Precursor-Directed Biosynthesis of 6-Deoxyerythronolide B Analogs in *Streptomyces coelicolor:* Understanding Precursor Effects

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A fermentation process employing precursor-directed biosynthesis is being developed for the manufacture of 6-deoxyerythronolide B (6-dEB) analogues. Through a plasmid-based system in *Streptomyces coelicolor*, 6-dEB synthesis is catalyzed by 6-dEB synthase (DEBS). 6-dEB synthesis is abolished by inactivation of the ketosynthase (KS) 1 domain of DEBS but can be restored by providing synthetic activated diketides. Because of its inherent catalytic flexibility, the KS1-deficient DEBS is capable of utilizing unnatural diketides to form various 13-substituted 6-dEBs. Here we characterize process variables associated with diketide feeding in shake-flask experiments. 13-R-6-dEB production was found to depend strongly on diketide feed concentrations, on the growth phase of cultures at feeding time, and on the R-group present in the diketide moiety. In all cases a major portion of the fed diketides was degraded by the cells.

Introduction

Macrolides are a class of natural products that have diverse biological activities, many of which have led to successful therapeutic applications (O'Hagan, 1995). Macrolide antibiotics include the widely used erythromycin, a product synthesized naturally by the actinomycete *Saccharopolyspora erythraea*. The polyketide synthase (PKS) of *S. erythraea* catalyzes synthesis of 6-deoxyerythronolide B (6-dEB), the core aglycone precursor of erythromycin. The 6-dEB synthase (DEBS) of *S. erythraea* has been successfully expressed in *Streptomyces coelicolor,* enabling production of 6-dEB in this heterologous host (Kao et al., 1994).

Incorporation of diketide as well as more advanced intermediates as *N*-acetylcysteamine (NAC) thioesters into polyketides has been demonstrated with several organisms possessing different PKSs (Cane et al., 1995; Cane and Luo, 1995; Tsantizos et al., 1995; Yoshizawa et al., 1990). *S. coelicolor* CH999/pCK7, harboring the complete set of DEBS genes of *S. erythraea,* seems especially efficient at incorporation of ^{13}C-labeled (2*S*,3*R*)-2-methyl-3-hydroxypentanoate into 6-dEB when fed the NAC thioester (Cane et al., 1995), but the natural pathway competes with the incorporation. *S. coelicolor* CH999/pJRJ2, harboring a mutant DEBS deficient in the active ketosynthase 1 domain (KS1°), is unable to produce 6-dEB unless supplemented with a synthetic activated diketide (Jacobsen et al., 1997). The mutant DEBS has been characterized in terms of substrate specificity and found to have some flexibility in processing synthetic precursors, especially in that part of the diketide corresponding to the normal starter unit in 6-dEB synthesis. These characteristics allow formation of 6-dEB analogues having various substitutions at the 13-position of the macrolide ring (Figure 1). Substitution at the 12-position of the 6-dEB backbone through diketide incorporation has also been demonstrated (Jacobsen et al., 1998).

In this work, use of *S. coelicolor* CH999/pJRJ2 in a fermentation process for manufacture of 6-dEB analogues is investigated. To become incorporated into 6-dEB, diketide NAC thioesters must be transported from the growth medium into the cells and must be acceptable substrates for DEBS. The inherent stability of these precursors and the competing degradative pathways in the organism are also important factors affecting successful incorporation. The feeding of synthetic diketide precursors is clearly a fundamental element of this process and represents the focus of this study. The effect of diketide feeding methodology is addressed, the fate of these fed precursors is evaluated, and the incorporation of several functionally different diketides is compared.

Materials and Methods

Synthesis of Diketide NAC Thioesters. Preparation of diketide NAC thioesters was performed as described previously (Weissman et al., 1998).

Strains and Culture Conditions. *S. coelicolor* CH999/pJRJ2 (Jacobsen et al., 1997) was used exclusively throughout this work. Cultures were grown in flasks using R6 medium (sucrose, 103 g/L; K_2S0_4, 0.25 g/L; $MgC1_2{\cdot}H_2O$, 10.12 g/L; sodium propionate, 0.96 g/L; casamino acids (Difco), 0.1 g/L; trace elements solution, 2 mL/L; yeast extract (Fisher), 5 g/L; pH 7) supplemented with bis-tris propane buffer (28.2 g/L). Trace elements solution contained $ZnC1_2$, 40 mg/L; $FeC1_3{\cdot}H_2O$, 200 mg/L; $CuC1_2{\cdot}H_2O$, 10 mg/L; $MnC1_2{\cdot}4H_2O$, 10 mg/L; $Na_2B_40_7{\cdot}1OH_2O$, 10 mg/L; $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$. All media were supplemented with 50 mg/L thiostrepton (Calbiochem) to select for plasmid-containing cells and with 5 mL/L Antifoam B (JT Baker) for control of foam.

A cell bank was prepared by adding glycerol (15% final concentration) to an exponentially growing culture (CH999/pJRJ2 in buffered R6 medium) and then freezing 1 mL aliquots at −80 °C. One milliliter from the frozen cell

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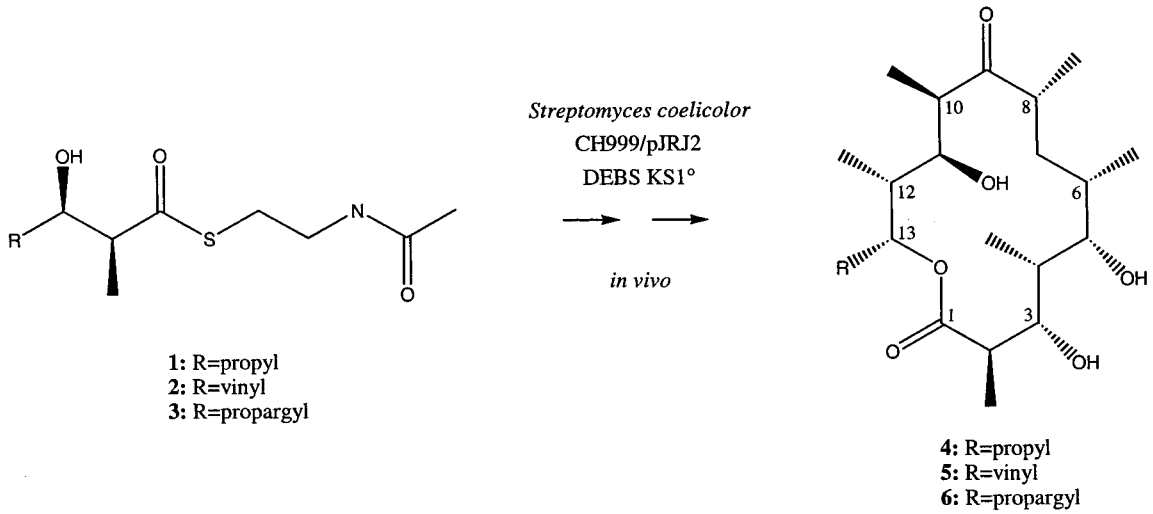

Figure 1. Scheme for precursor-directed biosynthesis of 6-dEB analogues.

bank was used to inoculate 50 mL of medium in a 250 mL baffled flask (Bellco 2540), which was incubated with shaking for 2 days at 240 rpm and 30 °C. This was then used directly for inoculation of flasks at 2% of final volume or transferred to 500 mL medium in a 2.8 L baffled Fernbach flask (Pyrex 4420) for 2 days when additional inoculum volume was required. All flask cultures were run in duplicate and sampled daily.

Diketides and thiostrepton were dissolved in DMSO prior to addition to cultures, giving a final DMSO concentration of approximately 7 mL/L of medium. This initial DMSO concentration was consistent in all experiments.

6-dEB and Diketide Analysis. Identification of various 6-dEB analogues was made using LC/MS and NMR. Quantitation of 6-dEB analogues was carried out using a Hewlett-Packard 1090 HPLC equipped with an Alltech 500 evaporative light scattering detector. Samples for LC were first centrifuged for 5 min at 12,000 × *g* to remove insolubles. Supernatant (20−50 μL) was injected, extracted on a 4.6 mm × 10 mm column (Inertsil, C18 ODS3, 5 μm) washed with water (0.75 mL/min for 2 min), and finally eluted onto the main column (4.6 mm × 150 mm, same stationary phase and flow rate) with a 9-min gradient starting with 100% water and ending with 100% acetonitrile. A hold time of several minutes with 100% acetonitrile eluted 13-R-6-dEBs between 11 and 13 min depending on the R-group. Standards were prepared using 13-R-6-dEB purified from fermentation broth. Quantitation error was ±10%. Diketides were quantitated using the HPLC system described above with UV detection at 250 nm.

To protect proprietary information, 13-R-6-dEB titers have been reported in arbitrary units. Arbitrary units are units of mass per volume, and the value of an arbitrary unit is consistent throughout all figures.

Results and Discussion

Effect of Diketide Feeding Methodology on Culture Productivity. Basic aspects of diketide feeding such as the initial feed concentration and the growth stage of the culture at the time of feeding were characterized. Using diketide thioester **2** (Figure 1) as a model substrate, the time-course of incorporation into the expected product **5** (Figure 1) was obtained at several different feed concentrations (Figure 2). These plots show a typical production period of 4−5 days. It is evident that

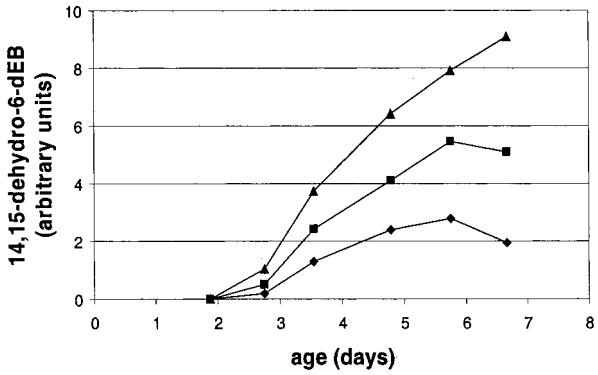

Figure 2. Time-course of 13,14-dehydro-6-dEB formation in shake flask cultures having different initial diketide thioester **2** feed concentrations: ◆, 1 mM; ■, 2 mM; ▲, 4 mM. All flasks were fed approximately 2 days after inoculation.

both the rate of product synthesis and the maximum achieved titer increased with increasing precursor concentration.

The effect of feed timing was evaluated by inoculating flasks in parallel and feeding 2 mM diketide thioester **2** on different days. Results indicate that supplying diketide within the first 2 days after inoculation led to equivalent product titers, while feeding after more than 2 days resulted in progressively lower productivity (Figure 3). This suggests that in batch culture of CH999/pJRJ2 there is a time window during which DEBS and supporting enzymes and metabolic pathways are active; maximum product titer is attained when diketide is made available throughout this active period.

To determine whether the diketide feeding methodology was generally applicable to the production of 6-dEB analogues, several differently substituted diketides were examined with respect to feed concentration effects. Figure 4 shows the relationship between product titer and feed concentration for diketide thioesters **1**, **2**, and **3** (Figure 1). The dependence of titer on diketide feed level varied with functional group and revealed substrate saturation behavior. Rates of disappearance in culture supernatants were similar for all three diketide substrates (data not shown). The diketide thioester **1** required the lowest feed concentration to obtain maximal productivity (about 2 mM), followed by **2** (>4 mM) and **3** (>8 mM). These differences are likely to reflect any combination of solubility, transport, or DEBS enzyme specificity effects. Product stability may also play a role,

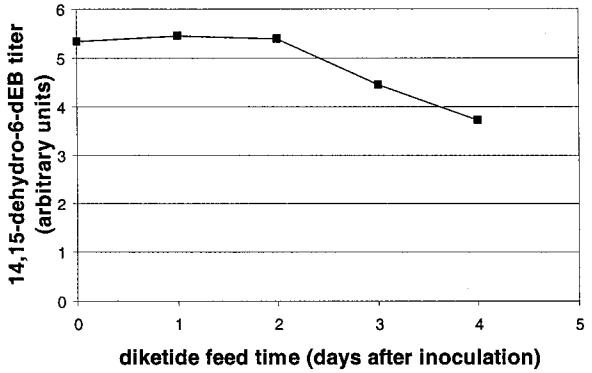

Figure 3. Maximum product titer as a function of diketide feed time; 50 mL flask cultures were fed diketide thioester **2** at 2 mM at inoculation or up to 4 days after inoculation.

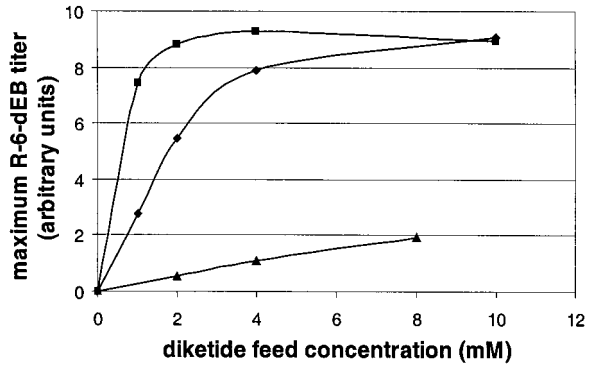

Figure 4. Comparison of feed concentration effect for several different diketides: ▲, 14,15-didehydro-6-dEB formed after feeding diketide thioester **3**; ◆, 14,15-dehydro-6-dEB formed after feeding diketide thioester **2**; ■, 15-methyl-6-dEB formed after feeding diketide thioester **1**. All titers represent maxima from time-course data.

evidenced by the observation that product **5** is much less stable than product **4** at pH 8.0 (data not shown). Interestingly, in vitro studies with a bimodular PKS derived from the first two processing modules of the DEBS KS1° mutant used here showed especially good incorporation of diketide thioester **1** into a triketide lactone (Chuck et al., 1997).

Feeding (2*S*,3*R*)-2-methyl-3-hydroxyacyl NAC thioesters to CH999/pJRJ2 results in formation of 6-dEB analogues such as **4**−**6** (Figure 1). However, racemic diketides containing both (2*S*,3*R*)- and (2*R*,3*S*)-enantiomers are relatively easier and less expensive to prepare. Therefore, the feasibility of using such racemic diketide thioesters as feedstocks for precursor-directed biosynthesis of 13-R-6-dEBs was investigated. Figure 5 shows a comparison of racemic and (2*S*,3*R*)-2-methyl-3-hydroxypentanoyl NAC thioester feeding in flask cultures. At a level of 1 mM, cultures fed racemic diketide produced some 30% less 6-dEB analogue as compared to those fed the single enantiomer. This is not surprising since only half of the racemic material would be expected to be available for incorporation into product. Doubling the racemic diketide concentration to 2 mM resulted in a production level approaching that observed for 1 mM feeding of the single enantiomer. This suggests that the (2*R*,3*S*)-enantiomer does not inhibit the processing of the (2*S*,3*R*)-compound by DEBS and demonstrates the feasibility of using racemic diketide thioesters in place of pure enantiomers in a process for production of 6-dEB analogues.

Process Yield and Degradation of Diketides. To better understand the process of diketide incorporation into R-6-dEBs, the time-course of diketide concentration

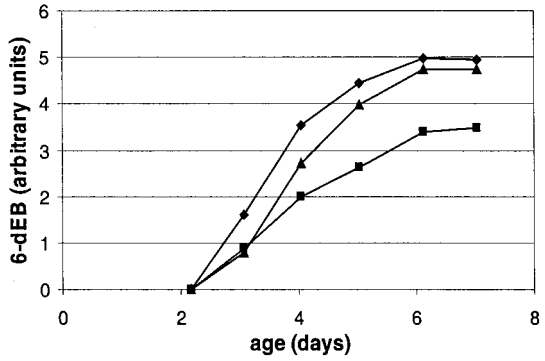

Figure 5. Comparison of single-enantiomer and racemic diketide NAC thioester feeding. (2*S*,3*R*)-2-Methyl-3-hydroxypentanoyl NAC thioester was fed at 1 mM (◆); the racemic counterpart was fed at 1 mM (■) and 2 mM (▲).

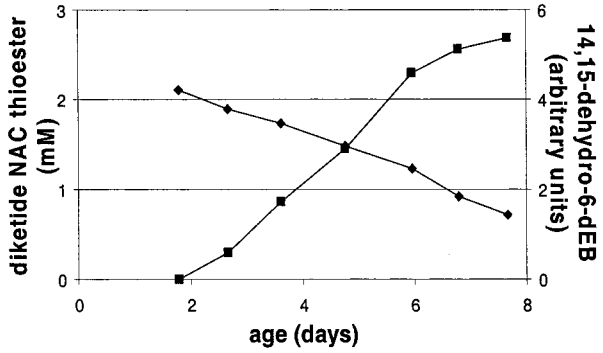

Figure 6. Typical time-course for diketide consumption in *S. coelicolor* flask cultures. Flasks were fed diketide thioester **2** (▲) on the second day following inoculation. Formation of 14,15-dehydro-6-dEB (■) is also shown.

was routinely determined. A typical time-course of diketide consumption along with the associated product formation is shown in Figure 6. In this flask culture, approximately 11% of fed diketide thioester **2** was incorporated into 14,15-dehydro-6-dEB, 34% remained in the culture, and more than half of the initial amount was degraded.

To rule out the possibility of medium induced hydrolysis, the stability of the diketides was tested in sterile R6 medium (data not shown). Diketide thioesters **1** and **2** were found to be stable for 5 days at 30 °C at pH 6.0 to 7.5, with some degradation occurring at pH 8.0 (<5% per day). The diketides would thus be expected to be stable under fermentation conditions (pH 6.4−7.6 in flasks). To examine the possibility of an extracellular enzyme catalyzing diketide degradation, stability tests included using sterile-filtered broth from a late stage production culture (pH 6.5), again showing **1** and **2** to be stable. Although diketide thioester **3** was not evaluated in these experiments, the rate of disappearance of this compound in culture is similar to that of the other diketide precursors. Finally, a strain comparison was performed to determine whether DEBS thioesterase activity could be responsible for diketide degradation. The rate of degradation of **2** was found to be the same with the host strain CH999 as with the recombinant CH999/pJRJ2 (data not shown), pointing to enzyme activities other than DEBS thioesterase as the cause of diketide degradation.

It has been suggested previously that the diketide substrates are degraded through oxidation enzymes and that supplying β-oxidation inhibitors in the growth medium improves incorporation of synthetic intermediates into polyketides (Yoshizawa et al., 1990; Li et al., 1992). While an exhaustive screening of β-oxidation inhibitors was not undertaken in this study, the addition

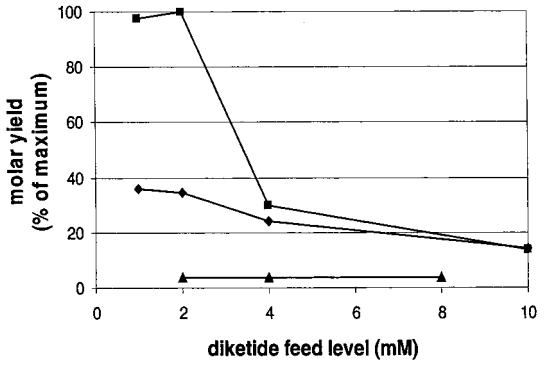

Figure 7. Overall process yield depends on diketide feed level and diketide functionality. Data are taken from the same experiment represented in Figure 4. 13-R-6-dEB yields from diketide thioesters **1** (■), **2** (◆), and **3** (▲) are shown. Molar yield is defined as moles product produced per mole diketide substrate initially fed to the culture.

of 25 or 75 mg/L 4-pentynoic acid to cultures at the time of diketide feeding was found to affect neither the rate of diketide degradation nor the production of R-6-dEB (data not shown).

While degradation rates were similar for the three diketides used in this study, their rates of incorporation into product were different (Figure 4). Because the synthetic diketides contribute significantly to process cost, it is of interest to look at the overall process yield. Data from flask cultures shown in Figure 4 were processed to determine molar yield from the diketides (Figure 7). Although culture productivity generally increased with increasing precursor feed concentration, there appears to be a tendency toward lower molar yield at higher feed levels, suggesting that alternative feeding strategies may improve the compromise between productivity and yield. Moreover, the differences in yield due to changes in precursor functional groups are striking.

Toward a General Process for Production of Erythromycin Analogues. The process described here for synthesizing 6-dEB analogues is currently employed as part of an overall process for preparation of the corresponding erythromycin analogues. It is clear that, despite some general trends, the precursor-directed biosynthesis process needs to be characterized for each individual precursor. The overall process requires the 13-R-6-dEB produced by *S. coelicolor* CH999/pJRJ2 in a primary fermentation to be converted to 13-R-erythromycin by an organism such as *S. erythraea* in a second fermentation. Jacobsen et al. (1997) have demonstrated such bioconversion for several 6-dEB analogues. Although feeding diketide directly to a *S. erythraea* KS1° mutant would eliminate one fermentation en route to erythromycins, the problem of diketide degradation seems to be more severe with this organism as compared to *S. coelicolor* (Cane et al., 1995; Frykman et al., manuscript in preparation). In all cases, a solution to the substrate degradation problem is likely to make any potential process more feasible.

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References and Notes

Cane, D. E.; Luo, G.; Khosla, C.; Kao, C. M.; Katz, L. Highly efficient incorporation of polyketide chain elongation intermediates into 6-deoxyerythronolide B in an engineered streptomyces host. *J. Antibiot.* **1995**, *48*, 647−651.

Cane, D. E.; Luo, G. Biosynthesis of polyketide antibiotics. Incorporation of a pentaketide chain elongation intermediate into nargenicin. *J. Am. Chem. Soc.* **1995**, *117*, 6633−6634.

Chuck, J.; McPherson, M.; Huang, H.; Jacobsen, J. R.; Khosla, C.; Cane, D. E. Molecular recognition of diketide substrates by a β-ketoacyl-acyl carrier protein synthase domain within a bimodular polyketide synthase. *Chem. Biol.* **1997**, *4*, 757−766.

Jacobsen, J. R.; Hutchinson, C. R.; Cane, D. E.; Khosla, C. Precursor-directed biosynthesis of erythromycin analogues by an engineered polyketide synthase. *Science* **1997**, *227*, 367−369.

Jacobsen, J. R.; Keatinge-Clay, A. T.; Cane, D. E.; Khosla, C. Precursor directed biosynthesis of 12-ethyl erythromycin. *Bioorg. Med. Chem.* **1998**, *6*, 1171−1177.

Kao, C. M.; Katz, L.; Khosla, C. Engineered biosynthesis of a complete macrolactone in a heterologous host. *Science* **1994**, *265*, 509−512.

Li, Z.; Martin, F. M.; Vederas, J. C. Biosynthetic incorporation of labeled tetraketide intermediates into dehydrocurvularin, a phytotoxin from *Alternaria cinerariae*, with assistance of β-oxidation inhibitors. *J. Am. Chem. Soc.* **1992**, *114*, 1531−1533.

O'Hagan, D. Biosynthesis of fatty acid and polyketide metabolites. *Nat. Prod. Rep.* **1995**, *12*, 1−32.

Tsantizos, Y. S.; Zhou, F.; Famili, P.; Yang, X. Biosynthesis of the hypotensive metabolite oudenone by *Oudemansiella radicata*. 1. Intact incorporation of a tetraketide chain elongation intermediate. *J. Org. Chem.* **1995**, *60*, 6922−6929.

Tsukamoto, N.; Chuck, J.; Luo, G.; Kao, C. M.; Khosla, C.; Cane, D. E. 6-deoxyerythronolide B synthase 1 is specifically acylated by a diketide intermediate at the β-ketoacyl-acyl carrier protein synthase domain of module 2. *Biochemistry* **1996**, *35*, 15244−15248.

Weissman, K. J.; Bycroft, M.; Cutter, A. L.; Hanefeld, U.; Frost, E. J.; Timoney, M. C.; Harris, R.; Handa, S.; Roddis, M.; Staunton, J.; Leadlay, P. F. Evaluating precursor-directed biosynthesis towards novel erythromycins through in vitro studies on a bimodular polyketide synthase. *Chem. Biol.* **1998**, *5*, 743−754.

Yoshizawa, Y.; Li, Z.; Reese, P. B.; Vederas, J. C. Intact incorporation of acetate-derived di- and tetraketides during biosynthesis of dehydrocurvularin, a macrolide phytotoxin from *Alternaria cinerariae*. *J. Am. Chem. Soc.* **1990**, *112*, 3212−3213.

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