

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 an ~3:1 equilibrium 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 *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*-acetylcysteaminethioester (**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 nonaromatic 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 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 accumula-

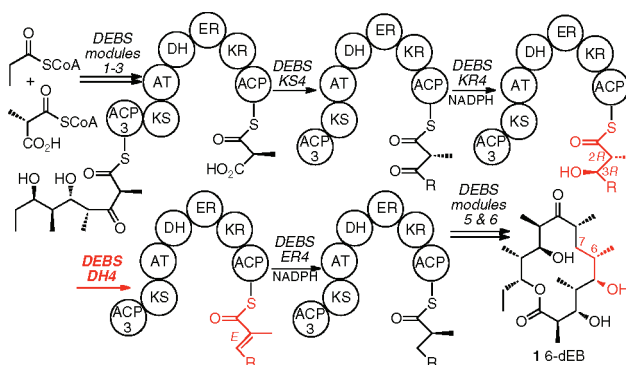


Figure 1. Proposed tetraketide substrate and pentaketide intermediates of DEBS module 4. The module has a KR, a DH, and an ER domain in addition to the obligate KS, AT, and ACP domains.

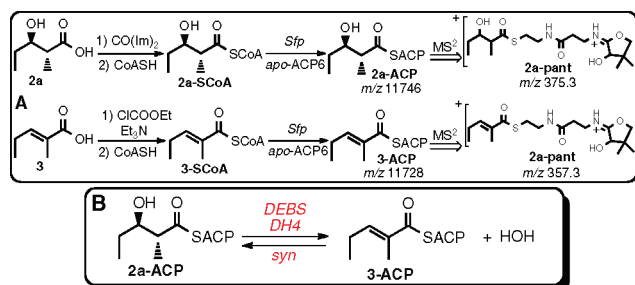
tion of a derivative of the corresponding (*E*)- $\Delta^{6,7}$ -anhydro-6-dEB by mutants of the erythromycin producer *Saccharopolyspora erythraea*.⁴ Although the stereochemistry of the substrate for the DEBS DH4 dehydratase is not known, the responsible ketoreductase, DEBS KR4, is predicted to generate 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 nonpimerizing 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 reversed-phase LC-ESI(+)-MS, both exhibited the expected molecular weights.⁷ The structures were each confirmed by the MS² 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 MS³ 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

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Scheme 1. (A) Synthesis and Analysis of ACP-Bound Substrates and (B) DEBS DH4-Catalyzed Interconversion of **2a-ACP** and **3-ACP**

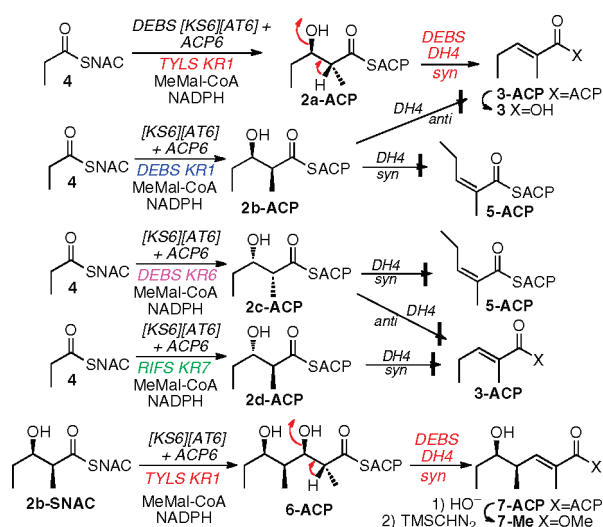


established by direct monitoring by LC-ESI(+)-MS³, including detection of the corresponding intact acyl-ACP and PPant ejection fragments for both **2a-ACP** and **3-ACP** (Scheme 1B). Similarly, incubation of DEBS DH4 with **3-ACP** resulted in the reverse enzyme-catalyzed hydration reaction, giving an ~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 four diastereomers of **2a–2d-ACP** (Scheme 2).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*-(2R,3R)-**2a-ACP**.^{11b} Addition of recombinant DEBS DH4, either simultaneously 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*-(2S,3R)-**2b-ACP** or *syn*-(2R,3S)-**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*-(2S,3S)-**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*-(2R,3R,4S,5R)-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}

Scheme 2. Stereochemistry of DEBS DH4-Catalyzed Dehydration



gave exclusively *E*-**7-ACP** (Scheme 2). 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 ²⁴⁰⁹HXXXGXXXXP and ²⁵⁷¹D(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 4.1 Å 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.

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Supporting Information Available: Experimental procedures, LC-ESI(+)-MS³, and GC-MS data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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