Polyketide Biosynthesis

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Polyketide-Chain Branching by an Enzymatic Michael Addition**

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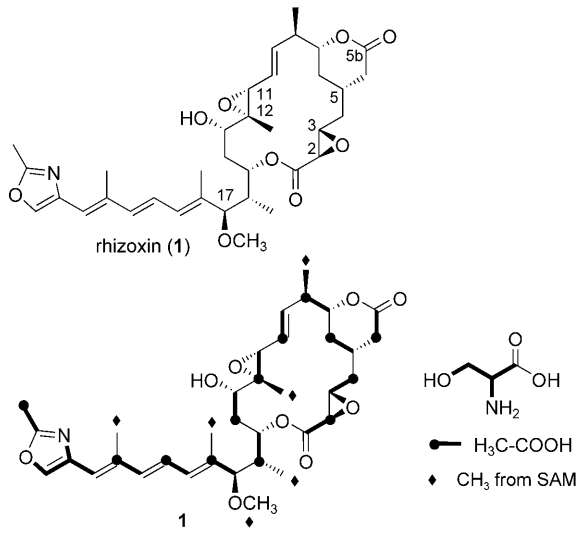
The class of polyketides comprises a wealth of natural products, many of which have important biological activities and complex chemical structures.[1] Irrespective of the producing organism, the type of polyketide synthase (PKS) involved, and the structure of the metabolite, polyketides are always formed by decarboxylative Claisen-type 1,2-head-to-tail condensations of thioesters with malonyl-derived extender units.[2] The resulting linear carbon backbone is then further processed and modified by various tailoring enzymes.[3] Carbon side chains α to carbonyl groups are typically derived from substituted malonyl units or methylene alkylation during or after chain elongation. In contrast, branches at the β position with one or two acetate-derived carbon atoms, which correspond to former acetyl carbonyl groups (C1), are rather scarce among polyketides. Structurally intriguing examples of β-alkylated polyketides (the biosynthesis of which involves isoprenoid-like biosynthetic logic) are bacillaene (methyl branches),[4,5] myxovirescin (methoxymethyl and ethyl branches),[6,7] pederin/onnamide (exomethylene group),[8–10] bryostatin (acrylic ester),[11,12] curacin (cyclopropyl group),[13,14] and jamaicamide (vinyl chloride).[15] In all hitherto examined pathways in which a β-branching event takes place, a biosynthetic strategy is employed that resembles early steps in mevalonate biosynthesis.[16] Recent investigations at the genetic and biochemical levels revealed that the enzymatic C–C coupling requires a set of enzymes that includes 3-hydroxy-3-methylglutaryl-CoA (HMG) synthase and enoyl-CoA hydratase (ECH) (or crotonase) homologues, as well as freestanding ketosynthase (KS) and acyl carrier protein (ACP) domains.[16]

Herein we present direct evidence for a novel biosynthetic strategy for the β-branching of a polyketide chain. The transformation involves a PKS-mediated Michael addition in the rhizoxin pathway. Rhizoxin (**1**) is a highly potent antimitotic agent and virulence factor of the rice-seedling-blight fungus *Rhizopus microsporus*.[17,18] In the course of biosynthetic studies, we found that rhizoxin is in fact not biosynthesized by the blight fungus but by bacteria that live within the fungal cytosol.[19–21] Through cloning and sequencing of the entire rhizoxin (*rhi*) biosynthesis gene cluster from the genome of the endosymbiont *Burkholderia rhizoxinica*, we gained a first insight into the giant modular rhizoxin assembly line.[22]

The most intriguing structural characteristic of rhizoxin is the rare acetate-derived δ-lactone side chain that branches off at a β position (Scheme 1).[23] However, our in silico analysis



Scheme 1. Structure of rhizoxin (**1**) and pattern of ^{13}C-isotope labeling according to Kobayashi et al.[23]. SAM = (*S*)-adenosylmethionine.

of the *rhi* gene locus indicated the absence of genes that encode enzymes required for isoprenoid-like β-branching.[22] Furthermore, the deduced architecture of the modular PKS as well as bioinformatic analysis of the *rhi* KS domains for their predicted substrate specificity provided strong evidence that the position at which branching takes place does not bear a keto group at C5, as one would expect, but more likely a double bond.[22,24] We thus postulated that the β-branch is introduced by a novel mechanism involving the conjugate addition of an acetate building block to the enzyme-bound enoyl moiety. Presumably, the downstream KS and the 530 aa spacer region (B domain), which does not show known domain motifs, catalyzes this unprecedented reaction.

To unravel the exact timing of polyketide branching and the nature of the precursor molecule, we aimed to manipulate the *rhi* PKS and analyze the structures of key pathway intermediates. For this purpose, we first targeted the thio-

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esterase (TE) domain located at the C terminus of the megasynthase. The TE domain is essential for off-loading and macrocyclization of the full-length polyketide chain. This approach seemed promising, as Piel and co-workers demonstrated recently that the ablation of the TE domain of a similar type of PKS involved in bacillaene biosynthesis, in which modules lack acyl transferase (AT) domains and are loaded by a free-standing AT (trans-AT PKS), results in the release of pathway intermediates.[5]

Although the handling and genetic manipulation of the frail endosymbionts in pure culture is a delicate issue, we finally managed to introduce a genetic construct into the bacteria that integrated site specifically into chromosomal DNA and induced a frame shift in the TE-coding region (see the Supporting Information). The correct location of the cross-over that dissects the gene region that codes for the TE domain was verified by PCR. HPLC–MS analysis of the total extract of the mutant culture broth showed that macrolide formation was fully abolished. Instead of the known rhizoxin derivatives **2**–**5** (see Scheme 2), which are typically produced by the symbionts, we observed the occurrence of a series of novel compounds with lower molecular masses. Analysis of the crude extract by HPLC with diode-array detection (DAD) indicated a conjugated polyene system (UV absorbance maxima at 298, 310, and 324 nm) in every compound eluted between 19 and 29 min. High-resolution ESIMS investigations suggested that these compounds are the predicted precursors as the free acids **7**–**15** (see Scheme 2). A reinvestigation of the metabolome of wild-type *B. rhizoxinica* revealed that these intermediates can also be detected as side products of the natural pathway, but at significantly lower titers (5–30%; Figure 1).

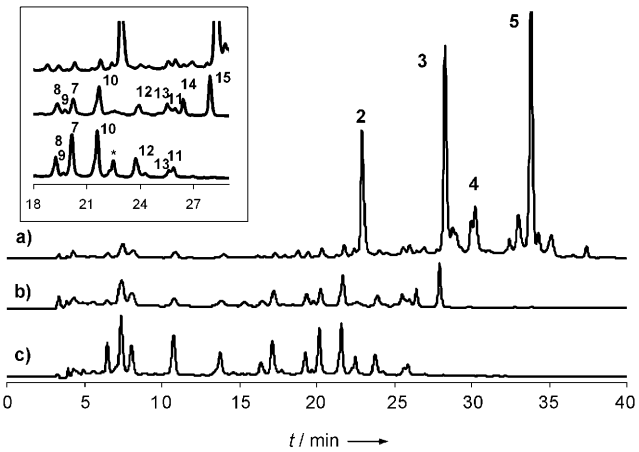
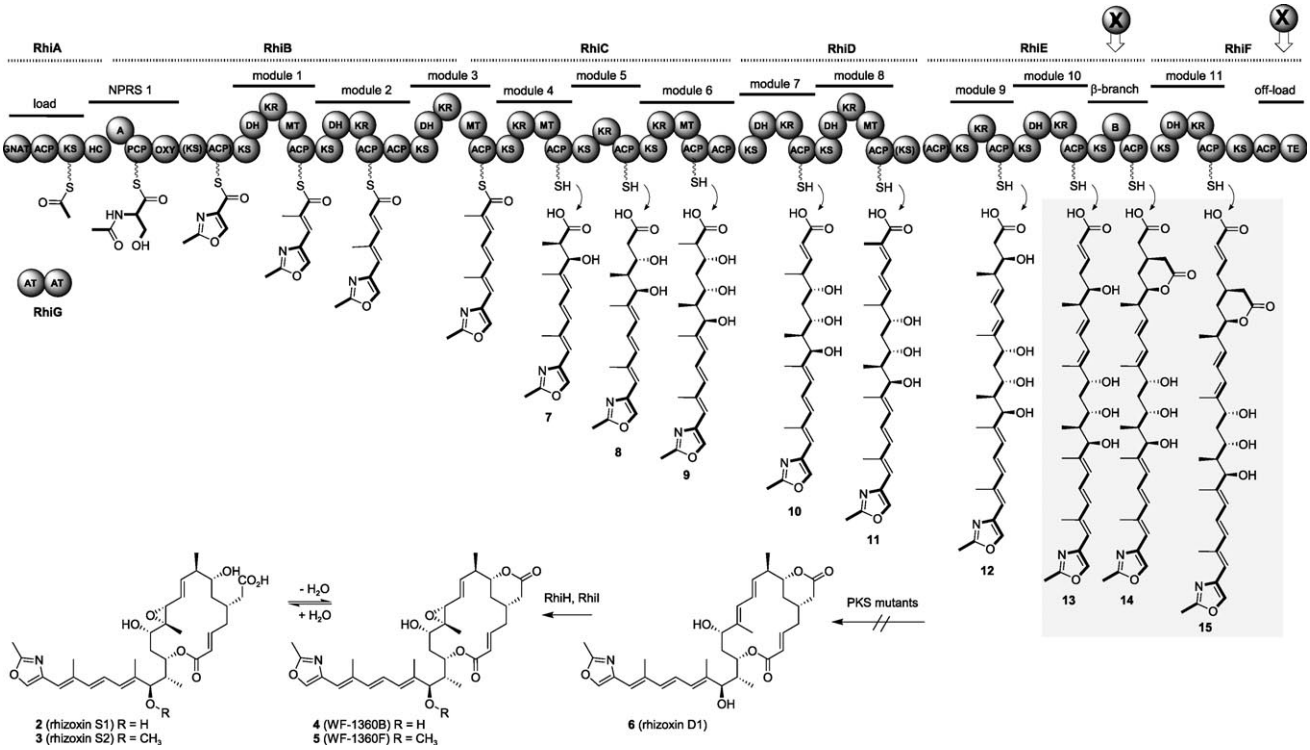


Figure 1. HPLC profiles of a) wild-type *B. rhizoxinica*, b) the TE-knockout mutant, and c) the β-branch-knockout mutant. Rhizoxin precursors are also present in the wild type (see the enlarged region in the inset). *Dehydration product derived from **9**.

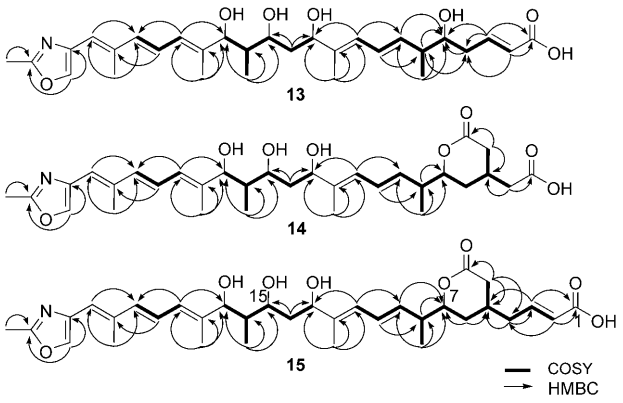
To gain insight into the branching event, we focused on the last three pathway intermediates (Scheme 2). From the HRMS data, the molecular formulae for **13** ($C_{30}H_{43}NO_7$), **14** ($C_{32}H_{45}NO_8$), and **15** ($C_{34}H_{47}NO_8$) were deduced. Although



Scheme 2. Rhizoxin biosynthetic assembly line and structures of the prematurely released pathway intermediates **7**–**15**. The polyketide synthase was mutated in the thioesterase (TE) and β-branching (B) domains. ACP = acyl carrier protein, DH = dehydratase, GNAT/AT = acyl transferase, KR = ketoreductase, KS = ketosynthase, MT = methyl transferase; NRPS is a nonribosomal peptide synthetase module consisting of A (adenylation), C (condensation), OXY (oxygenase), HC (heterocyclization), and PCP (peptidyl carrier protein) domains.

the HRMS data already provided compelling evidence for the presence of the predicted straight-chain intermediates, a full structural elucidation was essential to confirm the structures unequivocally. We therefore scaled up the fermentation of the engineered symbiont (to 50 and 200 L) to obtain enough material for thorough 1D and 2D NMR spectroscopic analysis.

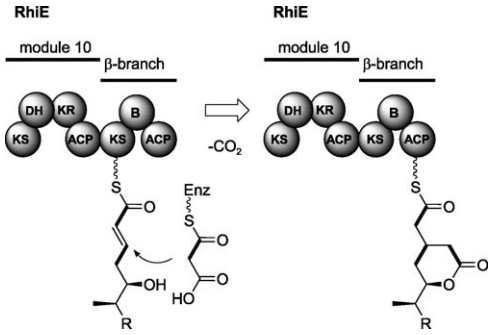
Through open column chromatography and repeated preparative HPLC we eventually obtained pure samples of the three compounds with the highest masses, **13**, **14**, and **15**, for full characterization (Scheme 3). These compounds are



Scheme 3. 2D NMR correlations that support the structures of isolated polyketides.

highly instable and prone to isomerization, degradation, and cyclization. The similarity between the NMR spectroscopic data of **15** and rhizoxin D1 (**6**)[20,25] indicated the presence of a didesepoxy rhizoxin skeleton (see Table 1 in the Supporting Information). The missing HMBC long-range coupling between 15-H and C1 suggests the presence of a linear rhizoxin precursor with a free carboxylic group at C1. A low-field chemical shift for C7 ($\delta = 82.7$ ppm) shows the existence of a δ-lactone between C5 and C7. Thus, compound **15** is the linear full-length polyketide product of the *rhi* assembly line. ^{1}H NMR spectroscopic data, including COSY geminal and vicinal couplings, revealed that **14** is the direct precursor of **15**, with the branching C_2 unit already incorporated. Since compound **14** decomposes readily with loss of water, its structural elucidation by NMR spectroscopy was hampered. ^{13}C NMR chemical shifts could only be obtained from the ^{13}C projections on HMBC/HSQC plots as a result of the small amount and instability of **14**. However, comparison of the NMR spectroscopic data of **14** and **15** established the structure of **14** unequivocally. Compound **14** is the penultimate PKS intermediate and contains the δ-lactone moiety. The structure of intermediate **13** ($C_{30}H_{43}NO_7$) was elucidated unambiguously by 1D and 2D NMR spectroscopic experiments. Accordingly, compound **13** has the same chain length as **14**, but features an acryloyl moiety instead of the β-branch. Thus, the noncanonical incorporation of acetate has not yet taken place in **13**. Furthermore, it is evident that the substrate for β-branching does not bear a β-keto group for isoprenoid-like branching, but a double bond (see Tables 1 and 2 in the Supporting Information).

To confirm the exact timing of chain branching, we next mutated the gene region that codes for the β-branching module. Through homologous recombination, we successfully generated variants with an engineered stop codon or an internal deletion within the coding region for the branching domain (see the Supporting Information). In both cases, the mutants produced intermediates **7**–**13**, which correspond to modules 1–10 of the rhizoxin assembly line, whereby the acrylic acid **13** was the most advanced intermediate. No β-branched compounds or longer chains from the downstream modules could be detected. We therefore conclude that the β-branching reaction takes place after PKS module 10 and before PKS module 11, according to the structures of the intermediates. The structures of **13** and **14** provide clear evidence that the branching acetate unit is incorporated by a conjugate addition reaction. A conceivable mechanism would be the nucleophilic addition of a malonyl unit to the Michael acceptor with concomitant decarboxylation. A nucleophilic attack of the δ hydroxy group may support the cleavage of the initial thioester bond with concomitant lactone formation (Scheme 4).



Scheme 4. Model for β-branching and δ-lactone formation in the biosynthesis of rhizoxin. Enz = enzyme (probably the same module: ACP).

In conclusion, we have successfully engineered the rhizoxin biosynthetic assembly line to gain insight into the timing of polyketide chain branching and the module involved. The isolation and full structural elucidation of the prematurely released pathway intermediates **13**–**15** enabled the reconstruction of the final steps in polyketide assembly. Accordingly, we found that the δ-lactone branch is introduced by the conjugate addition of a malonyl unit to an acryloyl precursor. Further mutation experiments provided strong evidence that the "branching module" (which consists of KS, B ("branching"), and ACP domains) downstream of module 10 is involved in this noncanonical enzymatic C–C coupling. Polyketide synthases generally catalyze Claisen condensation reactions for chain elongation. Until now, only three mechanisms used by nature for the introduction of carbon branches into polyketides were known: the incorporation of branched building blocks, SAM-dependent methylation, and a mechanism reminiscent of the isoprenoid pathway. The Michael-type addition proposed herein as the β-

branching mechanism for rhizoxin biosynthesis is fully unprecedented and a novelty in the field of polyketides. The enzymology behind this novel branching mechanism will be the focus of future studies in our laboratory.

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