effective. Furthermore, some of the country data used here are continually updated and improved in an effort to provide better regional analyses (for example, Australia) [S. Torok and N. Nicholls, *Aust. Meteorol. Mag.* **45**, 251 (1996)].
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20. We thank P. Ya. Groisman for his insightful comments. Partial support for this work was provided by the U.S. Department of Energy.

3 March 1997; accepted 2 May 1997

Precursor-Directed Biosynthesis of Erythromycin Analogs by an Engineered Polyketide Synthase

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A genetic block was introduced in the first condensation step of the polyketide biosynthetic pathway that leads to the formation of 6-deoxyerythronolide B (6-dEB), the macrocyclic precursor of erythromycin. Exogenous addition of designed synthetic molecules to small-scale cultures of this null mutant resulted in highly selective multimilligram production of unnatural polyketides, including aromatic and ring-expanded variants of 6-dEB. Unexpected incorporation patterns were observed, illustrating the catalytic versatility of modular polyketide synthases. Further processing of some of these scaffolds by postpolyketide enzymes of the erythromycin pathway resulted in the generation of novel antibacterials with in vitro potency comparable to that of their natural counterparts.

Polyketides comprise a large and diverse group of natural products, many of which possess important biological and medicinal properties (*1*), yet polyketide biosynthesis proceeds by simple, repetitive condensations of acetate or propionate monomers in a manner that closely parallels fatty acid synthesis (*2*). Structural complexity is introduced by variation in the stereochemistry and the degree of reduction after each condensation as well as by downstream enzymes that catalyze cyclizations, oxidations, alkylations, glycosylations, and other transformations. Although these compounds are an attractive target for drug discovery (*1*), the complexity of many interesting polyketides impedes the preparation of analogs. Genetic methods for manipulating polyketide synthases (PKSs) show considerable promise for the engineered biosynthesis of novel polyketide molecules (*3*) but are currently limited in the range of compounds that may be accessed. The challenges involved in total synthesis of macrolides (*4*) make this approach impractical for the preparation of derivatives. Synthetic modification of macrolides has led to the preparation of interesting compounds (*5–7*), but this method can also be extremely labor intensive and is limited in the range of transformations that can be selectively performed on these complex natural products. We report the development of a generally applicable, fermentation-based strategy in which chemically synthesized, cell-permeable, non-natural precursors are transformed into molecules resembling natural products by genetically engineered PKSs.

Deoxyerythronolide B synthase (DEBS) produces 6-deoxyerythronolide B (6-dEB) (**1** in Fig. 1), the parent macrolactone of the broad-spectrum antibiotic erythromycin. DEBS consists of three large polypeptides (each >300 kD), each containing ~10 distinct active sites. A one-to-one correspondence between active sites and chemical steps has been proposed (*8, 9*), leading to a model for the synthesis of 6-dEB in which each elongation step is handled by a separate enzyme "module" [see figure 1 of (*10*)]. The modular nature of DEBS and related PKSs (*11*) suggests potential strategies for genetic manipulation to generate novel natural products. Indeed, the feasibility of generating new polyketides has been demonstrated through the use of module deletion (*12*), loss-of-function mutagenesis within reductive domains (*9, 13, 14*), replacement of acyltransferase domains in order to alter starter or extender unit specificity (*15*), and gain-of-function mutagenesis to introduce novel catalytic activities within modules (*16*). Importantly, many experiments show that downstream enzymes can process non-natural intermediates.

Biochemical analysis has also revealed that DEBS has considerable tolerance toward non-natural substrates. For example, primer units such as acetyl and butyryl coenzyme A (CoA) (*17*), or *N*-acetylcysteamine (NAC) thioesters of their corresponding diketides (*18*), can be incorporated in vitro into the corresponding analogs of 6-dEB. However, in the course of these studies, it became clear that, even in the absence of externally added propionyl primers, a potential non-natural substrate must compete with propionate primers derived in situ by means of enzyme-catalyzed decarboxylation of methylmalonyl extender units (*19*). This competition puts severe limits on the priming of DEBS with unnatural thioesters because, for a poorly incorporated substrate, 6-dEB would be expected to be the dominant product.

We focused on incorporating substrates in vivo as cell-permeable NAC thioesters. Although exogenously fed NAC thioesters of advanced intermediates incorporate into several natural products derived from modular PKSs, the degree of specific incorporation was low (<3%) in all cases, presumably because of competing synthesis from metabolically derived intermediates (*20–23*). Mutational biosynthesis (*24*) offers the advantage of eliminating such competition. For example, a randomly generated mutant strain of the avermectin producer, in which biosynthesis of branched primer units is blocked, has been used to generate avermectin derivatives of commercial utility (*21, 25*). However, the unpredictability of random mutagenesis, coupled with the observation that incorporation efficiencies of natural and non-natural substrates by such a mutant are low (*26*), precludes the general applicability of this strategy. In contrast, the specific introduction of null mutations, facilitated by the modular nature of DEBS, provides a general method for construction of useful blocked mutants. For example, inactivation of the ketosynthase KS1 would be expected to abolish normal biosynthesis, but polyketide production might still occur if an appropriate diketide (such as **2** in Fig. 1) was supplied as an NAC thioester. This has been demonstrated in the case of an engineered bimodular derivative of DEBS (*27*). To evaluate the utility of such a mutational strategy for practical precursor-directed biosynthesis of novel, structurally complex molecules, we introduced the same KS1 null mutation in the context of the full DEBS system.

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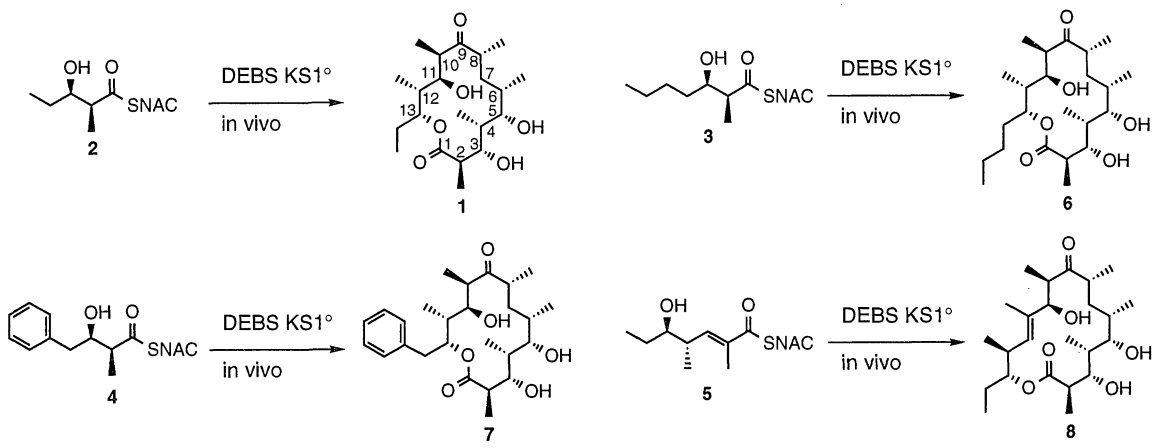

Fig. 1. Reactions catalyzed by an engineered DEBS KS1 null mutant. Lawns of CH999/pJRJ2 were grown at 30°C on R2YE agar plates containing 0.3 mg of sodium propionate per milliliter. After 3 days, each agar plate was overlaid with 1.5 ml of a 20 mM substrate solution in 9:1 water:dimethyl sulfoxide (DMSO). After an additional 4 days, the agar media (300 ml) were homogenized and extracted three times with ethyl acetate. The solvent was dried over magnesium sulfate and concentrated. Concentrated extracts were purified by silica gel chromatography (gradient of ethyl acetate in hexanes) to afford product **1** and products **6** through **8** from substrates **2** through **5**, respectively. Substrate **2** (where SNAC is *N*-acetyl cysteamine) was prepared by the method of Cane *et al.* (*22, 40*) . Substrates **3** and **4** were prepared using the same methodology, but with the substitution of the appropriate aldehydes (valeraldehyde and phenylacetaldehyde, respectively) for propionaldehyde in the aldol reactions. The preparation of substrate **5** has been described previously (*20*) . All substrates were characterized by nuclear magnetic resonance (NMR) spectroscopy. Product structures were determined by ^{1}H and ^{13}C NMR spectroscopy and by mass spectrometry (*41*).

With the use of methods described earlier, the active-site $Cys^{729}\rightarrow Ala$ mutation was introduced into the KS1 domain of the *ery*AI (DEBS1) gene on plasmid pCK7, which contains the *ery*AI, *ery*AII (DEBS2), and *ery*AIII (DEBS3) genes under the control of the *act*I promoter (*10*). The resulting plasmid, pJRJ2, was transformed into *Streptomyces coelicolor* CH999 (*28*), an engineered host that lacks the actinorhodin polyketide gene cluster. When grown on R2YE medium, CH999/pJRJ2 produces no detectable 6dEB-like products. However, when substrate **2** was added to growing cultures of this strain, 6dEB was produced in large quantities. Administration of 100 mg of substrate **2** to a small-scale culture of CH999/pJRJ2 [300 ml, grown on petri plates, as before (*10*)] allowed the isolation of 30 mg of 6dEB, an 18% yield relative to the substrate. Having established that the engineered PKS efficiently synthesizes 6dEB in a diketide-dependent manner, we then determined whether other analogs of **2** would be used by the PKS and converted into the corresponding analogs of 6dEB.

Compounds **3**, **4**, and **5** (Fig. 1) were chosen as initial targets for study to test the effects of a variety of alterations in substrate structure. Compound **3** has a chain length equivalent to that of a triketide but the functionality (methyl and hydroxyl groups) of a diketide. A bulky aromatic group replaces the terminal methyl group in substrate **4**; this substrate was designed to challenge enzyme tolerance of large steric changes at the chain terminus. Compound **5** is a triketide with non-natural stereochemistry at the γ position that has undergone α-β dehydration. This compound corresponds to an intermediate in the biosynthesis of tylosin but is unlike any of the intermediates in 6-dEB synthesis. Substrates **3** and **4** were administered to growing cultures of CH999/pJRJ2, and the expected products **6** and **7** (55 and 22 mg/liter, respectively) were isolated (Fig. 1). Unexpectedly, however, administration of substrate **5** led to the production of the 16-member lactone **8** (Fig. 1) (25 mg/liter).

The successful processing of these unnatural intermediates by the "downstream" modules of DEBS led us to investigate whether the post-PKS enzymes in the erythromycin biosynthetic pathway might also accept unnatural substrates. In the natural producer organism, *Saccharopolyspora erythrea*, 6dEB undergoes several enzyme-catalyzed transformations. Oxidation at carbon C6 and glycosylations at carbons C3 and C5 afford erythromycin D (**9**) (Fig. 2), and subsequent transformations afford erythromycins A, B, and C. To test the ability of the modification enzymes to act on unnatural derivatives of 6dEB, we used an *S. erythrea* mutant (*A34*) (*29*) that was unable to synthesize 6dEB. This strain produces no erythromycin when grown on R2YE plates (as judged by the ability of extracts to inhibit growth of the erythromycin-sensitive bacterium *Bacillus cereus*). However, when 6dEB (which has no antibacterial activity) was

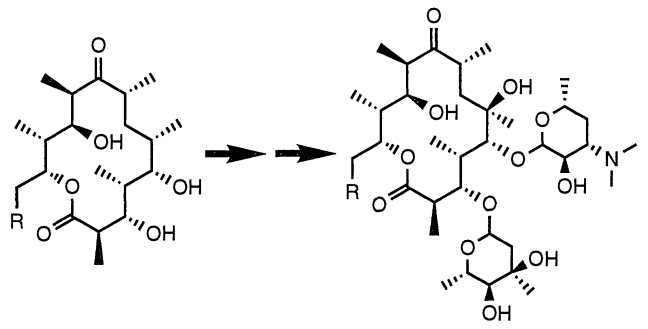

Fig. 2. Processing of 6dEB derivatives to erythromycin D derivatives. The 6dEB is converted to erythromycin D (**9**) by oxidation at C6 and glycosylation at C3 and C5. Purified **6** and **7** (5 mg dissolved in 7.5 ml of 50% aqueous ethanol) were layered onto R2YE plates (200 ml of media per experiment) and allowed to dry. Then, *S. erythrea A34* was applied so as to give lawns. After 7 days of growth, the media were homogenized and extracted three times with a 98.5:1.5 ratio of ethyl acetate:triethylamine. Pooled extracts from each experiment were dried over magnesium sulfate and concentrated. Extracts were partially purified by silica gel chromatography (gradient of methanol and triethylamine in chloroform). The partially purified extracts were examined by thin-layer chromatography and mass spectrometry (MS). Mass analysis suggests conversion of the 6dEB analog to the corresponding erythromycin D analogs **10** (from **6**) and **11** (from **7**). **10**: R_f = 0.51 (streak) (80:20:0.1 $CHCl_3$:MeOH:NH_4OH). MS (ESI^+): 732 (MH^+). MS (ESI^-): 766 (MCl^-). **11**: R_f = 0.49 (streak) (80:20:0.1 $CHCl_3$:MeOH:NH_4OH). MS (ESI^+): 766 (MH^+). MS (ESI^-): 800 (MCl^-).

added to the culture medium, extracts exhibited potent antibacterial activity. Samples of 6dEB derivatives **6** and **7** were assayed for conversion by this strain. Partially purified extracts demonstrated inhibition of *B. cereus* growth (*30*), and mass spectrometry was used to identify the major components of the extracts as **10** (from **6**) and **11** (from **7**) (Fig. 2), analogs of erythromycin D (*31*).

Our method provides the significant opportunity for structural variation afforded by diketide substrates [or more advanced intermediates that have, for example, been shown to selectively incorporate into related products of modular PKSs (*20*, *32*, *33*)]. The Evans method (*34*) was used in the preparation of substrates **2** through **4**, but other aldol methods (*35*) and alkylation chemistry (*36*) might be effectively applied to the generation of diketide substrates. There are no obvious limitations to the variety of substructures that might be incorporated in place of carbons 12 through 15 of erythromycin, a region of the molecule of particular medicinal interest (*6*, *37*). Substrates bearing reactive functional groups might be used to introduce "handles" for the synthetic modification of products. This use of a complex biosynthetic pathway to incorporate unnatural building blocks into polymeric products parallels an established method for the biosynthetic incorporation of unnatural amino acids into proteins (*38*).

The successful incorporation of substrates **3**, **4**, and **5** demonstrates significant tolerance of non-natural substrates by DEBS (modules 2 through 6) as well as other enzymes involved in erythromycin biosynthesis. Our results suggest that heterologous domains that produce diketide-like molecules may form functional chimeric complexes with DEBS (modules 2 through 6) to produce novel 6dEB or erythromycin derivatives by total biosynthesis. The unexpected incorporation of substrate **5** and the cyclization of the product to form a 16-membered macrolactone underscores the flexibility of the DEBS modules.

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41. The spectra of **1** were identical to that of an authentic sample. NMR spectra of products **6** through **8** were assigned using two-dimensional COSY spectroscopy as well as by comparison to the spectra of **1**. **6**: R_f = 0.64 (60% EtOAc/hexanes); ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.90 (d, 3H, J = 6.9 Hz, C12-Me), 0.91 (t, 3H, J = 7.0 Hz, H17), 1.02 (d, 3H, J = 6.8 Hz, C10-Me), 1.03–1.08 (m, 9H, C4-Me, C6-Me, C8-Me), 1.19–1.38 (m, 5H, H7a, H15, H16), 1.29 (d, 3H, J = 6.8 Hz, C2-Me), 1.43–1.52 (m, 1H, H14a), 1.59–1.88 (m, 4H, H4, H7b, H12, H14b), 2.00–2.07 (m, 1H, H6), 2.59–2.65 (m, 1H, H8), 2.73–2.82 (m, 2H, H2, H10), 3.68 (d, 1H, J = 9.8 Hz, H11), 3.92 (d, 1H, J = 10.7 Hz, H3), 4.01 (d, 1H, J = 3.0 Hz, H5), 5.24 (ddd, 1H, J = 9.7, 3.7, 1.4 Hz, H13); ^{13}C NMR (100 MHz, $CDCl_3$): δ 6.2 (C8-Me), 6.9 (C4-Me), 9.2 (C12-Me), 13.2 (C10-Me), 13.9 (C17), 14.7 (C2-Me), 16.5 (C6-Me), 22.3 (C16), 28.4 (C15), 31.9 (C14), 35.5 (C6), 37.4 (C7), 37.6 (C4), 39.2 (C8), 40.8 (C12), 43.4 (C2), 43.9 (C10), 70.9 (C11), 74.7 (C13), 76.4 (C5), 79.5 (C3), 178.3 (C1), 210.6 (C9); HRMS (FAB$^+$): Calculated for $(C_{23}H_{42}O_6)Na^+$: 437.2879. Found: 437.2881. **7**: R_f = 0.60 (60% EtOAc/hexanes); ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.96 (d, 3H, J = 6.6 Hz, C2-Me), 1.02 (d, 3H, J = 6.7 Hz, C12-Me), 1.03–1.05 (m, 9H, C6-Me, C8-Me, C10-Me), 1.07 (d, 3H, J = 7.0 Hz, C4-Me), 1.22–1.36 (m, 1H, H7a), 1.67 (ddd, 1H, J = 13.2, 11.7, 3.1 Hz, H7b), 1.79–1.92 (m, 2H, H12, H4), 1.97–2.06 (m, 1H, H6), 2.55–2.64 (m, 1H, H8), 2.66 (dq, 1H, J = 10.4, 6.7 Hz), 2.74–2.81 (m, 2H, H10, H14a), 3.06 (dd, 1H, J = 14.0, 10.7 Hz, H14b), 3.70 (d, 1H, J = 3.7 Hz, H11), 3.82 (d, 1H, J = 10.6 Hz, H3), 3.97 (d, 1H, J = 3.5 Hz), 5.43 (dd, 1H, J = 10.6, 2.0 Hz, H13), 7.19–7.31 (m, 5H, H-Phenyl); ^{13}C NMR (100 MHz, $CDCl_3$): δ 6.3 (C8-Me), 6.9 (C4-Me), 9.4 (C12-Me), 13.2 (C10-Me), 14.0 (C2-Me), 16.5 (C6-Me), 35.5 (C6), 37.5 (C14), 37.6 (C7), 38.6 (C4), 39.2 (C8), 40.7 (C12), 43.4 (C2), 43.8 (C10), 70.9, (C11) 75.2 (C12), 76.4 (C5), 79.5 (C3), 126.7 (C-Phenyl), 128.5 (C-Phenyl), 129.0 (C-Phenyl), 137.5 (C-Phenyl), 177.8 (C1), 213.4 (C9); HRMS (FAB$^+$): Calculated for $(C_{26}H_{40}O_6)Na^+$: 471.2723. Found: 471.2732. **8**: (Note that a ^{13}C label at the C4-methyl group of substrate **5** results in ^{13}C labeling of the C14-methyl group of **8**) R_f = 0.67 (50% EtOAc/hexanes); ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.36 (d, 1H, J = 8.2 Hz, H13), 4.71–4.77 (m, 1H, H15), 4.00 (d, 1H, J = 10.1 Hz, H11), 3.83 (d, 1H, J = 10.1 Hz, H3), 3.41 (dd, 1H, J = 10.1, 1.6 Hz, H5), 2.76 (q, 1H, J = 7.2 Hz, H2), 2.53–2.59 (m, 1H, H14), 2.19 (dq, 1H, J = 10.0, 6.5 Hz, H10), 1.96–2.01 (m, 1H, H8), 1.79–1.90 (m, 1H, H16a), 1.69–1.77 (m, 1H, H4), 1.75 (s, 3H, C12-Me), 1.51–1.68 (m, 1H, H6), 1.41–1.51 (m , 2H, H16b, H7a), 1.22–1.30 (m, 1H, 7b), 1.13 (d, 3H, J = 6.8 Hz, C10-Me), 1.11 (d, 3H, J = 7.2 Hz, C2-Me), 1.01 (d, 3H, J = 6.7 Hz, C4-Me), 0.99 (dd, 3H, J = 127.4, 6.9 Hz, C14-Me), 0.90 (t, 3H, J = 7.3 Hz, H17), 0.88 (d, 3H, J = 6.8 Hz, C8-Me), 0.75 (d, 3H, J = 6.5 Hz, C6-Me) ; ^{13}C NMR (100 MHz, $CDCl_3$): δ 178.9 (C1), 139.4 (C12), 133.8 (C13), 78.9 (C11), 78.8 (C15), 74.0 (C3), 72.5 (C5), 42.1 (C2), 39.6 (C10), 37.4 (C4), 36.8 (d, J_{CC} = 34.2 Hz) (C14), 34.8 (C6), 34.7 (C8), 31.8 (C7), 25.2 (C16), 16.7 (C14-Me), 16.6 (C6-Me), 15.7 (C2-Me), 12.7 (C12-Me), 10.7 (C10-Me), 9.3 (C17), 8.6 (C4-Me), 8.4 (C8-Me), (C9 not detected); MS (ESI$^+$): 450 (MNa^+). MS (ESI$^-$): 462 (MCl^-). HRMS (FAB$^+$): Calculated for $(C_{23}{}^{13}CH_{42}O_5\text{-}H_2O)Na^+$: 432.2807. Found: 432.2810.
42. We thank M. McPherson for mass spectrometry of 6dEB derivatives. Research in our laboratories was supported by grants from NIH (CA66736 to C.K., GM22172 to D.E.C., and GM31925 to C.R.H.), an NSF Young Investigator Award (to C.K.), and a David and Lucile Packard Fellowship for Science and Engineering (to C.K.). J.R.J. is a recipient of a National Institute of General Medical Sciences postdoctoral fellowship (1 F32 GM18590-01).

14 March 1997; accepted 29 May 1997