Dissecting the Evolutionary Relationship between 14-Membered and 16-Membered Macrolides

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Modular polyketide synthases (PKSs) 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. However, while the intermediates in fatty acid biosynthesis are fully reduced to generate unfunctionalized alkyl chains, the intermediates in polyketide biosynthesis are processed to varying degrees, giving rise to complex patterns of functional groups. Additional complexity arises from the incorporation of different starter and chain extension substrates, from the generation of stereogenic centers, and from further chemical modifications such as lactonization and glycosylation.

To control the biosynthesis of these complex molecules, modular polyketide synthases such as 6-deoxyerythronolide B synthase (DEBS)[2] and tylactone synthase (TS)[3] utilize a separate set of active sites for each condensation step. The biosynthesis of the 14-membered lactone 6-deoxyerythronolide B (6-dEB, **1**) 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 (Figure 1). Tylactone (**2**) is formed via seven condensation steps, carried out by the seven modules of TS. It has been shown in several cases that synthetic analogues of biosynthetic intermediates will enter the pathway of a modular PKS at the appropriate active site and be processed by the downstream active sites to yield a polyketide natural product.[4−7] To better understand the nature of this substrate specificity, we engineered a mutant of DEBS which can be used to assay the incorporation of "unnatural" substrates. We now report that, in at least one case, the configuration at a single stereocenter controls the uptake of a substrate molecule, directing it to either module 2 or module 3 of DEBS.

We recently described the development of a generally applicable, fermentation-based strategy in which chemically synthesized, cell-permeable, nonnatural precursors are transformed into novel, natural product-like molecules by a genetically engineered PKS.[8] DEBS KS1° carries an inactivating mutation in the ketosynthase domain of module 1. Because it is unable to synthesize the first (diketide) intermediate (**1a**), no 6-dEB (**1**) is produced. However, when provided with a synthetic analogue of this intermediate (**3**), DEBS KS1° efficiently converts the diketide substrate to 6-dEB (**1**) (Figure 2).

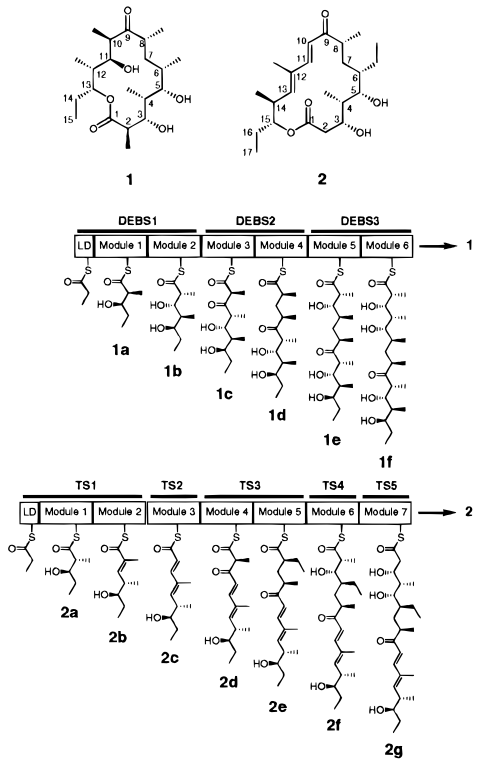

Figure 1. Biosynthesis of the macrolides 6-dEB (**1**) and tylactone (**2**). Genetic models of the two synthases are shown. DEBS consists of three large polypeptides, each of which contains two "modules". Each module catalyzes the addition of a single methylmalonyl-CoA extender unit along with any reductive steps. The enzyme-bound intermediates (**1a**−**1f**) which are generated by the corresponding six modules of DEBS are shown. The final linear intermediate (**1f**) is cyclized to form 6-dEB (**1**), a 14-membered lactone. Biosynthesis of tylactone (**2**) utilizes seven modules to generate the 16-membered lactone product.

During our initial studies of this system, we investigated the incorporation of substrate **4** by DEBS KS1°. This substrate corresponds to the triketide intermediate in the biosynthesis of tylosin (**2b**). Although the olefin functionality present in this substrate is found in the products of many modular PKSs, no such functional group is found in 6-dEB (**1**). However, we suspected that the methyl and hydroxyl groups at the 4- and 5-positions might be adequate for **4** to be recognized as a triketide substrate for module 3 of DEBS. Surprisingly, DEBS KS1° converted this substrate into lactone **5**, suggesting that **4** is directed to module 2 of DEBS and undergoes five extension cycles to yield an octaketide product.[8]

In addition to the presence of an olefin, substrate **4** differs from the biosynthetic intermediate **1b** in the stereochemistry at C-4. To examine the role of C-4 stereochemistry, we prepared substrate **6** in which the C-4 center is of the *R* configuration.[7] This substrate was fed to growing cultures of CH999/pJRJ2, and polyketide products were extracted from the growth media, as described previously.[8] The major product obtained after chromatography (2 mg obtained from 1 L of culture) was identified as 10,11-

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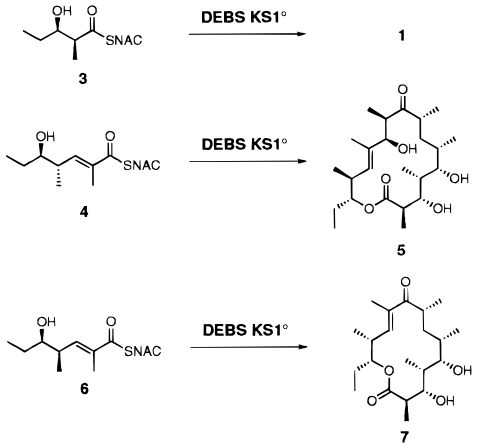

Figure 2. Conversion of diketide and triketide substrates by DEBS KS1°. DEBS KS1° contains an inactivating mutation in the ketosynthase domain of module 1. Biosynthesis of intermediate **1a** is blocked, but polyketide biosynthesis may proceed if a suitable substrate for one of the downstream modules is provided. For example, diketide substrate **3** which corresponds to intermediate **1a** is a substrate for module 2 and is elaborated to 6-dEB (**1**). The α,β-dehydrated triketide **4** was previously shown to be converted to octaketide lactone **5**, suggesting that it also enters the biosynthetic pathway at module 2. Triketide **6**, which differs from substrate **4** only by the stereochemistry at C-4, enters primarily via module 3 and is converted into 10,11-anhydro-6-dEB (**7**).

anhydro-6-deoxyerythronolide B (**7**) by NMR and mass spectrometry.[9] A minor product tentatively identified as a 16-membered lactone analogous to **5** was observed at low levels (<0.2 mg/L culture).[10] The observation that **7** is the major product suggests that the altered stereochemistry at C-4 directs substrate **6** to module 3, where it undergoes four subsequent extension reactions to afford heptaketide **7**.

The substrate specificity of modular PKSs is currently an area of considerable interest. Tolerance of unnatural intermediates has been widely observed in genetically engineered PKSs, leading to the production of novel polyketides.[11] Although yields of **7** are modest, these experiments provide further demonstration of the utility of precursor-directed biosynthesis as a method for generating derivatives of complex molecules. The ability to generate new derivatives at C10−C11 of erythromycin[12] may provide a useful route to the investigation of new pharmaceuticals since this region of the molecule is of particular medicinal interest.[13] Substrates which bear reactive functional groups such as olefins might be used to introduce "handles" for the synthetic modification of products.

The fact that the configuration at the C-4 stereocenter of substrates **4** and **6** directs these substrates to different modules in DEBS may reflect an evolutionary relationship between modular PKSs. Inspection of Figure 1 shows that the functions of DEBS modules 2−6 map closely onto the corresponding modules 3−7 of TS. Evolutionary conservation of this "core group" of modules in many PKSs would explain the stereochemical regularities noted by Celmer[14] and would suggest that the evolutionary divergence between DEBS and TS (and, more generally, between the 16-membered lactone synthases and the 14-membered lactone synthases) includes loss of (or addition of) an "early" module. A common feature of the 16-membered macrolides is the configuration at C-14 (derived from the *S*-configuration found at C-4 of intermediate **2b** or substrate **4**).[15] Our results suggest that this stereocenter plays a key role in the direction of substrate **4** to the "correct" module (module 3) of TS[4] and to the evolutionarily related module 2 of DEBS. Substrate **6**, which has the opposite stereochemistry at C-4, is not recognized as a substrate for DEBS module 2. It is instead directed to module 3, presumably due to its similarity with intermediate **1b** (identical functionality and configuration at C4 through C7). Extension of these genetic and chemical techniques should allow the specificity of individual modules in DEBS and other PKSs to be probed in detail. This will most likely lead to a better understanding of the evolutionary relationships between PKS modules, as well as provide valuable information for the genetic engineering of polyketide pathways.

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Supporting Information Available: Tables of ^{1}H and ^{13}C NMR data, including comparisons of **7** and **1** (3 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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(9) The crude extract from 1.0-L agar plates was initially purified on silica gel (1 × 6 cm silica gel, 35% EtOAc/hexanes). The major product was further purified by a second silica gel column (1 × 10 cm silica gel, gradient of 15 to 25% EtOAc/hexanes) to afford approximately 2 mg of lactone **7**. Physical data for **7** (refer to Figure 1 for numbering): ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.92 (t, 3H, J = 7.4 Hz, H15), 1.01 (d, 3H, J = 7.1 Hz, C6-Me), 1.03 (d, 3H, J = 7.1 Hz, C4-Me), 1.05 (d, 3H, J = 6.6 Hz, C8-Me), 1.06 (d, 3H, J = 7.1 Hz, C12-Me), 1.20−1.31 (m, 1H, H7b), 1.32 (d, 3H, J = 6.8 Hz, C2-Me), 1.52−1.62 (m, 2H, H7a, H14b), 1.73−1.82 (m, 1H, H14a), 1.74 (d, 3H, J = 1.1 Hz, C10-Me), 1.76−1.86 (m, 1H, H4), 2.02−2.10 (m, 1H, H6), 2.64−2.78 (m, 1H, H12), 2.77 (dq, 1H, J = 10.6 Hz, 6.8 Hz, H2), 3.08 (dq, 1H, J = 6.5 Hz, 2.8 Hz, H8), 3.97−4.00 (m, 2H, H3, H5), 5.09 (ddd, 1H, J = 9.4 Hz, 4.6 Hz, 1.6 Hz, H13), 6.46 (d, 1H, J = 9.2 Hz, H11); ^{13}C NMR (100 MHz, $CDCl_3$) δ 7.0 (C4-Me), 10.3 (C15), 11.3 (C8-Me), 12.0 (C12-Me), 14.0 (C10-Me), 14.9 (C2-Me), 16.5 (C6-Me), 25.1 (C14), 35.2 (C6), 36.6 (C4), 36.7 (C7), 37.8 (C8), 39.9 (C12), 43.9 (C2), 76.0 (C5), 77.2 (C13), 78.9 (C3), 134.6 (C10), 143.1 (C11), 176.4 (C1), 205.5 (C9). HRMS (FAB$^+$, NBA/NaI) calcd for $(C_{21}H_{36}O_5)Na^+$ 391.2460, found 391.2454. R_f = 0.65 (50% ethyl acetate/hexanes).

(10) A minor component isolated during the first chromatography appears to correspond to an octaketide lactone. This product was not present in sufficient amount or purity for thorough NMR characterization (<0.2 mg/L culture), but HRMS supports the assignment of a structure analogous to lactone **5**: HRMS (FAB$^+$, NBA/NaI) calcd for $(C_{24}H_{42}O_6)Na^+$ 449.2879, found 449.2865. R_f = 0.75 (50% ethyl acetate/hexanes).

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(12) To determine whether lactone **7** is a substrate for the "post-polyketide" enzymes which convert 6-dEB to erythromycin, we provided **7** as a substrate to a growing culture of *Saccharopolyspora erythrea* A34,[16] as described previously.[8] This mutant of the natural producer of erythromycin carries an inactivating mutation in the DEBS genes but retains all of the enzymes (glycosylases, oxygenases, and methylase) necessary for conversion of 6-dEB to erythromycin. Products **1** (1.7 mg) and **7** (1.0 mg) were dissolved in 0.5 mL of EtOH. Each was spread onto a single R2YE plate and allowed to dry. A control plate was spread with 0.5 mL of EtOH. *S. erythrea* A34 was applied so as to give lawns. After 8 days of growth, the plates were extracted three times with 1.5% Et_3N in ethyl acetate. The extracts were concentrated and dissolved in 1.4 mL of EtOH. Filter discs were soaked in these ethanolic solutions, dried under vacuum, and placed onto freshly plated lawns of *Bacillus cereus*.[17] After overnight incubation at 37 °C, zones of clearing were evident for the 6-dEB (**1**) and 10, 11-anhydro-6-dEB (**7**) conversion extracts but not for the control extract, suggesting that **7** is converted into an erythromycin analogue with antibacterial activity. Mass analysis suggests conversion of **7** to the corresponding erythromycin C analog: MS (APCI$^+$) 701 (M$^+$).

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