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Enantioselective microbial reduction of 2-oxo-2-(1′,2′,3′,4′-tetrahydro-1′,1′,4′,4′-tetramethyl-6′-naphthalenyl)acetic acid and its ethyl ester

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Abstract—The chiral ester ethyl (2*R*)-hydroxy-2-(1′,2′,3′,4′-tetrahydro-1′,1′,4′,4′-tetramethyl-6′-naphthalenyl) acetate **2** and the corresponding acid **4** were prepared as intermediates in the synthesis of the retinoic acid receptor gamma-specific agonist (*R*)-3-fluoro-4-[[hydroxy(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)acetyl]amino]benzoic acid **7**. Enantioselective reduction of ethyl 2-oxo-2-(1′,2′,3′,4′-tetrahydro-1′,1′,4′,4′-tetramethyl-6′naphthalenyl)acetate **1** to alcohol **2** was carried out using *Aureobasidium pullulans* SC 13849 in 98% yield and with an enantiomeric excess (e.e.) of 96%. Among microorganisms screened for the reduction of 2-oxo-2-(1′,2′,3′,4′-tetrahydro-1′,1′,4′,4′-tetramethyl-6′-naphthalenyl)acetic acid **3** to hydroxy acid **4**, *Candida maltosa* SC 16112 and two strains of *Candida utilis* (SC 13983, SC 13984) gave reaction yields of >53% with e.e.s of >96%. © 2002 Elsevier Science Ltd. All rights reserved.

1. Introduction

A number of studies have demonstrated that retinoids (vitamin A derivatives) are essential for normal growth, vision, tissue homeostasis, and reproduction.[1–5] Retinoic acid and its natural and synthetic analogs (retinoids) exert a wide variety of biological effects by binding to or activating a specific receptor or sets of receptors.[6,7] They have been shown to affect cellular growth and differentiation and are promising drugs for the treatment of cancers.[8–10] A few retinoids are already in clinical use for the treatment of dermatological diseases such as acne and psoriasis.[11,12] (*R*)-3-Fluoro-4-[[hydroxy(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)acetyl]amino]benzoic acid **7** is a retinoic acid receptor gamma-specific agonist which is potentially useful as a dermatological and anti-cancer drug.[12–16]

Herein, we describe the enantioselective microbial reduction of ethyl 2-oxo-2-(1′,2′,3′,4′-tetrahydro-1′,1′,4′,4′-tetramethyl-6′-naphthalenyl)acetate **1** and acid **3** to the corresponding (*R*)-hydroxy ester **2** and hydroxy acid **4**, potential intermediates in the synthesis of **7**. We also demonstrate the reduction of ketoamide **5** to the corresponding (*R*)-hydroxy amide **6**. Previously, the desired hydroxy acid **4**[17a] was prepared by chemical reduction of the corresponding keto acid **3** followed by resolution with an enantiomerically pure amine.[17b]

2. Results and discussion

Oxidoreductases from many organisms, including those from yeast,[18–23] horse liver,[24] *Thermoanerobic brockii*,[25,26] *Lactobacillus kefir*,[27] *Pseudomonas* sp.,[28] *Geotrichum candidum*,[29,30] *Hansenula polymorpha*,[31,32] *Mortierella rammaniana*,[33] and *Sulfolobus solfataricus*,[34] have been used for the enantioselective reduction of ketones to alcohols. A number of microorganisms were screened for the enantioselective reduction of keto ester **1** to alcohol **2**. The yields and product e.e.s obtained with the six best cultures are shown in Table 1. *A. pullulans* SC 13849 gave the highest yield (99%) and e.e. (97.9%) of the desired (*R*)-hydroxy ester.

Aureobasidium pullulans SC 13849 was investigated further. In a two-stage process: cells were grown in a 25 L fermentor for 40 h and then harvested by centrifugation and stored at −60°C until further use. Frozen cells were suspended in 50 mM potassium phosphate buffer (pH 6.8) and the resulting cell-suspensions were used to carry out bioreduction (as described in Section 4). After

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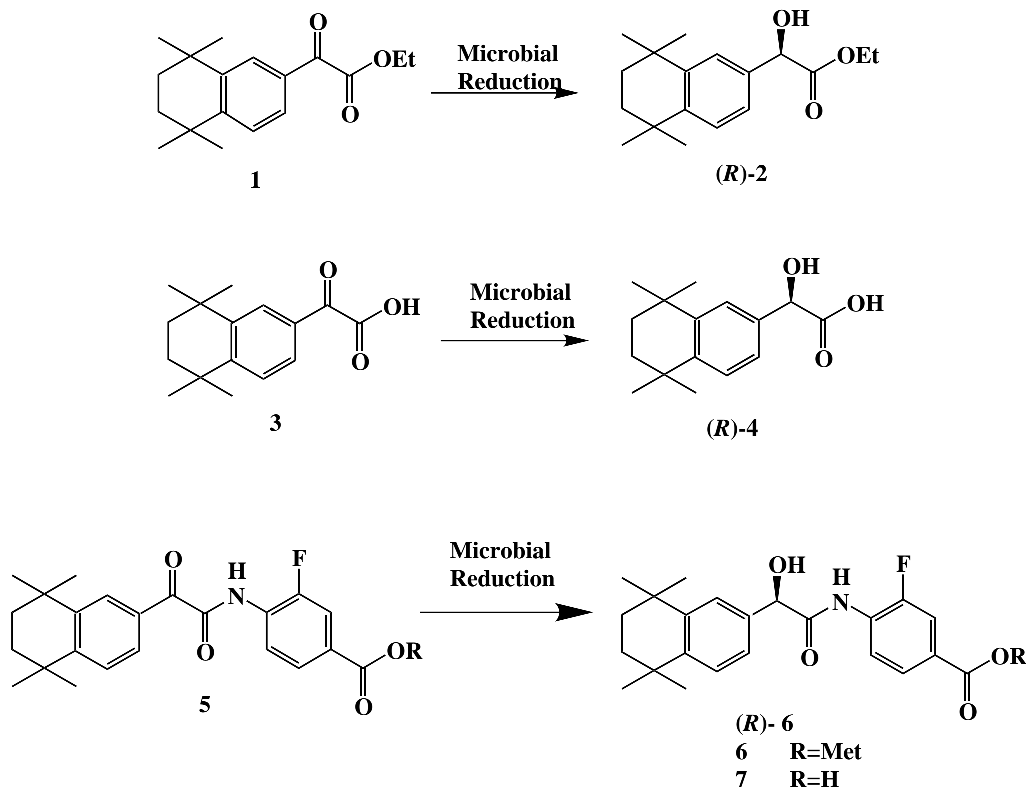

Table 1. Stereoselective microbial reduction of keto ester **1**

Microorganisms	Yield of hydroxy ester **2** (%)	E.e. of hydroxy ester **2** (%)
Aureobasidium pullulans SC 13894	98	98
Brevibacterium linens SC 13959	26	89
Hansenula polymorpha SC 13865	8	98
Pichia methanolica SC 16116	28	96
Pseudomonas oleovorans SC 13828	29	99
Trigonopsis variabilis SC 16071	13	93

The reaction yields and e.e.s were determined by HPLC using C-18 column and chiralcel OD column, respectively, as described in Section 4.

16 h, a yield of 99% and e.e. of 97% were obtained for (*R*)-hydroxy ester **2** (Table 2). At the end of the reaction, hydroxy ester **2** was adsorbed onto XAD-16 resin and, after filtration, it was recovered in 94% yield from the resin by acetonitrile extraction.

The recovered (*R*)-hydroxy ester **2** was treated with Chirazyme L-2 or pig liver esterase (as described in Section 4) to convert it to the corresponding (*R*)-hydroxy acid **4** in quantitative yield. The enzymatic hydrolysis was used to avoid the possibility of racemization under chemical hydrolytic conditions.

A simpler, single-stage fermentation–bioreduction process (fermentation of microbial cultures to complete growth and subsequent addition of substrate to the fermentor to continue biotransformation) was developed for the conversion of keto ester **1** (5 g/L) to the corresponding hydroxy ester **2** with cells of *pullulans* SC

Table 2. Stereoselective microbial reduction of keto ester **1** by *A. pullulans*: SC 13849: two-stage process

Reaction time (h)	Keto ester **1** (g/L)	Hydroxy ester **2** (g/L)	Yield (%)	E.e. of hydroxy ester **2** (%)
1	1.6	0.4	20	Not determined
3	0.84	1.1	55	Not determined
5	0.6	1.4	70	96
8	0.3	1.7	85	97
12	0.1	1.87	93	97
16	0	1.98	99	97

The reaction yields and e.e.s were determined by HPLC using C-18 column and chiralcel OD column, respectively, as described in Section 4.

13849 as described in the Section 4. A yield of 98% and an e.e. of 98% were obtained (Table 3). However, due to low cell concentration in this single-stage process, the reaction was completed in 107 h compared to 16 h for the two-stage process.

The enantioselective reduction of an alternative substrate, keto acid **3**, to hydroxy acid **4** was also investigated using various microorganisms. The yields and product e.e.s obtained with the three best cultures are shown in Table 4. *Candida utilis* SC 13983 gave the best reaction yield (64%) for the desired (*R*)-hydroxy acid **4**.

We also evaluated the microbial reduction of the more complex intermediate ketoamide **5** to the corresponding hydroxy amide **6**. Low reaction yields (<5%) were obtained, although several of the cultures tested positive for the reduction (Table 5). For example, *A. pullulans* SC 13894 and *H. anamola* SC 16158 gave 12 and 20% yields of the desired (*R*)-hydroxy amide **6** with e.e.s of 98.6 and 85.2%, respectively.

Conversion of ketoamide **5** to hydroxy amide **6** was improved by using cell extracts of *A. pullulans* SC 13984 and glucose dehydrogenase in the presence of glucose and NADP as shown in Table 6. The lower reaction yield obtained with a cell suspension may be due to the impermeability of ketoamide **5** through cells or may be due to limited NADPH-regenerating enzyme within cells of *A. pullulans* SC 13894. A higher yield of **6** (60%) was obtained using cell extracts and for when NADPH was used as a cofactor (Table 6). Data for reduction using these systems are also provided for the conversion of **1** to **2**.

3. Conclusion

The dehydrogenases from yeast,[18–23] horse liver,[24] and *T. brockii*[25] transfer the pro-*R* hydride to the *re* face of the carbonyl group to give (*S*)-alcohols, a process described by Prelog's rule.[36] In contrast, dehydrogenases from *Lactobacillus kefir*[27] and two *Pseudomonas* sp.[28] exhibit anti-Prelog specificity, transferring the Pro-*R* hydride to form (*R*)-alcohols. In this paper, we have described the enantioselective reduction of keto ester **1** and keto amide **5** to the corresponding (*R*)-hydroxy ester **2** and (*R*)-hydroxy amide **6** by *A. pullulans* SC 13848. NADPH is a better cofactor compared to NADH giving higher reaction yields when cell extracts were used for this bioreduction process. We have also demonstrated the enantioselective reduction of keto acid **3** to the corresponding (*R*)-hydroxy acid **4** by three different *Candida* strains. *A. pullulans* was not effective in catalyzing the reduction of keto acid **3**. The enzyme-catalyzed reduction is useful in the preparation of chiral alcohols, hydroxy acids and their esters, and amino acids which serve as key intermediates in the synthesis of pharmaceutical compounds.[35]

Table 5. Stereoselective microbial reduction of keto amide **5**

Microorganisms	Hydroxy amide **6** yield (%)	E.e. of (*R*)-hydroxy amide **6** (%)
Aureobasidium pullulans SC 13894	12	98
Aureobasidium pullulans SC 13925	4	Not determined
Brevibacterium citreum SC 14804	4	Not determined
Cunninghemella enchinulata SC 16024	5	Not determined
Hansenula anamola SC 16158	20	85
Mortierella ramanniana SC 13843	3	Not determined
Mycobacterium vacca SC 16204	3.5	Not determined
Rhizopus oligosporous SC 16197	9	50

The reaction yields and e.e.s were determined by HPLC using C-18 column and chiralcel OD column, respectively, as described in Section 4.

Table 3. Stereoselective microbial reduction of keto ester **1** by *A. pullulans* SC 13849: single-stage process

Reaction time (h)	Keto ester **1** (g/L)	Hydroxy ester **2** (g/L)	Yield (%)	E.e. of hydroxy ester **2** (%)
20	2.8	2.1	42	Not determined
44	1.15	3.8	76	98
71	0.4	4.3	86	98
95	0.14	4.6	92	98
107	Trace	4.9	98	98

The reaction yields and e.e.s were determined by HPLC using C-18 column and chiralcel OD column, respectively, as described in Section 4.

Table 4. Stereoselective microbial reduction of keto acid **3**

Microorganisms	Ketoacid **3** (g/L)	Hydroxy acid **4** (g/L)	Yield (%)	E.e. of hydroxy acid **4** (%)
Candida maltosa SC 16112	0.45	0.56	56	99
Candida utilis SC 13983	0.35	0.64	64	98
Candida utilis SC 13983	0.43	0.53	53	98

The reaction yields and e.e.s were determined by HPLC using C-18 column and chiralcel OD column, respectively, as described in Section 4.

Table 6. Stereoselective reduction of keto ester **1** and ketoamide **5** by cell extracts of *Aureobasidium pullulans* SC 13848:

Regenerating system	Substrate	Product	Reaction yield (%)	E.e. (%)
Glucose dehydrogenase	**1**	**2**	98	98
and NADP	**5**	**6**	60	96
Glucose dehydrogenase	**1**	**2**	12	98
and NAD	**5**	**6**	10	97

The reaction yields and e.e.s were determined by HPLC using C-18 column and chiralcel OD column, respectively, as described in Section 4.

4. Experimental

Substrates **1**, **3**, and **5** and reference compounds **2**, **4**, and **6** were synthesized by co-authors in the Process Research and Development Department, Bristol–Myers Squibb Pharmaceutical Research Institute. The physico–chemical properties including spectral characteristics (^{1}H, ^{13}C NMR, mass spectra), were in full accord for all these compounds.[17] The proton magnetic resonance (^{1}H NMR) and carbon magnetic resonance (^{13}C NMR) were recorded on a Brucker AM-300 spectrometer.

4.1. Preparation of keto ester 1

To a 250 mL, three-necked flask equipped with a magnetic stirrer, addition funnel, argon inlet adapter and temperature probe were charged in order 1,2,3,4-tetrahydro-1,1,4,4-tetramethylnaphthalene (20 g; 106.2 mmol), ethyl oxalyl chloride (13 mL; 116 mmol) and dichloromethane (110 mL). The reaction mixture was cooled to 0°C and aluminum chloride (22.6 g; 169 mmol) was added portionwise while maintaining the temperature at 0°C. The reaction mixture was allowed to warm to room temperature and stirred for 2 h, at which point the reaction was complete as indicated by TLC (1:1 ethyl acetate/hexane). The reaction mixture was poured into ice-water (500 mL) and the aqueous layer was extracted with dichloromethane (110 mL). The combined organic layers were washed with saturated aqueous bicarbonate solution (100 mL) and brine (100 mL). The organic layer was dried over sodium sulfate. Removal of the solvent gave the desired compound as an oil (28 g, 92%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.97 (d, $J=2$ Hz; 1H); 7.64 (dd, $J=8$, 2 Hz; 1H); 7.42 (d, $J=8$, Hz; 1H); 4.43 (q, $J=7$ Hz; 2H); 1.70 (s; 4H); 1.41 (t, $J=7$ Hz; 3H); 1.30 (s; 6H); 1.29 (s; 6H) ppm. MS (+ESI): 306 $(M+NH_4)^+$, 289 $(M+H)^+$.

4.2. Preparation of (±)-hydroxy ester 2

To a three-necked, round-bottomed flask equipped with a magnetic stirrer, addition funnel, argon inlet adapter and temperature probe was charged a solution of $NaBH_4$ (1.06 g; 0.35 mol equiv.) in ethanol (100 mL). The reaction mixture was cooled to 0°C. A solution of keto ester **1** (23.1 g) in ethanol (220 mL) was added to the reaction mixture while maintaining the internal temperature between 0 and 3°C and then the reaction mixture was stirred at 0°C for 1 h. The reaction was quenched with ice-water, acidified to pH 3 with 1N aqueous HCl and extracted with ethyl acetate (200 mL). The ethyl acetate extract was washed with saturated aqueous sodium bicarbonate solution (100 mL) and brine (100 mL). The organic extracts were dried over sodium sulfate. The crude product was purified by column chromatography using silica gel 60 (230–400 mesh, eluted with 20% ethyl acetate in hexanes) to give the desired product as an oil (18 g, 78%). ^{1}H NMR ($CDCl_3$; 400 MHz): δ 7.32 (d, $J=2$ Hz; 1H); d 7.28 (d, $J=8$ Hz; 1H); 7.15 (dd, $J=8$, 2 Hz; 1H); 5.10 (d, $J=6$ Hz; 1H); 4.15–4.35 (m; 2H); 3.29 (d, $J=2$ Hz); 1.20–1.28 (m; 15 H) ppm. MS (+ESI): 308 $(M+NH_4)^+$

4.3. Preparation of keto amide 5

To a 15 mL, two-necked flask equipped with a magnetic stirrer and nitrogen inlet adapter was charged keto acid **3** (0.5 g; 1.92 mmol) followed by anhydrous dichloromethane (5 mL) and DMF (two drops). The reaction mixture was cooled to 0°C and oxalyl chloride was added dropwise while maintaining the reaction temperature at 0°C. The reaction mixture was stirred at 0°C for 1 h and then allowed to warm to room temperature and stirred for a further 0.5 h. Methyl 3-fluoro-4-aminobenzoate (0.33 g; 1.92 mmol) was dissolved in a mixture of anhydrous dichloromethane (5 mL) and anhydrous pyridine (0.51 mL; 6.3 mmol). This solution was added dropwise to the reaction mixture while maintaining the internal temperature at –5°C. After completion of the reaction, the reaction mixture was transferred to a separatory funnel and washed with 1N aqueous HCl (2×25 mL) and brine (25 mL) and dried over sodium sulfate. Removal of the solvent gave the desired keto amide **5** as a yellow solid (0.66 g, 85%). Mp 131–133°C. ^{1}H NMR ($CDCl_3$; 400 MHz): δ 9.39 (br s; 1H); 8.59 (t, $J=8$ Hz; 1H); 8.41 (d, $J=2$ Hz; 1H); 8.15 (dd, $J=8$, 2 Hz; 1H); 7.90 (d; $J=8$ Hz; 1H); 7.82 (dd, $J=11$, 2 Hz; 1H); 7.43 (d, $J=8$ Hz; 1H); 3.92 (s; 3H); 1.70 (s; 4H); 1.34 (s; 6H); 1.31 (s; 6H) ppm. MS (-ESI): 410 $(M-H)^-$.

4.4. Preparation of keto acid 3

To a 250 mL, three-necked flask equipped with a magnetic stirrer, nitrogen inlet adapter and temperature probe were charged keto ester **1** (36 g; 125 mmol), THF (100 mL), water (50 mL) and $LiOH{\cdot}H_2O$ (15.7 g; 373 mmol). The reaction mixture was stirred at room temperature for 2.5 h. No starting material was detected at this point by thin-layer chromatography using precoated silica gel plates (GF-254; eluted with ethyl acetate/hexanes 1:1). The pH of the reaction mixture was adjusted to 2–3 with 1N aqueous HCl and extracted with MTBE. The MTBE layer was extracted with satd. $NaHCO_3$, acidified with 1N aqueous HCl to pH 2–3,

and extracted with ethyl acetate. Removal of the solvent followed by drying gave the desired acid as a yellow oil which solidified to a low melting solid upon cooling (30 g, 94% yield). ^{1}H NMR ($CDCl_3$; 400 MHz): δ 8.37 (d; $J=1$ Hz; 1H); 8.14 (dd, $J=8$, 1 Hz; 1H); 7.44 (d, $J=8$ Hz; 1H); 1.71 (s; 4H); 1.32 (s; 6H); 1.30 (s; 6H) ppm. HRMS calcd for $C_{16}H_{20}O_3$ 261.1491. Found 261.1501.

4.5. Preparation of (±)-hydroxy acid 4

To a 250 mL, three-necked flask equipped with a magnetic stirrer, nitrogen inlet adapter and temperature probe were charged the racemic hydroxy ester **2** (45.6 g; 157 mmol), THF (200 mL), water (100 mL) and $LiOH·H_2O$ (19.9 g; 473 mmol). The reaction mixture was stirred at room temperature for 2 h. No starting material was detected at this point by thin-layer chromatography using pre-coated silica gel plates (GF-254; eluted with ethyl acetate/hexanes 1:1). The reaction mixture was adjusted to pH 2–3 with 1N aqueous HCl and extracted with ethyl acetate (200 mL). Removal of the solvent followed by drying over magnesium sulfate gave the desired acid as a white solid (45 g). Recrystallization from 10% ethyl acetate in heptane gave the desired compound as a white solid (30 g, 73% yield). ^{1}H NMR ($CDCl_3$; 400 MHz): δ 7.35 (d $J=2$ Hz; 1H); 7.29 (d $J=8$ Hz; 1H); 7.16 (dd; $J=8$, 2 Hz; 1H); 5.19 (s; 1H); 1.67 (s; 4H); d 1.26 1.30 (overlapping singlets; 12 H). Anal. calcd for $C_{16}H_{22}O_3$: C, 73.25; H, 8.45. Found: C, 73.05; H, 8.36%.

4.6. Preparation of (±)-hydroxy amide 6

A 500 mL, three-necked flask equipped with a mechanical stirrer, gas inlet adapter and temperature probe was flushed with argon. To the flask were charged the hydroxy acid **3** (20 g; 76.3 mmol), 1,2,4-triazole (7.4 g; 106.8 mmol) and 150 mL of dichloromethane. The reaction mixture was stirred until it turned clear and then it was cooled to 0°C. Methyl 3-fluoro-4-(*N*-sulfinyl)aminobenzoate [$MeO_2CC_6H_3$(3-F)(4-NSO)] (23 g; 106.8 mmol) was charged in one portion and the reaction mixture was stirred at 0°C for 3 h. The dichloromethane was removed under reduced pressure at 35°C and further displaced by distillation with heptane (2×50 mL). The residual solids were dissolved in a 2:1 mixture of heptane/MTBE, washed with 3N HCl (5×70 mL), saturated $NaHCO_3$ (2×50 mL) and brine (50 mL). The organic layer was dried over sodium sulfate and the solvents were removed under reduced pressure to give the crude product as a solid (31 g). Recrystallization from cyclohexane gave the desired compound as a white solid (24.2 g, 77% yield). Mp 120–121°C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.25–1.30 (overlapping singlets; 12H); 1.68 (s; 4H); 3.51 (d, $J=2$ Hz; 1H); 3.89 (s; 3H); 5.17 (d, $J=2$ Hz; 1H); 7.22 (dd, $J=8$, 2 Hz; 1H); 7.33 (d, $J=8$ Hz; 1H); 7.4 (d, $J=2$ Hz; 1H); 7.75 (dd, $J=11$, 2 Hz; 1H); 7.80 (d, $J=11$ Hz; 1H); 8.44 (apparent t, $J=8$ Hz; 1H); δ 8.86 (d, $J=2$ Hz; 1H) ppm. HRMS calcd for $C_{24}H_{28}FN_4$ (+1) 414.2081. Found 414.2066.

4.7. Microorganisms

Microorganisms (Tables 1–5) were obtained from the culture collection of the Bristol–Myers Squibb Pharmaceutical Research Institute. Microbial cultures were stored at −90°C in vials.

4.8. Growth of microorganisms

For screening purposes, one vial 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 280 RPM for 48 h. Cultures were harvested by centrifugation at 18,000×g for 15 min, washed with 0.1 M potassium phosphate buffer pH 7.0, and used for reduction studies.

4.9. Reduction of keto ester 1 by cell-suspensions

Cells of various microorganisms were suspended separately in 60 mM potassium phosphate buffer (pH 7.0) at 20% (w/v, wet cells) cell concentration and supplemented with 1 mg/mL of keto ester **1** (in methanol) and 25 mg/mL of glucose. Reduction was conducted at 28°C and 150 RPM. Periodically, samples of 1 mL were taken and extracted with 2 mL of acetonitrile. The samples were filtered through a 0.2 µm LID/X filter and analyzed using a Hewlett Packard 1070 high pressure liquid chromatograph (HPLC). A Kromasil C-18 (150× 4.6 mm) column was used. The mobile phase consisted of solvent A (0.1% trifluoroacetic acid in water) and solvent B (0.1% trifluoroacetic acid in acetonitrile) used in a linear gradient over a 50 min run time at a flow rate of 1.0 mL/min as follows.

Time	% B
0	40
25	70
30	70
35	100
40	100
45	40
50	40

The detection wavelength was 210 nm. The retention times for keto ester **1** and hydroxy ester **2** were 29.3 and 19.6 min, respectively. Separation of the two enantiomers of the racemic hydroxy ester **2** was achieved on a Chiracel OD column. The mobile phase consisted of 2.5% isoproanol and 97.5% hexane. The flow rate was 1 mL/min and the detection wavelength was 230 nm. The retention time for the desired (*R*)-enantiomer was 12.7 min and that for the undesired (*S*)-enantiomer was 9.47 min.

4.10. Procedure for two-stage reduction of keto ester 1

A. pullulans SC 13894 culture was grown in a 25 L fermentor containing 15 L of medium A (1% malt

extract, 1% yeast extract, 2% glucose and 0.3% peptone) containing 0.025% UCON antifoam. Growth consisted of several inoculum development stages and fermentation. Inoculum development consisted of F1 and F2 stages. In the F1 stage, a frozen vial of culture was inoculated into 100 mL of medium A contained in a 500 mL flask. Growth was carried out at 28°C and 280 RPM for 48 h on a rotary shaker. In the F2 stage, 10 mL of F1 stage culture was inoculated into 1 L of medium A and incubated at 28°C and 280 RPM for 24 h. The fermentor containing 15 L of medium A was inoculated with 1 L of inoculum from an F2 stage. Fermentation was conducted at 25°C and 500 RPM with 15 LPM (liter per min) aeration for 48 h. After 48 h fermentation, cells were collected and stored at −90°C until further use. About 1 kg of wet cell paste was collected from the fermentation. *A. pullulans* SC 13894 culture was also grown in a very cheap medium in a 25 L fermentor containing 15 L of medium B (2% ardamine PH, 2% glucose, and 2% nutrisoy) without compromising activity for reduction of all substrates.

Frozen cells (600 g wet cells) from the above batches were suspended in 3 L of 70 mM potassium phosphate buffer (pH 7.0) and used to conduct the reduction of keto ester **1** in a 5 L reactor. Keto ester **1** (1 g/L or 5 g/L in methanol) and glucose (25 g/L) were added to the reactor and the reduction was carried out at 28°C and 200 RPM with 1 LPM aeration for 24–72 h. The pH was maintained between 6.8 and 7.0. Periodically, samples were prepared as described above and analyzed by HPLC to determine the percent conversion of keto ester **1** to hydroxy ester **2**. The e.e. of hydroxy ester **2** was determined by chiral HPLC as described earlier.

4.11. Single-stage process for reduction of 1

A. *pullulans* SC 13894 culture was grown in a 5 L fermentor containing 3 L of medium B as described above. After 16 h of growth, 15 g of keto ester **1** was added to the fermentor and the biotransformation process was continued for 107 h. The pH was maintained at 6.8 during the biotransformation process. Periodically, samples were prepared as described above and analyzed by HPLC to determine the % conversion of keto ester **1** to hydroxy ester **2**. The e.e. of hydroxy ester **2** was determined by chiral HPLC.

4.12. Isolation of hydroxy ester 2

At the end of the single-stage bioreduction, 150 g of wet XAD-16 (previously washed with methanol and then with 70 mM phosphate buffer pH 7.0) was added to the 3 L of reaction mixture to adsorb the product onto the resin under continuous mixing. After 4 h, the product rich-resin was recovered from the reaction mixture by filtration through a stainless steel screen (80 mesh). The separated resin was washed with 1 L of 70 mM phosphate buffer (pH 7.0), and hydroxy ester **2** was desorbed from the rich resin with 1 L of acetonitrile. The acetonitrile extract was evaporated under reduced pressure to obtain the crude product (12 g).

4.13. Hydrolysis of hydroxy ester 2 by pig liver esterase

Hydroxy ester **2** was hydrolyzed to the corresponding hydroxy acid **4** with pig liver esterase. At the end of the reduction of keto ester **1** to hydroxy ester **2**, 5 g/L of pig liver esterase was added to the reaction mixture and the mixture was incubated at 28°C for 16 h. Complete conversion of hydroxy ester **2** to hydroxy acid **4** was obtained.

4.14. Reduction of keto acid 3 and keto amide 5

For screening purposes, one vial of each culture was used to inoculate 100 mL of medium A. Cultures were grown at 28°C and 280 RPM for 48 h. Cultures were harvested by centrifugation at 18,000×*g* for 15 min, washed with 0.1 M potassium phosphate buffer pH 7.0, and suspended separately in 60 mM potassium phosphate buffer (pH 7.0) at 20% (w/v, wet cells) cell concentration. Cell suspensions were supplemented with 1 mg/mL of keto acid **3** or keto amide **5** (in methanol) and 25 mg/mL of glucose. The reduction was conducted at 28°C and 150 RPM. Periodically, samples of 1 mL were taken and extracted with 2 mL of acetonitrile. Each sample was filtered through a 0.2 μm LID/X filter and analyzed by HPLC as described earlier. The retention times for keto acid **3** and hydroxy acid **4** were 15.7 and 10.2 min, respectively.

Separation of the two enantiomers of the racemic hydroxy acid **4** was achieved on a Chiracel OD column as described earlier except the mobile phase consisted of 2.5% isopropanol, 98% hexane and 0.1% trifluoroacetic acid. The retention time for the desired (*R*)-enantiomer was 32.3 min and that for the undesired (*S*)-enantiomer was 36.5 min. To measure the concentration of ketoamide **5** and hydroxy amide **6**, a sample was extracted with 2 volumes of acetonitrile. The mixture was then dried under a stream of nitrogen and resuspended in 10% isopropanol:90% hexane mixture, sonicated, filtered through 0.2 μm LID/X filter and analyzed by HPLC as described earlier using 98% hexane and 2% isopropanol as the mobile phase. The retention times for keto amide, (*R*)-hydroxy amide, and (*S*)-hydroxy amide were 6.62, 22.9, and 26.6 min, respectively.

4.15. Preparation of cell extracts of *A. pullulans* SC 13894

Preparation of cell extracts were carried out at 4–7°C. Cells were washed with 50 mM potassium phosphate buffer (pH 7.0) and washed cells (30 g) were suspended in buffer (200 mL) containing 10% glycerol and 1 mM ethylenediamine tetraacetic acid (EDTA). Cell suspensions (20% w/v, wet cells) were disintegrated with a Microfluidizer (Microfluidics, Inc) at 12,000 psi (two passages), the disintegrated cells were centrifuged at 25,000×*g* for 30 min to obtain the cell extract. The protein in cell extracts was estimated using Bio-Rad protein reagent using bovine serum albumin as a stan-

dard. The assay mixture contained 1–10 μL of enzyme fraction, water (0.8 mL) and Bio-Rad reagent (0.2 mL). After mixing, the absorbance of the solution was measured at 595 nm. Cell extracts were evaluated for biotransformation of keto ester **1** and keto amide **5**. The reaction mixture contained 1 mg/mL substrate, 2 mM nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP), 8 units glucose dehydrogenase, and 2 mg/mL glucose to regenerate reduced cofactor (NADH or NADPH) required for the reduction reaction. Substrate and product concentrations were estimated by HPLC. The e.e.s of the products were determined by chiral HPLC as described earlier.

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