Articles

Characterization of the Aminocarboxycyclopropane-Forming Enzyme CmaC†

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ABSTRACT: The biosynthesis of the coronamic acid fragment of the pseudomonal phytotoxin coronatine involves construction of the cyclopropane ring from a γ-chloro-L-*allo*-Ile intermediate while covalently tethered as a phosphopantetheinyl thioester to the carrier protein CmaD. The cyclopropane-forming catalyst is CmaC, catalyzing an intramolecular displacement of the γ-Cl group by the α carbon. CmaC can be isolated as a Zn^{2+} protein with about 10-fold higher activity over the apo form. CmaC will not cyclize free γ-chloro amino acids or their *S*-*N*-acetylcysteamine (NAC) thioester derivatives but will recognize some other carrier protein scaffolds. Turnover numbers of 5 min^{-1} are observed for Zn−CmaC, acting on γ-chloro-L-aminobutyryl-*S*-CmaD, generating 1-aminocyclopropane-1-carbonyl (ACC)-*S*-CmaD. Products were detected either while still tethered to the phosphopantetheinyl prosthetic arm by mass spectrometry or after thioesterase-mediated release and derivatization of the free amino acid. In D_2O, CmaC catalyzed exchange of one deuterium into the aminobutyryl moiety of the γ-Cl-aminoacyl-*S*-CmaD, whereas the product ACC-*S*-CmaD lacked the deuterium, consistent with a competition for a γ-Cl-aminobutyryl α-carbanion between reprotonation and cyclization. CmaC-mediated cyclization yielded solely ACC, resulting from C−C bond formation and no azetidine carboxylate from an alternate N−C cyclization. CmaC could cyclize γ,γ-dichloroaminobutyryl to the Cl-ACC product but did not cyclize δ- or ϵ-chloroaminoacyl-*S*-CmaD substrates.

A large variety of nonproteinogenic amino acids found in natural products are constructed on nonribosomal peptide synthetase (NRPS) modular enzymatic assembly lines (*1*, *2*). The intersection of comparable polyketide synthase (PKS) with NRPS machinery leads to NRP-PK hybrid metabolites, many of which function in specific biological contexts. One such hybrid molecule, elaborated by phytopathogenic bacteria pathovars of *Pseudomonas syringae*, is coronatine (Scheme 1), composed of the amino acid, coronamic acid (CMA),[1] and the polyketide moiety, coronofacic acid (*3*, *4*). Coronatine may function as a molecular mimic of the plant hormone methyl jasmonic acid, (*5*) and bacterial secretion of coronatine elicits plant responses that enable *P. syringae* growth.

We have been interested in CMA biogenesis and the enzymatic construction of this particular cyclopropane scaffold densely functionalized with amino, carboxy, and ethyl substituents. Prior work (*4*) established that L-*allo*-isoleucine is the progenitor of CMA, but the mechanism for conversion of the unactivated γ-CH_3 of *allo*-Ile to the CH_2 of the cyclopropane ring was unobvious and suggested novel enzymology.

Indeed, recent studies elucidated a cryptic chlorination strategy to activate the L-*allo*-Ile γ-CH_3 as a γ-chloro intermediate, presumably now the subject of nucleophilic attack by the L-*allo*-Ile α carbon to construct the cyclopropane ring (Scheme 1) (*6*). The NRPS-like two-domain enzyme, CmaA, composed of an adenylation domain and a peptidyl carrier protein (A-PCP), activates L-*allo*-Ile as the

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[1] Abbreviations: CMA, coronamic acid; Cyt, cytotrienin; Cl-Aba, γ-chloro-L-2-aminobutyric acid; ACC, 1-aminocyclopropane-1-carboxylate; Cl-*allo*-Ile, γ-chloro-L-*allo*-isoleucine; Cl-Val, γ-chloro-L-valine; Cl-Nva, δ-chloro-L-norvaline; Cl-Nle, ϵ-chloro-L-norleucine; ACBC, 1-aminocyclobutane-1-carboxylate; ACPC, 1-aminocyclopentane-1-carboxylate; 4,4-diCl-Aba, 4,4-dichloro-L-2-aminobutyric acid; Cl-ACC, 2-chloro-1-aminocyclopropane-1-carboxylate; ICP-MS, inductively coupled plasma mass spectrometry; A, adenylation; PCP, peptidyl carrier protein; TE thioesterase; OPA, *o*-phthalaldehyde; FMOC, 9-fluorenylmethoxycarbonyl; TCEP, tris(2-carboxyethyl)phosphine; TFA, trifluoroacetic acid; SAM, *S*-adenosylmethionine; ABA, 2-aminobutyric acid; NAC, *N*-acetylcysteamine; FTMS, Fourier transform mass spectrometry.

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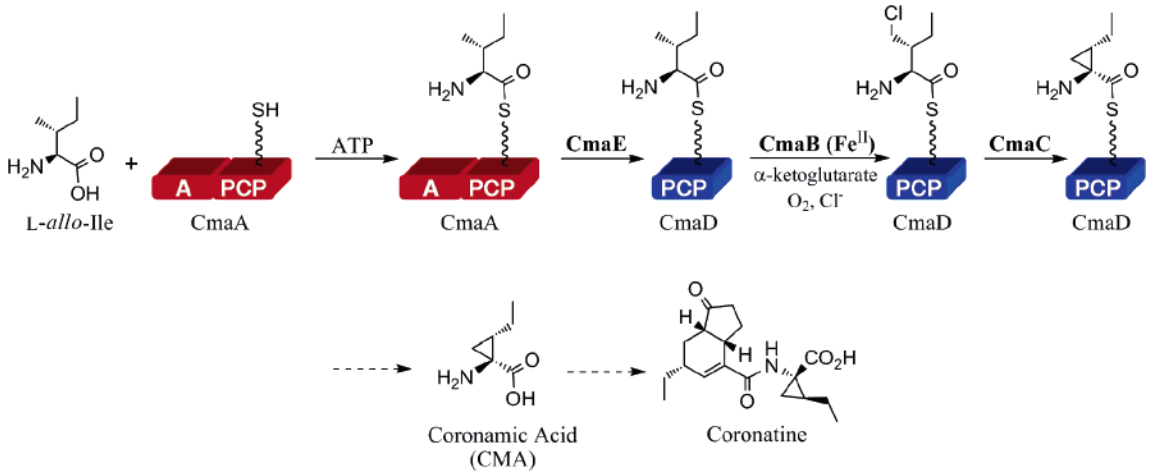
Scheme 1: Biosynthesis of the CMA Moiety of Coronatine



aminoacyl adenylate and then transfers that activated moiety to the HS-phosphopantetheinyl arm of its carrier domain (*7*). Before γ chlorination occurs, however, the *allo*-Ile moiety must be transferred to a free-standing 8 kDa carrier protein, CmaD, by an aminoacyl shuttle enzyme, CmaE (*6*). CmaB, a member of a novel class of O_2-dependent, mononuclear nonheme iron halogenases (*6*, *8*), chlorinates the γ-CH_3 group, creating the substrate for CmaC, the fifth protein in the Cma operon (*7*, *9*). It was briefly reported (*6*) that CmaC is the cyclopropane-forming catalyst, generating the coronamyl-*S*-CmaD nascent product that is then likely the substrate for the sixth and last protein of the operon, the thioesterase (TE) CmaT, to release free CMA.

CmaC, in addition to effecting a critical step en route to CMA, utilizes a previously unobserved mechanism for cyclopropane ring construction in naturally occurring metabolites. In this paper, we describe the characterization of CmaC as a zinc-containing catalyst for intramolecular displacement of the γ-chloro substituent by the stabilized α carbanion of γ-chloro-aminoacyl-*S*-CmaD substrates in cyclopropane ring formation.

MATERIALS AND METHODS

General. 4,4-Dichloro-L-aminobutyric acid (4,4-diCl-Aba) and γ-chloro-L-aminobutyric acid were synthesized as previously described (*10*, *11*). The preparation of δ-chloro-L-norvaline and ϵ-chloro-L-norleucine are described in the Supporting Information. All chemicals, unless otherwise indicated, were purchased from Sigma-Aldrich. Protein concentrations were determined by the method of Bradford, using bovine serum albumin as a standard (*12*). CmaC containing a mixture of metals was prepared as previously described (*6*). High-performance liquid chromatography (HPLC) analysis was performed on a Phenomenex Luna C18(2) (4.6 × 250 mm) with a Beckman System Gold instrument.

Expression and Purification of Apo-CmaC. Escherichia coli BL21(DE3) containing the expression plasmid for CmaC (*6*) was incubated at 37 °C overnight in 3 × 75 mL Luria Burtani medium (50 μg/mL kanamycin). The following morning, 8 × 20 mL of the overnight culture were used to inoculate 8 × 2 L Luria Burtani medium (50 μg/mL kanamycin), supplemented with potassium phosphate buffer as described for Terrific Broth (*13*), and the resulting cultures were grown at 30 °C until OD_{600} = 0.4. At this time, the temperature was reduced to 15 °C and protein expression was induced with the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cultures were incubated for an additional 18 h. CmaC was purified aerobically as follows: harvested cells were resuspended in 200 mL of lysis buffer [20 mM Tris (pH 8.0), 500 mM NaCl, 5 mM imidazole, 10% glycerol, 10 μg/mL deoxyribonuclease I, 1 mM $MgCl_2$, and 1 mM $CaCl_2$] and lysed by homogenization (Avestin EmulsiFlex-C5 homogenizer). The lysates were clarified by ultracentrifugation. The cell-free extract was incubated with 25 mL of Ni−nitrilotriacetic acid (NTA) resin (Qiagen) for 1 h at 4 °C. The resin was washed with an additional 200 mL of lysis buffer and 80 mL of wash buffer [20 mM Tris (pH 8.0), 500 mM NaCl, 20 mM imidazole, and 10% glycerol] and eluted with 35 mL of wash buffer containing 250 mM imidazole. A chelator cocktail [20 mM ethylenediaminetetraacetic acid (EDTA), 5 mM 1,1-phenanthroline, and 5 mM 2,2′-bipyridyl] was added to the eluted protein, incubated at 4 °C for 20 min, and concentrated to 10 mL before application to PD-10 desalting resin (GE Healthcare), equilibrated with 20 mM Tris (pH 8.5) and 50 mM NaCl. The N-terminal His_6 tag was cleaved by the addition of thrombin (2 units/mg of CmaC) and 2.5 mM $CaCl_2$ and incubated for 14 h at 4 °C. Any residual N-terminal His_6-CmaC was removed following Ni−NTA chelate chromatography. The protein solution was then loaded onto a MonoQ 10/100GL column (GE Healthcare), equilibrated with 20 mM Tris (pH 8.5) and 50 mM NaCl and eluted using a NaCl gradient of 50−500 mM over 30 column volumes. Fractions containing CmaC were pooled together, concentrated to 2 mL in 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.5), and again treated with a chelator cocktail (5 mM each of EDTA, 1,10-phenanthroline, and 2,2′-bipyridyl) for 2 h at 15 °C. Apo-CmaC was applied to a PD-10 desalting resin equilibrated with 25 mM HEPES (pH 7.5). The protein was concentrated to 2 mL, flash-frozen in liquid nitrogen, and stored at −80 °C.

Expression and Purification of Zn−CmaC. Expression of CmaC was the same as above, except for the addition of 200 μM $ZnCl_2$ to the expression culture 15 min prior to induction with IPTG. Purification of Zn−CmaC was the same as above, except for the addition of 100 μM $ZnCl_2$ to

all purification buffers and the omission of chelation treatments. After MonoQ chromatography, the protein was concentrated to a volume of 2 mL and dialyzed for 5 h against 4 L of 25 mM HEPES (pH 7.5). The protein was flash-frozen in liquid nitrogen and stored at −80 °C.

Generation of Fe/Zn−CmaC. Reconstitution was performed under an anaerobic atmosphere using a Mbraun Lambaster glovebox maintained at 2 ppm O_2 or less. To ensure an anaerobic solution of apo-CmaC, the protein was first applied to Bio-Gel P6 resin equilibrated with 25 mM HEPES (pH 7.5). Two 800 μL aliquots were prepared, each containing 100 μM apo-CmaC, 2 mM dithiothreitol (DTT), and 500 μM ferrous ammonium sulfate in 25 mM HEPES (pH 7.5). The solution was incubated at 15 °C for 20 min and applied to 8 mL of Bio-Gel P6 resin equilibrated in 25 mM HEPES (pH 7.5). The eluted protein was flash-frozen in liquid nitrogen and stored at −80 °C.

Metal Analysis. The metal content of apo-CmaC, Zn−CmaC, and Fe/Zn−CmaC was determined by inductively coupled plasma mass spectrometry (ICP-MS), performed by Elemental Research, Inc.

Preparation of Aminoacyl-S-CmaD. The priming of CmaD was achieved by incubating the carrier protein with surfactin phosphopantetheinyl transferase (Sfp) (*14*) and coenzyme A. A typical priming reaction mixture was incubated at 24 °C for 40 min and contained 100 μM CmaD, 25 mM HEPES (pH 7.5), 2 mM $MgCl_2$, 2 mM tris(2-carboxyethyl)phosphine (TCEP), 200 μM coenzyme A, and 1 mM Sfp. After the addition of 5 μM CytC1 (*11*), 3 mM ATP, and 5 mM chlorinated amino acid, reaction mixtures were incubated for an additional 90 min at 24 °C. The solution containing aminoacyl-*S*-CmaD was desalted using a Micro Bio-Spin 6 column (Bio-Rad) and, if needed, concentrated using an Ultrafree-0.5 centrifugal filter unit (Amicon).

Preparation of Aminoacyl-S-BarA. BarA was primed as indicated above for CmaD. After the addition of 5 μM BarD, 3 mM ATP, and 5 mM chlorinated amino acid, reaction mixtures were incubated for an additional 90 min at 24 °C before desalting using a Micro Bio-Spin 6 column (Bio-Rad).

CmaC-Dependent Cyclization Activity Assays. Reaction mixtures (150 μL) for detection of 1-amino-cycloalkane-1-carboxylate formation were prepared using 67 μM aminoacyl-*S*-CmaD (or aminoacyl-*S*-BarA) and 20 μM CmaC in 25 mM HEPES (pH 7.5). The reactions were incubated at 24 °C for 90 min. For reaction samples analyzed by Fourier transform mass spectrometry (FTMS), reactions were frozen in liquid nitrogen and stored at −80 °C, until ready for use. For reactions subject to derivatization and HPLC analysis, 10 μM type-II TE TycF was added and reactions were incubated for an additional 60 min. Protein components of the reaction mixtures were removed by filtration with an Ultrafree-0.5 centrifugal filter unit [5000 molecular weight cut-off (MWCO)], followed by a wash with 100 μL of H_2O pooled together with the initial filtrate.

Sample Preparation/Mass Spectrometry. All reaction volumes were raised to 150 μL with solvent A [5% acetonitrile and 0.1% trifluoroacetic acid (TFA) in water] prior to HPLC separation on a C4 Vydac 214 MS series column. Proteins were separated using a linear gradient [5−30% solvent B (95% acetonitrile and 0.09% TFA in water) over 5 min, 30−60% solvent B over 15 min, and 60−95% solvent B in 5 min] over 1.0 mL/min. Collected fractions were lyophilized and stored at −80 °C until use.

Immediately prior to FTMS analysis, samples were desalted by C4-ZipTip (Millipore) and suspended in electrospray ionization (ESI) solution (49% methanol, 49% water, and 2% formic acid). Mass analysis was performed on a custom-built 8.5 T quadrupole-enhanced Fourier transform-mass spectrometer (Q-FTMS) of the Marshall design (*15*). In general, a sample of interest was introduced to the mass spectrometer using a NanoMate 100 for automated nanospray (Advion Biosciences, Ithaca, NY). Targeted species were selected in the quadrupole, externally accumulated in an octopole ion trap, transferred to the ICR cell, and subsequently fragmented by infrared multiphoton dissociation (IRMPD) (*16*). Collected data were manually interpreted with the percent occupancy calculated as the sum weighted average of all detected charge states (accurate to within 5%) (*17*), and phosphopantetheinyl elimination products were used to confirm intermediate assignments (*18*).

o-Phthalaldehyde (OPA) Derivatization of TycF-Liberated Amino Acids (19). The OPA reagent was prepared with 10 mg of OPA, 200 μL of methanol, 19.6 μL of 3-mercaptoproprionic acid, and 880 μL of 0.4 M sodium borate (pH 10.4). Immediately prior to HPLC analysis, 12 μL of 0.4 M sodium borate (pH 10.4) and 2 μL of OPA reagent were added to 107 μL of reaction mixture filtrate and 100 μL of this was injected for HPLC analysis, employing a gradient of 20−100% solvent B (45:45:10 methanol/acetonitrile/H_2O) in solvent A (40 mM Na_2HPO_4 at pH 7.8) over 25 min. The absorbance was monitored at 338 nm.

9-Fluorenylmethoxycarbonyl (FMOC) Derivatization of TycF-Liberated Amino Acids (20). Samples were prepared as follows immediately prior to HPLC analysis: 50 μL of filtered reaction mixture, 100 μL of acetonitrile, 50 μL of 200 mM sodium borate (pH 8.0), and 20 μL of 10 mM FMOC (in acetonitrile) were added together and incubated at 24 °C for 2 min. At this time, 40 μL of 100 mM 1-aminoadamantane (in 1:1 acetonitrile/H_2O) was added, and the reaction was incubated for 1 min prior to centrifugation at 13 000 rpm for 1 min. The supernatant (200 μL) was analyzed by HPLC employing a gradient of 10−80% solvent B (acetonitrile) in solvent A (50 mM sodium acetate at pH 5.5) over 50 min. The absorbance was monitored at 263 nm.

Kinetics of 1-Aminocyclopropane-1-carboxylate (ACC)-S-CmaD Formation by CmaC. To obtain saturation kinetics for ACC-*S*-CmaD formation by various forms of CmaC, reaction mixtures typically contained 25 mM HEPES (pH 7.5), 1 μM CmaC, and varying concentrations of γ-chloro-L-2-aminobutyric acid (Cl-Aba)-*S*-CmaD. Reaction mixtures were incubated at 24 °C for 5 min, quenched by the addition of acetic acid to 30% (v/v), and immediately frozen in liquid nitrogen. The samples were then analyzed by FTMS as indicated above. The observed rate (k_{obs}) of ACC-*S*-CmaD formation by metal^{2+}−CmaC was measured with reactions containing 200 μM Cl-Aba-*S*-CmaD.

CmaC-Catalyzed Cl-Aba-S-CmaD Deuterium Incorporation. All reaction components were prepared in either D_2O or deuterated 25 mM HEPES (pD 7.5). Cl-Aba-*S*-CmaD was prepared as indicated above in 25 mM HEPES (pD 7.5). In 15 μL, 47 μM Cl-Aba-*S*-CmaD was incubated at 24 °C for 10 min either in the presence or absence of 1 μM Zn−CmaC.

Reactions were quenched by the addition of acetic acid to 30% (v/v) and immediately frozen in liquid nitrogen. Prior to FTMS analysis, the samples were prepared as described above and then introduced to the mass spectrometer. The incorporation of deuterium onto the aminoacyl-phosphopantetheinyl arm was monitored by inducing phosphopantetheinyl elimination products with IRMPD.

RESULTS

Preparation and Characterization of Protein-Bound Substrates and Catalyst CmaC. The substrates for the CmaC-mediated cyclization to cyclopropanes are γ-chloro-α-aminoacyl moieties presented as thioesters tethered to phosphopantetheinyl prosthetic groups on 10 kDa carrier protein domains. This raises challenges both in the generation of the chlorinated aminoacyl groups and in forming the aminoacylated forms of the carrier proteins. The various chlorinated amino acids were synthesized as described in the Materials and Methods. As noted below, the amino acids could be activated by adenylation domains and then installed on the holo forms of various carrier proteins. In turn, the holo forms of the carrier proteins, such as CmaD and BarA, were generated from the apo forms by transfer of the phosphopantetheinyl moiety from coenzyme A (CoA) by the *Bacillus subtilis* phosphopantetheinyl transferase, Sfp (*14*).

CmaC was overproduced heterologously in *E. coli* (*6*) with yields of 6 mg/L, as previously described. Efforts to generate apoprotein and reconstitute with divalent cations to an active form *in vitro* met with mixed reproducibility, noted below; therefore, we chose to add high levels of zinc (200 μM) to the growth medium to produce the zinc form of CmaC at 5 mg/L.

Substrate Tolerance of CmaC for Cyclopropane Ring Formation. The naturally occurring substrates for CmaC are γ-chloro-L-*allo*-isoleucine (Cl-*allo*-Ile)-*S*-CmaD and γ-chloro-L-valine (Cl-Val)-*S*-CmaD to provide CMA and norcoronamic acid, respectively (*6*). CmaA is ineffective for both the direct aminoacylation of CmaD and efficient activation of Cl-*allo*-Ile or Cl-Val; the activity as measured by [^{32}P]-PP$_i$-ATP exchange is reduced by more than 100-fold relative to that for L-*allo*-Ile (*6*). In our initial report, the chlorinated aminoacyl-*S*-PCP substrate was accessed through *in vitro* reconstitution from the sequential action CmaA, CmaD, CmaE, CmaB, and either L-*allo*-Ile or L-Val. Quantitative generation of the halogenated aminoacyl-*S*-CmaD, however, is problematic (*6*). Thus, *in vitro* reconstitution of the natural biosynthetic pathway would not be a viable entry point to generate the substantial quantities of homogeneous aminoacyl-*S*-CmaD needed for this study.

To this end, an additional method was sought to facilitate a more detailed analysis of the reaction catalyzed by CmaC. Other studies ongoing in our groups have established that CytC1, a free-standing A domain isolated from the cytotrienin-producing *Streptomyces* sp. RK95-74 (*11*), activates a broad range of amino acids, including Cl-Aba, and installs them on the holo form of the 10 kDa carrier protein CytC2. CmaC readily converts Cl-Aba-*S*-CytC2 to ACC-*S*-CytC2, indicating substrate tolerance not only toward the carrier protein scaffold but also toward the aminoacyl subject for carbocycle formation (*11*).

It was determined here that CytC1 efficiently aminoacylates the noncognate PCP CmaD to >95% occupancy (Figure 1), and the subsequent CmaC-dependent conversion of Cl-Aba-*S*-CmaD to ACC-*S*-CmaD was monitored by FTMS (Figure 1). In addition, PCP-bound amino acids were released following TE treatment, and ACC formation was confirmed by derivatization with OPA/3-mercaptopropionic acid (Figure 2A) (*19*) and co-chromatography with authentic standards.

Any generation of L-azetidine-2-carboxylic acid, whether enzyme-dependent or nonenzymatic, via nucleophilic attack of the Cl-Aba-*S*-CmaD α amine of the γ-chloro carbon, would possess a mass indistinguishable from that of ACC and fail to derivatize with the primary amine-specific OPA reagent. Therefore, to ascertain whether any L-azetidine-2-carboxylic acid was produced during the course of the reaction, the TE-liberated amino acids were subjected to FMOC derivatization. Reversed-phase HPLC analysis of the FMOC-derivatized reaction mixture indicated the absence of L-azetidine-2-carboxylic acid and established that ACC was the sole cyclization product of CmaC (Figure 2B).

We turned to a second carrier protein scaffold from a third biosynthetic pathway, BarA, from the barbamide system to further examine the tolerance of CmaC for Cl-aminoacyl moieties (*21*, *22*). In addition to its preferred substrate L-Leu, the free-standing adenylation domain BarD activates alternate chlorinated amino acids, loading them onto its cognate PCP BarA (*22*), and it was observed that this tolerance also extended to Cl-Aba (Supporting Information). Although BarD was unable to aminoacylate CmaD, with either Cl-Aba or leucine, CmaC did recognize Cl-Aba-*S*-BarA as a substrate for cyclopropane ring formation (Figure 2A), albeit at a reduced overall conversion (50 versus 85%) relative to CmaD-bound chlorinated amino acids under comparable reaction parameters (Figure S1 in the Supporting Information).

Test for CmaC Cyclization Activity on δ-Chloro- and ϵ-Chloroaminoacyl-S-CmaD Substrates. The fidelity of CmaC with respect to the size of the carbocyclic product formed during the reaction was also probed using δ-chloro-L-norvaline (Cl-Nva) and ϵ-chloro-L-norleucine (Cl-Nle) as potential substrates for the formation of 1-aminocyclobutane-1-carboxylate (ACBC) and 1-aminocyclopentane-1-carboxylate (ACPC), respectively. CytC2 was able to efficiently load CmaD with Cl-Nva (to 65% occupancy), but subsequent FTMS analysis indicated that substantial nonenzymatic cyclization of Cl-Nva to L-Pro had occurred during the course of the aminoacylation reaction (Figure S2 in the Supporting Information). CytC1 activates L-Pro poorly, about 50-fold less efficiently than for L-Val, and is ineffective for aminoacylation of CmaD with L-Pro under the reaction conditions employed (Figure S3 in the Supporting Information). Cl-Nva likely cyclizes nonenzymatically to provide L-Pro, following attachment as the thioester to CmaD. To test whether CmaC can mediate cyclopentane formation, BarD was utilized to generate Cl-Nle-*S*-BarA. However, only a small degree of nonenzymatic cyclization to yield pipecolic acid was observed, independent of the presence of CmaC (Figure S4 in the Supporting Information).

Finally, the ability of CmaC to recognize 4,4-diCl-Aba as yet another alternate substrate for cyclopropane ring formation was examined. The dichlorinated amino acid was efficiently loaded by CytC1 onto CmaD to yield >95% occupancy (Figure 3). When 4,4-diCl-Aba-*S*-CmaD was presented to CmaC, extensive conversion to 2-chloro-1-

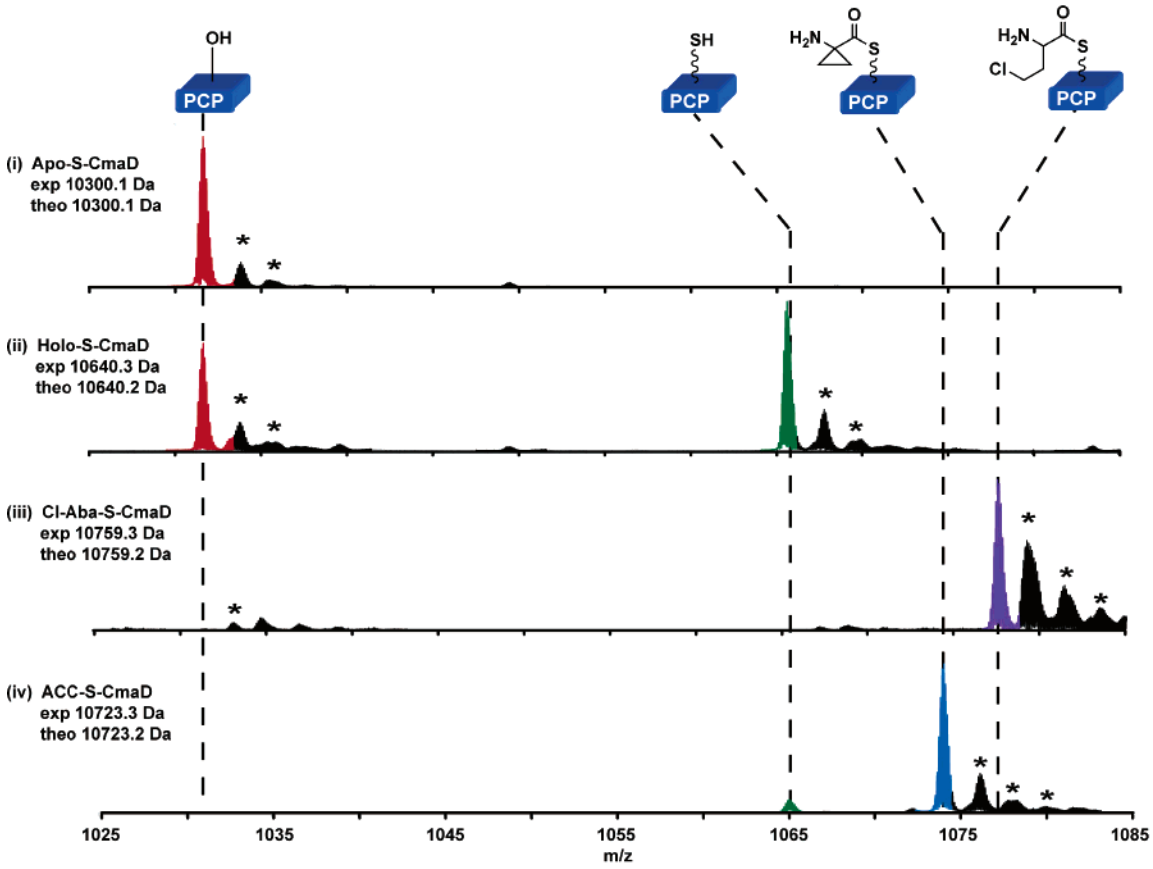


FIGURE 1: CmaC-catalyzed cyclization of Cl-Aba to ACC. FTMS spectra of the intermediates in the conversion of Cl-Aba-*S*-CmaD to ACC-*S*-CmaD in the presence of CmaC. The top of the figure depicts CmaD intermediates of interest [from left to right: apo- and holo- (+340 Da), ACC- (+423 Da), and Cl-Aba- (+459 Da)] and are aligned to the representative peaks in the mass spectra (vertical dashed lines). Shown is the *m*/*z* region from 1025 to 1085 encompassing ions with a +10 charge state. Asterisks indicate signals arising from artifactual adduction: sodium (+22 Da), potassium (+38 Da), phosphate (+98 Da), and oxidation of Met/Cys residues (+16 Da). (i) Apo-CmaD, (ii) holo-CmaD, (iii) Cl-Aba-*S*-CmaD, and (iv) reaction mixture containing Cl-Aba-*S*-CmaD and CmaC producing ACC-*S*-CmaD.

aminocyclopropane-1-carboxylate (Cl-ACC) was observed by FTMS (Figure 3), suggesting that CmaC tolerates this substitution at C_γ, the site of Cl displacement.

Requirement of a Divalent Metal Cofactor. An earlier report of CmaC indicated co-purification with a mixture of bound divalent metals at the following equivalent ratios to CmaC: Fe^{2+} (0.30), Zn^{2+} (0.24), Mn^{2+} (0.11), Ni^{2+} (0.06), and Cu^{2+} (0.01) (*6*). In an effort to determine whether one or more of these metals may serve as the preferred divalent metal cofactor, various forms of CmaC were prepared, either by *in vitro* chelation/reconstitution or by supplementation during growth conditions, and the metal content was determined via ICP-MS (Table 1). Zn−CmaC was successfully purified following heterologous protein expression in medium supplemented with $ZnCl_2$. Purification of CmaC, including treatment with a chelator cocktail (EDTA/2,2′-bipyridyl/1,10-phenanthroline) to remove protein-bound metals, provided apo-CmaC. Anaerobic reconstitution of apo-CmaC with ferrous ammonium sulfate was performed, but subsequent ICP-MS analysis revealed nearly equal ratios of CmaC-bound Fe and Zn (Fe/Zn−CmaC, Table 1). It is possible that CmaC may harbor a considerably higher affinity for Zn^{2+} than for Fe^{2+} and is able to scavenge Zn^{2+} from trace impurities in the components present in the reconstitution procedure.

The kinetic parameters for Zn−CmaC were determined with the Cl-Aba-*S*-CmaD substrate, providing a K_m of 188 ± 18 μM and a k_{cat} of 8.1 ± 0.5 min^{-1} (Figure 4) A comparison of the observed reaction rates, while holding Cl-Aba-*S*-CmaD constant at 200 μM, revealed that apo-CmaC retained significant activity, displaying only a 4-fold reduction in k_{obs} relative to Zn−CmaC (Table 2). After preincubation with $MgCl_2$, the k_{obs} of apo-CmaC was slightly enhanced, while the presence of EDTA did lead to a reduction in the rate (Table 2). Initial studies with CmaC containing a 1:1 mixture of Fe^{2+} and Zn^{2+} (Fe/Zn−CmaC) demonstrated k_{obs} values in the range of 5−10 min^{-1}, whether anaerobic or aerobic.

Incorporation of Solvent Deuterium in Cl-Aba during Catalytic Turnover. Cl-Aba-*S*-CmaD was prepared in deuterium oxide and presented to Zn−CmaC, and the extent of solvent deuterium incorporation into the α position of the Cl-Aba substrate was determined by FTMS analysis and by detection of the aminoacyl-phosphopantetheinyl elimination product (Figure 5) (*18*). The control reaction mixture lacking CmaC revealed only a small amount (10%) of deuterium incorporation. This is in contrast to the CmaC-containing reaction, where the shift in isotopic ratios (Figure 5) indicates 90% of the Cl-Aba-incorporated deuterium.

DISCUSSION

The strained three-membered cyclopropane ring is found in a diverse variety of natural products, and there are several documented modes of its enzymatic construction (*23*−*28*).

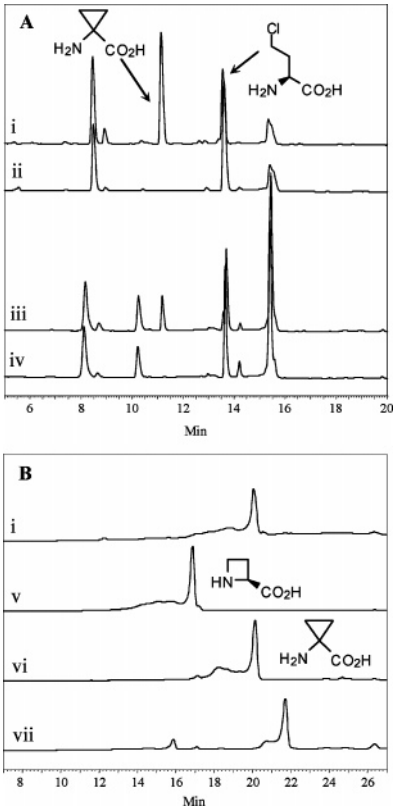


FIGURE 2: (A) OPA derivatization and HPLC analysis of thioesterase-cleaved amino acids. (i) Cl-Aba-*S*-CmaD with CmaC, (ii) Cl-Aba-*S*-CmaD without CmaC, (iii) Cl-Aba-*S*-BarA with CmaC, and (iv) Cl-Aba-*S*-BarA without CmaC. Indicated peaks correspond to the OPA-dervatized amino acids. (B) FMOC derivatization of thioesterase-cleaved CmaD and standards. (i) Cl-Aba-*S*-CmaD with CmaC, (v) azetidine-2-carboxylic acid, (vi) ACC, and (vii) Cl-Aba. Indicated peaks correspond to the FMOC-derivatized amino acids.

One route involves cyclopropane formation by the quenching of carbocationic intermediates in isoprenoid metabolism, for example, in the formation of the thujane monoterpenes (*28*) or in the generation of presqualene-pyrophosphate by squalene synthase (*29*, *30*). A second strategy is employed in the biogenesis of cyclopropane-containing fatty acids, where the π electrons of a 9,10-olefin attack *S*-adenosylmethionine (SAM) with the transfer of the activated CH_3 of SAM to fashion the three-membered ring (*31*−*33*). SAM is also involved in a very distinct mechanism, offering the aminobutyryl side chain as a scaffold for cyclopropane construction rather than the C_1 methyl attached to the sulfonium center. In this latter instance, the PLP-dependent enzyme ACC synthase generates a stabilized α carbanion and uses it as the nucleophile for intramolecular displacement on C_γ, with cleavage of the $C_\gamma-S^+$ bond and elimination of the thiomethyladenosine moiety from SAM (*34*) (Scheme 2A).

This strategy of enzymatic generation of an aminoacyl-α-carbanion equivalent to effect intramolecular capture of an activated $C_\gamma-CH_2$ group practiced by ACC synthase is parallel to the logic used by the enzyme CmaC (Scheme 2B). Indeed, CmaC can use not only *allo*-Ile and Val but even aminobutyrate to generate the same ACC product. However, two differences are immediately evident in comparison to the reactions in Scheme 2. ACC synthase uses the tightly bound PLP coenzyme for iminium ion catalysis to lower the energy barrier to make the C_α carbanion the nucleophile. Meanwhile, C_γ of SAM, as the sulfonium cation, is preactivated for the reaction as an electrophile. The CMA six-protein pathway solves the requirement for the polarization of C_α as a nucleophile and C_γ as a prospective electrophile with different molecular logic. The substrate for CmaC is the γ-chloro-α-aminoacyl-*S*-pantetheinyl thioester. The γ-Cl is functionally equivalent to the Ado-S^+-CH_3 cation of SAM in activating the methionine C_γ as an electrophile. The derivatization of the γ-CH_3 of *allo*-Ile, Val, and 2-aminobutyric acid (ABA) by the CmaB halogenase is a key functionalization step, and we have elsewhere commented on how powerful these oxygenative halogenation catalysts are for regiospecific functionalization of unactivated methyl groups (*6*, *8*, *22*, *35*).

The activation of C_α to get to a low-energy carbanion equivalent in the CmaC active site is solved by the NRPS logic of CmaA, installing the aminoacyl group as a *S*-phosphopantetheinyl thioester on a carrier protein domain. While the PLP coenzyme (Scheme 2A) stabilizes aminoacyl α-carbanion equivalents by delocalization into the PLP imine system, the *S*-phosphopantetheinyl prosthetic group stabilizes aminoacyl α carbanions by delocalization as the thioester enolates (Scheme 2B). In other NRPS assembly lines, this is the mechanism for L to D epimerization of aminoacyl/peptidyl-*S*-phosphopantetheinyl carrier proteins (*36*, *37*). The thioester enolate contributor to the C_α carbanion stabilization could be further assisted by divalent metal chelation as proposed in Scheme 2B and as observed in methylmalonyl CoA epimerase (*38*, *39*).

One test of the intermediacy of an aminoacyl α carbanion at some stage in the CmaC reaction is the evaluation of deuterium incorporation from D_2O. If carbanion formation is reversible and competitive with the intramolecular γ-Cl-displacing, cyclopropane-forming reaction in the front direction, then the back protonation could occur with a solvent-derived deuteron. Thus, if back protonation was fast relative to cyclopropane ring formation during catalytic turnover, the remaining substrate would become deuterated while waiting to again be deprotonated and partitioned in the forward direction. Mass spectrometric analysis of the γ-chloro-aminoacyl moiety tethered to the phosphopantetheinyl elimination product indeed shows that CmaC incorporates one deuterium into the "unreacted" γ-chloro-aminobutyryl-*S*-pantetheinyl arm. The simultaneous analysis of the product ACC-containing phosphopantetheinyl elimination product shows no incorporation of deuterium, fully consistent with ^2H being localized at C_α and thus removed in aminocyclopropane-1-carboxylate formation.

Bioinformatic predictions from the CmaC sequence suggests that it is homologous to the vicinal oxygen chelate (VOC) family of enzymes that use active-site divalent metal cations to position and stabilize substrates and intermediates in diverse reactions (*40*, *41*), as noted above for methylmalonyl CoA epimerase (*38*). Our initial effort to overproduce CmaC provided a mixed population of bound divalent cations. In this study, we found that supplementation of the growth medium with zinc chloride yields the zinc form of CmaC that has about a 10-fold higher turnover number (5 min^{-1}) than the apo form of the enzyme. Further studies will be required with other metals, such as Fe^{2+}, and with careful reconstitution studies to determine which divalent cation gives the highest catalytic efficiency and using Chelex-100-

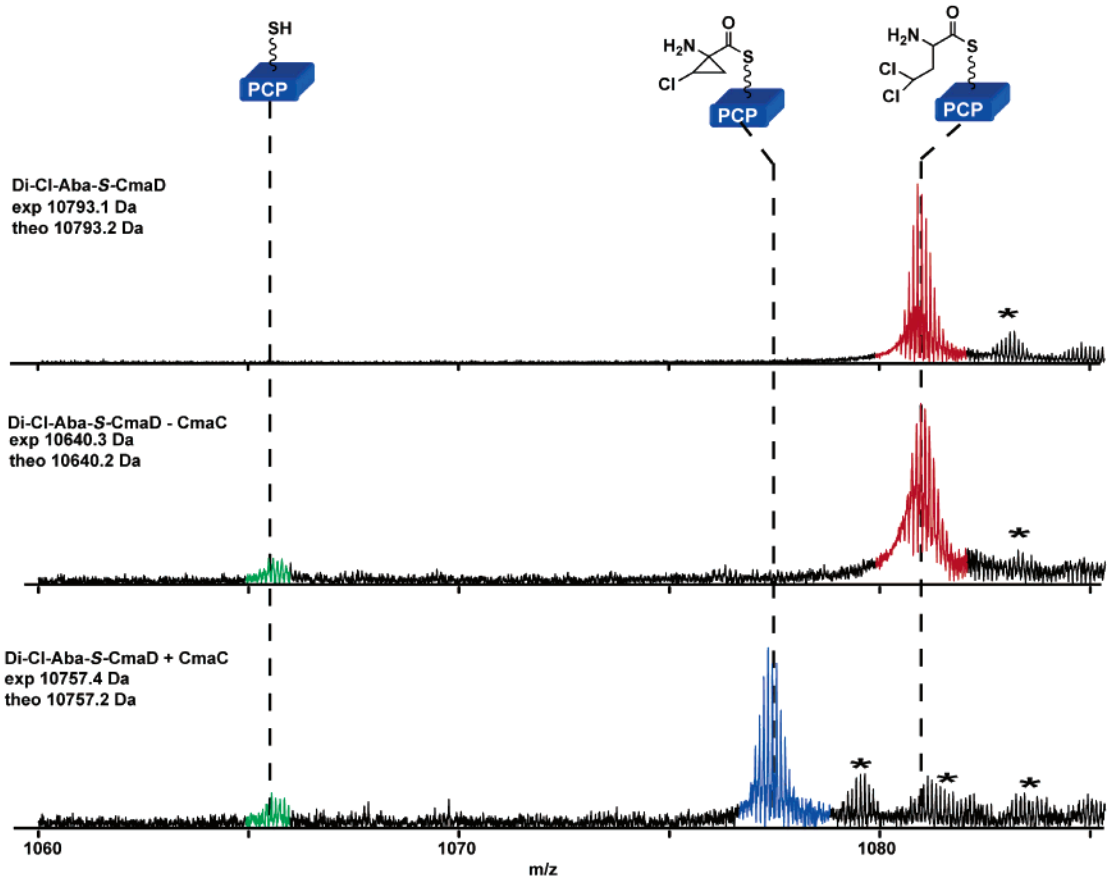


FIGURE 3: CmaC-catalyzed cyclization of 4,4-diCl-Aba-*S*-CmaD to provide Cl-ACC-*S*-CmaD. The top of the figure depicts CmaD intermediates of interest [from left to right: holo- (+340 Da), Cl-ACC- (+457 Da), and 4,4-diCl-Aba- (+493 Da)] and are aligned to the representative peaks in the mass spectra (vertical dashed lines). Shown is the *m*/*z* region from 1060 to 1085 encompassing ions with a +10 charge state. (i) Substrate formation, 4.4-diCl-Aba-*S*-CmaD, (ii) control incubation without CmaC, showing no nonenzymatic product formation, and (iii) incubation with CmaC yielding Cl-ACC-*S*-CmaD. Asterisks indicate signals arising from artifactual adduction: sodium (+22 Da), potassium (+38 Da), phosphate (+98 Da), and oxidation of Met/Cys residues (+16 Da).

Table 1: ICP-MS Analysis of CmaC Preparations

	divalent metal content (equivalents)			
CmaC form	Mn	Fe	Ni	Zn
apo-CmaC	0.00	0.00	0.00	0.03
Zn−CmaC	0.01	0.07	0.01	1.26
Fe/Zn−CmaC	0.00	0.56	0.00	0.57

Table 2: CmaC Reaction Rates

enzyme form/reaction	k_{cat} (min^{-1})
apo-CmaC	0.93 ± 0.10
apo-CmaC plus $MgCl_2$	1.56 ± 0.15
apo-CmaC plus EDTA	0.49 ± 0.04
Zn−CmaC	4.12 ± 0.61

treated buffers to determine the residual activity, if any, possessed by Mg^{2+}-free apo-CmaC.

One of the challenges for CmaC in three-membered ring construction on the aminoacyl-*S*-CmaD substrates is how to avoid competition by the α-NH_2 substituent to form the N−C_γ bond and generate the four-membered azetidine carboxylate. These two outcomes cannot be distinguished by MS analysis because these alternate products have the same molecular mass. To validate that CmaC was indeed making three-membered carbocycle and not the four-membered azetidine, the product thioester was hydrolyzed, converted to the FMOC derivative, and analyzed by HPLC. Only ACC was generated enzymatically. It is possible that the substrate amino group is either protonated or hydrogen-bonded in the CmaC active site to attenuate its nucleophilicity.

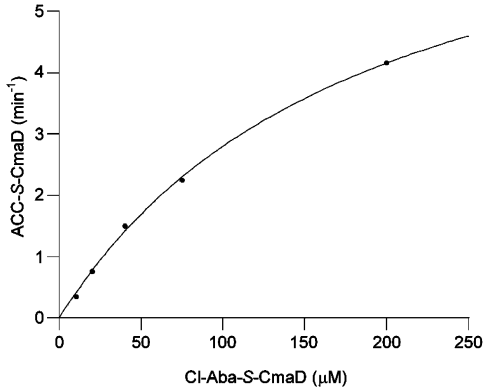


FIGURE 4: Michaelis−Menten analysis used to determine kinetic parameters for Zn−CmaC with Cl-Aba-*S*-CmaD.

When the γ,γ-dichloroaminobutyryl scaffold was presented to CmaC, it did catalyze the elimination of one of the two chlorines and the formation of a product with the mass of Cl-ACC. CmaC thus appears to accept various substitutions at the C_β and C_γ positions, as observed for its naturally occurring Cl-aminoacyl substrates, Cl-*allo*-Ile and Cl-Val, in addition to Cl-Aba. Further studies will be necessary to determine whether any larger substitutions

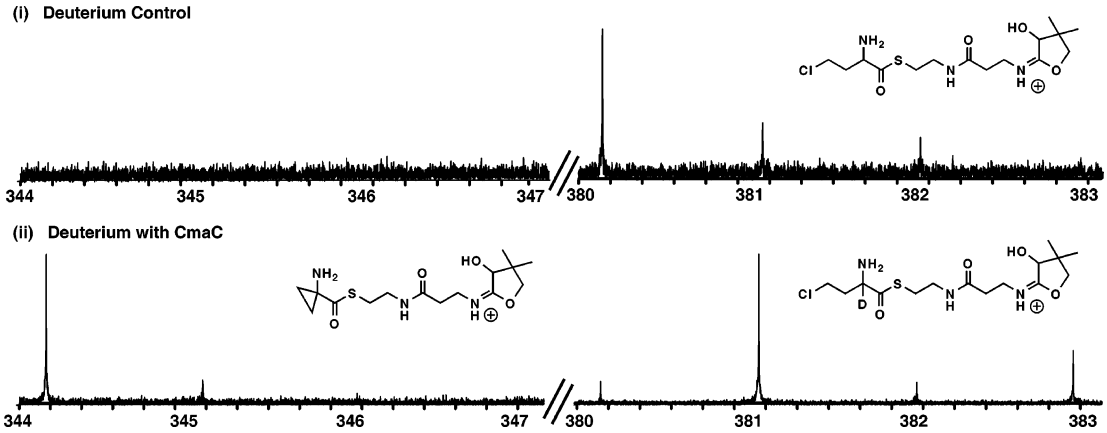
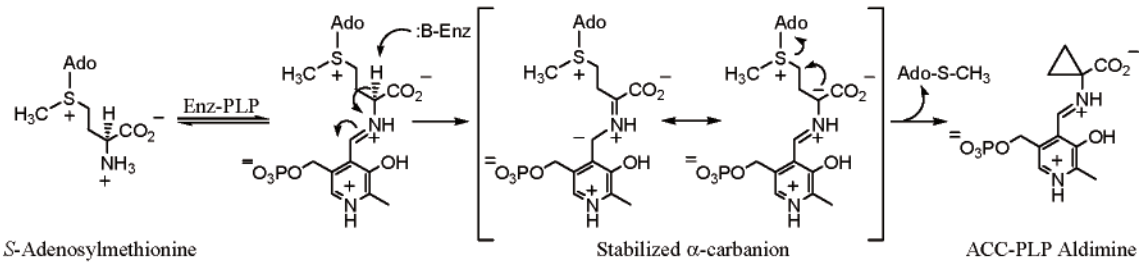


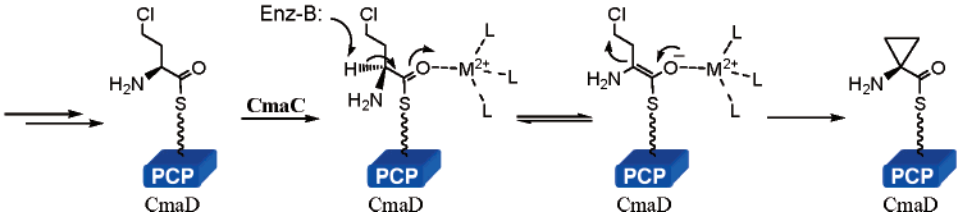
FIGURE 5: Solvent deuterium incorporation by CmaC into substrate Cl-Aba-*S*-CmaD. Mass spectra of the *m*/*z* region for predicted phosphopantetheinyl elimination products are shown: 344−347 ACC and 380−383 4-Cl-Aba. (i) Control incubation without CmaC, 10 min; and (ii) incubation with CmaC, 10 min.

Scheme 2: (A) Mechanism for 1-Aminocyclopropane-1-carboxylate Formation by ACC Synthase and (B) Proposed Mechanism for 1-Aminocyclopropane-1-carboxylate Formation by CmaC

(A) ACC Synthase (PLP coenzyme, SAM substrate: γ-sulfonium cation)



(B) CmaC (Aminoacyl thioester, γ-chloro substituent)



or functional groups at these positions will be tolerated by CmaC.

Our efforts to explore the scope of CmaC for cyclization of δ-chloro- and ϵ-chloro-aminoacyl substrates to the corresponding cyclobutanes and cyclopentanes, respectively, gave no evidence for catalysis of a larger ring formation. In the case of the δ-chloronorvalyl-*S*-CmaD protein, nonenzymatic cyclization intervened, exclusively with the α-NH_2 as a nucleophile to produce prolyl-*S*-CmaD. At a substantially slower rate, the ϵ-Cl substituent was displaced intramolecularly by the NH_2 to generate the pipecolyl-*S*-CmaD. These rates are in accordance with the known nonenzymatic cyclization propensities in which the cyclization of bromoalkylamines has a relative rate of 100:1:0.001 pyrrolidine/piperidine/azetidine (*42*, *43*). In particular, the 10^5 retardation to form azetidine versus pyrrolidine may provide a window for CmaC to effect C−C bond formation in cyclopropane construction but not be able to outcompete the five-membered ring N−C bond formation.

CmaC can accept γ-substituted and γ-branched aminoacyl moieties for cyclopropane formation. It can also recognize the aminoacyl groups when presented on noncognate carrier protein domains, raising the prospect for using CmaC to tailor aminoacyl moieties in other biosynthetic pathways. It remains to be seen how often nature accesses the Cma logic and enzymatic machinery to make aminocarboxycyclopropanes rather than using SAM and a PLP-dependent enzyme. We note the apoptotic agent cytotrienin (Cyt) A (*44*) has an ACC moiety, to which aminobutyrate is the precursor, and we have detected CmaA, CmaB, and CmaT homologues in the producer organism (*11*). Likewise, curacin has a cyclopropyl ring that may arise via a comparable halogenation and ring-closure route (*45*).

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SUPPORTING INFORMATION AVAILABLE

Additional Materials and Methods and Figures S1−S4. This material is available free of charge via the Internet at http://pubs.acs.org.

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