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Phytochemistry 67 (2006) 2256–2261
PHYTOCHEMISTRY
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Biotransformation of thymol, carvacrol, and eugenol by cultured cells of *Eucalyptus perriniana*

Kei Shimoda [a], Yoko Kondo [b], Tomohisa Nishida [b], Hatsuyuki Hamada [c], Nobuyoshi Nakajima [d], Hiroki Hamada [e,*]

[a] *Department of Pharmacology and Therapeutics, Faculty of Medicine, Oita University, 1-1 Hasama-machi, Oita 879-5593, Japan*
[b] *Department of Applied Science, Faculty of Science, Okayama University of Science, 1-1 Ridai-cho, Okayama 700-0005, Japan*
[c] *National Institute of Fitness and Sports in Kanoya, 1 Shiromizu-cho, Kagoshima 891-2390, Japan*
[d] *Industry, Government, and Academic Promotional Center, Regional Cooperative Research Organization, Okayama Prefectural University, Soja, Okayama 719-1197, Japan*
[e] *Department of Life Science, Faculty of Science, Okayama University of Science, 1-1 Ridai-cho, Okayama 700-0005, Japan*

Received 23 February 2006; received in revised form 1 June 2006
Available online 22 August 2006

Abstract

The biotransformations of aroma compounds of spices, such as thymol (**1**), carvacrol (**2**), and eugenol (**3**), were investigated using cultured plant cells of *Eucalyptus perriniana*. Besides a β-glucoside product (**4**, 3%), a biotransformation product, i.e., 5-methyl-2-(1-methylethyl)phenyl 6-*O*-(β-D-glucopyranosyl)-β-D-glucopyranoside (**5**, β-gentiobioside, 87%), was isolated from the suspension cells after the five-day incubation of **1**. On administration of **2**, a β-glucoside (**6**, 5%) and a β-gentiobioside, i.e., 2-methyl-5-(1-methylethyl)phenyl 6-*O*-(β-D-glucopyranosyl)-β-D-glucopyranoside (**7**, 56%), were produced. Furthermore, *E. perriniana* cells also converted **3** into the corresponding β-glucoside (**8**, 7%) and β-gentiobioside (**9**, 58%). The cultured cells of *E. perriniana* are able to convert these aroma compounds of spices into glycosides which are accumulated in the cells.

Keywords: *Eucalyptus perriniana*; Myrtaceae; Cultured plant cells; Biotransformation; Thymol; Carvacrol; Eugenol; Glycosides; β-Gentiobioside

1. Introduction

Biotransformation by cultured plant cells is considered to be an important method to convert cheap and plentiful organic compounds into more useful ones according to the ability of plant cell cultures to specifically produce secondary metabolites (Suga and Hirata, 1990). The reactions involved in the biotransformation of organic compounds by cultured plant cells include oxidation, reduction, hydroxylation, esterification, methylation, isomerization, hydrolysis, and glycosylation (Suga and Hirata, 1990; Ishihara et al., 2003). Glycosylation occurs readily in plant cells, i.e., many kinds of 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 (Furuya et al., 1989). Because one-step enzymatic glycosylation by cultured plant cells is useful for preparation of glycosides rather than chemical glycosylation which requires tedious steps (Mastelic et al., 2004; Namme et al., 2005), it has been the subject of increasing attention and has been carried out for many exogenous compounds, i.e., digitoxigenin, terpenoids, cineole, steviol, and tropic acid (Furuya et al., 1987b; Moyer and Gustine, 1987; Furuya et al., 1988; Tabata et al., 1988; Upmeier et al., 1988; Furuya et al., 1989; Ushiyama et al., 1989; Lewinson et al., 1996).

Thymol (**1**), carvacrol (**2**), and eugenol (**3**) are present in the essential oils from herbs and spices, such as thyme

* Corresponding author. Tel.: +81 86 256 9473; fax: +81 86 256 8468.
E-mail address: hamada@das.ous.ac.jp (H. Hamada).

0031-9422/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2006.07.006

(*Thymus vulgaris*), oreganum (*Origanum syriacum*), and clove (*Caryophilli flos*) (Cimanga et al., 2002). They are produced by these plant species as a chemical defence mechanism against phytopathogenic microorganisms (Vazquez et al., 2001). Therefore, these three compounds have attracted great attention in food industry, i.e., they have been used in foods such as cheese to prevent fungal growth as natural preservatives (Juven et al., 1994; Vazquez et al., 2001; Venturini et al., 2002). On the other hand, these compounds have been used as medicines because they have specific physiological activities such as antiseptic, antispasmodic, tonic, and carminative (Didry et al., 1994). Irrespective of such biological and physiological activities, their use as natural preservatives for foods and medicines has been limited, because of their water-insolubility, sublimation, and light decomposition (Mastelic et al., 2004). Glycosylation allows the conversion of water-insoluble and unstable organic compounds into the corresponding water-soluble and stable ones to improve their bio- and pharmacological properties, e.g., glycosides of volatile compounds such as aromatic and terpene alcohols have been widely used in folk medicines (Mastelic et al., 2004). From the physiological point of view, the glycosides of thymol (**1**), carvacrol (**2**), and eugenol (**3**), which are aroma compounds of naturally occurring herbs and spices, can be of pharmacological interest as well as usable for food additives and cosmetics. Recently, several attempts have been made to produce these glycosides such as β-glucosides by chemical glycosylation (Mastelic et al., 2004). However, little attention has been paid to the enzymatic glycosylation of **1**–**3** with cultured plant cells. We now report the biotransformations of **1**–**3** into β-glucosides and β-gentiobiosides with a higher water-solubility (Hamada et al., 2001) by the cultured plant cells of *Eucalyptus perriniana*.

2. Results and discussion

2.1. Biotransformation of thymol (*1*)

After a five-day incubation of thymol (**1**) with the cultured cells of *E. perriniana*, 5-methyl-2-(1-methylethyl)phenyl β-D-glucopyranoside (**4**) and 5-methyl-2-(1-methylethyl)phenyl 6-*O*-(β-D-glucopyranosyl)-β-D-glucopyranoside (**5**) were isolated from the cells by extraction with MeOH. No transformation products were detected in the medium. No additional conversion products were detected in the MeOH-extracts of the cells. On the basis of their HRFABMS, ^{1}H and ^{13}C NMR (Table 1), H–H COSY, C–H COSY, and NOE-spectra the products were determined to be 5-methyl-2-(1-methylethyl)phenyl β-D-glucopyranoside (**4**, 3%) and 5-methyl-2-(1-methylethyl)phenyl 6-*O*-(β-D-glucopyranosyl)-β-D-glucopyranoside (**5**, 87%). The β-gentiobioside product **5** has not been identified before. The HRFABMS spectrum of **5** showed a pseudomolecular ion $[M+Na]^{+}$ peak at *m*/*z* 497.2048 consistent with a molecular formula $C_{22}H_{34}O_{11}$ (calcd. 497.2000 for $C_{22}H_{34}O_{11}Na$). The ^{1}H NMR spectrum of **5** showed two anomeric proton signals at δ 4.91 (1H, *d*, $J = 8.0$ Hz) and 5.01 (1H, *d*, $J = 8.0$ Hz). The ^{13}C NMR spectrum of **5** exhibited 22 carbon signals including two anomeric carbon signals at δ 102.3 and 104.8. From the coupling pattern of the proton signals and the chemical shifts of the carbon resonances due to the sugar moiety, the sugar component in **5** was indicated to be β-D-glucopyranose (Shimoda et al., 2006). The ^{13}C NMR chemical shift of C-6′ was comparatively shifted downfield to δ 69.9. In addition, HMBC correlations were observed between the anomeric proton signal at δ 5.01 (H-1′) and the carbon resonance at δ 155.9 (C-3) and between the anomeric proton signal at δ 4.91 (H-1″) and the carbon resonance at δ 69.9 (C-6′) which confirms that the inner glucopyranosyl residue was attached to the phenolic hydroxyl group of thymol (**1**) and that the pair of β-D-glucopyranosyl residues was 1,6-linked. Thus, the structure of **5** was determined to be 5-methyl-2-(1-methylethyl)phenyl 6-*O*-(β-D-glucopyranosyl)-β-D-glucopyranoside. To investigate the biotransformation pathway, a time course in the conversion of **1** was followed. As shown in Fig. 1a, **1** was glucosylated to **4** following 6 h incubation, whereas the product **5** predominantly accumulated in the cells after 12 h incubation with a decrease in the amount of **4**. This indicated that the β-glucoside **4** was first formed and then β-gentiobioside **5** was produced as shown in Scheme 1. Recently, it has been reported that the cultured cells of *Achillea millefolium* converted exogenous thymol (**1**) to give limonene, carvacrol, and 1,8-cineol (Figueiredo et al., 1996). This is the first description of the glycosylation of thymol (**1**) by cultured

Table 1
^{13}C chemical shifts of the biotransformation products **4**–**9** in CD_3OD

Product	4	5	6	7	8	9
Aglycone						
1	137.2	137.4	126.0	125.9	146.3	145.9
2	117.0	116.8	157.1	156.7	150.4	150.4
3	155.9	155.9	114.3	114.3	114.0	113.8
4	136.3	136.0	148.9	148.8	136.4	136.2
5	126.6	126.5	120.9	120.8	121.8	122.1
6	124.1	124.0	131.2	131.1	118.0	118.3
7	21.3	21.3	16.1	16.1	40.8	40.7
8	27.0	27.1	35.0	35.0	138.8	138.7
9	23.5	23.2	24.3	24.5	115.8	115.7
10	23.5	23.2	24.3	24.5	56.7	56.6
Glc						
1′	102.8	102.3	102.5	102.6	102.9	102.8
2′	75.1	74.9	74.8	74.7	74.9	74.7
3′	78.1	77.9	78.0	77.7	78.0	77.7
4′	71.5	71.2	71.2	71.3	71.2	71.4
5′	78.6	77.0	78.0	76.8	78.0	77.1
6′	62.4	69.9	62.3	70.0	62.4	69.5
1″		104.8		104.8		104.4
2″		74.9		74.7		74.9
3″		77.9		77.7		77.7
4″		71.2		71.1		71.1
5″		77.9		77.7		77.4
6″		62.4		62.5		62.4

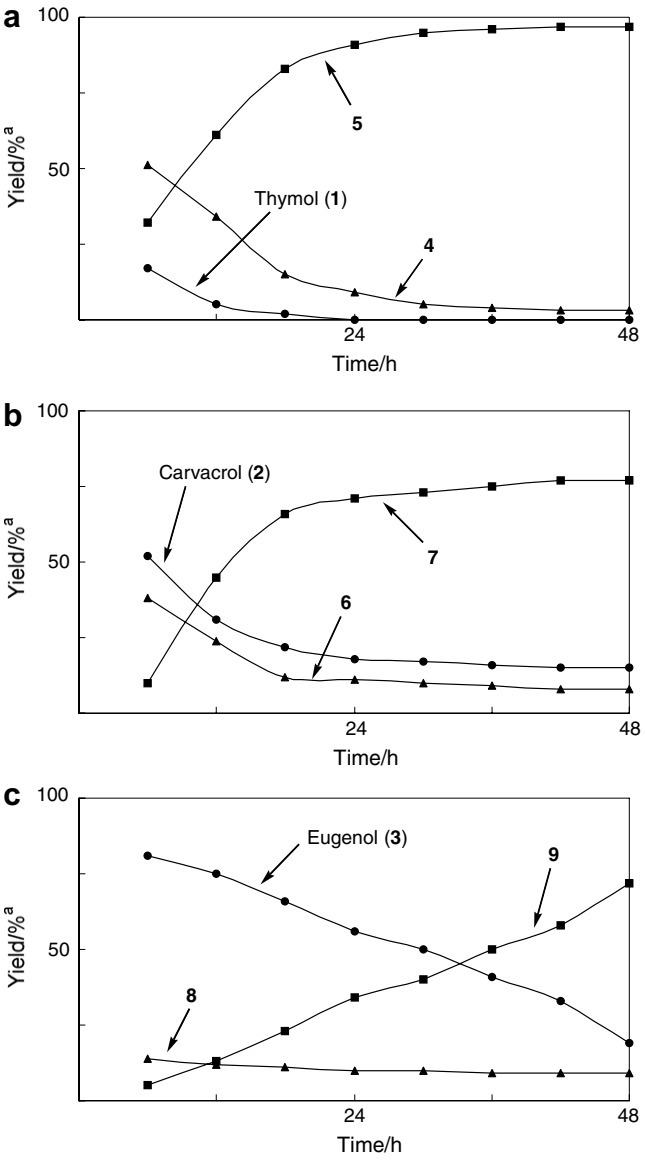


Fig. 1. Time course of the biotransformations of (a) thymol (**1**), (b) carvacrol (**2**), and (c) eugenol (**3**) by the cultured cells of *E. perriniana*. [a] Yield is expressed as relative percentage to the total amount of whole reaction products.

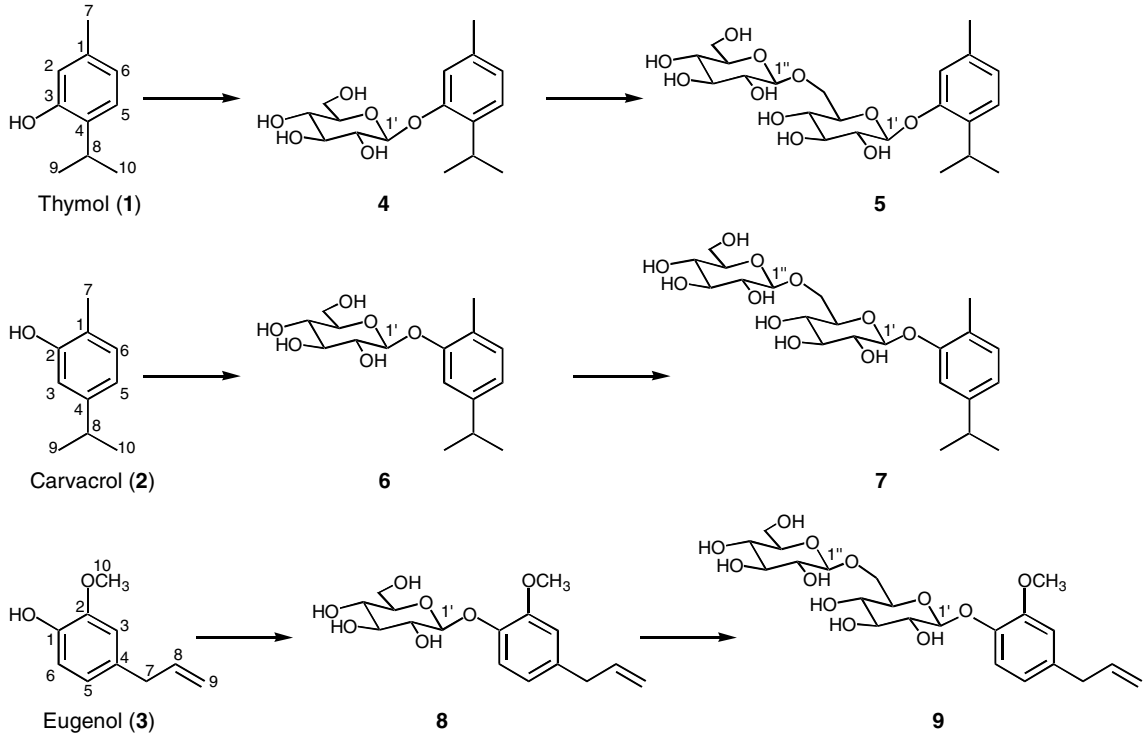
plant cells to produce the corresponding β-glucoside (**4**) and β-gentiobioside (**5**).

2.2. Biotransformation of carvacrol (2)

Carvacrol (**2**) was subjected to the same biotransformation system. After a five-day incubation period, products **6** and **7** were obtained from the MeOH-extracts of the cells. The yields of the products at 48 h (Fig. 1b) were slightly lower compared with the ones of the biotransformation of thymol (**1**) (Fig. 1a). The products were identified as 2-methyl-5-(1-methylethyl)phenyl β-D-glucopyranoside (**6**, 5%) and 2-methyl-5-(1-methylethyl)phenyl 6-*O*-(β-D-glucopyranosyl)-β-D-glucopyranoside (**7**, 56%). The disaccharide product **7** has not been identified before. The HRFABMS spectrum of the product **7** ($[M+Na]^+$ peak at *m/z* 497.2050) suggested the molecular formula $C_{22}H_{34}O_{11}$ (calcd. 497.2000 for $C_{22}H_{34}O_{11}Na$). From the coupling pattern of the proton signals and the chemical shifts of the carbon resonances due to the sugar moiety, the sugar component in **7** was indicated to be β-D-glucopyranose (Shimoda et al., 2006). The ^1H NMR spectrum of **7** showed two anomeric proton signals at δ 4.88 (1H, *d*, $J = 8.0$ Hz) and 4.90 (1H, *d*, $J = 8.0$ Hz), indicating the presence of β-anomers in the sugar moiety. The ^{13}C NMR data of **7** showed two anomeric carbon signals at δ 102.6 and 104.8. HMBC correlations were observed between the anomeric proton signal at δ 4.90 (H-1′) and the carbon resonance at δ 156.7 (C-2) and between the anomeric proton signal at δ 4.88 (H-1″) and the carbon resonance at δ 70.0 (C-6′). These results confirm that the inner β-D-glucopyranosyl residue was attached to the phenolic hydroxyl group of carvacrol (**2**) and that the pair of β-D-glucopyranosyl residues was 1,6-linked. Thus, compound **7** was identified as 2-methyl-5-(1-methylethyl)phenyl 6-*O*-(β-D-glucopyranosyl)-β-D-glucopyranoside. Earlier, the biotransformation of carvacrol (**2**), which has an antimicrobial activity, to the corresponding hydroquinone derivative by *Mucor hiemalis* has been reported (Herber et al., 1972). The result obtained here showed that the metabolism of carvacrol (**2**) in cultured plant cells of *E. perriniana* was quite different from that in microorganism such as *M. hiemalis*.

2.3. Biotransformation of eugenol (3)

Eugenol (**3**) was transformed by the same biotransformation system as thymol (**1**) and carvacrol (**2**). It has been reported that no formation of certain kinds of secondary metabolites occur in the cultured cells of higher plants (Suga and Hirata, 1990). Production of eugenol (**1**) in the cultured cells of *E. perriniana* was investigated. Extraction of the cultured cells of *E. perriniana* with MeOH revealed that no formation of eugenol (**1**) as the secondary metabolite occurred in the cultured *E. perriniana* cells. The products **8** and **9** were detected in the MeOH extract of the cells after a five-day incubation. A relatively slow formation of the β-gentiobioside **9** was observed in comparison with the cases of the conversion of **1** and **2** as shown in Fig. 1c. The structures of the products were determined as 2-methoxy-4-(2-propenyl)phenyl β-D-glucopyranoside (**8**, 7%) and 2-methoxy-4-(2-propenyl)phenyl 6-*O*-(β-D-glucopyranosyl)-β-D-glucopyranoside (**9**, 58%). The HRFABMS spectrum of **9** showed a pseudomolecular ion $[M+Na]^+$ peak at *m/z* 511.2183, suggesting the molecular formula $C_{22}H_{32}O_{12}$ (calcd. 511.2152 for $C_{22}H_{32}O_{12}Na$). The ^1H NMR spectrum of **9** showed two anomeric proton signals at δ 4.82 (1H, *d*, $J = 7.6$ Hz) and 4.90 (1H, *d*, $J = 7.6$ Hz). The ^{13}C NMR spectrum of **9** exhibited 22 carbon signals including two anomeric carbon resonances at δ 102.8 and 104.4. The sugar component in **9**



Scheme 1. Glycosylation of thymol (**1**), carvacrol (**2**), and eugenol (**3**) by the cultured cells of *E. perriniana*.

was determined to be β-D-glucopyranose from the coupling pattern of the sugar proton signals and the chemical shifts of the sugar carbon resonances (Shimoda et al., 2006). The ^{13}C NMR chemical shift of C-6′ comparatively shifted downfield to δ 69.5. Upon irradiation of the anomeric proton at δ 4.90 (H-1′), NOE was observed for the signal δ 7.13 (H-6). HMBC correlations were observed between the anomeric proton signal at δ 4.82 (H-1″) and the carbon resonance at δ 69.5 (C-6′). These results confirms that the inner glucopyranosyl residue was attached to the phenolic hydroxyl group of eugenol (**3**) and that the pair of β-D-glucopyranosyl residues was 1,6-linked. Thus, the compound **9** was identified as 2-methoxy-4-(2-propenyl)phenyl 6-*O*-(β-D-glucopyranosyl)-β-D-glucopyranoside.

3. Conclusions

The results of this experiment revealed that the cultured cells of *E. perriniana* are able to convert the compounds found in spices, such as thymol (**1**), carvacrol (**2**), and eugenol (**3**), into the corresponding β-glucosides and β-gentiobiosides which are accumulated in the cells. It is well known that glycosylation by plant cells serves for the detoxification of toxic phenolic compounds which could arise either from normal plant metabolism or from the environment (Tabata et al., 1976; Kamel et al., 1992). The results here suggested that exogenous phenolic compounds such as thymol (**1**), carvacrol (**2**), and eugenol (**3**) with high concentration act as chemical stress against the cultured *E. perriniana* cells and that defense system against chemical stress is different between plants and microorganisms (Herber et al., 1972). As shown in Fig. 1, the yield of glycosides of thymol (**1**) at 6 h was about 1.5 times higher than that of carvacrol (**2**) and about 4 times higher than that of eugenol (**3**). This suggests that glucosyltransferases in the cultured cells of *E. perriniana* have the highest specificity for thymol (**1**) among these substrates. The plant enzymes responsible for these biotransformations should be useful for the preparation of higher water-soluble derivatives of **1**–**3**. This procedure is a simple operation and is environmentally friendly. This method is useful for practical preparation of the glycosides as food additives. Studies of the physiological activities of the glycosides and the characterization of these enzymes from *E. perriniana* are now in progress.

4. Experimental

4.1. Substrates

Thymol (**1**), carvacrol (**2**), and eugenol (**3**) were purchased from Aldrich Chemical Co.

4.2. Cell line and culture conditions

Cultured suspension cells of *E. perriniana* were prepared as described previously (Furuya et al., 1987a). Just prior to use for this work, part of the callus tissues (fr. wt 40 g) was transplanted to freshly prepared Murashige and Skoog's medium (100 ml in a 300 ml conical flask, pH 6.2) contain-

ing 1×10^{-4}% of benzyladenine and 3% sucrose and grown with continuous shaking for 1 week on a rotary shaker (120 rpm) at 25 °C in the dark.

4.3. Biotransformation and purification of products

Each substrate (0.08 mmol) was administered to each of 10 flasks (0.8 mmol/l) containing the suspension cultured cells of *E. perriniana* and the cultures were incubated at 25 °C for five days on a rotary shaker (120 rpm) in the dark. After incubation, the cells and medium from each experiment were separated by filtration with suction. Each filtered medium (ca. 80 ml) was extracted with EtOAc (100 ml × 3), with the medium further extracted with *n*-BuOH (100 ml × 3). Each of the EtOAc and *n*-BuOH fractions were analyzed by the HPLC. Each of the cells were then extracted with MeOH for 12 h (100 ml × 3) and sonicated for 5 min. The yields of the products were calculated on the basis of the peak area from HPLC using the calibration curves prepared by HPLC analyses of the authentic glycosides. Each MeOH fraction was conc. and partitioned between H_2O (30 ml) and EtOAc (40 ml × 3), both the EtOAc fractions individually combined and analyzed by the HPLC. Each H_2O fraction was applied to a Diaion HP-20 column, thus being washed with H_2O followed by elution with MeOH. Each MeOH eluate was subjected to HPLC [column: YMC-Pack R&D ODS column (150 × 30 mm); solvent: MeOH–H_2O (9:11, v/v); detection: UV (280 nm); flow rate: 1.0 ml/min] to give products.

4.4. Analysis

The ^{1}H and ^{13}C NMR, H–H COSY, C–H COSY, NOE, and HMBC spectra were recorded using a Varian XL-400 spectrometer in CD_3OD solution and the chemical shifts were expressed in δ (ppm) referring to TMS. The HRFABMS spectra were measured using a JEOL The MStation JMS-700 spectrometer. The structures of the products were determined on the basis of their HRFABMS, ^{1}H and ^{13}C NMR, H–H COSY, C–H COSY, NOE, and HMBC spectra.

4.4.1. 5-Methyl-2-(1-methylethyl)phenyl β-D-glucopyranoside (**4**) (24 μmol)

White amorphous powder; HRFABMS: *m/z* 335.1820 $[M+Na]^+$; ^{1}H NMR (400 MHz, CD_3OD): δ 1.18 (6H, *d*, $J = 6.8$ Hz, H-9, 10), 2.27 (3H, *s*, H-7), 3.21 (1H, m, H-8), 3.30–3.48 (4H, *m*, H-2′, 3′, 4′, 5′), 3.70 (1H, *dd*, $J = 12.0$, 5.2 Hz, H-6a′), 3.90 (1H, *dd*, $J = 12.4$, 2.0 Hz, H-6b′), 4.86 (1H, *d*, $J = 7.6$ Hz, H-1′), 6.78 (1H, *d*, $J = 8.4$ Hz, H-6), 6.96 (1H, *s*, H-2), 7.05 (1H, *d*, $J = 7.6$ Hz, H-5); for ^{13}C NMR (100 MHz, CD_3OD), see Table 1.

4.4.2. 5-Methyl-2-(1-methylethyl)phenyl 6-O-(β-D-glucopyranosyl)-β-D-glucopyranoside (**5**) (696 μmol)

White amorphous powder; HRFABMS: *m/z* 497.2048 $[M+Na]^+$; ^{1}H NMR (400 MHz, CD_3OD): δ 1.18 (6H, *d*, $J = 6.8$ Hz, H-9, 10), 2.30 (3H, *s*, H-7), 3.20 (1H, m, H-8), 3.21–3.49 (8H, *m*, H-2′, 2″, 3′, 3″, 4′, 4″, 5′, 5″), 3.63 (1H, *dd*, $J = 12.0$, 5.9 Hz, H-6a″), 3.82 (1H, *dd*, $J = 12.0$, 6.0 Hz, H-6a′), 4.15 (1H, *dd*, $J = 12.0$, 2.0 Hz, H-6b″), 4.34 (1H, *dd*, $J = 12.0$, 2.0 Hz, H-6b′), 4.91 (1H, *d*, $J = 8.0$ Hz, H-1″), 5.01 (1H, *d*, $J = 8.0$ Hz, H-1′), 6.78 (1H, *d*, $J = 7.6$ Hz, H-6), 6.96 (1H, *s*, H-2), 7.06 (1H, *d*, $J = 7.6$ Hz, H-5); for ^{13}C NMR (100 MHz, CD_3OD), see Table 1.

4.4.3. 2-Methyl-5-(1-methylethyl)phenyl β-D-glucopyranoside (**6**) (40 μmol)

White amorphous powder; HRFABMS: *m/z* 335.1722 $[M+Na]^+$; ^{1}H NMR (400 MHz, CD_3OD): δ 1.22 (6H, *d*, $J = 6.8$ Hz, H-9, 10), 2.22 (3H, *s*, H-7), 2.84 (1H, *m*, H-8), 3.29–3.49 (4H, *m*, H-2′, 3′, 4′, 5′), 3.70 (1H, *d*, $J = 12.0$ Hz, H-6a′), 3.88 (1H, *d*, $J = 10.0$ Hz, H-6b′), 4.86 (1H, *d*, $J = 8.0$ Hz, H-1′), 6.77 (1H, *d*, $J = 8.0$ Hz, H-5), 6.99 (1H, *s*, H-3), 7.01 (1H, *d*, $J = 8.0$ Hz, H-6); for ^{13}C NMR (100 MHz, CD_3OD), see Table 1.

4.4.4. 2-Methyl-5-(1-methylethyl)phenyl 6-O-(β-D-glucopyranosyl)-β-D-glucopyranoside (**7**) (448 μmol)

White amorphous powder; HRFABMS: *m/z* 497.2050 $[M+Na]^+$; ^{1}H NMR (400 MHz, CD_3OD): δ 1.23 (6H, *d*, $J = 6.8$ Hz, H-9, 10), 2.22 (3H, *s*, H-7), 2.85 (1H, *m*, H-8), 3.22–3.69 (8H, *m*, H-2′, 2″, 3′, 3″, 4′, 4″, 5′, 5″), 3.76 (1H, *dd*, $J = 12.0$, 6.0 Hz, H-6a″), 4.01 (1H, *dd*, $J = 12.0$, 6.0 Hz, H-6a′), 4.17 (1H, *d*, $J = 12.0$ Hz, H-6b″), 4.33 (1H, *d*, $J = 12.0$ Hz, H-6b′), 4.88 (1H, *d*, $J = 8.0$ Hz, H-1″), 4.90 (1H, *d*, $J = 8.0$ Hz, H-1′), 6.77 (1H, *d*, $J = 7.2$ Hz, H-5), 6.98 (1H, *s*, H-3), 7.00 (1H, *d*, $J = 8.0$ Hz, H-6); for ^{13}C NMR (100 MHz, CD_3OD), see Table 1.

4.4.5. 2-Methoxy-4-(2-propenyl)phenyl β-D-glucopyranoside (**8**) (56 μmol)

White amorphous powder; HRFABMS: *m/z* 349.1861 $[M+Na]^+$; ^{1}H NMR (400 MHz, CD_3OD): δ 3.29–3.49 (6H, *m*, H-7, 2′, 3′, 4′, 5′), 3.69 (1H, *dd*, $J = 12.0$, 5.2 Hz, H-6a′), 3.83 (3H, *s*, H-10), 3.86 (1H, *dd*, $J = 12.4$, 2.0 Hz, H-6b′), 4.84 (1H, *d*, $J = 7.6$ Hz, H-1′), 4.99 (1H, *d*, $J = 19$ Hz, H-9a), 5.05 (1H, *d*, $J = 19$ Hz, H-9b), 5.94 (1H, *m*, H-8), 6.71 (1H, *d*, $J = 8.0$ Hz, H-5), 6.81 (1H, *s*, H-3), 7.09 (1H, *d*, $J = 8.4$ Hz, H-6); for ^{13}C NMR (100 MHz, CD_3OD), see Table 1.

4.4.6. 2-Methoxy-4-(2-propenyl)phenyl 6-O-(β-D-glucopyranosyl)-β-D-glucopyranoside (**9**) (464 μmol)

White amorphous powder; HRFABMS: *m/z* 511.2183 $[M+Na]^+$; ^{1}H NMR (400 MHz, CD_3OD): δ 3.20–3.52 (10H, *m*, H-7, 2′, 2″, 3′, 3″, 4′, 4″, 5′, 5″), 3.66 (1H, *dd*, $J = 11.6$, 5.2 Hz, H-6a″), 3.83 (3H, *s*, H-10), 3.85 (1H, *dd*, $J = 12.0$, 5.2 Hz, H-6a′), 4.13 (1H, *dd*, $J = 11.6$, 2.0 Hz, H-6b″), 4.37 (1H, *dd*, $J = 12.0$, 2.0 Hz, H-6b′), 4.82 (1H, *d*, $J = 7.6$ Hz, H-1″), 4.90 (1H, *d*, $J = 7.6$ Hz, H-1′), 4.99 (1H, *d*, $J = 19$ Hz, H-9a), 5.06 (1H, *d*, $J = 19$ Hz, H-9b),

5.95 (1H, *m*, H-8), 6.75 (1H, *dd*, $J = 8.0$, 1.2 Hz, H-5), 6.82 (1H, *s*, H-3), 7.13 (1H, *d*, $J = 8.4$ Hz, H-6); for ^{13}C NMR (100 MHz, CD_3OD), see Table 1.

4.5. Time course experiments

50 g (fr. wt) of the suspension cells of *E. perriniana* was partitionated to each of eight flasks containing 100 ml of the MS medium. Substrate (0.1 mmol) was administered to each of the flasks (1 mmol/l) and the mixtures were incubated on a rotary shaker at 25 °C. At a 6-h interval, one of the flasks was taken out from the rotary shaker, and the cells and medium were separated by filtration. The extraction and analysis procedures were same as described in Section 4.3. The yield of the products was determined on the basis of the peak area from HPLC and expressed as a relative percentage to the total amount of the whole reaction products extracted.

Acknowledgement

The authors thank Dr. Howard J. Williams of Texas A&M University for critical reading of the manuscript.

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