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Chemo-enzymatic route for (*R*)-terbutaline hydrochloride based on microbial asymmetric reduction of a substituted α-chloroacetophenone derivative

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ABSTRACT

To synthesize (*R*)-terbutaline hydrochloride, a potent β_2-adrenoceptor-stimulating agent, asymmetric reduction of a substituted α-chloroacetophenone derivative with cultured whole-cell biocatalyst of the yeast *Williopsis californica* JCM 3600 was developed as the key reaction. The reduction proceeded by a *si*-facial attack of hydride in a highly enantioselective manner. Co-factor generation was enhanced by applying glycerol as the carbon source.

1. Introduction

Among a number of aromatic aminoalcohols bearing phenolic hydroxy groups, terbutaline (**1a**, as hydrochloride salt) has been developed as a potent β_2-adrenoceptor-stimulating agent [1,2]. The activity is predominantly ascribable to (*R*)-**1a**, while the (*S*)-isomer has been reported to show side effects, causing airway hyper-reactivity [1]. To date, much effort has been devoted to produce (*R*)-isomer, either by fractional crystallization after derivatization to a chiral carboxylate salt [2], or by enzyme-catalyzed asymmetric hydrocyanation of an aldehyde precursor [3]. Additionally, enantiomerically enriched forms of aromatic aminoalcohols have a wide variety of uses in synthetic organic chemistry, especially in asymmetric syntheses [4], so the development of new methods of preparation is important. We planned to synthesize (*R*)-**1a** by nucleophilic displacement of the halogen atom with *tert*-butylamine on chlorohydrin (*R*)-**3**, whose two phenolic hydroxy groups are protected as methoxymethyl (MOM) group. Protection of the hydroxy groups is necessary, to avoid the oxidation of resorcinol moiety in the step of the introduction of nitrogen atom. For this purpose, MOM group seems to be advantageous, which is cleavable under acidic conditions and enables simultaneous formation of the requisite hydrochloride salt in (*R*)-**1a**. The stereochemistry of the secondary alcohol involved in (*R*)-**3** would be established by microbial reduction of the corresponding ketone **2a** (Scheme 1).

2. Experimental

All mps are uncorrected. IR spectra were measured as films for oils or KBr disks of solids on a Jasco FT/IR-410 spectrometer, and as ATR on a Jeol FT-IR SPX60 spectrometer. ^{1}H NMR spectra were measured at 270 MHz on a Jeol JNM EX-270 or at 400 MHz on a VARIAN 400-MR spectrometer. ^{13}C NMR spectra were measured at 63 MHz on a Jeol JNM EX-270 or at 100 MHz on a VARIAN 400-MR or at 125 MHz on a VARIAN INOVA-500 spectrometer. HPLC data were recorded on Jasco MD-2010 or SHIMADZU SPD-20A multi-channel detectors. Optical rotation values were recorded on a Jasco P-1010 polarimeter. Merck silica gel 60 F_{254} thin-layer plate (1.05715, 0.25 mm thickness) was used for thin-layer chromatographic analysis. 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., Inc. were used for preparative thin-layer chromatography and column chromatography, respectively. Racemic terbutaline sulfate, 2-bromo-1-(3′,5′-dihydroxyphenyl)ethanone **4a** and 1-(3′,5′-diacetoxyphenyl)ethanone **5a** were purchased from Wako Pure Chemical Industries, Ltd. (No. 208-12033), ChemPacific Co. (No. 32927), and Tokyo Chemical Industry Co. Ltd. (No. D1978), respectively. Yeast strains are available from Japan Collection of Microorganisms; Riken Bioresource Center, Planning Section, Research Promotion Division, RIKEN Tsukuba Institute, Tsukuba, Ibaraki, Japan, and to NITE Biological Resource Center; Department of Biotechnology, National Institute of Technology and Evaluation, Kisarazu, Chiba Japan. Microorganisms were pre-incubated in common medium [peptone (2%), yeast extract (0.5%), KH_2PO_4 (0.3%), K_2HPO_4 (0.2%) and pH 6.5] with carbon sources as indicated in each section. Peptone, malt extract and yeast extract were purchased

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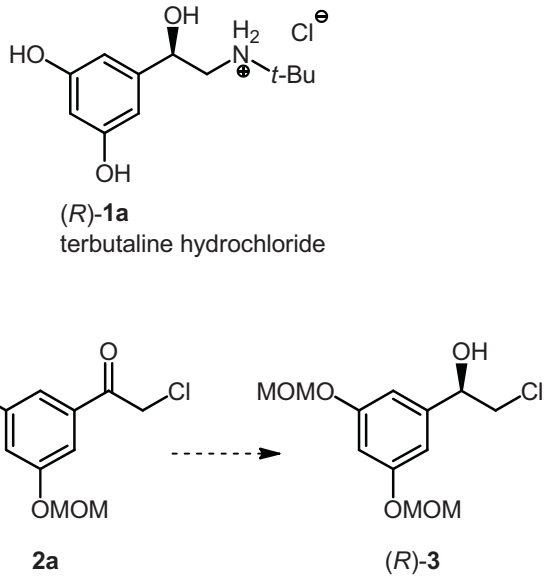


Scheme 1. Microbial asymmetric reduction of a substituted α-chloroacetophenone derivative **2a** toward (*R*)-terbutaline hydrochloride (**1a**).

from Kyokuto Pharmaceutical Industrial Co., Ltd. The growth was monitored by occasionally observing the OD (660 nm) of the broth on SP-300 spectrophotometer, Optima Inc., under suitably diluted conditions so that the OD was shown between 0.15 and 0.50. The ODs in the text and Sections 2.8 and 2.9 are the recalculated values.

2.1. 2-Chloro-1-[3′,5′-bis(methoxymethoxy)phenyl]ethanone (**2a**)

To a solution of **4a** (2.00 g, 8.7 mmol) in CH_3CN (86 mL) were added NaCl (2.78 g, 47.6 mmol) at room temperature. After cooling to 0 °C, MOMCl (1.58 mL, 21.7 mmol) and *i*-Pr_2NEt (4.08 mL, 23.4 mmol) were added dropwise to the mixture. The mixture was stirred overnight at room temperature. The reaction was quenched with phosphate buffer (pH 7.0, 0.5 M). The mixture was saturated with NaCl and 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 (100 g). Elution with hexane–AcOEt (5:1) afforded **2a** (1.96 g, 83%) as colorless needles, mp 47.0–47.5 °C. This was unstable upon contact with metals such as spatula, and was directly employed for the next step. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.48 (s, 6H, OCH_3), 4.65 (s, 2H, H2), 5.18 (s, 4H, CH_3O—CH_2—), 6.96 (t, $J_{2',4'}$ = 2.2 Hz, 1H, H4′), 7.24 (d, 2H, H2′,6′); IR: 666, 681, 692, 831, 922, 1040, 1088, 1150, 1324, 1453, 1606, 1707, 2826, 2937, 3079 cm^{-1}. When bromoketone **4b** was contaminated, a signal of δ 4.37 (s, 2H, H2) appeared in ^{1}H NMR spectrum.

2.2. 2-Bromo-1-(3′,5′-diacetoxyphenyl)ethanone (**5b**)

To a solution of **5a** (3.00 g, 12.7 mmol) in AcOH (10.4 mL) was added Br_2 (2.44 g, 15.3 mmol, 1.2 equiv.) in AcOH (3.4 mL) dropwise over 30 min at 20 °C. The mixture was stirred for 30 min. The reaction was quenched by the addition of ice and the mixture was extracted with AcOEt three times. The combined organic layer was washed successively with water twice, saturated aqueous $NaHCO_3$ solution, water, saturated aqueous $Na_2S_2O_3$ solution, and brine. The extract was dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The residual brown solid was recrystallized from ether to give **5b** (2.84 g, 71%) as slightly yellow needles. Further chromatographic purification of the concentrated mother liquor also provided the desired product (692 mg, 17%), and the combined yield was 88%. Mp 68.0–68.5 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.31 (s, 6H, Ac), 4.38 (s, 2H, H2), 7.18 (t, $J_{2',4'}$ = 2.2 Hz, 1H, H4′), 7.58 (d, 2H, H2′,6′). Calculated from ^{1}H NMR spectrum, this was contaminated with α,α-dibromo derivatives (8%) [δ 6.56 (t, $J_{2',4'}$ = 2.0 Hz, 1H, H4′), 7.69 (d, 2H, H2′,6′)], and was employed for next step without further purification.

2.3. 2-Chloro-1-(3′,5′-diacetoxyphenyl)ethanone (**2b**)

To a solution of above-mentioned **5b** (3.43 g, 92% purity, 10.0 mmol) in CH_3CN (100 mL) was added a solution of NaCl (4.12 g, 71.0 mmol, 7.1 equiv.) in water (34.3 mL), and the resulting mixture was vigorously stirred under reflux for 2.5 h. After cooling, the mixture was concentrated *in vacuo*, and the residue was diluted with brine and extracted with AcOEt three times. The combined organic layer was washed with saturated aqueous $Na_2S_2O_3$ solution and brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The residue was purified by recrystallization from ether to afford **2b** (1.71 g, 63%) as colorless fine needles, and further chromatographic purification of the concentrated mother liquor provided the desired product (455 mg, 17%). The combined yield was 80%. Mp 92.0–92.5 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.31 (s, 6H, Ac), 4.63 (s, 2H, H2), 7.19 (t, $J_{2',4'}$ = 2.0 Hz, 1H, H4′), 7.56 (d, 2H, H2′,6′); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 21.0 (×2), 45.8, 119.1 (×2), 121.1, 135.9, 151.4 (×2), 168.7 (×2), 189.3; IR: 872, 922, 1018, 1122, 1184, 1369, 1443, 1593, 1705, 1763 cm^{-1}. Anal. Calcd for $C_{12}H_{11}ClO_5$: C 53.25, H 4.10; found: C 53.37, H 4.17.

2.4. Candida antarctica lipase B-catalyzed hydrolysis of **2b**

To a mixture of **2b** (297 mg, 1.1 mmol), toluene (3 mL), and phosphate buffer (pH 7.0, 0.2 M, 15 mL) was added *C. antarctica* lipase B (Novozym 435, 300 mg). The mixture was vigorously stirred for 15.5 h at 50 °C under Ar. The mixture was diluted with AcOEt, filtered through a Celite pad and the aqueous layer was extracted four times with AcOEt. The combined organic layer was washed with saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (6 g). Elution with hexane/AcOEt = 4:1 afforded **2c** (160 mg, 78%) as pale yellow needles, mp 150.5–151.0 °C. ^{1}H NMR (400 MHz, CD_3OD): δ 4.81 (s, 2H, H2), 6.51 (t, $J_{2',4'}$ = 2.0 Hz, 1H, H4′), 6.87 (d, 2H, H2′,6′); ^{13}C NMR (CD_3OD, 125 MHz): δ 46.1, 106.3 (×2), 107.6, 136.2, 158.8 (×2), 191.8; IR: 795, 849, 1011, 1057, 1165, 1277, 1338, 1477, 1593, 1689, 3321 cm^{-1}. Anal. Calcd for $C_8H_7ClO_3$: C 51.49, H 3.78; found: C 51.53, H 3.74.

2.5. 2-Chloro-1-[3′,5′-bis(methoxymethoxy)phenyl]ethanone (**2a**)

To a solution of **2c** (148.0 mg, 0.79 mmol) in anhydrous CH_3CN (8.0 mL) was added MOMCl (159 mg, 1.97 mmol, 2.5 equiv.) at 0 °C, and then *i*-Pr_2NEt (296 mg, 2.29 mmol, 2.9 equiv.) was added dropwise at 0 °C. The mixture was stirred for 1 h at 0 °C. Then it was further stirred at room temperature, and finally for 4 h at 40 °C. The reaction was quenched by the addition of phosphate buffer (pH 7.0, 0.2 M) and the mixture was concentrated *in vacuo*. The residue was 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 (5.0 g). Elution with hexane/AcOEt = 3:1 afforded **2a** (138.0 mg, 63%) as colorless needles. The physical properties and spectral data were identical with those in Section 2.1.

2.6. (±)-2-Chloro-1-[3′,5′-bis(methoxymethoxy)phenyl]ethanol (**3**)

To a solution of **2a** (47.0 mg, 0.17 mmol) in anhydrous MeOH (260 μL) was added $NaBH_4$ (4.4 mg, 0.12 mmol, 0.71 equiv.) at 0 °C. The mixture was stirred for 5 h. The reaction was quenched with phosphate buffer (pH 7.0, 0.5 M) and the mixture was extracted with AcOEt. The organic layer was washed with water, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The residue was purified by preparative TLC (developed with hexane/AcOEt = 2:1) to afford (±)-**3** (36.6 mg, 77%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.46 (s, 6H, OCH_3), 3.61 (dd, $J_{1,2a}$ = 8.9 Hz, $J_{2a,2b}$ = 11.2 Hz, 1H, H2a), 3.72 (dd, $J_{1,2b}$ = 3.3 Hz, 1H, H2b), 4.81 (dd, 1H, H1), 5.14 (s, 4H, CH_3O—CH_2—), 6.71 (t, $J_{2',4'}$ = 2.1 Hz, 1H, H4′), 6.73 (d, 2H, H2′,6′); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 50.8, 56.1 (×2), 73.9, 94.4 (×2), 104.6 (×2), 107.3, 142.4, 158.4 (×2); IR: 650, 697, 854, 922, 1026, 1083, 1145, 1289, 1458, 1599, 2956 cm^{-1}. Anal. Calcd for $C_{12}H_{17}ClO_5$: C 52.09, H 6.19; found: C 51.89, H 6.20. HPLC [column, CHIRALCEL® OD-H, 0.46 cm × 25 cm; hexane-isopropyl alcohol (15:1); flow rate 0.5 mL/min; detected at 208 nm]: t_R (min) = 32.3, 38.8.

2.7. Screening of microorganisms for the reduction of **2a**

The microorganisms were incubated in the common medium (100 mL) with glucose as carbon source (5%) in a 500-mL baffled Erlenmeyer cultivating flask. The flasks were shaken on a gyratory shaker (180 rpm) for 48 h at 30 °C. The cells were harvested by centrifugation (3000 rpm, 1700 × *g*) for 15 min, and portion of cells (wet, *ca.* 500 mg) were re-suspended in phosphate buffer solution (pH 6.5, 0.1 M, 10 mL) in a test tube, together with **2a** (20.0 mg) and glucose (500 mg). The test tubes were shaken on a reciprocal shaker (210 cpm) for 48 h at 30 °C. The progress of the reduction was monitored by a TLC analysis: R_f for **2a**: 0.55; **3**: 0.45 (developed with hexane/AcOEt = 2:1). The broth was centrifuged (5000 rpm, 1200 × *g*) for 15 min. The precipitated cells were extracted three times with acetone. The supernatant in the step of centrifugation was saturated with NaCl and extracted with AcOEt four times. The combined extract was concentrated *in vacuo*. The residue was diluted with brine and extracted with AcOEt three times. The extract was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The conversion was estimated by comparing the area of signals in ^{1}H NMR: δ 3.72 (dd, 1H, H2b for **3**), 4.65 (s, 2H, H2 for **2a**), 6.71 (t, 1H, H4′ for **3**) and 6.96 (t, 1H, H4′ for **2a**) of this crude product. The residue was purified by preparative TLC (developed with hexane/AcOEt = 2:1) to afford **3**, whose ee was determined by HPLC analysis as described in Section 2.6.

2.8. Pre-incubation of Williopsis californica JCM 3600

W. californica was inoculated in a common medium. With glucose as carbon source (5%, w/v), the gyrotary-shaken culture (30 °C, 180 rpm) showed OD (660 nm) = 0.7 with an early logarithmic growing phase at 12 h. After being its OD to be 8.8 at 24 h, the growth become slower, but finally the OD reached to be 18 after 72 h, by way of 8.5 at 36 h. Cells (wet, 2.5 g/100 mL) could be recovered by centrifugation (3000 rpm, 1700 × *g*) for 15 min at the stage of 36 h incubation. The growth was accelerated, by adding antifoam (Dow-Corning, FS antiform AFE emulsion, diluted 10 times with water, 0.5 mL) to the broth (100 mL) and replacement of conventional urethane-foam stopper with perforated filter paper cap when OD reached 2.3 at 12 h. The amount of wet cells was increased to be 3.8 g from 100 mL of the broth at 33 h. The change of carbon source from glucose (5%) to glycerol (2%, w/v) brought about a moderate growth with an OD of 12 at 36 h under the stationary phase. Aerated condition as mentioned above also promoted the growth with OD of 18 at 36 h. Cells (wet, 4.0 g) were obtained from the broth (100 mL). A mixture of carbon source with glucose (2%) and glycerol (2%) resulted in a slower growth. The conventional incubation showed an OD (8.0) after reaching the stationary phase, while under the aerated conditions, the OD was 18.7 at 36 h to give cells (wet, 3.8 g/100 mL).

2.9. (R)-2-Chloro-1-[3′,5′-bis(methoxymethoxy)phenyl]ethanol (**3**)

Harvested cells (wet, 6.0 g) of *W. californica*, from incubation with glycerol for 37 h at 30 °C (OD = 20.0), were re-suspended in phosphate buffer (pH 6.5, 0.1 M, 30 mL) and glycerol (600 mg). The broth was divided into two test tubes, and each 75 mg (0.27 mmol) of **2a** was added to the tube. Two tubes were shaken on a reciprocal shaker (210 cpm) at 30 °C for 20 h. At 3 h, an insoluble precipitate was dissolved by the addition of EtOH (0.5 mL) for each test tube. After extractive workup as described in Section 2.7, the residue (150 mg) was charged on a silica gel column (10 g). Elution with hexane/AcOEt = 3:1 afforded (*R*)-**3** (118 mg, 80%). $[\alpha]_D^{25}$ –25.8 (*c* 1.00, EtOH). Its 98.4% ee was determined by HPLC analysis: t_R (min) = 32.3 [0.8%, (*S*)-**3**], 38.8 [99.2%, (*R*)-**3**]. The spectral data were identical with those in Section 2.6.

2.10. (R)-[3′,5′-Bis(methoxymethoxy)phenyl]oxirane (**10**)

To a solution of (*R*)-**3** (807 mg, 2.91 mmol) in ether (27.0 mL) were added TBAI (42.7 mg, 0.12 mmol, 0.04 equiv.) and aqueous NaOH solution (2 M, 13.5 mL). The mixture was stirred for 4 h at room temperature and quenched with phosphate buffer (pH 7.0, 0.5 M). The ether layer was separated and the aqueous layer 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 charged on a silica gel column (32 g). Elution with hexane/AcOEt = 5:1 afforded (*R*)-**10** (667 mg, 96%). This was employed for next step without further purification. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.69 (dd, $J_{1,2a}$ = 2.5 Hz, $J_{2a,2b}$ = 5.4 Hz, 1H, H1a), 3.03 (dd, $J_{1,2b}$ = 3.9 Hz, 1H, H2b), 3.40 (s, 6H, OCH_3), 3.74 (dd, 1H, H1), 5.08 (s, 4H, CH_3O—CH_2—), 6.57 (d, $J_{2',4'}$ = 2.4 Hz, 2H, H2′,6′), 6.60 (t, 1H, H4′); IR: 693, 836, 922, 1024, 1143, 1212, 1294, 1378, 1457, 1597, 2827 cm^{-1}.

2.11. (R)-1-[3′,5′-Bis(methoxymethoxy)phenyl]-2-(tert-butylamino)ethanol (**1b**)

To a solution of (*R*)-**10** (25.8 mg, 0.11 mmol) in anhydrous MeOH (2.0 mL) was added *tert*-butylamine (500 μL, 4.73 mmol, 43 equiv.). The mixture was stirred for 24 h at 50 °C. Then another portion of *tert*-butylamine (400 μL, 3.78 mmol, 34 equiv.) was added, and the mixture was further stirred for 5 h. The mixture was concentrated *in vacuo*, and the residue was purified by preparative TLC (developed with EtOH) to afford (*R*)-**1b** (26.6 mg, 77%). ^{1}H NMR (400 MHz, CD_3OD): δ 1.11 (s, 9H, *tert*-butyl), 2.65 (dd, $J_{1,2a}$ = 4.4 Hz, $J_{2a,2b}$ = 11.7 Hz, 1H, H2a), 2.71 (dd, $J_{1,2b}$ = 8.8 Hz, 1H, H2b), 4.61 (dd, 1H, H1), 3.43 (s, 6H, OCH_3), 5.15 (s, 4H, CH_3O—CH_2—), 6.62 (t, $J_{2',4'}$ = 2.0 Hz, 1H, H4′), 6.71 (d, 2H, H2′,6′). Regioisomer **11** on which amine attacked benzylic position: δ 1.02 (s, 9H, *tert*-butyl), 3.35 (dd, $J_{1,2a}$ = 8.8 Hz, $J_{2a,2b}$ = 10.9 Hz, 1H, H2a), 3.43 (s, 6H, OCH_3), 3.51 (dd, $J_{1,2b}$ = 4.9 Hz, 1H, H2b), 3.84 (dd, 1H, H1), 5.14 (s, 4H, CH_3O—CH_2—), 6.58 (t, $J_{2',4'}$ = 2.2 Hz, 1H, H4′), 6.73 (d, 2H, H2′,6′).

2.12. (R)-2-(N-tert-butylamino)-1-(3′,5′-dihydroxyphenyl)ethanol hydrochloride (**1a**)

Alcohol (*R*)-**1b** (26.6 mg, 0.085 mmol) was treated with a mixture of concentrated HCl (12 M) and ethanol (1:5, 1.5 mL), and stirred for 24 h at room temperature. The reaction mixture was concentrated *in vacuo* to afford (*R*)-**1a** (22.0 mg, quant, 99.4% ee). $[\alpha]_D^{25}$ −46.7 (*c* 1.10, MeOH) [lit. [3] $[\alpha]_D^{20}$ −32.5 (*c* 0.76, MeOH)]. ^{1}H NMR (400 MHz, CD_3OD): δ 1.38 (s, 9H, *tert*-butyl), 2.97 (dd, $J_{1,2a}$ = 10.7 Hz, $J_{2a,2b}$ = 12.7 Hz, 1H, H2a), 3.10 (dd, $J_{1,2b}$ = 2.9 Hz, 1H, H2b), 4.75 (dd, 1H, H3), 6.21 (t, $J_{2',4'}$ = 2.4 Hz, 1H, H4′), 6.37 (d, 2H, H2′,6′); ^{13}C NMR (CD_3OD, 100 MHz): δ 25.8 (×3), 49.8, 58.1, 70.7, 103.2, 105.2 (×2), 144.7, 159.8 (×2). NMR spectra were in good accordance with those of commercially available terbutaline sulfate. IR: 849, 1049, 1159, 1383, 1459, 1605, 2876, 2979, 3399 cm^{-1}.

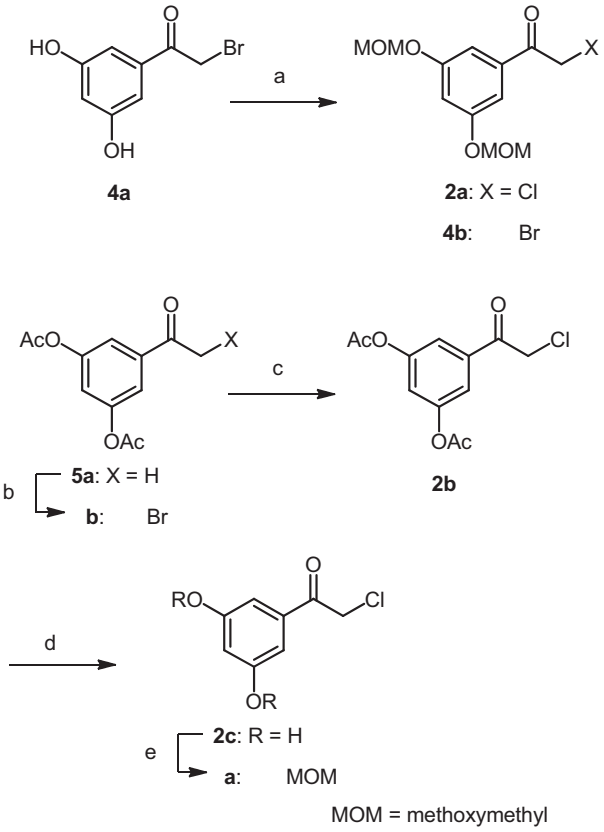
2.13. (R)-N-[2-acetoxy-2-(3′,5′-diacetoxyphenyl)ethyl]-N-tert-butylacetamide (**12**)

To a solution of (*R*)-**1a** (3.9 mg, 0.042 mmol) in pyridine (0.5 mL) was added acetic anhydride (0.5 mL, 5.3 mmol) and DMAP (0.2 mg), and the mixture was stirred for 16 h at room temperature. The reaction mixture was quenched by the addition of ice and the mixture was extracted with AcOEt three times. The combined organic layer was washed successively with water, HCl (1 M), water, saturated aqueous $NaHCO_3$ solution, and brine. The extract was dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The resulted oily residue was purified by preparative TLC (developed with hexane/AcOEt = 1:1) to afford pure **12** (0.4 mg, 7%, 99.4% ee). This was analyzed by HPLC [column, CHIRALCEL® OD-H, 0.46 cm × 25 cm; hexane/isopropyl alcohol = 10:1, flow rate 0.5 mL/min, detected at 212 nm]: t_R (min) = 29.7 [0.3%, (*S*)-**12**], 46.8 [99.7%, (*R*)-**12**]. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.44 (s, 9H, *tert*-butyl), 2.08 (s, 3H, $NCOCH_3$), 2.19 (s, 3H, 2-$OCOCH_3$) 2.27 (s, 6H, Ar—$OCOCH_3$) 3.53 (dd, $J_{1a,2}$ = 3.4 Hz, $J_{1a,1b}$ = 16.1 Hz, 1H, H1a), 3.72 (dd, $J_{1b,2}$ = 10.3 Hz 1H, H1b), 5.87 (dd, 1H, H2), 6.91 (t, $J_{2',4'}$ = 2.0 Hz, 1H, H4′), 6.93 (d, 2H, H2′,6′).

3. Results and discussion

Our first attempt was the direct synthesis of **2a** from commercially available **4a**. The desired product was, however, contaminated with certain amount of the corresponding bromide **4b**, whose strongly alkylating property was deleterious as an inhibitory effect in the subsequent microbial reduction. Due to the very similar chromatographic behavior between **2a** and **4b**, and poor crystalline nature of **2a** (mp 47.0–47.5 °C), we switched the synthetic route by way of another chloride **2c** with higher melting point (150.5–151.0 °C) as shown in Scheme 2. Cheaper starting material **5a** was treated with bromine in acetic acid to give α-bromoketone **5b**. The bromine atom was substituted with chlorine by applying NaCl in aqueous acetonitrile. The next requisite task was the hydrolysis of two phenolic acetates. Under basic conditions such as K_2CO_3/MeOH, the disappearance of **2b** was very fast, but some over-reaction was accompanied with the formation of more polar by-products. In turn, under strongly acidic conditions (hydrochloric acid), the hydrolysis was very slow. As this substrate has α-haloketone moiety which is susceptible to the nucleophilic displacement, biocatalytic transformation seemed to be advantageous under mild conditions.

For that purpose, *C. antarctica* lipase B (Novozymes, Novozym 435) was very effective catalyst to give **2c** (78%). MOM protection of the two phenolic hydroxy groups in **2c** was the next task. The reactive α-chloroketone itself worked as a good alkylating agent,



Scheme 2. Reagents and conditions: (a) MOMCl, NaCl, *i*-Pr_2NEt, CH_3CN (83%); (b) Br_2, AcOH; (c) NaCl, CH_3CN, H_2O (65%, two steps from **5a**); (d) *C. antarctica* lipase B (Novozym 435), pH 7.0 (78%); and (e) MOMCl, *i*-Pr_2NEt, CH_3CN (63%).

and we optimized the reaction conditions. The limited dose of *i*-Pr_2NEt (2.9 equiv.) in the treatment with MOMCl (2.5 equiv.) was the key for successful protection (63%).

Recently, a number of biocatalysts for the asymmetric reduction of substituted acetophenones have been studied [5–20]. We applied set of incubated microorganisms involving yeasts and fungi, which had been used for the reduction of ketone **6** with an aromatic ring [21] (Scheme 3). In our hand, out of six strains which showed a certain progress of the reduction, two exhibited the desired enantiofacial selectivity as shown in Table 1. The *si*-facial attack took place to give (*R*)-**3**. Moran and co-workers also reported a *si*-facial attack on similarly substituted α-haloacetophenone **8** to give (*R*)-**9** [12]. Another high, but reverse *re*-facial selectivity was shown in three strains.

In *Pichia minuta*, *Trichosporon cutaneum*, and *W. californica*, which had high enantiofacial preference on **6**, for the present substrate **3**, the attack of hydride occurred from the same enantioface as illustrated in Fig. 1.

The selected *W. californica* JCM 3600 showed high catalytic activity, however, there was observed a severe problem. The enantiomeric excess of the product (*R*)-**3** fluctuated (80–99% ee) even under the same incubation conditions, and we could not find out any reason in the incubation by applying conventional glucose medium for pre-incubation and reaction. In the incubated whole-cell microorganism, total enantioselectivities is the sum of contrasting enantiofacial preference shown by plural enzymes. For enhancing the major reductase with desired enantioselectivity, we embarked upon the optimization of the incubation and reduction conditions.

Table 1
Screening on the reduction of **2a**.

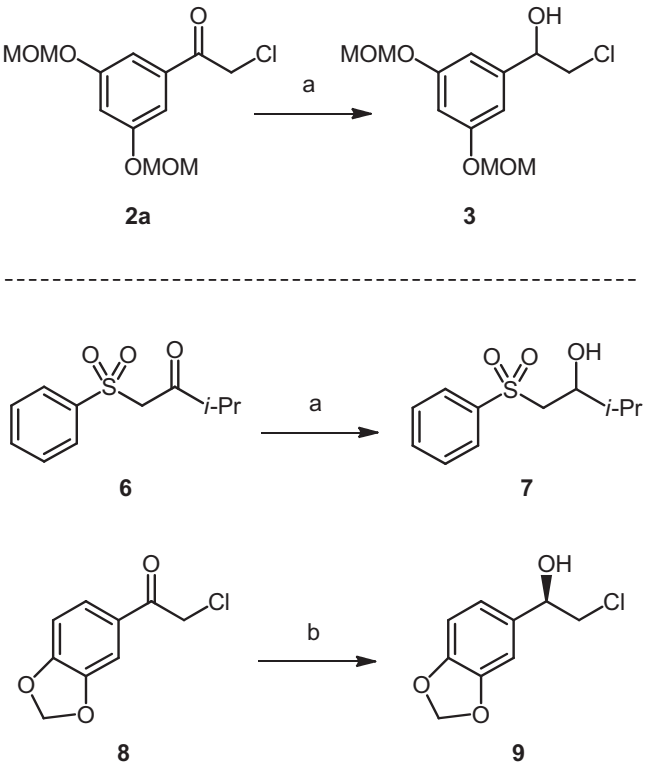
Microorganism (JCM No.)	Conversion (%)[a]	Recovery of **2a** (%)	**3**[b] isolated yield (%)	Abs. config.	%Ee
Candida kefyr (21874)	66	35	50	S	96.7
Candida nitratophila (9856)	8	90	5	S	83.8
Pichia farinosa (10896)[c]	15	85	10	S	45.1
Pichia minuta (3622)	12	74	12	R	69.7
Trichosporon cutaneum (1534)	86	13	69	S	90.4
Williopsis californica (3600)	97	3	94	R	99.3

[a] Determined by ^{1}H NMR analysis of the crude product. For detail, see Section 2.7.
[b] See Section 2.7.
[c] NBRC No.

First, we found that the growth strongly depended upon the supply of air during the pre-incubation, and the growth was promoted under aerobic conditions by applying antifoam and a perforated cap (see Section 2.8). Cells (wet, 3.8 g/100 mL of broth) were harvested at OD (660 nm) = 18 after 36 h. On the other hand, pre-incubation with a limited air supply retarded growth. For example, it dropped when the incubation flasks were capped with conventional urethane-foam stoppers. An apparent log phase was observed between 9 and 24 h, but after that, the growth slightly slowed down, finally reaching OD = 18 (3.0 g/100 mL of broth) after 36 h.

Among glucose, glycerol [22], and 2-propanol as the external carbon sources for the regeneration of NAD(P)H, only the addition of glycerol (2%, w/v) remarkably promoted the reduction. As the effect by the addition of glycerol showed a certain time lag, we suspected that the carbonyl reductases responsible for the reduction of **2a** were induced. Then, at the pre-incubation stage, the growth and the enzyme activity were examined by changing the carbon source [glucose (5%), glycerol (2%), and glucose (2%) + glycerol (2%)]. Although growth was slightly slower after pre-incubation with glycerol (2%) compared with the other two conditions involving glucose, in all three cases the OD eventually equally reached *ca.* 18 under intensively aerobic conditions as mentioned above. By applying whole-cell biocatalysts of *W. californica* harvested through the optimized pre-incubation conditions with glycerol, the reduction of **2a** by adding glycerol proceeded very smoothly to provide (*R*)-**3** with 98.4% ee in an 80% yield (Scheme 3).

We should mention some comments on the low concentration (5.0 g/L) of substrate **2a**. Due to the hydrophobic property of **2a**, its solubility in the broth was very low. When the charge of substrate is increased (10–20 g/L), certain amount remained intact as the precipitates in the incubation mixture. An increased concentration of **2a** by the addition of water-miscible solvent resulted in the poor enantioselectivity, as the chloroketone structure itself worked as an inhibitor for the reductase with *si*-facial preference. Such inhibitory effect was more prominent with the bromoketone **4b**. When the substrate **2a** was incubated in the presence of **4b** (1.7 g/L), the reduction of **2a** became very slow and the ee of (*R*)-**3a** was as low as 63%. Those observations recommend the keeping the substrate concentration to be low, according to the elaborated experimental procedure (Section 2.9).



Scheme 3. Reagents and conditions: (a) whole-cell microorganisms, see Table 1; and (b) *Rhodotorula glutinis* CCT 2182 [12] (98%, >99% ee).

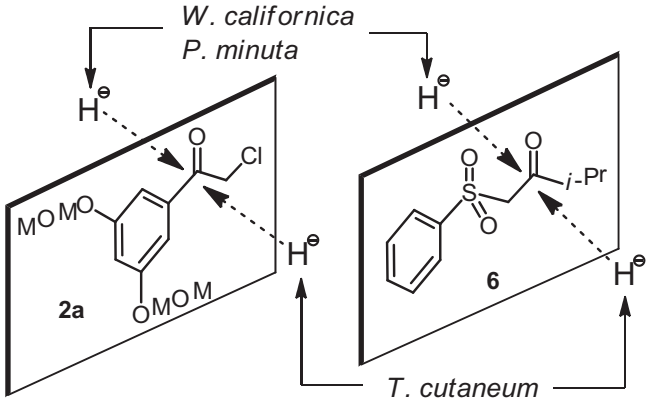
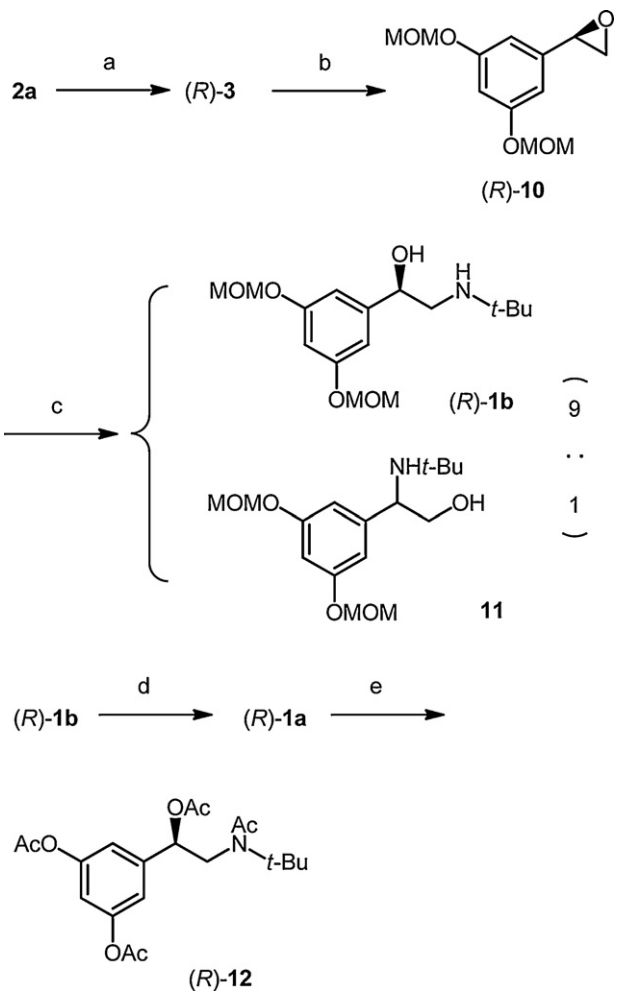


Fig. 1. Enantiofacial preference of microbial reductions on ketones.



Scheme 4. Reagent and conditions: (a) *Williopsis californica*, pH 6.5, glycerol (80%, 98.4% ee); (b) aq. NaOH (2 M), TBAI, Et_2O (96%); (c) *tert*-$BuNH_2$, MeOH; (d) aq. HCl [79%, two steps from (*R*)-**10**]; and (e) Ac_2O, pyridine, DMAP.

The requisite (*R*)-**3** was converted into epoxide (*R*)-**10** with a high yield of 96%, by the aid of a catalytic amount of TBAI under basic conditions. The attack of *tert*-butylamine proceeded predominantly (9:1) on the terminus of the epoxy ring over the regioisomer **11**. Acid treatment of the purified isomer to remove the MOM protective groups worked very well to provide terbutaline hydrochloride (*R*)-**1a** (Scheme 4) in 77% in two steps from (*R*)-**10**. The ee of (*R*)-**1a** was confirmed to be 99.4% by the HPLC analysis of (*R*)-**12**.

4. Conclusion

In this study, an expeditious approach for terbutaline hydrochloride was examined. Preparation of highly enantiomerically enriched key intermediate (*R*)-**3** was established, based on *W. californica*-catalyzed asymmetric reduction. *C. antarctica* lipase B-mediated hydrolysis under mild conditions enabled the choice of easily available and cheap starting material **5a**, whose two phenolic hydroxy groups were protected as acetates.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2012.01.020.

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