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Elucidating the Mechanism of *cis* Double Bond Formation in Epothilone Biosynthesis

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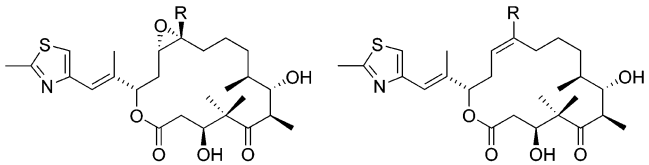
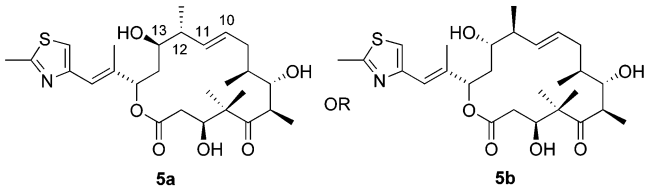
Epothilones A (**1**) and B (**2**), and their late-stage biosynthetic intermediates, epothilones C (**3**) and D (**4**), are a new class of naturally occurring microtubule depolymerization inhibitors with potent antitumor activity.[1] Epothilones D, B, and a derivative of B, are currently undergoing clinical trials. We and others have recently isolated and sequenced the epothilone gene cluster (epo) from *Sorangium cellulosum* and found that it encodes six multifunctional proteins comprising a mixed NRPS/type 1 modular PKS.[2] When moved into heterologous hosts, the six epothilone genes directed the production of epothilones C and D; addition of the adjacent gene *epoK* resulted in formation of epothilones A and B.[2,3]



It is now well established that type 1 modular PKSs are composed of multifunctional proteins comprising enzymatic domains that are organized into modules and that each module is responsible for a single complete cycle of polyketide chain extension and modification.[4] The domain composition and organization of the *epo* NRPS-PKS is consistent with the structure of epothilone, except for the absence of a DH function in module 4 that would produce the *cis* double bond between carbons 12 and 13 of **3** and **4**. We proposed previously that the *cis* double bond might be generated from the action of the DH of another domain, for example module 5.[2a]

To understand the role that it plays in epothilone biosynthesis, the DH5 domain was changed in two different ways: (a) inactivation: the highly conserved eight-amino acid segment 3944FLGDHLVF3951, located within the DH5 domain of EpoD, was replaced with the three-amino acid segment WLA; (b) removal: the entire DH/ER/KR segment in module 5 was replaced with a segment containing only a KR domain from either module 3 of the FK520 PKS or module 2 of the rapamycin PKS. All three recombinant strains produced (*E*)-10,11-didehydro-12,13-dihydro-13-hydroxyepothilone D (**5**) as the major product, with no detectable amount of either of 11-hydroxyepothilone C or D, the compounds expected if DH5 were not involved in forming the double bond at the 12,13 position. The structure of **5** was confirmed from MS and NMR data and the *anti* relationship of the 12-methyl and 13-hydroxyl groups established, but the absolute stereochemistry at C-12,13 was not determined. Trace amounts of the 12-desmethyl congener of **5** were detected by LC/MS.



Several independent experiments have yielded evidence consistent with the general correspondence between epothilone modules and epothilone structure. We have reported previously the production of (*E*)-10,11-didehydroepothilone D from an *Myxococcus xanthus* host carrying the *epo* PKS genes in which the ER domain of module 5 had been inactivated.[5] We have also independently inactivated the KR domains of modules 4 and 5 and have identified the expected 13-oxo- and 11-oxoepothilones from the corresponding strains and have executed AT exchanges in module 4 that have resulted in changes in the epothilone D to C ratios in the corresponding hosts.[6] These results indicate that modules 4 and 5 determine the structure of epothilone during the fourth and fifth elongation cycles of its biosynthesis, corresponding to positions 10−13 of the completed compounds. Production of an epothilone analogue containing the 13-hydroxy group (**5**) in the hosts in which the DH domain of module 5 was either inactivated or removed strongly implicates DH5 in the dehydration to yield the 12,13-double bond found in **3** and **4**. The unanticipated 10,11-double bond in **5** must have been produced by a DH function other than DH5, and probably beyond the fifth condensation cycle, either by the DH domain from another *epo* module or by a DH function not associated with the *epo* PKS. Thus, although likely, it is not currently possible to assert that DH5 carries out the dehydration that normally takes place during the fifth condensation cycle in the wild-type *epo* PKS. Nonetheless, the data presented here supports the proposal that, in the wild-type strain, the DH domain in the fifth module acts to dehydrate the alcohol function generated in the nascent chain during the fourth elongation cycle. Because it is very likely that DH5 also functions during the fifth condensation cycle, we propose that it acts twice during epothilone biosynthesis.

A biochemical mechanism for the iterative function of DH5 (Scheme 1) proposes that DH5 dehydrates two different chains, each tethered to ACP5. After the fourth elongation cycle (A), the acyl chain (**6**) is transferred from ACP4 to ACP5 without undergoing elongation, either directly or through intermediate transfer to KS5. With the chain attached to ACP5 and the backbone rotated to orient the 3*R*-OH and *pro*-2*S*-H for *syn* elimination, DH5-mediated dehydration generates **7**, containing the 2,3-*cis* double bond (B). Because ER5 would not be expected to reduce *cis* double

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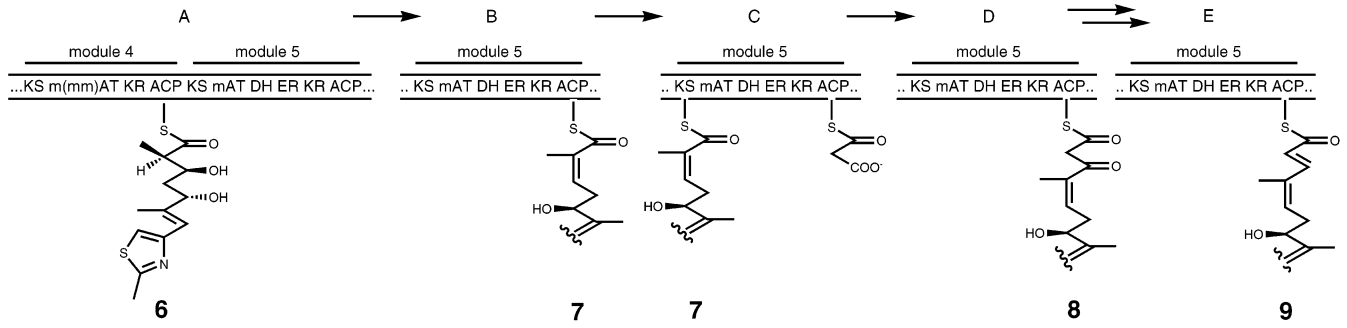
Scheme 1



bonds, **7** is not reduced, and the *cis* double bond persists throughout the succeeding steps of chain growth and maturation. At this point in the synthesis, **7** is passed backward from ACP5 to the KS domain of module 5 (either directly or via ACP4) to resume the fifth cycle of elongation (C−E), including the fifth cycle DH5-catalyzed dehydration that generates a *trans* double bond. In the DH5 mutant, after **6** is transferred from ACP4 to ACP5, it cannot undergo the first dehydration; thus, is backloaded to KS5 unchanged. Fifth-cycle elongation and β-ketoreduction takes place as normal, but the dehydration step at the 10,11 position is postponed until the chain has been passed to module 6 or beyond.

It is possible that the dehydrations could take place in the same chemical order as shown in Scheme 1 but with the acyl chain tethered to ACP4 for the first, and tethered to ACP5 for the second. For the first dehydration, therefore, ACP4 would have to make an unusual contact with DH5 to permit generation of the 2,3-*cis* double bond. Compound **7** would thus be attached to ACP4. Once the *cis* double bond is formed, ACP4 would pass **7** to the KS5 domain for normal fifth-cycle elongation and subsequent reduction. In the DH5 mutant, the acyl chain would not undergo dehydration and ACP4 would pass **6** to KS5. If this mechanism for the fourth-cycle dehydration occurs, however, one might expect that some amount of direct passage of **6** to KS5 would take place in the wild-type strain before it was acted upon by DH5, resulting in the production of at least a small amount of a 13-hydroxyepothilone analogue. Such a compound has never been observed, even under large-scale fermentation conditions of the wild-type strain.

For the first DH5-mediated dehydration, we have proposed ACP4-to-ACP5 acyl chain transfer. Examples of analogous ACP-to-ACP polyketide chain transfer have been proposed previously to account for the production of molecules two carbons shorter in length than that predicted by the structure of the corresponding PKS.[7] In each of the examples, the module is completely skipped and the chain is passed unaltered to the next downstream KS. In the *epo* PKS, we propose that not only is the condensation skipped but dehydration also takes place. Enoyl reduction does not occur due to the nature of the substrate for the ER5 domain. The second aspect of the model, reiteration of modular activity through backloading of the acyl chain from ACP5 to KS5 (termed "stuttering"), has also been reported previously to account for production of compounds two carbons longer than that predicted by the structure of the PKS.[8] Why the acyl chain is passed backward from the ACP to its cognate KS rather than forward to the KS of the next module on its first time through the module is not understood. In the *epo* PKS, we propose that stuttering must occur to ensure that molecules of correct chain length are produced. This is accomplished if KS6 acts as a gatekeeper and does not accept transfer of the pentaketide (**7**) that is attached to ACP5 at the end of the first pass through module 5. In the second pass through module 5, the hexaketide produced can be transferred to KS6 to allow continuation of epothilone biosynthesis. In a standard PKS, a KS5 domain would normally accept a pentaketide from its cognate ACP4, rather than ACP5. In the *epo* PKS, although KS5 receives the acyl chain from ACP5, the chain is still of the correct size. Thus, KS5 can also play a gatekeeper role in biosynthesis.

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Supporting Information Available: Diagram of the *epo* PKS gene cluster, experimental details for modification of the DH5 domain, and MS and NMR data for **5** (PDF). This material is available free of charge via the Internet at http:/pubs.acs.org.

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(2) Scheme 1 abbreviations: ACP, acyl carrier protein domain, AT, acyltransferase domain; DH, dehydratase domain; ER, enoyl reductase domain; KR, β-ketoreductase domain; KS, β-ketoacyl ACP synthase (condensing) domain. (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.; Strarmann, A.; Cyr, D. D.; Gorlach, J.; Mayo, J. M.; Hu, A.; Goff, S.; Schmid, J.; Ligon, J. M. *Chem. Biol.* **2000**, *7*, 97−108. (c) Julien, B.; Shah, S.; Ziermann, R.; Goldman, R.; Katz, L.; Khosla, C. *Gene* **2000**, *249*, 153−160.

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