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Biotransformation of valencene by cultured cells of *Gynostemma pentaphyllum*

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Abstract

It has been found that the suspension cultures of *Gynostemma pentaphyllum* convert valencene (**1**) into nootkatone (**2**) as the major product and nootkatol (**3**) as the minor product. Based on this finding, a further study was conducted to investigate the biotransformation of **1** by other cultured plant cells (*Caragana chamlagu*, *Hibiscus cannabinus*).

Keywords: Biotransformation; Plant catalyst; *Gynostemma pentaphyllum*; Nootkatone; Nootkatol; Epinootkatol

1. Introduction

Valencene (**1**) is an essential oil component of sesquiterpene obtained from *Citrus paradisi* Macfayden [1] and only a few compounds related to **1** show bioactivity. For example, nootkatone (**2**) is a mildly pungent sesquiterpene ketone valued for its contribution to the distinctive flavor of grapefruit (*C. paradisi*). Furthermore, **2** has an insecticidal activity against *Drosophila meranogaster* [2]. This compound has been isolated from *Alpinia oxyphylla* [3].

On the other hand, nootkatol (**3**), a sesquiterpene possessing a calcium-antagonistic activity, has been isolated from *A. oxyphylla* Miquel [4].

Some studies have already reported the chemical synthesis of **2** by chemical reagents [5,6]. However, an environmentally sensitive, milder, energy-saving and regio- and stereo-specific method has recently been required for synthetic chemistry. Plant cell cultures [7–9] and microbacteria are considered to be useful biocatalysts for reactions such as the hydroxylation at allelic positions, the oxidation–reduction of alcohols and ketones, and reduction of carbon–carbon double bonds.

We have reported the conversion of thujopsene into mayuron by the cultured cells of *Caragana chamlagu* (Leguminoceae), the degradation of bisphenol A and the biotransformation of ionones and diketones [10–13].

Takemoto et al. reported that bioactive gypenosides have been isolated from the leaves of *Gynostemma pentaphyllum* (Cucurbitaceae) [14]. However, no report has been made on the separation of gypenoside from callus tissues and the biotransformation of terpenes by the cultured cells of *G. pentaphyllum*. Previously, we succeeded for the first time in inducing the callus tissues from the leaves of *G. pentaphyllum*.

In this investigation, we studied the biotransformation of **1** in order to investigate the ability of cultured cells of *G. pentaphyllum* as a new biocatalyst. Furthermore, we compared the biotransformation activity of the cultured cells of *G. pen-*

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taphyllum with that of the cultured cells of *C. chamlagu* and *Hibiscus cannabinus*.

2. Experimental

2.1. Analytical and substrates

Melting points were determined on a Shimazu micro melting point apparatus. IR spectra were recorded using a Hitachi 270-50 and a Jasco FT-IR 230 spectrophotometer. Optical rotation was determined using a Horiba SEPA-200 polarimeter. ^{1}H and ^{13}C NMR spectra were recorded at 400 MHz using a JEOL GX-400 spectrometer with $SiMe_4$ as the internal reference. High resolution mass spectra (HREIMS) were obtained on a Hitachi M-80B spectrometer. GC–MS were recorded on a Shimadzu GC–MS QP5050 (EI-MS) 70 eV using DVI (0.25 mm × 30 m, 0.25 μm) capillary column. GLC was performed on Shimadzu GC-9A and 14A model equipped with 2% OV-17 column (5 mm × 3 m) at 200 °C.

Valencene (**1**) and nootkatone (**2**) were supplied by T. Hasegawa Co., Ltd. The purity of each was >98% by GLC analysis.

For inducing the callus tissues of *G. pentaphyllum*, the tissues were obtained by surface sterilization of leaves for 30 s in 70% EtOH. The tissues of leaves were washed twice with sterile distilled water and were then transferred to agar MS medium [15]. Callus tissues from leaves of *G. pentaphyllum*, *C. chamlagu* and *H. cannabinus* has been maintained our laboratory for approximately 10 years.

Unless otherwise stated all cultures were grown in MS medium plus 3% sucrose and 1 ppm of 2,4-dichlorophenoxyacetic acid (2,4-D) at 25 °C for 20 days in the dark on an orbital shaker (120 rpm).

2.2. Biotransformation of **1** by G. pentaphyllum

The callus tissues (4 g) were transferred to MS-medium (100 ml) containing 1 ppm of 2,4-D and 3% sucrose. Valencene (**1**, 90 mg) was added to the suspension in several 300 ml flask containing 100 ml MS-medium. The filtered culture medium was extracted with EtOAc, the solvent dried over Na_2SO_4, and removed in vacuo. The residue (83 mg) was chromatographed on SiO_2, using benzene–EtOAc (10:1) to give nootkatone (**2**, 72% by GLC). Elution with benzene–EtOAc (5:1) afforded nootkatol (**3**, 11% by GLC). Elution with benzene–EtOAc (5:1) gave epinootkatol (**4**, 5% by GLC).

2.3. Biotransformation of **1** by C. chamlagu

Valencene (**1**) (90 mg) was added to a suspension culture (from 4 g callus) in 300 ml flask containing 100 ml MS-medium. Metabolites (85 mg) were extracted as above and the yields of the products were determined on the basis of the peak area from GLC traces.

2.4. Biotransformation of **1** by H. cannabinus

Valencene (**1**) (90 mg) was added to a suspension culture (from 4 g callus) in 300 ml flask containing 100 ml MS-medium. Metabolites (83 mg) were extracted as above and the yields of the products were determined on the basis of the peak area from GLC traces.

2.5. Biotransformation of **4** by G. pentaphyllum

The callus tissues of *G. pentaphyllum* (2 g) were transferred to MS-medium (40 ml) containing 1 ppm of 2,4-D and 3% sucrose. Epinootkatol (**4**, 15 mg/0.3 ml EtOH) was added to the suspension in 100 ml flask containing 40 ml MS-medium for 15 days. The filtered culture medium was extracted with EtOAc, the solvent dried over Na_2SO_4, and removed in vacuo. The residue (10 mg) was chromatographed on SiO_2, using benzene–EtOAc (10:1) to give nootkatone (**2**, 93% by GLC).

2.6. Time course experiment

A callus of *G. pentaphyllum* (8 g) was transferred to 200 ml MS-culture medium in a 500 ml Erlenmeyer flask grown with continuous shaking for 3 days at 25 °C in the dark. The substrate (180 mg) was added to the suspension culture and incubated at 25 °C in a rotary shaker (120 rpm) in the dark. At regular intervals, several of the flasks were taken out and the incubation mixture was filtered and extracted with EtOAc. The yields of the products were determined on the basis of the peak area from GLC traces.

2.7. Reduction of **2**

A solution of **2** (109 mg) and $LiAlH_4$ (40 mg) in abs. ether (15 ml) was stirred for 10 h at room temperature. The suspension was treated as usual and extracted with ether. The residue was chromatographed on silica gel [benzene–EtOAc (10:1)]. Nootkatol (**3**) (4 mg) was eluted, followed by **4** (93 mg).

Nootkatone (**2**): mp 35–36 °C; $[\alpha]_D$: +191° (*c* 0.45, $CHCl_3$); IR ($CHCl_3$): ν 1660, 1620 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 0.97 (3H, s, H-15), 1.12 (3H, s, H-14), 1.74 (3H, s, H-13), 4.70 (2H, d, J = 8 Hz, H-12), 5.77 (1H, s, H-1); ^{13}C NMR ($CDCl_3$): δ 199.6 (s, C-2), 170.5 (s, C-11), 149.1 (s, C-10), 124.7 (d, C-1), 109.3 (t, C-12), 43.9 (t, C-9), 42.1 (t, C-3), 40.5 (d, C-7), 40.3 (d, C-4), 39.3 (s, C-5), 33.6 (t, C-8), 31.6 (t, C-6), 20.8 (q, C-13), 16.9 (q, C-15), 14.9 (q, C-14); HREIMS *m/z*: 218.1677 (calcd for $C_{15}H_{22}O$, 218.1671).

Nootkatol (**3**): mp 77–79 °C; $[\alpha]_D$: +203° (*c* 0.45, $CHCl_3$); IR ($CHCl_3$): ν 3251, 1615 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 0.89 (3H, s, H-15), 0.89 (3H, s, H-14), 1.71 (3H, s, H-13), 4.06 (1H, m, H-2), 4.68 (2H, m, H-12), 5.50 (1H, bs, J = 5.2 Hz, H-1); ^{13}C NMR ($CDCl_3$): δ 150.1 (s, C-10), 148.6 (s, C-11),

121.7 (d, C-1), 108.7 (t, C-12), 64.4 (d, C-2), 44.6 (t, C-9), 40.7 (d, C-7), 38.3 (s, C-5), 36.2 (t, C-3), 34.9 (q, C-13), 32.6 (t, C-8), 32.5 (t, C-6), 20.8 (d, C-4), 16.8 (q, C-15), 15.2 (q, C-14); HREIMS *m/z*: 220.1825 (calcd for $C_{15}H_{24}O$, 220.1827).

Epinootkatol (**4**): oil; $[\alpha]_D$: +90° (*c* 0.67, $CHCl_3$); IR ($CHCl_3$): ν 3260, 1625 cm^{-1}; ^1H NMR ($CDCl_3$): δ 0.88 (3H, s, H-15), 0.90 (3H, s, H-14), 1.71 (3H, s, H-13), 4.24 (1H, d, *J* = 7.8 Hz, H-2), 4.68 (2H, dd, *J* = 1.5, 2.4 Hz, H-12), 5.32 (1H, d, *J* = 1.7 Hz, H-1); ^{13}C NMR ($CDCl_3$): δ 150.3 (s, C-10), 146.1 (d, C-11), 124.3 (d, C-1), 108.6 (t, C-12), 68.1 (d, C-2), 44.6 (t, C-9), 41.8 (d, C-7), 39.3 (d, C-4), 38.2 (s, C-5), 37.3 (t, C-3), 32.9 (t, C-8), 32.4 (t, C-6), 20.8 (t, C-13), 18.2 (q, C-15), 15.4 (q, C-14); HREIMS *m/z*: 220.1828 (calcd for $C_{15}H_{24}O$, 220.1827).

3. Results and discussion

3.1. Biotransformation of **1** by G. pentaphyllum

The results of this biotransformation are shown in Fig. 1. The incubation of **1** with the suspension cultures of *G. pentaphyllum* for 20 days gave nootkatone (**2**) in a 72% yield. The spectral data for **2** agreed with those on the natural authentic sample from grapefruit oil (*C. paradisi*) [2].

The product (**3**) isolated in 11% yield had a band at 3251 cm^{-1} (OH) in its IR spectrum, and HREIMS gave M^+ 220.1825 ($C_{15}H_{24}O$). The ^{13}C NMR spectrum showed two tertiary methyls, one secondary methyl, three quaternary carbons, five methylenes, and four methine carbon signals. The ^1H NMR spectrum showed a methine proton signal at δ 4.06 (1H, br m, CH–OH) and an olefin proton signal at δ 5.50 (1H, bd, *J* = 5.2 Hz, CH=C). The IR, ^{13}C and ^1H NMR spectral data for compound **3** agreed with those of the authentic sample from the reduction of **2**. On the basis of these spectral data and its optical rotation, **3** was determined to be nootkatol.

The product (**4**) obtained in 5% yield had a band at 3260 cm^{-1} (OH) in its IR spectrum, and HREIMS gave M^+ 220.1828 ($C_{15}H_{24}O$). The ^{13}C NMR spectrum of **4** was the same as that of **3**. The ^1H NMR spectrum showed a methine proton signal at δ 4.24 (1H, d, *J* = 7.8 Hz, CH–O) and an olefin proton signal at δ 5.32 (1H, d, *J* = 1.7 Hz, CH=C). The IR, ^{13}C and ^1H NMR spectral data for compound **4** agreed with those of the authentic sample from the reduction of **2**. On the basis of these spectral data and its optical rotation, **4** was determined to be epinootkatol.

3.2. Biotransformation of **1** by C. chamlagu and H. cannabinus

In order to investigate the ability of other cultured plant cells, **1** was incubated with *C. chamlagu* and *H. cannabinus*. The results of this biotransformation are summarized in Table 1.

For the cultured cells of *C. chamlagu*, the reaction mixture (85 mg) was obtained after 20 days. According to the GC–MS data, **2** (25%) was obtained as the major product. The biotransformation of **1** using the cultured cells of *H. cannabinus* under the same conditions gave **2** (28%).

The metabolism of **1** by *G. pentaphyllum* is significantly higher than that by other cultures (*C. chamlagu* and *H. cannabinus*).

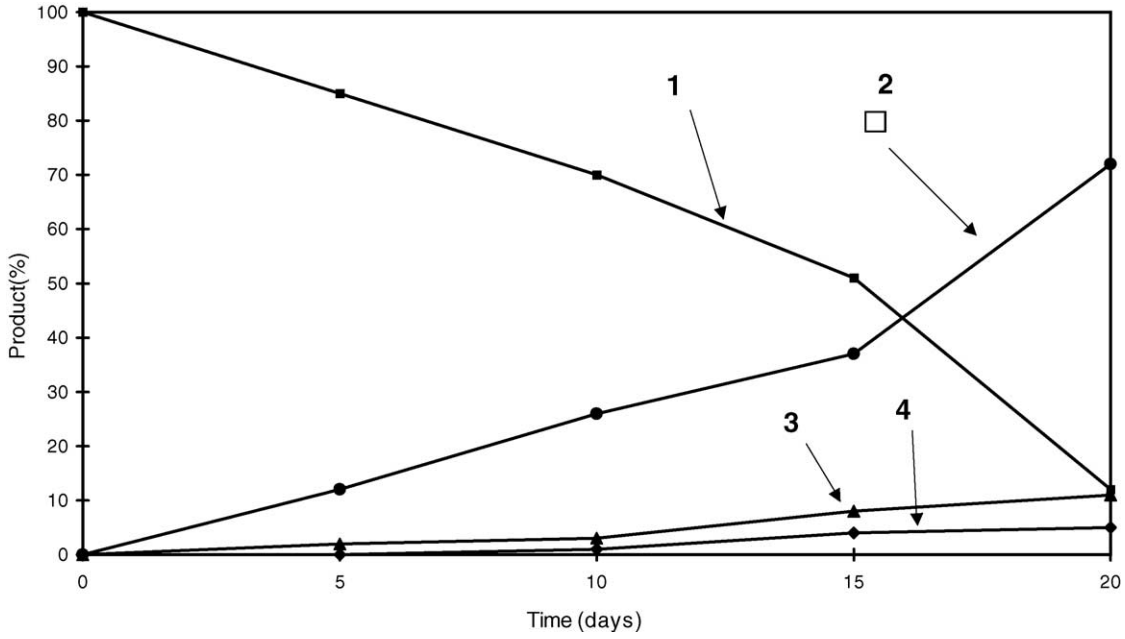
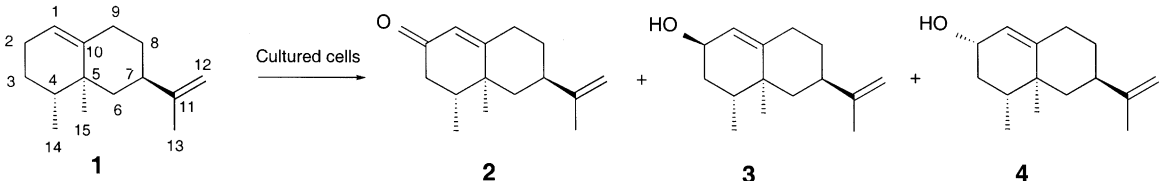
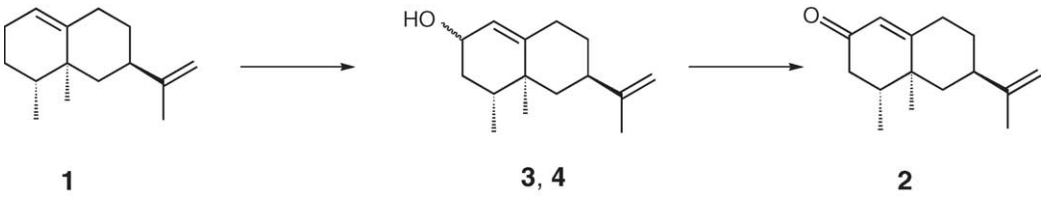


Fig. 1. Biotransformation of valencene (**1**) by *G. pentaphyllum*.

Table 1
Biotransformation of valencene (**1**) by plant cultured cells



Substrate	Cultured cells	Time (days)	Yields (%)
1	*C. chamlagu*	15	**2** (12), **3** (2), **4** (1)
1	*C. chamlagu*	20	**2** (25), **3** (4), **4** (2)
1	*H. cannabinus*	15	**2** (14), **3** (2), **4** (1)
1	*H. cannabinus*	20	**2** (28), **3** (3), **4** (1)
1	*G. pentaphyllum*	15	**2** (37), **3** (8), **4** (4)
1	*G. pentaphyllum*	20	**2** (72), **3** (11), **4** (5)



Scheme 1. Metabolic pathway of valencene (**1**) by plant cultured cells.

It is interesting to note that the biotransformation of **1** by *G. pentaphyllum* as a new biocatalyst preferentially produce **3** having bioactivity.

In order to investigate the course of the formation of nootkatone (**2**), transformation of **4** by *G. pentaphyllum* was performed to afford **2** with a yield of 86%. On the other hand, no transformation of **2** by *G. pentaphyllum* gave **3** and **4**. These experimental results showed that **3** and **4** were intermediates in the formation of **2**. On the base of these results, the metabolic pathway of the formation of **2** was assumed as shown in Scheme 1.

In conclusion, we have found that *G. pentaphyllum* cultures regioselectively convert the cycloolefinic part in the substrate into the corresponding unsaturated ketone and allyl alcohol.

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