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# Biosynthesis of 2-Nor-6-deoxyerythronolide B by Rationally Designed Domain Substitution

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Type 1 modular polyketide synthases (PKSs) catalyze the biosynthesis of structurally diverse polyketides possessing a wide range of biological activities.<sup>1,2</sup> These multifunctional enzymes are arranged in a modular fashion, with each module containing the activities responsible for one cycle of polyketide chain extension and  $\beta$ -keto group reduction. The apparent use of each enzyme activity in the PKS only once during biosynthesis suggests that novel polyketides can be generated by genetic manipulation of the individual activities. Further, the modularity of the PKSs has led to the idea that the concepts of combinatorial chemistry can be translated into "combinatorial biosynthesis" through rearrangement of the genetic modules.<sup>3,4</sup>

The erythromycin PKS of *Saccharopolyspora erythraea*, 6-deoxyerythronolide B synthase (DEBS; Figure 1) consists of six modules and catalyzes the biosynthesis of 6-deoxyerythronolide B (**1**) by extension of a propionyl starter unit with six methylmalonyl extender units.<sup>5,6</sup> Several novel analogs of **1** have previously been produced through inactivation of reductive domains in DEBS using chromosomal mutagenesis in *Sac. erythraea*.<sup>6,7</sup> The establishment of a heterologous expression system for DEBS in *Streptomyces coelicolor* CH999<sup>8</sup> has further allowed demonstration of gain-of-function<sup>9</sup> and change of extender unit specificity<sup>10</sup> through domain substitution in a truncated model system. The production of rationally-designed macrolactones through domain substitution has yet to be demonstrated, however. We describe here the production of designed macrolactones **2** and **3** through replacement of the acyltransferase in module 6 of DEBS with a heterologous malonyl-specific acyltransferase.

Plasmid pKOS015-22 was constructed by replacement of the DEBS module 6 acyltransferase with the module 2 acyltransferase from the rapamycin PKS<sup>11</sup> (Figure 2) and used to transform *S. coelicolor* CH999. A culture of the transformant was found to produce **2** (15 mg/L) and **3** (10 mg/L) upon

LC/MS spectrometric analysis. <sup>1</sup>H and <sup>13</sup>C NMR analysis of the purified compounds revealed the expected absence of signals for the 2-methyl group of **1** as well as shifts in the remaining resonances consistent with the proposed structures.<sup>12</sup> To confirm the origin of the last extender unit, the culture was grown in the presence of [1,2-<sup>13</sup>C]acetate to label malonyl-CoA. <sup>13</sup>C NMR analysis of the labeled **2** and **3** revealed enrichment at carbons 1 and 2 in both molecules, with  $J_{CC}$  = 56.2 and 56.4 Hz, respectively.

To demonstrate complete production of antibacterial agents using this technology, about 4 mg of the purified **2** was fed to a culture grown on 100 mL of R2YE medium<sup>13</sup> of *Sac. erythraea* WMH34, an *eryA* mutant containing a defective PKS and thus unable to synthesize **1**.<sup>14</sup> This led to production of a 2-norerythromycin complex, with the D component being the predominant product and no starting compound **2** being detected according to LC/MS analysis.<sup>15</sup> The antibacterial activity of this material was confirmed by filter disc diffusion assay against *Bacillus cereus*.<sup>16</sup> In contrast, extracts of *Sac. erythraea* WMH34 showed no growth inhibition in this assay.

The production of 2-norerythromycins by a clone of *Sac. erythraea* isolated from an experiment in which a nonproducing mutant of the organism was transformed with a genomic DNA library from *Streptomyces antibioticus* has been reported.<sup>17</sup> The mechanism by which a malonyl extender unit was specified in module 6 of the mutated DEBS in this case is unclear, and a means of extending this result to other structural modifications in the erythromycin series is not apparent. We have shown that such modifications can be rationally introduced through genetic manipulation of the PKS. An acyltransferase substitution similar to ours has been reported in a model system.<sup>10</sup> Replacement of the module 1 acyltransferase in a truncated two-module DEBS system with the module 2 acyltransferase of rapamycin synthase results in formation of the expected nortriketide lactones in low yield.

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(12) NMR characterization of KOS015-22a (**2**) and KOS015-22b (**3**). For KOS015-22a (**2**): <sup>1</sup>H NMR(CDCl<sub>3</sub>)  $\delta$  (ppm) 13-H 5.178–5.213 (1H, ddd, 1.2 Hz, 3.3 Hz, 9.2 Hz), 3-H 4.289–4.326 (1H, ddd, 0.92 Hz, 3.72 Hz, 11.96 Hz), 5-H 3.945–3.963 (1H, dd, 2.4 Hz, 4.8 Hz), 11-H 3.634–3.664 (1H, dd, 2.2 Hz, 10.1 Hz), 2,8,10-H 2.602–2.797 (4H, m), 6-H 1.942–2.390 (1H, m), 4,12,7a,14a-H 1.598–1.850 (4H, m), 14b-H 1.490–1.556 (1H, m), 7b-H 1.175–1.309 (1H, m), 4,6,8,10-CH<sub>3</sub> 1.065–1.082 (3H, d, 6.76 Hz), 1.050–1.065 (3H, d, 6.04 Hz), 1.027–1.045 (3H, d, 7.24 Hz), 1.004–1.022 (3H, d, 6.8 Hz), 15-CH<sub>3</sub> 0.902–0.939 (3H, d, 7.36 Hz), 12-CH<sub>3</sub> 0.874–0.891 (3H, d, 7.0 Hz); <sup>13</sup>C NMR (CDCl<sub>3</sub>)  $\delta$  (ppm) C-9 214.0, C-1 173.7, C-5 76.6, C-13 76.0, C-3 75.5, C-12 71.8, C-11 70.9, C-10 43.3, C-2 40.7, C-8 39.7, C-4 37.7, C-7 37.4, C-6 35.2, C-14 25.2, C-6Me 16.7, C-10Me 13.5, C-15 10.5, C-12Me 9.1, C-4Me 6.5, C-8Me 6.5. For KOS015-22b (**3**): <sup>1</sup>H NMR(CDCl<sub>3</sub>)  $\delta$  (ppm) 13-H 5.48–5.53 (1H, dq, 1.36 Hz, 6.6 Hz), 3-H 4.27–4.31 (1H, ddd, 1.24 Hz, 4.04 Hz, 10.92 Hz), 5-H 3.94–3.96 (1H, dd, 2.52 Hz, 4.80 Hz), 11-H 3.63–3.66 (1H, dd, 10.04 Hz), 10-H 2.78–2.84 (1H, dq, 1.76 Hz, 6.72 Hz), 2,8-H 2.58–2.74 (3H, m), 6-H 1.95–2.04 (1H, m), 4,12,7-H 1.56–1.69 (4H, m), 4,6,8,10,13-CH<sub>3</sub> 1.068–1.085 (3H, d, 6.96 Hz), 1.063–1.079 (3H, d, 6.4 Hz), 1.038–1.056 (3H, d, 7.04 Hz), 1.008–1.024 (3H, d, 6.8 Hz), 0.983–1.000 (3H, d, 7.12 Hz), 12-CH<sub>3</sub> 0.905–0.923 (3H, d, 6.96 Hz); <sup>13</sup>C NMR (CDCl<sub>3</sub>)  $\delta$  (ppm) C-9 214.2, C-1 173.3, C-5 75.4, C-3 73.8, C-13 71.0, C-11 70.1, C-12 41.8, C-10 43.1, C-2 41.0, C-8 40.0, C-4 37.6, C-7 37.6, C-6 35.3, C-14 18.2, C-6Me 16.8, C-10Me 13.6, C-12Me 8.7, C-4Me 6.4, C-8Me 6.4.

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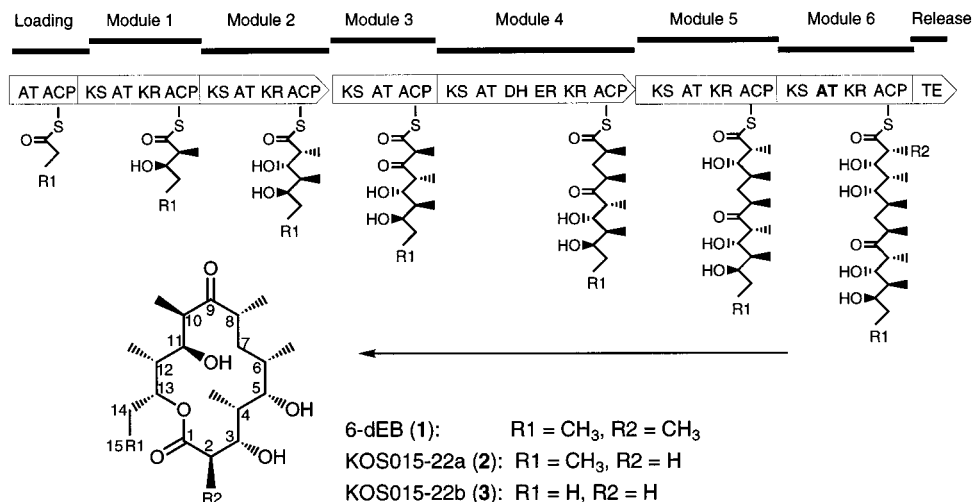
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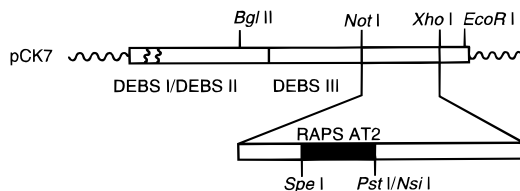
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**Figure 1.** The erythromycin polyketide synthase (DEBS). DEBS consists of three large multifunctional enzymes shown by thick arrows enclosing domain name abbreviations as follows: AT (acyltransferase), ACP (acyl carrier protein), KS (ketosynthase), KR (ketoreductase), ER (enoylreductase), DH (dehydratase). Each of the enzymes is composed of two modules shown by black bars above the arrows for a total of six modules. Each module catalyzes one cycle of chain extension and  $\beta$ -ketoreduction for the biosynthesis of 6-DEB (1). A primer loading domain as well as a product release domain (TE) are also shown by black bars. The growing polyketide chain is depicted below the DEBS enzyme. The polyketide products are indicated by the thin arrow. In plasmid pKOS015-22, **AT6** domain (shown in bold) was replaced with **AT2** domain from the rapamycin PKS and KOS015-22a (2) and KOS015-22b (3) were produced.



**Figure 2.** Construct design of plasmid pKOS015-22 containing the six-module DEBS AT6 substitution. A rapamycin PKS AT2 cassette was PCR amplified with flanking *Spe* I and *Nsi* I restriction sites (shown in bold) using the following pair of primers: forward, 5'-GTACTAGTCTTC-CCGGGTCAGGGTCGCAGCGTGCTG; reverse, 5'-CCATGCATCCACTCGACACTCACGCCGTTACGTACAGG. This cassette (designated by a black bar) was inserted into a subclone of the *Not* I/*Xho* I fragment of pCK7.<sup>8</sup> The subclone had been engineered by PCR to have *Spe* I and *Pst* I restriction sites flanking the AT6 using the following primers: for introducing *Spe* I 5'-GCCTCCGCCGGCGGCGTGGTACTAGTCT-TCCCAGGTCAGGGTGCTC; for introducing *Pst* I 5'-GGCGTGGCCGTGGACTGGCTGCAGGTCTTCCCGGCCGCACCTCCG. The *Not* I/*Xho* I subclone containing AT2 from rapamycin PKS at AT6 position of DEBS was subsequently introduced into a larger *Bgl* II/*Eco* R I subclone of pCK7 and finally ligated back into pCK7. pCK7 is the *Streptomyces* expression vector containing the genes encoding the six-module DEBS.

In summary, we report the first example of a functional domain substitution in a full-length modular PKS and demonstrate the production of an antibacterial erythromycin analog. This rational design approach could be used to generate a set of defined analogs by multiple substitutions of acyltransferase and reductive cycle domains in PKSs. Such analogs should prove useful in the generation and optimization of leads for new drug development.

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