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Cascade Reactions during Coronafacic Acid Biosynthesis: Elongation, Cyclization, and Functionalization during Cfa7-Catalyzed Condensation

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Modular polyketide synthases (PKSs) produce a myriad of pharmaceutically relevant natural products, for example, epothilones, erythromycin, and FK506.[1] Each PKS module comprises a set of catalytic and carrier protein domains that are responsible for carrying out a single polyketide elongation and modification. The modular nature of these enzymes results in the direct correlation between the functional groups present in the polyketide chain and the organization of catalytic domains. This one-to-one correlation, which is often referred to as the colinearity rule, has facilitated the prediction of polyketide structures directly from genome sequences.[2] Recently, however, there have been several examples of modular PKSs that do not follow the colinearity rule, suggesting that modular PKSs have a much greater potential for molecular diversity.[3] One possible context where the colinearity rule could fail, yet to be described, is that upon elongation of the polyketide chain, the resulting β-keto intermediate can undergo additional chemistry in a cascade-like fashion.[4] In this example, the structure of the polyketide could not be predicted by the sequence of the biosynthetic genes alone, as would be the case with canonical modular PKSs.

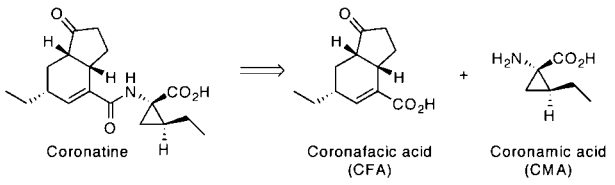
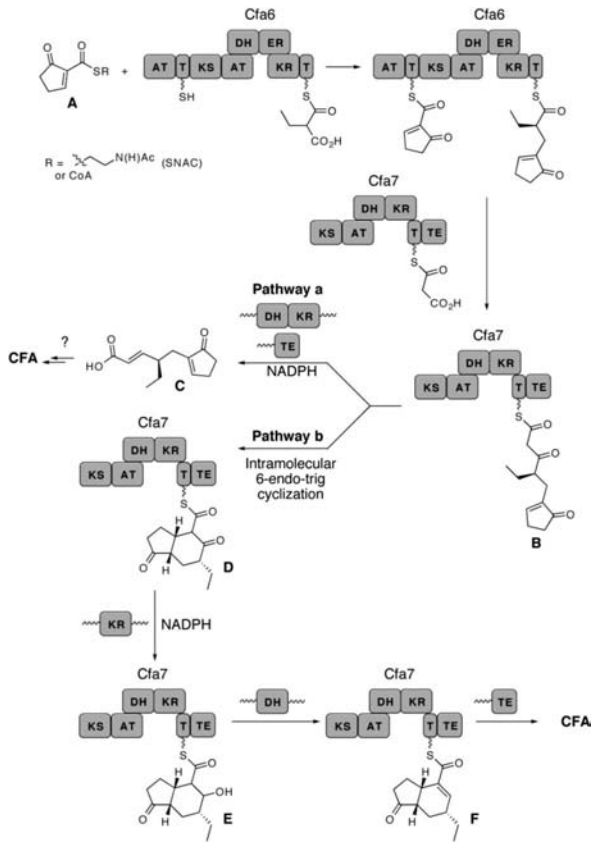


Figure 1. Coronatine and its two components, coronafacic acid (CFA) and coronamic acid (CMA).

To explore whether cascade reactions can occur during the elongation of a polyketide chain by a modular PKS, we chose to examine the biogenesis of the hydrindane ring system in coronafacic acid (CFA).[5] CFA is the polyketide portion of coronatine (Figure 1), a plant toxin produced by *Pseudomonas syringae* that subdues the innate immune response of the plant by mimicking a key hormone, jasmonate.[6,7] The gene cluster that is responsible for the biosynthesis of CFA contains two modular PKS units.[8] On the basis of the proposed biosynthetic pathway for CFA, the cyclopentenone starter unit **A** is shuttled to the first modular PKS, Cfa6.[8] Upon condensation with ethyl malonate via the action of Cfa6, the enzyme-bound intermediate is transferred to the downstream PKS, Cfa7, and condensed with malonate (Scheme 1). Two different products can potentially result from this condensation event. The first (pathway a in Scheme 1), would require the immediate tailoring of the enzyme-bound intermediate **B** via the catalytic action of the ketoreductase (KR), the dehydratase (DH), and the thioesterase (TE) embedded

Scheme 1. Proposed Biosynthetic Pathway for CFA



within Cfa7, to generate the linear product **C**. CFA can then be generated after further modification of **C**. The second possibility (pathway b in Scheme 1) involves the intramolecular 6-*endotrig* cyclization of the tethered intermediate **B** to form the hydrindane ring-containing intermediate **D**. In this case, the intramolecular cyclization of **B** occurs at a rate faster than the ketoreduction and dehydration. The significance of the latter pathway is that product **D** contains greater molecular complexity than that of the linear product **C**; that is, a 5,6-fused all-carbon ring system harboring two new stereogenic centers is formed in a single reaction. Moreover, the possible presence of intermediate **D** suggests that both the KR and DH domains are capable of modifying the polyketide once it has undergone the intramolecular cyclization.

To examine whether the hydrindane ring system of CFA is formed while a linear polyketide intermediate is bound to the enzyme, the 221 kiloDalton (kDa) protein, Cfa7, was required in both soluble and active form. After several trials, the only conditions under which soluble Cfa7 was obtained occurred while

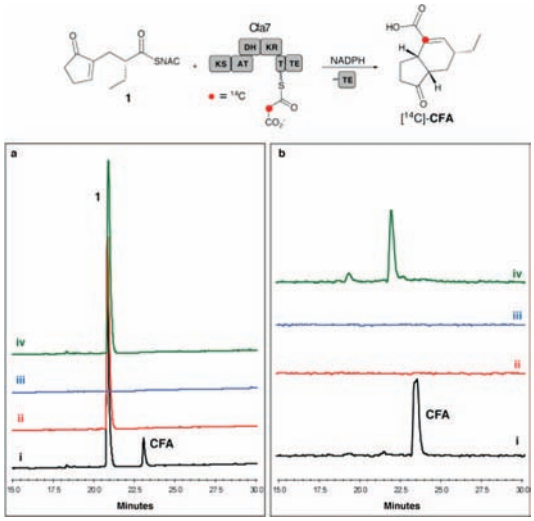


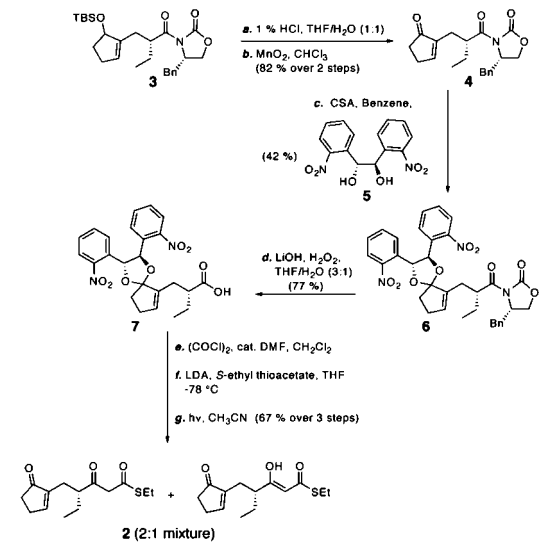
Figure 2. HPLC (a) and radio-HPLC (b) analysis of the Cfa7-catalyzed condensation between **1** and malonate: (i) Cfa7, **1**, and NADPH present in the reaction; (ii) Cfa7 is absent from the reaction; (iii) **1** is absent; and (iv) NADPH is absent.

using *Pseudomonas putida* KT2440 as an expression host. To subsequently determine whether Cfa7 was active, the autoacylation of Cfa7 using radio-labeled [2-^{14}C] malonyl CoA was performed. By monitoring the incorporation of [2-^{14}C] malonyl onto the holoform of Cfa7 by autoradiography we could establish that the acyltransferase (AT) domain of Cfa7 activates [2-^{14}C] malonyl CoA and loads the [2-^{14}C] malonyl group onto the phosphopantetheinyl group tethered to the carrier domain (T) (see Supporting Information).

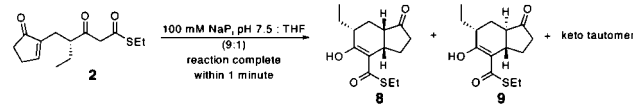
With the autoacylation ability of Cfa7 established, the condensation reaction between the [2-^{14}C] malonyl group and a thioester containing the cyclopentenone moiety was investigated. To examine the condensation, the *N*-acetylcysteamine (SNAC) derivative **1**[9] was synthesized as a surrogate for the proposed thioester intermediate that is bound to Cfa6. Upon incubating **1** with Cfa7, [2-^{14}C] malonyl CoA, and NADPH, a new product was observed by both HPLC and radio-HPLC (Figure 2a,b-i). This new product did not form, however, while performing the condensation in the absence of Cfa7 (Figure 2a,b-ii), **1** (Figure 2a,b-iii), or absence of NADPH (Figure 2a,b-iv), suggesting that product formation is dependent on the KR domain. Further analysis of the reaction product by LC−MS and ^{1}H NMR, indicated that the product is indeed CFA. These results suggest that the intramolecular cyclization to form the hydrindane ring system is faster than the subsequent ketoreduction[10] and dehydration.

The direct formation of CFA from the Cfa7-catalyzed elongation also indicates (1) that additional chemistry can occur other than that dictated by the catalytic domains embedded within the synthase and (2) the tethered intermediate (**D**) can be reduced and dehydrated on the assembly line. This observation is not without precedent, as a similar sequence of events has been observed during the biosynthesis of lovastatin.[11] In that case, an enzyme-bound hexaketide intermediate undergoes a Diels−Alder cyclization to generate the decalin ring system in lovastatin. The Diels−Alder cyclization is at least enzyme-assisted since the cyclization of the hexaketide SNAC thioester in aqueous media results in a mixture of diastereomers, while in the enzyme-catalyzed cyclization a single stereoisomer is formed.

Scheme 2. Synthesis of β-Keto Thioester **2**



Scheme 3. Intramolecular Cyclization of **2**



To ascertain what role Cfa7 plays in the intramolecular cyclization, the linear β-keto thioester **2** was synthesized (Scheme 2) and the enzyme-independent cyclization examined. The β-keto thioester **2** was subjected to a buffered aqueous solution (a 9:1 mixture of 100 mM sodium phosphate/THF) in the absence of Cfa7 to mimic the conditions in which the cyclization occurs in the Cfa7-catalyzed elongation of **1** with malonate (Scheme 3). In the event, a complex mixture of products results from this reaction as indicated by ^{1}H NMR analysis (see Supporting Information). However, **2** is completely consumed within this time scale and the major product (64% yield based on ^{1}H NMR) exhibits a *m/z* ratio identical to that of **2**, suggesting that the *6-endotrig* cyclization occurs in a facile manner. Upon further analysis of the reaction mixture by 2D ^{1}H NMR it is evident that a ca. 4:1 diastereomeric ratio of *cis*-**8** and *trans*-**9** is formed. Unfortunately, due to the heterogeneity of the reaction mixture, detailed kinetics of the cyclization could not be performed. Nonetheless, the observation that the 6-*endotrig* cyclization[14] rapidly occurs in the absence of Cfa7 indicates that the enzyme does not necessarily lower the energy barrier for cyclization. Rather, the formation of multiple stereoisomers in the absence of Cfa7 suggests that Cfa7 plays a role in controlling the stereochemistry of the cyclization.

The enzyme-independent cyclization was then utilized to investigate which product forms during the Cfa7-catalyzed condensation between **1** and malonate in the absence of NADPH. Since the ^{14}C-radiolabeled product shown in Figure 2b-iv could not be directly detected by LC-MS or ^{1}H NMR, hydrolysis of the cyclized β-keto thioester **8/9** was performed. As shown in Figure 3a, hydrolysis of **8/9** at pH 9.5 results in the formation of the cyclized β-keto acid **10**, which co-elutes with the ^{14}C-radiolabeled product from Figure 2b-iv. This result indicates that when the PKS is stalled due to the absence of NADPH, the intermediate **D** (Scheme 1) is formed and cleaved from Cfa7. Subsequently, the Cfa7-catalyzed reduction of **8/9** was examined using [^{3}H]-NADPH[15] (Figure 3b) and indeed [^{3}H]-CFA was

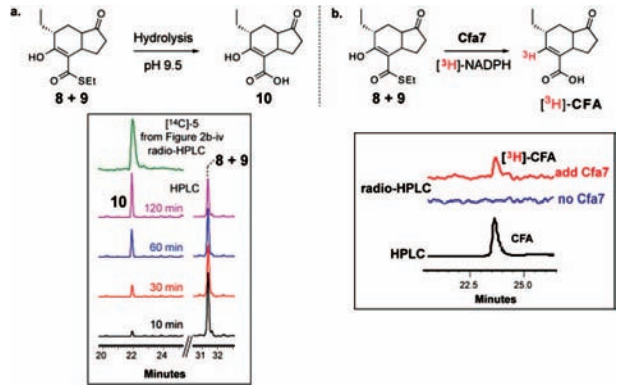


Figure 3. (a) Hydrolysis of **8**/**9** to afford **10** at pH 9.5 and co-elution with the ^{14}C-radiolabeled product from Figure 2b-iv; (b) radio-HPLC analysis of the Cfa7-catalyzed reduction of the β-keto thioester **8**,**9** using [^{3}H]-NADPH.

observed by radio-HPLC, providing additional support for the Cfa7-KR-catalyzed reduction of the cyclized β-keto thioester.

In summary, by examining how the hydrindane ring system of coronafacic acid is constructed, we have demonstrated that in addition to the canonical polyketide chain elongation and functionalization encoded by type I PKSs, cascade reactions can take place during assembly line-like biosynthesis.[16] Latent reactivity within the substrate must be unveiled upon elongation in order for cascade reactions to proceed at a rate faster than the functionalization reactions encoded by the catalytic domains within the type I PKS. Herein, the latency resides within the thioester intermediate harboring the cyclopentenone moiety such as **1**. Upon Claisen condensation with malonate, the reactive β-keto thioester intermediate can undergo a conformationally controlled 6-*endotrig* cyclization prior to the two subsequent steps of ketoreduction and dehydration encoded by the PKS that now work on the tethered bicyclic intermediates. The low reaction barrier for the 6-*endotrig* cyclization is further exemplified by the immediate formation of cyclized products **8** and **9** in the enzyme-independent reaction. This underlying reactivity, thus, generates molecular complexity in the form of a carbocyclic fused-ring system that is uncommon for type I PKSs. Whether or not cascade reactions during thio-templated type I PKS-catalyzed elongation are a general mechanism for creating molecular complexity remains to be seen. However, the biogenesis of complex molecular architectures by type I PKSs such as those observed in tetronasin[17] and spinosyn[18] suggests that it is possible for other type I PKS systems to utilize cascade reactions in a similar manner to that described for coronafacic acid.

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Supporting Information Available: Experimental procedures and NMR data for compounds **1**−**8**. This material is available free of charge via the Internet at http://pubs.acs.org.

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