The Molecular Basis of Celmer's Rules: The Stereochemistry of the Condensation Step in Chain Extension on the Erythromycin Polyketide Synthase†

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ABSTRACT: Modular polyketide synthases (PKSs), for example, the 6-deoxyerythronolide B synthase (DEBS) responsible for synthesis of the aglycone core of the macrolide antibiotic erythromycin, generate an impressive diversity of asymmetric centers in their polyketide products. However, as noted by Celmer, macrolides have the same absolute configuration at all comparable stereocenters. Understanding how the stereochemistry of chain extension is controlled is therefore crucial to determining the common mechanism of action of these enzymes. We aimed to elucidate the molecular basis of Celmer's rules through *in vitro* studies with DEBS 1-TE, a bimodular derivative of DEBS from *Saccharopolyspora erythraea*, which uses (2*S*)-methylmalonyl-coenzyme A to produce both D- and L-methyl centers in its triketide lactone product. We show here that condensation of (2*S*)-methylmalonyl-CoA in module 2 proceeds with decarboxylative inversion without cleavage of the C−H bond adjacent to the methyl group; in contrast, in module 1 the chain extension process involves loss of the hydrogen attached to C-2 of the methylmalonyl-CoA precursor. The production of the D-methyl center in module 2 without loss of hydrogen from the asymmetric center of the (2*S*)-methylmalonyl-CoA establishes that condensation takes place with inversion of configuration as in fatty acid biosynthesis. The loss of the key hydrogen from the (2*S*)-methylmalonyl-CoA to produce the L-methyl center generated in module 1 implies that an additional obligatory epimerization step takes place in that module. The nature and timing of the epimerization remain to be established.

Modular type I polyketide synthases (PKSs)[1] such as DEBS are giant, multifunctional enzymes that catalyze the biosynthesis of structurally complex and clinically important polyketide natural products. The multiple active sites within these enzymes are organized into sets or "modules," where each module catalyzes the stereospecific condensation of an extender unit onto a growing polyketide chain and determines the level of reduction of the β-keto group of the resulting intermediate (*1*−6). In the two PKS clusters for which the complete gene sequence has been published, there is, for almost all modules, a convincing correlation between the predicted constituent domains and the structure of the corresponding chain extension unit in the growing polyketide chain (*7*−*10*). A correlation has also been noted between the primary protein sequence of the acyltransferase (AT) domains and the nature (malonate or methylmalonate) of the unit selected for chain extension (*11*). It is possible, therefore, to predict with a high degree of confidence, the structure of the polyketide chain given the composition of the PKS responsible for its assembly.

In contrast, there is no discernible character of the PKS that can be correlated with the stereochemistry of the chiral centers in chain extension units. In both erythromycin and rapamycin the methyl centers at C-2 and hydroxyl centers at C-3 generated adjacent to the acyl center of each newly added chain extension unit can have either the D or L configuration. This intrachain variation was first noted by Celmer in formulating his empirical rules, which drew attention to the contrasting strong position-specific homology between diverse macrolide (*12*, *13*). This uniformity suggests a common mode of action among the macrolide synthases, as would be expected from their modular genetic origins. The stereochemical course of the processes of chain extension is therefore a central question in polyketide biosynthesis, and understanding how the stereochemical outcome is controlled will greatly facilitate attempts to design hybrid polyketide antibiotics with particular functionality and stereochemistry.

An early attempt to address the stereospecificity of condensation in erythromycin involved feeding isotopically labeled precursors to whole cells in order to generate (2*R*)- and (2*S*)-methylmalonyl-CoA *in situ* (*14*). Although the experiments were hampered by low levels of incorporation,

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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; NAC, *N*-acetylcysteamine.

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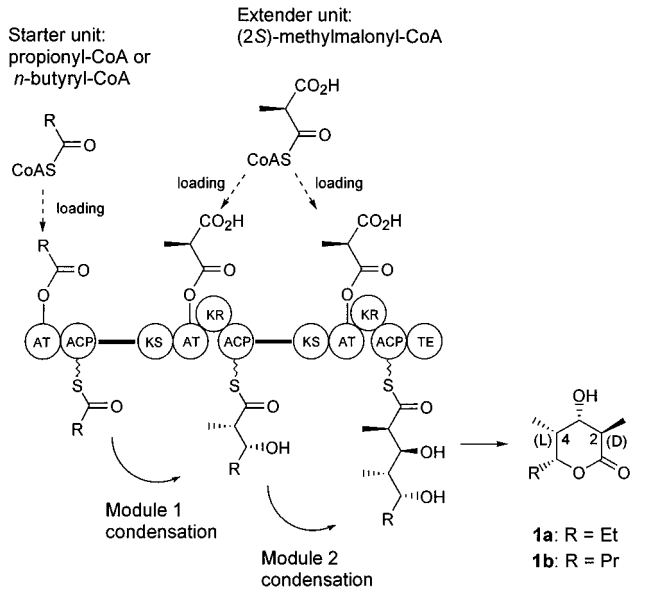


FIGURE 1: Operations to carry out two chain extension cycles by the "triketide lactone synthase", 6-deoxyerythronolide B synthase 1-thioesterase (DEBS 1-TE). DEBS 1-TE is organized into two modules which catalyze the stereospecific condensation of an extender unit onto the growing chain and set the reduction level of the β-keto group of the resulting intermediate. When supplied with NADPH and a suitable starter (*e.g.*, propionyl-CoA or *n*-butyryl-CoA) and the correct (2*S*)-stereoisomer of the chain-extending methylmalonyl-CoA, DEBS 1-TE produces the δ-lactones **1a** and **1b**, respectively.

there was evidence for labeling at C-2, C-4, and C-10 of the macrolide ring in the experiments where a precursor of (2*S*)-methylmalonyl-CoA was used. This result implicated the second, fifth, and sixth chain extension units as having arisen from (*S*)-methylmalonate, with inversion of configuration as found for fatty acid biosynthesis (*15*), but left the origin of the remaining units obscure. Attempts to incorporate labeled (2*R*)-methylmalonyl-CoA by feeding ethyl [2-$^2H_2$,2-^{13}C]-succinate, its direct precursor, were not successful.

The isolation and characterization of the DEBS proteins (*16*) has allowed polyketide chain extension to be investigated under more controlled conditions *in vitro*. Circumstantial evidence that chain extension involves exclusively (2*S*)-methylmalonyl-CoA at all sites was given by experiments which showed that acylation of all six AT domains is highly specific for the (2*S*) stereoisomer (*17*). Unambiguous confirmation that this preference is also exercised during chain extension came from turnover studies with isolated genetically engineered DEBS 1-TE (*18*). This mutant protein was created by adding a copy of the thioesterase (TE) domain to the end of bimodular DEBS 1 to effect chain release at the triketide stage giving the δ-lactone **1a** (*19*) (Figure 1). When supplied with NADPH and a suitable starter unit, such as propionyl-CoA, and (2*R*)-methylmalonyl-CoA for chain extension, the PKS did not produce product; in the presence of the (2*S*) isomer of methylmalonyl-CoA, product formation proceeded at a satisfactory rate, showing that both modules use this isomer. In the triketide lactone product the two methyl centers have opposite configurations. The proposal that one arises from (2*R*) and the second from (2*S*)-methylmalonyl-CoA was now firmly excluded. The studies confirmed the earlier acylation experiments for modules 1 and 2 (*20*), and it is therefore likely that all six modules employ (2*S*)-methylmalonyl-CoA for chain extension.

In the triketide lactone product of DEBS 1-TE, the methyl configuration at C-4 corresponds to condensation with retention of configuration of (2*S*)-methylmalonyl-CoA (catalyzed by module 1), and the configuration at C-2 to inversion of configuration (catalyzed by module 2) (Figure 1). There are four alternative explanations (mechanisms I−IV) for how a single enantiomer of methylmalonyl-CoA can give rise to opposite configurations at the methyl-bearing centers in the β-hydroxy intermediates: either (I) condensation occurs with retention of configuration of (2*S*)-methylmalonyl-CoA in module 1, and inversion of configuration in module 2 without epimerization at either center; (II) condensation occurs with retention of configuration at both centers, and includes an essential epimerization in module 2; (III) condensation occurs with inversion of configuration at both centers, and includes an essential epimerization in module 1; or (IV) condensation occurs with inversion of configuration in module 1, and retention of configuration in module 2, and includes an essential epimerization at both centers. In the first mechanism the stereochemistry at both methyl centers would be established directly by the condensation step. In mechanisms II and III one center would be established directly and one by epimerization. In mechanism IV, epimerase activity would be the agent of stereochemical control at both centers.

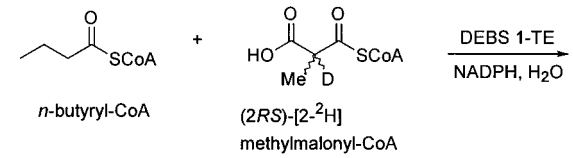
We hoped to distinguish between these possibilities by conducting triketide lactone biosynthesis with (2*S*)-[2-^{2}H]-methylmalonyl-CoA. Under ideal assay conditions, *i.e.* with no loss of deuterium through adventitious exchange, we expected one of the four following labeling patterns: retention of deuterium at both C-2 and C-4 methyl centers (mechanism I); retention of deuterium at C-4 only (mechanism II); retention of deuterium at C-2 only (mechanism III); or loss of deuterium at both C-2 and C-4 (mechanism IV) (Figure 2).

MATERIALS AND METHODS

Growth of Cells, Preparation of the Extract and Purification of DEBS 1-TE. Growth and harvesting of *Saccharopolyspora erythraea* JCB101 were carried out as described previously (*18*), and purification of the DEBS 1-TE was performed essentially as described for the DEBS multienzymes from *S. erythraea* (*16*) and for over-expressed DEBS 3 in *Escherichia coli* (*21*).

Synthesis of (2RS)-[2-^{2}H]Methylmalonyl-CoA. (2*R*,*S*)-[2-^{2}H]Methylmalonyl-CoA was synthesized by deuteration of (2*RS*)-[2-^{1}H]methylmalonyl-CoA using D_2O/1.0% v/v CD_3CO_2D (reaction complete as judged by 600 MHz ^{1}H NMR).

Synthesis of [2-^{2}H]-(3S,5R)-Dihydroxy-(2R,4R)-dimethyl-n-octanoic Acid δ-Lactone. Synthetic (3*S*,5*R*)-dihydroxy-(2*R*,4*R*)-dimethyl-*n*-octanoic acid δ-lactone (the synthesis of this compound will be given elsewhere) (2.4 mg, 13 μmol) (*18*) was dissolved in 0.75 mL of CD_3OD, and 3.2 μL of diazobicycloundecane (3.3 mg, 21 μmol, 1.7 equiv) was added. After 3.75 h (reaction complete as judged 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). ^{1}H NMR (600 MHz, $CDCl_3$) δ 4.22 (m,



n-butyryl-CoA (2*RS*)-[2-^{2}H] methylmalonyl-CoA

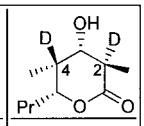
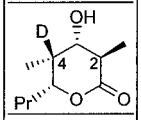
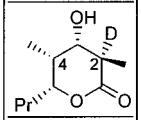
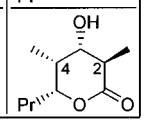
Mech.	Condense w/ retention	Condense w/ inversion	Epimeriz.(s)	Expected product
I	Module 1	Module 2	None	
II	Modules 1 + 2		Module 2	
III		Modules 1 + 2	Module 1	
IV	Module 2	Module 1	Modules 1 + 2	

FIGURE 2: Four alternative mechanisms for the generation of D- and L-methyl centers from (2*S*)-methylmalonyl-CoA by modules 1 and 2 of DEBS. We hoped to determine the correct mechanism by incubating DEBS 1-TE with (2*RS*)-[2-^{2}H]methylmalonyl-CoA *in vitro* and identifying the labeling pattern in the *n*-butyryl triketide lactone product.

1H), 3.82 (m, 1H), 2.12 (m, 1H), 1.72 (m, 2H), 1.44 (m, 2H), 1.41 (s, 3H), 0.97 (d, 3H), 0.96 (t, 3H).

Synthesis of (2S,3R)-2-Methyl-3-hydroxyhexanoyl-NAC Thioester. This compound was synthesized following the methodology of Evans (*22*−*24*). Full details of the synthesis will be given elsewhere.

Incorporation of (2RS)-[2-^{2}H]Methylmalonyl-CoA into n-Butyryl Triketide Lactones. Incubations (1.5 h, 30 °C) were carried out in the presence of 350 μM butyryl-CoA, 675 μM (2*RS*)-[2-^{2}H]methylmalonyl-CoA, 1.0 mM NADPH, in 400 mM potassium phosphate (pH 6.8−7.5), 1 mM EDTA. Protein concentrations (80 ± 10 μg/mL) were determined by the method of Bradford (*25*). For the NMR-scale experiment (5 h, 30 °C), the protein was concentrated by ammonium sulfate precipitation (50% saturation) to approximately 1 mg/mL, desalted on a PD-10 column (Pharmacia), and incubated with the above components at the stated concentrations. The reaction mixtures were quenched by extraction with two equal volumes of HPLC-grade ethyl acetate.

Incorporation of (2RS)-[2-^{1}H]Methylmalonyl-CoA and (2S,3R)-2-Methyl-3-hydroxyhexanoyl-NAC Thioester into n-Butyryl Triketide Lactones in the Presence of D_2O. Assays (1.5 h, 30 °C) were carried out in the presence of 350 μM butyryl CoA, 675 μM (2*RS*)-[2-^{1}H]methylmalonyl-CoA, 1.0 mM NADPH, in 400 mM D_2O potassium phosphate (pD 6.8−7.5/pH_{read} 6.4−7.1), 1 mM EDTA. When used, radioactivity was added in the form of [1-^{14}C]-*n*-butyryl-CoA (4.0 mCi/mmol, final concentration 67 μM) or DL-2-[*methyl*-^{14}C]-methylmalonyl-CoA (56.4 mCi/mmol, final concentration 6.9 μM). Protein concentrations (80 ± 10 μg/mL) were determined by the method of Bradford (*25*). For the incorporation of (2*S*,3*R*)-2-methyl-3-hydroxyhexanoyl-NAC thioester, the protein [∼1.5 mg/mL in 400 mM D_2O potassium phosphate (pD 7.7/pH_{read} 7.3), 1 mM ETDA] was incubated initially in the presence of 8.0 mM (2*S*,3*R*)-2-methyl-3-hydroxyhexanoyl-NAC thioester, 600 μM (2*RS*)-[2-^{1}H]methylmalonyl-CoA, and 8.0 mM NADPH. Methylmalonyl-CoA was added at 20 min (15 μL of 70 mM methylmalonyl-CoA) and 40 min (15 μL of 70 mM methylmalonyl-CoA), and half of the incubation mixture was removed and extracted with HPLC-grade ethyl acetate at 30 and 60 min, respectively.

Rate of Deuterium Exchange into Methylmalonyl-CoA. DEBS 1-TE was exchanged into D_2O assay buffer [400 mM potassium phosphate (pD 7.2/pH_{read} 6.8), 1 mM EDTA] to a final protein concentration of 80−100 μg/mL. A portion of the protein was inactivated by boiling for 10 min. *n*-Butyryl-CoA (350 μM), NADPH (1.0 mM), and then (2*RS*)-[2-^{1}H]-methylmalonyl-CoA (675 μM) were added to both protein samples. ^{1}H NMR analysis was performed on a Bruker 500 MHz spectrometer at 30 °C, with presaturation of the H_2O signal. The course of protium/deuterium exchange was followed via the methylmalonyl-CoA methyl doublet at δ 1.27 ppm. Spectra were acquired at 15 min intervals after addition of methylmalonyl-CoA into the assays. Deuteration at the methyl site generated a methyl singlet underneath the δ 1.26 ppm peak of the methyl doublet. The following formula was then used to calculate the percentage deuteration P of the methylmalonyl-CoA: $P = (A_{1.26ppm} - A_{1.28ppm})/(A_{1.26ppm} + A_{1.28ppm})$, where A = area of each peak as given by integration.

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 BPX5 5% phenyl polysilphenylene-siloxane column (inner diameter, 0.22 mm; film width, 0.25 μm; length, 25 m). The following temperature program was used to separate the *n*-propionyl and *n*-butyryl triketide lactones: 2 min at 70 °C, 20 min, 10 deg/min to 250 °C. MS/MS analysis was conducted at a collision energy of 50% (the optimal energy for fragmentation, as determined by control studies with synthetic C-2 deuterated *n*-butyryl triketide lactone). ^{1}H NMR spectra were acquired on Bruker 250, 400, 500, and 600 MHz spectrometers in 100% D_2O, CD_3OD, or $CDCl_3$.

RESULTS AND DISCUSSION

Design of the Experimental System. We chose to incubate purified DEBS 1-TE (*16*, *18*, *21*) with (2*RS*)-[2-^{2}H]methylmalonyl-CoA because it is known that only the (*S*)-isomer acylates the enzyme (*17*, *18*). We used *n*-butyryl-CoA rather than *n*-propionyl-CoA as the starter unit because we anticipated that deuterated *n*-propionyl-CoA would be produced *in vitro* by decarboxylation of (2*RS*)-[2-^{2}H]methylmalonyl-CoA (*26*, 27) and the resulting incorporation of deuterium into the propionyl starter group would complicate the analysis of deuterium levels at C-2 and C-4. The *n*-butyryl-derived lactones **1b** (designated *n*-butyryl lactones) could only incorporate deuterium at the C-2 and C-4 ring positions through chain extension reactions and could easily be separated from the small amount of propionyl-derived product **1a** produced as a consequence of decarboxylation of the methylmalonyl-CoA. The assays were conducted over a range of pH (6.8−7.5) in 400 mM phosphate buffer to maximize enzyme activity (*28*) while minimizing chemical epimerization of methylmalonyl-CoA, with concomitant loss

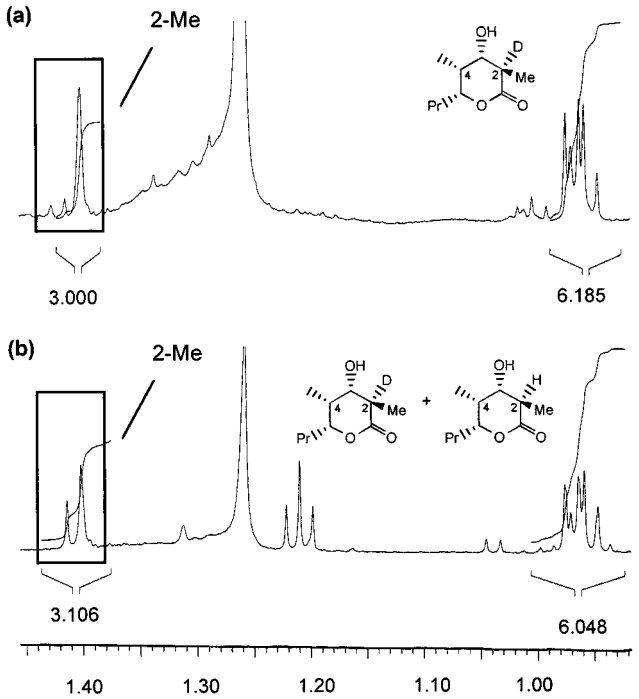


FIGURE 3: ^{1}H NMR (600 MHz) analysis of deuterium labeling in the *n*-butyryl lactone product of a (2*RS*)-[2-^{2}H]methylmalonyl-CoA/H_2O incubation. (a) ^{1}H NMR spectrum of biosynthetic *n*-butyryl lactone. Deuterium enrichment at C-2, calculated from the relative intensities of the methyl peaks at δ 1.40 and 1.42 ppm, is 90% ± 5%. (b) Synthetic *n*-butyryl lactones (1:1 mol/mol C-2 deuterated and undeuterated, at 10 μg/mL).

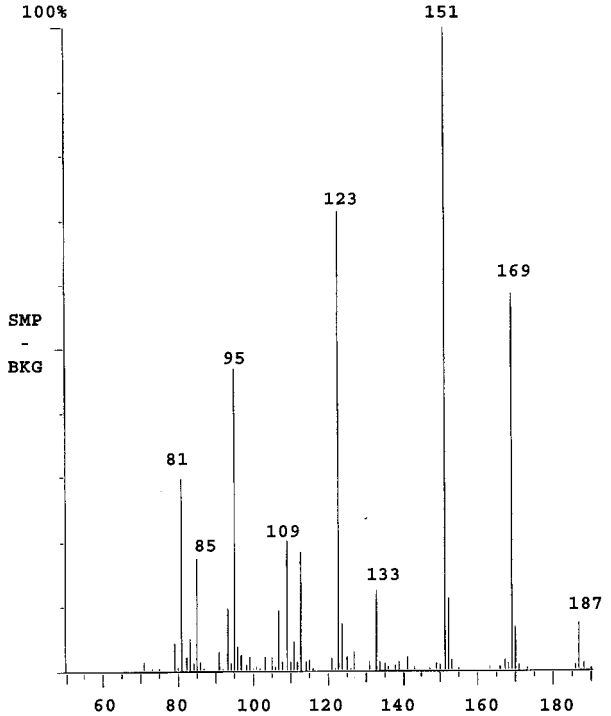


FIGURE 4: Mass spectrum of synthetic undeuterated *n*-butyryl triketide lactone under methane chemical ionization.

of the deuterium label (*29*). The incubations were quenched by extraction with ethyl acetate.

Analysis of Deuterium Labeling in n-Butyryl Triketide Lactones. The pattern of deuterium labeling at C-2 and C-4 was investigated by both ^{1}H NMR and gas chromatography/mass spectrometry (GC/MS). For maximum sensitivity in the NMR analysis we used ^{1}H NMR rather than ^{2}H NMR and looked for depletion of the ^{1}H NMR peaks arising from the C-2 and C-4 hydrogens, as indirect measurements of deuterium incorporation. In the ^{1}H NMR spectrum of synthetic *n*-butyryl triketide lactone (*18*), the chemical shifts for the C-2 and C-4 protons are δ 2.46 and 2.12 ppm, respectively. Integration of these signals relative to other proton signals in the spectrum of the labeled lactone gave a measure of the extent to which deuterium replaced protium at these positions. The measurement of deuterium enrichment at C-2 could be confirmed by observation of the signal pattern for the attached methyl group which appeared at δ 1.41 ppm (Figure 3). For molecules with a hydrogen at C-2, the methyl group appears as a doublet, $J = 7.1$ Hz, whereas for molecules with deuterium at that site, the methyl gives rises to a singlet, shifted upfield to δ 1.40 ppm. Although the singlet overlapped one of the doublet peaks, it was still possible to estimate the relative intensity of the two signals, using the well-resolved component of the doublet peak. The same check was not possible for the signals from the C-4 methyl group, due to overlap with the C-8 methyl triplet.

GC/MS/MS studies were also used to determine the relative amounts of deuterium at C-2 and C-4 based on fragmentation patterns. Under chemical ionization (CI) conditions and using methane as an ionization gas, synthetic unlabeled *n*-butyryl lactone showed ions at $[M + H]^+ =$ 187, 169, 151, 133, 123, 113, 109, 95, 85, and 81 (Figure 4). The following ions were identified in a control GC/MS study of synthetic mono-deuterated lactone, containing deuterium specifically at C-2 (as confirmed by ^{1}H NMR) $[M + H]^+ = 188$, 170, 152, 134, 124, 113, 110, 96, 95, 85, and 82. When key ions in the latter spectrum (170, 152, 124) were subjected to MS/MS, we observed enhanced $[M + 1 + H]^+$ peaks for some of the daughter ions relative to the undeuterated *n*-butyryl lactone, and $[M + H]^+$ peaks for others. The nature of the fragmentation leading to the daughter ions is not known, but the deuterium at C-2 is retained in the following fragments: 170, 152, 134, 124, 110, 96, and 82, and these can be used as diagnostic peaks for deuteration at C-2.

Both ^{1}H NMR and GC/MS/MS proved crucial to our analysis. ^{1}H NMR offered the advantage of giving a direct measure of the deuterium enrichments at C-2 and C-4, and so helped to validate our methods of MS/MS analysis via fragmentation patterns. A significant disadvantage of ^{1}H NMR is that it is a statistical technique which gives only the average distribution of ^{2}H at each site over all molecules in the sample. MS/MS allows a more specific analysis of deuterium labeling patterns, in which molecules carrying one deuterium can be analyzed independently of those carrying two. All samples were therefore routinely analyzed by the very sensitive GC/MS/MS technique, and ^{1}H NMR was used for a few important validation experiments which were run on a much larger scale.

Localization of Deuterium Label Incorporated into n-Butyryl Triketide Lactones by Purified DEBS 1-TE. In initial studies the reactions were carried out in H_2O at several pH values in the range pH 6.8−7.2, using (2*RS*)-[2-^{2}H]methylmalonyl-CoA as the substrate. It was found that at any pH, only singly-labeled (D1) and unlabeled (D0) *n*-butyryl lactones were present. At pH 7.2 MS analysis showed that 95% ± 5% of product molecules carried one deuterium. The fragmentation pattern of this labeled lactone exactly matched the pattern observed for the deuterated synthetic standard,

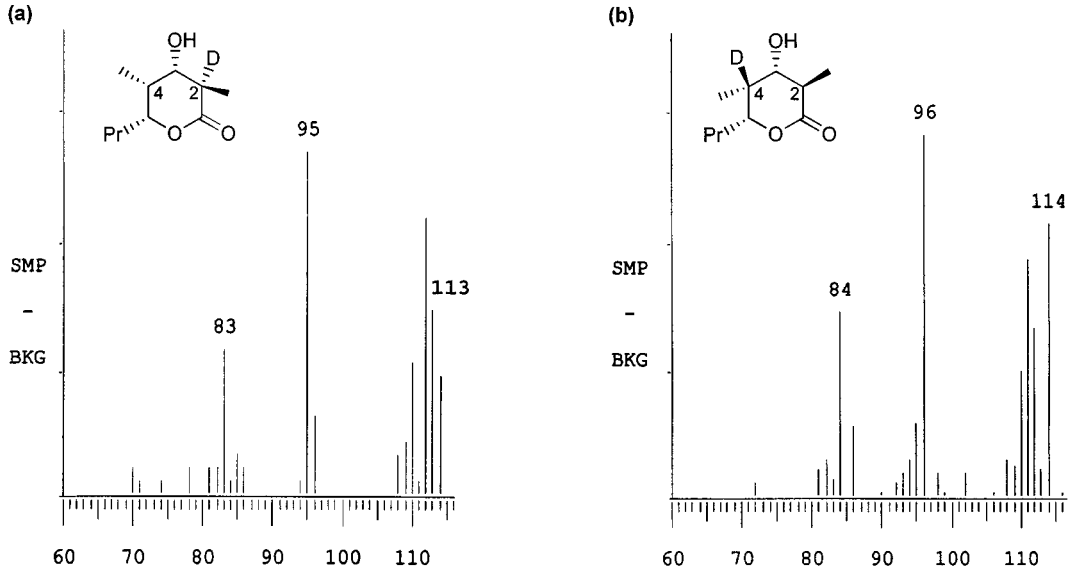


FIGURE 5: GC/MS/MS analysis of mono-deuterated *n*-butyryl triketide lactones from (a) D-methylmalonyl-CoA/H_2O incubation and (b) methylmalonyl-CoA/D_2O incubation. MS/MS was performed on the $[M + H]^+ = 170$ fragments of each sample, at 50% collision energy, under methane chemical ionization. The D1 species generated in D_2O has a significantly different fragmentation pattern than the C-2 deuterated D1 lactone produced in H_2O. This lactone therefore incorporates deuterium at C-4 exclusively, within detection limits.

consistent with the single deuterium being located at C-2. This result was checked by carrying out a larger scale reaction at pH 7.5 for a longer period, to obtain enough material for ^{1}H NMR analysis. The ^{1}H NMR spectrum also showed high levels of deuterium enrichment specifically at C-2 (90% ± 5%) (Figure 3). No evidence was found for significant amounts of deuterium at C-4, as judged by the relative integrals of the C-3, C-4, and C-5 protons.

Next, a complementary series of experiments was carried out in D_2O, using unlabeled (2*RS*)-methylmalonyl-CoA. When comparing experiments in H_2O and D_2O, equivalent conditions correspond to a difference of 0.4 pH units in the measured pH and the true pD values (*e.g.*, pH_{read} 7.0 is equivalent to pD 7.4) (*30*, *31*). These experiments were therefore conducted in the range pD 7.0−7.5 (pH_{read} 6.6−7.0). GC/MS analysis showed a significant portion of doubly-labeled species (D2), as well as D1, with double incorporation being greater at lower pD values. The products were subjected to MS/MS, which allowed analysis of D1-labeled molecules separately from D2. The D1 species gave a significantly different fragmentation pattern to the D1 compound generated in H_2O, which proved that there was no deuterium at C-2. For example, MS/MS analysis of the $[M + 1 + H]^+ = 170$ fragment for C-2 deuterated lactone gave daughter ions at $[M + H]^+ = 113$, 95, and 83, while fragmentation of a species of the same mass in the experiments conducted in D_2O gave peaks at $[M + 1 + H]^+ = 114$, 96, and 84 (Figure 5). Therefore, the D1-labeled molecules produced in D_2O incorporated deuterium at C-4 exclusively, within the limits of detection. The D2 molecules should be labeled at both C-2 and C-4, and the fragmentation patterns for the D2 lactone were consistent with this expectation.

Measurement of the Rate of Incorporation of Solvent into Substrate and Product During Catalysis by DEBS 1-TE. The observed deuteration at C-2 might be caused by chemical exchange into the methylmalonyl-CoA prior to loading onto the PKS. In order to measure directly the rate of incorporation of solvent deuterium into methylmalonyl-CoA, the DEBS 1-TE catalyzed reaction was monitored using 500 MHz ^{1}H NMR. The course of protium/deuterium exchange with substrate was followed at pD 7.2 (pH_{read} 6.8) via the methylmalonyl-CoA methyl doublet at δ 1.27 ppm rather than the quartet (δ 3.57 ppm) arising from the exchanging proton, as the proton signal was poorly resolved from the base line because of its low intensity and high multiplicity. Spectra were acquired at 15 min intervals after the addition of the methylmalonyl-CoA to the assay mixture. Deuteration at the methyl-bearing site generated a methyl singlet underneath the δ 1.26 ppm peak of the doublet, but again it was possible to integrate the two signals using the visible doublet peak and, therefore, to calculate the percentage deuterium at the methyl-bearing center.

Methylmalonyl-CoA is known to be relatively stable to epimerization at 0 °C during the short duration of these experiments (*29*), but at the higher temperature used (30 °C), incorporation of deuterium into the substrate was significant: protium/deuterium exchange followed first-order kinetics, giving 65% ± 5% deuteration of the methylmalonyl-CoA within the 1.5 h assay period. (2*RS*)-Methylmalonyl-CoA was used, but the chiral centers of the CoA moiety are remote from the site of exchange, so there should be no significant difference between the rate of deuterium exchange into the (2*S*) isomer and that measured for the total sample. Protein activity was not a factor in catalyzing this exchange, as a ^{1}H NMR experiment conducted in the presence of inactive protein produced the same result, within the limits of detection.

To correlate the deuteration levels of the substrate methylmalonyl-CoA and the product *n*-butyryl lactone, the time course of lactone formation was monitored under the assay conditions used in the ^{1}H NMR experiment; radioactivity was added in the form of either [^{14}C]-*n*-butyryl-CoA or [^{14}C]-(2*RS*)-methylmalonyl-CoA. The ^{14}C-labeled products were separated on TLC plates using diethyl ether as the mobile phase, and the plates were counted on a PhosphorImager (Molecular Dynamics) overnight. These counts were converted into dpm using a triketide lactone standard of known radioactivity. A parallel incubation was carried out in the absence of radiolabeled substrates, and samples of the

triketide lactone products were removed at 15 min intervals for GC/MS analysis.

The rate of synthesis of *n*-butyryl lactone was found to be linear during the 1.5 h assay period. The ratio of D2 to D1 produced as a result of chemical exchange in the substrate can therefore be calculated from the time course of deuterium incorporation into methylmalonyl-CoA. On the basis of this analysis, the following levels of deuteration at C-2 were predicted: 15 min (5.0%), 30 min (20%), 45 min (30%), 60 min (35%), 75 min (40%), and 90 min (44%). In order to observe the actual level of deuterium incorporation in the lactone products, the unlabeled assay extracts were again analyzed by GC/MS. Because the lactone fragmented readily under the original CI conditions, precluding accurate comparison of D1 and D2 molecular ion intensities, "softer" ammonia gas was used instead of methane as the ionization gas. Under ammonia CI, the molecular ions were observed at $[M + NH_4]^+$, with little fragmentation. In quantifying the level of D2 species present, allowance was made for equivalent ions arising from D1 molecules in the sample. This analysis showed that the actual level of deuteration at C-2 was significantly less (7−14%) than should have been observed on the basis of prior exchange into methylmalonyl-CoA. This discrepancy reflects the difficulty of calculating substrate exchange via incompletely resolved methyl signals. Clearly, however, all of the deuterium present at C-2 in the lactone product can be attributed to prior chemical exchange of the label into methylmalonyl-CoA, within these experimental errors.

Deuterium Incorporation at C-2 of an n-Butyryl Triketide Lactone Generated from a Diketide Intermediate and Methylmalonyl-CoA. To study the incorporation of solvent deuterium at C-2 independently of C-4, DEBS 1-TE was incubated with the *N*-acetylcysteamine (NAC) thioester of (2*S*,3*R*)-2-methyl-3-hydroxyhexanoic acid, prepared by an enantiospecific aldol condensation (*22*−*24*). This diketide already possesses the stereochemistry and functionality generated by module 1. We assumed that DEBS 1-TE would treat the substrate as an analog of its normal diketide intermediate, and subject it to one chain extension cycle as has been observed for other diketide analogs (*32*, *33*). The *n*-butyryl triketide lactone generated from this precursor and (2*S*)-[2-^{1}H]methylmalonyl-CoA in D_2O could then only incorporate deuterium from the solvent via exchange in module 2. To minimize deuterium exchange into the methylmalonyl-CoA, the assay was conducted with ∼1.5 mg/mL DEBS 1-TE at pD 7.7 (pH_{read} 7.3), and the methylmalonyl-CoA was replenished at intervals during the experiment; portions of the assay were quenched at 30 and 60 min. By GC/MS analysis, 90% ± 5% of the *n*-butyryl lactone product was produced without exchange at the C-2 position, confirming that methylmalonyl-CoA can be incorporated with its ^{1}H label intact. The observed level of deuterium incorporated from the solvent is no more than would be expected on the basis of exchange of the label into the methylmalonyl-CoA prior to loading onto the PKS.

The results in this paper, taken together, rule out alternative mechanisms in which epimerization occurs in the stereocenter created by module 2, C-2 of the triketide lactone (mechanisms II and IV, Figure 2). The fact that the C-H bond of the (2*S*)-methylmalonyl-CoA precursor is retained at this position proves that the ketosynthase domain in this module carries out the condensation with inversion of configuration.

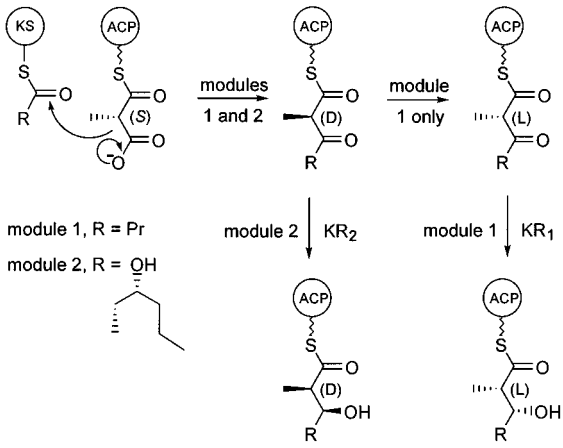


FIGURE 6: Stereochemistry of the condensation step in erythromycin biosynthesis. Our results with bimodular DEBS 1-TE *in vitro* have demonstrated that condensation of (2*S*)-methylmalonyl-CoA in module 2 proceeds with decarboxylative inversion without cleavage of the C−H bond adjacent to the methyl group. Our results are also consistent with the same stereochemistry of condensation, inversion, in module 1, followed by epimerization of the "wrong" D-methyl center to the correct L-methyl configuration in the β-keto intermediate.

This outcome has been observed for the equivalent reaction in fatty acid biosynthesis, underlying the mechanistic similarity between the two reaction pathways.

It is still formally possible that the stereochemistry of condensation catalyzed by the ketosynthase of module 1 is different (retention of configuration). However, this mechanism (mechanism I) would establish the L-configuration at C-4 directly without need for epimerization. Our results prove that the hydrogen at C-4 of the triketide is derived exclusively from water rather than the C-H bond of the methylmalonyl-CoA precursor, demonstrating that epimerization is obligatory in module 1. We note that mechanism III, condensation with inversion in both modules accompanied by an essential epimerization in module 1, would neatly account for the observed outcome (Figure 6).

SUPPORTING INFORMATION AVAILABLE

Syntheses of (3*S*,5*R*)-dihydroxy-(2*R*,4*R*)-dimethyl-*n*-octanoic acid δ-lactone and (2*S*,3*R*)-2-methyl-3-hydroxyhexanoyl-NAC thioester (12 pages). Ordering information is given on any current masthead page.

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