An asparagine oxygenase (AsnO) and a 3-hydroxyasparaginyl phosphotransferase (HasP) are involved in the biosynthesis of calcium-dependent lipopeptide antibiotics

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Nonribosomal peptides contain a wide range of unusual non-proteinogenic amino acid residues. As a result, these complex natural products are amongst the most structurally diverse secondary metabolites in nature, and possess a broad spectrum of biological activities. β -Hydroxylation of amino acid precursors or peptidyl residues and their subsequent processing by downstream tailoring enzymes are some of the most common themes in the biosynthetic diversification of these therapeutically important peptides. Identification and characterization of the biosynthetic intermediates and enzymes involved in these processes are thus pivotal in understanding nonribosomal peptide assembly and modification. To this end, the putative asparaginyl oxygenase- and 3-hydroxyasparaginyl phosphotransferase-encoding genes *hasP* and *asnO* were separately deleted from the calcium-dependent antibiotic (CDA) biosynthetic gene cluster of *Streptomyces coelicolor*. Whilst the parent strains produce a number of 3-hydroxyasparagine- and 3-phosphohydroxyasparagine-containing CDAs, the Δ *hasP* mutants produce exclusively non-phosphorylated CDAs. On the other hand, Δ *asnO* mutants produce several new Asn-containing CDAs not present in the wild-type, which retain calcium-dependent antimicrobial activity. This confirms that AsnO and HasP are required for the β -hydroxylation and phosphorylation of the Asn residue within CDA.

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INTRODUCTION

The calcium-dependent antibiotics (CDAs) from *Streptomyces coelicolor* (Hojati *et al.*, 2002; Kempter *et al.*, 1997) belong to the group of structurally related acidic

lipopeptide antibiotics, which include A54145 (Fukuda *et al.*, 1990; Miao *et al.*, 2006), daptomycin (Baltz *et al.*, 2005; Miao *et al.*, 2005; Raja *et al.*, 2003), friulimicins and amphomycins (Vértesy *et al.*, 2000). All of these nonribosomally biosynthesized lipopeptides contain *N*-terminal fatty acid side chains, which is a *trans*-2,3-epoxyhexanoyl moiety in the case of CDA (Fig. 1), along with decapeptide lactone or lactam cores. Within the decapeptide cores are a number of conserved amino acids, including acidic residues responsible for co-ordination of calcium ions, which are essential for antimicrobial activity. Notably, daptomycin was recently approved for clinical use, becoming the first new structural class of natural antibiotic to reach the clinic in over 30 years (Baltz *et al.*, 2005; Raja *et al.*, 2003). As a result of this, there has been considerable interest in establishing the biosynthetic origins of the acidic lipopeptide group of antibiotics, with the specific aim of engineering new lipopeptide variants with improved therapeutic properties. To this end we have focused on engineering the biosynthesis of CDAs, using adenylation domain active site modifications to replace Asp

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Abbreviations: CAS, clavaminic acid synthase; CDA, calcium-dependent antibiotic; D-HPG, D-hydroxyphenylglycine; EGF, epidermal growth factor; ESI, electrospray ionization; LC, liquid chromatography; 3-MeGlu, L-3-methylglutamic acid; *mw*, molecular weight; 3-OHAsn, (2*R*,3*S*)-3-hydroxyasparagine; 3-OPAsn, (2*R*,3*S*)-3-phosphohydroxyasparagine; PCP, peptidyl carrier protein.

Tables detailing the bacterial strains, plasmids, primers and PCR products used in this work, and also a figure showing the PCR analysis of Δ *asnO* and Δ *hasP* mutants, are available as supplementary data with the online version of this paper.

CDAx	R ₉	R ₁₀	R ₁₁	mw	Reference
CDA1b	OPO ₃ H ₂	H	H, H	1562.5	Kempter <i>et al.</i> (1997)
CDA2b	OPO ₃ H ₂	CH ₃	H, H	1576.5	Kempter <i>et al.</i> (1997)
CDA3b	OH	H	H, H	1482.5	Kempter <i>et al.</i> (1997)
CDA4b	OH	CH ₃	H, H	1496.5	Kempter <i>et al.</i> (1997)
CDA2a	OPO ₃ H ₂	CH ₃	π -bond	1574.5	Hojati <i>et al.</i> (2002)
CDA3a	OH	H	π -bond	1480.5	Hojati <i>et al.</i> (2002)
CDA4a	OH	CH ₃	π -bond	1494.5	Hojati <i>et al.</i> (2002)
CDA5a	H	H	π -bond	1464.5	This work
CDA6a	H	CH ₃	π -bond	1478.5	This work

Fig. 1. Structures of CDA variants. The b series CDAs contain L-Trp at position 11, whilst the a series possess Z-dehydrotryptophan residues at position 11 (Hojati *et al.*, 2002; Kempter *et al.*, 1997). CDA5a and 6a are novel biosynthetically engineered CDAs possessing Asn at position 9.

at position 7 in CDAs with Asn (Uguru *et al.*, 2004) and a mutasynthesis approach to replace the D-hydroxyphenylglycine (D-HPG) at position 6 with alternative arylglycines (Hojati *et al.*, 2002).

In addition to D-HPG, CDA contains a number of other non-proteinogenic amino acids, including L-3-methylglutamic acid (3-MeGlu; at position 10), which is also present at the same relative position in the decapeptide cores of daptomycin and A54145 (Milne *et al.*, 2006), and a C-terminal Z-dehydrotryptophan residue (Hojati *et al.*, 2002). CDA variants which contain either (2R,3S)-3-hydroxyasparagine (3-OHAsn) or (2R,3S)-3-phosphohydroxyasparagine (3-OPAsn) at position 9 are also known. Whilst 3-OHAsn is common to a number of peptide antibiotics, including A54145 (Fukuda *et al.*, 1990; Miao *et al.*, 2006), katanosin B (Kato *et al.*, 1988) and ramoplanin (Walker *et al.*, 2005; McCafferty *et al.*, 2002), 3-phosphohydroxyasparagine has not been found elsewhere in nature, to date.

Previously, we analysed the *cda* biosynthetic gene cluster and identified a gene, SCO3236, which encodes a protein that shows high similarity to clavamate synthase (CAS), an Fe(II)/2-oxoglutarate-dependent oxygenase from

Streptomyces clavuligerus. CAS catalyses the β -hydroxylation of the Arg side chain of deoxyguanidinoproclavaminic acid, and subsequent cyclization and dehydrogenation steps during the biosynthesis of clavulanic acid (Baldwin *et al.*, 1993; Salowe *et al.*, 1990). On this basis, we suggested that SCO3236 encodes an asparagine oxygenase (AsnO) that was predicted to catalyse the β -hydroxylation of an Asn residue in a CDA precursor to generate the 3-OHAsn found in CDA (3a, 3b, 4a and 4b variants) (Fig. 1).

β -Hydroxylation of amino acids is a common phenomenon in the biosynthesis of nonribosomal peptides (Chen *et al.*, 2001, 2002). Indeed the subsequent processing of the amino acid β -hydroxyl groups by further oxidation (Chen *et al.*, 2002), glycosylation (Lu *et al.*, 2004), macrolactonization (Kato *et al.*, 1988; Walker *et al.*, 2005; McCafferty *et al.*, 2002), methylation (Miao *et al.*, 2006) or in the case of CDA, phosphorylation, serves to increase the structural diversity of nonribosomal peptides and related products, resulting in a wide range of biological activities. Of the many β -hydroxylated amino acids and derivatives found in non-ribosomal peptides, some arise as a result of the oxidation of free amino acids prior to peptide assembly (Yin & Zabriskie, 2004; Ju *et al.*, 2004; Haltli *et al.*, 2005). For example, in the biosynthesis of viomycin and mannopeptimycin, L-Arg and the non-proteinogenic amino acid L-enduracididine are hydroxylated as free amino acids by non-haem Fe(II)/2-oxoglutarate-dependent oxygenases VioC (Yin & Zabriskie, 2004; Ju *et al.*, 2004) and MppO (Haltli *et al.*, 2005), respectively. In addition, there are a number of other non-haem Fe(II)/2-oxoglutarate-dependent oxygenases which are known to hydroxylate specific amino acid residues within ribosomal polypeptides. For example, mammalian asparaginyl hydroxylases EGFH and FIH are known, which catalyse the posttranslational β -hydroxylation of Asn residues within epidermal growth factor (EGF) domains (Jia *et al.*, 1994) and the hypoxia-inducible factor (HIF) C-terminal transactivation domains (Hewitson *et al.*, 2002). Finally, several monooxygenase enzymes have been characterized that function on amino acid thioesters that are tethered to carrier proteins via flexible phosphopantetheine prosthetic groups (Chen *et al.*, 2001, 2002). These include haem protein hydroxylases (ORF20, NovI and NikQ), which are responsible for the β -hydroxylation of peptidyl carrier protein (PCP)-tethered tyrosine and histidine residues during the biosynthesis of chloroeremomycin, novobiocin (Chen *et al.*, 2001) and nikkomycin (Chen *et al.*, 2002).

Downstream of *asnO*, in the CDA gene cluster, is a gene, SCO3234, which encodes a protein that shows sequence similarity to a number of ATP-dependent aminoglycoside phosphotransferases, including SpcN, which has been shown to phosphorylate spectinomycin as part of the host self-resistance mechanism in the producer strain *Streptomyces flavopersicus* (Lyutskanova *et al.*, 1997). Based on this, we suggested that the SCO3234 gene product is a putative 3-hydroxyasparagine phosphotransferase (HasP), which phosphorylates the 3-hydroxyasparaginyl

residue of CDA3a/b and CDA4a/b to produce the 3-phosphohydroxyasparaginyl residues found in CDA1b and CDA2a/b (Fig. 1). In this paper, we aim to determine the *in vivo* function of the *asnO* and *hasP* gene products, and test the hypothesis that these products are involved in the hydroxylation and subsequent phosphorylation of the asparaginyl residues in CDA. It was envisaged that this insight would provide a means to control the functionality of the Asn-derived residues in CDA which could be further utilized to engineer other therapeutically relevant acidic lipopeptides based around the A54145 and daptomycin biosynthetic templates.

METHODS

Bacterial strains, plasmids and culture conditions. The bacterial strains and plasmids used in this study are listed in Supplementary Table S1. *Escherichia coli* strains were propagated routinely in Luria–Bertani (LB) broth or on agar plates at 37 °C. Antibiotics were used in selective media at the standard concentrations (Sambrook *et al.*, 2000). For DNA manipulation, *Streptomyces* strains were routinely propagated in YEME liquid media or on R2YE agar plates at 30 °C (Kieser *et al.*, 2000).

DNA preparation, manipulation and sequencing. *S. coelicolor* cosmid ScE29 encompassing SCO3232–SCO3249 of the CDA biosynthetic gene cluster was obtained from the John Innes Centre, Norwich, UK, and was isolated from *E. coli* using the Qiagen Midiprep Kit. Small-scale plasmid preparation was carried out using the alkaline lysis method (Birnboim & Doly, 1979), unless a higher quality of DNA was required for sequencing, in which case DNA was prepared using a Midiprep Kit. *Streptomyces* genomic DNA was prepared as described elsewhere (Kieser *et al.*, 2000). Restriction enzymes were purchased from Roche, New England Biolabs and MBI Fermentas. Alkaline phosphatase, T4 DNA ligase and *Pwo* polymerase were all purchased from Roche. All enzymes were used according to the manufacturers' recommendations, unless otherwise specified.

PCR amplification was carried out on a GeneAmp PCR system 2400 (Perkin-Elmer) using 100 pmol of each primer, ~100 ng cosmid or 200 ng genomic DNA template per 100 µl reaction, and 1 unit of the high fidelity *Pwo* polymerase. PCR reactions also contained 5 % (v/v) DMSO to overcome potential secondary structure formation in the GC-rich *Streptomyces* DNA. Following amplification, PCR products were purified using the Qiaquick extraction kit (Qiagen). PCR products and primers used in this work are detailed in

Supplementary Table S2. DNA sequencing of PCR products was carried out by the chain-termination method using the ABI/PRISM dye terminator cycle sequencing core kit (Perkin-Elmer). *E. coli* plasmid construction and transformations were carried out using standard procedures (Sambrook *et al.*, 2000).

Construction of the *asnO* deletion cassette. An upstream region of SCO3236 was PCR-amplified using primers AsnOF2 and AsnOR2, containing *XhoI* and *PstI* restriction sites, respectively (Supplementary Table S2), with ScE29 as a template to generate a 1558 bp amplicon (fragment A) containing the first 226 bp of SCO3236. Fragment A and pMT3000 (Paget *et al.*, 1994) were digested with *XhoI* and *PstI*, and ligated to form plasmid pJN9a. Primers AsnOF3 and AsnOR3, containing *EcoRI* and *XhoI* restriction sites, were used in a PCR reaction with ScE29 as a template to generate a 1662 bp amplicon (fragment B) containing the last 227 bp of SCO3236. Fragment B and pMT3000 were digested with *EcoRI* and *XhoI*, and ligated to form pJN9b. Fragment A was excised from pJN9a via *PstI/XhoI* and ligated into similarly digested pJN9b to give the plasmid pJN10 containing the *asnO* (AB) deletion construct (Fig. 2). This was excised from pJN10 via the flanking *BglII* sites and ligated into the *BamHI* site of pKC1132 (Bierman *et al.*, 1992), resulting in pJN12, which was used for delivery of the truncated *asnO* into the chromosome of *S. coelicolor* 2377 (Hopwood & Wright, 1983) and MT1110 (Hindle & Smith, 1994) strains.

Construction of the *hasP* deletion cassette. An upstream region of SCO3234 was PCR-amplified using primers HasPF1 and HasPR1, containing *PstI* and *XbaI* restriction sites, respectively, with ScE29 as a template to generate a 1830 bp amplicon (fragment A') containing the first 70 bp of SCO3234. Fragment A' and pMT3000 were digested with *PstI* and *XbaI*, and ligated to form pMUT1. Primers HasPF2 and HasPR2, containing *XbaI* and *XhoI* restriction sites, respectively, and the template ScE29 were used in the PCR amplification of a 1939 bp amplicon (fragment B') containing the last 215 bp of SCO3234. Fragment B' and pMT3000 were digested with *XbaI* and *XhoI*, and ligated to form pMUT2, excised from pMUT2 via *XbaI/XhoI*, and ligated into similarly digested pMUT1 to give the plasmid pLG3 containing the *hasP* deletion construct (A'B') (Fig. 2). The deletion construct was excised from pLG3 via the flanking *BglII* sites and ligated into the *BamHI* site of pMAH (Paget *et al.*, 1994), resulting in pLG4, which was used for delivery of the truncated *hasP* into the chromosome of *S. coelicolor* 2377 and MT1110 strains.

Deletion of *asnO* and *hasP* from *S. coelicolor* MT1110 and 2377. Δ *asnO* and Δ *hasP* delivery plasmids pJN12 and pLG4 were first passed through *E. coli* strain ET12567 to remove Dam/Dcm methylation. The plasmids were then alkaline-denatured as described elsewhere (Oh & Chater, 1997) and introduced into *Streptomyces*

Fig. 2. Generation of *S. coelicolor* Δ *asnO* and Δ *hasP* mutant strains. Plasmids pLG4 and pJN12, which contain truncated *hasP* (A'B') and *asnO* (AB) deletion constructs, respectively, were used to perform standard double-crossover gene replacements, which resulted in mutants possessing in-frame truncated *hasP* and *asnO* genes, respectively.

protoplasts using the PEG-mediated transformation method (Kieser *et al.*, 2000). Primary integrants of pJN12 and pLG4 were selected for with apramycin and hygromycin, respectively, and grown for two generations on non-selective agar to allow double-crossover events to occur. Colonies were screened for the loss of antibiotic resistance by replica plating. Total genomic DNA was isolated from antibiotic-sensitive recombinants, and used as a template for PCR amplification using primers flanking the deleted region. Primers 29.05seqF2 and 29.05R2 amplified the region of *asnO*, giving products of size 1044 bp from strains that had not undergone gene replacement and 495 bp from strains that had a truncated form of *asnO* (Supplementary Fig. 1A). Similarly, primers HasPKO1 and HasPKO2 were used to amplify the region of *hasP*, resulting in products of size 1818 bp in the strains that had not undergone gene replacement and 1200 bp in the strains that contained a truncated form of *hasP* (Supplementary Fig. 1B). DNA sequencing of PCR products corresponding to truncated forms of *asnO* and *hasP* was also carried out, confirming that an in-frame deletion of 549 bp of the *asnO* and 618 bp of the *hasP* genes had occurred (data not shown). Two *S. coelicolor* 2377- Δ *asnO* strains and four *S. coelicolor* MT1110- Δ *asnO* strains were generated. In addition three *S. coelicolor* 2377- Δ *hasP* and *S. coelicolor* MT1110- Δ *hasP* strains were also generated.

CDA production, extraction and analysis. The MT1110 parent, Δ *asnO* and Δ *hasP* mutant strains were grown for 6 days at 30 °C in SV2 liquid medium (Hojati *et al.*, 2002). *S. coelicolor* 2377 parent, Δ *asnO* and Δ *hasP* mutant strains were grown for 6 days at 30 °C in SM22 liquid medium (Hojati *et al.*, 2002). The culture supernatants were acidified to pH 2 with 0.5 M HCl and passed through Varian MegaBond Elute SPE cartridges (Varian) as described previously (Hojati *et al.*, 2002). The CDA extracts were then eluted with 40 % acetonitrile in H₂O and dried under reduced pressure. Liquid chromatography (LC)-MS analysis of the crude extracts was carried out on a Micromass LCT orthogonal acceleration time of flight mass spectrometer equipped with an electrospray ionization source run in positive mode, combined with a Waters 2790 separation module. Gradient elution was carried out on a Luna C18 150 × 4.6 mm 3 µm analytical column (Phenomenex). Solvent A was 0.1 % formic acid and solvent B was acetonitrile/0.1 % formic acid. A gradient of 20 % B rising to 70 % over 10 min, followed by 100 % B held for 5 min at a flow rate of 1 ml min⁻¹ was applied.

Complementation of *S. coelicolor* MT1110- Δ *asnO* with plasmid-borne *asnO*. To construct a *Streptomyces* expression plasmid bearing *asnO*, the gene sequence was PCR-amplified from cosmid ScE29 with primers 29.05F2 and 29.05R2, containing restriction sites for *Xho*I and *Nde*I, respectively. The purified product was restricted and ligated into *E. coli* expression vector pET-15b, resulting in the 6708 bp plasmid pJN14. The *asnO* gene was excised from pJN14 by digestion with *Bam*HI and *Xho*I, and ligated into restriction-digested pIJ6021 (Takano *et al.*, 1995), resulting in the 8904 bp plasmid pJN15. The ligation products were used to transform *Streptomyces lividans* 1326, and positive transformants were selected by growth on medium containing kanamycin. pJN15 was isolated from transformants, checked by restriction analysis, and introduced into the *S. coelicolor* MT1110- Δ *asnO* mutant by protoplast transformation. The plasmid-complemented mutant was cultivated in SV2 medium supplemented with kanamycin, and thiostrepton was added at a concentration of 10 µg ml⁻¹ to induce expression of AsnO. CDA was extracted from the culture supernatant and analysed by LC-MS, as described above.

Cell-based bioassay of Asn-containing CDAs. Spores of *S. coelicolor* parent and Δ *asnO* mutant strains were used to inoculate nutrient agar plates in the presence and absence of Ca²⁺ (12 mM). After 2 days' incubation at 30 °C, an overnight culture of the CDA-sensitive *Bacillus mycoides* indicator strain was used to overlay the

plates in soft nutrient agar (per litre: 13 g nutrient broth, 7 g Bactoagar). Calcium-dependent bioactivity was observed as a zone of inhibition after one night of incubation at 30 °C (Fig. 5A).

Production and isolation of CDA6a from 2377- Δ *asnO*. Sterile SM22 liquid medium (2 l) was inoculated with *S. coelicolor* 2377- Δ *asnO* and then incubated at 28 °C and 180 r.p.m. for 5 days. The cells were removed by centrifugation and the CDA6a in the resulting supernatants was extracted onto Varian C-18 Bond Elute SPE cartridges (2 g), as described previously (Hojati *et al.*, 2002). Purification of the crude extracts was achieved by preparative and semi-preparative reversed-phase HPLC: Phenomenex C-18 10 µm, 250 × 21.2 mm and 5 µm, 250 × 10 mm columns; solvent A H₂O with 0.1 % HCO₂H; solvent B acetonitrile with 0.1 % HCO₂H. The flow rates were 20 and 5 ml min⁻¹, respectively, with a starting gradient of 0 % B and 100 % A, increasing to 100 % B over 30 min and then held for a further 5 min. Fractions for the peak containing mainly CDA6a (retention time ~17.3 min) were pooled and evaporated under reduced pressure to give CDA6a (1.0 mg, ~90 % pure).

Well-based bioassay of CDA6a. CDA-sensitive strain *Micrococcus luteus* was grown overnight at 30 °C in Oxoid nutrient broth (5 ml). This culture was used to seed Oxoid nutrient agar plates in the presence or absence of added Ca²⁺ (16 mM). Agar plugs were cut from the dried plates, creating wells into which a sterile aqueous solution of CDA6a (~100 µg in 100 µl) was added. The plates were then incubated overnight at 30 °C (Fig. 5B).

RESULTS

AsnO and HasP sequence analysis

BLAST sequence similarity searches on the SCO3236 (*asnO*) and SCO3234 (*hasP*) gene products were performed. In the case of HasP, no recently deposited protein sequences, in addition to the aminoglycoside phosphotransferases noted earlier (Hojati *et al.*, 2002), were identified which might provide insight into the function of the putative phosphotransferase. On the other hand, several new sequences, in addition to CAS, were identified which exhibit high similarity to the putative asparagine oxygenase (AsnO). For example, LptL possesses 45 % identity across 310 out of 330 amino acid residues, and is predicted to be involved in the hydroxylation of the Asn residue at position 3 of A54145 in *Streptomyces fradiae* (Miao *et al.*, 2006). In addition L-enduracididine hydroxylase, VioC (Yin & Zabriskie, 2004; Ju *et al.*, 2004), exhibits 33 % identity across a 292 amino acid alignment with AsnO, and the L-arginine hydroxylase MppO (Haltli *et al.*, 2005) possesses 31 % identity across 300 amino acid residues. All of these proteins possess the His-1 (HXE) and the C-terminal His-3 (DNXXXXH) motifs which make up the Fe(II) coordination sphere, which supports their known or putative function as Fe(II)/2-oxoglutarate-dependent oxygenase enzymes (Haltli *et al.*, 2005; Khaleeli *et al.*, 2000).

Generation of *S. coelicolor* Δ *asnO* and Δ *hasP* mutants

In order to establish the role of the *asnO* and *hasP* gene products, both genes were separately deleted from the *S. coelicolor* chromosome using a standard double-crossover

recombination approach (Fig. 2) (Kieser *et al.*, 2000). The successful generation of the required in-frame mutants was confirmed by PCR amplification across the truncated region (Supplementary Fig. S1), followed by DNA sequencing. Knockout experiments were performed in both 2377 and MT1110 strains, since these strains have different growth-media requirements and produce different profiles of CDAs. The combined information can thus provide valuable complementary information which can assist in validating the results of the separate experiments.

Analysis of *S. coelicolor* $\Delta hasP$ and $\Delta asnO$

The *S. coelicolor* 2377 parent strains and 2377- $\Delta hasP$ mutant strains were grown in liquid culture, using media and

conditions which we have previously shown (Hojati *et al.*, 2002) result in the production of phosphorylated CDAs. The culture supernatants were analysed by LC-MS, which confirmed the previous observation (Hojati *et al.*, 2002) that the parent strain 2377 produces D-3-phosphohydroxy-asparagine-containing peptide CDA2b as the major product, along with a minor amount of D-3-hydroxyasparagine variants CDA4b and CDA3b (Fig. 3A). In contrast, the 2377- $\Delta hasP$ mutants produced only the non-phosphorylated variants CDA3b and CDA4b under identical growth conditions (Fig. 3B). Indeed, repeated fermentation and analysis of several 2377- $\Delta hasP$ mutant strains clearly revealed a complete absence of CDA2b, or any other phosphorylated CDAs. This supports the hypothesis that the gene product HasP is the CDA 3-hydroxyasparaginyl

Fig. 3. LC-MS analysis of *S. coelicolor* 2377 parent and 2377- $\Delta hasP$ mutant strains. (A) *S. coelicolor* 2377 parent strain shows CDA2b ($[M+H]^+$ found 1577.5, requires 1577.5; $[M+Na]^+$ found 1599.5, requires 1599.5), CDA4b ($[M+H]^+$ found 1497.7, requires 1497.5; $[M+Na]^+$ found 1519.7, requires 1519.5), CDA3b ($[M+H]^+$ found 1483.7, requires 1483.5; $[M+Na]^+$ found 1505.7, requires 1505.5). The LC-MS of CDA2b standards also gave a very broad peak, with a retention time (R_t) of 10.5–12.0 min. This was due to the multiple ionization states of the phosphorylated peptide and the fact that it was not possible to use a buffered eluant, because this interferes severely with mass detection on LC-ESI-MS. (B) *S. coelicolor* 2377- $\Delta hasP$. Production of phosphorylated CDA2b is completely abolished in this mutant, which produces only CDA4b ($[M+H]^+$ found 1497.5; $[M+Na]^+$ found 1519.5) and CDA3b ($[M+H]^+$ found 1483.4; $[M+Na]^+$ found 1505.4). %, percentage of maximum intensity peak.

phosphotransferase. Analysis of the parent strain MT1110, grown on a variety of different media, showed only D-3-hydroxyasparagine-containing CDAs and failed to reveal any phosphorylated CDAs. Comparison of the phenotype change between MT1110 and MT1110- Δ hasP strains was therefore not possible in this case.

S. coelicolor MT1110 and MT1110- Δ asnO strains were grown in liquid media under identical conditions. LC-electrospray ionization (ESI)-MS analysis of MT1110 revealed the 3-MeGlu-containing peptide CDA4a as the major product along with a minor amount of the Glu-containing CDA3a (Fig. 4A). The mutant MT1110- Δ asnO, however, does not produce any CDA that is common to the MT1110 or 2377 strains (Hojati *et al.*, 2002; Kempter *et al.*, 1997), but instead exhibits a major new product with a retention time of 6.90 min, which exhibited protonated, sodiated and potassiated ($[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$) molecular ions in the ESI-MS consistent molecular weight (*mw*) 1478.5 Da (Fig. 4B). This CDA variant was therefore assigned as a non-hydroxylated Asn-containing variant, CDA6a (Fig. 1), which is related to CDA4a by the loss of the oxygen (1494.5–16.0 Da). Similarly, the *S. coelicolor* 2377 parent and 2377- Δ asnO mutant strains were grown in liquid culture. As above, the parent strain 2377 produced CDA2b as the major product with a minor amount of CDA4b and 3b. In contrast, the mutant 2377- Δ asnO produced none of the previously characterized CDAs. Instead, two new CDA products were identified. The faster-eluting compound, with an LC retention time of 6.90 min, exhibited protonated, sodiated and potassiated molecular ions in the ESI-MS consistent with CDA6a. In addition, a slower-eluting variant (R_t = 6.79) was observed with molecular ions that indicate *mw* 1464.5 (Fig. 4C). This product corresponds to a non-hydroxylated variant of CDA3a (1480.5–16.0 Da), which we have assigned as CDA5a (Fig. 1).

It is thus clear from these complementary results that deletion of *asnO* abolishes the normal CDA production profile of 3-OHAsn- and 3-OPAsn-containing peptides, and instead results in the production of two previously uncharacterized Asn-containing CDA variants (designated CDA5a and 6a).

Expression of plasmid-borne *asnO* in the MT1110- Δ asnO mutant

In order to gain further confirmation that AsnO is responsible for the hydroxylation of the Asn residue in CDA, the MT1110- Δ asnO strain was complemented by transformation with a thiostrepton-inducible *asnO* expression vector derived from the *Streptomyces* plasmid pIJ6021. The plasmid containing MT1110- Δ asnO mutants was grown on media containing the inducer thiostrepton. LC-MS analysis of the extracts revealed the same CDA profile as that of the MT1110 parent strain, with the production of 3-OHAsn-containing CDA4a and CDA3a being re-established. The results of the *asnO* knockout

and complementation experiments are thus entirely consistent with the AsnO, a single polypeptide, functioning as an asparaginyl oxygenase.

Bioactivity of Asn CDAs

Parent and Δ asnO mutant strains of *S. coelicolor* 2377 and MT1110 were each separately incubated with and without Ca^{2+} , and overlaid with the CDA-sensitive strain *B. mycoides*. A calcium-dependent zone of inhibition was seen around the two *S. coelicolor* 2377- Δ asnO strains, indicating that Asn-containing CDAs retained calcium-dependent antimicrobial activity (Fig. 5A). Similarly, all four of the *S. coelicolor* MT1110- Δ asnO strains exhibited calcium-dependent antibiosis with *B. mycoides* (data not shown). To further confirm that the bioactivity resulted from Asn-containing CDAs, 2377- Δ asnO was cultivated on a large scale (2 l), and the resulting CDA6a was purified from the culture supernatant using preparative and semi-preparative HPLC. High-resolution ESI-MS (*m/z* 1479.5333, $C_{67}H_{79}N_{14}O_{25}$ requires 1479.5340) and UV spectroscopy (λ_{max} 347 nm in H_2O) characteristic of Z-dehydrotryptophan-containing α -series CDA (Hojati *et al.*, 2002) further confirmed the structure of CDA6a. Well-plate bioassays of CDA6a with *M. luteus* as the indicator stain showed significant calcium-dependent zones of inhibition (Fig. 5B). Given that only ~1 mg of CDA6a could be purified from a 2 l culture of *S. coelicolor* 2377- Δ asnO, it was not practical to obtain sufficient quantities of CDA6a to determine accurate MIC values, for comparison with the other known CDAs. Nevertheless, from the size of the zones of inhibition and the concentrations of CDAs used, it is possible to conclude that CDA6a is less active than the corresponding 3-OHAsn-containing CDA4a and CDA3a (Milne *et al.*, 2006).

DISCUSSION

This work clearly shows that AsnO and HasP are involved in the biosynthesis of 3-OHAsn and 3-OPAsn in CDA. However, the results do not indicate directly whether the hydroxylation and phosphorylation reactions take place before, during or after peptide assembly. Despite this, clues about the true nature of the substrates for AsnO and HasP can be derived by analysing the amino acid specificity conferring code of the module 9 adenylation domain (A domain) of the nonribosomal peptide synthetase (cdaPS2) responsible for activating Asn or its derivatives. Indeed, the 10 essential active site residues of the module 9 A domain (DLTKIGEVGK) within CdaPS2 have similarity to many other Asn activating A domains from different sources (Stachelhaus *et al.*, 1999; Challis *et al.*, 2000). This indicates that the module 9 A domain will be able to activate Asn, and indeed this must necessarily be the case given the production of Asn-containing lipopeptides CDA5a and 6a in the Δ asnO mutants. Furthermore, the module 9 A domain might possess broad substrate specificity, allowing the activation of 3-OHAsn, which is only slightly larger than Asn. On the

other hand, it is unlikely that the A domain could activate both 3-OPAsn and Asn, given the very large difference in molecular volume and polarity of these two substrates. Furthermore, we have shown that it is possible to change the active site of the Asp-7 A domain (at the positions underlined: DLTKIGAVNK) to match exactly the sequence of the module 9 (Asn) A domain (DLTKIGEVGK). The resulting mutant produces a new lipopeptide product, CDA-7N, with Asn at position 7 instead of Asp (Uguru *et al.*, 2004). Conceivably, if free Asn was hydroxylated to 3-OHASn prior to A-domain activation, then this experiment could have resulted in CDAs with 3-OHASn or 3-OPAsn at position 7, which was not the case. However, the upstream condensation domain of module 7 was unaltered, and thus retained specificity for the Asp-S-PCP precursor. Thus, the condensation of any 3-OHASn-S-PCP intermediate (which may have accumulated) with the upstream hexapeptidyl intermediate would have been severely impaired. Indeed, this could account for the fact that the Asp-7 A domain double point mutant produces considerably more of the hydrolysed CDA hexapeptide intermediate than the Asn-containing CDA-7N (Uguru *et al.*, 2004).

Whilst the results presented here and previously (Hojati *et al.*, 2002; Uguru *et al.*, 2004) do not indicate the timing of the Asn hydroxylation, the evidence suggests that phosphorylation is most likely to occur after A-domain activation. Therefore, 3-OHASn-containing CDAs (e.g. CDA3a, 3b, 4a and 4b) are the most likely substrates for HasP. Alternatively, the possibility that phosphorylation occurs during peptide assembly cannot be ruled out, as other nonribosomal peptide-tailoring enzymes are suggested to modify peptidyl-thioester intermediates tethered to PCP domains. Indeed, there is evidence that oxidative coupling reactions between the phenolic side chains most likely occur on the peptidyl-thioester intermediates during the assembly of vancomycin and related glycopeptides (Zerbe *et al.*, 2004; Bischoff *et al.*, 2005).

It is also interesting to note that Asn-containing CDAs retain calcium-dependent antimicrobial activity (Fig. 5) and that 3-OHASn or 3-OPAsn motifs are therefore not essential for calcium binding. Interestingly, the 3-OHASn/Asp residues found in EGF are suggested to be involved in calcium binding (Valcarce *et al.*, 1999; Lancaster *et al.*, 2004). Thus,

Fig. 4. LC-MS analysis of MT1110 and Δ asnO mutants. (A) *S. coelicolor* MT1110 produces CDA3a ($[M+H]^+$ found 1481.9, requires 1481.5; $[M+Na]^+$ found 1503.8, requires 1503.5; $[M+K]^+$ found 1519.8, requires 1519.5), CDA4a ($[M+H]^+$ found 1495.9, requires 1495.5; $[M+Na]^+$ found 1517.8, requires 1517.5; $[M+K]^+$ found 1533.8, requires 1533.5). (B) *S. coelicolor* MT1110- Δ asnO produces CDA6a ($[M+H]^+$ found 1479.0, requires 1479.5; $[M+Na]^+$ found 1501.0, requires 1501.5; $[M+K]^+$ found 1517.1, requires 1517.5). (C) *S. coelicolor* 2377- Δ asnO produces CDA5a ($[M+H]^+$ found 1465.3, requires 1465.5; $[M+Na]^+$ found 1487.2, requires 1487.5; $[M+K]^+$ found 1503.2, requires 1503.5) and CDA6a ($[M+H]^+$ found 1479.2; $[M+Na]^+$ found 1501.2; $[M+K]^+$ found 1517.2). R_t =retention time (min). %, percentage of maximum intensity peak.

Fig. 5. Bioactivity of Asn-containing CDAs. (A) Cell bioassay of 2377- Δ asnO strains overlaid with *B. mycoides* as the indicator strain in the presence of Ca^{2+} (12 mmol). Strains 13 and 29 are Δ asnO mutant strains, whilst strains 23 and 34 had been transformed with the Δ asnO deletion construct, but proved not to possess the *asnO* deletion and therefore retained the same phenotype as the parent strain 2377. The parent 2377 strain is shown in the centre of the plate. No zones of inhibition were present in the control plates which contained no Ca^{2+} . (B) Well-plate bioassay with purified CDA6a (100 μg in 100 μl) and *M. luteus* as the indicator strain, in the presence of Ca^{2+} (16 mM). Again, zones of inhibition were completely absent in the control plates which possessed no Ca^{2+} .

whilst our results indicate that 3-OHAsn residues are not essential for coordination of Ca^{2+} ions to CDA, the possibility that β -hydroxylation of Asn in nonribosomal as well as ribosomal peptides is evolutionarily linked to the propensity of the β -hydroxylated peptide products to bind Ca^{2+} ions cannot be discounted (Lancaster *et al.*, 2004). In addition to this, it is also worth noting that the *S. coelicolor* MT1110- Δ hasP and 2377- Δ hasP strains were able to produce non-phosphorylated CDAs and maintain growth on media supplemented with Ca^{2+} . This suggests that HasP

is not part of the host self-resistance mechanism, as is the case for other phosphotransferases which share sequence similarity (Lyutskanova *et al.*, 1997).

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