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Structure, Biosynthetic Origin, and Engineered Biosynthesis of Calcium-Dependent Antibiotics from *Streptomyces coelicolor*

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Summary

The calcium-dependent antibiotic (CDA), from *Streptomyces coelicolor*, is an acidic lipopeptide comprising an N-terminal 2,3-epoxyhexanoyl fatty acid side chain and several nonproteinogenic amino acid residues. *S. coelicolor* grown on solid media was shown to produce several previously uncharacterized peptides with C-terminal *Z*-dehydrotryptophan residues. The CDA biosynthetic gene cluster contains open reading frames encoding nonribosomal peptide synthetases, fatty acid synthases, and enzymes involved in precursor supply and tailoring of the nascent peptide. On the basis of protein sequence similarity and chemical reasoning, the biosynthesis of CDA is rationalized. Deletion of SCO3229 (*hmaS*), a putative 4-hydroxymandelic acid synthase-encoding gene, abolishes CDA production. The exogenous supply of 4-hydroxymandelate, 4-hydroxyphenylglyoxylate, or 4-hydroxyphenylglycine re-establishes CDA production by the Δ*hmaS* mutant. Feeding analogs of these precursors to the mutant resulted in the directed biosynthesis of novel lipopeptides with modified arylglycine residues.

Introduction

With the advent of the post-genomic era, the sequences of numerous gene clusters required for the biosynthesis of many valuable secondary metabolites have become available for exploitation. Most notably, the genomes of *Streptomyces coelicolor* A3(2) [1] and *Streptomyces avermitilis* [2] from the prolific secondary metabolite-producing actinomycete genus have recently been sequenced. Each reveals more than 20 gene clusters predicted to be involved in the biosynthesis of secondary metabolites. With this wealth of new sequence information in hand and utilizing increasingly powerful genetic tools for the manipulation of microbial secondary metabolite genes, it should be possible to reprogram the biosynthesis of natural products in order to produce new molecules with altered and possibly improved biological activities [3, 4].

One class of natural products that are particularly suited to reprogrammed, engineered biosynthesis are the nonribosomal peptides [5, 6]. These secondary metabolites, which are among the most structurally diverse and widespread in nature, include linear and cyclic peptides comprised of both proteinogenic and unusual amino acids as well as many other structural moieties derived from fatty acid, polyketide, or carbohydrate building blocks [7]. Nonribosomal peptide-derived natural products display a wide range of biological activity and include the immunosuppressant cyclosporin, the antitumor agent bleomycin, and the current antibiotic of last resort, vancomycin. While there is always a need for new improved therapeutics of all kinds, the increase in the incidence of pathogenic bacteria with resistance to known antibiotics means that there has never been a greater need for new antimicrobial agents.

Recently, an 82 kb region of the *Streptomyces coelicolor* A3(2) genome that encodes the biosynthetic enzymes required for the production of the calcium-dependent antibiotic (CDA), an acidic lipopeptide, was identified, cloned, and partially sequenced at UMIST [8]. The CDA gene cluster, which comprises approximately 1% of the genome, was subsequently sequenced in entirety as part of the *S. coelicolor* genome project [1]. CDA is a cyclic-lactone undecapeptide which, in addition to an N-terminal 2,3-epoxyhexanoyl side chain, contains several D-configured as well as nonproteinogenic amino acids, including D-4-hydroxyphenylglycine, D-3-phosphohydroxyasparagine, and L-3-methylglutamic acid [9] (Figure 1).

CDA shares a similar structure and possibly a related mode of action to other acidic lipopeptide antibiotics, including daptomycin [10], friulimicins, and amphomycins [11] (Figure 1). Interestingly, all of these antibiotics are comprised of a decapeptide lactone or lactam ring derived from cyclization of L-threonine or L-*threo*-2,3-diaminobutyrate side chains onto the C-terminal carboxyl group. Noticeably, six of the amino acid side chains in the CDA lactone ring are found at the same positions in the other lipopeptide structures, including several acidic residues which are likely to be important for calcium binding and biological activity. The therapeutic importance of this class of antibiotics is best exemplified by daptomycin, which is active against a wide range of gram-positive pathogens including strains resistant to all current antibiotic treatments. At present, daptomycin, under the trade name Cidecin, is undergoing phase III clinical trials for the treatment of a range of life-threatening infections.

In this paper, the isolation and structural elucidation of novel CDA lipopeptides from wild-type *S. coelicolor* is described. A hypothesis, based on sequence analysis

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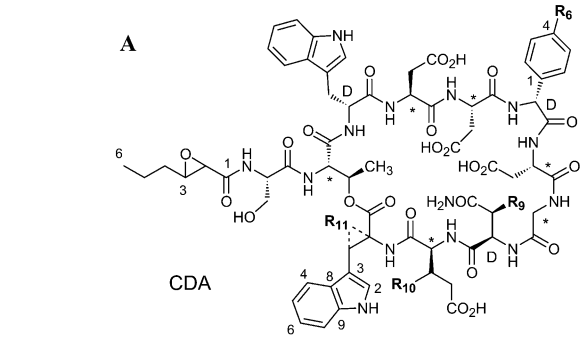
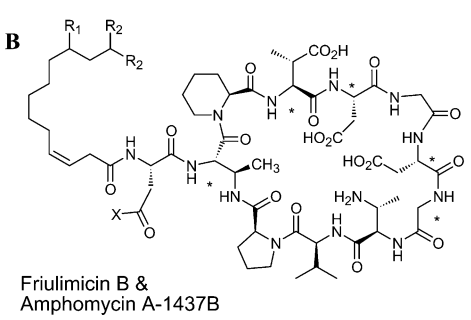


CDAx	R_6	R_9	R_{10}	R_{11}	Mol. wt.
CDA1b	OH	OPO_3H_2	H	H,H	1562
CDA2a	OH	OPO_3H_2	CH_3	π-bond	1574
CDA2b	OH	OPO_3H_2	CH_3	H,H	1576
CDA3a	OH	OH	H	π-bond	(1480) [a]
CDA3b	OH	OH	H	H,H	1482
CDA4a	OH	OH	CH_3	π-bond	1494
CDA4b	OH	OH	CH_3	H,H	1496
CDA2d	H	OPO_3H_2	CH_3	H,H	1560 [b]
CDA2fa	F	OPO_3H_2	CH_3	π-bond	1576 [b]
CDA2fb	F	OPO_3H_2	CH_3	H,H	1578 [b]



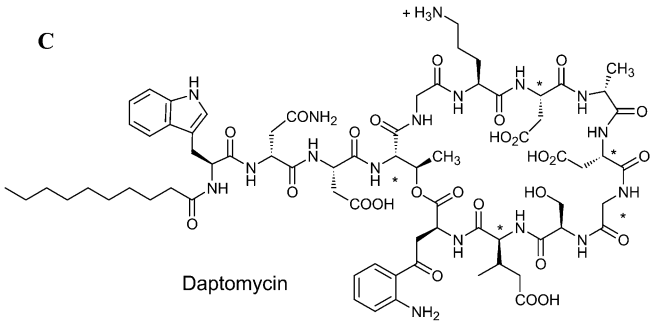


Figure 1. The Structures of Acidic Lipopeptide Antibiotics

(A) The calcium-dependent antibiotics isolated from *S. coelicolor*. The table shows the variability of amino acid residues at positions 6, 9, 10, and 11. Common amino acid residues found at the same positions in other acidic lipopeptides are indicated with an asterisk. The b-series CDA were isolated and characterized previously, and all were shown to possess a C-terminal L-tryptophan residue [9]. CDA2a and CDA4a were isolated from strain 2377 grown on solid media in this study and shown to differ by the presence of a C-terminal *Z*-2,3-dehydrotryptophan residue. [a]CDA3a was isolated previously, but not fully characterized [9]; [b]CDA2d, CDA2fa, and CDA2fb were isolated from the 2377Δ*hmaS* mutant supplemented with racemic phenylglycine or 4-fluorophenylglycine.

(B) Friulimicin B (X = NH_2, R_1 = H, R_2 = CH_3) and amphomycin A-1437B (X = OH, R_1 = CH_3, R_2 = H) from *Actinoplanes friuliensis* [11].

(C) Daptomycin (LY146032) derived from A21978C produced by *Streptomyces roseosporus* [10].

of the gene products from the CDA cluster, is proposed that accounts for the biosynthesis of CDA. Finally, the first rational engineered biosynthesis of novel modified lipopetides of this class is demonstrated using a mutasynthesis approach.

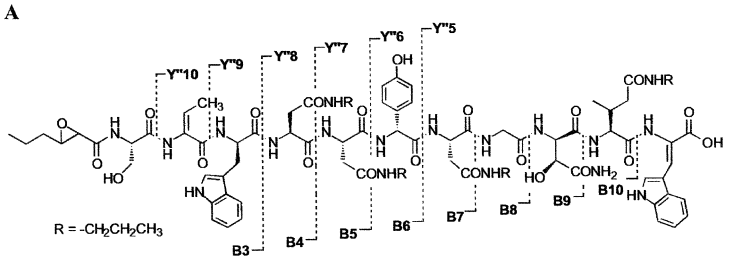
Results and Discussion

Isolation and Characterization of Natural CDA Variants

The structures of four calcium-dependent antibiotics, CDA1b, 2b, 3b, and 4b (Figure 1), produced by *S. coelicolor* A3(2) 2377 were determined previously [9]. These structures differ in the substitution pattern at amino acid residue 9, which is either D-3-hydroxyasparagine or D-3-phosphohydroxyasparagine, and residue 10, which is either L-glutamic acid or L-3-methylglutamic acid. Minor amounts of other peptides CDA3a and CDA4a, which differ in molecular weight by −2 Da, were also isolated but had not been characterized to date. In order to determine the structure of these minor metabolites and any other natural CDA variants, *S. coelicolor* strains 2377 [12] and MT1110 [13] were cultivated in a variety of different media. LC-MS of supernatants from many different liquid culture media revealed a similar profile of CDAs (data not shown) to that described previously [9]. However, strain 2377, when grown on oxoid nutrient agar (ONA), was shown, by LC-MS of the exudate, to produce previously uncharacterized CDA2a (1574 Da) and CDA4a (1494 Da) as major products (Figure 1). Solid-phase extraction on the exudate from large-scale solid media cultures followed by purification (HPLC) provided sufficient quantities of these peptides to allow detailed structural elucidation.

First, UV spectra of both CDA2a and CDA4a (see Supplemental Data) reveal a major absorption λ_{max} 349 nm (in H_2O) that is absent from previously characterized (b-series) peptides (λ_{max} 279 in H_2O). This is indicative of the presence of 2,3-dehydrotryptophan (ΔTrp) [14, 15]. High-resolution electrospray ionization mass spectrometry (HRESIMS) of CDA2a (*m/z* 1575.4955 $[M+H]^+$, $C_{67}H_{80}N_{14}O_{29}P$ requires 1575.4953) and CDA4a (*m/z* 1495.5238 $[M+H]^+$, $C_{67}H_{79}N_{14}O_{26}$ requires 1495.5290) are consistent with the molecular formula for the structures proposed. Both peptides are 2 Da lower than CDA2b and CDA4b, respectively, which implies that only one of the tryptophan-derived residues, at either position 3 or 11, has been dehydrogenated. In order to determine the position of the ΔTrp residue, the lower molecular weight peptide CDA4a, which is nonphosphorylated, was transformed to the corresponding *n*-propyl tetraam-

A



B

Figure 2. MS-MS Sequencing of CDA4a Derivative

(A) The linear peptide derived from CDA4a by tetramidation followed by basic hydrolysis, showing fragmentation pattern observed in the product ion MS-MS spectra (see Supplemental Data).

(B) The observed and calculated masses of the Y″ and B-series ions resulting from the product ion spectrum of the linear peptide derived from CDA4a. Accurate masses were determined for the majority of ions; the remainder were recorded at lower resolution. All are in agreement with the elemental formula derived from the predicted fragmentation pattern shown.

Product ion.	Mol. formula	Mass		Error	
		Calc'd	Observed	mDa	ppm.
Y"10	$C_{70}H_{94}N_{17}O_{18}$	1460.6963	1459.70	—	—
Y"9	$C_{66}H_{89}N_{16}O_{17}$	1377.6592	1377.70	—	—
Y"8	$C_{55}H_{79}N_{14}O_{16}$	1191.5798	1191.5846	4.7	4.0
Y"7	$C_{48}H_{67}N_{12}O_{14}$	1035.4900	1035.4872	-2.7	-2.7
Y"6	$C_{41}H_{55}N_{10}O_{12}$	879.4001	879.3976	-2.4	-2.8
Y"5	$C_{33}H_{48}N_9O_{10}$	730.3524	730.36	—	—
B10	$C_{68}H_{97}N_{16}O_{20}$	1457.7065	1457.7017	-4.8	-3.3
B9	$C_{59}H_{81}N_{14}O_{18}$	1273.5853	1273.58	—	—
B8	$C_{55}H_{75}N_{12}O_{15}$	1143.5475	1143.5470	-0.5	-0.4
B7	$C_{53}H_{72}N_{11}O_{14}$	1086.5260	1086.5253	-0.8	-0.7
B6	$C_{46}H_{60}N_9O_{12}$	930.4361	930.4392	-0.1	-0.1
B5	$C_{38}H_{53}N_8O_{10}$	781.3885	781.3840	-4.4	-5.7
B4	$C_{31}H_{41}N_6O_8$	625.2986	625.3033	4.8	7.6
B3	$C_{24}H_{29}N_4O_6$	469.2087	469.2105	1.8	3.9

ide using 1-propylamine, TBTU, and HOBt [9]. The major product of the reaction was purified by HPLC and shown to possess the expected molecular formula by HRESIMS (*m/z* 1659.7734 $[M+H]^+$, $C_{79}H_{107}N_{18}O_{22}$ requires 1659.7807). Hydrolysis of the peptide with 1 M aqueous NaOH and purification (RP-HPLC) gave a linear peptide (Figure 2A) of the same molecular formula (HRESIMS: *m/z* 1659.7792). The ESI MS-MS product ion spectra of this peptide (see Supplemental Data) and accurate mass measurements of key Y″ and B series ions (Figure 2B) were entirely consistent with the proposed structure of the linear peptide. Notably, the B10 ion (*m/z* 1457.7017) confirmed the presence of a C-terminal ΔTrp (position 11). The other observed ions also confirmed the sequence of the peptide and indicated that opening of the lactone ring proceeds with concomitant transformation of threonine, at position 2, to 2,3-dehydro-2-aminobutyric acid, probably via β-elimination (E1cB) of the C-terminal carboxylate.

The presence of the phosphohydroxyasparagine (position 9) in CDA2a was confirmed by ^{31}P NMR (δ_P = 1.28 ppm in $DMSO_{d6}$). Chemical derivatization and sequencing by mass spectrometry is complicated by the phosphoryl group and so further structural studies on CDA2a were carried out using NMR spectroscopy. Accordingly, the majority of ^1H and ^{13}C chemical shifts for CDA2a were assigned using ^1H-^{13}C HMQC, HMBC, and ^1H-^1H COSY experiments (see Table 1 in the Supplemental Data). The chemical shifts for the 2,3-epoxyhexanoyl side chain and amino acid residues 1–10 are in close agreement with those reported previously for CDA2b [9]. However, typical α and β chemical shifts for a second tryptophan residue are clearly absent. Instead, the ^1H and ^{13}C signals were observed, which agree closely with the chemical shifts for ΔTrp residues in other cyclic peptide natural products, including keramide F [14] and microsclerodermins G and I [16]. The stereochemistry of the ΔTrp residue in CDA2b is assigned *Z* configuration on the basis of the chemical shift of the β-proton ($\delta_{H\beta}$ = 8.00), which compares favorably with that observed for (*Z*)-ΔTrp containing keramide F ($\delta_{H\beta}$ = 7.83) [14] and *N*-acetyl-2,3-dehydrotryptophan ethyl ester [$\delta_{H\beta}$ = 7.60 (*Z*) and 6.90 (*E*)] [15]. The relative stereochemistry of the 2,3-epoxyhexanoyl side chain is assigned *trans* on the basis of the vicinal coupling constant between the H2 and H3 protons of the epoxide ($^3J_{HH}$ = 2.0 Hz), which is in close agreement with the coupling constant observed for ethyl 2,3-epoxyhexanoate [$^3J_{HH}$ = 1.9 Hz (*trans*) and 4.7 Hz (*cis*)] [17].

In summary, two previously uncharacterized peptides, CDA2a and CDA4a, which are produced by *S. coelicolor* 2377 in only very minor quantities in liquid culture, are

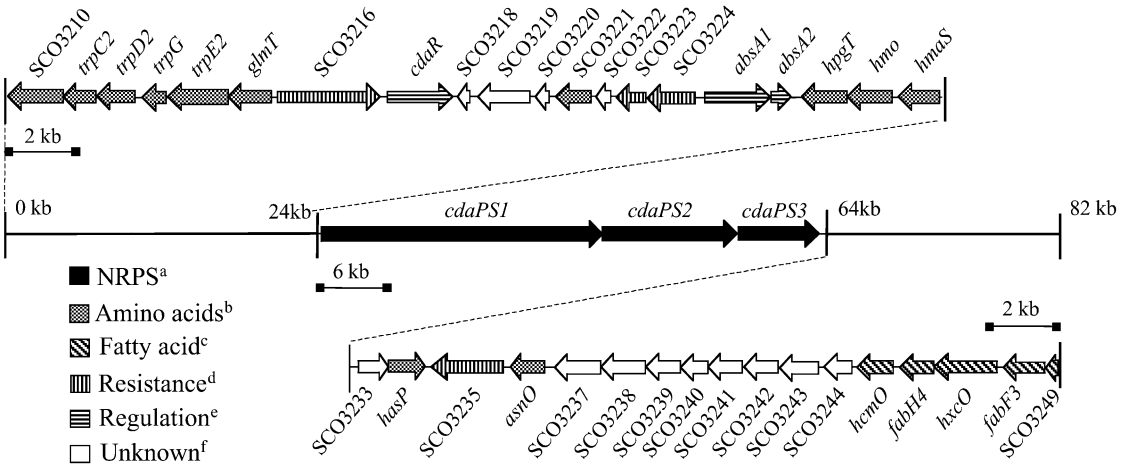


Figure 3. Organization of the CDA Biosynthetic Gene Cluster

[a]NRPS-encoding genes; [b]genes predicted to be involved in the biosynthesis of amino acid precursors or the amino acid tailoring enzymes; [c]genes proposed to be involved in the biosynthesis of the epoxyhexanoyl fatty acid side chain; [d]putative resistance genes; [e]putative regulation genes; [f]those genes which exhibit little or no similarity to genes of known function, or exhibit similarity with genes of known function but have no obvious role in CDA biosynthesis, resistance, or regulation.

in fact the major metabolites produced by this strain when cultivated on solid medium (ONA). The structures of these metabolites, which were determined using UV, NMR, and mass spectrometry, differ from the b-series of CDA [9] by the presence of an unusual *Z*-2,3-dehyrotryptophan residue rather than L-tryptophan at the C terminus. Few nonribosomal peptides have been reported that contain ΔTrp [14, 16]. On the basis of this work, the structure of CDA3a (1480 Da) [9] can also be assigned (Figure 1). Thus, seven CDA variants have been isolated so far. The most structurally modified peptide, CDA2a, probably constitutes the ultimate product of the CDA biosynthetic pathway.

Organization of the CDA Gene Cluster

The chromosomal location of the CDA gene cluster was first identified using degenerate probes against known conserved motifs of nonribosomal peptide synthetases (NRPSs) [8]. Targeted disruption of the putative NRPSs demonstrated their role in CDA biosynthesis. Regions within the CDA cluster were sequenced at UMIST, after which the entire cluster and indeed the whole genome were sequenced at the Sanger Institute (Cambridge, UK) [1]. The 82 kb CDA cluster (Figure 3) is located within the "core" region of the linear chromosome and consists of at least 40 ORFs. The cluster is contained on the overlapping cosmids ScE8-ScE63-ScE29 (SCO3210-3249) [18]. Using the publicly accessible sequence annotation (http://www.sanger.ac.uk/Projects/S_coelicolor/) and the results of more detailed similarity searches (see Experimental Procedures) performed on the deduced protein sequences encoded by each ORF, putative functions of the gene products are suggested (Table 1). From this, and taking into account the structure of CDA, a hypothesis for the mode of CDA biosynthesis is proposed. At the boundaries of the cluster are genes encoding DAHP synthetase (SCO3210) and acyl carrier protein (ACP) (SCO3249) homologs. The possibility that other genes exist beyond these boundaries that might be involved in regulation, resistance, or transport of CDA from the cell has been considered [19] and cannot be excluded at this stage. It is, however, notable that a recent global microarray-based study of gene expression identified the genes SCO3210-3249 as a specific cluster that is upregulated during the transition to CDA production [20]; this would fit the boundaries inferred here on the basis of predicted function.

Biosynthetic Origins of CDA Precursor Building Blocks

Adjacent to SCO3249, encoding the ACP homolog, are genes encoding β-ketoacyl-ACP synthase (KAS)-II and -III homologs (SCO3248 and SCO3246), respectively. KAS-III is likely to catalyze the first condensation reaction of acetyl and malonyl units, resulting in acetoacetyl-ACP, while KAS-II is likely to catalyze the second condensation reaction to give β-ketohexanoyl-ACP. Genes encoding the other FAS enzymes, acetyl and malonyl transacylases, β-ketoacyl reductase, β-hydroxyacyl dehydratase, as well as an enoyl reductase [21] are absent from the cluster. Presumably, these enzymes are recruited from primary metabolism and are responsible for the synthesis of the fatty acid side chain precursor, hexanoyl-ACP **1** (Figure 4A). *hxcO* (SCO3247) encodes a protein similar to many acyl-CoA oxidases, which utilize flavin adenine dinucleotide (FAD) in the desaturation of acyl-CoAs to *trans*-enoyl-CoAs during fatty acid degradation [21]. This suggests that hexanoyl-ACP **1** is first hydrolyzed to hexanoic acid, transformed to hexanoyl-CoA **2**, which is then desaturated by the *hxcO* gene product, hexanoyl-CoA oxidase (HxcO), to give *trans*-hexenoyl-CoA **3**. This would necessitate the involvement of a FAS thioesterase (TE) and acyl-CoA synthetase (ACS) [21], both of which may be recruited from primary metabolism.

In close proximity, *hcmO* encodes a protein with typi-

Table 1. Predicted Function of the Gene Products in the CDA Biosynthetic Cluster

ORF No.	Gene Symbol	Amino Acids	Highest Protein Sequence Similarity[1]	Putative Function in CDA Biosynthesis
SCO3210		484	Dhs1 *Arabidopsis thaliana*	class-II DAHP synthetase[2]
SCO3211	*trpC2*	258	TrpC *Pseudomonas aeruginosa*	indole-3-glycerol phosphate synthase[3]
SCO3212	*trpD2*	335	TrpD *Azospirillum brasilense*	anthranilate phosphoribosyltransferase[3]
SCO3213	*trpG*	200	TrpG *Rhodobacter sphaeroides*	anthranilate synthase component II
SCO3214	*trpE2*	511	TrpE *Pseudomonas putida*	anthranilate synthase component I[3]
SCO3215	*glmT*	338	PAB0269 *Pyrococcus abyssi*	glutamate-3-methyltransferase
SCO3216		796	CtpE *Mycobacterium tuberculosis* H37Rv	cation-transporting ATPase
SCO3217	*cdaR*	638	AfsR *S. coelicolor*	transcriptional activator/regulatory protein[4]
SCO3218		71	MbtH *M. tuberculosis* CDC1551	unknown (NRPS-associated protein)[5]
SCO3219		391	platelet activating factor *Cavia porcellus*	lipase
SCO3220		142	Sc8D11.8c encoded protein *S. coelicolor*	unknown
SCO3221		284	NovF *Streptomyces spheroides*	prephenate dehydrogenase
SCO3222		151	TbSP1 *Tuber borchii*	Ca^{2+} dependent phospholipase
SCO3223		264	ABC transporters *S. coelicolor*	ABC transporter integral membrane protein
SCO3224		317	ABC transporters *S. coelicolor*	ABC transporter ATP-binding protein
SCO3225	*absA1*	571	sensor kinase *S. coelicolor*	sensor kinase absA1 [19]
SCO3226	*absA2*	222	response regulator *S. coelicolor*	response regulator absA2 [19]
SCO3227	*hpgT*	431	HpgT *Streptomyces lavendulae*	4-hydroxyphenylglycine aminotransferase
SCO3228	*hmo*	377	Hmo *Amycolatopsis orientalis*	4-hydroxymandelate oxidase
SCO3229	*hmaS*	371	HmaS *A. orientalis*	4-hydroxymandelate synthase
SCO3230	*cdaPS1*	7463	multiple domains	CDA peptide synthetase I
SCO3231	*cdaPS2*	3670	multiple domains	CDA peptide synthetase II
SCO3232	*cdaPS3*	2417	multiple domains	CDA peptide synthetase III
SCO3233		272	PCZA361.30 *A. orientalis*	hydrolase/external thioesterase
SCO3234	*hasP*	300	SpcN *S. flavopersicus*	phosphotransferase
SCO3235		615	ABC transporter *S. avermitilis*	ABC transporter
SCO3236	*asnO*	333	Cas 1&2 *Streptomyces clavuligerus*	asparagine oxygenase
SCO3237		462	Sc3F9.14 encoded protein	phosphodiesterase[6]
SCO3238		386	Sc3F9.13 encoded protein	unknown[6]
SCO3239		289	Sc3F9.12 encoded protein	unknown[6]
SCO3240		236	Sc3F9.11 encoded protein	unknown[6]
SCO3241		290	Hyi *Sinorhizobium meliloti*	isomerase[6,7]
SCO3242		291	BchG *Chlorobium tepidum*	prenyltransferase[6,8]
SCO3243		388	Ino1 *Archaeoglobus fulgidus*	myo-inositol phosphate synthase[6,9]
SCO3244		265	none	unknown
SCO3245	*hcmO*	420	NahW *Pseudomonas stutzeri*	hexenoyl-CoA Monooxygenase
SCO3246	*fabH4*	330	mmyC *Streptomyces coelicolor*	β-ketoacyl-ACP synthase III
SCO3247	*hxcO*	600	ACX2 *Arabidopsis thaliana*	hexanoyl-CoA oxidase
SCO3248	*fabF3*	406	FabF *Aquifex aeolicus*	β-ketoacyl-ACP synthase II
SCO3249		81	NMB1696 *Neisseria meningitielis*	acyl carrier protein

[1] Proteins with high sequence similarity to deduced proteins, whose function is either unknown or has not been considered within the literature are not included.

[2] DAHP, 2-keto-3-deoxy-D-arabinoheptulosonate-7-phosphate.

[3] TrpC, D, and E paralogs are also encoded elsewhere in the genome [1].

[4] A.E. Hayes, P.P. Chong, Z.H., F.F., and C.P.S., unpublished data.

[5] Small highly conserved protein located within many NRPS-encoding biosynthetic gene clusters.

[6] These ORFs show very high similarity, even at the DNA sequence level, to seven genes in identical order elsewhere on the *S. coelicolor* genome (SCO6579-6573), but no clear role in CDA biosynthesis, resistance, or regulation can be proposed at this stage. Five of the gene products (encoded by ORFs SCO3237-3240 and SCO3242) display high similarity with proteins derived from genes clustered together in *Nostoc* species.

[7] Moderately similar to other isomerases, especially hydroxypyruvate isomerase in *Sinorhizobium meliloti*.

[8] Shows good alignment to the UniA prenyltransferase family and chlorophyll synthases.

[9] Similar to several putative myoinositol phosphate synthases from marine and parasitic organisms.

cal FAD binding motifs and sequence similarity to the flavin-dependent monooxygenases, particularly salicylate hydroxylase and zeaxanthin epoxidase [22]. It is anticipated that *hcmO* encodes a *trans*-hexenoyl-CoA monooxygenase (HcmO) and is responsible for epoxidation of the enone **3**. It is proposed that the distal oxygen of flavin-C4a-hydroperoxide (FADH-OOH) functions as an electrophile in the aromatic hydroxylases, resulting in hydroxylation of phenolic substrates [23]. However, the enone substrate **3** is more likely to undergo conjugate addition by the nucleophilic distal oxygen atom of flavin-C4a-peroxide (FADH-OO^-), resulting in an enol-peroxyflavin intermediate **4**, followed by elimination of flavin-C4a-hydroxide (FADH-OH). A similar mechanism has been proposed for the Baeyer-Villiger type oxidation of cyclic ketones catalyzed by FAD-dependent cyclohexanone monooxygenase [24]. Given that the epoxide in CDA is *trans*, as evidenced by the magnitude of the $^3J_{HH}$ coupling constant across the oxirane ring, the epoxidation reaction is expected to occur with *syn*-stereochemistry, resulting in a *trans*-2,3-epoxyhexanoyl-CoA **5**. The possibility that enzyme-bound hexanoyl-ACP **1** and *trans*-hexenoyl-ACP function as substrates for the putative oxidase (HxcO) and monooxygenase (HcmO),

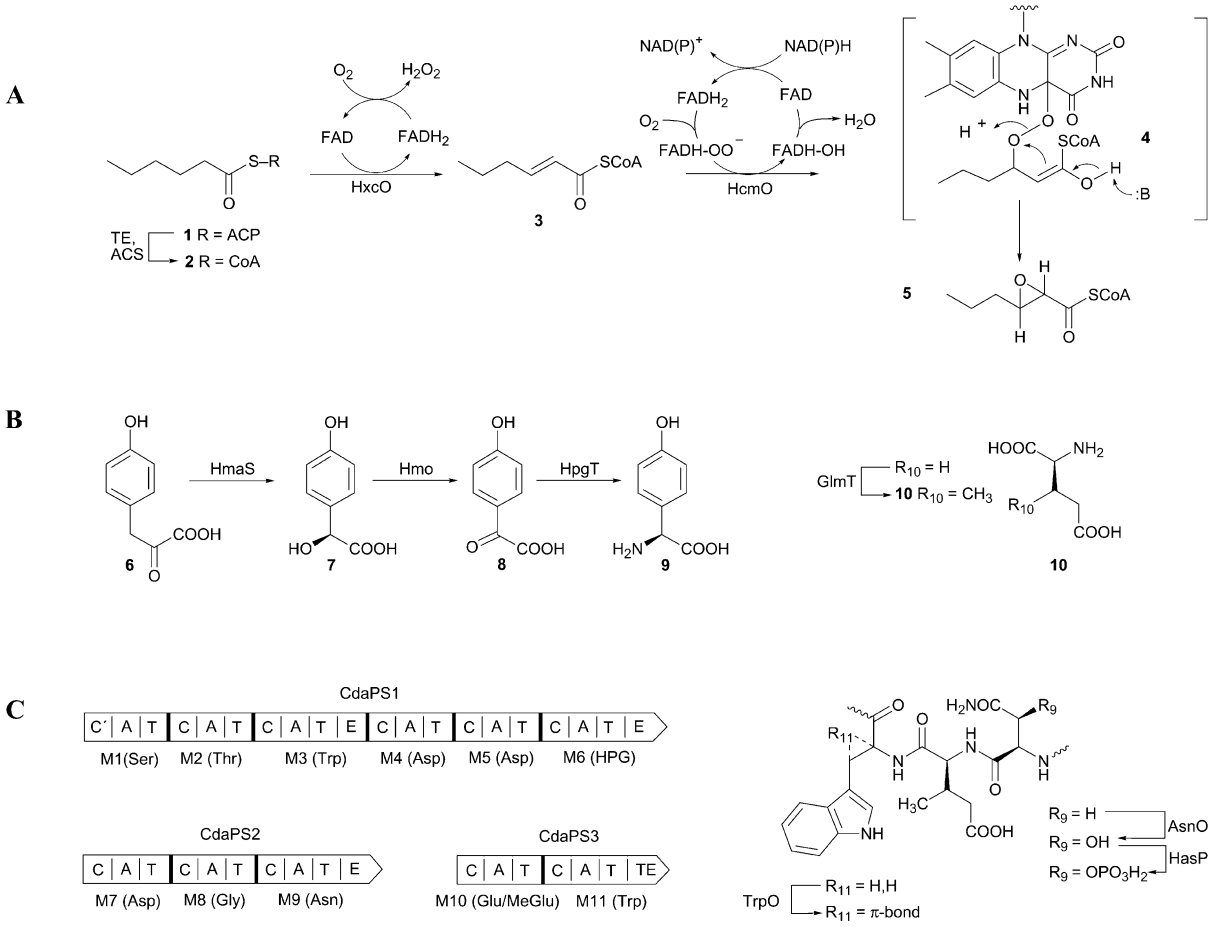


Figure 4. Proposed Biosynthesis of CDA

(A) Biosynthesis of *trans*-2,3-epoxyhexanoyl-CoA. TE, thioesterase; ACS, acyl-CoA synthetase; HxcO, hexanoyl-CoA oxidase; HcmO, *trans*-hexenoyl-CoA monooxygenase; FADH-OO$^-$, flavin-C4a-peroxide; FADH-OH, flavin-C4a-hydroxide.

(B) Biosynthesis of nonproteinogenic amino acids. HmaS, 4-hydroxymandelate synthase; Hmo, 4-hydroxymandelate oxidase; HpgT, 4-hydroxyphenylglycine transaminase; GlmT, glutamate-3-methyltransferase.

(C) Nonribosomal peptide synthetases and tailoring enzymes. C, condensation domain; A, adenylation domain; T, peptidyl carrier protein or thiolation domain; E, epimerization domain; AsnO, asparagine oxygenase; HasP, 3-hydroxyasparagine phosphotransferase; TrpO, tryptophan oxidase.

respectively, resulting in *trans*-2,3-epoxyhexanoyl-ACP, seems less plausible. First, the putative oxidase shows high similarity with many acyl-CoA oxidases and lower similarity to acyl-CoA dehydrogenases, both classes of enzyme turn-over acyl-CoA, not acyl-ACP substrates. Second, an intermediate hexenoyl-ACP is likely to be formed by the FAS β-hydroxyacyl dehydratase, and thus direct epoxidation of this intermediate would bypass the need for an oxidase activity in the first place.

In addition to the first enzyme of the shikimate pathway, DAHP-synthase encoded by SCO3210, there exists a second gene (SCO3221) that encodes a prephenate dehydrogenase, which is another enzyme of the shikimate pathway. Both are likely to be involved in the supply of precursors for the biosynthesis of the aromatic amino acids. In the case of tryptophan, there are four other genes (SCO3211–3214) which encode enzymes TrpC2, D2, G, and E2. Interestingly, there are no tryptophan synthase-encoding genes (*trpA* and *trpB*) within the cluster, so that to convert the local supply of anthranilate to Trp the organism needs to rely on the *trpA,B* genes of primary metabolism [1]. There are also three genes, *hmaS*, *hmo*, and *hpgT*, that display similarity to ORFs from the vancomycin-type antibiotic biosynthetic gene clusters in *Amycolatopsis orientalis* [25] and *Streptomyces lavendulae* [26]. These gene products have been shown to be involved in the biosynthesis of L-4-hydroxyphenylglycine (L-HPG) **9** [27, 28]. HmaS catalyzes the oxidative decarboxylation of 4-hydroxyphenylpyruvate **6** to give L-4-hydroxymandelic acid **7**, which is oxidixed by Hmo to give 4-hydroxyphenylglyoxylate **8**. Finally, transamination by HpgT gives L-HPG **9** (Figure 4B).

Nonribosomal Peptide Synthetases and Tailoring Enzymes

At the center of the CDA gene cluster are three large genes (*cdaPS1–3*) encoding nonribosomal peptide synthetase (NRPS) enzymes CdaPS1, 2, and 3. The peptide synthetases have a typical modular organization [7] with

repeating condensation (C), adenylation (A), and peptidyl carrier protein or thiolation (T) domains (Figure 4C). Additional epimerization domains (E) are also located at the end of modules 3, 6, and 9 that are responsible for the D-configuration of the amino acid residues in the corresponding positions in CDA. The N-terminal sequence of CdaPS1 to the first conserved motif of the module 1 A domain (~495 aa) shows little similarity with NRPS domains of known function. It is postulated here that this sequence constitutes a domain (C′) that is involved in the transfer of the *trans*-2,3-epoxyhexanoyl-CoA moiety to the first amino acid (Ser) attached to the thiolation domain of module 1. By analogy with the biosynthesis of the lipopeptide surfactin [29], it is anticipated that an additional acyl transferase might also be required for the acylation, which is likely to be the first step in the assembly of the peptide.

On the whole, the sequence of the putative amino acid binding pockets of each of the A domains of CdaPS1, 2, and 3 correspond well with the sequences of A domains from other NRPS that recognize the same amino acids [30, 31]. Noticeably, the A domain of module 10 is unlike other glutamic-acid-activating domains, suggesting that L-3-methylglutamic acid (L-3-MeGlu) **10** (Figure 4B) is the cognate substrate. Nevertheless, CDA with both Glu and L-3-MeGlu at position 10 have been isolated. It is possible that the A domain of module 10 has a broader substrate specificity with the ability to activate both amino acids. Furthermore, SCO3215 encodes a protein with a centrally positioned amino acid sequence (~90 aa) that exhibits similarity to regions of putative methyltransferases, including RapQ from the rapamycin biosynthetic gene cluster of *Streptomyces hygroscopicus* [32] and MitN from the mitomycin C biosynthetic gene cluster of *Streptomyces lavendulae* [33]. It is speculated that SCO3215, tentatively named *glmT* here, encodes a glutamate-3-methyltransferase required for C3-methylation of glutamic acid prior to activation by the tenth A domain.

The module 9 A domain is similar to other Asn-activating domains from different origins [30, 31]. Hence, it seems likely that oxidation and phosphorylation of Asn at position 9 in CDA occurs after assembly of the cyclic peptide (Figure 4C). This is consistent with the existence of two genes, *asnO* and *hasP*, which show similarity with known oxygenases and phosphotransferases, respectively. The former exhibits high similarity with clavaminate synthase (Cas) 1 and 2, an Fe(II)/2-oxoglutarate oxygenase that is known to catalyze the β-hydroxylation of the arginine side chain of a monocylic β-lactam precursor of clavaminic acid [34]. It is therefore likely that *asnO* encodes the required asparagine oxygenase (AsnO). The *hasP* gene product exhibits moderate similarity with the spectinomycin phosphotransferase (SpcN) [35], which is involved in the phosphorylation of an aminoglycoside antibiotic as part of the host's self-resistance mechanism; *hasP* is therefore likely to encode the required 3-hydroxyasparagine phosphotransferase and may also be involved in the CDA self-resistance mechanism. The discovery of the a-series CDA, which possess C-terminal *Z*-2,3-dehydrotryptophan residues, calls for a third tailoring enzyme tryptophan oxidase (TrpO). Although enzymes exist that are known to possess this function [15], a candidate TrpO-encoding ORF has not been identified at present within the CDA cluster. The possibility that the tailoring enzymes modify the Trp and Asn residues, as phosphopantetheinyl-thioesters, while attached to their respective adenylation domains cannot be ruled out at this stage [36].

Deletion of *hmaS,* the Hydroxymandelic Acid Synthase-Encoding Gene

There has been considerable effort to develop methods that will allow for the engineered and possibly even the combinatorial biosynthesis of novel nonribosomal peptides [3–6]. Notably, there has been significant progress in delineating rules through which it is possible to reprogram NRPSs by domain or module swaps and by active-site modification of adenylation domains in vitro. Despite this, there have been relatively few examples [37] where these approaches have been implemented in vivo leading to novel nonribosomal peptides.

CDA is a particularly good system for exploring in vivo engineered biosynthesis because the host genome has been fully sequenced, methods for the manipulation of the genome are well established, and the peptide backbone of CDA is not highly modified or crosslinked. Thus, issues of precursor supply can be addressed, and single amino acid changes might be readily introduced without disruption of overall structure or activity of the antibiotic. In a first attempt to engineer the biosynthesis of new CDA lipopeptides in vivo, a mutasynthesis approach [38] was adopted that focuses on modification of the 4-hydroxyphenylglycine residue. The strategy involves generation of a deletion of the putative 4-hydroxymandelate synthase-encoding gene *hmaS* to produce a mutant that would be deficient in L-HPG **9** and the precursors **7** and **8** (Figure 4B) and therefore unable to produce CDA. It was anticipated that the feeding of precursor mandelate, arylglyoxylate, and arylglycine analogs to this mutant should result in the production of modified CDA derivatives.

The *hmaS*, *hmo*, and *hpgT* genes putatively involved in L-HPG biosynthesis are considered likely to comprise a single operon (Figure 5A). Since *hmaS* is the promoter-proximal gene, it was important to design an *hmaS* gene disruption that should not interfere with transcription and translation of the downstream *hmo* and *hpgT* genes. Therefore, the bulk of the *hmaS* coding sequence (codons 10–324) was deleted, and an in-frame stop codon (TAG) was inserted at *hmaS* codon position 10. In this deletion, the terminal 47 codons of the *hmaS* gene will not be translated; this was considered unlikely to cause polarity because there is a 150 nucleotide untranslated sequence between the native stop codon of *hmaS* and the start codon of the downstream *hmo* gene. An appropriate deletion construct was first made in *E. coli* using standard methods (see Experimental Procedures) [39, 40] and used to generate a deletion of *hmaS* in *S. coelicolor* (Δ*hmaS*), as illustrated schematically in Figure 5A. The deletion was generated in both *S. coelicolor* MT1110 [13] (six isolates) and 2377 [12] (two isolates). The expected structure of the deletants was confirmed by PCR using primers flanking the deletion site (data not shown) and Southern analysis (Figure 5B).

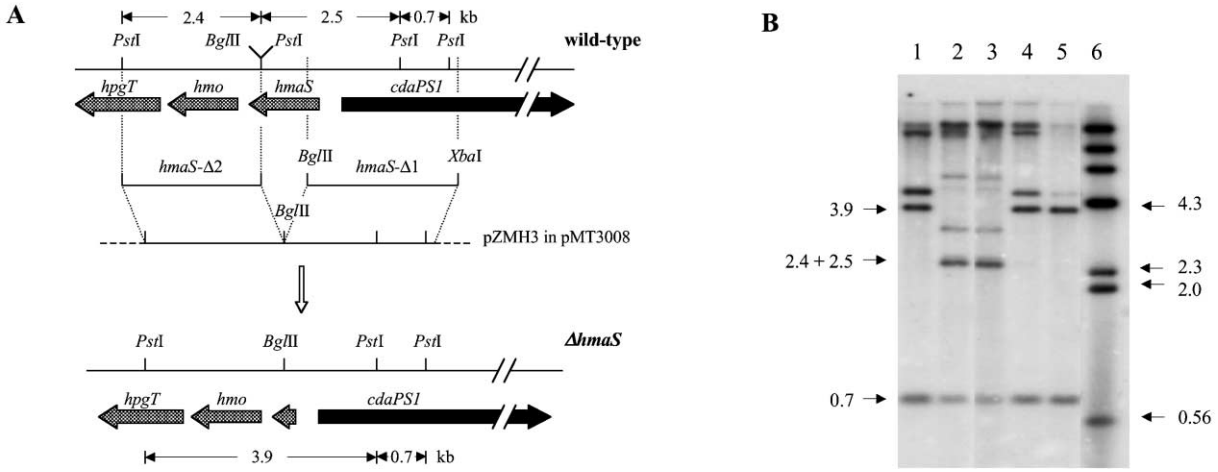


Figure 5. Deletion of the *hmaS* Gene in the *cda* Cluster of *S. coelicolor*

(A) Physical map of the *hmaS*-encoding region of the *cda* cluster. The 2.5 kb flanking sequence upstream of the *hmaS* gene, *hmaS*-Δ1, was generated by PCR from cosmid ScE63 [18], incorporating BglII and XbaI sites at the ends. The 2.4 kb *hmaS*-Δ2 flanking sequence was isolated as a PstI-BglII fragment from cosmid 6E4 [8]. The resulting plasmid, pZMH3, was used to delete the *hmaS* coding sequence by double-crossover gene replacement.

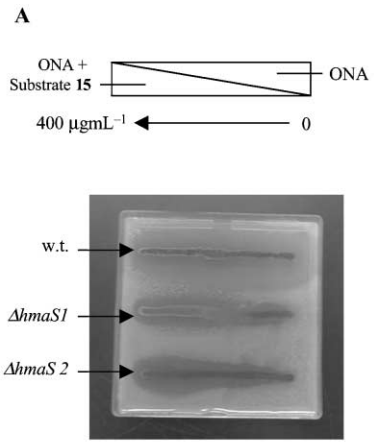
(B) Southern analysis. Chromosomal DNA from wild-type and selected Δ*hmaS* isolates was digested with PstI, and the Southern blot was probed with the 4.9 kb insert from from pZMH3 (random prime labeled with α-^{32}P-dCTP). The 2.4 kb and 2.5 kb PstI fragments of the wild-type are replaced by a 3.9 kb fragment in the Δ*hmaS* isolates. Lane 1, *S. coelicolor* 2377Δ*hmaS*; lane 2, 2377 wild-type; lane 3, *S. coelicolor* MT1110 wild-type; lanes 4 and 5, MT1110Δ*hmaS* isolates; lane 6, end-labeled HindIII-digested λ DNA size markers. The sizes of relevant fragments are indicated (in kb). Other hybridizing fragments on the blot represent partial digestion products.

Mutasynthesis of CDAs Possessing Modified Arylglycine Residues

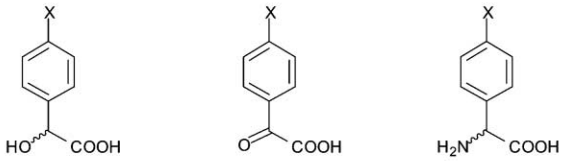
The Δ*hmaS* mutants derived from both 2377 and MT1110 were shown not to exhibit calcium-dependent antibiosis using standard plate bioassays [8]. LC-MS analysis of extracts derived from both mutant strains grown on solid and in liquid media also revealed a clear absence of any CDA metabolites of characteristic molecular weight and HPLC retention times. However, both Δ*hmaS* mutant strains, when grown in the presence of racemic 4-hydroxymandelic acid **7**, produced clear calcium-dependent zones of inhibition, which increased in size across a gradient plate bioassay with 0 to 400 μg/mL^{-1} of **7** (Figure 6A). The 2377Δ*hmaS* mutant was also grown in liquid media supplemented with either racemic 4-hydroxymandelate **7**, 4-hydroxyphenylglyoxylate **8**, or L-HPG **9** (25–200 μgmL^{-1}). In all cases, LC-MS analysis of the extracts clearly indicates the production of CDA2b as the major product (Figure 6B). These results indicate that the deletion of *hmaS* did not interfere with expression of the adjacent downstream *hmo* and *hpgT* genes. Large-scale fermentation of this mutant when supplemented with L-HPG allowed sufficient quantities of CDA2b to be isolated for characterization by NMR. The NMR data obtained (see Supplemental Data) were generally in close agreement with that previously reported [9], and HRESIMS (*m/z* 1577.5043 $[M+H]^+$, $C_{67}H_{82}N_{14}O_{29}P$ requires 1577.5110) further confirms the structure as CDA2b. As expected, the D-configured HPG enantiomer did not re-establish CDA production in the Δ*hmaS* mutants, which is consistent with activation of L-HPG and epimerization by the module 6 E domain.

A series of synthetic mandelate, arylglyoxylate, and arylglycine analogs (**11–22**) (Figure 6B) were also fed to the mutants and screened for the production of novel CDA derivatives using, in the first instance, gradient plate bioassays. For example, 4-fluorophenylglyoxylate **15** results in calcium-dependent antibiosis, with the effect increasing with substrate concentration (Figure 6A). Conversely, the 4-chlorophenylglyoxylate fails to restore antibiosis. Each substrate analog was then fed to liquid cultures of the 2377Δ*hmaS* mutant, and the production of novel CDAs was determined using LC-MS. In the case of the dehydroxy precursors (**11–13**), the major product, with typical CDA retention times on RP-HPLC, exhibits a molecular weight of 1560 Da, which is 16 Da down on CDA2b. This is consistent with the existence of a novel peptide CDA2d (Figure 1) possessing a phenylglycine residue, presumably D-configured, at position 6 rather than D-HPG. Similarly, the fluorinated precursors **15** and **16**, as was predicted from bioassays, gave rise to a new peptide of molecular weight 1578 Da (2 Da up) which is consistent with the structure CDA2fb possessing a 4-fluorophenylglycine residue (Figure 1). In contrast, none of the *para*-chloro **17–19** or *para*-methoxy **20–22** analogs gave rise to detectable CDA by LC-MS analysis. Presumably, increase in the size of the *para*-substituent results in failure of the A domain to recognize and activate the modified arylglycines.

It was also noted in these experiments that the arylglycines (**13** and **16**) resulted in the highest levels of mutasynthesized derivatives (CDA2fb and 2d). Therefore, separate large-scale fermentations of 2377Δ*hmaS*, supplemented with arylglycines **13** and **16**, were carried out, and milligram quantities (~2–5 mgL^{-1}) of the new CDAs were isolated and purified by HPLC. The structure of the peptides was confirmed using mass spectrometry and NMR. First, HRESIMS of CDA2d (*m/z* 1561.5148



B



X = OH	**7**→	CDA2b 1576	**8**→	CDA2b 1576[a]	**9** (L)→	CDA2b 1576[a]
					9 (D)→	no CDA
H	**11**→	CDA2d 1560	**12**→	CDA2d 1560[b]	**13**→	CDA2d 1560
F	**14**→	no CDA	**15**→	CDA2fb 1578[c]	**16**→	CDA2fb 1578[c]
Cl	**17**→	no CDA	**18**→	no CDA	**19**→	no CDA
OCH_3	**20**→	no CDA	**21**→	no CDA	**22**→	no CDA

Figure 6. Mutasynthesis of CDA with Modified Arylglycine Residues

(A) Typical gradient plate bioassay. MT1110 wild-type (w.t.) and MT1110Δ*hmaS* mutants (two separate isolates) were grown on a concentration gradient of between 0 to 400 μgmL^{-1} of fluorophenylglyoxylate **15** in ONA supplemented with $Ca(NO_3)_2$. The plate was overlaid with an indicator strain, typically *Bacillus mycoides*. The wild-type shows a uniform zone of inhibition, independent of the substrate concentration, while both mutants show a zone of inhibition that is dependent on the concentration of substrate **15**. In the absence of Ca^{2+}, no zones of inhibition are observed (data not shown).

(B) The table shows the molecular weight of the most abundant CDA variant produced by the 2377Δ*hmaS* mutant when supplemented with natural precursors **7–9** and substrate analogs **11–22** in liquid media, as determined by LC-MS. Small amounts of other CDA derivatives were also observed: [a]CDA1b (m.w. = 1562) and CDA2a (m.w. = 1574); [b]CDA (m.w. = 1558) consistent with the structure CDA2d but containing a single ΔTrp residue; [c]CDA2fa (m.w. = 1576) with a single ΔTrp residue (Figure 1).

$[M+H]^+$, $C_{67}H_{82}N_{14}O_{28}P$ requires 1561.5161) and CDA2fb (*m/z* 1579.5017 $[M+H]^+$, $C_{67}H_{81}N_{14}O_{28}FP$ requires 1579.5066) were consistent with the molecular formula for the structures proposed (Figure 1). In the case of CDA2d, assignment of ^1H and ^{13}C chemical shifts for all residues was possible with the aid of ^1H-^{13}C HMQC and ^1H-^1H COSY experiments (see Table 1 in the Supplemental Data). This reveals characteristic signals for the phenylglycine residue [δ_H/δ_C = 7.28/127.2 (4), 7.33/127.8 (3), 7.41/126.7 (2)] that are clearly distinct from those of HPG due to the absence of strong inductive and resonance effects of the *para*-hydroxyl substituent. Given that only 2 mg of pure CDA2fb was isolated, a complete assignment of all ^1H and ^{13}C shifts was not possible. However, stronger signals in the aromatic region of the NMR spectrum did allow assignments of the key fluorophenylglycine residue, and the presence of fluorine could clearly be detected by the characteristic magnitude of the ^1H-^{19}F coupling constants [δ_H/δ_C = 7.13 ($^3J_{HF}$ 8.5 Hz)/114.5 (3), 7.48 ($^4J_{HF}$ 4.5 Hz)/128.9 (2)]. Further confirmation of the presence of a fluorophenylglycine residue in CDA2fb was obtained through $^{19}F\{^1H\}$-NMR (see Supplemental Data), which shows a singlet ($\delta_F = -114.7$) that is typical for fluorophenylglycine [41]. During the isolation of CDA2fb, a minor amount (~0.1 mg) of another mutasynthesized peptide was isolated which exhibited *m/z* 1577.4878 ($[M+H]^+$, $C_{67}H_{79}N_{14}O_{28}FP$ requires 1577.4910). This is presumably ΔTrp-containing variant CDA2fa (Figure 1).

In conclusion, deletion of the *hmaS* gene abolished CDA production in *S. coelicolor*. Feeding the mutant 4-hydroxymandelate **7**, 4-hydroxyphenylglyoxylate **8**, or L-HPG **9** re-establishes the production of CDA. This clearly demonstrates that, as expected, the pathway responsible for the biosynthesis L-HPG in *A. orientalis* [27, 28] also operates in *S. coelicolor*. Moreover, feeding 4-fluoro and 4-dehydroxy analogs of these precursors to the Δ*hmaS* mutant results in new CDA peptides with modified arylglycines. In addition to generating new antibiotics, the work presented here clearly indicates that the enzyme Hmo, responsible for oxidation of 4-hydroxymandelate **7**, the transaminase HpgT, and the HPG-activating A domain of module 6, do not require the *para*-hydroxyl group for substrate recognition and will accommodate other *para*-substituents provided that they are no larger than the hydroxyl group. It can therefore be assumed that hydrogen bonding between the enzyme active sites and the phenolic-OH is not essential for the turn-over of these enzymes.

Significance

From the numerous microbial genome sequencing projects, it has become apparent that there is a wealth of unidentified secondary metabolites and biosynthetic machinery that might be utilized in an attempt to generate new therapeutic agents, particularly antibiotics. This paper describes the isolation and characterization of two new acidic lipopeptides of the calcium-dependent antibiotic (CDA) group from wild-type *S. coelicolor*. The structures differ from other CDAs by the presence of a C-terminal *Z*-2,3-dehydrotrypothan residue, a rare nonproteinogenic amino acid that has been found in only a few other nonribosomal peptide natural products to date.

Sequence analysis of the CDA gene cluster enabled a hypothesis that accounts for the biosynthesis of CDA to be proposed. On the basis of this hypothesis, experiments can be designed to characterize the individual enzymes proposed to be involved in the biosynthesis

of CDA. A number of these enzymes are likely to demonstrate novel activities that might be utilized in the modification of other valuable peptide natural products. The sequence analysis was also utilized to design experiments that have led to the first rational engineered biosynthesis of acidic lipopeptides antibiotics of this class. This is significant given that the acidic lipopeptide antibiotics, particularly the structurally related daptomycin, show significant potential as new antimicrobial agents.

The mutasynthesis strategy that was adopted to generate the new peptides resulted in the modification of the hydroxyphenylglycine (HPG) residue in CDA. HPG is a nonproteinogenic amino acid that is found in a number of nonribosomal peptide natural products, including vancomycin. Thus, this strategy may also be utilized to modify other important antibiotics. Alternatively, the methodology developed could be utilized to probe the later steps in the biosynthesis of HPG-containing peptides, such as the timing of phenolic crosslinking and glycosylation events in the biosynthesis of the vancomycin group of antibiotics.

Experimental Procedures

Cultivation and Extraction of CDA2a and CDA4a

The *S. coelicolor cda*$^+$ strain 2377 [12] is Act$^-$ and is commonly used for studies on CDA production [8]. An agar plug of the *S. coelicolor* 2377 was used to inoculate 50 ml of SV2 seed medium in a 250 ml conical flask containing two 6 mm diameter glass beads. This was incubated for 3 days at 28°C and 250 rpm. This culture was used to inoculate 100 petri dishes, each containing 30 ml of oxoid nutrient agar (ONA) (0.1 ml of culture was spread over the surface of each plate). After 7 days incubation at 28°C, the plates were frozen overnight at −20°C and then allowed to thaw at room temperature. The freeze-thaw exudate from these plates (~1.5 l) was collected by filtration, acidified to pH 2 with 0.5 M aq. HCl, and adsorbed onto Varian C-18 Megabondelute SPE cartridges (10 g). The cartridges were washed with H_2O (50 ml) and eluted with MeOH (50 ml). The MeOH eluant was concentrated to dryness in vacuo. SV2 contained, per liter: glucose, 15 g; glycerol, 15 g; soy peptone, 15 g; NaCl, 3 g; $CaCO_3$, 1 g. The medium was adjusted to pH 7.0 before sterilization.

Bioassay Guided Fractionation

A 5 mgmL^{-1} sample of this extract was prepared in DMSO. An aliquot (100 μl) was fractionated by analytical HPLC using the following conditions: Hypersil Elite C-18, 3 μ, 150 × 4.6 mm column; solvent A was H_2O with 0.1% HCO_2H; solvent B was 90% acetonitrile in H_2O with 0.1% HCO_2H. The flow rate was 1 mLmin^{-1} with a gradient elution of 0% B increasing to 100% B over 30 min, then held at 100% B for 10 min. Fractions were collected every minute, concentrated to dryness in vacuo, redissolved in DMSO (50 μl), and subjected to bioassay using *B. mycoides* as the indicator in the presence and absence of Ca^{2+} [8].

Purification of CDA2a and CDA4a

The crude extract (~200 mg) from the exudate prepared as described above was dissolved in DMSO (2 ml) and H_2O (20 ml). This suspension was adsorbed onto a Varian Megabondelute C-18 SPE cartridge (5 g). The cartridge was washed with H_2O (5 ml) and eluted with 30% acetonitrile in H_2O (20 ml). The eluant was concentrated to dryness in vacuo and dissolved in DMSO (2 ml). Purification was achieved using preparative RP-HPLC on a Gilson Unipoint system (2 × 1 ml injections) using the following conditions: Hypersil Elite C18, 5 μ, 150 × 20 mm column; solvent A was 25 mM aq. NH_4OAc with 0.3% TFA; solvent B was 25 mM NH_4OAc in 90% acetonitrile/H_2O with 0.3% TFA. The flow rate was 20 mLmin^{-1} with an isocratic elution of 35% B in A; detection by UV at λ = 280 nm. The two major peaks (retention times: 12.5 and 21 min) were collected and concentrated in vacuo. To remove inorganic salts, the fractions were dissolved in H_2O and passed through Varian C-18 Megabondelute SPE cartridges (200 mg). The cartridges were then washed with water (3 ml) and eluted with MeOH (3 ml). The MeOH eluant was then evaporated in vacuo to give CDA2a (3.8 mg) and CDA4a (10.5 mg).

Derivatization of CDA4a

CDA4a was derivatized according to the method of Kempter et al. [9] using 1-propylamine instead of 2-propylamine. Accordingly, CDA4a (2.0 mg) was dissolved in DMF (200 μl) and acetonitrile (5 ml). To this solution was added 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) (25.0 mg, 77.9 μmol), *N*-hydroxybenzotriazole (HOBt) (11.0 mg, 81.4 μmol), and 1-propylamine (10 μl, 122 μmol). The reaction was stirred at room temperature and monitored by LC-MS. After 6 hr, the reaction mixture was evaporated under reduced pressure and purified by semipreparative HPLC. The resulting CDA4a tetra-*n*-propylamide (~0.3 mg) was dissolved in 1 M aq. NaOH (1 ml) and stirred at room temperature for 2 hr. H_2O (10 ml) was then added to the reaction mixture, which was cooled to 0°C, adjusted to pH 2 with 0.5M aq. HCl, and then passed through a Varian C-18 Megabondelute SPE cartridge (100 mg). The cartridge was washed with H_2O (1 ml) and eluted with MeOH (1 ml). The eluant was evaporated under reduced pressure to give CDA4a tetra-*n*-propylamide linear peptide (Figure 2), which was sequenced by ESI MS-MS.

Characterization of CDAs

1D ^1H, ^{31}P, and 2D NMR spectra were recorded on a Bruker AMX500 or AV500 spectrometer fitted with a Nalorac 3 mm inverse broad band probe with z gradients. Standard Bruker pulse sequences were used. Elevated temperatures were used in order to obtain spectra sharp enough for interpretation. The signals were referenced to residual CD_3SOCD_2H at 2.52 δ and 39.5 δ for ^1H and ^{13}C, respectively. 1D ^{19}F NMR spectra were recorded on a Bruker AV400 spectrometer fitted with a 5 mm QNP probe with z gradients. The signals were referenced to $CFCl_3$ at 0.0 δ.

Electrospray ionization mass spectrometry was performed on a Micromass Q-Tof 2. The electrospray capillary voltage was 3.4 kV and cone voltage was 36V. The source temperature was maintained at 100°C. Mass measurements were performed at a resolution of 10,000 RP, and ESI MS-MS studies were performed at a resolution of 5000 RP.

Sequence Similarity Searches

Standard protein-protein BLAST searches on the NCBI database were inspected to identify proteins similar to those found within the CDA cluster. The default settings, excluding the low-complexity parameter, were used throughout. The putative function of genes within the cluster was deduced according to the similarity score with other known or consensus protein motifs, as well as the plausible influence of any conserved domains in determining a probable role in CDA biosynthesis and regulation. This analysis builds significantly on the original annotation of the *cda* cluster (contained within EMBL accession number AL645882) and has led to the assignment of specific biochemical functions for a large number of the predicted proteins (Table 1).

Construction of pZMH3 and Deletion of *hmaS*

S. coelicolor MT1110 is a prototrophic SCP1$^-$, SCP2$^-$ derivative of wild-type strain 1147 [13]. Unless stated otherwise, all methods and media used for growth and transformation of *Streptomyces* were as described previously [42]. Streptomycete strains were grown in YEME liquid medium for preparation of protoplasts and for isolation of chromosomal DNA. *E. coli* strains used in this study are as detailed by Bucca et al. [39], and the standard methods used for DNA manipulation are as described by Sambrook et al. [40]. A standard "double-crossover" replacement strategy was used to delete *hmaS* from the chromosome [13] using the delivery plasmid pZMH3. In brief, pZMH3 was generated by cloning two fragments that flank the chromosomal sequence destined for deletion into the *E. coli* plasmid pMT3008 (a derivative of pMac containing the *hyg* resistance gene for selection in *Streptomyces*). An "upstream" 2.5 kb flanking sequence desig-

nated *hmaS*-Δ1 was generated by PCR using cosmid ScE63 [18] as the template (EMBL accession number AL035640); primers hmasA1 (5′-GAAGATCTAATGAAGGAAGGGGAAAGG; including the nucleotide coordinates 2506–2524 and incorporating a BglII site) and hmasA2 (5′-GCTCTAGAACTCCCACACGGACAC; including the nucleotides 4818–4836 [complement], and incorporating a XbaI site) were used to amplify *hmaS*-Δ1 using standard conditions. The "downstream" 2.4 kb flanking sequence, designated *hmaS*-Δ2, was isolated as a PstI-BglII restriction fragment from cosmid 6E4 [8]; the BglII site corresponds to nucleotide coordinates 1555–1560 of cosmid ScE63. The location of these fragments relative to the *cda* cluster is shown in Figure 5A. Nonmethylated plasmid pZMH3 was NaOH denatured [39] and used to transform *S. coelicolor* 2377 and MT1110 protoplasts. Hygromycin-resistant transformants were isolated and passaged through two rounds of sporulation on nonselective media. Hygromycin-sensitive double-crossover recombinants were isolated, and the desired *hmaS* deletion derivatives were identified by PCR and Southern analysis of genomic DNA (Figure 5B).

Synthesis of Precursors and Analogs for Mutasynthesis

4-hydroxyphenylglyoxylic acid **8** was prepared by the oxidation of 4-hydroxyacetophenone [43]. *para*-substituted arylglyoxylates **15**, **18**, and **21** were prepared from the corresponding ethyl esters, which were synthesized using Friedel-Crafts acylation reactions [44]. Racemic 4-chlorophenylglycine and 4-methoxyphenylglycine were donated by GlaxoSmithKline. Other substrates were purchased from Sigma or Fluka.

***4-Hydroxyphenylglyoxylic Acid* 8**

mp 140°C (slow decomposition); ν_{max} (Nujol)/cm^{-1} 2926 (Ar-CH), 1658 (ketone C=O), 1608 (CO_2), 1370 (CO_2); λ_{max} (H_2O)/nm 250 (ϵ/dm^3 mol^{-1} cm^{-1} 12685); δ_H (400 MHz, DMSO-d_6) 6.95 (2H, d, *J* 9.0 Hz, H3/5), 7.82 (2H, d, *J* 9.0 Hz, H2/6); δ_C (75.5 MHz, DMSO-d_6) 116.4 (C3/5), 123.6 (C1), 132.6 (C2/6), 164.2 (C4), 167.2 (CO_2), 187.4 (Ar-*C*O); *m/z* (ESI$^-$) 165 ([M–H]$^-$, 100%); HRMS *m/z* (ESI$^-$) 165.0188 ([M-H]$^-$, $C_8H_5O_4^-$ requires *m/z* 165.0188).

***Sodium 4-Chlorophenylglyoxylate* 18**

Aluminium chloride (1.39 g, 10.4 mmol) was added to a mixture of chlorobenzene (0.51 ml, 5.0 mmol) and ethyl oxalyl chloride (0.82 ml, 7.3 mmol) in dichloromethane (10 ml) and stirred for 8 hr at between −10°C–0°C. The reaction mixture was warmed to room temperature, crushed ice (29 g) in concentrated aq. HCl (19 ml) was added, and the separated organic layer was washed with 0.1 M aq. NaOH (3 × 15 ml), dried over $MgSO_4$, and evaporated under reduced pressure. Purification by flash column chromatography on silica gel eluting with hexane/ethyl acetate (6:1→5:1) gave ethyl 4-chlorophenylglyoxylate (0.46 g) as a pale yellow oil. This was then dissolved in THF (1 ml), and 2.2 M aq. NaOH (1 ml) was added in two portions. The reaction mixture was stirred for 30 min and evaporated under reduced pressure to give the title compound (0.41 g, 40%) as a white crystalline solid, mp 230°C (slow decomposition); ν_{max} (Nujol)/cm^{-1} 2924 (Ar-CH), 1649 (ketone C=O), 1598 (CO_2), 1382 (CO_2), 787 (*p*-Ar); λ_{max} (MeOH)/nm 257 (ϵ/dm^3 mol^{-1} cm^{-1} 19074); δ_H (300 MHz, D_2O) 7.45 (2H, m, H3/5), 7.78 (2H, m, H2/6); δ_C (75.5 MHz, D_2O) 129.7 (C3/5), 130.9 (C1), 131.5 (C2/6), 141.2 (C4), 172.8 (CO_2), 196.1 (Ar-*C*O); *m/z* (ESI$^-$) 183 ([M–Na]$^-$, 100%), 110 ([M–$COCO_2$Na]$^-$, 10); HRMS *m/z* (ESI$^-$) 182.9848 ([M-Na]$^-$, $C_8H_4ClO_3^-$ requires *m/z* 182.9849).

***Sodium 4-Fluorophenylglyoxylate* 12**

Similarly prepared from fluorobenzene: mp 260°C (slow decomposition); ν_{max} (Nujol)/cm^{-1} 2918 (Ar-CH), 1662 (ketone C=O), 1612 (CO_2), 1388 (CO_2), 823 (*p*-Ar); λ_{max} (MeOH)/nm 226 (ϵ/dm^3 mol^{-1} cm^{-1} 9104), 265 (817); δ_H (300 MHz, D_2O) 7.15 (2H, m, H3/5), 7.84 (2H, m, H2/6); δ_C (75.5 MHz, D_2O) 116.7 (d, $^2J_{CF}$ 22.5 Hz, C3/5), 129.0 (C1), 133.0 (d, $^3J_{CF}$ 9.5 Hz, C2/6), 166.9 (d, $^1J_{CF}$ 255.1 Hz, C4), 173.1 (CO_2), 195.8 (Ar-CO); *m/z* (ESI$^-$) 167 ([M–Na]$^-$, 100%), 95 ([M–$COCO_2$Na]$^-$, 20); HRMS *m/z* (ESI$^-$) 167.0146 ([M–Na]$^-$, $C_8H_4FO_3^-$ requires *m/z* 167.0145).

***Sodium 4-Methoxyphenylglyoxylate* 21**

Similarly prepared from anisole: mp >300°C (slow decomposition); ν_{max} (Nujol)/cm^{-1} 2923 (Ar-CH), 1641 (ketone C=O), 1600 (CO_2), 1389 (CO_2), 810 (*p*-Ar); λ_{max} (H_2O)/nm 221 (ϵ/dm^3 mol^{-1} cm^{-1} 15401), 282 (28795); δ_H (300 MHz, D_2O) 3.71 (3H, s, OCH_3), 6.89 (2H, d, *J* 8.7Hz, H3/5), 7.76 (2H, *J* 8.7Hz, H2/6); δ_C (75.5 MHz, D_2O) 56.0 (OCH_3), 114.9 (C3/5), 125.3 (C1), 132.7 (C2/6), 164.9 (C4), 173.7 (CO_2), 196.1 (Ar-*C*O); *m/z* (ESI$^-$) 179 ([M–Na]$^-$, 100%); HRMS *m/z* (ESI$^-$) 179.0347 ([M–Na]$^-$, $C_9H_7O_4^-$ requires *m/z* 179.0344).

Mutasynthesis of CDA

The restoration of calcium-dependent antibiosis in the Δ*hmaS* mutants by the exogenous supply of 4-hydroxyphenylglycine **9**, precursors **7** and **8**, and analogs **11–22** was screened using gradient plate bioassay as follows: ONA (160 ml) supplemented with substrate (~400 μgmL^{-1}) was poured into square petri dishes (23 × 23 cm) held on an incline. After setting, the plates were placed horizontally, and an equal volume of nonsupplemented ONA was poured onto the plates. Plates were inoculated with single 50 μl streaks of *S. coelicolor* strains 2377 and 2377Δ*hmaS* or MT1110 and MT1110Δ*hmaS* from 3-day SV2 seed cultures prepared as described above. The plates were incubated for 3 days, then overlaid with soft ONA (100 ml) containing *Bacillus mycoides* and Ca_2NO_3 to give a final concentration of 12 mM Ca^{2+} as described previously [8]. Duplicate plates were similarly prepared but with no Ca^{2+}.

Small-scale liquid feeding experiments were carried out in 7 ml of SM22 media innoculated with 300 μl of 2377Δ*hmaS* from a 3-day SV2 seed culture. SM22 contained, per liter: monosodium glutamate, 18.7 g; NaCl, 0.5 g; K_2HPO_4, 0.5 g; 3-(*N*-morpholino)propane-sulphonic acid (MOPS), 21.0 g; lactose, 30.0 g; *0.2 M $MgSO_4$, 10 ml; *0.02 M $CaCl_2$, 10 ml; *1 M H_2SO_4, 5 μl; *$ZnSO_4.7H_2O$, 4.3 mg; *$MnSO_4.7H_2O$, 1.115 mg; *H_3BO_3, 0.31 mg; *$CuSO_4.5H_2O$, 0.625 mg; *$NaMoO_4.2H_2O$, 0.24 mg; *$CoCl_2.6H_2O$, 0.24 mg; *$FeSO_4.7H_2O$, 9 mg; *KI, 0.415 mg. The *S. coelicolor* strain 2377 is a histidine and uracil auxotroph; therefore, the media was also supplemented with histidine (50 μgmL^{-1}) and uracil (7.5 μgmL^{-1}). Ingredients indicated with an asterisk were added in order, and the medium was adjusted to pH 7.0 prior to sterilization. The cultures were then incubated at 28°C, 250 rpm, for 2 days. Filter-sterilized aqueous solutions of precursors **7–22** (5 mgmL^{-1}) were then added to give final concentrations of 25 μgmL^{-1}, 50 μgmL^{-1}, 100 μgmL^{-1}, and 200 μgmL^{-1}. After a total of 6 days incubation, the cultures were harvested by centrifugation, and the supernatants were adjusted to pH 2.0, extracted on Varian Megabondelute C-18 SPE cartridges (0.5 g), eluted with MeOH (2 ml), and evaporated in vacuo. Extracts were then analyzed by LC-MS using an LCQ DuO mass spectrometer in electrospray ionisation mode with positive/negative ion switching and the following LC conditions: Hypersil Elite C18, 3 μ, 150 × 4.6 mm column; solvent A was 10 mM aq. NH_4Oac with 0.1% HCO_2H; solvent B was 10 mM NH_4OAc in 90% acetonitrile/H_2O with 0.1% HCO_2H. The flow rate was 1 mLmin^{-1} with a gradient of 2% B and 98% A, increasing to 100% B over 20 min, then held for 5 min.

Large-scale incubations were carried out in an analogous fashion with 2377 Δ*hmaS* in SM22 medium. One-liter cultures (20 × 50 ml in 250 ml conical flasks) were fed the following precursors: L-4-hydroxyphenylglycine **9**, (+/−) phenylglycine **13**, and (+/−) 4-fluorophenylglycine **16** to a final concentration of 100 μgmL^{-1}. CDAs were extracted and purified using preparative RP-HPLC on a Gilson Unipoint system (4 × 1 ml injections) using the conditions described above. This gave CDA2d (5 mg), CDA2fb (2 mg), and CDA2fa (~0.1 mg)

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