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Richard H. Baltz · Paul Brian · Vivian Miao
Stephen K. Wrigley

Combinatorial biosynthesis of lipopeptide antibiotics in *Streptomyces roseosporus*

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Abstract Daptomycin is a cyclic lipopeptide antibiotic produced by *Streptomyces roseosporus*. Cubicin® (daptomycin-for-injection) was approved in 2003 by the FDA to treat skin and skin structure infections caused by Gram-positive pathogens. Daptomycin is particularly significant in that it represents the first new natural product antibacterial structural class approved for clinical use in three decades. The daptomycin gene cluster contains three very large genes (*dptA*, *dptBC*, and *dptD*) that encode the nonribosomal peptide synthetase (NRPS). The related cyclic lipopeptide A54145 has four NRPS genes (*lptA, lptB, lptC*, and *lptD*), and calcium dependent antibiotic (CDA) has three (*cdaPS1, cdaPS2*, and *cdaPS3*). Mutants of *S. roseosporus* containing deletions of one or more of the NRPS genes have been *trans*-complemented with *dptA, dptBC*, and *dptD* by inserting these genes under the control of the *ermEp** promoter into separate conjugal cloning vectors containing ϕC31 or IS*117* attachment (*attP int)* sites; delivering the plasmids into *S. roseosporus* by conjugation from *Escherichia coli*; and inserting the plasmids site-specifically into the chromosome at the corresponding *attB* sites. This *trans*-complementation system was used to generate subunit exchanges with *lptD* and *cdaPS3* and the recombinants produced novel hybrid molecules. Module exchanges at positions D-Ala_8 and D-Ser_{11} in the peptide have produced additional novel derivatives of daptomycin. The approaches of subunit exchanges and module exchanges were combined with amino acid modifications of Glu at position 12 and natural variations in lipid side chain starter units to generate a combinatorial library of antibiotics related to daptomycin. Many of the engineered strains produced levels of novel molecules amenable to isolation and antimicrobial testing, and most of the compounds displayed antibacterial activities.

Keywords Combinatorial biosynthesis · Daptomycin · Lipopeptide antibiotic · *Streptomyces roseosporus*

R. H. Baltz (✉) · P. Brian · V. Miao · S. K. Wrigley
Cubist Pharmaceuticals, Inc., 65 Hayden Avenue,
Lexington, MA, 02421 USA
E-mail: rbaltz@cubist.com
Tel.: +1-781-8608444
Fax: +1-781-8611164

Introduction

A21978C is an amphipathic acidic Ca^{2+}-dependent lipopeptide antibiotic complex produced by *Streptomyces roseosporus* [8]. A21978C is composed of a ten-membered cyclic peptide coupled by an ester bond between the *C*-terminus of kynurenine (Kyn_{13}) and the hydroxyl group of Thr_4 (Fig. 1). A21978C has a three amino acid exocyclic tail coupled by an amide linkage of the *N*-terminus of Trp_1 to different fatty acids. The fatty acids can be removed enzymatically by a deacylase produced by *Actinoplanes utahensis* [4, 9] and cloned in *Streptomyces lividans* on a multicopy plasmid for high level production [19], and the peptide can be reacylated to generate analogs of A21978C. Daptomycin is an analog of A21978C that contains an *N*-decanoyl fatty acid side chain, which can be added chemically [9] or by feeding decanoic acid to the fermentation [17].

Daptomycin and A21978C factors are composed of both D- and L-amino acids. NMR studies have shown that the position of certain D-amino acid and Gly residues facilitate the formation of a β-hairpin structure with intramolecular hydrogen bonds [33]. Recent studies have shown that the Asp_7 and Asp_9 residues (Fig. 1) are critical for antibacterial activity [11], and are probably involved in the binding of Ca^{2+}. Also, the D-Asn_2 residue in the tail is required for good antibacterial activity [27].

Cubicin® (daptomycin-for-injection) was approved in 2003 for the treatment of skin and skin structure infections caused by Gram-positive pathogens, including *Staphylococcus aureus* strains resistant to methicillin or vancomycin [1, 10, 22, 32]. Daptomycin has a novel

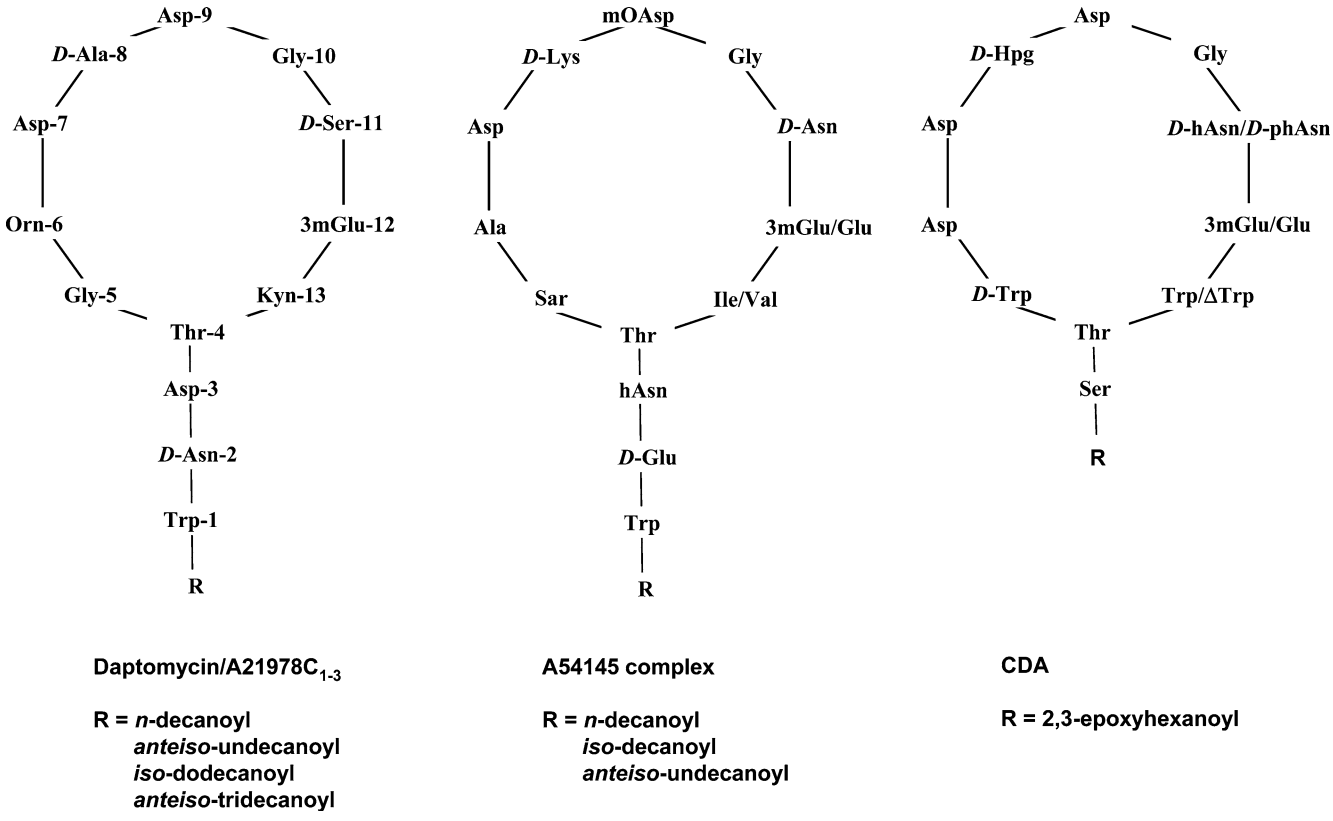

Fig. 1 Structures of A21978C factors, A54145 factors and CDA. Abbreviations for unusual amino acids: *hAsn* hydroxyasparagine, *phAsn* phosphohydroxyasparagine, *Hpg* hydroxyphenylglycine, *3mGlu* 3-methylglutamic acid, *OmAsp* 3-*O*-methyl-aspartic acid, *Orn* ornithine, *Sar* sarcosine, Δ*Trp Z*-2,3-dehydrotryptophan

mechanism of action that is not yet fully understood [18, 21, 36], but which must account for its bactericidal activity against antibiotic resistant pathogens [10, 22]. Although a number of derivatives of daptomycin modified at Orn_6 or D-Ser_{11}, or substituted with different fatty acid tails, have been synthesized and evaluated, none has proven superior to daptomycin [9, 13, 35]. The complex nature of daptomycin has presented difficulties for other chemical modifications; in particular, substitutions of different amino acids in the core cyclic peptide have not been explored by medicinal chemistry approaches, although a chemoenzymatic approach to generate small amounts of derivatives containing readily available amino acids looks promising [11].

Daptomycin is produced by a nonribosomal peptide synthetase (NRPS) [27, 40]. NRPS enzymes synthesize peptide secondary metabolites that frequently contain nonproteinogenic and D-amino acids, as well as L-amino acids, on large multi-subunit, multi-modular enzymes [23, 34]. NRPS enzymes are often composed of several giant multienzymes containing two or more modules for the incorporation of specific amino acids. Each module of a typical NRPS is comprised of an adenylation (A-domain) for activation of a specific amino acid, a condensation (C-domain) for coupling the amino acid to the nascent peptide, and a peptidyl carrier domain (thiolation or T–domain) arranged in the order CAT; accessory domains for methylation (M-domain) or epimerization (E-domain) that tailor individual amino acids may also be present (e.g., CATE). The first modules for certain peptides lack C domains, which is not the case with daptomycin. The modules, grouped in protein subunits, act in an ordered manner to build the final peptide, and a terminal thioesterase (Te) domain participates in the release of the completed peptide from the enzyme.

The genes for daptomycin biosynthesis were first localized to one end of the linear chromosome of *S. roseosporus* by transposition mutagenesis and pulsed field gel electrophoresis analysis [25, 26]; this facilitated the cosmid cloning of a large segment of the *dpt* gene cluster, and the initiation of gene sequencing. More recently, the complete *dpt* gene cluster was cloned in a BAC vector and expressed in *S. lividans* [27, 31]. This provided the starting material to complete the gene sequencing and explore the genetic engineering of the NRPS genes to produce novel derivatives of daptomycin not readily accessible by chemical synthesis.

Daptomycin and related lipopeptide biosynthetic gene clusters

The complete daptomycin gene cluster has been sequenced from cosmid and BAC clones [27]. The core

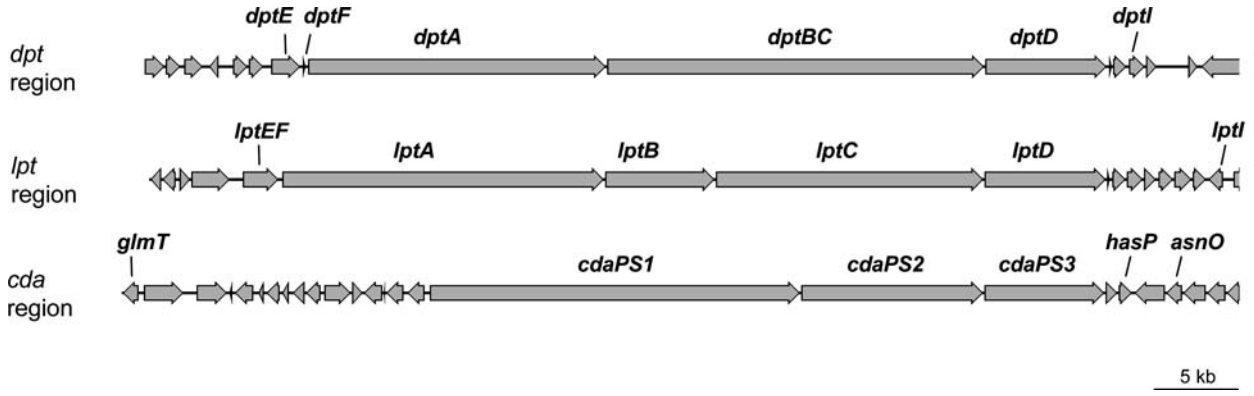

Fig. 2 The daptomycin, A54145 and CDA biosynthetic gene clusters. The *dpt* region from *S. roseosporus* contains genes for the daptomycin synthetase (NRPS genes *dptA*, *dptBC*, and *dptD*) and accessory functions such as *dptE*, *dptF*, and *dptI*. The *lpt* region from *S. fradiae* and the *cda* region from *S. coelicolor* likewise contain genes respectively for A54145 synthetase (*lptA*, *lptB*, *lptC*, and *lptD*) or CDA synthetase (*cdaPS1*, *cdaPS2* and *cdaPS3*) and accessory functions or tailoring enzymes

peptide is synthesized by a three subunit NRPS encoded by the *dptA*, *dptBC*, and *dptD* genes (Fig. 2). The *dptA* gene encodes the DptA multienzyme subunit involved in coupling the fatty acid and the first five amino acids; *dptBC* encodes the DptBC subunit involved in coupling of the next six amino acids; and *dptD* encodes the DptD subunit involved in coupling of the last two amino acids and the cyclization of the peptide. The *dptE* and *dptF* genes upstream of *dptA* encode homologs to acyl-CoA ligase and acyl carrier proteins, respectively, which are likely to participate in the coupling of the fatty acid starters to the amino group of Trp [27]. Downstream of the NRPS genes are a number of *dpt* genes, including *dptI*, which encodes the Glu-methyltransferase [27, Nguyen et al. manuscript in preparation].

Streptomyces fradiae and *Streptomyces coelicolor* produce ten-membered cyclic peptides somewhat related to daptomycin (Fig. 1). A cosmid library of *S. fradiae*, the producer of A54145 [5], was prepared and clones that contained the A54145 biosynthetic gene cluster were sequenced [28]. The A54145 NRPS has four subunits: the *lptA* gene encodes the LptA subunit involved in the coupling of fatty acids and the first five amino acids; *lptB* encodes the LptB subunit involved in coupling of the next two amino acids; *lptC* encodes the LptC subunit involved in coupling of the next four amino acids; and *lptD* encodes the LptD subunit involved in coupling of the final two amino acids and the cyclization of the peptide. It is noteworthy that the first and last subunits are identical in the number of modules and other critical functions (starter condensation domain in the first and thioesterase domain in the last) to those of the daptomycin NRPS (Fig. 2).

The biosynthetic gene cluster for the *S. coelicolor* lipopeptide CDA has also been sequenced and analyzed

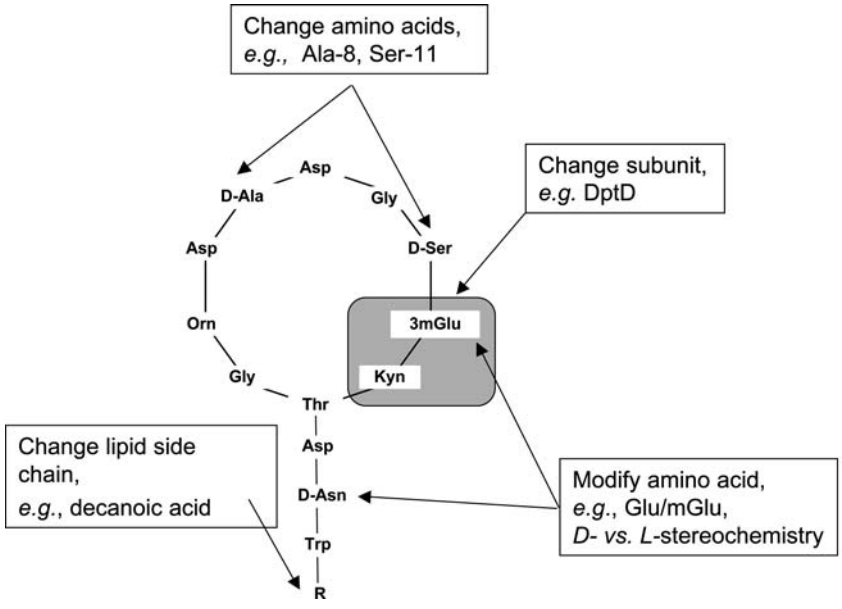

Fig. 3 Four dimensions for combinatorial biosynthesis. Daptomycin is used as an example

[14]. The CDA NRPS contains three subunits, encoded by *cdaPS1*, *cdaPS2*, and *cdaPS3* (Fig. 2). The *cdaPS3* gene encodes the CdaPS3 subunit involved in coupling the final two amino acids and ring closure, just as *dptD* and *lptD*. These three subunits all direct the incorporation of 3mGlu in the penultimate position of the three lipopeptides. It is likely that these genes in particular, and the lipopeptide pathways in general, evolved from a common ancestral NRPS pathway. The similar modular organization of these three gene clusters, the partial identities in subunit organization, the similarities and differences in the lipopeptide primary sequences, and the important antibacterial activities demonstrated by daptomycin make these NRPS pathways attractive starting materials for combinatorial biosynthesis.

Molecular genetic systems for combinatorial biosynthesis

There are four potential dimensions to combinatorial biosynthesis of lipopeptides (Fig. 3). These include manipulations of: (1) the individual modules of the NRPS; (2) the multi-modular NRPS subunits; (3) the lipid side chains; and (4) the amino acid modifications. It has been shown that *S. roseosporus* is a suitable host for a variety of molecular genetic manipulations [2, 15, 16, 25]. Importantly, *S. roseosporus* is a good recipient for conjugal transfer of plasmid DNA from *Escherichia coli* [25], using conjugal vectors that integrate site-specifically [3]. *S. roseosporus* mutants deleted for *dptD*, *dptBC*+*dptD*, and *dptA*+*dptD* can be complemented *in-trans* by inserting *dptD* into the IS*117* insertion site, and by inserting *dptA* or *dptBC* into the ϕC31 *attB* site (Fig. 4) [Nguyen et al. manuscript in preparation; Coeffet-Le Gal et al. manuscript in preparation; Miao et al. manuscript submitted]. In addition, a mutant deleted for the *dptBCDGHIJ* genes can be complemented *in-trans* by inserting the *dptBCD* genes into the ϕC31 *attB* site and different combinations of the *dptGHIJ* genes into the IS*117* insertion site [Nguyen et al. manuscript in preparation]. All of the genes inserted *in-trans* were expressed under the control of the strong constitutive promoter *ermEp**. This provided a system to engineer individual modules in *dptA, dptBC*, or *dptD* in *E. coli* [7; Brian et al. manuscript in preparation], then to introduce them into *S. roseosporus* by conjugation. It also provided a means to test for whole subunit *trans*-complementation with *lptD* and *cdaPS3* [Miao et al. manuscript submitted], and a means to delete the *dptI* (Glu-methyltransferase) gene.

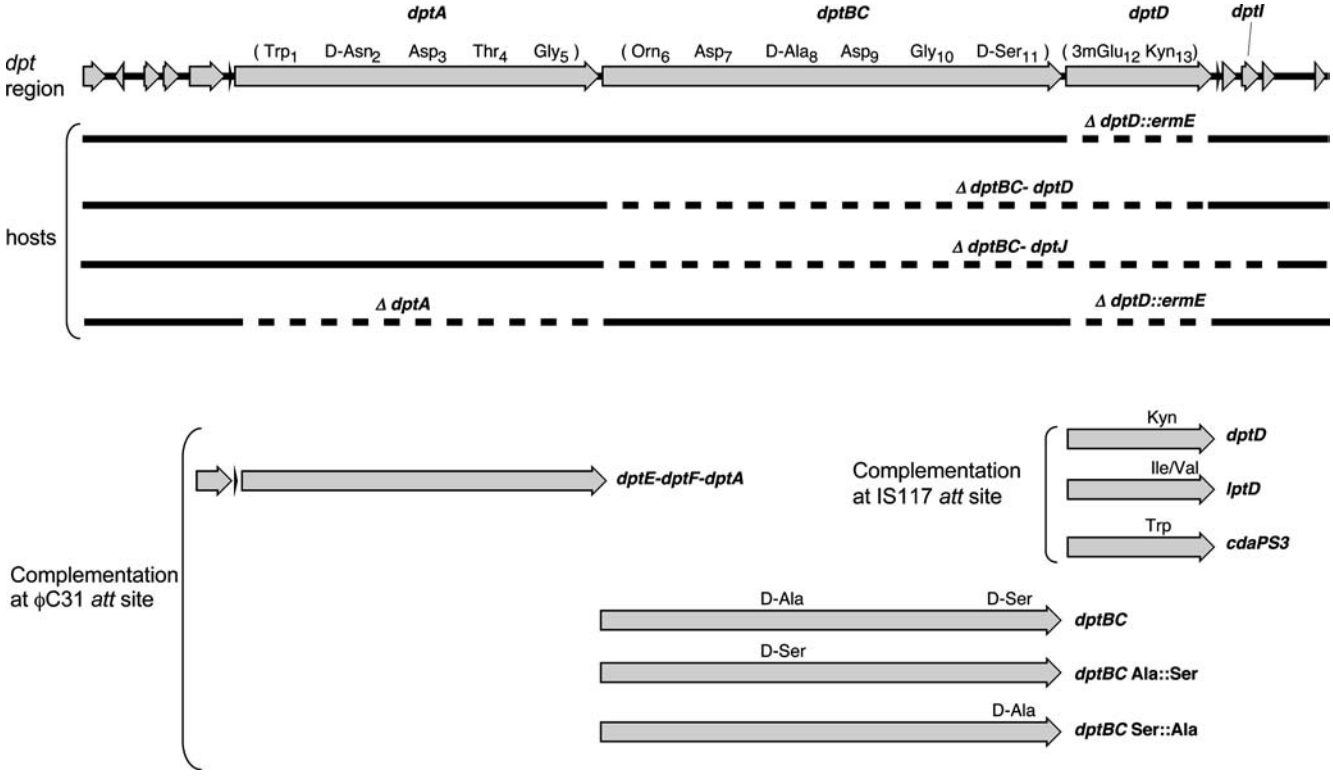

Fig. 4 Molecular genetic systems in *S. roseosporus*. The wild-type gene cluster is shown at the *top*: amino acids incorporated by modules in the respective subunits encoded by *dptA*, *dptBC*, and *dptD* are noted, in order, above the *arrow* representing the each gene. Deletion strains (*dashed lines* signify deleted regions) serve as hosts for complementation by plasmids with control (*dptE-dptF-dptA*, *dptA*, *dptBC*, or *dptD*), heterologous (*lptD*, *cdaPS3*) or engineered (*dptBC Ala::Ser*, *dptBC Ser::Ala*) genes. Significant positions subjected to changes are emphasized by *notation* above the appropriate *arrow*

Subunit exchanges

The production of hybrid polyketides by heterologous polyketide synthase (PKS) whole subunit exchanges has been demonstrated for the related 16-member lactones of tylosin and spiramycin [20] and the related 14-member lactones of erythromycin, picromycin, and oleandomycin [37]. It has been demonstrated more recently that the functional interactions between individual proteins in multi-subunit PKSs [38, 41, 42], NRPSs [12] and hybrid PKS/NRPS [24, 30] require the interaction between pairs of interpeptide linkers or recognition sequences located at the *C*-termini of donor proteins and at the *N*-termini of recipient proteins. Undoubtedly, the recognition sequences for tylosin and spiramycin, and for the three 14-membered macrolides, were conserved sufficiently to form functional protein–protein interactions in the multi-subunit PKS enzymes.

The daptomycin, A54145 and CDA biosynthetic pathways include NRPS genes encoding similar dimodular proteins that incorporate the final two amino acids $3mGlu_{12}$ and Kyn_{13}, $Ile(Val)_{13}$, or Trp_{13}, respectively (Fig. 5). We identified interpeptide recognition sequences, with similar distributions of charged amino acids, associated with the couplings of D-Ser_{11} of DptBC, D-Asn_{11} of LptD or D-$hAsn_{11}$ of CdaPS3 to the 3mGlu-12 recipients in the three respective proteins [Miao et al. manuscript submitted], and demonstrated subunit *trans*-complementation of the Δ*dptD* mutant by inserting the *lptD* gene or the *cdaPS3* gene into the IS*117* integration site under the control of the *ermEp** promoter. The yields of hybrid lipopeptides were quite high, about 50% of control for CdaPS3, and about 25% for LptD. This indicated that the genes were expressed well *in-trans* from the *ermEp** promoter, the heterologous protein–protein interactions facilitated by interpeptide recognition sequences were efficient, the Glu-methyltransferase encoded by the *dptI* gene efficiently methylated the hybrid molecules, and the thioesterase domains of CdaPS3 and LptD closed the hybrid peptide rings efficiently. All of the hybrid compounds displayed antibacterial activities [Miao et al. manuscript submitted]. The system is theoretically expandable to the *dptA* and *lptA* subunits [Coeffet-Le Gal et al. manuscript in preparation], and possibly to the middle subunits.

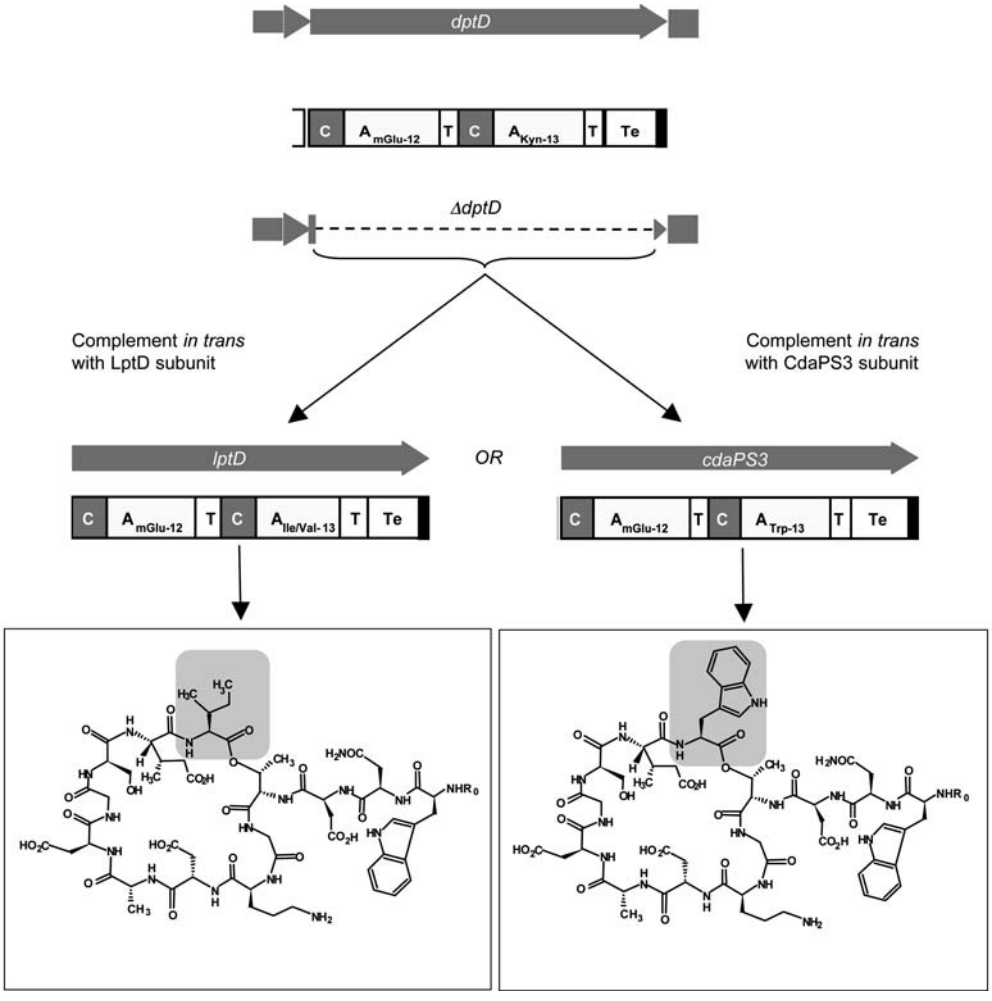

Fig. 5 Subunit complementations at DptD. The role of *dptD* can be replaced *in trans* by similar subunits from the A54145 or CDA biosynthetic pathways to result in elaboration of new compounds. The *dptD* gene in the Δ*dptD* strain used here was deleted and replaced by a resistance marker, resulting in a null production mutant

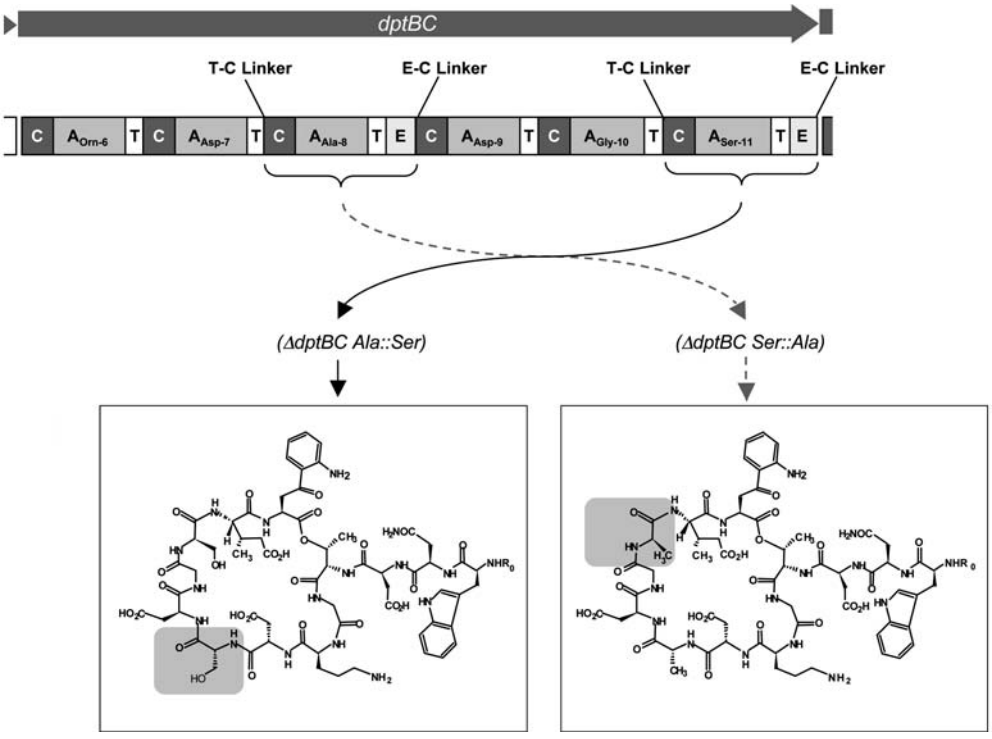

Fig. 6 Module exchanges in DptBC. A number of potential linker regions exist between individual and sets of domains. Selection of an appropriate linker pair allows precise excision of a module comprised either of CAT or CATE domains (*shown*), and subsequent replacement with a similarly delimited new module specifying a different amino acid. The new strains would produce a novel analog. Abbreviations for domains: *C* condensation, *A* adenylation (subscript indicates amino acid and residue position in daptomycin), *T* thiolation, and *E* epimerization

Module exchanges

We first attempted complete module exchanges between D-Ala_8 and D-Ser_{11} (Fig. 6), since these amino acids were similar in size and might not pose an excessive challenge for peptide coupling, based upon the known coupling constraints in NRPS multi-enzymes [25, 38]. These experiments were successful, and the product yields were surprisingly good. The strain with D-Ala_8 substituted for D-Ser_{11} produced nearly 60% of the amount of lipopeptide of the control strain. The recombinant with D-Ser_{11} substituted for D-Ala_8 produced close to 20% of control. Both novel molecules had in vitro antibacterial activities similar to daptomycin [Brian et al. manuscript in preparation]. It remains to be seen how well other module exchanges will work in this system.

Amino acid modifications

The *glmT* gene from the CDA pathway was proposed to encode the Glu-3-methyltransferase [14]. The daptomycin and A54145 pathways contain homologs of *glmT*, designated as *dptI* [27] and *lptI* [28]. Deletion of the *dptI* gene leads to the production of daptomycin analogs lacking the methyl group of $3mGlu_{12}$ [Nguyen et al. manuscript in preparation]. *S. fradiae* normally produces a mixture of lipopeptide factors containing similar ratios of Glu_{12} and $3mGlu_{12}$ [5]. A54145 factors containing Glu_{12} have somewhat less antibacterial activity than factors containing $3mGlu_{12}$, but they are considerably less toxic in mouse LD50 tests [6]. Thus coupling the $3mGlu_{12}$ to Glu_{12} modification with other engineered changes might be beneficial for some analogs.

Similarly, A54145 contains hydroxy-asparagine ($hAsn_2$) and 3-*O*-methyl-aspartic acid ($OmAsp_9$), and CDA contains hAsn and hydroxyphenylglycine (Hpg) (Fig. 1). Additional modifications of amino acids to be tested in combinatorial libraries may be achieved by exploitation of the genes involved in these biochemical steps, as well as by feeding analogs (mutasynthesis), as has been demonstrated with Hpg in CDA [14].

Lipid side chain modifications

A bonus to the genetic engineering by module exchanges, subunit exchanges and amino acid modifications is that *S. roseosporus* naturally produces a mixture of factors containing different fatty acid side chains [8] (Fig. 1). The factors containing *anteiso*-undecanoyl, *iso*-dodecanoyl and *anteiso*-tridecanoyl side chains accumulate in substantial quantities during fermentation. Thus every single genetic engineering modification generates three novel lipopeptides to evaluate. In addition, it has been shown that feeding different fatty acids or fatty acid esters to the fermentation can generate other compounds with new fatty acid side chains [17], so this approach is expandable.

Combinatorial biosynthesis

We have combined subunit exchanges with *lptD* and *cdaPS3*, module exchanges at position D-Ala_8 and

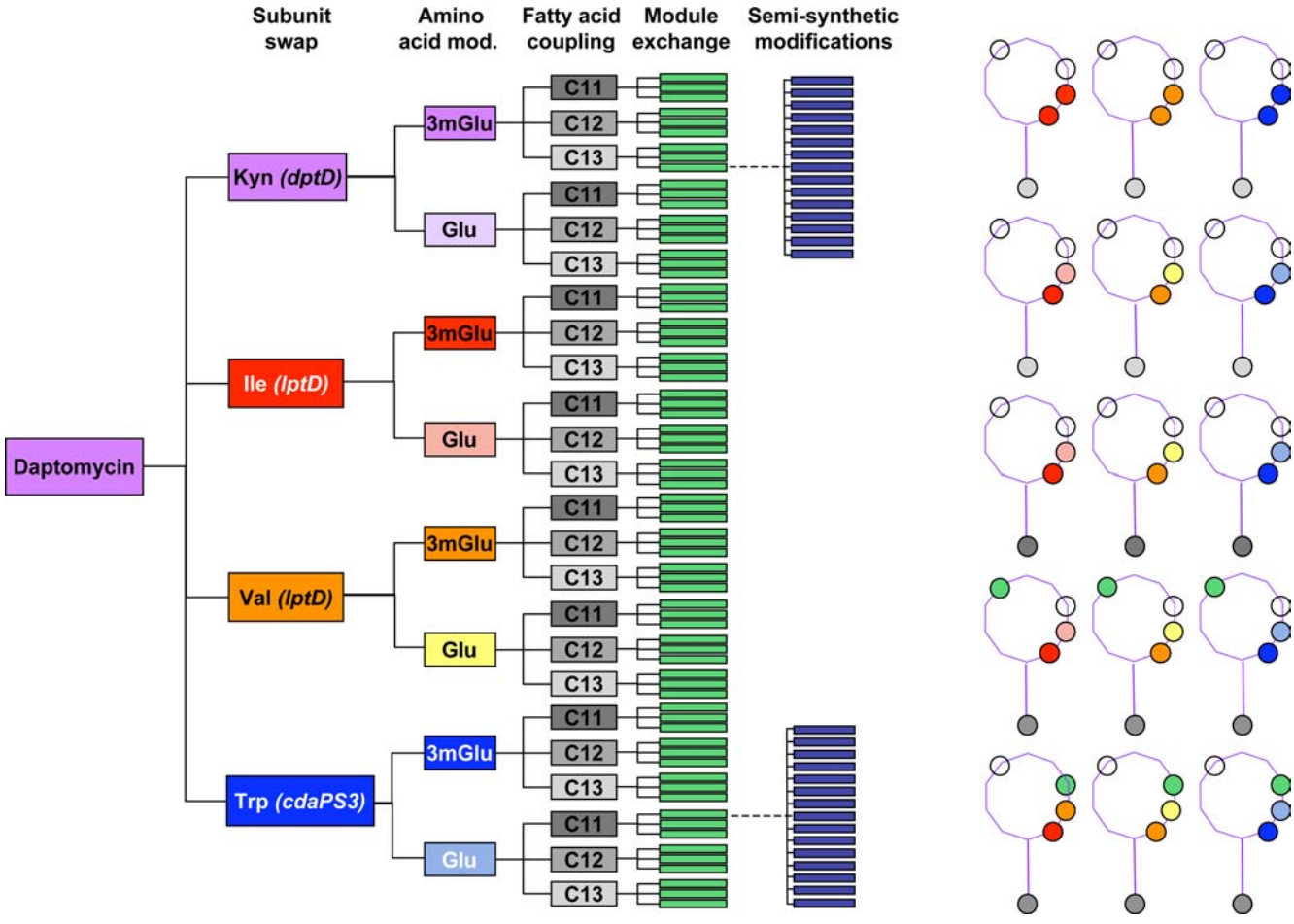

Fig. 7 Combinatorial libraries. (*Left*) Module exchanges, subunit exchanges and amino acid modifications alter lipopeptide templates; host cells supply three variations in the lipid tail. Semi-synthetic modifications can be added to novel cores through modifications of the lipid tail or the primary amines of Orn. (*Right*) Examples of novel compounds recently produced. The daptomycin/A21978C core in Fig. 1 is represented as a *purple outline*. Positions manipulated in the examples presented are *circles*, and *colors* representing various changes correspond to those on the *left panel*

D-Ser_{11}, modification of $3mGlu_{12}$ to Glu_{12}, and the natural lipid side chain modifications. Since *lptD* leads to the incorporation of Ile_{13} and Val_{13}, the potential biosynthetic combinatorial expansion should lead to $4\times3\times2\times3 = 72$ lipopeptides, all of which have been identified from recombinants (Fig. 7). Some are produced in abundance as discussed above, but some that required three genetic changes are produced in low quantities. All of the compounds tested to date have antibacterial activities, with minimal inhibitory concentrations of selected compounds on *S. aureus* ranging from 0.5 to 8 μg/ml [Brian et al. manuscript in preparation, Miao et al. manuscript submitted]. Those compounds containing the Glu_{12} for $3mGlu_{12}$ substitutions were the least active.

Prospects

This work demonstrates the potential for combinatorial biosynthesis of lipopeptide antibiotics related to daptomycin. We have just scratched the surface of potential module exchanges. Each additional module exchange at positions 1–11 can be readily leveraged to generate 24 new compounds by the methodology described here. Some of the positions in the daptomycin molecule may not be suitable for extensive module exchanges (e.g., the highly conserved Asp_7 and Asp_9), but others may be quite amenable. There is a considerable amount of variation in structure just based upon the depsipeptides daptomycin, A54145 and CDA. This might be further expanded by including ten-membered ring lipopeptides containing all amide linkages (e.g., friulimicin [39] or laspartomycin [29], and possibly others). Finally, combinatorial biosynthesis can be coupled with more extensive substitutions of the fatty acid tail and with modifications of the amino functions of Orn or Lys, or modifications of hydroxyl functions of D-Ser to couple combinatorial biosynthesis with medicinal chemistry to generate even larger sets of molecules to explore as potential candidates for clinical development. The combinatorial biosynthesis results reviewed here suggest that the methodology should be applicable to other peptide

secondary metabolites derived by NRPS or mixed NRPS/PKS pathways.

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