Chemoenzymatic Synthesis of Cryptophycin/Arenastatin Natural Products†

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ABSTRACT: Microbially derived modular polyketide synthase and nonribosomal peptide synthetase biosynthetic pathways are a rich source of novel natural products. Development of these systems for the engineered biosynthesis of diverse secondary metabolites continues to progress as a robust source of chemical diversity. Recent efforts that employ individual enzymes and catalytic domains for the production or modification of small molecules have met with growing success. In this study, the thioesterase domain from the cryptophycin biosynthetic pathway was isolated and its function evaluated with a series of linear chain elongation intermediates in developing a novel chemoenzymatic synthesis of the cryptophycin/arenastatin class of antitumor agents. The results show the high efficiency of the thioesterase in generating the 16-membered depsipeptide ring of this important natural product system. Moreover, analysis of selected substrates revealed considerable tolerance for structural variation within the *seco*-cryptophycin unit C β-alanine residue, but strict structural requirements at the phenyl group position of the unit A δ-hydroxy octadienoate chain elongation intermediates.

Cyanobacteria are a rich source of natural products with potential clinical applications. Although hundreds of bioactive compounds have been isolated from cyanobacteria (*1*), there has been only limited exploration of the genetics and enzymology of cyanobacterial secondary metabolism (*2*−*7*). Access to the biosynthetic pathways of these natural products is likely to facilitate the discovery and development of new biologically active small molecules. Furthermore, manipulation of the corresponding biosynthetic systems at the enzyme level can also lead to the development of new non-natural derivatives. Increasingly sophisticated efforts are being made to reengineer biosynthetic pathways for in vivo production of structural analogues (*8*−*11*); however, significant work remains to optimize library size and production yields of new compounds. These challenges have motivated development of chemoenzymatic methods for creation of structural diversity, by combining thioesterase (TE)[1] domains from selected biosynthetic clusters with substrates obtained through chemical synthesis (*12*, *13*).

Polyketide synthase (PKS), nonribosomal peptide synthetase (NRPS), and fatty acid synthase (FAS) TEs utilize an Asp(Glu)/His/Ser catalytic triad, analogous to serine proteases, to form an acyl intermediate with the Ser residue followed by either hydrolysis or intramolecular cyclization (*14*−*16*). Crystal structures of the PKS TEs of the pikromycin (*17*) and deoxyerythronolide B (*18*) biosynthetic clusters have been determined as well as the NRPS TE of the surfactin (*19*) biosynthetic cluster. Currently, no cocrystal structures of NRPS or PKS TEs with full-length native substrates or inhibitors incorporated into the active site binding pocket have been reported.

Extensive substrate specificity and chain length tolerance studies have been performed on the tyrocidine NRPS TE (*12*, *13*, *20*−*22*). By substituting alanine at each amino acid position in the molecule, Walsh and colleagues (*22*) determined that the tyrocidine TE is most sensitive to the amino acid changes near the site of ring closure. In addition, these investigators determined that peptide chains of 6, 8, 10, 12, and 14 amino acids could be cyclized with the tyrocidine TE (*21*). Ready access to synthetic polypeptide chains as substrates for NRPS-derived TEs has enabled their detailed analysis. By contrast, relatively little is known about the substrate specificity of PKS, or mixed PKS/NRPS TEs for native chain elongation intermediates, apparently because of the challenges involved in synthesizing these compounds (*23*, *24*).

During the 1990s, three research groups independently identified members of the cryptophycin (Crp) natural product family, each from different organisms (*25*−*28*). The first group to identify this secondary metabolite (in 1990), from the lichen-derived cyanobacterium *Nostoc* sp. ATCC 53789, discovered that the compound was active against the filamentous fungus of the genus *Cryptococcus*, hence the

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[1] Abbreviations: PKS, polyketide synthase; NRPS, nonribosomal peptide synthetase; TE, thioesterase; FAS, fatty acid synthase; SAR, structure−activity relationship; Crp, cryptophycin; PCP, peptidyl carrier protein; NAC, *N*-acetylcysteamine; HPLC, high-performance liquid chromatography; ESI-TOF, electrospray ionization time-of-flight; ORF, open reading frame; O/N, overnight; SD, standard deviation.

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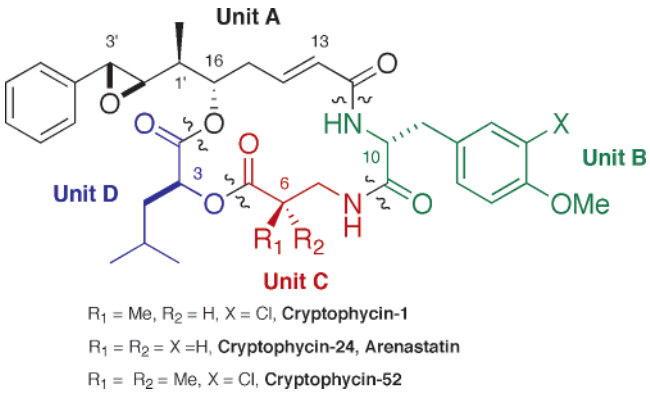


FIGURE 1: Chemical structures of cryptophycin-1, cryptophycin-24 (arenastatin), and cryptophycin-52.

name cryptophycin (Figure 1) (*26*). A related compound named arenastatin-A (cryptophycin 24, Figure 1) was later isolated from the marine sponge *Dysidea arenaria* by Kobayashi and Kitigawa (*27*). Subsequently, Moore and co-workers identified at least 29 closely related structures of the originally discovered cryptophycin in the extracts of the cyanobacterium *Nostoc* sp. GSV 224 (*25*, *28*, *29*). The major product isolated by Moore was cryptophycin-1 (Figure 1), which displayed selective tumor cytotoxicity (*30*). Furthermore, intravenous injection of cryptophycin-1 was found to be effective against mammary, colon, and pancreatic adenocarcinomas when assayed in mouse xenographs (*28*). Later, a main mode of action for this activity was found to be associated with inhibition of tubulin polymerization (*31*). The in vivo potency of cryptophycin-1 is in the picomolar range, making it 100−1000 times more potent than paclitaxel and vinblastine (*32*). This superior activity of cryptophycin-1 prompted the chemical synthesis of cryptophycin and extensive SAR studies (*33*−*36*). Recently, two new analogues, cryptophycin-309 and cryptophycin-249 (*37*), with improved stability and water solubility have shown significantly improved efficacy compared to cryptophycin-52 (LY 355073) (*38*), and are being considered as second-generation clinical candidates. However, the lengthy and low-yield synthesis of these important new derivatives has limited opportunities for clinical evaluation.

Previous total synthesis routes to the cryptophycins have employed cyclization at the C-9 or C-12 position of the molecule using standard peptide coupling methods (*39*−*44*). An alternative approach by Kobayashi utilized a Wittig−Horner cyclization at the C-12−C-13 position (*45*). Georg and co-workers pursued an alternative strategy that involved ester formation at the C-5 position (*46*). Significant challenges remain with respect to the efficient construction of cryptophycin natural products and analogues. Their continued promise as anticancer therapeutics motivated our development of native biosynthetic enzymes to enhance flexibility and expand strategy choices for their assembly and modification.

The biosynthesis of cryptophycin is particularly interesting because of its cyanobacterial origin, extensive functionalization, and use of mixed NRPS/PKS biosynthetic enzymes. Formation of cryptophycins is thought to originate with a phenyl acetate starter unit followed by addition of three acetyl groups catalyzed by PKS gene products (R. E. Moore, personal communication). The intermediate is then transferred to NRPS-type gene products for the addition of tyrosine (which is subsequently methylated on its hydroxyl group), methyl-β-alanine, and leucic acid. The full-length chain intermediate is then cyclized and released from the biosynthetic enzymes by the cryptophycin TE. During biosynthesis, tailoring enzymes catalyze chlorination of the OMe-tyrosine residue (unit B) and epoxidation of the unit A vinyl group (Figure 1). Interestingly, arenastatin lacks a chlorine atom and includes β-alanine (unit C) as opposed to the methyl-β-alanine incorporated in the most predominant species of cryptophycins (Figure 1).

Recently, we identified DNA encoding the biosynthetic cluster responsible for cryptophycin from the cyanobacterium *Nostoc* sp. ATCC 53789 (N. A. Magarvey and D. H. Sherman, unpublished observations). This was accomplished by comparative metabolomic analysis of the related cyanobiont *Nostoc punctiforme* that has an extensive array of presumed secondary metabolite gene clusters (N. A. Magarvey and D. H. Sherman, unpublished observations) determined from its genome sequence (http://genome.ornl.gov/microbial/npun/). Through comparison of the corresponding PKS/NRPS gene clusters cloned and sequence-scanned from *Nostoc* sp. ATCC 53789, we were able to identify a series of secondary metabolite biosynthetic systems unique to this organism. Analysis of the structure of cryptophycin and DNA sequenced from the localized cryptophycin gene cluster has provided the overall architecture and catalytic domain organization of the system.

In this paper, we describe efforts to explore the substrate tolerance of the cryptophycin terminal TE that mediates termination and cyclization of a mixed NRPS/PKS-derived chain elongation intermediate. This was accomplished through the cloning, expression, and use of the Crp TE domain in combination with chemically synthesized *seco*-cryptophycin and *seco*-arenastatin *N*-acetylcysteamine (NAC) thioester substrates. Specific structural analogues of the unit A δ-hydroxy octadienoate and unit C β-alanyl residue were generated in an effort to investigate TE substrate flexibility as well as its ability to catalyze hydrolysis or macrocyclization between C-2 ester and C-16 hydroxyl groups to provide an efficient chemoenzymatic synthesis of cryptophycin and arenastatin natural products.

EXPERIMENTAL PROCEDURES

Cryptophycin Thioesterase Cloning Strategy. DNA encoding the Crp TE is contained at the 3′-end of the 3′-terminal open reading frame necessary for biosynthesis of the backbone of cryptophycin. Identification of the DNA encoding the cryptophycin thioesterase was elucidated through use of the NCBI CDART program for identification of conserved domains. The nnpredict secondary structure prediction program (*47*) was used to determine the putative secondary structure of the gene product of the DNA between the putative thioesterase domain and a domain capable of being phosphopantetheinylated. The forward primer 5′-ATTTAT-CATATGGGTTCCGATTCCGGAGCCGA-3′ was designed immediately 3′ of DNA predicted to encode a protein capable of being phosphopantetheinylated in a region appearing to lack secondary structure based on the nnpredict program results and contained the NdeI endonuclease cleavage site.

The reverse primer 5′-AAATAAGGATCCTCATCATTTTTC-CAATTGATGGGT-3′ was constructed to anneal to the 3′-end of the open reading frame containing a BamHI cleavage site. DNA encoding the entire putative cryptophycin biosynthetic gene cluster was contained on the cosmid pDAM163. pDAM163 DNA was prepared using a Qiagen large construct DNA extraction kit from a 500 mL culture grown overnight at 25 °C in LB medium containing 50 μg/mL ampicillin. PCRs were performed with 0.1 μL of pDAM163 DNA from the extraction, 1 μM forward primer, 1 μM reverse primer, 1× ExTaq buffer (Takara), 1 μL of ExTaq polymerase (Takara), and 1 μM dNTP (Takara) in a final volume of 50 μL with water. The PCR program consisted of 30 cycles of the following amplification conditions: denaturation for 1 min at 95 °C, annealing for 1 min at 50 °C, and extension for 1.5 min at 72 °C. PCR fragments corresponding to the desired length were separated on a 1% agarose gel and purified from the gel using a Qiagen gel extraction kit. The PCR fragment was cloned into a pGEM T-Easy vector (Promega) using T-overhang cloning with the pGEM T-Easy kit (Promega). Clones were transformed into XL-1 Blue competent cells using heat shock protocols as described in the pGEM T-Easy kit. Constructs containing inserts were identified using blue/white screening according to the pGEM T-Easy kit protocol. Five clones containing the insert were replated, and half of the colony was subjected to PCR to verify the insert of the desired DNA size using the same PCR condition listed above, with the exception of a 5 min incubation of each clone at 96 °C prior to the amplification cycles. One clone containing the desired size DNA insert was grown in a 2 mL culture overnight in LB medium containing ampicillin (Research Products International Corp.) (50 μg/mL). DNA was purified using a Qiagen mini-prep kit. DNA sequenced at the University of Michigan DNA sequencing core lab was sequenced with 3-fold redundancy from the 5′-end using the T7 primer binding site and three times from the 3′-end using the SP6 primer binding site. DNA from the sequenced clone was ligated into the NdeI and BamHI sites in pET28b (Novagen) and transformed into BL21 electrocompetent cells using electroporation. All cells were plated on LB plates containing kanamycin (Research Products International Corp.) (50 μg/mL) and incubated O/N at 37 °C. Ten colonies were subjected to PCR verification of the desired DNA insert using the primers and protocols listed above.

Cryptophycin Thioesterase Expression and Purification. A clone containing the desired insert size, as visualized by agarose gel electrophoresis, was grown O/N in 25 mL of 2YT broth containing 50 μg/mL kanamycin at 37 °C. Five milliliters of the O/N culture were used to inoculate 1 L of 2YT medium containing 50 μg/mL kanamycin and grown at 37 °C. The culture was induced at an OD_{595} of 0.7 with 0.2 mM IPTG and grown overnight at 30 °C. Cells were harvested at 5000*g* for 30 min. The pellet was then resuspended in 20 mL of 0.1 M sodium phosphate buffer at pH 8 containing 20 mM imidazole and 300 mM NaCl; 4 mg of lysozyme and 2 g of sucrose were then added to the cell suspension and incubated at room temperature for 30 min until the solution viscosity increased. The solution was then placed on ice and subjected to sonication (5 × 20 s) (solution became less viscous). The suspension was then centrifuged at 17000*g* for 1 h at 4 °C. The supernatant was collected and incubated with 7 mL of Qiagen Ni agarose overnight at 4 °C. The agarose was then loaded into a column and washed with 10 column volumes of 0.1 M sodium phosphate buffer (pH 8) containing 20 mM imidazole and 300 mM NaCl. Next, the column was washed with 10 column volumes of wash buffer containing 50 mM imidazole. Protein was eluted with wash buffer containing 100 mM imidazole. The eluted sample contained ∼50 mg of protein as determined using a Bio-Rad Bradford assay kit. Samples were run on a 4 to 20% SDS−PAGE gel to check for purity. A band corresponding to the expected molecular weight was observed at >95% purity (Supporting Information, Figure S1). Protein was subjected to a PD-10 column prior to kinetic assays for buffer exchange to 100 mM sodium phosphate buffer at pH 8.

General Chemical Procedures. Analytical thin-layer chromatography (TLC) was performed on precoated Macherey-Nagel silica gel plates with a fluorescence indicator. Visualization was accomplished with UV light (254 nm) and by dipping into a solution of either vanillin (60 g of vanillin and 10 mL of concentrated H_2SO_4 in 1 L of 95% EtOH, stored at −20 °C) or $KMnO_4$ (3 g of $KMnO_4$, 20 g of K_2CO_3, and 0.25 g of NaOH in 300 mL of water) followed by heating. THF and ether were distilled from sodium benzophenone, and CH_2Cl_2 was distilled from CaH_2. Flash chromatography was performed with Fisher grade silica gel 60 (230−400 mesh) with the indicated solvent system. All reactions were performed under an inert atmosphere of dry argon in oven-dried (150 °C) glassware. Optical rotations were determined on an Autopol III (Rudolph Research) polarimeter using the sodium D line (λ = 589 nm) at 23 °C and are reported as follows: $[\alpha]_D$, concentration (*c* in grams per 100 mL), and solvent. ^{1}H and ^{13}C NMR spectra were recorded on either a Bruker-300 MHz or Bruker-500 MHz spectrometer. Proton chemical shifts are reported in parts per million from an internal standard of residual chloroform (7.26 ppm), and carbon chemical shifts are reported using an internal standard of residual chloroform (77.0 ppm). Proton chemical data are reported as follows: chemical shift, multiplicity (ovlp = overlapping, s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet, and br = broad), coupling constant, and integration. Mass spectra were obtained at the University of Michigan Mass Spectrometry Laboratory on a Waters Ultima magnetic sector mass spectrometer equipped with an electrospray interface. Chemical synthesis and characterization of each molecule are given in the Supporting Information.

*Kinetic Characterization of Cryptophycin Thioesterase Activity with Substrate **24***. A standard curve of the cleaved product was determined on a 10 to 67% acetonitrile/water (0.1% TFA) gradient over 30 min. Cleavage reactions were run for 15 min at 30 °C with 1.4 μM cryptophycin thioesterase with 0.312, 0.625, 1.25, 2.5, and 5 mM substrate containing 4% DMSO in 0.1 M NaH_2PO_4 buffer at pH 7, 8, and 8.75. Reaction product **25** was monitored at 245 nm in an effort to determine the rate of hydrolysis for the reactions. A standard curve at 245 nm was calculated for **25** in an effort to determine the kinetics of the reaction. All reactions were run in triplicate. Crp TE was incubated at 30 °C for 1 h followed by addition of substrate **24** and run in parallel with a reaction that included non-preincubated Crp TE to ensure

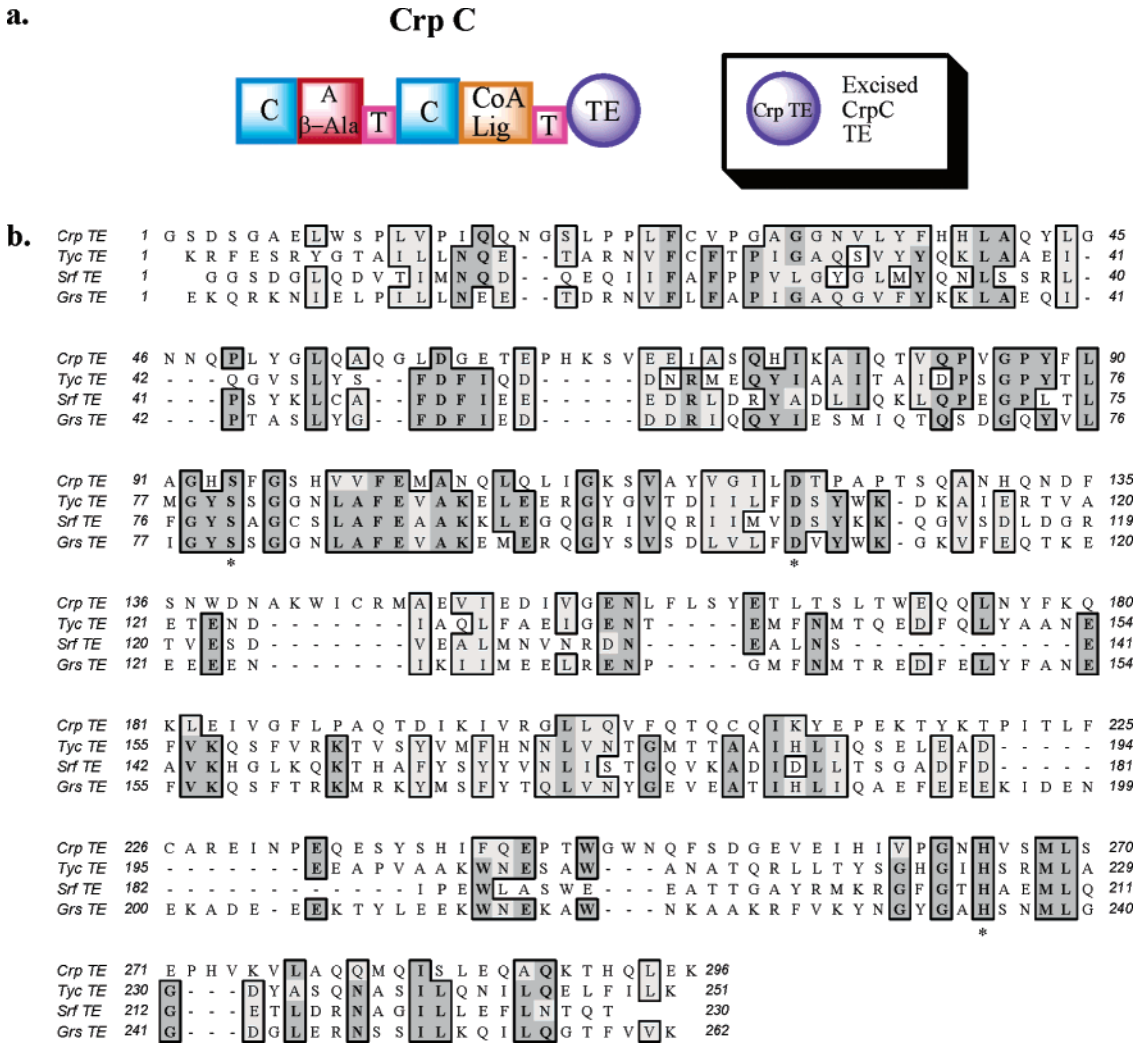


FIGURE 2: Comparative analysis of the cryptophycin thioesterase with NRPS thioesterases. (a) Representation of the CrpC polypeptide and the excised Crp TE. C represents the condensation domain, A the adenylation domain, T the peptidyl carrier protein, CoA Lig the CoA ligase, and TE the thioesterase. (b) Primary structure alignment of the thioesterase domains of the cryptophycin, tyrocidine, surfactin, and gramicidin biosynthetic clusters. The positions of the catalytic triad are indicated with asterisks. Dark gray regions are identical amino acids; light gray regions are similar amino acids based on hydrophobicity.

that the Crp TE was active for the entire time course of the reactions.

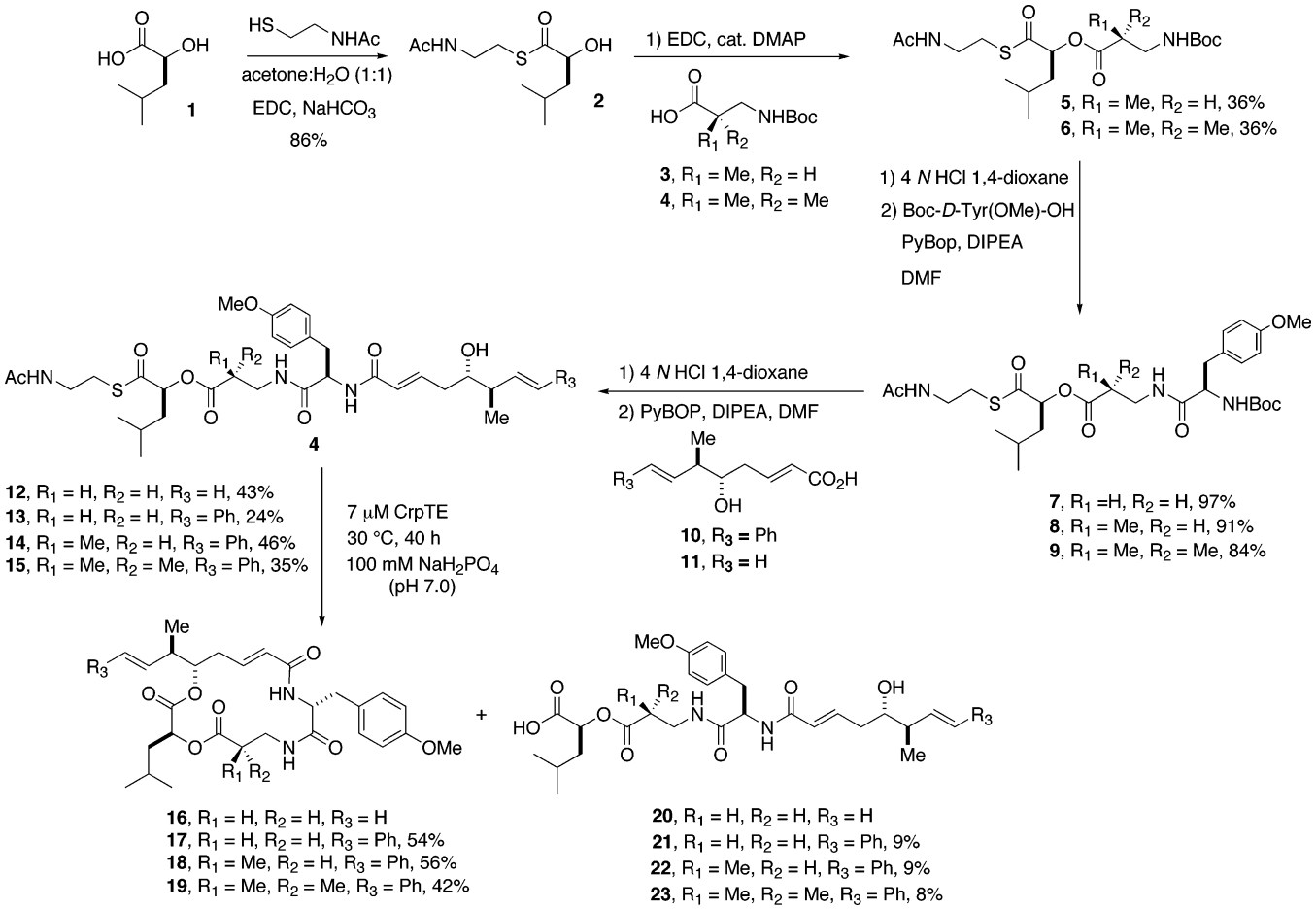
*Crp TE-Catalyzed Cyclizations of Substrates **12**−**15***. A 500 μL solution containing 25, 50, and 75 μM substrate **12** or substrate **14**, 12.5, 25, and 37.5 μM substrate **15**, and 6.25, 12.5, 25, 50, and 75 μM substrate **13** with 1 μM cryptophycin thioesterase, 0.095 M NaH_2PO_4 buffer (pH 7), and 5% DMSO was incubated at 30 °C; the reactions in 125 μL aliquots from each reaction mixture were terminated by addition of 125 μL of 10% TFA in water. The absorbance of the products of each reaction was measured at 245 and 260 nm. A standard curve for the cyclized product of **14** was determined at 245 and 260 nm. The absorbances of the substrates and products were determined to be approximately equal at 245 and 260 nm. The kinetics for cyclization of each substrate were determined at 245 or 260 nm. Each time course was repeated at least twice (Supporting Information, Figure S2).

*Large-Scale Cyclizations **12**−**15***. A 50 mL reaction mixture containing 100 μM **12**, **13**, **14**, or **15**, 0.1 M NaH_2PO_4 (pH 7.0), 5% DMSO, and 7 μM Crp TE was incubated overnight at 30 °C. A negative control for each reaction containing all reagents except for the Crp TE was run in parallel. The total contents of each reaction mixture were separated using reverse phase HPLC with a 10 to 100% gradient (acetonitrile and 0.1% TFA/water and 0.1% TFA) over the course of 37 min on an Alltech Econosil 10 μm C18 column with dimensions of 250 mm × 4.6 mm. The products were analyzed by ESI-TOF mass spectrometry in the positive ion mode. The relative concentration of products and substrates was determined by comparing absorption at 245 nm.

RESULTS

Crp TE Subcloning, Expression, and Purification. DNA harboring the Crp TE is contained within the larger framework of the 3′-terminal ORF necessary for biosynthesis of the backbone of cryptophycin (Figure 2). Therefore, truncation of the DNA in the final ORF was necessary to clone, overexpress, and purify Crp TE as an independent catalytic domain. Initially, the TE domain was identified using CDART (*48*). Immediately 5′ of the gene encoding Crp TE is a region that encodes a putative peptidyl carrier protein (PCP) domain. The expression plasmid was constructed to comprise the TE ORF only, beginning immediately 3′ of the DNA predicted to encode the PCP domain.

Scheme 1: Chemoenzymatic Synthesis of Cryptophycins/Arenastatins



Amplicons from PCRs were purified and inserted into pGEM T-Easy vectors using T-overhang cloning techniques. Resulting clones were screened using a blue/white screening strategy and subjected to DNA sequencing to identify the desired Crp TE product. The fragment was subcloned into a pET28b vector which incorporates an N-terminal six-His tag onto the corresponding gene product.

Recombinant Crp TE was purified on Ni agarose to >95% purity (Supporting Information, Figure S1). The molecular mass of the polypeptide was determined to be 35,424 Da by ESI mass spectrometry and 35,410 Da by MALDI-TOF mass spectrometry (Supporting Information, Figure S3). The calculated average mass for the Crp TE is 35,550 Da, and the monoisoptopic mass is 35,527 Da. The determined molecular mass corresponds to Crp TE that is missing its N-terminal methionine (calculated monoisotopic mass of 35,417 Da). The protein was further characterized by trypsin digestion followed by HPLC−MS analysis that confirmed the loss of the N-terminal methionine (data not shown).

Removal of the His tag with thrombin yielded a construct with an activity equivalent to that of the His tag-containing Crp TE (data not shown). Therefore, all subsequent studies were conducted using the His-containing Crp TE. In addition, the protein could be lyophilized and reconstituted in reaction buffer to give approximately the same activity as nonlyophilized Crp TE.

Primary sequence alignment of the Crp TE with the corresponding TE catalytic domains from the surfactin (*21*), gramicidin (*49*, *50*), and tyrocidine (*51*) gene clusters revealed Ser94, His265, and Asp121 of Crp TE as the amino acid residues comprising the presumed catalytic triad (Figure 2).

Chemical Synthesis of Substrates. Substrates were designed and synthesized to probe the ability of the Crp TE to cyclize unit A and unit C structural variants. Leucic acid (**1**) was converted to the *N*-acetylcysteamine (NAC) thioester (**2**) using EDCI (catalytic DMAP, CH_2Cl_2, 86%). The NAC thioester serves a dual role as both a C-terminal acid-stable protecting group and an activating group with respect to enzymatic macrocyclization. Esterification of **2** with Boc-*β*-alanine (**3**) employing EDCI (catalytic DMAP, CH_2Cl_2, 36%) afforded **5** in moderate yield. Removal of the N-terminal Boc (HCl, 1,4-dioxane, 25 °C, 1 h) and coupling of the crude amine hydrochloride salt with Boc-D-tyrosine methyl ether employing PyBOP as an activating agent (DIPEA, DMF, 25 °C, 1 h, 91%) afforded tridepsipeptide **8**. The Boc group was cleaved (HCl, 1,4-dioxane) followed by coupling to unit A (**10**) activated by PyBOP (DIPEA, DMF, 25 °C, 1 h, 46%) to afford the full-length *seco*-cryptophycin NAC thioester substrate (**14**) after HPLC purification. The full-length substrates **13** and **15** were prepared analogously (see Scheme 1). Substrate **12** was prepared from tridepsipeptide intermediate **7** by coupling with des-phenyl unit A analogue **11** (PyBOP, DIPEA, DMF, 1 h, 43%).

Cyclization of Unit A Derivatives. Previous studies of the tyrocidine TE have reported sensitivity of the enzyme to modification of substrate substituents in the proximity of the

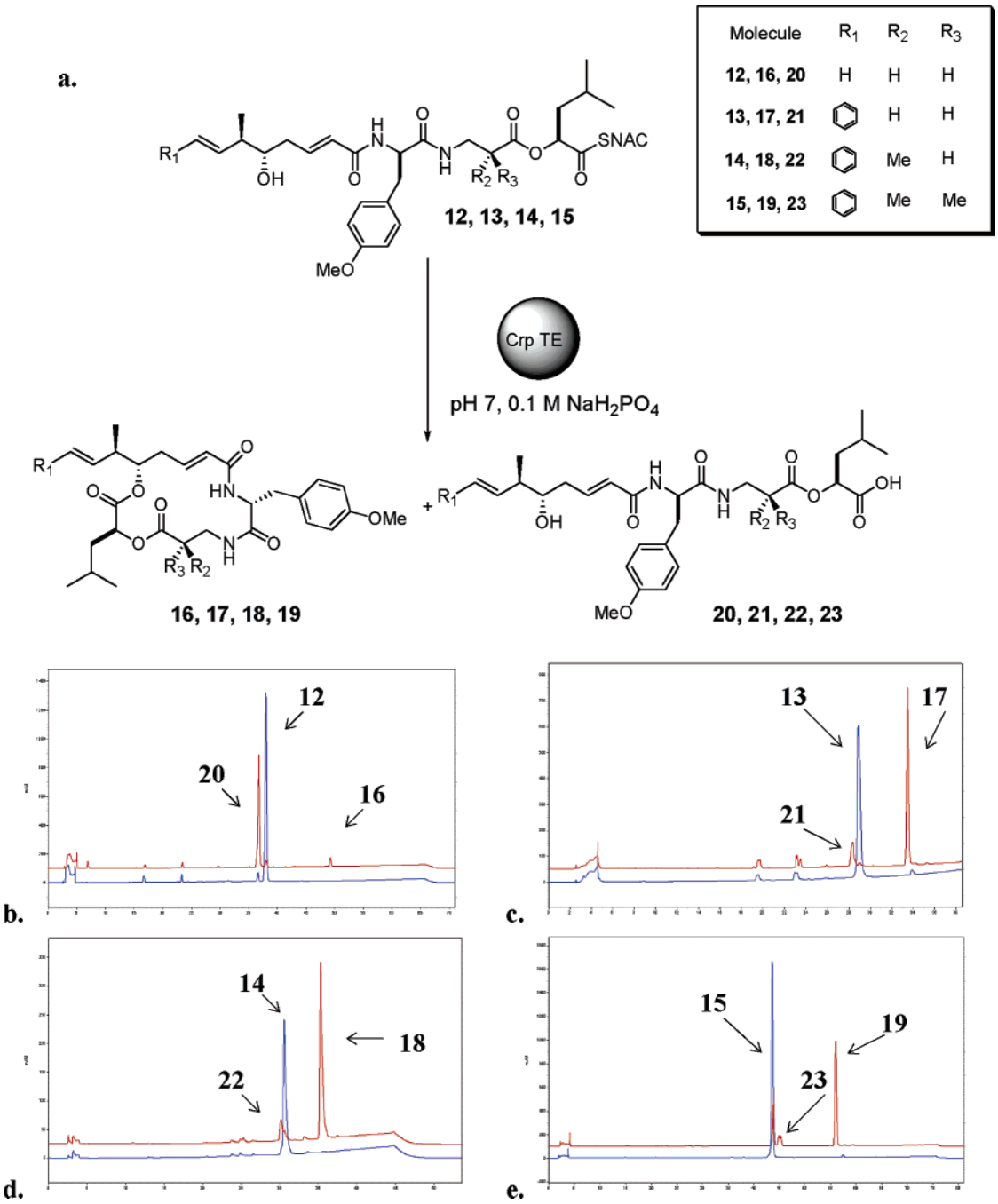


FIGURE 3: (a) Catalytic scheme for cyclization and hydrolysis reactions. (b−e) HPLC traces of cryptophycin thioesterase (TE)-catalyzed cyclization and hydrolysis of **12**−**15**, respectively. HPLC traces of reactions without Crp TE (red traces) and the products of cryptophycin thioesterase-catalyzed reactions (blue traces).

site of ring closure (*22*). Thus, we first attempted to test the flexibility of the Crp TE to cyclize substrates containing modifications to unit A of cryptophycin. Substrate **12** was designed to be identical to substrate **13**, except for the absence of a terminal phenyl group. Upon incubation of **13** with Crp TE, two new products were detected and analyzed by ESI-TOF mass spectrometry and found to correspond to **17** and **21** with a cyclization:hydrolysis ratio of 5:1 as calculated for small-scale reactions (Figure 4). The K_M for cyclization of **13** in the presence of Crp TE was 14 ± 3 μM, which was well below the maximal substrate concentration that was used (75 μM), while the k_{cat} for the reaction was determined to be 0.015 ± 0.001 s^{-1}, resulting in a k_{cat}/K_M value of 1.09 mM^{-1} s^{-1} (Table 1). The cyclization:hydrolysis ratio for the Crp TE-catalyzed cyclization of substrate **12** was determined to be 1:8.3 for **16** and **20** (more than 40 times less efficient than for Crp TE-catalyzed cyclization of substrate **13**). Calculation of the K_M for substrate **12** was precluded by the limited solubility of the substrate. The k_{cat}/K_M value for hydrolysis of substrate **12** was determined to be 0.296 ± 0.003 mM^{-1} s^{-1}. The k_{cat}/K_M value for cyclization was deduced on the basis of the k_{cat}/K_M for hydrolysis and the cyclization:hydrolysis ratio to be 0.0348 mM^{-1} s^{-1}.

Cyclization of Unit C Derivatives. The variation of methylation at the C-6 positions of arenastatin and cryptophycin (Figure 1) prompted us to test the ability of the Crp TE to cyclize NAC thioester substates containing no methyl

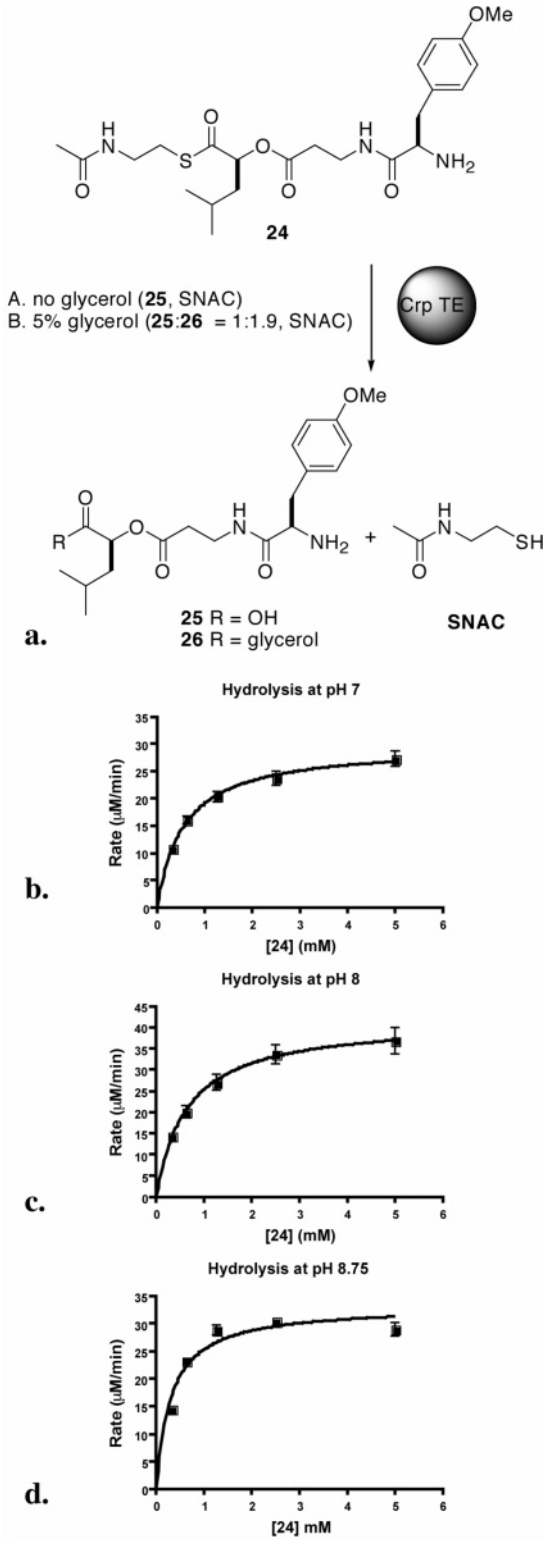


FIGURE 4: (a) Cryptophycin TE-catalyzed conversion of substrate **24** to products **25**, **26**, and SNAC in 0.1 M NaH_2PO_4 buffer (pH 8) containing 4% DMSO. (b−d) Rate vs [**24**] for the cryptophycin thioesterase-catalyzed hydrolysis of substrate **24** (5, 2.5, 1.25, 0.625, and 0.312 mM) at pH 7, 8, and 8.75, respectively. Reactions were conducted in triplicate, and standard errors are indicated by error bars on the graphs.

group (**13**), an R-methyl group (**14**), and a gem-dimethyl group (**15**), at the C-6 position. Cryptophycin-1 is the major natural product isolated from *Nostoc* sp. ATCC 53789, from which the cryptophycin biosynthetic cluster has been cloned (N. A. Magarvey and D. H. Sherman, unpublished observations), and resembles substrate **14** with regard to C-6 methylation. Upon incubation with Crp TE, substrate **14** was cyclized to produce **18** and hydrolyzed to provide **22** (Figure 3). The limited solubility of **14** precluded measurement of k_{cat} and K_M values for the cyclization reaction with Crp TE. Therefore, the k_{cat}/K_M was measured on the basis of the slope of the linear region of the rate versus [**14**] plot as 0.41 ± 0.01 mM^{-1} s^{-1}. The cyclization:hydrolysis ratio for the reaction was determined to be 10:1 for small-scale reactions. Because the cyclization reaction was assessed well below the K_M in the linear region of the rate versus [**14**] plot, the k_{cat}/K_M for hydrolysis was calculated on the basis of the cyclization:hydrolysis ratio to be 0.041 mM^{-1} s^{-1}.

Incorporation of a second methyl into the C-6 position of **14** yielded substrate **15**, which contains a gem-dimethyl, analogous to cryptophycin-52 (*52*). HPLC analysis followed by ESI-TOF mass spectrometry revealed two new peaks upon incubation of **15** with Crp TE corresponding to **19** and **23** (Figure 3). The cyclization:hydrolysis ratio was 6:1 for substrate **15**. Once again, the limited solubility of substrate **15** precluded measurement of individual k_{cat} and K_M values. The k_{cat}/K_M value for the cyclization of **15** was 0.15 ± 0.01 mM^{-1} s^{-1} and for hydrolysis 0.025 mM^{-1} s^{-1}.

To test the ability of these substrates to be cyclized by an alternate TE, substrates **13**−**15** were incubated in the presence of surfactin TE. The exclusive products of these reactions were the hydrolyzed forms of each thioester which yield the corresponding *seco*-acids **21**−**23**, respectively.

To verify the structures of the products from each cyclization reaction, large-scale reactions were conducted and the cyclized products were isolated and characterized by ^1H NMR (Supporting Information, Figure S4).

Hydrolysis of NAC-Depsipeptide (Units B−*D).* Both the linear and cyclized forms of the cryptophycins are fairly insoluble in water, and therefore, kinetic characterization of hydrolytic rate constants of the Crp TE was conducted using a substrate modeled after the depsipeptide fragment corresponding to the B−D units of cryptophycin-24 (Figure 4). Characterization of the Crp TE-catalyzed hydrolysis of substrate **24** was monitored by HPLC. The product of the reaction was determined using ES^+ mass spectrometry to be **25** (ES^+ = 380.6, calculated mass = 380.2).

Initially, the Crp TE was stored in buffer containing 5% glycerol. However, analysis by HPLC−MS of hydrolysis of substrate **24** with Crp TE in this buffer revealed that the glycerol adduct **26** was the major product of the reaction (ES^+ = 454.4, calculated mass = 454.2). Subsequent analysis of the Crp TE-catalyzed hydrolysis of substrate **24** in the absence of glycerol yielded **25** exclusively. The generation of a glycerol adduct has been observed previously with thioesterases (*21*), which is an important consideration when determining kinetics using buffers containing this solvent (especially when using indirect methods).

The hydrolytic activity of Crp TE was determined for substrate **24** using steady-state kinetic analysis utilizing HPLC analytical methods (Figure 4). Table 2 summarizes the catalytic rate constants for hydrolysis of substrate **24** with Crp TE at pH 7, 8, and 8.75.

These studies demonstrate that the Crp TE is efficiently expressed and robust in its stability and cyclization activity for *seco*-NAC-cryptophycins and *seco*-NAC-arenastatins. Initial specificity studies show that Crp TE is sensitive to

Table 1: Steady-State Kinetic Parameters for Crp TE-Catalyzed Cyclization and Hydrolysis

substrate	R_1	R_2	R_3	cyclization:hydrolysis	$k_{cat} \pm$ SD (s^{-1}) for cyclization	$K_M \pm$ SD (μM) for cyclization	$k_{cat} \pm$ SD (s^{-1}) for hydrolysis	$K_M \pm$ SD (μM) for hydrolysis	k_{cat}/K_M ($mM^{-1}\ s^{-1}$) for cyclization	k_{cat}/K_M ($mM^{-1}\ s^{-1}$) for hydrolysis
12	H	H	H	1:8.3	–	>75	–	>75	0.0348[a]	0.296 ± 0.003
13	Ph	H	H	5:1	0.015 ± 0.001	14 ± 3	0.0045 ± 0.0008	25 ± 11	1.09 ± 0.31	0.2 ± 0.1
14	Ph	Me	H	10:1	–	>75	–	>75	0.41 ± 0.01	0.041[a]
15	Ph	Me	Me	6:1	–	>37.5	–	>37.5	0.15 ± 0.01	0.025[a]

[a] The calculation of this value was based on the experimentally determined cyclization:hydrolysis ratio and value of the experimentally determined cyclization or hydrolysis for each molecule.

Table 2: Steady-State Kinetic Parameters for Cryptophycin TE-Catalyzed Hydrolysis of Molecule **24**

pH	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M ($mM^{-1}\ s^{-1}$)
7	0.35 ± 0.07	550 ± 70	0.64
8	0.6 ± 0.1	620 ± 30	0.97
8.75	0.39 ± 0.02	310 ± 50	1.26

deletion of the phenyl ring of unit A, but tolerant to methyl group variations in unit C. Furthermore, we have developed a unique and efficient method for the chemoenzymatic synthesis of cryptophycins/arenastatins.

DISCUSSION

TEs catalyze the release of natural product intermediates from many polyketide synthase, fatty acid synthase, and nonribosomal peptide synthetase biosynthetic systems (*14*−*16*). In the case of the cryptophycin mixed PKS/NRPS biosynthetic cluster, the TE domain also catalyzes the cyclization to the cryptophycins. The purpose of this study was to characterize the Crp TE domain from *Nostoc* sp. ATCC 53789 through semisynthesis of a series of *seco*-cryptophycin chain elongation intermediates. This paper describes the subcloning, expression, purification, chemical synthesis of full-length chain elongation intermediates, cyclization, and hydrolysis activity of the Crp TE.

To address the specificity of the Crp TE for unit A variants, derivatives of *seco*-cryptophycin-24 were designed, synthesized, and cyclized. Molecules **12** and **13** differ in the absence and presence of a terminal phenyl group in unit A, respectively. Crp TE catalyzed the cyclization of molecule **13** with a partition ratio of 5:1 (cyclized:hydrolyzed), whereas the partition ratio for cyclization of molecule **12** was 1:8.3 (cyclized:hydrolyzed). The greater than 40-fold difference indicates that the phenyl ring of the cryptophycins is essential for efficient cyclization. The k_{cat}/K_M for hydrolysis of **13** was much lower than the k_{cat}/K_M for hydrolysis of **12**. The rate of hydolysis for compound **12** was approximately the same as the rate of cyclization for compound **13**. In addition, the rate of hydrolysis of compound **13** was much lower than the rate of hydrolysis for compound **12**. It is possible that the phenyl group of **13** acts to exclude water that could potentially compete with the C-16 hydroxyl group as a nucleophile.

Arenastatin and cryptophycin-1 are presumably produced by very similar metabolic systems. To explore a potential divergence in the TE specificity for C-6 methylation within unit C, we tested the cyclization of β-alanine structural variants. Interestingly, the K_M for the methylated substrate **14** was at least 5 times greater than that of the β-alanine substrate **13**, resulting in an overall increased efficiency of cyclization for **13**. In enzyme systems that use diffusive loading, this may indicate that the substrate with the lower K_M and higher efficiency is the natural substrate. However, the significance of the lower K_M for substrate **13** in the context of the natural biosynthetic system is not readily apparent because instead of diffusive loading, the substrate is channeled from one enzyme to the next without breaking a covalent tether to the biosynthetic enzymes. Perhaps a better indicator of the natural substrate for NRPS and PKS systems is the ratio of cyclized to hydrolyzed product. In that case, molecule **14** as a substrate for Crp TE gave a cyclization:hydrolysis ratio of 10:1, while the ratio for **13** was 5:1.

Further modification of the unit C, C-6 methylene was performed to examine the utility of Crp TE for cyclization of substrates containing a C-6 gem-dimethyl, a functional group found in the clinical drug candidate cryptophycin-52 (*52*). The ability of the Crp TE to cyclize molecule **15** with a cyclization:hydrolysis ratio of 6:1 suggests that this route to cyclization is indeed viable despite the presence of two bulky methyl groups.

The limited solubility of the full-length cryptophycins made determination of the k_{cat} and K_M values for cyclization of these molecules difficult. Therefore, the hydrolytic activity of the cryptophycin thioesterase was explored with a NAC thioester depsipeptide modeled after the B−D units of the cryptophycins (**24**). The most efficient activity (k_{cat}/K_M = 1.26 $mM^{-1}\ s^{-1}$) for hydrolysis of substrate **24** was achieved at pH 8.75. However, over the pH range of 7−8.75, the catalytic efficiency varied less than 2-fold. The increased specificity constant at pH 8.75 was due to a decrease in k_{cat} from pH 8 to 8.75, and a decrease in K_M from pH 8 to 8.75. Interestingly, the k_{cat} of hydrolysis was far greater for these substrates than the k_{cat} for cyclization of molecules **14**−**16**. However, the K_M value for the depsipeptide was much greater than that for the proposed natural substrate **13**, resulting in a similar k_{cat}/K_M.

To evaluate the efficiency of the Crp TE relative to other lactone- and lactam-forming TEs, we compared the reported values for cyclization of the macrolactone-forming epothilone (*24*) and surfactin (*53*) terminal transferases and the lactam-forming tyrocidine (*21*) and gramicidin (*21*) TEs. Compared to the macrolactone-forming TEs, the Crp TE was >60 times more efficient at cyclization. The excised macrolactam-forming TEs are significantly more efficient at cyclization mainly because of their relatively low K_M values for their substrates (*21*). The two lactam-forming domains were greater than 30-fold more efficient at cyclization than the Crp TE. As noted previously, the meaning of K_M's, and therefore the k_{cat}/K_M's, for the excised domains is unclear in the context of the natural systems because the substrate is always covalently attached to an upstream domain prior to covalent attachment to the thioesterase in the biosynthetic systems.

Significantly, use of the Crp TE for the chemoenzymatic synthesis of cryptophycin/arenastatin natural products highlights a new route to depsipeptide cyclization and, hence, the synthesis of this important class of antitumor agents. These studies will aid in future work that is conducted on the efficient assembly of cryptophycins for development of anticancer drugs and will likely provide access to new structures by combining the tools of synthetic chemistry and natural product metabolic enzymes.

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SUPPORTING INFORMATION AVAILABLE

An SDS−PAGE gel of purified cryptophycin thioesterase, kinetic characterization of cyclization and hydrolysis for molecules **12**−**15**, mass spectrometry of cryptophycin thioesterase, ^{1}H NMR of molecules **17**−**19**, and details of the chemical synthesis and characterization for each molecule. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Burja, A. M., Banaigs, B., Abou-Mansour, E., Burgess, J. G., and Wright, P. C. (2001) Marine cyanobacteria: A prolific source of natural products, *Tetrahedron 57*, 9347−77.
2. Chang, Z., Flatt, P., Gerwick, W. H., Nguyen, V. A., Willis, C. L., and Sherman, D. H. (2002) The barbamide biosynthetic gene cluster: A novel marine cyanobacterial system of mixed polyketide synthase (PKS)-non-ribosomal peptide synthetase (NRPS) origin involving an unusual trichloroleucyl starter unit, *Gene 296*, 235−47.
3. Chang, Z., Sitachitta, N., Rossi, J. V., Roberts, M. A., Flatt, P. M., Jia, J., Sherman, D. H., and Gerwick, W. H. (2004) Biosynthetic pathway and gene cluster analysis of curacin A, an antitubulin natural product from the tropical marine cyanobacterium *Lyngbya majuscula*, *J. Nat. Prod. 67*, 1356−67.
4. Edwards, D. J., and Gerwick, W. H. (2004) Lyngbyatoxin biosynthesis: Sequence of biosynthetic gene cluster and identification of a novel aromatic prenyltransferase, *J. Am. Chem. Soc. 126*, 11432−3.
5. Edwards, D. J., Marquez, B. L., Nogle, L. M., McPhail, K., Goeger, D. E., Roberts, M. A., and Gerwick, W. H. (2004) Structure and biosynthesis of the jamaicamides, new mixed polyketide-peptide neurotoxins from the marine cyanobacterium *Lyngbya majuscula*, *Chem. Biol. 11*, 817−33.
6. Moffitt, M. C., and Neilan, B. A. (2004) Characterization of the nodularin synthetase gene cluster and proposed theory of the evolution of cyanobacterial hepatotoxins, *Appl. Environ. Microbiol. 70*, 6353−62.
7. Rouhiainen, L., Vakkilainen, T., Siemer, B. L., Buikema, W., Haselkorn, R., and Sivonen, K. (2004) Genes coding for hepatotoxic heptapeptides (microcystins) in the cyanobacterium *Anabaena* strain 90, *Appl. Environ. Microbiol. 70*, 686−92.
8. Chen, S., Xue, Y., Sherman, D. H., and Reynolds, K. A. (2000) Mechanisms of molecular recognition in the pikromycin polyketide synthase, *Chem. Biol. 7*, 907−18.
9. Reeves, C. D., Murli, S., Ashley, G. W., Piagentini, M., Hutchinson, C. R., and McDaniel, R. (2001) Alteration of the substrate specificity of a modular polyketide synthase acyltransferase domain through site-specific mutations, *Biochemistry 40*, 15464−70.
10. Ruan, X., Pereda, A., Stassi, D. L., Zeidner, D., Summers, R. G., Jackson, M., Shivakumar, A., Kakavas, S., Staver, M. J., Donadio, S., and Katz, L. (1997) Acyltransferase domain substitutions in erythromycin polyketide synthase yield novel erythromycin derivatives, *J. Bacteriol. 179*, 6416−25.
11. Yoon, Y. J., Beck, B. J., Kim, B. S., Kang, H. Y., Reynolds, K. A., and Sherman, D. H. (2002) Generation of multiple bioactive macrolides by hybrid modular polyketide synthases in *Streptomyces venezuelae*, *Chem. Biol. 9*, 203−14.
12. Kohli, R. M., Walsh, C. T., and Burkart, M. D. (2002) Biomimetic synthesis and optimization of cyclic peptide antibiotics, *Nature 418*, 658−61.
13. Xie, G. Y., Uttamchandani, M., Chen, G. Y. J., Bu, X. Z., Lin, S. S., Wong, K. M., Yan, W. L., Yao, S. Q., and Guo, Z. H. (2002) Substrate spectrum of tyrocidine thioesterase probed with randomized peptide *N*-acetylcysteamine thioesters, *Bioorg. Med. Chem. Lett. 12*, 989−92.
14. Aggarwal, R., Caffrey, P., Leadlay, P. F., Smith, C. J., and Staunton, J. (1995) The thioesterase of the erythromycin-producing polyketide synthase: Mechanistic studies in-vitro to investigate its mode of action and substrate-specificity, *J. Chem. Soc., Chem. Commun.*, 1519−20.
15. Lawson, D. M., Derewenda, U., Serre, L., Ferri, S., Szittner, R., Wei, Y., Meighen, E. A., and Derewenda, Z. S. (1994) Structure of a myristoyl-ACP-specific thioesterase from *Vibrio harveyi*, *Biochemistry 33*, 9382−8.
16. Shaw-Reid, C. A., Kelleher, N. L., Losey, H. C., Gehring, A. M., Berg, C., and Walsh, C. T. (1999) Assembly line enzymology by multimodular nonribosomal peptide synthetases: The thioesterase domain of *E. coli* EntF catalyzes both elongation and cyclolactonization, *Chem. Biol. 6*, 385−400.
17. Tsai, S. C., Lu, H., Cane, D. E., Khosla, C., and Stroud, R. M. (2002) Insights into channel architecture and substrate specificity from crystal structures of two macrocycle-forming thioesterases of modular polyketide synthases, *Biochemistry 41*, 12598−606.
18. Tsai, S. C., Miercke, L. J., Krucinski, J., Gokhale, R., Chen, J. C., Foster, P. G., Cane, D. E., Khosla, C., and Stroud, R. M. (2001) Crystal structure of the macrocycle-forming thioesterase domain of the erythromycin polyketide synthase: Versatility from a unique substrate channel, *Proc. Natl. Acad. Sci. U.S.A. 98*, 14808−13.
19. Bruner, S. D., Weber, T., Kohli, R. M., Schwarzer, D., Marahiel, M. A., Walsh, C. T., and Stubbs, M. T. (2002) Structural basis for the cyclization of the lipopeptide antibiotic surfactin by the thioesterase domain SrfTE, *Structure 10*, 301−10.
20. Kohli, R. M., Burke, M. D., Tao, J., and Walsh, C. T. (2003) Chemoenzymatic route to macrocyclic hybrid peptide/polyketide-like molecules, *J. Am. Chem. Soc. 125*, 7160−1.
21. Kohli, R. M., Trauger, J. W., Schwarzer, D., Marahiel, M. A., and Walsh, C. T. (2001) Generality of peptide cyclization catalyzed by isolated thioesterase domains of nonribosomal peptide synthetases, *Biochemistry 40*, 7099−108.
22. Trauger, J. W., Kohli, R. M., Mootz, H. D., Marahiel, M. A., and Walsh, C. T. (2000) Peptide cyclization catalysed by the thioesterase domain of tyrocidine synthetase, *Nature 407*, 215−8.
23. Aldrich, C. C., Venkatraman, L., Sherman, D. H., and Fecik, R. A. (2005) Chemoenzymatic synthesis of the polyketide macrolactone 10-deoxymethynolide, *J. Am. Chem. Soc. 127*, 8910−1.
24. Boddy, C. N., Schneider, T. L., Hotta, K., Walsh, C. T., and Khosla, C. (2003) Epothilone C macrolactonization and hydrolysis are catalyzed by the isolated thioesterase domain of epothilone polyketide synthase, *J. Am. Chem. Soc. 125*, 3428−9.
25. Subbaraju, G. V., Golakoti, T., Patterson, G. M., and Moore, R. E. (1997) Three new cryptophycins from *Nostoc* sp. GSV 224, *J. Nat. Prod. 60*, 302−5.
26. Schwartz, R. E., Hirsch, C. F., Sesin, D. F., Flor, J. E., Chartrain, M., Fromtling, R. E., Harris, G. H., Salvatore, M. J., Liesch, J. M., and Yudin, K. (1990) Pharmaceuticals from cultured algae, *J. Ind. Microbiol. 5*, 113−23.
27. Kobayashi, M., Aoki, S. J., Ohyabu, N., Kurosu, M., Wang, W. Q., and Kitagawa, I. (1994) Arenastatin-A, a potent cytotoxic depsipeptide from the Okinawan marine sponge *Dysidea arenaria*, *Tetrahedron Lett. 35*, 7969−72.
28. Golakoti, T., Ogino, J., Heltzel, C. E., Lehusebo, T., Jensen, C. M., Larsen, L. K., Patterson, G. M. L., Moore, R. E., Mooberry, S. L., Corbett, T. H., and Valeriote, F. A. (1995) Structure determination, conformational-analysis, chemical-stability studies, and antitumor evaluation of the cryptophycins: Isolation of 18 new analogs from *Nostoc* sp. strain GSV-224, *J. Am. Chem. Soc. 117*, 12030−49.
29. Chaganty, S., Golakoti, T., Heltzel, C., Moore, R. E., and Yoshida, W. Y. (2004) Isolation and structure determination of cryptophycins 38, 326, and 327 from the terrestrial cyanobacterium *Nostoc* sp. GSV 224, *J. Nat. Prod. 67*, 1403−6.

30. Corbett, T. H., Valeriote, F. A., Demchik, L., Lowichik, N., Polin, L., Panchapor, C., Pugh, S., White, K., Kushner, J., Rake, J., Wentland, M., Golakoti, T., Hetzel, C., Ogino, J., Patterson, G., and Moore, R. (1997) Discovery of cryptophycin-1 and BCN-183577: Examples of strategies and problems in the detection of antitumor activity in mice, *Invest. New Drugs 15*, 207−18.
31. Kerksiek, K., Mejillano, M. R., Schwartz, R. E., Georg, G. I., and Himes, R. H. (1995) Interaction of cryptophycin 1 with tubulin and microtubules, *FEBS Lett. 377*, 59−61.
32. Panda, D., Himes, R. H., Moore, R. E., Wilson, L., and Jordan, M. A. (1997) Mechanism of action of the unusually potent microtubule inhibitor cryptophycin 1, *Biochemistry 36*, 12948−53.
33. Al-Awar, R. S., Ray, J. E., Schultz, R. M., Andis, S. L., Kennedy, J. H., Moore, R. E., Liang, J., Golakoti, T., Subbaraju, G. V., and Corbett, T. H. (2003) A convergent approach to cryptophycin 52 analogues: Synthesis and biological evaluation of a novel series of fragment A epoxides and chlorohydrins, *J. Med. Chem. 46*, 2985−3007.
34. Barrow, R. A., Hemscheidt, T., Liang, J., Paik, S., Moore, R. E., and Tius, M. A. (1995) Total synthesis of cryptophycins: Revision of the structures of cryptophycin-A and cryptophycin-C, *J. Am. Chem. Soc. 117*, 2479−90.
35. Eggen, M., and Georg, G. I. (2002) The cryptophycins: Their synthesis and anticancer activity, *Med. Res. Rev. 22*, 85−101.
36. Tius, M. A. (2002) Synthesis of the cryptophycins, *Tetrahedron 58*, 4343.
37. Liang, J., Moore, R. E., Moher, E. D., Munroe, J. E., Al-awar, R. S., Hay, D. A., Varie, D. L., Zhang, T. Y., Aikins, J. A., Martinelli, M. J., Shih, C., Ray, J. E., Gibson, L. L., Vasudevan, V., Polin, L., White, K., Kushner, J., Simpson, C., Pugh, S., and Corbett, T. H. (2005) Cryptophycins-309, 249 and other cryptophycin analogs: Preclinical efficacy studies with mouse and human tumors, *Invest. New Drugs 23*, 213−24.
38. Stevenson, J. P., Sun, W., Gallagher, M., Johnson, R., Vaughn, D., Schuchter, L., Algazy, K., Hahn, S., Enas, N., Ellis, D., Thornton, D., and O'Dwyer, P. J. (2002) Phase I trial of the cryptophycin analogue LY355703 administered as an intravenous infusion on a day 1 and 8 schedule every 21 days, *Clin. Cancer Res. 8*, 2524−9.
39. Eggen, M., Mossman, C. J., Buck, S. B., Nair, S. K., Bhat, L., Ali, S. M., Reiff, E. A., Boge, T. C., and Georg, G. I. (2000) Total synthesis of cryptophycin-24 (Arenastatin A) amenable to structural modifications in the C16 side chain, *J. Org. Chem. 65*, 7792−9.
40. Gardinier, K. M., and Leahy, J. W. (1997) Enantiospecific total synthesis of the potent antitumor macrolides cryptophycins 1 and 8, *J. Org. Chem. 62*, 7098−9.
41. Rej, R., Nguyen, D., Go, B., Fortin, S., and Lavallee, J. F. (1996) Total synthesis of cryptophycins and their 16-(3-phenylacryloyl) derivatives, *J. Org. Chem. 61*, 6289−95.
42. Salamonczyk, G. M., Han, K., Guo, Z. W., and Sih, C. J. (1996) Total synthesis of cryptophycins via a chemoenzymatic approach, *J. Org. Chem. 61*, 6893−900.
43. White, J. D., Hong, J., and Robarge, L. A. (1999) Total synthesis of cryptophycins-1, -3, -4, -24 (arenastatin A), and -29, cytotoxic depsipeptides from cyanobacteria of the *Nostocaceae*, *J. Org. Chem. 64*, 6206−16.
44. Li, L. H., and Tius, M. A. (2002) Stereospecific synthesis of cryptophycin 1, *Org. Lett. 4*, 1637−40.
45. Kobayashi, M., Kurosu, M., Wang, W. Q., and Kitagawa, I. (1994) A total synthesis of arenastatin-A, an extremely potent cytotoxic depsipeptide, from the Okinawan marine sponge *Dysidea arenaria*, *Chem. Pharm. Bull. 42*, 2394−6.
46. Eggen, M. J., Nair, S. K., and Georg, G. I. (2001) Rapid entry into the cryptophycin core via an acyl-β-lactam macrolactonization: Total synthesis of cryptophycin-24, *Org. Lett. 3*, 1813−5.
47. Kneller, D. G., Cohen, F. E., and Langridge, R. (1990) Improvements in protein secondary structure prediction by an enhanced neural network, *J. Mol. Biol. 214*, 171−82.
48. Geer, L. Y., Domrachev, M., Lipman, D. J., and Bryant, S. H. (2002) CDART: Protein homology by domain architecture, *Genome Res. 12*, 1619−23.
49. Saito, F., Hori, K., Kanda, M., Kurotsu, T., and Saito, Y. (1994) Entire nucleotide sequence for *Bacillus brevis* Nagano Grs2 gene encoding gramicidin S synthetase 2: A multifunctional peptide synthetase, *J. Biochem. 116*, 357−67.
50. Turgay, K., Krause, M., and Marahiel, M. A. (1992) Four homologous domains in the primary structure of GrsB are related to domains in a superfamily of adenylate-forming enzymes, *Mol. Microbiol. 6*, 2743−4.
51. Mootz, H. D., and Marahiel, M. A. (1997) The tyrocidine biosynthesis operon of *Bacillus brevis*: Complete nucleotide sequence and biochemical characterization of functional internal adenylation domains, *J. Bacteriol. 179*, 6843−50.
52. Varie, D. L., Shih, C., Hay, D. A., Andis, S. L., Corbett, T. H., Gossett, L. S., Janisse, S. K., Martinelli, M. J., Moher, E. D., Schultz, R. M., and Toth, J. E. (1999) Synthesis and biological evaluation of cryptophycin analogs with substitution at C-6 (fragment C region), *Bioorg. Med. Chem. Lett. 9*, 369−74.
53. Tseng, C. C., Bruner, S. D., Kohli, R. M., Marahiel, M. A., Walsh, C. T., and Sieber, S. A. (2002) Characterization of the surfactin synthetase C-terminal thioesterase domain as a cyclic depsipeptide synthase, *Biochemistry 41*, 13350−9.

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