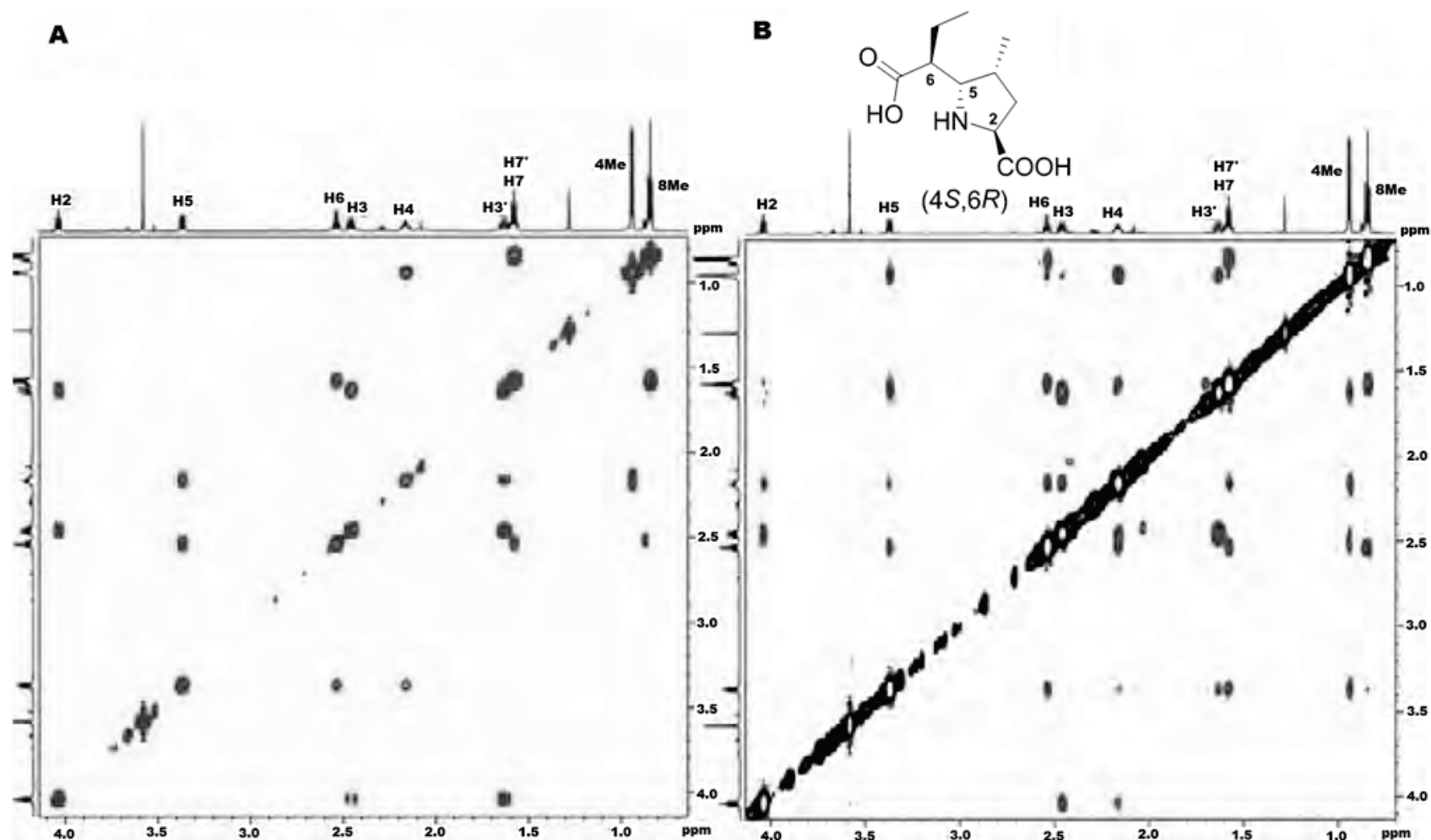


Figure 5.10. ^1H - ^1H COSY (A) and NOESY (B) spectra for (4S,6R)-4-methyl-6-ethyl-L-CMP produced from epimeric 4-methyl-L-GHP and epimeric ethylmalonyl-CoA by CarB W79F catalysis. Figure produced by Dr. R. Hamed.¹⁶

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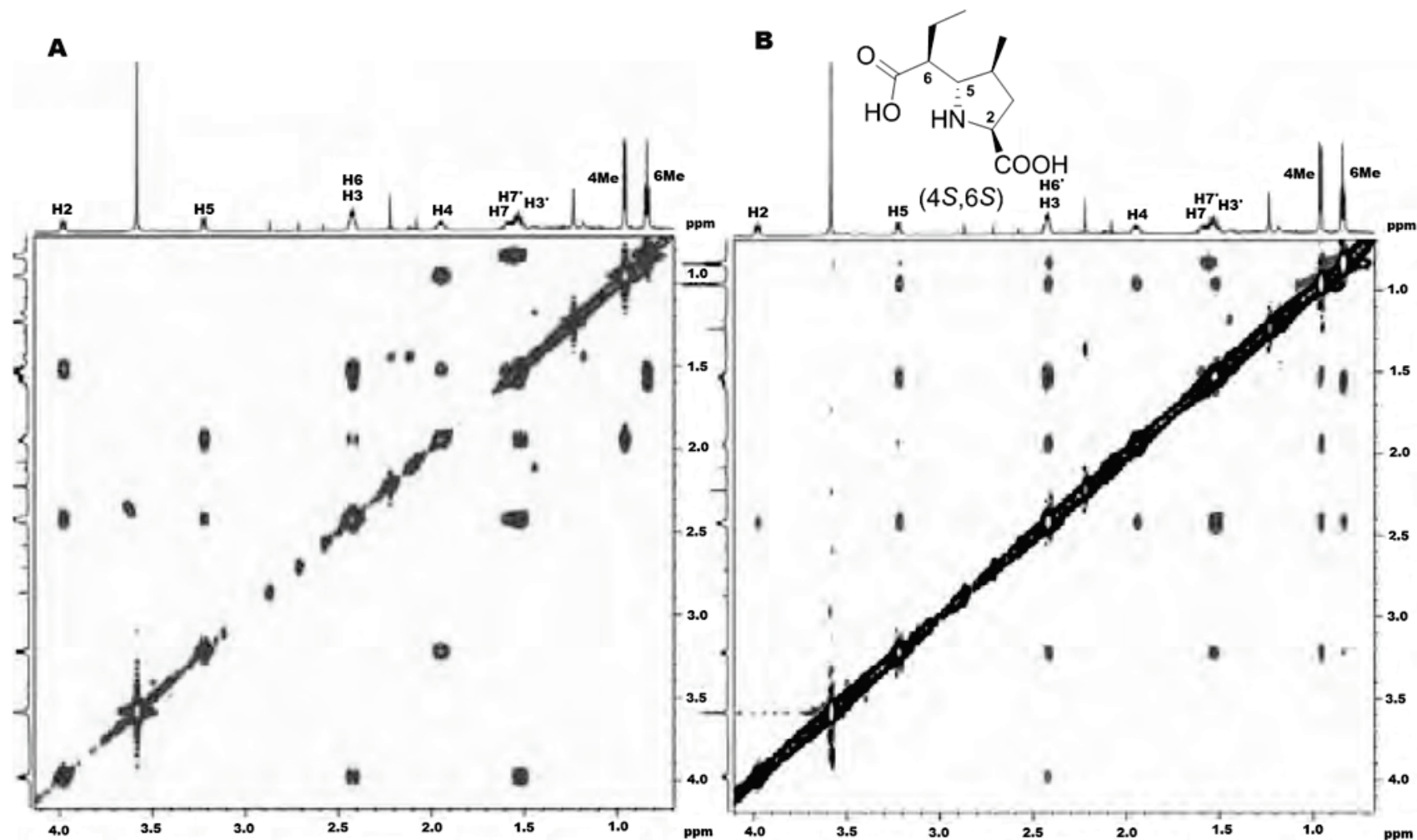


Figure 5.11. ^1H - ^1H COSY and NOESY spectra for (4S,6S)-4-methyl-6-ethyl-L-CMP produced from epimeric 4-methyl-L-GHP and epimeric ethylmalonyl-CoA by CarB W79F catalysis. Figure produced by Dr. R. Hamed.¹⁶

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Discussion.

Wild-type and engineered CMPSs provide access to a variety of 4,6-disubstituted derivatives of *t*-CMP from alkylmalonyl-CoA and 4-substituted-L-GHP. The results illustrate the utility of CMPSs as stereoselective biocatalysts and expand our knowledge of the residues that are amenable to productive modifications. Notably, these results demonstrate the potential of CarB variants to form compounds with three contiguous chiral centres in high diastereoselectivity.

As seen from the previous examples, future work should be aimed towards the engineering of CMPS variants (especially those of Trp79_{CarB} to make the most of the high yields they provide) to enhance the diastereoselectivity at C-4 to the (4*S*)-epimer, either by the sole action of CMPSs (*via* mutual dynamic kinetic resolution) or the coupled activity of Ccr/CMPS or Mat/CMPS.

Chirality is a key factor for the safety and efficacy of many drug products, including β -lactam antibiotics. The production of single diastereomers of drug intermediates has become increasingly important in the pharmaceutical industry. The results of this chapter further demonstrate the enormous potential of enzymes for the production of synthetic chemicals with high chemo-, regio-, and enantioselectivity. Recent developments in the area of directed evolution have enabled increases in activity and the selectivity of biocatalysts, thus rendering many enzymatic processes economically feasible, and allowing for these benefits to be extended to the study and engineering of CMPSs variants.

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Chapter 6. Conclusions.

In the preceding chapters, findings relating to mechanistic and biocatalytic studies of two carboxymethylproline synthases, CarB and ThnE, both members of the crotonase superfamily of enzymes have been presented. CarB catalysis has been reported to determine the stereochemical course of the C3C biosynthesis pathway.¹ The formation of *t*-CMP and (6*R*/6*S*)-6-methyl-*t*-CMP by CarB/ThnE catalysis has revealed the involvement of the two enzymes in a stereoselective C-C bond forming reaction. Because of the central importance of asymmetric C-C bond formation in organic synthesis, studies were conducted investigating C-C bond formation by CarB/ThnE. Analyses were carried out using both isolated CarB/ThnE, and with these enzymes in conjunction with other enzymes, *i.e.* crotonyl-CoA carboxylase/reductase (Ccr) the malonyl-CoA synthetase (MatB) and the methylmalonyl-CoA epimerase (MCE). Overall the results reveal that CarB/ThnE, including in engineered forms, are rather promiscuous with respect to their substrate selectivity and imply further biocatalytic studies on the crotonase family will be productive.

The biocatalytic studies aimed at exploring the capabilities of the catalytic machinery of wild-type and engineered CMPSs. Residue selection for site directed mutagenesis was on the basis of the knowledge gained from CarB crystal structure,² the mechanistic studies,²⁻⁵ docking studies, and comparison to other crotonases.⁶ Wild-type and variant CMPSs were used as biocatalysts for the preparation of functionalised 5-, and 6-membered *N*-heterocycles, in a diastereoselective fashion, from amino acid aldehydes and (alkylated)malonyl-CoA derivatives; the *N*-heterocycles produced were

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converted to the corresponding bicyclical β -lactams by carbapenam synthetase, CarA catalysis.

Overall, twenty-six variants (eighteen CarB-based, and eight ThnE-based) were generated and evaluated as biocatalysts for the preparation of *t*-CMP and *t*-CMPi derivatives. A single residue substitution involving one of the OAH residues (*i.e.* M108 in CarB for Val, and Val153 in ThnE for Ala) enabled control of tri-substituted enolate formation/reactivity and consequently control of the side-chain carbon (C-6) stereochemistry. CarB W79-based variants catalyzed the formation of 6-ethyl-*t*-CMP in good yields and displayed bias towards the formation of the (6*S*)-epimer.

Coupling CMPS catalysis with that of crotonyl-CoA carboxylase reductase (Ccr) enabled stereoselective formation of (6*R*)-6-ethyl-*t*-CMP. The biocatalytic promiscuity of malonyl-CoA ligase and the stereoselectivity of crotonyl-CoA carboxylase reductase were successfully coupled to the selective tri-substituted enolate forming capacity of engineered carboxymethylproline synthases for the preparation of functionalized 5- and 6-membered *N*-heterocycles substituted with a variety of alkyl side chains at the C-5/C-6 carboxymethyl-substituent at high diastereomeric excess. The effect of methylmalonyl-CoA epimerase on the diastereoselectivity of the carboxymethylproline synthase-catalysed enolated alkylation was also demonstrated.

It was also possible to simultaneously functionalize *t*-CMP with a 6-alkyl and a 4 β -methyl group in a stereoselective fashion by engineered CMPSs catalysis. Overall, twelve *t*-CMP derivatives substituted at C-4 or at C-4 and C-6 were produced employing wild-type and engineered CMPSs as biocatalysts. These products could

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serve as intermediates for the preparation of carbapenems with potentially enhanced stability profiles (e.g. carbapenems resistant to dehydropeptidase-I hydrolysis).

In total, more than thirty derivatives/analogues of *t*-CMP were generated employing CMPSs catalysis. Using the β -lactam synthetase, CarA, ten of the produced carboxymethyl-substituted *N*-heterocycles were converted into the corresponding bicyclical β -lactams. Overall, the work represents further advances towards the development of biocatalytic routes for the production of carbapenems or intermediates for their production, and highlights the potential of the crotonase superfamily of enzymes as stereoselective biocatalysts.

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Materials and Methods.

Materials.

Chemicals, unless otherwise stated, were obtained in the highest quality available from Sigma-Aldrich.

Plasmids and restrictions enzymes were from Sigma-Aldrich and New England Biolabs respectively. The *ccr/pET3d* plasmid was a kind gift from Prof. B.E. Alber, The Ohio State University.¹ The *matB/pET28b* and *mcee/pET28b* plasmids were a kind gift from Prof. A. Keatinge-Clay, The University of Texas at Austin.² Human MCE was a kind gift from Dr. Wyatt Yue, University of Oxford.³

Pfu Turbo[®] DNA polymerase and competent cells were from Stratagene.

Bacto tryptone, Yeast Extract and Bacto Agar for use in culture media were obtained from OXOID; sodium chloride for culture media was from Fisher BioReagents.

SeeBlue[™] Plus2 molecular weight markers were from Invitrogen, and 1kb DNA molecular weight markers were from New England Biolabs.

DTT and IPTG were obtained from Apollo Scientific, and electrophoresis grade agarose from Bioline.

Purified water was obtained from a Millipore Elix[®] Reverse Osmosis system which was further purified by a Millipore Milli-Q[®] Synthesis system (ultra pure water, 18.2 MΩ·cm). Water was sterilized for DNA applications by autoclaving.

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Molecular Biology

Standard sterile practices were followed thoroughly, with the aid of a Heraeus laminar flow hood when necessary. Media and equipment were sterilized by autoclaving at 121 °C for 20 min. Solutions of IPTG, antibiotic and other labile compounds were sterilized using 0.2 µm filters (Minisart[®], Sartorius Stedim)

Polymerase Chain Reaction

thnE was cloned from genomic DNA of *S. cattleya* by Mr. D. Harding and Dr. R. Hamed. PCR was performed in 0.5mL Eppendorf tubes, using the following components:

Component	Volume (µL)	Final concentration
Autoclaved MilliQ water	33.0	-
DMSO 100%	5.0	10% v/v
10x Pfu Buffer	5.0	1x
dNTPs mix (10mM each nucleotide)	1.0	200µM e.
MgCl ₂ (25mM)	1.5	375µM
Forward primer (10pmol/µL)	1.5	0.2 pmol/µL
Reverse primer (10pmol/µL)	1.0	0.2 pmol/µL
Genomic DNA template (100 ng/µL)	1.0	2 ng/µL
Pfu-Ultra polymerase (2.5 U/µL)	1.0	1 U/20µL

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All reagents were mixed, with the exception of DNA polymerase, and incubated at 95°C for 5 min for initial denaturation of DNA. This was followed by three loops of 12 cycles each consisting of:

	Temperature (°C)	Time (s)	
Loop 1			
	95	30	Denaturing
	71	30	Annealing
	72	1 min/kB	Extension
Loop 2			
	95	30	Denaturing
	66	30	Annealing
	72	1 min/kB	Extension
Loop 3			
	95	30	Denaturing
	61	30	Annealing
	72	1 min/kB	Extension

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This was followed by a final extension step of 10 min at 72°C. The samples were held at 4°C and stored at -20°C until further use.

Site Directed mutagenesis

CarB variants were prepared according to the QuikChange® Site-Directed Mutagenesis Protocol (Stratagene). A *pET24a/carB* construct was used as template for PCR mutagenesis. For preparation of the CarB M108A/W79F variant, the *pET24a/carB* M108A construct was used as a template. For preparation of the CarB M108V/W79F variant, the *pET24a/carB* W79F construct was used as a template. For the preparation of the CarB M108V(I) Q111N and CarB Q111A variants, the *pET24a/carB* Q111N construct was used as a template. For the preparation of the CarB M108V(I) Q111A variants, the *pET24a/carB* Q111A construct was used as a template. The oligonucleotide primers were obtained from Sigma-Genosys. Production of the desired mutant was verified by DNA sequencing.

Table 1. Primers used for CarB mutagenesis studies

CarB variant	Forward Primer	Reverse Primer (3' – 5')
M108A	gctatgcgattggtgcgggtttccagttcg	cctaccgatacgcctaaccacgcccaaaggt
M108V	gctatgcgattggtgtgggtttccagttcg	cctaccgatacgcctaaccacaccaaaaggt
M108L	gctatgcgattggtctgggtttccagttcg	cctaccgatacgcctaaccagacccaaaggt
M108I	gctatgcgattggtatcggtttccagttcg	cctaccgatacgcctaaccatagccaaaggt
Q111N	gggtttcaactcgcctgatgttgacc	ccatacccaaagttgaagcgggactacaa
Q111A	gggtttcctgttcgccctgatgttgacc	gggatgggttcgcgttcgccctgatgtt
Q111L	gggtttcgcgttcgccctgatgttgacc	gggatgggttcgcgttcgccctgatgtt
W79F	gacatcgaagagttcattgaccgcgtgatt	cgctcctgtagcttctcaagtaactggcgc
W79A	gacatcgaagaggcgattgaccgcgtgatt	cgctcctgtagcttctccgtaactggcgc
H229A	ccaaagctgtcgcgaaggcagcgttcagg	gaacgctgccttcgcgacagcttgggaagc
M108VQ111A	gctatgcgattggtgtgggtttcgcgttcg	gggatgggttcgcgttcgccctgatgtt

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For ThnE variant preparation, a *pET24a/thnE* construct was used as template. For preparation of the ThnE V153M/W124F variant, the *pET24a/thnE* W124F construct was used as a template.

Table 2. Primers used for ThnE mutagenesis studies.

CarB variant	Forward Primer	Reverse Primer (3' – 5')
V153M	ggttatgccatcggtatgggggtgcagatc	caacgagatctgcaacccataccgatgg
V153A	ggttatgccatcggtgcgggggtgcagatc	caacgagatctgcaacccgcaccgatgg
V153L	ggttatgccatcggtctgggggtgcagatc	caacgagatctgcaacccagaccgatgg
V153I	ggttatgccatcggtatcggggtgcagatc	caacgagatctgcaacccgataccgatgg
V153V	ggttatgccatcggtgtgggggtgcagatc	caacgagatctgcaacccacaccgatgg
W124F	ggtaacgcgttcacgcacacatcaccgac	gatgtcgtcgatgaacgcgttgacctcgtcc
H274A	gaggcgaaggagtcggcgcgcagagccttc	cgcgaaggctctgcgcgccgactccttcgc

Agarose Gel Electrophoresis

DNA samples were analyzed and purified by gel electrophoresis of the DNA mixture on 1% w/v agarose gels prepared with SYBR Safe™ DNA gel stain (Invitrogen). Samples were loaded after mixing with 5x loading buffer, and run in TAE buffer at a constant potential of 80V until suitable fragment separation was achieved. Gels were visualized by UV light and the size of the fragments referenced to a 1kb DNA ladder (New England Biolabs)

5x Sample loading buffer

Glycerol	30% (v/v)
Xylene cyanol FF	0.25% (v/v)
Bromophenol blue	0.25% (v/v)

50x TAE running buffer

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Per litre:

Tris	242.0g
Glacial acetic acid	57.0mL
EDTA (0.5M)	100.0mL

DNA electrophoresis gel

Per gel:

Agarose (electrophoresis grade)	0.5g
SYBR Safe™ DNA gel stain	3.0µl
TAE buffer (1x)	50.0mL

DNA Restriction Digest

After PCR, the methylated, non-mutated parental DNA template was digested by addition of DpnI (1 µL) to the reaction mixture, followed by incubation at 37 °C in a water bath for 1 h.

Small-scale restriction digests were carried out to determine the presence of an insert with the anticipated molecular weight by agarose gel electrophoresis. The following reagents were added in order:

	Large scale	Small scale
DNA	2 µg	0.2 µg

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Water	0 - 25 μL	0 – 2.5 μL
Buffer	5 μL	1 μL
Restriction enzyme A	1 μL	1 μL
Restriction enzyme B	2 μL	2 μL
Total	50 μL	10 μL

The mix was incubated at 37°C overnight. Digested DNA was purified using a QIAQuick Gel Extraction Kit (QIAGEN) following the instructions of the manufacturer.

DNA Ligation

Digested and purified DNA and vector with complementary sticky ends were ligated overnight at 4°C, under the following conditions to maximize the probability of success:

Component	A (μL)	B (μL)	C (μL)
Insert	6.5	5.0	4.0
T4 ligase buffer	1.0	1.0	1.0
Vector	1.5	3.0	4.0
T4 ligase	1.0	1.0	1.0

Purification of plasmid DNA from ligation was performed from 5mL of transformed bacterial culture using a QIAprep Spin Miniprep Kit (QIAGEN) following the instructions of the manufacturer.

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Transformation of Competent Cells.

Competent cells were thawed and resuspended on ice. Plasmid DNA (2µL) was transferred into a 1.5mL Eppendorf tube, previously chilled on ice. An aliquot of cells (20µL) was added, in the presence or absence of β-mercaptoethanol (2µL) according to the instructions of the specific competent cell line, with gentle mixing. The samples were incubated on ice according to the instructions of the specific cell line before a heat shock (42 °C, length of time depending on the competent cell line). After heat shock, the samples were incubated for 2 min on ice, upon which SOC medium (80µL) was added and the samples incubated for 1 hour at 37°C with shaking (300 r.p.m.). After incubation, samples were centrifuged, decanted to a final volume of 100 µL, and plated on solid LB medium containing the appropriate antibiotic and incubated overnight at 37°C.

E. coli strains used during this project possessed the following genotypes:

XL1-Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacIqZΔM15 Tn10</i> (Tet ^r)].
XL10-Gold	Tet ^r Δ(<i>mcrA</i>)183 Δ(<i>mcrCB-hsdSMR-mrr</i>)173 <i>endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte</i> [F' <i>proAB lacI^qZΔM15 Tn10</i> (Tet ^r) Amy Cam ^r].
BL21(DE3)	F ⁻ <i>ompT hsdS_B(r_B⁻ m_B⁻) gal dcm</i> (DE3)
BL21 CodonPlus (DE3)	<i>E. coli</i> B F ⁻ <i>ompT hsdS(r_B⁻ m_B⁻) dcm⁺ Tet^r galλ</i> (DE3) <i>endA Hte</i> [<i>argU proL</i> Cam ^r] [<i>argU ileY leuW</i> Strep/Spectr]

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BL21-Gold(DE3)	<i>E. coli</i> B F ⁻ <i>ompT hsdS</i> (r _B ⁻ m _B ⁻) <i>dcm</i> ⁺ Tet ^r <i>gal</i> λ(DE3) <i>endA</i> Hte
Rosetta2 (DE3)	F ⁻ <i>ompT hsdSB</i> (r _B ⁻ m _B ⁻) <i>gal dcm</i> pRARE2 ³ (Cam ^R)

Preparation of Glycerol Freeze

Bacterial cultures were preserved by the addition of 75% (v/v) glycerol (250μL) to cell culture (650μL), with gentle mixing. The glycerol freezes were stored at -80°C

Sequencing.

DNA sequencing was performed by Source BioScience LifeSciences using an Applied Biosystems 3730 DNA Analyzer.

N-terminal protein sequencing was carried out by Mr. A. Willis at the Protein Characterisation Facility of the Department of Biochemistry, University of Oxford, using an Applied Biosystems Procise 494A protein sequencer by the Edman degradation technique.

pH Measurements

The pH of solutions was determined using a Jenway pH Meter 3305, with an Aldrich glass/calomel combination electrode. Calibration was carried out between pH 4.0-7.0 or pH 7.0-10.0 immediately prior to use, with buffer solutions of phthalate (pH 4.0), phosphate (pH 7.0) and borate (pH 10.0) from Fischer Scientific. Electrodes were stored in a 4M potassium chloride solution.

Centrifugation.

Samples of volume less than 1.5 mL were centrifuged at room temperature using an accuSpin™ benchtop centrifuge (Fisher Scientific) at 3400 r.p.m. for 5 min. Cell growth

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media and cell lysates were centrifuged in a Beckmann Avanti J-25 (8500 r.p.m., 10 min, JA-10 rotor) and protein was concentrated using a Beckmann Allegra 21R (4000 r.p.m., SX4250 swing bucket rotor) or a Beckmann Avanti J-25 (24000 r.p.m., JA-25.5 rotor, respectively), at 4 °C and for 25 min periods. Expression trial growths (100 mL) were centrifuged in a Beckman GS6R at 3,600 rpm.

Incubations

Bacterial plate cultures were prepared using LB media supplemented with 2% (w/v) agar with the appropriate antibiotic. Following inoculation, plates were incubated overnight at 37°C in a Gallenkamp Duostat Incubator. Successful cultures were later stored in the dark at 4°C.

Liquid cultures of 2TY medium and the appropriate antibiotic were incubated at the relevant temperature, with shaking at 200 r.p.m. in a New Brunswick Scientific Innova® 44 Incubator.

Starter cultures were inoculated from a single bacterial colony or from a glycerol freeze. Small-scale starter cultures (5mL) were grown in 50mL Falcon tubes, while medium-scale cultures (100mL) were grown in 500mL Erlenmeyer flasks.

Inoculation of media for expression trials growths was *via* a 1% (v/v) addition of starter culture. Small-scale expression trials were carried out using 500mL Erlenmeyer flasks, containing 100mL of medium, while large-scale growths were performed in 2L Erlenmeyer flasks containing 600mL of medium.

Growth Media

All growth media were autoclaved at 121°C for 20 min before use.

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Luria-Bertani (LB) medium

Per litre:

Bacto tryptone	10.0g
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Yeast extracts	5.0g
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NaCl	10.0g
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Agar	15.0g
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2x Tryptone/Yeast extract (2TY) medium

Per liter:

Bacto tryptone	16.0g
----------------	-------

Yeast extracts	10.0g
----------------	-------

NaCl	5.0g
------	------

SOC medium

Per liter:

Bacto tryptone	16.0g
----------------	-------

Yeast extracts	10.0g
----------------	-------

NaCl	5.0g
------	------

MgCl, 1M	10.0mL
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Glucose, 2M

20.0mL

Cell Lysates

For expression trials, cell pellets were resuspended in Tris-HCl (50mM, pH 7.5) and lysed by sonication on ice, using a Soniprep 150 sonicator (10 μ amplitude). Sonication was for 3x30 s, separated by 30 s cooling intervals. Soluble and insoluble protein was separated by centrifugation of the lysate for 5 min in 50mL Falcon Tubes. Soluble fractions were further centrifuged for 5 min in 5mL Eppendorf tubes.

For protein purification the cell pellets were resuspended in the appropriate loading buffer and sonicated on ice using an Ultrasonic INC W-380 sonicator (60% amplitude) for 6x30 s, separated by 30 s cooling intervals. Soluble and insoluble protein was separated by centrifugation of the lysate. Soluble fractions were further centrifuged using a Beckmann Allegra 21R (4000 r.p.m., SX4250 swing bucket rotor).

Polyacrylamide Gel Electrophoresis (PAGE)

The purity and behaviour of proteins were analyzed by PAGE, following the procedure established by Laemmli.⁴ Gels were prepared using 70mm x 100mm glass plates, with 0.75 mm spacers. TEMED¹ and freshly prepared ammonium persulfate were added to gel mixtures immediately prior to casting. The separating gel was cast first, with the addition of a separate isopropanol layer to ensure a levelled surface. Following solidification of the separating gel, the isopropanol was removed and the stacking gel was cast. Gels were run on a Mini-PROTEAN Tetra Electrophoresis System (Bio-Rad) at a constant potential of 200 V (denaturing) and 50 V (native).

¹ TEMED. Tetramethylethylenediamine.

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Following electrophoresis, the gels were stained for 15 min with InstantBlue™ Coomassie® stain (Expedeon)

For denaturing PAGE analysis, samples were prepared by mixing with sample loading buffer (2x) and incubation at 95°C for 10 min. Sample volume was adjusted to load 10µg of protein per well.

Running buffer 10x

Per litre:

Tris	34.0g
Glycine	144.0g
SDS ²	10.0g

Separating gel 12.5% (v/v)

Per gel:

Acrylamide (30% w/v)	2.08mL
/bis acrylamide (1.034% w/v)	
Tris-HCl (1.5M, pH 8.8)	1.25mL
SDS (10% w/v)	50.0µL
H ₂ O	1.57mL
Ammonium Persulfate	50.0µL

² SDS. Sodium dodecyl Sulfate.

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TEMED	4.0 μ L
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Denaturing PAGE sample loading buffer 2x

Bromophenol Blue	0.01% (w/v)
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SDS	10.00% (w/v)
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Glycerol	50.0% (v/v)
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β -mercaptoethanol	1.0% (v/v)
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For native PAGE analysis, gels were prepared as denaturing gels, except for the replacement of SDS with H₂O and the use of an alternative sample loading buffer.

Native PAGE sample loading buffer 2x

Bromophenol Blue	0.01% (w/v)
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Glycerol	50.0% (v/v)
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Small-scale protein expression trials

Expression trials were set up at different temperatures, different IPTG concentrations and different incubation times in order to determine the optimum expression conditions. Cell pellets from a 100 ml cell growth mixture were resuspended in 5 ml of 50 mM Tris-HCl pH 7.5 and lysed on ice by sonication. The soluble and insoluble proteins were separated into 1.5 ml Eppendorf tubes after centrifugation of the lysate for 10 min; the soluble fraction were centrifuged for a second time and the level of expression of both soluble and insoluble protein was analyzed by SDS-PAGE .

Recombinant protein expression

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All *carB* and *thnE* constructs were initially transformed into *E. coli* XL1-Blue or XL10-Gold cells. The constructs were purified and then transformed into *E. coli* BL21 (DE3) GOLD cells. Cells were grown using 12 x 600 ml 2TY medium containing the appropriate antibiotic. A 1% (v/v) inoculation of overnight culture was grown at 37°C until OD₆₀₀ reached ~0.7, at which point protein expression was induced with IPTG. The temperature was adjusted when necessary and the cells were grown for the length of time required prior to harvesting *via* centrifugation at 8000 r.p.m. for 5 min at 4 °C in a Beckmann Avanti J-25 (10 min, JA-10 rotor).

Fast Protein Liquid Chromatography (FPLC)

Enzymes were purified at 4°C using an Amersham Pharmacia Biotech Äkta FPLC System (P920 pump system, IPC900 UV detector, Frac900 fraction collector, controlled by Amersham Pharmacia Unicorn Software v4.00.16) All buffers were freshly prepared with ultra-pure water, filtered and degassed prior to use.

Ccr was purified following the procedure established by Erb and co-workers,¹ with slight modifications. 10g of frozen cells were suspended in 100 mL of buffer A (20 mM Tris-HCl, pH 7.9) containing 0.1 mg of DNase I per ml, and lysozyme. The suspension was sonicated and centrifuged as described. Supernatants (100 mL) after the centrifugation step were applied at a flow rate of 2.5 mL/min onto a 30-ml DEAE-Sepharose Fast Flow column (Amersham Biosciences), which had been equilibrated with 60 ml of buffer A. The column was washed with 120 mL of buffer A and thereafter with 135 mL of buffer A containing 50mM NaCl. Activity was eluted with 100 mM NaCl in buffer A in a volume of 195 mL. Active fractions were pooled and concentrated to a final volume of 10 ml by ultrafiltration (Amicon[®] Ultra-15 10,000 NMWL; Millipore). Concentrated protein solution 10 mL obtained by DEAE chromatography

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was applied at a flow of 2.5 mL/min onto a 50 mL Blue Sepharose 6 Fast Flow (GE Healthcare) which had been equilibrated with 100 mL of buffer A. The column was washed with 100 mL of buffer A, followed by 185 mL of buffer A containing 100 mM NaCl and 185 mL of buffer A containing 200 mM of NaCl. Activity was eluted with 500 mM NaCl in buffer A in a volume of 150 mL. Active fractions were pooled, desalted, and concentrated to a final volume of *ca.* 1.5 mL by ultrafiltration (Amicon[®] Ultra-15 10,000 NMWL; Millipore). The protein was stored at -20°C.

CarB, ThnE and its variants were produced as reported.⁵ Following cell lysis, the enzymes were purified employing a two column protocol involving anion exchange (Q-sepharose) and hydrophobic interaction (phenyl sepharose) chromatography. A 55- or 14 mL Q-Sepharose FF column was washed and equilibrated in 50 mM Tris-HCl, pH 7.5. The filtered cell lysate was then loaded onto the column through the pumps. The column was then washed with 5 column volumes of 50 mM Tris-HCl, pH 7.5 to remove any unbound proteins. A gradient was then run from 0.0 to 0.6 M NaCl in 50 mM Tris-HCl, pH 7.5 over 10 column volumes. Selected samples were further analysed by SDS-PAGE. The fractions containing the target enzyme and of acceptable purity (>70% pure by SDS-PAGE analysis) were pooled and mixed with an equal volume of 2M ammonium sulfate in 50mM Tris-HCl, pH 7.5 before being loaded onto a 75- or 15 mL Phenyl-Sepharose HP column that had been washed and pre-equilibrated with 1M ammonium sulfate in 50 mM Tris-HCl, pH 7.5. Unbound protein was then removed by washing the column with another 3 volumes of ammonium sulfate in 50mM Tris-HCl, pH 7.5 before a gradient was run from 100-0% 1M ammonium sulfate in 50mM Tris-HCl, pH 7.5 over 10 column volumes. Target enzymes eluted at ~0.3M ammonium sulfate. The fractions containing the target enzyme and with purity >95%, as judged by

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SDS-PAGE analysis, were pooled and concentrated to 3 mL, then desalted and buffer-exchanged into 50mM Tris-HCl, pH 7.5. Samples were stored at -80°C in 100 µL aliquots.

MatB and MCE were produced as reported.² Starter cultures of *matB/pET28b* or *mcee/pET28b* in BL21 *E. coli* were grown overnight and used to inoculate 2TY broth, supplemented with 50 mg/L kanamycin. When OD₆₀₀ = 0.4, expression was induced with 0.5mM IPTG. After 16 hr, cells were harvested by centrifugation, resuspended in lysis buffer (100 mM HEPES-NaOH, pH 7.5, 10% (v/v) glycerol, 0.5M NaCl), sonicated, and centrifuged (30,000 relative centrifugal force for 45 min) to remove cellular debris. Cell-free lysate was passed over a nickel-NTA column equilibrated with lysis buffer. The column was washed with lysis buffer containing 15 mM imidazole and protein was eluted using lysis buffer containing 150 mM imidazole. MatB was further purified for crystallization via gel filtration using a Superdex 200 column equilibrated in gel filtration buffer (100 mM HEPES-NaOH, pH 7.5, 10% (v/v) glycerol, 0.5M NaCl). Fractions were collected and concentrated to 15 mg/ml in gel filtration buffer.

Protein Concentration Measurement.

Protein concentration was measured by a Nanodrop spectrophotometer ND-1000 (Thermo Scientific) at an absorbance of 280 nm, based on the calculated molecular weight and extinction coefficient of the sample enzymes (calculated by the ProtParam tool on the ExPASy server⁶), with as 2 µL sample, following the manufacturer instructions.

Enzymatic Activity Essays.

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A typical Ccr assay was prepared by the addition of reagents in the following order, from an established protocol:¹

Reagent	Quantity	Final Concentration
Tris HCl pH 7.9 (150 mM)	67 µl	100 mM
NADPH (80 mM)	5 µl	4 mM
Crotonyl-CoA (10 mM)	20µl	2 mM
CCR (6.25 µg/µl)	1 µl	+5 µg
NaHCO ₃ (0.3 M)	11 µl	33 mM

A typical CarB/ThnE assay consisted in the addition of reagents in the following order:

Reagent	Quantity	Final Concentration
Tris HCl pH 9.0 (600 mM)	35 µl	420 mM, pH 7.7
L-GHP (der)/10% v/v FA (~10 mM)	5 µl	2 mM
Malonyl-CoA (der) (10 mM)	8 µl	1.6 mM
CarB/ThnE	3 µl	+5 µg

Coupled Ccr-CMPSS essays were prepared as follows:

Reagent	Quantity	Final Concentration
Tris HCl pH 7.9 (150 mM)	67 µl	100 mM
NADPH (80 mM)	2.5 µl	2 mM
Crotonyl-CoA (10 mM)	10 µl	1 mM
CCR (6.25 µg/µl)	6 µl	+36 µg
NaHCO ₃ (0.3 M)	11 µl	33 mM
Tris HCl pH 9.0 (600 mM)	35 µl	420 mM, pH 7.7
L-GHP (der)/10% v/v FA (~10 mM)	5 µl	2 mM

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CarB/ThnE

3 μ l

+5 μ g

Assay reactions were incubated for 30 min in a 37 °C water bath. After this, reaction were quenched with 100 μ L of a 0.25 mM solution of *p*-aminosalicylic acid in methanol and incubated on ice for 10 min. Protein was precipitated by centrifugation and supernatants were aliquoted (~150 μ L) to a MS tube for analysis.

Coupled MatB-CMPS

- | | |
|--|--------------|
| - Tris 600 mM pH 9.0 | 35 μ L |
| - Aminoacid semialdehyde
in 10% v/v FA, 15 mM | 5 μ L |
| - Tris 600 mM pH 7.9 | 42.5 μ L |
| - Coenzyme A 10 mM | 5.5 μ L |
| - ATP in 50 mM Tris
pH 8.0, 100 mM | 1.3 μ L |
| - Malonic acid (der.) 1M | 2.2 μ L |
| - MgCl ₂ 200 mM | 4.5 μ L |
| - MatB 1.659 μ M | 1 μ L |
| - CMPs | 3 μ L |

Original protocol stipulates an overnight incubation at room temperature; this was optimized to 37 °C for 2h with equivalent yields as the original conditions, as observed by LC-MS.

MCE was used as 1 μ L of MCE_{human} @ 20 mg/mL; for MMCE_{bacterial} stock solution was diluted to 20 mg/mL (from a stock of 73.88 mg/mL) and use 1 μ L for all assays.

Large scale enzymatic product isolation, and preparation for NMR characterization

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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 μ L), centrifugation (13,000 $\times$ g) and freeze-drying of the supernatant. The resultant residue was resuspended in 15% aqueous methanol (200 μ L) and purified using a Waters Spherisorb column (250 mm $\times$ 10 mm, 5 μ) 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. Elution was monitored using a Micromass® Quattro micro™ API mass spectrometer (equipped with a Waters 1525 μ 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 D₂O (600 μ L), transferred to an Eppendorf vial and freeze-dried. The final residue was re-suspended in D₂O (12 or 6 μ L, according to the NMR system to be used), transferred into a 1mm NMR tube (Bruker) using a hand centrifuge, and analysed by NMR.

NMR Assignment of Products of CMPS catalysis

NMR analyses were recorded at 298 K using a Bruker AVIII 700 MHz spectrometer equipped with a ¹H TCI-inverse cryoprobe optimised for ¹H 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_{HH} coupling constants and NOEs. Chemical shifts are reported in ppm relative to D₂O (δ_{H} 4.72); the deuterium signal was used as an internal lock signal and the HDO signal was reduced by pre-saturation 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.

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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 ^1H -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 to (2*S*,5*S*)-carboxymethylproline. For all compounds reported, the assignment of the bridgehead carbon (C-5 of *t*-CMP/C-6 of *t*-CMPi) 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. Some of the products differ in their stereochemistry at C-6 (in the case of the 5-membered ring structures), or C-7 (in the case of the 6-membered ring structures). Assignment of the stereochemistry at C-6/C-7 is complicated because of rotation about the C5-C6/C6-C7 bond. Analysis of the NOE and coupling constant data, coupled to other NMR experiments (e.g. decoupling of selected protons and 1D-selective TOCSY experiments) were used in these assignments. 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 $\Phi \sim 130\text{-}155^\circ$) 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 (*gauche*) relationship between these two protons; (ii) The observation of a moderate NOE correlation between

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H-6 and H-4' (*t*-CMP)/ H-7 and H-5' (*t*-CMPi), together 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 7 weaker than) with H-4' (*t*-CMP).

Enzyme Kinetics

Time courses to study the kinetics of CCR and CarBW79F reactions were carried out by initiating 8 analytical assays (duplicate), followed by sequential quenching of individual assays after a set length of time of incubation (0, 10, 30 min, 1, 2, 5, 10, 24 h).

Time courses to study the kinetics of CarBW79F with a racemic mixture of ethylmalonyl-CoA were performed by incubating 8 analytical CarBW79F assays at 37°C for 0, 10, 30, 60 min, 2, 5, 10 and 24 h.

Time courses to study the effect of the equilibration of the two enantiomers of ethylmalonyl-CoA were performed by incubating 10-2x analytical CCR assays at 37°C. At the following time points, samples were reacted with CarBW79F to lock the stereochemistry of the compounds: 0, 10 and 30, 60 min, 2, 5, 10 and 24 h. Samples remaining after 30 min of incubation with CCR, were taken to 4°C to allow equilibration of the enantiomers, while preserving the thioester bond with CoA and further reacted with CarBW79F as described.

Time courses to study the effects of pH and temperature variations on the formation of the enolate of methylmalonyl-CoA *via* the formation of *t*-CMP were carried out by preparing a 10x normal assay of CarB, ThnEV153A or CarBM108V, followed by extraction of 50µL aliquots and quenching after a set length of time of incubation (30,

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60 sec, 2, 5, 10, 20, 30 min, 1, 2.5, 5 h). The pH employed for this measurements were 6.9, 7.3, 7.7 (normal pH for reaction of CarB/ThnE) and 8.0. To achieve the pH differing from the normal used, the following buffers and starting pHs were used (the use of L-GSA in 10% v/v formic acid acidifies the final reaction mixture):

Initial buffer concentration	Initial pH	Final pH
MOPSO 1M	7.7	6.9
Tris 600mM	8.7	7.3
Tris 600mM	9.0	7.7
Tris 600mM	9.6	8.0

Each assay was then analysed by LC-MS as described in the following section.

LC/MS Analysis

LC/MS was carried out using a Waters 1525 μ Binary HPLC Pump system coupled to a Waters 2777 Sample Manager, with mass spectrometry analysis being performed with a Micromass[®] Quattro micro[™] API mass spectrometer (positive electrospray ionization). A SiELC Primesep 100 column (100 Å, 5 μ m) with a column size of 4.6 x 250 mm, attached to a 10mm pre-column. The column was equilibrated at 1 mL/min with 5% eluent B. After 10 minutes, a gradient as run to 70% B over 20 minutes. The column was washed with 100 % B for 10 minutes before the column was re-equilibrated at 5 % B for 20 minutes.

Eluent A: 0.1 % HCOOH in H₂O (v/v)

Eluent B: 0.1 % HCOOH in MeCN (v/v)

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