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REVIEW

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Biosynthesis of depsipeptides, *or* Depsi: The peptides with varied generations

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Abstract

Depsipeptides are compounds that contain both ester bonds and amide bonds. Important natural product depsipeptides include the piscicide antimycin, the K^+ ionophores cereulide and valinomycin, the anticancer agent cryptophycin, and the antimicrobial kutzneride. Furthermore, database searches return hundreds of uncharacterized systems likely to produce novel depsipeptides. These compounds are made by specialized nonribosomal peptide synthetases (NRPSs). NRPSs are biosynthetic megaenzymes that use a module architecture and multi-step catalytic cycle to assemble monomer substrates into peptides, or in the case of specialized depsipeptide synthetases, depsipeptides. Two NRPS domains, the condensation domain and the thioesterase domain, catalyze ester bond formation, and ester bonds are introduced into depsipeptides in several different ways. The two most common occur during cyclization, in a reaction between a hydroxy-containing side chain and the C-terminal amino acid residue in a peptide intermediate, and during incorporation into the growing peptide chain of an α-hydroxy acyl moiety, recruited either by direct selection of an α-hydroxy acid substrate or by selection of an α-keto acid substrate that is reduced in situ. In this article, we discuss how and when these esters are introduced during depsipeptide synthesis, survey notable depsipeptide synthetases, and review insight into bacterial depsipeptide synthetases recently gained from structural studies.

KEYWORDS

biosynthesis, depsipeptide, enzyme, ester bond, nonribosomal peptide, synthetase

1 | INTRODUCTION TO NONRIBOSOMAL PEPTIDE SYNTHETASES AND THEIR PRODUCTS

Nonribosomal peptide synthetases (NRPSs) are multidomain microbial enzymes that synthesize natural products of medical and industrial importance.[1,2] Well-known examples of nonribosomal peptides include the antibiotics penicillin,[3] gramicidins S and D[4–7] and daptomycin,[8] the antifungal caspofungin,[9] the immunosuppressor cyclosporin,[10,11] and the emulsifier surfactin[12] (Figure 1a). Nonribosomal peptides are considered secondary metabolites, compounds that are not essential for organism survival in permissive growth conditions, but confer advantages.[13] They are known to function naturally in growth and reproduction in restrictive environments,[14,15] inhibiting, or killing competitor microbes[2,13] and modulating symbiont interactions.[16–18] Nonribosomal peptide biosynthesis is common in microbial organisms, with the most prolific producers being members of the bacterial phyla *Proteobacteria*,

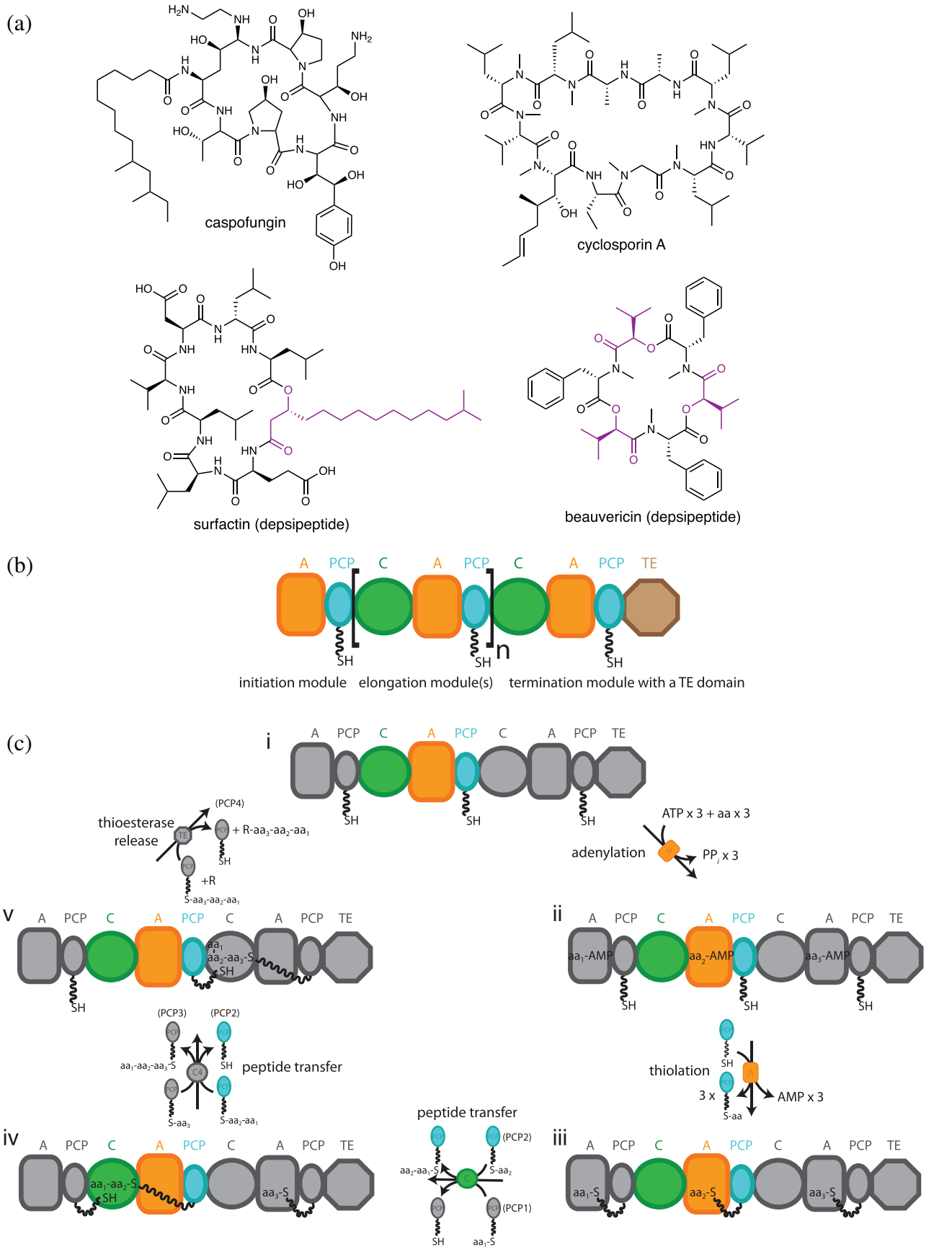


FIGURE 1 Legend on next page.

Actinobacteria, *Firmicutes*, and *Cyanobacteria*, and the fungal phylum *Ascomycota*.[19]

Nonribosomal peptides are small; typically containing ~2–20 residues. They often do not resemble ribosomally-synthesized peptides, as they can be cyclic, branched or linear, and contain many nonproteinaceous moieties (Figure 1a). More than 500 monomer substrates, including L- and D- amino acids, hydroxy acids, keto acids, fatty acids, and aryl acids, are known to be incorporated into these peptides.[20] Also, many co-synthetic modifications,[21,22] including methylation,[23,24] formylation,[7,25] epimerization,[26,27] residue cyclization,[28–30] oxidation,[31,32] reduction,[33,34] and hydroxylation,[35,36] are known to occur during peptide synthesis. This large substrate pool and set of tailoring modifications allow nonribosomal peptides to occupy an impressive volume of chemical space and achieve greatly diverse bioactivities. Furthermore, nonribosomal peptides can be postsynthetically modified,[22,35,37] and in nature NRPS systems are very commonly combined with polyketide synthases (PKSs) to make hybrid peptide-ketide products.[12,38] Incorporation of other metabolites such as lipids[39,40] and carbohydrates[41–43] increases chemical diversity even more.

NRPSs synthesize peptides using a modular synthetic scheme that resembles an assembly line. An NRPS module is defined as the set of domains that together possess all the activities required to add one acyl residue to the nascent nonribosomal peptide chain.[44–48] A standard elongation module includes a condensation (C) domain, an adenylation (A) domain, and a peptidyl carrier protein (PCP) domain arranged as C-A-PCP (Figure 1b). (Note that, the PCP domain is equally commonly called the T domain. In that abbreviation, T stands for thiolation, which is misleading because all other domains perform the reaction after which they are named, while the holo form of this domain is a substrate for, not the catalyst of, thioester formation ("thiolation"). We advocate that T should instead stand for transfer, since the role it plays is analogous to that of the transfer RNA (tRNA) in the ribosomal system.)

A minimal elongation cycle (Figure 1c) starts when the A domain selects the cognate monomer substrate, for example an amino acid, from the pool of cellular metabolites. Selectivity is hard-wired into each A domain by the set of residues that make up the substrate binding pocket of the A domain, known as "specificity code" residues.[49–52] The A domain then binds ATP and catalyzes the adenylation of the amino acid to make an activated aminoacyl-adenylate.[4,53] Next, the C-terminal subdomain of the A domain (A_{sub}) rearranges to enable the PCP domain to bind and insert its 4′-phosphopantheteinyl (ppant) moiety into the A domain active site.[54–57] The A domain catalyzes attack of the aminoacyl-adenylate by the ppant thiol, covalently linking the aminoacyl moiety to the PCP domain as a thioester (aminoacyl-*S*-ppant-PCP). The acyl thioester is then transported by the PCP domain to the C domain, which has binding sites for this acceptor aminoacyl-*S*-ppant-PCP and a donor peptidyl-*S*-ppant-PCP from the upstream module. The C domain will catalyze the condensation reaction, with the amino group from aminoacyl-*S*-ppant-PCP nucleophilically attacking the thioester carbonyl, which transfers and elongates the nascent peptidyl intermediate. The elongated peptidyl-*S*-ppant-PCP then moves to the C domain of the downstream module, where it acts as the donor substrate, passing off and further elongating the peptide and liberating the PCP to participate in the synthesis of the next peptide in the next catalytic cycle of its module.

The first and last modules have specialized domain composition and synthetic cycles. Initiation modules do not possess C domains, but only A-PCP, and after thiolation their aminoacyl-*S*-ppant-PCP intermediates are transported directly downstream to act as the donor substrate for the second module's C domain. Termination modules possess C, A, and PCP domains plus a specialized termination domain to which the PCP delivers the late-stage peptidyl intermediate for final processing and release. The most common termination domain, the thioesterase (TE) domain, typically releases the peptide through hydrolysis or cyclization,[58] while reductase (R) domains catalyze release by converting the thioester link to an aldehyde[59] or alcohol[60] and specialized C

FIGURE 1 (a) Some nonribosomal peptides (top) and depsipeptides (bottom). Depsipeptides contain amide and ester bonds. Monomers from which the bridging ester oxygen originates are highlighted in purple. (b) The modular organization of nonribosomal peptide synthetases (NRPSs). Brackets highlight an elongation module. NRPS domains are: A, adenylation; C, condensation; PCP, peptidyl carrier protein; TE, thioesterase. (c) A typical NRPS synthetic cycle. The cycle starts with A domains activating monomers by adenylation, followed by their attachment to PCP domains in a phosphopantetheinyl moiety (ppant) as a thioester. PCP domains transport intermediates throughout the cycle. C domains catalyze amide (or ester, see Section 3.1) bond formation between adjacent PCP-bound intermediates, elongating the peptide chain. Chain release occurs in termination modules, with TE domains the most common termination domains. Terminal reductase domains and terminal condensation domains also occur frequently. aa, amino acid; PP_i, inorganic pyrophosphate. Adapted from Huguenin-Dezot et al.[153]

domains[61] catalyze release by cyclization[62] and amide bond formation with small-molecule acceptor.[28,61,63,64]

There is typically a straightforward correspondence between the composition of a nonribosomal peptide and the NRPS which synthesizes that peptide[47]: Typically, the number of residues in the nonribosomal peptide corresponds to the number of modules in the NRPS, and the order and identity of the residues in the peptide corresponds to the order of the modules in the NRPS and each module's A domain substrate specificity. However, many exceptions to this correspondence are known, which include iterative[5] and module skipping NRPSs.[65] An NRPS system can consist of a single protein or can be split between multiple subunits which interact non-covalently through small docking or communication domains.[66–68] The genes for all the NRPS subunits, the in-trans tailoring or postsynthetic modifying proteins, and other accessory proteins[69–71] are often found together in the microbial genome, in a "biosynthetic gene cluster" (BGC). This clustering, combined with predictability of A domain substrates and activity of each domain, means that increasingly sophisticated algorithms[51,52,72–78] are becoming more and more accurate at predicting the final product of a BGC from gene sequence alone.

All of the common NRPS domains have been well characterized both biochemically and structurally. A domains are ~60 kDa Rossmann-like fold proteins that belong to the superfamily of adenylating enzymes.[4,55,79] They consist of a large subdomain of ~50 kDa (A_{core}, elsewhere called N-terminal subdomain) which contains binding sites for an acyl monomer and ATP, and a C-terminal small subdomain of ~10 kDa (A_{sub}, elsewhere called C-terminal subdomain) which contains a catalytic lysine for adenylation reaction[4,53] and which changes position radically during the synthetic cycle.[44,79] PCP domains are structured as 4-helix bundles, homologous to acyl carrier protein (ACP) domains of PKSs and fatty acid synthases.[80] PCP domains require phosphopantetheinylation on the serine of their GGHS motif in PCP helix 2 for the NRPS to be active. This posttranslational modification is imparted by a phosphopantetheinyl transferase (PPTase) using coenzyme A as substrate.[69,81] As described in detail in Section 3.1, C domains are bilobed pseudo-dimers with two binding sites for PCP domains, connected by a tunnel that accesses the active site.[61,62,82–86] TE domains, also described more extensively in Section 4.2, are α/β hydrolase superfamily proteins.[58,87–92]They catalyze two half reactions: acyl transfer onto their catalytic Ser, followed by nucleophilic off-loading, by promoting attack by water (leading to hydrolysis) or by a nucleophile in the peptide intermediate (leading to cyclization).[58,87] Another release strategy is reductive release, where R domains are termination domains that release the thioester peptide as an aldehyde or an alcohol using NADH or NADPH as a cofactor.[59,93,94] Furthermore, the broader structural view of NRPSs is starting to take shape with several recent studies of didomains, modules, or supra-modular NRPSs showing how domains and modules coordinate in peptide synthesis.[7,34,44,45,56,57,83,85,86,90,95–102]

2 | DEPSIPEPTIDES

Nonribosomal depsipeptides are compounds with both ester and amide bonds in their structures[103] (Figure 1a). They are common natural products with a vast array of biological activities of medical and industrial relevance.[104–107] For example, daptomycin is used as an antibiotic for severe infections,[8] antimycin is used as a piscicide in industrial fish farming,[108] valinomycin has anti-cancer and anti-coronavirus activity[109] and surfactin has potential as an industrial tenside.[12] Some depsipeptides are involved in microbial pathogenesis, like the emetic toxin cereulide.[110]

Depsipeptides have a variety of natural functions, from increasing organism fitness in K^+ deprived environments[14] to participating in symbiotic relationships both in marine[111] and terrestrial[18,112] organisms. Among the most interesting examples in symbiosis is the role of depsipeptides in the relationships between microorganisms and ants: Actinomycin, valinomycin, and antimycin participate in a tripartite symbiotic relationship between *Streptomyces sp*., a fungus, and leaf-cutter ants,[18,112] while dentigerumycin made in *Pseudonocardia* actinobacteria helps control pathogenic fungal growth in fungal gardens cultivated by ants.[113]

As with other NRPS products, the chemical diversity of depsipeptides is outstanding.[105–107] In addition to the occurrence of at least one ester and one amide bond in their structures, most depsipeptides contain a wide variety of acyl groups and other moieties added through the assembly line logic of NRPSs. Depsipeptides may be post-synthetically modified after release from the NRPS,[37] although it should be noted that this review concentrates on nonribosomal synthesis, rather than on downstream tailoring.

Intriguingly, with one exception (asperphenamate,[114] see Section 4.1.1.2), all nonribosomal depsipeptide synthetases characterized to date produce cyclic depsipeptides. Linear products are sometimes observed from cyclic depsipeptide synthetase systems, such as ring-opened antimycins,[115] but cyclic products predominate even in these systems. Cyclic natural products are very common, and their cyclic nature imparts advantages including reduced entropy costs in binding protein

targets,[116] preorganization for binding ions,[15,117] and protease resistance.[118,119] However, linear nonribosomal peptides are not uncommon, so it is somewhat surprising how few synthetases are known to produce linear nonribosomal depsipeptides.

Classification systems for depsipeptides

More than 1,300 naturally occurring cyclic depsipeptides are known.[104] De Spiegeleer and coworkers[104] have made a thorough classification and a curated database of these from their chemical features. This classification separates depsipeptides based on whether they have one or more ester bonds, whether multiple ester bonds occur regularly or irregularly, and whether the ester oxygen is in the α, β, or more distal position. It is extremely detailed and very useful. For this review, as a parallel and complementary system to Taevernier et al., we classify the biosynthetic systems based on the characterized or putative mode of introduction of the ester bond(s). Our system of classification is based on the timing of introduction of the ester, which NRPS domain catalyzes ester bond formation, whether a single-pass synthetic scheme or an iterative synthetic scheme was used by the NRPS to produce the compound, and the origin of the bridging ester hydroxy group (Figure 2). This classification system can be easily expanded if/when novel biosynthetic pathways are described. It is used to structure this review into sections, building from conceptually simpler depsipeptide pathways to more complex ones.

3 | DEPSIPEPTIDES WITH ESTER BONDS FORMED DURING ELONGATION

3.1 | The C domain catalyzes amide and ester bond formation

The condensation domain C_n (i.e., in module n) typically catalyzes peptide bond formation between a peptidyl-*S*-PCP_{n-1} donor substrate and an aminoacyl-*S*-PCP_n acceptor substrate (Figure 3a). In this condensation reaction, the α-NH_2 of the aminoacyl-*S*-PCP_n nucleophilically attacks the thioester carbon of the peptidyl-*S*-PCP_{n-1} donor substrate, which transfers the peptidyl moiety to the aminoacyl-*S*-PCP_n, and elongates the nascent chain by one amino acid residue.

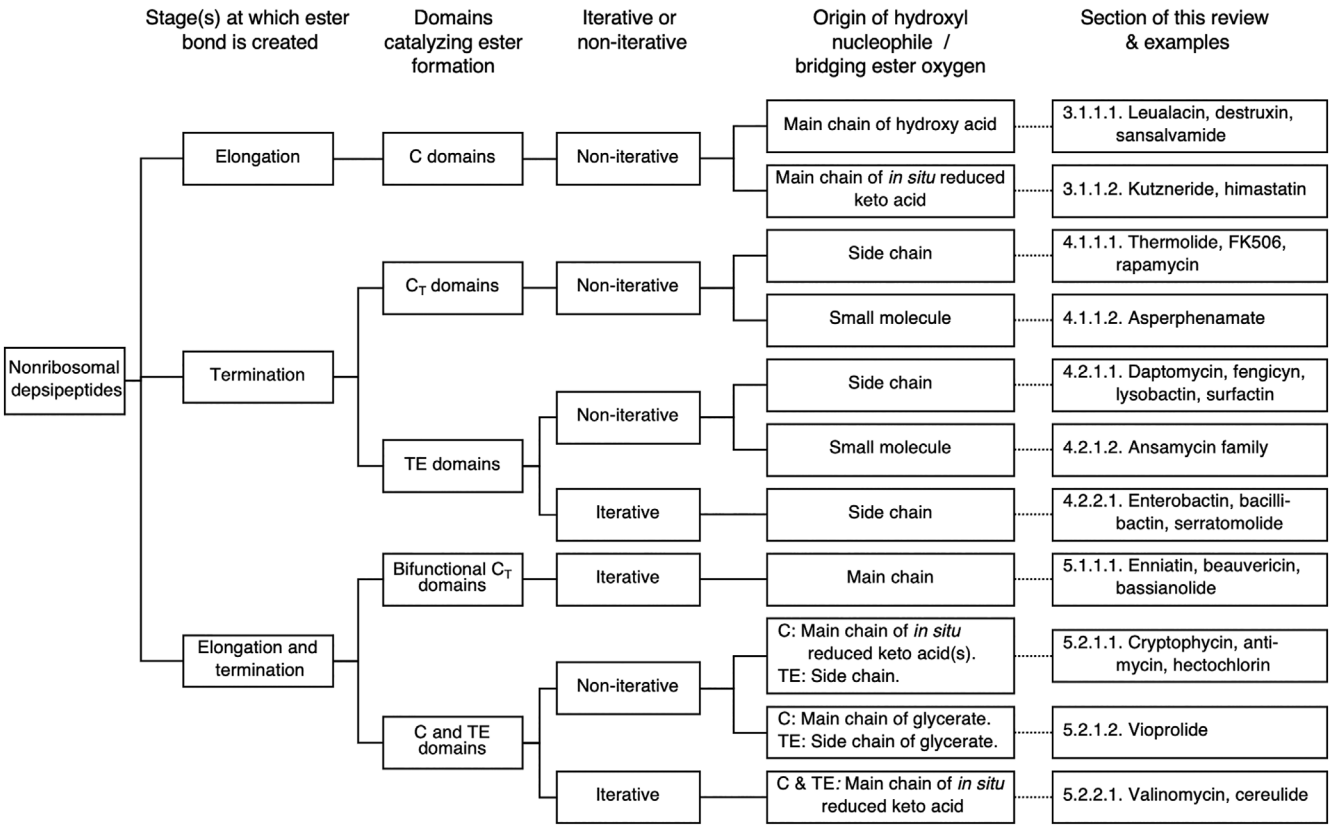


FIGURE 2 Nonribosomal depsipeptide classification by biosynthetic strategies of ester bond formation. Examples are not comprehensive

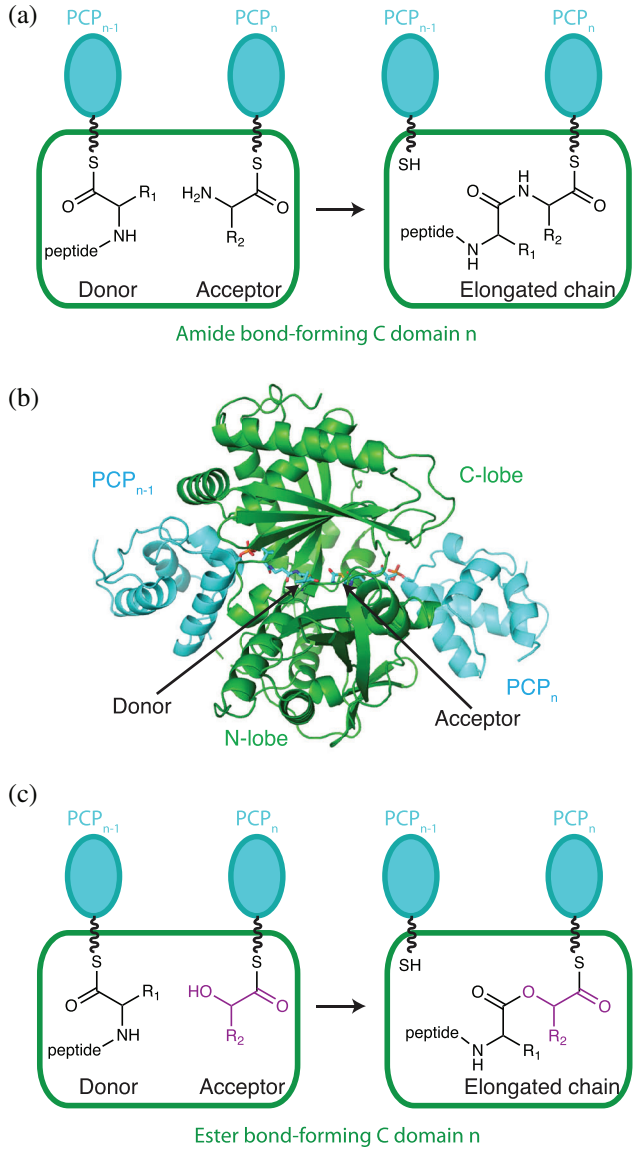


FIGURE 3 The condensation domain catalyzes amide and/or ester bond formation (a) Amide bond formation occurs in the active site of a condensation domain (green rectangle). Substrates are presented as thioesters attached to phosphopantetheine (wavy line) in PCP domains (cyan). The PCP in the preceding module ($n-1$) relays the donor substrate, while the acceptor substrate resides in the current module (n). (b) Structure of condensation conformation of the C domain with acceptor and donor PCP domains, extracted from the dimodular NRPS LgrA.[97] The domain has N-terminal and C-terminal lobes which each have chloramphenicol acetyl transferase-like folds. Donor peptidyl-ppant and acceptor aminoacyl-ppant are attached to PCP domains (cyan). (c) Some condensation domains can form ester bonds using OH groups as nucleophiles

Crystal structures reveal C domains to be pseudo-dimeric domains formed by C-terminal and N-terminal lobes[82] (Figure 3b). The active site, identifiable by the conserved motif HHxxxDG, lies in a tunnel that runs through the interface between the lobes. The second His in the motif (His157 based on numbering of first condensation domain of CDA, PDB ID: 4jn3[120]) is most important for reaction. It has been proposed to be a strong general base, deprotonating the α-amino group of the acceptor substrate in preparation for nucleophilic attack, or part of an electronic transition state stabilization,[121] or (by us) to principally act in substrate positioning.[84] Mutation of His157 leads to loss of condensation in some systems[122] but not others,[61,82,123] and heterocyclization domains, which are homologous and related to C domains and also catalyze the same condensation reaction, have a hydrophobic residue in place of His157.[28] The acceptor and donor PCP domain binding sites on the C domain are on the two ends of the active site tunnel.[62,83–86,97] Both PCP domain binding sites are made of portions of both N-terminal and C-terminal lobes, a reflection of the pseudosymmetry of those lobes[61] (Figure 3b).

Despite more commonly catalyzing amide bond formation, the ester forming activity of C domains has been confirmed directly in several systems. This activity was first verified biochemically for the biosynthesis of two molecules that happen not to have amide bonds in them and are thus not depsipeptides, the fungal metabolite fumonisin[124] and the antitumor antibiotic C-1027.[64,125] In fumonisin biosynthesis, the C domain Fum14p catalyzes ester bond formation between a donor polyketide thioester and an acceptor tricarballylic thioester.[124] In C-1027, the C domain SgC5 catalyzes ester bond formation between a donor enediyne core and the acceptor (*S*)-3-chloro-5-hydroxy-β-tyrosine thioester.[64,125] SgC5 is also capable of amide bond formation, suggesting C domains catalyze ester and amide bond formation with analogous catalytic mechanisms.[125] This suggestion is further supported by the conservation of either the full canonical HHXXXD motif.[64] or at least the important second histidine within the motif.[124] Experimental evidence of C domain installation of ester bonds into depsipeptides comes from domain deletion and substitution experiments[126,127] and in vitro biochemical assays of an ester bond forming module.[128]

3.1.1 | Ester bond formation during elongation by a C domain in noniterative synthesis

3.1.1.1 | Ester formed with main chain hydroxyl originating from hydroxy acid substrate

NRPS biosynthetic pathways which introduce hydroxy acid residues during elongation can closely resemble the typical NRPS that introduce amino acid residues. In the

conceptually simplest case, the A domain of an elongation module recognizes a hydroxy acid and this hydroxy acid is activated and transferred to the PCP domain. The resulting hydroxyacyl-*S*-PCP$_n$ is then used as the acceptor substrate in C domain catalyzed ester bond formation (Figure 3c). Systems that use this conceptually simple case are almost always fungal.[129]

To use a hydroxy acid as a building block substrate, both (1) availability and (2) selection of such substrates must be addressed by the BGC. (1) α-hydroxy acids are not common in primary metabolism, so in fungal systems that use α-hydroxy acids as NRPS substrates, the BGC often contains standalone ketoreductase (KR) proteins which convert α-keto acids to α-hydroxy acids in an NADPH-dependent reduction reaction[130–134] (Figure 4), with keto acids arising from branched-chain amino acid biosynthesis pathways[135] or from glycolysis. These KR proteins are not closely related to PKS KR domains,[128,136,137] but belong to the ApbA_C superfamily, which includes ketopantoate reductases.[130,133,138] Deletion of the KR protein abolishes production of the depsipeptide, which can be rescued by supplementing the media with α-hydroxy acids.[131] (2) A domain in these systems can discriminate between α-amino and α-hydroxy groups through a yet unknown mechanism (Figure 4a). In these A domains, the position of a highly conserved Asp (Asp235 in GrsA-PheA numbering used here for A domains[4]) is occupied by a Gly.[130] Asp235 makes key hydrogen bonds with the substrate's α-amino group in amino acid selecting A domains, and its mutation leads to loss of adenylation cognate amino acids.[139] It is unknown whether this substitution is enough to confer α-hydroxy acid selectivity, and without a substrate-bound structure of an α-hydroxy acid selecting A domain, the mode of α-hydroxy acid selection remains an open question.

Ester bond insertion by direct selection and incorporation of α-hydroxy acid occurs twice leualacin

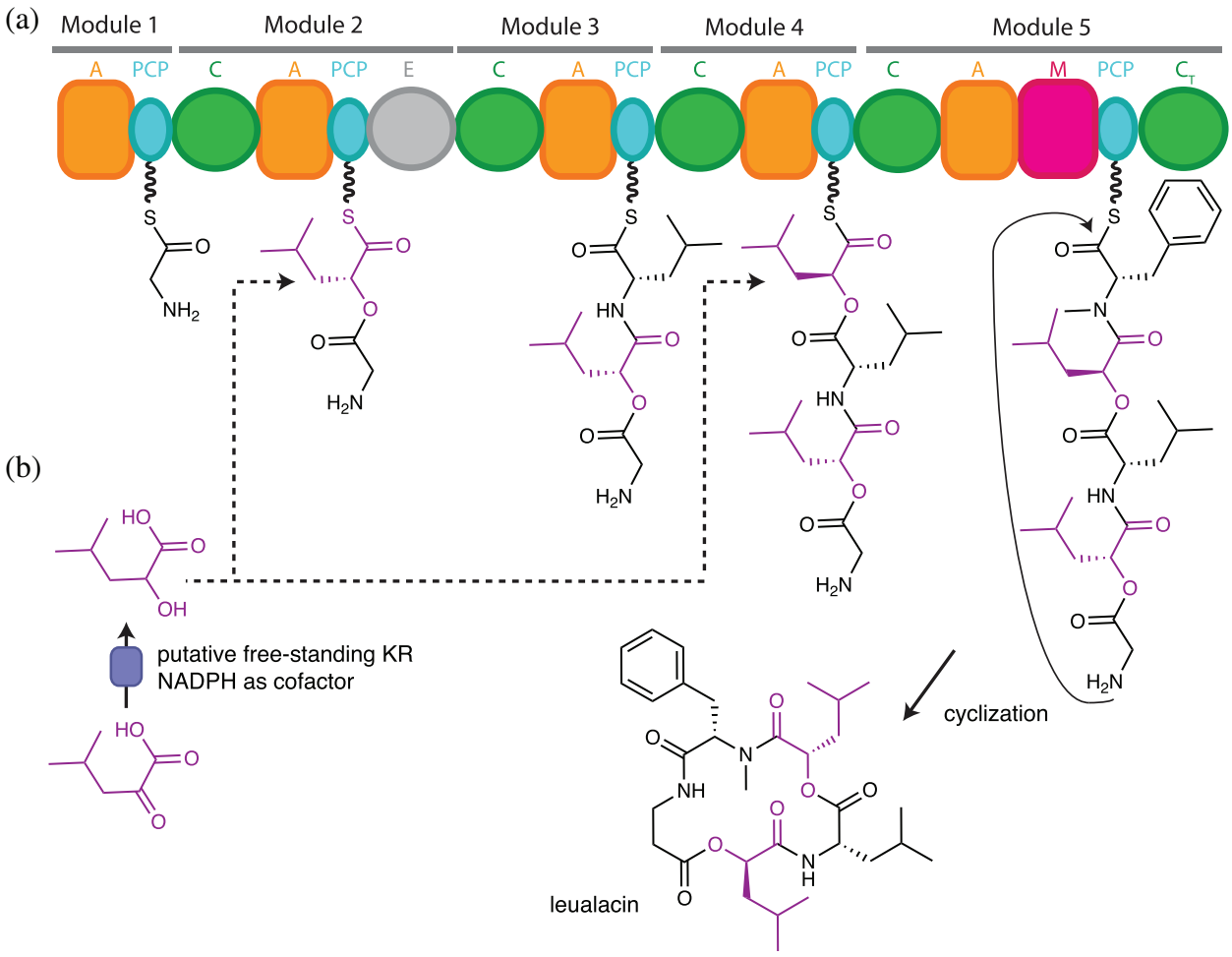


FIGURE 4 Proposed biosynthesis of leualacin. (a) Specialized C domains in Modules 2 and 4 catalyze ester bond formation using hydroxyacyl thioesters as acceptor substrates. Specialized A domains in the same modules activate the hydroxy acids. (b) Stand-alone ketoreductases (KR) produce hydroxy acids from keto acids in fungal systems. Figure based on Zhang et al.[140]

biosynthesis[140] (Figure 4): Leualacin is a cyclic depsipeptide which has two α-hydroxyacyl residues[140] and acts as a potent calcium channel blocker.[141] The five-module NRPS responsible for its biosynthesis was identified by genome sequencing and gene disruption.[140] Based on the collinearity rule, it is assumed that the A domains in Modules 2 and 4 select α-hydroxy-isocaproic acid (leucic acid), and the C domains of these modules almost certainly perform ester bond formation.

Other depsipeptides for which ester bond formation occurs during elongation and is catalyzed by a C domain include the destruxins[131,132,142,143] and sansalvamide.[132] Destruxins are cyclic insecticidal molecules synthesized in fungal entomopathogens by a 6-module NRPS.[142] Module 2 is likely responsible for inserting the ester bond, starting with direct selection of the α-hydroxy-isocaproic acid by the A domain.[132,143,144] Timing of the ester bond formation step was controversial, with elongation[143] and terminal cyclization[131] both proposed. Cluster analysis of a BGC for a similar molecule, sansalvamide[132] lent support to the elongation proposal, which is now broadly accepted.[144]

3.1.1.2 | Ester formed with main chain hydroxyl originating from keto acid substrate

Bacterial depsipeptide synthetases also form ester bonds during elongation. In contrast to the case in fungi, these hydroxy acids usually arise from specialized modules that select and reduce keto acids. As is clear from this review, the use of these specialized modules is not the only pathway NRPSs employ to insert esters, but their domain composition is dedicated for incorporation of hydroxy acid residues into dipeptides and are thus called "depsipeptide modules".[34]

Discovery, characterization, and an example of the "depsipeptide module" (C)-A-KR-PCP. Though the genes responsible for the production of the bacterial depsipeptide valinomycin were identified as early as 1990,[145] sequencing and annotation of the BGCs for valinomycin[109] and the similar compound cereulide[146] occurred ~15 years later. This sequencing suggested that a KR domain, related to KR domains of PKSs, was inserted into A domains in two modules of these depsipeptide synthetases. The KR insertion site in A domains of depsipeptide modules for cereulide[33] and other bacterial depsipeptides[38,147,148] was proposed to be between motifs A8 and A9 of the A_{sub} domain. This would create an interrupted A domain arrangement similar to that in the recently determined structure of an A-methyltransferase didomain.[23]

BGCs for both valinomycin and cereulide have two NRPS genes that encode 4-module, 2-subunit NRPSs.[109,146] The initiation module has domains A-KR-PCP, and the third module has domains C–A-KR-PCP. The domains in third modules are split between the two subunits, with the C domains the last domains of the first subunit (CesA/Vlm1) and A-KR-PCP the first three domains of the second subunit (CesB/Vlm2), meaning each subunit starts with domains A-KR-PCP. Magarvey et al. sub-cloned the two A-KR-PCP constructs from cereulide synthetase (Ces) and performed functional assays which revealed crucial information about depsipeptide synthetases.[33] It was shown that the A domains displayed high selectivity for α-keto acids over α-hydroxy acids and α-amino acids of either chirality,[33,147] and that the A domain also had side chain specificity.[34,147,149] In addition, keto reduction was shown to be NADPH-dependent and highly stereospecific.[33] The substrate for ketoreduction was shown to be the tethered ketoacyl-*S*-PCP, delineating the sequence of reactions in these modules (Figure 5b and 6a).

Since this initial characterization, scores of BGCs have been shown to include (C)-A-KR-PCP depsipeptide modules. These modules have an easily recognizable indicator of hydroxy acid incorporation in NRPSs. Among the bacterial depsipeptide synthetases with (C)-A-KR-PCP depsipeptide modules are kutzneride,[147,150] antimycin,[18] didemnin,[151] cryptophycin,[38,128] cereulide,[33] and valinomycin.[152,153] Most substrate α-keto acids originate in bacteria from branched-chain amino acid biosynthesis, while pyruvic acid originates from glycolysis.[154] Kutzneride synthetase[147] has a single depsipeptide module as its Module 2, and except for having a bypassed PCP domain situated after the C domain in KtzE (Figure 5a) is a straightforward example for hydroxy acid residue addition into bacterial depsipeptides:

The kutznerides are a family of cyclic hexadepsipeptides with antimicrobial and antifungal properties[147,150] (Figure 5a). Kutzneride synthetase has six modules in its four subunits, KtzE-G-H-N. The substrate for Module 1 is the unusual amino acid (1-methylcyclopropyl)-D-glycine (MecPGly; made by KtzB, D, A, F). Module 2, spread across KtzE and D, has the domain architecture C_2-PCP_{2a}–A_2-KR_2-PCP_{2b} (Figure 5b). A_2 selects α-ketoisovaleric acid (or 2-keto-3,3-dimethylbutanoic acid), activates it by adenylation, and transfers the ketoacyl moiety to PCP_{2b}. The α-ketoisovalyl-*S*-PCP_{2b} is reduced by the imbedded KR_2 domain using the co-substrate NADPH, producing α-hydroxyisovalyl-*S*-PCP_2 (HIV-*S*-PCP_2). This will act as the acceptor substrate for C domain-mediated ester condensation with donor MecPGly-*S*-PCP_1, giving MecPGly-HIV-*S*-PCP_2. MecPGly-HIV-*S*-PCP_2 will be donor substrate for C_3-mediated amide bond formation, liberating PCP_2 for the next round of depsipeptide synthesis[33,34,38,128,147,150,152,153] (Figure 5b). Notably, the only other characterized depsipeptide synthetase to insert a single hydroxy acid

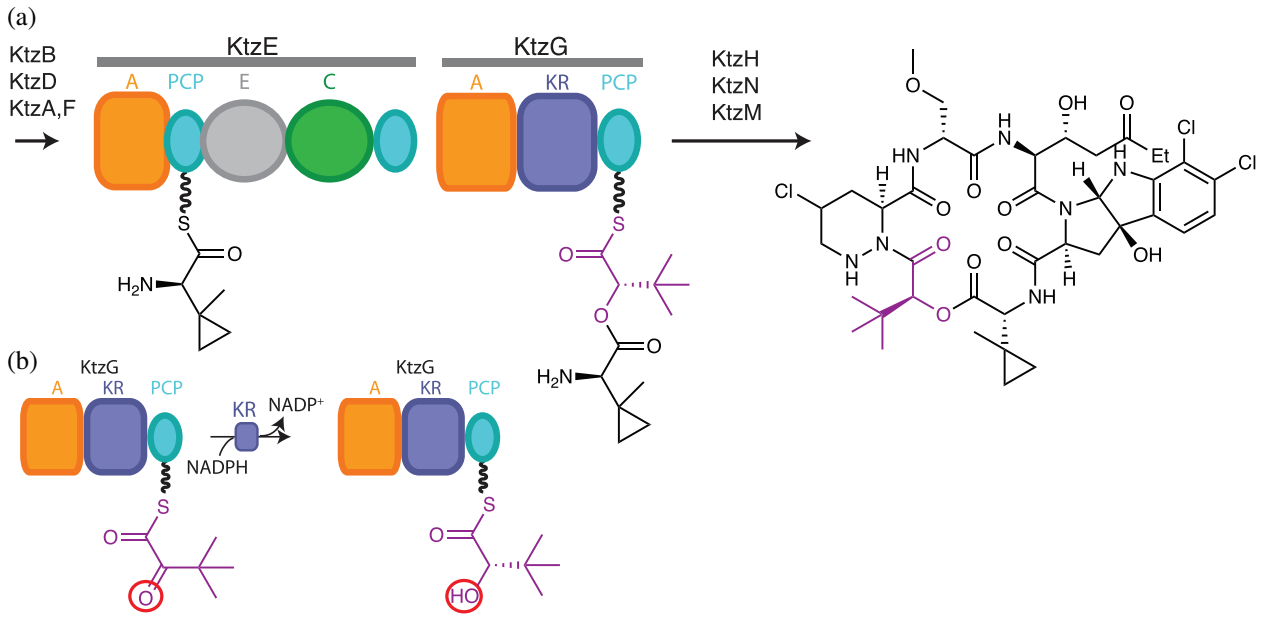


FIGURE 5 Proposed kutzneride biosynthesis.[147] (a) The C domain in KtzE catalyzes ester bond formation using an α-hydroxyacyl thioester (magenta) in KtzG as acceptor substrate. (b) The A-KR-PCP module KtzG generates α-hydroxy acids from α-keto acids. See also Figure 6

residue during NRPS elongation is involved in the biosynthesis of the antibiotic himastatin, which is shares >60% sequence identity and also features a C-PCP–A-KR-PCP module.[155,156] Mature himastatin has a peculiar dimeric structure introduced by postsynthetic tailoring.

Structural studies of a depsipeptide module. Until this year, no structural information was available for depsipeptide modules. That changed with our report of the crystallization of the initiation A-KR-PCP module from *Bacillus stratosphericus* LAMA 585 stratospherulide synthetase subunit A (StrA) and its structure determination[34] (Figure 6). This structure reveals a very surprising architecture for depsipeptide modules. Contrary to the predictions of an interrupted A domain,[33,38,147,148] StrA A_1 is completely intact, with full-length A_{core} and A_{sub} domains (Figure 6b). The KR domain is directly downstream of the intact A_1 and connected by a short linker. Even more surprisingly, there was clear density that allowed visualization of another small domain, located in space next to A_{sub} and between the A_{core} and KR domains (Figure 6b). This domain has the same fold as an A_{sub} domain and was termed a pseudo A_{sub} domain (Figure 6b–d). The pseudo A_{sub} is a dramatically split domain. Around two thirds of this domain comes from a sequence C-terminal of the KR domain, and approximately one third comes from the very N-terminus of the protein, meaning that residues over 1,100 amino acids apart in sequence assemble into a folded unit (Figure 6b, c). The N-terminal portion of the pseudo A_{sub} is connected to the intact A domain by a long linker. Deletion of the pseudo A_{sub} or shortening of the linker gives constructs that are unable to perform depsipeptide synthesis and have reduced stability. Bioinformatics strongly suggests that known depsipeptide modules feature an intact A domain and a split pseudo-A_{sub}.[34]

The revelation that the A domain is intact allowed us to interrogate the mechanism by which depsipeptide synthetase A domains are selective for α-keto acids.[34] The specificity-determining residues for keto acid-selecting A domains had been noted to contain a substitution of residue Asp235 (PheA numbering[4]) by an aliphatic residue,[147] but it was not clear if/how that allowed α-keto acid selection. Studies of the promiscuous A domain McyG, which selects unusual phenylpropanoids and which also has an aliphatic residue in place of Asp235, showed that Asp235 enhances binding of the unusual hydrophobic monomer, but the accompanying co-crystal structure had phenylalanine bound.[99] To elucidate the mechanism of keto acid selection, we excised the intact A domain and determined high resolution structures of it bound to its in situ formed α-keto adenylate (Figure 6e).[34] These structures revealed that the isoleucine (Ile306) in the analogous position to PheA Asp235[4] was not involved in α-keto acid binding. Moreover, there is no hydrogen bonding at all between the substrate α-keto acid and the A domain. Instead, the α-keto moiety interacts through an antiparallel carbonyl-carbonyl interaction with the backbone of Gly414. Antiparallel carbonyl-carbonyl interactions have been previously

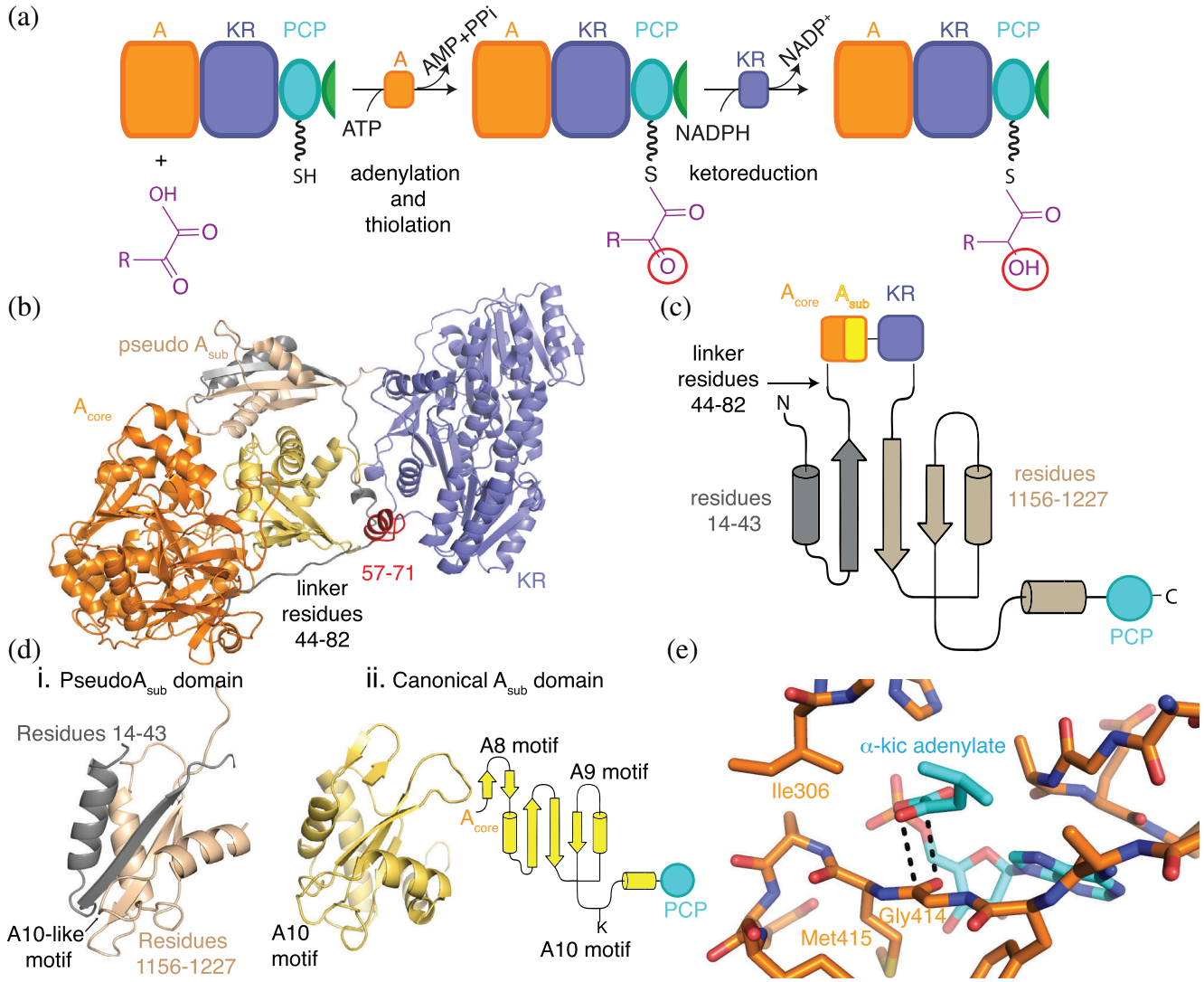


FIGURE 6 An initiation depsipeptide (A-KR-PCP) module. Note that elongation depsipeptide modules include the C domain (C-A-KR-PCP). (a) Specialized A domains activate α-keto acids by adenylation and attach the keto residue to the PCP domains for transport to ketoreductase (KR) domains within the same module. KR domains reduce the α-keto group, generating α-hydroxy acyl thioesters, which can be used in downstream reactions. Cartoon (b) and topology (c) representations show that the A domain has integral A_{core} (orange) and A_{sub} (yellow) domains in the monomer structural model of the A-KR-PCP module of StrA from *Bacillus stratosphericus* LAMA 585 (based on dimeric structure PDB ID: 6ULW[34]). A small linker connects the A_{sub} and KR (purple) domains, followed by a small domain (wheat and gray) with a tertiary structure (d) that resembles a canonical A_{sub} domain. (e) α-ketoacids bind to A domains through an antiparallel carbonyl-carbonyl interaction (PDB ID: 6ULX, 6ULY). Figure adapted from Alonzo et al.[34]

characterized in small molecules[157]and are involved in the stabilization of unfavorable protein tertiary structure.[158] We observed that the interaction in StrA is facilitated by the presence of an active site methionine that substitutes for a highly conserved proline found in amino acid-selecting A domains. The substitution effectively creates a flat conformation of Gly414 and Met415 (Figure 6e) that favors the stacking interaction. Mutation of the methionine to proline abolished keto acid selectivity, indicating that the disruption of the flat surface disrupts the carbonyl-carbonyl interaction. A domains selective for aryl acids (which lack and α-amino/hydroxy/keto motiety) also harbor the proline to methionine substitution.[57,159,160]

The larger structure from the crystals of the entire A-KR-PCP module also enabled the first visualization of an NRPS KR domain.[34] As predicted, the overall fold of the KR domain is similar to that of PKS KR domains and most closely resembles β-ketoreducing PKS KR domains.[33] These KR domains are pseudo-dimeric with the N-terminal Rossmann-like fold having a structural role and the C-terminal one containing the active site

with its Tyr-Ser-Lys catalytic triad.[161–163] They can be classified according to the stereochemistry of their product, A-type producing L-β-hydroxy acids and B-type producing D-β-hydroxy acids.[136,137] The type is distinguishable from sequence in PKSs, with a highly conserved Trp in type A and a Leu-Asp-Asp in type B KR.[164] In both types, the 4′-*pro*-S hydrogen faces the active site Tyr. The chirality of the product is determined by the approach of the β-ketoacyl-*S*-ACP, with the conserved motifs directing the β-ketoacyl-ACP to one or another ACP binding site with grooves reaching the active site from two opposite directions.[137,165] Depsipeptide KS domains have no identifiable sequence motifs[34,128] analogous to those found in PKS KR domains,[137] and both putative PCP binding sites and grooves to the active site appear feasible in the KR domain of StrA.[34] Despite being present in the crystals of StrA A-KR-PCP, the PCP was disordered. The mechanism of stereospecificity in NRPS KS domains awaits biochemical interrogation and structure determination of depsipeptide modules with each stereospecificity in their KR conformations, such as those underway in our lab. Nonetheless, the study revealed how keto acids can be used for ester bond formation in depsipeptide modules.

4 | DEPSIPEPTIDES WITH ESTER BONDS FORMED DURING TERMINATION

4.1 | The C domain can catalyze termination in depsipeptide synthesis

NRPSs employ a series of termination mechanisms, several of which can result in ester bond formation during peptide release. In fungal NRPSs, including peptide and depsipeptide synthetases, a common pathway of termination uses C-terminal, cyclizing C domains (C_T domains).[62,166] These C_T domains catalyze nucleophilic attack on the ester carbon of the terminal peptidyl-*S*-PCP by a nucleophile within the peptidyl intermediate, which cyclizes and releases the peptide. Cyclizing C_T domains cluster together phylogenetically,[166,167] suggesting one or few founding C_T domains may have first evolved this specialization. The best known nonribosomal peptide whose biosynthesis terminates in this way is cyclosporin A[10,168–170] (Figure 1) and the best characterized C_T domain responsible for terminal cyclization is that in the biosynthesis of fumiquinazoline F.[62,166,171] Structures including C_T domains show them to be very similar to elongating C domains, but with adaptations for an active site dedicated to cyclization instead of elongation, such as a narrower solvent channel, a blocked acceptor site and an overall more compact conformation, as observed in TqaA.[62] Neither cyclosporin nor fumiquinazoline F is a depsipeptide, but the knowledge of amide forming C_T domains should be relevant for ester forming C_T domains.

4.1.1 | Ester bond formation during termination by a C domain in noniterative synthesis

4.1.1.1 | Ester formed during cyclization with side chain hydroxyl

Several C_T domains which introduce ester bonds are found in hybrid PKS-NRPSs. Thermolides are nematocidal polyketide macrolactones which include a single amino acid that are produced by *Talaromyces thermophilus* strains (Figure 7).[172] The polyketide portion is generated by the highly reducing PKS ThmA, while the single amino acid in the structure is added by ThmB, an NRPS subunit with domains C-A-PCP-C_T. After amide bond formation between the amino acid and the polyketide intermediate in the first C domain, the C_T domain catalyzes cyclization by ester bond formation using a hydroxy group in the polyketide as a nucleophile. The cyclization activity of C_T was confirmed by site-directed mutagenesis of key residues in its active site.[172]

Other depsipeptides that are synthesized through ester bond forming cyclization are antalid,[167] the potent macrolide immunosuppressants rapamycin (made famous by its target, mammalian target of rapamycin [mTOR]), and the similar compounds FK506 and FK520. The latter molecules are pipecolate-containing polyketides[173,174] made by BGCs,[173] with three PKS genes and one four-domain NRPS with the domain arrangement C-A-PCP-C, named RapP or FkbP. Nielson et al. showed ATP-dependent pipecolate adenylating activity as early as 1991 for FkbP,[175] and sequencing, gene disruption and heterologous expression experiments characterized the analogous role for RapP in rapamycin biosynthesis a few years later.[174] The A domain activates pipecolate, the first C domain condenses the aminoacyl thioester to the polyketide core,[173] and it is believed that the second C domain catalyzes hydroxyl group 26 attack for ester bond forming cyclizing release.

4.1.1.2 | Ester formed during cyclization with a hydroxyl from a small molecule acceptor

Asperphenamate is a fungal linear depsipeptide[114,176,177] made of two phenylalanine and two benzoic acid residues assembled in an interesting way (Figure 8).[114] Through gene deletion, in vitro work, HPLC, NMR, and MS identification, Li and colleagues[114] showed that two dimodular

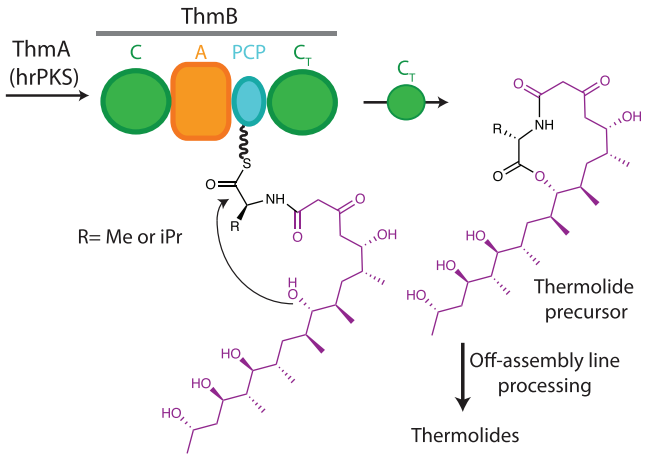


FIGURE 7 Thermolide biosynthesis. The C_T domain catalyzes cyclization using a hydroxy group from the polyketide moiety (magenta) as a nucleophile, forming the lactone portion of the thermolide precursor (right)

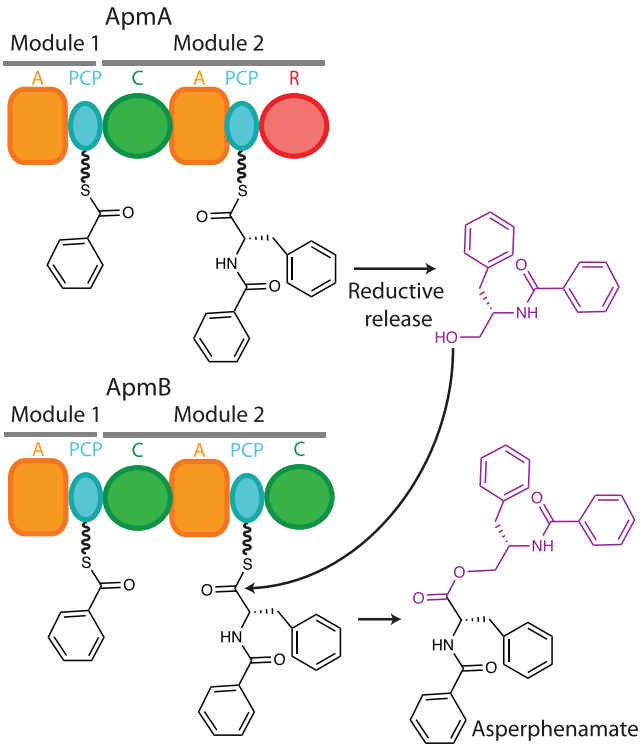


FIGURE 8 Proposed asperphenamate biosynthesis based on Li et al.[114] The R domain in ApmA releases *N*-benzoylphenylalaninol, which acts as a nucleophile in the ester bond forming reaction in the C-terminal C domain of ApmB, releasing asperphenamate. Note that the famous compound taxol has a depsipeptide moiety that resembles asperphenamate, but taxol is synthesized by yew trees in a completely different manner[251]

NRPS, ApmA, and ApmB, make asperphenamate and proposed a biosynthesis pathway. ApmA has domains A-PCP-C-A-PCP-R, and ApmB, domains A-PCP-C-A-PCP-C_T. ApmA assembles *N*-benzoylphenylalaninyl-$PCP_{2\text{-ApmA}}$, which its terminal R domain, via four electron reductive release, turns into free *N*-benzoylphenylalaninol. This is proposed to be the small molecule used by the C_T domain of ApmB as the acceptor molecule in reaction with the *N*-benzoylphenylalaninyl-$PCP_{2\text{-ApmB}}$ donor, which couples the two dipeptide intermediates to the final linear depsipeptide asperphenamate. Although this role for the C_T domain of ApmB has not been formally shown, it would not be unexpected, as terminal C domains which catalyze release though condensation with small molecule acceptors are known in bacillamide,[28,63] wortmanamide B[178] and C-1027[64,125] biosynthetic pathways.

There are sure to be many other depsipeptides and hybrid-PKS-depsipeptides for which C domains install ester bonds. Other review articles[104,106] highlight a variety of compounds which include single α-hydroxy acyl residues, but most of their BGCs have not been identified or characterized. Biochemical and biophysical characterization of these biosynthetic enzymes will shed more light on these processes in the years to come.

4.2 | The TE domain can catalyze ester bond formation in depsipeptide synthesis

One of most straightforward routes to produce a cyclic nonribosomal depsipeptide is to introduce the ester bond in the release step, exemplified in Section 4.1 by fungal C_T domains. In bacteria, this is very commonly catalyzed by the terminal TE domains. Since the TE domain is so central to depsipeptide synthesis (as well as other nonribosomal peptide and polyketide synthesis), a short general summary of TE structure and function is warranted here (Figure 9).

TE domains are α/β hydrolase proteins related by evolution and mechanism to serine proteases.[58,87] TE domains catalyze chain release through two half reactions using a catalytic Ser-His-Asp triad (Figure 9a).[58,87–92] In the first half reaction, the His residue increases nucleophilicity of the Ser hydroxy by proton abstraction, allowing attack on the thioester of the peptidyl-*S*-PCP, forming a covalent acyl-*O*-TE intermediate. In the second half reaction, a nucleophile (R in Figure 9a) attacks the acyl-*O*-TE intermediate, displacing the Ser oxygen and releasing the chain from the TE domain. The nucleophile can be water or a hydroxy or amine group, leading to carboxylic acid, ester, or amide formation.[94] If the nucleophile is water, linear peptide is released.[179] If the nucleophile is from within the peptide intermediate (side chain or first residue) a cyclic (depsi)peptide is released (as a branched or unbranched macrocycle, respectively).[180,181] If the nucleophile is contained within

the next peptide intermediate (peptidyl$_{n+1}$-*S*-PCP), oligomerization occurs (peptidyl$_n$-peptidyl$_{n+1}$-*S*-PCP).

Several TE domains from NRPS and PKS systems have been structurally characterized[58,88–92,153,182,183] (Figure 9b). They show the α/β hydrolase fold, the active site triad and a putative oxyanion hole to stabilize the negatively charged tetrahedral transition state. The structures also reveal an element inserted into the α/β hydrolase fold between strands 6 and 7, which is positioned on top of the active site, and was thus called "the lid" (Figure 9b). This element has high sequence and structural variability within TE domains, from a simple two-helix structure (e.g., DEBS-TE[88]), to subdomains of >100 residues (e.g., AB3403-TE[85]). The lid has been proposed to act as a solvent shield, to interact with the PCP domain, and/or interact with the peptide intermediate,[95] but correlation of lid character with substrate length, complexity or release mechanisms has not been forthcoming.[58] Lids can be mobile, having been observed in different positions or refolded in structures of the same TE domain[153,184] or be fully or partially disordered in structural studies.[89,185]

TE domains must interact with the PCP domain for their first half reaction. The best first views of this interaction come from apo PCP-TE NMR[90] and holo PCP-TE crystal[95] structures from enterobactin synthetase (Figure 9b). They show PCP-TE contacts and show a crevice for ppant binding formed by one of the helices of the lid and the active site canyon. The crystal structure shows putatively important contacts of the ppant arm with core and lid in the TE domain.

Attempts to generate intermediate-bound acyl-*O*-TE structures, representative of the product of the first half reaction and the substrate of the second half reaction, are hampered by the relative short lifetime of the acyl ester intermediates.[185,186] A few structures of TE-substrate complexes exist, using phosphonate analogues[182,183,187] or employing an expanded genetic code strategy to generate stable amide intermediates[153] (discussed in Section 5.2.2.1). In the analog-bound structures of the pikromycin TE bound to a pentaketide phosphonate,[187] the linear pikromycin intermediate curls back in the cavity between the lid and active site toward the catalytic Ser. It was proposed that a "hydrophilic barrier" formed by the lid and ordered water favors the cyclization conformation. There is very little residue-level conservation between lids of TE domains.

4.2.1 | Ester bond formation during termination by cyclization by TE domains in noniterative synthesis

4.2.1.1 | Ester formed during cyclization with side chain hydroxyl

TE-mediated cyclization commonly uses hydroxy groups from side chains of upstream residues in the peptide intermediate as final acceptor nucleophiles (Figure 10). In a very common case, the TE domain acts on a fully elongated linear peptidyl-PCP. In the first half reaction, the peptidyl moiety is transferred to the active site Ser of

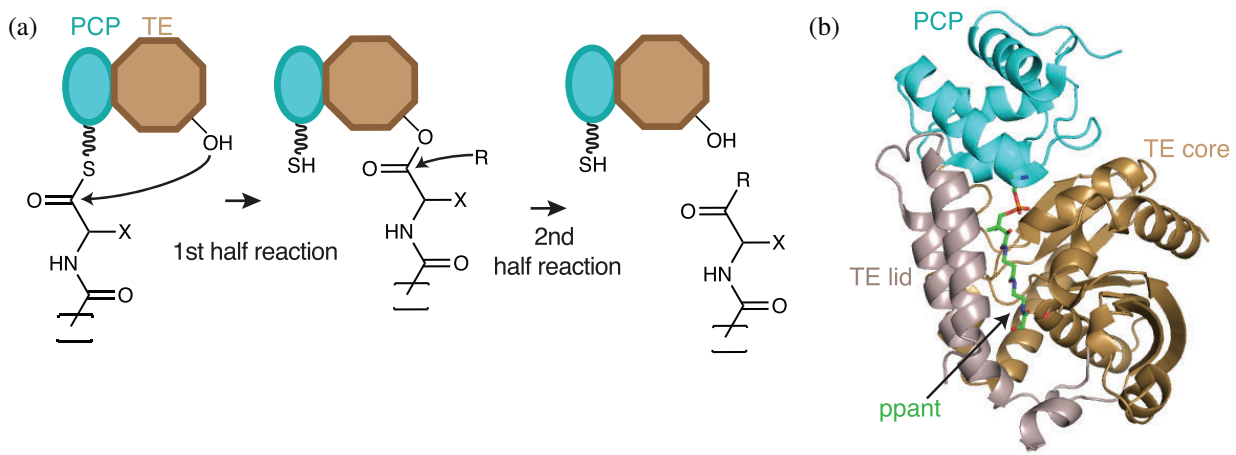


FIGURE 9 The thioesterase (TE) domain. (a) TE domains (brown) use an active site Ser (or, in rare instances, a Cys) residue to attack the elongated acyl thioester chain in the first half reaction. The resulting ester (thioester in case of Cys) is released by nucleophilic attack in the second half reaction. The nature of the incoming nucleophile determines the chemistry of the final product. (b) Cartoon representation of the holo-PCP-TE structure of entF (PDB ID: 3tej).[95] The TE domain has an α/β hydrolase fold formed by core (brown) and lid (grey) regions. TE lids are variable from protein to protein, and can be mobile

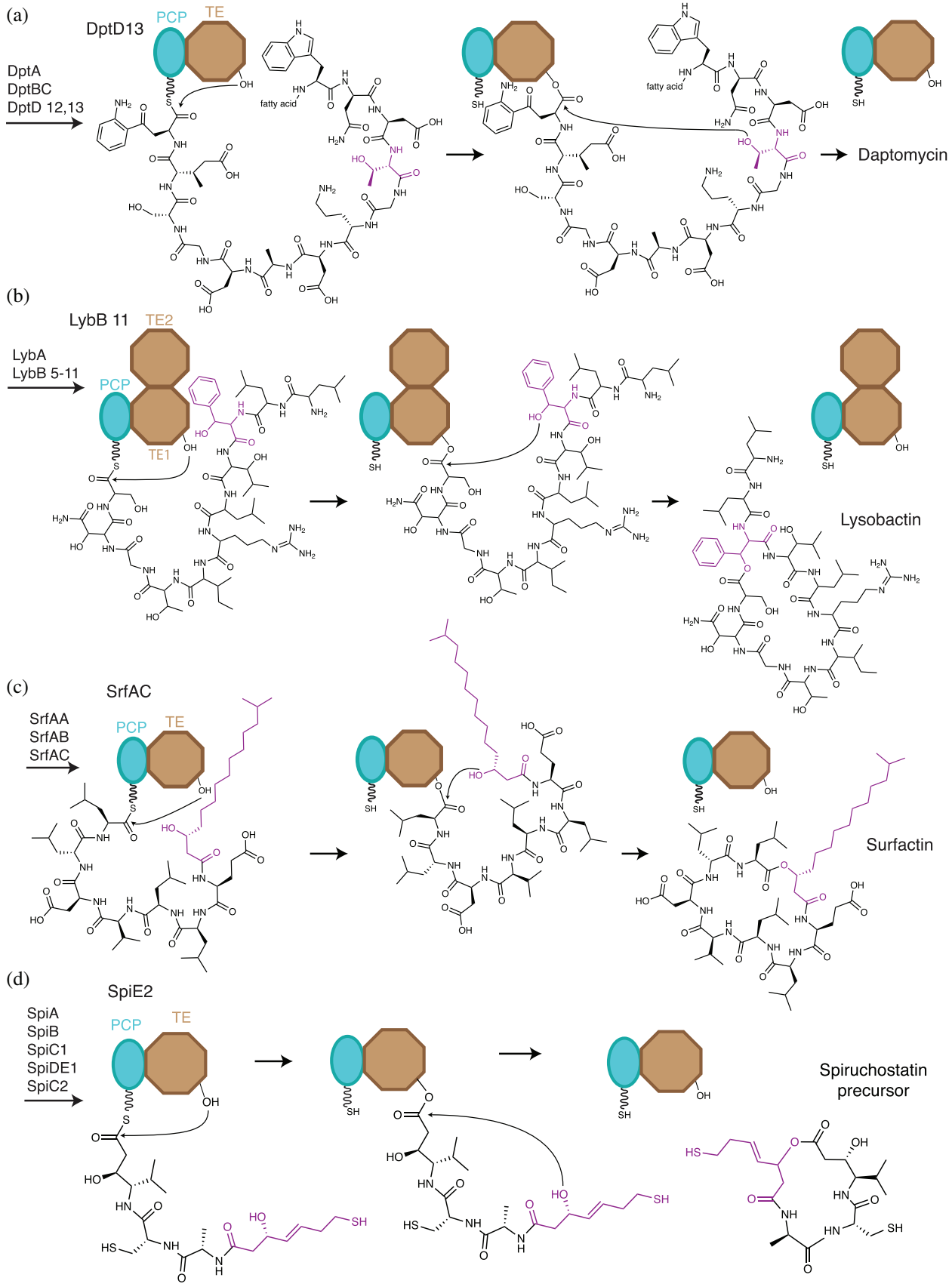


FIGURE 10 Legend on next page.

the TE domain forming a peptidyl-*O*-TE intermediate. In the second half reaction, the TE domain catalyzes ester bond formation by nucleophilic attack of the side chain OH on the peptidyl ester, which is followed by cyclization and release of the cyclic nonribosomal depsipeptide.[185] Two examples are in synthesis of daptomycin, where the nucleophile is a threonine side chain hydroxyl[188] (Figure 10a) and fengycin, where the nucleophile is a tyrosine side chain hydroxyl.[189]

Daptomycin is of high clinical relevance. Sold under the brand name Cubicin, it is a half-billion dollar cyclic lipodepsipeptide antibiotic used to treat severe skin infections.[8] Daptomycin synthetase is a 13-module, 3-subunit NRPS[190] whose TE domain can also accept small molecule thiophenol analogues of peptidyl-*S*-PCP substrates[40] for chemoenzymatic synthesis. Teixobactin, another potent antibiotic against Gram-positive bacteria,[191] is also released from its assembly line by cyclization with a Thr sidechain hydroxyl acceptor in a way very similar to daptomycin. Fengycin is a decadepsipeptide with antifungal activity that is cyclized through a mechanism similar to daptomycin, using the sidechain OH in residue Tyr4 as a nucleophile.[189] Structures of its TE domain show a single active site lid conformation and allowed the authors to unambiguously identify its oxyanion hole.[185]

The hydroxyl nucleophiles in the above two examples derive from proteinaceous amino acids, but other systems use moieties installed during co-synthetic hydroxylation tailoring. Cyclization through hydroxylated amino acids occurs in polyoxypeptin A, lysobactin and obafluorin synthesis.[102,192,193] In polyoxypeptin A biosynthesis, a P450 enzyme is proposed to catalyze the hydroxylation of leucine to produce a β-OH-Leu moiety containing the nucleophile for ester bond formation.[193] The nucleophilic hydroxyl in lysobactin biosynthesis[192] is installed in a similar way by a tailoring enzyme which hydroxylates Phe-*S*-PCP to β-OH-Phe-*S*-PCP (Figure 10b). A peculiarity of the lysobactin biosynthesis system is that the gene for the termination module has two TE domains in tandem. The first TE domain is the normal "type I" TE, catalyzing cyclization and release, while the second is likely proteolyzed to a bona fide free-standing type II TE domain in vivo. (Type II TE domains that hydrolyze misprimed PCP domains to rescue stalled NRPSs). In obafluorin biosynthesis, the TE domain has an altered triad composed of Cys-His-Asp, with a Cys in place of the normal Ser, and where the Asp is in a different topological position from Asps of other TE domains. This positioning is suggested to help in the transthioesterification reaction from PCP to TE thioester.[102]

The hydroxyl which is used in ester bond formation in depsipeptides need not come from an aminoacyl side chain. Cyclic lipo-depsipeptides and polyketide-depsipeptides are common, and cyclization often occurs through a hydroxy nucleophile from a hydroxylated acyl/ketide chain. Around 20% of depsipeptides in the De Spiegeleer chemical classification are hybrids.[104] For example, romidepsin, or FK228, is an FDA approved anti-cancer made in *Chromobacterium violaceum* as a cyclic polyketide-depsipeptide prodrug containing a disulfide, which is reduced to form the active drug upon cell entry.[194] Romidepsin acts as a histone deacetylase inhibitor and shows promise for treatment of schistosomiasis, a widespread parasite disease with few treatment options.[195,196]

Another well-known example is surfactin, a 7-member lipodepsipeptide with tenside, antitumoral, and antibiotic properties, which is produced by one of the best studied BGCs.[184,197–199] Surfactin is generated by the NRPS subunits SrfAA, SrfAB, and SrfAC working in conjunction with fatty acid biogenesis[12] (Figure 10c). The fatty acid substrate 3-hydroxy myristic acid is activated to a CoA thioester by a fatty acid CoA ligase to be used in surfactin biosynthesis.[12] Then, the N-terminal C domain in SrfAA catalyzes amide bond formation between the fatty acid and the glutamatyl-PCP. The synthesis proceeds through normal elongation steps in SrfAB and SrfAC until linear lipopeptidyl-*O*-TE is formed. Finally, the TE domain catalyzes cycle formation using the OH group in the distal hydroxylated lipid for cyclization and surfactin release (Figure 10c).[186,199]

The structure of SrfAC-TE was determined in 2002 by Bruner et al.[186] One of the most interesting findings in that study was that the active site lid was in two different conformations in the structure. This was the first structural evidence that the TE domain lid can be very mobile. The authors suggested that it could act as a solvent shield during the cyclization reaction. SrfAC was also a subject of the landmark study, which included the first full (termination) module structure of an NRPS.[86] Among many other vital insights, this and subsequent structures of termination modules,[85,96] showed that the TE domain does

FIGURE 10 Ester bond formation by cyclization in TE domains. Final steps of (a) daptomycin, (b) lysobactin, (c) surfactin, and (d) spiruchostatin biosynthesis. For simplicity, only the last two domains of the NRPS systems are represented. Cyclization occurs by nucleophilic attack of a hydroxy group within the acyl-ester intermediate on its own ester carbon linked to the active site serine. The hydroxy can be located in (a) the side chain of a proteogenic amino acid residue, (b), a β-hydroxylated amino acid residue (c) a fatty acyl moiety or a (d) polyketide moiety

not have a fixed position relative to the rest of the termination module. Although tethered to the C-A domain platform through the PCP domain, the TE domain operates independently of the other catalytic domains in the module.

The spiruchostatins are well-characterized examples of polyketide-depsipeptides (Figure 10d). Spiruchostatins are antineoplastics, originating from a hybrid PKS-NRPS biosynthetic cluster.[200] After condensation of the PKS and NRPS portions and chain elongation, the mixed linear ketide-peptidyl intermediate is released by the TE domain through ester bond formation with a hydroxyl in the ketide portion. The released polyketide-depsipeptide undergoes uncatalyzed disulfide bond formation to make mature spiruchostatin. Other polyketide-depsipeptides that very likely have analogous release steps include FK228 (romidepsin),[201] largazole,[202] and virginiamycin.[203]

4.2.1.2 | Ester formed using hydroxyl from a small molecule acceptor

Like terminal C domains, TE domains can employ small molecule acceptors to produce linear depsipeptide products, for example in ansatrienin biosynthesis. Ansatrienin is a depsipeptide antibiotic of the ansamycin family,[204–206] with a cyclic polyketide core attached through an ester bond to a cyclohexanoyl-D-alanine branch (Figure 11). The *Streptomyces collinus* ansatrienin BGC was identified as containing PKS and NRPS genes in 1999.[206] Through experiments including gene disruption of *astC* (which encodes an A-PCP-TE tri-domain NRPS), Shi and colleagues showed conclusively that AstC is responsible for the activation and addition of D-alanine to the cyclic polyketide core.[205] The TE domain would thus release a D-alanyl-polyketide, to which the cyclohexanoyl moiety is later attached, through the alaninyl amino group, by a standalone acyltransferase.

The TE-catalyzed release via a small molecule acceptor mirrors that in mycolic acid biosynthesis where trehalose is the acceptor.[207]

4.2.2 | Ester bond formation during termination by oligomerization and cyclization in iterative TE domains

4.2.2.1 | Ester formed with side chain hydroxyl

Another well-known mode of action for certain NRPS TE domains is multi-functional. Rather than acting only once per biosynthetic cycle, TE domains such as those in NRPSs which synthesize gramicidin S in *Bacillus brevis*,[5] enterobactin in *Escherichia coli*[208–210] and bacillibactin in *Bacillus subtilis*[98,211] introduce multiple bonds: They first oligomerize linear peptidyl intermediates, then cyclize the resulting multi-peptidyl intermediate to release it. In gramicidin S synthesis, the TE domain oligomerizes a pentapeptidyl intermediate via its N-terminal main chain amine, and then cyclizes the resulting decapeptidyl intermediate to give the macrocyclic decapeptide gramicidin S. By contrast, for depsipeptides enterobactin and bacillibactin, oligomerization and cyclization occur through a side chain hydroxyl, resulting in depsipeptide compounds.

Enterobactin is a siderophore with extreme affinity for Fe (III),[15] which is a trimer of the dipeptide 2,3-dihydroxybenzoate-Ser (DHB-Ser) linked through ester bonds between serine side chain hydroxyls to the serine carbonyl of the adjacent dipeptide[210] (Figure 12). The general synthetic pathway has been clearly delineated.[210,212,213] The NRPS machinery, including EntE, EntB, and EntF (C-A-PCP-TE), proceeds with standard synthesis, leading to an EntF holding a DHB-Ser-*S*-PCP intermediate. This is passed to the catalytic serine of the TE domain (DHB-Ser-*O*-TE). The upstream machinery then assembles a second DHB-Ser dipeptide on the PCP. The TE domain catalyzes dimerization, and through one or more steps, the dimer is placed on the TE domain ($(DHB\text{-}Ser)_2$-*O*-TE), and a third DHB-Ser-*S*-PCP is formed on the PCP. The TE domain catalyzes trimerization, and through one or more steps the trimer is placed on the TE domain ($(DHB\text{-}Ser)_3$-*O*-TE). This intermediate is the substrate of the cyclization half reaction by the TE, and in an analogous manner to other cyclization half reactions described above, the TE domain catalyzes attack of the free Ser side chain hydroxyl on the ester bond linking the intermediate to the TE domain, and mature, cyclic enterobactin is released.

There are two possible pathways for the oligomerization for EntF generally described above[210] (Figure 12). Because the TE domain performs oligomerization, the substrates of the first oligomerization reaction are identical, differing only by their acyl-enzyme attachment to the NRPS. Therefore, both DHB-Ser-*S*-PCP and DHB-Ser-*O*-TE contain a potential hydroxyl side chain nucleophile, and both have an activated (thio)ester linkage to the NRPS. Formally, either dipeptide could provide the nucleophile and either the electrophile. Should the hydroxyl of the DHB-Ser-*O*-TE attack the DHB-Ser-*S*-PCP thioester, the dimer is formed on the TE domain, and this dimer could simply wait for the next DHB-Ser-*S*-PCP to form to attack and make the trimer. This is referred to as forward transfer. Conversely, should the hydroxyl of the DHB-Ser-*S*-PCP attack the DHB-Ser-*O*-TE ester, the dimer is formed on the PCP domain and must be transferred to the TE domain to liberate the PCP to receive the next DHB-Ser dipeptide. Likewise, the dimer would be passed onto the new DHB-Ser-*S*-PCP, forming the trimer $(DHB\text{-}Ser)_3$ on the PCP. Again, this

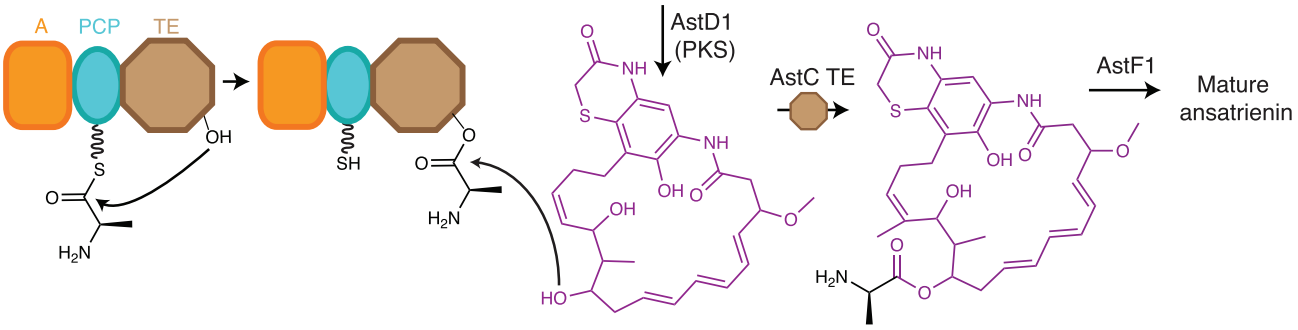


FIGURE 11 Proposed TE action in the Ast cluster in *Streptomyces sp*. XZQH13.[205] AstD1 generates a hydroxylated polyketide which acts as a small molecule acceptor, accepting *D*-Ala from the NRPS AstC. Mature ansatrienin requires downstream processing steps

would be passed to the TE domain for cyclization and release. This pathway is referred to as reverse transfer because intermediates are passed during oligomerization from the TE domain back to the PCP domain.

The forward transfer pathway (Figure 12b) was suggested by Walsh and colleagues[210] because they did not detect (DHB-Ser)$_2$-*S*-PCP or (DHB-Ser)$_3$-*S*-PCP intermediates in mass spectrometry of LysC digested EntF, but did detect DHB-Ser-*O*-TE and (DHB-Ser)$_2$-*O*-TE. (DHB-Ser)$_n$-*O*-TE are intermediates in both the reverse and forward mechanisms, and thioesters are more labile than esters, so the forward transfer pathway is not proven. In contrast, the reverse pathway has been definitively demonstrated for biosynthesis of valinomycin (see Section 5.2.2.1),[153] elaiophylin,[214] a macrodiolide side product from the erythromycin system,[215] conglobatin,[216] and gramicidin.[5] This precedence makes it more likely that biosynthesis of enterobactin and the similar compound bacillibactin employs reverse transfer.

As mentioned above, the EntF TE has been fundamentally important for structural understanding of PCP-TE interactions,[90,95] but the oligomerization pathway and the structural determinants of oligomer length are still not clear. Variations on the enterobactin biosynthetic theme include those for depsipeptides corynebactin, which is a trimer of tripeptides linked by the serine side chain hydroxyl,[15,217] and bacillibactin, a trimer of tripeptides linked by threonine side chain hydroxyls.[98,211] These are likely oligomerized and cyclized using the same iterative path as for enterobactin.

Somewhat further afield is serrawettin W1 (serratamolide), a surfactant lipodepsipeptide[218] of two symmetric fatty acyl-serinyl (FA-Ser) moieties joined through an ester bond between a ß-hydroxyl moiety in the fatty acid and the carboxyl group in Ser. As in enterobactin biosynthesis, its BGC includes an NRPS with domains C-A-PCP-TE.[219] It is likely that the A domain selects Ser and the C domain catalyzes amide bond formation with the activated fatty acyl to give FA-Ser-*S*-PCP, which is transferred to the TE domain, oligomerized by a forward or reverse pathway to (FA-Ser)$_2$-*O*-TE and cyclized.

5 | DEPSIPEPTIDES WITH ESTER BONDS FORMED DURING BOTH ELONGATION AND TERMINATION

Thus far, we have described BGCs for depsipeptides in which the ester bond is introduced in elongation (by C domains) or in termination (by C and by TE domains). Not surprisingly, these events can occur within the same biosynthetic cluster, and can be steps in either simple or complicated NRPS synthetic cycles. Bacterial NRPSs in which a C domain and the TE domain both install esters exist. These systems can be iterative or noniterative (see Section 5.2). It is likely that a simple, noniterative NRPS which has an ester installing elongating C domain and an ester installing terminating C domain, where each acts only once, also exists, but we are not aware of any.

5.1 | The same C domain can catalyze ester bond formation in elongation and termination

5.1.1 | Ester bond formation during elongation and termination by bifunctional iterative C_T domains

5.1.1.1 | Esters formed with main chain hydroxyls originating from hydroxy acid substrates

Enniatin, beauvericin,[129] and bassianolide[130] are structurally related entomopathogenic fungal toxins. They are cyclized oligomers of dipeptidols of an α-hydroxy acid and an amino acid[220] (Figure 13). Beauvericin and the enniatins consist of cycles of three dipeptidol residues, while bassianolide consists of a cycle of four dipeptidol residues. The biosynthetic clusters for enniatin,

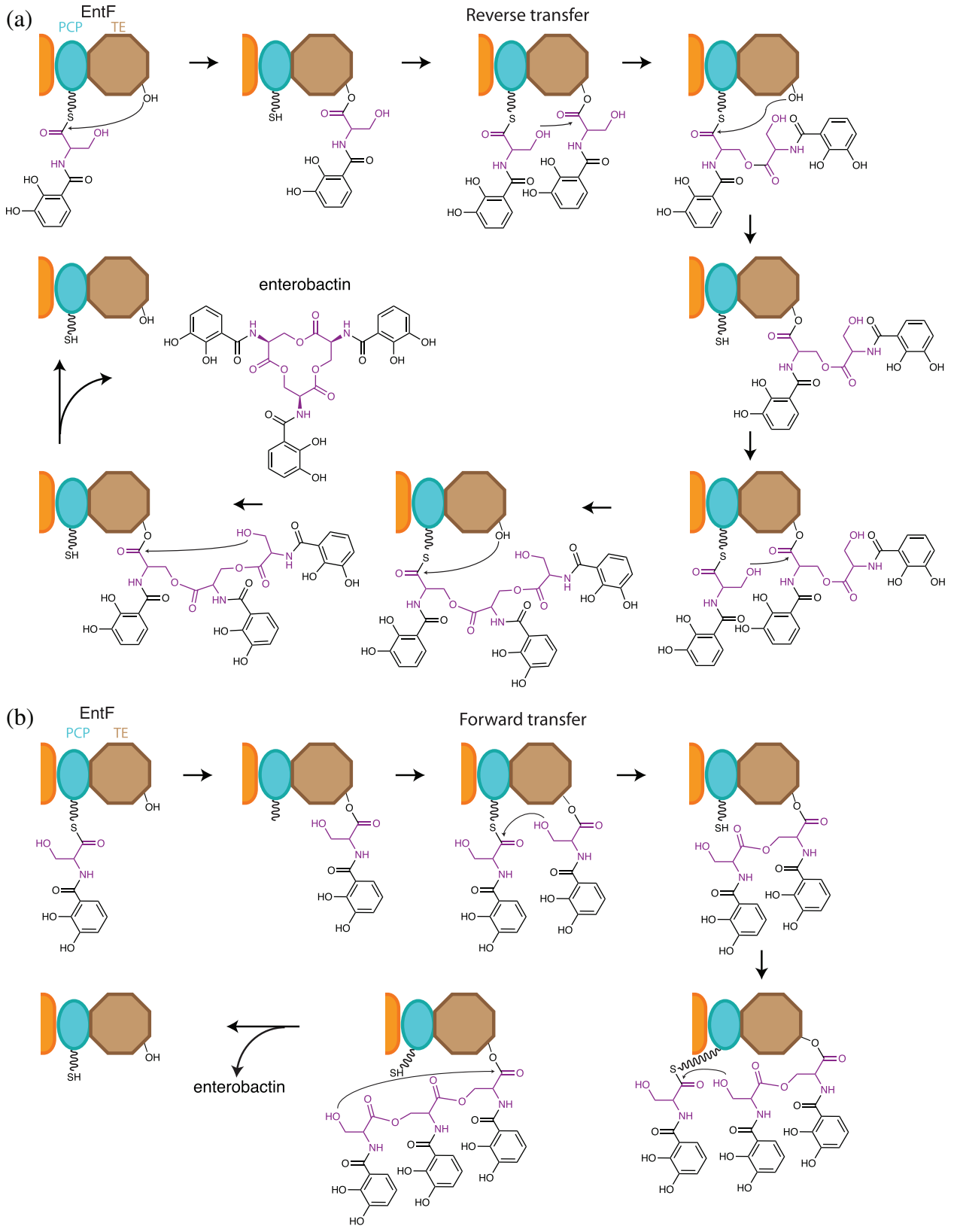


FIGURE 12 Legend on next page.

beauvericin, and bassionalide have been characterized.[126,127,129,130,221,222] Intriguingly, the three synthetases all have the same di-modular organization: C_1-A_1-PCP_1-C_2-A_2-MT-PCP_{2a}-PCP_{2b}-C_3[126,220] (Figure 13a), and a similar biosynthetic scheme, delineated independently by two groups[126,127] (Figure 13b): C_1 appears to be an inactive domain. A_1 adenylates its cognate α-hydroxy acid (D-Hiv for each synthetase) and transfers it to PCP_1. A_2 adenylates and transfers a cognate hydrophobic amino acid to either PCP_{2a} or PCP_{2b}. PCP_{2a} and PCP_{2b} are functionally redundant. *N*-methylation is likely installed at this point. Next, condensation by C_2 generates a dipeptidoyl intermediate on $PCP_{2a/b}$. Thereafter, an unusual elongation cycle is proposed, whereby PCP_1 and $PCP_{2a/b}$ alternate roles as acceptors and donor in condensation. First, A_1 produces a new hydroxyisovalyl-PCP_1, which acts as acceptor substrate in C_T-catalyzed condensation with dipeptidoyl-$PCP_{2a/b}$, installing the ester bond and making tridepsipeptidyl-PCP_1. Then, A_2 produces a new aminoacyl-$PCP_{2a/b}$, which is methylated and then acts as acceptor substrate in C_2-catalyzed condensation with tridepsipeptidyl-PCP_1 and making tetradepsipeptidyl-PCP_{2a2b} (i.e., an intermediate with two dipeptidol residues). One more analogous cycle of elongation occurs in biosynthesis of beauvericin and the enniatins, and two more in bassianolide, to produce the final linear hexadepsipeptidyl-PCP_{2a2b} (with three dipeptidol residues) or octadepsipeptidyl-PCP_{2a2b} (with four dipeptidol residues), respectively. These are substrates for C_T-catalyzed cyclization, with the terminal hydroxyl attacking the thioester and releasing the mature natural product.[223,224]

The fungal NRPS SidD also uses an unusual iterative pathway.[223,224] Fusarinine C is the precursor of the *Aspergillus fumigatus* virulence factor and siderophore triacetylfusarinine C. SidD has the architecture A_1-PCP_1-C_2-PCP_2-C_T. Notably, this includes only one A domain, which is specific for a very large, isopeptide bond-containing substrate, N^5-hydroxy-N^5-*cis*-anhydromevalonyl-ornithine. Fusarinine C is a cyclic trimer of this substrate. It will be assembled in an as yet unconfirmed pathway where A_1 performs all adenylation and thiolation, and the two elongation steps and final cyclization steps are performed by a combination of C_2 and C_T acting on PCP_1 and PCP_2 bound intermediates.

Both our biosynthetic classification and Taevernier's depsipeptide chemical classification[104] clusters enniatin, bassianolide and beauvericin together. However, it is notable that Taevernier includes bacterial depsipeptides such as antimycin, cereulide and valinomycin in the same class. This is appropriate chemically, but comparing the description of biosynthesis above to that for valinomycin in Section 5.2.2.1 illustrates how the biosynthetic pathways of fungal and bacterial depsipeptide synthetases can be strikingly different.

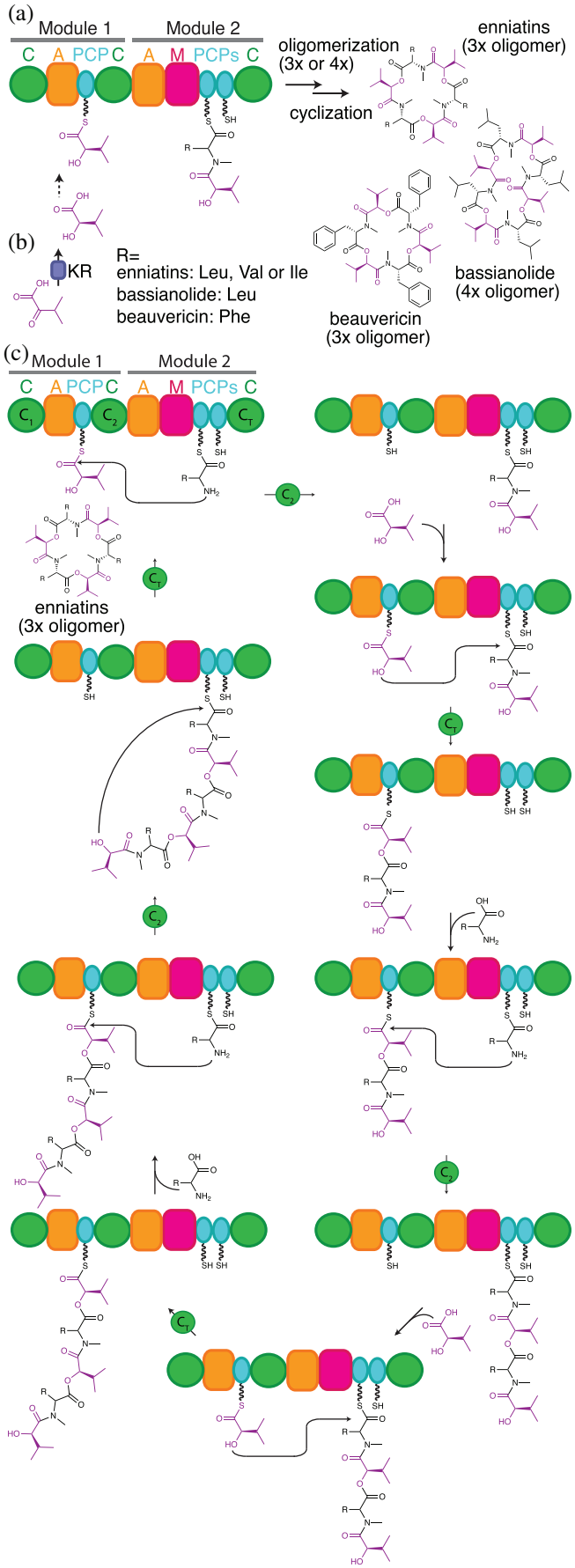
5.2 | The C domain and TE domain can both install esters into the same depsipeptide

5.2.1 | Ester bond formation during elongation by C domains and termination by TE domains in noniterative synthesis

5.2.1.1 | Ester formed with main chain hydroxyl originating from keto acid substrate (elongation) and from side chain hydroxyl (termination)

Cryptophycins are cyanobacterial depsipeptides with tubulin-depolymerizing properties,[225] whose synthetic analogues have been tested in phase II clinical trials for their anticancer properties.[38,226] There are more than 25 naturally occurring cryptophycin variants.[38,227] The two ester bonds in the structure are formed using an α-hydroxy acid and side chain hydroxyl, inserted during elongation and termination respectively. Subunits CrpA, CrpB, and CrpC synthesize a hybrid polyketide/peptide moiety, which is passed as a linear intermediate to CrpD (Figure 14), a di-modular NRPS subunit with a C-A-PCP-C-A-KR-PCP-TE architecture. The first module (CrpD1: C-A-PCP) adds β-alanine to the growing PKS-peptidyl chain, while the second module (CrpD2: C-A-KR-PCP-TE) adds α-hydroxyisocaproic acid (α-HIC) through α-keto acid activation and reduction in the A-KR domains[38,128,147](see below). The CrpD2 C domain uses the α-hydroxyacyl-*S*-PCP intermediate as an acceptor

FIGURE 12 Two proposed oligomerization mechanisms for EntF TE. In both scenarios DHB-Ser-*S*-PCP is formed on EntF and is transferred to its TE domain, generating DHB-Ser-*O*-TE. From there, the pathways differ. (a) Reverse transfer oligomerization pathway: A newly made DHB-Ser-*S*-PCP attacks DHB-Ser-*O*-TE, forming $(DHB\text{-}Ser)_2$-*S*-PCP. This is transferred to the serine of the TE domain. When a third DHB-Ser-*S*-PCP is made, another multistep cycle of this oligomerization mechanism takes place to make $(DHB\text{-}Ser)_3$-*S*-PCP and transfer it to the TE domain, giving $(DHB\text{-}Ser)_3$-*O*-TE. (b) Forward transfer oligomerization pathway: DHB-Ser-*O*-TE attacks the incoming DHB-Ser-*S*-PCP to make $(DHB\text{-}Ser)_2$-*O*-TE directly. When a third DHB-Ser-*S*-PCP is made, another iteration of the single step mechanism occurs to make $(DHB\text{-}Ser)_3$-*O*-TE. In both pathways the TE domain releases enterobactin by cyclization of the $(DHB\text{-}Ser)_3$ of $(DHB\text{-}Ser)_3$-*O*-TE



substrate in the ester bond forming condensation reaction. The ester bond forming activity of CrpD2 C domain was verified by heterologous expression and biochemical assays, making it the first characterized depsipeptide ester bond forming C domain.[128] The second ester bond in the cryptophycin structure arises from macrocyclization in a TE domain-catalyzed reaction, using the hydroxy group in the polyketide as a nucleophile.[128,227]

The antimycin-like family is a group of depsipeptides where ester bonds are also inserted during elongation and termination in an analogous manner to cryptophycin.[115,228,229] They possess an impressive diversity of bioactivities, including piscicidal,[108] insecticidal, nematocidal action[115,230] as well as symbiosis with ants.[18,231] Antimycin-like peptides are synthesized by mixed PKS-NRPS systems.[228,229] They all have at least one C-A-KR-T depsipeptide module for incorporation of keto acids into depsipeptides in elongation but can have multiple extras which increase the lactone ring size.[232–234] Awakawa and coworkers were able to generate novel antimycin-like compounds by employing a shuffling and deletions strategy between the JBIR-06 and neoantimycin NRPSs.[235] This led to functional chimeric depsipeptide synthetases, an elegant proof of concept of the bioengineering potential of these megaenzymes.

Didemnins are produced by sea bacteria from the *Tristrella* genus, and have antineoplastic, anti-viral and immunosuppressive activities.[151,236,237] Dehydrodidemnin B (plitidepsin or Aplidin®)[238,239] is approved for the treatment of multiple myeloma in Australia (Australian Register of Therapeutic Goods ID 291661) and has been

FIGURE 13 The proposed biosynthesis of enniatin, beauvericin and bassianolide. (a) Enniatin, bassianolide and beauvericin are made by di-modular NRPSs. The C_1 domain is inactive, and the adjacent PCP_{2a} and PCP_{2b} domains have redundant functions (b) Free-standing ketoreductases (KR), unrelated to PKS KRs, generate α-hydroxy acids from α-keto acids. (c) Chain expansion proceeds through alternating condensation reactions catalyzed by the C_2 (amide forming) and C_T (ester forming) domains, where all PCP domains can alternate between donor or acceptor roles depending on the context of the catalyzed reaction and the nature of the attached substrate. Amino acid acceptor monomers are attached to $PCP_{2a/b}$ during C_2 amide bond formation with elongated intermediate donors attached to PCP_1, while hydroxy acid acceptor monomers are always attached to PCP_1 in C_T-mediated ester bond formation with elongated intermediate donors attached to $PCP_{2a/b}$. When the elongated chain reaches a specific length, the C_T domain switches from elongation to cyclization activity through a yet unknown switching mechanism

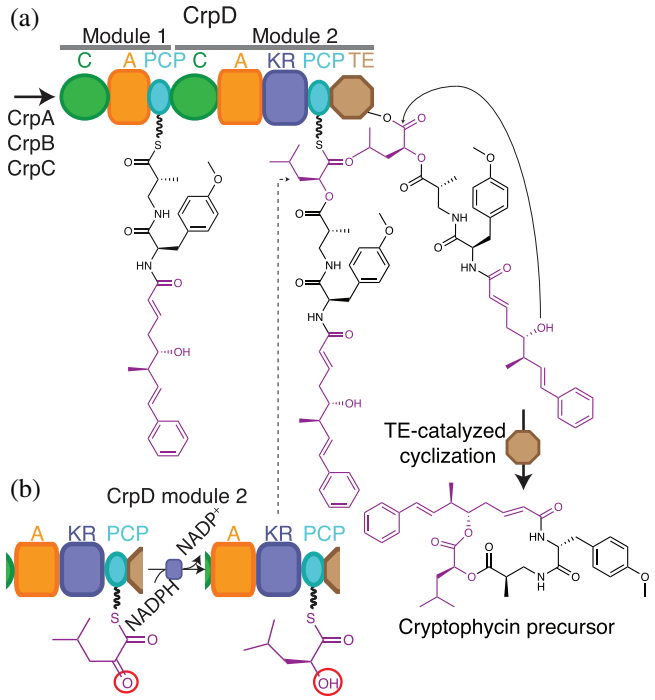


FIGURE 14 CrpD inserts ester bonds during elongation and termination steps in cryptophycin biosynthesis. (a) The C domain in CrpD Module 2 catalyzes ester bond formation between the growing polyketide-peptide chain and an α-hydroxy acid. The TE domain catalyzes cyclization using the OH group in the polyketide portion as nucleophile, releasing a cryptophycin precursor. The cycle proceeds in off-assembly line reactions. (b) The A-KR-PCP module in CrpD activates and reduces an α-ketoacid to generate an α-hydroxy acyl thioester. Figure based on Magarvey et al.[38]

reported by the company PhamaMar to show nanomolar in vitro inhibitory activity against coronavirus HCoV-229E, a similar virus to the one causing the current COVID-19 pandemic. Didemnins have two ester bonds formed by α-hydroxy acyl residues reduced in situ from keto acyl residues by depsipeptide modules in addition to an ester bond formed by a threonine side chain in termination.[151,236,238]

Another example in this depsipeptide classification, hectochlorin, is an antifungal compound isolated from the cyanobacterium *Lyngbya majuscula*.[240] The biosynthetic pathway also employs two elongating C-A-KR-PCP modules for ester bond formation during elongation.[241] The cyclizing TE domain catalyzes ester bond formation using a hydroxy group from the ketide portion of the molecule as a nucleophile.[241]

5.2.1.2 | Esters formed using main chain hydroxyl (elongation) and side chain hydroxyl (termination), both originating from bisphosphoglycerate

Vioprolide is a cyclic lipodepsipeptide (Figure 15) with an unusual origin for its bridging ester oxygens.[242] The 10-module NRPS system which produces the depsipeptide precursor of valpromide contains several tailoring domains and incorporates several non-proteinogenic amino acids. Of relevance for this review are the first elongation step and the termination step, which Mueller and coworkers described.[242,243] In Module 2, in place of an A domain there is a domain with homology to FkbH proteins of the haloacid dehalogenase superfamily. The FkbH domain in VioA, like the FkbH domain in the PKS OzmB,[244] has affinity for 1,3 bisphosphoglycerate. The phosphorylated carboxylate in 1,3 bisphosphoglycerate is energetically equal to the acyl-adenylates which A domains generate, so activation of 1,3 bisphosphoglycerate is not required for transfer to the PCP domain. The three-step reaction catalyzed by FkbH proceeds first with acyl-enzyme intermediate formation on an active site Cys in the FkbH domain (3-phosphoglyceryl-*S*-FkbG) displacing carboxylate phosphate, then dephosphorylation of the β-hydroxyl (to glyceryl-*S*-FkbG) and finally transthioesterification to glyceryl-*S*-PCP_2. This glyceryl moiety contains both the hydroxyls that will provide the bridging oxygens in the two ester bonds of vioprolide: the α-hydroxyl is the nucleophile in C_2-mediated condensation with an acyl-PCP_1 in the first elongation step, and the β-hydroxyl is the nucleophile in TE_{10}-mediated cyclization in termination (Figure 15).

5.2.2 | Ester bond formation during elongation by C domains and termination by TE domains in iterative synthesis

5.2.2.1 | Ester formed with main chain hydroxyls originating from keto acid substrates

Cereulide and valinomycin are dodecadepsipeptides formed by alternating arrays of α-amino and α-hydroxy acids.[145,146,245,246] They can also be described as trimers of a tetradepsipeptide unit. In spite of their strong structural resemblance to the fungal products enniatin, beauvericin, and bassianolide, their biosynthesis is very different (Figure 16).

The cereulide and valinomycin biosynthetic clusters are 2-subunit, 4-module NRPSs[33,109,152,247] (Figure 16a). CesA and Vlm1 have the architecture A_1-KR_1-PCP_1-C_2-A_2-PCP_2-E_2-C_3, while CesB and Vlm2 have A_3-KR_3-PCP_3-C_4-A_4-KR_4-PCP_4-TE_4. Depsipeptide initiation modules $CesA_1$ and $Vlm1_1$ and elongation modules $CesB_3$ and $Vlm2_3$ activate and reduce α-ketoacids through the action of their A-KR domains (Figure 16b).[33] However, the fate of each generated acyl thioester depends on whether the module is in an initiation or elongation position. The reduced hydroxyl in Residue 1 is not used immediately, but is first a passive part of the donor substrate (e.g., α-D-

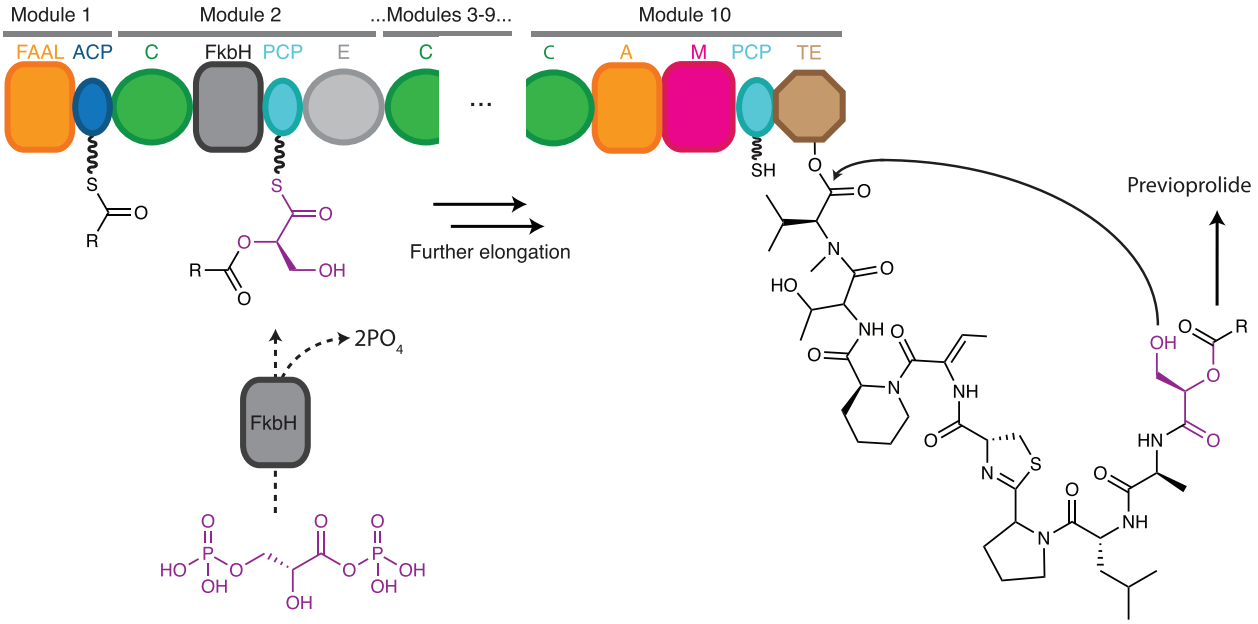


FIGURE 15 Proposed vioprolide biosynthesis. A decamodular NRPS generates previoprolide. It is suggested that the FkbH domain in Module 2 catalyzes stepwise covalent binding of D-1,3-biphosphoglycerate, dephosphorylation and transfer of glycerate to PCP_2, generating D-glyceryl-*S*-PCP_2. The α-hydroxy group serves as a nucleophile in the condensation reaction in the C_2, and the β-hydroxy group in the glyceryl side chain serves as the nucleophile in cyclic termination. This releases a previoprolide intermediate that undergoes further modification to generate mature vioprolide

HIC-*S*-PCP_1 in cereulide biosynthesis) for condensation in C_2 with L-Ala-*S*-PCP_2 as acceptor substrate. In contrast, the reduced hydroxyl in Residue 3 is used immediately after reduction to α-D-HIV-*S*-PCP_3, as it is the acceptor substrate for C_3 in that ester forming condensation reaction. The tridepsipeptidyl chain will then be elongated in the C_4 to a tetradepsipeptide intermediate with a free distal α-hydroxyl group and one internal ester bond. This tetradepsipeptide intermediate is then oligomerized and cyclized by the terminal TE domain,[5,248] but until recently, the details of this step were opaque.

Several outstanding questions pertaining to the oligomerization and termination cycles of these systems were addressed in a collaborative paper from our group, the Chin group and the Boddy group[153] (Figure 17). First, we addressed the question of whether the oligomerization mechanism proceeded through a forward[210] or reverse[5] transfer mechanism (Figure 17a; see also enterobactin Figure 12). Using Vlm2 TE heterologously expressed in *E. coli* and reacting it with a tetradepsipeptidyl-SNAC substrate, we confirmed that the reverse transfer mechanism is the most likely due to the detection of reaction intermediates of only part of the reverse transfer pathway. Then, we used a novel chemical biology approach to obtain co-complex structural information. It is classically challenging to obtain structural information on TE-intermediate complexes because of the instability of the ester bond in the acyl-enzyme intermediates.[185,186] Other groups have recently used phosphonate analogues to obtain analog co-complexes of the bifunctional nocardicin TE domain.[183] In the case of depsipeptide synthetases, we employed an expanded genetic code strategy[249,250] to replace the active site serine with the amine-containing amino acid 2,3-diaminopropionic acid (DAP)[153] (Figure 17b). This strategy effectively produced recombinant DAP-containing protein that, when incubated with SNAC analogues or valinomycin, formed stable amide TE-intermediate complexes analogous to the first and last steps of the oligomerization/cyclization reactions, whose structures we determined by X-ray crystallography.

The TE domain co-complex structures showed remarkable placidity in the lid. The Vlm TE lid is 88 residues, folded into an extended loop: bundled helices α1–3, short helix α4, long helix α5, and short helix α6. In the absence of substrates, one structure showed disordered lid and the other showed an ordered lid. The ordered position of the lid in the apo state was similar to that when bound with one tetradepsipeptidyl moiety (tetradepsipeptidyl-*NH*-TE). However, when the TE domain was bound with last-stage $(tetradepsipeptidyl)_3$-*NH*-TE the lid was not only in a different position, but also the secondary structure elements had markedly

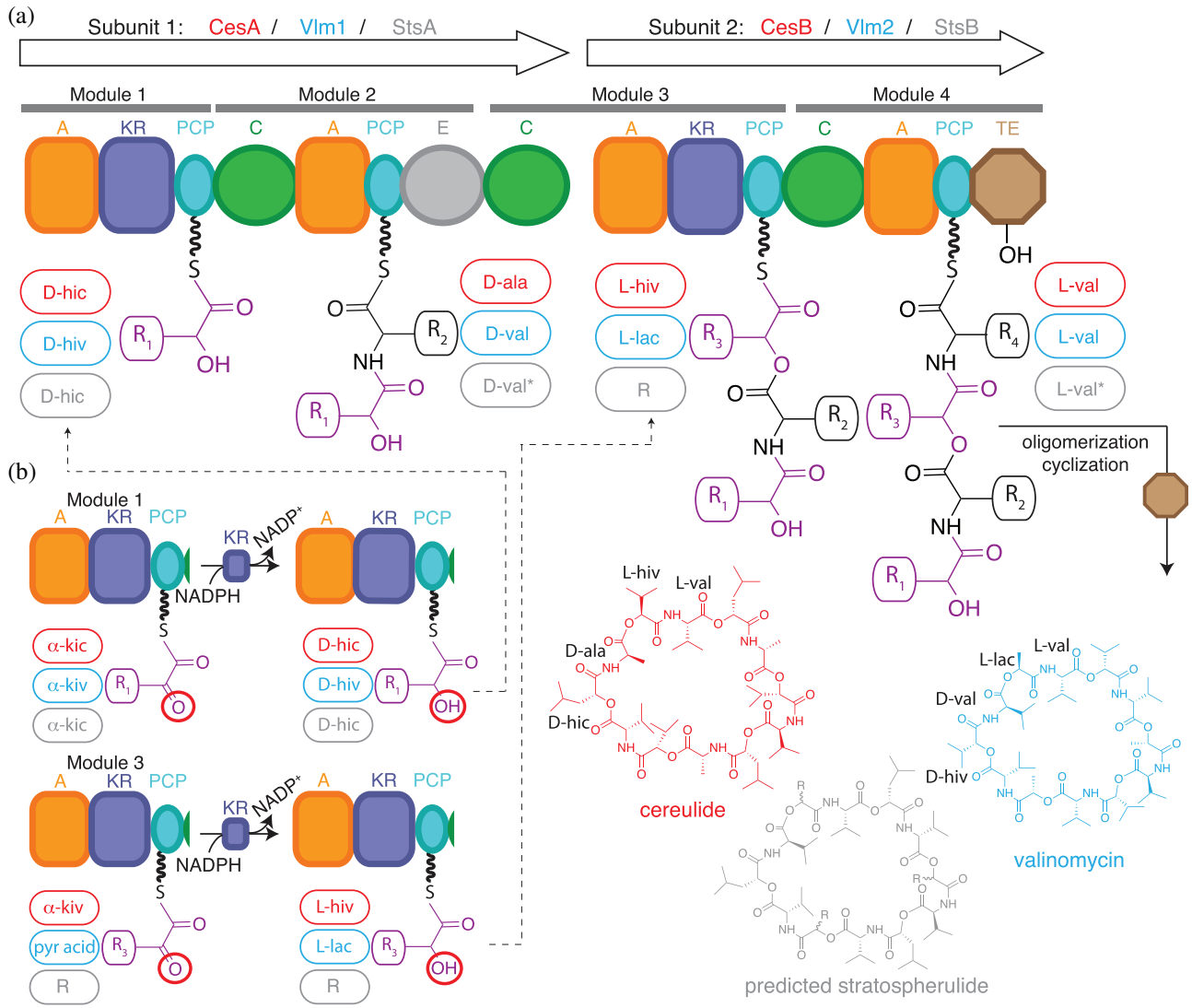


FIGURE 16 Proposed biosynthesis of cereulide, valinomycin and a hypothetical product from *Bacillus stratosphericus* LAMA 585. (a) During elongation ester bonds are formed by C_3 domains. During termination, the TE domains catalyzes oligomerization of tetradepsipeptidyl units, as well as cyclic release, using the distal α-hydroxy groups as nucleophiles (see also Figure 17). (b) Depsipeptide Modules 1 and 3 activate, thiolate and reduce α-keto acids to generate α-hydroxy acyl thioesters. Adapted from Alonzo et al.[34]

rearranged relative to each other (Figure 17d). This rearranged lid formed a hemisphere-like pocket, which likely helps curl the dodecadepsipeptide back toward the active site serine for cyclization (Figure 17e). Only a few of the substrate depsipeptidyl residues were visible in the density, which is perhaps not surprising considering the TE domain must accommodate a range of length of substrates during its cycle. Indeed, specific and strong binding interactions could slow the synthetic cycle, as the tetradepsipeptide must transition back and forth between being ligated to the PCP domain and ligated to the TE domain, and the same tetradepsipeptide must assume multiple different positions in the course of a cycle.

6 | CONCLUDING WORDS

The outstanding chemical diversity of depsipeptides and the array of ester bond forming strategies in depsipeptide synthetases create scientific and practical interest in the activities and biosynthesis of these compounds. Although the field has gained good knowledge of the biosynthetic pathways processes, there is still much to be discovered.

There are many orphan clusters and compounds throughout nature. Of note are the numerous uncharacterized fungal depsipeptide synthetases. If these act, as we believe, through a cycle that includes C domain-catalyzed ester bond formation, it will mean that ester-bond-forming-C domains are more widespread than

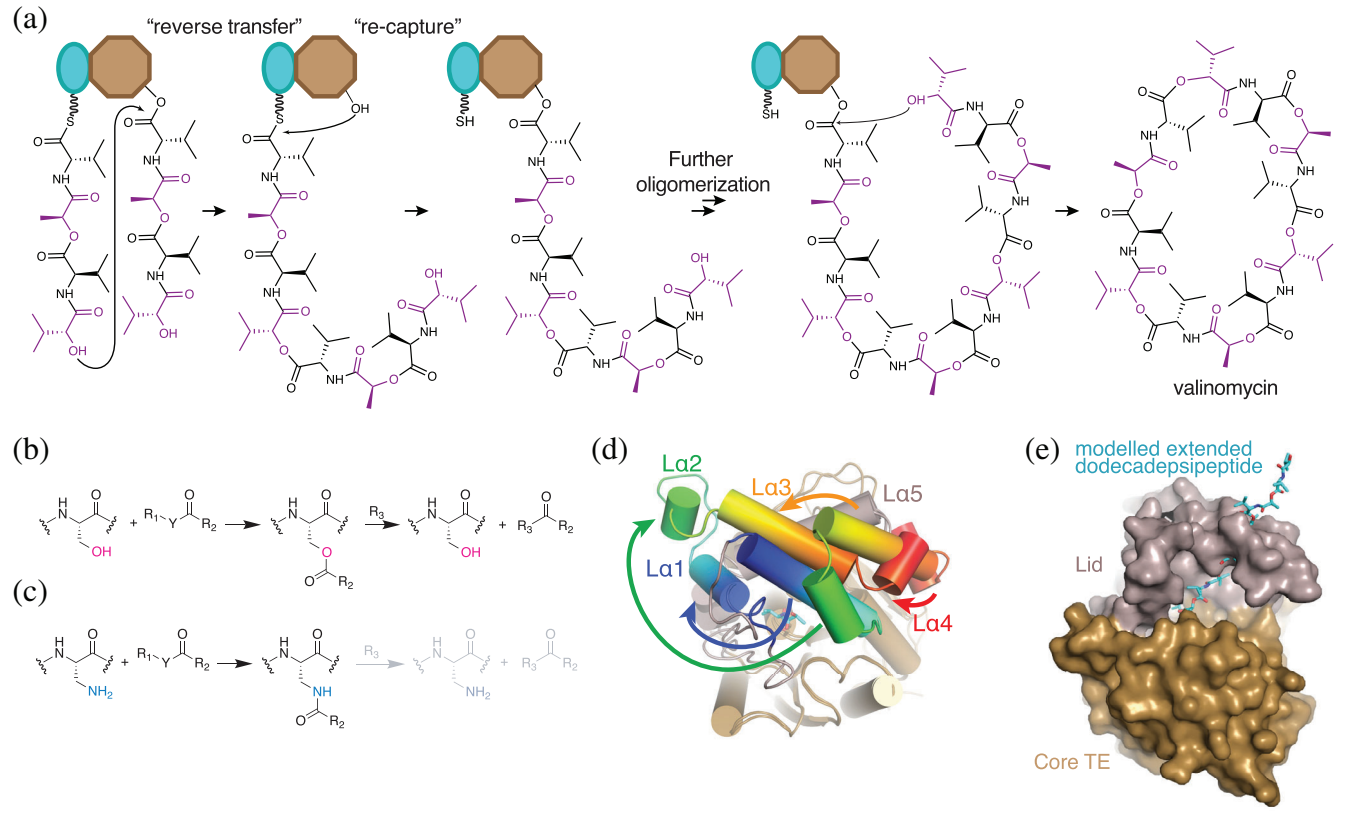


FIGURE 17 Valinomycin oligomerization and cyclization. (a) Oligomerization of tetradepsipeptidyl units proceeds through a reverse transfer pathway, where the distal α-hydroxyl in tetradepsipeptidyl-*S*-PCP_4 attacks the ester carbon in tetradepsipeptidyl-*O*-TE_4, generating $(tetradepsipeptidyl)_2$-*S*-PCP_4 after one oligomerization. This is transferred to TE_4 and the next tetradepsipeptidyl-*S*-PCP_4 is used to make $(tetradepsipeptidyl)_3$-*S*-PCP_4 in an analogous way. Cyclization of the $(tetradepsipeptidyl)_3$-*S*-PCP_4 via its α-hydroxyl releases mature valinomycin. (b) Vlm 2 TE forms ester intermediates during its reaction cycle. (c) Replacing the active site serine in Vlm2 TE by DAP generates a protein that can form amide bonds instead of ester bonds, making the intermediates more stable and amenable for structural and biophysical analysis. (d) The active site lid of Vlm2 TE undergoes massive conformational arrangements to favor the cyclization conformation of the dodecadepsipeptide in the active site. (e) A model of a fully elongated dodecadepsipeptide (cyan) shows that such conformation would clash with the active site lid (grey) observed in the X-ray structure of the dodecadepsipeptidyl-*NH*-TE complex, thus supporting the hypothesis that lid remodeling favors the cyclization reaction. Figure based on Huguenin-Dezot et al.[153]

once thought. Furthermore, if bifunctional C_T domains such as those in enniatin biosynthesis require promiscuity to catalyze both elongation and cyclization, they may be extremely promising targets for bioengineering efforts.[127] More structural, biophysical, and biochemical studies of the domain will increasingly shed light into the mechanistical details that separate the elongation and the cyclization activities of these domains, and whether they could eventually be exploited for combinatorial biosynthetic efforts.

Another open question in fungal systems concerns the production of α-hydroxyl acids in standalone KR domains. Do these KR domains transiently associate with the NRPSs to ensure a high local concentration of NRPS substrate, or is there another means of substrate channeling? It would seem wasteful to generate millimolar concentrations of hydroxy acids throughout the fungal cytoplasm. Answering this question could have relevance for many biosynthetic clusters.

An active topic of research in our laboratory pertains to the stereospecificity of KR domains in depsipeptide (C)-A-KR-T module. Because the motifs that identify stereochemistry outcomes in PKS KR domains are absent or degenerated in NRPS A-KR-PCP modules, additional information is required to predict depsipeptide stereochemistry. It is likely that stereochemistry motifs are different and that bioinformatic analysis and co-complex structure determination of these systems is necessary to elucidate the stereospecificity determinants.

In addition, the mechanisms by which the TE domains, with their very varied lid composition, direct the proper nucleophile for iterative oligomerization and/or cyclization requires further study. Only with a substantial number of co-crystal structures can excellent

generalizable insight be obtained. We are hopeful that the DAP expanded genetic code strategy employed for valinomycin TE will facilitate these studies. Controlling TE domain function is an important frontier for most comprehensive bioengineering efforts.

As much as fundamental insight into known pathways is needed, perhaps the most exciting question is that of what novel modes of depsipeptide biosynthesis are yet to be discovered. There are clear positions in the schematic (Figure 2) that could be filled in with yet-to-be discovered pathways (e.g., by a system that iteratively oligomerizes and cyclizes through the distal α-hydroxyl acid residue, similar to how the nondepsipeptide cyclic gramicidin is made). The filling in of those positions is probably merely a matter of time. However, these authors look most forward to the description of completely novel depsipeptide NRPS synthetic logic, awaiting discovery in earth's huge microbial diversity.

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AUTHOR CONTRIBUTIONS

Diego Alonzo: Visualization; writing-original draft; writing-review and editing. **T Schmeing:** Conceptualization; funding acquisition; project administration; resources; supervision; visualization; writing-original draft; writing-review and editing.

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