Ketoisophorone Transformation by *Marchantia polymorpha* and *Nicotiana tabacum* Cultured Cells

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Stereospecific olefin (C=C) and carbonyl (C=O) reduction of the readily available prochiral compound ketoisophorone (2,2,6-trimethyl-2-cyclohexene-1,4-dione) (**1**) by *Marchantia polymorpha* and *Nicotiana tabacum* cell suspension cultures produce the chiral products (6*R*)-levodione (**2**), (4*R*,5*S*)-4-hydroxy-3,3,5-trimethylcyclohexanone (**3**), and (4*R*,6*R*)-actinol (**4**) as well as the minor components (4*R*)-hydroxyisophorone (**5**) and (4*S*)-phorenol (**6**).

Key words: Cultured Plant Cells, Hydrogenation, Ketoisophorone

Introduction

The demand for stereoselective syntheses has increased owing at least in part to the efficacy and market pressure for optically pure metabolites. In pharmaceutical and agrochemical fields, chiral starting materials are increasingly required for chemical syntheses. Biological methods are often successfully adopted for the synthesis of optically active compounds since biocatalyzed transformations most often precede with high stereospecificities. Enzymatic dehydrogenation and reduction serve as important tools for the synthesis of chiral metabolites.

Ketoisophorone (4-oxoisophorone or 2,6,6-trimethyl-2-cyclohexene-1,4-dione) is an industrially important cyclic endione as it is a key intermediate in the synthesis of carotenoids and flavouring agents (Ernst, 2002). Catalytic hydrogenation and enzymatic reduction of ketoisophorone lead to many optically active hydroxycyclohexanone derivatives, such as (4*R*,6*R*)-4-hydroxy-2,2,6-trimethylcyclohexanone (actinol), that in turn is a useful chiral building block for naturally occurring xanthoxin (Burden and Taylor, 1970) and zeaxanthin (Leuenberger *et al*., 1976). (6*R*)-Dihydro-oxoisophorone (DOIP), also known as (6*R*)-levodione, is a key intermediate in the synthesis of some carotenoids and flavours, and synthesized via the enantioselective reduction of 4-oxoisophorone by *Saccharomyces cerevisiae* (baker's yeast), where *in situ* removal of the product is employed by an external crystallization step (Buque-Taboada *et al*., 2004).

Microbial production of actinol from 2,2,6-trimethylcyclohexanedione has been demonstrated previously by Nishi *et al*. (1989, 1990). Additionally, a two-step production of actinol from the commercially available ketoisophorone was reported by using old yellow enzyme and levodione reductase for enzymatic asymmetric reduction (Kataoka *et al*., 2002; Wada *et al*., 2003). In this work we report for the first time that cultured plant cells can catalyze the stereo- and regioselective reduction of C=C and C=O bonds at C-1 and C-4 position of ketoisophorone. The predicted scheme for the biotransformation of ketoisopho-

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rone is strongly supported by re-incubation of one of the products [(6*R*)-levodione] with the same cultured plant cells under identical conditions.

Results and Discussion

Biotransformation of ketoisophorone (**1**) by *Marchantia polymorpha* and *Nicotiana tabacum* cultured suspension cells was observed with products isolated from the cultured medium including: (6*R*)-levodione (**2**) and (4*R*,6*R*)-actinol (**4**) as major products as well as (4*R*,5*S*)-4-hydroxy-3,3,5-trimethylcyclohexanone (**3**), (4*R*)-hydroxyisophorone (**5**) and (4*S*)-phorenol (**6**) as the identified minor products. Structure elucidation was based on mass spectral fragmentation patterns and GC retention time matches with authentic standards. Enantiomeric excesses were calculated by chiral GC (Table I). The endocyclic olefin ketoisophorone (**1**) was initially reduced to afford **2** followed by carbonyl reduction to produce **3** and **4** (Scheme I). Additionally, carbonyl reduction of **1** was observed generating **5** and **6**. The sequence of product formation was estimated from time course studies (Fig. 1). An elevated conversion yield (%) for compounds **3** and **5** with *N. tabacum* (38.8 and 7.2%, respectively) was observed in comparison to *M. polymorpha* (20.5 and 4.8%, respectively). In contrast, the conversion yield (%) for compounds **2**, **4** and **6** (45, 14.8 and 13.8%, respectively) with *M. polymorpha* was higher than with *N. tabacum* (41.6, 7.4 and 3.7%, respectively), Table I.

(6R)-Levodione (**2**) has an optical rotation $[\alpha]_D^{25}$ of $(-39.0 \pm 0.6)°$. The ^{1}H NMR spectrum of **2** showed that H-7 (δ_H 1.12) has a characteristic coupling constant (J = 6.4 Hz) with H-6 (δ_H 2.98) which indicates olefin reduction; in addition, a disappearance of the δ_H 6.52 (1H, brs, H-5) signal in ketoisophorone (**1**) was observed. ^{13}C NMR spectral data confirmed the disappearance of signals at δ_C 136.9 (C-5) and 148.8 (C-6) with the concomitant appearance of δ_C 44.9 (C-5) and 39.8 (C-6) for **1** and **2**, respectively.

(4*R*,5*S*)-4-Hydroxy-3,3,5-trimethylcyclohexanone (**3**) has an *R* configuration and optical rotation $[\alpha]_D^{25}$ of +23.5° with enantiomeric excess of 17.5% calculated by the peak analysis of chiral GLC. The ^{1}H NMR spectrum of **3** showed that H-4 (δ_H 3.32, 1H) appears as a broad singlet. This result supports the prediction that reduction occurs at the C-1 carbonyl group (δ_C 207.8, C-1, **1**) which was confirmed by ^{13}C NMR spectral data in which there is a disappearance of the carbonyl signal and the appearance of a signal at δ_C 77.3 (C-4). Additionally, (4*R*,6*R*)-actinol (**4**) showed a close ^{13}C NMR spectrum with compound **3** which clarified the disappearance of the carbonyl group at C-4 (δ_C 214.0) instead of C-1 (δ_C 207.8) as is the case for **1** and the appearance of a signal at δ_C 65.7 (C-

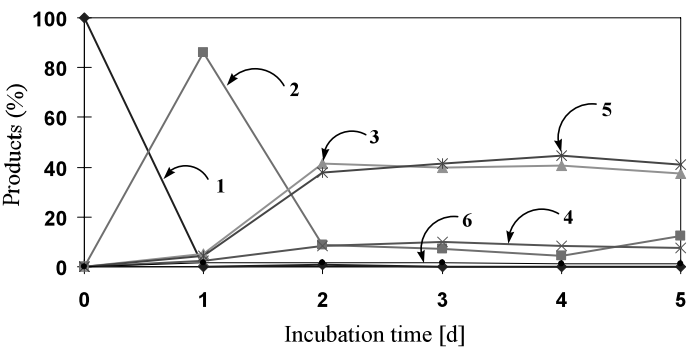
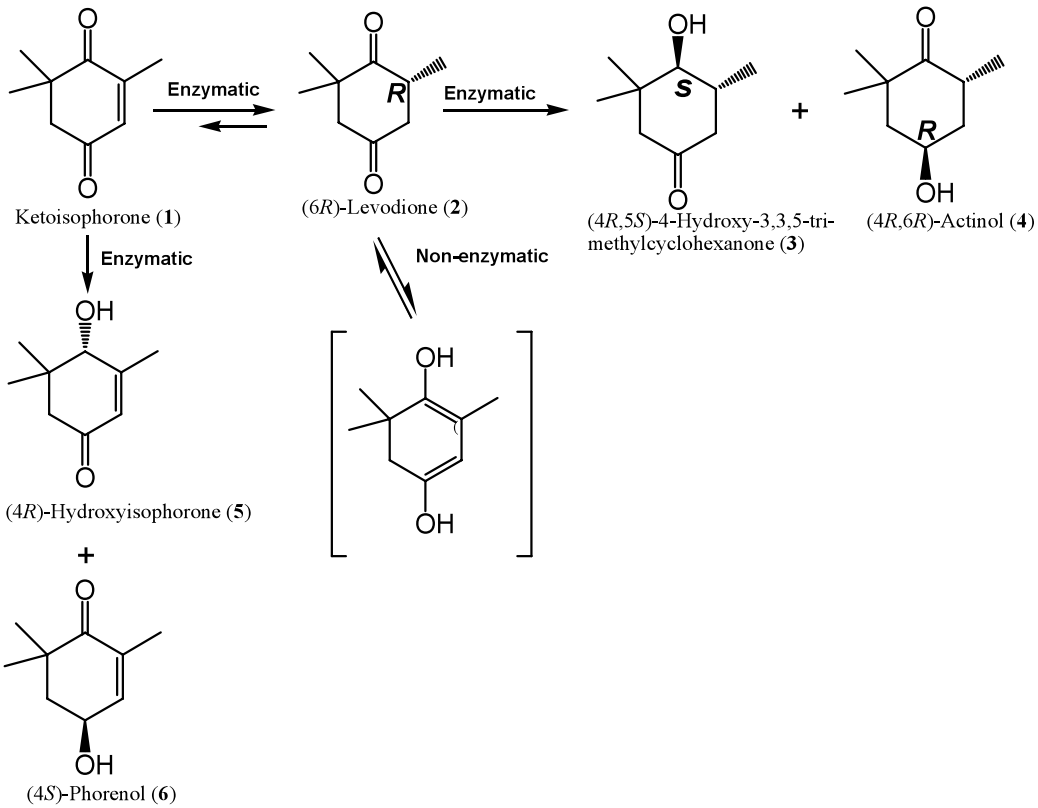


Fig. 1. Time-course of the biotransformation of ketoisophorone (**1**) with cultured cells of *M. polymorpha*.

Table I. Biotransformation of ketoisophorone (**1**) by cultured cells of *M. polymorpha* and *N. tabacum*.

Compound	Conversion (%)[a]		Enantiomeric excess (%)[b]		Configuration
	M. polymorpha	*N. tabacum*	*M. polymorpha*	*N. tabacum*	
2	45	41.6	37	76.4	6*R*
3	20.5	38.8	17.5	56.6	4*R*,5*S*
4	14.8	7.4	> 99	> 99	4*R*,6*R*
5	4.8	7.18	55.7	9.6	4*R*
6	13.8	3.7	> 99	9.1	4*S*

[a] Incubated for 7 days.
[b] e. e. is determined by GLC.



Scheme I. Predicted scheme for the biotransformation of ketoisophorone (**1**) by cultured cells of *M. polymorpha* and *N. tabacum.*

4). This result was confirmed by ^{13}C NMR spectral data which showed the disappearance of this signal and the appearance of a signal at δ_C 77.3 (C-4). This was supported by the ^1H NMR spectrum which showed that H-4 (δ_H 4.31) couples with H-5 (δ_H 1.37 and 2.24) and H-3 (δ_H 1.55 and 2.04) is a clear indication that the C=O group was reduced. Compound **4** had *R* configuration and an $[\alpha]_D^{25}$ value of −14.0° with enantiomeric excess of > 99% calculated by peak analysis of the GLC (chiral column) spectrum.

(4*R*)-Hydroxyisophorone (**5**) had *R* configuration and its $[\alpha]_D^{25}$ value was +10.7° with an enantiomeric excess of 55.7% calculated according the GLC (chiral column) spectrum. ^1H NMR spectrum showed the presence of signals at δ_H 5.84 (1H, brd, J = 0.6 Hz, H-2), 2.02 (1H, J = 1.2 Hz, H-7), 2.37 (1H, d, J = 16.4 Hz, H-6_b) and 2.71 (1H, d, J = 16.5 Hz, H-6_a) which were similar to those of **1** except for the appearance of a singlet signal at δ_H 4.01 (H-4). These results clarify that reduction occurred at C=O (C-1) of the incubated substrate **1**. Additionally, the ^1H NMR spectrum of (4*S*)-phorenol (**6**) showed that those signals had almost the same chemical shift as product **5** had but the signal splitting was different at δ_H 2.16 (1H, ddt, J = 12.8, 5.2 and 2.0 Hz, H-5_a), 1.82 (1H, dd, J = 13.0 and 10.0 Hz, H-5_b) and 4.58 (1H, m, H-4). These findings indicate that reduction occurred at C=O (C-4) of the incubated substrate **1**. Compound **6** had *S* configuration with an $[\alpha]_D^{25}$ value of −113.3° and enantiomeric excess of > 99%.

Many studies reported on the microbial transformation of ketoisophorone (Nishi *et al.*, 1990; Kataoka *et al.*, 2002; Wada *et al.*, 2003), but in this study we report for the first time the biotransformation of ketoisophorone (**1**) by suspension cultured cells, in addition to, compounds **3** and **4** are reported here for the first time as biotransformed products from microbial transformation.

Experimental

General

Optical rotations were measured with a JASCO DIP-370 digital polarimeter. The ^1H and ^{13}C NMR spectra were measured with a JEOL LA-500 spectrometer in chloroform solution with TMS as the internal reference. GLC was carried out with an FID and a capillary column (0.25 mm × 30 m) coated with 0.25 μm ZB-5 (Zebron-Phenomenex) using N_2 as carrier gas (60 cm^3/min) at a column temperature of 100–150 °C with chromatographic condition as follows: oven temperature was programmed from 100 to 150 °C at 5 °C/min; injector and detector temperatures were 250 °C; flow rate of nitrogen gas was 60.0 cm^3/s. Enantiomeric excess (e.e., %) of pure biotransformed products was detected by a chiral capillary column (0.25 mm × 20 m) (CHIRALDEX G-TA) using N_2 as carrier gas (60 cm^3/min) at a column temperature of 100–150 °C. Chromatographic conditions were as follows: oven temperature was programmed from 100 to 150 °C at 5 °C/min; injector and detector temperatures were 150 °C; flow rate of nitrogen gas was 60.0 cm^3/s. Analytical and preparative TLC were carried out on glass sheets (0.25 mm and 0.5 mm) coated with silica gel (Merck silica gel 60; GF 254), in addition to normal-phase silica gel (Sephadex LH-20). Compounds were visualized by spraying the plates with vanillin and brief heating. EIMS measurements were conducted using gas chromatography-mass spectroscopy (GC-MS) with the same chromatographic conditions as noted above. The temperature of the ion source was 255 °C, and the electron energy was 70 eV.

Plant material

The cells of *M. polymorpha* (Ono *et al.*, 1979) have been subcultured routinely every 3 weeks using MSK-II medium (Katoh *et al.*, 1980) containing 2% glucose, 0.1% *my*-inositol, 10 mM 2,4-dichlorophenoxyacetic acid (2,4-D) for more than 10 years in our laboratory. Prior to use for biotransformation experiments, the cultured cells were transplanted into a 300-ml conical flask containing 100 ml of MSK-II medium containing 2% glucose, 0.1% inositol, 10 mM 2,4-D and cultured on a rotary shaker (110 rpm) for 10 d at 25 °C under illumination (4000 lux) with white light. Suspension cells of *N. tabacum* (Hirata *et al.*, 1981) were cultured in 500-ml conical flasks containing 200 ml Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 1% sucrose and without auxin under illumination (4000 lux). Cells were cultivated on a rotary shaker (75 rpm) at 25 °C for 3 weeks prior to use in biotransformation experiments.

*Biotransformation of ketoisophorone (**1**) with cultured plant cells of Marchantia polymorpha*

To the flask containing the cells (about 20 g) of *Marchantia polymorpha* and *Nicotiana tabacum* suspended in the medium (100 ml) ketoisophorone (**1**) (20 mg; Aldrich) in DMSO (0.2 ml) was administered for each cultured plant cell, and the cultures were incubated at 25 °C on a rotary shaker (110 rpm) under illumination (4000 lux). After incubation, cells and medium were separated by filtration with suction. The filtrated medium was extracted with diethyl ether and the extract was concentrated by a rotary evaporator to give crude products. These crude products were subject to preparative TLC with diethyl ether/hexane (3:1, v/v) followed by Sephadex LH-20 chromatography to give products **2**–**6** in a pure form. The conversion yields were determined by GLC. The structure of each product was identified by NMR experiments and GC-MS analysis.

Time course experiments

Cultured cells of *M. polymorpha* (about 20 g) were transferred to a 300-ml Erlenmeyer flask containing 100 ml MSK-II medium, and cultured with continuous shaking for 10 d at 25 °C under illumination. Ketoisophorone (**1**) (20 mg) was added to the suspension culture and incubated at 25 °C on a rotary shaker (110 rpm) under illumination. At a regular time interval, one of the flasks was taken out and the incubation mixture was filtered and extracted with diethyl ether. The conversion yield of the product was determined on the basis of the peak ratio of the substrate and product in the GLC spectrum of the extract.

(6R)-Levodione (**2**): $[\alpha]_D^{25}$ (−39.0 ± 0.6)° (*c* 0.17, $CHCl_3$). – HR-EIMS: *m/z* (rel. int.) = 154 (100) $[M]^+$, 139 (100), 111 (12), 83 (29), 69 (93), 111 (12), 56 (100), 42 (100), 41 (100). – ^1H NMR (500 MHz, $CDCl_3$ TMS): δ = 1.09 (3H, s, H-9), 1.12 (3H, d, J = 6.4 Hz, H-7), 1.18 (3H, s, H-8), 2.31 (1H, dd, J = 14.5 and 17.5 Hz, H-5_{ax}), 2.49 (1H, d, J = 15.5 Hz, H-3_b), 2.70 (1H, d, J = 4.5 Hz, H-5_{eq}), 2.74

(1H, d, J = 4.5 Hz, H-3_a), 2.98 (1H, m, H-6_{ax}). – ^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ = 14.6 (C-7), 25.5 (C-9), 26.5 (C-8), 39.8 (C-6), 44.2 (C-2), 44.9 (C-5), 52.7 (C-3), 207.6 (C-1), 214.0 (C-4) (Leuenberger *et al.*, 1976; Majewski and Nowak, 1998).

(4R,5S)-4-Hydroxy-3,3,5-trimethylcyclohexanone (**3**): $[\alpha]_D^{25}$ +23.5° (*c* 0.02, $CHCl_3$). – HR-EIMS: *m/z* (rel. int.) = 156 (100) $[M]^+$, 141 (100), 98 (12), 98 (60), 83 (100), 69 (69), 58 (100), 41 (91). – ^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ = 0.87 (3H, s, H-9), 1.05 (3H, d, J = 6.7 Hz, H-7), 1.08 (3H, s, H-8), 1.87 (1H, dt, J = 13.7 and 1.0 Hz, H-2_{ax}), 2.05 (1H, dq, J = 13.5 and 2.0 Hz, H-6_{ax}), 2.21 (1H, m, H-5), 2.33 (1H, t, J = 13.4 Hz, H-6_{eq}), 2.57 (1H, d, J = 13.2 Hz, H-2_{eq}), 3.32 (1H, brs, H-4). – ^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ = 18.2 (C-7), 25.6 (C-9), 27.1 (C-8), 33.0 (C-2), 39.6 (C-3), 43.1 (C-6), 48.5 (C-5), 77.3 (C-4), 211.7 (C-1) (Khare *et al.*, 1988).

(4R,6R)-Actinol (**4**): $[\alpha]_D^{25}$ –14.0° (*c* 0.17, $CHCl_3$). – HR-EIMS: *m/z* (rel. int.) = 156 (100) $[M]^+$, 141 (100), 98 (12), 98 (60), 83 (100), 69 (69), 58 (100), 41 (91). – ^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ = 1.00 (3H, d, J = 6.4 Hz, H-7), 1.04 (3H, s, H-9), 1.18 (3H, s, H-8), 1.37 (1H, dt, J = 13.5 and 5.0 Hz, H-5_b), 1.55 (1H, t, J = 12.5 Hz, H-3_b), 2.04 (1H, dt, J = 13.0 and 4.0 Hz, H-3_a), 2.24 (1H, m, H-5_a), 2.71 (1H, m, H-6), 4.31 (1H, m, H-4). – ^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ = 14.7 (C-7), 25.7 (C-9), 26.4 (C-8), 37.9 (C-6), 44.3.6 (C-2), 44.4 (C-5), 49.5 (C-3), 65.7 (C-4), 215.3 (C-1) (Leuenberger *et al.*, 1976).

(4R)-Hydroxyisophorone (**5**): $[\alpha]_D^{25}$ +10.7° (*c* 0.03, $CHCl_3$). – HR-EIMS: *m/z* (rel. int.) = 154 (20) $[M]^+$, 121 (10), 98 (100), 70 (25), 31 (40). – ^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ = 1.00 (3H, s, H-9), 1.05 (3H, s, H-8), 2.02 (1H, t, J = 1.2 Hz, H-7), 2.19 (1H, d, J = 16.2 Hz, H-6_a), 2.37 (1H, d, J = 16.4 Hz, H-6_b), 4.01 (1H, s, H-4), 5.84 (1H, d, J = 0.6 Hz, H-2) (Hennig *et al.*, 2000).

(4S)-Phorenol (**6**): $[\alpha]_D^{25}$ –113.3° (*c* 0.01, $CHCl_3$). – HR-EIMS: *m/z* (rel. int.) = 154 (20) $[M]^+$, 121 (10), 98 (100), 70 (25), 31 (40). – ^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ = 1.10 (3H, s, H-9), 1.13 (3H, s, H-8), 1.77 (1H, t, J = 1.8 Hz, H-7), 1.82 (1H, dd, J = 13.0 and 10.0 Hz, H-5_b), 2.16 (1H, ddt, J = 12.8, 5.2 and 2.0 Hz, H-5_a), 4.58 (1H, m, H-4), 5.59 (1H, d, J = 0.6 Hz, H-3) (Li and Wu, 2002; Kiyota *et al.*, 1999).

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Buque-Taboada E. M., Straathof A. J., Heijnen J., Van Der Wielen J. J., and Luuk A. M. (2004), *In situ* product removal using a crystallization loop in asymmetric reduction of 4-oxoisophorone by *Saccharomyces cerevisiae*. Biotechnol. Bioeng. **86**, 795–800.

Burden R. S. and Taylor H. F. (1970), The structure and chemical transformation of xanthoxin. Tetrahedron Lett. **47**, 4071–4074.

Ernst H. (2002), Recent advances in industrial carotenoid synthesis. Pure Appl. Chem. **74**, 1369–1382.

Hennig M., Puntener K., and Scalone M. (2000), Synthesis of (*R*)- and (*S*)-4-hydroxyisophorone by ruthenium-catalyzed asymmetric transfer hydrogenation of ketoisophorone. Tetrahedron: Asym. **11**, 1849–1858.

Hirata T., Aoki T., Hirano Y., Ito T., and Suga T. (1981), The biotransformation of foreign substrates by tissue cultures. I. The hydroxylation of linalool and its related compounds with the suspension cells of *Nicotiana tabacum*. Bull. Chem. Soc. Jpn. **54**, 3527–3529.

Kataoka M., Kotaka A., Hasegawa A., Wada M., Yoshizumi A., Nakamori S., and Shimizu S. (2002), Old yellow enzyme from *Candida macedoniensis* catalyzes the stereospecific reduction of the C=C bond of ketoisophorone. Biosci. Biotechnol. Biochem. **66**, 2651–2657.

Katoh K., Ishikawa M., Miyake K., Ohta Y., Hirose Y., and Iwamura T. (1980), Nutrient utilization and requirement under photoheterotrophic growth of *Marchantia polymorpha*: Improvement of the culture medium. Physiol. Plant. **49**, 241–247.

Khare A., Moss G. P., and Weedon B. C. L. (1988), Carotenoids and related compounds. Part 42. Structure of isomytiloxanthin. J. Chem. Soc., Perkin Trans. 1 **6**, 1389–1395.

Kiyota H., Nakabayashi M., and Oritani T. (1999), Hydrolase-catalyzed preparation of (*R*)- and (*S*)-4-hydroxy-2,6,6-trimethyl-2-cyclohexen-1-one (phorenol), the key synthetic intermediates for abscisic acid. Tetrahedron: Asym. **10**, 3811–3817.

Leuenberger H. G. W., Boguth W., Widmer E., and Zell. R. (1976), Synthesis of optically active natural carotenoids and structurally related compounds. I. Synthesis of chiral key compound (4*R*,6*R*)-4-hydroxy-2,2,6-trimethylcyclohexanone. Helv. Chim. Acta **59**, 1832–1849.

Li C.-Y. and Wu T.-S. (2002), Constituents of the pollen of *Crocus sativus* L. and their tyrosinase inhibitory activity. Chem. Pharm. Bull. **50**, 1305–1309.

Majewski M. and Nowak P. (1998), Rate of deprotonation of a simple ketone by lithium diisopropylamide. Tetrahedron Lett. **39**, 1661–1664.

Murashige T. and Skoog F. (1962), A revised medium for rapid growth and bioassays with *Tobacco* tissue cultures. Physiol. Plant **15**, 473–497.

Nishi K., Sode K., and Karube I. (1989), Microbial conversion of dihydrooxoisophorone (DOIP) to 4-hydroxy-2,2,6-trimethylcyclohexanone (4-HTMCH) by thermophilic bacteria. J. Biotechnol. **9**, 117–128.

Nishi K., Sode K., and Karube I. (1990), Sequential two-step conversion of 4-oxoisophorone to 4-hydroxy-2,2,6-trimethylcyclohexanone by thermophilic bacteria. Appl. Microbiol. Biotechnol. **33**, 245–250.

Ono K., Ohyama K., and Gamborg O. L. (1979), Regeneration of the liverwort *Marchantia polymorpha* L. from protoplasts isolated from cell suspension culture. Plant Sci. Lett. **14**, 225–229.

Wada M., Yoshizumi A., Noda Y., Kataoka M., Shimizu S., Takagi H., and Nakamori S. (2003), Production of a doubly chiral compound, (4*R*,6*R*)-4-hydroxy-2,2,6-trimethylcyclohexanone, by two-step enzymatic asymmetric reduction. Appl. Environ. Microbiol. **69**, 933–937.