Alcohol Stereochemistry in Polyketide Backbones Is Controlled by the β-Ketoreductase Domains of Modular Polyketide Synthases

Camilla M. Kao,[†] Michael McPherson,[‡] Robert N. McDaniel,[§] Hong Fu,[§] David E. Cane,*[,‡] and Chaitan Khosla*[,†,||]

Departments of Chemical Engineering Chemistry, and Biochemistry Stanford University Stanford, California 94305-5025 Department of Chemistry, Box H, Brown University Providence, Rhode Island 02912 KOSAN Biosciences, Inc., 1450 Rollins Road Burlingame, California 94010

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Modular polyketide synthases (PKSs; MW > 300 000) catalyze the biosynthesis of polyketide natural products.[1,2] These enzymes program the complex series of reaction steps in polyketide biosynthesis in part by their modular active site organization, which has stimulated much interest in generating new molecules through the rational and combinatorial engineering of PKS genes.[2] While gain-of-function, replacement, and inactivation experiments have revealed the remarkably broad substrate specificity of PKS active sites,[3−15] the structural basis for stereochemical control by these multifunctional enzymes remains poorly understood.

In the monofunctional alcohol dehydrogenases, stereochemical control of carbonyl reduction has been extensively studied.[16,17] These enzymes (MW ≈35 000 per subunit) catalyze the reduction of aldehydes and ketones to the corresponding alcohols (as well as the reverse reaction), using NADH or NADPH as cofactors. During ketoreduction, a specific hydride ion (*pro-R* or *pro-S*) is transferred from the cofactor to one face of the carbonyl substrate, generating a D or L alcohol. Crystal structures have shown that this stereochemical control is achieved through oriented binding of both the cofactor and substrate, so that the hydride ion is always removed from and added to the same face of each molecule.

Similarities in stereochemical control mechanisms between the alcohol dehydrogenases and modular PKSs are not obvious a priori, due to the multidomain organization of the latter enzymes. In modular PKSs, stereochemistry might be controlled by the β-ketoreductase (KR) domains in a manner analogous to the monofunctional dehydrogenases, in that the KR binds NADPH and a polyketide intermediate in an oriented fashion and thus dictates β-hydroxyl stereochemistry. However, a typical reduced polyketide contains hydroxyl groups of both D and L stereochemistry, thereby requiring alternative modes of binding for the individual β-(ketoacyl)-ACP intermediates by the relevant KR domain. For example, the triketide lactone **1** (Figure 1), the product of a bimodular derivative of the 6-deoxyerythronolide B synthase (DEBS),[15,18] contains hydroxyl groups in both the L-(3*S*) and D-(5*R*) configurations.[10] To study the control of hydroxyl stereochemistry in reduced polyketides, we have carried out several KR domain replacements in a three-module derivative of the DEBS.[9] Our results demonstrate that β-hydroxyl stereochemical control is an intrinsic property of individual KR domains and is independent of the substitution pattern of β-(ketoacyl)-ACP substrates.

To examine the stereochemistry of β-ketoacyl thioester reduction, we constructed three derivatives of *Streptomyces coelicolor* CH999/pCK13[9] (Figure 1). Plasmid pKOS011-56 contains the KR domain of DEBS module 5 in place of the native KR2.[19] In plasmids pKAO392 and pKAO404, KR2 is replaced by the KR domains of the rapamycin synthase (RAPS)[20] modules 2 and 4, respectively.[21] The RAPS KR2 segment also contains a putatively inactive dehydratase ("null DH").[22] Each plasmid was introduced into *S. coelicolor* CH999,[23,24] and the resulting strains analyzed for polyketide production.[25]

Fermentation of *S. coelicolor* CH999/pKOS011-56 produced the tetraketides **2** and **3**, as determined by ^{1}H NMR spectroscopy (Figure 1). These products are identical to those synthesized by the wild-type three-module strain CH999/pCK13,[9] thus establishing that KR5 is fully functional in module 2. Similar results have also been reported for the bimodular DEBS derivative.[5] In contrast, fermentation of both *S. coelicolor* CH999/pKAO392 and CH999/pKAO404 yielded the triketide lactone (**4**) as the major product (∼20 mg/L; Figure 1). The structure of **4**, which is the

[†] Department of Chemical Engineering, Stanford University.
[‡] Brown University.
[§] KOSAN Biosciences, Inc.
[||] Departments of Chemistry and Biochemistry, Stanford University.

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(21) DNA sequences at the boundaries of KR2 domain replacements. In pKOS011-56: DEBS AT2 → DEBS KR5, TTCTGGCTGCAGATCCCC.; DEBS KR5 → DEBS ACP2, GAGGGCTCTAGAGACCGG. In pKAO392: DEBS AT2 → RAPS null DH2, TTCTGGCTGCAGGAGGGC.; RAPS KR2 → DEBS ACP2, GCCGGTTCTAGAGACCGG. In pKAO404: DEBS AT2 → RAPS KR4, TTCTGGCTGCAGGTCGACTCTAGCGTG; RAPS KR4 → DEBS ACP2, GGTGACTCTAGAGACCGG. In all cases, mutations were engineered via standard polymerase chain reaction (PCR) mutagenesis procedures. The engineered *Pst* I and *Xba* I sites were functionally validated in the control strain, CH999/pKAO263 (not shown).[3]
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(25) The transformed strains were grown on R2YE medium[24] at 30 °C under thiostrepton selection (50 mg/L) for 5−7 days. The mycelium was extracted three times with ethyl acetate (pH 7). Compound **4** was purified from *S. coelicolor* CH999/pKAO410 extracts on an isocratic silica gel column with 25% EtOAc/hexanes.

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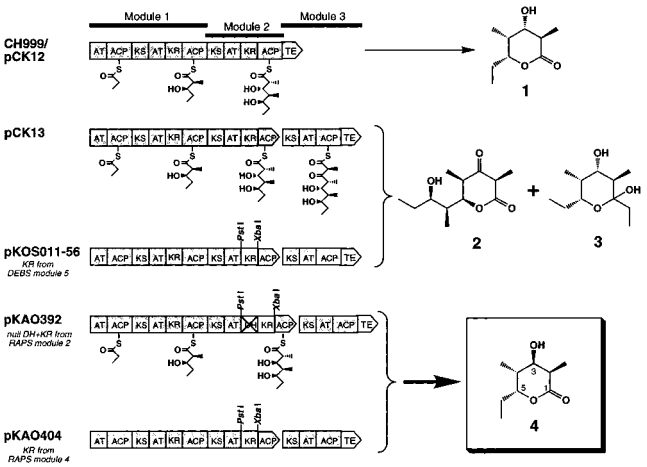
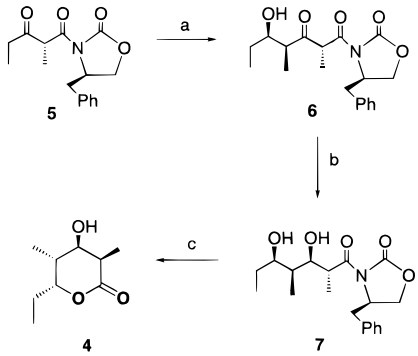


Figure 1. Polyketides produced by engineered derivatives of the 6-deoxyerythronolide B synthase (DEBS).[15,18] (See text for details.) The *S. coelicolor* CH999/pKOS011-56, which contains the DEBS KR5 in place of the native KR2 domain, produces a mixture of **2** and **3**. In *S. coelicolor* CH999/pKAO392 and KAO404, the native DEBS KR2 domain is replaced by the rapamycin polyketide synthase (RAPS)[20] reductive domains from modules 2 and 4, respectively. Both strains produce the aborted 3*R* triketide product (**4**), the C3-hydroxy diastereomer of the wild-type 3*S* triketide (**1**).[12] KR: β-ketoreductase; DH: dehydratase.

(3*R*)-hydroxy diastereomer of the wild-type triketide **1**,[12] was determined by ^{1}H and ^{13}C NMR spectroscopies,[26] including COSY and NOE, HRMS,[26] and isotopic labeling with [1-^{13}C]propionate and [1,2,3-$^{13}C_3$]propionate.[27] As a final confirmation of the structure of **4**, the ^{1}H and ^{13}C NMR spectra of biosynthetically derived **4** were identical to those of an authentic reference sample synthesized by the method of Evans (Scheme 1).[28] Stannous triflate-mediated aldol reaction of β-ketoimide[28b] **5** with propionaldehyde afforded the desired adduct **6** with high diastereoselectivity. Quantitative reduction of aldol adduct **6** with DIBAL gave the desired syn diol **7**.[28b] Treatment of **7** with LiOOH resulted in oxazolidinone cleavage and δ-lactonization to give (2*R*,3*R*,4*S*,5*R*)-2,4-dimethyl-3,5-dihydroxyheptanoic acid δ-lactone (**4**).

The polyketides reported in this study demonstrate that KR domains control the absolute stereochemistry of β-(ketoacyl)-ACP reduction. Furthermore, as illustrated in Figure 2, the KR domains operate regardless of the degree or stereochemistry of substitution of the β-ketoacyl thioester substrate. Thus CH999/pKOS011-56, which contains DEBS KR5 in place of KR2, generates the wild-type triketide (Figure 2(1b)), since both KR2 and KR5 naturally generate a (3*S*)-hydroxyl intermediate (cf. Figure 2(1a) and Figure 1). However, CH999/pKAO392, which contains

Scheme 1. Synthesis of **4**[a]



[a] $Sn(OTf)_2$, Et_3N, propionaldehyde, −78 °C, CH_2Cl_2; (B) DIBAL-H, −78 °C, THF; (C) LiOOH, 0 °C.

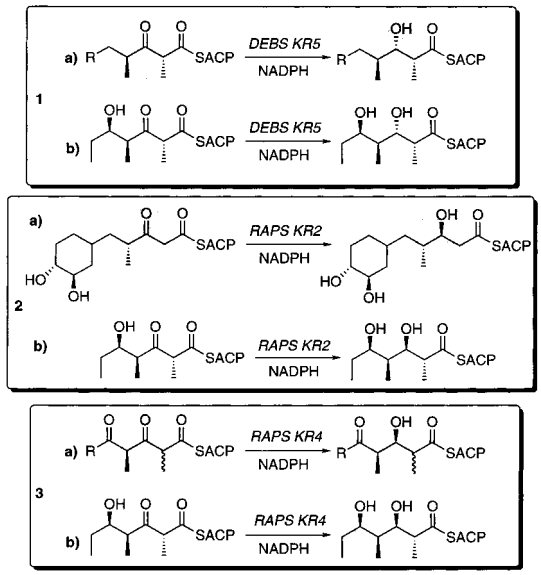


Figure 2. Comparison of structure and stereochemistry of native and engineered substrate−product pairs for DEBS KR5, RAPS KR2, and RAPS KR4. (1a,2a,3a) Native reduction of DEBS KR5, RAPS KR2, and RAPS KR4, respectively. (1b,2b,3b) DEBS KR4, RAPS KR2, and RAPS KR4 reduction, respectively, of the DEBS triketide. For the RAPS KR4 native reduction (3a), the β-hydroxyl stereochemistry has been inferred from the experiments described here, while the adjacent α-methyl configuration remains unknown.

RAPS KR2 in place of DEBS KR2, produces a 3*R* triketide intermediate (Figure 2(2b)), consistent with the RAPS KR2 stereochemistry in its native environment[20] (Figure 2(2a)). Note that the C-2 and C-4 substituents of the natural and engineered RAPS KR2 substrates are completely different in Figure 2(2). Finally, the β-hydroxyl stereochemistry of the RAPS module 4 intermediate, which (along with the α-methyl substituent) is obscured by subsequent dehydration during rapamycin biosynthesis[20] (Figure 2(3a)), is revealed to be 3*R* by CH999/pKAO404, which contains RAPS KR4 in module 2 (Figure 2(3b)). Further studies should reveal whether the isolation of **4**, an aborted triketide product from CH999/pKAO392 and pKAO404, reflects a stringent specificity of the ketosynthase domain of module 3 (KS3) for (3*S*)-hydroxy intermediates, a difference in lactonization rates of **1** and **4**, or other features of modular PKS structure and function.

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(26) For **4**: R_f = 0.47 (50% EtOAc/hexanes); ^{1}H NMR (400 MHz, CDCl3) δ (ppm) 4.68 (ddd, 1H, J = 2.9, 6.2, 8.1 Hz, H-5), 3.93 (dd, 1H, J = 3.7, 3.7 Hz, H-3), 2.64 (dq, 1H, J = 3.7. 7.2 Hz, H-2), 2.07 (ddq, 1H, J = 3.4, 3.8, 7.3 Hz, H-4), 1.78 (ddq, 1H, J = 7.9, 7.4, 14.5 Hz, H-6a), 1.52 (ddq, 1H, J = 6.3, 7.4, 13.8 Hz, H-6b), 1.33 (d, 3H, J = 7.2 Hz, C2−CH_3), 1.00 (t, 3H, J = 7.3 Hz, C7−CH_3), 0.97 (d, 3H, J = 7.2 Hz, C4−CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 173.8 (C-1), 79.61 (C5), 72.96 (C3), 36.52 (C2), 36.46 (C4), 24.99 (C6), 12.17 (C2−CH_3), 9.98 (C4−CH_3), 9.90 (C7−CH_3); HRMS (CI, isobutane) M + H^+, calcd *m/e* 173.1178, obsd 173.1178.

(27) Administration of sodium [1-^{13}C]propionate (300 mg/L) to *S. coelicolor* CH999/pKAO404 gave **4** labeled at C-1, C-3, and C-5. Administration of sodium [1,2,3-$^{13}C_3$]propionate (100 mg/L) gave **4** labeled at all nine carbons with ^{13}C NMR coupling patterns consistent with the derivation of **4** from four intact propionate units.

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