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## An unusual *Burkholderia gladioli* double chain-initiating nonribosomal peptide synthetase assembles 'fungal' icosalide antibiotics†

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*Burkholderia* is a multi-talented genus of Gram-negative bacteria, which in recent years has become increasingly recognised as a promising source of bioactive natural products. Metabolite profiling of *Burkholderia gladioli* BCC0238 showed that it produces the asymmetric lipopeptidolide antibiotic icosalide A1, originally isolated from a fungus. Comparative bioinformatics analysis of several genome-sequenced *B. gladioli* isolates identified a gene encoding a nonribosomal peptide synthase (NRPS) with an unusual architecture that was predicted to be responsible for icosalide biosynthesis. Inactivation of this gene in *B. gladioli* BCC0238 abolished icosalide production. PCR analysis and sequencing of total DNA from the original fungal icosalide A1 producer revealed it has a *B. gladioli* strain associated with it that harbours an NRPS with an identical architecture to that responsible for icosalide A1 assembly in *B. gladioli* BCC0238. Sequence analysis of the icosalide NRPS indicated that it contains two chain-initiating condensation (C<sub>i</sub>) domains. One of these is appended to the N-terminus of module 1 – a common architecture for NRPSs involved in lipopeptide assembly. The other is embedded in module 3, immediately downstream of a putative chain-elongating condensation domain. Analysis of the reactions catalysed by a tridomain construct from module 3 of the NRPS using intact protein mass spectrometry showed that the embedded C<sub>i</sub> domain initiates assembly of a second lipopeptide chain, providing key insights into the mechanism for asymmetric diolide assembly.

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## Introduction

Bacteria belonging to the *Burkholderia* genus thrive in various ecological niches, ranging from the plant rhizosphere to the human lung, and play critical roles in ecological interactions often through the secretion of specialised metabolites.<sup>1,2</sup> The

metabolic arsenal of these bacteria is employed to eliminate other bacterial competitors and suppress plant pathogenic fungi and nematodes. In recent years various *Burkholderia* species have been shown to produce numerous specialised metabolites, many of which are assembled by polyketide synthases (PKSs) and nonribosomal peptide synthetases (NRPSs) biosynthetic assembly lines.<sup>3</sup> Notable examples include: the respiratory toxin bongkrekic acid;<sup>4</sup> anti-proliferative agents such as thailanstatin and spliceostatin;<sup>5,6</sup> and the antibiotics enacyloxin IIa and gladiolin.<sup>7,8</sup>

*Burkholderia* participate in both antagonistic and mutualistic interactions with fungi. Much of the antagonistic behaviour can be attributed to an array of antifungal compounds secreted by many *Burkholderia* species.<sup>9–12</sup> However, it has also been reported that *Burkholderia* strains have beneficial effects on fungi, suggestive of symbiotic, or at least mutualistic interactions.<sup>13–15</sup> The exemplar study of this relationship is the symbiotic pairing of *Burkholderia rhizoxinica* (since reclassified as *Paraburkholderia rhizoxinica*) and the rice seedling blight pathogen *Rhizopus microspores*. *B. rhizoxinica* was identified as the producer of the antimetabolic macrolide rhizoxin, the causative agent of rice seedling blight.<sup>10,16</sup>

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In a preliminary communication, we disclosed that *Burkholderia gladioli* isolates from cystic fibrosis (CF) patients produce icosalide A1 (1),<sup>17</sup> a lipopeptidolide antibiotic originally isolated from a fungus.<sup>18</sup> We also showed that a non-ribosomal peptide synthetase (NRPS) with an unusual architecture is responsible for icosalide A1 assembly.<sup>17</sup> Subsequently, Hertweck and co-workers reported that a *B. gladioli* endosymbiont of a beetle, along with several other *Burkholderia* species from diverse sources, also produce icosalide A1 and showed that an NRPS with the same unusual architecture as that identified in the CF isolates is responsible for its biosynthesis.<sup>19</sup> Here we provide a full account of our work and show that a *B. gladioli* strain containing the icosalide A1 NRPS is associated with the originally reported fungal producer. Moreover, we demonstrate that a chain-initiating condensation (C<sub>1</sub>) domain embedded in the middle of the icosalide A1 NRPS selectively *N*-acylates a serinyl thioester covalently attached to the downstream peptidyl carrier protein (PCP) domain with a (3*R*)-3-hydroxydecanoyl panthetheine thioester, providing a mechanistic rationale for asymmetric lipopeptidolide formation.

## Results and discussion

We recently reported the discovery of gladiolin, a novel macrolide antibiotic with promising activity against *Mycobacterium tuberculosis* (MIC = 0.4 μg mL<sup>-1</sup>) and low mammalian cell cytotoxicity, from *B. gladioli* BCC0238 (LMG-P26202).<sup>7</sup> In the course of investigations of other specialised metabolites produced by this CF clinical isolate using UHPLC-ESI-Q-TOF-MS, compounds with the molecular formulae C<sub>36</sub>H<sub>64</sub>N<sub>4</sub>O<sub>10</sub> and C<sub>34</sub>H<sub>60</sub>N<sub>4</sub>O<sub>10</sub> were observed (Fig S1 and S2†). These molecular formulae correspond to a set of three structurally-related lipopeptidolides, known as icosalides A1 (1), A2 (2) and B (3), reported to be produced by fungi<sup>18</sup> (Fig. 1A).

The icosalides possess similar 20-membered lipopeptidolide structures consisting of two serine, leucine and 3-hydroxy acid residues. In icosalide A1 (1), one of the leucine residues is *D*-configured, whereas all of the amino acid residues in icosalides A2 (2) and B (3) are *L*-configured. Icosalides A1 (1) and A2 (2) both contain one eight-carbon and one ten-carbon 3-hydroxy acid residue, whereas icosalide B (3) contains two eight-carbon 3-hydroxy acid residues. The incorporation of a *D*-leucine residue into icosalide A1 (1) has a dramatic effect on its biological activity. While icosalide A1 (1) is active against *Streptococcus pyogenes* (MIC: 8–16 μg mL<sup>-1</sup>), icosalides A2 (2) and B (3) do not show any antibacterial activity but are cytotoxic towards replicating MDCK cells (CC<sub>50</sub>: 5 μg mL<sup>-1</sup>).<sup>18</sup>

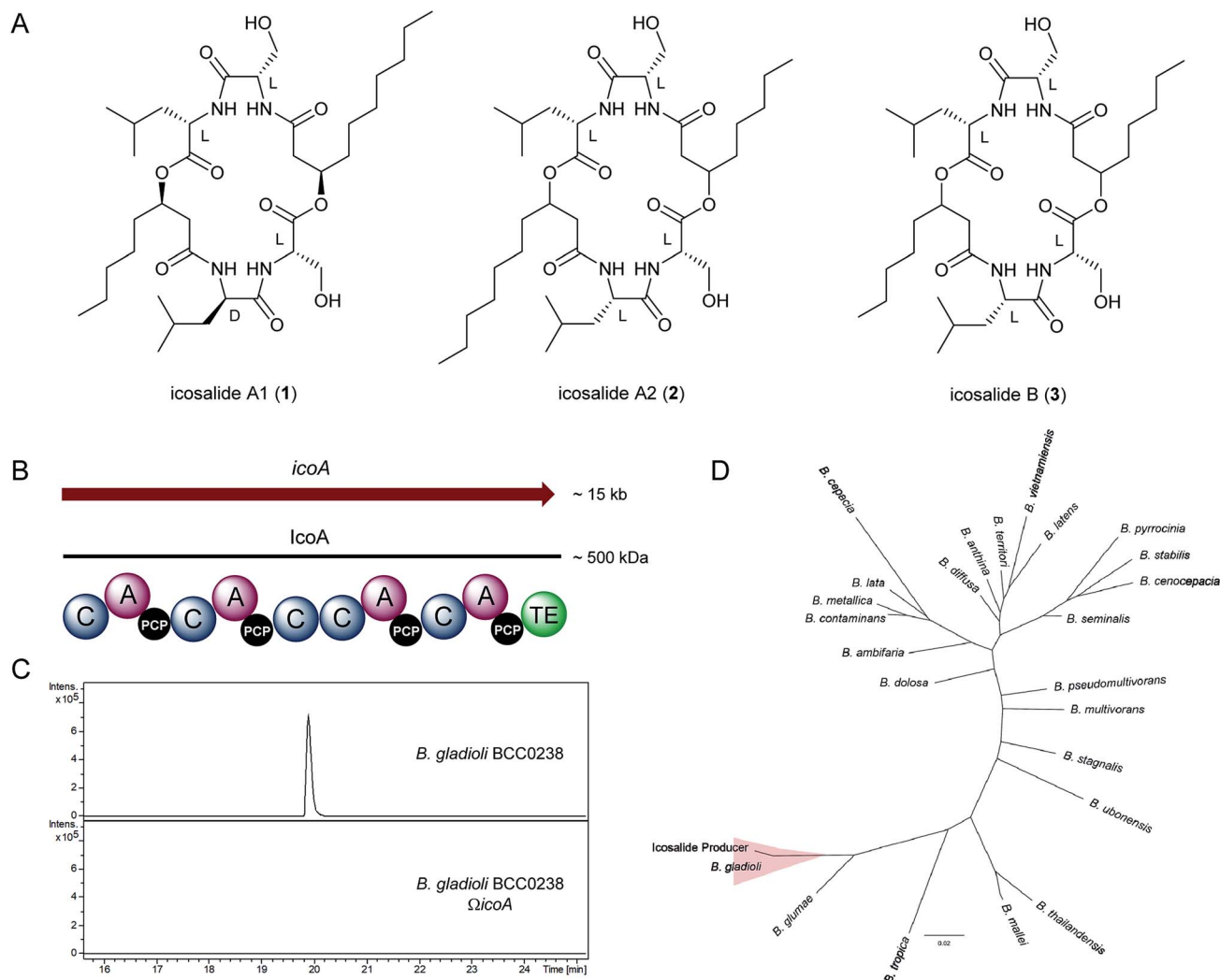
The compound with the molecular formula C<sub>36</sub>H<sub>64</sub>N<sub>4</sub>O<sub>10</sub> was purified using semi-preparative HPLC and its planar structure was shown to be identical to that of icosalide A1 (1) using a combination of <sup>1</sup>H, <sup>13</sup>C and HMBC NMR experiments (Fig. S3 and Table S1†). The absolute stereochemistry of the amino acid residues in the purified compound was determined *via* saponification of the esters, followed by semi-preparative HPLC purification and MS analysis of the resulting pair of 3-hydroxyacyl-dipeptides, acid hydrolysis to liberate the

constituent amino acids, derivatisation using Marfey's reagent and comparison with the corresponding *L* and *D*-configured leucine and serine standards (Fig. S4 and S5†). This revealed that the Leu-2 residue is *D*-configured, confirming the identity of the metabolite produced by *B. gladioli* as icosalide A1 (1). The production level of the compound with the molecular formula C<sub>34</sub>H<sub>60</sub>N<sub>4</sub>O<sub>10</sub> was too low to permit characterisation by NMR spectroscopy (Fig. S6†), but it seems likely that this has the same planar structure as icosalide B (3). The activity of purified icosalide A1 (1) against several Gram-positive and Gram-negative bacteria, including the ESKAPE panel of pathogens, and *Candida albicans* was tested. Modest activity against *E. faecium* (MIC: 16 μg mL<sup>-1</sup>) was observed (Table S2†).

Inspection of putative specialised metabolite biosynthetic gene clusters in the previously reported genome sequence of *B. gladioli* BCC0238,<sup>7</sup> identified a single ~15 kb gene (*icoA*) on chromosome 2 encoding a nonribosomal peptide synthetase (NRPS) with the requisite module and domain organisation for icosalide assembly (Fig. 1B). This NRPS contains four adenylation (A) domains, two of which are predicted to be specific for *L*-leucine and two for *L*-serine<sup>20</sup> (Table S3†). It also contains five condensation (C) domains and a thioesterase (TE) domain, which together account for the four amide and two ester bond-forming reactions required to assemble the lipopeptidolide structure of the icosalides<sup>21</sup> (Fig. 2A). We identified *icoA* in the genomes of several other *B. gladioli* isolates (BCC0252, BCC1677, BCC1698 and BCC1720), all of which were shown to produce icosalide A1 (1). In contrast, no icosalide A1 (1) production was detected in *B. gladioli* BCC1713 (Fig. S7†), which is lacking *icoA*. These data are consistent with the hypothesis that the NRPS encoded by *icoA* is responsible for icosalide biosynthesis. To verify this, we inactivated the *icoA* gene in *B. gladioli* BCC0238 using insertional mutagenesis. UHPLC-ESI-Q-TOF-MS analysis of extracts from the *icoA* mutant confirmed that it is no longer able to produce icosalides (Fig. 1C).

Icosalide A1 (1) was originally isolated from extracts of an *Aureobasidium* species fungus (strain MSX 59166), whereas icosalides A2 (2) and B (3) were isolated from a second less well characterized fungal species (MSX 74159).<sup>18</sup> The data presented above suggested that *B. gladioli* bacteria associated with these fungi might be the true producers of the icosalides isolated from them. To investigate this hypothesis, total DNA from the originally reported icosalide A1 (1) producer, *Aureobasidium* sp. MSX 59166, was screened by PCR for orthologues of *icoA* and the bacterial housekeeping gene *recA*. In both cases, we obtained amplicons of the expected size (Fig. S8†), confirming that a bacterium, likely harbouring an *icoA* orthologue, is associated with the fungus. *Burkholderia* species can be unambiguously identified *via* phylogenetic comparison of their *recA* sequences.<sup>22</sup> Thus, we compared the sequence of the *recA* amplicon with the sequences of *recA* genes from 25 representative *Burkholderia* species. This showed that the *Aureobasidium*-associated bacterium is *B. gladioli* (Fig. 1D). Illumina sequencing of the total DNA from the *Aureobasidium* sp. allowed an approximately 1 Mb contiguous sequence of bacterial DNA, encompassing the *icoA* orthologue, to be assembled (Fig. S9†). Comparison of this contiguous sequence with the





**Fig. 1** Structures of the icosalides, identification of the NRPS-encoding gene responsible for icosalide biosynthesis in *B. gladioli* BCC0238 and phylogenetic comparison of the *recA* sequence amplified from total DNA of the *Aureobasidium* sp. originally reported to produce icosalide A1 with various *Burkholderia* species. (A) Structures of icosalides A1 (1), A2 (2) and B (3). (B) Schematic representation of the ~500 kDa NRPS encoded by the ~15 kb *icoA* gene. Domain abbreviations are as follows: C, condensation; A, adenylation; PCP, peptidyl carrier protein; TE, thioesterase. (C) Extracted ion chromatogram at  $m/z$  713.46 ± 0.02, corresponding to  $[M + H]^+$  for icosalide A1, from LC-MS analyses of extracts of agar-grown cultures of *B. gladioli* BCC0238 (top) and *B. gladioli* BCC0238  $\Omega$ icoA, in which the NRPS-encoding gene has been disrupted (bottom). (D) Phylogenetic comparison of the *recA* sequence amplified from the total DNA extract of *Aureobasidium* sp. MSX 59166 (labelled 'icosalide producer') with *recA* sequences from 24 representative *Burkholderia* species, showing that the fungus-associated bacterium clades with *Burkholderia gladioli*. The scale bar indicates the number of substitutions per site.

corresponding region of the *B. gladioli* BCC0238 genome showed a high degree of synteny (Fig. S9†), confirming that the *Aureobasidium* sp. harbours a *B. gladioli* strain containing an *icoA* orthologue. Although a Gram-negative bacterium could be isolated from the *Aureobasidium* sp., it quickly lost viability and was not identifiable.

Inspection of the structure of icosalide A1 (1) suggests it is assembled *via* initial formation of 3-hydroxyoctanoyl-D-leucinyl-L-serinyl and 3-hydroxydecanoyl-L-serinyl-L-leucinyl thioesters, which are joined *via* a pair of ester linkages to form the lipopeptidolide. To gain insight into the role played by each of the five C domains of IcoA in the biosynthesis of icosalide A1 (1), we carried out phylogenetic analyses.<sup>23</sup> These indicated that the

first and fourth domains group with chain initiating ( $C_I$ ) domains, which are typically located at the N-terminus of the first module in NRPSs responsible for lipopeptide biosynthesis and initiate chain assembly *via* N-acylation of the amino acyl thioester bound to the downstream PCP domain with a fatty acyl thioester. The third and fifth C domains of IcoA belong to the  $C_L$  group, which catalyse formation of a peptide bond between L-configured amino acyl thioesters attached to the upstream and downstream PCP domains. In contrast, the second C domain of IcoA clusters with bifunctional epimerisation-condensation ( $C_E$ ) domains, which catalyse epimerisation of the  $\alpha$ -carbon in the aminoacyl thioester attached to the upstream PCP domain, followed by condensation of the resulting D-



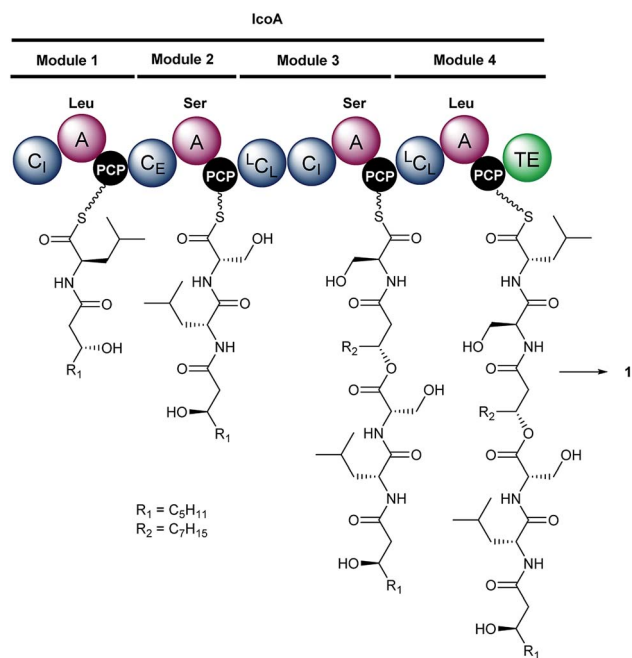


Fig. 2 Proposed mechanism for assembly of icosalide A1 (**1**) by IcoA. Domain and module organisation of the NRPS, showing the PCP-bound thioester intermediates proposed to be formed by each module. The  $C_1$  domains initiate lipopeptide chain assembly by *N*-acylating the aminoacyl thioesters attached to the downstream PCP domains with 3-hydroxyacyl thioester intermediates in fatty acid biosynthesis. Abbreviations are as follows: A, adenylation domain;  $C_1$ , chain initiating condensation domain;  $C_E$ , bifunctional epimerisation-condensation domain;  ${}^L C_L$ , condensation domain that catalyses condensation of *L*-configured aminoacyl donor and acceptor substrates; PCP, peptidyl carrier protein domain; TE, thioesterase domain. The residues used to predict the substrate specificity of A-domains are shown in Table S3†.

configured intermediate with an *L*-aminoacyl thioester attached to the downstream PCP domain. These C domain functional assignments led us to propose that modules 1 and 2 of IcoA are responsible for assembly of the 3-hydroxyoctanoyl-*D*-leucyl-*L*-serinyl thioester intermediate in icosalide A1 (**1**) biosynthesis (Fig. 2). The  $C_1$  domain appended to the N-terminus of module 1 initiates chain assembly *via* *N*-acylation of the *L*-leucyl thioester attached to the module 1 PCP domain with a 3-hydroxyoctanoyl thioester, likely donated by the primary metabolic fatty acid synthase (FAS). The C-domain in module 2 then catalyses epimerisation of the resulting *N*-acyl-leucyl thioester, followed by condensation with the *L*-serinyl thioester attached to the module 2 PCP domain. Module 3 of IcoA is very unusual, because it contains an  ${}^L C_L$  domain directly followed by a  $C_1$  domain. We hypothesize that the  $C_1$  domain in this module catalyses a second chain initiation event, resulting in *N*-acylation of the *L*-serinyl thioester attached to the downstream PCP domain with a 3-hydroxydecanoyl group, again likely provided by the primary metabolic FAS. The  ${}^L C_L$  domain then catalyses acylation of the hydroxyl group in the resulting 3-hydroxydecanoyl-*L*-serinyl thioester with the 3-hydroxyoctanoyl-*D*-leucyl-*L*-serinyl thioester attached to the module 2 PCP domain.

Elongation of the resulting intermediate with the *L*-leucyl thioester attached to the PCP domain in module 4, catalysed by the upstream  ${}^L C_L$  domain, followed by lactonisation catalysed by the downstream TE domain, yields icosalide A1 (Fig. 2).

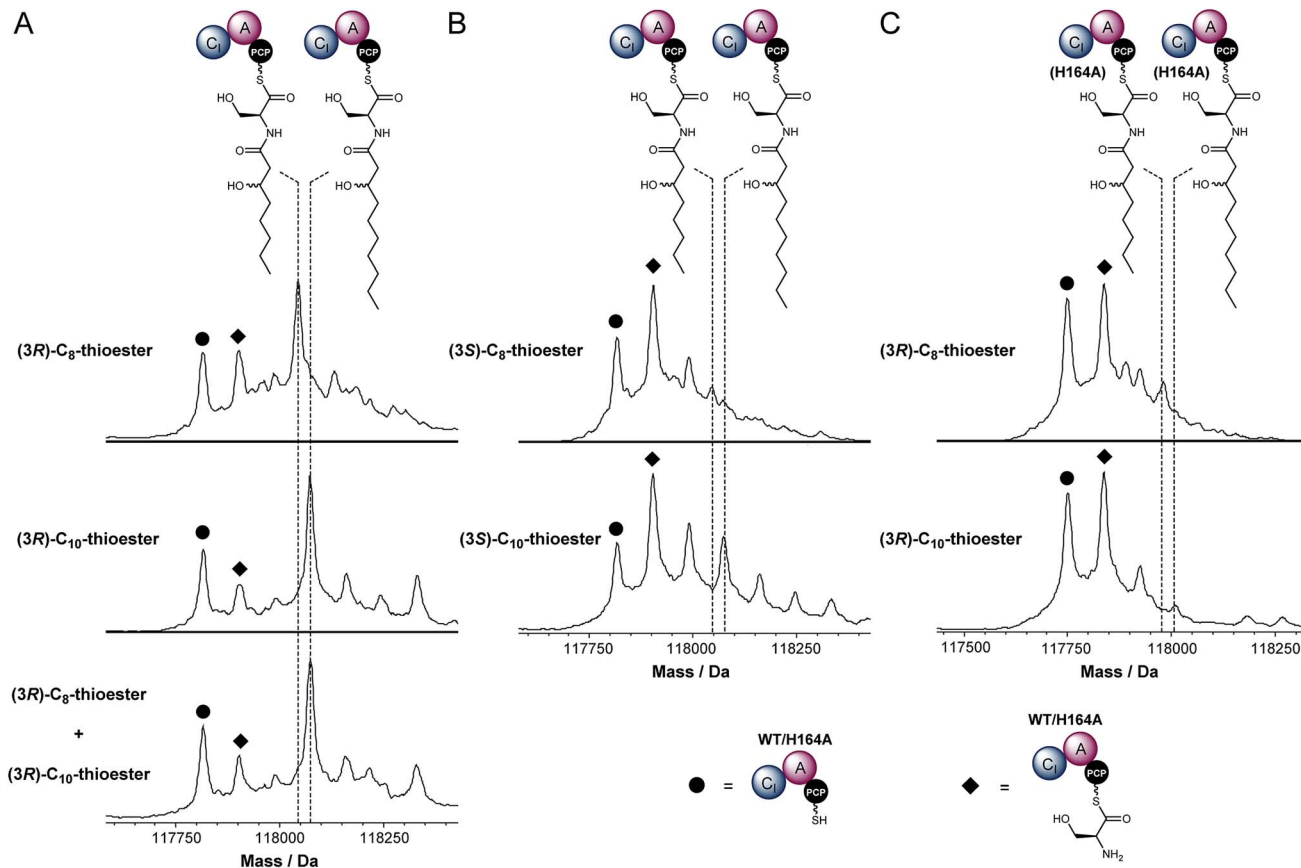
To the best of our knowledge, initiation of chain assembly by an embedded  $C_1$  domain like the one found in module 3 of IcoA is without precedent in NRPS enzymology. We thus sought to experimentally validate the proposed chain initiation reaction *in vitro* by overproducing the  $C_1$ -A-PCP tri-domain from module 3 of IcoA in *E. coli*. The resulting N-terminal His<sub>6</sub> fusion protein, was purified to homogeneity using immobilised metal-ion affinity chromatography and the hexahistidine tag was cleaved using thrombin (Fig. S10†). ESI-Q-TOF-MS analysis showed that the PCP domain of this protein had been partially converted to the *holo* form by an *E. coli* phosphopantetheinyl transferase (Fig. S10†). To fully convert the PCP domain into the *holo* form we incubated the protein with purified recombinant Sfp, a substrate tolerant phosphopantetheinyl transferase from *B. subtilis*,<sup>24</sup> and coenzyme A.

The *holo*- $C_1$ -A-PCP tri-domain was incubated with ATP, *L*-Ser and 3*R*-configured 3-hydroxyoctanoyl or 3-hydroxydecanoyl-pantetheine thioesters, which mimic the (3*R*)-3-hydroxyoctanoyl and (3*R*)-3-hydroxydecanoyl-ACP intermediates in fatty acid biosynthesis proposed to be intercepted by the  $C_1$  domains in modules 1 and 3, respectively, of the NRPS. UHPLC-ESI-Q-TOF-MS analysis of the  $C_1$ -A-PCP tri-domain showed mass shifts consistent with the formation of serinyl, 3-hydroxyoctanoyl-serinyl and 3-hydroxydecanoyl-serinyl PCP-thioesters (Fig. 3). When the  $C_1$ -A-PCP tri-domain was replaced with a mutant containing an Ala residue in place of the active site His residue in the  $C_1$  domain, only trace amounts of products were formed (Fig. 3).

Icosalide A1 contains an *N*-((3*R*)-3-hydroxydecanoyl)-*L*-serine residue.<sup>19</sup> The module 3  $C_1$  domain is thus expected to accept 3-hydroxydecanoyl thioesters in preference to 3-hydroxyoctanoyl thioesters. To test this hypothesis, we incubated the *holo*- $C_1$ -A-PCP tri-domain with ATP, *L*-Ser and equimolar mixture of the 3*R*-configured 3-hydroxyoctanoyl and 3-hydroxydecanoyl-pantetheine thioesters. UHPLC-ESI-Q-TOF-MS analysis showed that only the product resulting from condensation with the 10-carbon thioester is formed (Fig. 3). The incorporation of 3-hydroxyoctanoyl-*L*-serinyl-*L*-leucine into icosalide A2 (**2**), the major icosalide produced by the MSX 74159 fungus, suggests that the NRPS responsible for assembly of this metabolite contains an embedded  $C_1$  domain with a preference for 8-carbon thioesters. It would therefore be interesting to investigate the mechanisms underlying icosalide A2 biosynthesis.

The module 3  $C_1$  domain might also be expected to prefer *R*-configured 3-hydroxyacyl thioesters to their *S*-configured counterparts. (Note, however, that the ketoreductase in bacterial fatty acid biosynthesis produces exclusively 3*R*-configured 3-hydroxyacyl-ACP thioester intermediates.<sup>25</sup> There may therefore be no selective pressure to drive the evolution of stereoselectivity in  $C_1$  domains.) To investigate whether the module 3  $C_1$  domain is stereoselective, we incubated the *holo*- $C_1$ -A-PCP tri-domain with ATP, *L*-Ser and (3*S*)-3-hydroxyoctanoyl or (3*S*)-3-hydroxydecanoyl-pantetheine thioesters. Although the





**Fig. 3** *In vitro* reconstitution of aminoacyl thioester formation and *N*-acylation by the  $C_1$ -A-PCP tri-domain from module 3 of IcoA. Deconvoluted mass spectra of IcoA module 3  $C_1$ -A-PCP tri-domain. (A) Resulting from incubation with ATP, L-Ser and (3*R*)-3-hydroxyoctanoyl (top), (3*R*)-3-hydroxydecanoyl (middle), or (3*R*)-3-hydroxyoctanoyl and (3*R*)-3-hydroxydecanoyl (bottom) pantetheine thioesters. (B) Following incubation with ATP, L-Ser and (3*S*)-3-hydroxyoctanoyl (top), or (3*S*)-3-hydroxydecanoyl (bottom) pantetheine thioesters. (C) Resulting from incubation of the H164A mutant with ATP, L-Ser and (3*R*)-3-hydroxyoctanoyl (top), or (3*R*)-3-hydroxydecanoyl (bottom) pantetheine thioesters. Dashed lines indicate the peaks corresponding to each condensed species, and the *holo*- and L-Ser-loaded species are labelled as indicated in the bottom right corner. Loading of L-Ser onto the PCP domain results in an 87 Da mass increase. For the wild type IcoA  $C_1$ -A-PCP tri-domain, *N*-acylation of the serinyl-PCP thioester with the (3*R*)-3-hydroxyoctanoyl and (3*R*)-3-hydroxydecanoyl thioesters resulted in additional 142 and 170 Da mass increases, respectively. Analogous mass increases were observed when the (3*S*)-3-hydroxyoctanoyl and (3*S*)-3-hydroxydecanoyl thioesters were used, but the levels of product formation were lower, indicating that the  $C_1$  domain prefers *R*-configured 3-hydroxyacyl thioesters. A small amount of product formation was observed when the H164A mutant of the IcoA  $C_1$ -A-PCP tri-domain was used in place of the wild type protein, due to uncatalysed *N*-acylation of the serinyl thioester with the (3*R*)-3-hydroxyoctanoyl and (3*R*)-3-hydroxydecanoyl thioesters.

reaction with the 10-carbon thioester resulted in a significant degree of product formation, it was clearly less efficient than the reaction with the corresponding 3*R*-configured thioester (Fig. 3). In contrast, only trace amounts of product were formed in the reaction employing the eight-carbon thioester (Fig. 3). Thus, the  $C_1$  domain appears to possess a moderate to high degree of stereoselectivity, depending on the carbon chain length of the substrate. Overall, our data show that the module 3  $C_1$  domain of IcoA plays a key role in initiating the assembly of the (3*R*)-3-hydroxydecanoyl-L-serinyl-L-leucinyl moiety of icosalide A1.

## Conclusions

We have shown that the “fungal” antibiotic icosalide A1 (**1**), originally isolated from an *Aureobasidium* sp., is produced by a *B. gladioli* strain closely associated with the fungus. In addition, we have shown that *B. gladioli* isolates from a range of sources,

including CF lung infections, mushroom rot and insects also produce this metabolite. Comparative genome sequence analysis coupled with genetic manipulation allowed identification of a gene encoding the NRPS responsible for icosalide A1 biosynthesis. The third module of this NRPS has a very unusual architecture, containing a putative chain-initiating  $C_1$  domain in addition to a chain elongating  $^1C_L$  domain. Intact protein mass spectrometry showed that this  $C_1$  domain catalyses selective *N*-acylation of a serinyl thioester bound to the module 3 PCP domain with a (3*R*)-3-hydroxydecanoyl thioester. Thus, the asymmetric lipopeptidolide core of icosalide A1 (**1**) is assembled by a unique mechanism involving two distinct chain initiation events on a single NRPS subunit.

## Conflicts of interest

There are no conflicts to declare.



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