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Research paper

Chemoenzymatic approaches to the synthesis of the (1*S*,2*R*)-isomer of benzyl 2-hydroxycyclohexanecarboxylate

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ABSTRACT

We examined ten strains of cultured whole-cell yeasts for the asymmetric reduction of commercially available ethyl 2-oxocyclohexanecarboxylate, and found that the (1*S*,2*S*)-stereoisomer of ethyl 2-hydroxycyclohexanecarboxylate was the major stereoisomer produced by *Williopsis californica* JCM 3600. The ethyl group of the ester was then substituted with a benzyl group with low volatility and increased hydrophobicity to facilitate the isolation of the expected product. Incubation with *W. californica* furnished benzyl (1*S*,2*S*)-2-hydroxycyclohexanecarboxylate (>99.9% ee) in 51.0% yield together with its (1*R*,2*S*)-isomer (>99.9% ee) in 35.4% yield. Upon treatment of the same substrate bearing the benzyl ester with a screening kit of purified overexpressed carbonyl reductases (Daicel Chiralscreen® OH), two enzymes (E031, E078) furnished the (1*R*,2*S*)-isomer as the major product. With another enzyme (E007), the (1*S*,2*R*)-isomer was obtained, but its ee was very low (25.6%). The highly enantiomerically enriched (1*S*,2*S*)-isomer obtained by *W. californica* was transformed to the (1*S*,2*R*)-isomer (>99.9% ee), whose availability until now has been low, in 43.3% yield over two steps involving tosylation and subsequent inversive attack with tetrabutylammonium nitrite.

1. Introduction

The stereochemically enriched form of ethyl 2-hydroxycyclohexanecarboxylate (**1a**, Fig. 1) is a versatile synthetic starting material [1–5], taking advantage of two independent functional groups neighboring on the six-membered ring. By Fráter's stereoselective alkylation [6], which can introduce an all-carbon quaternary chiral center at the C-1 position, the resulting products (Fig. 1) have widened the usefulness of this starting material [7].

To generate stereochemically enriched forms, classical enantiomeric resolutions of the racemic mixture have been developed, such as preferential crystallization of the diasteromeric salt of the corresponding racemic hydroxy acid [5]. Also, much effort has been devoted to the development of enzyme-catalyzed kinetic resolution [3,8,9].

In addition, asymmetric reduction of the corresponding β-oxoester **2a** has been examined. The reduction is generally accompanied by the racemization of the substrate, as the acidity of the proton at C-1 is high [10–12]. Ruthenium-catalyzed asymmetric hydrogenation of **2a** produces **1a** with a varied ratio between *syn*- and *anti*-diastereomers with up to 90% ee in *syn*-**1a** [13,14] and with 91% ee in *anti*-**1a** [15].

Biocatalytic asymmetric reduction of carbonyl compounds is a potent way towards the production of enantiomerically enriched compounds [16]. As shown in recent examples, whole-cell microorganisms have the reservoir of various oxidoreductive enzymes with proper co-factor regenerating systems, and can be applied to wide range of substrates [17]. Once the desirable results in regard to the stereochemistry of the products and the catalytic activity are obtained, gene and protein engineering approaches enable large-scale production of the enzymes for the specific purposes [18,19].

In this context, the reduction of **2a** has a long history. Incubation with whole-cell *Saccharomyces cerevisiae* (bakers' yeast) [20,21] furnishes (1*R*,2*S*)-**1a** as the major isomer with 86% ee in 65% isolated yield [6]. Buison and Azerad examined many kinds of incubated whole-cell microorganisms to reduce **2a**, and found that *Mucor racemosus*, *M. circinelloides*, and *Colletotrichum gloeosporoides* exhibit high diastereoselectivity [22]. The former two furnish *syn*- (1*R*,2*S*)-**1a** with 96–97% ee, while the latter strain furnishes *anti*- (1*S*,2*S*)-**1a** with >99.9% ee. However, (1*S*,2*R*)-**1a** with high ee has so far been unattainable. Later, an isolated *S. cerevisiae* carbonyl reductase was overexpressed in *Escherichia coli*, and the ee of (1*S*,2*R*)-**1a** was 80%, although the ratio of the *syn*-isomer was

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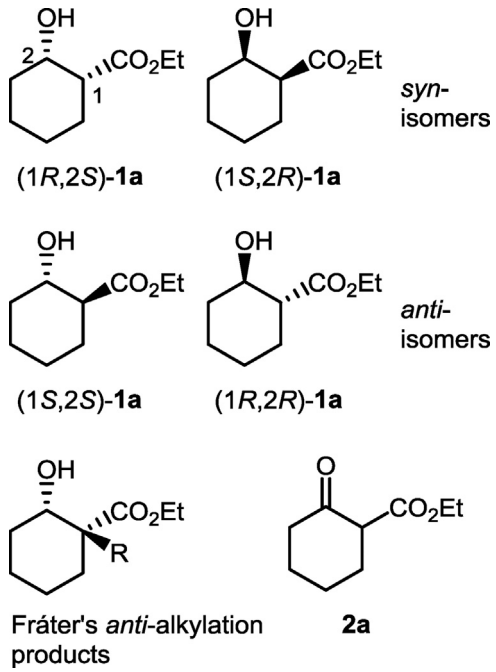


Fig. 1. Stereoisomers of ethyl 2-hydroxycyclohexanecarboxylate **1a**, the related alkylation product, and the corresponding β-oxoester **2a**, the reduction precursor.

quite high [23]. It has also been reported that the reduction of **2a** with an enzyme from a recombinant ketoreductase library, KRED-129, furnishes (1*S*,2*R*)-**1a** at 90% ee, but only at an analytical scale [24].

In this study, we performed biocatalytic asymmetric reduction by incubation with several whole-cell yeast strains [25–28] and with a commercially available carbonyl reductase screening kit, Daicel Chiralscreen® OH [29,30], to evaluate selectivity in the reduction and to find a chemo-enzymatic route to the (1*S*,2*R*)-stereoisomer.

2. Experimental

IR spectra were measured on a Jeol FT-IR SPX60 spectrometer. ^{1}H NMR spectra were measured in $CDCl_3$ at 400 MHz on a VARIAN 400-MR spectrometer or at 500 MHz on a VARIAN 500-MR spectrometer. ^{13}C NMR spectra were measured in $CDCl_3$ at 100 MHz on a VARIAN 400-MR spectrometer or at 125 MHz on a VARIAN 500-MR spectrometer. HPLC data were recorded on SHIMADZU SPD-M20A multi-channel detector. Optical rotation values were recorded on a Jasco P-1010 polarimeter. High resolution mass spectra (HRMS) were recorded on JEOL JMS-T100LP AccuTOF. TLC analysis and preparative TLC purification were performed with Merck Silica Gel 60 F_{254} plates (0.25 mm thickness, No. 5715 and 0.5 mm thickness, No. 5744), respectively. Silica gel 60 N (spherical, neutral, 63–210 μm, 37565-84) from Kanto Chemical Co. was used for column chromatography. Peptone and yeast extract were purchased from Kyokuto Pharmaceutical Co., for the cultivation of microorganism. Yeast strains are available at Japan Collection of Microorganisms; Riken Bioresource Center, Planning Section, Research Promotion Division, RIKEN Tsukuba Institute, 3-1-1 Koyadai, Tsukuba, Ibaraki 305-0074, Japan, and to NITE Biological Resource Center; Department of Biotechnology, National Institute of Technology and Evaluation, 2–5-8 Kazusakamatari, Kisarazu-shi, Chiba 292-0818, Japan. Primary kit of Chiralscreen® OH was purchased from Daicel Corp.

2.1. Ethyl (1R*,2R*)- and (1R*,2S*)-2-hydroxycyclohexanecarboxylate (**1a**), and (1R*,2R*)- and (1R*,2S*)-2-hydroxycyclohexanemethanol

To a solution of **2a** (197 mg, 1.16 mmol) in EtOH (6.5 mL) was added $NaBH_4$ (159 mg, 4.21 mmol) at 0 °C. The mixture was stirred for 1 h at that temperature. The reaction was quenched with cold aqueous 10% AcOH solution and the organic materials were extracted with AcOEt. The organic layer was washed with brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (750 mg). Elution with hexane/AcOEt (10:1) afforded *syn*-**1a** (12.1 mg, 6.1%) and *anti*-**1a** (20.2 mg, 10.1%) as colorless oil. For (1*R**,2*S**)-**1a** (*syn*-isomer): ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.24-1.36 (m, 5H), 1.39-1.51 (m, 2H), 1.64-1.75 (m, 2H), 1.83-2.01 (m, 2H), 2.47 (ddd, J = 2.7, 3.8, 11.2 Hz, 1H), 4.09-4.21 (m, 3H). For (1*R**,2*R**)-**1a** (*anti*-isomer): ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.18-1.40 (m, 7H), 1.69-1.79 (m, 2H), 1.99-2.07 (m, 2H), 2.24 (ddd, J = 3.7, 9.8, 12.3 Hz, 1H), 3.76 (ddd, J = 4.5, 9.8, 9.8 Hz, 1H), 4.17 (q, J = 7.0 Hz, 2H). There were unidentified signals: δ 3.70-4.04 in the ^{1}H NMR spectrum of crude product. Based on the benzoylation in the next step, those were assumed to be the contaminated diols caused by overreduction.

2.2. Ethyl (1R*,2R*)- and (1R*,2S*)-2-benzoyloxycyclohexanecarboxylate (**3a**), and (1R*,2R*)- and (1R*,2S*)-2-benzoyloxymethylcyclohexyl benzoate

To a solution of **1a** (12.1 mg, 7.03 μmol) in anhydrous pyridine (0.5 mL) was added benzoyl chloride (25 μL, 0.215 mmol) and catalytic amount of 4-(*N*,*N*-dimethylamino)pyridine (DMAP). The mixture was stirred for 4 h at room temperature. The reaction was quenched by the addition of saturated aqueous NH_4Cl solution and the organic materials were extracted with AcOEt. The 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 = 5:1) to give **3a** (8.5 mg, 43.6%) as colorless oil. For (1*R**,2*S**)-**3a** (*syn*-isomer): ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.11 (t, J = 7.1 Hz, 1H), 1.55-1.62 (m, 4H), 1.87-2.04 (m, 3H), 2.15-2.19 (m, 1H), 2.63 (ddd, J = 3.0, 4.6, 11.4 Hz, 1H), 4.00-4.14 (m, 2H), 5.64 (br, 1H), 7.39-7.46 (m, 2H), 7.52-7.57 (m, 1H), 8.00-8.03 (m, 2H). HPLC [column, Daicel CHIRALPAK® ID, 0.46 cm x 25 cm; hexane/*i*-PrOH = 85:15, flow rate 0.5 mL/min, detected at 237 nm], t_R (min) = 13.6, 14.4. In the ^{1}H NMR spectrum and HPLC chart, due to *cis*-diol dibenzoate, extra signals (δ 4.21 (dd, J = 8.2, 11.0 Hz, 1H), 4.34 (dd, J = 6.6 11.0 Hz, 1H), 5.49 (br, 1H)) and an extra inseparable peak (t_R = 18.8) appeared, respectively. Calculated from ^{1}H NMR spectrum, this product was contaminated with *cis*-diol dibenzoate (*ca.* 25%). For (1*R**,2*R**)-**3a** (*anti*-isomer): ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.12 (t, J = 7.1 Hz, 3H), 1.25-1.35 (m, 1H), 1.38-1.53 (m, 2H), 1.57-1.67 (m, 1H), 1.74-1.79 (m, 1H), 1.80-1.84 (m, 1H), 2.03-2.07 (m, 1H), 2.21-2.25 (m, 1H), 2.66 (ddd, J = 3.9, 10.3, 12.0 Hz, 1H), 4.02-4.11 (m, 2H), 5.21 (ddd, J = 4.4, 10.3, 10.3 Hz, 1H), 7.41-7.47 (m, 2H), 7.52-7.56 (m, 1H), 7.99-8.02 (m, 2H). HPLC analysis in the same conditions for *syn*-isomer, t_R (min) = 16.5, 17.9. In the ^{1}H NMR spectrum and HPLC chart, due to *anti*-diol dibenzoate, extra signals (δ 4.25 (dd, J = 6.0, 11.0 Hz, 1H) 4.45 (dd, J = 4.1, 11.0 Hz, 1H), 5.01 (ddd, J = 4.6, 10.1, 10.1 Hz, 1H)) and two peaks (t_R = 18.8, 21.6) appeared, respectively. Calculated from ^{1}H NMR spectrum, this product was contaminated with *anti*-diol dibenzoate (*ca.* 25%). The relationships between the retention time in HPLC analysis of each peak and stereoisomer of **3a** was assigned as follows, taking the results in Sections 2.3, 2.6, and with an authentic sample made by bakers' yeast-catalyzed reduction into account; 13.6 for (1*R*,2*S*)-**3a**, 14.4 for (1*S*,2*R*)-**3a**, 16.5 for (1*S*,2*S*)-**3a**, 17.9 for (1*R*,2*R*)-**3a**.

Table 1
Reduction of **2a** by incubation with whole-cell yeasts.[a]

yeast	strain No.	substrate.	isomeric ratio of the products			
			1*R*,2*S*	1*S*,2*R*	1*S*,2*S*	1*R*,2*R*
T. delbrueckii	NBRC 10921	**2a**	73.9	7.2	12.1	6.8
C. floricola	JCM 9439	**2a**	92.7	7.3	0.0	0.0
W. californica	JCM 3600	**2a**	16.6	2.4	81.0	0.0
W. californica	JCM 3600	**2b**	41.0	0.0	59.0	0.0

[a] Determined by ^{1}H NMR and HPLC analyses. For detail, see Sections 2.3 and 2.9.

2.3. Treatment of ***2a*** with incubated whole-cell yeast strains

According to the reported procedure [25–28], ten strains of yeasts from stock culture samples were incubated in glucose medium, and wet cells (ca. 3 g) were re-suspended in phosphate buffer (0.1 M, pH 6.5, 10 mL) in a test tube (2.5 cm ϕ x 20 cm). To each broth was added **2a** (50 mg, 0.294 mmol) and glucose (500 mg). The progress of the reduction was monitored by a TLC analysis: R_f for *anti*-**1a**: 0.10; *syn*-**1a**: 0.23 (silica gel, developed with hexane/AcOEt = 5:1), and we chose three strains (see text) for further analysis. Extractive workup of each three mixture was carried out in a similar manner to our previous examples. Ratio between *syn*-**1a** and *anti*-**1a** in the crude extract was estimated by comparing the area of signals in ^{1}H NMR spectrum: δ 2.45 (for *syn*-**1a**) and 2.24 (for *anti*-**1a**) of this crude product. For the determination of the product rate between each isomer of **1a**, the residue was treated with benzoyl chloride and DMAP in pyridine in the similar manner mentioned in Section 2.2 without further purification to give **3a**. The materials were analyzed by HPLC under the conditions as described in Section 2.2. The results were summarized in Table 1.

2.4. Ethyl (1*S*,2*S*)-2-hydroxycyclohexanecarboxylate (***1a***)

W. californica JCM 3600 was incubated in the medium (600 mL) as described above in six 500 mL baffled Erlenmeyer cultivating flasks. The flasks were shaken on an incubator shaker (180 rpm) for 48 h at 30 °C. The wet cells were harvested by centrifugation (3000 rpm x 10 min). The weight of combined wet cells was *ca.* 22.4 g from 600 mL of the broth. The whole of harvested cells was re-suspended with phosphate buffer solution (pH 6.5, 0.1 M, 130 mL). To the mixture was added **2a** (660 mg, 3.88 mmol) and glucose (2.7 g) and the resulting mixture was vigorously stirred for 48 h at 30 °C. The broth was centrifuged (3000 rpm x 10 min). The supernatant was saturated with NaCl and extracted with AcOEt three times. The precipitated cells were extracted with acetone. The mixture was filtered through a pad of Celite, and the filtrate was concentrated in vacuo. The reside was then extracted with AcOEt twice. The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (10.0 g). Elution with hexane/AcOEt (10:1) furnished (1*S*,2*S*)-**1a** (341 mg, 51.0%). $[\alpha]_D^{21.6}$ +47.4 (*c* 1.00, $CHCl_3$). Its ^{1}H NMR spectrum was identical with that for authentic sample of *anti*-**1a**. ^{13}C NMR (100 M Hz, $CDCl_3$): δ 14.1, 24.3, 24.9, 28.1, 33.6, 51.3, 60.5, 70.8, 175.2; IR: 867, 987, 1035, 1060, 1124, 1180, 1249, 1322, 1373, 1448, 1729, 2352, 2858, 2933, 2981, 3452 cm^{-1}. The ^{1}H NMR spectrum of the corresponding benzoate (1*S*,2*S*)-**3a** was identical with that for authentic sample of *anti*-**3a**. HPLC analysis under the same conditions in Section 2.2, t_R (min) = 16.5 [(1*S*,2*S*)-**3a**, >99.9%]

2.5. Ethyl (1*S*,2*S*)-2-(4-methylbenzenesulfonyl)cyclohexanecarboxylate (***3b***)

To a solution of (1*S*,2*S*)-**1a** (45.8 mg, 0.266 mmol) in anhydrous pyridine (0.9 mL) was added *p*-toluenesulfonyl chloride (252 mg, 1.32 mmol). The mixture was stirred for 3.5 h at 60 °C. The reaction was quenched by the addition of 2 M hydrochloric acid and the organic materials were extracted with AcOEt. The organic layer was washed with brine, dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give (1*S*,2*S*)-**3b** (81.6 mg, 94.0%) as yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.17 (t, *J* = 7.1 Hz, 3H), 1.19–1.27 (m, 1H), 1.29–1.38 (m, 1H), 1.47–1.58 (m, 2H), 1.59-1.64 (m, 1H), 1.72–1.76 (m, 1H), 1.91–1.95 (m, 1H), 2.14–2.19 (m, 1H), 2.44 (s, 3H), 2.52 (ddd, *J* = 4.1, 9.3, 11.2 Hz, 1H), 3.89–4.01 (m, 2H), 4.78 (ddd, *J* = 4.4, 9.8, 9.8 Hz, 1H), 7.31 (dd, *J* = 1.7, 6.6 Hz, 2H), 7.76 (dd, *J* = 1.7, 6.6 Hz, 2H); ^{13}C NMR (125 M Hz, $CDCl_3$): δ 14.0, 21.6, 23.6, 23.8, 28.1, 31.6, 48.7, 60.6, 82.0, 127.6 (x2), 127.8 (x2), 129.3, 144.4, 172.6; IR: 786, 811, 879, 948, 1172, 1267, 1361, 1729, 2360, 2863, 2940 cm^{-1}.

2.6. Ethyl (1*S*,2R)-2-hydroxycyclohexanecarboxylate (***1a***)

To a solution of (1*S*,2*S*)-**3b** (30.3 mg, 0.0928 mmol) in dimethyl sulfoxide (DMSO) (0.5 mL) was added KNO_2 (128 mg, 1.51 mmol) and 18-crown-6 (54.2 mg, 0.205 mmol). The mixture was stirred for 7 h at 90 °C. The reaction was quenched by the addition of phosphate buffer (0.2 M, pH 7.0) and the organic materials were extracted with AcOEt five times. The organic layer was washed with brine, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The crude material contained (1*S*,2*R*)-**1a**, judged from its ^{1}H NMR spectrum: δ 2.47 for *syn*-**1a**. According to the method mentioned above, a small portion of this sample was treated with benzoyl chloride and DMAP in pyridine to give (1*S*,2*R*)-**3a**. Its ^{1}H NMR spectrum was identical with that for authentic sample of *syn*-**3a.** HPLC analysis under the same conditions in Section 2.2, t_R (min) = 14.4 [(1*S*,2*R*)-**3a**, >99.9%].

2.7. Benzyl 2-oxocyclohexanecarboxylate (***2b***)

To a mixture of NaH (60% oil dispersion, 737 mg, 18.4 mmol) and dibenzyl carbonate (2.1 mL, 10.1 mmol) in anhydrous THF (10 mL) was added cyclohexanone (**4**, 0.5 mL, 5.0 mmol) dropwise over a period of 1 h. After 2 min followed by the initiation of the addition of cyclohexanone, catalytic amount of KH (30% oil dispersion) was added. The mixture was stirred for 2 h under reflux. The reaction was quenched by the addition of 2 M hydrochloric acid at 0 °C, and the organic materials were extracted with AcOEt three times. The organic layer was washed with brine, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (50 g). Elution with toluene/AcOEt (8:1) furnished **2b** (923 mg, 79.6%). For enol form of **2b**: ^{1}H NMR (400 MHz, $CDCl_3$): δ 1,57–1.64 (m, 2H), 1.65–1.72 (m, 2H), 2.26–2.29 (m, 4H), 5.21 (s, 2H), 7.31–7.34 (m, 5H), 12.1 (s, 1H). For keto form of **2b**: 1.58–1.71 (m, 2H), 1.77–1.90 (m, 2H), 1.94–2.00 (m, 1H), 2.10–2.20 (m, 1H), 2.32–2.40 (m, 1H), 2.46–2.54 (m, 1H), 3.43 (ddd, *J* = 1.2, 6.1, 9.6 Hz, 1H), 5.17 (d, *J* = 12.3 Hz, 1H), 5.22 (d, *J* = 12.3 Hz, 1H), 7.31–7.34 (m, 5H). The ratio between enol and keto forms was 5:4, judged from the area of signals in ^{1}H NMR spectrum: δ 12.1 (for enol form) and 3.43 (for keto form).

2.8. Benzyl (1R*,2R*)- and (1R*,2S*)-2-hydroxycyclohexanecarboxylate (***1b***)

In a similar manner as described for the preparation of **1a**, oxoester **2b** (187 mg, 0.805 mmol) was reduced with $NaBH_4$ (15.9 mg, 0.420 mmol) in EtOH (2.6 mL) at 0 °C for 1 h. The prod-

Table 2
Reduction of **2b** with carbonyl reductases from Chiralscreen® OH.[a]

enzyme No.	conv. (%)	isomeric ratio of **1b**			
		1*R*,2*S*	1*S*,2*R*	1*S*,2*S*	1*R*,2*R*
E007	58.2	37.2	62.8	0.0	0.0
E031	94.3	97.0	3.0	0.0	0.0
E078	54.1	96.9	3.1	0.0	0.0

[a] Determined by ^{1}H NMR and HPLC analyses. For detail, see Section 2.10.

uct was recovered in a conventional manner. The residue was purified by silica gel column chromatography (5.0 g). Elution with hexane/AcOEt (10:1) furnished *syn*-**1b** (65.5 mg, 34.7%) and *anti*-**1b** (56.0 mg, 29.7%) as colorless oil. For (1*R**,2*S**)-**1b** (*syn*-isomer): ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.24–1.37 (m, 1H), 1.39–1.51 (m, 2H), 1.63-1.78 (m, 3H), 1.83–1.97 (m, 2H), 2.54 (ddd, J = 2.7, 3.9, 11.2 Hz, 1H), 4.16–4.18 (m, 1H), 5.15 (s, 2H), 7.30–7.39 (m, 5H). HPLC [column, Daicel CHIRALPAK® ID, 0.46 cm x 25 cm; hexane/*i*-PrOH = 90:10, flow rate 0.5 mL/min, detected at 220 nm], t_R (min) = 19.2, 20.5. For (1*R**,2*R**)-**1b** (*anti*-isomer): ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.15-1.43 (m, 4H), 1.69-1.78 (m, 2H), 2.00-2.09 (m, 2H), 2.32 (ddd, J = 3.7, 10.0, 12.3 Hz, 1H), 3.79 (ddd, J = 4.5, 10.0, 10.0 Hz, 1H), 5.15 (d, J = 12.6 Hz, 1H), 5.18 (d, J = 12.6 Hz, 1H), 7.30–7.39 (m, 5H). HPLC analysis in the same conditions for *syn*-isomer, t_R (min) = 25.1, 32.4. The relationships between the retention time in HPLC analysis of each peak and stereoisomer of **1b** was assigned as follows, taking the results in Sections 2.2, 2.6 (ethyl ester), 2.9, and 2.12 (benzyl ester) into account; 19.2 for (1*R*,2*S*)-**1b**, 20.5 for (1*S*,2*R*)-**1b**, 25.1 for (1*S*,2*S*)-**1b**, 32.4 for (1*R*,2*R*)-**1b**.

2.9. Benzyl (1*S*,2*S*)-2-hydroxycyclohexanecarboxylate (**1b**)

The harvested wet cells of *W. californica* (38.7 g) were re-suspended with phosphate buffer solution (pH 6.5, 0.1 M, 200 mL). To the mixture was added a solution of **2b** (516 mg, 2.22 mmol) in DMSO (4.0 mL) and glucose (2.0 g) and the resulting mixture was vigorously stirred for 19 h at 30 °C. After similar workup with the reduction of **2a**, the residue was purified by silica gel column chromatography (50.0 g). Elution with hexane/AcOEt (10:1) furnished (1*S*,2*S*)-**1b** (249 mg, 47.9%). $[\alpha]_D^{21.8}$ +37.9 (c 1.01, $CHCl_3$). Its ^{1}H NMR spectrum was identical with that for authentic sample of *anti*-**1b**. ^{13}C NMR (100 M Hz, $CDCl_3$): δ 24.3, 24.9, 28.1, 33.6, 51.3, 66.3, 70.9, 127.9 (x2), 128.2, 128.6 (x2), 135.8, 175.0; IR: 742, 1012, 1060, 1261, 1448, 1716, 2358, 2858, 2939, 3399 cm^{-1}; HRMS (ESI +): m/z calcd. for $C_{14}H_{18}O_3Na$ [(M + Na)+] 257.1154, found 257.1195. HPLC analysis under the same conditions in Section 2.6, t_R (min) = 24.5 [(1*S*,2*S*)-**1b**, >99.9%].

2.10. Treatment of **2b** with carbonyl reductases in the primary kit of Chiralscreen® OH

According to the reported procedure [30], oxoester **2b** (56.6 mg) was dissolved in 2-propanol (285 μL) and each 50 μL of the solution was added to each reaction vessel of pre-adjusted reaction mixture of Chiralscreen® OH (E001, E007, E031, E039 and E078) involved in the primary kit. The mixture was stirred at 30 °C for 24 h. Then, AcOEt was added to each vial and stirred very well. The progress of the reduction was roughly estimated by a TLC analysis: R_f for *syn*-**1b**: 0.37; *anti*-**1b**: 0.27 (developed with hexane/AcOEt = 4:1). Except for the batch to which E001 and E039 were applied (no progress), the product was analyzed by HPLC as in Section 2.8, without any purification. The results were summarized in Table 2.

2.11. Benzyl (1*S*,2*S*)-2-(4-methylbenzenesulfonyl)oxycyclohexanecarboxylate (**5**)

To a solution of (1*S*,2*S*)-**1b** (162 mg, 0.691 mmol) in anhydrous pyridine (2.3 mL) was added *p*-toluenesulfonyl chloride (663 mg, 3.48 mmol) and DMAP (9.0 mg, 73.6 μmol). The mixture was stirred for 4 h at 70 °C. The reaction was quenched by the addition of 2 M hydrochloric acid and the organic materials were extracted with AcOEt. The organic layer was washed with brine, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (20.0 g). Elution with hexane/AcOEt (30:1) furnished (1*S*,2*S*)-**5** (233 mg, 86.8%) as yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.17–1.39 (m, 2H), 1.47–1.64 (m, 3H), 1.73–1.76 (m, 1H), 1.93–1.96 (m, 1H), 2.16–2.21 (m, 1H), 2.42 (s, 3H), 2.60 (ddd, J = 3.7, 9.8, 10.0 Hz, 1H), 4.80 (ddd, J = 4.3, 9.8, 9.8 Hz, 1H), 4.89 (d, J = 12.3 Hz, 1H), 4.97 (d, J = 12.3 Hz, 1H), 7.25-7.29 (m, 5H), 7.31–7.37 (m, 2H), 7.74 (d, J = 8.1 Hz, 2H); ^{13}C NMR (125 M Hz, $CDCl_3$): δ 21.5, 23.3, 23.6, 28.0, 31.7, 48.6, 66.3, 81.3, 127.7 (x2), 127.9 (x2), 128.1, 128.4 (x2), 129.5 (x2), 134.2, 135.6, 144.3 172.4; IR: 663, 744, 806, 875, 917, 973, 1018, 1099, 1174, 1249, 1355, 1461, 1598, 1735, 2360, 2865, 2940 cm^{-1}.

2.12. Benzyl (1*S*,2R)-2-hydroxycyclohexanecarboxylate (1b)

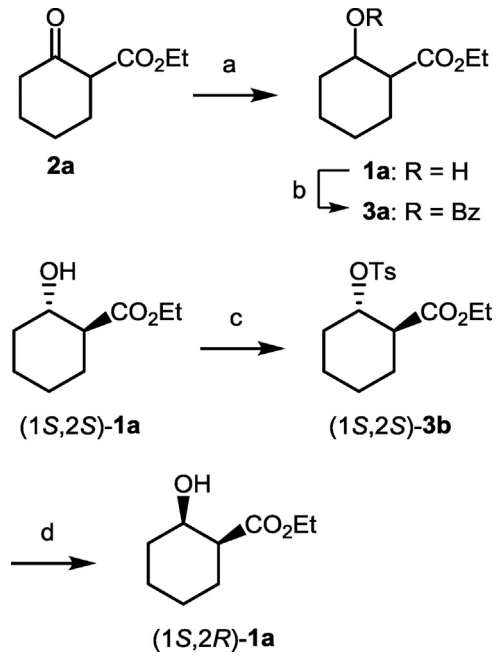
To a solution of (1*S*,2*S*)-**5** (108 mg, 0.278 mmol) in toluene (0.5 mL) was added tetrabutylammonium nitrite (429 mg, 1.49 mmol). The mixture was stirred for 48 h at 60 °C. The reaction was quenched by the addition of brine and the organic materials were extracted with AcOEt. The organic layer was washed with brine, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (10.0 g). Elution with hexane/AcOEt (10:1) furnished a yellow oil. Further purification with preparative TLC (developed with hexane/AcOEt = 5:1) yielded pure (1*S*,2*R*)-**1b** (32.5 mg, 49.9%) as a yellow oil. $[\alpha]_D^{22.1}$ −15.8 (c 1.00, $CHCl_3$). Its ^{1}H NMR spectrum was identical with that for authentic sample of *syn*-**1b**. ^{13}C NMR (100 M Hz, $CDCl_3$): δ 20.0, 23.8, 24.7, 31.7, 46.7, 66.3, 66.6, 128.0 (x2), 128.3, 128.6 (x2), 135.7, 175.6; IR: 734, 973, 1027, 1126, 1162, 1249, 1450, 1720, 2362, 2937, 3455 cm^{-1}; HRMS (ESI +): m/z calcd. for $C_{14}H_{18}O_3Na$ [(M + Na) +] 257.1154, found 257.1189. HPLC analysis under the same conditions in Section 2.8, t_R (min) = 20.5 [(1*S*,2*R*)-**1b**, >99.9%].

3. Results and discussion

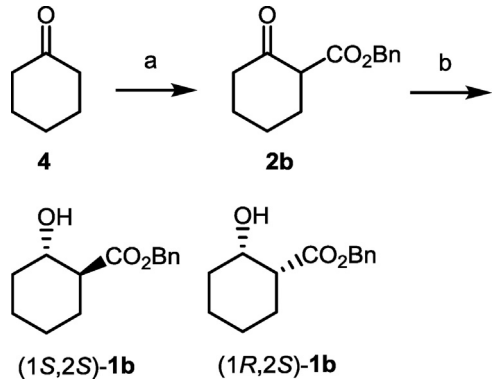
First, by incubating **2a** with ten strains of whole-cell yeasts (Section 2.3) as shown in Scheme 1, *Torulaspora delbruekii* NBRC 10921, *Williopsis californica* JCM 3600, and *Candida floricola* JCM 9439 were chosen based on the progress of reduction by TLC analysis.

The ratio of stereoisomers was determined by combining ^{1}H NMR analysis of the crude product and HPLC analysis after derivation to the corresponding benzoate **3a**, and the results are summarized in Table 1. In contrast to the other two strains, *W. californica* furnished *anti*-isomers as the major product, comparable to that reported for *C. gloeosporoides* [22].

We became interested in *W. californica*, because the combination of chemical transformation and the reduction product would furnish (1*S*,2*R*)-**1a** as shown in Scheme 1. Then the reduction at millimolar scale was attempted with *W. californica* several times. Although it always exhibited high ee for the *anti*-isomer form, the isolated yield of the products and the ratio between diastereomers often fluctuated. Moreover, when attempting stereochemical inversion at C-2 via the corresponding tosylate **3b**, (1*S*,2*R*)-**1a** was obtained at up to 75% yield, but the reproducibility



Scheme 1. Asymmetric reduction of β-oxoester **2a**, derivation of the product to benzoate **3a**, and the attempted stereochemical inversion. Reagents and conditions: (a) incubation by whole-cell yeast strains, (b) benzoyl chloride, 4-(*N*,*N*-dimethylamino)pyridine (DMAP), pyridine, (c) *p*-toluenesulfonyl chloride, pyridine, and (d) KNO_2, 18-crown-6, $(n\text{-}Bu)_4NNO_2$, dimethyl sulfoxide (DMSO), 90 °C.



Scheme 2. Preparation and incubation with whole-cell of *Williopsis californica* JCM 3600 to catalyze the asymmetric reduction of **2b**. Reagents and conditions: (a) NaH, KH, dibenzyl carbonate, THF and (b) *W. californica*.

of the yield was low. This was probably due to the high volatility of the products caused by intramolecular hydrogen bonding of **1a**.

We thus thought that lower volatility and increased hydrophobicity would facilitate the isolation of the reduction product from the incubation broth. Our previous experience showing that *W. californica* accepts bulky aromatic ketones [26,28] prompted us to combine this yeast strain with a substrate possessing a benzene ring. Then benzyl ester **2b** was synthesized and was incubated with *W. californica* as shown in Scheme 2. In the same manner as ethyl ester **2a**, (1*S*,2*S*)-**1b** (*anti*-isomer, >99.9% ee) in 51.0% yield and (1*R*,2*S*)-**1b** (*syn*-isomer, >99.9% ee) in 35.4% yield were obtained (Table 1). When the reaction was scaled-up to over 2 mmol of substrate, *anti*-isomer, (1*S*,2*S*)-**1b** (>99.9% ee) was isolated in 47.9% yield. The reduction, however, took longer time compared with the reduction of ethyl ester, probably due to the increase of hydrophobicity of the substrate. Then, DMSO was added as a co-solvent which enhanced the solubility of the substrate. The reaction time until the disappearance of the substrate could be shortened from several days to 18 h when applying the same wet weight of incubation cell mass per substrate.

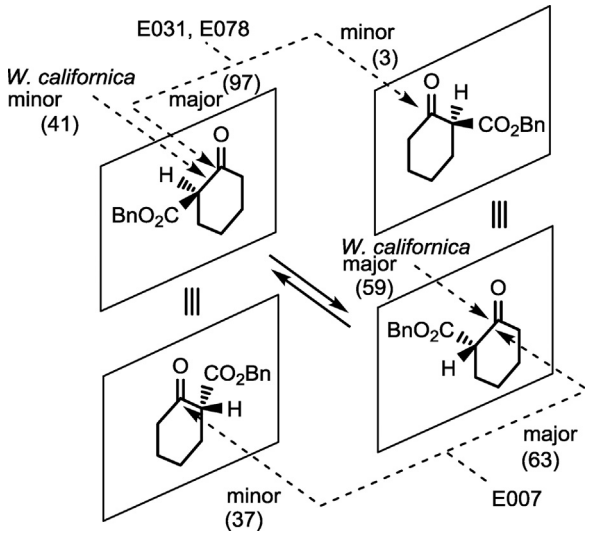


Fig. 2. Stereochemical preference in the attack of "formal hydride" with carbonyl reductases contained in *W. californica* and Chiralscreen® OH.

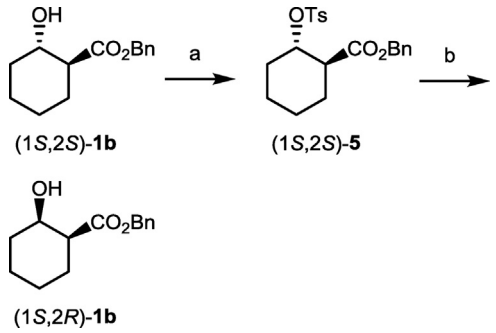
Recently we examined the reactivity and selectivity of carbonyl reductases using the primary kit of Chiralscreen® OH on ethyl 2-oxocyclopentanecarboxylate, which is an analogous five-membered ring substrate [31]. Among them, E039 preferred the formation of (1*S*,2*R*)-alcohol, although the conversion was as low as 21%. This result prompted us to reduce **2b** using these carbonyl reductases to compare with the use of whole-cell *W. californica*. In contrast to our previous results with five-membered ring substrate, two of the five enzymes (E001, E039) did not show any reduction on the present six-membered benzyl ester **2b**. As shown in Table 2, the other three reductases showed exclusive preference for the *syn*-isomer. In E031 and E078, the ee of product **1b** was almost the same at 94%, slightly lower than that of the enantiomerically pure product from the five-membered substrate with the same enzymes. With E007, an opposite enantiofacial preference was shown compared to the other enzymes, but the facial recognition was low (37.2:62.8). Such looser facial recognition in E007 was also observed for the five-membered substrate [31].

In Fig. 2, the diverse facial selectivity of the carbonyl reductases contained in *W. californica* and E007, E031, and E078 of Chiralscreen® OH is summarized. The enzyme(s) from *W. californica* showed the highest facial selectivity, with the orientation of the substrate with the large substituent located in the left position [26]. Such preference was effective in yielding highly enantiomerically enriched products in both *anti*- (major) and *syn*- (minor) diastereomers.

As the highly enantiomerically enriched (1*S*,2*S*)-**1b** (>99.9% ee) was obtained by *W. californica* at more than millimole scale, it was transformed to (1*S*,2*R*)-**1b** in two steps as shown in Scheme 3. First, the free hydroxy group was tosylated to give **5**. Under initially attempted conditions with KNO_2 and crown ether in DMSO [32], the inverted product (1*S*,2*R*)-**1b** was obtained but was accompanied by the undesired elimination of tosylate. This side reaction could be completely suppressed by changing to the less basic tetra-*n*-butylammonium nitrite in toluene [33], and (1*S*,2*R*)-**1b** with >99.9% ee was obtained in 43.3% yield over two steps.

4. Conclusion

W. californica JCM 3600 was found to be a useful whole-cell biocatalyst for the asymmetric reduction of a cyclic β-oxoester **2b** with



Scheme 3. Stereochemical inversion from (1*S*,2*S*)-**1b** to (1*S*,2*R*)-**1b**. Reagents and conditions: (a) *p*-toluenesulfonyl chloride, DMAP, pyridine and (b) $(n\text{-Bu})_4NNO_2$, toluene, 60 °C.

lipophilic benzyl ester. A scaled-up reaction with over 2 mmol of substrate worked efficiently, and (1*S*,2*S*)-**1b** (>99.9% ee) was isolated in 47.9% yield. An advantage of the use of incubated whole-cell microorganisms was that the cell mass worked as an immobilized form of enzymes, and the removal of the enzymes from the reaction mixture at the step of workup was easier than that in the use of isolated enzymes. The (1*S*,2*S*)-product was transformed to (1*S*,2*R*)-**1b** (>99.9% ee), in 43.3% yield over two steps by stereochemical inversion at the C-2 position. Our present chemoenzymatic study enables the preparation of the (1*S*,2*R*)-*syn*-isomer, whose availability until now has been low.

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References

[1] A.G.M. Barrett, N.K. Capps, Tetrahedron Lett. 27 (1986) 5571.
[2] M. Hamdani, B. De Jeso, H. Delueze, B. Maillard, Res. Chem. Intermed. 19 (1993) 681.
[3] M. Panunzio, R. Camerini, A. Mazzoni, D. Donati, C. Marchioro, R. Pachera, Tetrahedron: Asymmetry 8 (1997) 15.
[4] B. Bullbuck, W. Tochtermann, Tetrahedron 55 (1999) 7191.
[5] S. Furegati, W. Ganci, G. Przibille, P. Rüedi, Helv. Chim. Acta 81 (1998) 1127.
[6] G. Fráter, Helv. Chim. Acta 63 (1980) 1383.
[7] E.R. Alonso, K.A. Tehrani, M. Boelens, N. De Kimpe, Synlett (2005) 1726.
[8] Z.-F. Xie, I. Nakamura, H. Suemune, K. Sakai, J. Chem. Soc. Chem. Commun. (1988) 966.
[9] L.M. Levy, J.R. Dehli, V. Gotor, Tetrahedron: Asymmetry 14 (2003) 2053.
[10] R. Noyori, M. Tokunaga, M. Kitamura, Bull. Chem. Soc. Jpn. 68 (1995) 36.
[11] A.H. Kamaruddin, M.H. Uzir, H. Aboul-Enain, H.N.A. Halim, Chirality 21 (2009) 449.
[12] G.A. Applegate, D.B. Berlpwotz, Adv. Synth. Catal. 357 (2015) 1619.
[13] M. Kitamura, T. Ohkuma, M. Tokunaga, R. Noyori, Tetrahedron: Asymmetry 1 (1990) 1.
[14] S. Zeror, J. Collin, J.-C. Fiaud, L.A. Zouioueche, Tetrahedron: Asymmetry 21 (2010) 1211.
[15] J.P. Gênet, X. Pfister, V. Ratovelomanana-Vidal, C. Pinel, J.A. Laffitte, Tetrahedron Lett. 35 (1994) 4559.
[16] T. Matsuda, R. Yamanaka, K. Nakamura, Tetrahedron: Asymmetry 20 (2009) 513.
[17] A. Chadha, S. Venkataraman, R. Preetha, S.K. Padhi, Bioorg. Chem. 68 (2016) 187.
[18] R. Kratzer, J.M. Woodley, B. Nidetzky, Biotechnol. Adv. 33 (2015) 1641.
[19] N. Ito, Appl. Microbiol. Biotechnol. 98 (2014) 3889.
[20] B.S. Deol, D.D. Ridley, G.W. Simpson, Aust. J. Chem. 29 (1976) 2459.
[21] D. Seebach, S. Roggo, T. Maetzke, Helv. Chim. Acta 70 (1987) 1605.
[22] D. Buisson, R. Azerad, Tetrahedron Lett. 27 (1986) 2631.
[23] S.K. Padhi, I.A. Kaluzna, D. Buisson, R. Azerad, J.D. Stewart, Tetrahedron: Asymmetry 18 (2007) 2133.
[24] D. Zhu, C. Mukherjee, J.D. Dozzell, S. Kambourakis, L. Hua, Tetrahedron 62 (2006) 901.
[25] M. Furuta, M. Shoji, T. Sugai, J. Mol. Catal. B: Enz. 82 (2012) 8.
[26] S. Taketomi, M. Asano, T. Higashi, M. Shoji, T. Sugai, J. Mol. Catal. B: Enz. 84 (2012) 83.
[27] D. Tokoshima, K. Hanaya, M. Shoji, T. Sugai, J. Mol. Catal. B: Enz. 97 (2013) 95.
[28] K. Asami, T. Machida, S. Jung, K. Hanaya, M. Shoji, T. Sugai, J. Mol. Catal. B: Enz. 97 (2013) 106.
[29] M. Hayashi, Yukigosei kagaku kyokaishi, J. Synth. Org. Chem. Jpn. 69 (2011) 517.
[30] T. Nagai, S. Sakurai, N. Natori, M. Hataoka, T. Kinoshita, H. Inoue, K. Hanaya, M. Shoji, T. Sugai, Bioorg. Med. Chem. 25 (2017), http://dx.doi.org/10.1016/j.bmc.2017.03.067, published on-line.
[31] K. Kuwata, K. Hanaya, T. Sugai, M. Shoji, Tetrahedron: Asymmetry 28 (2017) 964.
[32] J.J. Shie, J.-M. Fang, S.-Y. Wang, K.-C. Tsai, Y.-S.E. Cheng, A.-S. Yang, S.-C. Hsiao, C.-Y. Su, C.-H. Wong, J. Am. Chem. Soc. 129 (2007) 11892.
[33] B. Raduchel, Synthesis (1980) 292.