Substrate Tolerance of Module 6 of the Epothilone Synthetase[†]

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ABSTRACT: The epothilone synthetase is a decamodular megasynthase responsible for the biosynthesis of a class of polyketide natural products with clinically promising antitumor activity. Recently, we developed a system comprised of modules 6−9 of the epothilone synthetase for the precursor-directed biosynthesis of epothilones in *Escherichia coli* [Boddy, C. N., Hotta, K., Tse, M. L., Watts, R. E., and Khosla, C. (2004) *J. Am. Chem. Soc. 126*, 7436−7437]. To systematically explore the biosynthetic potential of this system, we have now investigated the ability of the crucial first module in this engineered pathway, EpoD-M6, to accept, elongate, and process unnatural substrates. EpoD-M6 was expressed, purified, and demonstrated to accept both acyl-CoA and acylSNAC substrates. Of the substrates that were tested, octanoylSNAC and 3-octenoylSNAC proved to be excellent substrates in addition to the more complex natural substrate. Thus, this polyketide synthase module showed considerable tolerance, a feature that bodes well for the precursor-directed biosynthesis of epothilone analogues and related complex polyketides.

The ability to readily make analogues of complex molecules is essential to natural product drug development. Because polyketide synthases (PKSs)[1] are capable of generating molecules of great complexity, technologies that combine chemical and enzymatic synthesis will be of increasing benefit in the discovery of new polyketide drugs.

The epothilones are a family of polyketide natural products isolated from *Sorangium cellulosum* (*1, 2*). They are of great clinical interest as anticancer agents due to their taxane-like properties which are relatively unencumbered by recognition by export pumps that ordinarily make tumor cells resistant to drugs such as paclitaxel (*3−6*). The epothilone biosynthetic pathway is a modular megasynthetase comprised of a nonribosomal peptide synthetase module and nine PKS modules (*7, 8*). These enzymes construct the epothilone carbon backbone in a stepwise, linear process, tailoring the functionality and stereochemistry of each subunit at each step.

Polyketide biosynthesis is well suited to precursor-directed biosynthesis. Many unnatural substrates fed exogenously to polyketide-producing systems are capable of acylating the ketosynthase domain of an intermediate module and undergoing elongation and tailoring of functionality by that module. When the elongated, unnatural intermediate is passed to the next step, subsequent modules further elongate and
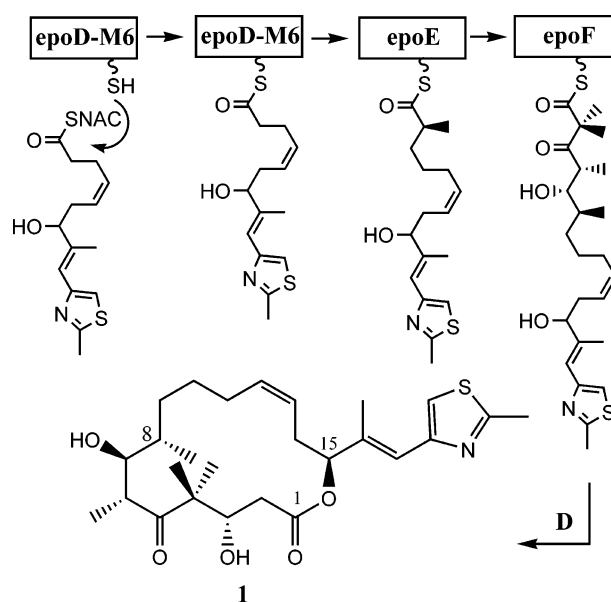

FIGURE 1: Precursor-directed biosynthesis of epothilone C, with an entry point at EpoD-M6.

tailor the growing chain, ultimately forming an analogue of the natural product (*9−13*).

In earlier studies on precursor-directed biosynthesis of epothilones, we fed an exogenous natural substrate mimic to a truncated version of the epothilone biosynthetic pathway and produced epothilone C (**1**, Figure 1) (*14*). We have extended this study by focusing on biochemical characterization of EpoD-M6, the entry module of this engineered PKS. In the process, we identified several unnatural substrates that are efficiently loaded and accurately processed by this critical module.

The KS domain of module 6 (M6) of the EpoD protein from the epothilone synthetase can be acylated in vivo by an activated precursor that mimics the native ACP5-bound

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[1] Abbreviations: ACP, acyl carrier protein; KS, ketosynthase; M6, module 6; PKS, polyketide synthase; SNAC, *N*-acetylcysteamine.

substrate for M6 (*14*). This substrate can then be processed and passed to downstream modules in the epothilone biosynthetic pathway, ultimately resulting in the precursor-directed production of epothilone C. It is important to understand more fully the flexibility of M6 toward novel precursors because the power of future precursor-directed biosynthesis of epothilone analogues lies largely in the ability of the KS domain of M6 of EpoD to accept various unnatural precursors. Structure−activity relationship studies on epo-thilones have shown that portions of this macrolactone synthesized in the late stages of biosynthesis are critical for its antimitotic activity but that portions synthesized prior to M6 can be modified for improved pharmacological proper-ties. Thus, M6 is a key entry point for precursor-directed biosynthesis of epothilone analogues. Thus, we sought to express M6 of EpoD as a stand-alone module to study its ability to accept and process various activated substrates. Here we describe our results from these investigations.

MATERIALS AND METHODS

Construction of Plasmids

Plasmid pMLT11 is based on pET28 and contains the *epoD-M6* gene, originally obtained from *S. cellulosum* genomic DNA containing cosmid pKOS35-70.1A2 (*7*). The N-terminal linker region from DEBS2 was appended to the beginning ketosynthase domain of module 6 (KS6) from EpoD to increase protein expression levels (*17*). To engineer this fusion construct, gene splicing by overlap extension (gene SOEing) was used, as described in detail by Horton et al. (*18*). This method enabled recombination of the 5′-end of the gene encoding DEBS2 with the downstream portion of the *epoD-M6* gene to yield a junction with the DNA sequence 5′-cgcgagctggaatccgagcccattgcggtgatcg (un-derlined bases comprise the two codons between which lies the precise fusion point). The recombined gene sequence was cloned into pET28 yielding pMLT11, which encodes a protein with both C- and N-terminal histidine tags. Plasmid pMLT17 is based on pET28 (Novagen) and is a polycistronic vector that contains the *epoD-M6* gene and the *pcc* gene cluster. The construction of plasmid pMLT17 has been described previously (*14*).

Synthesis of Standards

2-Methyldecanoic Acid. Decanoic acid (1 g, 5.8 mmol, 1 equiv) was dissolved in MeOH (25 mL) and treated with chlorotrimethylsilane (1.5 mL, 12 mmol, 2 equiv). The reaction mixture was stirred at room temperature overnight, after which the reaction was complete as determined by TLC (1:8 EtOAc/hexanes, $R_f = 0.26$ for the potential product). The mixture was concentrated in vacuo, affording a yellow oil (1.08 g). The crude material (105 mg, 0.564 mmol, 1 equiv) was dissolved in tetrahydrofuran (THF, 5 mL) and cooled to −75 °C. *N*-Sodium hexamethyldisilazane (2.0 M in THF, 370 μL, 1.3 equiv) was added, and the reaction mixture was stirred at −75 °C for 45 min. Methyl iodide (280 μL, 8 equiv) was added to the reaction mixture, and it was stirred for 2 h. The reaction was quenched by the addition of saturated aqueous NH₄Cl (1.5 mL) and the reaction mixture concentrated in vacuo to remove THF. The mixture was extracted with EtOAc (3 × 50 mL). The

combined organic phase was washed with brine (10 mL), dried over Na₂SO₄, and filtered. The solvent was removed in vacuo, affording 110 mg of a mixture containing both the methyl decanoate and the desired 2-methyl-methyl decanoate. A portion of this residue (52 mg) was dissolved in a 1:1 MeOH/H₂O mixture (4 mL). LiOH (25 mg, 3 equiv) was added. After 5 h, the reaction mixture was acidified with 10% HCl (1 mL) and extracted with EtOAc (3 × 50 mL). The combined organic phase was washed with brine (10 mL), dried with Na₂SO₄, and filtered. The solvent was removed in vacuo. Preparative TLC (1:5 EtOAc/hexanes with 1% acetic acid) performed on approximately 10% of the crude material afforded 2-methyldecanoic acid: ^{1}H NMR (400 MHz, CDCl₃) δ 2.44 (m, 1H), 1.65 (m, 1H), 1.44 (m, 1H), 1.25 (bm, 12H), 1.17 (d, $J = 6.8$ Hz, 3H), 0.86 (t, $J = 7.2$ Hz, 3H).

2-Methyl-4-decenoic acid was synthesized from 4-decenoic acid by the same procedures used for the synthesis of 2-methyldecanoic acid from decanoic acid: ^{1}H NMR (200 MHz, CDCl₃) δ 5.35−5.46 (m, 2H), 2.30−2.49 (m, 2H), 1.99−2.25 (m, 2H), 1.25−1.44 (m, 9H), 1.09−1.18 (dd, 1H, $J = 6.2, 13.0$ Hz), 0.84−0.908 (t, 3H, $J = 6.4$ Hz).

2-Methyl-5-decenoic acid was synthesized from 5-decenoic acid by the same procedures used for the synthesis of 2-methyldecanoic acid from decanoic acid: ^{1}H NMR (200 MHz, CDCl₃) δ 5.30−5.43 (m, 2H), 1.77−2.55 (m, 6H), 1.19−1.38 (m, 10H), 0.84−0.91 (t, 3H, $J = 6.8$ Hz).

Synthesis of N-Acetylcysteamine Thioesters

2-Octenoyl N-Acetylcysteamine Thioester. 2-Octenoic acid (530 μL, 3.5 mmol, 1 equiv) was dissolved in CH₂Cl₂ (35.2 mL). *N*-Acetylcysteamine (420 μL, 4.2 mmol, 1.2 equiv) was added followed by EDC (1.012 mg, 5.3 mmol, 1.5 equiv). The reaction mixture was stirred at room temperature overnight. The reaction was quenched with 10% HCl (5 mL) and the mixture extracted with 3 × 50 mL of EtOAc. The combined organic phase was washed with brine (10 mL), dried with Na₂SO₄, and filtered. The solvent was removed in vacuo. Column chromatography (silica gel, EtOAc) afforded 93 mg of 2-octenoyl *N*-acetylcysteamine thioester (0.38 mmol, 10.8% yield): $R_f = 0.5$ (EtOAc); ^{1}H NMR (500 MHz, CDCl₃) δ 6.89 (ddd, $J = 7.0, 7.1, 15.5$ Hz, 1H), 6.09 (ddd, $J = 1.6, 1.6, 15.5$ Hz, 1H), 5.85 (bs, 1H), 3.43 (dd, $J = 5.86, 12.45$ Hz, 2H), 3.06 (t, $J = 6.1$ Hz, 2H), 2.16 (ddd, $J = 1.6, 7.3, 14.8$ Hz, 2H), 1.27 (m, 4H), 0.86 (t, $J = 7.1$ Hz, 3H).

3-Octenoyl N-Acetylcysteamine Thioester. 3-Octenoic acid (Lancaster, 530 mL, 3.5 mmol, 1 equiv) was dissolved in CH₂Cl₂ (35.2 mL). *N*-Acetylcysteamine (415 μL, 4.2 mmol, 1.2 equiv) was added followed by dicyclohexylcarbodiimide (1.45 g, 7 mmol, 2 equiv). The reaction mixture was stirred at room temperature overnight. The reaction mixture was cooled to −78 °C and the dicyclohexylurea byproduct removed by filtration. The filtrate was concentrated in vacuo. Column chromatography (silica gel, EtOAc) afforded 611 mg of 3-octenoyl *N*-acetylcysteamine thioester (2.51 mmol, 71% yield): $R_f = 0.55$ (EtOAc); ^{1}H NMR (500 MHz, CDCl₃) δ 5.61 (m, 1H), 5.48 (m, 1H), 5.1 (bs, 1H), 3.27 (dd, $J = 6.7, 12.6$ Hz, 2H), 3.24 (m, 2H), 2.95 (t, $J = 6.7$ Hz, 2H), 1.85 (s, 3H), 1.31 (m, 6H), 0.87 (t, $J = 4.5$ Hz, 3H).

Octanoyl N-Acetylcysteamine Thioester. This compound was synthesized in the same manner as 2-octenoyl *N*-

acetylcysteamine thioester: ^{1}H NMR (200 MHz, CDCl$_3$) δ 5.75 (bs, 1H), 3.33 (dd, $J = 12.2$, 5.8 Hz, 2H), 2.93 (t, $J = 6.2$, 6.6 Hz, 2H), 2.48 (t, $J = 7.6$, 7.2 Hz, 2H), 1.91 (s, 3H), 1.22 (bm, 8H), 0.79 (t, $J = 6.8$, 6.2 Hz, 3H).

8-Bromooctanoyl N-Acetylcysteamine Thioester. This compound was synthesized in the same manner as 2-octenoyl *N*-acetylcysteamine thioester: ^{1}H NMR (200 MHz, CDCl$_3$) δ 5.7−5.9 (bs, 1H), 3.369−3.561 (ddd, $J = 6.8$, 6.8, 25.0 Hz, 2H), 3.389−3.481 (m, 2H), 2.991−3.055 (dd, $J = 6.6$ Hz, 2H), 2.538−2.612 (dd, $J = 7.8$ Hz, 2H), 1.966 (s, 3H), 1.251−1.919 (bm, 10H).

7-Hydroxyoctanoyl N-Acetylcysteamine Thioester. 7-Oxo-octanoic acid (915 mg, 5.8 mmol, 1 equiv) was dissolved in 20 mL of EtOH. Sodium borohydride (NaBH$_4$, 220 mg, 4 equiv) was added, and the reaction mixture was stirred at room temperature. After 1 h, additional NaBH$_4$ was added (220 mg, 4 equiv). After 1 h, the reaction was quenched with H$_2$O (10 mL) and the mixture concentrated in vacuo to remove EtOH. HCl was added to the mixture until the pH reached 4. The mixture was extracted with EtOAc (3 × 50 mL). The combined organic phase was washed with brine (10 mL), dried with Na$_2$SO$_4$, and filtered. The solvent was removed in vacuo to afford 1.04 g of crude acid. The residue was dissolved in CH$_2$Cl$_2$ (20 mL). *N*-Acetylcysteamine (800 μL, 8 mmol, 1.2 equiv) was added followed by dicyclo-hexylcarbodiimide (2.5 g, 12.1 mmol, 1.9 equiv). The reaction mixture was stirred at room temperature overnight. The reaction mixture was cooled to −78 °C and the dicyclohexylurea byproduct removed by filtration. The filtrate was concentrated in vacuo. Column chromatography (silica gel, EtOAc) afforded 1.425 g of 7-hydroxyoctanoyl *N*-acetylcysteamine thioester (5.46 mmol, 94% yield over two steps): $R_f = 0.167$ (EtOAc); ^{1}H NMR (400 MHz, CDCl$_3$) δ 5.90 (bs, 1H), 3.77 (m, $J = 5.8$ Hz, 1H), 3.40 (dd, $J = 12.4$, 6.0 Hz, 2H), 3.00 (t, $J = 6$, 6.8 Hz, 2H), 2.55 (t, $J = 8$, 7.2 Hz, 2H), 1.96 (s, 3H), 1.32 (bm, 8H), 1.17 (d, $J = 6$ Hz, 3H).

Expression and Purification of Holo and Apo EpoD-M6

The holo form of EpoD-M6 was expressed and purified from *E. coli* strain BAP1, containing pMLT11. BAP1 (*15*) is an engineered derivative of BL21(DE3) containing a chromosomally integrated copy of the *sfp* phosphopan-theinyl transferase gene from *Bacillus subtilis*, which is required for the posttranslational phosphopantheinylation of the acyl carrier protein (ACP) domain of a PKS module. The apo form of EpoD-M6 was expressed in *E. coli* strain BL21(DE3) containing pMLT11. Cultures were grown in 3 L of 2× YT medium (16 g/L peptone, 10 g/L yeast extract, and 5 g/L NaCl) containing 50 μg/mL kanamycin at 18 °C to an optical density (OD$_{600}$) of 3 (approximately 50 h). The cells were cooled on ice for 15 min, after which they were induced with 1 mM IPTG for 18 h and harvested by centrifugation. The cell pellets were resuspended in 20 mL of lysis buffer [50 mM Tris-HCl (pH 8.0), 200 mM NaCl, 2 mg/mL pepstatin, 2 mg/mL leupeptin, and 1.5 mM benza-midine] and lysed by sonication. The lysates were centrifuged for 1 h at 43000g. The supernatants were added to 10 mL of Ni-NTA resin (Qiagen) equilibrated with lysis buffer and rocked at 4 °C for 1 h. The resins were washed with 100 mL of lysis buffer, followed by 100 mL of wash buffer [50

mM Tris-HCl (pH 8.0), 200 mM NaCl, and 10 mM imidazole]. The bound proteins were eluted with 35 mL of elution buffer [50 mM Tris-HCl (pH 8.0), 200 mM NaCl, and 200 mM imidazole]. DTT to a concentration of 0.5 mM was added to the eluates. The eluates were concentrated to approximately 3 mL in a 100 000 MW cutoff concentrator (Amicon), resuspended in 35 mL of FPLC buffer A (100 mM Na$_2$HPO$_4$, 1 mM EDTA, 2.5 mM DTT, and 20% glycerol), and concentrated again to approximately 5 mL. The 5 mL eluate was diluted into 45 mL of FPLC buffer A (100 mM Na$_2$HPO$_4$, 1 mM EDTA, 2.5 mM DTT, and 20% glycerol) and further purified on a HiTrap Q anion-exchange column equilibrated with buffer A using a linear gradient from 100% FPLC buffer A to 100% FPLC buffer B (100 mM Na$_2$HPO$_4$, 1 mM EDTA, 2.5 mM DTT, 20% glycerol, and 500 mM NaCl) over 60 min at a flow rate of 3 mL/min. The appropriate fractions were collected and concentrated in a 100 000 MW cutoff to yield approximately 10 mg of purified holo or apo EpoD-M6 per liter of culture. Aliquots were flash-frozen in liquid nitrogen and stored at −80 °C.

Labeling of EpoD-M6

Labeling assays (20 μL) were performed either on ice for 5 min or at room temperature for 20 min. The reaction mixture contained 100 mM NaH$_2$PO$_4$ (pH 7.2), 1 mM EDTA, 10−20 μM [2-^{14}C]methylmalonyl-CoA, [1-^{14}C]acetyl-CoA, [1-^{14}C]octanoyl-CoA, or [1-^{14}C]lauroyl-CoA substrate (Ameri-can Radiolabeled Chemicals), and either 5 mM holo EpoD M6 or apo EpoD M6. The reactions were initiated by the addition of the CoA substrate. After 5 min on ice or 20 min at room temperature, the reactions were quenched with 10 μL of SDS−PAGE loading buffer. The samples (10 μL) were run on a 4 to 15% gradient gel (Bio-Rad), stained, destained, dried, and analyzed with a phosphoimager (InstantImager, Packard Instruments).

Assays for in Vitro Condensation by SDS−PAGE and Radio-TLC

Condensation assays (25 μL) were performed at 30 °C in a reaction mixture containing 400 mM NaH$_2$PO$_4$ (pH 7.4), 625 μM DTT, 250 μM EDTA, 5% glycerol, 4 mM NADPH, 18.4 μM [2-^{14}C]methylmalonyl-CoA, 2 mM SNAC thioester substrate, either 0 or 5 mM cerulenin, and 5 μM holo EpoD-M6. Immediately prior to initiation of the reaction, the enzyme was preincubated at room temperature for 15 min in the presence or absence of cerulenin and then added to the other reaction components. The reactions were initiated by the addition of [2-^{14}C]methylmalonyl-CoA. For SDS−PAGE analysis, 10 μL of the reaction mixture was removed after 20 min and the reaction quenched by adding 5 μL of SDS−PAGE loading buffer. The samples (10 μL) were run on a 4 to 15% gradient gel, stained, destained, dried, and analyzed with a phosphoimager. For radio-TLC analysis, 10 μL of the reaction mixture was removed after 20 min and the reaction quenched via addition of 20 μL of 500 mM KOH. The samples were heated at 65 °C for 20 min, after which 15 μL of 1.6 M HCl was added. The samples were then extracted with 2 × 100 μL of EtOAc, after which the extracts were dried in vacuo. The samples were resuspended in 10 μL of EtOAc and subjected to TLC analysis (silica gel, 1:5 EtOAc/hexane with 1% acetic acid for reactions with

octanoylSNAC, 8-bromooctanoylSNAC, 2-octenoylSNAC, and 3-octenoylSNAC, 3:1 EtOAc/hexane with 1% acetic acid for reactions with natural SNAC and 7-hydroxyoctanoyl-SNAC). The TLC plates were analyzed with a phospho-imager.

In Vitro Assay of Chain Elongation by GC−MS

Condensation assays (50 μL) were performed at 30 °C in a reaction mixture containing 400 mM NaH_2PO_4 (pH 7.4), 625 μM DTT, 250 μM EDTA, 5% glycerol, 4 mM NADPH, 150 μM methylmalonyl-CoA, 2 mM octanoylSNAC sub-strate, and 5 μM holo EpoD-M6. The reactions were initiated via the addition of methylmalonyl-CoA. After 2 h, the reactions were quenched by adding 100 μL of 500 mM KOH. The samples were heated at 65 °C for 20 min, after which 75 μL of 1.6 M HCl was added. The samples were then extracted with 2×200 μL of EtOAc, after which the extracts were dried in vacuo. The samples were resuspended in 10 μL of MeOH. Analysis by GC−MS was carried out on a Hewlett-Packard 6890 gas chromatograph fitted with a Hewlett-Packard 5973 mass spectrometer. A 30 m × 0.25 mm (internal diameter) HP5MS column was used with helium carrier gas at a flow rate of 1 mL/min. One microliter of sample was injected with a 5:1 split. The GC oven temperature was held at 50 °C for 5 min and then increased to 250 °C at a rate of 20 °C/min and held for 5 min.

In Vivo Production of Condensation Products

E. coli strain BAP1 containing pMLT17 was grown in 20 mL of LB containing 50 μg/mL kanamycin at 37 °C overnight. The overnight culture (10 mL) was used to inoculate 1 L of 2× YT medium (16 g/L peptone, 10 g/L yeast extract, and 5 g/L NaCl) containing 50 μg/mL kanamycin. This culture was grown at 18 °C to an OD_{600} of 3 (approximately 50 h) and cooled on ice for 15 min. Aliquots of cells (50 mL) were centrifuged and resuspended in 2.5 mL of fresh 2× YT medium containing 50 mg/mL kanamycin, resulting in an OD_{600} of ~60. The cultures were induced with 1 mM IPTG. SNAC thioester substrates (200 mM in DMSO) were added to a final concentration of 2 mM. As a negative control, DMSO was added in place of an SNAC substrate. Unlabeled propionate (0.4 g/mL, pH 7.0) or [1-^{13}C]propionate (0.2 g/mL, pH 7.0) was added to a final concentration of 2 g/L. The cultures were incubated at 18 °C. After 24 h, the cultures were extracted with EtOAc (1 × 20 mL and then 1 × 10 mL), washed with brine (2 mL), and dried over Na_2SO_4. The extracts were then dried in vacuo. The samples were resuspended in 10 mL of MeOH. Analysis by GC−MS was carried out as described above except with a sample split of 50:1.

RESULTS

Initial attempts to express the isolated M6 of EpoD resulted in lower than desired yields of protein production (C. N. Boddy, unpublished results). The level of protein expression of independent PKS modules has been shown to increase when the modules are fused to the linker regions that naturally reside on the N- and C-terminal ends of PKS polypeptides (*16, 17*). Therefore, we appended the N-terminal linker region from the protein DEBS2 of the 6-deoxyeryth-ronolide B synthase to the N-terminus of the ketosynthase
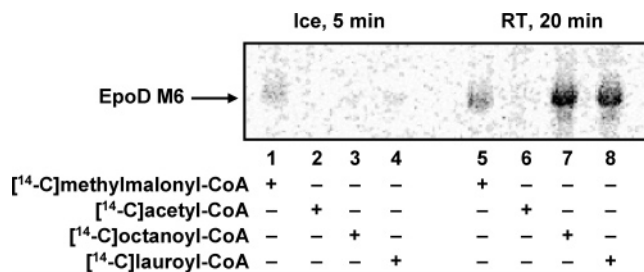

FIGURE 2: SDS−PAGE gel showing the ability of EpoD-M6 to load radiolabeled substrates.
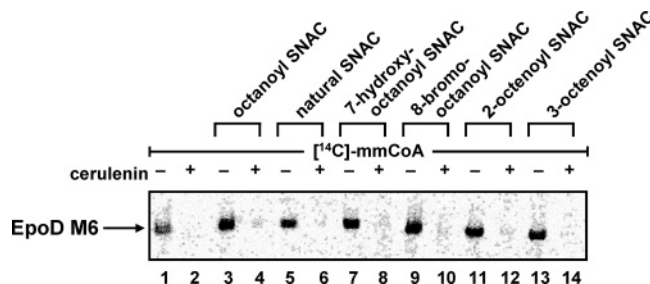

FIGURE 3: SDS−PAGE analysis of EpoD-M6 incubated at 30 °C for 20 min with [^{14}C]methylmalonyl-CoA and various SNAC thioester substrates in the presence and absence of cerulenin. The KS domain is active, as indicated by the presence of radiolabeling only in the absence of cerulenin. The extent of labeling was 1.5−3.5 times greater in the presence of SNAC thioester substrates as measured by scintillation counting. Lanes 1 and 2 correspond to addition of [^{14}C]methylmalonyl-CoA only with no SNAC thioester substrate. In lanes 5 and 6, "natural SNAC" refers to the SNAC thioester mimic that is converted into epothilone C by EpoD-M6, EpoE, and EpoF (see Figure 1).
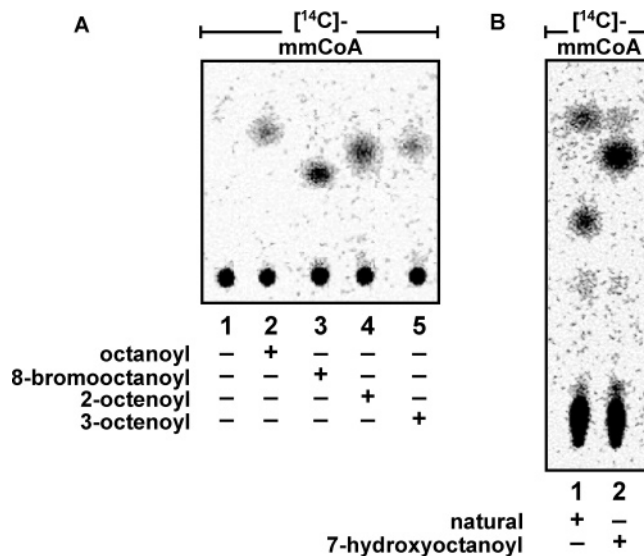

FIGURE 4: EpoD-M6 incubated with [2-^{14}C]methylmalonyl-CoA, and SNAC thioesters, followed by base hydrolysis and radio-TLC. (A) Lanes 1−5 were developed in 20% EtOAc in hexanes: (1) no SNAC substrate, (2) octanoylSNAC, (3) 8-bromooctanoylSNAC, (4) 2-octenoylSNAC, and (5) 3-octenoylSNAC. (B) Lanes 1 and 2 were developed in 75% EtOAc in hexanes: (1) natural SNAC and (2) 7-hydroxyoctanoylSNAC. The radiolabeled product that mi-grates faster than the expected elongation products of both substrates has not been characterized.

(KS) domain of module 6 of EpoD, creating the protein herein termed EpoD-M6. The holo form of this enzyme was obtained by expressing the *epoD-M6* gene in BAP1, an engineered derivative of BL21(DE3) that contains a chro-mosomally integrated copy of the *sfp* phosphopantetheinyl
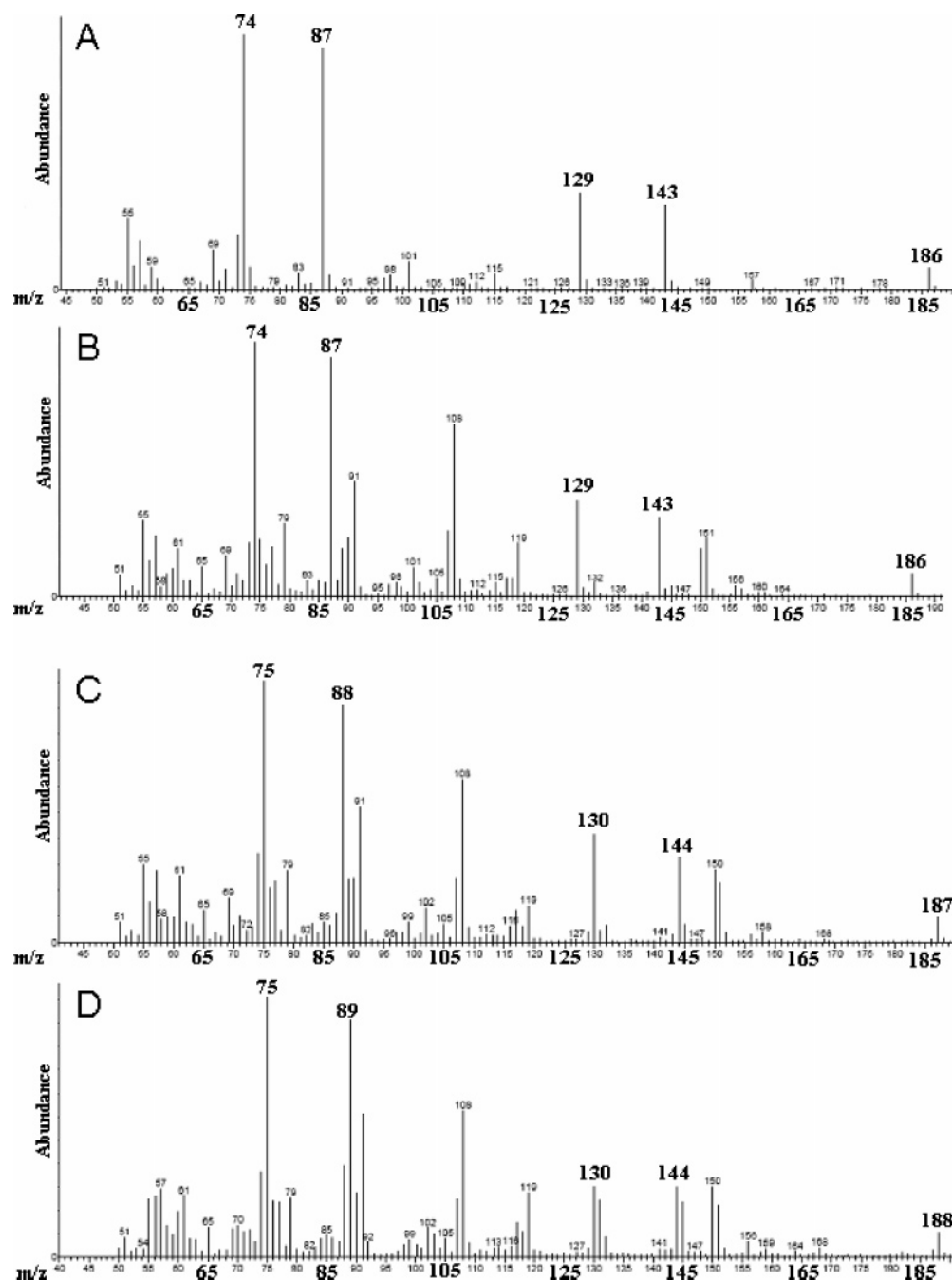
FIGURE 5: (A) 2-Methyldecanoic acid standard, with a retention time of 12.01 min and a parent ion at m/z 186, and parent ion and major fragments highlighted in boldface. (B) Extract of *E. coli* BAP1/pMLT17 fed with [^{12}C]octanoylSNAC and [^{12}C]propionate (retention time of 12.01 min, parent ion at m/z 186), and fragment peaks (m/z 143, 129, 87, and 74) correspond to the known standard. (C) An extract of the same strain fed with [^{13}C]octanoylSNAC and [^{12}C]propionate (retention time of 12.01 min, parent ion increases to m/z 187), and fragment peaks increase to m/z 144, 130, 88, and 75, consistent with incorporation of ^{13}C-labeled substrate. (D) An extract of the same strain fed with [^{13}C]octanoylSNAC and [^{13}C]propionate (retention time of 12.01 min, parent ion increases to m/z 188), and increases in fragment abundances for m/z 145 and 131 were observed and fragment peaks at m/z 89 and 75 observed, consistent with incorporation of both ^{13}C-labeled substrates into the product.

transferase gene from *B. subtilis*. The apo form of EpoD-M6 was expressed in *E. coli* strain BL21(DE3). The apo and holo forms of EpoD-M6 were purified as His-tagged proteins using Ni-NTA chromatography followed by anion-exchange chromatography to >90% homogeneity, as determined by SDS−PAGE (see the Supporting Information).

To test the flexibility of the KS domain of EpoD-M6 to accept substrates simpler than its natural substrate, we incubated purified holo EpoD-M6 with [2-^{14}C]methylmalo-nyl-CoA, [1-^{14}C]acetyl-CoA, [1-^{14}C]octanoyl-CoA, and [1-^{14}C]-

lauroyl-CoA. Substrates attached to AT and ACP domains are more hydrolytically labile than those attached to KS domains, so all of the labeled substrates mentioned above were incubated with EpoD-M6 both on ice for 5 min and at room temperature for 20 min (Figure 2). As predicted, after 5 min on ice, EpoD-M6 is weakly labeled with [2-^{14}C]-methylmalonyl-CoA. Interestingly, the labeling intensity is somewhat stronger after 20 min at room temperature, suggesting that some of the nucleophilic methylmalonyl substrate bound to the ACP may be decarboxylated and
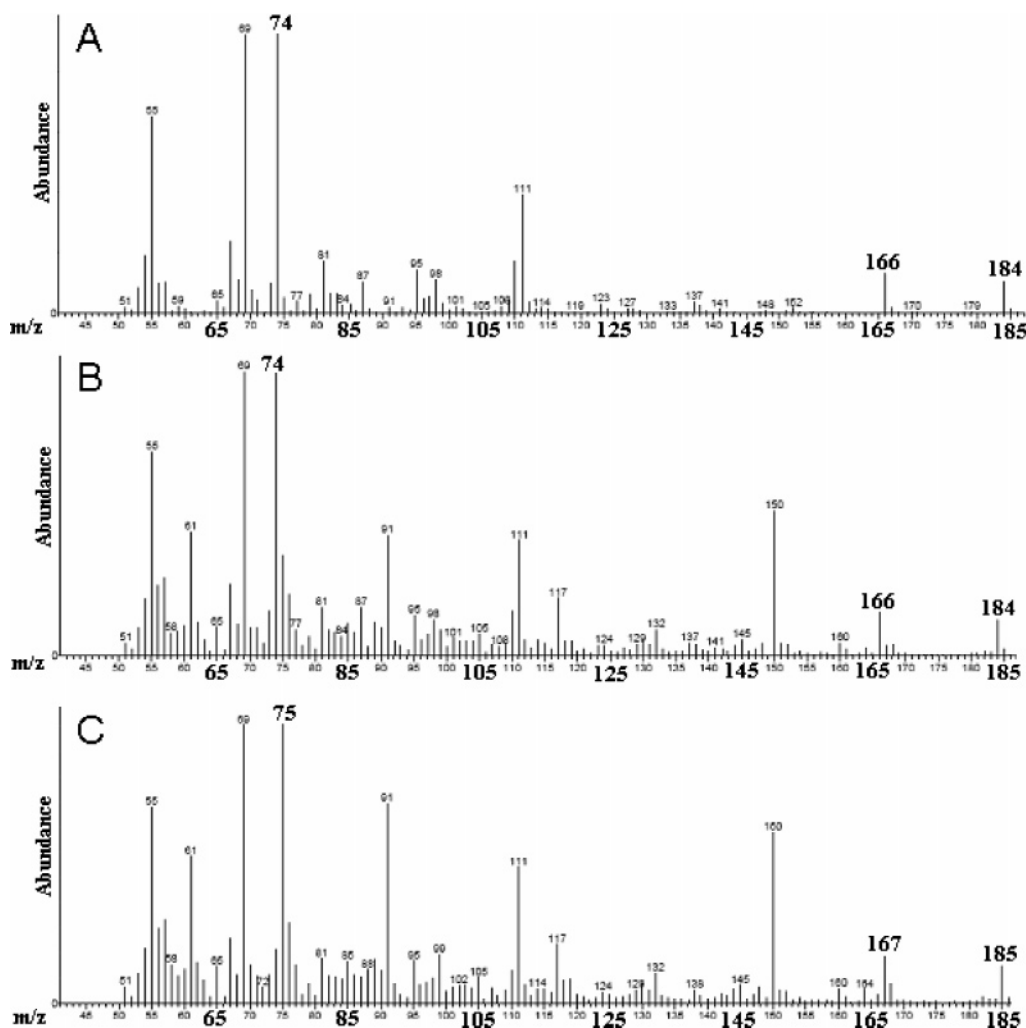
FIGURE 6: (A) 2-Methyl-5-decenoic acid standard, with a retention time of 11.96 min and a parent ion at *m/z* 184, and parent ion and major fragment peaks are highlighted in boldface. (B) Extract of *E. coli* BAP1/pMLT17 fed with [^{12}C]-3-octenoylSNAC and [^{12}C]propionate (retention time of 11.96 min, parent ion at *m/z* 184), and major fragment ions (*m/z* 166 and 74) correspond to a known standard. (C) Extract of the same strain fed with [^{12}C]-3-octenoylSNAC and [^{13}C]propionate (retention time of 11.96 min, parent ion at *m/z* 185), and major fragment ions (*m/z* 167 and 75) are consistent with incorporation of ^{13}C-labeled propionate into the product.

subsequently transferred to the KS. Consistent with this hypothesis, at either temperature, labeling of the apoprotein with [2-^{14}C]methylmalonyl-CoA was 3−4-fold weaker than labeling of the holoprotein under equivalent conditions (data not shown). The labeling of EpoD-M6 is considerably stronger in the presence of [1-^{14}C]octanoyl-CoA and [1-^{14}C]-lauroyl-CoA, which are presumably directly loaded onto the KS domain; however, acetyl-CoA does not label EpoD-M6.

Seeing that EpoD-M6 was labeled with [1-^{14}C]octanoyl-CoA, we tested the ability of EpoD-M6 to be acylated with a series of *N*-acetylcysteamine thioesters (SNAC), including octanoylSNAC, 2-octenoylSNAC, 3-octenoylSNAC, 8-bromooctanoylSNAC, and 7-hydroxyoctanoylSNAC, and to be condensed with a methylmalonyl extender unit to produce an elongated product. For this, we incubated EpoD-M6 with appropriate SNAC thioester substrates using the natural substrate mimic that yields epothilone C as a control (*14*). Incubations were performed at 30 °C for 20 min in the presence of [2-^{14}C]methylmalonyl-CoA, both with and without cerulenin, a known KS inhibitor. SDS−PAGE analysis followed by phosphorimaging showed that EpoD-M6 was labeled in the presence of [2-^{14}C]methylmalonyl-CoA alone but that the labeling was 1.5−3.5 times stronger

in the presence of various SNAC thioester substrates (Figure 3). As discussed above, weak labeling of EpoD-M6 in the presence of [2-^{14}C]methylmalonyl-CoA alone is presumably because its decarboxylated form is transferred back to the KS. Addition of an appropriate electrophilic SNAC thioester substrate presumably results in chain elongation, leading to formation of a relatively stable ACP-bound intermediate. That all observed labeling of the holoprotein in this experiment was blocked by cerulenin confirms that the observed labeling depends upon KS domain activity. Notably, cerulenin did not block the weak labeling of the apoprotein by [2-^{14}C]methylmalonyl-CoA alone (data not shown), a result that is also consistent with the requirement for methylmalonyl-ACP formation as a prerequisite for methylmalonyl decarboxylation.

The putative ACP-bound chain elongation products observed in Figure 3 can be released by base hydrolysis, as revealed by radio-TLC analysis (Figure 4). As expected, in a separate experiment, incubation of apo EpoD-M6 (the module without posttranslational phosphopanthetheinylation of the ACP domain) with [2-^{14}C]methylmalonyl-CoA in the presence of octanoylSNAC did not result in any visible product formation (data not shown).
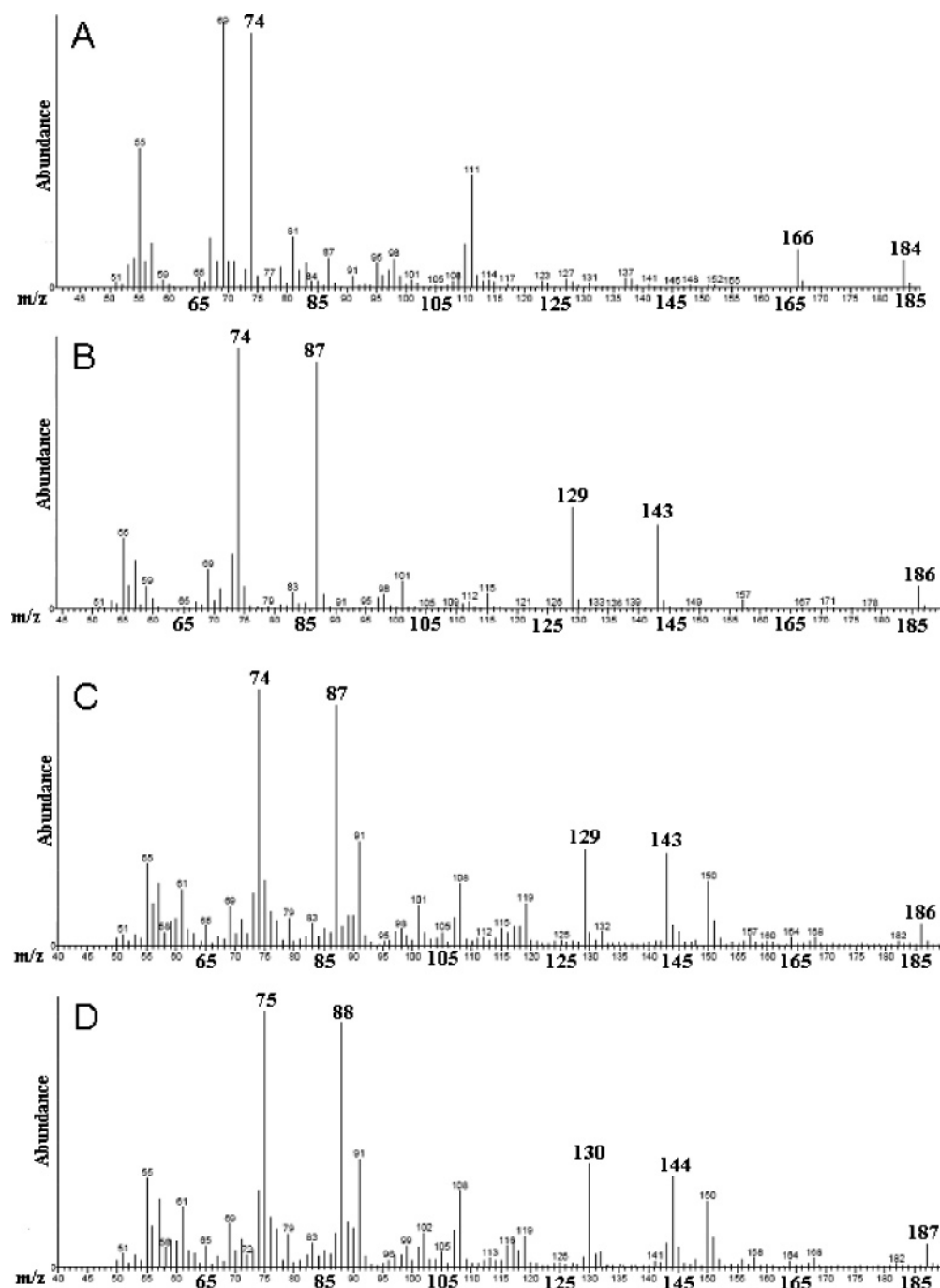
FIGURE 7: (A) 2-Methyl-4-decenoic acid standard, with a retention time of 12.00 min and a parent ion at *m/z* 184, and parent ion and major fragment peaks highlighted in boldface. (B) 2-Methyldecanoic acid standard, with a retention time of 12.01 min, and parent ion and major fragment peaks highlighted in boldface. (C) An extract of *E. coli* BAP1/pMLT17 fed with [^{12}C]-2-octenoylSNAC and [^{12}C]propionate (retention time of 12.01 min). The anticipated parent ion is not found (*m/z* 184); however, peaks corresponding to 2-methyldecanoic acid are found (*m/z* 186, 143, 129, 87, and 74) and verified. (D) The same strain is fed with [^{12}C]-2-octenoylSNAC and [^{13}C]propionate, where +1 peaks are found (*m/z* 187, 144, 129, 130, 88, and 75), indicating the product of 2-octenoylSNAC feeding is 2-methyldecanoic acid and that reduction of the starting material olefin has occurred.

The expected hydrolyzed product from the condensation of octanoylSNAC with methylmalonyl-CoA is 2-methyl-decanoic acid. To confirm that the product observed by radio-TLC (Figure 4) is in fact 2-methyldecanoic acid, a standard of this compound was synthesized. This standard comigrated alongside the in vitro reaction radioactive product. In addition, the nonradioactive condensation product was produced through incubation of EpoD-M6 with octanoyl-SNAC and unlabeled methylmalonyl-CoA, followed by base hydrolysis. Analysis by GC−MS showed this product had

the same retention time (12.01 min) and fragmentation profile as the 2-methyldecanoic acid standard. Thus, not only is the KS domain of EpoD-M6 acylated by octanoylSNAC and able to promote condensation, but the ketoreductase, dehydratase, and enoyl reductase domains are able to process this substrate and give the expected final product.

Identification of the expected product from in vitro incubation of EpoD-M6 with octanoylSNAC and methyl-malonyl-CoA encouraged us to attempt in vivo production of the same compound, as well as condensation compounds

from additional SNAC thioester substrates. To this end, cultures of *E. coli* strain BAP1 containing the plasmid encoding EpoD-M6 were fed individual SNAC thioester substrates (octanoylSNAC, 7-hydroxyoctanoylSNAC, 2-octenoylSNAC, or 3-octenoylSNAC) and the methylmalonyl-CoA precursor, sodium propionate. After incubation for 24 h, cell extracts were analyzed by GC−MS for the presence of condensation products.

We synthesized known standards of 2-methyldecanoic acid and 2-methyl-5-decenoic acid. Feeding sodium propionate and octanoylSNAC or 3-octenoylSNAC to EpoD-M6-expressing BAP1 cultures gave products with retention times and fragmentation patterns identical to those of known standards as determined by GC−MS (Figures 5 and 6). To confirm the identity of the products, feeding experiments were conducted with octanoylSNAC or 3-octenoylSNAC and ^{13}C-labeled sodium propionate. GC−MS analysis of the resultant cell extracts showed these experiments gave products with retention times identical to those of the known standards and with fragmentation patterns consistent with ^{13}C-labeled products. Further confirmation of the formation of 2-methyldecanoic acid was obtained by feeding experiments with ^{13}C-labeled octanoylSNAC and sodium propionate or ^{13}C-labeled sodium propionate. These assays resulted in products with retention times identical to those of known standards and fragmentation patterns consistent with products that were singly or doubly labeled with [13]C.

The expected product of feeding 2-octenoylSNAC and sodium propionate to BAP1 cultures expressing EpoD-M6 is 2-methyl-4-decenoic acid. No product with the retention time and fragmentation pattern of the synthetic 2-methyl-4-decenoic acid standard was found. Instead, we observed 2-methyldecanoic acid. That 2-octenoylSNAC is converted to 2-methyldecanoic acid was confirmed by feeding experiments with both ^{13}C-labeled and unlabeled sodium propionate. In the experiment with unlabeled sodium propionate, the product had the retention time and fragmentation pattern of the 2-methyldecanoic acid standard. In the experiment with ^{13}C-labeled sodium propionate, the product had the retention time of the 2-methyldecanoic acid standard and the fragmentation pattern observed when octanoylSNAC was fed with ^{13}C-labeled sodium propionate (Figure 7). Two possible explanations can be proposed for this observation. First, the olefin could be reduced by the PKS module, either before or after chain elongation. Although this explanation invokes an unusual behavior for a PKS module, it cannot be discounted because the 12−13 double bond in epothilone C (Figure 1) is known to be generated by a module downstream of Epo-M4 (*7*). Alternatively, the olefin in 2-octenoylSNAC is reduced to octanoylSNAC by the enoyl reductase of the endogenous fatty acid synthase, which is then elongated and processed as described above.

When EpoD-M6-expressing BAP1 cultures were fed 7-hydroxyoctanoylSNAC and sodium propionate, a product was identified that had the molecular weight of the expected product, 2-methyl-9-hydroxydecanoic acid. This was anticipated since evidence of incorporation had been seen in earlier in vitro assays (Figures 3 and 4). Identical feeding experiments in which ^{13}C-labeled sodium propionate was used as a substitute gave a product with an identical retention time and a molecular weight consistent with a ^{13}C-labeled product; however, anticipated fragment ions bearing heavy isotopes
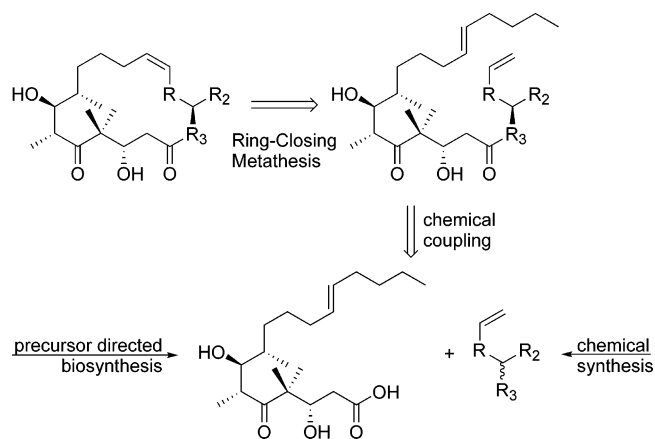

FIGURE 8: Combined chemical and enzymatic synthesis of epothilones.

were not found. The signal-to-noise ratio in the 7-hydroxy-octanoylSNAC feeding study was judged to be too poor to be conclusive in the absence of a synthetic standard. Similarly, evidence of incorporation of the 8-bromooctanoyl-SNAC had been produced by radio-TLC in in vitro assays; however, signal-to-noise ratios again prohibited definitive identification of the anticipated 2-methyl-10-bromodecanoic acid product by GC−MS (data not shown).

DISCUSSION

The epothilone system is especially well suited to precursor-directed biosynthesis. Structure−activity relationship studies of the epothilones have shown that modifications to the C1−C8 region of the natural product tend to eliminate microtubule stabilization but that changes to the rest of the molecule can be used to "tune" its activity (*3−6, 19−21*). We have shown that a mimic of the native substrate can be incorporated into the last four modules of the epothilone biosynthetic pathway, the portion of which synthesizes the critical C1−C8 segment (*14*). Developing non-native substrates that can gain entry into the last half of the epothilone system could enable the development of many epothilone analogues that could be tested for their pharmacological properties.

There are two ways to harness precursor-directed biosynthesis. The first involves synthesis of natural substrate analogues that are individually fed into the epothilone biosynthetic pathway. The advantage of this approach is that finished epothilone analogues are directly obtained from the fermentation medium. However, this approach is limited by the number of substrates that can be tolerated by the entire biosynthetic pathway. An alternative approach involves synthesizing and feeding a considerably simpler substrate with a chemically labile handle. The harvested product then becomes a starting material for the semisynthetic preparation of analogues of the natural product (Figure 8). The advantages of the latter approach are that a single efficiently incorporated precursor can yield a common intermediate for a variety of analogues of biological interest.

This work is a significant step toward the goal of producing epothilone analogues by precursor-directed biosynthesis. The promise shown by the ability of EpoD-M6 to accept and elongate 3-octenoylSNAC is brought into focus by the fact that several total syntheses of epothilone and its

analogues have used ring-closing metathesis to perform the crucial step of forming the macrolactone ring. We therefore envision the use of this or related substrates in combined chemoenzymatic syntheses of epothilones.

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SUPPORTING INFORMATION AVAILABLE

SDS−PAGE analysis of holo EpoD-M6 (lane 1) and apo EpoD-M6 (lane 2). This material is available free of charge via the Internet at http://pubs.acs.org.

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