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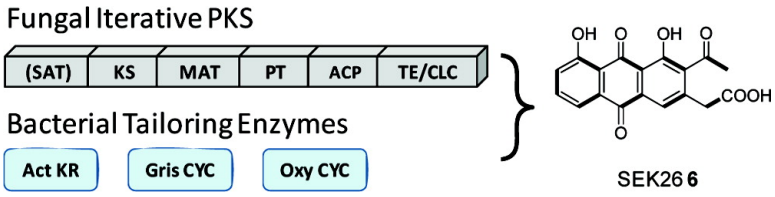

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Redirecting the Cyclization Steps of Fungal Polyketide Synthase

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Regiospecific cyclizations of the nascent poly-β-ketone backbones dictate the structures of polyketide natural products. Systematic reprogramming of the cyclization steps is therefore an important goal toward combinatorial biosynthesis of polyketides. Bacterial and fungal aromatic polyketide synthases (PKSs) employ different strategies to control the regiospecificities of cyclization reactions. Whereas the bacterial PKSs use dissociated enzymes to tailor the reactive poly-β-keto backbone synthesized by the minimal PKS,[1] the fungal iterative megasynthases use terminal thioesterase/claisen cyclase (TE/CLC) domains to direct the fate of the polyketide chains.[2] In this work, we present two strategies toward redirecting the cyclization steps of fungal PKSs. Most notably, we demonstrate that fungal and bacterial PKS components can be catalytically integrated *in trans*.

We focused our reprogramming efforts on the unreduced nonaketide synthase PKS4 from *Gibberella fujikuroi*.[3] We have recently successfully reconstituted the activities of the megasynthase purified from *Escherichia coli*.[4] PKS4 synthesized SMA76a **1** (Figure 1A), the precursor of the natural product bikaverin, from nine malonyl-CoA molecules. PKS4 is therefore a mechanistically complex fungal PKS, as it alone catalyzes eight iterations of claisen condensations, followed by regioselective cyclization of four linearly fused rings to yield **1**.

We first used site-directed mutagenesis and domain deletion to inactivate the PKS4 TE/CLC domain and investigate its role in controlling nonaketide cyclization. The TE/CLC domains from other fungal PKSs have been associated with the C1−C10 cyclization reactions that afford the second aromatic ring.[5] On the basis of sequence alignment, we mutated the putative active site (GW***S***AG) serine (S1830A) in PKS4. The mutant was solubly expressed from *E. coli* (8 mg/L), purified using Ni-NTA, and reconstituted in vitro (20 μM) in the presence of malonyl-CoA (2 mM). We observed complete loss of **1** and the emergence of a new predominant product SMA93 **2** in comparable yield (~1 mg/10 mL) to that of **1** synthesized by the PKS4 (Figure 1B). The mass of **2** ($[M - H]^-$ = 341) corresponds to a nascent nonaketide that has undergone two dehydrations, while the UV absorption of **2** strongly resembled that of a benzopyrone compound (Supporting Information). **2** was purified from a scaled-up in vitro reaction using MatB, and its structure was elucidated using 1D and 2D NMR spectroscopy, as shown in Scheme 1. Inactivation of the TE/CLC domain disrupted the C1−C10 cyclization step and led to the spontaneous esterification between O9−C1 to yield the benzopyrone moiety, followed by the aldol condensation between C12−C17 to yield the dihydroxyphenyl ring.

To test whether the inactivated TE/CLC domain has a template effect on polyketide cyclization, we constructed a PKS4 variant in which the TE/CLC domain was excised from the megasynthase.

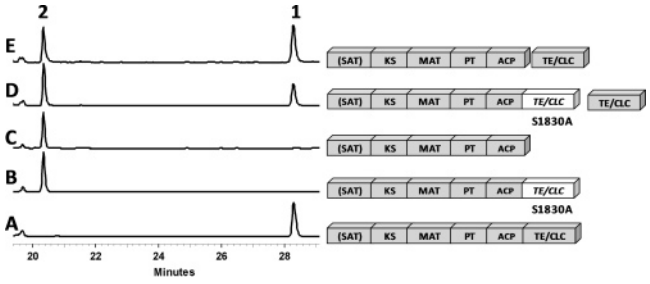
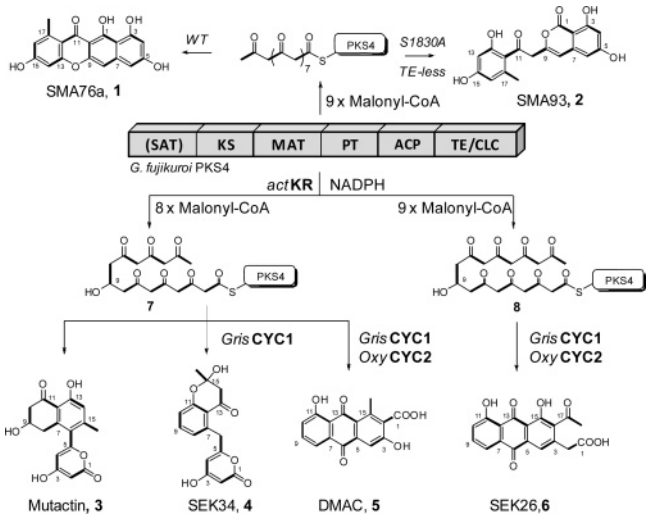


Figure 1. HPLC analysis (320 nm) of polyketides synthesized by (A) PKS4, (B) PKS4 S1830A mutant, (C) PKS4-99, (D) PKS4 S1830A mutant complemented with PKS4 standalone TE/CLC domain, and (E) PKS4-99 complemented with TE/CLC domain. Final concentrations for the megasynthases and TE/CLC are 20 and 40 μM, respectively.

Scheme 1



The domain boundary was chosen based on multiple sequence alignments of TE/CLC-containing PKSs (Supporting Information). The "TE-less" PKS4 mutant (PKS4-99) was expressed solubly from *E. coli* (2 mg/L), purified to homogeneity, and assayed for the synthesis of polyketide products in vitro. PKS4-99 synthesized **2** in comparable yield (0.9 mg/ 10 mL) as PKS4 S1830A (Figure 1C), confirming the TE/CLC domain is not required for proper PKS4 folding and catalytic formation of **2**.

We then tested whether the cyclization domain can recapture and modify the PKS4-bound polyketide *in trans*. We complemented both PKS4 S1830A and PKS4-99 with a standalone PKS4 TE/CLC protein expressed from *E. coli* (5 mg/L). The TE/CLC enzyme (40 μM) was able to partially restore the synthesis of **1** (Figure 1D,E) when added to the mutant megasynthases (20 μM). Complementation of PKS4 S1830A with TE/CLC yielded lower relative amounts of **1** than that between PKS4-99 and TE/CLC, most likely

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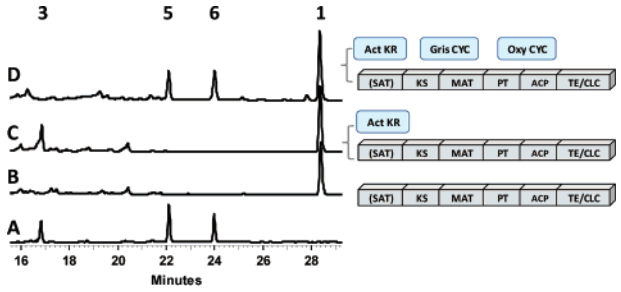

Figure 2. (A) Authentic standards of **3**, **5**, and **6**. HPLC analysis (280 nm) of polyketides synthesized by (B) PKS4, (C) PKS4 and *act* KR, and (D) PKS4, Gris ARO/CYC, and OxyN. Final concentrations for the megasynthases and bacterial enzymes are 20 and 40 μM, respectively.

due to competitive binding of the polyketide chain by the inactivated TE/CLC domain.

Results from the first approach show that, using *E. coli* as the expression host and an in vitro platform, we can systematically alter the cyclization steps of PKS4 to afford new polyketides. More importantly, we demonstrate that dissociated cyclization enzymes can interact with the megasynthase to produce regioselectively cyclized polyketides. This raises an intriguing possibility that the poly-β-ketone backbone synthesized by PKS4 may be accessed and tailored by ketoreductases (KRs) and cyclases from bacterial PKS pathways *in trans*. We tested this hypothesis by incubating PKS4 (20 μM) with different bacterial tailoring enzymes (40 μM) and assaying for the emergence of new polyketides using LC-MS. Interestingly, mixing PKS4 with the actinorhodin (*act*) KR and NADPH resulted in the emergence of a new compound ($\sim$0.2 mg/10 mL) along with **1** (Figure 2C). The R_f value, LC retention time, UV spectrum, and mass ($[M - H]^- = 301$) of the new compound all matched those of mutactin **3**,[6] which is an octaketide that has been subjected to regiospecific reduction at C9 (Scheme 1).[7] Removal of NADPH abolished the synthesis of **3**, which confirmed the catalytic involvement of *act* KR. **3** is the main shunt product synthesized by the *act* minimal PKS and *act* KR in *Streptomyces coelicolor*. The emergence of **3** in the heterologous enzyme mixture shows the dissociated *act* KR was able to interrupt the elongation steps of PKS4 at the octaketide stage and interface with the megasynthase productively to synthesize a *Streptomyces* polyketide. Therefore, the PKS4 megasynthase can substitute the role of the bacterial minimal PKS and provide an untailored polyketide backbone for modification by bacterial auxiliary enzymes.

We examined the stoichiometric requirements of the interaction between PKS4 and *act* KR using 2-^{14}C-malonyl-CoA and radio-TLC. Interestingly, significant amounts of **3** were observed only when *act* KR was added at equimolar or higher concentrations than PKS4. Nearly no **3** can be detected from the reaction mixture when *act* KR was supplied at lower concentrations than PKS4. Elevating the *act* KR concentration led to gradual increases and saturation in the amount of **3**. The maximum ratio of **3** to **1** observed in these studies was $\sim$1:2.5 when the final concentration of KR was 10 times that of PKS4.

We then assayed to see if the C9-reduced polyketides can be further tailored by dissociated cyclases *in trans* to yield regioselectively cyclized aromatic polyketides. Addition of the first ring aromatase/cyclase (Gris ARO/CYC[8]) from the griseusin PKS resulted in the appearance of the expected product SEK34[9] **4** (data not shown) and near complete disappearance of **3**. Inclusion of both Gris ARO/CYC and the recently characterized oxytetracycline second ring cyclase OxyN[10] resulted in the synthesis of two anthraquinone compounds **5** and **6** ($\sim$0.2 mg/10 mL each) in addition to **1** (Figure 2D). Compared to authentic standards, we identified the two compounds to be the octaketide DMAC[11] and the nonaketide SEK26,[12] respectively. The synthesis of **6** confirms that the *act* KR is also able to reduce a full-length nonaketide chain prior to the PKS4-catalyzed C2−C7 cyclization.

The interactions between PKS4 and bacterial tailoring enzymes reveal mechanistic details of both fungal and bacterial iterative PKSs: (1) During iterative chain elongation, the ACP-bound, growing polyketide must periodically exit the KS active site. The reactive poly-β-ketone backbone is shielded from spontaneous cyclization, by either the ACP domain or other domains in PKS4, such as the proposed product template domain.[13] Remarkably, the dissociated *act* KR is able to capture the protected nascent polyketide and perform the C9 reduction. (2) The C9 regioselectivity of *act* KR is precisely maintained in the presence of the fungal PKS, demonstrating that *act* KR has access to the *completed* ACP-bound polyketide chain. We observed no polyketides that were reduced at alternative ketone positions. (3) The synthesis of **3** in Figure 2C unequivocally proves that *act* KR does not require a C7−C12 cyclized polyketide as a substrate. Therefore, the commonly observed C7−C12 cyclization among bacterial aromatic polyketides does not have to take place within the KS-CLF active site.[14] Our results thus provide additional evidence that *act* KR controls the regioselective C7−C12 cyclization during polyketide synthesis.[15] When anchored in the KR active site, C7 and C12 of the nascent polyketide chain are proximally positioned to facilitate the aldol condensation.[1]

In summary, we have altered the complex cyclization steps of a fungal PKS in vitro. Our approaches have revealed new insights into the reactions catalyzed by iterative fungal PKSs. The cooperative activities of fungal and bacterial PKS components are especially important and enable synthesis of polyketides utilizing enzymes from two distinct families of PKSs.

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Supporting Information Available: Experimental details and NMR data. This material is available free of charge via the Internet at http://pubs.acs.org.

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