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Erythromycin Biosynthesis: Exploiting the Catalytic Versatility of the Modular Polyketide Synthase†

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Abstract—DEBS 1+TE is a recombinant modular polyketide synthase (PKS) in which the first two biosynthetic modules of the 6-deoxyerythronolide B synthase are linked to the thioesterase domain normally found at the *C*-terminus of DEBS 3. Incubation of DEBS 1+TE with propionyl-CoA, methylmalonyl-CoA, and NADPH gives the triketide lactone (2*R*,3*S*,4*S*,5*R*)-2,4-dimethyl-3,5-dihydroxy-*n*-heptanoic acid δ-lactone (**2**), the cyclized form of the normal triketide chain elongation product of DEBS 1. In order to probe the molecular recognition features of the PKS and to explore its synthetic versatility, [2,3-$^{13}C_2$]-(2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-NAC thioester (**3**), an analogue of the normal diketide chain elongation intermediate, and (2*RS*)-methylmalonyl-CoA were incubated with DEBS 1+TE, leading to the formation of the predicted labeled triketide ketolactone [4,5-$^{13}C_2$]-**8**, as established by ^{13}C NMR analysis and comparison with spectra of synthetic **8**. This stereoselective conversion illustrates the potential of using modular PKSs as multifunctional catalysts for the enzymatic synthesis of novel polyketides.

Introduction

The widely used broad spectrum antibiotic erythromycin A is representative of a large class of pharmaceutically active polyketide natural products.[1,2] The parent erythromycin macrolactone, 6-deoxyerythronolide B (**1**, 6-dEB), is biosynthesized by a modular polyketide synthase, known as DEBS, which has been shown to be composed of three large (ca. 3000 amino acids) multidomain proteins, DEBS 1, DEBS 2, and DEBS 3.[3] Genetic analysis has established that each protein consists of a group of catalytic domains, clustered into a pair of distinct functional units or modules, each of which contains the requisite set of active sites for one of six cycles of chain elongation and reduction of the resulting β-ketoacyl thioester.[4,5] During the course of the entire biosynthetic sequence leading to formation of 6-dEB, the various chain-elongation intermediates are never released from the synthase, but remain covalently bound as acylthioesters to the acyl carrier protein (ACP) or ketosynthase (KS) domains of the relevant biosynthetic modules. The biosynthetic sequence is presumably initiated by loading of a propionyl primer derived from propionyl-CoA onto the first module, catalysed by an acyltransferase (AT) domain at the *N*-terminal end of DEBS 1,[6,7] while macrolactonization and release of the 6-dEB product is catalysed by a thioesterase (TE) located at the *C*-terminus of DEBS 3.

The modular model for 6-dEB biosynthesis, which has been substantiated by extensive precursor incorporation[8] and molecular genetic experiments,[4,5,9] also appears to be applicable to the broad class of complex, reduced polyketides in general, as indicated by recent investigations of the oleandomycin,[10] avermectin,[11] and rapamycin[12] gene clusters. Recently, we reported the development of an expression system for recombinant DEBS,[13] and demonstrated that the resultant protein, containing at least 28 distinct active sites, could catalyse the cell-free formation of 6-dEB from propionyl-CoA, (2*RS*)-methylmalonyl-CoA, and NADPH.[14] (Scheme 1.) We also reported that a hybrid protein, DEBS 1+TE,[15] consisting of the first two biosynthetic modules, corresponding to DEBS 1, fused to the TE domain, derived from the *C*-terminus of DEBS 3, supports the in vitro conversion of propionyl-CoA, (2*RS*)-methylmalonyl-CoA, and NADPH to a triketide lactone, (2*R*,3*S*,4*S*,5*R*)-2,4-dimethyl-3,5-dihydroxy-*n*-heptanoic acid δ-lactone (**2**), the cyclized form of the normal triketide chain elongation product of DEBS 1[14,16,17] (Fig. 1 and Scheme 1). Since the DEBS 1+TE protein catalyses two full cycles of polyketide chain elongation and β-ketoacyl ester reduction, resulting in the generation of both D- and L-hydroxy functions and the introduction of both D- and L-methyl groups, this recombinant modular protein serves as a convenient vehicle for the study of the far more elaborate parent DEBS system. In the course of detailed examination of the specificity and kinetics of the reactions catalysed by DEBS 1+TE, we showed that in the presence of (2*RS*)-methylmalonyl-CoA and NADPH, DEBS 1+TE could also convert (2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-NAC thioester

†This paper is dedicated to Professor Sir Alan R. Battersby and Professor A. Ian Scott, recipients of the 1995 Tetrahedron Prize.
Key words: cell-free biosynthesis, modular polyketide synthase, erythromycin, diketide, triketide.

(**3**), a known diketide chain elongation intermediate,[8d,e] to the same triketide lactone **2**.[14]

Intriguingly, although DEBS is known to have an absolute specificity for (2*S*)-methylmalonyl-CoA,[6] we observed that DEBS 1+TE has a relaxed specificity both for the primer substrate and for chain elongation intermediates.[7] Thus DEBS 1+TE could not only be covalently acylated by both acetyl- and butyryl-CoA, in addition to the natural primer propionyl-CoA, but each primer analogue could be turned over catalytically as well, leading to the synthesis of **4** and **5**, respectively, the C_8 and C_{10} analogues of **2**, in comparable overall efficiency (Scheme 2). Competition experiments established a 32-fold preference in V_{max}/K_m for the propionate over the acetate primer but, surprisingly, only a 7.5-fold preference for the propionate over the butyrate primer.[17] A similar catalytic flexibility was also evident in the ability of DEBS 1+TE to recognize and process analogues of the natural chain elongation intermediates. For example, incubation of DEBS 1+TE with (2*S*,3*R*)-2-methyl-3-hydroxybutyryl-NAC thioester (**6**), an analogue of the diketide intermediate that would be derived from an acetyl primer, in the presence of (2*RS*)-methylmalonyl-CoA and NADPH led to formation of the expected C_8 lactone **4**.[7] It was also found that DEBS 1+TE could process unreduced chain elongation intermediates, as illustrated by the conversion of propionyl-CoA and (2*RS*)-methylmalonyl-CoA in the absence of NADPH to the pyranone **7**.[7,17] The latter findings were consistent with the results of earlier genetic experiments reported by Katz that established that a *Saccharopolyspora erythraea* mutant with a deletion engineered in the ketoreductase domain of module 5 produced the corresponding 5-oxo derivative of the erythronolide lactone.[5]

In order to exploit rationally the biosynthetic potential of DEBS 1+TE, we wish to probe further the catalytic specificity and synthetic versatility of this intriguing catalyst. It occured to us that incubation of DEBS 1+TE with methylmalonyl-CoA and the reduced diketide intermediate **3** in the absence of NADPH might provide access to the ketolactone **8** (or its enollactone tautomer **9**), an analogue that is intermediate in oxidation state between the pyrone **7** and the reduced triketide lactone **2**. We now report the successful achievement of this goal.

Results

An authentic sample of the requisite ketolactone **8** was conveniently prepared from the triketide lactone **2** itself. Initial attempts to oxidize **2** with the Swern reagent[18] led to a mixture of the pyrone **7** (30%), unreacted starting material (40%) and unidentified side products (30%), while treatment with Dess–Martin periodinane[19] gave only small amounts of unidentified products. On the other hand, reaction of **2** with 1.2 equiv of methyl(trifluoromethyl)dioxirane[20] for 2 h at 4 °C in CH_2Cl_2 gave the desired ketolactone (Scheme 3). The ^1H and ^{13}C NMR spectra of the crude product indicated the presence of a mixture of the ketolactone **8** and variable amounts of the corresponding enollactone tautomer **9**, with the precise ratio depending on the NMR solvent. For example, in acetone-d_6 the dominant form was **9**, whereas in $CDCl_3$ the usual ratio of **8**:**9** was ca. 1.6:1. Both ^1H and ^{13}C NMR of the crude oxidation product indicated that **8** thus isolated was initially present as a single diastereomer. The configuration of the C-2 methyl substituent was assigned to the kinetically and thermodynamically favored equatorial orientation. On one occasion, the less stable axial epimer of **8** was obtained as part of the crude reaction mixture, along with both **8** and **9**, but it rapidly rearranged to the more stable epimer. Preparative TLC and excision of a UV-active band, R_f 0.30, allowed isolation of essentially pure **8** in overall 80% yield. Treatment of **8** with dilute CF_3CO_2D directly in the NMR tube led to equilibration with the enol tautomer **9** over 15–30 min.[21] The high yield and the regioselectivity of the reaction of **2** with methyl(trifluoromethyl)dioxirane is notable. The oxidation of the hydroxyl function[20b] to a β-keto carbonyl function is accompanied by neither the formation of ring-opened

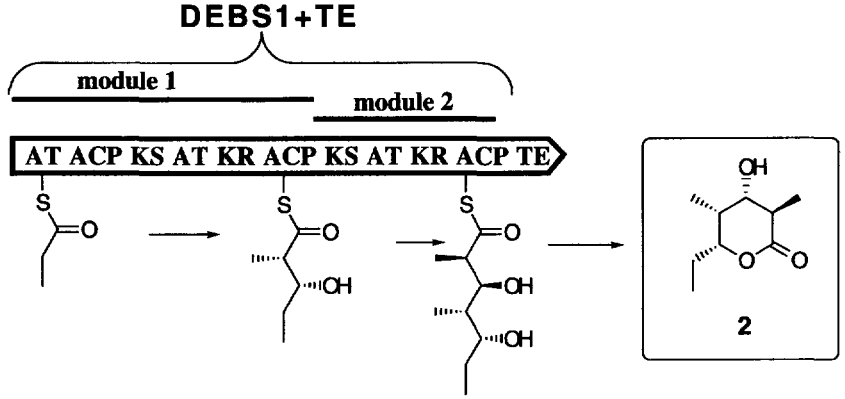
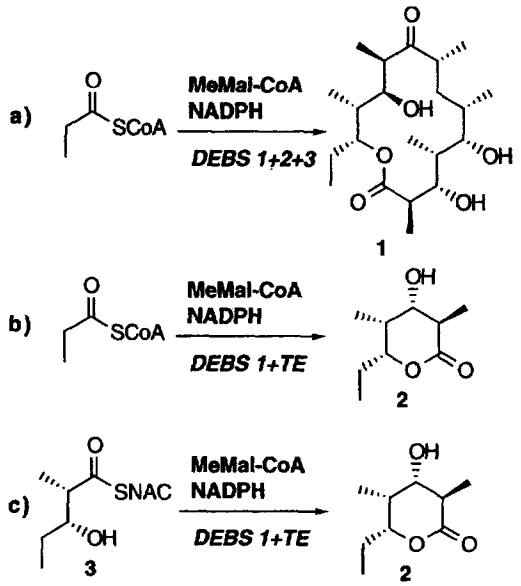


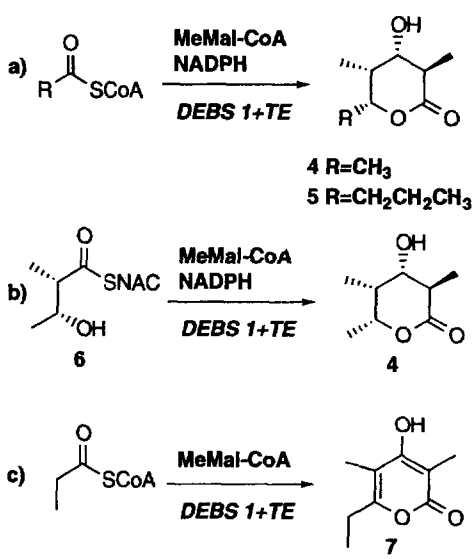
Figure 1. Modular model for the biosynthesis of **2** by DEBS 1+TE, containing the first two modules of DEBS 1 fused to the thioesterase domain from DEBS 3. The individual domains are designated as follows: acyltransferase (AT), β-ketoacyl-ACP synthase (KS), acyl carrier protein (ACP), β -ketoreductase (KR), and thioesterase (TE).



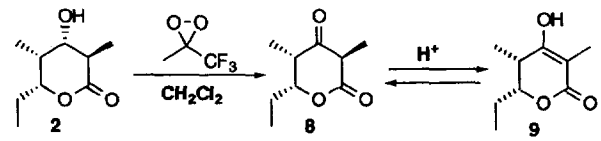
Scheme 1. Conversion of (a) propionyl-CoA, (2*S*)-methylmalonyl-CoA (MeMal-CoA), and NADPH to 6-deoxyerythronolide B (**1**) by DEBS 1+2+3; (b) propionyl-CoA, MeMal-CoA, and NADPH to **2** by DEBS 1+TE and (c) (2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-NAC thioester (**3**), MeMal-CoA, and NADPH to **2** by DEBS 1+TE.

products nor O-atom insertion into the δ-lactonic carbinyl C—H bond. By contrast, dioxirane oxidation of medium ring ethers (THP and THF) is known to lead to formation of the corresponding lactones.[20c]

For the preparative enzymatic incubation, [2,3-$^{13}C_2$] - (2*S*,3*R*) - 2 - methyl - 3 - hydroxypentanoyl - NAC thioester (**3**), prepared as previously described,[8d,e] and (2*RS*)-methylmalonyl-CoA were incubated for 12 h at 28 °C with partially purified DEBS 1+TE.[7,14,17] Analysis of the crude extract by ^{13}C NMR revealed, in addition to signals due to residual substrate and minor contaminants in the starting material, a new pair of doublets. Purification of the product by TLC and excision of the band with the appropriate R_f gave labeled ketolactone [4,5-$^{13}C_2$]-**8**, as evidenced by the appearance of a pair of enhanced and coupled doublets, J_{CC} = 36.2 Hz, centered at 78.52 (C-5) and 44.4 (C-4) ppm, respectively (Scheme 4).



Scheme 2. DEBS 1+TE-catalysed conversion of (a) Acetyl-CoA or Butyryl-CoA, MeMal-CoA, and NADPH to **4** or **5**, respectively; (b) (2*S*,3*R*)-2-methyl-3-hydroxybutyryl-NAC thioester (**3**), MeMal-CoA, and NADPH to **4**; and (c) propionyl-CoA and MeMal-CoA to **7**.

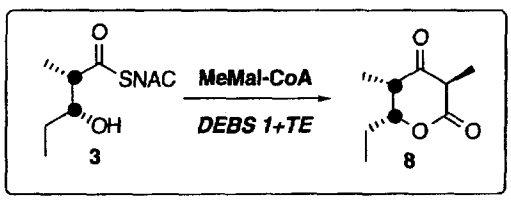


Scheme 3. Oxidation of triketide lactone **2** to ketolactone **8** and tautomerization of **8** to **9**.

Discussion

Conclusions

Over the past decade the tools of molecular biology have had a major impact on the use of enzymes in organic synthesis. Several complex molecules, such as vitamin B_{12}[22] and sialyl Lewis^x,[23] have been synthesized on milligram (and higher) scales with impressive yields. Although a few of the participant enzymes are known to have relatively broad specificities, the narrow overall selectivity of multistep enzymatic cascades derived from primary metabolism precludes their use as versatile multifunctional catalysts. In contrast, the potential of secondary metabolic pathways for the biosynthesis of 'unnatural' natural products is already well-documented.[24] Polyketide synthases appear to be notable in this regard. The structural complexity of their products, together with their remarkable tolerance towards altered substrates and advanced intermediates, makes these multienzyme assemblies attractive targets for exploitation in chemo-enzymatic synthesis. Here we have demonstrated this potential by using a chemically synthesized diketide thioester as a substrate for PKS-catalysed synthesis of a novel product. In the process, new insights into the molecular recognition properties of DEBS have been gained. Future studies along this direction will not only result in defining the precise stereoelectronic features of the substrate-binding site(s) within modular PKSs, but could also lead to the enzymatic synthesis of libraries of polyketide analogues with considerable medicinal potential.[25]



Scheme 4. DEBS 1+TE-catalysed conversion of (2*S*,3*R*)-2-methyl-3-hydroxybutyryl-NAC thioester (**3**) and MeMal-CoA to **4**.

Experimental

General methods

Reagents, chemicals, and buffers were obtained or prepared as previously described.[7] The recombinant strain of *Streptomyces coelicolor* CH999/pCK12, stored and cultivated as previously described, was used as a source of DEBS 1+TE.[7,15] All other biochemical methods for enzyme assay and protein quantitation were as previously described,[7] except as noted below. Proton (400 MHz) and ^{13}C NMR (100.4 MHz) spectra were acquired on a Bruker AM-400 spectrometer.

Isolation and partial purification of DEBS 1+TE

Mycelial pellets from *S. coelicolor* CH999/pCK12, prepared as previously described,[7] were resuspended in Buffer A (250 mM sodium phosphate pH 7.1, 0.3 M sodium chloride, 2 mM DTT, 1 mM benzamidine, 2 mM EDTA, and 30% glycerol) and disrupted by sonication (five bursts of 30 s, Branson Sonifier) at 0 °C. The lysate was centrifuged for 1.5 h at 192,000 × *g* and the supernatant was collected. Nucleic acids were precipitated with polyethylenimine (0.2%) and removed via centrifugation (20 min at 23,100 × *g*). The protein solution was saturated with ammonium sulfate to 50% and precipitated overnight. After centrifugation (30 min, 31.100 × *g*), the pellet containing the DEBS proteins was redissolved in buffer B (200 mM sodium phosphate pH 7.1, 2 mM DTT, 2 mM EDTA, and 10% glycerol) and desalted on a Sephadex G25 column using buffer B.

Enzymatic synthesis of [4,5-$^{13}C_2$]-(2*R*,4*S*,5*R*)-2,4-dimethyl-3-oxo-5-hydroxy-*n*-heptanoic acid δ-lactone (8). The desalted DEBS 1+TE preparation was adjusted to a total protein concentration of 8 mg/mL. Judging from densitometry scanning,[7] the DEBS concentration was estimated at 1 μM. DEBS 1+TE solution (4 mL) was incubated with 3 mg of [2,3-$^{13}C_2$]-(2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-NAC thioester (**3**), prepared as previously described,[8d,e] and 3 mg of (2*RS*)-methylmalonyl-CoA for 12 h at 28 °C. Subsequently, the solution was saturated with sodium phosphate and extracted with 3 × 4 mL ethylacetate. Preparative TLC (SiO_2, 1:1 ethyl acetate:hexanes) and excision of the band with an R_f (0.30) identical to that of a reference sample of synthetic ketolactone gave purified [4,5-$^{13}C_2$]-**8**, which was analysed by ^{13}C NMR: δ 78.52 (J = 36.24 Hz, C-5), 44.41 (J = 36.20 Hz, C-4).

(2*R*,4*S*,5*R*)-2,4-Dimethyl-3-oxo-5-hydroxy-*n*-heptanoic acid δ-lactone (8). The triketide lactone **2** (62 mg, 0.36 mmol), prepared as previously described,[16] was stirred at 4 °C in 2 mL CH_2Cl_2 while freshly prepared methyl(trifluoromethyl)dioxirane[20a] (0.26 M, 1.65 mL, 0.43 mmol, 1.2 equiv) was added. The solvent and reagent were removed by rotary evaporation and the resultant colorless oil was subjected to purification by TLC (1:1, ethyl acetate:hexanes) to give 49 mg (80%) of **8** (UV active spot, R_f 0.30). **8**: ^1H NMR (400 MHz, $CDCl_3$): δ 4.66 (ddd, J = 3.02, 5.30, 8.34 Hz, 1H, H-5), 3.62 (q, J = 6.66 Hz, 1H, H-2), 2.63 (dq, J = 3.02, 7.50 Hz, 1H, H-4), 1.83–1.95 (m, 1H, H-6a), 1.60–1.72 (m, 1H, H-6b), 1.37 [d, J = 6.66 Hz, 3H, C(2)-CH_3], 1.12 [d, J = 7.53 Hz, 3H, C(4)-CH_3], 1.08 (t, J = 7.41 Hz, 3H, H-7); ^{13}C NMR (assignments made by HETCOSY; (100 MHz, $CDCl_3$): δ 205.49 (C-3), 170.40 (C-1), 78.53 (C-5), 50.38 (C-2), 44.41 (C-4), 24.05 (C-6), 9.90 (C-7), 9.69 (C-4-CH_3), 8.25 (C-2-CH_3); HREIMS: calcd for $C_9H_{14}O_3$: *m/z* 170.0943, found: 170.0940. Analysis of a 1.6:1:0.5 mixture of **8**, **9**, and *epi*-(2*S*)-**8** allowed assignment of the ^1H NMR spectra of *epi*-(2*S*)-**8** and **9**. *epi*-(2*S*)-**8**: ^1H NMR (400 MHz, $CDCl_3$): δ 4.43 (dt, J = 4.45, 10.10 Hz, 1H, H-5), 3.23 (q, J = 7.47 Hz, 1H, H-2), 2.80 (dq, J = 4.62, 7.10 Hz, 1H, H-4), 1.5–1.7 (m, 2H, H-6), 1.46 [d, J = 7.50 Hz, 3H, C(2)-CH_3], 1.15 [d, J = 7.13 Hz, 3H, C(4)-CH_3], 1.05 (t, J = 7.26 Hz, 3H, H-7); **9**: ^1H NMR ($CDCl_3$): δ 4.32 (ddd, J = 3.27, 6.20, 8.11 Hz, 3H, H-5), 2.43 (dq, J = 3.26, 7.15 Hz, 1H, H-4), 1.75 [s, 3H, C(2)-CH_3], 1.5–1.7 (m, 2H, H-6), 1.13 [d, J = 7.15, 3H, C(4)-CH_3], 0.98 (t, 3H, J = 7.39 Hz, H-7); IR (**8** + **9**, 1.6:1): 3213 (br), 1728, 1652 cm^{-1}; (**8**+**9**, 1.6:1): $[\alpha]_D$ −83.9° (*c* 0.56, acetate).

Acknowledgments

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