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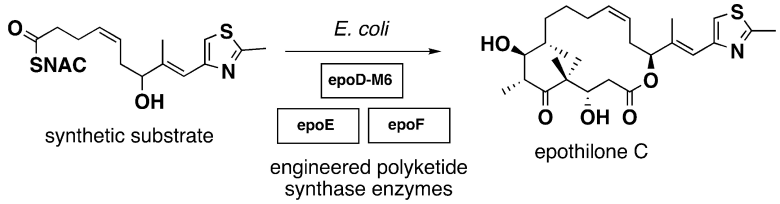

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Precursor-Directed Biosynthesis of Epothilone in *Escherichia coli*

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Combined chemical and enzymatic approaches to complex molecule synthesis may ultimately provide access to analogues with improved pharmacological properties in quantities sufficient for clinical development. To this end, we report the reconstitution of the epothilone biosynthetic pathway. An *E. coli* strain engineered to express the last half of the epothilone biosynthetic pathway is able to process exogenously fed synthetic substrate into epothilone C (Figure 1) at levels comparable to the native host.

The epothilone family of compounds is a group of polyketide natural products produced by the myxobacterium *Sorangium cellulosum*.[1] These small molecules are potent stabilizers of microtubules. Prolonged exposure to epothilones results in mitotic arrest followed by apoptotic cell death in a wide range of cell lines, making them interesting as anticancer agents.[2]

Epothilone C (**1**, Figure 1) is produced by the combined action of one nonribosomal peptide synthetase and nine polyketide synthase modules in a multienzyme system.[3] These proteins catalyze the formation and tailoring of the epothilone carbon−carbon backbone in a linear fashion starting with the heteroaromatic side chain.[4]

A key consideration when designing the engineered epothilone biosynthetic pathway was the desire to introduce chemical modifications into the polyketide backbone. Structure−activity studies have shown that microtubule stabilization activity is lost when the C1−C8 region of epothilone is modified.[2,5] The remainder of the molecule, which is produced in the early stages of the biosynthetic pathway, is relatively tolerant to modification.[2] Therefore, we focused on a precursor-directed approach because it provides a flexible mechanism for modifying the tolerant region of epothilone C.

Precursor-directed biosynthesis provides a powerful method to introduce non-native starting materials into biosynthetic pathways.[6] In polyketide biosynthesis, a non-native substrate acylates the ketosynthase domain active site of the first polyketide synthase, gaining entry into the pathway. By eliminating enzymes upstream of the desired entry point, it is possible to prevent the native substrate from out-competing the non-native precursor. Carrying out these experiments in vivo allows long biosynthetic pathways to be reconstituted and avoids difficult and costly protein purification and cofactor regeneration.

Identifying an appropriate precursor-directed entry point into the epothilone pathway required careful consideration. The likely rate-limiting step of this experiment is the initial acylation of the first polyketide synthase protein by the non-native precursor. Because this reaction has a very high K_M,[7] the reaction rate is maximized by having high concentrations of both protein and substrate. Therefore the precursor must be simple enough to be chemically accessible in large quantities and the polyketide synthase protein must be small enough (<200 kDa) to express well. Based on these considerations, we chose the final module from epoD (epoD-M6) as the optimal entry point into the pathway (Figure 1).

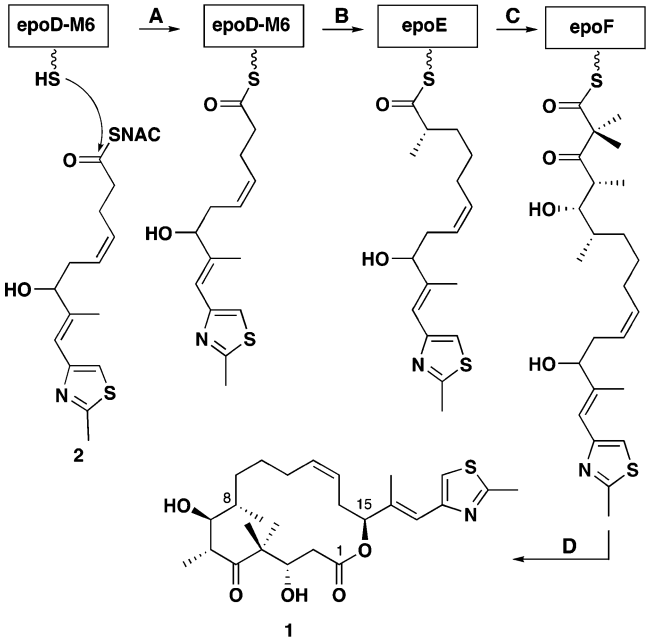
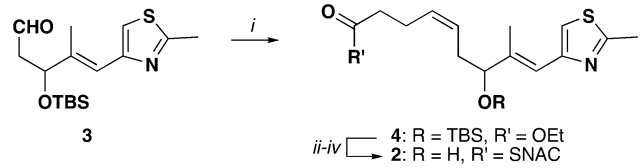


Figure 1. Mechanism for the precursor-directed biosynthesis of epothilone C (**1**). (A) The putative rate-limiting step is acylation of EpoD-M6 by **2**. (B) The acyl−enzyme intermediate is processed and transferred to epoE. (C) The acyl−enzyme intermediate is processed and transferred to epoF. (D) EpoF catalyzes the final round of elongation and cyclorelease.

Scheme 1. Synthesis of Enzymatic Substrate for Precursor-Directed Biosynthesis of Epothilone C[a]



[a] *i*. $EtO_2C(CH_2)_3PPh_3Br$, NaHMDS (48%). *ii*. TBAF. *iii*. LiOH. *iv*. HSNAC, DCC (87% over three steps).

To reconstitute the biosynthetic pathway starting at epoD-M6, a three-plasmid expression system was designed.[8] The genes *epoD-M6*, *epoE*, and *epoF* were cloned into three different *E. coli* expression vectors all under the control of the T7 promoter. *EpoE* and *epoF* encode for the final three modules of the pathway. The *pcc* gene cluster, necessary for production of the methyl malonyl-CoA extender unit,[9] was added to the *epoD-M6* expression vector.

The substrate required to complement the biosynthetic proteins was obtained by chemical synthesis. Scheme 1 shows the synthetic route starting from the racemic known aldehyde **3**.[10] The substrate was activated as the *N*-acetylcysteamine thioester, **2**, to mimic the native *S*-ACP bound substrate and can be obtained in gram quantities.

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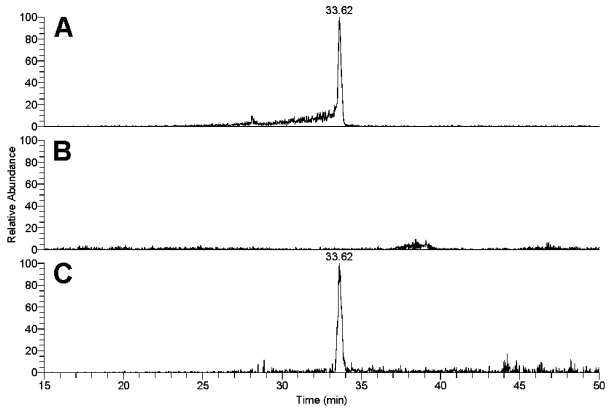

Figure 2. HPLC-MS analysis of the organic extracts from fermentation broths shows that epothilone C is produced by the engineered strain of *E. coli*. (ESI positive mode, ion extraction for $m/z = 478.3$): (A) epothilone C standard; (B) fermentation without substrate (**2**); (C) fermentation with 2 mM substrate (**2**).

Metabolically engineered *E. coli* strain BAP1[11] was co-transformed with the three plasmids described above. Cultures were grown to OD_{600} of 2 to 3 under conditions optimized for protein expression (18 °C, 2xYT media, approximately 48 h). The cultures were concentrated to $OD_{600} = 50$ and protein expression was induced with IPTG (1 mM). Sodium propionate, the precursor to methyl malonyl-CoA, was added to a final concentration of 2 g/L and the synthetic thioester substrate **2** was added to a final concentration of 2 mM. The high cell density during epothilone production phase minimizes the amount of substrate necessary to maintain acceptable initial acylation rates. The cultures were incubated for 24 h at 18 °C and the organic compounds isolated by ethyl acetate extraction.

Analysis of the organic extracts from the fermentation showed the production of epothilone C. When unlabeled propionate was used as the extender unit source, HPLC-MS analysis of the crude organic extracts from the fermentations clearly showed the presence of epothilone C (Figure 2). Additionally when 1-^{13}C propionate is fed to the fermentation cultures as the sole source of propionate, the expected triply labeled epothilone C is observed (see Supporting Information). Feeding of ^{14}C-labeled propionate provided ^{14}C-labeled epothilone C which could be detected by thin-layer chromatography followed by radioimaging (see Supporting Information).

Quantification of epothilone C production by HPLC analysis shows the production titer to be 0.7 mg/L. This titer is comparable to the native producing strain[12] and is significantly better than heterologous expression in *S. coelicolor* (<100 μg/L).[3a] Production of epothilone occurs predominantly during the first 12−24 h after substrate addition. After this time substrate **2** has been substantially depleted from the fermentation media by hydrolysis.[13] Addition of a second aliquot of **2** after 12 h of fermentation leads to an increase in epothilone C titer (1.0 mg/L). Increasing the stability of **2** in the fermentation media therefore provides an important mechanism for increasing epothilone C titer (also see Supporting Information).

A single diastereomer of epothilone C is produced by the engineered biosynthetic pathway (Figure 2C). Racemic **2** was used in the fermentations; however no (15-*epi*)-epothilone C is observed. We suspect that the non-native *R* configuration of this stereocenter impedes the cyclorelease activity of the thioesterase domain.[4d,14] Further elucidation of this mechanism is ongoing.

This work shows that large, complex biosynthetic pathways can be reconstituted in *E. coli* using precursor-directed biosynthesis. It is now possible to use this system to rapidly produce a large number of epothilone analogues. Because diversity elements can be introduced through the synthetic substrate, nonbiogenic functionality can be incorporated into the engineered epothilone analogues. In addition, since this diversity is not introduced through reengineering the polyketide synthase proteins, the rate at which new analogues are produced will be limited by the rate at which new chemical substrates can be synthesized.

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Supporting Information Available: plasmid Maps, experimental procedures, and new compound characterization (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(13) After 24 h approximately 85% of substrate **2** can be recovered as a mixture of **2** and the carboxylic acid produced by hydrolysis of **2**.

(14) The non-native 15*R* configured acyl chain is likely hydrolyzed by the thioesterase domain.[4d] This step is anticipated to be faster than the initial acylation of KS domain of epoD-M6.

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