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Type III Polyketide Synthase β -Ketoacyl-ACP Starter Unit and Ethylmalonyl-CoA Extender Unit Selectivity Discovered by *Streptomyces coelicolor* Genome Mining

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Polyketide synthases (PKSs) catalyze key carbon–carbon bondforming reactions in the biosynthesis of a diverse range of complex polyketide natural products, including many clinically useful antibacterial, anti-cancer, immunosuppressant, and anti-fungal compounds. 1 These reactions all involve the concomitant decarboxylation and condensation of a malonyl thioester with an acyl thioester and occur via a similar catalytic mechanism. 1 PKSs have been classified into different types according to their sequence and primary structure, as well as the nature of the extender units they utilize in chain assembly. 1 Type III PKSs occur commonly in plants, where they typically catalyze the iterative elongation of diverse acyl-CoA starter units with malonyl-CoA to form chalcone and stilbene products. 2 Recently, genes encoding type III PKSs have been identified in bacterial genomes. 2 These PKSs have been shown to typically catalyze the iterative decarboxylation and condensation of several malonyl-CoA molecules to form a poly- β -ketomethylene intermediate. This intermediate undergoes subsequent decarboxylation coupled with cyclization via Claisen and/or aldol reactions, followed by dehydration to give aromatic products such as 1,3,6,8-tetrahydroxynaphthalene, phloroglucinol, or 3,5-dihydroxyphenylacetyl coenzyme A, which is a precursor of the vancomycin non-proteinogenic amino acid residue 3,5-dihydroxyphenylglycine. 3,4 Analysis of the *Streptomyces coelicolor* A3(2) genome sequence has uncovered three open reading frames (ORFs) encoding potential type III PKSs, two of which are not associated with the production of known *S. coelicolor* metabolites. 5 Here we report that one of these PKSs (Gcs), encoded by the *sco7221* ORF, is required for key reactions in germicidin biosynthesis.

The sequence between the start and stop codons of *sco7221* was replaced on the chromosome of *S. coelicolor* M145 by an 81 bp in-frame “scar” sequence using a recently developed PCR-targeting method. 6 Cultures of the M145 strain and the Δ *sco7221* mutant were grown for 5 days in supplemented minimal medium (SMM). Comparative metabolic profiling of the culture supernatants of the wild type and the Δ *sco7221* mutant using LC-ESIMS identified two compounds with mass 196 and three compounds with mass 182 that were present in the wild type but lacking in the mutant (Figure 1). Complementation of the

Δ *sco7221* mutant with the plasmid pLS1 containing *sco7221* under the control of the *ermE** promoter restored production of these metabolites. High-resolution ESI-TOF-MS analysis gave $C_{11}H_{16}O_3$ and $C_{10}H_{14}O_3$ as the molecular formulas of the compounds of mass 196 and 182, respectively. A neutral loss of 44 mass units was observed in the negative-ion ESI-MS/MS spectra, indicating the presence of a pyran-2-one in each of the compounds. The compounds were purified by semi-preparative HPLC from culture supernatants of *S. coelicolor* M145. 1H , COSY, HMQC, HMBC, and NOE NMR analyses of the compounds of mass 196 identified them as germicidin A (**1**), previously isolated from *Streptomyces viridochromogenes* NRRL B-1551,7 and its novel isomer isogermicidin A (**2**). Similar analyses of the compounds with mass 182 indicated that they were germicidin B7 (**3**) and the new metabolites isogermicidin B (**4**) and germicidin C (**5**).

Inspection of the structures of **1–5** suggests that they may be assembled from 2-methylbutyryl-CoA, isovaleryl-CoA, isobutyryl-CoA, or butyryl-CoA starter units by elongation with a malonyl-CoA or malonyl-ACP extender unit, followed by elongation with an ethylmalonyl-CoA or methylmalonyl-CoA extender unit. To examine the metabolic origin of the ethyl group attached to C-3 of **1–4**, we fed $[^2H_7]$ butyric acid to cultures of *S. coelicolor* M145 grown in SMM. LC-ESIMS analyses of the culture supernatants revealed new compounds with the same retention time as **1** and **2** with $m/z = 201$ and 202 , and new compounds with the same retention time as **3** with $m/z = 187$ and 188 , consistent with incorporation of four or five of the deuterium atoms from $[^2H_7]$ -butyric acid into the ethyl groups attached to C-3 of **1**, **2**, and **3**. Similar analyses also revealed two new species with the same retention time as **4** with $m/z = 194$ and 195 , consistent with the incorporation of four or five of the deuterium atoms from $[^2H_7]$ -butyric acid into the C-3 ethyl group and incorporation of all seven of the deuterium atoms of $[^2H_7]$ butyric acid into the C-6 *n*-propyl group of **4**. These results indicate that butyric acid is incorporated intact into C-2, C-3, and the C-3 ethyl group of **1–4**, consistent with the proposal that the second condensation reaction in the biosynthesis of these metabolites utilizes ethylmalonyl-CoA as the extender unit. They also indicate that butyric acid is incorporated intact into C-6 and the *n*-propyl group attached to C-6 of **4**, consistent with the proposal that butyryl-CoA is the starter unit for biosynthesis of this compound. To investigate the role of 2-methylbutyryl-CoA as the starter unit for biosynthesis of **1**, we fed $[U-^{13}C]$ isoleucine to *S. coelicolor* M145. LC-ESIMS analysis of the culture supernatant identified a compound with the same retention time as **1** and $m/z = 202$, consistent with intact incorporation of five contiguous carbon atoms of $[U-^{13}C]$ isoleucine into C-6 and the 2-methylpropyl group attached to C-6 of **1**, indicating that 2-methylbutyryl-CoA is the starter unit for biosynthesis of **1**.

These results suggest two alternative models for the biosynthesis of **1–5** (Scheme 1). In the first model, the acyl-CoA starter unit is transacylated onto the active-site cysteine residue of Gcs, which subsequently catalyzes decarboxylation of malonyl-CoA and concomitant condensation with the starter unit to form the corresponding β -ketoacyl thioester **6** ($X = SCoA$). This thioester transacylates back onto the active-site cysteine residue of Gcs, which then catalyzes decarboxylation of ethyl- or methylmalonyl-CoA and concomitant condensation with the covalently bound β -keto thioester to give the corresponding β,δ -diketo thioester **7** ($X = SCoA$). Finally, Gcs catalyzes the cyclization of **7** to give **1–5**. In the second model, the β,δ -diketo thioester **7** ($X = SCoA$) is formed via transacylation of δ -ketoacyl thioester **6** ($X = SACP$) onto the active-site cysteine residue of Gcs. Decarboxylation

of ethyl- or methylmalonyl-CoA and concomitant condensation with **6** gives **7**, which undergoes the same cyclization reaction as in the first model to give the natural products. **6** (X = SACP) is a known intermediate in fatty acid biosynthesis in *Streptomyces* species that is formed by FabH- or FabF-catalyzed decarboxylation of malonyl-ACP and concomitant condensation with 2-methylbutyryl-, isovaleryl-, isobutyryl-CoA, or *n*-butyryl-ACP.⁸

To discriminate between these two models, we examined germicidin production in a recently reported strain of *S. coelicolor* M511 (a derivative of the M145 strain unable to produce actinorhodin) in which the *fabH* gene has been replaced by the orthologue from *Escherichia coli*.⁹ This strain produces predominantly straightchain fatty acids because the *E. coli* FabH is highly selective for acetyl-CoA as a starter unit.⁹ LC-ESIMS/MS analysis of culture supernatants from this strain grown in SMM showed that production of **1**, **2**, and **5** is abrogated, whereas small quantities (~5% relative to wild type) of **3** and **4** still appear to be produced, consistent with the second model for germicidin biosynthesis presented above. To further examine the role of Gcs in germicidin biosynthesis, we transformed *Streptomyces venezuelae* ISP5230 with the plasmid pLS1. The complete genome sequence of *S. venezuelae* does not contain any orthologues of *sco7221* (M. J. Bibb, personal communication), and LC-ESIMS analysis of culture supernatants of this organism grown in SMM indicated that it does not produce germicidins. LC-ESIMS/MS analysis of culture supernatants of *S. venezuelae*/pLS1 grown in SMM showed that it produces **1–5**, consistent with the hypothesis that only Gcs is required, in addition to enzymes of primary metabolism, for germicidin biosynthesis.

In conclusion, we have identified a novel bacterial type III PKS required for germicidin assembly that appears to catalyze elongation of specific β -ketoacyl-ACP thioester intermediates in fatty acid biosynthesis with ethyl- or methylmalonyl-CoA and subsequent cyclization of the resulting triketide. In vivo utilization of either ACP-tethered starter units or ethylmalonyl-CoA as an extender unit is unprecedented for any type III PKS.² Modular type I PKSs are known to incorporate ethylmalonyl-CoA,¹⁰ but only one other type III PKS has been proposed to utilize an extender unit other than malonyl CoA for the biosynthesis of a natural product. PstrCHS2, a plant type III PKS, is thought to incorporate methylmalonyl-CoA in a modular fashion during the poorly understood biosynthesis of C-methylated chalcones in the plant *Pinus strobus*.¹¹

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. LC-ESIMS extracted ion chromatograms at $m/z = 197$ and 183 for organic extracts of *S. coelicolor* M145 (blue and red, respectively) and the *sco7221::scar* mutant (black and purple, respectively).

Scheme 1.