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ENGINEERING THE BIOSYNTHESIS OF NONRIBOSOMAL LIPOPEPTIDE ANTIBIOTICS

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ABSTRACT

Biosynthetic engineering entails reprogramming the genes involved in the biosynthesis of natural products so as to generate new molecules, which would otherwise not exist in nature. Potentially this approach can be used to providing large numbers of secondary metabolites variants, with improved biological activities, many of which are too complex for effective total synthesis.

The calcium dependent antibiotic (CDA), from *Streptomyces coelicolor*, is nonribosomal lipopeptide. CDA is structurally related to the therapeutically important antibiotic daptomycin. The CDA producer, *S. coelicolor*, is also highly amenable to genetic modification, which makes CDA an ideal template for biosynthetic engineering. To this end we have probed the biosynthetic origins of CDA and utilized this information to develop methods which have enabled the first engineered biosynthesis of novel CDA-type lipopeptides. Notably a mutasynthesis approach was developed to generate CDAs with modified arylglycine residues. Active site modification of adenylation domains within the CDA nonribosomal peptide synthases also led to new lipopeptides.

INTRODUCTION

Nonribosomal peptides are secondary metabolites that are biosynthesized independently of the ribosome in prokaryotes and lower eukaryotes [1-3]. These complex natural products are linear or cyclic peptides comprised of many unusual as well as proteinogenic amino acids. Often the peptides contain fatty acid, polyketide, or carbohydrate building blocks. Oxidative cross-linking of amino acid side chains can further increase the diverse array of skeletal structures that are thus formed (Fig. 1). Indeed it transpires that the nonribosomal peptides are among the most structurally diverse and widespread secondary metabolites in nature [1-3]. Not surprisingly they display a wide range of biological activities and include many important therapeutic agents such as the immunosuppressive agent cyclosporin, the antitumour agent bleomycin and the antibiotic vancomycin (Fig. 1).

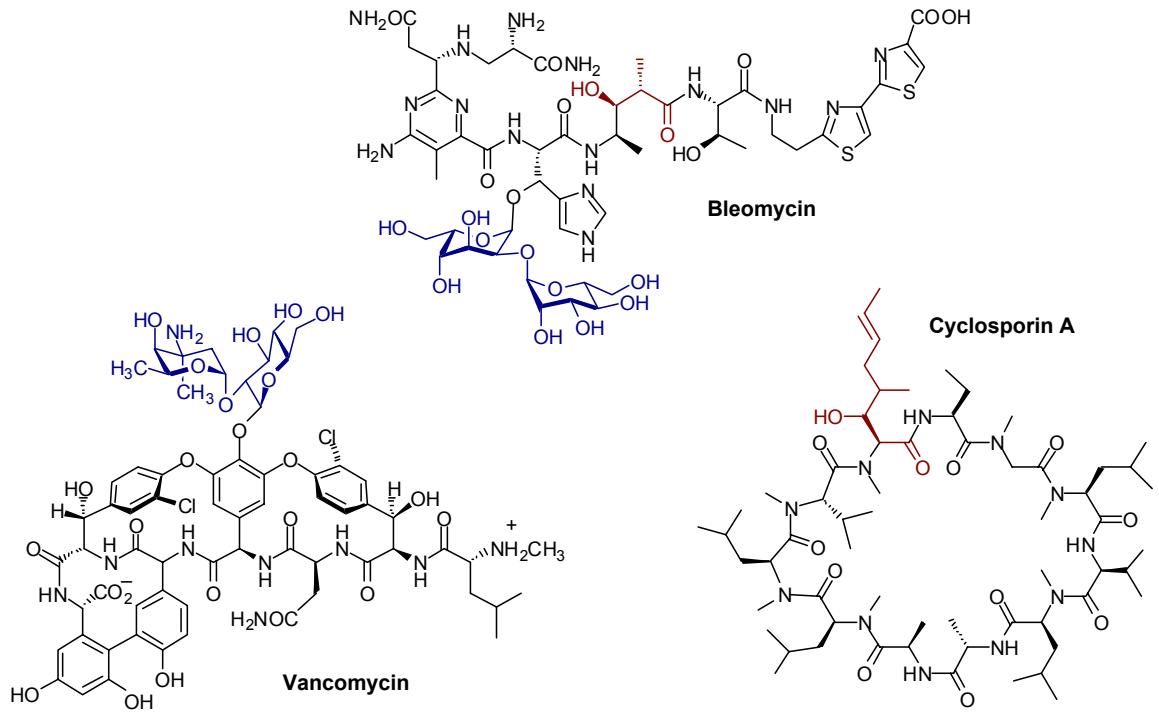


Figure 1. Nonribosomal peptide natural products of therapeutic importance. Bleomycin is produced by *Streptomyces verticillus*, vancomycin comes from *Streptomyces orientalis* and cylcosporin A is derived from *Tolypocladium niveum* and other fungi.

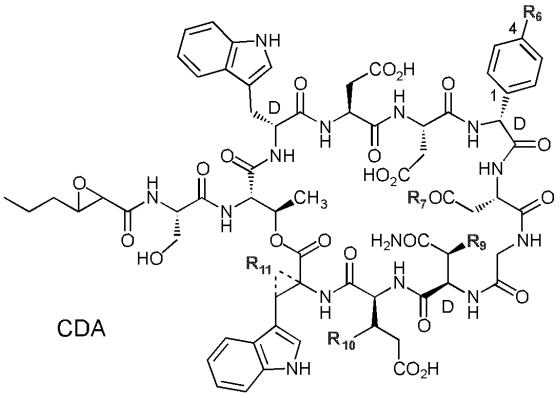
Whilst their structural complexity gives rise to their exquisite biological activities it also means that total synthesis is unable to supply the quantity or diversity of products required for drug development programmes. Consequently we are dependent on nature for our supply of these molecules and if we want novel structural variants of these existing natural products, with improved properties, then alternative biochemical methods of production must be developed.

To this end considerable attention has been focused on understanding how nature assembles the nonribosomal peptides, so that this insight might be used to develop methods which will lead to the engineered, possibly even combinatorial, biosynthesis of novel nonribosomal peptides with improved biological activities [4-7].

The nonribosomal peptide synthetases (NRPS) are the key assembly-line enzymes in the biosynthesis of the nonribosomal peptides [1-3]. NRPS are extremely large enzymes that contain multiple modules each of which is responsible for the activation and incorporation of a specific amino acid into the nascent peptide chain. A minimal NRPS module comprises a condensation (C), an adenylation (A) and a thiolation (T) domain, which is sometimes called the peptidyl carrier protein (PCP) domain. The T-domain is first post-translationally modified by a phosphopantetheinyl transferase (PPtase) enzyme which transfers the phosphopantetheine (Ppant) side chain of co-enzyme A to an active site serine residue. The A-domain then activates a specific amino acid substrate through the formation of amino-acyl adenylate intermediate, which is attacked by the thiol terminus of the Ppant group on the T-domain. It is upon this flexible Ppant side chain that the substrate amino-acyl thioesters can translocate to the upstream C-domain, which mediates the peptide bond forming reaction with the upstream intermediate. Release of the peptide from the NRPS, which is often accompanied by cyclization, is catalysed by a *C*-terminal thioesterase (Te) domain. Structural biology and enzymology studies have provided key insight into the specificities and mechanisms of these and other catalytic domains within NRPS modules [1-3]. From this a detailed picture has emerged which has made possible the engineered biosynthesis of new products [4-7]. Notably domains within NRPS modules can be surgically replaced or whole modules deleted by manipulation of the NRPS encoding genes, resulting in the mutant organisms that are capable of producing engineered products with different amino acid sequence and ring size [8, 9]. More recently, site directed mutagenesis of A-domains has been employed to change the specificity of the amino acids activated by the NRPS, which has also led to new peptide products [10].

THE ACIDIC LIPOPEPTIDE FAMILY OF ANTIBIOTICS

The calcium-dependent antibiotic (CDA), from *Streptomyces coelicolor* A3(2), is a cyclic-lactone undecapeptide which, in addition to an *N*-terminal 2,3-epoxyhexanoyl side chain, contains several D-configured as well as non-proteinogenic amino acids including D-4-hydroxyphenylglycine (D-HPG), D-3-phosphohydroxyasparagine and L-3-methylglutamic acid [11, 12] (Fig. 2). CDA shares a similar structure, and possibly a related mode of action [13], to other nonribosomally biosynthesized acidic lipopeptide antibiotics including daptomycin from *Streptomyces roseosporus* [14], A54145 from *Streptomyces fradiae* [15] and the friulimicins along with amphomycins from *Actinoplanes friuliensis* [16].



CDAx	R_6	R_7	R_9	R_{10}	R_{11}	Mol. wt.
CDA1b	OH	OH	OPO_3H_2	H	H,H	1562
CDA2a	OH	OH	OPO_3H_2	CH_3	π-bond	1574
CDA2b	OH	OH	OPO_3H_2	CH_3	H,H	1576
CDA3a	OH	OH	OH	H	π-bond	1480
CDA3b	OH	OH	OH	H	H,H	1482
CDA4a	OH	OH	OH	CH_3	π-bond	1494
CDA4b	OH	OH	OH	CH_3	H,H	1496
CDA2d	H	OH	OPO_3H_2	CH_3	H,H	1560
CDA2fa	F	OH	OPO_3H_2	CH_3	π-bond	1576
CDA2fb	F	OH	OPO_3H_2	CH_3	H,H	1578
CDA2a-7N	OH	NH_2	OPO_3H_2	CH_3	π-bond	1573

Figure 2. The structures of the calcium-dependent antibiotics (CDA). CDA1b, 2b, 3b and 4b were isolated and characterized previously [12]. CDA2a and CDA4a were isolated from S. coelicolor strain 2377 grown on solid media. CDA3a was isolated previously, but not fully characterized. CDA2d, CDA2fa and CDA2fb were generated by mutasynthesis [11]. CDA2a-7N is derived from active site modification of the module 7 Asp activating A-domain [35].

All of these lipopeptides contain common amino acid residues including several acidic residues which are important for calcium binding and subsequent antibiotic activity [13]. Remarkably, daptomycin recently became the first new class of natural antibiotic to reach the clinic in many years and is currently being used, under the trade name Cubicin, to treat skin infections with trials underway for its use to treat more serious life-threatening infections [17,18]. Thus there is a real need to develop methods that will enable the reprogrammed, engineered biosynthesis of new lipopeptide of the CDA/daptomycin class with altered and possibly improved antimicrobial activities.

Attempts to engineer new lipopeptides were made possible when the CDA biosynthetic gene cluster was identified, cloned and then sequenced [19,20]. This revealed open reading frames encoding 3 nonribosomal peptide synthetases (cdaPS1, 2 & 3) which are responsible for assembling 6, 3 and 2 amino acids respectively [11] (Fig. 3). Notably, there is an unusual *N*-terminal C′-domain that is likely to be involved in the transfer of the fatty acid to the first amino acid Ser of module 1. Also modules 3, 6 and 9 possess additional epimerization (E) domains which are responsible for the D-configured amino acids found at those positions in CDA. Finally there is a *C*-terminal Te-domain which catalyses the release of the peptide from the cdaPS3, through cyclization of the Ser-1 hydroxyl group on to the *C*-terminal carboxylate. In the flanking regions of the cluster are other genes that encode enzymes that are involved in the biosynthesis of some of the unusual amino acids and fatty acids building blocks, along with enzymes that are responsible for tailoring of the nascent peptide [11] (Fig. 3).

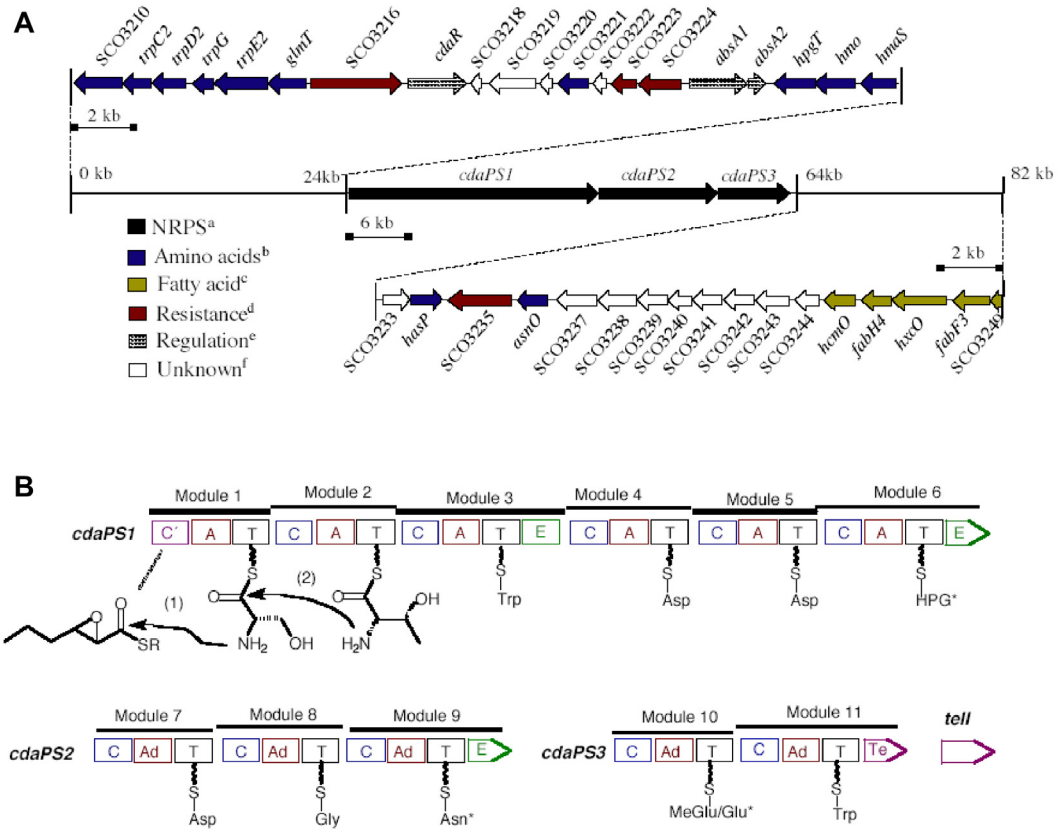


Figure 3. (A) Organization of the CDA biosynthetic gene cluster. **(B)** The CDA nonribosomal peptide synthase (NRPS).

WILD-TYPE CDAS WITH NOVEL Z-DEHYDROTRYPTOPHAN RESIDUES

Earlier work [12] had revealed the structures of four wild-type calcium-dependent antibiotics, CDA1b, 2b, 3b and 4b (Fig. 2), which differ in the substitution pattern at amino acid residues 9 and 10. Small amounts of other peptides were also isolated [12] but these were not characterized. Initially we set out to optimize the wild-type CDA production levels and to isolate and characterize any new natural CDA variants. Accordingly *S. coelicolor* strains 2377 [21] and MT1110 [21] were cultivated in a variety of different media and the supernatants where subsequently analysed by LC-MS. This revealed a similar profile of CDAs to that described previously [12]. On the other hand when strain 2377 was grown on solid media, Oxoid Nutrient Agar (ONA) LC-MS of the exudates revealed two previously uncharacterized CDA2a (1574 Da) and CDA4a (1494 Da) as the major products (Fig. 2).

Following large-scale solid media cultures and purification (HPLC) sufficient quantities of these peptides where isolated, which allowed a detailed structural elucidation using UV, NMR and mass spectrometry (MS). The structures of these metabolites differ from the b-series of CDA by the presence of an unusual *Z*-2,3-dehyrotryptophan (ΔTrp) residue rather than L-tryptophan. As there are two Trp derived residues in CDA at position 3 and 11, it was necessary to establish which of the two had been oxidized. This was achieved by ring opening CDA4a to generate a linear peptide, which was sequenced by tandem mass spectrometry. This clearly showed that the ΔTrp residue was at the *C*-terminus. Notably there are only a few other known nonribosomal peptides, including keramamide F and the microsclerodermins that contain ΔTrp [23, 24]. The structure of CDA3a (1480 Da) was also assigned (Fig. 2) on the basis of these findings.

MUTASYNTHESIS OF CDA WITH MODIFIED ARYLGLYCINE RESIDUES

In the *cda* gene cluster there are three genes *hmaS* (SCO3229), *hmo* (SCO3228), and *hpgT* (SCO3227) that encode proteins that display similarity to enzymes encoded by genes from the vancomycin-type antibiotic biosynthetic gene clusters in *Amycolatopsis orientalis* [25] and *Streptomyces lavendulae* [26]. These gene products are known to be involved in the biosynthesis of L-4-hydroxyphenylglycine (L-HPG) 3 [27,28]. HmaS catalyses the oxidative decarboxylation of 4-hydroxyphenylpyruvate to give L-4-hydroxymandelic acid 1 (Fig. 4). This then is oxidized by Hmo to give 4-hydroxyphenylglyoxylate 2, which is finally transaminated by HpgT resulting in L-HPG 3.

An initial attempt to engineer the biosynthesis of new CDA lipopeptides focused on the L-HPG pathway and utilized a mutasynthesis approach to generate new CDAs with modified arylglycine residues at position 6. Accordingly the first gene in the L-HPG pathway *hmaS* was deleted from the CDA cluster by standard 'double crossover' gene replacement. The resulting S. coelicolor mutant (Δ*hmaS*), is deficient in L-HPG 3 and the precursors 1 and 2 (Fig. 4) and was unable to produce CDA, as determined by both bioassays and LC-MS analysis. Feeding the mutant 4-hydroxymandelate 1,4-hydroxyphenylglyoxylate 2 or L-HPG 3 re-establishes the production of CDA in liquid and on solid culture media. This clearly demonstrates that, as expected, the pathway responsible for the biosynthesis L-HPG in *A. orientalis* [27,28] also operates in *S. coelicolor*.

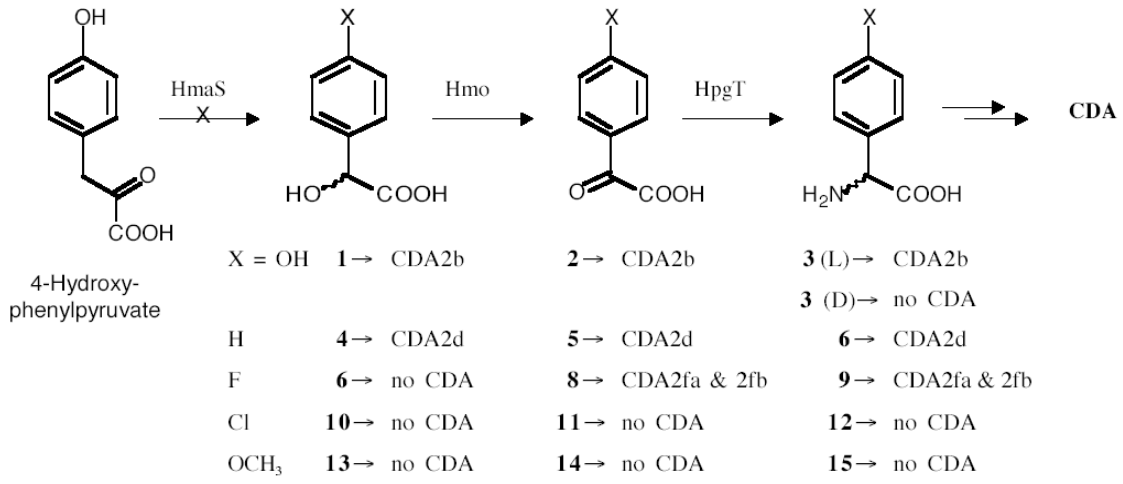


Figure 4. Mutasynthesis of CDA with modified arylglycine residues. HmaS = 4-hydroxymandelate synthase, Hmo = 4-hydroxymandelate oxidase, HpgT = 4-hydroxyphenylglycine transaminase. Both D- and L- enantiomers of HPG **3** were fed only the L-enantiomer is incorporated. All the other substrates were racemic.

Moreover, feeding 4-dehydroxy (4, 5, & 6) and 4-fluoro (8 &9) analogues of these precursors to the Δ*hmaS* mutant, grown in liquid culture media, resulted in new CDA peptides CDA2d, possessing modified phenylglycine (R_6 = H), as well as CDA2fa and CDA2fb with a 4-fluorophenylglycine (R_6 = F) (Fig. 4). The structures of these new peptides were confirmed by extensive NMR and mass spectrometry experiments [11]. In contrast 4-chloro 10-12 and 4-methoxy 13-16 analogues did not give rise to detectable CDA by LC-MS analysis. Presumably increasing the size of the C4-substituent, beyond the size of an hydroxyl group, results in failure of the NRPS L-HPG-activating A-domain to recognize and activate the modified arylglycines. This work is significant as it represents the first rational engineered biosynthesis of acidic lipopeptides antibiotics of this class. In addition this strategy could also be used to modify other important HPG-containing non-ribosomal peptides, such as those from the vancomycin group of antibiotics [29](Fig. 1).

SITE-DIRECTED MUTAGENESIS OF ADENYLATION DOMAINS

The next approach to engineer new CDAs focused on active site modification of the NRPS A-domains. Previously the 10 key residues at the active site of the A-domain that are responsible for binding the amino acid substrate were identified, using the X-ray crystal structure of (PheA), a Phe-activating A-domain, from gramicidin S synthetase [30,31].

Subsequently *in vitro* studies with recombinant A-domains showed that by changing as few as one of these residues it was possible to alter the specificity of the A-domain from activating one amino acid to another [30]. It was therefore anticipated, although at the outset unproven, that this approach might be extend *in vivo* in order to generate nonribsomal peptides with different amino acid sequences.

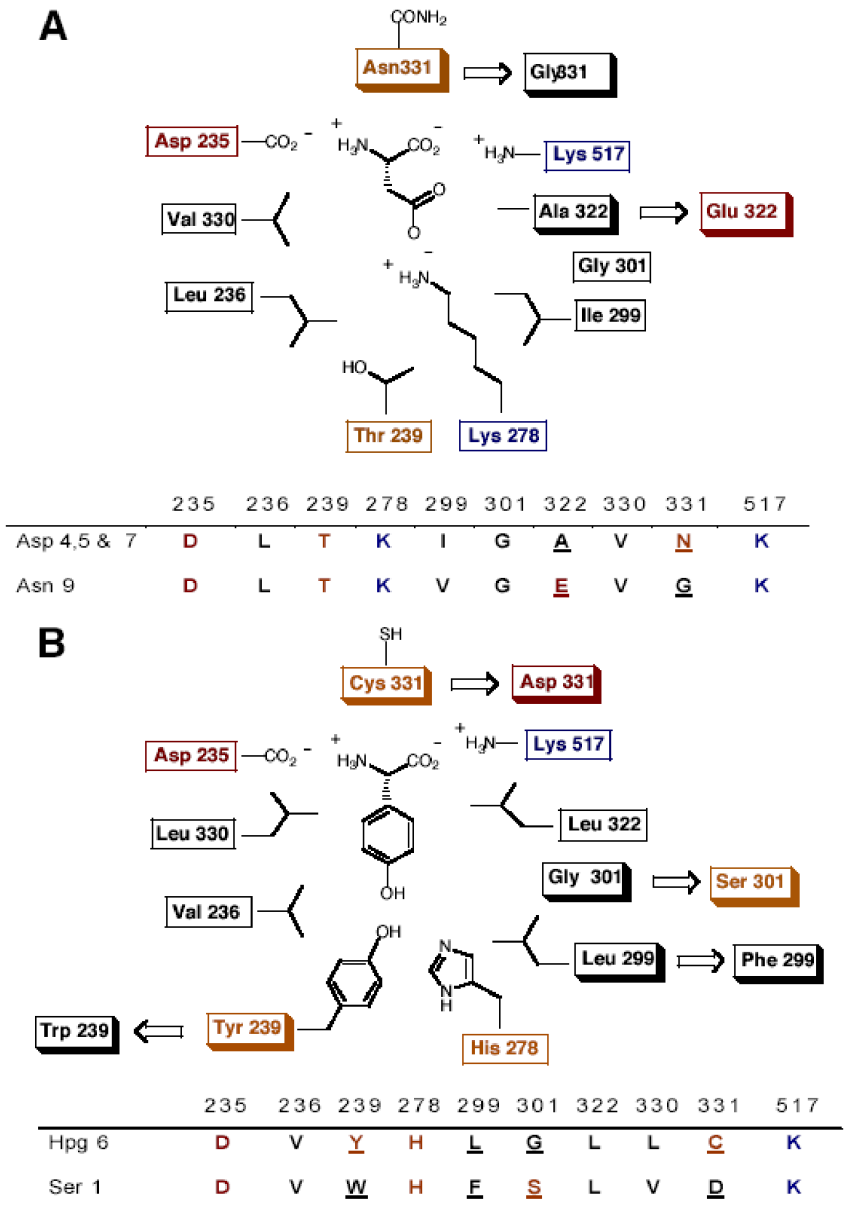
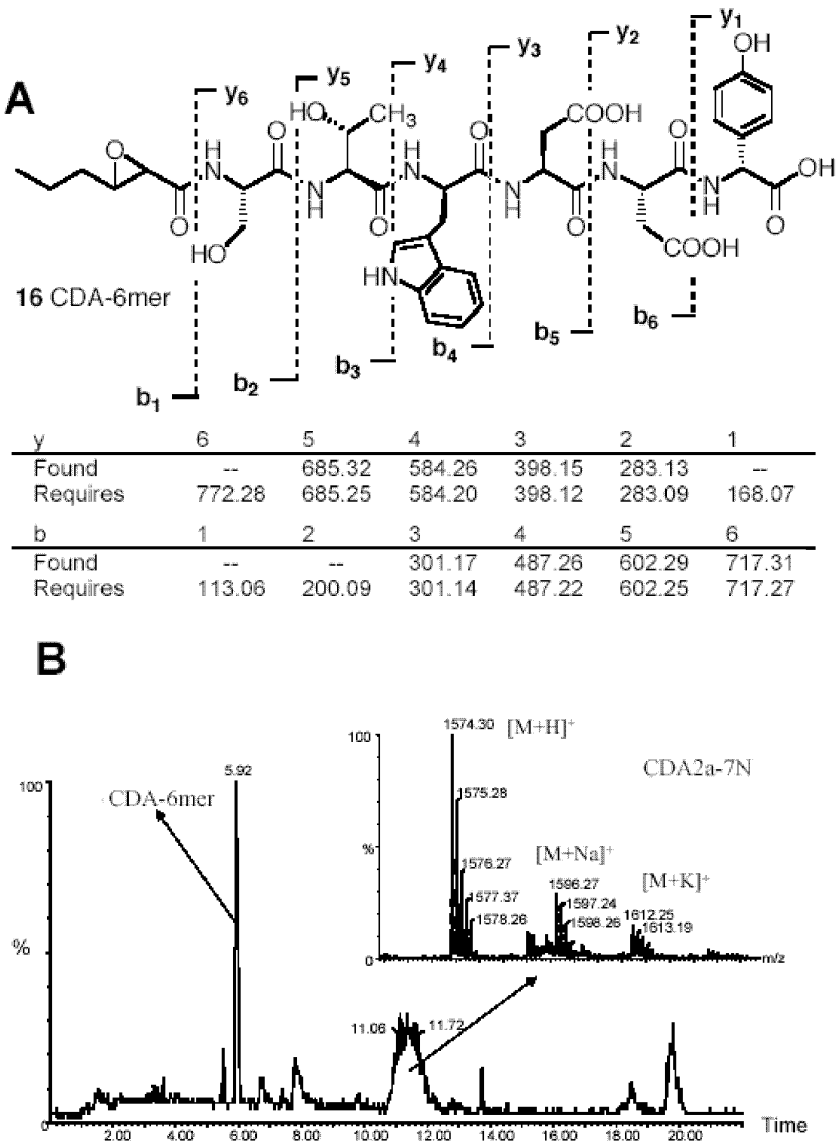


Figure 5. The active site architecture of **(A)** the module 7 Asp-activating A-domain and **(B)** the module 6 L-HPG activating A-domain. The residues of the amino acid binding pockets were determined through alignment with PheA [30, 31]. The tables below show the alignments of the Asp- and HPG-activating A-domains with the Asn and Ser activating A-domains of modules 9 and 1 respectively.

Alignment of the CDA NRPS A-domains with PheA enabled the key active site residues of each A-domain to be identified. Noticeably the active sites of the Asp-activating A-domains of modules 4, 5 and 7 are all identical to each other and also similar to the module 9 Asn activating A-domain differing at positions 299, 322 and 331 (Fig. 5A). Given that the residues at position 299 are similar (Val *vs* Ile) it was argued that changing Ala322→Glu and Asn331→Gly at one of the Asp-activating A-domains should result in a the incorporation of Asn into CDA instead of Asp. Accordingly, a DNA fragment encompassing module 7, was subjected to site directed mutagenesis to generate single (Ala322→Glu) and double-point (Ala322ÆGlu, Asn331→Gly) mutants which where cloned into a delivery plasmid to give pGUM7S and pGUM7D respectively. These plasmids were used to deliver the mutations to the appropriate regions of *cdaPS2* on the *S. coelicolor* chromosome through homologous recombination. The single-point mutant GUM7S failed to produce any CDA with Asn or Asp at position 7. A new product was however identified by LC-MS which was isolated and subsequently shown to the CDA-hexapeptide intermediate **16** by high resolution MS (*m/z* 884.3321 $[M+H]^+$, $C_{40}H_{50}N_7O_{16}$ requires 884.3314) and tandem MS (Fig. 6A). On the other hand the double-point mutant GUM7D produced both the linear 6mer **16** and a new product which exhibits *m/z* 1574.30 $[M+H]^+$, which is consistent with a new lipopeptide, CDA2a-7N (Fig. 2) possessing Asn at position 7 rather than Asp, that is not seen in the wild-type. Significantly, extracts containing CDAa-7N are inactive in bioassays, which suggests that Asp-7 is essential for calcium binding and antibiotic activity of CDA.

During this study a similar approach was used to generate modified variants of the lipopeptide surfactin in *Bacillus subtilis* [10]. In this case no peptidyl intermediates where identified. Therefore to test the generality of our findings another *S. coelicolor* mutant GUM6S was generated possessing a single-point mutation (Gly301→Ser) of the module 6 HPG-activating A-domain (Fig. 5B). It is predicted [31] that this mutation should change the specificity of the A-domain from HPG to Ser. LC-MS analysis of the mutant failed to show the production of CDA with Ser at position 6. However a new product was identified which corresponds in m.w. (734 Da) and HPLC retention time to the CDA 5mer intermediate 2,3-epoxyhexanoyl-Ser-Thr-D-Trp-Asp-Asp-OH 17. A module 6, A-domain quadruple point mutant (Gly301→Ser, Leu299→Phe, Cys331→Asp & Tyr239→Trp) was also prepared but this also failed to generate CDA with Ser at position 6 instead of HPG.



y	6	5	4	3	2	1
Found	--	685.32	584.26	398.15	283.13	--
Requires	772.28	685.25	584.20	398.12	283.09	168.07

b	1	2	3	4	5	6
Found	--	--	301.17	487.26	602.29	717.31
Requires	113.06	200.09	301.14	487.22	602.25	717.27

Figure 6. (A) The CDA-6mer **3** and the y and b series ions derived from the product ion MS-MS spectra. **(B)** LC-MS of extracts from the *S. coelicolor* double-point mutant GUM7D.

EVIDENCE OF THE EXISTENCE OF AN HITHERTO ELUSIVE NRPS PROOF-READING MECHANISM

It is possible, given the lability of the peptidyl thioester bond, that non-enzymatic hydrolysis could have caused the release of the CDA-6mer **16** and 5mer **17**. However, the clear absence of any of the other shorter intermediates in the culture supernatant means this is unlikely. It is more likely that changes to the active site of the A-domain result in failure or less efficient activation of non-cognate amino acids, which brings about a kinetic blockage on the NRPS that signals the enzymatic hydrolysis of the stalled upstream peptidyl chain.

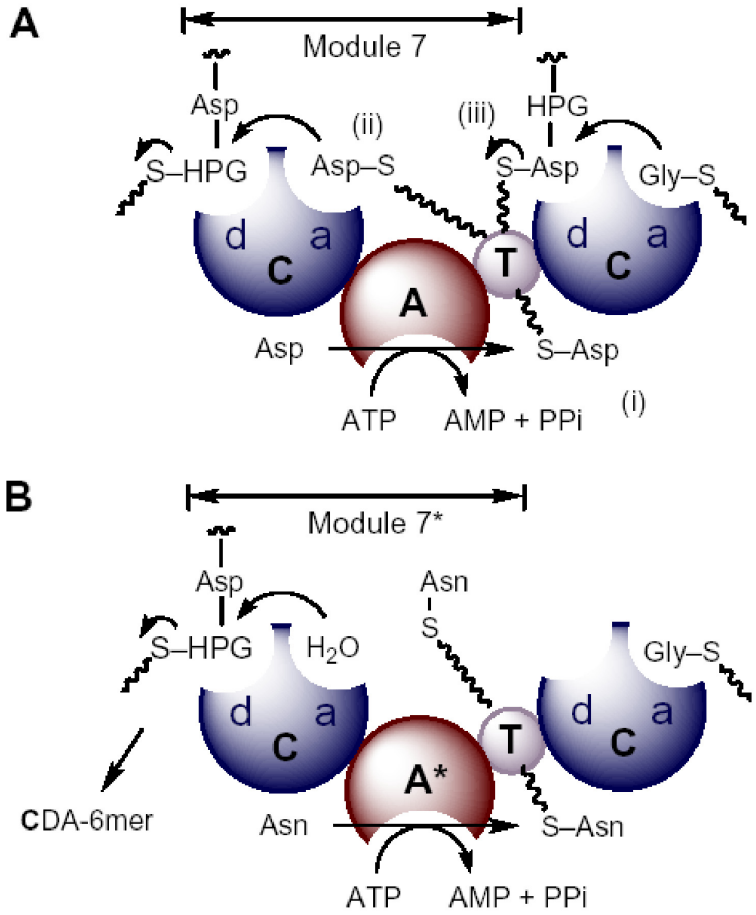


Figure 7. (A) A schematic description of the reactions catalysed by the module 7 of cdaPS2. (i) The A-domain activates and transfers the substrate amino acid to the Ppant side chain of the T-domain. (ii) The thioester intermediate translocates to the acceptor (a) site on the upstream C-domain, where it intercepts the upstream hexapeptidyl thioester intermediate in the donor (d) site. (iii) The resulting heptapeptidyl thioester is then translocated to the d-site on the down stream C-domain, before formation of the next peptide bond with Gly. **(B)** The mutant module 7 A-domain (GUM7D) activates Asn instead of Asp. However, the Asn-thioester intermediate is only weakly recognized by the upstream C-domain a-site. A water molecule can thus compete for the a-site and intercept the hexapeptidyl intermediate in the d-site.

In the *cda* gene cluster, down stream of *cdaPS3*, is gene *teII* that encodes a typical type II thioesterase (Fig. 3). It has been postulated that these type II thioesterases may be involved in proof-reading NRPS [32] and relate modular polyketide synthases (PKS) [33, 34]. A double mutant GUM7S-Δ*teII* was therefore generated where the *teII* gene was deleted from the *S. coelicolor* module 7, A-domain single-point mutant (GUM7S). This was also shown to produce the CDA-6mer 16, which suggests that TeII is not responsible for the hydrolysis of the stalled peptidyl intermediates. Instead we have proposed [35] that an alternative NRPS proof-reading mechanism exists where the upstream condensation (C) domain catalyses the hydrolysis of the stalled peptidyl intermediates (Fig. 7). According to this hypothesis a modified A-domain activates a non-cognate amino acid substrate, which is transferred to the phosphopantetheine group of the thiolation (T) domain. The amino acyl-*S*-Ppant group then translocates to the acceptor (a) site [36] on the upstream C-domain. However, the a-site is unable to, or only weakly able to, recognize the non-cognate substrate. Consequently a water molecule can bind to the a-site, then using the catalytic functionality of the C-domain, intercept the peptidyl-thioester intermediate in the donor (d) site of the C-domain. As a result of these findings we suggest that to incorporate a non-cognate amino acid into a nonribosomal peptide efficiently, it is likely that the specificity of the upstream C-domain acceptor site must be altered, as well as the A-domain.

CONCLUSION

We have isolated and elucidated the structures of several previously uncharacterized CDAs, which possess unusual *Z*-dehydrotryptophan residues at the C-terminus. In addition we were first to engineer the biosynthesis of novel lipopeptides of the CDA/daptomycin class. Our first approach involved a mutasynthesis strategy. Here a gene involved in the biosynthesis of L-hydroxyphenylglycine (L-HPG) was deleted. Analogues of HPG and its precursors were then fed to the resultant mutant to give several novel engineered CDAs with modified arylglycine residues at position 6. We also showed that site directed mutagenesis of the module 7 A-domain leads to a new engineered lipopeptide product (CDA2a-7N) with Asn at position 7 instead of Asp which is found in the wild type CDA. However the levels of CDA2a-7N were considerably reduced compared with the wild-type CDA. This was due to the premature hydrolysis of upstream peptidyl intermediate.

These findings are significant because they point to the existence of a hitherto elusive NRPS proof-reading mechanism, which must be further elucidated and eventually circumnavigated, if this approach is to be successful in delivering the quantities of modified products required for drug development.

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