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Crotonase Catalysis Enables Flexible Production of Functionalized Prolines and Carbapenams

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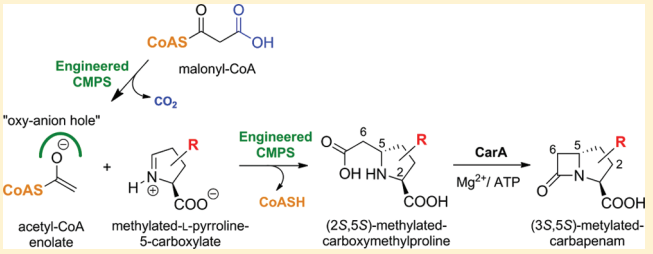
Supporting Information

ABSTRACT: The biocatalytic versatility of wildtype and engineered carboxymethylproline synthases (CMPSs) is demonstrated by the preparation of functionalized 5-carboxymethylproline derivatives methylated at C-2, C-3, C-4, or C-5 of the proline ring from appropriately substituted amino acid aldehydes and malonyl-coenzyme A. Notably, compounds with a quaternary center (at C-2 or C-5) were prepared in a stereoselective fashion by engineered CMPSs. The substituted-5-carboxymethyl-prolines were converted into the corresponding bicyclic β-lactams using a carbapenam synthetase. The results demonstrate the utility of the crotonase superfamily enzymes for stereoselective biocatalysis, the amenability of carbapenem biosynthesis pathways to engineering for the production of new bicyclic β-lactam derivatives, and the potential of engineered biocatalysts for the production of quaternary centers.



INTRODUCTION

Proline analogues are constituents of numerous natural products,[1] such as siderochelin A,[2] bottromycin A_2,[3] roseotoxin B,[4,5] kainic acid,[6−8] and lactacystin.[9,10] Proline derivatives are also widely used as pharmaceuticals, such as captropril and saxagliptin, and in biomedical research; for example, α-substituted prolines[11] are used as templates in structure−function relationship studies directed toward elucidation of biologically active conformations.[12] In organic synthesis, proline and proline analogues are also used as catalysts for asymmetric synthesis.[13]

Carbapenems are an important class of β-lactam antibiotics that are often used to treat serious infections including those involving multidrug resistant bacteria.[14] In contrast to many β-lactam antibiotics, that is, the penicillins and cephalosporins which are obtained by fermentation or from fermented precursors, clinically useful carbapenems are produced by chemical synthesis, which limits the range of compounds that can be produced efficiently. Hence, there is interest in optimizing biocatalytic routes to produce sufficient quantities of useful antibiotics or intermediates for use in semisynthetic approaches to carbapenems and other β-lactams. The substitution pattern on the bicyclic β-lactam antibiotics affects both their antibacterial activity and pharmacokinetic properties.[15] Structure−activity relationship studies on thienamycins have revealed that the introduction of a 1β-methyl substituent results in derivatives with superior antibacterial activity and increased resistance to inactivation by renal dehydropeptidases.[16,17] Therefore, most commercially available carbapenems, for example, ertapenem, meropenem, and doripenem, possess a 1β-methyl substituent (the α-methyl isomers are also resistant to dehydropeptidase hydrolysis but their antibacterial activities are reduced[17]).

Three enzymes (CarB, CarA, and CarC)[18,19] are required for biosynthesis of the simplest carbapenem (5*R*)-carbapen-2-em-3-carboxylate **1** in *Pectobacterium carotovorum* (Scheme 1). However, more enzymes are involved in biosynthesis of the C-2, C-6-substituted carbapenem thienamycin **2** in *Streptomyces cattleya*.[20] In both cases, an early ring-forming step is catalyzed by carboxymethylproline synthase (CMPS), a member of the crotonase superfamily:[21] CarB in *P. carotovorum*[22−25] and ThnE in *S. cattleya*.[26] CarB and ThnE catalyze conversion of malonyl-CoA **3** and L-glutamate semialdehyde/5-hydroxyproline/pyrroline-5-carboxylate (collectively L-GHP) to give (2*S*,5*S*)-carboxymethylproline (*t*-CMP) **4**. CarB/ThnE catalysis proceeds via decarboxylation of **3** to give an enolate, the anion of which is bound to an oxyanion hole formed by residues $Gly62_{CarB}$/$Gly107_{ThnE}$ and $Met108_{CarB}$/$Val153_{ThnE}$ (Figure 1). C−C bond formation then proceeds via reaction of the formed enolate with the imine form of L-GHP followed by thioester hydrolysis of the acyl intermediate to give **4** and CoASH[22,23] (Scheme 1).

The crotonase superfamily is ubiquitous and is characterized by a conserved repeated $\beta\beta\alpha$-crotonase fold.[21] In most cases, their catalytic mechanisms are proposed to proceed via an enolate intermediate, which is generated by decarboxylation of malonyl-CoA (derivatives), as for CarB and ThnE. The alkylation of enolates is an important reaction in organic chemistry, yet

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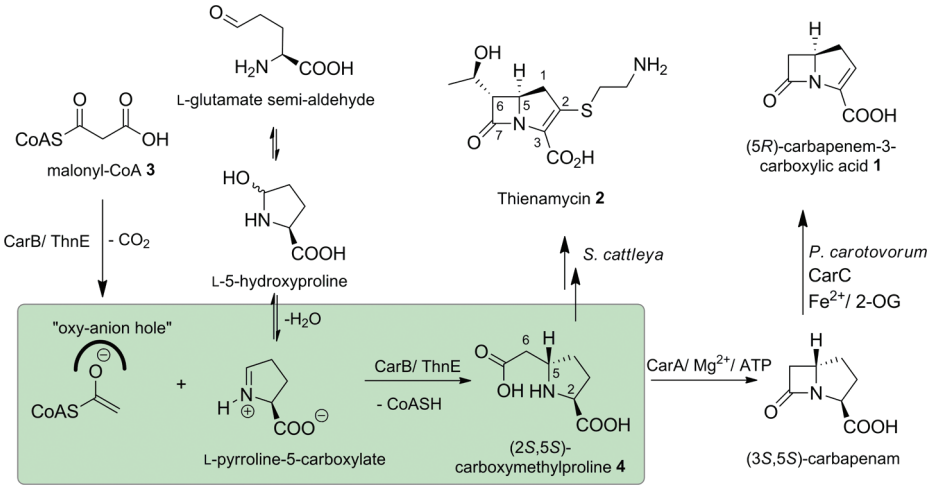
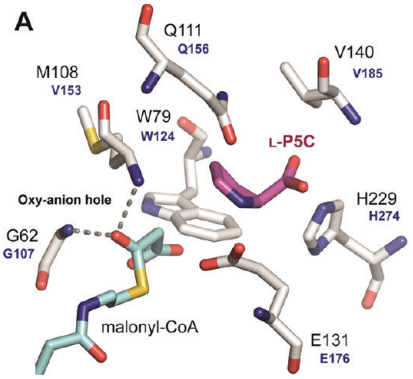
Scheme 1. Role of the Carboxymethylproline Synthases CarB and ThnE in the Biosynthesis of (5*R*)-Carbapen-2-em-3-carboxylic Acid 1 and Thienamycin 2, Respectively





B

CarB-based variants	ThnE-based variants
CarB M108A	ThnE V153A
CarB M108L	ThnE V153L
CarB M108V	ThnE V153M
CarB M108I	ThnE V153I
CarB Q111N	ThnE H274A
CarB H229A	ThnE W124F
CarB W79F	ThnE W124F/V153M
CarB W79F/M108A	
CarB W79A	

Figure 1. CarB and ThnE are crotonase superfamily enzymes. (A) View derived from a crystal structure of CarB[25] with malonyl-CoA **3** and pyrroline-5-carboxylate (L-P5C) modeled into the active site. The view highlights some of the residues proposed to be important in substrate binding/catalysis. Some of these residues were targeted for substitution. ThnE residues analogous to those of CarB active site are shown in blue;[26] (B) A list of the CarB/ThnE variants used in this study. The figure was generated using Pymol (www.pymol.org), Clustal W,[35] and Genedoc (http://www.nrbsc.org/gfx/genedoc/).

has been somewhat unexplored in biocatalysis (with one exception being the use of engineered aldolases[27−30] for stereoselective C−C bond formation), particularly with respect to the crotonase superfamily. Recently, we have reported that wildtype and variants of CarB/ThnE can accept analogues of L-GHP with different chain lengths to give 6- and 7-membered carboxymethyl-*N*-heterocycles.[31] Furthermore, use of appropriate variants enabled the stereoselective alkylation of enolates generated from C-2-alkylated malonyl-CoA.[32] These findings stimulated us to investigate other potential substrate analogues for CMPSs with a view to exploring their potential as biocatalysts for the production of 5-carboxymethylprolines substituted at any position of the proline ring including the production of compounds with a quaternary center at C-2 or C-5.

We now report on the use of CMPSs to prepare functionalized prolines substituted at C-2, C-3, C-4, and C-5 of the proline ring. The potential therapeutic utility of the resultant products is demonstrated by their conversion into the respective bicyclic β-lactam derivatives by carbapenam synthetase catalysis. The results exemplify the biocatalytic versatility of CMPSs and more generally the applicability of crotonases in biocatalysis.

RESULTS AND DISCUSSION

Preparation of C-4-methylated *t*-CMP derivatives. In order to test for the ability of CMPSs to accept C-4-methyl-substituted-L-GHP derivatives as substrates, and with a view to preparing 1β-methyl-carbapenams, we synthesized protected forms **5**−**7** of the C-4-methyl- and C-4-dimethyl-substituted-L-GHP derivatives **8**−**10** starting from pyroglutamate[33] (Scheme 2A); compounds **5**-**7** were deprotected under standard acidic conditions.[34] Upon incubation with CarB and **3**, 4,4-dimethyl-L-GHP **10** was converted to a single product (48% isolated yield, Table 1: entry 1, Table S1) assigned as 4,4-dimethyl-*t*-CMP **11** on the basis of MS and NMR analyses (Scheme 3I, Figures 2A and S1). The (*S*) stereochemistry at C-5 of the product, and all subsequently described *t*-CMP derivatives, was assigned based on coupling constant (*J*) values and 2D NOESY analyses (Figure S1), assuming retention of stereochemistry at C-2. When diastereomerically pure (>95% by ^{1}H NMR) (4*S*)-4-methyl-*N*-Boc-L-GHP *tert*-butyl ester **5** and (4*R*)-4-methyl-*N*-Boc-L-GHP *tert*-butyl ester **6** were deprotected and incubated with CarB and **3**, they each gave a ∼1:1 mixture of the C-4 epimeric products, (4*R*)-4-methyl-*t*-CMP **12**

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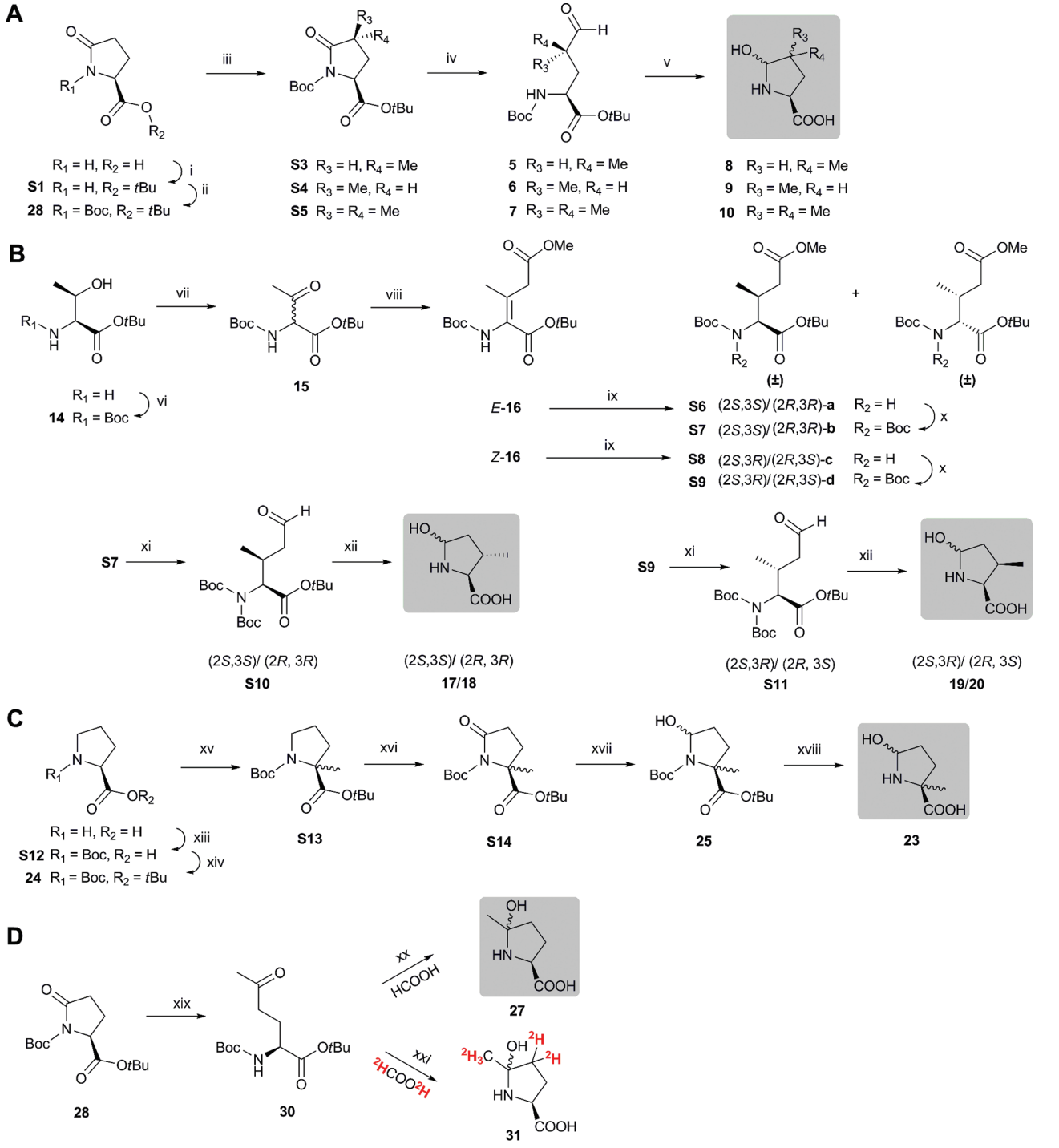
Scheme 2. Synthesis of Potential CMPS Substrates[a]



[a] (A) 4-methyl-L-GHP derivatives. (i) Isobutylene, H_2SO_4, 1,4-dioxane, −78 °C to rt, 6 d, 51%; (ii) di-*tert*-butyl dicarbonate, 4-dimethylaminopyridine, Et_3N, CH_2Cl_2, 3 d, rt, 85%; (iii) lithium bis(trimethylsilyl)amide, methyl trifluoromethanesulfonate, toluene/THF 2:1, −78 °C, 6 h, 21-62%; (iv) lithium triethylborohydride, THF, −78 °C, 84-87%; (v) 10% aq. HCOOH, 60 °C, 1 h, app. quant. An ~1:1 mixture of both **8** and **9** was obtained upon deprotection of either pure **5** or pure **6**. (B) 3-Methyl-L-GHP derivatives. (vi) Di-*tert*-butyl dicarbonate, Et_3N, CH_2Cl_2, rt, 97%; (vii) Dess-Martin periodinane, CH_2Cl_2, rt, 96%; (viii) methyl(triphenylphosphoranylidene)acetate, toluene, reflux, 62%; (ix) H_2 (g), palladium on carbon, ethyl acetate, rt, 98%; (x) di-*tert*-butyl dicarbonate, 4-dimethylaminopyridine, Et_3N, CH_2Cl_2, 85%; (xi) diisobutylaluminium hydride, Et_2O, −78°C, 70%; (xii) 10% aq. HCOOH, 60 °C, 1 h, app. quant. (C) 2-Methyl-L-GHP. (xiii) Di-*tert*-butyl dicarbonate, Et_3N, 1,4-dioxane, rt; (xiv) 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, 4-dimethylaminopyridine, 1:1 *tert*-butanol/CH_2Cl_2, rt, 78% over 2 steps; (xv) *n*-butyllithium, diisopropylamine, MeI, THF, −78 °C, 65%; (xvi) ruthenium tetroxide in situ from ruthenium(IV) oxide and sodium periodate, 1:4 ethyl acetate/water, rt, 85%; (xvii) lithium triethylborohydride, THF, −78 °C, 68%; (xviii) 10% aq. HCOOH/, 60°C, 1 h, app. quant. (D) 5-Methyl-L-GHP derivatives. (xix) MeMgBr, THF, 87%; (xx) 10% aq. HCOOH, 60 °C, 1 h, app. quant. (xxi) 10% deuterated-formic acid (^{2}HCOO^{2}H) in ^2H_2O, 60 °C, 1 h, app. quant.

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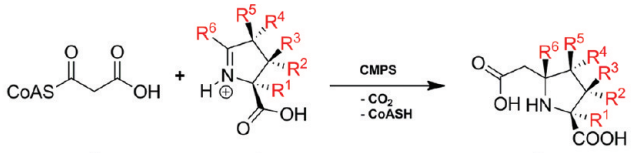
Table 1. Chemoenzymatic Synthesis of Functionalized 5-Carboxymethylprolines from Malonyl-CoA 3 and Methylated-L-GHP by CMPSs[e]



3 A B

Entry	Substrate A	Substrate R	Enzyme	Product B	Product d.r.[a]	Product R	Yield[b] (%)
1	10	$R^4 = R^5 = CH_3$	CarB	11	–	$R^4 = R^5 = CH_3$	48
2	10	$R^4 = R^5 = CH_3$	ThnE	11	–	$R^4 = R^5 = CH_3$	32
3	8/9	R^4 or $R^5 = CH_3$	CarB	12/13	49(12):51 (13)	$R^5 = CH_3$ (13)	31
4	8/9	R^4 or $R^5 = CH_3$	ThnE	12/13	8(12):92 (13)	$R^5 = CH_3$ (13)	44
5	8/9	R^4 or $R^5 = CH_3$	ThnE H274A	12/13	1(12):99 (13)	$R^5 = CH_3$ (13)	39
6	17	$R^2 = CH_3$	CarB	21	–	$R^2 = CH_3$	16 (32)[c]
7	17	$R^2 = CH_3$	ThnE	21	–	$R^2 = CH_3$	9 (18)[c]
8	19	$R^3 = CH_3$	CarB	22	–	$R^3 = CH_3$	12 (24)[c]
9	19	$R^3 = CH_3$	ThnE	22	–	$R^3 = CH_3$	2 (4)[c]
10	23	$R^1 = CH_3$	CarB	26	–	$R^1 = CH_3$	3 (6)[c]
11	23	$R^1 = CH_3$	ThnE	26	–	$R^1 = CH_3$	≥1 (2)[c]
12	23	$R^1 = CH_3$	CarB H229A	26	–	$R^1 = CH_3$	16 (32)[c]
13	27	$R^6 = CH_3$	CarB	29	–	$R^6 = CH_3$	NP[d]
14	27	$R^6 = CH_3$	ThnE	29	–	$R^6 = CH_3$	1
15	27	$R^6 = CH_3$	ThnE V153A	29	–	$R^6 = CH_3$	10

[a] d.r.: diastereomeric ratio as determined by ^{1}H NMR spectroscopy, under standard incubation conditions. [b] The % yield (isolated) was determined over the following steps: deprotection of amino acid aldehydes, incubation with enzyme, LC-MS purification, and lyophilization; products were quantified by ^{1}H NMR using $[^2H]_4$-trimethylsilylpropionate as an external standard.[31] [c] The starting materials (**17**, **19**, and **23**) were made as racemates; taking this into account, the yields are given in parentheses. [d] No product was detected. [e] CMPSs giving the highest yield and/or diastereomeric excess are shown (shaded) in comparison to wild-type CarB and ThnE.

and (4*S*)-4-methyl-*t*-CMP **13**, as shown by LC-MS and ^{1}H NMR analyses (Scheme 3**II**, Figures 2A, S2 and S3). Evidence that C-4 epimerization occurred during the acid mediated deprotection (likely via enol and/or enamine formation) came from deprotection of *N*-Boc-L-GHP *tert*-butyl ester in $^2HCOO^2H/^2H_2O$ which led to the incorporation of two deuterium atoms at C-4 of L-GHP.[34] Given that previous studies have shown the potential of CMPS variants to select from equilibrating mixtures of alkylmalonyl-CoA derivatives,[32] we then investigated whether the stereoselective production of 4-methyl-*t*-CMP from a mixture of 4-methyl-L-GHP epimers (**8**/**9**) is possible. A set of active site CarB variants (prepared as described,[32] Figure 1A) were then screened by analytical LC-MS for the formation of **12** and **13** from C-4 epimeric **8**/**9** and **3**, under standard incubation conditions. The results reveal that all tested CMPS enzymes are able to form the two C-4 epimers of 4-methyl-*t*-CMP, but with varying yields and diastereomeric ratios (4*S*:4*R*) as follows (Table S2, Figure 2B): CarB M108A (54:46), CarB M108L (56:44), CarB M108V (93:7), CarB M108I (90:10), CarB Q111N (70:30), CarB W79F (61:39), CarB W79F/M108A (62:38), CarB W79A (64:36), and CarB H229A (65:35). Likewise, screening of wild-type ThnE and active site ThnE variants (Table S2, Figure S4) reveals that they can catalyze the formation of the two C-4 epimers **12** and **13** with a particular bias toward the (4*S*)-epimer **13** (d.r. ≥ 90 (4*S*):10 (4*R*)) with the exception of ThnE V153A (d.r. = 74 (4*S*):26 (4*R*)). In case of both ThnE V153I and ThnE H274A, the d.r. was ≥98 (4*S*):2 (4*R*) (Table S2, Figures 2B and S4). For most of the CMPS variants tested, the reactions were scaled up and the diastereomeric ratio (d.r.) of the two epimers confirmed by ^{1}H NMR analyses (Table S2, Figure 2B). These results demonstrate the ability of CMPS to stereoselectively produce 4-methyl-*t*-CMP derivatives from equilibrating mixtures of semialdehyde substrates likely through a dynamic kinetic resolution process. It is notable that all CMPSs (including wildtype ThnE) with a β-branched residue (i.e., Val or Ile) at position 108_{CarB}/153_{ThnE} (one of the oxyanion hole forming residues) produced **13** as the major C-4-epimer, demonstrating that subtle active site variations can have large effects on the stereochemical outcome of CMPS-catalysis. Consistent with these results, the yield of **11** (Table S1) by CMPSs that favored the formation of **13** were considerably lower than that of wildtype CarB (which produced **12** and **13** in near 1:1 ratio). For example, the yield of **11** by CarB was ~4−5 times higher than that of CarB M108 V/I (Table S1). In case of CarB variants, a steric clash between the methyl-substituent on the (4*R*)-4-methyl-L-GHP **8** and β-branched residues at position 108_{CarB} provides a possible explanation for these selectivities.

Preparation of C-3-methylated-*t*-CMP derivatives. Interest in constructing a CMP-derivative substituted at C-3 arises because clinically useful carbapenems are substituted at an equivalent

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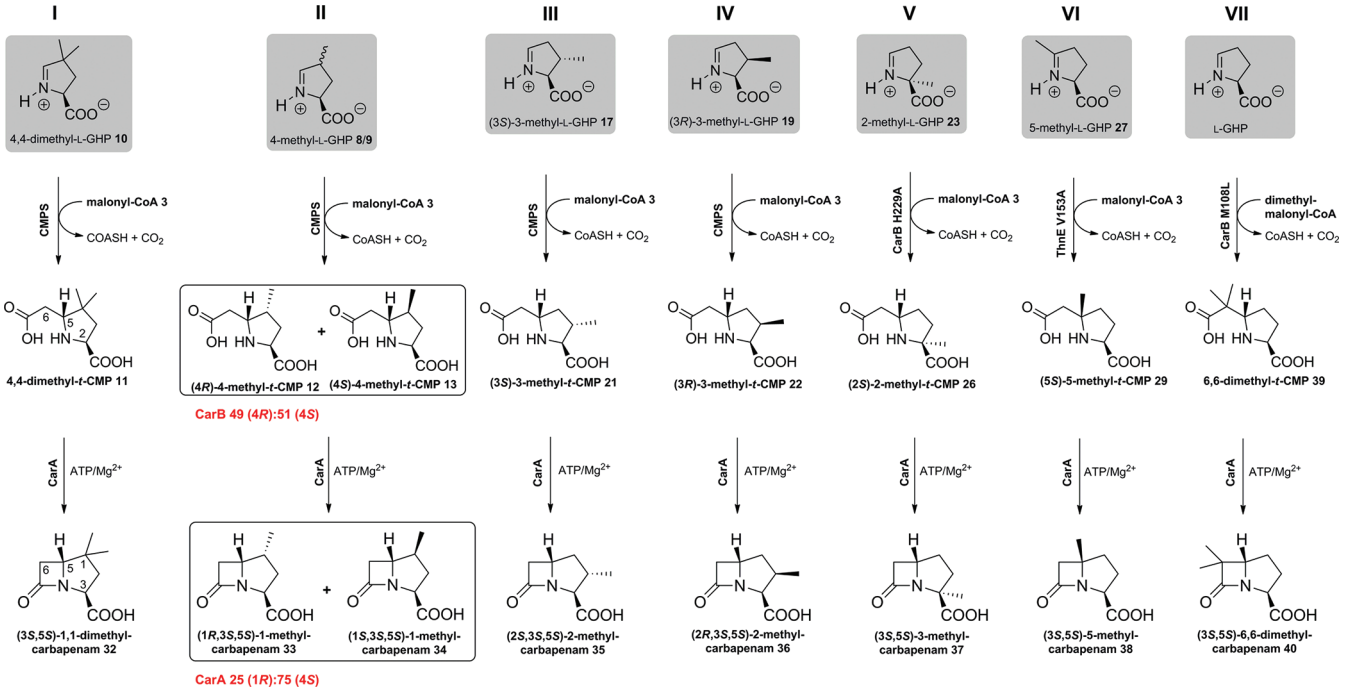
Scheme 3. Products of CMPS and CarA Catalysis (i.e., *t*-CMP and Bicyclic β-Lactam Derivatives, Respectively) Reported in This Study



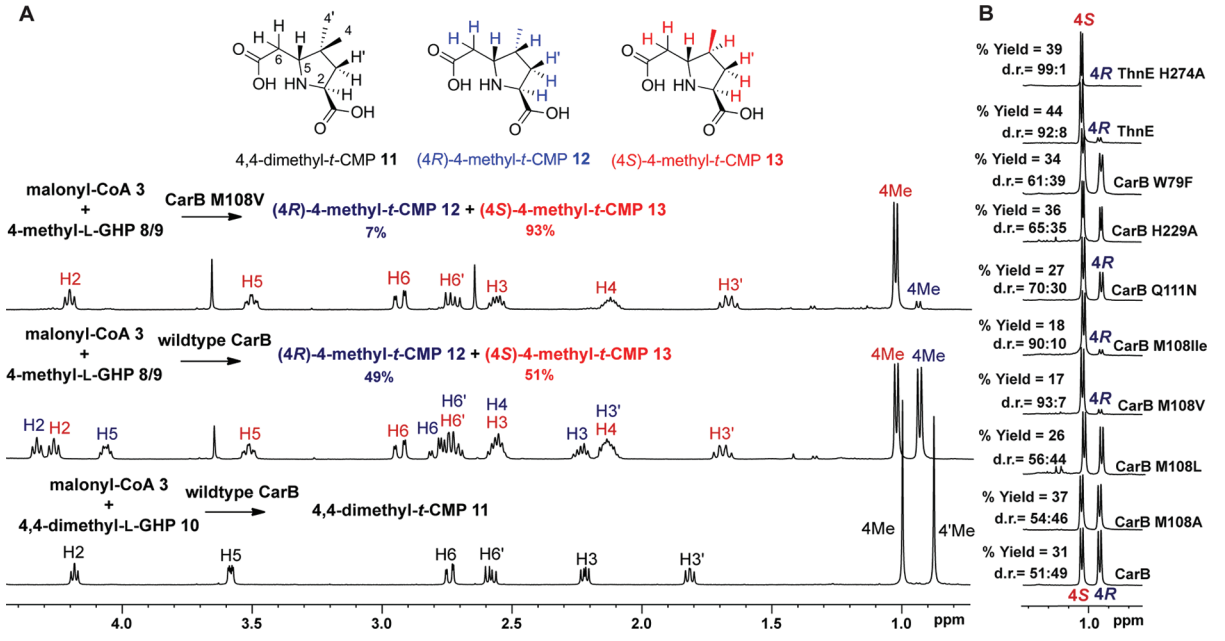


Figure 2. Improving the diastereoselectivity of CMPSs toward (4*S*)-4-methyl-*t*-CMP 13. (A) ^{1}H NMR spectra of the C4-methylated-*t*-CMPs resulting from incubation of malonyl-CoA **3** and C4-methylated-L-GHP derivatives (**8** to **10**) in the presence of the shown CMPSs. (B) Part of the ^{1}H NMR spectra for the two C4-epimers of 4-methyl-*t*-CMP (**12** and **13**) resulting from incubation of **3** and 4-methyl-L-GHP **8**/**9** in the presence of the shown CMPSs, under standard conditions, demonstrating that stereoselective production of C4-epimers can be achieved by use of appropriate variants.

position (with a substituted cysteaminyl moiety).[36] Both epimers of 3-methyl-L-GHP were obtained, in racemic form, from commercially available L-threonine-O*t*Bu ester in seven steps (23% overall yield, Scheme 2B). Oxidation of the protected amino acid **14** with Dess-Martin reagent[37] gave ketone **15** which was reacted under Wittig conditions to give a separable 1:1 mixture of *E*- and *Z*-isomers of alkene **16**. Hydrogenation, further protection, reduction, and finally deprotection of *Z*-**16** and *E*-**16** gave, respectively, racemic (3*S*)-3-methyl-L-GHP **17**/**18** and racemic (3*R*)-3-methyl-L-GHP **19**/**20** (Scheme 2B). Incubation of **17**/

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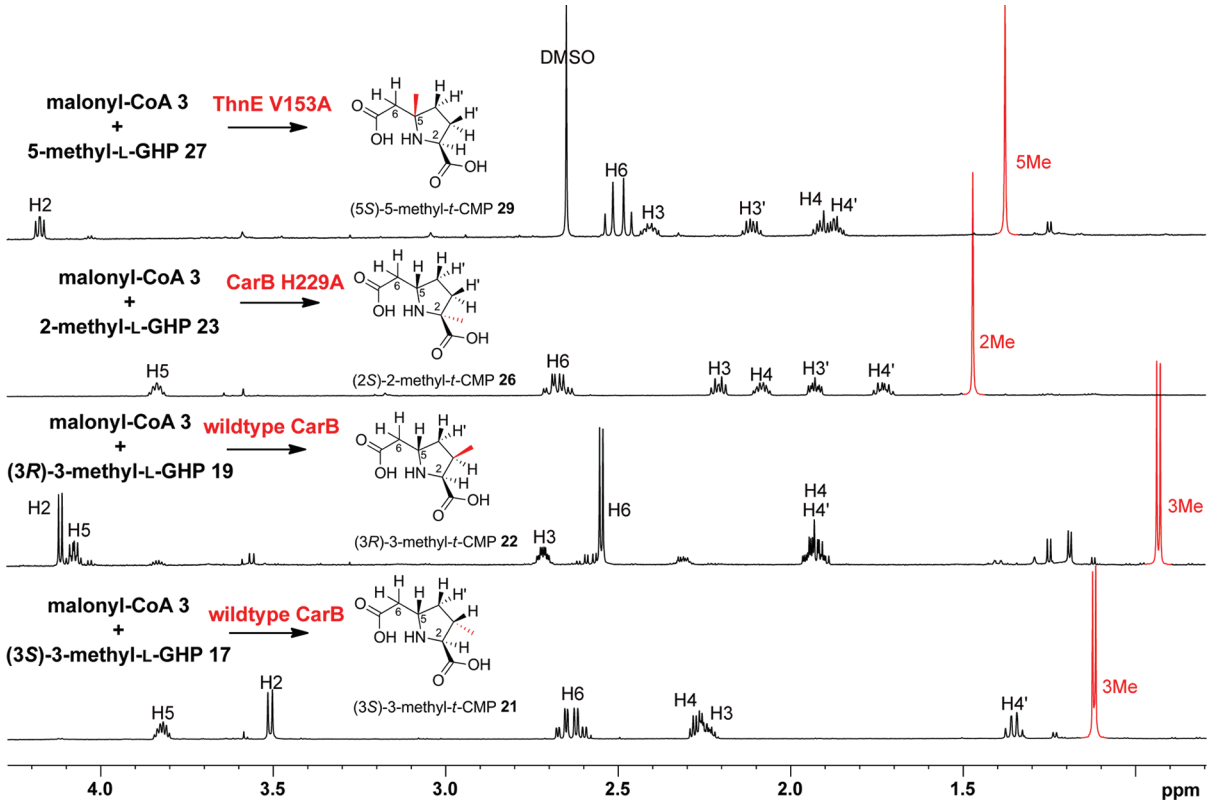

Figure 3. Spectroscopic analyses of the C2-, C3-, and C5-methyl-substituted-*t*-CMP products. ^{1}H NMR spectra of methylated-*t*-CMP derivatives resulting from incubation of malonyl-CoA **3** and the shown methylated-L-GHP derivatives in the presence of the shown CMPSs, under standard conditions.

18 and **3** in the presence of either wildtype or engineered CMPS variants resulted in the observation of a single major chromatographic peak with the anticipated mass (m/z = 188 $[M+1]^+$) as revealed by analytical LC-MS (Figure S5). Scale-up and LC-MS-guided purification of the product of catalysis of wildtype CarB, which produced the highest relative yield compared to the other tested CMPSs (Table 1: entry 6 and Table S3), led to isolation of a product which was then subjected to NMR analyses to assign its C-3 stereochemistry. Assuming the stereochemistry at C-2 is (*S*) based on reports that D-GHP is not a substrate for either CarB or ThnE,[26,38] 2D NOESY analyses enabled assignment of the product as (3*S*)-3-methyl-*t*-CMP **21** (Scheme 3III, Figures 3 and S6). Likewise, incubation of **19**/**20** and **3** in the presence of wildtype CarB provided a product that was identified as (3*R*)-3-methyl-*t*-CMP **22** (Scheme 3IV, Figures 3 and S7, Table 1: entry 8 and Table S3). These results demonstrate that CMPSs can catalyze the formation of C-3-substituted-*t*-CMP derivatives without loss of stereochemistry.

Preparation of C-2 and C-5-methylated-*t*-CMP derivatives. Construction of quaternary stereocenters is a significant challenge in the synthesis of heterocycles and natural products.[39] We therefore investigated whether CMPSs can catalyze the production of heterocycles with asymmetric quaternary centers, starting with the C-2 position. Racemic 2-methyl-GHP **23** was synthesized from L-proline in 6 steps (29% overall yield, Scheme 2C) via C-2 alkylation of protected L-proline **24** through the generation of a tertiary carbanion. Subsequent oxidation using ruthenium tetroxide[40] and reduction with lithium triethylborohydride[41] gave the protected form of 2-methyl-GHP **25**. Scale-up and purification of the product of incubation of **23** and **3** in the presence of the CarB H229A variant, which produced the highest relative yield compared to other CMPS variants tested (Figure 4A, Table 1: entry 12), followed by NMR analyses revealed a *trans*-relationship between H-5 and the methyl group at C-2 of the product (Scheme 3V, Figures 3 and S8). Therefore, the product of CarB H229A catalysis was assigned as (2*S*)-2-methyl-*t*-CMP **26**. This result demonstrates the capability of CMPSs to catalyze the formation of *t*-CMP derivatives with a quaternary center at C-2 and (at least for these substrate analogues) eliminates a mechanism involving proton abstraction at C-2. The high yield in the case of the CarB H229A variant may reflect (although other factors may contribute) an increased active site volume that enables productive binding of the quaternary C-2 stereocenter (Figure 4B). It is notable that the CarB H229A variant provides access to two *t*-CMP analogues (i.e., **26** and (2*S*,7*S*)-7-(carboxymethyl)azepane-2-carboxylic acid[31]), both not efficiently produced by other CMPSs tested. The common theme between the starting materials of the two products (i.e., **23** and L-aminopimelate semialdehyde[31]) is their increased steric demand compared to the natural substrate L-GHP.

To test for the production of C-5-methylated-*t*-CMP, 5-methyl-L-GHP **27** was synthesized by Grignard addition to the protected L-pyroglutamate **28** followed by deprotection (Scheme 2D). Screening of the CMPS variants led to the identification of ThnE V153A as the variant with the highest relative yield (Figure 4C, Table 1: entry 15). Notably, with wildtype CMPSs, yields for the formation of 5-methyl-*t*-CMP were very low (ThnE) or nonexistent (CarB) (Figure 4C, Table 1: entries 13 and 14) possibly

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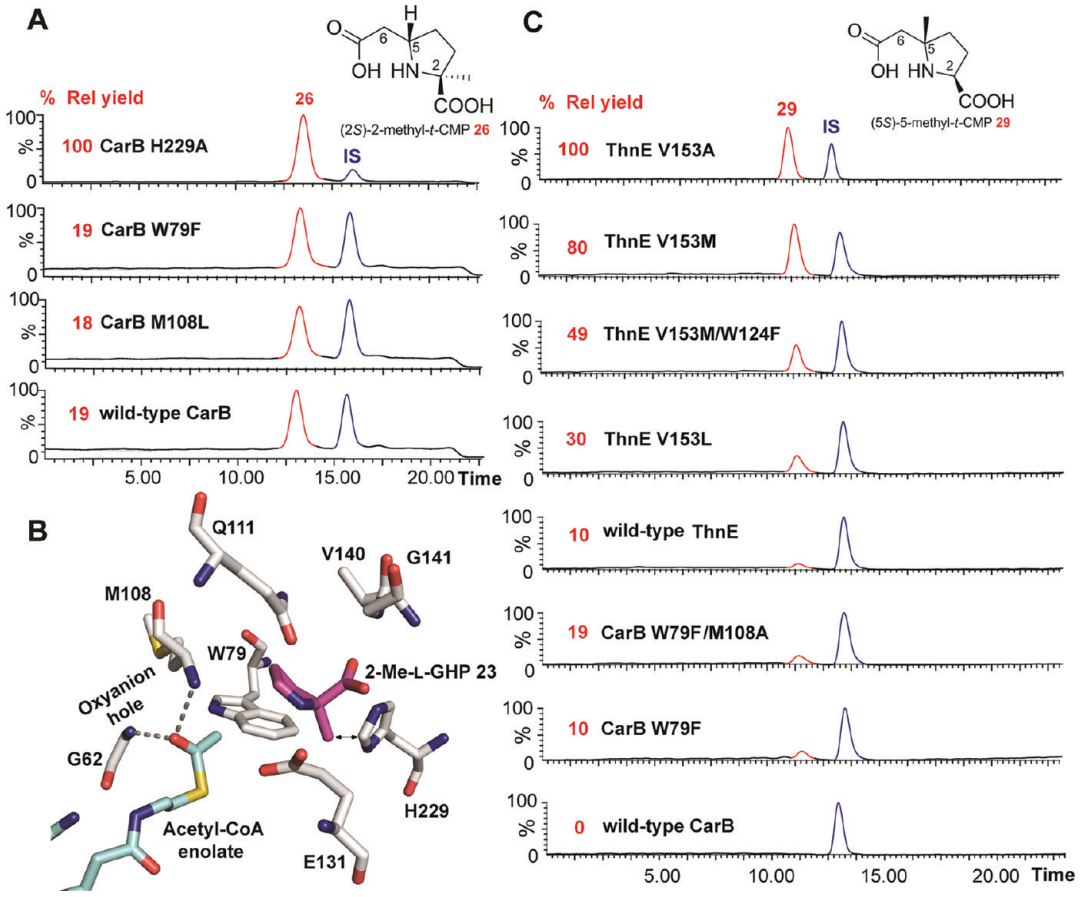

Figure 4. Improving the yield of quaternary center-containing *t*-CMP derivatives by engineered CMPSs. (A) Ion-extracted LC-MS chromatograms (positive electrospray ionization) displaying the relative yields for the formation of (2*S*)-2-methyl-*t*-CMP **26** (red peak) from racemic 2-methyl-GHP **23** and malonyl-CoA **3** catalyzed by the shown CMPSs. ThnE V153L, ThnE V153M, ThnE H274A, and ThnE V153A also produce **26**, and the relative yields are 17%, 13%, 12%, and 7%, respectively. (B) A view derived from a CarB crystal structure[25] with the acetyl-CoA enolate and the imine form of 2-methyl-L-GHP **23** modeled into the active site. His229 is predicted to be one of the closest residues to the imine form of **23** (~3 Å from τ-nitrogen of His229 to the methyl group at C-2 of **23**). (C) Ion extracted LC-MS chromatograms (positive electrospray ionization) showing the relative yields for the production of (5*S*)-5-methyl-*t*-CMP **29** (red peak) resulting from the incubation of 5-methyl-L-GHP **27**, **3** and the shown CMPSs. ThnE H274A and ThnE W124F also produce **29** and the relative yields are 7% and 3%, respectively. *p*-Aminosalicylic acid was used as an internal standard (IS, blue peak).

reflecting the fact that the nucleophilic attack of the intermediate enolate occurs at C-5 of the imine form (L-P5C)[22] (the electron donating effect of the methyl group can, in part, affect the C−C bond forming reaction). The yield of the ThnE V153A variant (Figure 4C, Table 1: entry 15) was ~10 times higher than that of wildtype ThnE revealing that substitution can alter yield substantially. Scale-up and LC-MS-guided purification of ThnE V153A product of catalysis led to isolation of 5-methyl-*t*-CMP, which is 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 ^{1}H NMR spectrum (Figure 3). 2D NOESY analyses (Figures S9 and S10) reveal a *trans*-relationship between H-2 and the methyl group at C-5, identifying the product as (5*S*)-5-methyl-*t*-CMP **29** (Scheme 3**VI**). It is notable that CMPS variants can accommodate such an increase in steric bulk adjacent to the site of C−C bond formation.

We also investigated whether the 5-methyl-L-GHP **27** substrate analogue follows the same mechanism as L-GHP,[22,23] employing labeled [^{2}H]-methylmalonyl-CoA (Figure S11) and 4,4,6,6,6-[^{2}H]$_5$-5-methyl-L-GHP **31**. The latter was prepared by deprotection of *N*-Boc-5-methyl-L-GHP *tert*-butyl ester **30** in 10% deuterated-formic acid (^{2}HCOO^{2}H) in ^2H_2O; substantial incorporation of five deuterium atoms (>90% ^{2}H) at C-4/C-6 of 5-methyl-L-GHP was detected by LC-MS analyses (Scheme 2D, Figure S12B). Incubation of C-2 [^{2}H]-methylmalonyl-CoA and **31** with ThnE/ThnE variants revealed the formation of a product observed as a single chromatographic peak with mass spectral data implying the formation of a product with six deuterium atoms as the major product (m/z = 208 [M+H]$^+$, Figure S12E). This result eliminates mechanisms involving attack of the enolate form, derived by decarboxylation of methylmalonyl-CoA, on the ring open form of **27** (5-methyl-L-GSA), followed by desaturation and intramolecular addition to form 5-methyl-*t*-CMP-CoA, and supports a mechanism involving attack of the enolate on the imine form of **27** (5-methyl-L-P5C) (Figure S12III), analogous to that of L-GHP.[22,23]

Conversion of the Prepared *t*-CMP Derivatives into Bicyclic β-Lactams. β-Lactams are finding new therapeutic applications in addition to their established use as antibiotics via inhibition of penicillin binding proteins (PBPs). β-Lactams are clinically used as β-lactamase inhibitors.[42] They have also been developed as protease inhibitors[43] (e.g., elastase), as cholesterol

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absorption inhibitors[44] (e.g., ezetimibe, a monobactam[45]), as a delivery vehicle for anticancer drugs,[43] and to regulate levels of the excitatory neurotransmitter glutamate[46] (via interaction with the glutamate transporter GLT1). The production of bicyclic β-lactams with quaternary centers on the ring core structure is of particular interest because such compounds can show improved stability to hydrolysis.[47,48] There is precedent for the biocatalytic production of bicyclic β-lactams with quaternary centers in work on isopenicillin N synthase (IPNS). IPNS is a promiscuous oxidase catalyzing production of the penicillin nucleus from a tripeptide precursor δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (ACV).[49,50] Substitution of L-cysteine for (3*R*)-methylcysteine in ACV and incubation with IPNS allowed the production of 5α-methyl penicillin.[48] The product showed no antibacterial activity but was noticeably more stable than penicillin G to β-lactamase 1 from *Bacillus cereus*.[48] The seven carboxymethyl-functionalized *N*-heterocycles, generated by CMPSs catalysis in this study (i.e., **11**, **12**, **13**, **21**, **22**, **26**, and **29**), were tested as substrates for the β-lactam ring forming enzyme carbapenam synthetase (CarA) from *P. carotovorum*.[41,51] CarA catalyzed the conversion of all tested *t*-CMP derivatives to the respective bicyclic β-lactam derivatives (**32** to **38**, respectively) as demonstrated by LC-MS analyses (Scheme 3, Figure S13). In the case of the CarA catalyzed reaction of a ~1:1 mixture of the C-4 epimers **12** and **13**, produced by wildtype CarB catalysis, CarA exhibited higher diastereoselective bias toward the (4*S*)-epimer **13** as a substrate (d.r. of products =24 (**33**):76 (**34**), as determined by LC-MS under standard assay conditions, Figure S14). Notably, the major product ((1*S*)-1-methyl-carbapenam **34**) has the same stereochemistry at C-4 as many commercially available carbapenems with improved stability toward dehydropeptidases.[52] We also found that 6,6-dimethyl-*t*-CMP **39**,[21] another CarB/CarB variant catalytic product, is a substrate for CarA producing the 6,6-disubstituted carbapenam **40** (Scheme 3**VII**, Figure S13). In the cases of **11** and **39**, the CarA reaction was scaled-up and the structures of the LC-MS isolated products (**32** and **40**, respectively) were confirmed by NMR analyses (Figures S15 to S17). The C-5 stereochemistry of the product of CMPS catalysis has to be inverted during carbapenem biosynthesis[53,54] to confer antibacterial activity to those compounds. Thus, as anticipated, the substituted carbapenams were not active antibiotics as found by holed-plate assays versus cephamycin C (data not shown). Although the yields obtained for the methylated bicyclic carbapenams varied, these results demonstrate the capability of the carbapenam synthetase CarA for converting substrate analogues of *t*-CMP, and it is likely that yields and/or diastereoselectivities can be improved by active site residue substitutions. 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[41] and the 6- and 7-membered ring analogues of *t*-CMP.[31]

With regard to the effects of substitution on the properties of carbapenams, during the NMR studies on the bicyclic products of CarA catalysis, we observed that functionalization with two methyl groups at C-6 (or to a lesser extent C-4) has a significant stabilizing effect on the carbapenams with respect to the rate of β-lactam hydrolysis: the $t_{1/2}$ for **40** was ~2 weeks; whereas that of **32** was ~2 days (as determined by ^{1}H NMR analyses at 4 °C and pH ~ 7). Although we did not carry out studies on the stability of 5-methylcarbapenam prepared by CMPS/CarA catalysis, 5-methylcarbapenems have been prepared by total synthesis and while possessing relatively low antibiotic activity are more stable to hydrolysis (e.g., by renal dehydropeptidase I) compared to the analogous 5-unsubstituted carbapenems.[47] Thus, CMPSs/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 wildtype enzymes (e.g., in case of C-2 and C-5-methylated-*t*-CMP, Figure 4, Table 1: entries 10−15). Certain variants are also capable of the stereoselective product formation including via preferential conversion of one diastereomer from an equilibrating mixture of C-4-methyl-L-GHP epimers **8**/**9** (Figure 2B, Table 1, entries 3−5), likely via a dynamic kinetic resolution process. It is notable that single residue substitutions in the wildtype 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 enzyme in cell cultures, due to the costs of coenzyme A derivatives. Despite these potential limitations, CMPS catalysis in vitro is useful for the preparation of functionalized prolines which are suitable for further modification as exemplified by the CarA-catalyzed production of functionalized bicyclic β-lactams from the *t*-CMP products. 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.

The crotonase superfamily is large and present in most, if not all, life forms. Somewhat surprisingly, to date, there have been only limited studies on their utility as biocatalysts.[31,32,55,56] The combined work on CMPSs coupled to knowledge that catalysis by most crotonase superfamily enzymes proceeds via conserved mechanisms involving the generation of stabilized enolates, suggests that the crotonase superfamily may be especially well adapted for protein engineering studies aiming at the production of useful small-molecules. Borrowing from a medicinal chemistry term,[57] we propose that certain enzyme families, including the crotonase superfamily, may be "privileged" from a biocatalytic perspective.

ASSOCIATED CONTENT

Supporting Information. Experimental procedures and compound characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ REFERENCES

(1) Mauger, A. B. *J. Nat. Prod.* **1996**, *59*, 1205–1211.
(2) Liu, W. C.; Fisher, S. M.; Wells, J. S., Jr. *J. Antibiot.* **1981**, *34*, 791–799.
(3) Waisvisz, J. M. *J. Am. Chem. Soc.* **1957**, *79*, 4520–4521.
(4) Springer, J. P.; Cole, R. J.; Dorner, J. W.; Cox, R. H.; Richard, J. L.; Barnes, C. L.; Van Der Helm, D. *J. Am. Chem. Soc.* **1984**, *106*, 2388–2392.
(5) Engstrom, G. W.; DeLance, J. V.; Richard, J. L.; Baetz, A. L. *J. Agric. Food Chem.* **1975**, *23*, 244–253.
(6) Murakami, S.; Takemoto, T.; Shimizu, Z.; Daigo, K. *Jpn. J. Pharm. Chem.* **1953**, *25*, 571–574.
(7) Johnston, G. A. R.; Curtis, D. R.; Davies, J.; McCulloch, R. M. *Nature* **1974**, *248*, 804–805.
(8) McGeer, E. G.; Olney, J. W.; McGeer, P. L. *Kainic acid as a tool in neurobiology*; Raven Press: New York, 1978.
(9) Omura, S.; Matsuzaki, K.; Fujimoto, T.; Kosuge, K.; Furuya, T.; Fujita, S.; Nakagawa, A. *J. Antibiot.* **1991**, *44*, 117–118.
(10) Omura, S.; Fujimoto, T.; Otoguro, K.; Matsuzaki, K.; Moriguchi, R.; Tanaka, H.; Sasaki, Y. *J. Antibiot.* **1991**, *44*, 113–116.
(11) Calaza, M. I.; Cativiela, C. *Eur. J. Org. Chem.* **2008**, 3427–3448.
(12) Karoyan, P.; Sagan, S.; Lequin, O.; Quancard, J.; Lavielle, S.; Chassaing, G. *Targets in Heterocycl. Syst. Chem. Prop.* **2005**, *8*, 216–273.
(13) List, B. *Tetrahedron* **2002**, *58*, 5573–5590.
(14) Frase, H.; Shi, Q.; Testero, S. A.; Mobashery, S.; Vakulenko, S. B. *J. Biol. Chem.* **2009**, *284*, 29509–29513.
(15) Dürckheimer, W.; Blumbach, J.; Lattrell, R.; Scheunemann, K. H. *Angew. Chem., Int. Ed.* **1985**, *24*, 180–202.
(16) Shih, D. H.; Baker, F.; Cama, L.; Christensen, B. G. *Heterocycles* **1984**, *21*, 29–40.
(17) Shih, D. H.; Cama, L.; Christensen, B. G. *Tetrahedron Lett.* **1985**, *26*, 587–590.
(18) Li, R. F.; Stapon, A.; Blanchfield, J. T.; Townsend, C. A. *J. Am. Chem. Soc.* **2000**, *122*, 9296–9297.
(19) McGowan, S. J.; Sebaihia, M.; O'Leary, S.; Hardie, K. R.; Williams, P.; Stewart, G. S. A. B.; Bycroft, B. W.; Salmond, G. P. C. *Mol. Microbiol.* **1997**, *26*, 545–556.
(20) Nunez, L. E.; Mendez, C.; Brana, A. F.; Blanco, G.; Salas, J. A. *Chem. Biol.* **2003**, *10*, 301–311.
(21) Hamed, R. B.; Batchelar, E. T.; Clifton, I. J.; Schofield, C. J. *Cell. Mol. Life Sci.* **2008**, *65*, 2507–2527.
(22) Batchelar, E. T.; Hamed, R. B.; Ducho, C.; Claridge, T. D. W.; Edelmann, M. J.; Kessler, B.; Schofield, C. J. *Angew. Chem., Int. Ed.* **2008**, *47*, 9322–9325.
(23) Gerratana, B.; Arnett, S. O.; Stapon, A.; Townsend, C. A. *Biochemistry* **2004**, *43*, 15936–15945.
(24) Sleeman, M. C.; Schofield, C. J. *J. Biol. Chem.* **2004**, *279*, 6730–6736.
(25) Sleeman, M. C.; Sorensen, J. L.; Batchelar, E. T.; McDonough, M. A.; Schofield, C. J. *J. Biol. Chem.* **2005**, *280*, 34956–34965.
(26) Hamed, R. B.; Batchelar, E. T.; Mecinović, J.; Claridge, T. D. W.; Schofield, C. J. *ChemBioChem* **2009**, *10*, 246–250.
(27) Schultz, P.; Lerner, R. *Science* **1995**, *269*, 1835–1842.
(28) Reymond, J. *J. Mol. Catal. B: Enzym.* **1998**, *5*, 331–337.
(29) Dean, S.; Greenberg, W.; Wong, C. *Adv. Synth. Catal.* **2007**, *349*, 1308–1320.
(30) Bolt, A.; Berry, A.; Nelson, A. *Arch. Biochem. Biophys.* **2008**, *474*, 318–330.
(31) Hamed, R. B.; Mecinovic, J.; Ducho, C.; Claridge, T. D. W.; Schofield, C. J. *Chem. Commun.* **2010**, *46*, 1413–1415.
(32) Hamed, R. B.; Gomez-Castellanos, J. R.; Thalhammer, A.; Harding, D.; Ducho, C.; Claridge, T. D. W.; Schofield, C. J. *Nat. Chem.* **2011**, *3*, 365–371.
(33) Charrier, J.-D.; Duffy, J. E. S.; Hitchcock, P. B.; Young, D. W. *J. Chem. Soc., Perkin Trans. 1* **2001**, 2367–2371.
(34) Ducho, C.; Hamed, R. B.; Batchelar, E. T.; Sorensen, J. L.; Odell, B.; Schofield, C. J. *Org. Biomol. Chem.* **2009**, *7*, 2770–2779.
(35) Thompson, J. D.; Higgins, D. G.; Gibson, T. J. *Nucleic Acids Res.* **1994**, *22*, 4673–4680.
(36) Sasaki, A.; Sunagawa, M. *Chem. Heterocycl. Compd.* **1998**, *34*, 1249–1265.
(37) Kelly, G. T.; Sharma, V.; Watanabe, C. M. H. *Bioorg. Chem.* **2008**, *36*, 4–15.
(38) Sorensen, J. L.; Sleeman, M. C.; Schofield, C. J. *Chem. Commun.* **2005**, 1155–1157.
(39) Christoffers, J.; Baro, A. *Quaternary stereocenters: challenges and solutions for organic synthesis*; Wiley-VCH Verlag GmbH& Co. kgAa: Weinheim, 2005.
(40) Yoshifuji, S.; Tanaka, K.-I.; Kawai, T.; Nitta, Y. *Chem. Pharm. Bull.* **1986**, *34*, 3873–3878.
(41) Gerratana, B.; Stapon, A.; Townsend, C. A. *Biochemistry* **2003**, *42*, 7836–7847.
(42) Page, M. G. P. *Drug Resist. Updates* **2000**, *3*, 109–125.
(43) Hamilton-Miller, J. *J. Antimicrob. Chemother.* **1999**, *44*, 729–734.
(44) Vaccaro, W. D.; Sher, R.; Davis, H. R. *Biorg. Med. Chem. Lett.* **1998**, *8*, 319–322.
(45) Earl, J.; Kirkpatrick, P. *Nat. Rev. Drug Discovery* **2003**, *2*, 97–98.
(46) Rothstein, J.; Dykes-Hoberg, M.; Pardo, C.; Bristol, L.; Jin, L.; Kuncl, R.; Kanai, Y.; Hediger, M.; Wang, Y.; Schielke, J. *Neuron* **1996**, *16*, 675–686.
(47) Onoue, H.; Narukawa, Y. *J. Antibiot.* **1989**, *42*, 1100–1113.
(48) Baldwin, J. E.; Adlington, R. M.; Moss, N.; Robinson, N. G. *J. Chem. Soc., Chem. Commun.* **1987**, 1664–1667.
(49) Baldwin, J. E.; Bradley, M. *Chem. Rev.* **1990**, *90*, 1079–1088.
(50) Robinson, J. A.; Gani, D. *Nat. Prod. Rep.* **1985**, *2*, 293–319.
(51) Miller, M. T.; Gerratana, B.; Stapon, A.; Townsend, C. A.; Rosenzweig, A. C. *J. Biol. Chem.* **2003**, *278*, 40996–41002.
(52) Nussbaum, F. v.; Brands, M.; Hinzen, B.; Weigand, S.; Häbich, D. *Angew. Chem., Int. Ed.* **2006**, *45*, 5072–5129.
(53) Sleeman, M.; Smith, P.; Kellam, B.; Chhabra, S.; Bycroft, B.; Schofield, C. *ChemBioChem* **2004**, *5*, 879–882.
(54) Stapon, A.; Li, R.; Townsend, C. A. *J. Am. Chem. Soc.* **2003**, *125*, 8486–8493.
(55) Grogan, G.; Graf, J.; Jones, A.; Parsons, S.; Turner, N. J.; Flitsch, S. L. *Angew. Chem., Int. Ed.* **2001**, *113*, 1111–1114.
(56) Siirola, E.; Grischek, B.; Clay, D.; Frank, A.; Grogan, G.; Kroutil, W. *Biotechnol. Bioeng.* **2011**, *108*, 2815–2822.
(57) DeSimone, R. W.; Currie, K. S.; Mitchell, S. A.; Darrow, J. W.; Pippin, D. A. *Comb. Chem. High Throughput Screening* **2004**, *7*, 473–94.