Origin of Starter Units for Erythromycin Biosynthesis†

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ABSTRACT: Modular polyketide synthases (PKSs), such as the 6-deoxyerythronolide B synthase (DEBS), are multifunctional proteins that govern the synthesis of a number of clinically important natural products. The modular arrangement of active sites within these enzymes suggests the possibility of a combinatorial approach to the synthesis of novel bioactive polyketides. The efficacy of combinatorial strategies toward altering the starter unit specificity of polyketide synthases critically depends on controlling the supply of competing endogenous starter acids. Using DEBS 1-TE, a bimodular derivative of DEBS, we aimed to determine whether the β-ketosynthase (KS) domain responsible for condensation in the first module also has the ability to prime its own biosynthesis by catalyzing the decarboxylation of methylmalonyl-CoA to produce propionyl-CoA. In contrast to earlier reports with a closely similar mini-PKS DEBS 1+TE, we have found that rigorously purified DEBS 1-TE does not catalyze the decarboxylation of methylmalonyl-CoA.

Complex polyketides are natural products that exhibit a wealth of important biological properties, including antibiotic, antiparasitic, immunosuppressive, and anticancer activities (*1*). These compounds are biosynthesized by polyketide synthases (PKSs[1]), which catalyze the repetitive condensation of simple monomers, in a process that resembles fatty acid biosynthesis (*2*, *3*). Although each PKS generates a single product or a small group of closely related products, PKS-derived compounds are structurally diverse. Variation between these molecules is derived from the way each PKS controls the number and type of units added, and the extent and stereochemistry of reduction at each cycle. In addition, the product of each PKS is frequently acted upon by post-synthase enzymes, including regiospecific glycosylases, methyltransferases, and oxidative enzymes, which introduce still greater diversity. The possibility of combining these functions through genetic engineering to produce a large number of novel compounds has generated considerable interest (*3*−*5*).

To determine the practicality of such combinatorial bioengineering, many studies have addressed the factors that control the ultimate structure of PKS products. These efforts have revealed that complex reduced polyketides, such as the macrolides and polyethers, are synthesized on multifunctional PKSs that contain a separate set, or module, of enzymatic activities for every round of chain extension (*6*−*10*). For example, the clinically important antibiotic erythromycin A is derived form propionyl-CoA and six molecules of (2*S*)-methylmalonyl-CoA (*11*), through the sequential action of six such sets of enzymes housed in three multienzyme polypeptides (DEBS 1, DEBS 2, and DEBS 3) (*12*) of the 6-deoxyerythronolide B synthase (DEBS) (Figure 1). In DEBS, as in all type I PKSs, each module determines the overall structure of its chain extension unit; that is, the functionality and stereochemistry are established by the joint action of multiple activities within the module. Attention has therefore been focused on the possibility of generating hybrid polyketides via the combination of suitable activities from different natural polyketide synthases.

An attractive target for combinatorial manipulation is the starter residue. There are several potential methods for altering the starter acid specificity of a particular polyketide synthase. First, the inherent flexibility of its loading domain may be exploited to introduce "non-natural" starter units. For example, although the loading domain of DEBS accepts only propionyl-CoA and acetyl-CoA in vivo, it tolerates *n*-butyryl-CoA and the branched starter isobutyryl-CoA in vitro (*13*). Remarkably, the loading module of the avermectin-producing polyketide synthase recognizes at least 44 different branched carboxylic acids or their derivatives when they are fed to *Streptomyces avermitilis* cells (*14*), and this broad specificity has been exploited to produce an avermectin analogue, doramectin (*15*), which is more active than the parent macrolide. Second, biosynthesis may be initiated directly through the KS domain of module 1 (KS1) in the absence of loading domains (*16*), potentially bypassing the strict specificity of the acyltransferase. However, a

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[1] Abbreviations: PKS, polyketide synthase; DEBS, 6-deoxyerythronolide B synthase; DEBS 1-TE, 6-deoxyerythronolide B synthase 1-thioesterase; AT, acyltransferase; ACP, acyl carrier protein; KS, β-ketoacyl synthase; KR, β-ketoacyl reductase; TE, thioesterase.

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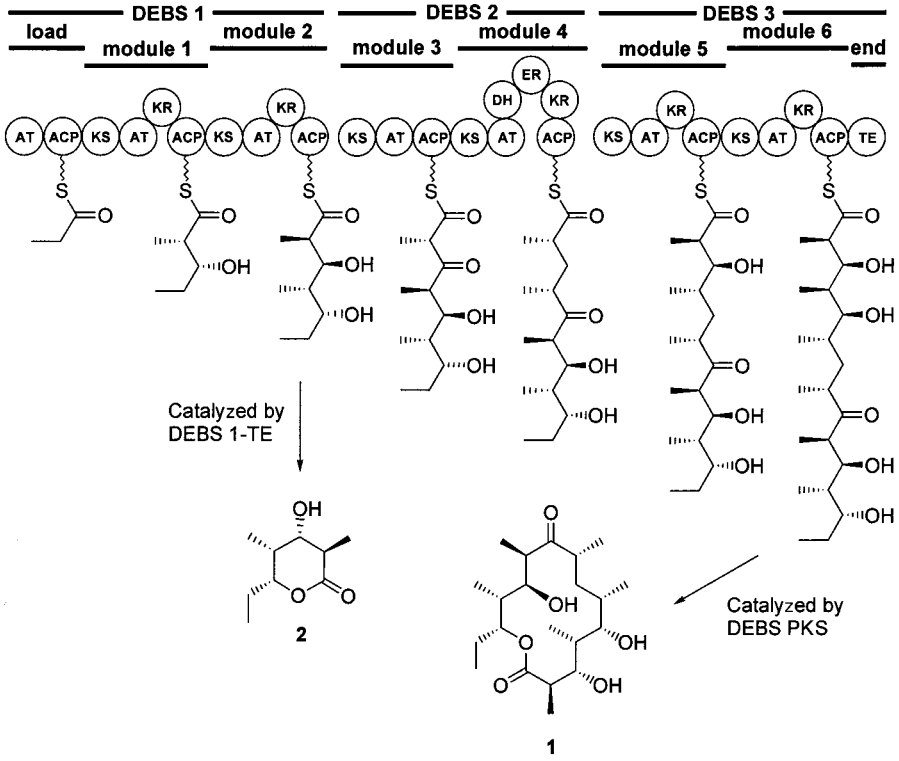

FIGURE 1: The erythromycin PKS consists of three giant multifunctional proteins, DEBS 1−3. The DEBS proteins are organized into modules, which catalyze the stereospecific condensation of an extender unit onto the growing chain, and set the level of reduction of the β-carbon of the resulting intermediate. DEBS 1 is fronted by a loading module, which initiates biosynthesis by transferring a propionate residue to the β-ketosynthase (KS) domain of module 1; DEBS 3 terminates in a thioesterase (TE) activity, which is responsible for formation of the macrolide ring, 6-deoxyerythronolide B **1**. DEBS 1-TE, which catalyzes the formation of the δ-lactone **2**, is a truncated version of DEBS, created by adding a copy of the TE domain to the end of bimodular DEBS 1.

mutant erythromycin PKS in which the initial AT and ACP domains had both been deleted produced a much lower level of erythromycins (0.5%) than was generated by the wild type protein (*16*). Even when the loading domains were provided in trans, the efficiency of erythromycin synthesis remained low (5%) (*16*), so direct loading of KS1 is not a practical strategy.

A third and more promising strategy is replacing the loading domains of one PKS with the comparable activities from another synthase. The viability of this approach was demonstrated for the erythromycin PKS, when its loading module was swapped with the comparable domains from the avermectin cluster (*17*). The resulting hybrid PKS produced a number of novel bioactive erythromycins, demonstrating that the broad specificity of the avermectin loading module can indeed be transferred to another modular polyketide synthase. Similarly, replacing the acetate-specific loading module of the spiramycin PKS with the propionate-specific counterpart from the tylosin PKS produced a hybrid 16-membered macrolide aglycone in good yield (*18*).

A fourth approach is precursor-directed biosynthesis, in which altered starter functionality is introduced through the incorporation of a synthetic non-natural diketide intermediate into the polyketide synthase (*19*−*21*). This strategy was used to produce analogues of the avermectins altered in the region of the starter unit (*22*) and more recently has been used successfully in the production of similarly altered erythromycin D analogues (*23*).

An important element for the success of these strategies is the suppression of incorporation of the natural starter unit or units, both in vivo and in vitro. For example, in the exploitation of the broad specificity of the avermectin loading domain (*24*), competition from the branched chain precursors normally present, isobutyryl-CoA and 2-methylbutyryl-CoA, was eliminated by carrying out the feedings of unnatural analogues using a mutant avermectin producer, defective in the ability to make branched chain acyl-CoAs (*14*, *25*). Similarly, the incorporation of synthetic polyketide intermediates in vivo is improved by eliminating competing biosynthesis from endogenous starter residues (*22*, *23*).

The control of starter unit supply in erythromycin biosynthesis may be an interesting special case, because the KS1 domain of DEBS has been reported to catalyze the decarboxylation of methylmalonyl-CoA to propionyl-CoA (*21*, *23*, *26*, *27*), in essence providing its own primer from the essential pool of chain extender units. The presence of an intrinsic methylmalonyl-CoA decarboxylase activity for KS1 was first reported for partially purified multienzyme DEBS 1+TE (5% of the total protein) (*26*). DEBS 1+TE is a truncated version of DEBS which catalyzes the formation of δ-lactone **2** (Figure 1) (*28*). More recently, the specific decarboxylase activity was reported to persist in purified DEBS 1+TE (*27*), without experimental details being given. As the authors pointed out, animal fatty acid synthase is known to catalyze an apparently analogous malonyl-CoA decarboxylation. The latter activity is stimulated by alkylation of the ketosynthase active site cysteine with iodoacetamide (*29*). If KS1 catalyzes endogenous decarboxylation of methylmalonyl-CoA, attempts to cut the supply of propionyl-CoA to erythromycin biosynthesis, by eliminating the enzyme or enzymes responsible for the alternative provision of propionyl-CoA, would be rendered futile.

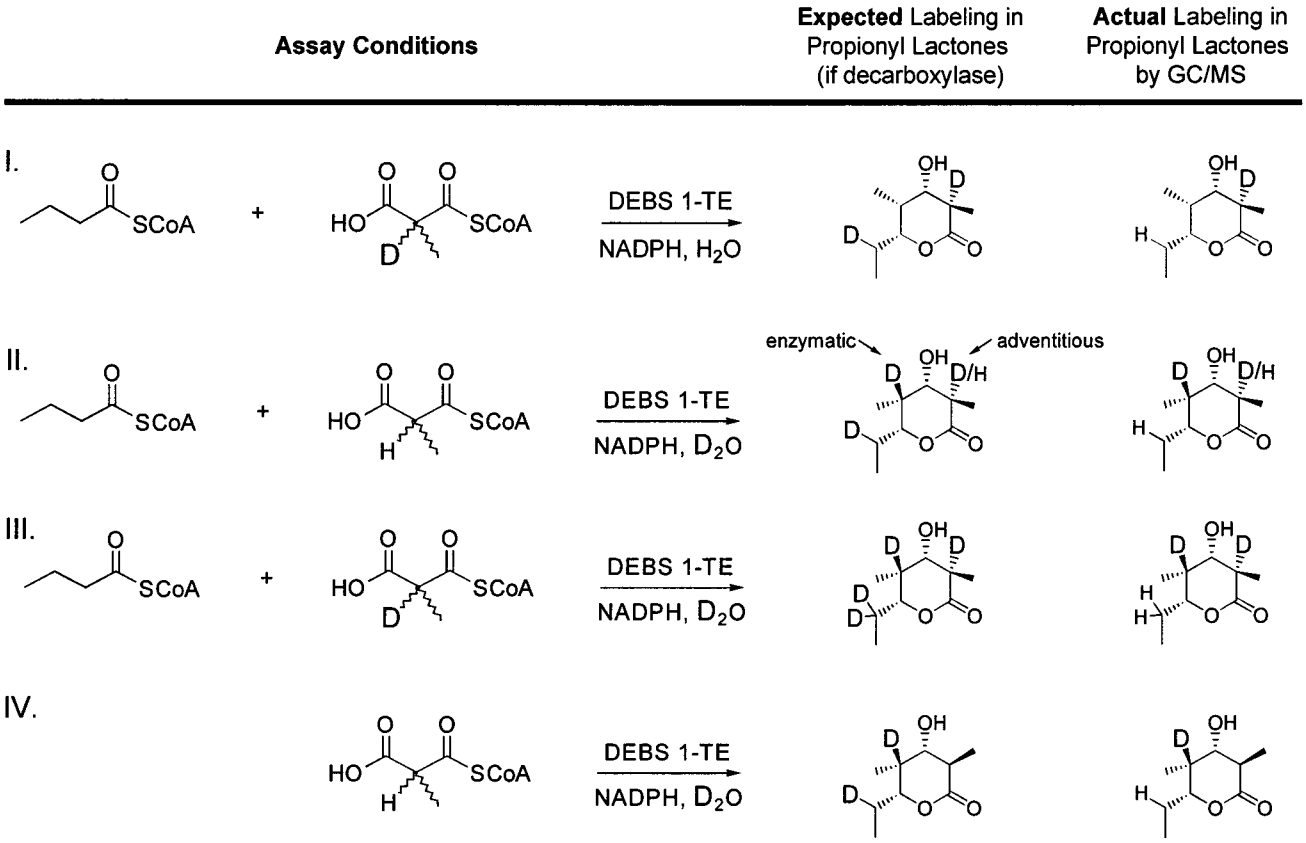

FIGURE 2: Design of assays for testing for decarboxylation of methylmalonyl-CoA catalyzed by DEBS 1-TE. We anticipated that a decarboxylative activity would incorporate deuterium into the starter unit of propionyl lactones, and that such labeling would be visible with GC−MS analysis. As decarboxylation has been reported in the presence of alternative primers, assays I−III included an *n*-butyryl-CoA starter unit. Assay IV was designed to evaluate the possibility that *n*-butyryl-CoA suppresses decarboxylation. GC−MS analysis gave no evidence for labeling of the side chain in any assay.

KS1 has already been shown to have three roles, transacylation of the growing polyketide chain from the acyl carrier protein (ACP) to its own active site cysteine residue (*30*), control of direct acylation (*16*), and decarboxylative condensation (*31*), and there is strong evidence for its ability to catalyze epimerization (*20*).

To gain mechanistic insight into this proposed additional role of the KS1 domain, we evaluated KS1-catalyzed decarboxylation in the context of purified DEBS 1-TE (*13*, *32*), a bimodular derivative of DEBS upon which DEBS 1+TE was originally modeled.

MATERIALS AND METHODS

Materials. Coenzyme A esters were purchased from Sigma and Fluka, and DL-2-[*methyl*-^{14}C]methylmalonyl-CoA was purchased from DuPont NEN. (2*RS*)-[2-^{2}H]Methylmalonyl-CoA was synthesized by deuteration of (2*RS*)-methylmalonyl-CoA using D_2O/1.0% v/v D-acetic acid (reaction judged to be complete by 600 MHz ^{1}H NMR) (*20*).

Synthesis of [2-^{2}H]-(3S,5R)-Dihydroxy-(2R,4R)-dimethyl-n-heptanoic Acid δ-Lactone (20). Synthetic (3*S*,5*R*)-dihydroxy-(2*R*,4*R*)-dimethyl-*n*-heptanoic acid δ-lactone (the synthesis of this compound will be given elsewhere) (2.1 mg, 12 μmol) was dissolved in 0.75 mL of CD_3OD, and 3.2 μL of diazobicycloundecane (3.3 mg, 21 mmol, 1.8 equiv) was added. After 24 h (reaction judged to be complete by 250 MHz ^{1}H NMR, >95% deuteration), the CD_3OD was removed in vacuo. The sample was dissolved in 10 mL of 1 M HCl and extracted with 3 × 10 mL of ethyl acetate, and the solvent was dried and removed in vacuo. The deuterated compound was purified on a 3 cm silica plug in ethyl acetate/hexane (4/1 v/v).

Growth of Cells, Preparation of the Extract, and Purification of DEBS 1-TE. Growth and harvesting of *Saccharopolyspora erythraea* JCB101 cells were carried out as described previously (*13*, *20*), and purification of DEBS 1-TE was performed essentially as described for the DEBS multienzymes from *S. erythraea* (*12*) and for overexpressed DEBS 3 in *Escherichia coli* (*33*).

Enzyme Assays. Incubations (8−24 h, 30 °C) were carried out in the presence of 600 μM methylmalonyl-CoA and 1.0 mM NADPH in 400 mM potassium phosphate. When used, *n*-butyryl-CoA was present at 300 μM and DL-2-[*methyl*-^{14}C]methylmalonyl-CoA (56.4 mCi/mmol) at 17 μM. Protein concentrations (80 ± 10 or 150 ± 20 μg/mL) were determined by the method of Bradford (*34*). Equal portions of the assays were removed at the following times: 30 min and 1.5, 3, 5, 8, and 24 h (for *n*-butyryl-CoA incubations only); the portions were extracted with 2 volumes of HPLC-grade ethyl acetate. Radiolabeled products were separated on TLC plates with diethyl ether as the mobile phase, and the plates were counted using a PhosphorImager (Molecular Dynamics) for 24−72 h. The counts were converted into disintegrations per minute using a propionyl triketide lactone standard with known radioactivity.

Gas Chromatography, Mass Spectrometry, and ^{1}H NMR Analysis. GC−MS analysis was carried out with chemical ionization (methane or ammonia as ionization gas) on a

Finnigan/MAT GCQ instrument, using an Annachem SGE BPXE 5% phenyl polysilphenylenesiloxane column (inner diameter, 0.22 mm; film width, 0.25 μM; and length, 25 m). The following temperature program was used to separate the *n*-propionyl and *n*-butyryl triketide lactones: 2 min at 70 °C, at 10 °C/min to 250 °C. The ^{1}H NMR spectra were acquired on a Bruker 250 or 400 MHz spectrometer in 100% $CDCl_3$.

RESULTS AND DISCUSSION

An intrinsic methylmalonyl-CoA decarboxylase activity was first proposed for DEBS 1+TE to account for the biosynthesis of propionyl triketide lactone in vitro in the absence of added propionyl-CoA; in these experiments, propionyl lactone production competed with synthesis of lactones from alternative *n*-butyryl-CoA or acetyl-CoA starter units (*26*). In assessing propionyl lactone biosynthesis by DEBS 1-TE, however, we considered three alternative explanations. (i) KS1 possesses an intrinsic decarboxylase activity [which is inactivated in a KS1° mutant (*21*, *23*)]. (ii) A decarboxylase is present in the crude cell extract, which is not specifically associated with DEBS, but which adventitiously provides the required propionyl-CoA starter units under the conditions of the assay, when partially purified enzyme is used; such a decarboxylase activity has been previously reported in *S. erythraea* (*35*), and conceivably, the recombinant *Saccharopolyspora coelicolor* used in the previous work with DEBS 1+TE contains an analogous enzyme (*21*, *26*−*28*). (iii) A decarboxylase activity is not present in the protein preparation, and instead, propionyl-CoA arises as a contaminant in the added methylmalonyl-CoA.

We assayed for a decarboxylase activity by conducting triketide lactone biosynthesis in the absence of added propionyl-CoA using purified DEBS 1-TE multienzyme, and under conditions where decarboxylation of methylmalonyl-CoA would incorporate deuterium into the resulting propionyl-CoA starter unit (Figure 2). The labeling pattern in the resulting propionyl-derived lactones (designated propionyl lactones) was then identified by gas chromatography−mass spectrometry (GC−MS) analysis.

In initial experiments, we chose to investigate propionyl lactone production under the conditions previously reported to encourage decarboxylation, using an alternative starter to prime biosynthesis (*26*). In an incubation of *n*-butyryl-CoA and (2*RS*)-[2-^{2}H]methylmalonyl-CoA in H_2O (pH 7.0 and 7.2), we anticipated that any propionyl lactone produced from the decarboxylation of methylmalonyl-CoA would incorporate at least one deuterium label (side chain), and the majority would contain two (side chain and C-2) (*20*). Analysis of the products, however, showed that >95% of propionyl lactones had only one deuterium (Figure 3), and the fragmentation pattern was identical with that of the synthetic C-2 deuterated standard. Similarly, in assays containing *n*-butyryl-CoA and (2*RS*)-[2-H]methylmalonyl-CoA in D_2O (pD 7.0 and 7.5), we anticipated that propionyl lactones produced via decarboxylation would incorporate a minimum of two deuteriums (side chain and C-4) and possibly an additional deuterium at C-2 through adventitious exchange of the label into methylmalonyl-CoA (*20*). In these assays, the propionyl lactones contained, according to GC−MS analysis, only one or two deuteriums, and had fragmentation patterns consistent with labeling at C-4 only, or at both C-4 and C-2. Again, the side chain was unlabeled. Furthermore, analysis of propionyl lactones from an assay in D_2O containing (2*RS*)-[2-^{2}H]methylmalonyl-CoA and *n*-butyryl-CoA in which four labels would be expected gave mass spectra consistent with incorporation of only two deuteriums, at C-2 and C-4 (Figure 4).

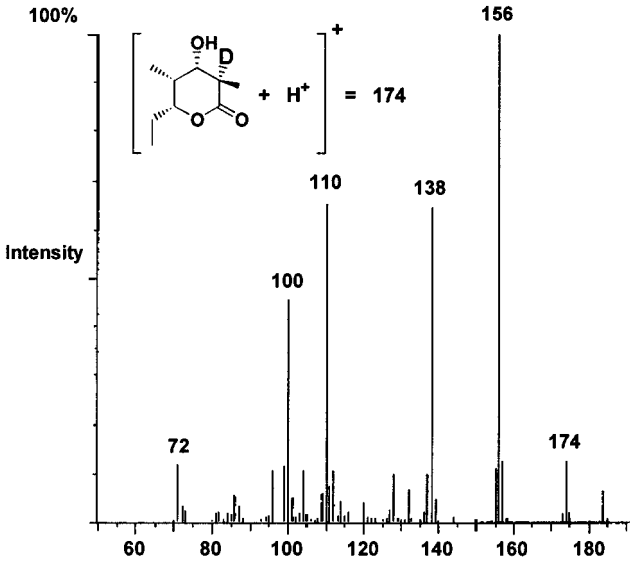

FIGURE 3: Mass spectrum of biosynthetic propionyl lactone produced in the presence of *n*-butyryl-CoA and [^{2}H]methylmalonyl-CoA in H_2O. Decarboxylation of [^{2}H]methylmalonyl-CoA to give propionyl-CoA would have produced deuterium-labeled starter. The resulting lactone would have a mass of 175, as a deuterium would also have been incorporated at C-2 during condensation. Instead, the lactone product had a mass of 174, and a fragmentation pattern consistent with this single deuterium being located at C-2. The intensity shown is background subtracted.

To rule out the possibility that decarboxylation is prevented by the presence of an alternative starter, we assayed (2*RS*)-[2-H]methylmalonyl-CoA in D_2O in the absence of any starter CoA thioester, and under conditions designed to minimize adventitious exchange of the deuterium label into the methylmalonyl-CoA (*20*). GC−MS analysis of the extracts showed that the propionyl lactone was formed and that it contained only a single deuterium label at C-4 (*20*).

Taken together, the GC−MS results pointed toward explanation iii, the presence of a propionyl-CoA contaminant in the methylmalonyl-CoA substrate. However, such a contaminant did not fully rule out the presence of a decarboxylase. It was still formally possible that the concentration of the propionyl-CoA contaminant was sufficiently high to inhibit the decarboxylation; such an effect on the putative decarboxylase activity has already been reported (*26*). To investigate this possibility, we monitored propionyl lactone formation in the absence of added propionyl-CoA; radioactivity was introduced in the form of [^{14}C]-(2*RS*)-methylmalonyl-CoA. In parallel experiments, we conducted lactone biosynthesis with propionyl-CoA present, to control for loss of enzyme activity over the assay period. Equal portions of the assays were removed at intervals (30 min and 1.5, 3, 5, and 8 h), and the ^{14}C-labeled products were separated on TLC plates and the plates counted using a PhosphorImager (Molecular Dynamics).

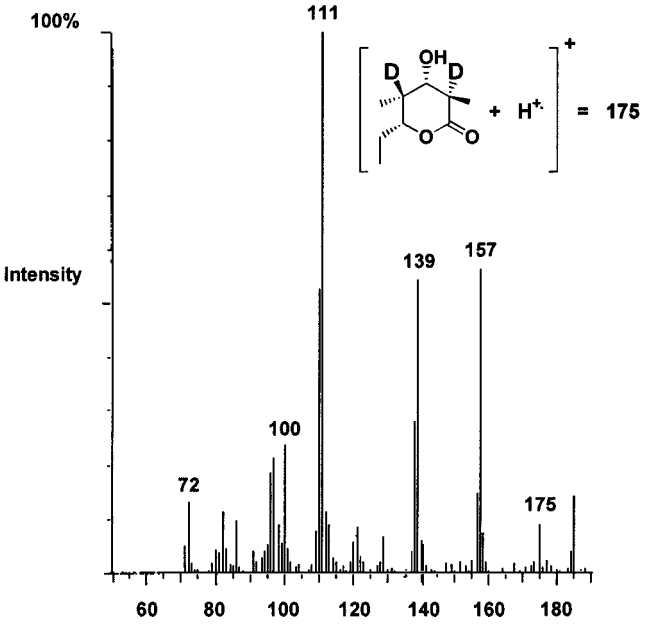

FIGURE 4: Mass spectrum of biosynthetic propionyl lactone produced in the presence of *n*-butyryl-CoA and [^{2}H]methylmalonyl-CoA in D_2O. Propionyl lactones produced via decarboxylation of [^{2}H]methylmalonyl-CoA were expected to contain four deuterium labels (mass of 177): two in the starter unit and one each at C-2 and C-4. With GC−MS analysis, the lactone product incorporated two deuterium labels (mass of 175) and had a fragmentation pattern consistent with labeling at C-2 and C-4 only. The intensity shown is background subtracted.

We anticipated that if purified DEBS 1-TE had no intrinsic decarboxylase activity, then in the absence of added propionyl-CoA lactone production would level off as the supply of contaminant starter was depleted. If however, the DEBS 1-TE preparation contained a decarboxylase, then lactone production was expected to continue throughout the course of the assay, as in the control incubation containing added propionyl-CoA. By PhosphorImager analysis, in the absence of added starter, lactone production occurred for 3−5 h and then ceased. However, with added propionyl-CoA, lactone product was biosynthesized through the 8 h assay. As lactone synthesis in the absence of starter failed to keep pace, it must have depleted a small pool of contaminant propionyl-CoA.

To confirm these results, assays were also carried out in the presence of *n*-butyryl-CoA and the products analyzed by GC−MS; the relative amounts of butyryl and propionyl lactones were calculated at the following time points: 30 min and 1.5, 3, 5, 8, and 24 h. In the absence of decarboxylase activity, we anticipated that the ratio of butyryl to propionyl lactones would increase over time, as the supply of propionyl-CoA was depleted. However, in the presence of a methylmalonyl-CoA decarboxylase, the enzyme should continually supply its own preferred starter unit once the initial pool was consumed, and the ratios of butyryl to propionyl lactones would remain constant. With GC−MS analysis, the proportion of butyryl lactones increased over time; whereas at 30 min the assay mixture contained an excess of propionyl lactone, by 24 h, the amount of butyryl lactone exceeded that of propionyl (Figure 5).

In a final experiment, we showed with 400 MHz ^{1}H NMR that the commercial sample of methylmalonyl-CoA (Sigma and Fluka, 90−95% pure) used in the assays contained sufficient amounts of propionyl-CoA (5−6%) to account for all of the observed propionyl triketide lactone production.

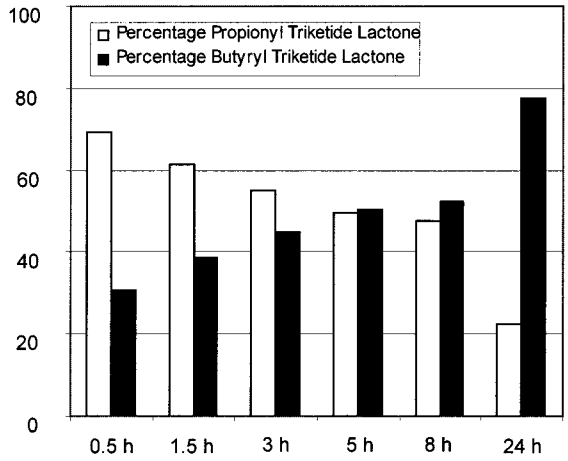

FIGURE 5: Time course for the relative amounts of propionyl and butyryl triketide lactones produced in the presence of *n*-butyryl-CoA, as determined by GC−MS. The increase in the percentage of butyryl triketide lactone over time demonstrated that the propionyl lactones must have arisen from an increasingly depleted pool of contaminant propionyl-CoA and that there was no methylmalonyl-CoA decarboxylase present capable of supplying additional primer.

Together, our results conclusively demonstrate that, in contrast to earlier reports with a similar bimodular PKS, DEBS 1+TE (*21*, *26*, *27*), fully purified DEBS 1-TE does not catalyze the decarboxylation of methylmalonyl-CoA in the absence of added propionyl-CoA. Although we cannot completely exclude the possibility that some damage occurs during purification that completely and specifically destroys such an activity, this seems less likely than the possibility that a contaminant decarboxylase has been removed more thoroughly than in previous work with the closely similar enzyme DEBS 1+TE (*26*, *27*). An active methylmalonyl-CoA decarboxylase has previously been characterized at least in *S. erythraea* (*35*). Because experimental details were absent from previous reports (*26*, *27*), we cannot comment further on the apparent contradiction between the two studies.

Our conclusions are completely consistent with recent results from Katz and co-workers (*16*). Using a DEBS construct that lacked loading domains, they demonstrated that the observed residual production of erythromycin A is likely due to direct acylation of KS1 by propionyl-CoA. They too found no evidence for the putative specific decarboxylase reported earlier. Together, these results imply that it should be possible to engineer an organism that lacks the ability to form propionyl-CoA, and thus erythromycin, by eliminating precursor enzymes [for example, the external methylmalonyl-CoA decarboxylase (*35*)], without risking damage to the synthetic operations of DEBS.

Finally, our results demonstrate that KS1°-blocked mutants of DEBS are unnecessary for in vitro experiments with purified enzymes.

SUPPORTING INFORMATION AVAILABLE

^{1}H NMR spectrum of commercially available methylmalonyl-CoA demonstrating its propionyl-CoA content (1 page). Ordering information is given on any current masthead page.

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