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Article

Thioesterase from Cereulide Biosynthesis Is Responsible for Oligomerization and Macrocyclization of a Linear Tetradepsipeptide

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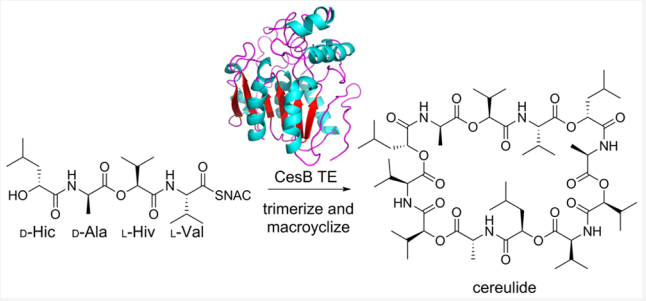
ABSTRACT: Cereulide is a toxic cyclic depsidodecapeptide produced in *Bacillus cereus* by two nonribosomal peptide synthetases, CesA and CesB. While highly similar in structure to valinomycin and with a homologous biosynthetic gene cluster, recent work suggests that cereulide is produced via a different mechanism that relies on a noncanonical coupling of two didepsipeptide-peptidyl carrier protein (PCP) bound intermediates. Ultimately this alternative mechanism generates a tetradepsipeptide-PCP bound intermediate that differs from the tetradepsipeptide-PCP intermediate predicted from canonical activity of CesA and CesB. To differentiate between the mechanisms, both tetradepsipeptides were prepared as *N*-acetyl cysteamine thioesters (SNAC), and the ability of the purified recombinant terminal CesB thioesterase (CesB TE) to oligomerize and macrocyclize each substrate was probed. Only the canonical substrate is converted to cereulide, ruling out the alternative mechanism. It was demonstrated that CesB TE can use related tetradepsipeptide substrates, such as the valinomycin tetradespipetide and a hybrid cereulide−valinomycin tetradepsipetide in conjunction with its native substrate to generate chimeric natural products. This work clarifies the biosynthetic origins of cereulide and provides a powerful biocatalyst to access analogues of these ionophoric natural products.



Cereulide, **1**, is a cyclic depsidodecapeptide produced by *Bacillus cereus* that was discovered in 1994.[1,2] It is a potassium ionophore, which rapidly depolarizes bacterial and mitochondrial membranes,[3] and as such it is a potent toxin.[4,5] At lower doses it is an emetic toxin impacting the 5-HT_3 receptor, stimulating the vagus afferent nerve leading to vomiting.[2] Due to the potential contamination of food products with *B. cereus* and the high chemical and temperature stability of cereulide, poisoning is a significant safety concern.[6−8]The biosynthetic gene cluster responsible for cereulide biosynthesis was identified in 2004.[9−11] Cereulide is produced by two nonribosomal peptide synthetases, CesA and CesB, encoded on the pXO1 megaplasmids.[12,13] CesA uses α-ketoisocaproate, which is reduced in the pathway to D-α-hydroxyisocaproate (D-Hic) and D-Ala to generate the peptidyl carrier protein (PCP)-linked D-Hic-D-Ala depsipeptide.[14−16] CesB uses α-ketoisovalerate, reduced in the pathway to L-α-hydroxyisovalerate (L-Hiv) and L-Val and in conjunction with CesA generates cereulide.[14,15] In the biosynthesis of the related ionophore valinomycin, **2**, Vlm1 generates a PCP-linked D-Hiv-D-Val, which is condensed with L-lactate and then L-Val to generate a PCP-linked D-Hiv-D-Val-L-Lac-L-Val.[14,17] Finally the tetradepsipeptide is trimerized and macrocyclized by the C-terminal thioesterase (TE) domain of Vlm2 to generate valinomycin.[18] Recent work suggests that despite the similarity between the structures of cereulide and valinomycin and their biosynthetic gene clusters, cereulide is produced via a different mechanism. It is proposed that a L-Hiv-L-Val-D-Hic-D-Ala PCP bound intermediate is generated (Figure 1B) rather than the expected D-Hic-D-Ala-L-Hiv-L-Val intermediate (Figure 1A).[19] Further it is suggested that some of the isocereulides[20−22] are produced by the unexpected coupling of a TE-bound depsidipeptide with a PCP-bound tetradepsipeptide.[19] This study sets out to test if valinomycin and cereulide have differing mechanisms of TE-mediated oligomerization and macrocyclization by characterizing the ability of the CesTE to oligomerize and macrocyclize tetradepsipeptide substrates.

RESULTS AND DISCUSSION

To test the TE-mediated oligomerization and macrocyclization step from cereulide biosynthesis, both the proposed cereulide TE substrate[19] as an *N*-acetyl cysteamine (NAC) thioester (**7**, L-Hiv-L-Val-D-Hic-D-Ala-SNAC) and the expected NAC substrate based on valinomycin homology (**12**, D-Hiv-D-Ala-L-Hiv-L-Val-SNAC) were synthesized. For **7** (Scheme 1), a Mitsunobu reaction was used to link Boc-L-Val to allyl L-α-

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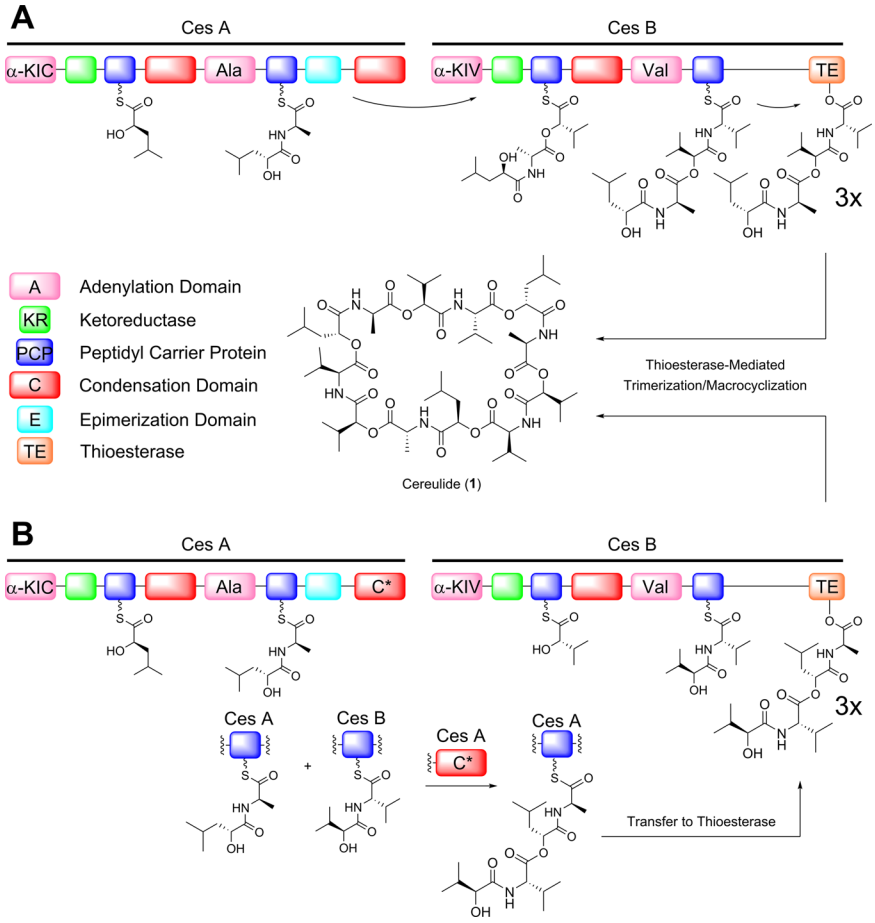

Figure 1. Two proposed pathways for the biosynthesis of cereulide by CesA and CesB. (A) This mechanism for cereulide formation is consistent with the expected activity of all the catalytic domains in CesA and CesB, generating a D-Hic-D-Ala-L-Hiv-L-Val-PCP bound intermediate on CesB that must be oligomerized and cyclized by the TE. (B) This proposed mechanism invokes a noncanonical use of the C* domain from CesA. Unlike mechanism A, mechanism B leads to the formation of a L-Hiv-L-Val-D-Hic-D-Ala PCP bound intermediate on CesA that must be oligomerized and cyclized by the TE.[19] Note: Linker regions between domains are not to scale. The extended distance between the PCP and TE domains in CesB is for clarity.

hydroxycaproate to generate the D-Hic-containing depsidipeptide. Deprotection of the allyl ester with $Pd(PPh_3)_4$ and coupling with D-Ala-SNAC generated the depsitripeptide. Finally Boc deprotection and coupling with L-Hiv furnished the tetradepsipeptide substrate **7**. Synthesis of **12** began with coupling Boc-D-Ala and allyl L-α-hydroxyisovalerate to form the ester linkage. From this intermediate the synthesis mirrored the route to access **7**, with allyl deprotection followed by coupling to L-Val-SNAC, Boc deprotection, and coupling with D-Hic to afford **12**. The SNAC thioester serves to activate the substrate for loading on the active site serine of the TE and mimic the phosphopantetheine arm of the PCP domain.[23]

The gene encoding the C-terminal TE domain from *cesB* was synthesized and cloned into an expression vector under the inducible T7 promoter. Ces TE was overexpressed in *E. coli* BL21 and purified to homogeneity by metal affinity chromatography. Treatment of Ces TE with **7** in 50 mM Tris buffer pH 7.4 led to no detectable formation of cereulide by LCMS (Figure 2). No oligomeric structures or hydrolysis of **7** was detected. This is consistent with Ces TE being unable to load **7**. In contrast, incubation of **12** with Ces TE generated cereulide, **1**, as well as dimeric, trimeric, and tetrameric SNAC products, **13**, **14**, and **15**, respectively (Figure 3). Both the no enzyme and the boiled enzyme controls showed no production of **1**, confirming its production is TE-dependent. This result clearly demonstrates that the Ces TE is capable of oligomerizing and macrocyclizing the expected tetradepsipeptide substrate derived from canonical NRPS activity, analogous to valinomycin biosynthesis.[18]

Kinetic characterization of Ces TE activity with **12** as monitored by thiol release using Elman's reagent provided an observed $k_{cat}/K_M = 0.28 \pm 0.02\ M^{-1}\ s^{-1}$. This is comparable, though on the low end, to specificity constants seen for other characterized TE domains,[24] such as the epothilone[25] and 6-deoxyerythronolide B[24] macrocyclizing TEs. The Ces TE could not be saturated even at 4 mM **12**, preventing k_{cat} and K_M from being independently determined. The K_M, which based on our kinetic characterization is >4 mM, is significantly higher than other TEs responsible for oligomerization and macrocyclization of nonribosomal peptides. For example, the tyrocidine TE (TycC TE) and the gramicidin TE (GrsB TE), which both dimerize D-Phe-L-Pro-L-Val-L-Orn-L-Leu-SNAC and cyclize the product to generate gramicidin S, show K_M's in the range of 0.3−0.5 mM.[26,27] We speculate that in order to effectively catalyze oligomerization and macrocyclization, these TEs likely possess enhanced affinity for oligomerized substrates over monomeric substrates as the oligomers have higher structural homology to the macrocyclization substrates and

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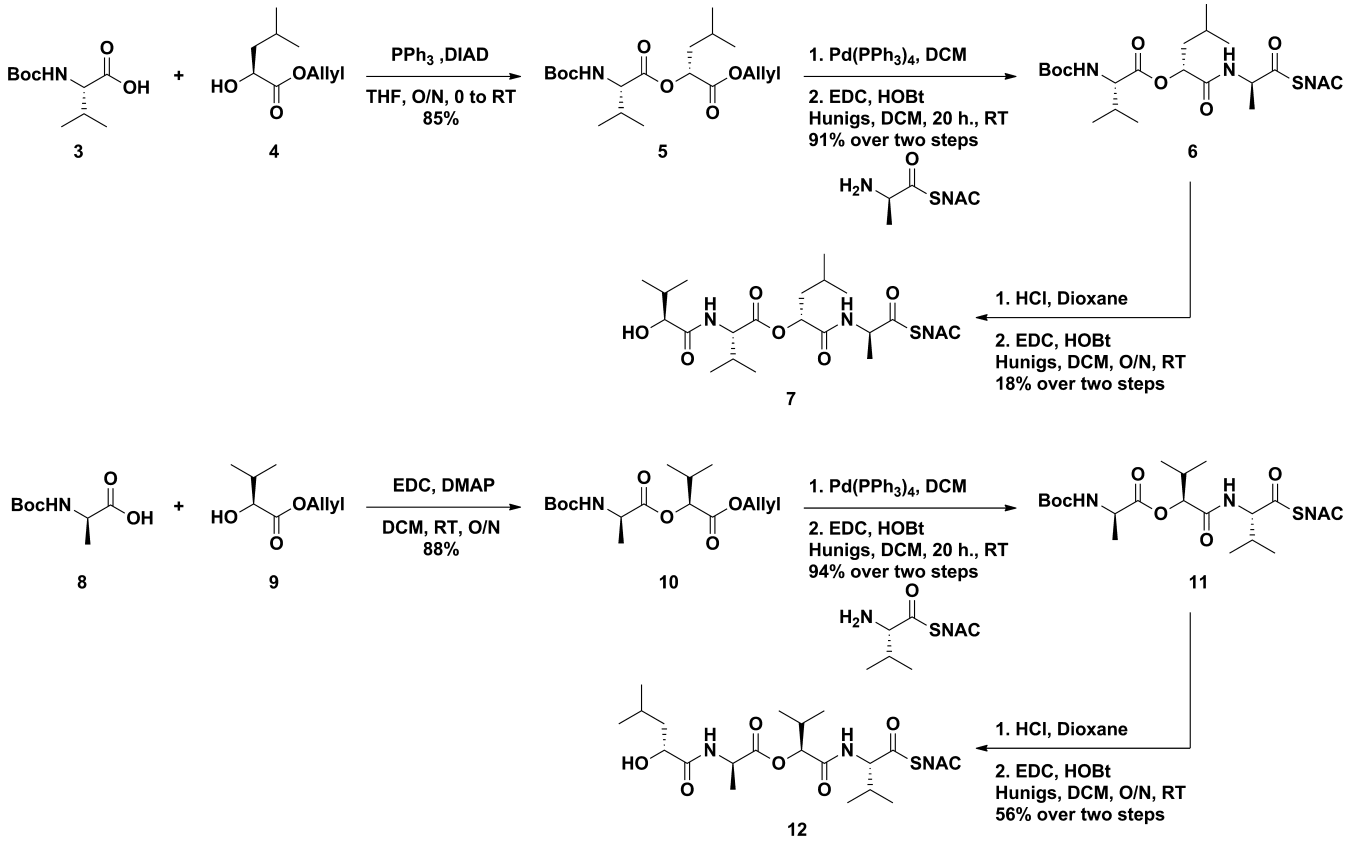
Scheme 1. Synthesis of SNAC Substrates 7 and 12



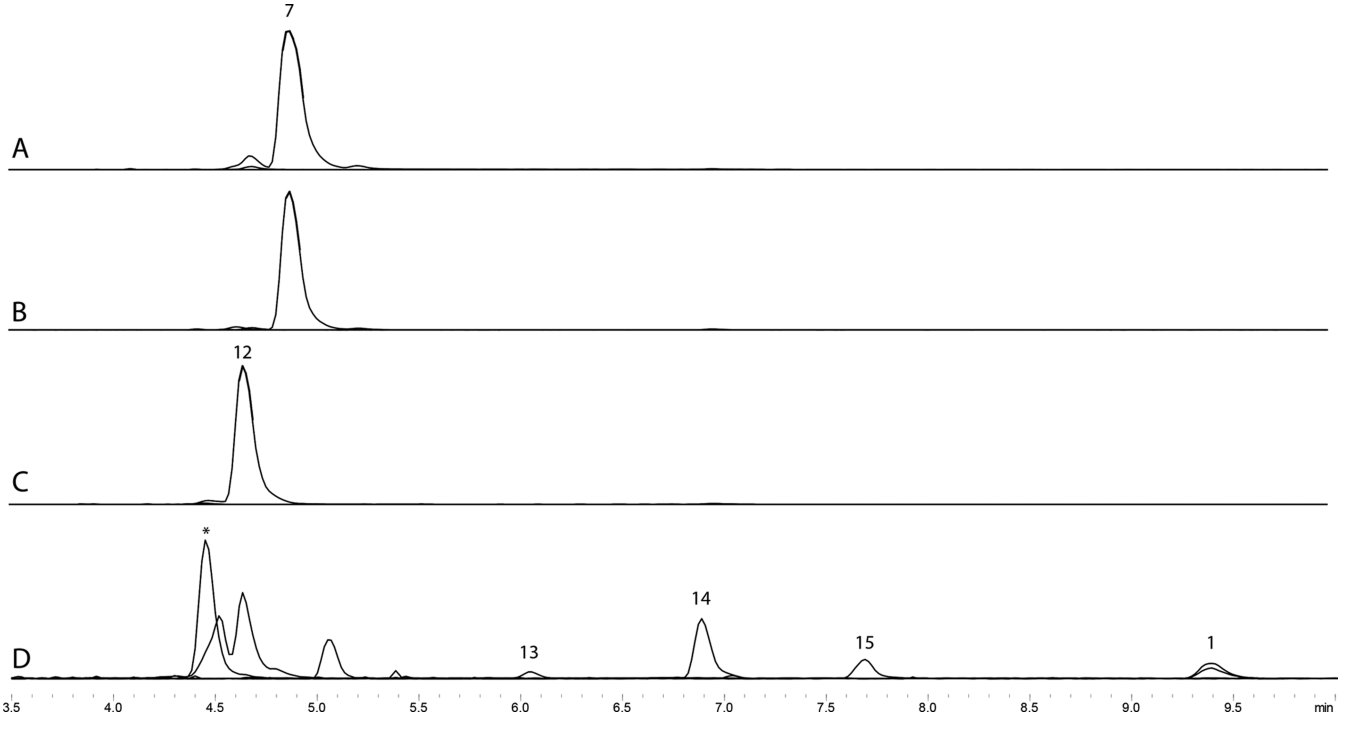


Figure 2. LCMS traces of incubation of synthetic tetradesipeptide *N*-acetyl cysteamine substrates with CesB TE. (A) Negative control. Boiled CesB TE (15 μM) with 1 mM **7** in 50 mM Tris pH 7.4, 14 h 22 °C. (B) CesB TE (15 μM) with 1 mM **7** in 50 mM Tris pH 7.4 for 14 h at 22 °C. No cereulide or oligomeric product is formed. (C) Negative control. Boiled CesB TE (15 μM) with 1 mM **12** in 50 mM Tris pH 7.4, 14 h 22 °C. (D) CesB TE (15 μM) with 1 mM **12** in 50 mM Tris pH 7.4 for 14 h at 22 °C. Cereulide (**1**) as well as dimeric, trimeric, and tetrameric *N*-acetyl cysteamine thioester products (**13**, **14**, and **15**, Figure 3) are formed. All traces are ion extraction for m/z = 504, 888, 1272, 1658, and 1170, which correspond to the $[M + H]^+$ for **7**/**12**, **13**, **14**, and **15**, and the $[M + NH_4]^+$ for **1**, respectively. *__12__ hydrolysis product, m/z = 403 $[M + H]^+$.

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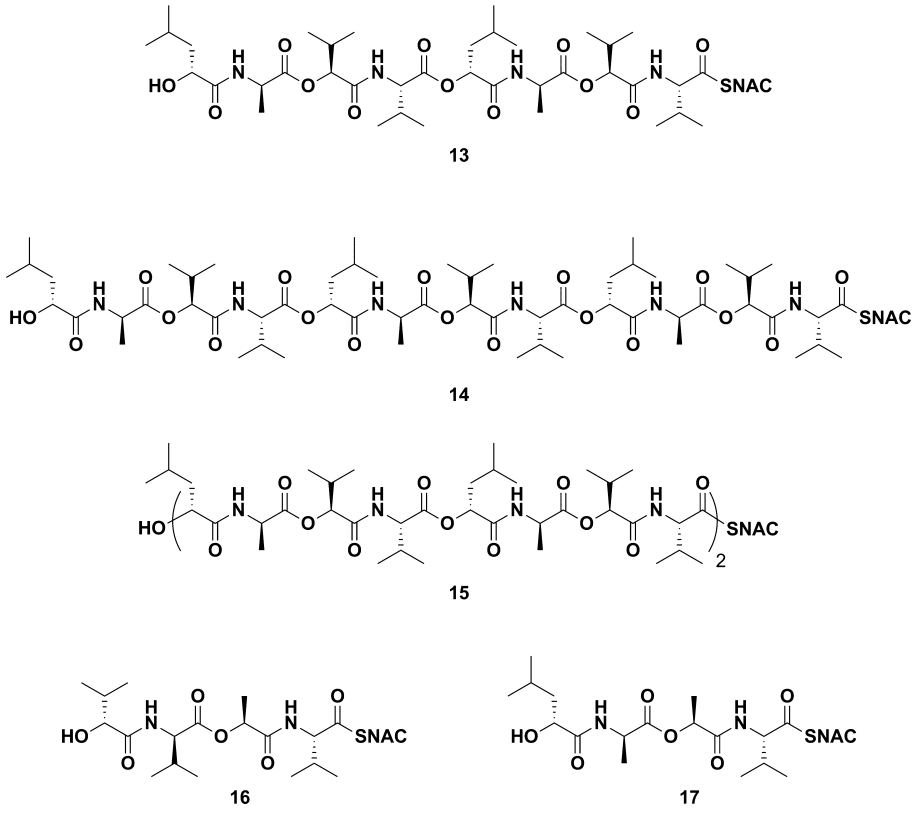

Figure 3. Structures of cereulide oligomers and additional SNAC substrates.

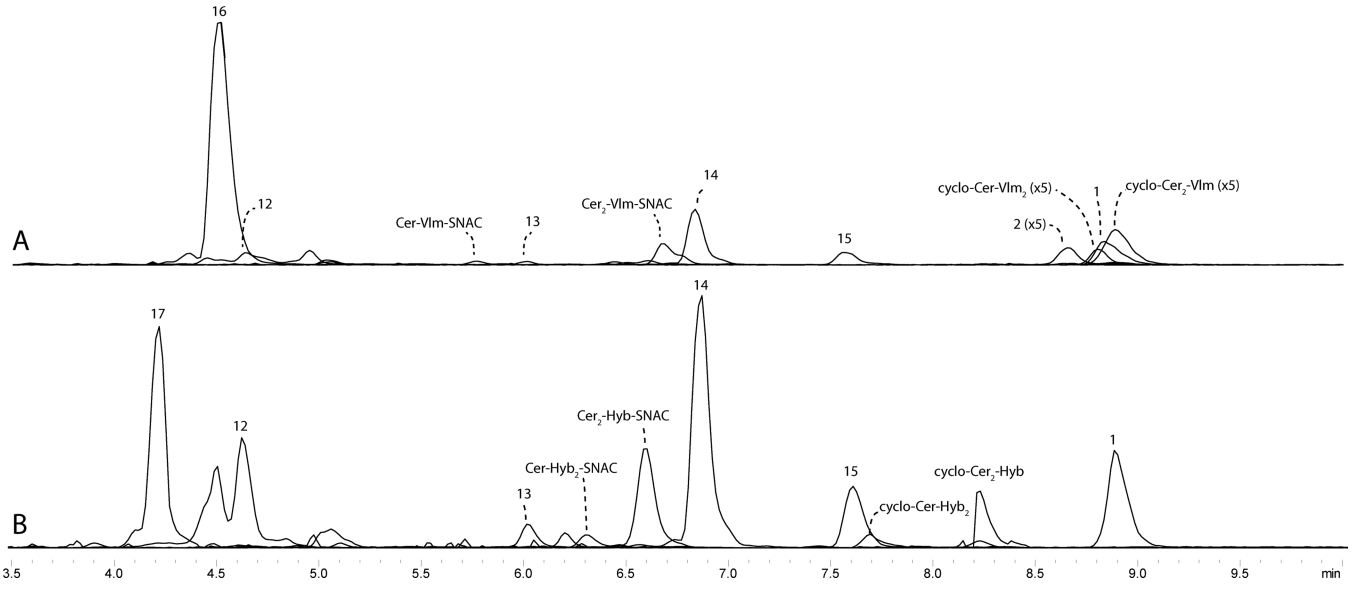

Figure 4. LCMS traces of incubation of two different synthetic tetradesipeptide *N*-acetyl cysteamine substrates with CesB TE. (A) CesB TE (15 μM) with 2 mM **12** and 1 mM **16** in in 50 mM Tris pH 7.4, 14 h at 22 °C. The expected natural products **1** and **2** were formed as well as two hybrid natural products comprising two cereulide monomers and one valinomycin monomer (cyclo-Cer_2-Vlm) and the other of two valinomycin monomers and one cereulide monomer (cyclco-Cer-Vlm_2). A boiled enzyme control showed no product formation. (B) CesB TE (15 μM) with 2 mM **12** and 1 mM **17** in in 50 mM Tris pH 7.4, 14 h at 22 °C. Cereulide and a complex mixture of oligomeric-SNAC compounds were generated. A boiled enzyme control generated no oligomeric or cyclic products. Traces are ion extraction for $[M + Na]^+$ ions with m/z = 526 (**12**), 512 (**16**), and 498 (**17**), $[M + H]^+$ ions with m/z = 889 (**13**), 875 (Cer-Vlm-SNAC), 861 (Cer-Hyb-SNAC), 1273 (**14**), 1259 (Cer_2-Vlm-SNAC), 1245 (Cer-Vlm_2-SNAC and Cer_2-Hyb-SNAC), 1217 (Cer-Hyb_2-SNAC), and 1658 (**15**), and $[M + NH_4]^+$ ions with m/z = 1170 (**1**), 1128 (**2**), 1156 (cyclo-Cer_2-Vlm), 1132 (Cyclo-Cer-Vlm_2), 1142 (cyclo-Cer_2-Hyb), and 1114 (cyclo-Cer-Hyb_2).

thus can be stabilized by more of the enzyme−substrate interactions involved in macrocyclization activity. In the case of Ces TE, we expect higher affinity for the more mature trimerized intermediate over the dimerized intermediate and monomeric substrate. This is consistent with the observation that the dimeric and trimeric SNAC intermediates do not

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accumulate to appreciable levels in the biochemical assays but are rather processed to products preferentially over the SNAC-activated monomer. Unlike the GrsB TE, which catalyzes dimerization and macrocyclization, Ces TE must trimerize and macrocyclize. This is also consistent with a higher K_M for the monomeric substrate of Ces TE than the monomeric substrate of GrsB TE.

Treatment of the Ces TE with the related tetradepsipeptide substrate from the valinomycin biosynthetic pathway (**16**, D-Hiv-D-Val-L-Lac-L-Val-SNAC) resulted in very little processing of the substrate (Figure S3D). While trace formation of valinomycin, **2**, and the trimeric intermediate is observable, the main product is the dimeric intermediate. Dimer formation shows that **16** is loaded onto the active site Ser of Ces TE. Attack by a second equivalent of **16**, which is required for formation of dimer and ultimately other higher order structures including **2**, occurs; however, the low processivity of Ces TE with this substrate suggests that attack by the second equivalent of substrate may be slow. Treatment of Ces TE with a mixture of **12** and **16** (Figure 4A) results in the formation of cereulide (**1**) and valinomycin (**2**) as well as hybrid cereulide−valinomycin macrocycles. Based on our data both the cereulide and valinomycin SNAC monomers can be loaded onto the active site Ser. We propose that a second equivalent of the cereulide monomer **12** is more effective than the valinomycin monomer **16** at acting as a substrate to effect off-loading for formation of dimeric and trimeric products. Following this mechanism, the hybrid cereulide−valinomycin product is generated from valinomycin monomer **16** loading onto Ces TE followed by a cereulide monomer **12** effecting off-loading via transesterification to make the activated valinomycin−cereulide dimer (D-Hiv-D-Val-L-Lac-L-Val-D-Hiv-D-Ala-L-Hiv-L-Val-SNAC). This substrate is then loaded back on the Ces TE and transesterified with the cereulide monomer **12**, generating the linear hybrid trimer with two cereulide monomers and one valinomycin monomer (Cer_2-Vlm-SNAC). Subsequent Ces TE loading and macrocyclization then gives the hybrid product. Because the valinomycin monomer **16** is not as effective a substrate to effect off-loading, misincorporation is limited, as clearly seen in the ratio of trimeric Cer_3-SNAC versus Cer_2-Vlm-SNAC products (Figure 4A).

On the basis of this working model, we investigated a hybrid cereulide−valinomycin hybrid monomer with the expectation that this substrate would load on the Ces TE but not be able to act as an off-loading substrate to make higher order oligomers. The hybrid tetradepsipeptide substrate, D-Hic-D-Ala-L-Lac-L-Val-SNAC, **17** (Scheme S1), was synthesized. Treatment of Ces TE with **17** led to minimal dimer formation and no macrocycle formation, consistent with our working model (Figure S3B). Treatment of Ces TE with a combination of **12** and **17** generated both cereulide and the hybrid product containing two cereulide monomers and one hybrid monomer from **17**, also fully consistent with our model (Figure 4B).

Our data and working model for Ces TE oligomerizing and macrocyclizing activity are consistent with the mechanism of formation for the closely related ionophore natural product valinomycin[18] and provide a mechanism for the biosynthesis of the isocereulides, which are produced along with cereulide in trace amounts.[20−22] The ability of Ces TE to cyclize the two non-native cereulide−valinomycin hybrids above clearly indicates that some degree of non-native side chain composition can be accommodated by the TE. Thus, isocereulides A, B, C, and D are generated by the single misincorporation of α-ketoisocaproate in place of α-ketoisovalerate by A1 of CesB, α-ketoisovalerate in place of α-ketoisocaproate by A1 of CesA, D-Ser in place of D-Ala by A2 of CesA, and L-Ala in place of L-Val by A2 of CesB, respectively.

The unexpected linear tetra-, hexa-, octa-, deca-, and dodecadepsipeptide esters isolated from alcoholic extracts of cereulide-producing strains were initially proposed to be due to alcoholic hydrolysis of PCP- and TE-linked biosynthetic intermediates.[19] To rationalize hexa- and decadepsipeptide formation, it was proposed that noncanonical C domain activity could be occurring to couple dipeptide products produced by CesA and CesB.[19] On the basis of our biochemical characterization of Ces TE, we suggest an alternative hypothesis fully consistent with the data and the canonical activity of C domains. In biochemical treatment of Ces TE with the native SNAC-activated substrate **12**, we observe formation of linear SNAC-activated tetramer (hexdecadepsipeptide). This occurs when the TE-O-trimer intermediate undergoes attack by another equivalent of monomer **12** before macrocyclization can occur. In our assays the linear activated tetramer is never observed to undergo macrocyclization. Thus, in the producing organism if the TE elongates the substrate beyond trimer, macrocyclization cannot occur and the tetramer will be either further elongated or hydrolyzed off. Unlike cereulide, these extra-long linear oligomers should be sensitive to alcoholic hydrolysis and readily generate the esters observed. Significantly, this mechanism provides a route to produce multiple equivalents of the linear esters, rather than being limited to between one and three equivalents relative to the amounts of CesA and CesB, as would be required if the esters were formed by alcoholic hydrolysis of the PCP- and TE-linked intermediates.

Thus, in summary this work demonstrates that cereulide is generated highly analogously to valinomycin where the TE mediates trimerization and macrocyclization of the expected tetradepsipeptide substrate based on canonical activity of the NRPS domains present in CesA and CesB. In addition the Ces TE is selective, in our biochemical assays, for the nucleophile that hydrolyzes the depsipeptidyl-TE intermediates. This can be used to access hybrid cereulide macrocycles, with a single non-native monomer incorporated into the final macrocycle. Lastly we propose a mechanism consistent with all data to account for the formation of an unexpected linear ester from cereulide-producing strains, which eliminates the need to resort to noncanonical activity of the CesB C domain.

EXPERIMENTAL SECTION

General Experimental Procedures. All solvents were purchased from Fisher Scientific. All reagents were purchased at the highest available purity and used without further purification. EDC was purchased from Oakwood Chemical (Estill, SC, USA), HOBt was purchased from GL Biochem (Shanghai, PRC), and all other chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada). All reactions were carried out using dry solvents under a nitrogen atmosphere unless otherwise noted. NMR spectroscopy was performed with either a Bruker Avance II operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C spectra or a Bruker Avance III with a cryoprobe operating at 600 MHz for ^{1}H and 150 MHz for ^{13}C spectra. LCMS analysis was carried out using a Shimadzu LC20A HPLC coupled with a Shimadzu LCMS2020 single quad mass spectrometer with an ESI source. High-resolution mass spectroscopy (HRMS) was conducted on a Micromass Q-TOF I (John L. Holmes Mass Spectroscopy Facility, Ottawa, ON, Canada).

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Boc-L-Val-D-Hic Allyl Ester (5). In a round-bottom flask, 453 mg of Boc-L-Val (2.08 mmol, 1.2 equiv) 172 mg of L-Hic allyl ester (1.74 mmol, 1 equiv), and 915 mg of triphenylphosphine (PPh_3, 3.49 mmol, 2 equiv) were dissolved in 18 mL of dry THF. The stirring solution was cooled to 0 °C on an ice bath for 10 min. To this was added 685 μL of diisopropyl azodicarboxylate (DIAD, 705 mg, 3.49 mmol, 2 equiv) dropwise, and the reaction was stirred on ice for an additional hour, then allowed to warm to ambient temperature and stirred overnight. At completion the reaction was concentrated and the title compound (550 mg, 85%) was purified by silica column chromatography (gradient 10% to 15% EtOAc in hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.87 (ddt, *J* = 17.2, 10.4, 5.8 Hz, 1H), 5.30 (dd, *J* = 17.2, 1.4 Hz, 1H), 5.23 (dd, *J* = 10.4, 1.1 Hz, 1H), 5.05 (dd, *J* = 10.0, 3.8 Hz, 1H), 4.96 (d, *J* = 9.0 Hz, 1H), 4.61 (dd, *J* = 5.8, 0.9 Hz, 2H), 4.29 (dd, *J* = 9.0, 4.6 Hz, 1H), 2.18 (dt, *J* = 12.5, 6.4 Hz, 1H), 1.82 (tt, *J* = 15.5, 5.1 Hz, 1H), 1.78−1.67 (m, 1H), 1.68−1.59 (m, 1H), 1.42 (s, 9H), 0.97 (d, *J* = 6.8 Hz, 3H), 0.93 (d, *J* = 6.5 Hz, 3H), 0.92−0.87 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.83, 169.96, 155.51, 131.47, 118.84, 79.72, 71.57, 65.88, 58.64, 39.72, 31.25, 28.30, 24.53, 23.07, 21.28, 19.08, 17.38; HRMS (ESI+) exact mass calculated for $C_{29}H_{33}NNaO_6$ 394.2200, found 394.2192.

Boc-L-Val-D-Hic-D-Ala-SNAC (6). In a round-bottom flask, 100 mg of **5** (0.27 mmol, 1 equiv) was dissolved in 2 mL of CH_2Cl_2 under a nitrogen atmosphere. To this solution were added 31 μL of morpholine (30 mg, 0.35 mmol, 1.3 equiv) and 16 mg of $Pd(PPh_3)_4$ in a single portion. The reaction was stirred at ambient temperature and monitored by TLC. At completion the reaction was quenched by the addition of 10% aqueous HCl, the organic layer was removed, and the remaining aqueous fraction was extracted 3× with CH_2Cl_2. The combined organic fractions were washed with brine, dried over Na_2SO_4, and concentrated in a dry round-bottom flask. This intermediate was used immediately in the subsequent reaction. To the flask were added 2 mL of CH_2Cl_2 and 61 mg of D-Ala-SNAC (as a HCl salt, 0.27 mmol, 1 equiv). To the resulting solution were added 140 μL of Hünig's base (104 mg, 0.81 mmol, 3 equiv), 55 mg of HOBt (0.4 mmol, 1.5 equiv), and 78 mg of EDC (0.4 mmol, 1.5 equiv). The reaction was stirred under argon at ambient temperature for 20 h. The reaction was quenched with saturated NH_4Cl(aq), extracted 3× with CH_2Cl_2, washed with $NaHCO_3$(aq), then with brine, dried over Na_2SO_4, and concentrated. The title compound (124 mg, 91%) was partially purified by silica column chromatography (40% acetone in hexanes) and taken into the subsequent reaction. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.09 (s, 1H), 5.32 (t, *J* = 6.7 Hz, 1H), 5.06 (d, *J* = 6.2 Hz, 1H), 4.57−4.45 (m, 1H), 3.92 (t, *J* = 6.7 Hz, 1H), 3.44−3.26 (m, 3H), 2.99 (td, *J* = 6.3, 3.6 Hz, 1H), 2.94 (t, *J* = 6.0 Hz, 2H), 2.02 (tt, *J* = 13.5, 6.7 Hz, 1H), 1.92 (s, *J* = 2.4 Hz, 4H), 1.80−1.75 (m, 2H), 1.67 (dt, *J* = 20.2, 6.6 Hz, 1H), 1.42 (d, *J* = 2.7 Hz, 3H), 1.37 (s, 10H), 1.02−0.96 (m, 6H), 0.93−0.88 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 201.33, 172.17, 170.77, 170.45, 156.23, 80.67, 72.66, 60.17, 55.59, 40.19, 39.20, 30.02, 28.48, 28.32, 24.41, 23.29, 23.12, 21.17, 19.08, 18.66, 16.99.

HO-L-Hiv-L-Val-D-Hic-D-Ala-SNAC (7). To a round-bottom flask was added 100 mg of **6** (0.2 mmol, 1 equiv) in a minimal amount of EtOAc and cooled to 0 °C. To this was added 2 mL of 4 M HCl in dioxane (Sigma), and the reaction allowed to warm to ambient temperature. The reaction was monitored by TLC, and at completion all solvent was removed by rotary evaporation. The unpurified intermediate was used immediately and was dissolved in 1 mL of CH_2Cl_2, and to this were added 105 μL of Hünig's base (78 mg, 0.6 mmol, 3 equiv), 26 mg of L-α-hydroxyisovaleric acid (0.21 mmol, 1.1 equiv), 40 mg of HOBt (0.3 mmol, 1.5 equiv), and 60 mg of EDC (0.3 mmol, 1.5 equiv). The reaction was stirred at ambient temperature overnight and at completion was quenched with saturated NH_4Cl(aq), extracted 5× with CH_2Cl_2, washed with $NaHCO_3$(aq), then with brine, dried over Na_2SO_4, and concentrated. The title compound (18 mg, 18%) was purified by silica column chromatography (50% acetone in hexanes). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.77 (d, *J* = 8.1 Hz, 1H), 7.35 (d, *J* = 6.8 Hz, 1H), 6.16 (s, 1H), 5.28 (dd, *J* = 10.6, 3.0 Hz, 1H), 4.62 (p, *J* = 7.4 Hz, 1H), 4.14 (dd, *J* = 9.0, 6.8 Hz, 1H), 3.92 (d, *J* = 3.3 Hz, 1H), 3.52 (td, *J* = 13.8, 6.6 Hz, 1H), 3.39−3.32 (m, 1H), 3.17 (dt, *J* = 13.4, 6.1 Hz, 1H), 2.16−2.07 (m, 2H), 1.88−1.70 (m, 3H), 1.48 (d, *J* = 7.3 Hz, 3H), 1.08 (d, *J* = 6.7 Hz, 3H), 1.04 (d, *J* = 3.1 Hz, 3H), 1.03 (d, *J* = 2.8 Hz, 3H), 0.97 (d, *J* = 6.3 Hz, 3H), 0.94 (d, *J* = 6.2 Hz, 3H), 0.90 (d, *J* = 6.9 Hz, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 201.79, 175.88, 172.10, 171.54, 171.13, 76.11, 72.94, 59.13, 55.02, 40.98, 40.29, 38.90, 31.82, 29.57, 28.58, 24.48, 23.33, 23.04, 20.86, 19.06, 19.03, 18.95, 17.00, 16.09; HRMS (ESI+) exact mass calculated for $C_{23}H_{41}N_3NaO_7S$ 526.2563, found 526.2551.

Boc-D-Ala-L-Hiv Allyl Ester (10). In a round-bottom flask, 200 mg of L-2-hydroxyisovalerate allyl ester (1.26 mmol, 1 equiv) and 264 mg of Boc-D-Ala (1.39 mmol, 1.1 equiv) were dissolved in 6.5 mL of CH_2Cl_2. To this solution were added 155 mg of 4-dimethylaminopyridine (DMAP, 1.26 mmol, 1 equiv) and 365 mg of 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide hydrochloride (EDC, 1.9 mmol, 1.5 equiv) at ambient temperature. The resulting solution was stirred for 14 h at ambient temperature. The reaction was quenched with saturated NH_4Cl(aq), extracted 3× with CH_2Cl_2, washed with $NaHCO_3$(aq), washed with brine, dried over Na_2SO_4, and concentrated. The title compound (368 mg, 88%) was purified by silica column chromatography (20% EtOAc in hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.90 (ddt, *J* = 17.2, 10.4, 5.8 Hz, 1H), 5.33 (dq, *J* = 17.2, 1.5 Hz, 1H), 5.25 (ddd, *J* = 10.4, 2.4, 1.2 Hz, 1H), 5.05 (d, *J* = 7.6 Hz, 1H), 4.68−4.59 (m, 2H), 4.49−4.36 (m, 1H), 2.36−2.21 (m, 1H), 1.44 (d, *J* = 6.0 Hz, 12H), 1.01 (d, *J* = 6.9 Hz, 3H), 0.98 (d, *J* = 6.9 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.79, 168.91, 131.50, 118.97, 84.84, 65.80, 56.04, 30.15, 28.33, 27.92, 18.74, 18.66, 17.10; HRMS (ESI+) exact mass calculated for $C_{16}H_{27}NNaO_6$ 352.1736, found 352.1736.

Boc-D-Ala-L-Hiv-L-Val-SNAC (11). In a round-bottom flask, 124 mg of **10** (0.37 mmol, 1 equiv) was dissolved in 2 mL of CH_2Cl_2 under a nitrogen atmosphere. To this solution were added 43 μL of morpholine (43 mg, 0.49 mmol, 1.3 equiv) and 30 mg of $Pd(PPh_3)_4$ in a single portion. The reaction was stirred at ambient temperature and monitored by TLC. At completion the reaction was quenched by the addition of 10% aqueous HCl, the organic layer was removed, and the remaining aqueous fraction was extracted 3× with CH_2Cl_2. The combined organic fractions were washed with brine, dried over Na_2SO_4, and concentrated in a dry round-bottom flask. This intermediate was used immediately in the subsequent reaction. To the flask were added 4 mL of CH_2Cl_2 and 120 mg of L-Val-SNAC (as HCl salt, 0.37 mmol, 1 equiv). To the resulting solution were added 197 μL of Hünig's base (146 mg, 1.12 mmol, 3 equiv), 77 mg of HOBt (0.56 mmol, 1.5 equiv), and 109 mg of EDC (0.56 mmol, 1.5 equiv). The reaction was stirred under argon at ambient temperature for 20 h. The reaction was quenched with saturated NH_4Cl(aq), extracted 3× with CH_2Cl_2, washed with $NaHCO_3$(aq), then with brine, dried over Na_2SO_4, and concentrated. The title compound (192 mg, 94%) was partially purified by silica column chromatography (40% acetone in hexanes) and taken into the subsequent reaction. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.15 (d, *J* = 8.6 Hz, 1H), 6.50 (d, *J* = 29.8 Hz, 1H), 5.47 (dd, *J* = 41.1, 5.9 Hz, 1H), 5.13 (t, *J* = 12.6 Hz, 1H), 4.42 (dd, *J* = 17.2, 9.8 Hz, 1H), 4.30−4.16 (m, 1H), 3.36−3.27 (m, 2H), 3.00−2.88 (m, 3H), 2.27 (td, *J* = 13.3, 6.6 Hz, 1H), 1.90 (d, *J* = 1.6 Hz, 4H), 1.40 (d, *J* = 7.2 Hz, 3H), 1.35 (s, 8H), 0.95−0.88 (m, 14H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 199.98, 172.91, 170.67, 170.01, 155.58, 80.13, 78.28, 64.55, 49.77, 39.05, 30.14, 30.03, 28.41, 28.24, 23.00, 19.38, 18.79, 17.84, 17.43, 16.54.

HO-D-Hic-D-Ala-L-Hiv-L-Val-SNAC (12). To a round-bottom flask was added 55 mg of **11** (0.11 mmol, 1 equiv) in a minimal amount of EtOAc and cooled to 0 °C. To this was added 1 mL of 4 M HCl in dioxane (Sigma), and the reaction allowed to warm to ambient temperature. The reaction was monitored by TLC, and at completion all solvent was removed by rotary evaporation. The unpurified intermediate was used immediately and was dissolved in 1 mL of CH_2Cl_2, and to this was added 60 μL of Hünig's base (44 mg, 0.33 mmol, 3 equiv), 17 mg of D-α-hydroxyisocaproic acid (0.12 mmol, 1.1 equiv), 19 mg of HOBt (0.16 mmol, 1.5 equiv), and 33 mg of EDC (0.16 mmol, 1.5 equiv). The reaction was stirred at ambient temperature overnight and at completion was quenched with

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saturated NH_4Cl(aq), extracted 5× with CH_2Cl_2, washed with $NaHCO_3$(aq), then with brine, dried over Na_2SO_4, and concentrated. The title compound (32 mg, 56%) was purified by silica column chromatography (50% acetone in hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.28−7.25 (m, 1H), 7.22 (d, J = 9.1 Hz, 1H), 6.12 (s, 1H), 5.11−5.04 (m, 1H), 4.65 (s, 1H), 4.55 (dd, J = 13.9, 7.0 Hz, 1H), 4.51−4.45 (m, 1H), 4.14 (d, J = 5.9 Hz, 1H), 3.49−3.35 (m, 2H), 3.35−3.20 (m, 1H), 3.14−3.04 (m, 1H), 2.84 (dt, J = 7.1, 6.5 Hz, 1H), 2.41−2.23 (m, 3H), 1.96 (s, 3H), 1.89−1.79 (m, 1H), 1.60−1.46 (m, 3H), 1.44 (t, J = 6.6 Hz, 3H), 1.03−0.86 (m, 26H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.88, 176.11, 172.67, 171.29, 169.99, 78.99, 70.45, 64.46, 48.18, 43.55, 38.72, 30.26, 29.94, 28.58, 24.50, 23.37, 23.09, 21.45, 19.48, 18.84, 18.05, 17.19, 16.74; HRMS (ESI+) exact mass calculated for $C_{23}H_{41}N_3NaO_7S$ 526.2563, found 526.2558.

Protein Expression and Purification. The CesB TE gene (UNIPROT Q20CI8 residues 2420-2681) was generated synthetically and cloned into pET21 (pGWH43). Expression conditions are modified from Alonzo et al.[15] Briefly, the CesB TE was expressed in *E. coli* BL21 Rosetta grown in LB media supplemented with 100 μg/mL ampicillin and 34 μg/mL chloramphenicol. Cells were induced at an OD_{600} = 0.45 with 0.1 mM IPTG and further incubated at 16 °C for 16 h. Cells were harvested by centrifugation and resuspended in buffer A (50 mM Tris pH 8.0, 300 mM NaCl, 10% v/v glycerol) and lysed by sonication. The lysate was centrifuged at 10 000 rpm for 45 min, and the clarified supernatant was incubated with 0.75 mL of Ni-NTA superflow resin (QIAGEN, Valenica, CA, USA) at 4 °C for 1 h. The resin was loaded into a column and first washed with buffer A supplemented with 20 mM imidazole; then CesB TE was eluted with buffer A supplemented with 150 mM imidazole. The purified protein was exchanged into dialysis buffer (25 mM HEPES, 100 mM NaCl, pH 8) and concentrated by centrifugation (Amicon 10 000 MWCO). The concentrated protein was flash frozen and stored at −78 °C. Of note, the original Alonzo et al. protocol calls for the addition of TCEP; this was omitted from our preparation to facilitate the use of a colormetric assay to monitor substrate loading. We observed more variability in activity and lower stability of the Ces TE in the absence of TCEP, indicating the enzyme may be redox sensitive. Protein concentration was determined by the Bradford assay (Biorad). Approximately 8 mg of purified protein was obtained per L of cell culture.

Enzyme Assays. Enzymatic assays were carried out in 50 mM Tris (pH 7.4) at 22 °C, with 15 μM Ces TE. Total substrate concentration was 1 mM (50 mM stock solutions in DMSO) for single substrate assays and 3 mM total substrate for mixed assays, and DMSO up to 10% solution volume was used as necessary. At completion (14 h) assays were quenched with an equal volume of 0.5% formic acid in acetonitrile before analysis by LCMS. Substrate mix and match assays were carried out as above but with 2 mM **12** and 1 mM of the appropriate non-native substrate **7** or **16**. LCMS analysis was carried out on a Shimadzu LC20A HPLC coupled with a Shimadzu LCMS-2020 using a Thermo Scientific Hypersil C_{18} column (100 × 2.1 mm). The HPLC conditions were 0.4 mL/min with a linear gradient from 5% B to 95% B over 5 min, holding at 95% B for 6 min, then returning to 5% B and re-equilibrating for 2 min. The column oven was set to 40 °C, and solvent composition was (A) H_2O + 0.05% formic acid and (B) MeCN + 0.05% formic acid.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jnatprod.0c00333.

Additional synthetic procedures, ^{1}H and ^{13}C NMR spectra, and kinetic characterization (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Agata, N.; Mori, M.; Ohta, M.; Suwan, S.; Ohtani, I.; Isobe, M. *FEMS Microbiol. Lett.* **1994**, *121*, 31.
(2) Agata, N.; Ohta, M.; Mori, M.; Isobe, M. *FEMS Microbiol. Lett.* **1995**, *129*, 17.
(3) Mikkola, R.; Saris, N. E. L.; Grigoriev, P. A.; Andersson, M. A.; Salkinoja-Salonen, M. S. *Eur. J. Biochem.* **1999**, *263*, 112.
(4) Shinagawa, K.; Konuma, H.; Sekita, H.; Sugii, S. *FEMS Microbiol. Lett.* **1995**, *130*, 87.
(5) Shinagawa, K.; Ueno, Y.; Hu, D.; Ueda, S.; Sugii, S. *J. Vet. Med. Sci.* **1996**, *58*, 1027.
(6) Schoeni, J. L.; Lee Wong, A. C. *J. Food Prot.* **2005**, *68*, 636.
(7) Ehling-Schulz, M.; Fricker, M.; Scherer, S. *Mol. Nutr. Food Res.* **2004**, *48*, 479.
(8) Agata, N.; Ohta, M.; Yokoyama, K. *Int. J. Food Microbiol.* **2002**, *73*, 23.
(9) Toh, M.; Moffitt, M. C.; Henrichsen, L.; Raftery, M.; Barrow, K.; Cox, J. M.; Marquis, C. P.; Neilan, B. A. *J. Appl. Microbiol.* **2004**, *97*, 992.
(10) Horwood, P. F.; Burgess, G. W.; Jane Oakey, H. *FEMS Microbiol. Lett.* **2004**, *236*, 319.
(11) Ehling-Schulz, M.; Vukov, N.; Schulz, A.; Shaheen, R.; Andersson, M.; Märtlbauer, E.; Scherer, S. *Appl. Environ. Microbiol.* **2005**, *71*, 105.
(12) Ehling-Schulz, M.; Fricker, M.; Grallert, H.; Rieck, P.; Wagner, M.; Scherer, S. *BMC Microbiol.* **2006**, *6*, 20.
(13) Rasko, D. A.; Rosovitz, M. J.; Økstad, O. A.; Fouts, D. E.; Jiang, L.; Cer, R. Z.; Kolstø, A. B.; Gill, S. R.; Ravel, J. *J. Bacteriol.* **2007**, *189*, 52.
(14) Magarvey, N. A.; Ehling-Schulz, M.; Walsh, C. T. *J. Am. Chem. Soc.* **2006**, *128*, 10698.
(15) Alonzo, D. A.; Magarvey, N. A.; Schmeing, T. M. *PLoS One* **2015**, *10*, e0128569.
(16) Alonzo, D. A.; Chiche-Lapierre, C.; Tarry, M. J.; Wang, J.; Schmeing, T. M. *Nat. Chem. Biol.* **2020**, *16*, 493.
(17) Jaitzig, J.; Li, J.; Süssmuth, R. D.; Neubauer, P. *ACS Synth. Biol.* **2014**, *3*, 432.
(18) Huguenin-Dezot, N.; Alonzo, D. A.; Heberlig, G. W.; Mahesh, M.; Nguyen, D. P.; Dornan, M. H.; Boddy, C. N.; Schmeing, T. M.; Chin, J. W. *Nature* **2019**, *565*, 112.
(19) Marxen, S.; Stark, T. D.; Rütschle, A.; Lücking, G.; Frenzel, E.; Scherer, S.; Ehling-Schulz, M.; Hofmann, T. *Sci. Rep.* **2015**, *5*, DOI: 10.1038/srep10637.
(20) Pitchayawasin, S.; Isobe, M.; Kuse, M.; Franz, T.; Agata, N.; Ohta, M. *Int. J. Mass Spectrom.* **2004**, *235*, 123.
(21) Stark, T.; Marxen, S.; Rütschle, A.; Lücking, G.; Scherer, S.; Ehling-Schulz, M.; Hofmann, T. *Anal. Bioanal. Chem.* **2013**, *405*, 191.

(22) Marxen, S.; Stark, T. D.; Frenzel, E.; Rütschle, A.; Lücking, G.; Pürstinger, G.; Pohl, E. E.; Scherer, S.; Ehling-Schulz, M.; Hofmann, T. *Anal. Bioanal. Chem.* **2015**, *407*, 2439.
(23) Franke, J.; Hertweck, C. *Cell Chem. Biol.* **2016**, *23*, 1179.
(24) Horsman, M. E.; Hari, T. P. A.; Boddy, C. N. *Nat. Prod. Rep.* **2016**, *33*, 183.
(25) Boddy, C. N.; Schneider, T. L.; Hotta, K.; Walsh, C. T.; Khosla, C. *J. Am. Chem. Soc.* **2003**, *125*, 3428.
(26) Trauger, J. W.; Kohli, R. M.; Mootz, H. D.; Marahiel, M. A.; Walsh, C. T. *Nature* **2000**, *407*, 215.
(27) Hoyer, K. M.; Mahlert, C.; Marahiel, M. A. *Chem. Biol.* **2007**, *14*, 13.

https://dx.doi.org/10.1021/acs.jnatprod.0c00333
J. Nat. Prod. 2020, 83, 1990−1997