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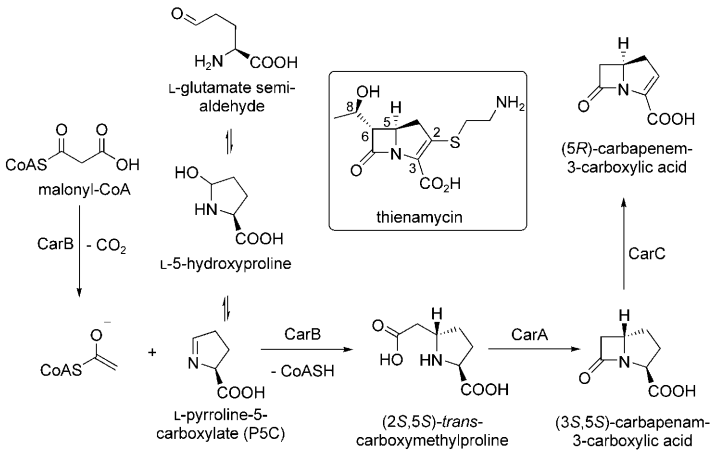
Evidence that Thienamycin Biosynthesis Proceeds via C-5 Epimerization: ThnE Catalyzes the Formation of (2*S*,5*S*)-*trans*-Carboxymethylproline

Refaat B. Hamed, Edward T. Batchelar, Jasmin Mecinović, Timothy D. W. Claridge, and Christopher J. Schofield*[a]

The biosynthetic pathways leading to most clinically important families of bicyclic β-lactam antibiotics and β-lactamase inhibitors involve epimerization steps.[1] In the penicillin/cephalosporin[2–4] and norcardicin[5] biosynthetic pathways, amino acid side chain epimerization can be catalyzed by enzymes from the well characterized pyridoxal phosphate-utilizing family. However, in the case of the pathways leading to clavulanic acid and the simplest naturally occurring carbapenem, (5*R*)-carbapenem-3-carboxylate, epimerization at the bridgehead C-5 position is likely to occur through unusual, if not unprecedented, reactions (for review see ref. [1]).

The biosynthesis of (5*R*)-carbapenem-3-carboxylate occurs in three steps[6,7] from malonyl-CoA and L-glutamate semialdehyde 5-hydroxyproline pyroline-5-carboxylate (L-GHP), with the epimerization step being catalyzed by the 2-oxoglutarate dependent oxygenase carbapenem synthase (CarC)[8–10] (Scheme 1). The first step in (5*R*)-carbapenem-3-carboxylate biosynthesis is catalyzed by the unusual crotonase superfamily (CS) enzyme carboxymethylproline synthase (CarB), which produces (2*S*,5*S*)-*t*-carboxymethylproline (*t*-CMP) from malonyl-CoA and L-GHP.[11–13] Carbapenam synthetase (CarA) then catalyzes the ATP-dependent β-lactam ring formation.[14–16] In the case of C-6 and C-2 functionalized carbapenems such as thienamycin, one of the most potent antibacterials known,[17] it is unclear whether C-5 epimerization is involved.



Scheme 1. Biosynthesis of (5*R*)-carbapenem-3-carboxylic acid in *Pectobacterium carotovorum*. L-GHP is a collective abbreviation for the equilibrium mixture of L-glutamate semialdehyde (GSA), L-5-hydroxyproline (5HP) and L-pyrroline-5-carboxylate (P5C).

The gene cluster for thienamycin biosynthesis has recently been sequenced and this sequencing revealed ThnE and ThnM as likely homologues of CarB and CarA, respectively.[13,18] However, a clear homologue of CarC was not identified.[19] Recently, evidence to support the incorporation of coenzyme A into the C-2 side chain of thienamycin after construction of the bicyclic nucleus has been reported.[20] Here, we report studies on recombinant ThnE. Like CarB, ThnE catalyzes the production of (2*S*,5*S*)-*t*-CMP derivatives; this implies a need for a subsequent C-5 epimerization step in thienamycin biosynthesis.

Initially, we cloned the wildtype (wt) *thnE* gene and expressed it in *E. coli* using the pET24a(+) expression vector to give, after purification, wt ThnE that was subsequently shown to be active (see below). However, we also found that deletion of the 45 amino acid residues from the N terminus of wt ThnE gave a protein (ThnEΔ2–46) of closer homology to CarB (based on the sequence alignment shown in Figure 1), which was more readily purified than wt ThnE. Both wt ThnE and ThnEΔ2–46 were purified to greater than 90% apparent purity (as judged by SDS-PAGE analysis, Figure S2). However, electrospray ionization mass spectrometry (ESI-MS, Figure S3) and Edman degradation analyses suggested partial proteolysis at the N terminus of wt ThnE (loss of eleven amino acid residues starting from Met1) and at the C termini of both ThnE and ThnEΔ2–46 (with cleavage between R288 and R289). ESI-MS analyses under non-denaturing conditions (trimeric wt ThnE without the eleven N-terminal amino acid residues: calculated mass = 93 525.6 Da, observed mass = 93 546.1 ± 4.9 Da; trimeric ThnEΔ2–46: calculated mass = 81 939.3 Da, observed mass = 81 943.8.2 ± 1.9 Da) and analytical gel filtration analyses revealed that, like many other members of the CS,[21] including CarB, both wt ThnE and ThnEΔ2–46 exist in a predominately trimeric form.

We then analyzed wt ThnE and ThnEΔ2–46 (hereafter referred to as ThnE because similar results were obtained with both enzymes) for activity with L-GHP prepared as described[22] and malonyl-CoA. The efficient conversion (> 90% under standard conditions) to a compound of *m/z* 174 Da $[M+H]^+$ was observed by LC-MS (Figure S4). Subsequent scale-up and LC-MS purification led to the isolation of sufficient product for ^{1}H NMR analyses (Figures 2, S6–7), which demonstrated that the product spectrum was near identical to spectra of *t*-CMP samples prepared by both CarB catalysis and by synthesis.[8] In

[a] *R. B. Hamed, E. T. Batchelar, J. Mecinović, Dr. T. D. W. Claridge, Prof. Dr. C. J. Schofield*
Department of Chemistry, Oxford University
Mansfield Road, Oxford, OX1 3TA (UK)
Fax: (+44) 1865-275674
E-mail: christopher.schofield@chem.ox.ac.uk

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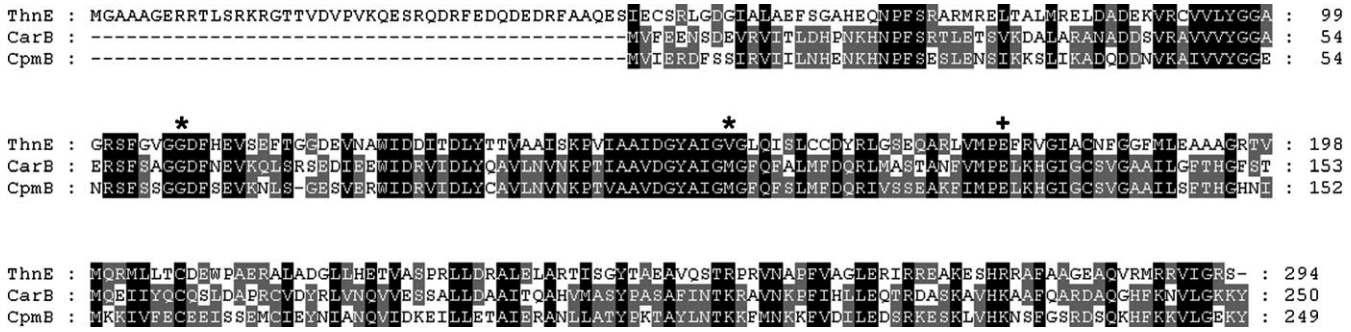


Figure 1. Sequence alignment for ThnE (*Streptomyces cattleya*), CarB (*Pectobacterium carotovorum*) and CpmB (*Photorhabdus luminescens*). Note the high degree of similarity between the three enzymes apart from the first 46 amino acid N-terminal residues in ThnE. The proposed oxyanion hole forming residues are marked with * and the catalytically important (at least in CarB) glutamate residue with +. The Figure was generated using Clustal W[23] and Genedoc.[24]

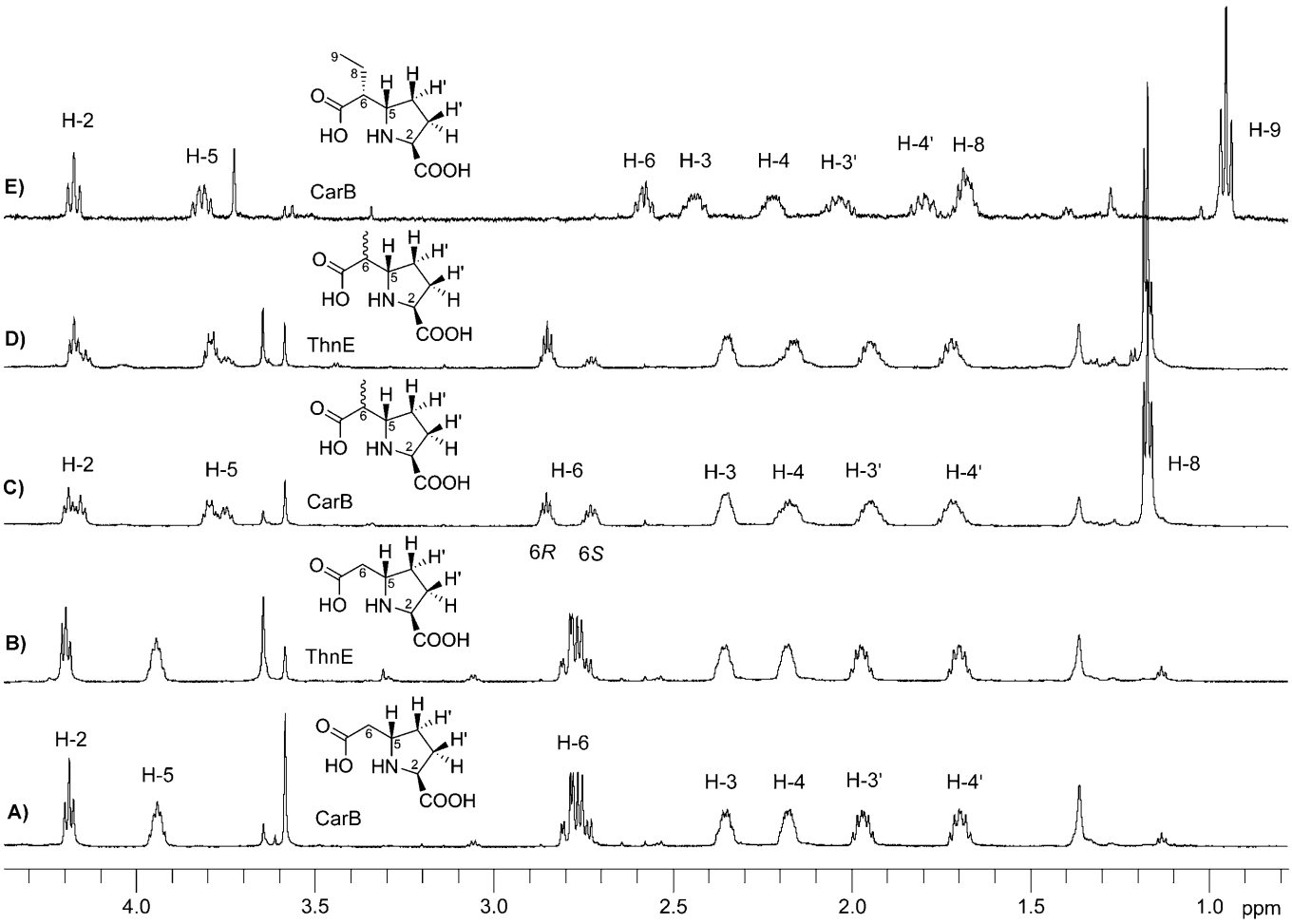


Figure 2. ^{1}H NMR spectra for purified carboxymethylprolines produced by CarB and ThnE. Spectra A) and B) represent *t*-CMP produced by CarB and ThnE, respectively. Spectra C) and D) represent the mixture of the two C-6 epimers of 6-methyl-*t*-CMP produced by CarB and ThnE, respectively. Note the different ratios of the two C-6 epimers produced by CarB and ThnE. Spectrum E) is (6*R*)-6-ethyl-*t*-CMP produced by CarB.

contrast, incubation of D-GHP with ThnE produced low levels of a product with *m/z* 174 Da $[M+H]^+$ corresponding to *t*-CMP, possibly arising from partial epimerization under the acidic deprotection conditions employed in the final step of D-GHP synthesis. These results, together with kinetic analyses using ^{1}H NMR spectroscopy (Table S4 and Figure S15), revealed that ThnE catalyzes the conversion of L-GHP and malonyl-CoA to *t*-CMP with a similar efficiency to CarB.[25] The production of *t*-CMP by ThnE implies that the thienamycin biosynthesis pathway, like that leading to (5*R*)-carbapenem-3-carboxylate, involves a (5*S*)- to (5*R*)- epimerization step.

Despite the lack of a clear CarC homologue in the thienamycin gene cluster, bioinformatic analyses indicate that it contains two potential 2-oxoglutarate dependent oxygenases (ThnG

and ThnQ).[19] Given the unusual nature of the CarC epimerization reaction, it is reasonable to propose that one of these oxygenases catalyzes the epimerization step in thienamycin biosynthesis. (The other might catalyze a hydroxylation reaction to produce the 6-hydroxyethyl side chain). However, the lack of close similarity between ThnG or ThnQ and CarC suggests that a single enzyme might not be responsible for both epimerization and desaturation activities in thienamycin biosynthesis.

It has been proposed 1) that the two carbon atoms of the C-6 hydroxyethyl side chain of thienamycin are introduced through separate methyl transfers,[26,27] and 2) that C-6 side chain hydroxylation occurs after introduction of at least one of the C-6 carbons because C-6 hydroxymethyl, ethyl and isopropyl carbapenems have been isolated from *Streptomyces* sp.[28,29] (Figure 3). The possibility that the C-6 (hydroxy)ethyl group of thienamycin is introduced at an early stage in the pathway led us to test whether ThnE could accept C-2 alkylated malonyl-CoA derivatives. Like CarB, ThnE was found to catalyze the conversion of methylmalony-CoA to a mixture of C-6 methyl *t*-CMP epimers. However, whereas CarB gave a 55:45 ratio of (6*R*)-:(6*S*)-epimers, ThnE gave an 80:20 ratio (60% diastereomeric excess) of the same epimers (determined by ^{1}H NMR analyses, Figures 2 and S8–9). This result is interesting because the stereochemistry at C−6 of the major epimer is consistent with the C-6 stereochemistry found in thienamycin (Figure 3).[30]

Ethylmalonyl-CoA was not converted by ThnE into the anticipated 6-ethyl-*t*-CMP under our standard conditions (Scheme 2); instead ThnE catalyzed decarboxylation of ethylmalonyl-CoA and hydrolysis of the resultant *n*-butyryl-CoA into CoASH and *n*-butyric acid. Interestingly, CarB catalyzed the conversion of ethylmalonyl-CoA into the two C-6 epimers of 6-ethyl-*t*-CMP (~20% yield) in a 2:1 ratio of (6*R*)-:(6*S*)-epimers (ratio determined by LC-MS, Figure S5) with the stereochemistry of the epimers determined by ^{1}H NMR analyses (Figures 2 and S10–14). This result contrasts with that for CarB and methylmalonyl-CoA where an 11:9 ratio of (6*R*)-:(6*S*)-epimers was observed.[25] Similarly to ethylmalonyl-CoA, dimethylmalonyl-CoA was not converted by ThnE into the anticipated 6,6′-dimethyl-*t*-CMP, whereas CarB did catalyze the conversion of dimethylmalonyl-CoA to 6,6′-dimethyl-*t*-CMP.[25] ThnE did, however, catalyze decarboxylation of dimethylmalonyl-CoA and hydrolysis of the resultant isobutyryl-CoA into CoASH and isobutyric acid. Incubation of isopropyl malonyl-CoA with either ThnE or CarB

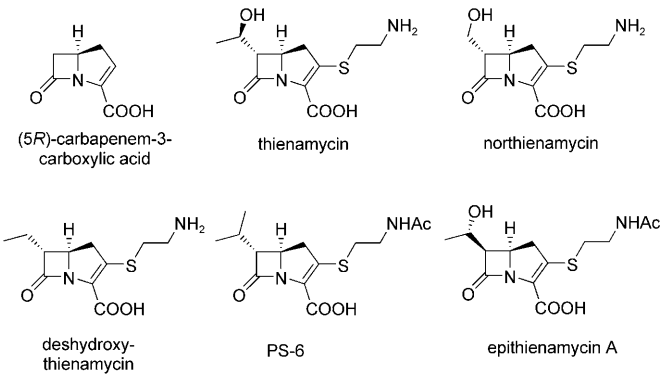
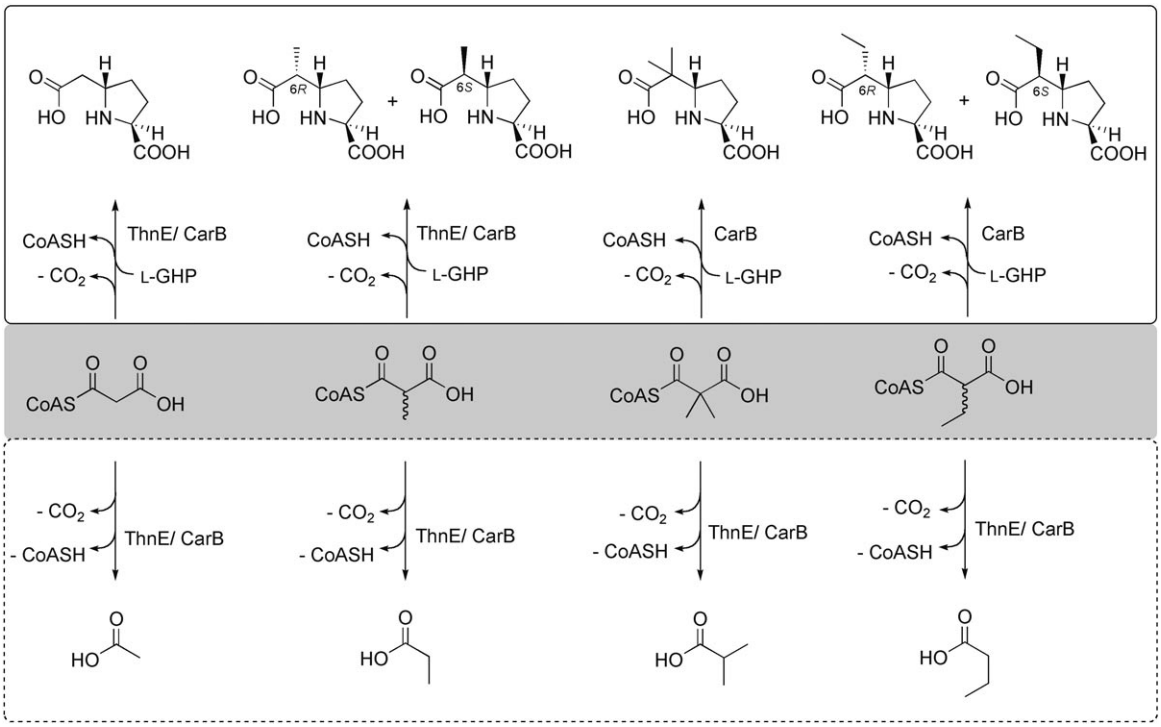


Figure 3. Some naturally occurring carbapenems that contain different C-6 alkyl moieties.



Scheme 2. Reactions catalyzed by ThnE and CarB. Reactions in the solid box were performed in the presence of L-GHP while those in dashed box were in the absence of L-GHP.

did not lead to production of 6-isopropyl-*t*-CMP. We also investigated the ability of ThnE to catalyze reactions with other coenzyme A derivatives. Incubation of acetyl-CoA, propionyl-CoA or isobutyryl-CoA with ThnE in the presence of L-GHP did not produce any *t*-CMP derivatives. Instead, ThnE catalyzed hydrolysis of these coenzyme A derivatives into CoASH and the corresponding organic acid. The specific activities for the decarboxylation and/or hydrolysis reactions of malonyl-, methylmalonyl-, acetyl- and propionyl-CoA were calculated by using ^{1}H NMR in the presence or absence of L-GHP (Scheme 2, Table S4, Figure S15).

Overall, our results reveal that thienamycin biosynthesis likely requires a C-5 epimerization step analogous to that occurring in the biosynthesis of (5*R*)-carbapenem-3-carboxylate. The ThnE catalyzed conversion of methylmalonyl-CoA to (6*R*)-methyl-*t*-CMP as the major product (60% *de*) suggests it is possible that the C-6 alkyl group is introduced at an early stage. However, the lack of conversion of ethylmalonyl-CoA to 6-ethyl-*t*-CMP by ThnE suggests subsequent further methylation and/or hydroxylation steps would be required if ThnE is responsible for the final C-6 stereochemistry in thienamycin. Attention can now focus on identification of the C-5 epimerase likely to be present in thienamycin biosynthesis; this epimerase might be encoded for by either *thnG* or *thnQ*.

Experimental Section

wt *thnE* cloning, expression, and purification: A PCR-amplified DNA product corresponding to the *Streptomyces cattleya thnE* gene was engineered as an Nde1-EcoR1 fragment into the pET24a(+) expression vector (Novagen, Nottingham, UK) using the following primers: forward, 5′-gag gag gcc ata tga tgg gcg cgg ccg ccg gcg-3′; reverse, 5′-gtg gtg gcg aat tct cag ctc cgc ccg atg acg-3′. The assigned *thnE* stop codon was mutated from UGA into UAA using the following primers: forward, 5′-gag ctc gaa ttc tta gct ccg ccc gat gac-3′; reverse, 5′- cgg gcg gag cta aga att cga gct ccg tcg-3′. The pET24a/*thnE* plasmid was transformed into *E. coli* BL21-CodonPlus® (DE3)-RP competent cells (Stratagene, Amsterdam, The Netherlands). Cells were grown in shaken flasks at 37 °C using 2TY medium containing 50 μg mL^{-1} kanamycin and 50 μg mL^{-1} chloramphenicol. When the cells had grown so that the media had an optical density OD_{600} 0.7, the temperature was reduced to 15 °C, isopropyl-1-thio-β-D-galactopyranoside (0.5 mM) was added and cells were allowed to grow for a further 16 h before harvesting by centrifugation at 9000 rpm for 15 min at 4 °C. The harvested cells were resuspended in Tris·HCl (50 mM, pH 7.5), with addition of protease inhibition cocktail (Sigma) and DNAse (Roche), before sonication and centrifugation. The resultant supernatant was filtered and then applied directly to a Q-Sepharose FF column, pre-equilibrated with Tris·HCl (50 mM, pH 7.5). Protein was eluted using a 0.0–0.6 M gradient of NaCl in Tris·HCl (50 mM, pH 7.5). The ThnE containing fractions, as judged by activity assays and SDS-PAGE analysis, were pooled. An equal volume of ammonium sulfate (2 M) was added to a final concentration of 1 M. This solution was applied to a phenyl-sepharose HP column, pre-equilibrated with Tris·HCl (50 mM, pH 7.5) containing ammonium sulfate (1 M). Protein was then eluted using a 1.0–0.0 M gradient of ammonium sulfate in Tris·HCl (50 mM, pH 7.5). ThnE containing fractions were identified by activity assays and SDS-PAGE analysis, and fractions greater than 90% pure (as judged by SDS-PAGE analysis) were pooled. The resultant protein solution was exchanged into Tris·HCl, (50 mM, pH 7.5), using a PD-10 gel-filtration column (Amersham Biosciences), concentrated to 20 mg mL^{-1} and stored at −80 °C until further use.

Construction of *thnEΔ 2–46*, expression and purification: To generate the N-terminal deletion ThnE variant (Figure 1), the 135 nucleotides (corresponding to the 45 amino acid residues at the N terminus of the protein) after the start codon of pET24a/*thnE* were deleted using the QuikChange® Site-Directed Mutagenesis Protocol (Stratagene). A pET24a/ThnEΔ2–46 plasmid was generated by PCR amplification using the pET24a/*thnE* plasmid as a template and the following primers: forward, 5′-ctt taa gaa gga gat ata cat atg atc gag tgc tcg cgg ctc ggc gac gg-3′; reverse, 5′-ccg tcg ccg agc cgc gag cac tcg atc ata tgt ata tct cct tct taa ag-3′ and Pfu Turbo DNA polymerase (Stratagene) according to the manufacturer's instructions. Expression and purification of ThnEΔ2–46 was as for wt ThnE.

ThnE assays and spectral characterization of the products from ThnE and CarB catalyzed reactions: ThnE incubations and LC-MS analyses were performed as reported for CarB.[13] See the Supporting Information for details of spectroscopic characterization of products. ^{1}H NMR monitoring of wt ThnE and ThnEΔ2–46 catalyzed reactions for specific activity determination was performed as described.[25]

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Keywords: antibiotics · biosynthesis · enzyme catalysis · NMR · stereochemistry

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