Polyketide Biosynthesis

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Enzymatic Polyketide Chain Branching To Give Substituted Lactone, Lactam, and Glutarimide Heterocycles**

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Dedicated to Professor Heinz G. Floss on the occasion of his 80th birthday

Abstract: *Polyketides typically result from head-to-tail condensation of acyl thioesters to produce highly functionalized linear chains. The biosynthesis of the phytotoxin rhizoxin, however, involves a polyketide synthase (PKS) module that introduces a δ-lactone chain branch through Michael addition of a malonyl extender to an α,β-unsaturated intermediate unit. To evaluate the scope of the branching module, polyketide mimics were synthesized and their biotransformation by the reconstituted PKS module from the Rhizopus symbiont Burkholderia rhizoxinica was monitored in vitro. The impact of the type and configuration of the δ-substituents was probed and it was found that amino-substituted surrogates yield the corresponding lactams. A carboxamide analogue was transformed into a glutarimide unit, which can be found in many natural products. Our findings illuminate the biosynthesis of glutarimide-bearing polyketides and also demonstrate the utility of this branching module for synthetic biology.*

Polyketides encompass an impressive range of natural products, many of which are in clinical use. Their structural diversity primarily emerges from simple head-to-tail condensations of activated acyl and malonyl building blocks. The resulting polyketide chains are enzymatically processed and may undergo enzymatic tailoring, for example, cyclization, oxygenation, halogenation, and glycosylation.[1] With respect to the assembly of the carbon backbone, it is remarkable that only a few variations to the unidirectional 1,2-condensation scheme have been observed. However, noncanonical substructures can be crucial for the biological activity of the polyketide, as exemplified by the remarkably potent antimitotic agent rhizoxin (**1**, Figure 1).[2] This phytotoxin is employed in a symbiosis of the plant-pathogenic fungus *Rhizopus microsporus* and its bacterial endosymbiont, *Burkholderia rhizoxinica*.[3] The structure of the macrolide is highly unusual because it features a δ-lactone ring that is tethered to the polyketide backbone. Notably, this chain branch is essential for the binding of rhizoxin to the β-tubulin subunit,[4] through which it efficiently halts cell division in the picomolar range.[3b] Isotope-labeling experiments have showed that the δ-lactone ring integrates an acetyl unit that branches off at a β-position relative to a former acetyl carbonyl in the polyketide chain (Figure 1).[5]

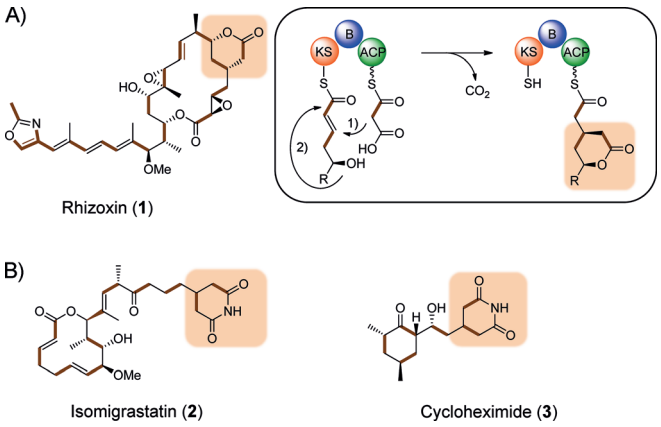

Figure 1. A) The structure of rhizoxin (**1**) and a model for vinylogous chain branching catalyzed by a specialized PKS module; 1) Michael addition, 2) lactonization. B) The structures of isomigrastatin (**2**) and cycloheximide (**3**) with the glutarimide motif highlighted. KS, ketosynthase; B, branching domain; ACP, acyl carrier domain; AT, acyltransferase.

Whereas alkyl substitutions at α-positions result from methylation or the incorporation of substituted malonyl units,[6] branches at β-positions typically involve enzymes that resemble those of the isoprenoid enzymatic machinery.[7] Surprisingly, genes for such components could not be found in the genome of the rhizoxin producer.[8] Instead, the architecture of the rhizoxin (*rhi*) type I polyketide synthase (PKS)[9] and the structures of isolated pathway intermediates[10] have suggested that the chain branch is introduced through a Michael addition. To shed light on the enzyme mechanism, we reconstituted the designated "branching module" of the *rhi* PKS, which consists of ketosynthase (KS), branching (B), and acyl carrier (ACP) domains, and a trans-acting acyl transferase (AT). By means of an in vitro enzyme assay, we successfully transformed a synthetic surrogate of the polyketide intermediate into the corresponding δ-lactone derivative. We thus confirmed that the pharmacophoric side chain

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is introduced through a vinylogous addition of a malonyl unit to an α,β-unsaturated thioester, followed by lactonization (Figure 1).[11] Notably, similar PKS modules are encoded in the biosynthetic gene clusters for glutarimide-bearing natural products such as isomigrastatin (**2**),[12] an important cell migration inhibitor (Figure 1). As in the structurally related antibiotic cycloheximide (**3**), the glutarimide residue is important for the biological activity of these compounds.[13] Although it had been proposed that an enzymatic Michael addition could give rise to the glutarimide structure, the roles of these PKS modules have remained elusive and the proposed mechanism still needs to be experimentally confirmed. Herein, we show that the chain-branching *rhi* PKS module is more versatile than expected. Through an in vitro multienzyme assay with synthetic polyketide mimics, we have demonstrated that the unusual KS–B–ACP module is also capable of forming lactams and even glutarimides.

To test the substrate specificity of the rhizoxin PKS branching module and to explore the scope of potential applications, we synthesized various polyketide analogues for in vitro multienzyme biotransformation experiments. Specifically, a series of δ-functionalized, α,β-unsaturated *N*-acetyl cysteamine thioester (SNAC) derivatives that mimic the activated intermediates were prepared. First, we explored the impact of the configuration of the δ-hydroxy group on the course of the reaction. For this purpose, we synthesized the *R* and *S* enantiomers of the 5-hydroxyhexenoic acid SNAC thioester from the corresponding protected hydroxybutanoates through a reduction–olefination sequence. The stereoisomers **4** and **5** were individually subjected to the in vitro enzyme assay, and the products were analyzed by HPLC–HRMS, with the synthetic δ-lactone as reference (Figure 2). Surprisingly, in both cases formation of the branched product was detected in only one diastereoisomeric form. From retention-time comparisons and deductions from the natural biosynthetic pathway, we inferred that in both cases the *syn*-substituted lactone was formed.

To shed light on the preferences of the KS, we determined the kinetic parameters of the branching reaction. For the *R* enantiomer, we found a K_M value of (2694 ± 627) μM and a v_{max} value of (20.9 ± 2.4) μM min^{-1}. By contrast, using the *S* enantiomer gave a K_M value of (1348 ± 315) μM and a v_{max} value of (2.1 ± 0.3) μM min^{-1}. Binding of the non-natural *R* enantiomer to the KS is thus only lowered twofold, whereas the transformation rate of this substrate is ten times lower compared to the *S* enantiomer. This result indicates that the KS basically does not differentiate between the configurations of the δ-hydroxy groups, but lactone ring formation is drastically reduced when the non-natural isomer is applied. It appears that this sequence of Michael addition and lactonization exclusively yields *syn*-substituted lactones and that the configuration of the hydroxy group determines the stereochemical course of the reaction.

To verify the proposed course of the reaction and to corroborate the idea that non-covalent interactions between the substrate and the malonyl unit define the stereochemical course of the reaction, we synthesized and tested the δ-hydroxy-substituted dihydro surrogate **8**, which cannot undergo a branching reaction because of the missing double bond. Furthermore, a phenol analogue (**10**) was prepared (Figure 3). The activated malonyl units and the rhizoxin polyketide chain mimics were individually added to the enzyme mixture that constitutes the functional PKS module in vitro. The reactions were monitored by MALDI analysis of the ACP with potentially bound products, as well as by high-resolution mass spectrometry (HRMS) of the hydrolyzed products. Unsurprisingly, the saturated alcohol **8** did not undergo any chain branching reaction, thus demonstrating once again that the Michael addition precedes ester bond formation. Furthermore, this experiment showed that the KS does not catalyze Claisen condensation with this substrate when a vinylogous attack is hampered. Changing the aliphatic alcohol to a phenol nucleophile did not give rise to the hypothetical product **11**. These results indicate a high degree of specificity for the KS–B–ACP module.

Next, we explored the possibility of replacing the nucleophilic δ-hydroxy substituent of the polyketide chain. It should be highlighted that this OH group is required for the release of the KS-bound intermediate by lactonization, which allows the propagation of the intermediate. Nonetheless, it is conceivable that other nucleophilic residues could also cleave the thioester bond, which would result in the formation of

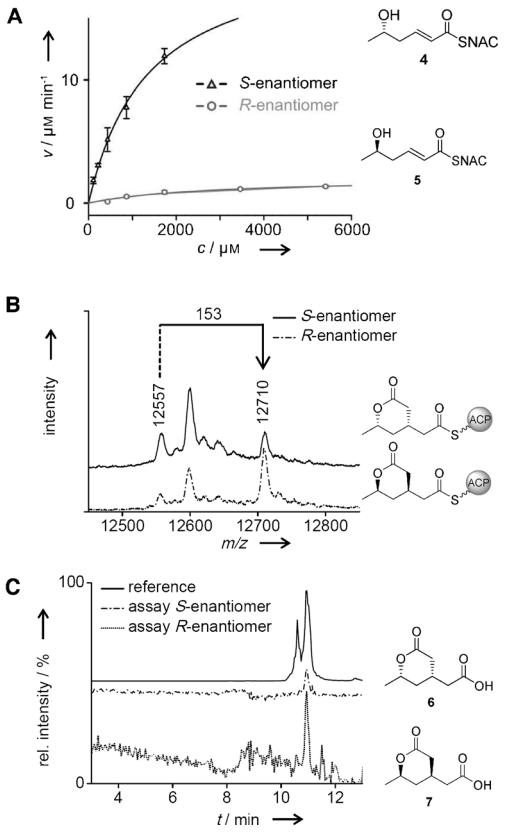

Figure 2. Vinylogous chain branching of pure *R* and *S* enantiomers of the *N*-acetyl cysteamine thioester of 5-hydroxyhexenoic acid, and subsequent lactone formation. A) Michaelis–Menten kinetics of the biotransformation. B) MALDI analysis of ACP-bound products (peak in middle relates to decarboxylated malonylated ACP species). C) SIM-LC–HRMS analysis of hydrolyzed products, and comparison with the racemic synthetic reference. SIM = selected ion monitoring.

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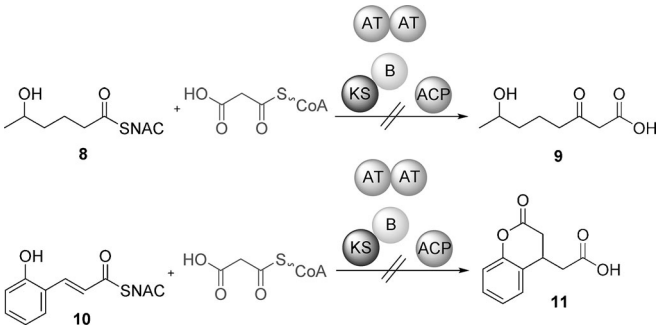

Figure 3. Probing the substrate tolerance of the branching module towards dihydro and phenol variants by using synthetic polyketide surrogates.

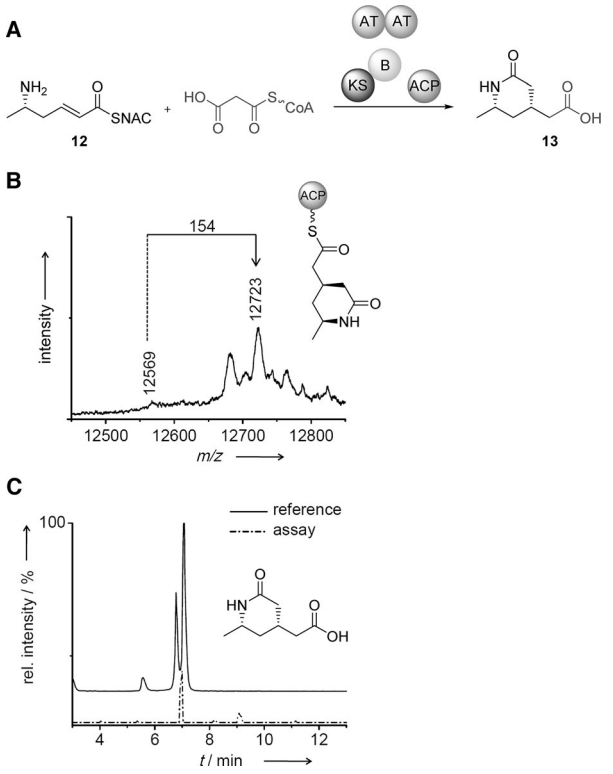

Figure 4. In vitro biotransformation of an amino-substituted polyketide surrogate into a lactam moiety. A) General Scheme of the enzyme assay. B) MALDI analysis of the ACP-bound product. C) SIM-LC–HRMS analysis of the hydrolyzed product and comparison with the synthetic reference (as a mixture of diastereoisomers); only one enantiomer is shown.

alternative heterocyclic systems. If the surrogate was accepted as a substrate for the Michael addition, aminolysis of the thioester would give a lactam ring. To evaluate the effect of an amino group in lieu of the hydroxy group, we synthesized the corresponding δ-amino-substituted surrogate of the natural polyketide precursor. To this end, *N*-Boc-protected ethyl D,L-3-aminobutanoate was reduced with DibalH. The amino-aldehyde was immediately subjected to a Horner–Wadsworth–Emmons (HWE) olefination, and subsequent deprotection gave the desired amine **12** (see the Supporting Information). This synthetic surrogate of the growing polyketide chain was employed in the in vitro assay and product formation was monitored by HRMS. The formation of a new compound with $m/z = 172.0966$ and a molecular formula of $C_8H_{14}O_3N$ $[M+H]^+$ as deduced from LC–HRMS suggested that the desired lactam compound was produced. To confirm the identity of the lactam, we prepared a synthetic reference. In brief, a protected D,L-β-aminoacid ester was subjected to a reductive Horner–Wadsworth–Emmons olefination to obtain the substituted α,β-unsaturated ester. A cesium carbonate catalyzed Michael addition of diethylmalonate followed by hydrolysis yielded the lactam as a diastereoisomeric mixture (see the Supporting Information). Comparison of the high-resolution mass spectra and HPLC retention times of the enzyme products and the synthetic reference confirmed the successful conversion of **12** into the δ-lactam **13** (Figure 4). Furthermore, only the (late-eluting) *syn*-diastereomer is formed, thus confirming that the enzymatic reaction is highly selective, yielding only one diastereoisomer from a mixture of enantiomeric δ-amino-substituted precursors, as has been observed for the δ-lactones.

The successful biotransformation of the amino analogue encouraged an expansion of this approach to carbonyl substituents such as carboxylic acid and amide residues. In principle, a carboxylate could give rise to an anhydride moiety or the respective dicarboxylic acid, whereas the carboxyamide would lead to the glutarimide moiety, which is found in many natural products. Unexpectedly, the synthesis of carboxy- and carboxamide-substituted SNAC derivatives proved to be highly challenging. Despite many attempts, the functionalization of glutaconic acid and HWE olefination were not feasible, possibly owing to the highly reactive α-proton at the allylic position. Finally, we succeeded in preparing the desired polyketide surrogates **14** and **16** (see the Supporting Information) through alkene metathesis of propenyl SNAC with but-3-enoic acid and but-3-enamide, respectively. Addition of the carboxy-substituted SNAC derivative **14** to the enzyme assay resulted in protein precipitation, and no biotransformation took place. To exclude the possibility of a pH effect, we repeated the experiment after accurately adjusting the pH value to 7.0 with Tris buffer. However, neither the respective anhydride **15** nor the hydrolyzed product could be detected.

By contrast, the carboxamide-substituted polyketide surrogate **16** was accepted as a substrate by the branching module. Through analysis of the enzyme mixture, we detected a MALDI signal corresponding to the ACP-bound glutarimide product (Figure 5). Moreover, acidic workup released the expected product with $m/z = 172.0610$ and a molecular formula of $C_7H_{10}O_4N$ (HRMS). The identity of the glutarimide **17** was rigorously confirmed through comparison of the HPLC retention times and high-resolution mass signals of the reaction product and a synthetic reference (Figure 5). A negative control experiment with a heat-inactivated KS–B didomain confirmed that the reaction is indeed enzyme-catalyzed.

Our results unequivocally show that the unusual KS–B–ACP module of the *rhi* PKS is not limited to lactone formation but is also capable of introducing branches that

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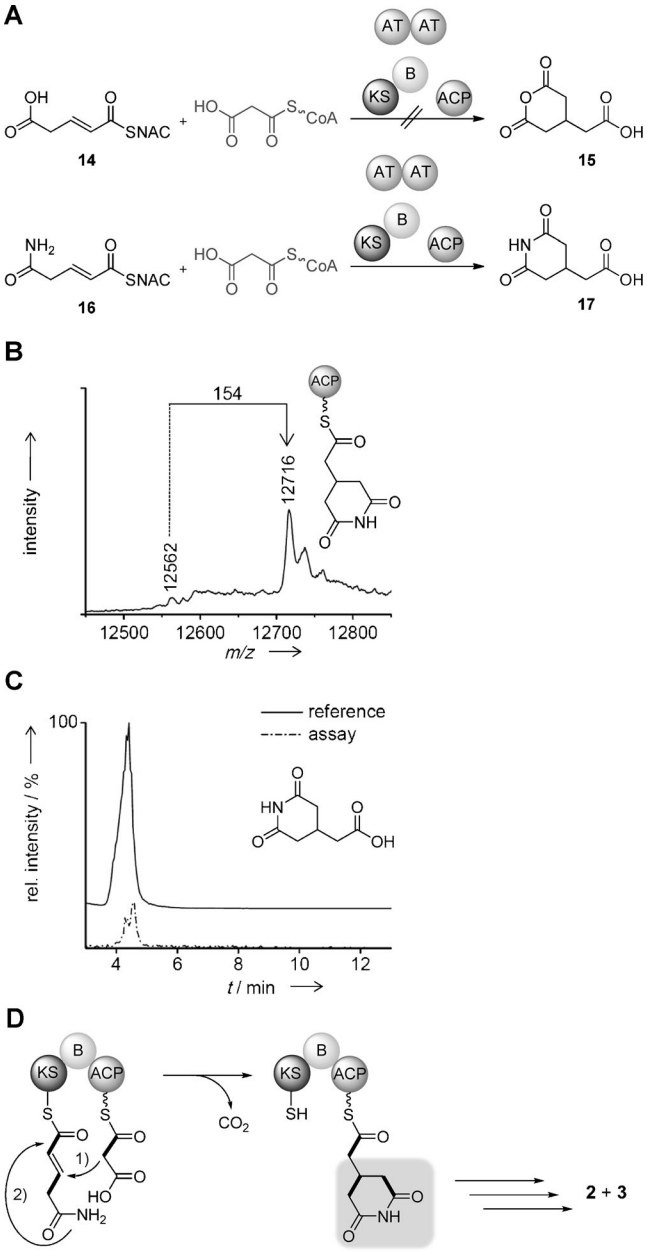

Figure 5. In vitro biotransformation of the amide-functionalized polyketide surrogate into a glutarimide moiety. A) General Scheme of the enzyme assay. B) MALDI analysis of the ACP-bound product. C) SIM-LC–HRMS analysis of hydrolyzed product and comparison with the reference. D) Proposed model for the biosynthesis of glutarimide-bearing polyketides such as **2** and **3** (see Figure 1).

constitute lactam and glutarimide moieties. The latter are particularly noteworthy since related modules (KS–"X"–ACP, or KS–B–ACP) were found in PKS complexes involved in the biosynthesis of the glutarimide-bearing polyketides such as 9-methylstreptimidone,[14] isomigrastatin[12] and cycloheximide.[15] The successful enzymatic synthesis of a glutarimide with the *rhi* PKS module for the first time confirms the proposed pathway involving Michael addition and heterocyclization.

In conclusion, we have shed light on the scope and versatility of a specialized PKS module that catalyzes the noncanonical vinylogous addition of a C2 extender unit to generate polyketide chain branches. Through the synthesis of a series of thioester surrogates with different δ-substituents, in vitro biotransformation experiments, and product analyses with synthetic references, we showed that the branching module generates lactone, lactam, and glutarimide heterocycles. We found that this set of domains exhibits a marked stereochemical substrate preference and that exclusively *syn*-substituted lactones and lactams are formed. However, there is a degree of flexibility that leaves room for various alternative conversions. Among these, the enzymatic formation of glutarimide provides first experimental support for postulated biosynthetic pathways to known glutarimide-bearing natural products. Although corresponding δ-lactam substructures have not yet been observed in natural polyketide structures, we postulate that the biosynthesis of polyketides with δ-lactam branches would be feasible and that such natural products await discovery. From a practical point of view, our findings are an important addition to our current understanding of non-terpenoid polyketide branching. Furthermore, the use of this branching module in synthetic biology or the engineering of enzymatic pathways could provide unique opportunities for compound diversification.

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