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Erythromycin Biosynthesis. The 4-*pro*-*S* Hydride of NADPH is Utilized for Ketoreduction by Both Module 5 and Module 6 of the 6-Deoxyerythronolide B Synthase

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Abstract—Incubation of chirally deuterated NADPH with 6-deoxyerythronolide B synthase (DEBS) modules 5 and module 6 and analysis of the derived triketide lactones established that the two ketoreductase domains, KR5 and KR6, are both specific for the 4-pro-*S* hydride of the nicotinamide cofactor.

Deoxyerythronolide B synthase (DEBS) catalyzes the biosynthesis of 6-deoxyerythronolide B (**1**), the parent macrolide aglycone of the widely used broad spectrum antibiotic erythromycin A.[1,2] DEBS is a modular Type I polyketide synthase (PKS), a large multifunctional enzyme with clusters of active sites grouped together in functional units called modules, each of which is responsible for a distinct round of carbon–carbon bond formation and functional group modification as part of the elaboration of the growing polyketide chain, using reactions closely related both mechanistically and genetically to the individual biochemical reactions of fatty acid biosynthesis (Fig. 1). DEBS itself consists of a specialized loading domain, responsible for the introduction of the propionyl-CoA primer or starter unit, plus six modules, each of which utilizes (2*S*)-methylmalonyl-CoA as the substrate for polyketide chain extension, and five of which contain functional β-ketoacyl-ACP reductase (KR) domains responsible for the formation of β-hydroxyacyl thioester intermediates of either D- or L-stereochemistry.[3,4] We have previously established that the actual hydroxyl group stereochemistry in these intermediates is an intrinsic property of each KR domain.[5]

DEBS1+TE is an engineered bimodular construct containing the AT and ACP loading domains plus modules 1 and 2 fused at the C-terminus to the thioesterase (TE) domain normally located at the C-terminus of module 6.[6] Incubation of DEBS1+TE with propionyl-CoA and methylmalonyl-CoA in the presence of NADPH results in two rounds of polyketide chain elongation and formation of the triketide lactone **2**. We recently used this system to demonstrate that the ketoreductase steps catalyzed by the β-ketoacyl-ACP reductase of module 1 (KR1) and module 2 (KR2) both utilize exclusively the *pro*-*S* face of the NADPH/$NADP^+$ cofactor, in spite of the fact that the two KR domains mediate the formation of β-hydroxyacyl thioester intermediates of either L-(3*S*) and D-(5*R*) stereochemistry, respectively.[7] We now report the extension of these results to the demonstration that both KR5 and KR6 are also 4-*pro*-*S* specific.

The recent high-level expression in *E. coli* of individual DEBS modules has provided a powerful tool for the investigation of the mechanism and specificity of discrete domains from the parent DEBS PKS.[8] Co-expression of each module carrying a TE domain fused to the C-terminus along with the phosphopantetheinyl transferase Sfp from *Bacillus subtilis*[9] gives active, pantetheinylated holoenzyme. Incubation of module 2+TE, module 5+TE, or module 6+TE with the natural diketide substrate analogue, (2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-SNAC (**3**) (*N*-acetylcysteamine), in the presence of methylmalonyl-CoA and NADPH gives the triketide lactone **2**[8] (Scheme 1). Intriguingly, the *syn*-(2*S*,3*R*)-stereoisomer is preferred by the KS domain of

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all three modules over the enantiomeric diketide substrate by a factor of as much as 100 to 1.[10] By contrast, neither of the corresponding anti-diastereomers is processed by any of the three modules. Module 5+TE and module 6+TE therefore provide a convenient system for the study of the coenzyme specificity of the KR5 and KR6 domains.

The requisite chirally deuterated samples of (4*R*)- and (4*S*)-[4-^{2}H]NADPH were prepared enzymatically from $NADP^+$ and purified as previously described.[7,11,12] Both (4*R*)- and (4*S*)-[4-^{2}H]NADPH were stereochemically and isotopically pure, as shown by ^{1}H NMR. The individual modules—module 5+TE and module 6+TE—were purified to apparent homogeneity by a Butyl Sepharose column followed by an Resource Q anion exchange column, as previously described.[8] For each preparative incubation, 1 mM of the individual chirally deuterated NADPH was incubated with 100 pmol of purified protein, 5 mM diketide thioester **3** and 2.5 mM [2-^{14}C]methylmalonyl CoA (spec. act 54 mCi/mmol) in a total volume of 100 μL for 30 min at 30 °C, as previously described, to yield triketide lactone **2** (Scheme 1). A control experiment with unlabeled NADPH as cofactor was also done in parallel. The formation of **2** was verified by TLC with detection by phosphoimaging and the purified triketide lactone was eluted from the TLC plate and then converted to the corresponding trimethylsilyl (TMS) ether by reaction with 10 μL of *N,O*-bis(trimethylsilyl)trifluoroacetamide. A 2-μL aliquot of each derivatized sample was analyzed by selected ion monitoring (SIM) GC–MS of the *m/z* of 245 (M^+), 246 ($[M+1]^+$), and 247 ($[M+2]^+$) peaks by CI-MS with CH_4 as reagent gas, using conditions previously described.[7] The deuterium content of each triketide lactone sample was calculated after correction for the natural abundance contributions to the M+1 (246) and M+2 (247) peaks (Table 1).

When (4*R*)-[4-^{2}H]NADPH was used as cofactor, no excess deuterium isotope could be detected in the triketide lactones **2a** that resulted from the incubations with either module 5+TE or module 6+TE. By contrast, the samples of triketide lactone, **2b**, derived from incubation of (4*S*)-[4-^{2}H]NADPH with each module were both deuterated. The position of deuteration at C-3 of **2** was assigned based on the well-established mechanism of formation of triketide lactone from the diketide substrate.[6,8] Although some excess washout of deuterium was observed in each sample of **2b**, as also found for the previously reported incubations with DEBS1+TE,[7] the results establish unambiguously that both the KR5 and KR6 β-ketoacyl-ACP domains utilize solely the 4-*pro-S* hydride of the NADPH cofactor.

The results described above demonstrate that the KR5 and KR6 domains are 'B-face' enzymes that have the same stereochemical preference for the nicotinamide cofactor as the previously investigated KR1 and KR2 domains.[7] Notably, KR2, KR5, and KR6 all generate L-(3*S*)-β-hydroxyacylthioesters, while KR1 produces the corresponding D-(3*R*)-β-hydroxyacylthioester intermediate. The observed stereochemical preference for the 4-*pro-S* hydride has also been established for the

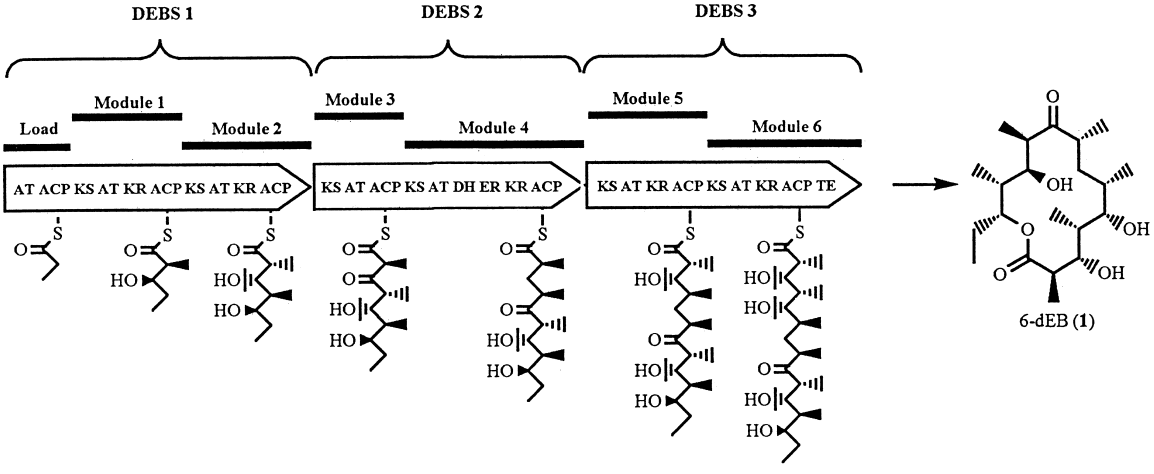
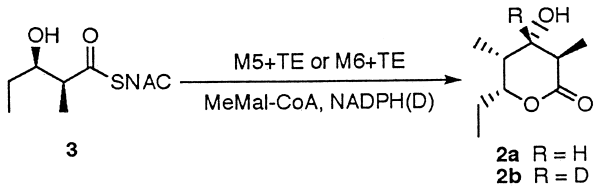


Figure 1. Modular organization of the 6-deoxyerythronolide B synthase (DEBS) that catalyzes the formation of 6-deoxyerythronolide B (6-dEB, **1**) The individual active site domains are the acyltransferases (AT), acyl carrier proteins (ACP), β-ketoacyl-ACP synthases (KS), β-ketoacyl reductases (KR), a dehydratase (DH), an enoyl reductase (ER), and a thioesterase (TE).



Scheme 1.

Table 1. Selected ion monitoring GC–MS analysis of triketide lactone **2** resulting from incubation of module 5+TE and module 6+TE with (4*R*)-[4-^{2}H]NADPH and (4*S*)-[4-^{2}H]NADPH

Enzyme	KR domain	TKL	NADPH	d_0 (%)	d_1 (%)
M5+TE	KR5	**2a**	(4*R*)-[4-^{2}H]	>90	<10
M5+TE	KR5	**2b**	(4*S*)-[4-^{2}H]	40	60
M6+TE	KR6	**2a**	(4*R*)-[4-^{2}H]	95	<5
M6+TE	KR6	**2b**	(4*S*)-[4-^{2}H]	45	55

mechanistically and genetically closely related KR domains of a variety of fatty acid synthases as well.[13] Since module 3 of DEBS harbors an inactive KR domain, only the stereochemical preference of the KR4 domain remains to be established. Based on the high level of sequence conservation among the 5 DEBS KR domains,[14] it is expected that KR4 will also show B-face specificity. Confirmation of this prediction awaits the development of an active module 4 + TE construct with an inactivated DH domain.

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