

A Branched Extender Unit Shared between Two Orthogonal Polyketide Pathways in an Endophyte**

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The large polyketide family of natural products impressively demonstrates how nature generates a wealth of diverse and complex compounds from simple building blocks.^[1] During the past two decades, a large body of knowledge on the various types of polyketide synthases (PKS) and their modes of action has been gained.^[1–3] In almost all cases, the polyketide backbone is assembled exclusively from malonyl-(MCoA) and methylmalonyl-CoA (mMCoA) units, and structural diversity is primarily governed through variations in chain length, processing of the β -keto group, versatile cyclizations, and numerous post-PKS tailoring reactions.^[1,4,5] While extender units other than MCoA and mMCoA are scarce, several complex polyketides in part composed of substituted malonyl building blocks have recently been discovered.^[6] Furthermore, it is known that modular polyketide synthases employ acyltransferase (AT) domains for the selection of the correct extender unit, and elegant studies have demonstrated that it is possible to graft alternative building blocks into complex polyketide backbones through domain swapping, mutagenesis, and mutasynthesis.^[7–10] Thus, with the aim to increase natural polyketide diversity, much effort is currently devoted to enlarging the repertoire of possible biosynthetic building blocks. Apart from ethylmalonyl-CoA^[11] and the recently discovered methoxymalonyl^[12] and hydroxy- and aminomalonyl^[13] building blocks, there is evidence for the utilization of longer alkyl residues that account for the propenyl and hexyl side chains of FK506^[14–16] and thuggacin,^[17] respectively. Although isotope labeling experiments have pointed to an α -methylbutyryl-derived extender in the polyoxypeptin pathway,^[18] to date the molecular basis for the formation of branched-chain malonyl units has remained fully unknown. Herein we report the discovery and biosynthetic origin of an unprecedented

isobutylmalonyl (ibMCoA) extender unit, which is surprisingly employed in two unrelated polyketide pathways in an endophytic *Streptomyces* species.

In the course of evaluating the metabolic capabilities of mangrove endophytes,^[19] we have discovered a set of unusual ansa macrolides, named divergolides, which differ markedly in their overall architecture but share an unprecedented branched side chain.^[20] This unusual residue suggested that the polyketide backbone has incorporated a novel type of branched extender unit that is likely derived from isobutyrate. Initial attempts to support this hypothesis through stable-isotope labeling failed because of the minute amounts of divergolides produced. We eventually succeeded in feeding experiments with [D₇]isobutyrate using a highly sensitive HPLC–HRMS (orbitrap) setup. In this way, we identified the expected mass shifts for the major components of the divergolide complex (e.g., divergolide C, **1**), thus providing evidence that the extender unit originates from the branched isobutyrate precursor (Figure 1).^[21]

Surprisingly, a closer inspection of the endophytes' metabolome in the presence or absence of [D₇]isobutyrate

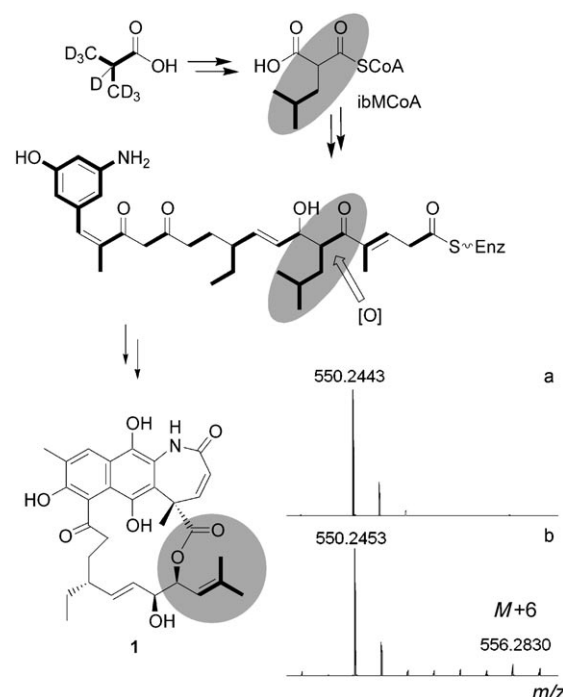


Figure 1. Stable-isotope labeling experiment showing that the branched side chain of divergolide C (**1**) and the putative linear precursor originates from isobutyrate. HRMS of a) **1** and b) **1** enriched with two CD₃ groups after feeding with [D₇]isobutyrate.

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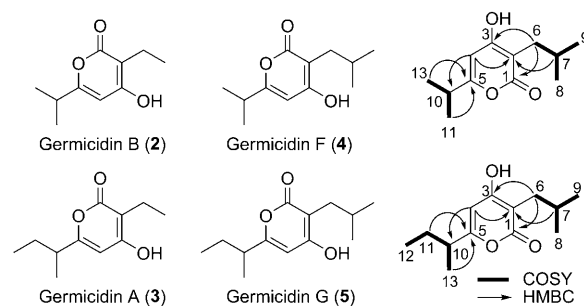
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revealed that the divergolides are not the only polyketides originating from this building block. To firmly elucidate the structures of these novel compounds, the ethyl acetate extract from an upscaled (200 L) fermentation of *Streptomyces* sp. HKI0576 was fractionated through open column chromatography and reversed-phase HPLC, yielding two new pyrone compounds, **4** and **5**, along with the known germicidins A (**3**) and B (**2**; Scheme 1).^[22] The structures of the two new metabolites were elucidated by HRMS and 1D and 2D NMR spectroscopy. HRMS (m/z 225.1489, $[M+H]^+$) and ^{13}C NMR spectroscopy data provided the molecular formula for **5** ($\text{C}_{13}\text{H}_{20}\text{O}_3$). The ^1H NMR spectrum showed signals for one olefinic proton ($\delta = 5.89$ ppm, H-4) and four methyl groups (three doublets: $\delta = 0.86$ ppm, H-8; $\delta = 0.86$ ppm, H-9; $\delta = 1.13$ ppm, H-13 and one triplet: $\delta = 0.82$ ppm, H-12). H,H COSY correlations established the partial structures for isobutyl and *sec*-butyl groups. ^{13}C NMR spectroscopy data also revealed a pyrone ring ($\delta = 167.6, 167.0, 166.5, 102.3, 99.3$ ppm), and HMBC correlations from H-6 to C-1 and C-3 and from H-11 and H-13 to C-5 (Scheme 1) eventually established the connections between the fragments to provide the structure of germicidin G (**5**). For compound **4**, a molecular formula of $\text{C}_{12}\text{H}_{18}\text{O}_3$ (m/z 211.1314, $[M+H]^+$) was found, suggesting that this metabolite could have one methyl group less than **3**. Indeed, diagnostic signals for the pyrone carbon atoms were conserved in the ^{13}C NMR spectrum, and the presence of isopropyl and isobutyl residues was deduced from the H,H COSY spectrum. Finally, the HMBC spectrum confirmed the connections between the fragments of germicidin F (**4**). The bioactivities of the germicidin variants were compared. Interestingly, **2**, **4**, and **5** showed significantly lower germination inhibition effects compared to germicidin A (**3**). However, the new germicidin G (**5**) showed potent antibacterial activities against pseudomonads, mycobacteria, and even multiresistant staphylococci and enterococci, thus contributing to the endophyte's antibiotic complex.

Both novel germicidins show unusual isobutyl residues, and through stable-isotope labeling using $[\text{D}_8]$ valine and $[\text{D}_7]$ isobutyrate, we confirmed that these side chains are derived from valine and isobutyrate, respectively (Figure 2). Considering that isobutyl-malonyl building blocks have been unprecedented for polyketides, the co-occurrence of two different classes of polyketide metabolites with branched side chains in a single microbial strain is intriguing. To shed light on the biogenetic origin of extender-unit biosynthesis, we subjected genomic DNA of the producer strain to shotgun/454 sequencing and analyzed contiguous sequences. A candi-



Scheme 1. Structures of germicidins isolated from the endophyte and schematic depictions of correlation data obtained from 2D NMR spectroscopy.

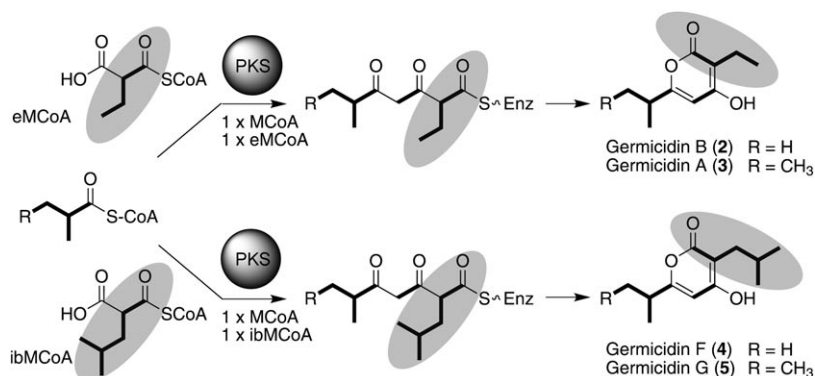
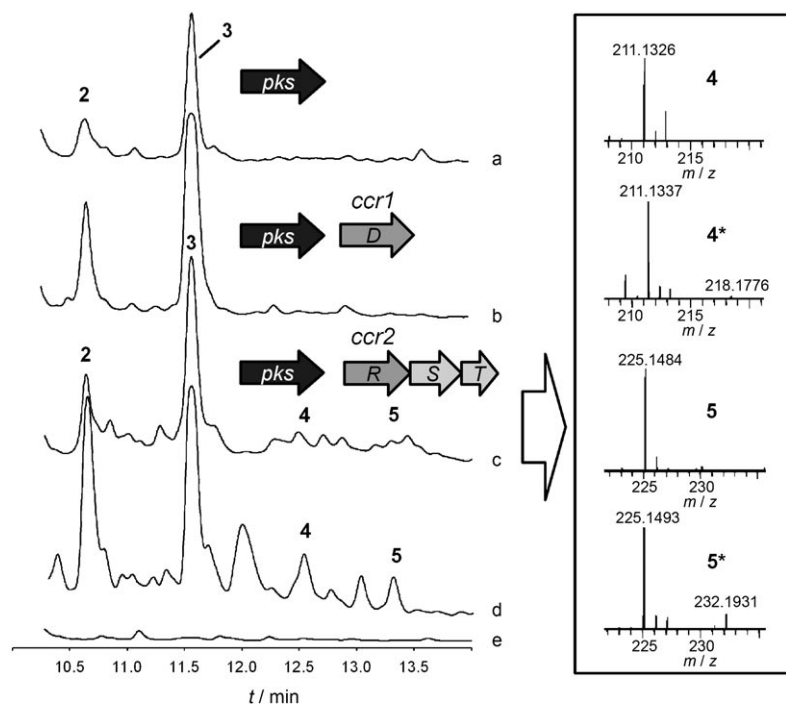
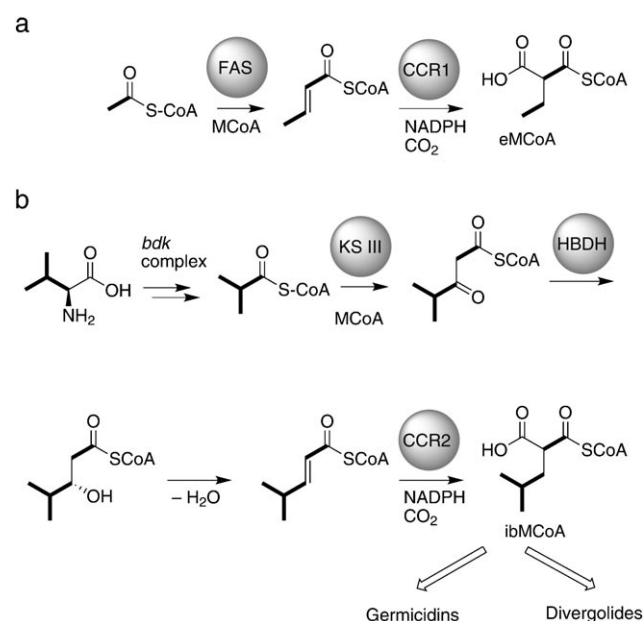


Figure 2. Top: HPLC–HRMS profiles of extracts from a) *S. albus*/pXU409 expressing the type III PKS gene; b) *S. albus*/pXU410 co-expressing the CCR1 gene for eMCoA formation; c) *S. albus*/pXU416 co-expressing the operon for ibMCoA biosynthesis; d) wild type; e) *S. albus* (negative control). HRMS for **4**, **5**, and isotopically enriched variants (**4*** and **5***) resulting from the incorporation of $[\text{D}_7]$ isobutyrate (similar results from $[\text{D}_8]$ valine). Bottom: Model for the biosynthesis of germicidins employing ethylmalonyl-CoA (eMCoA) and isobutylmalonyl-CoA (ibMCoA) extender units, respectively. MCoA: malonyl-CoA.

date gene (*pks*) coding for the germicidin synthase was identified by blasting the genome sequence against the *Streptomyces coelicolor* germicidin type III PKS gene (*sco7221*).^[23] Yet, no apparent extender-unit biosynthesis genes could be found immediately upstream or downstream of *pks*. To verify the function of the type III PKS gene, *pks* was cloned and heterologously expressed in *Streptomyces albus*. HPLC–HRMS analysis indicated that the resulting strain (*S. albus*/pXU409) gained the ability to produce the known germicidins A (**3**) and B (**2**), but not the branched derivatives (**4** and **5**). Consequently, the heterologous host is incapable of providing the unusual extender unit (Figure 2a). In the endophytic *Streptomyces* sp., the biosynthesis of the isobutylmalonyl-CoA building block could involve a crotonyl-CoA reductase/carboxylase (CCR), which was first characterized in the ethylmalonyl-CoA pathway.^[11,24] Indeed, through further genome analyses we discovered two copies of CCR genes, *ccr1* and *ccr2*, that are embedded in a large type I PKS gene cluster. Since this PKS gene locus also harbors genes for the biosynthesis of the 3,5-aminohydroxybenzoate (AHBA) ansamycin PKS starter unit,^[25,26] we reasoned that this gene cluster codes for the biosynthesis of the divergolides. As all attempts to introduce knock-out constructs into the endophyte strain using currently available protocols failed, we focused on functional studies by heterologous expression. Initially, we performed bioinformatic and phylogenetic analyses to predict the functions of the two CCR homologues. The deduced CCR1 (DivD) sequence has high sequence similarity (89%) to IdmF from the indanomycin pathway, where it was implicated in the formation of eMCoA.^[27] Thus, it appeared plausible that CCR1 provides the eMCoA extender required for divergolide backbone assembly. To confirm this hypothesis, *ccr1* was cloned and co-expressed with the germicidin synthase gene in *S. albus*. The production of germicidins in the expression strain (*S. albus*/pXU410) was monitored by HPLC–HRMS, showing that the titers of **2** and **3** were increased about two- to threefold, thus assigning the role of CCR1 in eMCoA biosynthesis (Figure 2b).

CCR2 (DivR) would be the best candidate for ibMCoA formation: In the phylogenetic tree, CCR1 and CCR2 fall into different clades, and CCR2 is closely related to SalG, which mediates the formation of chloroethylmalonyl-CoA in the salinosporamide A pathway.^[28] Moreover, *ccr2* is a component of a three-gene operon (*divRST/ccr2-ksIII-hbdh*), which could be responsible for the entire biosynthesis of the novel ibMCoA extender unit. The deduced ketoacyl-synthase (KS III) is related to FabH-like 3-oxoacyl-ACP synthases from fatty acid biosynthesis and non-acetate PKS starter unit pathways.^[25] However, the *div* KS III and its homologues from FAS and PKS differ significantly and group into individual clades (see the Supporting Information). While the KS III would be required to elongate the isobutyryl unit, the putative 3-hydroxybutyryl-CoA dehydrogenase (HBDH) would mediate β -keto processing en route to the crotonyl substrate for CCR2. To provide experimental evidence for the proposed function of the operon, the 3.6 kb cassette was PCR-amplified from the genome of *S. sp.* HKI0576 and co-expressed with the type III PKS gene (*pks*) in *S. albus*. Metabolic profiling of *S. albus*/pXU416 showed

that **4** and **5** are produced, thus revealing that the *ccr2-ksIII-hbdh* operon conferred to the host the ability to produce the branched extender unit (Figure 2c). These findings were complemented with successful isotope labeling experiments using [D₈]Val and [D₇]isobutyrate. In sum, the bioinformatic, genetic, and chemical analyses provide new insights into the formation of the unusual branched polyketide extender unit: the isobutyl moiety of ibMCoA originates from Val, which is processed into isobutyryl-CoA by the *bkd* complex. Isobutyryl-CoA is then elongated by a designated KS III and transformed into dimethylcrotonyl-CoA through HBDH-mediated β -keto processing before a specific CCR catalyzes reductive carboxylation (Scheme 2). To our knowledge, this is the first report on the biosynthesis of a branched PKS extender unit.



Scheme 2. Model for the biosynthesis of eMCoA and ibMCoA from acetyl-CoA and isobutyryl-CoA, respectively.

In light of the very rare utilization of non-malonyl-CoA extenders by type III PKS, it is remarkable that the germicidin synthase is capable of accepting the bulky isobutylmalonyl unit in lieu of the ethylmalonyl extender. However, even more intriguing is the observation that two fully unrelated polyketide biosynthetic pathways (a modular type I PKS and a type III PKS) share the same exotic extender unit in a wild-type strain. While it is known that an engineered PKS pathway may recruit fatty acid synthase starter units, for example, in benastatin biosynthesis,^[29] we are not aware of any other example where building blocks are naturally shared between two unrelated polyketide pathways. To the best of our knowledge, such naturally occurring combinatorial biosynthesis is fully unprecedented. In this instance, the utilization of the novel extender unit by the type III PKS even leads to a change in bioactivity, that is, to antibiotic activity. It is well conceivable that related cases of biosynthetic crosstalk may be found more often in secondary metabolism, as this

could contribute to the evolution of metabolic diversity. Finally, from a practical point of view, the novel PKS building block may be used for increasing the versatility of polyketides.

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