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Utilization of Alternate Substrates by the First Three Modules of the Epothilone Synthetase Assembly Line

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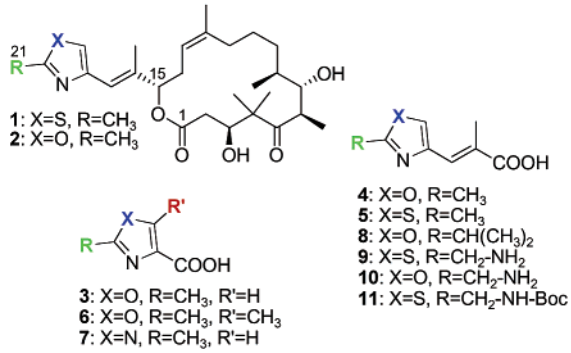
The epothilones, a family of macrolactone natural products produced by the myxobacterial species *Sorangium cellulosum*,[1] are of current clinical interest as antitumor agents. Like Taxol, this family of compounds stabilizes the polymerized state of microtubules, thereby halting cell cycle progression.[2] Inspection of the structure of the epothilones, such as epothilone D **1**, suggests a hybrid polyketide/nonribosomal peptide biosynthetic origin, and the recent sequencing of the epothilone biosynthetic gene cluster has validated this proposal.[3] Nine polyketide synthase (EpoA, EpoC−EpoF) and one nonribosomal peptide synthetase (EpoB) modules are distributed over six protein subunits. EpoA, the polyketide synthase (PKS) loading module, is proposed to load and decarboxylate malonyl-CoA to generate the acetyl-*S*-EpoA species, where *S* represents the thioester bond between the substrate and the posttranslationally attached phosphopantetheinyl arm of the carrier protein domain. EpoB catalyzes condensation of this acetyl with cysteinyl-*S*-EpoB to form the *N*-acetylcysteinyl-*S*-EpoB covalent intermediate. *N*-acetylcysteinyl-*S*-EpoB is then cyclized to form the methythiazolinyl-*S*-EpoB species that is oxidized to generate methythiazolyl-*S*-EpoB (Figure 1).[4] The remaining seven PKS modules (EpoC−F) extend the polyketide chain until synthesis is terminated by cyclization of the linear chain to the 16-membered macrolactone. Structure−activity studies have demonstrated that a thiazole or other aromatic moiety is absolutely required for antitumor activity.[5] Here we have examined unnatural substrates with EpoA and EpoB to investigate the enzymatic construction of alternate heterocyclic structures and the subsequent elongation of these products by EpoC.



Careful analysis of fermentation broths from epothilone-producing myxobacterial cultures reveals several minor products, among them the equally active oxazole-containing epothilone D analogue **2**.[6] The production of **2** presumably reflects some substrate permissivity of EpoB, and we have validated this expectation in vitro with purified EpoB and EpoC and the ACP domain from EpoA.[4,7] As previously reported, the reversible formation of L-seryl-AMP in the EpoB adenylation domain is supported by [^{32}P]-PP$_i$-ATP radioactive exchange at a rate of 11.0 min^{-1} as compared to a rate of 54.9 min^{-1} that is enabled by the native substrate cysteine.[4] When the covalent modification of EpoB with radiolabeled amino acid was monitored, 48% of the [^{3}H]-L-seryl-*S*-EpoB species was detected as compared to 79% formation of the [^{14}C]-L-cysteine-*S*-EpoB species (data not shown). Incubation of acetyl-*S*-EpoA-ACP with [^{3}H]-L-seryl-*S*-EpoB produced a covalent EpoB derivative that, after alkaline hydrolysis from the phosphopantetheine arm and radio-HPLC analysis, yielded the radiolabeled methyloxazole carboxylic acid **3**, as evidenced by co-injection with authentic standard and mass spectrometry[8] (for K$^+$ salt, 165.28 observed, 165.19 expected). Including EpoC, methylmalonyl-CoA, and NADPH in such incubations provided a covalent acyl-*S*-EpoC intermediate that, after hydrolysis and HPLC analysis, was shown to be the methyloxazolyl-methylacrylic acid **4** (Figure 2A), the oxazole analogue of the normal thiazolyl methylacrylate **5**. Thus EpoB can activate the alternative amino acid L-serine, use it as nucleophile for acylation by the acetyl-*S*-EpoA-ACP donor, cyclodehydrate, and aromatize to the methyloxazolyl-*S*-EpoB. Furthermore, the downstream methylmalonyl-*S*-EpoC module can use this alternate heterocycle as donor. The rates of the various steps are not readily amenable to steady state kinetic analysis since all of the intermediates are covalently bound acyl enzyme species and will require single turnover studies for kinetic deconvolution of relative catalytic efficiencies. However, a preliminary analysis indicates that the rate of oxazole formation is only slightly slower than that of thiazole formation.

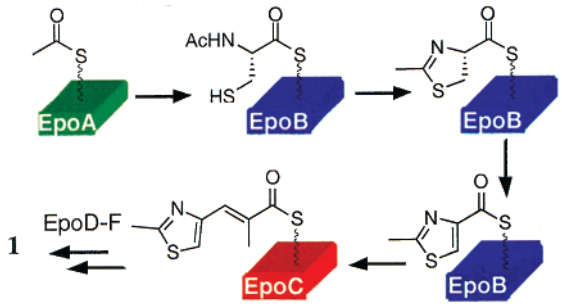


Figure 1. Covalent intermediates resulting from acetyl transfer from EpoA to EpoB, cyclodehydration, oxidation, and transfer to EpoC are shown. EpoD−F catalyze further polyketide chain elongation and cyclorelease of product.

Subsequent studies with L-threonine and L-2,3-diaminopropionate (Dap) in place of cysteine indicated that both substrates could be loaded onto EpoB (155 kDa) and acetylated by [^{3}H]-acetyl-*S*-ACP

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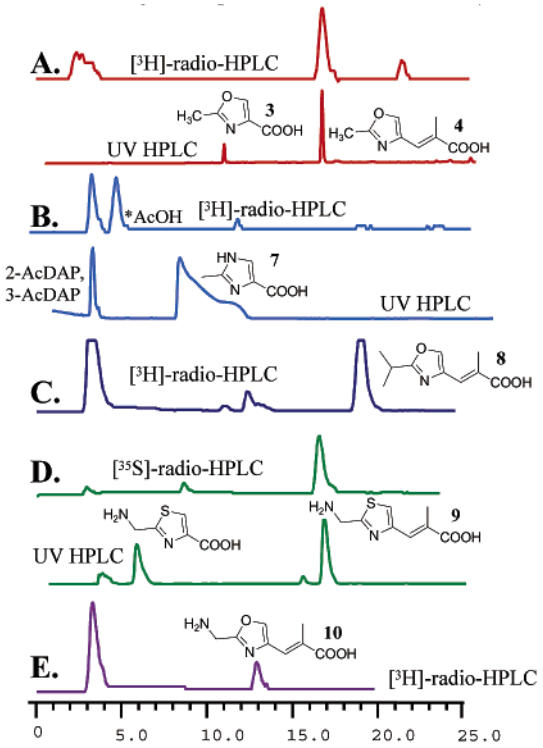

Figure 2. Radio-HPLC traces of EpoA/B/C reactions characterized by comparison to UV−HPLC of chemically synthesized standards or MS data. (A) Formation of **4** validated by co-injection. (B) Failure of reaction of Dap-*S*-EpoB to form methyl imidazole compound **7**, validated by co-injection. (C) Formation of **8**, validated by MS. (D) Formation of **9**, validated by co-injection and MS. (E) Formation of **10**, validated by MS.

(16.5 kDa) as evidenced by monitoring the shift of radioactivity on a protein gel using autoradiography (see Supporting Information). Further analysis of the alkaline-hydrolyzed products with radio-HPLC indicated that a very small amount of acetyl-Thr-*S*-EpoB appeared to cyclize to give the corresponding 2,5-dimethyloxazole **6** (data not shown), but no evidence for cyclization of the acetyl-Dap-*S*-EpoB to yield the analogous heteroaromatic imidazole **7** was detected (Figure 2B).

In prior studies, we have noted that EpoB will accept a variety of acyl group donors presented by the EpoA-ACP domain.[4] To test whether a double substrate substitution could be effected at the start of the epothilone synthetase assembly line, we utilized the priming phosphopantetheinyl transferase Sfp[7] to load the apo form of EpoA-ACP with isobutyryl-CoA in incubations with EpoB, ATP, and [^{3}H]-serine as well as methylmalonyl-CoA, NADPH, and EpoC. The isopropyloxazolyl methacrylate **8**, which was readily detected by radio-HPLC and characterized by mass spectroscopy (196.90 observed, 196.22 expected) after hydrolytic release from EpoC (Figure 2C), represents a double substitution that can be carried through the EpoA/B/C subunit machinery.

Introduction of polar functional groups at the epothilone C21 position may improve its pharmacological properties.[9] In an initial effort to introduce additional functionality into the epothilone molecule, we prepared and evaluated several aminoacyl-*S*-EpoA-ACP proteins[10] as potential donors for Cys-*S*-EpoB. Analysis by radio-HPLC suggested that a variety of aminoacyl groups were transferred across the EpoA/B/C subunit interfaces. As a representative example, the reaction of glycyl-*S*-EpoA-ACP, Cys-*S*-EpoB, and methylmalonyl-*S*-EpoC to yield the amino-alkyl-thiazolyl-methyacrylic acid product **9** was validated by mass spectroscopy (198.99 observed, 199.05, expected) and by co-injection with an authentic standard (Figure 2D). Additionally, product **10**, which represents another double substrate substitution, could be produced from Gly-*S*-ACP and Ser-*S*-EpoB as evidenced by mass spectroscopy (183.05 observed, 183.07, expected) (Figure 2E). When the cationic amino group of glycyl-CoA was blocked with *N*-Boc, acylglycyl transfer also occurred to yield the *N*-blocked-amino-alkyl-thiazolyl-methyacrylic acid product **11** (data not shown). Transfer of *N*-Boc protected intermediates among the protein interfaces appeared to be slightly slower than the corresponding processing of the free amine (data not shown), suggesting that steric bulk may slow substrate processing.

The first three enzymes of the epothilone biosynthetic machinery can utilize serine to install an oxazole in place of a thiazole in the epothilone structure. Furthermore, EpoB will tolerate functionalized donor groups from the EpoA-ACP domain to produce epothilone fragments modified at the C21 position. These studies with the early enzymes of the epothilone biosynthesis cluster suggest that combinatorial biosynthesis may be a viable means for producing a variety of epothilone analogues that incorporate diversity into the heterocycle starter unit.

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Supporting Information Available: Details of protein assays and chemical standard synthesis (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Hofle, G.; Bedorf, N.; Gerth, K.; Reichenbach, H. German Patent DE 4138042, 1993. (b) Gerth, K.; Bedorf, N.; Hofle, G. *J. Antibiot.* **1996**, 560−563.
(2) (a) Bollag, D. M.; McQueney, P. A.; Zhu, J.; Hensens, O.; Koupal, L.; Liesch, J.; Goetz, M.; Lazarides, E.; Woods, C. M. *Cancer Res.* **1995**, *55*, 2325−2333. (b) Florsheimer, A.; Altmann, K. H. *Expert Opin. Ther. Pat.* **2001**, *11*, 951−968.
(3) (a) Tang, L.; Shah, S.; Chung, L.; Carney, J.; Katz, L.; Khosla, C.; Julien, B. *Science* **2000**, *287*, 640−642. (b) Molnar, I.; Schupp, T.; Ono, M.; Zirkle, R. E.; Milnamow, M.; Nowak-Thompson, B.; Engel, N.; Toupet, C.; Stratmann, A.; Cyr, D. D.; Gorlach, J.; Mayo, J. M.; Hu, A.; Goff, S.; Schmid, J.; Ligon, J. M. *Chem. Biol.* **2000**, *7*, 97−109.
(4) (a) Chen, H.; O'Connor, S.; Cane, D. E.; Walsh, C. T. *Chem. Biol.* **2001**, *8*, 899−912. (b) O'Connor, S. E.; Chen, H.; Walsh, C. T. *Biochemistry* **2002**, *41*, 5685−5694.
(5) (a) Nicolaou, K. C.; Roschangar, F.; Vourloumis, D. *Angew. Chem., Int. Ed.* **1998**, *37*, 2014−2045. (b) Harris, C. R.; Danishefsky, S. J. *J. Org. Chem.* **1999**, *64*, 8434−8456. (c) Hofle, G.; Glaser, N.; Leibold, T.; Sefkow, M. *Pure Appl. Chem.* **1999**, *71*, 2019−2024.
(6) Hardt, I. H.; Steinmetz, H.; Gerth, K.; Sasse, F.; Reichenbach, H.; Hofle, G. *J. Nat. Prod.* **2001**, *64*, 847−856.
(7) Quadri, L. E. N.; Weinreb, P. H.; Lei, M.; Nakano, M. M. Zuber, P.; Walsh, C. T. *Biochemistry* **1998**, *37*, 1585−1595.
(8) For details of MALDI mass spectroscopy and for synthesis and characterization of authentic standards, refer to Supporting Information.
(9) (a) Lee, C. B.; Chou, T.-C.; Zhang, X.-G.; Wang, Z.-G.; Kuduk, S. D.; Chappell, M. D.; Stachel, S. J.; Danishefsky, S. J. *J. Org. Chem.* **2000**, *65*, 6525−6533. (b) Vite, G.; Hofle, G.; Bifano, M.; Fairchild, C.; Glaser, N.; Johnston, K.; Kamath, A.; Kim, S.-H.; Leavitt, K.; Lee, F. F.-Y.; Leibold, T. Long, B.; Peterson, R.; Raghavan, K.; Reguerio-Ren, A. In *Abstracts of Papers*, 223rd National Meeting of the American Chemical Society, Orlando, FL, 2002; American Chemical Society: Washington, DC, 2002; MEDI-018.
(10) Belshaw, P. J.; Walsh, C. T.; Stachelhaus, T. *Science* **1999**, *284*, 486-489.

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