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

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Abstract

Early acting cyclases play critical roles in programming the polyketide biosynthesis towards 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.

Abstract

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 mithramycin¹. Structural diversity of such polyketide scaffolds results from a number of factors, such as type of starter unit, the total amount of building blocks, but mostly from the type of polyketide synthase (PKS) with 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 the minimal PKS and PKS associated cyclases or aromatases, 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 non-reducing fungal PKSs are mainly dependent of a PT (product template) domain, uniquely found in fungal PKSs, the situation in aromatic polyketides synthesized by bacterial type II (or iterative)

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Supporting Information is Available. The supporting Information is included in the online version.

PKSs is less clear. A given rounds of iterative condensation reactions of simple substrates, typically acetate, propionate and/or malonate building blocks, generate a polyketide chain intermediate of certain length that is controlled by the β -subunit of the PKS (KS_{β} , therefore also called CLF = chain length factor)⁴. This is followed by PKS-associated enzyme 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 pathway⁷, though other factors are possibly involved as well, and the so-called minimal PKS, consisting of KS, CLF and ACP (acyl carrier protein) alone can be sufficient to control this first cyclization reaction. If cyclizations of 2nd-, 3rd-, 4th-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 4th ring is formed through a C2-C19 cyclization⁸. If the enzyme cluster is J-shaped, a C4-C17 cyclization is adopted for the 3rd-ring, and typical angucyclines emerge, which feature an angular skeleton⁹. Other scaffolds, e.g., tetracenomycins, 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 4 follow-up cyclizations resulting in a unique pentacyclic discoid scaffold¹⁰.

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 multi-enzyme 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 on understanding the exact functions of the interrogated cyclases. Protein crystal structures of cyclases revealed important structural features and mechanisms, 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 pre-steffimycinone, 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*¹¹, is an unusual anthracycline-type antitumor antibiotic because of its rich decoration with oxygen atoms in rings A and D¹². The compound has been shown to induce DNA damage, thus inducing high apoptotic response in p53-expressing HCT116 colon carcinoma cells¹³; several derivatives of steffimycin or chemically modified analogues also showed good carcinoma-repression effects¹⁴. Steffimycin contains several unique structural features which 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 4th-ring formation. Unlike other anthracyclines (daunorubicin and nogalamycin), which use a methyl ester substrate for the 4th-ring formation, the enzyme associated with the 4th cyclization in the steffimycin pathway, StfX¹², 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 towards 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.

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 established¹⁵. Two different ACPs, MtmS of the mithramycin and RavC of ravidomycin pathway¹⁶ also were expressed in *E. coli* and purified. These need first be 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*¹⁷ were also solubly expressed in *E. coli* and purified (Figure S1). During polyketide biosynthesis, simple molecules such as acetate and malonate are condensated 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 (MtmS) or ravidomycin (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 flawlessly.

Next, cyclase StfX and PKS-associated 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 HPLC (Figure 2A) and HR-MS analysis (Figure S3), suggesting StfXT are responsible for the C2-C19 cyclization of the fourth ring to form an anthracyclinone scaffold. 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), unambiguously indicating that a single cyclase StfX is able to shift the biosynthetic pathway towards anthracycline compounds (Scheme 2).

However we failed to shift the production toward a tetracycline/premithramycin scaffold (Scheme 2, towards premithramycinone). 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, although the first three rings were formed correctly. This fourth cyclization failed both (i) upon just adding MtmX and (ii) upon adding all post-PKS enzymes of the *mtm* pathway (Figure S5). Thus, even the addition

of MtmL, a homologue of the recently identified catalyst of the C1-C18 Claisen condensation that closes the 4th ring to complete the tetracycline framework¹⁸, did not accomplish the 4th ring cyclization. The intermediate **1** was always spontaneously converted to the quinone **2**, which is an intermediate of the steffimycin but not the mithramycin pathway (Scheme 2), suggesting prevention of spontaneous oxidation of the second ring to a quinone, e.g., through partly anaerobically operating enzymes, 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 oxidation¹⁹.

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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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.

Figure 2.

HPLC traces of enzymatic generations of pre-steffimycinone (**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-Hyg¹².

Scheme 1.
Cyclization patterns of different folded decahydrides

Scheme 2.
Biosynthetic pathways for steffimycin and mithramycin.