Engineered Biosynthesis of a Triketide Lactone from an Incomplete Modular Polyketide Synthase

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Polyketides are structurally diverse natural products possessing a broad range of biological activities. Polyketide synthases (PKSs)[2,3] and fatty acid synthases (FASs)[4] are structurally and mechanistically related, catalyzing the formation of carbon chain products through repeated decarboxylative condensations between acyl thioesters. However, unlike typical FASs, PKSs introduce structural variability into polyketides by catalyzing all, part, or none of a reductive cycle of ketoreduction, dehydration, and enoyl reduction on the β-keto group formed after each condensation reaction.[2,3] Modular PKSs[5-7] usually consist of several extremely large polypeptide subunits (300 000 kDa) that each include one or more repetitive patterns of active sites. These active sites are grouped into modules and appear to correspond to cycles of chain extension in polyketide biosynthesis.[5] Understanding the structural basis for the remarkable catalytic specificity of these enzymes is an important scientific challenge.

Recently, we described a potentially generally applicable strategy for the heterologous expression of recombinant modular PKSs using a specially constructed expression system.[8] Expression of the 6-deoxyerythronolide B synthase (DEBS)[5,6] from *Saccharopolyspora erythraea* (Figure 1) in *Streptomyces coelicolor* resulted in the biosynthesis of 6-deoxyerythronolide B (6dEB) (**1**) and 8,8a-deoxyoleandolide (**2**) (Figure 2).[8] The production of these molecules demonstrated the sufficient role of DEBS for polyketide biosynthesis in vivo, the presence in the heterologous host of all necessary precursors and auxiliary activities for PKS activity, and a relaxed specificity for the starter unit moiety in DEBS, confirming earlier reports of downstream active site tolerance toward abnormal polyketide intermediates.[5,9,10]

The modular PKS systems characterized thus far contain two (*ery*A, *avr*),[5-7] three (*avr*),[7] of four (*pks*X)[11] modules per polypeptide. However, the minimal functional unit of these enzymes, defined as the smallest component that can catalyze polyketide biosynthesis, remains unknown and must be identified before extensive rational manipulation of modular PKSs is possible. Here, we report the heterologous expression of

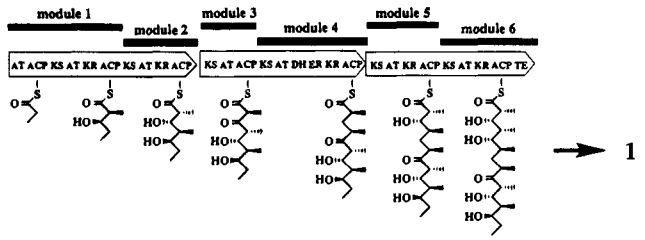
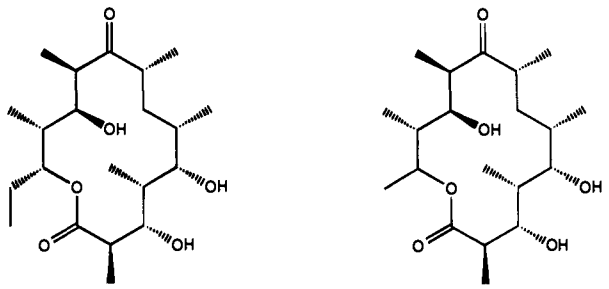


Figure 1. Genetic model for the 6-deoxyerythronolide B synthase (DEBS) from *S. erythraea*. The modules catalyze successive cycles of chain extension and β-keto reduction for the biosynthesis of **1**. Each module includes an acyltransferase (AT), a β-ketoacyl-acyl carrier protein synthase (KS), and an acyl carrier protein (ACP) as well as a subset of reductive active sites [β-ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER)]. The thioesterase (TE) catalyzes lactone formation of the final polyketide chain.



6-deoxyerythronolide **B** (**1**) 8,8a-deoxyoleandolide (**2**)

Figure 2. Polyketides synthesized by DEBS in *S. coelicolor* CH999.[9]

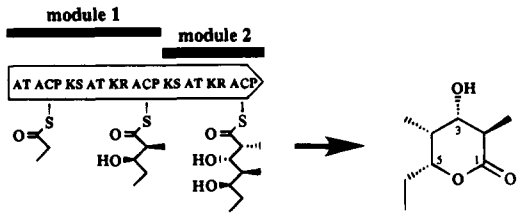


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Figure 3. Biosynthesis of (2*R*,3*S*,4*S*,5*R*)-2,4-dimethyl-3,5-dihydroxy-*n*-heptanoic acid δ-lactone (**3**) by DEBS1 in *S. coelicolor* CH999.

DEBS1 (3544 amino acids), the polypeptide containing the first two modules of DEBS, in *S. coelicolor* and the biosynthesis of the predicted triketide product (**3**) (Figure 3). These results demonstrate that a subset of two modules from a modular PKS can operate independently of the remaining modules and further support the "module hypothesis" for polyketide biosynthesis.[5,12]

To obtain a reference for the predicted product of DEBS1, (2*R*,3*S*,4*S*,5*R*)-2,4-dimethyl-3,5-dihydroxy-*n*-heptanoic acid δ-lactone (**3**) was readily synthesized by the method of Evans (Scheme 1).[13,14] Thus the protected aldehyde **4** (α_D 59.01° (*c* 1.92, $CHCl_3$)[15]), prepared in five steps as previously described,[16] was converted to the (2*R*,3*S*,4*S*,5*R*)-imide **6** in 87% yield by reaction with the chiral boron enolate of (4*R*)-*N*-propionyl-4-benzyloxazlidinone[13,14] (CH_2Cl_2, −78 °C, 2 h, then 0 °C, 1.5 h) followed by treatment of the resulting alcohol **5** with *tert*-butyldimethylsilyl triflate and diisopropylethylamine.[17] Hydrolysis of **6** (2 equiv of LiOH, 8 equiv of 30% H_2O_2, 3:1 THF/

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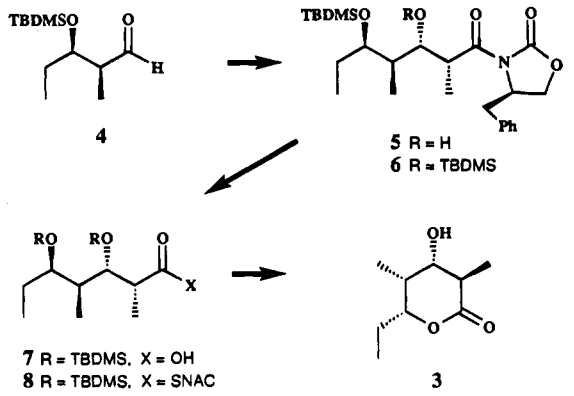
Scheme 1



H_2O, 0–25 °C, 12 h)[18] gave the free acid **7** (90%, α_D −11.68° (*c* 2.44, $CHCl_3$)). Conversion of **7** to the corresponding NAC thioester **8** was accomplished by successive treatment with diphenylphosphoryl azide and *N*-acetylcysteamine.[16] Finally, treatment of **8** with 49% aqueous HF in 1:1 CH_3CN/H_2O (room temperature, 12 h)[19] resulted in smooth removal of the TBDMS protecting groups and concomitant lactonization to give the desired lactone **3** in 90% purified yield from **7**.[20]

To heterologously express DEBS1, plasmid pCK9, which contains the *ery*AI gene, was constructed[21] and introduced into *S. coelicolor* CH999.[22] SDS–PAGE analysis of CH999/pCK9 revealed a single major protein with the same mobility as DEBS1 in a mixture of the three DEBS proteins.[23] Under growth conditions described earlier,[8] CH999/pCK9 produced the expected triketide lactone **3** at yields of 1–3 mg/L. ^1H and ^{13}C NMR spectra of **3** were identical with those of the synthetic sample. Propionic-*1-*^{13}C acid feeding experiments resulted in the enrichment of C1, C3, and C5, confirming the incorporation of three propionates into **3**.

The triketide product **3**, previously isolated from a mutant *S. erythraea* strain,[9] confirms the deduced biosynthetic function of DEBS1. These results show that a thioesterase domain is not essential for thiolysis and, more importantly, that DEBS1 can catalyze polyketide biosynthesis in the absence of the remaining four DEBS modules. Therefore the minimal functional unit of modular PKSs is at most a bimodular polypeptide and may eventually prove to be a single module.

Thus far, the production of two erythromycin analogs by *S. erythraea* chromosomal mutants[5,9,10] and the biosynthesis of 8,-8a-deoxyoleandolide by DEBS[9,24,25] have demonstrated the successful transfer of slightly altered intermediates as well as subsequent β-keto processing in the downstream modules of DEBS. However, the noncompetitive nature of polyketide biosynthesis with unnatural substrates in these experiments is probably important, since partially synthesized intermediates fed to macrolide producers were precisely incorporated into polyketide product,[16,26–28] hinting at relatively stringent substrate specificities in these enzymes. If a single module proves to be the functional unit of modular PKSs, polyketide molecules of desired chain lengths might be obtained through the controlled association of an arbitrary number of modules. Further genetic manipulation and chemical analysis of modular PKSs should reveal the criteria important for transferring and processing polyketide intermediates, eventually leading to the engineered biosynthesis of a wide range of novel polyketides.

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Supplementary Material Available: ^1H and ^{13}C NMR spectra of **3** (4 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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(20) **3**: R_f 0.28 (50% EtOAc/hexanes); IR (neat) ν 3444, 2972, 2942, 2884, 1732, 1696, 1456, 1360, 1292, 1216, 1111, 1056, 1023, 981, 926, 847, 813, 787, 719, 666 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 4.15 (ddd, 1H, *J* = 2.31, 6.28, and 8.21 Hz, C_5-H), 3.86–3.78 (m, 1H, C_3-H), 2.61–2.54 (br, 1H, OH), 2.53–2.42 (dq, 1H, *J* = 10.31 and 7.09 Hz, C_2-H), 1.88–1.75 (m, 1H, one of C_6-H_2), 1.64–1.51 (m, 1H, one of C_6-H_2), 1.40 (d 3H, *J* = 7.08 Hz, C_2-H_3), 1.00 (t, 3H, *J* = 7.46 Hz, C_7-H_3), 0.97 (d, 3H, *J* = 7.07 Hz, C_4-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.07, 81.49, 73.63, 39.73, 36.65, 25.18, 14.22, 9.79, 4.29; $[\alpha]_D$ = 114.44 (*c* 1.33, $CHCl_3$); HRMS (CI, isobutane), M + H^+, calculated *m/e* 173.1178, observed 173.1173.

(21) Plasmid pCK9 contains *ery*A DNA originating from pS1 (Tuan, J. S.; Weber, J. M.; Staver, M. J.; Leung, J. O.; Donadio, S.; Katz, L. *Gene* **1990**, *90*, 21). A fusion of *ery*A DNA containing the 3′ ends of *ery*AI and *ery*AIII (2.6 kb *Sph*I–*Bsa*BI and 5.2 kb *Bcl*I–*Sph*I fragments, respectively) was cloned into the *Sph*I site of pMAK705 (Hamilton, C. M.; Aldea, M.; Washburn, B. K.; Babitzke, P.; Kushner, S. R. *J. Bacteriol.* **1989**, 171, 4617) to give plasmid pCK8. The construction of pCK7, which contains the complete set of *ery*A genes, was described earlier.[8] Plasmid pCK9 was constructed from pCK8 and pCK7 using an in vivo recombination strategy described previously.[8]

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