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Stereospecificity of the Dehydratase Domain of the Erythromycin Polyketide Synthase

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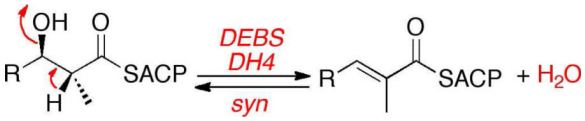
Abstract



The dehydratase (DH) domain of module 4 of the 6-deoxyerythronolide B synthase (DEBS) has been shown to catalyze an exclusive *syn* elimination/*syn* addition of water. Incubation of recombinant DH4 with chemoenzymatically prepared *anti*-(2*R*,3*R*)-2-methyl-3-hydroxypentanoyl-ACP (**2a-ACP**) gave the dehydration product **3-ACP**. Similarly, incubation of DH4 with synthetic **3-ACP** resulted in the reverse enzyme-catalyzed hydration reaction, giving a ~3:1 equilbrium mixture of **2a-ACP** and **3-ACP**. Incubation of a mixture of propionyl-SNAC (**4**), methylmalonyl-CoA, and NADPH with the DEBS β-ketoacyl synthase – acyl transferase [KS6][AT6] didomain, DEBS ACP6, and the ketoreductase domain from tylactone synthase module 1 (TYLS KR1) generated *in situ anti*-**2a-ACP** that underwent DH4-catalyzed *syn* dehydration to give **3-ACP**. DH4 did not dehydrate either *syn*-(2*S*,3*R*)-**2b-ACP**, *syn*-(2*R*,3*S*)-**2c-ACP**, or *anti*-(2*S*,3*S*)-**2d-ACP** generated *in situ* by DEBS KR1, DEBS KR6, or the rifamycin synthase KR7 (RIFS KR7), respectively. Similarly, incubation of a mixture of (2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-*N*-acetylcysteamine thioester (**2b-SNAC**), methylmalonyl-CoA, and NADPH with DEBS [KS6][AT6], DEBS ACP6, and TYLS KR1 gave *anti*-(2*R*,3*R*)-**6-ACP** that underwent *syn* dehydration catalyzed by DEBS DH4 to give (4*R*,5*R*)-(*E*)-2,4-dimethyl-5-hydroxy-hept-2-enoyl-ACP (**7-ACP**). The structure and stereochemistry of **7** were established by GC-MS and LC-MS comparison of the derived methyl ester **7-Me** to a synthetic sample of **7-Me**.

Of the more than 2000 non-aromatic polyketides, the vast majority contain one or more disubstituted or trisubstituted double bonds, most of which have *E* (*trans*) geometry.1 Moreover, essentially all polyketides that do not themselves display a double bond are biosynthesized by way of one or more unsaturated polyketide chain elongation intermediates. Thus although 6-deoxyerythronolide B (**1**, 6-dEB), the parent aglycone of the erythromycin family of antibiotics, does not have any double bonds in the final

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Supporting Information Available: Experimental procedures, LC-ESI(+)-MS^3, and GC-MS data This material is available free of charge via the Internet at http://pubs.acs.org.

macrolactone, the responsible modular polyketide synthase (PKS), 6-dEB synthase (DEBS), does in fact harbor a dehydratase domain in module 4, termed DEBS DH4 (Figure 1).2,3,

Direct evidence for the intermediacy of an unsaturated polyketide in erythromycin biosynthesis first came from disruption of the NADPH-binding motif of the ER4 domain, resulting in accumulation of a derivative of the corresponding (*E*)-$\Delta^{6,7}$-anhydro-6-dEB by mutants of the erythromycin producer *Saccharopolyspora erythraea*.4 Although the stereochemistry of the substrate for the DEBS DH4 dehydratase is not known, the responsible ketoreductase, DEBS KR4, is predicted to generate the (3*R*)-diastereomer of the 2-methyl-3-hydroxyacyl-ACP pentaketide, as deduced from the presence of a Leu-Ala-Asp triad closely correlated with the formation of (3*R*)-3-hydroxyacyl-ACP polyketide intermediates.5 Indeed, the vast majority of KR domains that are paired with a DH domain appear to harbor a conserved "Leu-Asp-Asp" motif.5a,b DEBS KR4 is also predicted to belong to the class of non-epimerizing ketoreductases, which would give rise to a (2*R*)-methyl group in the reduced product.5c

To establish the substrate specificity and stereochemical course of the DEBS DH4-catalyzed dehydration we used a chemoenzymatic strategy to prepare the requisite ACP-bound substrate and product analogues, **2a-ACP** and **3-ACP**. To this end the free acids **2a** and **3** were each converted to the corresponding –SCoA thioesters, **2a-SCoA** and **3-SCoA**, and thence to *anti*-(2*R*,3*R*)-2-methyl-3-hydroxypentanoyl-ACP6 (**2a-ACP**) and the expected dehydration product, (*E*)-2-methylpent-2-enoyl-ACP (**3-ACP**), from DEBS *apo*-ACP6 using the phosphopantetheinyl transferase Sfp (Scheme 1A).6 The two ACP derivatives, which were readily distinguished by reverse phase LC-ESI(+)-MS, both exhibited the expected molecular weights.7 The structures were each confirmed by the MS^2 phosphopantetheinate (PPant) ejection method which gave **2a-pant**, *m/z* 375.33, and **3-pant**, *m/z* 357.3, each with the predicted MW, as well as MS^3 analysis of each of the characteristic PPant ejection fragments.8

Incubation of recombinant DEBS DH49 with **2a-ACP** resulted in formation of the predicted dehydration product **3-ACP**, as established by direct monitoring by LC-ESI(+)-MS^3, including detection of the corresponding intact acyl-ACP and PPant ejection fragments for both **2a-ACP** and **3-ACP** (Scheme 1B). Similarly, incubation of DEBS DH4 with **3-ACP** resulted in the reverse enzyme-catalyzed hydration reaction, giving a ~3:1 equilibrium mixture of **2a-ACP** and **3-ACP**.10

We also carried out combinatorial incubations using mixtures of recombinant PKS domains in order to generate *in situ* each of the 4 diastereomers of **2a–2d-ACP**.11 In this manner, a mixture of the DEBS [KS6][AT6] didomain, DEBS ACP6, and TYLS KR1, the ketoreductase domain from module 1 of the tylactone synthase, was incubated with propionyl-SNAC (**4**), methylmalonyl-CoA, and NADPH to produce *anti*-(2*R*,3*R*)-**2a-ACP**. 11b Addition of recombinant DEBS DH4, either simultaneous with or subsequent to the formation of **2a-ACP**, resulted in dehydration to yield exclusively the predicted (*E*)-2-methylpent-2-enoyl-ACP (**3-ACP**), as confirmed by GC-MS analysis of the corresponding acid **3** and comparison with synthetic **3**.12 By contrast, DEBS DH4 did not dehydrate either *syn*-(2*S*,3*R*)-**2b-ACP** or *syn*-(2*R*,3*S*)-**2c-ACP** generated by DEBS KR1 or KR6, respectively,11a,c to either *E*-**3**-ACP or the corresponding *Z* isomer **5-ACP**, nor did DEBS DH4 dehydrate *anti*-(2*S*,3*S*)-**2d-ACP** produced by recombinant RIFS KR7,13 the KR domain from module 7 of the rifamycin synthase.

In further confirmation of the stereochemistry of the dehydration reaction, incubation of DEBS DH4 with *anti*-(2*R*,3*R*,4*S*,5*R*)-2,4-dimethyl-3,5-dihydroxyheptanoyl-ACP (**6-ACP**), generated *in situ* from **2b-SNAC**, methylmalonyl-CoA, and NADPH by DEBS [KS6][AT6]

+ ACP6 + TYLS KR1, as previously described,11b gave exclusively *E*-**7-ACP**. The structure and stereochemistry of **7-ACP** were determined by chiral GC-MS and LC-MS analysis of the derived methyl ester **7-Me**, obtained by basic hydrolysis and treatment of the liberated acid with TMS-diazomethane, and comparison with an authentic synthetic standard of **7-Me**.14

Sequence alignments of the DEBS DH4 domain with numerous PKS and FAS DH domains reveal conserved ^{2409}**H**XXXGXXXX**P** and ^{2571}**D**(A/V)(V/A)(A/L)(**Q**/H) motifs.2 Site-directed mutagenesis of the conserved active site His2409 of the DEBS DH4 domain abolished DEBS activity in *Sac. Erythraea*15a while the analogous His mutation also inactivates the homologous DH2 domain of the picromycin synthase.15b Together the conserved His and Asp residues comprise the catalytic dyad of the dehydratase, in which the active site His acts as a general base while the Asp2571, located 4.1Å from H2409 at the base of the substrate tunnel, is thought to serve as a general acid.9,16,17,,

Our results establish definitively that the DEBS DH4 domain catalyzes a *syn* elimination of water during erythromycin biosynthesis. The prototype dehydration catalyzed by the DH domain of the yeast FAS to give the characteristic disubstituted (*E*)-enoyl-ACP intermediates of fatty acid biosynthesis also takes place with net *syn* stereochemistry,18 as do the dehydrations catalyzed by the DH domains of module 2 of nanchangmycin synthase19 and module 2 of tylactone synthase.11b Indeed, the significant levels of overall sequence identity (>40%) and similarity (>55%) and the presence of the conserved motifs containing the catalytic dyad in more than 50 DH domains from a wide range of modular PKS systems, strongly suggest that the formation of all (*E*)-unsaturated polyketide intermediates involves a common *syn* dehydration mechanism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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3. Each PKS module typically has 3 core catalytic domains: the β-ketoacyl-ACP synthase (KS), the acyltransferase (AT), and the acyl carrier protein (ACP). Most modules also carry specific combinations of ketoreductase (KR), dehydratase (DH) and enoylreductase (ER) tailoring domains.
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12. The *E*-isomer **3** was cleanly resolved from the Z isomer **5** and the two compounds were not interconverted under the conditions of the reaction.
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14. Although DH4 processed both **2a-ACP** and **6-ACP**, DEBS DH4 does not dehydrate the corresponding SNAC thioester analog, *anti*-(2*R*,3*R*)-**2a-SNAC** (ref 9). This observation is consistent with the presence of a presumptive ACP-binding region on the DH surface adjacent to the entrance to the substrate binding tunnel, a feature also found in bacterial FabZ proteins (ref 17).
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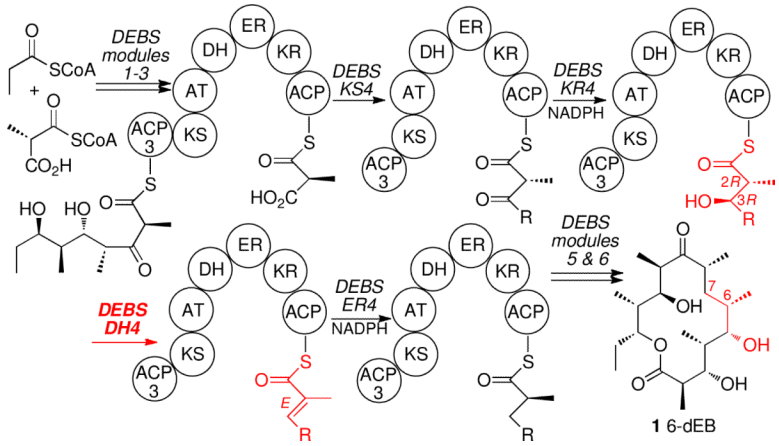
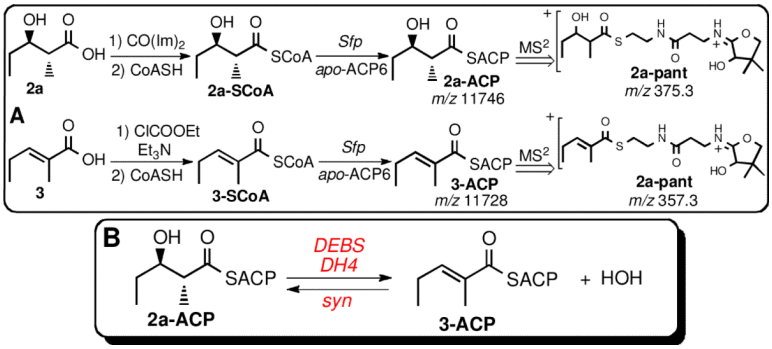
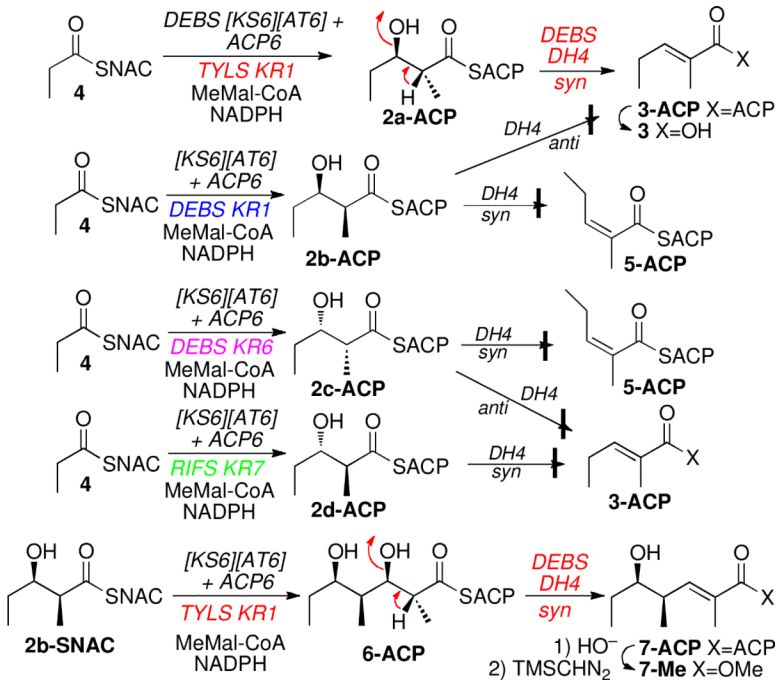


Figure 1.
Proposed tetraketide substrate and pentaketide intermediates of DEBS module 4. The module has a KR, a DH and an ER domain in addition to the obligate KS, AT and ACP domains.



Scheme 1.

A. Synthesis and analysis of ACP-bound substrates. B. DEBS DH4-catalyzed interconversion of **2a-ACP** and **3-ACP**.



Scheme 2.
Stereochemistry of DEBS DH4-catalyzed dehydration.