Biotransformation of sesquiterpenoids having α,β-unsaturated carbonyl groups with cultured plant cells of *Marchantia polymorpha*

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Abstract

The biotransformation of sesquiterpenoids having an α,β-unsaturated carbonyl group, such as α-santonin (**1**), lancerodiol *p*-hydroxybenzoate (**2**), 8,9-dehydronootkatone (**3**) and nootkatone (**4**), with cultured suspension cells of *Marchantia polymorpha* was investigated. It was found that the C-C double bond of **1** and **2** was hydrogenated to give 1,2-dihydro-α-santonin (**5**) and 3,4-dihydrolancerodiol *p*-hydroxybenzoate (**6**), respectively, while the allylic position of the C-C double bond of **3** and **4** was hydroxylated to give 13-hydroxy-8,9-dehydronootkatone (**7**) and 9-hydroxynootkatone (**8**), respectively.

Keywords: Biotransformation; *Marchantia polymorpha*; Hydrogenation; Hydroxylation; α-Santonin; Lancerodiol *p*-hydroxybenzoate; 8,9-Dehydronootkatone; Nootkatone.

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1. Introduction

Terpenoids are a large class of naturally occurring compounds, and are not only known as raw materials for flavor and fragrances but also biologically active substances against microorganisms, plants, insects and animals. Since a great majority of biologically active terpenoids are produced as plant secondary metabolites, many naturally occurring terpenoids were modified with biocatalysts to get substances with an enhanced biological activity [1-13].

The ability of biocatalysts to convert foreign substrates into chemo-, regio-, stereo- and enantioselectively useful substances under mild condition is one of great interest, as products may be formed which are difficult to prepare by synthetic chemical methods. Recently, we found that cultured plant cells contain several different enzymes participating in the asymmetric hydrogenation of the C-C double bond of monoterpene enones [14,15]. Recently, it was found that cultured cells of *Marchantia polymorpha* have bigger potentiality to hydrogenate the C–C double bond of maleimides in the several kinds of cultured plant cells [16]. In order to develop useful biocatalysts, we have further investigated the potentiality of the plant cells to convert natural sesquiterpenoids having more complex enone structures, such as α-santonin (**1**) [2,8,11], lancerodiol *p*-hydroxybenzoate (**2**) [17,18], 8,9-dehydronootkatone (**3**) and nootkatone (**4**) [12], as model compounds with cultured suspension cells of *Marchantia polymorpha*.

2. Experimental

2.1. Analysis

Analytical and preparative TLCs were carried out on glass sheets (0.25 mm and 0.5 mm) coated with silica gel (Merck silica gel 60; GF_{254}). GLC was carried out with FID and

a capillary column (0.25 mm x 30 m) coated with 0.25 μm ZB-5 (Zebron) using N_2 as carrier gas (60 cm^3 min^{-1}) at column temperature 100-250 ^oC. HPLC was carried out on Puresil C_{18} column (Waters) using $CH_3CN:H_2O$=2:3 (v/v) as the eluent. ^1H and ^{13}C NMR spectra were obtained using a JEOL LA500 spectrometer using tetramethylsilane as an internal standard. Mass spectra were performed using a JEOL SX-102A spectrometer with an ionizing energy of 70 eV.

2.2 Substrates

Substrates used for biotransformation experiments were α-santonin (Aldrich), lancerodiol *p*-hydroxybenzoate (isolated from *Ferula sinaica* species, Asteraceae), 8,9-dehydronootkatone (donated from Prof. Y. Noma of Tokushima Bunri University) and nootkatone (Aldrich).

2.3. Plant Material

The cells of *M. polymorpha* [19] have been subcultured routinely every 3 weeks using MSK-II medium [20], containing 2% glucose, 0.1% inositol, 10 mM of 2,4-dichlorophenoxyacetic acid (2,4-D), more than 10 years in our laboratory. Prior to use for biotransformation experiments, the cultured cells were transplanted to 300 ml conical flask containing 100 ml of MSK-II medium with 2% glucose, 0.1% inositol, 10 mM of 2,4-D and cultured on a rotary shaker (110 rpm) for 10 days at 25 ^oC under illumination (4000 lux).

*2.4. Biotransformation of sesquiterpenoids (**1**-**4**) with cultured plant cells of M. polymorpha*

To the flask containing the suspended cells (about 20 g) of *Marchantia polymorpha* in the medium (100 ml), each substrates **1**-**4** (20 mg) in methanol (0.2 ml) was administered, and the cultures were incubated at 25 ^oC on a rotary shaker (110 rpm) under illumination (4000 lux). After the incubation, the cells and medium were separated by filtration with

suction. The filtrated medium was extracted with CH_2Cl_2 and the extract was concentrated by rotary evaporator to give crude products. These crude products were subject to preparative TLC with ether : hexane (3:1) to give products **5-8** in a pure form. The conversion yields were determined by GLC and HPLC analysis, as shown in Table 1. The structure of each product was identified by NMR and MS analysis.

1,2-Dihydro-α-santonin (**5**): IR ($CHCl_3$) 1781, 1670 (C=O); EIMS m/z (rel. int.) 248 (100, $[M]^+$), 233 (30), 192 (96), 149 (53), 136 (41), 91 (40), 69 (45), and 55 (97); ^{1}H NMR ($CDCl_3$) δ 1.25 (3H, d, *J* = 7.0 Hz, 13-H), 1.31 (3H, s, 14-H), 1.53 (1H, dd, *J* = 4.5 and 13.0 Hz, 1-Hb), 1.65 (1H, dq, *J* = 3.5 and 13.0 Hz, 8-Hb), 1.72 (1H, dt, *J* = 13.5 and 3.0 Hz, 9-Hb), 1.79 (1H, dt, *J* = 13.5 and 5.0 Hz, 1-Hb), 1.89 (1H, qd, *J* = 11.5 and 3.5 Hz, 7-H), 1.90 (1H, td, *J* = 13.5 and 4.5 Hz, 9-Ha), 1.97 (1H, m, 8-Ha), 1.98 (3H, d, *J* = 2.0 Hz, H-15), 2.33 (1H, dq, *J* = 12.0 and 7.0 Hz, 11-H), 2.43 (1H, ddd, *J* = 16.5, 4.5 and 4.0 Hz, 2-Ha), 2.52 (1H, ddd, *J* = 16.5, 14.0 and 5.0 Hz, 2-Hb), 4.67 (1H, dd, *J* = 2.0 and 11.5 Hz, 6-H); ^{13}C NMR (see Table 2) [Lit. 2 and 11].

7,8-Dihydrolancerodiol *p*-hydroxybenzoate (**6**): EIMS, m/z (rel. int.) 356 (2, M-H_2O), 331 (10), 236 (12), 193 (16), 137 (13), and 121 (100); HR-FABMS 375.2178 ($[M]^+$+1) (calcd for $C_{22}H_{31}O_5$: 375.2171); ^{1}H NMR ($CDCl_3$) δ 0.83 (3H, d, *J* = 6.5 Hz, 12-H), 0.87 (3H, d, *J* = 6.5 Hz, 13-H), 1.18 (3H, d, *J* = 7.5 Hz, 15-H), 1.23 (1H, m, 3-Hb), 1.41 (3H, s, 14-H), 1.58 (1H, m, 3-Ha), 1.65 (1H, ddd, *J* = 3.0, 3.5, and 14.0 Hz, 7-Hb), 1.75 (1H, m, 11-H), 1.97 (1H, dd, *J* = 9.0 and 14.0, 7-Ha), 2.03 (1H, m, 2-Hb), 2.05 (1H, d, *J* = 11 Hz, 5-H), 2.17 (1H, dt, *J* = 14.5 and 3.5 Hz, 2-Ha), 2.58 (1H, dq, *J* = 3 and 7.5 Hz, 8-H), 2.59 (2H, brs, 10-H), 5.56 (1H, ddd, *J* = 3.5, 9.0 and 11.0 Hz, 6-H), 6.87 (2H, d, *J* = 10 Hz, 3',7'-H), 7.92 (2H, d, *J* = 10 Hz, 4',6'-H); ^{13}C NMR (see Table 2).

13-Hydroxy-8,9-dehydronootkatone (**7**): EIMS, m/z (rel. int.) 232 (0.5, $[M]^+$), 214 (43, M-H_2O), 172 (45), 129 (30), 115 (25), and 91 (39); ^{1}H NMR ($CDCl_3$) δ 0.96 (3H, d, *J* = 7.0 Hz, 15-H), 1.05 (3H, s, 14-H), 1.24 (1H, t, J = 13.0 Hz, 6-Hb) , 1.96 (1H, dq, J = 5.0 and 7.0 Hz, 4-H), 2.04 (1H, dd, *J* = 5.0 and 13.0 Hz, 6-Ha), 2.33 (2H, m, 3-H), 3.15 (1H, dt, J = 12.0 and 2.5 Hz, 7-H), 4.17 (2H, s, 13-H), 4.97 (1H, s, 12-Hb), 5.16 (1H, s, 12-Ha), 5.73 (1H, s, 1-H), 6.12 (1H, d, *J* = 10.0 Hz, 9-H), 6.21 (1H, dd, *J* = 3.0 and 10.0 Hz, 8-H) ; ^{13}C

NMR (see Table 2).

9-Hydroxynootkatone (**8**): EIMS, m/z (rel. int.) 234 (67, $[M]^+$), 216 (86, M- H_2O), 191 (88), 166 (55), and 137 (83); ^{1}H NMR ($CDCl_3$) δ 0.94 (3H, d, *J* = 7, 15-H), 1.08 (1H, t, *J* = 12.7, 6-Hb), 1.41 (3H, s, 14-H), 1.53 (1H, dt, *J* = 3.5 and 13.5 Hz, 8-Hb), 1.75 (3H, s, 13-H), 1.95 (1H, dt, *J* = 13.5 and 3.0 Hz, 6-Ha), 1.99 (1H, ddq, *J* = 14.0, 4.0 and 7.0 Hz, 4-H), 2.06 (1H, dq, *J* = 13.5 and 2.5 Hz, 8-Ha), 2.26 (1H, dd, *J* = 4.0 Hz, 17.5, 3-Hb), 2.35 (1H, dd, *J* = 14.0 and 17.0 Hz, 3-Ha), 2.76 (1H, tt, *J* = 6.0 and 13.5 Hz, 7-H), 4.45 (1H, t, *J* = 3.0 Hz, 9-H), 4.73 (1H, brd, *J* = 0.9 Hz, 12-Ha), 4.76 (1H, t, *J* = 1.5 Hz, 12-Hb), 5.86 (1H, s, 1-H) ; ^{13}C NMR (see Table 2) [Lit. 21].

2. 5. Time-course experiments

Cultured cells of *M. polymorpha* (about 20 g) was transferred to a 300 ml Erlenmeyer flask containing 100 ml MSK-II medium, and cultured with continuous shaking for 10 days at 25 ^{o}C under illumination. The substrates **1** or **2** (20 mg) were added to the suspension cultures and incubated at 25 ^{o}C in rotary shaker (110 rpm) under illumination. At a regular time interval, a part (10 ml) of the incubation mixture was taken out under sterile conditions and then extracted with CH_2Cl_2. The yields of the product were determined on the basis of the peak area from HPLC analyses and are expressed as a relative percentage to the total amounts of the whole reaction mixtures extracted.

3. Result and discussion

Biotransformation of sesquiterpenoids, **1**-**4**, with suspension cells of *M. polymorpha* gave products, **5**-**8**, respectively, as shown in Table 1.

Product **5** had a molecular formula $C_{15}H_{20}O_3$, estimated with its EIMS spectral data. Comparison of the ^{1}H- and ^{13}C NMR spectra of **5** with those of the authentic sample showed the product **5** to be 1,2-dihydro-α-santonin [2].

Product **6** had a molecular formula $C_{22}H_{30}O_5$, estimated with its HR-FABMS spectral data. Its ^{1}H- and ^{13}C NMR spectra were similar to those of substrate **2**, except for the appearance of new signals at δ 1.18 (3H, d, J=7.5 Hz) and 2.17 (1H, dt, J=14.5 and 3.5 Hz) in the ^{1}H NMR spectrum of **6**, instead of the methyl proton signal at δ 1.88 (3H, bs, 15-H) and the olefinic proton signal at δ 6.16 (1H, bs, 7-H) of substrate **2**, respectively. The complete ^{1}H and ^{13}C NMR analyses with ^{1}H-^{1}H COSY, HMQC and HMBC revealed the product **6** to be 7,8-dihydrolancerodiol *p*-hydroxybenzoate. The relative configuration at C-8 was determined by the NOE's experiments; irradiation of the signal at δ 5.56 (6-H) enhanced the signal at δ 2.58 (8-H) and no effect was observed on 5-H, 12-H, 13-H and 15-H. This indicates the orientation of the methyl group at C-8 of **6** to be *cis* against the hydroxybenzoyl group at C-6.

Product **7** had a molecular formula $C_{15}H_{20}O_2$, estimated with its EIMS spectral data. Its ^{1}H- and ^{13}C NMR spectra were similar to those of **3**, except the replacement of the olefinic methyl at δ 1.72 (13-H) in **3** by a signal at δ 4.17 (2H, s) which correlated with a carbon signal at δ 65.0 in ^{13}C NMR spectrum. These showed that the product **7** is 13-hydroxy-8,9-dehydronootkatone.

Product **8** had a molecular formula $C_{15}H_{22}O_2$, estimated with its EIMS spectral data. From ^{1}H- and ^{13}C NMR spectral analyses, it was clarified that substrate **4** was hydroxylated; appearance of a new signal at δ 4.45 (1H, t, *J* = 3.0 Hz) in the ^{1}H NMR spectrum, which indicated the presence of a CH(OH) group. The complete ^{1}H and ^{13}C NMR analyses with ^{1}H-^{1}H COSY, HMQC and HMBC revealed the product **8** to be 9-hydroxynootkatone [21]. The relative stereochemistry at 9-position was confirmed by the analyses of the coupling constants of related proton signals. The coupling constants of 3.0 Hz between H-9 (δ 4.45) and both of H-8a (δ 2.06) and H-8b (δ 1.53) indicated that the conformation of H-9 is equatorial. Additionally, the coupling constants of 13.5 Hz between H-7 (δ 2.76) and both of H-6b (δ 1.08) and H-8b (δ 1.53) indicated that the conformations of H-7 and H-8b are axial. These indicate that the orientation of 9-hydroxyl group is *trans* respective to the propenyl group at C-7.

Thus, it was found that the cultured cells of *M. polymorpha* hydrogenate the 1,2-

double bond of α-santonin (**1**) and the 7,8-double bond of lancerodiol *p*-hydroxybenzoate (**2**) to give dihydro derivatives, **5** and **6**, respectively, although the 4,5-double bond of α-santonin (**1**) was not reduced. The stereochemistry of hydrogen attack to the C-C double bond of **2** was determined. It appear that the addaition took place from the *si* face at the 8-position. This stereochemistry of hydrogen attack is analogous to that in the biotransformation of carvone with *Nicotiana tabacum* cells [22]. The time courses in the biotransformation of **1** and **2** were indicated that these substrates were easily reduced in 3 days incubation to give the dihydro derivatives in a 50% and 70% yield, respectively, as shown in Fig.1. It has been reported that biotransformation of α-santonin (**1**) by microbial biocatalysts gave 1,2-dihydro- and 3,4-dihydro derivatives in a low yield [8,11]. In the biotransformation by *M. polymorpha* cells, 1,2-dihidro derivative was obtained in a good yield, but no 3,4-dihydro derivative was obtained.

On the other hand, the C-C double bond of 8,9-dehydronootkatone (**3**) and nootkatone (**4**) were not hydrogenated. However, the allylic positions of the C-C double bond were hydroxylated to give 13-hydroxy-8,9-dehydronootkatone (**7**) and 9-hydroxynootkatone (**8**) in a 10% and 8% yield, respectively.

From above result, it was found that the cultured cells of *M. polymorpha* have potentiality of the hydrogenation of the conjugated C-C double bond and the hydroxylation of the allylic position of C-C double bond.

Acknowledgements

The authors thank Professor Yoshiaki Noma, Tokushima Bunri University, for a gift of the sample of 8,9-dehydronootkatone, and Professor Yoshinori Asakawa and Dr. Toshihiro Hashimoto, for a gift of the authentic samples of 1,2-dihydro-α-santonin and 3,4-dihydro-α-santonin.

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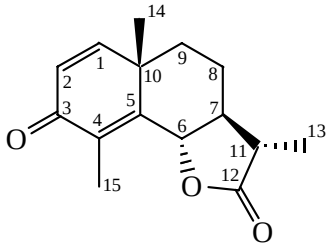

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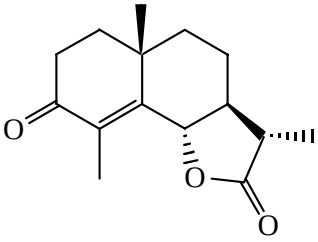

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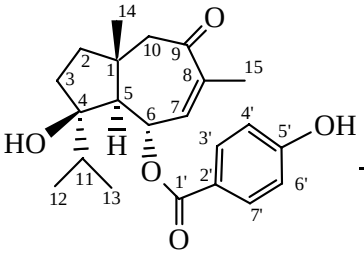

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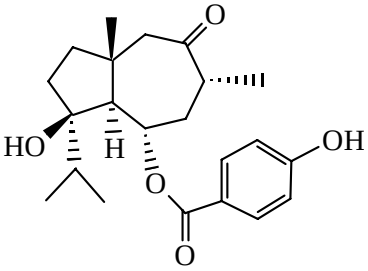

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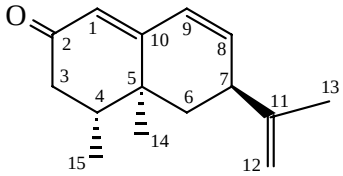

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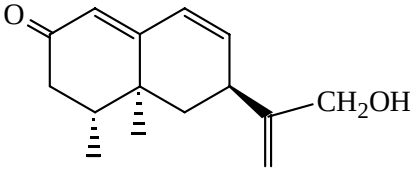

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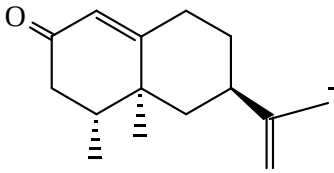

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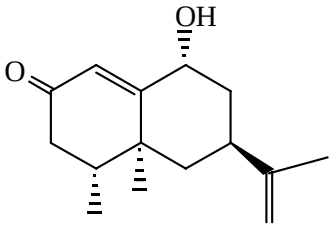

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Table 1
Biotransformation of sesquiterpenoids, **1**-**4**, by cultured cells of *M. polymorpha*

Substrates	Incubation time / day	Products	Yields / %
1	8	**5**	50
2	4	**6**	72
3	4	**7**	10
4	4	**8**	8

Table 2

^{13}C NMR spectral data for compounds **5-8**

Carbon	δ_C			
	5	**6**	**7**	**8**
1	38.2	42.7	124.4	127.3
2	33.6	39.3	199.6	200.6
3	198.8	41.6	42.3	42.4
4	128.6	85.5	38.8	41.2
5	152.4	57.4	50.9	38.7
6	81.9	72.8	40.3	43.6
7	52.9	31.8	37.7	34.0
8	24.6	55.5*	128.2	37.9
9	41.7	213.4	139.7	73.5
10	38.3	55.5*	163.3	168.0
11	41.2	36.9	151.4	149.0
12	177.7	18.4	111.5	109.4
13	12.4	17.3	65.0	20.9
14	24.3	21.3	16.0	18.1
15	11.2	18.0	14.8	14.9
1’	---	166.2	---	---
2’	---	122.3	---	---
3’	---	132.2	---	---
4’	---	115.5	---	---
5’	---	160.6	---	---
6’	---	115.5	---	---
7’	---	132.2	---	---

* Overlapped

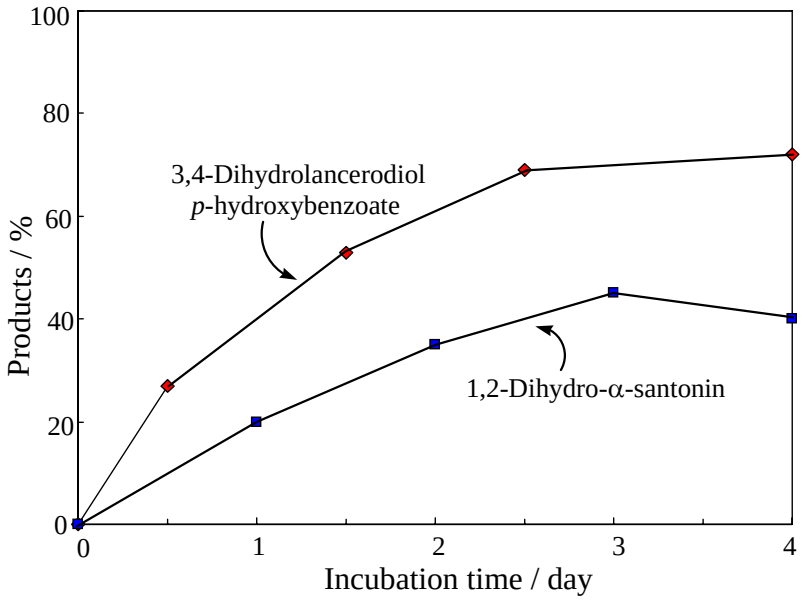

Fig. 1. Time-course experiments in the hydrogenation of α-santonin (**1**) and lancerodiol *p*-hydroxybenzoate (**2**) with the cultured cells of *M. polymorpha*