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BIOTRANSFORMATION OF 1,8-CINEOLE BY CULTURED CELLS OF *EUCALYPTUS PERRINIANA**

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Key Word Index—*Eucalyptus perriniana*; Myrtaceae; cell suspension culture; biotransformation; 1,8-cineole; 1,8-epoxy-*p*-menthan-2-yl *O*-β-D-glucopyranoside; 1,8-epoxy-*p*-menthan-3-yl *O*-β-D-glucopyranoside; hydroxylation; glucosylation.

Abstract—Four new biotransformation products, (1*R*, 2*R*, 4*S*)-1,8-epoxy-*p*-menthan-2-yl *O*-β-D-glucopyranoside, (1*S*, 3*R*, 4*R*)- and (1*R*, 3*S*, 4*S*)-1,8-epoxy-*p*-menthan-3-yl *O*-β-D-glucopyranosides, and (1*S*, 2*S*, 4*R*)-1,8-epoxy-*p*-menthan-2-yl *O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside, together with a known (1*S*, 2*S*, 4*R*)-1,8-epoxy-*p*-menthan-2-yl *O*-β-D-glucopyranoside were isolated from a cell suspension culture of *Eucalyptus perriniana* following administration of 1,8-cineole.

INTRODUCTION

Many *Eucalyptus* plants contain an essential oil, whose main component is 1,8-cineole (**1**) [1]. It has been shown that the biotransformation of **1** to 2- and 3-hydroxycineoles, hydroxylation products of **1**, can be carried out by a strain of *Pseudomonas flava* which was isolated from *Eucalyptus* leaves for its ability to utilize **1** as a carbon source [2] and by *Aspergillus niger* [3]. Chemical oxidation of **1** by *m*-chloroperbenzoic acid also produces hydroxycineoles [4]. There is no report on the biotransformation of **1** by cultured plant cells.

Previously, we reported that cultured cells of *Eucalyptus perriniana* are able to biotransform the monoterpenes, (−)-menthol [5], (+)-menthol [6] and (−)-borneol [7], the sesquiterpene, caryophyllene oxide [8], the diterpene, steviol [9], the triterpene, 18β-glycyrrhetinic acid [10], and the aromatic compounds, eugenol and isoeugenol [11], into their glycosylated and hydroxylated products.

We now report on the isolation and the structure determination of the biotransformation products of **1** produced by cell suspension culture of *E. perriniana*.

RESULTS AND DISCUSSION

Eucalyptus perriniana cells were cultured for three weeks in BA1 medium. Glucose (5 g per flask) and the substrate [1,8-cineole (**1**) 25 mg] were then administered to the culture, and the culture was continued for an additional seven days. The cells were then harvested and extracted as described in the Experimental.

The butanol fraction of the medium and cells afforded mixtures of **2** and **3** (69 mg, biotransformation yield 16%) and **4** and **5** (39 mg, 9.0%), and **6** (9 mg, 1.4%). The mixtures were separated into their component parts by HPLC.

Products **2**–**6** were not detected on TLC analysis of the shake flask culture of *E. perriniana* to which no substrate was fed, and of a mixture of **1** and the medium shaken for seven days.

The main products **2** and **3** were assigned a M_r of 332 (FAB-MS). In the ^{13}C NMR spectrum (Table 1), 16 carbon signals were observed. Ten carbon signals were assignable to the 1,8-cineole moiety and the remainder to a β-glucose moiety. The ^{13}C NMR spectrum showed that the 1,8-cineole moiety contained one more oxygenated methine and one less methylene carbon than the substrate (**1**). In the ^{1}H NMR spectra, the oxygenated methine protons of **2** and **3** were observed at δ3.80–3.94 and 4.08–4.19 and were overlapped by the H-2′ and H-5′ protons, respectively. ^{1}H–^{1}H COSY showed that couplings occurred between the oxygenated methine proton and a pair of methylene protons ($J_{H\text{-}2,\ H\text{-}3endo}$ = 3.0 Hz and $J_{H\text{-}2,\ H\text{-}3exo}$ = 9.5 Hz) and between the methylene protons and H-4 methine proton. On the basis of these findings the oxygenated methine proton was assigned to H-2$_{exo}$. Enzymatic hydrolysis of the mixture of **2** and **3** gave as aglycone the racemate **7** (^{1}H and ^{13}C NMR) indicating that **2** and **3** were the β-D-glucosides of a pair of enantiomers. It has been reported that the chirality of glycosylated oxygen-bearing carbons in cyclic secondary alcohol β-D-glucosides can be determined by analysis of their ^{13}C NMR spectra [12, 13]. Since the chemical shifts of C-1, C-2, C-3 and C-1′ of **2** were observed at lower field than those of **3**, the chirality at C-2 of **2** and **3** were *S* and *R*, respectively. According to the above results, it was

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Table 1. ^{13}C NMR (75 MHz) chemical shifts of 1,8-cineole (**1**) and its biotransformation products (**2**–**6**) in pyridine-d_5 and their aglycones (**7** and **8**) in $CDCl_3$.

C	1	7	2	3	8	4	5	6
1	69.7	72.5	72.5	72.0	70.9	70.8	70.7	72.4
2	31.9	71.2	80.2	76.3	42.9	41.6	40.9	80.3
3	23.3	34.6	34.4	32.2	65.3	71.9	73.0	34.6
4	33.2	34.3	34.6	34.4	40.4	37.0	39.4	34.5
5	23.3	22.2	22.7	22.5	13.9	14.9	15.3	22.7
6	31.9	24.9	26.5	26.6	31.1	31.6	31.6	26.5
7	27.9	24.1	25.1	25.3	27.1	27.5	27.6	25.0
8	73.6	73.5	73.4	73.3	73.3	73.3	73.3	73.4
9	29.3	29.0	29.3	29.3	29.0	29.4	29.2	29.5
10	29.3	28.6	29.1	29.0	28.4	28.7	28.7	29.1
1′			106.6	102.3		102.5	103.5	106.6
2′			75.7	75.3		75.6	75.4	75.5
3′			78.7	78.9		78.9	78.8	78.6
4′			71.9	72.0		71.9	71.9	71.9
5′			78.5	78.7		78.7	78.7	77.5
6′			63.1	63.1		62.9	63.0	70.4
1″								105.6
2″								75.5
3″								78.6
4″								71.9
5″								78.7
6″								63.0

concluded that the structures of **2** and **3** are those of (1*S*, 2*S*, 4*R*)- and (1*R*, 2*R*, 4*S*)-1,8-epoxy-*p*-menthan-2-yl *O*-β-D-glucopyranoside, respectively.

Products **4** and **5** were assigned a M_r of 332 (FAB-MS). Sixteen carbon signals were observed in the ^{13}C NMR spectra (Table 1). The latter also established that **4** and **5** were isomers of **2** and **3**. In the ^{1}H NMR spectra, the oxygenated methine protons of **4** and **5** were observed at δ4.73 and 4.67 each as a broad doublet, respectively. From the ^{1}H–^{1}H COSY, the couplings between the oxygenated methine proton and a pair of methylene protons [$J_{H\text{-}3,\ H\text{-}2endo} = 2.5$ Hz and $J_{H\text{-}3,\ H\text{-}2exo} = 10.0$ Hz (**4**) or 9.5 Hz (**5**)] and between the oxygenated methine proton and H-4 methine proton established that the oxygenated methine proton was H-3_{exo}. Additionally, NOEs between Me-10 and H-3_{exo} and between Me-9 and H-5_{exo} were also observed. The chiralities at C-3 of **4** and **5** were determined to be *R* and *S*, respectively. According to the above results, it was concluded that the structures of **4** and **5** are (1*S*, 3*R*, 4*R*)- and (1*R*, 3*S*, 4*S*)-1,8-epoxy-*p*-menthan-3-yl *O*-β-D-glucopyranoside, respectively.

Product **6** was assigned a M_r of 494 (FAB-MS), and 22 carbon signals were observed in the ^{13}C NMR spectrum (Table 1). The carbon signals assigned to the 1,8-cineole moiety agreed well with those of **2**, so that the aglycone part of **6** was determined to be (1*S*, 2*S*, 4*R*)-1,8-epoxy-*p*-menthan-2-ol. The remaining 12 carbon signals were assigned to be a β-gentiobiose moiety from their chemical shifts and ^{1}H NMR coupling constants. From the above data, it was concluded that the structure of **6** is (1*S*, 2*S*, 4*R*)-1,8-epoxy-*p*-menthan-2-yl *O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside.

The substrate **1** should be toxic to cultured cells of *E. perriniana* in spite of the fact that **1** is a main constituent of the essential oil of *Eucalyptus* plants. Thus, the biotransformations of **1** which take place in cultures of *E. perriniana* may be concerned with its detoxification. Based on the structures of the biotransformation products, the possible pathways for the metabolism of 1,8-cineole by cultured cells of *E. perriniana* are outlined in Fig. 1. Since all methylene carbons were hydroxylated (all *endo*-orientation), it was supposed that the regioselectivity of the hydroxylation enzyme (probably a P450-dependent monooxygenase) is low.

There is no report of the identification of hydroxycineoles or their glycosides from *Eucalyptus*. Though **2** was isolated from *Citrus unshiu* peel [14], other compounds **3**–**6** are new.

EXPERIMENTAL

^{1}H NMR: 300 MHz, ^{13}C NMR: 75 MHz, GC-MS: 70 eV, HP-1 5 m, temp. programme 60° for 2 min from 60 to 200° at 5° min^{-1}, injector temp. 220°, He 40 ml min^{-1}.

Cell line. The cells 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 [15] supplemented with sucrose (30 g l^{-1}), agar (9 g l^{-1}) and 6-benzylaminopurine (1 mg l^{-1})], as previously reported [16].

Feeding experiment. The *E. perriniana* cell suspension culture was initiated from static cultured cells in 1-l conical flasks each containing 250 ml BA1 liquid medium. The cultures were grown in a reciprocal shaker (90 strokes min^{-1}) at 25° in the dark. After 3 weeks culture, glucose (0.1 g ml^{-1} H_2O, 50 ml to each flask) and the substrate [1,8-cineole (**1**), 12.5 mg ml^{-1} EtOH, 2 ml to each flask] were administered. The cells were cultured for an additional 7 days after the addition of substrate.

Isolation of biotransformation products. The cells and the medium (4 flasks, total 200 mg of **1** was administered) were sepd by filtration with suction. The medium was passed through a Diaion HP20 column and the column was washed with H_2O and then eluted with MeOH. The MeOH eluate was concd under red. pres. and the residue was partitioned between H_2O and BuOH (M-HP-fr, 71 mg). The cells were extracted (×2) with MeOH at room temp. and the extract concd under red. pres. The residue was partitioned between EtOAc and H_2O to remove phytosterols and triterpenes, and the H_2O layer was further extracted (×2) with BuOH (C-Bu-fr, 1470 mg). Both BuOH frs were chromatographed on silica gel and the biotransformation products were further purified by HPLC [column; μBondasphere C18-100 Å, solvent; MeOH–H_2O (7:3 for **2**–**5** and 1:1 for **6**)]. Mixts of **2** and **3** (69 mg) and **4** and **5** (39 mg), and **6** (9 mg) were obtained. The mixts were further purified by HPLC [column; Senshu Pak ODS-H-4301, solvent; MeOH–H_2O (2:3)].

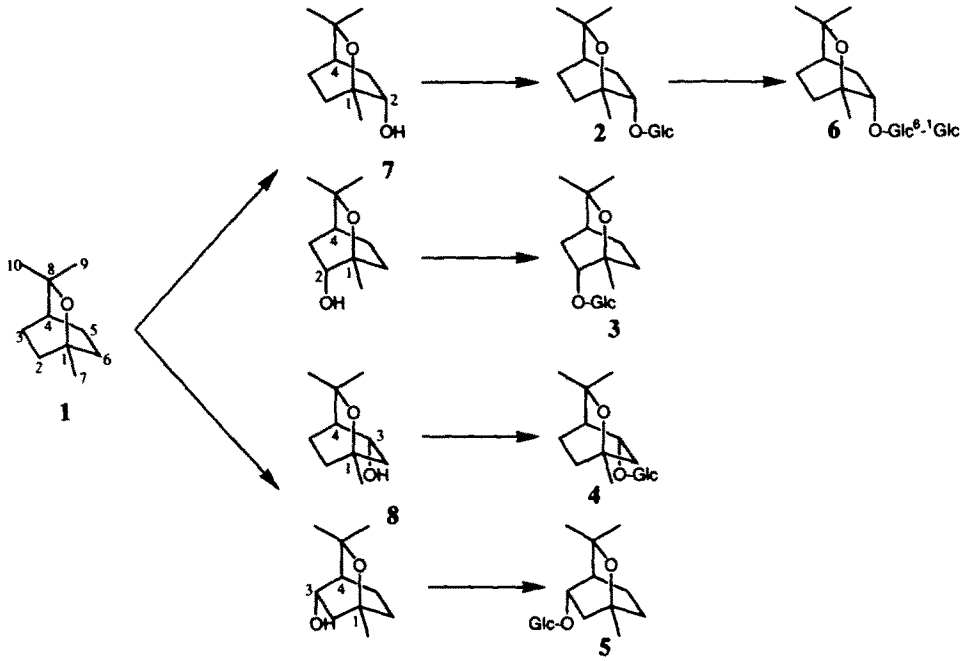

Fig. 1. Biotransformation of 1,8-cineole (**1**) by cultured cells of *E. perriniana*.

(1S, 2S, 4R)-1,8-*Epoxy*-p-*menthan*-2-*yl* O-β-D-*glucopyranoside* (**2**). Amorphous solid, $[\alpha]_D$ +7.2° (MeOH; *c* 3.1); ^{1}H NMR (pyridine-d_5): δ1.03 (3H, *s*, Me-9), 1.11 (3H, *s*, Me-10), 1.21 (1H, *m*, H-4), 1.34 (3H, *s*, Me-7), 1.30–1.42 (2H, *m*, H-5_{endo}, H-6_{exo}), 1.71 (1H, *m*, H-5_{exo}), 1.79 (1H, *ddd*, *J* = 14.0, 3.0, 3.0 Hz, H-3_{endo}), 1.94–2.05 (1H, *m*, H-6_{endo}), 2.48 (1H, *dddd*, *J* = 14.0, 9.5, 3.5, 3.0 Hz, H-3_{exo}), 3.80–3.94 (3H, *m*, H-2_{exo}, H-2′, H-5′), 4.07–4.18 (2H, *m*, H-3′, H-4′), 4.30 (1H, *dd*, *J* = 11.5, 5.0 Hz, H-6′a), 4.43 (1H, *dd*, *J* = 11.5, 2.5 Hz, H-6′b), 4.79 (1H, *d*, *J* = 8.0 Hz, H-1′); FAB-MS *m/z* 333 $[M+H]^+$.

(1R, 2R, 4S)-1,8-*Epoxy*-p-*menthan*-2-*yl* O-β-D-*glucopyranoside* (**3**). Amorphous solid, $[\alpha]_D$ −66° (MeOH; *c* 1.3); ^{1}H NMR (pyridine-d_5): δ1.02 (3H, *s*, Me-9), 1.12 (3H, *s*, Me-10), 1.22 (3H, *s*, Me-7), 1.29 (1H, *m*, H-4), 1.30–1.47 (2H, *m*, H-5_{endo}, H-6_{exo}), 1.64–1.75 (1H, *m*, H-5_{exo}), 1.73 (1H, *ddd*, *J* = 14.0, 3.0, 3.0 Hz, H-3_{endo}), 1.87–1.97 (1H, *m*, H-6_{endo}), 2.36 (1H, *dddd*, *J* = 14.0, 9.0, 3.0, 3.0 Hz, H-3_{exo}), 3.81–3.92 (2H, *m*, H-2′, H-5′), 4.08–4.19 (3H, *m*, H-2_{exo}, H-3′, H-4′), 4.25 (1H, *ddd*, *J* = 11.5, 6.0, 5.0 Hz, H-6′a), 4.42 (1H, *br d*, *J* = 11.5 Hz, H-6′b), 4.85 (1H, *d*, *J* = 7.5 Hz, H-1′); FAB-MS *m/z* 333 $[M+H]^+$.

(1S, 3R, 4R)-1,8-*Epoxy*-p-*menthan*-3-*yl* O-β-D-*glucopyranoside* (**4**). Amorphous solid, $[\alpha]_D$ −40° (MeOH; *c* 0.6); ^{1}H NMR (pyridine-d_5): δ0.90 (3H, *s*, Me-7), 1.04 (3H, *s*, Me-10), 1.14 (3H, *s*, Me-9), 1.29 (1H, *ddd*, *J* = 12.0, 12.0, 3.5 Hz, H-6_{endo}), 1.44 (1H, *dddd*, *J* = 12.0, 12.0, 5.5, 3.0 Hz, H-6_{exo}), 1.62 (1H, *br dd*, *J* = 13.0, 12.0 Hz, H-5_{exo}), 1.65 (1H, *dd*, *J* = 14.0, 2.5 Hz, H-2_{endo}), 1.83 (1H, *m*, H-4), 2.02 (1H, *ddd*, *J* = 14.0, 10.0, 3.0 Hz, H-2_{exo}), 2.42 (1H, *dddd*, *J* = 13.0, 12.0, 5.5, 2.0 Hz, H-5_{endo}), 3.86 (1H, *ddd*, *J* = 9.0, 5.0, 2.5 Hz, H-5′), 3.97 (1H, *m*, H-2′), 4.13–4.22 (2H, *m*, H-3′, H-4′), 4.29 (1H, *dd*, *J* = 12.0, 5.0 Hz, H-6′a), 4.44 (1H, *br d*, *J* = 12.0 Hz, H-6′b), 4.73 (1H, *br d*, *J* = 10.0 Hz, H-3_{exo}), 4.86 (1H, *d*, *J* = 8.0 Hz, H-1′); FAB-MS *m/z* 333 $[M+H]^+$.

(1R, 3S, 4S)-1,8-*Epoxy*-p-*menthan*-3-*yl* O-β-D-*glucopyranoside* (**5**). Amorphous solid, $[\alpha]_D$ −39° (MeOH; *c* 1.6); ^{1}H NMR (pyridine-d_5): δ0.91 (3H, *s*, Me-7), 1.01 (3H, *s*, Me-10), 1.09 (3H, *s*, Me-9), 1.28–1.51 (2H, *m*, H-6_{endo}, H-6_{exo}), 1.60 (1H, *m*, H-5_{exo}), 1.65 (1H, *dd*, *J* = 14.0, 2.5 Hz, H-2_{endo}), 1.83 (1H, *m*, H-4), 1.97 (1H, *ddd*, *J* = 14.0, 9.5, 3.0 Hz, H-2_{exo}), 2.27 (1H, *dddd*, *J* = 13.0, 12.0, 6.0, 2.0 Hz, H-5_{endo}), 3.86 (1H, *ddd*, *J* = 9.0, 5.0, 2.5 Hz, H-5′), 3.95 (1H, *m*, H-2′), 4.12–4.21 (2H, *m*, H-3′, H-4′), 4.27 (1H, *ddd*, *J* = 11.5, 6.0, 5.0 Hz, H-6′a), 4.44 (1H, *br d*, *J* = 11.5 Hz, H-6′b), 4,67 (1H, *br d*, *J* = 9.5 Hz, H-3_{exo}), 4.87 (1H, *d*, *J* = 8.0 Hz, H-1′); FAB-MS *m/z* 333 $[M+H]^+$.

(1S, 2S, 4R)-1,8-*Epoxy*-p-*menthan*-2-*yl* O-β-D-*glucopyranosyl*-(1→6)-β-D-*glucopyranoside* (**6**). Amorphous solid, $[\alpha]_D$ −16° (MeOH; *c* 0.9); ^{1}H NMR (pyridine-d_5): δ1.13, 1.15 (each 3H, *s*, Me-9, Me-10), 1.29 (1H, *m*, H-4), 1.35 (3H, *s*, Me-7), 1.38–1.52 (2H, *m*, H-5_{endo}, H-6_{exo}), 1.77 (1H, *m*, H-5_{exo}), 1.91 (1H, *ddd*, *J* = 14.0, 3.0, 3.0 Hz, H-3_{endo}), 1.98–2.10 (1H, *m*, H-6_{endo}), 2.82 (1H, *dddd*, *J* = 14.0, 10.0, 3.0, 3.0 Hz, H-3_{exo}), 3.87–4.23 (9H, *m*, H-2_{exo}, H-2′, H-2″, H-3′, H-3″, H-4′, H-4″, H-5′, H-5″), 4.27–4.36 (2H, *m*, H-6′a, H-6″a), 4.48 (1H, *br d*, *J* = 11.5 Hz, H-6″b), 4.76 (1H, *d*, *J* = 7.5 Hz, H-1′), 4.78 (1H, *br d*, *J* = 11.5 Hz, H-6′b), 5.15 (1H, *d*, *J* = 7.5 Hz, H-1″); FAB-MS *m/z* 495 $[M+H]^+$.

Enzymatic hydrolysis. Mixts of **2** and **3** (10 mg) or **4** and **5** (6 mg) and crude hesperidinase (10 mg) were each dissolved in citrate-Pi buffer (pH 4.0, 10 ml). The mixts were kept at room temp. for 8 hr and then they were extracted with CH_2Cl_2. The CH_2Cl_2 layers were dried over anhydrous Na_2SO_4 and evapd. The aglycones **7** (3 mg) and **8** (2 mg) were obtained.

(1SR, 2SR, 4RS)-1,8-*Epoxy*-p-*menthan*-2-*ol* (**7**). Amorphous solid; ^{1}H NMR ($CDCl_3$): δ1.10 (3H, *s*, Me-7), 1.19, 1.28 (each 3H, *s*, Me-9, Me-10), 1.30 (1H, *ddd*, J = 14.0, 4.0, 2.5, H-3_{endo}), 1.46–1.61 (3H, *m*, H-4, H-5_{endo}, H-6_{exo}), 1.82–2.03 (2H, *m*, H-5_{exo}, H-6_{endo}), 2.52 (1H, *dddd*, J = 14.0, 9.5, 3.5, 3.0 Hz, H-3_{exo}), 3.73 (1H, *br dd*, J = 9.5, 4.0 Hz, H-2_{exo}); GC-MS (R_t = 12.7 min) *m/z* (rel. int.): 170 $[M]^+$ (13), 126 (80), 111 (49), 108 (100), 93 (42), 83 (61), 71 (89), 69 (51), 55 (34).

(1SR, 3RS, 4RS)-1,8-*Epoxy*-p-*menthan*-3-*ol* (**8**). Amorphous solid; ^{1}H NMR ($CDCl_3$): δ1.07 (3H, *s*, Me-7), 1.21, 1.30 (each 3H, *s*, Me-9, Me-10), 1.32 (1H, *dd*, J = 14.0, 3.0 Hz, H-2_{endo}), 1.50 (1H, *ddd*, J = 13.5, 11.5, 3.5 Hz, H-6_{endo}), 1.55 (1H, *m*, H-4), 1.63 (1H, *dddd*, J = 13.5, 11.0, 6.0, 3.0 Hz, H-6_{exo}), 1.79 (1H, *ddddd*, J = 13.5, 11.0, 3.5, 3.0, 1.5 Hz, H-5_{exo}), 2.05 (1H, *dddd*, J = 13.5, 11.5, 6.0, 2.0 Hz, H-5_{endo}), 2.13 (1H, *ddd*, J = 14.0, 9.5, 3.0 Hz, H-2_{exo}), 4.47 (1H, *br d*, J = 9.5 Hz, H-3_{exo}); GC-MS (R_t = 13.0 min) *m/z* (rel. int.): 170 $[M]^+$ (34), 155 (29), 137 (31), 127 (36), 111 (45), 108 (74), 93 (91), 87 (86), 71 (68), 69 (100), 59 (74).

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