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REVIEW PAPER

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Polyketide-nonribosomal peptide epothilone antitumor agents: the EpoA, B, C subunits

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Abstract The epothilones are a family of macrolactone natural products from the myxobacterial species *Sorangium cellulosum*. Similar to taxol, they are of current clinical interest as anticancer agents. Sequence analysis of the epothilone gene cluster allowed the identification of polyketide synthase and nonribosomal peptide synthetase modules involved in catalyzing epothilone biosynthesis. Given this information, it has been possible to test the predicted functions of several modules to date. EpoA ACP, EpoB, and EpoC have been overproduced in *Escherichia coli*, allowing in vitro reconstitution of the EpoA/B/C interface and production of the expected epothilone precursor. Further experiments probed the tolerance of EpoB and EpoC for unnatural substrates. These studies of the first three modules of the epothilone biosynthetic cluster suggest that combinatorial biosynthesis may lead to the production of a variety of epothilone analogs that incorporate diversity into the heterocycle starter unit. Additional efforts with the remaining modules, coupled with increased understanding of the macrocyclizing thioesterase domain, may lead to the production of epothilone variants with improved clinical properties.

Keywords Epothilone · Anti-tumor agent · Biosynthesis · Hybrid polyketide-nonribosomal peptide

Introduction

The epothilones are a family of macrolactone natural products from the myxobacterial species *Sorangium cellulosum* [7, 12]. They are of current clinical interest due to their antiproliferative effects on animal cells which have led to the development of epothilone B and D for human trials as anticancer agents [3, 11, 17]. The epothilones bind to microtubules, stabilizing them in a polymerized state and thereby blocking cell cycle progression. They act analogously to taxol [3, 8], but are thought to bind to an adjacent or non-overlapping site on tubulin subunits [2, 9], explaining the activity of epothilones against taxol-resistant tumor cell lines.

The major metabolites, epothilones A–D, differ by the epoxidation of a double bond in the 16-membered macrolactone scaffold or by the presence or absence of a methyl group at the olefinic/epoxide terminus (Fig. 1). A large number of minor variants of epothilones have also been isolated from large-scale cultures [10]. The epothilone skeleton differs from a typical polyketide macrolactone by the presence of a methylthiazole ring in the acyclic portion of the molecule. Thiazole heterocycles can arise by two types of enzymatic processes: (1) the cyclization of glycine and cysteine residues in ribosomal peptides such as microcin B17 [18], and (2) the cyclization coincident with peptide bond formation by a nonribosomal peptide synthetase module [22]. The methylthiazole moiety of the epothilones has the hallmark of nonribosomal origin, resulting from the condensation of acetyl and cysteinyl moieties, and this was validated upon sequence determination of the biosynthetic gene cluster [20, 25].

EpoA–F encode a multimodular assembly line, where nine polyketide synthase (PKS) modules and one nonribosomal peptide synthetase (NRPS) module are distributed over six subunits (Fig. 2A). The EpoB subunit contains the NRPS module sandwiched between a malonyl CoA-utilizing starter module on the EpoA subunit, and a methylmalonyl CoA-utilizing EpoC subunit. Both NRPS and PKS modules use parallel logic for the selection of monomers and subsequent acyl chain growth [4]. Each module has a carrier protein domain, acyl carrier protein (ACP) in PKS modules and peptidyl carrier protein (PCP) in NRPS modules, that becomes posttranslationally modified with a 20 Å-long phosphopantetheine (Ppant)

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prosthetic group bearing a terminal thiol [26]. This thiol dissociates to the thiolate above neutral pH and serves as the nucleophile on which each acyl/aminoacyl monomer and each growing acyl/peptidyl chain is tethered during chain growth. The other domains in PKS and NRPS modules are catalytic in function, selecting a given monomer and attaching it through a thioester linkage to the Ppant thiolate, then catalyzing chain-elongation reactions and subsequent tailoring steps before the chain is transferred to the next downstream module for another round of chemistry [14]. The action of the assembly line is predicted to activate one cysteine moiety and nine acyl groups from malonyl or methylmalonyl CoA and to catalyze eight C–C-bond-forming steps and one amide-bond-forming step to produce a full-length linear acyl chain tethered on the most downstream ACP of the EpoF subunit (Fig. 2B). The last domain in the assembly line, the thioesterase (TE) domain at the C-terminus of EpoF, then releases the epothilone chain by intramolecular cyclization, thereby creating the 16-membered macrolactone. Post-assembly-line epoxidation of the olefin is effected by the EpoK cytochrome P450 monooxygenase, converting epothilone C or D to epothilone A or B, respectively.

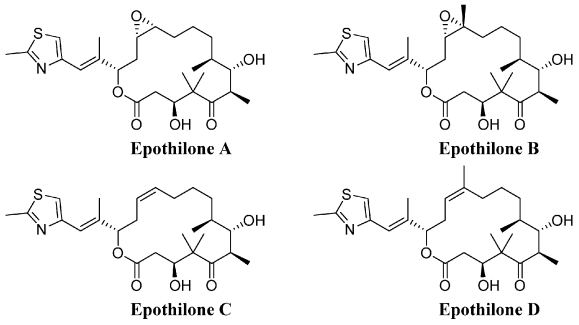

Fig. 1 The four major epothilone derivatives, epothilone A-D, produced by *Sorangium cellulosum*

Among the features of interest in the epothilone synthase assembly line is the meshing of PKS and NRPS assembly-line logics. The EpoA/B and EpoB/C subunit interfaces exemplify transitions from PKS to NRPS module and then from NRPS back to PKS module. This paper summarizes recent results that analyze the function of domains within the EpoA, B, and C subunits, including chain transfer across the A/B and B/C subunit boundaries.

The constituent functions of the EpoA, B and C subunits

Sequence analysis and bioinformatics predict that the first three subunits, EpoA–C, would each contain one functional module, in the order PKS, NRPS, PKS, with the 13 domains indicated in Fig. 3 [13, 20, 25]. EpoA would have a keto synthase (KS), acyltransferase (AT), enoylreductase (ER) and an acylcarrier protein (ACP) domain.

The EpoB subunit also has four predicted domains in the order Cy–A–Ox–PCP, where the Cy (cyclization) domain is homologous to other condensation/heterocyclization domains in thiazole-forming NRPS modules [15, 20]. The A (adenylation) domain is a typical amino acid activation domain, predicted to be specific for cysteine recognition and activation as Cys-AMP [13, 20, 24]. The Ox (oxidase) domain is analogous to flavoprotein oxidoreductase domains [13], and the PCP is the predicted peptidyl carrier protein domain, to be posttranslationally phosphopantetheinylated and

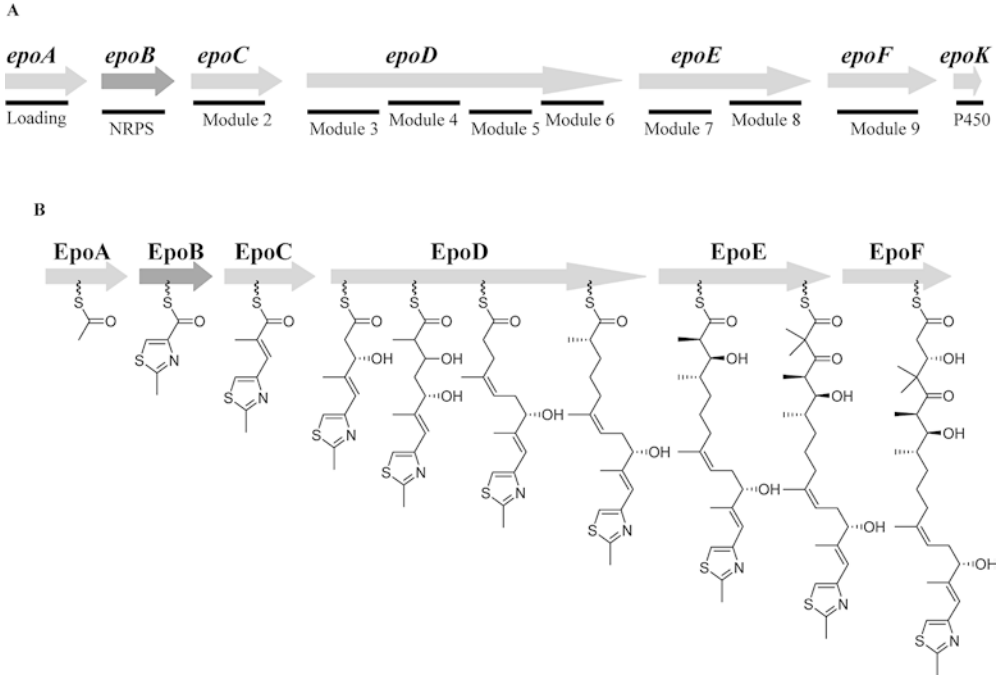

Fig. 2 **A** The open reading frames of the epothilone gene cluster. *epoA*, *epoC–epoF* are predicted to encode polyketide synthases, *epoB* encodes a nonribosomal peptide synthetase, and *epoK* encodes an epoxidase. **B** The predicted enzyme-tethered intermediates of epothilone biosynthesis

loaded with the Cys moiety from the Cys-AMP intermediate.

The EpoC subunit is predicted to have five domains in a functional PKS module, KS-AT-KR-DH-ACP. The two domains not present in the EpoA subunit are the keto reductase (KR) and dehydratase (DH) domains. The AT domain in the EpoC subunit contains the signature sequence of methylmalonyl CoA-specific acyl transferases while the AT domain in the EpoA module is predicted to be malonyl specific [13, 20].

With the assignment of predicted function to the 13 domains in the EpoA, B, and C subunits, the workings of the first three modules of the epothilone biosynthetic assembly line can be proposed. EpoA will use its AT domain to recognize malonyl CoA and transfer it to the thiolate of the Ppant moiety of the EpoA ACP domain. The EpoA KS domain is a variant that in other contexts is incompetent for condensation but able to catalyze decarboxylation of the intrasubunit malonyl–*S*–ACP to acetyl–*S*–ACP [1]. The fourth domain in EpoA, the ER domain, has mutations in key residues and is likely to be noncatalytic [20], perhaps reflecting evolution from a precursor module and/or playing an architectural role in EpoA subunit function.

The EpoB four domain NRPS module will use the A domain to select and activate Cys in an ATP-consuming step to produce bound Cys-AMP and then transfer the cysteinyl group to the Ppant thiolate of the PCP to yield Cys–*S*–EpoB. The remaining two domains, Cy and Ox, will be involved in amide bond formation and cyclization and then oxidation of the thiazoline to the thiazole, respectively. The Cy domain should use the acetyl group of acetyl–*S*–EpoA as donor and the amino group of Cys–*S*–EpoB as acceptor substrate to catalyze amide bond formation, with acetyl transfer across the subunit interface to yield, transiently, N-acetyl–Cys–*S*–EpoB and free EpoA-SH (Fig. 4A). The Cy domain will continue its operations and catalyze the cyclization of the Cys side-chain thiolate on the carbonyl group of the N-acetyl moiety to produce a tetrahedral adduct that, upon dehydration, generates a five-membered heterocyclic acyl enzyme, methylthiazolinyl–*S*–EpoB (Fig. 4B). This dihydroheterocycle will then be oxidized to the fully heteroaromatic methylthiazolyl–*S*–EpoB species by the oxidase domain of EpoB (Fig. 4B).

The EpoC subunit appears to be a conventional methylmalonyl-utilizing PKS module. The AT domain should transfer methylmalonyl from soluble methylmalonyl CoA to produce methylmalonyl–*S*–ACP in the EpoC subunit. The KS domain should carry out three functions. These are: (1) self-acylation at its active site cysteine with the donor in the C–C-bond-forming reaction, here the methylthiazolyl group from EpoB; (2) decarboxylation of methylmalonyl–*S*–ACP to produce the C_2 carbanion of propionyl–*S*–ACP as nucleophile; (3) catalysis of C–C bond formation by condensation of the donor methylthiazolyl onto the carbon nucleophile to yield methylthiazolyl-3-keto-2-methylpropionyl–*S*–EpoC (Fig. 4C). The two remaining domains of EpoC, KR and DH, would then work in tandem, reducing the 3-keto with NADPH to the 3-OH substituent (KR) and then elimination of the elements of water (DH) to create the 2,3-olefin in the product methylthiazolyl-methylacrylyl–*S*–EpoC (Fig. 4D).

Expression of epothilone synthetase domains and subunits in *Escherichia coli* for mechanistic analysis

To evaluate these predictions, understand the chemistry of specific steps, and analyze selectivity of the Cy domain of EpoB and the KS domain of EpoC as the key domains in intersubunit chain transfer and PKS/NRPS and NRPS/PKS interface catalysis, we have undertaken heterologous expression of the *S. cellulosum* EpoA, B, and C subunits in *E. coli* cells [5, 21]. EpoA is a 150-kDa protein and, while its expression could be detected, it was not obtained in reasonable quantity in soluble form after several attempts. It did prove possible to express the C-terminal domain of EpoA, the 16.5-kDa ACP domain, in high yield (10 mg/l) with good solubility such that preparative quantities of the apo ACP domain of EpoA were generated as a reagent for the incubations described below.

The 155-kDa EpoB subunit proved more tractable in *E. coli* expression (2 mg/l) and could be purified as a yellow flavoprotein with the FMN cofactor residing in the oxidase domain. Also, the 195-kDa five-domain EpoC subunit could be produced in 2.5 mg/l quantities in soluble, active form, sufficient for the experiments summarized below. All three carrier proteins, the ACP of EpoA, the ACP domain in EpoC, and the PCP domain in EpoB, could be posttranslationally primed with a phosphopantetheinyl group from cosubstrate CoASH via catalytic action of a phosphopantetheinyl transferase [16] (Fig. 5A), either in vitro (EpoA and EpoB) or by coexpression in vivo (EpoC).

Chain transfer between the EpoA/B subunits: PKS to NRPS module interface

Study of the catalysis of chain transfer and elongation across the EpoA/B subunit interface, a PKS/NRPS junction, requires the availability of the two acyl–*S*–enzymes that are the substrates for the Cy domain of EpoB, the acetyl–*S*–ACP of EpoA and the cysteinyl–*S*–EpoB. It also requires an analytical method to detect the product, methylthiazolyl–*S*–EpoB, the methylthiazolyl derivative of the Ppant prosthetic group attached to the PCP domain of EpoB. The acetyl–*S*–ACP donor was available through modification of the apo form of the ACP by posttranslational phosphopantetheinylation with acetyl CoA rather than the normal substrate, CoASH [5] (Fig. 5A). The

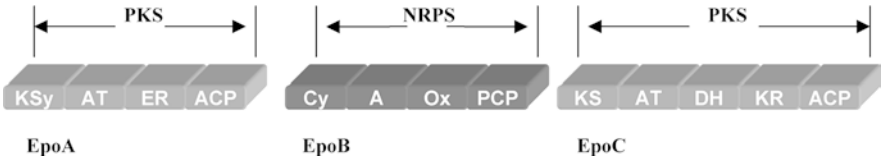

Fig. 3 The catalytic domains of EpoA, B and C

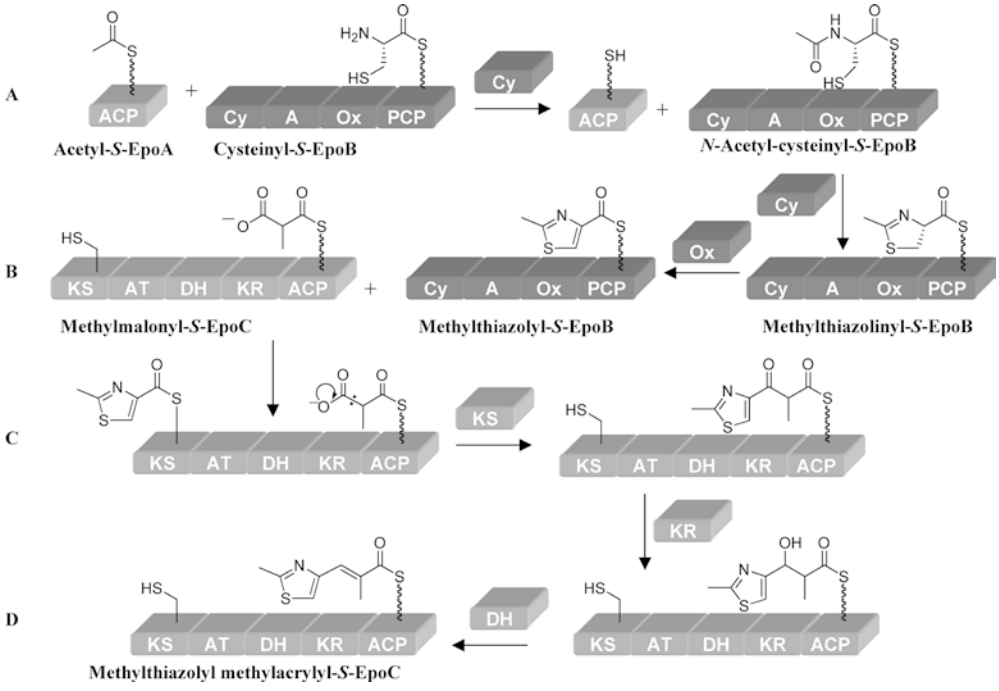

Fig. 4 Mechanism of epothilone biosynthesis. **A** Formation of *N*-acetyl-Cys–*S*–EpoB by acetyl transfer from acetyl–*S*–EpoA-ACP to Cys–*S*–EpoB. **B** Cyclization and oxidation of *N*-acetyl-Cys–*S*–EpoB to methyl thiazolyl–*S*–EpoB. **C** Transfer of methyl thiazolyl–*S*–EpoB to the active site cysteine residue of the KS domain of EpoC, decarboxylation of methylmalonyl–*S*–EpoC and formation of the β-keto methylthiazolyl condensation product. **D** Reduction and dehydration to form methylthiazolyl-methylacrylyl–*S*–EpoC product

acetyl–*S*–ACP thioester product could be isolated from the apo ACP by chromatography, and its composition was confirmed by mass spectrometry (Fig. 5B). Further, it was possible to prime the ACP with [^{14}C]-acetyl-CoA, enabling detection of the radiolabeled acyl group.

The cysteinyl–*S*–EpoB could be produced by letting the EpoB subunit carry out autoaminoacylation, which could be detected and quantitated with [^{35}S]-cysteine and ATP [5]. On mixing the two acyl enzyme species, the transfer of the labeled [^{3}H]-acetyl group from the 16.5-kDa ACP protein to the 155-kDa EpoB protein could be detected by SDS gel electrophoresis and autoradiography. The acetyl transfer was dependent on Cys and ATP, suggesting that enzymatically catalyzed amidation was occurring. To determine whether the [^{3}H]-acetyl group transferred to Cys–*S*–EpoB was present as [^{3}H]-N-acetyl–Cys–*S*–EpoB, [^{3}H]-methylthiazolinyl–*S*–EpoB, or [^{3}H]-methylthiazolyl–*S*–EpoB, the thioester bond was hydrolyzed by mild alkaline treatment and the released radioactive product was subjected to reverse-phase chromatography with comparison to the three standard acids. Only [^{3}H]-methylthiazole carboxylate was detected [5], indicating that all four domains of EpoB were acting as predicted and condensation, cyclization, and dehydration were occurring to yield the methylthiazolyl–*S*–EpoB acyl enzyme intermediate (Fig. 5C).

Chain transfer between the EpoB/C subunits: NRPS to PKS module interface

The methylthiazolyl–*S*–EpoB acyl enzyme available from the studies noted above could serve as acyl chain donor to the EpoC subunit and thus was used to probe the ability of the KS domain of EpoC to catalyze the NRPS to PKS interfacial transfer. In turn, this required as acceptor the methylmalonyl–*S*–EpoC acyl enzyme intermediate. We could demonstrate that purified EpoC was competent for autoacylation on its holo-ACP domain with [^{3}H]-methylmalonyl CoA via AT domain action [21]. When methylmalonyl–*S*–EpoC was mixed with methylthiazolyl–*S*–EpoB, with the donor labeled either with [^{3}H]-acetyl–*S*–ACP or ^{35}S-Cys, the label was transferred to EpoC. Since EpoB (155 kDa) and EpoC (195 kDa) were not well-resolved by SDS gel electrophoresis, the acyl enzyme species were hydrolyzed in mild base and the products compared with authentic standards using reverse-phase chromatography. The enzymatic product was methylthiazolyl-methylacrylic acid (Fig. 6A) and was obtained with a radioactive label when the reaction included a radiolabel on any substrate, either [^{3}H]-acetyl–*S*–ACP, [^{35}S]-cysteinyl–*S*–EpoB, or [^{14}C]-methylmalonyl–*S*–EpoC [21]. When NADPH was omitted as cosubstrate, the 3-keto intermediate

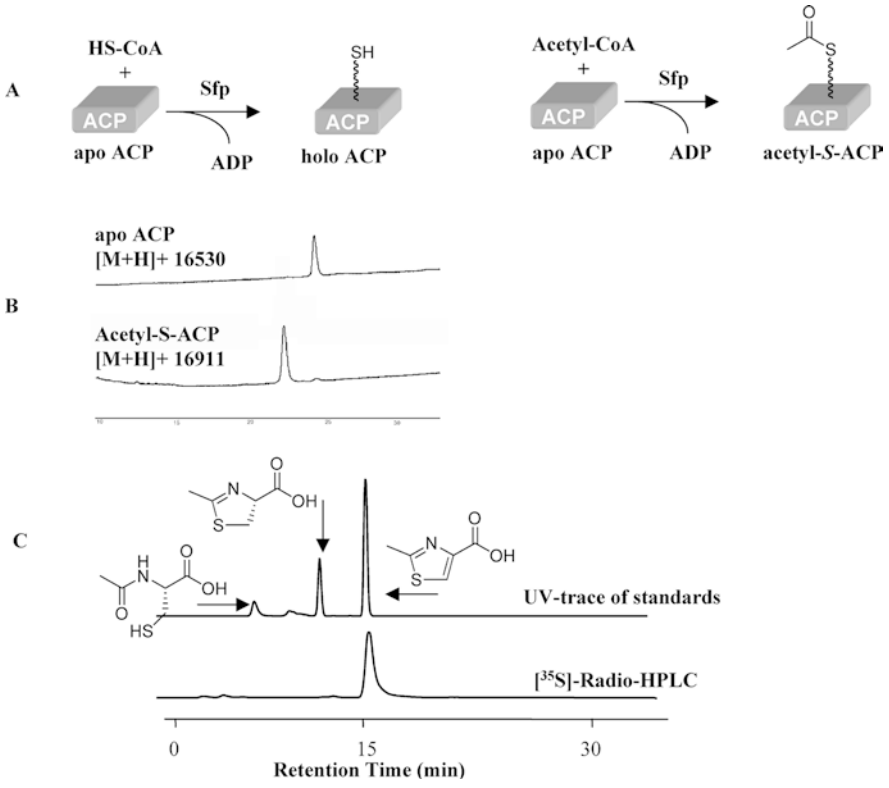

Fig. 5 **A** The phosphopantetheinyl transferase Sfp and HS-CoA or acetyl-CoA convert apo acyl carrier to holo carrier protein of acetyl–*S*–carrier protein. **B** Sfp transfers acetyl-CoA to an apo EpoA ACP as evidenced by HPLC retention time and MALDI mass spectrometry. **C** Hydrolysis of the reaction product of acetyl–*S*–EpoA-ACP and [^{35}S]-Cys–*S*–EpoB and analysis by radio-HPLC indicates formation of a product that co-elutes with methyl thiazole authentic standard

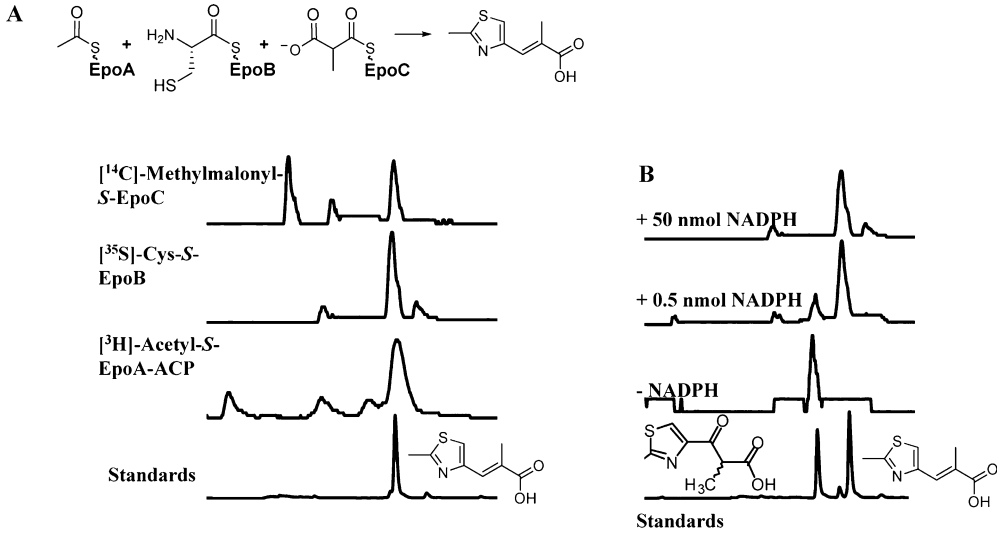

Fig. 6 **A** Reaction of acetyl–*S*–EpoA-ACP, Cys–*S*–EpoB and methylmalonyl–*S*–EpoC produce the expected product as evidenced by coelution with an authentic standard. The radiolabel was positioned on the acetyl group of EpoA, the cysteine of EpoB or the methylmalonyl of EpoC. **B** Removal of NADPH from the reaction mixture prevents formation of the final methylthiazolyl-methylacrylic acid product

acyl–*S*–EpoC accumulated, confirming C–C bond formation before reduction and elimination (Fig. 6B). At this point, starting from acetyl–*S*–ACP, five of the eight acyl enzyme intermediates proposed in the 13-domain, three-module initial portion of the epothilone synthetase assembly line had been directly detected. Both the PKS to NRPS interface (EpoA/B) and the NRPS to PKS interface (EpoB/C) had been reconstituted.

EpoD–F and post-assembly-line tailoring

The subsequent seven PKS modules in the three subunits EpoD–F are proposed to be conventional chain-elongation PKS modules, extending the acyl chain backbone by two carbons in each of seven cycles to build the full-length linear acyl chain docked covalently on the most downstream ACP domain of the EpoF subunit (Fig. 2). Perhaps the most unusual feature of those seven chain elongation steps is in module 8 of EpoE where a C-methyl transferase domain is predicted from bioinformatic analysis [13, 20]. The epothilones have a 4,4-dimethyl substituent pattern, consistent with use of a methylmalonyl CoA by the AT domain of this module, followed by a C-methylation event before the chain moves downstream. This is precedented by in vitro enzymatic analysis of a

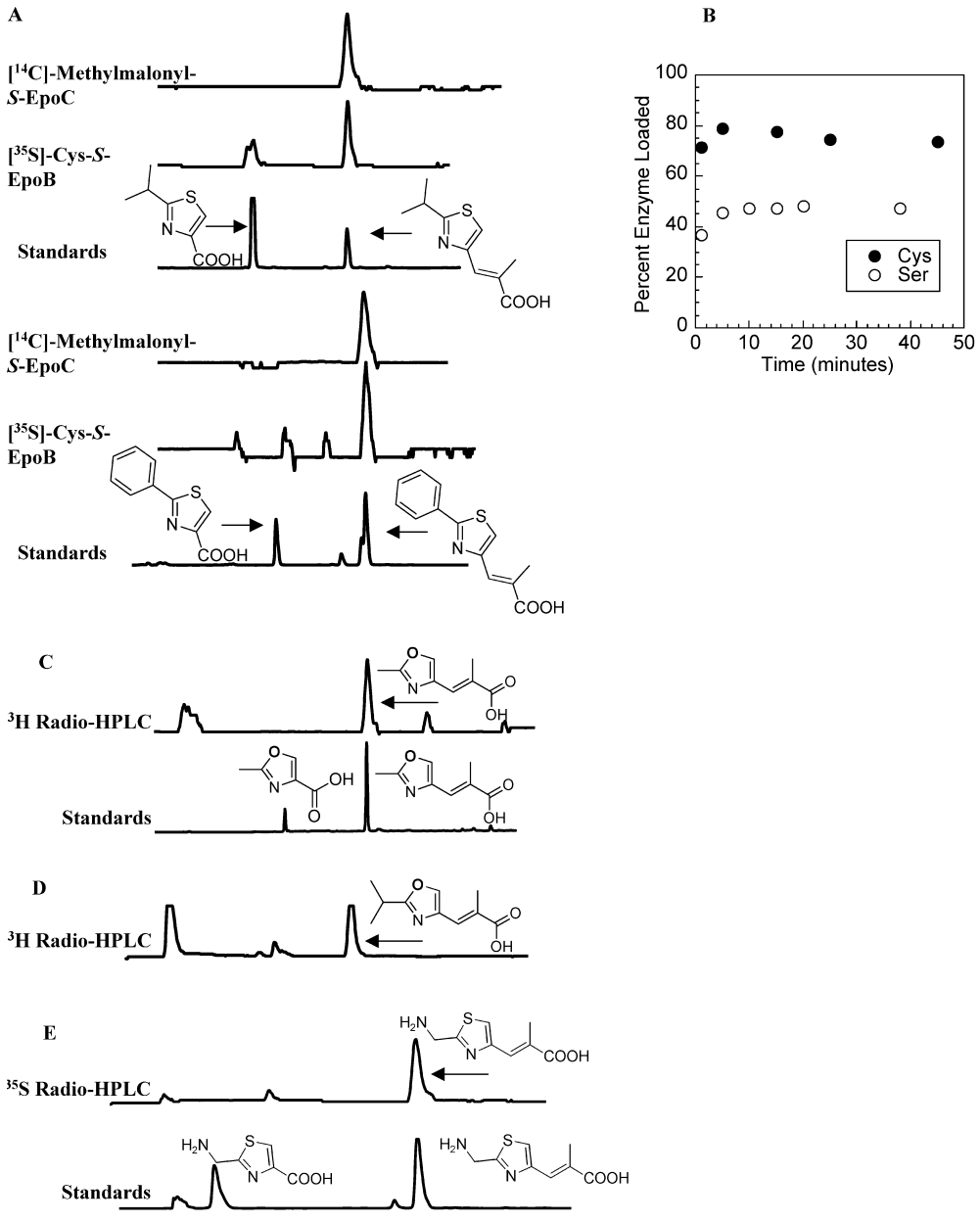

Fig. 7 **A** Substitution of acetyl-CoA with isobutyryl-CoA or benzoyl-CoA produces the isopropylthiazolyl-methylacrylic acid and phenylthiazolyl-methylacrylic acid as evidenced by coelution with authentic standards. **B** The A domain of EpoB catalyzes loading of the carrier protein domain with both cysteine (80% stoichiometry) and serine (50% stoichiometry). **C** Reaction of acetyl–*S*–EpoA-ACP, [^{3}H]-Ser–*S*–EpoB and methylmalonyl–*S*–EpoC produces the expected methyloxazolyl-methylacrylic acid product. **D** Reaction of isobutyryl–*S*–EpoA-ACP, [^{3}H]-Ser–*S*–EpoB and methylmalonyl–*S*–EpoC also forms the expected product, verified by MALDI mass spectrometry (observed: 196.90; expected: 196.22). **E** Reaction of acetyl–*S*–EpoA-ACP, [^{35}S]-Cys–*S*–EpoB and methylmalonyl–*S*–EpoC produces aminomethylthiazolyl-methylacrylic acid

related C-MT domain in the yersiniabactin synthetase assembly line [19]. The chain-terminating macrolactonizing thioesterase domain will release the 16-membered lactone from its covalent tethering on the ACP domain of EpoF. EpoK, corresponding to the open reading frame just downstream of EpoF, has the hallmarks of a cytochrome P450 catalyst, typical of many partner protein monooxygenases responsible for post-assembly-line oxidative tailoring of polyketide scaffolds. It was predicted that EpoK would be the epoxidase converting epothilone D to epothilone B and epothilone C to epothilone A, and this has been validated by knockout studies that increase yields of the epothilone C and D products in fermentations [25].

Utilization of alternate substrates by the first three modules

As noted above, while epothilones A–D are the major fermentation products, several variants in minute quantities can also be isolated from large-scale *S. cellulosum* fermentations [10]. Many of these arise by apparent post-assembly-line oxygenative tailoring of the epothilone products, but a methyloxazole variant suggested that serine could be used in place of cysteine by the EpoB NRPS module.

We have undertaken an initial evaluation of the tolerance of the Cy domain in EpoB and of the KS domain in EpoC in accepting and condensing acyl

enzymes with altered acyl substituents. The change in acyl groups on the EpoA ACP domain was the most straightforward since they could be introduced by replacement of acetyl CoA with various alkyl and aryl CoA analogs, enabling the transfer of the various acyl pantetheinyl phosphate moieties by the action of the posttranslational priming enzyme catalyst [5, 16]. For example, isobutyryl CoA and benzoyl CoA gave the isobutyryl–*S*–ACP and benzoyl–*S*–ACP thioesters which were reacted with Cys–*S*–EpoB to yield isopropylthiazolyl–*S*–EpoB and phenylthiazolyl–*S*–EpoB respectively (Fig. 7A). When these heterocyclic–*S*–EpoB species were utilized as donors to methylmalonyl–*S*–EpoC, the corresponding C–C bond formations ensued (Fig. 7A), validating that both the Cy domain of EpoB and the KS domain of EpoC, catalyzing amide and C–C bond formation respectively, would accept the altered acyl donor from EpoA.

The EpoB subunit was demonstrated to activate serine at about 10% of the rate of cysteine, and could load the activated seryl moiety of bound seryl-AMP on the holo PCP domain to 50% stoichiometry (Fig. 7B) [23]. On addition of acetyl–*S*–EpoA ACP to such incubations, the methyloxazolyl–*S*–EpoB was generated and then transferred on to methylmalonyl–*S*–EpoC to provide the oxazolyl analog of the normal acyl enzyme form of EpoC (Fig. 7C) [23]. This in vitro result is in accord with the ability to detect and even promote formation of the oxazole variants of epothilone in culture media [6, 10] and also indicates that the downstream PKS modules in EpoD–F subunits will accept the altered thiazole and oxazole donors on EpoC. Double variants could be produced in vitro by utilizing serine with EpoB and variant acyl–*S*–ACP donors: for example, the isobutyryloxazolyl–*S*–EpoB could be generated and then serve as the donor in C–C bond formation with methylmalonyl–*S*–EpoC (Fig. 7D) [23]. To introduce functionality to the alkyl substituent on the heterocyclic ring and increase water solubility, we synthesized glycyl CoA and used the phosphopantetheinyl transferase approach to prime the apo ACP with the glycyl–*S*–Ppant moiety. This primed ACP was accepted by EpoB and EpoC for tandem condensation with cysteine and methylmalonyl CoA to produce the aminomethylthiazolyl-methylacrylyl–*S*–EpoC acyl enzyme intermediate (Fig. 7E). It may be possible to further broaden the scope of acyl CoA, amino acids, and malonyl CoA accepted by EpoA, B, and C by mutagenesis of the active sites of these three modules.

Next steps

The reconstitution of the remainder of the epothilone synthetase assembly line in vitro requires expression of the seven PKS modules distributed in 4/2/1 fashion among the EpoD, E, and F subunits. These modules elaborate the carbon skeleton of the macrolactone with two unusual features, a *cis* double-bond at $C_{12}C_{13}$, and a 4,4-dimethyl substitution pattern. The C-terminal TE domain in EpoF is the macrolactonization catalyst. With a full EpoA–F assembly line in operation, it will be possible to probe what variations in substrates utilized by EpoA, B, C will be accepted for elongation and macrocyclization by EpoD-F.

Conclusions

The epothilones, a family of hybrid PK/NRP natural products, are of current biomedical interest as promising anticancer agents [3]. Sequence analysis of the epothilone gene cluster allowed the identification of PKS and NRPS modules involved in catalyzing epothilone biosynthesis. Initial testing of the predicted functions of these modules has included in vitro reconstitution of the EpoA/B/C interface, yielding the expected epothilone precursor, methylthiazolyl-methylacrylic acid. Further experiments indicated that EpoB and EpoC are tolerant of some unnatural substrates, yielding products including aminomethylthiazolyl-methylacrylic acid and methyloxazolyl-methylacrylic acid. These studies with the early enzymes of the epothilone biosynthetic cluster suggest that a combinatorial biosynthetic approach could produce a variety of epothilone analogs that incorporate diversity into the heterocycle starter unit. Additional efforts with the remaining PKS modules, coupled with increased understanding of the substrate requirements of the macrocyclizing TE domain, may lead to the production of epothilone variants with improved clinical properties.

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