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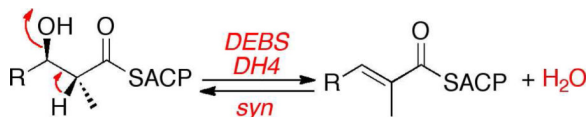
## Stereospecificity of the Dehydratase Domain of the Erythromycin Polyketide Synthase

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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 equilibrium mixture of **2a-ACP** and **3-ACP**. Incubation of a mixture of propionyl-SNAC (**4**), methylmalonyl-CoA, and NADPH with the DEBS  $\beta$ -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.<sup>1</sup> 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<sup>3</sup>, 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).<sup>2,3</sup>

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*.<sup>4</sup> 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.<sup>5</sup> Indeed, the vast majority of KR domains that are paired with a DH domain appear to harbor a conserved “Leu-Asp-Asp” motif.<sup>5a,b</sup> 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.<sup>5c</sup>

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).<sup>6</sup> The two ACP derivatives, which were readily distinguished by reverse phase LC-ESI(+)-MS, both exhibited the expected molecular weights.<sup>7</sup> The structures were each confirmed by the MS<sup>2</sup> 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<sup>3</sup> analysis of each of the characteristic PPant ejection fragments.<sup>8</sup>

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<sup>3</sup>, 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**.<sup>10</sup>

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**.<sup>11</sup> 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**.<sup>11b</sup> 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**.<sup>12</sup> 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,<sup>11a,c</sup> 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,<sup>13</sup> 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,<sup>11b</sup> 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.<sup>14</sup>

Sequence alignments of the DEBS DH4 domain with numerous PKS and FAS DH domains reveal conserved <sup>2409</sup>HXXXGXXXXP and <sup>2571</sup>D(A/V)(V/A)(A/L)(Q/H) motifs.<sup>2</sup> 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.<sup>15b</sup> 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.<sup>9·16·17,,</sup>

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,<sup>18</sup> as do the dehydrations catalyzed by the DH domains of module 2 of nanchangmycin synthase<sup>19</sup> and module 2 of tylactone synthase.<sup>11b</sup> 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.

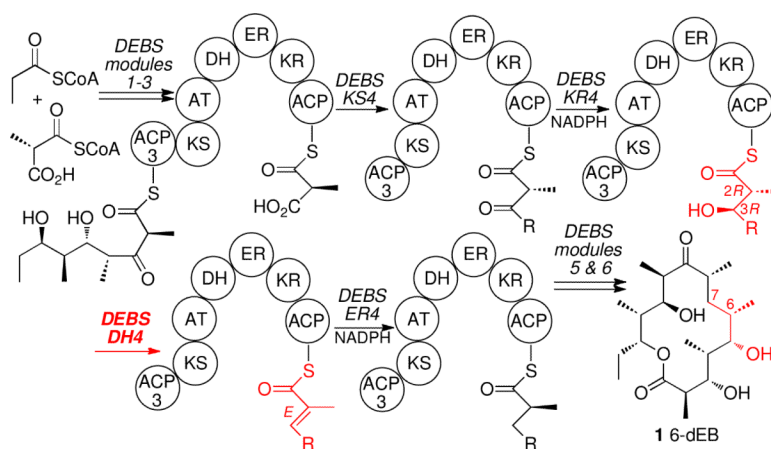
## Acknowledgments

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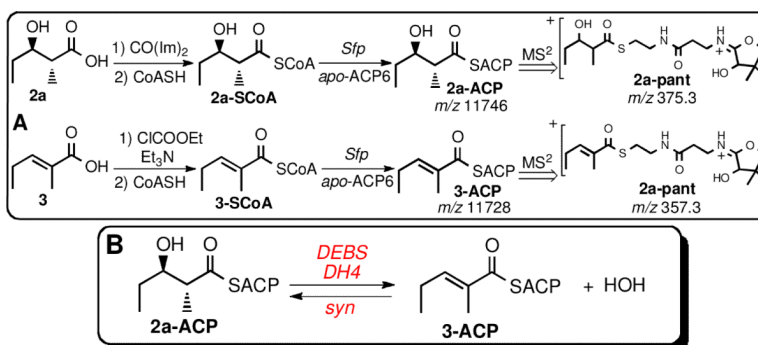
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3. Each PKS module typically has 3 core catalytic domains: the  $\beta$ -ketoacyl-ACP synthase (KS), the acyltransferase (AT), and the acyl carrier protein (ACP). Most modules also carry specific combinations of ketoreductase (KR), dehydratase (DH) and enoylreductase (ER) tailoring domains.
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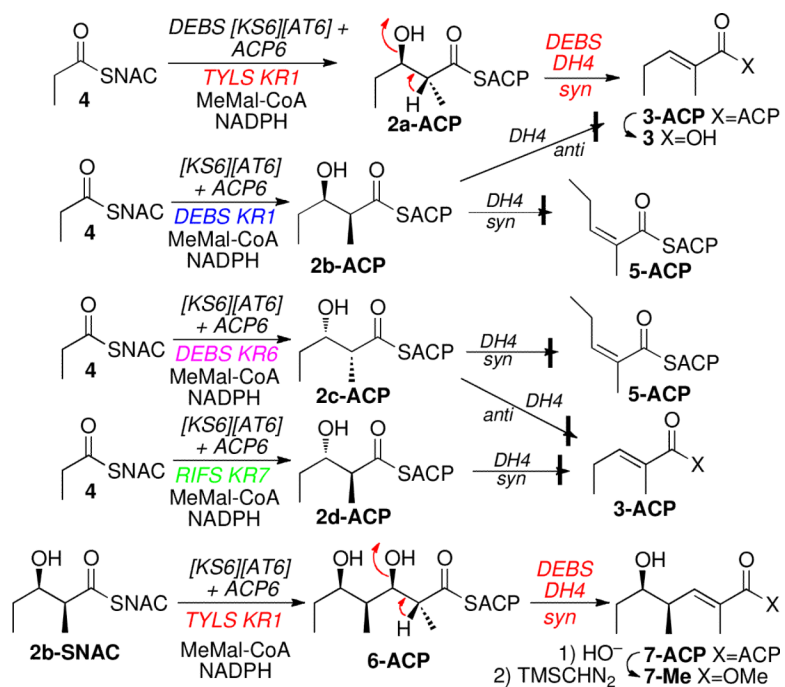
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12. The *E*-isomer **3** was cleanly resolved from the *Z* isomer **5** and the two compounds were not interconverted under the conditions of the reaction.
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14. Although DH4 processed both **2a-ACP** 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.