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Screening, substrate specificity and stereoselectivity of yeast strains, which reduce sterically hindered isopropyl ketones

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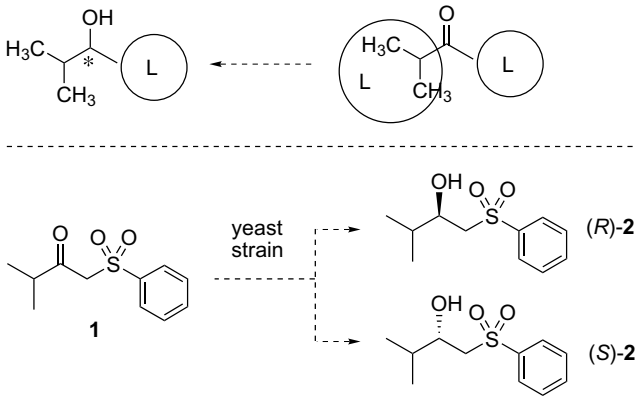
Abstract—Towards the synthesis of sterically hindered optically active secondary alcohol **2**, yeast strains (*Candida floricola* IAM 13115 and *Trichosporon cutaneum* IAM 12206) with *si*-face hydride attack on isopropyl phenylsulfonylmethyl ketone **1** were developed by screening. Strains with complementary *re*-facial selectivity (*Pichia angusta* IAM 12895 and *Pichia minuta* IAM 12215) were also found. Based on the substrate specificity studies of these four strains, microbial reduction was applied to the synthesis of (3*S*,5*S*)-2,6-dimethyl-3,5-heptanediol **12a**.

1. Introduction

Many kinds of enantiomerically enriched secondary alcohols are now available via biocatalytic methods.[1–11] So far, bakers' yeast and other microbial strain-catalyzed asymmetric reduction, and lipase-catalyzed enantiomeric resolution are very effective towards substrates with one small substituent, for example, methylcarbinols.

On the other hand, the asymmetric reduction of highly sterically hindered open-chain ketones still remains a challenging task. Although there has been a reported reduction of isopropyl ketones (Scheme 1),[12–15] the substrates are limited to ketoesters. Herein, we report an exploitation of yeast strains, which enable the preparation of enantiomerically enriched forms of isopropyl carbinols often encountered as the partial structure of naturally occurring products and chiral auxiliaries in organic chemistry.



Scheme 1.

2. Results and discussion

2.1. Screening of yeast strain on sulfonylketone 1

Towards this end, grown cells of more than 30 yeast strains from a culture collection (Institute of Applied Microbiology; IAM), in glucose medium were applied to the model substrate **1** (Scheme 1). Ketone **1** was designed along the following lines: (1) A bulky isopropyl group and a phenylsulfonyl group are directly attached to both ends of the carbonyl group. (2) The electron-withdrawing phenylsulfonyl group is expected to promote reduction. (3) The UV-absorbing phenylsulfonyl group would facilitate detection of the progress of the reduction as well as determination of the ee by HPLC analysis. (4) The sulfonyl group would assist C–C bond formation. So far, only lipase-catalyzed dynamic kinetic resolution of a racemic similar sulfonyl alcohol has been reported.[16]

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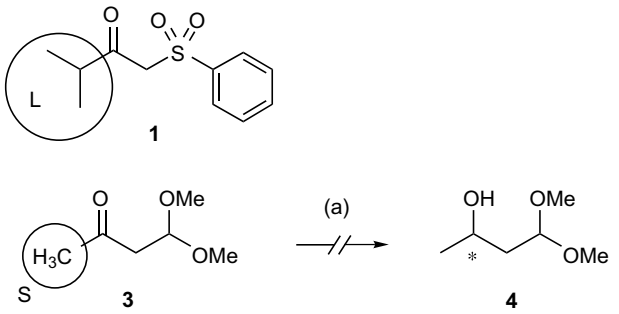
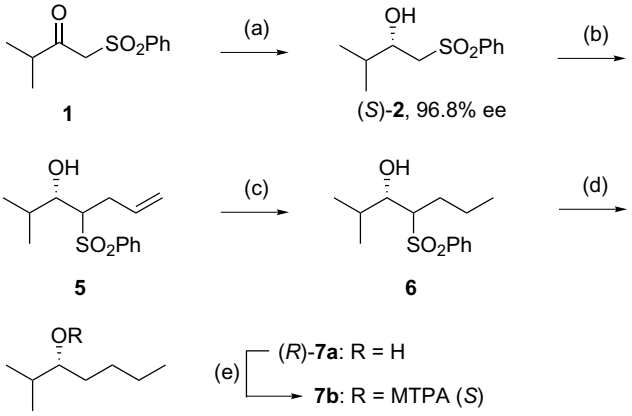
Table 1. Screening of the yeast strain on the reduction of **1**

Yeast strain	IAM No.	% ee of **2**[a]	Absolute configuration of **2**
Trichosporon cutaneum	12206	92.8	(*R*)
Candida floricola	13115	89.1	(*R*)
Rhodotorula minuta	12231	87.4	(*R*)
Rhodotorula aurantiaca	12885	71.0	(*R*)
Rhodosporidium sphaerocarpum	12261	61.3	(*R*)
Candida boidinii	12269	52.6	(*R*)
Pichia henricii	12891	29.7	(*R*)
Bullera alba	13475	28.3	(*R*)
Pichia finlandica	04982	23.1	(*R*)
Pichia jadinii	12214	9.2	(*R*)
Pichia minuta	12215	98.0	(*S*)
Pichia angusta	12895	95.0	(*S*)
Williopsis californica	04980	85.9	(*S*)
Candida nitratophila	12883	49.4	(*S*)
Yamadazyma farinosa	10896[b]	45.4	(*S*)
Fellomyces polyborus	13471	35.0	(*S*)
Yarrowia lipolytica	04947	25.7	(*S*)
Candida kefyr	04857	15.7	(*S*)
Saccharomyces cerevisiae	04125	12.6	(*S*)

[a] Daicel ChiralCel OD-H, see Section 4.4.
[b] NBRC No.



Scheme 2. (a) Four yeast strains used in this study.



Scheme 3. Reagents and conditions: (a) *P. minuta* IAM 12215, 92%; (b) LDA (2 equiv), THF, then allyl iodide (10 equiv), −78 °C to rt, 76%; (c) H_2, Pd–C, EtOH, 99%; (d) Li (5 equiv), ethylenediamine (7 equiv), Et_2O, 0 °C to rt, 26%; (e) (*R*)-MTPA chloride.

First, the preliminary screenings under the conditions with 50 mg of the substrate in test tube incubation were carried out. From 33 strains, nineteen exhibited the progress of the expected reduction (Table 1). *Trichosporon cutaneum* IAM 12206 and *Candida floricola* IAM 13115 yielded (*R*)-**2** and in contrast, both *Pichia minuta* IAM 12215 and *Pichia angusta* IAM 12895 provided (*S*)-**2**, in highly enantiomerically enriched states. *Yamadazyma farinosa* NBRC 10896,[17] which had shown catalytic activities towards the related sulfonyl methyl and trifluoromethyl ketones, only resulted in as low as 45.4% ee. The yeast strains showed diverse facial selectivity, as had been shown in a sulfonyl ketone[18] with different substituents and an α-ketoester.[19]

As already mentioned, isopropylketone **1** was designed for the screening of yeast enzymes, which are able to reduce sterically hindered carbonyl groups. A suggested high affinity towards hydrophobic substrates[15] was supported by the following result, the attempted reduction of a less hindered acyclic ketone **3** as the candidate (Scheme 2). Ketone **3** has so far been a good substrate for whole-cell mediated reduction by *Y. farinosa* NBRC 10896 to give (*R*)-**4** in 94.0% ee.[20] The above-mentioned four strains developed in this study, however, did not work on this substrate **3**.

2.2. Determination of absolute configuration of product 2

Since the enantiomerically enriched forms of the resulting alcohol **2** are not known in any literature, the absolute configuration of the enantiomerically enriched form was determined by derivatization as shown in Scheme 3.

(+)-Alcohol **2**, which was obtained by reduction with *P. minuta* IAM 12215, was treated with LDA (2 equiv) and then allyl iodide (10 equiv) in THF. The reaction temperature was gradually increased from −78 °C to room temp and the allylated product **5** was afforded in 76% yield. The smooth reaction required as much as 10 equiv of allyl iodide, even under an elevated temperature and the reaction was rather slow, probably due to the presence of a sterically hindered isopropyl group. The terminal double bond was then hydrogenated to provide saturated sulfone **6**. Birch reduction using a combination of lithium (5 equiv)–ethylenediamine (7 equiv) in diethyl ether[21] was applied to remove the phenylsulfonyl group of **6**. The very volatile product, 2-methyl-3-heptanol **7a** was identified, and (*R*)-absolute configuration was confirmed by a comparison of its (+)-sign of rotation with authentic data[22] and ^{1}H NMR analysis[23] of the corresponding (*S*)-MTPA ester **7b**. It was concluded that the absolute configuration of alcohol **2** obtained by the *P. minuta*-mediated asymmetric reduction was (*S*).

2.3. Towards the scale-up of the microbial reduction of sulfonylketone 1

Needless to say, an important aspect is the high catalytic activity, which is required for a whole-cell reduction enzyme. In this study, at the initial screening, a glucose concentration as high as 5% (w/v) was applied, so that yeast strains, which can grow and provide a high amount of cell

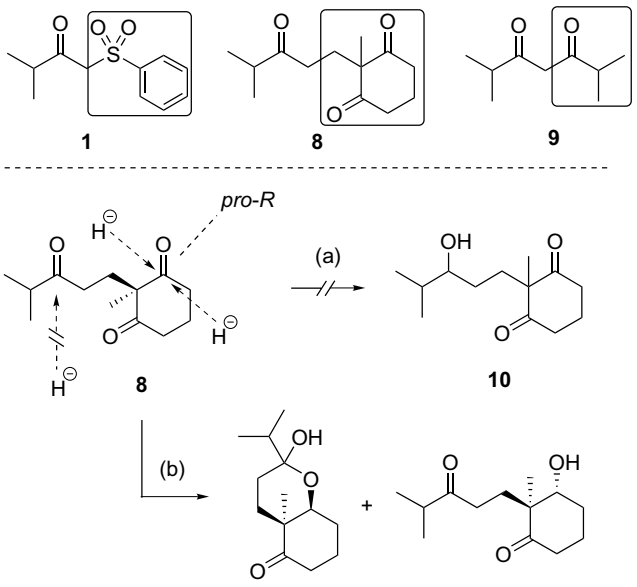
mass, were selected. Next, the large-scale production conditions for (*R*)- and (*S*)-alcohol **2** with high catalytic activities were further elaborated upon. For the (*R*)-isomer, *C. floricola* IAM 13115 was pre-incubated in glucose medium (pH 6.0) and ketone **1** (substrate concentration: 0.5 w/v%) was added. During the attempted scale-up reduction with 500 mg of the substrate, the incubation broth gradually turned acidic (pH 3.9), with the yield as low as 16%. Moreover, the ee of product **2** declined slightly to 86%. Occasionally adjusting the pH of the broth to 6.0 and lowering the substrate concentration (0.2%) was effective to enhance the conversion, so as to achieve 79% yield. In this case, the lower ratio between the substrate and wet cell weight (w/wcw, 0.03) also obviously affected the smooth reaction, compared with the original value (0.2). Finally, under a mechanically pH-controlled aerobic environment by adding antifoam, with a plentiful oxygen supply, a higher yield (93%) with a higher 91.9% ee of (*R*)-**2** was obtained.

There are some comments on another yeast strain, *T. cutaneum* IAM 12206, for the production of the (*R*)-isomer. An initial attempt for the use of the stationary phase harvested cells of *T. cutaneum* after prolonged incubation (48 h) only resulted in a low yield of the desired alcohol **2** (6%), and the ee of the product was unexpectedly low (88%). In contrast, direct introduction of substrate **1** after 48 h incubation without any harvesting of the cells improved the yield to 19% and the ee to 93.0%. In an independent experiment, the growth curve determination of this strain indicated the logarithmic growing phase terminates after 24 h, at an OD (660 nm) of 1.4. This result suggested that the desired reductive enzyme expresses at a higher level in younger growing phase cells. The introduction of the substrate (0.2%) at 24 h and the continuing incubation were indeed effective, to give 38% yield. Finally, the addition of antifoam towards the acceleration of oxygen uptake further enhanced the yield to 60% of (*R*)-**2** with 94.0% ee.

In contrast, excessive oxygen exposure showed a deleterious effect on the growth of *P. minuta* IAM 12215, and it was preferable to avoid the use of the antifoam at the stage of pre-incubation. The scaled-up reduction was reproducible with several grams of substrate by applying harvested wet cells (w/wcw, 0.13), and (*S*)-**2** of 96.8% ee was obtained in 92% yield. Although the pH of the medium turned acidic during the reaction (ca. 3.9), high enzyme activity was exhibited and mechanical pH adjustment was not necessary. With these three strains, the preparative-scale reduction to both (*R*)- and (*S*)-**2** was established, and the results are summarized in Table 2.

2.4. Substitution of the phenylsulfonyl group with alicyclic and aliphatic groups

Next, we modified the phenylsulfonyl group in **1**. Two new ketones **8** and **9** were the candidates, which have alicyclic and aliphatic ketones, respectively. A small modification from the phenylsulfonyl group to a similar six-membered ring as **8** brought about no reduction on the isopropyl ketone at all, towards **10**. In the case of *C. floricola* IAM 13115 the enzyme worked, however, the reduction on the *pro-R* carbonyl group located in the cyclohexane ring predominated and two new products were isolated[24] in a combined yield of 88% (Scheme 4).



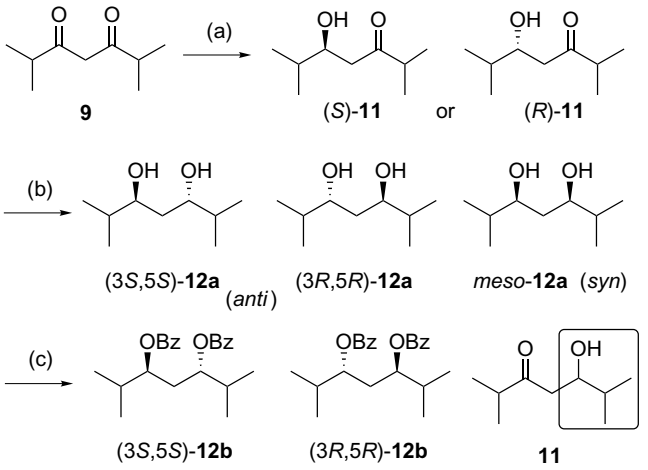
Scheme 4. (a) Four yeast strains in this study; (b) *C. floricola* IAM 13115.

In contrast, substitution by another bulky and electron-withdrawing isopropylcarbonyl group, the diketone-type substrate **9**, was accepted by all four strains of yeast as described above (Scheme 5). The reduction stopped at the stage of hydroxyketone **11**. This result means that the substitution of the phenylsulfonyl group in **1** with an isopropylcarbonyl group was acceptable, but with the isopropylhydroxymethyl group in **11**, it did not work. The results were in contrast to the fact that a similar hydroxyketone, 4-hydroxy-2-pentanone, was a good substrate to *Y. farinosa* NBRC 10896 to give diol.[25]

As the resulting hydroxyketone **11** was a rather labile compound, the enantiofacial selectivity was evaluated at the

Table 2. Comparison of facial selectivity between ketones **1** and **9**

Strain (IAM No.)	Product **2** from ketone **1**			Product **11** from ketone **9**		
	Absolute configuration	Yield (%)	% ee	Absolute configuration	Yield (%)	% ee
C. floricola (13115)	(*R*)	93	91.9	(*S*)	36	92.2
T. cutaneum (12206)	(*R*)	60	94.0	(*S*)	12	97.6
P. angusta (12895)	(*S*)	32	95.0	(*S*)	12	43.2
P. minuta (12215)	(*S*)	92	96.8	(*R*)	36	59.4



Scheme 5. Reagents and conditions: (a) Four yeast strains in this study; (b) $Me_4NHB(OAc)_3$, MeCN, AcOH, −20 °C; (c) BzCl, pyridine.

later stages as follows: the partially purified product **11** was immediately reduced with tetramethylammonium triacetoxyborohydride (Evans' reagent)[26] to give the enantiomerically enriched form of *anti*-diol **12a** as the major product, accompanied with a small amount of *meso* (*syn*)-**12a** (over 9:1). *anti*-Diol **12a** was then converted to the corresponding dibenzoate **12b** and was analyzed by HPLC with Daicel ChiralCel OD-H. The ee of **12b** represents the enantiofacial preference of the yeast-mediated reduction at the stage of the formation of **11**.

The results of the enantiofacial selection at the step of yeast enzyme-catalyzed reduction on **9** are summarized in Table 2, together with the results on the reduction of **1**. Three of the four yeast strains showed the same enantiofacial preference; however, *P. angusta* IAM 12895 showed a reversal of preference. In this case, it was supposed that plural enzymes, which have opposite selectivity, compete in the whole cell. One enzyme with *re*-facial selectivity on substrate **1**, and another with an inverted facial selectivity worked preferentially on **9** in whole cells, which are incubated under the same conditions (Fig. 1). At present, there is no information with regards to the type of coenzymes, such as NADH or NADPH.

So far, the chemical asymmetric reduction of **9**[27–29] has been elaborated towards **12a** (DMHD), a useful chiral auxiliary.[30,31] It was noted that the purification of the volatile and considerably labile hydroxyketone **11**, even in a partial state, lowers the two-step combined yield of yeast-catalyzed and Evans' reduction. We then considered Singh's one-pot approach[32] towards the diol, and an excess amount of $NaBH_4$ was directly added to the yeast-reduction broth after the consumption of starting material **9**, without any isolation of intermediate **11** (Scheme 6). Even though the Evans' reagent was switched with $NaBH_4$ for the second reduction, the preferential formation of *anti*-diol **12a** was still observed. All of the yields in the one-pot approach were improved over those of the aforementioned step-by-step conversion. The ees of the product were substantially lower, but the enantiofacial preference for

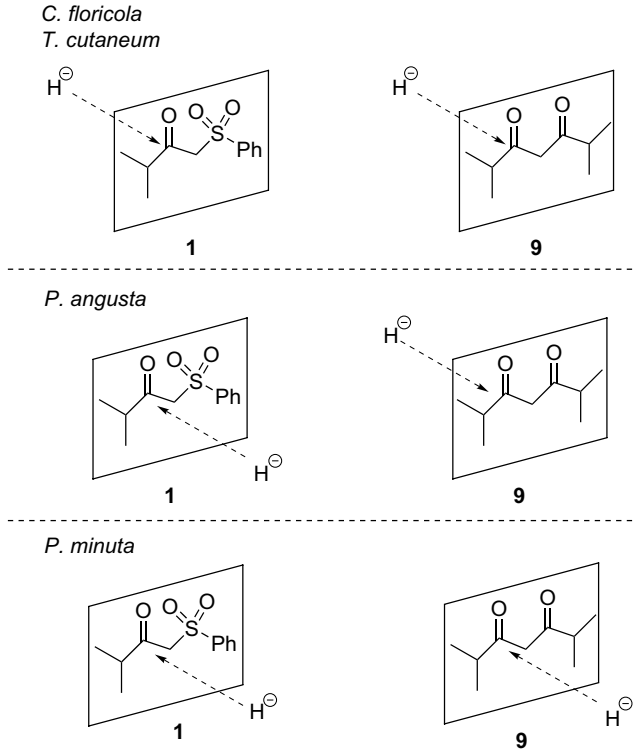
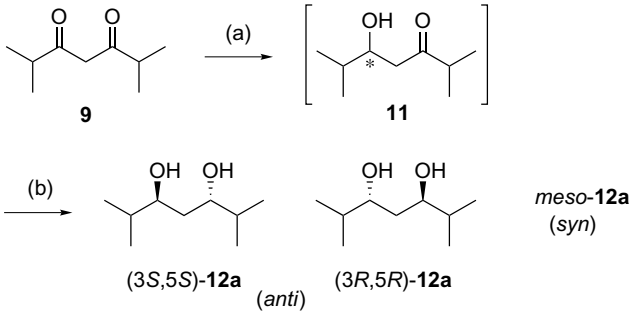


Figure 1. Change of enantiofacial selectivity in yeast-mediated reduction.



Scheme 6. Reagents: (a) Four yeast strains in this study; (b) $NaBH_4$.

strains was consistent (Table 3). *C. floricola* was the best, from the standpoint of the feasibility of the incubation and reproducibility in scaled-up conditions (63.4% ee, 61% yield) among the four strains.

Towards the enantiomerically pure (3*S*,5*S*)-**12a**, a complementary use of microbial reduction and an enzyme-catalyzed kinetic resolution was elaborated. There were no attempts on the enzyme reaction of the diol **12a** carrying large isopropyl substituents attached on both hydroxymethyl carbons, and indeed, the enzyme-catalyzed acylation was very slow. We then introduced two electron-withdrawing acyl groups[33] to provide the bis(chloroacetate) **12c**.

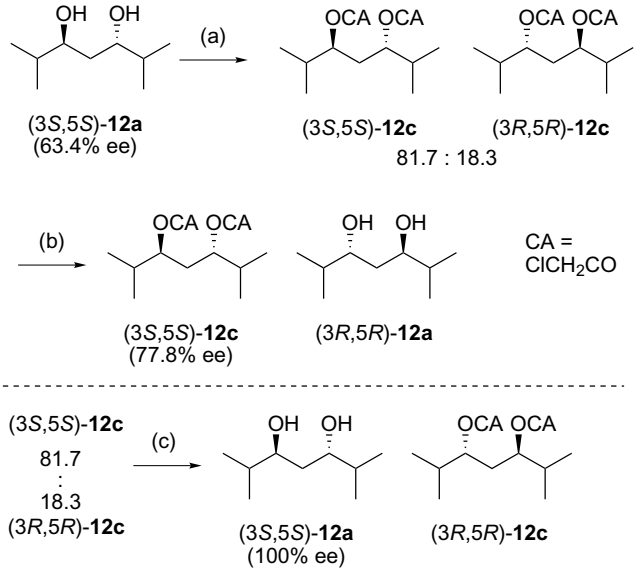
A mixture of (3*S*,5*S*)-**12c** and (3*R*,5*R*)-**12c** (81.7:18.3), prepared from **12a** (63.4% ee), was incubated with several

Table 3. Comparison between step-by-step and one-pot reduction of **9**

Strain (IAM No.)	Step-by-step		One-pot	
	Yield (%)	% ee	Yield (%)	% ee
C. floricola (13115)	36	92.2	61	63.4
T. cutaneum (12206)	12	97.6	22	81.6
P. angusta (12895)	12	43.2	17	32.0
P. minuta (12215)	36	59.4	55	69.4

commercially available hydrolytic enzymes. Our first candidate was *Bacillus licheniformis* protease (subtilisin), and the enzyme preferentially hydrolyzed the minor enantiomer, (3*R*,5*R*)-**12c**. The hydrolysis, however, always resulted in an incomplete manner, giving as low as 77.8% ee for the desired (3*S*,5*S*)-isomer as the unaffected substrate (Scheme 6). As from a preliminary independent experiment, the difference in the initial hydrolytic rate between (3*R*,5*R*)-**12c** and (3*S*,5*S*)-**12c** was very high. We concluded that the less reactive (3*R*,5*R*)-**12c** worked as the competitive inhibitor[34] with almost no k_{cat}. However, it showed a similar K_m with the preferred (3*S*,5*S*)-enantiomer.

Besides the enzymes with no enantioselectivity on **12c** (pig pancreatic lipase, *Candida rugosa* lipase: Meito OF), and *Candida antarctica* lipase: Chirazyme L-2 with very low activity on **12c**, there was found *Pseudomonas cepacia* lipase PS-C showing the contrasting enantio-preference with subtilisin.[35] In this case, we were successful in isolating the (3*S*,5*S*)-isomer in its enantiomerically pure state as diol **12a** in 55% yield by enzyme-catalyzed hydrolysis (Scheme 7). Moderate catalytic activity and enantioselectivity of yeast-catalyzed reduction of sterically hindered diketone **9** were overcome, by elaborating upon the combination with the subsequent one-pot reduction procedure and lipase-catalyzed kinetic resolution to give pure (3*S*,5*S*)-**12a**.



Scheme 7. Reagents: (a) $(ClCH_2CO)_2O$, pyridine; (b) *Bacillus licheniformis* protease (subtilisin); (c) *Pseudomonas cepacia* lipase PS-C.

3. Conclusion

So far, in both enzyme-catalyzed asymmetric reduction and the resolution of racemate, difficulties have often been experienced in the access to enantiomerically enriched forms of hindered alcohols due to the low reactivity caused by the sterically bulky group directly attached to the stereogenic centre. The present newly developed yeast strains, as a method for the preparation of enantiomers, would be a clue in how to overcome these problems.

4. Experimental

4.1. Materials and methods

Merck silica gel 60 F_{254} thin-layer plates (1.05744, 0.5 mm thickness) and silica gel 60 (spherical and neutral; 100–210 μm, 37560-79) from Kanto Chemical Co. were used for preparative thin-layer chromatography and column chromatography, respectively. Peptone and yeast extract were purchased from Kyokuto Pharmaceutical Co., for the cultivation of microorganism. Yeast strains are available from, the Institute of Applied Microbiology Culture Collection; the Institute of Molecular and Cellular Biosciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan.

4.2. Analytical methods

All mps are uncorrected. IR spectra were measured as films for oils or KBr disks of solids on a Jasco FT/IR-410 spectrometer. ^{1}H NMR spectra were measured in $CDCl_3$ at 270 MHz on a Jeol JNM EX-270 or at 400 MHz on a Jeol JNM GX-400 spectrometer. HPLC data were recorded on Jasco PU-2080 and MD-2010 liquid chromatographs. Optical rotation values were recorded on a Jasco DIP 360 and P-1010 polarimeter.

4.3. Preparation of substrate: 3-methyl-1-phenylsulfonyl-2-butanone 1

To a mixture of methyl phenyl sulfone (100 mg, 0.640 mmol) and THF (2.0 mL) cooled to −78 °C was added *n*-BuLi (1.56 M in hexane, 0.86 mL, 1.3 mmol). After stirring for 1 h, 2-methylpropanoyl chloride (80.0 μL, 0.764 mmol) was added to this solution at −78 °C. Stirring was continued for 1 h at −78 °C. The disappearance of the starting material was confirmed by a TLC analysis [silica gel, developed with hexane–AcOEt (1:1)]. The reaction mixture was poured into saturated aqueous NH_4Cl solution and extracted with AcOEt. The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The residue was charged on a silica gel column (8 g). Elution with hexane–AcOEt (4:1) afforded **1** (132 mg, 85%).

Further purification by recrystallization from hexane–AcOEt afforded **1** (114 mg, 73%) as colourless needles; mp 63.5–64.5 °C (lit.[36] mp 66.5–67 °C); IR ν_{max} 2936, 1714, 1449, 1383, 1319, 1151, 784, 687 cm^{-1}; ^{1}H NMR δ: 1.11 (d, $J = 6.9$ Hz, 6H), 2.92 (sept, $J = 6.9$ Hz, 1H), 4.23

(s, 2H), 7.56 (dd, $J = 7.1$, 7.4 Hz, 2H), 7.65 (t, $J = 7.4$ Hz, 1H), 7.88 (d, $J = 7.1$ Hz, 2H). Its IR and NMR spectra were identical with those reported previously.[36]

4.4. Screening of microorganisms

The screening of microorganisms was performed as follows. The microorganisms from stock culture samples were incubated in glucose medium [containing glucose (500 mg), peptone (200 mg), yeast extract (50 mg), KH_2PO_4 (30 mg), K_2HPO_4 (20 mg), at pH 6.5, total volume of 10 mL, in the test tube] for 2 days at 30 °C. Then ketone **1** (50 mg) and glucose (500 mg) were added and the mixture was shaken on a reciprocal shaker (250 cpm) for 2 days at 30 °C. The progress of the reduction was confirmed by TLC analysis [silica gel, developed with hexane–AcOEt (1:1)]. Each reaction mixture was separately worked up in the same way as described below. The reaction mixture was filtered through a Celite pad and extracted with AcOEt. The combined organic layer was washed with saturated aqueous $NaHCO_3$ solution and brine, dried over anhydrous Na_2SO_4 and concentrated in vacuo. The crude residue containing product **2** and unreacted starting material **1** was filtered through a short column of silica gel, analyzed by the HPLC analysis [column, Daicel Chiralcel OD-H, 0.46 cm × 25 cm; hexane–*i*-PrOH (15:1); flow rate 0.5 mL/min]: t_R (min) = 29.0 for (*R*)-**2**, 32.9 for (*S*)-**2**, 36.7 for **1**. Ee of the product **2** and conversion were estimated by this HPLC analysis.

4.5. Scaled-up reduction of 1

4.5.1. Pre-incubation of *C. floricola* IAM 13115. A small portion of yeast cells of *C. floricola* IAM 13115 grown on the agar-plate culture was aseptically inoculated to a glucose medium [containing glucose (20 g), peptone (8.0 g), yeast extract (2.0 g), KH_2PO_4 (1.2 g), K_2HPO_4 (0.8 g), at pH 6.5, total volume of 400 mL] in four 500-mL baffled Erlenmeyer cultivating flasks and then shaken on a gyratory shaker (180 rpm) for 35 h at 30 °C. The wet cells were harvested by centrifugation (3000 rpm) and washed with phosphate buffer (0.1 M, pH 6.5). The weight of combined wet cells was ca. 13 g from 200 mL of the broth.

4.5.2. Preparation of (*R*)-(−)-3-methyl-1-phenylsulfonyl-2-butanonol 2 by *C. floricola* IAM 13115 mediated reaction. The combined wet cells of *C. floricola* IAM 13115 (6.3 g) incubated as described above, were re-suspended in a reaction medium [containing glucose (5.0 g), phosphate buffer (0.1 M, pH 6.0), total volume of 100 mL] in a 200-mL round-bottomed flask, together with **1** (202 mg, 0.89 mmol), which was finely ground in advance. The mixture was stirred for 42 h at 30 °C. Throughout the reaction, its pH was kept at 6.0 by the occasional addition of aqueous NaOH solution (2.0 M, total 10 mL) with an automatic pH controller. The broth was then centrifuged (3000 rpm). The supernatant was saturated with NaCl and mixed with AcOEt (100 mL). The mixture was stirred for 1 h and filtered through a pad of Celite. The organic layer of the filtrate was separated and the aqueous layer was further extracted with AcOEt. On the other hand, the precipitated cell mass at the stage of centrifugation was mixed with acetone (5 mL). The mixture was stirred for 1 h and filtered through a pad of Celite. The combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4 and concentrated in vacuo. The residue was charged on a silica gel column (20 g). Elution with toluene–AcOEt (12:1) and the further purification of some contaminated fractions with preparative TLC with $CHCl_3$–MeOH (19:1) afforded (*R*)-**2** (189.9 mg, 93%). $[\alpha]_D^{20} = -17.8$ (*c* 0.98, EtOH). HPLC: t_R (min) = 31.6 (96.0%), 37.7 (4.1%); 91.9% ee. IR: ν_{max} 3521, 2964, 2877, 1446, 1304, 1146, 1086 cm^{-1}; ^1H NMR: δ 0.89 (d, $J = 4.5$ Hz, 6H), 1.69–1.81 (m, 1H), 3.18 (s, 1H), 3.20 (d, $J = 2.1$ Hz, 1H), 3.24 (d, $J = 2.3$ Hz, 1H), 3.93–4.00 (m, 1H), 7.57–7.72 (m, 2H), 7.93–7.96 (m, 3H). Anal. Calcd for $C_{11}H_{16}O_3S$: C, 57.87; H, 7.06. Found: C, 58.04; H, 7.031.

4.5.3. Incubation of *T. cutaneum* IAM 12206 and the preparation of (*R*)-(−)-2. *T. cutaneum* IAM 12206 were grown on the broth with the same ingredients with *C. floricola* (total volume of 100 mL) on a gyratory shaker (180 rpm) for 12 h at 30 °C. Then the antifoam AF emulsion (100 mg/mL, 0.5 mL) was added and the mixture was shaken for a further 12 h at 30 °C. Then substrate **1** (205 mg, 0.91 mmol), which was pre-treated as above, was introduced and the incubation was continued for 24 h. The extraction of products from the supernatant and cell mass, workup and purification were carried out in the same manner as described for *C. floricola*-mediated reaction, to give (*R*)-**2** (123.2 mg, 60%). $[\alpha]_D^{20} = -16.7$ (*c* 0.98, EtOH). The ee was estimated from HPLC analysis. HPLC: t_R (min) = 30.9 (97.0%), 36.7 (3.0%); 94.0% ee. Its IR and NMR spectra were identical with those mentioned above.

4.5.4. Incubation of *P. minuta* IAM 12215 and the preparation of (*S*)-(+)-2. The combined wet cells of *P. minuta* IAM 12215 (23 g), incubated as described for *C. floricola*, were re-suspended in a reaction medium [containing glucose (72 g), phosphate buffer (0.1 M, pH 6.0), total volume of 1438 mL] in a 5000-mL baffled Erlenmeyer cultivating flask, together with **1** (2.88 g, 12.7 mmol), which was pretreated as above, and shaken on a gyratory shaker (180 rpm) for 27.5 h at 30 °C. Then the broth was centrifuged (3000 rpm). The extraction of products from the supernatant and cell mass, workup and purification were carried out in the same manner as described for *C. floricola*-mediated reaction, to give (*S*)-**2** (2.67 g, 92%). $[\alpha]_D^{22} = +18.0$ (*c* 0.97, EtOH). HPLC: t_R (min) = 32.4 (1.6%), 36.3 min (98.4%); 96.8% ee. Anal. Calcd for $C_{11}H_{16}O_3S$: C, 57.87; H, 7.06. Found: C, 57.97; H, 7.038. Its IR and NMR spectra were identical with those mentioned above.

4.6. Determination of absolute configuration of 2

4.6.1. (3*S*,4*RS*)-2-Methyl-4-phenylsulfonyl-6-heptene-3-ol 5. To an LDA solution, separately provided by the addition of *n*-BuLi (2.71 M in hexane, 7.0 mL, 19 mmol) to a solution of *i*-Pr_2NH (2.9 mL, 21 mmol) in THF (26 mL) at 0 °C, a solution of (+)-**2** (2.1 g, 9.0 mmol, prepared by *P. minuta*-mediated reduction) in THF (20 mL) was added at −78 °C and the mixture was stirred for 30 min. Then \allyl iodide (8.2 mL, 91 mmol) was added and the mixture

stirred further at −78 °C for 30 min, and allowed to warm to room temperature over 2 h. The reaction was quenched with saturated aqueous NH_4Cl solution and the mixture extracted with AcOEt. The organic layer was washed with brine, dried over anhydrous Na_2SO_4 and concentrated in vacuo. The residue was charged on a silica gel column (170 g). Elution with hexane–AcOEt (4:1) afforded (3*S*,4*RS*)-**5** (1.8 g, 76%). $[\alpha]_D^{24} = +175.3$ (*c* 1.04, EtOH). IR ν_{max} 3527, 2962, 1446, 1302, 1146, 1084 cm^{-1}; ^1H NMR δ: 0.72 (d, $J = 6.8$ Hz, 3H), 1.00 (d, $J = 6.4$ Hz, 3H), 1.81 (dqq, $J = 9.1$, 6.4, 6.8 Hz, 1H), 2.66 (m, 2H), 2.95 (d, $J = 1.8$ Hz, 1H), 3.20 (t, $J = 5.4$ Hz, 1H), 3.77 (dd, $J = 1.8$, 9.1 Hz, 1H), 4.95 (dd, $J = 1.3$, 9.7 Hz, 1H), 5.00 (dd, $J = 1.5$, 16.0 Hz, 1H), 5.70 (m, 1H), 7.64 (m, 3H), 7.91 (m, 2H).

4.6.2. (3*S*,4*RS*)-2-Methyl-4-phenylsulfonyl-3-heptanol 6. The alcohol (3*S*,4*RS*)-**5** (1.7 g, 6.5 mmol) was dissolved in EtOH (64 mL) and then 10% Pd–C (340 mg) was added. The mixture was vigorously stirred under H_2 (1 atm) at room temperature for 15 h. The reaction mixture was filtered through a pad of Celite, and the Celite pad was washed with AcOEt. The combined organic solution was concentrated in vacuo to afford **6** (1.7 g, 99%) as a diastereomeric mixture (ca. 3:1). $[\alpha]_D^{24} = +14.7$ (*c* 1.01, EtOH). IR ν_{max} 3525, 2962, 1300, 1146, 1084, 729 cm^{-1}; ^1H NMR δ: major diastereomer; 0.70 (d, $J = 6.8$ Hz, 3H), 0.86 (t, $J = 7.3$ Hz, 3H), 0.99 (d, $J = 6.6$ Hz, 3H), 1.38 (m, 2H), 1.74 (dqq, $J = 9.6$, 6.6, 6.8 Hz, 1H), 1.88 (m, 2H), 2.98 (d, $J = 2.0$ Hz, 1H), 3.08 (t, $J = 5.1$ Hz, 1H), 3.66 (dd, $J = 2.0$, 9.6 Hz, 1H), 7.55–7.71 (3H, m), 7.89–7.92 (m, 2H); minor diastereomer; 0.79 (t, $J = 7.3$ Hz, 3H), 0.94 (d, $J = 6.8$ Hz, 3H), 0.99 (d, $J = 6.6$ Hz, 3H), 1.38 (m, 2H), 1.58 (m, 2H), 2.02 (dqq, $J = 5.2$, 6.6, 6.8 Hz, 1H), 3.18 (dt, $J = 5.1$, 5.0 Hz, 1H), 3.44 (d, $J = 5.4$ Hz, 1H), 3.72 (ddd, $J = 5.1$, 5.2, 5.4 Hz, 1H), 7.55–7.71 (m, 3H), 7.89–7.92 (m, 2H).

4.6.3. (*R*)-2-Methyl-3-heptanol 7a. A solution of the alcohol (3*S*,4*RS*)-**6** (694 mg, 2.6 mmol) in Et_2O (5.2 mL) and ethylenediamine (1.2 mL, 18 mmol) was cooled to 0 °C. The mixture was degassed to remove any trace of O_2 by applying ultrasonic (70–80 W) under evacuation. Lithium shot (Wako, 126-04913, 97 mg, 14 mmol) was added under the flow of N_2 and the mixture was vigorously stirred at 0 °C. The color of the reaction mixture immediately turned brown. After the proper period, the reaction was quenched by the addition of saturated aqueous NH_4Cl solution and 6 M HCl to adjust its pH to be 6. The mixture was then extracted several times with Et_2O. The combined extract was washed with water, 2 M aqueous NaOH solution, and brine and dried over anhydrous Na_2SO_4 with activated charcoal. The solvent was carefully removed by fractional distillation at atmospheric pressure. The residue was purified by preparative TLC with hexane–AcOEt (4:1) and further by micro-distillation apparatus to afford (*R*)-**7a** (87 mg, 26%). Bp 120 °C/80 mmHg; $[\alpha]_D^{24} = +25.7$ (*c* 0.46, EtOH) {lit.[22] $[\alpha]_D = +27.7$ (*c* 10, EtOH)} IR ν_{max} 3359, 2958, 2933, 2873, 1468, 991 cm^{-1}; ^1H NMR: δ 0.91 (m, 9H), 1.29–1.48 (m, 6H), 1.57–1.73 (m, 1H), 3.36 (br s, 1H); ^{13}C NMR δ: 14.2, 17.2, 19.0, 22.9, 28.3, 33.5, 33.9, 76.7.

4.6.4. (2*S*,1′*R*)-1′-Isopropylpentyl 3,3,3-trifluoro-2-methoxy-2-phenylpropanoate 7b. (*R*)-MTPA chloride (27 mg, 0.107 mmol) was added with stirring to a solution of (*R*)-**7a** (6 mg, 0.046 mmol) in pyridine (0.46 mL). After stirring overnight at room temp, the reaction was quenched with water (5 mL) and the mixture was extracted with AcOEt. The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The residue was dissolved in toluene (2 mL), concentrated in vacuo twice for removing pyridine, and purified by preparative TLC with hexane–AcOEt (9:1) to afford **7b** (14.4 mg, 99%). ^1H NMR δ: 0.83 (d, $J = 6.4$ Hz, 3H), 0.85 (d, $J = 6.1$ Hz, 3H), 0.88 (t, $J = 7.0$ Hz, 3H), 1.20–1.40 (m, 4H), 1.52–1.72 (m, 2H), 1.85–1.97 (m, 1H), 3.56 (s, 1H), 4.94 (dt, $J = 4.6$, 6.0 Hz, 1H), 7.37–7.42 (m, 3H), 7.55–7.58 (m, 2H). An authentic sample from racemic **7a** showed the additional signals ascribable to the (1′*S*)-isomer: 0.82 (t, $J = 7.0$ Hz, 3H), 0.91 (d, $J = 6.9$ Hz, 3H), 0.92 (d, $J = 6.9$ Hz, 3H), 1.10–1.37 (m, 4H), 1.49–1.69 (m, 2H), 1.85–2.00 (m, 1H), 3.56 (s, 1H), 4.97 (m, 1H), 7.38–7.42 (m, 3H), 7.55–7.56 (m, 2H). The ee of product **7a** from the microbial origin was 96%, judging from their NMR spectra.

4.7. Reduction of 9: step-by-step procedure

4.7.1. (3*S*,5*S*)-(−)-2,6-Dimethyl-3,5-heptanediol 12a by *C. floricola* IAM 13315. The combined wet cells of *C. floricola* (10 g) incubated as described above were re-suspended in a reaction medium [containing glucose (5 g), phosphate buffer (0.1 M, pH 6.0), total volume of 100 mL] in a 300-mL round-bottomed flask, together with **6** (200 mg, 1.28 mmol). The reaction mixture was stirred for 28 h at 30 °C. Antifoam AF emulsion (100 mg/mL, 0.5 mL) was then added to the reaction mixture and the mixture was azeotropically distilled in vacuo. The distillate was saturated with NaCl and extracted with Et_2O. The combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4 and concentrated in vacuo. The residue was employed for the next step immediately without further purification.

A solution of the crude product in anhydrous MeCN (0.85 mL) was added slowly with stirring under Ar to a solution of $Me_4NHB(OAc)_3$ (1.11 g, 4.2 mmol) in anhydrous MeCN (3.4 mL) and anhydrous AcOH (3.4 mL) at −20 °C. After stirring for 2 h at −20 °C, the reaction was quenched by the addition of saturated aqueous sodium potassium tartrate solution (2 mL) and stirred vigorously for 30 min. The mixture was extracted with AcOEt, and the organic layer was washed with saturated aqueous $NaHCO_3$ solution and brine, dried over anhydrous Na_2SO_4 and concentrated in vacuo. The residue was charged on a silica gel column (7 g). Elution with hexane–AcOEt (6:1) and the further purification of some contaminated fractions with preparative TLC with hexane–AcOEt (2:1), which was developed twice, afforded (3*S*,5*S*)-**12a** (73.7 mg, 36%) as a colourless solid; mp 88.5–90.0 °C; IR ν_{max} 3336, 2958, 1471, 1333, 1146, 1038, 795, 704 cm^{-1}; ^1H NMR δ: 0.91 (d, $J = 6.8$ Hz, 6H), 0.96 (d, $J = 6.6$ Hz, 6H), 1.59 (dd, $J = 5.6$, 5.7 Hz, 2H), 1.72 (dqq, $J = 5.9$, 6.6, 6.8 Hz, 2H), 2.34 (s, 2H), 3.63 (ddd, $J = 5.6$, 5.7, 5.9 Hz, 2H); ^{13}C

NMR δ: 18.16, 18.77, 33.79, 36.54, 74.20; $[\alpha]_D^{20} = -53.2$ (*c* 1.01, MeOH) {lit.[27] $[\alpha]_D^{25} = -64.5$ (*c* 1.00, MeOH)}. The ee of (3*S*,5*S*)-**12a** was determined by HPLC analysis of the corresponding dibenzoate **12b**. HPLC [column, Daicel Chiralcel OD-H, 0.46 cm × 25 cm; hexane–*i*-PrOH (19:1); flow rate 0.5 mL/min]: t_R (min) = 9.3 (96.1%), 10.3 (3.9%); 92.2% ee. Dibenzoate **12b**; ^{1}H NMR δ: 0.89 (d, $J = 6.7$ Hz, 6H), 0.92 (d, $J = 6.7$ Hz, 6H), 1.96 (dqq, $J = 5.9$, 6.7, 6.7 Hz, 2H), 1.98 (dd, $J = 5.5$, 5.6 Hz, 2H), 5.21 (ddd, $J = 5.5$, 5.6, 5.9 Hz, 4H), 7.27 (dd, $J = 7.3$, 7.5 Hz, 4H), 7.41 (tt, $J = 1.3$, 7.3 Hz, 2H), 7.88 (dd, $J = 1.3$, 7.5 Hz, 4H).

4.7.2. (3*S*,5*S*)-(−)-12a by *T. cutaneum* IAM 12206. *T. cutaneum* grown on the broth with the same ingredients with *C. floricola* (total volume of 100 mL) on a gyratory shaker (180 rpm) for 12 h at 30 °C. Then antifoam AF emulsion (100 mg/mL, 0.5 mL) was added and the mixture shaken for a further 12 h at 30 °C. Then substrate **9** (200 mg, 1.28 mmol) was introduced and the incubation was continued for 24 h. Extraction of the product and the subsequent Evans' reduction were carried out in the same manner as described for *C. floricola*-mediated reaction, to give (3*S*,5*S*)-**12a** (25.3 mg, 12%); $[\alpha]_D^{20} = -52.3$ (*c* 0.97, MeOH). The ee of (3*S*,5*S*)-**12a** was estimated from HPLC analysis of **12b** in the same manner as described previously; t_R (min) = 8.6 (1.2%), 9.5 (98.8%); 97.6% ee. Its IR and NMR spectra were identical with those described above.

4.7.3. (3*S*,5*S*)-(−)-12a by *P. angusta* IAM 12895. *P. angusta* was grown on the broth with the same ingredients with *C. floricola* (total volume of 200 mL) on a gyratory shaker (180 rpm) for 36 h at 30 °C. The combined wet cells (5 g) were re-suspended in the same reaction medium as above, in a 300-mL round-bottomed flask, together with **9** (200 mg, 1.28 mmol), and the incubation was continued for 43 h. The extraction and the subsequent reduction were carried out in the same manner as described for *C. floricola*-mediated reaction, to give (3*S*,5*S*)-**12a** (23.9 mg, 12%); $[\alpha]_D^{20} = -26.0$ (*c* 0.99, MeOH); HPLC of **12b** t_R (min) = 8.6 (28.4%), 9.5 (71.6%); 43.2% ee.

4.7.4. (3*R*,5*R*)-(+)-12a by *P. minuta* IAM 12215. *P. minuta* was grown on the broth with the same ingredients with *C. floricola* (total volume of 200 mL) on a gyratory shaker (180 rpm) for 36 h at 30 °C. The combined wet cells (10 g) were re-suspended in a reaction medium [containing glucose (5 g), phosphate buffer (0.1 M, pH 6.0), total volume of 100 mL] in a 300-mL round-bottomed flask, together with **9** (200 mg, 1.28 mmol). The mixture was stirred for 36 h at 30 °C. The extraction of the products and the subsequent Evans' reduction were carried out in the same manner as described for *C. floricola*-mediated reaction, to give (3*R*,5*R*)-**12a** (74.2 mg, 36%); $[\alpha]_D^{20} = +39.5$ (*c* 1.00, MeOH); HPLC of **12b** t_R (min) = 8.8 (79.7%), 9.9 (20.3%); 59.4% ee.

4.8. Reduction of 9: one-pot two step procedure

4.8.1. (3*S*,5*S*)-(−)-12a by *C. floricola* IAM 13315. The combined wet cells of *C. floricola* IAM (40 g from five 500-mL baffled Erlenmeyer cultivating flasks) incubated as described above were re-suspended in the same reaction medium as above in a 500-mL round-bottomed flask, together with **9** (400 mg, 2.56 mmol). After stirring for 14.5 h at 30 °C, the reaction medium was cooled to 15 °C and $NaBH_4$ (381 mg, 10.1 mmol) was occasionally added over 20 min, while monitoring the internal reaction temperature. Then the reaction mixture was centrifuged (3000 rpm). The supernatant was saturated with NaCl and mixed with AcOEt (ca. 130 mL). The mixture was stirred for 1 h and filtered through a pad of Celite. The organic layer of the filtrate was separated and the aqueous layer was further extracted with AcOEt. On the other hand, the precipitated cell mass at the stage of centrifugation was mixed with acetone (50 mL). The mixture was stirred for 1 h and filtered through a pad of Celite. The combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4 and concentrated in vacuo. The residue was charged on a silica gel column (4 g). Elution with hexane–AcOEt (6:1) and further purification of some contaminated fractions, preparative TLC with hexane–AcOEt (2:1, developed twice) afforded (3*S*,5*S*)-**12a** (249.1 mg, 61%) and *syn*-**12a** (58.3 mg, 14%).

Compound (3*S*,5*S*)-**12a**; $[\alpha]_D^{20} = -38.3$ (*c* 1.00, MeOH); HPLC of **12b** t_R (min) = 8.4 min (18.3%), 9.4 min (81.7%); 63.4% ee. Its IR and NMR spectra were identical with those described above. Compound *syn*-**12a**: IR ν_{max} 3357, 2960, 1468, 1385, 1151, 1063, 852, 604 cm^{-1}; ^{1}H NMR δ: 0.92 (d, $J = 6.9$ Hz, 12H), 1.41 (dt, $J = 14.0$, 10.2 Hz, 1H), 1.59 (dt, $J = 14.0$, 2.0 Hz, 1H), 1.67 (m, 2H), 3.12 (s, 2H), 3.62 (ddd, $J = 2.0$, 5.1, 10.2 Hz, 2H).

4.8.2. (3*S*,5*S*)-(−)-12a by *T. cutaneum* IAM 12206. *T. cutaneum* was grown in 100 mL of the broth in total 24 h at 30 °C as described above. Substrate **9** (200 mg, 1.28 mmol) was incubated for 24 h at 30 °C. The subsequent reduction with $NaBH_4$, workup and purification were done in the same manner as described above to give (3*S*,5*S*)-**12a** (45.6 mg, 22%) and *syn*-**12a** (7.3 mg, 4%). Compound (3*S*,5*S*)-**12a**: $[\alpha]_D^{20} = -40.2$ (*c* 0.80, MeOH); HPLC of **12b** t_R (min) = 8.4 (9.2%), 9.4 (90.8%); 81.6% ee. The NMR spectrum of *syn*-**12a** was identical with those described above.

4.8.3. (3*S*,5*S*)-(−)-12a by *P. angusta* IAM 12895. Diketone **9** (200 mg, 1.28 mmol) was incubated with the harvested wet cells of *P. angusta* (6 g from two 500-mL baffled Erlenmeyer cultivating flasks) for 70 h at 30 °C, and then further reduced with $NaBH_4$ affording (3*S*,5*S*)-**12a** (34.0 mg, 17%) and *syn*-**12a** (12.5 mg, 6.1%). Compound (3*S*,5*S*)-**12a**; $[\alpha]_D^{20} = -20.6$ (*c* 0.50, MeOH); HPLC of **12b** t_R (min) = 8.5 min (34.0%), 9.5 min (66.0%); 32.0% ee.

4.8.4. (3*R*,5*R*)-(+)-12a by *P. minuta* IAM 12215. Diketone **9** (200 mg, 1.28 mmol) was incubated with the harvested wet cells of *P. minuta* (8 g from two 500-mL baffled Erlenmeyer cultivating flasks) for 12 h at 30 °C. The subsequent reduction with $NaBH_4$, workup and purification were done in the same manner as described above to give (3*R*,5*R*)-**12a** (112.7 mg, 55%) and *syn*-**12a** (21.9 mg,

11%). Compound (3*R*,5*R*)-**12a**: $[\alpha]_D^{20} = +34.6$ (*c* 1.00, MeOH); HPLC of **12b** t_R (min) = 8.3 (84.7%), 9.2 (15.3%); 69.4% ee.

4.9. Enantiomeric enhancement of (3*S*,5*S*)-12a

4.9.1. (3*S*,5*S*)-2,6-Dimethyl-3,5-heptanediol bis(chloroacetyl)ester 12c. To a solution of (3*S*,5*S*)-**12a** (102.9 mg, 0.62 mmol) in anhydrous pyridine (1.0 mL) was added chloroacetic anhydride (267.0 mg, 1.56 mmol) and the mixture was stirred at room temperature for 1 h. Then the mixture was poured into phosphate buffer (1.0 mL, 0.1 M, pH 6.5) and extracted with AcOEt. The combined organic layer was washed with 2 M HCl, saturated $NaHCO_3$ solution and brine, dried over anhydrous Na_2SO_4 and concentrated in vacuo. The residue was charged on a silica gel column (15 g). Elution with hexane–AcOEt (20:1) afforded **12c** (190.7 mg, 94.8%); ^{1}H NMR δ: 0.91 (d, $J = 6.8$ Hz, 6H), 0.92 (d, $J = 6.8$ Hz, 6H), 1.80 (dd, $J = 5.6$, 5.6 Hz, 2H), 1.98 (dqq, $J = 7.9$, 6.8, 6.8 Hz, 2H), 4.04 (s, 4H), 4.86 (ddd, $J = 5.5$, 5.6, 7.9 Hz, 2H), ^{13}C NMR δ: 17.8, 18.1, 31.6, 32.0, 41.0, 76.1, 167.1. This was employed for the next step without further purification.

4.9.2. Subtilisin-catalyzed hydrolysis of 12c. To a mixture of (3*S*,5*S*)-**12c** [63.4% ee, (3*S*,5*S*):(3*R*,5*R*) = 81.7:18.3; 20.0 mg, total 0.064 mmol] phosphate buffer (0.5 mL, 0.2 M, pH 7.0) was added subtilisin (Sigma, P-5380, 50 mg) and the mixture stirred at 40 °C for 2 days. Then the insoluble materials were filtered off and the filtrate was extracted with AcOEt. The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4 and concentrated in vacuo. The residue was purified by preparative TLC [developed with hexane–AcOEt (4:1)] to give diol **12a** (1.1 mg, 11%), **12c** (10.9 mg, 55%) and the corresponding mono(chloroacetate) **12d** (1.3 mg, 9%). The recovered **12c** was hydrolyzed by applying K_2CO_3 in MeOH to give **12a**, and then converted to dibenzoate **12b**. HPLC t_R (min) = 8.4 (11.1%), 9.4 (88.9%); 77.8% ee.

4.9.3. *P. cepacia* lipase-catalyzed hydrolysis of 12c. To a mixture of (3*S*,5*S*)-**12c** [63.4% ee, as above, 108.6 mg, total 0.68 mmol] phosphate buffer (2.5 mL, 0.2 M, pH 7.0) was added lipase (Amano PS-C, 200 mg) and the mixture was stirred at 40 °C for 3 days. Then the enzyme was filtered off and the filtrate was extracted with AcOEt. After a similar workup as above, the residue was charged on a silica gel column (5 g). Elution with hexane–AcOEt (10:1) afforded diol **12a** (30.3 mg, 55%), **12c** (42.8 mg, 39%) and **12d** (3.2 mg, 4%); mp 89.5–90 °C (lit.[27] mp 89–91 °C); $[\alpha]_D^{22} = -64.3$ (*c* 1.01, MeOH) {lit.[27] $[\alpha]_D^{20} = -64.5$ (*c* 1.0, MeOH)}. Anal. Calcd for $C_9H_{20}O_2$: C, 67.45; H, 12.58. Found: C, 67.06; H, 12.26. HPLC of **12b** t_R (min) = 9.4 (100%) and no peak was detected at 8.4 min; 100% ee. Its IR and NMR spectra were identical with those of authentic sample.

Bis(chloroacetate) **12c** and mono(chloroacetate) **12d** were combined and hydrolyzed by applying K_2CO_3 in MeOH to give **12a**, and then converted to dibenzoate **12b**. HPLC t_R (min) = 8.4 (46.1%), 9.4 (55.9%); 11.8% ee as (3*S*,5*S*)-isomer.

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