Spontaneous Priming of a Downstream Module in 6-Deoxyerythronolide B Synthase Leads to Polyketide Biosynthesis†

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ABSTRACT: Modular polyketide synthases such as 6-deoxyerythronolide B synthase (DEBS) catalyze the biosynthesis of structurally complex natural products by repetitive condensation of simple carboxylic acid monomers. The synthase can be divided into groups of domains, called "modules", each of which is responsible for one cycle of chain extension and processing. The modular nature of these enzymes suggests that the biosynthetic pathway might be rationally reprogrammed by manipulation of synthases at the domain level. Although, several examples of successful engineering of DEBS have been reported, a critical issue which has not been well-studied is the tolerance of "downstream" active sites to nonnatural substrates. Here, we report that the terminal modules of DEBS, which normally process highly functionalized intermediates, are competent to carry out their natural functions on smaller, more simple substrates. Expressed in the absence of other DEBS proteins, the DEBS3 protein, which normally carries out the final two extension cycles in the synthesis of 6-deoxyerythronolide B (6-dEB), is spontaneously primed with a C_3 carboxylic acid. This substrate is then extended through two condensation cycles to form a triketide. Tolerance of the "shortened" intermediates in the biosynthesis of this triketide, in combination with results reported elsewhere [Jacobsen, J. R., Hutchinson, C. R., Cane, D. E., and Khosla, C. (1997) *Science 277*, 367−369], suggests that relaxed substrate specificity may be a common feature of modular polyketide synthases. Interestingly, priming of DEBS3 appears to proceed, not by acyltransfer from propionyl-CoA, but by decarboxylation of an enzyme-bound methylmalonyl extender unit. This is the second example of decarboxylative priming within DEBS [see also Pieper, R., Gokhale, R. S., Luo, G., Cane, D. E., and Khosla, C. (1997) *Biochemistry 36*, 1846−1851] and suggests that, in the absence of an acceptable primer (or transferred intermediate), decarboxylative priming of ketosynthase domains may be a general property of modular polyketide synthases.

Polyketide synthases (PKSs)[1] catalyze the assembly of complex natural products from simple precursors such as propionyl-CoA and methylmalonyl-CoA in a biosynthetic process which closely parallels fatty acid biosynthesis (*1*). Like fatty acids, polyketides are assembled by successive decarboxylative condensations of simple precursors. But while the intermediates in fatty acid biosynthesis are fully reduced to generate unfunctionalized alkyl chains, the intermediates in polyketide biosynthesis may be only partially processed, giving rise to complex patterns of functional groups. Additional complexity arises from the use of different starter and chain extension substrates, the generation of chiral centers, and by further functional group modifications such as lactonization or glycosylation.

To control the biosynthesis of these complex molecules, modular polyketide synthases such as 6-deoxyerythronolide B synthase (DEBS) utilize a separate set of active sites for each condensation step. The biosynthesis of 6-deoxyerythronolide B (6-dEB) involves six condensation steps, and consequently, DEBS is organized into six groups of active sites, called "modules", each of which is responsible for one cycle of extension and processing (*2*) (Figure 1A). Each module possesses domains analogous to the ketosynthase (KS), acyl transferase (AT), and acyl carrier protein (ACP) enzymes of fatty acid biosynthesis. Other domains analogous to ketoreductase (KR), dehydratase (DH), enoyl reductase (ER), and thioesterase (TE) enzymes are present in various combinations, giving rise to the complex final structure of 6-dEB (**1**). The six modules of DEBS are organized into three large polypeptides (each >300 kDa) with two modules contained in each of the DEBS1, DEBS2, and DEBS3 proteins.

The modular structure of these enzymes has made them attractive targets for genetic engineering despite a lack of 3-dimensional protein structural information. The feasibility

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[1] Abbreviations: PKS, polyketide synthase; DEBS, 6-deoxyerythronolide B synthase; 6-dEB, 6-deoxyerythronolide B.; KS, ketosynthase; KS1°, inactivated KS from module 1; AT, acyltransferase; ACP, acyl carrier protein; KR, ketoreductase; DH, dehydratase; ER, enoyl reductase; TE, thioesterase; CoA, coenzyme A.; NADPH, reduced nicotinamide adenine dinucleotide phosphate; Bu_2BOTf, dibutyl boron trifluoromethanesulfonate; TBDMSOTf, *tert*-butyldimethylsilyl trifluoromethanesulfonate; THF, tetrahydrofuran; DIBAL-H, diisobutylaluminumhydride; TLC, thin-layer chromatography.

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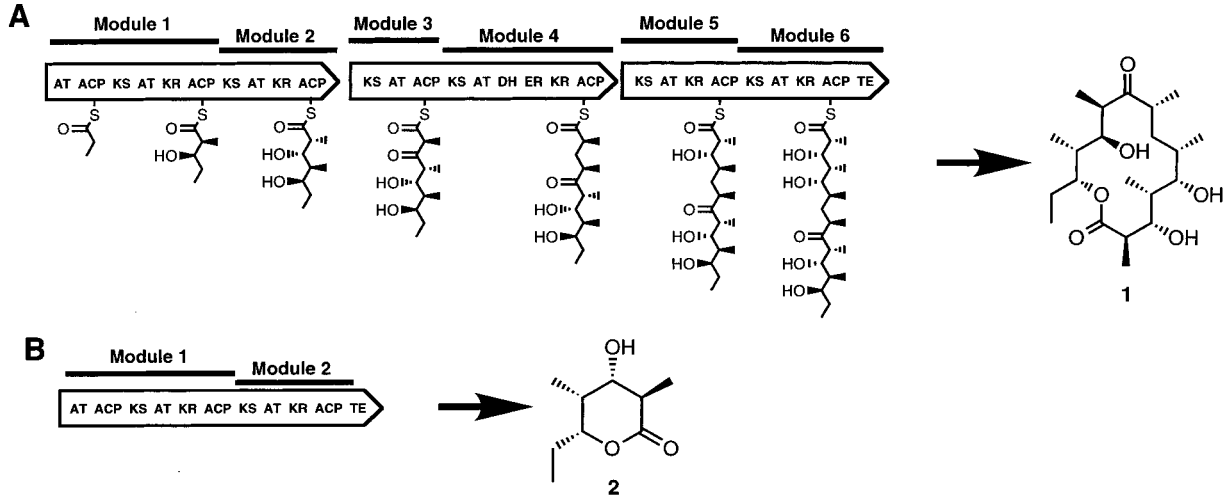

FIGURE 1: (A) Genetic model of DEBS. DEBS consists of three large polypeptides, each of which contains two "modules". Each module catalyzes the addition of a single methylmalonyl-CoA extender unit, along with any reductive steps. Each module contains a variety of domains including a ketosynthase (KS), acyltransferase (AT), and acyl carrier protein (ACP). All modules except module 3 have active ketoreductase (KR) domains, and module 4 contains dehydratase (DH) and enoyl reductase (ER) domains. Module 6 bears a thioesterase (TE) domain which catalyzes the cyclization of the heptaketide intermediate to from 6-dEB (**1**). Proposed enzyme-bound intermediates are shown for each module. (B) Genetic structure of DEBS1 + TE (pCK12). This engineered bimodular polyketide synthase (DEBS1 + TE) contains the first two modules of DEBS1 fused to the TE domain. This PKS produces triketide lactone **2**.

of generating new polyketides has been demonstrated via loss-of-function mutagenesis within reductive domains (*2*−*4*), replacement of acyltransferase domains to alter starter or extender unit specificity (*5*−*8*), replacement of a reductase domain to alter product stereochemistry (*9*), and gain-of-function mutagenesis to introduce novel catalytic activities within modules (*10*, *11*). Additional evidence for remarkably broad substrate specificity of modular PKSs has been obtained from in vitro enzymatic studies (*12*, *13*), and from precursor directed biosynthesis using a KS1-null (KS1°) mutant of DEBS (*14*). Although many of these experiments have emphasized the ability of downstream enzymes in a polyketide pathway to process nonnatural intermediates, the limits of this catalytic tolerance are very poorly understood.

As a dramatic example of the catalytic tolerance of downstream modules, we now report that the DEBS3 protein, expressed in the absence of other DEBS proteins, is competent for polyketide biosynthesis. This truncated multienzyme system can be spontaneously primed with a simple C_3 carboxylic acid in the absence of its far more complex natural substrate. Extension of this C_3 primer demonstrates considerable flexibility of modules 5 and 6. Remarkably, priming of module 5 with propionate appears to occur via DEBS-catalyzed decarboxylation of a methylmalonyl extender unit followed by intraenzyme acyl group transfer. Although these results show that improper priming can occur due to the high effective concentration of (enzyme-bound) propionate formed in situ, discrimination against priming by exogenous acyl donors, such as propionyl-CoA, may have implications for the mechanism by which synthetic fidelity is ensured.

MATERIALS AND METHODS

Reagents and Chemicals. DL-[2-*methyl*-^{14}C]Methylmalonyl-CoA (56.4 mCi/mmol) was obtained from ARC, Inc. [1-^{14}C]Propionyl-CoA (56.4 mCi/mmol) was obtained from Moravek Biochemicals. Reagents for chemical synthesis were obtained from Aldrich and used without purification except where noted.

Bacterial Strains and Plasmids. The construction of CH999/pJRJ2, which expresses a KS1° mutant of DEBS, has been described previously (*14*). Expression of the DEBS3 protein alone was achieved by cloning the DEBS3 gene from pJRJ2 in two portions. A short N-terminal portion was obtained by PCR using the primers 5′-TTAATTAAGGAGGACACAT**ATGAGCGGTGACAACGGCAT**-3′ and 5′-ACTAGT**AGATCTCCAGCATGATCCGCTGC**-3′ (portions which are complementary to DEBS DNA shown in bold). This allowed PCR amplification of a 395 bp segment extending from the ATG start site through an internal *Bgl*II site. Primer overhangs installed a *Pac*I site and ribosome binding site at the 5′-terminus and a *Spe*I site at the 3′-terminus. The resulting PCR fragment was cloned into an intermediate vector (which contains, in 5′ → 3′ order, *Pac*I, *Spe*I, and *Eco*RI sites) as a *Pac*I-*Spe*I fragment. The C-terminal portion of the DEBS3 gene was obtained as the *Bgl*II-*Eco*RI fragment of pJRJ2 (9.1 kb). Restriction of the intermediate plasmid with *Bgl*II and *Eco*RI allowed this fragment to be inserted to reconstitute the DEBS3 gene which was then excised as a *Pac*I-*Eco*RI fragment and inserted into Kao18′, a plasmid which contains all the backbone elements of pJRJ2. The resulting plasmid, pJRJ10, was transformed into *Streptomyces coelicolor* CH999 (*15*).

Production and Purification of Polyketides. Lawns of *S. coelicolor* CH999/pJRJ2 and CH999/pJRJ10 were grown on R2YE agar plates containing 0.3 mg/mL sodium propionate. After 7 days of incubation at 30 °C, the agar media (300 mL) were homogenized and extracted three times with warm (45 °C) ethyl acetate. The solvent was dried over magnesium sulfate, concentrated, and filtered through silica gel (1.2 × 5 cm, eluted with 25 mL of 50% ethyl acetate in hexanes). The extract was purified by silica gel chromatography (1.2 × 12 cm silica gel, gradient of 15 to 25% ethyl acetate in hexanes) to afford the product (**3**). $R_f = 0.36$ (50% ethyl acetate in hexanes). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.95 [d, 3H, $J = 7.4$ Hz, C(4)−CH_3], 1.02 [t, 3H, $J = 7.4$ Hz, C(7)−H_3], 1.34 [d, 3H, $J = 6.8$ Hz, C(2)−CH_3], 1.49−1.60 [m, 1H, one of C(6)−H_2], 1.68−1.81 [m, 1H, one of C(6)−

H_2], 1.94−2.02 [m, 1H, C(4)−H], 2.52−2.59 [m, 1H, C(2)−H], 3.41 [dd, J = 2.6 Hz, 7.6 Hz, C(3)−H], 4.38−4.44 [m, 1H, C(5)−H]. ^{13}C NMR (100 MHz, $CDCl_3$): δ 10.2 (C7), 12.0 (C4−CH_3), 13.9 (C2−CH_3), 24.4 (C6), 40.8 (C4), 42.3 (C2), 77.4 (C3), 79.3 (C5), 183.8 (C1). HRMS (FAB^+) MNa^+ calcd for $(C_9H_{16}O_3)Na^+$: 195.0997. Found 195.0991.

Synthesis of a Reference Compound. (*4R, 2′R, 3′S*)-*3*-(*2′-Methyl-3′-hydroxypentanoyl*)-*4-benzyl-2-oxazolidinone* (***4***). To 40 mL of dry CH_2Cl_2 was added 3.1 g (13 mmol) of (4*R*)-3-propionyl-4-benzyl-2-oxazolidinone, and the solution was cooled to 0 °C (*16*). Bu_2BOTf (16 mL of a 1.0 M solution in CH_2Cl_2) was added by syringe, followed by dropwise addition of diisopropylethylamine (3.2 mL, 18 mmol). The reaction was cooled to −78 °C, and freshly distilled propionaldehyde (1.1 mL, 15 mmol) was added. The reaction was stirred at −78 °C for 40 min then warmed to 0 °C and stirred for an additional 1.5 h. A mixture of 15 mL of phosphate buffer (1.0 M sodium phosphate, pH 7.4) and 45 mL of methanol was added to the reaction followed by dropwise addition of 45 mL 2:1 methanol:30% H_2O_2. After 1 h, organic solvents were evaporated in vacuo and the residual material resuspended in 10 mL of 5% aqueous $NaHCO_3$ and extracted (3 × 50 mL CH_2Cl_2). The combined extracts were washed (50 mL 5% aqueous $NaHCO_3$ followed by 50 mL brine), dried ($MgSO_4$), and concentrated in vacuo. Flash chromatography (3 × 15 cm silica gel, gradient of 15 to 25% ethyl acetate in hexanes) afforded 2.6 g (67%) of the desired product as a colorless oil. R_f = 0.58 (50% ethyl acetate in hexanes). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.98 [t, 3H, J = 7.4 Hz, C(5′)-H_3], 1.26 [d, 3H, J = 7.1 Hz, C(2′)-CH_3], 1.41−1.63 [m, 2H, C(4′)-H_2], 2.79 (dd, 1H, J = 9.5 Hz, 13.4 Hz, one of Ph-CH_2), 3.26 (dd, 1H, J = 3.3 Hz, 13.3 Hz, one of Ph-CH_2), 3.76−3.82 [m, 1H, C(2′)-H], 3.84−3.90 [m, 1H, C(3′)-H], 4.18−4.27 (m, 2H, OCH_2), 4.68−4.75 (m, 1H, N−CH), 7.19−7.37 (m, 5H, Ph-H_5).

(*2R,3S*)-*2-methyl-3*-(*tert-butyldimethylsiloxy*)-*N-methoxy-N-methylpentanamide* (***6***). To a suspension of 0.86 g (8.9 mmol) of *N*,*O*-dimethylhydroxylamine hydrochloride in 35 mL of CH_2Cl_2 at 0 °C was added trimethylaluminum (4.4 mL of a 2.0 M solution in hexanes, added dropwise over 5 min) (*17*). The reaction was gradually warmed to room temperature and, after 30 min, cooled to −15 °C. Imide **4** was dissolved in 8 mL of CH_2Cl_2, and the resulting solution was added dropwise to the reaction mixture. Residual imide was rinsed into the reaction with an additional 5 mL of CH_2Cl_2. After stirring for 30 min at −15 °C, the reaction was warmed gradually to room temperature. After 16 h, the reaction was quenched by cannulating into 25 mL of aqueous tartaric acid, with vigorous stirring. After stirring for 2 h, the layers were separated and the aqueous layer extracted (3 × 30 mL CH_2Cl_2). The organic layers were combined, washed with brine (50 mL), dried ($MgSO_4$), and concentrated in vacuo. Flash chromatography (2.5 × 14 cm silica gel, 30% ethyl acetate in hexanes) removed the majority of contaminants. The product was carried on directly by dissolving in 20 mL CH_2Cl_2 and cooling to −15 °C. TBDMSOTf (1.5 mL, 7.0 mmol) was added and the reaction was stirred for 10 min. Diisopropylethylamine (2.0 mL, 11 mmol) was added dropwise by syringe. The reaction was stirred for 30 min at −15 °C, then warmed to room temperature and stirred for an additional 1.5 h. The reaction was washed with brine (40 mL) and concentrated in vacuo. The residue was chromatographed (3 × 10 cm silica gel, 10% ethyl acetate in hexanes) to afford the product as a colorless oil (1.1 g, 86%, 2 steps). R_f = 0.83 (50% ethyl acetate in hexanes). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.04 (s, 3H, Si−CH_3), 0.05 (s, 3H, Si−CH_3), 0.87 [t, 3H, J = 7.4 Hz, C(5)−H_3], 0.89 [s, 9H, Si−C(CH_3)$_3$], 1.14 [d, 3H, J = 7.0 Hz, C(2)−CH_3], 1.39−1.59 [m, 2H, C(4)−H_2], 2.95−3.03 [m, 1H, C(2)−H], 3.16 [s, 3H, N−CH_3], 3.69 (s, 3H, O−CH_3), 3.85−3.91 [m, 1H, C(3)−H].

(*2R,3S*)-*2-methyl-3*-(*tert-butyldimethylsiloxy*)-*pentanal* (***7***). Amide **6** (1.1 g, 3.8 mmol) was dissolved in 30 mL of freshly distilled THF and cooled to −78 °C. DIBAL (7.8 mL of a 1.0 M in hexanes) was added dropwise by syringe (*18*). The reaction was stirred for 30 min then warmed to −45 °C and stirred for an additional 2 h. The reaction was quenched by cannulating into a vigorously stirred mixture of 75 mL of hexanes and 75 mL of 0.5 M tartaric acid at 0 °C. The resulting mixture was stirred for 30 min at 0 °C after which time the layers were separated and the organic phase was washed with water (30 mL) followed by brine (30 mL). The washes were combined with the aqueous layer from the quench mixture and extracted (2 × 60 mL of CH_2Cl_2). All organic layers were combined, dried ($MgSO_4$), and concentrated in vacuo. The residue was purified by chromatography (2.5 × 10 cm silica gel, gradient of 5 to 10% ethyl acetate in hexanes) to afford the desired product (670 mg, 77%). R_f = 0.49 (10% ethyl acetate in hexanes). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.03 (s, 3H, Si−CH_3), 0.06 (s, 3H, Si−CH_3), 0.83−0.93 [m, 12H, C(5)−H_3, Si−C(CH_3)$_3$], 1.05 [d, 3H, J = 7.0 Hz, C(2)−CH_3], 1.45−1.61 [m, 2H, C(4)−H_2], 2.44−2.48 [m, 1H, C(2)−H], 3.99−4.06 [m, 1H, C(3)−H], 9.76 (s, 1H, CHO).

(*4R, 2′R, 3′S, 4′R, 5′S*)-*3-[2′,4′-Dimethyl-3′-hydroxy-5′*-(*tert-butyldimethylsiloxy*)-*heptanoyl]-4-benzyl-2-oxazolidinone* (***8***). To 20 mL of dry CH_2Cl_2 was added 1.2 g (5.0 mmol) of (4*R*)-3-propionyl-4-benzyl-2-oxazolidinone, and the solution was cooled to 0 °C. Bu_2BOTf (4.8 mL of a 1.0 M solution in CH_2Cl_2) was added by syringe, followed by dropwise addition of diisopropylethylamine (0.92 mL, 5.3 mmol) (*16*). The reaction was cooled to −78 °C, and aldehyde (**7**) was added. The reaction was stirred at −78 °C for 30 min then warmed to 0 °C and stirred for an additional 1.5 h. A mixture of 5 mL of phosphate buffer (1.0 M sodium phosphate, pH 7.4) and 15 mL of methanol was added to the reaction followed by dropwise addition of 15 mL of 2:1 methanol:30% H_2O_2. After 30 min, the mixture was extracted (3 × 40 mL of CH_2Cl_2). The organic layers were combined, washed (50 mL of 5% aqueous $NaHCO_3$ followed by 50 mL of brine) and dried ($MgSO_4$). Flash chromatography (2.5 × 10 cm silica gel, gradient of 5 to 10% ethyl acetate in hexanes) afforded 380 mg (29%) of the desired product. R_f = 0.37 (20% ethyl acetate in hexanes). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.07 (s, 3H, Si−CH_3), 0.08 (s, 3H, Si−CH_3), 0.81 [t, 3H, J = 7.5 Hz, C(7′)-H_3], 0.86 [s, 9H, Si−C(CH_3)$_3$], 0.93 [d, 3H, J = 6.9 Hz, C(4′)-CH_3], 1.32 [d, 3H, J = 6.9 Hz, C(2′)-CH_3], 1.48−1.59 [m, 2H, C(6′)-H_2], 1.64−1.70 [m, 1H, C(4′)-H], 2.77 (dd, 1H, J = 9.6 Hz, 13.4 Hz, one of Ph-CH_2), 3.25 (dd, 1H, J = 3.3 Hz, 13.3 Hz, one of Ph-CH_2), 3.71−3.77 [m, 1H, C(5′)-H], 3.90−3.98 [m, 1H, C(2′)-H], 3.98−4.03 [m, 1H, C(3′)-H], 4.17−4.22 (m, 2H, OCH_2), 4.65−4.72 (m, 1H, N−CH), 7.19−7.37 (m, 5H, Ph-H_5).

(*4R, 2′R, 3′S, 4′R, 5′S*)-*3-[2′,4′-Dimethyl-3′,5′-di*(*tert-butyldimethylsiloxy*)-*heptanoyl]-4-benzyl-2-oxazolidinone* (***9***). Imide **8** (370 mg, 0.79 mmol) was dissolved in 10 mL of CH_2Cl_2 and cooled to 0 °C. Diisopropylethylamine (0.22 mL, 1.3 mmol) and TBDMSOTf (0.28 mL, 1.2 mmol) were added, and the reaction was allowed to warm slowly to room temperature and stirred for an additional 16 h. The reaction was washed (20 mL of brine, followed by 20 mL of saturated aqueous $NaHCO_3$, followed by 20 mL of brine), dried ($MgSO_4$), and concentrated in vacuo. The residue was chromatographed (2.5 × 12 cm silica gel, gradient of 5 to 10% ethyl acetate in hexanes) to afford the product as a colorless oil (380 mg, 82%). $R_f = 0.61$ (20% ethyl acetate in hexanes). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.03 (s, 3H, Si−CH_3), 0.04 (s, 3H, Si−CH_3), 0.09 (s, 3H, Si−CH_3), 0.10 (s, 3H, Si−CH_3), 0.84−0.96 [m, 24H, 2 × Si−C(CH_3)$_3$, C(7′)-H_3, C(4′)-CH_3], 1.26 [d, 3H, $J = 6.4$ Hz, C(2′)-CH_3], 1.50−1.72 [m, 3H, C(6′)-H_2, C(4′)-H], 2.77 (dd, 1H, $J =$ 9.6 Hz, 13.3 Hz, one of Ph-CH_2), 3.24 (dd, 1H, $J = 3.1$ Hz, 13.4 Hz, one of Ph-CH_2), 3.62−3.67 [m, 1H, C(5′)-H], 3.99−4.09 [m, 2H, C(2′)-H, C(3′)-H], 4.16−4.23 (m, 2H, OCH_2), 4.62−4.68 (m, 1H, N−CH), 7.19−7.37 (m, 5H, Ph-H_5).

(*2R, 3S, 4R, 5S*)-*2,4-Dimethyl-3,5-di*(*tert-butyldimethylsiloxy*)-*heptanoic acid* (***10***). Imide **9** (340 mg, 0.58 mmol) was dissolved in 9.8 mL of THF. Water (3.2 mL) was added and the solution was cooled to 0 °C with vigorous stirring. Hydrogen peroxide (0.56 mL of a 30% aqueous solution, 5.4 mmol) was added followed by lithium hydroxide (49 mg, 1.2 mmol) (*19*). The reaction was allowed to warm slowly to room temperature. After 24 h, the reaction was quenched by addition of sodium sulfite (5.1 mL of a 20% aqueous solution). After stirring for 10 min, the reaction was concentrated in vacuo to remove THF and then acidified to pH 2 by dropwise addition of 1 N HCl. The resulting cloudy mixture was extracted (3 × 30 mL of CH_2Cl_2). The organic layers were combined, dried ($MgSO_4$), and concentrated in vacuo. The residue was purified by chromatography (1.9 × 12 cm silica gel, 10% ethyl acetate in hexanes) to afford the desired product (220 mg, 91%). $R_f = 0.65$ (20% ethyl acetate in hexanes). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.01 (s, 3H, Si−CH_3), 0.04 (s, 3H, Si−CH_3), 0.05 (s, 3H, Si−CH_3), 0.06 (s, 3H, Si−CH_3), 0.83 [t, 3H, $J = 7.4$ Hz, C(7)−H_3], 0.85−0.93 [m, 21H, 2 × Si−C(CH_3)$_3$, C(4)−CH_3], 1.14 [d, 3H, $J = 7.0$ Hz, C(2)−CH_3], 1.49−1.58 [m, 2H, C(6)−H_2], 1.66−1.74 [m, 1H, C(4)−H], 2.83 [dq, 1H, $J = 3.7$ Hz, 7.0 Hz, C(2)−H], 3.53−3.59 [m, 1H, C(5)−H], 4.12 [dd, 1H, $J = 3.8$ Hz, 6.8 Hz, C(3)−H].

(*2R, 3S, 4R, 5S*)-*2,4-Dimethyl-3,5-dihydroxy-heptanoic acid-δ-lactone* (***3***). Acid **10** (100 mg, 0.24 mmol) was dissolved in 5.9 mL of acetonitrile. Water (2.2 mL) was added with vigorous stirring. To the cloudy mixture, HF (1.5 mL of a 48% aqueous solution, 47 mmol) was added dropwise (*20*). The mixture gradually cleared to a colorless solution and was stirred at room temperature for 24 h. The reaction was quenched by addition of saturated aqueous $NaHCO_3$ until the pH of the solution reached 7.5. The resulting mixture was extracted (3 × 15 mL of CH_2Cl_2). The organic layers were combined, dried ($MgSO_4$), and concentrated in vacuo. The residue was purified by chromatography (1.9 × 8 cm silica gel, 40% ethyl acetate in hexanes) to afford the desired triketide lactone (39 mg, 93%) whose physical properties were identical to those of the biosynthetic product (**3**).

In Vitro Assays. Protein extracts of CH999/pCK12 and CH999/pJRJ10 were prepared as described previously (*21*). Assay mixtures (230 μL) were composed of protein extract (200 μL in 100 mM sodium phosphate, pH 7.1, 2 mM DTT, 2 mM EDTA, and 10% glycerol) and 880 μM NADPH. Experiments with labeled propionyl CoA contained 250 μM propionyl-CoA (specific activity reduced to 5.4 mCi/mmol by dilution with unlabeled propionyl-CoA) and 560 μM methylmalonyl-CoA. Experiments with labeled methylmalonyl-CoA contained 17 μM [^{14}C]methylmalonyl-CoA (used without dilution) and no propionyl-CoA. Reactions were mixed and incubated at 30 °C for 1.5 h, diluted with 400 μL of 1.0 M NaH_2PO_4, and extracted with two 500 μL portions of ethyl acetate. The ethyl acetate layers were pooled, dried in vacuo, and loaded onto an analytical TLC plate. Due to the higher production levels from DEBS1 + TE, only 20% of the dried extracts were loaded for reactions of DEBS1 + TE. The plate was developed in 50% ethyl acetate in hexanes and visualized by electronic autoradiography (InstantImager, Packard Instruments). To confirm that the product observed by autoradiography was lactone **3**, the product of three in vitro reactions of DEBS3 with radiolabeled methylmalonyl-CoA was extracted from TLC plates into ethyl acetate and chromatographed (C-18 Ultrasphere IP 5 μM HPLC column, Beckman Instruments Inc., 1 mL/min flow rate, 5 min at 15% acetonitrile in water, followed by a gradient of 15 to 100% acetonitrile over 15 min). The radiolabeled material (detected by scintillation counting of 0.5 mL fractions) coeluted with a synthetic sample of **3** (detected by UV absorbance, 210 nm), with a retention time 14.1 min.

RESULTS

Isolation of a Triketide Lactone from Unfed Cultures of DEBS KS1°. CH999/pJRJ2 expresses a mutant of the complete DEBS multienzyme complex (DEBS1, DEBS2, and DEBS3) in which the ketosynthase domain of module 1 had been inactivated (*14*). This strain was not expected to produce polyketide products in the absence of a synthetic diketide substrate. To examine the biosynthetic capabilites of this mutant strain, extracts were made from agar plate cultures. After removal of the most polar components of the extract by silica gel chromatography, NMR of the partially purified material suggested the presence of a polyketide product (identified by characteristic methine and methyl group peaks). Further purification by silica gel chromatography led to the isolation of triketide lactone **3** (Figure 2A). NMR spectra of **3** showed considerable similarity to the previously identified product of DEBS1, triketide lactone **2** (Figure 1B), suggesting that the newly isolated product might be a stereoisomer of **2**. The biosynthesis of such a molecule would be predicted to require two consecutive PKS modules, each having a functional ketoreductase domain, but no additional reductase domains. These requirements are met by modules 1 and 2 (which are known to synthesize lactone **2**) and by modules 5 and 6.

Isolation of a Triketide Lactone from Cultures of DEBS3. To test the hypothesis that modules 5 and 6 are sufficient for the biosynthesis of lactone **3**, the DEBS3 protein was

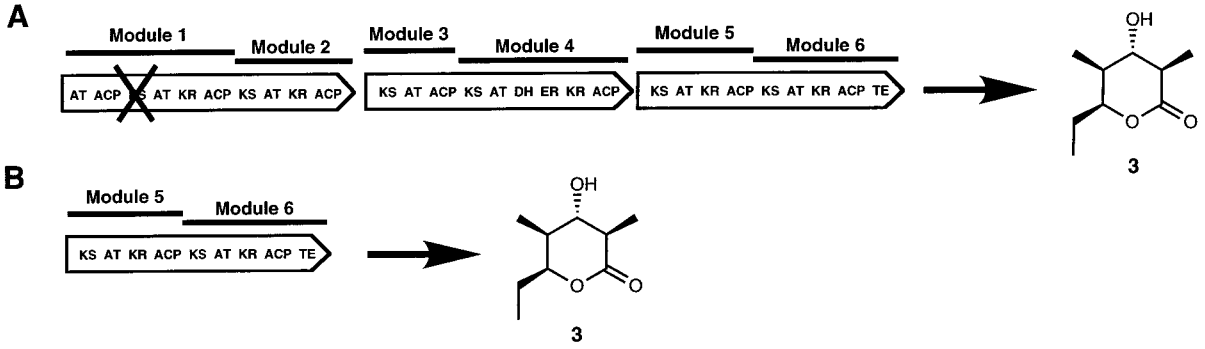

FIGURE 2: (A) KS1° mutant of DEBS (pJRJ2). Mutation of an active-site cysteine residue in the module 1 ketosynthase domain (KS1) inactivates this domain. No 6-dEB-like products are observed in fermentations of strains expressing this DEBS mutant, but triketide lactone **3** is produced at moderate levels. (B) DEBS3 expressed alone (pJRJ10). The DEBS3 protein, expressed in the absence of other DEBS proteins, synthesizes lactone **3**, confirming that modules 5 and 6 are sufficient for production of **3**.

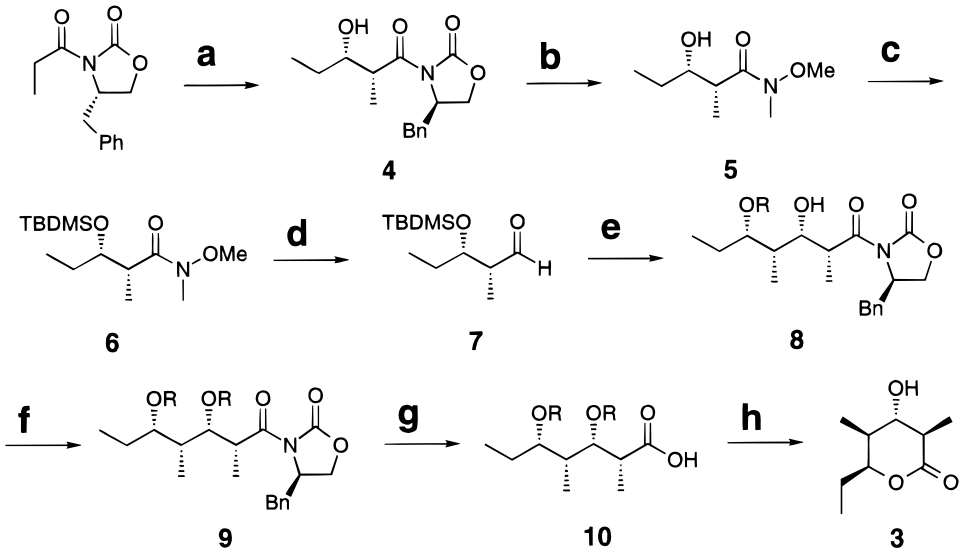

FIGURE 3: Synthesis of **3**. The stereochemistry of the biosynthetic triketide lactone **3** was predicted based on the structure of 6-dEB and the known mode of action of DEBS modules 5 and 6. To confirm the prediction, an authentic reference sample of **3** was prepared. Reagents for transformations: (a) Bu_2BOTf, $EtN(iPr)_2$, EtCHO. (b) $AlMe_3$, $MeN(OMe)H_2^+Cl^-$. (c) TBDMSOTf, $EtN(iPr)_2$. (d) DIBAL-H. (e) Propionyl oxazolidinone, Bu_2BOTf, $EtN(iPr)_2$. (f) TBDMSOTf, $EtN(iPr)_2$. (g) LiOH, H_2O_2. (h) HF.

cloned and expressed in the absence of other DEBS modules. The resulting plasmid (pJRJ10) was transformed into *S. coelicolor* CH999. Lawns of CH999/pJRJ10 were grown and extracted as described above. This strain was found to produce lactone **3** (Figure 2B). Silica gel chromatography allowed the isolation of 4.1 mg of **3**/L of agar media. Extracts from two control strains were also examined. CH999/Kao308 and CH999/pJL188 express engineered DEBS complexes consisting of modules 1 and 6 and modules 1−5, respectively. In the latter construct, inactivation of KS1 (KS1°) leads to a null phenotype. Careful analysis of the extracts from both strains by NMR showed no evidence of product **3**.

Synthesis of a Reference Compound. To assign the stereochemistry of the biosynthetic product, chemical synthesis was used to prepare a sample of the predicted triketide lactone (*20*, *22*). Assuming that module 5 is primed with propionate and that modules 5 and 6 carry out the same stereospecific transformations as are observed in 6-dEB biosynthesis, the predicted configuration of lactone **3** is 2*R*, 3*S*, 4*R*, 5*S*. This differs from the configuration of triketide lactone **2** at C4 and C5 due to the different stereospecificities of the PKS domains responsible for their biosynthesis. The Evans asymmetric aldol reaction (*16*) between the appropriate propionyl oxazolidinone and propionaldehyde was used to generate intermediate **4** (Figure 3). This intermediate was converted to the *N*,*O*-dimethylhydroxylamine amide **5** (*17*). Protection of the β-hydroxyl group afforded compound **6**, which was reduced to the aldehyde (**7**) with DIBAL (*18*) and used as a substrate for a second asymmetric aldol reaction, yielding intermediate **8** (*20*). Protection of the newly formed hydroxyl group afforded **9** which was cleaved from the chiral auxiliary with lithium hydroperoxide (*19*). Treatment of the resulting carboxylate, **10**, with HF cleaved both silyl ethers and catalyzed lactonization to form **3**. The NMR spectrum of the synthetic triketide lactone **3** was identical to that of the compound obtained from biosynthesis by CH999/pJRJ2 and CH999/pJRJ10.

In Vitro Studies of Triketide Lactone Biosynthesis. In an attempt to understand the biosynthetic mechanism for formation of **3**, active PKS preparations from *S. coelicolor* CH999/pJRJ10 were probed in vitro using radiolabeled methylmalonyl-CoA and propionyl-CoA. Protein extracts of CH999/pJRJ10 as well as CH999/pCK12 (a control strain which expresses DEBS1 + TE, an engineered derivative of the DEBS1 protein which produces triketide lactone **2**) were prepared as described previously (*21*). Previous work demonstrated that DEBS1 + TE can utilize propionyl-CoA

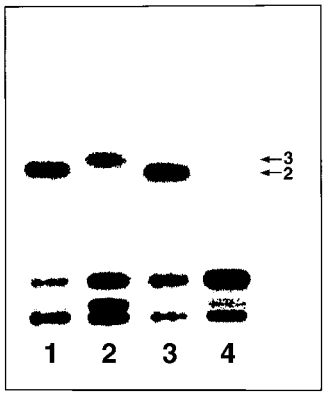

FIGURE 4: TLC autoradiograph of lactones formed in vitro. Crude extracts of CH999/pCK12 (DEBS1 + TE) and CH999/pJRJ10 (DEBS3) were incubated with NADPH and either ^{14}C-methylmalonyl-CoA (MMCoA) or ^{14}C-propionyl-CoA (PrCoA) and unlabeled MMCoA. (Lane 1) DEBS1 + TE, ^{14}C-MMCoA. (Lane 2) DEBS3, ^{14}C-MMCoA. (Lane 3) DEBS1 + TE, ^{14}C−PrCoA. (Lane 4) DEBS3, ^{14}C−PrCoA. Production of radiolabeled lactone **2** is evident in lanes 1 and 3. Radiolabeled lactone **3** is visible in lane 2.

as a primer for biosynthesis of lactone **2** (*23*) and that, in the absence of propionyl-CoA, the enzyme can decarboxylate methylmalonyl-CoA (or its enzyme-bound intermediate) to generate the same C_3 primer (*21*). As shown in Figure 4, both DEBS1 + TE and DEBS3 produce triketide lactone products which incorporate labeled methylmalonyl-CoA. The identity of the DEBS3 in vitro product was confirmed by extraction of the radiolabeled material from the TLC plate (Figure 4) and reversed-phase HPLC co-injection with a synthetic sample of compound **3** (data not shown). When radiolabeled propionyl-CoA and unlabeled methylmalonyl-CoA are used as substrates, DEBS1 + TE produces a labeled triketide lactone, consistent with the incorporation of [^{14}C]-propionate as a primer. However, no radiolabeled triketide lactone is formed by DEBS3 when propionyl-CoA contains the radiolabel, suggesting that DEBS3 is not primed by propionyl-CoA.

DISCUSSION

Polyketides comprise a large class of natural products, which are, despite tremendous chemical diversity, closely related in their biosynthesis. Small molecules derived from polyketide pathways include a significant number of important antibiotics, anticancer agents, and other pharmacologically active products. The considerable medicinal importance of polyketides has generated much interest in the study, and more recently the engineering, of polyketide biosynthesis. By adding, subtracting, replacing, and reorganizing the domains of modular polyketide synthases, new PKSs with novel, but predicted specificities have been generated. An essential requirement for reprogramming PKSs by manipulation at the domain level is that downstream domains faithfully process intermediates which differ from their natural substrates. Such tolerance of nonnatural substrates must however be reconciled with the observation that PKSs normally produce a single product or a small number of related products, suggesting the high degree of specificity typical of enzymatic processes.

As part of previous work, we reported the construction and analysis of pJRJ2, a DEBS derivative generated by site-directed mutagenesis to inactivate the ketosynthase of module 1 (KS1) (*14*). The resulting engineered strain was incapable of carrying out the first condensation step in the biosynthesis of 6-dEB, and therefore produces no 6-dEB-like products. However, when a synthetic analogue of the first (diketide) intermediate is administered to these cultures, it is efficiently converted to 6-dEB, demonstrating that all of the downstream domains remain functional. In the process of examining cultures of CH999/pJRJ2 which had not been provided any exogenous diketide substrates, a previously unknown polyketide product (**3**) was isolated. The NMR spectra of **3**, along with results from previous studies of DEBS, suggested that **3** might be a triketide lactone produced (from a propionate primer) by DEBS modules 5 and 6. Knowledge of the stereospecificties of these modules (evident in the final structure of 6-dEB) allowed the stereoconfiguration of **3** to be predicted and confirmed by chemcial synthesis of an authentic reference.

Confirmation that modules 5 and 6 are necessary and sufficient for the production of lactone **3** suggests that, in the absence of natural substrate (the enzyme-bound product of module 4), module 5 becomes primed with a propionyl unit. Once primed with this simple substrate, modules 5 and 6 carry out their native functions, extending the primer to a triketide. The observed product (**3**) displays the same functional groups and stereochemical configurations as that portion of 6-dEB produced by modules 5 and 6 (C1 through C5 of 6-dEB); however, the natural substrates for modules 5 and 6 are structurally complex penta- and hexaketides. Thus, the ability of these modules to process simple primer and diketide substrates demonstrates remarkably relaxed specificity and suggests that specificity may be focused on portions of the growing polyketide chain which are proximal to the carboxyl terminus. It should be noted, however, that product **3** is produced at relatively low levels (<10%), compared to those of lactone **2** produced by DEBS1 + TE. It is unclear whether this is due to inefficient priming of module 5 or due to inefficient processing of the unnatural substrates through modules 5 and 6. Nonetheless, our studies have yielded insights into the mechanism of priming in this truncated PKS.

Although the biochemical evidence clearly indicates that module 5 is primed with a propionyl unit, in vitro assay of the DEBS3 protein demonstrates that this primer cannot be derived from exogenous propionyl-CoA. Decarboxylation of methylmalonyl groups to form primer units has been observed in DEBS previously and is evidenced by the production of lactone **2** by DEBS1 + TE in the absence of propionyl-CoA. The absence of propionyl-CoA incorporation in the biosynthesis of **3** suggests that decarboxylation of a methylmalonyl group is the only method by which module 5 may become primed with a C_3 unit. This might occur by decarboxylation of an acyl carrier protein (ACP)-bound methylmalonyl group, followed by transfer of the resulting propionyl group to the ketosynthase where it could act as a primer. In the cases examined, the acyl carrier protein of module 4 (ACP4) was either not present (in pJRJ10 experiments) or unable to present the natural substrate for module 5 (in pJRJ2 experiments). Under these circumstances, slow decarboxylation of the methylmalonyl moiety and transfer of the propionyl residue from ACP5 may be the dominant process, leading to the synthesis of the observed product (**3**). This model suggests that the ketosynthase

domain may discriminate strongly against exogenous substrates such as propionyl-CoA, yet display a relaxed specificity toward enzyme-bound intermediates. Although the generality of this model of decarboxylative priming remains to be established, it is interesting to note its applicability to at least two modules of DEBS (modules 1 and 5).

The balance between substrate specificity and tolerance is critical to the productive recombination of PKSs at the domain level, either in the laboratory or over the course of evolution. An attractive model is that the constituent modules have a kinetic preference for intermediates passed from adjacent modules. This would allow downstream active sites to exhibit relaxed selectivity for substrate structure, thereby relying upon the fidelity of upstream active sites to guarantee presentation of the appropriate intermediate. It would also explain why formation of **3** is not observed with wild-type DEBS or with DEBS−KS1° that has been supplied with a diketide substrate. On the other hand, it is clear that KS domains are not completely promiscuous, as evidenced by our finding that the KS2 domain is specifically acylated by its natural diketide substrate (*24*) and, although KS2 tolerates a range of structural variations, it nonetheless exhibits a kinetic preference for processing of its natural substrate and closely related analogues (*20*). If discrimination in favor of enzyme-bound intermediates as substrates is a general rule, this might greatly facilitate the evolution of new biosynthetic domains by allowing the constituent domains to remain relatively tolerant of structural variations in the acylthioester substrate. The domains would therefore be truly "modular" since each would accept substrates from many combinations of upstream modules.

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