Biotransformation

Enantioselective Reduction of β-Keto Acids with Engineered *Streptomyces coelicolor***

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The soil bacterium *Streptomyces coelicolor* has long been studied owing to its production of antibiotics, the most significant of which is the dimeric benzoisochromanequinone

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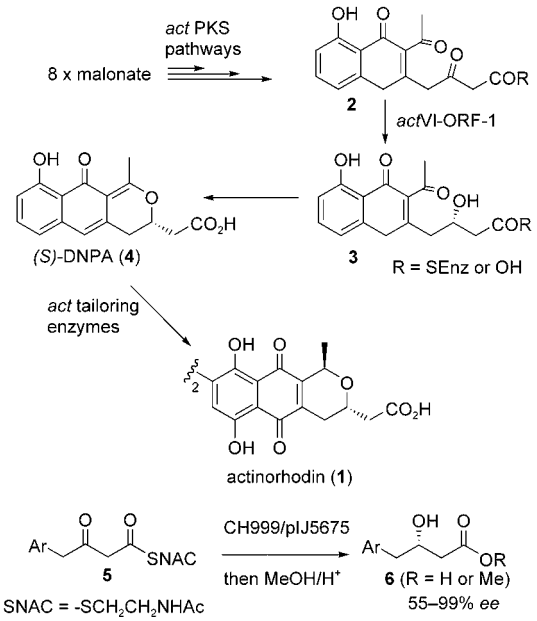
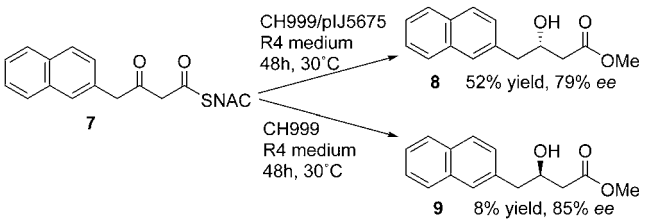
actinorhodin (ACT, **1**). *S. coelicolor* is generally regarded as the best genetically characterized of all the streptomycete bacteria. Recently its complete genomic sequence was published.[1] Over the years a clear picture has emerged for the biosynthesis of the skeleton of **1**.[2] It is generally accepted that **2** (or the phenol tautomer of **2**) is assembled from eight malonate units by a type II polyketide synthase (PKS). Whether **2** is released at this stage from the PKS as the free β-keto acid (R = OH) or is subject to further biotransformations as the enzyme-bound thiolate (R = SEnz) is a matter for speculation (see below). Reduction of **2** is thought to lead to the hydroxy acid **3**, which then undergoes cyclization followed by dehydration to give the yellow pigment (*S*)-DNPA (**4**, 4,10-dihydro-9-hydroxy-1-methyl-10-oxo-3*H*-naphtho[2,3*c*]pyran-3-(*S*)-acetic acid). Significantly, **4** has been isolated from both mutant[3] and recombinant[4] strains of *S. coelicolor*, thus strongly implicating its role as a true intermediate in ACT biosynthesis. It is thought that **4** then undergoes a series of enzyme-controlled reduction, oxidation, hydroxylation, and dimerization steps, which result in the formation of **1** (Scheme 1). A number of genes thought to encode the



Scheme 1. Biosynthesis of actinorhodin 1 and feeding studies with SNAC derivatives.

various enzymes have been identified (*act* tailoring genes), but at this stage the likely intermediates in the biosynthesis of **4** have yet to be confirmed. The reduction of **2** is believed to occur by the action of a reductase (RED1) encoded by the *act*VI-ORF-1 gene. Good evidence for this hypothesis was first obtained by Floss and co-workers,[3] who found that the B1 mutant strain of *S. coelicolor* accumulated significant quantities of **4**. More recently we showed[4] that a recombinant strain (CH999/pIJ5660) of *S. coelicolor* containing *act*PKS and *act*VI-ORF-1 genes resulted in the production of **4** on fermentation in a liquid medium. A further strain was engineered from the plasmid pIJ5660, from which the ketosynthase (KS) gene *act*I-ORF-1 had been deleted. This new strain (CH999/pIJ5675) did not have the ability to synthesize the polyketide skeleton from which **2** is derived, but could still express RED1 and thus serve as a whole-cell reduction medium. A number of *N*-acetylcysteamine β-ketothioester (SNAC) substrates were fed to this strain. They underwent enantioselective reduction to the corresponding β-hydroxy acids with moderate to high *ee* values (analyzed as the methyl esters **6**, R = Me). The fact that a range of nonnatural SNAC derivatives proved to be substrates for RED1, but that other β-ketoesters were not, provided convincing evidence at the time for the likely existence of **2** as the thiolate (R = SEnz). These results led us to conclude that "NAC thioesters are required for recognition by the *act*VIORF-1 reductase and/or they are required for successful transport across the cell membrane".[5] Herein we present results from further study that demonstrate that this statement is likely to be incorrect and that CH999/pIJ5675 is a much more general biotransformation system than previously thought.

In our previous studies on biotransformations with CH999/pIJ5675, it was found that some of the SNAC substrates underwent reduction with only moderate enantioselectivities. For example, the naphthyl substrate **7** was produced with just 55% *ee*. This low value was initially proposed to be a result of a lack of stereoselectivity of RED1. Previously we determined *ee* values by converting the reduced products into Mosher ester derivatives and analyzing the diastereomers by NMR spectroscopy. On reinvestigation of this reduction with a number of *act* strains it was found that the *ee* values of the derived hydroxyesters could be determined more reliably by HPLC on a chiral phase.[6] For example, the reduction of **7** with CH999/pIJ5675, followed by esterification, gave the *S* β-hydroxyester **8** as before, but the *ee* value was determined more accurately to be 79%, a result that was confirmed by repeated biotransformations. We were intrigued to find that the biotransformation of **7** with the host CH999 as a control gave the *R* β-hydroxyester **9** with 85% *ee* in consistently low chemical yield (Scheme 2). The fact that

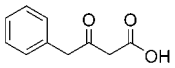
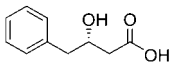
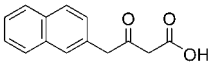
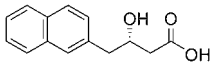
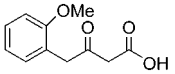
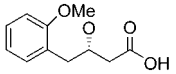
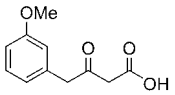
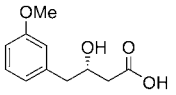
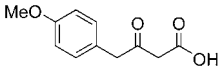
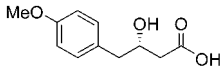
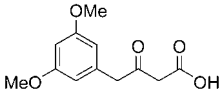
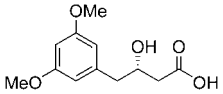
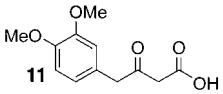
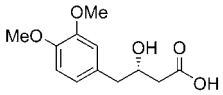
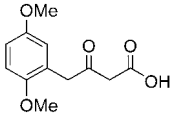
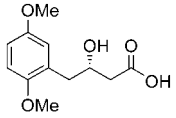
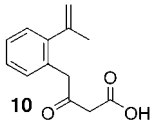
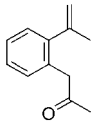
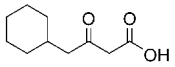
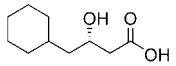
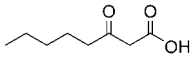
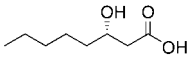


Scheme 2. Reduction of **7** with CH999/pIJ5675 and CH999.

the opposite absolute stereochemistry predominates in this case indicates that **7** is reduced by one or more non-*act* reductases within CH999. It is therefore very likely that the less-than-optimal *ee* values obtained previously with CH999/pIJ5675 and SNAC derivatives can be explained by the action of a non-*act* reductase, which competes with RED1 for the SNAC substrates.

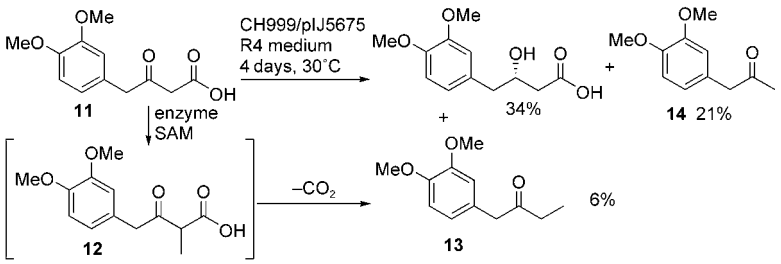
At about this time we were able to demonstrate[7] the functional expression of RED1 in *E. coli* and were intrigued to find that the reduction of **5** (Ar = Ph) appeared to proceed, following initial hydrolysis, to give the free β-keto acid **6**. This result prompted us to embark on an investigation into the generality of the CH999/pIJ5675 strain as a whole-cell biotransformation system for the enantioselective reduction of β-keto acids to the corresponding *S* β-hydroxy acids (Table 1). A range of β-keto acids were prepared by hydrolysis of the corresponding methyl esters, which themselves were prepared by general routes that we used previously for the synthesis of SNAC derivatives. These β-keto acids were then fed to CH999/pIJ5675 as the free acids and/or the sodium salts (0.8-mmol scale). Not unexpectedly, many of the β-keto acids proved to be labile towards decarboxylation, and in certain cases better results were obtained with the sodium salts. However, many of the lower yields were a reflection of unavoidable decarboxylation rather than the efficiency of the reductase itself, as is clear when yields are expressed based on recovered decarboxylated ketone. RED1 proved to be very tolerant of methoxy substitution at various positions on the aryl ring. Only when an alkenyl substituent was introduced at the *ortho* position (in **10**, Table 1, entry 9) was no reduction observed. This alkenyl compound **10** was prepared with the aim of synthesizing an analogue of **2** with an aryl methyl ketone moiety. Unfortunately, all attempts to convert **10** into the corresponding ketone (e.g. ozonolysis) proved fruitless, as the resulting product underwent spontaneous cyclization to a naphthyl derivative. It is reasonable to speculate that the failure of **10** to undergo reduction could be explained by the fact that the RED1 active site is likely to possess a binding domain that recognizes the polar methyl ketone residue in **2** rather than the lipophilic alkenyl group in **10**.[8] However, it was encouraging to note that the saturated cyclohexyl-substituted β-keto acid (Table 1, entry 10) and the *n*-pentyl-substituted β-keto acid (Table 1, entry 11) underwent clean reduction to the corresponding *S* hydroxy acids with high enantioselectivity. It is particularly interesting to observe that this latter β-keto acid has been reported to undergo reduction to the *R* hydroxy acid with baker's yeast.[9] These examples demonstrate the potential of RED1 for the reduction of non-aryl or non-actinorhodin-like substrates. In all cases biotransformation to the β-hydroxy acids occurred with essentially complete stereoselectivity, as evidenced by HPLC on a chiral phase and by comparison with synthetic samples of the racemates. The absolute stereochemistry was found to be *S* in all cases, as proved by correlation either with known compounds or with the *R* enantiomers, which were synthesized by Noyori hydrogenation[10] of the appropriate β-ketoesters.

Table 1: Enantioselective reduction of β-keto acids with engineered *S. coelicolor* (CH999/pIJ5675).

Entry	Substrate	Product	Yield [%]	*ee* [%]
1[b]			80	>99
2			77	97
3			24 (42)[a]	99
4[b]			61 (72)[a]	99
5			43 (59)[a]	98
6[b]			40 (56)[a]	98
7			34 (45)[a]	>99
8[b]			59 (80)[a]	99
9[b]			41[c]	
10			54	99
11[d]			52	>95

[a] Based on recovered decarboxylated ketone. [b] Fed as sodium salts. [c] In addition to 25% recovered **10.** [d] *ee* value determined by NMR spectroscopic analysis of Mosher ester derivative.

Reduction of the β-keto acid **11** also proved interesting. Along with the hydroxy acid and the decarboxylated ketone **14**, the ethyl ketone **13**[11] was obtained consistently as a minor component (5–6% yield). This ethyl ketone is probably formed by the adventitious action of an (*S*)-adenosylmethionine(SAM)-dependent methyltransferase on the β-keto acid **11**. It is likely that **11** is methylated to give **12**, which then undergoes decarboxylation to provide **13** (Scheme 3). No trace of the proposed methylated β-keto acid **12** was ever detected, despite several repeats of this biotransformation.



Scheme 3. Proposed ethyl ketone formation with SAM-dependent methyltransferase.

This methyltransferase is probably encoded somewhere on the CH999 host genome.[12] Strong evidence in favor of this hypothesis was found when **11** was incubated with the CH999 host strain and only **14** (26%) and **13** (5%) were obtained. No ethyl ketone products were detected from any of the biotransformations carried out with the other substrates in this study, thus suggesting that this methyltransferase has a high degree of substrate specificity.

In summary, a variety of aryl- and alkyl-substituted β-keto acids have been shown to be excellent substrates for RED1 from *S. coelicolor*. The products are formed in reasonable to good yields with complete selectivity for the *S* enantiomer. These results demonstrate that the engineered *S. coelicolor* strain CH999/pIJ5675 is a useful whole-cell biotransformation system for the reduction of β-keto acids with an enantioselectivity that is opposite to that of baker's yeast. Furthermore, these results, in conjunction with protein functional expression studies, provide the first clear evidence that the free β-keto acid **2** (R = OH) is the likely RED1 substrate in the biosynthesis of **1**, rather than an enzyme-bound β-ketothioester (**2**, R = SEnz) as previously thought. In vitro studies with purified RED1 are ongoing, and the results will be reported in the near future.

Experimental Section

Typical procedure: A suspension of CH999/pIJ5675 spores suspended in glycerol (15 μl) and thiostrepton solution (30 mM in DMF, 15 μl) was added to an R4 medium (10 mL) in a 25-mL universal tube under sterile conditions. The solution was incubated on an orbital shaker (30 °C, 220 rpm) for 3 days to form a seed culture. Six 500-mL conical flasks were then each charged with an R4 medium (50 mL), thiostrepton solution (5 μL), and seed culture (3 mL). A single flask containing CH999/pIJ5660 spores[4] was prepared in a similar way to be incubated simultaneously as an indicator of secondary-metabolite production. The cultures were incubated on a shaker (30 °C, 220 rpm) until the indicator had turned dark brown (approximately 2 days). 4-(Naphthalen-2-yl)-3-oxobutyric acid (0.18 g, 0.79 mmol) was dissolved in methanol (6 mL), and 1 mL of this solution added to each of the six flasks of CH999/pIJ5675 culture, which were then returned to the shaker and incubated for a further 4 days. The resulting solutions were filtered to remove cell debris, combined, and acidified to pH 3. The solution was extracted with ethyl acetate (3 × 100 mL), and the combined organic extracts were washed with water (2 × 50 mL) and brine (2 × 50 mL). The extracts were dried ($MgSO_4$), and the solvent was removed in vacuo to yield a brown solid. Purification by flash chromatography (eluant: 70% ethyl acetate/40–60 petroleum ether) gave (*S*)-3-hydroxy-4-(naphthalen-2-yl)butyric acid as a white solid (0.14 g, 77%). M.p.: 126–127 °C (lit.: 126–128 °C[13]); $[\alpha]_D$ +1.17 ($c = 1.03$, $CHCl_3$); IR (neat): $\tilde{\nu}_{max} = 3406, 2923, 1695\ cm^{-1}$; ^1H NMR (400 MHz, CD_3OD): $\delta = 2.56$ (dd, $J = 10.4$, 5.3 Hz, 1H, C*H*CO_2H), 2.63 (dd, $J = 10.4$, 2.3 Hz, 1H, C*H*CO_2H), 2.98 (dd, $J = 8.5$, 3.9 Hz, 1H, C*H*Ar), 3.03 (dd, $J = 8.5$, 4.4 Hz, 1H, C*H*Ar), 4.33–4.41 (m, 1H, C*H*OH), 7.29 (dd, $J = 5.3$, 0.9 Hz, 1H, ArH), 7.31–7.36 (m, 2H, Ar*H*), 7.59 (s, 1H, ArH), 7.67–7.71 ppm (m, 3H, ArH); ^{13}C NMR (100 MHz, CD_3OD, Me_4Si): $\delta = 40.9$ (*C*H_2CO_2H), 43.8 (*C*H_2Ar), 69.8 (*C*HOH), 126.0 (CH), 126.2 (CH), 126.3 (CH), 127.7 (CH), 127.9 (CH), 128.1 (CH), 128.3 (CH), 133.3 (C), 134.7 (C), 138.8 (*C*CH_2), 175.3 ppm (CO_2H); MS (EI): *m/z*: 230 ([*M*]$^+$, 50%), 212 (34), 167 (25), 142 (100), 128 (15), 115 (46), 89 (29), 83 (55), 71 (41), 63 (15).

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Keywords: bioorganic chemistry · biosynthesis · enantioselectivity · enzymes · reduction

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[6] Chiralcel OD column; heptane/2-PrOH/trifluoroacetic acid (90:10:0.1) for acids; heptane/2-PrOH (9:1) for esters.

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[11] Selected data for **13**: IR (neat): $\tilde{\nu}_{max} = 2926, 2853, 1713\ cm^{-1}$; ^1H NMR (400 MHz, $CDCl_3$, Me_4Si): $\delta = 1.02$ (t, $J = 4.6$ Hz, 3H, CH_3), 2.47 (q, $J = 4.6$ Hz, 2H, CH_2), 3.62 (s, 2H, CH_2), 3.87 (6H, $2 \times CH_3$), 6.71 (d, $J = 1.2$ Hz, 1H, ArH), 6.75 (dd, $J = 5.2$, 1.2 Hz,

1H, ArH), 6.84 ppm (d, $J = 5.2$ Hz, 1H, ArH); ^{13}C NMR (100 MHz, $CDCl_3$, Me_4Si): $\delta = 7.7$ (CH_3), 35.0 (CH_2CH_3), 49.4 (CH_2CO), 55.8 (CH_3OAr), 55.9 (CH_3OAr), 112.2 (CH), 112.4 (CH), 121.3 (CH), 126.9 (CCH_2), 148.0 ($COCH_3$), 149.0 ($COCH_3$), 209.4 ppm (CO); MS (EI): m/z: 208 ($[M]^+$, 12%), 151 (82), 107 (6), 87 (11), 83 (100), 57 (11).

[12] Two putative methyltransferases (ORFs (open reading frames) SCO2170 and SCO2098) have been identified on the *S. coelicolor* genome (from reference [1]).

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