Gain-of-Function Mutagenesis of a Modular Polyketide Synthase

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Modular polyketide synthases are multifunctional enzyme assemblies that catalyze the biosynthesis of the macrocyclic precursors of compounds such as erythromycin,[1,2] avermectin,[3] oleandomycin,[4] rapamycin,[5] and candicidin.[6] The active sites are clustered in "modules" that each perform a single cycle of condensation and β-ketoreduction in polyketide biosynthesis. The notion that this modular arrangement lends itself to combinatorial biosynthesis[7,8] has been tested through a variety of mutagenesis strategies including inactivation of reductive domains,[2,9,10] deletion of modules,[11−14] and replacement of an acyltransferase with methylmalonyl specificity with a corresponding domain with malonyltransferase specificity.[15] Notwithstanding these advances, however, the introduction of heterologous domains such as ketoreductases, dehydratases, and enoylreductases into modules that naturally lack these functions has not yet been demonstrated. Whereas this capability would be a powerful tool for generating structural diversity in unnatural polyketide libraries, it is particularly challenging due to the fact that the newly introduced enzymatic domain is nonessential and must successfully compete for its substrate with the next module.

Here we demonstrate the introduction of an unnatural enzymatic activity in a multistep biosynthetic pathway catalyzed by a modular polyketide synthase (PKS). Specifically, the β-ketoreductase domain of module 2 of the erythromycin PKS was replaced with a β-ketoreductase and a dehydratase domain derived from the rapamycin PKS. Not only does this chimeric multifunctional enzyme catalyze β-ketoreduction and regiospecific dehydration of the covalently bound triketide intermediate but also the resulting product is transferred to and faithfully processed by the next module of the erythromycin PKS. The chimeric module described here is also significant from a medicinal context, since it could considerably simplify access to a newly discovered class of semisynthetic antibiotics with potent activity against a broad range of drug-sensitive and resistant bacterial pathogens.

The deoxyerythronolide B synthase (DEBS; Figure 1) from *Saccharopolyspora erythraea* consists of six modules which catalyze formation of the erythromycin macrolactone, 6-dEB (**1**). In the current study we wished to test the feasibility of gain-of-function mutagenesis of modular PKSs by engineering a net gain of DH function within the reductive segment of module 2. Furthermore, we also wished to test the viability of the modified intermediate as a substrate for at least one downstream module. We therefore took advantage of a recently developed trimodular derivative of DEBS, which has been shown to synthesize compounds **2** and **3** *in vivo* (Figure 2).[14] In addition to simplifying mutagenesis procedures, it was rationalized that the absence of any reductive activity in module 3 would lead to spontaneous decarboxylation of the initially generated β-keto carboxylic acid, thereby facilitating the isolation and characterization of the (acyclic) reporter polyketide.

At the time of initiation of this study, rapamycin was the only modular PKS of known sequence that possessed modules containing functional KR and DH domains without accompanying enoylreductase domains.[5] Using the known sequence of the rapamycin gene cluster from *Streptomyces hygroscopicus* NRRL 5491 as a guide, the PKS genes were recloned. The reductive segment from rapamycin module 4, which contains a KR and DH domain, was targeted since the substrate seen by these enzymes was likely to be most related in structure to the corresponding substrate bound to ACP2 of DEBS. The recombinant strain, *Streptomyces coelicolor* CH999/pKOS009-7 (Figure 2), containing this rapamycin reductive segment in place of its DEBS module 2 homolog, produced two novel metabolites in good yields (ca. 5−10 mg/L, as compared to the yields of **2** and **3**, which were 20 and 5 mg/L, respectively). Both were purified and structurally characterized by NMR, mass spectroscopy and isotope labeling analysis.[16] The structures of KOS009-7a (**4**) and KOS009-7b (**5**) are consistent with a biosynthetic pathway in which the condensation product of module 2 undergoes β-ketoreduction and dehydration followed by transfer to module 3, whose catalytic properties remain unaltered. As expected, the acyclic product released by module 3 underwent spontaneous decarboxylation.

We note that a similar replacement of KR2 by the rapamycin KR-DH4 segment in the context of a full DEBS system could

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(16) Characterization of KOS009-7a (4) and KOS009-7b (5). **KOS009**-7a: ^{1}H NMR (400-MHz, $CDCl_3$) δ (ppm) 0.94 (t, 3H, J = 7.4 Hz, C-9), 1.05 (d, 3H, J = 6.7 Hz, C-6a), 1.06 (t, 3H, J = 7.3 Hz, C-1), 1.35 (m, 1H, C-8), 1.53 (m, 1H, C-8), 1.78 (d, 3H, J = 1.2 Hz, C-4a), 2.60 (ddq, 1H, J = 9.9, 6.6, 6.6 Hz, C-6), 2.66 (q, 2H, J = 7.4 Hz, C-2), 3.40 (ddd, H, J = 8.8, 6.3, 3.5 Hz, C-7), 6.47 (dd, H, J = 9.9, 1.3 Hz, C-5); ^{13}C NMR (100-MHz, $CDCl_3$) δ (ppm) 8.7 (C-1 or C-6a), 10.1 (C-9), 11.7 (C-1 or C-6a), 15.0 (C-4a), 27.9 (C-8), 30.5 (C-2), 39.3 (C-6), 76.6* (C-7), 136.3 (C-4), 143.8* (C-5), 202.8* (C-3). HRFAB-MS [M + H$^+$] 185.1549 (observed), 185.1542 (calculated). **KOS009**-7b: ^{1}H NMR (400-MHz, $CDCl_3$) δ (ppm) 1.08 (d, 3H, J = 7.0 Hz, C-6a), 1.09 (t, 3H, J = 7.4 Hz, C-1), 1.18 (d, 3H, J = 6.3 Hz, C-8), 1.82 (d, 3H, J = 1.3 Hz, C-4a), 2.60 (ddq, 1H, J = 9.9, 6.7, 6.7 Hz, C-6), 2.69 (q, 2H, J = 7.3 Hz, C-2), 3.72 (dq, H, J = 6.3, 6.3 Hz, C-7), 6.47 (dd, H, J = 9.9, 1.3 Hz, C-5); ^{13}C NMR (100-MHz, $CDCl_3$) δ (ppm) 8.7 (C-1 or C-6a), 11.8 (C-1 or C-6a), 15.4 (C-4a), 21.2 (C-8), 27.9 (C-8), 30.5 (C-2), 41.0 (C-6), 71.3 (C-7), 136.8 (C-4), 143.2* (C-5), 202.7* (C-3). CI-MS, [M + H$^+$] 171.5, [M + H$^+$, −H$_2$O] 153.5. *Peaks enriched in sample isotopically labeled by 1-^{13}C-propionate feeding.

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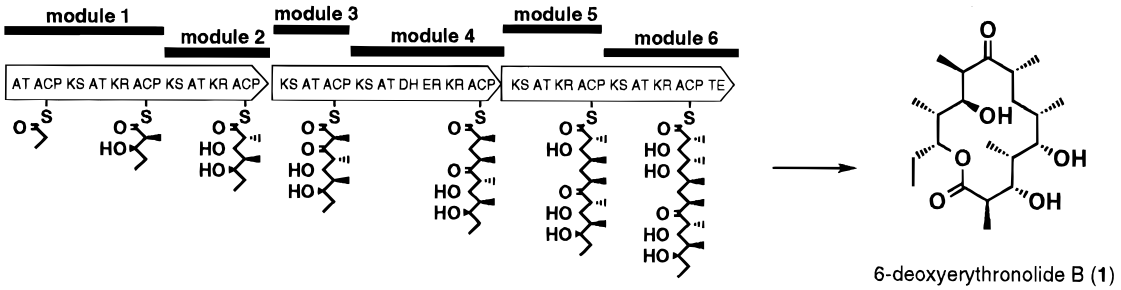

Figure 1. The erythromycin polyketide synthase (PKS). The PKS is encoded as three large multifunctional proteins and includes six modules as well as a primer loading segment and a product release segment. Each module catalyzes one cycle of chain extension and associated β-ketoreduction for the biosynthesis of 6-deoxyerythronolide B (6-dEB). All modules include ketosynthase (KS), acyltransferase (AT), and acyl carrier protein (ACP) domains; additionally they also possess a reductive segment comprising of all, some, or none of the set of ketoreductase (KR), dehydratase (DH), and enoylreductase (ER) domains. The loading segment consists of priming AT and ACP domains, whereas the release segment of the PKS is made up of a thioesterase (TE).

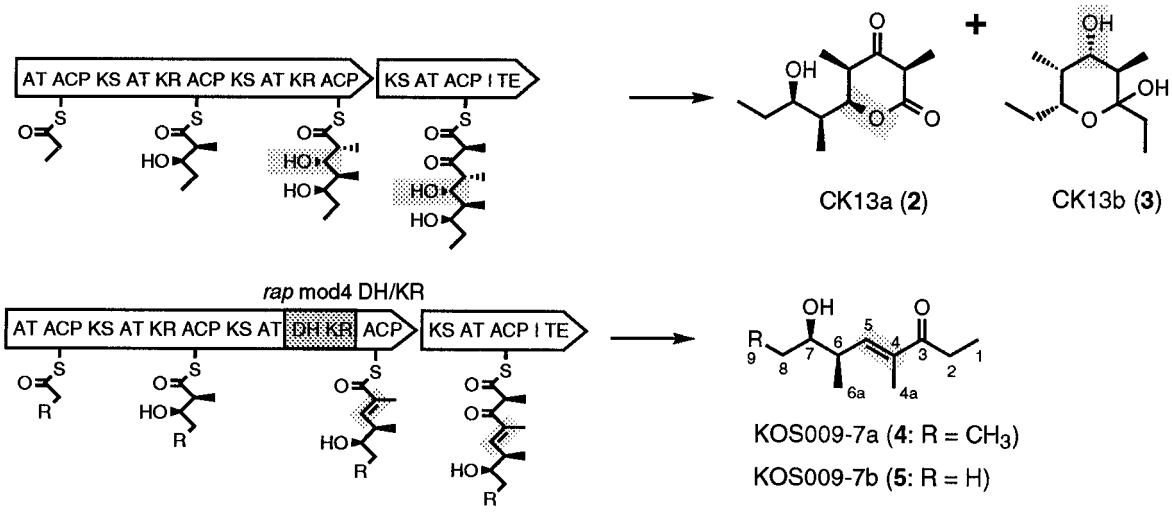

Figure 2. Polyketides biosynthesized by engineered erythromycin PKS genes. Expression of the first three DEBS modules in *S. coelicolor* CH999 results in production of the tetraketide lactone CK13a (**2**) and the decarboxylated tetraketide hemiketal CK13b (**3**).[14] Substitution of KR2 with a DH/KR cassette from module 4 of the rapamycin PKS[19] results in biosynthesis of the desired dehydrated tetraketides designated KOS009-7a (**4**) and KOS009-7b (**5**). As in the case of several other polyketides produced by DEBS PKSs in *S. coelicolor* CH999, both the propionate (**4**) and acetate (**5**) starter unit derived polyketides were isolated.[13,20,21] The acyclic form of the molecule, indicated by the C-3 carbonyl, presumably arises due to the presence of the trans double bond. KS, ketosynthase; AT, acyltransferase; ACP, acyl carrier protein; KR, β-ketoreductase; DH, dehydratase; TE, thioesterase.

potentially be combined with a loss-of-function mutation in the KR6 domain of DEBS to yield a 3-keto-10,11-anhydro derivative of 6-dEB. If so, then this intermediate should substantially simplify the preparation of existing as well as a variety of new ketolides, synthetic derivatives of erythromycin which show promising activity against a broad range of clinically relevant susceptible and resistant organisms.[17,18]

Our results reported here have two significant implications. On one hand, they illustrate the power of combinatorial biosynthesis for generating defined analogs of a known natural product pharmacophore. These analogs could in turn be further modified synthetically or biosynthetically to provide valuable insights into structure−activity relationships of the pharmacophore. More generally, we have demonstrated the feasibility of gain-of-function mutagenesis within the reductive segments of modular PKSs, which are largely responsible for the extraordinary steric and electronic features of the polyketide natural products. Further progress in harnessing the reductive and dehydrative variety in modular PKSs will undoubtedly increase the power of the combinatorial biosynthetic toolbox.

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(19) Plasmid pKOS009-7, containing the 3-module DEBS KR2 substitution, was constructed by inserting the rapamycin DH/KR domain into *Pst* I and *Xba* I restriction sites which had been engineered analogous to those in a previously described 2-module DEBS system.[10] The DH/KR cassette was PCR amplified with flanking *Pst* I and *Xba* I restriction sites (shown in bold) using the following primers: forward, 5′-**CTGCAG**AGCGTGGACCGGGCGGCT; reverse, **TCTAGA**GTCACCGGTAGAGGCGGCCCT.

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