Erythromycin Biosynthesis: The β-Ketoreductase Domains Catalyze the Stereospecific Transfer of the 4-*pro*-*S* Hydride of NADPH

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Polyketides represent a growing family of structurally diverse natural products which exhibit a wide array of medicinally important activities.[1] Members include erythromycin,[2] avermectin,[3] oleandomycin,[4] rapamycin,[5] and candicidin.[6] The macrocyclic portions of these compounds are biosynthesized by modular type I polyketide synthases (PKS's), large multifunctional enzymes whose arrangement of active sites correlates with the sequence of carbon−carbon bond construction and reductive modification in the elaboration of the growing polyketide chain. Controlled manipulation of the arrangement of enzymatic domains within the erythromycin PKS has allowed the rational design of novel polyketide products through modular deletion,[7] gain-of-function,[8] domain replacement,[9] and inactivation[9e,10] experiments and also helped to define the limits of substrate recognition and processing within these multidomain proteins.[11] One particularly useful construct has been DEBS1+TE,[7b,c] a bimodular derivative of the parent 6-deoxyerythronolide B (**2**) synthase that catalyzes the conversion of propionyl-CoA and methylmalonyl-CoA to triketide lactone **1** (Figure 1). This conversion requires two reductive steps that each utilize NADPH and that are mediated by specific β-ketoacyl-ACP reductases in each module of the PKS, KR1 and KR2, that generate β-hydroxyacyl thioester intermediates corresponding to the D-5(*R*) and L-3(*S*) stereochemistry, respectively, in the resultant triketide lactone **1**.[7,8a] We now report that the KR1 and KR2 domains of the erythromycin PKS conserve the same stereochemical preference for the *pro*-*S* face of the $NADP^+$/NADPH cofactor, although the two domains mediate the formation of β-hydroxyacyl thioester products of opposite configuration.

Although PKS's have been shown to exhibit relaxed substrate specificity, they still maintain a high degree of stereochemical control. For example, the six acyl transferase (AT) domains of the erythromycin PKS (DEBS) (Figure 1) have been shown to have a absolute specificity for the 2(*S*)-enantiomer of methylmalonyl CoA,[12] and the β-ketoacyl ACP synthase (KS) domain of module 2 has been shown to discriminate among structural and stereochemical analogues of its natural diketide substrate, with complete exclusion of the unnatural 2(*R*)-methyl enantiomer.[11] The factors which influence this striking mix of strict specificity and structural versatility are of considerable interest, not only from a mechanistic standpoint but also as they relate to the engineered production of novel polyketides.

In fatty acid synthases from all organisms studied to date, the β-ketoacyl thioester reductases are specific exclusively for the 4-*pro*-*S* hydride of NADPH.[13] To study the stereospecificity of the hydride transfers catalyzed by DEBS KR1 and by KR2, the required samples of 4(*R*)- and 4(*S*)-[4-^{2}H]NADPH were prepared enzymatically from $NADP^+$, using *Thermoanaerobium brockii* alcohol dehydrogenase[14a] and *Bacillus megaterium* glucose dehydrogenase[14b] to transfer deuteride from [$^2H_8$]-2-propanol and [1-^{2}H]glucose, respectively. The stereochemistry and isotopic purity of the resulting HPLC-purified, stereospecifically deuterated nicotinamides were determined by ^{1}H NMR and found to be greater than 95% in each case.

Both 4(*R*)- and 4(*S*)-[4-^{2}H]NADPH were incubated in separate experiments with [1-^{14}C]propionyl CoA and methylmalonyl CoA and partially purified DEBS1+TE,[15] obtained from *Streptomyces coelicolor* CH999/pCK12 as previously described.[7b] The resulting triketide lactone **1**, derivatized as the corresponding trimethylsilyl ether, was analyzed by selected ion monitoring GC-MS.[16] As a control, incubations were also carried out using unlabeled NADPH. When 4(*R*)-[4-^{2}H]NADPH was used as the cofactor, the resulting triketide lactone **1a** carried no deuterium (Table 1, Figure 2). On the other hand, incubation of 4(*S*)-[4-^{2}H]NADPH gave a triketide lactone sample **1b**, in which over half of the

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(15) (a) Typical incubations were performed overnight at 30 °C in 250 mM K phosphate buffer, at a protein concentration of approximately 4−5 mg mL^{-1}. After extraction with ethyl acetate, products were separated by thin-layer chromatography (60:40 EtOAc:Hex) and the radiolabeled triketide lactone detected by phosphoimaging (BioRad Molecular Imager GS-363). Radioactive bands were excised and eluted (EtOAc), and the solvent was evaporated under dry nitrogen. Residues were dissolved in *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (Pierce, 10 μL), and 2 μL aliquots were subjected to analysis by GC-MS. (b) DEBS1+TE and DEBS1+TE(KS1°) were partially purified by ammonium sulfate precipitation and gel filtration: Pieper, R.; Gokhale, R. S.; Luo, G.; Cane, D. E.; Khosla, C. *Biochemistry* **1997**, *36*, 1846−1851.

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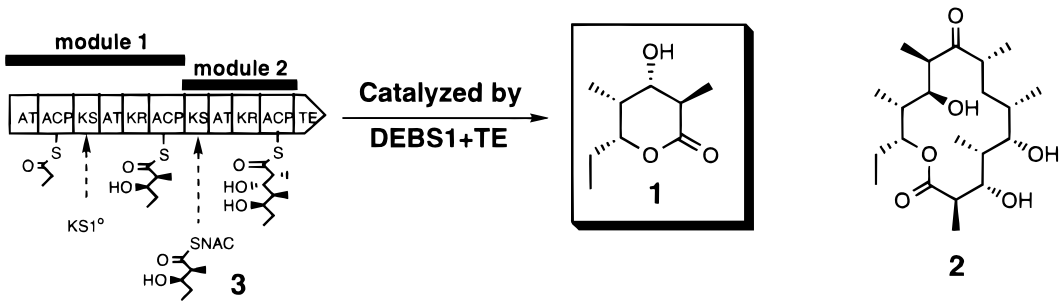

Figure 1. Genetic model for the biosynthesis of the C_9-triketide lactone (2*R*,3*S*,4*S*,5*R*)-2,4-dimethyl-3,5-dihydroxy-*n*-heptanoic acid δ lactone **1** catalyzed by DEBS1+TE. DEBS1+TE is a truncated derivative of 6-deoxyerythronolide B synthase (DEBS), the parent multidomain PKS which catalyzes the formation of 6-dEB **2**. Each module contains all of the active sites required for one round of polyketide-chain elongation and β-keto manipulation. The active sites are designated as follows: acyltransferase (AT), β-keto manipulation. The active sites are designated as follows: acyltransferase (AT), β-ketoacyl-ACP synthase (KS), acyl carrier protein (ACP), β-ketoreductase (KR), and thioesterase (TE).

Table 1. GC-MS Analysis of Triketide Lactones Produced by DEBS1+TE and DEBS1+TE(KS1°) with 4(*R*)-[^{2}H]NADPH and 4(*S*)-[^{2}H]NADPH

enzyme	KR domains	NADPH	product	d_0 (%)	d_1 (%)	d_2 (%)
DEBS1+TE	KR1, KR2	4(*R*)-[4-^{2}H]	**1a**	100	0	0
DEBS1+TE	KR1, KR2	4(*S*)-[4-^{2}H]	**1b**	12	32	56
DEBS1+TE(KS1°)	KR2	4(*R*)-[4-^{2}H]	**1c**	91	5	4
DEBS1+TE(KS1°)	KR2	4(*S*)-[4-^{2}H]	**1d**	31	55	14

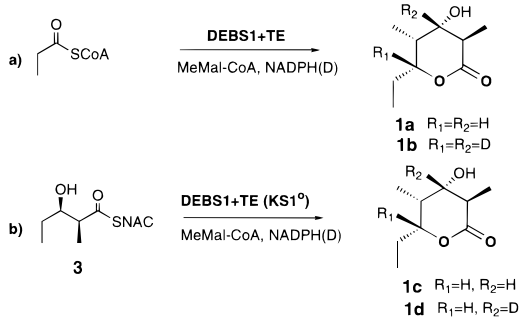

Figure 2. Incorporation of label from stereospecifically deuterated NADPH into triketide lactones **1b** and **1d** catalyzed by a) DEBS1+TE and b) DEBS1+TE (KS1°)

resulting molecules were doubly labeled with deuterium.[17] These results indicated that both KR1 and KR2 utilize exclusively the 4-*pro*-*S* hydride of the NADPH cofactor in the reduction of their cognate diketide and triketide β-ketoacyl-ACP substrates.

To further confirm these observations, a second set of incubations was carried out using DEBS1+TE(KS1°), a mutant of DEBS1+TE obtained from *S. coelicolor* CH999/pCK16 that carries an inactive β-ketoacyl-ACP synthase domain (KS1°) in module 1.[18] Although this enzyme cannot support triketide lactone production from the normal substrates, propionyl CoA and methylmalonyl CoA, due to the defect in module 1, it is able to recognize and convert a synthetic diketide *N*-acetyl cysteamine (NAC) thioester **3** into the triketide lactone **1** (Figure 1). Thus, incubation of partially purified DEBS1+TE(KS1°) with diketide **3**, in the presence of [2-^{14}C]methylmalonyl CoA and either 4(*R*)- or 4(*S*)-[4-^{2}H]NADPH gave the corresponding triketide lactones **1c** and **1d**, respectively. Whereas **1c** was effectively devoid of deuterium, as indicated by GC-MS analysis of the corresponding trimethylsilyl derivative (Figure 2, Table 1), **1d** was more than 50% monodeuterated at C-3.[19] Taken together, the incubations with DEBS1+TE(KS1°) mutant confirm the selectivity of the KR2 domain for the 4-*pro*-*S* hydride of NADPH.

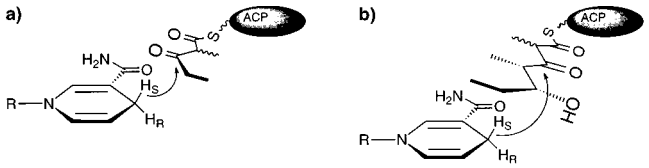

Figure 3. Proposed orientation of substrates in the β-ketoreduction of a) diketide and b) triketide chain elongation intermediates in the biosynthesis of triketide lactone by DEBS1+TE. R=adenine dinucleotide phosphate, ACP=acyl carrier protein.

In summary, we have established that both the KR1 and the KR2 domains of the bimodular PKS, DEBS1+TE, and by extension, the same two domains in the parent 6-deoxyerythronolide B (**2**) synthase (DEBS), are "B-face" enzymes that catalyze stereospecific transfer of hydride from the 4-*pro*-*S* face of the NADPH cofactor. Intriguingly, although both ketoreductases utilize the same face of NADPH, the KR1- and KR2-mediated reductions result in the formation of β-hydroxyacyl thioesters of D- and L-stereochemistry, respectively. We predict that the same stereospecificity for the 4-*pro*-*S* face of NADPH will be conserved in the downstream KR domains (KR4, KR5, and KR6) of the parent DEBS, itself a modular PKS that catalyzes the biosynthesis of the erythromycin aglycon, 6-dEB (**2**) (Figure 1). We have recently reported experiments which establish that the hydroxyl group stereochemistry in the polyketide product is an intrinsic property of each KR domain.[8a] The binding of the nicotinamide cofactor is presumably identical in each KR domain, which must therefore bind the individual β-ketoacyl thioester substrates in different orientations relative to the cofactor (Figure 3). The fact that both fatty acid synthase (FAS) and PKS KR domains utilize the same face of NADPH, regardless of the stereochemistry of the resulting β-hydroxyacyl thioester product, is consistent with a common ancestral link between polyketide and fatty acid biosynthesis. Extension of these results to additional PKS KR domains is in progress.

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(16) GC-MS analysis was performed on a Hewlett-Packard 5988A mass spectrometer interfaced to an HP 5890 II capillary gas chromatograph and using HP cross-linked silicone gum capillary column (12.5 m × 0.22 mm × 0.33 μm) with an oven temperature gradient of 80 to 250 °C at 25 °C/min and a solvent delay of 3 min. Under these conditions, with positive chemical ionization detection (CH_4) and selected ion monitoring at 245, 246, and 247 amu, triketide lactone trimethylsilyl ether had a retention time of 6.12 min. Deuterium content was calculated after correction for the natural abundance contributions to the M + 1 (246) and M + 2 (247) peaks.

(17) The excess washout of deuterium from **1b** may be explained by competing deuterium/hydrogen exchange catalyzed by contaminating diaphorase-like activities. The observed degree of deuterium loss was reduced with increasing purification of the DEBS1+TE and DEBS1+TE(KS1°) proteins.

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(19) The presence of a trace amount (ca. 10%) of apparently doubly deuterated triketide lactone in the sample of **1d** obtained from incubation with DEBS1+TE(KS1°) may be due to competing KR1-catalyzed redox exchange of the diketide NAC thioester **3**, prior to processing by module 2.