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Active-Site Modifications of Adenylation Domains Lead to Hydrolysis of Upstream Nonribosomal Peptidyl Thioester Intermediates

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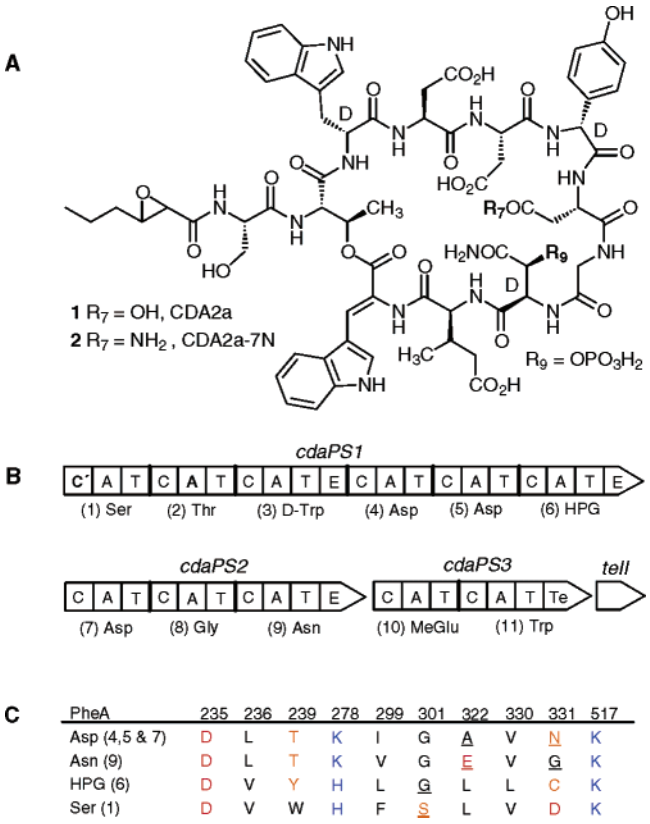
Nonribosomal peptides are among the most structurally diverse and widespread secondary metabolites in nature.[1] Many of these complex peptides are widely used therapeutic agents. There is therefore a great need to develop methods that will allow for the production of novel variants. Given their structural complexity, it is unlikely that total synthesis will supply this demand. On the other hand, the reprogrammed, engineered biosynthesis of modified nonribosomal peptides is more realistic.[2] Indeed, there has been significant progress in delineating rules through which it is possible to reprogram nonribosomal peptide synthetases (NRPS) leading to new peptide products.[3]

The calcium-dependent antibiotic (CDA) from *Streptomyces coelicolor* A3(2) is a nonribosomal acidic lipopeptide (Figure 1A).[4] CDA is similar in structure to daptomycin, which is the first antibiotic of this class to have received approval for clinical use.[5] Previously, we have used a mutasynthesis approach to engineer CDA variants with modified arylglycine residues at position 6.[4] Following this, we set out to engineer new CDAs by site-directed mutagenesis of adenylation (A) domains. The A-domain is responsible for the activation and thiolation of specific amino acids prior to peptide assembly.[1] On the basis of the crystal structure of PheA, a Phe-activating A-domain from gramicidin S synthetase, the 10 active-site residues that are responsible for binding the amino acid substrates were proposed.[6] By changing as few as one of these residues, it has been shown possible to alter the specificity from one amino acid to another, at least in vitro.[6a]

In CDA, there are 3 Asp residues, and the corresponding Asp-activating A-domains of modules 4, 5, and 7 have identical active sites (Figure 1B,C). These are similar to the module 9 Asn-activating A-domain differing at only three positions: Val299 versus Ile, Glu322 versus Ala, and Gly331 versus Asn. It was predicted that single (Ala322→Glu)- or double (Ala322→Glu, Asn331→Gly)-point mutations of one of the Asp A-domains should be sufficient to engineer new CDAs with Asn replacing Asp. Accordingly, a 1.7 kb DNA fragment encompassing the module 7 A-domain, derived by PCR, from *cdaPS2* was mutated and cloned into the plasmid pKC1132[7a] to generate plasmids pGUM7S and pGUM7D coding for single- and double-point mutants, respectively. Transformation of *S. coelicolor* 2377[7b] and MT1110[7c] protoplasts, followed by homologous recombination and subsequent screening of second crossover recombination events,[4] resulted in mutants GUM7S and GUM7D. The mutants were then grown in liquid culture under conditions that are favorable for CDA production,[4] and the supernatants were analyzed by LC-MS. The single-point mutant GUM7S failed to produce CDA with Asn at position 7, or indeed any wild-type CDAs. However, a new product was identified



PheA	235	236	239	278	299	301	322	330	331	517
Asp (4,5 & 7)	D	L	T	K	I	G	A	V	N	K
Asn (9)	D	L	T	K	V	G	E	V	G	K
HPG (6)	D	V	Y	H	L	G	L	L	C	K
Ser (1)	D	V	W	H	F	S	L	V	D	K

Figure 1. (A) Structures of wild-type CDA2a[4] **1** and engineered CDA2a-7N **2**. (B) Organization and order of domains in the CDA peptide synthetase encoding genes.[4] (C) Active-site residues of CDA A-domains identified by alignment with PheA.[6]

by LC-MS with a retention time of 5.9 min that exhibited protonated, sodiated, and potassiated singly charged ions, which are consistent with the linear CDA-hexapeptide intermediate **3** (mw 883 Da). This product was subsequently purified by HPLC and subjected to high-resolution MS, confirming the proposed molecular formula (*m*/*z* 884.3321 $[M + H]^+$, $C_{40}H_{50}N_7O_{16}$ requires 884.3314). The product **3** was also subjected to tandem MS, and the product ion spectrum was shown to possess the key y and b series ions that confirm the sequence of the peptide **3** (Figure 2A). Under identical fermentation conditions, this intermediate was clearly not evident in the original strains 2377 or MT1110.

In contrast, the double-point mutant GUM7D produced both the linear 6mer **3** and another new product with a retention time of ca. 11 min, which is close to the retention time of CDA2a produced by the wild-type (Figure 2B). The new product exhibits *m*/*z* 1574.30 $[M + H]^+$ along with sodiated, potassiated, and doubly charged ions that are all one mass unit lower than the wild-type CDA2a **1**

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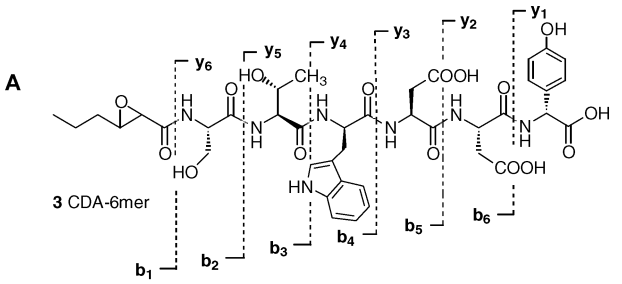

y	6	5	4	3	2	1
Found	--	685.32	584.26	398.15	283.13	--
Requires	772.28	685.25	584.20	398.12	283.09	168.07

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

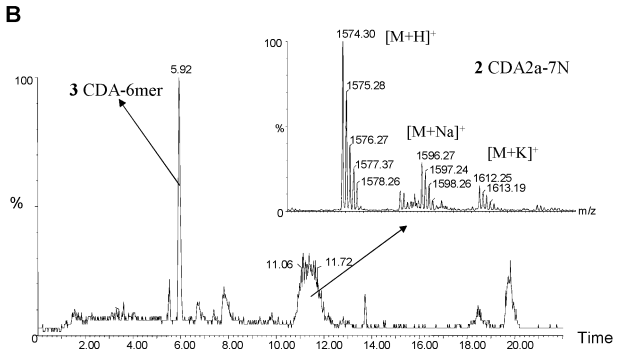

Figure 2. (A) The CDA-6mer **3** and the y and b series ions derived from the product ion MS-MS spectra. (B) LC-MS of the culture supernatant from the *S. coelicolor* 2377 double-point mutant GUM7D. The broad nature of the peak with a retention time of ca. 11 min is typical of phosphorylated CDA run under nonbuffered conditions.

(Figure 1A). This is consistent with production of a new lipopeptide, that is not seen in the wild-type, CDA2a-7N **2**, which as predicted possesses Asn at position 7 rather than Asp. Significantly, extracts of the mutant GUM7D containing CDA2a-7N are completely inactive in bioassays using *Bacillus mycoides* as an indicator strain. This suggests that Asp-7 is essential for calcium binding and subsequent antibiotic activity of CDA. While this work was in progress, a similar strategy was reported to modify the lipopeptide surfactin in *Bacillus subtilis*.[3a] However, this study does not report the release of peptidyl intermediates as a consequence of A-domain modification. To investigate the generality of these findings, we similarly generated another *S. coelicolor* 2377 mutant GUM6S possessing a single-point mutation (Gly301→Ser) of the module 6 hydroxyphenylglycine-activating A-domain. This mutation was predicted to alter the specificity of the A-domain to Ser.[6b] While no new CDA with Ser at position 6 was detected, a new product with a mw of 734 Da was identified that corresponds to the CDA pentamer 2,3-epoxyhexanoyl-Ser-Thr-D-Trp-Asp-Asp-OH **4**.

These findings are significant as they indicate the possible existence of a hitherto elusive proof-reading mechanism, whereby less efficient activation of noncognate amino acids results in a kinetic blockage on the NRPS, which signals the enzymatic hydrolysis of the stalled upstream peptidyl chain.[8] Directly downstream of *cdaPS3* in the *cda* cluster is a gene *teII* encoding a typical type II thioesterase (Figure 1B). It has been shown that related thioesterases are responsible for the hydrolysis of acetyl groups of peptidyl carrier proteins that have been misprimed with acetyl-CoA, instead of CoA.[9a] It has also been suggested that type II thioesterases may be involved in editing related polyketide synthases (PKS).[9b,c] To test whether this is also the case for the CDA NRPS, the putative *teII* gene was deleted in-frame, by homologous recombination, from the *S. coelicolor* MT1110 mutant that contains the module 7, A-domain single-point mutation (GUM7S). The resulting double mutant GUM7S−Δ*teII* was shown to also produce the 6mer **3**. This suggests that TeII does not catalyze the hydrolysis of the stalled peptide. Instead, a possible editing mechanism may involve hydrolysis catalyzed by the upstream condensation (C) domain. According to this proposal, a noncognate amino acid is activated and transferred to the phosphopantetheine group of the thiolation (T) domain; however, the acceptor (nucleophilic) site[10] on the upstream C-domain is unable to, or only weakly able to, recognize the noncognate amino acid. In its absence, a water molecule enters the acceptor site and attacks the peptidyl-thioester intermediate in the donor site[10] of the C-domain.

In conclusion, we have shown that it is possible to change the specificity of an A-domain in vivo leading to a new engineered lipopeptide product (CDA2a-7N). However, the yield of CDA2a-7N was reduced relative to that of wild-type CDA, because of the premature hydrolytic release of the upstream peptidyl intermediate. These findings have serious implications for the viability of using A-domain active-site modifications to engineer new nonribosomal peptides and suggest that it may be necessary to modify the specificity of the acceptor site of the upstream C-domain as well as the A-domain in order to incorporate a noncognate amino acid efficiently.

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Supporting Information Available: Procedures and data (PDF). See any current masthead page for ordering information and Web access instructions.

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(8) It is possible, given the lability of the peptidyl thioester bond, that nonenzymatic hydrolysis could be responsible for release of the CDA-6mer **3** and 5mer **4**. However, the clear absence of any of the other shorter intermediates in the culture supernatant means this is unlikely.

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