Gain of Function Mutagenesis of the Erythromycin Polyketide Synthase. 2. Engineered Biosynthesis of an Eight-Membered Ring Tetraketide Lactone

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Modular polyketide synthases (PKSs) catalyze the biosynthesis of polyketides, natural product molecules with remarkable structural complexity, and medicinal properties.[1,2] The modular organization of these multifunctional enzymes has created much interest in generating new polyketide molecules through the rational and combinatorial manipulation of PKS genes.[2] Experiments supporting these goals include module deletion experiments,[3−6] targeted inactivation of ketoreductase (KR) and enoyl reductase (ER) domains,[7−9] and more recently, functional replacement of KR[10−12] and acyltransferase (AT)[13,14] domains with heterologous domains possessing novel activities and specificities.

Here, we report the design, construction, and analysis of an engineered derivative of the 6-deoxyerythronolide B synthase (DEBS) that synthesizes a novel eight-membered ring lactone (**1**) *in vivo* (Figure 1). The chemical synthesis of functionalized eight-membered ring lactones (2-oxocanones) is extremely challenging,[15,16] and virtually no functionalized eight-membered ring lactones are known among natural products. We initially attempted to synthesize an eight-membered ring tetraketide lactone by constructing a simple three-module derivative of DEBS, designated "Mod123+TE".[6] However, the primary products were the isomeric six-membered ring lactone (**2**) and the decarboxylated 3-ketoacid (**3**) (Figure 1). We have now eliminated the δ-hydroxyl in the DEBS tetraketide intermediate via "gain-of-function" mutagenesis,[11] demonstrating the biosynthesis of a 2-oxocanone natural product and additional molecular recognition features of modular PKSs. Our results also suggest the existence of host enzymes that can selectively modify the products of engineered PKSs.

For this study, we prepared three new derivatives of Mod123+TE, originally expressed by *Streptomyces coelicolor* CH999/pCK13 (Figure 1).[6] Plasmid pKAO263 contains engineered *Pst* I and *Xba* I restriction sites flanking the KR domain of module 2 (KR2),[17] identical to those previously reported.[10] Plasmids pKAO410 and pKAO384 contain the dehydratase (DH)-enoyl reductase (ER)-KR domains of the rapamycin PKS (RAPS) module 1[18] and DEBS module 4, respectively, in place of KR2.[19] Each plasmid was introduced into *S. coelicolor* CH999,[20,21] and the resulting strains analyzed for polyketide production.[22] The control strain, *S. coelicolor* CH999/pKAO263, produced **2** and **3**, as determined by ^{1}H and ^{13}C NMR spectroscopy, at yields comparable to those from *S. coelicolor* CH999/pCK13.[6] Thus the engineered KR2 restriction sites do not interfere with PKS structure and activity.

Fermentation of *S. coelicolor* CH999/pKAO410, which contains the RAPS DH1-ER1-KR1 domains in place of the native KR2, generated a new metabolite, the tetraketide lactone KAO410a (**1**) (∼20 mg/L; Figure 1). The structure of **1** was determined by ^{1}H and ^{13}C NMR spectroscopy,[23] including COSY and NOE, and HRMS,[23] in combination with isotopic labeling by [1-^{13}C]- and [1,2,3-$^{13}C_3$]-propionate.[24] Analysis of the mass spectrum (CI, $[M + H]^+$ 215) revealed an unanticipated reduction of the expected 3-ketolactone, which was confirmed by a ^{13}C NMR signal at δ 84.1 for the C-3 hydroxyl-bearing carbon and a ^{1}H NMR peak at δ 3.20 for the H-3 carbinyl proton.[25] The ^{13}C and ^{1}H chemical shifts of the lactonic carbon,

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(19) DNA sequences at the boundaries of KR2 domain replacements. In pKAO410: DEBS AT2 → RAPS DH1, TTCTGGCTGCAGGGCGTG; RAPS KR1 → DEBS ACP2, GGTGATTCTAGAGACCGG. In pKAO384: DEBS AT2 → DEBS DH4, TTCTGGCTGCAGCCGCAC; DEBS KR4 → DEBS ACP2, CCGAACTCTAGAGACCGG. In all cases mutations were engineered via standard polymerase chain reaction (PCR) mutagenesis procedures.
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(22) The transformed strains were grown on R2YE medium[21] at 30 °C under thiostrepton selection (50 mg/L) for five to seven days. The mycelia was extracted three times with ethyl acetate (pH 7). **1** was purified from *S. coelicolor* CH999/pKAO410 extracts on an isocratic silica gel column with 25% EtOAc/Hexanes.
(23) **1**: R_f = 0.54 (50% EtOAc/hexanes); ^{1}H NMR (400 MHz, CDCl3) δ (ppm) 4.60 (ddd, 1H, J = 1.2, 4.6, 8.5 Hz, H-7), 3.42 (dq, 1H, J = 2.9, 6.6 Hz, H-2), 3.20 (dd, 1H, J = 3.0, 9.3 Hz, H-3), 1.98 (m, 1H, H-6), 1.76 (ddd, 1H, J = 3.1, 7.7, 15.1 Hz, H-5a), 1.66 (ddq, 1H, J = 7.3, 8.6, 13.9 Hz, H-8a), 1.60 (m, 1H, H-4), 1.48 (ddq, 1H, J = 4.7, 7.4, 13.8 Hz, H-8b), 1.42 (dd, 1H, J = 5.4, 15.1 Hz, H-5b), 1.17 (d, 3H, J = 6.6 Hz, C2-CH$_3$), 1.04 (d, 3H, J = 6.8 Hz, C4-CH$_3$), 0.93 (d, 3H, J = 7.1 Hz, C6-CH$_3$), 0.89 (t, 3H, J = 7.4 Hz, H$_3$-9); 13C NMR of [1,2,3-$^{13}C_3$]propionate-labeled **1** (100 MHz, CDCl$_3$) δ (ppm) 175.19 (d, J = 53.1 Hz, C-1), 84.12 (d, J = 41.5 Hz, C-3), 80.39 (d, J = 37.9 Hz, C-7), 38.76 (d, J = 34.6 Hz, C-5), 37.52 (dd, J = 37.4, 52.4 Hz, C-2), 35.69 (dd, J = 35.0, 35.0 Hz, C-6), 34.05 (dd, J = 36.5, 37.7 Hz, C-4), 27.47 (dd, J = 34.8, 39.3 Hz, C-8), 22.25 (d, J = 34.6 Hz, C6-CH$_3$), 14.89 (d, J = 37.7 Hz, C2-CH$_3$), 11.63 (d, J = 36.1 Hz, C4-CH$_3$), 10.86 (d, J = 34.6 Hz, C-9); HRMS (CI, isobutane) $[M + H]^+$: calculated *m/z* 215.1647, observed *m/z* 215.1639.
(24) Administration of sodium [1-^{13}C]propionate (300 mg/L) to *S. coelicolor* CH999/pKAO410 gave **1** labeled at C1, C3, C5, and C7. Administration of sodium [1,2,3-$^{13}C_3$]propionate (100 mg/L) gave **1** labeled at all 12 carbons with ^{13}C NMR coupling patterns consistent with the derivation of **1** from four intact propionate units.

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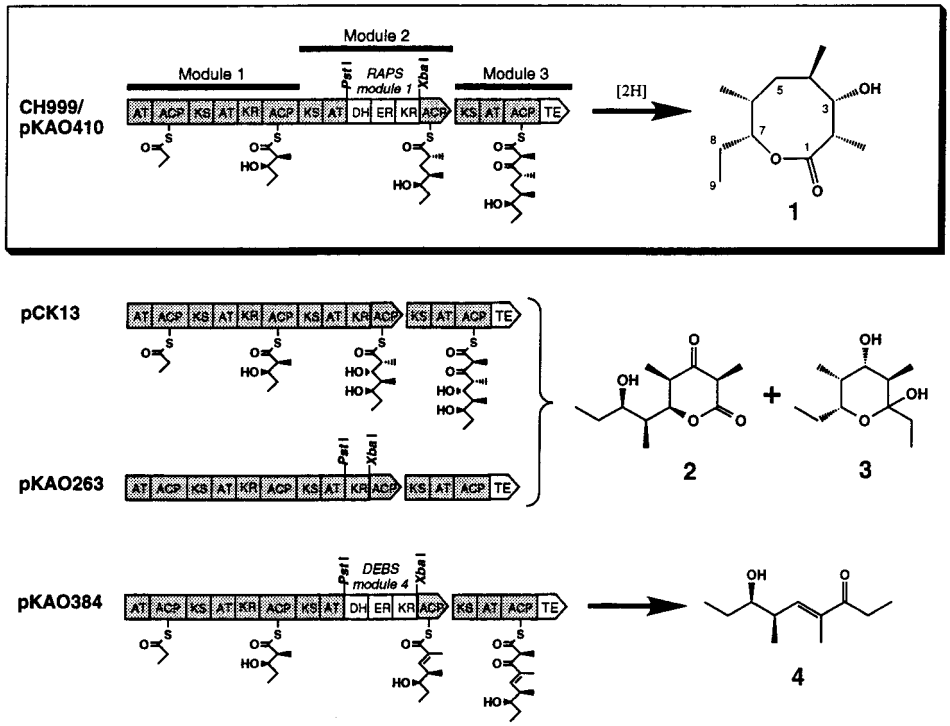

Figure 1. Polyketides produced by engineered three-module derivatives of DEBS. (See text for details.) *S. coelicolor* CH999/pKAO410, which contains the DH-ER-KR domains from module 1 of the rapamycin polyketide synthase (RAPS) in place of the native KR2 domain, produces the eight-membered ring tetraketide lactone (**1**).

C-7, and the attached proton, H-7, were δ 80.4 and δ 4.60, respectively. The H-5 methylene protons (δ 1.76 and 1.42) were assigned from their coupling patterns and by correlation with H-4 and H-6 in the COSY spectrum. Doublets for the three secondary methyl groups were present at δ 1.17 (C2-CH_3), 1.04 (C4-CH_3), and 0.93 (C6-CH_3), while the terminal methyl (H-9) protons appeared as a triplet at δ 0.89.

The stereochemistry of lactone **1** has been provisionally assigned from an analysis of coupling constants, ^{1}H NOE spectra, and biosynthetic considerations. The configurations at C-6 and C-7 were assigned (6*R*, 7*R*), given the genetically unaltered and known function of DEBS module 1. Irradiation of H-7 resulted in a 20% NOE enhancement of the H-2 signal, assigning the C-2 configuration as (2*S*). The (3*S*, 4*R*) configuration was assigned by the agreement between the observed ^{1}H coupling constants of the lactone ring protons with those predicted by molecular modeling of the (2*S*, 3*S*, 4*R*, 6*R*, 7*R*) diastereomer of **1**.[26] Experiments to verify the configuration of **1** by X-ray crystallography are in progress.

In contrast, *S. coelicolor* CH999/pKAO384, which contains the DEBS DH4-ER4-KR4 domains in place of KR2, produced the decarboxylated tetraketide KOS009-7a (**4**) (<10 mg/L), as determined by ^{1}H NMR spectroscopy (Figure 1). This product is identical to the *S. coelicolor* CH999/pKOS009-7 metabolite,[11] produced by a DEBS Mod123+TE mutant which contains the RAPS DH4-KR4 domains in place of KR2, and indicates that the ER4 domain of DEBS did not reduce the dehydrated triketide intermediate produced by the re-engineered module 2.

These experiments demonstrate the feasibility of DEBS KR2 domain replacements with domains from the same or heterologous PKSs, generating mutant PKSs with some or all of the newly introduced catalytic activities. The production of KAO410a (**1**) by *S. coelicolor* CH999/pKAO410 shows that the RAPS DH1-ER1-KR1 active sites are fully functional in the heterologous context of DEBS module 2, while the production of **4** by *S. coelicolor* CH999/pKAO384 reveals that the DEBS DH4-ER4-KR4 domains are only partially functional in the same context. The latter result may reflect higher substrate specificities of the DEBS ER4 or KS3 domains toward unnatural triketide intermediates.

The isolation of **1** vividly demonstrates the broad substrate specificity of the TE domain, which normally catalyzes cyclization of the 14-membered ring lactone, 6-deoxyerythronolide B. This TE has now been shown to mediate the formation of 6-, 8-, 12-, and 16-membered lactone rings.[4−6,27] The unexpected reduction of **1** also suggests the potential for recruiting additional metabolic enzymes to act upon "unnatural" natural products,[25] illustrating how post-PKS modification enzymes may have evolved in nature.

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(25) The observed reduction of **1** may result from the action of an endogenous *S. coelicolor* dehydrogenase or the cryptic KR3 domain of DEBS (not shown in Figure 1, but deduced from sequence analysis).[17] Preliminary HRMS data of a fully reduced analog of **3** isolated from CH999/pKAO410 (Fu, H., unpublished results) and cell-free incubations using purified PKS from the same strain (Gokhale, R., unpublished results) support the former hypothesis.

(26) Observed values of J_{HH} (Hz) between the ring protons of **1:** $J_{H2/H3}$ 2.9, $J_{H3/H4}$ 9.3, $J_{H4/H5a}$ 7.7, $J_{H4/H5b}$ 1.4, $J_{H5a/H6}$ 3.1, $J_{H5b/H6}$ 5.4, $J_{H6/H7}$ 1.2. MacroModel 6.0 (Columbia University; Mohamadi, F.; Richards, N. G. J.; Guida, W. C.; Liskamp, R.; Lipton, M.; Caulfield, C.; Chang, G.; Hendrickson, T.; Still, W. C., *J. Comput. Chem.* **1990**, *11*, 440−467) was used to analyze the conformations of (2*S*,3*S*,4*R*,6*R*,7*R*)-**1**, as well as the corresponding (3*S*,4*S*), (3*R*,4*R*), and (3*R*,4*S*) diastereomers, using an MM2* force-field with GB/SA solvation parameters for $CHCl_3$ (Still, W. C.; Tempczyk, A.; Hawley, R. C.; Hendrickson, T. *J. Am. Chem. Soc.* **1990**, *112*, 6127−6129) and the Low-Mode Conformational Search method (Kolassváry, I.; Guida, W. C. *J. Am. Chem. Soc.* **1996**, *118*, 5011−5019). Each diastereomer was searched through 1000 iterations. The Boltzmann-averaged J_{HH} coupling constants predicted for (2*S*,3*S*,4*R*,6*R*,7*R*)-**1** were in reasonable agreement with the observed values of J_{HH}: $J_{H2/H3}$ 3.9, $J_{H3/H4}$ 6.9, $J_{H4/H5a}$ 6.6, $J_{H4/H5b}$ 3.3, $J_{H5a/H6}$ 1.8, $J_{H5b/H6}$ 10.5, $J_{H6/H7}$ 4.7.

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