Erythromycin Biosynthesis

Highly Efficient Incorporation of Polyketide Chain Elongation Intermediates into 6-Deoxyerythronolide B in an Engineered *Streptomyces* Host†

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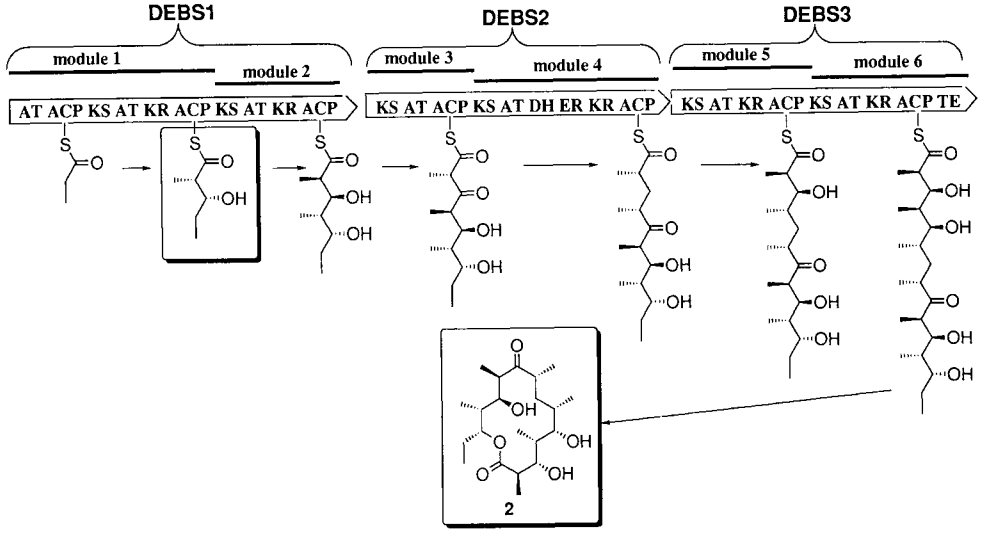
Feeding of (2*S*,3*R*)-[2,3-$^{13}C_2$]-2-methyl-3-hydroxypentanoyl NAC thioester (**1a**) to the recombinant organism *Streptomyces coelicolor* CH999/pCK7 harboring the complete set of *eryA* genes from *Saccharopolyspora erythraea* encoding the 6-deoxyerythronolide B synthase (DEBS) resulted in the formation of 6-deoxyerythronolide B (**2a**) labeled with ^{13}C at C-12 and C-13, as evidenced by the appearance of a pair of enhanced and coupled doublets in the ^{13}C NMR spectrum. The level of ^{13}C enrichment was 15~20 atom% ^{13}C, as much as 100 times higher than the usually observed efficiency of incorporation of NAC thioesters into polyketide metabolites. Similar incorporation of (2*S*,3*R*)-[3-^{2}H,3-^{13}C]-2-methyl-3-hydroxypentanoyl NAC thioester (**1b**) gave 6-deoxyerythronolide B (**2b**) labeled with both ^{13}C and deuterium at C-13. The intact incorporation of both precursors confirms the normal functioning of the recombinant DEBS proteins in the heterologous host.

The macrolides are among the most important antibiotics used in human and animal medicine. It is by now well-established that the characteristic branched chain, polyoxygenated skeletons of the macrolide aglycones are formed by a process closely related to fatty acid biosynthesis[1], in which each new carbon-carbon bond is formed by a decarboxylative condensation between a methylmalonyl or malonyl thioester and a fatty acyl thioester substrate[2]. In the reactions catalyzed by a polyketide synthase (PKS), the oxidation level and stereochemistry of the growing polyketide chain are adjusted subsequent to each step of chain elongation. Strong evidence in favor of this mechanistic scheme initially came from our successful incorporation of a labeled diketide intermediate, as the *N*-acetylcysteamine (NAC) thioester **1a**, into the macrolide antibiotic erythromycin[3]. This technique has since been successfully applied in our own and other laboratories to the incorporation of polyketide chain elongation intermediates into numerous other metabolites[4~11]; indeed recently we successfully incorporated a pentaketide precursor into the antibiotic nargenicin[12].

In spite of the success of these advanced precursor incorporation experiments, only recently has the genetic and biochemical basis for the programming of the complex series of biosynthetic transformations leading to the formation of partially reduced polyketides begun to be unraveled. A major advance in our understanding of PKS programming has come from the cloning and sequencing of the structural genes responsible for the formation of 6-deoxyerythronolide B (6-dEB, **2**), the parent aglycone of the erythromycin group of macrolide antibiotics[13,14]. It was thus shown that the *eryA* gene consists of three contiguous 10-kb open reading frames, each encoding a large (*ca.* 3,000 amino acid) multifunctional protein. These proteins, which have been designated as DEBS1, DEBS2, and DEBS3[15,16], appear to be organized into a series of functional domains, each with a combination of distinct active sites corresponding to the individual biochemical steps of polyketide chain elongation[17]. Even more remarkably, these domains are arranged in essentially the same order as the actual sequence of condensation and functional group modification reactions (Fig. 1).

† This paper is dedicated to Prof. SATOSHI ŌMURA on the occasion of his 60th birthday.

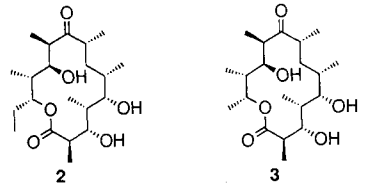
Fig. 1. Genetic model for the 6-deoxyerythronolide B synthase from *S. erythraea*.



Each module carries the appropriate combination of acyltransferase (AT), acyl carrier protein (ACP), β-ketosynthase (KS), β-ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) domains. The thioesterase (TE) at the *C*-terminus of module 6 catalyzes macrolactone formation. The (2*S*,3*R*)-2-methyl-3-hydroxypentanoyl ACP thioester intermediate is highlighted.

Recently, we reported the expression in a heterologous host, *Streptomyces coelicolor* CH999, of the complete 6-deoxyerythronolide B synthase (DEBS1, DEBS2, DEBS3) from *Saccharopolyspora erythraea*[18]. The resulting recombinant organism was found to produce substantial quantities of 6-dEB (**2**), accompanied by 8,8a-deoxyoleandolide (**3**), a closely related metabolite in which an acetate has replaced the normal propionate starter unit of the erythromycin aglycone (Fig. 2). It was speculated that this relaxed specificity might reflect a limiting concentration of the normal propionate starter moiety. We now describe experiments using this same recombinant strain that demonstrate the unusually efficient incorporation of diketide chain elongation intermediates into 6-dEB.

Fig. 2. Structures of 6-deoxyerythronolide B (**2**) and 8,8a-deoxyoleandolide (**3**).



Experimental

Materials and Methods

General analytical, spectroscopic, and synthetic methodology were as previously described[5,6]. ^{13}C (100.6 MHz) NMR spectra of labeled metabolites were recorded at 100.6 MHz as solutions in CD_2Cl_2 on a Bruker AM-400 NMR spectrometer. Chemical shifts are reported in parts per million relative to tetramethylsilane, CH_2Cl_2 as internal standard. Coupling constants are reported in Hz. (2*S*,3*R*)-[2,3-$^{13}C_2$]-2-methyl-3-hydroxypentanoyl NAC thioester (**1a**) and (2*S*,3*R*)-[3-2H,3-^{13}C]-2-methyl-3-hydroxypentanoyl NAC thioester (**1b**) were synthesized by a modification of the published procedures[5,6] in which the hydroxyl group was protected as the *tert*-butyldimethylsilyl ether prior to removal of the oxazolidinone chiral auxiliary. By incorporating the protection-deprotection steps into the hydrolysis and thioesterification sequence, the net yields of NAC thioester were increased from 50~60% to near quantitative.

Maintenance and Sporulation of *S. coelicolor* CH999/pCK7

Streptomyces coelicolor CH999/pCK7[18] was cultivated and maintained on R5 agar composed of sucrose (51.5 g), K_2SO_4 (125 mg), $MgCl_2 \cdot 6H_2O$ (5.06 g), glucose (5.0 g), Casamino acids (50 mg), trace element solution (1 ml of solution consisting of $ZnCl_2$ 8 mg, $FeCl_3 \cdot 6H_2O$ 40 mg, $CuCl_2 \cdot 2H_2O$ 2 mg, $MnCl_2 \cdot 4H_2O$ 2 mg, $Na_2B_4O_7 \cdot 10H_2O$ 2 mg, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 2 mg in 200 ml water), yeast extract (2.5 g), TES buffer (5.73%, adjusted to pH 7.2 by 2 N NaOH) (5.0 ml), distilled water up to 500 ml, and Difco Bacto agar (11 g). Following sterilization, the following sterilized solutions were added before pouring the plates: KH_2PO_4 (0.5%) (5.0 ml), $CaCl_2 \cdot 2H_2O$ (5 M) (2.0 ml), L-proline (20%) (7.5 ml), NaOH (1 N) (3.5 ml) and thiostrepton (50 mg/1 ml

DMSO) (0.5 ml). The plates were well-sporulated 6~7 days after inoculation.

Fermentation of *S. coelicolor* CH999/pCK7

Sterile water (5 ml + 5 ml wash) was poured into a well sporulated plate of *S. coelicolor* CH999/pCK7 and the spores were scratched off by a loop. The mixture was poured into a sterilized capped-bottle and vortexed very well for more than 2 minutes, then filtered twice through a cotton plug into a sterilized capped-bottle to remove mycelial fragments. After centrifugation (3,000 rpm, 10 minutes) the supernatant was discarded and the spores were suspended in 1 ml sterile water. The resulting spore suspension was used to inoculate 200 ml SMM medium in a 1-liter Erlenmeyer flask containing a 50 × 1.0-cm stainless steel spring. SMM medium composition: PEG 800 (6.1% w/v in H_2O) (163.8 ml), salt solution ($MgSO_4$ 24 g/liter, $(NH_4)_2SO_4$ 80 g/liter) (5 ml), TES buffer (0.25 M, pH 7.2) (20 ml), phosphates (0.05 M NaH_2PO_4, 0.05 M K_2HPO_4) (2.0 ml), glucose (80% w/v) (4.0 ml), trace elements (0.2 ml), Casamino acid (20%) (5.0 ml). The culture was incubated with shaking at 325 rpm and 29~30°C for 6 days before harvest. The mycelia and fermentation broth from the production medium were centrifuged at 8,000 rpm for 20 minutes in a Sorvall GS3 rotor (1,816 × *g*). The supernatant was decanted and saturated with NaCl and then extracted with EtOAc (5 × 100 ml). The combined organic solution was dried over celite, filtered and concentrated to afford a light yellow oil which was subjected to silica gel chromatography (20~30% EtOAc-hexanes). *Ca.* 1 mg of pure 6-dEB was obtained from each 200-ml fermentation culture.

Incorporation of Labeled Substrates

Each of the labeled diketide NAC thioesters (100 mg), (2*S*,3*R*)-[2,3-$^{13}C_2$]-2-methyl-3-hydroxypentanoyl NAC thioester (**1a**) or (2*S*,3*R*)-[3-^{2}H,3-^{13}C]-2-methyl-3-hydroxypentanoyl NAC thioester (**1b**) was administered in separate experiments along with 15 mg 4-pentynoic acid in 1 ml of DMSO, at intervals of 50 hours (40%), 62 hours (30%), and 86 hours (30%), to 200-ml cultures of actively growing *S. coelicolor* CH999/pCK7. The resulting labeled 6-dEB (**2a** and **2b**) was isolated and purified following the procedure described above to yield *ca.* 0.5 mg of aglycone in each case. *Ca.* 60 mg of [2,3-$^{13}C_2$]-**1a** diketide and 70 mg of [2-^{2}H,2-^{13}C]-**1b** were recovered from the respective fermentation broths.

Results

Both (2*S*,3*R*)-[2,3-$^{13}C_2$]-2-methyl-3-hydroxypentanoyl NAC thioester and (2*S*,3*R*)-[3-^{2}H,3-^{13}C]-2-methyl-3-hydroxypentanoyl NAC thioester were synthesized by improved procedures based on those already described[5,6], as illustrated in Scheme 1. In separate experiments, each of the resulting labeled substrates (100 mg total), plus the β-oxidation inhibitor 4-pentynoic acid (15 mg), were administered at intervals of 50 hours (40%), 62 hours (30%), and 86 hours (30%) to 200 ml of an actively fermenting culture of *S. coelicolor* CH999/pCK7. After purification by silica gel chromatography, the sample of labeled 6-dEB (**2a**) derived from

Scheme 1. Synthesis of a) (2*S*,3*R*)-[2,3-$^{13}C_2$]-2-methyl-3-hydroxypentanoyl NAC thioester (**1a**) and b) (2*S*,3*R*)-[3-^{2}H,3-^{13}C]-2-methyl-3-hydroxypentanoyl NAC thioester (**1b**).

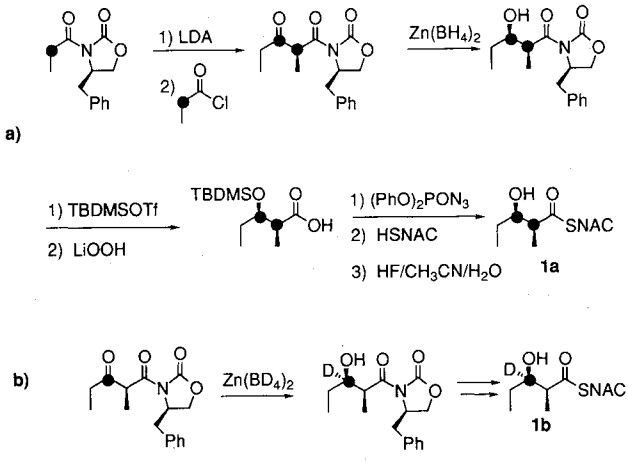
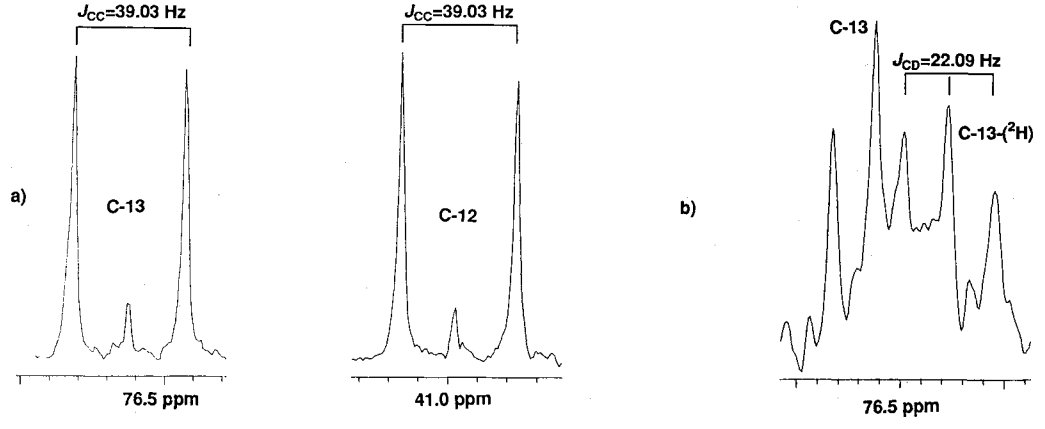
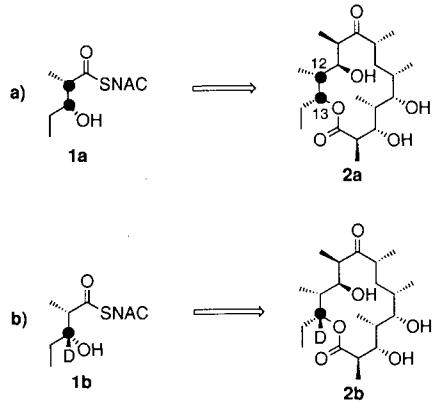


Fig. 3. 100.6 MHz ^{13}C NMR spectra of a) C-12 and C-13 of **2a**; b) C-13 of **2b**.



Scheme 2. Intact incorporation of labeled diketides **1a** and **1b** into 6-deoxyerythronolide (**2a**, **2b**).



(2*S*,3*R*)-[2,3-$^{13}C_2$]-**1a** was analyzed by 100 MHz ^{13}C NMR. The ^{13}C NMR spectrum of **2a** in CD_2Cl_2 displayed a pair of enhanced and coupled doublets (J_{CC} = 39.03 Hz) centered at 40.97 and 76.62 ppm, corresponding to enrichment at each of the expected sites of labeling, C-12 and C-13, respectively[3]. (Fig. 3a, Scheme 2a) The level of ^{13}C enrichment was a remarkable 15~20 atom%, a factor of nearly 20~100 times more efficient than the usual specific incorporation of NAC thioesters into complex polyketides[3~12]. A similar level of enrichment was achieved for the incorporation of (2*S*,3*R*)-[3-^{2}H,3-^{13}C]-**1b** into 6-dEB (**2b**). The ^{13}C NMR spectrum of **2b** exhibited a characteristic 1 : 1 : 1 triplet (J_{CD} = 22.09 Hz) shifted 0.35 ppm upfield of the natural abundance ^{13}C signal for C-13 at 76.62 ppm[19]. (Fig. 3b, Scheme 2b)

Discussion

According to the currently accepted genetic model for the 6-deoxyerythronolide B synthase, the three DEBS proteins are organized into a series of synthetic modules, each of which is responsible for a discrete condensation step (ketosynthase, KS) as well as the combination of keto-reduction (KR), dehydration (DH), enoyl-reduction (ER) reactions appropriate to each stage of polyketide chain elongation. Fig. 1 illustrates this model schematically and indicates the attachment of the presumed product of each successive round of chain elongation to the ACP domain of the relevant module. Under normal conditions of macrolide production, polyketide biosynthesis is initiated by acyltransferase (AT)-catalyzed loading of the propionyl CoA starter unit onto the active site Cys of the keto synthase, KS1, of module 1. When the shuttle plasmid pCK7, carrying the complete set of *eryA* genes, is expressed in *S. coelicolor* CH999, the resultant proteins DEBS1, DEBS2, and DEBS3 produce the erythromycin aglycone, 6-dEB, indicating that all the necessary auxiliary activities to support macrolide formation (*e.g.* pantothenyl transfer to generate functional ACP domains) are present in the host organism.

The fact that intermediates of polyketide chain elongation can be incorporated intact into complex polyketides implies that the relevant PKS can recognize key structural features of the exogenously administered thioester and load the intermediate onto the cognate keto-synthase domain so as to allow formation of the normal polyketide product. Thus the successful incorporation of both **1a** and **1b** suggests that the diketide substrates are loaded intact onto either the ACP of module 1 or directly onto the core cysteine of the ketosynthase of module 2 (KS2), from whence they are converted in the normal manner to 6-dEB. The intact incorporation of exogeneously added intermediates of polyketide chain elongation indicates that the proper programming of product formation is not exclusively controlled by the sequential organization of active sites on the multifunctional PKS protein but must also involve a degree of molecular recognition of as yet undefined structural or stereochemical elements of the various intermediates. In spite of the successes of advanced precursor feedings using the native Actinomycete or fungal hosts, however, feedings of partially elaborated polyketides as NAC thioesters has been hindered by the exceptionally low levels of incorporation of the labeled precursor, presumably due not only to the natural competition between exogeneously administered substrates and endogeneously generated precursors, but also to the rapid destruction of the precursor by competing degradative pathways such as fatty acid β-oxidation. Various clever devices have been developed to overcome the problems of substrate degradation[8,10], with varying degrees of success, but in general incorporation rates remain low. For example, administration of **1a** and **1b** to cultures of *S. erythraea* resulted in enrichments in erythromycin ranging from 0.1~1.0 atom% ^{13}C above natural abundance, with the majority of successful experiments giving incorporations at the lower end of this scale, even in the presence of various inhibitors of β-oxidation[3,19]. By contrast, feeding of **1a** to the engineered host *S. coelicolor* CH999/pCK7 gave 6-dEB enriched with ^{13}C to a level of 15~20 atom%, representing a nearly 100-fold increase in incorporation efficiency. Although feeding of the precursor depressed the net production of macrolide aglycone, this presented no problem due to the high levels of ^{13}C enrichment in the product. The recovery of 60~70% of the administered precursor also indicates that the competing degradation of the substrate is a much less severe problem in this heterologous host.

The successful incorporation of two labeled diketides into 6-dEB confirms the normal functioning of the engineered PKS in the heterologous host and provides a convenient system for directly testing the structural and stereochemical basis of molecular recognition in this system using substrate analogs.

Acknowledgments

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