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Mechanism and Engineering of Polyketide Chain Initiation in Fredericamycin Biosynthesis

Abhirup Das,[†] Ping-Hui Szu,[†] Jay T. Fitzgerald,[†] and Chaitan Khosla*[,†,‡,§]

Department of Chemistry, Department of Chemical Engineering, Department of Biochemistry, Stanford University, Stanford, California 94305

Received March 25, 2010; E-mail: khosla@stanford.edu

The actinomycetes produce structurally complex and diverse aromatic antibiotics using type II polyketide synthases (PKSs) and tailoring enzymes. In some cases, the presence of dedicated initiation modules in these PKSs leads to incorporation of atypical primer units into the corresponding polyketide products.[1,2] Two examples are the antibiotics hedamycin[3] (**1**) and fredericamycin[4] (**2**) (Figure 1). Genetic[3] and biochemical[5] studies have established that a multimodular (type I) PKS synthesizes a protein-bound C_6 intermediate in the hedamycin pathway, which is then transferred to the elongation PKS module. In contrast, the fredericamycin gene cluster lacks any type I PKS gene, even though it also synthesizes a very similar C_6 intermediate. Here we report on our biochemical analysis of polyketide chain initiation in the fredericamycin pathway and on the exploitation of this initiation PKS module to regiospecifically modify aromatic polyketides in a manner that cannot be readily achieved by other (bio)synthetic strategies.

The gene cluster involved in fredericamycin biosynthesis has been cloned and sequenced.[4] Among other genes, it encodes two ketosynthases (KS: FdmF and FdmS), a chain length factor (CLF: FdmG), an acyl carrier protein (ACP: FdmH), and two β-ketoreductases (KR: FdmC and FdmO); together, these proteins are thought to be responsible for polyketide chain initiation and growth. The presence of a single ACP in the gene cluster implies that the same protein serves this role in chain initiation and elongation (Figure S7).[6] A dedicated phosphopantetheinyl transferase (FdmW) has also been identified and shown to be required for polyketide biosynthesis.[7] We hypothesized two plausible mechanisms, outlined in Scheme 1, for biosynthesis of the hexadienyl-ACP intermediate by the initiation PKS module.[78] Both mechanisms start with FdmS-catalyzed decarboxylative condensation of a primer unit with a malonyl extender, followed by β-ketoreduction of the resulting intermediate by either FdmC or FdmO and dehydration of this β-hydroxythioester. As the *fdm* gene cluster lacks a dehydratase, the dehydration reaction is catalyzed by an extraneous dehydratase (DH) likely borrowed from the endogenous fatty acid synthase, as is the malonyl CoA:ACP transacylase (MAT). In mechanism 1, priming by acetyl-CoA leads to an initial round of elongation and processing, which is followed by a second identical round to yield the final hexadienyl priming unit. In contrast, in mechanism 2, priming by butyryl- or crotonyl-CoA necessitates only a single round of elongation and processing. In the final step of butyryl-CoA priming, oxidation and dehydration of the saturated chain occur to yield the expected hexadienyl moiety.

To test these hypotheses, FdmS, holo-FdmH, and FdmC were expressed, purified, and biochemically interrogated (for details, see Supporting Information). In the presence of malonyl-CoA, holo-FdmH, and MAT from *Streptomyces coelicolor*,[9] FdmS catalyzed

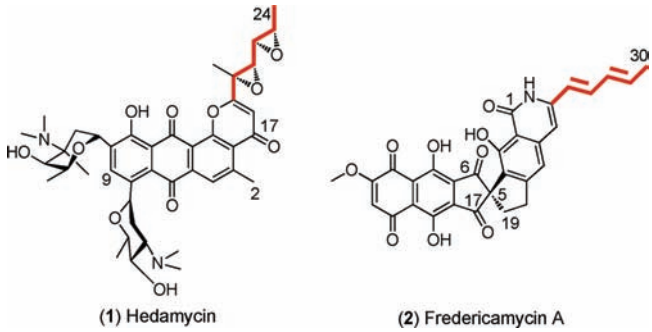


Figure 1. Natural products hedamycin and fredericamycin.

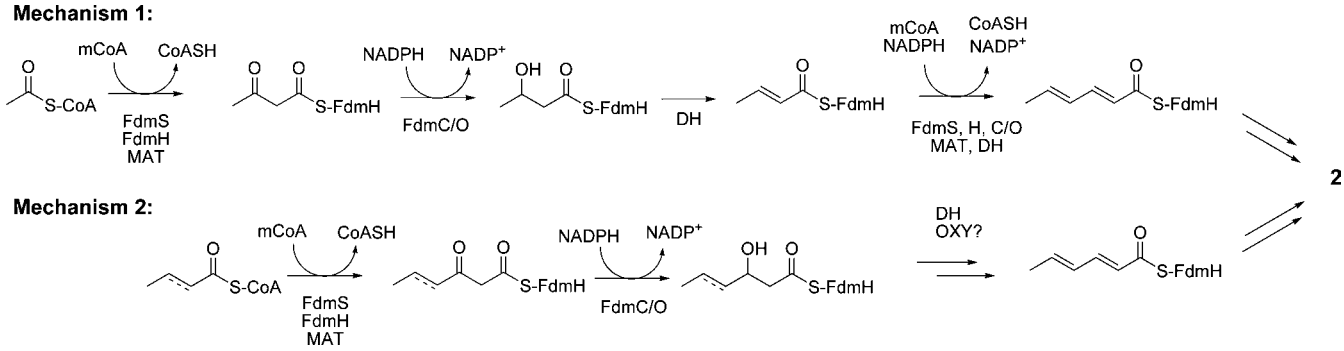
C−C bond formation in a reaction mixture containing either [1-^{14}C]acetyl-CoA or [1-^{14}C]butyryl-CoA, although the enzyme had >10-fold higher specificity for the latter substrate than the former. When the above reaction mixtures were supplemented with FdmC and NADPH, NADPH oxidation was spectrophotometrically observed. Malonyl-CoA was required to observe NADPH oxidation, indicating that reduction occurred on the product of the FdmS-catalyzed reaction. The steady-state rate of NADPH oxidation was comparable, regardless of whether the primer unit was acetyl-CoA, butyryl-CoA, crotonyl-CoA, or β-hydroxybutyryl-CoA. In the absence of any primer unit, the steady-state rate of NADPH oxidation was at least 5-fold lower; this low reaction rate is presumably due to either contaminating acetyl-CoA found in commercial malonyl-CoA or decarboxylative priming of FdmS with malonyl-CoA.

Together, these findings implied that either mechanism in Scheme 1 was feasible and that their relative balance would be likely dictated by intracellular concentrations of different acyl-CoA precursors. The viability of Mechanism 1 also suggests that FdmS is unique among initiation modules of type II PKSs in that it can catalyze two rounds of elongation before passing the nascent polyketide chain onto the elongation module. To verify this hypothesis, electrospray mass spectrometric analysis was performed on the FdmH ACP obtained from a reaction mixture containing either acetyl-CoA or $^{13}C_2$-acetyl-CoA, malonyl-CoA, MAT, holo-FdmH, FdmS, FdmC, and NADPH. The ACP showed a mass increase of 130 Da (132 Da for $^{13}C_2$-acetyl-CoA) from that of holo-FdmH, consistent with the presence of a covalently attached β,δ-dihydroxyhexanoyl intermediate on the pantetheinyl arm (Figures S3). Our biochemical data also suggested that FdmC alone was able to catalyze the reduction reactions ascribed to the initiation PKS module and that FdmO was unlikely to be required *in vivo*.

To evaluate the utility of the fredericamycin initiation PKS module for rationally designing novel polyketides, we sought to biosynthetically prepare regiospecifically modified analogues of YT84 (**3**), a simple 15-carbon polyketide obtained from the tandem

[†] Department of Chemistry.
[‡] Department of Chemical Engineering.
[§] Department of Biochemistry.

Scheme 1. Two Alternative Mechanisms for Chain Initiation of the Fredericamycin Polyketide Backbone



activities of the R1128 initiation module and the *act* PKS.[10] Coexpression of the *act* KS-CLF with FdmS, FdmC, FdmH, and FdmW[11] in *S. coelicolor* CH999/pAD284/pBOOST* resulted in formation of five major metabolites: SEK4[12] (**4**), SEK4b[13] (**5**), and three novel metabolites AD284a (**6**), AD284b (**7**), and AD284c (**8**) (Figure 2). NMR and mass spectrometric analyses (Supporting Information) verified the structure of the new metabolites. To confirm the necessity of FdmS in chain initiation, plasmid pAD282 was constructed lacking FdmS. Consistent with the *in vitro* data, the strain *S. coelicolor* CH999/pAD282/pBOOST* only produced SEK4 (**4**) and SEK4b (**5**).

Notably, AD284a and AD284c are regiospecifically modified analogues of YT84, the structures of which are consistent with the catalytic properties of the fredericamycin PKS initiation module, emphasizing the potential utility of the initiation module for combinatorial biosynthesis. They also reinforce the earlier conclusion that the minimal PKS controls polyketide chain length by recognizing the number of atoms incorporated into the backbone rather than the number of elongation cycles or other functional groups in the backbone.[14] The structures of AD284a and AD284c also implicate the involvement of an extraneous dehydratase and enoyl reductase both of which are presumably borrowed from the endogenous fatty acid synthase of *S. coelicolor*.[1,2,14]

The structure of AD284b also has interesting mechanistic implications. Specifically, two alternative biosynthetic mechanisms can be proposed (Figure S8). On one hand, AD284b may result from premature transfer of a C_4 butyryl intermediate between the initiation and elongation PKS modules of the hybrid synthase encoded on plasmid pAD284. Alternatively, FdmS could catalyze two rounds of chain elongation, but FdmC may have only reduced the first of the two β-ketoacyl-ACP intermediates. Both models are consistent with the available biochemical data summarized above; hence, additional experiments would be required to resolve these alternative models.

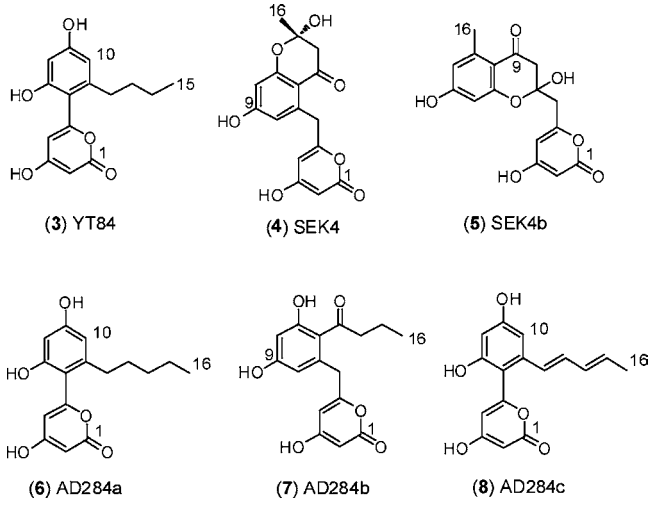


Figure 2. Structures of isolated polyketide products.

To disentangle the roles of FdmO and FdmC *in vivo*, plasmid pAD280 was constructed harboring the *fdmO* gene in place of *fdmC*. The strain *S. coelicolor* CH999/pAD280/pBOOST* produced SEK4 (**4**) and SEK4b (**5**) but none of compounds **6**−**8**. In addition the strain CH999/pAD242/pBOOST* containing both *fdmC* and *fdmO* was constructed as a positive control to ensure that FdmO did not play an inhibitory role. This strain produced a compound mixture identical to that of CH999/pAD284/pBOOST*. Taken together these results imply that FdmO has no role to play in the initiation module.

In summary, we have analyzed the properties of an unusual initiation PKS module from the fredericamycin biosynthetic pathway and have exploited its unique properties to develop a general strategy for replacing a methyl substituent in a polyfunctional aromatic product of a type II PKS with a hexanoyl or hexadienyl substituent. In the course of these studies, we have uncovered an unusual chain initiating KS that is capable of catalyzing two successive rounds of C−C bond formation before passing on the intermediate to the elongation module, and a dedicated KR that catalyzes β-ketoreduction on the product of each elongation cycle. In contrast to the much more elaborate (type I) chain initiation strategy of the hedamycin PKS, the fredericamycin PKS is simple and can therefore be readily exploited by the biosynthetic engineer. Parenthetically, we also note that our analysis of the initiation mechanism of the fredericamycin PKS has provided a clearer context to decode A-74528 biosynthesis.

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Supporting Information Available: Experimental procedures, and compound characterizations. This material is available free of charge via the Internet at http://pubs.acs.org.

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(8) Other mechanisms can also be proposed, such as formation of a C_{30} poly-β-ketoacyl chain, followed by reduction and dehydration of two terminal ketone functional groups. In this mechanism FdmS lacks an obvious function. We considered this mechanism unlikely, because we have been unable to identify a 30-carbon polyketide in a heterologous host that only expresses the minimal PKS comprising FdmF, FdmG, FdmH, and the FdmW phosphopantetheinyl transferase (data not shown).
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