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One-Pot Enzymatic Total Synthesis of Presteffimycinone, an Early Intermediate of the Anthracycline Antibiotic Steffimycin Biosynthesis

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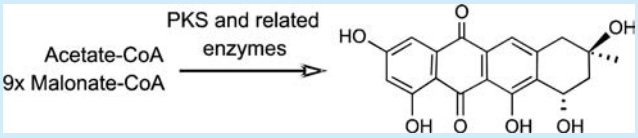
Supporting Information

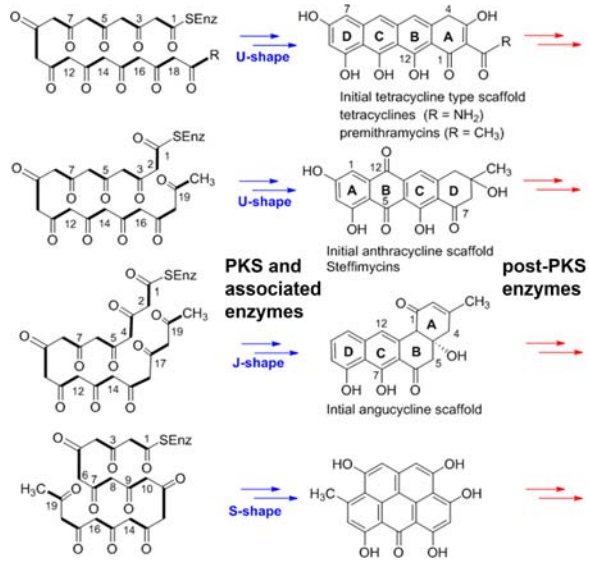
ABSTRACT: Early acting cyclases play critical roles in programming the polyketide biosynthesis toward certain, distinguished scaffolds. Starting from acetyl-CoA and malonyl-CoA, a one-pot enzymatic total synthesis of an anthracyclinone scaffold, presteffimycinone, was achieved by mixing polyketide synthase (PKS) and early post-PKS enzymes from the biosynthetic pathways of three different types of type II-PKS driven anticancer antibiotics, namely, the mithramycin (aureolic acid-type), gilvocarcin (rearranged angucycline-type), and steffimycin (anthracycline) pathways.



Aromatic polyketides represent a large group of important natural products with large structural diversity; some of them are clinically important therapeutics, including doxorubicin, tetracycline, and mithramycin.[1] Structural diversity of such polyketide scaffolds results from a number of factors, such as type of starter unit and the number of elongation cycles, but mostly from the type of polyketide synthase (PKS) and associated enzymes that lead to distinct cyclization patterns. Numerous decoration reactions after the formation of a core scaffold, so-called post-PKS tailoring steps, further enrich the diversity.[2,3] Cyclizations, which are catalyzed by PKS-associated cyclase or aromatases with assistive effect of the minimal PKS, play key roles in determining the initial polyketide framework that distinguishes the different classes of resulting natural products.[4−6] While the aromatic scaffolds produced by nonreducing fungal PKSs are mainly dependent on a PT (product template) domain, uniquely found in fungal PKSs, the situation in aromatic polyketides synthesized by bacterial type II (or iterative) PKSs is less clear. Given rounds of iterative condensation reactions of simple substrates typically derived from acetate, propionate and/or malonate generate a polyketide chain intermediate of certain length that is controlled by the β-subunit of the PKS (KS_β, therefore also called chain length factor (CLF)).[4] This is followed by PKS-associated enzymatic reactions that include a certain pattern of cyclization reactions to eventually afford an initial core structure (Scheme 1).

Among bacterial decaketides, a handful of typical cyclization reactions modify the 20-carbon poly-β-keto-ester chain into a few groups of initial, often tetracyclic scaffolds (Scheme 1).[5] Most of them adopt a C7−C12 aldol cyclization reaction to initiate the formation of the first ring, which can be catalyzed by an aromatase/cyclase, such as MtmQ in the mithramycin pathway,[7] though other factors are possibly involved as well, and the so-called minimal PKS, consisting of KS, CLF, and

Scheme 1. Cyclization Patterns of Different Folded Decaketides



ACP (acyl carrier protein) alone, can be sufficient to determine this first cyclization reaction.[8] If cyclizations of second-, third-, and fourth-ring occur in a U-shaped assembly of enzymes, e.g., between C5−C14, C3−C16, and C1−C18, linear polyphenols such as the tetracyclines and mithramycins are formed. Anthracycline antibiotics (e.g., doxorubicin, steffimycin) also require a U-shaped enzyme arrangement but differ from these only in that the fourth ring is formed through a C2−C19 cyclization.[9] If the enzyme arrangement is J-shaped, a C4−C17

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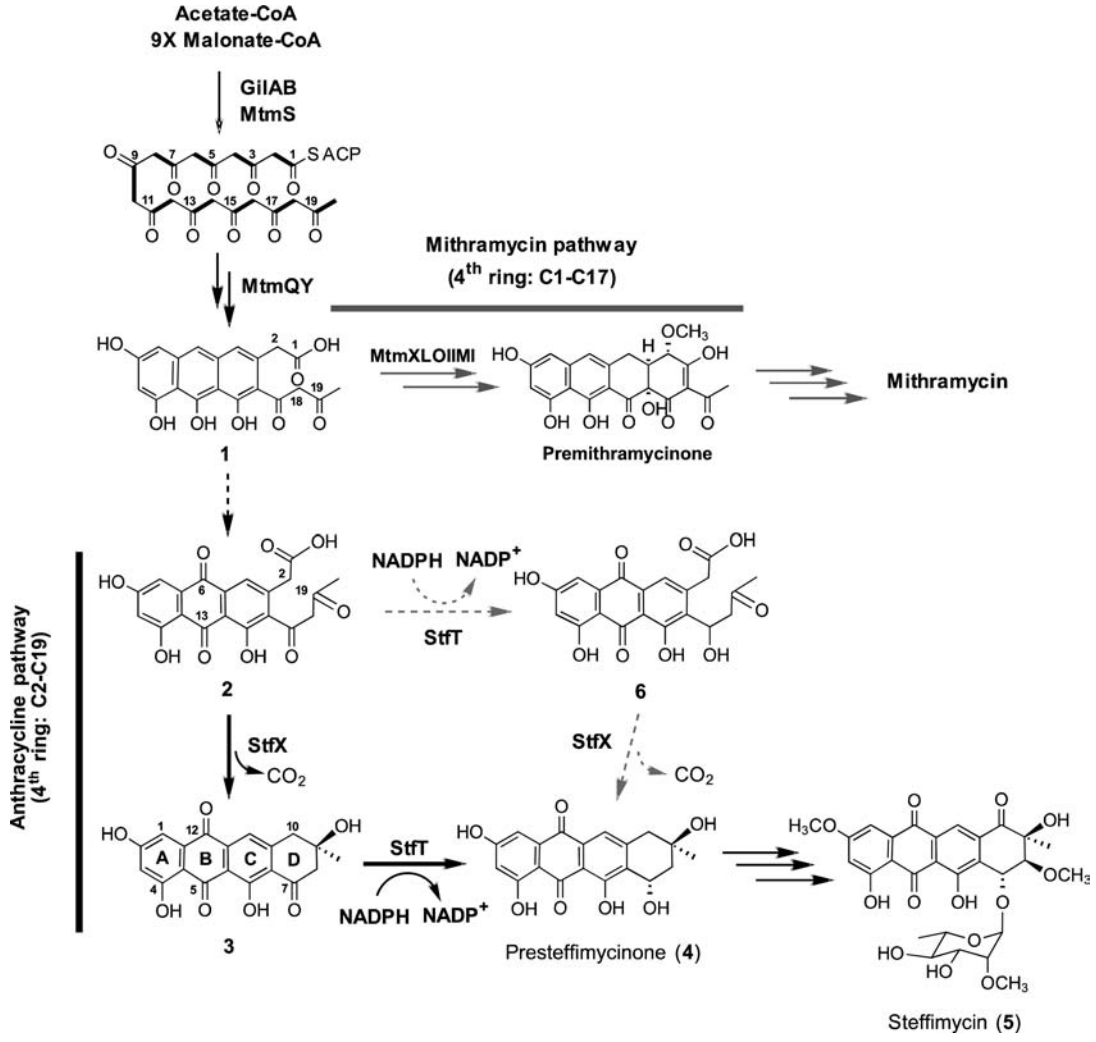
Scheme 2. Biosynthetic Pathways of Steffimycin and Mithramycin



cyclization is adopted for the third-ring, typical angucyclines emerge, which feature an angular skeleton.[10] Other scaffolds, e.g., tetracenomycins and resistomycins, arise from deviations already in their initial cyclization and undergo a C9−C14 initial cyclizations. While the PKS and associated enzymes are also assembled in a U-shape in case of the tetracenomycins, the resistomycins require a unique S-shaped enzyme assembly, leading to four follow-up cyclizations resulting in a unique pentacyclic discoid scaffold.[11]

We wanted to test whether combinatorial biosynthetic enzymology involving early framework-determining enzymes from different pathways is possible to establish a desired scaffold. Since all these enzymes have to assemble themselves into a functioning multienzyme complex, it was questionable whether such arrangements could work, especially if the enzymes were drawn from pathways fundamentally different in the normally resulting molecular frameworks. The studies also aimed to understand the exact functions of the interrogated cyclases. Protein crystal structures of cyclases revealed important structural features and mechanisms,[6] but suggest that an optimal cooperation of enzymes is only achieved when all enzymes stem from the same pathway. Here, we report the total synthesis of presteffimycinone, a key intermediate of the biosynthesis of the anthracycline antibiotic steffimycin, by combining PKS enzymes and cyclases of aureolic acid-, angucycline-, and anthracycline pathways.

Steffimycin (**5**), which was first isolated in 1967 from *Streptomyces steffiburgensis*,[12] is an unusual anthracycline-type antitumor antibiotic because of its rich decoration with oxygen atoms in rings A and D.[13] The compound has been shown to induce DNA damage, thus inducing high apoptotic response in p53-expressing HCT116 colon carcinoma cells;[14] several derivatives of steffimycin or chemically modified analogues also showed good carcinoma-repression effects.[15] Steffimycin contains several unique structural features that are absent in other anthracyclines, including the C10 keto group, two methoxy groups (C2 and C8), and a neutral deoxysugar instead of the more typical aminosugar (Scheme 2). Another attraction of this compound is its fourth-ring formation. Unlike other anthracyclines (daunorubicin and nogalamycin), which use a methyl ester substrate for the fourth-ring formation, the enzyme associated with the fourth cyclization in the steffimycin pathway, StfX,[13] acts on an ACP bound thioester. To further clarify details of this process and to check whether StfX alone can be used and is sufficient to turn other pathways toward anthracycline biosyntheses, we set up a total enzymatic synthesis, combining StfX with angucycline and mithramycin enzymes, to obtain the earliest anthracyclinone intermediate of the steffimycin pathway.

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First, we tried to express the PKS of the mithramycin pathway, MtmPK (polyketide synthase α- and β-subunit, respectively). MtmPK was unfortunately insoluble when expressed in *Escherichia coli*, and after repeated futile trials we used GilAB from the gilvocarcin biosynthetic pathway instead, for which soluble expression in *S. lividans* TK24 had been established.[16] Two different ACPs, MtmS of the mithramycin and RavC of ravidomycin pathway,[17] also were expressed in *E. coli* and purified. These are first subjected to a posttranslational phosphopantetheinylation modification to become holo-ACPs (see Supporting Information for details). Other PKS related enzymes including the aromatase/cyclase (ARO/CYC) MtmQ, the cyclase MtmY (CYC), the malonyl-CoA:ACP transacylase GilP (MCAT), and MatB (for malonyl-CoA synthesis) from *S. coelicolor*[18] were also solubly expressed in *E. coli* and purified (Figure S1). During polyketide biosynthesis, simple molecules such as acetate and malonate are condensed by PKS to form a long polyketide chain. We first evaluated the efficacy of *gil*PKS with heterologous ACP and CYCs.

As shown in Figure 1, the *gil* pathway PKS enzymes GilAB and GilP work well with the ACP from the mithramycin

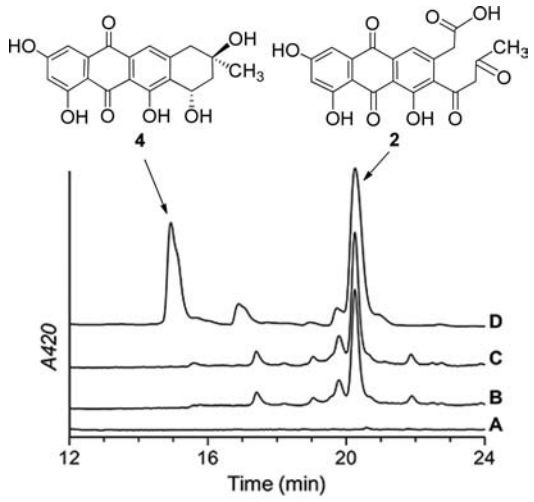

Figure 1. Efficacy assay of PKS with different acyl carrier proteins. (A) Enzymes GilP, MtmQY, RavC with acetyl-CoA, and malonyl-CoA (no PKS control reaction); (B) enzymes GilABP, MtmQY, RavC with acetyl-CoA, and malonyl-CoA; (C) enzymes GilABP, MtmQYS with acetyl-CoA, and malonyl-CoA; (D) **2** and **4** standards prepared from *S. albus* expressing pEM4KSEHX-Hyg.[13]

(MtmS) or ravdomycin (RavC) pathways; when including the *mtm* encoded cyclases MtmQ and MtmY, the first three rings were formed correctly between C7−C12, C5−C14, and C3−C16, respectively, to generate 2-hydroxy nogalonic acid (**2**), suggesting PKS and early associated enzymes of different pathways cooperate nicely.

Next, cyclase StfX and ketoreductase StfT were included into the enzyme mixture to test whether **2** will be converted to an anthracycline product. Presteffimycinone (**4**), the first intermediate of anthracycline pathway toward steffimycin (**5**), was observed in the reaction extract, as demonstrated by LC−MS analysis and ^{1}H NMR (Figure 2A, Figures S3 and S6), suggesting StfXT are responsible for the C2−C19 cyclization of the fourth ring to form an anthracyclinone scaffold. In this reaction, trace amount of the peak eluted at the same retention time as **2** was collected. Though they shared a similar retention time, this peak is not **2** as m/z = 399 was observed (Figure S4).

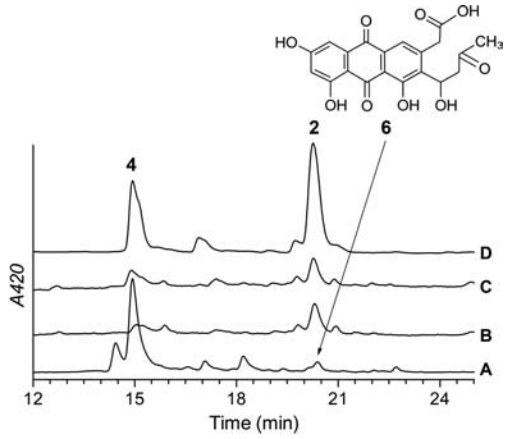

Figure 2. Enzymatic generations of presteffimycinone (**4**). (A) Enzymes GilABP, MtmQYS, StfXT with acetyl-CoA, and malonyl-CoA; (B) enzymes GilABP, MtmQYS with acetyl-CoA, and malonyl-CoA (no StfXT control reaction); (C) enzymes GilABP, MtmQYS, StfX with acetyl-CoA, and malonyl-CoA (no StfT control reaction); (D) **2** and **4** are standards prepared from *S. albus* expressing pEM4KSEHX-Hyg.[13]

We postulated that the 17-ketone group of **2** was reduced by StfT to yield compound **6** (Scheme 2). This finding is intriguing in that StfT is able to act on a fourth-ring open substrate indicating a significant substrate flexibility of this enzyme. The finding also proposed a possible new biosynthetic route for steffimycin, StfT acts before StfX (gray dashed arrows in Scheme 2), which is opposite to the previous proposed pathway based on gene knockout experiments (bold arrows in Scheme 2).[13] Notably, when only StfX was supplemented into the above-described reaction mixture, thus omitting StfT, **4** was still synthesized, albeit in a much lesser yield (Figure 2C); another reductase or hydrolase in the reaction mixture might act in the place of StfT to catalyze the reduction.

However, we failed to shift the production toward a tetracycline/mithramycin scaffold (Scheme 2, toward mithramycin). When adding MtmX, presumed to be the fourth cyclase of the *mtm* pathway, to the above-mentioned PKS and early associated enzymes mixture (GilABP + MtmQYS), the fourth ring failed to form; rather, the quinone compound **2** was produced (Figure S5), and neither the addition of MtmL, a homologue of the recently identified catalyst of the C1−C18 Claisen condensation that closes the fourth ring to complete the tetracycline framework.[19] Despite extensive in vitro enzymatic synthesis or in vivo heterologous expression experiments, all trials failed to reconstitute premithramycinone production, but led to a major production of the quinone **2**, which is an intermediate of the steffimycin but not the mithramycin pathway (Scheme 2), suggesting prevention of oxidation of the second ring to a quinone is critical for the mithramycin biosynthesis. In the closely related tetracycline pathway, OxyF introduced a methyl group on the second ring, which might help to prevent this oxidation.[20]

In summary, this work describes a one-pot reaction of eight PKS and associated enzymes including ACP and cyclases from three different pathways leading to the anthracyclinone presteffimycinone (**4**), the first intermediate of the steffimycin biosynthetic pathway.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b03708.

Methods and Materials, Table S1, and Figures S1−S6 (PDF)

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Notes

The authors declare no competing financial interest.

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