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Synthesis of ethyl and *t*-butyl (3*R*,5*S*)-dihydroxy-6-benzyloxy hexanoates via diastereo- and enantioselective microbial reduction

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Abstract—Previously we have demonstrated the reduction of ethyl diketoester **4** to the corresponding dihydroxy ester **6a** by *Acinetobacter* sp. SC13874. Recently we screened more than 100 cultures for microbial reduction of both the ethyl and *t*-butyl diketoesters **4** and **5**. Most yeast cultures showed a preference for reduction at the C-3 with low enantioselectivity. Among the three *Acinetobacter* strains screened, *Acinetobacter* sp. SC13874 reduced both compounds **4** and **5** to the corresponding (3*R*)- and (5*S*)-monohydroxy compounds. Monohydroxy compounds were isolated and their absolute configurations determined. (3*R*)- and (5*S*)-Monohydroxy compounds were reduced further to the corresponding dihydroxy esters **6a** and **8a** to provide alternate routes for the synthesis of compounds **14a** and **16a**, potential intermediates for the synthesis of HMG-CoA reductase inhibitors. Cell suspensions of *Acinetobacter* sp. SC13874 reduced the ethyl diketoester **4** to a mixture of desired *syn* and undesired *anti* diastereomers. The desired *syn*-(3*R*,5*S*)-dihydroxy ester **6a** was obtained with an enantiomeric excess (ee) of 99% and a diastereomeric excess (de) of 63%. Cell suspensions reduced the *t*-butyl diketoester **5** to a mixture of mono- and dihydroxy esters with the dihydroxy ester showing an ee of 87% and de of 51% for the desired *syn*-(3*R*,5*S*)-dihydroxy ester **8a**. Three different ketoreductases were purified to homogeneity, and their biochemical properties compared. Reductase I only catalyzes the reduction of ethyl diketoester **4** to its monohydroxy products **10** and **11**, whereas reductase II catalyzes the formation of dihydroxy products **6** and **7** from monohydroxy substrates **10** and **11**. A third reductase (III) was identified, which catalyzes the reduction of diketoester **4** to *syn*-(3*R*,5*S*)-dihydroxy ester **6a**.

1. Introduction

Ketoreductases are present in various bacteria, yeast, and fungi. Largely due to their high enantioselectivity, ketoreductases have been recognized and utilized as an important class of enzymes for biocatalytic applications in the chemical and pharmaceutical industries for the preparation of chiral alcohols.[1–7] Chiral β-hydroxy esters are useful intermediates for synthesizing bioactive chiral compounds. Biocatalytic reduction of ketoesters by ketoreductases offers an attractive route to optically pure β-hydroxy esters. Previously, we have shown that *Acinetobacter* sp. SC13874 contains a ketoreductase capable of stereoselectively reducing 3,5-dioxo-6-(benzyloxy)hexanoic acid ethyl ester **4** (Scheme 1) to its corresponding *syn*-diol **6a**.[8] In the present study, we have demonstrated reduction of both ethyl and *t*-butyl diketoesters. We have isolated corresponding monohydroxy compounds and established their absolute configurations. A more detailed investigation revealed that there are multiple ketoreductase enzyme systems with different properties for catalyzing the reduction of compound **4** to monohydroxy compounds **10** and **11** and the reduction of monohydroxy compounds **10** and **11** to dihydroxy compound **6a**. Three reductases with different substrate specificities in this microorganism have been purified to homogeneity and some of their biochemical properties have been characterized.

Kaneka alcohol **16a** (Scheme 1) with its two stereogenic centers is an essential building block for HMG-CoA reductase inhibitors.[9–13] Synthetic methods to prepare the Kaneka alcohol have attracted intensive attention.[14–16] Microbial reduction of the ethyl and *t*-butyl diketoesters **4** and **5** to the corresponding dihydroxy esters **6a** and **8a** could provide an alternate route to the Kaneka alcohol related compound **14a** and Kaneka alcohol **16a** itself. Herein we report an investigation of the microbial reduction of the ethyl and *t*-butyl diketoesters **4** and **5** by *Acinetobacter* sp. SC13874 and other microbial systems. The separation and purification of various ketoreductases from *Acinetobacter* sp. SC13874 are also reported.

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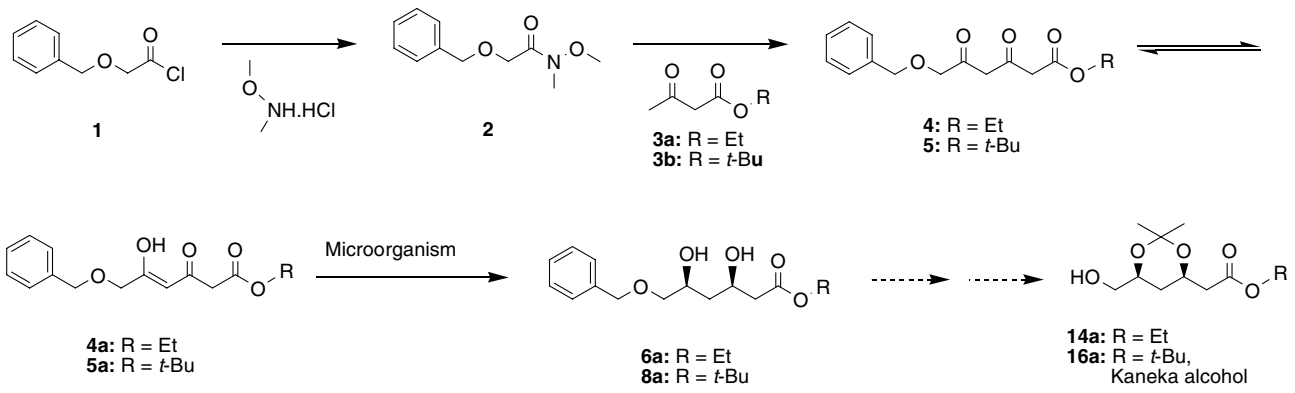


Scheme 1. Synthesis of Kaneka alcohol **16a** and ethyl ester **14a** via microbial reduction.

2. Results and discussion

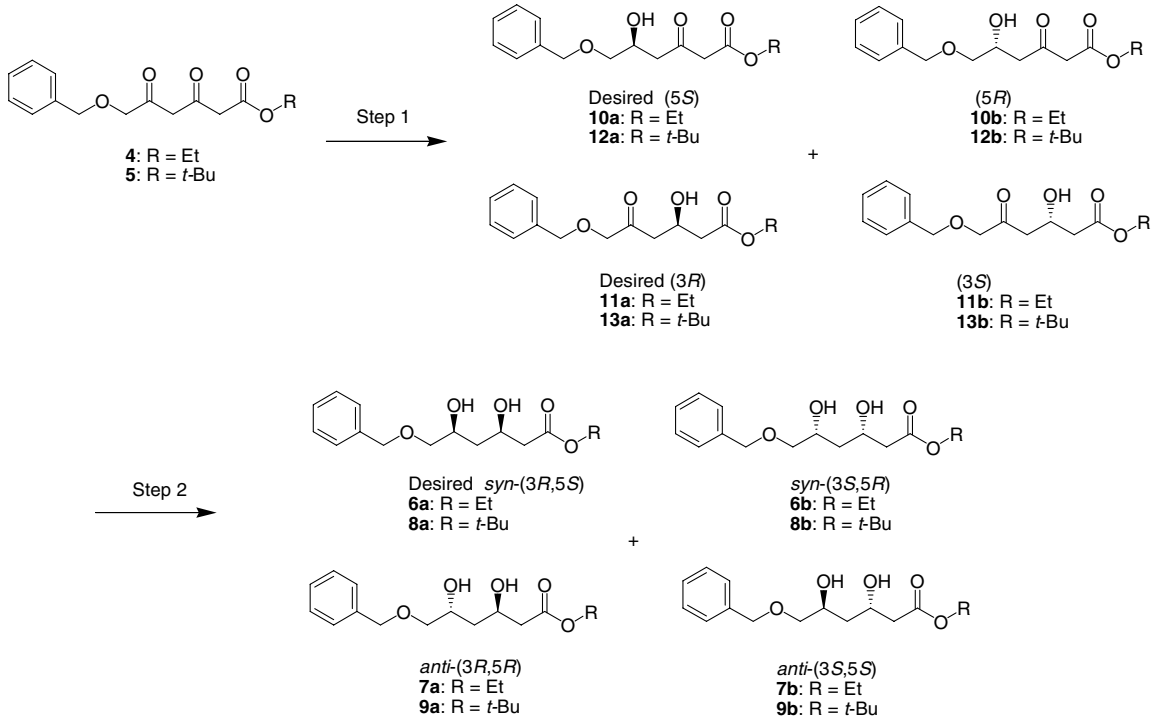
2.1. Chemical syntheses of the compounds in the ethyl ester series

Ethyl diketoester **4** was synthesized in two steps. The literature[16,17] procedure was modified. The first step gave compound **2** in 95% yield. Reaction of compound **2** with ethyl acetoacetate, NaH and butyl lithium gave ethyl diketoester **4** in 97% yield with HPLC AP 94. ^{1}H NMR (in $CDCl_3$) showed a 6:1 ratio of keto–enol form **4a**:diketo form **4** (Scheme 1).

(±)-*syn*-Dihydroxy ester **6** was prepared in 82% yield by reduction of compound **4** with diethyl(methoxy)borane and sodium borohydride. The mixture of four isomers, **6a**, **6b**, **7a**, and **7b**, was prepared by reduction of compound **4** with sodium borohydride to give a ratio of 4:1 for *syn*-**6**:*anti*-**7**. Chiral HPLC methods were developed to separate all four isomers (Scheme 2).

The monohydroxy ethyl ester mixture of four isomers (**10a**, **10b**, **11a**, and **11b**) was prepared by partial reduction of compound **4** with sodium borohydride. After flash chromatography, a mixture of racemic 3-keto-5-hydroxy-ester (±)-**10** (85%) and racemic 3-hydroxy-5-ketoester (±)-**11** (15%) was obtained. Further purification by preparative HPLC provided (±)-**10** with AP of 98 and (±)-**11** with AP of 99. Chiral HPLC methods were developed to separate all four monohydroxy isomers.

In order to evaluate the separation of the *syn*- and *anti*-isomers, a mixture of ethyl 3,5-dihydroxyacetonide-6-benzyloxyhexanoate **19** was prepared from a mixture of *syn*-**6**



Scheme 2. Chemical and microbial reduction of diketoesters.

and *anti*-**7** reacted with 2,2-dimthoxypropane. However, the separation of *syn*- and *anti*-acetonides was found to be more difficult than the separation of the *syn*- and *anti*-dihydroxy esters.

2.2. Chemical syntheses of compounds in the *t*-butyl ester series

The products can be directly converted to the Kaneka alcohol when R = *t*-butyl (Scheme 1). To evaluate this route, chemical syntheses of the starting material and product markers were carried out in the same way as those for the ethyl esters. During the synthesis of compound **5**, the product partially decomposed, probably due to aldol condensation. Pure product **5** was obtained in 9% yield by flash chromatography under basic condition with triethylamine (TEA). ^{1}H NMR (in $CDCl_3$) showed a 7:1 ratio of keto–enol form **5a**:diketo form **5** (Scheme 1).

Reduction of compound **5** with diethyl(methoxy)borane and sodium borohydride was performed under similar conditions for making compound **6** except that more reducing agent was needed. The ratio of *syn*-**8**:*anti*-**9** was found to be about 4:1. Chiral HPLC methods were developed to separate all four isomers **8a**, **8b**, **9a**, and **9b** (Scheme 2).

The monohydroxy *t*-butyl ester mixture of four isomers **12a**, **12b**, **13a**, and **13b** was prepared by partial reduction of compound **5** with DIBALH. (±)-5-Monohydroxy **12** was separated from (±)-3-monohydroxy **13** by preparative HPLC. All four isomer peaks in the chiral HPLC were tentatively assigned assuming that enantiomers of both the ethyl and *t*-butyl monohydroxy esters would show a similar mobility order in the same chiral HPLC system.

2.3. Microbial reduction of ethyl 3,5-diketo-6-benzyloxyhexanoate 4 to the ethyl dihydroxy ester

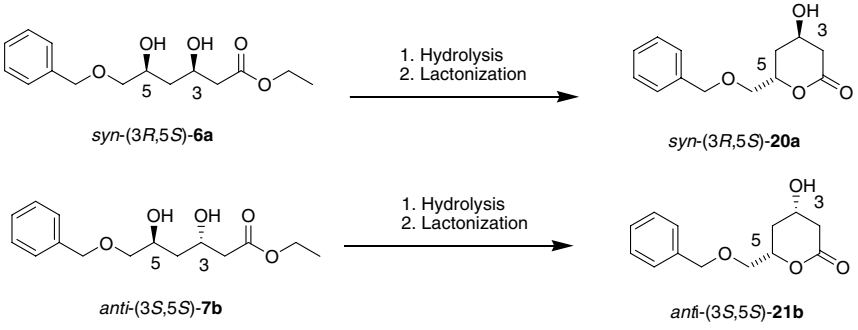
The reduction of ethyl diketoester **4** was carried out with cells of *Acinetobacter* sp. SC13874. The *syn*-**6** and *anti*-**7** dihydroxy esters were formed in a ratio of about 87:13, 83:17, 76:24 after 24 h at 2, 5, and 10 g/L of substrate input, respectively. There was no significant peak due to a monohydroxy ester. Chiral HPLC determined that the desired (3*R*,5*S*)-**6a** was the major product with 99.4% ee. Almost complete (>95%) conversion of the ethyl diketoester **4** to dihydroxy ester in 24 h was seen up to a substrate concentration of 10 g/L and cell concentration of 200 g/L. It was demonstrated that dihydroxyesters **6** and **7** could be adsorbed from the medium by XAD-16 resin after the reaction, thereby facilitating the recovery process. Only a negligible amount (0–2%) remained in the aqueous phase after the adsorption. Extraction of the XAD-16 by an organic solvent provided very high recovery of the product (83–100%).

2.4. Growth of *Acinetobacter* sp. SC13874 in pilot plant

In order to run larger scale reactions and isolate products, cells of *Acinetobacter* sp. SC13874 were grown in the pilot plant. Cells harvested after 24 h in the pilot plant showed lower activities for reduction than those grown for 12 and 18 h in the pilot plant. The shorter growth time was shown to provide better cells in terms of activity and product purity, although enantioselectivity was high in all cases, providing an ee of ⩾99% for the desired dihydroxy ester **6a**. The cell yields were lower at 12 h, as expected. These cells were used for preparative reduction of diketoesters **4** and **5** and also for the purification of enzymes.

2.5. Microbial reduction of 2.5 g of the ethyl diketoester 4 and the absolute configurations of the ethyl dihydroxy esters

The reduction of 2.5 g of the ethyl diketoester **4** with 50 g of *Acinetobacter* sp. SC13874 cells showed complete conversion of **4** in 24 h and provided 2.1 g of a yellow oil containing the dihydroxy ester **6a** as the major product with AP 40, de 63.3%, ee 99.3%. The crude microbial reduction product was subjected to repeated flash chromatography and crystallization to provide pure *syn*-**6a** (750 mg, AP 98, de 99%, ee 99%) and pure *anti*-**7b** (60 mg, AP 98, ee 97%, de >99%). Pure **6a** was converted to the corresponding known lactone **20a**, $[\alpha]_D = +9.0$ (*c* 0.40, $CHCl_3$). The absolute configuration of *syn*-(3*R*,5*S*) was determined by comparison of the specific rotation data, $[\alpha]_D = +6.5$ (*c* 1.56, $CHCl_3$), for **20a** *syn*-(3*R*,5*S*) reported in the literature (Scheme 3).[8,18] Similarly, pure **7b** was converted to the corresponding known lactone **21b**, $[\alpha]_D = +18.1$ (*c* 0.21, $CHCl_3$). The absolute configuration of *anti*-(3*S*,5*S*) was determined by comparison of the specific rotation data, $[\alpha]_D = +13.9$ (*c* 1.06, $CHCl_3$), for **21b** *anti*-(3*S*,5*S*) reported in the same literature.



Scheme 3. Conversion of microbial reduction products to known lactones.

2.6. Determination of the absolute configurations of the ethyl monohydroxy esters

To determine the absolute configurations of the ethyl monohydroxy esters, a mixture obtained in Section 2.1 containing 85% of (±)-**10** and 15% of (±)-**11** was analyzed by chiral HPLC method-3 (Section 4.4). The peaks at 43.5 and 41.2 min were due to the two enantiomers of 3-keto-5-monohydroxy (±)-**10** while those at 36.4 and 38.3 min were due to the two enantiomers of 5-keto-3-monohydroxy (±)-**11**.

A mixture of the ethyl monohydroxy esters containing negligible amounts of diketoester and dihydroxy esters was obtained during the screening of the microorganisms by recombinant *E. coli* R-Buspar ketoreductase (Section 2.9). To determine the absolute configurations of each monohydroxy ester peak in the chiral HPLC method-3, the above mixture was used as the starting material to be completely reduced to dihydroxy esters by SC13874 cells. The absolute configurations of the dihydroxy esters had already been established (Section 2.5). The results are listed in Table 1.

Assuming there is no inversion at any existing stereogenic centers, each monohyroxy ester isomer could provide two dihydroxy ester isomers, while each dihydroxy ester isomer could come from two monohydroxy ester isomers. In chiral HPLC Method-3, the peak at 38.3 min (85%) was the major source of the major product (3*S*,5*S*)-**7b** (83%) and its absolute configuration established as (3*S*)-**11b**. The peak at 36.4 min must be from the other 3-monohydroxy, (3*R*)-**11a**. The product (3*R*,5*R*)-**7a** (9%) could come from (3*R*)-**11a** (1%) and one of the two 5-hydroxy ester enantiomers. Since the peak at 43.5 min was only 1%, the peak at 41.2 min (13%) had to be the other source of (3*R*,5*R*)-**7a**. The peak at 41.2 min was therefore established as (5*R*)-**10b**, and the peak at 43.5 min the other 5-monohydroxy, (5*S*)-**10a**. The results also showed that microbial reduction by SC13874 cells of 3-hydroxy-5-ketoester proceeds with (5*S*)-selectivity while reduction of 3-keto-5-hydroxy ester proceeds with (3*R*)-selectivity.

2.7. Microbial reduction of the ethyl diketoester 4 to the ethyl monohydroxy esters by SC13874 cells

Microbial reduction to make the intermediate monohydroxy esters was evaluated using a lower cell to substrate ratio. Reduction of 0.1 g of ethyl diketoester **4** with 1 g of *Acinetobacter* sp. SC13874 cells mostly provided the monohydroxy esters. After 48 h, HPLC showed a relative ratio of 1.5% (*syn*-**6**), 0.4% (*anti*-**7**), 12% (5-hydroxy-3-keto **10**), 50% (3-hydroxy-5-keto **11**), and 36% (diketo-**4**). Chiral HPLC showed a relative ratio of the monohydroxy esters as 6% (5*S*-**10a**), 5% (5*R*-**10b**), 48% (3*R*-**11a**), and 41% (3*S*-**11b**). The results indicated that the *Acinetobacter* sp. SC13874 cells preferred the reduction at the 3-position over the 5-position of the diketoester. Both 5-hydroxy-3-keto **10** and 3-hydroxy-5-keto **11** obtained were nearly racemic, suggesting that the first reduction step showed negligible enantiomeric preference under the low cell to substrate ratio conditions.

2.8. Microbial reduction of the ethyl monohydroxy esters to the dihydroxy esters by *Acinetobacter* sp. SC13874 cells

A mixture of ethyl 3-keto-5-hydroxy (major) and 5-keto-3-hydroxy (minor) was obtained from partial microbial reduction. Another mixture of 3-keto-5-hydroxy (minor) and 5-keto-3-hydroxy (major) was obtained from partial chemical reduction. These two mixtures were subjected to microbial reduction by SC13874 cells for 6 h (not complete). The reduction provided the dihydroxy esters with the isomeric compositions listed in Table 2. The results indicated that the second reduction of the monohydroxy compound by SC13874 cells was quite enantiospecific. Reduction of the 3-keto-5-hydroxy predominantly provided the (3*R*)-hydroxy, while reduction of the 3-hydroxy-5-ketoester predominantly provided the (5*S*)-hydroxy.

2.9. Screening of other microorganisms for reduction of ethyl diketoester to monohydroxy esters

Thirteen yeast cultures, three *Acinetobacter calcoaceticus* strains, and four recombinant *E. coli* containing cloned

Table 1. Determination of absolute configurations of monohydroxy esters

Starting material (mixture of monohydroxy esters) Relative ratio and retention time by Chiral-HPLC Configurations need to be determined				Product (mixture of dihydroxy esters) Relative ratio and retention time by Chiral-HPLC Configurations known			
43.5 min	41.2 min	36.4 min	38.3 min	32.3 min	24.6 min	17.1 min	21.7 min
(5*S*)-**10a**	(5*R*)-**10b**	(3*R*)-**11a**	(3*S*)-**11b**	(3*R*,5*S*)-**6a**	(3*S*,5*R*)-**6b**	(3*R*,5*R*)-**7a**	(3*S*,5*S*)-**7b**
1%	13%	1%	85%	2%	6%	9%	83%

Table 2. Microbial reduction of ethyl monohydroxy esters by SC13874 cells

Substrate (mixture of monohydroxy esters) Relative % by Chiral-HPLC				Product (mixture of dihydroxy esters) Relative % by Chiral-HPLC			
(5*S*)-**10a**	(5*R*)-**10b**	(3*R*)-**11a**	(3*S*)-**11b**	(3*R*,5*S*)-**6a**	(3*S*,5*R*)-**6b**	(3*R*,5*R*)-**7a**	(3*S*,5*S*)-**7b**
6	5	48	41	66	0.8	0.8	32
42.5	42.5	7.5	7.5	62	5	26	7

Substrate 2 g/L, cells 200 g/L, 0.1 M, pH 7 phosphate buffer, 28 °C, 6 h.

ketoreductase enzymes from our culture collection were evaluated. In addition, 97 cultures from five multiwell plates (four yeast plates and one bacteria plate) were evaluated. In the event that there was any microbial reduction, the monohydroxy product was found to be the major product. Most yeast cultures showed a preference for reduction at the 3 position with low enantiospecificity. Selected results are listed in Table 3. The resulting mixture of ethyl monohydroxy esters from the reduction by R-Buspar reductase *rec E. coli* was used as the starting material to determine the absolute configurations of all ethyl monohydroxy esters.

2.10. Microbial reduction of *t*-butyl 3,5-diketo-6-benzyloxyhexanoate 5 by *Acinetobacter* sp. SC13874 cells

The *t*-butyl diketoester **5** was evaluated for the microbial reduction with *Acinetobacter* sp. SC13874 under the same conditions as the ethyl ester, except with lower substrate concentrations of 2 and 5 g/L. Reduction of the *t*-butyl diketoester was found to be slower than the ethyl ester. Even after 72 h, some diketoester was left, especially with the 5 g/L reaction. Monohydroxy esters resulting from reduction of either one of the two keto groups was the major product (area ratio 50–70%). Like the ethyl ester, *syn*-dihydroxy-**8** was produced in preference over *anti*-dihydroxy-**9** and one enantiomer of *syn*-(3*R*,5*S*)-**8a** was obtained preferentially. However, both the de and ee of the dihydroxy esters obtained in the microbial reduction of the diketo *t*-butyl ester were lower than those of the corresponding ethyl ester under similar conditions with the same batch of cells. For example, with 2 g/L substrate *t*-butyl diketoester input concentration the relative area ratios after 24 h were as follows: diketo 2%, monohydroxy 62%, dihydroxy 35%. The de for the *syn*-dihydroxy-**8** was 51% and the ee of the desired enantiomer *syn*-**8a** was 86.6%.

To isolate and identify the intermediates (monohydroxy esters) and help establish the stereochemistry of each reaction step, a microbial reduction reaction of 500 mg *t*-butyl diketoester was carried out. HPLC of the crude reaction product showed a total AP of 38 for four peaks with the ratio of 26:9:5:60 for **8**:**9**:**12**:**13**. By a combination of flash chromatography and preparative HPLC, compounds **8**, **9**, **12**, and **13** were separated, purified and characterized. The results are shown in Table 4.

The absolute configurations of the *t*-butyl dihydroxy esters were tentatively assigned on the basis of the assumption that the enantiomers of both the ethyl and *t*-butyl dihydroxy esters would show a similar mobility order with the same chiral HPLC method and the same direction of rotation (positive or negative) in the same solvent. On the basis of this assumption, the absolute configuration of the major enantiomer of **8** was tentatively assigned as *syn*-(3*R*,5*S*)-**8a**, while the major enantiomer of **9** was tentatively assigned as *anti*-(3*R*,5*R*)-**9a**.

The structures of 5-hydroxy **12** and 3-hydroxy **13** were determined by NMR analysis. The absolute configurations of the *t*-butyl monohydroxy esters were also tentatively assigned on the basis of the assumption that enantiomers of both the ethyl and *t*-butyl monohydroxy esters would show a similar mobility order with the same chiral HPLC method (See method-5 in Section 4.4). On the basis of this assumption, the major enantiomer of the isolated 5-hydroxy ester was (5*S*)-hydroxy **12a** while the major enantiomer of the isolated 3-hydroxy ester was (3*R*)-hydroxy **13a**.

2.11. Cofactor requirement

NADH and NADPH with their respective regeneration systems were used to examine the cofactor requirement of the ketoreductase. In the presence of NADH, *syn*-(3*R*,5*S*)-diol **6a** plus two monohydroxy compounds **10** and **11** were produced by the cell-free extract. After 18 h of incubation, the major product was *syn*-(3*R*,5*S*)-diol **6a** with low amounts of the two monohydroxy compounds (Fig. 1A).

Table 3. Screening of microoganisms for reduction of ethyl diketoester to ethyl monohydroxy esters

Microoranism		Relative % of monohydroxy esters			
		(5*S*)-**10a**	(5*R*)-**10b**	(3*R*)-**11a**	(3*S*)-**11b**
SC16413	*Pichia methanolica*	38	13	40	9
SC16414	*Pichia methanolica*	35	36	25	4
SC16441	*Candida Kefyr*	5	21	62	12
SC16296	*Candida famata*	6	1	7	86
R-Buspar	Recombinant *E. coli*	1	13	1	85

Table 4. Major products from microbial reduction of *t*-butyl diketoester **5** by SC13874 cells

Compound number	Relative ratio	After separation and purification					
		mg	AP	Ratio of **8**:**9**	ee (%)	$[\alpha]_D$ in $CHCl_3$	Major enantiomer
8	26	58	96	99.5:0.5	52	−9	*syn*-(3*R*,5*S*)-**8a**
9	9	4	96	1.5:98.5	79	−5.9	*anti*-(3*R*,5*R*)-**9a**
				Ratio of **12**:**13**			
12	5	8	85	96.5:3.5	77	11.2	(5*S*)-**12a**
13	60	120	99	0.5:99.5	64	1.3	(3*R*)-**13a**

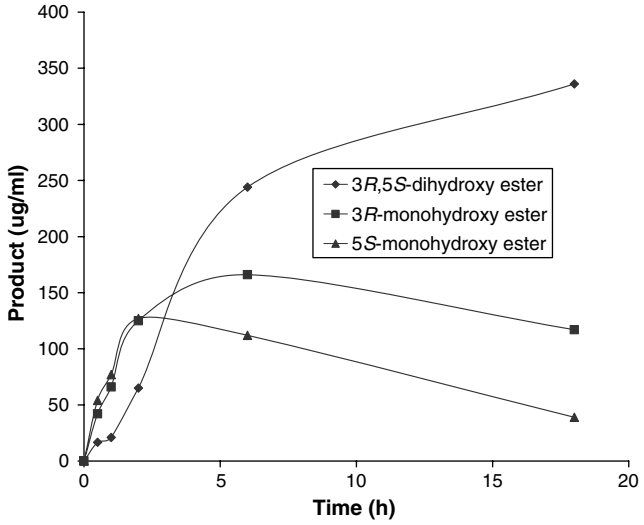


Figure 1A.

When NADPH was used as a cofactor, the *syn*-(3*R*,5*S*)-diol **6a** was produced in much less quantity, while the major product was one of the monohydroxy compounds **10**. After 18 h of incubation, the product profile did not change much, apart from the production of the *syn*-diol, which increased over the time period (Fig. 1B).

The difference between NADH and NADPH in monohydroxy and dihydroxy products formation over time implied that there were multiple reductases, each of which exhibited a different substrate specificity, reaction kinetics, product profile, and affinity for different cofactors. Both NADH and NADPH could be utilized by the ketoreductase as a cofactor, but NADH gave 4 times as much as *syn*-(3*R*,5*S*)-diol **6a** than NADPH. As a result, NADH was chosen as the cofactor for subsequent enzyme assay and purification.

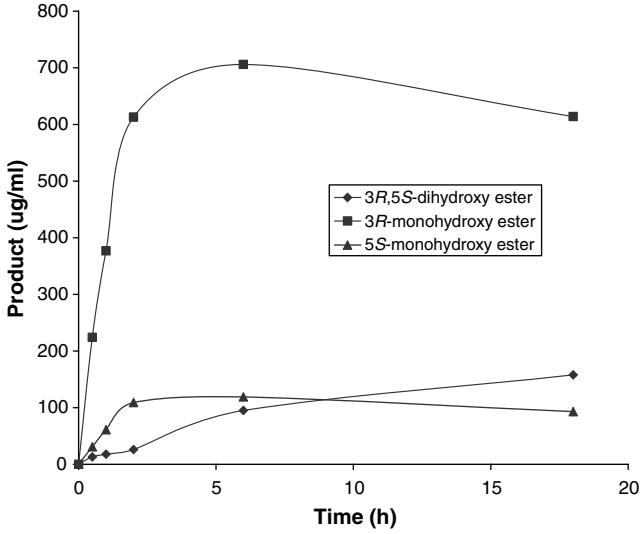


Figure 1B.

2.12. pH optimization

The reductase activity was assayed with buffers at different pH values. Based on the formation of *syn*-(3*R*,5*S*)-*cis*-diol **6a**, the optimal pH for the enzyme is pH 5.5 as shown in Figure 2.

2.13. Purification of reductase I

The reductase I catalyzing the formation of both monohydroxy products was purified more than 240-fold by a three-step procedure (Table 5). After the first UnoQ column, the active fraction was applied to a Cibacron Blue 3GA dye affinity column. The enzyme was eluted with 0.1 M NaCl and applied to a second UnoQ with NaCl gradient elution. The purified reductase I is a homodimeric protein with a native molecular weight of 50 kDa, and the molecular weight of the subunit is 28 kDa on SDS-PAGE.

2.14. Purification of reductase II

The reductase II catalyzing the conversion of the two monohydroxy compounds **10** and **11** to *syn*-(3*R*,5*S*)-*cis*-diol **6a** was assayed by utilizing a mixture of the two monohydroxy compounds **10** and **11** as substrate. The enzyme was purified more than 396-fold by two consecutive UnoQ columns as indicated in Table 6. The unbound fraction from the first UnoQ column was loaded onto a second UnoQ column after changing to a buffer with less salt and glycerol. The enzyme was eluted with a narrowed NaCl gradient to yield apparently homogenous protein. The purified reductase II appeared homogenous on SDS-PAGE with a subunit weight of 48 kDa. The native molecular weight was determined to be approximately 100 kDa by gel filtration, indicating that the enzyme is a homodimer.

2.15. Purification of reductase III

The reductase III that catalyzes the conversion of diketone **4** directly to *syn*-(3*R*,5*S*)-diol **6a** was purified and guided by the assays of activity on both diketone and monohydroxy substrates. Notably, there were three active peaks eluted from the Q-sephaose column. All three active peaks produced monohydroxy products while only one produced *syn*-(3*R*,5*S*)-diol **6a** along with monohydroxy compounds, which is in agreement with the reactions catalyzed by

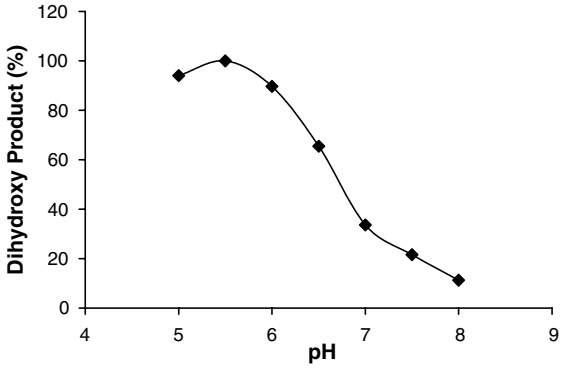


Figure 2.

Table 5. Purification of reductase I

Step	Total protein (mg)	Total activity (μg/h)	Specific activity (μg/h/mg)	Purification (fold)
Cell-free extract	38.9	339.4	8.7	1
1st UnoQ	8.2	179.2	21.8	2.5
Cabicron Blue	0.52	47.8	91.9	10.6
2nd UnoQ	0.021	45.1	2148.1	246.9

Table 6. Purification of reductase II

Step	Total protein (mg)	Total activity (μg/h)	Specific activity (μg/h/mg)	Purification (fold)
Cell-free extract	38.9	339.4	8.7	1
1st UnoQ	12.1	209.3	17.3	2.0
2nd UnoQ	0.03	103.4	3445.2	396.0

reductase I and II. Therefore, the peak producing *syn*-(3*R*,5*S*)-diol was pursued for further purification on a Superdex 200 gel filtration column. The native molecular weight of the enzyme was estimated to be approximately 35–38 kDa from gel filtration. Subsequently, two consecutive UnoQ columns were applied to purify the enzyme. The active fractions from the first UnoQ column were further purified by a second UnoQ column using a different NaCl gradient. After the second UnoQ, the enzyme was purified more than 560-fold and it appeared as a predominant band at 36 kDa on SDS-PAGE. Based on the molecular weight from Superdex 200, this enzyme appears to be a monomer (Table 7).

2.16. Protein and peptide sequencing and sequence analyses

The N-terminal sequence of reductase III was determined by Edman degradation, and two internal sequences were obtained after tryptic digestion and HPLC separation of the peptides. The sequencing work was performed by the Keck Biotechnology Center of Yale University (New Haven, CT). The sequences are as follows.

N-Terminal: TGITNVTVLGTGVLGSQIA,
Internal #1: DADLVIEAV,
Internal #2: LGELAPAK.

After searching the NCBI databases, all three sequences showed high homology to 3-hydroxyacyl-CoA dehydrogenase from different bacterial sources.

Table 7. Purification of reductase III

Step	Total protein (mg)	Total activity (μg/h)	Specific activity (μg/h/mg)	Purification (fold)
Cell-free extract	180.0	3,366.0	18.6	1
Q-Sepharose	5.6	2,855.0	509.8	27.4
Superdex 200	2.9	2,205.0	760.3	40.9
1st UnoQ (0–0.3 M NaCl)	0.84	2,071.2	2,465.7	132.5
2nd UnoQ (0.05–0.15 M NaCl)	0.13	1,358.3	10,448.5	561.7

2.17. Confirmation of stereochemistry of products

The enantioselectivity of reductase III was examined with 3-hydroxy-5-ketoester **10** and 5-hydroxy-3-ketoester **11**. After incubating with purified enzyme at 28 °C and 200 rpm for 24 h, the product was exclusively *syn*-(3*R*,5*S*)-diol **6a** in both cases. However, the (5*S*)-hydroxyester was a more preferable substrate for the enzyme than the 3*R*-hydroxyester based on the amount of remaining monohydroxy enantiomers. The different reaction rates for the (3*R*)- and (5*S*)-hydroxy esters suggests that reduction of the two carbonyl groups is likely to be sequential reactions by the same enzyme.

3. Conclusions

A cell suspension of *Acinetobacter* sp. SC13874 at a weight ratio of 20:1 for cells to substrate reduced the ethyl diketoester **4** completely to the desired *syn*-(3*R*,5*S*)-dihydroxy ester **6a**. *Acinetobacter* sp. SC13874 at a lower cell to substrate ratio partially reduced the ethyl diketoester **4** predominantly to the monohydroxy esters with negligible enantiomeric selectivity at both the 3 and 5 positions. When ethyl monohydroxy ester **10** or **11** was used as substrate, further reduction by *Acinetobacter* sp. SC13874 cells was quite enantiospecific. Reduction of the 3-keto-5-hydroxy ester **10** predominantly provided the (3*R*)-hydroxy, while reduction of the 3-hydroxy-5-ketoester **11** predominantly provided the (5*S*)-hydroxy. The absolute configurations of all mono- and dihydroxy esters from microbial reduction were determined and the corresponding peaks in chiral HPLC chromatograms were assigned. Three ketoreductases from *Acinetobacter* sp. SC13874 were purified. All three reductases can utilize both NADH and NADPH as cofactors, and their optimal pH values for the activity are around pH 5.5. The purification of the three reductases, especially reductase III, will lead to cloning and heterogeneous expression of the enzymes and applications in preparing enantiopure chiral intermediates for different drug candidates.

4. Experimental

4.1. Chemicals and general methods

Chemicals were purchased from VWR and/or Aldrich. NMR spectra were recorded in $CDCl_3$ except where indicated on a BRUKER-300 and/or a JEOL-400 NMR spectrophotometer. The proton assignments are based upon ^1H–^1H COSY experiments. LCMS data were recorded on a Shimadzu LCMS system with positive ion electrospray (ES+) or negative ion electrospray (ES−) methods. Rotation data were recorded in the solvent as indicated on a Perkin—Elmer 241 Polarimeter.

4.2. Microorganisms

Microorganisms were obtained from the Bristol-Myers Squibb (BMS) culture collection. The SC number denotes the number in the BMS culture collection. Microorganisms

were grown in F7 media, which contained 10 g malt extract, 10 g yeast extract, 1 g peptone, and 20 g dextrose per L of water, adjusted to pH 7 and autoclaved at 121 °C for 20 min. *Acinetobacter* sp. SC13874 is maintained in the culture collection of the Bristol-Myers Squibb Pharmaceutical Research Institute as frozen vials stored at −70 °C.

4.3. Growth of *Acinetobacter* sp. SC13874

Acinetobacter sp. SC13874 culture from a frozen vial (1 mL) was inoculated into 100 mL of F7 medium in a 500-mL flask and grown on a shaker at 28 °C and 200 rpm for 48 h. The entire stage I culture was transferred to a 4-L flask containing 1 L of F7 medium. The second stage was grown for 24 h under the same conditions. The second stage culture was used as an inoculum for the growth of cultures in a 20-L fermentor containing 15 L of F7 medium. The culture was grown at 28 °C and 300 rpm with 1 vvm aeration for 24 h. After 24 h growth, the cells were harvested by centrifugation. The harvested cells were washed with 15 L of 0.1 M potassium phosphate buffer (pH 7) and centrifuged again to collect the cell paste (~450 g). The cells were stored at −70 °C until further use.

4.4. Analytical methods

Analytical HPLC methods were performed with various gradients of solvent A (0.05% TFA in water/methanol 80:20) and solvent B (0.05% TFA in acetonitrile/methanol 80:20) at ambient temperature with UV detection at 220 nm except where otherwise indicated. HPLC methods 1 and 2 were performed on a Phenomenex Luna Phenyl-Hexyl column (5 μm, 150 × 4.6 mm) with a flow rate of 1 mL/min. Chiral methods 3, 4, and 5 were performed on a chiral column as indicated with a flow rate of 0.5 mL/min. Some modification of these HPLC methods was also used during the study for better separation or faster analysis.

Method-1 was used for achiral analysis of the ethyl esters with a gradient from 0% to 100% solvent B over 15 min. The retention times were 9.1 min for *anti*-**7**, 9.3 min for *syn*-**6**, 9.8 min for 3-OH-**11**, 10.1 min for 5-OH-**10**, and 11.9 min (broad) for diketo-**4**.

Method-2 was used for achiral analysis of the *t*-butyl esters with a gradient from 30% to 100% solvent B over 15 min. The retention times were 6.8 min for *anti*-**9**, 7.0 min for *syn*-**8**, 7.7 min for 3-OH-**13**, 7.9 min for 5-OH-**12**, and 10.0 min (broad) for diketo-**5**.

Method-3 was used for chiral analysis of the ethyl esters and was performed on a Chiralcel OD-RH column (150 × 4.6 mm) using an isocratic composition of A:B 90:10 for 60 min. The retention times were 17.1 min for *anti*-(3*R*,5*R*)-**7a**, 21.7 min for *anti*-(3*S*,5*S*)-**7b**, 24.6 min for *syn*-(3*S*,5*R*)-**6b**, 32.3 min for *syn*-(3*R*,5*S*)-**6a**, 36.4 min for (3*R*)-OH-**11a**, 38.3 min for (3*S*)-OH-**11b**, 41.2 min for (5*R*)-OH-**10b**, and 43.5 min for (5*S*)-OH-**10a**. Diketo-**4** gave a broad peak beginning at 58 min.

Method-4 was used for chiral analysis of the *t*-butyl dihydroxy esters and was performed on a Chiralcel OD-RH column (150 × 4.6 mm) using an isocratic composition of A:B 65:35 for 18 min. The retention times were 9.4 min for *anti*-(3*R*,5*R*)-**9a**, 12.0 min for *anti*-(3*S*,5*S*)-**9b**, 13.4 min for *syn*-(3*S*,5*R*)-**8b**, and 14.9 min for *syn*-(3*R*,5*S*)-**8a**.

Method-5 was used for chiral analysis of the ethyl and *t*-butyl monohydroxy esters and was performed on a Chiralpak AD-RH column (150 × 4.6 mm) with a gradient of 50–70% B over 30 min. The retention times of the ethyl monohydroxy esters were 8.3 min for (5*S*)-OH-**10a**, 10.0 min for (5*R*)-OH-**10b**, 12.1 min for (3*R*)-OH-**11a**, and 13.5 min for (3*S*)-OH-**11b**. The retention times of the *t*-butyl monohydroxy esters were 11.0 min for (5*S*)-OH-**12a**, 13.3 min for (5*R*)-OH-**12b**, 17.1 min for (3*R*)-OH-**13a**, and 19.7 min for (3*S*)-OH-**13b**.

Preparative HPLC was performed with a gradient of solvent A (water/methanol 80:20) and solvent B (acetonitrile/methanol 80:20) at ambient temperature on a Waters Prep 4000 system with a YMC-Pack ODS, s-5 μm column (250 × 20 mm).

4.5. Synthesis of *N*-methyl-*N*-methoxybenzyloxyacetamide 2

N-Methyl-*N*-methoxybenzyloxyacetamide **2** was synthesized according to the literature procedure[16] as shown in Scheme 1. LCMS 210 (M+1) by ES+ method. NMR ($CDCl_3$) ^1H δ 7.3 (m, 5H), 4.7 (s, 2H), 4.3 (s, 2H), 3.7 (s, 3H) and 3.2 (s, 3H) ppm; ^{13}C δ 169, 135, 126 (2C), 125.1 (2C), 125, 71, 65, 58, 30 ppm.

4.6. Synthesis of ethyl 3,5-diketo-6-benzyloxyhexanoate 4

Ethyl 3,5-diketo-6-benzyloxyhexanoate **4** was synthesized according to a literature procedure[16,17] with some modification. A dispersion of NaH (16.5 g, 60% in mineral oil) was washed with hexane and added to a 3-L, three-necked flask equipped with a mechanical stirrer under a nitrogen atmosphere, followed by 500 mL of anhydrous THF. A dry-ice bath was used and adjusted up and down as necessary to control the cooling. At 0–5 °C, ethyl acetoacetate (49.3 mL) was added over 20 min. The mixture was further cooled and butyl lithium (48 mL, 2.5 M in hexanes) added at −15 to −10 °C over 40 min. The mixture was then cooled down to <−50 °C. A solution of amide **2** (53.94 g) in 100 mL of anhydrous THF was added at <−45 °C over 30 min. After an additional 20 min, the reaction was carefully quenched with 1 L of 1 M HCl. The cooling bath was then removed and the two layers separated. The organic layer was washed with 0.1 M HCl (10 × 500 mL) and 500 mL of brine, dried over $MgSO_4$, and filtered. Solvent removal under reduced pressure at 25 °C gave 70.0 g of oily product **4**, yield 97%, AP 94.

^1H NMR ($CDCl_3$) showed the major product **4a** (Scheme 1) in keto and enol forms with a molar ratio of 152:25:7 for keto–enol product/diketo product/ethyl acetoacetate. This indicated that 1.8% of the weight of ethyl acetoacetate was an impurity. The peaks assigned to the keto–enol product were δ 7.38 (m, 5H, Ph), 5.97 (s, 1H, H-4), 4.59 (s, 2H, H-6), 4.20 (q, 2H, CO_2CH_2), 4.10 (s, 2H, Ph–CH_2), 3.37 (s, 2H, H-2), 1.28 (t, 3H, CH_3 in ethyl) ppm. The peaks as-

signed to the diketo product were δ 7.38 (m, 5H, Ph), 4.57 (s, 2H, H-6), 4.20 (q, 2H, CO_2CH_2), 4.10 (s, 2H, Ph–CH_2), 3.80 (s, 2H, H-4), 3.54 (s, 2H, H-2), 1.28 (t, 3H, CH_3 in ethyl) ppm. The peaks assigned to ethyl acetoacetate were δ 4.20 (q, 2H, CO_2CH_2), 3.45 (s, 2H, H-2), 2.28 (s, 3H, CH_3 in acetyl), 1.28 (t, 3H, CH_3 in ethyl) ppm. The keto–enol form gave δ 190, 186, 167, 137, 129 (2C), 128, 127.8 (2C), 98, 74, 71, 62, 45, 15 ppm in the ^{13}C NMR.

4.7. Preparation of racemic ethyl *syn*-3,5-dihydroxy-6-benzyloxyhexanoate 6 and racemic ethyl *anti*-3,5-dihydroxy-6-benzyloxyhexanoate 7

A solution of **4** (556 mg) in THF (20 mL) and MeOH (5 mL) was cooled in a dry-ice bath under a nitrogen atmosphere for 30 min. Diethyl(methoxy)borane (2.4 mL, 1 M in THF) was then added. The mixture was once warmed to room temperature (rt) and cooled again in a dry-ice bath. Sodium borohydride (378 mg) was added. After 20 min, the dry-ice bath was removed. The mixture was stirred for 3 h and then quenched with acetic acid (2 mL). The mixture was diluted with methyl *t*-butyl ether (MTBE, 20 mL), washed with aqueous $NaHCO_3$ twice and water, dried over $MgSO_4$, and concentrated to dryness. The crude residue showed a 9:1 ratio of *syn*-**6**:*anti*-**7**; flash chromatography, eluting with 10% acetone in dichloromethane (DCM) and gave 460 mg of pure product **6**, yield 82%, LCMS 283 (M+1). HPLC showed 99.5% de for *syn*-**6**. The two enantiomers gave a 1:1 ratio using chiral HPLC method-3. NMR ($CDCl_3$) ^{1}H δ 7.3 (m, 5H), 5.8 (s, 2H), 4.4 (m, 1H), 4.15 (m, 3H), 3.5 (dd, 1H), 3.4 (dd, 1H), 2.6 (dd, 1H), 2.4 (dd, 1H), 2.05 (m, 1H), 1.5 (m, 1H), 1.25 (t, 3H) ppm; ^{13}C δ 170.2, 137.9, 128.2 (2C), 127.5 (3C), 73.8 (2C), 70.6, 68.1, 61.0, 42.9, 35.7, 15.0 ppm.

Reduction of ethyl 3,5-diketo-6-benzyloxyhexanoate **4** by sodium borohydride without diethyl(methoxy)borane was carried out at 0.5 mmol scale. The crude product showed a 3:1 ratio of *syn*-**6**:*anti*-**7**. All four isomers were well separated using chiral HPLC method-3.

4.8. Preparation of the racemic ethyl 3-keto-5-hydroxy-6-benzyloxyhexanoate 10 and ethyl 3-hydroxy-5-keto-6-benzyloxyhexanoate 11

A solution of **4** (2.5 g) in THF (30 mL) was stirred at room temperature under a nitrogen atmosphere. Sodium borohydride (100 mg) was added. After an additional 30 min, the reaction was quenched with 0.1 M HCl (50 mL) and diluted with MTBE (30 mL). The organic phase was washed with 50 mL of brine and concentrated to dryness (2.32 g). HPLC method-1 showed a relative ratio of 34% of dihydroxy compounds **6** and **7**, 11% of 5-hydroxy **10**, 2% of 3-hydroxy **11**, and 53% of remaining diketo **4**. The crude residue was subjected to flash chromatography repeatedly to give 120 mg of a mixture of **10** and **11**. The relative ratio was 85% of **10** to 15% of **11**. In chiral HPLC Method-5, the mixture gave four peaks, which could be assigned to the two pairs of enantiomers based upon the relative area percentage. Thus, 5-hydroxy **10** gave two equal peaks at 8.3 and 10.0 min, while 3-hydroxy **11** gave two equal peaks at 12.1 and 13.5 min. LCMS showed 281 (M+1) by ES+ method and 279 (M−1) by ES− method. ^{1}H NMR showed 86% of 5-hydroxy **10** and 14% of 3-hydroxy **11**, in agreement with the HPLC analysis. The peaks for the major product of 5-hydroxy **10** were assigned as follows: ^{1}H δ 7.25–7.4 (m, 5H, Ph), 4.52 (S, 2H, Ph–CH_2), 4.26(m, 1H, 5-H), 4.16 (q, $J = 7.2$ Hz, 2H, CO_2CH_2), 3.46 (s, 2H, 2-H), 3.44 (m, 2H, 6-H), 3.25 (d, broad, 1H, OH), 2.72 (m, 2H, 4-H), 1.24 (t, $J = 7.2$ Hz, 3H, CH_3) ppm. ^{13}C δ 202.2, 166.9, 137.6, 128.2 (2C), 127.53, 127.51 (2C), 73.07, 73.02, 66.33, 61.14, 49.63, 46.12, 13.81 ppm.

4.9. Preparation of ethyl 3,5-dihydroxyacetonide-6-benzyloxyhexanoate 19 and attempted separation of the *syn* and *anti* isomers

A mixture of **6** and **7** (28 mg, *syn*-**6**:*anti*-**7** = 5:1) was dissolved in 5 mL of DCM. 2,2-Dimethoxypropane (123 μL) was added followed by *p*-toluenesulfonic acid monohydrate (2 mg). The mixture was stirred at room temperature overnight. TLC showed the reaction was complete and showed only one round spot with R_f 0.6 (DCM–acetone 9:1, R_f 0.2 for the starting material). The mixture was loaded onto a preparative TLC plate and developed in the same solvent system. The product band was collected and extracted with MTBE to give 18 mg of mixture **19**, LCMS 323 (M+H), 340 (M+NH_4) and 345 (M+Na). NMR peaks for the major *syn*-isomer were as follows: ^{1}H δ 7.25–7.4 (m 5H, Ph), 4.61 (d, $J = 12.2$ Hz, 1H, Ph–CH_2-A), 4.54 (d, $J = 12.2$ Hz, 1H, Ph–CH_2-B), 4.3 (m, 1H, 3-H), 4.1 (m, 3H, CO_2CH_2 and 5-H), 3.50 (dd, $J1 = 5.4$, $J2 = 9.8$, 1H, 6-H-A), 3.36 (dd, $J1 = 4.9$, $J2$=9.8, 1H, 6-H-B), 2.52 (dd, $J1 = 6.9$, $J2$=15.4, 1H, 2-H-A), 2.37 (dd, $J1 = 6.4$, $J2 = 15.4$, 1H, 2-H-B), 1.62 (m, 1H, 4-H-A), 1.47 (s, 3H, CH_3), 1.39 (s, 3H, CH_3), 1.24 (m, 4H, H-4-B and CH_3) ppm; ^{13}C δ 170.39, 137.95, 128.22 (2C), 127.59 (2C), 127.49, 98.99, 73.74 (2C), 68.70, 66.03, 60.88, 42.08, 33.90, 30.63, 20.39, 14.98 ppm.

Analysis of this mixture of *syn*- and *anti*-acetonides by HPLC and TLC methods did not show any separation of the two isomers.

4.10. Synthesis of *t*-butyl 3,5-diketo-6-benzyloxyhexanoate 5

To a 2-L, three-necked flask equipped with a mechanical stirrer was charged 200 mL of anhydrous THF. A dry-ice bath was used and adjusted up and down as necessary to control the cooling. A dispersion of NaH (5.8 g, 60% in mineral oil) was added in portions under a nitrogen atmosphere. At −5 to 0 °C, *tert*-butyl acetoacetate **3b** (23.9 mL) was added through an addition funnel over about 30 min. The mixture was stirred for an additional 10 min at the same temperature. The mixture was cooled further and butyl lithium (58 mL, 2.5 M in hexanes) was added at −15 to −10 °C over about 30 min. The mixture was stirred for an additional 10 min before being further cooled down to <−50 °C. A solution of amide **2** (10.0 g) in 50 mL of anhydrous THF was added at <−50°C. After addition, the dry-ice bath was lowered, and the mixture stirred for 30 min as the temperature rose slowly to about −10 °C. The reaction was quenched by the careful addition of 100 mL of water. The mixture was diluted with 250 mL

of MTBE. After shaking, the two phases were allowed to separate. The organic phase was washed with 5% aqueous $NaHCO_3$ (200 mL) and 100 mL of brine. The solvent was removed under reduced pressure. The major product **5** gave a broad peak between 9 and 11 min with HPLC Method-2.

The crude residue was subjected to flash chromatography. The first attempted purification, however, led to a partially decomposed mixture. A portion of this partially decomposed mixture (6.9 g) was dissolved in 40 mL of DCM and 1 mL of triethylamine (TEA). Silica gel (150 g) was mixed with DCM and loaded on to a column after the pH was adjusted to basic with TEA. The above solution mixture was loaded on to the column, eluted with 0.5% TEA in DCM (500 mL) followed by 0.5% TEA and 2% MeOH in DCM. Fractions related to desired product **5** were collected. Removal of the solvent provided a solid, 1.3 g, 9% yield, AP 84, LCMS 329 (M+Na). ^{1}H NMR showed a 7:1 ratio of keto–enol form/diketo form (Scheme 1). The keto–enol form **5a** gave ^{1}H NMR δ 7.4 (m, 5H), 6.0 (s, 1H), 4.6 (s, 2H), 4.1 (s, 2H), 3.3 (s, 2H), 1.5 (s, 9H) ppm and ^{13}C NMR δ 191, 187, 167, 138, 129 (2C), 128.2, 128 (2C), 98, 82, 74, 72, 46, 28 (3C) ppm.

4.11. Preparation of racemic *t*-butyl *syn*-3,5-dihydroxy-6-benzyloxyhexanoate 8 and racemic *t*-butyl *anti*-3,5-dihydroxy-6-benzyloxyhexanoate 9

A solution of **5** (306 mg) in THF (20 mL) and MeOH (3 mL) was cooled in a dry-ice bath under a nitrogen atmosphere for 30 min. Diethyl(methoxy)borane (1.2 mL, 1 M in THF) was added. The mixture was warmed to room temperature and cooled again in a dry-ice bath. Sodium borohydride (189 mg) was then added. Since the reduction was not complete, a second batch of diethyl(methoxy)borane (2.4 mL, 1 M in THF) and sodium borohydride (378 mg) was added and the mixture stirred at rt for 20 h. HPLC showed the reaction was complete and gave the dihydroxy esters as the major products. By chiral HPLC Method-4, four single isomers were well separated at 9.4 min (relative ratio 11%, **9a**), 12.0 min (11%, **9b**), 13.4 min (39%, **8b**), and 14.9 min (39%, **8a**).

4.12. Preparation of racemic *t*-butyl 3-keto-5-hydroxy-6-benzyloxyhexanoate 12 and *t*-butyl 3-hydroxy-5-keto-6-benzyloxyhexanoate 13

A solution of **5** (153 mg) in THF (10 mL) was stirred and cooled in a dry-ice bath under a nitrogen atmosphere for 30 min. DIBALH (5 mL, 1 M in THF) was added dropwise. The mixture was stirred overnight and gradually warmed up to rt. Work-up gave a mixture of *anti*-**9** (AP 2.5), *syn*-**8** (AP 2.2), 3-hydroxy **13** (AP 4.3), 5-hydroxy **12** (AP 4.5), and many by-products. The crude residue was subjected to preparative TLC (DCM/acetone/TEA 90:10:1) and the monohydroxy products **12** and **13** were enriched (R_f 0.3–0.5). The enriched product mixture was subjected to preparative HPLC and the *t*-butyl monohydroxy esters were collected in two portions. The relative ratios of **12**:**13** were 36:64 in the first portion and 89:11 in the second portion. Chiral HPLC method-5 showed two equal peaks at 11.0 min [(5*S*)-hydroxy **12a**] and 13.3 min [(5*R*)-hydroxy **12b**], while two equal peaks at 17.1 min [(3*R*)-hydroxy **13a**] and 19.7 min [(3*S*)-hydroxy **13b**].

4.13. Growth of *Acinetobacter* sp. SC13874 in flasks

Acinetobacter sp. SC13874 culture was grown in a 4-L flask containing 1 L of F7 medium as described earlier. The cells were harvested by centrifugation at about 10,000*g* for 20 min at 4 °C. The harvested cells were washed with 200 mL of 0.1 M phosphate buffer (pH 7) and then washed again in the same way. About 10 g of cells were obtained. The cells were stored in a freezer at −70 °C before use.

4.14. Reduction of ethyl 3,5-diketo-6-benzyloxyhexanoate 4 by *Acinetobacter* sp. SC13874 and general procedure for microbial reduction in a small flask

Acinetobacter sp. SC13874 cells (2 g) were suspended in 10 mL of 0.1 M phosphate buffer (pH 7) in a 50-mL flask. Glucose (750 mg) was then added followed by diketoester **4** (20 μL). The biotransformation was conducted by shaking at 200 rpm at 28 °C. After 20 h, a 1-mL sample was withdrawn from the biotransformation mixture. The sample was extracted with ethyl acetate (4 mL). The solvent was removed from the ethyl acetate extract, and the residue dissolved in acetonitrile/methanol (1 mL, 1:1), filtered and analyzed by HPLC.

In a second set of experiments, *Acinetobacter* sp. SC13874 cells (2 g) were suspended in 10 mL of 0.1 M phosphate buffer (pH 7) in each of the two 50-mL flasks. Glucose (750 mg) and 50 μL (reactor 1) and 100 μL (reactor 2) of ethyl 3,5-diketo-6-benzyloxyhexanoate **4** were added to the two flasks. The biotransformation was conducted by shaking at 200 rpm at 28 °C. After 24 h, a 1-mL sample was withdrawn from each biotransformation mixture. The samples were extracted with ethyl acetate (4 mL). Extracts equivalent to 2.5 μL of the substrate (2 mL from reactor 1 and 1-mL from reactor 2) were taken and the solvent removed. The residue was dissolved in acetonitrile/methanol (1 mL, 1:1), filtered and analyzed by HPLC. After 30 h, XAD-16 (20 times the substrate, 1 g for reactor 1 and 2 g for reactor 2) was added to the flask. Adsorption by shaking under the same conditions was continued and after 18 h, the resin was filtered to separate the aqueous layer from the resin. A 1-mL portion of the aqueous layer was analyzed as before. The resin was washed with water until the washing was clear. Each resin was extracted with MTBE (4 × 5 mL). After each MTBE addition, the mixture was mixed by vortexing for 30 seconds and then placed on a gentle tumble mixer for 30 min. A portion of the MTBE extract equivalent to 2.5 μL of the original substrate (1.11 mL for reactor 1, 555 μL for reactor 2) was evaporated and analyzed by HPLC.

4.15. Microbial reduction of 2.5 g of ethyl diketoester 4 and isolation and purification of the dihydroxy products *syn*-(3*R*,5*S*)-6a and *anti*-(3*S*,5*S*)-7b

In a jacketed, three-necked, 500-mL glass reactor, 50 g *Acinetobacter* sp. SC13874 was suspended in 250 mL of 0.1 M

phosphate buffer (pH 7) by stirring using an overhead stirrer (275 rpm) at 28 °C. Glucose (18.75 g) was added. Diketo ethyl ester (2.5 g) was added to the reactor. The stirring and temperature were maintained during the reaction. Samples were taken at various times and analyzed as described above.

After 24 h, XAD-16 resin (50 g) was added to the flask. The stirring speed was reduced to 180 rpm. After an additional 18 h, a 1-mL portion of the aqueous layer was analyzed as above. HPLC showed no detectable level of the dihydroxy ethyl ester in the water layer, suggesting complete adsorption by the XAD resin. The resin was filtered to separate the aqueous layer from the resin. The resin was washed with water until the washing was clear. The resin was extracted with MTBE (4 × 100 mL). After each MTBE addition, the mixture was stirred by an overhead stirrer for 20 min and then filtered through a stainless steel sieve (40 mesh). A water layer (about 50 mL) was seen in the first MTBE extract. The water was separated, extracted with MTBE (25 mL), and this MTBE extract was combined with the other MTBE extracts. Removal of solvent from the combined MTBE extract provided 2.1 g of a yellow oil. Analysis by various HPLC methods showed that the major component was the desired *syn*-(3*R*,5*S*)-dihydroxy ester **6a**, AP 40, de 63.3%, ee 99.3%.

The crude product was subjected to repeated flash chromatography. The first column (100 g silica gel) was eluted with 3% methanol in DCM. Four more columns (40–80 g silica gel each) were employed and eluted with 10% acetone in DCM to give 750 mg of pure *syn*-**6a**, AP 98, de 99% and ee 99%. The later eluted fractions from these columns were combined to give an *anti*-**7** enriched portion (140 mg) with AP 66 and de 83%, which was further purified by recrystallization twice using an MTBE–heptane solvent system to give pure *anti*-**7b** (60 mg), AP 98, de >99% and ee 97%.

The pure *syn*-**6a** had a specific rotation of $[\alpha]_D = -4.3$ (*c* 1.12, EtOH), $[\alpha]_D = -12.5$ (*c* 1.23, $CHCl_3$), which was in agreement with the literature[8] data $[\alpha]_D = -12.8$ (*c* 2.06, $CHCl_3$) for compound **6a**. ^{1}H NMR δ 7.25–7.4 (m, 5H, Ph), 4.56 (s, 2H, Ph–CH_2), 4.3 (m, 1H, 3-H), 4.17 (q, $J = 7.1$ Hz, 2H, CO_2CH_2), 4.08 (m, 1H, 5-H), 3.8 (d, 1H, D_2O exchangeable, OH), 3.44 (m, 2H, 6-H), 3.26 (d, 1H, D_2O exchangeable, OH), 2.5 (m, 2H, 2-H), 1.65 (m, 2H, 4-H), 1.27 (t, $J = 7.1$ Hz, 3H, CH_3) ppm; ^{13}C δ 171.9, 137.7, 128.3 (2C), 127.7, 127.6 (2C), 74.4, 73.7, 70.8, 68.5, 61.1, 42.2, 39.4, 14.9 ppm.

The pure *anti*-**7b** had a specific rotation of $[\alpha]_D = +7.2$ (*c* 1.50, $CHCl_3$); LCMS 283 (M+1); NMR ^{1}H δ 7.25–7.4 (m 5H, Ph), 4.54 (s, 2H, Ph–CH_2), 4.3 (m, 1H, 3-H), 4.15 (q, $J = 7.1$ Hz, 2H, CO_2CH_2), 4.1 (m, 1H, 5-H, overlap with CO_2CH_2), 3.49 (dd, $J1 = 3.9$, $J2 = 9.3$ Hz, 1H, 6A-H), 3.39 (dd, $J1 = 7.6$, $J2 = 9.5$ Hz, 1H, 6B-H), 2.9 (broad, D_2O exchangeable, OH), 2.48 (d, $J = 6.3$ Hz, 2H, 2-H), 1.59 (m, 2H, 4-H), 1.25 (t, $J = 7.1$ Hz, 3H, CH_3) ppm; ^{13}C δ 172.2, 137.7, 128.3 (2C), 127.7, 127.6 (2C), 74.6, 73.6, 67.9, 65.7, 61.1, 42.1, 39.4, 15.0 ppm.

4.16. Determination of absolute configuration of *syn*-(3*R*,5*S*)-dihydroxy ethyl ester 6a

The pure *syn*-**6a** (50 mg) was stirred in 2 mL of dioxane and 4 mL of aqueous 1 M NaOH at rt for 2 h. TLC showed the hydrolysis was complete. The mixture was extracted twice with MTBE. The aqueous layer containing the product was acidified with 4 M HCl and extracted with MTBE (3 × 5 mL). The extract was concentrated to dryness. The residue was refluxed in toluene for 2 h. Solvent was removed and the residue was subjected to preparative TLC (DCM/acetone 8:2) to give 8 mg of lactone **20a**, $[\alpha]_D = +9.0$ (*c* 0.40, $CHCl_3$). The literature[18] reports $[\alpha]_D = +6.5$ (*c* 1.56, $CHCl_3$) for *syn*-(3*R*,5*S*)-**20a**. LCMS 237 (M+1) was found by ES+ method. NMR ^{1}H δ 7.25–7.45 (m, 5H, Ph), 4.9 (m, 1H, 5-H), 4.6 (d, $J = 11.9$ Hz, 1H, Ph–CH_2-A), 4.5 (d, $J = 11.9$ Hz, 1H, Ph–CH_2-B), 4.4 (m, 1H, 3-H), 3.7 (dd, $J1 = 4.0$ Hz, $J2 = 10.7$ Hz, 1H, 6A-H), 3.6 (dd, $J1 = 4.1$ Hz, $J2 = 10.7$ Hz, 1H, 6B-H), 2.7 (m, 2H, 2-H), 2.2 (broad, D_2O exchangeable, OH), 2.0 (m, 2H, 4-H) ppm; ^{13}C δ 170.3, 137.7, 128.5 (2C), 127.9, 127.8 (2C), 75.0, 73.6, 71.6, 62.5, 38.6, 32.2 ppm.

4.17. Determination of absolute configuration of *anti*-(3*S*,5*S*)-dihydroxy ethyl ester 7b

Using the same procedure as described above, the pure *anti*-**7b** (40 mg) was converted to lactone **21b**, 4.2 mg, $[\alpha]_D = +18.1$ (*c* 0.21, $CHCl_3$). The literature[18] reports $[\alpha]_D = +13.9$ (*c* 1.06, $CHCl_3$) for *anti*-(3*S*,5*S*)-**21b**. LCMS 237 (M+1) was found by ES+ method. NMR ^{1}H δ 7.25–7.40 (m, 5H, Ph), 4.62 (d, $J = 12.0$ Hz, 1H, Ph–CH_2-A), 4.58 (d, $J = 12.0$ Hz, 1H, Ph–CH_2-B), 4.45 (m, 1H, 5-H), 4.25 (m, 1H, 3-H), 3.7 (dd, $J1 = 4.4$ Hz, $J2 = 10.4$ Hz, 1H, 6A-H), 3.6 (dd, $J1 = 4.4$ Hz, $J2 = 10.4$ Hz, 1H, 6B-H), 2.85 (m, 1H, 2A-H), 2.5 (dd, $J1 = 7.2$ Hz, $J2 = 17.2$ Hz, 1H, 2B-H), 2.5 (broad, overlap with 2B-H, D_2O exchangeable, OH), 2.3 (m,1H, 4A-H), 1.85 (m, 1H, 4B-H) ppm; ^{13}C δ 169.9, 137.3, 128.6 (2C), 128.0, 127.9 (2C), 76.1, 73.8, 71.5, 63.3, 39.4, 33.8 ppm.

4.18. Partial reduction of ethyl diketoester 4 to ethyl monohydroxy esters by SC13874 cells

In a 2-L flask, the pilot plant grown cells of SC13874 (100 g) and glucose (375 g) were added to 500 mL of 0.1 M phosphate buffer (pH 7). After shaking the mixture in a shaker at 225 rpm and 28 °C for 30 min, diketo ethyl ester **4** (10 g) was added. Microbial reduction was continued by shaking at 225 rpm and 28 °C. After 16 h, the mixture was acidified to pH 3 and extracted with MTBE. The MTBE extract was dried over $MgSO_4$, filtered, and concentrated to dryness. The crude residue (6.4 g) was subjected to flash chromatography (0–20% acetone in DCM). The fractions containing the monohydroxy products were collected. This mixture of the monohydroxy products (2.2 g) was subjected to repeated flash chromatography to give 290 mg of a mixture of **10** and **11**. The relative ratio determined by HPLC, ^{1}H and ^{13}C NMR was 15% 5-hydroxy-**10** and 85% 3-hydroxy **11**. The NMR peaks for the major product 3-hydroxy **11** were assigned as follows: ^{1}H δ

7.25–7.4 (m, 5H, Ph), 4.56 (s, 2H, Ph–CH_2), 4.50 (m, 1H, 3-H), 4.13 (q, $J = 7.2$ Hz, 2H, CO_2CH_2), 4.08 (s, 2H, 6-H), 2.66 (m, 2H, 4-H), 2.49 (d, $J = 6.2$ Hz, 2H, 2-H), 1.24 (t, $J = 7.2$ Hz, 3H, CH_3) ppm. ^{13}C δ 207.6, 171.5, 136.8, 128.2 (2C), 127.74, 127.65 (2C), 75.02, 73.01, 63.86, 60.43, 44.76, 40.71, 13.84 ppm.

4.19. Microbial reduction of *t*-butyl 3,5-diketo-6-benzyloxyhexanoate 5

The *t*-butyl diketoester **5** was subjected to microbial reduction with *Acinetobacter* sp. SC13874 under the same conditions as the ethyl ester except with lower substrate concentrations of 2 and 5 g/L. HPLC method-2 and 4 were used for achiral and chiral analysis, respectively, for the *t*-butyl dihydroxy esters. HPLC method-5 was used for chiral analysis of the *t*-butyl monohydroxy esters.

4.20. Isolation and purification of *t*-butyl monohydroxy 12 and 13 and dihydroxy 8 and 9 products from the microbial reduction

In a 500-mL flask, 50 g of pilot plant grown cells of SC13874 was suspended in 200 mL of 0.1 M phosphate buffer (pH 7) and shaken at 225 rpm and 28 °C for 1 h. Glucose (18.75 g) was added followed by *t*-butyl diketoester **5** (500 mg) and the reduction allowed to continue under the same conditions. After 28 h, the mixture was extracted with MTBE (3×300 mL). The combined MTBE extract was washed with 5% $NaHCO_3$ and brine. Removal of the solvent provided 0.5 g of a yellow oil, which was subjected to flash chromatography eluting with DCM/acetone/TEA (95:5:0.1) to give a monohydroxy ester mixture of **12** and **13** as the first eluted portion (180 mg), enriched *syn*-dihydroxy ester **8** as the second eluted portion (80 mg) and enriched *anti*-dihydroxy ester **9** as the third portion (38 mg). The monohydroxy ester portion was subjected to preparative HPLC to give pure 3-hydroxy ester **13** (120 mg) and enriched 5-hydroxy ester **12** (15 mg). The later was subjected to preparative HPLC again to give pure **12** (8 mg). The *syn*-dihydroxy ester portion was purified by flash chromatography and eluted with 5% acetone in DCM to give pure **8** (58 mg). The *anti*-dihydroxy ester portion was subjected to preparative HPLC to give pure **9** (4 mg). Chiral HPLC method-4 and 5 determined that the ee and the major enantiomers in the above purified products were *syn*-(3*R*,5*S*)-**8a**, *anti*-(3*R*,5*R*)-**9a**, (5*S*)-hydroxy **12a**, and (3*R*)-hydroxy **13a**.

Pure *syn*-(3*R*,5*S*)-**8a** (58 mg) showed AP 96, de 99%, ee 52%, $[\alpha]_D = -9.0$ (*c* 1.10, $CHCl_3$), LCMS 311 (M+H) and 328 (M+NH_4). NMR ^1H δ 7.25–7.4 (m, 5H, Ph), 4.55 (s, 2H, Ph–CH_2), 4.23 (m, 1H, 3-H), 4.07 (m, 1H, 5-H), 3.87 (broad, D_2O exchangeable, OH), 3.43 (m, 2H, 6-H), 3.4 (broad, overlap with 6-H and D_2O exchangeable, OH), 2.41 (m, 2H, 2-H), 1.63 (m, 2H, 4-H), 1.45 (s, 9H, *t*-Bu) ppm; ^{13}C δ 171.9, 138.0, 128.4 (2C), 127.8 (3C), 81.3, 74.1, 73.4, 70.4, 68.2, 42.6, 38.9, 28.1 (3C) ppm.

Pure *anti*-(3*R*,5*R*)-**9a** (4 mg) showed AP 96, de 97%, ee 79%, $[\alpha]_D = -5.9$ (*c* 0.37, $CHCl_3$), LCMS 311 (M+H), 328 (M+NH_4) and 333 (M+Na). NMR ^1H δ 7.25–7.4 (m, 5H, Ph), 4.56 (s, 2H, Ph–CH_2), 4.28 (m, 1H, 3-H), 4.13 (m, 1H, 5-H), 3.35–3.55 (m, 2H, 6-H), 3.5 (broad, overlap with 6-H and D_2O exchangeable, OH), 2.87 (broad, D_2O exchangeable, OH), 2.42 (m, 2H, 2-H), 1.60 (m, 2H, 4-H), 1.46 (s, 9H, *t*-Bu) ppm; ^{13}C δ 172.3, 138.0, 128.5 (2C), 127.8, 127.7 (2C), 81.3, 74.3, 73.3, 67.6, 65.4, 42.4, 38.7, 28.1 (3C) ppm.

Pure (5*S*)-OH-**12a** (8 mg) showed AP 85, ee 77%, a ratio of 96.5:3.5 for **12**:**13**, $[\alpha]_D = +11.2$ (*c* 0.84, $CHCl_3$). The LCMS was in agreement with the formula of the *t*-butyl monohydroxy ester: 307 (M−1) by ES− method and 331 (M+Na) by ES+ method. ^1H, ^1H-D_2O exchange, ^1H–^1H COSY, and ^{13}C NMR spectra determined the structure as the *t*-butyl 5-monohydroxy ester **12**. ^1H δ 7.3–7.5 (m, 5H, Ph), 4.56 (s, 2H, Ph–CH_2), 4.3 (m, 1H, 5-H), 3.48 (m, 2H, 6-H), 3.39 (s, 2H, D_2O exchangeable, 2-H), 2.86 (d, $J = 4.0$ Hz, D_2O exchangeable, OH), 2.76 (d, $J = 6.2$ Hz, 2H, 4-H), 1.46 (s, 9H, *t*-Bu) ppm; ^{13}C δ 203.04, 166.16, 137.82, 128.45 (2C), 127.81, 127.75 (2C), 82.20, 73.41, 73.09, 66.71, 51.20, 46.15, 27.94 (3C) ppm.

Pure (3*R*)-OH-**13a** (120 mg) showed AP 99, ee 64%, a ratio of 0.5:99.5 for **12**:**13**, $[\alpha]_D = +1.3$ (*c* 1.65, $CHCl_3$). The LCMS was in agreement with the formula of the *t*-butyl monohydroxy ester: 307 (M−1) by ES− method and 326 (M+NH_4) and 331 (M+Na) by ES+ method. ^1H, ^1H-D_2O exchange, ^1H–^1H COSY, and ^{13}C NMR spectra determined the structure as the *t*-butyl 3-monohydroxy ester **13**. ^1H δ 7.3–7.4 (m, 5H, Ph), 4.58 (s, 2H, Ph–CH_2), 4.45 (m, 1H, 3-H), 4.09 (s, 2H, 6-H), 3.48 (broad, D_2O exchangeable, OH), 2.65 (m, 2H, 4-H), 2.43 (d, $J = 5.8$ Hz, 2H, 2-H), 1.45 (s, 9H, *t*-Bu) ppm; ^{13}C δ 207.8, 171.2, 136.9, 128.4 (2C), 127.9, 127.8 (2C), 81.2, 75.2, 73.2, 64.2, 44.9, 41.7, 28.0 (3C) ppm.

4.21. Preparation of cell-free extract

Frozen cells of *Acinetobacter* sp. SC13874 (50 g) were suspended in 300 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM DTT and 10% glycerol. The cell suspensions were passed through a microfluidizer at 1000 psi 5 times to disrupt the cells, and the mixture was then centrifuged at 50,000*g* for 2 h to yield 250 mL supernatant as the cell-free extract.

4.22. Purification of reductase I

The cell-free extract was directly loaded onto a UnoQ column (1.5 mL, Bio-Rad) equilibrated with 18 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM DTT at a flow rate of 1 mL/min. After washing with 12 mL of the same buffer, the enzyme was eluted with 16 mL of NaCl using a linear gradient (0–0.5 M) while 1-mL fractions were collected. Active fractions were pooled, concentrated and desalted using an Amicon membrane (YM-10) to 5 mL. The preparation was then loaded onto a Cibacron blue 3GA (Sigma) column (1.5×15 cm) equilibrated with 100 mL of 25 mM potassium phosphate buffer (pH 7.0) containing 1 mM DTT, 1 mM $MgCl_2$ and

10% glycerol. The column was washed with 25 mL of the same buffer after loading the active fractions from the previous column (5 mL). The enzyme activity was eluted with 20 mL of the same buffer containing 0.1 M NaCl, and concentrated to 2 mL. Final purification was achieved by injecting the 2-mL concentrate onto a second UnoQ column (Bio-Rad). The column was pre-equilibrated with 24 mL of 25 mM potassium phosphate buffer (pH 7.0) containing 1 mM DTT, 10% glycerol and 0.1 M NaCl, washed with 12 mL of the same buffer after injection of the 2 mL sample, and eluted with 12 mL of a linear NaCl gradient (0.1–0.2 M) at a flow rate of 1 mL/min while 0.5 mL fractions were collected.

4.23. Purification of reductase II

The wash fractions (12 mL) of the first UnoQ column from the purification of reductase I as described above were pooled and concentrated to 2 mL. A second UnoQ column was used for the purification of reductase II. After equilibrating the column with 24 mL of 15 mM potassium phosphate buffer (pH 7.0) containing 1 mM DTT and 5% glycerol, 2 mL of the concentrated sample was injected onto the column. The column was washed with 12 mL of the same buffer and eluted with 12 mL of a linear NaCl gradient (0–0.1 M) at a flow rate of 1 mL/min while each 0.5 mL was collected as a fraction.

4.24. Purification of reductase III

The cell-free extract (90 mL) was applied onto a Q-sepharose 4B fast flow column (Pharmacia, 2.5×20 cm, 100 mL) equilibrated with 500 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM DTT and 10% glycerol. After washing with 150 mL of the same buffer, the column was eluted with 400 mL of a linear NaCl gradient (0–0.5 M). Fractions (7 mL each) were collected at a flow rate of 1.2 mL/min. Active fractions (45 mL) were pooled, concentrated, and desalted using an Amicon YM-10 membrane to 2 mL. This concentrate was loaded onto a Superdex 200 column (Bio-Rad, 1.2×25 cm) at a flow rate of 0.5 mL/min. The enzyme activity was eluted with 25 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM DTT and 0.1 M NaCl. Fractions of 1 mL each were collected, and the active fractions (4 mL) were pooled and concentrated to 2 mL. The concentrate was then injected onto a UnoQ column (1.5 mL, Bio-Rad) equilibrated with 18 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM DTT and 5% glycerol at a flow rate of 1 mL/min. After washing with 12 mL of the same buffer, the enzyme was eluted with 16 mL of a NaCl linear gradient (0–0.3 M) while collecting 1-mL fractions. Active fractions (4 mL) were pooled, concentrated, and desalted using an Amicon membrane (YM-10) to 2 mL. Finally, the concentrate from the UunoQ column was injected onto a second UnoQ column equilibrated with 16 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM DTT, 5% glycerol and 50 mM NaCl at a flow rate of 1 mL/min. After washing with 12 mL of the above buffer, the column was eluted with 16 mL of a linear NaCl gradient (50–150 mM) while collecting 0.75 mL fractions.

4.25. Enzyme assay

Enzyme assays were performed using a mixture containing 2.5 mM diketone **4** for reductases I and III (or a monohydroxy mixture of **10** and **11** for reductases II and III) dissolved in ethanol, 0.5 mM NAD^+, 2 units of formate dehydrogenase, 200 mM sodium formate, 0.1–2 mg enzyme solution, and 0.1 M potassium phosphate buffer (pH 6.0) in a final volume of 0.5 mL. All assay components were prepared freshly. The reaction was initiated by the addition of the enzyme, and terminated by adding 0.5 mL ethanol to the reaction mixture after incubating at 28 °C and 200 rpm for 18 h. After vortexing and centrifugation in a microfuge for 5 min, the resulting supernatant was subjected to HPLC analysis. The products for reductase I are monohydroxy products **10** and **11**, while the product for both reductases II and III is *syn*-(3*R*,5*S*)-dihydroxy ester **6a**. Over 18 h, under the conditions described, product formation was linear. Enzyme activity was expressed as product formation as μg/h/mg protein.

4.26. Determination of cofactor requirements and pH optimum

The cofactor requirement was examined by using NADH and NADPH regeneration systems in the enzyme activity assay. For NADH, the assay mixture contained the same components as described in the enzyme assay whereas the NADPH regeneration system (0.5 mM $NADP^+$, 2 units of glucose-6-phosphate dehydrogenase and 100 mM glucose-6-phosphate) substituted NAD^+, formate dehydrogenase and ammonium formate in the assay mixture for NADPH. The monohydroxy and diol product formation was monitored over 18 h to compare NADH and NADPH.

To determine the optimal pH for the reductases, 0.1 M potassium phosphate with different pH values ranging from 5 to 8 was used in the assay mixture with the same amount of enzyme. The *syn*-(3*R*,5*S*)-diol formation was quanitated to compare pH versus activity.

4.27. Determination of molecular weight and subunit size

A Superdex 200 column (1.2×30 cm, Bio-Rad) was used to determine the native molecular weight of the reductases at a flow rate of 0.5 mL/min with 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM DTT and 0.1 M NaCl. Molecular weight standards included blue dextran (250 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease (13.7 kDa). The native molecular weight was calculated based on the standard curve plotted from elution volume versus void volume.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the subunit molecular weight and was carried out with 10% NuPage pre-made gels from Invitrogen, Inc. and MOPS running buffer at 200 V. The gels were stained with SimplyBlue Safestain solution (Invitrogen, Inc.) and destained with distilled water. The molecular weight marker was SeaBlue Plus 2

from Invitrogen Inc. consisting of eight proteins (14–191 kDa); MOPS running buffer was used.

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