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Characterization of the Cereulide NRPS α-Hydroxy Acid Specifying Modules: Activation of α-Keto Acids and Chiral Reduction on the Assembly Line

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Several nonribosomal depsipeptide natural products are composites of α-hydroxy acid and α-amino acid monomers.[1] Cereulide (ces) (**1**), the emetic toxin from the human pathogen *Bacillus cereus*, and valinomycin (vlm) (**2**), from *Streptomyces* spp., are closely related macrocyclic K+ ionophores.[2,3] The macrocyclic core of each natural product contains alternating peptide (six) and ester (six) bonds and their cyclododecadepsipeptide structures consist of a tetradepsipeptide unit repeated three times. For ces (**1**) the tetradepsipeptide is D-α-hydroxyisocapryl-D-alanyl-L-α-hydroxyisovaleryl-L-valyl; for vlm (**2**), the repeat unit is D-α-hydroxyisovaleryl-D-valyl-L-lactyl-L-valyl (Figure 1). α-Hydroxy acids of ces (**1**) and vlm (**2**) are known to originate from their respective α-amino acid pools, but it has been unclear if hydroxy acids or amino acids are the monomer species that are selected and elongated by the nonribosomal depsipeptide enzymatic machinery.[4,5] In fact, as we show in this work it, is the corresponding α-keto acids that get selected, tethered as pantetheinyl thioesters and reduced to α-hydroxyacyl-*S*-pantetheinyl carrier protein intermediates in cis reductase domains.

Recently, the *ces* and *vlm* biosynthetic gene clusters have been cloned and sequenced.[6,7] Both of the *ces* NRPSs CesA (3391 aa) and CesB (2681 aa) begin with a module of the following domain arrangement, adenylation (A)-reductase/dehydrogenase (KR in Figure 1) peptidyl carrier protein (PCP).[6] The Vlm1 NRPS predicted from the *S. tsusimaensis* ATCC 15141 *vlm* biosynthetic gene cluster is annotated as having a module with the following domains: A-transaminase (TA)-dehydrogenase-PCP.[7] We have independently sequenced a second *vlm* cluster from *S. levoris* A-9 [9] (accession # DQ640825) (Figure S1, Supporting Information) and no such TA domain was found. Comparison of CesA, *S. levoris* A-9 Vlm1, and the *S. tsusimaensis* Vlm1 revealed them to be similar in size, sequence, and overall content (Figure S2). From our reanalysis, no TA domain exists within *S. tsusimaensis*, but a stretch of residues preceding the reductase/dehydrogenase domains of CesA/B and Vlm1/2 from *S. levoris* A-9 bears similarity to proposed "spacer regions" of melithiazol and myxothiazol type I polyketide synthases (PKSs).[8] Thus, both CesA/B NRPSs and Vlm1/2 NRPSs have similar domain organization (Figure 1). Most notable is the presence of predicted KR domains which are similar to *β*-ketoacyl reductase domains based on sequence analysis to KR domains found in PKS assembly lines, and further, these KR domains are inserted into the A domains (Figure S3). To characterize the CesA and CesB modules for monomer recognition and establish how the *ces* NRPS incorporates α-hydroxy acids, DNA fragments encoding the first CesA and B modules (A−KR−PCP) were obtained by PCR from *B. cereus* F4810/72 genomic DNA and cloned into *E. coli* expression vectors. The *cesA* fragment was cloned into pTrcHis-TOPO TA vector (Invitrogen), and the *cesB* fragment was cloned into the *Nco*I and *Xho*I sites of pET28b (Novagen). The CesA module was heterologously expressed in *E. coli* BL21 (DE3) as a doubly His-tagged (C and N-termini) protein, and the CesB module was expressed as an N-terminally His-tagged protein. Both proteins were purified to over 90% purity using Ni−NTA affinity and gel exclusion chromatographies (Figure S4). To determine the substrate of the A domains contained within the His-tagged CesA and CesB (A−KR−PCP) modules, the radioactive ATP−PPi exchange assay was used as a measure of reversible acyl-AMP formation. Test substrates for CesA were L- and D-isoleucine (Ile), L- and D-α-hydroxy isocaproic acid (L- and D-HIC), and α-ketoisocaproic acid (KIC). For CesB, test substrates were L- and D-valine (Val), both enantiomers of α-hydroxy isovaleric acid (L- and D-HIV), as well as α-ketoisovaleric acid (KIV). Both CesA and CesB were found to preferentially activate α-keto acids over their corresponding α-hydroxy acids and α-amino acids, establishing them as novel α-keto acid activating A domains (Figure 2, the CesA and B predicted NRPS codes are shown in Table S1). A trichloro-α-ketoisocaproic acid activating A domain was recently described in a barbamide NRPS.[10]

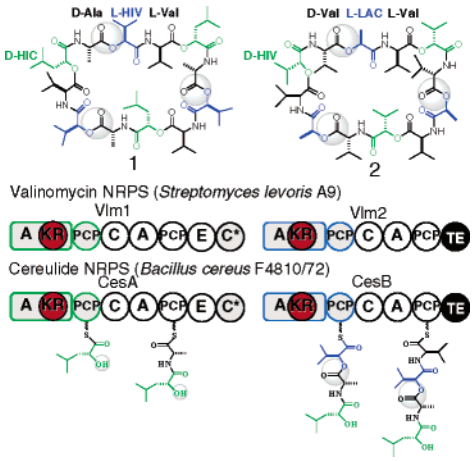

Figure 1. Cereulide and valinomycin and their NRPSs.

Next, the ability of CesA and CesB A domains to transacylate α-keto acyl-adenylates onto their adjacent PCPs was tested. CesA and CesB were converted from their purified apo-forms to their respective holo-forms by CesP, a phosphopantetheinyl transferase encoded by the *ces* cluster. CesP was also expressed and purified from *E. coli* as a His-tagged variant. Radio-labeled KIC and KIV were prepared from [^{14}C]-labeled Ile and Val, respectively, using snake venom L-amino acid oxidase. In each case the [^{14}C]-α-keto acids were loaded onto their cognate *holo*-PCPs by both the CesA and B A domains as assayed by radioactive incorporation into proteins via TCA-precipitation and subsequent liquid scintillation counting. The fate of the α-ketoacyl-S−PCPs was then examined.

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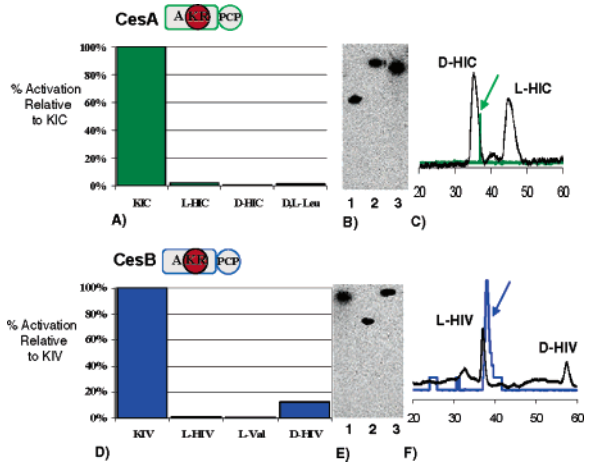

Figure 2. (A) ATP−PPi exchange assay with CesA A domain substrates; (B) radio-TLC of CesA products (lane 1, ^{14}C labeled α-KIC; lane 2, D-, L-HIC standard; lane 3, product of CesA KR domain); (C) chiral radio-HPLC of CesA KR domain product (green) with cold D-HIC and L-HIC (black); (D) ATP−PPi exchange assay with CesB A domain; (E) radio-TLC of CesB products (lane 1, ^{14}C labeled α-KIV; lane 2, D-, L-HIV standard; lane 3, product of CesB KR domain); **(**F) chiral radio-HPLC of CesB KR domain product (blue) and D-HIV and L-HIV (black).

In Figure 1 (and also Figure S3) we have noted the prediction of KR domains within CesA and B α-keto acid A domains. These are distinct from the KR domains found in PKS modules in two ways. First the PKS domains are *β*-ketoacyl-S-carrier protein reductases (*β*-KRs) while the CesA/B (and presumably VlmA/B) KR domains would be α-ketoacyl-S-carrier protein reductases (α-KRs). Second, while PKS *β*-KR domains are downstream of acyltransferase (AT) domains in modules where they are found, the reductase domains present in CesA and CesB are embedded within the α-keto acid A domains between A domain motifs A8 and A9 (schematized in Figure 1 and shown in Figure S3). The A domain region spanning sequence motifs A8−A9 is a flexible loop,[11] which must serve as a "stuffer region", accommodating diverse catalytic domains such as *N*- and *C*-methyltransferases, decarboxylase domains, and now α-KR domains. To establish α-KR domain function, [^{14}C]-labeled α-keto acyl-S−PCPs were generated as above on the CesA and CesB PCP domains, followed by the addition of NADPH. The thioester-bound products were liberated by TycF, the tyrocidine type II thioesterase and analyzed by radio-TLC versus KIV, KIC, L,D-HIV and L,D-HIC standards. The product obtained from the CesA module had an R_F identical to HIC, whereas the CesB product had an R_F equivalent to HIV (Figure 2). Chirality of α-hydroxy acids was established by radio-HPLC using chiral column chromatography of underivatized acids. The CesA α-hydroxy acid was identified as D-HIC, whereas L-HIV was the sole enantiomer formed by the CesB KR domain (Figure 2). Product chirality corresponds with the stereochemistry (D-HIC and L-HIV) of such residues within **1**. Thus the α-KR domain within CesA is a D-reductase and within CesB is an L-reductase. Structural analyses will be warranted to evaluate chirality and make comparisons to the D- and L-specific (more commonly referred to as *R*- and *S*-specific) *β*-KR domains in PKSs. Every other bond within the *ces* tetradepsipeptide unit (i.e., D-α-hydroxyisocapryl-D-alanyl-L-α-hydroxyisovaleryl-L-valyl) is an ester. The elongation module for the L-α-hydroxyisovaleryl monomer is the CesB A−KR−PCP and the condensation (C) domain found in trans at the C-terminus of CesA (Figure 1). We propose that the terminal CesA C domain is an ester synthase rather than an amide synthase working in trans with the HIC-D-Ala peptide as donor and the α-hydroxy group of the CesB thioester-bound L-HIV as nucleophilic acceptor. Elongation to the D-HIC-D-Ala-L-HIV-L-Val chain is presumably followed by transfer to the TE domain, while a second tetradepsipeptide builds up on the adjacent PCP of CesB. We anticipate the TE domain then acts like the enterobactin synthetase TE:[12] condensing two tetradepsipeptides to an octadepsipetidyl-O-TE and then a dodecadepsipetidyl-O-TE prior to macrolactonization to the cyclic twelve residue product cereulide.

The α-hydroxy acid incorporation strategy of α-keto acid selection, activation, tethering, and in situ chiral reduction should also hold for the *vlm* NRPSs (Figure 1), with the telltale α-KR embedded in the A domain. Two strategies appear to be utilized for α-hydroxy acid monomer incorporation into natural products via NRPS assembly lines: (1) The bacterial NRPS logic exemplified by the *ces* NRPSs and (2) the proposed fungal NRPS logic (e.g., enniatin and PF1022A)[13a,b] where an A domain activates and directly tethers the α-hydroxy acid generated from a nonassembly line associated α-keto acid dehydrogenase.[13c] As yet there is no evidence for a third variant where an aminoacyl-S-carrier protein would be oxidatively deaminated and then reduced to a hydroxy acyl moiety.

It will be of interest to see how many other nonribosomal products with alternating ester and amide bonds are generated by this coupled α-keto acid activation, tethering, and reduction logic. Three of the domains in the *ces* and *vlm* NRPSs are worthy of detailed study: the A domains for their ability to generate α-keto acyl-AMPs, the α-KRs that do chiral reduction of α-ketoacyl-S-carrier proteins as opposed to the *β*-ketoacyl-S-carrier proteins in PKS action, and the C domains that are chiral ester synthases rather than amide synthases.

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Note Added after ASAP Publication. After this paper was published ASAP on July 28, 2006, further changes were introduced to clarify sentence 3 of paragraph 2 and to correct the Vlm notation in sentences 5 and 6 of paragraph 2. The corrected version was published on the same date.

Supporting Information Available: Details of protein preparation, enzymatic, and chemical analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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