Engineered Biosynthesis of Structurally Diverse Tetraketides by a Trimodular Polyketide Synthase

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Deoxyerythronolide B synthase (DEBS) from *Saccharopolyspora erythraea* catalyzes the biosynthesis of the erythromycin macrolactone, 6-deoxyerythronolide B (6-dEB) (**1**). Genetic analysis,[1,2] site-directed mutagenesis,[1,3,4] and the incorporation of chemically synthesized intermediates[5] by this modular polyketide synthase (PKS) together have shown that the active sites for each cycle of condensation and β-keto reduction in 6-dEB biosynthesis are clustered in "modules" that operate successively and independently of the remaining modules (Figure 1). More recently, the biosynthesis of triketide lactones **2** and **3** by a deletion mutant comprising modules 1 and 2 fused to the TE[6−9] and the hexaketide lactone **4** by modules 1−5 fused to the TE[9] showed that the thioesterase domain (TE) from module 6 can catalyze the release of chains of diverse length (C_6−C_{15}) as well as the regiospecific lactonization of 12- and 14-membered lactones (Figure 1).

To further explore the catalytic specificity of the TE, we have engineered a trimodular DEBS mutant consisting of modules 1−3 fused to the TE (Figure 1). Plasmid pCK13 was constructed[10] and introduced into *Streptomyces coelicolor* CH999.[11] Under growth conditions described earlier,[12] CH999/pCK13 produced CK13a (**5**) (20 mg/L) and CK13b (**6**) (5 mg/L) (Figure 1).

The structure of **5** was determined by ^{1}H and ^{13}C NMR spectroscopies,[13] including COSY and HMQC, HRMS,[13] and isotopic labeling with [1-^{13}C]propionate and [1,2,3-$^{13}C_3$]-propionate.[14] In $CDCl_3$, **5** was present exclusively as the keto lactone,[13] while the presence of D_2O converted **5** almost exclusively to the corresponding enol lactone tautomer (unpublished results). Similar behavior has recently been reported for the triketide keto lactone **7**[15] (Figure 1). Signals for carbinyl protons H-5 and H-7[13] in **5** were assigned from the coupling patterns as well as by the COSY spectrum, and the 0.8 ppm downfield shift of H-5 relative to H-7 established the presence of a 6-membered ring lactone. The observed coupling between H-5 and H-4,[13] as well as comparisons with the corresponding signals in **7** (δ 2.63 (dq, 1H, J = 3.0, 7.5 Hz, H-4) and 4.66 (ddd, 1H, J = 3.0, 5.3, 8.3 Hz, H-5)[15] confirmed the expected *cis* stereochemistry of C-4 and C-5 substituents. The H-12 methyl appears to occupy the more stable equatorial position.[16] Configurations of C-6 and C-7 were not directly assigned but are based on (a) the known mode of action of DEBS1, (b) the configurations established for the corresponding C-6 and C-7 positions in the cometabolite **6** (see below), and (c) the 1.7 Hz coupling between H-6 and H-7 and the ^{13}C chemical shift (7.3 ppm) of the C-10 methyl, both typical of acyclic *erythro* stereochemistry.[17]

The sample of biosynthetic **6** was characterized using ^{1}H and ^{13}C NMR spectroscopy, including COSY, and [1-^{13}C]propionate labeling,[18] as well as by direct comparison with synthetic **6** and two derivatives.[19] To prepare the reference sample of **6**, the oxazolidinone derivative **8**, prepared similarly as before,[6] was first converted in overall 95% yield to the corresponding aldehyde **9** (MeONHMe·HCl, $AlMe_3$, CH_2Cl_2, −25 °C to room temperature (rt), 15 h;[20] TESOTf, DIEA, CH_2Cl_2, 0 °C to rt,

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(10) Plasmid pCK13 was constructed using an in vivo recombination strategy described earlier.[12] pCK13 is similar to pCK7[12] except for a deletion between the carboxy-terminal ends of ACP-3 and ACP-6 between residues L1466 of DEBS2 and Q2891 of DEBS3 and the insertion of a blunted *Sal*I fragment containing a kanamycin resistance gene (Kan^r GenBlock, Pharmacia) into the blunted *Hin*dIII site of pCK7. An *Spe*I site was engineered between L1466 and Q2891 so that the DNA sequence at the fusion is CTGACTAGTCAG.
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(13) **5**: R_f = 0.23 (60% EtOAc/hexanes); ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.82 (dd, 1H, J = 2.4, 10.4 Hz, H-5), 4.04 (ddd, 1H, J = 1.7, 4.5, 8.7 Hz, H-7), 3.70 (q, 1H, J = 6.6 Hz, H-2), 2.64 (dq, 1H, J = 2.6, 7.5 Hz, H-4), 1.89 (ddq, 1H, J = 1.8, 6.9, 10.3 Hz, H-6), 1.59 (ddq, 1H, J = 7.3, 8.8, 13.8 Hz, H-8), 1.43 (ddq, 1H, J = 4.9, 7.6, 13.7 Hz, H-8), 1.32 (d, 3H, J = 6.6 Hz, H-12), 1.10 (d, 3H, J = 7.6 Hz, H-11), 0.97 (t, 3H, J = 7.4 Hz, H-9), 0.85 (d, 3H, J = 7.0 Hz, H-10); ^{13}C NMR (labeled by [1,2,3-$^{13}C_3$]propionate, 100 MHz, $CDCl_3$) δ 205.8 (d, J = 39.1 Hz, C-3), 170.2 (d, J = 50.8 Hz, C-1), 77.2 (d, J = 39.7 Hz, C-5), 70.6 (d, J = 38.3 Hz, C-7), 50.3 (dd, J = 38.6, 48.9 Hz, C-2), 42.7 (dd, J = 33.6, 39.3 Hz, C-4), 37.5 (dd, J = 35.8, 40.1 Hz, C-6), 27.7 (dd, J = 35.0, 37.9 Hz, C-8), 10.6 (d, J = 34.8 Hz, C-9), 9.7 (d, J = 33.0 Hz, C-11), 7.9 (d, J = 39.1 Hz, C-12), 7.3 (d, J = 35.0 Hz, C-10); HRMS (FAB, NBA/NaI) $[M + Na]^+$ calcd 251.1260, found 251.1265.

(14) Administration of sodium [1-^{13}C]propionate (Cambridge Isotopes) to *S. coelicolor* CH999/pCK13 under conditions described previously[12] gave **5** labeled at C-1, C-3, C-5, and C-7. Administration of sodium [1,2,3-$^{13}C_3$]-propionate (Cambridge Isotopes) gave **5** labeled at all 12 carbons with ^{13}C NMR coupling patterns consistent with the derivation of **5** from four intact propionate units.

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(16) A 10% NOE interaction was observed between H-2 and H-5.

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(18) Administration of sodium [1-^{13}C]propionate (Cambridge Isotopes) to *S. coelicolor* CH999/pCK13 under conditions described previously[12] gave **6** labeled at C-3, C-5, and C-7. The presence of only three enriched carbons in **6** is consistent with its formation by decarboxylation of the full-length acyclic tetraketide, followed by hemiketal formation between the C(7)-OH and C-3.

(19) **6e**: R_f = 0.60 (60% EtOAc/hexanes); ^{1}H NMR (400 MHz, acetone-d_6) δ 4.03 (s, 1H, C(3)-OH), 3.86 (ddd, 1H, J = 5.1, 5.1, 10.3 Hz, H-5), 3.52 (d, 1H, J = 5.3 Hz, C(5)-OH), 3.44 (ddd, 1H, J = 2.7, 7.8, 10.4 Hz, H-7), 1.89 (dq, J = 4.9, 6.9 Hz, H-4), 1.55−1.69 (m, 2H, H-2, H-8), 1.46−1.55 (m, 1H, H-2), 1.34−1.46 (m, 1H, H-6), 1.27−1.44 (m, 1H, H-8), 0.84−0.91 (m, H-1, H-9, H-10, H-11 methyls); ^{13}C NMR (100 MHz, acetone-d_6) δ 100.40, 75.20, 71.61, 40.81, 36.89, 32.83, 26.25, 13.67, 9.87, 8.47, 7.47. **6a**: ^{1}H NMR (400 MHz, acetone-d_6, drop D_2O) δ 3.81 (ddd, 1 H J = 2.2, 6.1, 8.1 Hz, H-7), 3.73 (dd, 1H, J = 4.8, 10.9 Hz, H-5), 1.73−1.83 (m, 1H, H-8a), 1.25−1.39 (m, 1H, H-8b), 0.97 (d, 3H, J = 6.7 Hz, H-11), 0.91 (t, 3H, J = 7.6 Hz, H-1), 0.86 (t, 3H, J = 7.5 Hz, H-9), 0.81 (d, 3H, J = 6.9 Hz, H-10); ^{13}C NMR (100 MHz, acetone-d_6) δ 99.96, 72.75, 72.18, 39.23, 37.77, 33.43, 26.05, 12.19. 10.72, 7.94, 4.90; $[\alpha]_D$ = +105.36° (*c* 0.97, acetone); HRMS (FAB, NBA/NaI) $[M + Na]^+$ calcd 225.1467, found 225.1476; (EI, 16 ev) $[M - H_2O]^+$ calcd 184.1463, found 184.1464.

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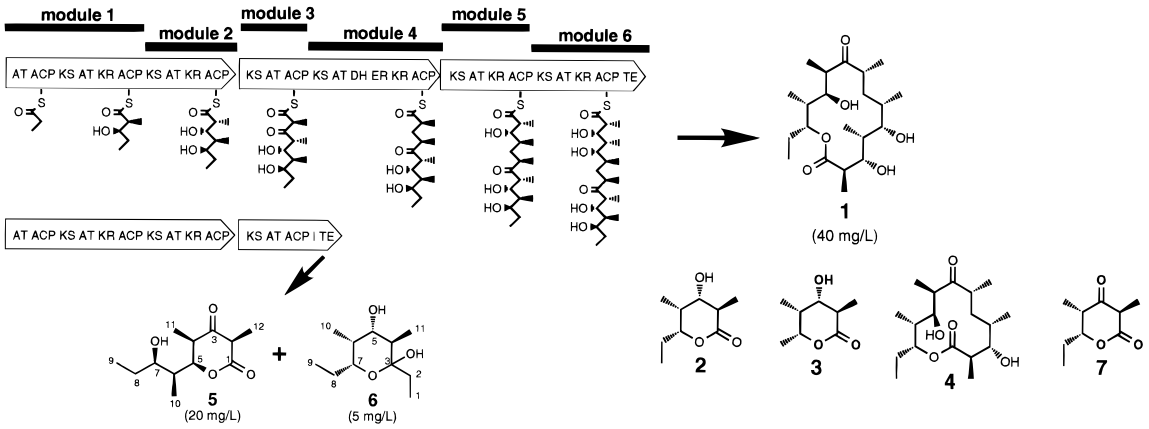
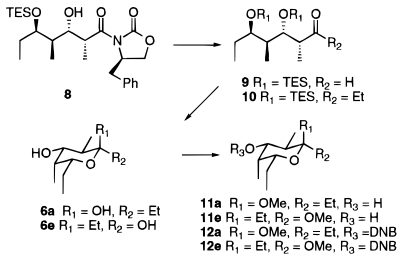


Figure 1. Polyketides produced by engineered *S. coelicolor* CH999 strains (see text); DEBS consists of the polypeptides DEBS1, DEBS2, and DEBS3 (each MW > 300 kDa) that each possess two modules.[1] Key: β-ketoacyl−acyl carrier protein synthase (KS); acyltransferase (AT): dehydratase (DH); enoyl reductase (ER); β-ketoreductase (KR); acyl carrier domain (ACP): thioesterase (TE); 6-dEB (**1**);[12] (2*R*,3*S*,4*S*,5*R*)-2,4-dimethyl-3,5-dihydroxy-*n*-heptanoic acid δ-lactone (**2**);[6,7,9] (2*R*,3*S*,4*S*,5*R*)-2,4-dimethyl-3,5-dihydroxy-*n*-hexanoic acid δ-lactone (**3**);[8,9] (8*R*,9*S*)-8,9-dihydro-8-methyl-9-hydroxy-10-deoxymethynolide (**4**);[9] CK13a (**5**); CK13b (**6**); (2*R*,4*S*,5*R*)-2,4-dimethyl-3-oxo-5-hydroxy-*n*-heptanoic acid δ-lactone (**7**).[15]

Scheme 1. Synthesis of **6a** and Conversion of Synthetic **6a** and Biosynthetic **6e** to **12a** and **12e**



1.5 h; DIBAL, THF, −78 to −40 °C, 1 h; see Scheme 1). Treatment of **9** with ethylmagnesium bromide (THF, 0 °C, 2 h) gave a mixture of epimeric alcohols which were oxidized with Dess−Martin Periodinane[21] (10:1 CH_2Cl_2/pyridine, 0 °C to rt, 1 h) to **10** (91%). Careful deprotection (CH_3CN/H_2O/HF, 0 °C, 10−15 min) afforded the axial anomer of the desired hemiketal **6a**, based on analysis of the ^{13}C NMR signal for C-3 and comparison of the ^1H NMR signals for the methyl groups with the corresponding resonances in a derivative, **12a**, of known configuration (see below). Interestingly, CK13b isolated from *S. coelicolor* CH999/pCK13 was predominantly the equatorial anomer **6e**. To confirm that **6a** and **6e** are anomers with identical stereochemistry at all other positions, each sample was treated with trimethyl orthoformate in MeOH with a catalytic amount of pyridinium *p*-toluenesulfonate (PPTS) (rt, 2 h) to give corresponding methyl ketals **11a** and **11e** in near quantitative yield.[22] Treatment of each mixture with 3,5-dinitrobenzoyl chloride (CH_2Cl_2, DMAP, 30 min) gave a mixture of axial and equatorial anomers of the corresponding dinitrobenzoate derivative, **12a** and **12e**, (combined yield >95%) that were readily separated by preparative TLC and identical in all respects by direct spectroscopic and chromatographic comparison. The axial anomer **12a** was readily identified by NOE enhancements between the methoxylmethyl group and both H-5 and H-7. The ^{13}C NMR shift of the anomeric carbon in **12a** (103.60 ppm) was 0.44 ppm upfield of the signal for C-3 in **12e** (104.06 ppm). Analogous differences were observed between the corresponding signals in **6a** (99.96 ppm) and **6e** (CK13b) (100.40 ppm), allowing unambiguous assignment of the anomeric configuration of each. These assignments were further confirmed by detailed chemical shift and coupling constant comparisons of the methyl resonances for **6a**/**12a** and **6e**/**12e** pairs (supporting material).

While the yields of **5** from CH999/pCK13 provide additional evidence for the TE-catalyzed release of diverse polyketide chain lengths, the limits of lactonization specificity remain less clear. The yields of **5** are comparable to the triketide **2** and hexaketide **4** lactone yields from CH999/pCK12 and CH999/pCK15[9] (Figure 1), respectively, and are a significantly greater than the ∼3 mg/L of **2** produced by CH999/pCK9[6] (DEBS1 without a TE). However, it remains to be determined whether the TE catalyzes the kinetically and thermodynamically favored δ-lactonization in preference to eight-membered lactone formation or whether hydrolysis to the acyclic tetraketide acid precedes nonenzymatic formation of the six-membered ring. The competing formation of **6** most likely results from nonenzymatic decarboxylation of this free keto acid, followed by formation of the hemiketal. Analogous metabolites of abortive polyketide intermediates are produced by wild-type and mutant strains of *Micromonospora griseorubida*[23] and *Streptomyces fradiae*[24] which produce the 16-membered macrolides mycinamicin and tylosin, respectively.

The engineered biosynthesis of CK13a and CK13b illustrate how intermediates of the 6-deoxyerythronolide B pathway can cyclize into structurally diverse products. Thus, these molecules present two additional scaffolds derived from truncated modular PKSs that could be combinatorially manipulated to generate molecular diversity in this medicinally important family of natural products.

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Supporting Information Available: NMR spectra of **5**, **6a**, **6e**, **12a**, **and 12e** and a summary of all NMR data for **5** and **6e** (20 pages). See any current masthead page for ordering information.

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