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Enantioselective microbial reduction of substituted acetophenones

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Abstract—The chiral intermediate (*S*)-1-(2′-bromo-4′-fluoro phenyl)ethanol **2** was prepared by the enantioselective microbial reduction of 2-bromo-4-fluoro acetophenone **1**. Organisms from genus *Candida*, *Hansenula*, *Pichia*, *Rhodotorula*, *Saccharomyces*, *Sphingomonas* and Baker's yeast reduced **1** to **2** in >90% yield and 99% enantiomeric excess (ee). In an alternative approach, the enantioselective microbial reductions of methyl, ethyl, and *tert*-butyl 4-(2′-acetyl-5′-fluorophenyl) butanoates **3**, **5**, and **7**, respectively, were demonstrated using strains of *Candida* and *Pichia*. Reaction yields of 40–53% and ee's of 90–99% were obtained for the corresponding (*S*)-hydroxy esters **4**, **6**, and **8**. The reductase, which catalyzed the enantioselective reduction of ketoesters was purified to homogeneity from cell extracts of *Pichia methanolica* SC 13825. It was cloned and expressed in *Escherichia coli* with recombinant cultures used for the enantioselective reduction of keto methyl ester **3** to the corresponding (*S*)-hydroxy methyl ester **4**. On a preparative scale, a reaction yield of 98% and an ee of 99% was obtained.

1. Introduction

Recently, much attention has been focused on the interaction of small molecules with biological macromolecules. Selective enzyme inhibitors and receptor agonists/antagonists are keys for target-oriented research in the pharmaceutical industry.[1] Chirality is a key factor in the efficiency of many drug products, and as a result the production of single enantiomers of molecules has become increasingly important in the pharmaceutical industry.[2] Single enantiomers can be produced by chemical or chemo-enzymatic synthesis. The advantages of biocatalysis over chemical synthesis are that enzyme-catalyzed reactions are often highly enantioselective and regioselective. They can be carried out at ambient temperature and atmospheric pressure, thus avoiding the use of more extreme conditions, which could cause problems due to isomerization, racemization, epimerization, or rearrangement. A number of review articles[3–10] have been published on the use of enzymes in organic synthesis.

Herein we report the enzymatic synthesis of (*S*)-1-(2′-bromo-4′-fluorophenyl)-ethanol **2** and (*S*)-hydroxy esters **4**, **6**, and **8** (Fig. 1A and B), as potentially useful intermediates in the synthesis of pharmaceutical products.[11–16]

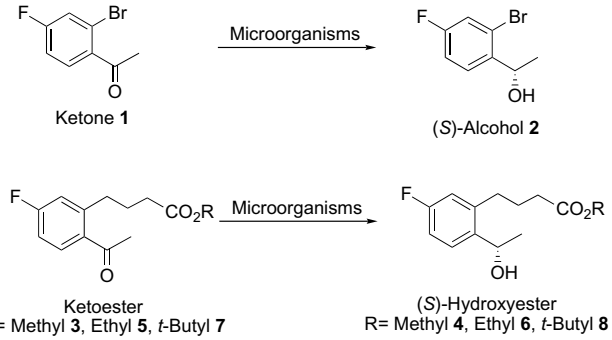

Figure 1.

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2. Results and discussion

A number of microorganisms were screened for the enantioselective reduction of ketone **1** to alcohol **2**. Results (yields, and ee's of product **2**) obtained with the best cultures are as shown in Table 1. Many cultures from *Candida*, *Hansenula*, and *Pichia* gave high reaction yields (99–100 M %) and ee's (>99%) of desired (*S*)-alcohol **2**. Furthermore, commercially available active dry yeast (Red Star) gave a reaction yield of 90% and an ee of 99.9% for desired alcohol **2**.

The dry yeast was investigated further with the reduction process carried out at a 1-L and 100-L scale as described in the Experimental section. A reaction yield of 90% and an ee of 99.5% were obtained for (*S*)-alcohol **2** in each experiment. At the end of the reaction, product **2** was adsorbed onto XAD-16 resin and, after filtration, recovered in 84% yield from the resin by acetonitrile extraction and silica gel chromatography.

Various microbial cultures were screened for the enantioselective reduction of keto methyl ester **3**. Results (yields, and ee's of product) obtained from the five best cultures are as shown in Table 2. Three strains of *Pichia* gave reaction yields of 33–41 M % and ee's of >96% of the desired (*S*)-alcohol **4**. The lower reaction yield relative to **1** was due to the hydrolysis of keto methyl ester **3** to the corresponding acid, which is not a substrate for reduction.

We also evaluated various cultures for the enantioselective reduction of ethyl ester **5** and *tert*-butyl ester **7**. Results with the three best cultures are as shown in Tables 3 and 4. As observed with the methyl ester, hydrolysis of the ethyl ester to the corresponding acid occurred with lower reaction yields being obtained. The *tert*-butyl ester proved more stable, with formation of the acid being less then 7%; however, a lower yield was obtained due to poor substrate specificity.

In order to circumvent the problem of ester hydrolysis, we decided to purify the reductase from *Pichia met-*

Table 2. Enantioselective microbial reduction of keto methyl ester **3**

Microorganism	% Conversion to (*S*)-alcohol **4**	Ee of (*S*)-alcohol **4** (%)
Pichia methanolica SC 13825	40	99.8
Pichia methanolica SC 13860	41	99
Pichia methanolica SC 16116	33	96
Candida boidinii SC 13821	11	99.4
Geotrichum candidum SC 16010	6	99

Microbial cultures were suspended in 10 mL of 100 mM phosphate buffer (pH 7.0) at 20% (w/v, wet cells) cell concentration supplemented with 1.0 mg/mL substrate **3** and 25 mg/mL of glucose. Reductions were carried out at 28 °C and 250 rpm on a rotary shaker for 24 h.

Table 3. Enantioselective microbial reduction of keto ethylester **5**

Microorganism	% Conversion to (*S*)-alcohol **6**	Ee of (*S*)-alcohol **6** (%)
Pichia methanolica SC 13825	18	93
Pichia methanolica SC 13860	51	>99.9
Candida boidinii SC 13821	33	98.5

Microbial cultures were suspended in 10 mL of 100 mM phosphate buffer (pH 7.0) at 20% (w/v, wet cells) cell concentration supplemented with 1.0 mg/mL substrate **5** and 25 mg/mL of glucose. Reductions were carried out at 28 °C and 250 rpm on a rotary shaker for 24 h.

Table 1. Enantioselective microbial reduction of 2-bromo-4-fluoroacetophenone **1**

Microorganism	% Conversion to (*S*)-alcohol **2**	Ee of (*S*)-alcohol **2** (%)
Candida sonorensis SC 16117	100	99.2
Candida Guilliermondi SC 13861	100	99
Candida boidinii SC 13821	100	97.4
Candida utilis SC 13983	99	99.6
Candida parapsilosis SC 16346	98	97.6
Rhodotorula glutinis SC 16267	100	99.9
Hansenula fabianii SC 13894	94	99
Hansenula polymorpha SC 13824	100	99.8
Hansenula saturnus SC 13829	100	99
Nocardia salmonicolor SC 6310	100	99.3
Pichia anomala SC 16142	100	99
Pichia methanolica SC 13860	100	99
Pichia pinus SC 13864	100	99
Pichia stiptis SC 13863	99	99
Pichia capsulata SC 16306	100	99
Pichia silvicola SC 16159	99	99
Sphingomonas paucimobilis SC 16113	100	99
Saccharomyces cerevisiae SC 13902	93	99.9
Active Dry Yeast (Red Star Co.)	90	99.9

Microbial cultures were suspended in 10 mL of 100 mM potassium phosphate buffer (pH 7.0) at 20% (w/v, wet cells) cell concentration supplemented with 1.5 mg/mL substrate **1** and 25 mg/mL of glucose. Reductions were carried out at 28 °C and 250 rpm on a rotary shaker for 24 h.

Table 4. Enantioselective microbial reduction of keto *tert*-butyl ester **7**

Microorganism	% Conversion to (*S*)-alcohol **8**	Ee of (*S*)-alcohol **8** (%)
Mucor rouxii SC 13920	53	>99
Mucor hiemalis SC 13974	43	93
Pichia methanolica SC 16116	12	92.6

Microbial cultures were suspended in 10 mL of 100 mM phosphate buffer (pH 7.0) at 20% (w/v, wet cells) cell concentration supplemented with 1.0 mg/mL substrate **7** and 25 mg/mL of glucose. Reductions were carried out at 28 °C and 250 rpm on a rotary shaker for 24 h.

hanolica SC 13825 and clone and overexpress this enzyme in a suitable host. The enzyme was purified 247-fold from the cell extract using Hi-Trap Blue affinity column chromatography (Table 5). The purified protein gave a single band on SDS-PAGE with a molecular weight of 40,000 Da. The molecular weight of the purified protein, as determined by size-exclusion column chromatography, was 38,000–40,000, indicating that the reductase was a monomeric protein.

The purified protein required NADPH or NADH as a cofactor for the reduction of keto methyl ester **3**. Reaction yields of 96% and 89% were obtained for product **4** with 0.1 mM NADP or NAD at 1 g/L substrate input, respectively, using glucose dehydrogenase as the cofactor recycling enzyme. The N-terminal and internal peptide sequences (generated by Lys-peptidase treatment) of the purified reductase were determined to allow the synthesis of oligonucleotide probes for cloning. The reductase was expressed as *Escherichia coli* strain SC 16445. The complete sequence of the ketoreductase is covered in Ref. 17.

The production of cloned ketoreductase in a 250-L fermentor was conducted as described in the Experimental section. Growth was completed in 48 h and about 50–52 g/L cells were obtained from the fermentation broth (Fig. 2). Cells harvested from the fermentor were used to conduct the bioreduction of keto methyl ester **3**.

The effect of substrate concentration was evaluated in flasks using 10% (w/v, wet cells) cell concentration of *E. coli* strain SC 16445. Their reaction went to completion in 24–30 h at 2–4 g/L substrate input. At 6 g/L substrate input, 48 h were required to complete the reaction (Fig. 3).

The biotransformation process was scaled up to 1-L and 500-L fermentors. Cells were suspended in 1 L of 50 mM

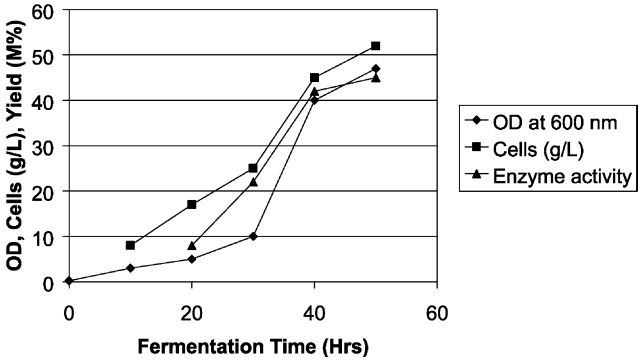


Figure 2.

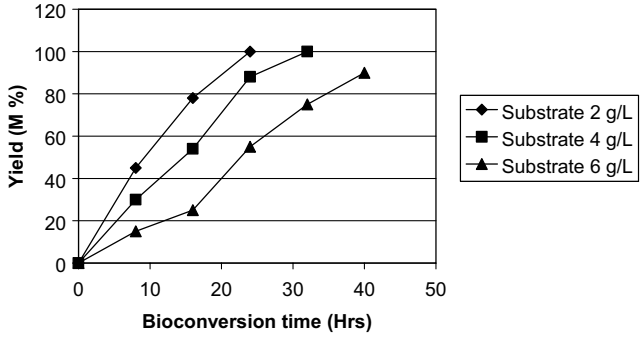


Figure 3.

phosphate buffer pH 7.0 at 10% (w/v) cell concentration. Cell suspensions were supplemented with nicotinamide adenine dinucleotide phosphate (NADP), glucose, glucose dehydrogenase, and 4.5 g/L substrate. Biotransformations were carried out at 500 rpm and 28 °C as described in the Experimental section. The reaction went to completion in 20 h with 95% reaction yield and 99.9% ee of product **4**. Significant hydrolysis of substrate **3** was not observed, with <2% of keto acid **5** being formed. This process was scaled-up to 500 L to prepare 2.0 kg of product **4**.

The dehydrogenases from yeast,[18–21] horse liver,[22] and *T. brockii*[23] transfer the pro-*R* hydride to the *re*-face of the carbonyl to give (*S*)-alcohols, a process described by Prelog's rule.[24] In contrast, dehydrogenases from *Lactobacillus kefir*[25] and two *Pseudomonas* sp.[26] exhibit anti-Prelog specificity, transferring the pro-*R* hydride to form (*R*)-alcohols. Previously we have used various dehydrogenases to catalyze the enantioselective reduction and reductive amination of ketones and α-ketoacids to

Table 5. Purification of ketoreductase from *Pichia methanolica* SC 13825

Step	Volume (mL)	Total protein (mg)	Total units	Sp. activity (units/mg protein)	Purification (fold)
Cell extracts	265	2170	9.4	0.0043	1
HiTrap affinity column (pH gradient)	51	19.5	1.2087	0.0619	14.4
HiTrap affinity column (NADP gradient)	2	0.06	0.0638	1.063	247

The reaction mixture for the enzyme assay contained 1 mg substrate **3**, 4 mM NADP, 4 units glucose dehydrogenase in 1 mL. The reaction was carried out at 30 °C for 5 h, and the concentration of product **4** determined by HPLC. One unit is defined as 1 μmol of **4** formed/h.

prepare alcohols and chiral amines required for the synthesis of antihypertensive, anticholesterol, anticancer, and antiviral drugs.[27–30]

3. Conclusion

Herein, we have described the enantioselective reduction of 2-bromo-4-fluoro-acetophenone **1** to the corresponding (*S*)-2-bromo-4-fluoro-phenyl ethanol **2** by Baker's yeast. We have also demonstrated the enantioselective microbial reduction of ketoesters **3**, **5**, and **7** to the corresponding (*S*)-hydroxy esters **4**,**6**, and **8**. The keto reductase from *P. methanolica* SC 13825 was cloned and expressed in *E. coli*. Recombinant *E. coli* expressing the keto reductase was used to catalyze the enantioselective reduction of keto methyl ester **3** to the corresponding (*S*)-hydroxy methyl ester **4** on preparative scale.

4. Experimental

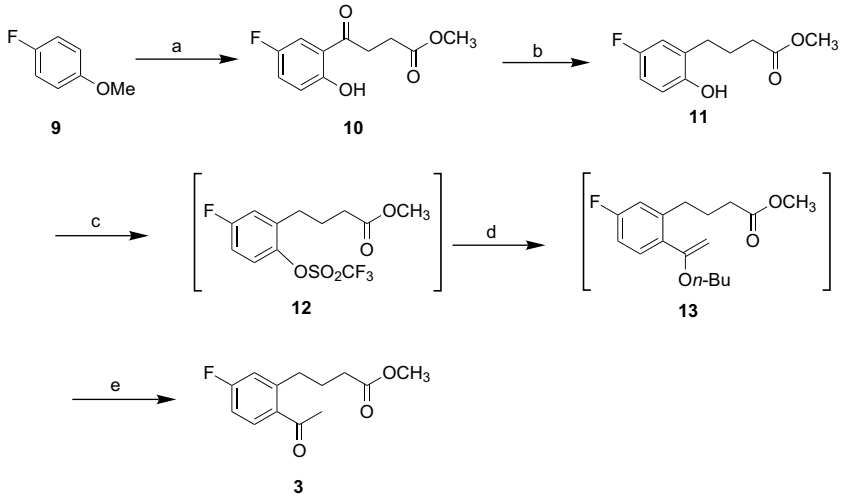
4.1. Materials

The 2-bromo-4-fluoroacetophenone was obtained from Lancaster Chemicals. Starting substrates **3**, **5**, **7** and reference compounds **2**, **4**, **6**, **8** were synthesized by colleagues in the Process Research and Development Department, Bristol-Myers Squibb Pharmaceutical Research Institute. The physico-chemical properties, including spectral characteristics (^{1}H NMR, ^{13}C NMR, mass spectra), were in full accord for all compounds.

4.2. Preparation of substrates 3, 5, and 7

All reactions were conducted under nitrogen unless mentioned otherwise. Commercially available reagents and solvents were used as received. Thin-layer chromatography was performed on silica gel F_{254}, 2.5×7.5 cm plates, obtained from EM Science. The HPLC conditions used for **10**, **11**, **12**, **13**, and **3** were as follows: Column: YMC ODS-AQ, 4.6×150 mm; UV detection at 225 nm; solvent A: water–CH_3CN (60:40 v/v); solvent B: water–CH_3CN (20:80 v/v), gradient program starting with 100% A for 10 min, changing to 100% B over 5 min, holding at 100% B for 15 min, changing to 100% A over 5 min and final equilibration with 100% A for 5 min; flow rate: 0.8 mL/min to follow the formation of **10** (rt 8.6 min) and 1.2 mL/min to follow the formation of **11** (rt 4.6 min), **12** (rt 17.3 min), **13** (rt 19.7 min), and **3** (rt 6.5 min). The HPLC conditions used for **15** and **19** were: Column: YMC S3 ODS-A, 6.0×150 mm; UV detection at 220 nm; solvent A: 0.2% H_3PO_4; solvent B: water–CH_3CN (10:90 v/v); gradient program starting at 30% of B and changing over to 100% B over 15 min and holding at 100% B for 5 min, changing over to 30% B over 5 min and final equilibration with 30% B for 5 min; flow rate: 1.5 mL/min; rt for **15** was 12.2 min and 14.5 min for **19**. Nuclear magnetic resonance (NMR) spectra were run on a Bruker AC-300 spectrometer at 300 MHz for proton and 75 MHz for carbon (Scheme 1).

4.2.1. Preparation of 4-(5-fluoro-2-hydroxy-phenyl)-4-oxo-butyric acid methyl ester 10. A 20-L reactor was charged with succinic anhydride (618 g, 6.17 mol) and aluminum chloride (2210 g, 16.57 mol). After inerting the vessel with nitrogen, 4-fluoroanisole **9** (650 g, 5.15 mol) dissolved in methylene chloride (2800 mL) was added under slow agitation while maintaining the reaction temperature below 35 °C. The transfer line was rinsed with additional methylene chloride (400 mL). The slurry was heated to 37–42 °C (reflux) and the reflux temperature maintained until the reaction was judged to have gone to completion by HPLC (about 9 h) to obtain the intermediate keto acid. The reaction mixture was cooled to room temperature and diluted with methylene chloride (3250 mL).



Scheme 1. Reagents and conditions: (a) CH_2Cl_2, $AlCl_3$, succinic anhydride; MeOH and HCl (g); (b) H_2, $Pd(OH)_2$, MeOH; (c) CH_2Cl_2, Tf_2O, DIPEA; (d) $Pd(OAc)_2$, DPPP, DIPEA, *n*-butyl vinyl ether; (e) 2 M HCl.

A separate 25-L reactor was charged with methanol (13,000 mL) and cooled to <10 °C at which point agitation commenced. The methylene chloride solution of the reaction mixture obtained above was charged slowly while maintaining the batch at <35 °C. Anhydrous HCl gas (650 g, 18 mol) was bubbled into the reaction mixture at ~10–25 g/min while maintaining the batch at <25 °C. The resulting slurry was then stirred at ambient temperature until the reaction to obtain the keto ester was determined to be complete by HPLC (18–24 h). The batch was concentrated to ~6500 mL, cooled to <10 °C, and then treated slowly with 1 M HCl (13,000 mL) and stirred for an additional 4 h. The solid obtained was filtered, washed with water (~6500 mL), and then dried in a vacuum oven at ⩽45 °C for 24 h to obtain 1064 g of the keto ester **10** as a pale yellow solid in 91.3% yield; ^{1}H NMR δ 7.5–6.8 (m, 3H), 3.6 (s, 3H), 3.3 (t, 2H, $J = 6.5$ Hz), 2.7 (t, 2H, $J = 6.5$ Hz) ppm; ^{13}C NMR δ 203.6, 173.2, 158.7, 156.7, 153.6, 124.4, 120.2, 116.9, 52.3, 33.5, and 27.8 ppm; FT-IR (KBr) 1746, 1649, 1490, 1449, 1378, 1285, 1173, 1009, 845, 794, and 676 cm^{-1}; Anal. Calcd for $C_{11}H_{11}O_4F{\cdot}0.04H_2O$: C, 58.21; H, 4.92. Found: C, 57.90; H, 4.68.

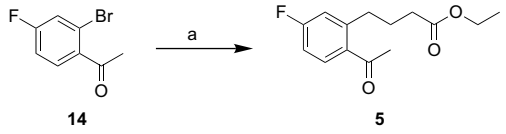
4.2.2. Preparation of 4-(5-fluoro-2-hydroxy-phenyl)-butyric acid methyl ester 11. A Buchi hydrogenator flask was charged with keto ester **10** (1049 g, 4.63 mol) and methanol (4140 mL) and stirred well. Pearlman's catalyst (105 g, 10 wt% loading, Degussa type, 50 wt% wet catalyst) was added to the resulting solution. Hydrogenation was conducted at ~60 psi for ~16 h. HPLC indicated that the reaction was complete. The reaction mixture was filtered on a bed of 40 g of Celite to remove the catalyst. The Celite bed was washed with methanol (6×200 mL). The combined washings and the initial product-rich filtrate were concentrated under reduced pressure, keeping the bath temperature at ⩽45 °C to obtain crude **11** as a solid (938 g). Toluene (1000 mL) was added and the slurry heated to ~50 °C to obtain a solution. After a polish filtration to remove some fine suspended particles, heptane (1000 mL) was added over a period of 60 min to the filtrate maintained at ~50 °C. The resulting hazy solution was stirred at the same temperature for 60 min and then treated with another portion of heptane (1000 mL). The cloudy solution was stirred for an additional hour at the same temperature and then for two more hours at ambient temperature. The solid obtained was filtered, washed with a toluene–heptane mixture (150 mL: 850 mL) and dried in a vacuum oven at ambient temperature for 24 h to obtain 759 g of **3** as a gray solid in 77.3% yield; ^{1}H NMR δ 6.7 (m, 3H), 3.6 (s, 3H), 2.6 (t, 2H, $J = 6.5$ Hz), 2.3 (t, 2H, $J = 6.5$ Hz), 1.9 (m, 2H) ppm; ^{13}C NMR δ 175.9, 158.7, 155.5, 150.6, 129.2, 114.9, 52.4, 33.2, 29.7, and 25.0 ppm; FT-IR (KBr) 1716, 1511, 1439, 1367, 1337, 1204, 1147, 1091, and 850 cm^{-1}; Anal. Calcd for $C_{11}H_{13}O_3F{\cdot}0.11H_2O{\cdot}0.1$MeOH: C, 61.32; H, 6.32. Found: C, 61.21; H, 5.88.

4.2.3. Preparation of 4-(2-acetyl-5-fluoro-phenyl)-butyric acid methyl ester 3. *Preparation of* **12***:* The first step consisted of the preparation of the triflate ester **12**. To a reactor containing the phenol **11** (650 g), diisopropylethyl amine (554.2 g, 747 mL), and dichloromethane (6500 mL) were added and the mixture cooled to −30 °C. Triflic anhydride (1039.4 g, 620 mL) was added slowly (~45 min) while maintaining the batch at <−20 °C. The reaction mixture was warmed and stirred at 20–22 °C for 15 min. The reaction was followed by TLC. After ~2 h, DMF (1950 mL) was charged slowly to the reactor and dichloromethane removed under vacuum while keeping the batch temperature at 50–60 °C until no condensation of dichloromethane was observed.

4.2.4. Palladium coupling to prepare the vinyl ether 13. Another reactor was charged with $Pd(OAc)_2$ (37.25 g), DPPP (70.86 g), diisopropylethyl amine (554.8 g, 747.7 mL), DMF (1.86 kg, 1.94 L) and agitated under nitrogen for 15 min. The triflate **12** prepared in the first step was added to the catalyst mixture over 5 min followed by *n*-butyl vinyl ether (462.58 g, 597.60 mL). The reaction mixture was heated to 83–85 °C over 90 min and the temperature held until the reaction was judged to have gone to completion by HPLC (~2 h). Water (1100 mL) was charged to the reaction mixture and the mixture stirred well. The product was extracted into heptane (1×3400 mL, 1×2000 mL, and 3×1500 mL). The combined heptane extract was washed with water (833 mL) and then concentrated under reduced pressure to remove solvent while maintaining the batch temperature at 50–60 °C to obtain 889 g of crude vinyl ether **13**. MTBE (4300 mL) was charged to the reactor containing **13** and agitated well to obtain a solution. HCl (2 M, 3430 mL) was added and the biphasic mixture was agitated vigorously at ambient temperature for about 3 h, at which time HPLC indicated that the hydrolysis was complete. The organic phase was separated, washed with water (3×1200 mL, final pH of the aqueous phase 3.1), and then concentrated under reduced pressure to remove solvent. The methyl ketone **3**, 769 g, was obtained as a light amber oil containing ~6 wt% of *n*-BuOH as a contaminant (NMR determination). The oil was further purified using an EVAPOR thin-film evaporator to remove *n*-BuOH. After two passes, the *n*-BuOH content was reduced to 2.2 wt% and the methyl ketone **3**, 671.8 g, was obtained as a light yellow liquid in 90% yield (corrected for *n*-BuOH content). ^{1}H NMR δ 7.8 (m, 1H), 7.0 (m, 2H), 3.6 (s, 3H), 2.9 (dd, 2H), 2.5 (s, 3H), 2.4 (dd, 2H), and 1.9 (m, 2H) ppm; ^{13}C NMR δ 200.3, 174.1, 166.1, 162.8, 146.2, 133.9, 132.6, 115.8, 51.8, 33.9, 33.7, 29.9, and 26.7 ppm; FT-IR (film) 1741, 1690, 1608, 1588, 1501, 1439, 1362, 1245, 1163, 1111, 1070, 983, and 825 cm^{-1}; Anal. Calcd for $C_{13}H_{15}FO_3{\cdot}0.17n$-BuOH: C, 65.52; H, 6.50. Found: C, 64.86; H, 6.40.

4.2.5. Preparation of 4-(2-acetyl-5-fluoro-phenyl)-butyric acid ethyl ester 5. A dry 1 L, round-bottomed flask was charged with $PdCl_2(PPh_3)_2$ (1.62 g). After flushing with nitrogen, DMA (120 mL) was charged to the flask followed by the addition of the bromo ketone **14** (10 g, 46.07 mmol). To the resulting suspension, 4-ethoxy-4-

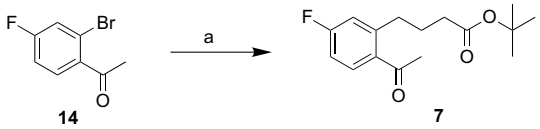
oxobutylzinc bromide (0.5 M solution in THF, 120 mL, 60 mmol) was added over a period of 20 min. The reaction mixture was stirred at ambient temperature for ~60 h. After cooling in an ice bath, the reaction mixture was quenched with 1 M HCl (100 mL) and then stirred for an hour at ambient temperature. THF was removed under reduced pressure and the resulting mixture extracted with MTBE (2×200 mL). The MTBE extract was washed with water (2×200 mL) followed by brine (200 mL) and dried over Na_2SO_4, and then concentrated under reduced pressure to remove solvent. The crude product was purified by flash chromatography on silica gel to obtain 9.2 g of ethyl ester **5** as a yellow liquid in 79.2 M % yield. ^{1}H NMR δ 7.8 (m, 1H), 6.9 (m, 2H), 4.1 (q, 2H, $J = 7$ Hz), 2.9 (dd, 2H), 2.6 (s, 3H), 2.4 (t, 2H), 1.9 (m, 2H), and 1.1 (t, 3H, $J = 6.8$ Hz) ppm; ^{13}C NMR δ 200.3, 173.7, 166.1, 162.8, 146.3, 134.0, 132.5, 115.8, 60.6, 34.2, 33.7, 30.0, 26.7, and 14.59 ppm; FT-IR (film): 1741, 1685, 1613, 1582, 1495, 1429, 1357, 1234, 1188, 1111, 800, and 973 cm^{-1}; Anal. Calcd for $C_{14}H_{17}FO_3$: C, 66.65; H, 6.79; F, 7.53; Found: C, 66.57; H, 6.59; F, 7.32 (Scheme 2).



Scheme 2. Reagents and conditions: (a) DMA, $PdCl_2(PPh_3)_2$, $BrZn(CH_2)_3COOEt$.

4.2.6. Preparation of 4-bromo-butyric acid *tert*-butyl ester 17. Trifluoroacetic anhydride (6 mL) was added slowly to a solution of 4-bromo butyric acid **16** (3.4 g, 20.48 mmol) in dry THF (30 mL), pre-cooled to −40 °C. The resulting solution was stirred at the same temperature for ~30 min. *tert*-Butyl alcohol (25 mL) was added and the solution allowed to warm to ambient temperature and stirred for an additional 16 h. The reaction mixture was poured slowly into a mixture of crushed ice and saturated sodium bicarbonate solution (50 mL). The product was extracted into EtOAc (100 mL) and the organic phase was washed with water (100 mL) and brine (100 mL). After drying over Na_2SO_4, the solution was concentrated under reduced pressure to obtain an oil. The oil was dissolved in MTBE (100 mL) and filtered through a short pad of silica-gel. The silica pad was washed with MTBE (100 mL). The combined MTBE filtrate and washing were concentrated under reduced pressure to obtain 4.1 g of **17** as a liquid in 90 M % yield (Scheme 3).

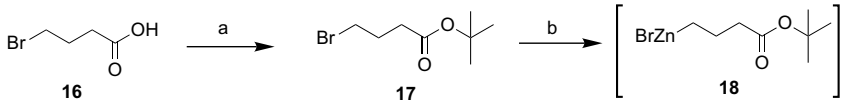
4.2.7. Preparation of 4-(2-acetyl-5-fluoro-phenyl)-butyric acid *tert*-butyl ester 7. A 250-mL, three-necked, round-bottomed flask was charged with zinc powder (1.7 g, 26.9 mmol) and lithium iodide (0.6 g, 4.5 mmol), then heated under vacuum at 100 °C for 1 h. After cooling to ambient temperature, dimethylacetamide (30 mL) was added to the flask followed by dibromoethane (0.25 g, 1.3 mmol). The reaction mixture was heated and held at 70 °C for 10 min. After cooling it back to ambient temperature, trimethylsilyl chloride (0.15 g, 1.3 mmol) was added and the reaction mixture was stirred for an additional 30 min. Bromo ester **17** (1.0 g, 4.48 mmol) was added and the mixture heated to 75 °C. The rest of the bromo ester (4.0 g, 17.92 mmol) was added over a 30 min period and heating continued for an additional 5 h. The reaction mixture was then cooled to ambient temperature and transferred over 15 min to a flask containing the bromo ketone **14** (3.0 g, 13.82 mmol) and $PdCl_2(PPh_3)_2$ (0.48 g, 0.69 mmol) in anhydrous THF (15 mL) and stirred for ~18 h. HCl (1M, 50 mL) was added and stirring continued. The reaction mixture was extracted with MTBE (100 mL). The organic phase was separated and the aqueous phase back-extracted with MTBE (50 mL). The combined organic phase was washed with water (2×100 mL) and brine (2×50 mL) and then stirred with deactivated carbon (2 g) for ~2 h. After drying over Na_2SO_4, the organic phase was concentrated under vacuum to a brown oil. The crude product was further purified by chromatography on silica gel to obtain 2.4 g of **7** as a yellow oil in 62% yield. ^{1}H NMR δ 7.8 (m, 1H), 6.8 (m, 2H), 2.8 (dd, 2H), 2.5 (s, 3H), 2.3 (t, 2H), 1.9 (m, 2H), 1.4 (s, 9H) ppm; ^{13}C NMR δ 200.3, 173.0, 166.1, 162.7, 146.4, 134.0, 132.4, 115.8, 80.4, 35.4, 33.6, 30.0, 28.4, and 26.8 ppm; FT-IR (film): 1726, 1690, 1603, 1593, 1367, 1239, 1157, and 968 cm^{-1}; Anal. Calcd for $C_{16}H_{21}FO_3$: C, 68.55; H, 7.55; F, 6.77; Found: C, 68.42; H, 7.46; F, 6.68 (Scheme 4).



Scheme 4. Reagents and conditions: (a) $PdCl_2(PPh_3)_2$, THF, and then $BrZn(CH_2)_3COOtBu$.

4.3. Microorganisms

Microorganisms (Tables 1–4) were obtained from the culture collection of the Bristol-Myers Squibb Pharmaceutical Research Institute. Microbial cultures were stored at −90 °C in vials.



Scheme 3. Reagents and conditions: (a) $(CF_3CO)_2O$, *t*-BuOH, THF; (b) DMA, Zn, LiI, $Br(CH_2)Br$, TMSCl.

4.4. Growth of microorganisms

For screening purposes, one vial (1 mL) of each culture was used to inoculate 100 mL of medium A (1% malt extract, 1% yeast extract, 2% glucose, and 0.3% peptone). The medium was adjusted to pH 6.8 before sterilization. Cultures were grown at 28 °C and 200 rpm for 48 h. Cultures were harvested by centrifugation at 18,000*g* for 15 min, washed with 100 mM potassium phosphate buffer (pH 7.0), and suspended in 10 mL of the same buffer, and then used for reduction studies.

4.5. Reduction of 2-bromo-4-fluoro acetophenone 1

Cells of various microorganisms were suspended separately in 100 mM potassium phosphate buffer (pH 7.0) at 20% (w/v, wet cells) cell concentration and supplemented with 1.5 mg/mL of acetophenone **1** and 25 mg/mL of glucose. Reduction was conducted at 28 °C and 150 rpm. Periodically, samples (1 mL) were taken and extracted with 4 mL of acetonitrile. The sample was filtered through a 0.2 μm LID/X filter and analyzed using a Hewlett Packard 1070 high pressure liquid chromatograph (HPLC) to determine the substrate **1** and product **2** concentration. The remaining solution was evaporated to dryness under a stream of nitrogen and the residue taken up in 1 mL of ethanol, filtered and analyzed by HPLC to determine the enantiomeric excess of product **2**. A phenylhexyl (150×4.6 mm, 5 μm Phenomenex) column was used. The mobile phase consisted of 1:1 acetonitrile and water, and was used at a flow rate of 1.0 mL/min. The detection wavelength was 210 nm and the column temperature 50 °C. The retention times for substrate **1** and product **2** were 6.3 and 5.4 min, respectively. Separation of the two enantiomers of the racemic product **2** was achieved on a Chiralpak AD (4.6 mm×250 mm, 10 μm, Diacel Chemical Industry Ltd.) column. The mobile phase consisted of 0.249% absolute ethanol in hexane. The flow rate was 1 mL/min and the detection wavelength 210 nm. The retention times for the desired (*R*)-enantiomer was 48 min and that for the undesired (*S*)-enantiomer 54 min.

4.6. Reduction of 2-bromo-4-fluoro acetophenone 1 using Baker's yeast

A 3-L bioreactor (Braun Biostat B) was equipped with a pH electrode and the impeller speed and temperature set at 500 rpm and 28 °C, respectively. Phosphate buffer (800 mL of 10 mM, pH 6.0) was added to the bioreactor and 150 g of Baker's yeast (from Red Star) added slowly over a 30 min period. The contents of the bioreactor were stirred at 500 rpm, with the temperature maintained at 28 °C, and the pH maintained at 6.0. Acetophenone **1** (5 g) and 192 mL of glucose solution (25%) were added to the reactor at the rate of 8 mL/h over a period of 24 h. Foaming was controlled by addition of 0.5 mL of SAG antifoam as required. Samples (1 mL) were taken at intervals and analyzed for substrate **1** and product **2** concentrations and ee of product **2**. Under similar conditions, this process was scaled-up to 200-L scale to reduce 1 kg of ketone **1**.

4.7. Recovery of product 2

At the end of the biotransformation (20 h), 50 g of XAD-16 resin (previously washed with 500 mL of water containing 50% methanol and then with 2×500 mL of water) was added to 1 L of the reaction broth. The mixture was stirred at room temperature at 300 rpm for 3 h and then filtered through 40 mesh stainless steel sieve. The collected resin was washed with 50 mL of water containing 20% methanol and filtered through a stainless steel sieve. The product-rich resin was treated with 50 mL of methyl *tert*-butyl ether (MTBE) to exude the desired alcohol **2**. The solution was dried over anhydrous sodium sulfate, filtered, and the solvent removed in vacuo to provide 4.25 g of an oil in 85% overall yield. HPLC analysis showed 98.9% HI for product **2** with an ee of 99.8%. The same procedure was used to recover alcohol **2** from a 200-L biotransformation batch. After adsorption of the product on XAD-16 and subsequent desorption of the product from the rich resin by methyl *tert*-butyl ether extraction and evaporation, about 1.03 kg of oil was recovered. The crude product was purified by silica gel chromatography to provide 707.4 g of product **2** in 99.6% purity with an ee of 99.9%.

4.8. Reduction of methyl-, ethyl, and *tert*-butyl 4-(2′-acetyl-5′-fluorophenyl) butanoate

Various microbial cultures (1 mL) were inoculated into 100 mL of medium A or medium B (1% malt extract, 1% yeast extract, 0.3% peptone, and 2% glycerol adjusted to pH 7) in a 500-mL flask and incubated at 28 °C and 200 rpm on a shaker for 48 h. Cells were harvested by centrifugation and suspended in 10 mL of 100 mM potassium phosphate buffer (pH 7.0) at 20% (w/v, wet cells) concentration. Glucose (25 mg/mL) and keto methyl ester **3** (1 mg/mL) were added to the cell suspensions. The reduction was carried out at 28 °C and 200 rpm on a shaker for 24–48 h. At predetermined times, the reaction mixtures were quenched with four volumes of acetone, mixed on a vortex mixer, filtered through a 0.2 μm filter, and collected, with a 1 mL sample analyzed by HPLC to determine the keto methyl ester **3** and hydroxy methyl ester **4** concentrations. The remaining solution was evaporated to dryness under a stream of nitrogen and the residue taken up in 1 mL of ethanol, filtered, and analyzed by chiral HPLC to determine the ee of product **4**. A YMC ODS-A (150×4.6 mm, 5 μm) column was used. The mobile phase consisted of a 40% acetonitrile and 60% H_3PO_4 (0.3%) mixture used at a flow rate of 1.0 mL/min. The detection wavelength was 210 nm and the column temperature 50 °C. The retention times for keto methyl ester **3** and hydroxy methyl ester **4** were 11.1 and 6.7 min, respectively. Separation of the two enantiomers of the racemic hydroxy methyl ester was achieved on a Chiralpak AD (4.6 mm×250 mm; 10 μm) column (Diacel

Chemical Industry Ltd). The mobile phase consisted of 95% solvent A (hexane/absolute ethanol, 98.5%:1.5%) and 5% solvent B (hexane/isopropanol, 95:5) mixture. The flow rate was 1 mL/min and the detection wavelength was 210 nm. The retention time for the desired (*R*)-enantiomer was 23.6 min and that for the undesired (*S*)-enantiomer was 31.1 min. Using similar conditions microbial reductions of keto ethyl ester **5** and keto *tert*-butyl ester **7** were carried out. The analysis of keto ethyl ester **5** and the hydroxy ethyl ester was carried out using a phenylhexyl (150×4.6 mm, 5 μm). The mobile phase consisted of a 50% acetonitrile and 50% water mixture used at a flow rate of 1.0 mL/min. The detection wavelength was 210 nm. The retention times for keto ethyl ester **5** and hydroxy ethyl ester **6** were 12.4 and 8.9 min, respectively. Separation of the two enantiomers of the racemic hydroxy ethyl ester was achieved on a Chiralpak AD (4.6 mm×250 mm; 10 μm) column (Diacel Chemical Industry Ltd). The mobile phase consisted of a hexane/absolute ethanol, and isopropanol (96.9%:2.85%:0.25%) mixture. The flow rate was 1 mL/min and the detection wavelength 210 nm. The retention time for the desired (*R*)-enantiomer was 15.0 min and that for the undesired (*S*)-enantiomer was 19.0 min. The analysis of keto *tert*-butyl ester **7** and hydroxy *tert*-butyl ester was carried out using a phenylhexyl (150×4.6 mm, 5 μm). The mobile phase consisted of a 50% acetonitrile and 50% water mixture used at a flow rate of 1.0 mL/min. The detection wavelength was 210 nm. The retention times for keto *tert*-butyl ester **7** and hydroxy *tert*-butyl ester **8** were 28.3 and 19.2 min, respectively. Separation of the two enantiomers of the racemic hydroxy *tert*-butyl ester was achieved on a Chiralpak AD (4.6 mm×250 mm; 10 μm) column (Diacel Chemical Industry Ltd). The mobile phase consisted of a hexane/isopropanol (90%:10%) mixture. The flow rate was 1 mL/min and the detection wavelength 210 nm. The retention time for the desired (*R*)-enantiomer was 23.6 min and that for the undesired (*S*)-enantiomer 32.8 min.

4.9. Growth of *Pichia methonica* in fermentor

P. methanolica SC 13825 was grown in 380-L fermentors containing 250 L of medium A containing 0.025% SAG and 0.025% Dow Corning antifoam. Growth consisted of two inoculum development stages and one fermentation stage. Inoculum development consisted of F1 and F2 stages. In the F1 stage, 1 mL of a culture of *P. methanolica* SC 13825 was inoculated into 100 mL of medium A contained in 500-mL flasks. Growth was carried out at 28 °C and 200 rpm for 24 h on a rotary shaker. In the F2 stage, 100 mL of F1 stage culture of the organism was inoculated into 1 L of medium A and incubated at 28 °C and 200 rpm for 48 h. A fermentor containing 250 L of medium A was inoculated with 2 L of F2 stage inoculum and grown at 28 °C and 200 rpm agitation with 250 LPM (liter per minute) aeration. During fermentation, cells were harvested periodically by centrifugation from 200 mL of culture broth and assayed for conversion of keto methyl ester **3** to hydroxy methyl ester **4**. At the end of the fermentation, 12 kg of wet cell paste was collected on a Sharples centrifuge and stored at −60 °C for future use.

4.10. Purification of ketoreductase from *P. methanolica*

Preparation of cell extracts was carried out at 4–7 °C. *P. methanolica* SC 13825 cells were washed with 10 mM potassium phosphate buffer pH 6.5 (buffer A). Washed cells (55 g) were suspended in 275 mL of buffer A containing 10% glycerol, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and than homogenized. A 20% cell suspension was disintegrated with a Microfluidizer (Microfluidics, Inc.) at 12,000 psi (three passages) and disintegrated cells were centrifuged at 25,000*g* for 30 min to obtain the cell extract. Protein in the cell extracts was estimated by Bio-Rad protein reagent using bovine serum albumin as standard. The assay mixture contained 1–10 μL of enzyme fraction, 0.8 mL water, and 0.2 mL Bio-Rad reagent. After mixing, the absorbance of the solution was measured at 595 nm. The activity of the enzyme was measured by reduction of keto methyl ester **3** to the corresponding hydroxy methyl ester **4** using the HPLC system described previously. The reaction mixture contained 5 mL of enzyme solution supplemented with 2 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), 20 units glucose dehydrogenase, 25 mg glucose, and 2.5 mg keto methyl ester **3**. The reaction mixture was incubated at 28 °C and 100 rpm. After 6 h incubation, the reaction mixtures were quenched with four volumes of acetone, mixed on a vortex mixer, and filtered through a 0.2 μm filter, with a 1 mL sample was analyzed by HPLC. The cell extract was loaded onto a Hi-Trap Blue-Sepharose affinity column, which was equilibrated with buffer A containing 10% glycerol and 2 mM DTT. The protein was eluted from the column by buffer A using a pH gradient running from pH 6.5–8.5. Active fractions eluted from the column were pooled and loaded again on to a Hi-Trap Blue-Sepharose affinity column and eluted with buffer A containing a NADP gradient running from 0.1 to 0.5 mM. Active fractions with similar specific activity of enzyme were pooled and analyzed by sodium dodecyl sulfate polyacrylamide (SDS/PAGE) gel electrophoresis to check the purity of the protein.

4.11. Sodium dodecyl sulfate polyacrylamide gel-electrophoresis

The active fractions from the second Hi-Trap Blue-Sepharose affinity column were evaluated by SDS-PAGE as described in the PhastSystem® procedure by Pharmacia[31] using the homogeneous 12.5% Phastgel. The enzyme samples were added to a buffer containing 10 mM Tris–HCl, 1 mM EDTA, pH 8, 2.5% SDS, and 5% β-mercaptoethanol. The mixture was heated at 100 °C for 5 min, and bromophenol blue was added to 0.01%. Gels were stained with silver stain and destained in 10% acetic acid solution. Markers with standard molecular weights were phosphorylase β (94,000), bovine serum albumin (67,000), ovalbumin (43,000),

carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400).

4.12. Determination of molecular weight

The molecular weight of the purified keto reductase was determined by size-exclusion chromatography using a Pharmacia Superose column® (15 cm × 1 cm). The column was equilibrated with buffer A. The reductase was applied to the column and eluted with buffer A at a flow rate of 0.4 mL/min. Fractions of 1 mL were collected. A standard protein mixture containing thyroglobulin (669,000 MW), ferritin (440,000 MW), human IgG (150,000 MW), human transferrin (81,000 MW), ovalbumin (43,000 MW), and myoglobin (17,600 MW) was also applied to the column and eluted with buffer A.

4.13. Cloning and expression of ketoreductase from *P. methanolica*

4.13.1. Construction of partial Sau3A1 library of *P. methanolica* SC 13825. *P. methanolica* SC 13825 chromosomal DNA was prepared from cultures grown on a YPD medium (1% yeast extract, 2% peptone, and 2% dextrose) in a 250-mL flask. The flask was incubated at 30 °C with shaking at 250 rpm for 20 h. The procedure for rapid isolation of *Saccharomyces cerevisiae* chromosomal DNA was used to prepare *P. methanolica* DNA. The precipitated DNA was washed with 70% ethanol, air-dried, and resuspended to a final concentration of 1.0 mg/mL in TE buffer (0.01 M Tris–HCl, 0.001 M EDTA, pH 8.0). DNA was partially cleaved with restriction endonuclease Sau3A1. The DNA was electrophoresed through a 0.8% agarose gel and the region containing DNA fragments of ca. 5–10 kb identified by comparison to a 1 kb DNA ladder (Life Technologies, Gaithersburg, MD) and excised from the gel. DNA was extracted using the QIAquick Gel Purification Kit (Qiagen Inc., Valencia, CA) following the recommended protocol. An aliquot was electrophoresed on a 0.8% TAE agarose gel for 20 h at 15 V to confirm that the desired fragment size range had been obtained and to determine the concentration of the fragment by comparison to a DNA mass ladder (Life Technologies).

The enriched *P. methanolica* DNA fragments (5–10 kb) were ligated to BamHI-cleaved pZero2 in a 0.02 mL reaction consisting of 0.1 μg chromosomal DNA, 0.03 μg plasmid DNA, 0.03 M Tris–HCl (pH 7.8), 0.01 M $MgCl_2$, 0.01 M dithiothreitol, and 0.0005 M adenosine-5′-triphosphate and 3 Weiss units of T4 DNA ligase (Promega). The reaction was carried out at 16 °C for 18 h. The ligated DNA was transformed into electrocompetent DH10B cells (Life Technologies) according to the vendor's recommendations. Following transformation, 0.96 mL of LB medium was added and the cells grown at 37 °C for 1 h. A 137 mm Hybond-N+ circle (Amersham-Pharmacia, Piscataway, NJ) was placed on top of a 150 mm Petri dish containing 75 mL LB agar containing kanamycin. An aliquot of the partial *Sau*3A1 library sufficient to give 5000 colony forming units was diluted into 1 mL LB medium and spread evenly on the filter. The plate was incubated at 37 °C for 24 h. Colonies were replicated onto two fresh filters, which were placed onto LB containing kanamycin agar medium and incubated at 37 °C for 6 h. Lysis of cells and neutralization of released DNA was performed according to directions that were provided with the filters. The DNA was crosslinked to the filters using a UV Stratalinker 2400 unit (Stratagene, Inc., La Jolla, CA) in the 'auto crosslink' mode. Cell debris was removed by placing the filters in a container with a solution of 3× SSC (20× SSC contains, per liter, 173.5 g NaCl, 88.2 g sodium citrate, pH adjusted to 7.0 with 10 N NaOH), 0.1% SDS and rubbing the lysed colonies with a wet kimwipe. The filters were then incubated with the same wash solution for at least 3 h at 65 °C.

4.13.2. Selection of clones containing the ketoreductase gene. Mixed oligonucleotide primers based on partial amino acid sequences of the purified *P. methanolica* ketoreductase were prepared. All possible combinations of sense and antisense primers were utilized in polymerase chain reactions (PCR). The reaction consisted of 0.05 M Tris–HCl (pH 8.3), 250 μg/mL bovine serum albumin, 2% (w/v) sucrose, 0.1 mM cresol red, 0.2 mM each dATP, dCTP, dGTP, dTTP, 4 mM $MgCl_2$, 0.0005 mM each primer, 0.25 μL (0.625 U) Takara Z-Taq DNA polymerase (PanVera, Madison, WI), and 0.1 μg *P. methanolica* chromosomal DNA in a total volume of 0.05 mL. Amplification was carried out in a Perkin–Elmer Model 480 Thermal Cycler under the following conditions: Denaturation at 94 °C for 4 min, followed by 30 cycles of 94 °C, 1 min; 50 °C, 1 min; 72 °C, 1.5 min, and a final extension at 72 °C for 5 min. Strong amplification of a 650- and 850-bp fragment, respectively, was observed using oligonucleotide pairs 183 and 186 and 185 and 188 after electrophoresis of a sample of each reaction on a 1.0% TAE agarose gel.

Fragments were isolated from the agaorse gel and purified using the QIAquick Gel Extraction Kit. The DNA was ligated to vector pCR2.1 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol and transformed into *E. coli* DH10B by electroporation. Cells were spread onto LB agar medium containing 50 μg/mL kanamycin and Bluo-gal (Life Technologies; 75 μL of a 2% [w/v] solution in dimethylformide) and incubated at 37 °C for 20 h. Five white colonies chosen at random from each ligation/transformation were inoculated into LB containing kanamycin liquid medium and grown at 37 °C, 250 rpm, for 20 h. Plasmid DNA was prepared from each sample using the QIAprep Spin miniplasmid Kit (Qiagen). The presence of the expected insert was confirmed by PCR using conditions described above. Partial amino acid sequences obtained from the purified enzyme but not used to synthesize oligonucleotides were also found encoded within the PCR fragments. Based on these results, digoxigenin-labeled probes were prepared using two sets of primers and the PCR DIG Probe Synthesis kit (Roche Biochemicals, Indianapolis, IN) according to the manufacturer's directions. Approximately 10 ng of the

isolated PCR fragment described above was included as template DNA. Amplification conditions were: Denaturation at 94 °C for 4 min, followed by 30 cycles of 94 °C, 1 min; 50 °C, 1 min, 72 °C, 1 min. Incorporation of the digoxigenin-dUTP nucleotide could be verified by a significant increase in the molecular weight of the labeled fragment. Superior incorporation was obtained using oligonucleotides 183 and 186.Duplicate filters containing lysed and denatured DNA from the *P. methanolica* Sau3A1 library were incubated with 10 mL of DIG EasyHyb solution (Roche) and 5 μL of the denatured, labeled PCR fragment in a roller bottle. Hybridization proceeded at 42 °C for 18 h. Filters were washed twice with 2× SSC (prepared from a 20× solution; 20× SSC contains, per liter, 173.5 g NaCl, 88.2 g sodium citrate, pH adjusted to 7.0 with 10 N NaOH), 0.1% sodium dodecyl sulfate (SDS) at room temperature for 5 min, then twice at 68 °C with 0.5% SSC, 0.1% SDS for 15 min. Anti-digoxigenin antibody binding, washing, and detection were performed using the DIG Labeling and Detection Kit reagents and protocols (Roche). The membranes were placed on Whatman 3 mm paper to remove excess liquid, covered with Saran Wrap, and exposed to autoradiography film (Kodak X-OMAT LS). A single hybridizing colony was picked from the master filter and streaked onto LB agar medium containing kanamycin and incubated at 37 °C for 24 h. The colony was grown LB liquid medium containing kanamycin for plasmid isolation using the QIAfilter Plasmid Midi Kit (Qiagen). Restriction mapping indicated an insert of ca. 5.0 kb was present in the recombinant plasmid. Isolated DNA was tested for its ability to support amplification using oligos 183 and 186 and 185 and 188, which was confirmed. Oligonucleotide primers based on the DNA sequence of the isolated PCR fragments were used for the analysis of the insert in pKR 5.0. An open reading frame of 1059 bp that encodes a protein of 353 amino acids with a molecular weight of 39,800 Da was found. This is in near agreement with the size of the isolated ketoreductase (40,000 Da by gel filtration).

4.13.3. Subcloning of the ketoreductase gene and expression in *E. coli*. The polymerase chain reaction was utilized to amplify the complete ketoreductase gene containing restriction sites suitable for cloning into expression vector pBMS2000. The primers used are given below:

*Bsp*HI

5′ TGC**TCATGA**ATTGGGAAAAAGTTCCACAAG 3′

Nucleotides in bold indicate the recognition sequence for the restriction enzyme BspHI;

*Bam*HI

5′ CTC**GGATCC**TTATAAAATTACAGAATATAAG 3′

Nucleotides in bold indicate the recognition sequence for the restriction enzyme BamHI; PCR conditions were identical to those described previously, except pKR 5.0 DNA was used as a template.

The BspHI/BamHI-digested PCR fragment was ligated to BspHI/BamHI-cleaved pBMS2000. The ligated DNA was transformed into electrocompetent DH10B cells (Life Technologies, Inc.) according to the vendor's recommendations. Following transformation, 0.96 mL of LB medium were added and the cells grown at 37 °C for 1 h. An aliquot of the cells was spread onto the LB agar medium containing 10 μg/mL neomycin sulfate (Sigma) and the plate incubated at 37 °C for 20 h. The presence of the correct insert was established by PCR using conditions described earlier except a portion of 16 randomly chosen colonies was the source of template DNA. Fourteen out of the 16 colonies amplified a fragment of the correct size. The plasmid from one of these isolates was named pBMS2000-PMKR (for ketoreductase). pBMS2000-PMKR was transformed into competent *E. coli* strain BL21-CodonPlus(DE3)-RIL cells (Stratagene) according the manufacturer's protocol. Cells were spread onto LB agar medium containing 30 μg/mL chloramphenicol and 10 μg/mL neomycin. Four colonies were randomly chosen and used as a source of template DNA for PCR using the conditions described earlier. All four reactions amplified a DNA fragment of the correct size, demonstrating that the BL21-CodonPlus(DE3)-RIL had been successfully transformed. One of these isolates was selected as the expression strain and used for all further experiments. Vial lots of the expression strain were prepared.

Expression of the ketoreductase gene was controlled by IPTG (isopropylthio-β-D galactoside) induction of the ptac promoter that originated on plasmid pBMS2000. The expression strain was grown in 25 mL of MT3/neo/chlor in a 250-mL flask at 15 °C, 225 rpm until it had reached $OD_{600\,nm}$ ~0.7. At this point, IPTG (Life Technologies) was added to a final concentration of 0.5 mM. The cultures were allowed to grow overnight (~16 h) to allow complete induction of the ketoreductase gene and production of the ketoreductase protein. Following overnight induction with IPTG, samples were analyzed on protein gels. The results indicated the strain's ability to overexpress the heterologous protein.

4.14. Reduction of keto methyl ester by recombinant *E. coli*

Cells of *E. coli* SC 16445 (expressing ketoreductase) were grown in a 250-L fermentor. Growth consisted of inoculum development stage, germinator stage and fermentation stage. In inoculum development stage, 1 mL culture of *E. coli* SC 16445 was inoculated into 1 L of medium B (1.0% NZ Amine A, 2.0% Yeastamin, 2.0% glycerol, 0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.125% $(NH_4)_2SO_4$, 0.0246% $MgSO_4 7H_2O$) containing a filtered sterilized solution (10 mg in ethanol) of neomycin sulfate, and a filtered sterilized solution (33 mg in water) of chloramphenicol. Growth was carried out in 4-L flasks at 15 °C and 150 rpm for 72 h. In a germinator stage, a 20-L fermentor containing 15 L medium B containing

neomycin sulfate and chloramphenicol (150 mg neomycin sulfate in 100 mL water and 495 mg chloramphenicol in 50 mL ethanol were separately filter sterilized using a 0.2 μm cellulose nitrate filter and added to the germinator after sterilizing the medium) was inoculated with 1 L inoculum. Cells were grown at 15 °C, 20 LPM (liter per min) aeration and 1000 rpm agitation for 24 h. A 380-L fermentor containing 250 L of medium B containing neomycin sulfate and chloramphenicol chloramphenicol (2.5 g neomycin sulfate in 600 mL water and 8.25 g chloramphenicol in 600 mL ethanol were separately filter sterilized using a 0.2 μm cellulose nitrate filter and added to the fermentor after sterilizing the medium) was inoculated with 15 L germinator grown inoculum. The growth was carried out at 15 °C, 250 LPM aeration, and 200 rpm agitation. Induction of the ketoreductase was carried out when the optical cell density (OD at 600 nm) of the culture reached close to 3.0 by the addition of a filter-sterilized solution (14.9 g in 1 L of water) of isopropyl-β-thiogalactoside (IPTG). Eight hours after addition of IPTG, the addition of nutrient feed (20 L of feed containing 1% NZ Amine A, 1% yeastamin, 1% glycerol, and 0.02% antifoam UCON) was initiated at 1 L/h over 20 h. During fermentation, cells were periodically harvested by centrifugation from 200 mL of culture broth and assayed for conversion of keto methyl ester **3** to hydroxy methyl ester **4**. Cells were harvested approximately 30 h after induction when the highest ketoreductase activity was observed. At the end of fermentation, the fermentor was cooled to 6–8 °C and cells collected in a Sharples centrifuge. About 10–11 kg of cell paste was collected and stored at −70 °C until further used in the biotransformation process.

Cells were used to catalyze the bioconversion of keto methyl ester **3**. Cells were suspended in 1 L of 50 mM pH 7.0 phosphate buffer at 10% (w/v, wet cells) cell concentration. The suspensions were supplemented with 100 μM nicotinamide adenine dinucleotide phosphate (NADP), 1 mM phenylmethanesulfonyl fluoride (PMSF), 20 g glucose, 34,000 units glucose dehydrogenase, and 4.5 g substrate **3**. Biotransformation was carried out at 500 rpm and 28 °C. The pH was maintained at 6.8–7.0. Glucose feed (solution containing 20% glucose, 1.92% K_2HPO_4, and 1.2% KH_2PO_4) was started after 1 h at rate of 6 mL/h/L of reaction mixture. The reaction was completed in 20 h with a 95% reaction yield of **4**. An ee of 99.9% was obtained for **4**. The biotransformation process was scaled up to 500 L to reduce 3 kg of substrate **3.**

4.15. Recovery of hydroxy methyl ester 4

At the end of the biotransformation, 40 g of XAD-16 resin (previously washed with 500 mL of a 1:1 (v/v) water and methanol mixture, and then washed with 2 × 500 mL of water) was added to the 1 L of reaction broth. The mixture was stirred at room temperature at 300 rpm for 8 h and finally filtered through a 40-mesh stainless steel sieve. The collected resin was washed with 50 mL of a water and methanol mixture (80:20 by vol) and filtered through a stainless steel sieve. The product-rich resin was treated with 50 mL of methyl *tert*-butyl ether (MTBE) to desorb the alcohol **4**. The solution was dried over anhydrous sodium sulfate and filtered and the solvent was removed on the rotary evaporator to provide 3.9 g of an oil in 86% overall yield. HPLC analysis showed 98.9 area percent for product **4** with an ee of 99.9%. The same procedure was used to recover alcohol **4** from a 500-L biotransformation batch.

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References and notes

1. Patel, R. N. *Enzyme Microb. Technol.* **2002**, *31*, 804–826.
2. Food & Drug Administration. *Chirality* **1992**, *4*, 338–340.
3. Buckland, B. C.; Robinson, D. K.; Chartrain, M. *Metab. Eng.* **2000**, *2*, 42–48.
4. Pesti, J. A.; Dicosimo, R. *Curr. Opin. Discov. Dev.* **2000**, *3*, 764–782.
5. Gotor, V. *Biocat. Biotrans.* **2000**, *18*, 87–103.
6. Stewart, D. *Curr. Opin. Chem. Biol.* **2001**, *5*, 120–129.
7. Zak, A. *Curr. Opin. Chem. Biol.* **2001**, *5*, 130–136.
8. Patel, R. N. In *Stereoselective Biocatalysis*; Patel, R. N., Ed.; Marcel Dekker: New York, 2000; pp 87–130.
9. Schnell, B.; Faber, K.; Kroutil, W. *Adv. Synth. Catal.* **2003**, *345*, 653–666.
10. Patel, R. N. *Adv. Synth. Catal.* **2001**, *343*, 527–546.
11. Churcher, I.; Nadin, A. J.; Owens, A. P. PCT Int. Appl. 2002; 56 pp. CAN 136:325575, AN 2002:293634.
12. Schenk, D.; Games, D.; Seubert, P. *J. Mol. Neurosci.* **2001**, *17*, 259–265.
13. Yang, M. G.; Liu, H. PCT Int. Appl. 2001; 170 pp. CAN 135:288707, AN 2001:747759.
14. Olson, R. E. PCT Int. Appl. 2001; 254 pp. CAN 136:20092, AN 2001:886081.
15. Felsenstein, K.; Smith, D. W.; Poss, M. A.; Chaturvedula, P. C.; Sloan, C. P.; Charles, P. Eur. Pat. Appl. 1997; 30 pp. CAN 127:109192, AN 1997:470004.
16. Rotella, D. P. *Chemtracts* **2000**, *13*, 626–629.
17. Patel, R. R. N.; Goswami, A.; Chu, L.; Nanduri, V.; Goldberg, S.; Johnston, R.; Donovan, M.-J ; Mirfakhrae, K. D. Patent Appl. WO 2002-US6783 20020306. CAN 137:277933. AN 2002:754608.
18. Ward, O. P.; Young, C. S. *Enzyme Microb. Technol.* **1990**, *12*, 482–493.
19. Csuk, R.; Glanzer, B. I. *Chem. Rev.* **1991**, *91*, 49–97.
20. Sih, C. J.; Chen, C. S. *Angew. Chem.* **1984**, *96*, 556–566.
21. Kula, M.-R.; Kragl, U. In *Stereoselective Biocatalysis*; Patel, R. N., Ed.; Marcel Dekker: New York, 2000; pp 839–866.
22. Jones, J. B.; Back, J. F. In *Applications of Biochemical Systems in Organic Synthesis*; Jones, J. B., Jones, C. J., Perlman, S. D., Eds.; John Wiley & Sons: NY, 1976; pp 248–376.
23. Keinan, E.; Hafeli, E. K.; Seth, K.; Lamed, R. *J. Am. Chem. Soc.* **1986**, *108*, 162–168.
24. Prelog, V. *Pure Appl. Chem.* **1964**, *9*, 119–123.

25. Bradshaw, C. W.; Hummel, W.; Wong, C.-H. *J. Org. Chem.* **1992**, *57*, 1532–1536.
26. Bradshaw, C. W.; Fu, H.; Shen, G.-J.; Wong, C.-H. *J. Org. Chem.* **1992**, *57*, 1526–1532.
27. Patel, R. N.; Banerjee, A.; McNamee, C. G.; Szarka, L. *J. Appl. Microbiol. Biotechnol.* **1993**, *40*, 241–245.
28. Patel, R. N.; Banerjee, A.; Howell, J. M.; McNamee, C. G.; Brzozowski, D.; Mirfakhrae, K. D.; Nanduri, V.; Thottathil, J. K.; Szarka, L. J. *Tetrahedron: Asymmetry* **1993**, *4*, 2069–2084.
29. Patel, R. N.; Banerjee, A.; Liu, M.; Hanson, R. L.; Ko, R. Y.; Howell, J. M.; Szarka, L. *J. Biotechnol. Appl. Biochem.* **1993**, *17*, 139–153.
30. Patel, R. N.; Banerjee, A.; McNamee, C.; Brzozowski, D.; Szarka, L. J. *Tetrahedron: Asymmetry* **1997**, *8*, 2547–2552.
31. Heukeshoven, J.; Dernick, R. *Electrophoresis* **1985**, *6*, 103–112.