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BIOTRANSFORMATION OF β-THUJAPLICIN BY CULTURED CELLS OF *EUCALYPTUS PERRINIANA*

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Key Word Index—*Eucalyptus perriniana*; Myrtaceae; cell culture; biotransformation; β-thujaplicin; hinokitiol; β-thujaplicin glucoside; β-thujaplicin gentiobioside; hinokitiol glucoside; hinokitiol gentiobioside; glucosylation; ^{1}H NMR; ^{13}C NMR.

Abstract—Four new biotransformation products, 4-isopropyltropolone 2-*O*-β-D-glucoside, 4-isopropyltropolone 2-*O*-β-D-gentiobioside, 6-isopropyltropolone 2-*O*-β-D-glucoside and 6-isopropyltropolone 2-*O*-β-D-gentiobioside, were isolated from cell suspension culture of *Eucalyptus perriniana* following administration of β-thujaplicin. The chemical structures of all products were determined by ^{1}H and ^{13}C NMR and MS spectroscopy. It was also observed that the production ratio of biotransformation products was higher in iron-deficient medium than in the medium containing iron ion. © 1997 Elsevier Science Ltd

INTRODUCTION

Plant cell culture is considered to be an important method for the propagation of rare plants and the production of useful secondary metabolites [1–3], such as saponins from *Panax ginseng* [4, 5] and shikonin from *Lithospermum erythrorhizon* [6]. Furthermore, cultured plant cells have the ability to specifically convert cheap and plentiful organic compounds into more useful compounds. The reactions involved in the biotransformation of organic compounds by plant cell cultures include oxidation, reduction, hydroxylation, esterification, methylation and isomerization [7–9].

Many kinds of useful secondary metabolites such as saponins and anthocyanins are produced in the form of glycosides in higher plants and most of them are accumulated in the vacuole of plant cells. It is known that cultured plant cells are also able to glycosylate exogenous compounds, and studies on glycosylation with cultured plant cells have been carried out for many compounds, e.g. digitoxigenin [10], terpenoids (menthol [11]), 1,8-cineole [12], steviol [13], 18 β-glycyrrhetinic acid [14]), eugenol [15] and tropic acid [16]. Glycosylation occurs readily in plant cells but with difficulty in microorganisms, and it is considered to be an important method for the conversion of water-insoluble compounds to water-soluble ones.

β-Thujaplicin (hinokitiol) is a naturally occurring aromatic seven-membered tropolone compound found in the heartwood of several cupressaceous plants such as *Chamaecyparis obtusa* var. *formosana* (Taiwan-hinoki in Japanese). *Thuja plicata* (Western red cedar) and *Thujopsis dolabrata* var. *hondai* (Hinokiasunaro in Japanese). β-Thujaplicin has antibacterial and antifungal activities, and a regulatory action on ethylene production and respiration in fruits and vegetables. It is used as a medicine, a food additive, a preservative and in cosmetics such as hair tonics [17]. However, the use of β-thujaplicin has been limited, as a consequence of its water-insolubility, sublimation, light decomposition, and chelation with metal ions.

In order to overcome some of these undesirable properties, we have investigated the biotransformation of β-thujaplicin into glycosylation products by cultured cells of *Eucalyptus perriniana* (Tsukinukiyukari in Japanese). In this paper, we describe the isolation and structure elucidation of the biotransformation products.

RESULTS AND DISCUSSION

Three-week-old cell suspension cultures of *E. perriniana* were administered β-thujaplicin **1** and incubated for a further 7 days. The methanol extract of the cells from these cultures yielded compounds **2**–**5**.

Enzymatic hydrolysis of products **2**–**5** afforded β-thujaplicin as the aglycone. In its FAB MS spectrum, product **2** showed a pseudo molecular ion peak at

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m/z 327 $[M+H]^+$. Four aromatic proton signals were observed at δ 6.66 (1H, *br d*, $J = 9.0$ Hz), 7.11 (1H, *dd*, $J = 12.0$, 9.0 Hz), 7.27 (1H, *br d*, $J = 12.0$ Hz), 7.52 (1H, *d*, $J = 1.0$ Hz) in the ^{1}H NMR spectrum of **2**. By analysis of ^{1}H-^{1}H COSY spectrum, these signals were assigned to H-5, H-6, H-7 and H-3, respectively. Furthermore, an anomeric proton signal was observed at δ 5.73 (1H, *d*, $J = 7.5$ Hz). The coupling constants of the signals of the sugar moiety of **2** established the presence of a β-D-glucopyranose. In the ^{13}C NMR spectrum, 16 carbon signals were observed and an anomeric carbon signal at δ 102.4, indicating that **2** was an *O*-glycoside. Enzymatic hydrolysis of **2** with β-glucosidase gave β-thujaplicin. β-Thujaplicin exists in two tautomeric forms, **1** and **1a**, so that glucoside was either compound **2** or compound **4**. The sugar linkage site in the aglycone was determined by NOE difference. Irradiation at the anomeric proton signal (δ 5.73) caused a NOE on the H-3 signal at δ 7.52 suggesting that the glucosyl moiety was attached to the 2-OH group of the aglycone. Therefore, the structure of **2** was 4-isopropyltropolone 2-*O*-β-D-glucoside.

Product **3** showed a pseudo molecular ion peak at *m/z* 489 $[M+H]^+$ which is larger by 162 mass units than that of **2**. In the ^{13}C NMR spectrum, **3** showed 22 carbon signals and two anomeric carbon signals were observed at δ 102.4 and 106.0 (Table 1). In the ^{1}H NMR spectrum of **3**, four assignable aromatic proton signals were observed at δ 6.64 (1H, *br d*, $J = 9.0$ Hz, H-5), 7.19 (1H, *dd*, $J = 12.0$, 9.0 Hz, H-6), 7.23 (1H, *br d*, $J = 12.0$ Hz, H-7) and 7.49 (1H, *d*, $J = 1.0$ Hz, H-3). The HOHAHA spectrum established the presence of two molecules of β-D-glucopyranose. A comparison of the ^{13}C NMR spectrum of **2** with that of **3** showed that the signals due to the aglycone moiety were similar and glycosylation shifts were observed for C-5 (−1.7 ppm) and C-6 (+7.4 ppm) of the inner glucose residue, confirming that **2** and **3** have the same aglycone and that gentiobiose was attached to the 2-OH group of the aglycone in **3**. Furthermore, HMBC correlation were observed between the following protons and carbons; H-6 and C-1; H-6 and C-4; H-7 and C-2; H-1′ and C-2. Based on the above spectral data, the structure of **3** was formulated as 4-isopropytropolone 2-*O*-β-D-gentiobioside.

Table 1. ^{13}C NMR spectral data of biotransformation products in C_5D_5N

Product		2	3	4	5
Aglycone	1	180.3	180.4	163.5	163.6
	2	163.5	163.3	180.4	180.5
	3	119.7	120.7	135.7	135.6
	4	154.5	154.6	157.1	157.3
	5	126.4	126.4	130.6	130.7
	6	136.5	136.6	132.3	133.1
	7	137.1	137.1	117.2	118.0
	8	39.1	38.8	38.4	38.4
	9	23.1	23.2	22.9	22.9
	10	23.3	23.4	23.0	23.0
Glc	1′	102.4	102.4	101.9	102.2
	2′	74.7	74.6	74.7	74.6
	3′	78.5	78.3	78.4	78.4
	4′	71.4	70.8	71.2	71.2
	5′	79.6	77.9	79.5	78.3
	6′	62.6	70.0	62.6	70.0
	1″		106.0		105.5
	2″		75.2		75.5
	3″		78.6		78.6
	4″		71.8		71.9
	5″		78.6		78.6
	6″		62.9		62.8

Table 2. Comparison of the biotransformation ratio* between BA1 and BA1 minus Fe media

Products	2	3	4	5	Total
Media					
BA1	2.4	0.6	6.6	1.3	10.9
BA1 minus Fe	6.8	1.6	7.4	7.6	23.4

* Wt of product/wt of substrate × 100 (%).

† BA1 is the medium containing iron ion and BA1 minus Fe is the one without iron ion.

Product **4** was found to have the same M_r (FAB-MS) as **2**. Its ^{13}C NMR spectrum showed 16 carbon signals. On analysis of the ^{1}H-^{1}H COSY spectrum of **4**, four assignable aromatic proton signals were observed at δ 6.66 (1H, *dd*, $J = 11.0$, 1.5 Hz, H-5), 6.87 (1H, *dd*, $J = 11.0$, 10.0 Hz, H-6), 7.29 (1H, *d*, $J = 1.5$ Hz, H-3) and 7.43 (1H, *d*, $J = 10.0$ Hz, H-7), and the component sugar was suggested to be β-D-glucopyranose. On irradiation of the H-1′ of glucopyranose at δ 5.72, a NOE was observed for H-7 of the aglycone unit at δ 7.43, showing that the glucosyl moiety was attached to the 1-OH group of the aglycone. Consequently, **4** was 6-isopropyltropolone-2-*O*-β-D-glucoside.

Product **5** gave a $[M+H]^+$ ion peak at *m/z* 489 in its FAB mass spectrum. In the ^{13}C NMR spectrum, **5** showed 22 carbon signals, and two anomeric carbons were observed at δ 102.2 and 105.5 (Table 1). In the ^{1}H NMR spectrum of **5**, four assignable aromatic proton signals were similar to those of **4**. The sugar moieties of **5** were shown to be two molecules of β-D-glucopyranose by analysis of the HOHAHA spectrum. In the ^{13}C NMR spectrum, the carbon signals due to the aglycone and sugar moieties of **5** were almost superimposable on those of **4** and **3**, respectively (Table 1). On irradiation of the H-1′ of inner glucopyranose at δ 5.63, a NOE was observed for H-7 of the aglycone unit at δ 7.72. Furthermore, the linkage site of the sugar moiety was ascertained to be the 1-OH group of the aglycone by analysis of the HMBC spectrum. Therefore, the structure of **5** was 6-isopropyltropolone 2-*O*-β-D-gentiobioside.

Based on the structures of biotransformation products, the pathway shown in Fig. 1 was proposed for

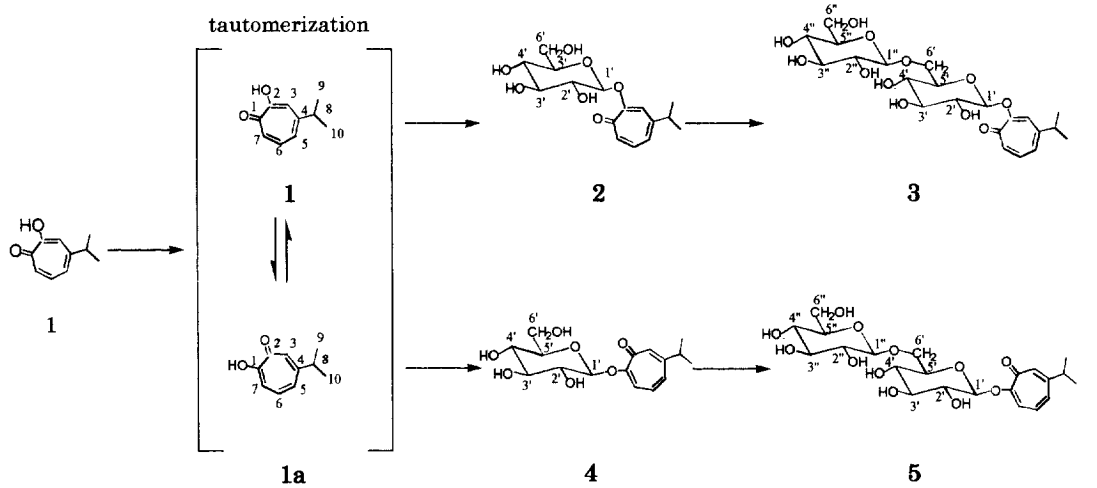

Fig. 1.

the metabolism of β-thujaplicin by cultured cells of *E. perriniana*. Since two types of products, products **2**, **3** and **4**, **5**, were produced, it was considered that β-thujaplicin formed a tautomeric mixture (**1** and **1a**) before glucosylation, and that compounds **3** and **5** were formed by glucosylation of compounds **1** and **1a** via **2** and **4**, respectively. It is noteworthy that the production ratio of glycosylated **1a** was higher than that of glycosylated **1**. It was also observed that the biotransformation ratio in BA1 minus Fe medium is higher than that using BA1 medium. This suggests that a hydroxyl group of β-thujaplicin is blocked and that glycosylation is inhibited, possibly as the result of chelation of iron by β-thujaplicin.

EXPERIMENTAL

^{1}H NMR and ^{13}C NMR spectra (C_5D_5N): Varian XL-300 and XL-400; Positive ion FAB MS (JEOL JMS DX-300): matrix; glycerol + thioglycerol.

Cell line. The cultured cells of *Eucalyptus perriniana* used in this investigation were derived from young stems of *E. perriniana* in 1980 and maintained on BA1 agar medium [Murashige and Skoog (MS) medium solidified with agar (9 g l^{-1}) and supplemented with sucrose (30 g l^{-1}) and 6-benzylaminopurine (1 mg l^{-1})].

Culture conditions and substrate feeding experiments. A cell suspension culture was initiated from static cultured callus in 500 ml Erlenmeyer flasks each containing 250 ml BA1 liquid medium(BA1 agar medium without agar) or 250 ml BA1 minus Fe liquid medium [BA1 agar medium without agar, iron ion ($FeSO_4 \cdot 7H_2O$) and Na_2EDTA]. After 3 weeks preculture on a rotary shaker (100 rotary min^{-1}) at 25° in the dark, a soln of the substrate (25 mg β-thujaplicin in 1 ml EtOH) was administered to each flask through a membrane filter and the cultures returned to the shaker for a further 7 days.

Extraction and purification of biotransformation products. The cells and medium were sepd by filtration with suction. The cells were extracted (×3) by homogenization with MeOH at room temp., and the extract was concd under red. pres. The residue was partitioned between H_2O and EtOAc. The H_2O layer was applied to a Diaion HP-20 column and the column was washed with H_2O followed by elution with MeOH. The MeOH eluate was chromatographed on ODS-silica gel column and the biotransformation products were further purified by HPLC [column: Waters μ Bondasphere C 18-100A, (19×150 mm); solvent; MeCN–H_2O (3:17), detection by UV (254 nm) absorption]. From these eluates, products **2**–**5** were obtained.

4-Isopropyltropolone 2-O-β-D-glucoside (**2**). Amorphous solid, $[\alpha]_D^{31} -75.9°$ (*c* 0.3, MeOH). Positive FAB MS (glycerol + thioglycerol) *m/z*: 327 $[M+H]^+$; ^{1}H NMR (C_5D_5N) δ_H (*J* in Hz): 1.11 (3H, *d*, *J* = 7.0, H-9), 1.14 (3H, *d*, *J* = 7.0, H-10), 2.69 (1H, *m*, H-8), 4.20 (1H, *ddd*, *J* = 9.0, 5.5, 2.0, H′-5), 4.28 (1H, *dd*, *J* = 9.0, 8.0, H′-4), 4.39 (1H, *dd*, *J* = 8.5, 8.0, H′-3), 4.38 (1H, *dd*, *J* = 12.0, 6.0, H′-6a), 4.45 (1H, *dd*, *J* = 8.5, 7.5, H′-2), 4.61 (1H, *dd*, *J* = 12.0, 1.5, H′-6b), 5.73 (1H, *d*, *J* = 7.5, H′-1) 6.66 (1H, *br d*, *J* = 9.0, H-5), 7.11 (1H, *dd*, *J* = 12.0, 9.0, H-6), 7.27 (1H, *br d*, *J* = 12.0, H-7), 7.52 (1H, *d*, *J* = 1.0, H-3).

4-Isopropyltropolone 2-O-β-D-gentiobioside (**3**). Amorphous solid, $[\alpha]_D^{32} -29.1°$ (*c* 0.3, MeOH). Positive FAB MS (glycerol + thioglycerol) *m/z*: 489 $[M+H]^+$; ^{1}H NMR (C_5D_5N) δ_H (*J* in Hz): 1.10 (6H, *d*, *J* = 7.0, H-9, 10), 2.72 (1H, *m*, H-8), 3.91 (1H, *ddd*, *J* = 9.0, 5.0, 2.0, H″-5), 4.04 (1H, *dd*, *J* = 8.0, 8.0, H″-2), 4.20 (1H, *dd*, *J* = 9.0, 8.5, H″-3), 4.25 (1H, *dd*, *J* = 9.0, 9.0, H″-4), 4.20–4.36 (4H, *m*, H′-2, 3, 4, 5), 4.36 (1H, *dd*, *J* = 11.5, 5.0, H″-6a), 4.44 (1H, *dd*, *J* = 11.5, 4.5, H′-6a), 4.51 (1H, *dd*, *J* = 11.5, 2.0, H″-6b), 4.81 (1H, *dd*, *J* = 11.5, 1.0, H′-6b), 5.03 (1H, *d*, *J* = 8.0, H″-1), 5.70 (1H, *d*, *J* = 7.0, H′-1), 6.64 (1H,

br d, $J = 9.0$, H-5), 7.19 (1H, *dd*, $J = 12.0$, 9.0, H-6), 7.23 1H, *br d*, $J = 12.0$, H-7), 7.49 (1H, *d*, $J = 1.0$, H-3).

6-*Isopropyltropolone* 2-O-β-D-*glucoside* (**4**). Amorphous solid, $[\alpha]_D^{30} -66.0°$ (*c* 0.3, MeOH). Positive FAB MS (glycerol + thioglycerol) *m/z*: 327 $[M+H]^+$; ^1H NMR (C_5D_5N) δ_H (*J* in Hz): 1.01 (6H, *d*, $J = 7.0$, H-9, 10), 2.56 (1H, *m*, H-8), 4.16 (1H, *ddd*, $J = 8.0$, 7.0, 2.0, H′-5), 4.31 (1H, *dd*, $J = 8.0$, 8.0, H′-4), 4.38 (1H, *dd*, $J = 8.0$, 8.0, H′-3), 4.38 (1H, *dd*, $J = 12.0$, 6.0, H′-6a), 4.44 (1H, *dd*, $J = 8.0$, 7.0, H′-2), 4.57 (1H, *dd*, $J = 12.0$, 2.0, H′-6b), 5.72 (1H, *d*, $J = 7.0$, H′-1), 6.66 (1H, *dd*, $J = 11.0$, 1.5, H-5), 6.87 (1H, *dd*, $J = 11.0$, 10.0, H-6), 7.29 (1H, *d*, $J = 1.5$, H-3), 7.43 (1H, *d*, $J = 10.0$, H-7).

6-*Isopropyltropolone* 2-O-β-D-*gentiobioside* (**5**). Amorphous solid, $[\alpha]_D^{31} -41.3°$ (*c* 0.3, MeOH). Positive FAB MS (glycerol + thioglycerol) *m/z*: 489 $[M+H]^+$; ^1H NMR (C_5D_5N) δ_H (*J* in Hz): 1.01 (3H, *d*, $J = 7.0$, H-9), 1.01 (3H, *d*, $J = 7.0$, H-10), 2.53 (1H, *m*, H-8), 3.89 (1H, *ddd*, $J = 8.5$, 4.5, 2.5, H″-5), 4.09 (1H, *dd*, $J = 8.5$, 8.0, H″-2), 4.15 (1H, *dd*, $J = 8.5$, 8.5, H′-4), 4.22 (1H, *dd*, $J = 8.5$, 8.5, H″-3), 4.27 (1H, *dd*, $J = 8.5$, 8.5, H″-4), 4.31 (1H, *dd*, $J = 8.5$, 8.0, H′-3), 4.32–4.39 (2H, *m*, H′-5 and H′-6a), 4.38 (1H, *dd*, $J = 8.0$, 7.5, H′-2), 4.36 (1H, *dd*, $J = 12.0$, 4.5, H″-6a), 4.52 (1H, *dd*, $J = 12.0$, 2.5, H″-6b), 4.83 (1H, *d*, $J = 10.0$, H′-6b), 5.12 (1H, *d*, $J = 8.0$, H″-1), 5.63 (1H, *d*, $J = 7.5$, H′-1), 6.59 (1H, *dd*, $J = 11.0$, 1.5, H-5), 7.21 (1H, *dd*, $J = 11.0$, 10.0, H-6), 7.27 (1H, *d*, $J = 1.5$, H-3), 7.72 (1H, *d*, $J = 10.0$, H-7).

Enzymatic hydrolysis. One mg of β-glucosidase was added to each soln of products **2**–**5** (0.2 mg) in McIlvaine's buffer (pH 5.0, 0.2 ml), and the mixts were incubated at 37° in the dark for 48 hr. The reaction mixts were subjected to TLC for identification of the aglycone.

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