Remarkably Broad Substrate Specificity of a Modular Polyketide Synthase in a Cell-Free System

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Received August 14, 1995

Polyketides, a large family of pharmaceutically important natural products, are synthesized by multifunctional polyketide synthases (PKSs) which catalyze a series of stereospecific/regiospecific condensation, reduction, and cyclization reactions.[1,2] Although these natural products exhibit a breathtaking range of structural diversity,[1] the striking similarities in stereochemistry within large classes of macrolide and polyether products have long been recognized,[3] leading to speculation that these stereochemical regularities might reflect an underlying modularity in the organization of the relevant biosynthetic enzymes. *N*-Acetylcysteamine (NAC) thioesters of increasingly elaborate intermediates of polyketide chain elongation have been incorporated into various macrolides using cultures of producing microorganisms.[4] However, these experiments do not address the ability of the PKS to process alternative substrates or intermediate analogs. Within the past few years, the application of genetic engineering strategies to this problem has facilitated a systematic exploration of the limits to this unusual combination of catalytic specificity and product diversity.[5−7] The functional modularity of active sites (or groups thereof) within PKSs is becoming increasingly apparent,[5,6,8] raising the possibility of designing libraries of novel polyketides via combinatorial biosynthesis.[9] Despite these advances, our understanding of the structural and mechanistic rules for the rational alteration of polyketide structure via mutagenesis remains rudimentary at best. In large part this has been due to a virtual lack of cell-free systems capable of providing direct insights into the mechanisms of molecular recognition and chain transfer within PKSs.[10]

Recently we reported[11] a method for cell-free biosynthesis of 6-deoxyerythonolide B, the parent macrolide aglycon of the broad spectrum antibiotic erythromycin A, using recombinant 6-deoxyerythronolide B synthase (DEBS),[7,12] a modular PKS which contains at least 28 distinct active sites.[5,6] In the presence of propionyl coenzyme A, (2*RS*)-methylmalonyl coenzyme A, and NADPH, the DEBS preparations synthesize 6-deoxyerythronolide B (6-dEB). We also showed that a truncated mutant of DEBS carrying the first two synthetic modules fused to the thioesterase domain (designated DEBS 1+TE; Figure 1) generates (2*R*,3*S*,4*S*,5*R*)-2,4-dimethyl-3,5-dihydroxy-*n*-heptanoic acid δ-lactone (**1**) *in vitro*.[13] Synthesis of the ^{13}C-labeled **1** from either propionyl-CoA or (2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-NAC thioester, a known diketide chain elongation intermediate in the formation of the erythromycin macrolactone, was confirmed by NMR spectroscopy. We now report that DEBS 1+TE can recognize a variety of unnatural primer units and related intermediates as well as unreduced intermediates, and convert each of them into triketide products in a cell-free system.

Cell-free preparations of DEBS 1+TE were prepared from recombinant *Streptomyces coelicolor* CH999/pCK12[13] as described earlier.[11] The enzyme was found to exhibit a relaxed specificity for primer unit analogs, as shown by both protein acylation and product formation. In addition to the expected[12] acylation by [1-^{14}C]propionyl-CoA, DEBS 1+TE also forms covalent adducts with [1-^{14}C]acetyl-CoA and [1-^{14}C]butyryl-CoA with comparable efficiencies, as judged by SDS−PAGE/autoradiography (supporting information). Since the protein labeling was unaffected by the presence of active site thiol inhibitors such as iodoacetamide, the substrates are presumably bound to the "loading" acyltransferase domain at the N-terminal end of DEBS 1 (Figure 1). In the presence of methylmalonyl-CoA and NADPH, both acetyl-CoA and butyryl-CoA serve as surrogate polyketide chain initiators for DEBS 1+TE, giving rise to **2**, the previously described C_8 analog of **1**[13] (2% yield, based on acetyl-CoA), and **3**, the C_{10} homolog of **1** (3% yield, based on butyryl-CoA), respectively. The identities of [^{14}C]-**2** and [^{14}C]-**3** were confirmed by synthesis of authentic reference samples by a variation of the procedure previously used for the preparation of **1**,[14] based on the chiral oxazolidinone method of Evans.[15,16] Thin layer chromatography−autoradiography revealed that the enzymatically generated products had R_f values identical to those of the corresponding reference samples of **2** and **3**. TLC-purified radiolabeled **2** and **3** were each diluted with authentic unlabeled samples and recrystallized repeatedly to constant activity.[17] While the ability of DEBS 1+TE to synthesize **2** *in vitro* is consistent with earlier reports describing *in vivo* engineered biosynthesis of 8,8a-deoxyoleandolide[7] and **2**[13] by the complete DEBS and DEBS 1+TE, respectively, **3** is a novel molecule not observed thus far in any natural or engineered polyketide producer. DEBS 1+TE can also recognize and properly process the NAC thioester **4**, corresponding to the diketide chain elongation intermediate generated from an acetyl starter in the course of the formation of the C_8 lactone **2**. Incubation of (2*S*,3*R*)-2-methyl-3-hydroxybutyryl-NAC thioester **4**, prepared by a simple variation of previously described methods,[4] with DEBS 1+TE in the presence of [2-^{14}C-methyl]-(2*RS*)-methylmalonyl coenzyme A and NADPH under the usual incubation conditions gave [^{14}C]-**2** (0.1% yield, based on methylmalonyl-CoA). The identity of the enzymatically

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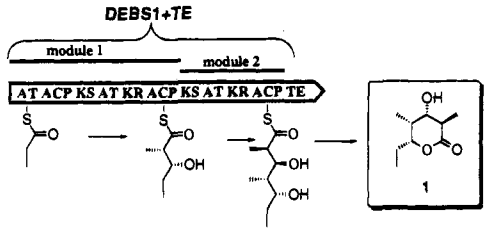
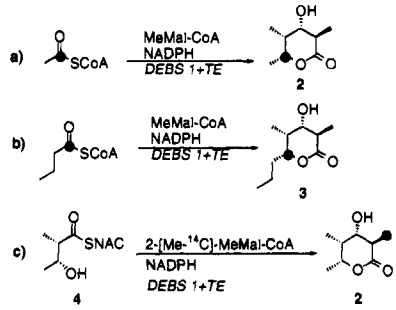
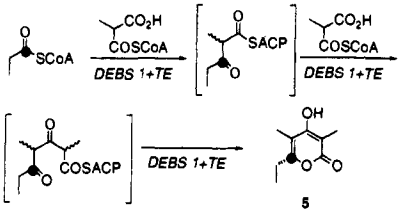


Figure 1. Model for biosynthesis of (2*R*,3*S*,4*S*,5*R*)-2,4-dimethyl-3,5-dihydroxy-*n*-heptanoic acid δ-lactone (**2**) by DEBS 1+TE which contains the first two modules of DEBS1 fused to the thioesterase domain from DEBS3. Each module accounts for one polyketide chain extension and reduction cycle. The active sites are designated as follows: acyltransferases (AT), β-ketoacyl-ACP synthases (KS), acyl carrier protein (ACP), β-ketoreductase (KR), and thioesterase (TE). For further details, see references 5 and 13a.

Scheme 1. Conversion of (a) [1-^{14}C]Acetyl-CoA to **2** by DEBS 1+TE, (b) [1-^{14}C]Butyryl-CoA to **3** by DEBS 1+TE, and (c) (2*S*,3*R*)-2-Methyl-3-hydroxybutyryl-NAC thioester **4** to **2**



Scheme 2. DEBS 1+TE Catalyzed Biosynthesis of **5**



generated **2** was verified by addition of unlabeled C_8 lactone to the chromatographically purified product and recrystallization to constant activity.[17] It is thus evident that while DEBS 1+TE treats butyryl-CoA as an analog of the normal propionyl-CoA primer, the (2*S*,3*R*)-2-methyl-3-hydroxybutyryl-NAC thioester **4** is recognized as an analog of the diketide *intermediate*. Had **4** been treated instead as an analog of the primer and prematurely released following the first condensation/reduction cycle, an entirely different diastereomer of the C_8 triketide lactone would have resulted from the enzymatic incubation.

In the absence of NADPH, DEBS 1+TE converted [1-^{14}C]-propionyl-CoA and (2*RS*)-methylmalonyl coenzyme A into the known product 6-ethyl-2-hydroxy-3,5-dimethyl-2*H*-pyran-2-one (**5**)[18] in 20% yield, based on propionyl-CoA. The identity of this radiolabeled product was confirmed by dilution with authentic unlabeled carrier and repeated recrystallization to constant activity.[19,20] This observation demonstrates that the KS domain in module 2 can accept and extend an unreduced intermediate.[21]

Our results demonstrate vividly the tremendous potential of cell-free systems for the study of modular PKS structure and mechanisms. In particular, the relative simplicity and high activity of DEBS 1+TE makes it an attractive model system for the exploration of properties fundamental to all modular PKSs. The two modules of this minilactone synthase harbor ketosynthases giving rise to both D and L stereochemistry at methyl-bearing carbons as well as ketoreductases of both D and L stereospecificity. Given that DEBS can accept a variety of substrates as primers and intermediates, it should now be possible to make quantitative assessments of its substrate specificity by determination of the relevant steady state kinetic parameters and to probe subtle details of the features which determine molecular recognition of substrates and intermediates with unparalleled power. Finally, these results demonstrate that cell-free systems provide a completely novel route for the controlled synthesis of new polyketides which might otherwise not be accessible via *in vivo* engineered biosynthesis.

Acknowledgment. This work was supported by a grant from the National Institutes of Health (GM22172) to D.E.C. and in part by a grant from the National Institutes of Health (CA 66736-01), a National Science Foundation Young Investigator Award, and a David and Lucile Packard Fellowship for Science and Engineering to C.K. We would like to thank Ms. Hai Huang for preparation of diketide **4**.

Supporting Information Available: Autoradiogram depicting covalent modification of DEBS 1+TE by ^{14}C-labeled starter units in the absence of chain extension reactions and scheme depicting synthesis of **2** and **3** (3 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

JA9527636

(16) **2**: mp 56.0 °C; R_f 0.17 (1:1 EtOAc/hexane); ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.41 (dq, 1 H, J = 2.43, and 6.56 Hz, C_5-H), 3.79 (dd, 1 H, J = 4.30, and 10.21 Hz, C_3-H), 2.42 (dq, 1 H, J = 10.21, 7.08 Hz, C_2-H), 2.12 - 2.00 (m, 1 H, C_4-H), 1.38 (d, 3 H, J = 7.08 Hz, C_2-CH_3), 1.31 (d, 3 H, J = 6.57 Hz, C_5-CH_3), 0.95 (d, 3 H, J = 7.08 Hz, C_4-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.99, 76.03, 73.66, 39.38, 38.29, 18.06, 14.18, 4.43; $[\alpha]_D$ +113.4° (*c* 1.45, $CHCl_3$); HRMS (CI, isobutane) [M + H]$^+$, calcd *m/z* 159.1021, obsd 159.1019. **3**: mp 49.5 °C; R_f 0.31 (1:1 EtOAc/hexane); ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.19 (ddd, 1 H, J = 2.32, 5.02, 7.81 Hz, C_5-H), 3.78 (dd, 1 H, J = 4.30, 10.31 Hz, C_3-H), 2.43 (dq, 1 H, J = 7.08, 10.31 Hz, C_2-H), 2.16−2.05 (m, 1 H, C_4-H), 1.80−1.68 (m, 1 H, one of C_6-H_2), 1.56−1.42 (m, 2 H, one of C_6-H_2 and one of C_7-H_2), 1.37 (d, 3 H, J = 7.08 Hz, C_2-CH_3), 1.42−1.29 (m, 1 H, one of C_7-H_2), 0.92 (d, 3 H, J = 6.98 Hz, C_4-CH_3), 0.91 (t, 3 H, J = 6.92 Hz, C_8-H_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.80, 79.69, 73.82, 39.79, 37.19, 34.28, 18.65, 14.24, 13.79, 4.48; $[\alpha]_D$ +109.6° (*c* 1.23, $CHCl_3$); HRMS (CI, isobutane) [M + H]$^+$, calcd *m/z* 187.1334, obsd 187.1326.

(17) (a) Typical incubations were performed overnight at 28 °C in 150 mM phosphate buffer, at a protein concentration of 8 mg/mL. After extraction and purification by preparative TLC, labeled **2** (43 840 dpm), from incubation of [1-^{14}C]acetyl-CoA (15 μM, 54 nCi/nmol) with DEBS 1+TE, methylmalonyl-CoA, and NADPH, was mixed with 26.6 mg of **2** and recrystallized four times from ether/hexane to constant ^{14}C activity, 831 ± 6 dpm/mg. (b) Labeled **3** (13 684 dpm), from incubation of [1-^{14}C]-butyryl-CoA (100 μM, 4 nCi/nmol) with DEBS 1+TE, methylmalonyl-CoA, and NADPH, was mixed with 11.6 mg of **3** and recrystallized four times from ether/hexane to constant ^{14}C activity 1149 ± 13 dpm/mg. (c) A portion (30 600 dpm) of the partially purified labeled **2**, from incubation of **4** (64.5 μM) plus [^{14}C]methylmalonyl-CoA (8.87 μM, 56.4 nCi/nmol) with DEBS 1+TE and NADPH (120 μM), was mixed with 16.1 mg of **2** and recrystallized eight times from ether/hexane to constant ^{14}C activity, 89 ± 3 dpm/mg.

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(19) Labeled **5** (130 000 dpm), from incubation of [1-^{14}C]propionyl-CoA (4 μM, 51 nCi/nmol) with DEBS 1+TE and methylmalonyl-CoA in the absence of NADPH, was mixed with 36.9 mg of **5** and recrystallized four times from acetone/hexane to constant ^{14}C activity, 2749 ± 9 dpm/mg.

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