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COMMUNICATION

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On-line Polyketide Cyclization into Diverse Medium-Sized Lactones by a Specialized Ketosynthase Domain

Srividhya Sundaram, Hak Joong Kim, Ruth Bauer, Tawatchai Thongkongkaew, Daniel Heine and Christian Hertweck*

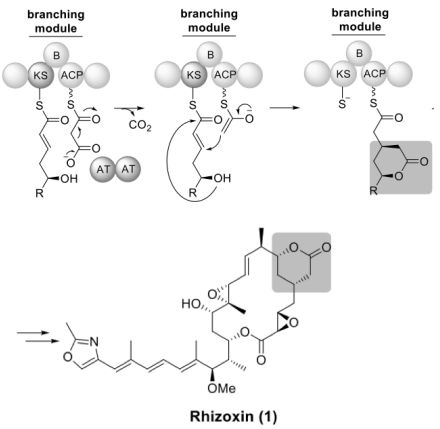
Abstract: *Ketosynthase (KS) domains of modular type I polyketide synthases (PKSs) typically catalyze Claisen condensation of acyl and malonyl units to form linear chains. In stark contrast, the KS of the rhizoxin PKS branching module mediates a Michael addition, which sets the basis for a pharmacophoric δ-lactone moiety. The precise role of the KS was evaluated by site-directed mutagenesis, chemical probes, and biotransformations. Biochemical and kinetic analyses helped to dissect branching and lactonization reactions, and unequivocally assign the entire sequence to the KS. Probing the range of accepted substrates with diverse synthetic surrogates* in vitro, *we found that the KS tolerates defined acyl chain lengths to produce 5- to 7-membered lactones. These results show that the KS is multifunctional as it catalyzes β-branching and lactonization. Information on the increased product portfolio of the unusual, TE-independent on-line cyclization is relevant for synthetic biology approaches.*

Modular polyketide synthases (PKS) are efficient biosynthetic machineries that generate highly diverse natural products with a broad range of biological activities. The motors of these molecular assembly lines are ketosynthase (KS) domains that promote sequential Claisen condensations to generate nascent polyketide chains tethered to acyl carrier protein (ACP) domains.[1] As soon as the polyketide has reached its final length, it is generally off-loaded by a thioesterase (TE) domain located at the N-terminus of the enzymatic processing line. Post-PKS enzymes optionally tailor the primary product to further functionalize the scaffolds.[2] There is growing evidence, however, that structural modifications of the polyketide backbone may be introduced while the intermediates are still tethered to the assembly line.[3] Among these on-line modifications, cyclization reactions are highly prolific as they alter the overall structure and add rigidity to the polyketide backbone.[4] This is well illustrated by the cycloether moieties found in a range of polyketides, which result from vinylogous cycloadditions catalyzed by pyran synthase (PS) domains.[5]

Another important example of a pharmacophoric polyketide-derived heterocycle is the δ-lactone residue of rhizoxin (**1**, Figure 1), a powerful phytotoxin produced by the bacterial endosymbiont of the rice seedling blight fungus *Rhizopus microsporus*.[6] Owing to its potent antiproliferative activity, this unusual bicyclic polyketide has been considered as an antitumoral therapeutic. In light of the complex overall structure of the macrolactone it is remarkable that the small carbon chain branch is totally essential for the binding of the toxin to the β-tubulin subunit and thus for antimitotic activity.[7]

According to text-book knowledge, polyketide (macro) lactonizations are generally catalyzed by TE domains, which promote an intramolecular attack of an alcohol residue onto the ACP-thioester.[8] In contrast to this scheme, the formation of the δ-lactone residue of rhizoxin does not involve any designated TE domain. Studies at the genetic, biochemical and chemical levels have revealed that the side chain is introduced by a non-canonical PKS module consisting of KS, branching (B), and acyl carrier protein (ACP) domains.[9] Unlike its homologues found in regular PKS modules, this KS domain catalyzes the vinylogous addition of ACP-bound malonyl units to an α,β-unsaturated thioester (**Figure 1**).[10] The branched chain can only propagate after cleavage of the KS-bound thioester, which yields the δ-lactone. By analogy, δ-lactam and glutarimide moieties are formed when the hydroxy nucleophile is replaced with amine and carboxamide nucleophiles, respectively.[11]

Domain substitution experiments have indicated that the B domain maintains the structural integrity of the module but does not have any significant impact on the choice of substrates used for heterocyclizations.[12] The biochemical basis for the cyclization reaction and its scope, however, has remained obscure. One may conceive that the lactonization is independent of enzyme catalysis. In lovastatin biosynthesis, for example, the hydroxy acid released by the TE cyclizes spontaneously under an acidic condition.[13] Here we show by *in vitro* assays, mutational studies and kinetic analyses that the lactonization is promoted by the branching KS domain and that the scope of cyclizations is broader than previously believed.



Rhizoxin (1)

Figure 1. Model of on-line branching and lactonization in rhizoxin (**1**) biosynthesis. KS, ketosynthase; AT, acyl transferase; B, branching domain; ACP, acyl carrier protein.

To evaluate the role of the chain-branching KS domain in the formation of the δ-lactone ring and to dissect branching and cyclization, we examined the primary sequence of the KS domain by bioinformatics. A phylogenetic analysis revealed that the KS domain of the rhizoxin PKS branching module (Rhi-KS14) falls into clade IX that corresponds to canonical KS domains utilizing olefinic substrates.[14] By multisequence alignments we compared the amino acid sequence of Rhi-KS14 with other KSs (both branching and elongating) from clade IX. Excluding the conserved catalytic residues, ten positions in the family of branching KSs differ considerably when compared against classical KSs mediating Claisen condensations (**Table S1 and Figure S1**). Conserved residues of the Rhi-KS14 (PDB code: 4kc5) were mapped and superimposed on the bacillaene KS5 structure (PDB code: 5erb**, Figure 2A**).[15] These residues

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were mutated to the corresponding residues represented in the canonical KSs. In the case of S3232, it was changed to Leu as well as to Ala. In total, eleven single mutations were introduced. To understand the cumulative effect of all selected amino acids, we designed a synthetic gene coding for KS'-B, in which all nine residues (except T3290) were mutated. All mutant KS domains were heterologously produced in *E. coli* BL21 (DE3) (**Figure S2)**. Except for KS'-B, of which soluble protein could only be obtained in minute amounts, CD spectra showed no significant differences between the secondary structures of all recombinant proteins (**Figure S3**).

We next tested the impact of these residues on the Michael addition and the subsequent lactonization. To identify residues that are involved in the Michael addition we probed all mutants *in vitro* with the coenzyme A mimicking *N*-acetylcysteamine (SNAC) thioester **2**.[16] Since this polyketide surrogate lacks the δ-hydroxy group, the branching reaction is arrested after the initial C–C bond formation.[10a] The trapped intermediate (**3**), which covalently links the KS-B with the ACP, can be visualized on the SDS gel (**Figure 2B**). All mutants, except C3228A, T3290Y and the KS'-B, retained the vinylogous C-C bond-forming activity (**Figure 2C**).

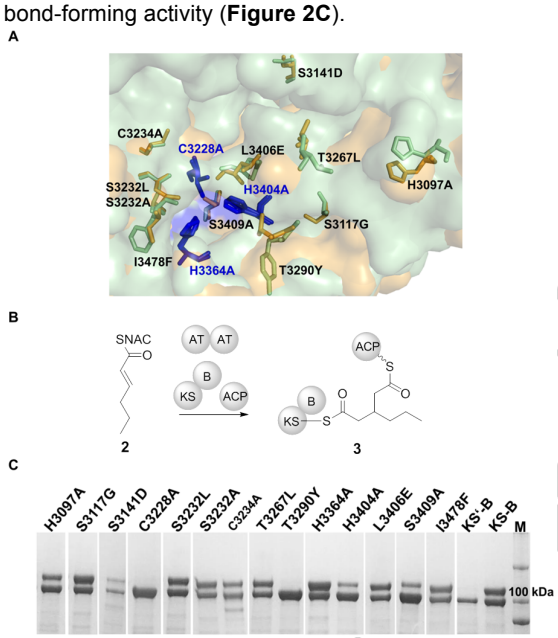

Figure 2. (**A**) Comparison of active-site residues of KS-B (orange) (PDB code 4kc5) with bacillaene KS5 (green) (PDB code 5erb). Residues chosen for mutational analysis are colored black. Residues in blue constitute catalytic triad. Numbers correspond to residues of Rhi–KS14 (PDB code 4kc5). (**B**) Chain-branching assay setup with deoxy variant of SNAC thioester substrate **2**. (**C**) SDS-PAGE analysis of the reaction mixture with **2**. The 100 kDa band corresponds to the KS-B didomain, upper band corresponds to fused KS-B and ACP domains (intermediate **3**). All proteins were verified by tryptic mass fingerprinting followed by MALDI analysis. M, protein molecular weight marker.

To investigate the role of the selected residues in the lactonization reaction, we performed *in vitro* assays with **4** to monitor the formation of lactone **5 (Figure 3A and 4A)**. Mutants H3097A, S3117G, S3141D, S3232L, S3232A, C3234A, T3267L, L3406E, S3409A and I3478F did not markedly hamper the cyclization activity (**Figure S4**). In all cases, **5** was observed in comparable amounts to KS-B (**Figure 3A, i**). Corroborating the SDS gel-shift experiments, mutants C3228A, T3290Y and KS'-B did not result in **5**, even when the corresponding enzymes were tested at 10 times higher concentrations for prolonged reaction times (16 h) (**Figure 3A, ii, iii and vi)**. The inactivity of C3228A mutation is not surprising (**Figure 3A, ii**) because the nucleophilic Cys residue is needed for binding the polyketide intermediate. However, the completely inactive T3290Y mutant (**Figure 3A, iii**) indicates that this residue is involved in the C-C bond formation.

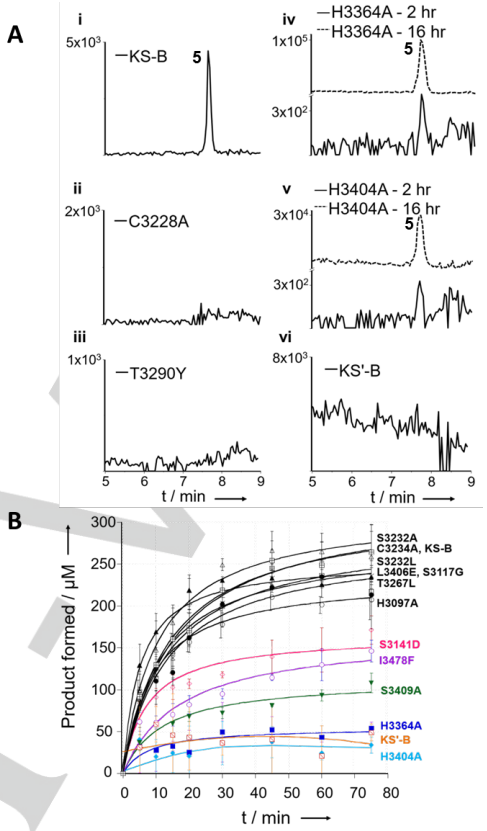

Figure 3. **(A)** LC-HRMS analysis of mutants showing extracted ion chromatograms of lactone **5** $[M+H]^+$) 173.0808, ($C_8H_{13}O_4$). **(B)** Rate of lactone (**5**) formation by wild-type KS-B and mutants. Data represent mean ± s.d from three independent experiments.

When testing mutants H3364A and H3404A, increasing the enzyme concentration and the duration of the assay also enlarged the amount of product formed (**Figure 3A, iv and v**). It is possible that both His residues complement each other in the branching reaction. A report on bacterial fatty acid synthases (FabF) suggested that the H3364 counterpart is not an absolute necessity for the C–C bond forming reaction, but enhances the overall rate of the reaction.[17] Analogous mutations of the catalytic His residues of the KS of 6-deoxyerythronolide B synthase (DEBS) module 1 and *E. coli* FabB resulted in moderate to very low turnover of the substrate, too.[17-18] A double mutant (H3364A and H3404A) stalled the formation of **5**, proving that these residues are indispensable for the Michael addition. According to functional studies with homologous KS domains,[17-19] H3404 activates the malonyl carboxylate and stabilizes the enolate. Reduction of the rates in the carbon-carbon bond formation as well as lactonization (**Figure 2C and 3A, v**) indicates that H3404 plays a major role in all steps of the reaction. To complement the LC-MS analysis, LC-ESI-MS of the intact ACP-bound proteins was also performed for KS-B, H3364A and H3404A and analyzed by the PPant-ejection method.[20] The lactone-bound PPant arm was observed in all the samples tested. However, compared to the wild-type KS-B (**Figure S5**), the intensities of the mass fragments were approximately 10-fold lower in H3364A and H3404A (**Figure S6 and S7**). Nonetheless, all mutants retained malonyl-CoA decarboxylation activity.

To identify other residues that could affect the reaction rate, the mutants (except for the inactive C3228A and T3290Y) were

further analyzed by kinetic experiments at saturating concentration of **4** (1 mM) and increased enzyme concentrations of KS-B and ACP, respectively. Combined kinetic data from three independent experiments showed that the mutants can be grouped into three types (**Figure 3B**). The largest group of mutants (represented by black curves) reached product saturation after approximately 45 min, thus showing that the lactonization is not impaired. In contrast, the rate of lactonization was substantially decreased in the S3141D, I3478F and S3409A mutants (**Figure 3B and Figure S8**). The H3364A, H3404A and KS'-B mutants exhibited only negligible activities within the specified time limit (75 min). The impact of the S3141 residue on the reaction is remarkable as it is distant from the active center and thus likely not directly involved in the branching/lactonization reaction. The mutation of I3478 to a bulkier phenyl alanine residue may have caused perturbations in the β-sheet where it is located.[10a] Phenyl alanine residues close to the active site are termed as gatekeeper residues that dictate the order of the reaction.[17] Apart from the active site mutants, the only mutant that showed attenuated reaction rate is S3409A. Since this serine moiety is located in the active site, we concluded that this residue is an important determinant for lactonization. However, it is conceivable that additional residues contribute to the lactonization reaction.

Our mutational and kinetic analyses demonstrated that the lactonization of the branched polyketide chain is catalyzed by the KS. To probe the flexibility of this multifunctional KS with respect to the ring size, we synthesized a range of SNAC thioester analogues. By metathesis we prepared substrates with γ-hydroxy groups (**6** and **7**, **Figure 4B**), which would give rise to ring-contracted, five-membered lactones. The substrate analogues were tested *in vitro* using the native KS-B didomain, ACP, AT and malonyl-CoA. The biotransformation was monitored by MALDI and LCMS to detect ACP-bound and hydrolyzed products. Analysis of the reaction mixture with **6** resulted in a new species with *m/z* of 12705.7, whereas the reaction with **7** resulted in a species with *m/z* 12722.5. These *m/z* values correspond to the expected ACP-bound five-membered lactones with methyl and ethyl side chains, respectively (**Figure S9,** lactone 5 and lactone 5*). High-resolution mass spectrometric analysis of the hydrolyzed products confirmed the formation of new compounds, **8**, $[M+H]^+$ 159.0652 ($C_7H_{11}O_4$) and **9,** $[M+H]^+$ 173.0808 ($C_8H_{13}O_4$) (**Figure 4D, ii and iii**). To unequivocally prove their structures we prepared synthetic references and used these authentic compounds for HPLC-MS comparisons. In both cases, the resulting double peaks indicated the formation of the expected *syn* and *anti* diastereomers.[11]

To test whether the branching module can also produce larger rings such as ε-caprolactones, SNAC thioesters **10** and **11** were used as probes. With **10** as the substrate, MALDI analysis of the reaction mixture revealed the formation of the 7-membered lactone bound to the ACP (**Figure S9,** lactone 7*). Analysis of the biotransformation products indicated the formation of a new compound **12**, with *m/z* $[M+H]^+$ 173.0808 ($C_8H_{13}O_4$) **(Figure 4D, iv)**. In contrast to **10**, compound **11** could not be transformed into the corresponding ε-substituted caprolactone. Even after prolonged reaction time (16 h at 23 °C) no ACP-bound intermediate could be detected. However, analysis of the hydrolyzed reaction mixture revealed a new compound (**13**) with *m/z* $[M+H]^+$ 205.1065 ($C_9H_{17}O_5$) (**Figure 4D, v**). According to the deviations in molecular masses and HPLC retention times, **13** differed from the expected 7-membered lactone **14**. The structure of **13** was elucidated as the dicarboxylic acid derivative by comparison with a synthetic reference (see SI). This finding showed that the initial Michael addition is working, but the subsequent lactonization is impaired for the secondary alcohol.

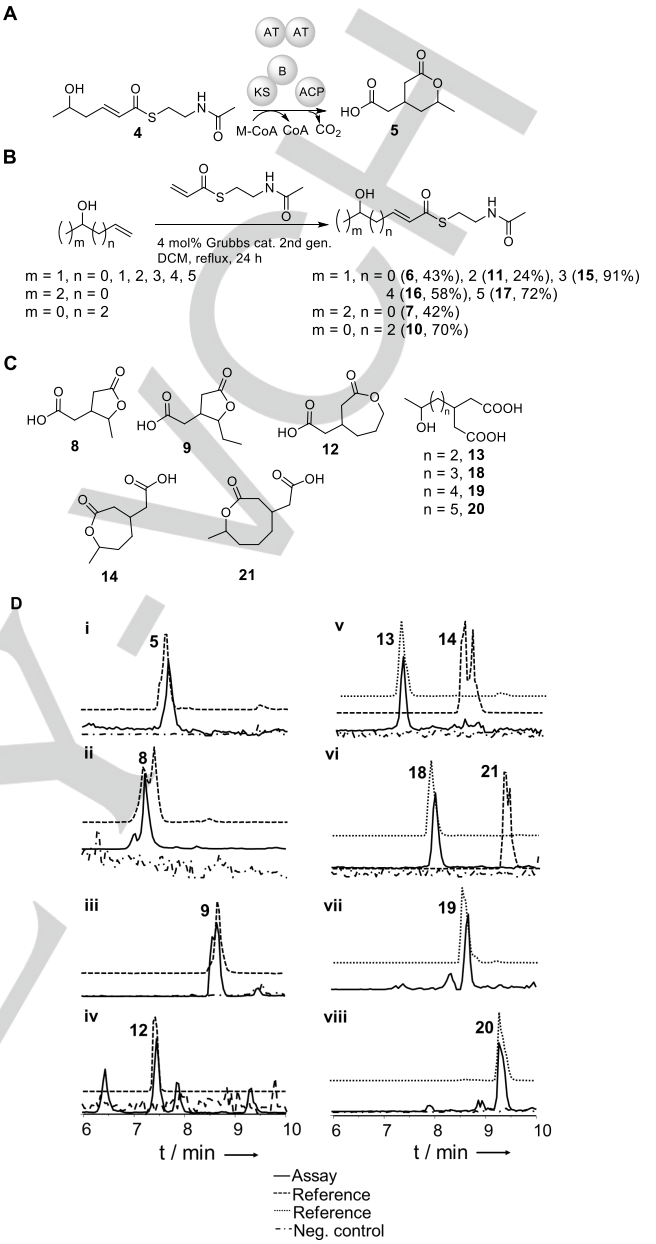

Figure 4. *In vitro* evaluation of KS-catalyzed branching/lactonization with synthetic surrogates**.** (**A**) Set-up of assay leading to δ-lactone. (**B**) Synthesis of substrate mimics (**C**) Schematic of observed biotransformation products. (**D**) LC-HRMS analysis of biotransformation assays. Solid lines, assay; dashed lines, synthetic references for lactones; dotted lines synthetic references for dicarboxylic acid derivatives; dash-dot lines, negative control. Negative control refers to the assays performed with heat-inactivated enzyme.

To corroborate this result, we synthesized and tested SNAC thioesters with extended carbon backbones (**15**, **16** and **17**). In all cases, the corresponding dicarboxylic acids, **18** (*m/z* $[M+H]^+$ 219.1219 ($C_{10}H_{19}O_5$), **19** *m/z* $[M+H]^+$ 233.1380 ($C_{11}H_{21}O_5$) and **20** *m/z* $[M+H]^+$ 247.1541 ($C_{12}H_{23}O_5$), were formed, as proven by comparison with synthetic references (**Figure 4D, vi-viii**). Notably, when the assays were performed with heat-inactivated KS-B (negative control), no products were observed, thus proving that the vinylogous carbon bond formation is always enzyme catalyzed. Since the OH group is necessary to form the lactone, we proposed that the farther the

OH group is from the thioester in the KS active site, the less feasible it is to form the lactone.

In conclusion, we have shed light on the versatility of the KS domain that catalyzes the vinylogous Michael addition and lactonization. Using a series of mutational and kinetic analyses, we showed the interplay of certain amino acid residues in catalyzing the enzymatic steps. Moreover, through the synthesis of a series of thioester surrogates, we showed that the KS is capable of forming 5- to 7-membered lactones. From a synthetic biologist's point of view, the range of possible ring sizes produced by the branching module holds vital importance to use its versatile KS domain to generate complex polyketides endowed with pharmacophoric heterocycles.

Keywords: Lactones • Macrolides • Michael addition • Natural Products • Polyketide Synthases

Experimental Section

Refer Supplementary Information.

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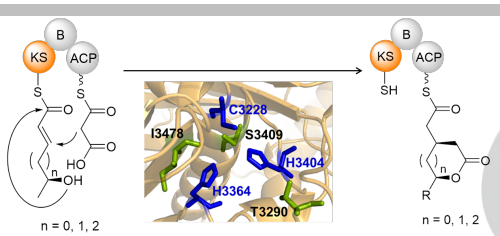
Entry for the Table of Contents

COMMUNICATION

Stepping out of line: Site-specific mutagenesis and kinetics of the versatile rhizoxin PKS branching module identified critical conserved residues and show that the KS domain promotes both Michael addition and lactonization. Moreover, the branching module is tolerant towards substrates with varied acyl chain lengths to form various medium-sized lactones.



Srividhya Sundaram, Hak Joong Kim, Ruth Bauer, Tawatchai Thongkongkaew, Daniel Heine and Christian Hertweck

Page No. – Page No.

On-line Polyketide Cyclization into Diverse Medium-Sized Lactones by a Specialized Ketosynthase Domain