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Chemo-biological preparation of the chiral building block (*R*)-4-acetoxy-2-methyl-1-butanol using *Pseudomonas putida*

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ABSTRACT

In order to develop new methyl substituted chiral building blocks which are useful for the synthesis of methyl branched natural products, the enantioselective bioreduction of an *exo*-methylene to a methyl group was investigated. 4-Acetoxy-2-methylene-1-butanol **3** was prepared from itaconic acid over four steps and converted to the chiral alcohol (*R*)-4-acetoxy-2-methyl-1-butanol **4**, by growing cells of *Pseudomonas putida*. The bioconversion achieved a high enantioselectivity (92% ee) and a high chemical yield (65%) within a relatively short reaction time (18–20 h).

1. Introduction

Optically active alcohols play a very important role as chiral building blocks.[1] In particular, chiral alcohols with methyl branches are widely used for the synthesis of antibiotics, such as azithromycin,[2] natural products such as muscone,[3–6] and many insect pheromones, for example, 5,9-dimethyl-heptadecane (*Leucoptera scitella*), 5,9-dimethyl pentadecane (*Perileucoptera coffeella*). and 4-methyl-1-nonanol (*Tenebrio molitor*).[7,8] Due to their high enantio- and regio-selectivities,[7] bioconversions are becoming essential approaches to prepare chiral compounds.[8] Many bioconversion processes are studied for the enantioselective reduction of carbon carbon double bonds (C=C).[9–11] The C=C bonds need to be activated by electron-withdrawing groups to cause the addition of hydrogen to occur in a *trans*-fashion across the double bond.[9] Since the enantioselectivity of the bioreductions of *endo* C=C bonds is influenced by the geometry of the substrate, the *E*:*Z* control of the substrate is essential.[12] From this point of view, the use of an *exo* C=C bond as a substrate is more attractive, because it is free from the *E*:*Z* control problem. There have been several attempts to reduce *exo*-olefins biocatalytically.[11–13] Even though high enantioselectivity has been achieved, the conversions rate are quite low.[13] As a result, the development of a bioreduction of *exo* C=C bonds with good conversion and high enantioselectivity is highly desired.

Recently, we have investigated the bioreduction of exo-methylenes to chiral methyl groups and found that *Pseudomonas putida* KCTC1644 has capabilities for the enantioselective reduction of the *exo*-methylene of **3** to yield 4-acetoxy-2-methyl-1-butanol **4** with good conversion.

2. Results and discussion

2.1. Synthesis of 4-acetoxy-2-methylene-1-butanol 3

The starting point of the chemo-biological synthesis was the inexpensive itaconic acid. Substrate **3** was synthesized by the sequence outlined in Scheme 1. Briefly, itaconic acid **1** was esterified with 2-propanol in the presence of sulfuric acid to produce diisopropyl ester. The reduction of the ester groups required a suitable reducing reagent to avoid an over-reduced product, which would result in low enantiomeric purity of the bioconversion product **4**. This was accomplished through the use of aluminum hydride, which was prepared from $AlCl_3$ and 3 equiv of lithium aluminum hydride (LAH). Diol **2** was acetylated with acetic anhydride to give the diacetate, and the allylic acetate was selectively hydrolyzed with LPS in a phosphate buffer (pH 7.0) to give **3**.

2.2. Screening of microorganisms for the synthesis of (*R*)-4-acetoxy-2-methyl-1-butanol 4

The abilities of the following six candidate microorganisms to perform the bioconversion were assessed: *Candida rugosa* KCTC 7282,[14] *P. putida* KCTC 1644,[15] *Alcaligenes* sp. KCTC 2338,[16] *Achromobacter* sp. KCTC 2757,[17] *Chromobacterium* sp. KCTC 2896,[18], and *Kluyveromyces fragilis* KCTC 7260.[19] Substrate **3** was added to their culture media, and the strains were cultured at the appropriate temperatures (*C. rugosa* at 25 °C, *P. putida* at 27 °C, *Alcaligenes* sp. at 30 °C, *Achromobacter* sp. at 30 °C, *Chromobacterium* sp. at 30 °C and *K. fragilis* at 25 °C). Cell suspensions were extracted with

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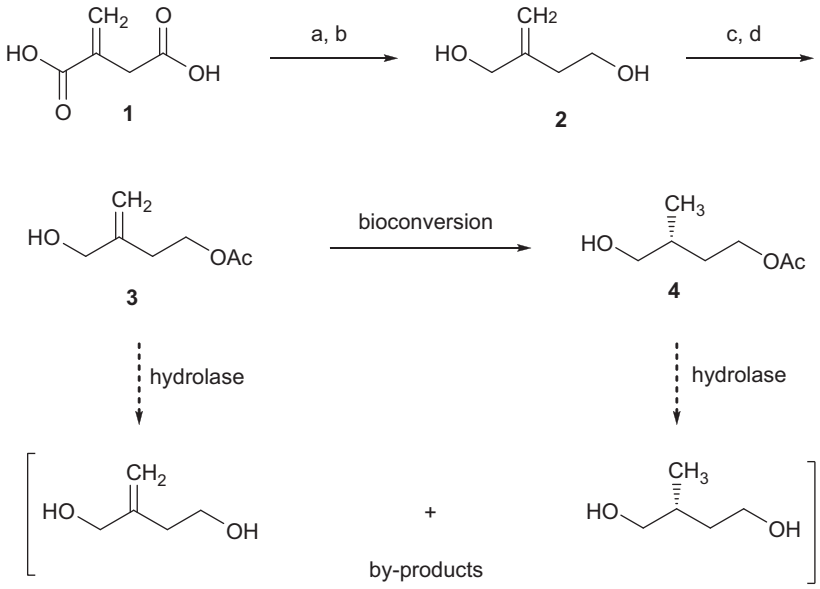

Scheme 1. Preparation and bioconversion of 4-acetoxy-2-methylene-1-butanol **3**. Reagents and conditions: (a) H_2SO_4, 2-propanol, reflux, 30 h, 98%; (b) AlH_3, Et_2O, −30 °C, 5 min; (c) Ac_2O, pyridine, CH_2Cl_2, 5 h, two steps 98%; (d) LPS*, P-buffer pH 7.0, 5 min, rt, 71%, *LPS = Lipase from *Pseudomonas* sp.

ethyl acetate (EtOAc) after 20 h, and the organic phases were analyzed by gas chromatography (GC). The results are summarized in Table 1. Acetate hydrolysis to produce by-products occurred in the cultures of *P. putida* and *K. fragilis* (Table 1, entries 2 and 6). The highest conversion to **4** was achieved with *P. putida* (entry 2), which was therefore chosen for further optimization.

Table 1
Screening of microorganisms for bioconversion

Entry	Microorganism	Product composition[a] (%)		
		3	**4**	By–products
1	*Candida rugosa*	95	5	0
2	*Pseudomonas putida*	12	73	15
3	*Alcaligenes* sp.	93	7	0
4	*Achromobacter* sp.	68	31	0
5	*Chromobacterium* sp.	75	25	0
6	*Kluyveromyces fragilis*	20	65	15

[a] Determined by GC.

2.3. Optimization of the bioconversion reaction conditions

The growth curve of *P. putida* is shown in Figure 1. To determine the optimal starting time for the biotransformation, **3** was added to the cultures at 0, 4, 6, and 10 h after inoculation. Conversion was analyzed by GC after 20 h (Table 2). The best results were achieved when the substrate was added immediately. In contrast, when the substrate was added after 6 h, only hydrolysis occurred without reduction of the C=C.

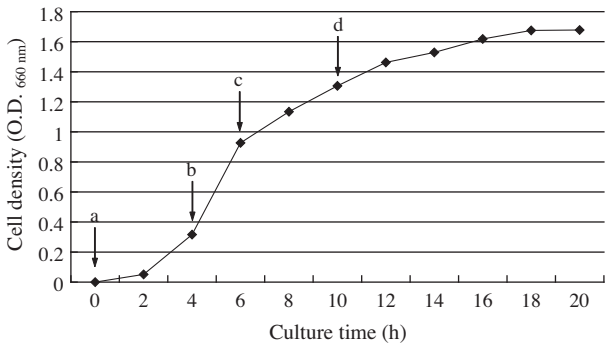

Figure 1. Cell growth of *P. putida*. Yeast peptone dextrose (YPD) media, 30 °C, 180 rotation per minute (rpm); Arrows indicate the time point of substrate addition, *a* = 0 h, *b* = 4 h, *c* = 6 h, *d* = 10 h; Optical density (OD) at 660 nm was measured.

Table 2
Effect of substrate **3** addition time point on the bioconversion by *P. putida*

Substrate addition time[a] (h)	Product composition[b] (%)		
	3	**4**[c]	By-products
0	11	73	16
4	30	40	30
6	60	2	38
10	73	2	25

[a] Marked on Figure 1.
[b] Determined by GC after culture for 20 h.
[c] The enantiomeric purity of products **4** was determined by chiral GC analysis, and all of them were found to be 92% ee.

The reaction conditions were further optimized with respect to the seed culture volume used for the inoculation (1–5%), substrate **3** concentration (7∼21 mmol/L), culture scale (5–100 mL), culture media (Nutrient broth, Luria-Bertani (LB) broth, yeast malt (YM) broth, YPD broth), pH of the media after adding substrate **3** (pH 6.5–8.1) and the cultivation temperature (27–35 °C). The best reaction conditions were obtained using 3% of seed culture with 0.10 g (0.70 mmol, 14 mmol/L) of substrate in 50 mL (in 500 mL Erlenmeyer flask) of YPD media (pH 7.1) at 30 °C. The time course of the biotransformation under these optimal conditions is shown in Figure 2. The formation of undesirable by-products gradually increased after 15 h of cultivation.

2.4. Determination of absolute configuration and enantiomeric excess of 4

To determine the absolute configuration of **4**, it was hydrolyzed to the corresponding diol and compared with the commercial compound (*R*)-2-methyl-1,4-butanediol (>98.0%, GC). The specific rotation value of deacylated **4** was measured to be $[\alpha]_D^{27} = +13.1$ (*c* 0.5, MeOH) while that of the commercial product was found to be $[\alpha]_D^{27} = +13.2$ (*c* 1.0, MeOH)/$[\alpha]_D^{27} = +13.6$ (*c* 3.3, MeOH). Hence,

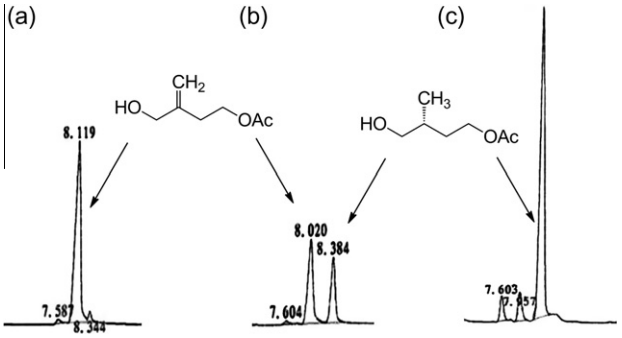

Figure 2. Time course of bioconversion of **5** by *P. putida*. YPD media, 30 °C, 180 rpm, analyzed by GC, (a) cultivation time 0 h, 8.1 min **3**; (b) cultivation time 10 h, 8.0 min **3**, 8.4 min **4**; (c) cultivation time 20 h, 8.0 min **3**, 8.4 min **4**.

the product of bioconversion **4** was determined to have an (*R*)-configuration. The enantiomeric purity of **4** was assessed by chiral GC, and it was found to be 92% ee [(*S*)-**4**, 4.1%, (*R*)-**4**, 95.9%].

2.5. Bioreduction mechanism of *P. putida*

It is well known that the asymmetric reduction of the C=C bond of allylic alcohol proceeds via three steps: oxidation of the primary alcohol to an aldehyde, enantioselective hydrogenation (1,4-reduction) of the activated C=C bond by enoate-reductase, and reduction of the aldehyde.[9] The reported mechanism of enoate-reductase[12] also satisfies the stereocontrol observed in the methylene reduction of **3** (Fig. 3). However, over the course of the bioreduction of **3** to **4** by *P. putida*, any peak of the possible intermediates (unsaturated and saturated aldehydes) was not detected in the GC (Fig. 2). This result cannot exclude the possibility that the rate-determining step is the first oxidation of **3** and that the intermediate aldehydes are quickly reduced to **4**. Therefore, further experiments for the elucidation of a detailed reaction mechanism are currently in progress and will be reported in due course.

3. Conclusion

The desired compound **4** is not commercially available, although a similar but costly (5 g, 162.50 USD) compound, (*R*)-2-methyl-1,4-butanediol (>98.0%, GC), is available from TCI (Tokyo Chemical Industry, Co., LTD). As a chiral building block, compound **4** is even more attractive than (*R*)-2-methyl-1,4-butanediol because one of the two hydroxy groups is protected as an acetate and thus individual transformations are possible.

Starting from the inexpensive itaconic acid, enantioenriched (*R*)-4-acetoxy-2-methyl-1-butanol **4** was readily prepared by a chemo-biological method. The bioconversion was achieved by growing cells of *P. putida*, within a relatively short reaction time (18–20 h), with high enantioselectivity (92% ee) and in high yield (65%). This bioreduction method is a convenient method for producing methyl-branched chiral building blocks. Our findings will give an opportunity for further applications of *exo*-methylene bioreductions.

4. Experimental

4.1. General

Unless otherwise stated, all chemicals were of reagent grade and purchased from Sigma–Aldrich (Yongin, Korea). Microorganisms were obtained from KCTC (Korean Collection for Type Cultures). All separations were conducted using open column chromatography with Merck silica gel 60 (40–63 mm). NMR spectra were recorded using a Varian 300 and 400 MHz FT-NMR. Chemical shifts were reported relative to TMS (δ 0.00), $CHCl_3$ (δ 7.26) was used as an internal standard and coupling constants (J) are reported in Hz.

4.2. Analytics

The bioconversion of **3** to **4** was determined by gas chromatography (Shimadzu GC 10) on a column code CBP1-M25-025 (Shimadzu). Method: column oven temperature 70–120 °C, 3 °C/min, injection port temperature 150 °C, detector temperature 280 °C, carrier gas N_2, and column flow rate 6.1 mL/min. The enantiomeric excess of **4** was determined by gas chromatography (Agilent 6890N) on a MOMTBDMSGCD column. Method: column oven temperature 70–150 °C, 3 °C/min, injection port temperature 150 °C, detector temperature 280 °C, carrier gas He, and column flow rate 0.7 mL/min. Optical rotation values were measured on a Jasco p-1020 polarimeter in a 10 mL cuvette.

4.3. 2-Methylene-1,4-butanediol 2

Concentrated H_2SO_4 (300 μL) was added to a stirred solution of itaconic acid **1** (130 g, 1.00 mol) and isopropanol (100 mL, 1.60 mol) in benzene (1 L). After stirring for 48 h at 70 °C, the solution was cooled to room temperature and extracted with EtOAc. The organic phases were combined and washed with aq $NaHCO_3$, dried over $MgSO_4$ and evaporated. The crude product was purified by distillation to yield the diisopropyl ester (210 g, 98%); ^{1}H NMR spectrum was identical to that reported in the literature; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.29 (s, 1H), 5.65 (s, 1H), 5.07 (dq, J = 6.3, 6.3, 1H), 5.02 (dq, J = 6.3, 6.3, 1H), 3.29 (s, 2H), 1.27 (d, J = 6.3, 6H), 1.24 (d, J = 6.3, 6H).[20]

A suspension of LAH (11.4 g, 0.30 mol) in Et_2O (200 mL) at 0 °C was added to a stirred solution of $AlCl_3$ (13.4 g, 0.10 mol) in dried Et_2O (300 mL). After 30 min, the ester (32.1 g, 0.15 mol) dissolved in Et_2O (100 mL) was slowly added at −30 °C. The reaction was immediately stopped after the addition of the ester, by adding 10% of aq H_2SO_4. The reaction mixture was extracted with EtOAc, and the organic phases were combined and washed with aq $NaHCO_3$, dried over $MgSO_4$ and evaporated to yield diol **2** (15.3 g, 98%); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 5.07 (s, 1H), 4.92 (s, 1H), 4.03 (s, 2H),

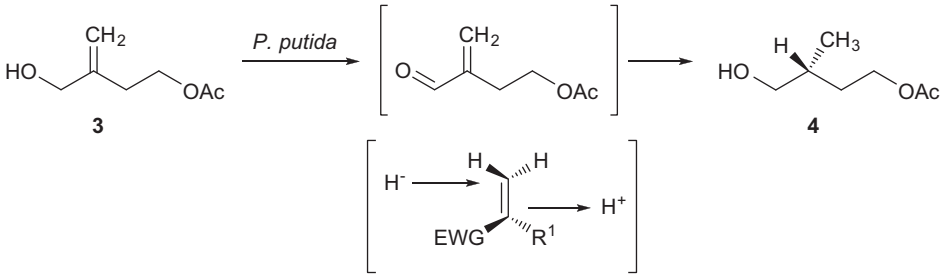

Figure 3. The plausible mechanism for the bioreduction of **3** to **4** by *P. putida*.

3.69 (t, J = 6.0 Hz, 2H), 2.31 (t, J = 6.0 Hz, 2H); the ^{1}H NMR spectrum was identical to that reported in the literature.[21]

4.4. 4-Acetoxy-2-methylene-1-butanol 3

At first, Ac_2O (25.0 g) was slowly added at 0 °C to a stirred solution of diol **3** (20.4 g, 0.20 mol) in pyridine (200 mL) and stirred for 5 h at room temperature. The solution was evaporated with MeOH to remove pyridine and extracted with EtOAc. The organic phase was washed with brine, dried over $MgSO_4$, and evaporated. The residue was purified by flash chromatography (petroleum hexane/EtOAc 4:1) to yield the diacetate (36.5 g, 98%); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 5.1 (s, 1H), 5.0 (s, 1H), 4.51 (s, 2H), 4.16 (t, J = 6.6 Hz, 2H), 2.36 (t, J = 6.6 Hz, 2H), 2.06 (s, 3H), 2.01 (s, 3H); the ^{1}H NMR spectrum was identical to that reported in the literature.[22]

The diacetate (200 mg, 1.10 mmol) was added to a stirred solution of LPS (Lipase PS, Amano, 200 mg) in a sodium phosphate buffer (pH 7.0, 10 mL). Stirring was continued for 5 min at room temperature. The reaction was filtered on Celite to remove the enzyme. The monoacetate was extracted with EtOAc. The organic phase was washed with aq $NaHCO_3$, dried over $MgSO_4$, and evaporated. The residue was purified by flash chromatography (petroleum hexane/EtOAc 4:1) to yield monoacetate **3** (113 mg, 71.4%); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 5.05 (s, 1H), 4.862 (s, 1H), 4.15 (t, J = 6.6 Hz, 2H), 4.02 (s, 2H), 2.34 (t, J = 6.6 Hz, 2H), 1.98 (s, 3H); ^{1}H NMR spectrum was identical to that reported in the literature.[22]

4.5. Screening of the bioconversion activity of microorganisms

Six microorganisms, *C. rugosa* (KCTC 7282, Difco™ YM broth, 25 °C), *P. putida* (KCTC 1644, Difco™ Nutrient broth, 27 °C), *Alcaligenes* sp. (KCTC 2338, Nutrient broth, 30 °C), *Achromobacter* sp. (KCTC 2757, Nutrient broth, 30 °C), *Chromobacterium* sp. (KCTC 2896, Nutrient broth, 30 °C), and *K. fragilis* (KCTC 7260, YM broth, 25 °C) were grown in culture tubes containing 5 mL of their appropriate medium at 180 rpm. After 24 h, substrate **3** (10 mg, 0.07 mmol) was added to each culture and incubated for another 24 h. Subsequently, 100 μL of each culture was collected, extracted with EtOAc (200 μL) and analyzed by GC.

4.6. Synthesis of 4-acetoxy-(*R*)-2-methyl-1-butanol 4

The seed cultured medium (1.5 mL), which was grown in YPD at 30 °C, 180 rpm, for 20 h, was added to 50 ml of YPD broth (Difco™) in a 500 ml Erlenmeyer flask. At the same time, substrate **3** (100 mg, 0.7 mmol) was also added, and the pH was adjusted to 7.1 with aq 1 M NaOH. Cultures were grown at 30 °C and 180 rpm. The bioconversion progress was checked every 5 h by GC. After approximately 18–20 h, the cell suspensions were extracted with EtOAc and the organic phases were washed with water, dried over $MgSO_4$, and evaporated. The residue was purified by flash chromatography (petroleum hexane/EtOAc 4:1) to yield (*R*)-**4** (66 mg, 65%, 92% ee, chiral GC on a MOMTBDMSGCD column). Method: column oven temperature 70–150 °C, 3 °C/min, injection port temperature 150 °C, detector temperature 280 °C, carrier gas He, and column flow rate 0.7 mL/min: 25.4 min [(*S*)-**4**, 4.1%], 25. 9 min [(*R*)-**4**, 95.9%]; ^{1}H NMR (300 MHz, $CDCl_3$) δ: 4.03–4.13 (m, 2H), 3.4 (d, J = 5.6 Hz, 2H), 2.0 (s, 3H), 1.65–1.78 (m, 2H), 1.33–1.41 (m, 1H), 0.87 (d, J = 6.8 Hz, 3H); the ^{1}H NMR spectrum was identical to that reported in the literature.[23]

4.7. Assignment of the absolute configuration: preparation and comparison of the specific rotation value of deacylated 4

The specific rotation value of **4** was compared with the value of the commercially available compound (*R*)-2-methyl-butane-1,4-diol (>98.0%, GC) by deacylation of **4**. The deacylation of **4** was achieved by adding K_2CO_3 (30 mg) to a stirred solution of **4** (60 mg, 0.4 mmol) in MeOH (1 mL). After stirring for 1 h, aq NH_4Cl was added, and the reaction mixture was evaporated to remove the MeOH and extracted with EtOAc. The organic phases were combined and washed with brine, dried over $MgSO_4$, and evaporated. The residue was purified by flash chromatography (petroleum hexane/EtOAc 2:1) to give the diol (35 mg, 84%); (*R*)-2-methyl-butane-1,4-diol, prepared from **4**: $[\alpha]_D^{27} = +13.1$ (c 0.5, MeOH), (*R*)-2-methyl-butane-1,4-diol, purchased from TCI: $[\alpha]_D^{27} = +13.2$ (c 1.0, MeOH)/$[\alpha]_D^{27} = +13.6$ (c 3.3, MeOH); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 3.63 (t, 2H, J = 5.4 Hz), 3.66 (m, 1H), 3.57 (dd, 1 H, J = 10.8, 4.8), 1.56–1.64 (m, 2H), 0.93 (d, J = 6.9, 3H); ^{1}H NMR spectrum was identical to that reported in the literature.[23]

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